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Curriculum of Biochemistry

MICROALGAE AS CELL FACTORIES: RESEARCH AND INDUSTRIAL APPLICATIONS

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FIGURE INDEX

TABLE OF CONTENTS

Chapter 1

Introduction

The current global economic landscape is witnessing a profound shift from fossil-based to renewable and biobased alternatives, propelled by policy incentives such as the European Union's 20-20-20 goals: 20% cut in greenhouse gas emissions (from 1990 levels) 20% of EU energy from renewables, 20% improvement in energy efficiency. These objectives, aiming for enhanced energy efficiency, reduced CO2 emissions, and increased renewable energy generation by 2020, have instigated a fundamental reevaluation of industrial practices. The APG Group, a vanguard in its domain, has proactively responded by integrating new and vanguardist company, with the purpose to reach these goals, have sponsored the participation of its R&D researchers to start an innovative Executive PhD program with the University of Genova and the Department of Experimental Medicine. This program, intricately woven with the company's dedication to circular economy principles, sustainability and microalgae research, has crystallized into a strategic endeavor to meet the escalating demands of research and development that the modern and climate sensitive market of the future requires.

Over a rigorous three-year journey, the program has successfully aligned academia and industry, yielding transformative accomplishments. One notable achievement involves the meticulous refinement of microalgae cosmetic extract production, unveiling new cosmetic properties that have positioned the APG Group at the forefront of the cosmetic microalgae sector. Simultaneously, the evolution of *Tetraselmis* biomass to cater to client demand within the competitive microalgae market exemplifies the program's commitment to adaptation and innovation. This chapter introduces the trajectory undertaken during this Executive PhD course,

signifying the entwined journey of academic exploration and industrial advancement within the fields of microalgae, circular economy, and sustainability.

The ensuing chapters dives into the intricate landscape of a biobased economy, elucidating the opportunities and challenges it presents. Microalgae biomass stands as a versatile contender, harboring lipids, proteins, carbohydrates, and pigments that span a broad spectrum of applications. This showcases the evolving demand for bio-based products, prompting a paradigm shift towards sustainable and renewable resources. With a focus on bio-lipids, bioenergy, and bio-based chemicals and materials, the transition towards bio-based products mandates a strategic knowledge of various feedstocks, conversion techniques, and production routes, encapsulated within the concept of biorefinery.

The potential of biorefining is far-reaching, with examples like oilseed mills, exemplifying the synthesis of food and feed products that the main company of the APG group, A&A Fratelli Parodi s.p.a., have been doing in the metropolitan area of Genoa since three generations. The biorefinery approach not only holds promise for sustainable microalgae biomass production but also for the creation of value-added products from biomass residues and extracts. Within this framework, photosynthetic pigments and carbohydrates emerge as a critical component, offering avenues for producing cosmeceutical products without preservatives or use of petroleum derivated chemicals and minimizing energy consumption.

In the realm of biobased resources, algae biomass emerges as a remarkable and versatile contender. Comprising lipids, proteins, carbohydrates, and pigments, algae biomass boasts a diverse array of valuable components[1], [2]. The spectrum of potential applications spans from high-value nutraceuticals, beneficial food

constituents, and cosmetics to lower-value commodities such as biofuels, fertilizers, and wastewater treatment applications [3], [4]. Historically, microalgae research predominantly centered on food and feed applications [5], [6], however, recent attention has markedly shifted towards the extraction of lipids and pigments for their cosmetic, antioxidant and nutraceutical applications [7]–[9]. This pivot is fueled by the extraordinary range of products that can be derived from algae biomass, facilitating the concept of a self-sustaining biorefinery model and a conscious use of the resources needed for their production; primarily: water, air and CO_2. Indeed, the biorefinery principle is deemed indispensable to attain sustainable and economically viable commercial algae production in the future [10].

This chapter sets the stage for an exploration of the intricate landscape of biobased resource utilization, as the subsequent sections will present the numerous steps of biorefinery operations, culminating in a comprehensive understanding of the APG Group's pioneering efforts in microalgae, circular economy, and sustainability.

The beginning of the Journey: 3.5 Billion Years Ago Microalgae's Vital Role

Algae, the lifeblood of Earth's atmospheric oxygen, plays an indispensable role in supporting human and animal respiration [11]. Within the realm of algae, two distinct types thrive: macroalgae, commonly known as seaweed, and microalgae, which take center stage in this thesis. Microalgae, encompassing microscopic eukaryotic forms (figure 1.) exhibit a vast spectrum of sizes – from solitary unicellular entities to intricate multicellular aggregations. In contrast to higher plants, microalgae lack specialized organs like roots, stems, flowers, and leaves, yet parallel the higher plants' ability for photosynthesis.

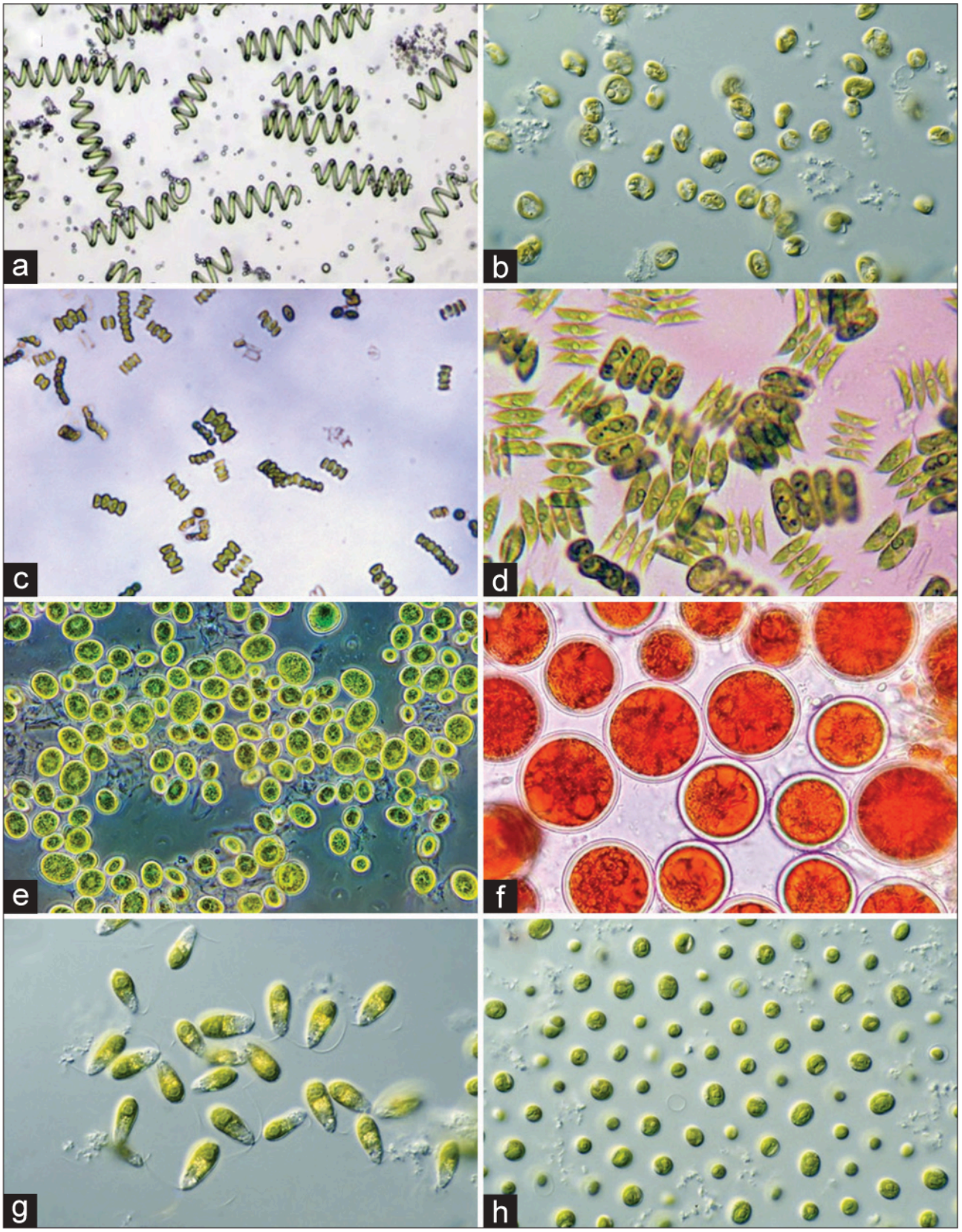


Figure 1 Microalgae of interest – (a) Spirulina spp., (b) Pavlova spp., (c) Desmodesmus spp., (d) Scenedesmus spp., (e) Chlorella vulgaris, (f) Haematococcus pluvialis, (g) Dunaliella spp., and (h) Nannochloropsis spp. The images reproduced in this figure are assigned to the public domain through a Creative Commons Zero (CC0) license or similar release – Wikimedia Commons.] [12]

The realm of microalgae teams with biodiversity, largely untapped and promising. While an estimated two hundred thousand species exist, a mere thirty-five thousand have been officially described [13], [14]. From algal biomass, over fifteen thousand novel compounds have been characterized chemically [15], endowing most microalgae with an array of unique products, spanning from carotenoids, antioxidants, fatty acids, enzymes, polymers, peptides, toxins, sterols, and more [16], [17]. The manipulation of environmental factors like temperature, illumination, pH, CO2 supply, salt, and nutrients enables the accumulation of these diverse products within microalgae [18], [19]

The distinctive array of products positions microalgae for a multitude of applications across agriculture, pharmaceuticals, cosmetics, and the food industry. Their contributions extend to livestock feed and as additives, enriching products with vital vitamins, pigments, and essential fatty acids. The commercial cultivation of microalgae emerges as an appealing mechanism to transmute light into both biomass and valuable compounds. [20], [21]

Microalgae biomass: biology and opportunities

The principal process propelling microalgae's energy acquisition is photosynthesis. Photosynthesis signifies the conversion of light energy into hydrocarbons through the assimilation of carbon dioxide, with oxygen as a byproduct:

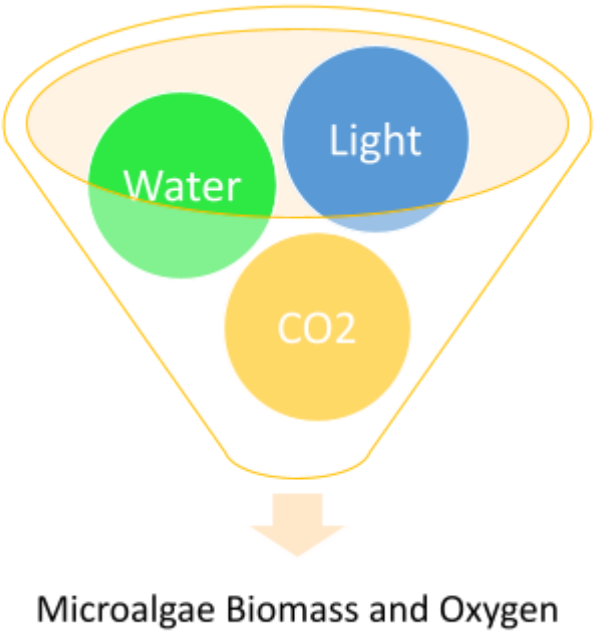


Figure 2- The principle of algae; algae require (sun-) light, water, and CO2, and through photosynthesis can produce oxygen and biomass, the latter of which can be used for a multitude of applications.

Comprising light and dark reactions, photosynthesis entails the absorption of photons within the range of 400 to 700 nm (Photosynthetic Active Radiation, PAR) by chlorophyll, supported by auxiliary pigments. This energy catalyzes the creation of ATP and NADPH, serving as chemical energy and reducing power for the ensuing dark reactions. In these dark reactions, enzymes within the Calvin cycle leverage ATP and NADPH to convert carbon dioxide into organic molecules [22].

For microalgae, the theoretical maximum efficiency of converting light energy to biomass, rooted in photosynthesis, stands at 21% on a PAR basis. [23]

Carotenoids and pigments: antennas for light

Pigments, those molecules that absorb specific portions of the visible light spectrum, play a pivotal role in defining colors as they reflect the unabsorbed light, perceived by the human eye. These pigments hold wide-ranging applications, spanning from food to cosmetics. While many pigments can be synthetically manufactured, often at

a lower cost compared to natural production using petroleum, organic acids, and inorganic chemicals, the demand for naturally derived pigments is surging [24].

Microorganisms, including microalgae, stand as a biotechnologically fascinating source of natural pigments due to their diverse array of these molecules. Among these pigments are chlorophylls (green), carotenoids (red, orange, yellow), and phycobiliproteins (red, blue). Microalgae boast a unique advantage as they can accumulate these pigments in concentrations often exceeding those found in higher plants by significant margins (Fig. 3).

Moreover, microalgae exhibit the ability to concurrently produce other high-value compounds like long-chain polyunsaturated fatty acids, vitamins, and bulk components such as starch, proteins, and lipids [25].

Despite the extensive advantages of employing microalgae for pigment production and their potential to yield a diverse spectrum of pigments, the commercial landscape currently only capitalizes on four specific pigments: β-carotene (*Dunaliella salina*), astaxanthin (*Haematococcus pluvialis*), phycocyanin (*Arthrospira platensis*) and fucoxanthin (*Tiso and Phaeodactylum tricornutum*). While β-carotene, astaxanthin and fucoxanthin have been associated with antioxidative benefits, rendering them valuable for nutraceutical applications, phycocyanobilin finds its utilization primarily in the food sector.

However, the broader utilization of these carotenoids and the expansion of their market presence necessitate a reduction in production costs. These costs primarily stem from cultivation, harvesting, and downstream processing expenditures, with photobioreactor investment costs prominently influencing the equation. Enhanced pigment yield in relation to light becomes imperative to diminish production

expenses, particularly in outdoor photobioreactor arrays, which directly link carotenoid yield on light to investment returns.[26], [27]

Unveiling optimal cultivation conditions and delving into pigment metabolic pathways and their regulation is pivotal for achieving maximal pigment yields in terms of light. Insightful research, such as that conducted by Zemke et al. [28]showcases the pronounced influence of initial biomass concentration on pigment yield for carotenogenic microalgae in outdoor photobioreactors. Moreover, fundamental discoveries concerning pigment metabolic pathways have illuminated key mechanisms. For instance, in *H.pluvialis*, it has been discerned that β-carotene acts as an astaxanthin precursor, with β-carotene ketolase upregulation relying on de novo protein synthesis [29], [30] Similarly, *D. salina* was found to accumulate β-carotene exclusively in chloroplastidic oil bodies [31], while in *P.tricornutum* the fucoxanthin production is tightly bonded with the type of the wavelength received [32]. These foundational insights serve as crucial touchstones for devising metabolic engineering strategies aimed at boosting carotenoid yield concerning light. While these pivotal findings represent significant strides, many aspects of pigment metabolism remain enigmatic, and substantial enhancements in pigment yield relative to light are yet to be fully realized. This leads to the primary objective of the research outlined in this thesis, which aims to gain deeper insights into the pigment metabolism of phototrophic microalgae across diverse process conditions, with a particular emphasis on secondary carotenoids. The goal is to pave the way for the optimization of pigment production derived from phototrophic microalgae and use their extract as a platform to develop new astonishing products.

Microalgal pigments, particularly chlorophylls and carotenoids, are crucial players in the photosynthetic process, forming intricate photosystems and light harvesting complexes that enable energy transfer for the primary photochemical reactions. These pigments serve as fundamental components in capturing and utilizing light energy, thus being fundamental to the growth and survival of microalgae. Beyond their intrinsic biological importance, these pigments have gained substantial recognition for their diverse applications in various industries, notably food and cosmetics. While synthetic pigment production remains prevalent, the surge in consumer demand for naturally sourced pigments has surged, driving the exploration of microalgae as a sustainable and efficient source of such compounds.[34], [35]

Microalgae, due to their extensive range of pigments including chlorophylls (green), carotenoids (red, orange, and yellow), and phycobiliproteins (red and blue), offer an array of colorful options that are highly valued in various applications. These pigments not only imbue products with vibrant hues but also possess functional attributes. For instance, carotenoids exhibit antioxidant properties, and the abundance of fucoxanthin enhances the capture of green light, a prevalent wavelength in aquatic environments. Carotenoids' capability to quench singlet oxygen increases with the number of conjugated double bonds, while the presence of functional groups in end rings further augments their antioxidant activity.[36]

Phaeodactylum tricornutum: the carotenoids' treasure chest

Phaeodactylum tricornutum, a marine microalgae categorized within the diverse group of diatoms (class Bacillariophyceae), thrives in temperate coastal waters, and adapts to varying salinity conditions. This single-celled organism exists in a range of morphologies, with the rod-shaped form being most prevalent, measuring around

10-12 µm. The genome of *P.tricornutum* has been sequenced, reflecting its significance for biological and applied research. This microalga is notably abundant in polyunsaturated fatty acids, particularly omega-3s, and the pigment fucoxanthin, which plays a pivotal role in both photosynthesis and oxidative stress mitigation [37], [38].

This microalga holds significant promise for large-scale cultivation due to its commercial viability and capability to achieve high cell densities [39]. Remarkably adaptable, it manifests three distinct morphotypes: oval, fusiform, and triradiate. Among these, the fusiform morphology (Figure 2) is the most consistent and stable. The morphological shifts observed within these types are influenced by external conditions in their habitat, and the underlying mechanisms driving these changes are intricate [40].

Figure 3 – Phaeodactylum tricornutum morphotypes

Each morphotype exhibits unique characteristics that contribute to their diversity. Notably, the fusiform and triradiate shapes possess higher buoyancy compared to the oval forms, a feature that enhances their adaptability to a more planktonic lifestyle. It is plausible that the species initially exhibited an oval shape and inhabited benthic communities; however, over the course of evolution, it transitioned to a planktonic lifestyle, a shift that conferred certain advantages [41]. The morphological differences of this microalga will be deeply analyzed through electron microscope

and fluorescent staining in order to better understand the differences between the forms.

Initially documented in 1897, this species has gained widespread cultivation as a vital feed in aquaculture practices but then gained a much more comprehensive application in the nutraceutical sector due to its carotenoids content [42]. Its optimal growth temperature range, typically between 18 and 22 °C, designates temperate climates as its favored habitat. The comprehensive composition of *P.tricornutum* comprises roughly 35-40% protein, 15-20% lipids, 25-30% carbohydrates, and 15% ash content.

P.tricornutum uniquely expresses chlorophyll a and c, as well as xanthophyll-type carotenoids, which lend their characteristic golden-brown coloring. Trimeric and oligomeric xanthophyll-chlorophyll-protein complexes contribute to blue-green light absorption in diatom thylakoids.

Notable xanthophylls produced by microalgae include lutein, astaxanthin, canthaxanthin, and fucoxanthin. While these pigments are integral to light-harvesting during the vegetative phase, their production is influenced by biotic and abiotic factors like light intensity, nutrient availability, pH, temperature, salinity, and heavy metals.

Fucoxanthin (Fx), constituting about 10% of total carotenoids, holds a pivotal role among microalgal carotenoids. The most abundant isomer, trans-fucoxanthin (E-Fx), exhibits superior biological activity. Nonetheless, extraction processes, especially involving high temperatures, can lead to isomerization alterations. Hence, the development of suitable extraction methods is vital to ensure optimal extraction yield without distorting the microalgae's native phytochemical profile. While research on Fx's health properties is extensive, information on microalgal

production, pretreatment, and emerging extraction technologies for optimal Fx yields remains limited.

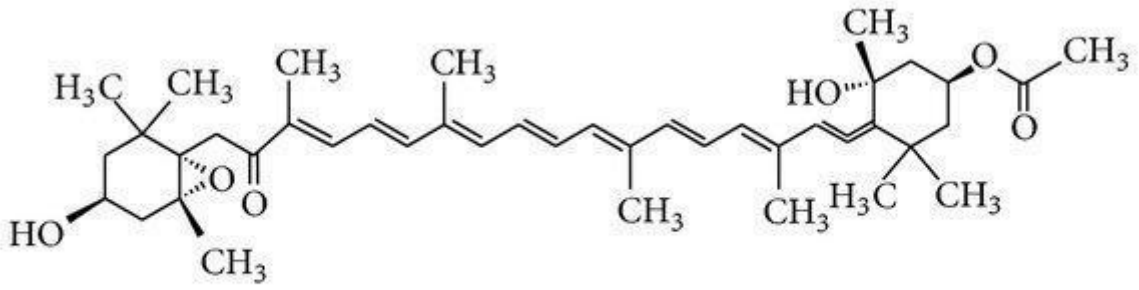


Figure 4 - Chemical structure of fucoxanthin (molecular formula: C42H58O6) - International Journal of Analytical Chemistry

To isolate pigments from microalgal biomass, various methods such as solvent, soxhlet-assisted, enzyme-assisted, and ultrasound-assisted extraction are employed. The choice of extraction method, solvents, and drying procedures significantly impacts bioactive compound yields.

Fucoxanthin: the carotenoids masterpiece

Xanthophylls, the most abundant class of carotenoids, add another layer of versatility with distinct chemical-physical characteristics. These oxygen-containing molecules are characterized by two cycles containing polar groups, which influence their interactions with membranes and peptides. [43]

Fucoxanthin, the most important pigment that we are going to study during this thesis, is a prominent xanthophyll, serves as a prime example with a distinctive role in conferring the characteristic golden-brown color to microalgae and certain

macroalgae. However, the delicate nature of fucoxanthin extracts and solutions, demands careful handling, as it degrades when exposed to heat and direct light.[44]

The potential health advantages of fucoxanthin are underscored by medical research, encompassing realms such as anti-cancer, anti-inflammatory, and anti-obesity properties. [45]

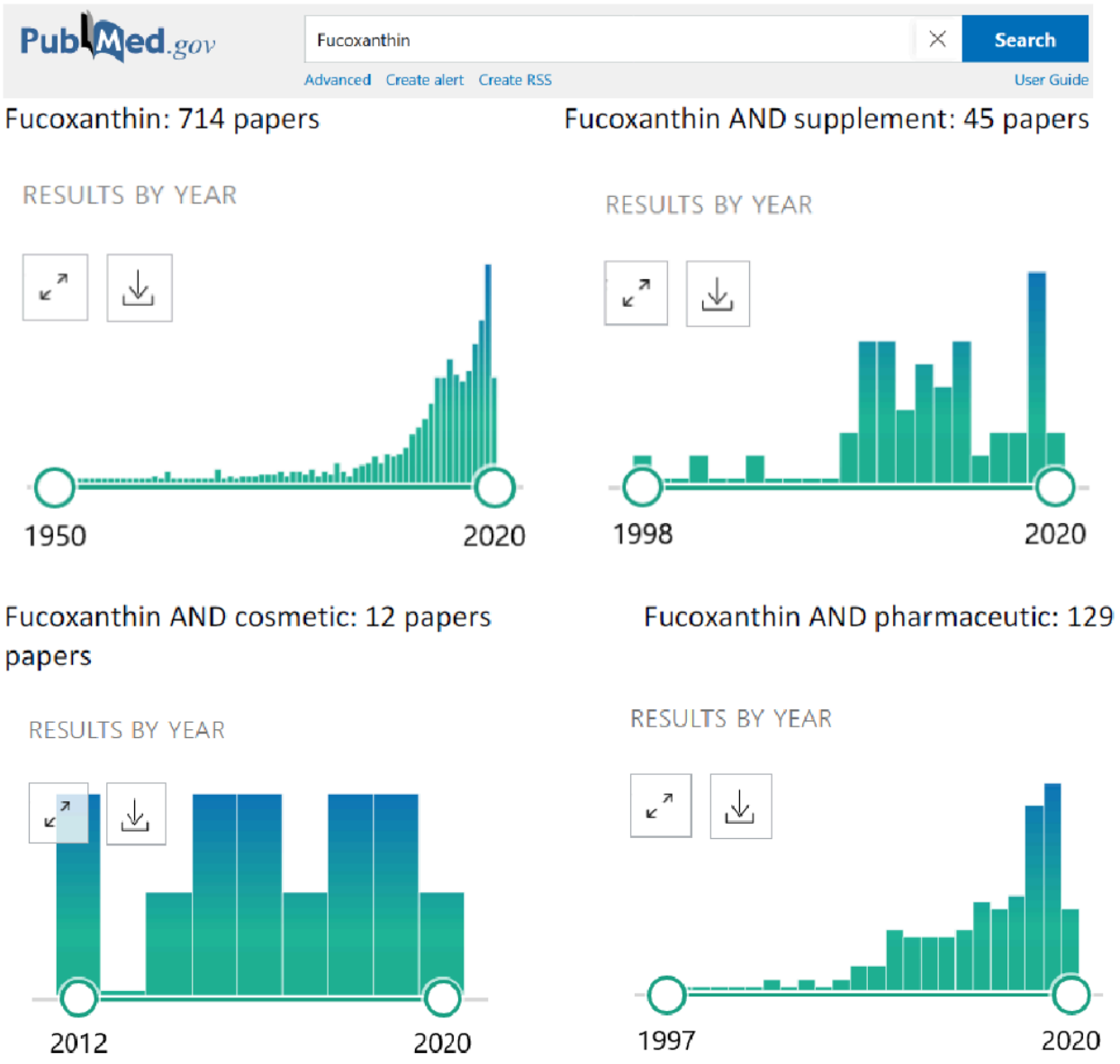


Figure 5 - Results obtained from searching "Fucoxanthin" on Pubmed.gov - All about algal fucoxanthin, S. Mangini - EABA

In the realm of pharmaceutical treatment, fucoxanthin's influence permeates multiple health dimensions. It exerts anti-type 2 diabetes effects, offers a shield against obesity, and counteractant cholesterol imbalances. Its potent anti-inflammatory properties contribute to a broader spectrum of health benefits. Additionally, fucoxanthin's diverse attributes extend to encompass antiangiogenic, antimalarial, and antihypertensive characteristics. Remarkably, its potential role in Alzheimer's disease treatment permits novel avenues for therapeutic interventions.

Food industry applications

Historically, humans have integrated fucoxanthin containing seaweed, such as *Saccharina latissima*, into their diets since antiquity. Such practices, at the very least, demonstrate that consuming fucoxanthin through seaweed consumption does not pose direct risks to human well-being. For cases of ingestion through dietary supplements, wherein varying doses of fucoxanthin were administered to male and female mice. In both single-dose and repeated-dose studies, encompassing doses of 1000 and 2000 mg/kg and 500 and 1000 mg/kg daily for 30 days, respectively, no heightened mortality or anomalous growth patterns were detected. [46] The food market landscape for fucoxanthin is populated with a multitude of products, with a notable presence of slimming food supplements. Yet, the availability of pure fucoxanthin is rare in these supplements. While studies have characterized fucoxanthin as a safe compound with limited bioavailability in humans, intriguing insights into its enhanced uptake have emerged. When consumed alongside lipids, either through natural incorporation or deliberate addition, fucoxanthin's bioavailability is shown to increase.

Considering the acknowledged low bioavailability of fucoxanthin from dietary sources, supplementation featuring the addition of specific lipids emerges as a superior strategy for facilitating its effective accumulation.[47] This approach offers a comprehensive overview of the intricate interplay between fucoxanthin, lipids, and their roles in bioavailability optimization.[48]

Fucoxanthin casts its influence over the realm of nutrition. As an intrinsic component of slimming food supplements derived from seaweeds, it aligns with the contemporary pursuit of holistic well-being. The remarkable journey traversed by fucoxanthin encompasses not only its discrete health-promoting attributes but also the intricate realm of microalgae extracts, where it unfurls extended health benefits with profound implications for human welfare. This panoramic exploration navigates the intricate landscape of fucoxanthin's diverse medical and nutritional facts, accentuating its intricate interplay with human health and well-being.

The realm of food safety intertwines complex regulatory scrutiny and the paramount objective of ensuring consumer well-being.

Notably, fucoxanthin's absence from the NF Union List constitutes a notable observation, hinting at the regulatory intricacies surrounding its inclusion in food products. However, a shine of clarity emerges with the presence of standardized 2% fucoxanthin in *Phaeodactylum tricornutum* extract, championed through a Novel Foods application by Microphyt. Delving into the realm of regulatory evaluations, the pursuit of Generally Recognized as Safe (GRAS) status for fucoxanthin brings testimony to the diligence exerted in assessing its safety profile. A scan through FDA's New Dietary Ingredient (NDI) database provides a dynamic perspective, unveiling two distinct NDINs (#1021 and #1048) from Algatechnologies, attesting to the evolving understanding of fucoxanthin's safety. Additionally, the emergence of a

new FDA NDI, namely *Phaeodactylum* extract "Phaeosol" (#1120) by Microphyt, adds a layer of intricacy to the discourse.

Fucoxanthin food supplements from microalgae		
Name	Company	Alga
Fucovital	Algatechnologies (Israel)	*Phaeodactylum tricornutum*
Phaeosol	Microphyt (France)	*Phaeodactylum tricornutum*

Figure 6 - Fucoxanthin food supplements on the market - All about algal fucoxanthin, S. Mangini – EABA

Cosmetic industry applications

Within the realm of cosmetic applications, which will be the focus of my study, fucoxanthin emerges as a noteworthy contender, showcasing its versatility beyond dietary contexts. Notably, CosIng, a significant regulatory entity, positions fucoxanthin as a "carotenoid isolated from the alga *Sargassum siliquastrum*."

INCI Name	FUCOXANTHIN
Description	Fucoxanthin is a carotenoid isolated from the alga, Sargassum siliquastrum
CAS #	3351-86-8
EC #	-
Identified INGREDIENTS or substances e.g.	
Cosmetics Regulation provisions	
Functions	SKIN CONDITIONING
SCCS opinions	

Figure 7 - Cosmetic Index research for Fucoxanthin - https://ec.europa.eu/growth/tools-databases/cosing/details/86428

This distinctive classification underscores its relevance as a pivotal cosmetic ingredient. Operating under the nomenclature "Skin conditioning" within CosIng, fucoxanthin assumes a multifaceted role in enhancing the overall skin experience. Its

presence, designated as INCI Fucoxanthin, accentuates its significance within the realm of cosmetic formulations.

Moreover, the profound impact of fucoxanthin resonates through its numerical positioning as the 9325th name out of 26491 entries in the EU glossary of common ingredient names. This cataloging, however, warrants careful interpretation, as it neither equates to approval nor qualification, signifying that an ingredient's classification may not inherently encompass all its potential applications. An essential facet in understanding the cosmetic landscape is the recognition that being bestowed a name or inventory inclusion doesn't necessarily imply an ingredient's suitability across all contexts; the intricate intricacies of the cosmetic realm necessitate a comprehensive safety assessment for each product formulation. As the allure of fucoxanthin expands into the world of cosmetics, it does so within a regulatory framework that demands meticulous assessment, unveiling a synergy of science and safety in the pursuit of enhancing skin health and beauty.

In summary, microalgal pigments have transcended their roles in photosynthesis to become valuable assets across diverse industries. From enhancing the visual appeal of food products to offering potential health benefits in pharmaceuticals and beautifying cosmetics, these pigments have proven their versatility and applicability. One of the main goals of this thesis, encapsulated in the R&D department of the APG group which encompasses different companies operating in distinctive but interconnected sectors for the food, feed, pharma and cosmetic production aim essentially to expand the knowledge and the use of fucoxanthin extracted from the microalga *P.tricornutum*, as research continues to unveil their intricate properties, the utilization of microalgal pigments is set to expand, fostering innovation and sustainability in various sectors.

Tetraselmis suecica: a broad platform for cosmetic applications

Tetraselmis suecica, a unicellular flagellated microalga, belongs to the Prasinophyceae class within the Chlorophyta phylum. This marine species is commonly found in oceanic phytoplankton, characterized by dimensions of approximately 7-8 µm by 12-13 µm. Rich in polyunsaturated fatty acids, particularly EPA and DHA, essential amino acids, trace elements, minerals, and vitamins, notably vitamin E, *T.suecica* holds significant nutritional value. Its versatility finds applications ranging from animal feed to the cosmetic industry, with particular emphasis on its mannitol content. [49]–[52].

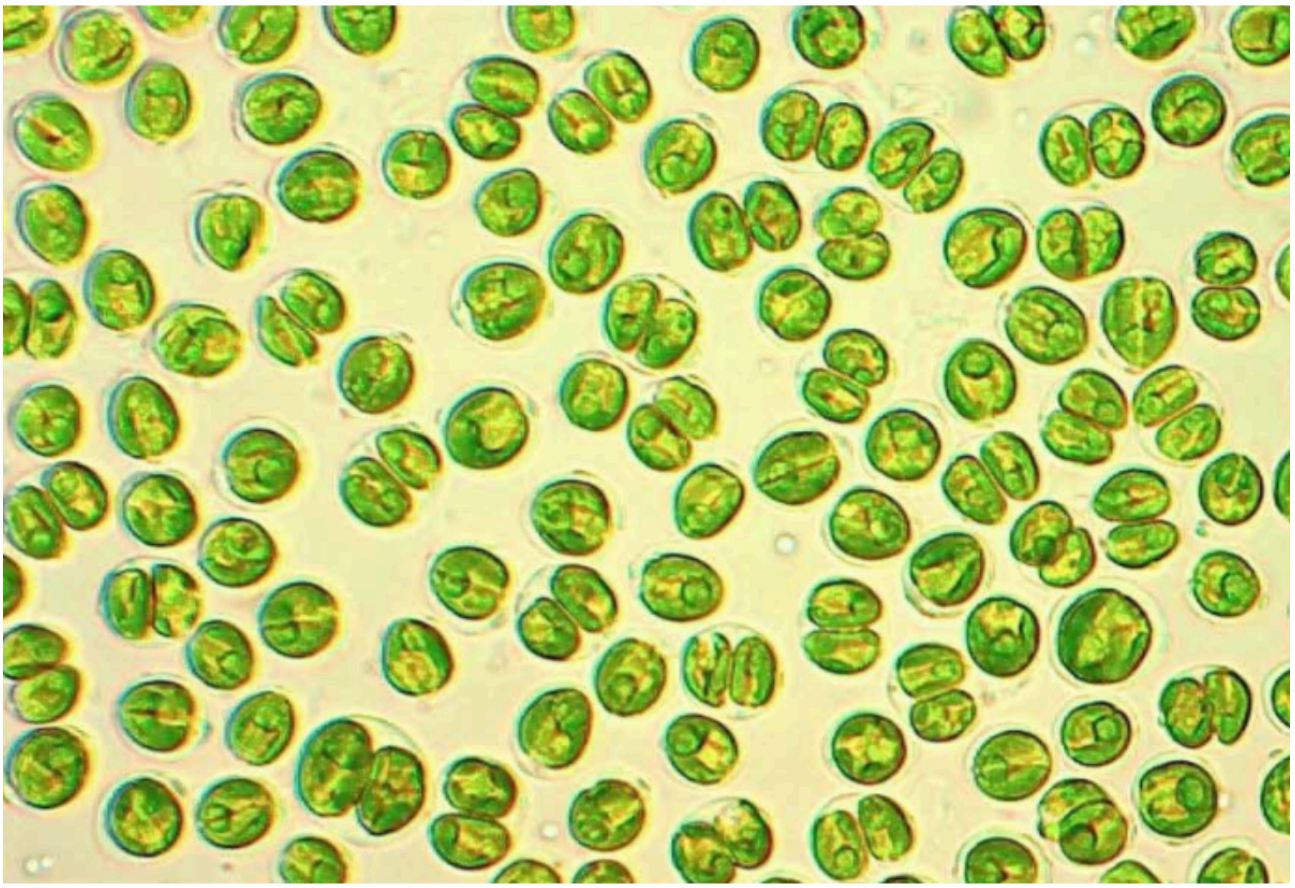


Figure 8 - Tetraselmis suecica culture By Eric C Henry, PhD, Research Scientist, Reed Mariculture Inc- Creative Commons

The species, possessing flagellated mobility, shows rapid growth and thrives across a wide spectrum of temperatures and pH levels. However, upon carotenoid extraction from the species, rapid degradation occurs, rendering it unsuitable for coloration purposes. Remarkably, *Tetraselmis suecica* features a delicate cell wall structure [53], potentially enhancing its components accessibility compared to microalgae with thicker cell walls.

Its composition comprises approximately 35-40% protein, 5-10% fat, 30-35% carbohydrate, comprising the poly-alcohol mannitol on which the studies of this thesis will look further upon, and 15% ash.

Mannitol: a new green source for cosmetic applications

Mmicroalgae possess the capability to produce carbohydrates through photosynthesis and sequester them for storage within starch granules known as pyrenoids. Notably, beyond this intracellular reservoir, the cell wall itself can function as an additional repository for these vital carbohydrates. This intricate carbohydrate landscape within microalgae is far from uniform, displaying variability both across genera and species, as well as through different stages of growth. [54]

The carbohydrate composition is notably different, encompassing a spectrum of sugars including arabinose, xylose, mannose, galactose, and glucose. Furthermore, the repertoire extends to include less conventional sugars such as rhamnose, fucose, and uronic acids [55], [56].

These sugars hold immense significance given the growing scarcity and escalating costs of carbohydrates and their derivates as commodities. This perspective underscores the urgency and the compelling incentive to consider microalgae as a

sustainable reservoir for these valuable carbohydrates, thereby initiating a growing market interest for its resourcing exploring new sources such as the microalgae world.

It is noteworthy that these carbohydrates can constitute a substantial proportion of microalgae biomass, presenting a remarkable prospect for sustainable resource utilization.[57], [58]

In this thesis I am going to concentrate my research on mannitol, the alditol of mannose

Mannitol stands as one of the most prevalent sugar alcohol compounds, with its presence spanning across bacteria, fungi, algae, and plants. Its versatility extends to acting as an organic penetrant, a compatible solute, an antioxidant, and a thermo-protective agent.[59]

Mannitol, known for its multifaceted nature, finds application in various domains. In food, it serves as a sweetener, while in the medical area, it holds roles as a drug to alleviate eye pressure and a medical filler. Additionally, it constitutes a pivotal constituent of mannitol salt agar, crucial for fostering bacterial growth cultures. Operating as a compatible solute within organisms, mannitol performs a myriad of functions encompassing osmolarity regulation, restoration of storage and reduction capacity, and the scavenging of reactive oxygen species [60], [61].

Mannitol's chemical structure renders it a principal contributor to the adaptability of certain algae to high saline concentrations. Its ability to retain substantial amounts of water within cells prevents them from succumbing to osmotic shock. This phenomenon is notably observed in green algae of the Platymonas genus, including *Tetraselmis*, where mannitol levels adjust during osmotic stress, possibly due to a specific enzyme pool.[62]

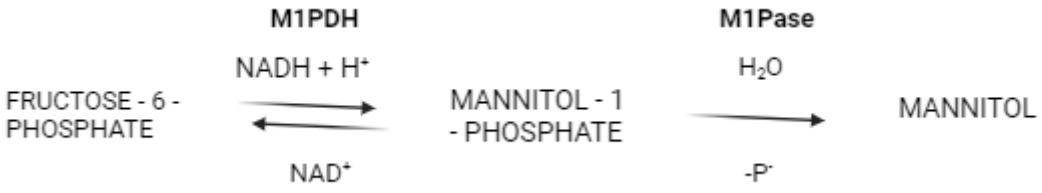


Figure 9 - Two steps in the production of mannitol from fructose-6-phosphate in algae.

Central to this synthesis pathway is the activation of the enzyme 1-mannitol phosphate dehydrogenase (M1PDH), which exhibits enhanced activity in the presence of elevated NaCl concentrations. The degradation pathway of mannitol also appears linked to decreasing sodium chloride concentrations in the culture medium.
In this thesis we aimed to investigate the metabolic shift caused by a range of different stimuli that can be applied to a large scale production of *T.suecica* in order to increase the amount of mannitol, while developing an in house method for its measurement.[64], [65]

Extraction technologies: solvents and applications

The extraction of microalgal pigments is a critical step in exploiting their diverse applications across industries. Various extraction technologies and solvents are employed to efficiently isolate these valuable compounds, unlocking their potential for incorporation into a wide range of products.
Extraction technologies play a pivotal role in obtaining microalgal added value molecules efficiently and effectively. Several methods, ranging from traditional to advanced techniques, are employed for this purpose.
Among these, supercritical fluid extraction using carbon dioxide (SF-CO_2) and solvent extraction are prevalent choices for the extraction and purification of lipids and

carotenoids, while various aqueous systems are employed to retrieve the polar fraction composed of carbohydrates, polysaccharides and proteins. [66]–[68]

SF-CO_2 extraction stands out for its ability to yield high-quality pigments while minimizing solvent residue in the final product. Carbon dioxide, under specific conditions of pressure and temperature, acts as a solvent to extract pigments without degrading their quality.

Solvent extraction, on the other hand, employs solvents like ethanol, methanol, acetone, and hexane. Each solvent offers distinct advantages, such as solubility and selectivity, in extracting specific pigments. Ethanol, for instance, is commonly used due to its effectiveness in extracting a wide range of pigments while being relatively safe and environmentally friendly.[70]

Due to the vast experience in the green chemistry and oil production of the APG group, despite having investigated the SF-CO2 method, it will not be treated in this thesis, while the experiments and the results that will be presented have been obtained using different combinations of solvent extraction for the fucoxanthin extract; on the other hand the mannitol increase in the biomass will not need to be extract since the product is sold as whole biomass for its synergic effects.

Apolar fraction extraction process

Several extraction media are reported in literature for analytical and preparative purpose; from industrial point of view, Methanol, Acetone, Ethyl acetate, Hexane and Ethanol are generally used.[71]–[73]

Table 3. Recent US-granted patents approaching cell disruption, extraction and recovery of microalgae biomolecules using organic solvents (FAME: fatty acid methyl esters).

Patent Purpose	Pretreament	Solvents	Main Results
Production and extraction of squalene from microalgae of the *Thraustochytriales* sp. Family [82]	Alkaline lysis (KOH 45%)	Hexane/ ethanol	6.7 g L^{-1} of squalene content
Production of pure microalgae extracts to modulate the metabolism of human skin and hair follicles [83]	-	Methanol/ ethanol/ ethyl acetate	Achieved an effective treatment using a composition comprising from 0.001 to 35% of dry matter content of an extract of *Monodus* sp.
Method for extraction of lipids in an organic phase and sugars by hydrolysis [84]	Homogenization	Acetone	Results suggest that it is possible to achieve an industrial-scale extraction yield of 96.3% of total lipids in the starting wet algal biomass
Biodiesel production and isolation of several valuable co-products from the marine alkenone-producing microalgae *Isochrysis* [85]	-	n-hexane/ethanol or methanol/ dichloromethane or toluene/acetonitrile	27:8:1 (FAME:alkenones: fucoxanthin) co-production

Figure 10 .Recent US-granted patents approaching cell disruption, extraction and recovery of microalgae biomolecules using organic solvents. [74]

This section aims to delineate the sequential stages that constitute a general extraction process, aimed at isolating apolar constituents, primarily carotenoids and lipids.

Initiating with the sample preparation, the microalgae biomass undergoes a dual approach to ensure analytical precision. Firstly, a controlled drying phase is executed, effectively eliminating the residual moisture content, and thwarting potential water-induced deviations in subsequent quantification. Subsequently, homogenization is pursued, wherein the dried biomass is subjected to mechanical mixing, to obtain a uniform and representative composite integral for statistically reliable analyses.

The subsequent phase, the mechanical transformation of the dried and homogenized sample is undertaken through a grinding procedure. This process serves to disintegrate cellular matrices, thereby facilitating efficient release of the target apolar compounds during the extraction phase. Following the grinding,

solvent extraction assumes a central role, underpinned by the careful choice of solvents like Methanol, Acetone, Hexane, and Ethanol. This strategic selection is based on the different affinities and solvating abilities of these solvents for different apolar molecules of interest.. The methods, time, temperature, and pressure for the contact between the biomass and the selected solvent are the key to a successful industrial process. During this thesis we refined the process used in the company. For reasons of confidentiality and data ownership, we will disclose the parts that are essential for the thesis without going through excessive details about the production process itself.

Following solvent extraction, the extract is subjected to a filtration step. This filtration operation is engineered to expunge particulate matter, cellular debris, and any residual matrix components, thereby yielding a refined extract of elevated purity. This filtrate stands as the foundation for ensuring quantitative analyses and purifications that need to take place in order to obtain a product with the desired composition and formulation.

To harness the full potential of the extracted apolar fraction, a judicious dilution step is introduced, harmonizing the extract concentration within a suitable range for subsequent analytical methodologies. This step not only mitigates potential solvent-induced matrix effects but also ensures the sample's compatibility with the dynamic range of detection instruments. In this step the residual solvents are completely eliminated using various removal technologies such as rotary evaporation under vacuum and heating.

The final step of the process must be compound detection and quantification. This is facilitated by interfacing the HPLC apparatus with a suitable detector, often taking

advantage of UV-Vis absorption, fluorescence, or mass spectrometric techniques. The detector transduces the eluting analytes into quantifiable signals, which are subsequently translated into concentration values through meticulous calibration processes.

In order to develop a robust method for extraction a solid knowledge of the target compound must be internalized.

Here are the main factor influencing extraction process:

- operation on wet or dry biomass.
- Fucoxanthin is damaged by heating: over 70°C it degrades by about 30% each day, while short term exposure at 80°C-100°C is generally well tolerated [75]
- Direct light should be avoided as much as possible, as this can cause rapid break down: exposure to a 60W light bulb degrades it by about 50% [76]

It is also necessary to know the initial content of the target compound, in this case fucoxanthin, in the target microalgae:

Specie	Fucoxanthin content (mg/g)	Sample	Extraction solvent	Reference
Odontella aurita	21,67	Dried	Ethanol	Xia *et al.*, 2013
Isochrysis aff. Galbana	18,23	Dried	MeOH	Kim *et al.*, 2012 (b)
Phaeodactylum tricornutum	15,33	Dried	Ethanol	Kim *et al.*, 2012 (a)
Phaeodactylum tricornutum	8,55	Dried	MeOH	Kim *et al.*, 2012 (b)
Isochrysis galbana	6,04	Dried	MeOH	Kim *et al.*, 2012 (b)
Cylindrotheca closterium	5,23	Dried	Microwave	Pasquet *et al.*, 2011
Nitzschia sp	4,92	Dried	MeOH	Kim *et al.*, 2012 (b)
Chaetoceros gracilis	2,24	Dried	MeOH	Kim *et al.*, 2012 (b)
Phaeodactylum tricornutum	1,81	Dried		Rebolloso-Fuentes *et al.*, 2011

Figure 11 Fucoxanthin occurrence in other microalgae species - All about algal fucoxanthin, S. Mangini – EABA

Analytical methods for compounds determination

It is essential to underline that the determination of carotenoids through HPLC for microalgal matrices has not been officially standardized, leaving the tuning for a reliable method for the determination at each research group in academia or industry.

It is worth of notice that a closer examination of established pharmacopoeial standards presents an HPLC method for *Zeaxanthin* within the USP41-NF36 (United States Pharmacopoeia). However, it is discernible that the realm of Fucoxanthin analysis by means of HPLC/SF is characterized by a dynamic landscape. Various research entities and stakeholder laboratories have actively developed and advanced Fucoxanthin test methodologies via the HPLC/SF framework.

One of the common grounds for all the fucoxanthin analysis methods is the integration of High-Performance Liquid Chromatography coupled with a spectrophotometric detector (HPLC/SF).

The development of a standardized methodology has earned a prominent place on the priority list of the CEN TC454 "Algae." [77]

Mannitol, despite being a commodity chemical, has never before been extracted and measured from a microalgal matrix hindering different possibilities on how to process the sample: it can be both considered as a plant or a powder opening different pretreatment methods. The analysis, however, has been carried out in HPLC coupled with a Refractive Index detector.

Key Elements in Algae Cultivation and Processing

Navigating the microalgae production chain demands consideration of numerous elements, ranging from resource production and supply to algae cultivation, product component formation, resource recycling, biomass processing, integration with production, transportation, and the consequential implications on economics, environment, community, and society [78]–[80]. At the heart of this chain lies microalgae biomass production, which plays a pivotal role. Algae cultivation has garnered significant attention over the past decades [81]. However, much of the research has been confined to laboratory-scale experimentation, primarily aimed at understanding the behavior of individual cells under controlled conditions [82], [83]. Given the centrality of cultivation in the production process, it is imperative to expand the scope of research to encompass the commercial or "large-scale" sector.

Microalgae Production Systems

Overcoming the barriers obstructing large-scale commercial microalgae production necessitates a multidisciplinary approach. One of the main issues of this approach revolves around devising appropriate reactor systems tailored for extensive commercial microalgae production. Among the reactor designs commonly employed and proven at a semi-industrial scale are open raceway ponds, horizontal and vertical tubular photobioreactors, and flat panel photobioreactors. After years of large-scale production at the Archimede Ricerche s.r.l. production plant, situated in the Ligurian riviera precisely at Camporosso (IM), the development of a patent pending closed photobioreactor have been the object of study of this doctoral program bringing new expertise to a field which is in constant evolution. Each design

comes with its distinct merits and drawbacks, elaborated upon in the subsequent discussion.[84]–[87]

Open Raceway Pond

The open raceway pond stands as a system where the microalgae culture remains in direct contact with the surrounding environment and ambient conditions. This design typically involves a culture depth of 10-30 centimeters and utilizes paddle wheels to facilitate mixing along elongated channels. Due to the extended optical path, open raceway ponds generally exhibit lower biomass concentrations compared to alternative systems. Operational parameters involve liquid velocities around 0.25 m/s, and an optimal length-to-width ratio of 10:1 is deemed ideal for raceway ponds. However, the openness of the raceway pond makes it more susceptible to contamination, limiting the viable species that can be cultivated to those capable of thriving under specialized and often extreme conditions that discourage contamination. While raceway ponds boast low initial investment costs, the lower biomass concentrations result in significant expenses associated with harvesting the microalgal biomass.[88]–[90]

Tubular Systems

Tubular photobioreactors comprise transparent tubes through which the microalgae culture circulates, effectively exposing it to sunlight. Such systems exhibit various configurations, including tubes arranged in a single horizontal plane, or in multiple vertical planes resembling a fence-like structure.

Tubular systems typically operate with liquid velocities ranging from 0.3 to 0.6 m/s, although higher values (0.6-0.9 m/s) are employed to counteract fouling. The adoption of increased liquid velocities corresponds to elevated production costs.

Tube diameters span from 0.02 to 0.15 meters, with narrower diameters correlating to higher biomass densities. Photosynthesis triggers the accumulation of dissolved oxygen in the pumped algae suspension within the tubes. Excessive dissolved oxygen concentrations have been identified to negatively impact growth. To mitigate this, a degasser forms an integral part of any tubular photobioreactor, typically, the degasser assumes the form of a vertical bubble column where air or an air/carbon dioxide mixture is introduced. Furthermore, the oscillation between light (tubes) and darkness (degasser) could detrimentally influence growth. To counteract this, efforts are directed at minimizing the dark volume within tubular photobioreactors.[91]–[94]

Flat Panel Photobioreactors

Flat panel photobioreactors adopt the form of slender rectangular vertical vessels, employing aeration for mixing the microalgal culture. These reactors are constructed from transparent materials such as glass or plastic plates, as well as plastic films. The aeration-driven mixing (approximately 1 L-1 min-1 or 1 vvm) effectively eliminates the dissolved oxygen where it's generated, eliminating the need for a separate degasser. The culture depth, representing the shortest optical path, tends to be relatively shallow, ranging from 0.01 to 0.1 meters. An inherent challenge with flat panel photobioreactors lies in their scalability, which typically involves increasing the number of units rather than the volume of each unit. This design leads to predominant mixing along the vertical axis, resulting in limited mixing across the horizontal axis of the reactor. Consequently, the maximal width of a unit is smaller than that of tubular reactors and raceway ponds. This characteristic translates to augmented infrastructure and labor requirements for constructing and operating a commercial plant with flat panels in contrast to tubular systems. However, certain

flat panel designs have effectively addressed these limitations, such as the, the Green Wall Panel from the University of Florence, and the Solix Biofuels flat panels.[95]–[99]

Aims of this thesis work

The primary aim of this thesis is addressing the evolving industrial needs of the APG group, which has been engaged in pioneering new products and technologies in the realms of oil and green chemistry.

The overarching goal is to seamlessly transition this expertise into the budding field of microalgae and the low-carbon footprint market, thereby contributing to increasing the company's already significant efforts to sustainability.

The incessant surge in demand for microalgae-based products, particularly in the nutraceutical and cosmetic markets, necessitates a continuous cycle of development and investments to meet client demands effectively. The focal point of our efforts revolves around the enhancement of a well-established extract produced by our company known as RED. This extract, derived from the diatom *P.tricornutum*, is enriched with the carotenoid Fucoxanthin, renowned for its promises in various applications, including the treatment of diabetes, liver diseases, and lipid metabolism. Additionally, it exhibits substantial cosmeceutical properties, serving as an antioxidant, UV damage protector, and anti-wrinkle agent, thus encapsulating the characteristics of an innovative and groundbreaking ingredient.

To realize the full potential of Fucoxanthin, our primary challenge lies in optimizing its production. A meticulous experimental plan has been devised to achieve this objective. This plan encompasses the development of a higher-yielding strain and an improved production method, optimizing both biomass production within the photobioreactors and the extract itself.

The cornerstone of improving Fucoxanthin production is the purification from microbes of the original microalgae strain used in the production plant. Various

isolation techniques and antibiotic mixtures have been rigorously tested to reduce the interference from other microorganisms during the inoculation process, resulting in a more efficient strain performance. Further, since purified strains exhibited inadequate growth on solid substrates, extensive studies were conducted to optimize colony formation, thereby increasing the number of obtainable strains. These strains were subsequently evaluated for their Fucoxanthin content, leading to the development of a rapid analysis method using thin-layer chromatography to identify promising strains with higher Fucoxanthin content.

The selected promising strains were subjected to different light spectra to assess their impact on growth and Fucoxanthin production. A two-step growth process was implemented to optimize biomass growth and Fucoxanthin accumulation. To gain a deeper understanding of the isolated morphology of the selected strain and its potential influence on growth vessel selection, a morphological study was conducted through electron microscopy. Additionally, a study on lipid droplet distribution was carried out by staining the microalgae with a fluorescent marker.

Following these laboratory phases, the strains were scaled up and grown in a closed photobioreactor within the production plant, ensuring optimal conditions based on temperature, light irradiance, and mixing parameters determined during the laboratory phases. The resulting biomass underwent downstream processes, including centrifugation and freeze-drying, to remove excess water and mitigate the potential heat damage associated with heat-based water evaporation systems.

The extraction process was carried on the resulting biomass powder, yielding a substantial Fucoxanthin extraction. Comprehensive analyses were conducted on the finished product, encompassing Fucoxanthin content, lipids, and the phytochemical profile of the extract. Simultaneously, the biomass was subjected to analyses for protein and lipid content.

The knowledge acquired during this endeavor paved the way for its application to a different microalgal strain, *Tetraselmis suecica*. Responding to a specific client request, we tailored the content of the alditol mannitol within the microalgae biomass. This was achieved through the manipulation of the medium composition, involving variations in salt concentrations to stress the osmotic balance in the microalgae without compromising viability. Moreover, a dedicated HPLC method for mannitol analysis was developed to support this experimental phase.

Chapter 2

Cultivation methods for *P.triconutum*

Culture and Growth Conditions of P.tricornutum in liquid and solid

An inoculum sample of *P.tricornutum*, isolated originally from Blackpool, England, was obtained from the Culture Collection of Algae and Protozoa (CCAP, SAMS Ltd, UK), identified as CCAP1055/1.

The liquid cultivation medium in laboratory consisted of Artificial Sea Water (ASW) supplemented with 30 g/L of Tropic Marin® Classic (Dr. Biener GmbH, Germany) and enriched with a modified f/2 Guillard's medium for diatoms as reported in the original article[101]. The salts per liter composing the medium were $NaNO_3$ 800 mg, NaH_2PO_4 60 mg, Na_2EDTA 0.8 mg, $FeCl_3$ 0.6 mg, $CuSO_4$ 125 µg, $ZnSO_4$ 440 µg, $CoCl_2$ 125 µg, $MnCl_2$ 3 mg, Na_2MoO_4 108 mg, Cyanocobalamin (Vitamin B12) 1 µg, Biotin 1 µg, Thiamine HCl (Vitamin B1) 200 µg. All these reagents were sourced from Sigma-Aldrich and used without further purification. The final pH was adjusted to 8.0 with 1M NaOH or 1M HCl prior to autoclaving at 15 psi for 15 minutes.

Solid medium for *P.tricornutum* was obtained additioning 15 g/l of Bacto agar (DIFCO, France) to the complete medium before sterilization.

Laboratory-scale cultivation of PT occurred over a 14-day period in Erlenmeyer flasks equipped with breathable cotton caps. These flasks contained 10% medium and 5% microalgae inoculum on a rotary flask shaker set to 150 rpm, 25 °C, and a light intensity of 250 µmol m–2 s–1. Cell concentration was determined through cell counting with a Thoma chamber. To grow P.tricornutum on a solid medium 15 g/l of Bacto agar (DIFCO, France) were added to the complete medium before sterilization.

Purification methods for microalgae in multiwell

Single cells deriving from the isolation on solid medium where micromanipulated using a sterile needle and implanted in different wells of a 24-Well Multiwell Plate for cells cultures (Thermo Fisher Scientific, Italy).

The micromanipulator is self-constructed and a picture of its components will be shown in the results section.

The isolation method consisted of picking from the solid medium plates (18 ml/plates) single colonies of isolated *P.tricornutum* (100 µL) inoculated with streak plate technique after a 21 days incubation under a 150-300 µmol/(m^2·s) of light. The single colonies were dissolved in 1ml of medium containing antibiotics.

The antibiotics concentration inside the wells that were used for the purification step are: Ampicillin sodium salt (Merck-Millipore, Italy) 7,5 mg/ml; Tetracycline hydrochloride 12,5 ug/uL; Kanamycin A sulfate salt 3 mg/ml; Streptomycin sulfate salt 1,5 mg/ml; Gentamycin sulfate salt hydrate 1,5 mg/ml, all from (Sigma-Aldrich, Italy).

The efficacy of the antibiotic treatment on the culture was evaluated based on the absence of microbial growth on specific microbiological mediums. Each well containing a single colony inoculum was tested for microbial absence on three distinct microbiological media. After 7 days (the method, for total microbial count UNI EN ISO 4833:2004, was extended from the prescribed 71h at 30°C of incubation to have a broader time of microbial detection). The medium used were: Plate Count Agar (PCA,20,5 g/l autoclaved 15 minutes at 121°C, Lab M, UK), one Tryptic Soy Agar (TSA, 40 g/l, Vwr chemicals, Italy), and Water Plate Count Agar (WPCA, 24 g/l, Lab M, UK) in order to account for different microbial strains.

Medium optimization for purified strains

Due to the slow growth of purified strains on solid medium, parallel growth experiments were conducted in order to reduce the incubation time of cultures. The variations were modulated as follows: Tropic marin salt was halved to 15 g/l; the complete medium was additioned of 300 mg/l of sodium metasilicate (Na_2SiO_3) 300, a filtered, with Minisart® Syringe Filter, Polyethersulfone (PES), Pore Size 0.22 µm, exhaust medium deriving from unpurified culture was added, at 0,1 and 0,5 %, in order to verify if some metabolites deriving from the commensalism with bacterial strains were fundamental for *P.tricornutum* grow.

Thin Layer Chromatography for fast fucoxanthin analysis

Adaptations of the extraction method used for HPLC on dried biomass was required due to the need for small volumes of wet microalgae obtained from the single cells isolation. Several gentle biomass drying techniques were tested (as fucoxanthin is heat-sensitive). Solvent volume (methanol), timing, and cell wall disruption methods were also adjusted to suit the sample type.

A rapid screening methodology for fucoxanthin-rich samples was established using Thin layer chromatography (TLC silica gel 60 f254, 5 x 10 cm, Merck KGaA, Germany). Suitable solvents were identified for proper separation of biomass components extracted with methanol (Reag. grade, Sigma-Aldrich,Italy) as reported in literature[102].

The extraction process was carried out as follows: 2 ml of culture samples obtained from the scale up of the multi-well cultures to 5 ml glass tubes grown under 24g cycles of light at 150 µmol/(m^2·s) for 7 days, were splitted in 2 Eppendorfs tubes in order to assess dry weight and perform the analysis.The samples were centrifuged at 3.000 rpm for 10 minutes and the supernatant discarded.

The dry weight assessment was conducted by weighting a thermally conditioned ellendorf tube (3h at 110°C), and letting the pellet dry for 8 hours at 110°C.

The sample for the fucoxanthin analysis was additioned with 100 µL of methanol and vortexed in pulses until the dissolution of the pellet. Then the tube was let sitting for 15 minutes repaired from direct light or heat in order to allow the extraction. The sample was then centrifuged again at 3.000 rpm for 10 minutes and the supernatant used to run the TLCs.

Different solvent polarity combinations were carried out in order to assess the best combination between resolving power of the solvents mixing. Here reported the solvent mixtures:

TLC n°	Toluene ml	Ethyl Acetate	Acetic Acid
2	7	2	1
3	9	0	1
4	0	9	1
5	9	0	1

Table 1 Solvent mixtures composition for TLC analysis

A standard solution of a commercial fucoxanthin standard (Supelco, Merck KGaA, Germany) was prepared at a concentration of 1% in order to run alongside the samples. After the run on TLC the plates were photographed and analyzed using software UN SCAN IT gel (ver 6.1, Silk Scientific, Inc., USA) to correlate band intensities with fucoxanthin concentration and obtain indicative percentages of fucoxanthin content.

Light waves determination for fucoxanthin production

Strains optimized through the cultivation process underwent assessment under four distinct light spectra irradiances during growth, using a dimmable LED strip light with pure RGB light sources (Bonve Pet, Number of lights 160, 5mx1cm, max voltage: 24 V, Max Power: 12 W, China) .

The selected light spectra were:

1. White light (R+G+B, as control): 80 to 350 µmol/(m²·s), 25°C
2. Blue light: 40 to 200 µmol/(m²·s), 24°C
3. Red light: 10 to 50 µmol/(m²·s), 30°C
4. Blue-Red light (magenta): 70 - 150 µmol/(m²·s), 28°C

The experiments were conducted in a glass jar equipped with air bubbling (0.5 vvm) and a cooling fan to keep the air temperature in the proposed range.

Morphological identification at SEM and fluorescent lipids staining

Microscopic investigations were carried out utilizing a Leica DM 2000 microscope, which was equipped with both transmitted light and epifluorescence capabilities. This microscope was paired with a DFC 320 camera sourced from Leica Microsystems in Wetzlar, Germany, facilitating the visualization and recording of cellular features and behaviors. To visualize neutral lipids within *P.tricornutum* cells, we employed the fluorescent dye BODIPY™ 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene). The BODIPY™ dye (Thermo Fisher Scientific, USA) was used at a concentration of 0.1 µg/mL in the culture medium. Aliquots of diatom cell suspensions (1×10^6 cells/mL) were incubated with the dye in darkness for 7 minutes. This staining procedure was conducted subsequent to the preparation of a stock solution of 0.5 mg/mL BODIPY™ in DMSO (Merck KGaA, Germany).

Following the staining process, the cells were meticulously collected, washed with 3-times the volume of culture medium, and then mounted as wet mounts on

microscope slides. For observations, the Leica DM 2000 microscope was employed, with excitation and emission wavelengths set at 488 nm and 510 nm, respectively.

Scanning Electron Microscopy (SEM)

To perform scanning electron microscopy (SEM) observations, preparations were made on samples of freshly cultured *P.tricornutum* following a protocol outlined by Veltkamp et al. [103]. The process consists in the fresh culture undergoing several treatments, including rinsing with distilled water to eliminate excess salt, centrifugation, washing with absolute ethanol at -18°C to mildly dehydrate the cells, followed by incubation at -18°C for a duration proportional to the sample volume. Subsequently, the sample was stored at 4°C overnight. This freezing and refrigeration cycle was repeated on the following day before proceeding with the drying of the algal biomass. The treated samples were then positioned on Whatman 22 µm filters and subjected to a gradual dehydration process using ascending concentrations of ethanol washes (50%–80%–90%–100%). Afterward, an air-drying method inspired by the research paper of Balbi et al.[104] was employed, with some adjustments due to the different nature of the sample. Following thorough dehydration and drying procedures, small sections of the filters were affixed to stubs using two-sided adhesive carbon tape. A 10-nanometer layer of gold was deposited on the samples through sputtering. The observations were conducted utilizing a SEM VEGA3-Tescan-type LMU microscope (Apollo, Tescan, France), enabling high-resolution imaging of the algal morphology and surface features.

Microalgae biomass production at the Archimede Ricerche s.r.l. production plant

Scale up, downstream and Biomass Dehydration

To generate a substantial biomass for processing and extraction, *P.tricornutum* was cultivated at a larger scale within the photobioreactors at the Archimede Ricerche

s.r.l. production plant in Camporosso, IM, Italy. The cultivation conditions mirrored those of the laboratory scale and followed the results obtained during the light wave tests, with the addition of air enriched with 1% of food grade CO_2 (Air Liquide Italia SpA). The culture of the purified strain was submitted to the following scaling up in order to start from a couple of ml of the initial inoculum up to several thousand liters inside closed photobioreactors. The initial inoculum was first diluted from 50 to 600 ml inside the production plant laboratory, cultivated in Guillard medium as reported in a previous section, inside glass tubes calla Kmeters where the particular geometry allows for an uniform irradiance from the LED lights, ranging from 150 to 600 µmol/(m^2·s), while the temperature is kept under control by an air climatizer set at 23°C. The mixing of the culture is guaranteed by an insufflation at the bottom of the glass tube at 0.5 vvm enriched with 1% CO_2 and the pH is kept under control by constant monitor from operators. Once the concentration of the inoculum reaches a set goal it can then be splitted and diluted in several other Kmeter in order to maintain the same dilution rate of 1 to 6 to keep the cells in an active phase of doubling. These operations need to be repeated for at least 3 weeks in the lab before taking the inoculum out to the natural light photobioreactors in the production plant where the first available volume will be 200 liters. In the plant the medium concentration and the dilution factors cannot be disclosed. After 14 days in the natural light the inoculum can be moved to the artificial light photobioreactor where it can begin the light modulation steps in order to produce fucoxanthin. Upon reaching a concentration of 1 g/L, the harvested algal biomass was freeze-dried in a Criofarma s.a.s machine. The dried biomass was then milled and stored at –20 °C until the extraction process.

Carotenoids Extraction Protocol

A two-step solvent extraction approach was employed to retrieve the pigments from the freeze-dried microalgal biomass obtained from the production plant. Initially, the

biomass underwent a 24-hour extraction with 95-100% ethanol (EMSURE®,Reag. Ph Eur, Italy) at room temperature. Subsequently, enological coal (Esseco s.r.l., Italy) was introduced at a ratio of 1:100 (w/w) to eliminate excess chlorophylls that are being extracted as well from the biomass. The ensuing mixture was vigorously agitated for 1 hour and subsequently passed through filter paper (VWR International Srl, Italy) to eliminate the coal and residual biomass. The resulting extract was subjected to evaporation until dryness, then reconstituted in a combination of water and ethyl acetate (1:1, v/v) to remove salts and purify the nonpolar fraction. This suspension was agitated at 150 rpm for 30 minutes and left to settle for an additional 30 minutes. The supernatant was collected, and this process was repeated twice more. Following the ultimate wash, the ethyl acetate fraction, enriched in carotenoids, was vacuum-evaporated at 40°C to isolate the nonpolar fraction.

Subsequently, the desiccated nonpolar fraction was suspended in food-grade ethanol to produce the final formulation consisting of 70:30 (w/v) extract/ethanol. This formulation was stored at 4°C until analysis.

Analytical Procedures

The analysis hereby reported are modifications, due to the different nature of microalgae, from the following internationally recognized methods:

Table 2 - International analysis adaptations for microalgae

Analysis	International method
Dry weight	*Pharmacopea Europea Section 2.2.32. " Loss on Drying "*
Fucoxanthin	internal method developed in collaboration with CNR (National Research Council) and the department of Industrial Chemistry of The University of Genova

Sterols	*Pharmacopea Europea Section 2.4.22 method A modified for sterols*
Lipids	*Pharmacopea Europea Section 2.4.22 method A "lipids in GC"*

Fucoxanthin analysis via HPLC

The method for fucoxanthin analysis on HPLC has been developed in house as follows: weight 25 to 50 mg of biomass on an analytical balance (Sartorius GMBH, Germany) and place it in a becker with 50 ml of methanol (HPLC grade, Sigma-Aldrich, Italy) stir it with a magnetic bar, repaired from light and heat for 30 minutes.

At the end of the extraction process filter the solution with a 0.22 µm filter with the aid of a syringe and place the sample inside an Eppendorf before injecting 70 µL in the injection loop.

HPLC settings

The U-HPLC ultimate 3000 (Thermofischer scientific, USA) equipped with quaternary pumps, UV-VIS lamps and a C18 column (Ace Equivalent 250x4,6 mm) was used with water (HPLC grade, Vwr international, Italy) and methanol (HPL grade, Sigma Aldrich, Italy) where used in gradient to analyze the samples

The conditions of the analysis were:

- Solvents ratio: 95% methanol, 5% water
- Flux: 1 ml/min
- Temperature: 35°C
- Volume of injection: 70 µL in a 10 µL loop
- Pressure: 270 bar

- Wavelength of detection: 450 nm

Calibration curve

The retention time and concentration of the fucoxanthin on this setup have been determined by using a standard solution and creating a calibration curve using the external standard method with Sigma-Aldrich fucoxanthin (HPLC purity 95%).

Three replicates were conducted for each standard solution, and the calibration curve was established using the mean area values, accounting for HPLC purity as weight purity.

Microalgal Fatty Acid Analysis using GC-FID

The method for the rapid acid methylation procedure needed to analyze the biomass sample is hereby reported: In a flask with a magnetic stir bar at the bottom, weigh: 1 g of Sample (residual moisture of the sample must be known), 30 mg of an odd methylated fatty acid standard that is not present in your sample, for example Methyl nonadecanoate C19:0 (Sigma-Aldrich, Italy), 30 g of methanol (HPLC grade, Vwr international, Italy), 5 ml of 95% Sulphuric Acid (Vwr International, Italy). To initiate the acid methylation process, put the flask, with a water cooled reflux, on a hotplate set at 90°C, with a stirring speed of 500 rpm for 1 hour. Once the required time has elapsed, allow the sample to cool for approximately 15 minutes and then add to the flask: 20 mL of deionized water (Sigma-Aldrich, Italy), 20 mL of Hexane (Sigma-Aldrich, Italy), close the flask and agitate and vent until the pressure drops.

Allow the components to separate, and then extract a sample from the organic phase and transfer it to a 1.5 mL Eppendorf tube. Prepare 0.1 µL for injection.

GC-FID conditions

The reported conditions are used for Fatty acid methyl esters (FAME) separation (C4 to C24) for 100 m columns on microalgae biomass and oils: Injector temperature: 250 °C, Detector temperature: 250 °C, Oven temperature: Ramp from 120 °C to 240 °C at a rate of 4 °C/min, holding for 7 min at 240 °C, Carrier gas helium: Column head

pressure of 220 kPa, Linear velocity: (30 to 40) cm/s, approximate flow rate of 1.0 ml/min, split ratio of 1:100, Injection volume: 0.1 µl of sample

Determination of Chlorophylls and Carotenoids Concentrations via spectroscopy

The extraction of Photosynthetic Pigments has been carried out following the method reported by the report issued from SCOR-UNESCO on the identification of photosynthetic pigments in sea water[105]

To extract the pigments from the microalgae cells, these steps were followed: Since dried biomass was used, about 20 mg were weighed. Then, around 1.5 to 2.0 g of small glass beads (0.4 mm in diameter, Vwr International s.r.l) were added to the cell pellet to help break the cell walls. Next, were poured 90% acetone in water (Sigma-Aldrich, Italy) over the glass beads until they were just covered, being careful not to add too much solvent that could affect the cell lysis. The tube was vortexed vigorously at 24 Hz for 3 minutes to disrupt the cells and release the pigments into the solvent. After that, was added a small volume of 90% acetone to the tube, mixed it well, and transferred the liquid part to a 25 mL volumetric flask using a glass Pasteur pipette. This step was repeated until the solvent in the tube became clear, indicating that no more pigments were left in the pellet. Then the volumetric flask was filled up with 90% acetone and shook it thoroughly. A sample of the solution was taken and centrifuged it at 3500 rpm for 10 minutes. The pellet should have a white-gray color, showing that all the pigments were extracted. Finally, the absorbance of the supernatant was measured in a spectrophotometer against a 90% acetone blank using a glass cuvette. This allowed us to quantify the pigment concentration in the solution.

Analysis and Calculation For Chlorophyll:

The concentration of various chlorophyll types is assessed by measuring absorbance at four different wavelengths (750, 663, 645, and 630 nm) against a 90% acetone

blank. The reading at 750 nm gauges sample turbidity and must be subtracted from other values. Chlorophyll type and amount (in mg L-1) are determined using Scor-Unesco's (1966) proposed formulas:

$$Chla\ (mg\ L\ -\ 1)\ = \frac{11.64\ (DO663 - DO750) - 2.16\ (DO645 - DO750) + 0.10\ (DO630 - DO750)}{V2}$$

$$Chlb\ (mg\ L\ -\ 1)\ = \frac{-3.94\ (DO663 - DO750) + 20.97\ (DO645 - DO750) - 3.66\ (DO630 - DO750)}{V2}$$

$$Chlc\ (mg\ L\ -\ 1)\ = \frac{-5.53\ (DO663 - DO750) - 14.81\ (DO645 - DO750) + 54.22\ (DO630 - DO750)}{V2}$$

where:

DO663, DO645, DO630, DO750 = optical density values at respective wavelengths

V2 = sample volume (L)

Analysis and Calculation For Carotenoids

The method calculates total carotenoid concentration using an average molar extinction coefficient and mean wavelength. The formula, derived from Parsons and Strickland [106], employs readings at 750 nm (turbidity) and 480 nm.

$$carotenoids\ (mg\ L\ -\ 1)\ = \frac{4.0\ (Abs480 - Abs750)}{Sample\ Volume\ L}$$

Biomass analysis at the University of Messina

During my PhD a collaboration between the company and Professor of Pharmaceutical Biology, Domenico Trombetta, from the Department of Chemical, Biological, Pharmaceutical and Environmental Sciences of the University of Messina, started. The cooperation between our labs was issued in order to characterize the biochemical profile of the produced biomass and derived extract. The collaboration was part of a broader project that aimed to develop innovative technologies for the

full utilization of microalgal biomass as a source of biofuels and raw materials for cosmetic and nutraceutical applications, following an integrated biorefinery approach. The analysis conducted by their laboratory were:

Aminoacidic qualitative analysis in LC-DAD-Flu

The measure was conducted on the biomass of *Phaedactylum tricornutum*, using LC-DAD-Flu, an analysis technique used in analytical chemistry to identify and quantify the components of a mixture. LC stands for liquid chromatography, DAD stands for diode array detection, and Flu stands for fluorescence detection. Three independent experiments were performed, each with three replicates, and calculated the mean and standard deviation of the biomass values. The results are expressed as grams per 100 grams of dry weight.

Fatty acids qualitative analysis in GC-MS

The measure was conducted on the biomass of *Phaedactylum tricornutum*, in GC-MS. In this technique, a sample is first separated into its individual components using Gas Chromatography.The separated components are ionized and fragmented into smaller molecules using Mass Spectrometry. The resulting fragments are then detected and analyzed to identify the individual components in the sample. They performed three independent experiments, each with three replicates, and calculated the mean and standard deviation of the biomass values.
The results are expressed as the mean area percentage of the total identified compounds.

Phytochemical profile of the *P.tricornutum* extract in LC-UV-Vis (DAD)/MS

The measure was conducted on the *P.tricornutum* extract using LC-UV-Vis (DAD)/MS. This is a technique used in analytical chemistry to identify and quantify the

components of a mixture. LC stands for liquid chromatography, UV-Vis stands for ultraviolet-visible spectroscopy, DAD stands for diode array detection, and MS stands for mass spectrometry. In this technique, a sample is separated into its individual components using liquid chromatography. The separated components are then detected using both UV-Vis and mass spectrometry detectors. The combination of these two detectors allows for the identification and quantification of the individual components in the mixture sample.

The results are expressed as the mean area percentage of the total identified compounds.

Medium optimization for *Tetraselmis suecica*

In order to enhance the endogenous mannitol production within *Tetraselmis suecica*, two distinct growth conditions were selected, as recommended in the papers of Iwamoto K. et al. [107] [108], to evaluate the potential increase in mannitol concentration. Using the same recipe here reported in the section for culture cultivation, were added 30 g/l of NaCl (Vwr International, Italy) leaving unaltered all the other components of the medium.

Following Iwamoto et al. research papers, also a second experimental trial was conducted modulating the light irradiance given to the culture: from 150 to 50 µmol/(m^2·s).

Mannitol Extraction Procedure from *Tetraselmis* Samples

The mannitol content of the *Tetraselmis*' biomass powder was determined by high-performance liquid chromatography (U-HPLC, Ultimate 3000, Thermo Fischer Scientific, USA) with refractive index detection (RefractoMax 521, Thermo Fischer Scientific, USA). A sample of about 200 mg of Tetraselmis powder was weighed and placed in a 50 Falcon tube. Glass beads (3 mm in diameter) were added to cover the powder completely. Then, 25 mL of demineralized water was added. The mixture

was vortexed for one minute at a frequency of 40 Hz using a manual setting. The tube was then immersed in a water bath at 90°C for 30 minutes to extract the mannitol from the powder. After the extraction, the mixture was vortexed again for one minute at 40 Hz and transferred to a 2 mL Eppendorf tube. The tube was centrifuged at 11.000 rpm for 3 minutes to separate the solid and liquid phases. The supernatant was filtered through a ministart filter (0.22 µm) attached to a syringe and collected in a new Eppendorf tube. A volume of 100 µL of the filtered extract was injected into an HPLC system equipped with a refractive index detector. The HPLC system had the following parameters:

- Mobile phase: water
- Flow rate: 0.4 mL/min
- Pressure: around 9 bar
- Column: PB++ resin (300x8 mm, 8 µm) (Hamilton Italia S.R.L., Italy)
- Column temperature: 80°C
- UV lamp wavelength: 210 nm
- Analysis time: 45 minutes

The HPLC analysis resulted in a chromatogram with retention time (min) on the x-axis and refractive index area (nRIU/min) on the y-axis. The retention time of the mannitol peak was 42 minutes with a specific area for each sample. The concentration of mannitol in the aqueous extract was calculated in mg/L using a previously acquired calibration curve equation. The concentration percentage of mannitol in the *Tetraselmis* powder was obtained by relating the concentration in mg/L to the sample weight using the following equation:

$$Concentration\,\% \;=\; \frac{200}{Weighted\ biomass} \times \frac{X}{80}$$

where weight is the weight of the Tetraselmis powder in mg and X is the concentration of mannitol in mg/L.

Chapter 3

Purification methods for *P.triconutum*

Starting from a Wild-type strain obtained from the CCAP the initial inoculum was scaled up in order to have enough biomass for the subsequent cleaning processes.

The first liquid inoculum was streaked on a petri dish plate in order to give birth to the first isolation generation from which another two new isolations were performed. This process was meticulously carried out to ensure the highest degree of purity and to minimize any potential contamination, as visually depicted in the subsequent image.

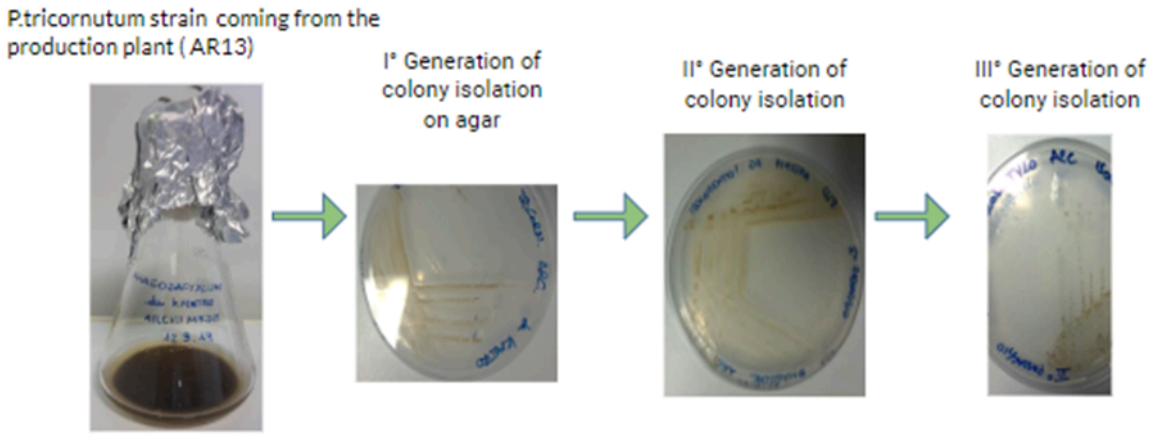


Figure 12 scheme of the amplification and purification steps of the P.tricornutum wildtype strain

Once the third generation was successfully isolated from the wild-type strain, the use of a custom-designed micromanipulator for the process of single colony selection yielded highly promising results. This simple but effective tool facilitated the precise and controlled selection of single colonies from the solid medium. The selected colonies were then transferred from the petri dishes to a 24-well plate. The primary goal of this procedure was to subject these isolated *P.tricornutum* colonies to various concentrations of antibiotics treatments. This step was undertaken with the specific aim of eliminating any microbial contamination that might have been present in the initial culture.

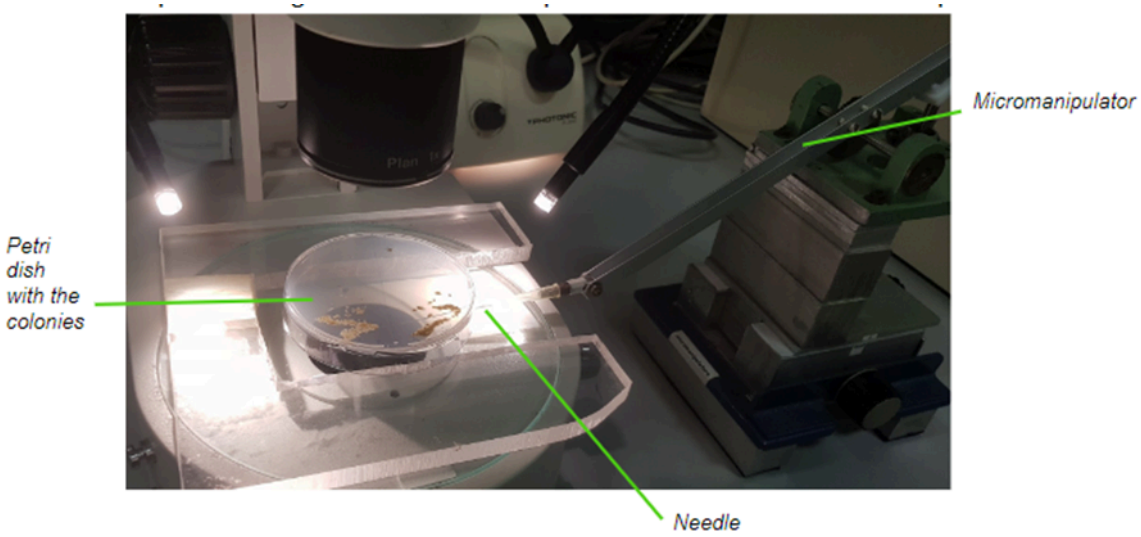


Figure 13 Scheme of the self constructed micromanipulator used for the single colony picking

The antibiotics tested were categorized into four Rows: A, B, C, and D. Each row included a selection of antibiotics with the goal of identifying the most effective treatment for decontaminating the culture.

Single colony picked from the plate was diluted in line 1 of each row and after 7 days of growth a serial dilution, as reported in the picture, was performed in order to dilute cells and eventual residual microbial contamination

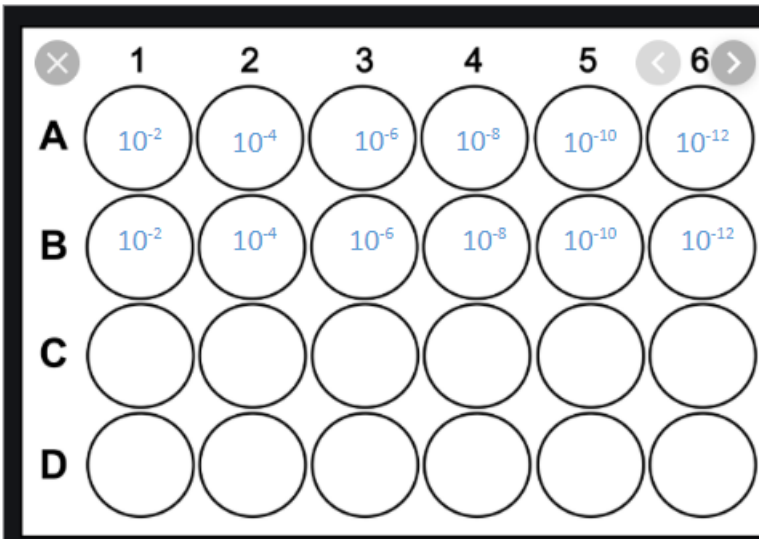
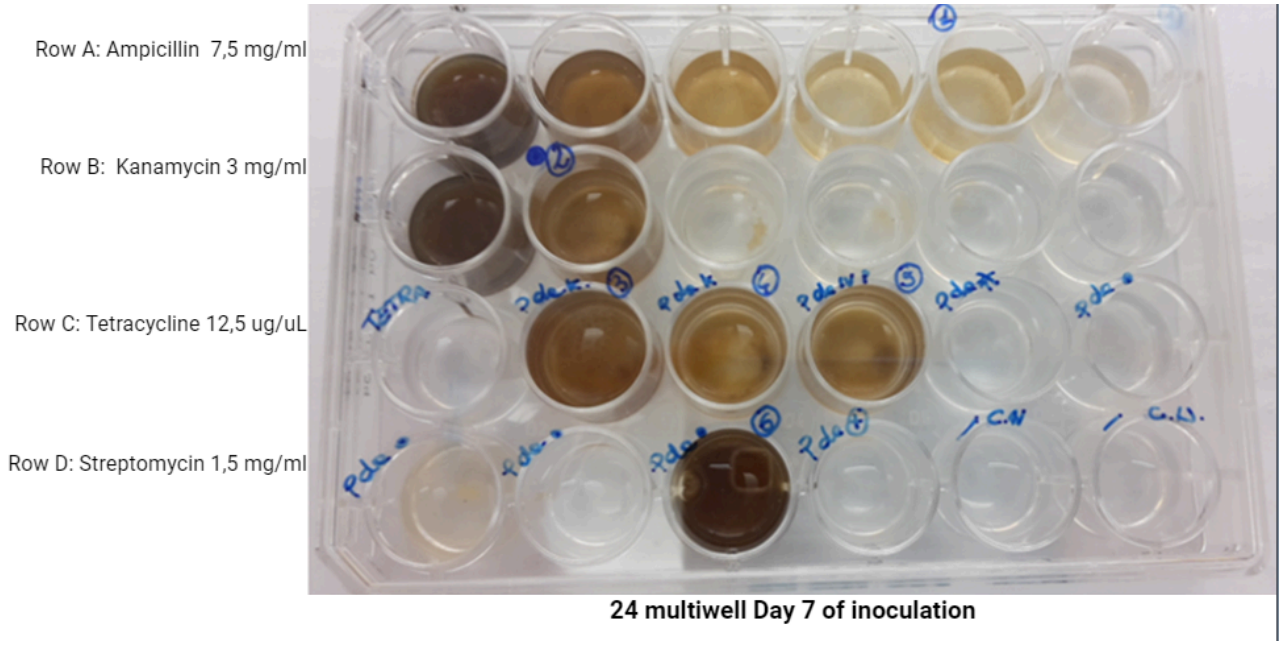
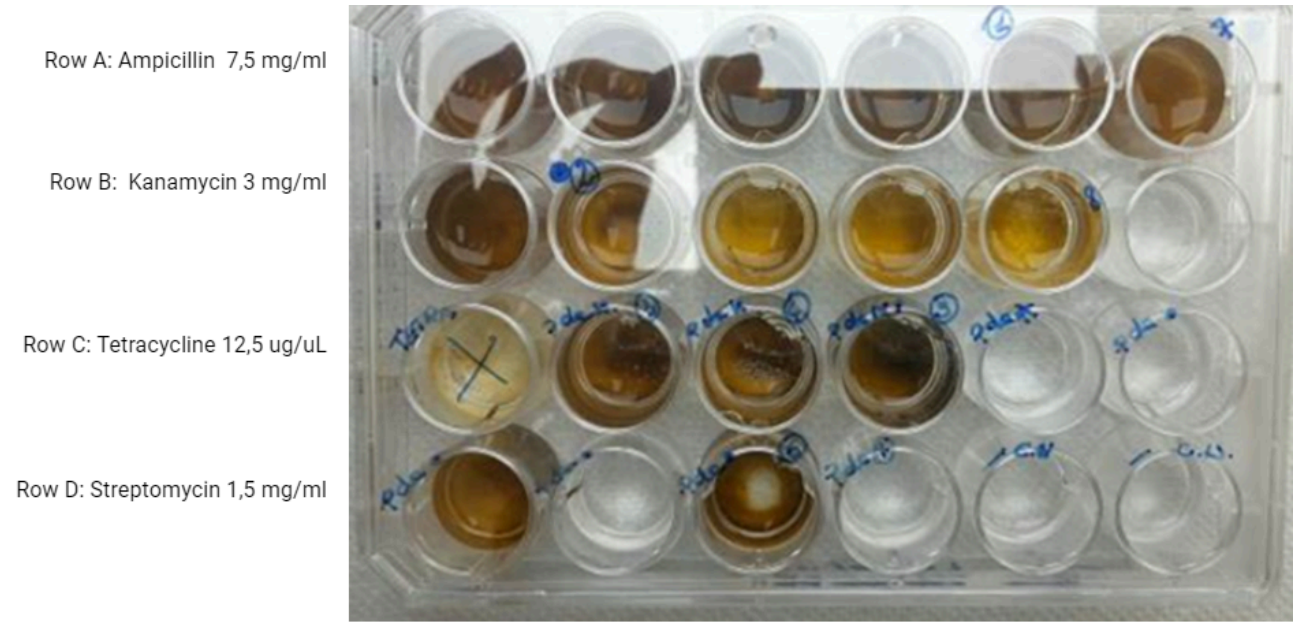


Figure 14 Serial Dilution scheme for the single colonies picked

After 7 and 24 days the wells looked like this:



24 multiwell Day 7 of inoculation



24 multiwell Day 21 of inoculation

Figure 15 Multiwell plates containing different antibiotics in the culture medium for the purification of the P.triconutum colonies

The wells that after microscopical observation showed no contamination were tested in order to assess the presence of residual microbiological contamination. The single colony culture in each well was inoculated onto three different microbiological media,PCA, TSA and WPCA, and these plates were incubated for 7 days. The results from this testing phase confirmed that there was no detectable contamination inside the culture.

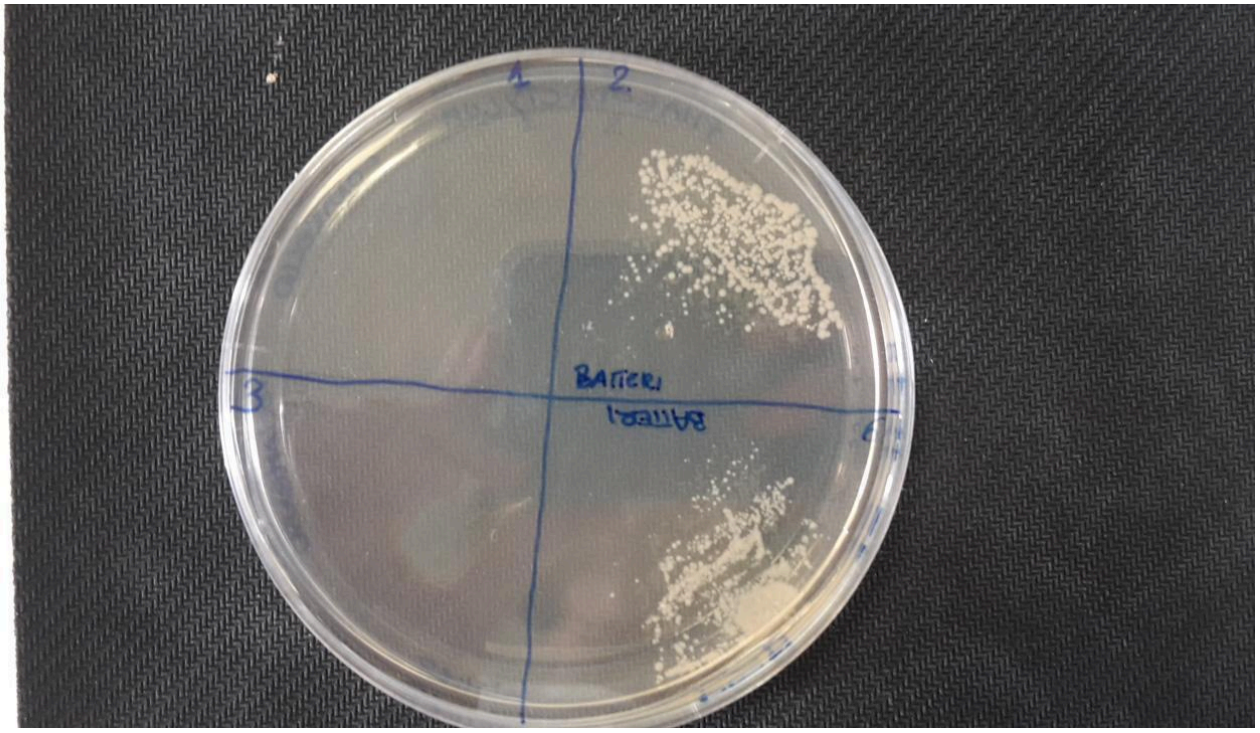


Figure 16 Absence of microbial contamination on samples 1-3 and presence on samples 2 and 4

The contamination tests confirmed that 11 strains were successfully isolated in axenic form as a result of the purification process. These strains have been cataloged and assigned a new collection name: "PHAEO" an abbreviation of *Phaeodactylum* and an increasing number. The single colony picking from the plates is outlined as follows:

Table 3 P.tricornutum axenic strains from the multiwell

Plate position	Antibiotic	Strain name
Row A Line 5	Ampicillin 7,5 mg/ml	PHAEO 101
Row A Line 6	Ampicillin 7,5 mg/ml	PHAEO 102
Row B Line 2	Kanamycin 3 mg/ml	PHAEO 103
Row B Line 3	Kanamycin 3 mg/ml	PHAEO 104
Row B Line 4	Kanamycin 3 mg/ml	PHAEO 105
Row B Line 5	Kanamycin 3 mg/ml	PHAEO 106
Row C Line 2	Tetracycline 12,5 ug/uL	PHAEO 107
Row C Line 3	Tetracycline 12,5 ug/uL	PHAEO 108
Row C Line 4	Tetracycline 12,5 ug/uL	PHAEO 109
Row D Line 1	Streptomycin 1,5 mg/ml	PHAEO 110
Row D Line 3	Streptomycin 1,5 mg/ml	PHAEO 111

The use of Kanamycin seemed to give the majority of successful purified strains but with an impaired growth while the Ampicillin had one of the lowest purification effects but did not seem to impair the *P.tricornutum* growth in liquid medium.

The purified strains were scaled up in glass tubes in order to increase their volume and concentration. Each newly isolated strain was splitted in two and grown under constant light for 14 days. The growth curve was monitored through optical density at 600 nm using a Spectrophotometer.

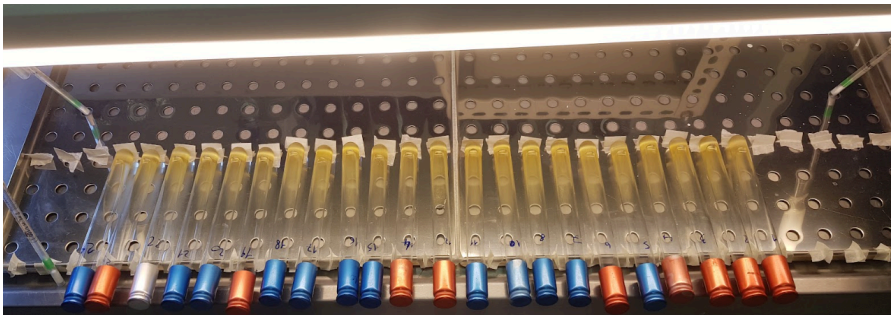


Figure 17 Tube vials of the isolated samples growing under steady light to asses the growing rate of the isolated strains

During this timeframe it was observed that certain colonies failed to exhibit any signs of growth within the liquid medium. This observation raises the possibility that mutations might have occurred within these particular colonies or that certains antibiotics may have weakened *P.triconutum*.

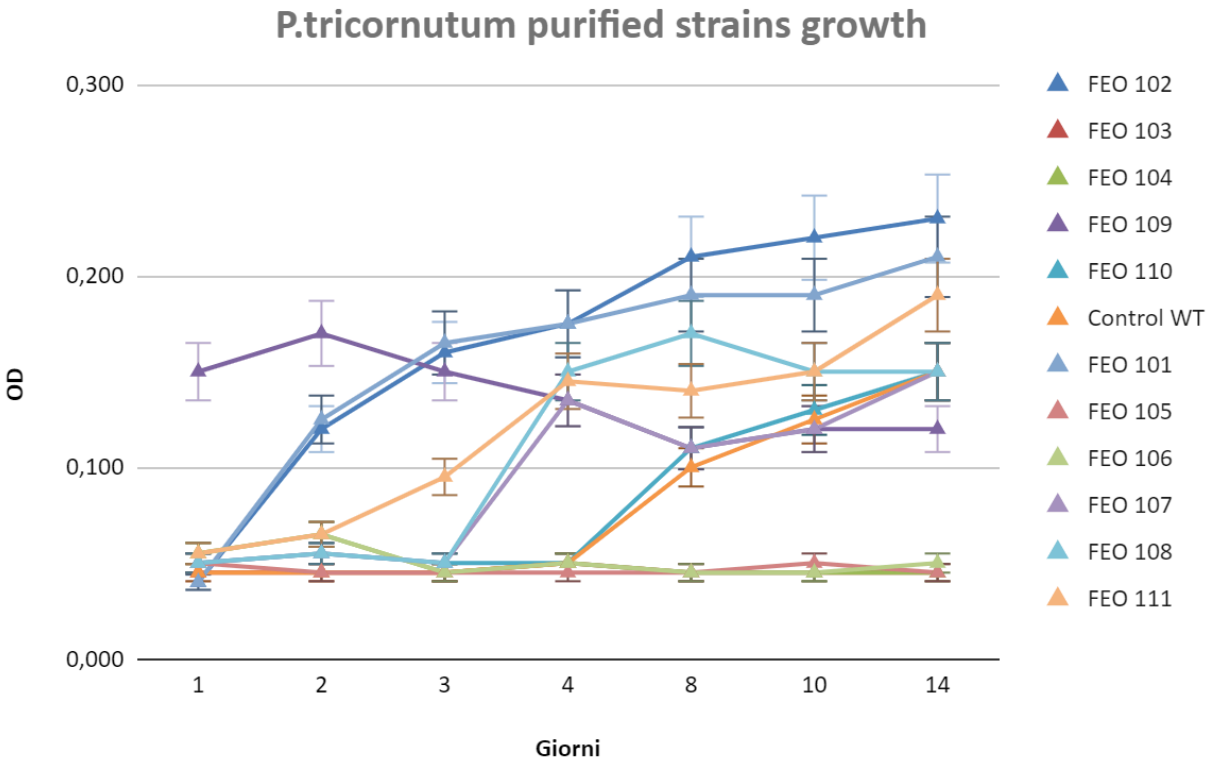


Figure 18 comparison between the differents strains obtained after antibiotics screening under constant light for 14 days

Following the cultivation of the promising strains in a liquid medium, we took another step, isolating them once again for a fresh round of selection (Standard deviation on 2 experiments value ± 10%) . Regrettably, it became evident that some of the strains displayed significantly reduced growth rates, with a few of them exhibiting no growth at all when placed on a standard solid medium in Petri dishes

In response to these unexpected results, we proceeded to evaluate the possibility of adding different kinds of metabolites to the growth medium in an attempt to address and understand the observed variations in growth behavior. A matrix of analysis was created to systematically explore the impact of various medium modifications on the growth of *P.tricornutum*:

Table 4 Table reporting the different medium modification tested

Piastra (6ml)	Agar (% m/v)	PO_4 (mg/L)	NO_3 (mg/L)	Vitamine (B12,Biotina, Tiamina)	Silicio (mg/L)	Digestato (% v/v)
ASW Half Tropic	15%	5	75	Conc. STD	x	x
ASW + SIlicon	15%	5	75	Conc. STD	30	x
ASW +Digestate	15%	5	75	Conc. STD	x	10%
ASW Half tropic + Silicon	15%	5	75	Conc. STD	30	x
ASW Half tropic + Silicon + Digestate	15%	5	75	Conc. STD	30	10%
ASW + Silicon + Digestate	15%	5	75	Conc. STD	30	10%
ASW control	1%	5	75	Conc. STD	x	x

The goal was to identify the most effective growth conditions that would promote faster and healthier growth of the purified strains. Parameters such as nitrate, phosphate, vitamins, and iron were kept constant, while other compounds were altered and tested to determine their influence on growth. The most favorable growth results on solid medium were achieved when the Petri dishes were supplemented with silicium. This promising outcome suggests that the incorporation of silicium into the solid medium could prove to be a valuable enhancement for expediting growth in future experiments conducted on solid and liquid substrates.

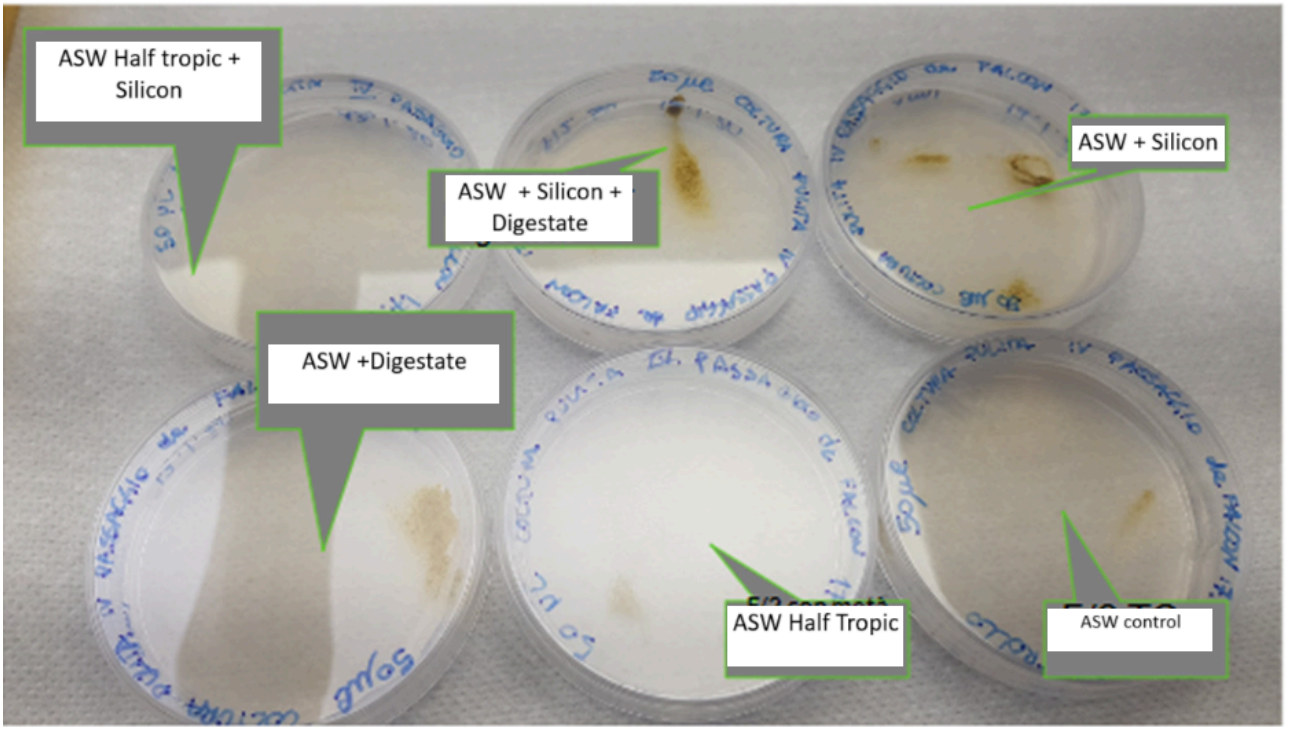


Figure 19 Results of the different condition tested

Thin Layer Chromatography for fast fucoxanthin analysis

This TLC-based method allowed for a rapid and qualitative assessment of fucoxanthin content in *P.tricornutum* purified samples. It facilitated the screening of strains for their fucoxanthin production potential without the need for time-consuming procedures such as HPLC. The ability to quickly and effectively analyze a wide range of strains is crucial for the early stages of the project, with more precise methods like HPLC being reserved for the refining and final steps. Ultimately, this approach aligns with the project's aim to identify promising strains capable of producing substantial amounts of fucoxanthin.

Among the numerous strains obtained through the preceding purification phases, only three exhibited the capability to thrive both on liquid and subsequently in solid medium, PHAEO 101, PHAEO 102 and PHAEO 111, yielding dry weight concentrations with a mean value of 1.9 g/l ± 0.2 SD over 3 repetitions. This enabled us to utilize them as samples for the fucoxanthin determination on TLC, following

the removal of the culture medium via centrifugation. The biomass was then resuspended at a 1/100 dilution, effectively concentrating the biomass.

Among the various solvent combinations tested, shown in the following picture, it was the composition of 7 parts toluene, 2 parts ethyl acetate, and 1 part acetic acid that provided the most distinct separation of components extracted from the *P.tricornutum* biomass. The resolution of photosynthetic pigments was further accentuated when observed under both UVB and UVA light wavelengths, enhancing the clarity of their differentiation.

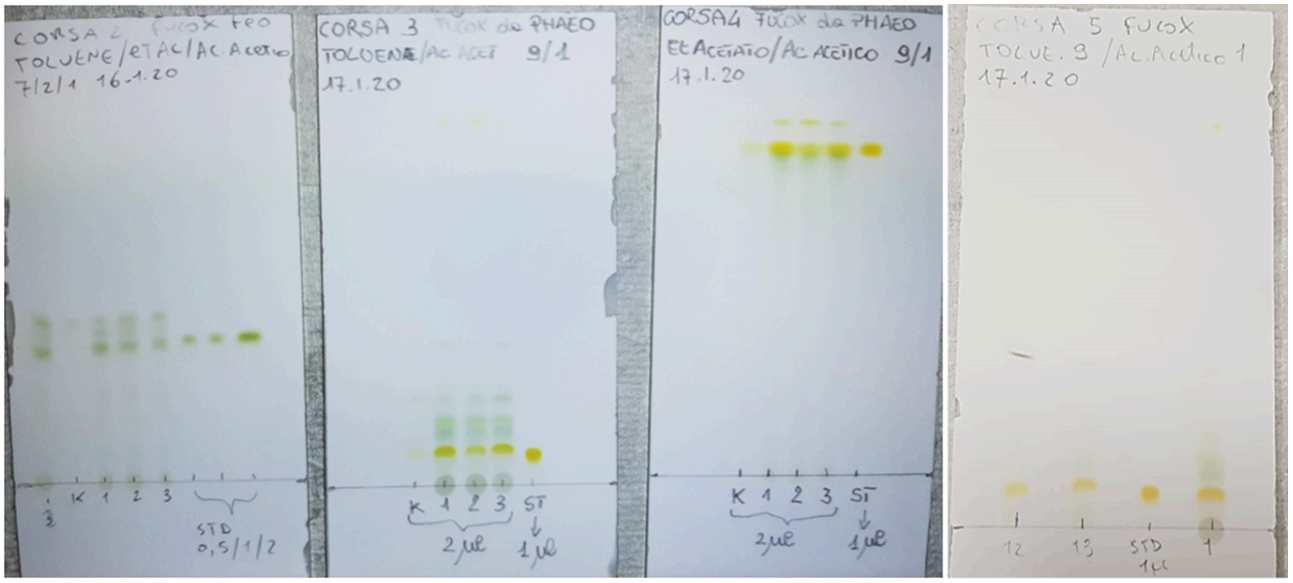


Figure 20 TLCs with different solvents combinations of the P.tricornutum samples that were tested for Fucoxanthin content

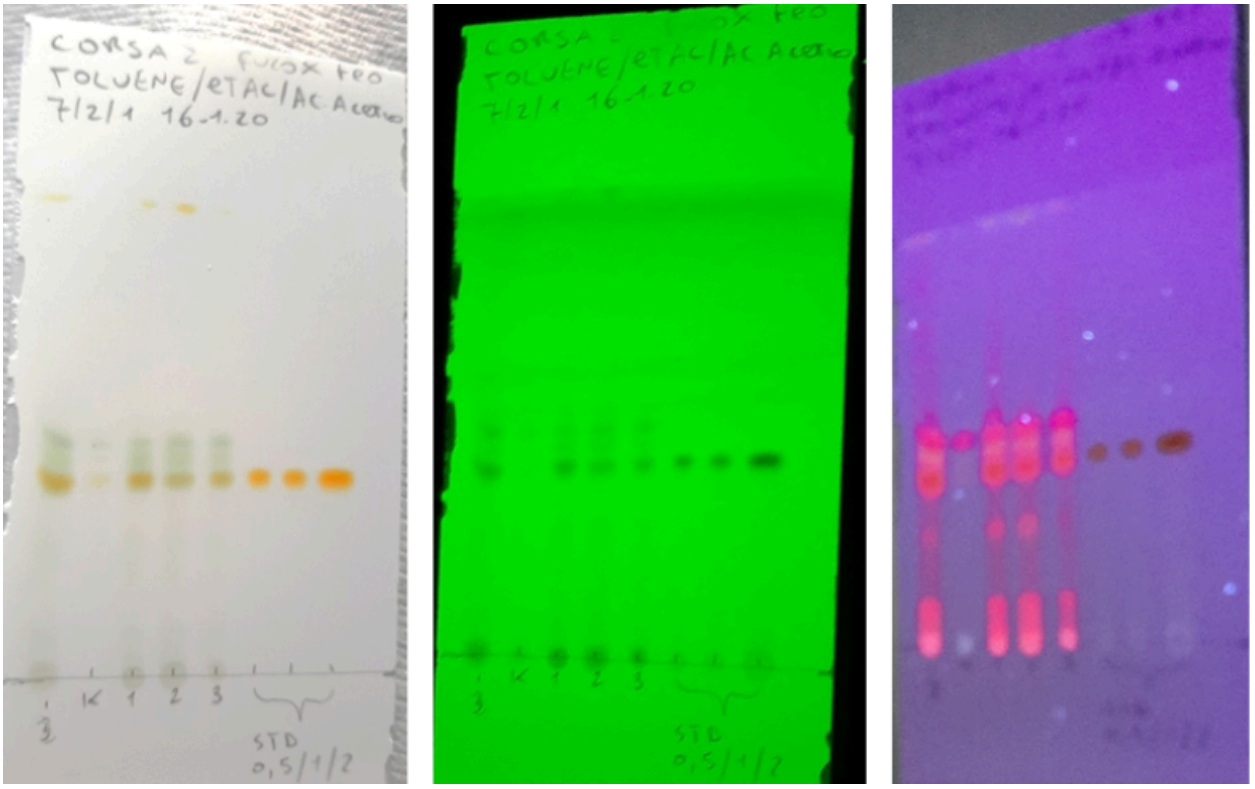


Figure 21 TLCs under White light, UVA and UVB wavelengths

By extrapolating the fucoxanthin content from the TLC run using a scale obtained from a purified fucoxanthin standard, we successfully determined the fucoxanthin content within the 3 isolated strains. This allowed us to construct a theoretical fucoxanthin percentage, which was subsequently validated through HPLC analysis.

Sample name	Sample used (µl)	Theoretical FX %	Real Fx % in HPLC
Phaeo 102	2	1%	0,9 ± 0,01
Phaeo 101	2	0,8%	0,7± 0,01
Phaeo 111	2	0,5%	0,6 ± 0,02
AR13 WT control	2	~0.6/0.9%	0,7 ± 0,06

The standard deviation was calculated on a 2 repetition analysis on HPLC. The three isolated and purified strains exhibited notably enhanced growth performance in

comparison to the original wild type. This enhanced growth can likely be attributed to the elimination of microbial competition within the culture medium. Moreover, the enrichment of the culture medium with silicium may have created a more favorable environment for the growth of *P.tricornutum*. While the improved growth performance was anticipated within the experimental design, the unexpected observation was the significantly increased fucoxanthin content during this stage. The Phaeo 102 strain, in particular, demonstrated a remarkable increase in fucoxanthin production. This promising result prompted us to choose this particular strain for the next phases of our study.

Determination of light waves for fucoxanthin production

As we progressed into the fucoxanthin production improvement stage, we chose to focus on the PHAEO 102 strain, which had displayed a substantial increase in fucoxanthin production. This strain was subjected to an enhanced cultivation system, specifically designed to assess the carotenoid production response under various light spectra conditions. For the test, a 0.7 L benchtop fermentor was employed, equipped with suspended magnetic stirrers and lower air sparging to ensure excellent mixing of the culture. This was achieved through both airlift and mechanical agitation. The fermenter was subsequently placed inside a glass cylinder, onto which the LEDs were mounted and secured to a magnetic stirring plate set at an average of 300 rpm. The bubbling and air/CO_2 mixture were ensured by an aquarium pump set at a constant air flow of 0.5 vvm (volume of air sparged per unit volume of growth medium per minute), while the CO_2 percentage was maintained at a constant level through daily pH range monitoring around 8.

Each individual light source consists of three LEDs, which can be independently selected and adjusted for intensity and brightness, enabling the creation of the desired color.

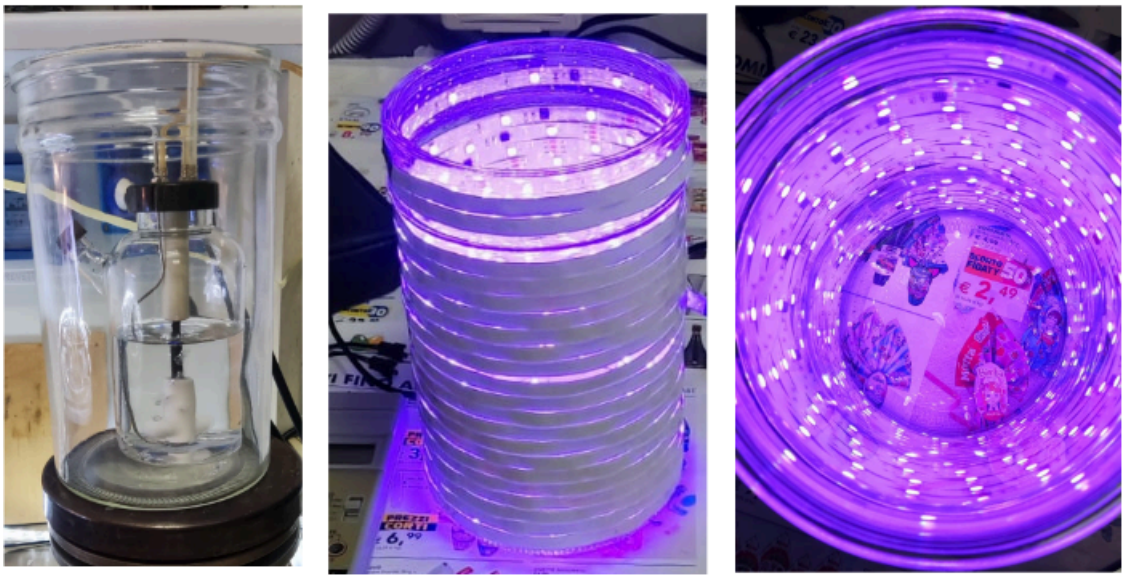


Figure 22 Fermenter and light source distribution

Being the light sources in close proximity to the glass of the bioreactor an increase in the internal temperature during the experiment was feared. To ensure the safety of the cultured cells, we carefully monitored the temperature conditions for each of the studied wavelengths for 24 hours prior to starting the experiment. This monitoring was essential, as excessively high temperatures (>35°C) could potentially harm the microalgae and impair their growth.

Figure 23 LED light source and control system for the RGB lights

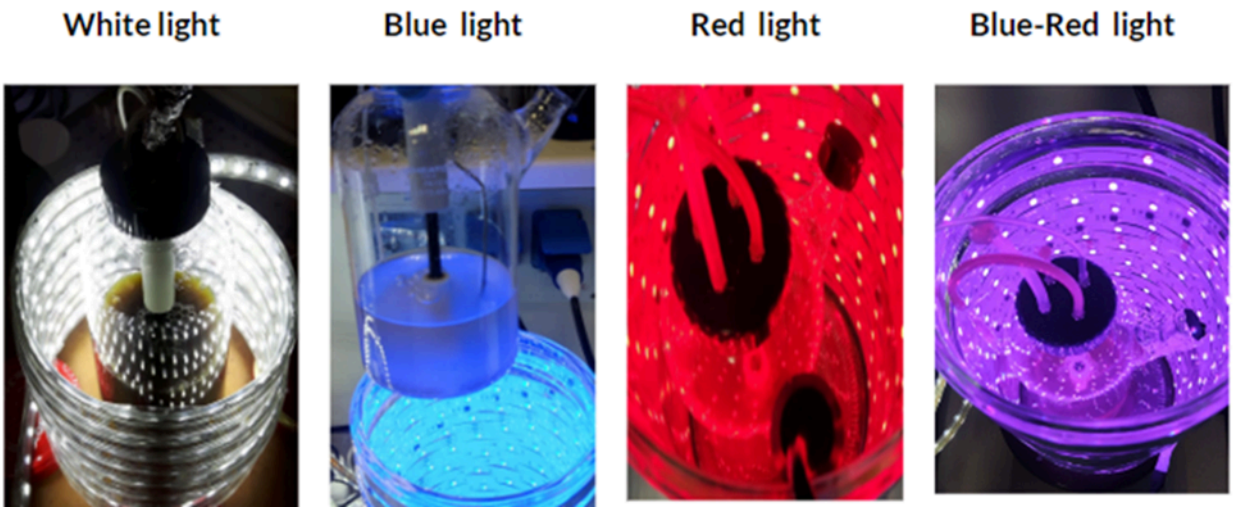


Figure 24 Fermenter under different types of light spectra

The chosen culture medium for the experiments was composed of ASW (artificial seawater), microelements, nitrates, phosphates, and vitamins enriched with silicon.

During the experiment, it was necessary to top up the fermenter's volume due to the physiological evaporation that the culture undergoes when air is insufflated at positive pressure, resulting in a broad headspace, owing to the cylindrical shape. Demineralized water is used for this purpose to avoid increasing the salt concentration within the culture.

The purpose of these experiments was to observe the effects of different wavelengths on biomass production, fucoxanthin production, and to determine the best combination of light spectra to achieve these results either together or sequentially.

In microalgae, fucoxanthin, chlorophyll a and c are associated with proteins, resulting in a complex called FCP. The absorption spectrum of fucoxanthin ranges from 450 to 540 nm in solution, but it absorbs blue-green light with wavelengths between 390 and 580 nm once it is bound within an FCP complex.

Therefore, microalgae that produce fucoxanthin are typically found in marine environments where blue-green light is prevalent. This is why we have selected these wavelengths for our biomass growth experiments. In contrast, red light, which is rare at the depths where *P.tricornutum* typically resides, over-stimulates the production of fucoxanthin as an adaptation to capture as much light as possible for survival.

To establish a correlation throughout the experiment while monitoring growth, we estimated a relationship between cell count and optical density. The principle was straightforward: a higher cell count indicated more cells present. However, optical density (OD) was not solely influenced by the number of cells; it was also affected by the fucoxanthin content within the cells, contributing to an increased turbidity value.

The results obtained are shown in the following graphs:

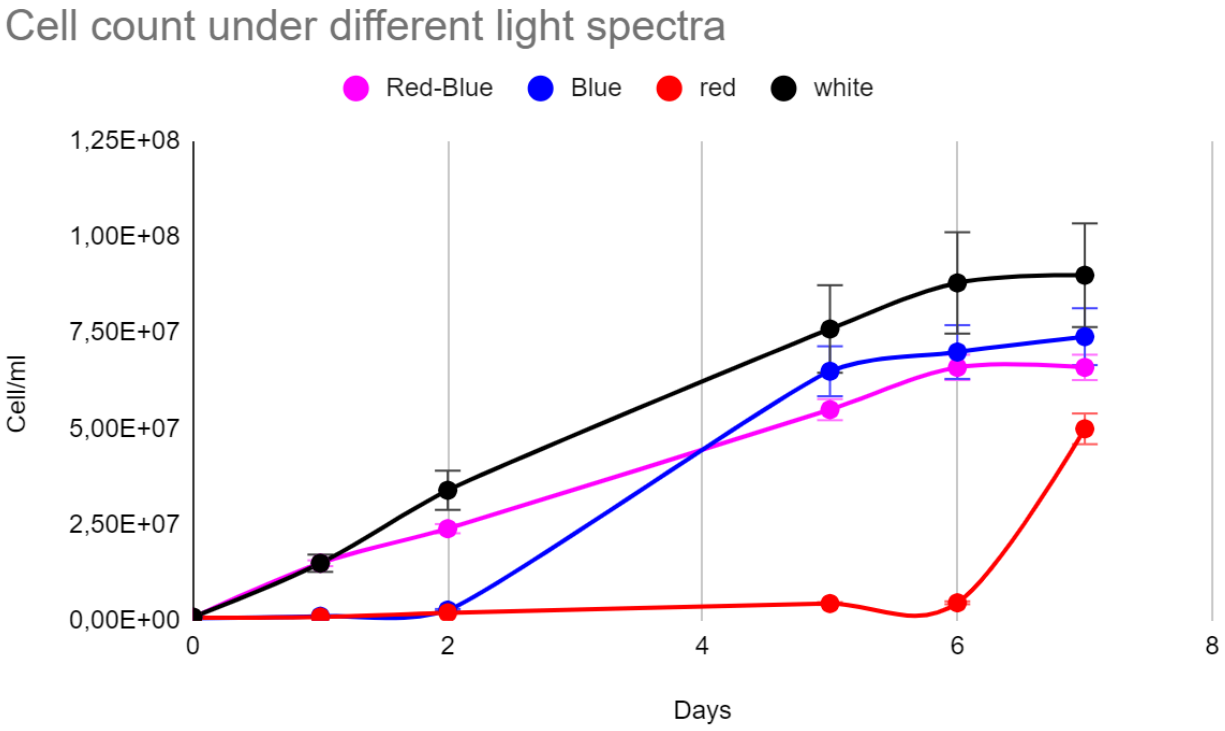


Figure 25 Cell count under different light spectra

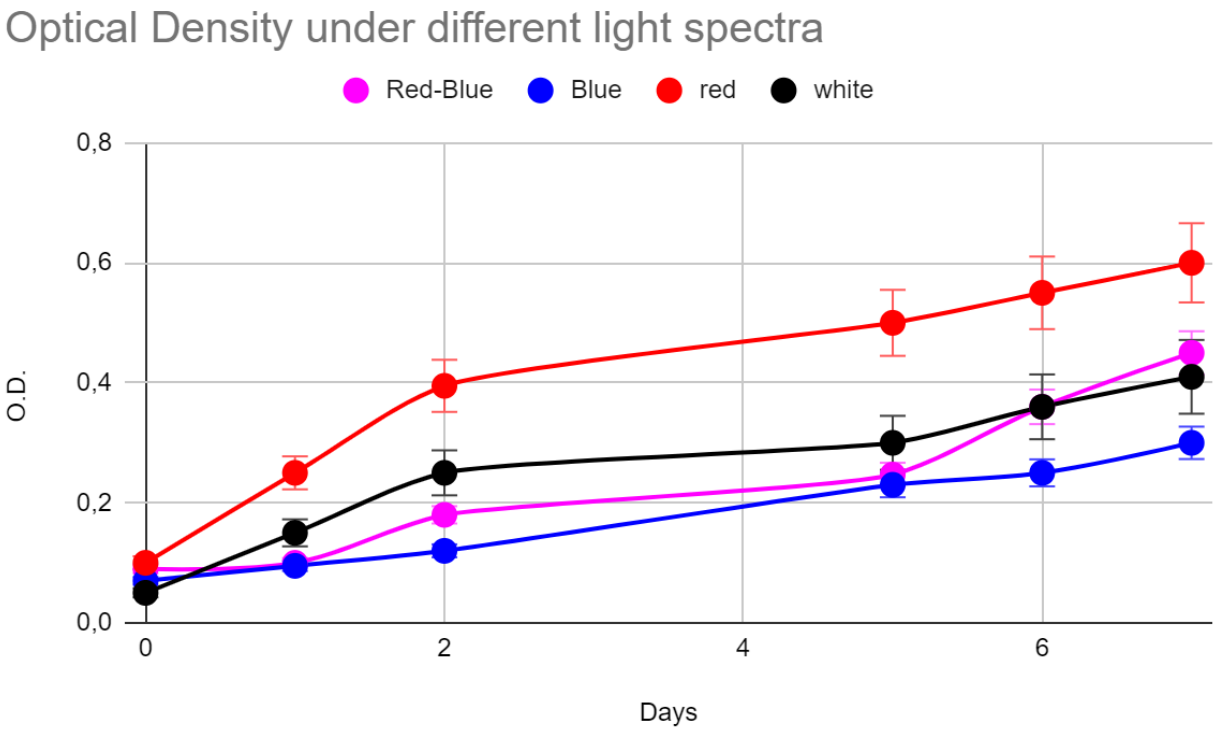


Figure 26 Optical density under different light spectra

Both measurements were performed twice for each light condition.

An overview of the experimental results provides valuable insights, as demonstrated in the table below. The data offers a glimpse into the different outcomes achieved under various lighting conditions. Notably, the highest biomass concentration was attained under white light. On the other hand blue light yielded the second-highest biomass concentration. This finding is of particular significance as we contemplate scaling up biomass production in the artificial light photobioreactor at the production plant.

Furthermore, examining the results from an optical density perspective reveals intriguing patterns. Red light, despite resulting in the lowest biomass concentration, produced the highest optical density. This observation raises an interesting hypothesis: the medium's increased turbidity may not be solely due to biomass but could be attributed to an accumulation of fucoxanthin within the cells. However, it's crucial to note that the red light experiment also recorded the highest temperature inside the fermenter. This insight underscores the necessity of implementing a cooling system when transitioning to larger-scale production in the plant.

The recorded data indicate that the internal temperatures were not a threat to the success of the experiments.

Measured values for each wavelength of the LED lights used.

Color	RGB	Temp	µmol/(m²·s) max	Ampere	Volt	Watt	Growth %	Max Cell/ml
White	R:255 G:255 B:255	30°C	350	0,70	22	15	100	9,5 E+07 ± 8,49E+06
Blue	R:0 G:0 B:255	28°C	200	0,37	22	8	70	7,4 E+07 ± 4,0E+06
Red	R:255 G:0 B:0	32°C	50	0,47	22	10	47	5,0 E+07 ± 7,07E+05

Magenta	R:255 G:0 B:255	30°C	150	0,58	22	13	63	6,6 E+07 ± 2,12E+06

It is well documented in literature that microalgae respond to different colors of light. This study aims to evaluate whether different light colors can be used as triggers to regulate biomass and carotenoids production by *P.tricornutum*.

To maximize the results of the experiments we employed a two-step approach to assess their effects on both biomass and fucoxanthin production. The method involves a two-phase strategy:

Initial Biomass Growth Phase: During this stage, we utilized blue light to stimulate biomass growth. This choice was made to minimize the energy input required to achieve a sufficient biomass concentration.

Fucoxanthin Accumulation Phase: Following the biomass growth phase, we transitioned to red light conditions. Red light was chosen for this phase as it promotes the accumulation of fucoxanthin within the cultivated cells. This approach allowed us to increase the percentage of fucoxanthin in the cells before the harvesting process.

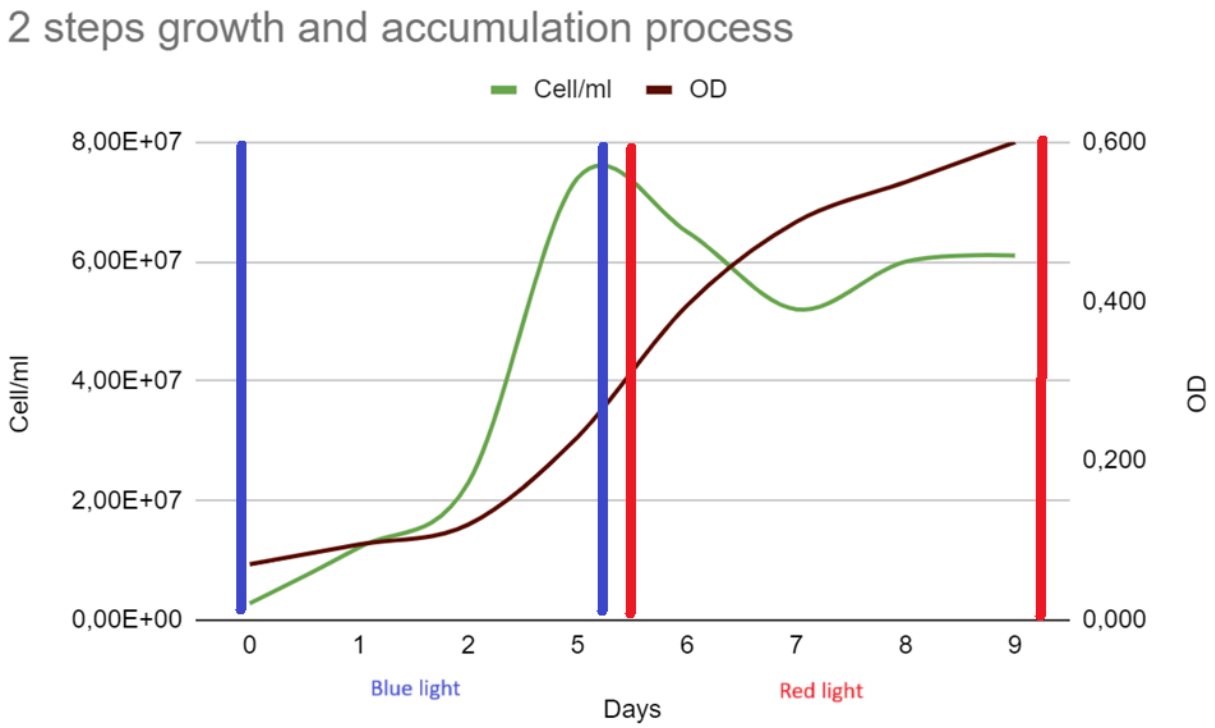


Figure 27 a proposed two step process to grow biomass and increase fucoxanthin using different light spectra

During the 6-day growth period, blue light resulted in a final cell count of 74 million cells per mL, which was 15% lower than the growth under white light in identical conditions. The experiment was repeated twice with a Standard deviation of ± 2,95E+06 cells/ml.

The switch to red light occurred when the recorded cell count showed a decline after the 6th day of exponential growth, marking the end of this phase and the beginning of the stationary phase.

At this juncture, a change in the correlation between cell count and optical density (OD) took place. The turbidity of the culture, besides reflecting the cell concentration, provided insights into the amount of fucoxanthin within the cells.

Upon concluding the growth phase, the increase in turbidity, considering the compensation for evaporation by adding water to prevent culture thickening, was attributed to fucoxanthin.

Microalgae biomass production at the Archimede Ricerche s.r.l. production plant

Benefiting from the proximity of a production plant directly linked to our R&D laboratories, I gained the unique opportunity to initiate mass production of the improved strain even before completing the development process. The swift adoption of the two-stage conditions identified through light spectra experiments by the production plant led to notable enhancements in biomass production and pigment concentrations. These improvements extended not only to *Phaeodactylum* cultures but also, with necessary adjustments, to various microalgae species, including green and red algae.

In the month of May 2022, the production plant successfully generated the first 10 kg batch of biomass from the novel strain within a sealed photobioreactor (patent pending). This advanced photobioreactor allowed meticulous control over critical parameters: air flow, bubbling, CO2 concentration, pH, temperature, and notably, light intensity and color. The newly employed strain was reclassified in accordance with the company's nomenclature and added to the company's collection. A genotypic identification process has been initiated in collaboration with the Photobiology department of CNR Firenze, co-founders of the microalgae production plant, and this work is currently underway.

Scale up, downstream and Biomass Dehydration

The cultivation of *P.tricornutum* was expanded to generate a substantial biomass for processing and extraction, and this was undertaken at the Archimede Ricerche S.r.l. production plant in Camporosso, IM, Italy. The cultivation conditions at the production plant closely resembled those utilized in the laboratory, although adjustments were made based on the results obtained from the light wave tests. Notably, air enriched with 1% food-grade CO_2, supplied by Air Liquide Italia SpA, was introduced to enhance the growth environment.

Scaling up the cultivation process the microalgae were cultivated in the laboratory within glass tubes known as Kmeters. These tubes featured a unique geometry designed to ensure a uniform distribution of irradiance provided by LED lights, ranging from 150 to 600 µmol/(m²·s). Additionally, the temperature was carefully controlled at 23°C using an air climatizer.

The pH of the culture was continuously monitored and adjusted by skilled operators.

The laboratory-scale cultivation proceeded for 20 days, during which the inoculum concentration was progressively increased. Upon achieving a specified concentration goal, the inoculum could be divided and diluted into several other Kmeters to maintain a consistent dilution rate of 1 to 6.

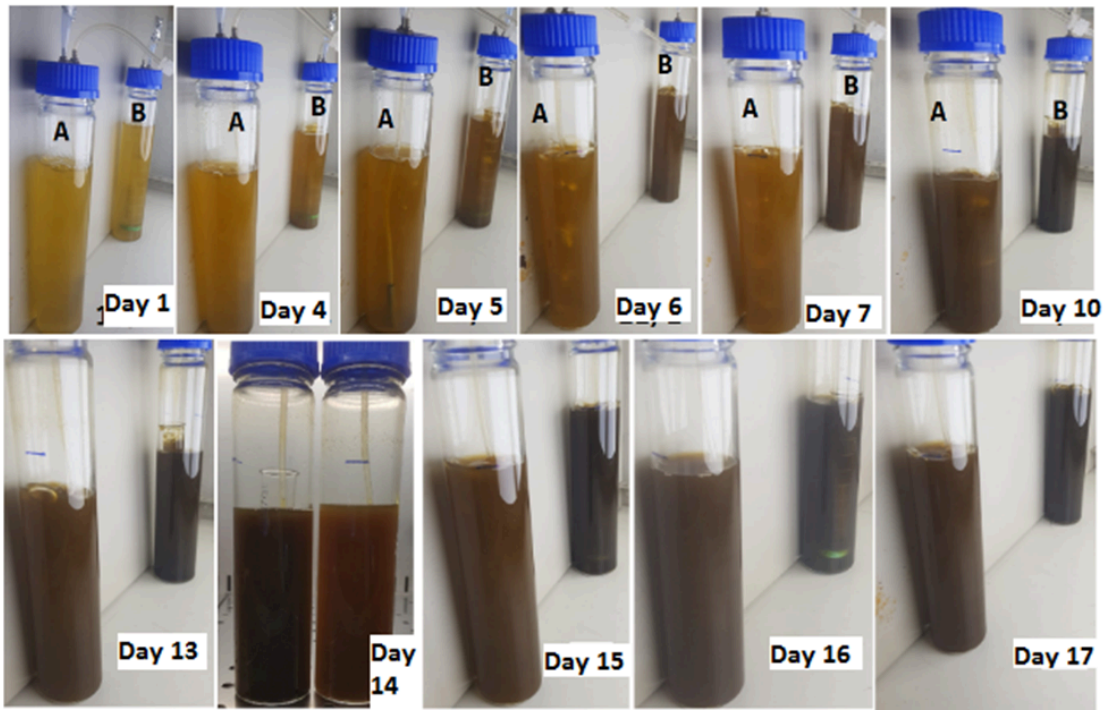


Figure 28 Growth of the newly purified Phaeo 102 (B) and the wild type strain (A) in the production plant laboratory

Following this laboratory phase, the inoculum was transferred to the natural light photobioreactors within the production plant. The initial available volume for cultivation was 200 liters.

Details regarding the medium concentration and specific dilution factors employed in the production plant are not disclosed.

Figure 29 The GWP®-II is a low-cost photobioreactor suitable for pilot plants and commercial installations at the Archimede Ricerche Production site.

After an additional 14 days of cultivation in natural light, the inoculum was transferred to an artificial light photobioreactor. In this phase, light modulations for the reproduction of the two steps process were initiated to encourage the production of fucoxanthin after an initial biomass growth phase.

Once the algal biomass reached a concentration of 1 ± 0.3 g/L, it was harvested for further processing. The harvested biomass was then subjected to freeze-drying in a specialized Criofarma s.a.s. machine.

Following the freeze-drying process, the dried biomass was milled to prepare it for subsequent storage at a temperature of -20°C. This scaling-up process enabled the production of a significant biomass of *P.tricornutum*, setting the stage for the extraction and processing of valuable compounds such as pour main goal fucoxanthin.

Analysis on the PHAEO 102 biomass produced

After obtaining the biomass, an extensive biochemical analysis was conducted on the newly produced batch. We collaborated with external certified laboratories to gain a comprehensive understanding of its merceological composition. We also performed internal analyses to determine the fatty acid composition using GC-FID and conducted fucoxanthin analysis through HPLC. Furthermore, our recent partnership with the University of Messina enabled us to deeply understand the amino acid composition and explore the apolar fraction inside the biomass.

Biochemical composition

An initial biochemical composition analysis of the biomass from an external certified laboratory gave us the following results:

Table 5 Analysis of freeze dried biomass

Batch n°	**PHAEO 102 - 21**	**Unit**
Moisture	3,0	% w/w
Ash	21,6	g/100 g
Protein	34,8	g/100 g
Carbohydrate	10,5	g/100 g
Lipid	22,2	% w/w

Amino acids composition in LC-DAD-Flu

The results presented in this study were achieved through our collaboration with the University of Messina, where a biomass analysis was conducted. Additionally, a qualitative analysis of amino acids was performed using LC-DAD-Flu.

Table 6 Quantitative and qualitative characterization of the amino acids present in the biomass of Phaedactylum tricornutum. The results, which represent the mean ± the standard deviation of three independent experiments performed in triplicate, are expressed in g/100 g of biomass.

Amino acid	g/100 g
Cysteine	3.62 ± 0.11
Histidin	0.21 ± 0.07
Threonine	1.95 ± 0.13
Tyrosine	1.10 ± 0.02
Valine	0.60 ± 0.05
Methionine	0.37 ± 0.01
Phenylalanine	0.32 ± 0.02
Leucine	2.41 ± 0.08
Isoleucine	2.46 ± 0.14
Aspartic Acid	16.50 ± 1.12
Glutamic Acid	8.47 ± 0.76
Serine	0.95 ± 0.65
Glycine	0.20 ± 0.06
Arginine	0.42 ± 0.12
Alanine	3.18 ± 0.21
Proline	0.03 ± 0.00

The fatty acid composition of the biomass was analyzed by the Department of Pharmacology at the University of Messina.

Table 7 qualitative and quantitative characterization using Gas Chromatography-Mass Spectrometry (GC-MS) for 37 fatty acids in the biomass of Phaedactylum tricornutum.

Fatty Acids	Area %
Saturated Fatty Acids (SFA)	18.71%
C14:0 - Myristic Acid	3.81 ± 0.02
C15:0 - Pentadecanoic Acid	0.22 ± 0.01
C16:0 - Palmitic Acid	12.60 ± 0.21
C18:0 - Stearic Acid	0.36 ± 0.01
C24:00 - Tetracosanoic Acid (Lignoceric):	1.72 ± 0.02
Monounsaturated Fatty Acids (MUFA)	18.25%
C16:1n-7 - Palmitoleic Acid	17.03 ± 0.15
C18:1n-9 - Oleic Acid	1.22 ± 0.02
Polyunsaturated Fatty Acids (PUFA)	63.06%
C16:2n-7 - Hexadecadienoic Acid	0.59 ± 0.02
C16:2n-9 - 9,12-Hexadecadienoic Acid	4.40 ± 0.04
C16:3n-6 - 6,9,12-Hexadecatrienoic Acid	9.91 ± 0.02
C18:2n-6 - Linoleic Acid	3.22 ± 0.03
C18:3n-6 - γ-Linolenic Acid	0.61 ± 0.02
C18:3n-3 - α-Linolenic Acid	1.10 ± 0.01
C18:4n-3 - Eicosatetraenoic Acid (ETA)	1.40 ± 0.02
C20:4n-6 - Arachidonic Acid	0.66 ± 0.01
C20:5n-3 - Eicosapentaenoic Acid (EPA)	39.90 ± 0.32
C22:6n-3 - Docosahexaenoic Acid (DHA)	1.27 ± 0.01
n-3 PUFA	43.67%
n-6 PUFA	14.40%

Morphological identification at SEM and fluorescent lipids staining

Our introduction to the research group at the University of Messina came through Professor Cornara of the Botanical Department at the University of Genova, DISTAV. Professor Cornara's keen interest lies in the morphological study of the novel *Phaeodactylum* strain. A synergistic collaboration has been initiated, focused on obtaining fluorescence microscopy images of PHAEO 102. These images aim to provide insights into the distinct lipid accumulation patterns within microalgal cells alongside variations in the proportion of photosynthetic pigments. Ongoing endeavors encompass the application of staining techniques and the collection of microscopy images to unravel these intriguing features.

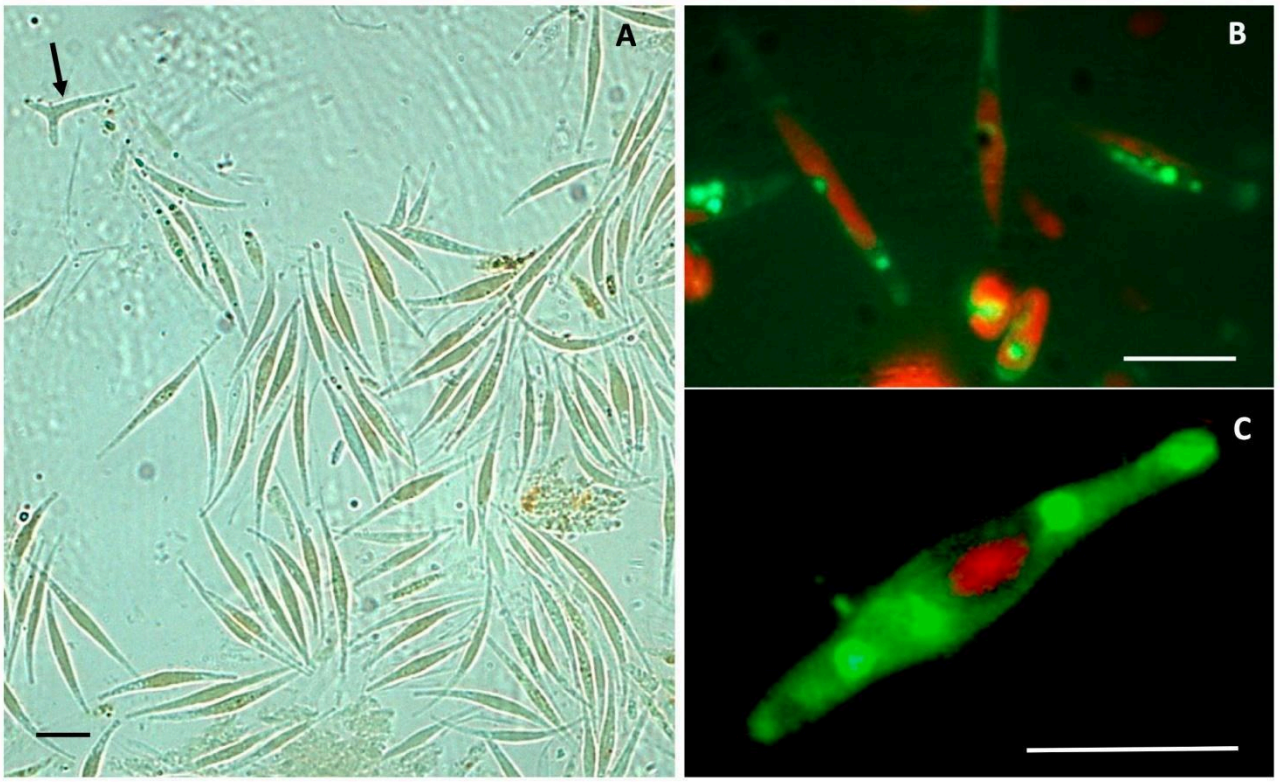


Figure 30 Light micrographs of P.tricornutum from a freeze-dried sample diluted in water for observation. (A) Many fusiformis cells dominate the field of observation, while only one isolated triradiate form is visible (dark arrow). (B,C) Magnified fluorescence This image is from an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

The study of the morphology of the newly isolated *P.tricornutum* reveals how these cells adapt to different stages of their life cycle. During the growth phase, the cells accumulate large amounts of lipids, which are visible as red spots in the image. Lipids are important for energy storage and membrane synthesis. In contrast, in older cells, the amount of fucoxanthin increases and covers the entire cell, shown by the color green. Fucoxanthin is a carotenoid pigment that gives the cells a brownish color and protects them from oxidative stress and excess light. The image shows the different colors of the cells depending on their age and metabolic state.

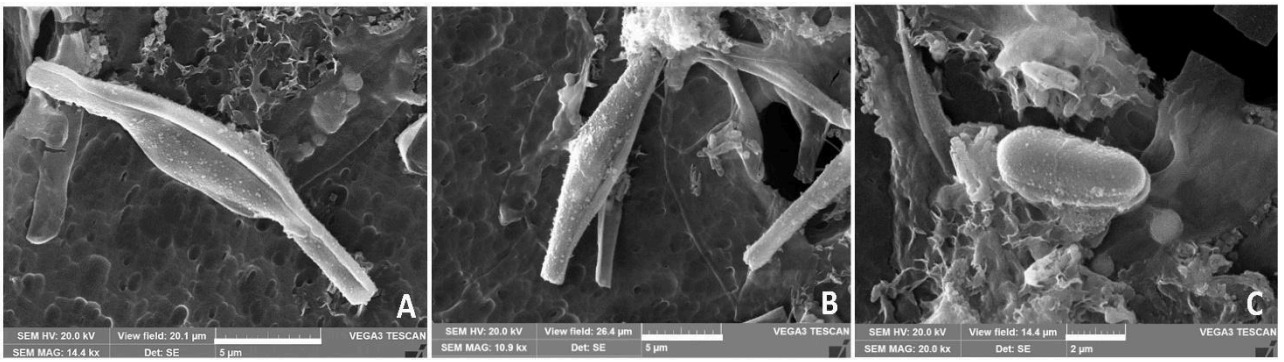


Figure 31 Scanning electron micrographs of a P.tricornutum cell culture. (A,B) Cells of the fusiformis morphotype in the process of cytodieresis. (C) Detail of a cell of the ovoidal morphotype. (A,B) Scale bar = 5 µm; (C) Scale bar = 2 µm. This image is from an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

The SEM microscope allowed us to observe the diversity of cell shapes in PHAEO 102, that we purified from the original wild type. We noticed that PHAEO 102 had two distinct morphologies: a pennate shape, which is typical of diatoms, and an ovoid shape, which is unusual for this stage of development. We hypothesized that the ovoid shape was a result of the faster growth rate of PHAEO 102 compared to the wild type diatoms, which only had the pennate shape. The ovoid shape might be an adaptation to increase the surface area to volume ratio and enhance the nutrient uptake and photosynthesis of PHAEO 102.

Production of the Fucoxanthin enriched extract: RED

The biomass batch obtained from the newly purified PHAEO 102 cultivated with the two steps process was subsequently processed into a highly fucoxanthin-enriched extract, creating a proprietary cosmetic ingredient known as RED. This extract stands as the core behind the inception of this research project. The desire for a deeper understanding of the components within the extract, other than reinforcing the already internalized knowledge about this extract, prompted the company to create a collaboration with the University of Messina. The objective of this collaboration is to identify all the carotenoids present in the cosmetic product. The ongoing partnership has thus far identified over 14 distinct pigments, with ongoing studies exploring the potential positive impacts stemming from this newfound knowledge.

Extraction process

The extraction process was carried out on the microalgae biomass obtained from the Camporosso plant. Initially, the biomass was freeze-dried and then milled to form a coarse powder.

The extraction procedure can be summarized as follows and is depicted in the following image:

1. The first step involved the extraction with 95% ethanol.
2. The ethanolic extract underwent a decoloration process using activated charcoal to remove chlorophylls.
3. The solvent was evaporated to obtain the crude extract.
4. The crude extract was treated in a biphasic system consisting of water and organic solvent to remove salts present in the crude extract.
5. The organic fraction was separated, and the solvent was evaporated until dryness.

6. The final step involved dilution with 95% ethanol to produce the ultimate REDOX V2 extract, with an approximate concentration of 70%.

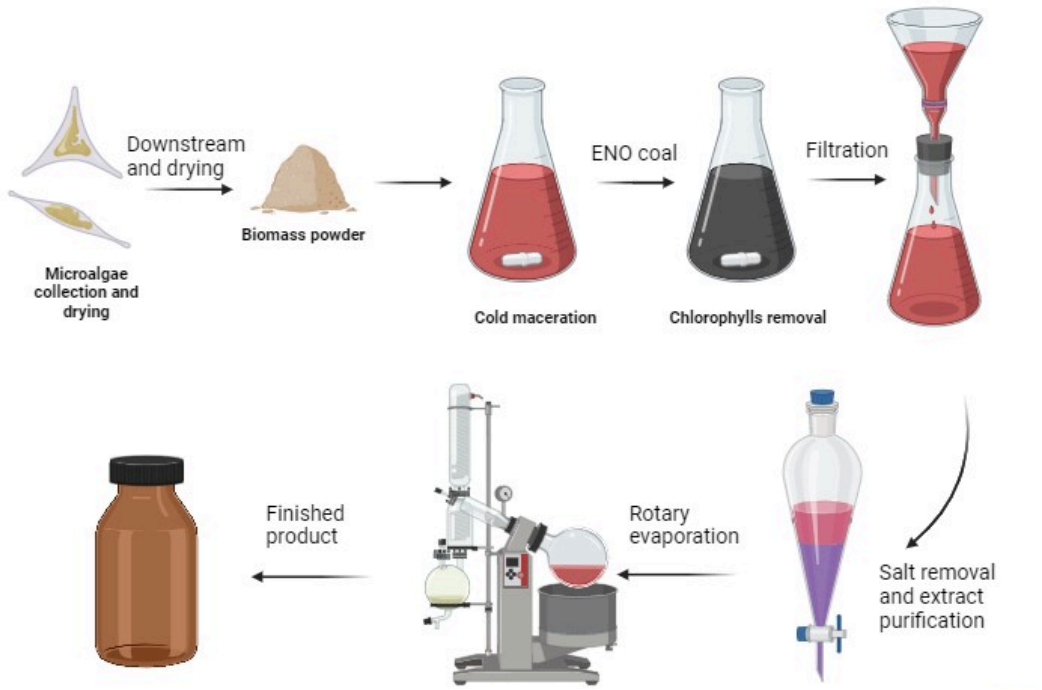


Figure 32 Diagram for the production of the Fucoxanthin enriched extract RED

The altered composition and production process of the new red extract, when compared to the previous one obtained from the wild type, resulted in a significantly higher recovery rate and improved extraction performance:

	WT *P.tricornutum*	**PHAEO 102**
% EXTRACTION YIELD	RED V1	RED V2
Crude extract/biomass	18,2 ± 1,0 %	25,0 ± 1,0 %
Final extract/biomass	6,0 ± 0,2 %	8,8 ± 0,2 %
Final extract/crude extract	33,1 ± 1,4 %	35,6 ± 1,2 %

Figure 33 Extraction yield (dry extract)

The table above indicates variations in the extraction yield among the previous wild-type strain and the newly purified PHAEO 102 cultivated with the 2 steps process involved in this project.

Several factors could contribute to these differences:

1. Distinct biochemical compositions of microalgae (variations due to different strains, biomass composition, and cell wall structure).
2. Minor variations in the extraction process, which could be influenced by factors like the operator, external temperature, and process variability.
3. Differences in the particle size of the biomass powder.

To ensure process consistency and repeatability, further work is required to assess and optimize the extraction procedure for each individual strain, potentially minimizing variations in yield.

Fucoxanthin enriched extract composition analysis

The chemical composition of RED V2 extract was examined with a focus on identifying and quantifying the classes of substances that are likely to be extracted using ethanol.

Lipids

Microalgae are known to produce a wide array of fatty acids and lipids. The biochemical composition of algae can vary significantly between species and can be influenced by factors like light, temperature, and growth stage. Changes in the biochemical composition of algae due to different growth stages are often associated with culture age and nutrient availability, especially in batch cultures.

Lipid class composition has also been observed to change with the growth stage. In general, phospholipids and glycolipids tend to decrease, while triacylglycerols and free fatty acids increase. The types and quantities of fatty acids found in algae can vary significantly among different species. Additionally, sterols, which are lipid components found in eukaryotes, have important functions related to hormone synthesis and regulation.

The extraction of lipids from algae requires careful consideration of their polarity, as this is related to how lipids are distributed within the algal cell and their associations with non-lipid components. Lipids found in algae can be classified into two main categories: neutral and polar lipids, which are further subdivided as shown in the table below:

Neutral lipids	**Polar lipids**	
	Phospholipids	**Glycolipids**
Triglycerides	Phosphatidylcholine	Sulphoquinovosyldiglyceride
Wax esters	Phosphatidylethanolamine	Monogalactosyldiglyceride
Hydrocarbons	Phosphatidylserine	Digalactosyldiglyceride
Free fatty acids	Phosphatidylglycerol	
Sterols	Phosphatidylinositol	

Figure 34 Classification of lipids

Polyunsaturated fatty acids (PUFA) can be found in triglycerides, phospholipids, and glycolipids. According to Molina Grima et al. (1994), ethanol at 96% concentration is an effective solvent for extracting lipids from *Isochrysis galbana*. This is particularly relevant for this microalgae, as approximately 73.5% of its lipids are polar in nature. Similarly, *P.tricornutum* has a high percentage of polar lipids, accounting for 76.8% of its total lipids.

The analysis of fatty acids was performed using the FAME (Fatty Acid Methyl Ester) using an internal method in GC-FID. This analysis was conducted on the extract RED V2, as well as on the freeze-dried biomass of *P.tricornutum* PHAEO 102. The following table presents a summary of the fatty acid composition, represented as a percentage of the total fatty acid content, in the dry biomass of *P.tricornutum* and its related extract, along with reference data from the literature for comparison.

	PHAEO 102 freeze dried biomass	RED V2
	% of total fatty acid	
C14 Myristic acid	5,07	4,95
C16 Palmitic acid	12,03	10,10
C16:1 Palmitoleic acid	16,09	17,11
C16:4 hexadecatetraenoic	**8,26**	**10,00**
C18 Stearic acid	0,29	0,34
C18:1 Oleic acid	1,59	1,40
C18:2 Linoleic acid	3,62	3,67
C18:3 Linolenic acid (ALA)	0,58	0,68
C18:4 Octadecatetranoic acid	1,01	1,21
C20:4 eicosatetraenoic (n-6) ARA	1,01	0,89
20:5n-3 EPA Acid	**34,49**	**35,04**
22:6n-3 DHA Acid	1,59	1,23
C24 Lignoceric acid	2,17	0,68
TOTAL FATTY ACID (% d.w.)	7,35	57,51

Figure 35 Fatty acid composition (as percentage of total fatty acid content) of P.Tricornutum dry biomass and extract

The fatty acid composition of *P.tricornutum*, as produced in Camporosso, aligns with existing literature data. The RED extract composition also closely matches the percentage composition of the dry biomass. Eicosapentaenoic acid (EPA 20:5(n-3)), a

polyunsaturated fatty acid (PUFA), is the predominant fatty acid in *Phaeodactylum tricornutum*, making up approximately 35% of the lipids and serving as the principal constituent of the glycolipids fraction.

Sterols

Sterol analysis was conducted using the method of Innovhub - SSOG laboratory in Milan on *P.tricornutum PHAEO 102* biomass and the RED V2 extract. The results, presented in the next Table are compared to existing literature data.

		PHAEO 102 freeze dried biomass	RED V2 (ethanol extract)
Unsaponifiable	% w/w on dry matter	3,3	17,32
cholesterol	%	/	0,3
brassicasterol	%	95,8	95,4
	g/kg on dry matter	1,92	6,93
24-methylcholesterol	%	0,5	0,2
campesterol	%	3,1	3,5
β-sitosterol	%	/	0,6
Total sterols	g/kg on dry matter	2,01	7,26

Figure 36 – Sterols content in Phaeodactylum T. and RED extracts (% on total sterols)

The results highlight that brassicasterol is the major component of the sterolic fraction in *P.tricornutum*.

Carotenoids

Fucoxanthin, a prominent carotenoid, is abundant in both macro and microalgae. It has been identified as the major carotenoid in the diatom *Phaeodactylum tricornutum*, making it a rich source of fucoxanthin, with concentrations at least ten

times higher than those in macroalgae. Fucoxanthin holds significant potential for applications in human and animal food, health, and cosmetics.

The analyses of fucoxanthin content on the biomass and the RED V2 extract were conducted using an internally developed HPLC method. The chlorophylls were analyzed spectroscopically, meanwhile the analysis on the carotenoids composition were performed by the university of Messina.

Fucoxanthin analysis via HPLC

A calibration curve was established using the external standard method with Sigma-Aldrich fucoxanthin (HPLC purity 95%). The procedure involved the preparation of various standard solutions:

1. A master standard solution was created by weighing 10.0 mg of Fucoxanthin and transferring it to a Class A 50 mL volumetric flask. Ethanol (approximately 96%) was added to reach a final concentration of 200 µg/mL.
2. A 2 µg/mL standard solution was prepared by transferring 0.5 mL of the master standard solution into a 50 mL Class A volumetric flask and diluting it with ethanol (approximately 96%) to achieve a final concentration of 2 µg/mL.
3. Similarly, a 4 µg/mL standard solution was prepared by transferring 1.0 mL of the master standard solution into a 50 mL Class A volumetric flask and diluting it with ethanol (approximately 96%) to achieve a final concentration of 4 µg/mL.
4. A 6 µg/mL standard solution was created by transferring 1.5 mL of the master standard solution into a 50 mL Class A volumetric flask and diluting it with ethanol (approximately 96%) to achieve a final concentration of 6 µg/mL.

5. Finally, a 8 µg/mL standard solution was prepared by transferring 2.0 mL of the master standard solution into a 50 mL Class A volumetric flask and diluting it with ethanol (approximately 96%) to achieve a final concentration of 8 µg/mL.

For each standard solution, three replicates were performed. The calibration curve was then constructed using the average area values obtained from the replicates, taking into consideration the declared HPLC purity standard as if it were a weight purity.

		PHAEO 102 freeze dried biomass	**RED V2 extract 70% in ethanol**
FUCOXANTHIN	% w/w	1,1 ± 0,01	4,32 ± 0,05
% on dry extract		/	5,57

Figure 37 Fucoxanthin content in biomass and related extract

Here the chromatogram of the fucoxanthin analyzed through the HPLC method.

Figure 38 HPLC chromatogram of the PHAEO 102: Fucoxanthin at 4,26 minutes, othere unknown carotenoids at 2,95 and 6 minutes

The final extract, comprising 70% biomass extract and 30% ethanol, exhibited an exceptionally high fucoxanthin content. This remarkable outcome can be attributed

to the enhanced cultivation process and the isolation of the *P.tricornutum* strain known as PHAEO 102. The improved techniques employed in the cultivation and isolation of this specific strain have yielded a concentrated and potent source of fucoxanthin within the extract.

Phytochemical profile of the *P.tricornutum* extract in LC-UV-Vis (DAD)/MS

Table 8 Phytochemical profile of a hydroalcoholic extract of Phaedactylum tricornutum. The results, which represent the mean ± the standard deviation of three independent experiments performed in triplicate, are expressed as the average percentage area (%) relative to the total identified compounds.

Compound	Area %
Chlorophyllide A	0.11 ± 0.01%
(E)-Fucoxanthin	77.45 ± 0.55%
Chlorophyll c1	0.41 ± 0.02%
(9Z)-Fucoxanthin	15.44 ± 0.24%
(13Z)-Fucoxanthin	5.18 ± 0.03%
(E)-Diadinoxanthin	0.47 ± 0.01%
(E)-Diatoxanthin	0.45 ± 0.02%
Chlorophyll a	0.28 ± 0.01%
β-Carotene	0.22 ± 0.01%
Chlorophylls	0.8%
Carotenoids	99.20%
Xanthophylls	98.99%
Carotenes	0.22%
Fucoxanthin	98.07%

The composition of the ethanolic extract reveals a notable presence of cis-fucoxanthin, accounting for up to 77% of the total fucoxanthin content, while trans-fucoxanthin comprises approximately 15% of the composition. This significant concentration of fucoxanthin, a natural carotenoid found in microalgae, holds great promise for various applications, particularly in the cosmetic industry. In addition to fucoxanthin, the extract contains a total of ten photosynthetic pigments. This diverse pigment profile provides the extract with a broad spectrum of potential uses in cosmetic formulations.

Chlorophylls

Batch	Clf A mg/l	Clf B mg/l	Clf C mg/l
RED V2 extract 70% in ethanol	5,19 ± 0,13	0,00	4,22 ± 0,15

Figure 39 Chlorophylls content in RED V2

As anticipated for an extract originating from a diatom, the absence of chlorophyll B was consistent with expectations. Meanwhile, the proportions of chlorophyll a and chlorophyll C are closely aligned with the known quantities found in *P.tricornutum*. This valuable insight provides assurance that the cultivation system and the strain have been effectively shielded from contamination by other microalgae species throughout the entire process. This purity and specificity of the extract are vital for maintaining the desired characteristics and qualities, ensuring its suitability for various applications and research purposes.

Percentage Recovery of the Extraction Process

The analytical data can be processed to assess the efficiency of the extraction process by considering the yield of the purified extract. This allows us to evaluate the process's effectiveness for each substance or class of substances.

In the next Table are compiled the percentage recovery of the most important substances from *Phaeodactylum tricornutum* biomass. The first column indicates the content of each substance in 100 grams of biomass, based on analytical data.

As mentioned in figure 41, the extraction process from 100 grams of PHAEO 102 biomass yielded 8.8 grams of purified extract. In the second column, you can find the

quantity of each substance in 8.8 grams of dry extract. Finally, the third column represents the percentage of recovery.

The results reveal a recovery of about 70% for the most abundant fatty acids and fucoxanthin, and a recovery of around 30% for the sterolic fraction. This difference can be attributed to the polarity of substances and the choice of the extraction solvent. Ethanol exhibits a stronger affinity for polar lipids and fucoxanthin compared to non-polar sterols.

Substance	Content in 100 g of PHAEO 102	RED V2 content in 8,8 g of dry matter (obtained from 100 g of biomass)	% of recovery
g of Palmitic acid	0,83	0,47	56,6 %
g of palmitoleic (n-7) acid	1,1	0,80	72,7 %
g of hexadecadienoic (n-6) acid	0,14	0,076	54,3 %
g of hexadecatrienoic (n-3) acid	0,3	0,22	73,3 %
g of hexadecatetraenoic (n-6) acid	0,57	0,47	82,4 %
g of oleic (n-9) acid	0,11	0,066	60,0 %
g of linoleic (n-6) acid	0,25	0,17	68,0 %
g ofeicosapentaenoic (n-3) acid (EPA)	2,38	1,64	68,9 %
g of docosahexaenoic (n-3) acid (DHA)	0,11	0,058	52,7 %
g of total fatty acid	6,2	4,28	69,0 %
g of Unsaponifiable	3,3	1,52	46,1 %
mg of Total sterols	200,6	63,89	31,8 %
mg of brassicasterol	192,3	60,96	31,7 %

g of FUCOXANTHIN	1,1	0,73	66,2 %

Figure 40 : percentage of recovery

Shelf life of the ethanolic extract after 1 year

No specific tests were conducted to assess the shelf life of the ethanolic extracts, which were stored at +4°C in dark plastic bottles. RED V2 was produced in September 2022. The fucoxanthin content of RED V2 was initially evaluated at the time of production and after one year, yielding lower results and a new peak, probably a degradation byproduct.

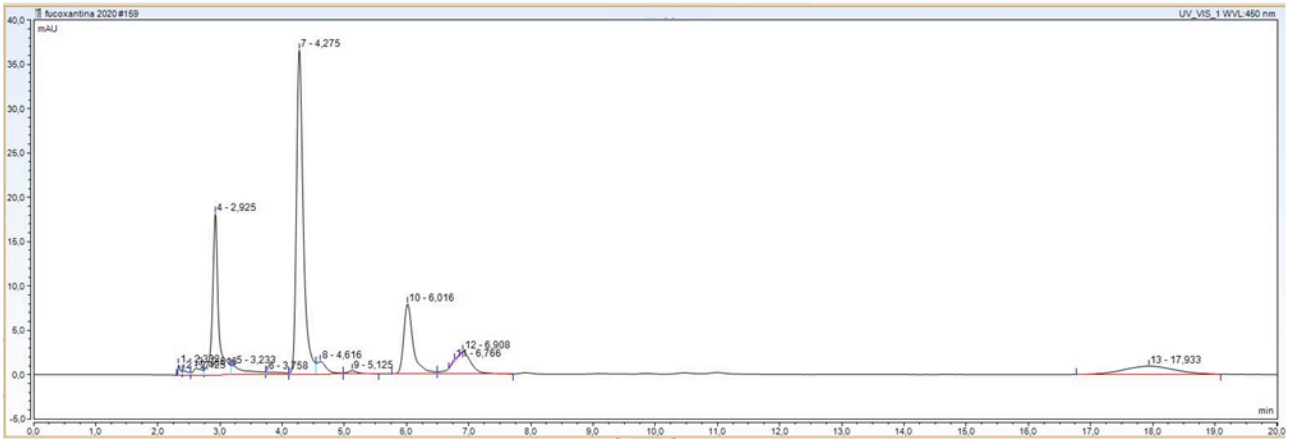


Figure 41 Fucoxanthin peak (retention time 4,2 min) of the RED V2 extracta after a year from the production

The significant decrease in fucoxanthin content, about 43%, may be attributed to a degradation process occurring over time due to the absence of preservatives.

To determine the exact shelf life of the extract, further investigations will be required.

Medium optimization for *Tetraselmis suecica*

In order to enhance the endogenous mannitol production within *Tetraselmis suecica*, two distinct growth conditions were selected, as recommended in existing literature, to evaluate the potential increase in mannitol concentration.

These experimental conditions, as informed by pertinent literature, were chosen by modifying one fundamental parameter in each setup to assess its influence on microalgal cultures. Specifically, one condition entails a reduced light exposure, while the other involves the utilization of double the concentration of sodium chloride, the primary component of seawater, at 60 g/L instead of 30 g/L. (Iwamoto K, et al 2003)

The control culture medium utilized is F/2 medium (Guillard et al. 1975). For every liter of medium, 950 mL of artificial seawater (ASW) is combined with essential nutrient salts, trace metals, and various vitamins. The pH is adjusted to 8 using either 1M NaOH or 1M HCl. After preparation, the medium is sterilized in an autoclave for 20 minutes at 15 psi and subsequently employed once room temperature is reached. Cultures were monitored for growth by measuring their absorbance at 600 nm using a spectrophotometer at 24/48-hour intervals. The resulting growth graph is presented below for visual reference.

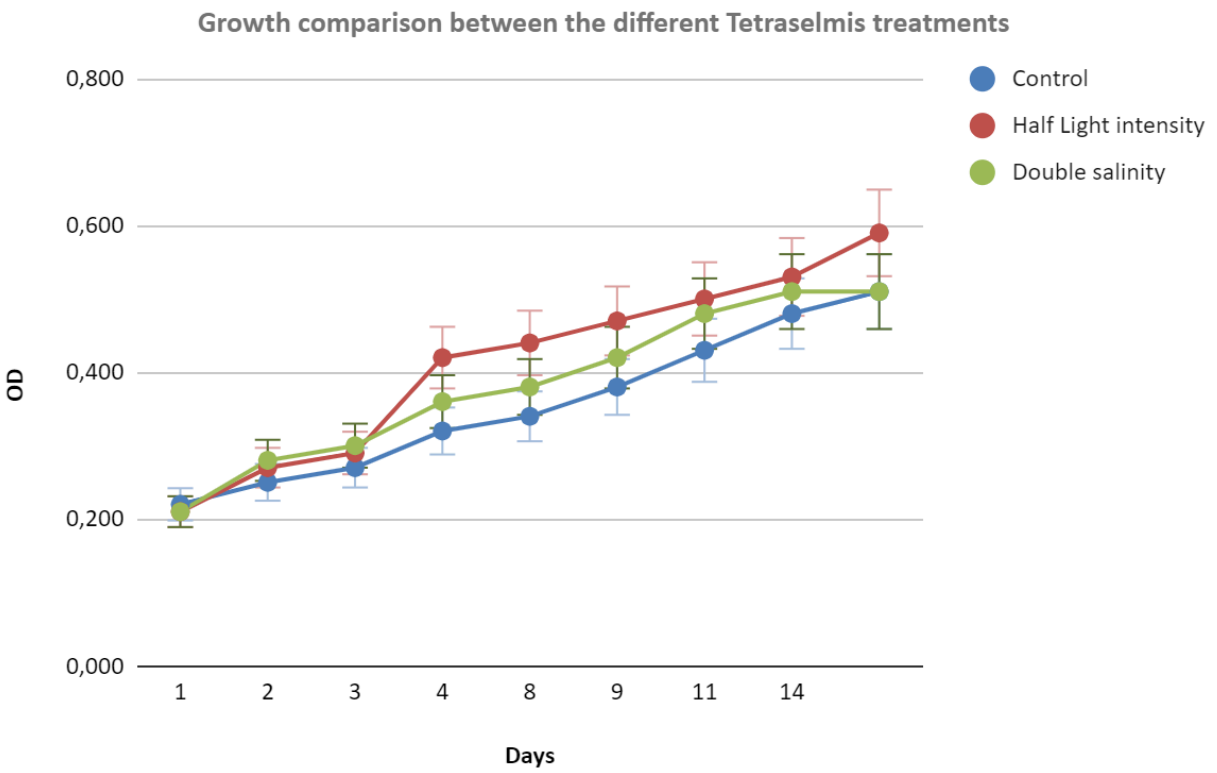


Figure 42 Optical Density of the T.suecica cultures under 3 different conditions

We measured the optical density of the *Tetraselmis* cultures to evaluate their growth under different conditions. The growth measurements for each treatment were repeated twice. The standard deviation of the trials ranged from 5 to 10 % of the mean value for each point. In the case of green microalgae which doesn't accumulate carotenoids, the optical density is a direct correlation for the biomass concentration of the algae. We expected that some conditions would enhance the growth rate of *Tetraselmis*, while others would inhibit it. However, the results showed that there was no significant difference in the optical density among the tested conditions. This means that the conditions tested did not affect the growth capacity of *Tetraselmis*. This finding leaves open the possibilities for both techniques in the scaling up of the cultivation in the production plant.

To analyze the mannitol content extracted from *Tetraselmis* samples, a detailed procedure was devised, aiming to ensure accurate measurements of this compound.

Mannitol analysis from *Tetraselmis* Samples

At the end of the exponential growth phase, biomass samples are collected from all the experimental setups. The method of biomass treatment and the settings for the analysis by HPLC are not reported due to being an internal company method. The chromatograms of the tested conditions are shown below.

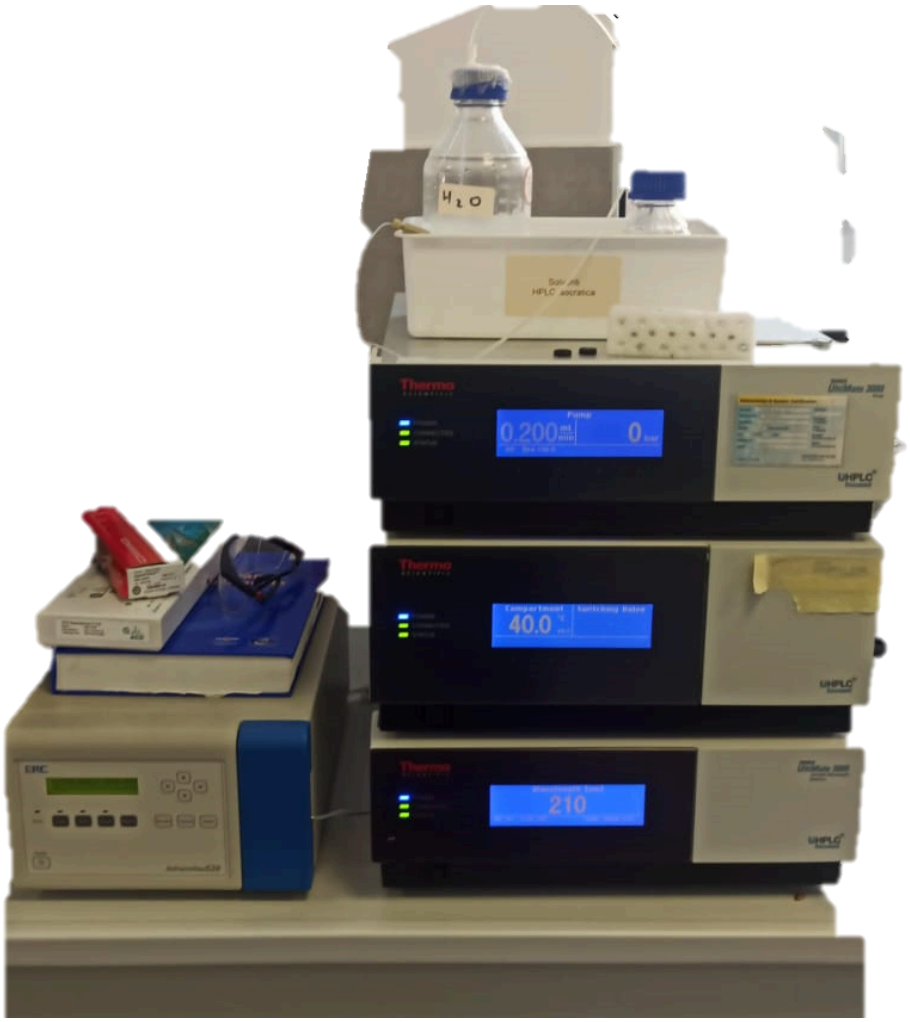


Figure 43 UHPLC set up with the refractive index system connected available at the APG group

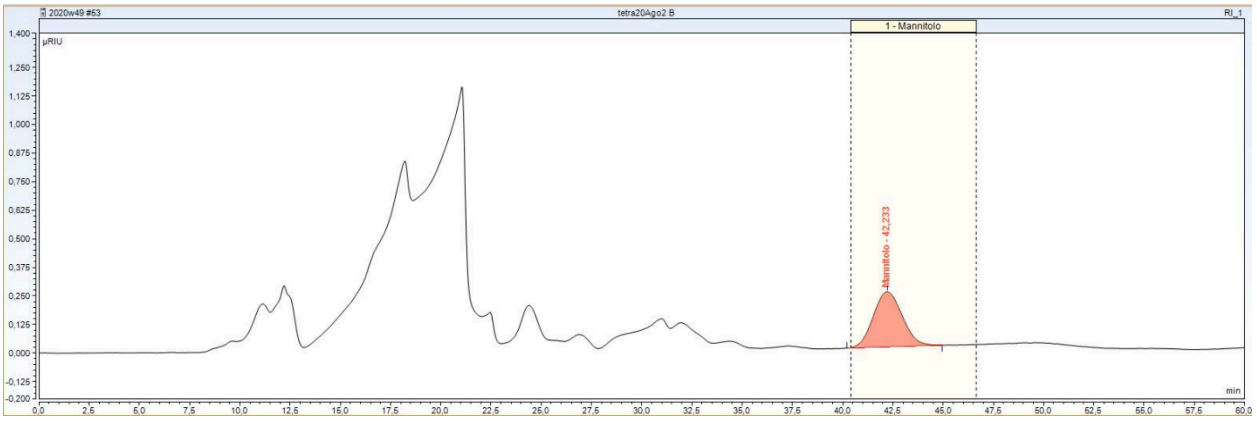


Figure 44 HPLC Chromatogram of the Tetraselmis Control sample

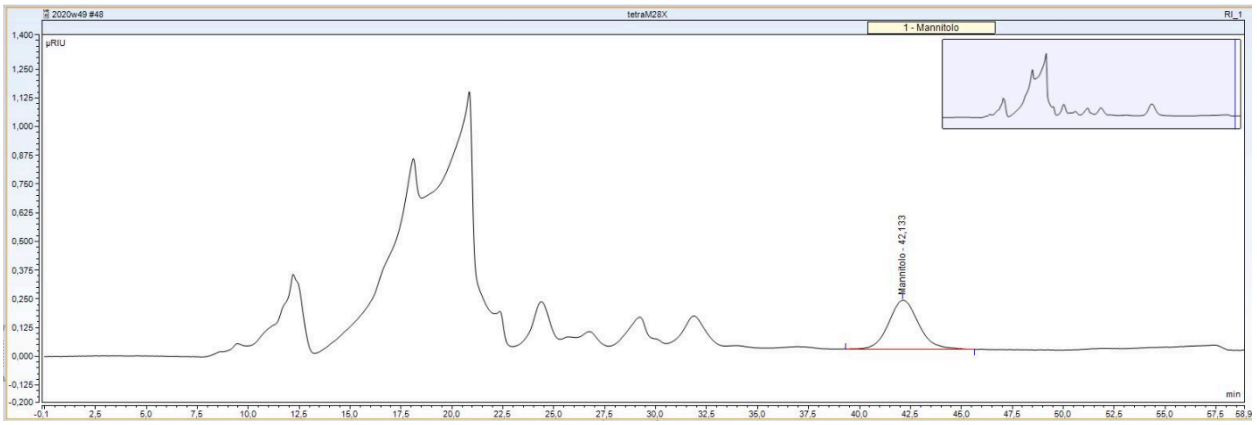


Figure 45 HPLC CHromatogram of Tetraselmis sample with halved light

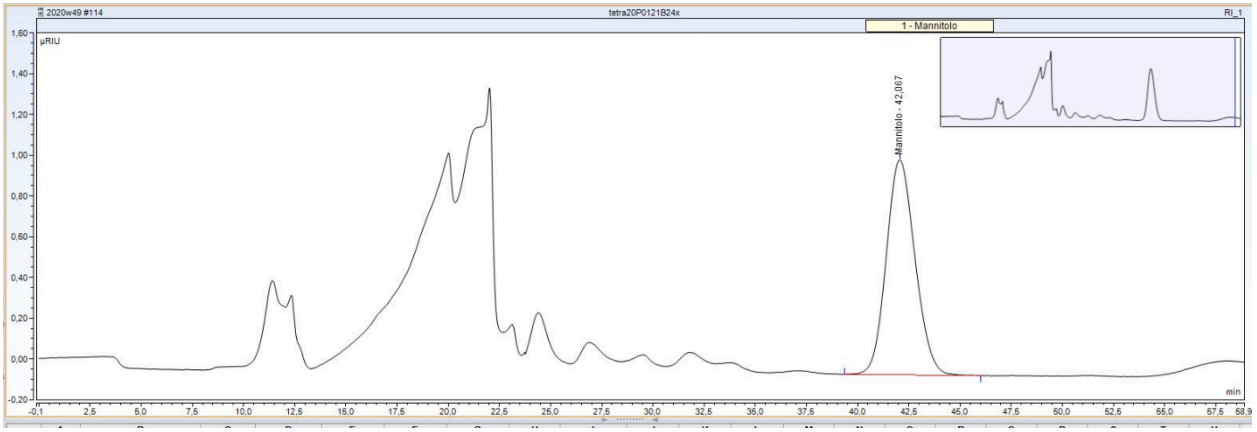


Figure 46 HPLC Chromatogram of Tetraselmis with doubled salinity sample

Chromatograms are shown without reporting the concentration of mannitol as this is a parameter currently under discussion with the buyer of the biomass and is a crucial condition for price definition. It is however noticeable that the set-up involving the doubling of the medium salinity positively influenced the amount of mannitol content.

Chapter 4

Conclusions

The culmination of three years of dedicated work within the laboratory of Active Cells, the research facility at A&A Fratelli Parodi, and the production plant of Archimede Ricerche, in collaboration with the University of Messina and in partnership with the University of Genova, has resulted in groundbreaking achievements and breakthroughs that are presented in this thesis. The inception of this project stemmed from the necessity to establish a more sustainable process for obtaining new ingredients derived from microalgae. The scientific work undertaken during the development of this industrial Ph.D. project have lead to a true innovation in the cutting-edge realm of microalgae, which are already recognized as innovative endeavors in the environmental, biotechnological, and cosmetic sectors.

Being the APG group of companies in the domain of green chemistry, the pursuit of advance and create new products tailored to our clients' needs and enhancing sustainability in production has remained at the forefront of our objectives. Transitioning Archimede Ricerche from the business of raw material production, such as biomass, to the creation of an improved photoprotective extract, was an intricate journey not shy of challenges. Each step was meticulously determined, following a process of development and feedback. Opting to purify the initial wild-type strain was an unconventional choice, given the recurring issue of microbial contamination in industrial production plants that undergo scaling up processes in open environments, like the Camporosso site.

Since the purification of PHAEO 102, the strain has been maintained under axenic conditions through monthly culture renewal, ensuring both cleanliness and preserving the unique metabolic assets that made it an excellent candidate for this project. Similar purification steps have been applied to other microalgae in our

collection, including *Isochrysis lutea*, *Porphyridium cruentum,* and *Nannochloropsis*. These microalgae have proven to be valuable platforms for the development of new extracts and high-value products in various sectors.

Analyzing microalgae products is a complex endeavor, given the lack of standardized methods in European, American or world Pharmacopeas, leading to discrepancies between laboratories and research centers while trying to align the analytical landscape of the many metabolites coming from microalgae. The use of thin-layer chromatography, while a simple method, provided a quick and efficient way to compare the various strains that emerged from the isolation steps, enabling a high-throughput assessment that was further validated using the more rigorous, internally developed HPLC method.

The incorporation of different light wavelengths into the cultivation process represents a significant advancement, particularly for the production plant that traditionally relied on natural light. This innovation mitigates challenges related to seasonality and low light availability during rainy periods, ensuring a more consistent and reproducible production. Understanding the light requirements for widespread microalgae cultivation, through the regulation of different light types, was a pivotal step during reactor development.

Studying the effects of red, blue, white, and magenta lights has been fundamental in comprehending the development for various microalgae, extending beyond *P.tricornutum* and *Tetraselmis s.*. The use of blue light for biomass growth and the utilization of red light to induce carotenoid production have become integral tools in our daily production plan, enabling the production of high-end microalgae biomass, such as Astaxanthin from *Haematococcus*.

This multifaceted journey has not only contributed to the advancement of our company but has also added valuable knowledge to the broader scientific community, setting the stage for future innovations in the microalgae industry.

The collaborations stemming from this project, particularly with the Botanic Department of UNIGE and the University of Messina, have afforded us a previously unseen perspective on our microalgae. The application of fluorescent staining techniques provided invaluable insights into the behavior of the new strain, Phaeo 102, allowing us to observe how it accumulates fatty acids and fucoxanthin in distinct regions of the cell. Additionally, electron microscopy images revealed the ovoidal shape of *P.tricornutum*, indicating a new morphology and biochemical profile for the cells. This transformation is reflected in the enhanced fucoxanthin content of PHAEO 102.

The biochemical analysis and amino acid composition provided valuable information about the protein content and nutritional profile of the biomass. This knowledge could be instrumental in the reuse and circular economy of the spent biomass once carotenoids have been extracted and fatty acids recovered, as it can serve as a beneficial protein substrate for hatcheries or aquaculture. The rich PUFAs content in the fatty acid composition offers a significant advantage for the nutritional profile and anti-inflammatory effects of nutraceutical products derived from this biomass.

The extraction process for the carotenoid fraction, a well-established method in our company, has seen improvements, particularly in terms of increased recovery percentages for apolar fractions, attributed to the unique shape and carotenoid enrichment of PHAEO 102. For the first time, the sterolic fraction has been analyzed,

revealing the potential of microalgae as a source of phytosterols. The refinement of HPLC fucoxanthin analysis using a calibration curve represents a notable improvement over previous internal methods. The comprehensive analysis of other photosynthetic pigments and carotenoids, previously unexplored, has provided us with a deeper understanding of the extract's composition and, consequently, the biomass used.

The recovery percentages of lipids, sterols, and carotenoids are vital aspects of optimizing the development of a successful product. Evaluating the shelf life and stability of the extract is an ongoing study and will be a focal point of a new research project, considering various preservation aspects. Finally, the optimization of mannitol metabolite within *Tetraselmis suecica* has been a long-awaited achievement for our company, enabling us to tailor the metabolite content to our specific requirements.

Moreover, the development of this analysis from a complex matrix like microalgae represents a breakthrough, and we intend to seek European approval to establish a standardized analysis for such products.

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