

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**IMPROVING PROCESS-EFFICIENCY OF MICROALGAE
CULTIVATION: HETEROTROPHIC, ATTACHED GROWTH AND
SIMULTANEOUS WASTE UTILIZATION AND LIPID
PRODUCTION**

ZEYNEP BÜŞRA ÇAKIR
**A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY**
DEPARTMENT OF ENVIRONMENTAL ENGINEERING

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PROF. DR. MELEK ÖZKAN

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2023

**T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ**

**MİKROALG KÜLTÜRLEMEDE PROSES
VERİMİNİN İYİLEŞTİRİLMESİ:
HETEROTROFİK, BAĞLI BÜYÜME VE
EŞZAMANLI ATIK KULLANIMI VE LİPİT
ÜRETİMİ**

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**DANIŞMANI
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**GEBZE
2023**

GEBZE TEKNİK ÜNİVERSİTESİ	DOKTORA JÜRİ ONAY FORMU
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SUMMARY

Heterotrophic metabolism was combined with attached cultivation of *Chromochloris zofingiensis* for co-production of lipids and astaxanthin to simplify processing, improve efficiency, and mitigate cost. Microalgae were cultivated on agar media surface homogeneously with high solid content (40%). Parameters for pigment extraction were optimized and consequently, lipids and astaxanthin could be co-extracted. Cultivation parameters were optimized; 30 days room temperature incubation with 40 g/L initial glucose, 4% inoculum, and wet extraction of the harvested biomass yielded 8.2 g/L lipids and 6.5 mg/L astaxanthin. Advantages of agar-attached cultivation include: no energy input during incubation, simple harvesting, and no drying of biomass.

Growth and lipid production of *Auxenochlorella protothecoides* (*Ap*) was studied and the synthetic growth medium was optimized, yielding a 90% improvement in lipid output. Carrot pomace, a waste by-product of the juice industry, was utilized to fully replace synthetic growth medium in the heterotrophic cultivation of *Ap*. With carrot medium (CM) alone, 4.9 g/L total lipids, and with an addition of 5 g/L glucose to the CM, 5.9 g/L lipids could be produced – the latter being equivalent to the lipid output achieved with the optimized synthetic growth medium.

Heterotrophic, attached growth of *Ap* was tested with various materials and string was found to support the highest biomass attachment. Biomass and lipid production of *Ap* was tested using CM as a growth medium and string for attachment. String-attached biomass samples could be extracted directly for lipids. Culture of *Ap* using CM was tested in a bioreactor to determine the potential for scaleup.

Key Words: Microalgal Cultivation, Heterotrophic, Attached Growth, Processing Efficiency, Lipids, Astaxanthin.

ÖZET

Heterotrofik ve bağılı büyüme koşullarında *Chromochloris zofingiensis* türü kullanılarak eşzamanlı olarak lipit ve astaksantin üretilmiş, böylece prosesin sadeleşmesi, verimliliğin artması ve maliyetin düşürülmesi hedeflenmiştir. Mikroalgler agar besiyeri yüzeyinde homojen bir şekilde büyütüldüğünde elde edilen biyokütlenin yüksek katı oranına (%40) sahip olduğu gösterilmiştir. Pigment ekstraksiyon parametrelerinin optimizasyonu sonucunda lipit ve astaksantin eşzamanlı ekstraksiyonu mümkün olmuştur. Ayrıca, kültür parametrelerinin de optimizasyonu gerçekleştirilmiştir. Bu sayede, 40 g/L başlangıç glikozu, %4 inokulum ve 30 gün süre ile oda sıcaklığında inkübasyon sonucunda hasatlanan biyokütlenin ıslak ekstraksiyonundan 8,2 g/L lipit ve 6,5 mg/L astaksantin elde edilmiştir. Agar yüzeyinde kültivasyonun şu avantajları ortaya konmuştur: (i) inkübasyonun enerji gerektirmemesi, (ii) hücrelerin basit ve manuel olarak hasatlanması ve (iii) biyokütle kurutma aşamasının olmaması.

Auxenochlorella protothecoides (Ap) türünün büyüme davranışı ve lipit üretimi sentetik besiyerinde optimize edilerek lipit üretiminde %90 iyileştirme elde edilmiştir. Ardından, meyve suyu endüstrisinin atık yan ürünü olan havuç posası, Ap türünün heterotrofik kültivasyonunda sentetik besiyeri yerine kullanılmıştır. Tek başına havuçlu besiyer (CM) ile toplam 4,9 g/L lipit üretilirken, 5 g/L glikoz ilavesiyle 5,9 g/L lipit üretilmiştir. Glikoz ilavesi yapılan CM ile elde edilen lipit, optimize edilmiş sentetik besiyeri ile elde edilen lipit ile eşdeğerdir.

Ap türünün heterotrofik ve bağılı büyümesi çeşitli malzemelerle test edilip bağılı büyüme için en iyi destekleyen malzemenin ip olduğu gösterilmiştir. Besiyeri olarak CM ve bağılı büyüme için ip kullanılarak Ap türünün biyokütlesi ve lipit üretimi incelenmiştir. İpe bağlanan biyokütlenin doğrudan ekstrakt edilmesiyle lipit elde edilebilmiştir. Ap türünün CM besiyerinde kültivasyonu biyoreaktörde test edilmiş ve ölçek büyütme potansiyeli gösterilmiştir.

Anahtar Kelimeler: Mikroalg Kültürleme, Heterotrofik, Bağılı Büyüme, Proses Verimliliği, Lipit, Astaksantin.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
°C	: Degrees Celsius
$\mu\text{E m}^{-2} \text{s}^{-1}$	: microeinsteins per second per square meter
AA	: Arachidonic acid
ASTM	: American Society for Testing and Materials
BDWG	: Biodiesel industry waste glycerol
BOD	: Biochemical oxygen demand
C/N	: Carbon:nitrogen
CM	: Carrot medium
CM5	: Carrot medium plus 5 g/L glucose
CM10	: Carrot medium plus 10 g/L glucose
COD	: Chemical oxygen demand
DCW	: Dry cell weight
DE	: Dairy effluent
FAME	: Fatty acid methyl esters
H ₂ O ₂	: Hydrogen peroxide
HCl	: Hydrochloric acid
HPLC	: High-performance liquid chromatography
HPTLC	: High-performance thin layer chromatography
HRAP	: High-rate alga pond
ID	: Inner diameter
MAE	: Microwave-assisted extraction
MBBR	: Moving bed biofilm reactor
NaClO	: Sodium hypochlorite
NaOH	: Sodium hydroxide
OD	: Outer diameter
ORP	: Open raceway pond
PBR	: Photobioreactor
PSU	: Practical salinity units
PUFAs	: Polyunsaturated fatty acids
PW	: Piggery wastewater

ROS	:	Reactive oxygen species
SC-CO ₂	:	Supercritical carbon dioxide
TAG	:	Triacylglycerol
TLC	:	Thin layer chromatography
TN	:	Total nitrogen
TP	:	Total phosphorus
TPBR	:	Tubular photobioreactor
UAE	:	Ultrasound-assisted extraction
WNF	:	Waste nitrogen fertilizer

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1. INTRODUCTION

With the world's population and consequent energy demands increasing every year, the need to develop alternative renewable energy sources is impending. As the third generation of biofuels, microalgae offer many advantages that deem them worth pursuing as a potential biodiesel feedstock. Their photosynthetic efficiency, ability to grow in brackish and waste waters without requiring farmable land and their potential to reduce CO₂ emissions has made them a topic of interest. Microalgal cultivation is being pursued in many facilities worldwide, including the USA, Japan, Germany, France, Sweden, Israel, China and India for products such as dry biomass, micronutrients or fatty acids [1]. However, based on the processing costs, microalgae-based biodiesel does not yet compete well with fossil fuels.

Differing strategies continue to be researched in an effort to improve the processing efficiency and reduce the costs associated with microalgae-derived biodiesel. Cultivation of microalgae relying on their autotrophic metabolism has its advantages, such as, their usage of CO₂ as an inorganic carbon source and their reduction of nitrogen and phosphorus present in municipal wastewaters, making them environmentally advantageous. However, these cultures tend to have low biomass and lipid yields resulting in a costly feedstock for biodiesel production. Alternatively, heterotrophic cultivation of microalgae offers the advantages of faster microbial growth, higher biomass and lipid outputs, no dependence on light and, therefore, the potential for more efficient processing. The primary disadvantage associated with heterotrophic cultivation is the cost of an organic carbon source.

Alternative methodologies are investigated to improve the cost-efficiency and feasibility of microalgal lipids production. The biorefinery approach is one avenue of interest, where microalgae are considered for their potential to produce any combination of biomass, protein, starch, lipids or high-value products simultaneously, to shift the cost-benefit balance. Co-production of lipids for biodiesel and high-value metabolites is an attractive option if a microalgal species has the ability to produce lipids and the metabolite in a positively correlated manner. *Chromochloris zofingiensis* is a strain that has been shown to produce lipids with high triacylglycerol (TAG) content applicable as a biodiesel feedstock and a high-value carotenoid, astaxanthin, simultaneously and with a positive correlation. That is, cultivation parameters can be optimized to improve both lipids and astaxanthin production. The

high value of astaxanthin has the potential to improve the cost-effectiveness of producing lipids-derived biodiesel from this microalga.

Utilization of organic wastes or wastewaters with high organic carbon content is another avenue of research geared to improve the cost-effectiveness and environmental impacts of heterotrophic cultivation of microalgae. Dairy effluent, piggery wastewater, cheese whey permeate, volatile fatty acids and municipal wastewaters are just some examples of the waste streams that microalgae have been successfully cultivated with [2]. *Auxenochlorella protothecoides* is a microalgal strain that is well-studied for its fast growth and lipid production rates. It has been shown capable of utilizing nutrients from a variety of wastes and wastewaters [3]–[9], making it a great candidate to improve the cost-benefit balance and feasibility of heterotrophic cultivation of microalgal lipids.

Another strategy for advancing the feasibility of microalgae-based biodiesel production is reducing the cost of energy-intensive processing steps. Traditionally, the process involves cultivation of microalgae, harvesting (thickening and dewatering), drying, extraction for lipids and transesterification to biodiesel. Harvesting can account for 20-30% of the total cost of production [10]. Attached growth of culture is a valuable option that can allow for effective and economical harvesting of biomass [11]. Attached growth has generally been applied in microalgal cultivation to improve the cells' access to light and to avoid self-shading, which is a growth-limiting factor associated with photosynthetic cultivation of algae [12], [13]. To our knowledge, attached growth of microalgae under heterotrophic condition (no light) was not previously studied.

In this thesis, the aim was to investigate various strategies for improving processing efficiency and positively impacting the feasibility of producing microalgal lipids-derived biodiesel. One goal was to examine the effect of cultivation parameters and to explore the possibility of combining heterotrophic and attached growth of microalgae. The second goal was to study the co-production of lipids and astaxanthin to improve the cost-benefit balance. Finally, the third goal was to research the use of carrot pomace waste to replace the carbon and nutrients needed to heterotrophically cultivate microalgae. Lipid and astaxanthin production of *C. zoofingiensis* was investigated under varying initial glucose conditions and its heterotrophic, attached growth on agar media surface was demonstrated. Operational parameters were tested to optimize lipids production under agar-attached condition. Extraction parameters

were tested to optimize astaxanthin recovery. The feasibility of heterotrophic, attached growth on agar media surface for co-production of lipids and astaxanthin was established. The lipid production of *A. protothecoides* was investigated initially in suspended cultivation by optimizing synthetic growth medium and cultivation parameters, including initial glucose, pH, growth medium constituents and temperature. Then, a process was developed to use carrot pomace waste to replace the growth medium, including optimization of acid hydrolysis parameters. *A. protothecoides* growth was also researched in heterotrophic, attached form with various materials added to the growth medium for biomass attachment and direct lipid extraction of attached biomass was investigated. Growth and lipid production of *A. protothecoides* was researched using carrot medium with the addition of string for biomass attachment. Finally, cultivation of *A. protothecoides* using carrot medium was also tested in a bioreactor to investigate the potential for scalability.

2. BACKGROUND

2.1. Sustainability and Renewable Energy

Traditionally, fossil fuels are widely used to meet the continually growing energy demand. With the world's population rising and a great rate of industrialization, the consumption of fossil fuels, including coal, oil and gas, has increased on a large scale globally, having more than doubled in the last 50 years (Figure 2.1, [14]). In 2019, fossil fuels were used to supply ca. 84% of the world's primary energy demand. They take millions of years to form in the earth's crust as organic matter is decomposed and converted to materials high in hydrocarbons. Contrary to the time that it takes for their formation, fossil fuels are used at a much greater pace. Utilization of any resource at a greater rate than its formation will inevitably lead to its depletion. Therefore, vast research and development efforts are geared towards the utilization of more readily renewable sources of energy, leading to a more sustainable cycling of carbon.

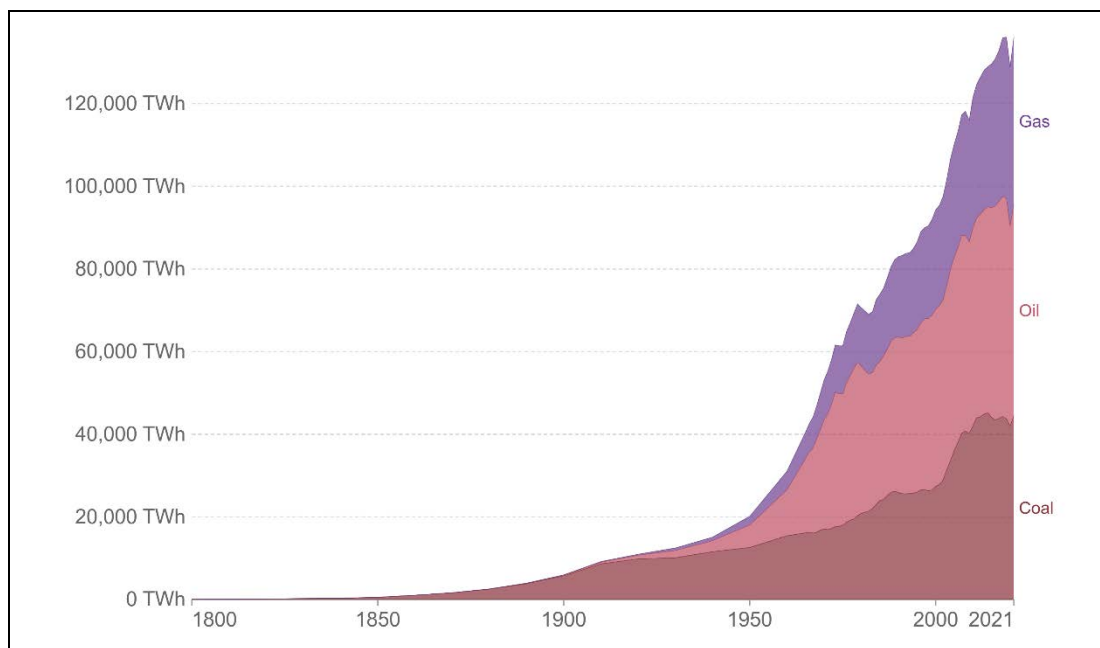


Figure 2.1: Global primary energy consumption by fossil fuel source, measured in terawatt-hours (TWh).

Renewable energy sources (RES) are gaining interest as a necessity to combat the issues of fossil fuel depletion, increasing greenhouse gas emissions, environmental pollution and global warming [15]. These sources include wind, solar, hydro-power,

biomass and geothermal. In 2017, these RES accounted for 26.5% of electricity usage globally, with hydro-power and wind in the lead at 16.4 and 5.6%, respectively [15]. Solar thermal energy is abundantly used domestically, including in water heaters, cookers and refrigeration. Biomass energy has great potential as a RES since it can be used to produce a variety of fuels, including biodiesel, biomethane, bioethanol, biohydrogen and biohythane [16]. Primarily used for heat and power generation and transportation fuels, biomass energy can be derived from various feedstocks, such as household waste, paper waste, sawdust, wood, municipal and industrial effluents, and microalgae and cyanobacteria. With further improvements in processing technology and reduction in production costs, biomass-derived energy sources have the potential to promote a larger dependence on renewable sources and thus, a more sustainable cycling of energy and materials.

2.2. Microalgal Lipids Derived Biodiesel

Microalgae as a renewable source of energy is a widely studied topic of research. They are part of the third generation of biofuels, as illustrated in Figure 2.2 [17]. The first generation, based on agricultural yields, is not considered a viable option since a scaleup would compete directly with land and resources available for the production of foods for human and animal feed [18]. The second generation, based on non-food agricultural products, such as straw, forest residues, manure, inedible parts of plants, etc., is not considered feasible due to high processing costs necessitated by pre-treatment steps [19]. Microalgae, as the third-generation biofuel, is thought to have the potential to overcome the challenges associated with first and second generations. One of the advantages associated with cultivation of microalgae is that there is no need for arable land, therefore, no direct competition with agricultural resources. Additionally, the culture can be grown using brackish or wastewaters [17]. Microalgae have also been shown to have a much higher areal productivity – 15-300 times more oil can be produced on an area basis, when compared to traditional plants [17]. Furthermore, compared to agricultural harvests, which occur at most twice a year, the short cultivation time of microalgae allows for 30+ harvesting cycles to be possible in a year depending on the cultivation period that is preferred.

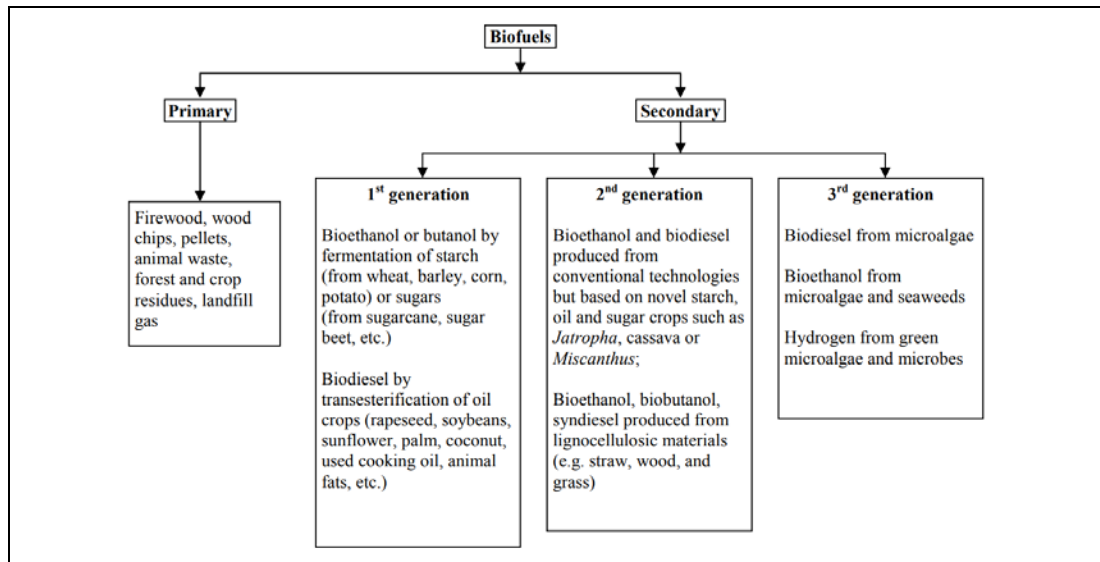


Figure 2.2: Classification of biofuels.

Microalgae can be utilized for their carbohydrate or protein content, however, their use as a biodiesel feedstock depends on their lipid content. The lipids that are stored either intracellularly or forming their cell membranes can be extracted from their cell structures and converted to biodiesel [20]. Traditionally, the process entails cultivation of microalgae, harvesting of the cells, concentration or drying of the biomass (cells), extraction for lipids and transesterification of lipids into biodiesel [21]. A flowchart demonstrates the process steps and the many factors involved, which ultimately affect process throughput and efficiency (Figure 2.3 [21]).

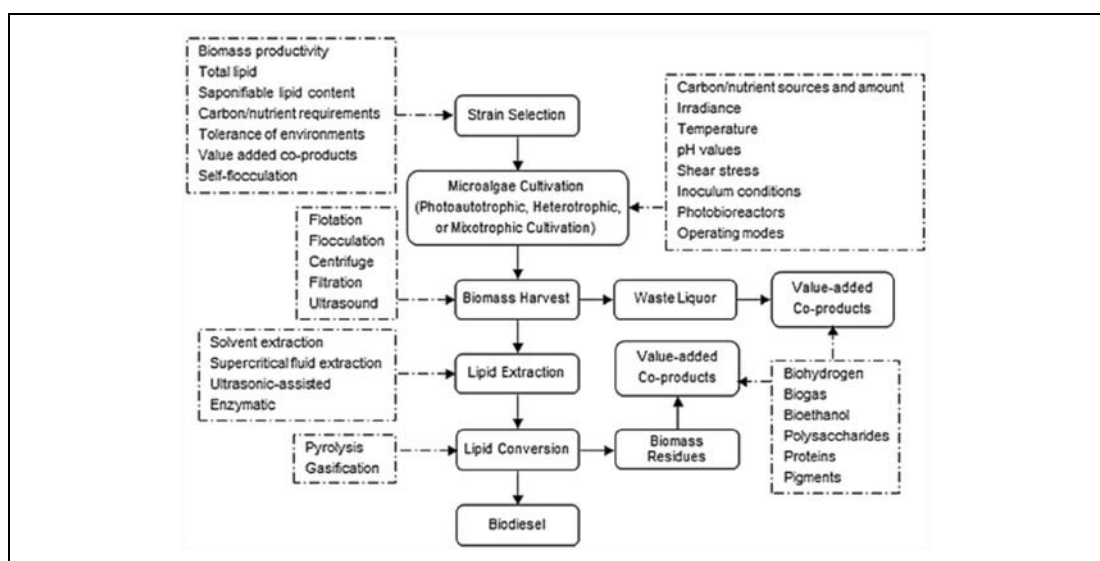


Figure 2.3: Flowchart of processing steps for producing biodiesel from microalgae.

2.2.1. Cultivation of Microalgae

The process of producing biodiesel essentially begins with cultivation of either a single strain or a consortium. Axenic strains are preferred from the perspective of quality and process control that can be accomplished since the culture's biomass and lipid production behavior become more predictable. However, a consortium is inevitable if the cultivation is scaled up with an open pond system, where the culture is exposed to the atmosphere and a mixture of microalgae and bacterial growth may ensue. For closed systems strain selection is an important step that will inevitably dictate the process yields. The general considerations are, that the strain should be easy to cultivate, resilient to contamination, have a high growth rate along with high lipid content and ideally, should be amenable to harvesting and extracting for lipids. If the alga is intended for use with waste or wastewater, its compatibility and ability to utilize the available nutrients should also be considered.

Once a strain is identified for the process, there are a number of optimizations that can be determined to improve the lipid production rate. Well known for their impact on microalgae's accumulation of lipids are parameters such as, pH, temperature, light intensity, salinity, aeration, and nutrient levels [22]–[24]. Like all cultivation parameters the preference for culture pH can be strain-specific. One study of the marine microalga, *Nannochloropsis salina*, tested pH between 5 and 10 demonstrating that the best growth was achieved at a pH of 8 and 9 [25]. In this study the strain was cultivated autotrophically using natural lighting and buffer solutions were used to control pH rather than the injection of CO₂, in order to differentiate the effect of pH alone. They also discovered that the pH and buffering could help protect the culture against invading organisms, such as ciliates and diatoms. This is a worthwhile revelation as it is such information that can lead to the setup of a robust and sustainable process. Another study of the growth of *Chlorella vulgaris* with culture pH ranging from 3 to 11 revealed that the strain grew and accumulated lipids optimally at a pH of 7.5, achieving a maximum lipid content of 53.4% [26]. The strain was shown to tolerate a pH range of 5–8 well. The culture conditions were heterotrophic; i.e., no light was used and glucose was provided as a carbon source. Additionally, the sulfur in the growth medium was limited to promote higher lipid content. Such a case illustrates how more than one parameter can be strategically optimized, in this case, pH and sulfur-limitation, to improve the lipid output. Beyond

the common strains, there are also extremophilic microalgae, such as *Galdieria* strains, which are known for their ability to tolerate extremely acidic (pH of 1-2) environments [27]. These strains are also highly tolerant of metal-polluted wastewaters and are frequently investigated for their use in a variety of waste streams, including treatment of acid mine drainage [28]–[30]. These benefits, along with the algae's ability to accumulate lipids and a valuable pigment compound, phycocyanin, their capacity to utilize various (50+) carbon sources and their tolerance of high temperatures (up to 56°C) make them great candidates for simultaneous treatment of non-sterile waste streams and production of valuable bio-products, all the while resisting contamination due to their extreme cultivation conditions [27], [31].

Temperature is also an important factor in cultivation of microalgae, affecting growth rates, lipid content and even the profile of fatty acids produced [32]. Most commonly microalgae prefer a cultivation temperature between 20-30°C. A study of *N. salina* investigated varying nitrogen concentrations along with a temperature range of 15-35°C [33]. They found that supplementing 25% nitrogen compared to the original growth medium (f2) and 30°C cultivation temperature yielded microbial biomass with 59.3% lipid content. Once again, this demonstrates that a combination of stress factors can be implemented to increase microalgal lipid production. Another group studying *Spirulina platensis*, *Chlorella vulgaris* and *Botryococcus braunii* in batch culture revealed that all three strains responded to a temperature increase in a similar way, i.e., with a corresponding shift in the composition of fatty acids accumulated intracellularly such that the unsaturated fats were relatively decreased [34].

The effects of light intensity are often researched in the autotrophic or mixotrophic culture of microalgae since it impacts biomass and lipid production of various strains. One group analyzed the effect of applying three different light intensities, 50-300 $\mu\text{E m}^{-2}\text{s}^{-1}$, on the growth and fatty acid content of *Chlorella*, *Desmodesmus*, *Ettlia* and *Scenedesmus* strains [35]. They found that all four strains had higher biomass as a result of increasing light intensity and, that two strains, *Desmodesmus* and *Scenedesmus* species, also had an increasing content of fatty acids with a corresponding decrease in their protein content. Relevant to note is that the group used municipal wastewater as the growth medium and demonstrated that all strains could remove more than 75% of the total nitrogen (TN) and total phosphorus

(TP) at a light intensity $>50 \mu\text{E}$ and that there was a tangible increase in the nutrient removal capability of *Desmodesmus* with an increase in light intensity from 50 to 150 μE . This study illustrates how a cultivation parameter can be used to improve biomass and lipid yield, while also serving the benefit of advancing wastewater treatment.

Salinity of the growth medium is yet another variable that can be controlled to improve the lipid production capability of microalgae. One group studying *N. salina* investigated the effect of growth medium salinity on the growth and lipid accumulation of the strain, as well as its impact on contaminating organisms [36]. They found that the salinity, measured in practical salinity units (PSU), appeared optimal at 22 and 34 PSU in terms of improving algal growth. They also demonstrated that 22 PSU was the salinity corresponding to the lowest presence of invading organisms. They further tested a two-phase cultivation strategy implementing an initial salinity of 22 PSU to promote algal growth, followed by 34 PSU to improve the alga's lipid content to 36%. Once again, this study shows how a cultivation parameter could be used to serve multiple advantages, in this case, to improve lipid output as well as decreasing the possibility of culture contamination.

Aeration is another factor that has been observed to impact microalgal cultivation output. In the case of autotrophic cultivation, the effect of aeration is due to the CO_2 being supplied to the culture, which is the cells' carbon source. A group studying *Dunaliella salina* tested different levels of CO_2 aeration and found that the highest amount of aeration (12%) led to the greatest lipid content and lipid productivity of 40.65% and $59.23 \text{ mg}\cdot\text{L}^{-1}\text{d}^{-1}$, respectively [37]. In contrast, heterotrophic cultures have a dependence on air for the oxygen it contains, which is necessary to degrade the organic carbon sources in the growth medium. A study of a *Micractinium* species cultivated with an organic carbon source, molasses, demonstrated that the culture's biomass production could be significantly improved by 30% when the aeration rate was doubled [38]. Regardless of the culture's metabolic preference, aeration can be used to optimize microalgal biomass and lipid outputs.

Nutrient stress is a widely studied variable as it is known to significantly affect the lipid accumulation behavior of many microalgal species [39]. Nitrogen and phosphorus are essential nutrients for cell growth, which when limited in the growth medium put the cells in a state of oxidative stress and promote their production of TAGs [39]–[41]. One group studied the effects of nitrogen deficiency on the lipid production of *Dunaliella*, *Chlorella* and *Desmodesmus* species [42]. They cultivated

the strains for 20 days and when comparing the behavior in growth medium deficient vs. sufficient in nitrogen, they found that all the strains had an increase in lipid content in the deficient medium. Most notably, the lipid content of the *Desmodesmus* species increased 2-fold, from 20 to 40% by day 20. Another group evaluated the effect of phosphorus stress on the growth and lipid accumulation of a *Scenedesmus* species [43]. Having tested various phosphorus concentrations, they found that implementing 1 vs. 50 mg/L phosphorus resulted in a significant increase in the alga's lipid content, from 22.3 to 42.5%. Thus, limitations of some essential nutrients can be used to promote lipid accumulation in many microalgal strains. However, it is also relevant to note that these nutrient deficiencies can adversely affect microalgal cell growth. In the mentioned study for example, the phosphorus stress also corresponded to a decrease in biomass, such that the overall lipids produced were nearly the same [43]. For this reason, cultivation of microalgae in two stages is also researched as a strategy to improve lipid production [44]. The principle behind two-stage cultivation is, that initially nutrients can be optimally supplemented to support cell proliferation and then the culture can be exposed to a stress condition to trigger lipid production [45].

2.2.2. Harvesting

Once the cultivation stage is completed, the microalgal cells must be concentrated to enable efficient post-processing. Low cell density, the small size of cells, negative surface charge and culture density being close to that of water are critical factors that make harvesting of microalgal culture difficult and costly [46]. Sedimentation, centrifugation, filtration, flocculation and flotation are commonly studied methods for harvesting microalgae.

Sedimentation of microalgal cells is governed by gravitational force. The settling rate depends directly on particle size and the difference in density when compared to water [47]. The density of microalgae (998 – 1,230 kg/m³) being so close to that of water and their cell size being on the order of μm 's leads to very low settling velocities (0.05 – 0.2 m/d) [47]–[49]. Further, the settleability of microalgae can be highly strain-specific; due to their morphology some strains can be more resistant to settling [50]. Although, sedimentation is a cheap and simple option, its application can require long processing time and can be limited to only high-density algal strains.

Centrifugation is a commonly used procedure at lab-scale for harvesting of microalgae. It offers many advantages including quick processing, high biomass recovery and being generally effective independent of the strain [51]. The main hindrance for its large-scale use is the high cost of equipment and operation and the possibility of cell damage. Considerations that could make this process more cost-effective would be a biorefinery approach, where more components of the algal biomass are utilized (not just lipids) or pre-concentration of the algal culture so that throughput and energy expenditure of centrifugation could be minimized [47].

Filtration is an effective process that can be used to concentrate microalgal cells. In its simplest form – pressure can be applied to the algal culture to pass it through a membrane with a certain pore size, designed to capture the cells. Micro-filtration (0.1-10 μm) is generally appropriate to collect common species of microalgae [49], [52]. Some downsides are the operational cost and maintenance since membrane clogging may occur and periodic membrane replacement is required [51]. Various technologies can be combined to improve filtration operation and throughput. Examples include filter presses, rotary vacuum filters and belt filters, which have been shown effective with *Scenedesmus*, *Spirulina*, and *Micractinium* species [47], [53], [54].

Microalgal cells generally have negative surface charge that prevents them from aggregating and settling. Flocculation is a method that requires the addition of a flocculant, which can neutralize surface charge and allow cells to aggregate [51]. As cells form flocs they grow in size and have a higher tendency to settle. Flocculation can be implemented with various options such as, auto-flocculation, where the culture's pH is raised to destabilize surface charge, chemical flocculation with the addition of either organic or inorganic flocculants, or bio-flocculation [46]. Addition of organic flocculants involves the addition of a polymer, which becomes attached to the microalgal cell by way of electrostatic force. Chitosan is a popular example, whose efficacy has been demonstrated with *Chlorella*, *Chaetoceros*, *Ankistrodesmus* and *Scenedesmus* species [55]–[57]. Ferric and alum salts are commonly used as inorganic flocculants. They are highly effective and cost-efficient, however, the high metal concentration that results in the sediment can limit the post-processing options with the microalgal biomass [58]. Bio-flocculation can involve the use of plant-based compounds, such as guar gum or eggshell powder, which are low-cost, not toxic, environmentally friendly options shown to be effective with *Chlamydomonas* and *Chlorella* species [59]–[62]. Alternatively, co-culture of microalgae is studied with

bacteria or fungi that have filamentous structure or have a general tendency to aggregate with microalgal cells and promote biomass settling [63]–[66].

Flotation is a time- and space-efficient method for microalgal harvesting. Since the algal cells' density is close to that of water, “pushing” the cells to the surface of the water for collection can be effective. Various technologies exist such as, dissolved air, electrolytic or dispersed air flotation [47], [67]. Air bubble size is an important factor that can affect harvesting efficiency. Flocculants or surfactants may be needed to improve collection output [47]. The primary drawbacks of flotation are the high initial and operational costs and high energy expenditure, which limit the feasibility of its implementation at large-scale [47], [51], [52].

2.2.3. Lipid Extraction

Once the microalgal cells are collected they may or may not require subsequent drying, depending on their concentration and intended post-processing. Cells that are harvested with the intent of serving as a biodiesel feedstock must be extracted for lipids. The fats accumulated by microalgae are either stored intracellularly or within their membrane structures. This requires that the cells' structure is disrupted in order for the lipids to be thoroughly removed and separated.

Chemical extraction of biomass is widely studied, involving the use of organic solvents. A mixture of chloroform and methanol is commonly researched and dates back to the methods of Folch and Bligh and Dyer [68], [69]. Ideally, the solvent used should:

- have high volatility making it easy to separate from the lipids,
- be free of toxic impurities,
- be able to have a separation of phases with water conducive to removal of non-lipids,
- be ineffective at extracting undesired compounds, and
- should have a polarity and solubility parameter that make it appropriate for extraction of the desired lipid class [70].

The general procedure for organic solvent extraction involves some exposure or contact time between the biomass sample and solvent, during which other methods may be used to aid in extraction efficiency. Afterwards phase separation may be

implemented between solvent and water to separate lipids in the solvent, such as chloroform, and non-lipids in the water phase. Chloroform, methanol, ethanol, hexane and isopropanol are well studied for lipid extraction [70]. Efficiency of the method can depend greatly on the strain, its cell structure and lipid content. Generally, solvent extraction can be highly effective, however, costly and not environmentally friendly.

A variety of options exist that can make solvent extraction more practicable. Implementation of a mechanical or chemical pre-treatment step is well known to improve the effectiveness of solvent extraction [71]. Mechanical pre-treatment can be as simple as milling, bead beating or homogenization, which can disrupt cell structure and improve a solvent's access to cellular compounds. Alternatively, the use of ultrasound or microwave to improve extraction are also options (discussed below). Chemical pre-treatment options include acid or base hydrolysis, oxidation or the use of surfactants [71]. All steps combined, with their cost and environmental implications must be considered when comparing overall extraction efficiency of various methodologies.

Use of the Soxhlet apparatus during lipid extraction is an attractive option because it allows for more efficient use of the solvent [71]. The method involves continuous heating and condensation of the solvent, hence, re-applying it to the biomass sample being extracted (Figure 2.4.a [72]). Additional energy expenditure is an associated disadvantage. One group utilizing Soxhlet extraction driven by solar energy illustrated the lipid extraction of *Chlorella* biomass, saving ca. 11 times more energy compared to the conventional method (Figure 2.4.b [73]).

Mechanical extraction of microalgae is also possible with methods such as, oil expeller, ultrasonically-assisted or microwave-assisted extraction [74]. An oil expeller relies on applying pressure to the biomass to the extent that cells are disrupted and oil can be displaced. One study demonstrated that 75% of lipids could be extracted from filamentous algae using an expeller press [74], [75]. Ultrasonic-assisted extraction (UAE) relies on the ultrasonication of the liquid creating high- and low-pressure cycles resulting in shear forces breaking the cells. Mainly used to leave the cells more susceptible to being extracted of their fat content, ultrasonic pre-treatment of biomass can significantly increase extraction efficiency and reduce the required treatment time [74], [76]. Similarly, microwave-assisted extraction (MAE) can improve the penetration of cells and has the added advantage of being a non-contact method so that residual sample loss can be avoided [74]. MAE has also been shown to improve

extraction efficiency; one study increased oil recovery of *Scenedesmus obliquus* from 43-47% to 76-77% [74], [77]. Disadvantages of UAE and MAE are, high energy expenditure and, that overheating of the sample can result in denaturing of cellular components [71].

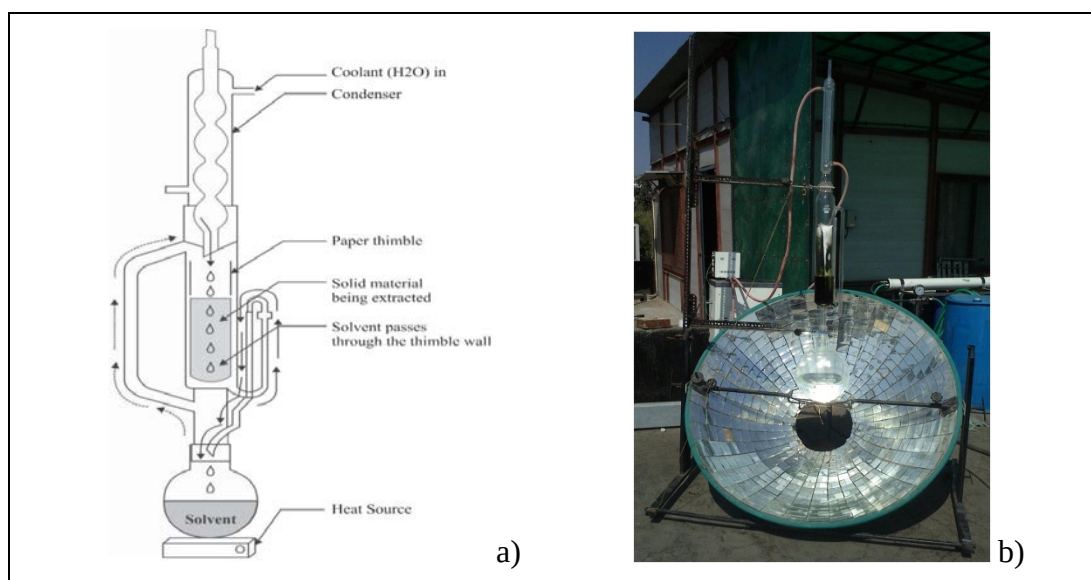


Figure 2.4: a) Diagram of the Soxhlet apparatus illustrating the flow and recycling of solvent and b) a photograph of a Soxhlet apparatus powered by solar energy.

Supercritical fluid extraction has gained popularity as a lipid extraction method for microalgae. The method involves using a compound at a temperature and pressure that are above its critical point, where the compound does not exist in a distinct phase, liquid or gas [71]. Exposing microalgal cells to this supercritical fluid allows for the transfer of lipids to this fluidic phase and later, condensation allows for extract recovery. Carbon dioxide is commonly studied for extraction of microalgae due to its low toxicity, recoverability and non-flammability, making it more environmentally friendly than traditional organic solvents [71], [78]. Carbon dioxide is also preferred for its selectivity for lipophilic compounds. Supercritical CO₂ (SC-CO₂) has been shown to be an effective lipid extraction method; one group studying *Scenedesmus* biomass demonstrated that SC-CO₂ parameters could be optimized to significantly alter lipid yield and that a scale-up of the operation was possible [71], [79]. Further, adding organic solvent while implementing SC-CO₂ has been shown to improve lipid selectivity and recovery [71], [80]. A more environmentally clean option, SC-CO₂, does have the downside of being costly to set up and operate.

Lipid extraction using bio-derived green solvents is another topic of interest. The bio-derived solvents in consideration are terpenes, which are hydrocarbon compounds that are primarily produced by plants, such as citrus plants and pine trees [71], [81]. Examples of solvents that are being studied due to their effectivity, cost, renewability, eco-friendliness, and non-toxicity are, gum turpentine, p-cymene and D-limonene [81]. One study of *C. vulgaris* biomass extraction using the Soxhlet method illustrated that the amount of crude oil that could be extracted with terpenes was higher when compared to n-hexane, possibly owing to the compounds' higher polarity [82]. Some disadvantages of using bio-derived solvents are that a consistent supply of the compound may be difficult to acquire and the lack of baseline data, requiring more experimentation to find optimal parameters for their use [81].

Alternatively, other strategies exist that allow for bypassing the traditional lipid extraction process step. OriginOil developed a technology, Quantum Fracturing™, utilizing pH modification and pulsed electromagnetic fields to disrupt microalgal cells. The intracellular lipids float upwards due to their lower density, while the remaining cell debris settles downward, allowing for simultaneous harvesting, dewatering and lipid extraction of microalgae (Figure 2.5, [83], [84]). The practical separation of phases is conducive to the reuse of water for algal cultivation. The processing appears energy-efficient and environmentally friendly due to a lack of solvent use.

Another concept that has been explored to improve process efficiency is “milking.” The idea behind it is that cells can be cultivated and stimulated to release their secondary metabolites, while they themselves remain intact and viable to keep producing – similar to the milking of a cow [85]. All microalgal cells may not be appropriate for such a method, depending on their cell structures and how their lipids are stored. Most commonly studied for milking is *Botryococcus braunii*, a freshwater microalga that is known to store lipids in an extracellular matrix, i.e., outside the cell [86]. This makes them a good candidate for being “extracted” of their lipids without damaging the cells – this in-situ milking can eliminate the need for harvesting and re-cultivation and has great cost-saving implications. There are many technologies and concepts worthy of consideration when determining an optimal, efficient flow for the downstream processing of microalgal lipids as a biodiesel feedstock.

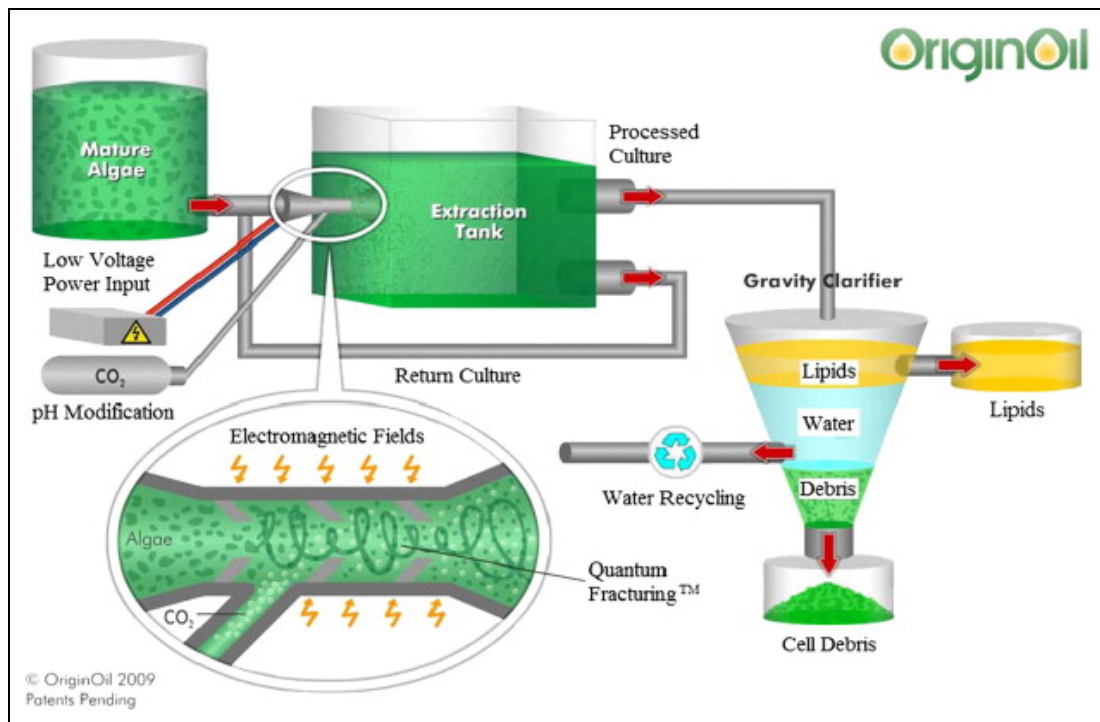


Figure 2.5: A schematic illustrating the technology utilized by OriginOil for Single-Step Extraction™ of microalgae.

2.2.4. Transesterification of Lipids

The final step of the process is the transesterification of microalgal lipids to produce biodiesel. The crude algal oil may need to be washed and refined to remove impurities. The transesterification proceeds with the additions of an alcohol and a catalyst; the triglycerides are esterified into fatty acid methyl esters (FAMES) and glycerol is also produced as a waste by-product (Figure 2.6.a, [87]). Transesterification also requires further downstream processing, including the removal of glycerol and the alcohol, neutralization, washing and drying before achieving a usable biodiesel product (Figure 2.6.b, [88]).

Direct transesterification is well studied for its potential to significantly improve process efficiency and reduce associated energy demands. The method involves directly trans-esterifying microalgal biomass so that lipid extraction and transesterification are combined in one step [88]. This concept has been tested with a variety of microalgal species, including *Chlorella*, *Nannochloropsis* and *Schizochytrium* [88]. A number of parameters must be studied to optimize the biodiesel yield. The alcohol chosen for the process and the molar ratio at which it is applied are particularly important since the alcohol will replace the solvent responsible

for extracting lipids *and* will trans-esterify the lipids. The catalyst must be chosen accordingly, to complement the alcohol. A combination of methanol and sulfuric acid as the catalyst are commonly studied [89]–[91]. The major disadvantage is that the transesterification process requires an excess of alcohol to be used. This could be balanced by ensuring that solvents are recovered and reused in the process. Alternatively, solvent demand could be reduced by incorporation of microwave or ultrasonic assistance [92]–[95]. Direct transesterification could potentially lead to major processing advantages for the scaleup of microalgal lipids-derived biodiesel [96].

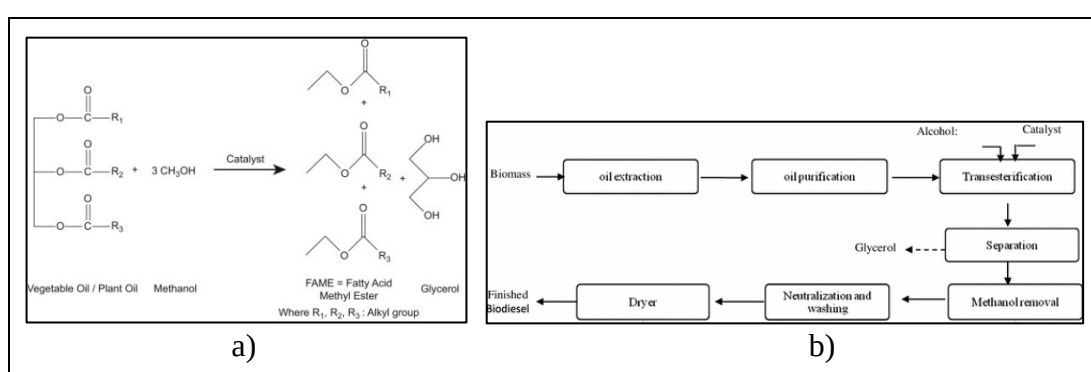


Figure 2.6: a) Transesterification of a triglyceride molecule and b) a schematic overview of producing biodiesel from microalgal lipids.

2.3. Autotrophic and Heterotrophic Cultivation

Traditionally microalgae are known and studied for their autotrophic metabolism. Their ability to harness the energy of light – photosynthesis – is what allows them to utilize carbon dioxide in the atmosphere. This ability makes microalgae an attractive microorganism as a biodiesel feedstock because they represent a more carbon-neutral and environmentally-sound option. This, along with microalgae’s ability to use nitrogen and phosphorus in municipal or agricultural wastewaters also makes them a good choice for tertiary treatment [97]–[99]. Besides the research ongoing at lab-scale, microalgae are currently cultivated using their photoautotrophic metabolism in various facilities worldwide. They are usually cultivated using open raceway ponds (Figure 2.7.a, [100]), tubular photobioreactors (Figure 2.7.b, [100]) or a combination of both [101].

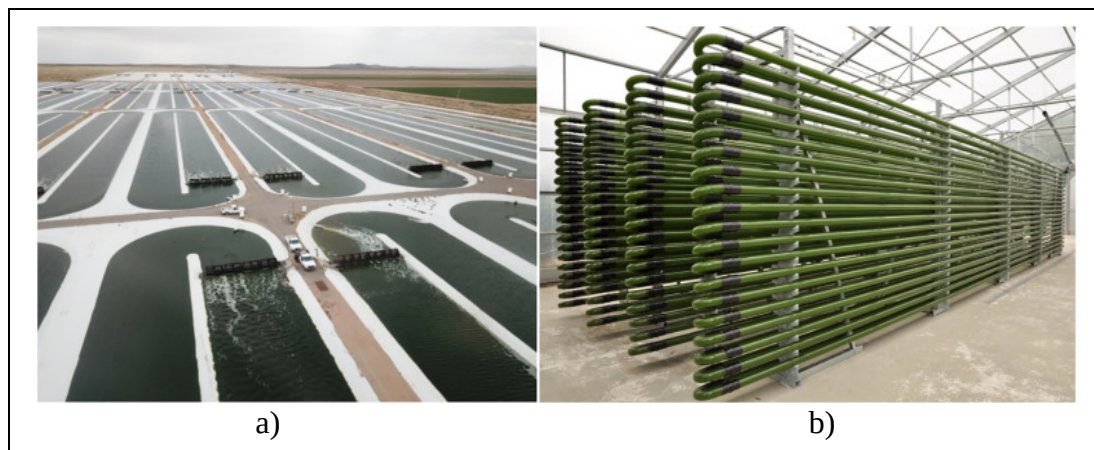


Figure 2.7: a) Large-scale microalgae cultivation in open raceway ponds and b) tubular photobioreactors.

The open raceway ponds (ORPs) and photobioreactors (PBRs) each have their advantages and disadvantages. ORPs are cheaper to install, easy to operate, have low energy demand and can have large throughput [101]. PBRs have high capital cost, require more energy to operate and are more difficult to clean and maintain [101]. ORPs have limited productivity per unit area because they must be shallow by design since light penetration becomes growth-limiting at greater depths, while PBRs can be designed vertically for more efficient use of space. Because ORPs are open to the atmosphere they are highly prone to contamination, and they are subject to local weather conditions. Therefore, ORPs have lower biomass and lipid outputs and nutrient removal compared to PBRs, where much greater process control can be implemented. One group compared the operational performances of a high-rate alga pond (HRAP) and an airlift tubular photobioreactor (TPBR) using the effluent of a municipal wastewater treatment plant. They found that the TPBR had significantly greater TN (89.7 vs. 65.1%) and TP (86.7 vs. 58.8%) removal and higher maximum areal productivity (21.8 vs. 8.3 g SS m⁻² d⁻¹) [102]. They did note however that the TPBR had severe biofouling, to the extent that the experiment had to be ended after 157 days of operation. Once again, both operating systems come with their benefits and drawbacks.

Heterotrophic metabolism of microalgae is also widely studied. In this case, microalgae utilize dissolved organic carbon sources in the water. Many microalgal species are able to degrade organic carbon, including glucose, fructose and acetate. To achieve strictly heterotrophic metabolism the cells would have to be cultivated under dark conditions to avoid them additionally utilizing CO₂. If cells are being cultured

under light while an organic carbon source is also provided, this would be referred to as mixotrophic cultivation.

Heterotrophic cultivation is generally known to promote faster growth and lipid accumulation of microalgae when compared to autotrophic condition. One group studying four strains of green microalgae demonstrated that they produced significantly higher biomass with heterotrophic vs. autotrophic metabolism [103]. Another study of *Chlorella minutissima* demonstrated that the strain could utilize industrial waste products, acetate and glycerol, as carbon sources [104]. Further, they found that the alga produced the greatest amount of biomass and highest lipid content under heterotrophic condition. A study of *Chlorella sorokiniana* illustrated that the strain achieved higher biomass (4.0 vs. 2.5 g/L) and higher lipid content (36 vs. 26%) in heterotrophic vs. phototrophic condition using BG-11 medium [105]. They noted a similar trend when culturing the strain in aquaculture wastewater.

Overall, there is much evidence to support the idea that microalgae can be cultivated heterotrophically to produce biomass and lipids faster and in greater amounts as compared to autotrophic cultivation. The primary reason being that the availability of CO₂ in the water limits autotrophic cell proliferation and in the cases where concentrated CO₂ is supplemented, growth is enhanced, however, still limited eventually by light penetration. Additionally, supplementation of concentrated CO₂ is an added expense for autotrophic cultivation [106]. In an ideal world, industrial flue gas rich in CO₂ can be treated, while also promoting autotrophic algal growth. However, progress is still needed in CO₂ sequestration technologies to make such scenarios practicable. The additional advantage of heterotrophic cultivation is its independence of a light source. This factor critically affects reactor design when it comes to autotrophic cultivation – either open ponds are used, which use space inefficiently and have lower lipid production rates, or photobioreactors are used, which have high investment costs and difficult maintenance. Alternatively, heterotrophic cultivation of microalgae can be scaled-up in compact bioreactors, such as fermenters. That being said, heterotrophic cultivation comes with the cost of an organic carbon source, which must be mitigated by using an appropriate waste stream. Research on the topic of combining heterotrophic growth of microalgae with waste remediation is ongoing (see 2.5).

2.4. Attached Cultivation

Traditionally, microbial cultures are grown in suspended form in liquid growth medium. Attached growth of microorganisms is an interesting alternative that offers many processing advantages. The concentrated biofilm formed by the cells can be easily harvested for post-processing depending on the attachment configuration used. Hence, the bottleneck and cost associated with harvesting and dewatering of the biomass can be significantly reduced. Attached growth systems are also documented to have higher areal productivity and nutrient removal values compared to suspended cultivation. One group demonstrated the performance of a bacterial-microalgal consortium in the treatment of poultry waste using photobioreactors tested under suspended and attached-growth conditions [107]. Higher nutrient removal performance was recorded for the attached-growth conditions compared to suspended, and high biomass productivity was achieved with a maximum lipid content of 19.6%. Another group developed a rotating algal biofilm reactor designed to promote attached growth of *C. vulgaris* [108]. They tested the strain's affinity for attachment on various materials, including microfiber, muslin cheesecloth and velvet and found that cotton sheet best supported attached growth. They showed that the attached biomass could be easily manually harvested and had water content similar to that of centrifuged biomass, indicating a simplification and cost-savings for post-processing. Further, they tested retrofitting an open raceway pond with the biofilm reactors and illustrated that higher areal biomass productivity was achievable compared to the control suspended system. *C. vulgaris* was tested in another study for its attached growth behavior. The authors designed a biofilm membrane photobioreactor with an array of flexible fiber bundles that were intended to support algal biomass attachment and could be harvested with an appropriate separator system (Figure 2.8, [109]). They were able to achieve 44% higher biomass production with the same reactor when attached growth was implemented and 72% of that biomass was grown in attached condition. Once again, these studies indicate that attached cultivation of microalgae is a promising option that could improve areal productivity and reduce processing costs.

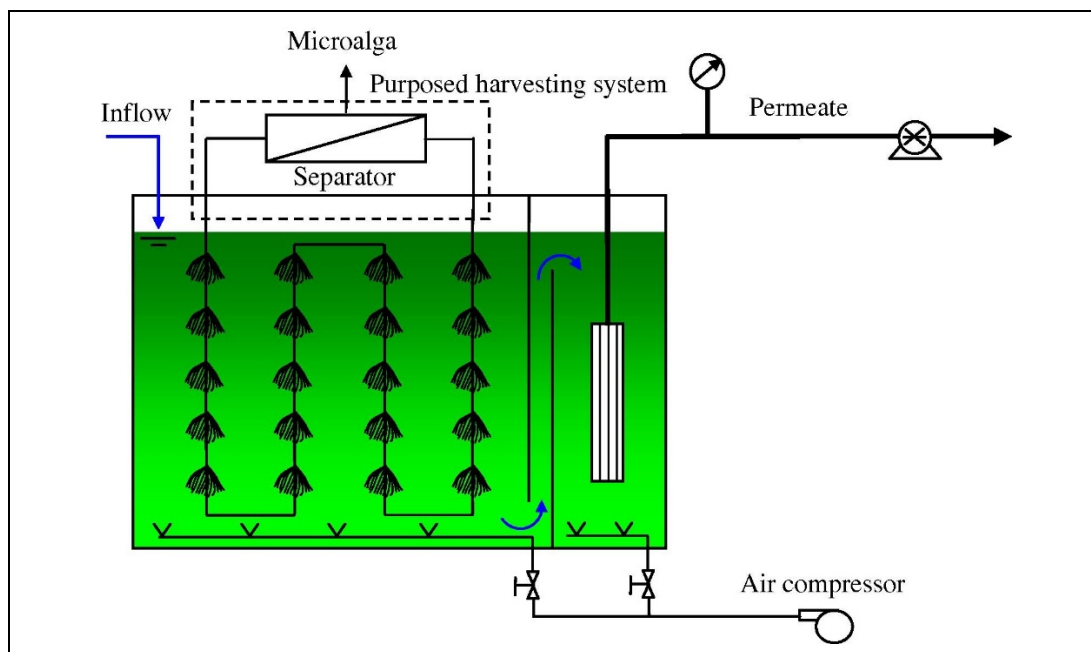


Figure 2.8: A diagram of the biofilm membrane photobioreactor.

Agar is a compound consisting of polysaccharide agarose and agaropectin. It is widely used in the laboratory for solidification of growth medium for cultivation of microorganisms. The solidified media, also known as agar media, can be used for long-term maintenance of axenic cultures with low risk of contamination. To our knowledge, the use of agar media for attached cultivation, harvesting and lipid production of microalgae was not previously studied.

2.5. Waste Remediation in Microalgal Cultivation

The prospect of combining wastewater treatment with simultaneous microalgal lipid production as a biodiesel feedstock continues to draw attention for its potential energy and environmental benefits [110], [111]. Piggery wastewater with its high organic content was demonstrated as a potential source of nutrients for the mixotrophic cultivation of *Chlorella pyrenoidosa* [112]. The solids in the wastewater were settled out by gravitational sedimentation and the supernatant was diluted to contain varying concentrations of chemical oxygen demand (COD). The highest concentration tested, 1000 mg/L COD, resulted in the highest proportions of nitrogen, phosphorus and COD removal, and the highest lipid productivity of $6.3 \text{ mg L}^{-1} \text{ d}^{-1}$, indicating that dual benefit can be achieved.

The use of dairy effluent (DE) to support the growth and lipid accumulation of a *Chlorococcum* species was investigated [113]. The DE was used without pretreatments or pH adjustments and was found adequate to support the alga's growth in heterotrophic and mixotrophic conditions. Since the DE sugar content was low (0.02 g/L), the authors also tested additionally supplementing with biodiesel industry waste glycerol (BDWG). Supplementing the DE with 6% BDWG resulted in achievement of the highest biomass (1.94 g/L), lipid content (42%) and lipid yield (0.8 g/L). Additionally, as a result of microalgal cultivation, the DE post-cultivation had a pH, BOD and COD within discharge limits and was also rid of its initially offensive odor. Simultaneous reduction of pollution load and production of microalgal lipids with a fatty acid profile appropriate as a biodiesel feedstock was demonstrated.

Domestic wastewater is also evaluated as a growth medium option for microalgae cultivation, providing essential macronutrients needed for growth, including nitrogen and phosphorus. One study illustrated that domestic wastewater could be used to cultivate a *Scenedesmus* species in a bubble column PBR [114]. They observed a maximum productivity for biomass and lipids of 196 and 65 mg L⁻¹ d⁻¹, respectively, with 2.5% CO₂ supplementation. This resulted in simultaneous removal of phosphate, nitrogen and COD from the wastewater at a proportion of 70-98%. The fatty acid content of the algal oil was suitable to use for biodiesel production. In another study, whey permeate (WP) was used to enrich growth medium for cultivating *Scenedesmus obliquus* [115]. The strain was selected specifically for its ability to use lactose, which was found to constitute more than 80% of the WP. Substituting the WP for 40% of the growth medium was found to be an optimal ratio to support the strain's growth and resulted in a maximum lipid productivity of 37.8 mg L⁻¹ d⁻¹, while the ammonium from the WP was found to be depleted. The study also demonstrated that microalgal cultivation costs could be reduced with the added benefit of wastewater treatment.

In addition to the use of various wastewaters, utilization of solid wastes to support microalgal cultivation is also widely studied [116]. One study showed that *Galdieria sulphuraria* could be cultivated heterotrophically with the enzymatic hydrolysates of restaurant and bakery wastes, which were rich in various sugars and peptides [117]. They also found that the strain's production of phycocyanin, a valuable pigment compound, could be maximized with the addition of ammonium. Another study evaluated the potential use of waste nitrogen fertilizers (WNFs) produced by a

fertilizer company to replace the nitrogen in the growth medium [118]. They tested three microalgal strains and found that the *Tetraselmis* species grew well using the WNFs. The authors suggested that while the *Tetraselmis* biomass could be used as an ingredient in animal feed, the added benefit would be alleviating the burden of landfilling the WNFs. One group studied the use of human, poultry and cow wastes (excreta) to replace the nutrients needed for cultivation of three microalgal strains [119]. The solid wastes were digested with sulfuric acid solution and diluted at ratios of 5-20%. They found that *C. sorokiniana* grew well in all variations of media and achieved maximum biomass and lipid production rates of 140 and 45.5 mg L⁻¹ d⁻¹, respectively, with 20% poultry waste media. There is a vast accumulation of research that establishes the use of wastewaters' and solid wastes' nutrient content to promote microalgal cultivation, with the combined benefit of reducing the processing costs in the production of microalgal biomass and lipids and the alleviation of waste treatment costs.

2.5.1. Carrot Pomace

Carrot pomace is an abundantly nutritious waste that is produced at great rates as a by-product of the juice industry. In addition to its high carbohydrate content, it contains minerals, metals and vitamins [120]. Carrot pomace is researched for a variety of potential purposes including, to produce dietary fiber powder[121], enriched baked goods [122]–[124], animal feed [125], [126], pectin [127], carotenoids [128], [129], bioethanol [130], [131] and biodiesel [132], [133]. Sharoba *et al.*, demonstrated that various fruit and vegetable wastes, including carrot pomace, could be used as raw material for producing fiber powder. The powder could be supplemented in place of flour for making cakes with higher fiber content intended for diabetic or obese people [121]. Similarly, another study showed that dried carrot pomace could be used to replace 12% of the wheat flour for making nutritious cookies higher in fiber content [123]. One group evaluated the potential for fruit and vegetable wastes to be used in ruminants' feed and found that carrot and apple pomaces were the highest in some parameters, including metabolizable energy and short-chain fatty acids, making them good candidates to reduce costs associated with producing animal feed [126]. Besides its dietary value, carrot pomace is also a good source of carotenoids; Umair *et al.*, illustrated that ultrasonic-assisted extraction of carrot pomace could be optimized to

maximize the yield of β -carotene, lutein and lycopene. These compounds are known for their function as colorants as well as their health-promoting value.

In addition to its applications in human and animal feed products, carrot pomace is also studied for its potential to be microbially converted to biofuels. One group demonstrated that dried and ground carrot pomace could be enzymatically hydrolyzed to replace the carbon content of the synthetic growth medium used to cultivate the yeast, *Kluyveromyces marxianus* [130]. They established that simultaneous saccharification and fermentation was possible with this thermotolerant strain and that the carrot pomace could be used to produce a maximum ethanol output of 37 g/L after 42 h of fermentation without requiring the addition of a synthetic carbon source. Another study showed that carrot pomace could be used to support the growth and lipid production of fungal strain, *Coniochaeta hoffmannii* [134]. Optimization of carrot pomace loading, pH, nitrogen source and time yielded biomass with 52% lipid content that could serve as a potential biodiesel feedstock. Finally, the use of carrot pomace as a carbon source was also studied for the cultivation of microalgal strain *C. vulgaris* [133]. With the addition of 0.5 g/L carrot pomace-derived sugar they were able to achieve 0.699 g/L biomass under mixotrophic condition. They were able to produce higher algal biomass with carrot pomace sugar compared to the use of glucose – a double benefit considering the savings in cultivation costs. The lipids could be transesterified to biodiesel with high efficiency. Considering that the USA alone produces 175,000 tons of carrot pomace waste annually [135], there is great potential to alleviate the organic carbon cost associated with heterotrophic cultivation of microalgae as a biodiesel feedstock.

2.6. *Chromochloris zofingiensis*

Chromochloris zofingiensis is a strain known for its high growth rates, easy culturing and potential to be scaled up in indoor and outdoor applications [136]. It is a unicellular, freshwater green microalga able to grow under autotrophic, mixotrophic and heterotrophic conditions. It can accumulate a large fraction of lipids suitable as a biodiesel feedstock and is also documented for its accumulation of carotenoids under stress-inducing cultivation conditions [137]. The culture generally appears in a shade of green when provided appropriate nutrients and shifts towards an orange shade as

nutrients become limiting, inducing stress and carotenoid production (Figure 2.9, [137]).

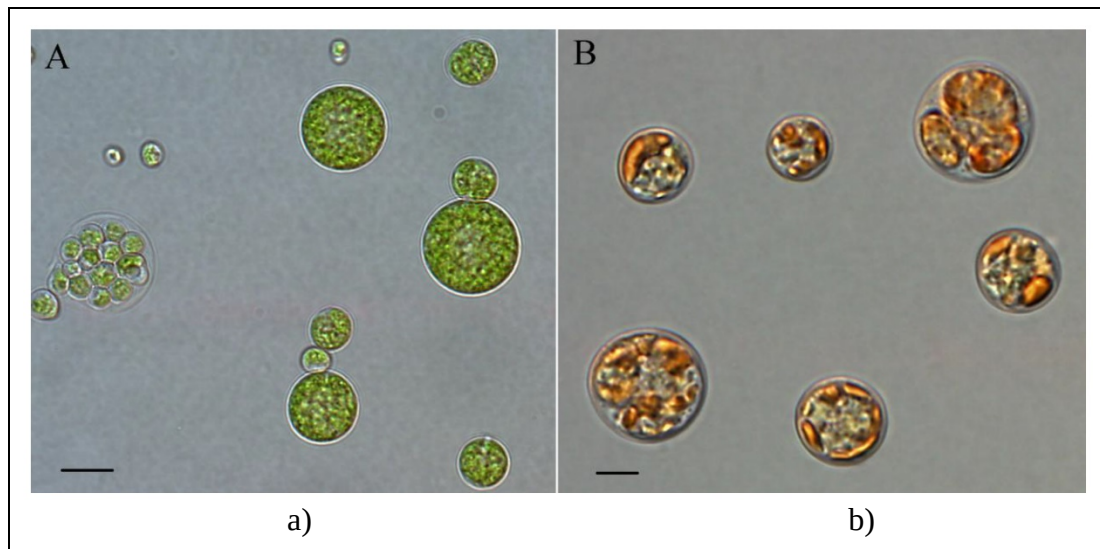


Figure 2.9: a) Microscope images of *C. zofingiensis* under favorable and b) stress conditions. Bars represent 5 µm.

C. zofingiensis has been studied for its high lipid accumulation capacity. A group investigating the biomass and lipid production of *C. zofingiensis* compared the strain's performance under autotrophic and heterotrophic conditions [138]. After testing in batch culture, they found that the heterotrophic condition yielded a growth rate and maximum biomass that was, respectively, more than 3- and 5-times greater than the results of the autotrophic condition. The heterotrophic condition they tested included the same basal medium plus 30 g/L initial glucose, which they found was 78% consumed by the end of batch cultivation. At the end of 14 days of cultivation they noted that the autotrophic and heterotrophic cultures had lipid contents of 25.8 and 51.1%, respectively, of which the proportion of neutral lipids was 29.4 and 80.9%, respectively. Not only did the cells grow faster under heterotrophic condition, but they grew to achieve much higher biomass with higher lipid content of more suitable content for a biodiesel application. Overall, the lipid output of heterotrophic cells was 10-fold the amount achieved by autotrophic condition (4.96 vs. 0.49 g/L).

The reuse of harvest water was investigated to support the cultivation of *C. zofingiensis* [139]. The term harvest water is used to denote the supernatant collected from centrifugation of a previous cycle of biomass growth. Nutrients were added to this water to constitute the growth medium. Various conditions were tested including

nitrogen and phosphorus deficiencies. The cells were found to grow well in photobioreactors using the recycled harvest water. The conditions of nitrogen and phosphorus deficiency yielded higher lipid contents compared to the nutrient-sufficient medium (41-46% vs. 26%), however due to the lower biomass produced these conditions did not yield the maximum amount of lipids. The authors concluded that the harvest water from microalgal cultivation could be reused without hindering lipid production, which would serve as a cost- and environmental-benefit in biodiesel processing.

Combining microalgal lipid production with wastewater treatment is an important topic of research, as it is directly associated with improving feasibility from a cultivation-cost perspective. Cultivation of *C. zofingiensis* has been shown to be successful using a number of wastewaters. One group demonstrated that piggery wastewater (PW) could be used to support growth and lipid production of *C. zofingiensis*, optimally so, when the wastewater was diluted to contain a COD of 1900 mg/L [140]. Naturally, the added benefit was the simultaneous treatment of the PW; after a 10-day cultivation period 80, 83 and 98%, respectively, of COD, TN and TP were removed. In another study the feasibility of using PW was further established by showing that the wastewater could be disinfected with NaClO in place of autoclaving and, consequently, the biomass and lipid production and nutrient removal performance of *C. zofingiensis* was not negatively impacted [141]. They were also able to cultivate the alga outdoors, indicating potential for a more scalable process.

Besides its high lipid production potential, *C. zofingiensis* has also been established as a producer of astaxanthin, a health-promoting carotenoid compound. There are many studies that document this strain's ability to accumulate lipids and astaxanthin in a positively correlated manner. One group tested the autotrophic cultivation of *C. zofingiensis* under nutrient-stress conditions. Nitrogen- and sulfur-starvation were both found to stimulate TAG and astaxanthin accumulation [142]. The highest astaxanthin content (3.9 mg/g) was found in cells cultivated under nitrogen-starvation. Another group implemented high light and salinity stresses to influence the astaxanthin and lipid production of *C. zofingiensis*. They demonstrated that while high light and salinity stress individually promoted the cells' accumulation, combining these stresses had a greater impact and resulted in the highest productivities [143]. Their transcriptional analysis revealed that the metabolic pathways leading to TAG and astaxanthin synthesis were coordinated. This positive correlation makes *C.*

zofingiensis a good candidate for simultaneous production of biodiesel feedstock and a high-value compound, which can positively influence the cost-benefit balance.

C. zofingiensis is widely studied under mixotrophic and heterotrophic cultivation conditions, where it is also able to produce astaxanthin. One study illustrated that under heterotrophic, batch culture *C. zofingiensis* was capable of producing 10.3 mg/L astaxanthin with an initial glucose concentration of 50 g/L [144]. Having tested the effect of varying the initial carbon:nitrogen (C/N) ratio of the growth medium, they found that a higher C/N promotes cellular astaxanthin production. Another group studied the use of different carbon sources, including glucose, mannose, fructose, sucrose, galactose and lactose [145]. Their results revealed that glucose and mannose were the best utilized by *C. zofingiensis* during heterotrophic cultivation, yielding the highest biomass (ca. 10 g/L) and astaxanthin content (1 mg/g). This finding indicated that, while the cultivation condition promoted cell proliferation, it enhanced astaxanthin accumulation as well – in contrast to the classic problem, where inducing secondary metabolite production results in a decrease in culture biomass. Further, testing of fed-batch cultivation while maintaining the glucose concentration between 5-20 g/L resulted in ultra-high cell density of ca. 53 g/L and an astaxanthin output of ca. 32 mg/L. The successful co-production of oil and astaxanthin with heterotrophic cultivation of *C. zofingiensis* has also been established [146]. The authors tested pretreated cane molasses as a source of carbon and noted that it yielded higher production rates compared to the use of glucose. The genes that were up-regulated during molasses metabolism were involved in the biosynthesis of fatty acids and astaxanthin. Similarly, another group evaluated the cultivation of *C. zofingiensis* mutant with diluted pretreated cane molasses as the growth medium [147]. Their fed-batch testing under heterotrophic condition revealed that the molasses-based medium resulted in an astaxanthin productivity nearly the same as the glucose-based medium (5.35 vs. 5.59 mg/L-day). Once again, the ability of *C. zofingiensis* to produce biomass, lipids and astaxanthin in a correlated manner under heterotrophic conditions, and its potential to use waste-derived carbon sources makes it a great candidate as a more economical and scalable biodiesel feedstock.

2.7. *Auxenochlorella protothecoides*

Auxenochlorella protothecoides is well studied and documented for its ability to grow under autotrophic, mixotrophic and heterotrophic cultivation conditions. *A. protothecoides* is a unicellular green microalga of the Chlorellaceae family. The cells are spherical in shape (Figure 2.10.a [148]) and can appear in shades of green or yellow depending on the culture conditions. Cells cultivated heterotrophically, in the absence of light, appear yellow in hue, while autotrophic cells appear green due to the change in their chlorophyll content (Figure 2.10.b [149]).

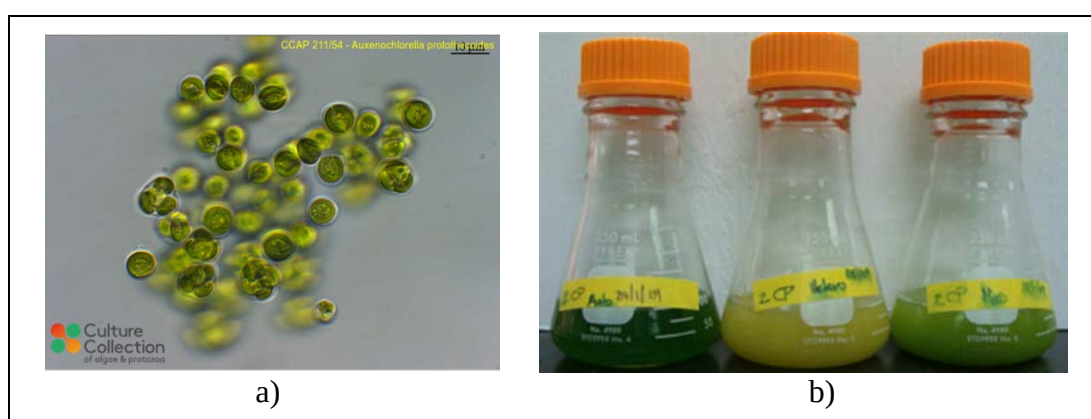


Figure 2.10: a) A microscope image of *A. protothecoides* and b) appearance of the algal culture when cultivated under various conditions: from left to right – autotrophic, heterotrophic and mixotrophic.

Besides its production of biomass and lipids and consideration as a biofuel product, *A. protothecoides* is also investigated for its lutein content. Microalgal biomass with high lutein content could have potential as a health supplement. Lutein is a pigment that can also be used in the coloration of animal products, cosmetics, drugs, and foods. One study of *A. protothecoides* showed that when cultured autotrophically and exposed to nutrient, salinity and light stresses the culture's hue shifted to orange-red due to its significant accumulation of carotenoids, including canthaxanthin, zeaxanthin, astaxanthin, echinenone and lutein [150]. Another group investigated using a shift in cultivation mode as a strategy to increase the lutein produced by *A. protothecoides* [151]. They showed that first using heterotrophic cultivation condition to increase biomass, followed by autotrophic condition with nitrogen supplementation could stimulate the cells' lutein accumulation, maximizing

the lutein output. They also noted the down-regulation of genes associated with lutein synthesis during heterotrophic condition, as compared to autotrophic cultivation.

A. protothecoides is well known for its potential to grow at fast rates and accumulate high biomass and lipids, making it a strong contender for biodiesel applications. One group investigated various nutritional and cultivation parameters to optimize the biomass and lipid production of *A. protothecoides*. They demonstrated that the culture's biomass and lipid output was not significantly different between heterotrophic and mixotrophic condition but were nearly 5 times the values achieved with autotrophic cultivation [149]. They also observed that the culture exhibited nearly the same growth behavior under heterotrophic and mixotrophic conditions, possibly indicating that the strain does not utilize CO₂ when organic carbon is present.

Another group evaluated scaling up the heterotrophic cultivation of *A. protothecoides*. They tested the culture's performance with 5-, 750- and 11,000-L capacity bioreactors [152]. The culture achieved 15.5 and 14.2 g/L biomass with 46.1 and 44.3% lipid content, respectively, using the 5- and 11,000-L reactors. Accordingly, they established that scale-up was possible with very little reduction (11%) in lipid production. They also showed that the biodiesel produced from the algal oil complied with U.S. biodiesel standards, demonstrating the feasibility and scalability of producing heterotrophically cultivated microalgal lipids for biofuel. Another study of *A. protothecoides* compared the alga's growth and lipid accumulation behavior in autotrophic versus heterotrophic cultivation. They found that there was a dramatic shift in the proportions of proteins and lipids [153]. The autotrophic and heterotrophic protein contents, respectively, were 53 and 10%, while their lipid contents were 15 and 55%. The authors also tested the use of corn powder hydrolysate as a source of carbon and found that it yielded higher biomass and lipids in batch culture compared to the use of glucose. The resulting microalgal oil was converted to biodiesel through acid transesterification and was found to comply with most ASTM biodiesel standards.

As is the case with many microalgal strains, *A. protothecoides* has also been shown capable of using the nutrients from various wastes. One group demonstrated that the hydrolysate of sweet sorghum juice could be used in place of glucose and in 5 days heterotrophic batch culture the cells could achieve significantly higher biomass (5.1 vs. 3.7 g/L) with similar lipid content, resulting in a maximum lipid production rate of 536 mg L⁻¹ d⁻¹ [154]. Another study illustrated that crude glycerol, which is a

waste by-product of the biodiesel industry, could be used to support heterotrophic growth of *A. protothecoides* [155]. The batch cultivation test revealed that the maximum achieved biomass values were 15.3, 19.2 and 23.5 g/L and lipids were 7.7, 9.8 and 14.6 g/L, respectively, with growth medium containing glucose, pure glycerol and crude glycerol. Not only was crude glycerol able to replace the organic carbon, it also significantly improved the lipid production of the culture by 90% compared to the use of glucose. Testing of fed-batch cultivation showed that biomass and lipid outputs could be further increased to 45.2 and 24.6 g/L, respectively, resulting in a maximum lipid production rate of 3 g L⁻¹ d⁻¹. Such studies indicate *A. protothecoides* as a robust strain able to grow and accumulate lipids at high rates under diverse circumstances. The additional consideration of the strain's ability to use waste-derived nutrients make it a cost-effective contender for large-scale microalgae-derived biodiesel production.

2.8. High-Value Products by Microalgae

Thus far the use of microalgae as a feedstock for biodiesel production has limited applicability due to its lacking economic feasibility. Accordingly, there is a growing interest in the potential of microalgae to produce high-value compounds. Microalgae biomass as a whole is high in nutrition and *Chlorella* and *Spirulina* species are often used for their carbohydrate, protein or lipid content as a dietary supplement [156], [157]. They are also known to produce a wide variety of pigment compounds, such as chlorophylls, astaxanthin, β -carotene, lutein, canthaxanthin, fucoxanthin, phycocyanin and phycobilins [158]. Microalgae also produce many bioactive compounds, such as, polyphenols, fatty acids and carotenoids, that are known for their anti-inflammatory, antioxidant and anticancer health benefits.

Polyunsaturated fatty acids (PUFAs) are one category of lipids produced by microalgae that are also known for their human health benefits [159]. *Porphyridium purpureum*, a red marine microalga, has been shown to produce arachidonic acid (AA) under stress-inducing nutrient conditions, such as nitrogen limitation [160]. This strain's production of AA could also be optimized by varying the concentrations of mineral salts in the growth medium [161]. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are ω -3 PUFAs that can be produced in concentrated amounts by *Schizochytrium*, *Aurantiochytrium*, *Thraustochytrium* and *Cryptocodinium* species [162]. Besides their cardiovascular benefits, these fatty

acids also serve against asthma, arthritis, and depression [162]. Not only are microalgae a good source of PUFAs themselves, they are also the primary producers responsible for the accumulation of these fatty acids in fish via the food chain.

Pigments are valuable compounds for which microalgae are an important source serving a natural alternative to synthetic compounds. A recent bibliometric review found phycocyanin, β -carotene, astaxanthin and chlorophylls as the pigments with the most relevance [163]. Microalgae producers worldwide process 19,000 tons of biomass annually generating ca. 5.7 billion USD in revenue [164]. Such a revenue stream could serve a major benefit when coupled with the production of biodiesel derived from microalgal lipids, with the potential to improve the cost-benefit balance.

Various microalgal species continue to be investigated for their potential to simultaneously produce lipids as a biodiesel feedstock and high-value metabolites. *Dunaliella* species are well studied for their production of β -carotene, a health-promoting pigment compound [165]. Studies also show that *Dunaliella* species are able to accumulate fatty acids that can be utilized for biodiesel. *Dunaliella tertiolecta* was observed to increase its neutral lipid content in a time-dependent manner in response to a growth medium lacking in nitrogen [166]. A study of *Dunaliella parva* demonstrated that with optimized growth medium through response surface methodology a maximum of 39.1% lipid content could be achieved [167]. Another group revealed that CO₂ enrichment enhanced lipid and carotenoid accumulation of *D. tertiolecta* [168]. They further showed that the CO₂ produced from beer fermentation could be integrated to improve the microalga's lipid and product output, making these species promising candidates for co-production of lipids and carotenoids with added environmental benefits [165]–[168].

2.8.1. Astaxanthin

A powerful antioxidant xanthophyll compound, astaxanthin, is known for its red color and health benefits with protective and therapeutic effects against many diseases [169], [170]. Microalgae-derived astaxanthin has been shown to be safe for human consumption as a nutritional supplement, and a study comparing its antioxidant activity to that of synthetically derived astaxanthin showed it to be 20 times more effective in the elimination of free radicals [171]. Astaxanthin is also well-known and used for pigmentation purposes in the aquaculture industry [172]. It is a lipid-soluble

metabolite with a molecular structure resembling that of β -carotene (Figure 2.11, [173]).

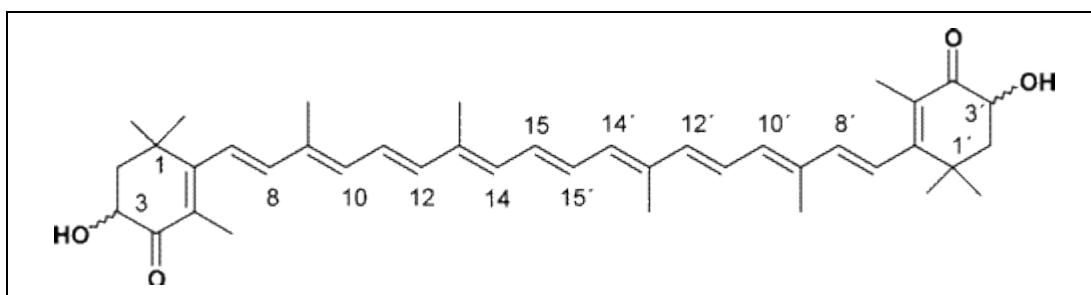


Figure 2.11: Molecular structure of astaxanthin.

Production of the secondary carotenoid, astaxanthin, has been widely studied with microalgal strains, *Haematococcus pluvialis* and *C. zoofingiensis*. While the former is capable of producing astaxanthin at high concentrations, its large-scale use is hindered by its slow growth rate and requirement for a lengthy cultivation period [174], [175]. *C. zoofingiensis*, on the other hand, despite its lower astaxanthin content, is able to produce astaxanthin at similar rates due to its higher growth rate and cell densities. Additionally, this species has been shown to produce astaxanthin under photoautotrophic, heterotrophic and mixotrophic conditions allowing greater flexibility to optimize its cultivation parameters.

Microalgae are generally known to produce astaxanthin under stress conditions, such as nutrient deficiency, nonoptimal lighting, exposure to reactive oxygen species (ROS), metal ions or salt. One study tested low light, high light, nitrogen deficiency and nitrogen deficiency plus high light conditions to compare their promotion of astaxanthin production in *C. zoofingiensis* [176]. In semi-continuous culture they were able to achieve astaxanthin and TAG productivities of 3.3 and 297 mg·L⁻¹d⁻¹ under photoautotrophic conditions. Another study cultivating *C. zoofingiensis* photoautotrophically demonstrated that the astaxanthin content per cell decreased with increasing nitrate concentration in the range of 5-40 mM, while it increased with increasing NaCl concentration up to 200 mM [174].

Another stress condition that has been explored is the addition of ROS to the growth medium. One such study attempted to improve the astaxanthin yield by introducing hydrogen peroxide or sodium hypochlorite to the cultivation medium of *C. zoofingiensis* under heterotrophic conditions [177]. Up to a certain concentration,

both compounds stimulated a decrease in cell dry weight coupled with an increase in astaxanthin content. Although the biomass concentration decreased, the overall result was a greater yield of astaxanthin with the addition of hydrogen peroxide; the yield was increased from 9.9 to 12.6 mg/L with exposure to 0.1 mM H₂O₂. Similarly, a study cultivating a *Chlorococcum* sp. under heterotrophic and mixotrophic conditions found the astaxanthin content per cell dry weight to increase from 1.03 to 1.78 mg/g and 5.46 to 7.09 mg/g, respectively, with the addition of 0.1 mM H₂O₂ [178].

Different cultivation strategies have been studied in an effort to attain higher astaxanthin productivities. Generally, astaxanthin production is associated with stress conditions, which are inversely correlated with biomass growth – so that, while an increase in astaxanthin content is stimulated a concurrent decrease in biomass productivity is found. Therefore, two-stage cultivation is often utilized to first increase biomass concentration in a growth phase and then induce astaxanthin accumulation in a stress phase that is suboptimal for cell growth. One such study implemented 14-day heterotrophic cultivation of *C. zofingiensis* in a fermenter, followed by a 4-day phase in an outdoor photobioreactor to improve astaxanthin output [179]. Medium with glucose was provided as a carbon source during the heterotrophic phase in fed-batch mode. By the end of the 14-day phase a biomass concentration of 98.4 g/L and astaxanthin yield of 73.3 mg/L was achieved, resulting in an astaxanthin productivity of 5.24 mg·L⁻¹·d⁻¹. The 4-day outdoor phase led to an increase of the astaxanthin content by 1.5-fold with a similar astaxanthin productivity of 5.26 mg·L⁻¹·d⁻¹. The results here indicate that although the astaxanthin content was increased, a nearly equivalent productivity resulted after the photoinduction phase, demonstrating that heterotrophic cultivation alone may be sufficient to produce astaxanthin at a high rate.

In contrast, there are also attempts to cultivate microalgae efficiently in the production of astaxanthin in a single-stage process to avoid the added costs of two-stage cultivation. One such study of *C. zofingiensis* in heterotrophic, batch culture with initial glucose and nitrate concentrations of 20 g/L and 0.75 g/L, respectively, demonstrated that after 2 days the nitrate in the medium was consumed and after this point the astaxanthin content of the cells began to increase [180]. This increase was concurrent with a continuing increase in the biomass concentration since the glucose was not yet depleted, indicating that astaxanthin could be accumulated while the cells are still growing. A related study cultivating *C. zofingiensis* under heterotrophic conditions tested lactose, sucrose, glucose, fructose, galactose and mannose as carbon

sources to determine their effect on the biomass and astaxanthin yields of the culture [145]. Glucose and mannose supported the highest biomass and astaxanthin yields. Furthermore, fed-batch cultivation with replenishment of glucose yielded a concurrent increase in biomass and astaxanthin outputs, the highest values achieved being 53 g/L and 32 mg/L, respectively; based on 16 days of cultivation time this corresponds to an astaxanthin productivity of 2 mg·L⁻¹·d⁻¹. These studies all support the idea that *C. zoefingiensis* can be cultivated heterotrophically or mixotrophically in a single-stage process to produce astaxanthin efficiently with a high C/N ratio provided by a simple sugar as the carbon source, such as mannose or glucose.

3. SUSPENDED AND ATTACHED GROWTH OF *CHROMOCHLORIS ZOFINGIENSIS*

3.1. Materials and Methods

3.1.1. Microalgal Strain and Growth Conditions

An axenic strain of *C. zofingiensis* (SAG 211-14) was acquired from the Culture Collection of Algae at Göttingen University (SAG, Göttingen, Germany). The stock culture was maintained on 1.5% agar media, incubated at room temperature (RT) in a well-ventilated, dark space and replicated every 3-6 weeks. Modified Bristol's medium, referenced as CZ-M1 [144](Ip & Chen, 2005), was used throughout all the experiments and prepared to include the following ingredients per liter: 0.75 g NaNO₃, 0.175 g KH₂PO₄, 0.075 g K₂HPO₄, 0.037 g MgSO₄, 0.025 g CaCl₂·2H₂O, 0.025 g NaCl, 0.005 g FeCl₃ and 1 mL of the trace metal solution (TMS). The TMS contained the following per liter: 0.287 g ZnSO₄·7H₂O, 0.169 g MnSO₄·H₂O, 0.061 g H₃BO₃, 0.0025 g CuSO₄·5H₂O and 0.00124 g (NH₄)₆Mo₇O₂₄·7H₂O. The iron and trace metal solutions were added after the medium was autoclaved and cooled to avoid any precipitation reactions. The stock and starter cultures were cultivated using media containing 10 g/L glucose, while the media prepared for the experiments had varying glucose content, as later specified. Media pH was 6.5 pre-autoclaving, unless noted otherwise. All chemicals used were of the highest purity available.

Starter culture was cultivated in two phases. First, 25 mL of medium in a 100 mL flask was inoculated from stock culture and incubated at RT with a 12h:12h light:dark cycle under 150 $\mu\text{mol photons/m}^2\cdot\text{s}$ with 300 rpm magnetic stirring. After 5-7 days 20 mL of the starter culture was added to 200 mL of medium in a 500 mL bottle, which was incubated in darkness in a shaking incubator set to 28°C and 150 rpm. After 5 days this seed culture was used to inoculate the media used in the experiment. The purpose of the two phases was to increase the starter culture volume sufficiently to inoculate all media used in the experiment while acclimating the cells for growth under suspended, heterotrophic conditions. All conditions were tested, at a minimum, with biological duplicates.

3.1.2. Effect of Initial Glucose on Suspended Cultivation

Cultures were cultivated in 500 mL bottles with autoclavable sponge plugs to allow for air transfer while minimizing contamination risks. Media, CZ-M1, was prepared to contain 10, 20, 30 or 40 g/L glucose, and were autoclaved directly in the culture bottles. Media pH was set to 7.5 prior to autoclaving. An inoculation ratio of 10% (v/v) was applied; i.e., 10 mL of starter culture was added to 190 mL of sterile media making a total working volume of 200 mL. The cultures were incubated in a dark shaking incubator set to 150 rpm and 28°C for a total of 30 days. Samples were taken throughout the experiment to determine optical density (OD), biomass, lipid and astaxanthin contents.

Optical density was determined on a spectrophotometer at a wavelength of 680 nm. Culture samples were diluted as necessary to ensure that the absorbance values were in the range of 0.1-1. Absorbance and biomass values were later compared to ascertain if a reliable biomass-OD correlation could be established.

3.1.3. Optimization of Culture Conditions and Comparison of Suspended vs. Agar-Attached Cultivation

This experiment was designed to optimize the parameters associated with agar-attached cultivation and to compare the biomass and product outputs of suspended vs. agar-attached growth. The parameters tested for agar-attached growth include the initial glucose concentration, cultivation time and inoculation ratio since it was theorized that these factors could impact the biomass, lipids and astaxanthin produced by the cultures. The initial glucose concentration of the liquid and agar media was 10 or 40 g/L. The cultivation times tested were 15 and 30 days – i.e., samples were taken from suspended (liquid) cultures and in the case of agar-attached cultivation, the biomass on petri dishes were fully harvested. The inoculation ratio (v/v) was implemented as 10% for the suspended cultures and varied, as 4, 10 or 20%, for the agar-attached. All cultures were incubated in darkness at 28°C; the suspended were in a shaking incubator set to 150 rpm, while the agar-attached were kept stationary. Biomass samples were freeze-dried and later extracted for lipids and astaxanthin. Each condition was tested with 2-3 culture replicates.

3.1.4. Process Improvements for Agar-Attached Cultivation

In order to determine if further improvement of product output or process efficiency was possible, a 60-day cultivation experiment of agar-attached cultivation was conducted with initial glucose concentrations of 10, 20, 30 and 40 g/L. Agar media was inoculated with 4% (v/v) liquid seed culture. The cultures were incubated in a dark cupboard at RT (20-22°C) and harvested after 30 and 60 days, to ascertain if a lower temperature combined with a longer cultivation time could yield equivalent or higher product outputs. Additionally, the harvested samples were stored at -32°C in either wet or dry form to establish if drying could be eliminated as a process step. The biomass samples denoted as “dry” were freeze-dried to constant weight, while the “wet” were stored as is (without drying) until lipids and astaxanthin extraction. Each condition was tested with 2-3 replicates.

3.1.5. Reuse of Agar

The potential to reuse the chemical agar was tested. Three consecutive culture batches were grown in agar-attached condition. Liquid starter culture was used to seed all agar media at an inoculation ratio of 4% (v/v). The media used contained 10 g/L glucose. Each batch contained five replicates, was incubated in a dark cupboard at RT (22-28°C) and harvested after 15 days. A cultivation term of 15 days was implemented based on data indicating that agar-attached cultures with 10 g/L initial glucose did not continue to grow after 15 days. Hence, the expectation was that the essential nutrients in the agar media would already be expended by day 15 and only the agar content would be reutilized.

On the day of harvesting the first set of cultures, the solid (spent) media that remained in the petri dishes were rinsed off with a few mL of deionized water to ensure that any residual biomass was removed. These media fragments were transferred to a bottle, to which glucose and 100x concentrated mineral stock solutions were added, per the CZ-M1 recipe. The mixture was autoclaved and poured into new petri dishes. Care was taken to ensure consistency with reuse of the agar. For example, if the spent media of 5 petri dishes was being reused, then glucose and mineral solutions were added appropriate for 125 mL of media (5 × 25 mL) and the autoclaved media was poured evenly into 5 new petri dishes. After the second set of cultures was harvested,

the spent media was again reused to make the next batch of agar media, which was used to grow the third culture set. Thus, the first, second and third sets of cultures were cultivated on agar media, where the chemical agar was being used for the first, second and third time, respectively. All harvested samples were freeze-dried to determine the dry biomass output of the culture sets.

3.1.6. Agar-to-Agar Inoculation

The possibility of inoculating agar media directly with culture harvested from agar media – agar-to-agar inoculation – was investigated to determine if the preparation of a liquid starter culture could be avoided. Four consecutive batches of agar-attached cultures were each cultivated on media containing 40 g/L initial glucose for 30 days in a dark cupboard at RT (22-28°C). The culture batches are referred to as I-IV in the order of their cultivation term. Culture set I was started per the previous procedures – 4% (v/v) inoculation was implemented with liquid seed culture. On day 15 of cultivation, one agar culture was fully harvested aseptically and suspended in 5 mL of sterile saline solution. This mixture was used as the seed culture for culture set II; 200 µL was transferred to the surface of each agar medium (petri dish) and spread-plated. The amount of seed culture used for each petri dish, 200 µL, was intended to produce an inoculation ratio of 4%. Since 200 µL is 4% of 5 mL, this would indicate that in a continuous production scenario, 25 new petri dishes could be inoculated with the biomass harvest of one culture. Similarly, culture sets III and IV were inoculated with the seed mixture prepared from a 15-day harvest of the preceding culture set. A period of 15 days was chosen to harvest the inoculum-culture based on previous data that showed that agar-attached cultures with 40 g/L initial glucose and 4% inoculum were still in active growth phase after 15 days of cultivation. Culture sets I-III were cultivated on fresh (unused) agar medium, while set IV was grown on reused agar media, as described in 3.1.5. The purpose of this experiment was to establish if consistent product outputs could be achieved without the use of a liquid starter culture and to ascertain the added effect of agar reuse. All harvested samples were stored at -32°C in wet form and later extracted for lipid and astaxanthin analyses. Each batch contained 5-6 replicates.

3.1.7. Biomass Determination

The biomass content of suspended (liquid) cultures was determined with a filter test. Culture samples were filtered through pre-dried (105°C) 1.2 µm glass microfiber filter papers and rinsed with de-ionized water. The filters were then dried at 105°C until constant weight and cooled in a desiccator before weighing. Dry cell weight (biomass content) of the cultures was determined by the final weight of the filters.

In the case of agar-attached cultivation, each petri dish was treated as one culture unit and was fully harvested in order to estimate the biomass achieved with each culture. For example, if 100 mg dry biomass was acquired from a culture, then its biomass output was estimated as 4 g/L ($100 \text{ mg} \div 25 \text{ mL}$).

3.1.8. Inoculation and Harvesting of Agar-Attached Cultures

Liquid starter culture was used to inoculate agar media, unless noted otherwise (see 3.1.6). Starter culture was initially concentrated via centrifuge. That is, a specified volume of starter culture was transferred to a sterile test tube using aseptic technique, centrifuged at 6000 rpm and the majority of the supernatant was discarded. The remaining supernatant, ca. 200 µL, was used to resuspend the sedimented cell pellet carefully with a micropipette. This mixture was transferred to the surface of agar media (petri dish) and spread-plated evenly with the round bottom of a sterile test tube. The volume of seed culture used to inoculate each petri dish was determined based on the desired inoculation ratio. The volume of growth medium in each petri dish was estimated to be approximately 25 mL. Therefore, either 1, 2.5 or 5 mL of seed culture was used to yield an inoculation ratio of 4, 10 or 20% (v/v), respectively.

Harvesting of the agar-attached cells was conducted manually by scraping up the biomass with a metal instrument (Figure 3.1) and transferring it to a pre-weighed test tube. Biomass transfer was done as thoroughly as possible, although there was always some residual cell loss in the process. This loss could either be avoided in the future with customized tooling or it could be determined as negligible with a scaleup of the process. Harvested biomass samples were freeze-dried until constant weight to determine dry biomass values, except when wet extraction was indicated.

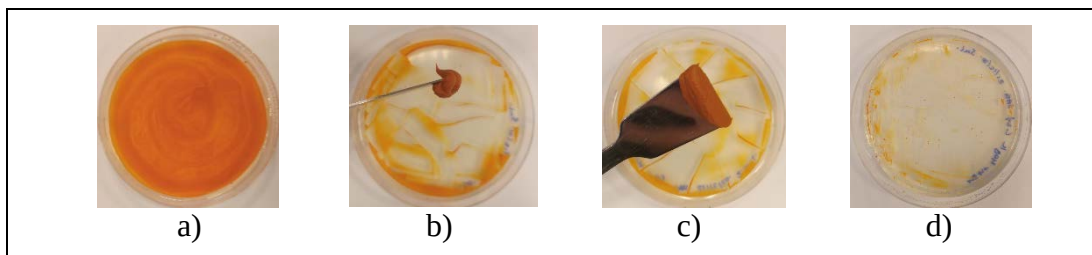


Figure 3.1: Photographs of agar-attached *C. zofingiensis* cultures during manual harvesting; a) agar-attached culture prior to harvesting, b-c) examples of attached cultures as they are being manually harvested and d) spent media after thorough harvesting of attached cells.

3.1.9. Lipid Extraction

Liquid culture samples were collected in test tubes, which were centrifuged at 9000 rpm for 5 minutes. The supernatants were decanted and the cell pellets were rinsed with deionized water and centrifuged again. The sedimented cells (biomass) were lyophilized with a freeze-dryer (Christ Alpha 1-2 LDplus, Germany) until constant weight. The samples harvested from agar media were either lyophilized for dry extraction procedures or maintained in wet form (-32°C) for wet extraction testing.

Samples were extracted for lipids with an established protocol (see 4.1.8). Briefly, samples were transferred to glass test tubes, weighed, and extracted with chloroform:methanol (1:1). Initially, samples were wet-ground with solvent and then magnetically stirred at 300 rpm in a water bath set to 50°C for 5 h. It should be noted that only dry samples were ground, whereas wet biomass samples were directly extracted with solvent (i.e., $50^{\circ}\text{C}/5\text{h}$). Phase separation was facilitated with the addition of 0.73% aqueous NaCl solution and centrifugation of the mixture at 3000 rpm. The chloroform phases containing the extracted lipids were further clarified, transferred to beakers, where the chloroform was volatilized and the remaining lipids were dried and weighed. All solvents used were analysis-grade.

Final lipid weights were used to determine the lipid content, as a percentage of dry cell weight (DCW) and the lipid output of the cultures, according to the equations below:

$$\text{Lipid content (\%DCW)} = 100 \times \left[\frac{\text{Lipids (mg)}}{\text{Extracted biomass (mg)}} \right] \quad (3.1)$$

$$\text{Lipid output (g/L)} = \text{Biomass (g/L)} \times \text{Lipid content (\%DCW)} \quad (3.2)$$

3.1.10. Pigment Extraction Procedure Optimization

Heterotrophically grown *C. zofingiensis* suspended culture was divided into 5 mL aliquots and freeze-dried. These samples were used to test variations in the pigment extraction procedure to establish the parameters that would yield maximum astaxanthin recovery. Samples were extracted with the lipid extraction procedure, as previously described (3.1.9), with the exception of the parameters that were varied. Acetone, dichloromethane:methanol (1:3) and chloroform:methanol (1:1) were tested as the extraction solvents. The temperature and time applied for the water bath were varied as RT/40°C/50°C and 1/2/3/4/5 h, respectively. A higher extraction temperature was not tested, as it is known that astaxanthin is more susceptible to degradation at higher temperatures [181]. All conditions were tested with duplicate samples.

3.1.11. Pigment Extraction and Analysis

Based on the optimization results (3.2.1), the pigment extraction procedure was implemented with chloroform:methanol (1:1) as the solvent, and 50°C/4 h as the water bath conditions. The procedure was conducted as described for lipid extraction, except for the extraction time of 4 h and the use of aluminum foil around the water bath to shield the samples from exposure to light in order to avoid degradation of astaxanthin. Since astaxanthin is a lipid-soluble pigment compound primarily present in the form of fatty acid esters [182], [183], it is contained in the lipids extracted as a result of this procedure.

The dried lipids (pigments) were resuspended with 3 mL of acetone and filtered with a 0.45 µm PVDF filter. Two mL of the filtered extract was transferred to a sealed container for saponification, to which 0.4 mL of saponification fluid, 0.021 M NaOH dissolved in methanol, was added for a 5:1 ratio [184]. Care was taken to ensure an inert environment; nitrogen (g) flushing was applied to the empty container, to the added extract and after the addition of saponification fluid [185]. Flushing of the mixture with nitrogen (g) was also intended to bring the final volume from 2.4 back down to 2 mL with the volatilization of solvent. The sealed containers were kept in a

dark, 4°C chamber for 15 hours, after which they were either analyzed directly or transferred to -18°C for later analysis.

Saponified extracts were analyzed on HPLC (Prominence, Shimadzu, Kyoto, Japan) using a SVEA[®] 5µm C18 Gold 4.6 x 250 mm column (Nanologica, Södertälje, Sweden) and a diode array detector according to an established protocol [185]. Measurements were collected at a wavelength of 480 nm. A column temperature of 30°C, isocratic flow with a mobile phase containing methanol:dichloromethane:acetonitrile:water (85:5:5.5:4.5) and a flowrate of 1 mL/min were applied. It should be noted that since the astaxanthin esters were hydrolyzed (i.e., the extracts were saponified) the intent was to only measure trans-astaxanthin; therefore, a single mobile phase was used. Pure astaxanthin standard ($\geq 97\%$, Sigma) was used to establish a standard curve for the evaluation of HPLC measurements. A stock solution was prepared by dissolving 5.5 mg of astaxanthin in 35 mL of acetone. A series of dilutions was implemented with acetone to yield a standard curve in the range of 0.3-15.7 mg/L ($R^2 = 1.000$), which was referenced for all astaxanthin measurements. Astaxanthin contents (mg/g) were determined based on the volume of solvent used to dissolve the extracts (3 mL) and the mass of the samples that were extracted. The saponified samples were measured volumetrically immediately prior to analysis and if their volume differed from 2 mL, then a dilution or concentration factor was used to take that difference into account.

3.1.12. Microscopy

Cells harvested from agar-attached cultures were suspended aseptically with deionized water. Cell suspensions were viewed on a light microscope (Axio Scope.A1, Zeiss, Germany) with 100-fold magnification. The field of view was determined based on the total magnification (1000x).

3.1.13. Statistical Analysis

Statistical analyses were conducted using a student's t test with unequal variance. A p value threshold of 0.05 was used to determine significance.

3.2. Results

The experiments conducted with *C. zofingiensis* were intended on ascertaining the strain's biomass, lipid and astaxanthin production behavior under heterotrophic (dark) condition. Preliminary experiments revealed that the astaxanthin recovered from biomass samples could vary depending on the pigment extraction procedure used. Therefore, variations of the pigment extraction procedure were initially tested to determine the optimal extraction protocol to maximize the astaxanthin recovered from the samples. Accordingly, the astaxanthin data in the subsequent studies was acquired using this optimized protocol. The effect of initial glucose concentration was investigated first in suspended cultivation. Then, the effect of cultivation conditions (initial glucose, time, inoculation ratio) were tested in agar-attached condition and the strain's performance was compared with suspended cultivation. Finally, further process improvements (initial glucose, wet extraction, temperature) were investigated to enhance process efficiency.

3.2.1. Pigment Extraction Procedure Optimization

The effects of solvent, temperature and time on pigment extraction efficiency were investigated. The solvent used appeared to have the greatest impact on the astaxanthin that could be recovered from the biomass samples (Table 3.1). Although acetone and dichloromethane:methanol (1:3) are commonly used in pigment extraction protocols of *Chlorella zofingiensis* samples [144], [145], [174], [186], here we found chloroform:methanol (1:1) to be the most effective, with significance (p value < 0.05). Accordingly, chloroform:methanol (1:1) was chosen as the pigment extraction solvent. Variations of temperature and time did not show great differences in the astaxanthin recovered from the samples. Based on the maximum astaxanthin content measured, the extraction temperature of 50°C and time of 4 hours were selected.

Table 3.1. Astaxanthin content achieved with a *C. zoofingensis* sample extracted using various solvent, temperature and time parameters.

Solvent	Temperature (°C)	Time (h)	Astaxanthin Content (mg/g)
Acetone			0.37
Dichloromethane:methanol (1:3)	50	3	0.31
Chloroform:methanol (1:1)			0.45
	RT		0.40
Chloroform:methanol (1:1)	40	3	0.40
	50		0.42
		1	0.37
		2	0.38
Chloroform:methanol (1:1)	50	3	0.37
		4	0.41
		5	0.40

3.2.2. Effect of Initial Glucose on Suspended Cultivation

All of the biomass and corresponding optical density (OD) data acquired throughout the study were plotted to determine if a reliable correlation could be established (Figure 3.2). The biomass data was acquired with a filter test, where a 1.2 µm filter was used to collect each culture sample with slight vacuum applied using filtering equipment. The filters were later oven dried to determine final weights. Since this sampling procedure is costly and time-consuming, the ability to use absorbance (OD) measurements to estimate culture biomass would be quick and economical. However, it is known that some microalgal cultures' biomass cannot be accurately estimated with OD due to the shift in pigmentation that occurs during the cultivation cycle, which affects the corresponding absorbance values. It is evident that a few outliers exist in the dataset (Figure 3.2.a). These can be eliminated based on the phase of the cultivation cycle. That is, if only the data collected during the exponential growth phase are plotted (Figure 3.2.b) a linear correlation can be established. The equation determined for the correlation was, $y = 0.7152x + 0.7068$, with a R^2 value of 0.9739, where y is the biomass (g/L) and x is the absorbance measured at 680 nm. This correlation could be referenced to estimate culture biomass during the exponential

growth phase of *C. zofingiensis* under the given conditions, however, a different correlation may be needed if any culture conditions are varied.

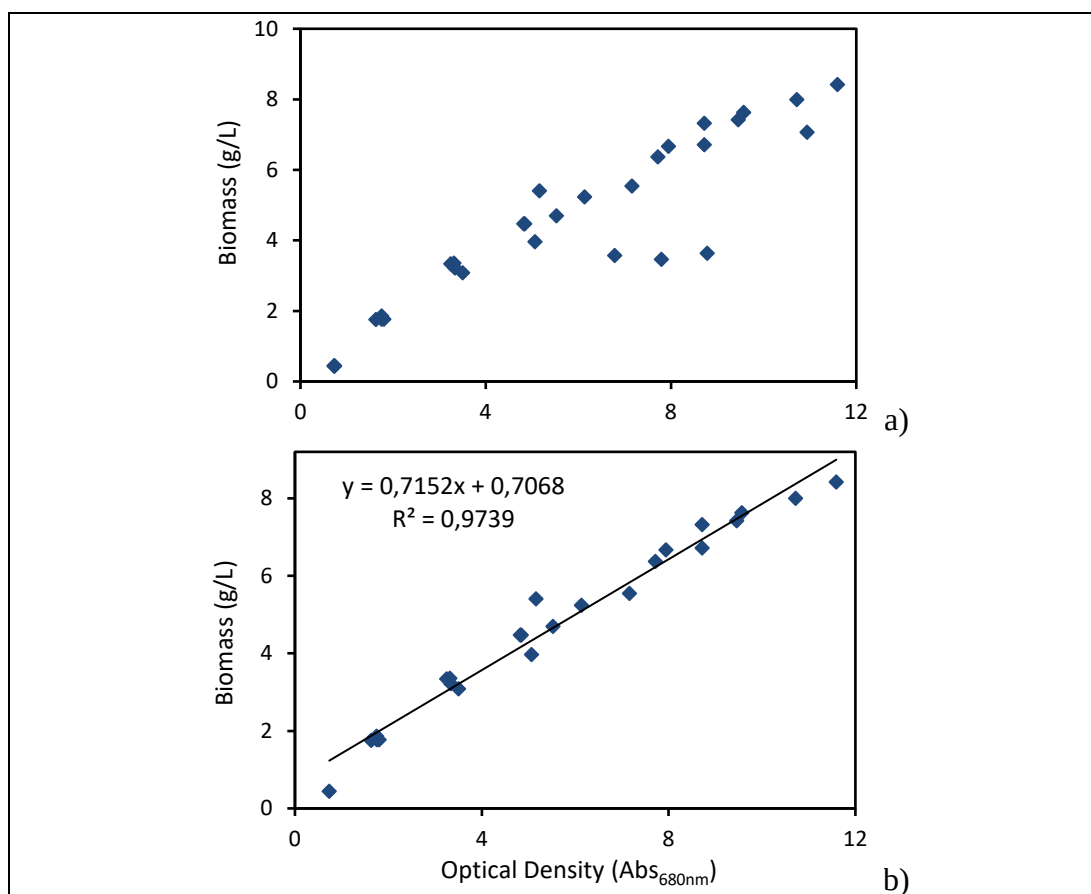


Figure 3.2. Optical density (OD) measured at a wavelength of 680 nm plotted against the biomass of the *C. zofingiensis* cultures to determine a linear correlation. a) full dataset, b) outliers from the stationary phase were eliminated.

The biomass of the cultures was drastically lower with 10 g/L initial glucose compared to all other initial glucose conditions (Figure 3.3.a). The maximum biomass achieved with 10, 20, 30 and 40 g/L initial glucose was 4.0, 7.4, 8.0 and 8.4 g/L, respectively. Accordingly, the biomass:glucose yield was much higher for the 10 and 20 g/L initial glucose conditions as compared to 30 and 40 g/L – 0.44 and 0.41 vs. 0.30 and 0.23, respectively. Similarly, the lipid content and output of the suspended cultures was much higher with 20-40 g/L as compared to 10 g/L initial glucose. The maximum lipid contents achieved with 10, 20, 30 and 40 g/L glucose were 35.2, 47.3, 51.5 and 50.2% (Figure 3.3.b), respectively, which were estimated as a percentage of dry cell weight (DCW). The maximum lipid output achieved was nearly equivalent, 4.1 and 4.2 g/L, with 30 and 40 g/L initial glucose (Figure 3.3.c), respectively, which

was at the end of 30 days of cultivation. This would indicate that 30 g/L initial glucose may be preferred with respect to maximizing lipid production at a lesser cost of glucose.

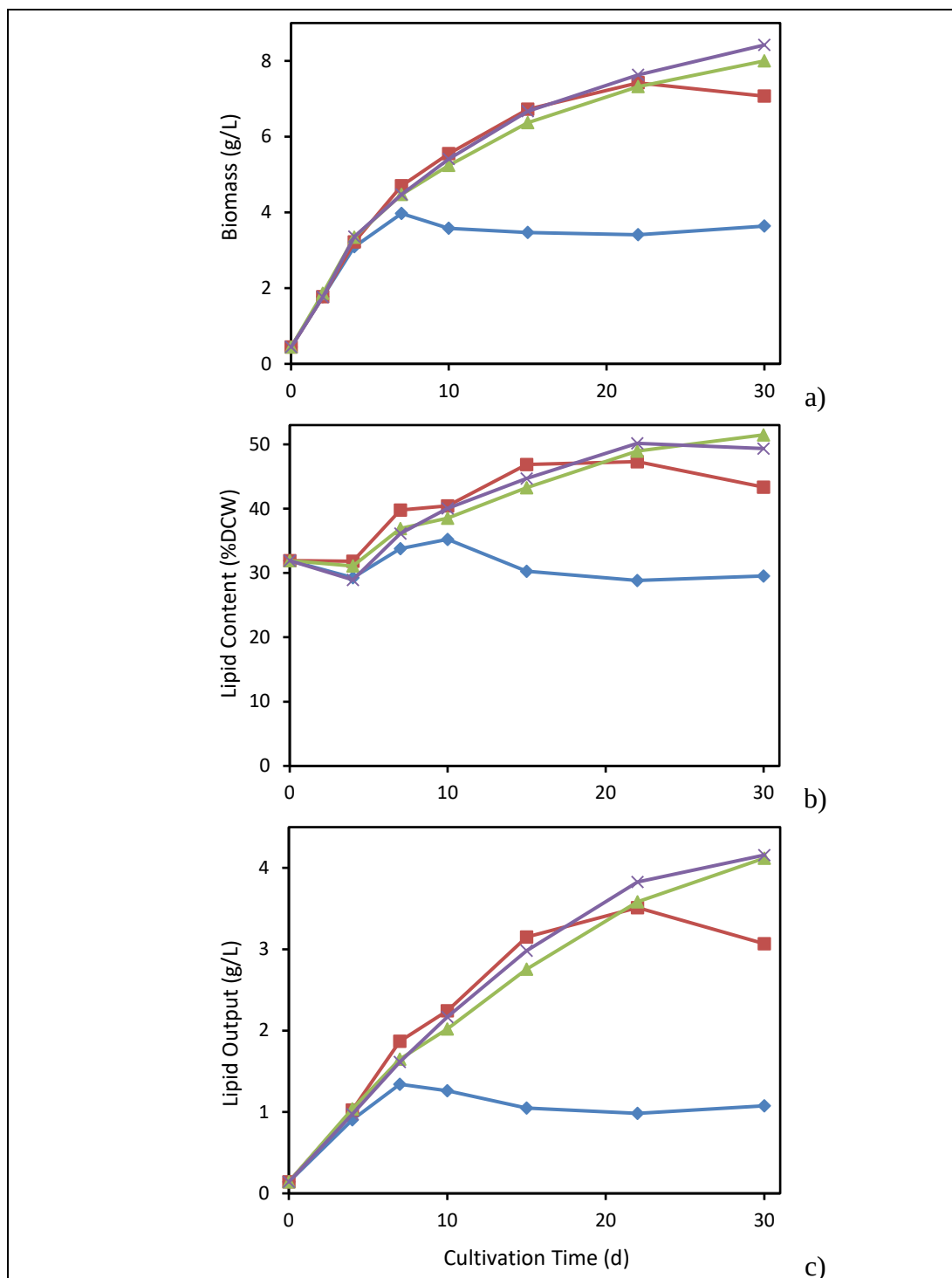


Figure 3.3: a) Biomass, b) lipid content and c) lipid output of heterotrophic, suspended *C. zofingiensis* cultures cultivated with various initial glucose concentrations: 10 (diamonds), 20 (squares), 30 (triangles) and 40 g/L (×'s).

3.2.3. Optimization of Culture Conditions and Comparison of Suspended vs. Agar-Attached Cultivation

The purpose of the initial agar-based experiment, conducted in an incubator set to 28°C, was to determine the effects of initial glucose concentration (10 and 40 g/L), cultivation time (15 and 30 days) and inoculation ratio (4, 10 and 20%, v/v) to see how the parameters for agar-attached cultivation could be optimized. Further, the lipid and astaxanthin production performance of the microalga in suspended versus agar-attached condition was compared. The agar-attached cultures cultivated using 10 g/L initial glucose were not greatly affected by cultivation time or inoculation ratio (Figure 3.4.a). The biomass output for these conditions were all in the range of 3.2-3.5 g/L with no significant differences between the 4, 10 and 20% inoculation ratios or 15- and 30-day cultivation periods. The biomass produced by day 15 with the agar-attached cultures (3.2-3.5 g/L) was slightly lower than that achieved by the suspended cultures (3.8 g/L), without statistical significance (p value > 0.10). The biomass output by day 30 however, was significantly lower in the agar-attached cultures when compared to suspended – 3.2-3.5 vs. 4.3 g/L (p value < 0.05).

Similarly, the lipid accumulation behavior of the agar-attached cultures using 10 g/L initial glucose was unaffected by inoculation ratio or cultivation period, having a lipid content range of 31.0-34.2% (Figure 3.4.b). The lipid content achieved by the corresponding suspended cultures on days 15 and 30, 36.0 and 31.6%, respectively, was comparable to that of the agar-attached cultures. The decrease in lipid content from day 15 to 30 may be a result of the cells consuming their stored lipids as the organic carbon available becomes limiting. There were no significant differences between the lipid content of the agar-attached and suspended cultures on days 15 or 30 (p value > 0.30). The lipid output (g/L) for the 10% inoculum condition of the 10 g/L initial glucose agar-attached cultures was compared to the suspended since the latter were also inoculated at a ratio of 10%. The lipid output was not different for the 15- and 30-day harvests (Table 3.2). The agar-attached cultures produced 1.0 g/L lipids, which was significantly lower than the 1.4 g/L output of the suspended (p value < 0.05). Overall, the biomass and lipid output for the various agar-attached conditions using 10 g/L glucose were 7-27% less than the corresponding suspended cultures.

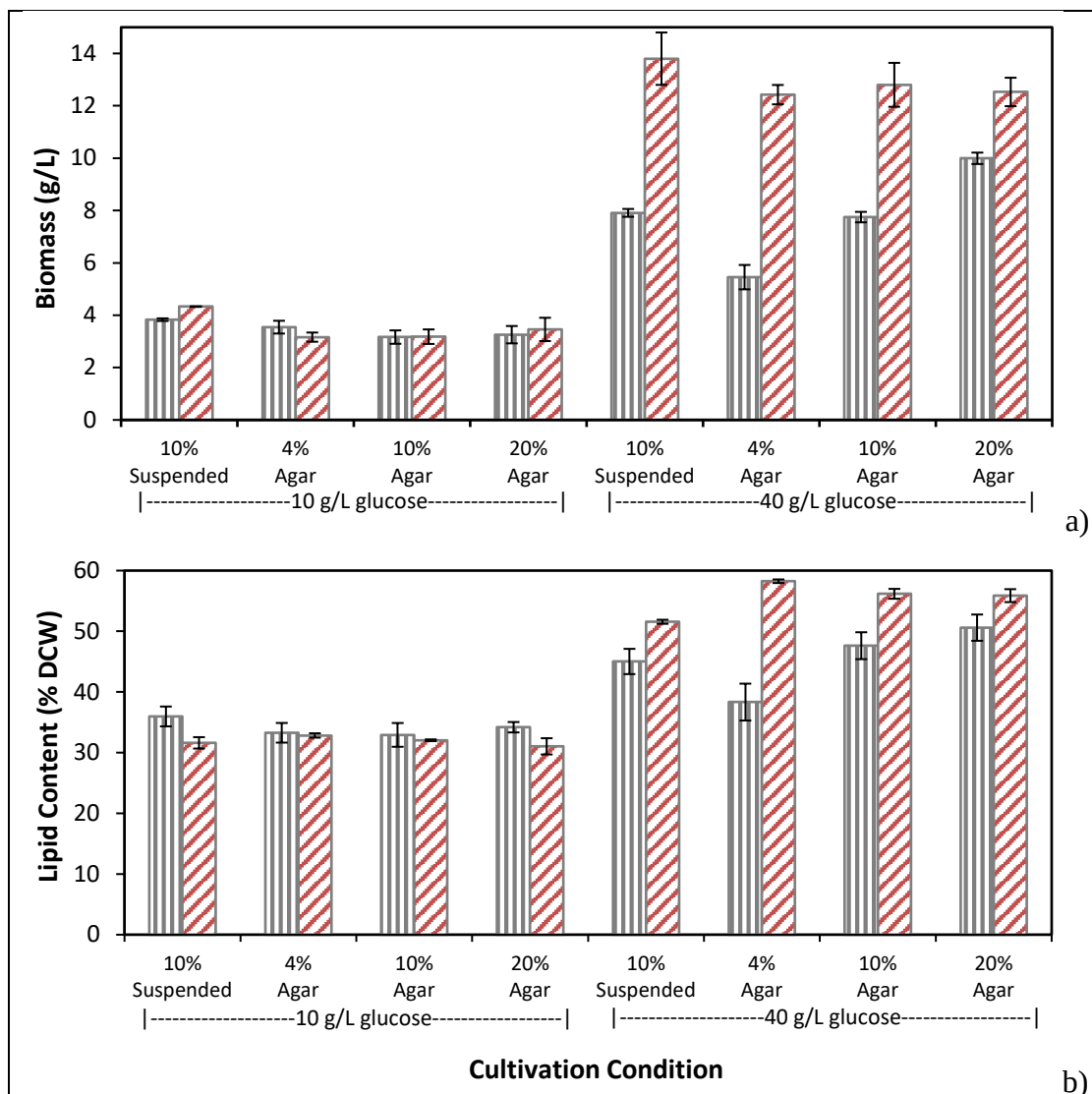


Figure 3.4: a) Biomass and b) lipid content as a percentage of dry cell weight produced by *C. zoefingiensis* cultures under suspended and agar-attached cultivation conditions. Ten or 40 g/L initial glucose was implemented. Cultures were harvested after 15 (vertical) or 30 days (diagonal stripes) of cultivation. For suspended cultures, 10%, and for agar-attached, either 4, 10 or 20% inoculum was used to start each culture – as indicated below the data.

The astaxanthin content for the agar-attached cultures utilizing 10 g/L glucose were significantly lower on day 15 than on day 30 (p value < 0.05 , Table 3.3). However, the astaxanthin content on day 30 was not significantly different based on cultivation mode – 0.45 and 0.43 mg/g were achieved for the suspended and 10% inoculum agar-attached cultures, respectively (p value > 0.50). On the other hand, the astaxanthin produced was 29.3% lower for the agar-attached 10% inoculum condition (1.4 mg/L) compared to suspended cultivation (1.9 mg/L, Table 3.2).

Table 3.2: Summary of lipid and astaxanthin outputs and yields based on the supplemented glucose for various growth conditions under 28°C incubation.

Growth Condition	Initial Glucose (g/L)	Inoculation Ratio (%)	Lipid Output (g/L)	Lipid: Glucose Yield (g:g)	Astaxanthin Output (mg/L)	Astaxanthin: Glucose Yield (mg:g)
Suspended	10	10	1.4	0.14	1.9	0.19
Agar	10	4	1.0	0.10	1.3	0.13
		10	1.0	0.10	1.4	0.14
		20	1.1	0.11	1.5	0.15
Suspended	40	10	7.1	0.18	8.8	0.22
Agar	40	4	7.2	0.18	7.5	0.19
		10	7.2	0.18	8.0	0.20
		20	7.0	0.18	8.4	0.21

Table 3.3: Astaxanthin content of cultures grown under various conditions – suspended vs. agar-attached cultivation with 10 or 40 g/L initial glucose supplementation.

Growth Condition	Initial Glucose (g/L)	Inoculation Ratio (%)	Astaxanthin Content (mg/g)		
			Day		
			0	15	30
Suspended	10	Seed Culture	0.22		
Suspended	10	10		0.44	0.45
Agar		4		0.08	0.43
Agar		10		0.08	0.43
Agar		20		0.10	0.43
Suspended	40	10		0.42	0.64
Agar		4		0.05	0.61
Agar		10		0.06	0.62
Agar		20		0.10	0.67

With growth medium containing 40 g/L initial glucose there was a dramatic increase in the biomass produced from day 15 to 30 for both agar-attached and suspended cultures (Figure 3.4.a). The biomass output of the suspended cultures was 7.9 and 13.8 g/L on days 15 and 30, respectively – a 74% increase. This indicates that the cultures were still in their exponential growth phase on day 15. When cultivated

using 40 g/L glucose the agar-attached cultures' biomass output showed a strong dependence on inoculation ratio for only the day 15 harvest. The biomass achieved with inoculation ratios of 4, 10 and 20%, respectively, was 5.5, 7.7 and 10.0 g/L on day 15, with significant differences (p value < 0.05), and 12.4, 12.8 and 12.5 g/L on day 30. This indicates that the agar-attached cultures were also in active growth phase on day 15 and, that the higher inoculation ratio led to faster growth rates. However, by day 30 the biomass produced by the agar-attached cultures showed no significant difference based on inoculation ratio (p value > 0.60). The biomass output under agar-attached condition (12.4-12.8 g/L) was lower than that of suspended cultivation (13.8 g/L), although without statistical significance.

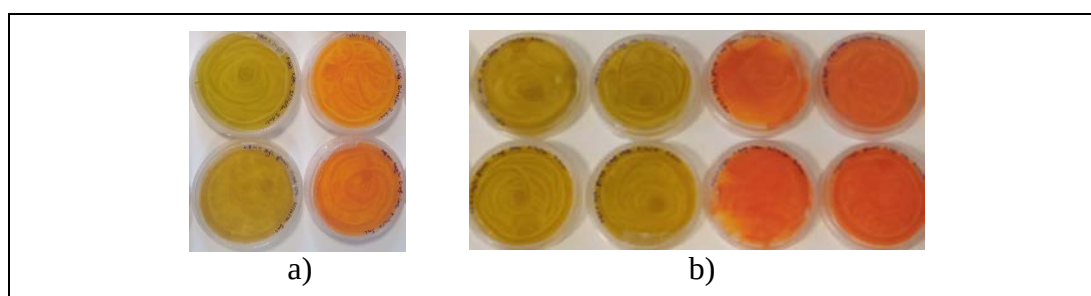


Figure 3.5: Photographs of some *C. zofingiensis* cultures that were cultivated heterotrophically for a) 15 and b) 30 days. The cultures of green hue were grown with 10 and those with orange hue on 40 g/L initial glucose.

Similarly, the lipid content of agar-attached cultures with 40 g/L initial glucose showed a positive correlation based on inoculation ratio on day 15, with a maximum of 50.6% being achieved with 20% inoculum (Figure 3.4.b). Once again, this initially strong dependence of lipid content on inoculation ratio may have been due to the higher rate of nutrient consumption associated with the faster growth rate, in turn, promoting lipid production as nutrients become limiting. In contrast, by day 30 the 4% inoculum cultures achieved the highest lipid content, 58.3%, with statistical significance (p value < 0.05). In both cases, days 15 and 30, the lipid content of the suspended cultures, 45.0 and 51.6%, respectively, was lower than the maximum achieved under the agar-attached conditions. The lipid output of the cultures followed the lipid content trend, with a 95-100% increase (p value < 0.05) from day 15 to 30 for all cultures with 40 g/L initial glucose. Although the biomass output of the agar-attached conditions was lower compared to suspended cultivation, the lipid content of their biomass being higher made up the difference. Accordingly, the maximum lipids

produced on day 30 were nearly equivalent, 7.1 and 7.2 g/L (p value > 0.90), for suspended and agar-attached cultivation (10% inoculum), respectively.

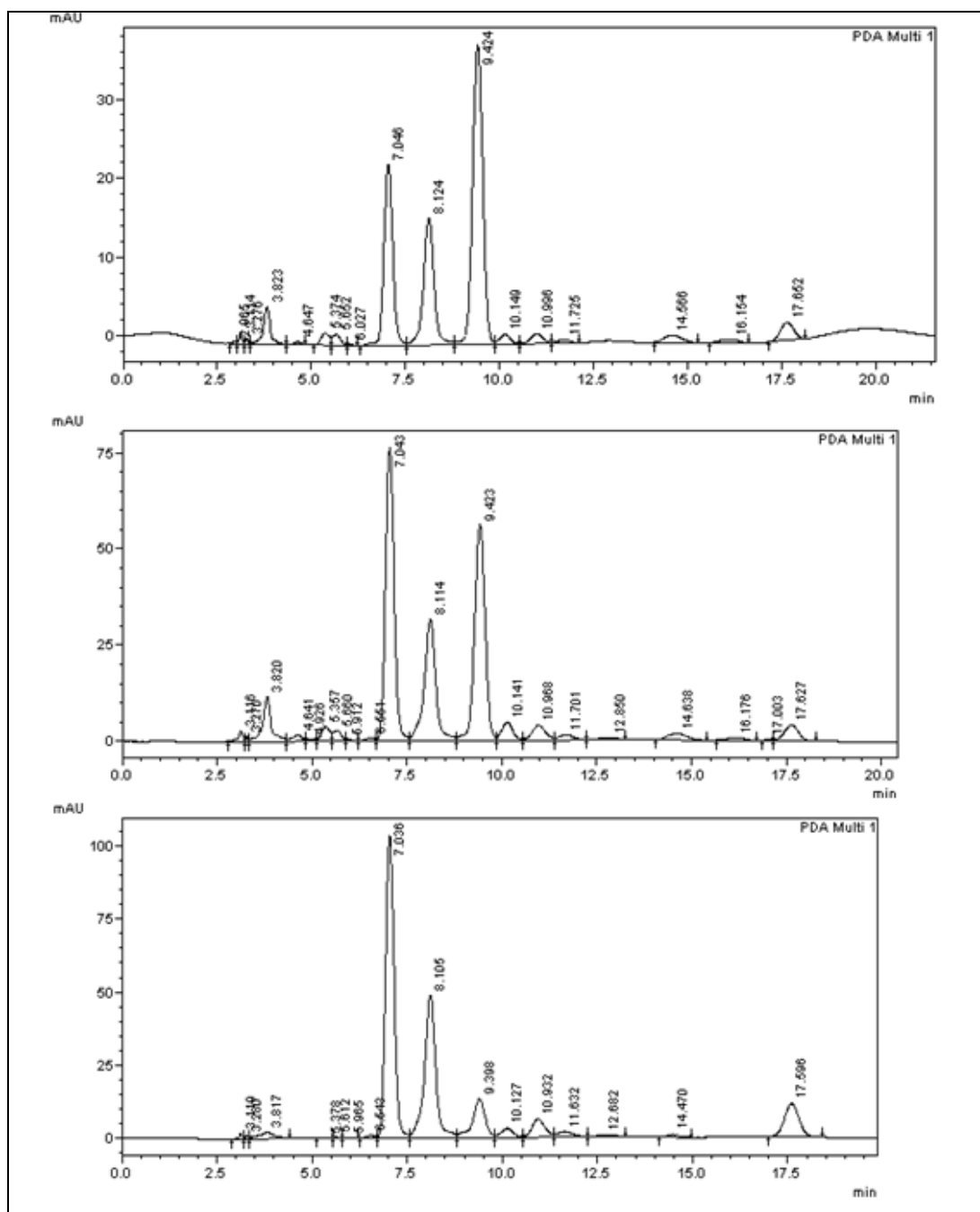


Figure 3.6: Chromatograms from the measurements of saponified *C. zoefingiensis* extracts: liquid seed culture (top) and 30-day agar-attached cultures with 10 g/L (middle) and 40 g/L initial glucose (bottom). The peak with the retention time of 7.0 minutes was identified as astaxanthin based on the pure standard.

The astaxanthin content of the biomass grown using 40 g/L initial glucose showed a steep increase between day 15 and 30 for the agar-attached cultures (Table 3.3). This finding was supported by the visual appearance of the cultures; the cultures

cultivated for 15 days (Figure 3.5.a) appear lighter in hue than those of the 30-day harvest (Figure 3.5.b). The maximum astaxanthin content was achieved on day 30, 0.64 and 0.67 mg/g with significant difference (p value < 0.05), respectively, for the suspended and the 20% inoculum agar conditions. The visual appearance of the cultures on day 30 (Figure 3.5.b) illustrates a strong green and orange hue for those cultivated with 10 and 40 g/L initial glucose, respectively, which parallels the higher content of the red-pigmented compound, astaxanthin, achieved with the 40 g/L glucose condition (0.67 vs. 0.43 mg/g). Further, the chromatograms of the extracts indicate that the ratio of astaxanthin produced compared to the other carotenoids was higher with 40 versus 10 g/L initial glucose (Figure 3.6). There was a slight positive correlation between the inoculation ratio and astaxanthin output of the agar-attached cultures (Table 3.2), with 8.4 mg/L astaxanthin being the maximum produced under the 20% inoculum condition.

Light microscope images of the biomass samples harvested from agar-attached condition show a wide variation in cell size (Figure 3.7). While many cells were ≤ 1 μm in diameter, there were also some with a 1-5 μm diameter. Overall, cell size appears greater when *C. zoofingensis* was cultivated with 40 compared to 10 g/L initial glucose. A thicker cell wall is visible with some larger cells, which is also noted to occur in the literature when microalgal cells are stressed [187].

The various conditions for agar-attached cultivation were tested to determine what parameters could lead to higher lipid and astaxanthin production with, ideally, more efficient processing. When considering the cultivation time, the lipid and astaxanthin outputs were significantly higher (p value < 0.05) on day 30 compared to day 15 in the case of 40 g/L initial glucose. The cost of culture incubation in the case of agar-attached cultivation is minimal – only a temperature-controlled environment of 28°C was provided. Therefore, maintaining a longer cultivation time of 30 days can be well offset by the increased product output. When reviewing 30-day outputs, the concentration of initial glucose supplemented, 10 vs. 40 g/L, can be compared by way of the lipid:glucose and astaxanthin:glucose yields (Table 3.2). The lipid:glucose (g:g) yields were significantly different (p value < 0.05) for the 10 and 40 g/L initial glucose conditions, 0.10-0.11 and 0.18, respectively. Similarly, the astaxanthin:glucose (mg:g) yields were significantly higher with 40 compared to 10 g/L glucose – 0.19-0.21 vs. 0.13-0.15 (p value < 0.05). Accordingly, 40 g/L appears a more beneficial initial glucose concentration to use in this cultivation system and

was considered optimal for the discussion herein. However, a further comparison with intermediate initial glucose concentrations, 20 and 30 g/L, was also tested (see 3.2.4).

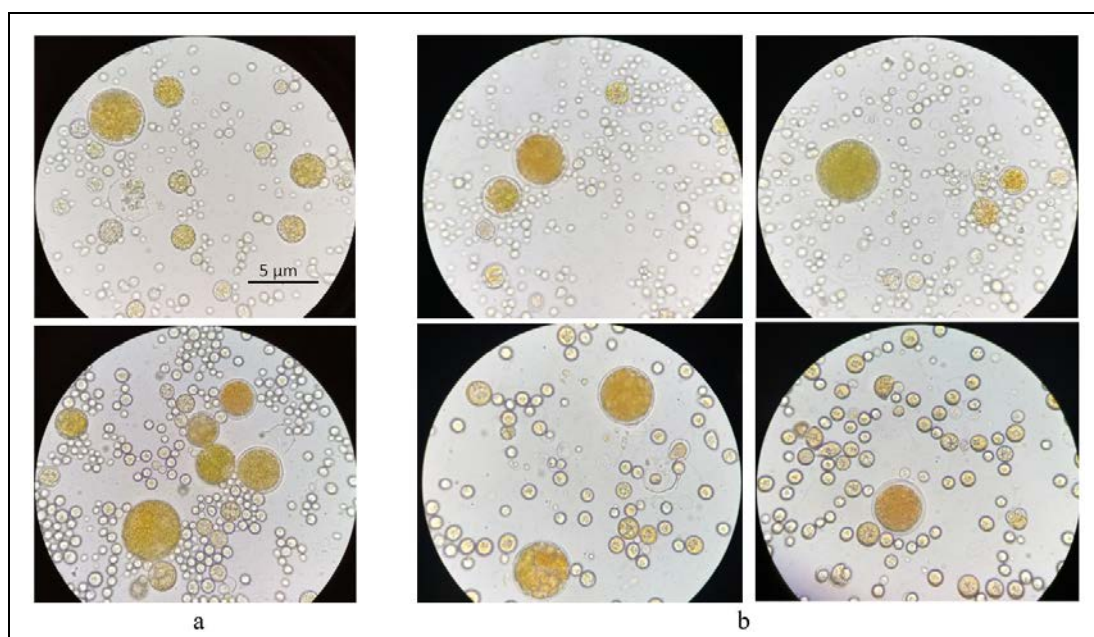


Figure 3.7: Light microscope images of agar-attached culture samples suspended with deionized water for viewing purposes. Cultures were harvested after a) 15 and b) 30 days. Either 10 (top) or 40 g/L initial glucose (bottom) was supplemented. The 5 µm scale bar shown applies to all images.

When evaluating inoculation ratio, it is relevant to consider how the product output would ultimately be affected if a continuous process were to be designed. That is, if an inoculation ratio of 20% is being applied, this implies that 20% of any biomass harvest must be utilized to inoculate the next batch of media and therefore, only 80% of the harvested biomass would be available for product extraction. In order to take this into account the lipid and astaxanthin outputs for 30-day, 40 g/L initial glucose cultures were adjusted to estimate the actual product outputs that could be attained under a continuous production scenario (Table 3.4). In this case, the maximum lipid and astaxanthin outputs for agar-attached condition, 6.9 g/L and 7.2 mg/L, respectively, were achieved with the 4% inoculum condition. Based on this initial study, the most favorable operating parameters for agar-attached cultivation appear to be, 30 days incubation, 40 g/L initial glucose and 4% inoculation ratio. Based on its potential to lead to a more sustainable and continuous production process, the 4% inoculation ratio was considered optimal and was implemented consistently in all subsequent studies.

The lipid and astaxanthin output for suspended cultivation was also adjusted to account for the 10% inoculation ratio to project its continuous production potential. Accordingly, suspended culture with 40 g/L initial glucose produced 6.4 g/L lipids and 7.9 mg/L astaxanthin after 30 days of cultivation (Table 3.4). When comparing the maximum continuous output of agar-attached cultivation to that of suspended, the lipids produced were 8.5% higher (6.9 vs. 6.4 g/L) and the astaxanthin was 8.4% lower (7.2 vs. 7.9 mg/L).

Table 3.4: Continuous lipids and astaxanthin production potential of suspended and agar-attached cultures cultivated for 30 days with 40 g/L initial glucose, estimated based on the inoculation ratios used.

Growth Condition	Inoculation Ratio (%)	Continuous Lipid Output (g/L)	Continuous Astaxanthin Output (mg/L)
Suspended	10	6.4	7.9
Agar	4	6.9	7.2
Agar	10	6.5	7.2
Agar	20	5.6	6.7

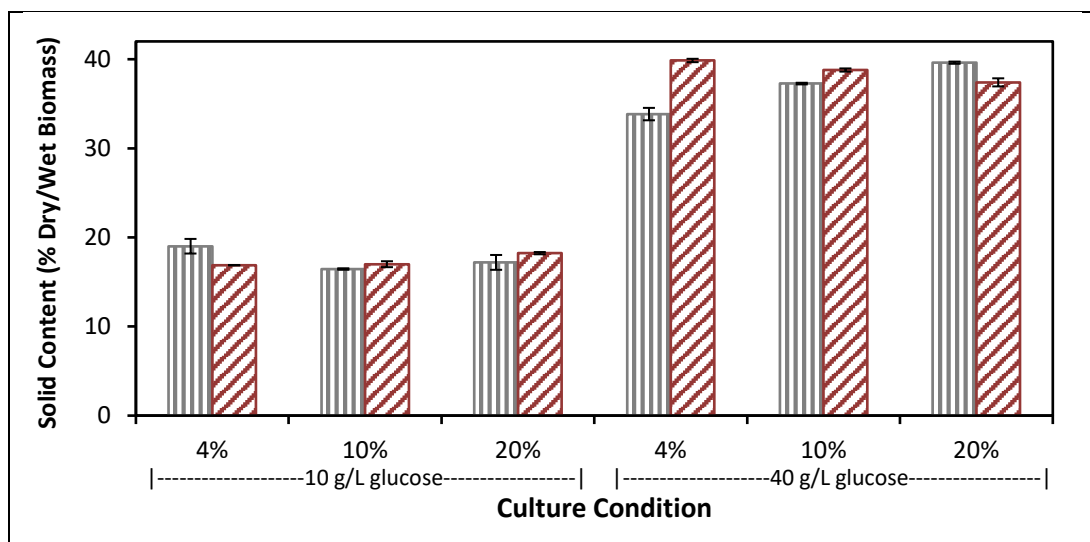


Figure 3.8: Solid content of the biomass samples cultivated under agar-attached condition for 15 (vertical) or 30 days (diagonal stripes). Either 10 or 40 g/L initial glucose was used. The cultures were inoculated with 4, 10 or 20% inoculum, as indicated below the data.

The solid content of the agar-attached biomass samples was determined based on the wet weight immediately after harvesting and final dry weight of the samples

after freeze-drying. The biomass harvested from the 10 g/L initial glucose conditions had a solid content in the range of 16.5-19.0%, while the 40 g/L conditions were significantly higher, 33.8-39.9% (p value < 0.05, Figure 3.8). No other consistent trend was noted with solid content based on cultivation time or inoculation ratio. The solid content ratio of the harvested samples being so high seemed promising – such biomass samples could lend well to being extracted directly without drying of the biomass. Therefore, a comparison of product yield with dry vs. wet extraction was investigated (see 3.2.4).

3.2.4. Process improvements for agar-attached cultivation

The biomass output of the cultures was positively correlated with initial glucose concentration, and showed a decrease associated with the longer cultivation time, 60 days (Table 3.5). The highest biomass, 12.3 g/L, was achieved after 30 days of cultivation; this was accomplished with 40 g/L initial glucose, with statistical significance when compared to the other glucose conditions (p value < 0.05). The lipid content of the 10, 20 and 30 g/L initial glucose conditions appears lower on day 60 vs. 30. The decrease in lipid content is a recurring trend and can be associated with the depletion of organic carbon and the culture's tendency to use its stored lipids. In contrast, the 40 g/L initial glucose cultures maintained the same lipid content on days 30 and 60 – 57.0% – the highest achieved in this experiment. In accordance with the decline in biomass and lipid content from day 30 to 60, the lipid output was also lower. The maximum lipids produced on days 30 and 60, respectively, were 7.0 and 6.0 g/L with 40 g/L initial glucose and dry extraction condition. Apart from the 30 g/L initial glucose condition there was a decrease in the astaxanthin output from day 30 to 60. The most dramatic decline was in the astaxanthin produced with the 40 g/L initial glucose condition, which was 5.9 and 4.9 mg/L on days 30 and 60, respectively. Based on the general drop in lipid and astaxanthin outputs it was established that 60 days served no advantage over 30 days of cultivation.

The term “wet” implies that the biomass samples were harvested from the agar media surface, frozen (-32°C) and defrosted just prior to the extraction analyses, whereas the dry samples were freeze-dried until constant weight. Due to their water content, the wet samples have higher biomass and lower lipid and astaxanthin contents, making these values incomparable with the dry-extraction data. However, the lipid

and astaxanthin output values could be compared and revealed that the 30-day product outputs were improved when samples were wet- rather than dry-extracted, except for the 30 g/L initial glucose condition (Table 3.5). The maximum lipids and astaxanthin, 8.2 g/L and 6.5 mg/L, respectively, were produced with the wet-extracted samples cultivated for 30 days utilizing 40 g/L initial glucose, with statistical significance when compared to the other glucose conditions (p value < 0.05). In addition to the increase in product output, wet extraction brings about the benefit of improving process efficiency since the drying of biomass samples is made unnecessary.

Table 3.5: Biomass, lipids and astaxanthin achieved with 4% inoculation (v/v), after 30- and 60-days incubation of cultures at RT (20-22°C); dry and wet extraction was compared for samples harvested after 30 days.

30 DAY HARVEST							
Initial Glucose (g/L)	Dry Biomass Extraction					Wet Biomass Extraction	
	Dry Biomass (g/L)	Lipid Content (%DCW)	Lipid Output (g/L)	Astaxanthin Content (mg/g)	Astaxanthin Output (mg/L)	Lipid Output (g/L)	Astaxanthin Output (mg/L)
10	3.1	36.0	1.1	0.43	1.4	1.4	1.5
20	6.4	48.2	3.1	0.39	2.6	3.7	2.9
30	10.4	54.5	5.7	0.41	4.3	5.2	3.3
40	12.3	57.0	7.0	0.50	5.9	8.2	6.5

60 DAY HARVEST					
Initial Glucose (g/L)	Dry Biomass Extraction				
	Dry Biomass (g/L)	Lipid Content (%DCW)	Lipid Output (g/L)	Astaxanthin Content (mg/g)	Astaxanthin Output (mg/L)
10	3.0	30.5	0.9	0.39	1.2
20	5.5	45.1	2.5	0.45	2.4
30	10.1	52.1	5.5	0.45	4.6
40	10.5	57.0	6.0	0.47	4.9

Lipid:glucose and astaxanthin:glucose yields can be used to compare the efficiency with which the glucose was utilized under the various initial glucose conditions. There is no clear trend between initial glucose concentration and product yield (data not shown). However, the highest lipid and astaxanthin yields, 0.21 g:g and 0.16 mg:g, respectively, were achieved with wet extraction of 30-day, 40 g/L initial glucose cultures. Having the highest yields on glucose, in addition to the highest lipid and astaxanthin outputs continues to indicate the use of 40 g/L as the optimal

initial glucose concentration for agar-attached cultivation of *C. zofingiensis* using growth medium CZ-M1.

When comparing the maximal product outputs achieved at RT (20-22°C, 4% inoculum, wet extraction) with the 28°C experiment (4% inoculum, dry extraction), we see an improvement in the lipids (8.2 vs. 7.2 g/L) and a reduction in the astaxanthin produced (6.5 vs. 7.5 mg/L). This indicates that with the RT incubation and wet extraction, ca. 1 gram more lipids and 1 mg less astaxanthin are produced per liter of growth medium used. Based on the scale, the greater production of lipids appears a more valuable benefit and is further justified by the processing advantages of not requiring temperature-controlled incubation or any drying of the harvested biomass. Further comparison of these outputs to 28°C suspended cultivation (10% inoculum) again reveals higher lipids (8.2 vs. 7.1 g/L) and lower astaxanthin produced (6.5 vs. 8.8 mg/L). Similarly, lipids are produced at a greater amount on a gram-scale, while astaxanthin output is affected on a mg-scale.

3.2.5. Reuse of Agar

The compound agar used to solidify the growth medium yielded the surface on which the attached growth experiments were conducted. The addition of agar adds cost to the cultivation process. Therefore, the reuse of agar was studied to determine if this additional cost could be minimized. The successful remelting of agar media, which remained after harvesting the microalgal lawn grown on its surface, allowed for the same agar compound to be used for 3 consecutive cultivation cycles. The biomass achieved with this series of 15-day growth cycles using 10 g/L initial glucose was 3.3, 3.2 and 3.2 g/L with no significant differences. Further, this experiment demonstrated that while the agar was being reused, so was the water content contained within the spent media. The agar media could be remelted with only the addition of the concentrated (100x) mineral solutions, indicating the additional advantage with the reuse of water.

3.2.6. Agar-to-Agar Inoculation

The experiments conducted thus far had been started with liquid seed culture grown over a 10-12 day period, as previously described. In order to establish the practicability of a continuous, self-sustaining system of microalgal growth in agar-

attached form, the possibility of inoculating agar media directly with cells grown on agar media, i.e., agar-to-agar inoculation, was investigated. The first set of cultures (I) was inoculated with liquid seed culture, while the 3 subsequent set of cultures (II-IV) was inoculated consecutively with the harvest of one culture from the preceding culture set. Culture sets I-III were all grown on fresh agar media while set IV was grown on reused agar media.

The lipids produced after 30 days of cultivation with agar media containing 40 g/L initial glucose were of a consistent value, 7.4 g/L, for culture sets I-III, with no statistical differences (p value > 0.60 , Figure 3.9). However, culture set IV had a significantly lower lipid output of 6.6 g/L (p value < 0.05). The astaxanthin produced was slightly lower for the cultures inoculated directly from agar culture; the astaxanthin output of culture sets I-IV were 5.2, 4.9, 4.7 and 5.1 mg/L, respectively, with no significant differences. Overall, it was established that agar-to-agar inoculation could yield consistent lipid and astaxanthin outputs and that preparation of liquid seed culture could be avoided. Further, culture set IV, which was grown on reused agar media, had nearly equivalent astaxanthin output (5.1 vs. 5.2 mg/L) and 10.3% lower lipid output as compared to culture set I, demonstrating that reuse of agar can also be implemented with only minimal reduction in product output. Further testing can be pursued to see if this drop in lipid production could be avoided.

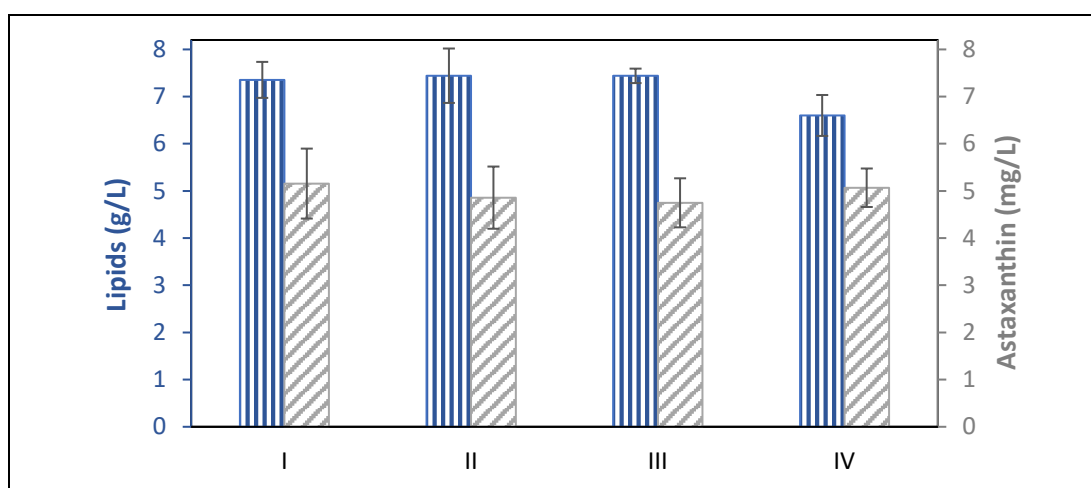


Figure 3.9: Lipids (vertical) and astaxanthin (diagonal stripes) produced by 4 consecutive microalgal growth cycles, each cultivated at RT (22-28°C) for 30 days with 40 g/L initial glucose. Culture set I was inoculated with liquid seed culture, while the others were seeded consecutively using cells harvested from the preceding set of cultures. Culture set IV was cultivated with reused agar media.

3.3. Discussion

Microalgae can be described as microscopic plants, able to metabolize CO₂ in the atmosphere using energy from sunlight (photosynthesis). However, the dependence on a light source, natural or synthetic, leads to the phenomenon known as “self-shading” – as cell density increases so does turbidity, limiting the cells’ access to light and as a result, limiting the culture’s growth. Either shallow open ponds with inefficient use of space or complex photobioreactor systems with high capital cost are used to overcome this handicap. Alternatively, heterotrophic cultivation of microalgae can yield much higher biomass and lipid production rates compared to photoautotrophic metabolism [188], [189] and can be scaled up in traditional equipment, such as fermentors [152], [190]. Organic carbon, being dissolved and readily available in the growth medium, is what allows for the higher growth rates seen in heterotrophic cultivation and is also the cause of added cost. This can be alleviated with the use of wastes or wastewaters containing organic carbons, such as, crude glycerol [191], [192], molasses [193], [194], dairy effluent [195], [196], or fruit pomaces [197], [198].

For its mentioned benefits, here we focused only on heterotrophic metabolism with *C. zofingiensis*, a strain able to produce lipids and astaxanthin simultaneously independent of a light source. Further, by combining heterotrophic and attached modes of cultivation we endeavored to combine their processing advantages. First, we demonstrated that *C. zofingiensis* could be cultivated on agar media homogeneously and consistently with spread-plating of concentrated liquid seed culture and that the attached biomass could be easily and thoroughly harvested with minimal manual effort. Variations of cultivation parameters revealed 30 days harvesting period, 40 g/L initial glucose concentration and 4% inoculation ratio (v/v) to be optimal based on having the highest lipid and astaxanthin outputs and yields on glucose. Further testing showed that with RT incubation (20-22°C) and wet extraction of harvested biomass, the lipid output could be improved (8.2 vs. 7.2 g/L) with some reduction of the astaxanthin produced (6.5 vs. 7.5 mg/L). This indicated that higher lipid output could be achieved with additional process benefits – no need for temperature-controlled incubation or drying of the harvested biomass. Compared to suspended cultivation at 28°C, the RT agar-attached experiment resulted in higher lipids (8.2 vs. 7.1 g/L) and lower astaxanthin output (6.5 vs. 8.8 mg/L). Besides the

advance in lipid production, the prospect of agar-attached cultivation offers many processing advantages – no energy expenditure during incubation, 4 vs. 10% inoculation indicating a greater ratio of “usable” product output, and easy harvesting of biomass with high solid content that does not require any concentration or drying.

In addition to parameter optimization, two other concepts were tested to establish a more practicable agar-attached cultivation process. First, the possibility of reusing agar was demonstrated. It is known that the addition of agar, a naturally derived mixture of polysaccharides and agaropectin used to solidify growth medium [199], is an added expense. Therefore, the reuse of agar would reduce the added cost. Harvested (spent) media were remelted in the autoclaving process so that the same agar and the water it retained were used sequentially for three independent cultivation cycles. There was no significant reduction in the produced biomass indicating that agar and water could be used at least three times without hindering culture growth and the cost of agar could be reduced to one-third. Further testing could potentially prove more cycles of reuse to be possible. Second, agar-to-agar inoculation was tested to determine if the need for a liquid seed culture could be eliminated from the process. Sequential cultivation cycles showed no difference in produced lipids and a statistically insignificant drop in astaxanthin output. Thus, the potential to cultivate *C. zofingiensis* in agar-attached condition for consistent lipid and astaxanthin production, without requiring a liquid starter culture and the equipment involved therein, was illustrated – ultimately presenting a more sustainable continuous process.

Beyond the process improvements, we also indicated the potential cost-benefit associated with simultaneous production of lipids and astaxanthin. The monetary value of astaxanthin helps to tip the scale for economic viability of microalgae-based biodiesel. In our study, we optimized pigment extraction parameters to ensure that astaxanthin was recovered maximally from the biomass samples. The added advantage of the optimization was that the final procedure for pigment extraction was nearly the same as that for lipid extraction; chloroform:methanol (1:1) was used in both cases with 50°C heat treatment. This indicates that both lipids and astaxanthin can be co-extracted efficiently and with minimal operational effort, which is not always demonstrated in co-production studies [200], [201]. As a future endeavor, a practical and economical method for efficient separation of lipids and astaxanthin could be researched.

The maximum astaxanthin produced in our suspended cultivation experiment with 40 g/L initial glucose and 28°C incubation temperature was comparable to the results of another study of *C. zofingiensis* using the same glucose concentration and 30°C [144]. They achieved 9.7 g/L biomass with 0.97 mg/g astaxanthin content, yielding 9.4 mg/L astaxanthin. Although we achieved higher biomass (13.8 g/L), due to a lower astaxanthin content (0.64 mg/g) our maximum astaxanthin output (8.8 mg/L) was 6.4% lower. The astaxanthin content in our cultures being lower could be explained by the fact that the batch cultures in the Ip and Chen study were cultivated in 100 mL medium, while ours were in 200 mL. A study evaluating the effect of culture volume showed a significant difference in astaxanthin content after 7 days heterotrophic cultivation of *C. zofingiensis*; they found ca. 0.66 and 0.36 mg/g astaxanthin, for 100- and 200 mL- culture volumes, respectively [180]. It is also relevant to note that the Ip and Chen study only demonstrated the biomass and astaxanthin production of the culture, while we focused our efforts on showing co-production of lipids and astaxanthin for the potential cost-benefit.

Another group cultivating *C. zofingiensis* heterotrophically in 25°C batch culture with 30 g/L initial glucose for 8 days produced 11.3 g/L biomass, 5.3 g/L lipids and 12.8 mg/L astaxanthin [146]. The 30 g/L initial glucose agar-attached condition in our RT experiment (20-22°C) yielded 10.4 g/L biomass with slightly higher lipids (5.7 g/L) and significantly lower astaxanthin output (4.3 mg/L), owing to the higher lipid and lower astaxanthin contents of the biomass. The C/N ratio (mol/mol) in our experiment being higher than the mentioned study (113 vs. 100) does not explain the lower astaxanthin content we achieved since a higher C/N has been shown to promote astaxanthin production [144]. The cultivation time used in our study to achieve these results was 30 days, which inevitably leads to lower productivity values. However, we would argue that agar-attached cultivation is still a more cost-efficient option for cultivation because, despite being longer, the incubation period has no practical cost-implications – the cultures were grown in a stationary space, literally a cupboard, with no dependence on light and no energy expenditures since neither stirring, shaking nor agitation was necessary.

The mentioned study [146] also demonstrated that *C. zofingiensis* could be cultivated heterotrophically using cane molasses, a sugar industry waste by-product, as the sole source of carbon. Their results demonstrated that when pretreated with cation exchange, molasses in the growth medium yielded higher biomass, lipid and

astaxanthin outputs in batch culture as compared to the supplementation of glucose. The use of cane molasses in the growth medium could be investigated in the future for agar-attached cultivation of *C. zofingiensis* to further diminish processing costs.

The harvesting procedure is known to have a large impact on the cost and efficiency of processing microalgal biomass as a biodiesel feedstock. Various solutions continue to be developed. Centrifugation, though known for its high harvesting efficiency at lab-scale, is not feasible to scaleup due to its operational energy expenditure [202]. Flocculation is an active area of research as it is believed to have the potential to be implemented at large scale either alone or in combination with other processes to concentrate biomass and reduce operational costs [203]. Traditional chemical flocculants, such as metal salts, or organic flocculants, including chitosan and protamine, can facilitate the aggregation and settling of microalgal cells [46]. Some limitations are, that the flocculant, as in the case of metal salts, can be toxic and can limit the bioconversion processes possible with the lipid-extracted-biomass (post-processing) and some flocculants can be highly pH-sensitive requiring drastic pH changes to be effective [204], [205]. The harvesting efficiency of these methods are sometimes strain-specific. Overall, these approaches require the use of an additional compound and pH adjustments, which entail monitoring and management to ensure harvesting efficiency. Alternatively, our proposal of agar-attached cultivation requires only the additional compound agar, which we demonstrated to be reusable along with the water content of the growth medium. Further, agar-attached growth allows for thorough and simple harvesting of the cells with minimal effort. While a combination of flocculation for initial harvesting and centrifugation for further dewatering yields algal paste with 25% solid content [206], our harvested samples were shown to have a 40% solid content and could be extracted directly for lipids and astaxanthin.

Attached growth of microalgal cultures is an ongoing topic of research due to the advantages it can present, including higher areal productivities, more efficient harvesting of already concentrated microalgal cells and, potential water- and cost-savings [207], [208]. One study showed that it was possible to cultivate *Spirulina platensis* attached onto electrostatic flocking cloth [209]. A sprayer to keep the attachment material wetted with growth medium and a CO₂ injector were used to support microalgal growth in outdoor conditions. The study was focused on improving biomass production and they were able to show high areal biomass productivity.

Another group demonstrated the attached cultivation of *Chlorella vulgaris* onto a ceramic filter material [210]. Active pumping of the growth medium through the material, an air sparger and LED illumination were used to support the microalgal growth. The biomass achieved with their system had a solid content of ca. 20%, while applying 0.6 bar vacuum increased this value to 41%. In contrast to these systems, a perk of agar-attached cultivation is that the surface for cellular attachment and the growth medium are one and the same. Therefore, no mechanical interference, such as pumping of the growth medium, is needed to ensure that the cells access the nutrients. Furthermore, the major advantage of implementing attached growth with heterotrophic metabolism is that there is no dependence on light. Accordingly, the culture's areal productivity can be greatly multiplied by simply stacking the "plates" holding the agar media, as was done during our experiments. Scaleup of agar-attached cultivation can be envisioned with a tray bioreactor, where trays of media could be cultured and harvested simultaneously and independently.

4. GROWTH MEDIUM OPTIMIZATION, WASTE UTILIZATION and ATTACHED GROWTH WITH *AUXENOCHLORELLA PROTOTHECOIDES*

4.1. Materials and Methods

4.1.1. Microalgal Strain and Growth Conditions

A. protothecoides was obtained from SAG (Göttingen, Germany). The stock culture was maintained on agar media, incubated at room temperature near a North-facing window and was replicated every 2-4 weeks. The synthetic growth medium, referred to as PM1[211], for the control condition of all initial studies included the following ingredients per liter: 0.7 g KH_2PO_4 , 0.3 g K_2HPO_4 , 0.147 g MgSO_4 , 25 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 3 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.1 g glycine, 4 g yeast extract, 0.01 mg vitamin B1 and 1 mL A5 trace mineral (TM) solution. Glucose was added as later specified. The media was prepared, autoclaved and sterile FeCl_3 , vitamin B1 and A5 TM stock solutions were added after the media were cooled to room temperature. All chemical compounds used were of the highest purity available.

All cultures were grown in 500 mL bottles, where 190 mL sterile media was inoculated with 10 mL of seed culture for a 5% inoculation ratio (v/v). The seed cultures were grown with the same media constituents (PM1) plus 10 g/L glucose and were inoculated with culture on agar media. The same seed culture was used to start all cultures during any given study to avoid variability. The seed cultures were cultivated for 4-5 days before use and generally had a biomass content of 4-5 g/L at the time of their use for inoculation. All cultures including seeds were incubated in darkness in a shaking incubator set to 28°C and 150 rpm, unless noted otherwise. Conditions in the optimization experiments were run with biological duplicates.

4.1.2. Optimization Experiments

4.1.2.1. Effect of Initial Glucose

The media were prepared to contain 10, 20, 30 or 40 g/L glucose. Since all media contained the same amount of yeast extract (4 g/L), this difference in glucose concentration allowed for a comparison of various C/N ratios. The pH of all media was adjusted to be 6.5 post-autoclaving.

4.1.2.2. Effect of Initial pH

The titrant amounts necessary to adjust the medium pH to 4.5, 6.5, 7.5 and 8.5 were pre-determined. The pH adjustments were made aseptically post-autoclaving with filter-sterilized HCl and NaOH solutions and samples were taken to verify the resulting initial pH of the growth media. Accordingly, based on the actual initial pH values, the conditions tested were noted as 4.4, 6.4, 7.4 and 8.4 ± 0.1 . All media contained 30 g/L initial glucose.

4.1.2.3. Effect of Varying Growth Medium Constituents and Temperature

In accordance with examples in the literature, the composition of growth medium PM1 was varied to ascertain if the lipid yield could be improved by way of increasing the biomass output and/or the lipid content. The changes applied were as follows: the addition of NaCl, the addition of urea as a nitrogen source rather than yeast extract, a decrease in sulfate and an increase in iron content (Table 4.1). In addition to these variations, the control medium, PM1, was also tested for cultivation at 20-22°C (room temperature, RT) to evaluate the impact of temperature. All media contained 30 g/L initial glucose and initial pH was adjusted to 7.5 post-autoclaving.

Table 4.1: Summary of changes in growth medium composition.

Group Name:	Description of Change:
Control	PM1, as previously specified
NaCl ^a	1 g/L NaCl
Urea ^b	1.35 g/L urea, no yeast extract
Sulfate ^c	9.8 mg/L MgSO ₄
Iron ^d	13.5 mg/L FeCl ₃ ·6H ₂ O
RT	PM1, 20-22°C

^a[212], ^b[213], ^c[214], ^d[215]

4.1.3. Carrot Medium Experiments

4.1.3.1. Carrot Pomace Pre-Treatment

Acid hydrolysis is an effective method to improve the total reducing sugar yield attainable from carrot pomace [216]. Temperature, time and acid concentration were explored as parameters to improve the hydrolysis of the sucrose found in carrot pomace. Initially carrot pomace was tested at a concentration of 200 g/L to compare the effect of temperature and time. 20 g samples of defrosted carrot pomace were each blended with 80 mL of 0.4% H₂SO₄. It should be noted that 80 mL was used, rather than 100, to account for the water released as a result of carrot pomace hydrolysis. The mixtures were retained at room temperature (RT), 60°C or 90°C. The RT and 60°C samples were incubated for 1 hour, while the 90°C samples were incubated for 3 hours and hourly samples were taken. The samples were neutralized, strained and their reducing sugar content (glucose and fructose) was estimated. Afterwards, 600 g/L carrot pomace was tested with 90°C, 1 hour heat treatment as described, and the concentration of acid was varied to observe the effect and to finalize acid hydrolysis conditions to be used in the carrot medium studies. The total reducing sugar content of all carrot medium samples was determined using the DNS method [217] and total Kjeldahl nitrogen using standard methods [218].

4.1.3.2. Use of Carrot Pomace for Microalgae Cultivation

Synthetic growth medium PM2 is a modified version of PM1, where 1.35 g/L urea is supplemented rather than 4 g/L yeast extract. PM2 containing 30 g/L glucose was used as the medium for the control condition. The general procedure for preparing the carrot medium (CM) was as follows: carrot pomace was flash frozen (-40°C) by the manufacturer (GE-TA Tarım, Adana, Turkey) on the day of processing and shipped to our facility for testing. The pomace was stored at -18°C and defrosted at the time of use. Based on the carbon content, the intended concentration to use the carrot pomace was 600 g/L. In a 2-L beaker 900 g of the pomace was combined with 900 mL of 0.8% H₂SO₄ (Figure 4.1). The mixture was blended for about 3 minutes with a handheld blender (Multiquick 7, Braun, Germany). The blended mixture was kept at 90°C for 1 hour, cooled to room temperature, then strained through two layers of cheesecloth to separate out most of the solid content and the pH of the filtrate was

neutralized to 6.5. Note that the total volume of the filtrate was measured as 1530 mL so that the actual concentration of carrot pomace used to constitute the CM was 588 g/L. This filtrate was either used as is for the 100% CM condition or mixed with the synthetic growth medium, PM2, at a 3:1 or 1:1 ratio to formulate the 75% and 50% CM conditions, respectively. All media were autoclaved, cooled and titrated to a pH of 8.5. For the control condition, the sterile FeCl_3 , vitamin B1 and A5 TM stock solutions were added as usual after autoclaving. These additions were not made in the case of the 50 and 75% CM conditions based on the preliminary carrot medium testing, which demonstrated that they were not necessary. The experiment was run with biological triplicates.



Figure 4.1: Photographs of the processing of carrot medium (CM); defrosted carrot pomace used to make the CM (left), carrot pomace blended with dilute sulfuric acid (middle) and the CM and resulting pomace after the cheesecloth straining (right).

4.1.3.3. Glucose Addition to 100% Carrot Medium

HPTLC analysis of the CM from the initial study showed that in addition to fructose and glucose the medium also contained sucrose. The intent had been to prepare a medium using carrot pomace such that all of its sucrose content had been hydrolyzed; however, that was not the case. A higher acid concentration, 1.1%, was found sufficient to breakdown the sucrose. In fact, due to the more thorough hydrolysis it was confirmed that 500 g/L carrot pomace was sufficient to produce a medium containing about 30 g/L reducing sugar. Therefore, the procedure for this study was modified to use 1.1% sulfuric acid at a volume equivalent to about 60% of the final intended volume with a final carrot pomace concentration of 500 g/L.

One-thousand grams of defrosted carrot pomace was blended with 1250 mL of 1.1% H_2SO_4 as described previously. After cheesecloth filtration and neutralization

the volume of the mixture was measured and determined to be 1930 mL, due primarily to the water content contributed by the carrot pomace. The volume was topped off to make 2 L so that the final concentration of carrot pomace used to formulate the CM was 500 g/L, as intended.

Synthetic growth medium, PM2, containing 30 g/L glucose was used for the control condition. The condition testing only the carrot medium was referred to as “CM,” while the addition of 5 g/L glucose to the CM as “CM5” and the addition of 10 g/L glucose as “CM10.” All media were autoclaved, cooled, and titrated to a pH of 8.5. The experiment was run with biological triplicates.

4.1.4. Attached Growth Experiments

4.1.4.1. Attached Growth on Various Materials

The purpose of this experiment was to test the tendency of *A. protothecoides* cells to grow in attached form on the surface of various materials (Figure 4.2). The materials tested for biomass attachment were:

- Cotton string, about 1mm in diameter
- Polydimethylsiloxane non-porous hollow fibers, 0.3/0.5 mm (ID/OD)
- Porous sponge
- Reticulated polyether sponge with 20 parts per inch holes
- Sand with 0.3-0.7 mm diameter grain size, and
- Polyethylene biocarriers (MBBR, Aquaflex).



Figure 4.2: Samples of the materials used for attached growth testing; from left to right: string, hollow fiber, sponge, reticulated sponge, sand and biocarrier.

All materials were submerged in water to determine their amount equivalent to 5 mL in volume. Accordingly, a 5 mL amount of each material was added per culture. The materials were rinsed with deionized water, oven-dried (100°C), cooled and weighed before being placed in the 100 mL bottles intended for culturing and were autoclaved together. Synthetic growth medium PM1 containing 30 g/L initial glucose was prepared and autoclaved and seed culture was cultivated in advance, as previously described. Thirty-eight mL sterile media and 2 mL seed culture were added aseptically to each bottle, making an inoculation ratio of 5% (v/v). Each material was tested in duplicate. Additionally, 2 cultures were cultivated with no added materials to serve as a control making a total of 14 cultures.

Cultures were cultivated for 10 days in a dark shaking incubator set to 28°C and 120 rpm. At the end of the cultivation period all cultures were sampled to determine biomass. In the case of the control cultures, since no materials were added only suspended culture biomass was determined. Where materials were tested, the cultures were filtered with a filter set and light suction was applied. Additionally, the materials were pressed lightly to ensure that any remaining suspended culture would be displaced. The material samples were then transferred to pre-weighed beakers and freeze-dried until constant weight – about 2 days. The final beaker weights and the material samples' initial weights were used to determine the net biomass attached in each case. In addition, the suspended cultures were all sampled, measured for OD at 540 nm and their biomass was determined based on a previously established biomass-OD correlation (see 4.2.1.1).

4.1.4.2. Direct Lipid Extraction of String-Attached Biomass

The intent with the attached growth of the microalgal biomass was to simplify culture harvesting. With that, the expectation was that the biomass could be extracted for lipids directly in attached form without having to separate the biomass from the string. Accordingly, variations of the lipid extraction procedure were tested to determine how the direct extraction of string-attached biomass could be improved. *A. protothecoides* was cultivated heterotrophically with synthetic growth medium PM1 containing 30 g/L glucose and string. Duplicate cultures were run. Each culture contained four pieces of string, ca. 5 m in length, each weighing 1.1717 ± 0.0012 g. All strings were placed in methanol in an ultrasonic bath for 15 minutes to remove any residues that could potentially impact lipid extraction results downstream. They were

then rinsed with deionized water and oven dried (100°C) before weighing. The cultures were cultivated for 9 days in a dark shaking incubator set to 28°C and 150 rpm. At the time of harvesting the cultures were filtered using a filter set with light suction and the collected strings were pressed lightly to displace residual suspended culture. The string samples were collected in pre-weighed beakers and freeze-dried. The remaining suspended cultures were measured volumetrically and they were centrifuged, rinsed with deionized water, centrifuged again and freeze-dried in pre-weighed test tubes. Net dry weights were used to determine the suspended and attached biomass growth of the cultures.

Ten mL of chloroform:methanol (1:1) was added to each string-biomass sample and five variations of the lipid extraction procedure were applied, as summarized (Table 4.2). All procedures were ended with the previously described lipid extraction protocol, including separation of phases and evaporation of the chloroform phase to determine final lipid weight. It should be noted that procedures A and B both retained the samples in a water bath set to 50°C for 5 hours, the only difference being that procedure A had a magnetic stir bar stirring the string-solvent mixture and in procedure B the bottles containing the mixtures were shaken. In both cases, the purpose of the motion was to increase contact between the string and solvent and to improve the solvent's diffusion into the cells. The ultrasonic cleaner bath (SK3310HP, Kudos) was operating at a frequency of 53 kHz and ca. 35°C. Procedure D involved two complete rounds of extraction, meaning that phases were separated – methanol-water phases were discarded, chloroform phases were transferred and fresh solvent was added to the string samples for the second round of extraction. Finally, procedure E tested the use of procedure C as a pre-treatment for procedure A; phase separation was only conducted at the end of both procedures.

Table 4.2: Variations in the direct lipid extraction of string-attached biomass samples.

Procedure:	Description
A	50°C/5h with 500 rpm magnetic stirring with a stir bar
B	50°C/5h with 150 rpm shaking
C	15 minutes in an ultrasonic bath
D	Two rounds of extraction with Procedure C
E	Procedure C plus procedure A

4.1.4.3. String Amount to be Used with Carrot Medium

The purpose of this 10-day cultivation experiment was to test the addition of various amounts of string for attached culture growth, to determine what amount would be most appropriate. Carrot medium (CM) was prepared with 500 g/L carrot pomace, as previously described. Each condition was tested in 250 mL bottles containing 95 mL sterile CM plus 5 mL exponentially growing seed culture of *A. protothecoides*, making a total of 100 mL initial culture volume. All conditions were tested with duplicate cultures. The control had no string added, while the others had either 12, 6 or 3 g of string added. The string samples were placed in the culture bottles along with the CM prior to autoclaving so that all components would be sterile. The cultures were cultivated in a dark shaking incubator set to 25°C and 150 rpm for 10 days. At the end of the cultivation term suspended culture samples were transferred to pre-weighed test tubes, which were centrifuged. The cell pellets were rinsed with deionized water, centrifuged again and freeze-dried to determine final weights. At the time of harvesting the volume of suspended culture that remained for each was measured volumetrically and noted. This data was used to determine the amount of biomass that had accumulated in suspended form for each culture. The string samples were collected with a filter set using light vacuum, where the strings were pressed lightly to displace the residual suspended culture. The strings were later freeze-dried in pre-weighed containers to determine the attached biomass that had accumulated.

The string-attached biomass samples were extracted for lipids using chloroform:methanol, 1:1. Per the results of 0, lipid extraction with 50°C, 5 h heat treatment and magnetic stirring (Procedure A) was determined to be the most effective. When implementing this procedure here however, the stir-bars were found unable to stir the string-solvent mixture due to the larger amounts of string being extracted. Therefore, Procedure B, with slightly lower lipid extraction efficiency (statistically insignificant difference), involving 50°C, 5 h heat treatment in a shaker was implemented for the lipid extraction of string-attached biomass samples in this experiment. The amount of solvent added to the string samples was proportioned based on the amount of attached biomass to provide equal conditions between the groups. Accordingly, the 12, 6 and 3 g string samples were extracted with 100, 54 and 43 mL of solvent, respectively. They were all extracted in 100 mL bottles with tightly sealed caps to avoid solvent evaporation. At the end of the heat treatment the NaCl

solution was added and the mixtures were thoroughly stirred to ensure contact between all phases. The strings were then left submerged in the solvent-water mixtures overnight and phases were separated the following day. Strings were strained as best as possible to separate out the liquid fractions, which were centrifuged for phase separation. Per usual procedures the chloroform phases were transferred to beakers for drying and final weighing of lipid extracts. In this experiment chloroform phases were passed through a cotton filter to avoid any solid residues in the extract.

4.1.4.4. Suspended and Attached Growth Comparison using CM5

The purpose of this 7-day experiment was to compare the biomass and lipid production behavior of *A. protothecoides* cells when cultured heterotrophically with and without string. Carrot medium plus 5 g/L glucose (CM5) was used as the growth medium; it was prepared using 500 g/L carrot pomace, as previously described. Media was prepared so that 95 mL was autoclaved in 250 mL bottles, to which 5 mL exponentially growing seed culture was added making a 5% (v/v) inoculation ratio. A dark shaking incubator was used, set to 28°C and 150 rpm. Strings were washed with deionized water and oven dried (105°C) before taking final weights. Each piece of string weighed 0.9990 ± 0.0007 g and was wound up to avoid the pieces getting entangled (Figure 4.3.a). Based on previous testing, ca. 12 g of string was added per 100 mL of culture for the attached-growth condition. No string was added to the control cultures. The conditions were tested with duplicate cultures. Suspended culture samples were taken and centrifuged in pre-weighed test tubes to determine biomass content. After 7 days the remainder of the cultures were harvested, and the strings were thoroughly strained using a 60-mL syringe (Figure 4.3.b). All string samples were weighed on the day of harvesting. Afterwards, a portion of the samples were freeze-dried until constant weight, denoted as “dry.” The remaining samples were kept frozen (-18°C) and defrosted prior to extraction – referred to as “wet”. The string-attached biomass samples and suspended samples (harvested on day 7) were extracted for lipids in wet and dry form, as previously described.

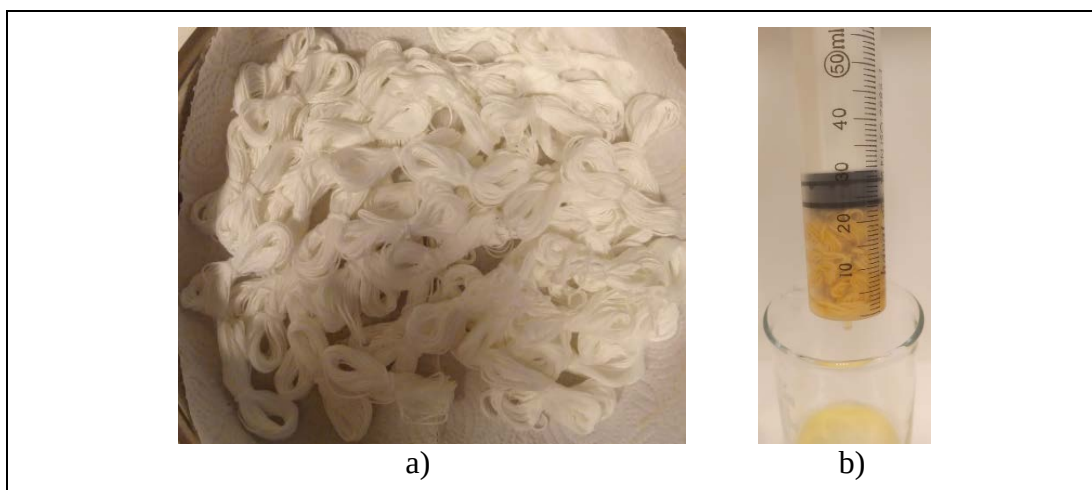


Figure 4.3: a) Pre-weighed string pieces that were wound up to avoid entanglement, b) string sample being strained in a 60-mL syringe.

4.1.5. Reactor Experiments

4.1.5.1. Batch Reactor Experiments with Carrot Medium

These initial batch experiments were conducted to establish the biomass and lipid production behavior of *A. protothecoides* when cultivated heterotrophically using carrot medium in an aerated and stirred system. Additionally, the stirring speed was varied as a parameter to optimize it with respect to maximizing lipid production.

A 1000 mL autoclavable amber bottle with a silicone stopper was used as the reactor chamber. The stopper had 4 vertically placed holes, through which autoclavable polyurethane tubing was passed (Figure 4.4). The tubes were dedicated ports for the transfer of air-in, air-out, fluid-in and fluid-out. The air source was connected to a pneumatic regulator set to 2 bar and the air flowrate was measured with a mass flowmeter at 0.32 L/min. The air was passed through a 0.45 μm sterile filter before entering the reactor. The tubing supplying the air was connected to a spherical aeration stone to provide fine bubbles. A 60x9 mm magnetic stir-bar was used to stir the culture on a stir-plate. The stirring speed was tested as 200, 300 and 400 rpm. The reactor was placed in a 2 L beaker containing water, which was used to monitor the temperature of the culture.

All experiments were conducted with carrot medium plus 5 g/L glucose denoted as CM5. The medium was prepared using defrosted carrot pomace as previously described. A total volume of 600 mL of CM5 was freshly prepared for each

experiment, where 475 mL was transferred to the reactor and 95 mL to another bottle. Both bottles were autoclaved and the extra CM5 was used to test the pH adjustment, which was conducted with filter-sterilized 4M NaOH solution. A pre-determined amount of titrant (220 μ L) was added to the extra CM5 in order to adjust its pH to 8.5 ± 0.2 ; after this pH was confirmed, a proportional amount of titrant (1.1 mL) was added to the reactor chamber. If pH was not found to be in the desired range, then the amount of titrant was modified slightly as needed.

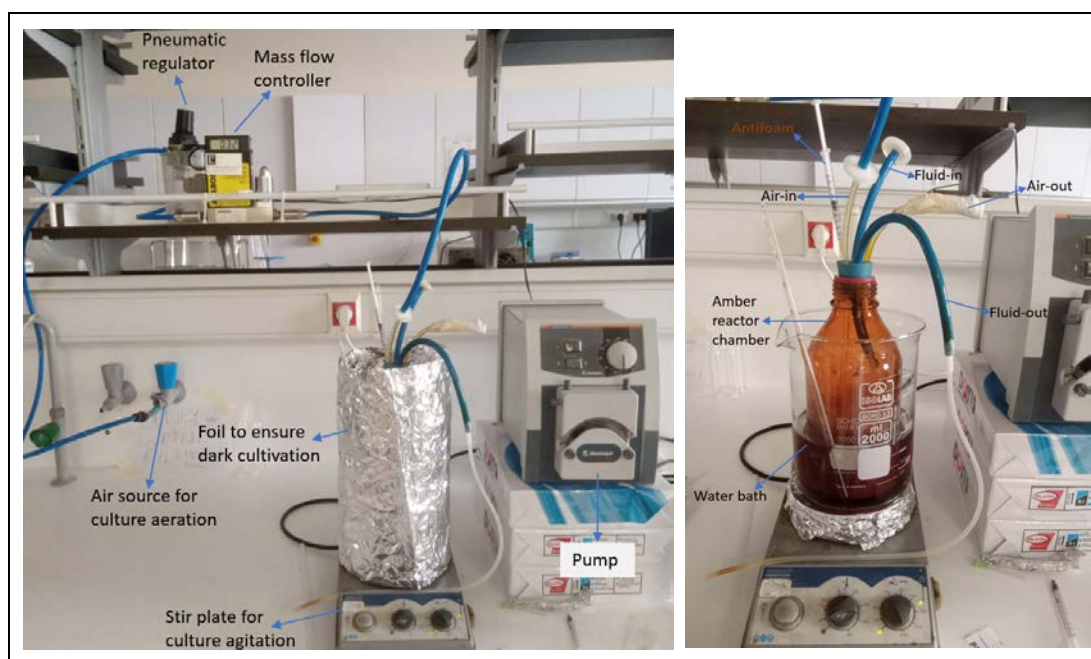


Figure 4.4: Reactor configuration overview (left) and detailed view (right).

Based on previous trials it was determined that upon aeration the culture had a tendency to produce a rising layer of foam. Various mechanical and chemical solutions were tested to resolve this issue. Use of a chemical foam-breaker, Antifoam Y-30 Emulsion (Sigma) containing 30% silicone, was determined to be effective and was implemented in all experiments. Antifoam solution was prepared by aseptically diluting the chemical foam-breaker to a concentration of 10% using sterile deionized water for easier handling. The antifoam solution was added to make an initial concentration of 0.3-0.4%; e.g., for a 0.3% concentration 1.5 mL of 10% antifoam solution was added.

Twenty-five mL seed culture was added for a 5% (v/v) inoculation ratio, making a final volume of 500 mL in the reactor chamber. The fluid-in port was used to add the titrant, antifoam solution and seed culture under a biosafety flow cabinet. After

each of these additions, sterile deionized water was used to flush the port. The amount of water added was pre-determined based on the autoclaving process, which was known to yield ca. 3% evaporation of water content.

Foam levels occasionally rose during the first 3 days of cultivation, at which times antifoam solution was added minimally, just enough to subside the foam, with a sterile syringe-needle transgressing the stopper. Samples were drawn using the fluid-out tube and a peristaltic pump. The first mL of culture drawn was discarded and about 10 mL was pumped into a pre-weighed test tube. The sampling tube was then flushed with autoclaved deionized water. The water added was intended to keep the tubing clean against the risk of contamination and to offset evaporation. The culture sample was measured for pH and was centrifuged at 9000 rpm. The sedimented cell pellet was rinsed with deionized water, centrifuged again and freeze-dried to constant weight. The dried biomass samples were later extracted to determine the lipid content and lipid output of the cultures.

4.1.5.2. Effect of Antifoam Solution

Preliminary experiments of culturing *A. protothecoides* in a reactor chamber with active aeration of the culture showed that there was a rising foam formed during cultivation. Testing demonstrated that a chemical foam-breaker, Antifoam Y-30 Emulsion containing 30% silicone, was effective. Foam-breaking chemicals are sometimes known to have a toxic effect on cultures when used at high levels. The purpose of this experiment was to determine a range of the foam-breaker that could be added to *A. protothecoides* culture without hindering growth or lipid production.

The microalga was cultured heterotrophically for 7 days. Synthetic growth media, PM2, was prepared containing 30 g/L initial glucose and its pH post-autoclaving was 7.7. The antifoam solution was prepared aseptically by diluting the chemical, Antifoam Y-30, at a 1:10 ratio using sterile deionized water. The 10% antifoam solution was added to the sterile media at concentrations of 0.1, 0.2 and 0.5%. That is, for each 100 mL batch of media the antifoam solution was added in the amount of 100, 200 or 500 μ L. As a control, media with no antifoam addition was also tested. Each condition was run with duplicate cultures. At the start of the experiment, it was noted that the antifoam addition affected the optical density of the media and therefore absorbance was not a reliable measure of biomass. Culture growth was tracked by taking 5 mL samples in pre-weighed test tubes, and, per previous procedures, rinsed

cell pellets were freeze-dried until constant weight. The final culture samples taken on day 7 were extracted for lipids, as described (4.1.8).

4.1.6. Biomass Determination

Culture samples were taken and filtered through pre-dried (100°C) 1.2 µm filter paper with light suction and rinsed with deionized water. The filtered samples were dried until constant weight in an oven set to 100°C for 12-24 hours, cooled in a desiccator and weighed to determine biomass content. Throughout the cultivation period samples were measured for absorbance on a spectrophotometer at a wavelength of 540 nm. Samples were diluted as needed to ensure that their absorbance was between 0.1-1. The linearity of measurement in this absorbance range was verified. Actual absorbance values were estimated by accounting for the dilution factors. These values were used to determine a linear correlation with biomass content so that biomass could be estimated based on absorbance.

For the studies involving carrot medium an alternative method was used to determine biomass. The culture samples were centrifuged in pre-weighed test tubes, rinsed with deionized water, centrifuged again and cell pellets were freeze-dried until constant weight (ca. 2 days). Centrifugation was applied at 9000 rpm. The difference in weights were used to determine the biomass content of the cultures.

4.1.7. Lipid Extraction Procedure Comparison

Ultrasonication is frequently used to facilitate the breakdown of microalgal cell walls to improve lipid extraction efficiency[219]–[221]. It has been our observation however, that ultrasonication alone can be ineffective in breaking up thoroughly freeze-dried biomass samples. A comparison was made using an *A. protothecoides* biomass sample to evaluate lipid extraction efficiency of various procedures (Table 4.3). Chloroform:methanol 1:1, an effective lipid extraction solvent [222], was used in all cases.

Table 4.3: Lipid extraction procedures implemented with *A. protothecoides* biomass to compare efficiency.

Lipid Extraction Procedures	Explanation
A	1 round of extraction with 20 minutes ultrasonication 2 rounds of extraction with 20 minutes ultrasonication
B	(The extraction procedure was completed with phase separation before applying the 2 nd round of ultrasonication.)
C	Wet grinding followed by 20 minutes ultrasonication
D	Wet grinding followed by 5 hours, 50°C with 300 rpm magnetic stirring

4.1.8. Lipid Extraction

Procedure D was implemented (Table 4.3). The term “wet grinding” was used to express that a small volume of solvent, ca. 500 μ L, was added to the biomass before grinding – just enough to wet the sample. This appeared to aid in fragmentation of the sample through shear force. Freeze-dried biomass was weighed and wet ground in a ceramic mortar using the round-bottom of a glass test tube. Then, more solvent was added and the mixture was transferred to a glass test tube with a magnetic stirrer. The total amount of solvent in each test tube was 7.5 mL. The test tubes were placed in a pre-heated, 50°C water bath on a hot plate, where stirring of 300 rpm was applied for 5 hours. The stirrers were then removed and 3 mL of 0.73% aqueous NaCl solution was added to each, making a final chloroform:methanol:water ratio of 1:1:0.8 [223]. The mixtures were mixed and centrifuged at 3000 rpm for 3 minutes for phase separation. The resulting top phases (methanol-water) were discarded and the bottom, chloroform phases were transferred to clean test tubes. These were centrifuged again to help separate any remaining biomass residues from the extract. Finally, the chloroform phases were transferred to individual beakers for final drying. These were left overnight on a hot plate set to 40°C under a venting fume hood to facilitate the evaporation of chloroform. The extracts were then placed in a 50°C oven for 1-2 hours and cooled in a desiccator before weighing. Extracts were carefully decanted using chloroform to resuspend the lipids, taking care to leave behind any biomass residues that were noticed on the beaker surfaces [224]. These beakers were again placed in an

oven set to 50°C for 1-2 hours, cooled in a desiccator and weighed. The decanted weights were used to determine the net extracted lipids and the biomass sample weights were used to determine the samples' lipid contents as a percentage of dry cell weight (%DCW). All solvents used for lipid extraction and lipid analysis were analysis-grade.

4.1.9. Lipid Analysis

Biomass samples that were harvested on day 10 of cultivation were used for this analysis. Approximately 170-200 mg of freeze dried biomass samples were extracted and the lipid samples were stored at room temperature until the following procedure was applied.

Lipid samples were separated using columns containing 1.5 g silica powder suspended in chloroform. The samples were dissolved in chloroform and loaded. 25 mL each of chloroform, acetone and methanol were applied in series to acquire the neutral lipid (NL), glycolipid (GL) and phospholipid (PL) fractions, respectively [146]. Samples were kept under a venting fume hood to ensure that the solvents were evaporated. They were then placed in a 50°C oven for 1-2 hours and cooled in a desiccator before weighing to determine the lipid fractions. The NL fractions were stored at room temperature until further analysis.

NL samples were separated on thin layer chromatography (TLC) plates (TLC silica gel 60, Merck, Germany) that were activated in a 100°C oven for 2 hours before use[146]. The TLC tank was prepared with a solvent mixture of n-hexane:diethyl ether:acetic acid (70:30:1). The NL samples were dissolved in chloroform and applied to the plates, which were kept in the tank to separate out the lipid classes. The plates were removed, dried, and placed in a second tank saturated in iodine vapor to visualize the bands. The bands were later scratched off, dissolved in chloroform, filtered, dried and weighed to determine the triacylglycerol (TAG) content of the NLs.

4.1.10. Statistical Analysis

Student's t test with unequal variance was used for the statistical analyses. A p value threshold of 0.05 was used to determine significance.

4.2. Results

4.2.1. Optimization Experiments

Based on preliminary growth experiments and the literature it was noted that the biomass and lipids produced with *A. protothecoides* could be improved by varying growth medium parameters. Therefore, the effects of initial glucose, pH, various growth medium components and temperature were investigated with the aim of maximizing the heterotrophic lipid output of *A. protothecoides*. Additionally, variations of the lipid extraction procedure were tested to maximize the lipids that could be recovered from the biomass samples. The optimized procedure was implemented for all experiments of suspended biomass samples.

4.2.1.1. Effect of Initial Glucose

Biomass data acquired with a filter test were plotted against absorbance values measured at 540 nm to determine a correlation (Figure 4.5). A linear relationship with an R^2 value of 0.976 was established as follows: $y = 0.144x + 0.746$, where y is biomass (g/L) and x is the OD at 540 nm. It should be noted again that culture samples were diluted as needed to achieve absorbance measurements between 0.1-1 and the dilution factors were accounted for to calculate the actual OD. This correlation was used to estimate the culture's biomass throughout the experiment. All data was acquired and used under heterotrophic cultivation conditions, where cultures had a yellow hue. When cultivated with light *A. protothecoides* cultures have a strong green hue, which may affect their absorbance values. Therefore, the correlation was only intended for use under consistent cultivation conditions.

The effect of initial glucose concentration on biomass and lipid output was determined. The growth phase was prolonged based on glucose content of the medium; growth continued until day 3, 4, 5 and 6 for the cultures with an initial glucose concentration of 10, 20, 30 and 40 g/L, respectively (Figure 4.6.a). There was an increase in the produced biomass corresponding to the increase in glucose. In contrast, the biomass:glucose yield decreased from 0.45-0.46 for 10 and 20 g/L initial glucose down to 0.38 and 0.32 for the 30 and 40 g/L conditions, respectively. This is expected since other essential nutrients become growth-limiting as the glucose content increases

beyond the stoichiometrically required amount. The maximum biomass achieved was 12.2 g/L, which was with the 40 g/L initial glucose condition.

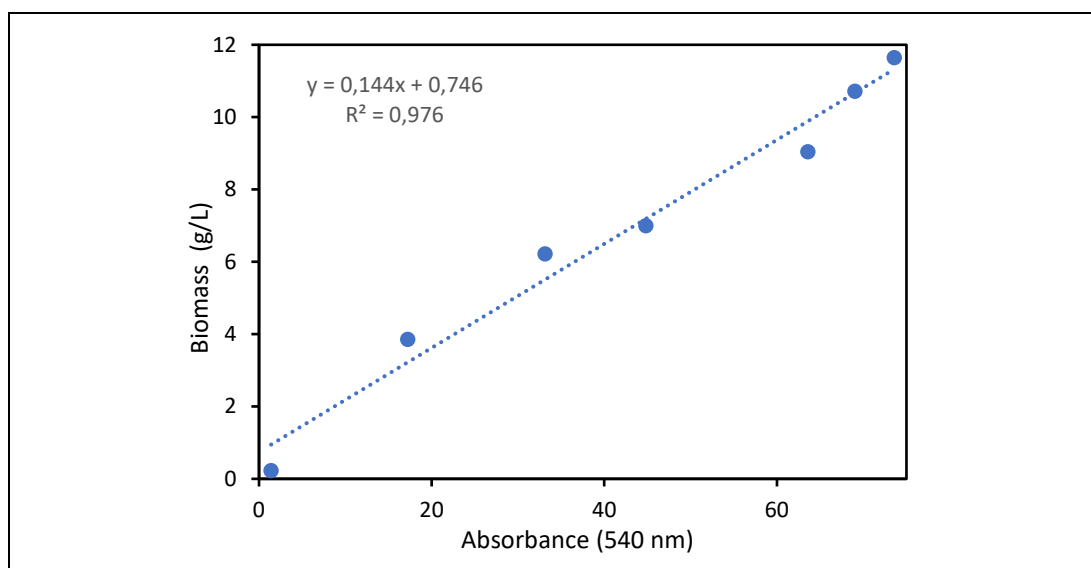


Figure 4.5: Linear correlation determined between the absorbance values and biomass of heterotrophically cultivated *A. protothecoides* culture samples.

There was a direct correlation between the initial glucose concentration of the growth medium and the resulting lipid content of the cultures. The highest lipid contents recorded for the 10, 20, 30 and 40 g/L glucose conditions were, respectively, 19.7, 22.9, 28.9 and 31.5, as a percentage of dry cell weight (%DCW). Such a trend is common since the increase in C/N ratio leaves the cells with an excess of glucose that is converted to lipids [225]. The 10 g/L glucose condition showed a decrease in lipid content throughout the cultivation period, while the 20 and 30 g/L conditions showed an increase until day 5 followed by a decrease. The exception was with 40 g/L glucose, where the lipid content increased until day 10. The decrease in lipid content may indicate that the cells are sufficiently depleted of carbon and start to use up their stored lipids. The maximum lipid output was also directly correlated with the initial glucose of the culture medium. The highest lipid:glucose yield was 0.11 and was achieved with the 30 g/L initial glucose condition. Therefore, 30 g/L was selected as the optimal glucose concentration for the growth medium and was implemented accordingly in the subsequent studies.

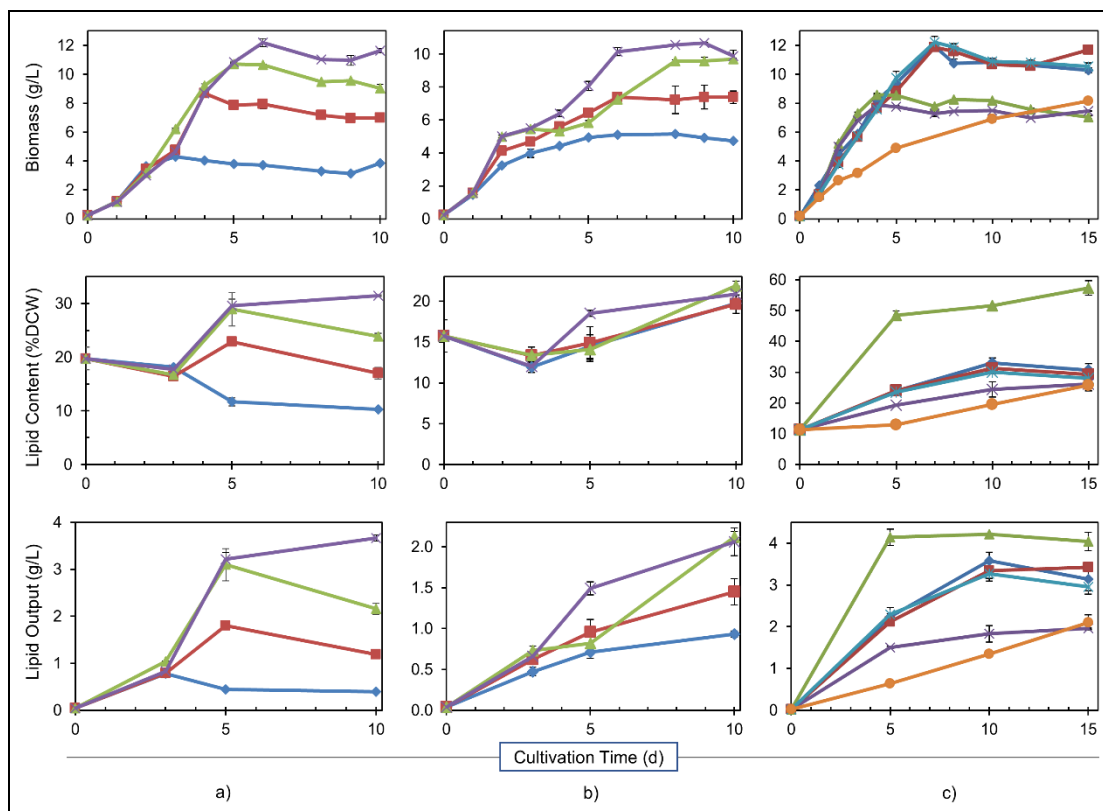


Figure 4.6: Biomass and lipids achieved during heterotrophic cultivation of *A. protothecoides* under various conditions. a) Effect of initial glucose (g/L) of the growth medium; 10 (diamonds), 20 (squares), 30 (triangles), 40 (X's). b) Effect of initial pH of the growth medium; 4.4 (diamonds), 6.4 (squares), 7.4 (triangles), 8.4 (X's). c) Effect of growth medium components and temperature; Control (diamonds), NaCl (squares), Urea (triangles), Sulfate (X's), Iron (Ж's), Room Temperature (circles).

4.2.1.2. Effect of Initial pH

The effect of growth medium initial pH on biomass and lipid yield were determined. The biomass gained was positively correlated with an increase in initial pH (Figure 4.6.b). The maximum biomass content achieved in the cultures during a 10-day cultivation period were 5.2, 7.4, 9.7 and 10.7 g/L with initial pH values of 4.4, 6.4, 7.4 and 8.4, respectively. The higher initial pH may have better supported cell growth due to the sensitivity of cellular enzymes to pH [226] and because a higher pH could better balance the acidic compounds produced during the exponential growth phase. The lipid content of the cultures were close in value and did not show a correlation to the initial pH. The highest lipid content determined was 21.9%, which was lower than the 30 g/L condition in the comparison of initial glucose (see 4.2.1.1). This can be explained by the fact that the lipid contents in this pH study were still on

the rise by day 10 and may have continued to rise given a longer cultivation time. This difference in lipid content trend may be due to the metabolic state of the seed culture used to start the experiment. The lipid yield of the cultures improves with an increase in initial pH; the highest lipid output, 2.1 g/L, was achieved on day 10 by the cultures with an initial growth medium pH of 7.4 and 8.4 (p value < 0.05).

Overall, the most conclusive effect we deduced was, that an increase in the initial growth medium pH in the range of 4.4 to 8.4 resulted in a positively correlated increase in biomass but had no discernible impact on lipid content. Moreover, based on the maximal lipid yield, the optimal initial pH range was determined as 7.4-8.4 and was implemented as such in subsequent studies.

4.2.1.3. Effect of varying growth medium and temperature

Variations of the growth medium components were tested with the aim of improving the culture's lipid production capacity. Variations tested were based on the literature. The conditions will be referred to shortly as follows: NaCl (1 g/L added), Urea (1.35 g/L added in place of yeast extract), Sulfate (9.8 instead of 147 mg/L), Iron (13.5 instead of 3 mg/L) and RT (22°C cultivation rather than 28°C, control media).

The NaCl and Iron groups exhibited growth behavior very similar to the control. All three showed exponential growth until day 7, followed by a stationary phase until day 15 (Figure 4.6.c). The maximum biomass content of the NaCl, Iron and Control groups were 11.9, 12.2 and 12.0 g/L, respectively. In contrast, the Urea and Sulfate groups grew steadily until day 4, followed by a stationary phase with a slight decrease in biomass until day 15. The highest biomass achieved in the Urea and Sulfate groups was 8.6 and 7.8 g/L, which was 72 and 65% of the control, respectively. Finally, the RT cultures grew significantly slower and reached a maximum biomass of 8.2 g/L on day 15, which was 68% of the control. The RT cultures were tracked for three more days and it was confirmed that their growth was complete (data not shown).

The lipid content of the NaCl and Iron groups were very close in value to the control throughout the cultivation period (Figure 4.6.c). The highest lipid content determined in the NaCl, Iron and control groups were 31.3, 30.0 and 33.0%, respectively, achieved on day 10. The Sulfate and RT groups were significantly lower than the others and had a maximum lipid content of 26% on day 15. The RT group showed a much more gradual rise in lipid content, possibly due to the slower biomass growth and depletion of nutrients. In contrast, the Urea group had a significantly

higher lipid content compared to all others (p value < 0.05); a maximum of 57% was achieved on day 15. No correlation was noted between the final pH and lipid content of the cultures.

The lipid output of the NaCl and Iron groups followed closely in value to the control (Figure 4.6.c). The maximum yields achieved for the NaCl, Iron and control groups were 3.4, 3.3 and 3.6 g/L lipids, respectively. In contrast, the Sulfate and RT groups outputted a significantly lower amount of lipids (p value < 0.05), 2.0 and 2.1 g/L, respectively, which were both achieved by day 15. The highest amount of lipids was produced by the Urea group due to it having the highest lipid content – 4.2 g/L lipids by day 10, which is only slightly higher than the 4.1 g/L achieved by day 5. The results of the Urea group are encouraging – potentially even higher lipid yields could be accomplished if the biomass output were also improved. The growth medium recipe was updated to include 1.35 g/L urea without the addition of yeast extract; the medium was referred to as PM2 and used as the control in the studies that followed.

Since all cultivation studies were conducted at 28°C, the RT condition was tested to evaluate if this temperature difference would have a significant impact on results. The maximum lipid produced under RT was 59% of that produced by the control. Adding to that, the lipids were produced at a slower rate in the RT group, only reaching a maximal value by day 15, as opposed to the control peaking by day 10. Therefore, 28°C remained as the preferred cultivation temperature.

4.2.2. Lipid Extraction Procedure Comparison

In comparing lipid extraction procedures the use of one round ultrasonication (Procedure A) was found to be the least effective, yielding only 11.0% lipid content. Two rounds of extraction with ultrasonication (Procedure B) improved the lipid content to 13.5%; however, the addition of a wet grinding step preceding the ultrasonication (Procedure C) yielded a larger improvement, resulting in 14.8% lipids. Compared to the complete re-extraction of a sample, wet grinding is a simple step that can be added to increase the lipid extraction efficiency of an existing procedure. A significantly higher lipid content, 19.0%, was achieved with wet grinding followed by a 5-hour, 50°C heat treatment with 300 rpm magnetic stirring (Procedure D, p value < 0.05). This method's effectivity is presumably owing to the mixing that promotes good contact between the biomass and solvent phases and the application of heat,

which increases vapor pressure and diffusion kinetics. From a practicality standpoint, the method does not require much manual effort and allows for the extraction of many samples simultaneously without the use of a device prone to overheating. In terms of scalability, appropriate equipment could be utilized to automate such a grinding procedure. Procedure D was implemented in all subsequent studies of suspended culture samples.

4.2.3. Carrot Medium Experiments

Utilization of carrot pomace, a waste by-product of the juice industry, was investigated with the aim of reducing the cultivation cost of microalgae-derived biodiesel. Our preliminary studies showed that liquid medium produced using carrot pomace contained glucose, fructose and sucrose. *A. protothecoides* was seeded in such a carrot medium under heterotrophic conditions and it was observed that the species utilized the glucose and fructose but the sucrose content remained unchanged. Therefore, it was decided that acid hydrolysis could be applied as a practical and economical method to hydrolyze the sucrose. Acid hydrolysis parameters were tested to maximize the monosaccharide content achievable from the carrot pomace-derived growth medium.

The use of carrot medium (CM) for cultivation was initially tested by partially replacing the synthetic growth medium – in proportions of 50, 75 and 100%. Once it was confirmed that 100% CM could be used for cultivation, minimal additions of glucose were tested to determine if the lipid output could be improved.

4.2.3.1. Carrot Pomace Pre-Treatment

In the acid hydrolysis trials for the preparation of carrot medium, RT, 60°C and 90°C were compared as a treatment condition for 200 g/L carrot pomace with 0.4% sulfuric acid. The reducing sugar content for the RT, 60°C and 90°C groups were 3.2, 8.5 and 10.2 g/L as glucose (Figure 4.7). The 90°C condition was determined to be the most effective to promote the hydrolysis of sucrose (p value < 0.05). One hour treatment with 100°C was also tested and showed no significant improvement in the total reducing sugar yield compared to 90°C. A comparison of heat treatment time at 90°C showed a slight increase between 1, 2 and 3 hours (p value > 0.10). This may

have been due to the increasing evaporation from the samples and based on the minimal increase in sugar yield, 1-hour heat treatment was deemed sufficient.

The concentration of carrot pomace was increased to 600 g/L to yield sugar content close to 30 g/L to produce a medium comparable to the synthetic growth medium (control). The effect of sulfuric acid concentration was compared for 600 g/L carrot pomace with 90°C, 1-hour heat treatment. Reducing sugar content was improved up to 0.8% acid – 31 g/L as glucose (Figure 4.7). There was no significant difference in the sugar content achieved between 0.8 and 1.2% acid (p value = 0.92). Therefore, the acid hydrolysis conditions to prepare the carrot medium were set as 600 g/L carrot pomace, 90°C for 1 hour with 0.8% acid. Note that the intent was to use a minimal concentration of acid due to the potential growth inhibition that may occur with increased salt concentration after acid neutralization.

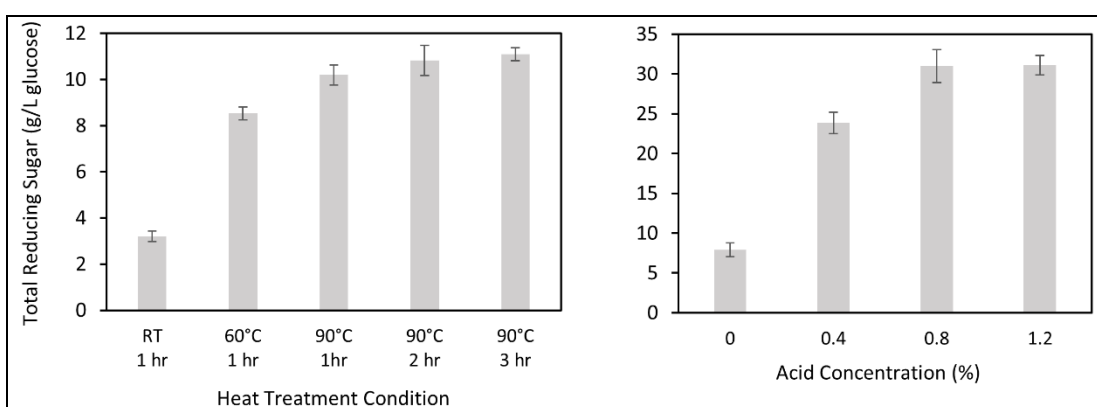


Figure 4.7: Total reducing sugars as a result of varying acid hydrolysis conditions; effect of temperature and time with 0.4% sulfuric acid (left), effect of sulfuric acid concentration with 90°C, 1 hour heat treatment (right).

4.2.3.2. Use of carrot pomace for microalgae cultivation

The initial study with carrot medium (CM) intended to explore whether it was possible to replace the synthetic growth medium that is used to produce *A. protothecoides* biomass and lipids. The carrot pomace used to constitute the CM was obtained directly from a juice producing company, GE-TA Tarım. A comparison was made between the use of 100% PM2, 100% CM and combinations of the two, where 50:50 and 75:25, CM:PM2, were tested. PM2 contained 30 g/L glucose. The CM reducing sugar content was estimated to be 30 g/L as glucose. The algal biomass produced during cultivation was estimated by subtracting off the “carrot mass” that

was known to exist as part of the autoclaved CM. That is, it was determined that the CM had a 1.88 g/L solid content after being centrifuged and freeze-dried and therefore, this amount was deducted depending on the CM fraction of the culture group. The results were promising. All cultures had achieved their highest (algal) biomass content by day 5 – 10.5 g/L for the control and 12.3, 10.8 and 10.7 g/L for the 50, 75 and 100% CM conditions, respectively (Figure 4.8). Especially the use of 100% CM indicates that growth medium containing carrot pomace waste alone as the sole source of nutrients and minerals could produce algal biomass equivalently to synthetic growth medium, contributing a great savings in the production cost of this potential biodiesel feedstock. It should also be noted that the biomass produced with the control (PM2) was improved from a maximum of 8.6 g/L to 10.5 (see 4.2.1.3), due to the increase in initial pH from 7.5 to 8.5. Once again, this supports our finding that *A. protothecoides* favors a more basic initial pH.

The algal lipid content of the cultures grown using CM were estimated by adjusting for the lipid contribution of the CM. The lipid content of the CM conditions was significantly lower than the control (p value < 0.05) – the maximum for the 50, 75 and 100% CM being 35.4, 35.9 and 32.3%, while the control was 47.7%. Naturally, the lipid output of the CM cultures was also lower than the control (p value < 0.05); the 50, 75 and 100% CM groups yielded a maximum of 3.6, 3.4 and 3.2 g/L lipids, respectively, while the control outputted 4.9 g/L. Nevertheless, the fact that 3.2 g/L microalgal lipids could be produced with a growth medium made using only a juice industry waste is still remarkable.

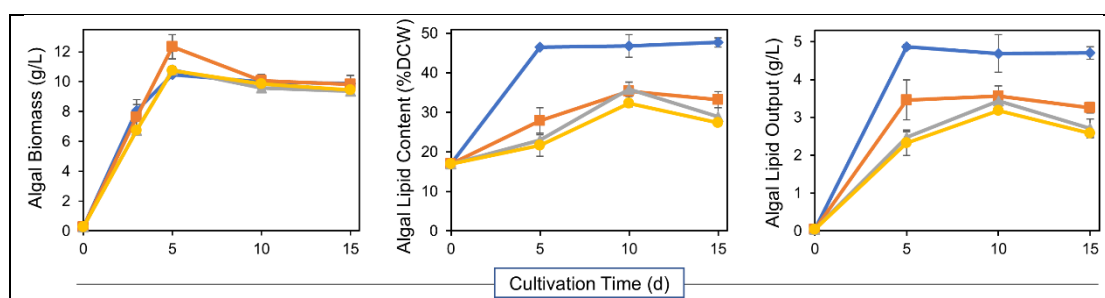


Figure 4.8: Biomass and lipid results of *A. protothecoides* culture grown using various combinations of carrot medium (CM) and synthetic growth medium (PM2); 100% PM2 (diamonds), 50:50 (squares), 75:25 CM:PM2 (triangles) and 100% CM (circles).

Furthermore, an advantage of heterotrophic cultivation of microalgae is that there is no dependence on light. Therefore, the growth medium does not need to be translucent. The carrot medium produced in our experiments was fairly opaque and this did not serve any hindrance to the culture's growth. Photographs of the cultures at the end of the experiment and the microscope images are shown (Figure 4.9). Microscopically it can be seen that *A. protothecoides* cells have regular morphology when growing alongside fragments of carrot pomace.

The *Chlorella* culture having grown so well in 100% CM with no need for trace mineral or vitamin additions implies that the CM provided a rich, nutritious medium, which may explain why the lipid content was lower. Additionally, the C/N ratio for the CM was determined to be 19, while it was 22 for the synthetic growth medium. This could also be a reason for the lower lipid content associated with the CM. Therefore, on the basis of these results it seemed worthwhile to explore if a small addition of glucose could improve the lipid content and yield to be achieved utilizing 100% CM. Accordingly, a CM study with an addition of glucose ensued (see 4.2.3.3).

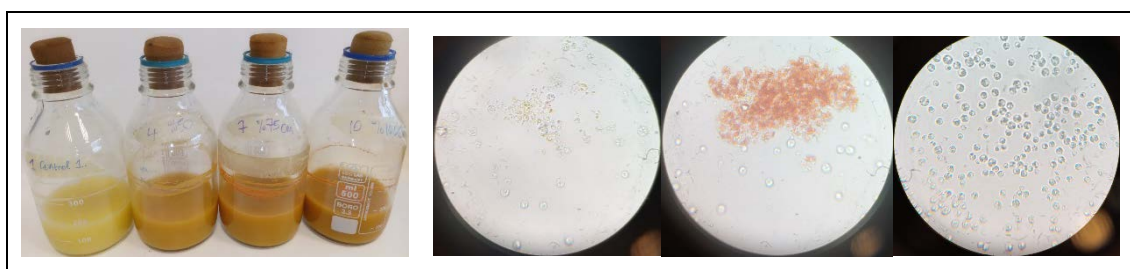


Figure 4.9: Photographs of the cultures at the end of the cultivation experiment (left), and microscope images of the microalgal cells (right).

4.2.3.3. Glucose addition to 100% carrot medium

The CM used for this experiment had a total reducing sugar content of 31 g/L as glucose. Control and CM cultures continued to grow until day 5, followed by a slight decrease in biomass until day 10 (Figure 4.10). The other groups displayed a more prolonged growth phase; CM5 reached peak biomass by day 7 and CM10 by day 10. This is logical since those groups had a higher sugar content. The algal biomass values were estimated, as previously explained, by deducting the centrifugable solids contributed by the CM, which was determined to be 1.98 g/L. The maximum algal biomass achieved by the control, CM, CM5 and CM10 groups, respectively, was 11.6,

10.9, 12.3 and 12.0 g/L. This corresponds to 21.8, 24.6 and 24.0 g algal biomass produced per kg wet carrot pomace for the CM, CM5 and CM10 conditions, respectively. The CM5 condition yielding a higher biomass than the CM indicates that the CM can support greater algal growth based on its non-carbon nutrient content.

The algal lipid content of the CM cultures showed a similar trend – a continuous increase until day 10 with maximum values of 49.9, 54.4 and 48.1%, respectively, for CM, CM5 and CM10 conditions (Figure 4.10). The control group achieved its highest lipid content of 53.1% by day 7. Although the lipid content of the CM groups was slower to rise compared to the control, the values are close in range. Additionally, it was noted that the lipid content of the CM alone was greatly improved (see 4.2.3.2) from 32.3 to 49.9%. This can be explained by the increase in C/N ratio from 19 to 31 owing to the change in acid hydrolysis conditions.

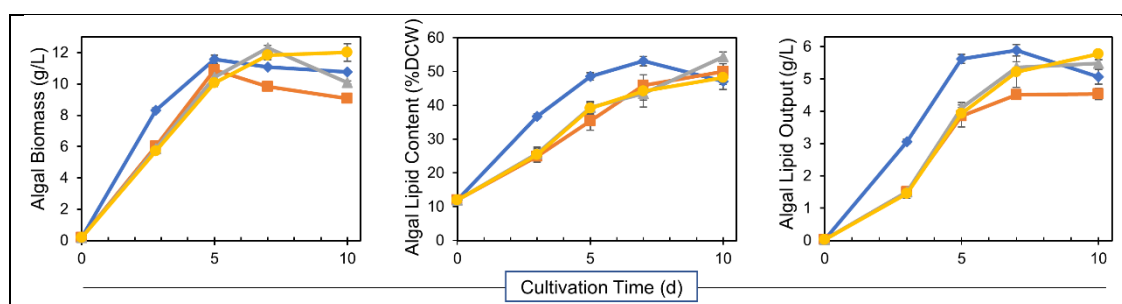


Figure 4.10: Biomass and lipid results of cultures grown using 100% carrot medium (CM) and additions of glucose; synthetic growth medium PM2 (diamonds), CM (squares), CM plus 5 g/L glucose (triangles) and CM plus 10 g/L glucose (circles).

Similarly, the algal lipids outputted by the CM culture groups showed an increase until day 10, while the control had a quicker rise and peaked by day 7. The maximum algal lipids produced for the control, CM, CM5 and CM10, were 5.9, 4.5, 5.5 and 5.8 g/L, respectively. Overall, the addition of glucose to the CM has led to an increase in the lipid produced by those cultures. Whether or not the increase in lipid output is worth the additional cost of the added glucose should be considered thoroughly with respect to the costs of the entire process. Here, the lipid:glucose yield was compared based on the glucose supplemented and the lipids produced as a result of that additional glucose. That is, the lipid amount produced by the CM group, 4.5 g/L, was deducted from the results of the CM5 and CM10 groups in order to assess the impact of only the added glucose. Accordingly, the lipid:glucose yields were 0.21, 0.20 and 0.13 for the control, CM5 and CM10 conditions, respectively (Table 4.4).

Compared to the optimized synthetic growth medium, the CM5 condition has nearly an equivalent yield (0.20 vs. 0.21, p value: 0.69) from the supplemented glucose with no other nutrients added. This indicates that the 5 g/L glucose added under the CM5 condition was efficiently converted to lipids.

Table 4.4: Summary of results with various carrot medium (CM) conditions, including the lipid:glucose yield.

Group	Max. Algal Lipids (g/L)	Glucose Supplemented ^a (g/L)	Increase in Lipids ^b (g/L)	Lipid:Glucose Yield
Control	5.88	28.5	-	0.21
CM	4.53	0	-	-
CM5	5.48	4.75	0.95	0.20
CM10	5.78	9.5	1.25	0.13

^aThe glucose supplemented was estimated based on the inoculation ratio of 5%, v/v, with a seed culture containing negligible glucose.

^bThe increase in lipid output due to the addition of glucose – this was estimated by deducting the lipid output of the CM group from the results.

As mentioned before, the CM contains some amount of solids that settle after being centrifuged (1.98 g/L), and these CM solids have a lipid content of 20.5% – so that, the carrot medium itself makes a 0.4 g/L lipid contribution. When the culture samples were centrifuged, it was consistently noted that the algal biomass settled at the bottom, while the CM solids settled above the algal biomass (Figure 4.11). Therefore, it is always an option that the CM solids could be removed if not desired as part of the final product, i.e., the biodiesel feedstock. For CM and CM5 conditions, two separate lipid analyses were conducted: (i) with algal biomass only and (ii) with algal biomass and CM solids together.



Figure 4.11: A photograph of centrifuged culture samples that were cultivated with carrot medium; the yellow algal biomass settles at the bottom, while the orange carrot solids settle on top.

Lipid analysis was initially conducted in columns of plastic material, which were later deemed inappropriate as the solvents could leach residues from these materials.

The chromatography analysis was then repeated with glass columns to separate the lipid samples into their NL, GL and PL fractions (Figure 4.12.a). The NL samples were later run on TLC plates to further separate the lipid classes (Figure 4.12.b-d). The plates shown are from the control (b), CM5 (c) and CM5 including CM solids (d) groups. The distribution of the lipid bands looks similar between the groups. The lipid bands were visualized with exposure to iodine gas. The iodine only forms bonds temporarily and later volatilizes, thus, not impacting the weight of the bands. The thick bands known as TAGs were outlined lightly as shown and scratched off along with all other bands on the plates to determine the TAG content of the NLs.

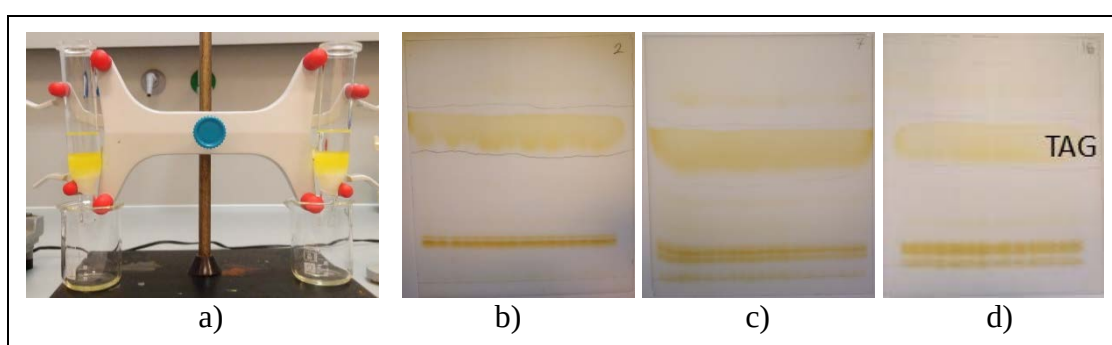


Figure 4.12: a) Photographs of lipid analysis; glass column chromatography being used to separate the lipid fractions and b-d) TLC plates after NL samples were run.

The lipid analysis revealed that the neutral lipid (NL) content was quite high, > 90% for all groups (Figure 4.13). The CM group had a slightly lower NL content compared to the control, but not significantly so (91.6 vs. 93.9%, p value: 0.14). The CM and CM5 samples including carrot solids had NL contents only 1% lower than when algal biomass alone was analyzed (p value > 0.20). The remaining lipid content was composed more so of phospholipids and minimally of glycolipids. The TAG/NL fraction was also fairly high for all conditions, 73-87%. Overall, the fraction of TAGs in the lipids was 76.4, 70.8, 81.3, 78.6, 66.5, 78.4% for the control, CM, CM5, CM10, CM (incl. CM solids) and CM5 (incl. CM solids), respectively. As expected, the TAG fractions were slightly lower when carrot solids were included in the analysis, compared to when algal biomass alone was sampled. Nevertheless, the CM5 and CM10 groups had higher TAG content than the control. High TAG content is essential for efficient transesterification of lipids to biodiesel fuel [227].

The TAG content of the CM5 group being so high, even when CM solids are included, would indicate that the CM solids do not need to be separated from the algal

biomass and can be included as part of the biodiesel feedstock. Therefore, with a 78.4% TAG content the *total* lipid yield of the CM5 group was 5.9 g/L (11.8 g lipids / kg wet carrot pomace), while the synthetic growth medium (control) yielded 5.9 g/L lipids with a 76.4% TAG content. The total lipid yield and corresponding TAG content while utilizing carrot pomace with only the addition of 5 g/L glucose was equivalent (p value > 0.50) to that of synthetic growth medium containing 30 g/L glucose.

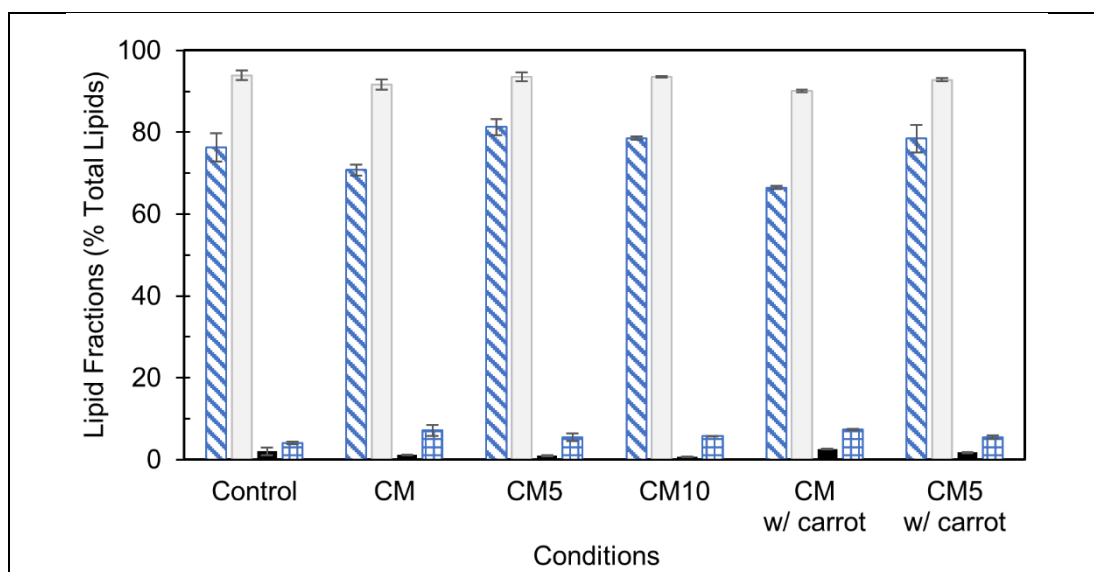


Figure 4.13: Distribution of lipid fractions as a percentage of lipids. All samples analyzed were from day 10 of the glucose addition experiment. Control: synthetic growth medium PM2, CM: carrot medium, CM5: CM plus 5 g/L glucose, CM10: CM plus 10 g/L glucose, CM w/carrot: CM with carrot medium solids, CM5 w/carrot: CM5 with carrot medium solids. Triacylglycerol (diagonal stripes), neutral lipids (gray), glycolipids (black), phospholipids (grid).

4.2.4. Attached Growth Experiments

The prospect of cultivating microalgae in attached form rather than suspended in the growth medium offers the primary advantage of alleviating the harvesting bottleneck from the processing of microalgae-derived biodiesel. The potential for heterotrophic, attached growth of *A. protothecoides* was investigated. Initially, various materials were tested to determine the cells' affinity for attachment. Then, with the chosen material (string), direct lipid extraction of attached biomass and variations in the amount of material used were tested to determine how usage of attached biomass could be optimized.

4.2.4.1. Biomass Attachment on Various Materials

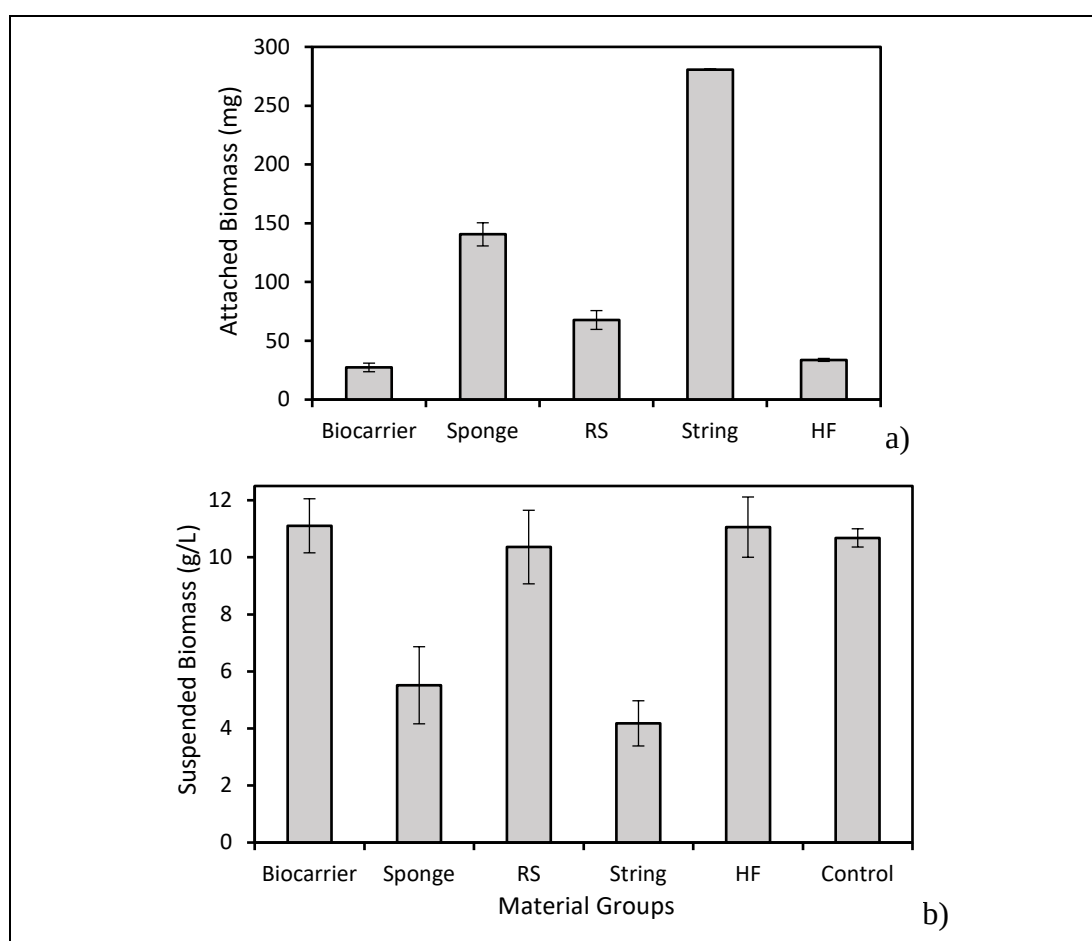


Figure 4.14: a) Attached and b) suspended biomass growth of *A. protothecoides* after 10 days heterotrophic cultivation with the addition of various materials. RS: reticulated sponge, HF: hollow fiber.

The amount of biomass attachment achieved with the different materials during the cultivation experiment was compared. Of the 6 materials tested, sand was eliminated from consideration due to the limited attached growth and the difficulties in handling and transferring the material. With an average of 280.7 mg increase in dry weight, cotton string had significantly higher biomass attachment than all other materials (p value < 0.05 , Figure 4.14.a). Accordingly, the suspended biomass of the cultures with added string was significantly lower than the control – 4.2 vs. 10.5 g/L (p value < 0.05 , Figure 4.14.b). This is expected; a portion of the biomass accumulated in attached form, therefore, there was less biomass in suspended form. In contrast, the biocarrier, reticulated sponge (RS) and hollow fiber (HF) groups had low attached biomass but their suspended culture values were in the same range as the control with

no significant differences (p value > 0.20). Based on these results, the cotton string appeared to be the best material choice for cellular on-growth. Additionally, string has the added advantages of being environmentally friendly, of minimal cost, potentially reusable and easy to handle and dimension.

4.2.4.2. Direct Lipid Extraction of String-Attached Biomass

Five variations of the lipid extraction procedure were tested in an effort to maximize the lipids that could be recovered from the string-attached biomass samples. Use of the ultrasonic bath alone appears the least effective; the lipid content recovered with one and two extraction cycles using the ultrasonic bath (procedures C & D) resulted in only 6.7 and 9.2%, respectively (Figure 4.15). The highest lipid content, 23.2%, was recovered with procedure A, which implemented 5 hours in a 50°C water bath with magnetic stirring to mix the string and solvent. This outcome was only slightly higher than procedure B, where the bath conditions were the same but shaking of the bottles was applied. Finally, procedure E, where procedure C was used as a pre-treatment for procedure A, yielded 22.5% with no significant difference compared to procedure A alone (p value > 0.30). Therefore, use of the ultrasonic bath as a pre-treatment step was determined to serve no advantage. Procedure A was deemed the most effective for direct lipid extraction of string-attached biomass samples.

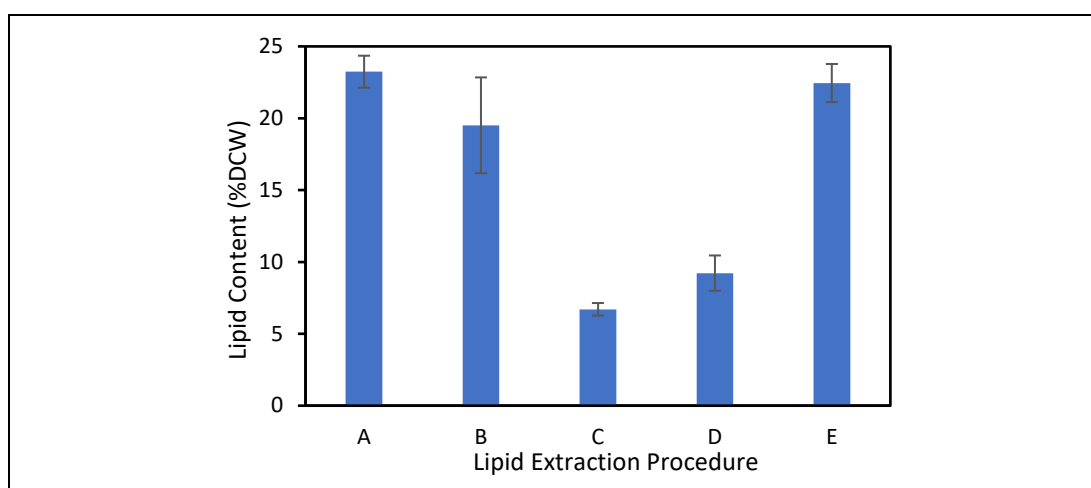


Figure 4.15: Lipid content achieved from the lipid extraction of string-attached biomass samples using various procedures; 50°C/5h with magnetic stirring (A), 50°C/5h with shaking (B), 15 min in ultrasonic bath (C), 2 rounds of ultrasonic bath extraction (D) and a combination of procedures C and A (E).

In addition to the testing with the string samples the suspended biomass collected from the cultures were also extracted for lipids. The previously established protocol was used, which involved wet-grinding of samples followed by a 50°C, 5 h heat treatment with magnetic stirring in chloroform:methanol, 1:1. In this case, the lipid extraction protocol is the same as Procedure A in the comparison of direct extraction of string-attached biomass samples, making the results very comparable. The lipid content achieved with the extraction of the suspended samples was 38.6%, which is significantly higher than the lipids recovered with Procedure A, 23.2% (p value < 0.01). If we assume that the cells attached onto the string material have the same lipid content as the cells growing in suspended form, this would indicate that a greater amount of lipids could be recovered from the string-attached biomass samples and that the current procedure does not sufficiently extract lipids from the cells. Further testing with the lipid extraction protocol of string-attached biomass samples can shed light on this. It is also possible that the cells in attached form have lower lipid content than the suspended cells. It has been observed that the cotton string absorbs the growth medium, which probably facilitates growth of cells on its surface. For that same reason, the attached cells might have better access to nutrients than the suspended cells, possibly taking longer to reach a nutrient-deprived, stressed, lipid-accumulation-promoting metabolic state.

4.2.4.3. String Amount to be Used with Carrot Medium

The amount of string to be added to the growth medium was tested to determine if an optimal amount could better promote biomass attachment. The amount of biomass that was accumulated in attached and suspended forms for the various conditions was compared (Figure 4.16). There was a slight increase in the attached biomass accumulated with 6 g compared to the 3 g condition (p value < 0.05). However, there was a steep increase in attached biomass when the amount of string used was 12 g (p value < 0.05). The percentage of attached biomass when compared to the total increases with increasing amount of string; the highest ratio of attached biomass, 87.0%, was achieved with the 12-g string group. The total amount of biomass accumulated was 1.3 grams for the 3- and 6-g string groups and 1.8 grams for the 12-g string group. This would indicate that the presence of more string in the carrot medium promoted the culture's growth in the case of adding 12 g per 100 mL. Note

that a higher amount of string (> 12 g / 100 mL) was not tested since the string takes up significant volume and can eventually exceed the water level.

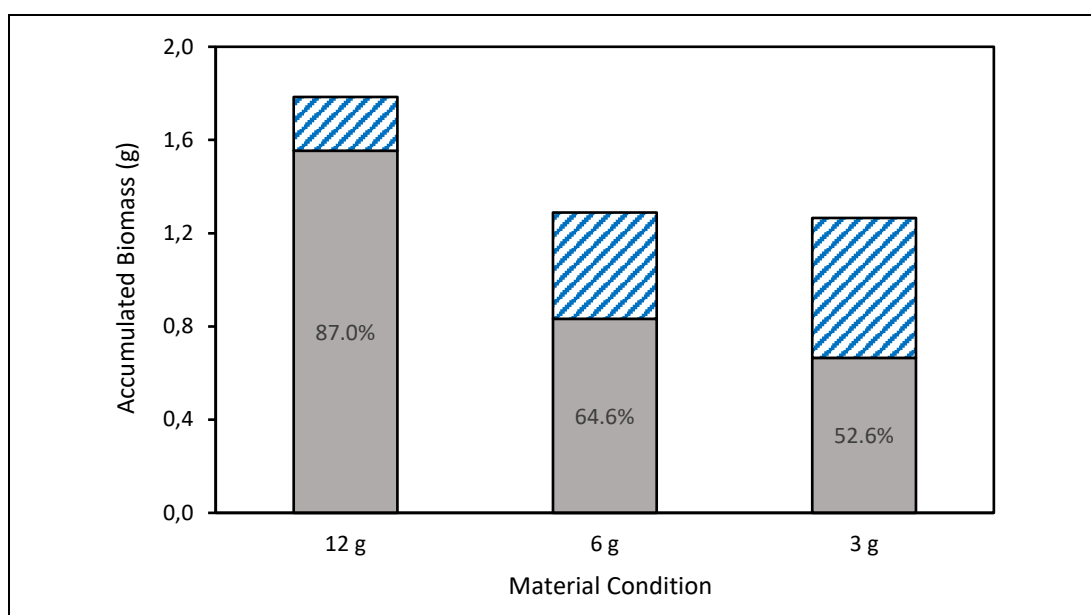


Figure 4.16: Biomass accumulated after 10 days heterotrophic cultivation of *A. protothecoides* using carrot medium with string added for biomass-ongrowth. Both attached (solid) and suspended (striped) fractions are shown. Either 12, 6 or 3 g of string were added for 100 mL of culture, as labeled. The percentages indicate the fraction of attached:total biomass.

The string samples were extracted directly for lipids. The amount of organic solvent used was proportional to the amount of string-attached biomass in order to provide equal extraction conditions. The average lipids extracted were 297 mg for the 12-g and 149 mg for the 3- and 6-g string groups (Figure 4.17). Once again, this shows the advantage of using 12 g of string per 100 mL of carrot medium. A higher total amount and attached fraction (87.0%) of biomass was accumulated, which did not need to be harvested with traditional methods used for suspended biomass. Instead, merely straining the string was sufficient to collect the biomass and after drying, this string-attached biomass could be extracted directly for lipids without any need for separating the biomass from the string.

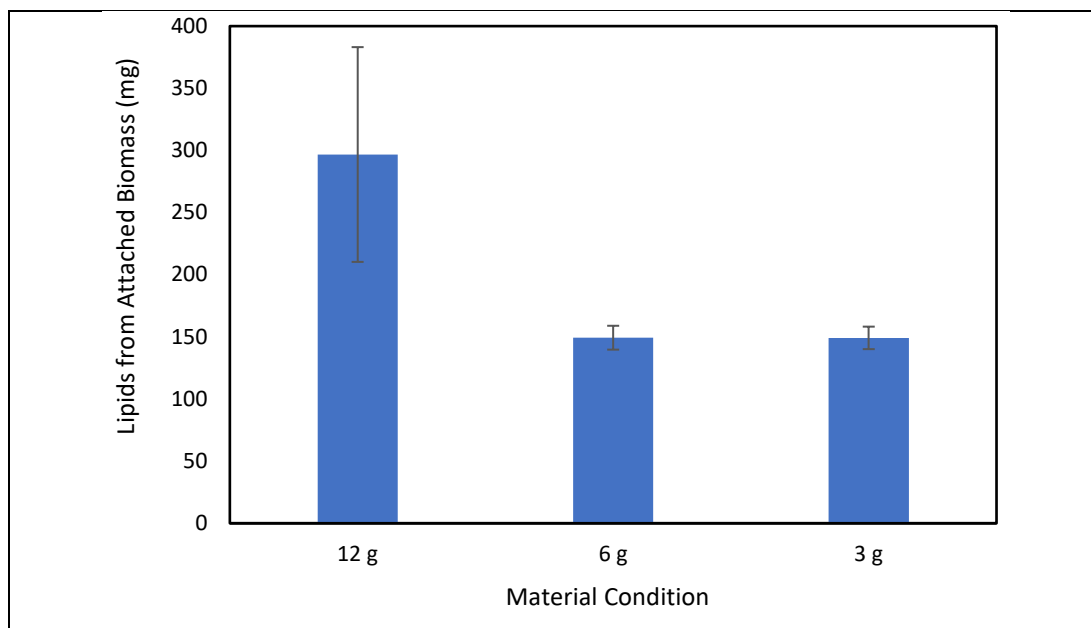


Figure 4.17: The amount of lipids that were extracted directly from string-attached biomass samples. Either 12, 6 or 3 g of string were added per 100 mL of CM.

A general evaluation of cotton string as a material for biomass attachment is that it is easy to handle, economical and an environmentally friendly choice. Although long-term usage means that the string would be periodically renewed, as a waste, it is biodegradable. Our tests of the material show that cotton string can be autoclaved or put through the lipid extraction procedure multiple times without apparent damage to the structure. These considerations make cotton string a good material choice for attached growth.

4.2.4.4. Suspended and Attached Growth Comparison using CM5

Suspended growth of the cultures with and without string addition were compared. The control cultures not containing string demonstrated biomass accumulation as expected, while the cultures with added string had very little suspended growth throughout the experiment (Figure 4.18.a). This difference could also be observed with the appearance of suspended samples after centrifugation (Figure 4.18.b). There was hardly any cell pellet collected during the experiment for the string-condition when 5 mL samples were taken. The attached biomass harvested on day 7 was estimated by deducting the weight of the string used. Accordingly, the average amount of dry string-attached biomass per 100 mL of culture was 1.299 g, while the suspended biomass in these cultures was only 20 mg. This indicates that 98.5% of the total biomass accumulated in these cultures was attached on the string

and consequently, were easily harvested when the string was strained. The total biomass of the cultures (suspended plus attached) with the string-condition can be estimated as 13.2 g/L based on the initial culture volume (100 mL). This was only 5.3% lower than the biomass achieved with the group where string was not added (13.9 g/L).

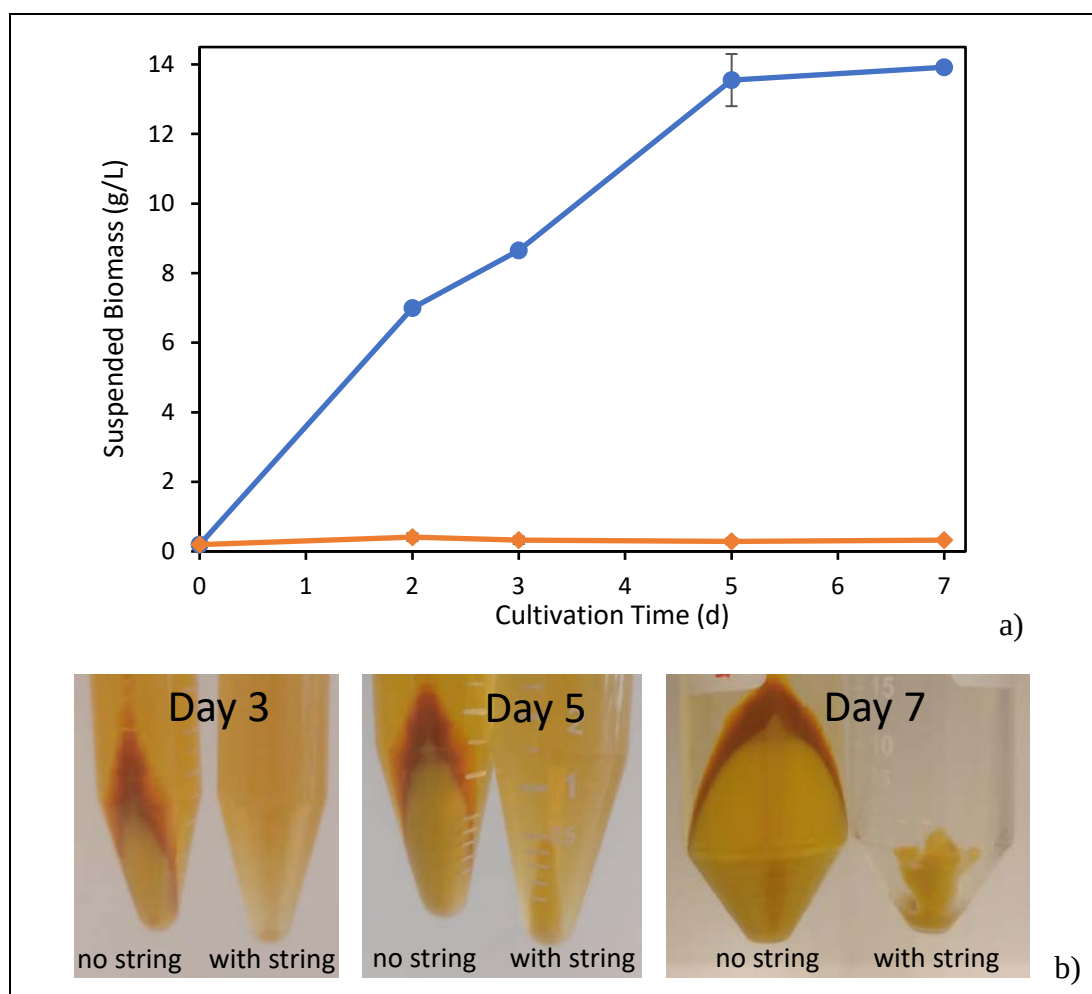


Figure 4.18: a) Suspended biomass of cultures cultivated with string (diamonds) and without string (circles) and b) images of centrifuged suspended samples.

Based on the wet and dry weights, the solid content of the string-attached biomass samples was 49.2%, w/w. In contrast, the centrifuged suspended biomass samples from the no-string condition only had a solid content of 19.6%, w/w. The lipid content of the samples was determined as a percent of dry cell weight with both wet and dry samples to compare the recoverability of lipids. The samples denoted as “dry” were thoroughly freeze-dried before lipid extraction, while the “wet” samples were frozen (-18°C) in wet form and only defrosted prior to lipid extraction. The lipid

content recovered from the string-attached biomass samples (15.5%) was statistically equivalent (p value > 0.80) when samples were extracted in dry versus wet form. On the other hand, the lipid content of the suspended biomass samples from the no-string condition were 38.6 and 28.9%, respectively, when extracted in dry and wet form. This indicates that the string-attached biomass samples could be extracted for lipids without a need for drying since lipid recovery was not affected. However, the suspended biomass – despite being centrifuged – requires freeze-drying to achieve full lipid recovery. The lipid content of the string-attached biomass samples was significantly lower than the suspended samples (15.5 vs. 38.6%). This may be due to a difference in the lipid accumulation behavior of the cells in attached form. Since the string soaks up a large amount of the carrot medium, it is possible that the cells attached on the string surface have greater access to nutrients and consequently, could take a longer time to be depleted of nutrients and to accumulate lipids. This could potentially be resolved by lengthening cultivation time. Overall, the prospect of cultivating *A. protothecoides* in string-attached form offers the advantages of easy, manual harvesting of biomass with high (98.5%) biomass recovery and direct lipid extraction of wet string-attached biomass without any need for a drying step.

4.2.5. Reactor Experiments

Determining the scalability of a microbial process is important for establishing its practicability. By scaling the cultivation of *A. protothecoides* using carrot medium to a 1-L reactor we aimed to investigate its lipid production performance outside of a shaking incubator – in an aerated chamber, which is more representative of a scalable configuration. Batch reactor experiments were initially conducted to gain baseline cultivation data and to fix stirring speed. Antifoam solution concentration was tested to ensure that the amounts used in the reactor would not hinder growth or lipid output.

4.2.5.1. Batch Reactor Experiments with Carrot Medium

During initial testing it was determined that the rotational speed with which the reactor culture was mixed was an important factor that affected culture growth. Stirring speeds of 200, 300 and 400 rpm were tested. The initial batch test run with 200 rpm indicated a slower accumulation of biomass, the maximum value, 8.5 g/L, being achieved by day 12 of cultivation. That, in combination with a maximum lipid

content of 29.9% yielded 2.6 g/L lipids (Figure 4.19). It should be noted that the first experiment with 200 rpm was cultivated for longer (12 days) since culture growth was slower, while the other experiments were ended after 10 days of cultivation. When the stirring speed was increased to 300 rpm the biomass and lipid outputs were greatly improved; a maximum biomass of 13.3 g/L was achieved by day 5 with 42.0% lipid content yielding 5.6 g/L lipids. Increasing the stirring speed to 400 rpm did not appear to make a significant difference. The maximum lipids produced were also 5.6 g/L on day 5 of cultivation. The use of higher stirring speed did result in more foam produced during cultivation and required the addition of more antifoam solution. Therefore, the use of 300 rpm appeared the optimal setting and was implemented for subsequent reactor experiments. Furthermore, based on the maximum lipid output, 5 days could be an ideal cultivation time to harvest the culture.

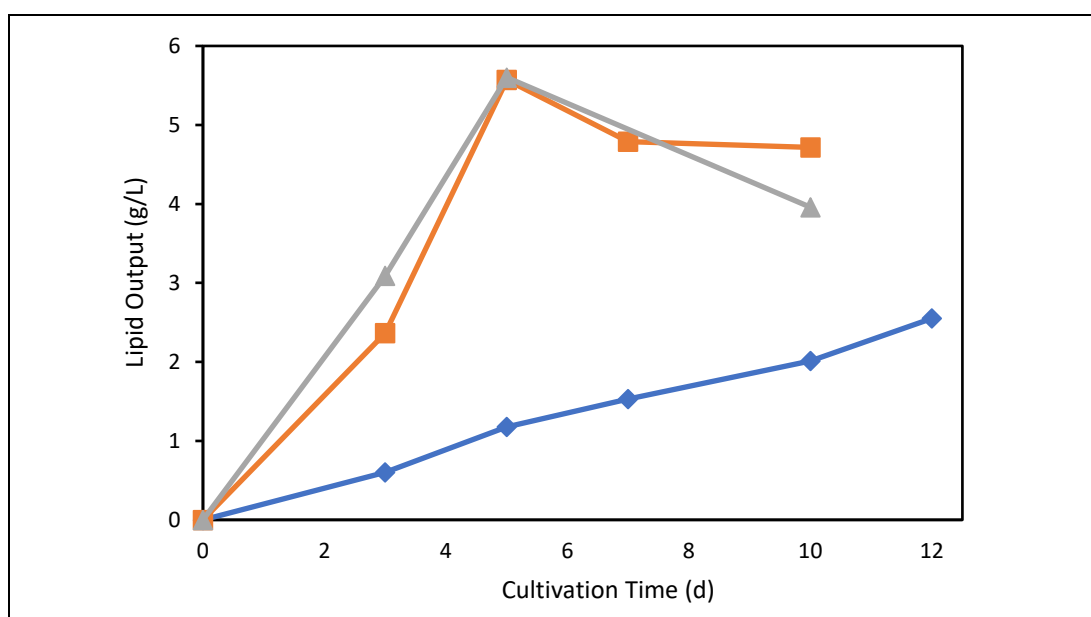


Figure 4.19: The lipids produced during batch reactor experiments with carrot medium plus 5 g/L glucose (CM5) using various stirring speeds; 200 (diamonds), 300 (squares) and 400 rpm (triangles).

4.2.5.2. Effect of Antifoam Solution

The biomass of the cultures showed a very consistent trend between all groups, independent of the amount of antifoam that was added. The exponential growth appeared completed by day 3, followed by a stationary phase until day 7 (Figure 4.20.a). The maximum biomass achieved by the cultures was 8.5 g/L for the control

group, which contained no antifoam solution and 8.5, 8.9 and 8.5 g/L for the groups with 0.1, 0.2 and 0.5% addition of antifoam solution, respectively. Although the group with 0.2% antifoam solution had a higher biomass, it was not significantly greater than the others (p values > 0.29).

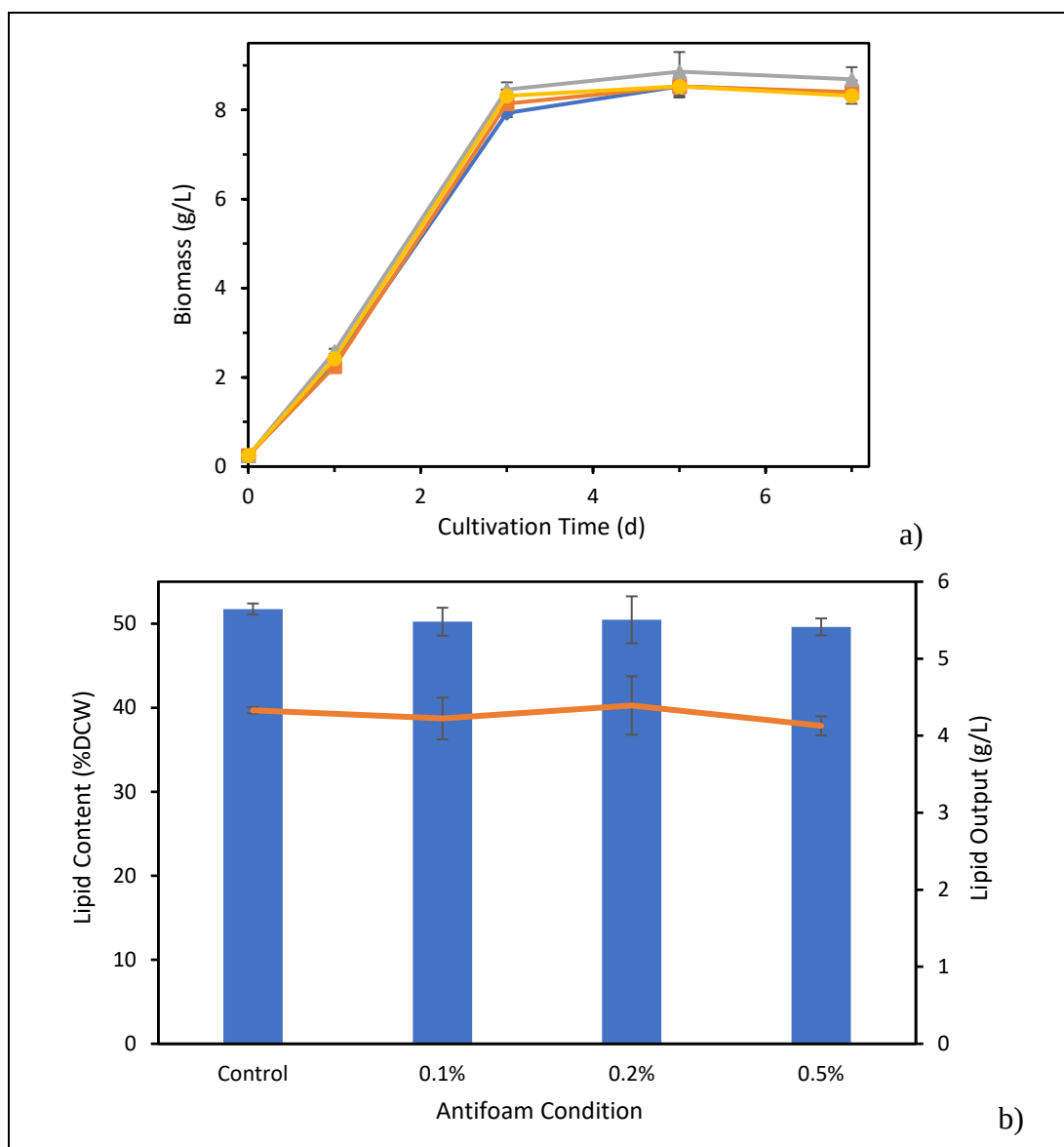


Figure 4.20: a) Biomass of *A. protothecoides* cultures grown heterotrophically over a period of 7 days with varying initial amounts of antifoam solution added: Control (diamonds), 0.1% (squares), 0.2% (triangles) and 0.5% (circles). b) Lipid content (bars) and lipid output (line) achieved with day 7 cultures.

The lipid content of the day 7 biomass samples was determined as a percentage of dry cell weight (DCW). The lipid contents were very similar in value (Figure 4.20.b) – all in the range of 49.6-51.7% with no significant differences between the

groups (p value > 0.10). The lipid outputs of the cultures were also close in range – 4.1-4.4 g/L with no significant differences between the conditions (p value > 0.30). Accordingly, the addition of 10% antifoam solution in the range of 0.1 to 0.5% was determined to be acceptable since it did not hinder biomass growth or lipid output.

4.3. Discussion

The biomass and lipid results achieved in our optimization experiments are comparable to similar studies in the literature. In our study, with 30 g/L initial glucose a maximum biomass of 10.7 g/L with 28.9% lipid content was achieved, yielding 3.1 g/L lipids. In one study with *A. protothecoides* grown heterotrophically using 30 g/L glucose, 4 g/L yeast extract and an initial pH of 6.5 – they achieved ca. 10 g/L biomass with a 23% lipid content [149]. The group tested initial pH values between 6.3 and 7.1 and did not note any significant differences in biomass or lipid content based on that pH range. Another study explored the prospect of enhancing the lipid content of *A. protothecoides* while exposing it to a pH of 10 in addition to nitrogen starvation [228]. The additionally applied alkaline condition showed an increase in lipid productivity and TAG content. Furthermore, their examination of initial pH in the range of 6 to 11 showed a general decrease in biomass corresponding to an increase in pH. This contradicts our findings that *A. protothecoides* favors a more basic initial pH. However, since their cultivation mode was primarily photoautotrophic, this could explain why a higher pH would limit growth due to the consequently lower concentration of CO₂ available in aqueous phase.

In our experiment testing variations in growth medium components, all media initially had a pH of 7.5. However, the final pH values of the cultures ranged from 4 to 7.2. The culture groups that achieved a higher biomass content of about 12 g/L (NaCl, Iron and Control) had a final pH of 7.1-7.2, while the cultures that grew 65-72% of that amount (Urea, Sulfate and RT) had a lower final pH of 4-4.9. This could lead to the conclusion that low pH caused a hindrance of cellular growth. This was likely the case for the Urea group since the final culture pH was 4. Studies have shown that $\text{pH} \leq 4$ deters microalgal growth, perhaps since it is an unfavorable condition for certain metabolic functions [229], [230]. Urea is known to be taken up by microalgal cells and degraded to NH₃ and CO₂ with the aid of enzymes [231]–[233]. This excess CO₂ production could have caused the more severe pH drop seen in the Urea group. In the case of the Sulfate group, it is probable that the sulfate concentration applied,

0.08 mM, had limited algal growth. A group evaluating the effect of sulfate concentration on the growth of a freshwater microalga determined that an amount lower than 0.1 mM hindered biomass growth [234]. We have observed that with heterotrophic cultivation of *A. protothecoides* the culture has a drop in pH during the growth phase followed by an increase in pH in the stationary phase. The initial drop in pH may be due to the production of acidic metabolites, which in turn may be degraded during the stationary phase after glucose has been depleted, resulting later in a rise in pH. This phenomenon could explain why the pH remained low in the Sulfate group since all acidic compounds could not be degraded due to growth-hindrance. The biomass output of the RT group was also lower than control, which is an expected outcome of a lower, non-optimal cultivation temperature [235], [236]. The same phenomenon could explain the low pH of the RT cultures since they displayed slower and more limited growth.

The original growth medium recipe containing 4 g/L yeast extract (PM1) and the revised recipe containing 1.35 g/L urea (PM2) resulted in steep differences in lipid content and lipid output. Their C/N ratios were estimated on a mole per mole basis and the assumption that yeast extract has 11% nitrogen. With the addition of 30 g/L glucose the growth medium PM1 has a C/N ratio of 32 and PM2 a ratio of 22. This difference does not explain the improvement in lipid content between PM1 and PM2 since an increase in C/N ratio generally supports higher lipid accumulation [237]–[239]. Yeast extract is a rich source of nutrients, including amino acids, carbohydrates, minerals, and vitamins. This abundance of nutrients may have had a growth-promoting effect and could explain the lower lipid content achieved in the presence of yeast extract.

Urea, yeast extract, ammonium and nitrate are commonly researched as a nitrogen source for microalgae and the optimal choice with respect to maximizing biomass and lipid yield varies even when considering the same strain. One such study comparing the effect of nitrogen source on 4 different strains of *A. protothecoides* found that nitrate had the greatest outcome [213]. In particular, they found that growth medium containing 40 g/L glucose and 2.4 g/L KNO₃ had the highest lipid yield, 5.9 g/L, and lipid productivity, 654 mg/L-day. The maximum lipid productivity achieved in our optimization studies was higher, 820 mg/L-day, after 5 days cultivation with only 30 g/L glucose and 1.35 g/L urea. In contrast, another study of *A. protothecoides* in heterotrophic, batch culture demonstrated a lipid yield of 8.28 g/L after 7 days,

resulting in an even higher lipid productivity of 1183 mg/L-day utilizing a growth medium containing 30 g/L glucose and 4 g/L yeast extract [211]. The nitrogen source that could optimize lipid yield is still up for debate based on the literature, and it is worthwhile to test it for oneself with the strain on hand.

The experiments for utilizing carrot pomace demonstrated that this waste can be economically processed with only acid hydrolysis and coarse filtering to produce a carrot medium (CM) that was successfully used for microalgal cultivation. The CM alone was sufficient in supporting the cultivation and lipid production of *A. protothecoides*; 4.9 g/L total lipids were produced (9.9 g lipids / kg wet carrot pomace). Considering as an example that the USA produces 175,000 tons of carrot waste annually [135], and assuming a 98% transesterification efficiency [240], we estimate that 1,698 tons of biodiesel could be produced yearly using this waste with no cost of synthetic nutrients for cultivation. Moreover, the 5 g/L glucose addition to the CM increased the total lipid output by 19% (4.9 to 5.9 g/L, p value < 0.05) and the TAG content by 18% (66.5 to 78.4%, p value < 0.05). These results support the finding that carrot pomace, a waste by-product of the juice industry, could successfully be utilized with minimal pre-treatment to cultivate microalgae heterotrophically and produce lipids suitable as a potential biodiesel feedstock. Based on the improvement in the TAG content of the produced lipids and the high lipid:glucose yield, the addition of 5 g/L glucose to the CM may be preferred over the use of CM alone.

The biomass and lipid results achieved in our CM experiments are comparable to others that have utilized organic wastes as a nutrient and/or carbon source for microalgae cultivation. The use of sugarcane bagasse hydrolysate was demonstrated as a potential carbon source to cultivate *A. protothecoides*; with the 30 g/L reducing sugar condition they were able to achieve ca. 9 g/L biomass with a 46% lipid content [241]. The sugarcane bagasse was treated with cellulase and β -glucosidase enzymes and the hydrolysate was centrifuged and filtered in order to use the supernatant, which makes for expensive pre-treatment. In our study only acid hydrolysis and coarse filtering with a common material (cheesecloth) was applied, and the carrot medium was not centrifuged in order to establish an economical, practical and scalable approach for utilizing carrot pomace. Another study of *A. protothecoides* showed that waste molasses could be used to support culture growth and lipid accumulation, in fact, even better than a glucose-based synthetic medium; they achieved 6.98 g/L lipids after 120 hours of batch culture [242]. Once again, the medium was prepared by

centrifugation and enzymatic hydrolysis of the waste molasses, which are added costs that could reduce the benefit to be gained from this waste.

Research is also ongoing to explore the conversion of carrot pomace to biodiesel through microbial processes. It was demonstrated that carrot pomace could be used as a carbon source to cultivate the fungus *Coniochaeta hoffmannii* with high lipid content [134]. They achieved 52.0% lipid content after 48 hours cultivation with the addition of 0.5 g/l whey as a nitrogen source. The carrot pomace was prepared in 300 g/L concentration and diluted to 10% for optimal results. In another study carrot pomace was used as a carbon source to produce lipids with *Chlorella vulgaris* [133]. In the cultivation experiments BG-11 was used as the base growth medium and acid hydrolyzed carrot pomace was added as the carbon source to provide a sugar concentration of 0.5 g/L. As a result of 10 days photoheterotrophic cultivation the culture produced 0.699 g/L biomass with a 40.0% lipid content, and therefore, a 0.28 g/L lipid output. Comparatively speaking, the primary advantage of our study is that we were able to demonstrate that carrot pomace could be used as the sole source of carbon and nutrients for the production of 4.5 g/L algal lipids in a period of 7 days heterotrophic cultivation, eliminating the need for light and expensive synthetic growth medium ingredients.

Attached growth experiments with *A. protothecoides* revealed cotton string as the material on which the highest biomass was collected. Use of materials as a surface for microbial attachment for the purpose of improving process efficiency is a commonly studied phenomenon. One group evaluated the use of glass rods supported by a metal grid for attached growth of filamentous cyanobacteria for poultry litter waste treatment and simultaneous lipid accumulation [107]. The attached biomass was harvested by scraping the rods and had a maximum of 19.6% lipids. The lipid content achievable from the string-attached biomass in our experiments was 23.2%. A primary advantage to our experimental setup is that the attached biomass did not need to be “harvested” from the string and could be extracted directly in attached form. The string was also noted to be intact and usable again for another cycle of cultivation. Further, a comparison of suspended versus attached growth condition revealed that when string was added to the culture medium, 98.5% of the biomass could be easily harvested in string-attached form. The lipid content of the string-attached biomass was lower than suspended biomass, indicating that longer cultivation times may be needed to accumulate lipids under attached condition. The high solid content (49.2%)

of the wet string-attached biomass allowed for direct extraction without drying, yielding the same amount of lipids. Hence, attached growth of culture offers two main advantages – simple, manual harvesting of biomass and no need for a drying step.

Batch reactor experiments were conducted with heterotrophic cultivation of *A. protothecoides* using CM5 as the growth medium. The results showed that 5.6 g/L lipids could be produced in the reactor by day 5 of cultivation. In our previous experiments in the shaking incubator, cultivation with CM5 yielded 4.5 and 5.9 g/L lipids, respectively, by days 5 and 10. The maximum lipids produced in the reactor were only 5% lower than that achieved in the shaking incubator (5.6 vs. 5.9 g/L). Furthermore, when estimated based on 5-day outputs, the lipid production rate was improved from 903 to 1,114 mg/L·d, making the reactor an attractive option for lowering cultivation time. Accordingly, the potential to implement the cultivation of *A. protothecoides* using carrot waste derived growth medium in an aerated chamber that does not rely on high energy expenditure (like a shaking incubator) was demonstrated. The potential to scale up this process was established.

5. CONCLUSIONS

Cultivation of *C. zofingiensis* heterotrophically on agar media surface for homogeneous and consistent culture growth was shown. Parameter optimization allowed for production of 8.2 g/L lipids and 6.5 mg/L astaxanthin with 40 g/L initial glucose, room temperature incubation, and direct co-extraction of the wet biomass. The prospect of agar-attached culture growth has several benefits, including the energy-free incubation without any aeration, agitation or pumping of the growth medium, simple and efficient manual harvesting and no need for dewatering or drying of the already concentrated biomass. Based on the additional proofs of concept of agar reuse and agar-to-agar inoculation, heterotrophic, agar-attached cultivation of *C. zofingiensis* appears practicable for the efficient and economical co-production of lipids and astaxanthin.

The series of optimization studies were of great value in improving the lipid yield that could be achieved with *A. protothecoides* using a synthetic growth medium. The initial growth medium (PM1) containing yeast extract and 30 g/L glucose had yielded 3.1 g/L lipids, while the optimized medium (PM2) containing urea with a higher initial pH yielded 5.9 g/L lipids – a 90% improvement. Furthermore, the potential to use carrot pomace as the sole source of carbon and nutrients (100% carrot medium) for the heterotrophic cultivation of *A. protothecoides* for lipid production was demonstrated. An improved biomass output (12.3 g/L), lipid content (54.4%), total lipid output (5.9 g/L) and TAG content (78.4%) were achievable with an addition of 5 g/L glucose to the carrot medium. This addition of glucose to the 100% carrot-pomace-based growth medium may be preferable as it improves the quantity and quality of lipids produced. This still indicates a significant cost-savings considering that 30 g/L glucose, minerals and trace elements are used to formulate the synthetic growth medium, yielding the same amount of lipids. Carrot pomace represents a cost-efficient resource for more economical production of microalgal lipids potentially suitable as a biodiesel feedstock.

Various materials were tested for the attached, heterotrophic growth of *A. protothecoides* and string was found to support the highest biomass attachment. Simple manual harvesting of string-attached biomass was demonstrated and the ratio of biomass attachment was improved by increasing the amount of string added to the carrot medium. The direct extraction of string-attached biomass for lipids was

possible; extraction procedure variations were tested to improve lipid recovery. The addition of string to carrot medium was shown to support 98.5% attached growth, which could be easily harvested since the string was manually strained. The wet string-attached biomass could be extracted directly for lipids without any drying of samples, indicating additional potential cost-savings associated with this attached growth configuration. Further, cultivation of *A. protothecoides* using carrot medium was tested in an aerated bioreactor chamber. Successful microalgal growth and lipid production independent of a shaking incubator, and accordingly, the potential for scalability, was demonstrated.

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BIOGRAPHY

Zeynep Büşra Çakır completed her elementary, middle and high school education in Troy, NY, USA. She studied biomedical engineering, earning her B.Sc. degree at Rensselaer Polytechnic Institute in 2007 and M.Sc. degree at Johns Hopkins University in 2010. Afterwards, she was employed by Synthes (West Chester, PA, USA) in their Engineering Rotation Program from 2010 to 2013, including rotations in the USA and Switzerland branches. She returned permanently to Istanbul, Turkey at the end of 2013. After some work experience in the healthcare industry, she decided to pursue a doctoral degree in environmental engineering. She has been completing her studies in environmental microbiology at Gebze Technical University since 2016.

APPENDICES

Appendix A: Articles Published During the Thesis Study

Çakır, Z. B., Yılmaz H., Ertan F., Tanrıseven A., Özkan M., (2022), "Carrot pomace alone supports heterotrophic growth and lipid production of *Auxenochlorella protothecoides*", Biomass Conversion and Biorefinery, 1-13.