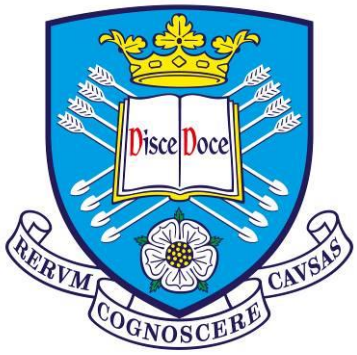

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Carbon Dioxide Uptake by Microalgae *Chlorella vulgaris* and *Porphyridium purpureum* and Its Development towards Bioplastics Production

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PhD

Sep 2020

Carbon Dioxide Uptake by Microalgae *Chlorella vulgaris* and *Porphyridium purpureum* and Its Development towards Bioplastics Production

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Contents

Abbreviations

2*f*	A medium modified from *f*/2 (refer below), in which its nitrate and phosphate concentration has been increased by a factor of 4.
4*f*	A medium modified from *f*/2 (refer below), in which its nitrate and phosphate concentration has been increased by a factor of 8.
BDUG	Name of a microalgae culture collection affiliated to Bharathidasan University.
C_0	Microalgae culture condition, in which no additional carbon is added.
$C_{0.01M}$	Microalgae culture condition, in which initial 0.01M of bicarbonate was added during inoculation.
$C_{1\%}$	A microalgae culture condition which is aerated with a continuous 1% CO_2.
C_{air}	A microalgae culture condition which is aerated with continuous air.
CCAP	Acronym of The Culture Collection of Algae and Protozoa, a microalgae culture collection located in Oban, UK.
CO_2	Carbon dioxide.
CO_3^{2-}	Carbonate ion. One of the DIC species.
D	Day. For example, D0 refers to Day 0, the day when the experiment started.
DIC	Dissolved inorganic carbon. It is sometimes referred to as T_{CO2} in this thesis. Among its species are CO_2, HCO_3^- and CO_3^{2-}.
DIN	Dissolved inorganic nitrogen.
DIP	Dissolved inorganic phosphorous.
f	A medium modified from *f*/2 (refer below), in which its nitrate and phosphate concentration has been increased by a factor of 2.
f/2	A type of medium used to grow microalgae, in which its initial concentration of nitrate and phosphate are 0.88 mM and 0.036 mM respectively.
HCO_3^-	Bicarbonate ion. One of the DIC species.
H_20	Chemical formula for water
mM	Millimolar - unit for measuring molar concentration. One mM = one-thousandth of a mole per litre.
OH^-	Hydroxide ion.
H_2CO_3	Carbonic acid
ppm	parts per million.
T_{CO2}	Total carbon dioxide. Synonymous to DIC in this thesis.
TPS	Thermoplastics Starch.

Abstract

Increasing levels of atmospheric carbon dioxide (CO_2) and plastics waste pollution are among the environmental problems that need to be addressed. Several potential candidates have been identified to mitigate these problems, including microalgae cultivation, which also offers parallel benefits such as the ability to remediate industrial wastes and produce value-added chemicals. This thesis focuses on assessing CO_2 uptake in selected strains of microalgae towards developing a general understanding of microalgae carbon sequestration and development of microalgae feedstock for bioplastics production, as a simultaneous solution for addressing the two environmental issues mentioned.

This research has performed an optimisation towards the assessment of dissolved inorganic carbon (DIC) in media in terms of analysis volume and storage duration. Assessment of DIC availability in two cultures of *Chlorella vulgaris* strains found that their behaviours are different in terms of carbon uptake, growth, nutrient consumption and biochemical production. Interestingly, it was found that the DIC saturation levels in both algae culture medium are higher than in a blank medium, suggesting a greater carbon sequestration capacity of the culture medium in the presence of microalgae. Later, the influence of nutrients towards carbon consumption was examined by increasing the amount of nitrate and phosphate supplied, at a constant carbon load. To achieve that, we described a dynamic method to quantify carbon uptake in a growth culture, based on the assessment of DIC. It was observed that carbon uptake rates did not increase with increased nitrate and phosphate. The rate was also independent of their growth phase. The increase of nutrients increased the growth, up until a point, with no significant change beyond.

A similar treatment of red algae (*Porphyridium purpureum*) showed carbon uptake profiles of slightly lower value. Increasing nutrient does not change the carbohydrate proportion. Instead, it increases the protein percentage. Later, both intracellular polysaccharides and exopolysaccharides synthesised by the red algae were incorporated into bioplastics production. The best biofilm produced have similar mechanical properties to conventional low-density polyethene plastics, but with shorter elongation capability and less hydrophobicity. We also found that the use of extracted polysaccharides from the microalgae to produce microalgae bioplastics will result in higher tensile strength and elasticity, rather than incorporating the microalgae as whole cells.

CHAPTER 1: Introduction

1.1 Background

The atmospheric level of carbon dioxide (CO_2), which is increasing alarmingly, has been known to contribute towards global warming and the greenhouse effects. Among sustainable ways to mitigate this phenomenon is the cultivation of microalgae. Microalgae are photosynthetic microorganisms that fix atmospheric CO_2 with the potential to convert it to organic carbon of value. Microalgae also help to reduce greenhouse gases by taking CO_2 for photosynthesis and turning it to value-added biochemical products (Kassim & Meng, 2017).

Microalgae has several properties that make them outstanding option such as relatively faster growth to higher plants, high photosynthetic efficiency, high CO_2 fixation rate, broad biomass application and high tolerance towards high concentration level of carbon and nutrient (Singh & Ahluwalia, 2013). Besides, microalgae reactors can be situated in most type of lands, thus avoiding competition with food crops. Microalgae can also live in fresh, brackish or seawater.

In the past decade, interest in microalgae has been sustained by the need to find alternatives for renewable energy generation. However, the economics of microalgae cultivation do not yet meet the requirements to be in the position to significantly impact fossil fuel replacements (Markou & Nerantzis, 2013). Nevertheless, microalgae cultivation is not about biofuels production alone. Instead, it has bio-refinery potential to source multiple value-added chemicals (Laurens et al., 2017). By providing light and nutrients, even sourced from wastewater and flue gases, some species of microalgae will be able to produce high-value pharmaceutical products, food supplement for human and animals and bioplastics precursors, while at the same time help in nutrient bioremediation and CO_2 sequestration.

Microalgae cultivation is currently done in both open and closed system, in which in the latter, essential parameters can be carefully controlled (Bhola et al., 2014). The parameters include CO_2 delivery to the algae, which can be done in several ways, such as in the form of gas or soluble bicarbonate. Nevertheless, several challenges revolve around carbon delivery issue such as the economics of CO_2 supply and storage, the inefficiency due to CO_2 loss to the atmosphere as well as the effects that inorganic carbon brings to the culture.

This research will study two aspects related to the microalgae capability, i.e. CO_2 sequestration behaviour and production of bioplastics precursors. The aim is to develop strategies for reduction of CO_2 in the environment and at the same time, explore environmentally sustainable alternative routes to bioplastics production as a replacement for conventional plastics.

In another issue, plastics waste, due to improper disposal, has spread through every part of our land, water and air (Law, 2017). They are known to take hundreds of year to degrade. While plastics waste can be recycled (which will incur energy and money), a recent report in July 2018 (theguardian.com) has pointed to irregularities that lead to environmental pollution. Instead of being recycled, plastics waste are left to pollute rivers and oceans, or dumped in other countries as waste. Thus there is an increasing drive and desire to minimise plastics usage (especially single-use plastics) and cut down on plastic waste generation.

Although some plastics are bio-based and biodegradable, its biodegradability often happens only under controlled heat and humidity conditions, which are different from the natural environment (Iles & Martin, 2013). Thus proper disposal is required. Nevertheless, this should not discourage the use of bio-based plastics to be part of the solution. By having bio-based plastics decomposing facilities, it will help in improving soil health, water quality, carbon storage in soil (Kuenkel et al., 2016), and thus reducing the need for fertilisers. Composting also brings economic benefits in terms of job opportunity compared to using landfills.

Although the consumer trend points to high demand for plastics usage, there is increasing support towards cutting down single-use plastic and use of bioplastics, as reported in April 2019 by YouGov, an international research data and analytics group (yougov.co.uk). The same report informed that consumers are willing to pay more for environmentally sustainable bioplastics.

Based on these projections, there is a considerable prospect in investigating the potential of microalgae for sequestrating CO_2 and producing bioplastics precursors. Most works on carbon mitigation by microalgae focus on the apparent supply of CO_2 and infer its uptake through the production of biomass or value-added products. However, given that CO_2 is a gas with poor solubility in aqueous environments, supply does not necessarily mean availability. There is still not much information in terms of dissolved inorganic carbon availability in the media and its uptake; thus, more researches are needed.

The research here aims to address this gap by monitoring the benefits of microalgae in terms of the available DIC capacity in the culture medium. Besides, it addresses the use of microalgae biomass in bioplastics production. Although there are different ways microalgae can be involved in bioplastics production (Mathiot et al., 2019; Dong et al., 2013; Talukder, Das, & Wu, 2012; Hempel et al., 2010; Zhang et al., 2000), this research will focus on the accumulated polysaccharides (starch) towards the production of thermoplastics starch (TPS), considering its broad and straightforward application in industry. Since this will be a biological source of polysaccharide, it is hypothesised that it can show similar characteristics with polysaccharides from other sources that form TPS. Alongside these motivations, microalgae response towards different carbon utilisation and growth conditions will be studied together to understand their behaviour and preference in mitigating CO_2 and bioplastics production.

Researching this topic is part of developing sustainable strategies for effective CO_2 utilisation and its reduction in the environment, in parallel to minimising current reliance on fossil fuel-based sources. Monitoring microalgae carbon uptake will help us understand how microalgae culture respond to CO_2 supply and route the supply to biochemical of value. Incorporation of microalgae or microalgae-sourced precursors into plastics production is hoped to be part of the solution towards developing bio-based biodegradable plastics and mitigating the environmental concern that urgently needs addressing.

1.2 Aims and Objectives

The primary aim of this research is to understand carbon uptake in two brackish water strains of the green alga *Chlorella vulgaris* (CCAP 211/21A and BDUG 91771), isolated from a similar habitat but from two distinct culture collections, and the red alga *Porphyridium purpureum* CCAP 1380/1A, towards developing their potential in addressing the mitigation of two global environmental concerns, i.e. CO_2 emissions and plastics pollution.

The specific objectives of this research are outlined below.

Objective 1 is to study the availability of DIC in the culture media of two green alga strains upon different CO_2 supply regimes. As part of this objective, carbon limited and carbon sufficient conditions were investigated. The behaviours of the two strains of *Chlorella vulgaris* were compared by exposing them to culture (1) without active carbon supply (allowing passive

diffusion of atmospheric CO_2), (2) with an initial supply of 0.01M bicarbonate, (3) continuous active supply of atmospheric CO_2 (0.04%) and (4) continuous active supply of CO_2 at a higher level (1% CO_2). This objective will be discussed in Chapter 4.

Objective 2 is to examine the influence of important nutrients on carbon uptake by the two *Chlorella. vulgaris* strains. To address this, culture media with increased nitrogen and phosphorus contents were examined. Discussion on this topic will be reported in Chapter 5.

Objective 3 is to examine and compare the behaviour of a red microalga as a producer of bioplastics precursors (i.e. intracellular polysaccharides and exopolysaccharides) upon similar carbon and nutrient treatments. To achieve this, condition of carbon and nutrient treatments similar to those employed with the *Chlorella vulgaris* cultivation were examined. This objective will be covered in Chapter 6.

Objective 4 is to examine and assess the red microalgae polysaccharide fraction and biomass towards bioplastic film formation. To do this, bioplastic films that incorporated the microalgae components were produced. Later, the mechanical data comprising of maximum elongation, tensile strength and elasticity were evaluated. This objective will be reported in Chapter 7.

CHAPTER 2: Literature Review

2.1 Introduction

This review will look into the relevance of microalgae in addressing current environmental issues, the carbon uptake mechanisms, methods for carbon delivery, estimation of carbon uptake in culture, factors influencing the carbon uptake and the production of bioplastics precursor. The hope is to develop strategies for CO_2 reduction in the environment, while at the same time providing developmental avenues for a sustainable alternative to conventional plastics.

2.1.1 Environmental Issues

Greenhouse Gas Problems

Combustion of fossil fuel for energy production has increased environmental concerns as it contributes to pollution and an increase in global warming due to the increase of greenhouse gases such as CO_2. Increasing levels of CO_2 in the atmosphere is a growing environmental concern that has a broad global impact on energy policy and the way we manufacture chemicals. Since the industrial revolution, CO_2 concentration in the atmosphere, which was around 270 ppm by volume, has increased more than 40% and is currently rising about two ppm/year (Moreira & Pires, 2016). In 2016, CO_2 levels crossed the 400 ppm mark (Kahn, 2016) in which the monthly value failed to drop below this. In 2019, the average peaked in May at 414.7 ppm (National Oceanic and Atmospheric Administration, 2019). This build-up is believed to be contributed by anthropogenic burning of fossil fuels such as during energy production, which cannot be balanced within the natural carbon cycle (North, 2014).

Countries have started to adopt policies on carbon mitigation and use of green energy. Several strategies were explored to reduce CO_2 in air, which can be classified either as carbon capture and storage (CCS) or carbon capture and utilisation (CCU) (Jones et al., 2017). CCS, which predominantly involves geological or underground CO_2 storage, is a temporary mitigation strategy in lowering the CO_2 emissions and does not produce a permanent solution - although it is also welcomed to be part of the solution. On the other hand, CCU uses CO_2 to create new or value-added products which can promote sustainability. One approach to develop strategies for carbon utilisation is to explore biological CO_2 utilising processes such as photosynthesis and develop them in sustainable processes. Among this approach, one common public opinion

is to plant more forests which will act as a carbon sink, as can be seen in a recent initiative called #teamtrees, which aimed to plant 20 million trees by 2020 (teamtrees.org/).

Plastics Degradation

It is hard to imagine our life without plastics since they make up almost all the things around us. Plastics are polymers derived from monomeric units. Its properties such as lightweight, low cost, ease of moulding and durability make it a suitable choice in many applications such as packaging, clothing and instrument manufacturing. Polystyrene, nylon and silicone are some of the materials that originate from conventional plastics used widely around us, which can exist as bags, insulation, coatings and others.

The global plastic production has exceeded 380 million metric tons per year (Groh et al., 2019). Manufacturing of conventional plastics utilised natural gas and hydrocarbon gas liquids (HGL), which is a by-product of petroleum refining and natural gas processing (US Energy Information Administration, 2016). In fact, plastics are the second-largest petroleum application, which comes after energy (Mekonnen et al., 2013). At this amount of plastics production, it requires petrochemicals processing of about 150 million tons a year (Noreen et al., 2016). At the same time, petroleum is a depletable source of energy that contributes to pollutions and an increase in greenhouse gases. Thus, the increase of plastics production number gives concerns due to the heavy dependence of these applications on the fuel resources (Slav, 2019) and the pollution issue during manufacturing.

Another drawback of plastics is that they are non-biodegradable, which later will contribute towards waste pollution. This massive amount of waste can take thousands of years for complete biodegradation (Zia et al., 2017). The petroleum consumption by plastics manufacturing contributes to several environmental concerns such as accumulation of non-biodegradable debris in landfills, leaching of toxic chemicals and physical problems to wildlife due to ingestion or entanglement of plastic (Barnes et al., 2009; Thompson et al., 2009; Derraik, 2002). Additives such as plasticisers which leach out from disposed plastics may also exert their toxicity towards organisms that live around. (Krueger, Harms, & Schlosser, 2015).

These situations create alarming questions on how to minimise the dangers of plastics and thus, develop environmentally sustainable solutions. There is a growing demand for bioplastics that are environmentally friendly and capable of solving current environmental concerns. Realising

the pollution that plastic contributes, people are looking for sustainable alternatives. Bioplastics which is originated from biological sources and easier to biodegrade are an excellent substitute for conventional or fossil fuel-based plastic (Zia et al., 2017).

2.1.2 Microalgae as Potential Solution to Environmental Problems

Microalgae have been seen as a potential answer to these two problems since these organisms consume CO_2 for photosynthesis and growth while being able to accumulate products such as polysaccharides that can be developed as plastic precursors, although the technology is in its infancy and requires in-depth studies. The term microalgae itself has a broad definition. In phycology, it refers to both microscopic eukaryotic green algae and cyanobacteria (Troschl, Meixner, & Drosg, 2017). Others defined it as a functional group of organisms that carry out oxygenic photosynthesis (Raven & Giordano, 2014). They are responsible for around 50% of the global carbon fixation (Chen et al., 2016). However, its contribution to the bioplastics market is yet to be explored.

Advantages of Algae Utilization

Research on algae as an option towards CO_2 mitigation has been increasing lately. Compared to higher plants, microalgae can have higher photosynthetic efficiency and biomass productivity (Singh & Ahluwalia, 2013; Douskova et al., 2009). Moreover, microalgae cultivation is more sustainable as opposed to replanting higher plants, which needs a considerable amount of land, nutrients and water resources and time for biomass generation.

Photosynthetic efficiency, which is defined as the energy stored as new biomass per unit of the incident light energy, is usually between 1 – 2% among terrestrial plants (Singh & Ahluwalia, 2013). However, in microalgae, they are report of photosynthetic efficiency between 8 – 15% in *Spirulina platensis* (Richmond & Zou, 1999), 20% in *Chlorella* and 15 – 20% in *Phaeodactylum tricornutum* (Huntley & Redalje, 2007).

Besides, microalgae can have high biomass productivity, which can be 50 times more than that of switchgrass, i.e. the fastest growing terrestrial plant. Microalgae species such as *Chlorella* have short doubling times, which later translates to high productivities compared to other species (Singh & Ahluwalia, 2013).

Microalgae have several characteristics that make it an outstanding option for CO_2 utilisation too, such as the ability to fix atmospheric CO_2 as well as CO_2 from more concentrated sources, such as flue gases. Microalgae also have the capability to resource nutrients from waste streams for conversion to value-added chemicals of commercial value. These value-added chemicals include biofuels, fertilisers, nutritional supplements, pharmaceutical products, cosmetics, colourants and bioplastics.

Some species of microalgae also show tolerance to high levels of CO_2 concentrations. There are records of *Chlorella* sp. being grown in 100% CO_2 (Maeda et al., 1995) and wastewater containing a high amount of nitrogen and phosphorus (Wang et al., 2010).

The ability of microalgae to utilise the nutrients and convert them into multiple value-added biobased and bioenergy products can be conceptualised as a biorefinery (Laurens et al., 2017), similar to petroleum refinery that transforms crude oil to multiple useful products. Nevertheless, as it is still new, this concept has many challenges to be attended to such as operating cost, selection of suitable strains, control of culture conditions, avoidance of bacterial contamination and others (Yen et al., 2013).

Cultivation of Microalgae

Cultivation of microalgae can be done in different media of varying salinity, nutrient level or pH. As an example, *Chlorella* can be grown in brackish water aside from freshwater (Kapoore et al., 2019; Sadeghizadeh et al., 2017; Matos et al., 2016; B. Zhao et al., 2015; Chen et al., 2014). Differences between freshwater and seawater cultivation of the same species have been observed, for example, in terms of lipid production and calorific value (Luangpipat & Chisti, 2017). There are also reports of cultivation in extreme pH conditions ranging from alkaline of pH >10 (for example, *Chlorella sorokiniana* (Vadlamani et al., 2019)) to very acidic media of pH 1 (for example, *Galdieria partita* (Kurano et al., 1995)). There are also reports on the influence of media formulation including BG11 (Blue-Green medium), BBM (Bolds Basal Medium) and *f*/2 towards microalgae cultivation (de Carvalho et al., 2019; Thangamani et al., 2016; Crofcheck et al., 2012). These different formulations contain different forms and concentrations of nutrients, which will results in different yields in terms of growth rate, oil productivity and others.

Similar to the growth of other microorganism, microalgae can be cultivated in batch, semi-continuous or continuous modes. Growth of microalgae in batch cultivation can be segmented into several stages: lag phases, exponential, linear, declining, stationary and death phase (Lee, Jalalizadeh, & Zhang, 2015; Pires, 2015). In the lag phase, the growth of the inoculated cell is slightly delayed due to adaptation in new media. Next, they multiply as an exponential function of time with the support of available nutrients and lights. As light and nutrient become limited, they enter a linear growth phase. This growth phase is followed by a declining phase in which cell growth stops, and the cells eventually die.

Future and Current Challenges in Microalgae Cultivation

Microalgae have attracted interest in major companies, especially for its potential use in biofuel production and CO_2 utilisation. For example, recent work funded by ExxonMobil and Synthetic Genomics has modified *Nannochloropsis gaditana* strain to enhance its oil content from 20% to more than 40% (Ajjawi et al., 2017). ExxonMobil is targeting to produce daily production of 10000 barrels of biofuels by algae by 2025 (exxonmobil.co.uk). Besides, there is also a project by CO2Bio AS Technology for development of omega-3 production from microalgae. For this project, Technology Center Mongstad (TCM) has agreed to the use of 30000 tons of CO_2 that it captures (co2bio.no). Several other industries involved in the study of the application of microalgae could be found in a document in 2018 entitled Assessing the Cost Reduction Potential and Competitiveness of Novel (Next Generation) UK Carbon Capture Technology (service.gov.uk).

Despite its potential to capture CO_2 and function as biorefinery for the production of value-added chemicals, microalgae cultivation requires development concerning its limitations and challenges to address the environmental problems mentioned before significantly. The realisation of the microalgal biotechnological potential for product formation depends on optimising four key areas: (1) growth dynamics, (2) biomass productivity, (3) biomass harvesting and (4) product extraction & purification (Khan, Shin, & Kim, 2018; Gomaa, Al-Haj, & Abed, 2016; Seth & Wangikar, 2015). Besides, there is also a need to review the life cycle assessment of the process so that minimal energy input is required during cultivation, harvesting and drying (Lam, Lee, & Mohamed, 2012).

This chapter will look into the carbon uptake mechanism by microalgae, methods for carbon supply, estimation of carbon consumption by microalgae as well as market demand and its

production for bioplastics made from the microalgae. Several ways of getting the plastics from microalgae will also be reviewed, in which the routes and strains for bioplastics production for this research will be identified. The objective is to understand the carbon uptake mechanism by microalgae to optimise it, while also relating it to the production of bioplastics of desired properties with commercial potential.

2.2 Mechanism and Theory on Carbon Uptake by Microalgae

Carbon makes up around 50% of the whole microalgae cell, and its acquisition by microalgae can be observed through autotrophic, heterotrophic and mixotrophic behaviour (Pires, 2015).

Autotrophism (self-nourishment) is a behaviour in which the algae utilises light energy and inorganic nutrients (carbon, nitrogen, phosphorus and others) to produce biomass components such as carbohydrates, protein, lipids and pigments (Markou & Nerantzis, 2013). One type of autotrophism is by photosynthesis (photoautotrophism), where energy is derived from light or solar radiation, while another is chemoautotrophism, which is the use of inorganic chemicals (Bae et al., 2001) for energy and synthesis of all necessary organic compounds.

Photosynthesis is part of the natural carbon cycle which regulates CO_2 level in the atmosphere and hence the surface temperature of the earth (North, 2014). It is also an essential part of the growth of green plants. During photosynthesis, CO_2 is utilised for conversion into organic molecules such as sugars, lipids and other hydrocarbons, thus defining the CO_2 fixation process term. The common empirical equation used to describe the overall photosynthesis reaction is as follows:

$$6H_2O + 6CO_2 \xrightarrow{Light\ Energy} C_6H_{12}O_6 + 6O_2$$

Photosynthesis overall reaction can be divided into two pathways: (1) light-dependent reaction and (2) light-independent or dark reaction (Klinthong et al., 2015) as illustrated in Figure 1.

Light-dependent reaction captures the light energy and converts ADP and NADP+ into ATP (energy carriers) and NADPH via the electron transport chain and produces oxygen (Zhao & Su, 2014). Excited electrons are transferred to electron acceptors, leaving the reaction centre in an oxidised state. The overall equation for the light reaction is as follows:

$$2H_2O + 2NADP^+ + 3ADP + 3P \xrightarrow{Light\ Energy} 2NADPH + 2H^+ + 3ATP + O_2$$

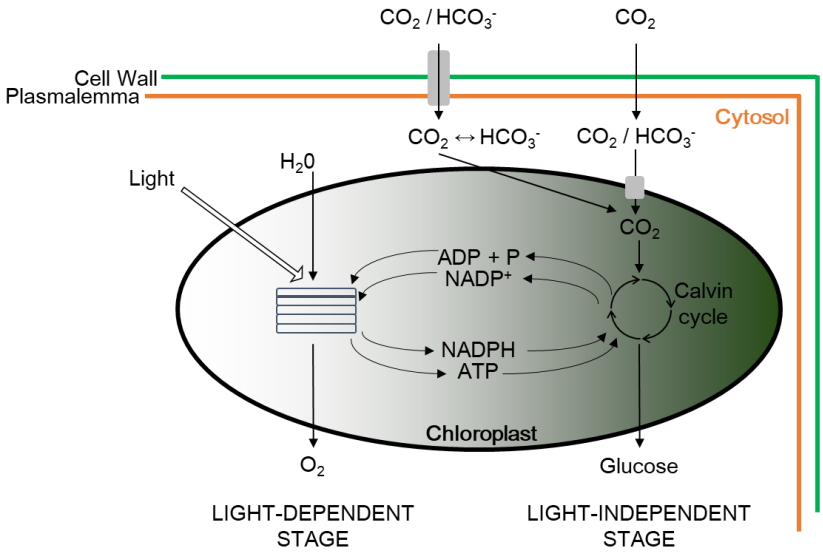


Figure 1 Illustration of carbon influx pathways into the microalgae, together with the light-dependent and light-independent reactions in the photosynthesis process. The shaded boxes indicate the active transport of CO_2. The illustration is modified from a model by Giordano, Beardall, & Raven (2005).

Next, the dark reaction captures CO_2 using RuBisCO enzyme and produce the precursors of carbohydrates by the Calvin-Benson cycle with the presence of ATP and NADPH formed previously. Dark reaction overall equation is as follows:

$$3CO_2 + 9ATP + 6NADPH + 6H^+ \rightarrow C_3H_6O_3\text{-phosphate} + 9ADP + 8P + 6NADP^+ + 3H_2O$$

The sugars produced during this carbon metabolism generate carbon skeletons which can be further used for other metabolic reactions such as amino acids and lipids production (Razzak et al., 2013).

Meanwhile, in heterotrophic behaviour, the organisms have to acquire carbon in the form of reduced organic substances such as glucose or acetate to grow and accumulate starch. One example is what is reported by Choix et al. (2014) to *Chlorella vulgaris*.

In the other hand, mixotrophism refers to the combination of both autotrophism and heterotrophism. Microalgae consume CO_2 in the presence of light as well as organic carbon in the growth media. Mixotrophic cultures are known to have several advantages such as allowing oxygen re-utilisation, which is to prevent photo-oxidative damage due to accumulation of oxygen produced by photosynthesis (Pires, 2015). Besides, they usually have a higher growth rate compared to autotrophic or heterotrophic culture (Mitra, van Leeuwen, & Lamsal, 2012;

Chojnacka & Noworyta, 2004). However, the use of organic compounds in the medium may also increase the production cost besides allowing heterotrophic organisms to contaminate the culture easier (Pires, 2015).

2.2.1 Autotrophic Carbon Delivery

At least three methods are known to direct carbon towards microalgae culture, i.e. (1) actively bubbling the medium with CO_2 (Sydney et al., 2010), (2) dissolving the inorganic carbon, e.g. HCO_3^- into the culture (Lam & Lee, 2013) and (3) changing the external pH of the culture to passively diffuse atmospheric CO_2 into the medium (Moazami-Goudarzi & Colman, 2012).

Actively bubbling CO_2 to the medium is usually done by sparging captured CO_2 to the medium. Usually, this method is associated with the problem of expensive CO_2 capture and transportation, as well as significant CO_2 loss during cultivation (Chi, O'Fallon, & Chen, 2011). Besides, the low solubility of CO_2 in water also creates limitations towards CO_2 bio-fixation by microalgae (Lam & Lee, 2013). As CO_2 has low solubility in water, i.e. approximately 1.45 g/l at 25 °C and 1 atm, it easily escapes to the atmosphere. Thus a continuous supply of air is required to ensure the microalgae has a carbon source to carry out photosynthesis. Henry's Law can be related to this step. At equilibrium, when the partial pressure of CO_2 is the same in both the air and the medium (i.e. $p = P$), CO_2 molecules enter and leave each phase at the same rate. However, as the partial pressure of CO_2 in one reservoir is higher than that in the other, there will be a net diffusive flow of gas into or out of the medium in response to the concentration gradient across the air/sea interface (Chester, 1990).

Another method of delivering CO_2 to microalgae is by supplying inorganic carbon in the form of bicarbonate (HCO_3^-), taking advantage of their high solubility in water. For instance, the solubility of sodium bicarbonate at 25 °C is 9.32% (w/w) (Chi, O'Fallon, & Chen, 2011). One of its advantages is that it is an effective lipid accumulation trigger (Gardner et al., 2012), especially in marine Chlorophytes (White et al., 2013). Gardner et al. (2013) compared CO_2 and bicarbonate as inorganic carbon sources in *Chlamydomonas reinhardtii*. They found that high CO_2 concentration produced the highest amount of biofuel precursors among the experimental set, while high bicarbonate gave a stable TAG and starch production, but with the effect of cessation of cell cycling. A study done in a culture of *Dunaliella salina* using

0.06M bicarbonate found an increase of 2.8 folds in specific growth rate; however, pH of the culture need to be controlled to achieve this high biomass productivity (Kim et al., 2017).

Dissolved inorganic carbon (DIC) exists as CO_2 gas, carbonate and bicarbonate. Altogether, the summation of these components will be referred to in this report as the total carbon dioxide, TCO_2. As noted by Chen et al. (2016), the possibility that microalgae only use carbon in CO_2 gas form or also take up the HCO_3^- and CO_3^{2-} forms is not a critical issue, as reactions that interconvert CO_2, H_2CO_3, HCO_3^- and CO_3^{2-} in the soluble form below are fast enough not to be limiting steps in cells' CO_2 demand.

$$HCO_3^- + H_2O \rightleftharpoons H_2CO_3 + OH^-$$
$$H_2CO_3 \rightleftharpoons CO_2 + H_2O$$

However, there are also challenges in supplying inorganic carbon in this form as it is essential to choose strains that are tolerant and resistant to high ionic strength (Chi et al., 2013, 2014). As this will also create a culture medium of high alkalinity, one needs to find a photosynthetic organism that can grow under such conditions. This problem may lead to reduced efficiency that is too low to have commercial value.

Another way of delivering carbon is by adjusting the pH. According to Bjerrum's plot (Figure 2), the DIC in seawater at 25 °C of around pH 7.5 exist as bicarbonate, while at pH 4, almost all of them exist as CO_2, (Chen et al., 2016). There is general agreement on microalgae preference for CO_2 as the inorganic carbon source, as it is easily controlled and results in a small reduction in pH (Carvalho, Meireles, & Malcata, 2006).

As pH increases, the solubility of essential nutrients such as phosphorus, iron and many trace elements decreases, and the growth of the microalgae decreases (Goldman, 1973). Thus there is a need to maintain the culture at optimum pH levels that satisfy the microalgae preference to deliver the carbon to them optimally. Moazami-Goudarzi & Colman (2012) had observed effect of changing the pH in *Stichococcus minor* and *S. cylindricus* and found that in low pH, active uptake of HCO_3^- and CO_2 is absent; instead, the CO_2 is taken up by diffusion.

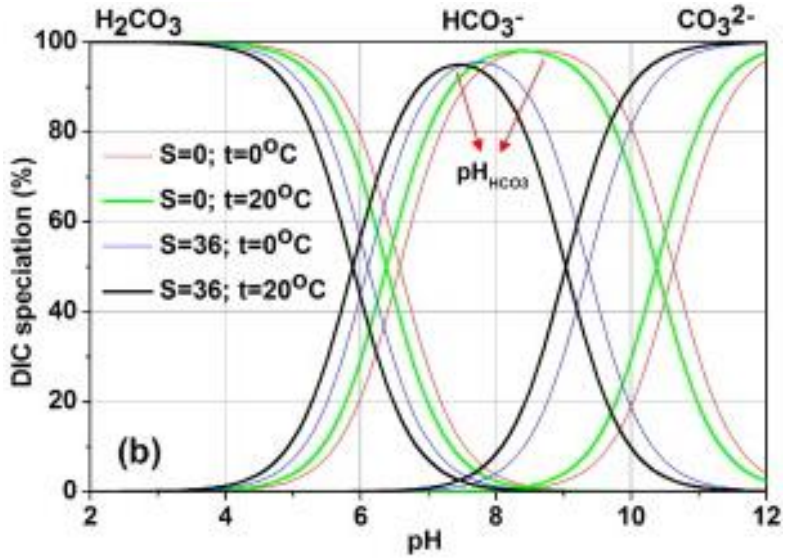


Figure 2 Bjerrum plot describes the proportion of DIC species with respect to pH, salinity (‰) and temperature (Chen et al., 2016)**.**

Besides, since Bjerrum's plot is a function of salinity and temperature, it is observed that in freshwater, the curve is shifted to the left. As microalgae are also classified according to their habitat (i.e. range of freshwater to marine), information on the proportion of the carbon is essential when accessing their growth condition.

2.2.2 Calvin Cycle and Carbon Concentrating Mechanism (CCM)

Most of the dissolved inorganic carbon influx into microalgae use the C_3 pathway, i.e. photosynthetic carbon reduction cycle, also known as the Calvin Cycle (Figure 1) (Giordano, Beardall, & Raven, 2005). The carbon acquisition and conversion to organic carbon are helped by Rubisco enzyme. During this process, a Carbon Concentrating Mechanism (CCM) plays a vital role to keep CO_2 concentration at the active site of Rubisco at a higher concentration than oxygen (Zhao & Su, 2014). This mechanism increases the rate of photosynthesis.

As CO_2 diffuses into the microalgae culture medium, the CO_2 exists as CO_2 gas or aqueous HCO_3^- or $CO3^{2-}$. The dominant carbon species in a medium of pH between 6.4 and 10.3 is bicarbonate (>50%) while in low pH, the dominant species is CO_2. Detailed studies on the effect of the carbon source on the productivity of microalgae have shown that, while HCO_3^- is easily absorbed by cells, it is a weak carbon source compared to CO_2 (Carvalho, Meireles, & Malcata, 2006). However, HCO_3^- is also being utilised, which is assumed to be either with the help of carbonic anhydrase or via direct HCO_3^- transport using transmembrane bicarbonate transporter (Sydney et al., 2019; Sayre, 2010). For example, in *Chlorella vulgaris*, cellular

carbon uptake is done through the carbonic anhydrase enzyme, which catalyses the hydration of CO_2 that produces HCO_3^- and a proton (Sydney et al., 2019).

Bicarbonate is actively pumped into cells to increase internal CO_2 concentrations to levels above those reached by equilibrium with air, and competitively suppress photorespiration (Sayre, 2010). This photorespiration process may decrease the efficiency of photosynthetic carbon fixation by 20% to 30% (Zhu, Long, & Ort, 2008).

2.2.3 Assessing Carbon Uptake by Microalgae

One of the ways to improve the growth dynamics of microalgae is by increasing the efficiency of carbon fixation by the algae. Carbon uptake by cells will be used for at least three purposes, i.e. respiration, a source of energy and formation of cells (Berman-frank & Dubinsky, 1999). Its dynamic can vary according to multiple factors such as the state of the algae physiology, medium chemistry, reactor design and temperature.

Biological & Chemical Equilibrium

There are two equilibria to consider when assessing carbon uptake inside the culture, i.e. chemical and biological equilibrium (Rodríguez-Maroto et al., 2005) as shown in Figure 3. Chemical equilibrium considers the change of carbon content as it interacts with the culture medium. For example, carbon might be supplied as $NaHCO_3$ or CO_2 gas. Besides, CO_2 solubility is influenced by salinity too. H. Zhao et al. (2015) observed that there is a difference in CO_2 solubility between CO_2-H_2O and CO_2-brine system, in which the latter has a higher solubility value.

Once the media is filled with the algae, the condition involves a combination of chemical and biological equilibrium. The differences between the media and the algae culture will help us understand the behaviour of the biological equilibrium.

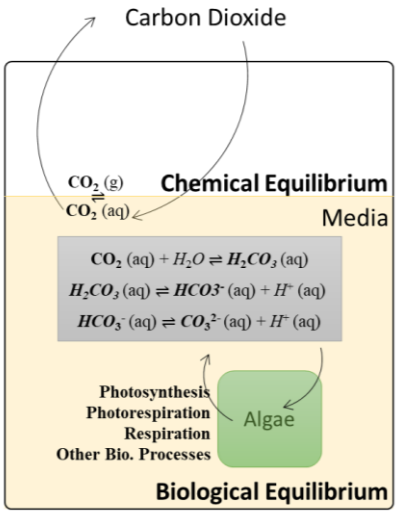


Figure 3 Biological and Chemical Equilibria among the DIC species in Microalgae Cultivation. Arrows indicate carbon transfer (i) between outside and inside of the media and (ii) between algal extracellular and intracellular environments. Equations represent interconversions between carbon species.

In terms of biological equilibrium, several processes have been known to involve in microalgae carbon uptake. One of them is photosynthesis, in which the algae uses CO_2 and light to produce glucose and oxygen. The details on the reaction have been described at the beginning of Section 2.2. This detail comprises of both light-dependent and independent reaction. Regardless of the carbon species being incorporated into the cultivation, all of them will be converted to CO_2 by intracellular carbonic anhydrase before reaching the reaction centre of Rubisco (Chen et al., 2016).

Another biological process is photorespiration, in which due to low CO_2 solubility, the RuBisCo enzyme binds with O_2 rather than CO_2 (Cheah et al., 2015). It inhibits CO_2 fixation and subsequently reduces photosynthetic efficiency (Kesaano et al., 2015). Another related process is respiration, which is when the algae consume O_2 while giving out CO_2. This process is assumed to be constant and occurs both in the light and dark, whereas photorespiration is mostly active in the light (Kesaano et al., 2015). Depending on the CO_2 concentration or pH during development, many unicellular algae may exist in both states, with cells growing at high CO_2 appearing to be less efficient in their photosynthetic carbon assimilation (Spalding, 1989).

Several parameters can be used to study the efficiency and productivity of microalgae in sequestrating CO_2: (1) Difference of CO_2 concentration in gas inlet and outlet (2) availability of carbon in liquid media and (3) carbon fixed into biomass. Methods to measure these

parameters are sometimes known as gas measurement, dynamic method and yield method, respectively (Garcia-Ochoa & Gomez, 2009).

Method 1: Gas Measurement Method

By monitoring gas inlet and outlet of the reactors, the efficiency of CO_2 removal and its rate of reduction can be estimated, as shown by Chiu et al. (2008). In their report, calculations were made by observing percentage difference in outlet and inlet, which gave efficiencies ranging between 20 – 60% and CO_2 consumption rate between 0.3 – 0.6 g/L/h (equivalent to 7 – 14 mM/h). In another report, Jacob-Lopes et al. (2010) applied this same method and obtained a maximum CO_2 sequestration rate of 18.7 mg/L/min (equivalent to 26 mM/h).

To apply this method, a blank trial using only sterile media in the vessel is needed. For example, Sydney et al. (2010) ran the blank trial for five days with data acquisition to define the sensors baselines for O_2 and CO_2. This baseline will be used later as a basis to calculate CO_2 consumption.

Method 2: Carbon Dissolution into Culture

The solubility of CO_2 gas at 25 °C is approximately 1.5 g CO_2 / kg water (equivalent to 35 mM). In microalgae cultivation, DIC readings are expected to be around 1.3 – 2.9 mM upon CO_2 supply (Moheimani & Borowitzka, 2011). As carbon is being dissolved, it is used by the algae as multiple species, i.e. CO_2, HCO_3^-, CO_3^{2-} and H_2CO_3. The Bjerrum plot (Figure 2) models the concentrations of these species as a function of the culture pH at equilibrium. For example, in normal seawater where the equilibrium is controlled by pH, more than 99% of the CO_2 exist in the form of carbonate and bicarbonate ions (Chester, 1990). Algae growth rates are controlled by the total concentration of these species (Goldman, 1973). The preferred form of CO_2 consumed is dependent on the algae strain and the condition of media they grow in (Chen et al., 2016).

Gattuso et al. (2010) described five main perturbation techniques towards carbonate chemistry. CO_2 bubbling and mixing with high CO_2 water increases the DIC while not changing the total alkalinity. This perturbation is opposite to the addition of strong acid, which lowers the total alkalinity (TA), but does not change the DIC. Meanwhile, both addition of HCO_3^- and CO_3^{2-} increase the TA and DIC concentration. The combined addition of acid and bicarbonate/carbonate is similar to the first case – DIC increases at constant TA.

One of the methods to measure total CO_2 in culture is by titration. Chen et al. (2016) proposed a better approach towards the estimation of carbon species of higher accuracy and precision. They performed the titration in a 10 ml sample, besides recommending to measure pH (thus to begin the DIC analysis) as soon as possible once the sample was collected. Measurement can also be done with the help of devices such as Multi N/C 3000 analyser and Mettler Toledo lnPro5000 (Tang et al., 2011; Jacob-Lopes et al., 2010).

To quantify CO_2 uptake in the liquid phase, its transfer is interrupted so that its concentration can be measured as a function of time (Jacob-Lopes et al., 2010). This method also requires the establishment of baseline. The total variation in CO_2 concentration is observed. The difference is known to be contributed by the microorganism and by the losses of CO_2 through the outlet.

Method 3: Carbon Assimilated by Biomass

Carbon may be fixed as organic compounds in the cell, mostly as carbohydrates, proteins and lipids (Mondal et al., 2017). In this method, carbon uptake is measured based on dry weight increase and its carbon content which is around 50% (Douskova et al., 2009). By knowing the carbon uptake, its utilisation efficiency can also be calculated:

$$\text{Utilization Efficiency (\%)} = \frac{Productivity \times Carbon\ Content\ Percentage \times \frac{44}{12}}{CO_2\ Aeration\ Rate} \times 100\%$$ (Zhang, Kurano, & Miyachi, 2002).

Judd et al. (2015) discussed the algae remediation of CO_2 and nutrient, in which they registered carbon fixation rates between 0 – 6 g/L/d. Sydney et al. (2019) however recorded lower CO_2 fixation rate by several microalgae strain which is between 0.1 – 1.5 g/L/d.

However, to consider one particular type of carbon fixation such as lipid productivity as carbon uptake parameters will only give incomplete observation. This observation is incomplete because microalgae cultivation may have high lipid productivity but at the same time having low cell growth, such as in widely recognised cultivation strategy to induce lipid production by the influence of nutrient level (Chen et al., 2015).

Estimation of carbon uptake by using biomass will give lower reading since it does not consider carbon being dissolved into the culture medium (Van Den Hende, Vervaeren, & Boon, 2012).

Besides, the carbon in biomass can constitute big range, between 25 -60% of its dry weight (Adamczyk, Lasek, & Skawińska, 2016; Krishnan, Uemura, & Mansor, 2015; Posadas et al., 2015), depending on culture conditions; thus the estimation can be variable. Nevertheless, the approach can be useful if biomass accumulation is the end goal.

2.3 Strategies to Influence Carbon Uptake in Microalgae

There are several factors affecting carbon fixation by the microalgae. For example, under optimised conditions, high CO_2 capture efficiencies between 80 - 99% with short gas residence times at two seconds are achievable (Keffer & Kleinheinz, 2002). Therefore, this section will cover the topic of optimising the carbon uptake by microalgae to reach their highest potential. Strategies to enhance carbon uptake in microalgae cultures can be divided between biological strategies and non-biological strategies. Each of these strategies is discussed below.

2.3.1 Biological Strategies

Strain Selection

Different microalgae have different ability to sequester CO_2 (B. Zhao et al., 2015). A review by Mondal et al. (2017) studied the selection of efficient strain for biodiesel production through carbon capture. They also listed strains that can fix CO_2 suitably such as *Galdieria*, *Chlorella*, *Chlorococcum*, *Synechococcus* and *Nannochloropsis*. Zhao & Su (2014) too found that *Chlorella* was among microalgae species with the most efficient CO_2 utilisation capacity. Besides, different strains often produce highly significant variation in their behaviour; thus, it is sensible to examine not only exemplar taxa but also multiple strains from specific species (Slocombe et al., 2015).

Microalgae Evolution

Microalgae can adapt to different carbon conditions so it can increase its CO_2 fixation capability and biomass production, which can be done using special methods including selective isolation, pre-culture and habituated culture (B. Zhao et al., 2015). This phenomenon can be approached using scientific adaptive laboratory evolution technique (Dragosits & Mattanovich, 2013). Thus there is potential in finding strains that can adapt best to uptake more carbon.

Co-culturing

Mixing microalgae culture with other microorganism is a strategy adopted to obtain a symbiotic relationship between them, such as in terms of biomass growth, growth production, or carbon consumption. For example, Shu et al. (2013) observed a 195% improvement in CO_2 bio-fixation rate in a mixed culture of *Chlorella* sp. and *Saccharomyces cerevisiae* compared to a monoculture of *Chlorella* sp.

Inoculation Density

Varying inoculation (initial culture) density could influence microalgae CO_2 fixation rate (B. Zhao et al., 2015). Chiu et al. (2008) observed that when they grow *Chlorella* sp. of high initial density at 5% CO_2 for eight days, its biomass productivity increased by 0.8 g/l, in which the low initial density showed increased of biomass by four-folds, while the high initial density has ten folds increase. Nevertheless, having high cell density will result in less exposure to light due to shading effects.

2.3.2 Non-Biological Strategies

Carbon Treatment

Carbon is one of the major nutrients needed by the microalgae (Pires, 2015). Carbon treatment can be done either by varying the CO_2 supply concentration or by adding more amount of inorganic or organic carbon into the cultures. Mokashi et al. (2016) recorded that when extra 1 g/L of inorganic carbon (bicarbonate) was added to the culture, extra CO_2 fixation increase of 0.3 g/mL/d was observed. In another report, CO_2 fixation rate of *Chlorella vulgaris* was found to be higher by 0.5 g/l when supplied with a gas of higher carbon concentration (6%) compared to air (Chen, Xu, & Vaidyanathan, 2020).

Nutrient Treatment

Nitrogen and phosphorous are among macronutrients needed by the microalgae. Nitrogen is needed for the production of proteins and nucleic acids while phosphorous is needed for essential components of cells such as DNA, RNA, phospholipids and ATP (Pires, 2015). Since the increase of nutrient will usually lead to more carbon being fixed and also increase in biomass growth (Pereira et al., 2016; Singh & Ahluwalia, 2013), it is hypothesised that increase of nutrient will also increase the carbon uptake too. This hypothesis is in parallel with Yun et

al. (1997) who stated that CO_2 fixation by microalgae requires an abundant supply of nutrients, but not in excess that will result in an environmental problem such as eutrophication.

Although there are studies on the relationship between uptake of nutrients such as nitrogen or phosphorus with the uptake of carbon, most of them are about whether the latter affecting the former (Gonçalves et al., 2016; Arbib et al., 2012; Obaja, Mace, & Mata-Alvarez, 2005; Kargi & Uygur, 2003; Cramer & Myers, 1948). Nevertheless, some reports indicate high carbon fixation happened at the same time when more nitrogen being removed (Nayak, Karemore, & Sen, 2016), or more biomass productivity when more nutrients are being made available (Kuo et al., 2016), or high carbon removal percentage during high nutrient removal percentage (Almomani et al., 2019). However, in all these cases, carbon fixation is not mentioned to be directly affected by the amount of nutrient being consumed nor the condition of the nutrients (replete or deplete).

Chemical Introduction

There is a report of the introduction of zeolite into microalgae culture, in which it increases the DIC concentration by a factor of 1.5, while in blank media, its introduction increases CO_2 capacity by 1.7 (Zainal et al., 2017). CO_2 can also be captured by reacting it with sodium hydroxide (NaOH) aqueous solutions. Yoo, Han, & Wee (2013) reported that 0.9 gram NaOH is capable of capturing a gram of CO_2. They also reported a theoretical amount of carbon capture for MEA, which is 1.4 gram per g CO_2. Lime $Ca(OH)_2$ is also a chemical that can capture CO_2 with a capacity of 0.3 g of lime per g CO_2 (Han et al., 2011). Similarly, there is also a report of using limestone CaO in which cultivation with 2.7 mM of it showed a higher rate of carbon utilisation compared with culture with 10 mM $Ca(OH)_2$ (Zawar et al., 2016). Thus it is has been shown that introducing CO_2 absorbent or adsorbent may increase carbon uptake by the culture.

Bioreactor Design

Bioreactor design (Figure 4) may help towards microalgae carbon uptake by enabling more transfer and delivery of CO_2 supply to the cell. Use of vertical tubular reactors is known to give high efficiency of CO_2 utilisation (Miyamoto, Wable, & Benemann, 1988). Another example is by the introduction of the membrane to photo-bioreactor, in which Fan et al. (2007) found that *Chlorella vulgaris* can demonstrate more CO_2 fixation by two folds, compared to bubble column and airlift reactor. There is also a strategy of delivering CO_2 in the form of

microbubbles, such as of terminal rise velocity 10^{-3} m/s and residence time in minutes, to enhance CO_2 mass transfer rate into water (Al-mashhadani, Wilkinson, & Zimmerman, 2016; Zimmerman et al., 2011), and thus to the microalgae.

Light Intensity and Photo-period

Light is a crucial parameter in autotrophic cultivation as it is required for the microalgae to do photosynthesis. For optimum CO_2 fixation, light intensity must be at an acceptable range in which it is not low to become a limiting factor and not high to cause photoinhibition (Mondal et al., 2017). In a study using a mixed culture of *Chlorella* sp. and *Saccharomyces cerevisiae*, it is found that their CO_2 bio-fixation rate increases from 1000 to 5000 lux, before it decreases afterwards (Shu et al., 2013). In another study, Jacob-Lopes et al. (2010) studied the effect of photoperiod cycles (day/night) in *Aphanothece microscopica Nägeli* culture and found that its CO_2 sequestration capacity during the day/night (12:12) experiment was about 25% lesser compared to continuously illuminated set.

Cultivation pH

pH value is an instrumental tool in CO_2 dissolution. Variation in pH will change the availability of carbon, nutrients and enzymes activity as well as electron and substrate transportation across membranes during respiration and photosynthesis (Singh & Ahluwalia, 2013). For example, in an experiment on pH variation between 7 – 10 using *Thalassiosira*, it was noted that maximum uptake was observed at approximately pH 8, in which they suggested that at high pH levels, CO_2 availability to the algae growth and photosynthesis may become limited (Chen & Durbin, 1994).

Other Physicochemical Factors

Other physicochemical properties of microalgae cultivation also have been identified to contribute toward CO_2 such as temperature, aeration flowrate, mixing and gas hold up (Zhao & Su, 2014). In a study about the influence of temperature towards *Chlorella vulgaris* cultivation under 6% CO_2 supply, Chinnasamy et al. (2009) discovered that CO_2 uptake is reduced by 40% when the temperature is increased by from 30 to 40 °C, and further 10% decrease with further temperature increase by 10 °C.

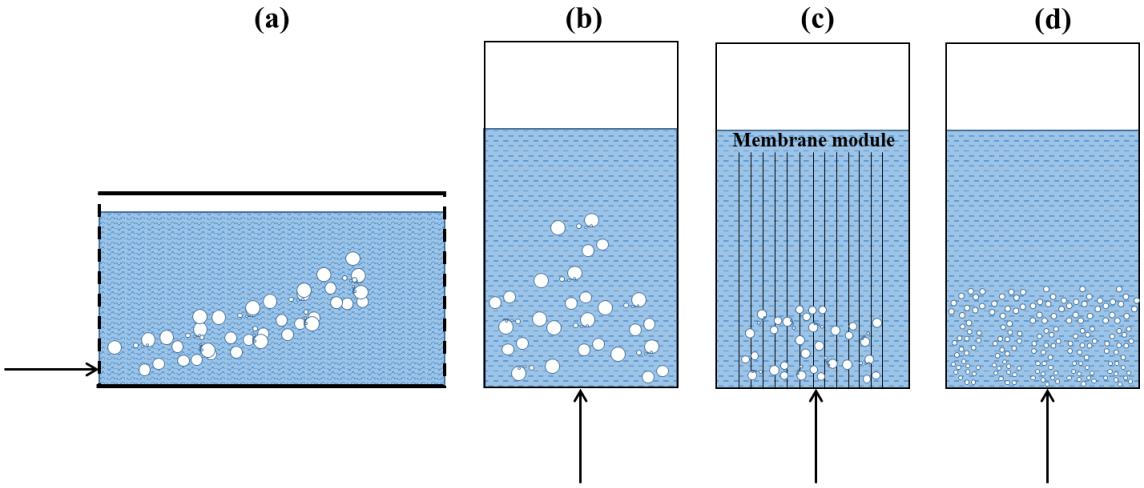


Figure 4 Examples of different configurations of bioreactors which will affect the flow and the dissolution of the CO_2 supply. The arrow indicates the feed of gas into the reactor. (a) Horizontal reactor. The dotted lines indicate that the figure is a cross-section of the horizontal reactor. (b) Vertical tubular reactor (c) Reactor with a dead-end hollow fibre membrane to increase the gas-liquid contact area and reduce the diameter of the gas bubble (d) Reactor with a fluidic oscillator to generate microbubbles.

2.4 Production of Bioplastics Precursors by Microalgae

2.4.1 Introduction to Bioplastics

Plastics are synonymous with polymers, which consists of long chains of molecules polymerised together (Halden, 2010). Although plastic may be referred to a polymeric material that may contain other substances (for better performance and cost), International Union of Pure and Applied Chemistry (IUPAC) however recommends the use of the term "polymer", since the term plastic is generic and also a source of confusion (Vert et al., 2012). Nevertheless, the term "plastics" has been used widely by researchers and manufacturers around the world. These amorphous organic solids cover a wide range of polymerisation products suitable for the manufacture of diversified products (Mekonnen et al., 2013). In terms of its composition, they are usually composed of base polymers and additives such as plasticiser, stabiliser, fillers and colourants (Šprajcar, Horvat, & Kržan, 2012).

Polymerisation refers to a process in which a monomer or mixture of monomers, is converted into macromolecules (Vert et al., 2012). There are two primary polymerisation approaches, i.e. step reaction (also referred to as condensation polymerisation) and chain reaction (also referred to as addition polymerisation). During condensation polymerisation, the reaction often liberates a small molecule such as water, while for addition polymerisation, it requires an initiator such as free radical to occur (Harper, 2002).

Plastics can be categorised into two types, i.e. thermoset or thermoplastics. In terms of chemical structure, a thermoset is cross-linked and will not flow; however, a thermoplastic can be softened and melted by heating it at an appropriate temperature (Harper, 2002).

The term bio in bioplastics or biopolymers indicates that the plastics are either bio-based, biodegradable or having both properties. Bio-based products are products derived in whole or in part from biomass, including intermediates, materials, and semi-finished or final products. Meanwhile, biodegradability refers to the ability to degrade by biological activity that leads to a significant change in the chemical structure of a product (The British Standards Institution, 2013).

Similar to a polymer, there are at least four ways of characterising bioplastics, i.e. mechanical, optical/morphological, thermal and structural properties (Freile-Pelegrín & Madera-Santana, 2017). Mechanical properties of bioplastics are usually illustrated using stress-stain curves. Meanwhile, optical properties can be observed using spectro-calorimeter or microscope. Thermal properties are assessed using four standard techniques, namely differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), thermomechanical analysis (TMA), and dynamic mechanical analysis (DMA). The structural characterisation is based on an infrared technique to elucidate the structure at the molecular scale.

Bioplastics make up nearly 331,000 tons of the plastics market globally (Law, 2017). The main driver for this growing market is the presence of biobased and non-biodegradable plastics, such as PET and PE. Another motivation for bioplastics is that since starch and cellulose have successfully emerged as biobased plastics for non-food purposes (with million-tonne-scale in the industry), it is technically manageable to produce million-tonnes of bioplastics or at a scale that can substitute petrochemical plastics (Shen, Worrell, & Patel, 2010). Although its technical substitution potential of replacing petrochemical plastics is estimated at 90% (Shen, Worrell, & Patel, 2010), this thousand tons of bioplastics represents less than 1% of all the plastics produced annually (Docksai, 2012).

In biodegradable bioplastics market, TPS (thermoplastic starch) recorded the highest global capacity, i.e. over 250 thousand tonnes per year, with an estimation that it has reached around 400 thousand tonnes per year in 2017 (Polyglobe, 2015). Its long history may contribute to this TPS market position compared to other types of bioplastics (Byun & Kim, 2013). TPS, which

are polymers which can flow when heated above a melting or vitrification temperature (Shank & Kong, 2011), are widely used in many sectors such as packaging, catering, gardening and others. Its basic unit, i.e. starch, is easily seen as biodegradable, renewable, of low cost and widely available worldwide (Carissimi, Flôres, & Rech, 2018). TPS global capacity is followed by biobased PE and PLA with global manufacturing capacities of 200 thousand tonnes per year (Kaeb et al., 2016; Polyglobe, 2015).

Bioplastics are used in a wide range of markets in which packaging remains the single largest application sector with approximately 70% (1.2 million tonnes) of the total bioplastics market, which is followed by textiles, consumer goods and agriculture & horticulture (European Bioplastics, 2015). The most profitable type of conventional plastics to substitute is polystyrene, according to the trend in the past ten years. It has the highest market price compared to other polymers at the most time (€ 1000 per tonne), followed by ethylene (€ 900 per tonne) (Polyglobe, 2015). This lightweight material with excellent insulation properties will make a good prospect if it can be produced in biobased and biodegradable form.

Advantages and Disadvantages of Bioplastics

The attractive feature of bioplastics is its biodegradability. Biodegradation refers to the degradation of a polymeric item due to cell-mediated phenomena (Vert et al., 2012). The breaking down process is carried out by microorganism either with the presence of oxygen or without oxygen and at different periods (The British Standards Institution, 2013). It is dependent on several factors such as the exposed surface area, molecular weight polymer composition, nature of monomer unit, crystallinity, moisture, temperature, pH of the environment and cellular activity (Bugnicourt et al., 2014). Later it converts the carbon of plastic to CO_2, water and biomass (Emadian, Onay, & Demirel, 2017). In Europe, European standard EN13432 is used to outline requirements for biodegradation of packaging materials through composting (Zhang, Heaven, & Banks, 2018).

Figure 5 shows examples of plastics classified according to its origin (i.e. biobased or fossil fuel-based) and the ability to decompose after usage (biodegradability). The term “bioplastics” thus refers to plastics that are either biobased or biodegradable. In the table, they belong to Q1, Q2 and Q3. Conventional or petrochemical plastics are grouped in Q4.

	Origin	
Biodegradability	**Q1: Biodegradable & Biobased**	**Q2: Biodegradable & Fossil Fuel Based**
	PLA (Polylactic Acid) PHA (Polyhydroxyalkanoates) PBS (Polybutylene succinate) Polysaccharides / Starch Blends	PBAT (Polybutyrate) PCL (Polycaprolactone)
	Q3: Non-biodegradable & Biobased	**Q4: Non-biodegradable & Fossil Fuel Based**
	PA (Polyamide) Biobased PE (Polyethylene) PET (Polyethylene terephthalate) PTT (Polytrimethylene terephthalate)	Conventional PE PP (Polypropylene) Conventional PET

Figure 5 Example of Plastics, Classified to Their Origin and Biodegradability (European Bioplastics, 2016; Iwata, 2015; Mohd Yusof et al., 2015; Šprajcar, Horvat, & Kržan, 2012)

Among other advantages of bioplastics is that it does not emit toxic remains. This advantage gives a favourable view towards bioplastics amid the widely publicised effects of climate change, higher prices of raw materials and increased reliance on renewable fuels (european-bioplastics.org/). Moreover, since bioplastics are derived from biological processes that utilise CO_2, producing bioplastics provides multiple advantages of saving fossil resources, minimising the greenhouse gases and reducing the dangers of non-biodegradable waste. For example, Grushkin (2011) reported that in a Braskem's green polyethene plant, production of 1 kg of polyethene emitted roughly 2.5 kg CO_2, however, its counterpart, i.e. bio-polyethylene removed 2.5 kg of CO_2 per kg production. This difference is because the sugarcane used as the feedstock is not only a renewable source but also utilises CO_2 for growth.

2.4.2 Bioplastics Made of Microalgae

There are at least three ways in which microalgae can contribute towards bioplastics production, i.e. (1) by directly blending the microalgae with polymers (Zhang et al., 2000) (2) through the production of biochemical components such as polysaccharides (e.g. thermoplastic starch TPS) (Mathiot et al., 2019) and poly-(R)-3-hydroxybutyrate (PHB) (Hempel et al., 2010) that can be used to construct bioplastics and (3) as feedstock that helps bioplastics production, such as hydrolysis of *Nannochloropsis salina* to produce lactic acid for the production of polylactic acid (PLA) (Talukder, Das, & Wu, 2012) or production of ethylene by catalytic pyrolysis, which can be later polymerised to polyethene (Dong et al., 2013).

Polysaccharides as Bioplastics Precursors

Synthesis of bioplastics from polysaccharide can be traced back to the photosynthesis process, in which the polymer is a product of the assimilation of carbon in CO_2 to starch or polysaccharides. Similar to biofuels, the source of this precursors can be classified into four generations (Cuellar-Bermudez et al., 2015), i.e. the first-generation belongs to food crops, the second generation to non-food crop such as their waste, the third-generation belongs to algae while forth refers to genetically modified organisms.

Polysaccharides from microalgae enable the production of industrial and low-grade plastics that can be derived either from starch, cellulose or glycogen, which are all the polymers of glucose (Jabeen, Majid, & Nayik, 2015; Brányiková et al., 2011). Its production can be considered as simple since there is no tedious process required, such as separating the monomers from the algae or genetically modifying them to get better quality and quantity of bioplastics precursors. Bioplastics can be produced by harvesting the biomass into powder before mixing it with polymers, plasticiser and additives to have the plastic property.

PHB as Bioplastics Precursors

PHB, similar to polysaccharides, is a biopolymer produced as a carbon and energy storage molecule and has material properties similar to synthetic plastics such as polypropylene and polystyrene (Sathish et al., 2014). Extraction can be done by rupturing the cells by alkaline treatment or by solvent and water before precipitation into fine powders (Reddy, Reddy, & Gupta, 2013). PHB synthesis by microbes starts with the condensation of two acetyl-CoA molecules to yield acetoacetyl-CoA, which then reduced to hydroxybutyryl-CoA, followed by polymerisation of the latter compound (Anjum et al., 2016; Suriyamongkol et al., 2007), in which all of this processes occur apparently in response to conditions of physiological stress (Ackermann et al., 1995). A review by Miyake et al. (2000) listed several cyanobacteria that are capable of producing PHB such as *Spirulina platensis* (6% of cell dry wt), *Gloeothece* sp. (6% of dry cell weight) and *Synechococcus* sp. MA19 (~30% of cell dry wt). However, in microalgae, bacterial genes (e.g. from *E. coli*) need to be inserted to enable the PHB synthesis. A summary by Somleva, Peoples, & Snell (2013) stated that current production of PHB by genetically engineered *Chlamydomonas reinhardtii* is at 6×10^{-4}% dry weight, while genetically engineered *Phaeodactylum tricornutum* is at a much higher percentage, i.e. 10.6% dry weight.

PHB properties are best summarised by Chanprateep (2010) by comparing it to conventional plastic polymer. The former has higher crystalline melting temperature, Young's modulus and tensile strength, but with a lower percentage of elongation. Pure PHB has thermoplastic processability, absolute resistance to water and completely biodegradable, making it attractive for bioplastics market (Balaji, Gopi, & Muthuvelan, 2013). Its melting point is also near to that of polypropylene, also has better oxygen barrier property (Zia et al., 2017). However, similar to other bioplastic precursors, PHB production by bacterial fermentation is still very costly; thus, its contribution towards the overall plastic market is meagre. The only way to expand its market share is through more approaches that are biotechnological (Balaji, Gopi, & Muthuvelan, 2013).

Microalgae as Feedstock for PLA Production

Polylactic acid is a product of polymerisation of lactic acid, which can be produced by fermentation of algae feedstocks with bacteria. For example, Talukder, Das, & Wu (2012) reported a method for lactic acid production and lipid extraction using *Nannochloropsis salina*, with bacterium *Lactobacillus pentosus*, which gave a maximum lactic acid yield of 92.8%. However, this experiment was done in low volumetric productivity, thus needing improvement for cost-effective production of lactic acid.

PLA physical properties can be tailored through material modification. Besides having the advantage of water resistance, PLA is capable of producing compostable hybrid paper-plastic packaging. It can also be recycled back to its monomer lactic acid by hydrolysis or alcoholysis (Jamshidian et al., 2010). However, the weakness at this moment is the uneconomic viability for the degradation of the carbohydrate from algae or cyanobacteria biomass into necessary feedstocks for PLA productions (Coates, Trentacoste, & Gerwick, 2013).

Microalgae as A Feedstock in Polyethylene Polymerization

One example of plastics produced by monomers obtained from microalgae is biobased polyethene. In this route, the monomer ethylene is produced from microalgae-produced ethanol

$$\text{Ethanol produced by algae} \xrightarrow{dehydration} \text{Ethylene} \xrightarrow{polymerization} \text{Biobased polyethene}$$

Ethylene, which is a primary component in most plastics, can be produced in high yield through various methods such as catalytic pyrolysis of microalgae *Chlorella pyrenoidosa* (Dong et al.,

2013). However, the problem lies at the storage and transportation of ethanol between facilities and feeding of ethanol to reactors for bioplastic production. This method of ethanol production is already a well-established fuel source in many countries; however, it is not economically competitive and feasible compared to conventional ethanol production. A plant producing 500,000 tonnes of ethylene per year would require $150 million of capital cost, which is a lower value compared to $700 million for a cracking plant (Seddon, 2010). To make the conversion of ethanol to ethylene cost-effective, it is suggested to combine the algae technology with the former catalyst (Fan, Dai, & Wu, 2013). Among promising catalyst that they found was a nanoscale catalyst HZSM-5, which has 630h lifespan and 99.7% ethylene selectivity at 240 °C.

2.4.3 Challenges to Bioplastics

Several reviews have been written on challenges and disadvantages of bioplastics such as by Arikan & Ozsoy (2015). Among them are their high cost, the misconception on their biodegradability properties, contamination with conventional plastics at recycling site, possible reduction in raw materials in case bioplastics are made from food crops, and lack of legislation such as on waste management.

All the routes also have economic constraints in delivering bioplastics to the market. Several properties are needed to be addressed in making bioplastics cheaper and more attractive, thus more widely usable. For example, at the upstream level, efforts must be made to obtain bioplastics precursors at a cheap and straightforward method. High costs are also required in obtaining biomass and extracting the saccharides. At downstream level, bioplastics properties such as thermal instability, difficult heat sealability and high water vapour permeability limit their use as films for food packaging. Another example is properties of PHB, in which its stiffness, thermal instability and weak impact resistance restrict its application in food packaging (Jabeen, Majid, & Nayik, 2015).

On topics of degradation or decomposition, not all bioplastics such as PLA can be decomposed, thus requiring a solution at the recycling site. On this topic, there is a recommendation to install new systems that can identify PLA so that it will not mix with other degradable plastics (Docksai, 2012), although there will be a question on whether recycling plants will be willing or able to invest in this.

Despite the disadvantages, among the three routes, polysaccharides, which are the most abundant macromolecules, have been widely used primarily in food packaging as it shows acceptable mechanical and barrier properties against oxygen and CO_2 (Ferreira, Alves, & Coelhoso, 2016). However, to compete with conventional plastics, they are still behind, as it is highly hydrophilic and thus having high water vapour permeability (Galgano et al., 2015). Topics on improving its properties are still ongoing (Orsuwan & Sothornvit, 2018; Cazón et al., 2017; Guo et al., 2017), in which Michaud (2018) believes that there is potential in obtaining polysaccharides from autotrophic growth, in which it has a low relative cost of production and extraction.

2.5 Conclusions

Worsening environmental problems such as increasing amount of greenhouse gases, specifically CO_2, and plastics pollution needs to be addressed by any reasonable methods. Microalgae cultivation is among the potential mitigation methods to these problems while at the same time, it shows other exceptional properties such as fast growth and potential for development as a biorefinery. Different methods have been employed to measure carbon sequestration by microalgae culture. Besides, several factors have also been identified to influence carbon uptake by microalgae, which have been categorized to biological strategies (e.g. strain selection) and non-biological strategies (e.g. carbon and nutrient influence).

Besides, there have been reports found on the incorporation of microalgae in bioplastics production. At least three ways have been found, i.e. by directly blending the cells, by extracting biochemical components which have been identified as bioplastics precursors and as a side feedstock that helps bioplastics production. Polysaccharides are among widely used precursors for bioplastics which can be found in industries such as food packaging. Nevertheless, several significant disadvantages still exist with relative to conventional plastics such as in terms of mechanical properties and hydrophobicity.

Therefore this research will look into microalgae behaviour in sequestrating CO_2. Two different strains of *Chlorella vulgaris*, a species which have been known with high CO_2 sequestration capacity, will be tested. They will be exposed to different carbon and nutrient treatment to see how these differences will affect CO_2 sequestration. Later, red algae, *Porphyridium purpureum* which is known to produce intrapolysaccharides and exopolysaccharides will be tested for carbon sequestration too. At the same time, polysaccharides synthesised from this autotrophic

culture will be utilized to study its potential in incorporating them to produce bioplastics, with a focused assessment towards their mechanical and hydrophobicity properties.

CHAPTER 3: Materials and Methods

General methods that will be used throughout this research will be described. This chapter aims to describe essential procedures, which will include microalgae cultivation, growth measurement and assays of several parameters related to its growth, such as nitrate and carbohydrate profile.

3.1 Microalgae Maintenance and Cultivation

3.1.1 Microalgae Strains

Three microalgae strains were investigated; two of them belong to *Chlorella vulgaris* species (*Chlorella vulgaris* CCAP 211/21A and *Chlorella vulgaris* BDUG 91771) while the third is *Porphyridium purpureum* CCAP 1380/1A (Figure 6). All of them were obtained from the respective culture collection - CCAP is the acronym for The Culture Collection of Algae and Protozoa, which based in the UK. Meanwhile, BDUG refers to culture collection maintained by a university in India, i.e. Bharathidasan University. In its website, CCAP acknowledged that their *Chlorella vulgaris* is also known as *Chlorella ovalis* while *Pophyridium purpureum* is synonymous with *Porphyridium cruentum*.

Chlorella, which belongs to green algae "chlorophyta" is spherical in the shape of average 2 - 10 µm diameter size (Kim, Lee, & Lu, 2014; Phukan et al., 2011) while *Porphyridium* belongs to red algae "rhodophyta" which is spherical too with a slightly larger diameter, i.e. 10 – 18 µm (Sánchez-Saavedra et al., 2018). *Chlorella* is favoured for its high growth rate, ability to assimilate CO_2 at high concentration (Judd et al., 2015) and possessing high CO_2 utilization efficiency (Zhao & Su, 2014). Besides, it can be easily grown in relatively low-cost media and produces significant lipid content (Šoštarič et al., 2009). Meanwhile, *Porphyridium* is known to produce and excrete polysaccharides with bioplastics potential. Both are common species of microalgae reported in the literature. It is known that different strains often produce highly significant variation in yields; thus it is sensible to examine not only exemplar taxa but also multiple strains from specific species (Slocombe et al., 2015). We chose the two *Chlorella* strains to be those isolated from similar habitats, albeit from two geographically distinct culture repositories.

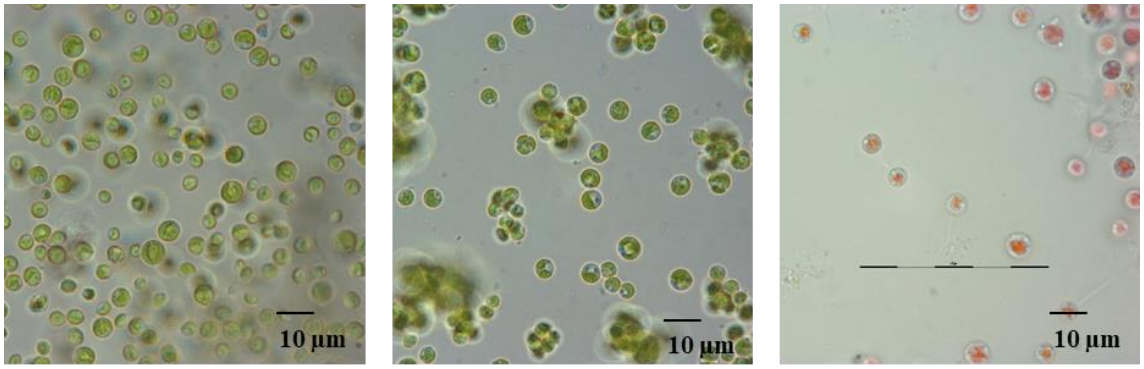


Figure 6 Morphology of (a) *Chlorella vulgaris* BDUG 91771, (b) CCAP 211/21A and (c) *Porphyridium purpureum* CCAP 1380/1A. Images are of same microscopic 100x magnification, in which the scale bar represents 10 µm

For molecular confirmation of strains, their DNA was extracted and used for PCR amplification using a protocol provided by CCAP in their website (ccap.ac.uk). Briefly, cultures were grown in *f*/2 medium (as detailed in Section 3.2.2) until they reached lag phase. 10 ml of the cultures were later centrifuged at 5000 rpm, 25 ˚C, for 20 minutes (details) and the pellets of algae obtained were recovered. The pellets were later subjected to glass bead-beating before genomic DNA extraction using DNeasy Plant Mini Kit (© QIAGEN) as in their instruction.

PCR amplification was done using primer pairs of EAF3 and ITS055R in 50 µl reaction mixture based on the same method by CCAP. The PCR products were later sent to the sequencing service centre, i.e. The Core Genomic Facility of The University of Sheffield. The sequencing results were compared with the database of the National Centre of Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST).

Adjustment and correction to the alignment sequences were made using Geneious Prime® software, which was also used to construct their phylogenetic tree (Figure 7). Sequences were compared with their reference, i.e. *Chlorella vulgaris* BDUG 91771, *Chlorella vulgaris* CCAP 211/21A while for *Pophyridium purpureum* CCAP 1380/1A, a source from its website indicates that the strain is synonymous to *Porphyridium purpureum* SAG 1380-1a.

Analysis of similarity with BLAST result showed a reading of at least 96% similarity with the referenced sequences of strains in the NCBI database. Therefore, this confirmed the identity of microalgae strains used in this study.

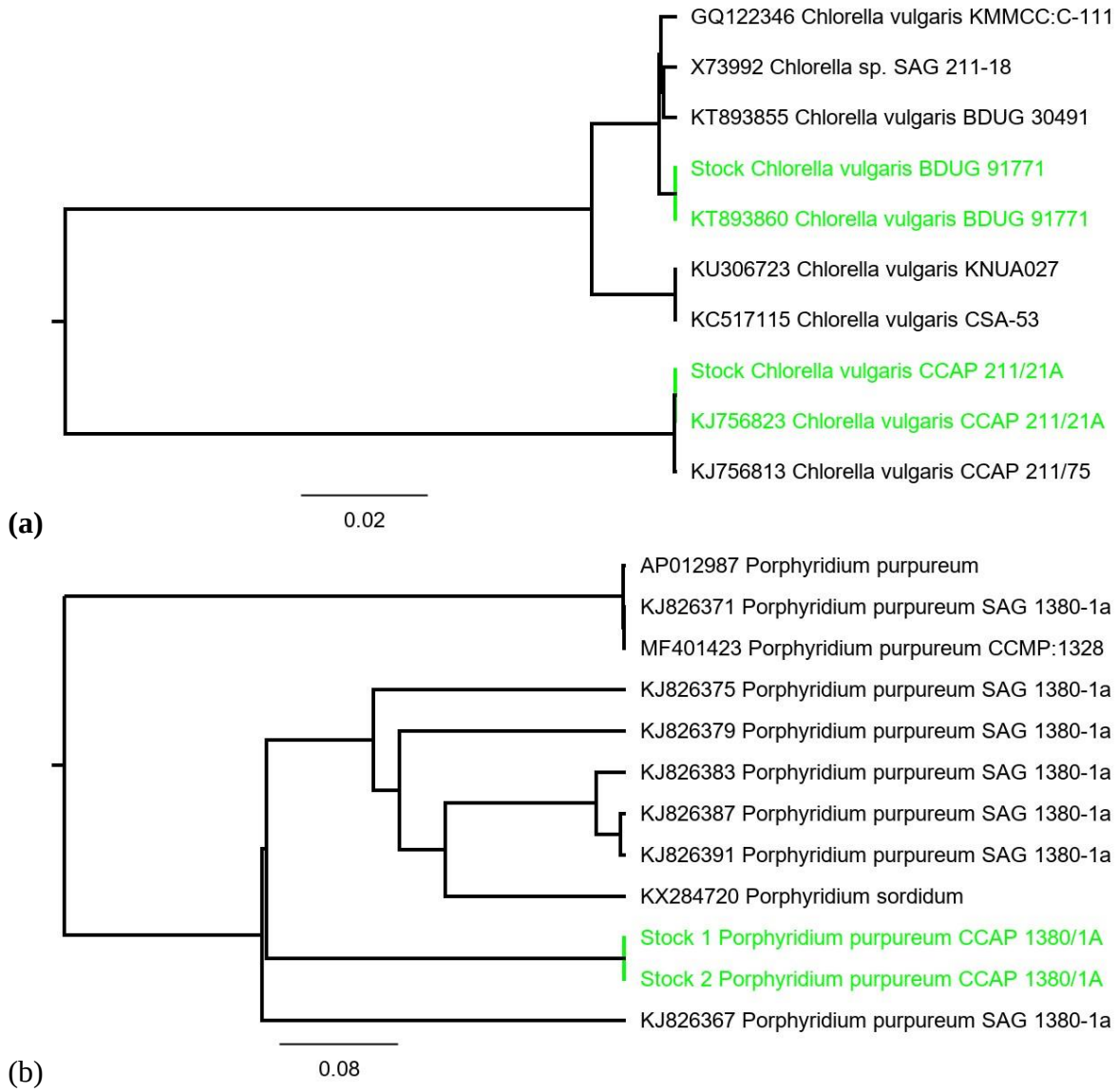


Figure 7 Phylogenetic tree of (a) *Chlorella vulgaris* and (b) *Porphyridium purpureum* by Tamura-Nei distances, with reference to same species of other culture collection. Species used in this research are prefixed by "Stock" while other species are prefixed by their GenBank accession numbers. Images are generated by Geneious Prime® 2020.0 software.

3.1.2 Cultivation Medium

All the microalgae were maintained in *f*/2 medium (Guillard, 1975), which is suitable for marine microalgae species and also among commonly reported standard media. The *f*/2 medium is made per litre by mixing 33.6 g artificial seawater salts (Ultra Marine Synthetic Sea Salt, Waterlife), 75 mg $NaNO_3$, 5.65 mg $NaH_2PO_4.2H_2O$, 1 ml trace elements stock and 1 ml vitamin mix stock. The trace elemental solution (per litre) consists of 4.16 g Na_2EDTA, 3.15 g $FeCl_3.6H_2O$, 0.18 g $MnCl_2.4H_2O$, 10 mg $CoCl_2.6H_2O$, 10 mg $CuSO_4.5H_2O$, 22 mg $ZnSO_4.7H_2O$ and 6 mg $Na_2MoO_4.2H_2O$. The vitamin mix solution (per litre) consists of 100 mg vitamin B1, 0.5 mg vitamin B12 and 0.5 mg biotin. Cultures were maintained on orbital shaker of 150 rpm.

3.1.3 Aeration

Two types of aeration were used throughout this research, i.e. continuous supply of air and 1% CO_2. Aeration was obtained from gas lines (air and CO_2) in the laboratory through Teflon tubing. The flowrate is ensured by connecting rotameter between gas supply and the reactor.

To get specific 1% CO_2 concentration, it was obtained either from the gas cylinder tank of 1% CO_2 or by mixing the pure 100% CO_2 and air at different flowrate. The CO_2/air mixture was adjusted to achieve the desired concentration of carbon dioxide in the airstream through three rotameters that measured the flow rates of the carbon dioxide, the air, and the mixture of gases, respectively. To monitor the CO_2 concentration, gas supply to the culture was split to create one extra flow to BlueInOne gas analyser (BlueSens, GmbH, Germany) which will log the CO_2 concentration every hour using its infrared sensor. Calibration was done as described in its website (bluesens.com) using ambient air as 0.04 vol% CO_2.

3.1.4 Light and Temperature

All the microalgae were cultivated at 200 µE/m2/s for *Chlorella* (Alketife, Judd, & Znad, 2017; Cheng, Labavitch, & Vandergheynst, 2015) and 100 µE/m^2/s for *Porphyridium* (Guihéneuf & Stengel, 2015; Velea, Ilie, & Filipescu, 2011; Gasljevic et al., 2009). This reading will be average for at least six positions in reactors.

Red microalgae are known to have a low tolerance to high irradiances as they have deep habitat (Gaignard et al., 2019). Research by Sánchez-Saavedra et al. (2018) found that 50 µE/m^2/s light is optimum for lipid and biomass production, while 200 µE/m^2/s is suitable for pigments synthesis.

They were exposed under 24:0 daylight regime to have a high growth rate and carbon uptake (Prasetyo, Setyaningsih, & Ratih Agungpriyono, 2015; Keffer & Kleinheinz, 2002; Iqbal & Zafar, 1993). Illumination was provided by strips of LED attached to the frame of hollow cylindrical, positioned around the reactors. Photosynthesis activated radiance (PAR) irradiance sensor (QSL-2100, Biospherical Instruments Inc.) was used to measure the light intensity.

Regulating temperature in the laboratory is quite challenging to avoid significant differences during day/night and summer/winter. Thus, the experiments were done in an enclosed structure with front opening, equipped with temperature controller, heater, fan and thermostat (Figure

8). After adjustment to the set temperature of the thermostat, continuous monitoring of the culture showed a consistent reading of 23 ± 3°C.

3.1.5 Acclimatisation

All strains are maintained in *f*/2 medium aerated by air. Few days before experimentation, the cells were acclimatised to the CO_2 concertation and nutrient condition of the experiment. Also known as acclimation, this procedure is to aid the microalgae adaptation in the new environment and also to reduce the effect of overfeeding of the algal cell, and that may lead to the growth inhibition (Chen, Xu, & Vaidyanathan, 2020), thus skewing the results.

3.2 Microalgae Harvest and Analysis

3.2.1 Biomass Concentration Measurement

Biomass concentration was determined based on their optical density (OD), measured using UV/Visible Spectrophotometer at 595 nm and 760 nm for *Chlorella* and *Porphyridium*, respectively. These standard wavelengths were used to avoid interference with absorbance by chlorophyll or other photosynthetic pigments, in which at these wavelengths, the absorption of the pigment is at a minimum (Kapoore et al., 2019; Moheimani et al., 2013). This optical density can be converted to dry weight (g/l) using calibration equation which will be described in Chapter 5 on Nutrient Treatments in *Chlorella* and Chapter 6 on Carbon and Nutrient Treatments in *Porphyridium*.

Calibration equation is established by using cultures at the end of the experiment of each chapter. This equation is established based on the assumption that during the short duration of the experiment, there is no significant difference in cell weight per OD between treatments. The cultures were serially diluted to represent several optical densities. These cultures were later centrifuged to remove the supernatant, washed with PBS buffer and froze, before being freeze-dried in lyophilizer overnight. Dried samples were then weighed. Later, the dried weights were plotted against their representative optical densities to obtain the straight-line equation, which was used for the dry weight – optical density conversion.

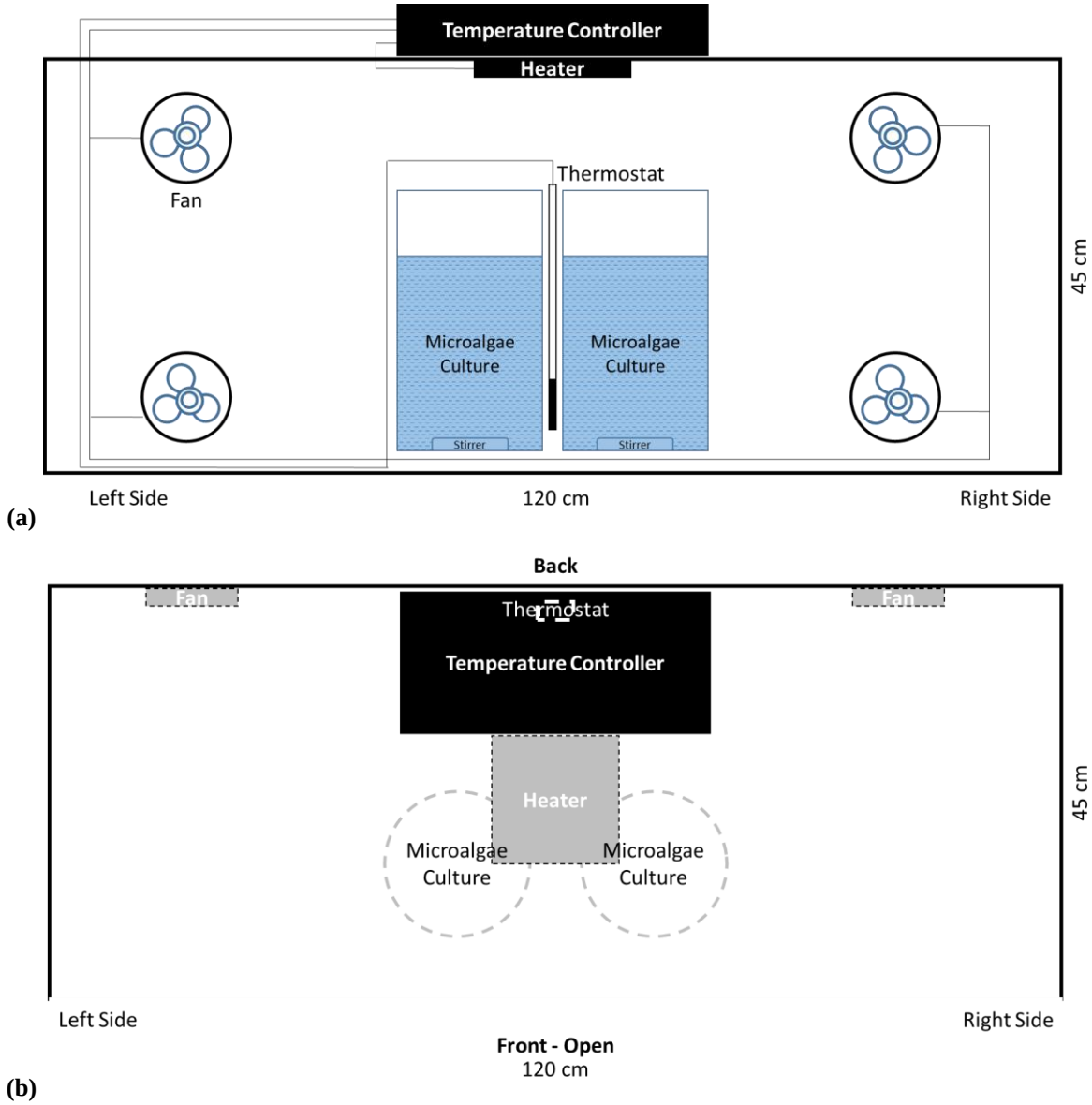


Figure 8 Regulation of Temperature in The Experiment Setup by using Thermostat, Fan, Heater and Temperature Controller. (a) Front and (b) Top View of the Structure. Drawings are not to scale.

3.2.2 Dissolved Inorganic Nitrogen and Phosphorous Measurement

Dissolved inorganic nitrate (DIN) and phosphate (DIP) concentration in culture were determined spectrophotometrically using the culture supernatants based on the methods by Collos et al. (1999) and Strickland & Parsons (1972) respectively. In brief, DIN in the media was determined by measuring the absorbance of 0.5 ml supernatant at 220 nm. DIP was determined by measuring the absorbance at 885 nm after reacting 1.0 ml of the supernatants with 0.1 ml of composite reagent containing molybdic acid, sulphuric acid, ascorbic acid and trivalent antimony of 2:5:2:1 ratio. The absorbance was read about half an hour after the reaction to allow the blue colour developed to remain stable.

3.2.3 pH Measurement

The pH of the sample was measured using a pH meter (S40 SevenMulti™ pH meter, Mettler Toledo). Before each measurement, the vial used to contain the microalgae sample were rinsed using deionized water. pH meter was calibrated with buffer added with a similar salt concentration to avoid error due to liquid junction potential, which occurs due to difference in ionic strength between the samples and buffers (Illingworth, 1981).

3.2.4 Dissolved Inorganic Carbon (DIC) Measurement

DIC concentration in culture was estimated based on methods by Chen et al. (2016). This method eliminates non-carbonate alkalinity in consideration (e.g. those contributed by OH^-, NH_3^+ and H_3PO_4). In brief, samples were harvested, and several relevant parameters (i.e. salinity, pH and temperature) were measured. Based on the salinity and temperature, the pH where bicarbonate species is dominant (pH_{HCO3}) was determined. The samples were later brought to this pH. The first titration was carried out to pH 3.5, while the second titration was done back to its initial pH_{HCO3} after nitrogen purging. The amount and concentration of titrant were recorded, which was translated to Total Alkalinity and Non-carbonate Alkalinity. Based on the correlation, Carbonate Alkalinity obtained was converted to total CO_2 (TCO_2) or DIC. Speciation of this value to CO_2, bicarbonate and carbonate were done by inputting the parameters to CO2SYS program available online.

Positive DIC indicates that there is carbon available in the media for microalgae consumption. This situation happens when carbon consumption is less than the amount of carbon being supplied. Meanwhile, zero DIC indicates that no carbon is available in the media. This condition may be because carbon consumption is more than the carbon available or the carbon being supplied. At least two sources of carbon supply are identified: by algae (respiration) and external source, e.g. from inlet to surrounding. However, there is no possibility of negative DIC.

3.2.5 Carbon Uptake Measurement

Carbon uptake by the microalgae is calculated based on the difference in DIC concentration between two time-points, assuming the carbon losses from the media are all being consumed by the microalgae. This method is applicable for culture without continuous gas supply.

For example, carbon uptake in the first day of the experiment was calculated by observing the difference in the value of DIC at D0 and D1.

$$Carbon\ Uptake,\ mmol = (DIC_{D1} - DIC_{D0}) \times Culture\ Volume$$

A control experiment on carbonated blank media (without the algae) in reactors left for several days was run to monitor the changes in DIC concentration, if any. It is observed that there is no difference in DIC concentration.

3.2.6 Biochemical Properties Measurement

Carbohydrate, protein, lipid, chlorophyll and carotenoid concentration of the biomass were assayed simultaneously based on the method detailed by Chen et al. (2013). The concentration of all these biochemical components was measured spectrophotometrically. In brief, harvested pellets were resuspended in a mixture of methanol and sodium hydroxide before being subjected to beads beating. Aliquots were taken for total carbohydrate measurement using the anthrone method. Later, the remaining sample was saponified. The resulting supernatant was later used for estimating the proteins content. Next, its aliquot was added to a solvent mixture of chloroforms/methanol, 2:1 v/v. The mixture was later centrifuged to get two phases. The top phase was used for the assay of chlorophyll. Half of the lower organic phase was used for the assay of carotenoids while another half was further kept for reaction with triethanolamine–copper salts for the assay of lipid.

3.2.7 Statistical Analysis

Each experiment was conducted in a minimum of three replicates. In all graphs in the following chapters, the data points are the mean values of those replicates, while the error bars denote the standard errors. When comparing two data, those with a significant difference of $p > 0.05$ were only reported, which is calculated using t-test of "Data Analysis" in Microsoft Excel.

3.3 Summary

General methods have been discussed. However, to satisfy research objectives and making comparisons, there may be slight modification towards the methods in the following chapters, such as the introduction of bicarbonate or gas into microalgae culture as well as modification in nutrient concentration, which will be detailed therein.

CHAPTER 4: Dissolved Inorganic Carbon in Cultivation of Two Microalgae Strains of *Chlorella vulgaris* upon Different Carbon Treatments

* Part of this chapter has been presented in two conferences

1. Ahmad Shuhaili, Faqih & Vaidyanathan, Seetharaman (2018). CO_2 and Nutrient Uptake in Two Brackish Water Strains of Chlorella vulgaris. 16th International Conference on Carbon Dioxide Utilization (ICCDU XVI), 29 Aug, Rio de Janeiro.
2. Ahmad Shuhaili, Faqih & Vaidyanathan, Seetharaman, (2018). Carbon Dioxide Uptake by Brackish Water Strains of Chlorella vulgaris. ChemEngDayUK 2018, 27 Mar, Leeds.

Abstract

Two brackish water strains of *Chlorella vulgaris* from two geographically distinct culture repositories, i.e. BDUG and CCAP were compared. The objective of this research was to assess the carbon and nutrient uptake behaviour of the two strains exposed to different regimes of carbon availability. Their response was monitored by measurement of growth, dissolved inorganic carbon, nitrate and phosphate in the media and their biochemical profiles. The BDUG strain showed higher biomass production as well as higher growth rate compared to the CCAP strain. In terms of pH, cultivation of BDUG showed a slightly more alkaline reading. However, in terms of nutrient consumption, faster consumption of nitrate and phosphate was observed in culture with CO_2 aeration. We also noted that for active nutrient uptake, a sufficient carbon, appropriate pH and mixing will help to maximise mass transfer. In terms of carbon availability, it is noticed that the presence of algae increased the amount of DIC in the culture, which to the best of our knowledge, has been acknowledged here for the first time. Among BDUG and CCAP, the former showed a faster rate of carbon consumption, although their difference is not apparent among different carbon treatment. More amount of lipid was produced in CCAP culture, but no significant difference is observed in protein and carbohydrate production. In terms of carbon treatment, cultivation with continuous carbon supply gives the best growth compared to the remaining three cultivation, i.e. without carbon addition (C_0), with an initial supply of 0.01M of bicarbonate ($C_{0.01M}$) and with continuous air supply (C_{air}), except for lipid production, which showed higher concentration in C_0 and $C_{0.01M}$. pH in cultivation CO_2 is acidic, while cultivation with air, bicarbonate and no carbon supply is alkaline.

4.1 Introduction

Recently, microalgae cultivation has gained attention as an alternative means towards CO_2 sequestration, alongside with its bio-refinery potential, i.e. a mini version of a traditional petroleum refinery. Compared to terrestrial plants, microalgae have higher photosynthetic efficiency, produces more biomass yield and needs a smaller land area for cultivation (Klinthong et al., 2015). Besides, it can utilize nutrient resources from waste (Razzak et al., 2017) to produce chemicals with value-addition, such as biofuels, pharmaceuticals bioplastics and many more.

Carbon, which is essential in algae growth and reproduction is one of the essential elements required for microalgae nutrition. In photoautotrophic growth, carbon is typically available as dissolved inorganic carbon (DIC). Seawater has an approximate DIC concentration of 2.5 mM (Sauvage et al., 2014). Interchangeability of species that constitute DIC is based on the following chemical equilibria:

$$CO_2\ (g) \rightleftharpoons CO_2\ (aq)$$
$$CO_2\ (aq) + H_2O \rightleftharpoons H_2CO_3\ (aq)$$
$$H_2CO_3\ (aq) \rightleftharpoons H^+\ (aq) + HCO3^-\ (aq)$$
$$HCO_3^-\ (aq) \rightleftharpoons H^+\ (aq) + CO_3^{2-}\ (aq)$$

In growth media such as *f*/2, more difference is expected. Addition of buffer, i.e. TRIS causes significant deviations from natural carbonate chemistry as it increases total alkalinity towards high values (Gattuso et al., 2010). However, nitrate and phosphate addition does not alter the alkalinity system since these species do not contribute to the total alkalinity.

According to Moazami-Goudarzi & Colman (2012), in seawater of pH between 8.0 – 8.3, bicarbonate (HCO_3^-) is the predominant form, i.e. around 2 mM, while CO_2 represents less than 1% (20 µM) of the T_{CO2}. This statement is agreed by Nakajima, Tanaka, & Matsuda (2013), who found that the concentration of CO_2 gas in seawater under the present atmosphere is below 15 µM at 20 °C.

The proportion of DIC in aquatic environments is best illustrated as a function of pH, salinity and temperature (Chen et al., 2016). At pH 9, there is almost no CO_2, while HCO_3^- and CO_3^{2-}

exists at an equal ratio approximately. At pH 7.7, salinity 33 ppt and temperature 20 °C, more than 90% of carbon exist as bicarbonate while less than 10% is present as CO_2 and CO_3^{2-}. At low pH, e.g. 5.0, most of the total DIC is in the form of CO_2. It is reported that at this pH, there is no active HCO^{3-} and CO_2 uptake, and cells appear to take up CO_2 by diffusion (Moazami-Goudarzi & Colman, 2012).

Theoretically, in CO_2-H_2O-NaCl system of 33.5 g of NaCl per 1 kg water, CO_2 solubility is 33 mM at 20 C and 0.1 MPa (Akinfiev & Diamond, 2010), which is equivalent to 1.5 g/L (CO_2 molecular mass = 44 g / mol). However, it seems that DIC readings are unable to capture the saturation value of 33 mM of CO_2 in bubbling regimes, and only barely approaches these in a media containing NaOH (a CO_2 absorbent). In this case, the pH is adjusted to high enough value of > 10. Another method is by changing the volumetric flow rate (vvm - gas volume flow per unit of liquid volume per minute) or pCO_2 (Chang et al., 2016). In the case of bubbling CO_2 into the medium, as the amount of CO_2 increases, more hydrogen ion is present; thus, the pH decreases. On saturation, the medium is unable to take CO2 anymore; thus, pH stops decreasing.

In microalgae, carbon is a significant nutrient that makes up the cell, apart from nitrogen and phosphorus. It represents about 50% of the cell dry weight (Pires, 2015). Thus, increasing the amount of DIC will favour microalgae growth. The efficiency of carbon uptake by microalgae is highly related to the concentration of CO_2 supplied. Usually, the higher the concentration of CO_2 supplied, the better the growth and biomass productivity. However, the supply of CO_2 to the microalgae culture will also change the environmental condition that the algae live. Thus, a balance between maximising carbon capacity in the culture and keeping optimal conditions for growth must be defined and observed.

Algae utilise the carbon supplied to them differently according to many variable factors such as strain type (Singh & Singh, 2014), carbon source, nutrient supply (e.g. nitrate and phosphate) as well as the condition that they live in (e.g. pH, salinity, temperature and light conditions). This variation will result in different growth characteristics and biochemical composition of the algae.

It is known that different species of microalgae have a different level of adaptation towards CO_2 uptake. Among them, *Chlorella vulgaris*, highly productive microalgae is a species widely

used in algae sequestration of CO_2. *Chlorella*, which belongs to the phylum of Chlorophyta, has been reported to show carbon fixation rates between 0.25 to 6.24 g/L/d (Lam, Lee, & Mohamed, 2012).

Carbon supply to microalgae mass culture systems is among the principal challenges and constraints that require strain-specific characterisations. This aspect will not only influence carbon consumption by microalgae but also its survival under carbon starvation and use of stored carbon. Thus, a proper understanding of this aspect is essential to develop strategies to achieve high carbon uptake and in turn, carbon routing to products of interest.

Chlorella vulgaris can be grown in seawater based media (Luangpipat & Chisti, 2017). The pH of the culture is usually alkaline (Kassim & Meng, 2017). However, highly alkaline conditions will reduce the availability of carbon in the form of CO_2 gas, which will inhibit the microalgae growth later. This reduction in the carbon availability is because inorganic carbon is nearly always supplied as CO_2, but is taken up by the cells mainly as bicarbonate (Shene et al., 2016).

In this work, we evaluated CO_2 uptake by two closely-related brackish water strains of *Chlorella vulgaris*, originating from two different culture collections (CCAP in the UK and BDUG in India) and studied it with respect to different carbon availability regimes. This chapter also aims to investigate the characteristics of carbon saturation in media used for microalgae cultivation. Understanding the microalgae response towards carbon supply shall assist in developing strategies for CO_2 sequestration and sustainable production of value-added chemicals.

We also tried to optimise the earlier published method of DIC determination (Chen et al., 2016) referring to reducing sample volume and enabling storage of samples before analysis. This study is necessitated primarily in experiments when frequent sampling is required. The objective is to reduce the amount of sample volume needed to be taken from culture, thus reducing time for sample processing and the amount of titrant needed. Besides, enabling mid-term storage will allow for analysis of several samples together and decreasing sampling intervals, when needed.

4.2 Methods

CO_2 Dissolution Behaviour in Blank Media

Three levels of CO_2 gas (volumes in the air) were tested, i.e. 100%, 10% and 1%. Before pumping of CO_2, pH was set to the desired value by using 0.1M sodium hydroxide, to have approximately similar speciation of carbon in the media at the beginning. CO_2 was sparged into the culture medium (with no algae in it), and the DIC measured at intervals until it saturates. Saturation is defined when three recorded readings are within the 10% range.

Microalgae Cultivation

The starting inoculum of OD_{595} 0.2 of *Chlorella vulgaris* of two different strains BDUG 91771 and CCAP 211/21A was grown in 1.0-litre culture for ten days using standard *f*/2 media (see Chapter 3 for details of medium composition), at initial pH of 8.3, adjusted with different inorganic carbon treatment (refer below). In this report, both strains will be referred by their culture collection ID, i.e. BDUG and CCAP. The reactor was stirred at 200 rotations per minute (rpm) while the aeration rate was set at 0.2 vvm. Light intensity was maintained throughout the experiment at an average of 200 µE m^{-2} s^{-1} using cool-white LED.

All the reactors and media were autoclaved before the usage throughout the experiment. Sterile practices were applied when dealing with the culture during the experiment.

Carbon Treatment

Four different carbon supply regimes were examined (Figure 9), i.e. (1) without active carbon supply (allowing passive diffusion of atmospheric CO_2) (C_0), (2) with an initial supply of 0.01M bicarbonate ($C_{0.01M}$), (3) continuous active supply of atmospheric CO_2 (0.04%) (C_{air}) and (4) continuous active supply of CO_2 at a higher level (1% CO_2 in air) ($C_{1\%}$).

Dissolved Inorganic Carbon (DIC), Nitrogen (DIN) and Phosphorus (DIP) Measurement

DIC was estimated by back-titration method, while DIN, DIP was spectrophotometrically measured as detailed in Chapter 3

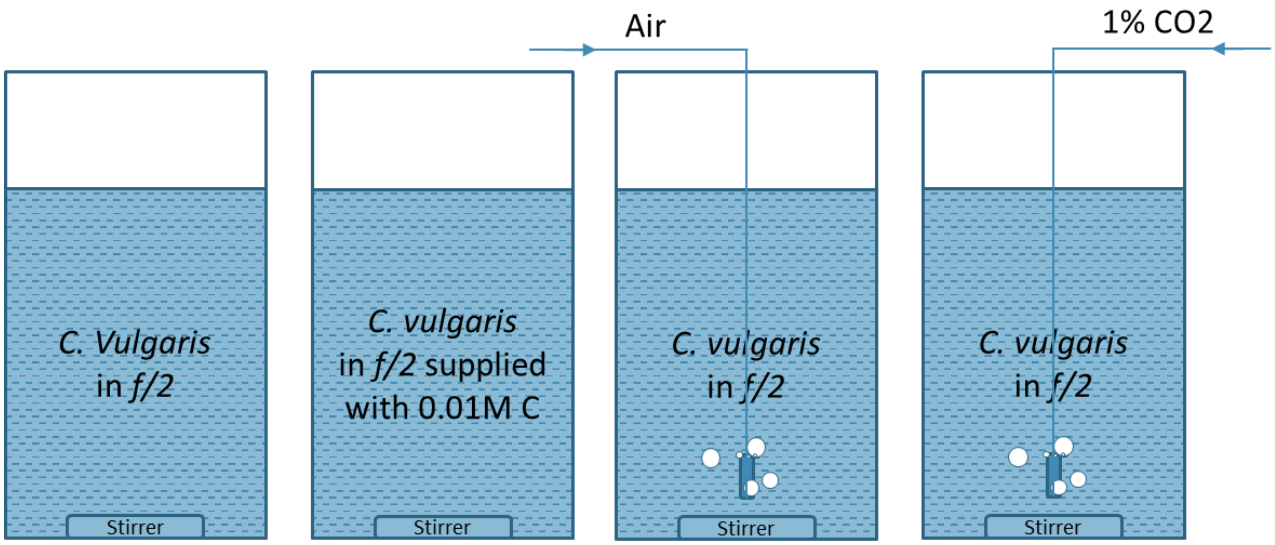


Figure 9: Experimental setup of (from left to right) C_0, $C_{0.01M}$, C_{air} and $C_{1\%}$

Carbon, Nitrogen and Phosphorus Uptake Rate Measurement

Nutrient, i.e. carbon, nitrogen and phosphorus uptake rate were measured based on the difference between each initial and final concentration of DIN and DIP.

$$\text{Removal rate} = \frac{C_i - C_0}{t_i - t_0}$$

C_0 and C_i refer to the initial and the final concentration of the nutrient, respectively. Meanwhile, t_o and t_i each refer to its particular time.

Specific to carbon uptake rate in this chapter, since continuous aeration were made to C_{air} and $C_{1\%}$, its rate could not be measured by using the formula. Thus, only the carbon uptake rate in the C_0 and $C_{0.01M}$ cultivation will be reported.

Folds change in DIC, DIN and DIP were calculated by using the formula:

$$\text{Folds Change} = \frac{Concentration\ Value}{Initial\ Concentration\ at\ Day\ 0}$$

Growth Measurement

Two methods of monitoring growth were carried out in this experiment, i.e. optical density (OD) at 595 nm and cell count. OD was measured by a UV / Visible Spectrophotometer (Ultrospec 2100 Pro) while cell count was done using 1 ml sample, using a haemocytometer under a microscope.

The growth rate is measured by using the following formula,

$$Growth\ Rate = \frac{\ln(N_{t_2}/N_{t_1})}{t_2 - t_1}$$

in which N_1 and N_2 are the optical densities or cell counts at time 1 (t_1) and 2 (t_2) respectively (Moheimani et al., 2013).

pH Measurement

pH measurements were made every day by Orion Star pH Meter A211 Manual (Thermo Fisher).

Biochemical Assay

Samples were frozen at -20 °C for storage before analysis for simultaneous carbohydrate (intrapolysaccharides), protein, lipid and pigment analysis spectrophotometrically, as detailed by Chen & Vaidyanathan (2013). The details have been explained previously in Chapter 3.

Statistical Analysis

The experiments were conducted in triplicates. Details on the statistical analysis carried out have been mentioned in Chapter 3.

4.3 Results and Discussion

4.3.1 DIC Availability and Saturation in Growth Media

Effects of Volume Reduction and Storage on DIC Determination

The earlier published method for DIC determination (Chen et al., 2016) was carried out with 10 mL sample volume and was analysed soon after sampling without storage. Here, we investigated reducing the sample volume and allowing for sample storage at -20 °C. Results showed that there is no significant change in DIC concentration between the optimized set and the control (Figure 10). By reducing titration volume from 10 ml to 5 ml, and by storing the sample under -20 °C for up to 7 several days, the DIC concentration of the samples was not found to be significantly affected.

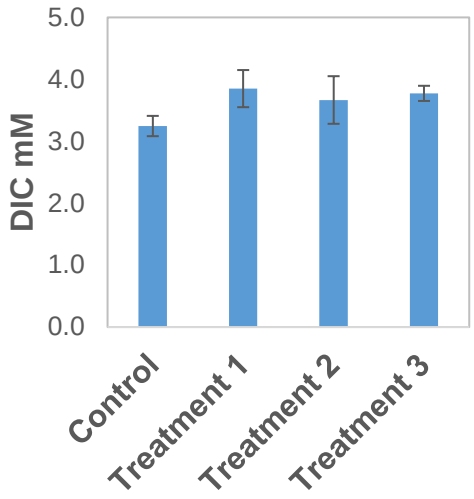


Figure 10 DIC Concentration Before and After Different Optimization Treatments. Treatment 1 is by reducing the volume to 5 ml; Treatment 2 is by storing in -20 °C for three days while Treatment 3 is by both volume reduction and storage, i.e. 5 ml after seven days. Values are means (*n*=3), and error bars correspond to the standard error about the mean. No statistically significant difference could be noted between the treatments and control.

By reducing the volume for analysis, the number of samples that can be taken from the experiment can be increased, and the total volume removed from the culture can be minimised for a given number of samples, since sampling more than 10% of initial volume can be avoided. However, this small volume should not be so little that it will affect the chemistry significantly when titrant is added. As for storage optimization, this will enable many samples to be taken for analysis that runs in parallel and involves a short time difference. The optimised condition (5 mL samples which can be stored at -20 °C for up to 7 days) was employed throughout this investigation.

CO_2 Dissolution Behaviour in Blank Media

The solubility of CO_2 in algal growth media is studied by measuring its DIC content. Upon supply with increasing concentrations of CO_2 gas, DIC concentration in the blank media showed increasing saturation values. However, none of them achieved the theoretical maximum of 33 mM, as saturation by 100% CO_2 at 0.2 vvm gave a DIC saturation value of 16 mM.

As shown in Figure 11a, media aerated with 100% CO_2 showed an 8-fold increase in DIC, at saturation, while 10% CO_2 gave a 2.5-fold increase and 1% CO_2 a 2-fold increase in DIC at saturation, compared to the starting values. Meanwhile, sparging the medium with air decreased the DIC by 30%.

A supply of pure CO_2 of 0.2 vvm for an hour in 1-litre media is equivalent to a supply of 500 mM CO_2. Similarly, a supply of 1% CO_2 is equivalent to 5 mM carbon (0.218 g/l). However, this is not always the value being absorbed into the medium. There is always a chemical equilibrium between dissolved and gas-phase CO_2, with the gas escape to the surrounding. In the experiment, the supply of specified CO_2 concentration and vvm results in a maximum of 2.6 mM (CO_2 of 0.114 g/l) of DIC that media can retain. Thus, in this one hour supply, there is an efficiency of around 50% of CO_2 dissolution into the medium. As the medium reached carbon saturation level, this efficiency decreases since more and more carbon supplied to the media will escape to the surrounding.

Most of the saturation kinetics happened in the first hour of carbon supply. Supply of 100% CO_2 gives fluctuating readings and takes slightly longer time to settle down, i.e. 40 minutes, compared to 10% CO_2, at the employed volumetric flow rate. This fluctuation may be due to the high proportion of CO_2 gas being present in the media, which may easily diffuse out from media to the surrounding.

It is also found that sparging with air (0.04% CO_2, 21% O_2, 78% N_2) does not contribute towards the increase in CO_2 dissolution value, but instead results in loss of DIC from the medium (Figure 11b). After all, aeration is known as one of the methods of CO_2 removal (Gauntlett, 1980). As pH decreases, the amount of DIC loss also increases. This decrease may be due to the CO_2 gas of T_{CO2} being purged due to the aeration. At pH 9.0, there is no DIC loss, since almost of T_{CO2} exists as HCO_3^-.

CO_2 saturation curves were also obtained under different initial pH. The results are plotted in Figure 11c, Figure 11d and Figure 11e, for 100%, 10% and 1% CO_2 in the air. It is found that as the initial pH of the medium increases, the DIC value at saturation also increases. It is also observed that as the concentration of CO_2 supply increases, the DIC saturation value also increases. Besides, it can be seen that the graph of 100% CO_2 has the highest gradient, i.e. 2.7 mM/pH unit, followed by 10%, CO_2 (0.9 mM/pH), then 1% CO_2 (0.5 mM/pH), following the trend of the amount of CO_2 dissolved into the medium. In literature, there is a report of higher saturation value, i.e. 9 mM of DIC by aerating 1% CO_2 at 0.2 vvm (Chang et al., 2016). However, that is without indication of the type of media and initial pH.

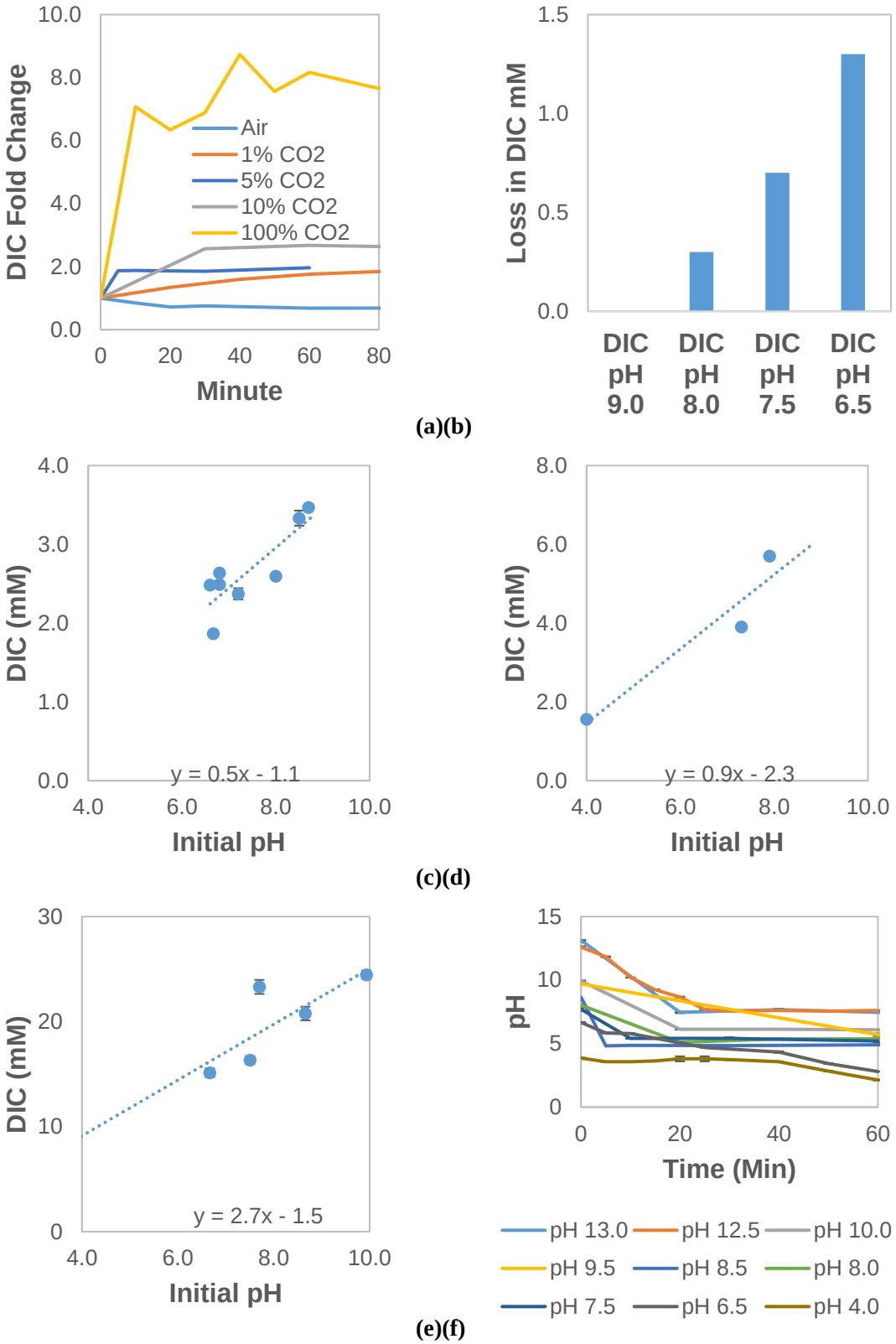


Figure 11 (a) Folds change in DIC in Media pH 7.5 after aeration with air, 1%, 10% and 100% CO_2 (b) Loss in DIC value at saturation, upon saturation supply by air. (c) (d) (e) DIC saturation value upon supply of CO_2 at different concentration 1%, 10% and 100% respectively. (f) pH Changes in Media of the different pH starting point, upon supplied with 100% CO_2. Values in (c)-(f) are means (n=3), and error bars correspond to the standard error about the mean.

Figure 11f shows changes in pH with time after the dissolution of 100% CO_2. It can be seen that as initial pH decreases, the final pH when DIC saturates also decreases. It is noted too that as CO_2 increases from 1% to 100%, the final pH also decreases.

4.3.2 DIC Availability in Microalgae Culture

Initially, DIC molarity in all sets of experiments was measured and known to contain 2 mM (standard carbonate system in seawater (Guihéneuf & Stengel, 2015)) on average. Later, this amount was modified according to the treatments experimented.

$C_{0.01M}$ culture was further supplied with the high DIC of 10 mM of bicarbonate. As for the amount of carbon being supplied by air and 1% CO_2 (0.2 vvm for nine days), gravimetric analysis shows that carbon supplied to the culture is equivalent to 0.04 M and 1.07 M of T_{CO2} respectively. This conversion is made assuming a pressure of 1 atm and a temperature of 23 °C.

CO_2 Concentration (mol/litre) = Total CO_2 supply in 9 days at 0.2 litre/minute (g) ÷ CO_2 molecular weight (= 44 g/mol) ÷ experimental volume (litre)

However, this amount is not fully available for consumption as it includes the gas that escapes to the surrounding, which accounts for about 80 to 90% of the total gas supplied (see Chapter 5.3.6). Thus, we may estimate the amount of carbon supplied in C_0 as negligible, 10 mM in bicarbonate-supplied, a low insufficient < 4 mM (~0.4-0.8 mM) in air and < 1070 mM nevertheless sufficient (~100-200 mM) in 1% CO_2.

In C_0 and $C_{0.01M}$ cultures, carbon was utilized rapidly at the beginning of the experiment, i.e. during their exponential phase, as shown in Figure 12a and Figure 12b. In BDUG, C_0 cultures become carbon limited at Day 6 (D6), while for $C_{0.01M}$, carbon-limitation happened at D8. In CCAP, DIC in C_0 culture was consumed fast at first two days before the uptake rate slowed. At D8, the culture becomes carbon limited. Meanwhile, $C_{0.01M}$ culture becomes carbon limited at D4, coinciding with a stop in growth (Figure 13c and Figure 13d). This observation indicates that 10 mM bicarbonate is not enough for consumption in cultures of both strains in the given duration, or in other words, they showed that their carbon consumption capacity is higher than this value.

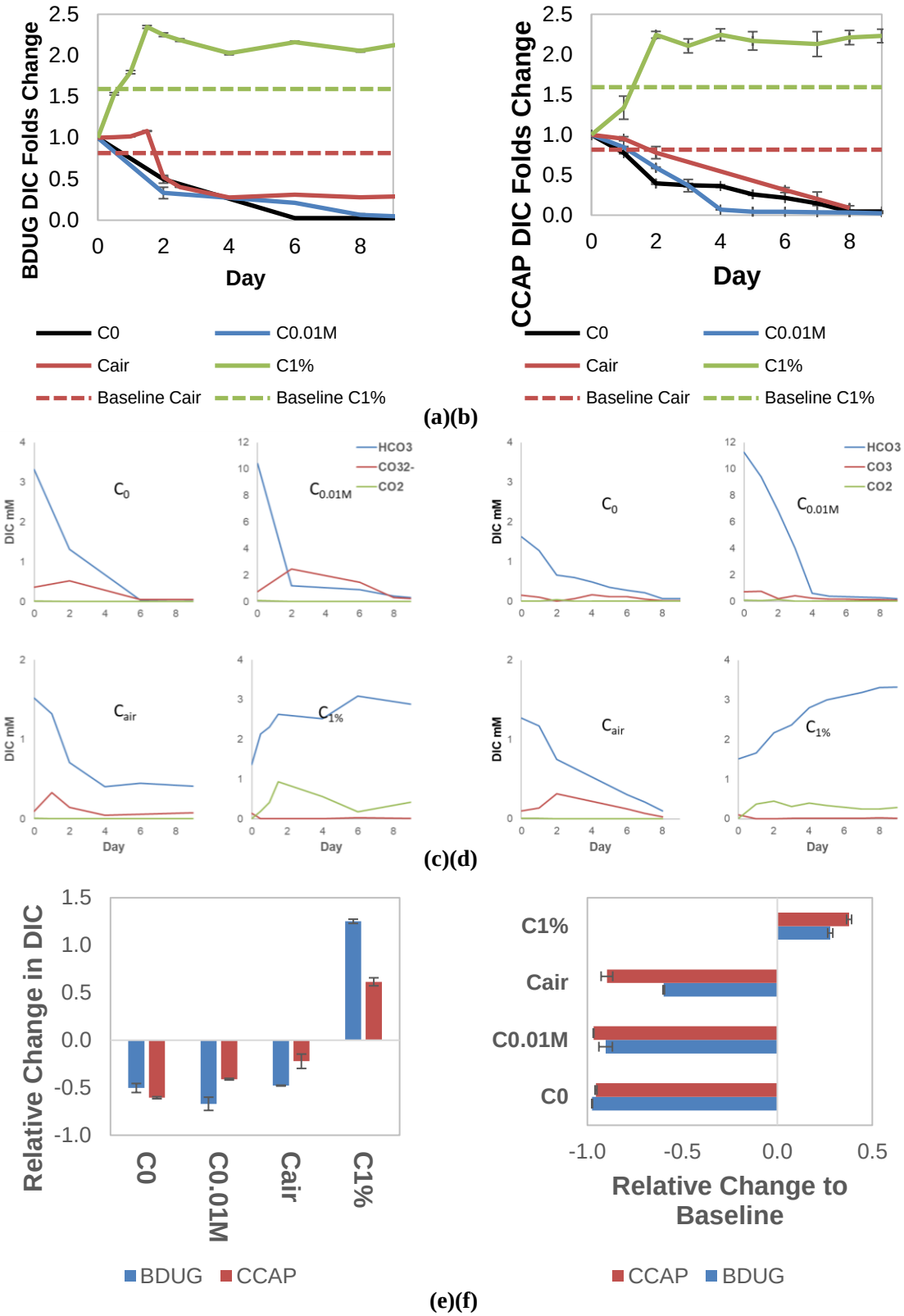


Figure 12 DIC profiles in (a) BDUG and (b) CCAP strains with its respective carbon speciation; (c) and (d). (e) Relative change in DIC at D2 compared to D0, in which minus reading indicates a decrease in DIC compared to D0. (f) Relative change in final day DIC to Baseline. Reading of -1 indicates full usage of DIC, while the reading above 0 indicates that more DIC are trapped in the media. Values in (a)(b)(e) and (f) are means (*n*=3) and error bars correspond to the standard error about the mean.

In C_{air} culture, carbon starvation was observed in both cultures. In BDUG culture, DIC decreases at D2 before plateauing at D4 at around 0.5 mM DIC. In CCAP, the DIC decreases slowly from 1.4 mM at the beginning to nearly zero at the end of the experiment (D8).

In $C_{1\%}$ culture, DIC equilibrium was reached for both BDUG and CCAP culture after D2. In CCAP culture, the DIC increases by more than two folds to 3.3 mM at D2 and saturates at this value. Meanwhile, in BDUG $C_{1\%}$, DIC increases from 1.5 mM to 3.6 mM at D2, before getting saturated around 3.3 mM. Both saturation values in microalgae culture, i.e. 3.3 mM, are above the saturation value in blank media, which is around 2.6 mM. Thus, the presence of algae has increased the amount of DIC in the culture, which to the best of our knowledge, has been noticed here for the first time.

The baselines for both C_{air} and $C_{1\%}$ are displayed in the graph (Figure 12a and Figure 12b). The baseline is obtained by passing either air or 1% CO_2 to the media without any culture. Initial pH was adjusted to be the same as the initial pH of the algal culture. Later, the difference between the reading and baseline can be compared (Figure 12f). We interpreted the reading higher than the baseline as an indicator that carbon being consumed by the algae is less than the carbon being supplied. This condition may also show that the algae can make space for more DIC in the culture, which can explain the observation in which saturation values of BDU and CCAP culture, i.e. 3.3 mM are above the saturation value in blank media which is around 2.6 mM. On the contrary, a reading lower than the baseline shows that the amount of carbon being consumed is higher than the carbon being supplied.

Figure 12c and Figure 12d give details on the speciation of the DIC in the BDUG and CCAP culture, respectively. Most of the carbon in media existed as bicarbonate at all times. However, there was an exception in $C_{0.01M}$ of BDUG in which there is a significant presence of carbonate CO_3^{2-}, which peaked at D2. This observation can be related to the high pH in $C_{0.01M}$ culture during its active growth. It can also be seen that in $C_{1\%}$ of both cultivations, although CO_2 gas is being supplied, it only makes up about 15% of the DIC.

Figure 13a and Figure 13c show that the carbon-limited conditions (such as in D6 of C_0, D8 of $C_{0.01M}$ of BDUG and D5 of C_0 of CCAP) coincide with cessation in growth. To avoid this, a continuous supply of carbon is necessary since the mass transfer of CO_2 from surrounding to culture is very slow.

4.3.3 Carbon Sequestration

Depending on the amount of T_{CO2} in culture, carbon uptake rate in both species is different, in which the culture with a higher amount of DIC concentration can exhibit carbon uptake rate of around five times faster.

Figure 12e shows changes in DIC after two days of cultivation. In C_0 and $C_{0.01M}$, DIC has been used up by 50%. Since initial DIC in $C_{0.01M}$ is around five times than initial value in C_0, thus the rate is five times higher in $C_{0.01M}$. In C_{air}, the DIC decreases half to its initial value in BDUG, showing active uptake rate, while in CCAP, the value decreased by around 25%. In $C_{1\%}$ of BDUG, positive change indicates that the DIC is higher than its initial value by a factor of one (100%), while in CCAP, the increase is only by 50%.

In both BDUG and CCAP, maximum carbon uptakes rates in C_0 and $C_{0.01M}$ were recorded as 1.0 mM/day and 4.0 mM/day respectively, both at D2 for BDUG, but at D4 for CCAP. This value is much higher compared to experiment by Aishvarya et al. (2012) using freshwater *Chlorella* sp., in which HCO_3^- concentration changed at a rate of 0.63 mM/day and in an experiment by Chen et al. (2016), which showed rate around 0.7 mM/day in *N. salina* culture initially supplied with 5 mM bicarbonate.

For culture aerated with air and CO_2, change in DIC could not be taken as the carbon uptake since the carbon is being supplied continuously. Nevertheless, their behaviour can be estimated based on the difference between DIC reading in cultures and blank media, i.e. comparison with their respective baseline. Figure 12f compares DIC values on the final day with their baseline, which is based on the following formula:

$$\frac{DIC_{final\ day} - DIC_{baseline}}{DIC_{baseline}}$$

For the sake of comparison between the four treatments, the baseline of C_0 and $C_{0.01M}$ is their initial DIC value. A negative reading indicates that the DIC value in the final day is less than the baseline. Value of -1 for C_0, $C_{0.01M}$ and C_{air} shows that the DIC is fully consumed. This is the case mostly observed, except in C_{air} of BDUG, in which there is around 60% difference between saturation and its final DIC value. A different case is observed with $C_{1\%}$ of both cultures, in which DIC in the final day exceeds the baseline of the media by 30% in BDUG and 40% in CCAP.

For C_{air} and $C_{1\%}$ culture, both strains showed similar characteristics. DIC of C_{air} of both strains dropped below the baseline since D2 (Figure 12a and Figure 12b), with a maximum difference of 0.8 mM in BDUG and 1.1 mM for CCAP. When supplied with 1% CO_2, DIC in both strains, culture passed above the baseline at D1, showing a maximum difference of 1.0 mM with the media without the algae.

In other words, the kinetics of the carbon sequestration in this particular chapter is only reliably calculated for C_0 and $C_{0.01M}$ cultures, considering the rate for C_{air} and $C_{1\%}$ could not be calculated because of the continuous supply of carbon. Carbon uptake rates in C_0 and $C_{0.01M}$ was 1.0 and 4.0 mM day^{-1} respectively at maximum, in which the kinetics resembles reaction that obeys the first-order rate law.

4.3.4 pH Profile

pH is among essential factors in microbial growth as it affects the cell growth and the metabolism of the microalgae. For most of the microalgae, the suitable pH for growth of the microalgae is in the neutral to a slightly alkaline range (7 – 10) (Nayak et al., 2018). In this pH region, most of the carbon exists as bicarbonate rather than CO_2 gas or carbonate ion. Very acidic media will prevent CO_2 from forming bicarbonate for the microalgae, while very alkaline media will turn the carbon to inaccessible carbonate form.

Table 1 shows the pH reading of the BDUG and CCAP cultures upon different treatments. pH is expected to increase in microalgae cultivation due to the loss of CO_2 during photosynthesis since carbon consumption during this process results in an accumulation of OH^- (Nayak et al., 2018; Rangel et al., 2003; Chester, 1990). In the BDUG cultivation, pH in the C_0, $C_{0.01M}$ and C_{air} cultures are slightly more alkaline (pH between 9.0 and 9.5) than in the CCAP cultivation (pH between 8.0 and 9.0) at the final day. However, in the $C_{1\%}$ cultivation, the cultures turned acidic, and their pH stayed in the range of 6.0 to 7.0 for the rest of the experiment.

To compare, in an experiment by Lohman et al. (2015) of *Chlorella vulgaris* UTEX 395, they found that the pH stays between 8.0 to 8.5 in the C_0 cultivation while for the cultures supplied with 0.05M $NaHCO_3$, the pH went up to 10. This C_0 behaviour is similar to CCAP strain, but pH 10 observed by UTEX is too alkaline compared to this experiment.

Table 1 pH of the BDUG and CCAP cultivation during the initial and final day of experiments of respective treatments. Values are means ± standard error (*n*=3).

pH	BDUG		CCAP	
	Initial	Final	Final	Final
C_0	8.4 ± 0.0	9.4 ± 0.0	8.3 ± 0.0	8.9 ± 0.1
$C_{0.01M}$	8.2 ± 0.0	9.3 ± 0.1	8.2 ± 0.0	9.0 ± 0.0
C_{air}	8.2 ± 0.0	8.7 ± 0.0	8.3 ± 0.0	8.8 ± 0.0
$C_{1\%}$	8.4 ± 0.0	6.8 ± 0.0	8.2 ± 0.0	7.1 ± 0.1

Comparing C_{air} reading, similar behaviour was observed by Kuo et al. (2016) when *Chlorella* sp. is exposed to air as the pH increased from 8.5 to 9.5. This is similar to BDUG pH profiles. However, there is a report of lower pH reading, i.e. 8.5 (Nayak, Karemore, & Sen, 2016), which is also similar to CCAP profiles.

Kuo et al. (2016) also reported similar reading when the culture was exposed to 2% CO_2, in which the pH stayed between 6.5 and 7.0. This is not far from BDUG reading in this experiment, since we supplied the culture with 1% CO_2. In another experiment using lower CO_2 concentration, i.e. 0.5%, pH stays around 7.0 (Ji et al., 2017). Thus, supplying CO_2 in the gas state increases the dissolved CO_2 availability to the microalgae, and counters the rise in pH.

However, very low pH is associated with reduced efficiency of photosynthesis and also carbonic anhydrase activity due to acid formation (Thomas, Mechery, & Paulose, 2016; Lam, Lee, & Mohamed, 2012). Carbonic anhydrase is the enzyme responsible in carbon concentration mechanism and thus its sequestration capacity. As it presents on the surface of the cell, it catalyses the CO_2 hydration in microalgae by rapidly converting bicarbonate to CO_2, which later will be transported into the cell for assimilation. In very low pH, however, most of the bicarbonate species will not be abundant.

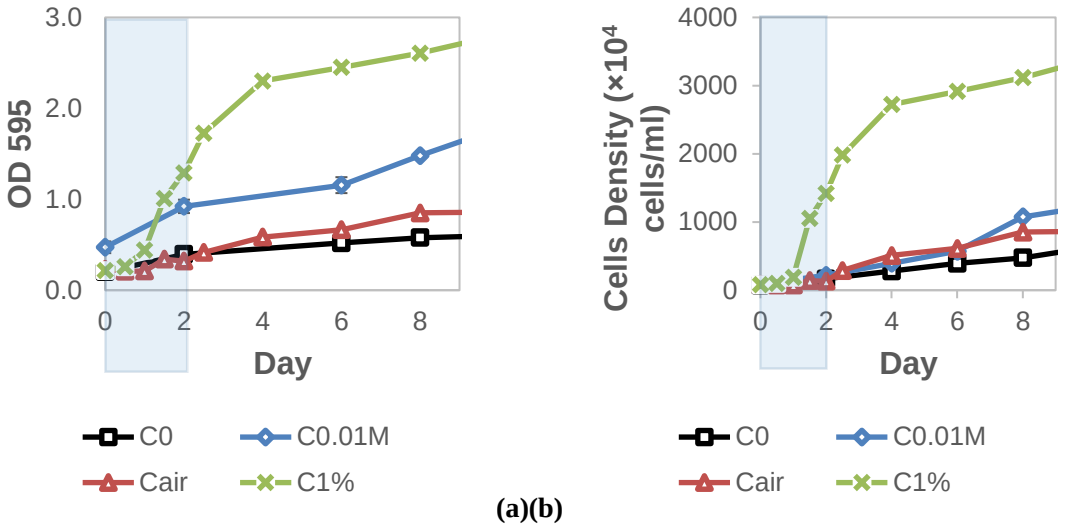
In C_{air} culture, although air contains roughly 0.04% v/v CO_2 (accurate to 2 decimal place), this value is not enough to combat the rise in pH due to microalgae activity (Kuo et al., 2016; Shene et al., 2016). Besides, by supplying with 1% CO_2 concentration level, it did not reduce pH to a very acidic condition that will inhibit algal growth. It is known that low pH is associated with

a reduction in the activity of extracellular carbonic anhydrase by microalgae, thus not helping the carbon concentration mechanism (Lam, Lee, & Mohamed, 2012).

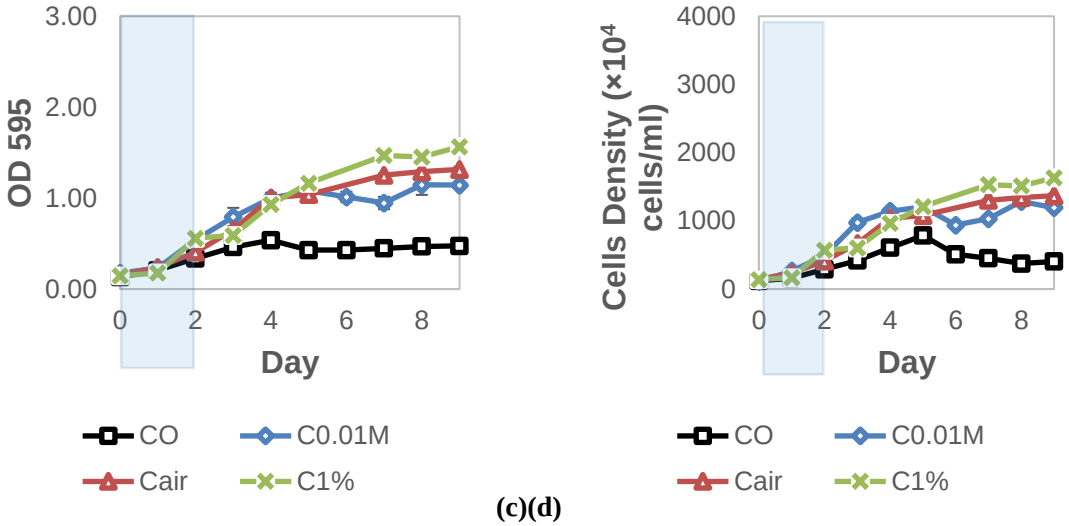
4.3.5 Microalgae Growth

During ten days of the experiment, the highest amount of biomass is produced by BDUG culture supplied with 1% CO_2, i.e. increase by 10 and 40 folds in terms of optical density (Figure 13a) or cell abundance (Figure 13b) respectively. The lowest amount of biomass was produced in the C_0 culture of both strains, inferring that the mass transfer has limited the microalgae growth. Algae has grown well in $C_{1\%}$ cultures, which indicates that the algae experienced sufficient carbon conditions in this supply regime. By changing the method of carbon supply from C_0 to $C_{1\%}$, the biomass increased by five folds and four folds in BDUG and CCAP (Figure 13c and Figure 13d) respectively. By changing to 0.01M C, the difference is nearly 2 and 3 folds, respectively.

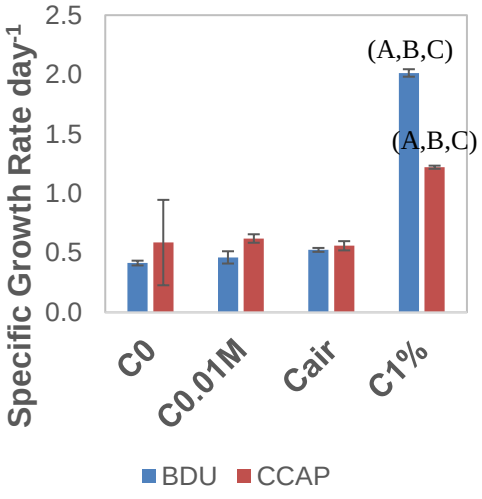
Thus, supplementing the culture with carbon either through initial bicarbonate supply or through aeration of air or CO_2 will lead to an increase in the production of biomass. Both strains showed that when supplied with CO_2, the amount of biomass produced is the highest. The set that produced second-highest biomass in BDUG is $C_{0.01M}$ culture, while in CCAP, its $C_{0.01M}$ culture is not statistically different with $C_{1\%}$ culture. In experiments by Lam & Lee (2013), they found that CO_2 bubbling increases biomass productivity by five folds compared to the use of $NaHCO_3$. Despite this, they also reported significantly lower CO_2 removal efficiency. In this experiment, the difference observed was by three folds, which happened in BDUG set of experiment.



(a)(b)



(c)(d)



(e)

Figure 13 (a) Optical density reading and (b) cell density measurement in BDUG. (c) Optical density reading and (d) cell density measurement in CCAP culture. (e) The specific growth rate of the microalgae culture at D2 with respect to their cell density. The first two-days is selected as it is when the growth rate is at maximum. Values are means (*n*=3), and error bars correspond to the standard error about the mean. For specific growth rate (Figure (e)), an analysis was carried out to estimate the significance of differences between treatments, showing significant difference when compared to (A) C_0 (B) $C_{0.01M}$ (C) C_{air} treatment.

This similarity in growth rate, however, does not contribute towards biomass produced. BDUG seems to consume carbon slowly (as shown in growth rate by C_0, $C_{0.01M}$ and C_{air}) but assimilated it into biomass more effectively, i.e. more increase in biomass. This is understood from the data wherein the $C_{1\%}$ culture, BDUG showed the highest growth rate compared to CCAP. In the remaining carbon treatments, i.e. C_{air}, $C_{0.01M}$ and C_0, BDUG showed a lower growth rate than the CCAP. It appears that CCAP can grow better than BDUG at low carbon availability, but BDUG appears to require more carbon to be available for more significant growth. When sufficient carbon is available, it can convert it to biomass, better than CCAP does.

4.3.6 Nutrient Uptake

Figure 14a and Figure 14c illustrate the folds-change profile of nitrogen concentration in both strains. For all the treatments, the initial nitrogen concentration is at 880 µM as in *f*/2 media. By changing the carbon treatments, it has changed the nutrient consumption behaviour of the microalgae. It is observed than in both strains, cultures without gas bubbling appear to be slow in consuming the nitrate available in the media. This is supported by Paes et al. (2016), who discovered that the aerated cultures tend to run out of nitrogen faster than non-aerated cultures, due to the carbon availability. In C_0 and $C_{0.01M}$, the overall consumption is only 20% on average. However, for the C_{air} and $C_{1\%}$ setup in both CCAP and BDUG, nitrate is consumed at least to 80% of initial concentration at the end of the experiment.

In BDUG, nitrate is wholly removed at D3 and D9 for $C_{1\%}$ and C_{air} cultures respectively. While in CCAP, nitrate is removed at D3 and D4 for $C_{1\%}$ and C_{air} respectively.

The highest maximum nitrate uptake rate is recorded in the $C_{1\%}$ culture of BDUG (Figure 14e), which happened at D2. Maximum nitrate uptake occurred at D2 for C_0 and C_{air} culture, but D3 for $C_{0.01M}$ culture. In CCAP, the highest maximum uptake rate is recorded by cultures with $C_{1\%}$, which happened at D2. Maximum uptake rate in aerated culture and $C_{0.01M}$ happened at D3, while for C0 culture, at D8.

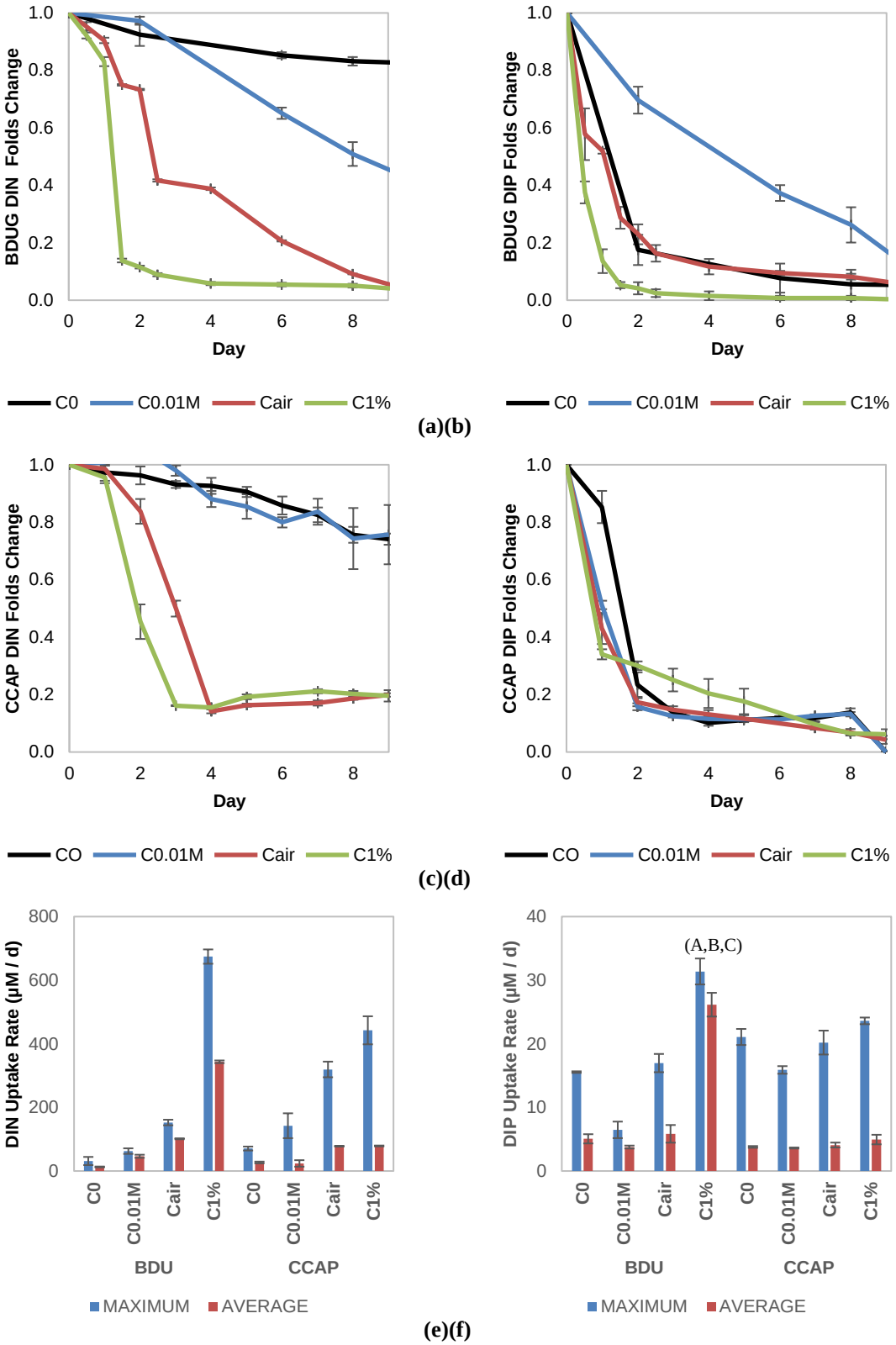


Figure 14 (a) and (b): Dissolved Inorganic Nitrogen and Phosphate Profile by BDUG. (c) and (d): Equivalent profile by CCAP strains. (e) Nitrate Removal Rate in all Set of Experiments (f) Equivalent Phosphate removal rate in all set of Experiments. (g) and (h): Internal utilization efficiency of the nitrate and phosphate, respectively. Values are means (*n*=3), and error bars correspond to the standard error about the mean. For the average phosphate uptake in BDUG (Figure (f)), an analysis was carried out to estimate the significance of differences between treatments, showing significant difference when compared to (A) C_0 (B) $C_{0.01M}$ (C) C_{air} treatment.

Figure 14b and Figure 14d show phosphorus concentration profile of BDUG and CCAP culture respectively. Phosphate is removed completely in all set of experiments, except in $C_{0.01M}$ of BDUG. In BDUG, phosphate is removed completely at D2 in $C_{1\%}$ culture, and at D6 in C_0 and C_{air}. In CCAP, phosphate was entirely removed at D7 in air and $C_{1\%}$ culture, while for C_0 and $C_{0.01M}$ culture, phosphate was removed entirely at D9.

In BDUG, the rate of phosphate consumption is the fastest in $C_{1\%}$, followed by remaining cultures which did not show a significant difference between them (Figure 14f). Highest maximum uptake rate is recorded in BDUG setup of $C_{1\%}$, which happened at D1. Maximum uptake rate for culture aerated with air also happened at D1. For the $C_{0.01M}$ and C_0 cultivation, the maximum phosphate uptake rate happened at D2. In CCAP, phosphate uptake rate is independent of the type of carbon supplied. The maximum phosphate uptake rate of C_0 culture happened at D2, while in the remaining cultures, it happened earlier, i.e. at D1.BDUG takes up phosphate less readily with respect to $C_{0.01M}$. Phosphate uptake is the most rapid in $C_{1\%}$.

BDUG may favour slightly acidic conditions, which is reflected in the fast nitrate and phosphate consumption at low pH ($C_{1\%}$) and slow consumption at high pH ($C_{0.01M}$). Previously on the discussion about growth, there is a drastic increase in growth for $C_{1\%}$, which is when BDUG was grown in acidic condition. In alkaline cultivation, i.e. $C_{0.01M}$, BDUG showed slower average carbon uptake, nutrient consumption and growth compared to CCAP. As carbon consumption will be assisted by carbonic anhydrase enzyme usually in alkaline pH, it is inferred that its concentration in BDUG culture is lower compared to CCAP culture.

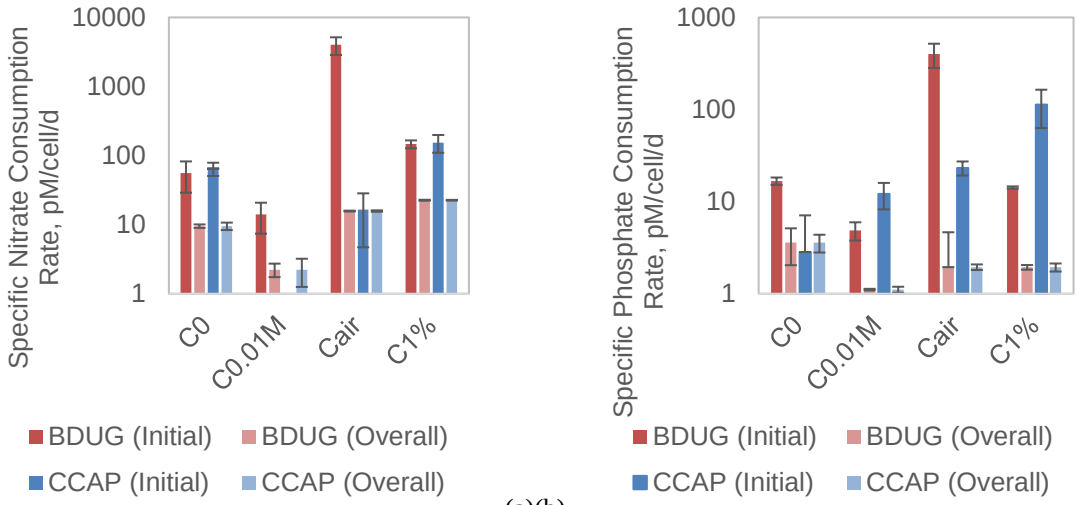
The calculation for correlation has been attempted for nitrate and phosphate assimilation profiles (Figure 14a-d) with biomass productivity (Figure 13a-b), giving an average coefficient value of 0.9 ± 0.1 and 0.9 ± 0.0 respectively. This value indicated that the increase in nutrient assimilation happens in parallel with biomass productivity. Meanwhile, the correlation between nitrogen and CO_2 consumptions showed an average coefficient value of 0.9 ± 0.0, indicating a strong relationship between them. This correlation was calculated for the consumption in C_0 and $C_{0.01M}$ only since C_{air} and $C_{1\%}$ are continuously aerated, in which their carbon consumption could not be calculated based on the difference in DIC only.

Based on these correlations, it can be said that in the event when the carbon is limited, and the biomass productivity is low, the nitrate consumption also turns low, suggesting the nitrate

assimilation mechanism in the microalgae was affected. This is although a similar amount of nitrate is available in the beginning. Thus, for the nutrient to be consumed, adequate carbon must be present to allow the process. An attempt to find and compare works of literature that studied the relationship between nitrate and carbon assimilation in microalgae, however, did not give specific result, indicating that the topic is not widely researched.

In terms of nutrient use efficiency (NUE), among standard terms used to quantify it are the partial factor productivity (PFP), the partial nutrient balance (PNB) and the internal utilization efficiency (IE) (Fixen et al., 2015). PFP addresses the question of how productive is the algae in comparison to its nutrient input, i.e. the ratio of yield per nutrient input. By applying this method, it is evident that the $C_{1\%}$ culture is the most efficient since it produced the most yield (Figure 13a-d) given that all cultures have the same amount of nutrient input. Meanwhile, PNB addresses the question on the amount of nutrient being taken out of the system in relation to the input amount. The trend can be seen in Figure 14a-d, in which the nitrate use efficiency is the best in the $C_{1\%}$ and C_{air} culture, while the phosphate use efficiency is nearly the same for all cultures at the end of the experiment.

However, if IE is used, i.e. by how much the nutrient consumption was achieved by the biomass production (Figure 15a-b), the values will be different since the dynamic of consumption and growth are different in each day, i.e. there are cultures of low nutrient consumption with low yield (e.g. C_0), while there are also cultures with immediate nutrient consumption (e.g. phosphate) during the early cultivation, but the biomass production is still at early exponential stage (e.g. $C_{1\%}$). In terms of nitrate consumption (Figure 15a), the BDUG C_{air} (initial) cultivation showed the highest specific nitrate consumption. This is followed by the $C_{1\%}$ cultivation, which does not show a significant difference with the C_0 and $C_{0.01M}$ cultivation. High specific nitrate consumption in the early stage of growth in the BDUG C_{air} cultivation might show the preference of this strain towards the aeration by air, as it has been maintained in this aeration before. Besides, it also records the highest specific phosphate consumption (Figure 15b). In terms of specific phosphate consumption, CCAP and BDUG readings do not show a significant difference in terms of overall reading. Rapid phosphate consumption in the early cultivation was observed in the CCAP $C_{1\%,}$ followed by C_{air}.



(a)(b)

Figure 15 Specific (a) nitrate and (b) phosphate consumption by BDUG and CCAP strain upon the four carbon treatments. Initial consumption represents the reading in the first two days in which rapid consumptions were observed, while overall consumption represents the reading during nine days of cultivation.

Generally, the first two calculation methods are more suitable in this context, since among microalgae cultivation goals is nutrient removal and biomass production. However, the latter may be suitable to use in the case of maximizing yield while using a minimum amount of nutrient.

Nitrate is an essential nutrient needed by algae for major metabolic pathways, production of proteins and nucleic acids (Pires, 2015; Tebbani, Filali, & Lopes, 2014). The availability of carbon will make the consumption of nitrogen faster since the cells are supplied with carbon for amino synthesis (Paes et al., 2016). An increase in nitrogen consumption leads to more cellular and protein productivity. This can be seen clearly in $C_{1\%}$ cultivation, in which it showed maximum growth (Figure 13) and nutrient consumption (Figure 14).

Meanwhile, phosphate is an essential element in essential molecules such as ATP, DNA and RNA. It is hypothesized that the uptake of phosphate was dependent on the inorganic carbon level, similar to nitrate uptake (Huertas, Montero, & Lubián, 2000). Since all sets of experiments begin with initial 2.0 mM DIC and phosphate in f/2 is only 36 µM, in most of the cases the phosphate was entirely consumed. A shortage of phosphorus will results in a reduction in photosynthetic efficiency as it is among building components of vital molecules such as energy carrier ATP, DNA, RNA and phospholipids (Pires, 2015). As phosphorus and nucleic acid synthesis become limited, rate of synthesis and regeneration in of substrates in

Calvin-Benson cycle will be affected, thus reducing the rate of light utilization for carbon fixation (Barsanti & Gualtieri, 2014).

To summarize, highest uptake of nitrate and phosphate were recorded in culture with $C_{1\%}$ in both strains, suggesting that for active nutrient uptake sufficient carbon, appropriate pH, and mixing to maximise mass transfer help. Both strains demonstrated that there is a strong positive correlation between nutrient uptake, carbon availability and biomass productivity. Although in general, all significant nutrients are taken up effectively, there are differences in the uptake rate that is influenced by carbon supply and availability. These variations later manifested in different biochemical composition of the algae.

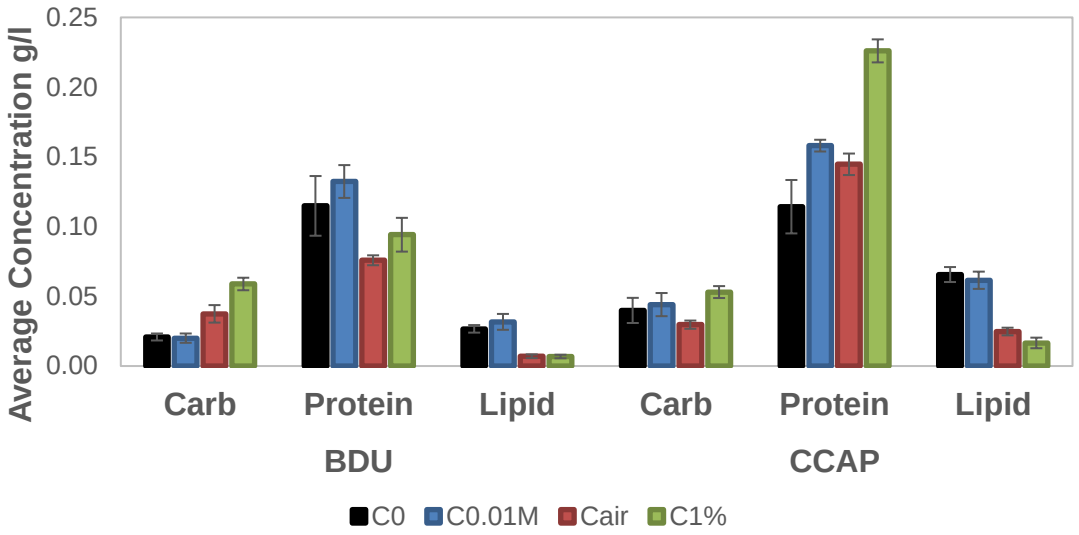
4.3.7 Biochemical Components

Figure 16a represents variation in biochemical components in microalgae after four different carbon treatments. The average reading for the last four days was taken and compared.

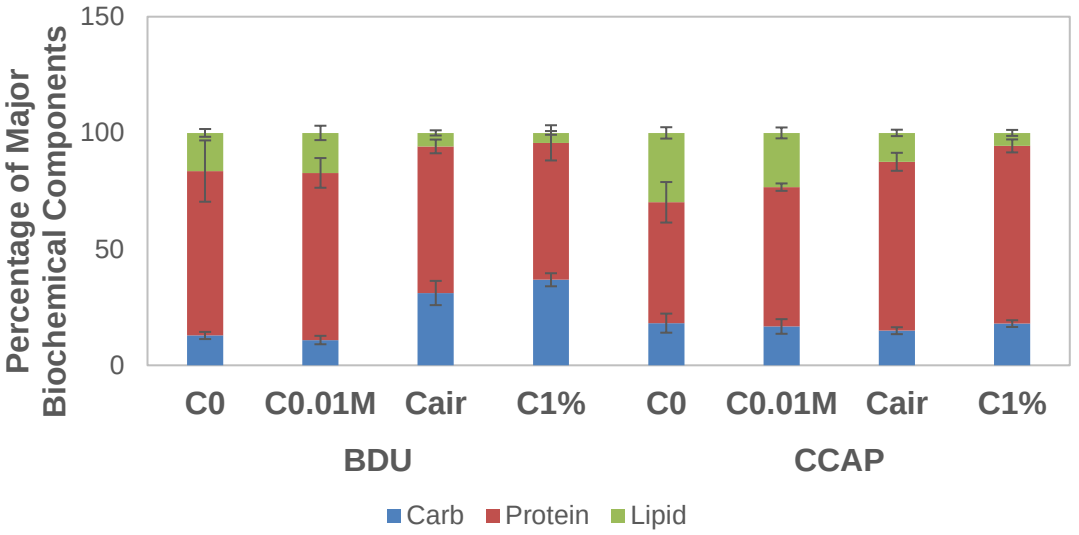
The highest concentration is showed by protein in CCAP $C_{1\%}$ culture, i.e. nearly 0.25 g/l. However, proteins in BDUG do not show significant difference among a different set of carbon treatments which are in the range 0.10 to 0.15 g/l. The lowest reading was recorded by C_{air} culture, i.e. 0.08 g/l. Day to day reading showed that the protein concentration of both C_0 and $C_{0.01M}$ decreases with time while in C_{air} and $C_{1\%,}$ the concentration does not change much.

For carbohydrate, the highest reading in BDUG is observed in $C_{1\%}$ culture (0.06 g/l), followed by C_{air} (0.04 g/l). In CCAP cultivation, it looks like there is no variation due to carbon treatment in which the concentration stays around 0.05 g/l.

Lipid gave the highest reading in C_0 and $C_{0.01M}$ culture of both strains, i.e. 0.05 g/l. In C_{air} and $C_{1\%}$, the values are low, i.e. less than 0.02 g/l. It is stated by Akita & Kamo (2015) that lipid accumulation in microalgae culture is caused by carbon starvation instead of nitrate. Increase in lipid is also recorded by White et al. (2013) who discovered that cultivation by adding bicarbonate results in an increase of lipid in *T. suecica* and *N. salina* cultures.



(a)



(b)

Figure 16 (a) Average concentration of major biochemical component (Carbohydrate, Protein and Lipid) of BDUG and CCAP cultivation. The reading represents the average concentration in the last four days. Values are means (*n*=3), and error bars correspond to the standard error about the mean.

Comparing this biochemical component's data with nutrient consumption (Figure 14a-d), it can be observed that the culture with the most amount of nitrate consumption, i.e. $C_{1\%}$ and C_{air} showed different concentration in biochemical components among them. This situation is similar to phosphate consumption, in which although almost all of them consumed the phosphate available, their biochemical components concentrations are also different. This indicates that the change in carbon treatments plays a critical role in determining the concentration of the biochemical components. For example, an active and excessive supply of carbon ($C_{1\%}$) resulted in a high concentration of protein but a low concentration of lipid (Figure 16a).

Figure 16b represents the proportion of biochemical components in terms of the percentage. The values were obtained by dividing the concentration of the component with the sum of the carbohydrate, protein and lipid. It is also important to note that the bar represents the proportion and not the total concentration. For example, although the proportion of C_0 and $C_{0.01M}$ are similar, their total concentrations are different, in which $C_{0.01M}$ showed higher concentration value.

It can be seen that protein made up the biggest proportion (50 – 60%) of biochemical components in all samples as expected (Hirotsu & Otsuki, 2017). In BDUG, the proportion of protein in C_0 and $C_{0.01M}$ is around 70% while in C_{air} and $C_{1\%,}$ the proportion is around 60%. In CCAP, protein is around 50% in C_0, 60% in $C_{0.01M}$, 70% in C_{air} and 80% in C_{air}.
Carbohydrate proportion is high in BDUG $C_{1\%}$ and C_{air}, while in the remaining cultivation, their proportion is nearly the same, i.e. 20%. It is also noted that there is no variation in carbohydrate proportion in CCAP cultivation in the different carbon treatments.

Lipid proportion is higher in CCAP compared to BDUG. The highest proportion of lipid is usually produced by C_0 culture, while the lowest proportion of lipid happened in $C_{1\%}$. However, care is required in interpreting these results, as the concentration of the culture in C_0 was low and the lipid concentrations determined are also on the lower side, so a small variation in lipid at low cell concentrations shows up as a relatively high proportion.

As a comparison, research by Matos et al. (2016) found that dried biomass of *Chlorella vulgaris* (Brazil) at the beginning of stationary growth phase in f/2 media and exposed to CO_2, contained 94% dry matter and 6% moisture. Dry matter can be further classified into 7% ash and 6% fibre, while the remaining 81% belongs to protein (41%), carbohydrates (27%) and lipid (13%). Comparing these three, protein made up 50%, carbohydrate 30% and lipid 20%, i.e. when considering only these three components, i.e., as a proportion of just these three.

The carbohydrate, protein and lipid proportion may look similar to data obtained in this experiment; however, higher carbohydrate and lipid percentage in their report may be contributed by the continuous saturation of CO_2. For example, *Chlorella* cultivation by Guil-Guerrero & Rebolloso-Fuentes (2008) in freshwater gives increasing protein proportion from 28% to at D2 to 53% at D7. Zhang, Zhang, & Yan (2015) obtained 60% protein from *Chlorella* at D10. Spolaore et al. (2006) reported a range of 51 – 58% of proteins from *Chlorella* vulgaris.

Kassim & Meng (2017) reported protein percentage 50% and 70% for cultivation by air and 5% CO_2 respectively; however, this increase compensated a percentage of both carbohydrate and lipid in cultivation.

Proportionally, carbohydrate has the most significant proportion in the $C_{1\%}$ culture for BDUG (37%). Carbohydrate does not show many changes in concentration at the end of the experiment. Decreases were observed in C_0 cultures of both strains and two CCAP cultivation, i.e. $C_{0.01M}$ and C_{air} (Figure 16a). Quantitatively, highest carbohydrate concentration reading is 0.07 g/l, recorded by $C_{1\%}$ of BDUG. In CCAP culture, carbohydrate reading for C_{air} culture is 0.03 g/l on average. $C_{1\%}$ culture has an average of 0.06 g/l carbohydrate concentration.

In C_0 and $C_{0.01M}$, the concentration of lipid increased with time, in contrast to carbohydrate. Culture aerated with air and 1% CO_2 showed lower reading compared with the non-aerated sets. In other words, the lipid/carbohydrate ratio increases in C_0 and $C_{0.01M}$ compared to C_{air} and $C_{1\%}$ for both strains. Increase in lipid in $C_{0.01M}$ also discovered by Li et al. (2018) who found that growing $NaHCO_3$ in the range of pH 7.5 to pH 9.5 would increase lipid production. This agrees with data from this experiment which reported the highest reading by the culture of both strains with $C_{0.01M}$. BDUG cultures reported average pH reading of 9.5 while for CCAP, it is 8.7.

It is also interesting to note that although there is no carbon being added, there is a production of lipid happening. This may be due to the initial presence of carbon in the media. Furthermore, the value reported was minimal, i.e. a maximum of 0.07 g/l, which also agrees with Chen et al. (2014). The absence of this nutrient will lead to the accumulation of lipids and reduction of protein content (Pires, 2015).

4.4 Summary and Conclusion

Dissolution of carbon in growth media was studied. Supply of continuous CO_2 gas is not fully efficient as there is always gas escape to the surrounding. We recorded an efficiency of 50% of CO_2 that goes into the media during the first hour of gas supply. DIC readings were typically well below the theoretical saturation value of 33 mM for CO_2 in water unless the media contained alkali, such as NaOH, which is a CO_2 reactant. DIC reading in f/2 media saturated by 1% CO_2 gives reading around 2.5 mM. When supplied with 1% CO_2, the pH of the media

is set around 7.0. Increasing supply of CO_2 percentages increases DIC saturation. It is also noted that as the initial pH of the media increases, the DIC value at saturation also increases. In the presence of algae, the DIC value is more than in blank. Comparison between strains found that both have similar DIC saturation value in f/2; however, the saturation dynamics differed depending on the carbon supply regime used.

Microalgae *Chlorella vulgaris* of two different strains were examined under four different carbon treatments. DIC availability in those experiments was also examined. Although they are of the same species, they show different characteristics in terms of nutrient uptake and growth response, when subjected to differing carbon supply regimes. It is also observed that carbon supply dynamics can enhance microalgae CO_2 sequestration. Although the cultures grew well in all setup, BDUG strains supplied with 1% CO_2 is the best setup to achieve higher biomass, growth rate, carbohydrate and protein production. In terms of pH, cultivation of BDUG resulted in slightly more alkalinity of the medium. pH in cultivation with CO_2 is acidic, while cultivation with air, bicarbonate and no active carbon supply is alkaline. In terms of nutrient consumption, BDUG showed faster consumption of nitrate and phosphate when cultured with CO_2. We also noted that for active nutrient uptake, sufficient carbon, appropriate pH, and mixing to maximise mass transfer help. In terms of carbon consumption, it is noticed that the presence of algae has increased the amount of DIC in the culture, which to the best of our knowledge, has been noticed here for the first time. Among BDUG and CCAP, the former shown faster rate of carbon consumption, although their difference is not apparent among different carbon treatment. More amount of lipid was produced in CCAP culture. In terms of carbon treatment, cultivation with continuous carbon supply gives the best chemical production compared to remaining three cultivation, except for lipid production, which showed higher concentration in cultivation without C_0 and $C_{0.01M}$, which is cultivation without carbon addition and cultivation with initial addition of 0.01M bicarbonate. Carbon supply and availability are, therefore, has shown to play an essential role in nutrient uptake and biochemical composition.

Among the four treatments, $C_{1\%}$ seems the best configuration as the carbon supply is active and excessive. In general, it recorded the highest and fastest growth, aside from the highest amount and fastest uptake of nitrate and phosphate in both strains. The increase in CO_2 concentration and DIC availability are known to positively influence pigments production in green microalgae (Thawechai et al., 2016; White et al., 2013).

This information can be used to further research on optimal initial carbonate concentration or CO_2 percentage for algal growth. Analysis of carbon utilization of microalgae at the metabolic level could be an avenue for further research, besides obtaining more details on the production of value-added chemicals, e.g. fatty acid methyl ester (FAME) production. It is also recommended to study the best form of carbon species for efficient carbon supply and resultant uptake.

CHAPTER 5: Influence of Nutrients towards Carbon Uptake by Two Strains of *Chlorella vulgaris*

Abstract

Here we report responses by two brackish strains of *Chlorella vulgaris,* i.e. BDUG 91771 and CCAP 211/21A upon exposure to a different level of nutrients. Their responses were monitored based on carbon and nutrient consumption in cultivation with continuous 1% CO_2 supply. Three different nutrient levels were experimented, i.e. *f*/2 and the same media with nitrogen and phosphorous level increased by 4 and 8 folds, labelled 2*f* and 4*f*, respectively.

It was found that both strains responded differently upon different nutrient exposure. In terms of carbon uptake, BDUG responded negatively to nutrient increase, in which cultivation in *f/2* recorded the highest carbon uptake, i.e. 1.2 mM/h. This value is translated into average carbon sequestration of 13 and 9 g CO_2 / g algae for BDUG and CCAP respectively, which is above the common (theoretical) reported value of 1.83 g CO_2 / g algae, as the estimate in our investigation is based on the amount of carbon being dissolved in the medium. Comparing this value with sequestration capacity by chemical means, the capacity by microalgae has a higher value, which highlights its additional advantages and usefulness, thus the potential for CO_2 sequestration. In both strains, an increase in nutrient supply from *f/2* to 2*f* and 4*f* does not increase the total carbon consumption; instead, it shows a decreasing trend. In parallel with this, our data on carbon and nutrient consumption suggested that carbon consumption is best at limiting nitrogen and phosphate concentrations. In terms of growth, BDUG showed an increase in biomass production with an increase in nutrients, where the highest reading was recorded in 4*f* cultivation. In CCAP, there is an increase in biomass production in 2*f*, but doubling the nutrients in 4*f* resulted in a similar production as in 2*f*. The highest amount of nitrate removal was recorded in CCAP in the 2*f* cultivation, followed by BDUG in the 4*f* cultivation. Meanwhile, phosphate consumption increased with supply, for both strains. Despite all the differences, both strains demonstrated an increase in the saturated DIC values in the culture upon an increase in nutrient level, and that carbon consumption is best at nutrient limiting conditions. This experiment demonstrates that two strains from the same species isolated from similar habitats can have different capability to sequester CO_2 and removing nutrient.

5.1 Introduction

Carbon is among the essential elements needed for consumption by microalgae. It is used for growth, reproduction, as well as for energy storage (Pires, 2015). This makes microalgae among the potential candidates for carbon sequestration strategies. At the same time, microalgae possess several advantages such as the commercial application of its biomass and tolerance level towards high carbon and nutrient level, which makes microalgae a suitable candidate for flue gas utilization and wastewater treatment also (Singh & Ahluwalia, 2013). In wastewater treatment, microalgae utilize the nitrogen, phosphorus and minor nutrients of a variant form of wastewater including domestic sewage, farm drainage and industrial wastewater, bringing the extra benefit of wastewater bioremediation.

In the previous chapter, we have found that microalgae can utilize all the nitrogen and phosphorus available in the medium in the most set of experiments. It is known too that different species of microalgae have different levels of adaptation towards CO_2 condition. Besides, the culture supplied with 1% CO_2 gave the best result in terms of nutrient uptake and growth. In the scenario of the increasing amount of nutrients, it has been shown that this will result in different nutrient removal kinetics (Wang et al., 2014).

It is interesting to examine the influence of increasing nutrient levels on the carbon uptake behaviour of the two strains. Since an increase of nutrient will generally lead to an increase in biomass (Pereira et al., 2016), it is hypothesized that this will also increase the carbon uptake rate. Moreover, it is noted in the previous experiments that DIC readings are higher than their baseline, suggesting that this excess could be taken up if more nutrients (nitrogen and phosphorous) are available.

Although studies on CO_2 sequestration by microalgae usually reports that 1 kg of biomass can fix approximately 1.83 kg of CO_2 (Chisti, 2007), there are also studies saying this simplified method is not accurate, arguing that the carbon content in microalgae biomass after cultivation should be accounted for, to determine CO_2 bio-fixation (Adamczyk, Lasek, & Skawińska, 2016). Despite the contradictions, both estimates are based on biomass yield, which does not consider the amount of carbon being dissolved into the culture medium, which will contribute to a higher reading (Van Den Hende, Vervaeren, & Boon, 2012). In this investigation, we extend our earlier DIC estimations to arrive at carbon uptake rates using a dynamic method.

Based on these issues, this experiment aims to observe whether an increase in nutrient availability will affect their carbon uptake rate in the two strains of *Chlorella vulgaris*. For doing this, nutrient concentration in *f*/2 media will be chosen as the base. Two modified media will be tested by increasing the nitrate and phosphate by 4 and 8 folds, respectively. A similar approach of multiplying the nutrient concentrations have been mentioned in works of literature before to create a condition with an excessive supply of nutrients (de Jesús-Campos et al., 2020; Becerra-Dórame et al., 2010; Kang, Kim, & Lee, 1996). The capacity of CO_2 that these microalgae cultures can contain and their nutrient consumption response will also be studied. Similarly, this will influence any strategy developed for CO_2 utilization and chemical production.

5.2 Experimental Method

Experimental Setup

Chlorella vulgaris CCAP 211/21A and BDUG 91771 were inoculated at OD_{595} 0.2 as before, in 1L photobioreactors with the appropriate media. 1% CO_2 in the air was bubbled continuously for seven days, and the reactor was stirred at 200 rpm. The aeration rate was set at 0.2 vvm. Light intensity was maintained throughout the experiment at ~200 μE m^{-2} s^{-1} using cool-white LED.

Experiments were done by observing algal response towards different media, i.e. *f/2*, 2*f* and 4*f*. 2*f* and 4*f* media were made by increasing the nitrate and phosphate contents to 4-fold and 8-fold, respectively while keeping the other ingredients the same as in *f/2*. 2*f* media had 3.6 mM nitrate and 0.15 mM phosphate, while 4*f* had 7.2 mM nitrate and 0.29 mM phosphate. These media, without the presence of any microalgae, will be called blank media, henceforth.

All the reactors and media were autoclaved before the usage throughout the experiment. Sterile practices were applied when dealing with the culture during the experiment.

Growth Measurement

Samples were taken for OD_{595} and pH measurement every day. OD was measured by a UV / Visible Spectrophotometer (Ultrospec 2100 Pro) while pH was measured by Orion Star pH Meter A211 Manual (Thermo Fisher).

OD obtained was later translated to dry weight measurement based on the conversion equation where:

$$\text{BDUG dry weight (g/l)} = 0.1579OD_{595} - 0.0064 \ (R^2 = 0.99).$$

$$\text{CCAP dry weight (g/l)} = 0.1719OD_{595} - 0.0229 \ (R^2 = 0.99).$$

This equation was obtained by generating the standard curve of the relationship between dry weight and optical density (as detailed in Section 3.3).

The specific growth rate was calculated by the following formula, based on the OD measurements:

$$\mu = \frac{\ln(x) - \ln(x_0)}{t}$$

Biomass produced was obtained by finding the difference between the initial and final dry weight of the culture. Productivity is another way of measuring algal growth. Average productivity refers to the total amount of biomass produced (mg) in this duration of the experiment (10 days), expressed as mg/l/d, while maximum productivity is obtained by taking the highest reading of productivity on a specific day.

Removal Rate Calculation

Phosphate removal is based on the difference between the initial and final concentration of DIP. The removal rate is calculated based on the following equation:

$$Removal\ rate, R = \frac{C_0 - C_i}{t}$$

C_0 refers to the initial concentration of phosphate, while C_i refers to the final concentration of phosphate. In most cases, final concentration is 0. *t* refers to the total duration of the experiment or duration needed to remove the phosphate completely.

The specific DIP removal rate is obtained by dividing the phosphate removal rate with the biomass present.

Dissolved Inorganic Carbon Measurement

This was carried out by double titration as detailed in Section 3.3.

Determination of Carbon Uptake by Microalgae

When determining for the carbon uptake rate, a dynamic method was employed, where the supply of CO_2 was stopped at a given time point and switched to nitrogen supply for up to 60 minutes, and the DIC readings were recorded at intervals. The carbon supply was resumed after 60 minutes. Carbon uptake by algae is measured by considering the difference in DIC change in both blank media (treated similarly) (starting pH 7.2) and also the algal culture as shown in the formula below.

$$Algal\ DIC\ Uptake\ rate\ (\frac{mM}{hr}) = \frac{DIC_0 - DIC_t}{t_t - t_0}_{culture} - \frac{DIC_0 - DIC_t}{t_t - t_0}_{media}$$

where DIC_0 and DIC_t are the DIC readings taken at time t_0 (when the carbon supply was switched to nitrogen and at time t_t, after which the carbon supply was resumed (Figure 17a).

Carbon Sequestration

Carbon sequestration by microalgae is measured in grams of CO_2 sequestered per gram of algae biomass produced. In this investigation, the amount of carbon dioxide produced is calculated from the amount of carbon lost during the dynamic method, assuming the loss is all directed towards the algae. The amount of algae biomass produced is calculated on a dry weight basis – the difference in DW on the desired day and that at the beginning.

Carbon Balance

Assuming steady state condition, carbon balance was calculated based on the following equation,

Input + *Generation* = *Accumulation* + *Consumption* + *Output*.

Input refers to carbon being present in the culture at the beginning of the calculation, which is equivalent to DIC concentration the previous day, mM * 44.01 g / mol * volume of the culture, litre. Generation is the amount of carbon supplied, which is measured gravimetrically. Amount of CO_2 = volume in the flow * carbon concentration. Carbon generated in two days is twice the amount of carbon generated in a single day. Accumulation refers to DIC at the present day, mM * 44.01 g / mol * volume of culture, litre. Consumption refers to the carbon uptake by the algae determined through the dynamic method detailed above. Since Carbon uptake is calculated per hour, the value will be multiplied with the time difference from the previous time-point. The output is the amount of carbon escaped to the surrounding. Since the output is

difficult to determine since gas may escape through small holes, it is determined by using the equation above.

Effectivity of carbon supply is determined by subtracting the ratio of the amount of carbon escaped (output) to the amount of carbon supplied (generated) from 1.

5.3 Results and Discussion

5.3.1 Determination of Carbon Uptake by Microalgae

For cultures with the continuous gas supply, carbon uptake estimation is based on the dynamic method (Figure 17a), similar methods have been employed by (Jacob-Lopes et al., 2010; Garcia-Ochoa & Gomez, 2009) to measure gas uptake. This requires the CO_2 supply to stop and substituted with N_2 gas for a while, e.g. one hour, so its uptake by algae can be monitored.

The first step is to establish the baseline, i.e. apply the method to the blank media. We initially started the analysis by just switching off the gas supply, so there is no gas sparging. It was observed that as the gas supply stopped, the DIC readings observed were not stable. We inferred that this is due to equilibration of the supplied carbon with that in the headspace of the reactor, which was dissolving back into the media, due to a lack of active gas supply (negative pressure). Continuous aeration is ensured to avoid this error from happening by supplying inert gas, i.e. nitrogen gas (N_2) as a substitute to the culture at the same flowrate, in place of air containing 1% CO_2, during the duration of the dynamic method. This is to maintain the same flow condition of the previous gas supply.

When selecting for the duration to apply the dynamic method, four time-points were investigated, i.e. 10, 20, 30 and 60 minutes (Figure 17b). As N_2 is being substituted, there is some loss of DIC (as dissolved CO_2) due to the purging action of the nitrogen. This caused a more significant deviation in the readings in the first minutes that decreased as the time passed (Figure 17b). Thus 60 minutes duration was chosen since this time-point showed the least deviation among replicates compared to the other time points examined (i.e. 10, 20 and 30 minutes).

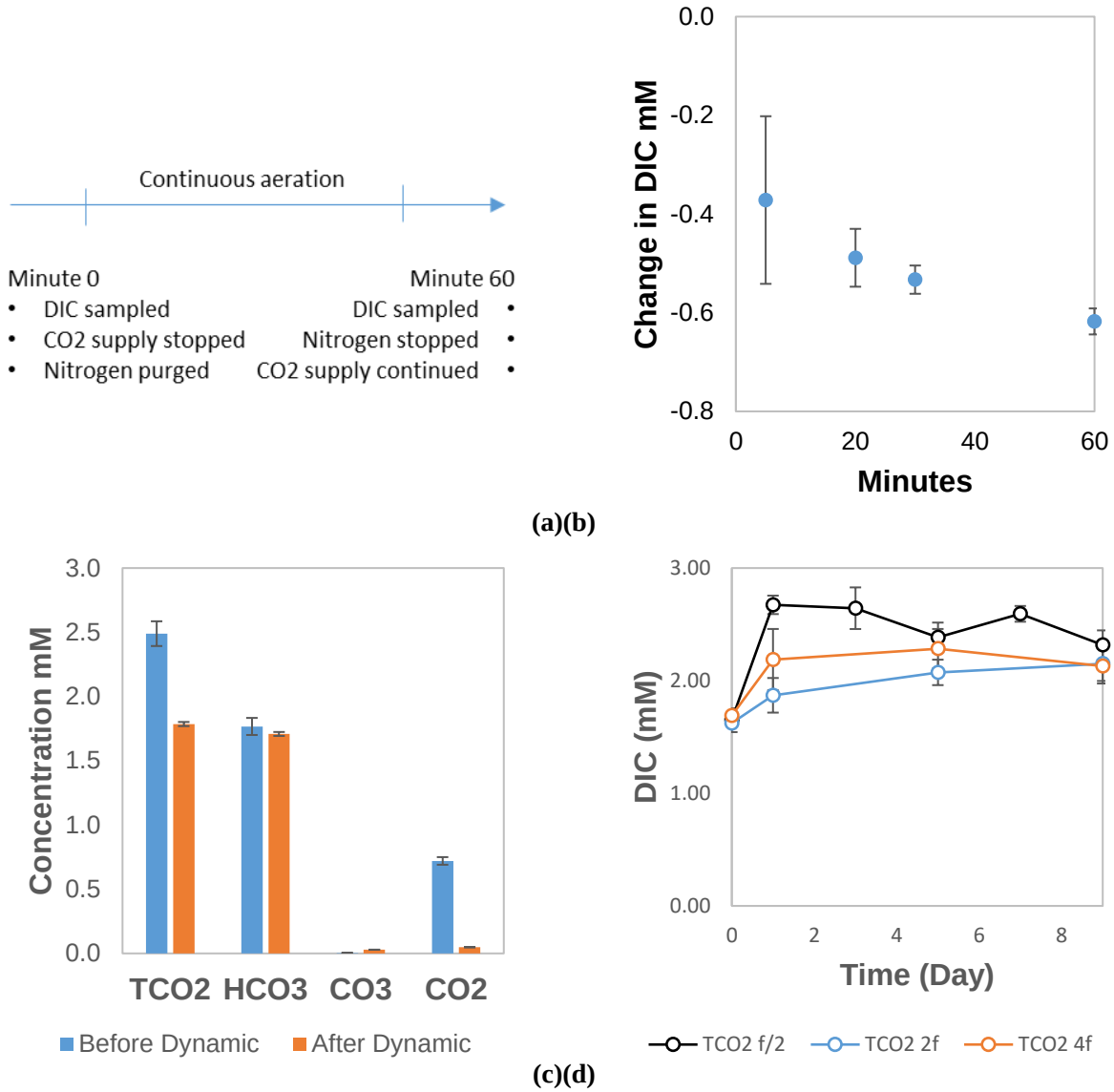


Figure 17 (a) Description of Dynamic Methods. (b) Change in DIC concentration after N2 Purge. (c) Change in TCO_2 and its Components after Dynamic Method applied to Blank Medium. (d) DIC in blank media of different nutrient levels for nine days, continuously supplied by 1% CO_2, indicating no statistically significant difference in DIC during the experimental period. Values are means (*n*=3), and error bars correspond to the standard error about the mean.

It was observed that after one hour, the media which had been aerated with 1% of CO_2 recorded average DIC loss of 0.6 mM to the surrounding (at the pH studied). Speciation study (Figure 17c) reveals that this is due to CO_2 gas in the media being driven off by the N_2. Although CO_2 is being driven off, it has been known that the microalgae are still able to use other carbon species for consumption, while at the same time, the species interchangeability is fast. As stated by (Chen et al., 2016), the possibility that microalgae only use carbon in CO_2 gas form or also take up the HCO_3^- and CO_3^{2-} forms is not a critical issue, as reactions that interconvert CO_2, H_2CO_3, HCO_3^- and CO_3^{2-} in the soluble form are fast enough not to be limiting steps in the CO_2 demand of the microalgae.

Thus, to apply this dynamic method to culture aerated with 1% CO_2, there are several requirements to be met. First, initial DIC concentration before applying the method must be above 0.6 mM. This is because control experiment without the presence of microalgae found that there is an average loss of 0.6 mM DIC when N_2 is purged, which is accounted for by the loss of CO_2 gas.

Second, DIC concentration after the 1-hour duration must be above zero. Zero DIC indicates that the culture consumed an equal amount or more than the value of inorganic carbon present during the dynamic method period. This may create uncertainty on the amount of carbon being consumed. If this situation happened, more carbon must be supplied and made available in the culture before applying the method.

Among nutrient-adjusted media (*f/2*, 2*f* and 4*f*), it was found that there is no statistically significant difference between T_{CO2} in the culture media (Figure 17d). This is supported by Gattuso et al. (2010), who stated that nitrate and phosphate addition does not alter alkalinity, since these species per se do not contribute to the carbonate alkalinity measured.

5.3.2 Microalgae Growth

High biomass production is observed in the 4*f* and 2*f* cultures (Figure 18a). During nine days of experiments, cultures in 4*f* and 2*f* achieved a biomass concentration of 0.6 g/l. Increase of nutrient concentration by four folds from *f/2* to 2*f* only increases biomass by 1.5 fold in BDUG and 2.1 folds in CCAP. However, in both strains, the increase from 2*f* to 4*f* does not increase the amount of biomass produced. Increase from *f/2* to 2*f* results in a significant increase in growth, while an increase to 4*f* shows no significant increase, and is deemed unnecessary from the point of biomass production. Statistically, in terms of biomass produced, there is no difference between BDUG and CCAP in each media. In other words, BDUG and CCAP in *f/2* produced an equal amount of biomass, and similar was the case in 2*f* and 4*f*.

Maximum growth rate (Figure 18b) was captured immediately after D1 in all set of experiments. The highest growth rate was observed by both BDUG *f/2* and CCAP 4*f* (0.9 d^{-1}). Other cultures showed a similar amount of maximum growth rate that was lower than these two. The growth rate was similar to that reported by Montoya et al. (2014), where a growth rate of 0.99 d^{-1} was reported for *Chlorella vulgaris* CCAP 211 aerated by 2% CO_2.

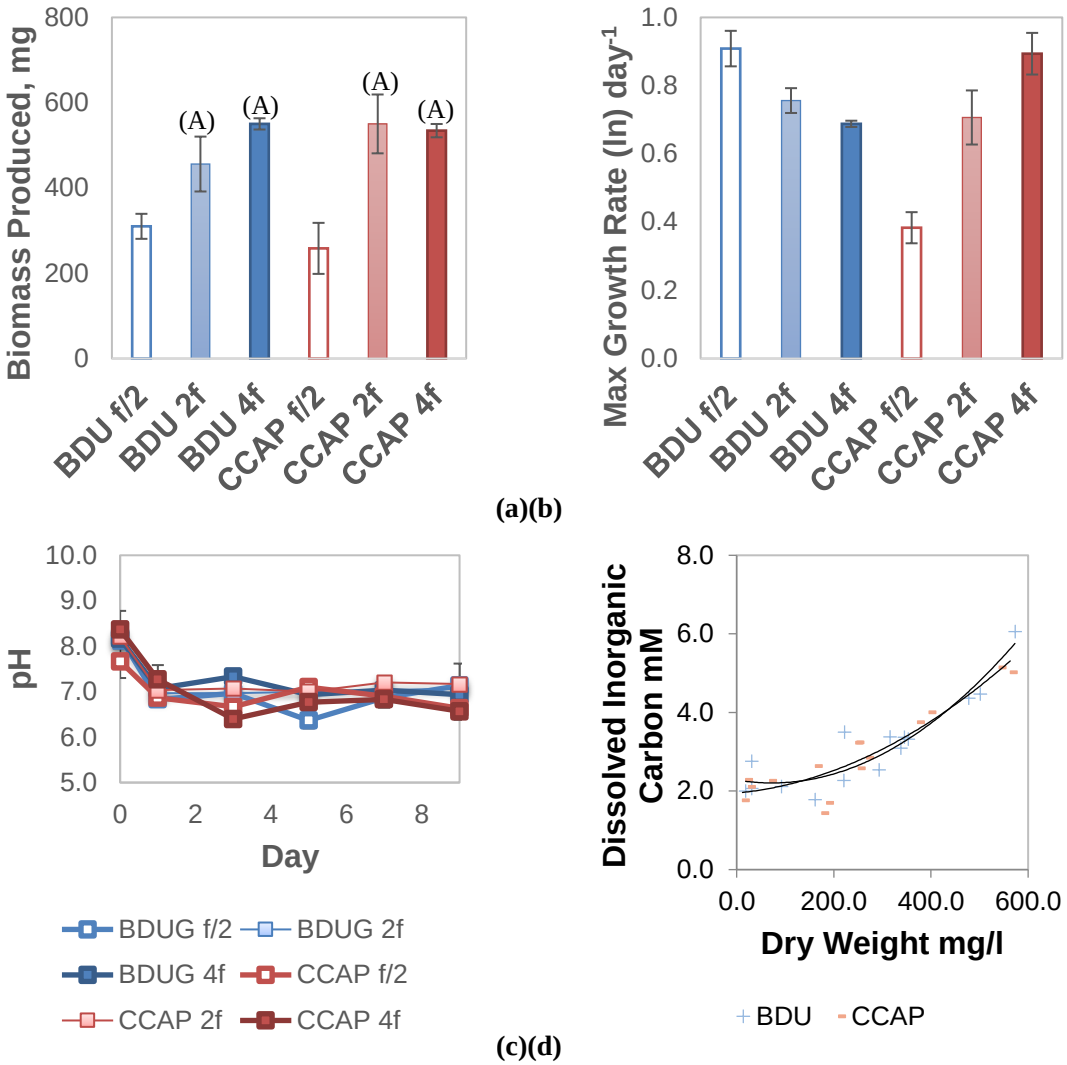


Figure 18 (a) Biomass Produced at Day 9. (b) Maximum Growth Rate (ln) (c) pH changes during growth. (d) relationship between DIC availability in media with its dry weight. Values in (a)-(c) are means (*n*=3) and error bars correspond to the standard error about the mean. For biomass production (Figure (a)), an analysis was carried out to estimate the significance of differences between different levels of nutrients for each species, showing significant difference when compared to (A) *f*/2 (B) 2*f* (C) 4*f*.

It can also be noted that there is an opposite trend between the two strains in term of the change in the maximum growth rate. While the maximum growth rate of BDUG cultivation decreases with the increase in nutrient level, an opposite case happened in CCAP cultivation. This highlighted another difference between the two strains.

Besides, it is essential to state that the data in Figure 18b represents the maximum growth rate observed during the nine days of the experiment. Specifically to BDUG *f*/2 cultivation, although the cultivation with fewer nutrients showed less biomass production (Figure 18a), its maximum growth rate is higher compared to 4*f* and seems to contradict. This can be understood by observing the growth rate of other days. For example, at D3, the growth rate of the 4*f*

cultivation is the highest compared to the remaining cultivations, while for the *f*/2 cultivation, its growth rate was among the lowest.

5.3.3 pH Change

pH in all cultures begins at around pH 8 ± 0.25 (Figure 18c). No significant difference in pH was observed between sets of the experiment. A supply of 1% CO_2 resulted in the pH of the media to stay between 6.5 and 7.0. This value is reasonable as observed by Liu et al. (2016), when the pH stayed around 7.0, on supplying the media with 1% CO_2. Shene et al. (2016) also observed that when culture is supplied with 0.81 or 1.34% of CO_2, the pH oscillated in a range of 7.0 to 8.5.

5.3.4 DIC and Biomass Concentration

Here we observed that as the algae grow and increase in dry weight, it can capture more carbon into the culture, making the carbon available for the algae to grow more. Thus carbon is not a limiting factor in their growth.

Correlation between dry weight and DIC concentration in this experiment gave an R^2 value of 0.88, which shows that there is a strong relationship between the two. In other words, as dry weight increases, DIC also increases and vice versa. However, this increase in DIC saturation value does not necessarily mean high carbon uptake rate, which needs to be observed using different parameters.

Sketching the trendline using the software gives the highest R^2 value as 0.844 for a sixth-order polynomial (Figure 18d). However, this equation indicates that only forward extrapolation might be valid since backward extrapolation will give high DW for a low amount of DIC, which is not the case.

There is also a strong correlation between maximum dry weight and maximum DIC of R^2 value of 0.96. This number is obtained by a logarithmic trendline, which makes more sense – there will be asymptote of the minimum threshold of DIC for algae to grow and maximum DW will be obtained however high DIC is available.

Although nitrate consumption may increase the alkalinity of the culture (Nguyen & Rittmann, 2016; Zeebe & Wolf-Gladrow, 2001; Brewer & Goldman, 1976), it will not be added to the

DIC value calculated by this method (Chen et al., 2016) as the DIC estimation are based on the carbonate alkalinity. The alkalinity that will be increased on nitrate consumption will be non-carbonate alkalinity, thus will not be affecting the DIC reading.

5.3.5 Carbon Uptake Rate

Calculation of the carbon uptake rate indicates that BDUG has faster consumption compared to CCAP (Figure 19a). Both BDUG *f*/2 and BDUG 2*f* recorded the fastest consumption. Increasing from *f*/2 to 2*f* in BDUG did not result in a significant change in carbon uptake rate while increasing nutrient to 4*f* seems to decrease the value. All three set for CCAP gave the lowest readings, with little difference between the conditions. This result signals that by increasing the nutrients in BDUG culture, the carbon uptake rate is not increased, but rather decreases, while in CCAP, the rate stays the same.

By comparison, the study by Moazami-Goudarzi & Colman (2012) using *Stichococcus minor* and *S. cylindricus* reported CO_2 depletion rate of 0.5 mM/h, while El-Ansari & Colman (2015) reported CO_2 uptake of 0.9 mM/h for *Chlorella kessleri* grown in pH 6. These data showed that the value obtained by *Chlorella vulgaris* in this experiment is not far from the readings reported in the literature.

A relationship between carbon uptake rate and its dry weight was investigated, but no clear relation could be deciphered (Figure 19b). In other words, although the dry weight increases, the speed with which carbon is consumed does not increase. The graph indicates that the peak in carbon uptake rate happened in the middle of the graph, i.e. when the dry weight is around 300 mg/l.

5.3.6 Carbon Balance and Sequestration by Microalgae

CO_2 consumption by the culture was studied based on the carbon uptake rate measured at each day. Figure 19c indicates the cumulative CO_2 amount consumed by 1-litre cultivation for a total of 9 days of the experiment, i.e. an average of 0.3 - 0.8 g/l/d. It is observed that in BDUG, as the nutrient increases, total CO_2 consumed decreases. A similar trend is observed in CCAP culture; however, the difference between the values recorded for *f*/2 and 2*f* is not statistically significant.

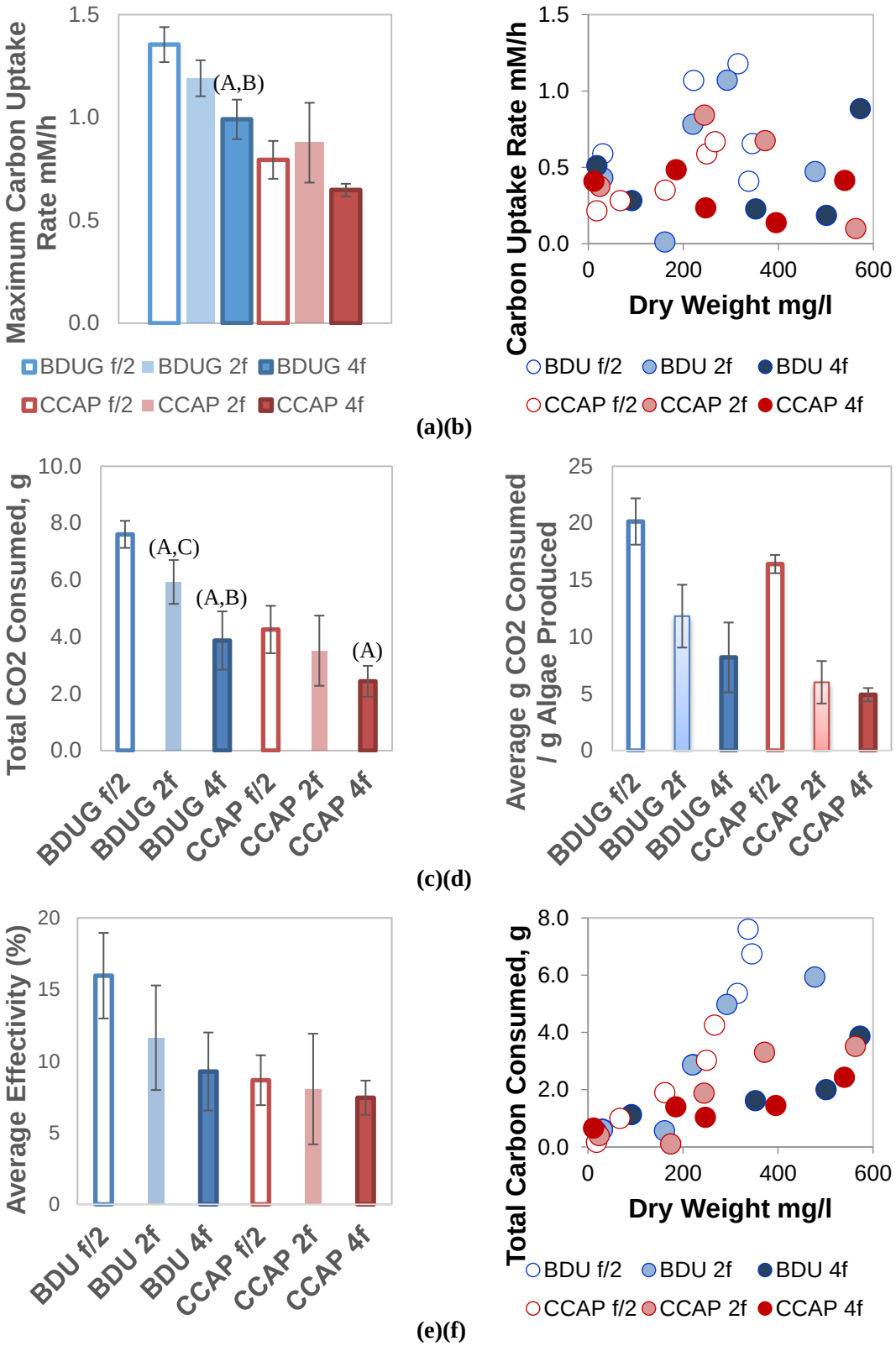


Figure 19 (a) Comparison of maximum carbon uptake. (b) Relationship between carbon uptake rate and its dry weight. (c) Amount of CO_2 consumed after nine days of cultivation. (d) Average sequestration capacity, i.e. CO_2 consumed per microalgae production. (e) Average effectivity (f) Relationship between Total Carbon Removed and its dry weight. No relationship between CUR and DW was observed. Values in (a),(c)-(e) are means (*n*=3), and error bars correspond to the standard error about the mean. For maximum carbon uptake in BDUG and total CO_2 consumption (Figure (a) and (c)), an analysis was carried out to estimate the significance of differences between different levels of nutrients for each species, showing significant difference when compared to (A) *f*/2 (B) 2*f* (C) 4*f*.

Comparison with other reports shows similar order of consumption values such as 3.6 g/l/d (Fan et al., 2008), 2.75 g/l/d (Adamczyk, Lasek, & Skawińska, 2016), 2.4 g/l/d (Sydney et al., 2010), 1.25 g/l/d (Yun, Park, & Yang, 1996) and 1.23 g/l/d (García-Cubero, Moreno-Fernández, et al., 2018), all recorded by *Chlorella vulgaris*. There is also a report of 0.865 g/l/d by *Chlorella* sp. (Hirata et al., 1996).

The amount of carbon consumed was then compared to the number of microalgae produced (Figure 19d). There are many ways of expressing this parameter in which the common cited theoretical amount of CO_2 sequestered by the algae is 1.83 kg CO_2 per kg algae (Chisti, 2007), disregarding the loss to the atmosphere. If CO_2 loss to atmosphere is counted together, this ratio will be lower. Since there is no explanation found on how this value is arrived at, it is inferred that it is based on the carbon composition in the algae, which is around 50%, thus 50% × 44 g/mol (CO_2 molecular mass) ÷ 12 g/mol (carbon atomic mass) = 1.83 (Buehner et al., 2009).

The highest value of carbon sequestration ratio is recorded by *f*/2 culture of both strains. The second-highest reading is by BDUG 2*f* while the lowest sequestration capacity is by CCAP 4*f*, i.e. 4 kg CO_2 / kg algae produced. Although the value recorded is ten times the theoretical amount reported previously, it is expected, since calculated sequestration ratio from works of literature is in broad ranges, such as 9 (Fan et al., 2008), 1 – 2 (Ramaraj, Tsai, & Chen, 2014), 1 (Adamczyk, Lasek, & Skawińska, 2016), 0.4 – 1.3 (Zhou et al., 2017), 0.2 (Sydney et al., 2010) and as little as 5.63×10^{-7} g CO_2 removed / g algal culture in a report by Keffer & Kleinheinz (2002) using *Chlorella vulgaris*.

Alternatively, if total CO_2 being supplied towards the algae culture is regarded including those that escape to the atmosphere, the value obtained gives average value 0.1 – 0.3 g of CO_2 sequestered by 1 gram of microalgae. It roughly indicates the amount of CO_2 needed to produce 1 gram of algae. CCAP *f/2* sequesters more CO_2 than other media conditions.

A relationship studied between amounts of carbon being removed (based on the carbon uptake rate) and dry weight is shown in Figure 19f. Data analysis by Microsoft Excel gives R^2 value of 0.5, which indicates that there is a moderate relationship between them. Although the coefficient value is not keen to imply a perfect relationship, it can be said approximately that as the size of biomass increases, the amount of carbon being consumed also increases.

Table 2 provides a comparison of carbon uptake capacity between the chemical means and biological mean (this microalgae experiment) on a gram per gram basis. Among chemical means, the highest recorded sequestration capacity is shown by NaOH (1.1 g CO_2 / g). This value is 0.7 g lesser than the usual theoretical sequestration capacity reported, i.e. 1.8 g CO_2 / g algae produced.

This mass difference may highlight the potential of microalgae as an agent for CO_2 utilization. Besides, microalgae have the advantage of producing value-added chemicals, not involving high temperature nor pressure. Moreover, less storage is needed since the value-added chemicals are stored in the cell wall in dry powder form. These advantages highlighted the usefulness and potential of microalgae cultivations for CO_2 sequestration. This table, however, does not necessarily suggest that the gram per gram basis is the only criteria for comparison since there are other aspects to consider such as overall cost, process complexity and their lifecycle assessment.

Figure 19e shows the effectivity of the carbon supply. In general, the effectivity of the supply is in the range of 5 – 20%. Effectivity of carbon supply is determined by subtracting the ratio of the amount of carbon escaped (output) to the amount of carbon supplied (generated) from 1. The values reported here were obtained by picking the maximum values recorded by each cultivation based on the nine days of the experiment. For example, both 4*f* cultures have the highest effectivity on the last day of the experiment.

A study by Keffer & Kleinheinz (2002) reported CO_2 removal of 14 g / hour upon loading of 20 g / hour. This 70% removal efficiency is based on the difference in CO_2 gas concentration. Doucha, Straka, & Lívanský (2005) previously demonstrated that only 38.7% of supplied carbon dioxide was utilized by the algal cells. Jacob-Lopes et al. (2010) has shown that microalgae effectively fixed about 3% of carbon into biomass, which suggests that there are other CO_2 conversion routes in the reactor.

Table 2 Comparison of Carbon Uptake Capacity with Several Chemicals. NaOH: Sodium hydroxide. MEA: Monoethanolamine. IPA: Isopropylamine. DA: Diethylamine. PZ: Piperazine. AMP: aminomethyl propanol. AEEA: Aminoethylethanolamine. MDEA: Methyl diethanolamine. DET: Diethyltryptamine. TETA: Triethylenetetramine.

Sequestration Agents	Amount Reported in the Literature	g CO_2 / g Equivalent Produced / Present	Note
Chlorella vulgaris (This experiment)	4 – 25	4 – 25	Calculation based on the DIC in the culture media
Microalgae (in general)	1.8 ton/ton CO_2	1.8	Commonly Cited (Chisti, 2007)
NaOH	0.9 ton/ton CO_2	1.1	Theoretical Value (Yoo, Han, & Wee, 2013)
MEA	1.39 ton/ton CO_2	0.7	Theoretical Value (Yoo, Han, & Wee, 2013)
	581.3 g CO_2/kg MEA	0.6	Experimental Value (Huertas et al., 2015)
IPA / DA	0.499 g CO_2/g amine	0.5	Experimental Value (Cheng et al., 2019)
PZ / AMP	1.0 mol CO_2/mol	0.5	Experimental Value (Yu, Huang, & Tan, 2012)
AEEA / MDEA / DET	1.0 mol CO_2/mol	0.4	Experimental Value (Yu, Huang, & Tan, 2012)
Graphene	7.95 mmol CO_2/g	0.4	Adsorption capacity (Yu, Huang, & Tan, 2012)
Ionic liquids [TETAH][BF4] containing 40% water	2.04 mol CO_2/mol	0.4	Experimental Value (Luis, 2016)
TETA	170 g CO_2/kg	0.2	Experimental Value (Bernhardsen & Knuutila, 2017)

5.3.7 Nutrient Removal

Generally, the rate of nitrate uptake depends on three factors, i.e. the nitrogen status of the cells (e.g. whether in starvation or excess), the presence of other nitrogen compounds and light conditions (Hellebust & Ahmad, 1988). As nitrates are being actively transported into the microalgae cells, it is reduced to ammonium by nitrate and nitrite reductases and later incorporated into amino acids by the glutamine synthetase pathway (Taziki, Ahmadzadeh, & A. Murry, 2016). Its assimilation is regulated at three levels, i.e. the activity of nitrate transport

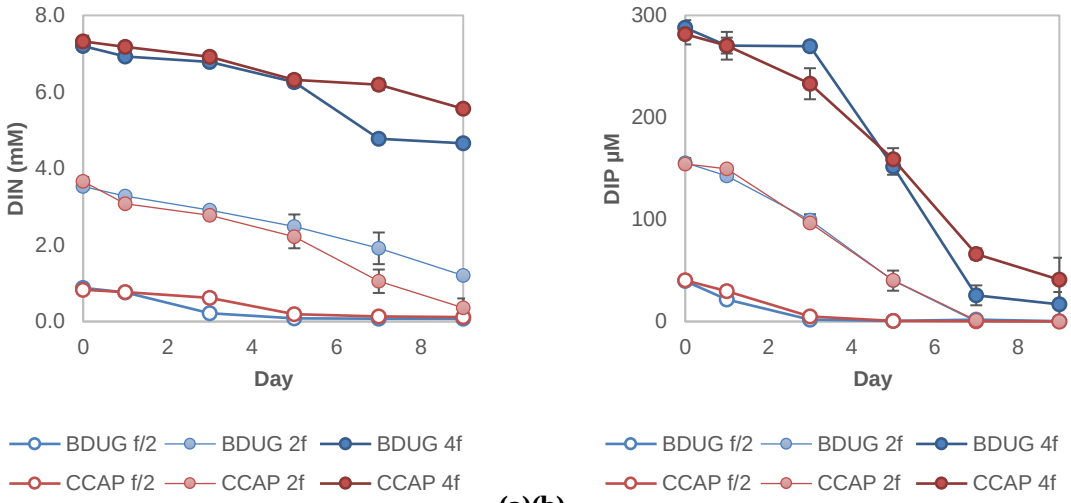
system, the activity of nitrate reductase and the amount of nitrate of reductase in the cells (Hellebust & Ahmad, 1988).

Concurrently, phosphate uptake in microalgae is influenced by factors such as status of the cells, light condition, temperature, pH, salinity and growth (Eixler, Karsten, & Selig, 2006). It is also directly influenced by growth, known as the metabolic uptake. Their uptake can be characterized in many ways, such as starvation uptake and storage uptake. During storage uptake, the excess of phosphate accumulated is usually stored as inorganic polyphosphate, which serves as internal storage and can be utilized when needed (Singh, Nedbal, & Ebenhöh, 2018). This storage molecule has been suggested to play a role in regulating the synthesis of compounds such as ATP.

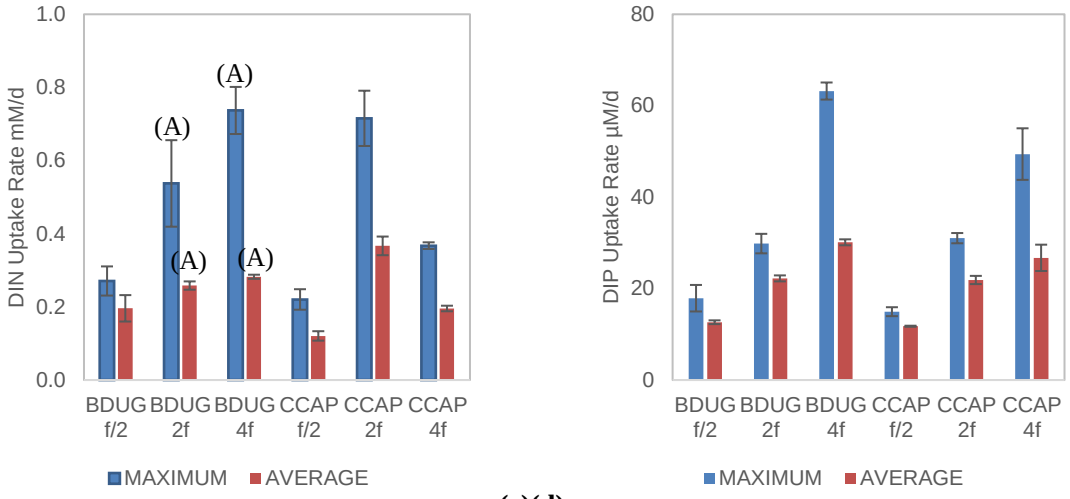
The amount of phosphate and nitrate consumed for the production of algae are different among the set of experiments (Figure 20a). The amount of nitrate removed up to Day 9, sorted in ascending order is as follow: 20% in CCAP 4*f* (equivalent to 1.4 mM nitrate being removed), 40% in BDUG 4*f* (= 2.9 mM), 70% in BDUG 2*f* (= 5.0 mM) and 90% in CCAP 2*f* (= 6.5 mM). This result supports the idea that when the same strain of algae is exposed to different nutrient concentration, their removal behaviour will be different (Wang et al., 2014).

Earliest nitrogen deprived condition is observed at D5 in BDUG *f/2*. Only three cultures removed all the nitrate, i.e. *f/2* and CCAP 2*f*, in which complete removal is considered once the concentration reached 10% of its initial value.

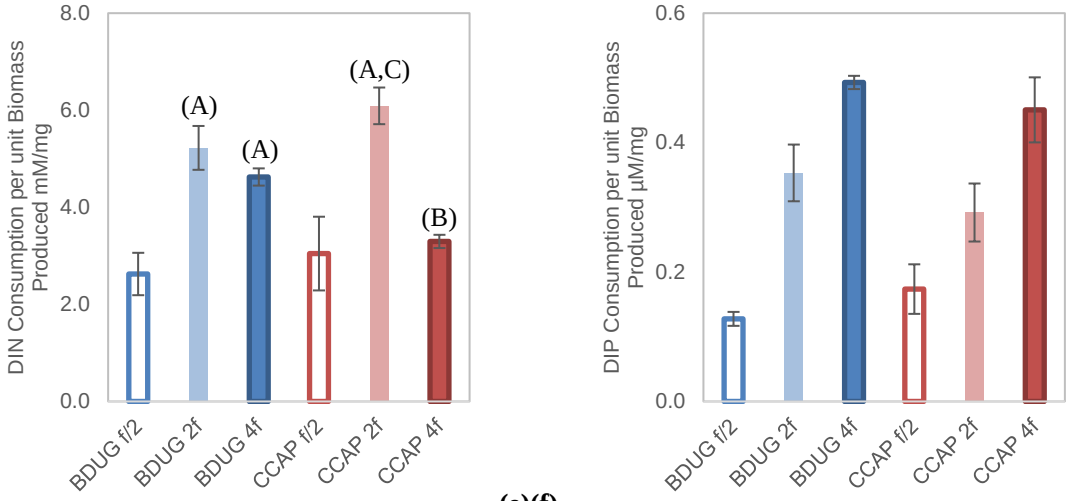
Based on the profile, the nitrate removal rate was calculated, as shown in Figure 20c. Removal rates are based on nine days of the experiment, except for BDUG *f*/2, in which the nitrate was removed entirely at Day 5. Nitrate average removal rate is the fastest in CCAP 2*f*. Among BDUG, fast maximum and average nitrogen consumption are recorded by both 2*f* and 4*f*, in which there is no significant statistical difference between them.



(a)(b)



(c)(d)



(e)(f)

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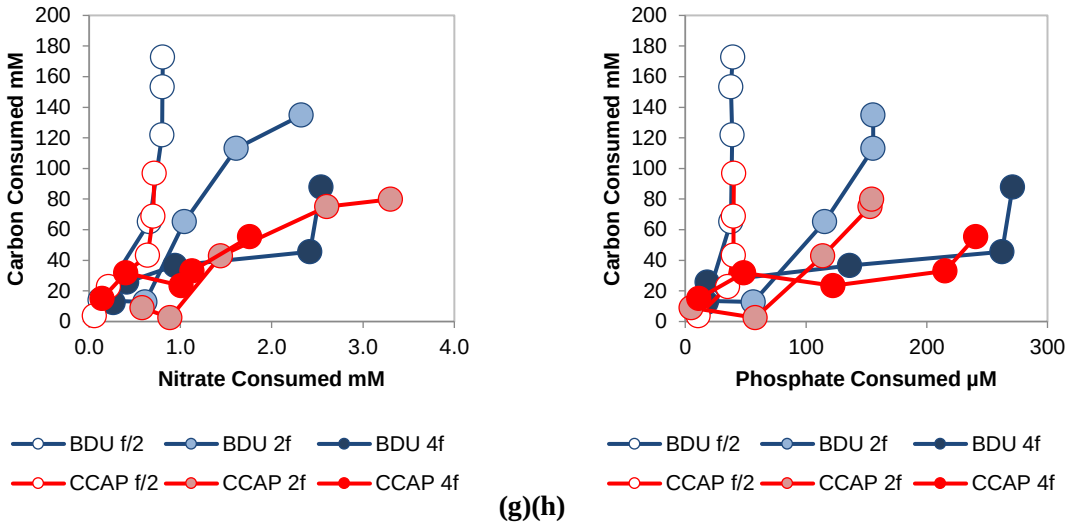


(g)(h)

Figure 20 Change in (a) DIN and (b) DIP concentration. Rate of (c) nitrate and (d) phosphate removal by the respective microalgae culture. Amount of (e) DIN and (f) DIP consumed per biomass produced. Relationship between the amount of carbon consumed per (g) nitrate and (h) phosphate consumed. Values in (a)-(f) are means (*n*=3), and error bars correspond to the standard error about the mean. For nitrogen uptake rate in BDUG and DIN consumption per unit biomass produced (Figure (c) and (e)), an analysis was carried out to estimate the significance of differences between different levels of nutrients for each species, showing significant difference when compared to (A) *f*/2 (B) 2*f* (C) 4*f*.

Shene et al. (2016) observed that pH influences nitrate uptake in microalgae, in a way that the pH that allows a higher proportion of bicarbonate will favour the uptake. This is displayed in this experiment as well, in which pH in which bicarbonate is shown to be high (BDUG 4*f* for example) (Figure 18c), also showed biggest and fastest consumption of nitrate (Figure 20c). Besides, high nitrate uptake rate is connected with the photosynthetic apparatus (Tischner & Lorenzen, 1979). If this can be related to biomass production, then this statement appears correct in this experiment, in which highest biomass production is also recorded by BDUG 4*f* culture.

It is observed in *C. reinhardtii* cultivation that when it is exposed to 4 mM of nitrate, less than 0.5 mM of it gets consumed (Gardner et al., 2013). This shows that *Chlorella* in this experiment is better in taking up nutrient at high concentration, as it removed 70% and 90% of 4 mM nitrate in BDUG and CCAP respectively.

Specific DIN uptake rate measurement reveals that the highest reading was observed at Day 1 for all setups, similar with DIC and DIP. This was when the cells pulled the carbon available for growth and reproduction. Although the maximum value is observed at D3 for nitrate CCAP 2*f*, its D1 value (0.08 mM/d/g) is not far from the maximum. Maximum specific uptake recorded at Day 1, which is the beginning of the exponential phase of all set of experiments.

During this stage, although the cell has low dry weight, it acquires the highest amount of carbon, nitrogen and phosphorus. In both BDUG and CCAP, 2*f* culture shows the highest value of nitrate consumption per unit of biomass produced (Figure 20e). However, for BDUG, this value is not significantly different from 4*f* culture.

Phosphate is entirely removed in all media, except in CCAP f4 (85% removed) (Figure 20b). Both strains in *f/2* entirely removed phosphate in D3, while this happened in D7, for the other conditions. Earliest phosphorous deprived condition is observed at D3 in both strains of *f/2*. Fastest phosphate removal is in 4*f*, which is around 30 µM / day, followed by 2*f* (20 µM / day) and *f/2* (10 µM / day) (Figure 20d). In terms of the difference between strains, BDUG has a higher phosphate removal rate in 4*f*, but a similar rate in 2*f* and *f/2*. In comparison, Liang et al. (2013) recorded 80% removal of 30 µM phosphate by *Chlorella vulgaris* FACHB-6 (China) at D3. They also recorded maximum phosphate removal rate of 21 µM / d. Further analysis on the specific phosphate uptake rate (mM/d/g) reveals that the highest reading was observed at Day 1 for all setups, in which there is no statistically significant difference between all sets of experiments, concerning phosphate uptake rate. It is also found that microalgae in 4*f* culture consumed more amount of phosphate per biomass produced.

Figure 20e and Figure 20f shows the amount of DIN and DIP consumed per unit biomass produced in each culture, respectively. Cultures in *f/2* of both strains required a lesser amount of phosphate and nitrate for production of 1 gram of biomass. In terms of nitrate consumption for biomass production, both 2*f* cultures show the highest reading. From the graph, CCAP 2*f* needed 6 mM of nitrate, while *f/2* needed only half of that amount. This number increases from *f*/2, later decreases in 4*f*, showing the same trend between the cultures of both strains. A different trend is observed in the DIP consumption per unit of biomass produced. A high amount of phosphate is needed for biomass production in 4*f* cultures As the supply amount of phosphate increases, the DIP consumption per unit biomass increases for both strain cultures.

The difference in trends between nitrate and phosphate may be due to their concentration, since the highest amount of phosphate being supplied (i.e. in 4*f*) is 0.3 mM, while the highest amount of nitrate concentration is 7.2 mM Even nitrate concentration in *f/2* is already 0.8 mM. This suggests that nitrate in the medium is already saturating at 2*f*, but phosphate is not even saturated at 4*f*.

The last two graphs in Figure 20 show the analysis of nutrient consumptions with reference to carbon sequestration. Figure 20g shows the relationship between the total concentration of carbon and nitrate being consumed, in which in *f*/2 cultivation, carbon is still being consumed, although nitrate has been consumed completely. A plot for 4*f* cultivation gave a low gradient indicating that although the amount of carbon being consumed is low, the nitrate is continuously being consumed. A consumption graph of a similar trend can be seen in phosphate consumption (Figure 20h). It is parallel with the idea that the phosphorus uptake rate is not strongly influenced by CO_2 concentrations (Gonçalves et al., 2016). Our data even suggested that carbon consumption in *Chlorella* is the best at limiting nitrogen and phosphate concentrations, which is not explicitly observed and mentioned by works of literature before. This, however, does not denies another observation that nutrient consumption is better when carbon is available, which has been recorded in the previous chapter.

5.4 Summary and Conclusion

Increase of nutrient supply from *f/2* to 2*f* (4-fold increase in nitrogen and phosphorous) results in a significant increase in growth, while a further 2-fold increase to 4*f* does not show a significant increase, therefore may not be necessary, from a growth perspective. In terms of biomass produced, there is no difference between strains of BDUG and CCAP. Highest specific DIC, DIN and DIP uptake was observed at Day 1 for all setups. Cultures in *f/2* of both strains required a lesser amount of phosphate and nitrate for production of 1 gram of biomass. Nitrate overall removal rate is the fastest in 2*f*. Fastest phosphate removal is in 4*f*.

In terms of sequestration capability, there are differences observed between cultures of different strains of similar species. In this experiment, the carbon uptake rate in *Chlorella vulgaris* is found to be between 0.5 – 1.2 mM/h, in which BDUG strains have roughly higher maximum uptake rate compared to CCAP. The effectivity of continuous carbon supply is found to be between 10 – 25%. In both strains, an increase in nutrient supply from *f/2* to 2*f* and 4*f* does not increase the total carbon consumption; instead, it shows a decreasing trend. In parallel with this, our data on carbon and nutrient consumption suggested that carbon consumption is best at limiting nitrogen and phosphate concentrations. On an average, carbon sequestration is 13 and 9 g CO_2 / g algae for BDUG and CCAP respectively, which is higher than the usually reported value of 1.83, since this calculation considers the amount of carbon being dissolved in the media too. Also, the media contributes to a massive proportion of the culture compared

to the microalgae cells. Comparing this value with sequestration capacity by chemical means, microalgae have a higher value, which highlights its additional advantages and usefulness, thus the potential of for CO_2 sequestration. It is also found that the amount of DIC availability in cultures increases with microalgae growth. Altering growth or culture condition will bring effects towards an increase in DIC, which also implies an increase in CO_2 sequestration. Highest recorded increase in DIC is in 4*f* (BDUG then CCAP). By manipulating nutrient condition, the DIC increases by a significant fold in 2*f* and 4*f*.

CHAPTER 6: Carbon Uptake in Red Microalgae *Porphyridium purpureum* upon Different Carbon and Nutrient Treatments

Abstract

Previously, we have examined the influence of carbon and nutrient treatment in green microalgae *Chlorella vulgaris*. It has been found that more DIC was able to be contained in the culture of higher biomass. This is shown in DIC concentration in the cultures aerated with 1% CO_2, which rises above their baseline. Thus we wished to see whether a similar scenario happens in a species from a different lineage (i.e., red as opposed to green algae) with respect to the similar changes that have been observed.

This chapter aims to study red microalgae *Porphyridium purpureum* carbon consumption behaviour and identify an optimum condition that enables high production of bioplastics precursor, i.e. polysaccharides. Two types of bioplastics precursors were investigated, i.e. intracellular polysaccharides and exopolysaccharides (EPS). The algae were subject to the same four different carbon treatment as was investigated for *Chlorella vulgaris* (Chapter 4), i.e. no carbon supply (C_0), with initial 0.01M carbon supply ($C_{0.01M}$), continuous air (C_{air}) and carbon dioxide aeration ($C_{1\%}$) and three nutrient treatments, i.e. 2, 4 and 8 times increase of nitrate and phosphate initial concentration (labelled *f*, 2*f* and 4*f* cultivation respectively).

P. purpureum culture showed the highest carbon uptake rate in 4*f*, which is around 1 mM/h. This cultivation also showed the highest level of DIC saturation compared to others. Calculation considering biomass production reveals carbon sequestration of 7 g CO_2 per g algae produced as the highest. *P. purpureum* grew slowly with a μ_{max} of around 0.35 day^{-1} but was able to produce six-fold biomass concentration in 10 days. Highest nitrate removal recorded was only 80% by *f* culture while phosphate is removed entirely in all cultures except those with nutrient increase. The 4f culture recorded both the highest nitrate and phosphate uptake values. Carbohydrate concentration was highest in the culture with continuous aeration (C_{air}). Increasing nutrient does not change the carbohydrate proportion; instead, it increases the protein percentage of its dry weight, given that the conditions become nutrient replete. Lipid is highest in culture without carbon supply (C_0). Normalized pigment concentrations to their dry weights showed high value for culture in high pH and high nutrient value (peaked at *f*), while EPS is secreted the highest in 2*f* culture.

6.1 Introduction

Microalgae cultivation has several potential applications such as for carbon consumption and synthesis of value-added products, e.g. food supplements, pharmaceuticals and bioplastics precursors. To achieve optimal condition for these applications, among necessary steps are selecting the best strains and also providing the best strategies to deliver their growth requirement.

Previously, we have examined the influence of carbon and nutrient treatment in the green microalga *Chlorella vulgaris*. It has been found that more DIC was able to be contained in the culture of higher biomass. This is shown by DIC concentration in the cultures aerated with 1% CO_2, in which the DIC level rose above their baseline. Since microalgae belong to a diversified group of different families, such as red algae, green algae, blue-green algae and diatoms (Cheah et al., 2015), it is interesting to see whether this behaviour can also be observed in other groups of microalgae.

Rhodophyta, also known as red algae family is known to produce bioplastics precursors, i.e. polysaccharides of two different types, i.e. exopolysaccharides (EPS) and intracellular polysaccharides as part of their growth (Delattre et al., 2016). During microalgae growth, the EPS that encapsulated the cell dissolves into the medium, increasing the medium viscosity (Bernaerts et al., 2018). Among those which belong to this family of red algae are *Porphyridium purpureum*. Generally, they are grown in the Mediterranean area, where the abiotic parameters are favourable for growth (García-Cubero, Cabanelas, et al., 2018).

Microalgae produce the EPS of distinctive and diverse composition as part of its survival in variable marine conditions (Freitas, Torres, & Reis, 2017; Roca et al., 2016; Finore et al., 2014). The EPS are composed of multiple monosaccharides; the most abundant being xylose, glucose and galactose (Marcati et al., 2014; Villay et al., 2013). Others include deoxy-monosaccharides, methylated sugars and uronic acids. They are negatively-charged because of the presence of glucuronic acid and sulphate groups (Patel et al., 2013).

The EPS is considered more as by-products rather than as value-added chemicals, although they have potential in food and pharmaceutical applications (Douskova et al., 2009). For

achieving a high amount of EPS, it is required to have good culture growth and optimized culture condition (Iqbal & Zafar, 1993).

Data on optimization of EPS by red algae are not abundant, while parameters used to enhance EPS production are strain-dependent (Markou & Nerantzis, 2013). There is only a limited number of research that concentrate on the influence of culture media and physicochemical parameters towards biomass and EPS production (Klinthong et al., 2015).

Several reports on conditions for carbon fixation in *P. purpureum* (as intra-polysaccharides, exopolysaccharides and biomass) through the influence of nutrient has been found (Assunção, Varejão, & Santos, 2017; Coward et al., 2016; Fuentes-Grünewald et al., 2015; Soanen et al., 2015; De Jesus Raposo, De Morais, & De Morais, 2014; Razaghi, Godhe, & Albers, 2014; Villay et al., 2013; Gasljevic et al., 2009). In summary, high intracellular polysaccharides are closely related to high production of biomass. For overproduction of carbohydrate in the culture, it is suggested to have nitrogen starvation. Meanwhile, to achieve high EPS, it is by having limited phosphorous and magnesium, higher ion concentration, glucose as a carbon source and temperature at 28 $^{\circ}$C. For achieving both high biomass and intracellular polysaccharide, a high N:P ratio helps. As for the production of both biomass and EPS, semi-continuous cultivation with high salt content (3 to 5 M NaCl, higher than that in *f*/2 medium) and low light intensity might be preferable (Xiao & Zheng, 2016; Mishra & Jha, 2009).

Less information is available on biochemical composition concerning bioplastics precursors on a daily basis and strain-specific behaviour. Even less is known about the carbon sequestration capacity of this organism.

This chapter aims to examine the behaviour of *P. purpureum* as one of producers of bioplastics precursors (i.e. intracellular polysaccharides and exopolysaccharides) upon carbon and nutrient treatments, so a comparative assessment with that of *C. vulgaris* can be effected. For achieving this, similar conditions of carbon and nutrient treatments as in the previous chapters were used for comparison. As previously mentioned, the four treatments represent limiting vs excessive and active vs passive carbon supply. As for the increase in fold amount of nutrients, it is based on the concentration in *f*/2 medium, as mentioned in Chapter 5.

6.2 Experimental Method

Microalgae Cultivation

Porphyridium purpureum CCAP 1380/1A was grown in 1 litre *f/2* media with a continuous natural white light of 100 µE/m^2/s. Mechanical stirring was at 200 rpm while the temperature was at 23 ± 3 ˚C. Initial pH was set to 8. The reactor was inoculated with OD_{760} 0.2 microalgae and grown for ten days.

Manipulating Carbon and Nutrient Source

Microalgae were exposed to the four different carbon treatments, i.e. C_0, $C_{0.01M}$, C_{air} and $C_{1\%}$, as detailed in Chapter 4. When manipulating the nutrient source, a similar setup as $C_{1\%}$ was used. The concentrations of nutrients in the media were adjusted by increasing the folds of nitrate and phosphate. *f* medium refers to an increase of 2 folds of both nitrate and phosphate from *f/2*, while 2*f* and 4*f* media contain the addition of 4 and 8 folds increase respectively.

Microalgae Harvesting

6 ml of the sample was harvested at each time point. 0.5 ml samples were taken for growth measurement by OD. Another 0.5 ml was taken for DIN, DIP analysis. The remaining 5 ml of sample was for DIC measurement, for which the sample was centrifuged at 5000 rpm, 25 ˚C, for 10 minutes – the supernatant was analysed for DIC and pH while the pellet was analysed for biochemical components.

Growth Measurement

Samples were taken for optical density measurement at wavelengths of 760 nm. Dry weight was determined through its relationship with optical density. This relationship was established by using culture at the end of cultivation, which was diluted serially to represent samples of several optical densities.

"Amount of algae produced" was measured by obtaining the difference between initial and final dry weight. Since dry weight was measured as concentration g/l, total biomass was obtained by multiplying it by the volume of culture.

DIC, DIN and DIP Measurements

These were carried out as detailed in Chapter 3. DIC calculation was done by double titration method, while DIN and DIP were determined spectrophotometrically.

Carbon Uptake

For C_0 and $C_{0.01M}$ culture, the uptake rate is calculated directly from the change in DIC in their media. For C_{air} and $C_{1\%}$, carbon uptake rate is measured by the dynamic method detailed in Section 5.2.

Biochemical Assay

Samples were frozen at -20 °C for storage before analysis for simultaneous carbohydrate (intrapolysaccharides), protein, lipid and pigment analysis spectrophotometrically, as detailed by Chen & Vaidyanathan (2013). The details have been explained previously in Chapter 3.

Exopolysaccharide Measurement

Exopolysaccharide concentration was determined by anthrone-sulphuric acid method (Chen & Vaidyanathan, 2013) based on the supernatant obtained, which is similar to the method by Wang et al. (2007). Briefly, the supernatant was reacted with anthrone reagent and a high concentration of sulphuric acid before being boiled at 100 °C for 15 minutes. After cooling, the absorbance was read at 578 nm by using a spectrophotometer.

Statistics

Each experiment was done in triplicates to ensure reliability. A test for statistical significance was performed at 95% significance level with the help of "Data Analysis" in Microsoft Excel.

6.3 Results and Discussion

6.3.1 DIC Availability and Carbon Uptake

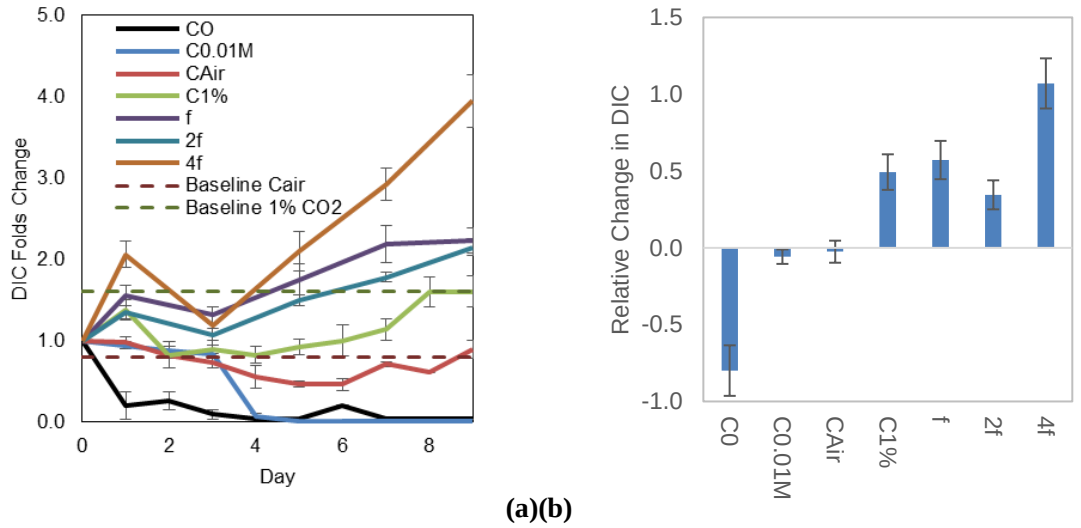
Figure 21a shows DIC availability in *P. purpureum* culture upon carbon and nutrient treatment. The culture without active carbon supply (C_0) contained an initial amount of 1.4 mM DIC. It was utilized immediately during the first day of the experiment creating a carbon-starved condition. There was one record of DIC increase to 0.2 mM, i.e. D6, however, it went back down the following days. Although there is no active supply of CO_2, there is passive diffusion of air into the culture medium, which could have provided with the carbon for the growth observed in C_0. Whatever CO_2 was diffused into the culture was taken up for algae growth, which is reflected by its lowest biomass compared to others (Figure 22a). In $C_{0.01M}$ culture, carbon was utilized slowly in the first three days before rapid consumption until it reached limited carbon condition in a single day. Throughout the culture, DIC in C_{air} stays around 1.0

mM, near its baseline. This value indicates a steady consumption of carbon concerning its supply. As for $C_{1\%}$, it stays around 2.0 mM, which is below its baseline. This value indicates the carbon is being consumed actively up to a condition where the carbon supplied could not help achieve the saturation conditions achieved with the blank media.

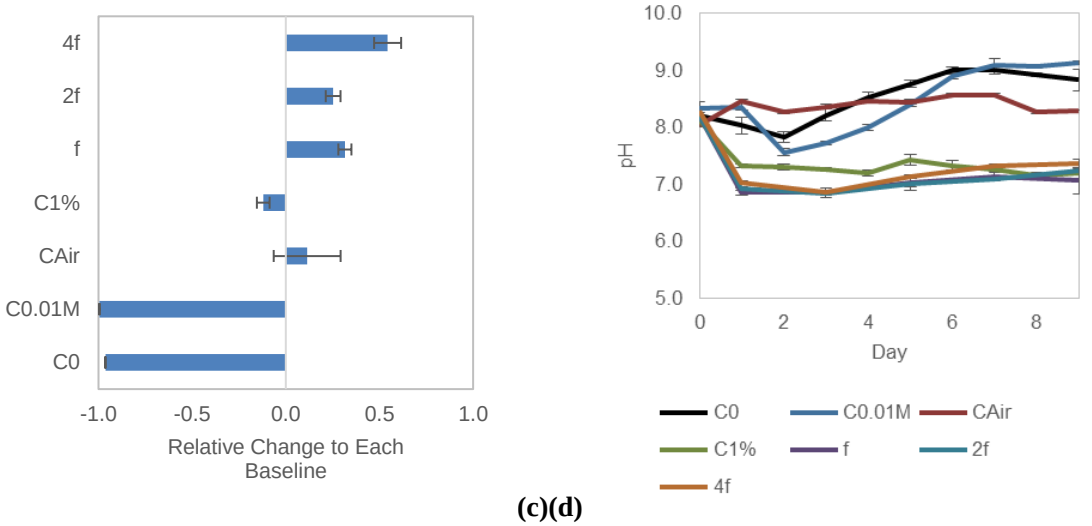
Among nutrient-adjusted cultivations, the highest DIC value was recorded by the culture with the highest nutrient concentration, i.e. 4*f*. This value is followed by both 2*f* and *f* cultivations, in which no significant difference in final DIC value was observed between them. All three cultures recorded DIC values beyond their baseline value, indicating that the activities of the microalgae had enabled more CO_2 being captured into the media.

Figure 21b describes changes in DIC after one day of cultivation. It shows that C_0 has consumed around 75% of its initial carbon concentration. DIC in $C_{0.01M}$ reduced a little while in C_{air}, it still was at the same level, despite the continuous supply of 0.04% of CO_2 in air. $C_{1\%}$ displayed an increase in 50% of DIC in *f*/2, *f* and 2*f* cultivation, while in 4*f*, there are changes with a higher percentage, i.e. a further 100%.

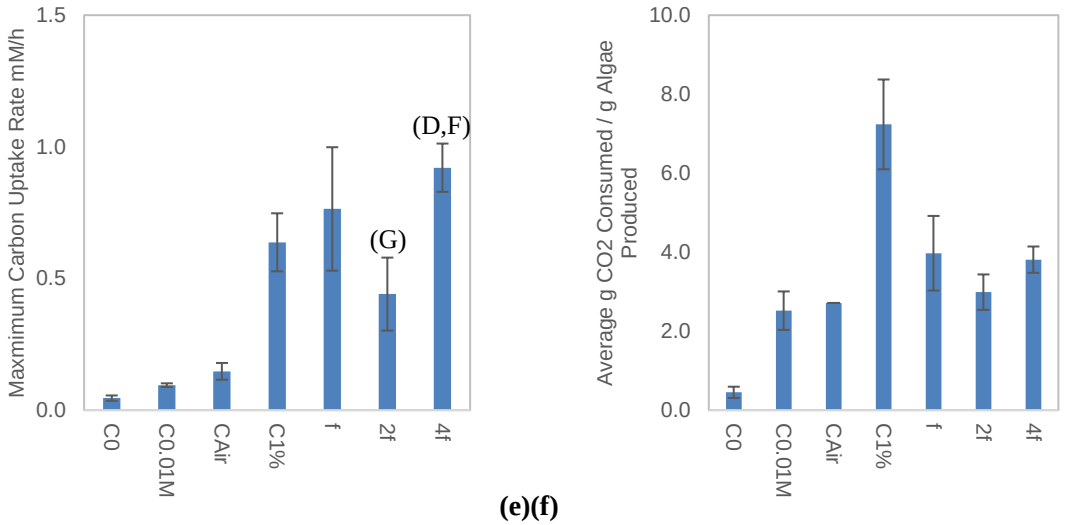
The biggest negative change in DIC was observed in D4 for C_{air} culture and D3 for $C_{1\%}$ culture. A negative change indicates that there is more demand for inorganic carbon by the algae than the supply. This situation agrees with the determination of carbon uptake, which shows that the maximum carbon uptake rate happened at D3. On the contrary, the biggest positive change in DIC was observed in D7 for C_{air} culture and D8 for $C_{1\%}$. A positive change indicates that there is more supply of inorganic carbon than the demand by the algae. In other words, the microalgae consume a lesser amount of carbon. Thus, the media was able to contain more inorganic carbon for the algae to consume. This situation might also indicate that the algae consume a lesser amount of carbon. To say that this DIC change is the effect of added nutrients is not necessarily right, since (1) chemically nutrient does not contribute to the change in DIC in blank media, as described in Chapter 3 and (2) added nutrients supposed to increase the microalgae activity and thus the carbon consumption; instead the amount of DIC in the media increases.



(a)(b)

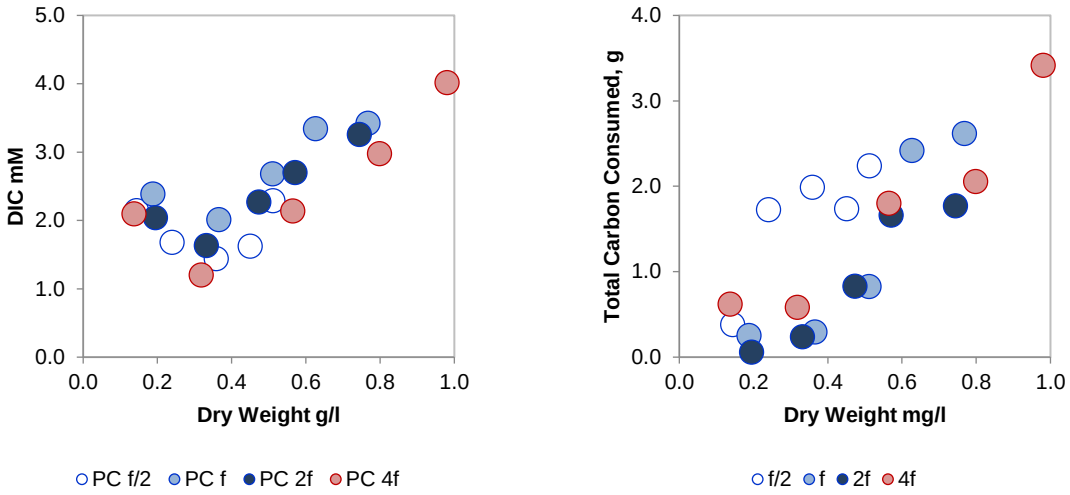


(c)(d)



(e)(f)

Figure 21 continued on next page.



(g)(h)

Figure 21 (a) Change in DIC concentration in each cultivation with respect to time. (b) Relative change in DIC of D1 to D0, in which minus reading indicates a decrease in DIC compared to D0. (c) Relative change in final day DIC to Baseline. Reading of -1 indicates full usage of DIC, while a reading above 0 indicates that more DIC are trapped in the media. (d) Change in pH in each cultivation with respect to time. (e) Comparison of maximum carbon uptake in each culture. (f) Average sequestration capacity, i.e. CO_2 consumed per microalgae production. (g) Relationship between DIC availability in media and (h) total carbon being consumed with its dry weight. Values in (a)-(f) are means (*n*=3), and error bars correspond to the standard error about the mean. For maximum carbon uptake rate (Figure (e)), an analysis was carried out to estimate the significance of differences between different levels of nutrients for each species, showing significant difference when compared to (D) *f*/2 (E) *f* (F) 2*f* (G) 4*f*.

In Figure 21c, the DIC concentration in the final day is compared to their respective baseline, which is taken as the initial DIC supply for C_0 and $C_{0.01M}$ cultivation. We can see that all DIC in C_0 and $C_{0.01M}$ have been used up, while in C_{air}, the DIC stops at its baseline. Meanwhile, in $C_{1\%}$, DIC was actively being consumed, in which DIC is still available, but under its supposed baseline level. DIC available in *f* and 2*f* cultures is higher than its baseline by 30%, while in 4*f*, it is higher by 50%.

Cultivation with an increased amount of nutrients shows an increase in the DIC availability in the media (Figure 21g), which also means that there is an increased amount of carbon being sequestered into the culture. It creates a situation in which microalgae can make space for more DIC to diffuse into the medium. However, it only happened around D5 (Figure 21a). Figure 21h shows the relationship between total carbon consumed and the total dry weight. We can see that as the amount of biomass increases, the total carbon consumption also increases.

P. purpureum is known to utilize bicarbonate as in $C_{0.01M}$ as the source of inorganic carbon for photosynthesis (Colman & Gehl, 1983). Carbonic anhydrase works by interconverting HCO_3^- into CO_2 intracellularly (Gaignard et al., 2019). Dixon, Patel, & Merrett (1987) found that in C_{air}, intracellular carbonic anhydrase increases the steady-state flux of CO_2. Phosphorous

availability enables synthesis and regeneration of substrates in the Calvin-Benson cycle (Figure 1), thus increasing the photosynthetic efficiency (Pires, 2015), as described in Section 2.2 before. This later translated into more consumption of carbon to allow photosynthesis to happen.

Figure 21e shows the maximum carbon uptake recorded by each culture. C_0 exhibited the least maximum carbon uptake rate, i.e. less than 0.1 mM / h, followed by $C_{0.01M}$ and $C_{air,}$ i.e. 0.1 mM / h. The $C_{1\%}$ culture exhibited a higher degree of maximum carbon uptake rate. The highest carbon uptake rate is by $C_{1\%}$ culture, i.e. 0.6 mM / h, followed by $C_{air,}$ i.e. 0.5 mM / h. Meanwhile, the increase of nutrient level did not give significant change towards the value of maximum carbon uptake. For C_0 culture, maximum carbon uptake rate happened at D1, as seen in Figure 22a, while for $C_{0.01M}$, it is at D4. For C_{air} and $C_{1\%}$, maximum carbon uptake happened at D3.

Figure 21f represents average sequestration capacity of each culture. Maximum value, i.e. 7 g of CO_2 consumption per biomass produced, was recorded by $C_{1\%}$ culture (*f*/2). Commonly reported value in literature is 2 g CO_2 per g biomass produced (Chisti, 2007); however, the value in this experiment is expected to be higher, since it considers sequestration by both media and the microalgae. It can also be seen that as the concentration of nutrient supply increases, it does not increase the amount of CO_2 sequestration. It appears that *f*/2 represents the ideal medium for maximum CO_2 sequestration.

6.3.2 pH

Figure 21d shows changes in pH for all cultures with respect to time. All cultures begin at an average value of pH 8.2. In C_0, the pH went slightly down until D2, before going up to pH 9.0. This pattern was also observed in $C_{0.01M}$ culture. In C_{air} culture, the pH stays around 8.5. In all $C_{1\%}$ culture, pH is reduced to a neutral condition of pH around 7. In this pH region, the proportion of CO_2 out of TCO_2 is higher compared to the alkaline region.

Alkaline pH in C_0, $C_{0.01M}$ and C_{air} culture might be due to the activity of carbonic anhydrase in the absence of supplemental CO_2 (Seyed Hosseini, Shang, & Scott, 2018). Colman & Gehl (1983) found that the rate of photosynthesis for *Porphyridium cruentum* was optimal at pH 7.5. A review by Gaignard et al. (2019) reported that *Porphyridium* is usually cultivated in pH

between 7 and 8 with a maximum CO_2 supply concentration of 4%. There are also reports on growth in 0.5 – 3% CO_2 supply (Soanen et al., 2015; Kumar et al., 2010). This explains the favourable growth in all cultures supplied with 1% CO_2. These reports may indicate the tolerance level of *Porphyridium* towards low pH and high concentration of CO_2. Our experimental data support that once the 5% CO_2 is being supplied to the culture, the pH was reduced to 6.0 – 6.5, while the culture produced lower biomass and slower growth rate.

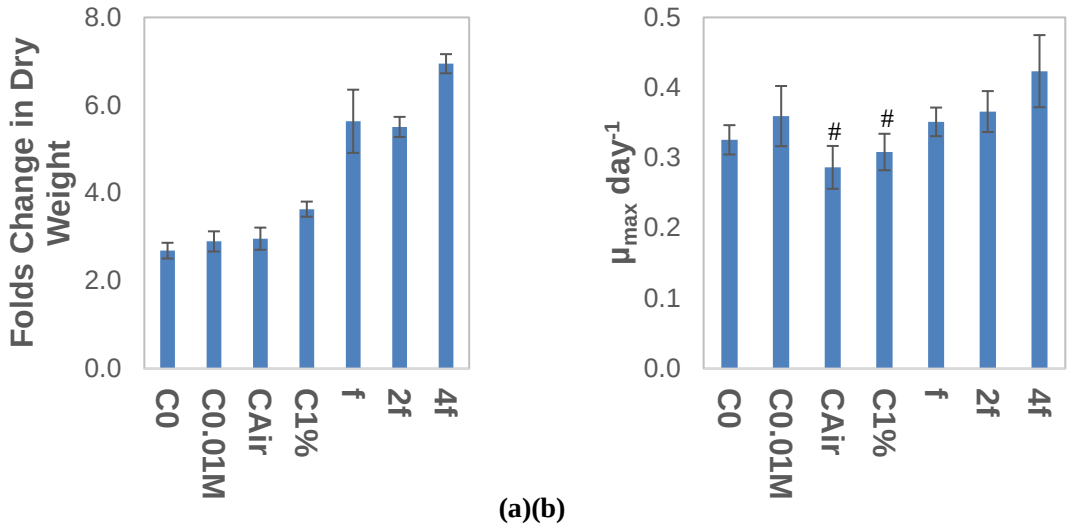
6.3.3 Microalgae Growth

Figure 22a represents the folds change in the dry weight of each culture. C_0 gives the lowest value along with $C_{0.01M}$ and C_{air}. $C_{1\%}$ rises steadily, producing the highest biomass reading among the first four sets of cultivations of different carbon treatments, approximately 0.8 g. Upon the increase of nutrient supply, the biomass increases drastically, showing a 100% increase compared to C_0. There was no increase in biomass when nutrient was increased to 2*f*, however as the nutrient further increase to 4*f*, there is a slight increase by 20%.

The highest maximum growth rate is observed in the 4*f* culture, as shown in Figure 22b. However, this value is not significantly different from most of the other cultures. The μ_{max} values are between 0.3 – 0.5 day^{-1}. In the *f* cultivation by Sánchez-Saavedra et al. (2018), they recorded a growth rate of 0.55, which is slightly higher than this report. Their more alkaline pH may contribute this.

6.3.4 Nutrient Uptake

Figure 23a shows nitrate removal percentage in each culture. In C_0 culture, the algae appear not to consume the nitrate. On D9, only 10% of the initial dissolved inorganic nitrogen was removed by the algae in this culture. In $C_{0.01M}$ and C_{air}, 30% of DIN was removed. In $C_{0.01M}$ culture, nitrogen was consumed slowly from the beginning. In C_{air} culture, the nitrate was consumed slower than in $C_{0.01M}$; however, its active uptake begins at D7. In $C_{1\%}$ culture, 60% of DIN were removed. Rapid uptake is observed beginning D4. The highest percentage of DIN removal is in 2*f* culture (80% = 1.4 mM). However, the culture of 2*f* and 4*f* only recorded around 20% of nitrogen removal (0.7 and 1.4 mM respectively).



(a)(b)

Figure 22 Growth of *Porphyridium purpureum*. (a) Folds change in dry weight between final and the first day of cultivation. (b) Maximum specific growth rate recorded by each culture. Values are means (*n*=3), and error bars correspond to the standard error about the mean. For the maximum specific growth rate (Figure (b)), an analysis was carried out to estimate the significance of differences between different treatments for each species, in which # indicates significant difference when compared to the maximum.

This reduction in the percentage of nitrate uptake may be explained by its high concentration since Tischner (2000) reported that if nitrate is supplied in high concentrations, it may hinder its net uptake by the microalgae. As the nitrate uptake matches the growth rate, the uptake was inhibited due to reduced demand for nitrate during continuous growth. This inhibition at high nitrate concentrations could not be displayed by Monod or Michaelis-Menten model; instead, the Haldane-like model is more suitable (Kasiri, Ulrich, & Prasad, 2015).

Figure 23c represents nitrate uptake rate of each culture. Highest maximum nitrate uptake rate was observed in 4*f* culture, i.e. 1 mM/d. This value is far from other cultures by more than three folds. However, this maximum uptake is not far from literature, since there is also a report on 0.7 – 1.0 mM of nitrate consumption per day (Adda, Merchuk, & Malis, 1986). It may be explained by its high available concentration during inoculation, i.e. 7 mM, thus enabling high consumption in a short time. Cultures of $C_{1\%}$ and $C_{0.01M}$ removed approximately 0.15 mM/d nitrate at maximum, while cultures of C_0 and C_{air} removed approximately 0.10 mM/d. Comparing the average nitrate consumption rate between C_{air} and $C_{1\%}$, the latter is faster by two folds.

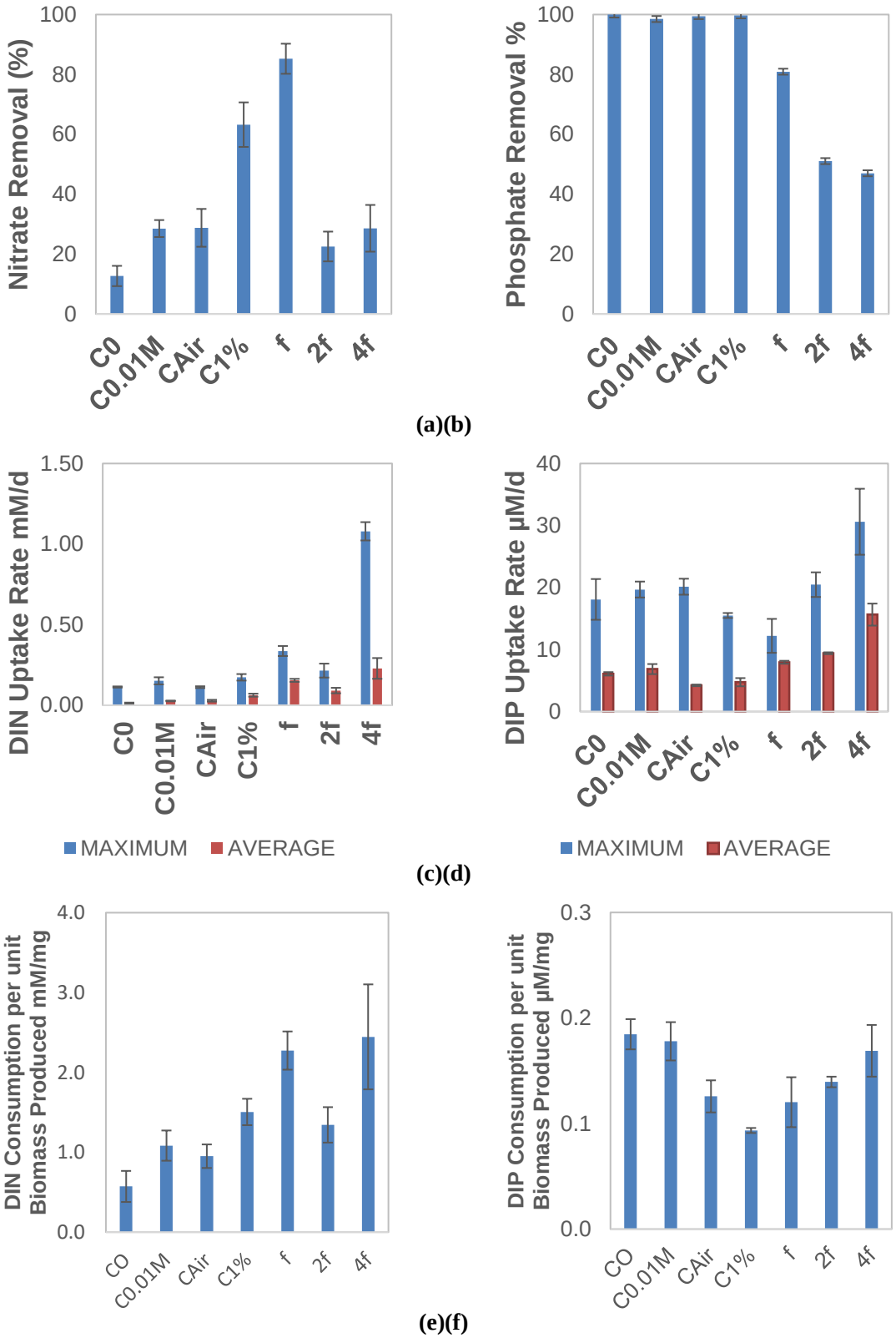


Figure 23 Nutrient profile in each culture. (a) Percentage of nitrate and (b) phosphate being removed in each culture. The uptake rate of (c) nitrate and (d) phosphate in each culture, reported by their maximum and average reading. Amount of (e) DIN and (f) DIP consumed per biomass produced. Values are means (*n*=3), and error bars correspond to the standard error about the mean.

Figure 23b illustrates phosphate removal percentage in each culture. 40 µM of phosphate seems just sufficient as it is being removed entirely in all *f/2* culture. C_0 and $C_{0.01M}$ culture experienced phosphate limitation at D6, while with C_{air} and $C_{1\%}$ this was at D8. Once the phosphate increased twice, i.e. *f* culture, the removal percentage decreases to 80%. This, however, is still higher than *f*/2 in terms of the amount, i.e. 0.06 mM. This removal of 0.06 mM in 10 days is similar to cultivation by Fuentes-Grünewald et al. (2015). In 2*f* and 4*f* culture, the removal percentage later decreases to 40%.

Meanwhile, Figure 23d indicates phosphate uptake rate in each culture. Similar to nitrate removal, the fastest phosphate removal rate is recorded by culture supplied with 4*f*, i.e. 30 µM/d. The removal rates in other cultures are between 10 – 20 mM/d.

Figure 23e and Figure 23f reported the nitrate and phosphate consumption per unit biomass produced respectively. C_0 gave the lowest nitrate consumption per biomass production, i.e. 0.6 mM/mg, while the highest reading was recorded by both *f* and 4*f* culture, i.e. 2 mM/mg. By supplying an excessive carbon to *Porphyridium* culture, the consumption per biomass production increases. In terms of phosphate consumption per biomass production, their readings are in the small range between 0.1 and 0.2 µM/mg.

6.3.5 Biochemical Composition

Figure 24a shows the proportion of biochemical components of *P. purpureum* at D9, at which time the microalgae cultures are all in stationary phase. C_0 culture contains approximately 30% carbohydrate, 50% protein and 20% lipid. Carbohydrate content in C_0 stays around 0.05 g/l while protein content in C_0 stays around 0.2 g/l, similar in $C_{0.01M}$ culture (data not reported).

By supplying the initial amount of 0.01M bicarbonate, both carbohydrate and lipid composition stays at 20%, while protein percentage increases by 10%. Both aeration with air and 1% CO_2 seems to favour carbohydrate while reducing protein percentage, i.e. 60% carbohydrate, 30% protein and 10% lipid.

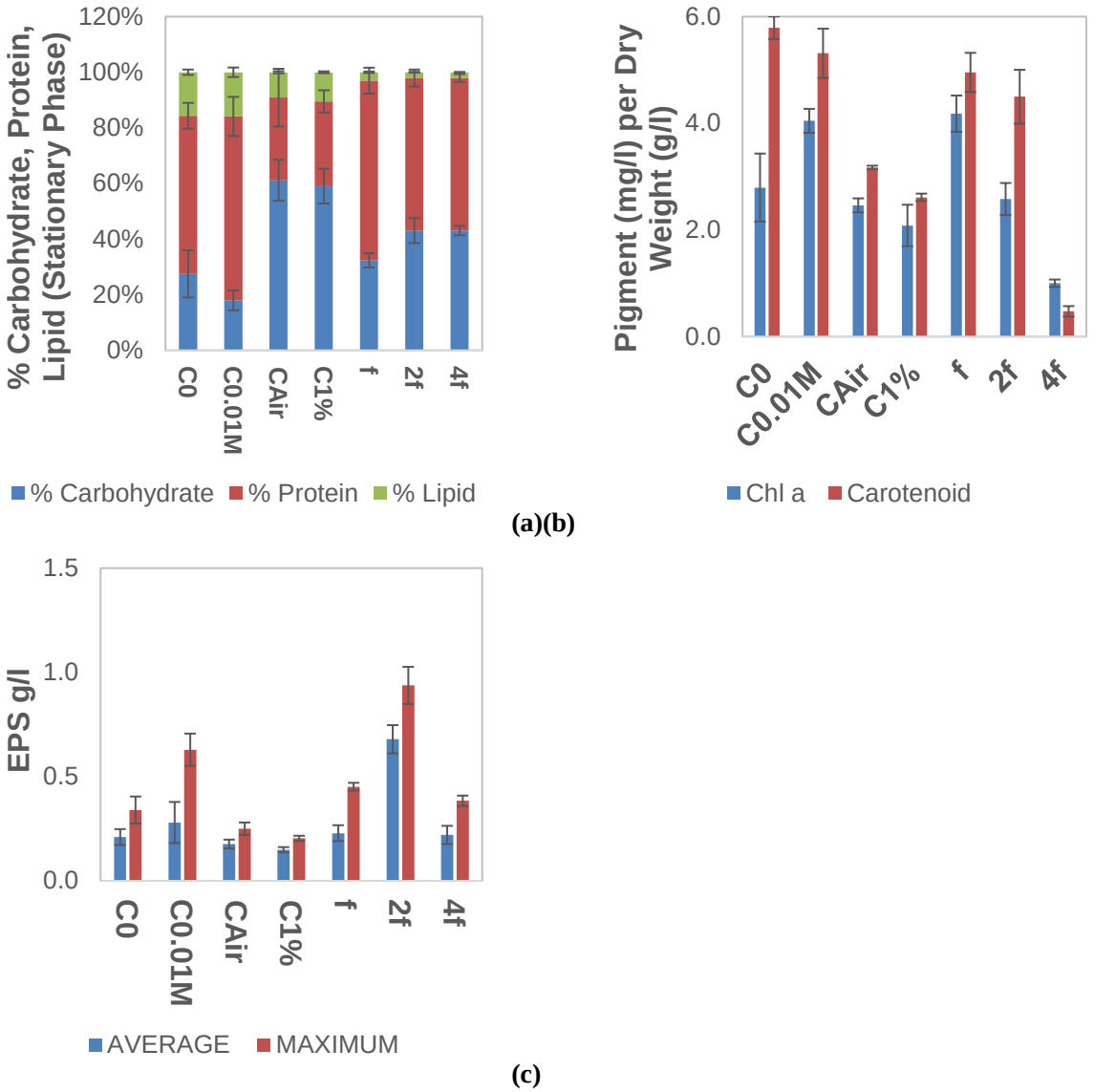


Figure 24 (a) Proportion of biochemical components of *Porphyridium purpureum* at its stationary phase. (b) Chlorophyll a and Carotenoid concentration on the final day of cultivation, normalized to their dry weight (c) Exopolysaccharide concentration assayed from cultivation media. Values are means (*n*=3), and error bars correspond to the standard error about the mean.

In both cultures, carbohydrate content increases to 0.2 g/l concentration at the end of cultivation (data not reported). This concentration and percentage are nearly similar to research by Razaghi, Godhe, & Albers (2014). Meanwhile, cultivation by Assunção, Varejão, & Santos (2017) obtained 0.30 g/l carbohydrate at D21 of cultivation, which represented 30% of its dry weight content.

The highest recorded lipid content is by both C_0 and $C_{0.01M}$ cultures, which is around 0.1 g / l at D5 before it decreased to 0.05 g / l towards the end of the experiment. For C_{air} and $C_{1\%}$ cultures, lipid content was recorded at 0.05 g/l throughout the experiment. A high concentration

of lipid is expected as inorganic carbon supply is an effective lipid accumulation trigger (Gardner et al., 2012).

By increasing the nutrients to *f*, 2*f* and 4*f*, we can see that carbohydrate stays at values between 30 – 40%, and lipids are nearly 0%, however the protein percentage increases to 60 – 70%. This high protein value is equivalent to 0.4 – 0.5 g/l. Similar protein levels (0.3 – 0.5 g/l protein) can be seen in other reports using nitrate values as used in this experiment (Li et al., 2019; Adda, Merchuk, & Malis, 1986). Readings from this experiment also support the fact that nitrogen deficiency reduces the production of primary metabolism compounds such as protein (Costa et al., 2018).

Comparing the percentages of the components, research by Matos et al. (2016) found that dried biomass of *P. cruentum* at the beginning of stationary growth phase in *f/2* media, exposed to CO_2 contained 85% dry matter. Dry matter can be further classified into 15% ash and 20% fibre, while the remaining 55% belongs to carbohydrates (30%), protein (60%) and lipid (10%). Meanwhile, Spolaore et al. (2006) reported a higher carbohydrate percentage in a general composition of *P. cruentum*, i.e. 40 – 55% carbohydrate, 30 – 40% protein and 10 – 15% lipid. Coward et al. (2016) discovered that for *P. cruentum* aerated with air in *f/2* for ten days, with 60 uE / m2 RGB light, the lipid stays at 15%, carbohydrate increased to 30% from 20% while protein decreases to 20% from 25%. Thus, values obtained in this experiment is not far from the literature data for C_0, $C_{0.01M}$ and C_{air}, while the $C_{1\%}$ culture contained more carbohydrate and protein.

Figure 24b represents the concentrations of pigments (chlorophyll and carotenoids) at D9, which have been normalized to their dry weight. For $C_{1\%}$ culture, the normalized value is similar to that reported by Coward et al. (2016), in which both recorded 2 mg/l chlorophyll per g/l dry weight. The *f* culture reported the highest pigment concentration, i.e. 4, while it was 3 and 1 in 2*f* and 4*f* cultures, respectively.

Not many works of literature are found reporting total carotenoids concentration specifically by *P. purpureum*, except by Fuentes-Grünewald et al. (2015), who reported 0.2 µg carotenoids per mg of biomass, which is lower compared to readings in this report, i.e. 0.5 – 6.0 µg / mg.

Chlorophyll is the most common pigment used by microorganisms for light absorption while carotenoids complement the energy absorbance by utilizing the centre of the light spectrum (Drosg, 2015; Yen et al., 2013). It seems that the concentrations are not entirely following the growth curves. Among the cultures with different carbon treatments, the decrease in the pigment production might be associated with the decrease in pH (Figure 21d), which were also found in other reports before (Chen, Xu, & Vaidyanathan, 2020; Khalil et al., 2010). Furthermore, bicarbonate addition is known to contribute a significant positive effect on cellular levels of pigments (White et al., 2013). When the level of the nutrients was increased, the pigments also increased, since nitrogen is among the requirements for pigment synthesis (Chen et al., 2015). The increase in pigments production peaked at the *f* cultivation before it drops significantly in 4*f* cultivation. Extra formation of carotenoids, as observed in Figure 24b, was reported as a protection for the cells against photo-oxidative damage of the chlorophyll (Raso et al., 2012).

6.3.6 Exopolysaccharide Content

It can be seen that the cultivation of *P. purpureum* in *f*/2 with low light allows the production of both IPS and EPS. Figure 24c represents the average and maximum EPS concentrations in each culture. Highest maximum reading was recorded by 2*f* culture, which is at 0.9 g/l. Other cultures showed similar average concentration at around 0.25 g/l, although their concentration dynamics are different. For example, $C_{0.01M}$ culture showed the highest amount of EPS the day after the experiment started. However, it decreased to 0.1 g/l towards the end of the experiment. C_0 culture shows an increase of EPS content from 0.1 to 0.3 g/l, before a decrease to 0.2 at the end of the experiment. For *f/2* $C_{1\%}$, EPS contents stay around 2 g/l. Meanwhile, average 2*f* readings are also the highest, i.e. 0.7 g/l. This shows that 2*f* is the optimum formula to produce a high concentration of EPS in *Porphyridium* culture.

These values are not far from several literature reports which record 0.7 g/l EPS (Assunção, Varejão, & Santos, 2017; Villay et al., 2013). However, there are also literature data that recorded production of EPS of about 1 - 4 g/l (Coward et al., 2016; Fuentes-Grünewald et al., 2015; Soanen et al., 2015), where they applied semi-continuous cultivation and low light as their production method. There are also reports on the increasing concentration of bicarbonate, i.e. to 0.02M, which yields EPS production of 1.3 g/l (Velea, Ilie, & Filipescu, 2011).

6.4 Summary and Conclusion

P. purpureum culture showed the highest carbon uptake rate in 4*f*, which is around 1 mM/h. This cultivation also showed the highest level of DIC saturation compared to others. Calculation considering biomass production reveals carbon sequestration of 7 g CO_2 per g algae produced as the highest. *P. purpureum* grew slowly with μ_{max} around 0.35 day^{-1} but was able to produce a six-fold biomass concentration in 10 days. Highest nitrate removal recorded was only 80% by *f* culture, while phosphate is removed entirely in all cultures except those with nutrient increase. Both highest nitrate and phosphate uptake were recorded by 4*f* culture. Carbohydrate concentration is highest in culture with continuous aeration. Increasing nutrient does not change the carbohydrate proportion; instead, it increases the protein percentage of its dry weight. Lipid is highest in culture without carbon supply. Normalized pigment concentrations to their dry weights showed high value for culture in high pH and high nutrient value (peaked at *f*), while exopolysaccharide was secreted the highest in 2*f* culture.

CHAPTER 7: Incorporation of Microalgae *Porphyridium purpureum* and Its Components for Bioplastics Production: Production and Mechanical Characterization

Abstract

This chapter aims to study characteristics of bioplastics and see whether incorporation of microalgae *P. purpureum* or its components as base polymer will give prospects in developing alternatives for current conventional plastics. Changes were made to the base polymer to identify one that can give reliable properties as a biofilm. Later additives such as hydrophobicity enhancer and crosslinking agent were tested to obtain improvement in biofilm properties. The effect of glycerol as a plasticizer was also studied. Finally, factors that gave the best properties to biofilm were combined to see whether the combination will give a biofilm with better characteristics.

It is found that between corn starch and alginate, the latter gave better mechanical properties and got better as its weight percentage was increased to 5%. Incorporation of whole microalgae containing different carbohydrate weight percentage (polysaccharides) and at different percentages of polymer resulted in no significant difference between sets of data. However, by extracting the polysaccharide from microalgae and incorporating it alone, the mechanical properties improved positively. Adding oil improves hydrophobicity of the algae; however, it reduced the mechanical properties of the biofilm. Besides, the addition of citric acid as crosslinking agent gave increased tensile strength and elasticity of the biofilm; however, sodium borate (commonly used for the purpose) does not do the job as well as the former agent. Increasing the glycerol percentage increases the mechanical properties except for elongation at break. Optimising the microalgae incorporation found that the combination of previous factors does not mean improvement towards biofilm properties. Based on these testing, incorporation of 1% exopolysaccharides of *P. purpureum*, 5% alginate and 1% glycerol emerged as the best option thus far (of the options tested) for producing biofilms made, in terms of elongation at break, tensile strength, elasticity and moisture content.

7.1 Introduction

Among the advantages of microalgae is its potential for operating as a biorefinery (Laurens et al., 2017). This concept in which they can produce different types of value-added chemicals is attractive. One of the products are polysaccharides which can be used as bioplastics precursors.

Plastics, apart from its base polymer (such as polysaccharides, polyethene and polylactic acid), are also made up of additives to improve performance or reduce costs. For example, plasticizers give mouldable properties and lower the processing temperature, while compatibilizers may be added to improve the mixing of plastics components (Gao, Pollet, & Avérous, 2017; Azapagic, Emsley, & Hamerton, 2003).

Polysaccharides such as starch, cellulose and alginates have been known as building basics for many biopolymers applications such as food nutrients, feed productions and materials (Persin et al., 2011). They can be originated from many sources such as plants, animals and microbes (Mano et al., 2007). It is proposed that since polysaccharides have been successfully incorporated as biopolymers, then a similar case for polysaccharides from microalgae can be observed.

Among the polysaccharides base polymers, alginate has gained attention due to its excellent film-forming properties (Gao, Pollet, & Avérous, 2017). These polysaccharides, which are extracted from different species of brown algae, have been developed to different application such as in food, biomedical and printing industry. In terms of molecular structure, alginate $(C_6H_8O_6)n$) is a rectilinear polymer composed of b-D-mannuronic acid and α-L-guluronic acid and also considered as polyuronide having tetrahydropyran ring (Kamal et al., 2017). Alginate is a supreme material for chemical modifications (such as oxidation, sulfation and esterification) since it has a large number of free carboxyl and hydroxyl groups (Figure 30a), which allow for chemical modifications such as crosslinking.

Several routes have been identified for the microalgae to be made as bioplastics (Section 2.4). The next step after identifying the biological source of polysaccharides for polymer production is to find an appropriate strategy to extract and polymerize the bioplastics precursors that the algae produce to produce the biomaterials. At least there are four methods to characterize algae-

based materials, i.e. optical properties, thermal properties, mechanical properties and structural properties (Freile-Pelegrín & Madera-Santana, 2017).

Thermoplastic starch (TPS) is a type of plastics that can be related to microalgae since the latter can produce the starch during their growth. Studies into properties of starch reveal that this macromolecule is made by the combination of amylose and amylopectin. In terms of the structure, amylose are linear polysaccharides linked by α-1,4 glycosidic linkages while amylopectin is branched polysaccharides linked by α-1,6 glycosidic linkages (Orsuwan & Sothornvit, 2018)

The combination of amylose and amylopectin is different across starch resources. In most plants, amylose is the less abundant form compared to amylopectin (Bhagavan & Ha, 2011). For example corn starch contains 28% amylose, wheat starch contains 25% amylose, while rice contains starch of amylose percentage range 7 – 32% (Fabra et al., 2018; Basiak, Lenart, & Debeaufort, 2017; Zavareze et al., 2010).

Basiak, Lenart, & Debeaufort (2017) found that the amylose/amylopectin ratio in starch films contributes significantly towards their physical and chemical properties, and thus whether they are useful as specific components or not. In this case, a slight change in percentage results in a change to the mechanical properties, even by 50 folds. Their research, however, does not involve microalgae as the producer of the base polymer.

There are also researches on macroalgae to make bioplastics film such as by Chiellini et al. (2008) who used *Ulva* at different temperature and composite percentage. Apart from visual, thermal and mechanical characterization, they also buried the film under the soil to measure films ability to degrade. They found that *Ulva* can be used as filler for the production of composite materials with poly(vinyl alcohol) (PVA). Its thermal stability is compatible for melt processing with PVA, while its degradation could be represented by a first-order reaction. Besides, the addition of glycerol was found to allow the composites to be flexible, while its mechanical properties are promising for the production of tools in the packaging and agricultural sector.

Some reports exist on polysaccharides by algae such as by Shi, Wideman, & Wang (2012) who applied *Nannochloropsis* and *Spirulina* of the different starch percentage to make bioplastics

by extrusion. In this research, they incorporated microalgae into a non-biodegradable polymer, i.e. polyolefin before dry blending starch and algae, including surfactant first, and the mixture was subjected to melt-extrusion. Zeller et al. (2013) studied the effect of pressure, temperature, the content of plasticizer, and processing time towards thermoplastics blends from *Spirulina* and *Chlorella*. They used 100% algae-based bioplastics and the thermoplastic blends of microalgae, polyethene and glycerol. Fabra et al. (2018) studied the incorporation of *Nannochloropsis*, *Spirulina* and *Scenedesmus* to corn starch biocomposites. They found that when the microalgae were added, the water vapour permeability and the mechanical properties of the biocomposites dropped by 54%. When *Nannochloropsis* were added, it reduced the matrix rigidity and improved the oxygen permeability, while when Spirulina or Scenedesmus were added, the oxygen permeability and the mechanical properties were not improved.

Porphyridium is among the microalgae that can produce polysaccharides as an energy storage mechanism. However, as it does not belong to the family of green algae, but red algae, i.e. Rhodophyta, it produces a different type of starch known as floridean starch, which is made up of mostly amylopectin (Yu et al., 2002). Structure of the floridean starch granule is similar to plant starch but more variable in size and shape (Al Abdallah, Nixon, & Fortwendel, 2016).

At the same time, *Porphyridium* also excretes exopolysaccharides to the growth medium. These polysaccharides are usually made of xylose, glucose, galactose and other minor types of monosaccharides (Patel et al., 2013). A review by Xiao & Zheng (2016) reported that the presence of both L- and D-forms of galactose alongside with aldobiuronic acid 3-O-(α-D-glucopyranosyluronic acid)-L-galactopyranose appears to be a characteristic common to the exopolysaccharides of red algae.

This chapter aims to study mechanical characteristics of bioplastics and see whether incorporation of microalgae *P. purpureum* will give prospects for developing alternatives to current conventional plastics. The investigation focuses on the utilization of bioplastics precursors extracted. Several factors have been identified to affect characteristics of microalgae-based biofilms, based on the ingredients of bioplastics, i.e. base polymer and additives. The plastics produced will be tested later for its maximum elongation, tensile strength, elasticity with comparison to conventional plastics. Hydrophobicity and moisture content are also among essential bioplastics properties to assess. The suitability of bioplastics produced can be analysed and compared to the standards.

7.2 Experimental Method

Materials

Corn starch (27% amylose), sodium alginate and glycerol ≥ 99% used in this experiment are all supplied by Sigma Aldrich. The lecithin used for lipid formulation was supplied by Alfa Aesar. The coconut oil (organic virgin) and olive oil (extra virgin) used were commercially available from Sainsbury's.

Plastics Films Preparation

Plastics were prepared based on modified methods by Fabra et al. (2018). The required amount of base polymers, glycerol (density 1.25 g / ml) and other components of plastics was weighed out and poured into the test tube in the water bath, which was preheated to 50 °C to help the mixing. The ratio of the base polymers and additives used will be detailed in each section. Water was added to make up the final volume of 15 ml. The stirring was done using a spatula until the mixture was evenly mixed. This mixture was later poured into silicon moulds for drying and settling. The samples were put in 25 ˚C incubator for two days to dry.

Microalgae Incorporation

Microalgae *Porphyridium purpureum* CCAP 1380/1A cultures were centrifuged at 4500 rpm for 15 minutes. The supernatant was discarded, while the pellets were washed with PBS buffer. It was later frozen overnight before being freeze-dried. The freeze-dried microalgae samples were then weighed before being mixed into the plastic solution required, which were pre-heated in the water bath at 85 ˚C.

Polysaccharide Extraction

500 ml of cultures were centrifuged at 4500 rpm for 15 minutes before being subjected to polysaccharides extraction as outlined by Assunção, Varejão, & Santos (2017). Briefly, the supernatant was precipitated with ethanol for exopolysaccharide extraction. The mixture was sieved so that the exopolysaccharide could be collected, dried at 60 °C and stored before being incorporated to the plastics, using similar procedures as whole microalgae above. For intra-polysaccharides, the samples underwent multiple treatments, e.g. methanol-acetone treatment in an ultrasonic bath, alkaline treatment and vacuum filtration. The polysaccharides were later dried and stored before being incorporated into the bioplastics (Refer methods on Plastics Preparation above).

Carbohydrate Content Measurement

Carbohydrate content was varied by changing the nutrient and carbon supply towards the microalgae. The content was later measured spectrophotometrically based on the method by Chen & Vaidyanathan (2013). Before the assay, the samples were frozen at -20 °C for storage before analysis for carbohydrate. The details have been explained previously in Chapter 3.

Oils Incorporation

Oils, also known as lipids, were incorporated according to proportion relative to the total amount of mixtures. Two types of oils were tested, i.e. coconut oil and olive oil. For ensuring even mixing between oils and bioplastics mixture, 1wt% lecithin was added as an emulsifier.

Crosslinking the Films

Two crosslinking agents were used to improve the strength of bioplastics, i.e. citric acid and sodium borate. Their proportion was measured relative to the amount of base polymer used. The crosslinking reaction was carried out similar to methods by Reddy & Yang (2010) and Seligra et al. (2016), which was confirmed by FTIR, swelling and ^{1}H NMR analysis. Briefly, the mixture of plastics components was added with the crosslinking agent and stirred at room temperature until it formed an even mixture. It was later heated to 75 - 85 $^{\circ}$C in a water bath for 30 minutes before being cooled. The solution was later stirred again for 3 minutes before being poured into an empty mould to dry, similar to the drying process of the plastics samples above.

Polymers Mechanical Characterization

Film characteristics were assessed using Teslen 5KN and Zwick Proline Z020 tensile testing machine. All measurements and ratios were kept the same for all samples tested. Testings were done according to International Standards for Testing ISO 527-3:2018, which specifies the conditions for determining the tensile properties of plastics films less than 1 mm thick (The British Standards Institution, 2018). Good value for each parameter for a plastics will be different for different applications. Several values were reported in Table 3 and discussed.

Elongation at break, measured as a percentage, is the maximum elongation of the film before breaking. This parameter, which indicates the stretching ability of the bioplastics, is obtained by dividing the length before the break with the initial length before getting its percentage.

Tensile strength, which is measured in MPa, refers to the maximum stress that the film able to withstand before breaking. It is calculated by dividing the maximum tensile force (N) by the cross-sectional area of the film (m^2).

Young's Modulus represents the stiffness or the elasticity of the film, which also represents the ability of the plastics to return to its initial position after being stretched. It is obtained based on the slope of the stress-strain graph. It is measured in the unit of MPa.

Hydrophobicity Test

Hydrophobicity of the film may be observed based on the water dropping test (Gourson et al., 1999). As it is commonly perceived that the film will break upon the introduction of water (thus the reason of not using the contact angle method); therefore a simple test was applied to detect whether improvement on the hydrophobicity of the plastics could be made. For doing that, a drop of water was placed on the film to see its behaviour. The interpretation of the test is based upon whether the film will break in 10 minutes, keeping all other parameters (volume of water, sample size, the distance where the water was dropped and others) constant. If the water does not break the film within the duration, the hydrophobicity of the film can be considered as improved. This test method is not quantitative; instead, more of a detection method.

Moisture Content Measurement

The moisture content of the films was measured according to British Standard for determining water content by drying in a ventilated oven (The British Standards Institution, 2008). It measures the total water, which can be from the film surface and also from water accessible pores. In brief, the sample was weighed before being dried in an oven at 110 °C for 24 hours. Successive weighings are used to determine the constant mass of the dried portion. The determination of water content is based on the difference in the wet sample and dried sample and expressed as a percentage of the dried sample.

Plastics Film Appearance

Morphological analysis was done by using Hitachi TM3030 Hitachi TM3030Plus Tabletop Scanning Electron Microscope (SEM), located in The Diamond, University of Sheffield, to observe film homogeneity and surface appearance. This equipment has a charge-up reduction feature, which allows for no sample preparation; thus, there is no need for specimen coating

before imaging. Before the imaging procedure, the films need to be dried overnight. The images were recorded at 100 and 1000 times magnification using a 5 kV accelerating voltage. At 5kV, the electron beam does not penetrate far into the sample; thus, the images show more surface detail in excellent resolution. Several images at different parts of the sample were taken for each set to select a representative one, i.e. the most similar to all of the others.

Statistics

Each experiment was repeated a minimum of three times to ensure reliability. The only statistically significant difference will be reported. Test for statistical significance was performed at 95% significance level with the help of "Data Analysis" in Microsoft Excel.

7.3 Results and Discussion

7.3.1 Effects of Type of Base Polymers

Before this experiment, experiments were done using a different type of starches such as wheat and rice, however, when they are mixed with microalgae *Porphyridium*, they formed a brittle and non-uniform film, which were difficult to characterise. This may be due to their high sensitivity to water (Peelman et al., 2013).

In opposite, sodium alginate by 2% and 5% incorporation and corn starch by 5% incorporation are two base polymers that can mix well with this algae biomass. Figure 25a shows changes in elongation at break in each film, with reference to films made of 2 wt% alginate. An increase in this parameter was observed when 1% of algae being incorporated into the film only, i.e. by 20%. Further incorporation reduces the parameters by around 50%.

Figure 25b indicates that the increase in tensile strength can be observed when more percentage of base polymers were added, i.e. to 5 wt%. Change of polymer base to corn starch also increases the tensile strength; however, when microalgae were incorporated, the tensile strength decreased.

Figure 25c compares changes in the elasticity (Young's modulus) of the film. The highest increase was observed when 5% of microalgae is being incorporated in corn starch film. It seems that 5% is a good percentage as an increase in elasticity is also observed in both percentages of alginate.

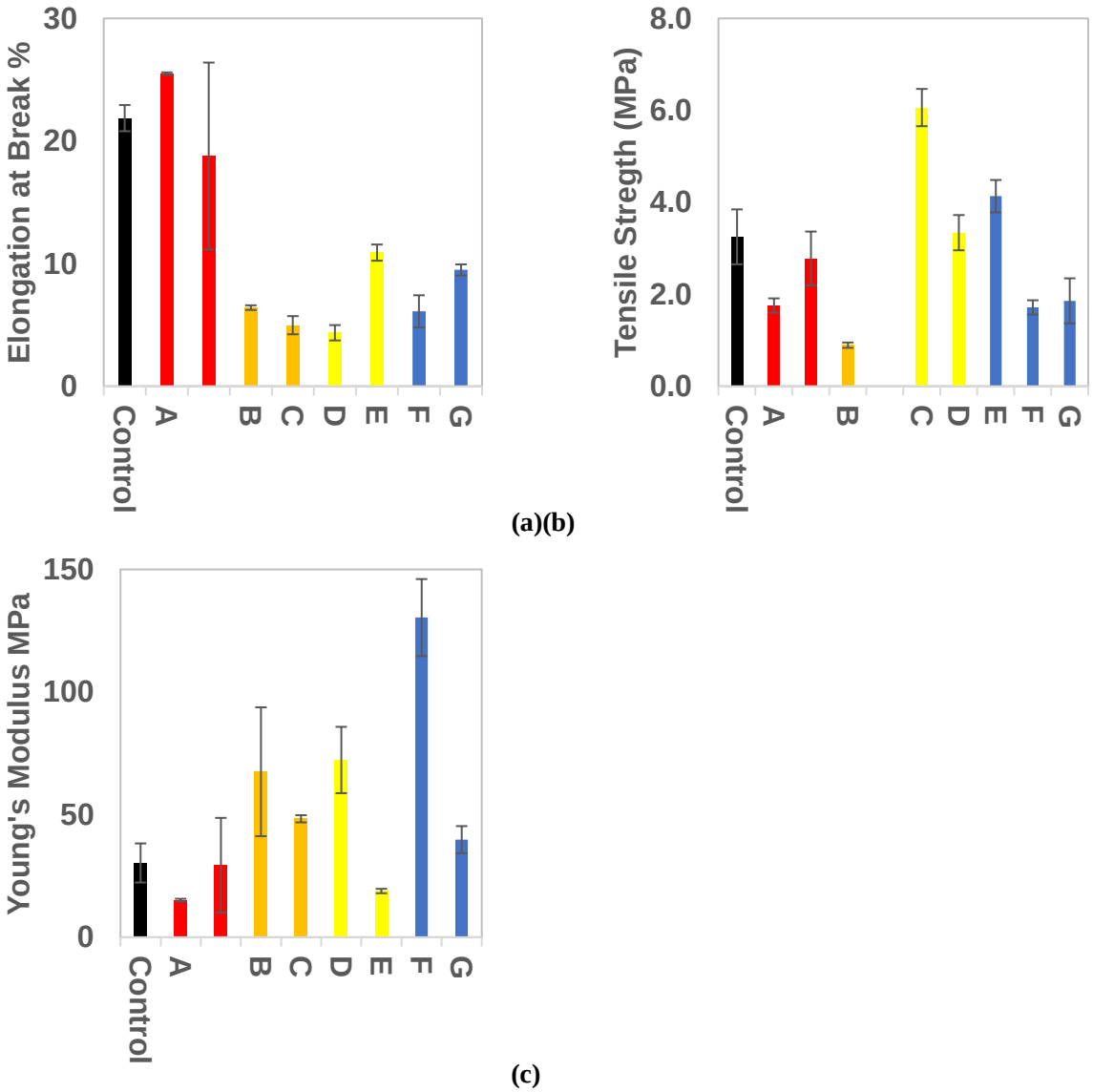


Figure 25 Mechanical properties of films made from different base polymer (alginate and corn starch) and microalgae concentration (0, 20 and 50%): (a) Elongation at Break. (b) Tensile strength. (c) Elasticity modulus. Control: 2% alginate, 0% algae. A: 2% alginate, 1% algae. B: 2% alginate, 2% algae. C: 5% alginate 0% algae. D: 5% alginate 1% algae. E: 5% corn starch 0% algae. F: 5% corn starch 1% algae. Values are means (*n*=3), and error bars correspond to the standard error about the mean.

Initially, we can see that only corn starch and alginate were able to make good films with *P. purpureum* bioplastics films, while the use of wheat and rice starch does not yield favourable results. This difference shows us that different types of starch can have different chemical properties and capabilities to blend with the microalgae. In literature, this observation is usually explained by the difference in the amylose/amylopectin ratio in both polysaccharides (Basiak, Lenart, & Debeaufort, 2017; Luchese, Spada, & Tessaro, 2017). They found that a slight change to the amylose-amylopectin ratio will result in a significant change in the mechanical properties of the film. This is because the resulted change in the microstructure affects the viscosity of the film-forming suspensions, and thus affecting the retraction of the network

during the film drying and also the final thickness. They also noted that the higher the amylopectin content is, the greater the mechanical properties are.

Comparing properties of 2% and 5% alginate as a base film, we can see that there is an increase in tensile strength and elasticity modulus when more alginate is being used. This is supported by Luchese, Spada, & Tessaro (2017), who found that an increase in the starch content promoted an increase in these two parameters. However, in almost all cases, the elongation at break decreases, thus we can say that incorporation of microalgae will affect this parameter negatively. This situation is also the main concern raised in other reports as a contributor to minimising competitiveness against synthetic polymers (Cazón et al., 2017).

7.3.2 Effects of Carbohydrate and Its Percentage

The changes in the mechanical properties of the biofilms were assessed by considering two parameters (1) weight percentage of incorporation (2) percentage of carbohydrate in the biomass. The carbohydrates here referred to the intrapolysaccharides of the *Porphyridium*, which are floridean starches, which is made up of mostly amylopectin.

Figure 26a describes the changes in the elongation at break. We can see that by increasing the percentage of carbohydrate in the biomass incorporated to the film, differences among them are not significant. In terms of the tensile strength (Figure 26b) and elasticity (Figure 26c), almost all the samples show a decrease in performance compared to the controls.

Furthermore, the hydrophobicity test carried out found that the water drop breaks all the films, indicating that the introduction of carbohydrate does not improve their hydrophobicity. Time-wise, as the percentage amount of algae being incorporated increases, the time that would be taken for the water to break the films shortens.

In other words, changing the percentage or increasing the incorporation amount of carbohydrate does not bring positive impact towards bioplastics mechanical properties.

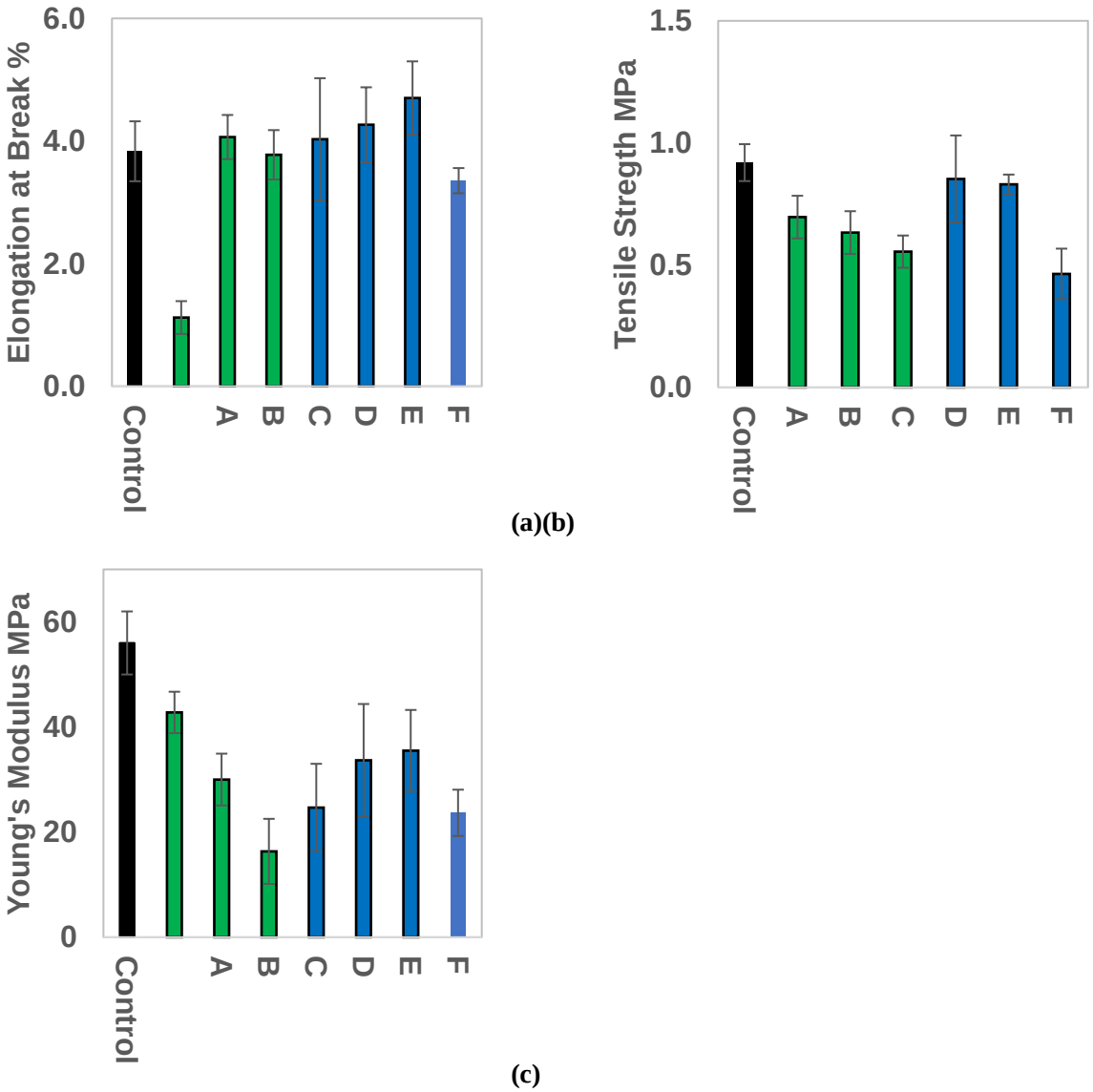
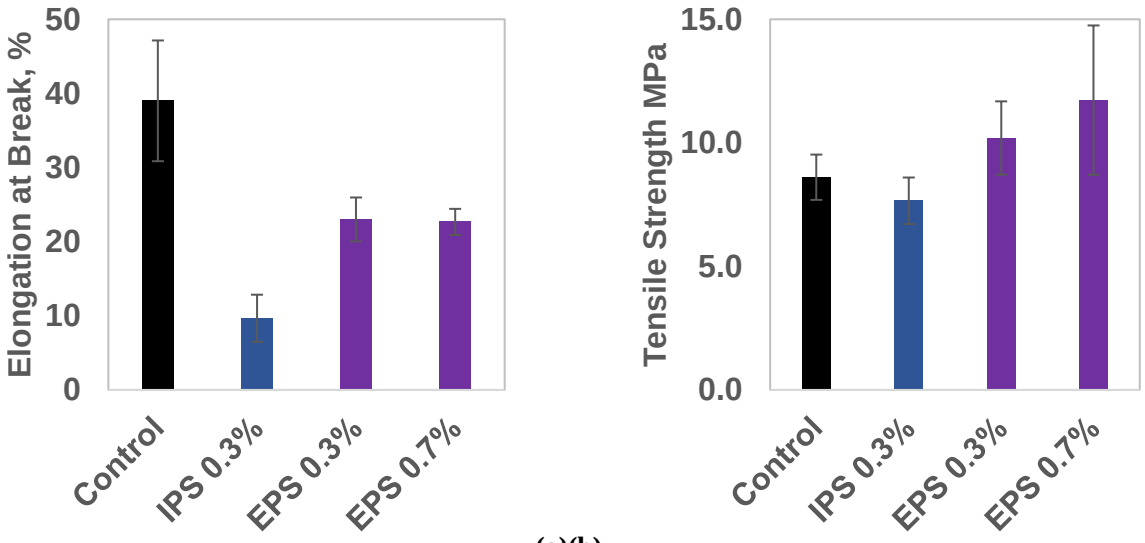


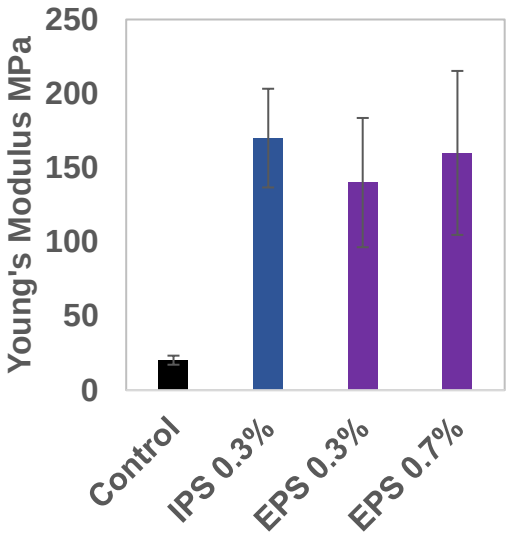
Figure 26 Mechanical properties of the films made from different amount of microalgae of different concentration of polysaccharides: (a) Elongation at Break. (b) Tensile strength. (c) Elasticity modulus. Control: 2% alginate 0% algae. A: 0.1% algae of 15wt% carb. B: 0.2% algae of 15wt% carb. C: 0.5% algae of 15wt%. D: 0.1% algae of 30wt% Carb. E: 0.2% algae of 30wt% carb F: 0.5% algae of 30wt% carb. Values are means (*n*=3), and error bars correspond to the standard error about the mean.

7.3.3 Incorporation of Exopolysaccharides and Intracellular Polysaccharides

Figure 27 reported the changes to film properties in terms of the elongation at break and the tensile strength of the films compared to the control when the polysaccharides were specifically extracted from the microalgae for the incorporation to the films, instead of using the whole dried biomass. To summarize, the use of EPS gives films slightly more elongation properties and tensile strength compared to IPS. Elasticity (Young's) modulus is not significantly different.



(a)(b)



(c)

Figure 27 Mechanical properties of the film made of different types of polysaccharides (a) Elongation at the break. (b) tensile strength. (b) Elasticity modulus. Control: 5% alginate 0% algae. Values are means (*n*=3), and error bars correspond to the standard error about the mean.

Once the polysaccharides were incorporated, the elongation at break reduces by more than 75% in IPS film. This reduction decreases when IPS are replaced by EPS of the same amount. A similar reduction was observed when the number of EPS was doubled. In terms of tensile strength, incorporation of IPS nearly reduces this parameter; however, there is an increase in tensile strength when EPS is being used. Similar to elongation at break, increasing the amount does not change the tensile strength of the EPS film. Figure 3c reports the change in the elasticity (Young's modulus) of the film. It shows that there are increases in the elasticity of the films incorporated with extracted polysaccharides, compared to the film without the algae. However, among the three treatments, differences among them are not statistically significant.

The morphology of the films incorporated with the microalgae polysaccharides was studied by the use of scanning electron microscopy (SEM) imaging. The images provide observation and information on the surface topography and composition of the film after the polysaccharides incorporation and how the particles interact with the base components of the polymer (Reis et al., 2015). Comparing images of Figure 28a with Figure 28c and Figure 28e, we can say that the IPS film can be easily distinguished from EPS. IPS were seen as small particles under 1000× magnification (Figure 4b) while EPS looks like a rougher and bigger structure than IPS (Figure 28d and Figure 28f). Particles in the film made by IPS is more homogenously distributed compared to EPS. In other words, the images have shown more inclusion of IPS into the plastics, compared to EPS. Figure 28g and Figure 28h are the images of conventional commercial plastics under magnification, which shows a smoother and more homogenous structure.

7.3.4 Effects of Oils as Additives

The intention here is to address oil incorporation to microalgae polysaccharide film. Plastics made from polysaccharides usually have the problem of breaking down under wet condition. To solve that, Sutherland (2009) suggested coating the plastics with a hydrophobic agent. In these experiments, oils were added to give hydrophobicity properties to the biofilms. Two types of oils were examined, which showed different degree of saturation, i.e. coconut oil has 90.5% saturated fat, while olive oil has 85.4% unsaturated fat.

Figure 29 shows the mechanical properties of films after incorporation of coconut and olive oil as hydrophobic agents. The three mechanical properties did not increase. Plastics of 10% coconut oil is unsuitable for testing by the mechanical test machine due to its relatively weak structure. However, hydrophobicity test carried out to the four samples found that only films with 10% incorporation of oil (i.e. coconut and olive oil) successfully passed the test, i.e. water does not break the film in 5 minutes of contact time. Thus, by incorporating the oils, only the hydrophobicity index of the film increases, but not the mechanical. Use of olive oil for a long term usage is always negatively perceived due to its rancidity (Ghanbari Shendi et al., 2018).

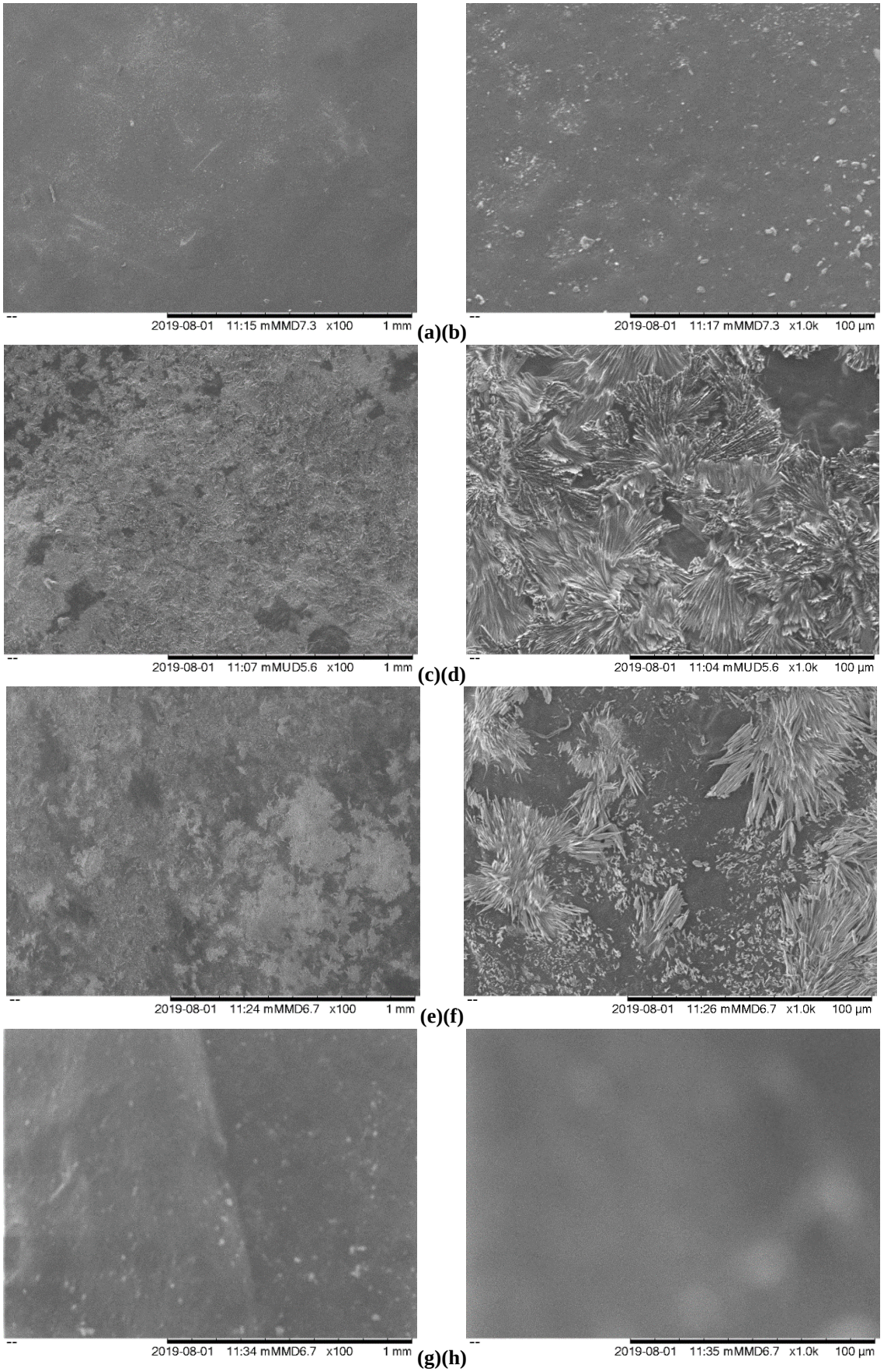


Figure 28 Representative SEM images of films incorporated with (a) 0.3% intrapolysaccharides, (c) 0.3% exopolysaccharides (e) 0.7% exopolysaccharides and (g) commercial plastics under 100× magnification. Image (b) (d) (f) (h) are similar image of their left counterparts, in 1000× magnification.

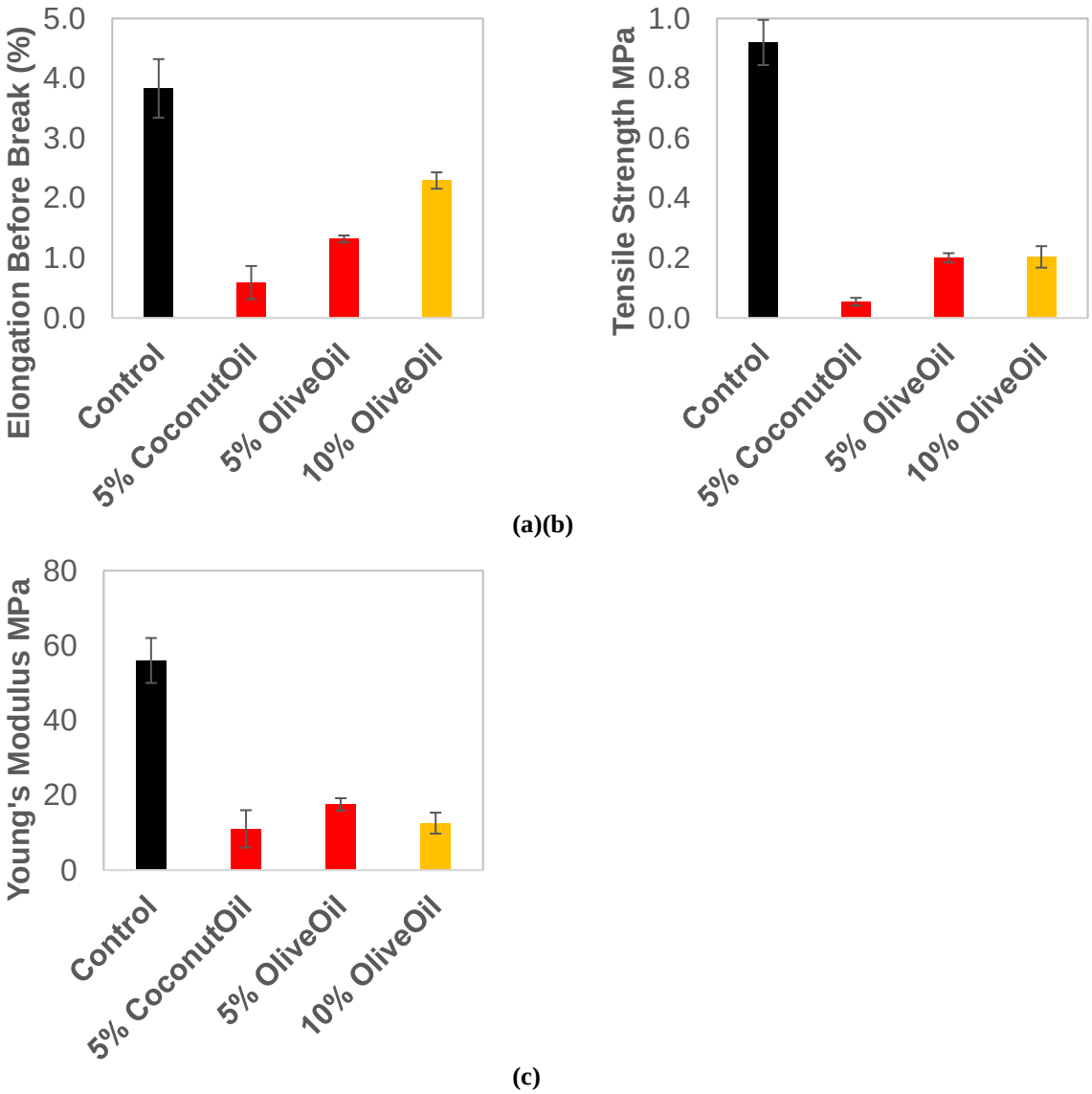


Figure 29 Mechanical properties of the film after incorporation of coconut and olive oil as a hydrophobic agent. Control: 2% alginate 0% algae 0% oil. Values are means (*n*=3), and error bars correspond to the standard error about the mean.

7.3.5 Effects of Citric Acid and Sodium Borate as Crosslinking Agent

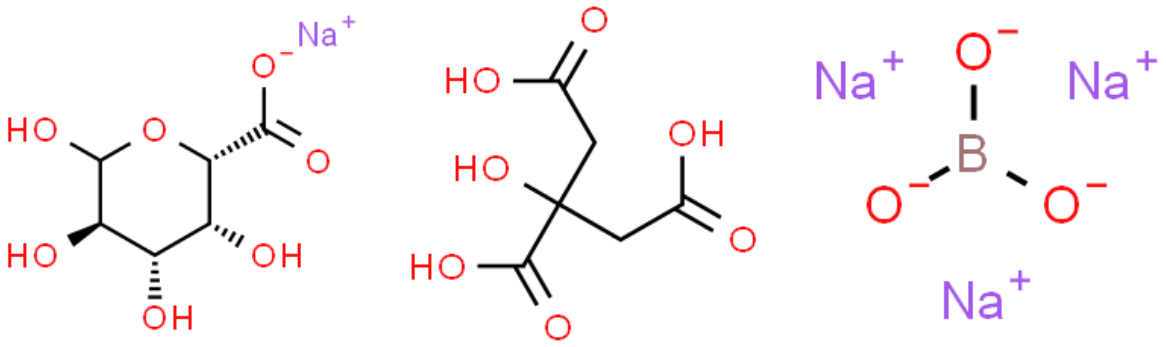
The possibility of crosslinking agent to be added to polysaccharide film as its additive is explored. In this context, crosslinking refers to the chemical linkage between two hydroxyl groups on neighbouring starch molecules, to have mechanical properties of higher value and to reduce the film sensitivities to water (Bastioli, 2005). The crosslinking agent has multifunctional groups to react with the hydroxyl groups of starch, thus increasing its hydrophobicity, and leading to new chemical bonds between molecular starch chains (Seligra et al., 2016). The cross-linking process involves the formation of covalent bonds between function groups and functional crosslinking agents (Efthimiadou, Metaxa, & Kordas, 2014), which is stronger than the former intermolecular forces that attract the polymer chains.

Two different cross-linking agents were tested, i.e. citric acid and sodium borate. Both of them has a multi-carboxylic structure (Figure 30b and Figure 30c) that enables them to be used as a starch crosslinking agent. This is done by strengthening the hydrogen bonding interactions with those of floridean starch. Similarly, borate ion (BO_3^{3-}) of sodium borate can be subjected to a condensation reaction with the hydroxyl groups on the floridean starch chains, to form crosslinking bridges of B-O-C (Lim et al., 2015).

Among the works of literature that used citric acid as a crosslinking agent for starch, they reported different ways of citric acid incorporation (Seligra et al., 2016). One of them is by mixing the citric acid with water, glycerol and starch before the gelatinization process, similar to the method applied in this experiment. It was found in several cases that when citric acid or sodium borate were added, they did not mix well with the film components, thus making the cross-linking procedures unable to proceed and not allowing them to be tested for mechanical characterization, so their value is not reported in the figure.

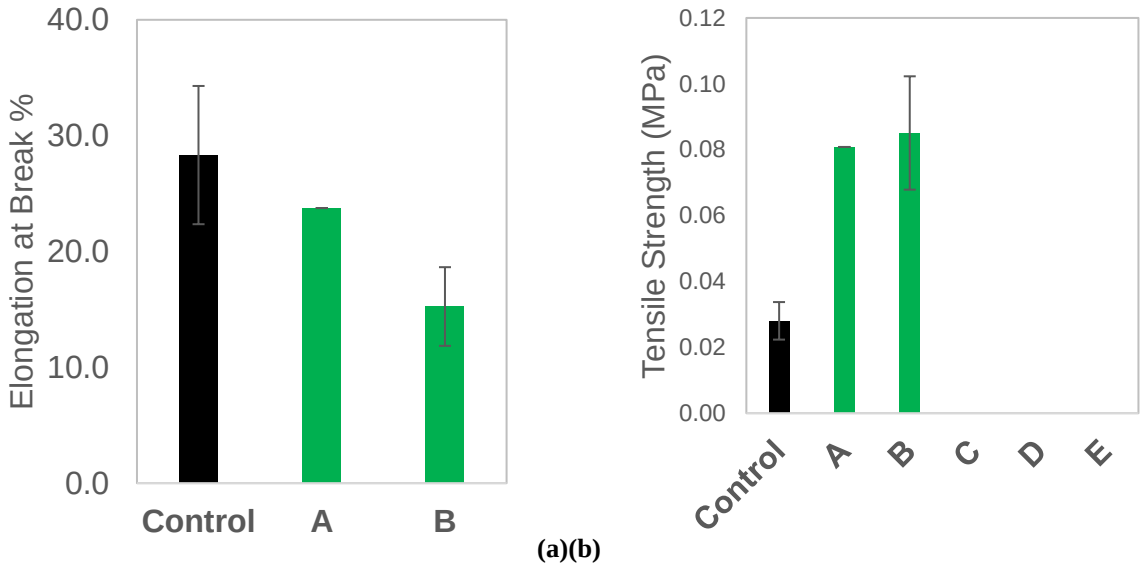
Figure 31 reports the characteristics of the film after incorporation of crosslinking agent. It is found that the introduction of citric acid as a crosslinking agent reduces the elongation at break, but gives improvement towards its tensile strength and Young's Modulus. By introducing sodium borate, the mechanical properties were not improved. Hydrophobicity test was done towards the film, and it was found that all of them passed, i.e. the water did not break the film during 5 minutes duration. An analysis of the moisture content shows that on average, 30% of the film made of the sodium borate is constituted by water.

In other words, incorporation of citric acid does add desired value towards better plastics properties, but this is not true with sodium borate. Although citric acid increases the hydrophobicity of the film, this property can also be obtained through incorporation of coconut oil (previous section), at a higher value. Possible reasons might contribute to this low improvement in mechanical properties such as crosslinking agent concentration or reaction temperature, which might also affect the degree of crosslinking. Thus in the future, it is advised that the products undergo structural analysis to confirm that the crosslinking has occurred at a high degree and the polymer structure has been enhanced.

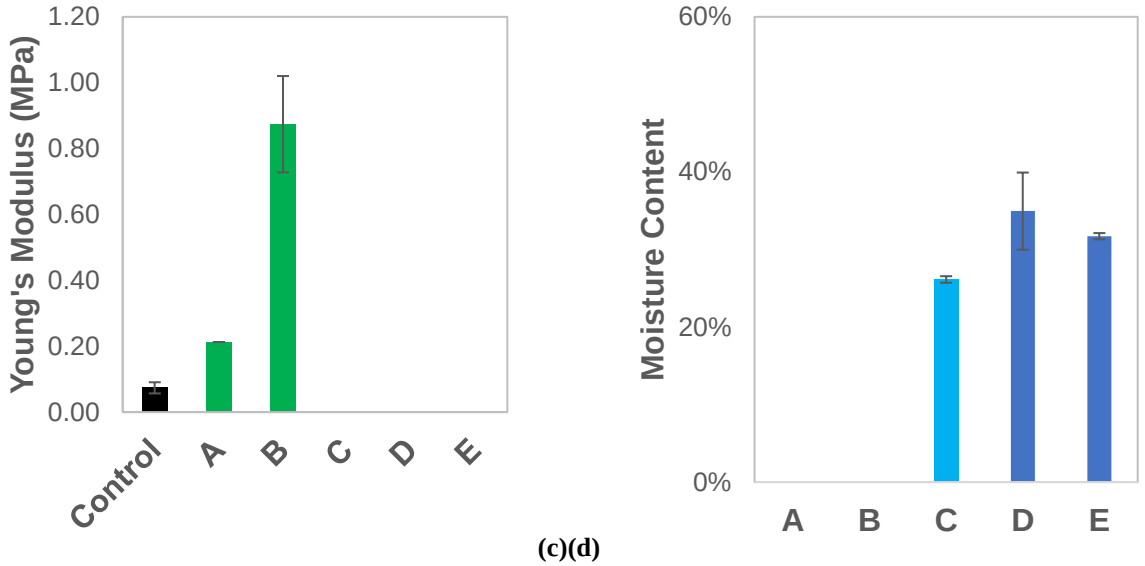


(a) **(b)** **(c)**

Figure 30 Structure of (a) sodium alginate (b) citric acid and (c) sodium borate showing their functional groups that can involve in the crosslinking process. Source of images: chemspider.com



(a)(b)



(c)(d)

Figure 31 Mechanical Properties of film after incorporation of different crosslinking agent at different concentration of glycerol. (a) Elongation at break (%). (b) Tensile Strength. (c) Control: 5% alginate 0% algae 0% crosslinking agent. A: 0.2% Citric Acid. B: 0.5% Citric Acid. C: 0.2% Sodium Borate. D: 0.5% Sodium Borate. E: 0.2% Citric Acid, 0.2% Sodium Borate. Values are means (*n*=3) and error bars correspond to the standard error about the mean.

7.3.6 Effects of Glycerol Concentration

Here, the effect of glycerol as the plasticizing agent of the film is studied. This is done by increasing the glycerol concentration while keeping the same ratio of alginate, glycerol and water. Initially, the plasticizer was added at a weight ratio of 0.3:1 glycerol:starch, according to Basiak, Lenart, & Debeaufort (2017). This ratio is later normalized to the total amount of ingredients to make the bioplastics film, which is equivalent to 1 wt%. Three values of percentage were chosen, i.e. 1%, 3% and 5%, in which each of them is incorporated with either 0.2% or 0.5% citric acid.

Figure 32 reports the mechanical properties of the film, together with the moisture content. It can be seen that the increase of glycerol reduced the elongation at break (%). A high percentage of glycerol gives a more reduction in the value of elongation at break. Despite that, the increase of glycerol increased the tensile strength, elasticity modulus and hydrophobicity too. The highest increase in tensile strength was recorded by the highest amount of glycerol, i.e. 5 wt%. Moisture content recorded a lesser amount (i.e. 20% on average) compared to when they are incorporated with cross-linking agent only. In terms of the change in the elasticity, there were significant increases recorded. As the glycerol and the citric acid percentage increased, Young's modulus value increases too, although the difference between them may not be significant enough. The increase of Young's modulus value with the increase of crosslinking degree can be related to the properties of an amorphous material, which shows elasticity when the crosslinking degree is not too high, based on the theory of rubber-like elasticity (Charlesby & Hancock, 1953). Furthermore, this increase might be contributed to the increase in the amount of plasticizer too. Hydrophobicity test was done towards the film, and it was found that all of them showed improvements, i.e. the water did not break the film during 5 minutes duration.

7.3.7 Selection of Optimum Factors

Previous subtopics have examined the effects of adjusting the components of polysaccharide film, i.e. by adjusting (1) base polymers and its polysaccharides content, (2) additives content, i.e. hydrophobicity and crosslinking agents and (3) glycerol content as the plasticizer.

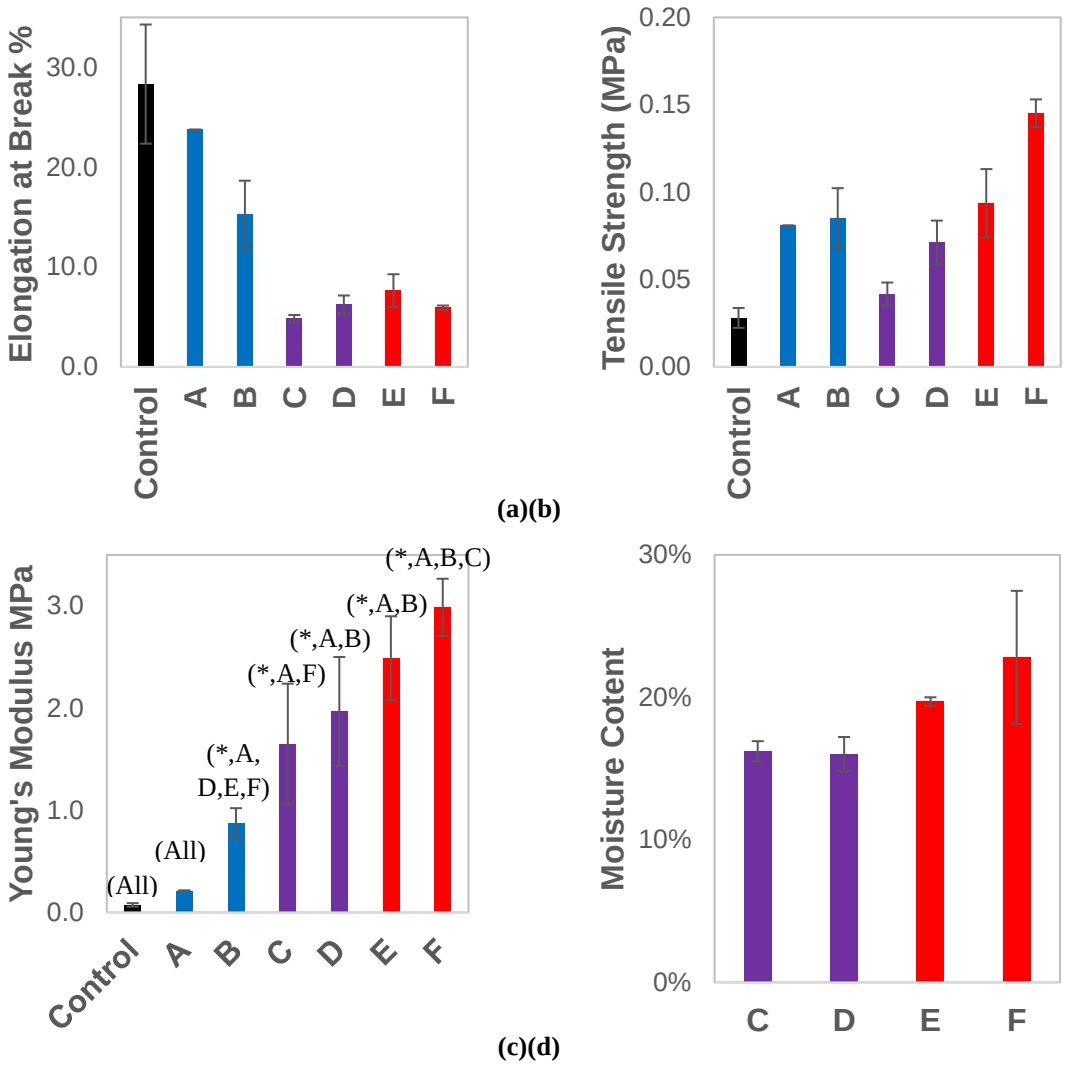


Figure 32 (a) Elongation at Break (b) Tensile Strength (c) Young's modulus (d) Moisture Content. Control: 5% alginate 0% algae 1% glycerol 0% crosslinking agent. A: 1% glycerol 0.2% citric acid. B: 1% glycerol 0.5% citric acid 0. C: 3% glycerol 0.2% citric acid. D: 3% glycerol 0.5% citric acid. E: 5% glycerol 0.2% citric acid. F: 5% glycerol 0.5% citric acid. Values are means (*n*=3), and error bars correspond to the standard error about the mean. For maximum carbon uptake rate (Figure (e)), an analysis was carried out to estimate the significance of differences between different levels of nutrients for each species, showing significant difference when compared to the respective film in which * denotes control.

Since testing on samples for additives and plasticizer variation in previous experiments were done without any incorporation of *P. purpureum*, it is best to use the best combination of base polymers to see how microalgae will interact with additives and plasticizers of the film. EPS at 0.7% was chosen as a base polymer for further film making. Olive oil was chosen as a hydrophobicity agent since it produces more stable films compared to coconut oil. Citric acid was chosen as a crosslinking agent for its better properties compared to sodium borate. Glycerol at high percentage was chosen since it results in better tensile strength and elasticity.

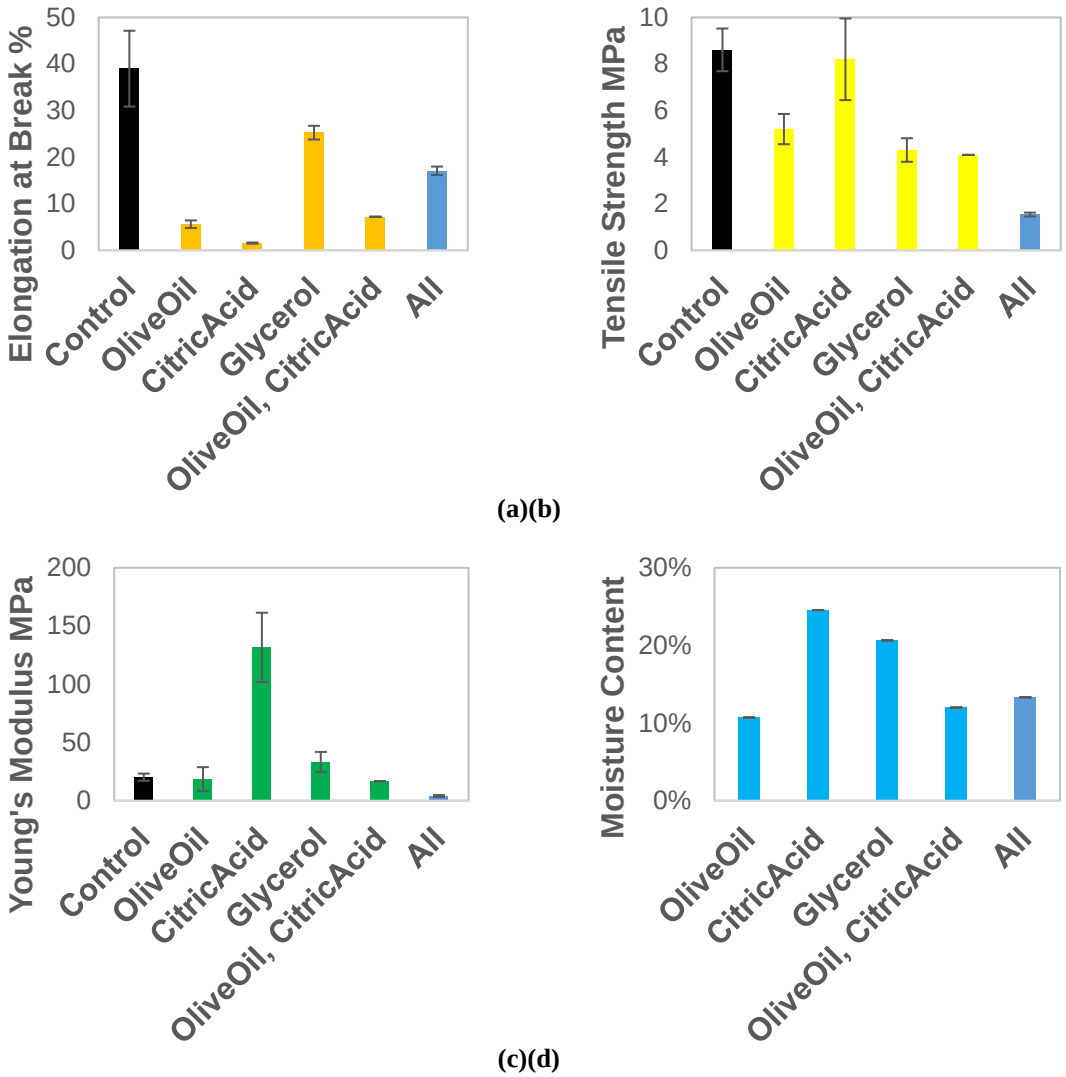


Figure 33 Bioplastics mechanical properties (a) elongation at break (b) tensile strength (c) Young's modulus and (d) moisture content after improvement towards its several components. Control i.e. 5% alginate 0% algae. Values are means (*n*=3), and error bars correspond to the standard error about the mean.

Figure 33 illustrates the effect of optimizing each selected components towards *P. purpureum* film properties. EPS at 0.7 wt% was chosen as a base polymer in this further research. Based on the graph, it can be said that EPS of *P. purpureum* does not blend well with the hydrophobicity agent, crosslinking agent and plasticizers. As expected, all the films showed a reduction in the elongation at break. A similar case was observed in the tensile strength, although there is no change in the citric acid treatment. The same treatment produced six-folds of increase in the elasticity modulus. High incorporation of glycerol increases the elasticity too. The increase of Young's modulus while not for the elongation at break indicates that there is an improvement in the elasticity region of the film. However, the mixing of all the treatments only reduces the elasticity of the film. In terms of the moisture content, there is a slight increase

in it by 10% in both sets with the olive oil treatment. In this case, the oil may contribute to this improvement.

Table 3 compares mechanical data from this study with data on conventional plastics from other reports. In terms of elongation at break, the values recorded in this study are the lowest. The case of lower elongation at break is observed in almost all samples examined in this study. Meanwhile, tensile strength recorded in this study can be said as average, which are also similar to LDPE values. In terms of elasticity (Young's Modulus value), control values are below average. However, as EPS is incorporated, the values are above those recorded by corn starch-based film (Luchese, Spada, & Tessaro, 2017), but still far below HDPE.

Table 3 Comparison of mechanical data from this study to works of literature. Average readings were taken for comparison.

	Elongation at Break %	Tensile Strength MPa	Young's Modulus MPa	References
Control	39	9	20	This study
EPS 0.7%	23	12	160	
Corn starch-based film	120	4	65	(Luchese, Spada, & Tessaro, 2017)
LDPE	99	4	71	
PVC	45	17	81	
LDPE	625	10	300	(Abolibda, 2015)
HDPE	590	26	1000	
LDPE Blends		10	144	(Tai, Li, & Ng, 2000)

When comparing with the current market, LDPE based film can be used in food packaging (Luchese, Spada, & Tessaro, 2017). Thus for the *P. purpureum* bioplastics to be more competitive, more studies are needed to improve its elongation at break and water barrier.

7.4 Summary and Conclusion

It is found that between corn starch and alginate, the latter gave better mechanical properties and got better as its weight percentage was increased to 5%. Incorporation of whole microalgae containing different carbohydrate weight percentage (polysaccharides) and at different

percentages of the polymer showed no significant difference between sets of data. However, by extracting the polysaccharides from microalgae and incorporating it alone, the mechanical properties improve positively. Adding oil improves the hydrophobicity of the algae; however, it reduced the mechanical properties of the biofilm. Besides, the addition of citric acid as a crosslinking agent increased tensile strength and elasticity of the biofilm; however, sodium borate does not do the job as well as the former agent. Increasing the glycerol percentage does increase the mechanical properties but not the elongation at break. Optimizing the microalgae incorporation showed that the combination of previous factors does not mean improvement towards biofilm properties. Based on these testing, incorporation of 1% exopolysaccharides of *P. purpureum*, 5% alginate and 1% glycerol emerged as the best biofilm made in terms of elongation at break, tensile strength, elasticity and moisture content.

CHAPTER 8: Conclusions and Future Works

8.1 Research Summary

This research aimed to assess carbon sequestration, nutrient uptake and biochemical production in the cultures of selected strains of microalgae with the hope that these microalgae can be a simultaneous solution towards the two environmental problems mentioned before. Here are several essential novel findings that have been reported in the chapters before.

1. This research has optimized the assessment of dissolved inorganic carbon (DIC) in media in terms of analysis volume and storage duration.
2. This research also described a novel method to quantify carbon uptake in the culture based on the assessment of DIC.
3. Assessment of different DIC availability in two cultures of *Chlorella vulgaris* strains found that their behaviours are different in terms of carbon uptake, growth, nutrient consumption and biochemical production.
4. The DIC saturation levels in both algae culture medium are higher than in blank medium, suggesting a greater carbon sequestration capacity of the culture medium in the presence of microalgae.
5. The carbon uptake rate does not necessarily increase with increasing nitrate and phosphate. Carbon consumption is best at limiting nitrogen and phosphate concentrations.
6. An increase of nutrients increases the growth up until a point, with no significant change beyond.
7. A similar treatment of red algae (*Porphyridium purpureum*) shows carbon uptake profiles of slightly lower value.
8. Carbohydrate concentration is highest in culture with continuous aeration. EPS is secreted the highest in 2f culture.
9. The best biofilm produced have similar mechanical properties to conventional low-density polyethene plastics, but with shorter elongation capability and less hydrophobicity.
10. For producing microalgae bioplastics of higher tensile strength and elasticity, it is better to use its polysaccharides per se, rather than incorporating them as whole cells.

These findings will be further discussed in the next section with the conclusion that we can benefit from this research.

8.2 Conclusion and Recommendations

8.2.1 Different Strains and Behaviours

Microalgae cultivation of three different strains have been examined in this research, i.e. *Chlorella vulgaris* CCAP 211/21A, *Chlorella vulgaris* BDUG 91771 and *Porphyridium purpureum* CCAP 1380/1A, which will be referred in this chapter as BDUG, CCAP and PP respectively. Both strains of *Chlorella* are of the same species which belongs to the group of Chlorophyta (green algae); however, they are isolated initially at different geographical locations, but from a similar habitat. *Porphyridium* belongs to group Rhodophyta (red algae) and is also from CCAP, UK. Their phylogenetic tree arrived by molecular typing has been illustrated in Chapter 3.

As can be seen from this work, even strains from the same species can be seen to display different behaviour. For example, in cultivation without aeration, pH in BDUG can reach up to 9.7; however, in CCAP, it is 9.0 and PP is 9.1. More observation in terms of their growth, carbon and nutrient consumption as well as biochemical production will be highlighted in the following sections.

8.2.2 DIC Availability

Available DIC in the media and culture was analysed based on the back titration method, which only considers carbonate alkalinity. Attempt to optimize the measurement by reducing the sample volume by half and observing the effect of storage at -20 C was shown to be effective in retaining DIC content. Reducing the volume allows for more sampling time points and less usage of the titrant. As for storage optimization, this will enable several samples to be taken for analysis that can be run in parallel or sampling at short time intervals. Data on this optimization has been reported in Chapter 4.

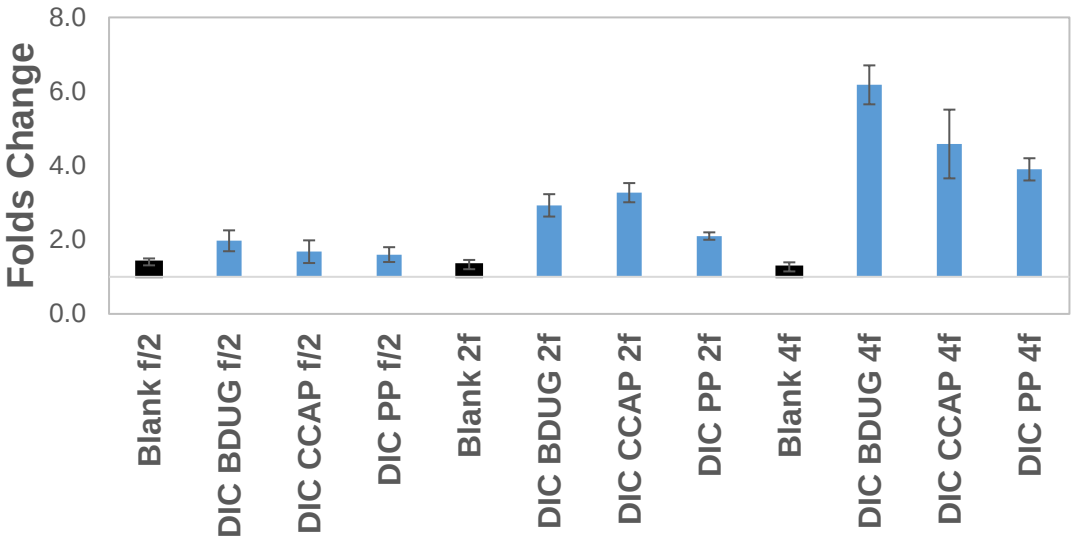
It has been established that cultivation media used in this research is similar to seawater in terms of initial available DIC. When supplied with CO_2 at 1% concentration, the DIC increases and becomes saturated and stabilised at values of around 2 mM, which is about a 30% increase compared to initial values (unsaturated medium). In a culture with a continuous supply of 1%CO_2, this value will be referred to as the baseline.

It has also been established that changes in DIC may be interpreted in two main perspectives, i.e. carbon sequestration by medium and also carbon incorporation into the microalgae. When the same supply of gas being channelled to microalgae cultivation, the amount of DIC increases above what being contained in the blank media that does not contain the microalgae (Figure 34a). This situation happened although all the cultures were supplied similarly with 1% CO_2. To the best of our knowledge, this relative comparison is a finding that has not been reported in the literature before.

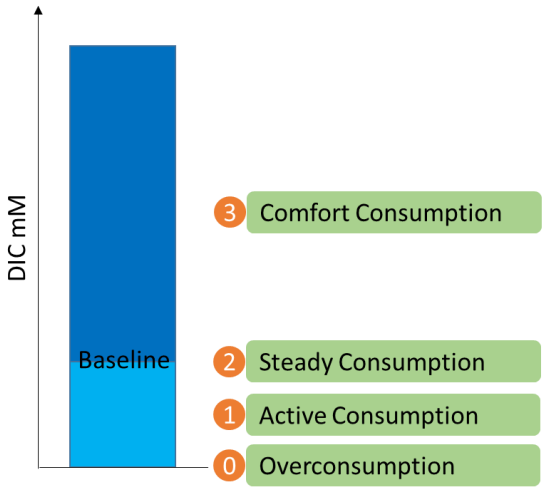
In cultivations using *f*/2 media, DIC in all three cultures was observed to increase by two folds while in 2*f*, it is by three folds in *C. vulgaris*, but only two folds in *P. purpureum* (slightly higher than in *f*/2). In 4*f* media, the increase is by six folds in BDUG, five folds in CCAP and four folds in PP. An increase in nutrient availability was translated to an increase in biomass concentration and also an increase in DIC available in the culture. It is also important to note that the fold increase in the graph is based on the reading at D9. Only BDUG and CCAP in *f*/2 appears to be saturated with respect to DIC at D2, while DIC in other cultures is still seen to be rising even at D9.

A relationship has been attempted between DIC availability and the microalgae biomass accumulated. An R^2 value of 0.88 is observed in *Chlorella* while in *Porphyridium*, it is 0.80, showing a strong correlation between these two variables. In other words, when the dry weight increases, DIC is also seen to increase and vice versa.

Since the DIC has risen above the baseline, the situation in which the microalgae is consuming the CO_2 can be conceptualised (Figure 34b). We described the consumption behaviour in this region as “Type 3” or comfort consumption. This is when the system has more DIC available for the microalgae to consume, more than what the media blank contains. In other words, the system has managed to create more availability for carbonate to dissolve into the media so that it is available for consumption by microalgae. At the same time, the microalgae consumption of CO_2 is not active enough for the level of CO_2 to go below the baseline.



(a)



(b)

Figure 34 (a) Change in DIC after 9 Days of Cultivation in 1%CO_2. Readings have been normalized to their initial DIC. (b) Attempt to describe DIC availability in culture with a continuous gas supply.

It should be noted that the increased dissolution is despite a decrease in medium pH, thus cannot be attributed to pH or components contributing to the pH per se. Comparing the CO_2 dissolution (chemical equilibrium) to the microalgae biological uptake (biological equilibrium), the former is still faster and approaches a new equilibrium value that is higher than that present without the microalgae. This situation may be referred to as the luxury uptake, similar to a phenomenon reviewed by Solovchenko et al. (2019). However, they may refer to the phosphate storage in the cell, as in this research, it is about the excessive availability of carbon in the media and outside of the cell.

The following situation is “Type 2” or steady consumption. This is when the DIC level has equal value to the baseline. In other words, the same amount of CO_2 that has been supplied is

utilized by the microalgae. In this scenario, it appears that the chemical and biological equilibria are balanced. “Type 1” or active consumption is when DIC availability is below the baseline. In this scenario, CO_2 supply is not able to keep up with the biological consumption, due to the uptake by the microalgae. However, this active supply is not to the level that DIC is not available in the media. “Type 0” or overconsumption is when DIC is not available for the microalgae to consume, which leads to carbon starvation. This situation is observed in cultures without active carbon supply (C_0) after several days, in which simple diffusion from the surrounding is not enough to keep up with microalgae consumption. Interestingly, this situation also happened when air is being supplied continuously; however, it was only observed in the BDUG culture grown in *f/2*. However, the absence of carbon in the media or presence in a low concentration, may not necessarily mean the cells are carbon-deficient, but it can indicate a close coupling between uptake and consumption.

Although several factors are known to affect DIC measurement in the culture, e.g. pH, aeration rate, the supply of CO_2 or initial DIC content (Shene et al., 2016), this factor has been kept constant throughout the experiment. It is believed that the differences observed are primarily of biological origin, which is also noted by Gattuso et al. (2010) who stated that biological processes, e.g. photosynthesis, respiration and nutrient uptake could distort the carbonate chemistry. Further insights into the cause of the increased DIC needs development for a proper understanding of the phenomenon.

8.2.3 Carbon Uptake

Our study suggested that the use of the dynamic method for assessing change in carbon content in the culture can be used to measure carbon uptake rates. This method considers not only the carbon consumed by the microalgae but also that transferred into the media and available for the algae to use for growth. This method, which involves stopping the supply of CO_2 for up to an hour, has been described in Chapter 5.

Carbon uptake rate in BDUG has been observed to be 1.2 mM/h at its maximum, while in CCAP it is 0.8 mM/h. *Porphyridium*, meanwhile recorded values in between, i.e. 0.9 mM/h. Our observation also indicates that the maximum carbon uptake rate happened at different stages of growth, which is also supported by Amoroso et al. (1998). By observing uptake in 9 days, we manage to record average carbon sequestration of 13, 9 and 5 g CO2 / g algae for

BDUG, CCAP and PP respectively, which is significantly above the commonly reported value of 1.83, since this estimation considers the amount of carbon being dissolved in the media too. Comparing this value with sequestration capacity by chemical means, microalgae have a higher value, which highlights its additional advantages and usefulness, thus the potential for development towards CO_2 sequestration. By this method too, we managed to track the effectivity of continuous aeration in our cultivation, which is between 10 – 20%. This leaves for a considerable scope to develop strategies for improving the effectiveness of carbon supply.

In this research, we can observe the carbon consumption differences among different setup. Highest uptake rate was observed in culture with active CO_2 aeration. In culture initially supplied by carbon, the uptake rate is slightly lower, while in cultivation without carbon supply, the uptake rate is the slowest. In other words, although there is a high amount of DIC available at the initial time point, the microalgae consume that slowly in 3 – 5 days, rather than in bulk, perhaps restricted by mass transfer limitations.

Besides, we observed that increasing the nutrient availability does not necessarily translate to an increase in carbon uptake rate. This is because the highest uptake rate is observed in *f*/2 in BDUG, 2*f* in CCAP and 4*f* in PP. In terms of overall consumption, in BDUG, *f*/2 sequesters the most, while in CCAP, maximum CO_2 sequestration is in both *f/2* and 2*f*. In PP it is by 4*f* culture. Our data on carbon and nutrient consumption also suggested that carbon consumption is best at limiting nitrogen and phosphate concentrations.

8.2.4 Nutrient Uptake

We have noted that for active nutrient uptake, sufficient carbon supply, appropriate pH, and mixing will help to maximise mass transfer. Highest nitrate uptake rate is observed in 4*f* culture of PP, which is 1.0 mM/d, while among *Chlorella*, the highest rate was at 0.7 mM/d, which is recorded by BDUG *f/2* and CCAP 2*f*. In terms of phosphate, the highest uptake rate was observed in 4*f* for all cultures. The fastest consumption was with BDUG at 60 µM/d. We can also observe a trend in phosphate consumption, which is as the amount of phosphate supplied increases, its consumption rate also increases. This is not observed in nitrate consumption.

8.2.5 Microalgae Growth

Among the three strains, the gravimetric analysis showed that biomass production in PP is the highest. Between BDUG and CCAP, there is not much difference in biomass production. Based on their final dry weight, *f/2* cultivation shows the lowest biomass; however, among 2*f* and 4*f*, the difference is not statistically significant.

8.2.6 Bioplastics from Microalgae

Chapter 7 addresses topics on the incorporation of polysaccharides of *Porphyridium purpureum* into bioplastics production. Almost all of our attempts to include the microalgae results in a reduction in its elongation at break. We found that by extracting the polysaccharides from the microalgae, rather than incorporating them as whole cells, results in improvements in tensile strength and elasticity. Incorporating oil with the hope of increasing its hydrophobicity level achieved the objective; however, it reduced the mechanical properties significantly. Citric acid is functioning well as a crosslinking agent by increasing the tensile strength and elasticity, so does glycerol as the plasticizer.

Optimizing the microalgae incorporation found that the combination of changing the base polymers, plasticizers and additives shows that it does not mean improvement towards biofilm properties. Based on these results, incorporation of 1% exopolysaccharides of *P. purpureum*, 5% alginate and 1% glycerol emerged as the best biofilms made in terms of elongation at break, tensile strength, elasticity and moisture content.

8.3 Future Directions

The understanding of CO_2 uptake in microalgae and its efficient routing to relevant biochemical products such as bioplastics precursors is the big topic that requires continuing research efforts. More frequent discussions on this topic will help people not only to develop the microalgae for the commercialization purpose but also alongside its efficiency as nutrient removal and carbon utilization agent (specifically along the photoautotrophic way in this research), amid current global environmental problems. The use of these novel routes for the green biotechnology will optimistically bring positive sustainable impacts on the global carbon balance.

Topics on the sustainability, life cycle assessment and economic feasibility of the microalgae cultivation and commercialization should also be discussed in parallel (Fabris et al., 2020;

Khan, Shin, & Kim, 2018). This will cover aspects such as resource management, operational productivity, minimization of natural effect and socio-economic contemplations (Kamyab et al., 2019). By having precise monitoring on the cultivation performances such as carbon supply and uptake, biomass output and harvesting, energy requirement and others, it will able to increase people perception towards the potential that the microalgae cultivation have.

Optimal carbon supply has to be a key focus of optimizing the microalgae growth. It is suggested to find a supply of CO_2 that increases DIC availability in the media, while at the same time suit algae growth. Carbon saturation in media is among aspects where the attention can be given too, such as optimizing the time, amount and effectivity of carbon saturation. Besides, our report recorded aeration effectivity between 10 – 20% in 9 days of cultivation, suggesting considerable room for improvement.

Study of carbon sequestration in *C. vulgaris* and *P. purpureum* cultures have shown that their carbon sequestration behaviours are different. Levels of DIC increase in the cultures of both strains are also different. Thus, it is recommended that similar study be conducted for microalgae of different classes such as in diatoms (e.g. *Phaeodactylum tricornutum*) or acidophilic strains (e.g. *Galdieria sulphuraria*).

Microalgae preference for specific carbon species is also a topic that can be addressed. It is interesting to see if changes in specific carbon species abundance will affect the microalgae CO_2 sequestration or biochemical productivity.

Besides, the influence of physicochemical factors towards carbon uptake and production of bioplastics precursors are also recommended. This includes the use of a different type of light (e.g. green light) and different light intensity. This is known to affect the photosynthetic process (Choix et al., 2014; Garcia-Ochoa & Gomez, 2009), and probably CO_2 sequestration and biochemical production too. Range of salinity can also be tested to represent a range of habitats and hence resources for cultivation too, i.e. freshwater, brackish to the marine environment.

The method to assess carbon uptake in this research, i.e. the dynamic method is open to improvement too. This includes probably steps that not involve more effective N_2 gas purging, or methods to overcome the requirements mentioned. Its suitability to be used in different CO_2 concentration can be expanded too.

Besides, our study did not look much into the characterization of the bioplastics precursor. Its relationship with carbon and nutrient uptake is not studied too. In this experiment, polysaccharides of different carbon and nutrient treatment were collected as one. However, it is suggested to characterize the polysaccharides synthesized under different carbon and nutrient treatments, and observe how it affects the characteristics of bioplastics made. Our study also did not examine the use of polysaccharides (starch) of *Chlorella*. Although it is known to produce EPS (Xiao & Zheng, 2016) and IPS, we have not found reports of incorporation of its polysaccharides for bioplastics production.

Another recommended tools to achieve this is the use of metabolomics analysis, such as to understand how the microalgae fix the CO_2 to value-added chemicals.

Thus it is hoped by understanding the microalgae physiology and behaviour will contribute to the production of bioplastics with high quality. By expanding this investigation further incorporating a more significant scale operation, it should help in addressing the two environmental problems mentioned, i.e. the increasing level of CO_2 and plastics waste pollution.

References

Al Abdallah, Q., Nixon, B. T. & Fortwendel, J. R. (2016). The Enzymatic Conversion of Major Algal and Cyanobacterial Carbohydrates to Bioethanol. *Frontiers in Energy Research*, 4(November), 1–15.

Abolibda, T. Z. (2015). Physical and Chemical Investigations of Starch Based Bio-Plastics. *PhD Thesis, University of Leicester*.

Ackermann, J. uwe, Müller, S., Lösche, A., Bley, T. & Babel, wolfgang. (1995). Methylobacterium Rhodesianum Cells Tend to Double the DNA Content under Growth Limitations and Accumulate PHB. *Journal of Biotechnology*, 39(1), 9–20.

Adamczyk, M., Lasek, J. & Skawińska, A. (2016). CO2 Biofixation and Growth Kinetics of Chlorella Vulgaris and Nannochloropsis Gaditana. *Applied Biochemistry and Biotechnology*, 179(7), 1248–1261.

Adda, M., Merchuk, J. C. & Malis, S. (1986). Effect of Nitrate on Growth and Production of Cell-Wall. Polysaccharide by the Unicellular Red Alga Porphyridium. *Biomass*, 10, 131–140.

Aishvarya, V., Pradhan, N., Nayak, R. R., Sukla, L. B. & Mishra, B. K. (2012). Enhanced Inorganic Carbon Uptake by Chlorella Sp IMMTCC-2 under Autotrophic Conditions for Lipid Production and CO2 Sequestration. *Journal of Applied Phycology*, 24(6), 1455–1463.

Ajjawi, I., Verruto, J., Aqui, M., Soriaga, L. B., Coppersmith, J., Kwok, K., Peach, L., Orchard, E., Kalb, R., Xu, W., Carlson, T. J., Francis, K., Konigsfeld, K., Bartalis, J., Schultz, A., Lambert, W., Schwartz, A. S., Brown, R. & Moellering, E. R. (2017). Lipid Production in Nannochloropsis Gaditana Is Doubled by Decreasing Expression of a Single Transcriptional Regulator. *Nature Biotechnology*, 35(7), 647–652.

Akinfiev, N. N. & Diamond, L. W. (2010). Thermodynamic Model of Aqueous CO2-H2O-NaCl Solutions from -22 to 100 °C and from 0.1 to 100MPa. *Fluid Phase Equilibria*, 295(1), 104–124.

Akita, T. & Kamo, M. (2015). Theoretical Lessons for Increasing Algal Biofuel: Evolution of Oil Accumulation to Avert Carbon Starvation in Microalgae. *Journal of Theoretical Biology*, 380, 183–191.

Al-mashhadani, M. K. H., Wilkinson, S. J. & Zimmerman, W. B. (2016). Carbon Dioxide Rich Microbubble Acceleration of Biogas Production in Anaerobic Digestion. *Chemical Engineering Science*, 156, 24–35.

Alketife, A. M., Judd, S. & Znad, H. (2017). Synergistic Effects and Optimization of Nitrogen and Phosphorus Concentrations on the Growth and Nutrient Uptake of a Freshwater Chlorella Vulgaris. *Environmental Technology (United Kingdom)*.

Almomani, F., Al Ketife, A., Judd, S., Shurair, M., Bhosale, R. R., Znad, H. & Tawalbeh, M. (2019). Impact of CO 2 Concentration and Ambient Conditions on Microalgal Growth and Nutrient Removal from Wastewater by a Photobioreactor. *Science of the Total Environment*, 662, 662–671.

Amoroso, G., Sultemeyer, D., Thyssen, C. & Fock, H. P. (1998). Uptake of HCO3- and CO2 in Cells and Chloroplasts from the Microalgae Chlamydomonas Reinhardtii and Dunaliella Tertiolecta. *Plant Physiology*, 116(1), 193–201.

Anjum, A., Zuber, M., Zia, K. M., Noreen, A., Anjum, M. N. & Tabasum, S. (2016). Microbial Production of Polyhydroxyalkanoates (PHAs) and Its Copolymers: A Review of Recent Advancements. *International Journal of Biological Macromolecules*, 89, 161–174.

Arbib, Z., Ruiz, J., Alvarez, P., Garrido, C., Barragan, J. & Perales, J. A. (2012). Chlorella Stigmatophora for Urban Wastewater Nutrient Removal and CO2 Abatement. *International Journal of Phytoremediation*, 14(7), 714–725.

Arikan, E. B. & Ozsoy, H. D. (2015). A Review: Investigation of Bioplastics. *Journal of Civil Engineering and Architecture*, 9(2), 188–192.

Assunção, M. F. G., Varejão, J. M. T. B. & Santos, L. M. A. (2017). Nutritional Characterization of the Microalga Ruttnera Lamellosa Compared to Porphyridium Purpureum. *Algal Research*, 26(September 2016), 8–14.

Azapagic, A., Emsley, A. & Hamerton, L. (2003). Polymers in Everyday Use: Principles, Properties and Environmental Effects. *Polymers, the Environment and Sustainable Development*, 9, 17–46.

Bae, S., Kwak, K., Kim, S., Chung, S. & Igarashi, Y. (2001). Isolation and Characterization of CO2-Fixing Hydrogen-Oxidizing Marine Bacteria. *Journal of Bioscience and Bioengineering*, 91(5), 442–448.

Balaji, S., Gopi, K. & Muthuvelan, B. (2013). A Review on Production of Poly β Hydroxybutyrates from Cyanobacteria for the Production of Bio Plastics. *Algal Research*, 2(3), 278–285.

Barnes, D. K. A., Galgani, F., Thompson, R. C. & Barlaz, M. (2009). Accumulation and Fragmentation of Plastic Debris in Global Environments. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364(1526), 1985–1998.

Barsanti, L. & Gualtieri, P. (2014). Biogeochemical Role of Algae, in: *Algae: Anatomy, Biochemistry, and Biotechnology*, (pp. 199–220).

Basiak, E., Lenart, A. & Debeaufort, F. (2017). Effect of Starch Type on the Physico-Chemical Properties of Edible Films. *International Journal of Biological Macromolecules*, 98, 348–356.

Bastioli, C. (2005). Starch-Based Technology, in: *Handbook of Biodegradable Polymers*, (pp. 257–286).

Becerra-Dórame, M. J., López-Elías, J. A., Enríquez-Ocaña, F., Huerta-Aldaz, N., Voltolina, D., Osuna-López, I. & Izaguirre-Fierro, G. (2010). The Effect of Initial Cell and Nutrient Concentrations on the Growth and Biomass Production of Outdoor Cultures of Dunaliella Sp. *Annales Botanici Fennici*, 47(2), 109–112.

Berman-frank, I. & Dubinsky, Z. (1999). Balanced Growth in Aquatic Plants: Myth or Reality? *BioScience*, (January), 29–37.

Bernaerts, T. M. M., Kyomugasho, C., Van Looveren, N., Gheysen, L., Foubert, I., Hendrickx, M. E. & Van Loey, A. M. (2018). Molecular and Rheological Characterization of Different Cell Wall Fractions of Porphyridium Cruentum. *Carbohydrate Polymers*, 195(3), 542–550.

Bernhardsen, I. M. & Knuutila, H. K. (2017). A Review of Potential Amine Solvents for CO2 Absorption Process: Absorption Capacity, Cyclic Capacity and PKa. *International Journal of Greenhouse Gas Control*, 61, 27–48.

Bhagavan, N. V. & Ha, C.-E. (2011). Simple Carbohydrates. *Essentials of Medical Biochemistry*, 65–74.

Bhola, V., Swalaha, F., Ranjith Kumar, R., Singh, M. & Bux, F. (2014). Overview of the Potential of Microalgae for CO2 Sequestration. *International Journal of Environmental Science and Technology*, 11(7), 2103–2118.

Brányiková, I., Maršálková, B., Doucha, J., Brányik, T., Bišová, K., Zachleder, V. & Vítová, M. (2011). Microalgae-Novel Highly Efficient Starch Producers. *Biotechnology and Bioengineering*, 108(4), 766–776.

Brewer, P. G. & Goldman, J. C. (1976). Alkalinity Changes Generated by Phytoplankton Growth. *Limnology and Oceanography*, 21(1), 108–117.

Buehner, M. R., Young, P. M., Willson, B., Rausen, D., Schoonover, R., Babbitt, G. & Bunch, S. (2009). Microalgae Growth Modeling and Control for a Vertical Flat Panel Photobioreactor, in: *Proceedings of the American Control Conference*, (pp. 2301–2306).

Bugnicourt, E., Cinelli, P., Lazzeri, A. & Alvarez, V. (2014). Polyhydroxyalkanoate (PHA): Review of Synthesis, Characteristics, Processing and Potential Applications in Packaging. *Express Polymer Letters*, 8(11), 791–808.

Byun, Y. & Kim, Y. T. (2013). *Utilization of Bioplastics for Food Packaging Industry*. Elsevier Ltd.

Carissimi, M., Flôres, S. H. & Rech, R. (2018). Effect of Microalgae Addition on Active Biodegradable Starch Film. *Algal Research*, 32(April), 201–209.

Carvalho, A. P., Meireles, L. A. & Malcata, F. X. (2006). Microalgal Reactors: A Review of Enclosed System Designs and Performances. *Biotechnology Progress*, 22(6), 1490–1506.

de Carvalho, J. C., Sydney, E. B., Assú Tessari, L. F. & Soccol, C. R. (2019). *Culture Media for Mass Production of Microalgae*. Elsevier B.V.

Cazón, P., Velazquez, G., Ramírez, J. A. & Vázquez, M. (2017). Polysaccharide-Based Films and Coatings for Food Packaging: A Review. *Food Hydrocolloids*, 68, 136–148.

Chang, H. X., Huang, Y., Fu, Q., Liao, Q. & Zhu, X. (2016). Kinetic Characteristics and Modeling of Microalgae Chlorella Vulgaris Growth and CO2biofixation Considering the Coupled Effects of Light Intensity and Dissolved Inorganic Carbon. *Bioresource Technology*, 206, 231–238.

Chanprateep, S. (2010). Current Trends in Biodegradable Polyhydroxyalkanoates. *Journal of Bioscience and Bioengineering*, 110(6), 621–632.

Charlesby, A. & Hancock, N. H. (1953). The Effect of Cross-Linking on the Elastic Modulus of Polythene. *Proceedings of the Royal Society of London. Series A. Mathematical and Physical Sciences*, 218, 245–255.

Cheah, W. Y., Show, P. L., Chang, J. S., Ling, T. C. & Juan, J. C. (2015). Biosequestration of Atmospheric CO2 and Flue Gas-Containing CO2 by Microalgae. *Bioresource Technology*, 184, 190–201.

Chen, C. & Durbin, E. (1994). Effects of PH on the Growth and Carbon Uptake of Marine Phytoplankton. *Marine Ecology Progress Series*, 111(1992), 83–94.

Chen, Y., Tang, X., Kapoore, R. V., Xu, C. & Vaidyanathan, S. (2015). Influence of Nutrient Status on the Accumulation of Biomass and Lipid in Nannochloropsis Salina and Dunaliella Salina. *Energy Conversion and Management*, 106, 62–72.

Chen, Y. & Vaidyanathan, S. (2013). Simultaneous Assay of Pigments, Carbohydrates, Proteins and Lipids in Microalgae. *Analytica Chimica Acta*, 776, 31–40.

Chen, Y., Xu, C. & Vaidyanathan, S. (2020). Influence of Gas Management on Biochemical Conversion of CO2 by Microalgae for Biofuel Production. *Applied Energy*, 261(June 2019), 114420.

Chen, Y., Zhang, L., Xu, C. & Vaidyanathan, S. (2016). Dissolved Inorganic Carbon Speciation in Aquatic Environments and Its Application to Monitor Algal Carbon Uptake. *Science of the Total Environment*, 541, 1282–1295.

Chen, Z., Zhang, J., Lei, X., Zhang, B., Cai, G., Zhang, H., Li, Y., Zheng, W., Tian, Y., Xu, H. & Zheng, T. (2014). Influence of Plaque-Forming Bacterium, Rhodobacteraceae Sp. on the Growth of Chlorella Vulgaris. *Bioresource Technology*, 169, 784–788.

Cheng, J., Zhu, K., Lu, H., Yue, H., Liu, C., Liang, B. & Tang, S. (2019). Quantitative Relationship between CO2 Absorption Capacity and Amine Water System: DFT, Statistical, and Experimental Study. *Industrial and Engineering Chemistry Research*, 58(31), 13848–13857.

Cheng, Y. S., Labavitch, J. M. & Vandergheynst, J. S. (2015). Elevated CO2 Concentration Impacts Cell Wall Polysaccharide Composition of Green Microalgae of the Genus Chlorella. *Letters in Applied Microbiology*, 60(1), 1–7.

Chester, R. (1990). Dissolved Gases in Seawater, in: *Marine Geochemistry*, (pp. 233–271). Springer Netherlands.

Chi, Z., Elloy, F., Xie, Y., Hu, Y. & Chen, S. (2014). Selection of Microalgae and Cyanobacteria Strains for Bicarbonate-Based Integrated Carbon Capture and Algae Production System. *Applied Biochemistry and Biotechnology*, 172(1), 447–457.

Chi, Z., O'Fallon, J. V. & Chen, S. (2011). Bicarbonate Produced from Carbon Capture for Algae Culture. *Trends in Biotechnology*, 29(11), 537–541.

Chi, Z., Xie, Y., Elloy, F., Zheng, Y., Hu, Y. & Chen, S. (2013). Bicarbonate-Based Integrated Carbon Capture and Algae Production System with Alkalihalophilic Cyanobacterium. *Bioresource Technology*, 133, 513–521.

Chiellini, E., Cinelli, P., Ilieva, V. I. & Martera, M. (2008). Biodegradable Thermoplastic Composites Based on Polyvinyl Alcohol and Algae. *Biomacromolecules*, 9(3), 1007–1013.

Chinnasamy, S., Ramakrishnan, B., Bhatnagar, A. & Das, K. C. (2009). Biomass Production Potential of a Wastewater Alga Chlorella Vulgaris ARC 1 under Elevated Levels of CO2 and Temperature. *International Journal of Molecular Sciences*, 10(2), 518–532.

Chisti, Y. (2007). Biodiesel from Microalgae. *Biotechnology Advances*, 25(3), 294–306.

Chiu, S. Y., Kao, C. Y., Chen, C. H., Kuan, T. C., Ong, S. C. & Lin, C. S. (2008). Reduction of CO2 by a High-Density Culture of Chlorella Sp. in a Semicontinuous Photobioreactor. *Bioresource Technology*, 99(9), 3389–3396.

Choix, F. J., Bashan, Y., Mendoza, A. & De-Bashan, L. E. (2014). Enhanced Activity of ADP Glucose Pyrophosphorylase and Formation of Starch Induced by Azospirillum Brasilense in Chlorella Vulgaris. *Journal of Biotechnology*, 177(1), 22–34.

Chojnacka, K. & Noworyta, A. (2004). Evaluation of Spirulina Sp. Growth in Photoautotrophic, Heterotrophic and Mixotrophic Cultures. *Enzyme and Microbial Technology*, 34(5), 461–465.

Coates, R. C., Trentacoste, E. & Gerwick, W. H. (2013). Bioactive and Novel Chemicals from Microalgae, in: *Handbook of Microalgal Culture: Applied Phycology and Biotechnology*, (pp. 504–531).

Collos, Y., Mornet, F., Sciandra, A., Waser, N., Larson, A. & Harrison, P. J. (1999). An Optical Method for the Rapid Measurement of Micromolar Concentrations of Nitrate in Marine Phytoplankton Cultures. *Journal of Applied Phycology*, 11(2), 179–184.

Colman, B. & Gehl, K. A. (1983). Physiological Characteristics of Photosynthesis in Porphyridium Cruentum: Evidence for Bicarbonate Transport in A Unicellular Red Alga. *Journal of Phycology*, 19, 216–219.

Costa, S. S., Miranda, A. L., Andrade, B. B., Assis, D. de J., Souza, C. O., de Morais, M. G., Costa, J. A. V. & Druzian, J. I. (2018). Influence of Nitrogen on Growth, Biomass Composition, Production, and Properties of Polyhydroxyalkanoates (PHAs) by Microalgae. *International Journal of Biological Macromolecules*, 116, 552–562.

Coward, T., Fuentes-Grünewald, C., Silkina, A., Oatley-Radcliffe, D. L., Llewellyn, G. & Lovitt, R. W. (2016). Utilising Light-Emitting Diodes of Specific Narrow Wavelengths for the Optimization and Co-Production of Multiple High-Value Compounds in Porphyridium Purpureum. *Bioresource Technology*, 221, 607–615.

Cramer, M. & Myers, J. (1948). Nitrate Reduction and Assimilation in Chlorella. *Journal of General Physiology*, (1), 93–102.

Crofcheck, C., Monstross, M., Xinyi, E., Shea, A., Crocker, M. & Andrews, R. (2012). Influence of Media Composition on the Growth Rate of Chlorella Vulgaris and Scenedesmus Acutus Utilized for CO2 Mitigation. *Journal Of Biochemical Technology*, 4(2), 532–549.

Cuellar-Bermudez, S. P., Garcia-Perez, J. S., Rittmann, B. E. & Parra-Saldivar, R. (2015). Photosynthetic Bioenergy Utilizing CO2: An Approach on Flue Gases Utilization for Third Generation Biofuels. *Journal of Cleaner Production*, 98, 53–65.

Delattre, C., Pierre, G., Laroche, C. & Michaud, P. (2016). Production, Extraction and Characterization of Microalgal and Cyanobacterial Exopolysaccharides. *Biotechnology Advances*, 34(7), 1159–1179.

Derraik, J. G. B. (2002). The Pollution of the Marine Environment by Plastic Debris: A Review. *Marine Pollution Bulletin*, 44(9), 842–852.

Dixon, G. K., Patel, B. N. & Merrett, M. J. (1987). Role of Intracellular Carbonic Anhydrase in Inorganic-Carbon Assimilation by Porphyridium Purpureum. *Planta*, 172(4), 508–513.

Docksai, R. (2012). Market for Bioplastics. *The Futurist*, (November-December), 9–13.

Dong, X., Chen, Z., Xue, S., Zhang, J., Zhou, J., Liu, Y., Xu, Y. & Liu, Z. (2013). Catalytic Pyrolysis of Microalga Chlorella Pyrenoidosa for Production of Ethylene, Propylene and Butene. *RSC Advances*, 3(48), 25780–25787.

Doucha, J., Straka, F. & Lívanský, K. (2005). Utilization of Flue Gas for Cultivation of Microalgae (Chlorella Sp.) in an Outdoor Open Thin-Layer Photobioreactor. *Journal of Applied Phycology*, 17(5), 403–412.

Douskova, I., Doucha, J., Livansky, K., MacHat, J., Novak, P., Umysova, D., Zachleder, V. & Vitova, M. (2009). Simultaneous Flue Gas Bioremediation and Reduction of Microalgal Biomass Production Costs. *Applied Microbiology and Biotechnology*, 82(1), 179–185.

Dragosits, M. & Mattanovich, D. (2013). Adaptive Laboratory Evolution -- Principles and Applications for Biotechnology. *Microb Cell Fact*, 12, 64.

Drosg, B. (2015). Photo-Autotrophic Production of Poly(Hydroxyalkanoates) in Cyanobacteria. *Chemical and Biochemical Engineering Quarterly*, 29(2), 145–156.

Efthimiadou, E. K., Metaxa, A. & Kordas, G. (2014). Modified Polysaccharides as Drug Delivery, in: *Polysaccharides*, (pp. 1–26).

Eixler, S., Karsten, U. & Selig, U. (2006). Phosphorus Storage in Chlorella Vulgaris (Trebouxiophyceae, Chlorophyta) Cells and Its Dependence on Phosphate Supply. *Phycologia*, 45(1), 53–60.

El-Ansari, O. & Colman, B. (2015). Inorganic Carbon Acquisition in the Acid-Tolerant Alga Chlorella Kessleri. *Physiologia Plantarum*, 153(1), 175–182.

Emadian, S. M., Onay, T. T. & Demirel, B. (2017). Biodegradation of Bioplastics in Natural Environments. *Waste Management*, 59, 526–536.

European Bioplastics. (2015). *Bioplastics Packaging - Combining Performance with Sustainability*.

European Bioplastics. (2016). *What Are Bioplastics?*

Fabra, M. J., Martínez-Sanz, M., Gómez-Mascaraque, L. G., Gavara, R. & López-Rubio, A. (2018). Structural and Physicochemical Characterization of Thermoplastic Corn Starch Films Containing Microalgae. *Carbohydrate Polymers*, 186(October 2017), 184–191.

Fabris, M., Abbriano, R. M., Pernice, M., Sutherland, D. L., Commault, A. S., Hall, C. C., Labeeuw, L., McCauley, J. I., Kuzhiuparambil, U., Ray, P., Kahlke, T. & Ralph, P. J. (2020). Emerging Technologies in Algal Biotechnology: Toward the Establishment of a Sustainable, Algae-Based Bioeconomy. *Frontiers in Plant Science*, 11(March), 1–22.

Fan, D., Dai, D. J. & Wu, H. S. (2013). Ethylene Formation by Catalytic Dehydration of Ethanol with Industrial Considerations. *Materials*, 6(1), 101–115.

Fan, L. H., Zhang, Y. T., Zhang, L. & Chen, H. L. (2008). Evaluation of a Membrane-Sparged Helical Tubular Photobioreactor for Carbon Dioxide Biofixation by Chlorella Vulgaris. *Journal of Membrane Science*, 325(1), 336–345.

Fan, L., Zhang, Y., Cheng, L., Zhang, L., Tang, D. & Chen, H. (2007). Optimization of Carbon Dioxide Fixation by Chlorella Vulgaris Cultivated in a Membrane-Photobioreactor. *Chemical Engineering and Technology*, 30(8), 1094–1099.

Ferreira, A. R. V., Alves, V. D. & Coelhoso, I. M. (2016). Polysaccharide-Based Membranes in Food Packaging Applications. *Membranes*, 6(2), 1–17.

Finore, I., Di Donato, P., Mastascusa, V., Nicolaus, B. & Poli, A. (2014). Fermentation Technologies for the Optimization of Marine Microbial Exopolysaccharide Production. *Marine Drugs*, 12(5), 3005–3024.

Fixen, P., Brentrup, F., Tom Bruulsema, F. G., Norton, R. & Zingore, S. (2015). Nutrient/Fertilizer Use Efficiency: Measurement, Current Situation and Trends, in: *Managing Water and Fertilizer for Sustainable Agricultural Intensification*.

Freile-Pelegrín, Y. & Madera-Santana, T. J. (2017). Characterization Techniques for Algae-Based Materials. *Algae Based Polymers, Blends, and Composites: Chemistry, Biotechnology and Materials Science*, 18, 649–670.

Freitas, F., Torres, C. A. V. & Reis, M. A. M. (2017). Engineering Aspects of Microbial Exopolysaccharide Production. *Bioresource Technology*, 245(PartB), 1674–1683.

Fuentes-Grünewald, C., Bayliss, C., Zanain, M., Pooley, C., Scolamacchia, M. & Silkina, A. (2015). Evaluation of Batch and Semi-Continuous Culture of Porphyridium Purpureum in a Photobioreactor in High Latitudes Using Fourier Transform Infrared Spectroscopy for Monitoring Biomass Composition and Metabolites Production. *Bioresource Technology*, 189, 357–363.

Gaignard, C., Gargouch, N., Dubessay, P., Delattre, C., Pierre, G., Laroche, C., Fendri, I., Abdelkafi, S. & Michaud, P. (2019). New Horizons in Culture and Valorization of Red Microalgae. *Biotechnology Advances*, 37(1), 193–222.

Galgano, F., Condelli, N., Favati, F., Di Bianco, V., Perretti, G. & Caruso, M. C. (2015). Biodegradable Packaging and EDIBLE COATING for Fresh-Cut Fruits and Vegetables. *Italian Journal of Food Science*, 27(1), 1–20.

Gao, C., Pollet, E. & Avérous, L. (2017). Innovative Plasticized Alginate Obtained by Thermo-Mechanical Mixing: Effect of Different Biobased Polyols Systems. *Carbohydrate Polymers*, 157, 669–676.

García-Cubero, R., Cabanelas, I. T. D., Sijtsma, L., Kleinegris, D. M. M. & Barbosa, M. J. (2018). Production of Exopolysaccharide by Botryococcus Braunii CCALA 778 under Laboratory Simulated Mediterranean Climate Conditions. *Algal Research*, 29(December 2017), 330–336.

García-Cubero, R., Moreno-Fernández, J., Acién-Fernández, F. G. & García-González, M. (2018). How to Combine CO2 Abatement and Starch Production in Chlorella Vulgaris. *Algal Research*, 32(May 2017), 270–279.

Garcia-Ochoa, F. & Gomez, E. (2009). Bioreactor Scale-up and Oxygen Transfer Rate in Microbial Processes: An Overview. *Biotechnology Advances*, 27(2), 153–176.

Gardner, R. D., Cooksey, K. E., Mus, F., Macur, R., Moll, K., Eustance, E., Carlson, R. P., Gerlach, R., Fields, M. W. & Peyton, B. M. (2012). Use of Sodium Bicarbonate to Stimulate Triacylglycerol Accumulation in the Chlorophyte Scenedesmus Sp. and the Diatom Phaeodactylum Tricornutum. *Journal of Applied Phycology*, 24(5), 1311–1320.

Gardner, R. D., Lohman, E., Gerlach, R., Cooksey, K. E. & Peyton, B. M. (2013). Comparison of CO2 and Bicarbonate as Inorganic Carbon Sources for Triacylglycerol and Starch Accumulation in Chlamydomonas Reinhardtii. *Biotechnology and Bioengineering*, 110(1), 87–96.

Gasljevic, K., Hall, K. A., Oakes, S., Chapman, D. J. & Matthys, E. F. (2009). Increased Production of Extracellular Polysaccharide by Porphyridium Cruentum Immobilized in Foam Sheets. *Engineering in Life Sciences*, 9(6), 479–489.

Gattuso, J.-P., Gao, K., Lee, K., Rost, B. & Schulz, K. G. (2010). Approaches and Tools to Manipulate the Carbonate Chemistry, in: *Guide to Best Practices for Ocean Acidification Research and Data Reporting*, (pp. 41–52).

Gauntlett, R. B. (1980). *A Literature Review of CO2 Removal by Aeration*.

Ghanbari Shendi, E., Sivri Ozay, D., Ozkaya, M. T. & Ustunel, N. F. (2018). Changes

Occurring in Chemical Composition and Oxidative Stability of Virgin Olive Oil during Storage. *OCL - Oilseeds and fats, Crops and Lipids*, 25(6), 4–11.
Giordano, M., Beardall, J. & Raven, J. A. (2005). CO2 Concentrating Mechanisms in Algae: Mechanisms, Environmental Modulation, and Evolution. *Annual Review of Plant Biology*, 56(1), 99–131.
Goldman, J. C. (1973). Carbon Dioxide and PH: Effect on Species Succession of Algae. *Source: Science, New Series*, 182(4109), 306–307.
Gomaa, M. A., Al-Haj, L. & Abed, R. M. M. (2016). Metabolic Engineering of Cyanobacteria and Microalgae for Enhanced Production of Biofuels and High-Value Products. *Journal of Applied Microbiology*, 121(4), 919–931.
Gonçalves, A. L., Rodrigues, C. M., Pires, J. C. M. & Simões, M. (2016). The Effect of Increasing CO2concentrations on Its Capture, Biomass Production and Wastewater Bioremediation by Microalgae and Cyanobacteria. *Algal Research*, 14, 127–136.
Gourson, C., Benhaddou, R., Granet, R., Krausz, P., Saulnier, L. & Thibault, J. F. (1999). Preparation of Biodegradable Plastic in Microwave Oven and Solvent-Free Conditions. *Comptes Rendus de l'Academie des Sciences - Series IIc: Chemistry*, 2(2), 75–78.
Groh, K. J., Backhaus, T., Carney-Almroth, B., Geueke, B., Inostroza, P. A., Lennquist, A., Leslie, H. A., Maffini, M., Slunge, D., Trasande, L., Warhurst, A. M. & Muncke, J. (2019). Overview of Known Plastic Packaging-Associated Chemicals and Their Hazards. *Science of the Total Environment*, 651, 3253–3268.
Grushkin, D. (2011). Breaking the Mold. *Nature Publishing Group*, 29(1), 16–18.
Guihéneuf, F. & Stengel, D. B. (2015). Towards the Biorefinery Concept: Interaction of Light, Temperature and Nitrogen for Optimizing the Co-Production of High-Value Compounds in Porphyridium Purpureum. *Algal Research*, 10, 152–163.
Guil-Guerrero, J. L. & Rebolloso-Fuentes, M. M. (2008). Nutrient Composition of Chlorella Spp. and Monodus Subterraneus Cultured in a Bubble Column Bioreactor. *Food Biotechnology*, 22(3), 218–233.
Guillard, R. R. L. (1975). Culture of Phytoplankton for Feeding Marine Invertebrates, in: *Culture of Marine Invertebrate Animals*, (pp. 29–60).
Guo, M. Q., Hu, X., Wang, C. & Ai, L. (2017). Polysaccharides: Structure and Solubility, in: *Solubility of Polysaccharides*, (pp. 7–21).
Halden, R. U. (2010). Plastics and Health Risks. *Annual Review of Public Health*, 31(1), 179–194.
Han, S. J., Yoo, M., Kim, D. W. & Wee, J. H. (2011). Carbon Dioxide Capture Using Calcium Hydroxide Aqueous Solution as the Absorbent. *Energy and Fuels*, 25(8), 3825–3834.
Harper, C. A. (2002). Thermoplastics, in: *Handbook of Plastics, Elastomers, and Composites*.
Hellebust, J. A. & Ahmad, I. (1988). Regulation of Nitrogen Assimilation in Green Microalgae. *Biological Oceanography*, 6(3), 241–255.
Hempel, F., Bosarth, A. S., Lindenkamp, N., Klingl, A., Zauner, S., Linne, U., Steinbuchel, A. & Maier, U. G. (2010). Microalgae as Bioreactors for Bioplastic Production. *Microbial Cell Factories*, 2–7.
Van Den Hende, S., Vervaeren, H. & Boon, N. (2012). Flue Gas Compounds and Microalgae: (Bio-)Chemical Interactions Leading to Biotechnological Opportunities. *Biotechnology Advances*, 30(6), 1405–1424.
Hirata, S., Hayashitani, M., Taya, M. & Tone, S. (1996). Carbon Dioxide Fixation in Batch Culture of Chlorella Sp. Using a Photobioreactor with a Sunlight-Collection Device. *Journal of Fermentation and Bioengineering*, 81(5), 470–472.
Hirotsu, T. & Otsuki, T. (2017). Chlorella-Based Composites, in: *Algae Based Polymers, Blends, and Composites: Chemistry, Biotechnology and Materials Science*, (pp. 531–564). Elsevier Inc.

Huertas, E., Montero, O. & Lubián, L. M. (2000). Effects of Dissolved Inorganic Carbon Availability on Growth, Nutrient Uptake and Chlorophyll Fluorescence of Two Species of Marine Microalgae. *Aquacultural Engineering*, 22(3), 181–197.

Huertas, J. I., Gomez, M. D., Giraldo, N. & Garzón, J. (2015). CO2 Absorbing Capacity of MEA. *Journal of Chemistry*, 2015(2).

Huntley, M. E. & Redalje, D. G. (2007). *CO2 Mitigation and Renewable Oil from Photosynthetic Microbes: A New Appraisal*.

Iles, A. & Martin, A. N. (2013). Expanding Bioplastics Production: Sustainable Business Innovation in the Chemical Industry. *Journal of Cleaner Production*, 45, 38–49.

Illingworth, J. A. (1981). A Common Source of Error in PH Measurements. *Biochemical Journal*, 195(1), 259–262.

Iqbal, M. & Zafar, S. I. (1993). Effects of Photon Flux Density, CO2, Aeration Rate, and Inoculum Density on Growth and Extracellular Polysaccharide Production by Porphyridium Cruentum. *Folia Microbiologica*, 38(6), 509–514.

Iwata, T. (2015). Biodegradable and Bio-Based Polymers: Future Prospects of Eco-Friendly Plastics. *Angewandte Chemie - International Edition*, 54(11), 3210–3215.

Jabeen, N., Majid, I. & Nayik, G. A. (2015). Bioplastics and Food Packaging: A Review. *Cogent Food & Agriculture*, 1(1), 1–6.

Jacob-Lopes, E., Gimenes Scoparo, C. H., Queiroz, M. I. & Franco, T. T. (2010). Biotransformations of Carbon Dioxide in Photobioreactors. *Energy Conversion and Management*, 51(5), 894–900.

Jamshidian, M., Tehrany, E. A., Imran, M., Jacquot, M. & Desobry, S. (2010). Poly-Lactic Acid: Production, Applications, Nanocomposites, and Release Studies. *Comprehensive Reviews in Food Science and Food Safety*, 9(5), 552–571.

de Jesús-Campos, D., López-Elías, J. A., Medina-Juarez, L. Á., Carvallo-Ruiz, G., Fimbres-Olivarria, D. & Hayano-Kanashiro, C. (2020). Chemical Composition, Fatty Acid Profile and Molecular Changes Derived from Nitrogen Stress in the Diatom Chaetoceros Muelleri. *Aquaculture Reports*, 16(August 2019), 100281.

De Jesus Raposo, M. F., De Morais, A. M. M. B. & De Morais, R. M. S. C. (2014). Bioactivity and Applications of Polysaccharides from Marine Microalgae, in: *Polysaccharides*.

Ji, C., Wang, J., Li, R. & Liu, T. (2017). Modeling of Carbon Dioxide Mass Transfer Behavior in Attached Cultivation Photobioreactor Using the Analysis of the PH Profiles. *Bioprocess and Biosystems Engineering*, 40(7), 1079–1090.

Jones, C. R., Olfe-Kräutlein, B., Naims, H. & Armstrong, K. (2017). The Social Acceptance of Carbon Dioxide Utilisation: A Review and Research Agenda. *Frontiers in Energy Research*, 5(JUN), 1–13.

Judd, S., van den Broeke, L. J. P., Shurair, M., Kuti, Y. & Znad, H. (2015). Algal Remediation of CO2 and Nutrient Discharges: A Review. *Water Research*, 87, 356–366.

Kaeb, H., Aeschelmann, F., Dammer, L. & Carus, M. (2016). *Market Study on the Consumption of Biodegradable and Compostable Plastic Products in Europe 2015 and 2020*.

Kahn, B. (2016). Earth's CO2 Passes the 400 PPM Threshold — Maybe Permanently. *Scientific American*, (September), 1–9.

Kamal, S., Rehman, M., Rehman, S., Nazli, Z. i. H., Yaqoob, N., Noreen, R., Ikram, S. & Min, H. S. (2017). *Blends of Algae With Natural Polymers*. Elsevier Inc.

Kamyab, H., Lee, C. T., Chelliapan, S., Khademi, T., Talaiekhozani, A. & Rezania, S. (2019). Role of Microalgal Biotechnology in Environmental Sustainability-a Mini Review. *Chemical Engineering Transactions*, 72(October 2018), 451–456.

Kang, J.-S., Kim, H.-S. & Lee, J. H. (1996). Morphological Variations of the Marine Diatom Thalassiosira Weissflogii under Culture Conditions. *The Korean Journal of Phycology*,

11(1), 23–34.
Kapoore, R. V., Huete-Ortega, M., Day, J. G., Okurowska, K., Slocombe, S. P., Stanley, M. S. & Vaidyanathan, S. (2019). Effects of Cryopreservation on Viability and Functional Stability of an Industrially Relevant Alga. *Scientific Reports*, 9(1), 2093.
Kargi, F. & Uygur, A. (2003). Effect of Carbon Source on Biological Nutrient Removal in a Sequencing Batch Reactor. *Bioresource Technology*, 89(1), 89–93.
Kasiri, S., Ulrich, A. & Prasad, V. (2015). Kinetic Modeling and Optimization of Carbon Dioxide Fixation Using Microalgae Cultivated in Oil-Sands Process Water. *Chemical Engineering Science*, 137, 697–711.
Kassim, M. A. & Meng, T. K. (2017). Carbon Dioxide (CO2) Biofixation by Microalgae and Its Potential for Biorefinery and Biofuel Production. *Science of the Total Environment*, 584–585, 1121–1129.
Keffer, J. E. & Kleinheinz, G. T. (2002). Use of Chlorella Vulgaris for CO2 Mitigation in a Photobioreactor. *Journal of Industrial Microbiology and Biotechnology*, 29(5), 275–280.
Kesaano, M., Gardner, R. D., Moll, K., Lauchnor, E., Gerlach, R., Peyton, B. M. & Sims, R. C. (2015). Dissolved Inorganic Carbon Enhanced Growth, Nutrient Uptake, and Lipid Accumulation in Wastewater Grown Microalgal Biofilms. *Bioresource Technology*, 180, 7–15.
Khalil, Z. I., Asker, M. M. S., El-Sayed, S. & Kobbia, I. A. (2010). Effect of PH on Growth and Biochemical Responses of Dunaliella Bardawil and Chlorella Ellipsoidea. *World Journal of Microbiology and Biotechnology*, 26(7), 1225–1231.
Khan, M. I., Shin, J. H. & Kim, J. D. (2018). The Promising Future of Microalgae: Current Status, Challenges, and Optimization of a Sustainable and Renewable Industry for Biofuels, Feed, and Other Products. *Microbial Cell Factories*, 17(1), 1–21.
Kim, G. Y., Heo, J., Kim, H. S. & Han, J. I. (2017). Bicarbonate-Based Cultivation of Dunaliella Salina for Enhancing Carbon Utilization Efficiency. *Bioresource Technology*, 237, 72–77.
Kim, J., Lee, J. Y. & Lu, T. (2014). Effects of Dissolved Inorganic Carbon and Mixing on Autotrophic Growth of Chlorella Vulgaris. *Biochemical Engineering Journal*, 82, 34–40.
Klinthong, W., Yang, Y. H., Huang, C. H. & Tan, C. S. (2015). A Review: Microalgae and Their Applications in CO2 Capture and Renewable Energy. *Aerosol and Air Quality Research*, 15(2), 712–742.
Krishnan, V., Uemura, Y. & Mansor, N. (2015). Carbon Content and Lipid Content of Marine Microalgae Cultivated in Different Carbon Dioxide Concentration, in: *SOJO-UTP Conference*.
Krueger, M. C., Harms, H. & Schlosser, D. (2015). Prospects for Microbiological Solutions to Environmental Pollution with Plastics. *Applied Microbiology and Biotechnology*, 99(21), 8857–8874.
Kuenkel, A., Becker, J., Borger, L., Hamprecht, J., Koltzenburg, S., Loos, R., Schick, M. B., Schlegel, K., Sinkel, C., Skupin, G. & Yamamoto, M. (2016). Polymers, Biodegradable. *Ullmanns's Encyclopedia of Industrial Chemistry*, 29.
Kumar, A., Ergas, S., Yuan, X., Sahu, A., Zhang, Q., Dewulf, J., Malcata, F. X. & van Langenhove, H. (2010). Enhanced CO2 Fixation and Biofuel Production via Microalgae: Recent Developments and Future Directions. *Trends in Biotechnology*, 28(7), 371–380.
Kuo, C. M., Jian, J. F., Lin, T. H., Chang, Y. Bin, Wan, X. H., Lai, J. T., Chang, J. S. & Lin, C. S. (2016). Simultaneous Microalgal Biomass Production and CO2 Fixation by Cultivating Chlorella Sp. GD with Aquaculture Wastewater and Boiler Flue Gas. *Bioresource Technology*, 221(75), 241–250.
Kurano, N., Ikemoto, H., Miyashita, H., Hasegawa, T., Hata, H. & Miyachi, S. (1995). Fixation and Utilization of Carbon Dioxide by Microalgal Photosynthesis. *Energy Conversion and*

Management, 36(6–9), 689–692.
Lam, M. K. & Lee, K. T. (2013). Effect of Carbon Source towards the Growth of Chlorella Vulgaris for CO2 Bio-Mitigation and Biodiesel Production. *International Journal of Greenhouse Gas Control*, 14, 169–176.
Lam, M. K., Lee, K. T. & Mohamed, A. R. (2012). Current Status and Challenges on Microalgae-Based Carbon Capture. *International Journal of Greenhouse Gas Control*, 10, 456–469.
Laurens, L. M. L., Markham, J., Templeton, D. W., Christensen, E. D., Van Wychen, S., Vadelius, E. W., Chen-Glasser, M., Dong, T., Davis, R. & Pienkos, P. T. (2017). Development of Algae Biorefinery Concepts for Biofuels and Bioproducts; a Perspective on Process-Compatible Products and Their Impact on Cost-Reduction. *Energy and Environmental Science*, 10(8), 1716–1738.
Law, K. L. (2017). Plastics in the Marine Environment. *Annual Review of Marine Science*, 9(1), 205–229.
Lee, E., Jalalizadeh, M. & Zhang, Q. (2015). Growth Kinetic Models for Microalgae Cultivation: A Review. *Algal Research*, 12, 497–512.
Li, J., Li, C., Lan, C. Q. & Liao, D. (2018). Effects of Sodium Bicarbonate on Cell Growth, Lipid Accumulation, and Morphology of Chlorella Vulgaris. *Microbial Cell Factories*, 17(1), 111.
Li, T., Xu, J., Wu, H., Jiang, P., Chen, Z. & Xiang, W. (2019). Growth and Biochemical Composition of Porphyridium Purpureum SCS-02 under Different Nitrogen Concentrations. *Marine Drugs*, 17(2).
Liang, Z., Liu, Y., Ge, F., Xu, Y., Tao, N., Peng, F. & Wong, M. (2013). Efficiency Assessment and PH Effect in Removing Nitrogen and Phosphorus by Algae-Bacteria Combined System of Chlorella Vulgaris and Bacillus Licheniformis. *Chemosphere*, 92(10), 1383–1389.
Lim, M., Kwon, H., Kim, D., Seo, J., Han, H. & Khan, S. B. (2015). Highly-Enhanced Water Resistant and Oxygen Barrier Properties of Cross-Linked Poly(Vinyl Alcohol) Hybrid Films for Packaging Applications. *Progress in Organic Coatings*, 85, 68–75.
Liu, N., Yang, Y., Li, F., Ge, F. & Kuang, Y. (2016). Importance of Controlling PH-Depended Dissolved Inorganic Carbon to Prevent Algal Bloom Outbreaks. *Bioresource Technology*, 220, 246–252.
Lohman, E. J., Gardner, R. D., Pedersen, T., Peyton, B. M., Cooksey, K. E. & Gerlach, R. (2015). Optimized Inorganic Carbon Regime for Enhanced Growth and Lipid Accumulation in Chlorella Vulgaris. *Biotechnology for Biofuels*, 8(1), 1–13.
Luangpipat, T. & Chisti, Y. (2017). Biomass and Oil Production by Chlorella Vulgaris and Four Other Microalgae — Effects of Salinity and Other Factors. *Journal of Biotechnology*.
Luchese, C. L., Spada, J. C. & Tessaro, I. C. (2017). Starch Content Affects Physicochemical Properties of Corn and Cassava Starch-Based Films. *Industrial Crops and Products*, 109(September), 619–626.
Luis, P. (2016). Use of Monoethanolamine (MEA) for CO2 Capture in a Global Scenario: Consequences and Alternatives. *Desalination*, 380, 93–99.
Maeda, K., Owada, M., Kimura, N., Omata, K. & Karube, I. (1995). CO2 Fixation from the Flue Gas on Coal-Fired Thermal Power Plant by Microalgae. *Energy Conversion and Management*, 36(6–9), 717–720.
Mano, J. F., Silva, G. A., Azevedo, H. S., Malafaya, P. B., Sousa, R. A., Silva, S. S., Boesel, L. F., Oliveira, J. M., Santos, T. C., Marques, A. P., Neves, N. M. & Reis, R. L. (2007). Natural Origin Biodegradable Systems in Tissue Engineering and Regenerative Medicine: Present Status and Some Moving Trends. *Journal of the Royal Society Interface*, 4(17), 999–1030.

Marcati, A., Ursu, A. V., Laroche, C., Soanen, N., Marchal, L., Jubeau, S., Djelveh, G. & Michaud, P. (2014). Extraction and Fractionation of Polysaccharides and B-Phycoerythrin from the Microalga Porphyridium Cruentum by Membrane Technology. *Algal Research*, 5(1), 258–263.

Markou, G. & Nerantzis, E. (2013). Microalgae for High-Value Compounds and Biofuels Production: A Review with Focus on Cultivation under Stress Conditions. *Biotechnology Advances*, 31(8), 1532–1542.

Mathiot, C., Ponge, P., Gallard, B., Sassi, J.-F., Delrue, F. & Le Moigne, N. (2019). Microalgae Starch-Based Bioplastics: Screening of Ten Strains and Plasticization of Unfractionated Microalgae by Extrusion. *Carbohydrate Polymers*, 208(December 2018), 142–151.

Matos, Â. P., Feller, R., Moecke, E. H. S., de Oliveira, J. V., Junior, A. F., Derner, R. B. & Sant'Anna, E. S. (2016). Chemical Characterization of Six Microalgae with Potential Utility for Food Application. *JAOCS, Journal of the American Oil Chemists' Society*, 93(7), 963–972.

Mekonnen, T., Mussone, P., Khalil, H. & Bressler, D. (2013). Progress in Bio-Based Plastics and Plasticizing Modifications. *Journal of Materials Chemistry A*, 1(43), 13379–13398.

Michaud, P. (2018). Polysaccharides from Microalgae, What's Future? *Advances in Biotechnology & Microbiology*, 8(2).

Mishra, A. & Jha, B. (2009). Isolation and Characterization of Extracellular Polymeric Substances from Micro-Algae Dunaliella Salina under Salt Stress. *Bioresource Technology*, 100(13), 3382–3386.

Mitra, D., van Leeuwen, J. (Hans) & Lamsal, B. (2012). Heterotrophic/Mixotrophic Cultivation of Oleaginous Chlorella Vulgaris on Industrial Co-Products. *Algal Research*, 1(1), 40–48.

Miyake, M., Takase, K., Narato, M., Khatipov, E., Schnackenberg, J., Shirai, M., Kurane, R. & Asada, Y. (2000). Polyhydroxybutyrate Production from Carbon Dioxide by Cyanobacteria. *Applied Biochemistry and Biotechnology*, 84, 991–1002.

Miyamoto, K., Wable, O. & Benemann, J. R. (1988). Vertical Tubular Reactor for Microalgae Cultivation. *Biotechnology Letters*, 10(10), 703–708.

Moazami-Goudarzi, M. & Colman, B. (2012). Changes in Carbon Uptake Mechanisms in Two Green Marine Algae by Reduced Seawater PH. *Journal of Experimental Marine Biology and Ecology*, 413, 94–99.

Mohd Yusof, Z. A., Ya, H., Mohd Ishak, Y., Rha, C. K., Mohd Ali, H. & Kumar, K. S. (2015). *Malaysia's Bioplastics Transformation*.

Moheimani, N. R. & Borowitzka, M. A. (2011). Increased CO2and the Effect of PH on Growth and Calcification of Pleurochrysis Carterae and Emiliania Huxleyi (Haptophyta) in Semicontinuous Cultures. *Applied Microbiology and Biotechnology*, 90(4), 1399–1407.

Moheimani, N. R., Borowitzka, M. A., Isdepsky, A. & Sing, S. F. (2013). Standard Methods for Measuring Growth of Algae and Their Composition. *Algae for Biofuels and Energy*, 265–284.

Mokashi, K., Shetty, V., George, S. A. & Sibi, G. (2016). Sodium Bicarbonate as Inorganic Carbon Source for Higher Biomass and Lipid Production Integrated Carbon Capture in Chlorella Vulgaris. *Achievements in the Life Sciences*, 10(1), 111–117.

Mondal, M., Goswami, S., Ghosh, A., Oinam, G., Tiwari, O. N., Das, P., Gayen, K., Mandal, M. K. & Halder, G. N. (2017). Production of Biodiesel from Microalgae through Biological Carbon Capture: A Review. *3 Biotech*, 7(2), 1–21.

Montoya, E. Y. O., Casazza, A. A., Aliakbarian, B., Perego, P., Converti, A. & de Carvalho, J. C. M. (2014). Production of Chlorella Vulgaris as a Source of Essential Fatty Acids in a Tubular Photobioreactor Continuously Fed with Air Enriched with CO2 at Different Concentrations. *Biotechnology Progress*, 30(4), 916–922.

Moreira, D. & Pires, J. C. M. (2016). Atmospheric CO2 Capture by Algae: Negative Carbon Dioxide Emission Path. *Bioresource Technology*, 215, 371–379.

Nakajima, K., Tanaka, A. & Matsuda, Y. (2013). SLC4 Family Transporters in a Marine Diatom Directly Pump Bicarbonate from Seawater. *Proceedings of the National Academy of Sciences*, 110(5), 1767–1772.

National Oceanic and Atmospheric Administration. (2019). Carbon Dioxide Levels in Atmosphere Hit Record High in May. *National Oceanic and Atmospheric Administration*. Retrieved May 13, 2020, from https://www.noaa.gov/news/carbon-dioxide-levels-in-atmosphere-hit-record-high-in-may

Nayak, M., Karemore, A. & Sen, R. (2016). Performance Evaluation of Microalgae for Concomitant Wastewater Bioremediation, CO2 Biofixation and Lipid Biosynthesis for Biodiesel Application. *Algal Research*, 16, 216–223.

Nayak, M., Suh, W. I., Lee, B. & Chang, Y. K. (2018). Enhanced Carbon Utilization Efficiency and FAME Production of Chlorella Sp. HS2 through Combined Supplementation of Bicarbonate and Carbon Dioxide. *Energy Conversion and Management*, 156(August 2017), 45–52.

Nguyen, B. T. & Rittmann, B. E. (2016). Effects of Inorganic Carbon and PH on Growth Kinetics of Synechocystis Sp. PCC 6803. *Algal Research*, 19, 363–369.

Noreen, A., Zia, K. M., Zuber, M., Ali, M. & Mujahid, M. (2016). A Critical Review of Algal Biomass: A Versatile Platform of Bio-Based Polyesters from Renewable Resources. *International Journal of Biological Macromolecules*, 86, 937–949.

North, M. (2014). What Is CO2? Thermodynamics, Basic Reactions and Physical Chemistry, in: *Carbon Dioxide Utilisation: Closing the Carbon Cycle: First Edition*, (pp. 3–17). Elsevier B.V.

Obaja, D., Mace, S. & Mata-Alvarez, J. (2005). Biological Nutrient Removal by a Sequencing Batch Reactor (SBR) Using an Internal Organic Carbon Source in Digested Piggery Wastewater. *Bioresource Technology*, 96(1), 7–14.

Orsuwan, A. & Sothornvit, R. (2018). Polysaccharide Nanobased Packaging Materials for Food Application, in: *Food Packaging and Preservation*, (pp. 239–270).

Paes, C. R. P. S., Faria, G. R., Tinoco, N. A. B., Castro, D. J. F. A., Barbarino, E. & Lourenço, S. O. (2016). Growth, Nutrient Uptake and Chemical Composition of Chlorella Sp. and Nannochloropsis Oculata under Nitrogen Starvation. *Latin American Journal of Aquatic Research*, 44(2), 275–292.

Patel, A. K., Laroche, C., Marcati, A., Ursu, A. V., Jubeau, S., Marchal, L., Petit, E., Djelveh, G. & Michaud, P. (2013). Separation and Fractionation of Exopolysaccharides from Porphyridium Cruentum. *Bioresource Technology*, 145, 345–350.

Peelman, N., Ragaert, P., De Meulenaer, B., Adons, D., Peeters, R., Cardon, L., Van Impe, F. & Devlieghere, F. (2013). Application of Bioplastics for Food Packaging. *Trends in Food Science and Technology*, 32(2), 128–141.

Pereira, S. F. L., Gonçalves, A. L., Moreira, F. C., Silva, T. F. C. V, Vilar, V. J. P. & Pires, J. C. M. (2016). Nitrogen Removal from Landfill Leachate by Microalgae. *International Journal of Molecular Sciences*, 17(11).

Persin, Z., Stana-Kleinschek, K., Foster, T. J., Van Dam, J. E. G., Boeriu, C. G. & Navard, P. (2011). Challenges and Opportunities in Polysaccharides Research and Technology: The EPNOE Views for the next Decade in the Areas of Materials, Food and Health Care. *Carbohydrate Polymers*, 84(1), 22–32.

Phukan, M. M., Chutia, R. S., Konwar, B. K. & Kataki, R. (2011). Microalgae Chlorella as a Potential Bio-Energy Feedstock. *Applied Energy*, 88(10), 3307–3312.

Pires, J. C. M. (2015). Mass Production of Microalgae. *Handbook of Marine Microalgae: Biotechnology Advances*, (1), 55–68.

Polyglobe. (2015). *Biodegradable Plastics: Global Capacities*.
Posadas, E., Morales, M. del M., Gomez, C., Acién, F. G. & Muñoz, R. (2015). Influence of PH and CO2 Source on the Performance of Microalgae-Based Secondary Domestic Wastewater Treatment in Outdoors Pilot Raceways. *Chemical Engineering Journal*, 265, 239–248.
Prasetyo, H., Setyaningsih, I. & Ratih Agungpriyono, D. (2015). Growth and Extracelluler Polysaccaride Production of Porphyridium Cruentum In Various Photoperiod. *Jurnal Pengolahan Hasil Perikanan Indonesia*, 18(2), 220–230.
Ramaraj, R., Tsai, D. D. W. & Chen, P. H. (2014). Freshwater Microalgae Niche of Air Carbon Dioxide Mitigation. *Ecological Engineering*, 68, 47–52.
Rangel, C. de O., Pelizer, L. H., Carvalho, J. C. M., Moraes, I. O., Sassano, C. E. ., Danesi, E. D. G. & Sato, S. (2003). Influence of Inoculum Age and Concentration in Spirulina Platensis Cultivation. *Journal of Food Engineering*, 56(4), 371–375.
Raso, S., van Genugten, B., Vermuë, M. & Wijffels, R. H. (2012). Effect of Oxygen Concentration on the Growth of Nannochloropsis Sp. at Low Light Intensity. *Journal of Applied Phycology*, 24(4), 863–871.
Raven, J. A. & Giordano, M. (2014). Algae. *Current Biology*, 24(13).
Razaghi, A., Godhe, A. & Albers, E. (2014). Effects of Nitrogen on Growth and Carbohydrate Formation in Porphyridium Cruentum. *Central European Journal of Biology*, 9(2), 156–162.
Razzak, S. A., Ali, S. A. M., Hossain, M. M. & deLasa, H. (2017). Biological CO2 Fixation with Production of Microalgae in Wastewater – A Review. *Renewable and Sustainable Energy Reviews*, 76(February), 379–390.
Razzak, S. A., Hossain, M. M., Lucky, R. A., Bassi, A. S. & De Lasa, H. (2013). Integrated CO2capture, Wastewater Treatment and Biofuel Production by Microalgae Culturing - A Review. *Renewable and Sustainable Energy Reviews*, 27, 622–653.
Reddy, N. & Yang, Y. (2010). Citric Acid Cross-Linking of Starch Films. *Food Chemistry*, 118(3), 702–711.
Reddy, R. L., Reddy, V. S. & Gupta, G. A. (2013). Study of Bio-Plastics As Green & Sustainable Alternative to Plastics. *International Journal of Emerging Technology and Advanced Engineering*, 3(5), 82–89.
Reis, K. C., Pereira, L., Melo, I. C. N. A., Marconcini, J. M., Trugilho, P. F. & Tonoli, G. H. D. (2015). Particles of Coffee Wastes as Reinforcement in Polyhydroxybutyrate (PHB) Based Composites. *Materials Research*, 18(3), 546–552.
Richmond, A. & Zou, N. (1999). Efficient Utilisation of High Photon Irradiance for Mass Production of Photoautotrophic Micro-Organisms. *Journal of Applied Phycology*, 11(1), 123–127.
Roca, C., Lehmann, M., Torres, C. A. V., Baptista, S., Gaudêncio, S. P., Freitas, F. & Reis, M. A. M. (2016). Exopolysaccharide Production by a Marine Pseudoalteromonas Sp. Strain Isolated from Madeira Archipelago Ocean Sediments. *New Biotechnology*, 33(4), 460–466.
Rodríguez-Maroto, J. M., Jiménez, C., Aguilera, J. & Niell, F. X. (2005). Air Bubbling Results in Carbon Loss during Microalgal Cultivation in Bicarbonate-Enriched Media: Experimental Data and Process Modeling. *Aquacultural Engineering*, 32(3–4), 493–508.
Sadeghizadeh, A., Farhad dad, F., Moghaddasi, L. & Rahimi, R. (2017). CO2capture from Air by Chlorella Vulgaris Microalgae in an Airlift Photobioreactor. *Bioresource Technology*, 243, 441–447.
Sánchez-Saavedra, M. del P., Castro-Ochoa, F. Y., Nava-Ruiz, V. M., Ruiz-Güereca, D. A., Villagómez-Aranda, A. L., Siqueiros-Vargas, F. & Molina-Cárdenas, C. A. (2018). Effects of Nitrogen Source and Irradiance on Porphyridium Cruentum. *Journal of Applied*

Phycology, 30(2), 783–792.
Sathish, A., Glaittli, K., Sims, R. C. & Miller, C. D. (2014). Algae Biomass Based Media for Poly(3-Hydroxybutyrate) (PHB) Production by Escherichia Coli. *Journal of Polymers and the Environment*, 22(2), 272–277.
Sauvage, J., Spivack, A. J., Murray, R. W. & D'Hondt, S. (2014). Determination of in Situ Dissolved Inorganic Carbon Concentration and Alkalinity for Marine Sedimentary Porewater. *Chemical Geology*, 387(1), 66–73.
Sayre, R. (2010). Microalgae: The Potential for Carbon Capture. *BioScience*, 60(9), 722–727.
Seddon, D. (2010). Other Routes To Olefins From Hydrocarbons, in: *Petrochemical Economics*, (pp. 179–200).
Seligra, P. G., Medina Jaramillo, C., Famá, L. & Goyanes, S. (2016). Biodegradable and Non-Retrogradable Eco-Films Based on Starch-Glycerol with Citric Acid as Crosslinking Agent. *Carbohydrate Polymers*, 138, 66–74.
Seth, J. R. & Wangikar, P. P. (2015). Challenges and Opportunities for Microalgae-Mediated CO2 Capture and Biorefinery. *Biotechnology and Bioengineering*, 112(7), 1281–1296.
Seyed Hosseini, N., Shang, H. & Scott, J. A. (2018). Biosequestration of Industrial Off-Gas CO2 for Enhanced Lipid Productivity in Open Microalgae Cultivation Systems. *Renewable and Sustainable Energy Reviews*, 92, 458–469.
Shank, R. & Kong, I. (2011). Thermoplastic Starch, in: *Thermoplastic Elastomers*, (pp. 95–114).
Shen, L., Worrell, E. & Patel, M. (2010). Present and Future Development in Plastics from Biomass. *Biofuels, Bioproducts & Biorefining*, 4.
Shene, C., Chisti, Y., Bustamante, M. & Rubilar, M. (2016). Effect of CO2 in the Aeration Gas on Cultivation of the Microalga Nannochloropsis Oculata: Experimental Study and Mathematical Modeling of CO2 Assimilation. *Algal Research*, 13(3), 16–29.
Shi, B., Wideman, G. & Wang, J. H. (2012). A New Approach of BioCO2 Fixation by Thermoplastic Processing of Microalgae. *Journal of Polymers and the Environment*, 20(1), 124–131.
Shu, C. H., Tsai, C. C., Chen, K. Y., Liao, W. H. & Huang, H. C. (2013). Enhancing High Quality Oil Accumulation and Carbon Dioxide Fixation by a Mixed Culture of Chlorella Sp. and Saccharomyces Cerevisiae. *Journal of the Taiwan Institute of Chemical Engineers*, 44(6), 936–942.
Singh, D., Nedbal, L. & Ebenhöh, O. (2018). Modelling Phosphorus Uptake in Microalgae. *Biochemical Society Transactions*, 46(2), 483–490.
Singh, S. P. & Singh, P. (2014). Effect of CO2 Concentration on Algal Growth: A Review. *Renewable and Sustainable Energy Reviews*, 38, 172–179.
Singh, U. B. & Ahluwalia, A. S. (2013). Microalgae: A Promising Tool for Carbon Sequestration. *Mitigation and Adaptation Strategies for Global Change*, 18(1), 73–95.
Slav, I. (2019). How Much Crude Oil Does Plastic Production Really Consume ? *Oilprice.com*. Retrieved October 11, 2019, from https://oilprice.com/Energy/Energy-General/How-Much-Crude-Oil-Does-Plastic-Production-Really-Consume.html
Slocombe, S. P., Zhang, Q., Ross, M., Anderson, A., Thomas, N. J., Lapresa, Á., Rad-Menéndez, C., Campbell, C. N., Black, K. D., Stanley, M. S. & Day, J. G. (2015). Unlocking Nature's Treasure-Chest: Screening for Oleaginous Algae. *Scientific Reports*, 5, 9844.
Soanen, N., Da Silva, E., Gardarin, C., Michaud, P. & Laroche, C. (2015). Improvement of Exopolysaccharide Production by Porphyridium Marinum. *Bioresource Technology*, 213, 231–238.
Solovchenko, A. E., Ismagulova, T. T., Lukyanov, A. A., Vasilieva, S. G., Konyukhov, I. V., Pogosyan, S. I., Lobakova, E. S. & Gorelova, O. A. (2019). Luxury Phosphorus Uptake

in Microalgae. *Journal of Applied Phycology*, 31(5), 2755–2770.
Somleva, M. N., Peoples, O. P. & Snell, K. D. (2013). PHA Bioplastics, Biochemicals, and Energy from Crops. *Plant Biotechnology Journal*, 11(2), 233–252.
Šoštariè, M., Golob, J., Bricelj, M., Klinar, D. & Pivec, A. (2009). Studies on the Growth of Chlorella Vulgaris in Culture Media with Different Carbon Sources. *Chem. Biochem. Eng.*, Q. 23 (4)(December).
Spalding, M. H. (1989). Photosynthesis and Photorespiration in Freshwater Green Algae. *Aquatic Botany*, 34(1), 181–209.
Spolaore, P., Joannis-Cassan, C., Duran, E. & Isambert, A. (2006). Commercial Applications of Microalgae. *Journal of Bioscience and Bioengineering*, 101(2), 87–96.
Šprajcar, M., Horvat, P. & Kržan, A. (2012). *Biopolymers and Bioplastics*.
Strickland, J. D. . & Parsons, T. R. (1972). *A Practical Handbook of Seawater Analysis*. Fisheries Research Board of Canada.
Suriyamongkol, P., Weselake, R., Narine, S., Moloney, M. & Shah, S. (2007). Biotechnological Approaches for the Production of Polyhydroxyalkanoates in Microorganisms and Plants - A Review. *Biotechnology Advances*, 25(2), 148–175.
Sutherland, I. W. (2009). Bioplastic and Biopolymer Production. *Encyclopedia of Life Support Systems (EOLSS)*.
Sydney, E. B., Sturm, W., de Carvalho, J. C., Thomaz-Soccol, V., Larroche, C., Pandey, A. & Soccol, C. R. (2010). Potential Carbon Dioxide Fixation by Industrially Important Microalgae. *Bioresource Technology*, 101(15), 5892–5896.
Sydney, E. B., Sydney, A. C. N., de Carvalho, J. C. & Soccol, C. R. (2019). *Potential Carbon Fixation of Industrially Important Microalgae*. Elsevier B.V.
Tai, C. M., Li, R. K. Y. & Ng, C. N. (2000). Impact Behaviour of Polypropylene/Polyethylene Blends. *Polymer Testing*, 19(2), 143–154.
Talukder, M. M. R., Das, P. & Wu, J. C. (2012). Microalgae (Nannochloropsis Salina) Biomass to Lactic Acid and Lipid. *Biochemical Engineering Journal*, 68, 109–113.
Tang, D., Han, W., Li, P., Miao, X. & Zhong, J. (2011). CO2biofixation and Fatty Acid Composition of Scenedesmus Obliquus and Chlorella Pyrenoidosa in Response to Different CO2 Levels. *Bioresource Technology*, 102(3), 3071–3076.
Taziki, M., Ahmadzadeh, H. & A. Murry, M. (2016). Growth of Chlorella Vulgaris in High Concentrations of Nitrate and Nitrite for Wastewater Treatment. *Current Biotechnology*, 4(4), 441–447.
Tebbani, S., Filali, R. & Lopes, F. (2014). Microalgae, in: *CO2 Biofixation by Microalgae: Modeling, Estimation and Control*, (p. 191).
Thangamani, P., Chellappan, A., Citarasu, T. & Thangaswamy, S. (2016). Effect of Different Culture Media for the Growth and Oil Yield in Selected Marine Microalgae. *Journal of Aquaculture in the Tropics*, 31(3–4), 165–177.
Thawechai, T., Cheirsilp, B., Louhasakul, Y., Boonsawang, P. & Prasertsan, P. (2016). Mitigation of Carbon Dioxide by Oleaginous Microalgae for Lipids and Pigments Production: Effect of Light Illumination and Carbon Dioxide Feeding Strategies. *Bioresource Technology*, 219, 139–149.
The British Standards Institution. (2008). Tests for Mechanical and Physical Properties of Aggregates. Part 5: Determination of the Water Content by Drying in a Ventilated Oven. *British Standard*.
The British Standards Institution. (2013). *PAS 600:2013: Bio-Based Products – Guide to Standards and Claims*.
The British Standards Institution. (2018). *Plastics – Determination of Tensile Properties Part 3: Test Conditions for Films and Sheets (ISO 527-3:2018)*.
Thomas, D. M., Mechery, J. & Paulose, S. V. (2016). Carbon Dioxide Capture Strategies from

Flue Gas Using Microalgae: A Review. *Environmental Science and Pollution Research*, 23(17), 16926–16940.
Thompson, R. C., Moore, C. J., Saal, F. S. V. & Swan, S. H. (2009). Plastics, the Environment and Human Health: Current Consensus and Future Trends. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364(1526), 2153–2166.
Tischner, R. (2000). Nitrate Uptake and Reduction in Plants. *Plant, Cell and Environment*, 23, 1005–1024.
Tischner, R. & Lorenzen, H. (1979). Nitrate Uptake and Nitrate Reduction in Synchronous Chlorella. *Planta*, 146(3), 287–292.
Troschl, C., Meixner, K. & Drosg, B. (2017). Cyanobacterial PHA Production — Review of Recent Advances and a Summary of Three Years ' Working Experience Running a Pilot Plant. , 1–20.
US Energy Information Administration. (2016). How Much Oil Is Used to Make Plastic? Retrieved May 17, 2017, from https://www.eia.gov/tools/faqs/faq.php?id=34
Vadlamani, A., Pendyala, B., Viamajala, S. & Varanasi, S. (2019). High Productivity Cultivation of Microalgae without Concentrated CO2 Input. *ACS Sustainable Chemistry and Engineering*, 7(2), 1933–1943.
Velea, S., Ilie, L. & Filipescu, L. (2011). Optimization of Porphyridium Purpureum Culture Growth Using Two Variables Experimental Design: Light And Sodium Bicarbonate. *U.P.B. Scientific Bulletin*, 73(4).
Vert, M., Doi, Y., Hellwich, K. H., Hess, M., Hodge, P., Kubisa, P., Rinaudo, M. & Schué, F. (2012). Terminology for Biorelated Polymers and Applications (IUPAC Recommendations 2012). *Pure and Applied Chemistry*, 84(2), 377–410.
Villay, A., Laroche, C., Roriz, D., El Alaoui, H., Delbac, F. & Michaud, P. (2013). Optimisation of Culture Parameters for Exopolysaccharides Production by the Microalga Rhodella Violacea. *Bioresource Technology*, 146, 732–735.
Wang, J., Chen, B., Rao, X., Huang, J. & Li, M. (2007). Optimization of Culturing Conditions of Porphyridium Cruentum Using Uniform Design. *World Journal of Microbiology and Biotechnology*, 23(10), 1345–1350.
Wang, L., Min, M., Li, Y., Chen, P., Chen, Y., Liu, Y., Wang, Y. & Ruan, R. (2010). Cultivation of Green Algae Chlorella Sp. in Different Wastewaters from Municipal Wastewater Treatment Plant. *Applied Biochemistry and Biotechnology*, 162(4), 1174–1186.
Wang, M., Kuo-Dahab, W. C., Dolan, S. & Park, C. (2014). Kinetics of Nutrient Removal and Expression of Extracellular Polymeric Substances of the Microalgae, Chlorella Sp. and Micractinium Sp., in Wastewater Treatment. *Bioresource Technology*, 154, 131–137.
White, D. A., Pagarette, A., Rooks, P. & Ali, S. T. (2013). The Effect of Sodium Bicarbonate Supplementation on Growth and Biochemical Composition of Marine Microalgae Cultures. *Journal of Applied Phycology*, 25(1), 153–165.
Xiao, R. & Zheng, Y. (2016). Overview of Microalgal Extracellular Polymeric Substances (EPS) and Their Applications. *Biotechnology Advances*, 34(7), 1225–1244.
Yen, H. W., Hu, I. C., Chen, C. Y., Ho, S. H., Lee, D. J. & Chang, J. S. (2013). Microalgae-Based Biorefinery - From Biofuels to Natural Products. *Bioresource Technology*, 135, 166–174.
Yoo, M., Han, S. J. & Wee, J. H. (2013). Carbon Dioxide Capture Capacity of Sodium Hydroxide Aqueous Solution. *Journal of Environmental Management*, 114, 512–519.
Yu, C. H., Huang, C. H. & Tan, C. S. (2012). A Review of CO2 Capture by Absorption and Adsorption. *Aerosol and Air Quality Research*, 12(5), 745–769.
Yu, S., Blennow, A., Bojko, M., Madsen, F., Olsen, C. E. & Engelsen, S. B. (2002). Physico-Chemical Characterization of Floridean Starch of Red Algae. *Starch/Staerke*, 54(2), 66–

74.
Yun, Y., Lee, S. B., Park, J. M., Lee, C. & Yang, J. (1997). Carbon Dioxide Fixation by Algal Cultivation Using Wastewater Nutrients. *Journal of Chemical Technology & Biotechnology*, 69(4), 451–455.
Yun, Y. S., Park, J. M. & Yang, J. W. (1996). Enhancement of CO2 Tolerance of Chlorella Vulgaris by Gradual Increase of CO2 Concentration. *Biotechnology Techniques*, 10(9), 713–716.
Zainal, A., Yahya, L., Chik, M. N., Hussien, N. K. & Mohd, H. (2017). Effect of Zeolite 4A to Marine Microalgae Culture. *Chemical Engineering Transactions*, 56, 487.
Zavareze, E. da R., Storck, C. R., de Castro, L. A. S., Schirmer, M. A. & Dias, A. R. G. (2010). Effect of Heat-Moisture Treatment on Rice Starch of Varying Amylose Content. *Food Chemistry*, 121(2), 358–365.
Zawar, P., Javalkote, V., Burnap, R., Mahulikar, P. & Puranik, P. (2016). CO2 Capture Using Limestone for Cultivation of the Freshwater Microalga Chlorella Sorokiniana PAZ and the Cyanobacterium Arthrospira Sp. VSJ. *Bioresource Technology*, 221, 498–509.
Zeebe, R. E. & Wolf-Gladrow, D. (2001). Equilibrium, in: *CO2 in seawater: Equilibrium, kinetics, isotopes*, (pp. 1–84).
Zeller, M. A., Hunt, R., Jones, A. & Sharma, S. (2013). Bioplastics and Their Thermoplastic Blends from Spirulina and Chlorella Microalgae. *Journal of Applied Polymer Science*, 130(5), 3263–3275.
Zhang, F., Kabeya, H., Kitagawa, R., Hirotsu, T., Yamashita, M. & Otsuki, T. (2000). Exploratory Research of PVC-Chlorella Composite Material (PCCM) as Effective Utilization of Chlorella Biologically Fixing CO2. *Journal of Materials Science*, 35(10), 2603–2609.
Zhang, K., Kurano, N. & Miyachi, S. (2002). Optimized Aeration by Carbon Dioxide Gas for Microalgal Production and Mass Transfer Characterization in a Vertical Flat-Plate Photobioreactor. *Bioprocess and Biosystems Engineering*, 25(2), 97–101.
Zhang, W., Heaven, S. & Banks, C. J. (2018). Degradation of Some EN13432 Compliant Plastics in Simulated Mesophilic Anaerobic Digestion of Food Waste. *Polymer Degradation and Stability*, 147(November), 76–88.
Zhang, W., Zhang, Z. & Yan, S. (2015). Effects of Various Amino Acids as Organic Nitrogen Sources on the Growth and Biochemical Composition of Chlorella Pyrenoidosa. *Bioresource Technology*, 197, 458–464.
Zhao, B. & Su, Y. (2014). Process Effect of Microalgal-Carbon Dioxide Fixation and Biomass Production: A Review. *Renewable and Sustainable Energy Reviews*, 31, 121–132.
Zhao, B., Su, Y., Zhang, Y. & Cui, G. (2015). Carbon Dioxide Fixation and Biomass Production from Combustion Flue Gas Using Energy Microalgae. *Energy*, 89, 347–357.
Zhao, H., Dilmore, R., Allen, D. E., Hedges, S. W., Soong, Y. & Lvov, S. N. (2015). Measurement and Modeling of CO2 Solubility in Natural and Synthetic Formation Brines for CO2 Sequestration. *Environmental Science and Technology*, 49(3), 1972–1980.
Zhou, W., Wang, J., Chen, P., Ji, C., Kang, Q., Lu, B., Li, K., Liu, J. & Ruan, R. (2017). Bio-Mitigation of Carbon Dioxide Using Microalgal Systems: Advances and Perspectives. *Renewable and Sustainable Energy Reviews*, 76, 1163–1175.
Zhu, X. G., Long, S. P. & Ort, D. R. (2008). What Is the Maximum Efficiency with Which Photosynthesis Can Convert Solar Energy into Biomass? *Current Opinion in Biotechnology*, 19(2), 153–159.
Zia, K. M., Tabasum, S., Nasif, M., Sultan, N., Aslam, N., Noreen, A. & Zuber, M. (2017). A Review on Synthesis, Properties and Applications of Natural Polymer Based Carrageenan Blends and Composites. *International Journal of Biological Macromolecules*, 96, 282–301.

Zimmerman, W. B., Zandi, M., Hemaka Bandulasena, H. C., Tesař, V., James Gilmour, D. & Ying, K. (2011). Design of an Airlift Loop Bioreactor and Pilot Scales Studies with Fluidic Oscillator Induced Microbubbles for Growth of a Microalgae Dunaliella Salina. *Applied Energy*, 88(10), 3357–3369.