Fermentation CO_2 biosequestration by microalgae for the production of health beneficial natural compounds

by

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A thesis submitted in fulfillment
of the requirements for the degree of
Master of Applied Science (M.A.Sc) in Engineering Science

The Faculty of Graduate Studies
Laurentian University
Sudbury, Ontario, Canada

THESIS DEFENCE COMMITTEE/COMITÉ DE SOUTENANCE DE THÈSE
Laurentian Université/Université Laurentienne
Office of Graduate Studies/Bureau des études supérieures

Title of Thesis Titre de la thèse	Fermentation CO_2 biosequestration by microalgae for the production of health beneficial natural compounds
Name of Candidate Nom du candidat	Yavari, Nasim
Degree Diplôme	Master of Science
Department/Program Département/Programme	Engineering
Date of Defence Date de la soutenance	April 21, 2022

APPROVED/APPROUVÉ

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Abstract

In this work, three different microalgal strains were investigated for effective biosequestration of CO_2 generated by beer (yeast) fermentation. They were a culture collection *Chlamydomonas reinhardtii* and two bioprospected strains, *Coccomyxa* sp. (P-918) obtained from a polishing pond at an operational smelter (pH 2.8) and *Chlamydomonas* sp. (M-23) obtained from a low pH abandoned mine site (pH 3). The three strains were investigated for use in beer fermentation CO_2 fixation and their production of both lipid and protein. The pH of beer fermentation CO_2-enriched *Chlamydomonas reinhardtii* cultures varied between 4.5 and 7.3 throughout the experiment. For the *Chlamydomonas* sp., pH varied between 4.6 and 7.1 and for *Coccomyxa* sp. between 5.3 and 7.4. As expected, during higher fermentation activity (day 1 to 3 for each beer kit), more CO_2 was released to the microalgal cultures causing a drop in pH. When the rate of fermentation slowed in day 4, there was an increase in pH.

For all three microalgal cultures which were grown only under atmospheric CO_2 (controls), the pH increased continuously along with microalgal growth over 16 days of experiment. *Chlamydomonas reinhardtii* control culture pH was 6.7 at the start of the experiment and reached 8.7 at day 16. For the *Chlamydomonas* sp. control culture, pH increased steadily from 6.6 to 8.4, and for *Coccomyxa* sp. from 7 to 8.6.

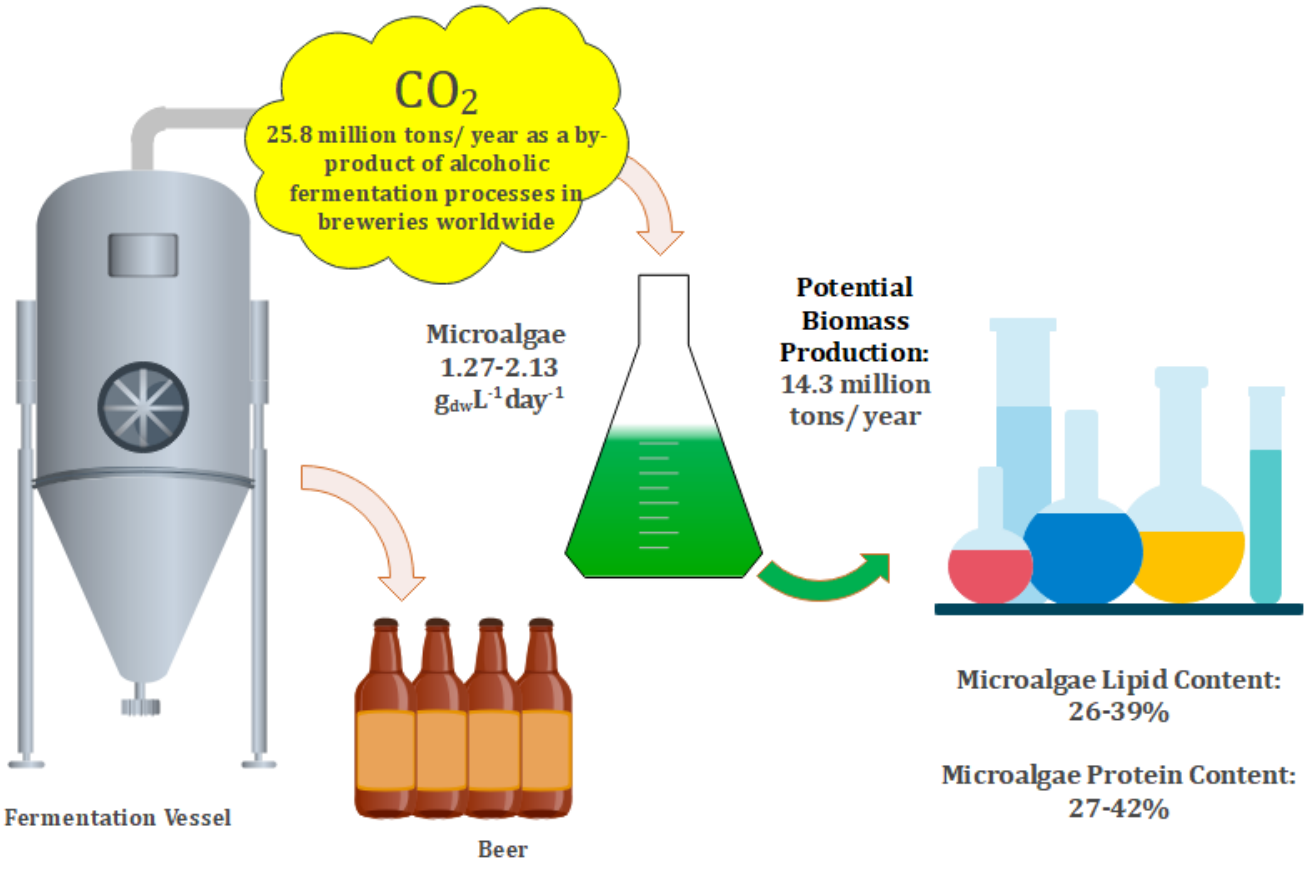
Experimental results indicated that the bioprospected *Coccomyxa sp.* adapted well to the low pH created by sparging in beer fermentation CO_2. Its volumetric biomass productivity was 0.124 $g_{dw}L^{-1}day^{-1}$, which was higher than both *Chlamydomonas reinhardtii* (0.072 $g_{dw}L^{-1}day^{-1}$) and bioprospected *Chlamydomonas* sp. (0.086 $g_{dw}L^{-1}day^{-1}$). The *Coccomyxa sp.* when exposed to fermentation CO_2 reached a maximum specific growth rate of 0.167 day^{-1}, which was 29% higher than achieved without sparging in fermentation CO_2. Moreover, its carbon fixation rate increased from 122.1 to 227.9 mg_{CO2} L^{-1} day^{-1} with fermentation CO_2.

However, lipid synthesis occurred more rapidly and efficiently in *Chlamydomonas* sp. and *Chlamydomonas reinhardtii* rather than *Coccomyxa* sp., reaching 39% and 35% of biomass dry weight after 16 days of beer fermentation CO_2 exposure. Whereas the amount of lipid in *Coccomyxa sp.* was 26% of the biomass dry weight at 16 days. This would indicate that the bioprospected *Chlamydomonas* sp. was a better candidate for biofuel production as its dry weight lipid content increased from 20% to 39% when exposed

fermentation CO_2. While the lipid content of *Chlamydomonas* sp. culture that grew under atmospheric CO_2 reached 24% of biomass dry weight at the end of experiment (day 16) from its initial 20%. It was found that protein content with fermentation CO_2 was 42.5% of *Coccomyxa sp.* biomass dry weight. Protein content of *Chlamydomonas reinhardtii* and *Chlamydomonas* sp. dry weight were 30.7% and 27.4%, respectively.

Keywords

Microalgae, CO_2 Sequestration, Fermentation, Biodiesel, health beneficial natural compounds



Graphical Abstract

Acknowledgments

I would like to express my sincere gratitude to my supervisor Dr. John Ashley Scott, whose expertise was invaluable in formulating the research questions and methodology. For providing this project with funding and entrusting me with his time, invaluable ideas and constructive criticism. His vision, sincerity and motivation have deeply inspired me. I could not have asked for a better mentor for my M.A.Sc studies. I very much appreciate Dr. Gerusa Senhorinho for all the unwavering support, guidance and friendship she provided when I was trying to figure things out. Her insight and knowledge into my thesis subject steered me through this research. I would also like to extend my thanks to Dr. Corey Laamanen, his insightful feedback brought my work to a higher level and his valuable contribution to my project paved my way throughout this journey. I would also like to give my special thanks to my external examiner, Dr Suzana T Cunha Lima for her insight and advice. I want to thank my lab mates in the Ongen group, Jacob Comley, and Claire Fawcett, for such a supportive atmosphere in the lab, you made it memorable, thank you.

I am extremely grateful to my family, their trust and support made it all possible. To my parents, Monireh Jannat and Naser Yavari for their caring and sacrifices, for believing in me and inspiring me to follow my dreams and to each of my brothers Poria and Kian for being all I could have needed as a sibling and a friend.

My appreciation also goes out to my friends Monica Motamedi, Mohammad Safavi and Farzaneh Sami for their profound belief in my work and encouragement through all the ups and down of my studies.

Last but not least, I would like to thank Sudbury Integrated Nickel Operations (a Glencore Company) for their generous funding through the Mitacs program and making this project possible.

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List of Abbreviations

Abbreviation	Description
ABV	Alcohol by Volume
BBM	Bold's Basal Medium
BSA	Bovine Serum Albumin
$CaC_{l2}.2H_2O$	Calcium Chloride Dihydrate
CO_2	Carbon dioxide
$C_6H_{12}O_6$	Glucose
C_2H_6O	Ethanol
CR	*Chlamydomonas reinhardtii*
$C_{b,i}$	Initial Dry Biomass
$C_{b,f}$	Final Dry Biomass
COOH	Carboxyl group
CH_3	Methyl group
CO	Carbon Monoxide
DCW	Dry Cell Weight
EAA	Essential Amino Acid
FG	Final Gravity
K_2HPO_4	Dipotassium phosphate
KH_2PO_4	Monopotassium phosphate

Abbreviation	Description
HSO^{3-}	Hydrogen sulfite
HCL	hydrochloric acid
M-23	*Chlamydomonas* sp.
$MgSO_4.7H_2O$	*Magnesium sulfate heptahydrate*
NO_X	Nitrogen Oxides
NaOH	Sodium hydroxide
$NaNO_3$	Sodium nitrate
NaCl	Sodium chloride
OG	Original Gravity
P-918	*Coccomyxa* sp.
Pv	volumetric biomass productivity
RC	CO2 fixation rates
SO_4^{2-}	sulfate
SO_3^{2-}	Sulfite
SO_X	Sulfur oxides
SD	Standard Deviation
μ	Microalgal specific growth rate
Δt	Growth Phase Duration

Chapter One: Literature Review

1.1 Introduction

Global warming, with an approximate rate of 0.2°C per decade, is occurring even with the implementation of greenhouse gases mitigation policies around the world. Carbon dioxide (CO_2) emitted from various industrial processes plays a key role in this global environmental issue. As historical emission reduction has not done enough to limit global warming, carbon capture, combined with emission reduction, is required. For the combined approach to be successful, collaboration between countries and industries is necessary [1]. Specifically, to meet the Copenhagen Accord target (reducing global emissions to keep the rise of average global temperature below 2°C) it is suggested that the cumulative carbon emissions should not exceed 1100 Gt from 2011 to 2050 [2]. According to global energy review, till the end of 2020, approximately 323 Gt CO_2 has been emitted to the atmosphere, which does not show the expected progress in carbon capture to reach Copenhagen Accord target [3, p. 2020].

Over the past decade, the environmental impact of beverage production has drawn more attention as a result of noticeable expansion of ethanol-based industries [4]. A joint commitment was formed in 2015, the Beverage Industry Environmental Roundtable, with 19 major commercial companies participating and aiming to reduce greenhouse gasses emissions. Mandatory and voluntary actions are taken internationally towards climate change mitigation and beer producers are allocating funds for conducting research on the advantages and difficulties of improving sustainable production methods to reduce emissions for both craft and large scale breweries [5]. While industry wide, it has been reported that a growing number of beverage companies have begun to measure and reduce greenhouse gas emissions through their operations [5].

To regulate and reduce greenhouse gases emissions, carbon capture and storage technologies have been developed. These methods can serve as a commercially feasible ways to capture and fix CO_2 emissions, owing to its considerable potential [6]. There are various types of carbon capture, including cryogenic methods, pressure swing adsorption, gas membrane separation and solvent absorption. The most expensive stage is capturing the CO_2, which contributes 80-90% of the total expenditure when including subsequent storage and transportation [7]. However, biological CO_2 fixation has been introduced as a more cost effective and sustainable strategy [8]. The reason is that current, non-biological technologies have large

energy demands and are not cost effective when scaled to commercial sizes [9]. Terrestrial sequestration, oceanic fertilization, and phytosequestration are three common categories of biological sequestration techniques [8]. It is anticipated that over time, microalgal sequestration, which is an environmentally sustainable CO_2 sequestration technology will become economically feasible. As a result, microalgae have attracted a growing attention due to their interesting and unique characteristics.

The beverage industry is a prime target for biological carbon capture. For example, beer is the fifth most consumed beverage worldwide, at a rate of 9.6 L/capita annually with a growing consumption trend [5] [10]. According to Moreno et al., approximately 25.8 million tons of CO_2 is produced worldwide as a by-product of alcoholic fermentation processes on a yearly basis [11]. Interestingly, CO_2 produced during alcoholic fermentation can be used as a highly available and clean carbon source for cultivation of photosynthetic microorganisms, such as eukaryotic microalgae. Phototrophic microalgae have the ability to convert the energy from sunlight to chemical energy and utilize CO_2 as a carbon source, with a 10 to 50 times greater efficiency than terrestrial plants. Biomass that is generated through photosynthesis is a value added material that can be used in a wide range of applications, such as food or biofuel production [12].

Stoichiometrically, for the production of 1 L of ethanol, 0.76 kg of carbon dioxide will be released during the fermentation process [2]. It has been reported that carbon accounts for about 50% of microalgal biomass and by producing 1 kg of microalgal biomass, approximately 1.8 kg of CO_2 is fixed [9], [13]–[15]. This means that for every liter of ethanol produced in fermentation approximately 0.42 kg of microalgal biomass could be produced.

Microalgae have attracted attention due to their potential for carbon capture, particularly arising from their fast growth rate, which is approximately 100 times faster than terrestrial plants and their significant contribution to the global carbon cycle stabilization [16]. It has been reported that microalgal biomass can double in less than 24 hours [9]. Moreover, arable land is not required for microalgal biomass production. Some microalgae have the potential to grow efficiently in wastewater and saline waters, which are promising features in order to produce feedstock compared to terrestrial crops [12]. The microalgal large surface-to-volume ratio and simple cellular structure give them the advantage of uptaking considerable amounts of nutrients from water, which contributes to their fast growth rates. They can also easily adapt and thrive in harsh environmental conditions [8] [17].

Apart from the aforementioned cultivation advantages, microalgae are also able to convert CO_2 into organic carbon and synthesize various biomolecules, like carbohydrates, lipids, proteins, and secondary metabolites. The produced biomass has the capacity to be used to produce biofuels, food, pharmaceuticals and nutraceuticals, animal feed, and other value-added products [12]. Of particular interest are a diverse group of secondary metabolites produced by microalgae. These molecules are not required for basic cell function and are responsible to help cells deal with environmental stressors [18], [19]. Secondary metabolites are of particular interest as they have been linked to therapeutic properties and application as immunostimulator, antioxidant and anti-inflammatory agents [20].

Microalgae are also considered as a promising feedstock for biodiesel production due to their combination of shorter cultivation times in comparison with territorial plants and high oil contents [19]. As energy security has become a growing concern over past decades, many countries are encouraging policies that emphasize renewable energy production. Currently, renewable energy accounts for 13% of the total energy production worldwide and bioenergy constitutes almost 10% of all renewable energy production. Bioenergy is referred to gaseous, liquid, solid energy content that is derived from biological raw materials. Bioenergy is categorized in biofuels that are used for transportation, such as bioethanol and biodiesel, wood chips and pellets that are used to produce electricity and heat, and biogas that are formed by processing municipal and industrial waste biological materials [21].

However, in spite of many studies that have been carried out on biological CO_2 sequestration through microalgal cultivation, there is a lack of information on the mitigation of CO_2 from fermentation processes and microalgal production utilizing clean sources of CO_2 to produce valuable products. As such, this review focused on addressing that gap by outlining the microalgal biosequestration developments, restrictions and the prospective potential of fermentation processes as a source of clean CO_2 to produce value-added products and biofuel.

1.2 A short history of microalgae-based systems development

Historically, the idea that microalgae could be considered as a source of renewable fuels and a potential protein source for food production was proposed during 1940s and 1950s. Larger-scale production and investigation of the engineering requirements for microalgal production systems started in 1948–1950 at the Stanford Research Institute. Utilization of flue gas CO_2 from industry was an option in Germany and Japan using strains of *Chlorella*. The first important pilot-scale outdoor system was designed for *Chlorella*

production in 1951 in Massachusetts. Large-scale microalgal culture production was soon tested in other countries, including Germany, Netherland and Japan [22].

By the end of 1950s, fundamental advances were made in the use of algae in wastewater treatment, such as understanding of algal light capture and photosynthesis, optimization of algal productivity, autoinhibitory effect and the sexuality and genetics of microalgae. Following that, the commercial production of microalgae, to be used specifically as nutraceuticals began in the 1960s, and evaluating the possibility energy production carried out in 1970s. *Chlorella* was the first algae that was used for large-scale commercial production in Japan in the 1960's, followed by *Spirulina* in Mexico and Thailand in the 1970's. By 1980, 46 large-scale factories were producing more than 12,000 kg of microalgae annually around the world [12], [22]. *Dunaliella salina* as a source of β-carotene, was established in Australia in the late 1980s. During 1990s, the freshwater green alga *Haematococcus pluvialis* started to be produced as a source of the carotenoid astaxanthin, which was mainly sold as an antioxidant and nutraceutical [23].

In order to increase photosynthetic efficiency, genetic manipulation of algal strains has been performed. One example of such a manipulation is reducing the size of the light-harvesting antenna, which remained at the laboratory scale in late 1990s. It was concluded that the ideal algae for production of biofuels would probably be different for various locations, especially for those growing in outdoor ponds. It was also revealed that to find highly productive strains, microalgal growth rate should be determined, the growth conditions optimized for large-scale systems, and more than one strain should be used for maximizing productivity throughout the year [22]. According to studies during 1990s [24], [25] technical feasibility of microalgal cultivation and harvesting was not limited by fundamental engineering issues such as energy, water and nutrient (e.g., CO_2) requirements, but by general system considerations or harvesting technologies. At that time, although total biomass and algal lipids productivities were acceptable or even high, they were not economically viable. Eventually, it was concluded that microalgal biofuels production would not be economically feasible unless it is integrated with treatment of wastewater [26].

In parallel with these studies, in 1990 a research program was launched at the Research Institute of Innovative Technology for the Earth aimed to develop sustainable methods of CO_2 biological fixation. The proposed scheme focused on five important areas including development of high-density, large-volume photosynthetic microalgae with high CO_2 fixation capability; utilization of solar radiation for photosynthesis; energy and useful compound production [27]. This program alongside other studies [28] [29] lead to the conclusion that microalgae could be grown using untreated flue gas, which contained CO_2

coming from power stations. This was a major result both in CO_2 bioremediation and for future work on microalgal growth in order to gain biofuels using a flue gas from an industrial plant as a source of CO_2 [22]. It was found that the green alga *Chlorococcum littorale* and the rhodophyte *Galdie*ria *partita* were a high-CO_2 and temperature tolerant strains, respectively [30]–[33]. Moreover, some pond studies at small scale were done close to heavy industries and electric utilities and a green alga strain called *Tetraselmis* was found stable throughout all seasons with a mean productivity of about 11 g m^{-2} day^{-1} per year[34]. Interestingly, studies involving *Tetraselmis* and lipid extraction and transesterification for biodiesel production have been attracting significant attention recently [35] [36].

CO_2 bio-mitigation and biodiesel production efficiency have been largely investigated and developed to date either with pure CO_2 or simulated flue gas [37] [38]. It is stated that the microalgal growth rate was significantly enhanced for microalgae exposed to 1 to 15% of CO_2 in comparison with atmospheric air (0.04% CO_2) during a quite short time frame like 18 days. Lipid and carbohydrate in the resulting biomass can be processed to produce biodiesel and bioethanol, respectively. A sustainable carbon cycle can be reached by applying this method, as CO_2 emission from biofuel combustion can be absorbed for microalgal cultivation [9].

It has been reported during the last decade that the global biomass market is dominated by *Spirulina* with the production rate of 5000 tonnes per year. This number is followed by *Chlorella* and *Dunaliella*, with production rates of 2000-4000 and 1000 tonnes per year, respectively [39]. It is anticipated that the global market of microalgae-based products will reach about US $ 5.343×10^{10} by 2026, while it was US $ 3.26×10^{10} in 2017, which would represent 65% increase [40].

The industrial scale production of microalgal valuable fractions is still under development, and it is anticipated that this limited market will expand with the improvement of low-cost biomass production and more efficient processing methods [34].

1.3 Bioprospecting

When deciding to establish a specific microalgae production plant, it is of great importance to select a proper microalgal strain for the targeted purposes of the production. Typically, it is not a good solution to select a traditional strain simply because its characteristics are well-understood. Metabolic pathways and growth characteristics of the chosen strain should be close to the final phenotype. Microalgae are a diverse

group of microorganisms and make bioprospecting a first and essential step in identifying the optimal bioproduct for a special purpose [41]. Bubbling flue gases in photobioreactors will cause acidification of the culture, which can result in a sharp decrease in microalgal growth productivity. Although it can be a stressor to induce the accumulation of targeted metabolites, additional process complications will be necessary in order to neutralizing the medium, such as costs from adding neutralizing chemicals, like lime. As such, bioprospecting certain water bodies with low pH, like those historically influenced by mining operations, provides potential to isolate acidophilic or acid-tolerant cells that could be used, to make systems more economically effective, and reduce the risk of undesired invasive species and contaminant organisms.

Acidophilic microalgae have the ability to consume CO_2 equally compared to when they are cultivated at neutral pH, but at lower bicarbonate concentrations. CO_2 Bio-fixation rates varies among acidophilic microalgae and depends on both pH and CO_2 concentrations [42, p.]. For example, it was reported that acidophilic *Chlamydomonas acidophila* showed higher net photosynthetic rates in comparison with neutrophilic *Chlamydomonas reinhardtii* [43]. However, under nutrient deprivation, lipid and carbohydrate accumulation in neutrophilic microalgae showed similar trends as acidophilic microalgae [44]. This study focuses on green microalgal response to acidic conditions, to obtain organisms that can be cultivated with an accepted productivity in low pH environments originating from acidic water bodies in order to biosequestration of fermentation CO_2 sources.

1.4 Industrial flue gas utilization for microalgal growth

Research has been conducted in the area of combination of industrial carbon sequestration and microalgal biomass production [16] [45] [12] [8] [46] [47] as a source of biofuel and value-added products. It is reported that CO_2 mole percentage in examined flue gas can vary from 7-30% and still be effective for microalgal cultivation. For instance, natural gas combustion fume includes 9%, or less, carbon dioxide. While for coal-fired power generation, cement production and steel and iron production plants are 10%, 20% and about 30%, respectively [48]. Growth of microalgae through photosynthesis will not occur without carbon dioxide [16], and as atmospheric CO_2 concentration is approximately 360 ppm and CO_2 diffusion into liquid phase is slow, insufficient CO_2 supply to the microalgae culture may result in a slower microalgae growth and productivity. As such, it follows that sparging CO_2 to the cultivated medium would accelerate CO_2 transfer to microalgae and increase the growth rate. To achieve this, a CO_2 pump will be

required, however this will also raise the total cost of biomass and biofuel production. It has been reported that the mass transfer of CO_2 between gas and liquid phases can be increased by direct CO_2 bubbling from an anaerobic biological source [64].

However, using industrial flue gases as CO_2 sources can impose microalgae with various new environmental stressors, as flue gases can contain heavy metals, other particulate matter, and can acidify the growth medium. Acidification can occur as a result of the CO_2 content in flue gas, but can also be increased significantly as the gas may contain more than 100 ppm NO_X and SO_X, which results in further acidification of the microalgal culture [49]. While SO_X and NO_X tolerant strains have been identified, they are reported to be effective only at lower concentrations of these acidic gases. At higher concentrations of SO_X and NO_X, controlling the pH drop by addition of a basic component (such as sodium hydroxide (NaOH)) is required, which is costly and causes additional adverse effects on the culture due to high ionic strengths [50] [51]. Actually, flue gas composition can lead to an extreme change of pH varying from pH 7 to pH 2 [52]. Moreover, as flue gas is often released at more than 150°C, microalgae can be exposed to a sudden stress of high temperature and growth rate will be inhibited [53] [54]. Putting microalgae under these condition decreases their growth rate causing a reduction in their ability to fix CO_2 [55] [52].

Due to the potential stressors resulting from the application of industrial flue gas, microalgal species selection is of utmost importance or some modification in the microalgae strain may be needed [52] [16]. However, in comparison with most industrial flue gas, fermentation process flue gas is a mostly pure source of CO_2 without any contamination that may threaten human health, which allows for the use of the produced biomass in food or pharmaceutical production. In comparison with mentioned sources, fermentation processes can be considered a small source of CO_2 emission, but a highly pure and clean one [7]. Also, as a result of tightening CO_2 regulations and vast growth in the ethanol industry, more attention is needed to CO_2 emissions from fermentation processes.

1.5 Estimation of CO_2 released during fermentation

Ethanol concentration is the factor that can be used to classify the three main kinds of alcoholic beverage, beer, wine, and spirits, with approximate ethanol percentage of 4-6, 10-16 and 40, respectively. The World Health Organization (WHO) reviewed alcohol consumption and reported that the total pure alcohol consumed per capita was 6.2 L in 2010 with the participation of people older than 15 years in 194 countries. This amount would correspond to approximately $34{,}177.5 \times 10^6$ L of pure ethanol per annum. More than

half of the consumption was in the form of spirit and approximately a quarter of this amount was not sold in normal controls of government. An estimation of approximately 25.8 million tons of CO_2 production as fermentation by-product per annum can be driven from (Eq. (1)) [11].

Fermentation takes place according to the reaction equation (Eq. (1.1)). Glucose is used by yeast under anaerobic reaction conditions to produce two moles of CO_2 and two moles of ethanol per mole of glucose. From moles to mass conversion, it can be calculated that each gram of glucose theoretically forms 0.51 and 0.49 g of ethanol and CO_2, respectively [2], [11].

$$C_6H_{12}O_6 \xrightarrow{yeast} 2C_2H_6O + 2CO_2 \qquad \text{Eq.1.1}$$

The produced CO_2 is a saturated gas that can be considered as a pure CO_2 stream [7].

For the biosynthesis of one ton of dried biomass of microalgae, 1.8 ton CO_2 can be mitigated [53], theoretically, which is a potential resource for various end-products like biofuels and food additives [41]. Therefore, on average, 14.3 million tons of dried microalgal biomass could be produced by utilizing all the CO_2 from fermentation worldwide.

Utilizing the CO_2 from alcoholic fermentation processes as a raw material to produce beneficial products is an effective strategy to produce chemical feedstock that is a clean source of food. In comparison with industrial flue gas utilization, little work has been done on examining the integrated system of photo-bioreactors and yeast fermenters. Three outstanding merits of this strategy are that the CO_2-rich gas is released from fermentation close to room temperature; produced CO_2 is extremely pure; and thus, the large-scale equipment for the pretreatment of flue gas prior to utilization is not required [4]. Generally, in all other, non-biological, CO_2 mitigation methods it is required to separate CO_2 from the industrial flue gas, whereas biological mitigation using microalgae does not required that separation [12]. Moreover, there is no SO_X and NO_X in the fermentation flue gas, which results in more ideal cultivation conditions without reduced environmental stressors for microalgae growth. While compared to operations that are not coupled to industrial CO_2 sources, the need for CO_2 storage and processing is eliminated, which reduces 40% of the cost of raw materials required for microalgae cultivation [57].

1.6 Effect of CO_2 on microalgal species

CO_2 concentrations greater than atmosphere levels can stimulate microalgal growth, allowing the use CO_2 from different industrial flue gases to enhance production [58]. Microalgae are able to fix CO_2 through photosynthesis to reduce carbon emissions to the atmosphere and the subsequently harvested microalgal biomass is a source of potential value-added compounds. However, it should be considered that high concentrations of carbon dioxide can be toxic to some microalgal species, while other strains such as *Chlorella* sp. [16], *Scenedesmus* sp. and *Spirulina* sp. have the capacity to tolerate high CO_2 concentrations. The tolerance of various strains of microalgae to CO_2 seems to be dissimilar [59]. According to previous studies, the CO_2 concentration that microalgae can tolerate ranges from 14 to 100 % [60] [41] [61]. For instance, studies revealed that *Chlorella* sp. and *Scenedesmus* sp. were able to grow with 100% and 80% CO_2 concentration, respectively. Although 10 to 20 % of CO_2 concentration was sufficient to achieve the maximum growth rate [62]. Achieving higher biomass and lipid productivities is possible by applying optimum CO_2 quantities and concentration to the microalgal culture. However, it was suggested that in order to get better results, parameters such as microalgal species, pH, light intensity, temperature and medium composition should be taken into account [63] [13] [50].

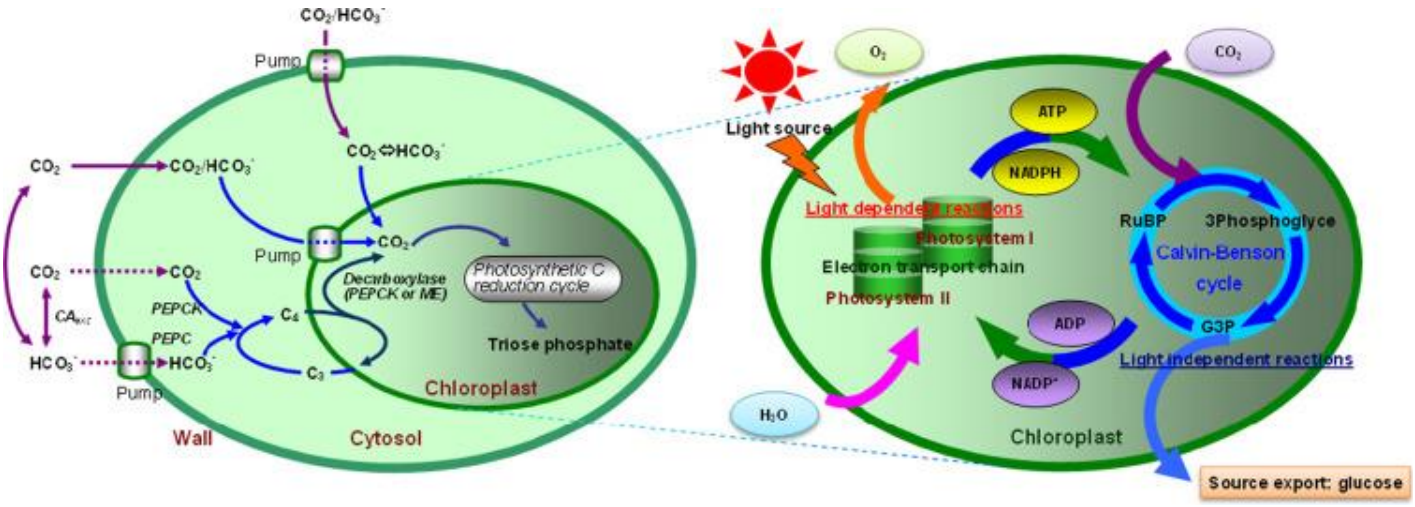

Figure 1-1: Photobiochemical principles of microalgal-CO2 fixation: (left) typical carbon concentrating mechanism and (right) photosynthesis process [64].

CO_2 can be fixed inside the microalga chloroplast by Rubisco to produce two molecules of 3-phosphoglycerate (Figure 1-1). Through some steps including a series of reactions, these two organic acids

are formed as substrates for production of oil and starch. for Calvin Cycle by Rubisco, oxygen can compete with CO_2. The enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the rate-limiting enzyme in the Calvin cycle.

Products of the oxygenase reaction are 2-phosphoglycolate and 3-phosphoglycerate. 2-Phosphoglycolate is metabolized to glycine subsequently, which can result in the CO_2 loss when condensed with another glycine molecule to synthesize serine. This loss of carbon inhibits the Calvin cycle to regenerate the five-carbon sugar substrate ribulose bisphosphate, which are required for Calvin cycle by Rubisco. This will cause further efficiency reduction of photosynthesis. This overall process is called photorespiration owing to the fact that it occurs mainly in the presence of light. It is reported that the photorespiration process reduces efficiency of photosynthetic carbon fixation by 20–30% [65]. Microalgae have the ability to pump and store adequate CO_2 reducing the effect of this competition between oxygen and CO_2 on Calvin cycle by Rubisco. This ability will elevate internal CO_2 concentrations above equilibrium levels with air [66].

Three categories of microalgae, according to their CO_2 tolerance, have been defined. It should be emphasized that CO_2 tolerance differs greatly not only between species, but also within a single species as well, depending on microalgal strain growth conditions. Generally, species inhibited by less than 2 to 5% CO_2 are considered to be CO_2-sensitive. Strains that withstand up to 20% CO_2 are denoted to as CO_2-tolerant, and the species can cope with higher CO_2 concentrations (up to 100%) are referred as extremely CO_2-tolerant [67].

Many researchers have investigated microalgal CO_2 biosequestration based on characteristics of different cultivation conditions (Table 1-1). According to studies [36], [52], [56], [57], [58] CO_2 enrichment, which is the CO_2 amount that let microalgae meet their photosynthesis potentials, is an efficient technique to enhance microalgal cultures productivity, provided that an appropriate approach is taken. It is essential to consider an optimal CO_2 feeding rate to the cultivation system. However, CO_2 injection rate is usually limited by CO_2 tolerance of microalgae as described above. Mass transfer between the liquid culture medium and the gaseous phase is another factor that plays a crucial role in the efficiency of CO_2 utilization by microalgae.

Apart from that, the rate of gas application to the culture and the intensity of agitation will influence the microalgal growth, becoming a negative contributor above a certain shear stress level. There are also some concerns regarding the capture of CO_2 from flue gases directly because flue gases usually contain some sulfur and nitrogen oxides. These contents can form acids when dissolved in the culture medium. Therefore, special treatment such as pre-filtering and neutralization is required before they are fed to microalgae [68]. Having said that, fermentation CO_2 is relatively pure and a clean source of CO_2 for microalgal growth, these characteristics can cut the extra expenditure that is needed for other types of flue gases. As an overall conclusion, all these parameters need to be considered for the design of photobioreactors in order to capturing CO_2 by microalgae [70].

Table 1-1: Comparison of different cultivation conditions to investigated microalgae CO_2 biosequestration rates

Microalgal species	**CO_2 (%) Conc.**	**CO_2 source**	**Bioreactor type & size**	**Light intensity (µmol m^{-2} s^{-1})**	**Biomass productivity (g L^{-1} d^{-1})**	**CO_2 fixation rate (g L^{-1} d^{-1})**	**Controlled pH?**	**REF.**
***Chlorella* Sp.**	5%	Pure CO_2	Bubble column photobioreactor, 0.5 L working volume	106.6 µmol m^{-2} s^{-1} during the entire batch experiment in a 12:12 h (light/dark)	0.314	0.57	Yes, pH was controlled for initial 36 h	[71]
***Chlorella* Sp.**	5%	Flue gas (61 ppm NO_X, 0.3% v/v SO_X)	Bubble column photobioreactor, 0.5 L working volume	106.6 µmol m^{-2} s^{-1} during the entire batch experiment in a 12:12 h (light/dark)	0.273	0.5	Yes, pH was controlled for initial 36 h	[71]
Chlorella vulgaris	2.5%	Pure CO_2	Bubble column LED-based PBRs, 0.5 L working volume, using low dose of sugars for promoting photosynthetic efficiency	300 µE/ m^{-2} s^{-1}	1.86	3.51	No	[72]
***Spirulina* Sp.**	6%	Pure CO_2	three 2 L (1.8 L working volume) column photobioreactors (CPBR)	12 h dark/light photoperiod with 3200 lx of illumination provided by 40 W daylight-type fluorescent lamps	0.22	0.43	No	[73]

1.7 The effect of CO_2 on culture pH

The pH plays a key role in the distribution of inorganic carbon and essential nutrients in the growth medium, as well as their availability to the microalgae. It also has influence on the photosynthetic process, microalgal metabolism, the enzyme activities within cells, and the biomass and lipid yield of microalgae [74]. Commonly, during inoculation, the growth medium is maintained at neutral pH and after regulation of the carbonaceous species in medium, the pH increases steadily as a result of inorganic carbon utilization [75]. According to Kumar et al. [76], without supplementing CO_2, the pH will increase to about 8 resulting from the activity of the carbonic anhydrase enzyme, which transfers hydroxide ions from the cell into solution. The CO_2 absorption rate will be elevated at higher a pH as a result of the reaction between hydroxyl ions and CO_2 to produce bicarbonate [12].

Most published studies identify neutral pH as favorable, for microalgal strains such as *Chlorella protothecoides* and *Chlorella vulgaris* which are optimally grown at pH 6.5 and 7, respectively [74]. While some microalgae, such as *Spirulina platensis*, *Chroococcus minor* and *Tetraselmis suecica*, are tolerant to higher pH and are grown at pH 9 [74]. While some species favor lower pH, such as *Chlorococcum littorale* which can grow at pH 4 [75]. The relationship between CO_2 concentration and pH in microalgal systems is quite complicated, as a result of the underlying chemical equilibrium among such chemicals as CO_2, CO_3^{2-}, HCO^{3-} and H_2CO_3 [56]. A decrease in pH will occur in the presence of high concentrations of CO_2 as unused CO_2 converts to carbonic acid in the culture [64].

With CO_2 content of 5–10% (v/v), which is the concentration of many industrial flue gases, the culture pH commonly decreases to approximately 5, while higher SO_X concentrations will drop culture pH even further, to around 2.6 due to the formation of SO_4^{2-}, SO_3^{2-}, and HSO^{3-} in the culture. It can be concluded that the pH change resulting from CO_2 does not have major impact on the growth of common microalgal strains. Whereas, the significant pH change caused by SO_X will commonly inhibit microalgal growth as most strains will not survive at pH lower than 4 [64] [77]. It is suggested that with buffered medium, like sodium hydroxide or calcium carbonate, the pH can be adjusted to the optimal level for the target species [43].

1.8 Microalgal biomass composition

Variation in the biomass composition of microalgal species is reported to be significant. Moreover, biomass composition of the microalgal strain noticeably depends on cultivation conditions, such as nutrient levels in the culture, light intensity, growth phases and temperature [78] [79]. As an example, the protein content typically shows an increase-decrease pattern across the growth phases, where maximum content is reached in the exponential phase, based on the microalgal species and cultivating conditions [80]. The challenge for protein production is that the overall concentration of biomass during the exponential phase is lower than during the stationary phase. However, the biomass that is harvested in the stationary phase is commonly low in protein [79]. Therefore, it is of a great importance to precisely choose the harvesting time in order to optimize the production efficiency of a specific component, whether a common cell component or a specific secondary metabolite. Understanding the composition of biomass is also essential in identifying potential by-products to be used in a biorefinery process. Composition of microalgal species that are used at an industrial level, which are mostly grown in nutrient-rich media, is reported to be from 30 to 75% for proteins, from 25 to 60% for carbohydrates, and 20 to 50% for lipids. It should be noted that these fractions can differ for biomasses that are subjected to heterotrophic growth or stress conditions [39].

1.9 Microalgae as a source of high-value health beneficial natural compounds

Using microalgae as a source of food and food products is not a new concept, but it has been gaining more attention and interest recently. Moreover, as the global population continues to grow, microalgae can be an available and valuable replacement for chemical-based compounds. Detection, purification, isolation and identification of compounds in complex microalgal extracts has been made possible with the development of technologies in this field and has paved the way to efficiently screen natural products like protein, lipid and carbohydrates [18].

The beneficial effects of microalgae as part of a diet are wide ranging and can improve overall health, while specifically lowering the risk of disease like cardiovascular disease and increasing life expectancy. Microalgae contain components that are beneficial food supplements, including minerals, carotenoids, proteins, fatty acids, vitamins and antioxidants [81], [82]. Important vitamins and minerals that can be found in the cellular structure of microalgae are essential for body health and viability [83], [84]. For instance, all the vital amino acids required for holistic human health can be extracted from microalgal

proteins. Also, the amount of protein in microalgae is considerably higher than what could be found in meat (26%), poultry (11%), or milk (3.4%) [85].

Based on previous studies, protein and total lipids content can reach 71% and 75% in special cultivation condition or applying genetic modification, respectively, which is significantly higher than in many terrestrial plants [86] [87] [13] [40]. For example, soybeans, a common food crop, contain 15-25% lipids and 30-50% protein [40]. Despite the illustrated benefit, it is clear that significant development is required to get microalgae to be a staple in people's daily diet [88]. Currently, only a few species of microalgae, including *Spirulina*, *Dunaliella, Chlorella*, *Nostoc* and *Aphanizomenon* are used as food for humans due to the market demand, economic factors and strict food safety regulations [89]. Therefore, the algae-based food products for human consumption must be prepared as per food and drug administration safety guidelines and proper product stability information should be provided for each product. Economic and sustainable aspects of algal biomass production lines should be evaluated too [90].

1.9.1 Lipid

As mentioned earlier, dry weight lipid content of many species is approximately 20–50%, significantly higher than the 5% content common in many terrestrial crops [21]. The promising ability of microalgae to produce lipids and the diversity of the composition of lipids by microalgae makes them suitable for a wide range of applications including food products and additives, biofuel production, aquaculture, and feed for farm animals and birds [91], [92].

As per Michalak et al., lipids are extracted most frequently in comparison to other microalgal compounds and show the enormous potential for commercialization[40]. Currently, *Botryococcus*, *Dunaliella, Thalassiosira, Arthrospira, Nostoc, Crypthecodinium, Aphanizomenon, Schizochytrium, Haematococcus, Chaetoceros, Nannochloropsis, Chlorella, Isochrysis, Cylindrotheca, Pavlova, Neochloris, Porphyridium* and *Phaeodactina* are all used in the commercial scale production of lipids [40].

Phospholipids, glycolipids and betaine are polar constituents of the lipids present in microalgae and acylglycerols, sterols and free fatty acids are non-polar constituents [93] [17]. The polar lipids are essential structural components of cell membranes and intracellular thylakoid membranes. The thylakoid membranes are formed of the digalactosyl diacylglycerol and monogalactosyl diacylglycerol, which

contain high amounts of palmitic acid and polyunsaturated fatty acids. Most of the non-polar storage lipids that are found inside the cells are triacylglycerols [94]. Lower percentage of polyunsaturated fatty acids can be found in triacylglycerols, because they are mainly made up of monounsaturated and saturated fatty acids[95] [69].

The fatty acid molecule consists of a carboxyl group (COOH) and a methyl group (CH_3) at the ends of the hydrocarbon chain. It can be said that in living organisms the fatty acid type is mainly determined by the length of its hydrocarbon chain. Short-chain, medium-chain, long-chain and very-long-chain fatty acids show various application and characteristics. Presence and number of double bonds and their location are other factors that can categorize fatty acids to monounsaturated fatty acids and polyunsaturated fatty acids[40].

All of the necessary lipids cannot be synthesized in the human body and externally supplied lipids are needed to be metabolized into different bioactive molecules. So, supplying lipids in a proper diet is of a great importance to maintain lipid homeostasis in the body [96] [95].

It is reported that polyunsaturated fatty acids, including ω-3 and ω-6 fatty acids, are categorized as essential nutrients for human body metabolism [60]. One of the main drawbacks in producing polyunsaturated fatty acids rich lipid at large scale from microalgae is that is the lipid content in algal cell and the biomass density in the reactor are relatively low. Under industrial culture conditions, biomass density in existing bioreactors usually does not exceed 400–600 mg/L, which affect the harvesting cost noticeably [59]. Although short chain fatty acids are classified to be unusual in microalgae, long chain fatty acids are produced commonly in microalgal cells [97]. However, to optimize cultivation condition of microalgae for a specific target like fatty acid production, understanding existing patterns in microalgal composition and selecting strains with maximum productivity plays a crucial role.

1.9.2 Short-chain and medium-chain fatty acids in microalgae

Short chain fatty acids are defined as having a carbon number between 6 and 10 [97]. There are infrequent reports on the presence of C6–C8 chain fatty acids in microalgae, however it is reported that the content of short chain fatty acids is not considerable in microalgae under normal growth conditions [98], [99]. However, some microalgae are able to produce short chain fatty acids, while they are stressed, but the

underlying mechanism is unclear [97]. For instance, in *Nannochloropsis* sp. and *Arthrospira platensis* in stressed condition, the amount of C6:0 and C8:0 was 1.48–3.93% and 0.04%, respectively [40].

This pattern also is repeated for medium-chain fatty acids (C10-C14), which are rarely reported in significant quantities in microalgae. Medium-chain fatty acids have higher energy density than short-chain fatty acids, and using them as antimicrobial agents, flavor additives, animal feed, and precursors of biofuels is some of their applications [100].

As nutrients and metabolism regulators, short-chain and medium-chain fatty acids can play a crucial role in the human body. Also, long-chain fatty acid hydrolysis disorders patients can benefit from them as a nutritional resource. When ingesting triacylglycerol that contains medium-chain fatty acids, triacylglycerol will be well-absorbed [40].

1.9.3 Long-chain fatty acids in microalgae

Long-chain polyunsaturated fatty acids with 16-18 carbons or more, are health beneficial fatty acids that are proved to be effective in modulating nervous and immune systems, maintaining sturdy skeletal muscle and preventing cardiac diseases[101]. As a result of mentioned health benefits, the global demand for long-chain polyunsaturated fatty acids has been increasing during the last few years. Arachidonic acid[102] [103], eicosapentaenoic acid[104] and docosahexaenoic acid[105] are long-chain polyunsaturated fatty acids that can be found within the microalgae cells. Long-chain fatty acids represent a wide range of applications and are of greatest commercial interest. Saturated and unsaturated Long-chain fatty acids of microalgae are valuable in order to producing oils with different properties, which can be used in various industrial fields [40].

1.9.4 Very-long-chain fatty acids in microalgae

Very-long-chain fatty acids with 20 or more carbons can be found in many commercial fish, for example cod or tilapia, as a convenient and usual source of food. However, only small amounts of oil are stored in fish fillets. According to some studies [17] [106] [107] certain algae species contain considerable amounts of very-long-chain polyunsaturated fatty acid. Very long chain polyunsaturated fatty acids known to have beneficial effects on human health including being involved in the anti-inflammatory signaling molecules production and hasten the inflammation resolution [103].

1.9.5 Microalgal pigments

Pigments are molecules that can absorb visible spectrum light and are used for producing a range of products from food additives and colorants to nutraceutical and pharmaceutical agents. Industrial pigments are commonly produced from non-renewable synthetic sources like inorganic chemicals, organic acids and petrochemicals for reduced costs. However, pigments production from natural resources like microalgae is in rising demand because of the safety and environmental concerns associated with synthetically produced pigments [108]. Some microalgae produce valuable pigments, like β-carotene, astaxanthin and lutein, which can prevent and treat diseases. They have beneficial biological activities, such as anticancer, anti-obesity, antioxidant, anti-inflammatory, anti-angiogenic, and neuroprotective. At least three classes of pigments are produced in microalgae: carotenoids, phycobilins, and chlorophylls. Different types of microalgae can contain from 0.5 - 1.0% of chlorophyll per dry weight [109].

1.9.6 Carotenoids

Carotenoids are microalgal pigments with unique coloring properties and antioxidant activities. They cannot be synthesized in human organs and a sufficient amount of them must be obtained from our diet. Microalgae represent a high capacity of producing carotenoids and all known xanthophylls that can be found in terrestrial plants [110]. Additionally, microalgae are capable of synthesizing a variety of other pigments that can be found only in algae, cyanobacteria, and some species of yeast [111] [112]. During photosynthesis, carotenoids are responsible for important cellular functions, such as photoprotection and light harvesting. Carotenoids are able to harvest available light and pass it as energy to the chlorophyll molecules when the light intensity is low. While they accept excess energy from chlorophyll and dissipate it to protect chlorophyll from photodamage during being exposed to high light intensities.

Carotenoids' ability to neutralize reactive oxygen species and free radicals give them interesting antioxidant characteristics – as human health application can slow cellular damage, chronic diseases and aging [113] [114]. Food, feed, cosmetics and nutraceutical industries are all using carotenoids because of their nutritional properties and color. Carotenoids are added to various food products including yoghurts and confectionary, as a safe natural dye to enhance their color [110]. In the human body, carotenoids act as provitamin A and an amount of 0.1–0.2% is commonly present in the total microalgae dry matter [115]. Similarly, in cosmetics, carotenoids have applications as active ingredients with biological activity and

nutritional value in creams, lotions and hair products. When they are applied on skin, carotenoids work as a protection from UV-light damage [116] [117].

1.9.7 Chlorophylls

Photoautotrophic microalgae can synthesize green pigments, which account for 0.5–1.5% of the dried biomass and show antioxidant, antigenotoxic and anticarcenogenic properties. It is reported that chlorophyll has the potential to stimulate the liver recovery [108], [118] and improve metabolism of carbohydrates, proteins and lipids in humans body. Chlorophyll contains chlorophyllin, which is a sodium-copper-salt derivative and can be easily absorbed by human body. Studies have revealed that Chlorophyll has a potential to make the cancer progression and development slower [119].

1.9.8 Algal polysaccharides

Polysaccharides are natural polymers that can potentially be extracted from microalgae instead of land plants or macroalgae. Microalgal species, like *Spirulina platensis* and *Chlorella vulgaris*, present carbohydrate contents of 8–14% and 12–17%, respectively, under standard cultivation conditions[120]. While stressed conditions and differing cultivation parameters such as light intensity, CO_2 concentration and nitrogen content may increase carbohydrate content up to 77% in *Chlorella* sp. [121]. Microalgal polysaccharides have been examined for their antioxidant activity more than any other biological activity [122] [20]. The results of studies on microalgal polysaccharides antioxidant activity suggest that they can be considered for pharmaceutical purposes such as for the treatment of oxidative disorders. Several factors including polysaccharides' molecular weight and polysaccharide chemical structure can influence their antioxidant activity [20]. Moreover, polysaccharides exhibit an enormous potential in pharmaceutical sector to be used as immunomodulatory and antitumor agents [123]. In addition to being applied in the medical sectors, food and cosmetic industries have used microalgal polysaccharides as stabilizers, flocculants, viscosifiers and hydrating and gelling agents to improve food products quality and texture [81]. based on the composition of cell wall, some species like *Diacronema lutheri, Tisochrysis lutea, Nannochloropsis* sp., *A. platensis, C. vulgaris and Odontella aurita* are being used in snacks , pastas and candy bars[82].

1.9.9 Vitamins

Two large groups of vitamins are those which are soluble in water (vitamins B and C) and the others that are fat-soluble compounds (vitamins A, D, E and K). Photosynthetic organisms are mostly responsible for synthetizing vitamins. However, some vitamins like vitamin K and some vitamins B are mainly produced by bacteria and should be bioaccumulated through diet [124] [125]. Recommendations say that a diet with high number of various vitamins should be followed to avoid vitamins deficit. Some vitamins are hard to find in plants and some, including vitamins K, D or B, can be found in low concentration [126]. While microalgae is able to provide many necessary vitamins (A, B1, B2, B6 and C) required in a healthy diet in higher contents and better quality rather that terrestrial plants [127]. So, microalgae cultivation as a natural vitamin source represents a promising future for human consumption.

1.9.10 Proteins and amino acids

Proteins are known as building blocks of the human body. Proteins are long chains of amino acids responsible for overall growth of a person. A complete profile of essential amino acids can be found in the common foods, like eggs, poultry and red meat, fish and dairy. However, these sources are not suitable for a growing population around the world and the growing interest in vegetarianism. With respect to vegetarianism, it should be noted that a complete essential amino acid (EAA) profile cannot be found in most of the plant derived proteins [86]. On the other hand, microalgae are a valuable and excellent source of proteins that can be utilized as nutritional and therapeutic products [108]. Approximately, 40 to 50% of microalgal biomass is protein, but percentages differ based on growth conditions and species. According to the protein content and the degree of refining, there are five categories of protein products that are obtained from microalgae including protein concentrates, whole-cell protein, hydrolysates, isolates, and bioactive peptides [128].

Whole-cell protein that is consumed directly is the densest product and has an intact cellular structure. Other products are isolates, protein concentrates, bioactive peptides and hydrolysates [128].

1.9.11 Antibacterial compounds

It seems that a study carried out by Pratt et al. [129] is the initial point of considering microalgae as a source of antibiotics. They found that chlorellin, which can be produced by green microalgae is able to inhibit both Gram-negative and Gram-positive growth. Many studies after that have shown that microalgae can be

a source of compounds such as indole alkaloids, terpenes, phenolics, glycolipids, β-diketone and fatty acids that exhibit antibacterial activity. [18] [59]–[61]. Among them, long chain unsaturated fatty acids show the most antibacterial activity [131] [18]. According to studies done by our research group [18], green microalgae from extreme environments such as fresh or salt water bodies with low pH or municipal wastewater contamination exhibit antibacterial activity against human pathogens.

1.9.12 Antioxidants

Preferences for antioxidants from natural sources are growing fast all over the world. Especially after finding that synthetic antioxidants have toxic effects on human body[114]. So, microalgae are considered as a replacement for synthetic antioxidants with higher stability. Currently, astaxanthin, β-carotene and lutein are high value market antioxidants from microalgae. Abiotic stress in microalgal cultivation can instigate the production of a unique cellular defensive antioxidant response. It should be considered that the enzymatic and non-enzymatic antioxidants that can be produced by stress response will differ between each stressor and algal species [133].

1.10 Biofuel from microalgae

Biofuels are classified to four generations, Based on the feedstock. The first generation is made from oil-based plants, sugar and starch crops. The first generation is known for CO_2 emissions reduction potential, while they are criticized for causing inefficient utilization of resources and land usage.

Second generation is derived from non-food crops including lignocellulosic biomass and waste animal oils as more efficient renewable alternatives. Whereas, second generation overcomes the disadvantages of the first generation, to produce sufficient biofuels at a competitive cost more studies should be carried out. An excellent alternative in fuel market can be aquatic feedstock such as algal biomass[134]. Just first and second-generation biofuels are commercially produced. However, if competition for available land were to arise, the second generation biofuel production could become environmentally and economically unsustainable [135].

Third generation biofuels are derived from algae and have attracted a growing attention due to their relatively simple processing, high yield and carbon dioxide sequestration ability. Algae can be cultivated in unproductive drylands and marginal farmlands as well as in wastewater and seawater. Therefore, they

do not compete with food crops on arable land [135]. As a comparison, from one-hectare microalgae containing 20–50% lipids in dry biomass can yield up to 14000L of oil annually, while this number for corn, soy and canola are 172, 636 and 974 L, respectively [37].

However, to reach the target of considering microalgae as biodiesel alternative feedstock more steps should be taken. Fourth-generation biofuels use bioengineered microorganisms like algae. Actually, microorganisms are genetically altered to consume a higher amount of CO_2 in comparison with the amount they emit to the atmosphere while burning as biofuel [134].

Biodiesel is an important biofuel in form of liquid consisting of mono alkyl esters of long chain fatty acids, which are derived from animal fats or vegetable oils, and also micro and macro algal oil [46]. Currently biodiesel production at large scales still cannot compete with petroleum diesel because of the current challenges such as the mass cultivation, high operational cost, harvesting, and dewatering.

Based on the location and scale of the production, biodiesel that is extracted from microalgae can cost US$0.42–9.11/L and US$0.9–22.55/L from open pond and photo-bioreactors, respectively. While to be competent enough to the price of petroleum diesel, the cost should not exceed US$0.526–1.619/L [124]. Dewatering of algal biomass accounts for 89% of the total energy input according to a life cycle analysis carried out on biodiesel production from microalgae [136].

The maximum oil content of microalgal biomass varies widely based on the cultivation condition and different microalgal characteristics. The ratio and amount of unsaturated and saturated fatty acid, which are the compounds that are suitable to be extracted and converted into biofuels, is important [84]. Throughout different processes, microalgae are capable of producing several biofuels including biodiesel, bioethanol, methane and hydrogen. These fuels are compatible with existing engines without requiring modifications, as the properties of microalgae-based biodiesel fuels is similar to petroleum-based biofuels like viscosity, flash point, heating value, density and cold filter plugging point. Therefore, microalgae-based biodiesel is compatible with the standards of both the International Biodiesel Standard for Vehicles and American Society for Testing and Materials. Moreover, microalgae-based biodiesel fuel emits less pollution, such as SO_X and CO, compared to petroleum-based fuel [134].

Chapter Two: Hypothesis and Objectives

Microalgae can be a source of valuable food ingredients and they can produce those while capturing carbon dioxide (CO_2) from many industrial processes, such as the alcoholic beverage industry. However, to be able to capture fermentation CO_2 and achieve increased microalgal growth rate and lipid, fatty acid and protein productions more information about cultivation under these conditions is required.

2.1 Hypothesis

Microalgae will respond positively to being cultivated with the addition of alcoholic yeast (beer) fermentation CO_2. The exposure to the fermentation CO_2 will usefully increase microalgal bioproductivity of lipids, fatty acids and proteins.

2.2 Objectives

The objective of this work was to investigate three microalgal strains including the culture collection *Chlamydomonas reinhardtii* and two bioprospected strains (P-918 (*Coccomyxa* sp.) and M-23 (*Chlamydomonas* sp.)) and estimate their potential to be used with the off-gas from a yeast fermentation. Nutrition removal and CO_2 fixation were analyzed and compared, and the productivity of potential value-added bioproducts analyzed.

Chapter Three: Methodology

3.1 Microalgae and culture medium

The three microalgal strains utilized in this study were:

- *Coccomyxa* sp. (P-918) obtained from a polishing pond at an operational smelter (pH 2.8)
- *Chlamydomonas* sp. (M-23) obtained from a low pH abandoned mine site environment (pH 3)
- C*hlamydomonas reinhardtii* (CPCC11) obtained from the Canadian Phycological Culture Centre (Waterloo, Canada)

The first strain investigated was from the genus *Chlamydomonas* specifically *Chlamydomonas reinhardtii*, which has been studied widely as an attractive microalgal model. This species has genetic tractability, short life cycle, haploid growth, and the flexible metabolic features that allow for studying algal physiology, metabolism, photosynthesis and flagellar structure and function [137]. While the number of studies on *Coccomyxa* sp. were significantly fewer than the ones focusing on *C. reinhardtii*, *Coccomyxa* sp. cells are small with an approximate size of 3–14 μm and have no flagella. They also have an irregular globular to elliptical shape, and contain a parietal chloroplast without a pyrenoid [138]. After isolation and identification, both strains *Coccomyxa* sp. and *Chlamydomonas* sp. had been kept at neutral pH for long-term storage.

The cell cultivation was performed in modified Bold's Basal Medium (BBM) [139], for which all components were purchased from Fisher Scientific Canada. Sterile Milli-Q water was utilized in all experiments. For 1 L of Total BBM, 936 mL Sterile Milli-Q water was used. Then, 10 mL of each macronutrient's solutions including $NaNO_3$, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, K_2HPO_4, KH_2PO_4, NaCl was added. Also, 1 mL each of alkaline EDTA, acidified iron, boron and trace metals were added to the solution[140].

Each microalgal culture was initially inoculated in 250 mL Erlenmeyer stock flasks containing 150 mL medium at 25 °C (room temperature) with continuous light irradiation of approximately 60 μmol m^{-2} s^{-1}. Cultures were cultivated for 14 days on a shaker table at 120 rpm. Before and during the cultivation experiments, the flask's media, glassware, and pipettes were sterilized before use by autoclaving at 121°C and 100 kPa gauge pressure.

3.2 Measurement of cell growth

To evaluate the effect of carbon dioxide (CO_2) from beer fermentation on the growth parameters of the three microalgae, experiments were conducted over the growth periods of 16 days. The cultures were connected directly to the beer fermentation process to be exposed to CO_2. For each strain, a control culture growing under atmospheric CO_2 was considered. Each microalgal culture was grown in biological triplicate in 500 mL of BBM in 1000 mL Erlenmeyer flasks at 25 °C (room temperature) with continuous light irradiation (~60 μmol m^{-2} s^{-1}) and continuous mixing at 80 rpm. Mixing of the culture media prevented cells from settling and improved the transfer rate of CO_2, avoided nutrient concentration gradients, increasing light utilization efficiency, keeping temperature and pH homogeneous, and eliminating accumulated dissolved oxygen [61].

Throughout the experiment samples were collected every 3 days for the measurement of dry weight biomass (mg_{dw} L^{-1}). The pH of the solutions was uncontrolled, but it was assessed every other day throughout the experiment using a Folio Instruments PHS-38W microprocessor pH/MV/Temperature Meter (Kitchener, ON). For each algal strain, a negative control culture flask, not subjected to CO_2 exposure, was included in the experiments.

Microalgal biomass from each flask was harvested by centrifugation, frozen at -80°C and subsequently freeze-dried at -50°C under vacuum for 24 h. Microalgal specific growth rate (μ) was calculated from the initial dry biomass ($C_{b,i}$) and final dry biomass ($C_{b,f}$) concentration over the logarithmic growth phase (Δt) (Eq. 3.1) [139]:

$$\mu = \frac{ln\left(\frac{C_{b,f}}{C_{b,i}}\right)}{\Delta t} \qquad \text{Eq. 3.1}$$

Also, volumetric biomass productivity (P_v) was calculated from Eq. 3.2:

$$P_v = \frac{C_{b,f} - C_{b,t}}{\Delta t} \qquad \text{Eq. 3.2}$$

3.3 Assessment of CO_2 bio-fixation rates and removal efficiencies by various microalgal species

The CO_2 fixation rates (g d^{-1}) were evaluated using Eq. 3.3 [141]:

$$RC = C_C \times P \times \left(\frac{M_{CO_2}}{M_C}\right) \times V \quad \text{Eq. 3.3}$$

Where P is Biomass productivity (g L^{-1} d^{-1}), C_c is the mass fraction of carbon in dry microalgal biomass (%w/w), which is approximately 50% [4], M_{CO2} is the molar mass of carbon dioxide (44 g mol^{-1}), M_C is the molar mass of carbon (12 g mol^{-1}), and V is the working volume (L).

3.4 Lipid analysis

To investigate the effect of CO_2 on the lipid productivity of the microalgal strains, lipid content was measured for each sample throughout the experiment. The lipid content of the dried biomass was extracted applying a modification of the method first described by Folch et al. [142]. In Folch's original method, to separate precipitated non-extractable residues, the crude extract is filtered, whereas in the modified method, this step is omitted due to practical aspects in the microscale extraction [142].

At first step, freeze-dried biomass was mixed with 1.2 mL of chloroform: methanol with 2:1 volume proportion in a centrifuge tube. The mixture was then sonicated using a cell disrupter, Mini-Beadbeater-16 (BioSpec, USA) for 1 minute. Then, the samples were centrifuged using a centrifuge (Sorvall ST8, Thermo Scientific, Suzhou, China) and the supernatant was placed in a weighed tube (Figure 3.1). Extraction was repeated in triplicate for each strain. At the next stage, the solvent was evaporated in a vacuum drier and the mass of the remaining lipid was gravimetrically determined. The lipid content was expressed as percentage on dry weight basis using Eq. 3.4. Where $W_{Extracted\ Lipid}$ is weight of dried extract and W_{DCW} is weight of dried biomass.

$$Lipid\ content\ (\%) = \frac{W_{Extracted\ Lipid}}{W_{DCW}} \times 100\% \quad \text{Eq.3.4}$$

Figure 3-1: Extracted lipids of *Coccomyxa* sp. and *Chlamydomonas* sp.

3.5 Protein Analysis

As the majority of microalgal proteins are immobilized inside the cell, an efficient protein extraction method was required to produce a functional extraction of protein. In the current study, total protein content was estimated using Lowry's method, which is considered suitable for microalgal protein extraction [143].

At the first step, mother solutions were prepared with approximately 500 mL of distilled water and 2 N NaOH was added to adjust the solution to pH 12 for maximum protein solubility. A sample of freeze-dried biomass was added to 25 mL of mother solution. The mixture was then stirred for 2 h at 40 °C. The separation of the supernatant from the pellet was conducted by centrifugation at 10,000 g for 10 min at 20 °C. The supernatant was then adjusted to pH 3 with 0.1 M HCl to precipitate the proteins. The protein isolate was collected after centrifugation at 10,000 g for 10 min at 20 °C and the pellet was neutralized with 0.01 M NaOH. Samples were taken for protein analysis.

The Lowry's method involves protein reaction with cupric sulfate and tartrate in an alkaline solution, which results in formation of tetradentate copper-protein complexes. By adding the Folin-Ciocalteu reagent, it is effectively reduced in proportion to these chelated copper complexes, producing a water-soluble product. The solution blue color can be measured at 750 nm.

Protein concentration was estimated by making a standard curve with bovine serum albumin (BSA) at a concentration range of 0 to 1500 μg mL^{-1}. The Lowry kit, a prepared mixture of 2 N Folin-Ciocalteu reagents, Lowry reagent and bovine serum albumin (BSA) standards, was purchased from Thermo Fisher Scientific. 2N NaOH and 0.1M HCl were also purchased from Thermo Fisher Scientific. 20 mg dried biomass of each sample was used for total protein analysis. The blue color solution was then measured at 750 nm with a UV-1700 UVVIS (Shimadzu, Tokyo, Japan) to give an optical density that could be compared back to the standard BSA curve.

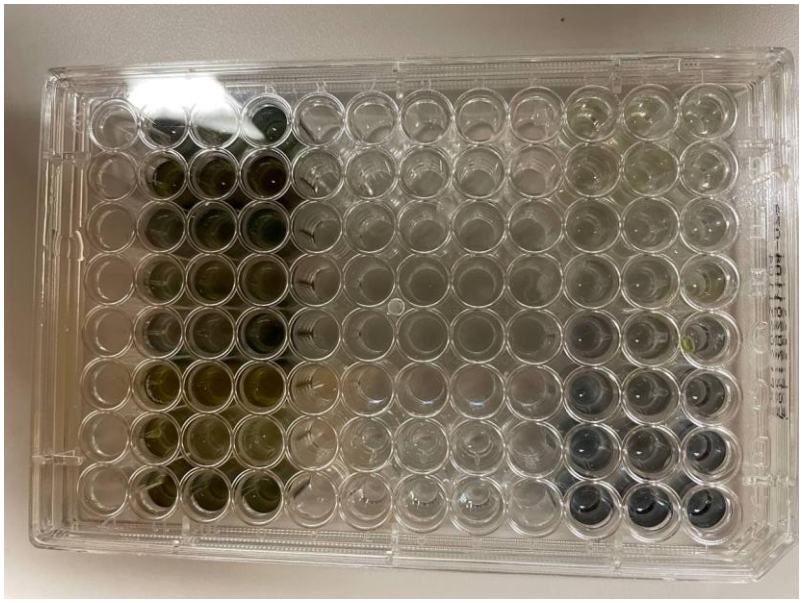

Figure 3-2: Micro plate containing unknown prepared samples of *Coccomyxa* sp. and *Chlamydomonas* reinhardtii strains for protein analysis

The first step was preparation of BSA Standards according to Table 3-1.

Table 3-1: Preparation of Diluted Albumin (BSA) Standards, Dilution Scheme for Microplate Procedure (Working Range = 1-1500µg/mL)

Vial	Volume of diluent	Volume source of BSA	Final BSA content
A	125 µL	375 µL	1500 mg/ml
B	312 µL	312 µL	1000 mg/ml
C	155 µL	155 µL of vial A	750 mg/ml
D	312 µL	312.5 µL of vial B	500 mg/ml
E	312 µL	312.5 µL of vial D	250 mg/ml
F	312 µL	312.5 µL of vial E	125 mg/ml
G	400 µL	100 µL of vial F	25 mg/ml
H	400 µL	100 µL of vial G	5 mg/ml
I	400 µL	100 µL of vial H	1 mg/ml
J	500 µL	0 µL	0 mg/ml

The contents of one bovine albumin standard (BSA) ampule were diluted into several clean vials. The pooled contents of one ampule of 2.0 mg/mL albumin standard was sufficient to prepare three replications of each diluted standard for the working ranges given in Table 3-1, which were calculated for the microplate procedure. The second step was preparation of 1X Folin-Ciocalteu Reagent by diluting the supplied 2X (2N) reagent 1:1 with ultrapure water. After that, the microplate procedure was started by Pipetting 40 µL of each standard and unknown sample replicate into a microplate well. Then, 200 µL of modified Lowry reagent was added to each well using a multi-channel pipettor. The microplate was covered and incubated at room temperature for exactly 10 minutes. Following that, 20 µL of prepared 1X Folin-Ciocalteu Reagent was added to each well by a multi-channel pipettor and immediately, microplate was mixed on plate mixer for 30 seconds. Another incubation was done for 30 minutes after that and the absorbance at 750 nm on a plate reader was measured [141].

3.6 Beer fermentation set-up

Two 5-gallon (20 L) beer kits of Brewer's Best® Kölsch were used for each round of experiments. The beer was made from hops, malt yeast and water. Before commencing an experiment, all the brewing equipment was thoroughly cleaned and sanitized. Tap water (9.5 L) was heated to the appropriate steeping temperature (65-75°C) when crushed grains (560 g) were added. The crushed grains were steeped for 20 minutes and then removed, with the water now known as wort. After bringing the wort to a gentle boil, all the liquid malt extract (3 kg) and corn sugar (454 g) were added to the boiling wort. The hops (140 g) were then mixed into the solution, followed by a further 60 minutes of boiling. The wort was then cooled to 21°C and the wort siphoned onto 6 fermentation flasks (transfer of the sediment (turb) was avoided).

At this point 9.5 L of 20°C tap water was added to the six fermenters equally (1.58 L for each fermenter). Using a sanitized hydrometer, the original gravity was read and recorded for the alcohol by volume (ABV%) calculation. Following this stage, the contents of the yeast sachet (1.92 g for each fermenter) were sprinkled over the top of the wort surface. Manual agitation and aeration of the wort were performed for 2 min at the beginning of the process and then the fermenters moved to a dark, and temperature stable area (room temperature ~22°C), where they were connected to the microalgal culture flasks. The wort began to ferment within 24 to 48 hours and released CO_2 was noticed visually. The specific gravity of the fermentations was read on day 7. It was compared to the final gravity instruction given by the beer kit recommended procedure, which was 1.013~1.017. If the gravity was not in the given range (1.013~1.017) as final gravity, the fermentation was allowed to continue if it was still producing CO_2 until reaching the final recommended (by the kit supplier) gravity range. In the current work, the primary fermentation lasted between 5 to 7 days and a direct indicator of the fermentation activity of the yeast was the amount of released CO_2. The first beer kit was replaced by the second beer kit on day 8 of the experiment.

According to the fermentation stoichiometric equation (Eq. 3.5) and the percentage of ethanol produced (Eq. 3.6), per each 100 L of beer that is produced, around 2.5~3.8 kg CO_2 is formed [7] [144]. In the current study, original gravity was 1.055 and final gravity was 1.017 after 7 days. As each beer kit was converted to 19 L of beer, it can be estimated that 0.475 kg CO_2 was produced from each kit. The values for alcohol by volume (ABV), original gravity (OG) and final gravity (FG) were all obtained.

$$C_6H_{12}O_6 \xrightarrow{yeast} 2C_2H_6O + 2CO_2 \qquad \text{Eq.3.5}$$

ABV (%) = (OG – FG) x 131.25 Eq.3.6

Each beer kit was equally divided to 6 fermentation flasks, and each microalgal cultivation flask was connected to a fermentation flask containing 3.17 L of beer (Figure 3-3 and Figure 3-4).

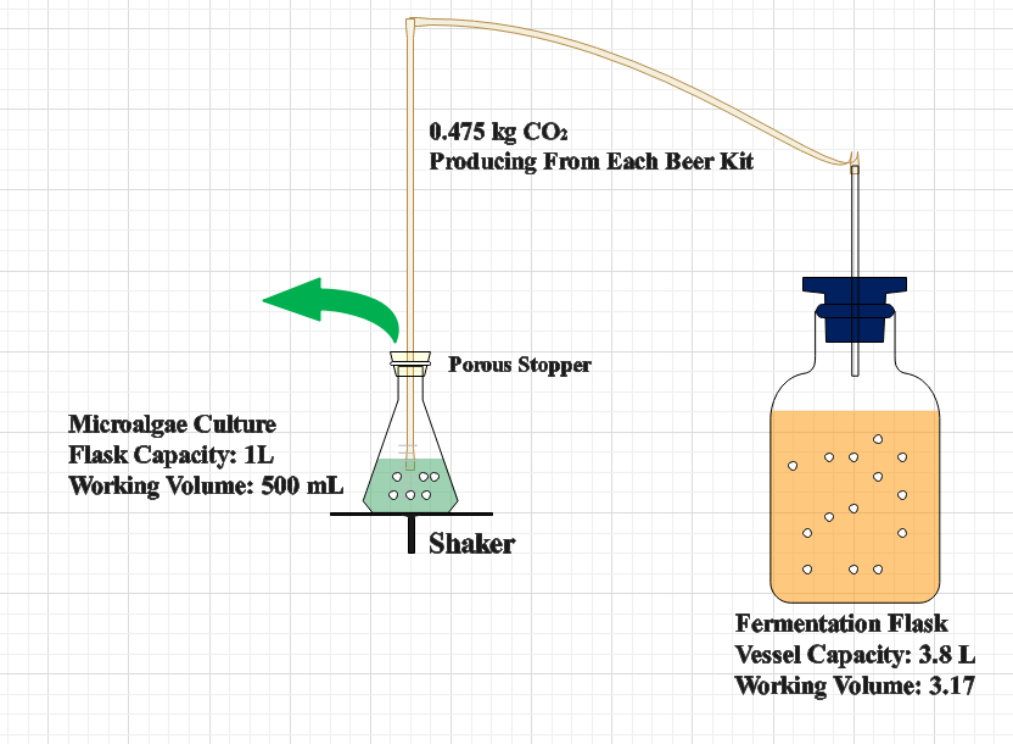

Figure 3-3: Fermentation and microalgal culture connection schematic diagram

The fermenter gas exits were connected to the microalgal flasks, so that total estimated 0.95 kg CO_2 produced by two sets of fermentation was bubbled into the bottom of each algal culture throughout the experiment (16 days) based on the estimated 5% ABV obtained by using Equation 3.6. A control culture, without CO_2 injection, was always performed in parallel for each microalgal strain. An image of a microalgal culture connected to a fermentation flask is provided in Figure 3-4.

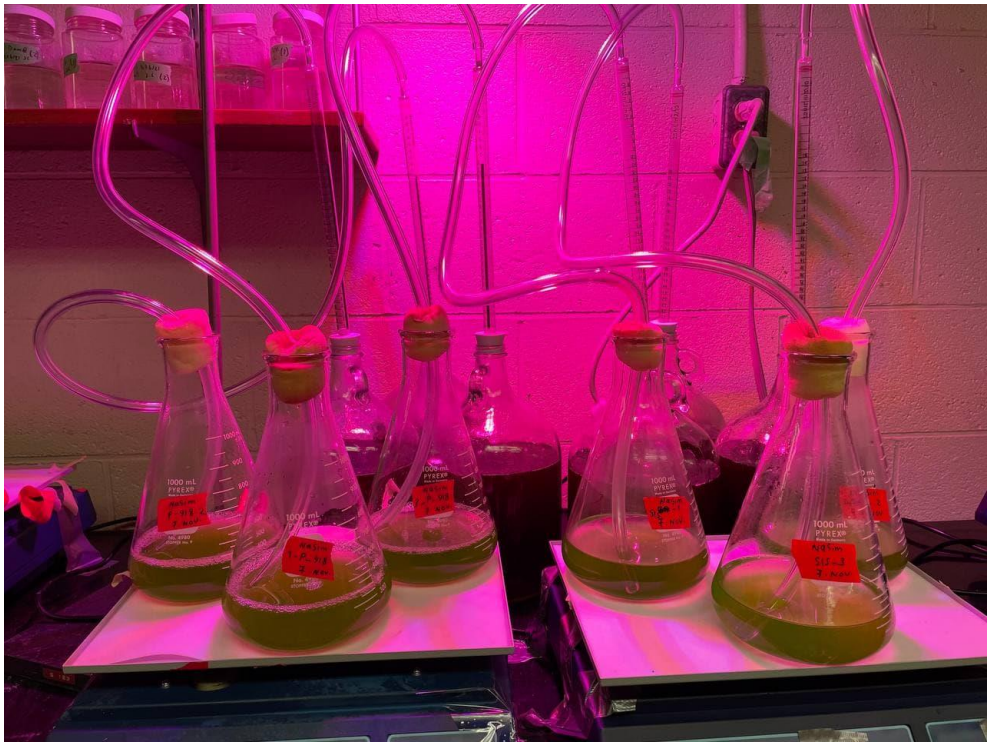

Figure 3-4: Fermentation and microalgal culture connection system

3.7 Statistical Analysis

All values are reported as means values ± standard error. Statistical analyses were performed using a "test_1sapm class in scify's stats" library in a Python 3.8 code. Since we had one control sample for each microalga, we performed the T-test considering this value as an expected value of a sample of independent observations. T-tests were performed to discriminate significant differences.

Chapter Four: Results and discussion

4.1 Effect of CO_2 released during fermentation on the growth of algal culture

The amount of CO_2 sparged into each algal culture from the fermentation setup (estimated by the fermentation Eq.3.5) was approximately 0.475 kg. When two beer fermentations "back-to-back" were used, this gave a total of 0.95 kg of CO_2 over the whole experiment (16 days). Due to substrate consumption by yeast, CO_2 evolution rate continuously increased up to 72 h for each beer kit and then started decreasing [4]. So, the concentration of CO_2 was not constant during fermentation and microalgal growth. The influence of CO_2 supplementation on biomass yield ($g_{dw}\ L^{-1}$), lipid content (% dry cell weight (dcw)), and protein content (% dry cell weight (dcw)) of the *Chlamydomonas reinhardtii*, *Chlamydomonas* sp. and *Coccomyxa* sp. are illustrated in Table 4-1.

The *Chlamydomonas reinhardtii* strain, which is a unicellular freshwater green microalgal species [145] was examined for its growth and biomass production when exposed to fermentation CO_2. Studies on *Chlamydomonas reinhardtii* strain could reveal its mechanism of CO_2 sequestration and it is widely reported that high CO_2 concentration can lead to an enhancement of carbon metabolism. It also can improve biomass and fatty acids accumulation in *Chlamydomonas reinhardtii* [146].

As stated in Table 4-1, it was clearly see that specific growth rate, volumetric biomass productivity, CO_2 sequestration rate and lipid content percentage of *Chlamydomonas reinhardtii* increased, by 10, 38, 38 and 16%, respectively, when the microalgal cultures were exposed to CO_2 from fermentation. However, as it can be seen in the

Table 4-1, protein content of the final biomass increased from 30.3 to 30.7%, which is not a significant difference.

All CO_2-enriched cultures showed higher concentrations of dried biomass in comparison with the control culture. However, there were no significant differences in specific growth rate (with average standard deviation of 0.01) among the dry weight biomass concentrations of the *Chlamydomonas reinhardtii* cultures.

Table 4-1: Specific growth rates, volumetric productivities, CO_2 sequestration, lipid and protein content at different CO_2 content in the feed gas for three microalgal strains at day 16 of experiment

Microalgal strain	**CO_2 Origin**	**Specific growth rate (day^{-1}) +/-SD**	**Volumetric biomass productivity ($g_{dw}L^{-1}day^{-1}$)**	**Average CO_2 sequestration rate (mg_{CO2} L^{-1} day^{-1})**	**Dry weight lipid content (%)**	**Dry weight protein content (%)**
***Chlamydomonas reinhardtii* (CR)**	Atmosphere	0.129	0.052	95.1	19%	30.3%
	Fermentation	0.143±0.01	0.072	131.1	35%	30.7%
***Chlamydomonas* sp. (M-23)**	Atmosphere	0.132	0.062	113.7	24%	28.8%
	Fermentation	0.159±0.04	0.086	158.5	39%	27.4%
***Coccomyxa* sp. (P-918)**	Atmosphere	0.129	0.067	122.1	13%	36.7%
	Fermentation	0.167±0.01	0.124	227.9	26.3%	42.5%

As presented in Table 4-1, specific growth rate increased approximately 20% in a CO_2 enriched environment for the *Chlamydomonas* sp. strain. Also, the *Chlamydomonas* sp. volumetric biomass productivity, CO_2 sequestration rate and lipid content showed respectively 39%, 39% and 147% increases when the microalgae were exposed to fermentation CO_2. In this strain, protein content decreased from approximately 28.8 to 27.4%. The increased biomass yield and total lipid content in both *Chlamydomonas* strains could be because the carboxylation activity is increased by elevated CO_2 concentration and the oxygenating activity of Rubisco is suppressed, which leads to a higher photosynthesis rate[4]. The culture medium CO_2 enrichment improved the electron transport between Photosystem I and Photosystem II, which can lead to enhanced fixation of CO_2 and growth acceleration[145]. According to many studies [147], a CO_2-rich environments can improve lipid accumulation in the microalgal cell. By comparing the lipid percentage for samples that were exposed to CO_2 with the controlled samples, it can be concluded that less available CO_2 slows down microalgal metabolism, which causes a reduction in lipids formation.

For the *Coccomyxa* sp. strain (P-918), this study showed that specific growth rate, volumetric biomass productivity, CO_2 sequestration rate and lipid content percentage increased significantly when the microalgae were exposed to the fermentation CO_2. The maximal biomass productivity with fermentation CO_2 was 0.124 mg L^{-1} day^{-1}, which was 67% higher than that for atmospheric CO_2. Carbon fixation rate was also markedly increased with fermentation CO_2 as compared to atmospheric CO_2, which was attributed to the boosted biomass productivity rendered by CO_2 (Table 4-1). The carbon fixation rate was raised to 86% (from 122.1 to 227.9 mg_{CO2} L^{-1} day^{-1}) during 16 days of the experiment.

These results indicate that the *Coccomyxa* sp. adapted well to fermentation gas containing a high level of CO_2. This is probably due to the fact that this microalga is an acid-tolerant strain. *Coccomyxa* sp. belongs to the class Trebouxiophyceae [148], and was obtained from a mine site polishing pond at pH 2.8 at the Sudbury Integrated Nickel Operations smelter. A few strains of this genus have been isolated from extremophile environments by other researchers, and all of them showed resistance to cold adaptation, high metal concentrations, low pH, high doses of ionizing radiation, and heavy metal ions [149]. The findings in this work are consistent with previous studies that reported growth of acid tolerant and acidophilic microalgae depending on the dissolved inorganic carbon, CO_2 and HCO_3^- availability. The bioavailability of dissolved inorganic carbon varies significantly depending on the culture medium pH. However, most of the dissolved inorganic carbon is available in the form of CO_2 at acidic pH. The inorganic carbon low concentration that is present in acidic waters would inhibit the algal photosynthesis. In order to cope with carbon bioavailability limitations, the microalgae originated from acidic habitats commonly have high-affinity mechanisms for uptake of CO_2 and Rubisco enzymes [138].

4.2 Growth rate

Two stages are required for microalgae to utilize CO_2. This includes mass transfer and absorption of CO_2 from the gas phase, which occur by chemical reaction and CO_2 fixation through photosynthesis. CO_2 diffusion into the media from the atmosphere hinders growth of microalgae because of the water high surface tension and a low presence of CO_2 in air. Therefore, an additional supply of carbon can increase production of biomass [61] and the use of free and pure CO_2 from fermentation processes can be considered as an appealing economic option. It should be taken into consideration that although low concentrations of CO_2 can be a limiting factor in algal growth, high CO_2 concentrations or continuously sparged CO_2 to the

culture can also hinder the growth rate for sensitive strains. This results from denaturing cellular enzymes and the associated reduction in capacity to uptake CO_2 [12].

4.2.1 *Chlamydomonas reinhardtii* (culture collection strain)

This work revealed an increase of 38% in volumetric biomass productivity of the *Chlamydomonas reinhardtii* algal culture when supplemented with CO_2 from fermentation, as shown in Figure 4-1. Two "back-to-back" beer kits were used and the first fermentation kit was replaced by the second one on day 8 of the experiment (Figure 4-1). From the start of the experiment to day 14, there was a significant increase in biomass dry weight concentration (9.4 times higher) for samples that were exposed to CO_2. By continuing the cultivation, a decrease in biomass growth was observed from day 14 to day 16 for both CO_2 enriched and control samples. However, the decrease for CO_2 enriched samples was sharper, which is likely due to lack of nutrients in the culture, as during higher productivity more nutrient is consumed. As CO_2 enriched samples grew faster, they turned to a yellowish color compared to those grown under atmospheric CO_2 conditions, which may be a marked decrease in chlorophyll content [137]. A decrease in chlorophyll content occurs when the cells are nitrogen-starved [50]. As fermentation CO_2 was intermittent and CO_2 concentration went up for three days and then decreased gradually, it allowed the *Chlamydomonas reinhardtii* culture, as a low pH sensitive strain, to acclimatize and did not limit its growth rate.

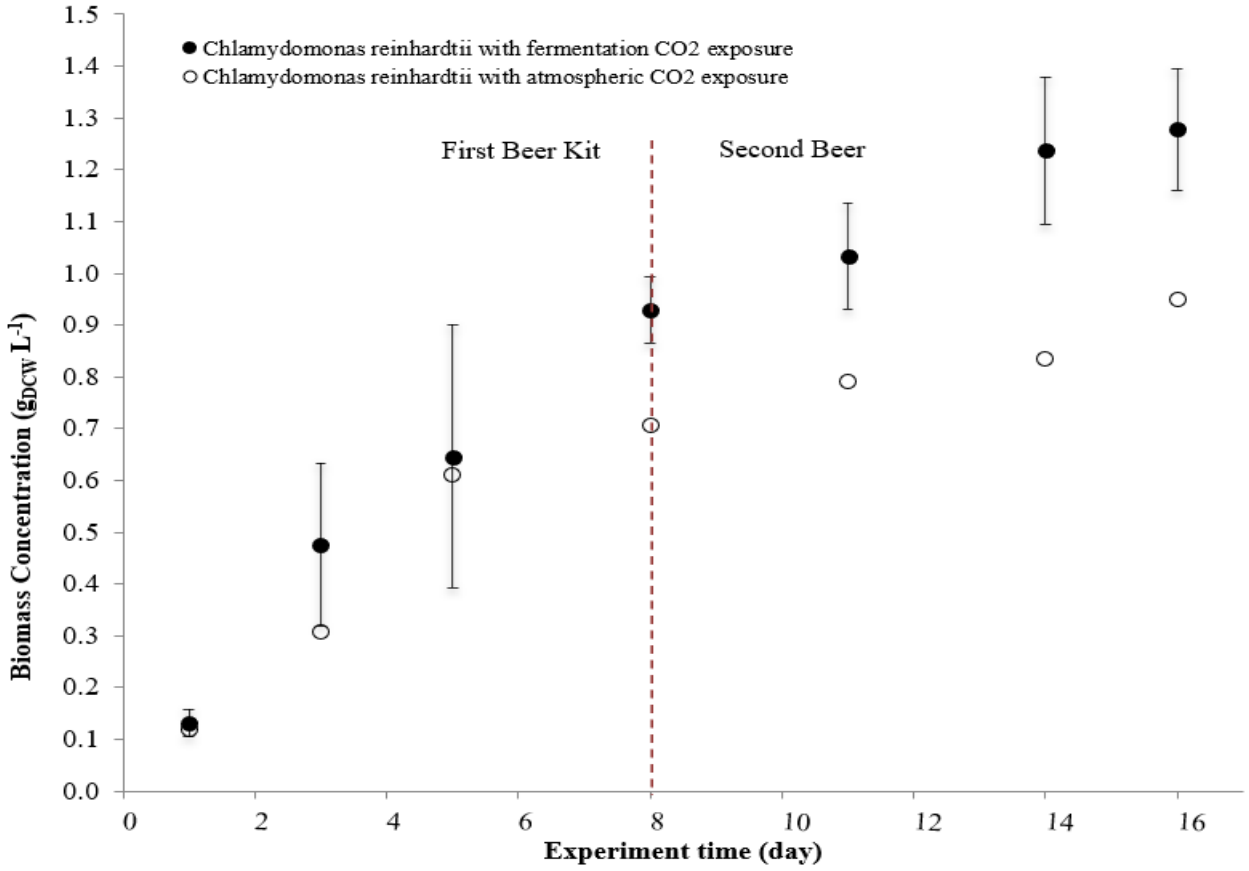

Figure 4-1: *Chlamydomonas reinhardtii* growth rate with and without exposure to fermentation CO_2

CO_2 injection in *Chlamydomonas reinhardtii* cultures lead to coagulation and flocculation even when well mixed, causing aggregation into clumps that can be filtered easily from the medium (Figure 4-2). Cheng et al. [139] investigated the effects of CO_2 on the cell wall carbohydrate composition of microalgae. Knowing the fact that cell wall composition has an influence on cell flocculation and the recovery of intracellular products, it was reported that increasing CO_2 level can result in high uronic acid and polysaccharide contents of microalgal cell wall, which favorably affects cell flocculation and the recovery of intracellular products [139]. As harvesting and dewatering account for a significant portion of microalgae production costs, having microalgae self-flocculate could be economically beneficial.

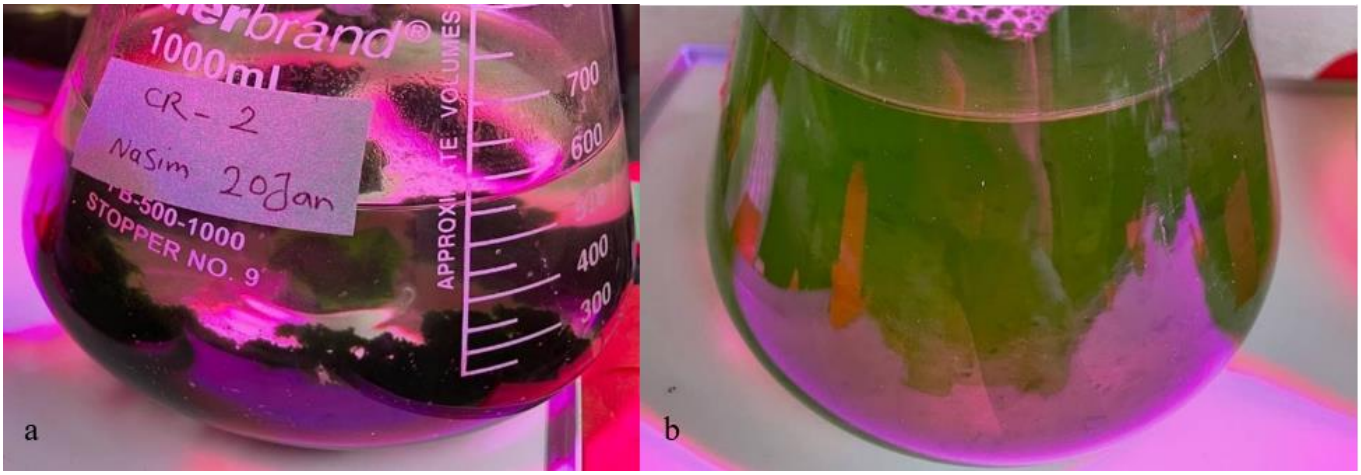

Figure 4-2: a) *Chlamydomonas reinhardtii* cultivation with CO_2 from beer fermentation, b) *Chlamydomonas reinhardtii* growing only under atmospheric CO_2 (both on day 8)

4.2.2 *Chlamydomonas* sp. and *Coccomyxa* sp. (bioprospected strains)

Similar to the previous section, the first fermentation kit was replaced by the second one on day 8 of the experiment (Figure 4-3 and Figure 4-4). It can be seen from Figure 4-3 that a similar growth trend to *Chlamydomonas reinhardtii* was found with the *Chlamydomonas* sp strain.

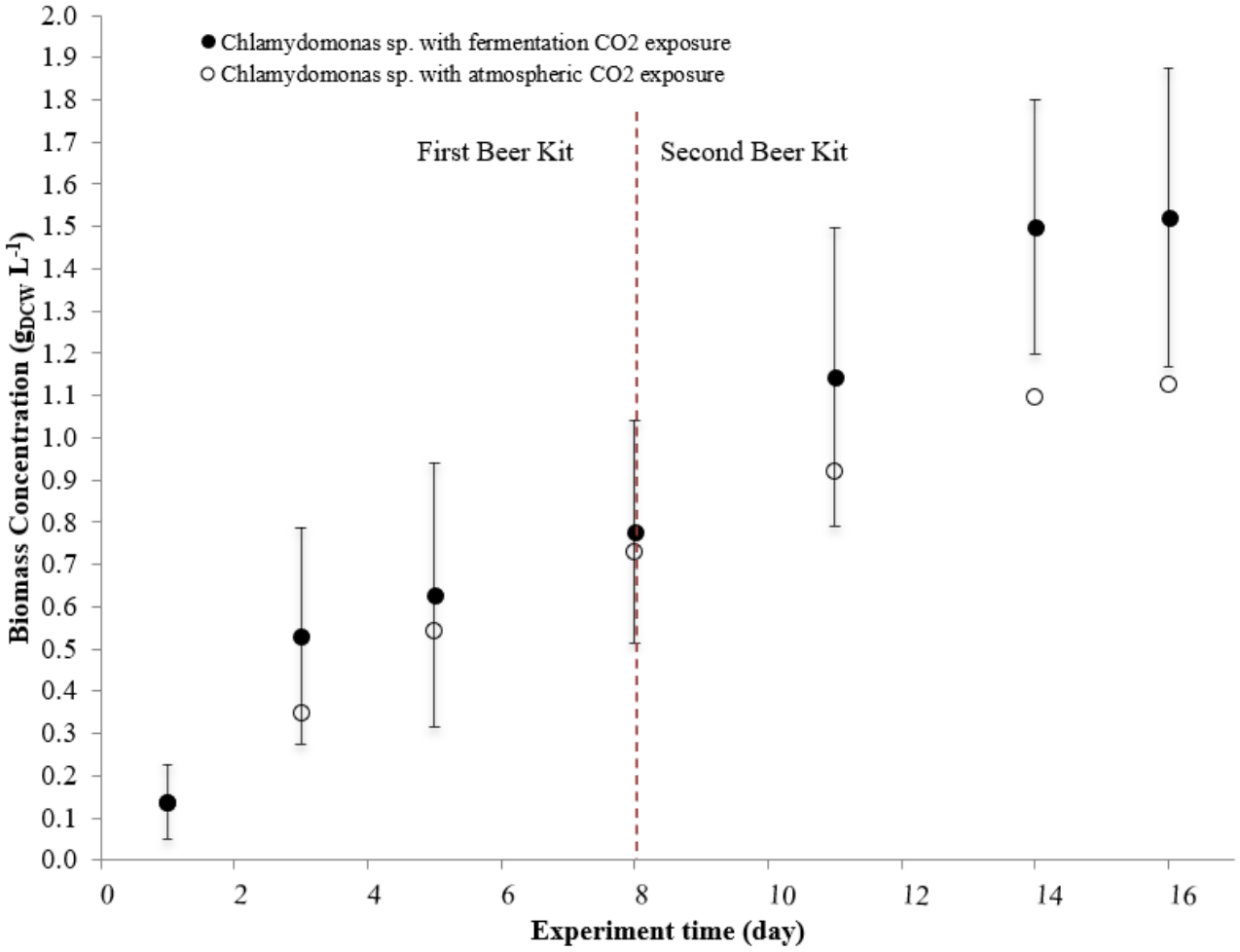

Figure 4-3: *Chlamydomonas* sp. growth rate with and without exposure to fermentation CO_2

The biomass concentration of *Coccomyxa* sp. increased 14.2 times by the end of the experiment when supplemented with CO_2 from the fermentation (Figure 4-4), which is a very promising result. This equivalent values for *Chlamydomonas reinhardtii* and *Chlamydomonas* sp. strains were 9.7 and 11.1, respectively.

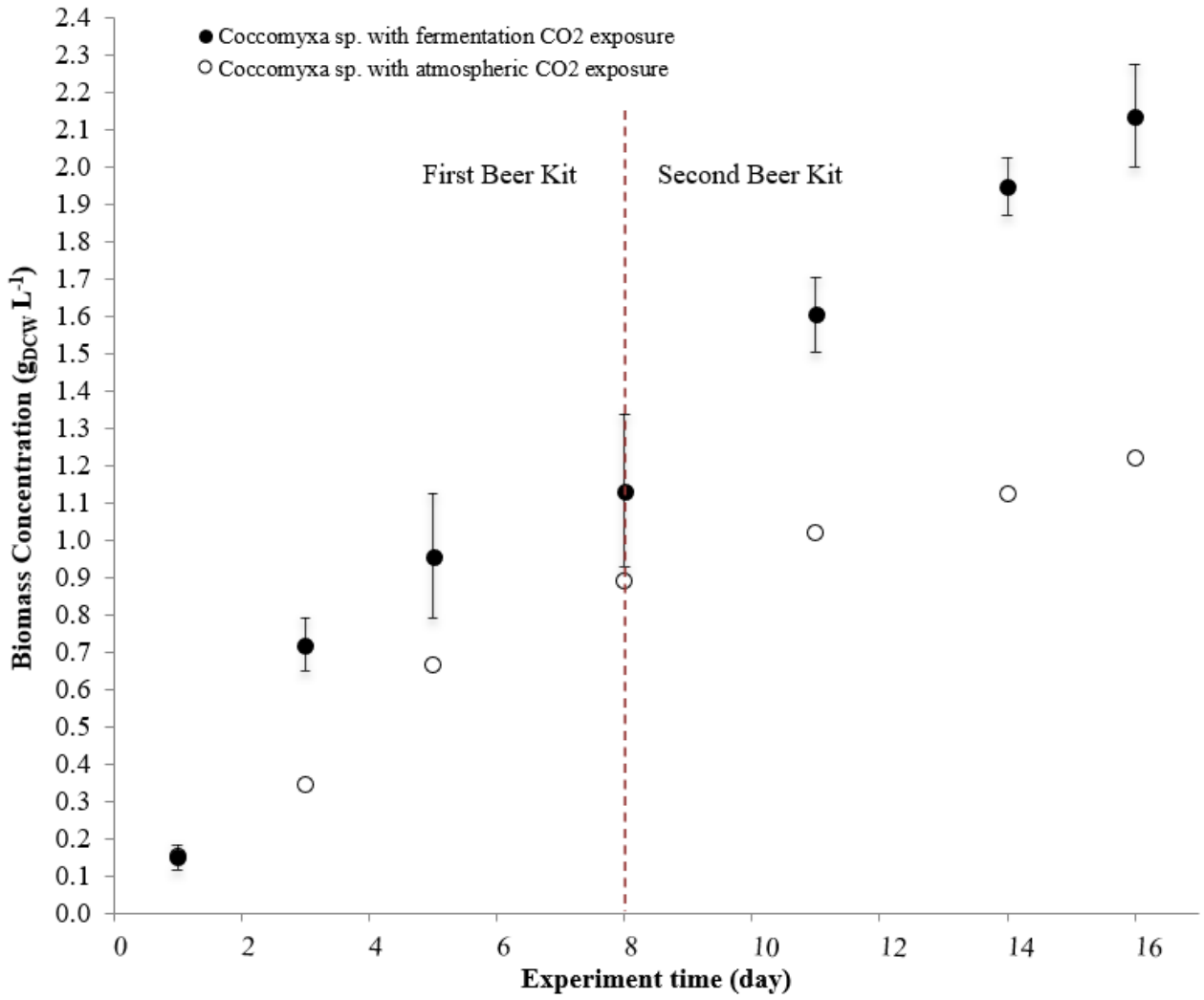

Figure 4-4: *Coccomyxa* sp. growth rate with and without exposure to fermentation CO_2

From this, it can be concluded that fermentation process CO_2 is an ideal source for microalgal strains that are not sensitive to low pH. From Figure 4-4, it can be observed that the *Coccomyxa* sp. culture starts growing exponentially until about 5 d, when decelerating growth takes place, probably because of decreasing CO_2 supply from the fermenters. After 8 days of cultivation, which is the start of the second beer kit, the culture again showed exponential growth. It achieved a more steady-state corresponding to a dry weight biomass concentration of 1.95 g L^{-1} at 14 days and 2.13 g L^{-1} at day 16. From these findings, it can be concluded that *Coccomyxa* sp. is able to provide high biomass productivity with an enhanced CO_2 supply and it could, therefore, be a promising strain to be used in carbon fixation systems. The same

clumping that was mentioned in the previous section was seen in *Coccomyxa* sp. culture. However, *Chlamydomonas* sp. culture did not show any coagulation and clumping.

4.2.3 Comparison of the three strains' growth

Figure 4-5 illustrates the dry weight biomass concentration versus the cultivation time of each of the three species (*Chlamydomonas reinhardtii*, *Chlamydomonas* sp. and *Coccomyxa* sp.) during the cultivation period (16 days) in the presence of the fermentation CO_2. As discussed earlier, it was found that dry weight biomass concentration was higher for the cultures that were exposed to the fermentation CO_2 rather than control cultures. However, dry weight biomass concentration of *Coccomyxa* sp. was 2.13 g L^{-1} on day 16 of the experiment, which was higher than *Chlamydomonas reinhardtii* (1.27 g L^{-1}) and *Chlamydomonas* sp. (1.52 g L^{-1}) concentration on the same day. It can be seen from Figure 4-5 that the overall trend of the three strains was similar showing a higher growth rate from day 1 to 3 and 8 to 11, which were in response to an elevation in fermentation CO_2 formation rate. Both *Chlamydomonas reinhardtii* and *Chlamydomonas* sp. growth rate slowed down from day 14 to 16 reaching stationary phase. While *Coccomyxa* sp. tended to continue its exponential phase.

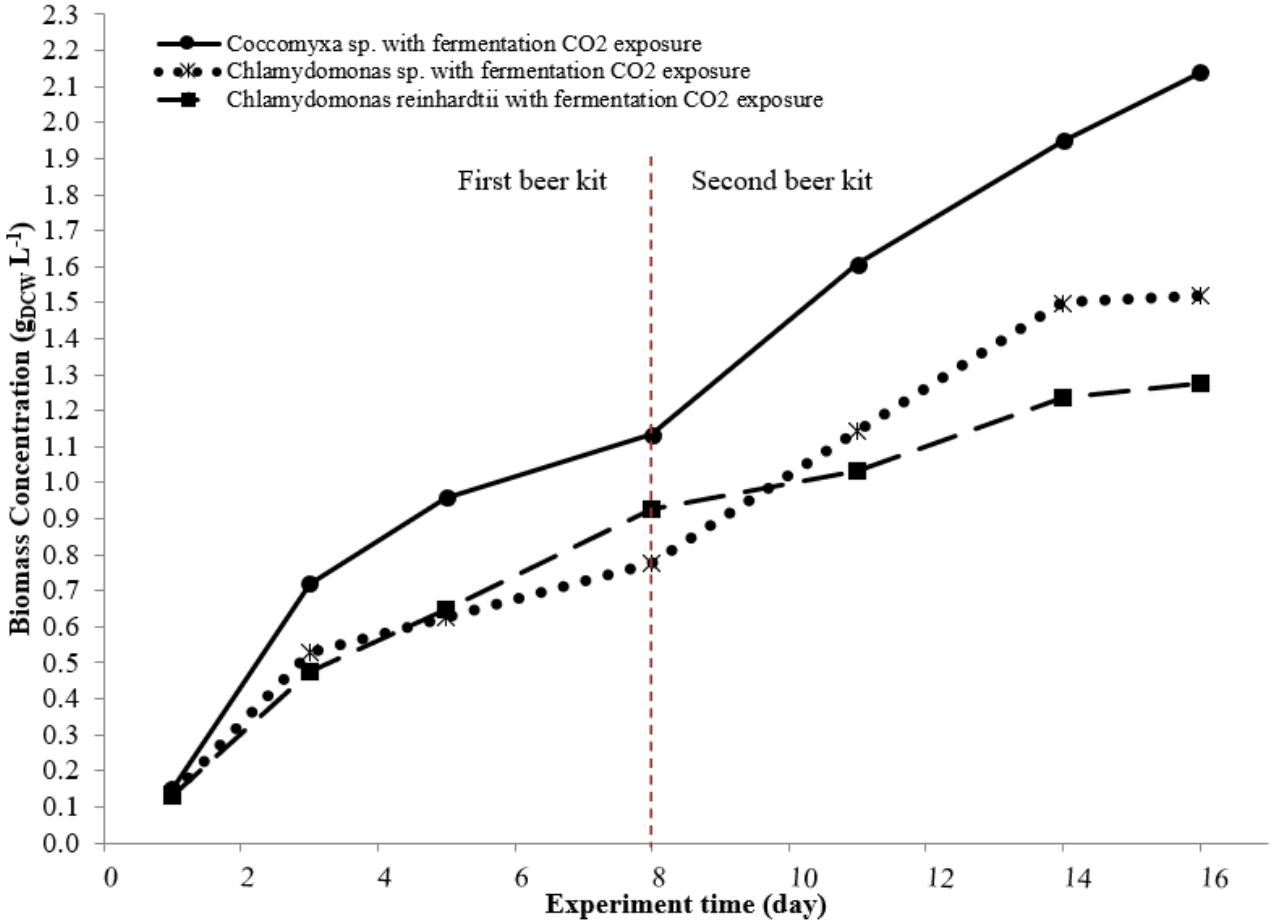

Figure 4-5: Comparison of biomass concentrations (g_{DCW} L^{-1}) from the three strains exposed to fermentation CO_2

4.3 pH of the medium during microalgal growth

The connection of fermentation flasks to the microalgal cultures was started after 14 days of microalgal cultivation, to allow sufficient time to increase microalgal biomass to a level capable of fixing the incoming CO_2. In this way, excessive medium pH decrease was avoided by utilization of the dissolved carbon by the microalgal cells, which results in increasing pH. The microalgal cultures pH was measured coinciding with the highest CO_2 production peaks, with the pH variation a result of bicarbonate–carbonate equilibrium. The bicarbonate–carbonate equilibrium in the culture depends on the balance between CO_2 uptake rate by the microalgae and CO_2 supplied in the inlet gas stream [141]. This excess CO_2 is the cause of the decrease in the pH, resulting in conditions not suitable for growth of pH-sensitive microalgal strains [145].

4.3.1 *Chlamydomonas reinhardtii*

In the current study, the pH of CO_2-enriched *Chlamydomonas reinhardtii* cultures oscillated between 4.6 and 7.3 for those samples that were exposed to CO_2. The pH decreased to 4.6 on day 3 of fermentation CO_2 exposure indicating an excess of CO_2 production by yeast in comparison with CO_2 rate of uptake by the microalgae. As the rate of fermentation slowed down after day 3 and the rate of microalgal growth increased, pH began to increase. It can be seen from Figure 4-6, that after replacing the first beer kit with the second one on day 8 of experiment, pH decreased again to 4.8, but as the fermentation progressed, the amount of injected CO_2 decreased and the pH again rose to 7.3. Although neutral pH to weak alkalinity conditions is generally preferred by most microalgae , *Chlamydomonas reinhardtii* can grow in relatively low pH, although this may affect growth rate [150]. It can be concluded that for microalgal strains that are sensitive to low pH, it is better to control the culture pH by keeping a balance between CO_2 supply and consumption to have a better biomass productivity[151]. A buffer like calcium carbonate or sodium hydroxide can be used to adjust the pH to optimal level when fermentation CO_2 is being utilized, which can enhance productivity as well as CO_2 fixation rate [61], but this comes with additional cultivation costs.

It can be seen from the Figure 4-6 that pH in the control flask gradually increases and reaches 8.7. According to previous studies [76], the pH is elevated to 8 and above, without supplemental CO_2, due to the carbonic anhydrase enzyme transferring hydroxide ions to outside the algal cell. Another reason for increasing pH in cultures that are growing without supplemental CO_2 is the consumption of nitrate as nitrogen source by the microalgae. To assimilate nitrate, microalgal cells transport it across the membrane and then reduce it to ammonia. During this process, large amounts of energy, carbon, and protons are consumed and consequently pH increases [79], [95], [150] [152].

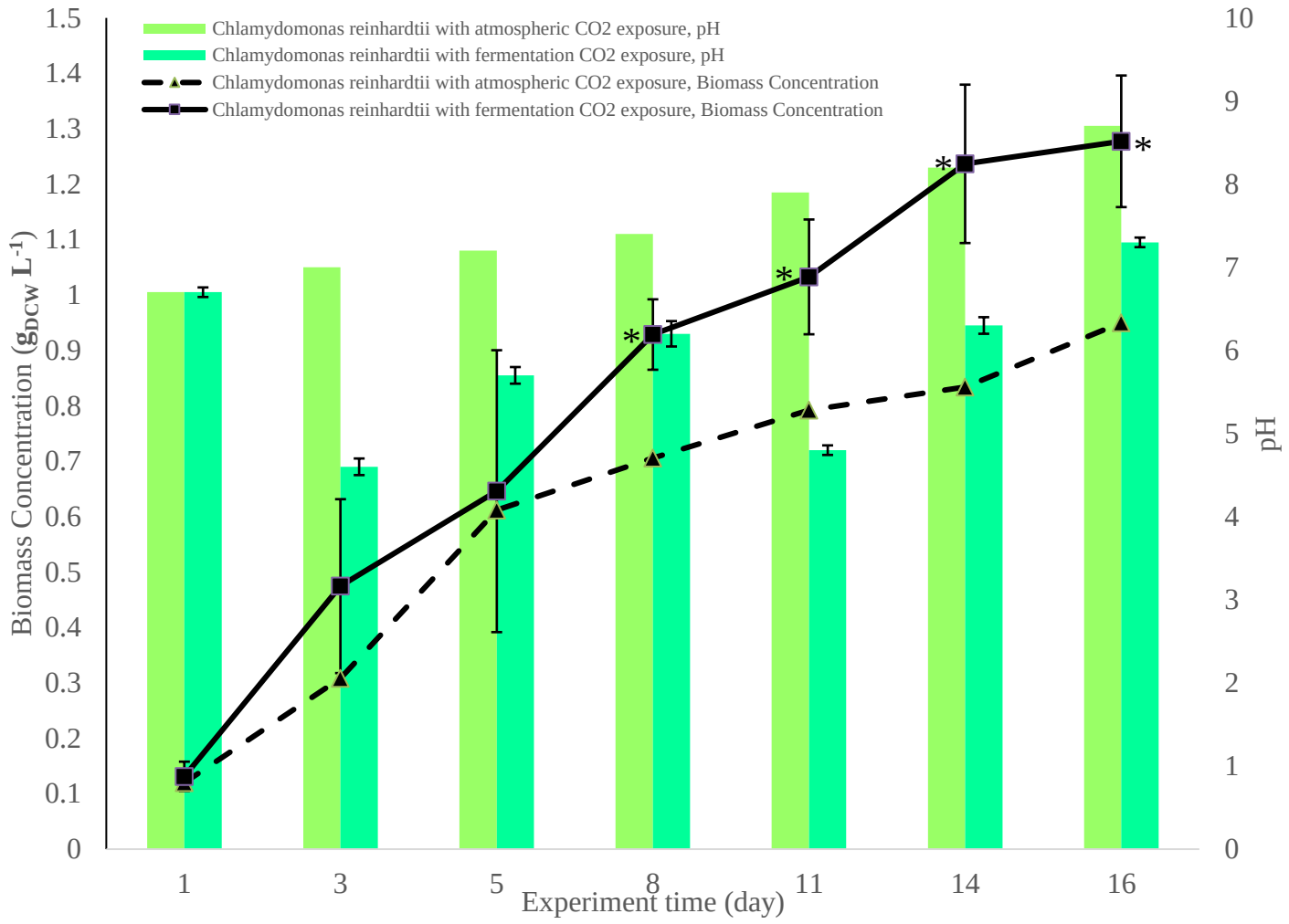

Figure 4-6: Biomass concentration (lines, g_{DCW} L^{-1}) and pH (bars) from *Chlamydomonas reinhardtii* exposed to fermentation and atmospheric CO_2. An asterisk represents a significant difference to the control ($P < 0.05$, t-test).

4.3.2 *Chlamydomonas* sp.

Similar to *Chlamydomonas reinhardtii* strain, in the CO_2-supplemented runs of the *Chlamydomonas* sp., occasional periods of low pH were recorded due to excessive CO_2. The pH pattern during the experiment was like that of the *Chlamydomonas reinhardtii* strain. *Chlamydomonas* sp. pH culture oscillated from 4.6 to 7.1. The growth rate was slow from day 3 to 8, which could be the period that the microalga was trying to adapt to the acidic culture (Figure 4-7). However, it seems that due to *Chlamydomonas* sp. being obtained from low pH environment, elevated CO_2 concentration and subsequently low pH had less effect on its biomass concentration at the end of the experiment in comparison with the *Chlamydomonas reinhardtii*

strain. It is obvious that after day 8, which is the start of the second beer kit, pH started to decrease again and reached 4.6 on day 11 of the experiment. It can be noted on Figure 4-7 that the pH in the control flask gradually increased and reached 8.4 on day 16 of the experiment.

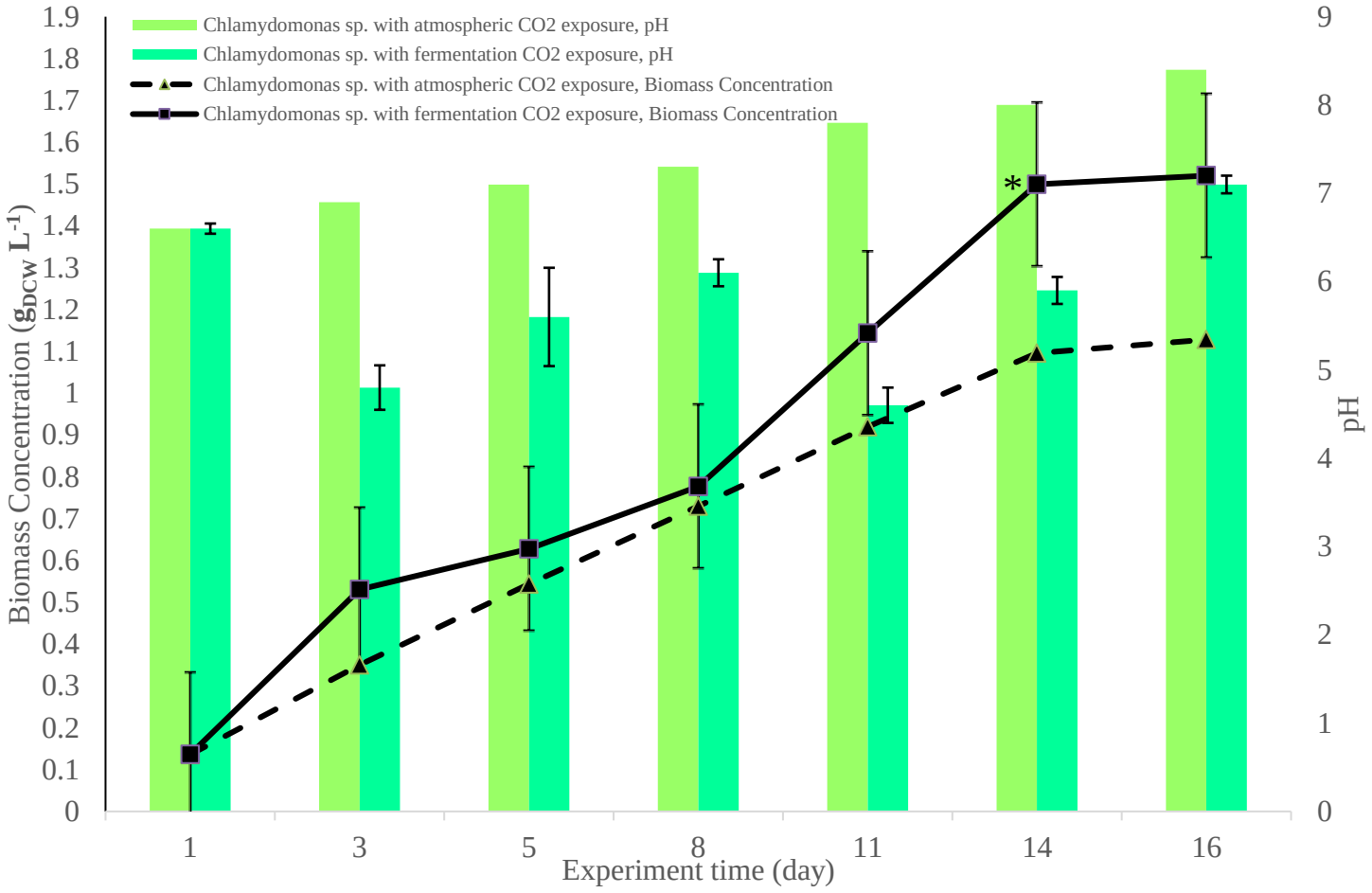

Figure 4-7: Biomass concentration (lines, g_{DCW} L^{-1}) and pH (bars) from *Chlamydomonas* sp. exposed to fermentation and atmospheric CO_2. An asterisk represents a significant difference to the control ($P < 0.05$, t-test).

4.3.3 Coccomyxa sp.

From Figure 4-8 it can be observed that the pH pattern over the experiment as a result of consumption of dissolved CO_2 by the *Coccomyxa* sp. was similar to the other strains tested. From the initial value of 7 it decreased to reach a value of 5.3 after 3 days of cultivation under fermentation CO_2, but then started to increase until the second fermentation batch was attached and started to release CO_2 when it dropped to 5.9. On the final day of the experiment, the CO_2 enriched samples' pH was about 7.4, compared to the

control sample pH of 8.6. *Coccomyxa* sp. is not very sensitive to changes of pH and can be cultivated at low pH, under which the chance of phototrophs and protozoa contamination is minimized, especially when it is cultivated in open ponds [148]. In a study [138] of the effect of pH on *Coccomyxa* sp. (*Coccomyxa onubensis ACCV1*) productivity, it was revealed that immediate adaptation in terms of cell density and dry weight occurred during a shifted pH from 2.5 to higher pH values. The results showed that *Coccomyxa onubensis ACCV1* biomass productivity at alkaline, acidic and neutral pH, if the cultures were previously adapted, differ only slightly. Therefore, it can be concluded that as the *Coccomyxa* sp. used was bioprospected from an extreme environment, it already has the mechanisms to adapt in low pH without inhibiting growth rate and productivity. Therefore, its capacity to fix higher CO_2 concentrations is higher and it could be a good algal candidate for sequestration of high CO_2 concentrations.

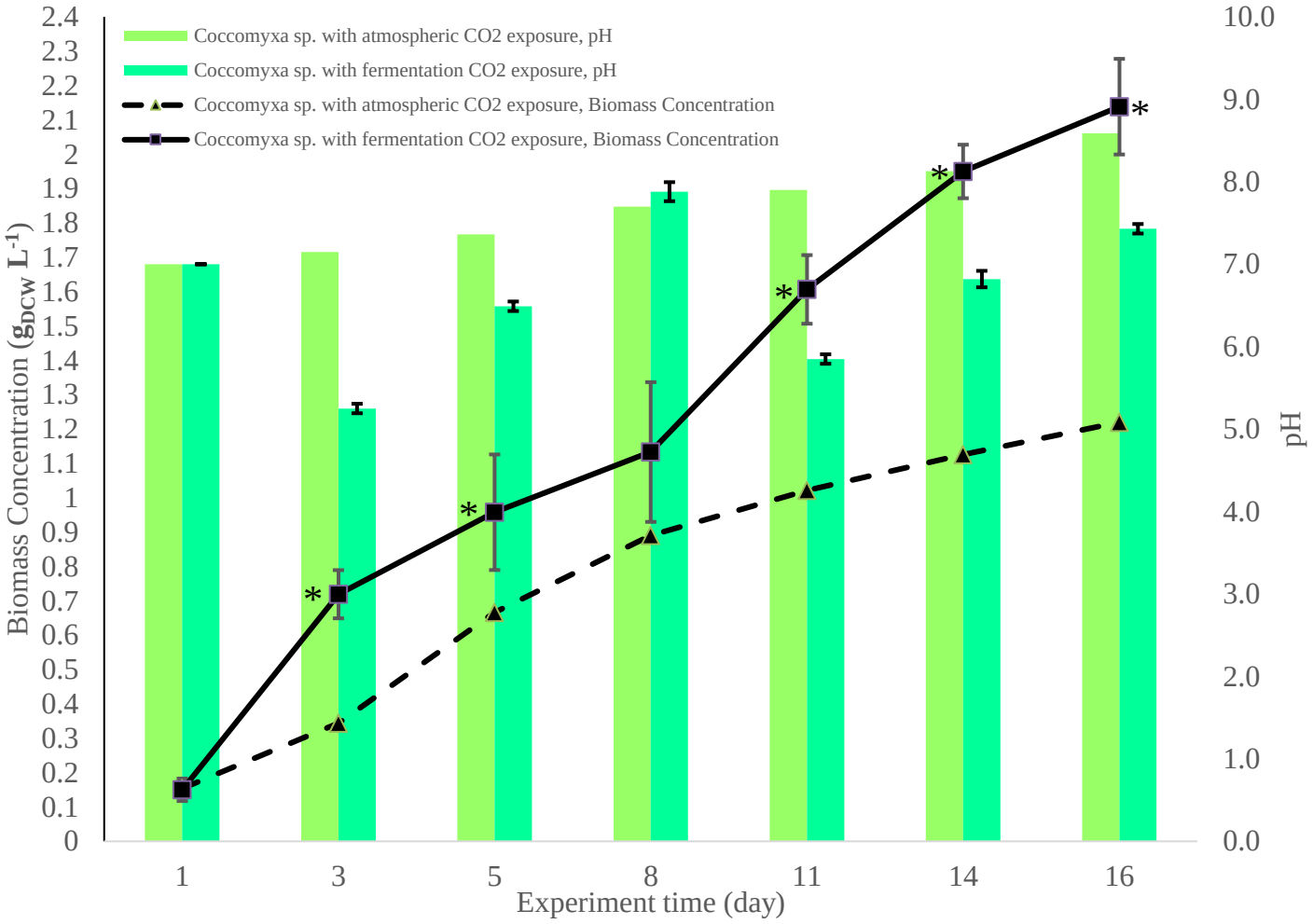

Figure 4-8: Biomass concentration (lines, g_{DCW} L^{-1}) and pH (bars) from *Coccomyxa* sp. exposed to fermentation and atmospheric CO_2. An asterisk represents a significant difference to the control ($P < 0.05$, t-test).

4.3.4 Comparison of the three strains' growth pH

On the start day of the experiment pH of *Chlamydomonas reinhardtii*, *Chlamydomonas* sp. and *Coccomyxa* sp. were 6.7, 6.6 and 7 respectively. However, by starting the first beer kit and being exposed to CO_2, the pH in all cultures reduced. This reduction was partially compensated by microalgal growth, which lead to an increase of culture pH. By starting the second kit, this trend was repeated. Controlled cultures' pH increased continuously and reached 8.7 for *Chlamydomonas reinhardtii*, 8.4 for *Chlamydomonas* sp. and 8.6 for *Coccomyxa* sp. at the end of the experiment. As can be seen in Figure 4-9, the pH of *Coccomyxa* sp. culture is higher thorough the experiment due to its higher growth rate, which leads to pH increment.

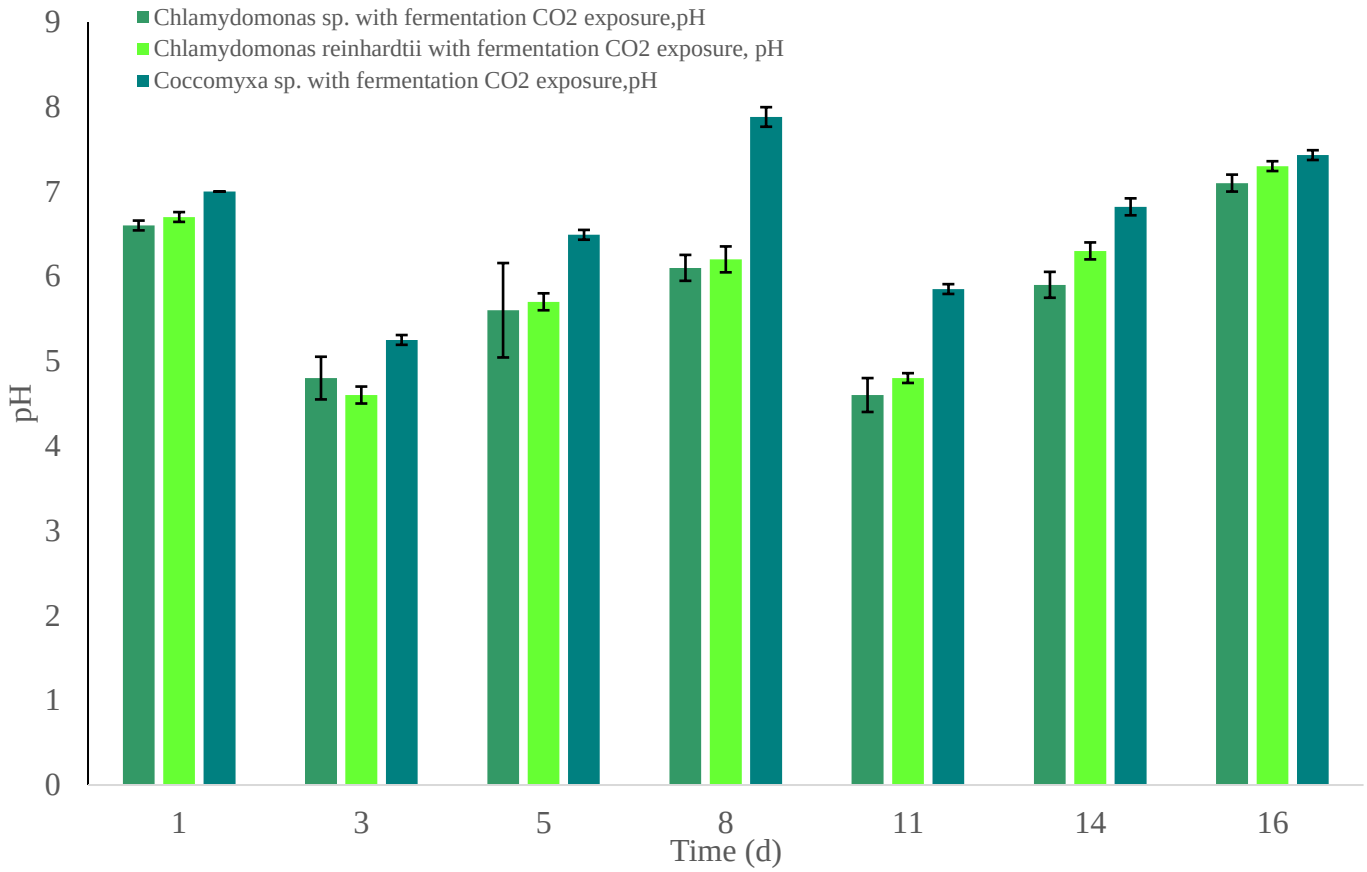

Figure 4-9: pH Comparison of the three microalgal strains exposed to fermentation CO_2

4.4 Lipid production

As discussed in Section 1.9.1, several strains of green microalgae have received attention because of their high biomass production rates, while also producing high concentrations of lipids [79], [94]. Due to its high lipid content and the variety of lipid composition, green algae are a potential oil producer [104], [150]. The availability of carbon sources has a positive impact on the quality and quantity of the microalgal lipid accumulation. In this study, the lipid content of the dried biomass was extracted applying modified Folch. method [142] and stated as percentage and lipid productivity ($mg_{lipid}\,L^{-1}$).

4.4.1 *Chlamydomonas reinhardtii*

In this study, the lipid accumulation of *Chlamydomonas reinhardtii* under 0.04% (atmospheric CO_2 concentration) and fermentation CO_2 was investigated. As shown in Figure 4-10, lipid production was 18% (23.2 $mg_{lipid}\,L^{-1}$) of the dried biomass at the start date of experiment and reached over 35% (447 $mg_{lipid}\,L^{-1}$) on day 16 for samples that were exposed to the fermentation CO_2.

From being exposed to CO_2 continuously for 16 days, lipid content of the dried biomass increased 19.2 times, while lipid content in the sample that was growing under atmospheric CO_2 concentration did not exceed 8.8 times in comparison with the start of the experiment. Actually, the lipid percentage of dry cell weight (DCW) only increased to 19% (180.5 $mg_{lipid}\ L^{-1}$) during 16 days of the experiment when *Chlamydomonas reinhardtii* was growing under atmospheric CO_2. There were no significant differences among cultures enriched with CO_2 in terms of lipid content of dry weight biomass. Similar observations of an increase in lipid content with higher concentration of CO_2 was reported in several resources [152] [69] [150].

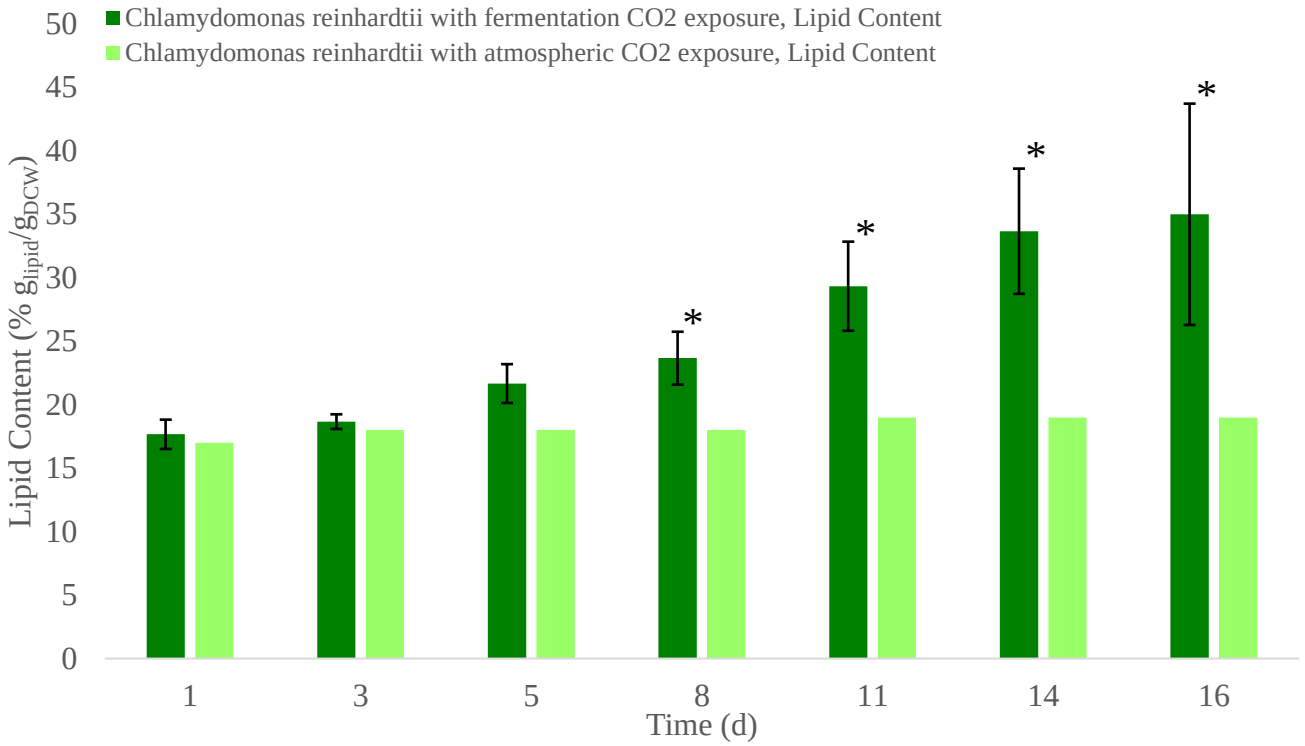

Figure 4-10: Changes in lipid content in *Chlamydomonas reinhardtii* exposed to fermentation and atmospheric CO_2. An asterisk represents a significant difference to the control ($P < 0.05$, t-test).

4.4.2 *Chlamydomonas* sp.

As discussed before, the availability of other carbon sources has a positive impact on the quality and quantity of the microalgal lipid accumulation [75]. As can be seen in Figure 4-11, without fermentation CO_2, the lipid content of dry cell weight of *Chlamydomonas* sp. increased from 27.2 $mg_{lipid}\,L^{-1}$ (20%) to 270.7 g L^{-1} (24%) in 16 days. But with fermentation CO_2, lipid content in *Chlamydomonas* sp., increased from 27.3 $mg_{lipid}\,L^{-1}$ (20%) to 592.8 $mg_{lipid}\,L^{-1}$ (39%), which was the highest lipid production among the examined microalgal strains in this study. So, fermentation CO_2 aeration proved to be an effective factor for the elevation of lipid accumulation in microalgae.

The decrease in lipid content on day 11 can be related to the algal growth phase, as on day 11 cultures that were exposed to CO_2 showed a sharp increase in biomass growth and they were still in the logarithmic phase. According to a study done by Huerlimann et. al [153], lipid content for a given species depends on growth phase and medium composition, lowest lipid yields are common for logarithmic phase and nutrient

deprived cultures. While, lipid yields are higher at the end of logarithmic phase [153]. As per previous studies [154], *Chlamydomonas* sp. has been investigated by many researchers to be a potential strain for production of lipid, but there is not much work regarding the effect of different carbon sources on its lipid production.

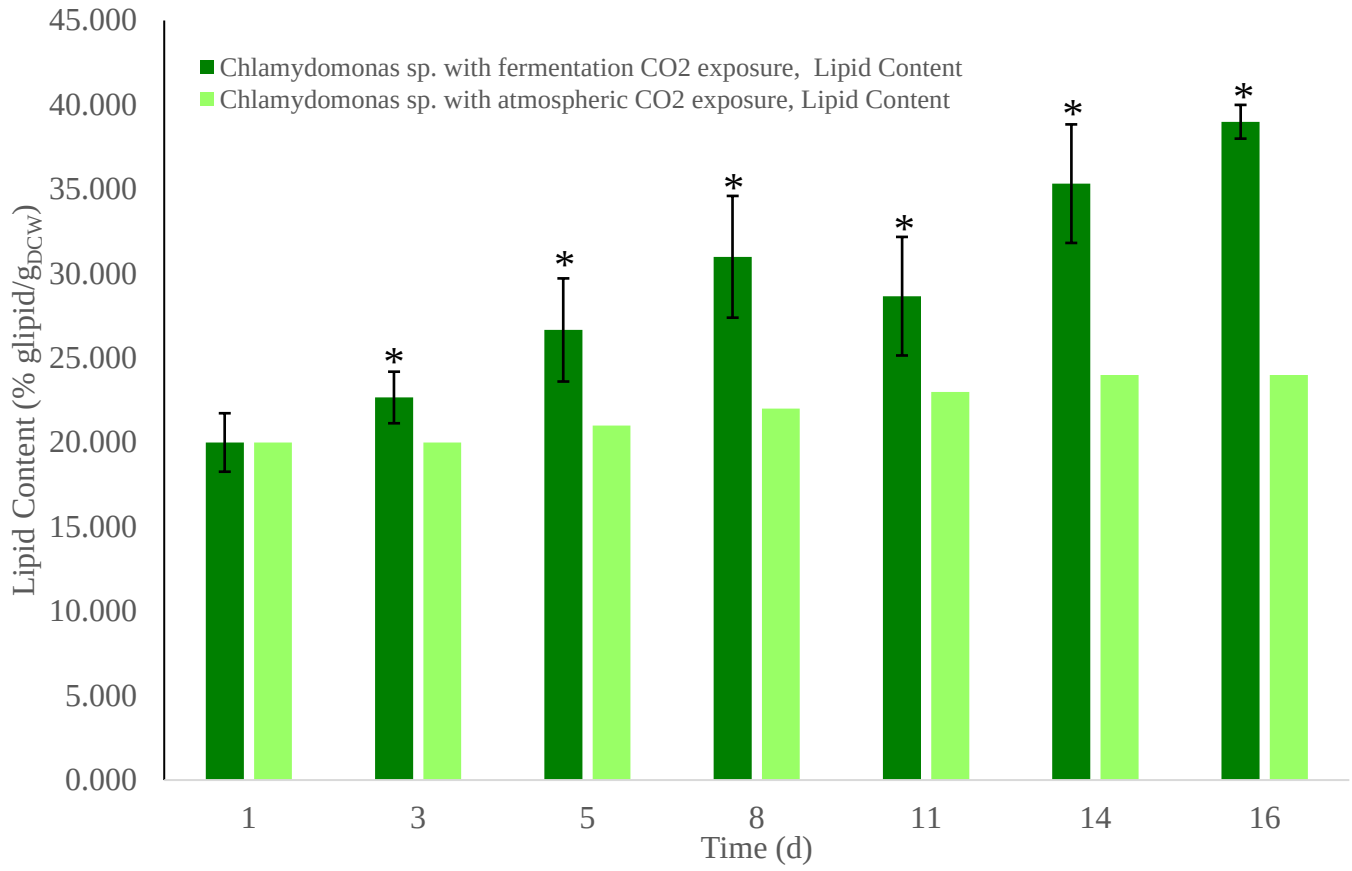

Figure 4-11: Changes in lipid content in *Chlamydomonas* sp. exposed to fermentation and atmospheric CO_2. An asterisk represents a significant difference to the control ($P < 0.05$, t-test).

4.4.3 *Coccomyxa* sp.

The results showed that the total lipid content, described as percentage of dry mass of *Coccomyxa* sp. ranged from 9% for the control to 26.3% for the samples exposed to fermentation off-gas (Figure 4-12), which was slightly higher than other reported lipid contents of 20-25% for *Coccomyxa* sp. [155]. However, under nitrogen depletion conditions, the unicellular alga *Coccomyxa subellipsoidea* strain can rapidly grow and accumulate triacylglycerols in lipid bodies at levels higher than 60% (w/w) of its dry weight in minimal

mineral media. This algal strain has been used as a model organism for biofuel production due to its rapid growth rate and high-quality lipids. This microalga also lacks a rigid cell wall, which make lipid extraction relatively straightforward [149].

The effect of environmental factors such as CO_2 concentration on lipid metabolism is poorly understood in *Coccomyxa* sp. [137]. In this study, dry weight biomass lipid content reached 19% after only 5 days of cultivation, and 26.3 % at the end of cultivation under fermentation CO_2. These outcomes are consistent with those observed with another extremophile *Coccomyxa* sp. strain [155]. However, compared to cells cultured with atmospheric CO_2, lipid content of CO_2-supplemented cells was 3.5 times higher, demonstrating that CO_2 supplementation was an effective trigger for lipid accumulation in *Coccomyxa* sp. As can be seen in Figure 4-12, without fermentation CO_2, the lipid content of dry cell weight of *Coccomyxa* sp. increased from 14 $mg_{lipid}\ L^{-1}$ (9%) to 158.6 g L^{-1} (13%) in 16 days. But with fermentation CO_2, lipid content in the *Coccomyxa* sp., increased from 13.6 $mg_{lipid}\ L^{-1}$ (9%) to 563.1 $mg_{lipid}\ L^{-1}$ (26.3%).

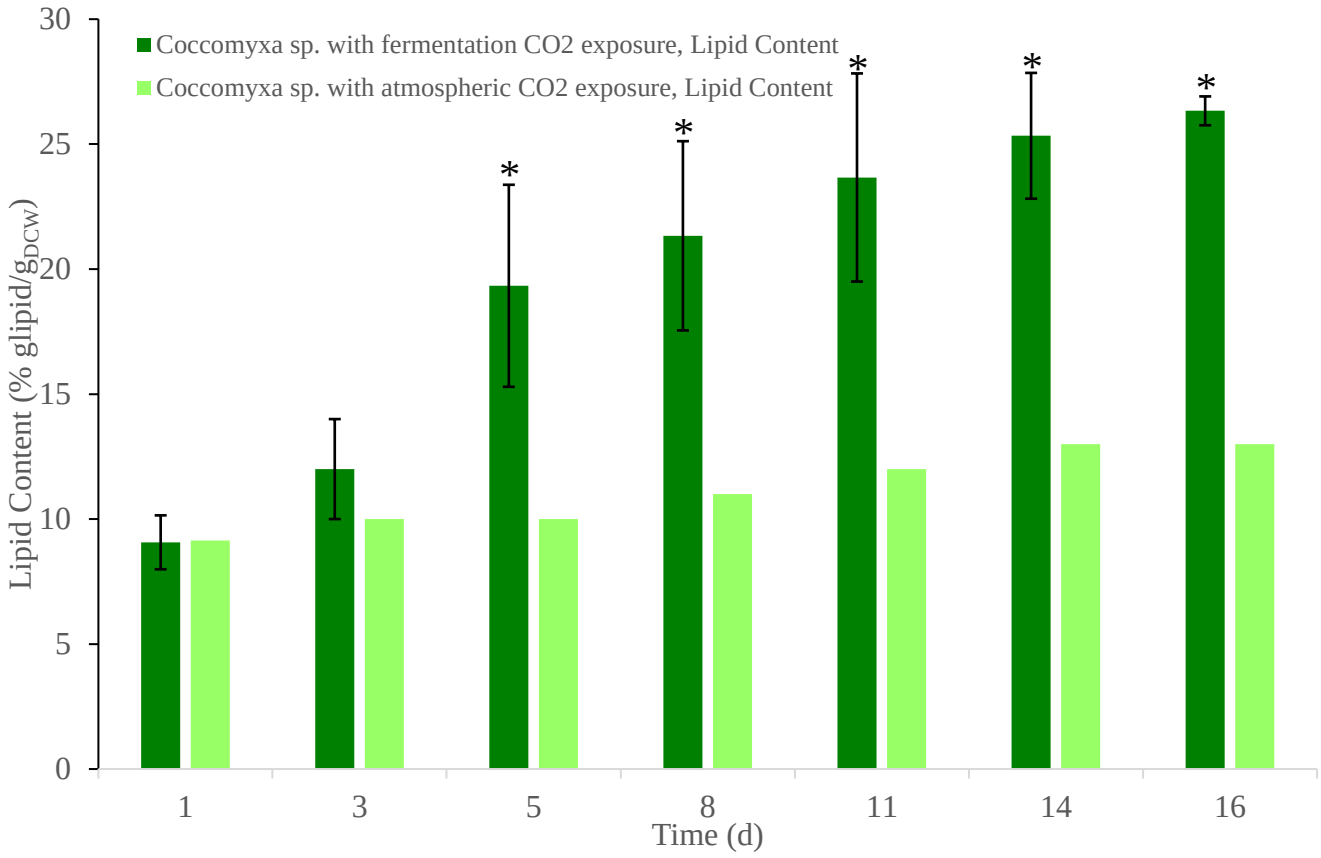

Figure 4-12: Change in lipid content in *Coccomyxa* sp. exposed to fermentation and atmospheric CO_2. An asterisk represents a significant difference to the control ($P < 0.05$, t-test).

4.4.4 Comparison of the three strains' lipid content

Figure 4-13 a comparison of dry weight biomass lipid content of the three microalgal strains that were exposed to fermentation CO_2 is given. It can be noted that all the strains' lipid content followed a similar trend and increased during the experiment except *Chlamydomonas* sp., which showed a decrease in its lipid content on day 11. This decrease was probably a technical issue or possibly related to the algal growth phase. However, two other strains' lipid content showed a gradual increase without any reduction. It is known that during microalgal growth, nitrogen concentration in the culture reduces [50]. It can be concluded that longer time of nitrogen starvation obviously resulted in higher accumulation of lipid inside the three microalgal cells. It is clear from Figure 4-13 that *Chlamydomonas* sp. is a better candidate for lipid production in enriched CO_2 environment as its dry weight biomass lipid content reached 39% at the end of the experiment. This number for *Chlamydomonas reinhardtii* was 35% and for *Coccomyxa* sp. was 26.3%.

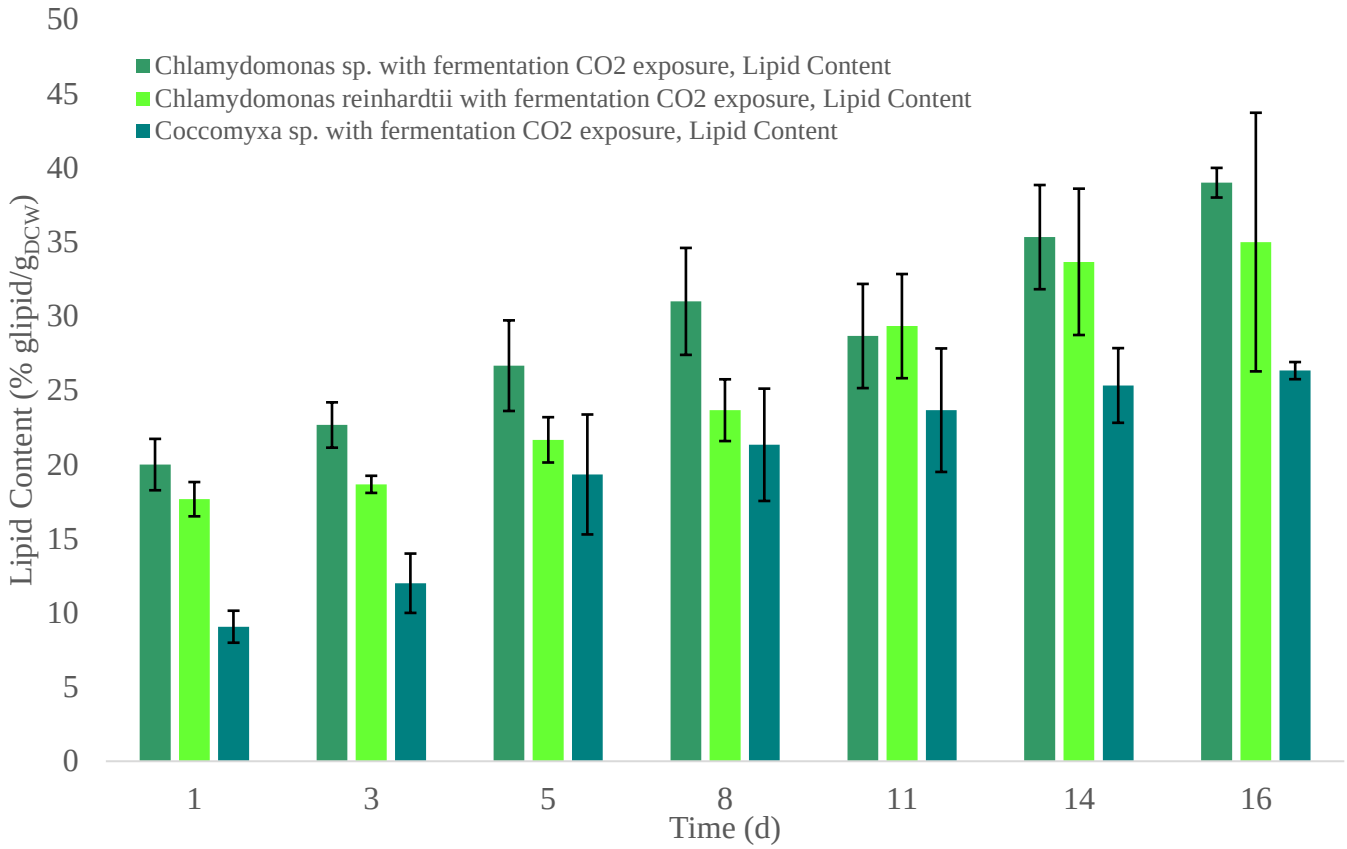

Figure 4-13: Comparison of lipid content in the three microalgal strains exposed to fermentation CO_2

Figure 4-14 compares the lipid productivity of three cultures with fermentation CO_2. *Chlamydomonas* sp. lipid productivity indicated a maximum amount of 592.8 ($mg_{lipid,\ DCW}\ L^{-1}$). Although dry weight biomass lipid content of *Coccomyxa* sp. was much lower than *Chlamydomonas* sp., its lipid productivity was 563.2 ($mglipid_{,\ DCW}\ L^{-1}$), which is close to *Chlamydomonas* sp. lipid productivity. This is due to *Coccomyxa* sp. high growth rate under elevated CO_2 concentration. It must be noted that *Coccomyxa* sp. was the strain that showed the best combination of biomass productivity and lipid content. From Figure 4-14, lipid productivity for all strains showed a similar trend and increased from the start to the end of the experiment.

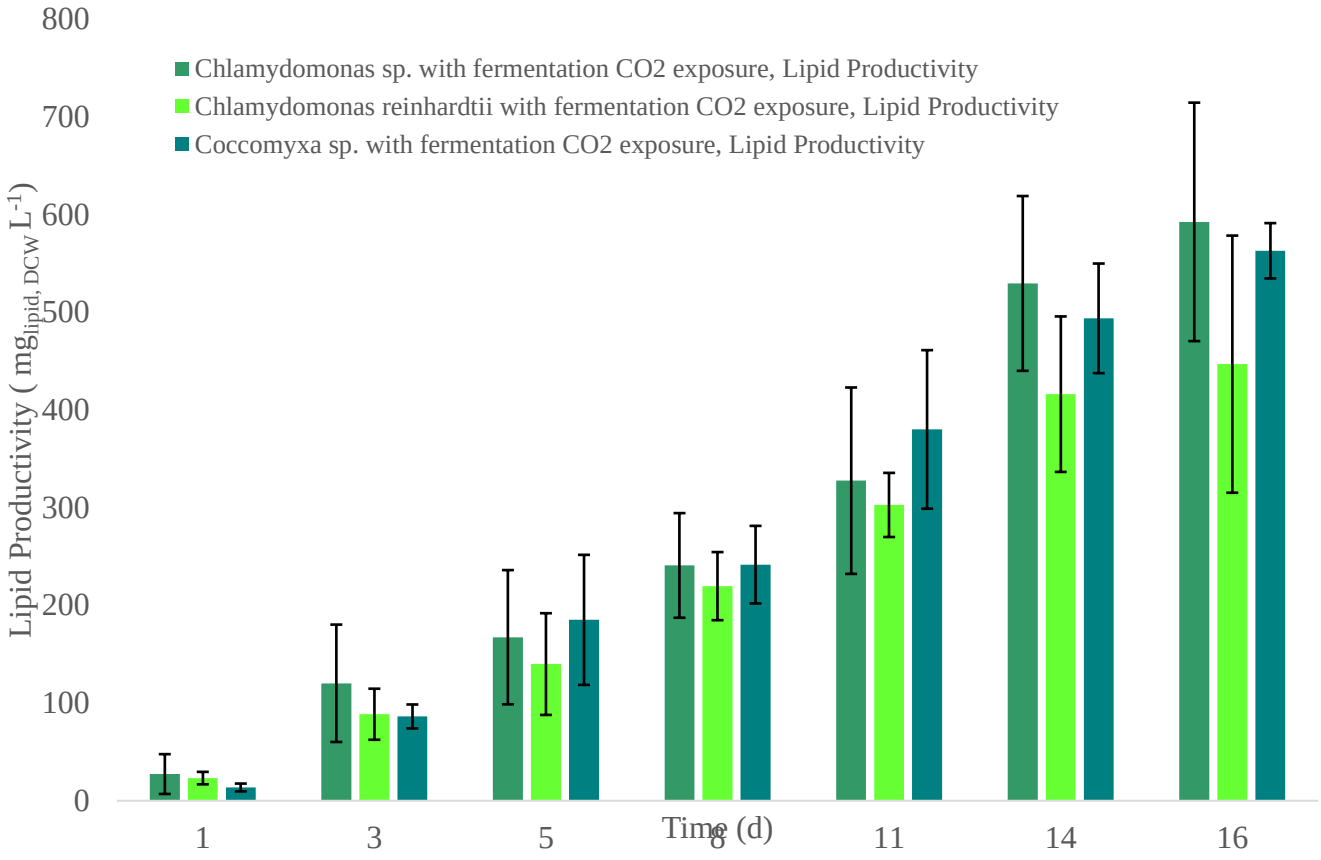

Figure 4-14: Comparison of lipid productivity in the three microalgal strains exposed to fermentation CO_2.

4.5 Protein production

Nitrogen present in the microalgal culture is consumed, converted into amino acids and packaged as proteins under normal conditions. So, adjusted concentrations and chemical forms of nitrogen within the growing culture of microalgal has a positive influence on cell growth and protein content [122]. However, due to microalgal unicellular nature, their protein composition is diverse and depends on the cultivation conditions. Many studies have been carried out to evaluate the impact of environmental factors on microalgal growth and biochemical profiles of proteins. Along with genetic traits, microalgal protein content can be affected by various factors such as CO_2 levels, temperature, light intensity and spectrum, nutrient media composition and pH [86] [148]. Furthermore, the effect of each factor varies among different species. In order to reach a favorable biochemical composition, it should be considered that each microalgal species has an optimal pH range. It has been reported that many proteins, including enzymes, are affected by the growth medium pH [109] [128]. pH variation may alter the active structure of enzymes or proteins

and lead to changes of the ionic charges on the molecule. In this case, the metabolism is inhibited and enzyme activities are usually reduced [109]. In this study, the protein content of the dried biomass was extracted applying Lowry's method, which is considered suitable for microalgal protein extraction [143] and stated as percentage.

4.5.1 *Chlamydomonas reinhardtii*

The protein content of *Chlamydomonas reinhardtii* can be up to 46.9% of dry biomass [156]. Some studies [157] reported that microalgal protein content was affected substantially by the percentage of CO_2 supplied to the culture. In this study, *Chlamydomonas reinhardtii* biomass protein percentage was 30.7% of dry weight biomass for the cultures exposed to fermentation CO_2 and 30.3% those that grew under atmospheric CO_2. This result can be related to the low pH caused by injected CO_2, as *Chlamydomonas reinhardtii* is sensitive to pH, its protein content in the biomass may not increase as expected with the decrease in pH in comparison with neutral pH. Moreover, little change in protein content may be related to consumption of nutrients specially nitrogen. Msanne et.al (2012) reported that the content of total and soluble proteins decreases substantially in nutrient deprived cells.

As can be seen in Figure 4-15, protein content reached 31.5% for CO_2 enriched samples by day 11 and then started to decrease. This reduction may be a result of nitrogen depletion as the CO_2 enriched samples' growth rates were much higher than the control. It could also be due to the low pH of the culture. The results in Figure 4-15 showed that the pH and the CO_2 source did not affect the protein content of the dry weight biomass noticeably, as protein content remained quite constant in all experiments (29.5% ±2.4%).

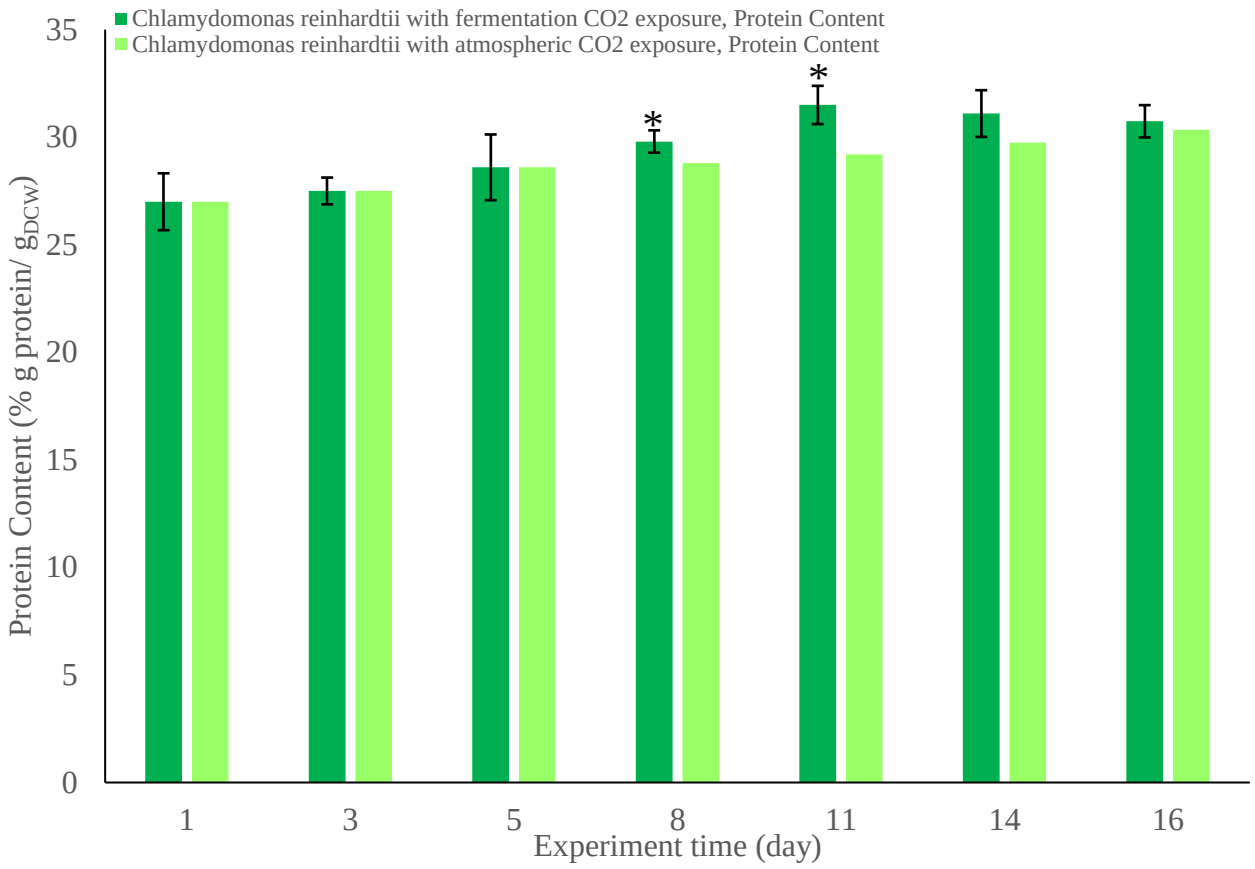

Figure 4-15: Change in protein content in *Chlamydomonas reinhardtii* exposed to fermentation and atmospheric CO_2. An asterisk represents a significant difference to the control ($P < 0.05$, t-test).

From previous studies [137], it is known that in the absence of nitrogen, which is necessary for cell growth and protein synthesis, excess carbon from photosynthesis can be channeled into storage molecules, such as starch and lipids (especially triglycerides). Therefore, a decrease in protein concentration can result in increasing of the carbon content in the cell, which is caused by forcing cells to alter from protein production to carbon storage pathways [141].

4.5.2 *Chlamydomonas* sp.

The results in Figure 4-16 showed that protein content of the dry weight biomass of *Chlamydomonas* sp. was 25% at the beginning of the experiment and reached 27.4% for samples that were exposed to the fermentation CO_2 and 28.8% for those that grew under atmospheric CO_2. It can be concluded that the low pH adversely affected the protein content of *Chlamydomonas* sp.

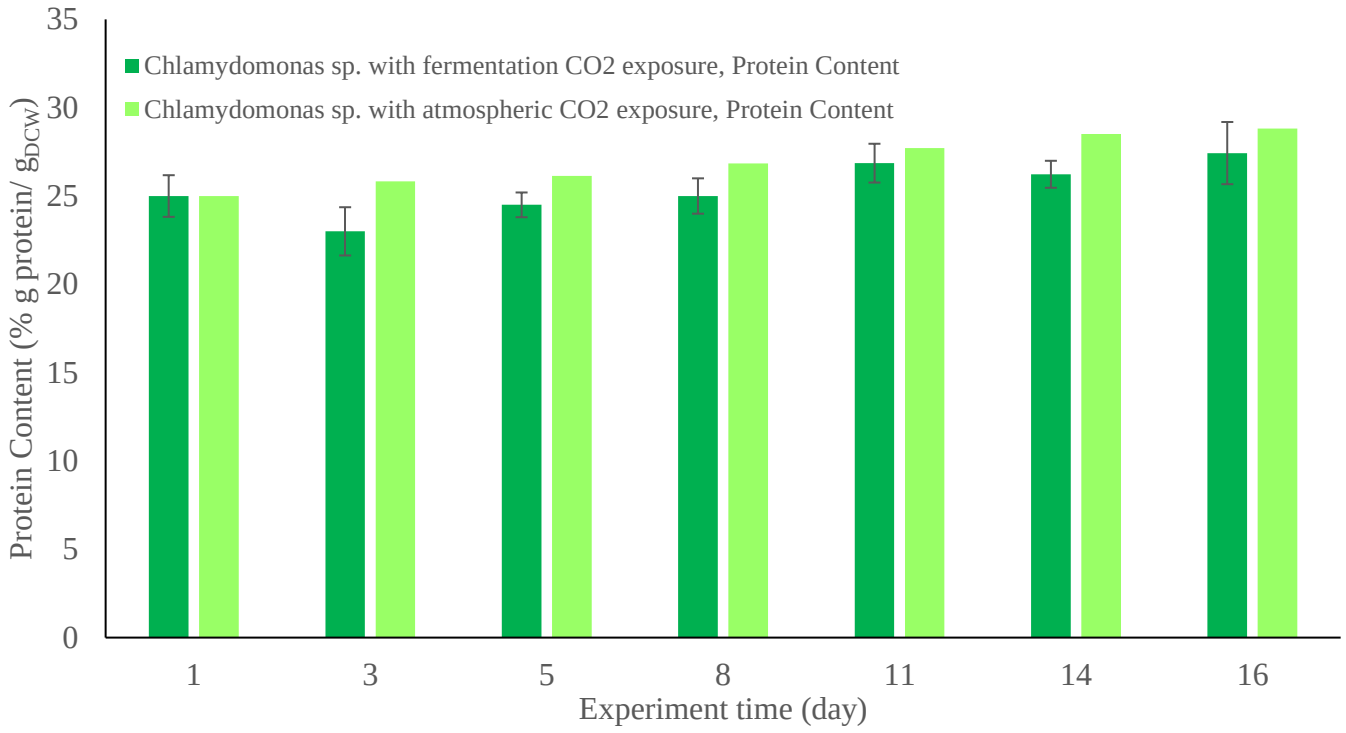

Figure 4-16: Changes in protein content in *Chlamydomonas* sp. exposed to fermentation and atmospheric CO_2. An asterisk represents a significant difference to the control ($P < 0.05$, t-test).

4.5.3 *Coccomyxa* sp.

As can be seen in Figure 4-17 protein content of dry biomass weight increased from 29 to 36.7% for control batch and increased from 29% to 42.5% for CO_2 enriched samples. It has been suggested in previous studies [14] [128] [158] that higher growth rates lead to an increase in protein biosynthesis due cells needing increased amounts of proteins for cellular division. On the contrary, if microalgae are cultivated in a nitrogen-depleted culture, cell division reduces, and the cells start to have a lower protein content and instead accumulate energy-rich compounds such as polysaccharides or lipids. It has been also reported that higher protein contents are mostly achieved in nitrogen-rich media [137]. From Figure 4-17, it is obvious that protein content of dry biomass weight was the highest percentage (44.4%) on day 11 of the experiment and because of nitrogen depletion, it decreased to 43.1% on day 14 and 42.5% on day 16 of the experiment.

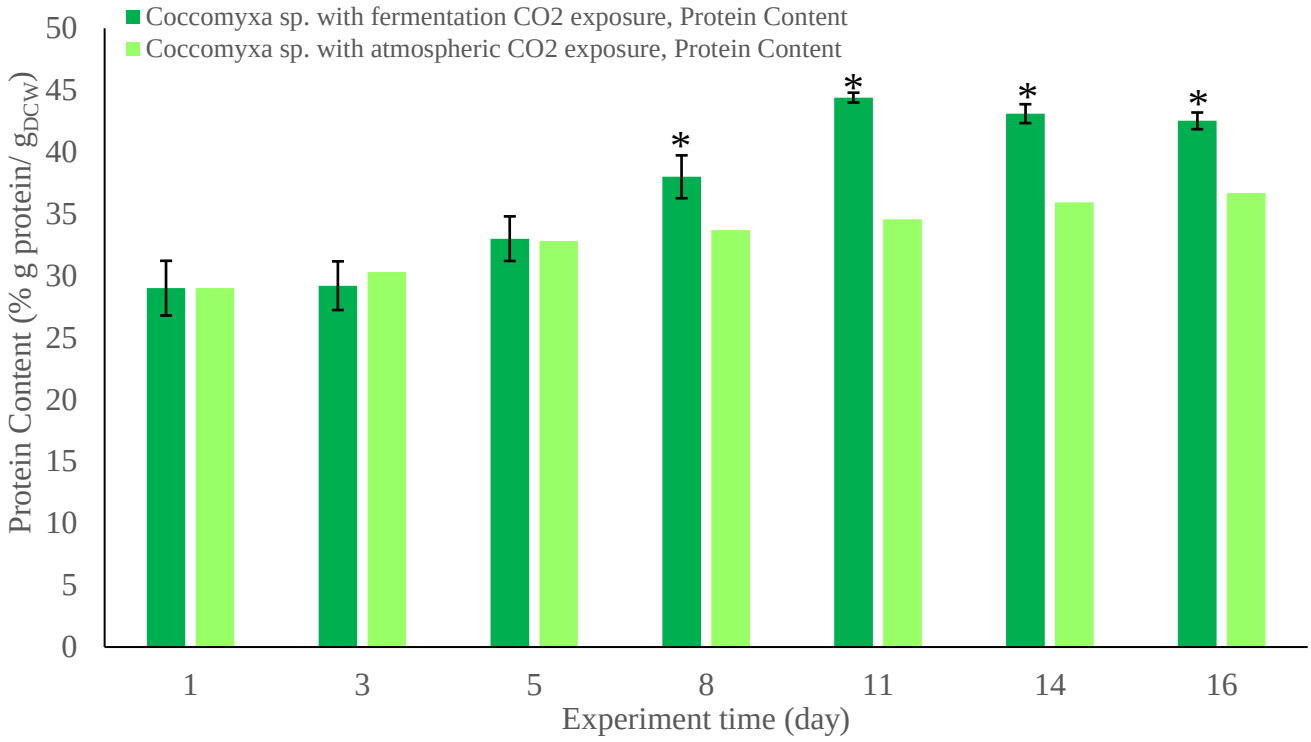

Figure 4-17: Changes in protein content in *Coccomyxa* sp. exposed to fermentation and atmospheric CO_2. An asterisk represents a significant difference to the control ($P < 0.05$, t-test).

The total protein content of dry biomass weight generated by *Coccomyxa* sp. doubled when cultures fed with fermentation CO_2 (Figure 4-17). The protein content in the biomass of *Coccomyxa* sp. on the 16th day of incubation was 909.3 mg L^{-1} at a fed CO_2 of fermentation and 447.7 mg L^{-1} at atmospheric CO_2, respectively. These results are consistent with previous studies on other microalgal strains including *Scenedesmus* sp. and *B. braunii* [158].

4.5.4 Comparison of the three strains' protein content

The maximum protein content of 44.4% of DCW was attained in the exponential phase of *Coccomyxa* sp. cells that were cultivated using fermentation CO_2. The protein content of the two other strains was almost the same and remained in the range of 27 to 30.7% for *Chlamydomonas reinhardtii* and 25 to 27.4% for *Chlamydomonas* sp. of DCW. As can be seen in Figure 4-18. trend of the protein content was not similar to each other for three types of microalgae. For instance, as the experiment progressed from day 1 to 11, protein content of *Chlamydomonas reinhardtii* sp. increased from 27 to 31.54% of DCW. However, it showed a reduction from day 11 to 16 of the experiment. While for *Chlamydomonas* sp., a reduction

occurred from day 1 to day 3 (from 25 to 23%) of the experiment and after that the microalgal protein content started to increase again till day 11 to 26.8% of DCW. After that another reduction to 27.4% of DCW was observed at the end of the experiment.

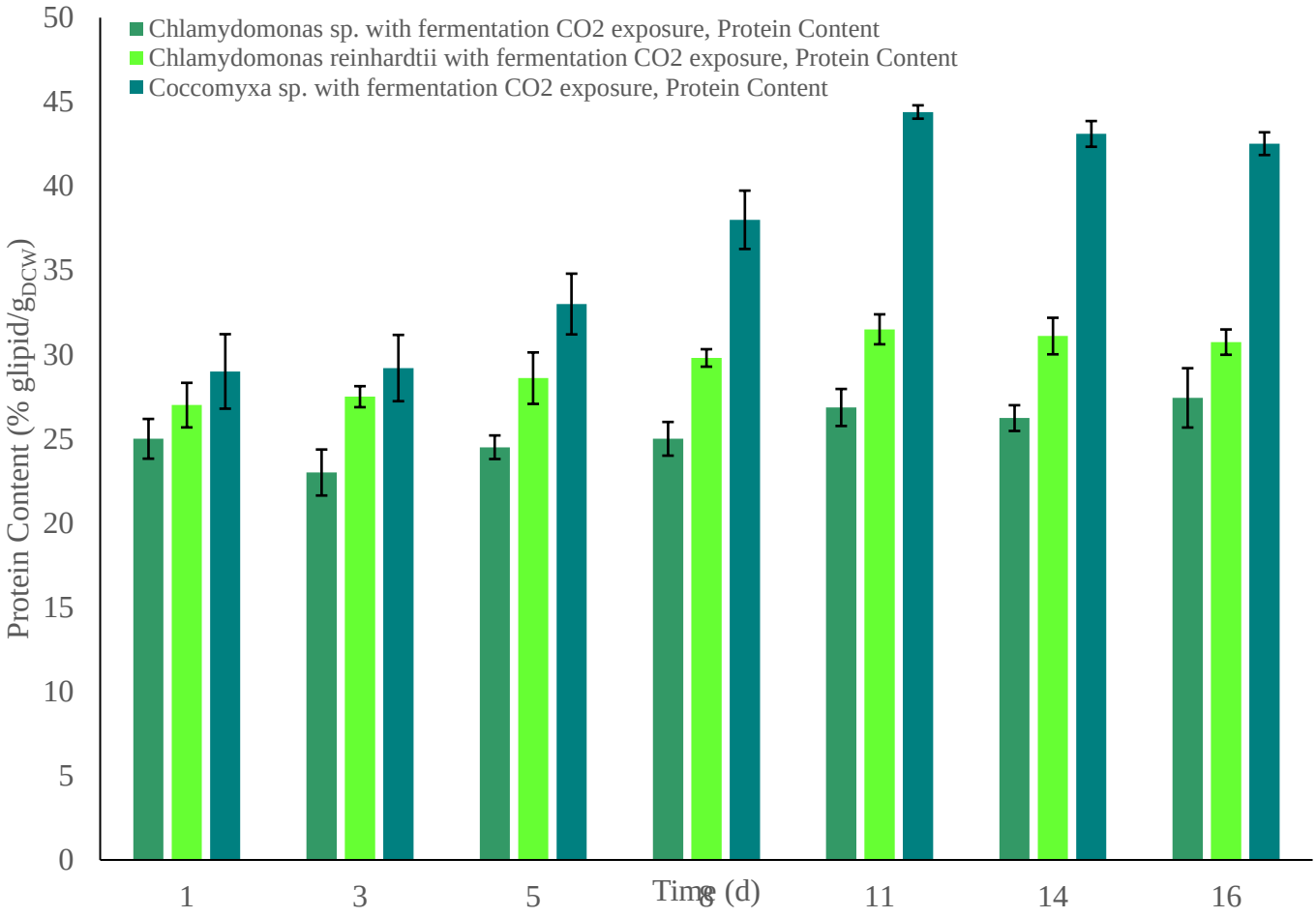

Figure 4-18: Comparison of the protein content in the three microalgal strains exposed to fermentation CO_2. CO_2 sequestration rate

4.6 CO_2 sequestration rate

CO_2 sequestration rate was calculated by considering that microalgal dry biomass contains 50% of carbon [141] and using Equation 3.3, CO_2 bioremediation is the microalgal ability to use CO_2 as the sole source of carbon through the photosynthesis process, which results in the CO_2 removal from the atmosphere. The maximum CO_2 bioremediation rate and maximum productivity are parameters that play a significant role in assessment of the application of microalgal species in CO_2 sequestration.

As discussed in section 1.6, microalgae have the ability to pump and store adequate CO_2 reducing the effect of the competition between oxygen and CO_2 on Calvin cycle by Rubisco. This ability will elevate internal CO_2 concentrations above equilibrium levels with air and the adverse effect of reduced photorespiration process efficiency will be compensated [65]. According to previous studies [67], CO_2 tolerance differs greatly not only between species, but also within a single species as well, depending on microalgal strain growth conditions.

4.6.1 *Chlamydomonas reinhardtii*

Figure 4-19 shows the CO_2 sequestration rate of *Chlamydomonas reinhardtii* throughout the cultivation period with atmospheric and fermentation CO_2 exposure. For samples that were exposed to fermentation CO_2, the maximum sequestration rate occurred from day 1 to 3 at 314.7 $mg_{CO2}\,L^{-1}\,day^{-1}$ (Figure 4-19). Average CO_2 uptake rates were 131.3 and 95.1 $mg_{CO2}L^{-1}day^{-1}$ for enriched CO_2 and the control samples respectively, which is a 38% increase in CO_2 fixation rate.

The results obtained in this study were in agreement with the investigation of Sharma et al.[4] and Ferreira et al. [159], who investigated the fermentation CO_2 influence on different algal strains. They similarly reported that elevation of CO_2 levels made available improved the specific growth rate and microalgal photosynthetic activity, which results in higher CO_2 sequestration potential. The higher CO_2 sequestration rates during days 1 to 3 were likely due to the high rate of capture early in the exponential phase prior to the CO_2 levels decreasing the pH. However, this pattern was reversed in the second fermentation batch probably due to microalgal adaption to low pH.

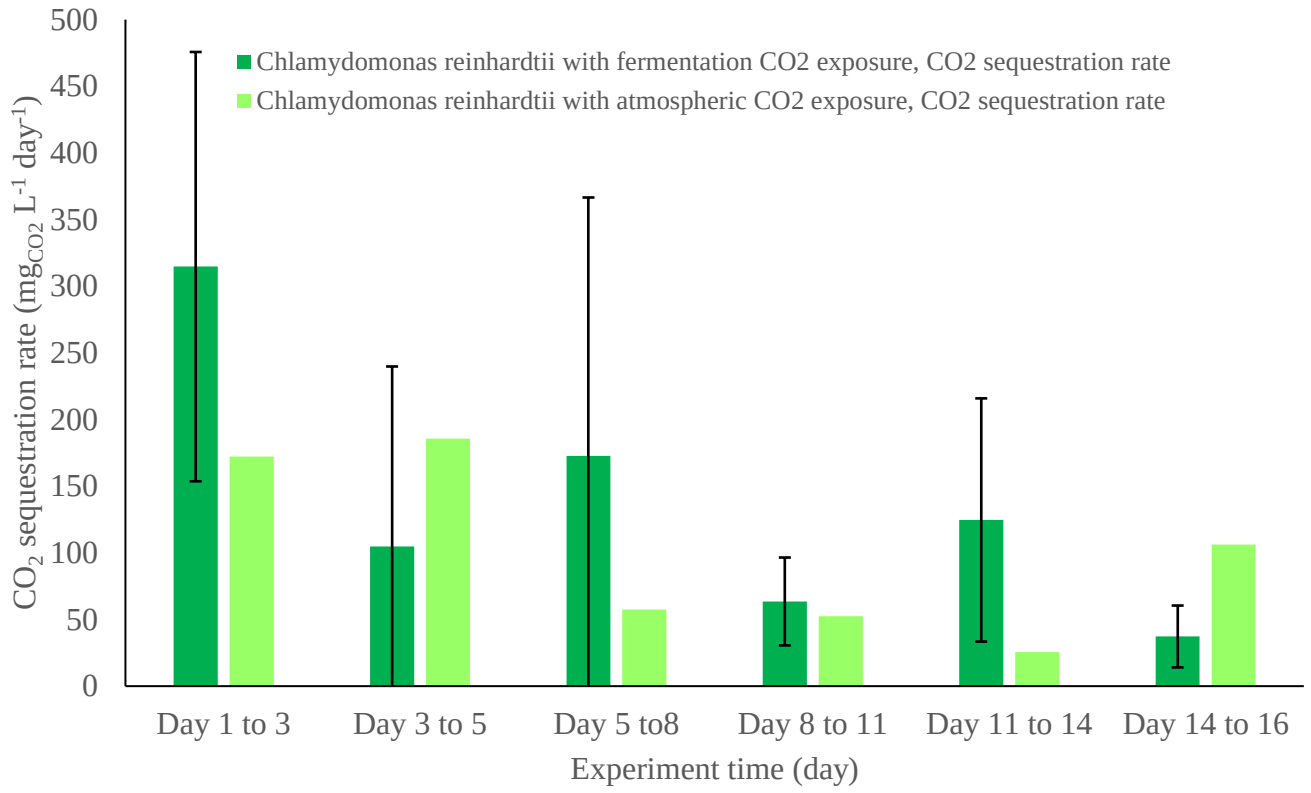

Figure 4-19: Changes in CO_2 sequestration rate in *Chlamydomonas reinhardtii* exposed to fermentation and atmospheric CO_2.

4.6.2 *Chlamydomonas* sp.

It can be observed from Figure 4-19 and Figure 4-20 that although *Chlamydomonas reinhardtii* and *Chlamydomonas* sp. are from the same genus, their response to similar CO_2 concentration differs in terms of CO_2 capture. Average CO_2 sequestration rate was 158.5 and 131.3 $mg_{CO2}\,L^{-1}\,day^{-1}$ for *Chlamydomonas* sp. and *Chlamydomonas reinhardtii*, respectively, which was 20% higher for *Chlamydomonas* sp. This higher CO_2 sequestration rate as well as higher growth rate than *Chlamydomonas reinhardtii* likely occurred as a result of better adaption to low pH caused by elevated CO_2. Also, similar to *Chlamydomonas reinhardtii* Sp. CO_2 sequestration rate for samples that were exposed to fermentation CO_2, the maximum CO_2 sequestration rate occurred from day 1 to 3 at 361.1 $mg_{CO2}\,L^{-1}\,day^{-1}$ (Figure 4-20).

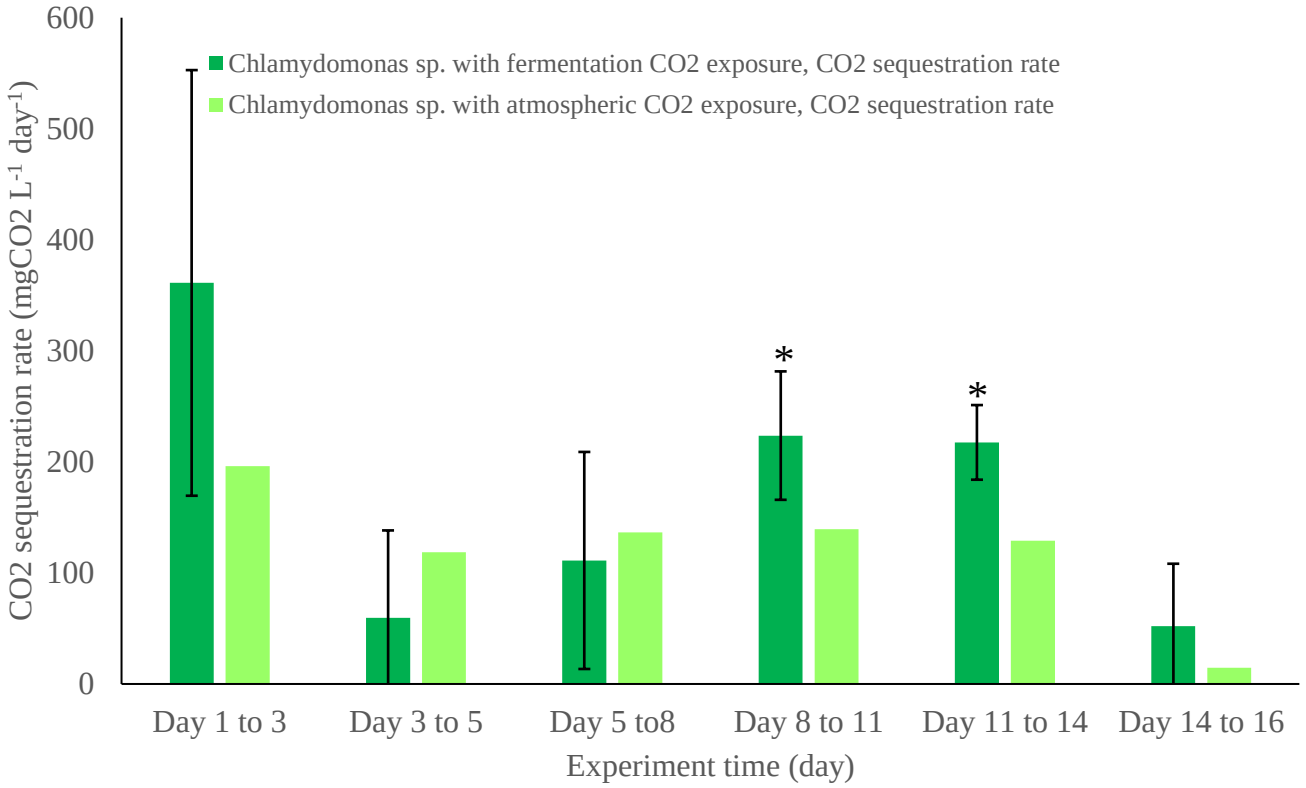

Figure 4-20: Changes in CO_2 sequestration rate in *Chlamydomonas* sp. exposed to fermentation and atmospheric CO_2. An asterisk represents a significant difference to the control ($P < 0.05$, t-test).

4.6.3 *Coccomyxa* sp.

As discussed in this thesis, many free-living algae possess a photosynthetic CO_2 concentrating mechanism, which enables microalgae to accumulate dissolved inorganic carbon within the cells. This let increasing the CO_2 concentration in the carboxylating enzyme Rubisco. The accumulation of dissolved inorganic carbon is achieved by the active uptake of HCO^{3-} and/or CO_2 and the action of an external carbonic anhydrase. In this way, intracellular CO_2 concentration increases, which is in favor for the carboxylation reaction and inhibits the Rubisco oxygenase activity and subsequently reduces photorespiration. However, a limited number of eukaryotic microalgae such as *Coccomyxa* sp. do not have a CO_2 concentrating mechanism. These species take up CO_2 directly from the air or grow at neutral or acidic pH. It is known that *Coccomyxa* sp. grows well over a pH range of 3.0-9.0, which indicates that this species maintains a constant internal pH and maintains photosynthesis over this range of pH [160].

Although *Coccomyxa* sp. is a species that lacks a CO_2 concentrating mechanism, its biosequestration rate was higher than both tested *Chlamydomonas* strains.

Average CO_2 bioremediation rates were approximately 227.6 and 122.1 $mg_{CO2}\ L^{-1}\ day^{-1}$ for enriched CO_2 and control samples, respectively (Figure 4-21). Also, it is obvious from Figure 4-21 that with elevating the produced CO_2 from fermentation to the microalgal cultures on day 1 to 3 and day 8 to 11, CO_2 sequestration rate increases. This result suggests that *Coccomyxa* sp. is more suitable to be grown with fermentation off-gases to mitigate the CO_2 and producing value-added products as well.

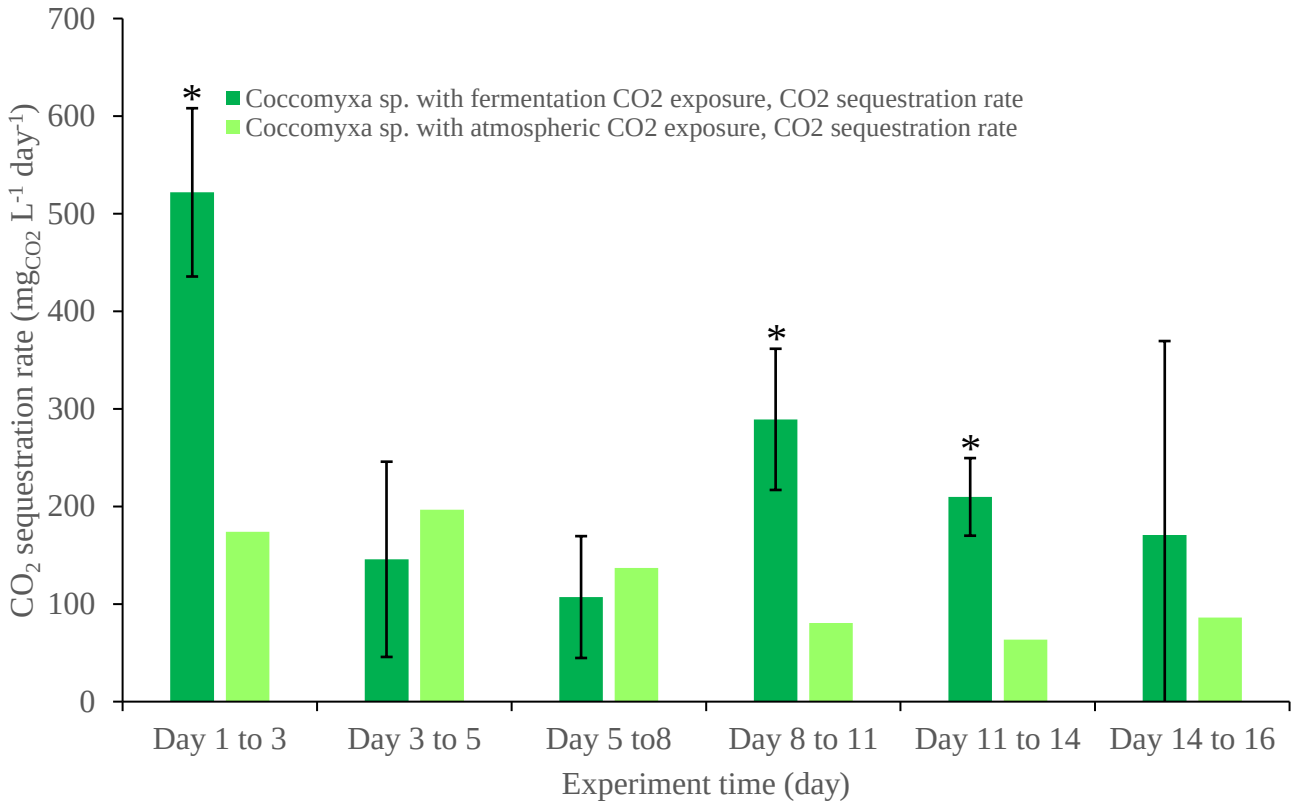

Figure 4-21: Changes in CO_2 sequestration rate in *Coccomyxa* sp. exposed to fermentation and atmospheric CO_2. An asterisk represents a significant difference to the control ($P < 0.05$, t-test).

4.6.4 Comparison of the three strains' CO2 sequestration rate

The rates of the sequestration of CO_2 for three microalgal strains can be seen in Figure 4-22. The values of maximum rate of CO_2 removed varied from 314.7 $mg_{CO2}\ L^{-1}\ day^{-1}$to 521.8 $mg_{CO2}\ L^{-1}\ day^{-1}$ from *Chlamydomonas reinhardtii* sp. to *Coccomyxa* sp. Thus, the results indicated that *Coccomyxa* sp. that has

been isolated from a polishing pond at an operational smelter is a promising strain in taking up excess CO_2 and converting it into biomass which can be further used for extraction of valuable bioproducts.

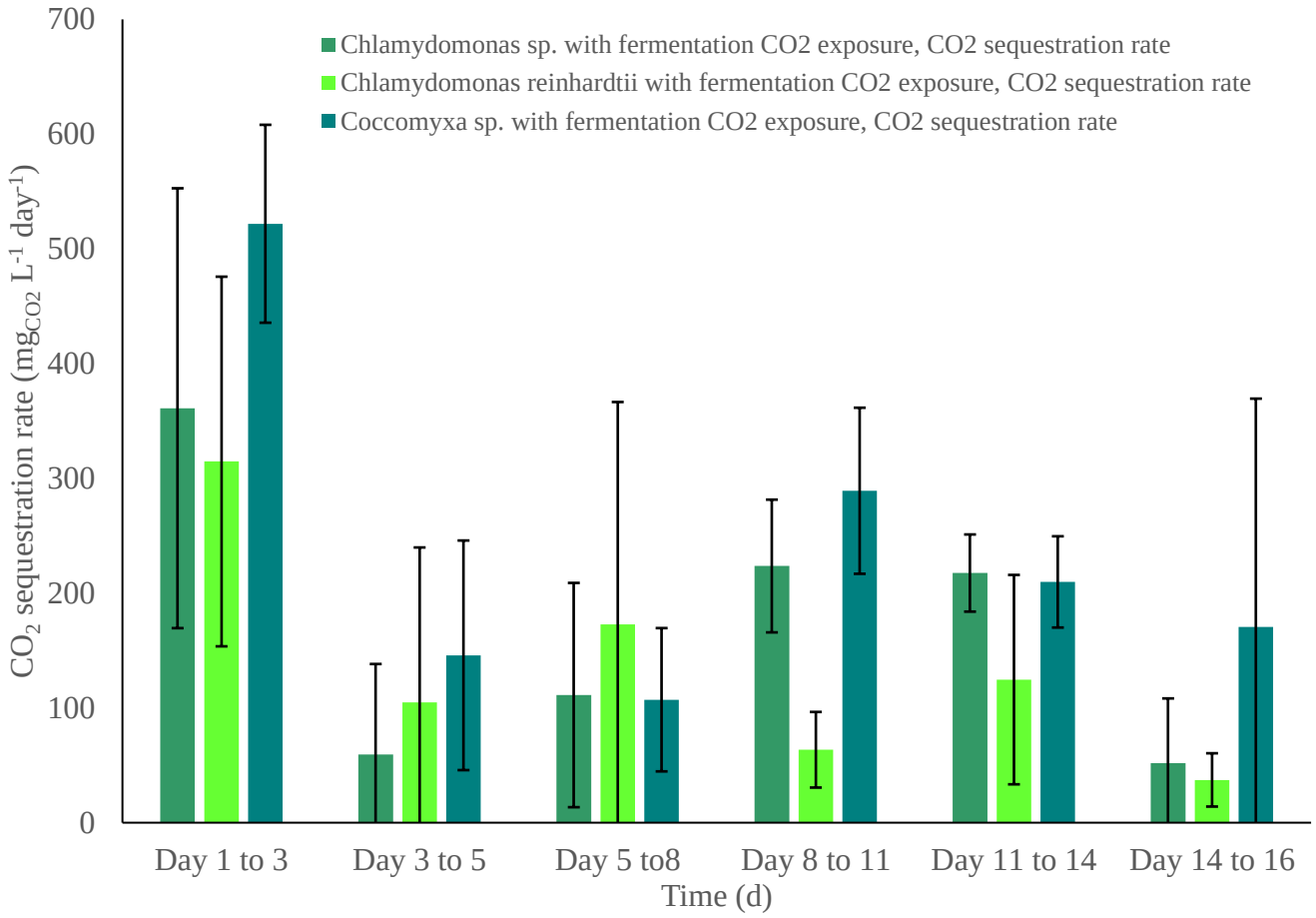

Figure 4-22: Comparison of exposure sequestration rate from the three microalgal strains exposed to fermentation CO_2

Chapter Five: Conclusion and Future Directions

Capturing CO_2 from ethanol beverage fermentation will be advantageous with strict CO_2 emission regulations. An integrated culture system between microalgal cultivation and yeast fermentation used in this work demonstrated a potential environmentally beneficial process, not least as CO_2 released during fermentation is a relatively clean source of carbon for the microalgae. As shown in our work, CO_2 of wort fermentation supplied to microalgal cultivations can positively impact microalgal biomass productivity and lipid and protein content of the biomass. This technology can help meet the worldwide need to reduce greenhouse gas emissions by utilizing it to grow photosynthetic microorganisms, which convert CO_2 into highly valuable compounds, which has the potential to be used in food and pharmaceutical industry. In this study, three strains were studied, two of which were *Chlamydomonas* sp. (one from culture collection and one bioprospected) and one was *Coccomyxa* sp. (bioprospected).

Two different microalgal genera that were obtained from distinct extreme environments with low pH exhibited similar positive responses to a CO_2 enriched environment. These results suggest that microalgae from extreme environments involving low pH could be potential targets for investigation involving CO_2 capturing. According to a previous study [137], *Coccomyxa* sp. cells grow slower photoautotrophically under ambient levels of CO_2 and are smaller than *Chlamydomonas* sp. This may be related to the lack of a carbon concentrating mechanism (CCM) in *Coccomyxa* sp. [137]. However, in the current study, we observed a higher growth and CO_2 fixation rate for *Coccomyxa* sp. than with the two *Chlamydomonas* sp. strains, which may be correlated with a relatively more efficient Rubisco of this algae. According to Kristin et. al. [160], the absence of CCM in *Coccomyxa* sp. is related to Rubisco being more efficient in comparison with *Chlamydomonas reinhardtii*, which has a carbon concentrating mechanism. *Coccomyxa* sp. has no pyrenoid in its cell structure and Rubisco is evenly distributed in its chloroplast, resulting in more efficient Rubisco enzymes for the fixation of carbon derived from CO_2 [160].

Microalgae are considered a renewable biofuel promising source. Yet, there is a long way for the production of algal biofuels to become feasible, economically. Many studies [21], [56], [134] have emphasized the necessity of low-cost culture systems, using sunlight as the main or sole source of energy for biomass synthesis and production. Thus, in the current study, metabolite accumulation in *Chlamydomonas* sp. and *Coccomyxa* sp. cells subject to pure CO_2 from fermentation systems was examined under strictly

photoautotrophic conditions, so that the information that is gained may be useful for understanding and predicting the strains' response in the similar condition.

Lipid synthesis occurred much rapidly and efficiently in *Chlamydomonas reinhardtii* rather than protein accumulation, reaching a peak of 35 and 39 after 16 days of fermentation CO_2 exposure for *Chlamydomonas reinhardtii* and *Chlamydomonas* sp., respectively. However, in relative terms, the percentage of synthesized lipid of dry biomass weight was much lower in *Coccomyxa* sp. (26.3%) than in *Chlamydomonas* sp. cells. *Chlamydomonas* sp., the fastest growing species, had the highest overall lipid content, followed by *Chlamydomonas reinhardtii* and *Coccomyxa* sp. It should be considered that the lipid productivity obtained in the present study is not necessarily superior to those reported in the previous studies using various microalgal strains. However, the inexpensive and simple method that was conducted in this study, resulted in a significant increase of lipid content while maintaining the productivity at a high value that can be easily applied to other microalgal strains at larger scales.

Excess CO_2 also resulted in a significant increase in protein content dry biomass weight, in *Coccomyxa* sp. Nonetheless, the observations suggest that lipid accumulation, triggered by excess CO_2, followed similar overall patterns in *Chlamydomonas* sp. and *Coccomyxa* sp. grown photoautotrophically.

Having all the above results, it can be concluded that the selection of suitable species of microalgae is the basis for high-efficiency CO_2 fixation. Bioprospected strains, especially those originated from acidic environments, may fulfill this role. As although CO_2 is a key component for the microalgal growth, by increasing the concentration of CO_2, the culture medium pH drops. A significant decrease in pH usually has significant effects on the growth of microalgae. Therefore, by selecting strains that can tolerate low pH from exposure to high concentrations of CO_2, more favorable results may be achieved. Strains obtained from acidic mine water bodies are shown to be potential candidates.

5.1 Future directions

Current studies on microalgal biomass production mostly reported experiments that have been performed on the bench-scale under strictly controlled conditions. So, little is known about the reactor scale-up feasibility.

In order to the system being feasible in commercial scale, it is of a great importance to consider the photobioreactors, the required footprint, and the required investment. Further studies on methods that can

help lower the cost of the selected microalgal production systems on a commercial scale is necessary. While reducing costs, financial income from obtaining higher-value products as well as biofuel should not be overlooked. Potential collaborative processes include production of useful biomass, wastewater treatment, pharmaceutical industry, animal feed and biofertilizers. Microalgal sourced biofuels may play an important role in the future, although they are not currently competitive with present fossil fuels.

Also, most investigations focus on closed bioreactors, while open ponds that are directly exposed to sunlight should be considered for future research, because open systems provide a better possibility of more widespread use of biological CO_2 mitigation. Among these, combination of photobioreactors and technologies that support the supply of adequate amounts of CO_2 for the microalgal growth should be investigated on a scale-up scale. For these systems, characterized bioprospected microalgal strains, which can adapt to low pH and higher concentration of CO_2, can be utilized.

Enhanced CO_2 level is needed for efficient growth and metabolism of microalgae, and currently is a major contributor to the microalgal cultivation overall cost. However, different sources of CO_2 contain different compounds and applying fermentation CO_2 will greatly decrease the overall process cost, because it is quite pure and does not need further treatment before injecting to the photobioreactors. So, CO_2 fermentation can be directly connected to photobioreactors and the produced biomass can be used in pharmaceutical and food industry without any concern on contamination risk. Furthermore, nutrients could easily be extracted from brewery wastewater, which is a clean source containing a rich amount of nitrogen and phosphorus. Therefore, extraction of value-added products from the algae biomasses such as lipids, carbohydrates, proteins, pigments and even cellulose should be studied further.

Moreover, more research is required to optimize the cultivation conditions of the combination of introduced microalgae in this thesis and fermentation CO_2. It could include investigation of the effect of nutrient supply, light intensity, photobioreactor geometry and location, temperature and mixing. After selecting a proper strain, it is necessary to optimize the culture conditions, such as temperature, light intensity, medium composition, salinity to obtain maximum productivity. Furthermore, understanding the physiological response of microalgae to stress in various environments plays a crucial role in optimizing the design of microalgal culture systems. Genetic modification potential of the selected strains to improve the characteristics of microalgae is another parameter that can be influential in the later stages. The use of modern technologies such as genetic engineering to improve the characteristics of microalgae for an efficient CO_2 fixation in complex environments could be very useful.

Overall, for maximizing the economic feasibility of CO_2 fixation through microalgae, this process can be combined with other synergistic processes including production of useful biomass, wastewater treatment production of biofuels, animal feed and biofertilizers. Although they are not competitive with present fossil fuels, steps should be taken to reduce microalgal biomass production cost.

References

[1] H. Schweitzer *et al.*, "Innovating carbon-capture biotechnologies through ecosystem-inspired solutions," *One Earth*, vol. 4, no. 1, pp. 49–59, 2021, doi: https://doi.org/10.1016/j.oneear.2020.12.006.

[2] J.-B. Beigbeder, M. Sanglier, J. M. de M. Dantas, and J.-M. Lavoie, "CO2 capture and inorganic carbon assimilation of gaseous fermentation effluents using Parachlorella kessleri microalgae," *Journal of CO2 Utilization*, vol. 50, p. 101581, 2021, doi: https://doi.org/10.1016/j.jcou.2021.101581.

[3] "IEA (2021), Global Energy Review: CO2 Emissions in 2020, IEA, Paris https://www.iea.org/articles/global-energy-review-co2-emissions-in-2020".

[4] J. Sharma *et al.*, "Upgrading of microalgal consortia with CO2 from fermentation of wheat straw for the phycoremediation of domestic wastewater," *Bioresource Technology*, vol. 305, p. 123063, 2020, doi: https://doi.org/10.1016/j.biortech.2020.123063.

[5] R. Shin and C. Searcy, "Evaluating the Greenhouse Gas Emissions in the Craft Beer Industry: An Assessment of Challenges and Benefits of Greenhouse Gas Accounting," *Sustainability*, vol. 10, no. 11, 2018, doi: 10.3390/su10114191.

[6] C.-Y. Chen, K.-L. Yeh, R. Aisyah, D.-J. Lee, and J.-S. Chang, "Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: a critical review," *Bioresource technology*, vol. 102, no. 1, pp. 71–81, 2011.

[7] Y. Xu, L. Isom, and M. A. Hanna, "Adding value to carbon dioxide from ethanol fermentations," *Bioresource Technology*, vol. 101, no. 10, pp. 3311–3319, 2010, doi: https://doi.org/10.1016/j.biortech.2010.01.006.

[8] S. Zhang and Z. Liu, "Advances in the biological fixation of carbon dioxide by microalgae," *Journal of Chemical Technology & Biotechnology*, vol. 96, no. 6, pp. 1475–1495, 2021, doi: https://doi.org/10.1002/jctb.6714.

[9] M. K. Lam, K. T. Lee, and A. R. Mohamed, "Current status and challenges on microalgae-based carbon capture," *International Journal of Greenhouse Gas Control*, vol. 10, pp. 456–469, 2012, doi: https://doi.org/10.1016/j.ijggc.2012.07.010.

[10] A. A. Olajire, "The brewing industry and environmental challenges," *Journal of Cleaner Production*, vol. 256, p. 102817, May 2020, doi: 10.1016/j.jclepro.2012.03.003.

[11] C. Alonso-Moreno and S. García-Yuste, "Environmental potential of the use of CO2 from alcoholic fermentation processes. The CO2-AFP strategy," *Science of The Total Environment*, vol. 568, pp. 319–326, 2016, doi: https://doi.org/10.1016/j.scitotenv.2016.05.220.

[12] N. S. Hosseini, H. Shang, and J. A. Scott, "Biosequestration of industrial off-gas CO2 for enhanced lipid productivity in open microalgae cultivation systems," *Renewable and Sustainable Energy Reviews*, vol. 92, pp. 458–469, 2018, doi: https://doi.org/10.1016/j.rser.2018.04.086.

[13] Y. Chisti, "Biodiesel from microalgae," *Biotechnology Advances*, vol. 25, no. 3, pp. 294–306, 2007, doi: https://doi.org/10.1016/j.biotechadv.2007.02.001.

[14] J. N. Rosenberg, A. Mathias, K. Korth, M. J. Betenbaugh, and G. A. Oyler, "Microalgal biomass production and carbon dioxide sequestration from an integrated ethanol biorefinery in Iowa: A

technical appraisal and economic feasibility evaluation," *Biomass and Bioenergy*, vol. 35, no. 9, pp. 3865–3876, 2011, doi: https://doi.org/10.1016/j.biombioe.2011.05.014.

[15] B. Wang, Y. Li, N. Wu, and C. Q. Lan, "CO2 bio-mitigation using microalgae," *Applied Microbiology and Biotechnology*, vol. 79, no. 5, pp. 707–718, Jul. 2008, doi: 10.1007/s00253-008-1518-y.

[16] H. Yen, S. Ho, C. Chen, and J. Chang, "CO2, NOx and SOx removal from flue gas via microalgae cultivation: a critical review," *Biotechnology journal*, vol. 10, no. 6, pp. 829–839, 2015.

[17] Y. Li-Beisson, J. J. Thelen, E. Fedosejevs, and J. L. Harwood, "The lipid biochemistry of eukaryotic algae," *Progress in Lipid Research*, vol. 74, pp. 31–68, 2019, doi: https://doi.org/10.1016/j.plipres.2019.01.003.

[18] Little Shannon M. Senhorinho Gerusa N. A. ,. Saleh Mazen, Basiliko Nathan, Scott John A., "Antibacterial compounds in green microalgae from extreme environments: a review," *Algae*, vol. 36, no. 1, pp. 61–72, 2021, doi: 10.4490/algae.2021.36.3.6.

[19] A. Ghosh *et al.*, "Progress toward isolation of strains and genetically engineered strains of microalgae for production of biofuel and other value added chemicals: A review," *Energy Conversion and Management*, vol. 113, pp. 104–118, 2016, doi: https://doi.org/10.1016/j.enconman.2016.01.050.

[20] L. Sun, L. Wang, J. Li, and H. Liu, "Characterization and antioxidant activities of degraded polysaccharides from two marine Chrysophyta," *Food chemistry*, vol. 160, pp. 1–7, 2014.

[21] D. P. Ho, H. H. Ngo, and W. Guo, "A mini review on renewable sources for biofuel," *Bioresource Technology*, vol. 169, pp. 742–749, 2014, doi: https://doi.org/10.1016/j.biortech.2014.07.022.

[22] M. A. Borowitzka, "Energy from Microalgae: A Short History," in *Algae for Biofuels and Energy*, M. A. Borowitzka and N. R. Moheimani, Eds. Dordrecht: Springer Netherlands, 2013, pp. 1–15. doi: 10.1007/978-94-007-5479-9_1.

[23] T. R. Sommer, F. M. L. D'souza, and N. M. Morrissy, "Pigmentation of adult rainbow trout, Oncorhynchus mykiss, using the green alga Haematococcus pluvialis," *Aquaculture*, vol. 106, no. 1, pp. 63–74, 1992.

[24] J. Neidhardt, J. R. Benemann, L. Zhang, and A. Melis, "Photosystem-II repair and chloroplast recovery from irradiance stress: relationship between chronic photoinhibition, light-harvesting chlorophyll antenna size and photosynthetic productivity in Dunaliella salina (green algae)," *Photosynthesis research*, vol. 56, no. 2, pp. 175–184, 1998.

[25] A. Melis, J. Neidhardt, and J. R. Benemann, "Dunaliella salina (Chlorophyta) with small chlorophyll antenna sizes exhibit higher photosynthetic productivities and photon use efficiencies than normally pigmented cells," *Journal of Applied Phycology*, vol. 10, no. 6, pp. 515–525, 1998.

[26] J. Sheehan, T. Dunahay, J. Benemann, and P. Roessler, "A look back at the US Department of Energy's aquatic species program: biodiesel from algae," *National Renewable Energy Laboratory*, vol. 328, pp. 1–294, 1998.

[27] H. Michiki, "Biological CO2 fixation and utilization project," *Energy conversion and management*, vol. 36, no. 6–9, pp. 701–705, 1995.

[28] M. Negoro, N. Shioji, K. Miyamoto, and Y. Micira, "Growth of microalgae in high CO 2 gas and effects of SO x and NO x," *Applied biochemistry and biotechnology*, vol. 28, no. 1, pp. 877–886, 1991.

[29] M. Negoro, N. Shioji, Y. Ikuta, T. Makita, and M. Uchiumi, “Growth characteristics of microalgae in high-concentration CO 2 gas, effects of culture medium trace components, and impurities thereon,” *Applied biochemistry and biotechnology*, vol. 34, no. 1, pp. 681–692, 1992.

[30] N. Hanagata, T. Takeuchi, Y. Fukuju, D. J. Barnes, and I. Karube, “Tolerance of microalgae to high CO2 and high temperature,” *Phytochemistry*, vol. 31, no. 10, pp. 3345–3348, 1992.

[31] N. Kurano, H. Ikemoto, H. Miyashita, T. Hasegawa, H. Hata, and S. Miyachi, “Fixation and utilization of carbon dioxide by microalgal photosynthesis,” *Energy Conversion and Management*, vol. 36, no. 6–9, pp. 689–692, 1995.

[32] M. Kodama, “A new species of highly CO_2 tolerant fast growing marine microalga suitable for high-density culture.,” *J Mar Biotechnol*, vol. 1, pp. 21–25, 1993.

[33] K. Uemura, S. Miyachi, and A. Yokota, “Ribulose-1, 5-Bisphosphate Carboxylase/Oxygenase from thermophilic red algae with a strong specificity for CO2Fixation,” *Biochemical and biophysical research communications*, vol. 233, no. 2, pp. 568–571, 1997.

[34] A. Hamasaki *et al.*, “Carbon dioxide fixation by microalgal photosynthesis using actual flue gas from a power plant,” *Applied biochemistry and biotechnology*, vol. 45, no. 1, pp. 799–809, 1994.

[35] P. Singh, S. Kumari, A. Guldhe, R. Misra, I. Rawat, and F. Bux, “Trends and novel strategies for enhancing lipid accumulation and quality in microalgae,” *Renewable and Sustainable Energy Reviews*, vol. 55, pp. 1–16, 2016, doi: https://doi.org/10.1016/j.rser.2015.11.001.

[36] K. Kumar *et al.*, “Recent developments on biofuels production from microalgae and macroalgae,” *Renewable and Sustainable Energy Reviews*, vol. 65, pp. 235–249, 2016, doi: https://doi.org/10.1016/j.rser.2016.06.055.

[37] S.-H. Ho, C.-Y. Chen, K.-L. Yeh, W.-M. Chen, C.-Y. Lin, and J.-S. Chang, “Characterization of photosynthetic carbon dioxide fixation ability of indigenous Scenedesmus obliquus isolates,” *Biochemical Engineering Journal*, vol. 53, no. 1, pp. 57–62, 2010, doi: https://doi.org/10.1016/j.bej.2010.09.006.

[38] S.-H. Ho, W.-M. Chen, and J.-S. Chang, “Scenedesmus obliquus CNW-N as a potential candidate for CO2 mitigation and biodiesel production,” *Bioresource Technology*, vol. 101, no. 22, pp. 8725–8730, 2010, doi: https://doi.org/10.1016/j.biortech.2010.06.112.

[39] J. C. de Carvalho *et al.*, “Microalgal biomass pretreatment for integrated processing into biofuels, food, and feed,” *Bioresource Technology*, vol. 300, p. 122719, 2020, doi: https://doi.org/10.1016/j.biortech.2019.122719.

[40] Y. Maltsev and K. Maltseva, “Fatty acids of microalgae: diversity and applications,” *Reviews in Environmental Science and Bio/Technology*, vol. 20, no. 2, pp. 515–547, Jun. 2021, doi: 10.1007/s11157-021-09571-3.

[41] S.-H. Ho, C.-Y. Chen, D.-J. Lee, and J.-S. Chang, “Perspectives on microalgal CO2-emission mitigation systems—a review,” *Biotechnology advances*, vol. 29, no. 2, pp. 189–198, 2011.

[42] S. M. Desjardins, C. A. Laamanen, N. Basiliko, and J. A. Scott, “Selection and re-acclimation of bioprospected acid-tolerant green microalgae suitable for growth at low pH,” *Extremophiles*, vol. 25, no. 2, pp. 129–141, Mar. 2021, doi: 10.1007/s00792-021-01216-1.

[43] A. GERLOFF-ELIAS, E. SPIJKERMAN, and T. PRÖSCHOLD, “Effect of external pH on the growth, photosynthesis and photosynthetic electron transport of Chlamydomonas acidophila Negoro, isolated from an extremely acidic lake (pH 2.6),” *Plant, Cell & Environment*, vol. 28, no. 10, pp. 1218–1229, 2005, doi: https://doi.org/10.1111/j.1365-3040.2005.01357.x.

[44] K. M. Tan, M. A. Kassim, Z. J. Ng, and J. Lalung, “Isolation and characterization of novel acidophilic microalgae from abandoned mining site area for carbohydrate biosynthesis and its kinetic growth study in photobioreactor,” *IOP Conference Series: Materials Science and Engineering*, vol. 716, no. 1, p. 012011, Jan. 2020, doi: 10.1088/1757-899x/716/1/012011.

[45] Q. Zheng, G. J. Martin, and S. E. Kentish, “Energy efficient transfer of carbon dioxide from flue gases to microalgal systems,” *Energy & Environmental Science*, vol. 9, no. 3, pp. 1074–1082, 2016.

[46] Y. Jiang, W. Zhang, J. Wang, Y. Chen, S. Shen, and T. Liu, “Utilization of simulated flue gas for cultivation of Scenedesmus dimorphus,” *Bioresource technology*, vol. 128, pp. 359–364, 2013.

[47] M.-K. Ji, H.-S. Yun, J.-H. Hwang, E.-S. Salama, B.-H. Jeon, and J. Choi, “Effect of flue gas CO2 on the growth, carbohydrate and fatty acid composition of a green microalga Scenedesmus obliquus for biofuel production,” *Environmental technology*, vol. 38, no. 16, pp. 2085–2092, 2017.

[48] R. Bounaceur, N. Lape, D. Roizard, C. Vallieres, and E. Favre, “Membrane processes for post-combustion carbon dioxide capture: A parametric study,” *Energy*, vol. 31, no. 14, pp. 2556–2570, 2006, doi: https://doi.org/10.1016/j.energy.2005.10.038.

[49] R. Praveenkumar *et al.*, “Improved biomass and lipid production in a mixotrophic culture of Chlorella sp. KR-1 with addition of coal-fired flue-gas,” *Bioresource technology*, vol. 171, pp. 500–505, 2014.

[50] S.-Y. Chiu *et al.*, “Microalgal biomass production and on-site bioremediation of carbon dioxide, nitrogen oxide and sulfur dioxide from flue gas using Chlorella sp. cultures,” *Bioresource technology*, vol. 102, no. 19, pp. 9135–9142, 2011.

[51] K. Kumar, D. Banerjee, and D. Das, “Carbon dioxide sequestration from industrial flue gas by Chlorella sorokiniana,” *Bioresource technology*, vol. 152, pp. 225–233, 2014.

[52] S. M. Desjardins, C. A. Laamanen, N. Basiliko, and J. A. Scott, “Selection and re-acclimation of bioprospected acid-tolerant green microalgae suitable for growth at low pH,” *Extremophiles*, vol. 25, no. 2, pp. 129–141, Mar. 2021, doi: 10.1007/s00792-021-01216-1.

[53] J.-S. Lee and J.-P. Lee, “Review of advances in biological CO 2 mitigation technology,” *Biotechnology and Bioprocess Engineering*, vol. 8, no. 6, pp. 354–359, 2003.

[54] C.-Y. Kao *et al.*, “Utilization of carbon dioxide in industrial flue gases for the cultivation of microalga Chlorella sp.,” *Bioresource technology*, vol. 166, pp. 485–493, 2014.

[55] F. M. Santos, L. P. Mazur, D. A. Mayer, V. J. P. Vilar, and J. C. M. Pires, “Inhibition effect of zinc, cadmium, and nickel ions in microalgal growth and nutrient uptake from water: An experimental approach,” *Chemical Engineering Journal*, vol. 366, pp. 358–367, 2019, doi: https://doi.org/10.1016/j.cej.2019.02.080.

[56] A. Kumar *et al.*, “Enhanced CO2 fixation and biofuel production via microalgae: recent developments and future directions,” *Trends in Biotechnology*, vol. 28, no. 7, pp. 371–380, 2010, doi: https://doi.org/10.1016/j.tibtech.2010.04.004.

[57] E. M. Grima, E.-H. Belarbi, F. G. A. Fernández, A. R. Medina, and Y. Chisti, "Recovery of microalgal biomass and metabolites: process options and economics," *Biotechnology Advances*, vol. 20, no. 7, pp. 491–515, 2003, doi: https://doi.org/10.1016/S0734-9750(02)00050-2.

[58] D. Tang, W. Han, P. Li, X. Miao, and J. Zhong, "CO2 biofixation and fatty acid composition of Scenedesmus obliquus and Chlorella pyrenoidosa in response to different CO2 levels," *Bioresource Technology*, vol. 102, no. 3, pp. 3071–3076, 2011, doi: https://doi.org/10.1016/j.biortech.2010.10.047.

[59] C. Song, X. Hu, Z. Liu, S. Li, and Y. Kitamura, "Combination of brewery wastewater purification and CO2 fixation with potential value-added ingredients production via different microalgae strains cultivation," *Journal of Cleaner Production*, vol. 268, p. 122332, 2020, doi: https://doi.org/10.1016/j.jclepro.2020.122332.

[60] J. Doucha, F. Straka, and K. Lívanský, "Utilization of flue gas for cultivation of microalgae Chlorella sp.) in an outdoor open thin-layer photobioreactor," *Journal of Applied Phycology*, vol. 17, no. 5, pp. 403–412, 2005.

[61] W. Y. Cheah, P. L. Show, J.-S. Chang, T. C. Ling, and J. C. Juan, "Biosequestration of atmospheric CO2 and flue gas-containing CO2 by microalgae," *Bioresource technology*, vol. 184, pp. 190–201, 2015.

[62] K. Maedal, M. Owadai, N. Kimura, and I. Karubd, "Pergamon CO2 fixation from the flue gas on coal-fired thermal power plant by microalgae To screen microalgac which arc suitable for direct CO2 fixation, microalgae were sampled from," *Energy Convers. Mgmi.*, vol. 36, pp. 717–720, 1995.

[63] B. E. Rittmann, "Opportunities for renewable bioenergy using microorganisms," *Biotechnology and bioengineering*, vol. 100, no. 2, pp. 203–212, 2008.

[64] B. Zhao and Y. Su, "Process effect of microalgal-carbon dioxide fixation and biomass production: A review," *Renewable and Sustainable Energy Reviews*, vol. 31, pp. 121–132, 2014, doi: https://doi.org/10.1016/j.rser.2013.11.054.

[65] X.-G. Zhu, S. P. Long, and D. R. Ort, "What is the maximum efficiency with which photosynthesis can convert solar energy into biomass?," *Current Opinion in Biotechnology*, vol. 19, no. 2, pp. 153–159, 2008, doi: https://doi.org/10.1016/j.copbio.2008.02.004.

[66] R. Sayre, "Microalgae: The Potential for Carbon Capture," *BioScience*, vol. 60, no. 9, pp. 722–727, Oct. 2010, doi: 10.1525/bio.2010.60.9.9.

[67] S. Miyachi, I. Iwasaki, and Y. Shiraiwa, "Historical perspective on microalgal and cyanobacterial acclimation to low- and extremely high-CO2 conditions," *Photosynthesis Research*, vol. 77, no. 2, pp. 139–153, Sep. 2003, doi: 10.1023/A:1025817616865.

[68] A. D. Kroumov *et al.*, "A systems approach for CO2 fixation from flue gas by microalgae—Theory review," *Process Biochemistry*, vol. 51, no. 11, pp. 1817–1832, 2016, doi: https://doi.org/10.1016/j.procbio.2016.05.019.

[69] B. Sajjadi, W.-Y. Chen, A. A. A. Raman, and S. Ibrahim, "Microalgae lipid and biomass for biofuel production: A comprehensive review on lipid enhancement strategies and their effects on fatty acid composition," *Renewable and Sustainable Energy Reviews*, vol. 97, pp. 200–232, 2018, doi: https://doi.org/10.1016/j.rser.2018.07.050.

[70] A. Solovchenko and I. Khozin-Goldberg, “High-CO2 tolerance in microalgae: possible mechanisms and implications for biotechnology and bioremediation,” *Biotechnology Letters*, vol. 35, no. 11, pp. 1745–1752, Nov. 2013, doi: 10.1007/s10529-013-1274-7.

[71] G. Yadav, A. Karemore, S. K. Dash, and R. Sen, “Performance evaluation of a green process for microalgal CO2 sequestration in closed photobioreactor using flue gas generated in-situ,” *Bioresource Technology*, vol. 191, pp. 399–406, 2015, doi: https://doi.org/10.1016/j.biortech.2015.04.040.

[72] W. Fu *et al.*, “Sugar-stimulated CO2 sequestration by the green microalga Chlorella vulgaris,” *Science of The Total Environment*, vol. 654, pp. 275–283, 2019, doi: https://doi.org/10.1016/j.scitotenv.2018.11.120.

[73] M. G. de Morais and J. A. V. Costa, “Biofixation of carbon dioxide by Spirulina sp. and Scenedesmus obliquus cultivated in a three-stage serial tubular photobioreactor,” *Journal of Biotechnology*, vol. 129, no. 3, pp. 439–445, 2007, doi: https://doi.org/10.1016/j.jbiotec.2007.01.009.

[74] K. Brindhadevi, T. Mathimani, E. R. Rene, S. Shanmugam, N. T. L. Chi, and A. Pugazhendhi, “Impact of cultivation conditions on the biomass and lipid in microalgae with an emphasis on biodiesel,” *Fuel*, vol. 284, p. 119058, 2021, doi: https://doi.org/10.1016/j.fuel.2020.119058.

[75] R. Qiu, S. Gao, P. A. Lopez, and K. L. Ogden, “Effects of pH on cell growth, lipid production and CO2 addition of microalgae Chlorella sorokiniana,” *Algal Research*, vol. 28, pp. 192–199, 2017, doi: https://doi.org/10.1016/j.algal.2017.11.004.

[76] K. Kumar and D. Das, “Growth characteristics of Chlorella sorokiniana in airlift and bubble column photobioreactors,” *Bioresource Technology*, vol. 116, pp. 307–313, 2012, doi: https://doi.org/10.1016/j.biortech.2012.03.074.

[77] M. K. Lam, K. T. Lee, and A. R. Mohamed, “Current status and challenges on microalgae-based carbon capture,” *International Journal of Greenhouse Gas Control*, vol. 10, pp. 456–469, 2012, doi: https://doi.org/10.1016/j.ijggc.2012.07.010.

[78] Y. Sui, Y. Jiang, M. Moretti, and S. E. Vlaeminck, “Harvesting time and biomass composition affect the economics of microalgae production,” *Journal of Cleaner Production*, vol. 259, p. 120782, 2020, doi: https://doi.org/10.1016/j.jclepro.2020.120782.

[79] M. Piorreck and P. Pohl, “Formation of biomass, total protein, chlorophylls, lipids and fatty acids in green and blue-green algae during one growth phase.,” *Phytochemistry*, vol. 23, no. 2, pp. 217–223, 1984, doi: https://doi.org/10.1016/S0031-9422(00)80305-2.

[80] Y. Sui, M. Muys, P. Vermeir, S. D’Adamo, and S. E. Vlaeminck, “Light regime and growth phase affect the microalgal production of protein quantity and quality with Dunaliella salina,” *Bioresource Technology*, vol. 275, pp. 145–152, 2019, doi: https://doi.org/10.1016/j.biortech.2018.12.046.

[81] R. Sathasivam, R. Radhakrishnan, A. Hashem, and E. F. Abd_Allah, “Microalgae metabolites: A rich source for food and medicine,” *Saudi journal of biological sciences*, vol. 26, no. 4, pp. 709–722, 2019.

[82] J. Matos, C. Cardoso, N. Bandarra, and C. Afonso, “Microalgae as healthy ingredients for functional food: a review,” *Food & function*, vol. 8, no. 8, pp. 2672–2685, 2017.

[83] M. G. de Morais, B. da S. Vaz, E. G. de Morais, and J. A. V. Costa, “Biologically Active Metabolites Synthesized by Microalgae,” *BioMed Research International*, vol. 2015, p. 835761, Aug. 2015, doi: 10.1155/2015/835761.

[84] F. Camacho, A. Macedo, and F. Malcata, “Potential Industrial Applications and Commercialization of Microalgae in the Functional Food and Feed Industries: A Short Review,” *Marine Drugs*, vol. 17, no. 6, 2019, doi: 10.3390/md17060312.

[85] L. Bhatia, R. K. Bachheti, V. K. Garlapati, and A. K. Chandel, “Third-generation biorefineries: a sustainable platform for food, clean energy, and nutraceuticals production,” *Biomass Conversion and Biorefinery*, Jul. 2020, doi: 10.1007/s13399-020-00843-6.

[86] E. W. Becker, “Micro-algae as a source of protein,” *Biotechnology Advances*, vol. 25, no. 2, pp. 207–210, 2007, doi: https://doi.org/10.1016/j.biotechadv.2006.11.002.

[87] M. Plaza, M. Herrero, A. Cifuentes, and E. Ibáñez, “Innovative Natural Functional Ingredients from Microalgae,” *J. Agric. Food Chem.*, vol. 57, no. 16, pp. 7159–7170, Aug. 2009, doi: 10.1021/jf901070g.

[88] B. Godman *et al.*, “Barriers for access to new medicines: searching for the balance between rising costs and limited budgets,” *Frontiers in public health*, vol. 6, p. 328, 2018.

[89] O. Pulz and W. Gross, “Valuable products from biotechnology of microalgae,” *Applied microbiology and biotechnology*, vol. 65, no. 6, pp. 635–648, 2004.

[90] R. Chandra, H. M. N. Iqbal, G. Vishal, H.-S. Lee, and S. Nagra, “Algal biorefinery: A sustainable approach to valorize algal-based biomass towards multiple product recovery,” *Bioresource Technology*, vol. 278, pp. 346–359, 2019, doi: https://doi.org/10.1016/j.biortech.2019.01.104.

[91] S. Bellou, M. N. Baeshen, A. M. Elazzazy, D. Aggeli, F. Sayegh, and G. Aggelis, “Microalgal lipids biochemistry and biotechnological perspectives,” *Biotechnology Advances*, vol. 32, no. 8, pp. 1476–1493, 2014.

[92] X.-M. Sun, L.-J. Ren, Q.-Y. Zhao, X.-J. Ji, and H. Huang, “Enhancement of lipid accumulation in microalgae by metabolic engineering,” *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, vol. 1864, no. 4, pp. 552–566, 2019, doi: https://doi.org/10.1016/j.bbalip.2018.10.004.

[93] R. Natarajan, W. M. R. Ang, X. Chen, M. Voigtmann, and R. Lau, “Lipid releasing characteristics of microalgae species through continuous ultrasonication,” *Bioresource Technology*, vol. 158, pp. 7–11, 2014, doi: https://doi.org/10.1016/j.biortech.2014.01.146.

[94] I. A. Guschina and J. L. Harwood, “Lipids and lipid metabolism in eukaryotic algae,” *Progress in lipid research*, vol. 45, no. 2, pp. 160–186, 2006.

[95] I. A. Guschina and J. L. Harwood, “Algal lipids and effect of the environment on their biochemistry,” in *Lipids in aquatic ecosystems*, Springer, 2009, pp. 1–24.

[96] J. Lupette and C. Benning, “Human health benefits of very-long-chain polyunsaturated fatty acids from microalgae,” *Biochimie*, vol. 178, pp. 15–25, 2020, doi: https://doi.org/10.1016/j.biochi.2020.04.022.

[97] Y. Gong and X. Miao, “Short Chain Fatty Acid Biosynthesis in Microalgae Synechococcus sp. PCC 7942,” *Mar Drugs*, vol. 17, no. 5, p. 255, Apr. 2019, doi: 10.3390/md17050255.

[98] C. Härtig, N. Loffhagen, and W. Babel, “Glucose stimulates a decrease of the fatty acid saturation degree in Acinetobacter calcoaceticus,” *Archives of microbiology*, vol. 171, no. 3, pp. 166–172, 1999.

[99] J. Huang *et al.*, “Profile of polyunsaturated fatty acids produced by Thraustochytrium sp. KK17-3,” *Journal of the American Oil Chemists’ Society*, vol. 78, no. 6, pp. 605–610, 2001.

[100] N. Chu *et al.*, “Microbial Electrosynthesis for Producing Medium Chain Fatty Acids,” *Engineering*, 2021, doi: https://doi.org/10.1016/j.eng.2021.03.025.

[101] X. Wang *et al.*, “Enrichment of Long-Chain Polyunsaturated Fatty Acids by Coordinated Expression of Multiple Metabolic Nodes in the Oleaginous Microalga Phaeodactylum tricornutum,” *J. Agric. Food Chem.*, vol. 65, no. 35, pp. 7713–7720, Sep. 2017, doi: 10.1021/acs.jafc.7b02397.

[102] Y. Gong, X. Guo, X. Wan, Z. Liang, and M. Jiang, “Characterization of a novel thioesterase (PtTE) from Phaeodactylum tricornutum,” *Journal of basic microbiology*, vol. 51, no. 6, pp. 666–672, 2011.

[103] K.-T. Peng *et al.*, “Delta 5 fatty acid desaturase upregulates the synthesis of polyunsaturated fatty acids in the marine diatom Phaeodactylum tricornutum,” *Journal of agricultural and food chemistry*, vol. 62, no. 35, pp. 8773–8776, 2014.

[104] E. M. Trentacoste *et al.*, “Metabolic engineering of lipid catabolism increases microalgal lipid accumulation without compromising growth,” *Proceedings of the National Academy of Sciences*, vol. 110, no. 49, pp. 19748–19753, 2013.

[105] T. Kobayashi *et al.*, “Increase of eicosapentaenoic acid in thraustochytrids through thraustochytrid ubiquitin promoter-driven expression of a fatty acid Δ5 desaturase gene,” *Applied and environmental microbiology*, vol. 77, no. 11, pp. 3870–3876, 2011.

[106] G. Wu *et al.*, “Stepwise engineering to produce high yields of very long-chain polyunsaturated fatty acids in plants,” *Nature biotechnology*, vol. 23, no. 8, pp. 1013–1017, 2005.

[107] J. Harwood and T. S. Moore Jr, “Lipid metabolism in plants,” *Critical reviews in plant sciences*, vol. 8, no. 1, pp. 1–43, 1989.

[108] A. K. Koyande, K. W. Chew, K. Rambabu, Y. Tao, D.-T. Chu, and P.-L. Show, “Microalgae: A potential alternative to health supplementation for humans,” *Food Science and Human Wellness*, vol. 8, no. 1, pp. 16–24, 2019, doi: https://doi.org/10.1016/j.fshw.2019.03.001.

[109] A. Shekh, A. Sharma, P. M. Schenk, G. Kumar, and S. Mudliar, “Microalgae cultivation: photobioreactors, CO2 utilization, and value-added products of industrial importance,” *Journal of Chemical Technology & Biotechnology*, vol. n/a, no. n/a, doi: https://doi.org/10.1002/jctb.6902.

[110] L. Novoveská, M. E. Ross, M. S. Stanley, R. Pradelles, V. Wasiolek, and J.-F. Sassi, “Microalgal Carotenoids: A Review of Production, Current Markets, Regulations, and Future Direction,” *Marine Drugs*, vol. 17, no. 11, 2019, doi: 10.3390/md17110640.

[111] E.-S. Jin, J. E. Polle, H.-K. Lee, S.-M. Hyun, and M. Chang, “Xanthophylls in microalgae: from biosynthesis to biotechnological mass production and application,” *Journal of microbiology and biotechnology*, vol. 13, no. 2, pp. 165–174, 2003.

[112] R. R. Ambati, S.-M. Phang, S. Ravi, and R. G. Aswathanarayana, “Astaxanthin: sources, extraction, stability, biological activities and its commercial applications—a review,” *Marine drugs*, vol. 12, no. 1, pp. 128–152, 2014.

[113] B. Demmig-Adams and W. W. Adams, “Antioxidants in photosynthesis and human nutrition,” *Science*, vol. 298, no. 5601, pp. 2149–2153, 2002.

[114] K. Griffiths, B. B. Aggarwal, R. B. Singh, H. S. Buttar, D. Wilson, and F. De Meester, "Food antioxidants and their anti-inflammatory properties: a potential role in cardiovascular diseases and cancer prevention," *Diseases*, vol. 4, no. 3, p. 28, 2016.

[115] E. Christaki, P. Florou-Paneri, and E. Bonos, "Microalgae: a novel ingredient in nutrition," *International journal of food sciences and nutrition*, vol. 62, no. 8, pp. 794–799, 2011.

[116] R. Sathasivam and J.-S. Ki, "A review of the biological activities of microalgal carotenoids and their potential use in healthcare and cosmetic industries," *Marine drugs*, vol. 16, no. 1, p. 26, 2018.

[117] W. Stahl and H. Sies, "β-Carotene and other carotenoids in protection from sunlight," *The American journal of clinical nutrition*, vol. 96, no. 5, pp. 1179S-1184S, 2012.

[118] W. M. Bishop and H. M. Zubeck, "Evaluation of microalgae for use as nutraceuticals and nutritional supplements," *J Nutr Food Sci*, vol. 2, no. 5, pp. 1–6, 2012.

[119] S. Nagini, F. Palitti, and A. T. Natarajan, "Chemopreventive potential of chlorophyllin: a review of the mechanisms of action and molecular targets," *Nutrition and cancer*, vol. 67, no. 2, pp. 203–211, 2015.

[120] R. Harun and M. K. Danquah, "Enzymatic hydrolysis of microalgal biomass for bioethanol production," *Chemical Engineering Journal*, vol. 168, no. 3, pp. 1079–1084, 2011.

[121] D. Cheng *et al.*, "Improving carbohydrate and starch accumulation in Chlorella sp. AE10 by a novel two-stage process with cell dilution," *Biotechnology for biofuels*, vol. 10, no. 1, pp. 1–14, 2017.

[122] A. H. El-Baky, H. K. El Baz, and S. El-Latife, "Induction of sulfated polysaccharides in Spirulina platensis as response to nitrogen concentration and its biological evaluation," *Journal of Aquaculture Research & Development*, vol. 5, no. 1, p. 1, 2014.

[123] L. Sun, J. Chu, Z. Sun, and L. Chen, "Physicochemical properties, immunomodulation and antitumor activities of polysaccharide from Pavlova viridis," *Life sciences*, vol. 144, pp. 156–161, 2016.

[124] M. T. Croft, M. J. Warren, and A. G. Smith, "Algae need their vitamins," *Eukaryotic cell*, vol. 5, no. 8, pp. 1175–1183, 2006.

[125] A. R. Vicente, C. H. Crisosto, G. O. Sozzi, and G. A. Manganaris, "Nutritional quality of fruits and vegetables 2009," Academic Press, 2009.

[126] A. Del Mondo, A. Smerilli, E. Sané, C. Sansone, and C. Brunet, "Challenging microalgal vitamins for human health," *Microbial Cell Factories*, vol. 19, no. 1, p. 201, Nov. 2020, doi: 10.1186/s12934-020-01459-1.

[127] M. Rizwan, G. Mujtaba, S. A. Memon, K. Lee, and N. Rashid, "Exploring the potential of microalgae for new biotechnology applications and beyond: A review," *Renewable and Sustainable Energy Reviews*, vol. 92, pp. 394–404, 2018, doi: https://doi.org/10.1016/j.rser.2018.04.034.

[128] L. Soto-Sierra, P. Stoykova, and Z. L. Nikolov, "Extraction and fractionation of microalgae-based protein products," *Algal Research*, vol. 36, pp. 175–192, 2018, doi: https://doi.org/10.1016/j.algal.2018.10.023.

[129] R. Pratt *et al.*, "Chlorellin, an Antibacterial Substance from *Chlorella*," *Science*, vol. 99, no. 2574, pp. 351–352, 1944, doi: 10.1126/science.99.2574.351.

[130] S. Sinha, K. Bisaria, T. Mehrotra, and R. Singh, “Applications and Efficacy of Exceptional Bioactive Compounds from Microalgae,” in *Bioprocess Engineering for Bioremediation: Valorization and Management Techniques*, M. Jerold, S. Arockiasamy, and V. Sivasubramanian, Eds. Cham: Springer International Publishing, 2020, pp. 161–176. doi: 10.1007/698_2020_581.

[131] R. Kholssi, P. V. Ramos, E. A. N. Marks, O. Montero, and C. Rad, “2Biotechnological uses of microalgae: A review on the state of the art and challenges for the circular economy,” *Biocatalysis and Agricultural Biotechnology*, vol. 36, p. 102114, 2021, doi: https://doi.org/10.1016/j.bcab.2021.102114.

[132] J. A. V. Costa, B. F. Lucas, A. G. P. Alvarenga, J. B. Moreira, and M. G. de Morais, “Microalgae Polysaccharides: An Overview of Production, Characterization, and Potential Applications,” *Polysaccharides*, vol. 2, no. 4, pp. 759–772, 2021, doi: 10.3390/polysaccharides2040046.

[133] M. R. Gauthier, G. N. A. Senhorinho, and J. A. Scott, “Microalgae under environmental stress as a source of antioxidants,” *Algal Research*, vol. 52, p. 102104, 2020, doi: https://doi.org/10.1016/j.algal.2020.102104.

[134] H. A. Alalwan, A. H. Alminshid, and H. A. S. Aljaafari, “Promising evolution of biofuel generations. Subject review,” *Renewable Energy Focus*, vol. 28, pp. 127–139, 2019, doi: https://doi.org/10.1016/j.ref.2018.12.006.

[135] B. Abdullah *et al.*, “Fourth generation biofuel: A review on risks and mitigation strategies,” *Renewable and Sustainable Energy Reviews*, vol. 107, pp. 37–50, 2019, doi: https://doi.org/10.1016/j.rser.2019.02.018.

[136] K. Sander and G. S. Murthy, “Life cycle analysis of algae biodiesel,” *The International Journal of Life Cycle Assessment*, vol. 15, no. 7, pp. 704–714, 2010.

[137] J. Msanne *et al.*, “Metabolic and gene expression changes triggered by nitrogen deprivation in the photoautotrophically grown microalgae Chlamydomonas reinhardtii and Coccomyxa sp. C-169,” *Phytochemistry*, vol. 75, pp. 50–59, 2012, doi: https://doi.org/10.1016/j.phytochem.2011.12.007.

[138] J. L. Fuentes *et al.*, “Phylogenetic characterization and morphological and physiological aspects of a novel acidotolerant and halotolerant microalga Coccomyxa onubensis sp. nov. (Chlorophyta, Trebouxiophyceae),” *Journal of Applied Phycology*, vol. 28, no. 6, pp. 3269–3279, Dec. 2016, doi: 10.1007/s10811-016-0887-3.

[139] R. A. Andersen, *Algal culturing techniques*. Elsevier, 2005.

[140] J. A. Hellebust and J. S. Craigie, *Handbook of phycological methods: volume 4: ecological field methods: macroalgae*, vol. 4. Cambridge University Press, 1985.

[141] E. Aghaalipour, A. Akbulut, and G. Güllü, “Carbon dioxide capture with microalgae species in continuous gas-supplied closed cultivation systems,” *Biochemical Engineering Journal*, vol. 163, p. 107741, 2020, doi: https://doi.org/10.1016/j.bej.2020.107741.

[142] “FOLCH, J., LEES, M., & SLOANE STANLEY, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. The Journal of biological chemistry, 226(1), 497–509.”.

[143] O. Lowry, N. Rosebrough, A. L. Farr, and R. Randall, “PROTEIN MEASUREMENT WITH THE FOLIN PHENOL REAGENT,” *Journal of Biological Chemistry*, vol. 193, no. 1, pp. 265–275, 1951, doi: https://doi.org/10.1016/S0021-9258(19)52451-6.

[144] A. Elshani, K. Pehlivani, B. Kelmendi, and I. Cacaj, “Possibility and determination of the use of CO2 produced by the production of beers,” *Journal of Pharmaceutical Sciences and Research*, vol. 10, pp. 1229–1230, May 2018.

[145] Y. Yang and K. Gao, “Effects of CO2 concentrations on the freshwater microalgae, Chlamydomonas reinhardtii, Chlorella pyrenoidosa and Scenedesmus obliquus (Chlorophyta),” *Journal of Applied Phycology*, vol. 15, no. 5, pp. 379–389, Sep. 2003, doi: 10.1023/A:1026021021774.

[146] B. Zhu, G. Chen, X. Cao, and D. Wei, “Molecular characterization of CO2 sequestration and assimilation in microalgae and its biotechnological applications,” *Bioresource Technology*, vol. 244, pp. 1207–1215, 2017, doi: https://doi.org/10.1016/j.biortech.2017.05.199.

[147] A. Solovchenko and I. Khozin-Goldberg, “High-CO 2 tolerance in microalgae: possible mechanisms and implications for biotechnology and bioremediation,” *Biotechnology letters*, vol. 35, no. 11, pp. 1745–1752, 2013.

[148] Y. Yoshimitsu, J. Abe, and S. Harayama, “Cas9-guide RNA ribonucleoprotein-induced genome editing in the industrial green alga Coccomyxa sp. strain KJ,” *Biotechnology for Biofuels*, vol. 11, no. 1, p. 326, Dec. 2018, doi: 10.1186/s13068-018-1327-1.

[149] N. Aburai, R. Kunishima, F. Iijima, and K. Fujii, “Effects of light-emitting diodes (LEDs) on lipid production of the aerial microalga Coccomyxa sp. KGU-D001 under liquid- and aerial-phase conditions,” *Journal of Biotechnology*, vol. 323, pp. 274–282, 2020, doi: https://doi.org/10.1016/j.jbiotec.2020.09.005.

[150] A. Nakanishi *et al.*, “Development of lipid productivities under different CO2 conditions of marine microalgae Chlamydomonas sp. JSC4,” *Bioresource Technology*, vol. 152, pp. 247–252, 2014, doi: https://doi.org/10.1016/j.biortech.2013.11.009.

[151] A. L. Chagas, A. O. Rios, A. Jarenkow, N. R. Marcílio, M. A. Z. Ayub, and R. Rech, “Production of carotenoids and lipids by Dunaliella tertiolecta using CO2 from beer fermentation,” *Process Biochemistry*, vol. 50, no. 6, pp. 981–988, 2015, doi: https://doi.org/10.1016/j.procbio.2015.03.012.

[152] O. Perez-Garcia, F. M. E. Escalante, L. E. de-Bashan, and Y. Bashan, “Heterotrophic cultures of microalgae: Metabolism and potential products,” *Water Research*, vol. 45, no. 1, pp. 11–36, 2011, doi: https://doi.org/10.1016/j.watres.2010.08.037.

[153] R. Huerlimann, R. de Nys, and K. Heimann, “Growth, lipid content, productivity, and fatty acid composition of tropical microalgae for scale-up production,” *Biotechnology and Bioengineering*, vol. 107, no. 2, pp. 245–257, 2010, doi: https://doi.org/10.1002/bit.22809.

[154] M. Mondal *et al.*, “Mixotrophic cultivation of Chlorella sp. BTA 9031 and Chlamydomonas sp. BTA 9032 isolated from coal field using various carbon sources for biodiesel production,” *Energy Conversion and Management*, vol. 124, pp. 297–304, 2016, doi: https://doi.org/10.1016/j.enconman.2016.07.033.

[155] S. Soru, V. Malavasi, A. Concas, P. Caboni, and G. Cao, “A novel investigation of the growth and lipid production of the extremophile microalga Coccomyxa melkonianii SCCA 048 under the effect of different cultivation conditions: Experiments and modeling,” *Chemical Engineering Journal*, vol. 377, p. 120589, 2019, doi: https://doi.org/10.1016/j.cej.2018.12.049.

[156] R. Darwish, M. A. Gedi, P. Akepach, H. Assaye, A. S. Zaky, and D. A. Gray, “Chlamydomonas reinhardtii Is a Potential Food Supplement with the Capacity to Outperform Chlorella and Spirulina,” *Applied Sciences*, vol. 10, no. 19, 2020, doi: 10.3390/app10196736.

[157] S. P. Singh and P. Singh, “Effect of CO2 concentration on algal growth: a review,” *Renewable and Sustainable Energy Reviews*, vol. 38, pp. 172–179, 2014.

[158] L. I. Rodas-Zuluaga *et al.*, “Bio-capture and influence of CO2 on the growth rate and biomass composition of the microalgae Botryococcus braunii and Scenedesmus sp,” *Journal of CO2 Utilization*, vol. 43, p. 101371, 2021, doi: https://doi.org/10.1016/j.jcou.2020.101371.

[159] A. Ferreira *et al.*, “Scenedesmus obliquus mediated brewery wastewater remediation and CO2 biofixation for green energy purposes,” *Journal of Cleaner Production*, vol. 165, pp. 1316–1327, 2017, doi: https://doi.org/10.1016/j.jclepro.2017.07.232.

[160] K. Palmqvist, A. de Los Rios, C. Ascaso, and G. Samuelsson, “Photosynthetic carbon acquisition in the lichen photobionts Coccomyxa and Trebouxia (Chlorophyta),” *Physiologia Plantarum*, vol. 101, no. 1, pp. 67–76, 1997, doi: https://doi.org/10.1111/j.1399-3054.1997.tb01821.x.