IN-SITU DISINFECTION AND ALGAL LIPID EXTRACTION USING OZONATION IN NOVEL MICROBUBBLE BIOREACTOR FOR BIOFUEL PRODUCTION

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ABSTRACT

The scaling up and downstream processing costs of biodiesel from microalgae are major concerns. This study focuses on developing a new method by integrating ozone-rich microbubbles in both the production of microalgae and in downstream processes such as biomass harvesting and lipid extraction. A bacterial contaminant of a green algal (*Dunaliella salina)* culture was successfully screened, isolated and identified using 16S rRNA gene sequencing as a member of the *Halomonas* genus (gram-negative). Ozonation of mixed cultures of *D. salina* and *Halomonas* for 10 minutes at 8 mg L^{-1} reduced the bacterial contaminant without harming the microalgal cells. The sterilisation efficiency reached 66% after 5 minutes and increased to 93% after 10 minutes of ozonation. The algal cell growth performance (biomass concentration) was decreased by over 50% at 10% (v/v) contaminant concentration. Ozonation for 10 minutes at the beginning of the experiment resulted in a biomass reduction of 28.6%, which suggests that ozonation at the beginning of experiment can control the contamination. The optimum values for three parameters (culture media volume, ozone concentration and ozonation time) suggested by the statistical software were 30.63 mL, 8.20 mg L^{-1} and 37.7 min, respectively.

Harvesting of *D. salina* cells through microflotation resulted in a 93.4% recovery efficiency. Ozonation of the harvested microalgae for 40 minutes produced three main saturated compounds [2-pentadecanone 6, 10, 14-trimethyl; n-hexadecanoic acid (palmitic acid); and octadecanoic acid (stearic acid)] that consist of 16 to 18 carbons. The main products increased significantly around 156%, 88.9% and 150% for 2-pentadecanone, 6, 10, 14-trimethyl; palmitic acid and stearic acid, respectively when the temperature was increased (60 ˚C), and smaller bubbles (generated by a fluidic oscillator) were introduced during the extraction process. By integrating microbubbles and ozonation into an airlift-loop bioreactor-processing system, this thesis describes a microbubble photobioreactor that delivers *in-situ* disinfection with microflotation harvesting and lipid extraction in an easily scalable and energy-efficient process.

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Nomenclature

ALB	airlift-loop bioreactor
BOD	biochemical oxygen demand
CFU	colony forming unit
DO	dissolved oxygen
FA	fatty acid
FAME	fatty acid methyl esters
GC-MS	gas chromatography-mass spectrometry
K_La	mass transfer coefficient
NMR	Nuclear Magnetic Resonance
OD	Optical density
PUFA	poly-unsaturated fatty acid
pH	pH value of liquid
RSM	response surface methodology
16s rRNA	a section of prokaryotic DNA found in all bacteria and archae

CHAPTER 1

INTRODUCTION

Five key elements are presented in this introduction. It begins with an overview of the study topic and subsequently focuses on the problem statement. The research hypothesis is then comprehensively explained, as are the aims and objectives of the investigation. Next, the research scope and limitations are highlighted. Lastly, the significance of the research is considered before the study structure is explained.

1.1 Overview and Problem Statement of the Study

Over the past decade, the majority of the research on sustainable, environmentally friendly energy sources has focused on biofuels (Salam et al., 2016). Apart from that, pharmaceuticals and nutraceuticals are other crucial co-products in addition to biofuels that are obtainable from microbial biomass (Harun et al., 2010). The production of biofuels and their associated co-products from microalgae basically consists of three main unit operations: culturing (including sterilisation), harvesting (including dewatering) and lipid extraction. All of these operations are largely uneconomical due to the high energy cost of processing (Brennan and Owende, 2010). For biofuels to be sustainable, current practices must seek to increase the production efficiency of all key unit processes and increase the profitability of integrated

processing plants with co-products.

The successful production of the low cost microalgal biomass at industrial scale is hugely dependent on an axenic (bacteria-free) culture and avoidance of the cross contaminations by other species (Wang et al., 2013). However, ensuring contaminant-free cultivation can prove challenging because conventional methods by using chemicals can be ineffective for commercial scale (McBride et al., 2014). Resource competition (Twiner et al., 2005), direct cell-to-cell contact (Twirler et al., 2001) and allelopathy (Twiner et al., 2005) are the three foremost contamination mechanisms involved if the starter culture is not pure and the contaminant strain is more dominant (Vasudevan and Briggs, 2008). Therefore, it is important to study the contaminations impact towards the performance of microalgae growth including a method of controlling the contaminant.

Prior to conversion to useful end-products, the biomass is harvested from cultures and dewatered. Several methods to achieve this goal exist, including filtration, centrifugation and flotation (Kim et al., 2013). Recovery by flotation is the most effective industrial technique for colloidal particle recovery (Hanotu et al., 2012). The process entails generating bubbles that attach to the cells and results in the rise of the consortium to the surface of the column, where skimming is performed (Hanotu et al., 2013). In dissolved air flotation, pressure levels of 87-116 psi are employed, including the use of saturators to make microbubbles. These combined with the vacuum pumps required to push water into saturators, further increases both

the capital and operating costs associated with microbubble production (Hanotu et al., 2013). With the fluidic oscillation approach by contrast, only air, approximately 1000 times less dense than water, is pushed at less friction loss than steady flow through the same piping (Zimmerman et al., 2011). So the energy efficiency is a crucial benefit. But capital efficiency is nearly as important as only a low pressure blower is required rather than a compressor. To date, there is no report regarding the energy consumption of the flotation method by using microbubbles produced by fluidic oscillator. Therefore estimation of the energy usage of the microflotation technique will be calculated and discussed.

Harvesting and extracting lipids from the microalgal biomass are the most expensive processes (Mubarak et al., 2015). The cost of harvesting itself contributes up to 30% of the cost of the entire process (Kim et al., 2013). Brentner et al. (2011) has reported that the process of microalgal biomass harvesting through centrifugation and press filtration, requires 90% and 79% of the total energy gained from the biofuel production, while lipid extraction through supercritical CO_2 and ultrasonication requires 66% and 110%. It is economically acceptable if the extracted compounds were high value and low volume products (pharmaceutical industry) (Salam et al., 2016). However, it is not sustainable if the compounds (lipids) are solely extracted for biofuel production (Surendhiran and Vijay, 2014). Therefore, a more energy efficient algal lipid extraction method should be conducted in order to make the biofuel economically sustainable.

1.2 Research Hypothesis, Aims and Objectives

This thesis reports on the development of a novel airlift loop bioreactor in which the microbubble dispersal can be switched from a CO_2-rich stack gas to air blown through the plasma reactor to disperse ozone. Furthermore, by tuning only the bubble size (depending on types of diffuser), the rig is readily adaptable to harvesting algae. The expectation is that the algae will grow to a greater cell density and exhibit higher growth rates with intermittent disinfection. A new downstream process method that can contribute both to *in-situ* disinfection and to the development of an easy disruption method that can be applied on an industrial scale is developed and tested.

Therefore, the aims of this thesis are to report, (1) on the development of axenic conditions at the beginning of the process and of intermittent disinfection during the growth phase (used to eliminate or reduce contamination), and (2) on the development of an easy and cheap method of disruption and lipid extraction using ozonation that is applicable on a large scale. Thus, the objectives of this research are as follows: (1) to screen, isolate and identify the microorganism contaminating *D. salina* cultures, (2) to study the effectiveness of ozonation methods for disinfection of the contaminated culture, (3) to identify and optimize the products extracted with the ozonation extraction method and, (4) to estimate the energy usage and cost of the ozonation extraction method.

1.3 Research Scope and Limitations

The scope of this study is to develop *in-situ* disinfection and a new downstream processing scheme from algal harvesting for lipid extraction that uses ozone-rich microbubbles for biofuel production. Thus, information about the current and suggested scheme is discussed in the chapters ahead. Nevertheless, several limitations are worth mentioning. First, disinfection by ozonation was conducted on a small scale (i.e., a shake flask) due to incompatible bioreactor material (polyacrylamide) that can be oxidized by ozone. Apart from that, most of the experiments were conducted twice due to lack of bioreactors (budget constraint) and to the time-consuming cultivation of microalgae. For these reasons, a low concentration of harvested algal biomass (algal slurry) was used in the lipid-extraction experiments.

1.4 Significance of Research

Given the scaling up and downstream processing (high energy consumption) associated with costs in biofuels production, the findings of this work can significantly reduce overall costs. Furthermore, modulating the size of the microbubbles created by the fluidic oscillator to match particle size ensures recovery of the cells after culture (microflotation harvesting). All three key operations; disinfection, harvesting and lipid extraction, are assembled in a scalable, relatively energy-efficient process. Moreover, if lipid extraction and direct esterification can be achieved by an ozonation method, it will simplify and eliminate the energy intensive methods that are still being used nowadays.

1.5 Study Structure

This study is structured into eight chapters. The thesis begins with an introductory chapter that offers a research overview and problem statement followed by a statement of research hypothesis, aims, objectives, scope, limitations and significance. Chapter 2 reviews recent publications about biofuel production; it starts with a consideration of algal cultivation and of the processes and methods involved in it. Chapter 3 details the materials and methods used to conduct this research. Chapter 4 presents the first results and discussion chapter. Here, the isolation and identification of contaminants by using 16S rRNA sequencing are discussed. Chapter 5 discusses the optimization of the ozonation effect towards reducing chlorophyll content with statistical analysis. Here, response-surface-methodology (RSM) software was used to optimize three parameters: culture volume, ozone concentration and ozonation time. The relationships between these parameters were also discussed. Chapter 6 presents results and discussion on the effect of CO_2 flowrate towards biomass production. It also discusses the harvesting method (i.e., microflotation) and the comparison with a previous study. Chapter 7 presents results and discussion on the products gained and optimized via the direct ozonation-extraction method. Finally, Chapter 8 presents the summarized main findings and offers recommendations for future work.

CHAPTER 2

LITERATURE REVIEW

The beginning of this chapter introduces algal biofuels and the mass production of microalgae before it discusses the morphology of the *Dunaliella salina* strain and explains why it was chosen. Next, recent problems on the downstream (especially on algal lipid extraction) processes are highlighted, and the different methods used for disruption of microalgal cells are described and summarized. Lastly, it describes ozonation and ozone generation followed by a description of a novel microbubble photobioreactor used in this study.

2.1 Biofuels Generation

The world's primary energy consumption levels have soared over the last five decades in line with the growth of the global population. According to Jones and Mayfield (2012), global consumption of primary energy reached peak growth in 2010, increasing by 5.6% - the highest it has been since the early 1970s. Consequently, as Gupta and Tuohy (2013) point out, environmental pollution and the energy crisis (caused by global price volatility and reduced fossil fuel reserves) pose two serious

threats to the global population today. Acheampong et al. (2016) explain that in order to minimise the detrimental effects of global warming and protect future generations, it is crucial that carbon dioxide (CO_2) emissions – currently at an alarmingly high rate due to excessive consumption of fossil fuels – are significantly reduced. The 2014 United Nations' Intergovernmental Panel on Climate Change fifth assessment report (AR5) (IPCC, 2014) outlined serious plans to abandon the use of fossil fuels over the next 80 years. In order to achieve this by 2100, the report prescribes that renewable energy should represent 80% of global energy supply by 2050, at minimum, reflecting a significant increase compared to today's standards, with renewable energy representing just 30% of global energy supply. According to Kirschbaum (2003) and Eurostat (2015), biofuel will eventually become the main source of renewable energy, with Shell International Petroleum Co. anticipating the role of biofuel as a key energy source from 2020 onwards.

Agency (2007) and Senauer (2008) highlight the rise in global biofuel production (mainly from food crops) over recent years, with a threefold increase witnessed in 2007 compared to 2000, representing 16 billion gallons compared to 5 billion gallons. Biofuel has therefore become an area of focus for a number of the most developed nations around the globe. For instance, Demirbas (2009) and Buyx and Tait (2011) report that the United States plans to produce more than 36 billion gallons of biofuel by 2022 as per the 2007 Energy Independence and Security Act. Similarly, Demirbas (2009) and Bonin and Lal (2012) add that the European Union aims for a 20% decrease in greenhouse gas (GHG) emissions by 2020 (compared to

1990) and to ensure that 20% of the EU's energy consumption is represented by renewable energy, with 10% being biofuel.

Acheampong et al. (2016) explain that biofuels are considered as first, second, third and fourth generation biofuels according to the technology used to produce this type of fuel and the manner in which it is sourced. Figure 2.1 outlines the different biofuel categories and their association with specific production technologies. As illustrated, the transformation of feedstock (i.e. sugar beet cereals, cassava and the starch and sugar components of sugarcane) into ethanol for the production of biofuel is categorised as first generation biofuel. As Pedroli et al. (2013) point out, first generation biofuels also include those produced from palm oil, soybean oil, sunflower oil, rapeseed oil and other seed oil crops.

Pedroli et al. (2013) and Senauer (2008) explain that biofuel technologies using more energy-efficient sources (e.g. marginal land based crops, bagasse, straw, jatropha, trees, etc.) and lignocellulosic feedstock (i.e. forest and farming biomass) are classified as producing second generation biofuels. As Buyx and Tait (2011) explain, second generation biofuel is produced through the conversion of the above feedstocks into methanol and ethanol. According to Senauer (2008), one of the main differences between first and second generation biofuels is the difference in the total amount of feedstock used – with second generation biofuels produced out of all plant parts, including fats, sugars and grains, and first generation biofuels produced out of

only the latter three parts. Consequently, land use is more efficient when producing second generation biofuel.

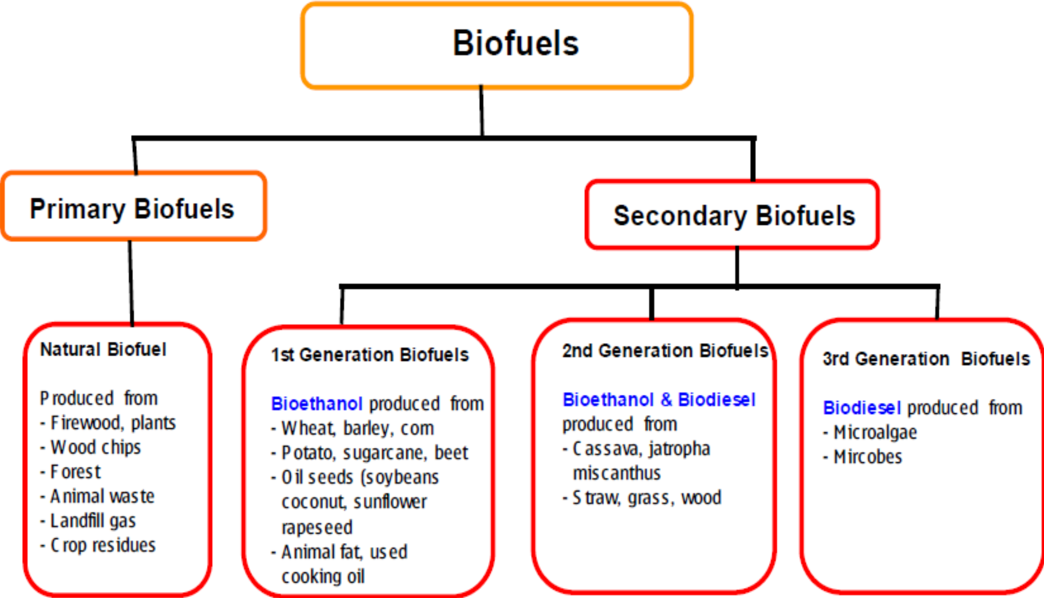

Figure 2.1: Biofuels generations (Alam et al., 2015).

As Chisti (2007) and Rawat et al. (2013) explain, the next level of biofuel production is achieved using sophisticated biotechnology that enables feedstock such as algae to be used to create what is classified as third generation biofuel. Compared to sugarcane, corn and other traditional crop feedstock, algae offers a much higher capacity in terms of land efficiency, producing more inexpensive, higher-energy and 100% renewable feedstock (Chisti, 2007).

2.1.1 The cost of Biofuel from Microalgae

As Taleb et al. (2016) emphasise, every country in the world today is threatened by the risks of a fossil fuel price rise, petroleum shortage and global warming. Consequently, as Chisti (2007) and Hu et al. (2008) point out, the exploration of renewable fuels has become a hot topic over the last few decades, with microalgae highlighted as a potential solution given their ability to produce biodiesel and other biofuels. Mata et al. (2010) and Taleb et al. (2015) explain that the benefit of microalgae, as photosynthetic microorganisms, is their significantly higher capacity to produce biomass (thus, higher land efficiency), the higher level of intracellular lipids, and their metabolic adjustment. For instance, Hu et al. (2008) and Kandilian et al. (2014) explain that algae are an ideal source for biodiesel generation due to their ability to amass high levels of neutral lipids (as TAGs) when deprived of nitrogen or other nutrients.

As reported by Scott et al. (2010) and Li et al. (2011), various studies have explored the issues involved in generating biodiesel out of microalgae, mainly in terms of the sustainability and level of expense associated with the method. As Chisti (2013) and Rawat et al. (2013) emphasise, the realisation of microalgal biodiesel achieving widespread adoption and surpassing other fuels depends upon the ability of the technology supporting it to deal with these challenges and provide adequate supply to fulfil global transportation needs. As Chisti (2008) and Hannon et al. (2010) note, the core issue is cost. Alabi et al. (2009) report that whilst crude petroleum can

be produced at USD $40-$120 per barrel, or $0.24-$0.73 per litre, microalgal biofuel cost $300-$2,600 per barrel, or $1.83-$15.85 per litre, based on technology used within the last decade. However, it should be highlighted that if algal biomass with a dry weight lipid content of 70% is used, the cost decreases significantly to $115 per barrel.

In a more recent study, Amaro et al. (2011) report a $2.38-$10.58 per litre cost of microalgal biodiesel, with Sun et al. (2011) noting a $3 per litre average cost for the production of baseline lipid (triacylglycerol). Thus, as Wijffels and Barbosa (2010) assert, if microalgal biofuel is to compete effectively with current fuels, it must be produced at a cost 10 times lower than current estimates. According to Singh and Gu (2010), one of the main ways to achieve this would be to introduce biorefinery plants that are able to produce both biofuel and value added products. Additionally, costs could be further reduced if biorefineries were introduced with more efficient operational procedures and cost-effective designs. Thus, as Chisti (2007) points out, microalgal biodiesel can only become competitive if biomass production can be achieved at a lower cost. The current thesis discusses three main production factors with the aim of addressing this issue: algal biomass disinfection at the time of production; the harvesting of algal biomass; and ozonation-based lipid extraction through the use of a microbubble bioreactor.

2.1.2 Introduction of Microalgae

Richmond (2004) defines algae as non-vascular organisms that contain *chlorophyll a* (photosynthetic pigment) with no differentiation into stems, roots and leaf systems. According to Lundquist et al. (2010), algae are of two types: macro algae (for example, sea weeds), and microalgae. Scientists have recently considered the morphological, cytological and genetic characteristics in classifying algae. This approach has made classification more specific and has significantly contributed to the current algae classification system (Richmond, 2004). With time, microalgae have evolved into three major cellular organizations: unicellular, colonial and filamentous. Different types of the microalgae have developed different adaptations; for instance, flagella have been developed for motility. Algae have also developed different rates of doubling. For instance, green algae double every 24 hours while the prokaryotic blue-green algae (cyanobacteria) double every 6-12 hours. Other algal taxa have a typical doubling rate of 18 hours (Griffiths and Harrison, 2009). Richmond (2004) notes that unlike terrestrial plants, microalgae grow very well in areas with varying temperatures, salinity and pH because of their simple structure.

Microalgae are microscopic, but their photosynthetic machinery and action is the same as that of higher plants that are found in fresh, brackish and marine waters (Walker et al., 2005). Classification of these microbes is usually made on the basis of their pigment dominance (e.g., blue-green, brown, red and green pigments). Thousands of microalgal species have been characterized. These photosynthesizing

microbes are able to exhibit accelerated growth because of their simple structure (Mata et al., 2010). Tredici (2010) reports that the rate of growth of microalgae is a hundred times greater than that of terrestrial plants and that microalgal mass can be doubled in less than 24 hours. Richmond (2004) provides an in-depth illustration of microalgae, specifically cyanobacteria (which are prokaryotic) and chlorophytes (which are eukaryotic). Water, sunlight and inorganic nutrients are required for the growth of these photoautotrophic microbes (Borowitzka and Borowitzka, 1988).

Since the 1960s, these microbes have been used to produce valuable compounds that are of clinical importance on a commercial scale (Walker et al., 2005). However, numerous recent studies report that these microorganisms produce biodiesel better and more efficiently than other feedstocks (Rosenberg et al., 2008 and Rodolfi et al., 2009). As of today, 30,000 species of algae have been discovered by researchers. Other new species have been found by bioprospecting, which seeks desirable qualities in algal strains (Mutanda, et al., 2011 and Richmond, 2004). This study focusses on oleaginous algal species, which have the capability to accumulate neutral lipids whose properties resemble those of oils derived from first and second-generation fuel crops (Chisti, 2007).

2.1.3 Strains of Interest

Several desirable characteristics of the algal strains are usually considered when bio-prospecting so as to come up with the best algal biofuels. The characteristics

include the ability to grow in extreme conditions (high shear force, wide range of temperature and CO_2 tolerance/ uptake), high growth rate, and high content of desired product (Borowitzka, 1992 and Griffiths and Harrison, 2009). However, *Dunaliella salina* has been used in this study since it has demonstrated valuable results in earlier studies (Zimmerman et al., 2011). This species has been used widely in projects that range from small scale studies in laboratories to raceway pond system for commercial production (Del Campo et al., 2007 and Prieto et al., 2011). The benefits of using this organism are many. For instance, the organism demonstrates tolerance to extreme environmental conditions and can grow in non-sterile environments. They can survive and grow in extremely alkaline or salty environments where many other microbes like bacteria cannot live (Borowitzka and Borowitzka, 1988).

2.1.4 Dunaliella salina

This strain of green algae with an ovoid shape is mainly found in marine environments. It belongs to the group unicellular biflagellates and comprises only one chloroplast. *Dunaliella salina* cells have a pair of flagella of equal length at their anterior end. According to Van Den Hende, et al. (2012), this characteristic shows that *Dunaliella* is a very active motile microalga. Additionally, Attaway and Zaborsky (1993), note that the plasma membrane of *Dunaliella* has a mucous envelope but its cell is not covered by a cell wall. This characteristic is important given that a change in the extracellular osmotic pressure of the environment inhabited by *Dunaliella*

means that it can easily adapt by rapidly changing its cellular volume though the synthesis or degradation of intracellular glycerol.

The *Dunaliella* strains can actually thrive in saline environments with concentrations as low as 0.05 M NaCl to saturated concentration (5 M NaCl) due to the utilisation of intracellular glycerol (Chen and Jiang, 2009). Additionally, the tolerance to varying pH value of the *Dunaliella* species is very high. Reports by Borowitzka (1988) and Visviki and Santikul (2000) show that the strains of *Dunaliella* algae can grow in very acidic conditions (pH=1) as well as very alkaline conditions (pH=11). Also, the *Dunaliella* algal strains are temperature resilient. According to Wegmann, et al. (1980), *D.salina* growth is possible in high temperatures up to 40 °C. Moreover, studies by many authors such as Borowitzka (1988) and Tsuji et al. (2002) indicate that *Dunaliella* is tolerant to the chlorinated hydrocarbons and heavy metals.

2.1.5 Morphology of Dunaliella salina

Characteristically, *D. salina* is an unicellular green alga with a pair of flagella and is able to synthesize and accumulate β-carotene in high quantities (Browitzka, 1995). β-carotene is not only a vitamin, but an antioxidant, colorant and a food additive. It can be utilized in improving several cosmetic and body care products (Edge et al., 1997 and Preito, 2011). Besides carotene, the organism contains high concentration of lipid. The lipid content of *D. salina* ranges from 16-44% of the dry

weight of the cell and can serve as the raw material for production of bio-fuel (Borowitzka and Borowitzka, 1988). Due to these reasons, *D. salina* is amongst the most widely studied algae for mass culturing.

Morphological characters of *D. salina* are shown in Figure 2.2. *Dunaliella salina* lives in highly saline/salty environment. Colour of the microalgal cells is green to dark red depending on the concentration of *β*- carotene in the cells (Ben-Amotz and Avron, 1983). Cells are cylindrical, pyriform, ellipsoid or ovoid in shape. Typical cells are 5 to 29 µm long and 3.8 to 20.3 µm wide. Cells are not surrounded by cell walls; however, each cell has a pair of flagella that facilitates its locomotion. Length of each flagellum is the same as that of the cell itself. Moreover, *D. salina* reproduces sexually as well as asexually. Zygospores having a round shape are produced by meiosis during sexual reproduction which is heterothallic (Oren, 2005).

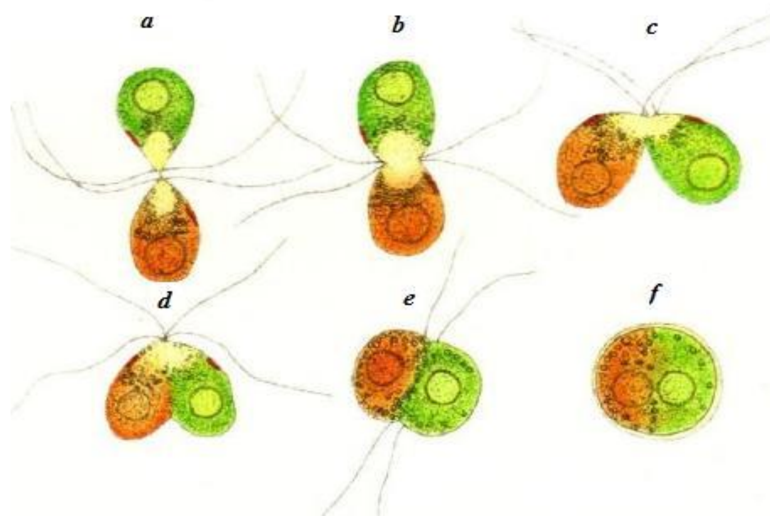

Figure 2.2: Morphology of *Dunaliella salina*, (*a-f*) Zygote formation (Oren, 2005).

2.1.6 Industrial Application of Dunaliella salina

Researchers have also been studied *Dunaliella* for industrial applications. *Dunaliella* can be cultivated in large quantities given that they can adapt to various environmental conditions. In this algal genus, *D. salina* is the best known species because it can synthesise carotenoids in large quantities. Carotenoids have commercial applications in cosmetics and food supplements (Burri, 1997). When grown in areas with high levels of salinity, *D. salina* has the capacity to produce a significant amount of glycerol which has wide applications in the pharmaceutical and food industries, according to the Soap and Dergent Association (1990). As such, in chemical harvesting applications, *D. salina* is among the first mass cultured algae.

The production of glycerol and carotenoids by *D. salina* are actually means of protecting itself from harsh environmental conditions. The synthesis of glycerol by *D. salina* is a means to balance its osmotic pressure while the generation of the highly concentrated β-carotene shields it from intensive light. While *D. salina* synthesizes these chemicals to shield itself from harmful environmental conditions, human beings have found a way of manipulating *D. salina* growth conditions to optimizing these chemicals. As such, higher yields of β-carotene can be produced from *D. salina* through the application of higher light intensities and salinity. Additionally, *D. salina* biomass can also form the source of other valuable products such as amino acids, enzymes, and vitamins among others (Borowitzka, 1988). The tolerance of *D. salina*

to different environmental conditions and the preference of *D. salina* to high salinity medium allows only rare contamination of outdoor cultures.

The potential of *D. salina* to be a successful biofuel crop is very high. However, various authors have shown that the productivity of lipid in this algal strain varies greatly. In every unit dry weight biomass, lipid content has been found to vary from 10 to 35%, but the overall biomass yield have been found to be high (Griffiths and Harrison, 2009). Nonetheless, Guzman et al. (2012) note that other strains of *D. salina* have also been found to possess a lipid profile rich in C16:0 and C18:0 FAs in addition to PUFA C18:3 that are detrimental to activities for the production of biodiesel. According to Mendoza et al. (1999), the production of high levels of β-carotene could be associated with an increase in C18:1 fatty acids and a reduction in PUFA which implies that it is more favourable to overproduce the *D. salina* 19/18-like strains. In particular in NaCl stress experiments, a shift in the contents of fatty acids has also been observed (Xu and Beardall, 1997).

D. salina has also been found to grow very well in customized photo-bioreactors that make use of the industrial bio reactors; this was established by the Sheffield's University Zimmerman research group (Zimmerman, et al., 2010). As such, in spite of the relatively poor lipid performance demonstrated by this algal species, it has other characteristics that include the tolerance to salt conditions and the easy extraction of oil that give it certain advantages as a fuel crop. Since tolerance to salt conditions and extraction of oil contributes significantly to the balance of energy

and water in the oil production process, it is worth to consider *Dunaliella* in biofuel research (Christi, 2013).

2.2 Microalgae Mass Culture

Algae can be grown in mass culture through a wide range of different techniques including continuous or batch culture, closed or open culture and outdoor or indoor culture. Source and intensity of light serve to be crucial factors that significantly influence the growth of microalgae in outdoor and indoor cultures (Mata et al., 2010). Sunlight serves to be the main source of light for outdoor cultures whereas indoor photobioreactors are usually provided with light through solar-energy-excited optical fibre (Chen et al., 2011). However, the optimal light intensity for *D. salina* growth were around 100 µmol m^{-2} s^{-1} and increasing the light intensity over 200 µmol m^{-2} s^{-1} will induced β-carotene production (Wu et al., 2016). The conventional fluorescent lamp can provide the light intensity required for *D. salina* cells growth. Different benefits and drawbacks of various techniques for algal mass culture are summarized in Table 2.1.

Table 2.1: Pros and cons of different algal cultivation methods (Coutteau, 1996).

Cultivation methods	Pros	Cons
Outdoors	-Inexpensive	-Difficult to predict
Indoors	-Easy to control	-Costly
Open	-Inexpensive	-Easily to be contaminated
Closed	-Less contamination	-Costly
Non-axenic	-Easy to applied and inexpensive	-More susceptible to crashes
Axenic	-Less likely to crashes, easy to predict	-Expensive and difficult to applied
Batch	-The easiest and most reliable cultivation technique	-Less efficient and inconsistent quality
Continuous	-Produce consistent product and efficient.	- Expensive equipment due to complexity. Usually applicable to small quantities

Batch culture serves to be the most common technique for mass cultivation of microalgae. Batch culture system has been widely used for commercial purposes since it is simple, extremely reliable and can be completely sterilized conveniently. In this system, a culture vessel containing a suitable quantity of culture medium is inoculated with algal cells. Inoculation is followed by incubation in conditions which are suitable for the algae. When maximum density of algal biomass is attained, the algal biomass can either be transferred to a culture vessel of larger size so that the algal cells can grow further or the algal mass can be used to inoculate a subsequent batch of culture. During cultivation of cells, medium is shaken constantly to facilitate

the supply of nutrients and gases to all cells in the medium (Richmond, 2004). The mass cultivation of microalgae in batch system is shown in Figure 2.3.

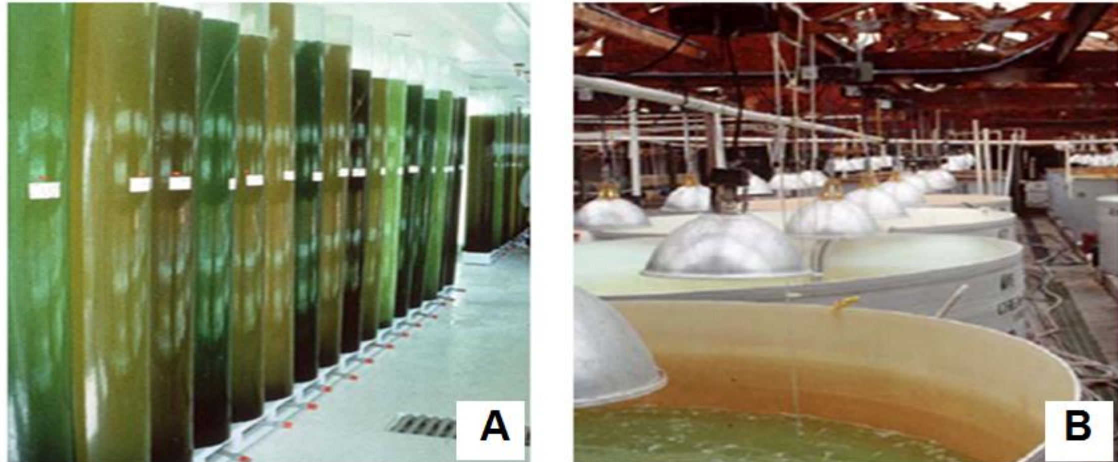

Figure 2.3: Image of the mass production in batch culture systems. A) production in 150 L bioreactors, (B) production in 20,000 L tanks. (Coutteau, 1996).

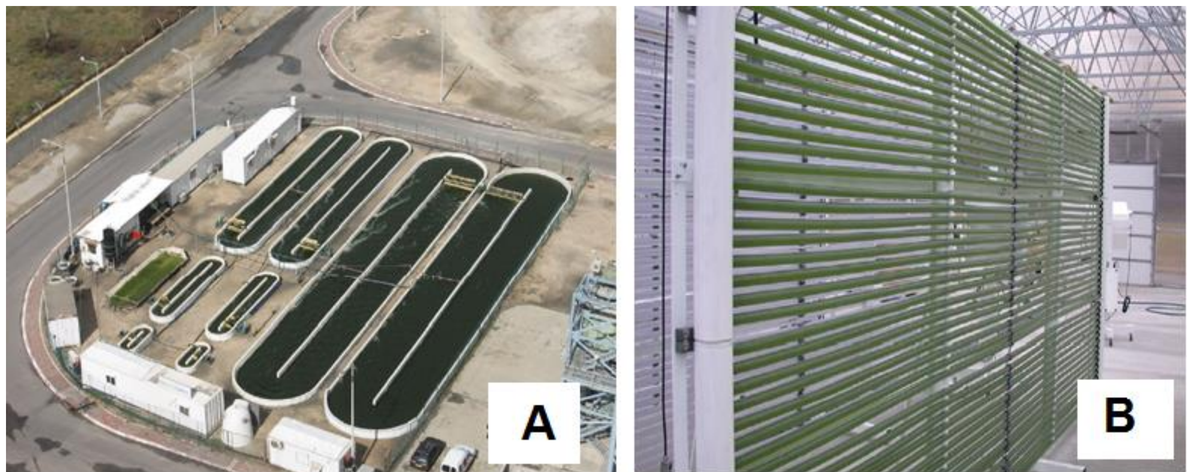

Figure 2.4: Image of large scale culture ponds (A) and tubular photobioreactor in operation (B) (Greenwell et al., 2010).

Large size culture ponds together with operating tubular photo bioreactors are shown in Figure 2.4. An efficient culture system demonstrates several characters such as low level of contamination, convenient operation, minimum requirement for land area, reduced production cost, optimum gaseous supply and an effective arrangement for lighting (Xu et al., 2009). Raceway ponds are most widely used for commercial cultivation of microalgae mostly for manufacturing products of high nutritive value for feeding animals. The system is equipped with a paddle wheel to prevent sedimentation of microalgal mass and CO_2 which is used as a carbon source is sparged at the bottom (Stephenson et al., 2010).

In the biofuel production process, the ratio of total energy output to total energy input also known as energy-efficiency ratio (EER) has been a common method to describe the energy conversion efficiency, where positive net energy is indicated by ratios higher than 1 while ratios below 1 indicate negative net energy (Lam and Lee, 2012). The EER of various techniques of microalgal cultivation are given in Table 2.2. The studies reported by Jorquera et al. (2010) and Stephenson et al. (2010) on EER value of *Chlorella* and *Nannochloropsis* cultivation in raceways pond are higher than 1 (positive value), while the cultivation of the same organisms in tubular photobiorectors gave value lower than 1 (negative value). The information shows that cultivation in raceways pond is more energy efficient than the cultivation in tubular photobiorectors.

Table 2.2: Energy efficiency ratio for different methods of microalgae culture.

Microalgae	Methods culture	Energy Efficiency Ratio (EER)	Reference
Chlorella vulgaris	Tubular photobioreactors	0.35	(Stephenson et al., 2010)
Nannochloropsis sp.		0.07	(Jorquera et al., 2010)
Chlorella vulgaris	Raceways pond	1.46	(Stephenson et al., 2010)
Nannochloropsis sp.		3.05	(Jorquera et al., 2010)

The open raceway pond system is an effective technique for the production of microalgae biomass in large scale due to the low costs and its simplicity (Sheehan, 1998). However, the main drawback of growing some microalgae (*Tetraselmis* sp., *Scenedesmus* sp., and *Chlorococcum* sp.) in raceway ponds is population crashes and culture contamination (Rodolfi, 2009). Contamination is a big restriction to the aim of producing low cost microalgae biomass at industrial scale (Wang et al 2013). There are many microalgae contaminats such as protozoans, fungi, virus and other microalgae which compete for the nutrients, space and light (Mayali and Azam, 2004 and Tillmann, 2004).

2.3 Bacterial Contamination

It have been reported broadly that the cross contaminations by other species of microalgae are unavoidable (Piazzi and Ceccherelli, 2002 and Wang et al., 2013). The contaminants mode of contamination is depicted in Table 2.3. Resource competition (Twiner et al., 2005), direct cell-to-cell contact (Twirler et al., 2001) and allelopathy (Twiner et al., 2005) are the three foremost contamination mechanisms involved if the starter culture is not pure and the contaminant strain is more dominant (Vasudevan and Briggs, 2008). Cells collisions (cell-to-cell contact) between two microalgae species at high cell density and space competition is the reason for the survival of the more dominant microalgae species (Yasuhiro et al., 2007). It has been reported that *Gymnodinium mikimotoi* cells were damaged and destroyed after several contacts with *Heterocapas circularisquama* cells (Uchida et al., 1999).

Table 2.3: The contaminants mode of action

Contaminant	**Mode of action**	**References**
Harmful algae	Competing for nutrients and resources	(Twiner et al., 2005)
Zooplankton	Competing for nutrients and resources (grazing)	(Huo et al., 2008)
Bacteria	Discharge lytic compounds; Competing for nutrients and resources	(Shi et al., (2006)
Virus	Infection	(Wu et al., (2011)

The resource competition of different microalgae strain occurs when the resources (nutrients, CO_2, light) are lower than the actual demand. The dominant microalgae is usually able to adapt and absorb nutrients (such as magnesium, nitrogen, phosphorus, calcium and potassium) better as has been observed by Litchman, (2003). The ability of certain microalgae to produce biochemicals (one or more) for its survival is known as allelopathy (Suikkanen et al., 2004). It has been reported that a substance secreted by the dinoflagellate *Peridinium* can kill competing *Rhodomonas lacustris* (Cryptophyceace) cells (Rengefors and Legrand, 2001).

The common predatory species of zooplankton are *Cladocera* and copepods (Frederiksen et al., 2006), rotifers (Lurling and Beekman, 2006), and ciliates (Rosetta and McManus, 2003). The cell concentration of *Dunaliella salina* was decreased from 2.7 x 10^4 cells/ml to 1.2 x 10^4 cells/ml when incubated with ciliates for 2 days (Li et al., 2006). While Moreno-Garrido and Canavate, (2001), also reported a decrease in *D. salina* cells concentration during outdoor mass cultivation within 2 days due to grazing of ciliates. It has been reported that, behavior mechanism and mechanical mechanism are the feeding mechanism involved (Vanderploeg and Paffenhofer, 1985). The copepods will activate the behavior mechanism during low nutrient condition. While mechanical feeding mode was selected during excess nutrient conditions (Kleppel et al., 1996).

Some lytic bacteria species are able to suppress microalgal growth (Wang et al., 2010) and can destroy mass cultivation of microalgae (killing the algae) (Zhou et al., 2011) through direct attack (cell-to-cell contact) (Shi et al., 2006) or indirect attack (extracellular compounds) (Kang et al., 2005). Sakai et al., (2007) have reported that the damaged of DNA to the double helix structure was the reason for the microalgae death. Furthermore, lytic bacteria caused the breakage of microalgae cell walls resulting in the leakage of intracellular material (Nakashima et al., 2006). Several reported species were *Flavobacterium* sp. (Fukami et al., 1992), *Alteromonas* sp. (Imai et al., 1995), M*yxobacter* sp. (Junichi et al., 1998), *Pseudomonas* sp., and *Bacillus* sp. (Kim et al., 2007). Viruses can infect microalgae culture (eukaryotic algae and prokaryotic cyanobacteria) quickly due to their high specificity and fast replication cycle (Wang et al., 2013). It has been reported that the microalgae cell concentration was reduced significantly (within a few days) due to virus infection (Kagami et al., 2007). While the HaVo1 algicidal activity has been reported which caused all microalgae cells to lose motility within 24 hours (Nagasaki et al., 1999).

There are several techniques that have been reported to alleviate the contamination impact such as maintaining high alkalinity, high salinity and high nutrients (Lee, 1986), but only work for certain microalgae (*Spirulina*, *Dunaliella* and *Chlorella*) (Lee, 2001). The most common technique is by adding chemicals into the open pond system to alter the pond conditions. To control protozoa in *Nannochloropsis* cultures, the use of hyperchlorite has been deployed (Weissman, 2010). To control zooplankton in *Chlorella* sp. cultures, pesticides (Parathion,

dichlorodiphenyltrichloroethane and Dipterex) has been practiced (Loosanoff, 1957). The use of glyphosate to control contaminants in *Nannochloropsis* culture has also been reported (Vick, 2010 and Weissman et al., 2010). However, none of the above mentioned chemical methods has been effective for long periods of time (commercial scale) (McBride et al., 2014).

2.3.1 Halomonas sp.

Normally, halophilic bacteria exist naturally in saline ecosystems such as saline soils, saline lakes and seas (Arahal and Ventosa, 2006). The genus *Halomonas* (members of *Halomonadaceae*) have been described as heterogeneous genus, ubiquitous and with the largest number of species in its family (Arahal and Ventosa, 2006). Previously, many novel species of *Halomonadaceae* have been reported such as *Halomonas illicicola* (found in saltern, Spain) (Arenas et al., 2009), *H. jeotgali* (found in fermented seafood, Korea) and *H. salifodinae* (found in salt mine, China) (Wang et al. 2008). In order to grow, halophilic bacteria require salt and it has been reported that they perform well at salt concentrations > 100 g L^{-1} (Margesin and Schinner, 2001 and Oren, 2002).

Halomonas sp has been chosen in this study due to its special relationship with *D. salina* and has been reported to live together in high salt culture (Cao et al., 2013). The rod-shaped (12.5-21.6 µm), gram-negative and nonmotile *Halomonas* is believed to benefits from glycerol released by *D. salina* (Bardavid et al., 2008). It has been

reported that, the halophilic microbes primarily consumed glycerol (released by microalgae) as their carbon sources (Borowitzka, 1981). Scientist have suggested that the lysis and membrane leakage of microalgae cells as the reasons of glycerol availability in the environs (Fujii and Hellebust, 1992 and Hard and Gilmour, 1991). Kazamia et al. (2012) has shown a symbiotic phenomenon where the algae can benefit from vitamins produced by bacteria. However, there is no solid proof that microalgae requires vitamins from the bacteria (Croft et al., 2006). It has been mentioned in the Section 2.3 above that when the bacteria become dominant, they will compete with microalgae for nutrients and resources which will affect the microalgae biomass production. Therefore, it is important to study the contamination impact towards the performance of microalgae growth including a method of controlling the contaminant.

2.4 Algal Lipids

Any biological molecule that dissolves in an organic solvent can be classified as a lipid. For this reason, Halim et al. (2012) note that lipids comes in different structures and thus classified based on types. In the production of biodiesel, fatty acids (FA) lipid types and some of their variants are of more significance. A molecule of a fatty acid is made up of a RCOO- (a hydrophilic carboxylate group) which has hydrophobic hydrocarbon chains attached. According to Nelson and Cox (2004), the number of carbon atoms and that of the double bonds in a chain of a fatty acid is the basis upon which different classification systems have been developed. Fatty acids

with no double bonds are classified as saturated FAs while those with at least one double bond are classified as unsaturated FAs. FAs with many (more than one) bonds are classified as poly-unsaturated (PUFA).

Additionally, some lipids have a charge while others do not. This yields another classification of neutral or net polar classification. For instance, when a charged FA molecule (carboxylate end of a FA) bonds to a glycerol (uncharged head), a neutral lipid singular free fatty acid (FFA) or triacylglycerol) is formed. On the other hand, a polar lipid (for instance a phospholipid) is created if a FA creates a bond with glycerol or phosphate complex which are charged heads. In the cells of microalgae, neutral charged lipids act as a primary source of energy. On the other hand, polar lipids combine to yield cell membrane made of phospholipids. Though a significant proportion of lipids contain FAs, others such as pigments (carotenes and chlorophylls), hydrocarbons (HC), ketones (K), and sterols (ST) do not contain FAs and it is not possible to convert them into biodiesel (Halim et al., 2012). As discussed in the previous sections, the dry biomass of total lipid content in microalgae differs a lot from one algal species to another ranging from 5 to 77 wt. % (Chisti, 2007). According to Zhukova and Aizdaicher, (1995), within this range, the type of lipid can also vary significantly between the various species where some may have a higher affinity for the production of biodiesel due to the high quantities of neutral lipids.

The variation in lipid becomes more complex based on the growth stage and the environment to which the microalgae is exposed. Based on the various exposures

such as temperature, ratio of light/dark cycle, medium composition, illumination intensity, and aeration rate, the type of FA that is produced is altered significantly (Guzman et al., 2010). In the log growth of the microalgae, the amount of polar lipids is usually higher than in a microalgal cell in the stationary phase (Lv et al., 2010). The composition of lipids in microalgae is also majorly affected by nutrient starvation and other environmental stresses (Solovchenko, 2012). From these variations which arise from different causes, it is critical that the choice of the most suitable algal lipid for the production of biodiesel be assessed on the basis of conditions, time or strain. In the production of biodiesel, the neutral acylglycerol lipid are the, most preferred because of two major reasons. First, neutral acylglycerol lipids are capable of producing biodiesel with higher oxidation stability as their structure has low amounts of unsaturation than that of the polar type lipids. Secondly, the commercial processes (alkaline-catalysed transesterification) are designed specifically for acylglycerols in the production of biodiesel from lipids and are not appropriate for the processing of polar lipids and FFA (Lang et al., 2001).

According to Hu et al. (2008), oleaginous green algae under average environmental conditions accumulate lipid to approximately 25.5% dry cell weight (DCW). However, under stressful environmental conditions, the lipid content can double or triple. Also, most accumulation of lipid takes place at the end of the life cycle i.e. in stationary phase. In either of these two cases, the increase in the content of the lipid specifically comes as neutral acylglycerols. This increase in the neutral lipid content arises due to the shift in the lipid metabolism from membrane lipid

synthesis as the division of cells is given the lowest priority (Hu et al., 2008). Additionally, according to Tonon et al. (2002), the increase in the neutral lipid content is associated with *de novo* biosynthesis and the formation of triacylglycerols from the existing membrane polar lipids. In the formation of a range of lipids (either saturated or unsaturated) with varying lengths from 12 to 22 carbons, the first building blocks mainly arise from the algal synthesis of FAs.

However, according to Medina et al. (1998), the FA chains have double bonds that do not go beyond 6 and that the majority of the unsaturated FAs are cis isomers. Similar to the higher plants, Ohlrogge and Browse, (1995) note that C16 to C18 are the most common chain lengths. In these FAs, the majority of the variants that are saturated and mono-saturated are found in algal species (Borowitzka, 1988). Table 2.4 shows a list of the main types of fatty acid and poly-unsaturated fatty acid which are produced from the major groups based on the classification of the algae. *Dunaliella salina* is classified in the group Chlorophyceae with the fatty acids containing C16:0 and C18:1 as the dominant types. The classification of *D. salina* in Chlorophyceae (green algae) group is well known and have been reported by many researchers (Assunção et al., 2012 and Giordano et al., 2014).

Table 2.4: Different algae classes with their main lipid types (Hu et al., 2008).

Algal class	Fatty Acids	PUFAs
Bacillariophyceae	C16:0 and C16:1	C20:5ω3 and C22:6ω3
Chrysophyceae	C16:0, C16:1 and C18:1	C20:5, C22:5 and C22:6
Chlorophyceae	**C16:0 and C18:1**	**C18:2 and C18:3ω3**
Cyanobacteria	C16:0, C16:1 and C18:1	C16:0, C18:2 and C18:3ω3
Cryptophyceae	C16:0 and C20:1	C18:3ω3, C18:4 and C20:5
Dinophyceae	C16:0	C18:5ω3 and C22:6ω3
Eustigmatophyceae	C16:0 and C18:1	C20:3 and C20:4ω3
Euglenophyceae	C16:0 and C18:1	C18:2 and C18:3ω3
Prymnesiophyceae	C16:0, C16:1 and C18:1	C18:2, C18:3ω3 and C22:6ω3
Prasinophyceae	C16:0 and C18:1	C18: 3ω3 and C20:5
Xanthophyceae	C14:0, C16:0 and C16:1	C16:3 and C20:5
Rhodophyceae	C16:0	C18:2 and C20:5

2.4.1 Biosynthesis of Fatty Acids

In comparison to the study of higher plants such as *Arabidopsis,* the study of lipid metabolism and the pathways for biosynthesis of FAs have been little studied in microalgae (Liu and Benning, 2013). Recently, Hu et al (2008), has discovered the

common biochemical characteristics of several enzymes and their genes especially those isolated from *Chlamydomonas reinhardtii*. The work by Hu et al. (2008) provides detailed understanding about this pathway. It is in the chloroplast that the formation of the FAs occurs through pyruvate kinase (PK) catalyzing the production of pyruvate from PEP (phosphoenolpyruvate) which is then transformed to acetyl CoA. As demonstrated in Figure 2.5, C16 and/or C18 FAs are created by the *de novo* synthesis pathway. The creation of malonyl CoA from acetyl CoA and CO_2 through the catalysis of ACCase forms the first step. The synthesis of the chloroplast, cellular membranes, and neutral storage lipids (for example, TAG) occurs from the FAs that are originally made as illustrated in Figure 2.6.

A substrate (Acetyl CoA) enters the pathway for acetyl CoA carboxylase (Reaction 1) similarly during the initial condensation reaction (Reaction 3). The reaction of CoA to form malonyl ACP are catalyzed by transfers malonyl and malonyl CoA:ACP transferase (Reaction 2). The subsequent elongation reactions is contributed by Malonyl ACP carbon. After subsequent condensations, the 3-ketoacyl ACP product is reduced by 3-ketoacyl ACP reductase, dehydrated by 3-hydroxyacyl ACP dehydrase and reduced again by enoyl ACP reductase (Reaction 4, Reaction 5, Reaction 6, respectively)(Hu, et al., 2008).

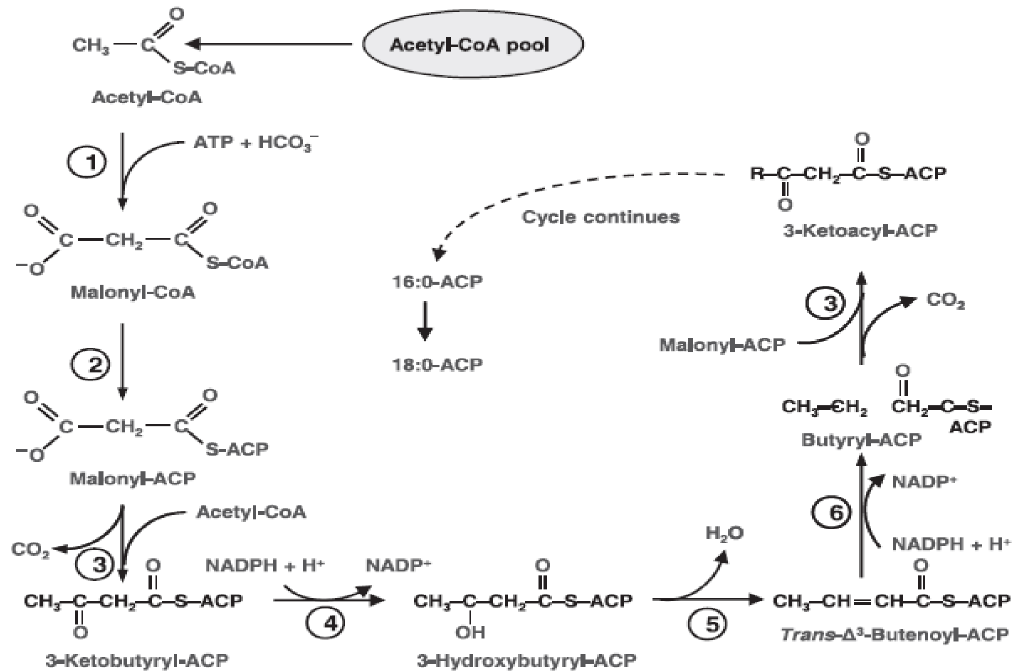

Figure 2.5: Pathway of fatty acid synthesis in the chloroplast (Hu et al., 2008).

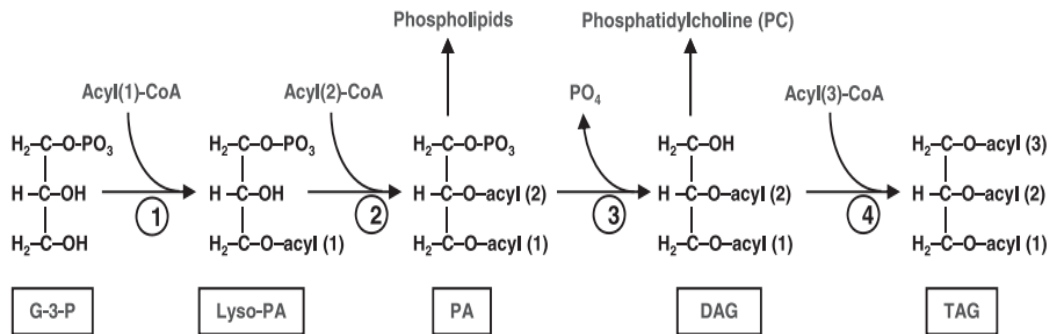

Figure 2.6: Triacylglycerol biosynthesis pathway in algae (simplified schematic). Cytosolic glycerol-3-phosphate acyl transferase, (1); lyso-phosphatidic acid acyl transferase, (2); phosphatidic acid phosphatase, (3); and diacylglycerol acyl transferase, (4) (Hu et al., 2008).

2.4.2 Algal Biomass Harvesting

Algal biomass for the generation of biofuel is extracted from water in a process called harvesting. This two-stage procedure is described in the work of Brennan and Owende, (2010) and Chen et al., (2011). First, the techniques of gravity sedimentation, flocculation and flotation are used to extract the algae from the aqueous suspension, then the product slurry is concentrated by means of centrifugation and filtration. As pointed out by Lam and Lee, (2012) and Suali and Sarbatly, (2012), the small size of the algal cells (typically 1 - 20 μm) and the condition of aqueous suspension make the process of harvesting a formidable task.

The harvest of the algae and the extraction of the lipid occurs when the algal growth attains enough biomass yield and has the necessary lipids. The attainment of these algal characteristics is important for the balance of energy and for the financial viability of the entire process of the production of the biofuel (Chisti, 2013). For this reason, a lot of academic and commercial research and funding has gone into this area as illustrated by the up-to-date summary shown in Table 2.5. Technologies developed for water treatment and purification purposes form the basis of the methods of harvesting that are widely used currently. These technologies, according to Kim et al. (2013) include centrifugation, flotation and filtration. However, the major limitation of these technologies is that they are not designed based on the physiology of the algae.

Table 2.5: Several algal harvesting methods, with advantages and disadvantages

Harvesting method	Description	Pros or Cons	References
Centrifugation	concentrating biomass based using centrifugal force	-High efficiency and short time. -Intensive energy consumption.	(Beach et al., 2012)
Electrolysis	The hydroxides formed will stick/bind to microalgae cell followed by electrocoagulation induction	-Applicable to wide range of pH with high efficiency -Problem to recycle the media	(Kim et al., 2012), (Gao et al., 2010)
Flocculation	Flocculating algal cells at the bottom to enable separation.	-Low energy consumption. -Slow process, less effective, expensive flocculants.	(Farooq et al., 2013), (Banerjee et al., 2012)
Filtration	Separation through membrane filtration or porous mesh	-Effective separation, easy to set up -Problems with biofouling	(Zhang et al., 2010), (Ahmad et al., 2012)
Flotation	Cells are pushed up by rising gas bubbles	-High efficiency dependent on bubble size. -Low cost with fluidic oscillation -Toxicity of flocculants	(Garg et al., 2012), (Hanotu et al., 2012)
Magnetic Separation	The magnetic particles stick/bind to the surface of microalgae cell followed by harvesting using magnetic field	-Fast harvest time -Too many recycling process and chemicals consumed	(Toh, et al., 2012), (Lim et al., 2012)
Immobilization	Cells are grown in alginate matrix, which can be easily harvested.	- Low energy consumption. -Costly to set up.	(Zhang and Hu, 2012), (Lam and Lee, 2012)
Ultrasound	The cells were forced to aggregate by low ultrasound frequency	-Able to aggregate and disrupt the cells -High energy consumption, low separation efficiency	(Zhang et al., 2009), (Bosma et al., 2003)

Chen et al. (2011) have noted that the mass ratio of algal biomass to water is small even when the product is cultivated using a closed photobioreactor. For instance, taking the biomass productivity to be 0.05 - 3.8 g L^{-1} day^{-1}, the mass ratio of algal biomass to water after cultivation in a closed photobioreactor for seven days is in the range 0.00035 – 0.027. Consequently, a significant quantity of water - about 73 tonnes on average, is required to harvest 1 tonne of algal biomass when the cultivation process is adapted to large-scale production. Hence, the development of a commercially practical harvesting technique is vitally important to the future of algal biofuel.

Sedimentation relies on gravity to divide particles or materials according to their size and density which can be described by the Stoke's Law (Liss et al., 2004). According to Milledge and Heaven (2013), since the algal cells' density and radius are responsible for the precise sedimentation of a suspended solid. They go on to assert that the greater the variation in two particles' densities, the greater the rate of sedimentation, and vice versa. Eventually, the harvest outflow can gather up the biomass which has sunk in the sedimentation process. Since this method is purely reliant on gravity, it is relatively low-cost. However, Uduman et al. (2010) note that flocculating agents are necessary to ensure consistent results, and there are energy costs involved to transfer the slurry. Taher et al. (2011) also point out that sedimentation is by no means the quickest process available.

Another option is centrifugation technique which, as outlined by Al Hattab et al. (2015), exploits a centrifugal force to turn the liquid suspension at high speeds, resulting in a pressure differential which encourages separation. Grima et al. (2003) assert that the strength of the centrifugal force determines the effectiveness of the separation. Studies by Borowitzka and Moheimani (2013) and Enviropro (2014) find that the disc stack is the most popular choice of centrifuge for most large-scale, high-value production lines, including algal biofuel. Density is still a key factor in the separation process. The disc stack centrifuge has a higher rate of removal of microalgae than other types of centrifuge. However, it does have some drawbacks, as outlined by Sharma et al. (2013): not only is it quite an intricate mechanism, with various nooks which are difficult to keep clean, even with chemicals, but also the discharge system has a low dry substance content. Apart from that, centrifugation at high G force increased the cells disruption due to hydrodynamic forces occurred within the centrifuge (Chulp et al., 2008).

Alternatively, filtration techniques pass the suspension through a permeable membrane which allows the liquid to pass through, but stops the algae biomass. Al Hattab et al. (2015) describe how a vacuum, pressure or gravity creates a variation in pressure on either side of the membrane; the larger the pores, the lower the pressure necessary for separation. Decalite (2012) and Sanderson (2014) offer definitions of vacuum filtration, both of which focus on the suction created by the vacuum around the membrane. Grima et al. (2003) and Brennan and Owende (2010) note that, since the size of microalgae ranges from 2 to 30 µm, these particles can be caught in the

vacuum filtration process. There are also membranes suitable for larger microalgae cells or flocculated cells (Milledge and Heaven, 2013). Petrusevski et al. (1995) argue that the greatest benefit of vacuum filtration is that the cells are preserved when removed from the membrane. Though this is a more resourceful method of harvesting microalgae than sedimentation, the membrane can have maintenance problems - outlined in a study by Gonzalez-Fernandez and Ballesteros (2013) - as it needs to be cleaned or changed regularly to maintain its efficiency.

Edzwald (1993) offers flotation technique as another option, one which relies on low density of microalgal cells. Microalgae attach themselves to bubbles of gas which float through the suspension; Sing et al. (2011) thus label this process as physiochemical gravity separation. It is easier for smaller particles to attach themselves to bubbles of gas. There are four subcategories of flotation which are distinguished by bubble formation, including dispersed air, dissolved air, electrolytic flotation, and microbubble generation. Hanotu et al. (2012) remark that a huge amount of energy is necessary to achieved the high pressure used in dissolved air flotation. Zimmerman et al. (2009) and Hanotu et al. (2012), on the other hand, remark on the relatively small amount of energy needed by the fluidic oscillator: 2 or 3 times less that that used in dissolved and dispersed air flotation (see section 2.6). Though flotation is viable harvesting option (Garg et al., 2012 and Hanotu et al., 2012), others advocate magnetic separation (Toh et al., 2012 and Lim et al., 2012). Whichever method one considers, it will still be in need of development, since both method still at the lab stage and thus not yet at the peak of their commercial viability.

2.4.2.1 Algal Harvesting Energy Consumption

As Uduman et al. (2010) and Al Hattab et al. (2015) point out, the harvesting stage of production represents the main challenge in the generation of value added products from microalgae biomass. As Grima et al. (2003) explain, since between 0.1g and 2.0g of dry microalgae biomass is gained per litre of microalgae cultures, the cultures must be concentrated in order to be used for this purpose. Thus, as Zitelli et al. (2006) note, the overall cost of producing microalgal biofuel is impacted significantly by the cost of removing water content from the biomass (20-30% of the overall cost). As Danquah et al. (2009) report, the higher the level of concentration, the less expensive it is to extract and purify the biomass. Table 2.6 illustrates the various harvesting techniques that can be used for microalgal biofuel production and the level of energy consumption associated with each technique.

Al Hattab et al. (2015) points out that very few biofuel plants currently use sedimentation tanks for microalgal concentration despite their ability to achieve 1.5% total suspended solids (TSS) concentration. Uduman et al. (2010) explain that this is largely due to the fact that if flocculating agents are not utilised, this technique is unreliable even if gravitational settling serves as a less expensive option compared to other methods. Additionally, the researchers add that slurry pumping requires additional energy consumption, whist Taher et al. (2011) highlight the fact that compared to other methods, the use of sedimentation tanks is significantly more time consuming. The latter point is echoed in other research, wherein it is also noted that

cell composition can alter using this method (Gonzalez-Fernandez and Ballesteros, 2013). Furthermore, other researchers point out that this method can yield low cell concentrations (Mata et al., 2010) and cell recovery rates (60-65%) (Ras et al., 2011).

Table 2.6: Harvesting methods and their energy consumption

Harvesting Method	Energy usage (KWh m^{-3})	Algal biomass yield (% solid)	References
Centrifugation	8.0	22	(Girma et al., 2010)
Coagulation (electro)	1.5	-	(Bektas et al., 2004)
Filtration (natural)	0.4	6	(Semerjian et al., 2003)
Filtration (pressurized)	0.88	27	(Semerjian et al., 2003)
Filtration (Vacuum)	5.9	18	(Girma et al., 2003)
Flocculation (polymer)	14.81	15	(Danquah et al., 2009)
Flocculation (electro)	0.33	-	(Edzwald, 1995)
Gravity sedimentation	0.1	1.5	(Shelef et al., 1984)
Flotation (dissolve air)	7.6	5	(Wiley et al., 2009)

Depending on the filter type, vacuum filtration has been found to consume somewhere between 0.1 and 5.9 kWh m^{-3} of energy, according to early research (Shelef et al., 1984). Other studies have shown that the use of a vacuum belt filter results in energy consumption of 0.25 kWh m^{-3} (Milledge and Heaven, 2013); vacuum

drum filtration results in an energy consumption of 3 kWh m^{-3} (Mohn, 1988); and cross-flow filtration results in an energy consumption of between 3 kWh m^{-3} and 10kWh m^{-3} (depending on the feed, system and pressure) (Rossignol et al., 1999). The latter method has also been highlighted as an inexpensive option, resulting in 0.38-2.06 kWh m^{-3} of energy consumption for suspended solids with a 2.5-8.9% concentration (Danquah et al., 2009). In more recent work, 5 kWh m^{-3} energy consumption was reported using cross-flow filtration (Crittenden et al., 2012).

Energy consumption of 0.53-5.5 kWh m^{-3} has been noted by researchers using the disc-stack centrifuge method for microalgal biofuel production, and 1.3-8 kWh m^{-3} when using decanter centrifuge. For instance, the use of the decanter centrifuge technique has been reported to result in an energy consumption of 1.3 kWh m^{-3} and 8 kWh m^{-3} with 0.04% to 4.00% biomass concentration and 22% (w/v) concentration, respectively (Sim et al., 1988 and Grima et al., 2003). In terms of disc centrifugation, 1.4 kWh m^{-3} energy consumption was reported by Goh (1984) when harvesting microalgae from the waste matter of pigs. Additionally, 5.5 kWh m^{-3} energy consumption has been reported when harvesting *Chlorella sp.* (Sharma et al., 2013).

The cationic organic polyacrylamide-based flocculant, Praestol, has been found to achieve 70% efficiency in the flocculation of *Teraselmis* and *Spirulina* (Pushparaj et al., 1993). In other research, a positive association is reported between salinity levels and the amount of flocculant needed to extract microalgae from liquid suspension with a 90% recovery rate (Sukenik et al., 1988). Polymer flocculation has

been shown to consume energy at 14.81 kWh m^{-3} with a 15% (w/v) concentration of microalgae (Danquah et al., 2009). Compared to all other approaches to flocculation, electrophoresis-based flocculation has been found to reduce energy consumption. For instance, 2.1 Wh g^{-1} energy consumption has been noted in the flocculation of *C. vulgaris* (freshwater microalgae) and 0.2 Wh g^{-1} in *P. tricornutum* (marine microalgae), both given in dry weight (Vandamme et al., 2011). It has been proposed that lower energy consumption can be achieved in the harvesting of marine microalgae due to its higher level of compatibility with electrocoagulation as a result of greater conductivity (Gonzalez-Fernandez and Ballesteros, 2013).

As Haarhoff and Steinbach (1996) and Féris and Rubio (1999) explain, the dissolved air flotation method is known to be a costly but efficient approach to algae harvesting. The reason for the high cost involved in this method is the use of a compressor that operates at 390 kPa pressure, which demands high energy consumption. This point is also highlighted in the work of Hanotu et al. (2012). Hanotu et al. (2012) and Zimmerman et al. (2009) that compared to dispersed air flotation and dissolved air flotation, lower costs and energy consumption can be achieved through the use of a fluidic oscillator (see section 2.6). However, the microbubbles created by the fluidic oscillator have not been utilised to perform the flotation method in any published study that reports its specific energy consumption level. The current study aims to address this gap in the literature by testing and reporting the energy consumption associated with this approach.

2.4.3 Algal Lipid Extraction

According to Chisti (2007), the major importance of algal cultivation is in the transformation of algal lipids into biodiesel which is the lipid extraction step following on from the algal biomass dehydration stage. Efforts for the development of alternatives or the improvement of this infrastructure are currently underway. Table 2.7 shows several algal lipid extraction methods, with advantages and disadvantages.

Traditionally, the extraction of lipids has been based on mechanical principles such as disruption alongside the solvent fractionation, but these procedures are waste a lot of biomass and are slow (Halim et al., 2012). Great potential has been illustrated by the latest approaches such as electroporation which consume energy efficiently in addition to producing higher outputs (Halim et al., 2012). Lam and Lee (2012), point out that a mechanical press of the type that is effective in extracting oil for soil-grown crops is not likely to be effective for lipid extraction from algal biomass because the presence of the thick cell wall impedes the release of intracellular lipids.

Halim et al. (2012), argue that an optimal methodology for algal lipid extraction must be strongly specific and selective with respect to algal lipids such as acylglycerol without resulting in the concurrent extraction of other compounds (proteins, carbohydrates, ketones, carotenes) that are not useful for conversion to biodiesel. The fact that chemical solvent displays strong specificity toward algal lipids, along with the solubility of algal lipids in such solvent, makes this common laboratory-scale method and the most useful methodology for algal lipid extraction.

According to Halim et al. (2012) and Ranjan et al. (2010), this methodology even facilitates diffusion of intracellular lipids across the algal cell walls, allowing these to be successfully extracted. Halim et al. (2012) and Lam and Lee (2012), list solvents including n-hexane, methanol, ethanol, and mixed polar/nonpolar chemical solvents such as methanol/chloroform and hexane/ isopropanol as being applicable to algal lipid extraction, although the effectiveness of the extraction is strongly conditional on the algal strain.

A number of problems must be resolved before chemical solvent extraction can be commercially employed. These include the necessary use of significant amounts of chemical solvent for practical lipid extraction, the health and safety considerations relating to the toxicity of these solvents, the further energy consumption required for solvent recovery, and further costs arising from the need for wastewater treatment. Lee et al. (2010), have described autoclaving as a possible alternative technology for enhanced algal lipid extraction; Halim et al. (2011), suggest the application of supercritical CO_2, while Adam et al. (2012), Lee et al. (2010) and Prabakaran and Ravindran (2011), suggest ultrasonication. All of these techniques remain in the research stage (lab scale) and there is pressing need for them to be fully optimised before they can be commercially applied.

Table 2.7: Several algal lipid extraction methods, with advantages and disadvantages

Lipid extraction method	Description	Advantages or disadvantages	References
Bead mill	The bead interaction through friction/ collision disrupted the cell culture	-Simple and rapid treatment -Low efficiency, small scale only	(Sheng et al., 2012), (Zheng et al., 2011)
Biological disruption	The enzymes ruptured the algal cells through cell envelope degradation	-Effective disruption -Expensive enzyme cost	(Jin et al., 2012)
Direct transesterification	Combines extraction and biodiesel conversion.	-Simple industrial process -Need to remove by-products.	(Patil et al., 2011), (Ranjan et al., 2010)
High pressure homogenization (HPH)	The cell breakage is triggered by the compression of the hydraulic shear force	-Simple and effective -Slow and high energy costs	(Halim et al., 2012), (Zheng et al., 2011)
Electroporation	The interaction with the dipole moments causing the algal cell to disrupt when the electromagnetic fields are activated	-Simple with high energy efficiency -Limited information. -Commercial trials (OriginOil)	(Halim et al., 2012), (Sheng et al., 2011)
Chemical Treatment	The algal cells lysis were induced by chemicals, detergents, acids and alkalis.	-Highly effective method -High chemicals cost	(Sathish and Sims, 2012), (Vaara, 1992)
Osmotic shock	The algal cell ruptures were activated due to osmotic pressure (sudden increase or decrease in salinity)	-Easy to set up and inexpensive. -Ineffective and wasting a lot of water.	(Prabakaran and Ravindran, 2011), (Yoo et al., 2012)
Microwave	Microwave radiation heats cell envelopes and breaks down hydrogen bonds	-Quick penetration and rapid disruption -High energy consumption	(Balasubramanian et al., 2011), (Prabakaran and Ravindran, 2011)
Soxhlet extraction	The algal cells disruption is performed through solvent evaporation and re-condensation.	-Widely used process -Slow and energy inefficient -Small scale	(Halim et al., 2012), (Kanda and Li, 2011)
Ultrasonication	The algal cells were lysed due to bubble cavitation created by the ultrasound	-Effective cell disruption -High energy consumption, small scale only	(Adam et al., 2012)
Milking	The desired products are extracted out from algal cells without cell disruption process	- Ineffective (slow process) -More study and data required.	(Kleinegris et al., 2010), (Wijffels and Barbosa, 2010)

2.4.4 Production of Biodiesel

After the process of the extraction of the lipids is done, another method is used to separate/fraction the various components of the crude oil lipids such as pigments, acylgycerols, and polar lipids among others so as to be left with acylgycerols which is primarily used for the production of biodiesel (Medina et al., 1998). The process of transesterification (or alcoholysis) is used for converting the purified lipids into biodiesel. During transesterification, an alcohol, for example, methanol is used to react the various varieties (tri, di and mono) of acylgycerols alongside a catalyst so as to produce biodiesel or FAME (fatty acid methyl esters). Glycerol is produced as a by-product, during the process. The catalyst for the alcoholysis can either be an acid, an alkali (KOH upwards), or lipase enzymes. Each catalyst comes with its own advantages and disadvantages may arise (Kim, et al., 2013 and Xiao et al., 2009).

According to Huang et al. (2010) in chemical industries, alkali catalyst are preferred over the acid catalysts as they exhibit superior reaction rates with plant and animal lipids in addition to having higher efficiencies than the acid-based catalysts. After the transesterification reaction is over, techniques such as bi-phasic fractionation separation are used to purify the product further so as to remove the excess solvents, catalysts or glycerol (Griffiths et al., 2010). After that, a gas chromatography (GC) system fitted with a lipid specific column is then used to check the constituents of the biodiesel/FAME (Laurens et al., 2012). There are several factors affecting the entire biodiesel/FAME conversion process. This include the acylglycerol to methanol mole ratio, the temperature for reaction, acylglycerol to

catalyst mole ratio, the time of exposure, and the crude lipid extract water content. FFAs can also be formed from the reaction of water with monoacylglycerols. A further reaction between FFAs with an alkaline catalyst results in the formation of soaps. The efficiency of the alkali catalysts in converting non-acylglycerol FAs with lipid fractions (for example polar lipids) is low. For this reason, the commercial scale production of neutral lipid feedstock is the most preferred (Halim et al., 2012).

2.4.5 Fuel requirements

The FA composition of algae varies greatly as compared to the other first generation and second generation biofuel crops. Though this algal property come with some benefits especially in nutrient (such as nutraceuticals) production, Spolaore et al. (2006) note that in some cases it has negative effects for biodiesel production cases. The structure and FAME feedstock composition of the biodiesel determines its quality and viability (Knothe, 2005). Fuel with desirable properties such as cold flow, low pour point, and low cloud point are mainly produced form of FAME derived from *cis*-unsaturated fatty acids. On the other hand, FAME which are extracted from saturated FA, according to Halim et al. (2012), yield biodiesel with very good properties such as higher cetane number and superior oxidative stability, but come with poor properties when the temperatures are low. This is because, with low or a decrease in temperature the saturated chains pack closely to produce tight semicrystalline structures that gel. The good low temperature properties in the *cis*-

unsaturated FAMEs are as a result of the bends that the *cis* double bonds impose to prevent molecular packing leading to very low freezing points (Lang et al., 2001).

Further, PUFAs produce FAMEs with good flow properties, but are prone to oxidation in addition to having poor volatility. In this case, prolonged storage can result in instability challenges such as the formation of gum (Ramos et al., 2009). These reactions explain why acylglycerols acting as feedstock are best suited for the FAME production; the degree of unsaturation in the acylglycerols (C18:1) is generally low as compared to that of polar lipids (C18:3, C18:4). Though some algal lipids are considered unsuitable for biodiesel production (low oxidative stability due to higher double bonds composition), they are useful for biodiesel production when processed through additional steps using catalytic hydrogenation (Dijkstra, 2006 and Chisti, 2007).

2.5 Disruption of Microalgae Cells

It has recently been reported that currently available techniques for commercial production of biodiesel from lipid content of microalgal mass are not cost-effective as their cost of operation in downstream processing is quite high (Lee et al., 2012 and Uduman et al., 2010). There are several steps of downstream processing required in the production of biodiesel. The process of biodiesel production from microalgae can be divided into four main stages i.e. growing

microalgal mass, separating the algal growth from culture medium, extracting lipid content and finally transesterificating lipids to end products. Lipid content of all microalgal species is found inside the cells in the form of sterols, glycolipids, triacylglycerols and fatty acids. However, *Botryococcus braunii* serves to be an exceptional case (Banerjee et al., 2002). Otherwise, cell membranes and in some cases cell walls surround the intracellular components including lipid making their extraction quite difficult from intact cells (Barsanti et al., 2007). Disruption of cells can lead to 3-4 times greater extraction of lipid content from the biomass of microalgae (Boldor et al., 2010).

Figure 2.7.1 shows the image of cell rupturing extraction mechanism and Figure 2.7.2 images of *Chlorococcum* species before and after cell disruption executed by high-pressure homogenizer. Cell disruption is considered as an essential process for extracting intracellular entities of a cell and it has been explored extensively for various microbes of industrial importance like *Saccharomyces cerevisiae* and *Escherichia coli*. However, this process has not proved to be efficient in terms of energy and cost since the cell membranes and cell walls of microalgae are very tough. Cell disruption can be executed by four basic mechanisms i.e. physical, mechanical, enzymatic and chemical (Lee et al., 2012).

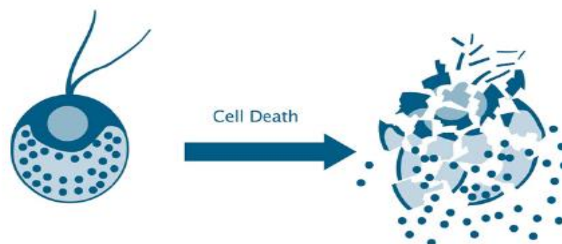

Figure 2.7.1: Schematic drawing of the extraction mechanism: cell death and rupture (Kleinegris et al., 2011).

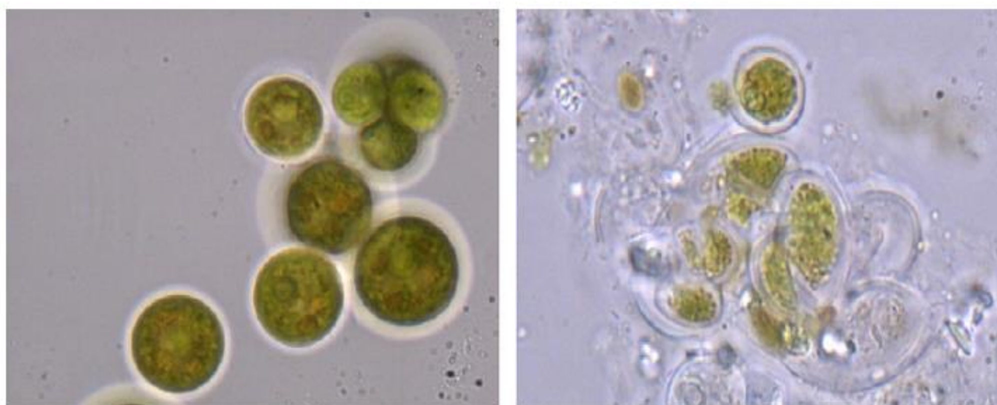

Figure 2.7.2: Microscopic images of the intact and disrupted cells of *Chlorococcum*. Cells are disrupted by high-pressure homogenizer (Halim et al., 2012).

The microalgae cells are small (<20 µm) which makes the cell disruption process through mechanical presses difficult, resulting in high energy intensive process (Surendhiran and Vijay, 2014 and Salam et al., 2016). A summary of cell disruption methods and their energy consumptions is shown in Table 2.8. The highest energy consumed (cell disruption) was from high pressure homogenization (HPH) method with 529 MJ kg^{-1}, while the lowest was from hydrodynamic cavitation method

with 33 MJ kg^{-1}. Even though the combustion energy derived from microalgae biomass is estimated around 27 MJ kg^{-1}, theoretically the minimum energy consumption should be lower (factor of 10^5) as compared to hydrodynamic cavitation method value (Salam et al., 2016). It is economically acceptable if the extracted compounds were aimed for high value and low volume products (pharmaceutical). However, it is not sustainable if the compounds (lipids) are solely extracted for biofuel production (Surendhiran and Vijay, 2014). Therefore, a more energy efficient method should be conducted in order to make the biofuel economically sustainable.

Table 2.8: Cell disruption methods and their energy consumptions.

Extraction Method	Microalgae	Energy consumption, (MJ kg^{-1} dry mass)	References
Bead mills	*Chlorella, Botryococcus, Scenedesmus,* (Laboratory, industrial)	504	(Lee et al., 2010)
Microwave	*Chlorella, Botryococcus, Scenedesmus,* (Laboratory)	420	(Lee et al., 2010)
Sonication	*Chlorococcum sp.,* (Laboratory, industrial)	132	(Halim et al., 2012)
High pressure homogenization (HPH)	*Chlorococcum sp.* (Laboratory)	529	(Halim et al., 2012)
Hydrodynamic cavitation	*Saccharomyces cerevisiae* (Laboratory, industrial)	33	(Balasundaram and Pandit, 2001)

2.5.1 Microbial Inactivation Mechanism by Ozone

Ozone in very low concentration causes inactivation of microbes in a short time (Poynter et al., 1973). It is a complicated process as various components of the cells are attacked by ozone such as peptidoglycan layer surrounding the cell, respiratory enzymes, unsaturated lipids and proteins in cell membranes, cytoplasmic nucleic acids and enzymes, capsids of viruses and peptidoglycan and proteins in the coat of bacterial spores (Khadre et al., 2001). Ozone is a highly unstable substance as it decomposes into superoxide ($\bullet O_2^-$), hydroperoxy ($\bullet HO_2$) and hydroxyl ($\bullet OH$) radicals in aqueous as well as gaseous phase. High reactivity of ozone is due to the oxidation potential of these free radicals (Manousaridis et al., 2005). Ozone causes inactivation of microbial cells by disrupting their cell membranes or cell lysis by disintegration of cells. Bacterial cells are more susceptible to inactivation by ozone as compared to fungi and yeasts. Moreover, spore forms of bacterial cells are more resistant to ozone than vegetative forms. Similarly gram positive bacterial strains demonstrate more sensitivity to ozone (Pascual et al., 2007).

The mechanism of bacterial cells inactivation by ozone is depicted in Figure 2.7. Ozone inactivates bacterial cells through oxidation reaction (Bringmann, 1954). Cell membranes of bacterial cells are first attacked by ozone and unsaturated lipids and proteins present in the membrane serve to be the prime targets (Pryor et al., 1983). Thanomsub et al. (2002) has proposed that ozone inactivates bacterial cells by destroying cell membranes leading to cell lysis. However, Cho et al. (2010) suggested that inactivation is mainly due to damage to cell surface as shown in Figure 2.7.3. It

has been established that cultivation of algae and extraction of its metabolites are significantly affected by the concentration of ozone and the competitive reactions among organic substances and toxins. Hammes et al. (2007) has determined that treatment of algal mass with ozone causes the release of extracellular organic substances. Recent research has also supported this finding by suggesting that ozonation of *M. aeruginosa* causes damage to cell wall leading to release of intracellular substances (Sharma et al., 2012).

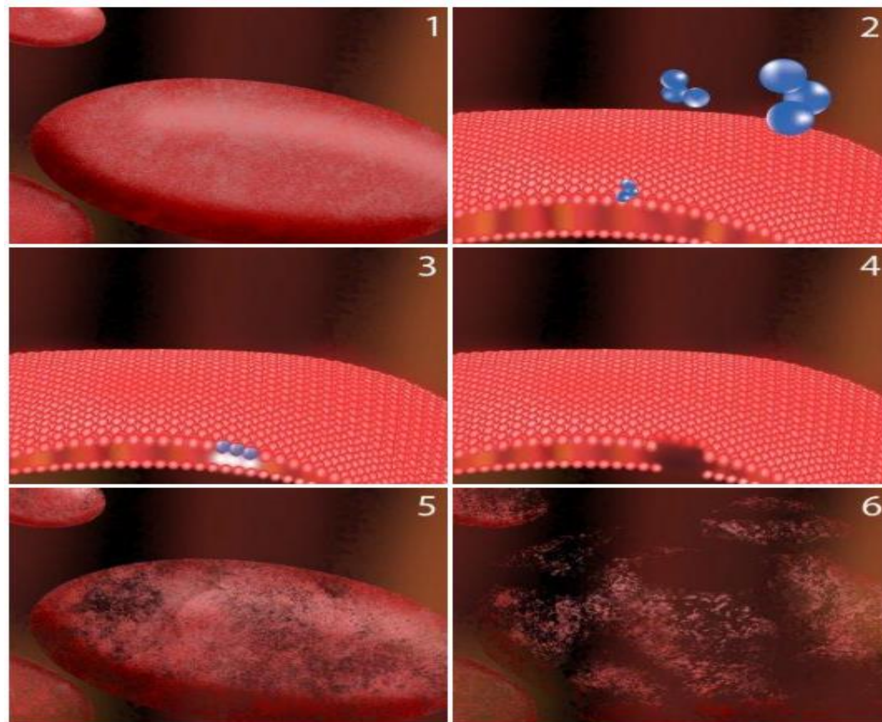

Figure 2.7.3: Effect of ozone on bacteria: (1) A normal bacterial cell, (2) Contact of ozone molecule with the cell wall, (3) A reaction (oxidative burst) occurs which creates a tiny hole, (4) A hole created in the cell wall has injured the bacteria, (5) The bacteria lose its shape while ozone molecules continues attacking the cell wall, (6) After several seconds of ozone collisions, bacterial cell lysis occurs and the cell dies. (Ozone Solution, 2016).

Ozonation has been used in food industries as a sanitation method to reduce microbial contamination and treating fruits with ozone has been reported to increase shelf life (Kim et al., 1999). Table 2.9 contains a brief overview of some studies related to inactivation of bacterial cells by ozone. Selma et al. (2007) reported that the ozonation of lettuce at 5 ppm for 5 min reduced 1.8 log units of *Shigella sonnei* colonies. Significant reductions of bacterial colonies have been reported when ozonation treatment for 3 to 5 h at 5 or 10 ppm were applied to dried figs (Oztekin et al., 2005). The ozonation treatment have been reported as an efficient method in killing vegetative cells such as *Bacillus* species (Khadre and Yousef, 2001) and *E. coli* (Akbas and Ozdemir, 2008). Furthermore, ozonation has been reported as efficient method in killing Gram-negative bacteria (such as *Yersinia enterocolitica* and *Pseudomonas aeruginosa*) and Gram-positive bacteria (such as *Staphlyococcus aureus* and *Listeria monocytogenes*) (Restaino et al., 1995). Therefore the effectiveness of ozonation treatment in controlling bacterial contamination should be further explored and utilized.

Table 2.9: Inactivation of bacteria by ozone

Bacterium	Reduction (log 10)	Ozone concentration	Time (min)	Reference
Escherichia coli	6.0	0.9g h^{-1}	45-75	(Williams et al., 2004)
Leuconostoc mesenteroides	0.7-7	0.3-3.8 μg mL^{-1}	0.5	(Kim and Yousef, 2000)
Salmonella	<5.0	0.9g h^{-1}	240	(Williams et al., 2004)
Shigella sonnei	3.7-5.6	1.6-2.2 ppm	1	(Selma et al., 2007)

2.5.2 Ozone

A European scientist Schonbein discovered ozone in 1839. It was 1907, when ozone was commercially employed for treating the municipal water supply in Nice. Later on, in 1910, it was used in St. Petersburg for the same purpose (Guzel-Seydim et al., 2004). In 1983, it was used for treating drinking water in the Netherlands. Ozonation has been widely used in Europe for disinfection of drinking water for past several decades. However, it took a long time to be employed in United States. In the United States, ozone was basically used for different purposes other than disinfection such as removing colours or controlling odour and taste (Robson et al., 1990). Later on, various commercial uses of ozone were recognized such as treatment of waste water, protecting cooling towers from fouling and disinfection of bottled water and swimming pools (Guzel-Seydim et al., 2004). Ozone has a number of industrial applications. Some uses of ozone in chemical, food and other industries are given in Table 2.9.1.

Ozone is an unstable gas having high oxidation potential. It appears faintly blue; however, at room temperature it is colourless. It has pungent smell. Molecule of ozone is triangular in shape and has molecular weight of 47.9982. Bond angle of ozone is 117° and bond length is 0.128 nm. At temperature lower than -193 °C, it gives rise to a deep blue-violet solid. In liquid form it is indigo blue in colour and highly explosive at temperature below -112 °C (Becker, 2005). Ozone should be generated by application of energy to dried air or oxygen at the time of use. Ozone has proved to be the most potent oxidant after free hydroxyl radical. It can oxidize a variety of inorganic and organic substances in aqueous environment (Li, 2004). Reaction cycle comprises of 3 main stages i.e. initiation,

propagation and termination. It usually starts with the reaction between ozone and a hydoxide ion (OH^-) or any other entity like H_2O_2 and UV. Decomposition of ozone, once initiated, is self-sustaining and quick and lasts until any other entity reacts with hydroxyl radical (OH•) and does not give rise to a radical (Glaze, 1986 and Beltrán, 2004). The reaction cycle of ozone decomposition in aqueous solution is shown in Figure 2.8.1.

Table 2.9.1: Application of ozone in industries
(Becker, 2005 and Tiwari et al., 2010).

Food industry	Chemical industry	Other industries
- Food preservation	- Oxidizing agent in the organic chemical industry	- Disinfectant agent in drinking water and air
- Shelf-life extension	- Bleaching flour, paper pulp, starch, and sugar	- Treating industrial wastes
- Equipment sterilization	- Processing certain perfumes, vanillin, and camphor	- Deodorizing of feathers, air and sewage gases
- Improvement of food plant effluent	- Rapid drying of varnishes and printing inks	- Bactericide
- Disinfectant agent of food in cold storage rooms	- Producing peroxides	- Producing steroid hormones
- Food preservative for cold storage of meats	- Removal of chlorine from nitric acid	- Grain processing
- Inhibit the growth of yeast and mold in fruit storage		- Extraction of some heavy metals from soil

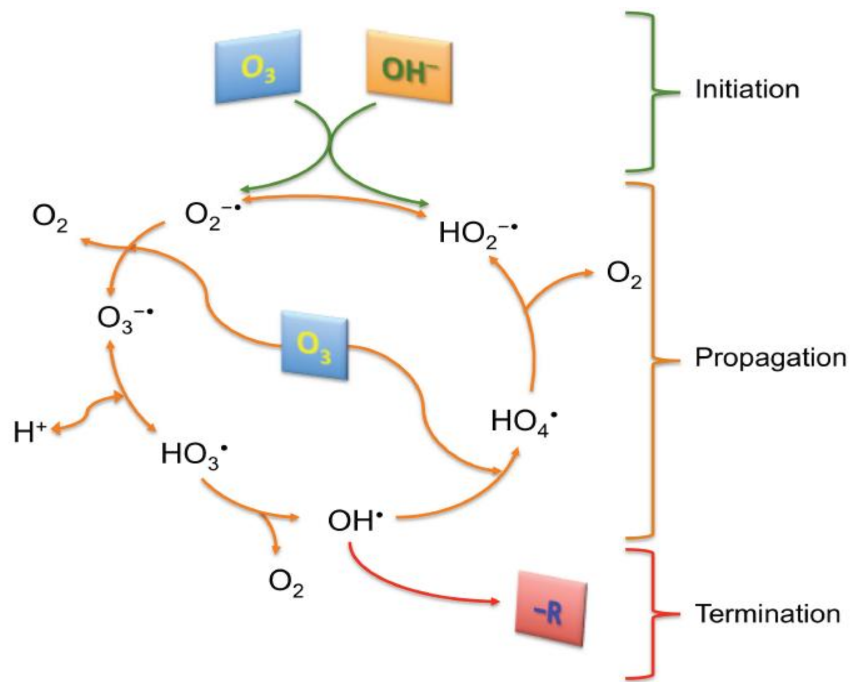

Figure 2.8.1: Generalized ozone decomposition pathway in aqueous solution (Beltrán, 2004).

2.5.3 Generation of ozone

Since handling, storage and transportation of ozone is difficult, it is always produced at the site of use (Becker, 2005). Free oxygen radicals are generated when the strong O-O bonds are broken by using high amount of energy. Ozone can be produced by using corona discharge techniques and ultraviolet radiation for starting formation of free oxygen radicals. Beside photochemical methods based on using ultraviolet radiation, ozone can also be generated by other techniques like electric discharge methods, thermal, chemical, electrolytic and chemonuclear techniques (Kim et al., 1999).

Majority of the commercially available ozone generators are based on corona discharge technique for ozone generation. During this technique, oxygen or dried air is allowed to pass between two electrodes. High voltage is applied to the electrodes which are separated by a dielectric medium mostly glass. It is important that concentrated oxygen or air should not contain particulate substances and must be dried to a dew point of at least -60 °C so that the corona discharge system remains protected. In the case of concentrated oxygen, the ozone/gas mixture released from the ozone generator comprises of 3-6% ozone, whereas, when dry air is used as the feed gas, the proportion becomes 1-3% (Muthukumarappan et al., 2000).

The process of ozone generation using an ambient air ozone generator is shown in Figure 2.8.2. A corona discharge is formed between the electrodes when a high voltage is applied to them resulting in conversion of the discharge gap to ozone. Hence the corona discharge can be characterized as a physical process during which a gas present in the gap of a voltage gradient is electrically discharged. First, atomic oxygen is formed by splitting molecular oxygen. Then atomic oxygen reacts with oxygen molecules resulting in formation of ozone (O_3) (Muthukumarappan et al., 2008).

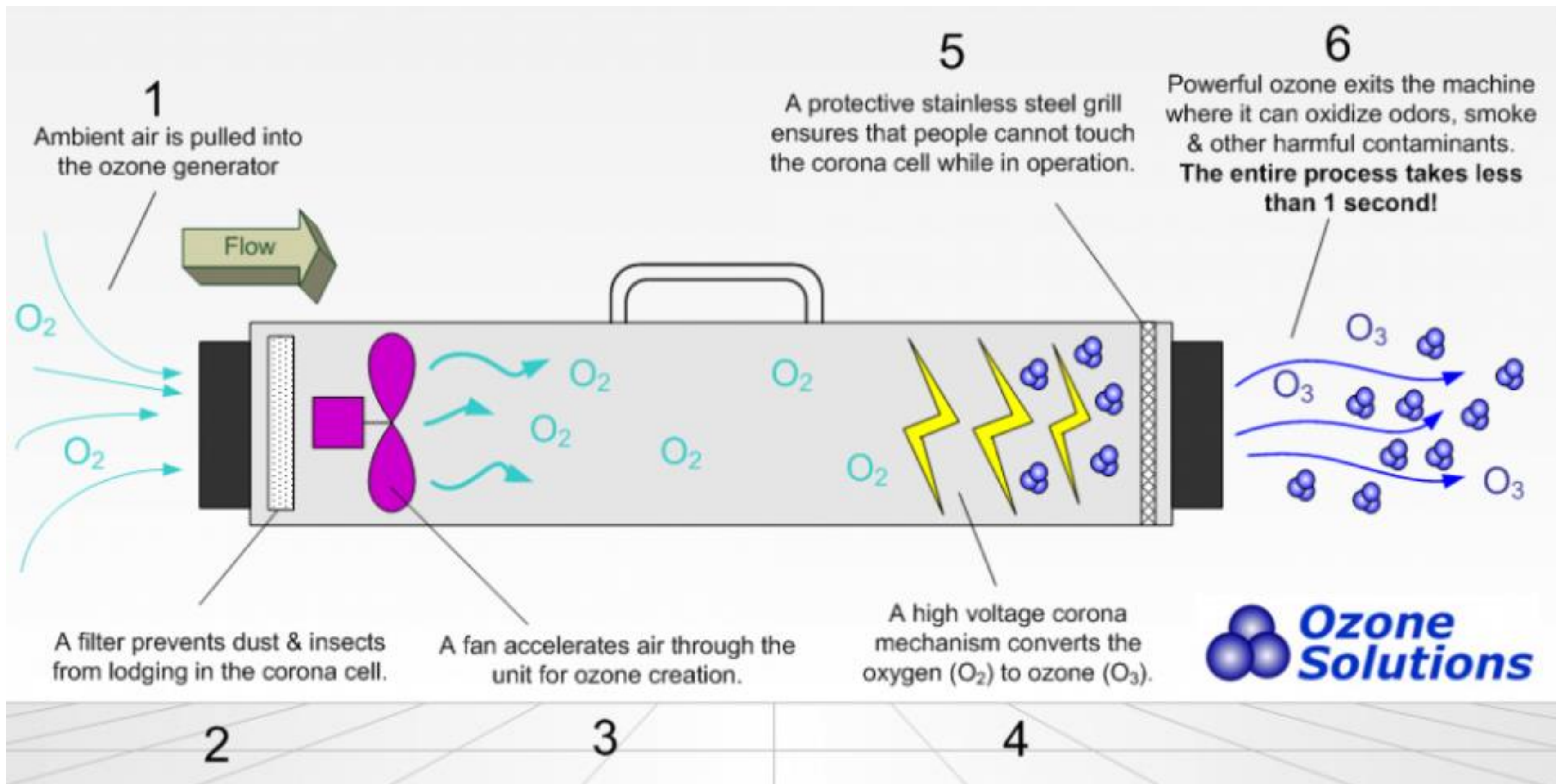

Figure 2.8.2: Process of generating ozone from an ambient air ozone generator ("How ozone generators work," 2016).

2.6 Introduction of Novel Microbubble Bioreactor

Earlier, Zimmerman et al. (2008) presented a novel oscillator together with a microchip and micro porous diffuser. It was shown to be capable of delivering good results in microbubble generation. The main biological concerns relating to inhibition by oxygen, CO_2 efficiency and mixing were significantly resolved by using microbubbles (Zimmerman et al., 2011). The reason behind this is the high mass transfer and prolonged holdup of the gas by microbubbles. Carbon dioxide is considered to be dissolved quickly and adequately leading to increase in algal biomass and decreased release of CO_2 in the atmosphere in case of commercial applications. In addition to resolving the problem of oxygen inhibition, microbubbles are capable of extracting dissolved O_2 because of its high mass transfer and the O_2 gradient existing between the phases. Hence, O_2 is brought to the surface of water and released into the atmosphere by these bubbles (Zimmerman et al., 2008). A microbubble diffuser, an air source and a fluidic oscillator constitute the instrument for microbubble generation.

Apart from that, a miniature plasma reactor capable of operation at low power has been developed recently. According to an estimate, ten times less power was required as compared to the conventional ozone generators for generating the same quantity of ozone. Earlier, a high voltage was required by the corona discharge generators for the dissociation of oxygen. It has been established that the efficiency of ozone production by negative coronas ranges between only 16-20% as described by Lozano-Parada and Zimmerman (2010). However, the device produced low concentration of ozone during stable state. Increasing the power will lead to unstable plasma generation and cause arcing. Thus, a conventional ozone generator was used in this research. A

combination of both technologies was therefore integrated resulting in development of an ozone-rich microbubble bioreactor system which has not been reported in the past. A novel airlift loop photobioreactor would be developed in this research project. This system will allow switching of microbubble distribution from a nutrient gaseous medium like CO_2-rich (stack gas) to air introduced by the ozone generator to distribute ozone.

2.6.1 Microbubbles generated by fluidic oscillation

Microbubbles having a diameter of 1-999 μm have been employed for various industrial purposes as their surface to volume ratios are quite high as compared to fine bubbles whose diameter ranges between 1-2mm (Tesarˇ, 2013). This high surface to volume ratio is responsible for increased mass transfer as well as mixing rate efficiencies due to the upward and downward flow of the gas/liquid mixture when performed in airlift loop bioreactor (Bangert, 2013). Microbubles are usually formed by three methods. There are: 1) compressing air by high pressures to dissolve it in liquid phase, 2) through ultrasonic waves and 3) low power microfluidic method comprising of flow focusing, fluidic oscillation and porous substance (Zimmerman et al., 2008). It has been reported by Zimmerman et al. (2008) that microbubbles with ten times smaller diameter can be produced with fluidic oscillation technique in comparison with other conventional techniques. It also caused considerable reduction in energy requirement. Since this technology comprises of immobile parts leading to reduced cost of maintenance, it is appropriate for industrial applications (Lozano-Parada and Zimmerman, 2010). Figure 2.9 illustrates the production of microbubbles by oscillator at different flow frequency.

Zimmerman together with his co-workers was the pioneer developer of a fluidic oscillator instrument combined with airlift loop bioreactor for the production of microbubbles (Zimmerman et al., 2008). The fluidic oscillator generates microbubbles all with the size of the pore, when it is connected to a suitable frequency of air. Benefits of using the fluidic oscillator are many including the cost-effectiveness, robustness, reliability, immobile parts and no requirement of electricity (to produce flow frequency) (Zimmerman et al., 2011). Capacity of this device for diverting the jet passing through the supply nozzle is regulated by terminal X_1 and X_2 as shown in Figure 2.9.1 and Figure 2.9.2 shows an image of an actual fluidic oscillator (FO). A remarkable feature of this system is that the frequency of the oscillation is adjustable through alteration in the air flow rate and the length of the feedback loop (Tesarˇ et al., 2006).

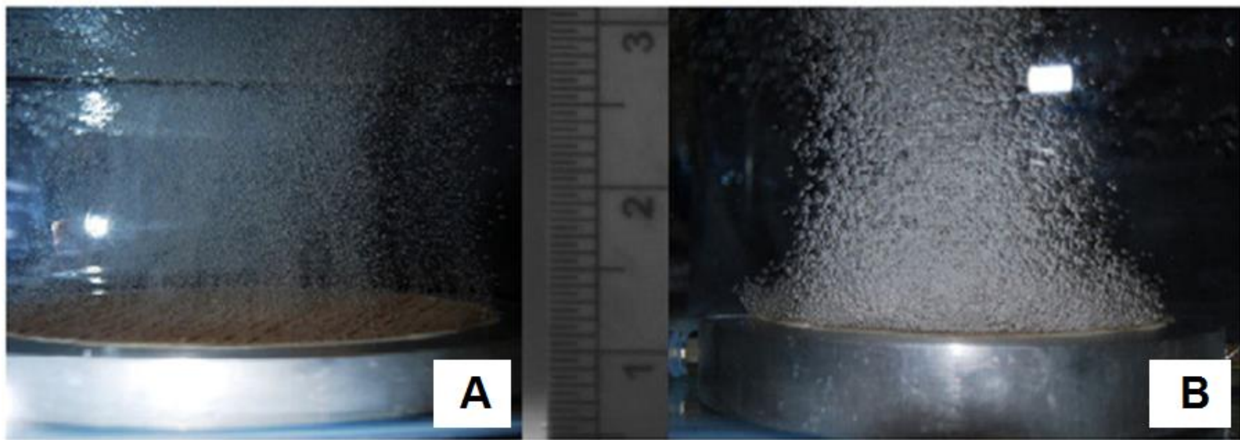

Figure 2.9: Microbubbles generated by fluidic oscillator (FO). A) Microbubbles generated by fast oscillation, B) Steady flow microbubbles.

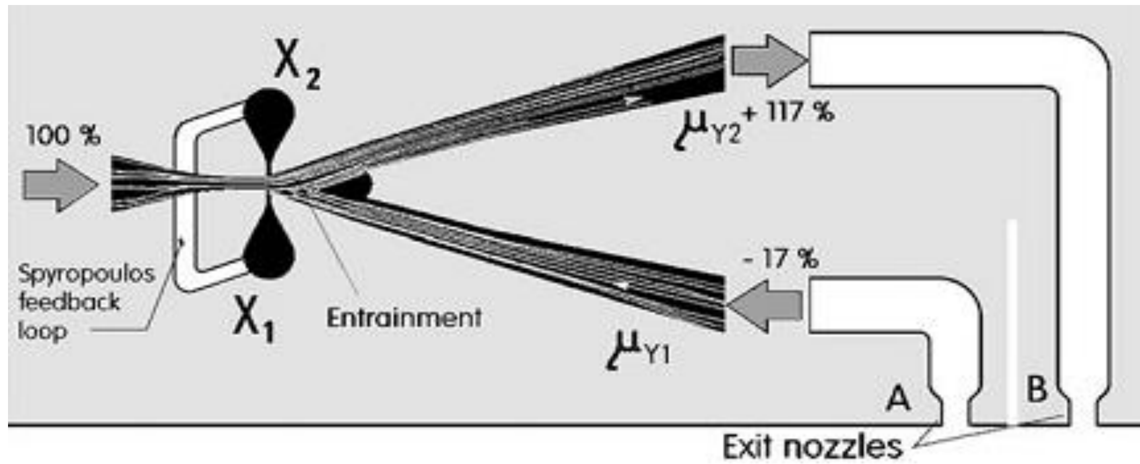

Figure 2.9.1: Schematic design of fluidic amplifier (Zimmerman et al., 2008)

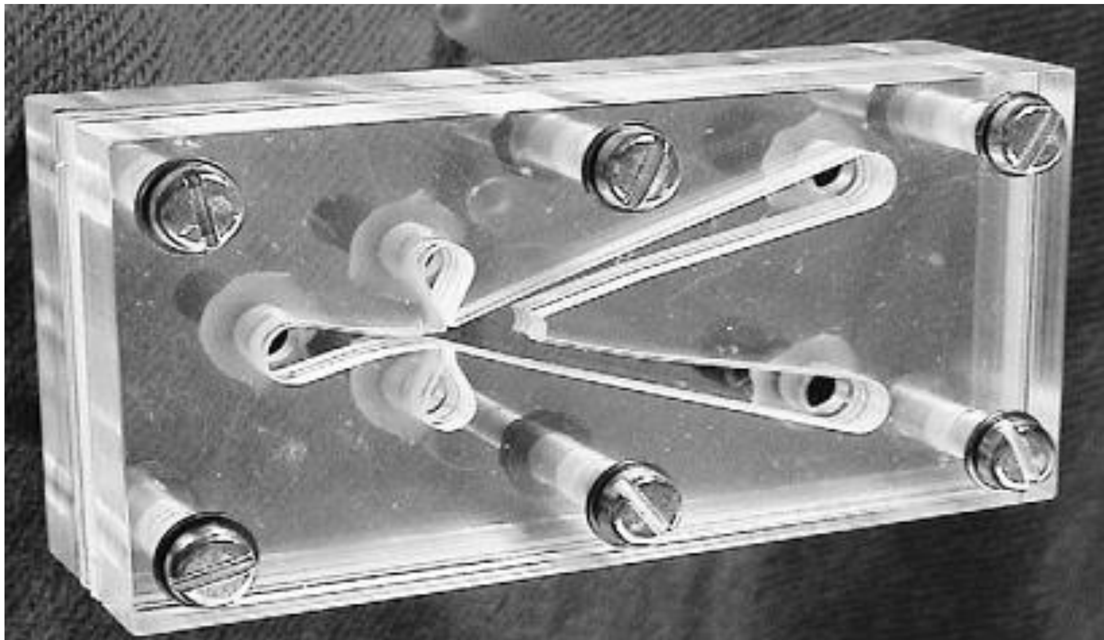

Figure 2.9.2: Actual picture of fluidic amplifier (Zimmerman et al., 2008)

CHAPTER 3

GENERAL MATERIALS AND METHODS

This chapter focuses on the experimental design and experimental techniques used. The research design and methodology are described in detail at the beginning of this chapter. It explains all of the steps or stages that must be done in this research. This is followed by a description of the experimental setup of small bioreactor (0.1 L), 2 L airlift-loop photobioreactor and 0.2 L ozonation extraction reactor. Finally, brief descriptions are given of the experimental techniques and medium preparation that were used in this research.

3.1 Research Design and Methodology

Figure 3.1 summarizes the research design and methodology of this study. There are four main parts involved. First, the sterility of the bioreactor was studied in a small-scale bioreactor (0.1 L). By using an ozone generator, the culture inside the bioreactor was ozonated (bubbling) with a certain concentration of ozone and the visible growth of the microorganism was calculated (colony forming unit, CFU). For the second part, the effects of ozonation on the cell-culture morphology was studied in detail under a scanning electron microscope (SEM). This is essential to determine whether ozonation can become a method for disruption of microalgae cell culture. Response-surface-methodology (RSM) software was used to study the relationship of all

parameters and to optimize the algal cell disruption rate by ozonation. Finally, optimization of the lipid-extraction process by ozonation (algal slurry in methanol) at different temperatures and with or without a fluidic oscillator was studied in a 0.2 L ozonation-extraction reactor.

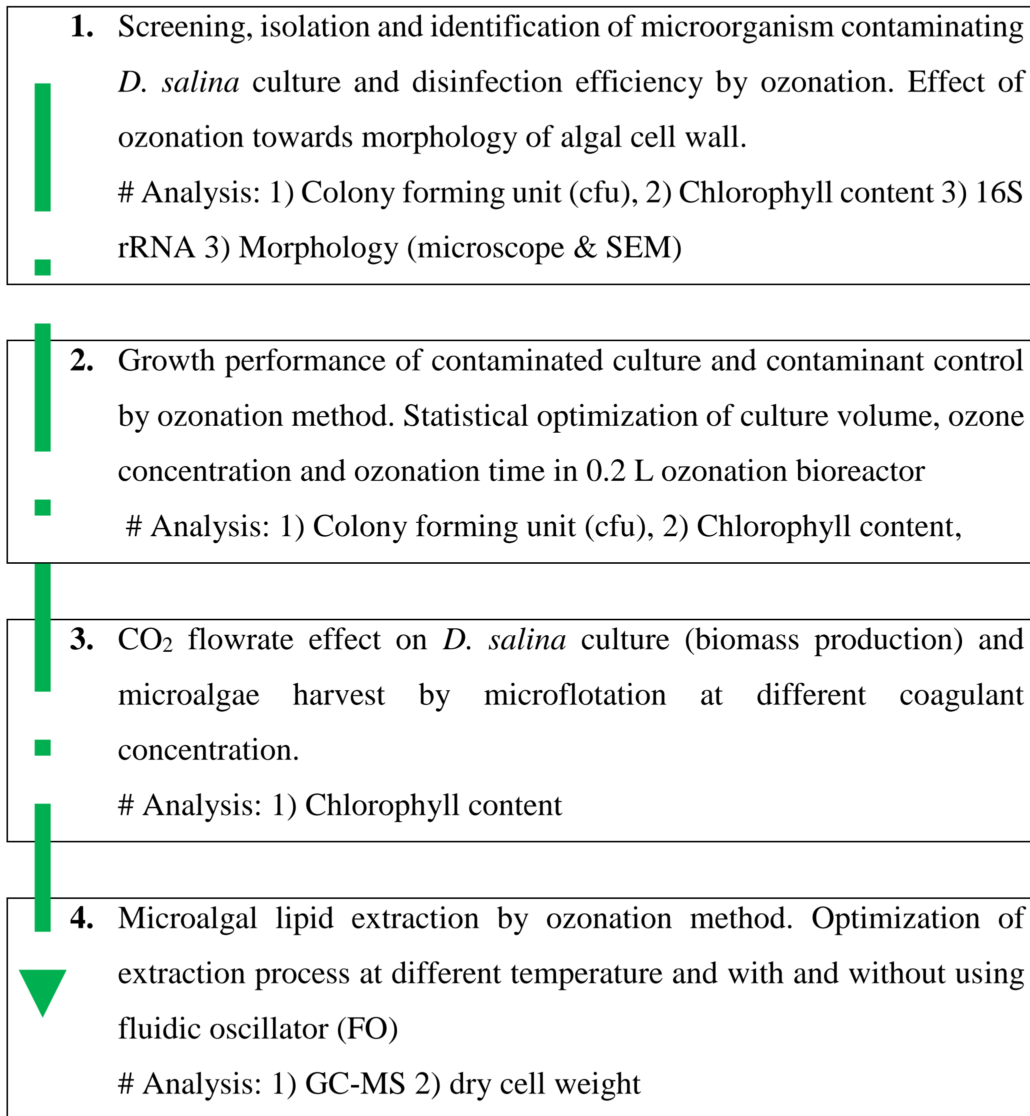

Figure 3.1: Experimental design

3.2. Microalgae culture and bioreactor set-up

The *Dunaliella salina* strain 19/30 used in the study was obtained from the Culture Centre of Algae and Protozoa, Oban, UK. The culture was grown for 14 days in 250 mL shake flask with 100 mL of working volume during preparation of inoculum. While for the mass production, the strain was grown in 2 L photobioreactor with 1.5 L working volume and both cultivation methods used artificial seawater as the culture medium (Zimmerman et al., 2011). A 10% (v/v) inoculum size (14 days old) was used in all *D. salina* culturing processes. A mixture of 5% CO_2 and 95% N_2 was directed into the photobioreactor for 30 minutes every day to serve as a carbon source and agitation. The main reason 5% CO_2 and 95% N_2 gas is used is to mimic the stack gas (flue gas) composition. The photobioreactor is equipped with 20 µm ceramic diffuser which reported to produce an average size of 600 µm in diameter of microbubbles (Ying et al., 2013). Continuous illumination of the shake flasks and photobioreactor cultures was accomplished using a fluorescent lamp at 90 µmol quanta m^{-2} s^{-1}; this measurement was obtained using a quantum sensor (Hansatech Instrument Ltd., UK). The experimental set up and parameters were based on previous studies conducted by Ying et al. (2014), as it produced the highest algal biomass concentration. The *D. salina* cultures were maintained at room temperature (23-25 °C).

Generally, there were 4 different bioreactors employed in this study (Figure 3.2). Firstly, in the study of contaminant effects on algal growth performance, 0.1 L small bioreactor equipped with 16 µm sintered glass diffuser was used to perform the 10 min ozonation. Secondly, 2 L airlift loop bioreactor (ALB) equipped with 20 µm ceramic diffuser was used to study the different gas flowrate effects towards *D. salina* growth performance. Then, 1 L microflotation bioreactor

equipped with 50 µm steel mesh diffuser was used during harvesting of the algal cells. Lastly, the cell disruption and lipid extraction equipped with 16 µm sintered glass diffuser was performed in 0.2 L ozonation extraction bioreactor. The piping and instrumentation schematic for the novel bioreactor rig consisting of the airlift loop bioreactor (ALB) and microflotation units is shown in Figure 3.3. While the processing scheme from cultivated microalgae to algal lipid extraction is illustrated in Figure 3.4.

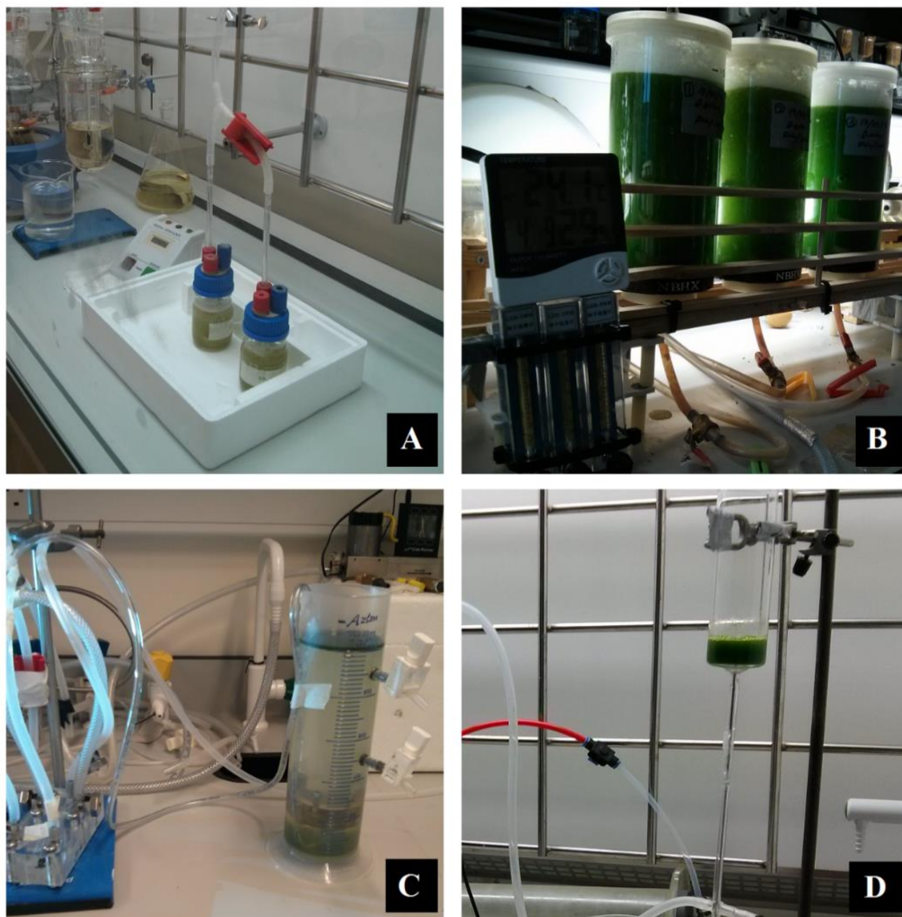

Figure 3.2: Bioreactor set-up: A; 0.1 L small bioreactor (disinfection), B; 2 L airlift loop bioreactor (biomass production), C; 1 L microfloatation bioreactor (harvesting), D; 0.2 L ozone extraction bioreactor.

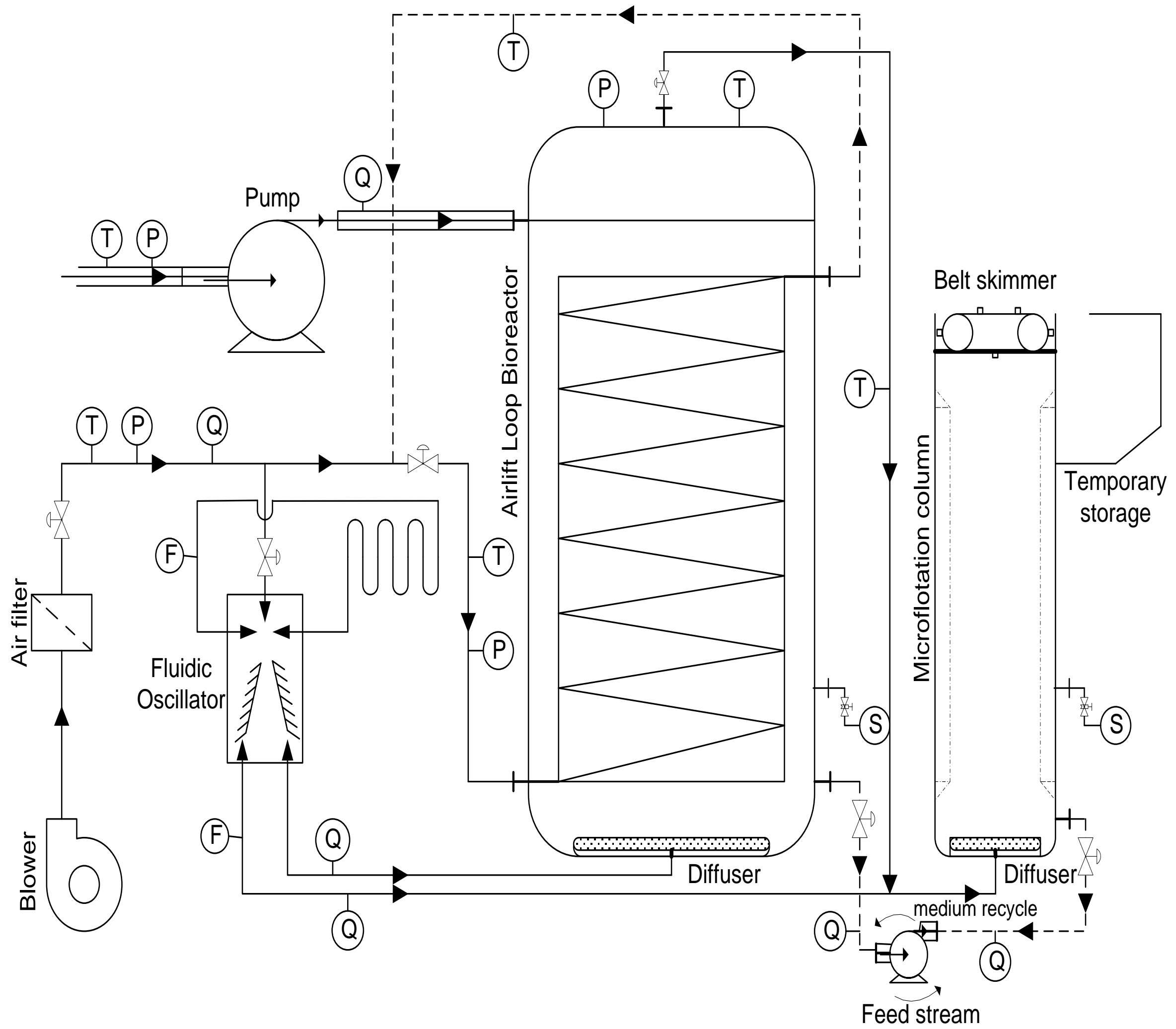

Figure 3.3: Piping and instrumentation schematic for the novel bioreactor rig for biomass production. The main units in the production system are the Airlift Loop Bioreactor (ALB) and the Microflotation (MF) units. Where Q, T P and S represent Air Flowrate, Temperature, Pressure and Sampling port respectively. The zig zag line is a heat exchanger pipe around the draft tube.

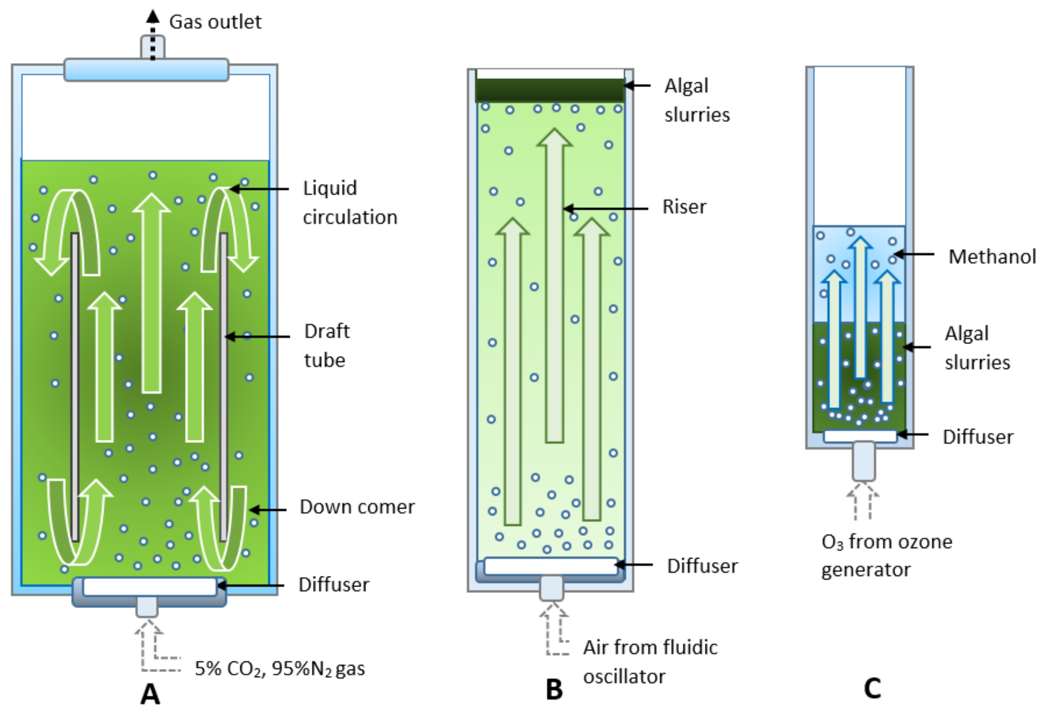

Figure 3.4: Processing scheme from cultivated microalgae to algal lipid extraction. A; 2 L airlift loop bioreactor (ALB), B; 1 L microfloatation bioreactor and C; 0.2 L ozone extraction bioreactor.

3.3. Screening of contaminants and disinfection efficiency

The screening and isolation of contaminants was accomplished using two methods: the spread plate and streak plate techniques. First, a 100 µL sample was collected from an old microalgal culture (>3 months) due to higher contamination rate and transferred onto a 1 M NaCl nutrient agar plate. The purpose of using 1 M NaCl in the nutrient plate agar is to simulate the high saline condition of the *D. salina* growth medium. Then, the sample was spread evenly using a glass spreader. The plates were incubated in a 25 °C growth room for 3 to 4 days. Visible contaminants

were transferred onto new fresh plates via the streak technique to allow the identification of contaminants. The process of screening and isolation of contaminants is shown in Figure 3.5.

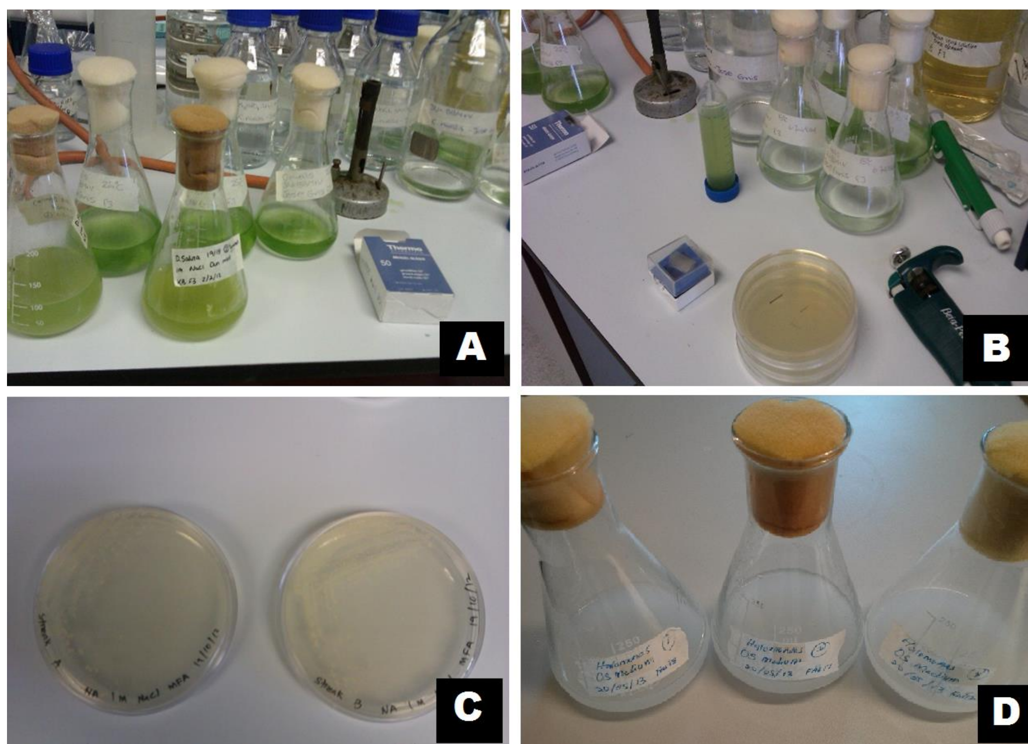

Figure 3.5: A screening and isolation of contaminants: (A) A matured *Dunaliella salina* culture in 250 mL shake flask, (B) The plates after spread plate technique, (C) Two pure colonies isolated and named as strain A and Strain B, and (D) The isolated bacteria were cultivated in 250 mL shake flask.

The disinfection efficiency was determined by mixing 50 mL of bacterial culture (5 days old) with 50 mL *D. salina* culture (14 days old) in 0.1 L small bioreactor and ozonated for 10 min. Five mL samples was taken for chlorophyll content analysis while 100 μL samples (after serial dilutions) were pipetted onto agar plates and left in the dark at room temperature for 3-5 days. The

disinfection efficiency was determined by counting colony forming units (CFU) on the initial nutrient agar spread plates containing 1 M NaCl. The experiments including the controls were conducted in triplicate. Finally, by using the formulation as shown below, the disinfection efficiency was calculated.

$$S\,(\%) = \frac{No - N1}{No} \times 100$$

Where N_o is bacterial colonies (CFU) before ozonation and N_1 are the numbers of bacterial colonies (CFU) after the ozonation.

3.4. Identification of bacterial contaminants

Identification of bacterial contaminants was achieved using 16S rRNA gene sequencing. DNA was extracted using the Qiagen DNA purification kit. The PCR was performed using 16S gene universal primers (forward, AGAGTTTGATGCTCAG and reverse, GGTTACCTTGCGACTT). The sequencing was performed by Eurofins Genomics (*http://www.eurofinsgenomics.com*). A BLAST (Basic Local Alignment Search Tool) search (*blast.ncbi.nlm.nih.gov)* was performed using the obtained partial 16S rRNA sequence as a query against the complete ribosomal database project library.

3.5. Chlorophyll content and specific growth rate determination

A 15 ml Falcon tube containing a 5 mL microalgal sample was subjected to centrifugation (Hettich Universal 320, UK) at 3000 rpm for ten minutes to separate the cells. The pellet was resuspended in 1 mL of distilled water after the supernatant discarded. Subsequently, 4 mL of acetone was added to every tube and adequately mixed by vortexing. The tubes were subjected to full-speed centrifugation at 15000 rpm for five minutes, and the process was repeated until the pellet became entirely white. The spectrophotometer was zeroed using acetone prior to the measurement of the supernatant's optical density at 645 nm and 663 nm (OD_{645} and OD_{663}). The experiments including the controls, were conducted in triplicate. The chlorophyll content was calculated using the following equation:

$$\text{Chlorophyll concentration } (\mu\text{g/mL}) = \frac{OD_{645} \times 202 + OD_{663} \times 80.2}{2 \times 5}$$

The specific growth rate (μ·day^{-1}) was calculated based on method described by Levasseur et al. (1993) using the following equation:

$$\mu = \frac{\ln(c_2 / c_1)}{(t_2 - t_1)}$$

Where c_1 and c_2 are chlorophyll concentrations (µg/mL) at time intervals t_1 and t_2 (day).

3.5.1. The growth performance of contaminated culture

The investigation of the impact of the bacterial contaminant on algal biomass development was conducted using 250 mL shake flask cultures with 100 mL working volume and 10% (v/v) inoculum size. The contaminated old culture (>3 months) contains approximately $3.33 \pm 1.5 \times 10^3$ CFU. However *Halomonas* cells at low concentration do not affect the algal growth much and grew together with algae due to limitation of carbon source. Thus, the introduction of higher *Halomonas* cell concentration at the beginning of cultivation is essential in order to create the worst case scenario of contamination. *Halomonas* bacteria was grown using induced *D. salina* media (2% (w/v) of glucose) in 250 mL shake flask (100 mL working volume) for 3 days at room temperature. The initial number of *Halomonas* bacteria was approximately 5×10^7 CFU mL^{-1} ($5.08 \pm 1.27 \times 10^7 \approx 5 \times 10^7$ CFU mL^{-1}, $OD_{600} = 0.7$). Thus, we assumed that approximately 1×10^6, 2×10^6, 3×10^6, 4×10^6 and 5×10^6 CFU mL^{-1} of *Halomonas* will be present in concentrations of 2%, 4%, 6%, 8% and 10% (v/v) bacterial contamination. This experiments were conducted in small scale to prove that ozonation can control or reduce bacterial contamination during production stage. However, further studies on controlling bacterial contamination in large scale by ozonation should be conducted.

After the introduction of the bacterial contaminant (depending on the concentration) with 10% (v/v) 14 day old *D. salina* cells in 100 mL culture medium at the beginning of the experiment, the heterogeneous culture (*D. salina* and *Halomonas*) was left to develop for 14 days at room temperature (23-25 ˚C). The continuous illumination of the cultures were accomplished using a fluorescent lamp at 90 μmol quanta m^{-2} s^{-1}. On the other hand, the effect of ozonation towards

heterogeneous culture was investigated by bubbling 8 mg L^{-1} of ozone for 10 minutes on day one. The ozonation was performed in 0.1 L small bioreactor and conducted under sterile conditions. Then, the culture was transferred to 250 mL shake flaks and left to develop (same conditions with heterogeneous culture without ozonation treatment were applied). The experiments including the controls, were conducted in triplicate.

3.6. Microflotation harvesting

After accumulation, the *D. salina* biomass was harvested via microflotation (Hanotu et al., 2012). A litre of the *D. salina* culture was obtained (diluted to 1.00 OD_{682}) and pretreatment was performed using aluminium sulphate as a coagulant. A flocculator (Stuart, UK) was used to induce rapid mixing at a speed of 250 rpm for 10 min to ensure particle contact with the aluminium sulphate. Thereafter, the mixing speed was reduced to 100 rpm for 5 min to allow the interaction of the particles and the growth of the floc. After this step, the sample was transferred to the one litre microflotation column. According to Hanotu et al. (2012) microflotation is a fluidic oscillator-driven system of flotation. The microflotation rig is fitted with a steel mesh diffuser with 50 µm-sized pores producing microbubbles with an average size of 100 µm (Hanotu et al., 2013). Due to limited availability of algal culture, every experiment was run in duplicate for 12 minutes. Samples were collected every 2 minutes to assess the recovery efficiency using the DR 2800 spectrophotometer (HACH Lange, UK) to evaluate the associated absorbance at 682 nm. The algal layer on top of the reactor was scooped out into 50 mL centrifuge tube and stored in 4 ˚C

refrigerator prior to use in cell disruption and extraction by ozonation. The actual pictures of the whole microflotation process are shown in Figure 3.6 and Figure 3.6.1.

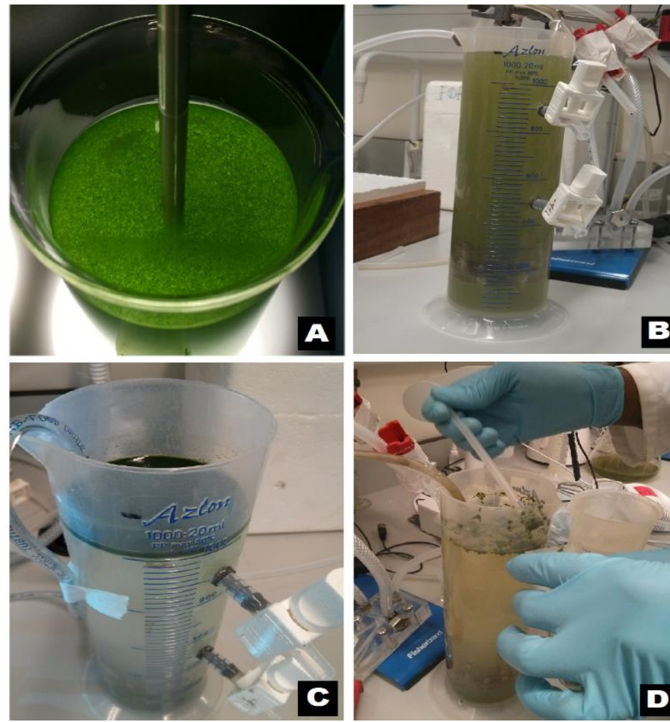

Figure 3.6: Harvesting of *D. salina* culture by microflotation process. A) Mixing coagulant with algal culture, B) 1 L microflotation bioreactor, C) Algal slurry floating on top of the culture, D) Collection of the algal slurry by scooping out using spatula.

Figure 3.6.1:

A flocculator (Stuart, UK) to induce rapid mixing to ensure particle contact with the aluminium sulphate.

3.7. Cell disruption and lipid extraction by ozonation

Approximately, 5 mL of algal slurries were obtained from every 1 L of *D. salina* culture harvested by microflotation process. The cell disruption process was performed in a 0.2 L ozonation bioreactor equipped with a glass diffuser with a pore size of 16 µm which can produce an average size of 46 µm in diameter of microbubbles at 0.1 L min^{-1} of air (Kokoo, 2015). Firstly, 10 mL of microalgal slurries were mixed with 20 mL of methanol (1:2 v/v) and the ozonation process was performed at 8 mg L^{-1} for 20, 40 and 60 minutes. Due to limited availability of algal slurries, the experiments were conducted in duplicate. The air flow rate was 0.1 L min^{-1} to ensure that it produced the smallest microbubbles. After the ozonation process, 1 mL of the sample was transferred to 15 mL centrifuge tube. One mL of chloroform was added to the tube and inverted twice (gentle mixing) prior to centrifugation (Hettich Universal 320, UK) at 1000 rpm for 10 min to separate the solvent, water and algal cells.

The separation method is based on Bligh and Dryer (1959), with modification to limit the extraction to easily accessible lipids. The bottom layer containing the products in chloroform was transferred to 2 mL centrifuge tube (Eppendorf). The chloroform was evaporated by leaving the tube in the fume hood (air dried) at room temperature (>24 hours). However, drying under nitrogen is recommended and will be applied in future studies as air drying may cause some oxidation problems. Lastly, 1 mL of methanol was added to dissolve the pellet and transferred to 2 mL glass vial with cap prior to GC-MS analysis. No catalyst (acid) was involved in any step in order to study the potential of direct esterification by ozonolysis.

3.7.1. Gas Chromatography-Mass Spectrometry (GC-MS)

The identification of the main fatty acids and products present in the ozonated mixture, was accomplished by the gas chromatography mass spectroscopy (GC-MS). The GC-MS employed were an AutoSystem XL Gas Chromatograph, Perkin Elmer and a TurboMass Mass Spectrometer (Perkin Elmer) built in with a Zebron ZB-5MS GC capillary column. The software (Perkin Elmer's Turbomass) that linked to a NIST database was used to identify the GC-MS chromatogram peaks. Several main compounds detected with high probability were reconfirmed by comparing their retention times to GC-MS standards bought from Sigma Aldrich (UK). All the standard chemicals chosen in the table were based on the highest probability suggested by the NIST database. The settings highlighted below were used for the analyses: Auto sampler method: 2 µL injection volume; 2 µL pre-injection solvent washes; 6 µL post-injection solvent washes; Split mode: 20:1 µL; Temperature Program: 60 to 300 °C; Ramp 1: 5 to 300 ˚C min^{-1}; Carrier gas flow: constant 20 ml min^{-1} He gas.

Table 3.1: The standard chemicals used for product confirmation

Compound detected (NIST database)	Molecular formula	Group	Standard chemicals (confirmation)
1-Hexadecene $C_{16}H_{32}$	$C_{16}H_{32}$	Alkene	Sigma Aldrich, UK
8-Heptadecene + isomers	$C_{17}H_{34}$	Alkene	Sigma Aldrich, UK
Hexadecanoic acid $C_{16}H_{32}$	$C_{16}H_{32}O_2$	Fatty acid	Sigma Aldrich, UK
2-Pentadecanone-6,10,14-trimethyl	$C_{18}H_{36}O$	Hexahydrofarnesyl acetone	Sigma Aldrich, UK
Phytol + isomers	$C_{20}H_{40}O$	Acrylic diterpene alcohol	Sigma Aldrich, UK
Octadecanoic acid	$C_{18}H_{36}O_2$	Fatty acid	Sigma Aldrich, UK

3.7.2. Scanning Electron Microscopy (SEM) sample preparation

After processing for a period of 3 hours at a temperature of 4 °C in 2-3% glutaraldehyde in 0.1 M sodium phosphate, the specimens were washed twice at 4 °C in 0.1 M phosphate buffer at ten minutes intervals. Then, the specimens were suspended for 1 hour at ambient temperature in 1-2% aqueous osmium tetroxide. Sample dehydration was subsequently undertaken using a consecutive series of ethanol gradients (75%, 95% and 100%) for a period of 15 minutes; the samples were left to dry for 15 minutes in 100% ethanol over anhydrous copper sulphate. Afterwards, the specimens were introduced to an equal-part solution of 100% ethanol and 100% hexamethyldisilazane for half an hour and then 100% hexamethyldisilazane for another half hour before being left to dry overnight. The dry samples were affixed onto carbon sticky stubs measuring 12.5 mm in diameter and covered with approximately 25 nm of gold using an Edwards (UK) S150B sputter coater. Finally, the samples were examined in a Philips (UK)/FEI XL-20 scanning electron microscope (SEM) at a 20 KV accelerating voltage.

3.8. Ozone generation and measurement

Ozone was generated by a Dryden Aqua ozone generator (corona discharge type) connected by silicone tubing to a glass diffuser type 4 with a pore size of 16 µm**.** These type 4 diffusers will produce an average size of 46 µm microbubbles at 0.1 L min^{-1} of air (Kokoo, 2015). The actual picture of ozone generator volume control is shown in Figure 3.7. The ozone concentration in the liquid phase is determined by the potassium iodide titration method proposed

by Eaton et al. (1999). According to Rakness et al. (1996), these procedures have previously been used in many water treatment plants and are relevant for all O_3 concentration ranges. Moreover, these iodometric titrations are suitable for both phases, are inexpensive, and the detection limit is dependent on the system (Gottschalk et al., 2009). First, a constant flow rate of ozone gas passes through a solution containing a certain concentration of potassium iodide. The products react with sodium thiosulphate ($Na_2S_2O_3$) to produce a pale yellow-coloured solution. Then, starch solution is added and a titration is conducted until the blue colour fades. All experiments were conducted in triplicate. Finally, the concentration of ozone is calculated as follows:

$$\text{Ozone concentration (mg/L)} = \frac{24 \times Vs \times Ns}{V}$$

V is the volume of air bubbled (L), V_s is the volume of sodium thiosulfate (mL), and N_s is the normality of sodium thiosulfate (mg/me). The ozone generator calibration at minimum, medium and maximum volume setting is summarized in Table 3.2.

Table 3.2: Ozone generator calibration at minimum, medium and maximum volume setting. The medium setting of ozone generator producing 7.68 ± 0.48 ≈ 8 mg L^{-1} was chosen as it produced consistent ozone concentration within 60 minutes.

Volume setting	**Gas flow at 0.1 L min^{-1}**	**Gas flow at 0.2 L min^{-1}**
Maximum	9.63 ± 0.06 ≈ 10 mg L^{-1}	22.08 ± 2.54 ≈ 22 mg L^{-1}
Medium	7.68 ± 0.48 ≈ 8 mg L^{-1}	12.00 ± 2.49 ≈ 12 mg L^{-1}
Minimum	5.92 ± 0.28 ≈ 6 mg L^{-1}	9.60 ± 1.66 ≈ 10 mg L^{-1}

Figure 3.7: Ozone generator volume control (minimum, medium and maximum).

3.9 Statistical Analysis using Response Surface Methodology (RSM)

The process of optimization of chlorophyll reduction (algal cell disruption) by ozonation employs the Design Expert Version 6 software. This software uses statistical concepts to set up experiments. A central composite face-centred (CCF) set up was used to calculate the best possible values of culture volume, ozonation time and ozone concentration using a technique different from the conventional method. A CCF set up can help to increase the efficiency of an experiment. Restrictions were posted on the CCF matrix (Table 3.3). In this technique each factor has to be considered at 3 levels, the true limits are equivalent to the parameters related to the factors. For the purpose of this research the set-up must be able to predict the results produced by interaction among culture volume, ozone concentration and ozonation time.

The set-up of a CCF matrix which had a central point was prepared. The experiment was able to utilize the central composite design to decrease the chlorophyll content to the highest possible value since it can adhere to the quadratic surface which helps in the optimization procedure. A total of 20 experimental set ups were suggested (Table 3.4) in context of 3 factorial (2^3) designs and at the center point there were 6 replications, the second order polynomial model saw the use of 6 star points. In Table 3.3 the highest and lowest values of culture volume, ozone concentration and time used in the research are presented. The lowest culture volume selected is 30 mL and the highest is 100 mL. The lowest ozone concentration selected is 6 mg L^{-1} and the highest ozone concentration is 10 mg L^{-1}. The minimum time selected is 10 min and the maximum time is 40 min. On the other hand, 20 cycles that had to be completed as predicted by the software are shown in Table 3.4. The response gained (chlorophyll content) value are inserted prior to statistical analysis generated by the software and discussed in Chapter 5.

Table 3.3: Parameters restrictions of the CCF matrix.

	Independent Variable	Level		
		-1	**0**	**+1**
A	Culture volume (mL)	30	65	100
B	Ozone concentration (mg L^{-1})	6	8	10
C	Time (min)	10	25	40

Table 3.4: Numbers of experiments suggested by the RSM software

Run	Block	Factor 1 A: C.volume	Factor 2 B: O.conc	Factor 3 C: O.time	Response Chlorophyll
1	Block 1	65.00	8.00	25.00	
2	Block 1	65.00	8.00	25.00	
3	Block 1	30.00	10.00	10.00	
4	Block 1	65.00	8.00	25.00	
5	Block 1	100.00	6.00	40.00	
6	Block 1	100.00	6.00	10.00	
7	Block 1	65.00	8.00	25.00	
8	Block 1	100.00	8.00	25.00	
9	Block 1	65.00	8.00	10.00	
10	Block 1	65.00	6.00	25.00	
11	Block 1	30.00	6.00	40.00	
12	Block 1	30.00	10.00	40.00	
13	Block 1	30.00	6.00	10.00	
14	Block 1	65.00	10.00	25.00	
15	Block 1	100.00	10.00	40.00	
16	Block 1	65.00	8.00	25.00	
17	Block 1	100.00	10.00	10.00	
18	Block 1	65.00	8.00	40.00	
19	Block 1	65.00	8.00	25.00	
20	Block 1	30.00	8.00	25.00	

CHAPTER 4

IDENTIFICATION OF CONTAMINANT AND ITS EFFECT TO ALGAL GROWTH PERFORMANCE

4.1 Introduction

The chapter discusses the development of an in-situ disinfection method by ozonolysis. Initially, the process involves bacterial (contaminant) identification, which starts with screening, isolation and identification through 16S rRNA. Then, a fresh algal culture is contaminanted with certain concentrations of bacteria that are isolated from an old *D. salina* culture to study the performance of algal growth. Next, the effect of ozonation (at certain concentrations and time) on the contaminated culture is discussed. A morphological study on the effect of ozonation on the algal cell wall is also presented.

The chapter is organized starting with the screening and isolation of the bacteria contaminating the old *D. salina* culture. This is followed by the identification of the contaminant through the 16S rRNA method. Next, the effect of the contaminant on algal growth performance and ozonation of the heterogeneous culture are considered. Finally, the ozonation and characterisation of the heterogenous culture is presented.

4.2 Screening and isolation of contaminants for identification

The screening and isolation of contaminants was done by using two methods: the spread-plate and streak-plate techniques. First, 100 μL of sample was removed from the old microalgal culture (more than 3 months culture) and transferred onto a 1 M NaCl nutrient agar plate. The purpose of using 1 M NaCl of nutrient-plate agar is to mimic the salty condition of the *D. salina* medium, which is high in salt. Then the sample was spread evenly with a glass spreader. Next, the plates were incubated for 3 to 4 days at 25 °C. Then the visible contaminants were transferred onto new fresh plate with a streaking technique. The *D. salina* medium described by Vonshak (1986) was used to grow *D. salina* in batch culture for 7 days. Figure 3.5 depicts the screening and isolation process used to get pure colonies of the bacteria that contaminated the microalgal culture.

The next part involves the identification of species-specific oligonucleotides (molecular identification) using the 16S rRNA method, which is the most powerful method existing in the biological world for distinguishing between two different species (Olmos et al., 2009). Basically, molecular characterization is achieved by using ribosomal RNA (16S rRNA and 18S rRNA) (Alonso et al., 2012). The 16S rRNA (about 1500 base pairs) encodes for the small subunit of the prokaryotic ribosome (Cai et al., 2003). Previously, classical methods known as phenotype methods were used for prokaryotic classification, which were based on physiology, morphology and biochemical features. Biochemical and physiological description comprises information on growth at different pH, temperature, salinities, antimicrobial resistance, metabolic pathways and activity of different enzymes. Morphological description is based on motility, colony colour, cell shape, endospore viability, gram staining and type of inclusion bodies (Rosselló-Mora and Amann,

2001). Most of these characteristics are irrelevant but still helpful for taxa recognition (Vandamme et al., 1996). In this work, DNA was extracted with a Qiagen DNA purification kit. Then, the amplification of specific regions was performed via PCR reaction. Finally, purified PCR products were quantified before they were sent for sequencing (section 2.3).

4.3. Identification of contaminants by 16S rRNA

A bacterial contaminant was successfully isolated from an old culture of *D. salina* (>3 months) cultivated in a 250 mL shake flask. Then, the isolated strains were disrupted by using a bead-beating method (different times) to extract the DNA, and this procedure was followed by 16S rRNA amplification using PCR. The existence of 16S rRNA gene products was observed by analysing the PCR product on 1% agarose gel stained with ethidium bromide (Figure Appendix 1). The gene ruler ladder (molecular weights ranging from 250 to 10000 bp) was used to compared with the products for easy qualitative and DNA size determination (Mwirichia et al., 2011). All of the fragment band size of 16S rRNA that were successfully amplified is roughly 1 kb followed by PCR products purification (PCR purification kit). The sequencing was performed by Eurofins Genomics. The sequences are 577 and 951 bp long, respectively (Table Appendix 2). The different read lengths reflect the fact that different time of cell disruption treatment damaging the genomic DNA.

Sequencing of the 16S rRNA gene shows that the 16S rDNA sequence of the contaminant strain is 100% identical to a group of bacterial strains within the *Halomonas* genus (Table 4.1).

Halomonas bacteria are Gram-negative, rod-shaped cells that are usually unpigmented or yellow-tinted in colour (Lee et al., 2005). These bacterial strains are moderate halophiles (salt loving) and grow well with high levels of NaCl. They are also highly versatile in terms of their ability to grow successfully in a wide range of pH and temperature (Quillaguamán et al., 2005). Previous work has shown that *Dunaliella* cultures are easily contaminated with *Halomonas* bacteria even though the medium contains a high concentration of salt (Cummings and Gilmour, 1995).

Table 4.1: Top ten matches from the NCBI database based on similarity with the 16SrRNA gene sequence of the contaminant strain. Sequencing was done using the reverse primer.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JQ421326.1	*Halomonas* sp. GQ43 16S ribosomal RNA gene, partial sequence	1277	1277	100%	0.0	100%
JQ421325.1	*Halomonas* sp. GQ31 16S ribosomal RNA gene, partial sequence	1277	1277	100%	0.0	100%
JQ421322.1	*Halomonas* sp. GQ42 16S ribosomal RNA gene, partial sequence	1277	1277	100%	0.0	100%
JQ421319.1	*Halomonas* sp. GQ11 16S ribosomal RNA gene, partial sequence	1277	1277	100%	0.0	100%
JQ028719.1	*Halomonas* sp. GQ30 16S ribosomal RNA gene, partial sequence	1277	1277	100%	0.0	100%
JN998404.1	*Halomonas* sp. GQ1 16S ribosomal RNA gene, partial sequence	1277	1277	100%	0.0	100%
JN998396.1	*Halomonas* sp. GQ47 16S ribosomal RNA gene, partial sequence	1277	1277	100%	0.0	100%
JN998393.1	*Halomonas* sp. GQ34 16S ribosomal RNA gene, partial sequence	1277	1277	100%	0.0	100%
JN998392.1	*Halomonas* sp. GQ32 16S ribosomal RNA gene, partial sequence	1277	1277	100%	0.0	100%
JN998391.1	*Halomonas* sp. GQ29 16S ribosomal RNA gene, partial sequence	1277	1277	100%	0.0	100%

4.4. Halomonas sp. Growth Profile

To study the sterility of the media in the small bioreactor, the growth profile (growth phases) of the contaminants was determined first. This is a crucial step in identifying all different phases of the bacteria culture other than doubling time and growth rate of the bacteria. Figure 4.1 shows the growth profile of *Halomonas* sp. after 7 days of incubation with two different media used which are normal *D. salina* media and induced *D. salina* media (addition of 2% (w/v) of glucose as mentioned in Section 3.5.1). All of the *Halomonas* growth profile experiments were performed in triplicate. Mata et al. (2010) clearly explained that the growth phases of bacteria in a closed batch culture system consists of a lag phase (adaptation stage), log phase (exponential stage), stationary phase and lastly the death phase.

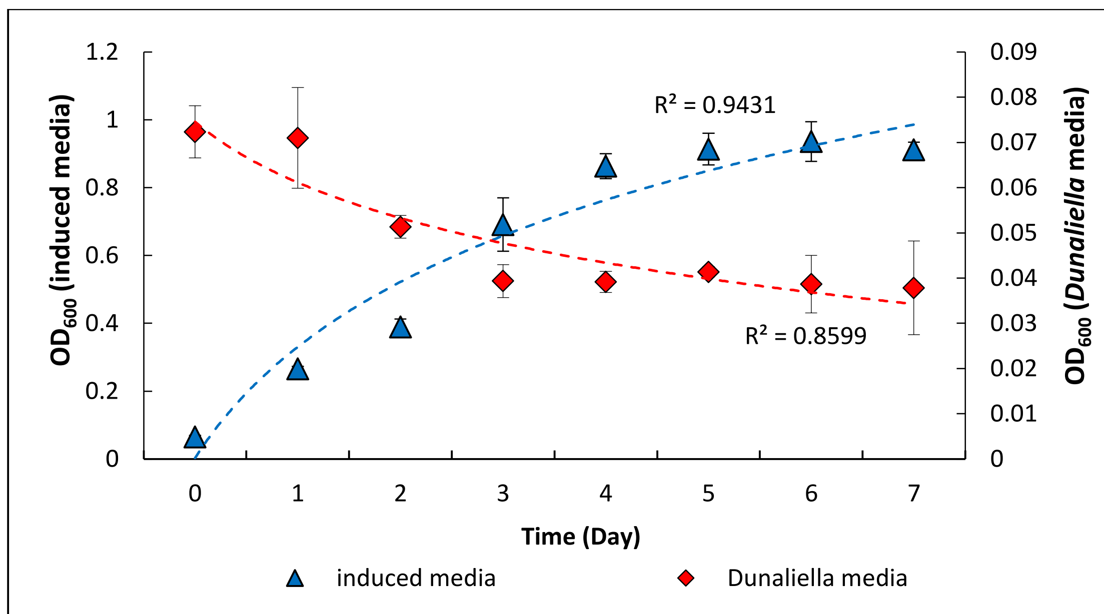

Figure 4.1: *Halomonas* growth profile using induced and normal media.

In this experiment, the *Halomonas* strain was grown inside 250 mL shake flask for 7 days at room temperature with 100 rpm of agitation. At the beginning of this experiment, one loop full of bacterial was introduced. The graph clearly shows that the log phase (exponential phase) for *Halomonas* culture, using induced *D. salina* media, was within 2 days before it entered the stationary phase on the fourth day and reached its maximum cell concentration on the sixth day (OD_{600} reading 0.936 ±0.059). While the *Halomonas* cell concentration that used normal *D. salina* media (no glucose) is far too low (OD_{600} reading 0.039 ±0.006) compared to the cell concentration that used induced *D. salina* media on the same day. This is probably due to the fact that normal *D. salina* media has a limited carbon source. Thus, the induced *D. salina* medium (plus glucose) was chosen as the best medium and was used in the next part of the experiment where *Halomonas* sp. was cultured for 5 days. The cell concentration was determined quantitatively in the form of a colony-forming unit (cfu).

4.5 Contaminant Effect to Algal Growth Performance

Shake flask cultures (a 100 mL volume of algae in a 250 mL flask) were used to investigate the impact of the contaminant on biomass development. The growth performance of *D. salina* (10% inoculum size) was monitored over 14 days of cultivation following contamination with various concentrations of *Halomonas* bacteria (Figure 4.2). After the introduction of the bacterial contaminant at the start of the experiment, the heterogeneous culture was left to develop for 14 days. The results showed that the increase in the contaminant concentration occurred concomitant with a decrease in the algal growth performance. *Halomonas* bacteria (approximately$1x10^6$, $2x10^6$,

$3x10^6$, $4x10^6$, and $5x10^6$ CFU representing concentrations of 2%, 4%, 6%, 8% and 10% (v/v)) resulted in a biomass decrease of 16.3, 29.9, 32.8, 43.9, and 52.9%, respectively.

The algal biomass concentration was decreased by over 50% at the 10% (v/v) contaminant concentration, which corroborated the results of earlier research that revealed that bacteria and microalgae were in competition for inorganic nutrients (Grover, 2000). Zhang et al. (2012) reported that microalgae photosynthesis could not occur because the microorganisms and bacterial films covering the internal photo-bioreactor wall reduced the amount of available light. Algae development is hindered by algicidal bacteria directly via cell-to-cell contact or indirectly through extracellular compound secretion (Furusawa et al., 2003 and Nakashima et al., 2006). This phenomenon was reported when a combination of factors such as nutrient competition, algicidal bacteria, and insufficient light contributed to *C. pyrenoidosa* growth suppression in piggery wastewater exposed to ozonation (Gan et al., 2014).

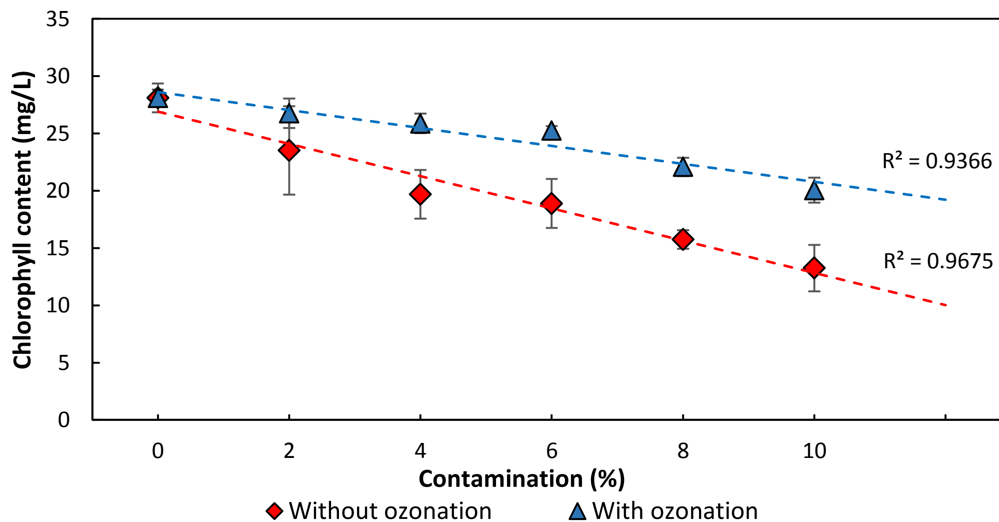

Figure 4.2: The growth performance of *D. salina* with *Halomonas* contamination with and without ozonation.

4.6. Ozonation and Characterisation of the Heterogeneous Culture

The effect of ozonation on the heterogeneous culture (*D. salina* and *Halomonas*) was investigated in a 0.1 L bioreactor. Figure 4.3 shows the graph of the effect of ozonation of the mixed culture at the 8 mg L^{-1} ozone concentration for 10 minutes. The sterilisation efficiency reached 66% after 5 min and increased to 93% after 10 min of ozonation (based on the cfu number). The chlorophyll concentration can be used to estimate the *D. salina* cell concentration because ozone can oxidize chlorophyll. A reduction in the chlorophyll concentration would suggest that the algal cells are damaged, thus exposing the chlorophyll to ozone attack. The results in Figure 4.3 show a reduction in the number of *Halomonas* colonies, whereas the chlorophyll content of *D. salina* remains relatively constant with slight decrease towards the end of 10 min with R^2 of 0.8548. R^2 is used to statistically measure the fit of a model and the value of 1 (R^2) indicates the line perfectly fits the data. This result demonstrates that at a lower ozone dosage and short period of ozonation time, the intermittent disinfection can be applied to eliminate or reduce contaminants, with minimal damage to the microalgae. This result is in agreement with the findings of Choi et al. (2006), who applied a dielectric barrier discharge (DBD) treatment for more than 1 min to kill *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas* and obtained a sterilisation efficiency of 99.99%. Additionally, Gan et al. (2014), treated piggery wastewater by bubbling 300 mL min^{-1} of ozone gas for 5 min and reported a sterilisation efficiency of 98%.

Ozone is a powerful oxidising agent that rapidly kills the microorganism by damaging the cytoplasmic membrane, nucleic acids, and cell wall (Benita, 2014 and Gan et al., 2014). According to Pascual et al. (2007), ozone causes inactivation of microbial cells by disrupting their cell

membranes or cell lysis by disintegration of the cell walls. Thanomsub et al. (2002) proposed that ozone inactivates bacterial cells by destroying cell membranes, leading to cell lysis. However, Cho et al. (2010) suggested that inactivation was mainly due to damage to cell surfaces. To achieve sterile conditions such as those obtained with an autoclave, approximately six (6)-log reductions are required (Miller and Hansen, 2002). However, this process will kill the entire microbial consortium, including the microalgae. Thus, determining the optimum conditions between the ozone concentration and time is important to reduce the contamination with a minimal or no effect on the microalgal cells.

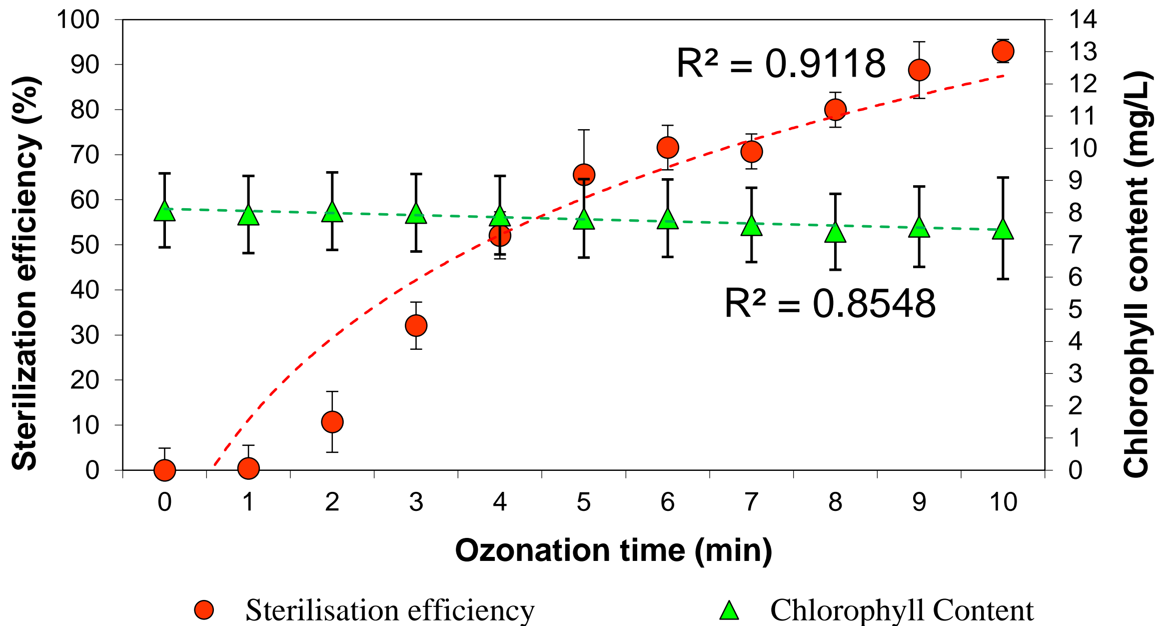

Figure 4.3: *Halomonas* colony forming unit (initial number: 2.15 ± 0.11 x10^6 CFU) and chlorophyll content (*Dunaliella salina*) of the mixed culture (1:1 (v/v)) after up to 10 minutes of ozonation at 8 mg L^{-1}. The experiments were conducted three times for each parameter and error bars represent standard deviation.

Exposure of a 14 day old *D. salina* culture to ozone for a relatively long period of time (>60 minutes) completely destroyed the microalgal cells. Microscopic study revealed that the *D. salina* cells burst and released their intracellular organelles into the culture medium. This result was in agreement with Sharma et al. (2012), who showed that O_3 treatment on *Microcystis aeruginosa* caused a discharge from within the cells due to harm to the cell wall. Figure 4.4 shows light microscope images of the morphology of the *D. salina* cells before and after cell disruption caused by ozonation.

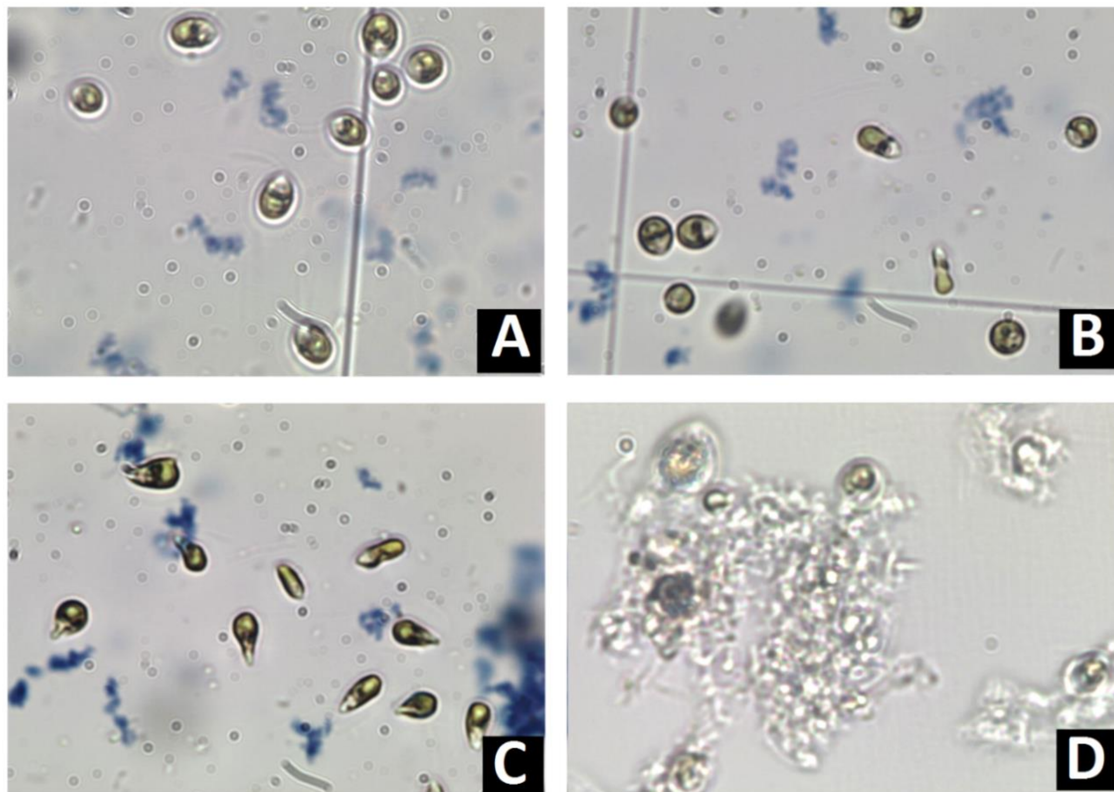

Figure 4.4: Morphology of *Dunaliella salina* after ozonation at 8 mg L^{-1} under light microscope (1000x magnification). A; control, B; 30 minutes of ozonation, C; 60 minutes of ozonation and D; 90 minutes of ozonation.

These images clearly showed that the *D. salina* cells were ovoid, green and healthy prior to ozonation (Figure 4.4 A). After 30 minutes, the shape of the cells was distorted and they had shrunk, and some were completely damaged (Figure 4.4 B). Moreover, ozonation for 60 minutes resulted in dramatic shrinkage of the anterior cytoplasmic compartment of the cells, whereas the posterior chloroplast still looked largely intact (Figure 4.4 C). However, the *D. salina* cells were totally disrupted and colourless after 90 minutes of ozonation, which indicated that the cell contents were released into the culture medium and probably oxidized (Figure 4.4 D). The SEM images in Fig. 4.5 show normal cell structures versus damaged cells due to the ozone treatment. The cultivation of algae and the extraction of their metabolites are significantly affected by the concentration of ozone and the competitive reactions among the organic substances and toxins. Hammes et al. (2007) reported that treatment of the algal mass with ozone caused the release of intracellular organic substances.

In order to study the improvement of ozonation treatment towards contaminated culture, the heterogeneous cultures were ozonated with 8 mg L^{-1} of ozone concentration for 10 minutes on day one. The growth performance of *D. salina* with *Halomonas* contamination treated with ozone are shown in Figure 4.2. The production of the microalgal biomass at increased microbial contamination concentrations of 2%, 4%, 6%, 8% and 10% (v/v) of *Halomonas* resulted in a biomass reduction of 4.8%, 7.9%, 10.1%, 21.4% and 28.6%, respectively. The results shows that ozonation at the beginning of experiment can control the contamination as compared to without ozonation and slightly increased the biomass production. It has been reported that ozonation at 2.59 to 3.11 mg L^{-1} was associated with excellent disinfection effects that were able to suppress the growth of bacterial cells and their spores (Sadatullah et al., 2012). Although a 93% efficiency

of disinfection was attained within 10 minutes of ozonation, the growth of residual bacterial persisted along with the growth of the microalgae. These results agree with those obtained by Gan et al. (2014), whose study focused on the use of ozone for the treatment of piggery wastewater and recorded a 98% efficiency of disinfection attained after a five-minute ozonation process at 8 mg L^{-1}. The study also recorded the growth of remaining bacteria (survived from the ozonation) in the piggery wastewater together with the microalgae.

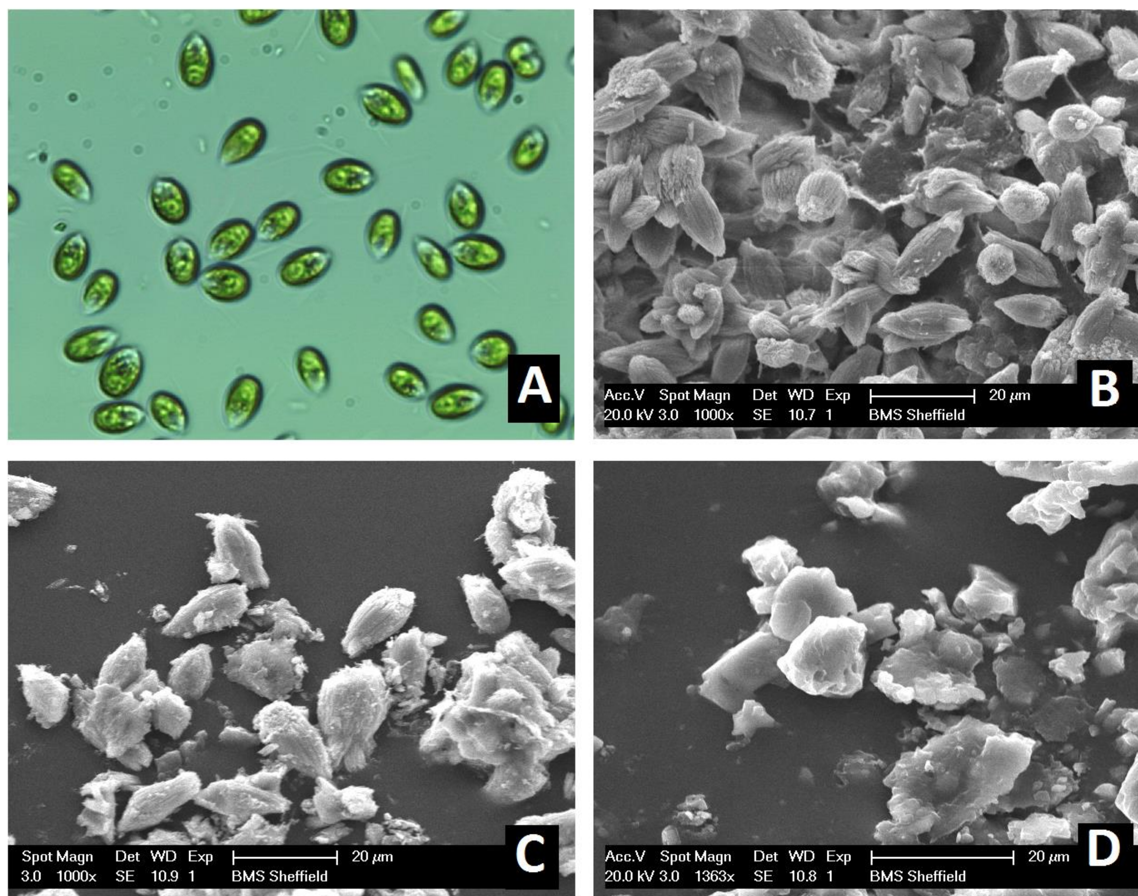

Figure 4.5: SEM images of *Dunaliella salina* before and after ozonation at 8 mg L^{-1}. A; Control, under light microscope (1000x magnification), B; Control, SEM image, C; after 60 min of ozonation, D; after 120 min of ozonation.

4.7 Summary

In this chapter, the bacterial contaminant of *D. salina* culture was successfully screened, isolated and identified using the 16S rRNA method which shown 100% similarity with *Halomonas* spp. As a result, the first objectve of this study is achieved. The *Halomonas* culture was induced with glucose to increase the growth of the bacterial cells, as the bacteria did not effect algal growth at low concentration. The increase in the contaminant concentration occurred concomitant with a decrease in algal growth. Due to competition for inorganic nutrients, the algal biomass concentration was decreased by over 50% at the 10% (v/v) contaminant concentration. However, ozonation for 10 minutes at the beginning of the experiment resulted in an algal biomass reduction of only 28.6% (compared to 52.9%), which shows that ozonation at the beginning of the experiment can control the contamination. The sterilisation efficiency reached 66% after 5 minutes and increased to 93% after 10 minutes of ozonation. Exposure of a 14-day-old *D. salina* culture to ozone for 10 minute did not harm the microalgae. However, ozonation for a relatively long period of time (>60 minutes) completely destroyed the microalgal cells. The second objective of this study is accomplished as the intermittent disinfection during the growth phase manage to control the contaminant and resulting in slightly higher algal biomass production compared to without disinfection.

CHAPTER 5

STATISTICAL ANALYSIS OF ALGAL CELL DISRUPTION RATE USING RESPONSE SURFACE METHODOLOGY (RSM)

5.1 Introduction

This chapter considers the optimization of the algal cell disruption rate. Three parameters (volume of culture, ozone concentration and ozonation time) were statistically optimized using response-surface-methodology software (RSM). Twenty experiments suggested by the software were run, and the highest probabilty experiments suggested by the software (desirability functions) were then repeated. Finally, the optimum conditions for all three parameters were tested for *Halomonas* culture (ozonation for 20 minutes and 60 minutes), and the reduction of the colony (cfu) was plotted and discussed.

5.2 Optimization of Algal Cell Disruption using Response Surface Methodology

Design Expert Version 6.0.4 Software was employed to maximize algal cell disruption by analysing the reduction of chlorophyll content. According to Montgomery (2001), the Design Expert software uses statistics to formulate experiments. The range of ozone concentration, ozonation time and culture volume were optimized by using a central composite design (CCD).

According to this methodology, each of the three elements must have three levels to set their true limit to allow the interaction and effects of those three factors to be assessed. The highest and lowest values of those factors were then determined (Material and Methods 3.9).

Traditional methods of optimization require fixing other variables (parameters) while changing one independent variable at a certain level. This single dimensional search is time consuming, painstaking and cannot reach a true optimum limit because it is unable to estimate interactions among experimental parameters. This statistical method is more practical, as it is developed from an experimental method that embraces interactions among the parameters (Bas and Boyaci, 2007). The use of a statistical methodology for product optimization has been widely employed. Wang and Lan (2011) reported on optimising the lipid production of green algae. Renita et al. (2014) reported optimizing algal methyl esters using RSM. In this study, a statistical method that uses RSM was applied to optimize chlorophyll reduction by ozonation processes in a 0.2 L ozonation extraction bioreactor. The factors analysed are culture volume, ozone concentration and ozonation time.

5.3. Statistical Analysis

Table 5.1 enumerates the various results produced in the central composite face centred (CCF) in terms of the chlorophyll content reduction of *D. salina*. Since the lowest culture volume level was 30 mL (15% minimum working volume), we decided to employ the central composite face centred design (CCF). During CCF, the value of alpha was kept constant at 1. However, the situation varies in central composite design (CCD) when the value of alpha is taken as greater than 1 and varies per the factors being used. Quadratic varieties of models were utilized for the response,

and the different P levels of linear and interactive variables are given below in the model equation (as shown in Table 5.4). Important variables were produced in the form of responses, and reduced models were prepared. The different properties of models were studied to predict the efficiency of the models.

A model considered as significant when the F-value is significant, the R^2 value is high, the standard deviation is low and the lack-of-fit F value is significant. Analysis of variance (ANOVA) was used to determine whether the model is statistically significant or not, as depicted in Table 5.2. The response (chlorophyll content) demonstrates that statistical significance can be accorded to the regression in which the probability value was > F (>0.0001). The F value obtained for the response is 4.75. With respect to all variables considered, the possibility of this Model F-value being produced due to chance is only 1.15%. Any values of "probability > F" less than 5% is statistically significant.

The optimum condition for the highest chlorophyll reduction suggested by the software was repeated three times. Comparison of experimentally predicted values of chlorophyll reduction at optimal levels predicted by RSM, and comparison of reduction obtained from RSM and the preliminary study is depicted in Table 5.3. The chlorophyll reduction predicted by the software for the optimum condition is 20.01%. On the other hand, only 18.58% chlorophyll reduction was gained after the optimum condition was repeated three times. The error could have occurred during the chlorophyll content analysis, but the difference (chlorophyll reduction) is not much. However, the reduction during the preliminary study is far too low (5.25%). and the improvement under

optimum conditions is 254%.Thus, it is important to optimize the parameters to get the maximum effect, which in this case is the chlorophyll reduction.

Table 5.1: Optimization using statistical method.

Run	A	B	C	Chlorophyll (μg mL^{-1})
1	65	8	25	27.766
2	65	8	25	27.957
3	30	10	10	27.469
4	65	8	25	28.719
5	100	6	40	26.813
6	100	6	10	28.973
7	65	8	25	28.867
8	100	8	25	27.745
9	65	8	10	29.227
10	65	6	25	28.084
11	30	6	40	24.716
12	30	10	40	24.356
13	30	6	10	27.829
14	65	10	25	28.274
15	100	10	40	27.554
16	65	8	25	28.634
17	100	10	10	28.931
18	65	8	40	23.657
19	65	8	25	28.317
20	30	8	25	23.975

** A; Culture volume (mL), B; Ozone concentration (mg/L) and C; Ozonation time (min)

Table 5.2: Analysis of variance (ANOVA) for the surface quadratic model

Response	Model F-value	Prob > F	R^2 Value	Adjusted R^2 Value	Lack-of-fit	Standard Deviation
Chlorophyll reduction	4.75	0.0115	0.8104	0.6398	10.44	0.050

Table 5.3: Comparison of experimental, predicted value of chlorophyll reduction at the optimal levels predicted by RSM and comparison of reduction obtained from RSM and preliminary study.

Response	Response predicted	Response obtained (RSM)	Preliminary study	Improvement
Chlorophyll reduction	20.01%	18.58%	5.25%	254%

**Preliminary study was conducted in 0.2 L Ozonation bioreactor with 100 mL culture volume, 10mg L^{-1} of ozone concentration and 40 min of ozonation.

Table 5.4: Final equation in terms of coded factors (A) and actual factors (B)

Final Equation in Terms of Coded Factors:		Final Equation in Terms of Actual Factors:	
Chlorophyl	=	Chlorophyl	=
+1.32		+1.31698	
+0.055	* A	+6.84217E-003	* volume
+8.000E-004	* B	-0.21537	* concentration
-0.072	* C	+9.62410E-005	* time
-0.061	* A^2	-5.00186E-005	* $volume^2$
+0.048	* B^2	+0.048227	* $concentration^2$
-0.034	* C^2	-1.50101E-004	* $time^2$
+8.375E-003	* A * B	+2.39286E-004	* volume * concentration
+0.016	* A * C	+3.02381E-005	* volume * time
+4.625E-003	* B * C	+3.08333E-004	* concentration * time
	A		B

5.4. Optimization using the Desirability Functions

The expected value for every response and variable was picked up from the menu during numerical optimization. The different milestones were the following: none, lying within the range, highest, lowest, target and for factors it would be to reach a precise value. Every variable must have a defined highest and lowest value. If the shape of the desirability function must be modified, then every milestone should be flagged with a weight. All of the milestones are considered in totality to obtain the total desirability function. Desirability measures the achievement of a milestone. 1 indicates that the milestone is reached; 0 indicates that the milestone is not reached. The aim of the program is to make the process more efficient. A random initial point is taken, and a steep slope is climbed to the highest value. Because the response surface has a curvature of more

than 1, the highest value may be present and the net sum is extrapolated to the desirability function (Malihe et al., 2008). With the help of numerical optimization, a point that helped in maximizing the desirability function was determined. All milestones are ranked in the context of other milestones (desirability ramp).

In Figure 5.1, three parameters (culture volume, ozone concentration and ozonation time) are defined and depicted in the context of numerical optimization. The software produced six different solutions, which were used along with high desirability of 100%. The values at which the optimum local occurred are 30.63 mL of culture medium, 8.20 mg L^{-1} of ozone concentration and 37.7 minute of ozonation time. The expected values for the highest chlorophyll reduction is 23.39 μg mL^{-1} (control = 29.23 μg mL^{-1}). The value of desirability at 1.00 shows that the function is representative of both the optimum and experimental set ups.

The RSM 3D graph that shows the relationship of all the parameters is illustrated in Figure 5.2. The lower the culture volume, the more it reduces the chlorophyll content due to highest surface contact. However, the highest ozone concentration (10 mg L^{-1}) does not produce the highest chlorophyll content reduction due to unstable (fluctuated) production of ozone. It has been reported that continuous corona discharges is not compatible for many applications due to a very low power. The voltage can be increased to raise the power level, unfortunately this leads into arcs. We believe that at the maximum volume of ozone generator, the device is tuned to prevent it from arcing. Stable ozone concentration is produced at the middle volume, which generates 8 mg L^{-1} of ozone concentration.

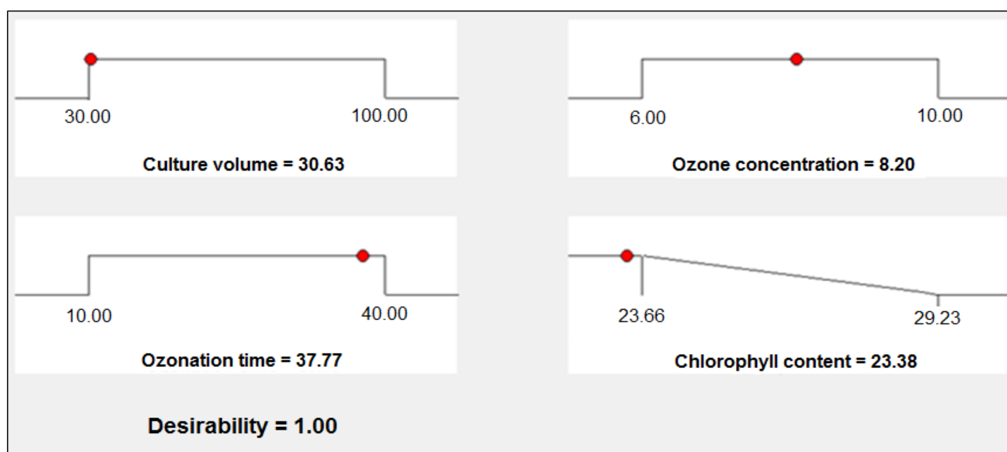

Figure 5.1: Desirability ramp for numerical optimization of three factors, namely culture volume, ozone concentration and ozonation time.

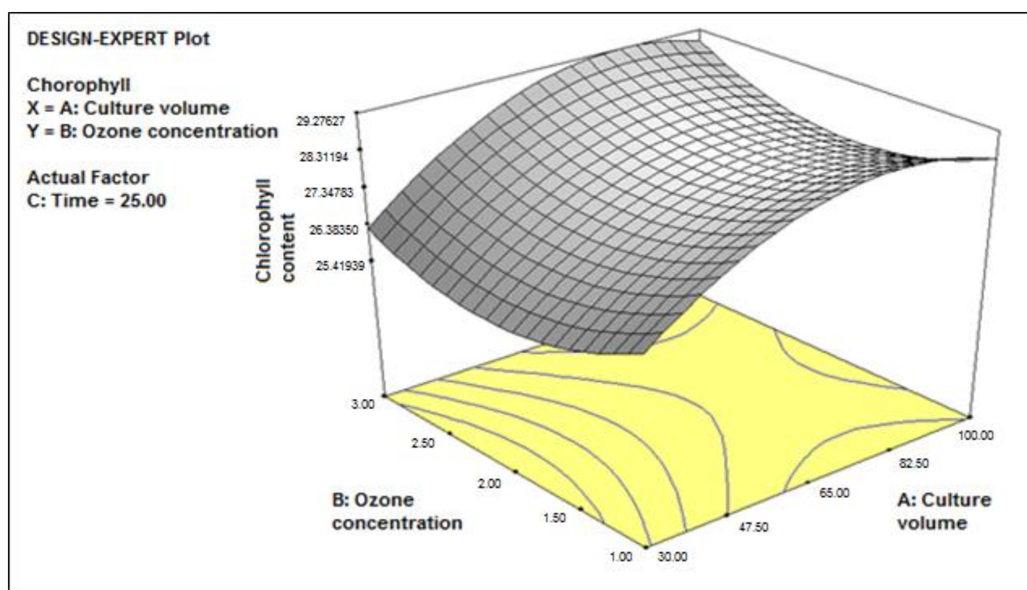

Figure 5.2: RSM 3D graph

The optimized conditions (culture volume: 30 mL, ozone concentration: 8 mg L^{-1} and ozonation: 5 to 60 min) were then applied to *Halomonas* culture. The aim is to study the potential of ozonation process to sterilize the bacterial culture. The *Halomonas* culture was grown using *D. salina* media with addition of glucose (20 g L^{-1}) in 250 mL shake flask for 6 days at room

temperature (20 ˚C – 25 ˚C). The ozonation of the *Halomonas* culture was conducted for 60 min and 100 µL of sample was taken out every 5 min. Then a serial dilution was performed up to 10^6 times followed by spread plate method and incubation at room temperature for 3 days. Lastly, the number of colonies of bacteria observed was measured and the graph is plotted (Figure 5.3). The *Halomonas* CFU number gradually decreased as the ozonation time increased. The *Halomonas* colony was greatly reduced from $1.96 \times 10^9 \pm 8.73 \times 10^8$ to $3.33 \times 10^3 \pm 1.53 \times 10^3$. The log reduction (5.77) was nearly as effective as sterilization by using autoclave (6 log reduction). However, the *Halomonas* culture volume used in this experiment was only 30 mL. The results suggests that, it is possible to sterilize a small bioreactor (0.1 L) using ozonation (8 mg L^{-1}) for 60 min. Furthermore, it seems a good option to reduce the number of bacterial cells contaminating an algal culture especially an inoculum culture (low volume).

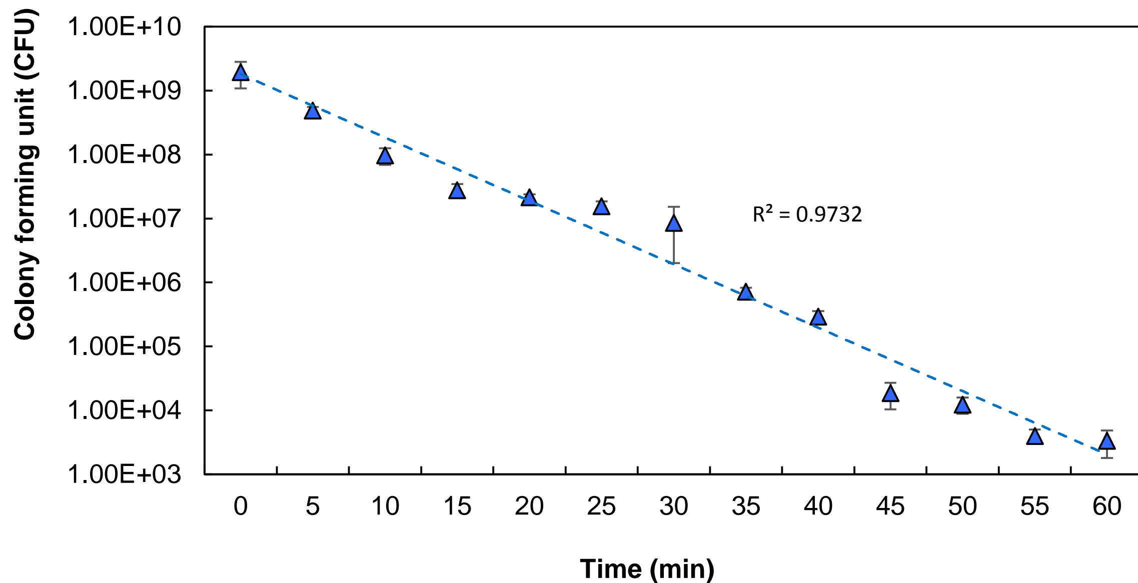

Figure 5.3: Ozonation of *Halomonas* culture for 60 min

5.5 Summary

In this chapter, the optimum conditions for *Dunaliella salina* cells disruption were investigated using a statistical method. This study is important in order to achieve maximum cell disruption and the optimum parameters that are suggested by the software will be used in the next stage which is algal lipid extraction (Chapter 7). Three parameters (volume of culture, ozone concentration and ozonation time) were statistically optimized using response-surface-methodology software (RSM). Twenty experiments suggested by the software were run, and the higest probabilty experiments suggested by the software (desirability functions) were repeated in triplicate. The F value obtained for the response is 4.75. The chance of this F-value being produced due to chance is only 1.15% (occur due to noise). Any probability of F less than 5% is normally considered statistically significant. The optimum value suggested by the software is 30.63 mL of culture medium, 8.2 mg L^{-1} of ozone concentration and 37.7 minute of ozonation time. The chlorophyll reduction predicted by the software for the optimum condition is 20.01%. On the other hand, only 18.58% chlorophyll reduction is gained after 3 repetitions of the optimum condition. However, the reduction during preliminary study is far too low (5.25%), and the improvement using optimum conditions is 254% suggesting that a low volume culture and prolongation of ozonation time is needed to maximize the ozone effect. Therefore, the optimum condition for all three parameters (culture medium: 30 mL; ozone concentration: 8 mg L^{-1}; ozonation time 60 min) were then tested with *Halomonas* culture to study the ozone sterilization. The *Halomonas* colony was greatly reduced from $1.96 \times 10^9 \pm 8.73 \times 10^8$ to $3.33 \times 10^3 \pm 1.53 \times 10^3$. The log reduction (5.77) is approximately equivalent to sterilization by using an autoclave (6 log reduction) proving that this method can be used to sterilize bacterial contamination especially for low volume culture in a small container.

CHAPTER 6

THE EFFECT OF CO_2 FLOWRATE ON ALGAL CULTURE AND HARVESTING USING FLOTATION

6.1 Introduction

This chapter considers the optimization of algal cell biomass by studying the effect of CO_2 flowrate during the production stage. The effect of 5% CO_2 and 95% N_2 gas flow rate (0.1, 0.3, 0.5, 0.7 and 0.9 L min^{-1}) on algal growth was studied in two-litre airlift bioreactors (ALB). Then, the gas-flow rate that produced the highest microalgae biomass was chosen. To extract lipids from an algal cell, the cell must be separated (harvested) from the culture. Here, a microflotation technique proven to have a high recovery efficiency on algal harvesting was chosen. Five different concentrations of aluminium sulphate (300, 400, 500, 600 and 700 mg L^{-1}), which acts as a flocculant, were tested and discussed. Lastly, the energy usage estimation for the harvesting technique using microflotation is presented.

6.2 CO_2 Flowrate Effect on Dunaliella salina Culture

Three cultures for each parameter (triplicate) in the cultivation experiments were carried out over a long period of time (1 month for 1 batch). Here the effect of 5% CO_2 and 95% N_2 gas flow rate on algal growth was assessed as is illustrated in Figure 6.1. The selected flow rates were

0.1, 0.3, 0.5, 0.7 and 0.9 L min^{-1}. Two litre airlift bioreactors (ALB) with 1.5 L working volume were employed in the experiment. The results of the experiment demonstrated that the ALB attained a higher accumulation of biomass compared to the bubble column bioreactor within 25 days of culture. The bubble column (ALB without the draught tube) at the optimum algal concentration attained a chlorophyll content of 33.4 mg L^{-1}, whereas the airlift loop at the same flow rate (0.5 L min^{-1}) highly exceeded this concentration by attaining a maximum concentration of 42.9 mg L^{-1}, which represented a 28.4% increment. The maximum concentration of chlorophyll (54.78 mg L^{-1}) was obtained at a flow rate of 0.9 L min^{-1}, representing a 55.4% increase compared to the lowest concentration attained at the 0.1 L min^{-1} flow rate (35.25 mg L^{-1}).

In general, the specific growth rate (μ) of *D. salina* cultures grown in ALB were increased with the increment of gas flow rate starting from 0.1 L min^{-1} (0.23 day^{-1}), 0.3 L min^{-1} (0.24 day^{-1}), 0.5 L min^{-1} (0.29 day^{-1}), 0.7 L min^{-1} (0.31 day^{-1}) and 0.9 L min^{-1} (0.31 day^{-1}), respectively. Both 0.7 and 0.9 L min^{-1} showed 25% improvement as compared to the lowest (0.1 L min^{-1}). However the culture grown in bubble column with 0.5 L min^{-1} showed higher specific growth rate (0.28 day^{-1}) when compared to cultures grown in ALB with 0.1 and 0.3 L min^{-1} gas flowrate. The airlift loop bioreactor fitted with microbubble dosing allowed a high mass transfer of carbon dioxide dissolution and oxygen elimination (Ying, 2013). A study by Zimmerman et al. (2011) showed a pilot scale microalgal culture (2200 L) similarly designed to the type used in this study, was neither carbon dioxide-limited nor oxygen-inhibited, resulting in a high growth rate of the algal cells. According to Ying et al. (2013), an optimum rate of growth (μ) of *D. salina* was achieved in their slightly larger 3 L airlift loop bioreactor fitted with a fluidic oscillator at a 0.9 L min^{-1} flow rate. Nevertheless, the rate of growth was abruptly reduced by increasing the flow rate up to 1.1 L min-

[1]. This result was attributed to the production of a high degree of turbulence that caused damage to the wall-less algal cells. Surprisingly, the maximum chlorophyll content gained (32.65 mg L^{-1}) at a 0.9 L min^{-1} flow rate in their study was far lower than that found in this study (54.78 mg L^{-1}). This may be due to different algal biomass concentrations introduced during the inoculation process. Apart from that, the contamination during inoculum preparation, sampling or during cultivation itself possibly occurred. Thus, the axenic conditions during cultivation should be prioritised to prevent great loss of algal biomass.

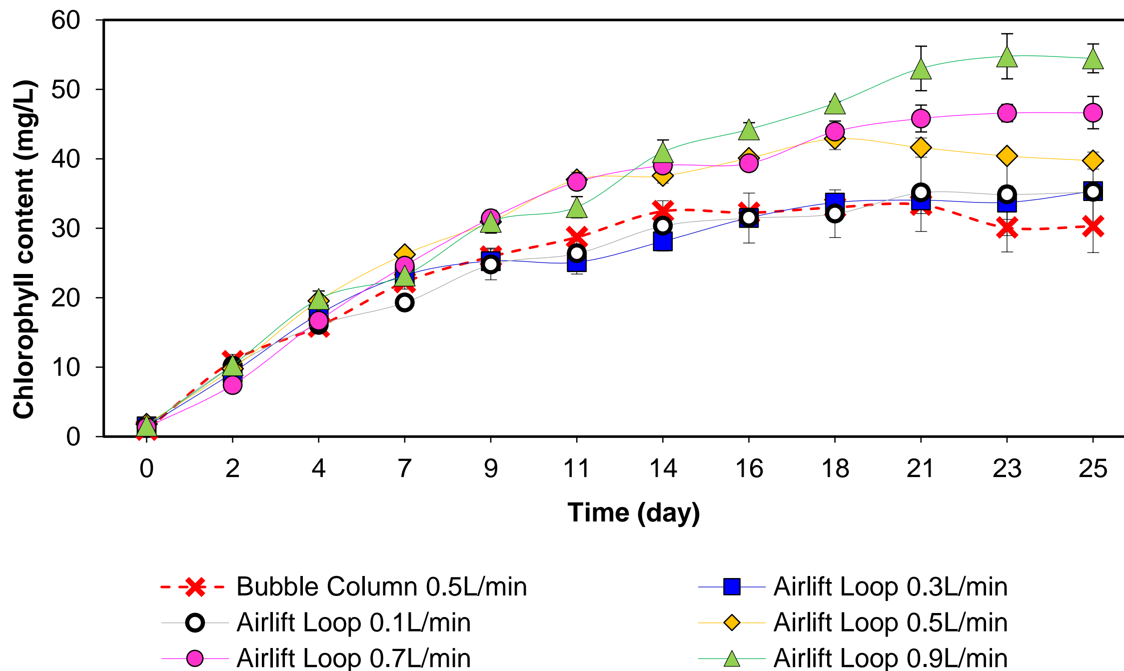

Figure 6.1: Growth performance of *D. salina* culture in a 2 L photobioreactor with different flow rate. A 30 minutes gas (5% CO_2: 95% N_2) bubbling (depending on flow rate) was performed every day. The experiments were conducted three times for each parameter error bars represent standard deviation.

6.3 Microalgae Harvest by Microflotation

In addition to the advantageous mass-transfer properties discussed above, microbubbles have important and useful momentum transfer and coordination properties. Microbubbles can attach to algal cells, giving the whole complex greater buoyancy. Such flotation brings flocculated algal cells to the surface where they are more readily harvested by skimming. Figure 6.2 shows the effect of different coagulant concentrations over time. The lowest recovery efficiency obtained was 44.6% at 300 mg L^{-1} aluminium sulphate, followed by 71.3% at 400 mg L^{-1}, 84.1% at 500 mg L^{-1}, 88.9% at 600 mg L^{-1} and 93.4% at 700 mg L^{-1}. The results shows an increase in the recovery of microalgal cells as the concentration of the flocculant (aluminium sulphate) increases.

This increase in recovery efficiency is due largely to the high presence of trivalent ions at increased coagulant dosage. These potent trivalent ions are responsible for particle destabilisation. Charge neutralisation and double-layer compression are commonly associated with inorganic coagulants as the main particle-destabilisation mechanisms, thus leading to particle agglomeration (Ralston and Dukhin 1999). The agglomerated cells readily attach to the rising microbubbles and are transported to the top of the flotation separator for collection. This result is in agreement with Hanotu et al. (2012), who obtained higher recovery efficiency (95.2%) using a similar setup.

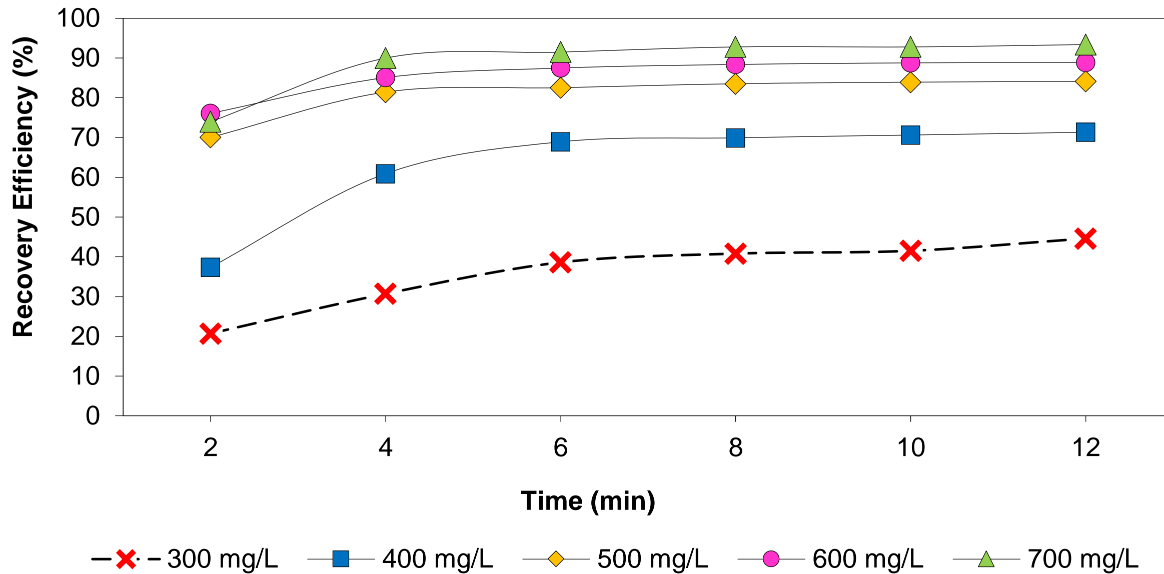

Figure 6.2: Microflotation harvesting with different aluminium sulphate concentrations. It would have been relevant to test further flocculant concentration to ascertain optimality but given that the priority is demonstrating microflotation performance as a modular unit, the results provide sufficient information. The experiment was conducted twice for each parameter (duplicate) and each point representing the mean of the value.

In these experiments, a pH of 5 was chosen due to previous studies conducted by Hanotu et al. (2013), who showed the best performance for the treatment of the algae-water suspension in the dispersed air-flotation process. The reason behind this is that the degree and the rate of adsorption of these trivalent and other complex ions onto colloidal surfaces depends on the pH. According to Wyatt et al. (2012) and Pernitsky and Edzwald (2006), Al^{3+} is the dominant species in the continuous phase at room temperature and under acidic pH. In the colloidal-charge neutralization, Al^{3+} is the most effective and tends to attach to the negatively charged algae. Trivalent metal species are further released when the hydroxides of these metals react with excess H^+, which is present at low pH. Consequently, the species of Al^{3+} is made available to neutralize

the charges, but the quantity of the hydroxide species is significantly reduced. As the pH becomes more alkaline, the concentration of OH^- increases while that of H^+ decreases which results in a decrease in the amount of trivalent ions. The large number of OH^- then freely reacts with the trivalent ions to form the metallic hydroxides of these trivalent metals. For this reason, the now alkaline conditions of the solution produce hydroxide species as the predominant ions. These attach to the cells and allow spontaneous formation of flocs (cell aggregates) of the algae (Chatsungnoen and Chisti, 2016). At room temperature and with the increase in the pH far away from 7, the concentration of the hydroxide species of the aluminium salts increased, according to Wyatt et al. (2011) and Pernitsky and Edzwald (2006).

6.4 Energy Consumption of Algal Harvesting by Flotation

The harvesting of microalgal biomass is costly due to high energy intensity processes as mentioned in Section 2.4.2. Till now, there are no specific best algal harvesting methods for commercial scale (large scale). Thus, the energy consumption of different algal harvesting methods that have been reported previously are discussed in this section. The summary of energy consumption and biomass yield of different microalgae harvesting methods from recent studies is depicted in Table 6.1.

Obviously gravity sedimentation method is using the lowest energy with 0.1 KWh m^{-3}. This is due to only a few things (pump, motor and settling tank) being needed for harvesting process. However, according to Singh et al. (2013), due to slow sedimentation, several large volume tanks (about 100 000 L) are required in order to reach commercial scale production. The highest energy consumption method with 14.81 KWh m^{-3} is polymer flocculation method.

Conversely, electro flocculation method uses low energy as compared to polymer flocculation method with 0.33 KWh m^{-3}. However the electro flocculation method remains in the research stage and large scale testing is needed to validate whether it is cost effective method or not (Singh et al., 2013). The pressurized filtration method harvested the highest solid algal biomass with 27% and using quite a low energy (0.88 KWh m^{-3}). However the long term maintenance and lifetime cost of the filter should be considered and validated in the future. While centrifugation, which is the most common method for harvesting, was in second place with 22% solid algal biomass. However the centrifugation process is quite energy intensive with 8 KWh m^{-3}.

Table 6.1: Energy consumption and biomass yield of different microalgae harvesting methods compares to recent study

Harvesting Method	Energy usage (KWh m^{-3})	Algal biomass yield (% solid)	References
Centrifugation	8.0	22	Girma et al., (2010)
Filtration (natural)	0.4	6.0	Semerjian et al., (2003)
Filtration (pressurized)	0.88	27	Semerjian et al., (2003)
Filtration (Tangential flow)	2.1	8.9	Danquah et al., (2009)
Filtration (Vacuum)	5.9	18	Girma et al., (2003)
Flocculation (polymer)	14.81	15	Danquah et al., (2009)
Flocculation (electro)	0.33	-	Edzwald, (1995)
Gravity sedimentation	0.1	1.5	Shelef et al., (1984)
Flotation (dissolve air)	7.6	5.0	Wiley et al., (2009)
Flotation (fluidic oscillation)	6.8	5.0	Current study

The current study, when air compressor was used in the lab to supply the air (65 L min^{-1} at 0.5 bar), the flotation method using microbubbles produced by fluidic oscillator consumed about 3.4 KWh cm^{-3} of energy. This estimation is based on the average power usage of 17 kWh that has been reported by Schmidt (2005), on the studies of 4 air compressors (30-hp rotary) producing 85 to 92 psig of pressures. However, if the current fluidic oscillator was able to work with a conventional air pump producing 65 L min^{-1} at 4.35 psi, the energy usage will be around 6.8 KWh m^{-3}. This estimation is based on the average power usage of 0.0068 KWh when an air pump model AP-60 from Thomas is used (Apendix 2.1).

Furthermore, both of the flotation methods also achieved 5% of solid algal biomass (Table 6.1). However, the energy usage of FO flotation is slightly lower than dissolved air flotation method. The current fluidic oscillator need a high air flow rate (65 L min^{-1} at 0.5 bar) due to air bleeding being needed in order to work. Even though it is not the most energy-efficient method for the time being, the energy usage of this method can be reduced when the future fluidic oscillator that are able to work at lower air flow rate (no air bleed required) is tested which is still in the development stage by Zimmerman research group. Additionally, the current algal harvesting by using microflotation was conducted in 1 L bioreactor. Scaling up of microflotation bioreactor from 1 L to 10 L or 100 L definitely will reduce the energy usage if the air pressure and flow rate are the same. The scaling up of the microflotation bioreactor is currently being tested.

6.4 Summary

In this chapter, there is no objective to be achieved as the microflotation technique has been extensively studied and reported by Hanotu et al. (2013). However, obtaining the maximum biomass should be prioritized as the extraction method requires a large quantity of algal slurry. Therefore, the study of the optimum CO_2 gas flowrate and optimum aluminium sulphate concentration were conducted. The effect of CO_2 gas flowrate was measured to optimize the algal cell production during the biomass production stage. The airlift loop bioreactor fitted with a microbubble doser allowed a high mass transfer of carbon dioxide dissolution and oxygen elimination which resulted in higher biomass production than the bubble-column bioreactor. The best flowrate of CO_2 gas during biomass production in an airlift loop bioreactor is 0.9 L min^{-1}; the lowest is at 0.1 L min^{-1}. However, increasing the gas flowrate above 0.9 L min^{-1} reduces algal cell (biomass) production. This result is attributed to the production of a high degree of turbulence that damaged the wall-less *D. salina* algal cells used in this test. The lowest recovery efficiency obtained was 44.6% at 300 mg L^{-1} aluminium sulphate; the highest was 93.4% at 700 mg L^{-1}, which is in agreement with the previous study (Hanotu et al., 2013). The aim of this part of the work was to harvest the microalgae and the algal slurry was collected prior to use in the lipid extraction part (Chapter 7). The aluminium sulphate was chosen as a flocculant because of it availability and it is cheaper than the others. Furthermore the energy usage of the current microbubbles flotation method generated by fluidic oscillator is estimated and calculated. Even though the current microflotation method is not the most energy efficient method, further improvement by using low flow rate fluidic oscillator and up scaling of the flotation bioreactor in the future should be promising.

CHAPTER 7

MICROALGAL LIPID EXTRACTION BY OZONOLYSIS

7.1. Introduction

This chapter considers the development of the lipid-extraction process by direct ozonolysis in a 0.2 L ozonation-extraction bioreactor. In aqueous solution, the solubility of ozone is relatively unstable. Its decomposition to oxygen in aqueous solution is continuous but slow based on a pseudo first-order reaction (Tomiyasu et al., 1985). Thus, the harvested algal slurries were mixed with methanol during the extraction process. Here different ozonation times (20, 40 and 60 minutes) were tested. The samples were analyzed by GC-MS and the main compounds detected were compared with NIST database and standard chemicals (Sigma Aldrich, UK). Temperature and microbubble effects (produced by fluidic oscillator) on product formation in the ozonation-extraction process were also considered.

This chapter is organized as follows: the next section presents the microalgal lipid extraction and yields (ozonation for 20, 40 and 60 minutes). The main compounds detect and the mechanisms involved are discussed. Next, a trace of methyl ester detected in 20 minute ozonation sample is discussed along with further transesterification processes using standard methods. Then, the extraction process with and without a fluidic oscillator is discussed, as are the effects of

temperature on lipid extraction. Finally, the energy usage and cost estimation of the ozonation extraction method is estimated and discussed.

7.2. Microalgal lipid extraction and yields

The harvested microalgal biomass (known as algal slurries) was ozonated in a 0.2 L ozonation bioreactor to extract the lipids from the cells. Figure 7.1 shows GC-MS chromatograms of compounds detected after the ozonation process. While the chemical compounds detected with highest probability based on NIST Database are summarized in Table 7.1. Ozonation of the mixture for 20 minutes produced several compounds (2-pentadecanone, 6, 10, 14-trimethyl; n-hexadecanoic acid (also known as palmitic acid); phytol and octadecanoic acid (also known as stearic acid). Ozonation for 40 and 60 minutes clearly produced 3 main compounds (2-pentadecanone, 6, 10, 14-trimethyl; palmitic acid; stearic acid). The obtained fatty acids include C16:0 and C18:1 which in agreement with the classification of *D. salina* in Chlorophyceae group. The classification of *D. salina* in Chlorophyceae group is well known and have been reported by many researchers (Assunção et al., 2012 and Giordano et al., 2014).

Due to the sensitivity of polyunsaturated fatty acid to oxidation (Lamers et al., 2012), we can observe the accumulation of saturated fatty acid (hexadecanoic acid, octadecanoic acid) along with the time of ozonation. Lin and Hong (2013), reported that ozonation of *Chlorococcum aquaticum* with methanol in a sand filtration reactor generated several products in the forms of long-chain largely saturated hydrocarbons with 16 to 20 carbons. They also suggested that with

ozonation, the composition of biodiesel can be controlled and would be beneficial for utilization in cold regions (unsaturated hydrocarbons) or more oxidation resistant (saturated hydrocarbons).

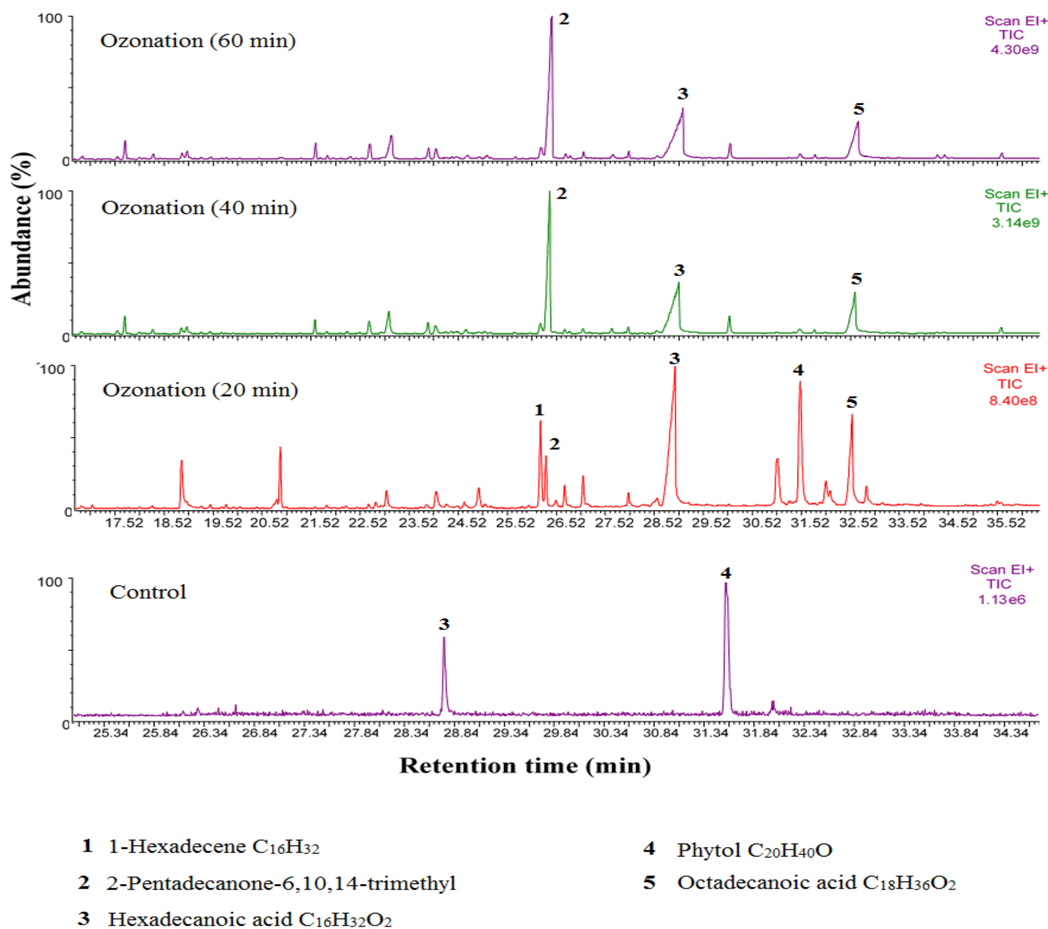

Figure 7.1: GC-MS chromatograms of the identified compounds in microalgae extracts collected after the ozonation process.

In this part, transesterification was not performed in order to study the possibility of direct transesterification by ozonation. It has been reported by Lin and Hong (2013), due to elevated

boiling and melting points, saturated compounds elute slowly in the GC column relative to their unsaturated compounds, resulting in their absence under conditions used for unsaturated hydrocarbons. However, with the setting used (GC-MS method, materials and methods), fatty acids were detected. This information was confirmed with the expert, Simon Thorpe from Faculty of Science Mass Spectrometry Centre. Unfortunately, without the esterification, the sensitivity is reduced. Thus, resulting in low % probability form the NIST Database. However, we have reconfirmed the peak with the standard compounds (Sigma Aldrich, UK).

Table 7.1: The chemical compounds detected with highest probability (NIST Database) and its concentration. Control represent sample without ozonation, while final is sample after 60 minutes of ozonation. These 3 compounds are the highest produced after 60 min of ozonation. The main products were reconfirmed with GCMS standards chemicals (Sigma Aldrich, UK).

Compound detected (NIST database)	Molecular formula	Probability (%)	Control concentration (g/g dry biomass)	Final concentration (g/g dry biomass)
2-Pentadecanone-6,10,14-trimethyl	$C_{18}H_{36}O$	83.5	-	0.114
Hexadecanoic acid	$C_{16}H_{32}O_2$	82.8	0.0007	0.099
Octadecanoic acid	$C_{18}H_{36}O_2$	78.9	-	0.061

The control for the present study produced low concentrations of hexadecanoic acid and phytol which are due to minimal breakage of the cells during separation process (solvent and centrifugation). Phytol is an acrylic diterpene alcohol which originates from chlorophyll

metabolism and is used in industries as a fragrance agent (flowery odor) (Yamamoto et al., 2014). The increase of 2-pentadecanone, 6, 10, 14-trimethyl, which has been previously reported in *Scenedesmus* and *Chlorella vulgaris* cells extracted by steam distillation (Rzama et al., 1995), could be due to the degradation of higher hydrocarbon compounds which is in this case, phytol. Figure 7.2 shows the possible degradation mechanism of phytol to 2-pentadecanone, 6, 10, 14-trimethyl by oxidation process.

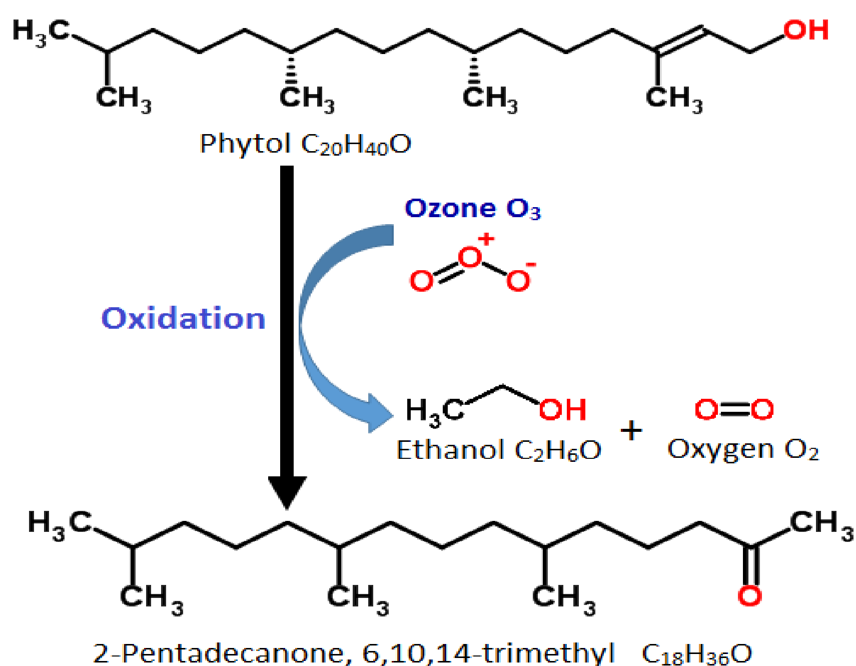

Figure 7.2: Degradation mechanism of phytol to 2-pentadecanone, 6, 10, 14-trimethyl by ozone oxidation.

The possible disruption mechanism for *D. salina* cells by oxidation process is illustrated in Figure 7.3. The cell membranes of *D. salina* cells are first attacked by ozone and unsaturated lipids and protein present in the membrane serve to be the prime target (Figure 7.3 C). A reaction called

oxidative burst occurred when the ozone molecule contacted with the cell membrane which creates a tiny hole resulting the cell to lose it shape. The presence of methanol will further disrupt and extract the internal lipids when in contact with the lipids inside of the *D. salina* cell (Figure 7.3 D).

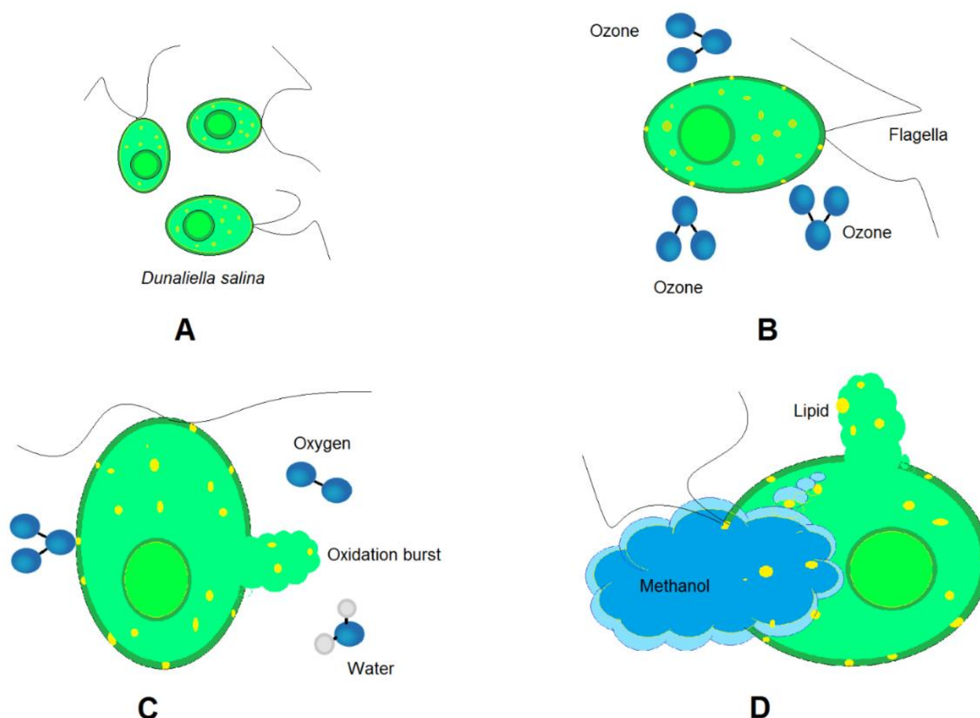

Figure 7.3: Disruption mechanism of *Dunaliella salina* cells by ozone oxidation.

Based on the findings, the lipid extraction by ozonation can possibly bypass the needs of energy intensive pretreatment methods such as microwave, bead mills, osmotic pressure, autoclaving, electroporation, and ultrasonication which previously have been reported to improve the efficiency of the solvent-extraction process (Garoma and Janda 2016). Moreover, ozonation-extraction process might possibly be the solution to the solvent diffusion limitation and lipids polarity problems faced due to the high water content in solvent extraction process (Fajardo et al.,

2007). However, further studies should be conducted as the present study was performed using *D. salina* known to lack a rigid cell wall (Oren, 2005).

7.3 Direct ozonation to produce FAME

In this study, no catalysts (such as acid, alkali or enzymes) were used in the lipid extraction process to analyse the potential of direct transesterification by ozonolysis (mentioned in section 2.7). Normally, in the production of fatty acid methyl esters (FAME), the transesterification reaction occurs only when the mono, di and tri varieties of acylglycerols are reacted with methanol in the presence of a catalyst (Xio et al., 2009 and Kim et al., 2013). In the chemical industry, alkali catalysts are widely used for their higher reaction rates and conversion efficiency compared with acid catalysts (Huang et al., 2010).

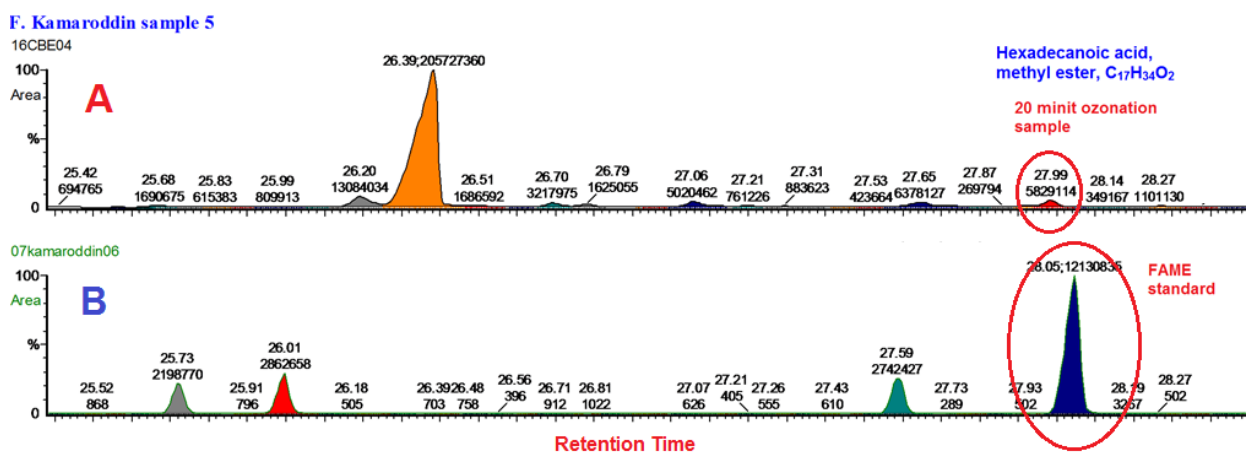

Figure 7.4: GC-MS chromatograms of the; A) 20 min ozonation sample, B) Methyl ester standard.

Based on comparison of the chromatogram peak with NIST database and methyl ester standards, a small trace of Hexadecanoic acid, methyl ester $C_{17}H_{34}O_2$, was detected in the 20 minute ozonation sample (Figure 7.4). However, the amount is too little and is insignificant compared with the main products gained through the same process (Table 7.1). A smaller trace of methyl ester compounds detected in a sample after 40 and 60 minutes of ozonation suggests that the compound might been oxidized by the ozonolysis process. However, further esterification of 20 minute samples produced two ester products (hexadecanoic acid methyl ester and octadecanoic acid methyl ester), as expected (Figure 7.5). Therefore, an experiment was performed to study the temperature effect (20 ˚C and 60 ˚C) versus ozonation time. In addition, an experiment was also conducted to study the bubbles size effect with and without an oscillator during ozonation at the same time.

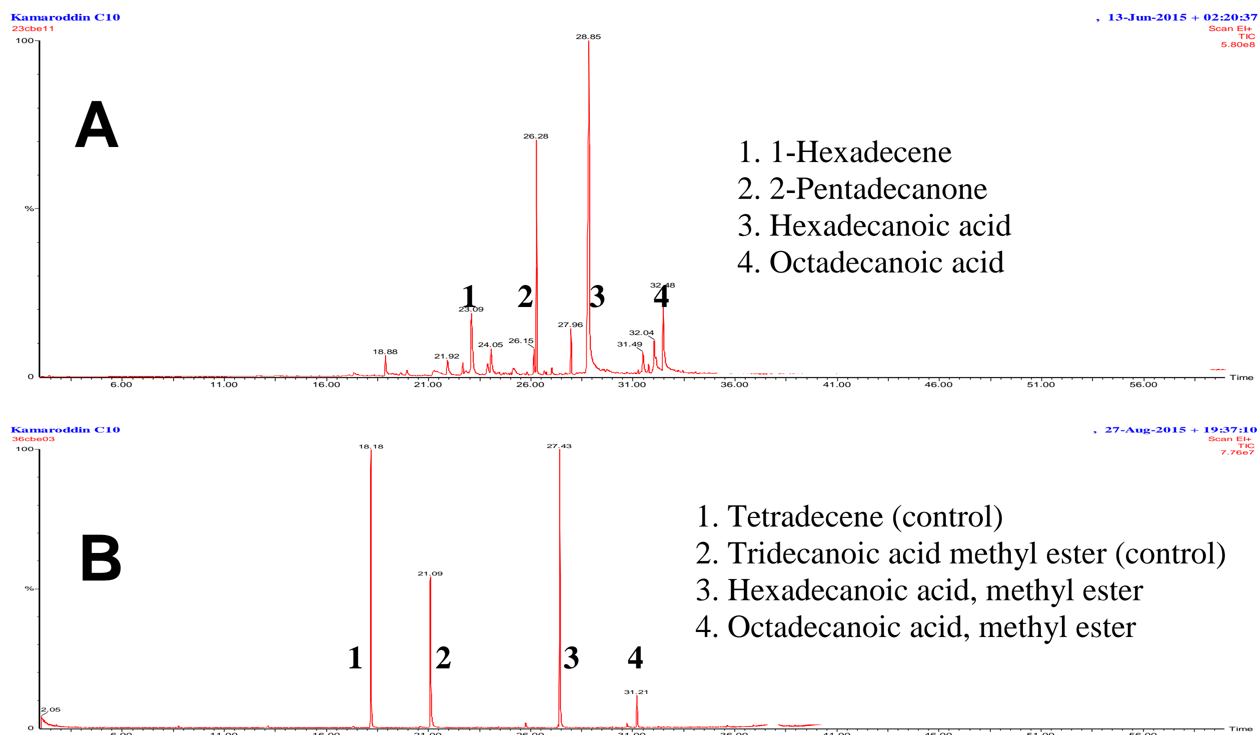

Figure 7.5: GC-MS chromatograms of the A) 20 min ozonation sample, B) Further esterification of 20 min ozonation sample

7.4 Optimization of Algal Lipid Extraction

The study continued with the optimization of extraction processes to gain higher algal lipids. Two main factor were studied in this part: the temperature and the effect of smaller bubbles (generated by a fluidic oscillator). The experimental set up was the same as in the previous experiment (section 7.3); however, a 0.1 L Schott bottle was heated on the hot plate with the maintained and monitored temperature (Figure Appendix 2). The experiments were divided into two parts: 1) ozonation for 80 minutes at 20 ˚C with and without a fluidic oscillator, 2) ozonation for 80 minutes at 60 ˚C with and without a fluidic oscillator. Due to an insufficient algal slurry, every experiment was conducted twice and with more diluted ratio of algal slurry over methanol than the previous experiments (1:5 v/v).

The ozonation at 20 ˚C increases the products (2-pentadecanone, 6, 10, 14-trimethyl; n-hexadecanoic acid and octadecanoic acid) with prolongation of ozonation time (Figure 7.6 A). Starting with 2.59 x 10^{-4}, 9.53 x 10^{-4} and 2.12 x 10^{-4} (g/g dry biomass) for 2-pentadecanone, 6, 10, 14-trimethyl; n-hexadecanoic acid and octadecanoic acid, respectively after 20 minutes of ozonation. It concludes with a maximum gained of 9.49 x 10^{-4}, 1.99 x 10^{-3} and 4.07 x 10^{-4} (g/g dry biomass) for 2-pentadecanone, 6, 10, 14-trimethyl; n-hexadecanoic acid and octadecanoic acid, respectively after 80 min of ozonation. However, with the integration of a fluidic oscillator (previously proven to produce smaller bubbles), the concentration of the 3 products slightly improved (Figure 7.6 B). The products that start with 3.78 x 10^{-4}, 6.82 x 10^{-4} and 6.31 x 10^{-5} (g/g dry biomass) after 20 minutes of ozonation end up with 1.17 x 10^{-3}, 2.87 x 10^{-3} and 6.37 x 10^{-4} (g/g dry biomass) for 2-pentadecanone, 6, 10, 14-trimethyl; n-hexadecanoic acid and octadecanoic

acid, respectively (after ozonation for 80 min). The product increments are 23.7%, 44.5% and 56.6% for 2-pentadecanone, 6, 10, 14-trimethyl; n-hexadecanoic acid and octadecanoic acid, respectively as summarized in Table 7.2.

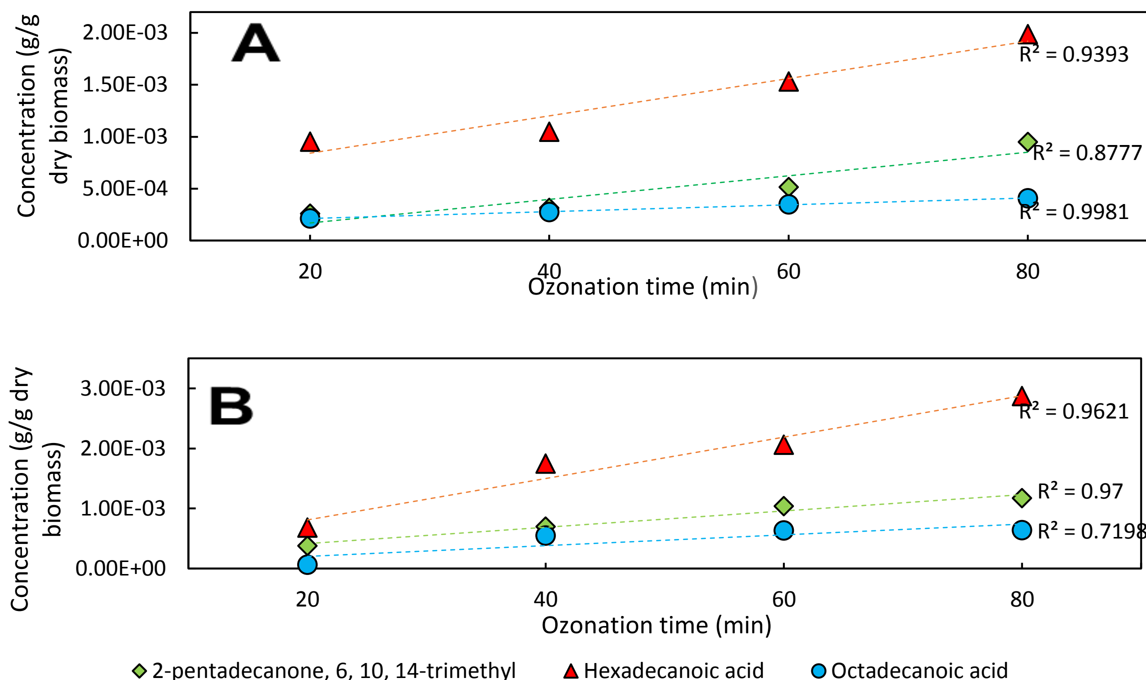

Figure 7.6: Ozonation of microalgal slurry with methanol (1:5 v/v) in 0.1 L ozonation extraction bioreactor. A) Ozonation at 20 ˚C without using fluidic oscillator, B) Ozonation at 20 ˚C with fluidic oscillator.

Table 7.2: Products improvement of ozonation lipid extraction at 20 ˚C for 80 min

Ozonation lipid extraction at (20˚C)	2-pentadecanone, 6, 10, 14-trimethyl (g/g dry biomass)	n-hexadecanoic acid (g/g dry biomass)	octadecanoic acid (g/g dry biomass)
Without FO	9.49×10^{-4}	1.99×10^{-3}	4.07×10^{-4}
With FO	1.17×10^{-3}	2.87×10^{-3}	6.37×10^{-4}
Improvement	23.7%	44.5%	56.6%

Further optimization of the algal lipid extraction process was carried out at 60 ˚C (Figure 7.7). Generally, the ozonation at 60 ˚C increased the products yields with prolongation of ozonation time (almost similar trend with ozonation at 20 ˚C). Starting with 6.93×10^{-4}, 1.23×10^{-2} and 3.05×10^{-3} (g/g dry biomass) for 2-pentadecanone, 6, 10, 14-trimethyl; n-hexadecanoic acid and octadecanoic acid, respectively after 20 min of ozonation, it ends with the maximum gained of 1.14×10^{-3}, 2.28×10^{-2} and 4.41×10^{-3} (g/g dry biomass) for 2-pentadecanone, 6, 10, 14-trimethyl; n-hexadecanoic acid and octadecanoic acid, respectively after 80 minutes of ozonation (Figure 7.7 A). However, with the integration of a fluidic oscillator, the concentrations of the 3 products were again slightly improved (Figure 7.7 B). The products start with 8.79×10^{-4}, 1.26×10^{-2} and 4.63×10^{-3} (g/g dry biomass) after ozonation for 20 minutes and end with 2.91×10^{-3}, 4.30×10^{-2} and 1.10×10^{-2} (g/g dry biomass) for 2-pentadecanone, 6, 10, 14-trimethyl; n-hexadecanoic acid and octadecanoic acid, respectively (after ozonation for 80 minutes). The products increased significantly with the use of the fluidic oscillator by around 156%, 88.9% and 150% for 2-pentadecanone, 6, 10, 14-trimethyl; n-hexadecanoic acid and octadecanoic acid, respectively as summarized in Table 7.3.

Based on the experimental results, the advantages of using microbubbles are highlighted. Smaller sized bubbles require less time to dissolve gaseous compounds (ozone) than larger bubbles. Apart from that, the mass transfer also increases when the gas-flow rate increases. The integration of a fluidic oscillator significantly increased product formation compared to lipid extraction processes that do not use it. Even though pressure-assisted ozonation (PAO) methods have been reported to disrupt 80.3% of *Chlorella* cells and to produce 24% (w/w) of lipid yield (Huang et al., 2016), we still believe that our method is more energy efficient, because our method

does not use a pressure mechanism. However, further study using our method should be conducted to make an apple-to-apple comparison.

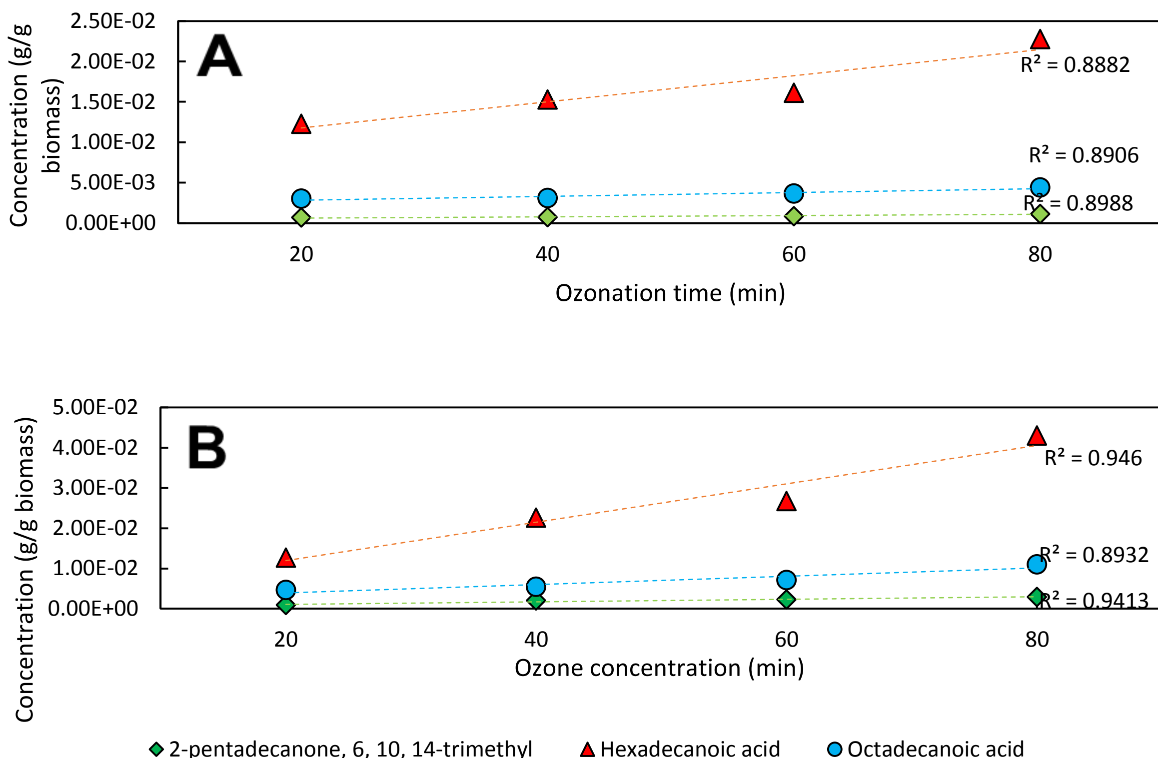

Figure 7.7: Ozonation of microalgal slurry with methanol (1:2 v/v) in 0.2 L ozonation extraction bioreactor. A) Ozonation at 60 ˚C without using fluidic oscillator, B) Ozonation at 60 ˚C with fluidic oscillator.

Table 7.3: Products improvement of ozonation lipid extraction at 60 ˚C for 80 min

Ozonation lipid extraction (60˚C)	2-pentadecanone, 6, 10, 14-trimethyl (g/g dry biomass)	n-hexadecanoic acid (g/g dry biomass)	octadecanoic acid (g/g dry biomass)
Without FO	1.14×10^{-3}	2.28×10^{-2}	4.41×10^{-3}
With FO	2.91×10^{-3}	4.30×10^{-2}	1.10×10^{-2}
Improvement	156%	88.9%	150%

7.5 Energy Consumption and Cost of Lipid Extraction by Ozonation

In obtaining algal lipids, two major and expensive steps are involved: algal harvesting and lipid extraction (Lin and Hong, 2013). The energy demand for the centrifugation process is 90% of the energy gained in the production of the biofuel; for press filtration the energy demand is approximately 79% of total energy and a further step that follows dewatering in the extraction of the solvent requires an extra 10% of the energy used in the production process (Brentner et al. 2011). Other routes such as ultrasonication and supercritical CO_2 also require large amounts of energy: about 110% and 66% respectively of the total biodiesel production energy (Brentner et al. 2011). From this explanation, it is clear that the whole of the energy budget in the production of biodiesel is consumed by the dewatering and lipid-extraction processes. Thus, it is crucial that the harvesting and extraction of lipids should be made more efficient in terms of the consumption of energy.

Energy consumption of different microalgae extraction methods taken from recent studies is summarized in Table 7.4. The method developed and utilized in this study uses microflotation (harvesting) followed by algal-ozone rupturing in methanol (disruption and extraction). According to Barathraj (2013), normally 8 watt of power is required by a good ozone generator (medium frequency, 800 Hz - 1000 Hz) to produce 1 gram of ozone. In this study 48 mg of ozone is required to extract 1 g of dry mass (within 60 min) which will required 48 g of ozone to extract 1 kg of dry mass. Therefore the energy consumption of the ozone generator is about 48 kWh which is equivalent to 172.8 MJ (1 kWh = 3.6 MJ). Based on an electricity price of £0.12 per kWh (UK

Power, 2016), the cost of the ozonation extraction using ozone generator is about £5.76 per 1 kg of algal dry mass.

Table 7.4: Energy consumption of different microalgae extraction methods taken from recent studies

Extraction Method	Microalgae	Energy consumption, (MJ kg^{-1} dry mass)	References
Bead mills	*Chlorella, Botryococcus, Scenedesmus,* (Laboratory, industrial)	504	(Lee et al., 2010)
Microwave	*Chlorella, Botryococcus, Scenedesmus,* (Laboratory)	420	(Lee et al., 2010)
Sonication	*Chlorococcum sp.,* (Laboratory, industrial)	132	(Halim et al., 2012)
High pressure homogenization (HPH)	*Chlorococcum sp.* (Laboratory)	529	(Halim et al., 2012)
Hydrodynamic cavitation	*Saccharomyces cerevisiae* (Laboratory, industrial)	33	(Balasundaram and Pandit 2001)
Ozone-rich Microbubbles	*Dunaliella salina* (Laboratory)	2.16	Recent study

However, according to Lin and Hong (2013), commercially the amount of electricity required to generate ozone is in the range of 8–17 kWh kg^{-1} O_3. In this study, an ozone dose of approximately 48 g O_3 per kg dry algae was used to rupture algae and extract lipids. Thus, approximately 1.38 to 2.94 MJ kg^{-1} dry algae (384 Wh to 816 Wh) electrical energy would be required to generate ozone for cell disruption and lipid extraction. As such, the cost of energy for rupturing algae with ozone at 2.16 MJ kg^{-1} algae (average of 1.38 and 2.94 MJ kg^{-1} algae) is a small fraction of the energy that is used in the production of biodiesel (see Table 7.4). Additionally, the cost of algal lipid extraction using a commercial ozone generator is about £0.05 per 1 kg of algal dry mass which is based on an electricity price of £0.12 per kWh (UK Power, 2016).

According to Lin and Hong (2013), approximately 40 MJ kg^{-1} are used for the production of biodiesel and approximately 6 MJ kg^{-1} for the rupturing of algae. This is based on the assumption that 15% of the content of lipids can be converted to a similar amount of biodiesel. Unlike centrifugation and solvent extraction methods, which consume more than 90% of the energy, this method consumes only 36% (2.16/6) of the energy. Though the technique used in this study does not consider all the energy that a full-scale system may require, it is clear that employing this technique makes it possible to use less energy than that used in centrifugation and solvent extraction. However, this process has been tested on a non-cell wall microalgae with the assumption that the energy required to disrupt and extract lipids from *D. salina* cells is less than for a microalgae with a cell wall. Further studies should be conducted on microalgae containing cell walls such as *Chlorella* sp., *Chlorococcum* sp., *Botryococcus* sp. and *Scenedesmus* sp. in order to fairly compare the energy consumption and cost estimation for the cell disruption and algal lipid extraction for each microalgae.

7.6 Summary

In this chapter, there are 2 objectives that have been achieved which are (1) identification and optimization of the the products extracted with the ozonation extraction method and, (2) the estimation the energy usage and cost of the ozonation extraction method. The lipid extraction of a *D. salina* slurry with methanol (1:2 v/v) was performed in a 0.2 L bioreactor at room temperature with direct ozonation (8 mg L^{-1}). Ozonation for 60 minutes accumulates three main compounds: 2-pentadecanone, 6, 10, 14-trimethyl, palmitic acid and stearic acid. A small trace of hexadecanoic acid, methyl ester $C_{17}H_{34}O_2$, was detected in a 20 minute ozonation sample. Unfortunately, the amount is too small and is not significant compared to the main products gained through the same process. When the temperature was increased (60 ˚C) and smaller bubbles were introduced during extraction, the concentration of products increased significantly to around 156%, 88.9% and 150% for 2-pentadecanone, 6, 10, 14-trimethyl; n-hexadecanoic acid and octadecanoic acid, respectively. The energy usage for extracting *D. salina* lipid with ozone has been estimated to be around 2.16 MJ kg^{-1} dry algae which is a small fraction of the energy that is used in the production of biodiesel (40 MJ kg^{-1}). Unlike centrifugation and solvent extraction methods, which consume more than 90% of the energy, this method consumes only 36% (2.16/6) of the energy. Further studies should be carried out with microalgae containing cell walls and up scaling of the bioreactors to a larger volume is crucial in order reduce the biofuel production cost commercially.

CHAPTER 8

CONCLUSION AND FUTURE WORKS

8.1 Conclusion

A novel microbubble-driven photobioreactor system that integrates ozonation into the production stage, microflotation during harvesting and lipid extraction by direct ozonation has been developed and tested. Study of the effect of ozone on the mixed algal/bacterial culture showed that intermittent disinfection can be used to eliminate or reduce bacterial contaminants. The sterilisation efficiency reached 66% after 5 minute and increased to 93% after 10 minutes of ozonation. Exposure of a 14-day-old *D. salina* culture to ozone for 10 minutes did not harm the microalgal cells. Three significant factors (culture volume, ozone concentration and ozonation time) have been optimized and applied to achieve a higher cell disruption efficiency. The optimum value suggested by the RSM software is 30.63 mL of culture medium, 8.2 mg L^{-1} of ozone concentration and 37.7 minutes of ozonation time. The optimum conditions were then tested on *Halomonas* (the main bacterial contaminant of *D. salina* cells) and a 5.77 log reduction was achieved which is near to the sterilization value achieved by using an autoclave (6 log reduction) proving that this method is applicable to sterilize a small volume container or bioreactor.

The study of CO_2 gas-flow rate suggests that the best flow rate for algal biomass production in an airlift loop bioreactor is 0.9 L min^{-1}. Increasing the gas flow rate above 0.9 L min^{-1} reduces algal cell (biomass) production due to a high degree of turbulence, which is detrimental to the wall-less *D. salina* cells. Further, harvesting using microflotation results in a high recovery efficiency with the highest being 93.4% at 700 mg L^{-1}, which is in agreement with the previous study (Hanotu et al., 2013). While the ozonation of harvested microalgal cells in methanol ruptures the cells, extracts the algal lipids and accumulates saturated fatty acid. Ozonation for 60 minutes accumulates three main compounds: 2-pentadecanone, 6, 10, 14-trimethyl, palmitic acid and stearic acid. The integration of a fluidic oscillator to produce smaller bubbles at higher temperatures during the algal lipid-extraction process significantly increases the lipid yield to around 156%, 88.9% and 150% for 2-pentadecanone, 6, 10, 14-trimethyl; n-hexadecanoic acid and octadecanoic acid, respectively. The energy usage for extracting *D. salina* lipid with ozone has been estimated around 2.16 MJ kg^{-1} dry algae (costing around £0.05 per 1 kg of algal dry mass) which is a small fraction of the energy that is used in the production of biodiesel (40 MJ kg^{-1}). Overall, these results are readily scalable by matching local bubble flux rates on a large scale.

8.2 Future Works

The results of this research show that ozone-rich microbubbles can significantly reduce bacterial contamination and simplify algal lipid extraction process. However, the study does not show whether this method is applicable to all microalgae strains, due to different cell wall characteristics. Therefore, I would like to give a few recommendations for future work:

1. The disinfection efficiency in a larger bioreactor should be conducted as the current study was conducted on a small scale due to incompatible bioreactor material (acrylamide material). A larger glass or stainless steel bioreactor (3 L) should be build and tested.
2. The ozonation of other microalgal strains such as *Chlorella, Chlamydomonas* and *Tetraselmis* sp which have all been reported to have high lipid content should be conducted. This will give a fair comparison whether the method is applicable to wider range of microalgae or not.
3. The ozonolysis method did not esterify the algal lipid much in fact it is too little to be useful. Thus, the addition of catalysts during the algal lipid extraction process should be carried out in order to perform direct transesterification of algal lipid to fatty acid methyl ester (FAME).
4. Life Cycle Assessment (LCA) of microalgae biofuel using the method described in this thesis should be conducted in order to fairly compare the energy efficiency and cost with the other reported methods. This was not done in the current study due to insufficient information on the ozone generator used.
5. The bioreactor system can be simplified (combination of all system in one bioreactor) as illustrated in Figure Appendix 22.

8.3 Publication

Kamaroddin, M. F., Hanotu, J., Gilmour, D. J. & Zimmerman, W. B. (2016). In-situ disinfection and a new downstream processing scheme from algal harvesting to lipid extraction using ozone-rich microbubbles for biofuel production. *Algal Research,* 17**,** 217–226. (Q1, IF 5.01). Available at: http://dx.doi.org/10.1016/j.algal.2016.05.006

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In-situ disinfection and a new downstream processing scheme from algal harvesting to lipid extraction using ozone-rich microbubbles for biofuel production

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ABSTRACT

The scaling up and downstream processing costs of biofuels from microalgae are major concerns. This study focuses on reducing the cost by using energy efficient methods in the production of microalgae biomass and the downstream processes (biomass harvesting and lipid extraction). Ozonation of *Dunaliella salina* (green alga) and *Halomonas* (Gram-negative bacterium) mixed cultures for 10 min at 8 mg/L resulted in a reduction in the bacterial contaminant without harming the microalga. Harvesting of *D. salina* cells through microflotation resulted in a 93.4% recovery efficiency. Ozonation of the harvested microalgal cells for 60 min produced three main saturated hydrocarbon compounds (2-pentadecanone, 6, 10, 14-trimethyl; hexadecanoic acid; octadecanoic acid) consisting of 16 to 18 carbons. By systematically switching the carrier gas from CO_2 to O_3, the microbubble-driven airlift loop bioreactor (ALB) delivers nutrient to the culture and in-situ disinfection respectively. Further, modulating the bubble size to match particle size ensures recovery of the cells after culture. All three key operations (disinfection, harvesting and lipid extraction) are assembled in a scalable, relatively energy efficient process.

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Zitelli, G. C., Rodolfi, L., Biondi, N. and Tredici, M. R. (2006) 'Productivity and photosynthetic efficiency of outdoor cultures of *Tetraselmis suecica* in annular columns', *Aquaculture*, 261, pp. 932-943.

APPENDICES

Table Appendix 1: *D. salina* growth media

The stock solutions are prepared in distilled water	
2.0 M MgCl2.6H20	91.5 g in 225 ml
4.0 M NaNO3	34.0 g in 100 ml
0.1 M NaH2PO4	3 g in 250 ml
1.5 mM FeEDTA pH 7.6	0.055 g in 100 ml
2.4 M MgSO4.7H20	133.1 g in 225 ml
1.0 M CaCl2.2H20	33.1 g in 225 ml
0.5 M Na2SO4	63.9 g in 900 ml
2.0 M KCl	74.6 g in 500 ml
1.0 M HEPES pH 7.6	59.6 g in 250 ml

Trace elements		
185 mM H3BO3	4.576 g	all in a total of 400ml
0.8 mM ZnCl2	0.044 g	
7 mM MnCl2.4H20	0.554 g	
0.02 mM CoCl2	4 ml of 2 mM	
CoCl2.6H20 (2 mM)	0.107 g in 225 ml	
CuCl2.2H20 (0.2 mM)	0.031 g in 900 ml	
0.0002 mM CuCl2	0.4ml of 0.2 mM	

Algal culture medium (Vonshak, 1986).		
Stock Solution	Concentration wanted (mM)	Volume of stock for 1litre (ml)
2 M KCl	10	5
1 M $CaCl_2$	10	10
4 M $NaNO_3$	5	1.25
100 mM NaH_2PO_4	0.1	1
2 M $MgCl_2$	20	10
2.4 M $MgSO_4$	24	10
0.5 M Na_2SO_4	24	48
Solid NaCl	e.g. 1.5 M	87.75 g
1.5 mM FeEDTA	0.002	1
1 M HEPES pH 7.6	20	20
$NaHCO_3$ (solid)	1 g litre-1	1 g
Trace elements	1 ml litre-1	1

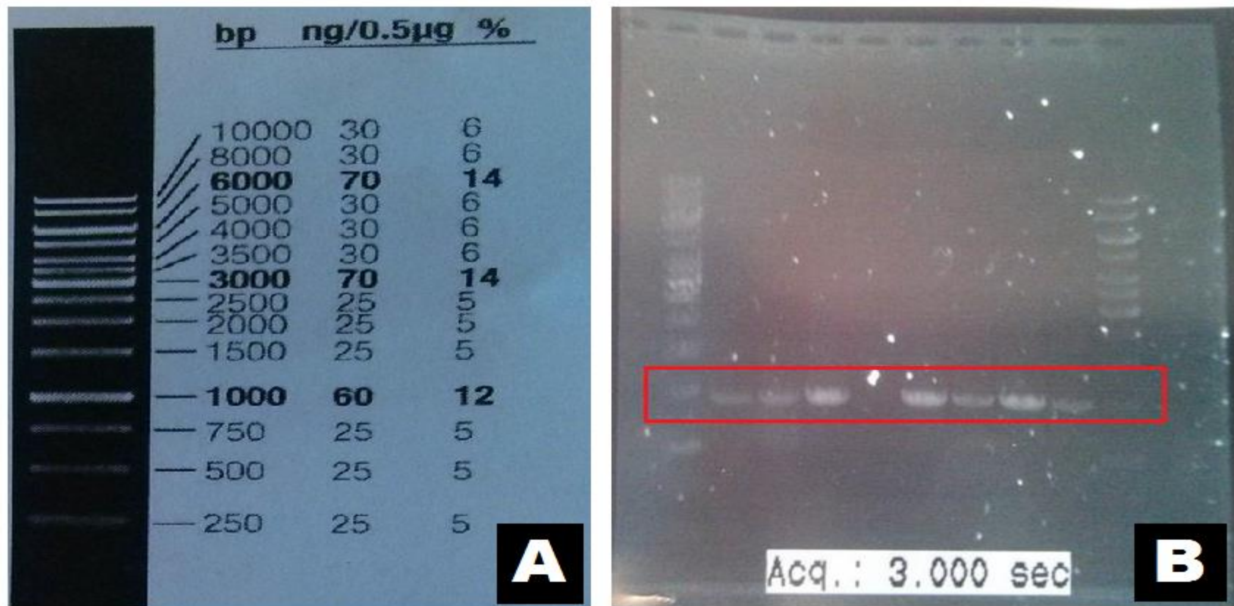


Figure Appendix 1: A) Bands of 10 kb DNA ladders and B) Total genomic DNA extracts with sizes around 1000 base pairs from the same strains (different bead beating time).

Table Appendix 2: Forward Primer for amplification of 16S rRNA

Template	Primer	Left Clip	Right Clip	Length
F1 Forward	16S Forward	208	866	658
F1 Forward	16S Forward	269	959	690
G1 Forward	16S Forward	259	836	577
G1 Forward	16S Forward	21	972	951
G3 Forward	16S Forward	22	912	890

Table Appendix 3: Fasta Sequences of *Halomonas* gene

Fasta sequences:

>F1 For_16S-For -- 208..866 of sequence
GCTTGTTGGTGAGGTAAAGGCTCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGA
TCAGCCACATCGGGACTGAGACACGGCCCGAACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCTTTCGGGT
TGTAAAGCACTTTCAGTGAGGAAGAAGGCCTTGGGGCTAATACCCCCGAGGAAGGACATC
ACTCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCG
AGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGCGGCGTGATAAGCCGGTTGTG
AAAGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACTGTCAGGCTAGAGTGCAGGAGAG
GAAGGTAGAATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAATACCAGTGGC
GAAGGCGGCCTTCTGGACTGACACTGACGCTGAGGTGCGAAAGCGTGGGTAGCAAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGGTCCTAGAG
ACCTTTGTGGCGCAGTTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTT

>F1 For_16S-For -- 269..959 of sequence
AGCCACATCGGGACTGAGACACGGCCCGAACTCCTACGGGAGGCAGCAGTGGGGAATATT
GGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCTTTCGGGTTG
TAAAGCACTTTCAGTGAGGAAGAAGGCCTTGGGGCTAATACCCCCGAGGAAGGACATCAC
TCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAG
CGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGCGGCGTGATAAGCCGGTTGTGAA
AGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACTGTCAGGCTAGAGTGCAGGAGAGGA
AGGTAGAATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAATACCAGTGGCGA
AGGCGGCCTTCTGGACTGACACTGACGCTGAGGTGCGAAAGCGTGGGTAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGGTCCTAGAGAC
CTTTGTGGCGCAGTTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAA
ACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCA
ACGCGAAGAACCTTACCTACCCTTGACATCG

>G1 For_16S-For -- 259..836 of sequence
ATGATCAGCCACATCGGGACTGAGACACGGCCCGAACTCCTACGGGAGGCAGCAGTGGGG
AATATTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCCTC
GGGTTGTAAAGCACTTTCAGCGAGGAAGAACGCCTGTCGGTTAATACCCGTCAGGAAAGA
CATCACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGG
TGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGCTTGATAAGCCGGT
TGTGAAAGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACTATGAGGCTAGAGTGCAGG
AGAGGAAGGTAGAATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAATACCAG
TGGCGAAGGCGGCCTTCTGGACTGACACTGACACTGAGGTGCGAAAGCGTGGGTAGCAAA
CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACCAGCCGTTGGGTGCCT
AGCGCACTTTGTGGCGAAGTTAACGCGATAAGTCGACC

>G1 For_16S-For -- 21..972 of sequence
TGCAAGTCGAGCGGTAACAGGTCCAGCTTGCTGGATGCTGACGAGCGGCGGACGGGTGAG
TAACGCATAGGAATCTACCCGGTAGTGGGGGATAACCTGGGAAACCCAGGCTAATACCG
CATACGTCCTACGGGAGAAAGGGGGCTTAGGCTCCCGCTATTGGATGAGCCTATGCCGGA
TTAGCTGGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGA
TGATCAGCCACATCGGGACTGAGACACGGCCCGAACTCCTACGGGAGGCAGCAGTGGGGA
ATATTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCCTCG
GGTTGTAAAGCACTTTCAGCGAGGAAGAACGCCTGTCGGTTAATACCCGTCAGGAAAGAC
ATCACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGT
GCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGCTTGATAAGCCGGTT
GTGAAAGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACTATGAGGCTAGAGTGCAGGA
GAGGAAGGTAGAATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAATACCAGT
GGCGAAGGCGGCCTTCTGGACTGACACTGACACTGAGGTGCGAAAGCGTGGGTAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACCAGCCGTTGGGTGCCTA
GCGCACTTTGTGGCGAAGTTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGG
TTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG
ATGCAACGCGAAGAACCTTACCTACCCTTGACATCCTGCGAACTTGTGAGAG

>G3 For_16S-For -- 22..912 of sequence
CAGTCGAGCGGTAACAGGTCCAGCTTGCTGGATGCTGACGAGCGGCGGACGGGTGAGTAA
CGCATAGGAATCTACCCGGTAGTGGGGGATAACCTGGGAAACCCAGGCTAATACCGCAT
ACGTCCTACGGGAGAAAGGGGGCTTAGGCTCCCGCTATTGGATGAGCCTATGCCGGATTA
GCTGGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGA
TCAGCCACATCGGGACTGAGACACGGCCCGAACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCCTCGGGT
TGTAAAGCACTTTCAGCGAGGAAGAACGCCTGTCGGTTAATACCCGTCAGGAAAGACATC
ACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCA
AGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGCTTGATAAGCCGGTTGTG
AAAGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACTATGAGGCTAGAGTGCAGGAGAG
GAAGGTAGAATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAATACCAGTGGC
GAAGGCGGCCTTCTGGACTGACACTGACACTGAGGTGCGAAAGCGTGGGTAGCAAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACCAGCCGTTGGGTGCCTAGCG
CACTTTGTGGCGAAGTTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTAA
AACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTAA

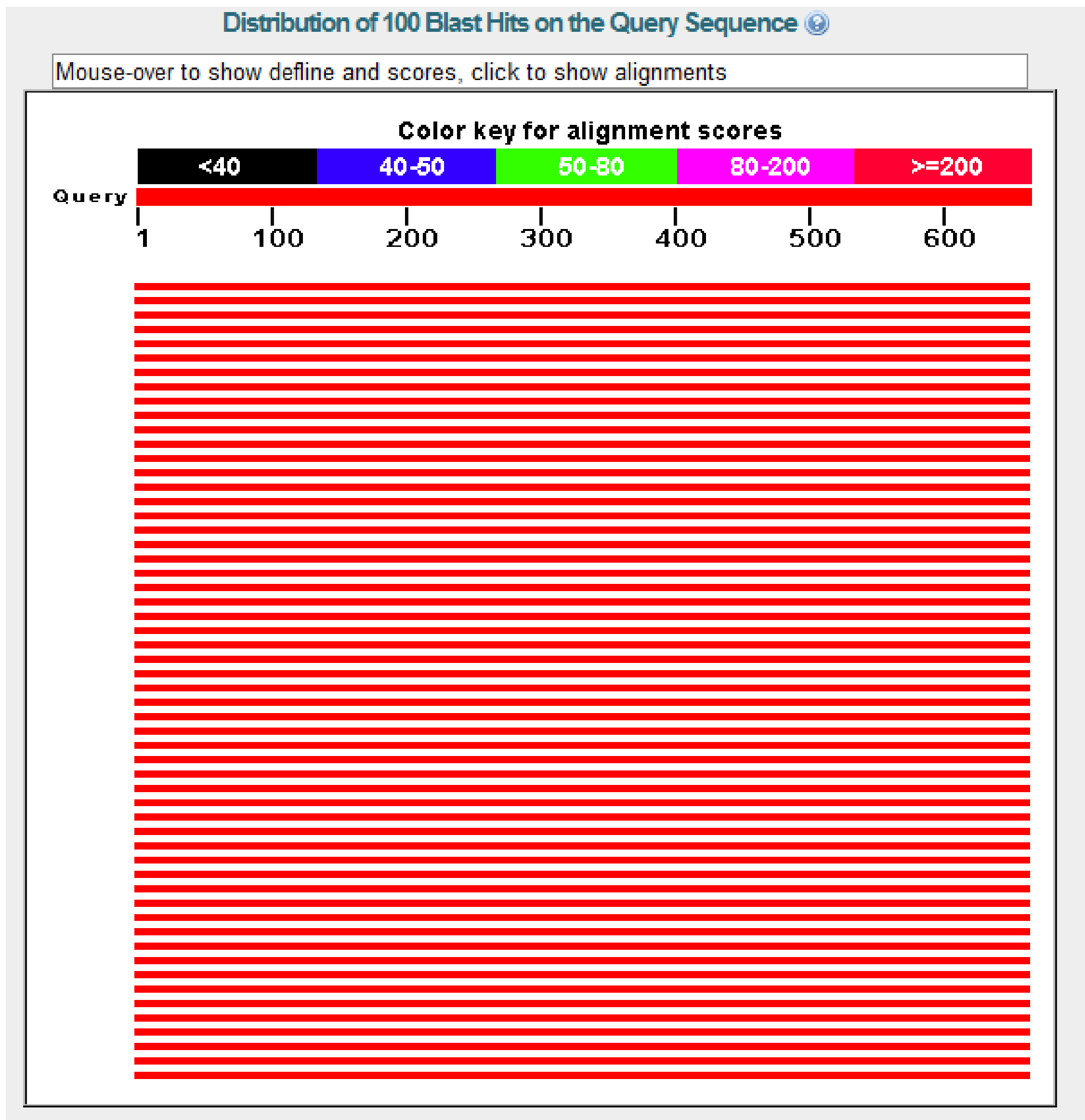


Figure Appendix 1.1: DNA sequence received was run through BLAST database and shows 100% similarity with *Halomonas* sp.

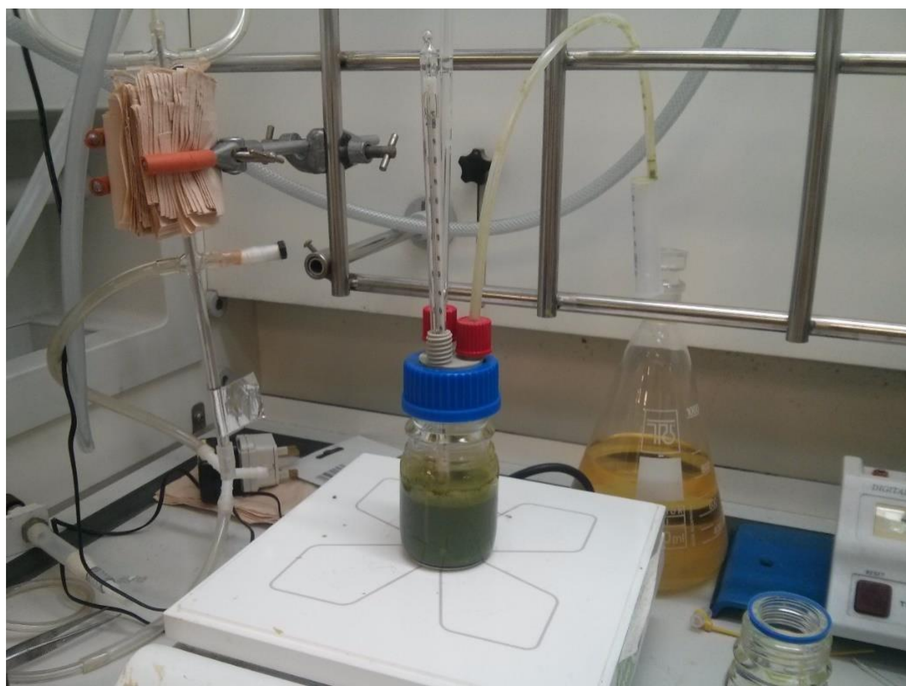


Figure Appendix 2: Ozonation of algal slurry at 60 ˚C

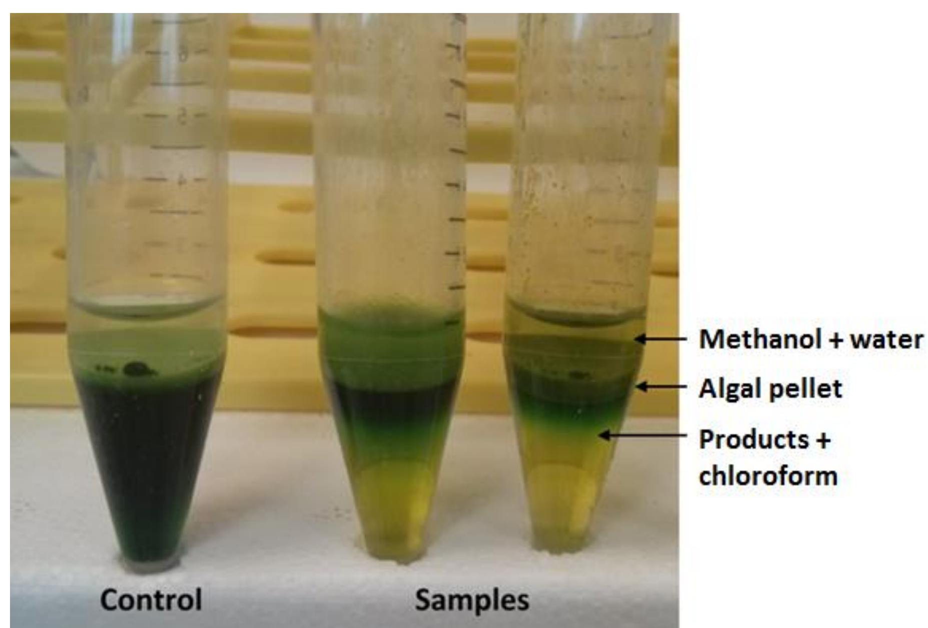


Figure Appendix 3: Products seperation

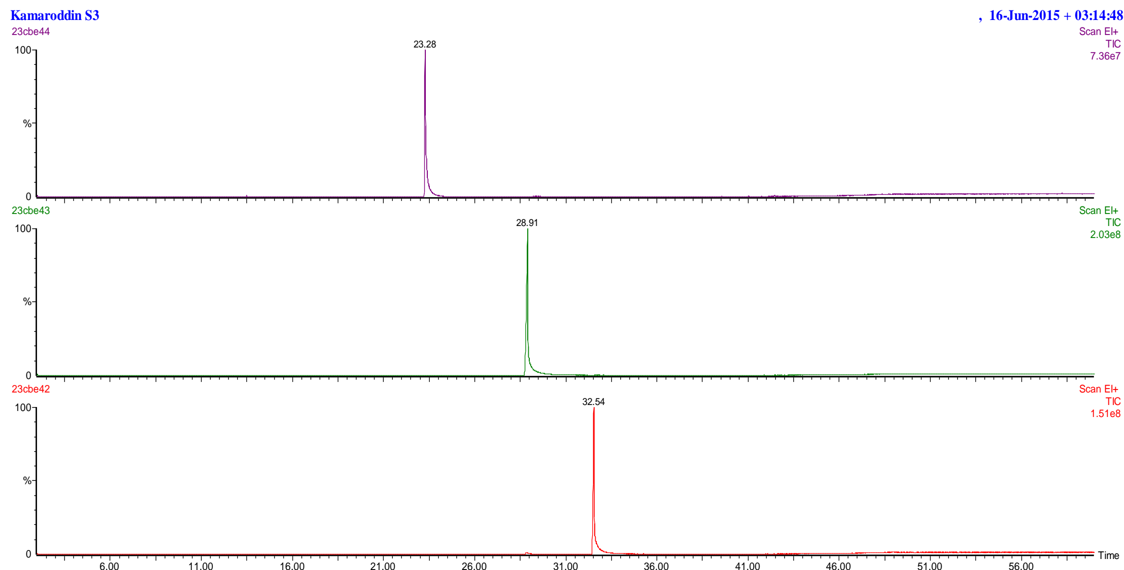


Figure Appendix 4: GC-MS chromatogram of main compounds (2-Pentadecanone, n-hexadecanoic acid, Octadecanoic acid)

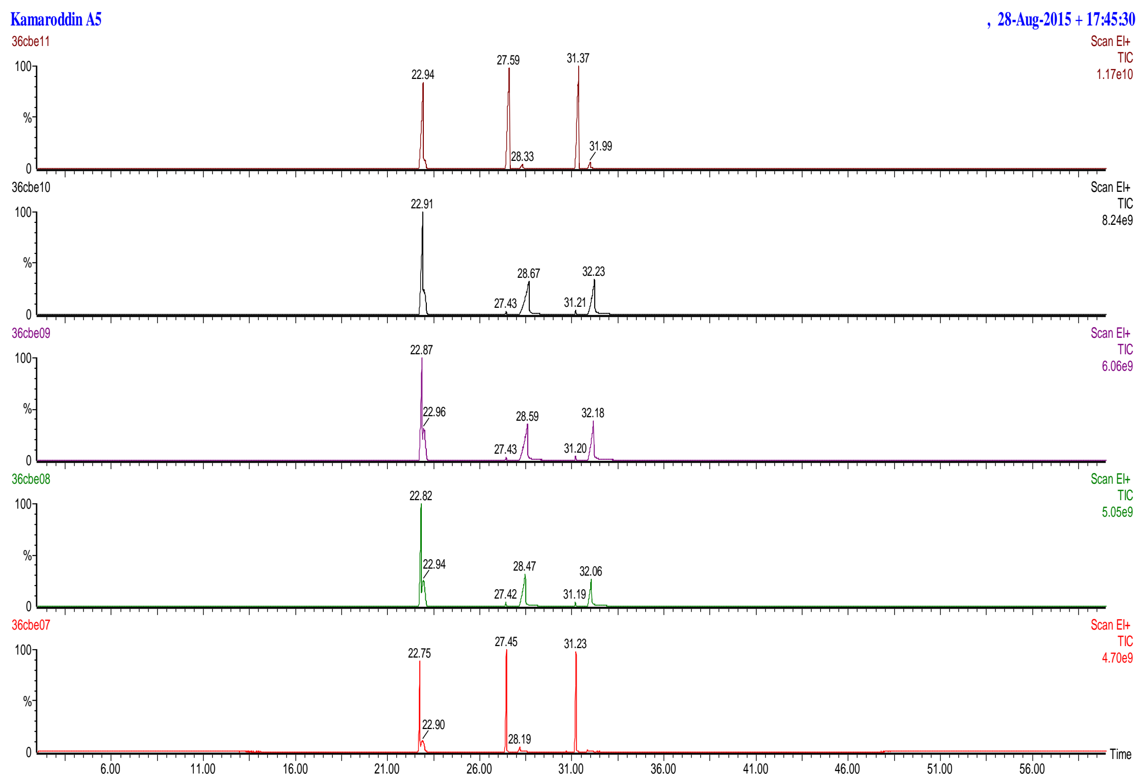


Figure Appendix 5: GC-MS chromatogram of main compounds (Standards)(2-Pentadecanone, n-hexadecanoic acid, Octadecanoic acid)

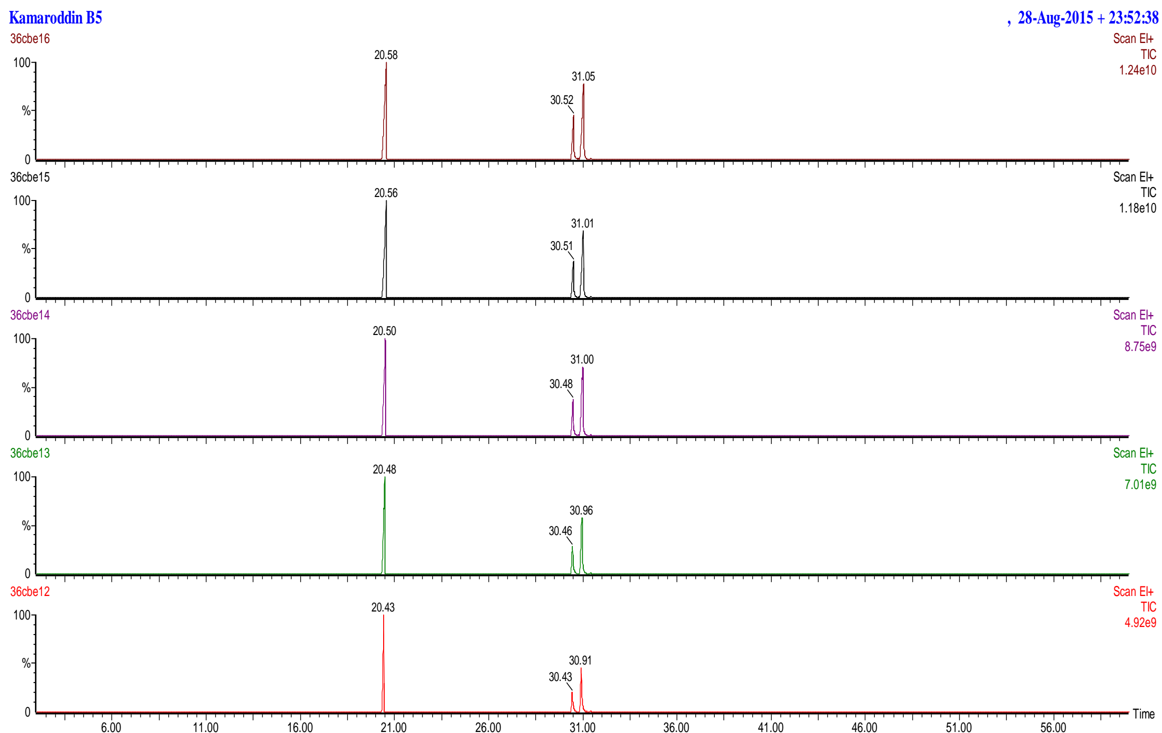


Figure Appendix 6: GC-MS chromatogram of main compounds (Standards)

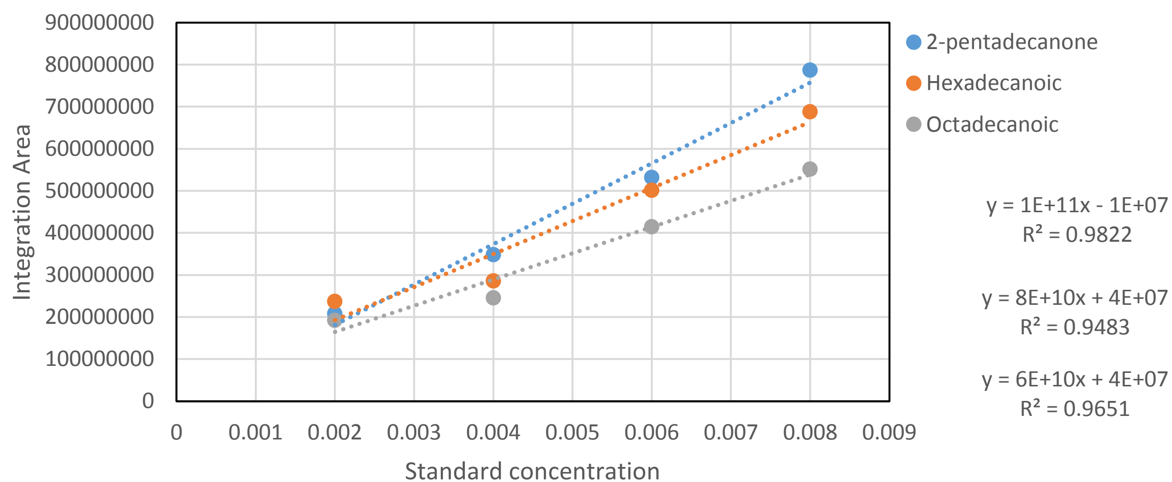


Figure Appendix 7: Standard curve of main compounds (Standards)

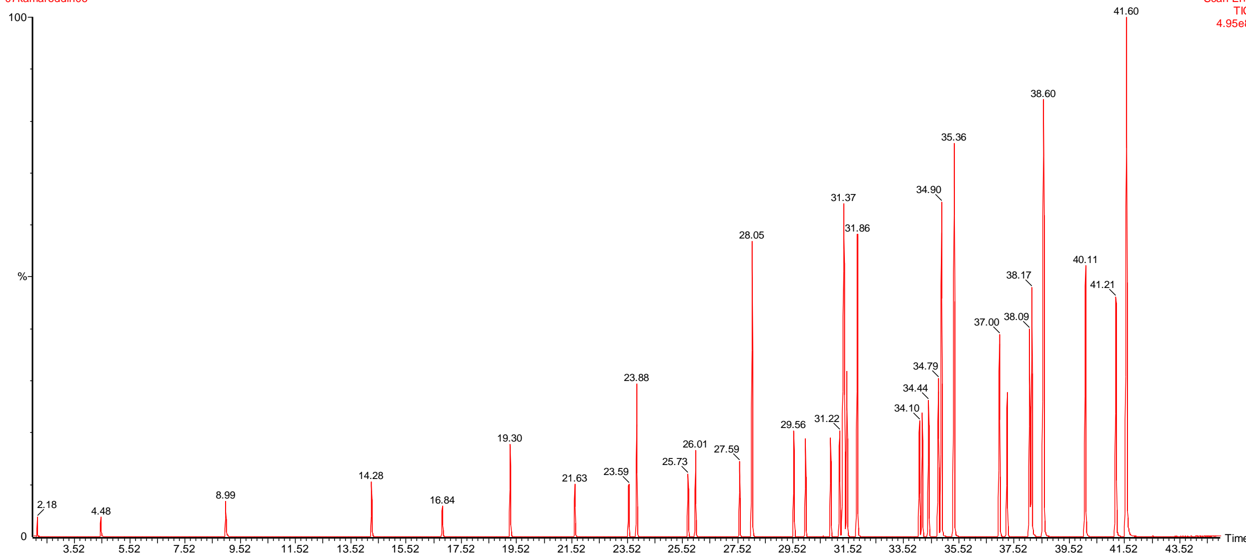
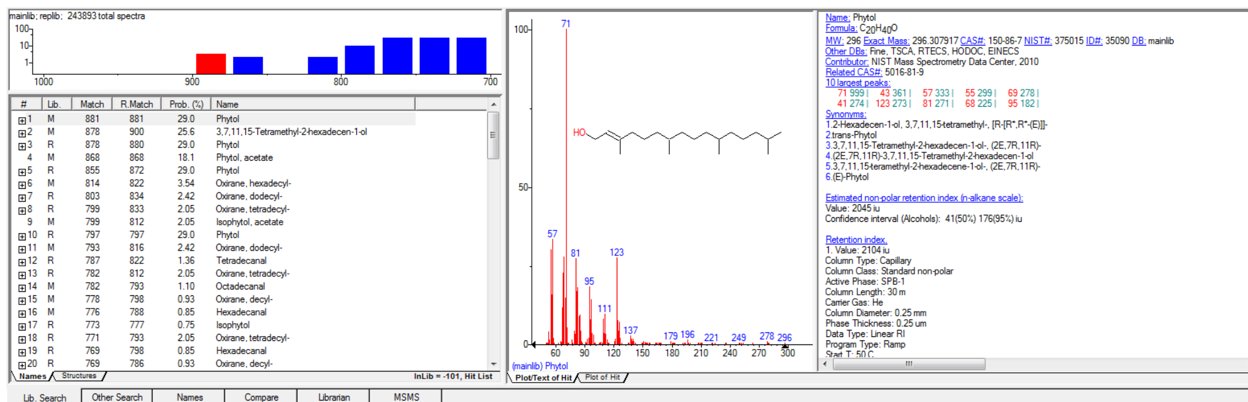


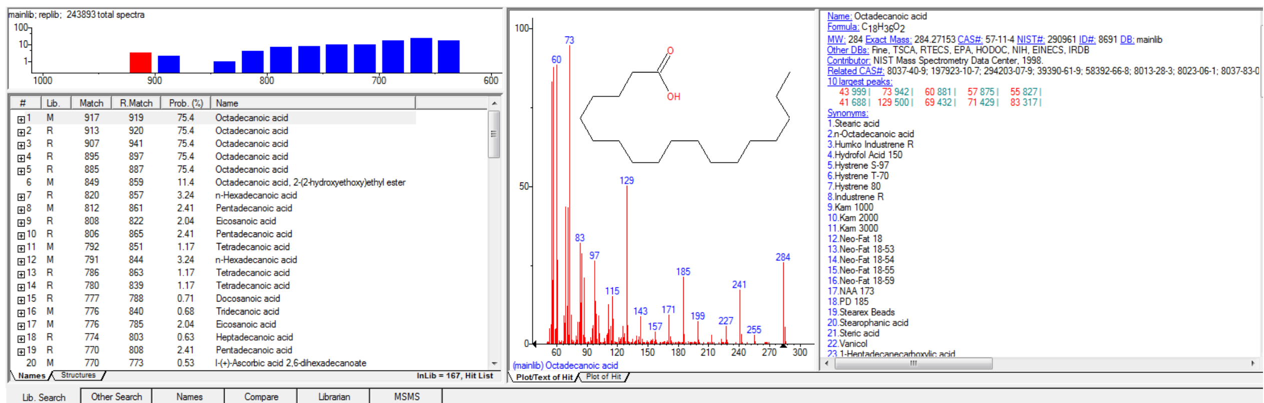
Figure Appendix 8: GC-MS chromatogram of FAME (Standards)

Figure Appendix 9: Comparison of GC-MC peak with NIST Database

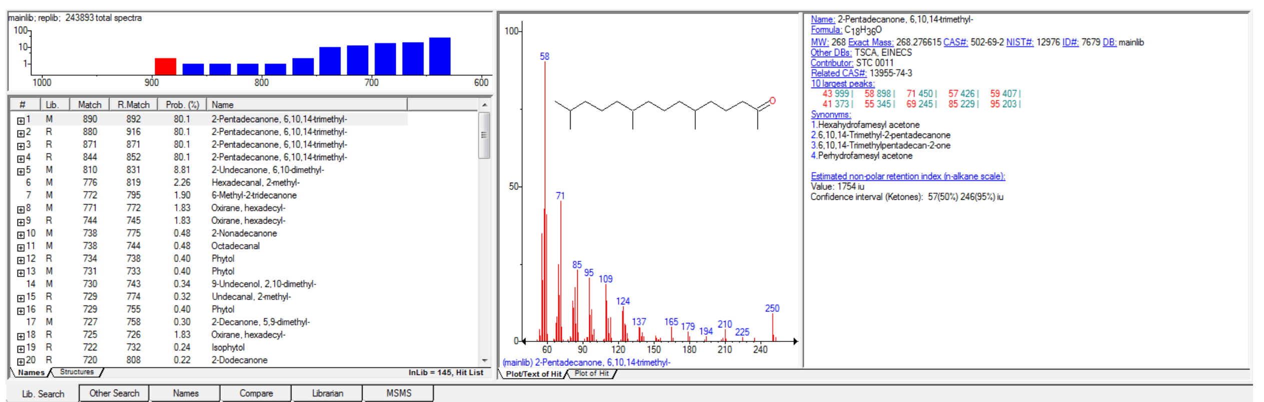


#	Lib.	Match	R.Match	Prob. (%)	Name
1	M	881	881	29.0	Phytol
2	M	878	900	25.6	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
3	R	878	880	29.0	Phytol
4	M	868	868	18.1	Phytol, acetate
5	R	855	872	29.0	Phytol
6	M	814	822	3.54	Oxirane, hexadecyl-
7	R	803	834	2.42	Oxirane, dodecyl-
8	R	799	833	2.05	Oxirane, tetradecyl-
9	M	799	812	2.05	Isophytol, acetate
10	R	797	797	29.0	Phytol
11	M	793	816	2.42	Oxirane, dodecyl-
12	R	787	822	1.36	Tetradecanal
13	R	782	812	2.05	Oxirane, tetradecyl-
14	M	782	793	1.10	Octadecanal
15	M	778	798	0.93	Oxirane, decyl-
16	M	776	788	0.85	Hexadecanal
17	R	773	777	0.75	Isophytol
18	R	771	793	2.05	Oxirane, tetradecyl-
19	R	769	798	0.85	Hexadecanal
20	R	769	786	0.93	Oxirane, decyl-

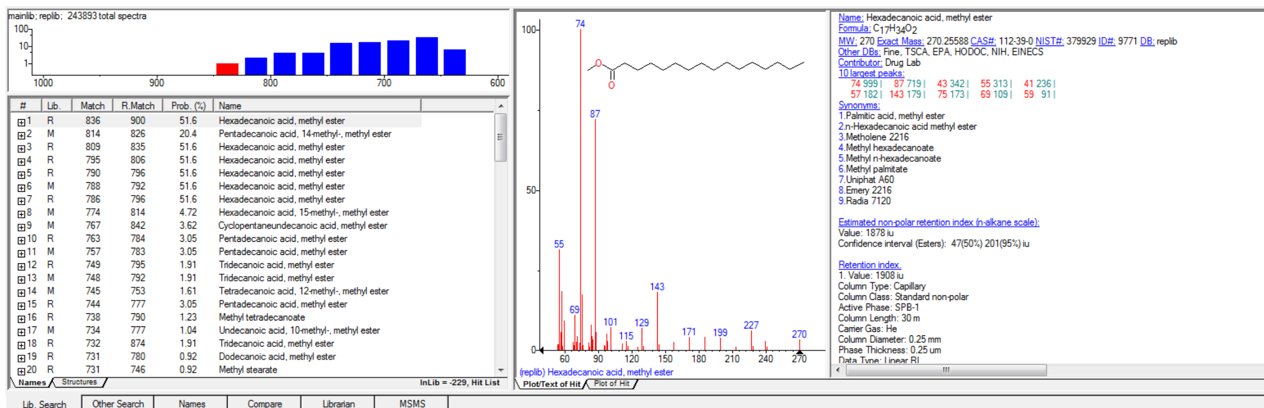
Phytol: 29%, Peak: 32.91



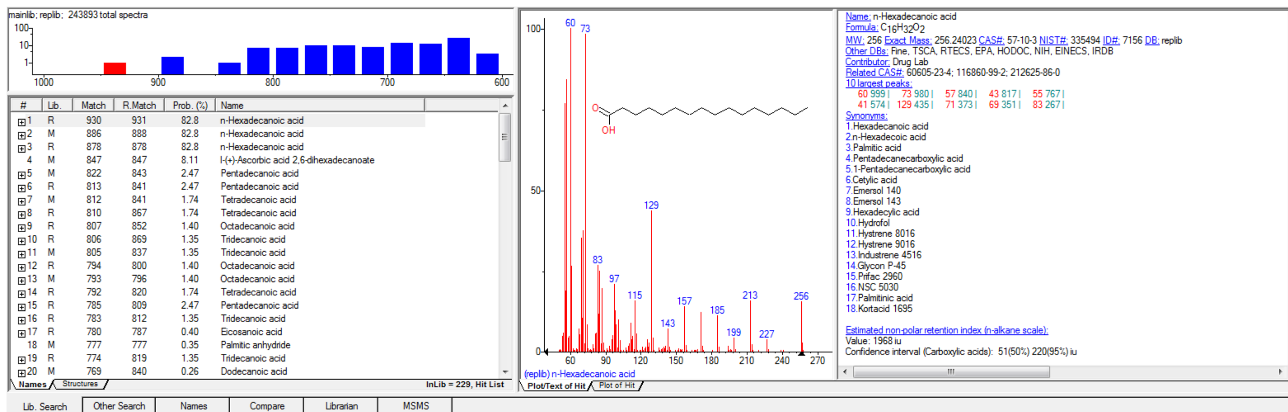
Octadecanoic acid



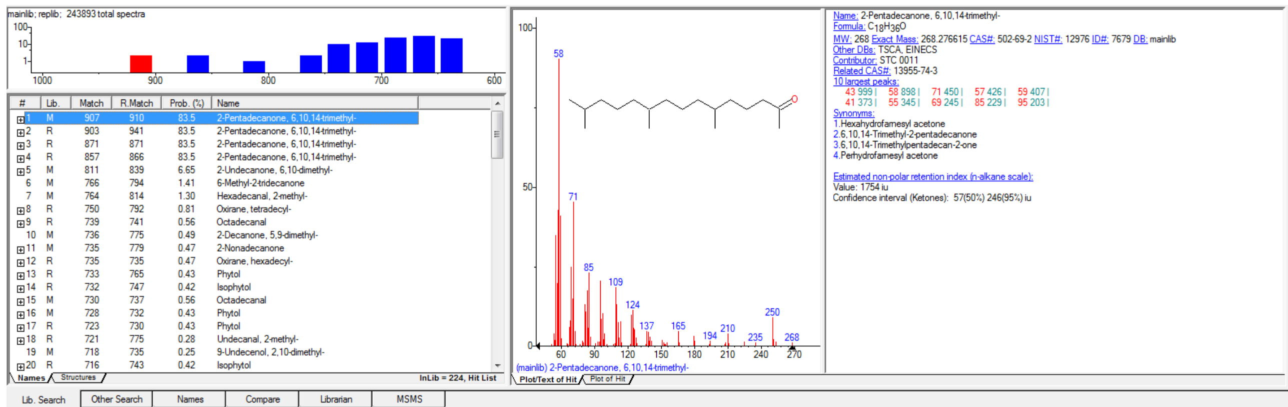
Pentadecanone, 6,10, 14 trimethyl, peak: 26.31 2



Hexadecanoic acid, methyl ester (51.6%), $C_{17}H_{34}O_2$, peak: 27.99



n-hexadecanoic acid (80%), peak: 26.02



2-Pentadecanone, 6,10,14- trimethyl (83.5 %), peak: 26.31

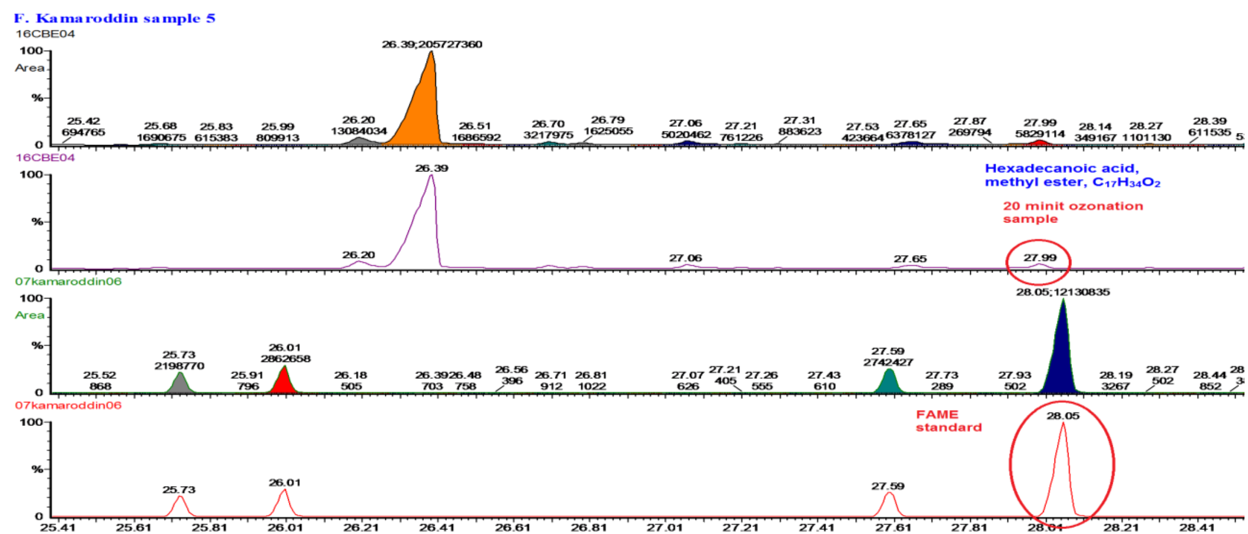


Figure Appendix 10: Comparison of GC-MC peak (20 min sample) with FAME standard

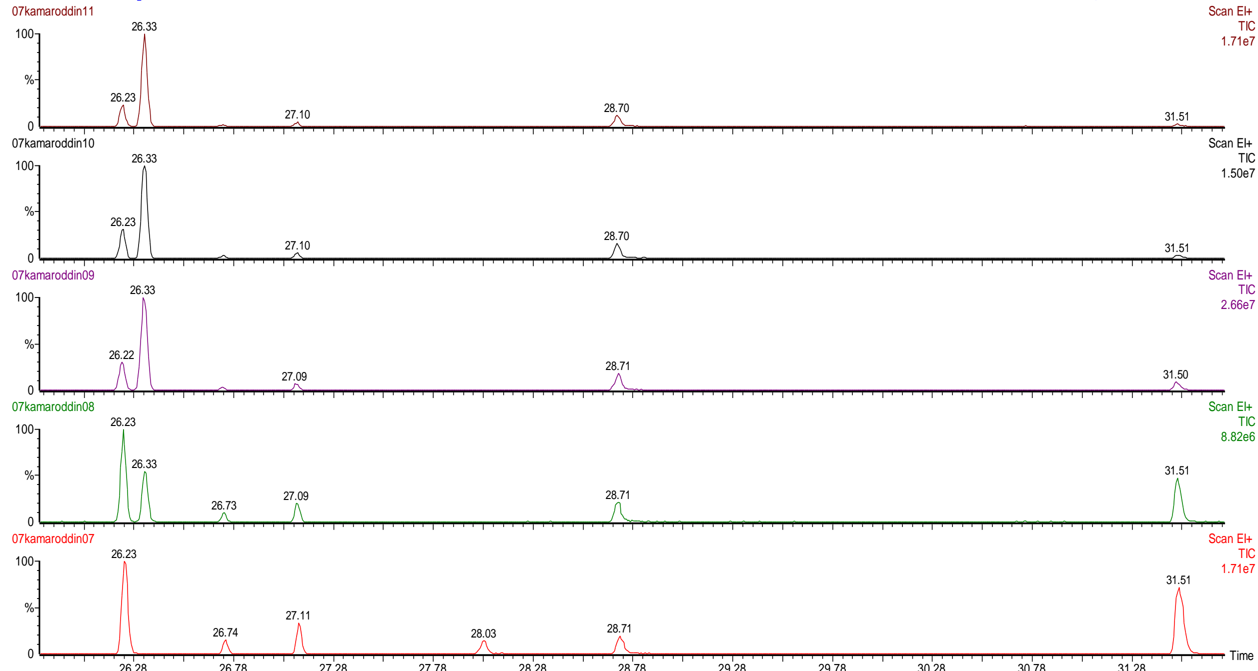


Figure Appendix 11: GC-MC chromatogram peaks (low lipid concentration) (1:10 v/v)

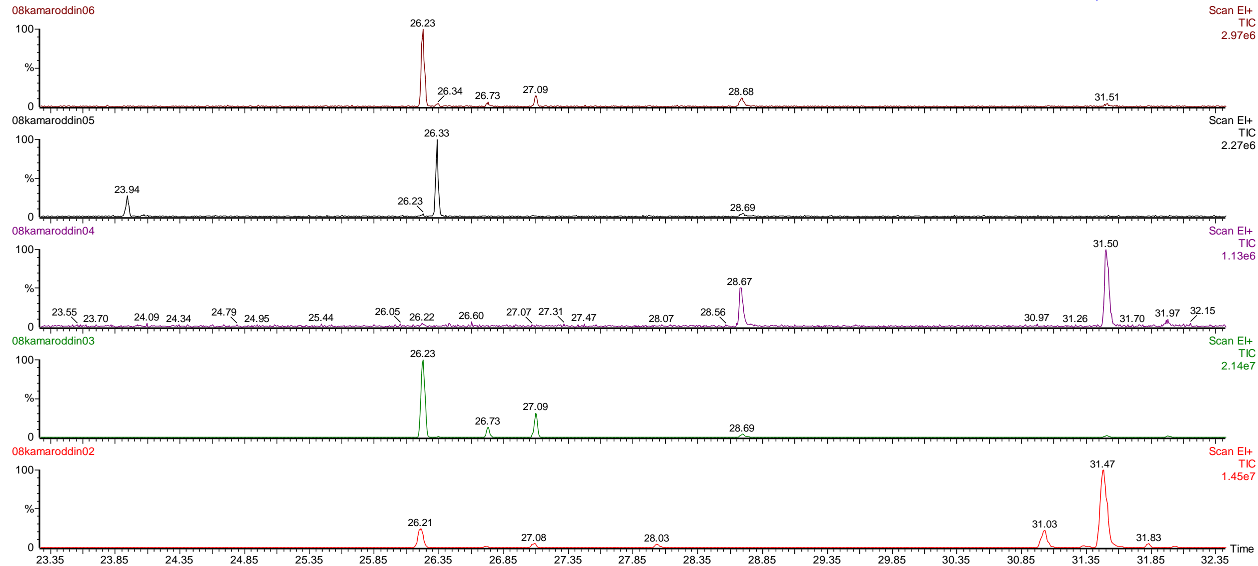


Figure Appendix 12: Standard mixture (02), Acetone extraction (03), Methanol extraction (04), Methanol ozonation 8 min (05).

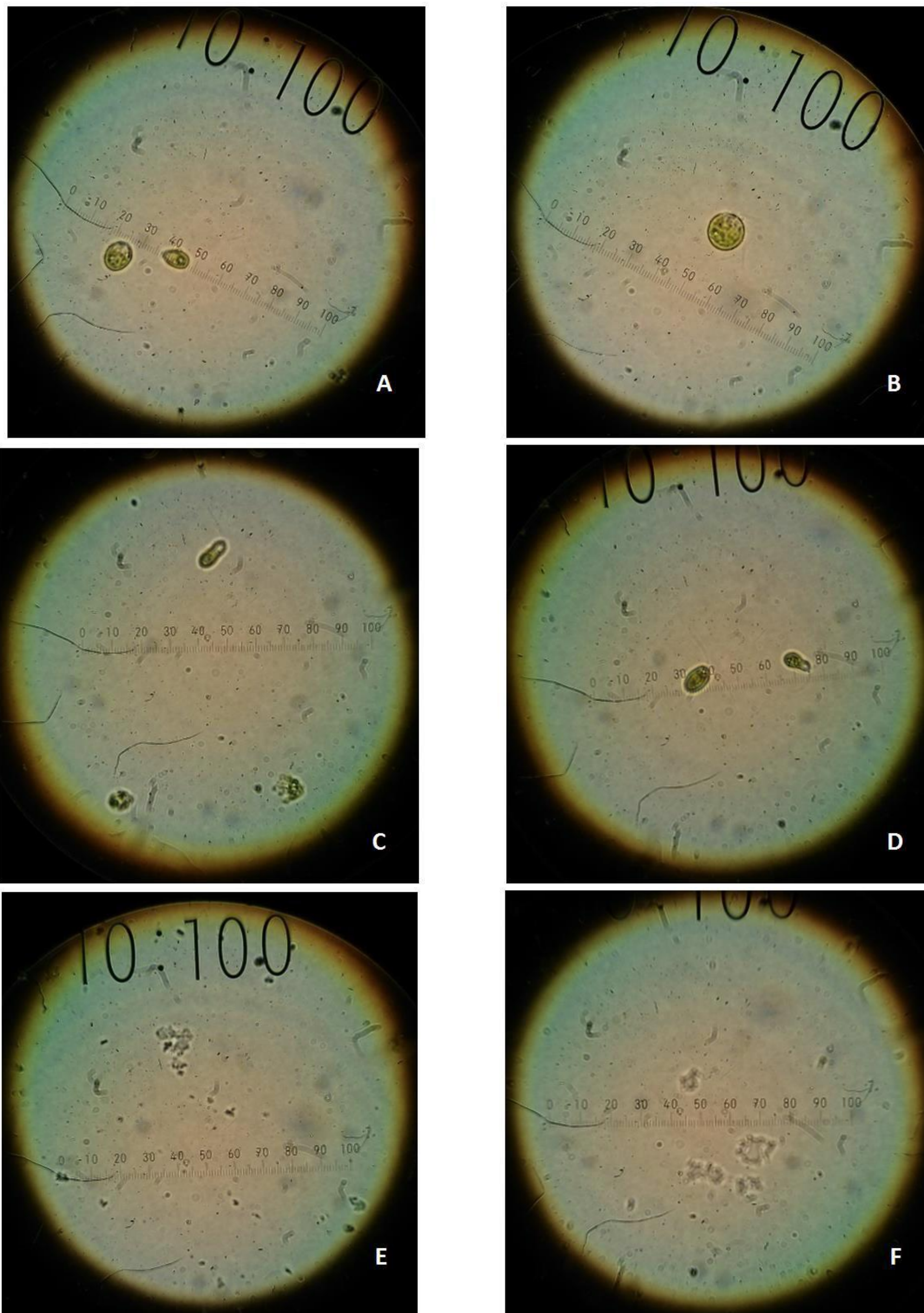


Figure 13. Morphology of *Dunaliella salina* after Ozonation at 250 mg/h (1000x magnification). A and B, Control; C and D, 30 minutes; E and F, 60 minutes.

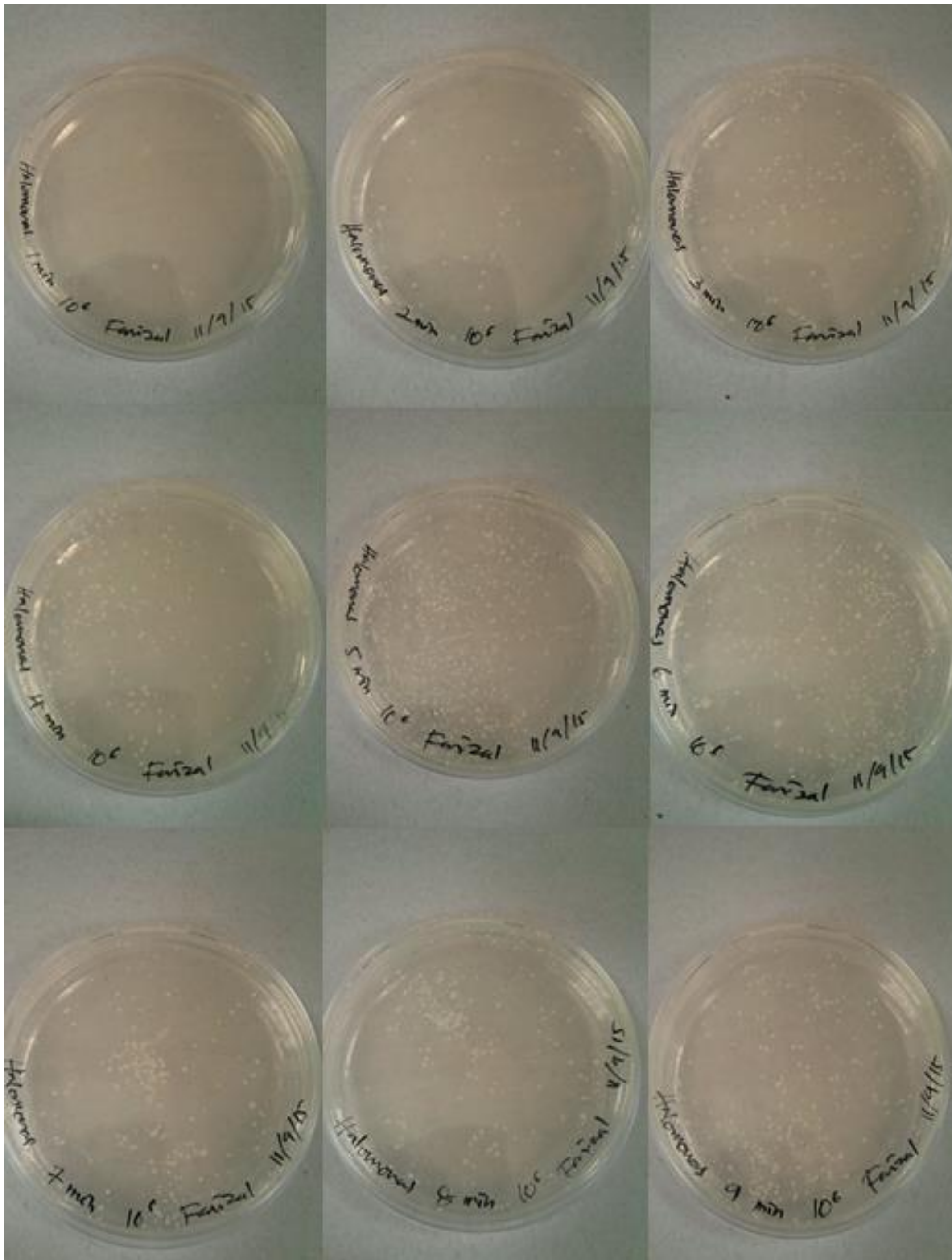


Figure Appendix 14: Ozonation of *Halomonas* culture for 20 min

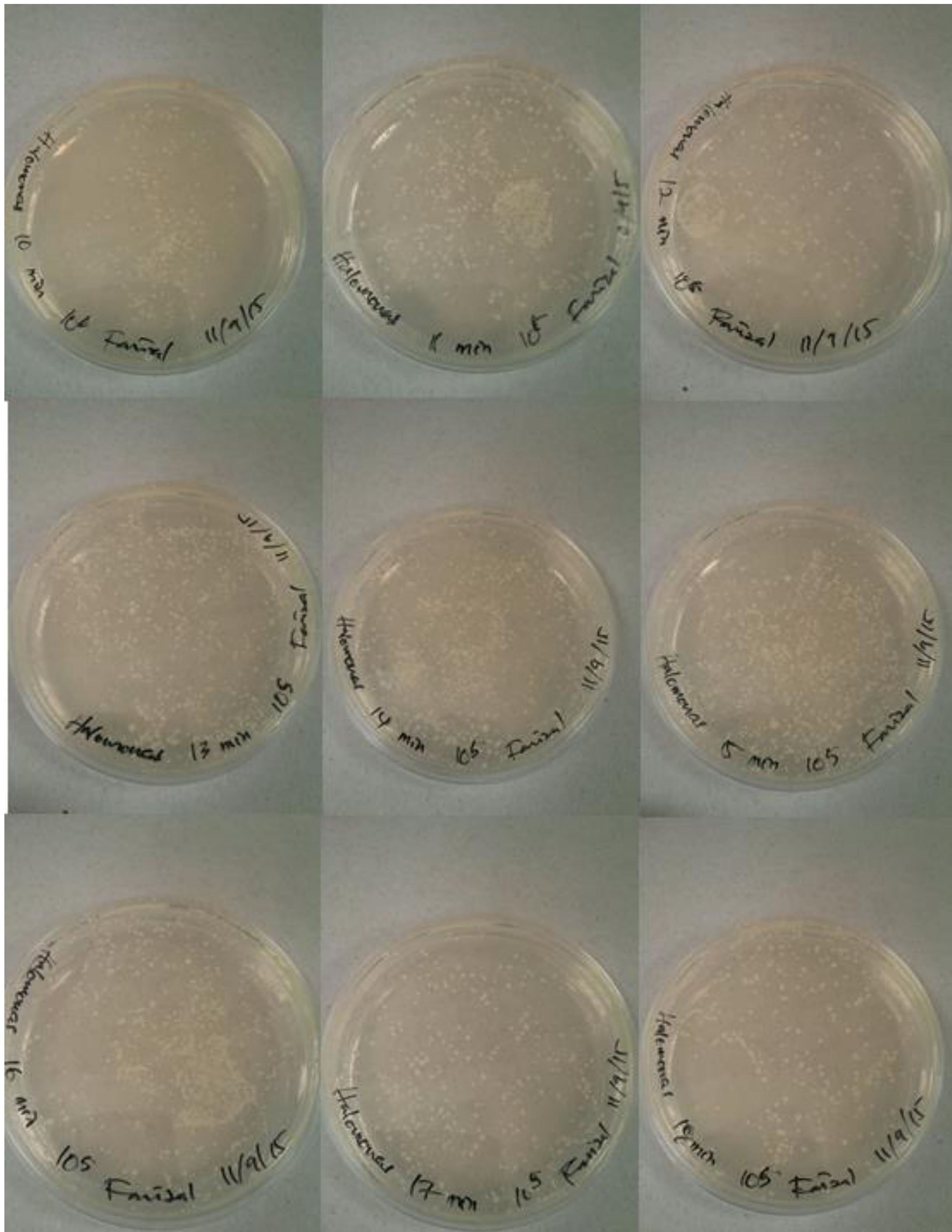


Figure Appendix 15: Ozonation of *Halomonas* culture for 20 min

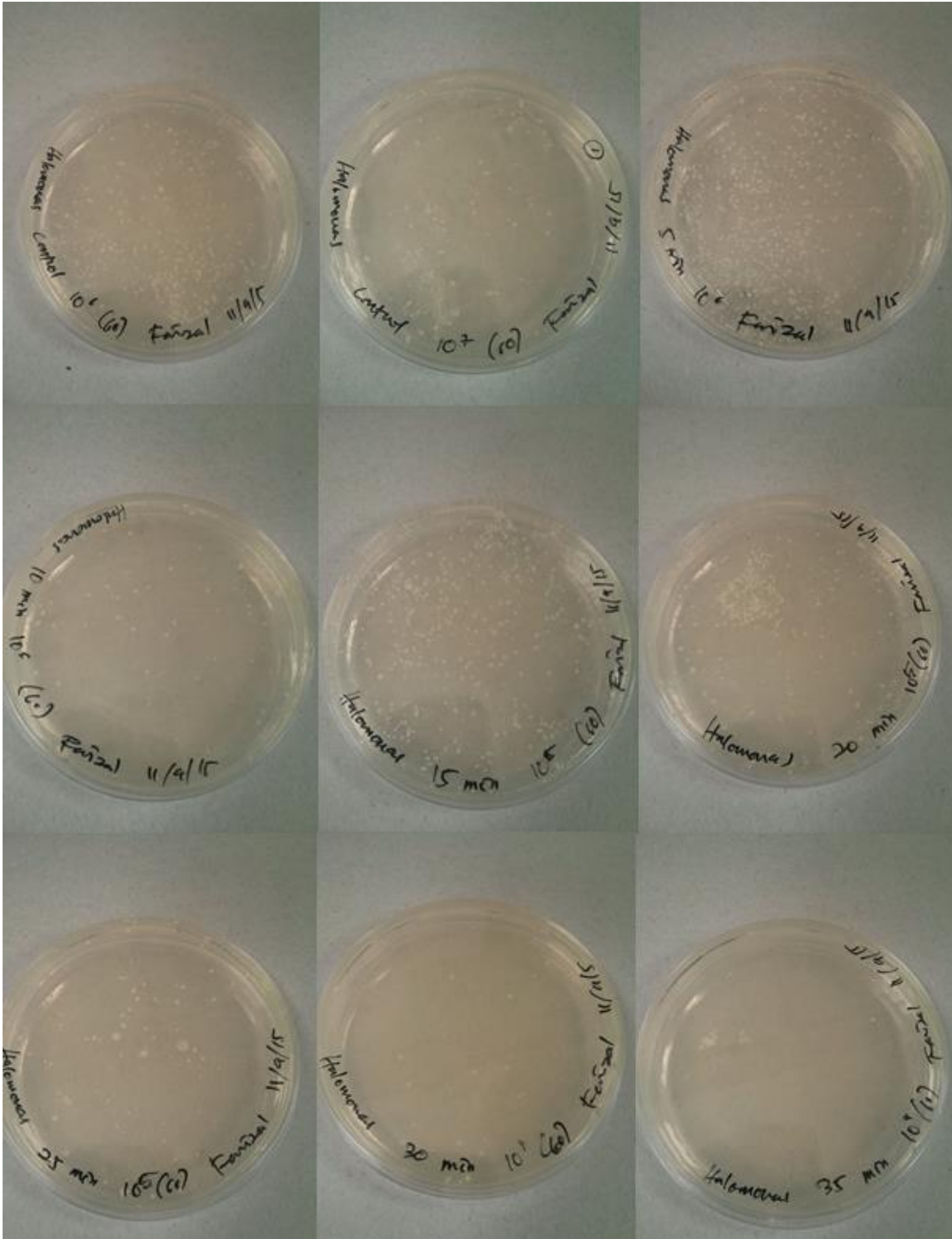


Figure Appendix 16: Ozonation of *Halomonas* culture for 60 min

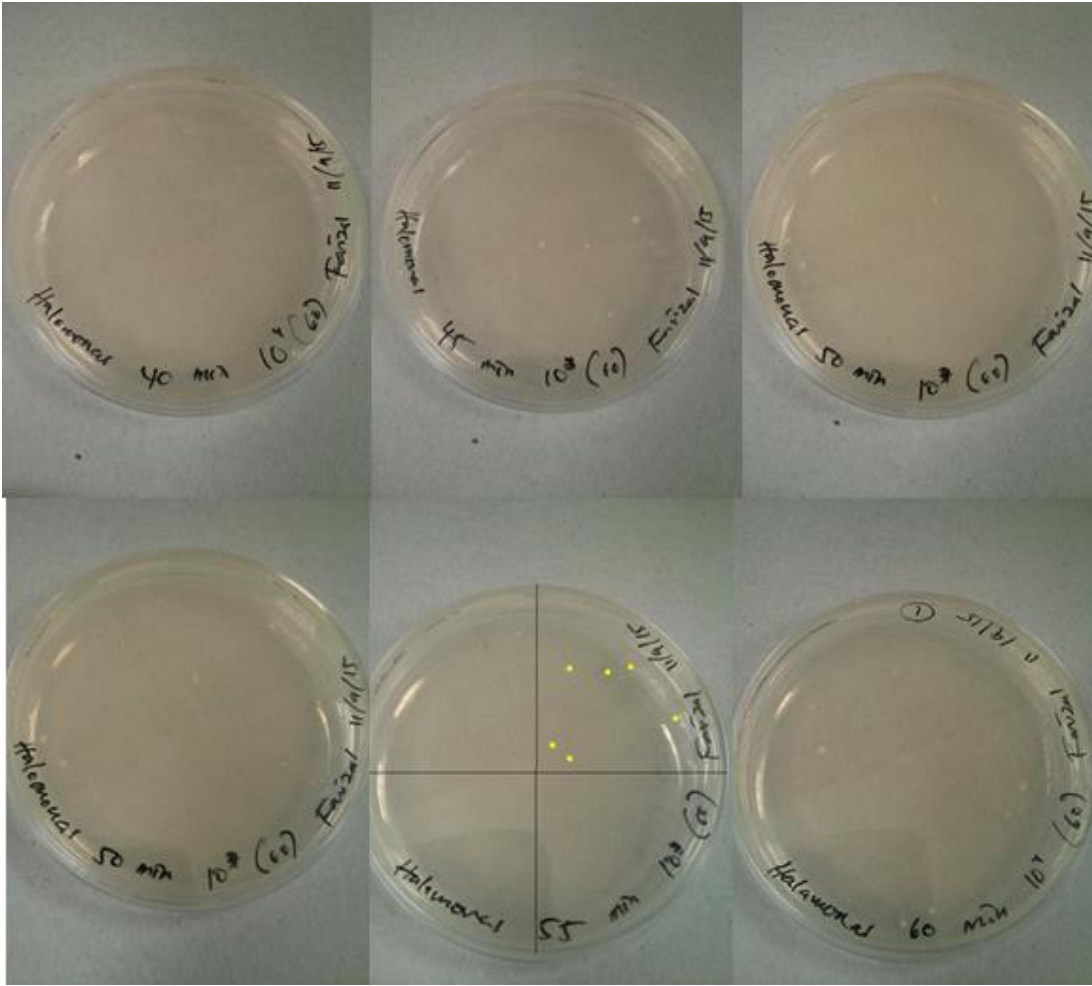


Figure Appendix 17: Ozonation of *Halomonas* culture for 60 min

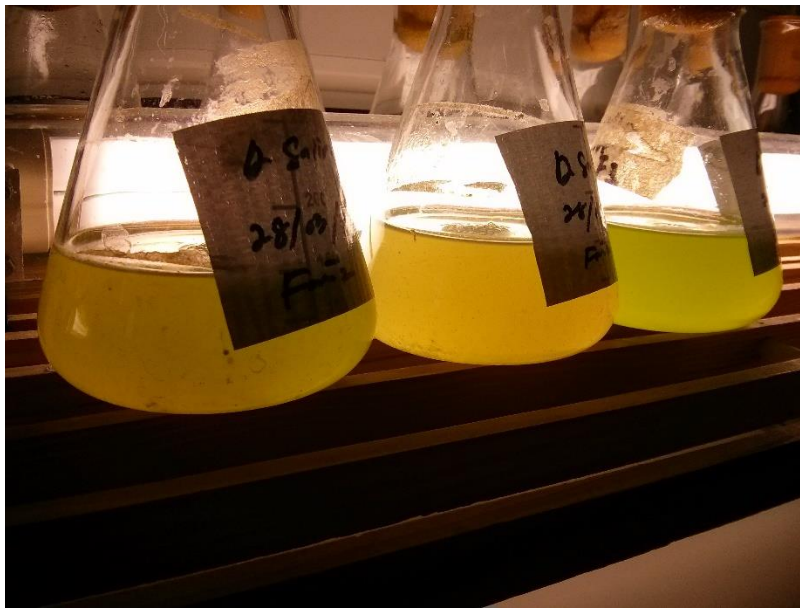


Figure Appendix 18: Contaminated algal culture with Halomonas in photobioreactor and shake flasks

Response: Chlorophyl

ANOVA for Response Surface Quadratic Model

Analysis of variance table [Partial sum of squares]

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	0.11	9	0.012	4.75	0.0115	significant
A	*0.030*	*1*	*0.030*	*12.18*	*0.0058*	
B	*6.400E-006*	*1*	*6.400E-006*	*2.568E-003*	*0.9606*	
C	*0.052*	*1*	*0.052*	*21.03*	*0.0010*	
A^2	*0.010*	*1*	*0.010*	*4.14*	*0.0692*	
B^2	*6.396E-003*	*1*	*6.396E-003*	*2.57*	*0.1402*	
C^2	*3.137E-003*	*1*	*3.137E-003*	*1.26*	*0.2881*	
AB	*5.611E-004*	*1*	*5.611E-004*	*0.23*	*0.6453*	
AC	*2.016E-003*	*1*	*2.016E-003*	*0.81*	*0.3896*	
BC	*1.711E-004*	*1*	*1.711E-004*	*0.069*	*0.7986*	
Residual	0.025	10	2.492E-003			
Lack of Fit	*0.023*	*5*	*4.549E-003*	*10.44*	*0.0111*	*significant*
Pure Error	*2.179E-003*	*5*	*4.358E-004*			
Cor Total	0.13	19				

Figure Appendix 19: RSM Anova analysis

The Model F-value of 4.75 implies the model is significant. There is only
a 1.15% chance that a "Model F-Value" this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.
In this case A, C are significant model terms.
Values greater than 0.1000 indicate the model terms are not significant.
If there are many insignificant model terms (not counting those required to support hierarchy),
model reduction may improve your model.

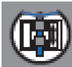
The "Lack of Fit F-value" of 10.44 implies the Lack of Fit is significant. There is only a
1.11% chance that a "Lack of Fit F-value" this large could occur due to noise.
Significant lack of fit is bad -- we want the model to fit.

Std. Dev.	0.050	R-Squared	0.8104
Mean	1.29	Adj R-Squared	0.6398
C.V.	3.86	Pred R-Squared	-0.0861
PRESS	0.14	Adeq Precision	7.436

A negative "Pred R-Squared" implies that the overall mean is a better predictor of your
response than the current model.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your
ratio of 7.436 indicates an adequate signal. This model can be used to navigate the design space.

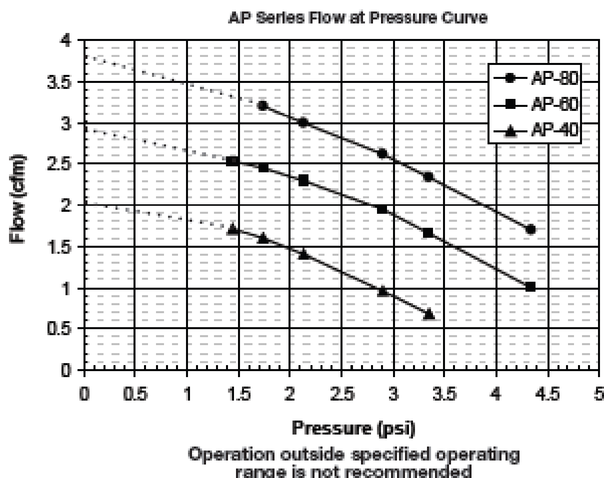
Figure Appendix 20: RSM Anova analysis



Linear AP Series - 115 V 60 Hz

THOMAS
A Gardner Denver Product

Max. Flow	3.20 cfm
Max. Pressure	4.35 psi



Model Number	AP-40	AP-60	AP-80
Manufacturing Code	150131	150132	150133
Performance Data			
Flow at rated pressure	1.60 cfm	2.30 cfm	3.00 cfm
Rated pressure	1.74 psi	2.13 psi	2.13 psi
Operating range	1.45 to 3.35 psi	1.45 to 4.35 psi	1.74 to 4.35 psi
Electrical Data			
Motor type	Linear drive	Linear drive	Linear drive
Nominal voltage	115 V 60 Hz	115 V 60 Hz	115 V 60 Hz
Consumption at rated pressure	0.67A / 34W	0.54A / 43W	0.78A / 64W
General Data			
Operating conditions	14° - 104°F	14° - 104°F	14° - 104°F
Safety certification	UL listed	UL listed	UL listed
Net weight	10.3 lb	10.9 lb	10.9 lb
Sound level at rated pressure	30 dB(A)	34 dB(A)	35 dB(A)

Figure Appendix 21: Conventional linear air pump

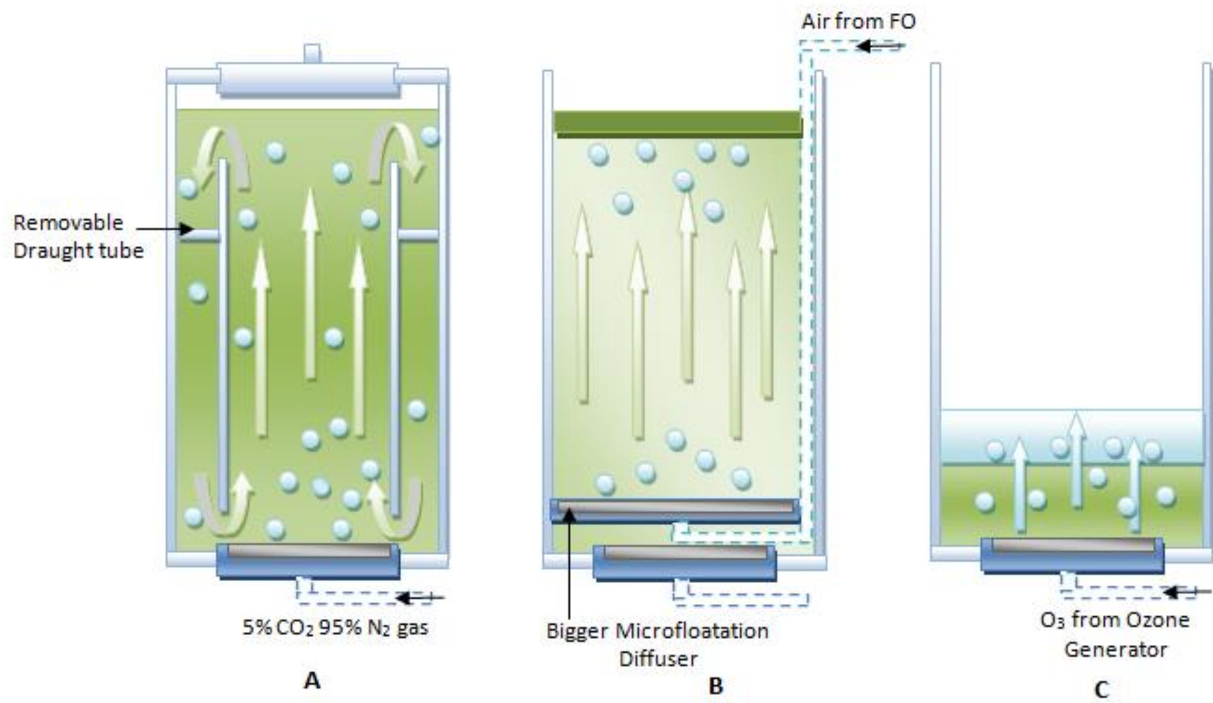


Figure Appendix 22. Suggested processing scheme from cultivated microalgae to algal lipid in one bioreactor system. A; 1.5 L photobioreactor, B; 1.5L microfloatation bioreactor and C; 1.5L Extraction bioreactor.