Algae Screening and Acclimation for Acetaldehyde Removal and Fermentation Gas Effluent Treatment

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Abstract

Microalgae cultivation is considered as a promising solution for the treatment of gaseous effluent from bioethanol production. However, acetaldehyde which is a common pollutant in the effluent is believed to be able to inhibit the growth of many algae strains. Therefore, the aims of this doctoral research were to determine the acetaldehyde tolerance of different algae species and acclimate microalgae to adapt to acetaldehyde exposure.

To ensure the efficiency of screening and acclimation processes, a novel microbubble induced algae screening system was developed. According to the results, the mixing, circulation and mass transfer of this system were largely enhanced compared with conventional shake flasks and bubble columns. The K_La values of the microbubble induced system are 4 times higher than the conventional bubble columns. The results of this research have also revealed that higher algae growth rate can be achieved by applying this system, which was approximately 20% higher than the specific growth rate in conventional systems. The screening experiment determined that at flux of 1.22 m/s, highest algae growth rate can be observed.

Five algae strains were screened in this research namely *Dunaliella salina*, *Chlorella*, *Teraselmis*, *Chlamydomonas* and *Nannochloropsis oculata*. The research discovered that acetaldehyde exposure (124 mg/L, 62.8 mg/L) could cause severe inhibition to almost all strains. However, *Dunaliella salina*, *Chlorella* and *Teraselmis* showed better tolerance to acetaldehyde. These three strains were further investigated for their tolerance to acetaldehyde and their acclimation capacities.

The impact of acetaldehyde on *Dunaliella* and *Chlorella* growth was correlated by measuring cell density of algal cultures under different acetaldehyde dosage (31.4 mg/L, 62.8 mg/L). Under these dosages, acetaldehyde caused growth inhibition to *Dunaliella* but barely affected the growth of *Chlorella*. However, *Dunaliella* and *Chlorella* showed remarkable

enhancement of their tolerance to acetaldehyde after the acclimation. Moreover, acetaldehyde was observed to be able to enhance the growth of acclimated *Chlorella*. The influence of acetaldehyde on photosynthesis of *Dunaliella* and *Chlorella* are also studied using an oxygen electrode. Acetaldehyde increased the photosynthesis rate of *Chlorella* but caused inhibition of the photosynthesis of *Dunaliella*. However, for both strains the acclimated cultures achieved better growth and higher photosynthesis rate than those without acclimation. The acetaldehyde influence on microalgae lipid production was also investigated by NMR and GC-MS and discussed in this thesis.

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Nomenclature

A/a	interfacial area
ALB	airlift-loop bioreactor
Bo	Bodenstein number
BOD	biochemical oxygen demand
C_0	initial concentration
Ct	instantaneous concentration
C*	equilibrium concentration
C_g	molar concentration in gas phase
C_l	molar concentration in liquid phase
CCS	carbon capture and Sequestration
Cr (VI)	hexavalent chromium
DO	dissolved oxygen
D_Z	axial dispersion coefficient
EC_{50}	half maximal effective concentration
F	additional volume force (N/ m^3)
GC-MS	gas chromatography-mass spectrometry
GHG	greenhouse gases
J	interphase mass transfer rate
K_l	masa transfer coefficient

K_La	volumetric mass transfer coefficient (min^{-1}), where 'K_L' is the CO_2 gas-liquid mass transfer coefficient (m min^{-1}); 'a' means the gas-liquid interfacial area (m^{-1})
L	characteristic length
LC_{50}	lethal concentration 50
NMR	Nuclear Magnetic Resonance
OD	Optical density
PAN	peroxyacylnitrates
pH	pH value of liquid
T1	acclimation treatment 1
T2	acclimation treatment2
U_L	liquid velocity (m/s)
V_B	average volume of a single bubble
V_G	gas volume inside bioreactor
V_L	total volume of liquid inside the reactor
VOC	volatile organic compound
$a_{c13\ std}$	integrated area of the C13 FAME standard in algal sample
$a_{c19\ std}$	integrated area of the C19 FAME standard in the algal samples
$c_{c13\ yint}$	y-axis intercept of the line from the appropriate C13 calibration curve
$c_{c13\ std}$	concentration of C13 FAME in the sample
d_B	diameter of bubbles

$e_{trans-ext}$	standard external transesterification efficiency
$e_{trans-int}$	standard internal transesterification efficiency
g	the gravity vector (m/s2)
$m_{c13\ RF}$	gradient of the C13 calibration curve or RF
$m_{c13\ IRF}$	gradient of the C13 calibration curve (or IRF)
-mgl	mass transfer rate from gas to liquid
p	pressure (Pa)
u	denotes velocity (m/s)
v_r	rising velocity
vvm	volume of air per unit of medium per minete
ε	the gas holdup, (V/V ratio)
μ	viscosity of the liquid
μ_1	dynamic viscosity of the liquid (Pa•s)
μ_T	turbulent viscosity (Pa•s)
υ	specific growth rate
ρ	density (kg/ m^3)
ΔG	the Gibbs free energy change for the reaction, $J \cdot mol^{-1}$
ΔG_{f1}	Gibbs free energy of formation for converting acetaldehyde to acetate J•mol-1
ΔG_{f2}	Gibbs free energy of formation for converting acetate into CO_2 and H_2O

$\Delta G°$	standard change of the reaction in Gibbs free energy $J \bullet mol^{-1}$
ΔP	pressure difference between riser and downcomer
$\emptyset R$	diameter of the riser
$\emptyset D$	diameter of the downcomer
ϕ	phase volume fraction (m^3/ m^3)
$[\Delta Na+]$	concentration of additional cations

CHAPTER 1 INTRODUCTION

1.1 Research Background

Humanity is facing two major challenges - the energy crisis and climate change. Due to the abuse of fossil fuels, these two challenges have deteriorated and grown into greater threats that put mankind's very survival and future development in the balance. Therefore, discovering alternative energy sources became an urgent quest. However, among all the attempts of finding ideal alternative fuels, bioethanol is proven to be a promising replacement. Bioethanol is a renewable, easily available, clean and efficient fuel. It is produced by very common agricultural feedstock such as sugar cane, corn, potato, beetroot, manioc, etc (Rudolf *et al*., 2009: 722). During the growth of these feedstocks, CO_2 from atmosphere was captured and fixed into the biomass that will be fermented in order to produce bioethanol. Although the same amount of CO_2 will be eventually released back into atmosphere after burning bioethanol as fuels, there is no net greenhouse gas generated. Therefore, under the pressure of the energy crisis and global warming, industrial production of bioethanol fuels has been developed dramatically. In 2007, 52 billion litres of ethanol was produced and used for transport fuel around the world. It is more than three time as much as the ethanol produced in 2000, which was 17 billion litres (Stefan *et al*., 2009). In 2010, the world wide ethanol production had reached to 86.9 billion litres (Renewable Fuels Association-RFA, 2014). The table 1 shows the world's top 10 ethanol producing countries.

Table 1.1 Annual Fuel Ethanol Production by Country (2007–2010)

Top 10 countries/regional blocks (Millions of U.S. liquid gallons per year) (RFA, 2014)

World rank	**Country/Region**	**2010**	**2009**	**2008**	**2007**
1	United States	13,230.00	10,600.00	9,000.00	6,498.60
2	Brazil	6,921.54	6,577.89	6,472.2	5,019.2
3	European Union	1,176.88	1,039.52	733.60	570.30
4	China	541.55	541.55	501.90	486.00
5	Thailand		435.20	89.80	79.20
6	Canada	356.63	290.59	237.70	211.30
7	India		91.67	66.00	52.80
8	Colombia		83.21	79.30	74.90
9	Australia	66.04	56.80	26.40	26.40
10	Other		247.27		
	World Total	**22,946.87**	**19,534.993**	**17,335.20**	**13,101.7**

Although the utilization of bioethanol fuels generates zero net CO_2 emissions, the production processes of bioethanol will still emit noticeable amounts of CO_2 from culturing and harvesting feedstock, transporting raw material and products, heating fermenters and so on (Department of Transport, 2008). Taking the emissions of sugarcane ethanol production as an example, the production process could generate 1540 kg/ha greenhouse gas each year. The majority of the CO_2 emissions came from the fossil fuels combusted for maintaining heat and operating machineries while producing bioethanol. However, compared to the production of traditional fossil fuel such as gasoline, producing bioethanol generates much less greenhouse gas. For instance, producing bioethanol from sugarcanes could emit 56% less CO_2 than

producing gasoline. For producing corn ethanol, 22% less CO_2 is generated compared with gasoline production (Bourne, 2007). However, due to the rapid expansion of bioethanol manufacturing industries, the emitted greenhouse gas should not be ignored.

The exhaust gas emitted from bioethanol productions contains not only CO_2 but also various volatile organic compounds (VOCs) (Brady and Pratt, 2007). These VOCs could cause environmental problems if emitted without treatment. Acetaldehyde is one of the most common VOCs found in the gaseous effluent of bioethanol production. It is reported to be harmful to human health. Short term exposure could cause irritation, dizziness, vomiting, etc. The long term exposure is more dangerous, which could greatly increase the chances of getting cancer and malformed foetuses. If the acetaldehyde is released into environment without proper treatment, it may endanger the aquatic ecosystem and cause serious odor problems (Chemicals Evaluation and Research Institute-CERI, 2007).

The production of bioethanol also generates significant amount of waste water, which contains mainly agricultural waste, various biodegradable impurities and soil stone particles (Chandel, 2007). The major effluent is discharged from fermentation-distillation processes of the bioethanol production which is generally known as stillage. It almost contains everything fed into the fermenter such as less fermentable sugar plus yeast metabolites and yeast contents (Driessen and Vereijken, 2003). Compared to the wastewater discharged from gasoline production, the stillage has much less toxicity. However, if directly discharge the stillage into water bodies without proper treatment, it can still lead to many serious environmental impacts such as depletion of dissolved oxygen, discoloration, odours, eutrophication, salinization, acidification, increasing the water temperature, changes in species composition of aquatic flora and fauna, and in some extreme situations, leading to fish kill (CERI, 2007).

Traditionally, the stillage and VOCs can be treated in a general municipal wastewater treatment plant. However, many organic compound contained in the stillage have the potential to produce bio-fuels, but are not recycled and reused by the traditional process (Blonskaja *et al*., 2006). Moreover, none of the existing treatments could process the wastewater and VOCs while abating the CO_2 emissions. To minimize the environmental impacts of bioethanol production, the waste treatment would have to be combined with CO_2 fixing technologies such as Carbon Capture and Sequestration which is believed to be as expensive processes in terms of financial cost and energy consumption (Folger, 2009). To process the waste water and the exhaust gas from bioethanol production by traditional means, multiple separate processes have to be built. Which will lead to an increase of the capital cost. Therefore, processing the wastewater and the exhaust gas from bioethanol production more economically and environmental friendly has attracted the attention of many researchers and engineers (Sayre, 2010).

1.2 Algal Cultivation as a Solution

Growing algae can reduce greenhouse gas emission, process wastewater and producing valuable biomass. The CO_2 emitted from the bioethanol production could be the main carbon source of algal photosynthesis. In addition, the nitrogen, phosphorous and sulphur content found in the stillage could be used by algae as nutrients after certain treatments had been carried out (Oilgae, 2009). Moreover, the growth of the microalgae need only simple cultivation systems (pond or bubble column) and requires only solar energy and essential nutrients. Compared with other carbon fixation technologies such as CCS which requires the rebuilding of existing production systems, with the installation of expensive equipment and the consumption of extra energy (Folger, 2009), algal carbon bio-fixation is a much more economical solution. The biomass produced by algal cultivation can be used as the raw

material for many industries such as bio-fuels, food supplements and various fine chemicals (Borowitzka and Borowizka, 1988). Unlike terrestrial plants, microalgae have higher growth rates, higher solar conversion efficiencies and occupy much less space (Borowitzka and Borowitzka, 1988).

Acetaldehyde is one of the main by-products of the bioethanol production (Enviromental Protection Agency-EPA, 1992). It is a problematic impurity and pollutant which may affect the quality of the product and also cause many environmental issues (CERI, 2007). Therefore, acetaldehyde needs to be removed. However, the effectiveness of algal based treatment may be limited and even damaged by acetaldehyde, because acetaldehyde exposure could lead to photosynthesis and growth inhibition to various algal strains (CERI, 2007). Nevertheless, for many mixotrophic algal strains, simple organic carbons such as ethanol and acetic acid can be taken up and utilized as supplementary carbon source. Moreover, It is an established fact that microalgae are able to adjust, even completely change their metabolic pathway through biochemical and physical acclimation that allow them in general to adapt and tolerate a wide range of conditions (Richmond, 2004). Therefore, with careful selection and appropriate acclimation certain algal strains are believed to be able to tolerate and utilize acetaldehyde.

However, the practical application of this algae-based solution faces many challenges. First of all, the CO_2 mass transfer rate in many current microalgae-based carbon capture technologies is considered to be insufficient (Lam *et al*., 2012); secondly, although it is believed that increasing CO_2 concentrations in the gas stream can stimulate microalgae to grow faster, depending on strains, excessively high CO_2 concentrations could also inhibit the algal growth (80 to 98% v/v of CO_2 can be found in the flue gas from ethanol production) (Filho *et al.*, 2013); Thirdly, the industrial flue gas contains various pollutant and impurities such as NOx, SOx, VOCs etc, which could cause inhibitions to microalga. In order to overcome these challenges, a novel microbubble induced microalga screening and

acclimation system was developed, which could considerably increase the CO_2 mass transfer rate due to the advantages of microbubble, and improve the tolerance of microalgae to various conditions and pollutants by selecting and acclimating different algal strains.

1.3 Research Hypothesis and Objective

In this research, five different algal strains were selected as potential candidates for acetaldehyde removal and carbon bio-fixation. These five strains are all well studied, easily available and have high commercial values. It is believed that optimal algal strains can be selected among them. The ideal strains should have relatively high tolerance to acetaldehyde exposure and potential to take up acetaldehyde as supplementary carbon source. Through gradual exposure to acetaldehyde, the acclimation of selected algae strains should enhanced their tolerance to acetaldehyde, and the results of acclimation should reveal their potential to remove acetaldehyde.

In this study, microbubble induced airlift loop bioreactors were used as the algal cultivation system which was applied for screening and acclimation experiments. This is a scaled down approach compared to that reported for pilot scale studies on stack gas (Zimmerman *et al*., 2011). The microbubble technology allows higher carbon dioxide mass transfer rate, better mixing and higher algal growth rates to be achieved (Zimmerman *et al*., 2009), which is believed to be able to enhance the effectiveness of the acclimation system.

The major hypothesis of this thesis is that, with the desirable growth condition provided by microbubble bioreactor, microalgae can be efficiently selected and acclimated. The acclimated algal strains should be able to tolerant acetaldehyde exposure and even take it up as a supplementary carbon source. The objectives of this research are listed below:

1. Build and test screening system. Compare the microbubble bioreactor induced screening system with conventional bubble column growth system. Evaluate the performance of the screening system.
2. Algal screening. Determine optimal algal strains for the research. Study the growth rates of different algae strains under acetaldehyde exposure.
3. Algal acclimation. Gradually increase the dose of acetaldehyde into the culture medium, and study algae responses in terms of growth rate and photosynthesis rate
4. Lipid content production. Uncover the influence of acetaldehyde on algal lipid production. Reveal the mechanisms behind the effects caused by acetaldehyde.

CHAPTER 2 LITERATURE REVIEW

2.1 Microalgae

2.1.1 Microalgae properties and applications

Microalgae are a large group of omnipresent photosynthetic microorganisms which can be discovered in wide range of ecosystems typically in freshwater and marine systems. Microalgae have enormous biodiversity, it is estimated that about 200,000-800,000 species existed around world including both prokaryotic and eukaryotic species (Senne, 2012). Unlike higher terrestrial plants, microalgae do not have roots, leaves and stems, but only have a unicellular or simple multicellular structure. Microalgae are very important lifeforms on earth and they have played significant roles in terraforming primitive earth into today's landscape (Biello, 2009). They also produce half of the atmospheric oxygen and simultaneously fix an equal amount of carbon dioxide, which are crucial processes that allow the survival of many other lifeforms and also enhance the stability of global climate and ecosystems (Dunning, 2012).

Microalgae are adaptable and resilient and are able to thrive in diverse conditions. Their growth requires only inorganic carbon, essential nutrients, water and a light source (Borowitzka and Borowitzka, 1988). However some strains are able to perform mixotrophic growth i.e they can adapt their metabolic processes in order to use organic carbon sources as their supplementary or, in some extreme cases, primary carbon and energy sources. For instance, certain *Chlorella* species can actively utilize acetate as their carbon source (Heredia-Arroyo *et al*., 2011). Such species can grow mixotrophically and produce more cell biomass than their autotrophic and heterotrophic growth combined (Heredia-Arroyo *et al*., 2011). This trait provides them with enough flexibility to survive under changeable environments, which gives them significant advantages in various industrial applications. In

this particulate research, this characteristic is valuable, because the research aims to study the algal response to acetaldehyde exposure as well as to select the algal strains suitable for carbon bio-fixation and acetaldehyde removal. For mixotrophic microalgae, there are possibilities that they could take up acetaldehyde as their carbon source and achieve higher yields in the meantime.

Microalgae have already been widely studied and utilized in academic research and industrial application for decades (Christenson and Sims, 2011). Algal cultivation only requires simple equipment, minimal energy consumption and basic nutrients, but it rewards greatly with renewable and valuable biomass (Borowitzka and Borowizka, 1988). Therefore, it has attracted a lot of attention from researchers and investors.

For a long time, microalgae have been culturing for aquaculture and animal consumption (Borowitzka, 1997). Algal cell biomass is rich in protein, hydrocarbon and lipid, which could provide sufficient energy and nutrients for different live-stock (Brown, 2002). For instance, some protein enriched algal strains are reported to be able to replace soybean meal as a protein source for animal feed. Israeli researchers discovered that sewage grown algae could provide enough proteins to replace 25% soybean in poultry feed (Borowitzka and Borowitzka, 1988; Lipstein *et al*., 1980). It is also reported that 33% of protein can be successfully replaced by adding *Spirulina* and *Chlorella* in the pig feed (Yap *et al*., 1982). Moreover, with algal supplemented feed, a pig can grow 10% of its body weight every day (He *et al*., 2002). Microalgae also played significant role in aquaculture. For example, diatoms are widely used as fish and shellfish feed as well as *Isochrysis, Chaetoceros, Nannochloris, Chlorella* and *Pavlova*. Algae played essential roles in culturing marine *Bivalve mollusks*, seawater shrimp larvae, certain fish species and zooplankters (Brown, 2002). Microalgae are not only provide nutrient and energy for these species, they also serve as a growth factors, possibly as bactericidal agent, inside the culture (Brown, 2002).

Microalgae can produce important raw materials for various fine chemical productions such as food supplements, medicine and food additives. Vitamins are essential for human health, however cannot be synthesized sufficiently by ourselves. Therefore, vitamins have to be obtained from diet. However, many algae species are able to excrete such vital organic compounds. For example, *Chlorella*, *Spirulina* and *Porphyria* have been found to contain Vitamin B_{12} which is obtained through symbiotic interactions with bacterial (Croft *et al*., 2005). Vitamin B_{12} is a key nutrient for maintaining the functions of human brain and neural system. Vitamin E plays a significant role in the prevention of light-induced pathologies and degenerative disorders. However, vitamin E can only be synthesised by photosynthetic organisms such as various microalgae. It was reported that *Nannochloropsis* is able to excrete vitamin E under nitrogen limited conditions (Durmaz, 2007). Moreover, many researchers have conducted investigation on the medical applications of microalgae. For instance, the extract of the brown algae *Sargassum oligocystum* was dicscovered to have remarkable antitumor activity (Zandi *et al*., 2010), and *Chlorella* is reported to have anti-inflammatory effect in chronic and acute inflammation (Renju *et al*., 2013). In addition, Algae can also be used to produce natural colourings for food industry (Emodi, 1978).

Algae play many important roles in environmental protection and renewable energy development. Microalgae are rapid growing photoautotrophic organisms which can capture 10 – 50 times higher carbon dioxide than terrestrial plants (Li *et al*., 2008). Microalgae consume nitrogen, phosphorous and sulphur compounds to grow, which can be used for wastewater treatment to prevent the eutrophication in water bodies (Mallick, 2002). Some algae can even tolerate NO_X and SO_x, and utilize them as nutrients (Kim *et al*., 2014). Many algal strains are able to remove heavy metals and other pollutants from the water body (Mallick, 2002). The growth of algae could also provide oxygen for aerobic bacteria to meet the BOD. Some algal strains are very sensitive to the change of the environment which were

suggested to be utilized as environmental indicators of ecotoxicity (Becker, 1994). There is a research on using *Chlorella* and *Klebsormidium* to develop a biosensor for monitoring the concentrations of volatile organic compounds (Podola *et al*., 2004).

Algal biomass can be used for bio-fuel production. Many algal strains contains high level of lipid content which can be processed to produce biodiesel. Some research indicated that microalgae can be much greater oil source than soybeans and palms. Microalgae can generate 250 times and 31 times larger amount of oil than soybean and palms respectively (Hossain *et al*., 2008). For some microalgae strains, 16 to 24% of their dry weight is free fatty acid which can be directly extracted to produce a good quality of fuels (Aresta *et al*., 2005). Moreover, the algal bio-fuels are considered much safer and cleaner than petroleum. Algal bio-diesel contains much less heavy metals and other impurities (Gao *et al*., 2012).

2.1.2 Microalgae carbon bio-fixation.

Carbon dioxide is considered to be the largest contributor to greenhouse effect. Due to humanity's activities, the carbon dioxide atmospheric concentrations have been increasing rapidly since the beginning of last century, which have caused a series of global climate alterations. It is believed that 60% of total CO_2 emissions are released by industrial activities (Bradshaw *et al*., 2005). CO_2 sequestration is becoming a pressing challenge that has raised global attention. Among various technologies developed to mitigate CO_2 emissions, algae based bio-fixation is an efficient and sustainable solution.

Industrial flue gas contains high levels of highly accessible CO_2, but is currently regarded as waste and discharged without utilization. However, CO_2 is an absolute requirement for microalgae growth. Such flue gas could be ideal carbon source for algal cultivation. Flue gases emitted from many industries can be directly injected into the algal cultivation without causing serious inhibition for algae because they are resilient and adaptable organisms. Many

algal strains showed enough tolerance to NO_x and SO_x which commonly exist in combustion emissions (Mastumoto *et al*., 1997; Olaizola, 2003). Some strains can even utilize these inorganic nitrogen and sulphur compounds as nutrient (Mastumoto *et al*., 1997). For biomass yield production, using industrial flue gas as carbon source could greatly enhance the production rate with lower cost. However, strains that can produce desired bio-products might not be the strains which can tolerate impurities in the flue gas. For instance, NO_x and SO_x may cause inhibitions to many high lipid yielding strains (Negoro *et al*., 1991). Therefore, the feasibility of such application is considered limited and doubtful by some researchers. However, others believed due to the large bio-diversity of microalgae, strains that are both capable of producing targeted product and tolerating the toxins are not impossible to find. Moreover, the adaptable nature of microalgae allows them to alter themselves and thrive in difficult conditions. Therefore, with proper acclimation, it is believed algae may adjust to the environment remarkably quickly (Richmond, 2004). A study carried out by a Japanese researcher discovered that power station flue gas that directly injected into an algal raceway pond did inhibit algal growth, but strains such as *Nanochloris* and *NANNO2* still grew after a period of lag phase (Negoro *et al*., 1991). A pilot scale trial was conducted in UK using flue gas directly from Tata Steal plant for *Dunaliella salina* cultivation. During the trial, the alga was observed to be able to grow exponentially under high concentrations of CO_2 and exposures of toxic impurities. CO_2 capture rate of the microalgae was reported to be 0.1 g/L/h (Zimmerman *et al*., 2011).

Different types of industrial flue gas have already been studied as carbon source for algal culture. Pre-treated emissions from an industrial boiler fuelled by natural gases were suggested to be used for growing *Chlorella* sp. (Doucha *et al*., 2005). Off-gas from municipal waste incinerator was also considered as potential carbon source for *Chorella* sp. cultivation (Douskova *et al*., 2009). Untreated coal-fired power station flue gas was reported to be used

successfully for an algal culture (Moheimani, 2012). It should be noticed that the flue gases vary with different industries. However, the majority of the researches focused on combustion emissions, while the off-gas from fermentation processes was barely mentioned. The fermentation exhaust gas contains almost pure carbon dioxide, which should be recognised as a carbon rich source for algae cultivation.

2.1.3 Microalgae strains used in this research

Generally, to ensure the suitability for the purpose of microalgae cultivation, the algal strains selection should consider the following features: algal growth rate, resistance to environmental changes, nutrient requirements, and valuable qualities such as the accumulation of bio-products. In this work, the adaptability and carbon dioxide assimilation of the microalgae were specifically considered, because the purpose of this research is to determine which strains show high tolerance and acclimation to acetaldehyde exposure and can be used for fermentation waste gas treatment.

2.1.3.1 Dunaliella salina

Dunaliella is a genus of unicellular, biflagellate, marine green algae which presents an ovoid-shape and contains only one chloroplast. In the anterior of the *Dunaliella* cell, there are two flagella of equal length and this morphological features indicates that the *Dunaliella* genus are actively motile microalga (Hende *et al*., 2012). The *Dunaliella* cell is not covered by a cell wall, however a mucous surface envelope has been observed over its plasma membrane (Attaway and Zaborsky, 1993). When extracellular osmotic pressure changes, such a cellular structure means that the cell volume changes rapidly and *Dunaliella* cells adapt to a wide range of salinity by synthesising or degrading intracellular glycerol. The utilisation of glycerol as a compatible solute allows *Dunaliella* strains to thrive at salinities from 0.05M to almost the saturation point (5 M NaCl) (Chen and Jiang, 2009). *Dunaliella* species are also found to be highly tolerant to different pH values. There are reports indicated that *Dunaliella*

strains can grow under strongly acidic conditions (pH=1) and alkaline conditions (pH=11) (Borowitzka and Borowitzka, 1988; Visviki and Satikul, 2000). In addition, *Dunaliella* are resilient to temperature as well, the halophile *D.salina* is able to grow at temperatures up to 40°C (Wegmann *et al*., 1980). Moreover, many investigations have reported that *Dunaliella* has high tolerability to heavy metals and chlorinated hydrocarbons (Borowitzka and Borowitzka, 1988; Tsuji *et al*., 2002). Such adaptabilities has been studied both for their cultivation and for better understanding of the mechanisms behind their adaptability.

Dunaliella are also well exploited for industrial application. As noted above, they can adapt to various environmental conditions, which make them ideal candidates for mass cultivations. *D. salina* is the best-known species among the genus due to its ability to synthesis large amount of carotenoids which are antioxidant agents commercially used in food supplements and cosmetics (Burri, 1997). It is also able to generate considerable amounts of glycerol when grown at high salinities, glycerol is widely used in food and pharmaceutical industries (The Soap and Dergent Association, 1990). Therefore, *D. salina* is among the first mass cultured algae for fine chemicals harvesting. Producing carotenoids and glycerol are actually protective mechanisms for *D. salina.* As one of few organisms known to be able to survive in highly saline conditions such as salt evaporation ponds, high concentration of β-carotene produced by *D salina* can shield them from intensive light, and high glycerol contents maintain their osmotic balance. These characteristics provided commercial opportunities to produce valuable chemicals biologically. To achieve maximized β-carotene yield production, higher light intensities and salinities should be applied in *D. salina* cultivations. In addition to β-carotene and glycerol, other valuable products can also be extracted from *D. salina* biomass such as enzymes, vitamins, amino acids, etc (Borowitzka and Borowitzka, 1988). Like other species in the genus *Dunaliella*, *D. salina* is a robust alga which has remarkable tolerance to

various conditions, which make it easy to culture even in the outdoor environment. Due to the high salinity medium that *D.salina* prefer, outdoor cultures are only rarely contaminated.

In this research, *D. salina* was chosen due to its well-known adaptability. Exposure to acetaldehyde could cause growth inhibition to aquatic organisms. However, it is established that *D. salina* has high tolerance to many toxic chemicals. It is very possible that with proper acclimation *D. salina* can survive and even thrive when acetaldehyde is present in the medium.

2.1.3.2 Chlorella sp.

Chlorella is a genus of unicellular, non-motile, spherical, generally freshwater green algae. *Chlorella* was among the earliest algal strains to be discovered and studied. Since late 19th century, *Chlorella* has been isolated as a pure culture. (Borowitzka amd Borowitzka, 1988) Its photosynthesis and cultivation have been researched for almost 100 years and it is one of the first algae which was studied for industrial mass culture. (Belasco, 1997) *Chlorella* species are fast growing algae able to multiply rapidly and the chloroplast of *Chlorella* (like all green algae) contains photosynthetic pigments chlorophyll-a and –b. *Chlorella* can grow photoautotrophically by using carbon dioxide, water, sunlight and a small amount of minerals (Borowitzka and Borowitzka, 1988).

Chlorella is rich in protein and other nutrients which makes it an ideal alternative food source. There are approximately 45% protein, 20% carbohydrate, 5% fibre and 10% minerals and vitamins in the dry weight of *Chlorella*. Research indicated that around 42-58% of *C. vulgaris* dry weight is protein (Belasco, 1997). Compared to standard nutrition profile provided by World Health Organization, the amino acid profile of *C. vulgaris* protein is even more favourable (Safi *et al.*, 2014). *Chlorella* has the potential for medical uses since it is believed to have antioxidant and anticancer abilities. Mohd *et al.* (2008) revealed that extracts from *C. vulgaris* can successfully, restrain the proliferation and increase the apoptosis of rat's

liver cancer cells. The active component extracted from *C. vulgaris* is reported to be able to inhibit lung cancer metastasis (Wang *et al*., 2010). The antitumor activities of polysaccharides extracted from *C. pyrenoidosa* has also been identified by Sheng *et al.* (2007).

The biomass of some *Chlorella* species contains high levels of lipids, which make them potential feedstock for bio-energy production. *Chlorella* sp. MP-1 was reported to be a strong candidate for bioenergy production due to its high energy content (18.59MJ/kg) (Phukan *et al*., 2011). Zhao *et al*. (2014) reported that co-cultivating *Chlorella* U4341 and *Monoraphidium* sp. FXY-10 can largely increase the yield of lipids, which suggests that *Chlorella* could be a more competitive source for biofuel production.

Chlorella also has great potential for carbon bio-fixation and wastewater treatment. When cultivated in photobioreactors, *Chlorella* is able to capture 74% of carbon dioxide from gas flow and remove 45 to 97% nitrogen, 28-95% phosphorus from growth medium (Safi, et al., 2014). Such ability can greatly reduce the chemical oxygen demand for wastewater treatment and contribute to carbon dioxide abatement. *Chlorella* can also remove heavy metals and control pathogens that exist in wastewater (Safi *et al*., 2014). Moreover, co-culture of *Chlorell*a with some microalgae growth promoting bacteria can largely enhance the performance of wastewater treatment. It is reported that for these mixed cultures 100% of ammonium (NH_4^+) can be removed during four consecutive cycles of 48 h and 83% of phosphorus after only one cycle (Safi *et al*., 2014).

Chlorella is able to perform mixotrophic growth and utilize various organic compound as its carbon source. Glucose and acetate are reported to be used for *Chlorella* mixotrophic cultivation (Wan *et al*., 2011). This capacity of *Chlorella* is particularly interesting for this research, which will study and discuss the possibility of *Chlorella* utilizing acetaldehyde as a

carbon source. There is research discovered that the oxidant stress in aldehyde dehydrogenase enzyme deactivated mice was relieved by feeding *Chlorella* and the declining of cognitive ability was also prevented (Nakashima *et al*., 2009). Researches on using *Chlorella* to break down acetaldehyde formed by alcohol metabolisms in the body of alcohol drinking volunteers has also been reported (Sun Chlorella Corporation, 2011). The research discovered that *Chlorella* injested subjects have significantly lower blood acetaldehyde level than the control group, which indicated that *Chlorella* effectively promoted the acetaldehyde metabolism in humans. Although, these studies are not focused on uptake of acetaldehyde from water bodies or gas emissions, their results suggest the great potential of *Chlorella* for acetaldehyde removal.

2.1.3.3 Tetraselmis

Tetraselmis is a unicellular, ellipsoid, motile, marine phytoplankton, which has four equal length flagella that allows it to swim rapidly (Borowitzka and Borowitzka, 1988). However, during the cell division, *Tetraselmis* generally cease to be motile and some species tend to attach to a surface. This genus of algae normally has a medium to thick cell wall, which gives them remarkable adaptability to various environmental conditions. They can tolerate wide range of salinity, pH and temperatures (Fabregas *et al*., 1984). Therefore, they are widely spread in most coastal and estuarine waters. *Tetraselmis* requires simple nutrient to grow and can outcompete many other species when in mixed culture. It can utilize various chemicals as nitrogen source such as nitrate, urea, ammonium and amino acids (Ricketts, 1990).

Tetraselmis is considered to be a relatively easy algae to be mass cultured as a quality food source for animal and aquaculture. It can provide essential nutrients to many commercially valuable animal larvae such as clams, oysters, abalone, etc. It is reported that *Tetraselmis suecia* rich in docosahexaenoic acid, which is known to be essential to various larvae, Wikfors et al. reported that *Tetraselmis* contains high concentrations of lipid which is ideal

for increasing the growth rate of oyster larvae. *Teraselmis* has also been applied for penaeid prawn cultivation (Brown, 2002).

Due to high yield of lipid content, *Tetraselmis* could be a promising source for bio-fuel production. It was reported that 50% of *Tetraselmis* lipids can be converted into biodiesel (Chamoumi *et al*., 2012) and *Tetraselm*is *suecica* is reported to produce 25% of its dry weight lipid (Moheimani, 2013). Bondioli *et al*. (2012) discovered that, under stressed condition, *Tetraselmis seucica* F&M-M33 can produce biomass 7.6 $gm^{-2}day^{-1}$ and lipids 1.76 $gm^{-2}day^{-1}$. Research indicates that *Tetraselmis chuii* is able to produce 230-450 $mgL^{-1}day^{-1}$ lipid and *Tetraselmis tetrathele* is able to produce 905-1333 $mgL^{-1}day^{-1}$ of lipid. (Liu *et al*., 2011). Biodiesel produced from *Tetraselmis chuii* has kinematic viscosity of 3.2 mm^2s^{-1}, cetane number of 66.6% and heating value of 41.3 MJ/kg, which fully meet the US (ASTM 6751) and European (EN14214) standards (Kumaran *et al*., 2014).

Tetraselmis was also reported to be a genus of mixotrophic algae. Organic carbon present in the culture medium benefits the growth of *Tetraselmis*. Yeast extract, glucose and peptone are all discovered can be their carbon sources (Cid *et al*., 1992). *Tetraselmis* species are promising candidates for this research, the cell wall it processes can shield it from harmful conditions which gives it more tolerance to harsh environment, and mixotrophic growth provides possibilities of acetaldehyde utilization and removal.

2.1.3.4 Chlamydomonas

Chlamydomonas is a genus of well-known, well-studied, unicellular and motile green algae. *Chlamydomonas* are a spherical shaped algae usually covered by a cell wall and contains a single chloroplast and two flagella. It is generally considered ubiquitous, since it is widely spread throughout all types of water body such as stagnant water, damp soil, freshwater, seawater, and even snow. For half a century, *Chlamydomonas* has served as a model organism for molecular biology and genetic research in Biogenesis, flagella motility,

metabolic pathways and chloroplast dynamics (Borowitzka and Borowitzka, 1988). Its nuclear genome has been fully sequenced in 2007and it was discovered that there are approximately 15,000 genes encoded in the genome. Moreover, the mitochondrial and chloroplast genomes also have been sequenced (Brunner and Merrow, 2008). For decades, the study of *Chlamydomonas* provided important information which deepened the understanding of green algae at both cellular and molecular levels (Harris, 2011).

Chlamydomonas is also a mixtrophic green algae and it was reported that mixotrophic growth of *Cs humicola lucksch* can achieve 6 times higher growth rate than heterotrophic growth and 2.5 times higher than autotrophic growth (Laliberte and Nouie, 1993). The mixotrophic culture produced more protein and chlorophyll content, but less lipid and carbon hydrate compared to autotrophic and heterotrophic growth. Generally, acetate is used as carbon source for *C* mixotrophic and heterotrophic growth. *C reinhartii* grows very well mixotrophiclly in acetate-supplemented TAP medium. The mixotrophically grown C. *reinhartii* are more resistant to photo-inhibition compared to autotrophic one under high light intensity (Roach *et al*., 2013).

Due to the extensive research carried out on *Chlamydomona*s, the understanding of these strains is greater and more comprehensive than many other taxa. In this research, *Chlamydomona*s is a competitive selection and ideal control group. However, the only drawback of this genus is that they have been reported to be less stable in mass cultivation (Borowitzka and Borowitzka, 1988).

2.1.3.5 Nannochloropsis oculata

Nannochloropsis oculata is one of 6 species in the genus of *Nannochloropsis*. This genus of algae is considered to be rich in lipid content, which make the species of this genus ideal candidates for bio-fuel production. It was reported that *Nannochloropsis* strains could produce 28.7% dry weight lipid

which are mainly unsaturated fatty acid and significant amount of palmitic acid. Sufficient amount of linolenic and other polyunsaturated acid can also be found in the biomass (Gouveia and Oliveira, 2009).

However, the main reason for selecting *Nannochloropsis oculata* for this research is this strain of algae can remove formaldehyde from its culture medium as Yoshida *et al*. (2009) reported that the growth of *Nannochloropsis oculata* was increased after adding formaldehyde into its medium and the concentration of formaldehyde also reduced during the experiment. They also confirmed that *N. oculata* can remove 99.3 % of the formaldehyde within 22 days. This reports evidenced that *N. oculata* is able to assimilate chemicals similar to acetaldehyde, which suggests that *N.* oculata could be a promising candidates for this research.

2.2 Gas Emissions from Bioethanol Fermentation Process

2.2.1 Compositions

Gaseous effluent released from fermentation processes mainly contains carbon dioxide and volatile organic compounds (VOCs) which could either be simple hydrocarbons such as aldehydes or complex aromatic compounds. Take a yeast production plant as an example, such facility can release 90 tons of VOCs ever year and the composition varies in different industries (EPA, 1992). The most common VOCs generated from bioethanol fermentations are acetaldehyde, formaldehyde, acetic acid and acrolein (EPA, 1992). The biological processes as well as biomass and fermentation conditions employed by different plants could result in different concentrations of VOCs (Brady and Pratt, 2007). Therefore, a comprehensively study of the compositions of these VOCs is difficult. VOCs are considered as troublesome impurities which can affect the quality of bioethanol products, and they may also cause serious environmental issues. For example, many of them are greenhouse gas and some of them can lead to health problems， because of their toxicity to many species

including human being. Therefore, it is important to regulate and reduce the emissions of VOCs (Munoz *et al*., 2007; Khan *et al*., 2000).

2.2.2 VOCs treatment

Conventionally, VOCs from fermentation processes can be treated by many methods such as wet scrubber, carbon adsorbers, incinerators and condensers. However, these methods are far from perfect. The wet scrubber is reported to have limited and variable efficiency when treating emissions of acetaldehyde, ethanol, and ethyl acetate. Carbon adsorber are costly method, the replacement of active carbon is expensive. Carbon adsorber can only achieve best performance when treating low a moisture gas stream. For incinerators, further CO_2 emission will be generated (Tata *et al*., 2003). Therefore, it is recognized that a more effective approach should be developed for VOCs treatment (EPA, 1992).

In addition to the methods listed above, various biological means can be applied for VOCs abatement. For example, bioscrubbers, biofilters and trickle bed reactors are all sustainable processes to limit VOCs emissions. Microorganisms are reported to have successfully digested different organic pollutants and converted them into carbon dioxide, water, minerals and biomass (Munoz *et al*., 2007; Berthe-Corti *et al*., 1998). However, these methods have two major disadvantages: first, the extra carbon dioxide which will be produced by the metabolism of microorganisms; second, the microorganisms may be inhibited by some of the VOCs with high toxicities (Munoz *et al*., 2007).

Although largely unexplored, microalgae could contribute to VOCs removal sustainably and efficiently (Hende *et al*., 2012). In many investigations, microalgae have already partially or indirectly participated in the biodegradation of VOCs. *Chlorella* and *Scenedesmus* are introduced into VOCs treatment to provide oxygen for meeting the BOD and to remove CO_2 generated by other microorganisms (Berthe-Corti *et al*., 1998). Microalgae are suggested to

be fully capable of removing VOCs directly e.g. *N. oculata* is reported to be able to reduce 99.3% formaldehyde in the medium within 22 days (Yoshida *et al*., 2009), exposed *N oculata* to formaldehyde inside arm shake flasks and discovered that the growth rate of the alga increased.

2.3 Acetaldehyde

2.3.1 Acetaldehyde emissions from bioethanol production and its properties

Acetaldehyde is one of the major VOCs emitted from bioethanol production. However, determining the concentrations of acetaldehyde in the exhaust gas is difficult due to ethanol and acetaldehyde being constantly transformed into each other (EPA, 1992). Therefore, the proportions of acetaldehyde and ethanol are variable depending on many factors such as the sugar input into the fermenters and agitation. However, generally speaking, acetaldehyde makes up approximately 10 to 20% of total VOCs emitted from yeast based fermentation processes (EPA, 1992).

Acetaldehyde is problematic in the ethanol production industry. Acetaldehyde inside the final product can be easily oxidized to acetate which increases the acidity of bioethanol. For ethanol products such as biodiesel and wines, increasing acidity may significantly affect their qualities (Batista and Meirelles, 2009). According to European standard EN 14104, acid number of biodiesel should not be higher than 0.50 mg KOH/g. During bioethanol production, generally, acetaldehyde inside the raw stream from the fermentation process can be separated through stripping columns and degassing system. However, the separated acetaldehyde will be in the gaseous phase and become a part of exhaust gas emissions (Batista and Meirelles, 2009).

Although it is reported that chemical and food industry sectors produced highest acetaldehyde emissions, acetaldehyde can also be released due to incomplete combustions. It can be found

in gaseous effluents from many industries such as power generatiors, steal manufacture and waste incinerators. Acetaldehyde is considered as one of the most common industrial carbonyl emissions (Kim *et al*., 2008). Internal combustion engine of automobiles is also a main source of acetaldehyde emissions, and the emission can be even doubled if bioethanol is added into the fuels (Poulopoulos *et al*., 2001).

As the emissions of acetaldehyde continuously increase, its negative effect to environment has "sounded" an alarm to the society. Acetaldehyde is reported to be a greenhouse gas and it also plays significant part in forming photochemical smog. Nowadays, acetaldehyde has already been classified as a pollutant required to be regulated (Poulopoulos *et al*., 2001). Acetaldehyde is a transparent, extremely volatile liquid which has a boiling point at 21°C and high vapour pressure of 99 kPa at 20 °C. It can be dissolved in water at any proportion. Acetaldehyde has a unique penetrating smell, which can be detected at 0.05 ppm minimal (Von-Burg and Stout, 1991; World Health Organization-WHO, 1994). However, at low concentrations acetaldehyde has a fruity scent. In chemical industry, acetaldehyde is the raw material for synthesis of ethyl acetate and a total of 370,000 tons of acetaldehyde was produce in 2001 in Japan (CERI, 2007). Acetaldehyde can be also used in flavouring and aroma in cosmetic and food industry and in the production of pesticides, lubricants and adhesives (CERI, 2007).

2.3.2 Environmental impacts of acetaldehyde

2.3.2.1 Photochemical smog

Acetaldehyde played significant role in forming photochemical smog. Acetaldehyde can react with NO_2 to form peroxyacylnitrates (PAN). The reactions are shown in:

$$CH_3CHO + OH^{\cdot} \longrightarrow CH_3{}'CO^{\cdot} + H_2O$$

$$CH_3{}'CO^{\cdot} + O_2 \longrightarrow CH_3{}'C(O)O_2^{\cdot}$$

$$CH_3'C(O)O_2^{\cdot} + NO_2 \longrightarrow CH_3'C(O)O_2NO_2(PAN) \quad (1)$$

PAN is a serious health hazardous chemicals and one of the main components of photochemical smog (Rani *et al*., 2011). PAN can cause powerful irritation to both eyes and respiratory tracks even at low levels of exposure. It can readily dissolve in water and lead to a large amount of lachrymation when in contact with eyes. At higher concentrations, PAN can cause damage to exposed skins. It is dangerous to vegetation and animals. There are reports that extensive damage is observed when plants and animals are exposed to high concentrations of PAN (Rani *et al*., 2011). PAN is also considered as mutagenic chemicals. It is believed that PAN can be a factor that increases the risk of skin cancer (Rani *et al*., 2011).

2.3.2.2 Effects on living organisms

Acetaldehyde has been reported that to cause growth inhibition for many microorganisms and Yuen *et al.* (1995) reported that the minimal concentration of acetaldehyde that could cause inhibition to 11 species of fungi is 540 mg/m^3. At acetaldehyde concentration of 357,000 mg/m^3, death of these fungi can be observed. They also observed that *Penicillium italicum* and *P. digitatum* are the most acetaldehyde sensitive fungal species with 95% and 91% growth inhibition respectively after 5 days of exposure to acetaldehyde vapour at a concentration of 540 mg/m^3. Similar inhibition can be found in aquatic microorganisms, for instance Bioluminescence inhibition was found in luminescent bacterium *Photobacterium phosphoreum* and 342 mg/L was obtained as the 0.5 -h EC50 (CERI, 2007). Growth inhibition was also found in *Tetrahymena pyriformis* at toxicity of 44 mg/L as the 9 -h EC_{50} (CERI, 2007).

Acetaldehyde can cause colour change and necrosis to vegetation. Such effects can be observed in lettuce after 4 hours exposure to acetaldehyde (54,000-108,000 mg/m^3). (Aharoni *et al*., 1979; Stewart *et al*., 1980). Acetaldehyde can also prevent germination to many species and acetaldehyde concentration of 1,520 mg/L, 50% and more seeds of onion, carrot, palmer amaranth and tomato had germination inhibition (Bradow and Connic, 1988).

Acetaldehyde is considered toxic to many animal species. 100% mortality at all life stages can be observed in *Myzus persicae* and *Acythosiphon kondai* when exposed to acetaldehyde at concentrations of 3,600 and 4,500 mg/m^3 respectively (Aharoni *et al*., 1979). The acute toxicity of acetaldehyde has been reported in many fish species such as fathead minnow, pinfish, bluegill, guppy and rainbow trout. Take fathead minnow as an example, the 96 -h LC_{50} is 30.8 mg/L, and for pinfish, the 24 -h LC_{50} is 70mg/L (Brooke *et al*., 1984).

Many researchers concluded that acetaldehyde has hazardous effects on organisms in the environment. The CERI (2007) suggested that the acute toxicity value of acetaldehyde should correspond to the globally harmonized system of classification and labelling of chemicals (GHS) acute toxicity hazard category III (harmful).

2.3.2.3 Effects on human health

Acetaldehyde can be absorbed into human body through many ways. For instance, acetaldehyde can be inhaled through the lungs, taken up through gastrointestinal tract, or even adsorbed through skin due to its physico-chemical properties inside human body (WHO, 1995), acetaldehyde is distributed through blood into liver, kidney, spleen, heart, muscle and possibly foetal environment (Hobara *et al*., 1985). Irritations in the eyes and respiratory tracts could be caused by exposure to acetaldehyde, erythema can be developed on exposed skin (CERI, 2007). However, due to lack of sufficient data, the exact effects of acetaldehyde on human reproduction/development, neurological system and immune system are yet unknown,

although the animal experiments suggested that acetaldehyde could be hazardous to human health and should a concern (CERI, 2007).

According to experiments on rats, it was discovered that the acute toxicity of acetaldehyde with inhalation exposure is much higher than that with oral dose. For oral administration, the LD_{50} is from 660 to 1930 mg/Kg. For inhalation, the LC_{50} is between 13,100 (4 hours) and 20,200 (0.5 hours) ppm (CERI, 2007). Rats that have absorbed acetaldehyde show many symptoms such as accelerated in heart rate, increased in blood pressure, pulmonary edema and effects on their central nervous system (CERI, 2007). Hyperkeratosis in the forestomach of the rats can be observed after 4 weeks of acetaldehyde oral administration at a dose of 675 mg/Kg/day (Til *et al*., 1988). For inhalation exposed rats, acetaldehyde could damage the respiratory tracts, and most of the damaged tissues were found from upper respiratory tract.

In order to study the reproductive and developmental toxicity of acetaldehyde, intravenous and intraperitoneal injection were conducted on female pregnant mice. The foetuses of the injected mice showed foetal resorptions, body weight loss, neural tube defect and malformation. Foetal skeletal defects can be observed in female rats treated by oral administration of acetaldehyde at a dose of 200 mg/Kg/day on gestation days 6 to 18 (Fadel and Persaud, 1990).

Acetaldehyde is considered mutagenic. In various in vitro studies, positive responses to acetaldehyde have been reported. It is established that acetaldehyde is able to cause gene mutation, chromosomal aberration and sister chromatid exchanges. Acetaldehyde is believed to be genotoxic. At a concentration of 13 μg/mL, acetaldehyde can cause human lymphocytes hprt gene locus mutation. (He *et al.*, 1990) Acetaldehyde can also cause chlromosomal aberration on human lymphocytes from a dosage of 7.8 μg/mL and cause sister chromatid exchange from a dosage of 4 μg/mL (Obe and Beek, 1979; Bohlke *et al*., 1983).

Acetaldehyde is also reported to cause DNA strand breaks on human leukocytes and DNA-DNA cross-links of human lymphocytes (Lambert *et al*., 1985).

Animal tests also indicated that acetaldehyde could have carcinogenicity. After 28 months, 6 hours / day, 5 days / week inhalation exposure, carcinoma can be discovered in the nasal cavity of both male and female rats (Woutersen *et al*., 1986). Respiratory tract tumours can be found in Syrian hamster after 52 weeks, 7 hours / day, 5 days / week inhalation exposure (Feron *et al*., 1982). The dose dependent increases in carcinoma indicated that acetaldehyde should be considered as carcinogenic chemicals. Acetaldehyde is classified as Group 2B (possible carcinogenic to humans) by the International Agency for Research on Cancer (IARC) (CERI, 2007).

2.3.2 The Effects of Acetaldehyde on Microalgae Growth

Microalgae are sensitive to environmental changes such as temperature, pH, light intensity and toxic compounds. Therefore, they are often used as indicators for chemical risk assessments. Due to such high sensitivity, the response of microalgae to acetaldehyde exposure may be drastic. According to the toxicity test on the microalgal strain *Peseudokirchmeriella subcapitata*, acetaldehyde showed extremely high toxicity to *P. subcapitata* among 90 different organic compounds tested. At acetaldehyde concentration of 4,439 mg/L, the growth rate of *P. subcapitata* could only achieve half of its unaffected rate (Tsai and Chen, 2007). Acetaldehyde is reported to cause growth and photosynthesis inhibition to microalga. An acute toxicity test indicated that growth inhibition can be observed in marine diatoms when EC_{50} is from 237 to 249 mg/L (CERI, 2007). According to the research conducted by Brack and Frack (1998), the photosynthesis of *C. reinhardtii* can be affected at an acetaldehyde concentration of 23 mg/L, the decline of both photochemical and non-photochemical quenching can be discovered when the microalgae was exposed to acetaldehyde.

Although all these investigations indicated that acetaldehyde is strongly toxic to microalgae, they are toxicity studies which involve high levels of acetaldehyde exposure, as well as specific and sensitive strains. This is how the risk of emitting acetaldehyde into environment can be determined. However, microalgae are diverse and adaptable life forms, different algal species will have various responses when exposed to acetaldehyde because of different cytology, physiology and genetics.

2.4 Microalgae Screening and Acclimation

2.4.1 Microalgae response to toxic compounds

Many researchers reported the toxicity of acetaldehyde to algal growth; however, few of their studies involved the acclimation of microalgae. It is established that microalgae are resilient organisms, they are repeatedly discovered thriving in extreme environment, and they also tend to alter themselves metabolically in order to adapt to new environments.

It is an established fact that the evolutionary adaptability of some microalgae allow them tolerate compounds and pollutants that are far more toxic than acetaldehyde. The growth of *D. salina* and *T. suecica* are reported to be slowed at early days of cadmium and copper exposure; however, such inhibition did not maintain permanently. The growth rate of both strains' caught up later and achieved similar rates with that of the control cultures (Rodriguez and Rivera, 1995). Some pollutants such as heavy metals are extremely toxic when released into the environment; however, these compounds might provide crucial micronutrient for algal growth. For instance, *Scenedesmus incrassatulus* is reported to be able to remove chromium, cadmium, and copper. It is believed that cadmium played a significant role in energy related processes required by *S. incrassatulus*, which explained the remarkable cadmium removal ability that *S. incrassatulus* possessed (Pena-Castro *et al.*, 2004). *Dictyosphaerium chlorelloids* presented another ideal example of the microalgal tolerance to toxic environments. By gradually increasing the exposure *D. chlorelloids* to TNT, the alga

became tolerant to this contaminant. This research showed the growth rate of treated algae even declined when TNT was taken out of the medium. Moreover, significant reduction of its photosynthesis rate was also observed (Garcia-Villada *et al*., 2002).

The above examples provide evidence that, although acetaldehyde is regarded as a harmful chemical to algal growth, it is quite possible certain strains can develop sufficient tolerance that allow them to thrive under the exposure of acetaldehyde. Algae such as *Dunaliella*, *Tetraselmis* and *Chlorella* are repeatedly reported to be able to resist the influences of various toxins. Screening methods should be introduced into the research in order to identify those microalgae with high tolerance to acetaldehyde. As evolving life forms, microalgae have never ceased to mutate and adjust themselves, which have made them the fittest organisms in every aquatic environments since eons ago. Therefore, tolerating and utilizing acetaldehyde should not be considered as an impossible challenge to microalgae for which acclimation could be a promising solution.

2.4.2 Algal screening techniques

Algae screening is a strategy applied to an algal population to identify and select the optimal strains with required bio-functions such as capability of producing a target bio-product, containing high level of nutrients or abilities to remove unwanted pollutants, etc. For instance, many investigations were conducted in order to determine the ideal algal species for bio-fuel productions recently. Nascimento *et al.* (2012) carried out a screening experiment for 12 microalgae strains in order to determine the suitable strains for biodiesel production. The algae were cultured in Erlenmeyer flasks which were kept under constant temperature of 25 °C and agitation of 90 rpm. Cultures were aerated with CO_2 enriched air (5%) at flow rate of 0.5 vvm, and 12:12 h light dark cycle was also provided to the screening cultures. The biodiesel fuel properties of these microalgae were measured and evaluated by their volumetric lipid productivity and fatty acid profiles. The study reported that the highest lipid

yields were found for *Chlorella* and *Botryococcus*. However, *Kirchneriella lunaris*, *Ankistrodesmus fustiformis*, *Chlamydocapsa bacillus*, and *Ankistrodesmus falcatus* showed highest level of polyunsaturated FAME, which indicated potential for high quality biodiesel production.

Another example of microalgae screening research was conducted by Doan *et al*. (2011) in Singapore, they reported that *Nannochloropsis* strains contained highest concentrations of lipid. During the research, 96 marine microalgae strains were isolated from the coastal water of Singapore, and 21 strains among these microalgae were characterized for biodiesel feed stock. During the screening process, the isolated algae were grown inside flasks and maintained in a photoincubator at a temperature of 25±1 °C, light intensity of 60 µmol photon m^{-2} s^{-1}, and 12:12 h light / dark cycle with manual shaking twice a day. Microalgae with cellular lipid droplets were selected and further screened for cell division and lipid production. The cell growth rates were measured by optical density, and the lipid productions were determined by total lipid content and fatty acid profile which can be measured respectively by Nile Red fluorescence and GC - MS. It was concluded on the basis of this screening experiment that *Nannochloropsis* sp. have rapid growth rate, high lipid content and favourable fatty acid profile, which suggested that *Nannochloropsi*s is a suitable candidate for biofuel production.

In addition, many researchers have focused on screening microalgae for valuable bio-product. For example, Nair *et al*. (2007) screened twenty-six marine algae all collected from coastal regions of Gujarat for their possession of antibacterial active agents. The active compounds of these microalgae are extracted by acetone and methanol and used against 5 clinically important bacterial strains, namely *Bacillus cereus, Micrococcus flavus, Citrobacter freundii, Klebsiella pneumonia* and *Pseudomonas testosterone*. Agar disc diffusion and agar dish methods were employed for testing the antibacterial activity of extracted algal compounds.

The macroalga *Enteromorpha intestinalis* proved to be the most promising candidate for antibacterial agent production among the 26 strains.

Plaza *et al*. (2010) also screened microalgae for bioactive compounds. According to their research, bioactive compounds were extracted from the cyanobacterium *Synechocystis* sp. and *Himanthalia elongate* and characterized by GC-MS and HPLC-DAD. The extracts were discovered to have high antioxidant activities. Many antimicrobial active compounds such as phytol, fucosterol, neophytadiene, palmitoleic and oleic acids were identified.

Microalgae are believed to have great potential for treating waste water, exhaust gas and other waste materials. Therefore, it is important to study the tolerance of microalgae to various pollutants. Hence, many researchers focused their studies on the microalgae screening for high resistance to toxic compounds present in waste materials. Sivasubramanian *et al*. (2011) screened 8 different microalgae for their tolerance to the sludge of hypochlorite manufacturing industry. The microalgae, namely *Chroococcus turgidus, Chlamydomonas pertusa, Chlorella vulgaris, Chlorococcum humicog, Dactylococcopsis raphidioides* and two isolated wild strains were cultured in test tubes which contained culture medium and two different concentrations of effluent sludge namely 2.5% and 5%. This effluent sludge contains mainly acid insoluble, carbonate, hydroxides and sulphates. The tolerance of microalgae was determined by their growth rates which were measured by cell counts. The screening experiment reported that *Chlorococcum humicola* and *Chroococcus turgidus* had highest growth rates. This indicated that they have significant tolerance to the effluent sludge and great potential for hyposludge remediation.

An algal screening project resarching for chromium tolerance revealed that the cyanobacterium *Nostoc* sp. can tolerate Cr (VI) at concentrations of 5 and 10 mg/L. The algae were screened in 250ml Erlenmeyer flasks under 5 different concentrations of Cr (VI) (5, 10,

15 and 20 mg/L). After 10 days of incubation, the growth rates were measured and compared with control group which was grown without Cr (VI) exposure. Tolerance to chromium was determined by dry weight, chlorophyll-a and protein content. It is reported that the cyanobacterium showed highest growth rate at a chromium concentration of 5 mg/L. However, the highest chlorophyll-a, protein and phycocyanin concentration was found in the cyanobacterium culture exposed to 10 and 15 mg/L Cr (VI) (Shivram and Vishwanath, 2012).

Zhang *et al*. (2013) screened microalgae for CO_2 tolerance. Seventeen algal strains were cultured in flasks, and pure CO_2 blended with air was injected into the culture. The effect of CO_2 concentration in the inlet gas on algal growth was screened from 2 to 20% (v/v). They discovered that, among the strains they screened, strains closely related to the species *Scenedesmus* and *Chlorococcum* were the most CO_2 tolerant ones.

As noted earlier in this thesis, microalgae show great diversity in speciation and function and this has attracted a lot of attention from various industries. Different culture collection centres all over the world have maintained thousands of algal cultures. Such diversity creates difficulties for identifying the most suitable algal candidates for required functions. Therefore, proper and efficient screening processes are desperately needed. However, as can be seen in the cases listed above, the majority of screening experiments use plain shake flasks to grow microalgae which are simple and economical, but do not support the most efficient growth. Cultivation system is important to the screening process. Applying ideal algae culture system could provide a desirable environment for algal growth and reduce external interference to the screening.

2.4.3 Acclimation for increasing tolerance to toxic substances

Acclimation is the method which can allow microalgae to maintain growth across a range of environmental conditions by creating a gradual change of the environment which forces

microalgae to adjust their behaviour and physiology. Such adjustment generally occurs within the generation time of the organism, and the capability of acclimation is presumably genetically transmitted. It is a well-known fact that microalgae have large acclimation capacities to various conditions which allow them to adapt and tolerate a wide range of exposure to different toxic compounds. In order to perform bio-assimilation of many harmful pollutants in bodies of water, algal acclimation has been introduced into the water treatment researches.

Research into algal acclimation for phenol tolerance revealed that algae strains could gradually gain the ability to grow in phenolic medium. Three algal strains, *Oocystis pusilla, Chlorella pyrenoidosa* and *Oscillatoria quadripunctulata* were isolated from a local waste stream. They were grown in 50ml culture tubes. The algae were exposed to a series of phenol concentrations which were 0.1, 1, 2, 4, and 5 mg/L. After 8 days growth, the cells were centrifuged, incubated and exposed to successively higher concentrations of phenol in the order of 7, 10, 12, 15, 17, 20, 22 and 25 mg/L. Initially, growth inhibition can be observed in all strains due to increase of phenol concentration. However, the algal cell yields after second exposure were nearly the same as the control group or even higher (Joseph and Joseph, 1999).

Wang (1985) studied the acclimation and response of algae to zinc toxicity. Algae samples (*Selenastrum*, *Microcystis*, *Anabaena, Diatoms*, etc.) collected from Peoria and Farmington, Illinois were placed into BOD bottles and exposed to zinc. Increasing the zinc concentration after each acclimation experiment showed that algae from different regions responded differently to zinc exposure. However, the algal response to zinc exposure could change after acclimation mainly due to the tolerance microalgae developed during acclimation.

Johnson *et al*. (2007) reported that tolerance of microalgae to copper could be developed after acclimation. Two strains *Nitzschia closterium* and *Chlorella* sp. were cultured at copper

concentrations of < 2 µg/L and <1 µg/L respectively. Then transferred into media containing copper concentrations of 5 or 25 µg/L for *N. chlosterium* and 2 µg/L for *Chlorella* sp. The researchers concluded that acclimation had little effect on the tolerance of these two strains to copper, however, for long time exposure, acclimated samples showed more resistance.

Compared to aromatic compounds and heavy metals tested in the cases described above, acetaldehyde has been proven to be less hazardous to microorganisms. Acetaldehyde can be generated by metabolism of various organisms and even reported can be digested by many bacteria and fungi. Therefore, it is not completely unreasonable that microalgae could be acclimated to tolerate or even assimilate acetaldehyde.

2.5 Microalgae Cultivation

2.5.1 Culture techniques

For industrial scale culture of algal biomass, there are two distinct concepts which have been emerged since early 1950s. According to Chaumont (1993), the first is the closed photobioreactor described by Burlew in 1953; the second is large, channelized, mixed open ponds as pioneered by Oswald and Golueke in 1960. Generally, the most widely applied microalgal cultivation systems in the world are these two types. For open pond systems, as their size expands, they will be much easier affected and contaminated by the environment. Therefore, there is often a huge gap between the theoretical and the actual productivities. Only a few algal strains can be cultured in an industrial scale open pond system (Richmond, 2004). By contrast, the closed photobioreactors are relatively more reliable and stable and can prevent the external contamination and disturbance. Their productivities can be very close to the theoretical value. However, many problems such as O_2 inhibition, mixing, circulation and CO_2 supply could still restrain its development and popularization. According to the photosynthesis process ($6CO_2 + 6H_2O \xrightarrow{Light} C_6H_{12}O_6 + 6O_2$), oxygen will be generated and

accumulated in the medium during the growth of microalgae. High oxygen concentration could limit the photosynthesis rate. In addition, proper mixing of the medium is also important to algal growth. Generally, mechanical stirrers and pumps are used for mixing. However they will consume energy and may break delicate algal cells (Miron *et al*., 1999). Moreover, CO_2 dosing can significantly affect the performance of the bioreactor, it is because that CO_2 is the most important carbon source for photosynthesis. However, the CO_2 is generally dosed into the medium by normal sized bubbles (over 500 μm), the resident time and the mass transfer rate are not ideal, which will considerably limit the CO_2 uptake by microalgae.

An airlift photo-bioreactor is a closed or semi-closed biomass production system. The airlift photo-bioreactor can provide a stable and reliable condition for algal growth with little external contamination. It has relatively simple structures, which means the capital cost, operational cost and maintenance cost are low. Due to its unique geometries, it can also achieve very fine gas dispersal and mixing with low energy consumption. The airlift photo-bioreactor has no moving parts, low power consumption, high mass transfer rate, homogeneous shear force, rapid mixing rate, and less land requirement biomass production system (Miron *et al*., 2002).

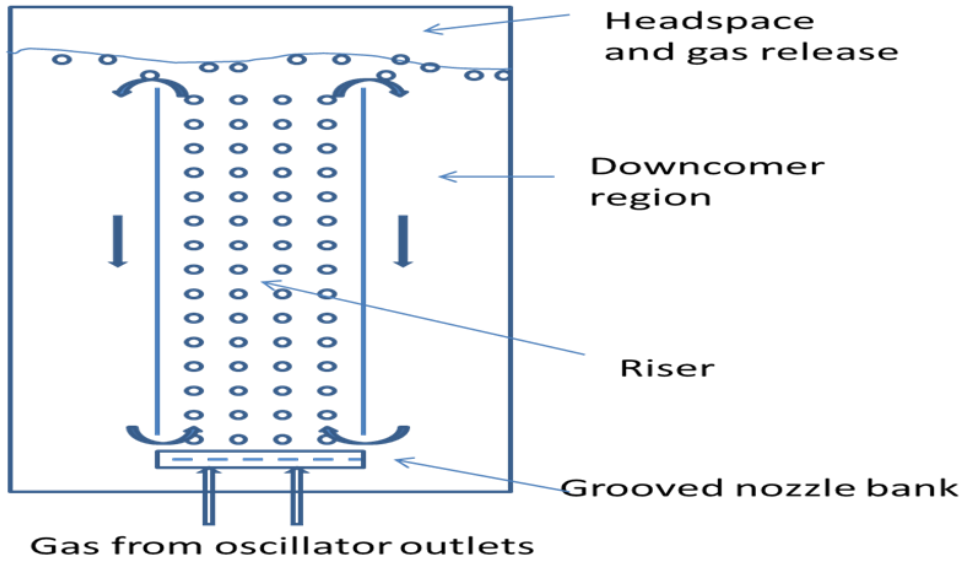


Figure 2.1. The principle of the circulation of gas and liquid within an airlift bioreactor

Figure 2.1 shows the principle of gas and liquid circulation in the airlift bioreactor. The gas flow will be injected into the bioreactor from the diffuser placed at the bottom of the reactor. When gas stream entered into the draft tube, water will be dragged up and formed a rising flow. Due to more gas in the draft tube than the rest of the bioreactor, the density of the fluid in the centre of the reactor is lower. A pressure difference is generated due to this density difference, which drives the flow to circulate in the bioreactor. This pressure difference can be expressed by following formula (Merchuk and Yunger, 1990).

$$\Delta P = \rho L\, g(\emptyset R - \emptyset D) \quad \text{Eq 2.1}$$

Where, ΔP is the pressure difference between the riser and downcomer; ρ is the liquid density, L is the height of the liquid; ØR is the diameter of the reactor; ØD is the diameter of the draft tube. The circulation generated by the pressure difference between the riser region and the downcomer region is more homogeneous. This circulation provides very fine mixing of the medium and the bubbles, which prevents the sedimentation of the algal cells. The circulation can move algal cell from the dark zone of the reactor to the light zone, which makes more

cells exposed to sufficient light. Compared to mechanical stirring, the circulation generated in the airlift bioreactor has very low shearing force, which minimizes the potential damage to the algal cells (Merchuk and Yunger, 1990).

2.5.2 Growth factors

The light conditions, pH value, temperature, nutrient and salinity are some of the most important aspects that affect the growth of the algae. Light is the key input that determines the energy density of the biomass produced by the algae photosynthesis. The enzyme activity of the microalgae is vitally important for almost every biochemical process occurring in the algal cell. It is highly determined by the pH value and the temperature of the cultural medium. The nutrients are key elements which maintain the survival of the algae. The metabolism pathways greatly depend on the composition of the nutrients in the medium.

2.5.2.1 Light intensity

Generally, increasing the light intensity can increase the energy amount received by algal pigments, which means more solar energy can be harvested by the algal cell (Tamiya, 1957). Chlorophyll molecules act as reaction centres which converts the photo energy into chemical energy. With more light harvesting pigments, more photons can be captured and utilized. However, if the amount of photons exceeds the capability of the pigments, then the excess photons cannot be processed and will be wasted as heat energy. Therefore, when the light intensity becomes saturated, increasing the intensity will not increase the rate of photosynthesis. Moreover, very high light intensity can lead to light inhibition of the microalgae, and it can even damage the algal cells through photoinhibition. The increase heat energy can affect the enzyme activity of the algae, and the algal cell apparatus can be damaged by continually increasing temperature (Samuelsson and Richardson, 1982). Therefore, sufficient light intensity benefits the algal growth. However, increasing the light intensity excessively will cause light inhibition. Generally, the light density between 5000 to

10000 lux in large container and 1000lux in small flask is desirable for algal cultivation (Borowitzka and Borowitzka, 1988).

Algal cultivation in photobioreactors may easily achieve high density of algal culture, however, such dense culture could also lead to problems of light attenuation or inhibition. The algal cell remains in the shaded dark zones may not receive sufficient light flux for its growth. However, the algal cells at the light zones close to the wall of the reactors may have light flux that exceeds the saturation point. Such maldisribution of light could affect the algal growth in the reactor and eventually reduce the productivity of the cultivation. It is reported that optimal light-dark cycle period could improve the photosynthetic efficiencies (Richmond *et al*, 2003). When algae growth in continuous lighting, the cells in dark zones of the reactor are brought to the illuminatated region while those former 'light' cells are shifted to dark regions, in an airlift loop bioreactor. By this circulaton pattern, the light-dark cycle could be achieved. In the case of cultivation in photobioreactors, this light-dark cycle generally is driven by gas bubbling. Through proper adjustment of the circulation time and design of bioreactor geometry, the optimal light-dark cycle can be achieved (Sforza *et al*. 2012).

2.5.2.3 The influence by Temperature

Enzymes are the bio-catalysts in algae metabolism and enzyme activity is very sensitive to temperature. At low temperature, the enzymatic reactions are slow or even stopped. As the temperature rises the activity of the enzyme will recover and increase till it reaches the optimal range. However, over the optimal temperature, the enzymatic activity drops rapidly, because the high temperature will cause enzyme inactivation or even denaturation. (Tamiya, 1957)

Generally, algal metabolism will stop when the temperature is 10 to 25℃ below the optimal value. However when the temperature is just 2 to 3 ℃ higher than the optimal temperature, algal growth will be rapidly decreased. Overall, the biomass production by microalgae will be

affected by the temperature. At the optimal temperature, the algae cell size tend to be small and the biochemical contents are the lowest. Generally, when the temperature is slightly higher than the optimal temperature, the best biomass production can be achieved (Borowitzka and Borowitzka, 1988).

2.5.2.4 pH

The pH can affect the CO_2 dosing, dispersing and chemical form in the medium. In higher pH medium, there is more CO_3^{2-} than HCO_3^- and free CO_2. As the pH increases, the HCO_3^- and free CO_2 keep decreasing. Generally, except cyanobacteria, high pH value may restrain the growth of the algae. However, as growth of the algae keeps utilizing CO_2 species, the pH value in the medium will increase. The high pH may affect the algal uptake of some key nutrients such as magnesium which is significant to the synthesis of chlorophyll. The magnesium ions will sediment under high pH condition. Generally, for most species, the pH range between 6.0 and 8.0 is appropriate (Richmond, 1986).

2.5.2.5 Salinity

The salinity of the culture medium is important to maintain the osmotic balance of the algal cell. Increasing the salinity of the medium may cause osmotic water loss. To keep the cell hydrated, the salinity must be maintained at an appropriate level. The salinity can also affect the synthesis of some organic products in the algal cell. At higher salinity conditions, the production of the lipid contents can be increased. For *Dunaliella* species, increasing the salinity can enhance the production of glycerol and carotenoids (Borowitzka, 1997). However, both high salinity (hypertonic stress) and low salinity (hypoosmotic stress) may reduce the photosynthesis rate (Borowitzka and Borowitzka, 1988).

2.5.2.6 Nutrients

Carbon

Carbon dioxide is essential to algal photosynthesis. Aquatic species of algae which cannot directly capture CO_2 from the atmosphere, can only uptake the CO_2 dissolved in water. For achieving higher photosynthesis rates in photobioreators, the CO_2 has to be injected into the medium by concentrated flow in order to provide sufficient carbon source. The CO_2 can also affect the pH value. As the CO_2 is dosed into medium, the pH value will reduce. Normally, the metabolism of the algae can increase the pH in the medium which may harm the algae photosynthesis. The pH value can be adjusted to optimal range by changing the amount of CO_2 dosing (Borowitzka and Borowitzka, 1988).

Nitrogen

Nitrogen is a key constituent of nucleic acid and proteins and during the cultivation of algal biomass, nitrogen is usually introduced into the culture medium in inorganic forms. Generally the N source for algal culture is nitrate (NO_3^-). However, many researchers have reported that the ammonia (NH_3) can be an alternative N source for algae growth. The ammonia can be assimilated by algal cell at very low energy cost compared to nitrate and it can be acquired from many industrial and municipal waste (Borowitzka and Borowitzka, 1988).

Phosphorus

Phosphorus plays a significant role in energy transfer in algae cells as a key element in the composition of ATP and ADP. It is also an important constituent of nucleic acid. The microalgae can only uptake the inorganic phosphorus ($H_2PO_4^-$ and HPO_4^{2-}). Generally, the optimal concentration of phosphorus in the medium is between 0.02 to 0.025 g/l (Borowitzka and Borowitzka, 1988).

Trace elements

Trace elements are normally metal ions (Mg, Ca, Mn, Zn, Cu and Mo) and vitamins which play important roles in algal photosynthesis and metabolism. The requirement for trace elements is very small and the amount of the trace elements added into the medium should be carefully measured. Even a very small amount of the trace element in excess of the requirement will be acutely toxic to algae (Borowitzka and Borowitzka, 1988).

2.5.3 CO_2 supply

2.5.3.1 CO_2 mass transfer

The efficiency of CO_2 supply is principally the efficiency of CO_2 gas-liquid mass transfer, and subsequently the mass transfer of CO_2 trough cell-liquid interface. Moreover, the accumulated dissolved oxygen generated by algal photosynthesis is also removed via same two phase mass transfer process.

The two-phase mass transfer process could be explained by two-film theory. As can be seen in Figure 2.2, the region where mass transfer occurs consists of three parts a gas film, a liquid film and a gas-liquid interface (Whitman, 1962). It can be assumed that all the resistance to mass transfer were lies in gas film and liquid film, and the phase interface is assumed to offer no resistance. According to Henry's law, the mole fraction of the species in liquid phase should be directly proportional to the mole fraction of that species in gas phase when equilibrium achieved, which could be described by following equation:

$$C_{Gi} = H' \times C_{Li} \quad \text{Eq 2.2}$$

Where the C_{Gi} is the gas mole fraction at the interface, and C_{Li} is the liquid mole fraction at the interface. The H' is a dimensionless form of Henry's law coefficient (BIOTOL, 1992). This equation can be used to determine the interfacial concentrations of the studying species. Chisti (1989) reported that it can be assumed that the mass transfer occurred in both films is solely molecular diffusion. Moreover, such diffusion is believed to be driven by the by the concentration gradient in the films.

Therefore, the driven force of the mass transfer in gas film and liquid film could described by (C_G - C_{Gi}) and (C_L – C_{Li}) respectively. However, the diffusivities of gas phase are much larger than those in liquid phase. Therefore, compared to liquid film the resistances in gas film could be considered to be negligible. Hence, it can be safely assumed that all the resistance to mass transfer lies in the liquid film. The mass transfer in liquid film is the control stage of the overall mass transfer. The overall mass transfer can be expressed by：

$$\frac{dC_L}{dt} = K_L a(C^* - C_L) \qquad \text{Eq 2.3}$$

Where dC_L/dt is the instant mass transfer rate, C* is the equilibrium concentration and K_La is the so-called volumetric or overall mass transfer coefficient.

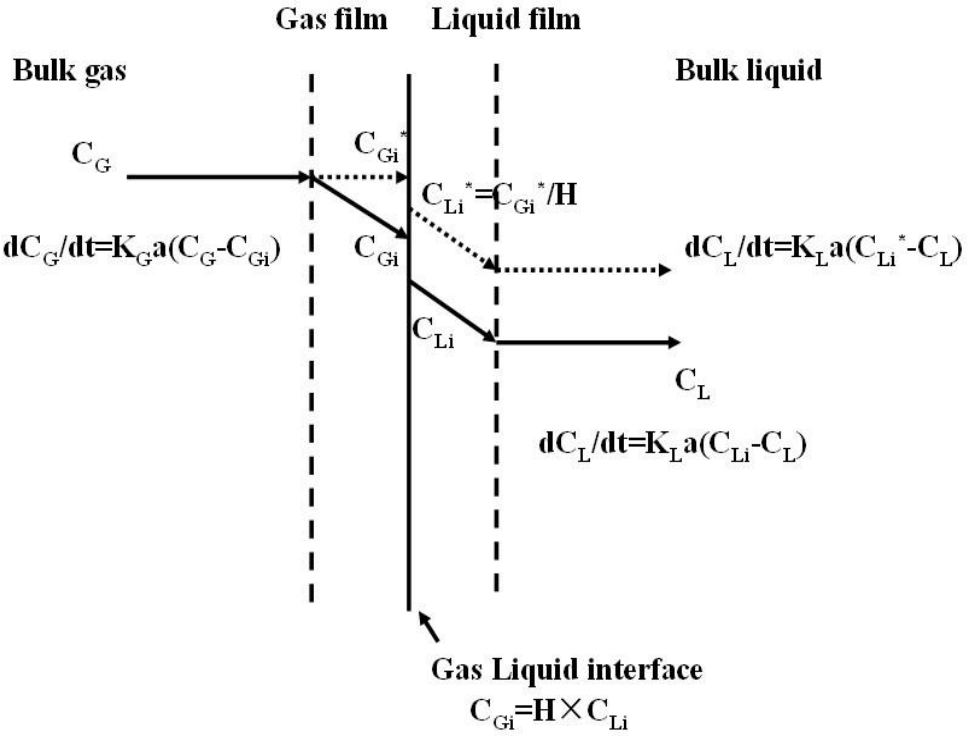


Figure 2.2: Gas-liquid mass transfer explained by two-film theory

(Adapted from Chisti, 1989)

2.5.3.2 Introducing CO_2 by microbubbles

Microbubbles refer to the bubbles with diameter ranges from 1 μm to 1mm. Due to many properties of microbubbles such as relatively high mass and heat transfer rate and slow rising time, microbubbles have been reported to have many important applications in various industrial processes, for example, separation process, special porous polymer synthesis, waste water treatment and many biological processes.

According to equation 2.3, K_La and C* are most important factors that determine the mass transfer rate. In the case CO_2 gas-liquid mass transfer, the C* is directly proportional to the CO_2 concentration in inlet gas, which indicates that dosing gases with higher CO_2 concentrations could effectively enhance the mass transfer rate. For K_La, K_L is usually a constant only depends on the gas-liquid properties. Therefore, the interfacial area 'a' is a key factor which could affect the effectiveness of mass transfer.

The interphase mass transfer has direct proportion relationship with the interphase area. For bubbles, the interphase area is its specific surface area which can be calculated by:

$$Specific\ Surface = \frac{Suface\ Area}{volume} = \frac{4\pi r^2}{\frac{4}{3}\pi r^3} = \frac{3}{r} \qquad \text{Eq 2.4}$$

As can be seen in equation 2.4, the bubble size and gas hold up are the governing parameters for mass transfer rate.

The Figure 2.3 shows the advantages of the micro-bubble, the graph (a) demonstrates the surface area increase by dividing large bubble to micro-bubbles. The diagram (b) shows that the bubble volume is proportional to the square of bubble size, and the surface and transfer rate are proportional to the cube of the bubble size. The diagram (c) shows the relationship of the total transfer rate and the bubble size (Zimmerman *et al.*, 2008).

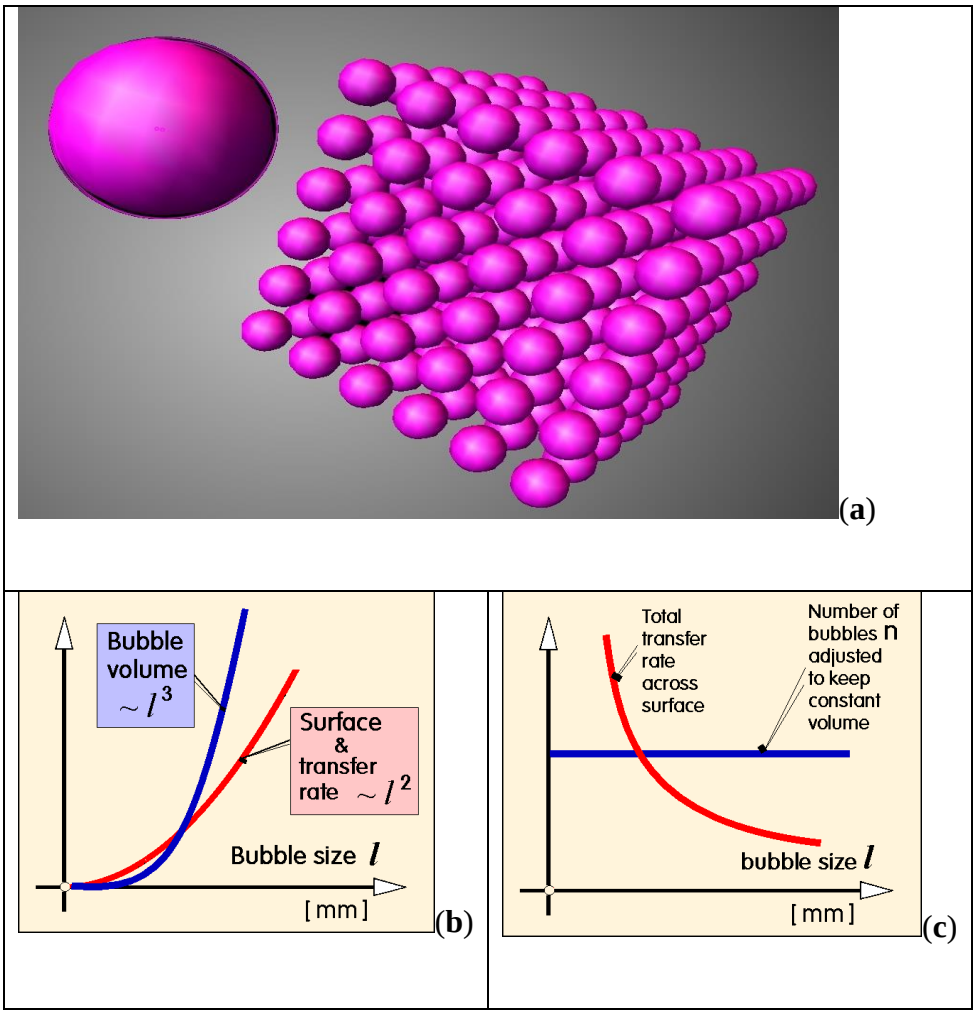


Fig 2.3 The advantages of micro-bubble

One of the key limitations of CO_2 supply for algal cultivation is lack of sufficient mass transfer efficiency. By traditional aeration methods, only 13-20% of dosed CO_2 can be utilized by microalgae, however, most of the CO_2 is wasted and released into atmosphere. There are reports claimed that this low mass transfer efficiency is determined by the bubbles size. (Carvalho and Malcata, 2006) It is believed that utilizing microbubble instead of big conventional bubbles could achieve relatively higher surface to volume ratio which could result in desirable mass transfer rate.

Moreover, the bubble with smaller size have longer residence time in liquid than larger size bubbles. According to Stokes law, the rising velocity of bubbles can be calculated through the following equation:

$$v_r = \frac{2(\rho_L - \rho_G)gr^2}{9\mu} \quad \text{Eq 2.5}$$

Where v_r is the terminal rising velocity for a single bubble with the diameter of r, μ is the viscosity of liquid, ($\rho_L - \rho_G$) is the density difference between liquid and gas. (Chisti 1989; Zimmerman *et al*, 2008) Therefore, reducing the size of bubbles can considerably reduce the rising velocity, which means much longer residence time. Zimmerman *et al*. (2009) reported that microbubble have lower momentum rising however could increase the momentum for liquid rising which is considered to be a benefactor for liquid mixing.

The size of microbubbles is generally similar to the size of an algal cell or even smaller. Therefore, the micro bubble can attach to the algal cell and provide more buoyant force to the cell, Along with the long residence time of the microbubbles, good suspension of the algal cell in the medium can be achieved.

For CO_2 supply in the algae biomass cultivation, more CO_2 dosing can be achieved by high mass transfer rate and long residence time of microbubbles. Due to the enhancement of liquid –gas mass transfer, the microbubble could help trip out accumulated dissolved oxygen.

Factors such as fluidic properties, geometry and material of orifice, etc. could all affect the formation of bubbles. In this research, the marine algae are studied. Therefore, the influence of liquid salinity and viscosity to bubble formation should be discussed. The liquid viscosity is considered as a controversial parameter of bubble formation. The reports contradicted each other. Siemes and Kaufmann (1956) reported that the liquid viscosity could not affect bubble size when the studied viscosity are relatively low. Kuma and Kuloor (1970) reported that

viscosity has no effect on bubbles formation. However, Schäfer *et al*. (2002) and Mouza *et al*. (2005) both reported the bubbles size could increase follow the increase of viscosity. During the research, it is discovered that as the algae growing the viscosity of the culture increased. However, the CO_2 dosage haven't been affected (see section 4.3 of this thesis), which means the viscosity did not affect the CO_2 mass transfer in this research.

Salinity of culture medium was reported to be able to affect the bubble formation and mass transfer. It is believed that salinity could alter the gas hold-up, mass transfer and hydrodynamic characteristics in the airlift reactor. Sajjadi *et al*. (2011) reported that the bubble size will decrease with increase in the salinity. They believe that salinity could increase the Lapalace pressure difference which could inhibit the coakescence of bubbles and improve the bubble breakage. Similar phenomenon was reported by Ruen-ngam *et al*. (2008). They discovered that increasing the salts concentration in the airlift reactor could increase the overall volumetric specific area. The mean bubble size in high salinity water is reported to be smaller than that in fresh water.

2.5.3.3 Microbubble generation by novel fluid oscillator

Conventional microbubble generation technologies are always considered to have high energy consumption and to be expensive. However, a new microbubble generation technology developed by Professor Will Zimmerman can greatly reduce the energy requirement, capital cost and the operational cost which the conventional technologies require. It is achieved by introducing a novel fluid oscillator, patented by Prof Zimmerman, into the system.

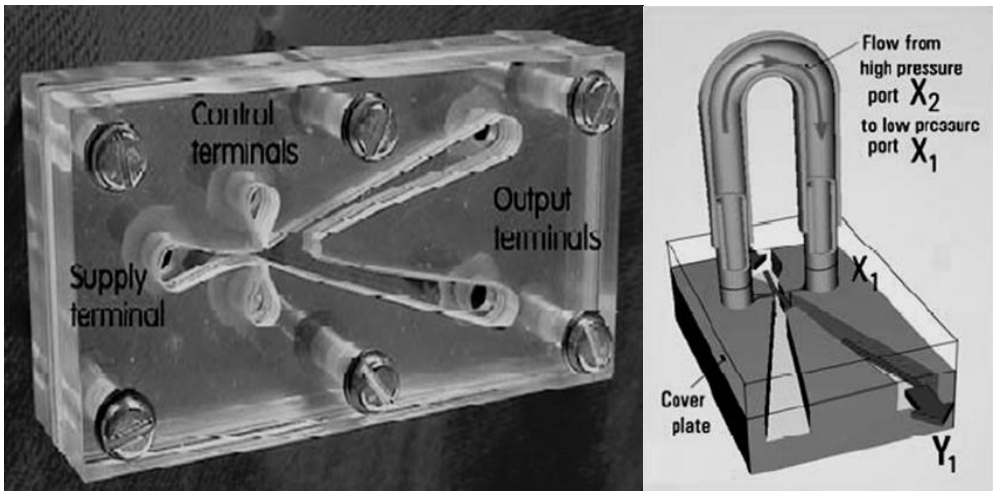


Figure 2.3 the operation principle of the fluidic oscillator

Figure 2.3 shows the basic structure and operation principle of the fluidic oscillator. It achieves oscillation by switching the continuous flow input into alternative exit channels at very high frequency.

The inlet gas flow enters into the oscillator through supply terminal. According to Coanda effect, the fluid jet turns to attach itself to nearby surface and remain attached even when the surface curves away from the initial jet direction. The flow will not come out from the both outlet terminal at the same time. It will randomly remain attached to one channel and pass through it. When the gas flow passes by one of the two control terminals, a small low pressure zone will be created near the side of the nearby control terminal due to intermolecular forces and shearing force. Therefore a pressure difference is generated between two control terminals, which are connected by a feedback. The pressure difference between the control terminals will make the gas in the feedback loop form a gas flow and move from the high pressure zone to low pressure zone. This flow can switch the input gas flow to the opposite side. Then, the whole process will repeat again and go round and round. Thereby, the oscillating flows are generated.

According to the Young-Laplace law, reducing the size of orifices will not necessarily reduce the bubble size. It can be explained by equation 2.6:

$$\Delta p = \frac{2\sigma}{r} \qquad \text{Eq 2.6}$$

Where ΔP is the pressure difference between inside and outside of a bubble, σ is surface tension and r is curvature radius of bubble. When bubbles start to form on the orifices, they have infinite curvature radius. As the bubbles growing, the radius reduced and the bubbles tend to form spherical shape. However, after the bubbles grow in to hemispherical shape, the radius start to increase as the growth of bubbles volume. As can be seen in equation 2.6, when radius reduced, the pressure difference which air flow needs to overcome is also reduced. Therefore, air could be much easier to flow into the bubbles. Hence, the bubbles are going to grow faster, and its size will be much larger than the size of orifice (Zimmerman *et al.*, 2009).

In order to stop the growth of bubbles, and reduce the bubble size, the bubble should be break off when it is still a hemispherical cap. The fluidic oscillator introduced above could generate oscillated gas flow which switching off gas supply for bubble growth. After cutting off gas supply, the surrounding water would flood the orifice and force bubbles breaking off the apertures. After, the bubbles come off the diffuser, the gas supply is switched back and the next bubbles start anew (Zimmerman *et al.*, 2009). Therefore, the bubble size will be similar to the orifice in ceramic diffuser, which are much smaller than conventional large bubbles. Thus the microbubbles are generated.

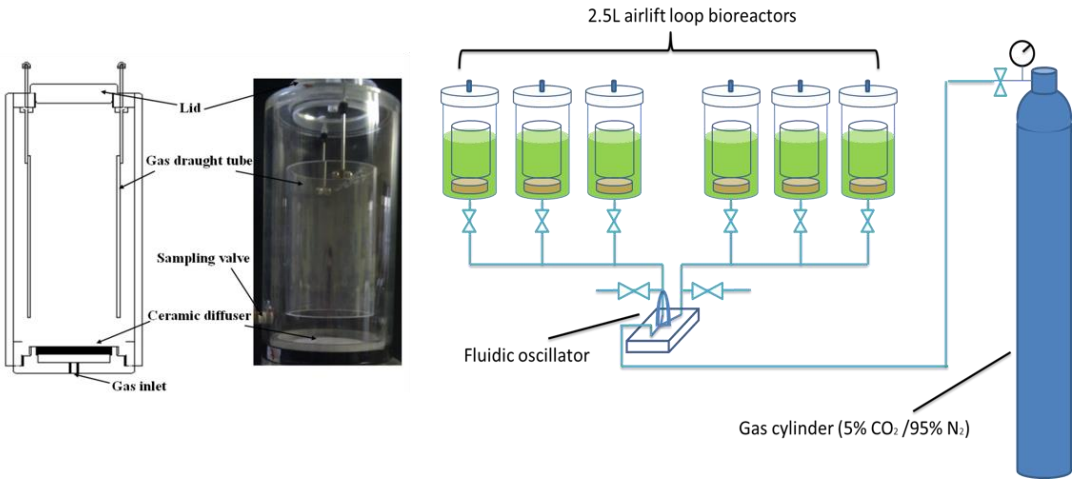
The following chapters of this thesis will report and discuss the experiments carried out by the author which examine the possibility of microalgae acclimation for acetaldehyde removal using screening based on the use of airlift photo-bioreactor systems employing microbubbles.

Chapter 3 Methodology

3.1 Equipment and Materials

3.1.1 Screening and acclimation system

The cultivation system for acclimation consisted of six 2.5L microbubble induced airlift loop bioreactors. This is a scaled down approach compared to that reported for pilot scale studies on stack gas (Zimmerman *et al*., 2011). The microbubble technology allows higher carbon dioxide mass transfer rate, better mixing and higher algal growth rate to be achieved (Zimmerman *et al*., 2009), which increases the efficiency of the acclimation system. Figure 3.1 shows the setup of the acclimation system and the basic structure of the bioreactor.



(a) (b)

Figure 3.1 The structure of the 2.5L airlift bioreactor (a) and the schematic setup of acclimation system (b).

The inlet gas contains 5% CO_2 and 95% Nitrogen. Gas flux is 1.22 m/s and the duration of the aeration is 30 minutes per day. 30 min aeration time is determined by previous research done by Ying (2013), the CO_2 concentration in the medium will be saturated within 30 min, and therefore, further dosing would not affect the mass transfer and CO_2 uptake. However,

continuous dosing would waste more energy for pumping gas flow into the culture system, moreover, continuous bubbling will maintain the pH value at acidic level which may inhibit many strains. Hence, after repeated experiment, the optimal dosing time is determined to be 30 min to 40min. (Ying, 2013a and Ying 2013c)

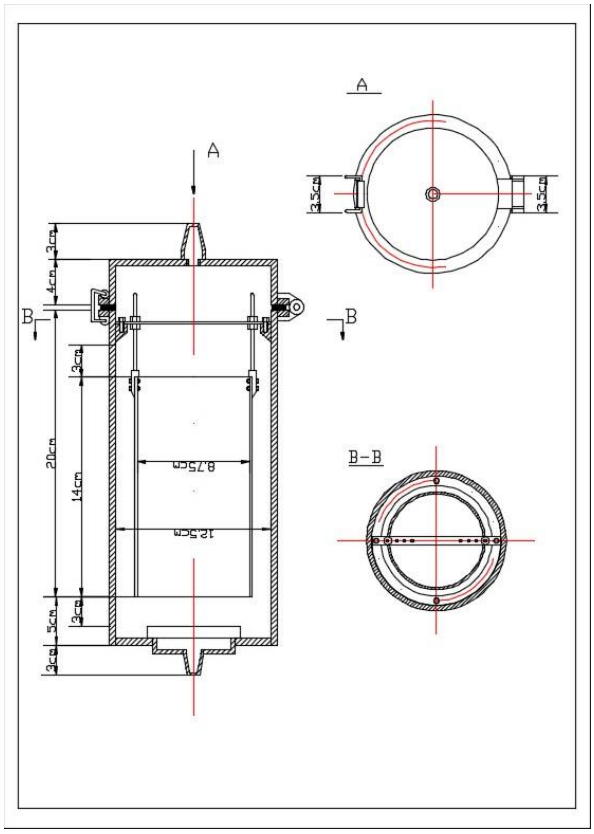
The acetaldehyde was added into the medium in liquid phase. The properties of the acetaldehyde: grade-puriss.p.a, assay-≥99.5% (GC), quality-anhydrous. Product Number: 402788, company: Sigma – Aldrich.

3.1.2 Design of screening and acclimation system

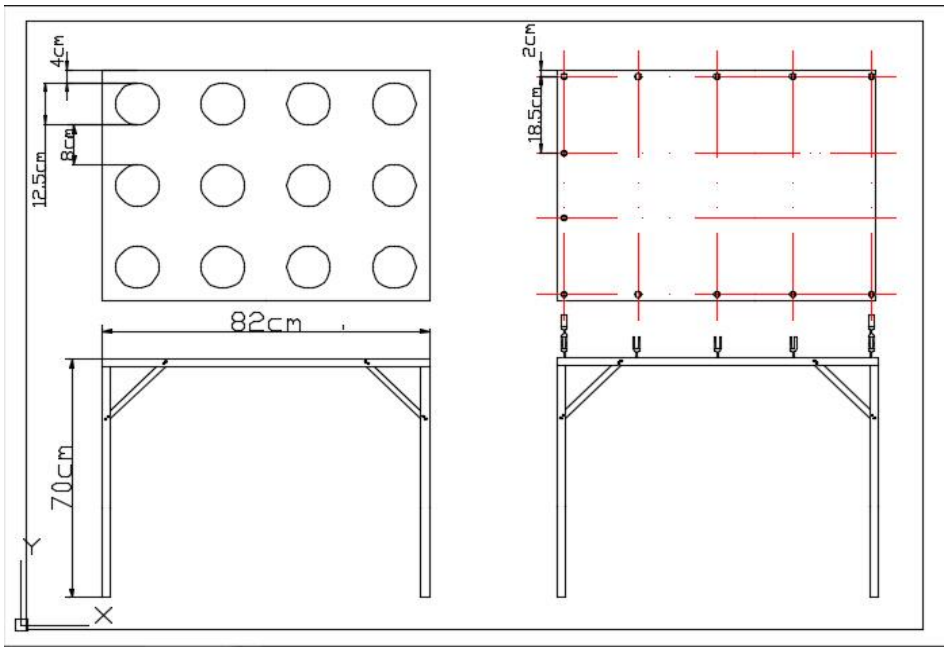
The fluidic oscillators developed by Professor Zimmerman were introduced into the design of the screening system to generate oscillated gas flow which feed through ceramic diffuser to produce microbubbles with diameters around 400 μm to the bioreactors. Microbubbles have a much larger surface to volume ratio which means higher gas and liquid mass transfer efficiency and larger dragging force when floating in the medium. These features could be exploited to enhance the CO_2 dosage and bioreactor circulation.

The bioreactor was designed to be a closed culture system due to two reasons. First is prevent contamination and reduce external influence. Second is the experiment requires dosage of acetaldehyde into the medium. Acetaldehyde is volatile and considered as harmful pollutants, therefore it should not be emitted directly into atmosphere. Hence, when designing the bioreactor a filter system should be added at the gas outlet on the top of the reactors.

As the growing of microalgae, light penetration becomes weaker, which could limit the algal growth. Therefore, the concept of airlift loop bioreactor was incorporated into the design of the screening system. The airlift loop bioreactor could generate gentle circulation which could bring algae to the shining surfaces more frequently.



(a)



(b)

Figure 3.2. (a) the design of the airlift loop bioreactors. (b) achematic management of screening/acclimation system.

The Fig 3.2 (a) shows the structure of the bioreactor applied for building the screening system. The bioreactors are made of transparent Perspex so that light can easily penetrate the bioreactors. The main column of the bioreactor is 3 L (2.5 L when operating). The diameter of the bioreactor is 12.5cm. The total height is 33cm. The diameter of the draft tube is 8.75 and it is 14cm high. The bottom of the draft tube is 2.5cm higher above the diffuser. The diffuser will be placed at the bottom of the reactor and share the same diameter with the draft tube. A 4cm high lid is fixed at the top of the bioreactor in order to prevent contamination and evaporation. Gas outlet will be collected at the top of the lid. The inlet gas from cylinder will be sent to fluid oscillator and be converted into high frequency oscillating flow. The inlet gas flow eventually is injected in the bioreactor through a ceramic diffuser. The key dimensions such as the diameter and the height of the draft tube, the height of the gas separator and the height of the liquid surface are adjustable in order to study the bioreactor geometric influence to the algal growth.

The Fig 3.2 (b) shows the 3×4 arrangement of the screening system. Each circle represents an independent 3 litre airlift bioreactor. This system can test 12 different algal strains simultaneously. Each dash dot line represents a light tube horizontally placed on the 14 racks and the 4 top light fixed above the bioreactors to provide even light distribution for each bioreactor.

3.1.3 Algal strains and culture media

All microalgae studied in this research were provided by Doctor Gilmour, Department of Molecular Biology and Biotechnology, University of Sheffield.

Dunaliella salina: It has the ability to synthesis large amount carotenoids which can be used in cosmetic and dietary supplement. It is robust and can thrive in extreme environments, where it can resist various harmful compounds, survive in highly salty conditions, and can tolerate intense light. *Dunaliella salina* is one of the most common algal strains used in commercial production. Culture medium is *Dunaliella Growth* Medium with a salinity of 1 M NaCl shown in Table 3.1. (Hajibagheri *et al*., 1986)

Table 3.1 Culture medium for *Dunaliella salina*

***Dunaliella* Growth Medium**	
Stock solution	Volume of solution for 1L of medium (ml)
1M NaCl	87.75g
2M KCl	5
1M $MgCl_2$	10
1M $CaCl_2$	10
2.4M $MgSO_4$	10
4M $NaNO_3$	1.25
0.5 M Na_2SO_4	48
100 mM NaH_2PO_4	1
1.5 mM FeEDTA	1
Trace elements	1
1M HEPES (pH 7.6)	20
$NaHCO_3$	1g

Chlorella sp.: *Chlorella* are a group of algae strains which also have high commercial value, as mentioned in previous Chapters. *Chlorella* cells contain high concentration of proteins and many other essential nutrients. They have impressive growth rates, and can reproduce rapidly requiring only carbon dioxide, water, sunlight and small amount of minerals, although many species of *Chlorella* also grow mixotrophically and heterotrophically. *Chlorella* strains have high carbon dioxide tolerance up to 40% CO_2

concentration (Hanagata *et al.*, 1992). Mixotrophic growth of *Chlorella* allows the removal of various toxic chemicals from the medium.

Chlorella sp. were cultured in 3N-BBM (modified Bold Basal Medium with 3-fold Nitrogen) plus vitamins medium shown in Table 3.2.

Table 3.2 Culture medium for *Chlorella* sp.

3N-BBM + V Medium	
Stock solution	Volume of solution for 1L of medium (ml)
25g/L $NaNO_3$	30
2.5 g/L $CaCl_2 \cdot 2H_2O$	5
7.5 g/L $MgSO_4 \cdot 7H_2O$	10
7.5 g/L $K_2HPO_4 \cdot 3H_2O$	10
17.5 g/L KH_2PO_4	10
2.5g NaCl	10
Trace element solution	6
97 mg/L $FeCl_3.6H_2O$ **41 mg/L $MnCl_2.4H_2O$** **5 mg/L $ZnCl_2$** **2 mg/L $CoCl_2.6H_2O$** **4 mg/L $Na_2MoO_4.2H_2O$**	
12 mg/L Vitamin B_1	1
10 mg/L Vitamin B_{12}	1

Tetraselmis: Tetraselmis is a genus of green algae that contains strains that accumulate very high lipid levels. Lipid content is major source for bio-fuel production.

Nannochloropsis oculata is a promising strain for this research. It is microalgae can grow mixotrophically and reported to be able to assimilate aldehyde.

The culture medium for *Tetraselmis* and *Nannochloropsis oculata* is F/2 medium shown in Table 3.3.

Table 3.3 Culture medium for *Tetraselmis* sp and *Nannochloropsis oculata*.

f/2 medium	
Stock solution	Volume of solution for 1L of medium (ml)
75 g/L $NaNO_3$	1
5.65 g/L $NaH_2PO_4 \cdot 2H_2O$	1
Trace element solution	1
4.16 g/L Na_2EDTA	
3.15 g/L $FeCl_3 \cdot 6H_2O$	
0.01 g/L $CuSO_4 \cdot 5H_2O$	
0.022 g/L $ZnSO_4 \cdot 7H_2O$	
0.01 g/L $CoCl_2 \cdot 6H_2O$	
0.18 g/L $MnCl_2 \cdot 4H_2O$	
0.006 g/L $Na_2Mo_4 \cdot 2H_2O$	
Vitamin mix	1
0.0005 g/L Cyanocobalamin (B_{12})	
0.1 g/L Thiamine HCl (B_1)	
0.0005 g/L Biotin	
Artificial Seawater	Make up to 1 L

Chlamydomonas: can use photosynthesis, heterotrophy and mixotrophy. It is well studied and used as a model organism in various research for many decades.

Chlamydomonas sp. were cultured in TAP medium shown in Table 3.4

Table 3.4 Culture medium for *Chlamydomonas* sp.

TAP medium		
Compound	**Quantity**	**Solution protocol**
Tris	2.42 g	
Solution 1 (TAP salts)	25 ml	NH_4Cl 15.0 g $MgSO_47H_2O$ 4.0g $CaCl_2.2H_2O$ 2.0g Water to 1 litre
Solution 2 (Phosphate solution)	0.374 ml	K_2HPO_4 28.8 g KH_2PO_4 14.4 g Water to 100 ml
Solution 3 (Hunter's trace elements)	1 ml	EDTA disodium salt 200 g $ZnSO_4.7H_2O$ 220 g H_3BO_3 57 g $MnCl_2.4H_2O$ 101g $CoCl_2.6H_2O$ 32.3 g $CuSO_4.5H_2O$ 31.4 g $(NH_4)_6Mo7O_{24}.4H_2O$ 22 g $FeSO_4.7H_2O$
Glacial acetic acid	1 ml	
dH_2O	To 1 litre	

3.1.4 Oxygen electrode for photosynthesis measurement

The photosynthesis rate was measured through measuring the concentration changes of oxygen in the medium. A Clark-type oxygen electrode was used to measure the dissolved oxygen concentration in algae samples contained in the temperature controlled chamber of the apparatus. A Teflon membrane permeable to oxygen forms the base of the chamber. Underneath this membrane is a shallow compartment containing a 2.3 M KCl solution and two electrodes, a platinum cathode and a silver anode. A fixed polarizing voltage is applied between the electrodes and the resulting tiny current is proportional to the oxygen

concentration. The current is determined using a chart recorder. The method is essentially polarographic, oxygen being reduced at the platinum cathode:

$$O_2 + 2e^- + 2H_2O \rightarrow H_2O_2 + 2OH^-$$

The current depends upon the rate of the diffusion of oxygen to the cathode, and this rate is proportional to the dissolved oxygen concentration. Figure 3.2 shows the basic principle and structure of the oxygen electrode.

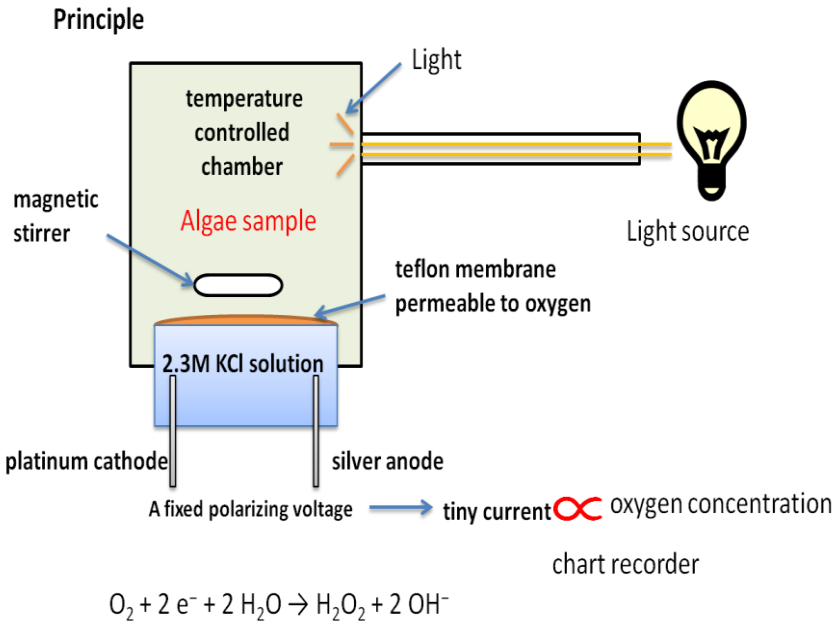


Figure 3.3. Basic working principle of oxygen electrode

3.2 Research Methods

3.2.1 Performance study of novel screening system

3.2.1.1 COMSOL simulations

A Comsol model was built for simulating the circulation and mixing of the bioreactor. It is based on the bubbly flow model provided by COMSOL Multiphysics CFD module package. This bubbly flow model is a simplification of the two-fluid model, relying on the following assumptions:

1. The gas density is negligible compared to the liquid density.

2. The motion of the gas bubbles relative to the liquid is determined by a balance between viscous drag and pressure forces.

3. The two phases share the same pressure field.

According to above assumptions, a momentum equation for liquid velocity, a continuity equation, and a transport equation for the volume fraction of gas phase can be given by summing the momentum equation for both liquid and gas phase.

The momentum equation is:

$$\Phi_1\rho_1\frac{\partial u_1}{\partial t}+\Phi_1\rho_1 u_1\cdot\nabla u_1=-\nabla p+\nabla\cdot\left[\phi_1(\mu_1+\mu_T)\left(\nabla u_1+\nabla u_1^T-\frac{2}{3}(\nabla\cdot u_1)\mathbf{I}\right)\right]+\phi_1\rho_1\mathbf{g}+F \quad (3\text{-}1)$$

Where, u denotes velocity (m/s), p pressure (Pa), Φ phase volume fraction (m^3/m^3), ρ density (kg/m^3), g the gravity vector (m/s^2), F any additional volume force (N/m^3), μ_1 dynamic

viscosity of the liquid (Pa·s), and μ_T turbulent viscosity (Pa·s). The subscripts "I" and "g" denote quantities related to the liquid phase and the gas phase, respectively.

The continuity equation is:

$$\frac{\partial(\rho_l\phi_l+\rho_g\phi_g)}{\partial t} + \nabla\cdot\left(\rho_l\phi_l u_l + \rho_g\phi_g u_g\right) = o \qquad (3\text{-}2)$$

The equation for the transport of the volume fraction of gas is:

$$\frac{\partial\rho_g\phi_g}{\partial t} + \nabla\cdot\left(\rho_g\phi_g u_g\right) = -m_{gl} \qquad (3\text{-}3)$$

Where $-m_{gl}$ is the mass transfer rate from gas to liquid (kg/(m^3·s)).

For low gas volume fractions (ϕ_g <0.1), it is in general valid to replace the continuity equation, equation 3-2, by:

$$\nabla\cdot u_l = 0 \qquad (3\text{-}4)$$

This bubbly flow model solves for u_l, p, and $\bar{\rho}_g = \rho_g\phi_g$, the effective gas density. The gas velocity u_g is the sum of the following velocities: $u_g = u_l + u_{slip} + u_{drift}$

Where u_{slip} is the relative velocity between the phases, and u_{drift} is an additional contribution when it is including a turbulence model. In this research, the bubbly flow is

assumed laminar. The bubbly flow model calculates the gas density from the ideal gas law. Liquid volume fraction is calculated from $\phi_l = 1 - \phi_g$.

3.2.1.2 Methods for microbubble bioreactor mass transfer determination

An airlift bioreactor with exchangeable diffusers was used for studying the influences of bubble size to mass transfer rate. Different diffusers were used to generate different sizes of bubbles. Five different bubble sizes were tested namely 440, 755, 790, 910, and 1020 micron. Nitrogen (99%, in 200 bar, 50 L cylinder) was bubbled into the reactor in order to strip out dissolved oxygen. The dissolved oxygen (DO) was measured with a DO meter (placed in down comer, 50 mm beneath the water) every 30 seconds until the readings stopped changing. Then, air was bubbled into the reactor, the DO measured as above and the DO reading was recorded every 30 seconds until saturated. When complete, the diffuser was change and above procedure repeated.

3.2.1.3 Methods for studying the effects of microbubble to algae growth

Two types of bioreactor were utilized, one type was equipped with fluidic oscillator which will generate microbubble for the CO_2 injection, and the other type was traditional bubble column. Two fluorescent lamps provided constant light intensity of 90±2 µmol·m^{-2}·s^{-1}.

In microbubble induced bioreactors, CO_2 was injected at bubble size of 300 µm. In traditional bioreactors, CO_2 was injecting at bubble size of 600 µm. *Dunaliella salina* was selected as the model strain for this experiment which were pre-cultured in 250 ml shake flasks and then inoculated into the reactors. The aeration duration is 30 min per day and inlet gas was composed of 5% CO_2 and 95% nitrogen. 1052+ ml samples were taken from every bioreactor

each day and stored in the fridge for further measurement. The cultivation lasted 20 days and the growth rate of algal cells in each bioreactor was measured by chlorophyll content.

3.2.2 Algal screening method

3.2.2.1 Experiment methods for screening of optimal gas flow rate to algae growth

The screening system consisted of 6 bioreactors which were equipped with a fluidic oscillator which generated microbubbles for CO_2 injection. The illumination conditions were the same as section 3.2.1.3.

Figure 3.4 The experimental set-up

6 different flux (0.14, 0.41, 0.68, 0.95, 1.22 and 1.49 m/s) were applied in order to determine the optimal gas flow rate for algal culture. *Dunaliella salina* was selected as the model strain for this experiment. The cells which were pre-cultured in 6 × 250 ml shake flasks and then transferred into the reactors as inocula. The aeration plan and inlet gas composition were the same as the experiment described in section 3.2.1.3.

3.2.2.2 Algal screening for determination of optimal strains

Five unialgal strains namely *Dunaliella salina, Chlorella* sp., *Tetraselmis*, *Chlamydomonas* and *Nannochloropsis oculata* were pre-cultured in 250 ml shake flasks and inoculated into the screening system. During the growth of microalgae, the light intensity was maintained at 84.8 µmol/m^2/sec and the temperature was 24 ± 2°C. Inlet gas contained 5% CO_2 and 95% N_2 was bubbled into the system for 30 min/per day at flow rate of 0.9 L/min.

Acetaldehyde was added into the system in liquid phase by pipette. Due to the low boiling point of acetaldehyde (21°C), it would be very hard to inject it by gas flow. The dosage of acetaldehyde was determined by actual concentrations measured in the exhaust gas from bioethanol production. The VOC compounds in the exhaust gas of a typical fermentation process is around 10000 ppm, and 20 % of these VOCs are acetaldehyde. Therefore, there is approximately 0.2% of acetaldehyde in the exhaust gas. The aeration duration for each reactor is 30 min per day and the flow rate is 0.9 L/min. Hence, if using actual exhaust gas as the gas feed for algal growth, the amount of acetaldehyde dosed into each reactor each day should be:

$$0.2\ \% \times 0.9\ L/min \times 30\ min/day = 0.054\ L/day$$

Which equals to adding liquid acetaldehyde:

$$0.054\,L/day \times \left(22.4\,L/mol \times \frac{273.15K + lab\ temperature°C}{273.15K}\right) \times \frac{acetaldehyde\ molecular\ weight}{density\ of\ liquid\ acetaldehyde} =$$

$$0.054 \times \left(22.4 \times \frac{295.15}{273.15}\right) \times \frac{44.05}{0.784 \times 10^3}\ L = 100\ \mu L$$

In order to study the microalgae response to various levels of acetaldehyde exposure, the screening processes contains two groups. The first group planned to discover how microalgae

respond to extreme acetaldehyde exposure. The dosage of acetaldehyde increased rapidly on a daily basis and the highest acetaldehyde dosage is 4 times higher than the amount would be if delivered by actual bioethanol effluent gas. The acetaldehyde addition scheme for the group 1 is shown in Table 3.5.

Table 3.5 Acetaldehyde addition scheme for screening group 1

day	**volume (μL)**
1,2	50
3,4	75
5,6	100
7	125
8	150
9	175
10	200
11	250
12	300
13	350
14 - 18	400

Group 2 aimed to determine the promising candidates from the five microalgae strains. The algae were grown under relatively gentle acetaldehyde exposure. The acetaldehyde dosage gradually increased during the cultivation and the highest dosage was set twice higher than delivered directly from effluent gas in order to set a buffering zone for the considerations of future industrial applications. The acetaldehyde addition scheme for group 2 is shown in table 3.6.

Table 3.6 Acetaldehyde addition scheme for screening group 2

day	Volume (µL)
1,2	50
3,4	75
5,6	100
7	125
8	150
9	175
10 - 18	200

15 ml of samples were taken every day from each bioreactor. The samples then measured by spectrophotometer for optical density at wavelength of 595 nm. The cell numbers were counted under microscope for each sample in order to draw the calibration curves between optical density and cell density. The growth rate of microalgae in different bioreactors were determined by cell density which can be calculated from optical density through the calibration curves.

3.2.3 Microalgae acclimation

Acetaldehyde can cause photosynthesis inhibition and growth inhibition to many algae strains. In order to increase algae tolerance to acetaldehyde and prevent rapid inhibition, the acclimation in this research was divided into two stages: treatment 1 and treatment 2.

In treatment 1, the pure algae samples were inoculated into the assay system. During the 21 days cultivation, the acetaldehyde dosage was gradually increased from 50µL to 100µL in each bioreactor. The acetaldehyde dosing procedure is shown in Table 3.6.

Table 3.6 Acetaldehyde dosing plan for treatment 1

Day	Acetaldehyde dosage (volume per 2.5L bioreactor)
3rd, 4th	50 μL
5th, 6th	75 μL
7th – 21th	100 μL

After treatment 1 finished, the algae samples taken from treatment 1 were inoculated into the assay system to continue the acclimation for treatment 2. In treatment 2, the acetaldehyde dosage was gradually increased from 100 μL to 200 μL in each reactor. The acetaldehyde dosing plan is displayed in the Table 3.7.

Table 3.7 Acetaldehyde dosing plan for treatment 2

Day	Acetaldehyde dosage (volume per 2.5L bioreactor)
3rd, 4th	100 μL
5th, 6th	125 μL
7th-8th	150 μL
8th-21th	200 μL

During the acclimation, algae samples were taken every day, and the optical density of the samples was measured by spectrophotometer. The wave-length of the OD measurement was set at 600 nm. The absorption readings acquired from spectrophotometer were calculated and converted in to cell density through cell count versus OD calibration curve.

When treatment 1 or treatment 2 finished, the acclimated algae samples were taken and inoculated into another assay system and cultivated for 21 days in the same way as the treatment 1 and treatment 2. Acetaldehyde 100 μL or 200 μL (depending on which treatment the innocula were taken from) was added into each bioreactor every day from the first day. This part of the experiment aims to evaluate the performance of the acclimation by studying the tolerance of the algal strains to acetaldehyde after each acclimation treatment. Samples were taken every day and measured by the same methods as in treatment 1 and treatment 2. Figure 3.4 showed the flow chart of acclimation processes.

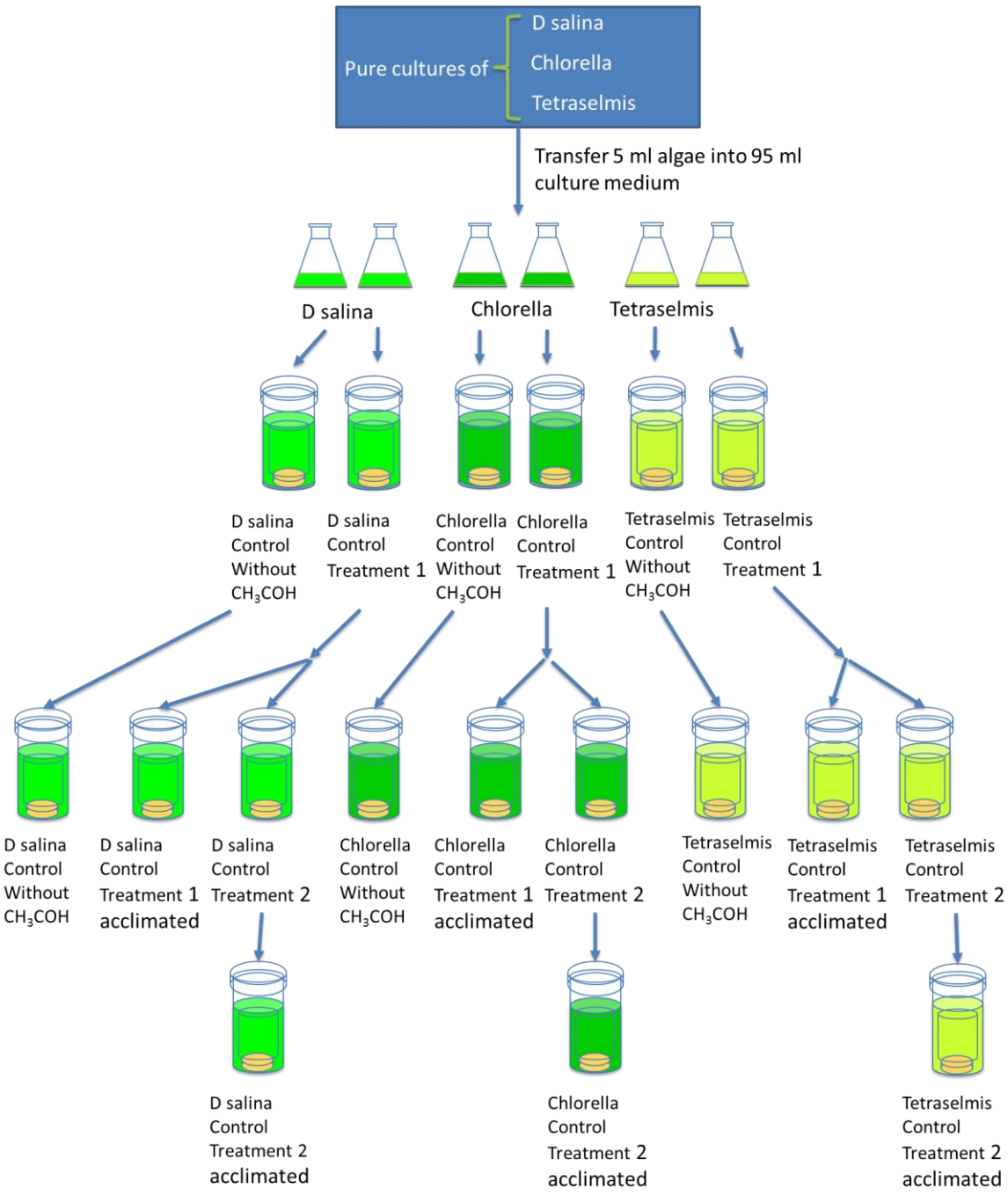


Figure 3.5 The flow chart of acclimation process

3.2 Analysis methods

3.3.1 Determination of microalgal growth

Generally, the microalgal growth is determined by specific growth rate. Scragg (1991) described the method for the calculation of overall specific growth rate of an algal culture. The relationship between the algal growth rate dC_t/d_t and the specific growth rate υ could be explained by following equation:

$$\frac{dC_t}{dt} = \mu C_t$$

Where C_t is instantaneous concentration and μ is the overall specific growth rate.

Integrate this equation, the following form can be acquired:

$$\ln\left(\frac{C_t}{C_0}\right) = \mu t$$

Where C_0 is the initial concentration. Plot $\ln(C_t/C_0)$ against t, then the overall specific growth rate can be obtained as the slope of the plot.

3.3.2 Chlorophyll content

1. Transfer 5 ml sample collected every day from bioreactors in to a 15ml Falcon tube and centrifuge it at full speed (3000 g) in a bench top centrifuge for 10min

2. Pour off the supernatant immediately. And add 1ml distilled water into the tube in order to re-suspend sediments.

3. Add 4 ml acetone into each tube. Leave the tube on the bench (avoiding direct sunlight) for a few minutes to let the acetone broke the algal cell and dissolve the chlorophyll. Then centrifuge at full speed (3000 g) for another 5 min.

4. Check whether the sediments on the bottom of the tube are totally white. If they are not, then repeat step 3.

5. Use spectrophotometer to measure the chlorophyll content of the sample. Use 80% acetone solution to zero the spectrophotometer. Then measure the optical density of the supernatant from each tube at 645 and 663nm

The chlorophyll concentration can be calculated by:

$$OD_{645} \times 202 = \quad y$$

$$OD_{663} \times 80.2 = \quad Z$$

$$(y + z)/2 = \ \mu g \text{ chlorophyll } 5 * ml^{-1}$$ (Hardwick and Baker, 1973)

3.3.3 Determination of Photosynthesis rate

1. Add 2 ml of the algae sample into the temperature controlled chamber and close the chamber. Switch off the light source but turn on the chart recorder and measurement box, and leave for 3min till the system is stable.

2. Switch on the light source and wait 5min for photosynthesis of the algae. According to the chart recorder, oxygen is increasing inside the chamber.

3. Switch off the light source and leave for 5 min. the oxygen in the chamber is decreasing because of the respiration.

4. Calculate the photosynthesis rate. The following equation is used to calculate the rate of oxygen evolution in the light and oxygen uptake in the dark, the rate of photosynthesis is calculated by adding the two rates together ignoring + and – values.:

$$oxygen\ changes = Standard/range \times number\ of\ units/time \times 60/cell\ density$$

Where the standard is the amount of oxygen at the 100% saturation point, the range is the number of chart recorder units between 0% oxygen point and 100% oxygen saturation point (given by calibration). The number of units/time is read directly from the chart recorder.

The photosynthesis experiment contains two treatments as well. Treatment 1: acetaldehyde concentration in the medium is 31.4 mg/L (equals 100 µL acetaldehyde into 2.5 L medium); and the Treatment 2: acetaldehyde concentration in the medium is 62.8mg/L (equals 200 µL acetaldehyde into 2.5 L medium)

3.3.4 Lipid measurement

3.3.4.1 Nuclear magnetic resonance (NMR)

Samples (15-30 ml) taken from each bioreactor at the end of the cultivation were centrifuged at 3000 g for 10 mins. The microalgae can be seen to be separated from growth medium and formed pellets after centrifugation. The supernatant was discarded and the pellets were re-suspended in 5 ml distilled water then transferred into 15 ml Falcon tubes. The tubes were centrifuged again at full speed (3000 g) for 5 mins. Then the supernatant water was poured off and the microalgae pellets were re-suspended in 0.75 ml of distilled water. The re-suspended samples were transferred into 1.5 ml pre-weighed and labelled microfuge (Eppendorf) tubes. The Falcon tubes were washed by 0.75 ml distilled water which was later added into the microfuge tubes in order to give a total volume of 1.5 ml.

The microfuge tubes were centrifuged for 10 mins at full speed. Pipette was used to remove supernatant, and each pellet was re-suspended in 1 ml distilled water. Same numbers of unused microfuge tubes were taken. The lids were cut off and a hole was drilled in each lid by dissecting needle. These lids were put on top of the microfuges which contained the samples.

The samples were then stored in freezer at temperature of -80°C for more than 1 hour (longer e.g. overnight is fine). The samples were taken out afterwards and freeze dried (lyophilized) for 24-48 hours. After lyophilization, the holed lids were discarded and tubes were sealed by their own lids. Re-weigh the Eppendorf tubes in order to estimate the weight of algal biomass. The samples could be stored at room temperature on the bench waiting for NMR measurement.

However, for some algal strains such as *Chlorella* sp. and *Tetraselmis* sp., centrifuging and freeze drying alone cannot ensure algal lysis. Therefore, before freeze drying the sample, sonication was applied to *Chlorella* and *Tetraselmis* samples in order to further disrupt the cells.

The freeze dried samples were dissolved into 500 µL 1:6 deuterated chloroform ($CDCl_3$): deuterated (CD_3Cl_3) with 5 µL chloroform ($CHCl_3$) used as an internal standard for reference. Spectra could be acquired by using a Bruker Avance 600 which is equipped with a cryoprobe inti 16k complex data points. A simple pulse-acquire programme was used with a 3 second recycle time with four blank scans prior to eight scans of the sample. The acquired spectra were Fourier transformed using a 1 Hz line broadening followed by manual baseline correction. Bruker Topspin 1.3 was used to process and integrate final spectra.

3.3.4.2 GC-MS measurement for FAME

The method was developed by Doctor Krys Bangert (University of Sheffield) based on the published work by Ichihara *et al*., 2010. Algal samples were collected in 50 ml Falcon tubes from the screening system. Samples were centrifuged at 3000g for 10 min. The supernatant was poured off and the algae pellets were re-suspended in 5 ml distilled water. Then, the Falcon tubes were centrifuged again at 3000 g for 5 min. The supernatant water were discarded and pellets were re-suspended in 1 ml distilled water. Eppendorf tubes were selected, weighted and labelled, which were fitted with separate holed lids. The centrifuged microalgae samples were transferred into these Eppendorf tubes and then stored in the freezer at -80 °C overnight (or 12 hours minimum). The samples were taken out next day and sent for lyophilisation for 24 hours. The freeze dried samples were then weighed for determination of biomass yield. Approximately 7 to 10 mg of the dried sample were taken and transferred into clean glass vials (11 mm, 2ml, 12×30 mm Kinesis CRV12-02).

190 µL chloroform / methanol (2:1, v/v) were added into the vials for lipid solubilisation. 10 µL (0.1 mg) of tridecanoic acid (C13 lipid) in chloroform / methanol (2:1, v/v) were added in to the vials as internal standards for determination of solubilisation efficiency. The vials were then sealed with PTEE lined cap (Kinesis CRC11-02 B2) and placed on a hot plate set at 85 °C for 1 hour for the lipids transesterification. When finished, the vials were taken out and placed on the bench for 5 min in order to let the samples cool to room temperature. The caps were removed and 975 µL hexane were added into each vial for FAME extraction. The vials were re-sealed and left on the bench for another hour. Then, the vials were unsealed and 487.5 µL of the top hexane layer were transferred into fresh vials. 12.5 µL (0.125 mg) of 10 mg / mL methyl nonadecanoate (C19 FAME) in hexane internal standard were added into each new vials and PTEE lined caps were used for sealing the vials.

Gas chromatography mass spectroscopy (GC-MS) were used for identifying and quantifying the lipids extracted from algae samples. The model of the GC-MS used in this research is Perkin Elmer – AutoSystem XL Gas Chromatography (CHM-100-790) combined with Perkin Elmer – TurboMass Mass Spectrometer (13657). The GC Capillary column equipped in this machine is Zebron – ZB – 5MS – 30 m 0.25 mm ID and 0.25 µm FT (7HG – G010 - 11). The GC-MS setting is listed below:

Autosampler Method:

Injection volume: 5 µL

Preinjection solvent washes: 2

Post injection solvent washes: 6

Temperature Program (FAME03 _ 100 min): 100 – 300 °C, Ramp 1:2 °C/min to 300,

20 ml/min He Constant carrier gas flow

MS Scan: EI+,

Start mass: 50

End mass: 600

Scan time: 0.5 s

Interscan time: 0.1 s

Start time: 0

End time: 100 min

The Perkin Elmer's Turbomass software linked to NIST data base was used to identify and integrate the resultant peaks.

In order to calibrate the FAMEs quantification in algal biomass, the transesterification efficiency should be calculated. It can be acquired based on the C13 lipid standard. The related equations are listed below:

Molecular weight (M_w) C13 fatty acid = 214.4 g/Mol, Molecular weight C13 FAMW = 228.4 g/Mol.

$$\text{Moles in 0.1 mg of C13 FA} = \frac{\left(0.1\frac{mg}{1000}\right)}{214.4\frac{g}{M}} = 4.66 \times 10^{-7} M,$$

the coverted weight should be

$$(Mol_{C13FA} \times M_W C13FAME) \times 1000 = (4.66 \times 10^{-7} M \times 228.4\frac{g}{M} \times 1000 = 0.107\ mg$$

Therefore, the external standard transesterification efficiency should be:

$$\frac{\left(a_{C13std} - c_{C13yint}\right)}{m_{C13RF}} = c_{c13\ std}$$

$$\left(\left(\frac{c_{c13stg/mg}}{0.107mg}\right) \times 100\right) = e_{trans-ext}$$

The internal std transesterification efficiency should be:

$$\left(\frac{\left(a_{c13\ std}/a_{c19\ std}\right) - C_{c13\ yint}}{m_{c13\ IRF}}\right) \times C_{c19\ std} = C_{C13\ std/mg}$$

$$\left(\frac{c_{c13\ std/mg}}{0.107\ mg}\right) \times 100 = e_{trans-int}$$

Where $e_{trans\text{-}ext}$ is the standard external transesterification efficiency, $e_{trans\text{-}int}$ is the standard internal transesterification efficiency, $a_{c13\ std}$ is the integrated area of the C13 FAME standard in algal sample, $c_{c13\ yint}$ is the y-axis intercept of the line from the appropriate C13 calibration curve, $a_{c19\ std}$ is the integrated area of the C19 FAME standard in the algal samples, $m_{c13\ RF}$ is gradient of the C13 calibration curve or RF, $c_{c13\ std}$ is the concentration of C13 FAME in the sample, 0.107 mg is the theoretical maximum concentration of C13 FAME transesterified in the samples and $m_{c13\ IRF}$ is the gradient of the C13 calibration curve (or IRF).

In order to calculate the concentrations of algal FAMEs requires to make calibration curve by using pre-mixed set of FAME standard (Supelco 37 Component FAME mix 47885-U) combined with a 0.25 mg methyl nona-decanoate (C19 FAME) internal standard. Five concentration of the standard solution were prepared (0, 2, 4, 6 and 8 mg/ml) to create a 5 point calibration curve.

The Response Factor (RF) can be calculated by following equation:

$$RF = \frac{FAME\ area_{supelco}}{FAME\ conc_{supelco}} = \frac{y\ axis}{x\ axis}$$

The Internal Response Factors (IRF):

$$IRF = \frac{RF_{supelco}}{RF_{C19\ int\ std}} = \frac{FAME\ area_{supeico}/FAME\ area_{C19\ int\ std}}{FAME\ conc_{supeico}/FAME\ conc_{C19\ int\ std}}$$

Where, FAME $area_{supeico}$ is the integration area of the FAME in Supeico solution; FAME $conc_{supeico}$ is the actual concentration of the FAME in Supeico solution; FAME $area_{C19\ int\ std}$ is the integration area of the C19 internal standard; FAME $conc_{C19\ int\ std}$ is the concentration of the C19 internal standard.

Then the FAME in algae samples can be calculated by folloing equations

$$\frac{a_{AlgFAME} - c_{Alg\ FAME\ yint}}{m_{AlgFAME\ RF}} = C_{algae\ FAME}$$

$$\left(\left(C_{algae\ FAME}/e_{trans-ext}\right) \times 100\right) = C_{algae\ FAME\ compensated}$$

$$\left(\left(\frac{C_{algae\ FAME\ compensated}}{W_{BM}}\right) \times 10\right) = C_{algae\ FAME\ per\ biomass}$$

$$C_{algae\ FAME\ per\ biomass} \times 0.1 \times 100 = FAME\ as\ \%\ Biomass$$

Where $C_{algae\ FAME}$ is the concentration of FAME in the sample; $C_{algae\ FAME\ compensated}$ is the FAME concentration compensated for the transesterification efficiency by using C13 standard; $C_{algae\ FAME\ per\ biomass}$ is the FAME concentration per unit biomass.

3.3.5 Statistical analysis

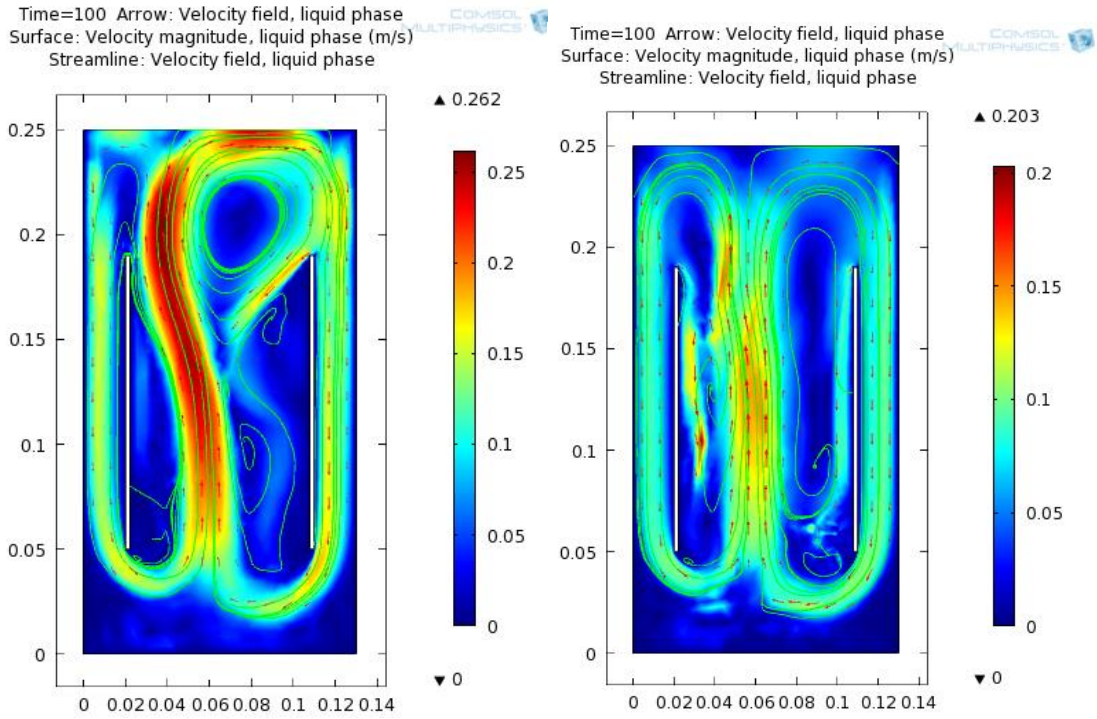
In order to prove the scientificity, validity and repeatability of the findings of this research, independent replicates were performed for the mass transfer, algal screening and algal acclimation experiment. The results of the experiments were demonstrated correspond to the

calculated mean from these replicates. The data variation were represented by the standard deviation which graphically presented as error bar.

In order to determine the statistical significance of the data acquired from acclimation trial of *Dunaliella salina* and *Chlorella* sp., the software minitab 17 were introduced for the statistical analysis. A general linear model was generated for the analysis of variance (ANOVA). The specific growth rates, photosynthesis rates and lipid production of the algal cultures of same strains under different acclimation treatments (control, treatment 1 and treatment 2) and different stages of acclimation (before acclimation, acclimating, acclimated) were compared and evaluated to determine whether there was any significant statistical difference. The statistical analysis provided more precise and statistically significant evidences on how the two microalgae strains respond to acetaldehyde exposure, and evaluate the effectiveness of the acclimation scientifically. Thus, the more suitable algal strain with high acetaldehyde tolerance and potential for the development of acetaldehyde and CO_2 sequestration system could be determined.

CHAPTER 4 PERFORMANCE STUDY OF THE SCREEENING SYSTEM

4.1 COMSOl Simulation of the Circulation and Mixing of Microbubble Bioreactor



with oscillator (Flux 0.95m/s, 300 micron bubble size)

without oscillator (Flux 0.95m/s, 600 micron bubble size)

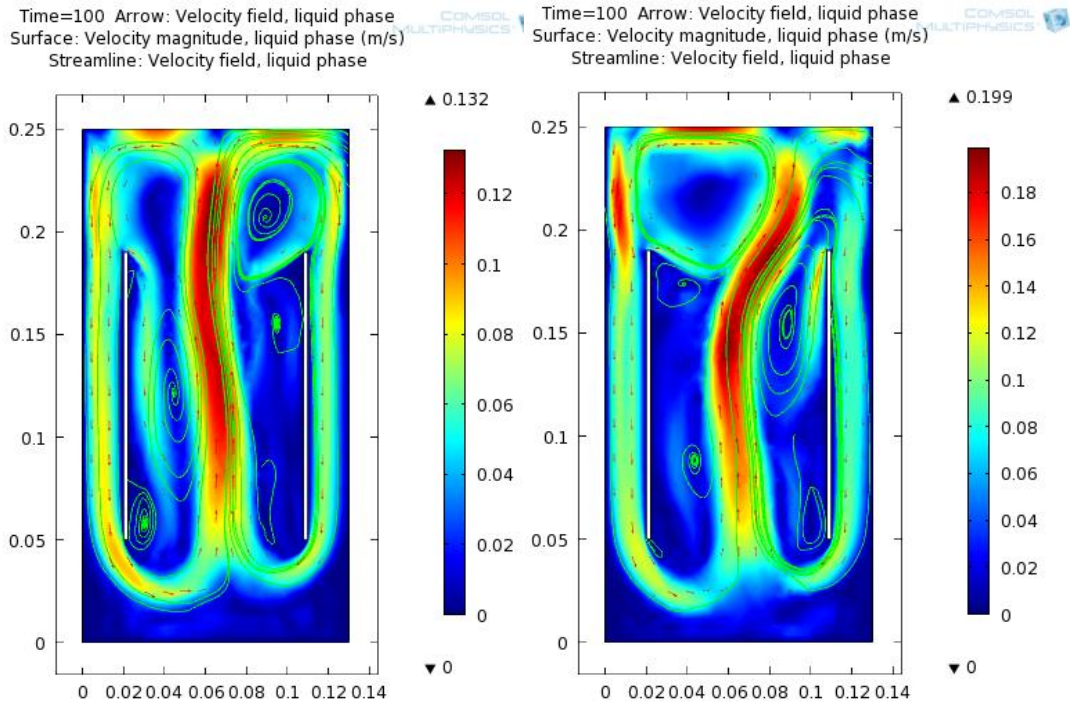
Figure 4.1 The simulation of the circulation in the bioreactor with / without oscillator at a flux of 0.95 m/s

A COMSOL Multiphysics model was built in order to simulate the circulation and mixing of the bioreactors applied in the screening system. As Figure 4.1 demonstrates, both the bioreactors with oscillator and the ones without should be able to achieve homogeneous circulation and mixing. However, the model predicted that the liquid velocities in the bioreactors with oscillator are much higher than the velocities in the bioreactors without oscillator under the same gas flux. Due to the oscillation, the bubbles injected into the

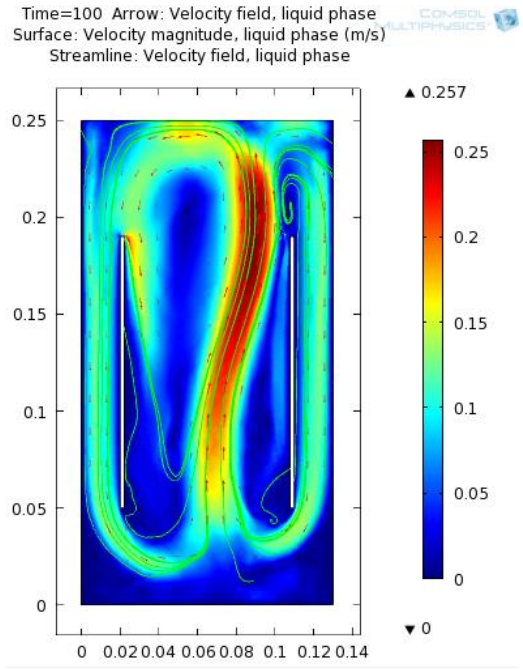
reactors with oscillator are micro-bubbles (100 to 300 micron). Micro-bubbles have much larger specific surface areas which can generate larger dragging force to the surrounding liquid. The smaller bubbles have longer residence time in the water. The gas hold-up in the draft tube of microbubble reactor should be larger. Therefore higher pressure differences are generated, which leads to higher liquid velocity. According to the equation:

$$Bo = \frac{U_L L}{D_Z}$$ (Asterio, 2000)

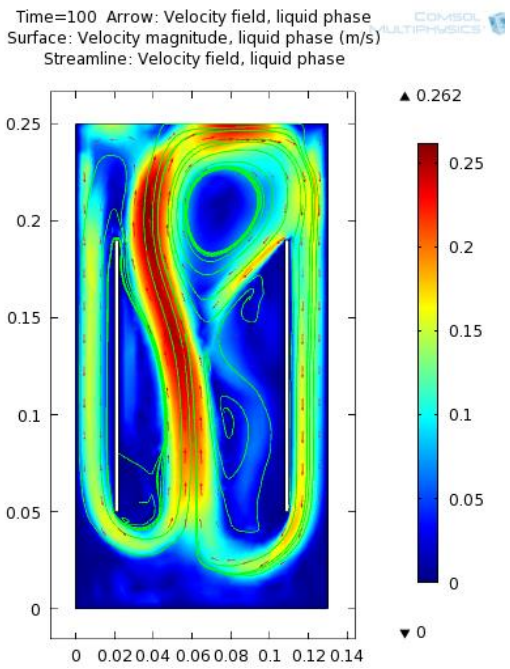
Where *Bo* is Bodenstein number which is used to describe the mixing in the reactor, *L* is the characteristic length, U_L is the liquid velocity and D_Z is axial dispersion coefficient. Increasing the liquid velocity can enhance the mixing of the medium in the bioreactor.



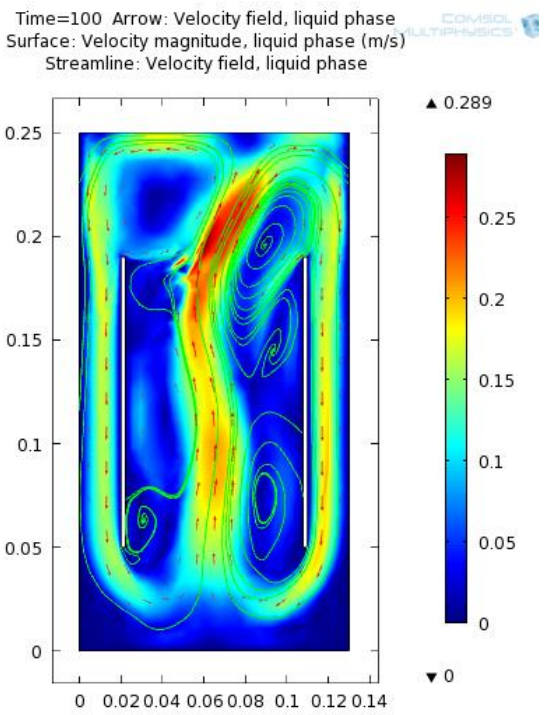
(a) Flux 0.14 m/s (with oscillator) (b) Flux 0.41m/s (with oscillator)



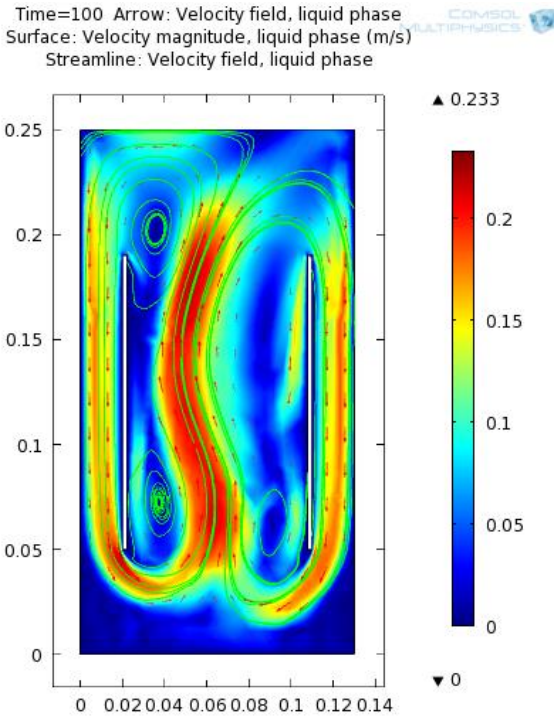
(c) Flux 0.68 m/s (with oscillator)



(d) Flux 0.95 m/s (with oscillator)



(e) Flux 1.22 m/s (with oscillator)



(f) Flux 1.49 m/s (with oscillator)

Figure 4.2 The simulation of liquid circulation in the bioreactor with oscillator

As can be seen in the Fig 4.2, the model predicted that very fine circulation can be observed under all different gas fluxes. The liquid velocities in different bioreactors should be increased following the increase of the inlet gas flux. The driving forces of the liquid circulation in the reactors are the drag forces between rising bubbles and surrounding waters as well as the pressure differences between the riser and the down comer region. As the gas flux increased, more bubbles will enter into the draft tube. Therefore, the fluid density inside the draft riser is decreased which increases the pressure difference between inside and outside the draft tube. Larger drive force is generated.

When the gas flux is low, the liquid velocity inside the draft tube is higher than the velocity outside. Due to lower gas flux, the pressure difference between riser and down comer regions is not sufficient. However, the rising bubbles still cause a dragging effect to its surrounding waters. Due to more bubbles concentrated inside the draft tube, larger drag force created by the bubbles will be generated inside the draft tube. Hence, higher velocity should be inside the tube. It may benefit the algal growth. The algal cells can be circulated with the liquid streams. Higher velocity in the centre of the bioreactor means shorter residence time in for algal cells the dark zone, and lower velocity in the down comers means longer residence time in the light zone. However, when the gas flux increased, pressure differences become more significant driving force. More homogeneous velocity distribution will be spotted.

The model predicted that both types of bioreactor should have decent mixing and circulations. However, the microbubble bioreactors have faster liquid velocity which could lead to stronger mixing and circulation, whether this estimation is accurate and what effects such characteristics could have to microalgae growth remained to be proven by experiments.

4.2 Mass Transfer Enhancement by Microbubble Bioreactors

4.2.1 Hypothesis

According to two-film theory, the mass transfer of two phases can be described by the following equation:

$$\frac{dC_t}{dt} = K_L a \cdot (C^* - C_t)$$

Where, K_L is mass transfer coefficient which only depends on fluidic properties such as density, viscosity diffusivity and temperature etc., and *a* is the interfacial area. K_L and *a* are generally combined together as one parameter which can reflect the effectiveness of mass transfer. In the case of bubbly flow, a can be calculated by following equations:

$$a = \frac{V_G}{V_B} \times a_B$$

$$\rightarrow \quad a = \frac{\frac{\varepsilon}{1-\varepsilon} \times V_L}{\frac{4}{3}\pi\frac{d_B}{8}^3} \times 4\pi \frac{d_B}{4}^2$$

$$\rightarrow \quad a = \frac{6\varepsilon V_L}{(1-\varepsilon) d_B}$$

Where, V_G is total gas volume inside bioreactor, V_B is the average volume of a single bubble, a_B is the mean surface area of each bubble, Ɛ is the gas holdup, V_L is the total volume of liquid inside the reactor and d_B is the average diameter of each bubble. Ɛ is inversely proportional to bubbles rising velocity which will increase following the bubble size. V_L is determined by the volume of the reactor. According to the equation, under the same inlet gas flow rate, reducing the diameters of the bubbles can increase the interfacial area which could

enhance the effectiveness of gas – liquid mass transfer. Therefore, introducing microbubble into air lift bioreactors might largely benefit their mass transfer efficiency.

Moreover, bubbling oxygen free gas flow into the system can effectively remove the dissolved oxygen. Due to the high mass transfer efficiency of microbubbles, the dissolved oxygen inside the medium may effectively transfer into the gaseous phase. During the cultivation, O_2 is generated by photosynthesis reactions and accumulates in the medium. According to the relationship between the concentrations of products and the change of Gibbs free energy, the high dissolved oxygen level may affect the photosynthesis rate. It can be explained by following equations:

Photosynthesis reaction: $6CO_2 + 12H_2O \xrightarrow{photons} C_6H_{12}O_6 + 6O_2 + 6H_2O$

The change of Gibbs free energy: $\Delta G = \Delta G° + RTln\frac{[C_6H_{12}O_6][O_2]^6[H_2O]^6}{[CO_2]^6[H_2O]^{12}}$

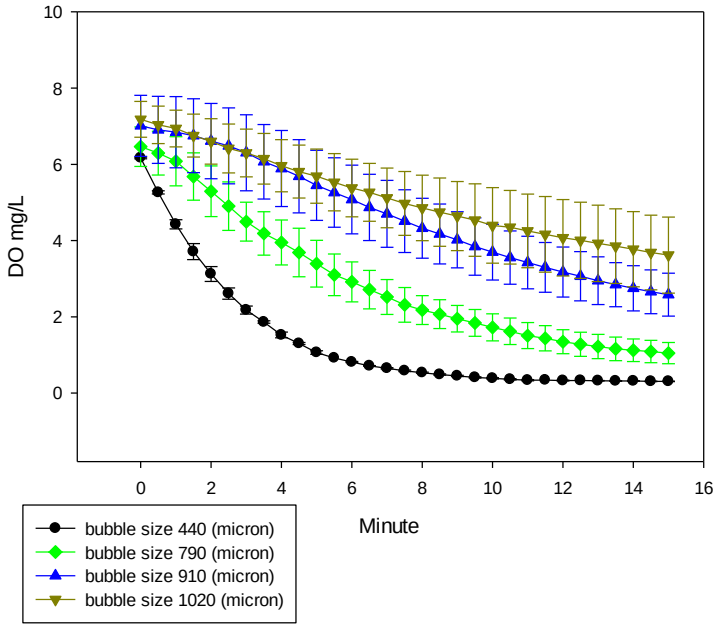
Where ΔG° is the standard change of the reaction in Gibbs free energy, it equals to $-RTlnK$.

K is equilibrium constant which only depends on the temperature. Therefore, oxygen accumulation in the medium will lead to an increase in the ΔG. When ΔG<0 the reaction can happen spontaneously. However, when ΔG=0 the reaction reaches equilibrium, and ΔG>0 the reaction cannot happen without extra work. Therefore, increasing the concentration of oxygen in the growth medium may restrain or even inhibit the photosynthesis process. Therefore, stripping out oxygen from the culture is believed to benefit the algal growth.

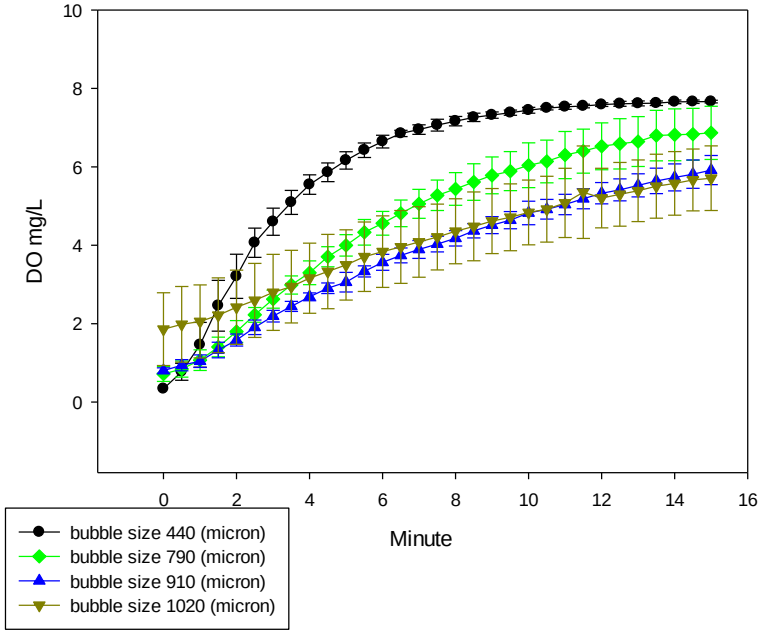
4.2.2 Results and discussion

The experiment has two stages. First bubbling nitrogen by different sizes of bubbles in to water to study the effectiveness of oxygen striping. Then, bubbling air into oxygen striped water in order to determine the mass transfer rate of gas dosing. Four different bubble sizes

namely 440, 790, 910 and 1020 micron were studied. All the groups were conducted under same gas flux (0.14 m/s).



(a) Oxygen striping



(b) Oxygen dosing

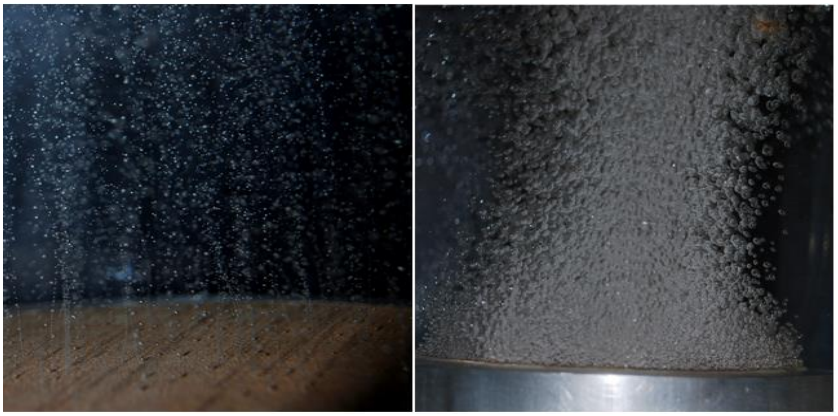
Figure 4.3 Oxygen mass-transfer driven by different sized bubbles

As can be seen in the Figure 4.3, the highest oxygen exchange rate was driven by 440 micron sized bubbles. In both oxygen depletion and dissolution experiments, the mass transfer rate were inversely proportional to the size of bubbles, which supported the hypothesis that reducing bubble size may significantly improve the effectiveness of gas-liquid mass transfer. At inlet gas bubble size of 440 micron, the dissolved oxygen level dropped (or rose, depending on depletion or dissolution) rapidly for first 6 minutes of aeration, and then reached equilibrium concentration. However, all other test groups with larger bubble sizes failed to achieve equilibrium within 15 minutes of bubbling.

Another interesting phenomenon can be observed in Figure 4.3. The groups with smaller bubbles turns out have less deviation. Replicate experiments indicated that the results of mass transfer driven by larger bubble had lower consistency. As equation 4.2 indicated, the larger bubble size lead to higher rising velocities:

$$v_r = \frac{2(\rho_L - \rho_G) g d_B^2}{9\mu} \qquad \text{Eq. 4.2}$$

Where, v_r is the rising velocity, $(\rho_L - \rho_g)$ is the density differences between liquid and gas, d_B is the diameter of bubbles, and μ is the viscosity of the liquid. According to this equation increasing the bubble size can significantly increase the rising velocity. As shown in Figure 4.4 (b), the large fast rising bubbles formed crowd swarms which more easily collide with each other and generate even larger bubbles. The diameter distribution of these bubble swarms are wide. Therefore, their mass transfer rate could be diverse and inconsistent.



(a) (b)

Fig 4.4 Photos of bubble swarms (a) bubble size around 388 micron (b) bubble size around 719micron

As can be seen in Figure 4.4 (a), the smaller sized bubbles have more uniform distribution. The bubbles were even sized and have low rising velocity which means longer residence time and higher gas hold-up. Therefore, more efficient mass transfer and less deviation can be achieved.

K_La is an essential parameter in two film theory, it directly reflects the effectiveness of mass transfer. The relationship between the mass transfer rate and K_La can be described by following equations:

$$\frac{dC_t}{dt} = K_L a(C^* - C_t) \qquad \text{Eq. 4.3}$$

Where C_t is instantaneous concentration, C^* is equilibrium concentration, and t is the aeration duration. This equation can be transformed into:

$$ln\frac{(C^*-C_0)}{(C^*-C_t)} = K_L a \cdot t \qquad \text{Eq. 4.4}$$

Where,

$$\frac{C^* - C_0}{C^* - C_t} = \frac{1}{\frac{C^* - C_t}{C^* - C_0}} = \frac{1}{\frac{C^* - C_t + C_0 - C_0}{C^* - C_0}} = \frac{1}{1 - \frac{C_t - C_0}{C^* - C_0}}$$

Where C_0 is the initial concentration. Make $E = \frac{C_t - C_0}{C^* - C_0}$, then the equation can be converted into:

$$ln\frac{1}{1-E} = K_L a \cdot t \qquad \text{Eq. 4.5}$$

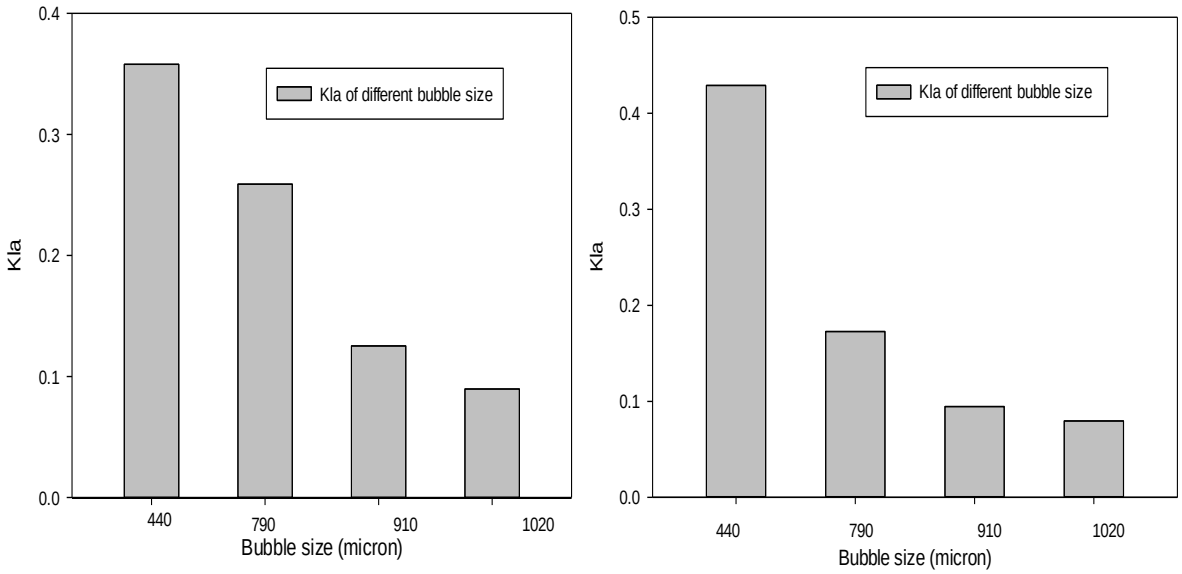
Plot $ln\frac{1}{1-E}$ against t, and the slope of the plot is $K_L a$.

The $K_L a$ of different bubble sizes were calculated and are shown in Figure 4.5. According to this bar-chart, higher $K_L a$ can be achieved by aeration through microbubbles (defined as diameter < 500 micron) compared with fine bubbles (diameter >500 micron but < 1000 micron) and normal bubbles (diameter > 1000), under same flow rate. For oxygen dissolution (Figure 4.5 (a)), the highest $K_L a$ (0.3580 min^{-1}) which was driven by microbubbles of size 440 micron was 3 times higher than the $K_L a$ (0.0897 min^{-1}) achieved by normal bubble (size of 1020 micron). The fine bubbles at size of 790 micron and 910 micron respectively had the second and third highest $K_L a$ (0.2590 and 0.1253 min^{-1}). Similarly, for the oxygen removal (Figure 4.5 (b)), the highest $K_L a$ (0.4290) was also obtained at a bubble size of 440 micron which was 4 times higher than the $K_L a$ (0.0796) of normal bubble. In conclusion, under same flow rate, reducing bubble size can improve the $K_L a$. With nonlinear regression, the relationship between $K_L a$ and the bubble size can be described by the following equations:

For oxygen dissolution $K_L a = -0.3096 \times \log(0.0007 d_B)$ $resnorm = 5.8 \times 10^{-3}$

For oxygen removal

$$K_L a = -0.4316 \times \log(0.0008 d_B) \qquad resnorm = 5.97 \times 10^{-4}$$



(a) oxygen dissolution (b) oxygen removal

Fig 4.5 K_La of different bubble sizes

According to the experimental results, the advantages of reducing bubble size are well supported. To achieve required concentration of dissolved gaseous compound, smaller sized bubble need much shorter time to dose or remove target components under the same flow rate than larger bubbles. Much research has indicated that increasing the gas flow rate can also increase the gross mass transfer. However, maintaining the gas flow rate requires consuming energy to overcome the pressure drop of the system, and increasing the flow rate means extra energy is needed. Moreover, high flow rate could cause intensive agitation which may damage the algal cells. Therefore, applying smaller bubbles for mass transfer is obviously a more energy efficient method.

4.3 Algal Growth Enhancement by Microbubble Bioreactors

4.3.1 Hypothesis

According to above experiment and simulations, microbubble airlift loop bioreactor is believed to largely benefit the growth of microalgae. Due to the large specific surface area of the microbubble, the mass transfer rate between gas phase and the liquid phase is much higher than normal bubbles. CO_2 from the bubbles can be rapidly dosed into the medium, which increases the CO_2 concentration in the medium and stripes the dissolved oxygen out of the liquid phase.

A microbubble can attach to an algal cell and provide more buoyant force to the cell and along with the long residence time of the microbubble, this means that a nice suspension of algal cells in the medium can be achieved. Furthermore the airlift bioreactor can provide fine mixing and circulation with gentle shearing force.

In order to assess the feasibility of the theory, an experiment was conducted to compare the microalgae growth rate between microbubble and fine bubble induced reactors.

4.3.2 Results and discussion

In this experiment, the algal growth was determined by chlorophyll content which generally is directly proportional to the concentration of algal cells. The model strain for this experiment was *Dunaliella salina*. Figure 4.6 shows the growth curves of *D. salina* cultured in microbubble bioreactors and fine bubble reactors. Both of the cultures were growing well, the algae chlorophyll content multiplied more than 30 times within 20 days. As can be seen in Figure 4.6, the lag phase and exponential growing phase commonly observed in microorganism cultivations were not obvious in this experiment. The growth curves were almost linear. The lack of an obvious lag phase can be explained by desirable environments provided by the airlift loop bioreactor. It would take much less time for inoculated

microalgae to adapt to the new environment. The linear growth curve is actually one of the signature features of dense algal culture thriving under the constant illumination. (Douskova, et al., 2009)

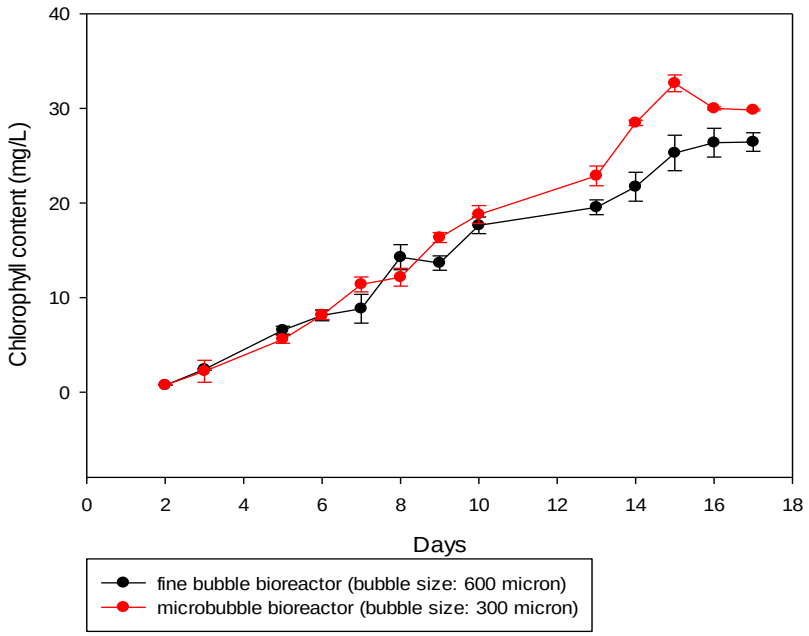


Figure 4.6 Growth of *Dunaliella salina* in microbubble and fine bubble induced reactors

As can be seen from Figure 4.6, the average growth rate of *D. salina* cultured in microbubble bioreactors was better than the one in fine bubble bioreactors, which proved the hypothesis that aeration through smaller bubbles could benefit the growth of microalgae. The chlorophyll contents of microbubble treated cultures increased from 0.74 mg/L to the highest point of 32.65 mg/L i.e., multiplied 44 times within 15 days. However, for the fine bubble cultures, the chlorophyll content only increased from 0.73 mg/L to 25.28 mg/L, which increased roughly 34 times within same period of time. The specific growth rate of these two groups can be calculated by the method described in section 3.4.1. The specific growth rate of microbubble induced cultures was 0.15 day^{-1}, and the specific growth rate of fine bubble induced cultures was 0.125 day^{-1}. By reducing the bubble size by half, a 20% improvement in growth rate was achieved.

The main advantage of microbubble aeration is rapid mass transfer. It is noteworthy that the difference in growth rates between the microbubble and fine bubble cultures was seen towards the end of the growth period (days 13 to 15), when the cultures had reached a high cell density, It is likely that some of improvement in growth due to the microbubble is caused by reducing the oxygen levels in densely populated cultures (Figure 4.6).

CO_2 dosing rate is also largely enhanced by reducing bubble size, which means more carbon source available for microalgae photosynthesis. This could be the key reason of algal growth enhancement in densely populated cultures. However, in order to prove this assumption, the relationship between CO_2 dosage and algae growth rate should be studied. Different CO_2 dosage could be achieved through different bubble size and flow rate, and the corresponding algal growth determined by the specific growth rate.

In the experiment, the CO_2 dosage is defined as the total sum of daily CO_2 transferred into each bioreactor during the period of cultivation. The daily transferred CO_2 is actually the concentration differences between CO_2 levels before and after bubbling. According to the research done by Ying (2013b), CO_2 concentration can be calculated by pH value of the culture medium:

$$[CO_2] = \frac{(10^{-pH} - 10^{(pH-14)} + \Delta[Na^+])10^{(-2\,pH)}}{10^{(-6.381-pH)} + 2\times 10^{(-16.758)}} \qquad (mol/L)$$

Where, $[\Delta Na^+]$ is the concentration of additional cations due to hydrolyzation

The relationship between algal growth and CO_2 dosages is shown in Figure 4.7. As can be seen in this illustration, the assumption was well supported by the experimental data. The algal growth rate was observed to be directly proportional to the CO_2 dosage. This should not be difficult to understand, due to increasing the concentration of CO_2 and striping out oxygen

could reduce the Gibbs free energy of photosynthesis reaction, which could favourably shift the equilibrium of the photosynthesis.

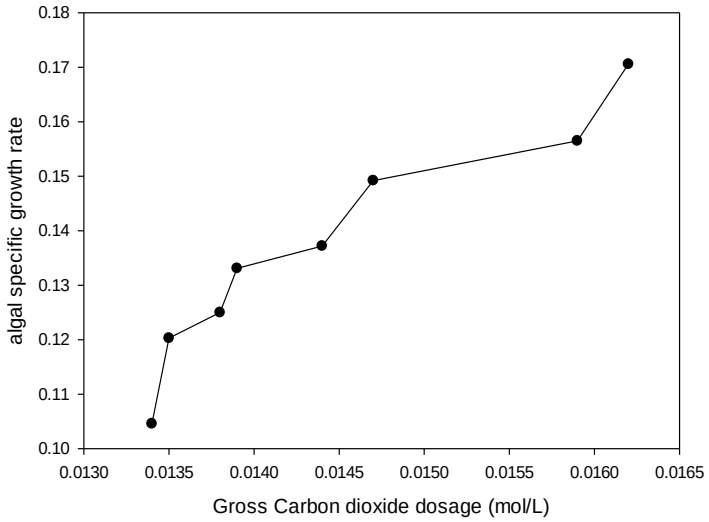


Figure 4.7. The relationship between algal growth rate and CO_2 dosage

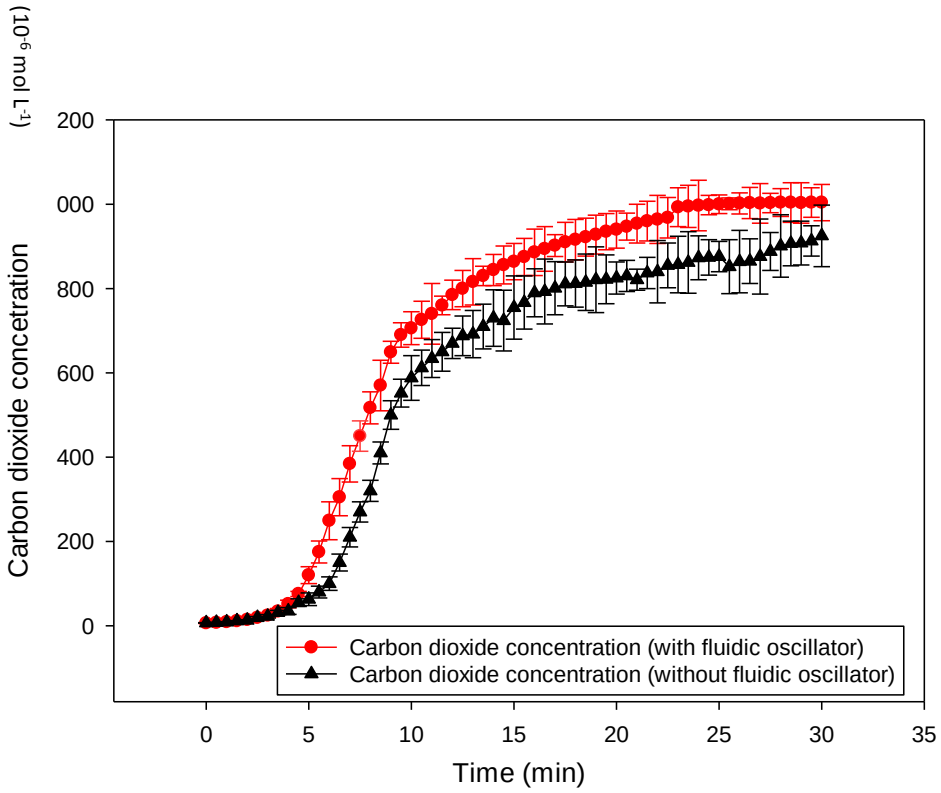


Figure 4.8. CO_2 concentration changes during the aeration

The Figure 4.8 shows the concentration changes of CO_2 in the culture medium during the aeration. As can be seen in this chart, the CO_2 concentration increased faster in bioreactor with oscillator than that in reactor without oscillator. This is because that the fluidic oscillator helps generate smaller sized bubbles which could increase the effectiveness of the mass transfer in the reactors. According to figure 4.8, the CO_2 concentration increased rapidly in first 10 minutes from 6.12±0.5 10^{-6} mol L^{-1} to 709±39 10^{-6} mol L^{-1} in the reactors with oscillator and from 6.88±0.5 10^{-6} mol L^{-1} to 588±53 10^{-6} mol L^{-1}. Then the dosing of CO_2 slowed. For the reactors with oscillator, the CO_2 concentration reached to saturation point at around 25 min of duration. The saturated concentration is 1004±42 10^{-6} mol L^{-1}. However, for reactors without oscillator, the CO_2 concentration failed to reach saturation point within 30min due to lack of mass transfer efficiency. The figure 4.8 also indicated that, for microbubble bioreactors, 30min aeration is sufficient.

4.4. Conclusion

The experiments and analysis of this chapter supported the hypothesis that algal growth could be enhanced by introducing microbubble technology. According to the experiments, the specific growth rate in the microbubble bioreactors was increased 20 to 40% compared to the fine bubble induced bioreactors at the same flow rate. Such improvements have been proved to be due to the advantages of microbubble aeration such as rapid mass transfer rate, fine mixing and circulation and oxygen striping. Moreover, the microbubbles in this research were generated by fluidic oscillators which cost no extra energy and can be manufactured economically. It is believed that culturing microalgae by the innovative microbubble airlift bioreactor is a cost effective and highly efficient algal cultivation technology.

The microbubble bioreactor is also believed by the author to have significant potential for algae screening and acclimation, since these processes essentially are simply algae cultivation under different conditions. According to the previous experiment, the algae grew faster inside microbubble bioreactors and appeared to have no lag phase (Figure 4.6), which means the time needed for reaching the goal of screening and acclimation can be shortened. Therefore, increased efficiencies of these two processes can be achieved. In addition, uniform bubbly flow consisting of microbubbles could create gentle and homogenous circulation and mixing which contributes to the distribution of nutrients without damaging the cells. For toxicity studies, such circulation and mixing can prevent sudden local overexposure which may affect the accuracy of the studies. For acclimation research, the adaptability of microalgae may not be limited only by target conditions, they could also be stressed by the cultivation environment. However, cultured inside the ‘well served’ circumstances provided by microbubble reactors (Zimmerman *et al.*, 2011), the potential of microalgae could be fully developed with minimized external interference.

CHAPTER 5 ALGAE SCREENING

5.1 Algae Screening for Determination of Optimal Inlet Gas Flux

5.1.1 Hypothesis

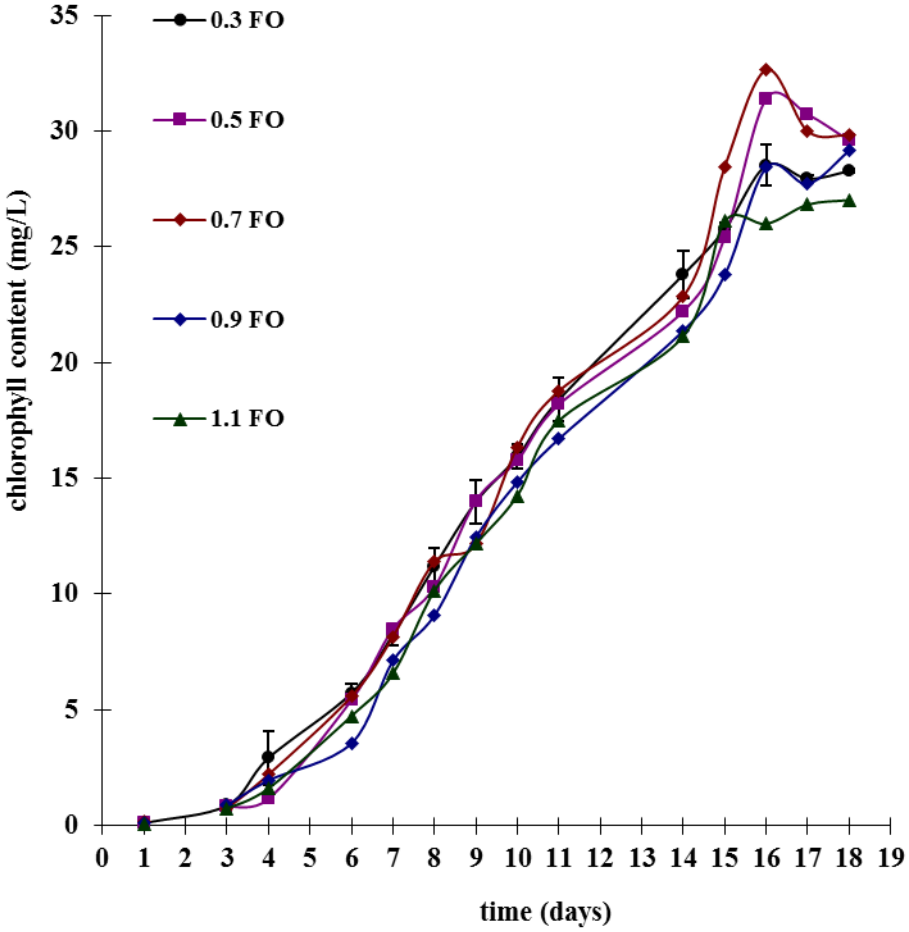
To fully explore the potential of the microbubble induced screening system, the optimal operation conditions should be studied and determined. Much research has already been done on operation conditions such as pH, temperature and light intensities. However, few studies have discussed the relationship between inlet gas flow rate and the algal specific growth rate.

Repeated experiments have indicated that inlet gas flux can affect the growth rate of microalgae (Becker, 1994). Firstly, flux can influence the CO_2 dissolution. Higher flux means larger volumes of gas injected into the reactor, which might lead to increase of the gross mass transfer. However, high flux can result in larger bubble size due to the growth and colliding of bubbles, which could reduce the mass transfer capacities of individual bubbles and eventually the CO_2 dissolution. However, for low flux, smaller bubbles in uniform gentle swarms can be observed, but aeration with such flux may lack sufficient gas hold-up to achieve the required mass transfer rate. Secondly, high flux could cause excessive turbulence which may disturb the growth of microalgae and even damage the algal cells. However, low flux may not be able to generate desired circulation inside the bioreactors, which lead to less homogenous nutrient and light distribution. In conclusion, gas flux is important parameter for algal cultivation, however neither simply increasing nor decreasing the flux will automatically benefit the algal growth rate. The optimal flow rate should be determined.

5.1.2 Results and discussion

The experiment studied how algae growth was affected by 5 different gas flux (0.41, 0.68, 0.95, 1.22 and 1.49 m/s). The model strain used is *Dunaliella salina*. Six 2.5 L microbubble airlift bioreactors were used to build the screening system. The composition of the inlet gas

was 5% CO_2 and 95% N_2. All the bioreactors were working under same light intensity (90±2 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and temperature (23 °C). The pH of the cultivation medium was adjusted to neutral (pH=7). Samples (10 ml) were taken from each bioreactor, and the algae growth was determined by chlorophyll content. The specific growth rates of each cultivation were calculated by equation 4.6. The relationship between algae growth and inlet gas flux is demonstrated in Figure 5.1.



n=?

Figure 5.1 Growth curves of *D.salina* under different flow rates, all reactors generated microbubbles

As can been seen in Figure 5.1, the *D. salina* cultures under all testing flow rates grew very well. The chlorophyll content in all cultures multiplied more than 200 times, and all the

cultures entered into the stationary growth phase at the 16th day. Under gas flux of 0.95 m/s (flow rate 0.7 L/min), the microalgae culture achieved the highest chlorophyll concentration of 32.65 mg/L. The second highest chlorophyll concentration (31.48 mg/L) was found in the culture exposed to 0.68 m/s of gas flux (0.5 L/min of flow rate), the culture under gas flux of 1.49 m/s (1.0 L/min of flow rate) produced the lowest chlorophyll content (26.83 mg/L) before entering the stationary phase. However, after the 16th day, the chlorophyll content in cultures under flux of 0.95 and 0.68 m/s decreased but were still higher than algae cultures under other flow rates (Figure 5.1). Microalgae cells in very dense cultures often start to form clusters which could easily settle to the bottom of the reactors. Such a phenomenon could be the explanation of the chlorophyll content decrease observed in the cultures under gas flux of 0.95 and 0.68 m/s.

It is difficult to fully determine the influence of flow rate to algae growth merely by chlorophyll content. This is because, in addition to the flow rate, the chlorophyll content could also depend on the inoculum concentrations. Due to the limitation of the research conditions, the inoculum concentration could not be easily controlled. In order to reflect the actual relationship between algae growth and flow rate, the specific growth rate should also be considered as an important parameter.

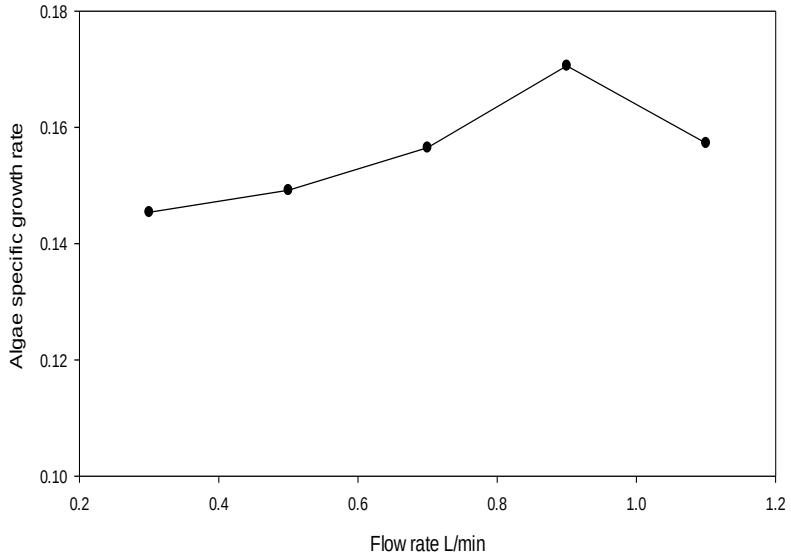


Figure 5.2. The relationship between flow rate and algae specific growth rate for *D. salina* cultures

As can be seen in Figure 5.2, in the flow rate range between 0.3 and 0.9, the specific growth rate of *D. salina* increased in tandem with the increasing flow rate. However, at flux of 1.49 m/s (flow rate of 1.1 L/min), the specific growth rate dropped rapidly. As shown in Figure 5.2, the highest specific growth rate was observed at a flux of 1.22 m/s (flow rate of 0.9 L/min). However, Figure 5.1 showed that the highest concentration of chlorophyll was produced by the *D. salina* culture under flux of 0.95 m/s (flow rate of 0.7 L/min). This was because the inoculum of cultures under flux of 1.22 L/min had lower concentration than the rest of the cultures (due to an accident). Therefore, although the microalgae grown under 1.22 m/s achieved much higher growth rate, the total chlorophyll content they produced were less than cultures under flux of 0.95 and 0.68 m/s (flow rate of 0.7 and 0.5 L/min) but still higher than the ones under 0.41 and 1.49 m/s (flow rate of 0.3 and 1.1 L/min). According to these results, it can be concluded that, when gas flux is low, increasing it could benefit the growth of microalgae. However, very high gas flux could slow the algae growth. Therefore, a plot of

CO_2 dosage versus the gas flux was made to further uncover the mechanism of this phenomenon.

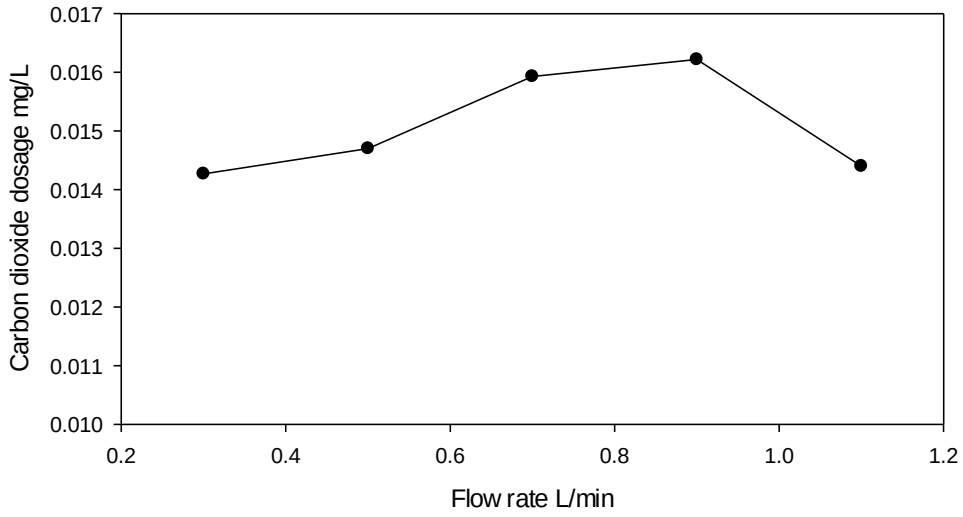


Figure5.3. The relationship between flow rate and CO_2 dosage for *D. salina* cultures

Similar trend shown in Figure 5.2 can be observed in Figure 5.3. When the flow rate is in the range of 0.3 to 0.9 L/min (gas flux of 0.41 to 1.22 m/s), the CO_2 dosage was directly proportional to the flow rate. However, when the flow rate exceeded 0.9 L/min (gas flux of 1.22 m/s), the CO_2 dosage decreased dramatically to 0.0144 mol/L. The highest CO_2 dosage was achieved at a flow rate of 0.9 L/min, and the lowest CO_2 was observed at flow rate of 0.3 L/min (gas flux of 0.41 m/s). By comparing Figures 5.2 and 5.3, it can be concluded that the inlet gas flow rate affected algae growth by influencing the CO_2 mass transfer inside the bioreactors.

Generally, an increase in the flow rate means more carbon source could be dosed into the medium, which would benefit the algal growth. However, increasing of flow rate could also increase the bubble size. Although larger amounts of CO_2 could enter the reactors through inlet gas stream when flow rate is high, the effectiveness of mass transfer was reduced due to an increase of bubble size. Therefore, when flow rate increased to a certain point, in this case

it was 0.9 L/min (gas flux of 1.22 m/s), further increases could not only stop benefiting CO_2 dosage but also cause a decline the mass transfer, which would slow the growth of the microalgae. According to the results of this experiment, the optimal flow rate for this cultivation system is 0.9 L/min (gas flux 1.22 m/s). Under this flow rate / gas flux, the highest CO_2 mass transfer rate can be achieved.

5.2 Determination of Algal Acetaldehyde Tolerance

5.2.1 Hypothesis

Effluent gas from bioethanol production contains mainly CO_2 and VOCs such as acetaldehyde. Acetaldehyde is a problematic impurity which affects the qualities of products of fermentation processes. Its emissions could cause many environmental problems. Exposure to acetaldehyde is considered harmful to both human health and aquatic ecosystems. Current treatment methods cannot effectively process such effluent gas without expensive and complex facilities which generate more CO_2.

Microalgae are believed by the author to be the solution. Algal photosynthesis could reduce CO_2 emission and produce valuable biomass to compensate for the capital costs. The only problem remaining is acetaldehyde removal. Generally acetaldehyde is considered harmful to microalgae. There are a few examples of toxicology research which reported that acetaldehyde could cause growth inhibition and photosynthesis inhibition. (CERI, 2007) However, it must be taken into consideration that such toxicity studies employed intensive acetaldehyde exposure (around 250 mg/L) and sensitive strains (see section 2.3.2). The expected exposure caused by industrial gas effluent is much lower at around 32 mg/L. The effects of low acetaldehyde exposure to microalgae have hardly been reported. However, considering the vast diversity and remarkable adaptabilities of microalgae, it is highly possible to find strains with high acetaldehyde tolerance.

Searching suitable algae candidates among tens of thousands of strains is not economical in terms of cost and time. Because the purpose of this research is future industrial application, the algae selected should be well studied, easily available and very robust to ensure that the process could be profitable. Therefore, in this research, the pool of the algae candidates was narrowed to 5 strains namely: *Dunaliella salina*, *Chlorella*, *Teraselmis*, *Chlamydomonas* and *Nannochloropsis*. All of these 5 strains are well researched and understood, and are considered to be flexible and commercially valuable. Their unique characteristics and the potentials of being optimal candidate were thoroughly discussed in Chapter 2.

5.2.2 Results and discussion

Five strains were grown separately in the screening system under their preferred light intensity, culture medium and temperature. The acetaldehyde dosed followed the two addition scheme listed in Table 3.5 and 3.6, Chapter 3. The first dosing scheme aims to reveal the limits of algae tolerance to acetaldehyde, which employed extreme acetaldehyde exposure. The second scheme is to study the algae response to the acetaldehyde exposure at the levels in the industrial effluent, by increasing acetaldehyde exposure from zero to twice as high as the concentration from the effluent.

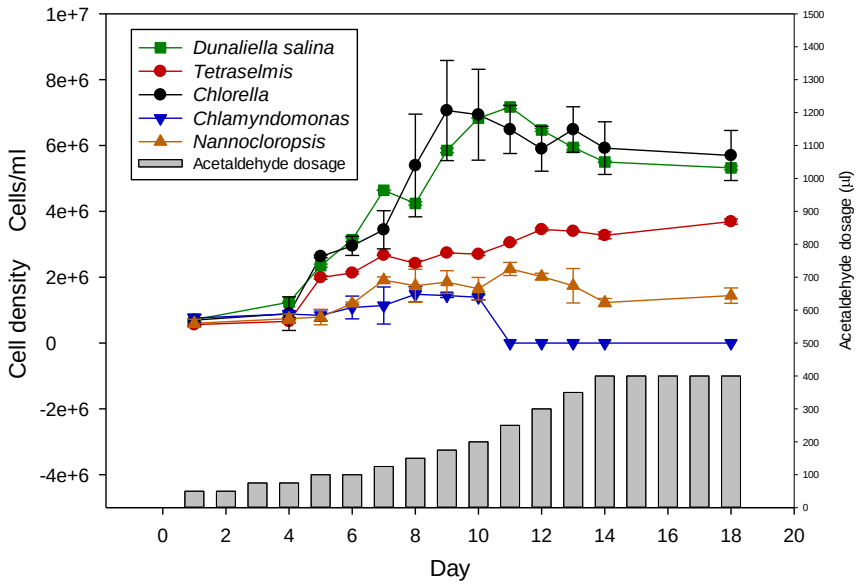


Figure 5.4. The algal response to acetaldehyde exposure follow the dosing scheme 1. Growth was estimated by measuring cell density

As can be seen in Figure 5.4, growth inhibition was observed in all 5 strains. *Chlorella* and *Dunaliella* were able to multiply respectively from 6.97×10^6 and 7.23×10^6 to 7.20×10^7 and 7.17×10^7 when daily acetaldehyde dosage was lower than 200 µL. However, the cell densities of these two strains dropped rapidly after the 9th and 11th day following the further increase of acetaldehyde daily dosage. Figure 5.4 also showed that the *Tetraselmis* culture ceased to grow after daily acetaldehyde dosing reached 125 µL. The growth of *Chlamydomonas* and *Nannochloropsis* seemed to be extremely slow from the very beginning of the experiment. All experiment conditions were maintained as each microalga preferred, such decreasing of growth rate can be explained by the influence of acetaldehyde exposure. However, as shown in Figure 5.4, *Chlorella, D. salina* and *Tetraselmis* showed better growth than the other two strains. This is *Chlorella* which was observed no obvious inhibition caused by acetaldehyde before the daily dosage reached to 200 µL. The highest cell density (7.20×10^7) was achieved by *Chlorella* at the 9th day of the experiment. After the 10th day of the experiment, *Chlamydomonas* was observed to be heavily contaminated by bacteria or fungi. This may because acetaldehyde and poorly grown algae provided carbon source for heterotrophic microorganisms to grow, which lead to serious contamination. However, marine algae such as *Dunaliella*, *Tetraselmis* and *Nannochloropsis*, are less likely be contaminated by common fresh water bacteria. However no contamination was observed in *Chlorella* cultures during the experiment. Even when *Chlorella* was inhibited by acetaldehyde, it still remained the dominant species in the medium.

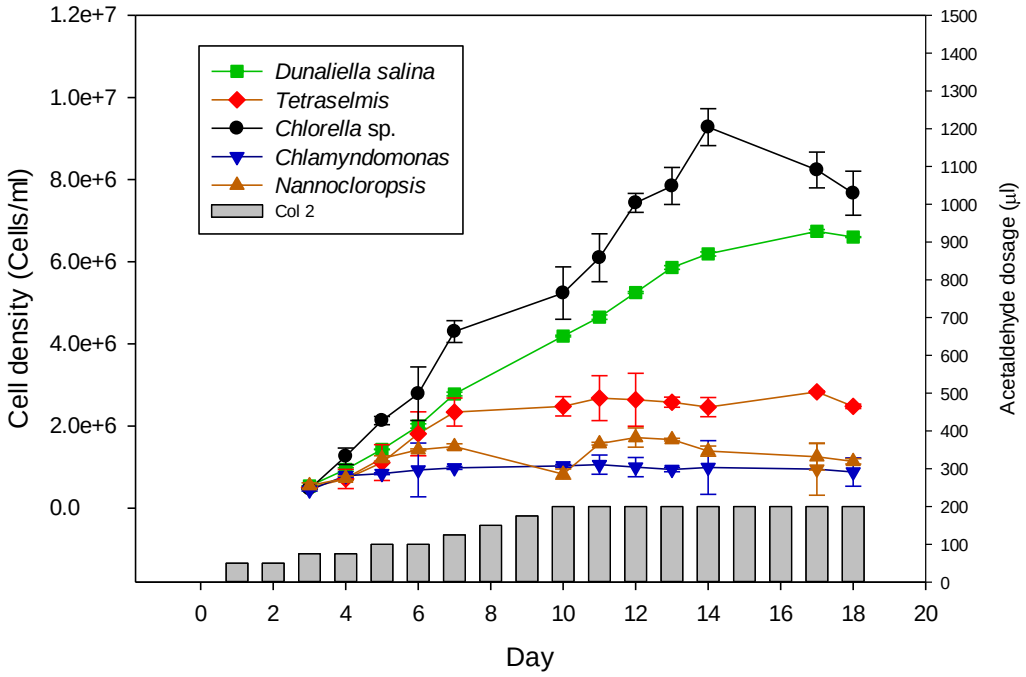


Figure 5.5. Algae growth response to acetaldehyde exposure under dosing scheme 2. Growth was estimated by measuring cell density

Figure 5.5 shows microalgae growth under acetaldehyde exposure at the level of industrial effluent. By comparing Figure 5.5 with Figure 5.4, it can be discovered that the growth of *Dunaliella* and *Chlorella* were obviously increased when acetaldehyde dosage was reduced. The cell density of *Chlorella* multiplied from 4.63×10^6 to 9.28×10^7, which was the fastest growing strain in this experiment. The growth of *Dunaliella* also seemed to be improved under lower acetaldehyde exposure. The cell density of *Dunaliella* increased from 4.95×10^6 to 6.74×10^7. It can be observed that *Tetraselmis* was able to grow positively during the experiment. However, when acetaldehyde daily dose reached 125 µL, the cell density of *Tetraselmis* stopped increasing and maintained around 2.64×10^7 (Figure 5.5). It is similar to the trend shown in Figure 5.4, which suggested that the maximum acetaldehyde daily dosage that *Tetraselmis* can endure is around 125 µL. Strong growth inhibition was still observed in

Nannochloropsis and *Chlamydomonas* cultures, both strains showed no positive growth rate, and little change in cell density can be measured during the experiment (Figure 5.5).

As Figure 5.4 and Figure 5.5 show, acetaldehyde inhibition can still be observed among strains even when the exposure levels were much lower than that employed by toxicology studies. The mechanisms of acetaldehyde toxicity to algae cells are rarely reported. However, a few investigations on acetaldehyde toxicity to terrestrial plant cells indicated that acetaldehyde is able to disrupt multiple cellular bio-functionsfor instance, acetaldehyde could disturb the oxide/redox balance, which could cause oxidative stress (Kotchoni *et al*., 2006). Research done by Tadege *et al*. (1998) showed that, acetaldehyde accumulated in potato leaf cells and resulted in degradation of starch and necrosis. Through animal and human cells study, acetaldehyde is proved to be a genotoxicant. This is because highly electronegative oxygen atom of acetaldehyde molecule can draw electron density from carbon and hydrogen atoms, which make the two carbon atoms become hard electrophiles. Therefore, the acetaldehyde molecule could preferentially react with hard nucleophiles such as nitrogen of deoxyguanosine. Because DNA of all eukaryotes shares same basic structure, there are reasons to believe that acetaldehyde could have similar genotoxicity to microalgae (LoPachin and Gavin, 2014).

However, the results shown in Figure 5.4 and Figure 5.5 also indicated that algal response to toxicants can be diverse. Different strains have different tolerance level towards acetaldehyde. *Chlorella* and *Dunaliella* seemed to be more resistant to acetaldehyde than other strains. The acute toxicity of acetaldehyde to *Chlorella* and *Dunaliella* was only expressed when acetaldehyde dosage reached 200 µL per day. This was not surprising, because both strains are known to be extreme adaptable. For *Chlorella*, some medically related studies suggested *Chlorella* contains the aldehyde dehydrogenase enzyme which could be used as an antioxidants (Nakashima *et al*., 2009; Sun Chlorella Corporation, 2011). The results (Figure

5.4 and Figure 5.5) showed that *Tetraselmis* possessed a certain level of tolerance to acetaldehyde. Positive growth rate can be observed in *Tetraselmis* cultures when the acetaldehyde daily dosage was lower than 125 µL. However, acetaldehyde tolerance was not observed in cultures of *Nannochloropsis* and *Chlamydomonas* in these experiments. Low growth rates of both strains only suggested that acetaldehyde toxicity was expressed by inhibition of cell multiplying. Algicidal effect was observed in *Chlamydomonas* culture during the experiment.

5.3 Conclusion

This chapter studied the influence of gas flow rate on algae growth. Algae cultures were screened under five gas flux (0.41, 0.68, 0.95, 1.22 and 1.49 m/s). It was observed that increasing gas flux could indeed increase the specific growth rate of microalgae. However, when gas flux exceeded a certain point, it could slow the algae growth rapidly. The screening experiment also discovered that changing the mass transfer efficiency was the reason that gas flux could affect microalgae growth. The results of this experiment suggested that, for the system employed by this research, the optimal flux is 1.22 m/s which could achieve highest CO_2 mass transfer efficiency.

According to the results of the screening experiments for the determination of algal acetaldehyde tolerance, acetaldehyde could indeed cause negative effects on microalgae growth even at much lower exposure levels compared to toxicology studies. However, *Chlorella* and *Dunaliella* showed promising resistance to acetaldehyde exposure. Both strains were considered by the author as potential candidates for developing algae based treatment of fermentation gaseous effluent. Moreover, *Tetraselmis* also showed certain tolerance to acetaldehyde. Under daily dosage of 125 µL (still higher than the level of industrial effluent), positive growth can be observed. Although *Nannochloropsis* strains were reported could take up formaldehyde as supplementary carbon source, there was no evidence revealed by this

research to support the hypothesis that *Nannochloropsis oculata* possesses sufficient acetaldehyde tolerance for the treatment of gaseous effluents which contains acetaldehyde.

CHAPTER 6 ALGAE ACCLIMATION FOR ACETALDEHYDE TOLERANCE AND REMOVAL

6.1 Hypothesis

As shown in Chapter 5, *Chlorella* and *Dunaliella salina* showed better tolerance to acetaldehyde exposure. Therefore, this part of the research aims to explore their capacities of acclimation and discuss the possibility of utilizing them for treatment of gaseous effluent that contains acetaldehyde. Acclimation is a mechanism that could improve living organisms' survivability in a changeable environments. Organisms across all species are known to be able to adjust their morphological, behavioural, and physical and biochemical traits to adapt to the changes of their environment. Especially for microalgae, which can be found thriving in many extreme conditions. Through acclimation, the algae tolerance to acetaldehyde could be further improved, and their ability to remove acetaldehyde could potentially be developed.

For mixotrophic strains such as *Chlorella*, it is not difficult to believe that acetaldehyde could be utilized as a supplementary carbon source. It is reported that *Chlorella* could grow mixotrophically by feeding acetate into the medium. Moreover, the mixotrophic growth showed higher growth rate than both autotrophic and heterotrophic growth combined. Theoretically, acetaldehyde contains more chemical energy than acetate, uptake and utilization of acetaldehyde would benefit algae more. For organisms that metabolize acetaldehyde, it will be converted into acetate through dehydrogenase reaction, and then acetate will be further oxidized into CO_2 and H_2O. (Bullock, 1990; Umulis *et al*., 2005) The following thermodynamics calculation will reveal the energy changes during this process.

The flow chart of acetaldehyde metabolism:

Acetaldehyde $\longrightarrow$ acetate $\longrightarrow$ Acetyl-CoA $\longrightarrow$ H_2O+CO_2

The Gibbs Free Energy of formation for acetaldehyde is -174.8 kJ/mol.

The Gibbs Free Energy of formation for acetate is -389.9 kJ/mol.

The Gibbs Free Energy of formation for CO_2 + H_2O is -1500.1 kJ/mol.

Therefore:

The Gibbs Free Energy of formation for converting acetaldehyde into acetate should be:

$$\Delta G_{f1} = -389.9 + 174.8 = -215.1\ kJ/mol$$

The Gibbs Free Energy of formation for converting acetate into carbon dioxide and water should be:

$$\Delta G_{f2} = -1500.1 + 389.9 = -1110.5\ kJ/mol$$

As can be seen in above calculations, the acetaldehyde metabolism could release 215.1 kJ/mol more energy than directly taken up acetate as carbon and energy source.

There is little evidence that *Dunaliella* genus can perform mixotrophic growth (Hard, Gilmour, 1996). However, according to the results found in section 5.2, *Dunaliella* showed noticeable tolerance to acetaldehyde, which indicated that *Dunaliella* could be used for carbon bio-fixation of gas effluent that contains acetaldehyde. Although *Dunaliella* might not be able to remove acetaldehyde from the effluent, the benefits of CO_2 emission abatement and biomass production could compensate for the costs of acetaldehyde treatments as long as high growth rates can be achieved. Besides, *Dunaliella* could be co-cultured with the organisms that could metabolize acetaldehyde.

6.2 Results and Discussion

6.2.1 Acclimation of *Dunaliella salina* and *Chlorella* sp.

The methodology of acclimation experiments was described in Chapter 3. The results of the two acclimation treatments are shown in the Figure 6.1, where the curves demonstrate the algae growth and the bar charts are volumes of the acetaldehyde dosage. As can be seen in these diagrams, during the treatment 1 (or T1), both strains presented very similar trends and cell density with their control groups (growing without any acetaldehyde dosage) which means that they are hardly affected by acetaldehyde exposure. However, as the acetaldehyde is increasingly accumulated in the medium, an obvious inhibition can be spotted in the *D. salina* T1 cultures after 14 days of cultivation. However, the opposite phenomenon was observed in the *Chlorella* T1 group. After 14 days, while the *Chlorella* control group entered the stationary phase, the T1 group continued growing and surpassed the control group (Figure 6.1).

For treatment 2 (or T2), growth inhibition can be observed in both strains, but especially, for *D. salina* T2. The *D. salina* T2 cultures also showed similar trend with their control cultures but with much slower growth. The growth slowed as soon as the acetaldehyde dosing began, and it produced 25% less cell density than the control group (Figure 6.1). For *Chlorella* T2, it shared similar cell density and growth trends with its control until the 10^{th} day of the cultivation. Then, its growth slowed down due to the increasingly mount of acetaldehyde in the medium. However, the growth trend of *Chlorella* T2 cultures showed a level of adaptation. They grew steadily and eventually reached same level of cell density with their control group (Figure 6.1).

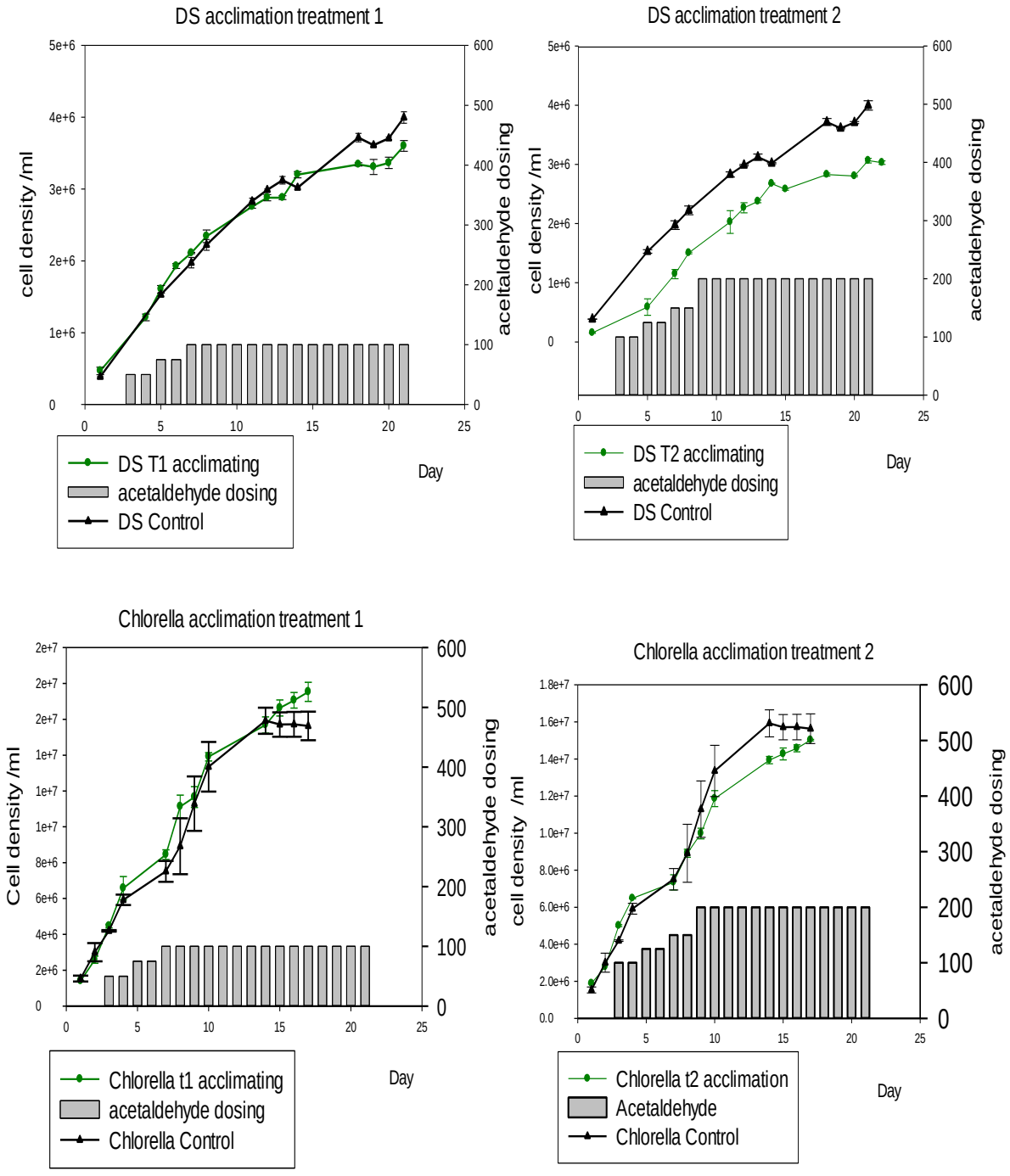


Figure 6.1 Growth of *Dunaliella salina* and *Chlorella* sp. on two acetaldehyde acclimation treatments. T1: acetaldehyde increasing to 100 µl; T2: acetaldehyde increasing to 200 µl.

As the results shown in Figure 6.1 indicated, growth inhibitions were observed during the acclimation. This can be explained by the detrimental effects of acetaldehyde. Acetaldehyde

is reported to be toxic to some microalgae such as *Pseudokirchneriella subcapitata* (Tsai and Chen, 2007). It is able to cause photosynthesis inhibition and growth inhibition in many other strains (CERI, 2007). Acetaldehyde as a reactive molecule with its side groups can be found in multiple biological tissues and processes. It is able to form acetals with alcohol, thiol and amino groups, which may affect the metabolism of the microalgae. (Slatyer *et al.*, 1983). However, microalgae, as a very diverse group of organisms, may have variable sensitivity responses to acetaldehyde due to their different cytology, physiology and genetics as illustrated here by lesser inhibition observed in *Chlorella* compared with *Dunaliella* (Figure 6.1).

6.2.2 Evaluate the performance of acclimation

To evaluate the performance of the acclimation, the algae cultures from treatment 1 and treatment 2 were taken and inoculated into a new system. Instead of gradually increasing the dose of acetaldehyde, in this part of experiment the acetaldehyde was added directly at the beginning of the growth period at 100 μL or 200 μL (depending on which treatments the inocula came from). By comparing the growth of these acclimated groups with treatment 1 and 2, we can discover whether the algae tolerance to acetaldehyde improved after acclimation. The results are shown in Figure 6.2.

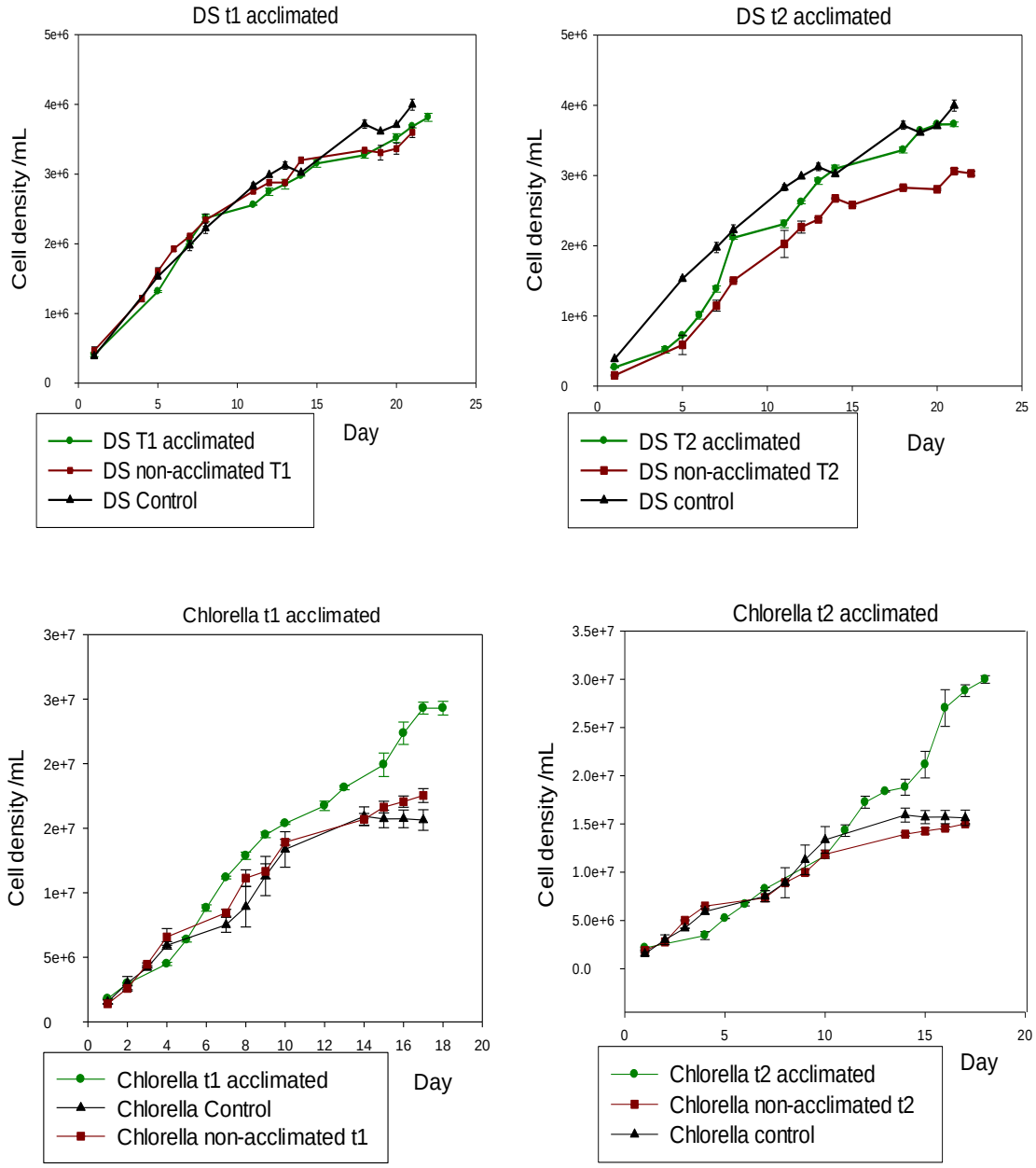


Figure 6.2 Comparison of the growth of acclimated *D salina* and *Chlorella* sp. cultures with the non-acclimated cultures.

According to Figure 6.2, both the acclimated *D salina* and *Chlorella* showed improvement in their growth and their tolerance to acetaldehyde compared with non-acclimated cultures exposed to treatment 1 and 2. Especially for *Chlorella*, under the same or even higher levels of acetaldehyde exposure the acclimated groups achieved much better growth than treatments 1 and 2 cultures (non-acclimated), and they even surpassed the control group (Figure 6.2).

For the *Chlorella* T1 acclimated group (inoculum is from treatment 1, dose acetaldehyde 100 μL per day), 40% higher cell density was achieved at the end of the cultivation compared with the control group. The *Chlorella* T2 acclimated group (inoculum was from treatment 2, dose acetaldehyde 200 μL per day) achieved 50% higher cell density than the control group. Moreover, the cell densities of acclimated *Chlorella* groups were continuously increasing even after the control groups reached the stationary phase.

However, for *D. salina*, such dramatic improvement in growth as observed in *Chlorella* was not seen. The *Dunaliella* T1 acclimated group shared almost the same trend and growth rate with the Dunaliella non-acclimated cultures. This is maybe because the growth of *Dunaliella* T1 was already very close to the control group. Therefore, there is not much space for further improvement. However, the improvement of the growth of *Dunaliella* T2 acclimated group was obvious. Compared with non-acclimated T2 cultures, the *Dunaliella* T2 acclimated group showed strong tolerance to acetaldehyde. It achieved a very similar growth curve and cell production with the control group (Figure 6.2). However, the inhibition can be still observed in the *Dunaliella* T2 acclimated group at the early stage of the cultivation, this may be due to the changes in the acetaldehyde dosing plan. Instead of gradually adding acetaldehyde, in the acclimated experiment the acetaldehyde was dosed directly at the maximum volume of acetaldehyde dosage of treatment 2. However, the acclimated group gradually caught up with the control group and eventually reached the same level of the cell production (Figure 6.2).

According to the results of acclimated groups shown in Figure 6.2, the acetaldehyde seems to have different influence towards different strains. For *D. salina*, acetaldehyde can cause obvious growth inhibition. The more acetaldehyde present in the medium the more serious the inhibition. However, with acclimation, the tolerance of *D. salina* to acetaldehyde can be increased. After acclimation, the inhibition can be reduced largely, as is shown in Figure 6.3.

However, for *Chlorella*, on low level of exposure, the acetaldehyde seems to have little negative effect, it can even slightly improve the growth of *Chlorella*. However, for higher concentrations of acetaldehyde such as 62.8 mg/L (equals to 200 µL acetaldehyde in 2.5 L medium), minor inhibition can be observed (Figure 6.3).

More interestingly, after the acclimation, acetaldehyde seems to be able to increase the growth of *Chlorella* sp. at both higher and lower level of exposure. Moreover, higher acetaldehyde concentration can lead to higher cell production, which can be seen in Figure 6.3.

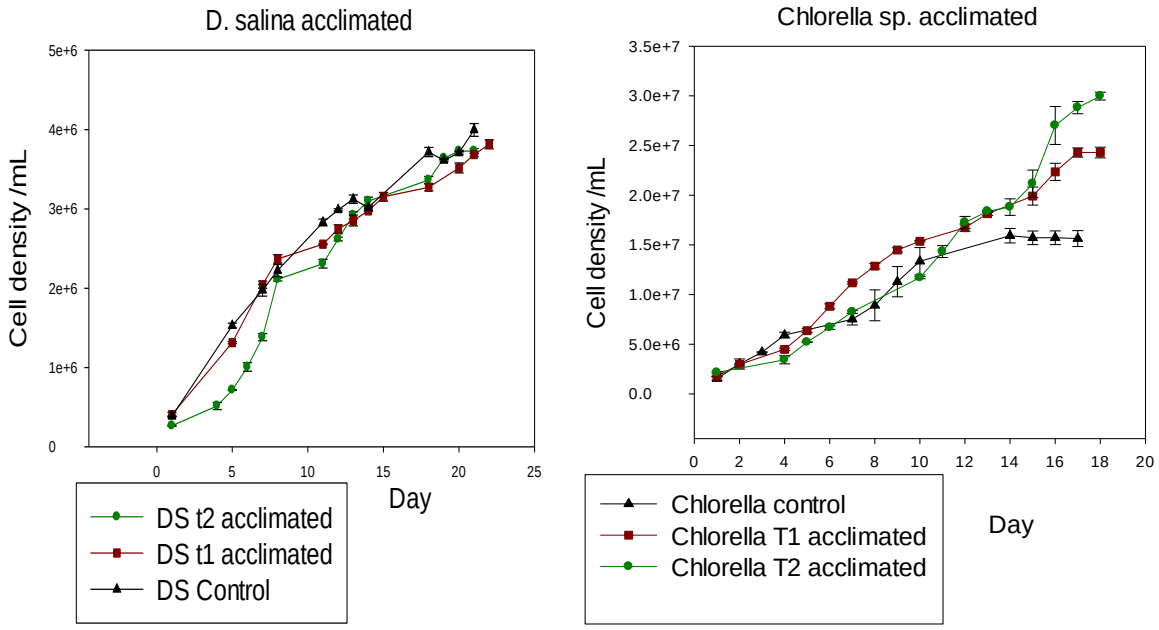


Figure 6.3 The growth of acclimated *D salina* and *Chlorella*.

Comparison of the acclimated algae cultures with the acclimation treatments indicate that acclimation benefits the growth of both *D. salina* and *Chlorella sp.*, and improves the *D. salina* tolerance to acetaldehyde. Microalgae are believed to be able to adapt to the environment they are living in and can even modify their metabolism completely through biochemical and physical acclimation, which allows them to tolerate and survive a wide range of conditions (Richmond, 2004). That is why the growth inhibition in the acclimated *D.*

salina reduced considerably compared to the acclimation treatment 2. However, for *Chlorella*, after the acclimation, it is not only tolerating the presence of acetaldehyde but its growth is also largely enhanced.

As the results shown in Figure 6.3 demonstrated, unlike *D salina*, *Chlorella* showed remarkable tolerance to acetaldehyde exposure and significant growth enhancement after acclimation. This may be because that *Chlorella* sp. is reported to be able to grow under mixotrophic conditions. It is reported that considerable growth improvement and biomass productivity of *Chlorella* cultured under mixtrophic conditions were observed compared with the photoautotrophic cultures (Wan *et al*., 2011). There is no report in the literature that *Chlorella sp.* is able to use acetaldehyde as its organic carbon source for mixotrophic growth. Moreover, during the acclimation *Chlorella* did not present noticeable growth improvement even with the presence of acetaldehyde. However, in the acclimated groups the growth enhancement was observed. The results of this experiment suggest that *Chlorella* is able to utilize acetaldehyde as organic carbon source due to acclimation.

Table 6.1 Algal specific growth rate of different acetaldehyde treatment

	Dunaliella salina		***Chlorella* sp.**	
Treatments	Specific growth rate (Cells/ml/day^{-1})	Standard divisions	Specific growth rate (Cells/ml/day^{-1})	Standard divisions
Control	0.147	0.000746	0.166	0.0109
Non – acclimated Treatment 1	0.109	0.00606	0.172	0.00154
Non-acclimated Treatment 2	0.0903	0.00046	0.142	0.00168
Acclimated Treatment1	0.142	0.00490	0.205	0.00380
Acclimated Treatment2	0.147	0.00214	0.197	0.115

Finally, the specific growth rates of different acetaldehyde treatments were calculated to provide more concrete overview of the growth of algae. The specific growth rates were shown in the Table 6.1.

According to the table, the specific growth rates of non-acclimated *Dunaliella salina* slowed considerably when exposed to acetaldehyde. The specific growth rates of *D. salina* non-acclimated treatment 1 and non-acclimated 2 were 26% and 39% lower than that of the control group respectively. According to the statistical analysis described in Chapter 3, with more than 95% confidences, there were significant decreasing of the specific growth rates of *D. salina* non-acclimated treatments compared to the control group. However, the specific growth rates of the acclimated treatments were improved and reached to the same level as the control group. With more than 90% of confidences, there were no significant differences between the specific growth rates of *D. salina* acclimated treatments and that of the control group.

For Chlorella strains, when the non-acclimated algae culture exposed to the lower level of acetaldehyde such as treatment 1, the specific growth rate showed no obvious reduction. With 82% of confidence that there was no significant differences between the specific growth rates of non-acclimated *Chlorella* sp. treatment 1 and the control. As the acetaldehyde exposure level increased, the inhibition of algal growth can be observed. The non-acclimated *Chlorella* sp. treatment 2 showed 14% of reduction of specific growth rate compared to that of the control group. However, after acclimation the specific growth rates of cultures that exposed to acetaldehyde were largely improved. Compared to the control group, the acclimated treatment 1 showed 23% improvement of specific growth rate and the acclimated treatment 2 showed 19% of improvement. With a confidence level of 95%, these improvements of specific growth rates were significant.

The result of the specific growth rate analysis was consistent with the result of growth curve discussion. The acetaldehyde could inhibit the growth of microalgae *Dunaliella salina* and *Chlorella* sp.. However, acclimation could significantly improve the tolerance of the alga. Moreover, after acclimation, the acetaldehyde exposure could benefit the growth of *Chlorella* sp.

During the experiment, it is revealed that the results of acclimation were genetically heritable. Samples taken from the acetaldehyde acclimation treatments were revived in cultures without acetaldehyde exposure, however, the improved tolerance can still be observed once the acetaldehyde dosage started. The acclimated algae cultures can grow in medium without acetaldehyde for weeks and still possess the abilities developed during the acclimation treatments. It appears that the results in acclimation is due to genetic mutations. In order to acquire solid prove of this conclusions, future works are recommended for instance, DNA sequencing and comparison could provide concrete evidence of the mutations.

6.2.3 Response of algae photosynthesis rate to acetaldehyde

Figure 6.4 shows the effect of the exposure to two acetaldehyde treatments on the photosynthesis rate of *D salina* and *Chlorella* sp. cultures. The non-acclimated group in Figure 6.4 are the algae samples which were taken from control group and the same concentrations of acetaldehyde were added as in corresponding treatments directly before the photosynthesis measurement. As can been seen, the two microalgae strains showed opposing response to the presence of acetaldehyde. For *D salina*, the photo synthesis rate declined following the increase of the acetaldehyde concentration. The control group growing without any acetaldehyde presented the highest average photosynthesis rate of 1.10 μmoles O_2 per 10^6 cells per h. However, for Treatment 1 acclimated cultures, their photosynthesis dropped by approximately 10% compared to control group which is 1.0 μmoles O_2 per 10^6 cell per h. Compared with the acclimated cultures, the non-acclimated group showed 28% lower of

photosynthesis rate which was 0.859 μmoles O_2 per 10^6 cell per h. According to the statistic analysis, there was 90% of confidence that no significant difference between the acclimated treatment 1 and the control, however, 95% of confidence that the differences between non-acclimated treatment 1 and the control group were significant. All the treatment 2 cultures showed the lower photosynthesis rates including acclimated and non-acclimated groups. The photosynthesis rate of the T2 acclimated group is 0.77 μmoles O_2 per 10^6 cell per h which is 30% lower compared with the control. However, it is still higher than the non-acclimated group, which is 0.24 μmoles O_2 per 10^6 cell per h, which is only 70% lower than the photosynthesis rate of T2 acclimated group and 80% lower than the control group. The differences of photosynthesis rate among the control and the two treatments are significant, with a confidence level of 95%. It is attributed to the acetaldehyde exposure. The decline of the photosynthesis rate was dependent and inversely proportional to the acetaldehyde dosage. However, in both treatments, the acclimated culture showed noticeable improvement of their photosynthesis rate compared to non-acclimated ones, which means the acclimation is beneficial.

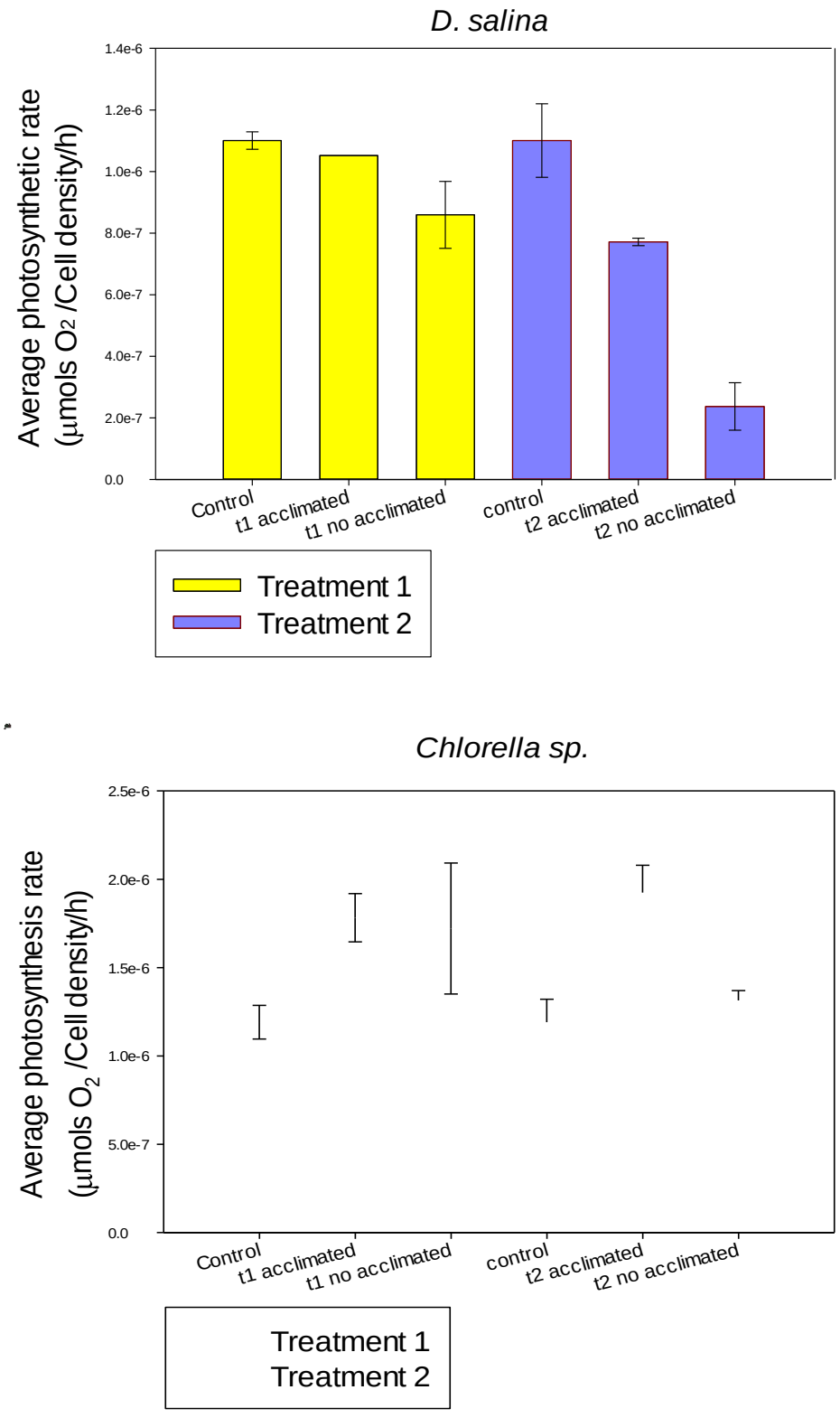


Figure 6.4 Average photosynthesis rate of the acclimated and non-acclimated *D. salina* and *Chlorella* sp. cultures at different acetaldehyde treatments. T1 cultures had 100 µl of acetaldehyde added and T2 cultures had 200 µl of acetaldehyde added.

For *Chlorella* sp., the response to the acetaldehyde is totally opposite to that of *D salina*. The cultures of acclimated *Chlorella* presented an acetaldehyde concentration-dependent enhancement in their photosynthesis rate. The T2 acclimated culture with highest acetaldehyde concentration (62.8 mg/L, 200µl) achieved the highest photosynthesis rate of 1.93 µmoles O_2 per 10^6 cell per h. The acclimated T1 culture achieved the second highest photosynthesis rate of 1.78 µmoles O_2 per 10^6 cell per h. Compared to the control group which had a photosynthesis rate of 1.19 µmoles O_2 per 10^6 cell per h, the photosynthesis rates of acclimated T1 and T2 increased 50% and 62% respectively. The non-acclimated group of T1 showed similar photosynthesis rate with the T1 acclimated group with an average photosynthesis rate of 1.72 µmoles O_2 per 10^6 cell per h is slightly lower than the acclimated one, but non-acclimated groups have higher deviations among the replicates. However, the acetaldehyde T2 *Chlorella* sp. without acclimation did not present much noticeable change in photosynthesis compared with the control; although it still has a slightly higher average rate of photosynthesis than the control group. The improvement of the photosynthesis rates of acclimated treatments were proven to be significant with confidence level of 95%. However, there was little difference between the non-acclimated treatment 1 and acclimated treatment 1 with an 80% confidence, and the non-acclimated treatment 2 showed no significant difference compared with control group with a 90% confidence. Comparing the photosynthesis rates of control, acclimated and non-acclimated groups suggests that acetaldehyde does not cause photosynthesis inhibition in *Chlorella* sp., on the contrary, acetaldehyde is able to interact with the metabolism of *Chlorella* sp., and contribute positively to its photosynthetic rate (Figure 6.4).

The results of the photosynthesis experiemts are consistent with the results of growth acclimation. For *D salina*, photosynthesis inhibition was observed inversely proportional to

the concentration of acetaldehyde, and acclimation appears to be able to reduce such inhibition. Due to all the conditions remaining unchanged, this inhibition should be explained by the negative influence of acetaldehyde. According to the research undertaken by Slatyer (1983), acetaldehyde did not affect the photosynthetic electron flow in algae (or *cyanobacteria*) cells. However, acetaldehyde is proven to be able to irreversibly inhibit the CO_2 fixation which could lead to a reduction of the photosynthesis rate. This is because acetaldehyde could react with many chemical groups commonly found in living cells such as thiol and amino groups which can be found in various protein and enzymes. Such reactions could possibly block the electron-accepting pathways of CO_2 fixation (Slatyer *et al*., 1983; LoPachin and Gavin, 2014). For *Chlorella*, the photosynthesis inhibition was not observed when exposed to acetaldehyde. On contrary, acetaldehyde could largely benefit the acclimated *Chlorella* culture. This might be because *Chlorella* was grown mixotrophically that the existence of the organic carbon source may alter both the photosynthetic and heterotrophic metabolism. Therefore, there are researches indicated that although photosynthesis and heterotrophic growth happened simultaneously and independently in mixotrophic strains. The mixotrophic growth was still higher than simply combined growth of photosynthesis and heterotrophy. It is believed that photosynthesis could be stimulated by the presence of organic carbon through high level of intracellular CO_2 generated by the metabolisms of these organic carbons (Cheirslip and Torpee, 2012).

6.3 Conclusion

Acetaldehyde can cause growth inhibition and photosynthesis inhibition to *Dunaliella salina*. The growth rates and photosynthesis rates of *D salina* decline with increasing levels of exposure to acetaldehyde. However, after acclimation, *D salina* showed remarkable tolerance to acetaldehyde exposure. The acclimated *D salina* from both treatments achieved similar biomass productivity to the control group. Although an obvious photosynthesis inhibition

was spotted in the T2 acclimated group, its photosynthesis rate increased 70% compared with the non-acclimated group.

However, for *Chlorella*, acetaldehyde can barely affect its growth and photosynthesis. Moreover, with the acclimation, the acetaldehyde can considerably increase the cell productivity and the photosynthesis rate. In the T 1 experiment, the acclimated group generated 40% more biomass than the control. In the treatment 2, even higher growth was observed; the acclimated group achieved 50% higher cell density than control group. It is likely that the acclimated *Chlorella* sp. is able to utilize acetaldehyde as an organic carbon source for mixotrophic growth.

CHAPTER 7 INFLUENCE OF ACETALDEHYDE ON ALGAL LIPID PRODUCTION

7.1 Hypothesis

The Lipid composition of algal cells plays significant roles in many bio-functions. It is believed that the ability to produce an exceptional variety of lipid compositions is the key reason that algae are able to adapt to a vast range of environmental conditions (Guschina and Harwood, 2006). Lipids and proteins are key structural components of all kinds of membranes in algae cells. Such membranes not only protect cells and organelles from damage, they also provides indispensable reaction sites for almost all the biochemical reactions (Thompson, 1996). Lipids participate in algal photosynthesis directly, for instance, pigments such as chlorophyll and β-carotene are involved in the absorption of light. Lipids can also serve as energy reserves inside algal cells (Wada and Murata, 2009). Algal lipids are also considered as alternative fuel sources with high potential. Many scientists believe that harvesting algal fatty acid could be the answer to the growing oppression of the energy crisis and climate change. Many component of algal lipids are also fine chemicals with high commercial values such as β-carotene and polyunsaturated fatty acids (PUFAs). Therefore, studying the algal lipid production holds great commercial opportunities.

According to previous chapters, it has been shown that acetaldehyde could have significant effects (positive or negative) on algae growth and metabolism. Due to the fact that lipids are involved in almost all the algal bio-functions, it is not difficult to believe that acetaldehyde could also influence the lipid production and composition. Acetaldehyde is not uncommon in the cells of living organisms, because it is an intermediate of many cellular pathways. However, due to its chemical highly reactive nature, excessive accumulation of acetaldehyde could cause many problems. For instance, acetaldehyde was reported to be able to cause

genotoxic effects, lipid peroxidation and interactions with important proteins (Kotchoni *et al*, 2006; Worrall *et al*, 1993). The mechanisms of lipid peroxidation can be explained by the following flow chart:

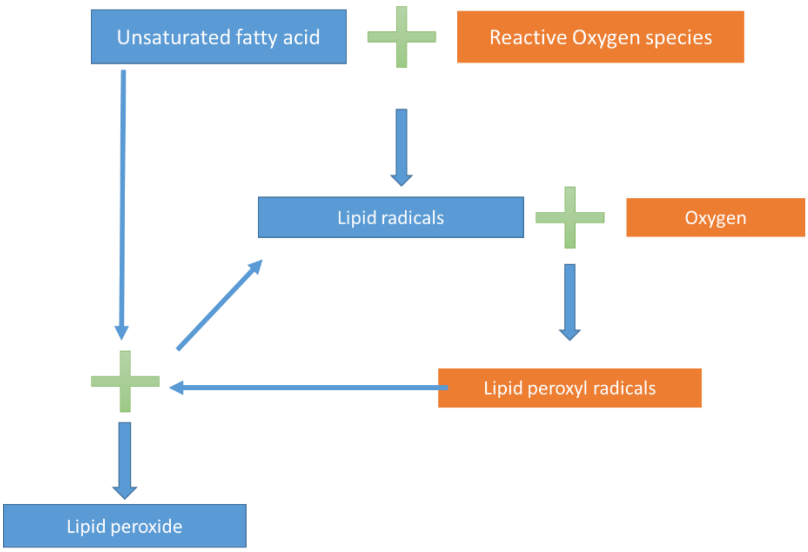


Figure 7.1 the mechanisms of lipid peroxidation

When acetaldehyde enters into the organisms cells, it will undergo a dehydrogenase reaction. Due to the catalytic effect of dehydrogenase enzymes such as xanthine oxidase, acetaldehyde will be converted into acetate and release free radicals known as oxygen reactive species. As can be seen in Figure 7.1, these free radicals could react with unsaturated fatty acid by capturing the hydrogen atom and produce H_2O and fatty acid radicals. The fatty acid radical is not stable and is further oxidized into fatty acid peroxyl radical which is also unstable. The peroxyl radical can react with other unsaturated fatty acid and release more fatty acid radicals. This is a chain reaction which could largely damage the cells and disrupt bio-functions if not terminated fast enough (Marnett, 1999). If such a phenomenon happened in the algae samples, the lipid contents could be expected to be reduced by acetaldehyde exposure.

However, it is reported that algae are able to accumulate lipid contents under stressed nutrient or environmental conditions (Yilancioglu *et al*., 2014). The numerous bio-functions of lipid contents allows algae to survive through difficult situations and produce reserve energy for growth after the stress. For instance, nitrogen stress is widely recognized as an effective way to increase algal lipid contents. It is generally believed that carbon normally allocated to protein synthesis is channelled into increasing lipid contents and hydrocarbon levels because of insufficient nitrogen. However, according to later research, it is noticeable that nitrogen starvation can also cause oxidative stress similar to that caused by acetaldehyde (Yilancioglu *et al*., 2014). In addition this study also discovered that oxidative stress could induce lipid accumulation. The research reported by Zhang *et al.* (2013) supported this opinion, they discovered that the natural lipid accumulation was significantly increased by artificially adding H_2O_2 to *Chlorella sorokiniana C3* cultures. The lipid accumulation caused by the artificial exogenous oxidative stress was even higher than under nitrogen starvation. They believe that oxidative stress should be recognized as more noteworthy benefactor to algae lipid accumulation than nitrogen stress.

Therefore, the expected influence of acetaldehyde on algal lipid production is not easily determined. Acetaldehyde could damage the lipid content inside algae cells but also stimulate the accumulations of lipids. Hence, experiments are required to uncover the relationship between algal lipids production and acetaldehyde exposure. The results could possibly question the traditional views of how best to produce microalgal lipid cost-effectively.

7.2 Effect of Exposure to Acetaldehyde on Algal Lipid Production

Algal samples were taken from the control, treatment 1 acclimated and treatment 2 acclimated cultures (see Chapter 6). Samples were prepared following the procedures described in Chapter 2. A Bruker Avance 600 NMR was used for measuring the photosynthetic pigments and general lipid contents in the algae samples.

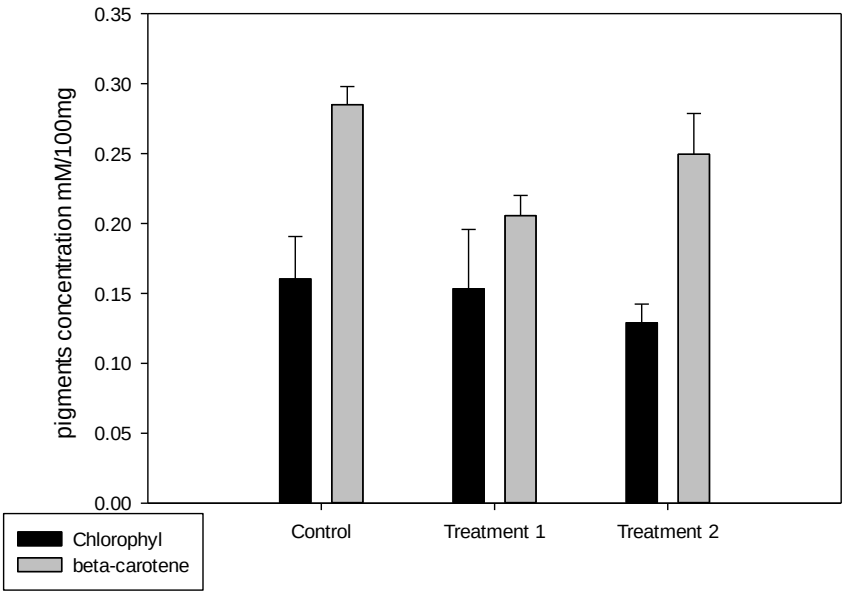


Figure 7.*2 Dunaliell salina* pigment concentration under acetaldehyde exposure

Figure 7.2 shows the chlorophyll and beta-carotene contents of *Dunaliella salina* in different acetaldehyde treatments. As can be seen in this chart, the chlorophyll content was reduced from around 1.6×10^{-3} mM/mg to 1.3×10^{-3} mM/mg due to the increase of acetaldehyde exposure. The decrease of chlorophyll content is consistent to the changes of photosynthesis rate described in the Chapter 6, but the change in chlorophyll content is small. Following the increase of acetaldehyde dosing, the photosynthesis rate of *Dunaliella* declined. It is suggested that acetaldehyde may affect photosynthesis through influencing the synthesis of chlorophyll. As discussed above, the reduction of chlorophyll content could be the result of lipid peroxidation caused by acetaldehyde. However, the changes of beta-carotene content showed different trend (Figure 7.2). Although samples from both acetaldehyde treatments showed lower concentration of beta-carotene (2.1×10^{-3} mM/mg for treatment 1; 2.5×10^{-3} mM/mg for treatment 2) than the control group (2.9×10^{-3} mM/mg), the concentrations in treatment 2 (which contained higher level of acetaldehyde) was higher than treatment 1. According to this result, beta-carotene production in algae cells could be affected by

acetaldehyde because acetaldehyde is able to react with multiple functional groups of essential molecules inside cells of various organisms. However, beta-carotene is also an antioxidant agent (Dummermuth *et al*., 2003). Algae might increase the production of beta-carotene and other antioxidant vitamins to relieve the oxidase stress caused by acetaldehyde, which could be the reason why higher carotene content can be measured in samples from treatment 2 (Figure 7.2).

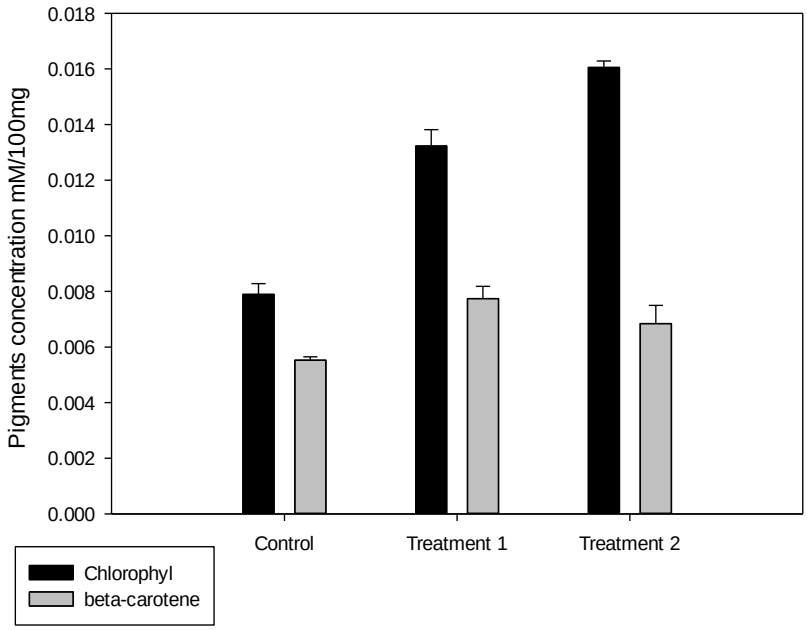


Figure 7.3 *Chlorella* pigment concentration under acetaldehyde exposure.

According to Figure 7.3, the chlorophyll changes of *Chlorella* samples showed opposite trend to *Dunaliella.* Acetaldehyde appeared to be a benefactor to the accumulation of chlorophyll in *Chlorella* cells. The chlorophyll content in treatment 1 samples were measured to be 0.13×10^{-3} mM/mg which was much higher than the concentration in control groups (0.08×10^{-3} mM/mg). The highest concentration of chlorophyll (0.16×10^{-3} mM/mg) was found in samples of treatment 2. The positive effect of acetaldehyde on chlorophyll accumulation could be the reason why photosynthesis rate of Chlorella cells was increased under acetaldehyde exposure (Chapter 6). The beta-carotene contents also increased when exposed

to acetaldehyde, which is possibly due to the need to reduce oxidase stress. However, the highest beta-carotene concentration was observed in samples of treatment 1 (0.077×10^{-3} mM/mg) rather than in samples of treatment 2 (0.068×10^{-3} mM/mg), which was still higher than the control group (0.053×10^{-3} mM/mg). The reasons for this reduction could be various, for instance: beta-carotene could be damaged by high levels of acetaldehyde exposure; acetaldehyde could react with enzymes involved in synthesis of carotene (LoPachin and Gavin, 2014); or it could be simply because acetaldehyde was utilized by *Chlorella* as a carbon source so that large amounts of antioxidant were no longer required to counter the oxidative stress. The mechanisms of this phenomenon could only be revealed by further studies, however, such phenomenon provided a novel insight for potentially increasing the yield of valuable chemicals such as β-carotene.

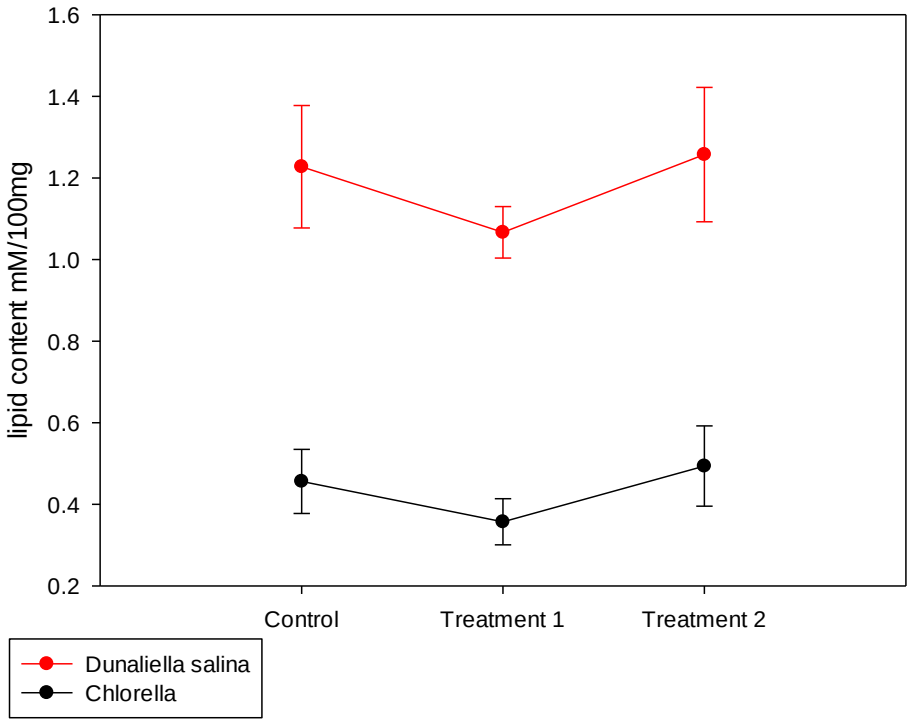


Figure 7.4 Lipid production of both strains under acetaldehyde exposure

Figure 7.4 shows the total lipid concentration in both *Dunaliella salina* and *Chlorella* strains. The diagram indicated that *Dunaliella salina* contains higher lipid content than *Chlorella sp*.. This might be because *Chlorella* strains in all treatments achieved higher specific growth rate according to the experimental results discussed in Chapter 6. It is believed algae tend to accumulate lipid content when their growth rate is reduced. For *Chlorella* in this experiment, the rapid growth suggested that metabolic pathways were directed into synthesising protein and other structural macromolecules for rapidly reproducing cells (Fan *et al*., 2014). Another possible reason for this outcome could be the lysis of *Chlorella* cells before the NMR measurement. *Chlorella* cells were reported to be difficult to break, therefore, the extraction of lipid content may not be complete in these experiments. However, due to the good consistency of repeated experiments, the tendency of the lipid fluctuation of *Chlorella* strain under different acetaldehyde exposure should be reliable.

As can be seen in Figure 7.4, lipid content of both strains showed similar trend. The lowest concentration (1.067×10^{-2} mM/mg for *Dunaliella*, 0.357×10^{-2} mM/mg for *Chlorella*) of methyl esters can be observed in treatment 1 which were followed by control group (1.228×10^{-2} mM/mg for *Dunaliella*, 0.456×10^{-2} mM/mg for *Chlorella*). The highest of the methyl contents (1.257×10^{-2} Mm/mg for *Dunaliella*, 0.494×10^{-2} mM/mg for *Chlorella*) were measured in samples from treatment 2 which were exposed to highest acetaldehyde dosage. According to the results of both strains, increasing the acetaldehyde exposure does not significantly increase or decrease the lipid accumulation, which contradict research evidence that the lipid concentration in algal cells increase due to various types of stress. Many researchers also indicated that the tolerance algae developed against certain conditions could allow them to tolerate other situations. For instance, *Dunaliella* was reported to be able to tolerate intensively high salinities by synthesising significant amounts of β-carotene. However, the same mechanisms could also shield algae from high light intensities and help

algae overcome nitrogen starvation or endure the toxicity of heavy metals. It is believed that all different stresses no matter whether they be nutrient starvation, toxic pollutants or changes of environment, eventually come down to the oxidative stress. Therefore, the correct level of acetaldehyde exposure might be able to induce desirable stress responses in microalgae. However, as shown in Figure 7.4, there might not be sufficient acetaldehyde induced stress in treatment 1 to stimulate the microalgae to respond by accumulating lipid to protect itself from the damage. However, as the exposure level increased in treatment two, a possibly more desirable acetaldehyde stress triggered the algal mechanisms to produce more lipid contents. Especially for *Chlorella*, it achieved relatively higher lipid accumulation (compared with its control group) while growing rapidly in treatment 2. The lipid concentration of treatment 2 was not much higher than that of control group. However, with over 50% more cell density produced by treatment 2 cultures (Figure 6.3), the total lipid productivity would no doubt be higher than the control. Although the lipid concentration seemed to be much less than *Dunaliella*, the large biomass *Chlorella* cultures produced would compensate this difference.

7.3 The Effects of Acetaldehyde Exposure to Algal Fatty Acid Methyl Ester (FAMEs)

Algae samples were taken from control, acclimated treatment 1 and acclimated treatment 2 cultures and prepared following the method explained in Chapter 3. Samples were then sent to the mass spectrometry lab for FAMEs analysis. Table 7.1 and 7.2 show the identified FAMEs and their concentrations in *Dunaliella salina* and *Chlorella* samples. The chromatograph spectrum of each sample can be found in appendix.

Five different FAMEs were identified in *Dunaliella salina* samples which are listed in Table 7.1. Among these 5 FAMEs, *methyl 4,7,10,13-hexadecateraenoate* (16:4(n-3)) is a type of polyunsaturated fatty acid (PUFA) which is considered as part of the group of important fatty acids that including many essential fatty acids. *Hexadecateraenoic acid* (16:4(n-3)) is reported to be able to inhibit the production of leukotriene in the mouse mast cell line

(Ishihara *et al*., 2000). The *palmitic acid* methyl ester is one of the saturated FAMEs. It is considered as the most common fatty acid found in many organisms (Frank *et al*., 2007). Palmitic acid can be used for producing many products such as soaps, cosmetics, and release agents. *Linolenic acid* and *linoleic acid* are both essential polyunsaturated fatty acid, which are vital to the health of humans and animals. These fatty acids can only be ingested though food source but cannot be synthesised inside the human body. The *linolenic* and *linoleic fatty acids* are classified respectively as omega-3 and omega-6 fatty acids. *Trans-13-octadecenoic acid* is a trans fatty acid which is edible but considered as a long-term risk to human health. However, it is believed to be favourable (18:1) for biodiesel (Durrett *et al*., 2008).

As Table 7.1 suggested, all identified FAMEs in *Dunaliella* samples, except *linolenic acid* methyl ester seemed to be reduced under the exposure to acetaldehyde. The results suggested that acetaldehyde could inhibit the synthesis of FAMEs in *Dunaliella salina*. The inhibition could be the results of peroxidation and other harmful effects (as mentioned and discussed in previous chapters) caused by acetaldehyde. However, algae samples taken from treatment 1 showed higher concentration of *linolenic acid* methyl ester (0.39%) than that of the control groups (0.18%). The *linolenic acid* methyl ester in samples from treatment 2 was still lower than the control, however, the degree of reduction was measured to be less than other FAMEs in same samples. Linoleic acid methyl ester cannot be found in algae samples from treatment 1 and treatment2. Such phenomenon could suggest that the influence of acetaldehyde may alter the linoleic acid synthesis into producing *linolenic acid.* *Linolenic acid* is reported to be a strong trigger to oxidative burst which is a mechanism that organisms use to protect themselves from pathogens and toxic material. However, the principle of this mechanism is similar to lipid peroxidation, which can be summarised as the free fatty acids (FFAs) driven by enzyme activity or direct reaction with reactive oxygen species (ROS) to cause a chain reaction to mass produce ROS to inhibit pathogens and detoxify harmful molecules (Kupper

et al., 2009). In the case of algae response to acetaldehyde exposure, the author believes similar protective mechanisms might be triggered. *Linolenic acid* was produced to trigger the oxidative burst to react with acetaldehyde and form less reactive products such as acetate, lipid peroxide and long carbon chain aldehydes (Ishige *et al.*, 2000), and eventually reduce the level of toxicity.

Table 7.1 The identified FAMEs in *Dunaliella* samples

Identified FAMEs	*Dunaliella salina*		
	Control	**Treatment 1**	**Treatment 2**
	% of dry biomass	**% of dry biomass**	**% of dry biomass**
Methyl 4,7,10, 13-hexadecateraenoate	0.30	0.28	0.13
Palmitic acid methyl ester	1.57	1.36	0.85
Linolenic acid methyl ester	0.18	0.39	0.15
Trans-13-octadecenoic acid methyl ester	0.35	0.21	0.16
Linoleic acid methyl ester	0.84	-	-

For *Chlorella* samples, the influence of acetaldehyde on FAMEs production seemed to be beneficial. As Table 7.2 demonstrates, only one fatty acid *Palmitic acid* can be identified in control samples. However, many more species of fatty acid can be measured in acetaldehyde exposed samples. Seven fatty acids can be observed in the chromatograph of treatment 1 and 8 can be found in samples of treatment 2. *linolenic acid methyl ester*, *linoleic ester* and *trans-13-octadecenoic acid methyl ester* can also be found in acetaldehyde exposed samples. However, linoleic acid cannot be identified in all *Chlorella* samples. Trace amount of *2.2-dimethylocta-3,4-dienal, methyl 2-hydroxy-octadeca-9,12,15-trienoate* and *ISO C17 methyl ester* can be detected in samples from both treatments 1 and 2. The major component of the FAMEs in acetaldehyde exposed *Chlorella samples* seemed to be *methyl (7E)-7-hexadecenoate, linoleic ester, palmitic acid methyl ester* and *(9Z,12Z)-9,12-octadecadienoate.*

Among these identified FAMEs, methyl 2-hydroxy-octadeca-9,12,15-trienoate is a omega-3 polyunsaturated fatty acid which has very similar molecular structure to linolenic acid except it has a hydroxy functional group at its α carbon atom. Studies suggested that hydroxy fatty acids may play a roles in animal and human reproduction and possibly habe anti-cancer abilities (Martinez *et al*., 2005; LIado *et al*., 2009; Llado *et al*., 2010). *Methyl (7E)-7-hexadecenoate* is an omega-9 monounsaturated fat which is not considered as an essential fatty acid. However it can still be utilized for bio-fuel production (Pazouki *et al*., 2010). Iso c17 methyl acid is a saturated fatty acid which can be found in many organisms (Richardson and Williams, 2013). Though ingesting large amounts of saturated fat could raise health issues, saturated fatty acids could make excellent biodiesel due to high oxidative stabilities (Puhan *et al*., 2010).

Table 7.2 The identified FAMEs in *Chlorella* samples

Identified FAMEs	*Chlorella*		
	Control	**Treatment 1**	**Treatment 2**
	% of dry biomass	**% of dry biomass**	**% of dry biomass**
2.2-Dimethylocta-3,4-dienal	-	0.09	0.06
Methyl 2-hydroxy-octadeca-9,12,15-trienoate	-	0.07	0.04
Methyl (7E)-7-hexadecenoate	-	1.26	1.33
Palmitic acid methyl ester	0.98	1.61	1.51
ISO C17 methyl ester	-	0.10	0.03
Linoleic acid methyl ester	-	3.48	3.22
Linolenic acid, methyl ester	-	0.45	0.45
trans-13-octadecenoic acid methyl ester	-	0.29	0.27

As shown in Table 7.2, the FAMEs profile of this *Chlorella* species is not impressive. There is only one fatty acid that can be identified by GC-MS in control samples. This may be because, the algae showed continuously fast growth rate during the whole cultivation, which

normally would not accumulate lipid content but multiply more cells. However, the detected FAMEs profiles were significantly improved in acetaldehyde treated *Chlorella* samples. Not only the quantities of the fatty acid methyl ester has been improved, but the numbers of FAMEs species detected was also increased. Moreover, the growth rate of cultures of the two treatments was not inhibited but improved by acetaldehyde. Six more fatty acids were identified in samples from treatments 1 and 2. The *palmitic acid ester* in both treatments (1.61% and 1.51% respectively) was also largely increased compared to control group (0.98%). The fatty acid with highest concentrations in samples of treatments 1 and 2 is *linoleic acid* which was 3.48% of dry biomass weight in treatment 1 and 3.22 % of dry biomass weight in treatment 2. It is desirable because *linoleic acid* is valuable essential fatty acid which could be used for producing food supplement. The fatty acids levels in treatment 2 sample were observed to be slightly lower than that of treatment 1 samples, especially for *methyl 2-hydroxy-octadeca-9,12,15-trienoate* and *ISO C17 methyl ester*. The explanations for the improvement of FAMEs production in *Chlorella* sp. under acetaldehyde exposure could be various. According to the research done by Benjamas et al. (2012), for mixotrophic grown strains, organic carbon sources in the cultures may trigger the alteration of algae photosynthetic and heterotrophic metabolisms. Such alteration could results in increased fatty acid accumulation because the carbon and energy required for lipid production may not necessarily come from photosynthesis, they can also be provided by heterotrophic metabolism. Arroyo et al. (2010) also discovered *Chlorella vulgaris* could achieve much higher growth rate under mixotrophic growth while maintaining consistent levels of lipid production. They believed that microalgae have the potential to fully utilize all the organic and inorganic resources to obtain maximum biomass and lipid production. Another possible explanation of improving the FAMEs profile under acetaldehyde exposure is mentioned in the hypothesis part of this thesis. Acetaldehyde may cause the appropriate level of oxidative

stress that could trigger the accumulation of fatty acids. However, due to the advantages of possibly mixotrophy, the growth rates of *Chlorella* cultures of treatment 1 and 2 were uninhibited.

7.4 Conclusion

The influence of acetaldehyde on algal lipid production was varied and depends heavily on the species of the microalgae and the levels of acetaldehyde exposure. The results of this part of the thesis suggested that acetaldehyde may affect algae photosynthesis through influencing the synthesis of photosynthetic pigments. β-carotene production showed different response to acetaldehyde exposure in *Dunaliella* and *Chlorella* cultures. Higher levels of acetaldehyde dosage triggered more β-carotene to accumulate inside *D. salina* samples. On the contrary, highest level of β-carotene was produced under relatively lower acetaldehyde exposure for *Chlorella*. The total lipid production of both strains shared similar tendencies, however, *D. salina* achieved much higher productivity.

For fatty acid methyl ester determinations, acetaldehyde can be considered as an inhibitor to the FAMEs production of *D. salina.* However the FAMEs profiles of *Chlorella* were largely improved under acetaldehyde exposure. It was discovered that *Chlorella* could improve both its biomass production and FAMEs accumulation due to the presence of acetaldehyde, which substantiated the assumption that *Chlorella* could be performing mixotrophic growth by utilizing acetaldehyde as a carbon source. This *Chlorella* strain also revealed the possibility that high yield of FAMEs can be achieved without stressing and limiting the growth of the microalgae.

CHAPTER 8 RECOMMENDATIONS FOR FUTURE WORK

8.1 Scope of this Chapter

The results of this research are very inspiring, which demonstrated the grand potentials of the algae utilization. The results indicate the possibility that effluents from bioethanol productions could be treated economically and efficiently by cultivating microalgae. Algae strains such as *Dunaliella salina,* and *Chlorella* presented remarkable capability of acclimations which allow them to develop sufficient tolerance to acetaldehyde. Moreover, acetaldehyde was found could even benefit the growth and lipid production of *Chlorella* sp.. However, the outcomes of the research also raised many questions which are interesting to explore further in order to deepen the understanding of algae acclimation, to reveal the mechanisms of acetaldehyde influence and uncover the metabolic pathways of acetaldehyde inside the algae.

On the bases of the results of this research, *Chlorella* were especially found promising for bioethanol gaseous effluent treatment. This finding inspired more initial point of view towards the prospective that study the interaction of acetaldehyde and *Chlorella* metabolisms. The particular interest of the author is finding the solid proof that *Chlorella* sp. could grow mixotrophically by utilizing acetaldehyde as its supplementary carbon source. This could be realized by conducting isotope labelling experiments. Through feeding labelled acetaldehyde and measure the isotope distribution inside the cell could not only support the hypothesis of growth improvement of *Chlorella* but also could reveal the metabolic pathway of acetaldehyde inside algae cells. Moreover, identify the species of this *Chlorella* strain could also provide important information to interpret its response to acetaldehyde exposure.

Although acetaldehyde was observed as mainly an inhibitor to *Dunaliella salina,* it is still premature to believe that *D. salina* is not suitable for acetaldehyde removal. The tolerance of

D. salina to acetaldehyde improved after acclimation. Good growth rate can be observed in *D. salina* culture. To explore the potential of *D. salina* may not necessarily has to rely solely on pure *D. salina* culture. Mutualisitic could provide solutions. Due the acclimation capacity and sufficient acetaldehyde tolerance of *D. salina* shown in the reseach, it is not difficult to believe that removing and utilizing acetaldehyde can be achieved by co-culturing with desired organisms.

8.2 DNA Sequencing of *Chlorella* sp.

8.2.1 Introduction

The *Chlorella* strain used in this research was isolated from local water body. It was identified as *Chlorella* through its morphology. However, *Chlorella* is a large genus including numerous strains, and each strain has its unique characteristics. To understand the acclimation capability and the acetaldehyde beneficial effects, fully identifying this strain is necessary. To do so, DNA sequencing is the most reliable way. Moreover by comparing the results with identified DNA sequences, the bio-function of the strain can be identified, which is valuable for understanding the mechanisms of the response of tested microalgae to acetaldehyde exposure.

The DNA sequestration could also determine the natures of the *Chlorella* acclimation. It could provide important evidence to determine whether the acclimation was the results of genetic mutation. To do so, the DNA sequence of the acclimated *Chlorella* strain and the control group should be compared.

8.2.2 Presumably Methodology

Genomic DNA can be extracted from fresh liquid algae cell culture by QIAgen genomic tip kit. Prepare the gel and run gel in order to ensure the presence of gDNA and the absence of

RNA. When RNA observed 1 µl RNAse could be added into the samples and incubated at 4 °C over night. Heat samples 20 minutes at temperatures of 37 °C. Then extract the genomic DNA and ethanol precipitate by phenol-chloroform.

PCR mix should be prepared by following recipe:

Distilled Water	39µl
10×Buffer	5 µl
50 mM $MgCl_2$	2.5 µl
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
dNTPs	1 µl
genomic DNA	1 µl
Bioline Taq	0.5 µl

After preparation, the PCR mix will be run by following cycle (Named 16s Standard):

Initial denature:	94 °C	
Denature:	94 °C	repeat for 30 cycles
Anneal:	60 °C	
Elongation :	72 °C	
Final elongation:	72 °C	then hold at 10 °C

The reacted samples will be sent to Medical school, university of Sheffield, for sequencing and identification.

8.3 ^{13}C-labelling for Determination of Acetaldehyde Metabolic Pathway in Microalgae Cells

8.3.1 Introduction

The research discovered that acetaldehyde can affect microalgae metabolism significantly. The photosynthesis and lipid accumulations were both observed to be altered due to the exposure of acetaldehyde. Moreover, strains such as *Dunaliella salina* and *Chlorella* developed significant resistance to acetaldehyde after acclimation. Furthermore, *Chlorella* was seen potentially capable of utilizing acetaldehyde as its supplementary carbon and energy source. The hypothesis were made based on the results and literatures to explain the influence of acetaldehyde exposure, however the exact mechanisms are yet uncertain. Isotope labelling provides reliable tool to probe the metabolic flux of microalgae. It is considered as one of the standard methods for metabolic studies of organisms (Crown and Antoniewicz, 2012). Introducing ^{13}C labelled acetaldehyde into algae medium and tracing the isotope through NMR can discover and determine the reaction acetaldehyde involved, the pathways of acetaldehyde metabolisms and the product acetaldehyde eventually become. It could also provide valuable insight of algae acclimation by comparing acclimated samples with non-acclimated ones. The results of isotope labelling could also be the solid proof of acetaldehyde removal and utilization by *Chlorella* sp..

When ^{13}C labelled acetaldehyde present in the medium, if *Chlorella* and *Dunaliella* could utilize it then the isotope could be detected from the output metablolite. Because isotope atom has different gyromagnetic radios from general carbon atom, therefore it can be detected by nuclear magnetic resonance. The NMR could even identify the location of isotope atom in the

structures of the metabolic products (Dickin, 2005). Through the isotope patterns of the output metabolite, the distribution of acetaldehyde in microalgae's metabolic network can be determined as well as the conversion rate (Lee *et al*., 2011).

8.3.2 Presumably Methodology

Purchase ^{13}C labelled acetaldehyde, preferably labelled on both carbon atom. Both *Chlorella* and *Dunaliella* should be cultivated over the lag phase in bioreactors and then inoculated into 250ml shake flasks. ^{13}C labelled substrates could be expensive. Therefore, algae samples would be better to be cultivated till the metabolic steady stage is reached before adding labelled substrate (Tang *et al*., 2009). Both acclimated and non-acclimated cultures should be included in the experiment in order to determine the performance of acclimation. Place the flasks under preferred temperature and light intensity. Add isotope labelled acetaldehyde at the concentration of 32 mg/L and 64 mg/L respectively into the corresponded flasks. Cultivate the microalgae for around 1 week (a quasi-steady state) (Wiechert, 2001).

Samples should be taken and prepared for NMR measurement. The ^{13}C NMR is considered as preferable techniques than Proton NMR. With NMR measurement, each single isotope carbon atom and its position can be detected in a metabolite pool and the distribution of the isotopomers can be observed in details. It is realized because the hyperfine splitting signals generated by labelled carbon atom is different from the neighbouring non-labelled that atoms. Therefore, when there is no labelled atom in neighbouring area then only a single peak can be detected. However, doublet peak can emerges because one neighbouring atom is labelled. The size of the peaks is depended on the function group of the neighbouring carbon atom (Wiechert, 2001). With the information provided by NMR, the percentage of labelled isotopomers at different positions can be calculated, which could be used for quantification of acetaldehyde uptake. Through the results of NMR measurement and the stoichiometric analysis, the metabolic fluxes of acetaldehyde can be identified (Tang *et al*., 2009).

8.3 Mutualisitic Research of *Dunaliella salina*

8.3.1 Introduction

Although there is no evidence to support that *Dunaliella salina* could utilize acetaldehyde for its growth, there are many bacteria and fungi which are, for instance *Halomonas* gunas were reported repeatedly that it could perform acetaldehyde dehydrogenase (Sripo *et al*., 2002). Moreover, many *Halomonas* strains have been commonly found to coexisted with *D. salina* in *D.S* cultures (Chao *et al*., 2013). Both *D. salina* and *Halomonas* prefer high salinity environment. *Halomonas* is generally harmless to *D. salina*. Halomonas could also perform both anaerobic and aerobic growth. All these characteristics make *Halomonas* an ideal candidate for symbiosis research with *D. salina. Halomonas* could reduce level of acetaldehyde exposure minimize the influence to *D. salina* and process organic carbon into inorganic nutrient for the growth of *D. salina*. In return, the photosynthesis of *D. salina.* provides sufficient oxygen for *Halomonas* aerobic metabolisms when aldehyde dehydrogenase process is active. Therefore, the symbiosis of *D. salina* and *Halomonas* strain could be very promising.

8.3.2 Presumable methodology

The *Halomonas* strain will be acquired through isolating *D. salina* culture. A screening experiment should be conducted in order to study the optimal compositions of the mixed culture. Test *D. salina*. to *Halomonas* concentration ratios from 9:1 to 3:7. Provides glucose for *Halomonas* growth during the cultivation initially, stop if affect the growth of *Dunaliella salina*.

After the optimal ratios determined, add acetaldehyde into selected mix cultures, study the changes of acetaldehyde concentrations through high performance liquid chromatograph (HPLC). Measure the photosynthesis rate, lipid profile and protein profile of the culture. When acetaldehyde removal observed, adding isotope labelled acetaldehyde for isotope

labelling analysis. Then isolate two strains and measure the NMR in order to analysis the metabolic flux.

CHAPTER 9 CONCLUTION

The objectives of this doctorial research were achieved. A novel screening system was designed and built. It is proven to be beneficial to algae growth and the efficiency of screening and acclimation process. The thesis also discussed the relationship between inlet gas bubble size, mass transfer rate and algae growth rate. It revealed that with improving the effectiveness of mass transfer are could improve the algae growth considerably. The influence of gas flow rate to algae growth was also analysed and optimal gas flow rate was determined. 5 different algae strains were screened in order to determine the most promising candidates with high acetaldehyde tolerance. *Dunaliella salina* and *Chlorella* were selected due to their higher growth rate when exposed to acetaldehyde. The performance of both strain also showed significant improvement due to the acclimation. Additionally, the effect of acetaldehyde on algae lipid accumulation were also studied and discussed

9.1 The Performances of the Novel Microbubble Induced Screening System

A COMSOL simulation were built during the research to predict the performance of the systems. The model suggested that with the geometry of the bioreactor and the smaller bubble size better mixing and circulation were expected to be achieved. The model also predicted higher liquid velocity could be achieved by microbubble, which could result in stronger mixing and circulation.

Two experiments were conducted in order to reveal the relationship between mass transfer rate and inlet gas bubble size. It is determined that the reducing bubble size could largely increase the effectiveness of mass transfer under the same flow rate. The K_La of microbubble was achieved more than 4 times higher than the K_La of the normal bubbles. Through nonlinear regression, the relationship of K_La and bubble size could be obtained:

For oxygen dissolution $K_L a = -0.3096 \times \log(0.0007 d_B)$ $resnorm = 5.8 \times 10^{-3}$

For oxygen removal

$$K_L a = -0.4316 \times \log(0.0008 d_B) \qquad resnorm = 5.97 \times 10^{-4}$$

The advantages of smaller bubble size in mass transfer is well supported by the results.

To prove the beneficial effect of microbubble on algae growth, model strains were cultured in microbubble bioreactors and conventional reactors. The experiment results indicated that the specific growth rate in microbubble bioreactors were found to be 20 to 40 % higher than the growth rate in conventional reactors. The experiment also determined that microbubble contribute to algae growth through its rapid mass transfer rate. More carbon source were provided to microalgae and the oxygen were striped out. The advantages of the microbubble bioreactor is believed could also improve the efficiency of screening and acclimation processes.

9.2 Algae Screening

The relationship of the inlet gas flow rate and microalgae growth rate was studied. 5 different flow rate were injected into 5 bioreactors respectively. The specific growth rate of microalgae from each bioreactor were calculated and compared. It is discovered that increasing the flow rate did not necessarily increase the growth rate of the microalgae. The experiment discovered that under flow rate of 0.9 L/min, highest algae growth rate can be achieved. It is also revealed that the improvement of algae growth is due to the increase of mass transfer rate. When the flow rate was near 0.9 L/min, the highest CO_2 mass transfer rate could be reached.

Algae strains namely: *Dunaliella salina, Chlorella, Tetraselmis, Chlamyndomonas* and *Nanochloropsis oculata* were screened under two set of acetaldehyde exposure plans. Under

high level of acetaldehyde exposure, inhibitory effects can be observed in all strains. However, at lower level of exposure, *Chlorella* and *D. salina* showed highest tolerance to acetaldehyde, which were determined as the most promising candidates. Both strain were grown without obvious inhibition at lower level of exposure. Positive growth can also be observed in *Tetraselmis* cultures; however, the strain cease to grow when acetaldehyde daily dosage reached 125 µl. Both strains of *Chlamyndomonas* and *Nannocloropsis* showed no growth which were presumably dead because of the exposure of acetladhyde.

9.3 Algae Acclimation

The inhibitory effects of acetaldehyde to *Dunaliella salina* could be observed during the experiment. Both of the growth rate and photosynthesis rate of *D salina* suffered decline when exposed to acetaldehyde. However, acclimation could significantly reduce the inhibition. The acclimated cultures showed little difference from the control group. Especially for photosynthesis rate, the T2 acclimated group showed 70% improvement compared to the non-acclimated groups.

For *Chlorella,* the effect of acetaldehyde was almost entirely beneficial. The *Chlorella* strains during the acclimation showed little difference from the control. The acetaldehyde did not seem to affect the growth of *Chlorella.* Moreover, acetaldehyde exposure seemed to be able to improve the photosynthesis rate of Chlorella. After the acclimation, considerable increase of both the growth and photosynthesis rate can be observed. The T2 acclimated group achieved 50% higher cell density and improved more than 60% of photosynthesis rate compared to the control groups.

The results proved that the both algae strains have remarkable capacities of acclimation. Sufficient tolerance can be developed through acclimation. However, the inhibition of photosynthesis can still be observed in acclimated *Dunaliella,* though largely improved. The

beneficial effects of acetaldehyde on *Chlorella* supported the hypothesis that *Chlorella* could potentially utilize acetaldehyde as its supplementary carbon source for mixotrophic growth.

9.4 The Determination of Acetaldehyde Influence on Algal Lipid Production

According to the results acquired by NMR measurement, the influence of acetaldehyde on algal lipid production was various. For *Dunaliella salina*, the Chlorophyll contents were observed to reduce when exposed to acetaldehyde. The tendency is consist to the photosynthesis rate under acetaldehyde exposure, which hinted that acetaldehyde influence the photosynthesis of *Dunaliella salina* through affecting the synthesis of Chlorophyll content. The Change of the Chlorophyll content of *Chlorella* showed opposite trend. As the level of acetaldehyde exposure increased, more Chlorophyll content accumulated inside the cell, which explained why acetaldehyde could benefit the photosynthesis rate of *Chlorella.*

The concentration of beta-carotene inside *Dunaliella* cells fluctuated when exposed to acetaldehyde. The initial decreasing of β-carotene concentration in *D. salina* cells when exposed to acetaldehyde treatment 1 may be due to acetaldehyde toxicity. Acetaldehyde is considered very reactive inside the system of organisms. It could react with many essential molecule which could lead serious disorder to the organisms. This might be the reason for the change of the β-carotene concentrations. However, β-carotene is also an antioxidant which could be excreted to relief the oxidative stress cause by acetaldehyde. Different trend can be observed in *Chlorella* cultures. The highest β-carotene concentration was achieved when samples exposed to acetaldehyde treatment 1. The *Dunaliella salina* samples were found to contain much higher concentrations of lipid than *Chlorella*. This could due to lower growth rate *Dunaliella* achieved. Lipid content was believed to be able to accumulate when the growth of microalgae was stressed. Due to the fast growth rate of *Chlorella*, the carbon and energy source might be directed to reproducing the cells rather than accumulating lipid.

Reductions of FAME accumulation can be observed in *Dunaliella* due to acetaldehyde exposure. However, acetaldehyde could largely improve the lipid profile of *Chlorella*. More fatty acids can be identified in *Chlorella* samples when exposed to acetaldehyde. Moreover, the quantities of the FAMEs were also increased. Due to the influence of acetaldehyde, *Chlorella* could manage to grow its biomass rapidly while accumulating fatty acids. Generally, microalgae only mass accumulate lipid content when the growth is stressed. According to the analysis, the oxidative stress might still exist inside the *Chlorella* cells; however, the benefit of the mixotropic growth compensated the inhibition caused by the stress. Moreover, the acetaldehyde level inside *Chlorella* cells could also reduce due to its metabolisms.

9.5 Feasibility of future industrial application

As discussed in Chapter 2, acetaldehyde is a troublesome side product of bioethanol production. It could affect the quality of the ethanol fuels and cause environmental issues. The manufacturers of ethanol fuels are seeking effective and environmental friendly approaches to treat acetaldehyde emissions. Therefore, this research was carried out in order to provide an algae based solution to remove acetaldehyde from the exhaust gas of bioethanol production.

According to previous experiments and discussions, the potential of algae based treatment has been proven to be promising. Algal strains such as *Dunaliella salina* and *Chlorella* sp. showed sufficient tolerance to acetaldehyde exposure and the growth rate of *Chlorella* enhanced after acetaldehyde involved acclimation, which may suggest the ability of utilization of acetaldehyde as carbon source. Such algae based treatment has many advantages in industrial application. Both algal strains *Dunaliella salina* and *Chlorella* sp. are well studied and widely used, which are considered to be adaptable strains and can be easily acquired and cultivated. Moreover, both strains can produce bio-products with high

commercial values. The algae based technology also has advantages in reducing capital cost and land use. Unlike traditional VOC treatment process which requires large financial investment and space for installation of complex facilities, the algal acetaldehyde removal system needs only simple photo-bioreactors. These bioreactors are cheap to manufacture and easy to maintain. This novel algal system also requires lower energy consumption. The sunlight can be used to provide light source for algal photosynthesis, and the geometry of the bioreactor could generate sufficient circulation to provide required mixing, therefore, the energy cost can be reduced due to less electricity needed for illumination and agitation.

In terms of industry applications, the batch culture techniques should be considered in the design of the removal system. Batch culture is the most common method for microalgal mass cultivation due to its simplicity, high reliability and low requirement for complex sterilization. In practice, the batch culture systems for algae based acetaldehyde removal should consist of different batches of microbubble bioreactors. The microalgae is grown in one batch of bioreactors until it reaching the maximum biomass density, then they will be retained as inoculum for next culture batch. The carbon source for the algal photosynthesis will be provided by injecting exhaust gas from bioethanol production. The outlet gas should be recycled in order to further reduce the acetaldehyde and CO_2 emissions.

The cultivation system could installed on roof top in order to further reduce the land requirement and achieve better sun illumination. To reduce the capital cost, the pre-treated municipal wastewater can be used to replace cultural medium. The processes such as ozonation could break the large molecule in the wastewater and perform decontamination, which could convert the municipal wastewater into ideal algal growth medium. According to the discussion, the industrial application of algal based acetaldehyde removal technology is believed by the author to be a promising, economical and feasible approach.

9.6 Final thoughts

It can be established that this research generated a number of novel impacts and raised many unexpected, yet significant questions. Firstly, a novel microbubble induced screening system were built and tested. The experiment results fully supported the advantages of this system over traditional shake flasks based screening techniques. It not only suitable for lab scale research but also ideal for industrial scaled-up trails due to the stable and desired cultivation environment the system provides.

Secondly, the algal response of 5 different strains to acetaldehyde exposure were studied. After a thorough examination of literature, there are few reports on acetaldehyde influence to microalgae except a handful of toxicity researches which involving extremely high levels of exposure. The thesis provided first-hand information of different algal responses when exposed to acetaldehyde at the level of industrial effluent. The traditional point of views believed acetaldehyde would cause serious inhibition to microalgae even lead to algaecide. On contrary, the results of this dissertation revealed that some strains are naturally more resilient to acetaldehyde influence and remarkable capacities of acclimation. It is proved that after acclimation *Dunaliella* could grow unaffected at presence of acetaldehyde. Moreover, the growth of acclimated *Chorella* sp. was observed increased significantly under acetaldehyde exposure. These findings overturned the traditional ideas of the interaction between microalgae and acetaldehyde and also demonstrated the possibility of algae based techniques of acetaldehyde treatment.

Last not the least, the thesis also discussed the influence of acetaldehyde on the lipid production *Dunaliella* and *Chlorella.* The beneficial effect of acetaldehyde on *Chlorella* FAMEs profile is the most unexpected and interesting outcome. It is worth to mention the growth rate of the *Chlorella* cultures is described in Chapter 6, which also increased due to the effect of acetaldehyde. The traditional perspective of FAMEs accumulation is

contradictory to the growth of microalgae. It was commonly recognized that stressing the growth of microalgae is the necessary cost to ensure the yield of algal FAMEs. However, the results of this thesis offered a novel insight that the increase of both biomass and lipid production of microalgae is not impossible to be achieved simultaneously. Presumably because the mixotrophic growth provided microalgae extra carbon and energy pathway which compensated the influence of the stresses. However, the toxicities of the acetaldehyde stimulated the protective mechanisms of microalgae which normally results lipid accumulation.

These outcomes and impacts are significant and the author offered explanations based on literatures and deductive thinking. However, all these conclusions, deductions, assumptions and hypothesis would be more convincing if supported by more analysis on cellular and molecular level for instance isotope labelling analysis. By the methods mentioned in the future work chapter, the understanding of the microalgae behaviour under acetaldehyde exposure could be much deepened. In conclusion, this research is served as an essential and fundamental study that could be developed into a novel algae based technology that would play significant roles in many area such as lipid harvesting, waste material treatment and biomass production.

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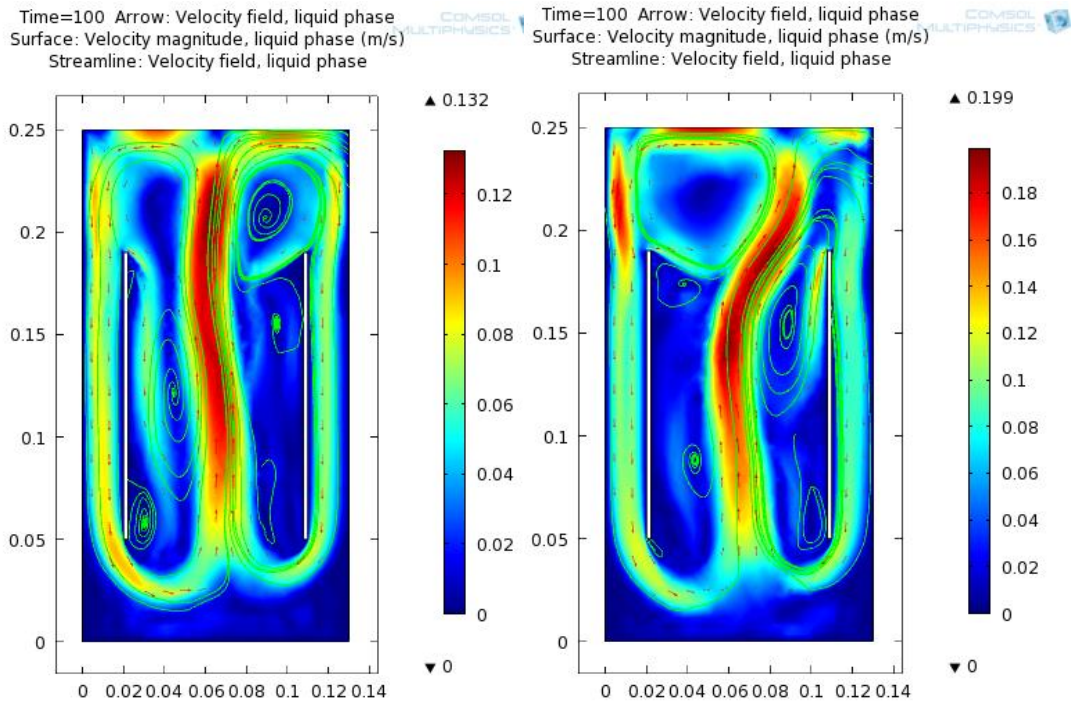
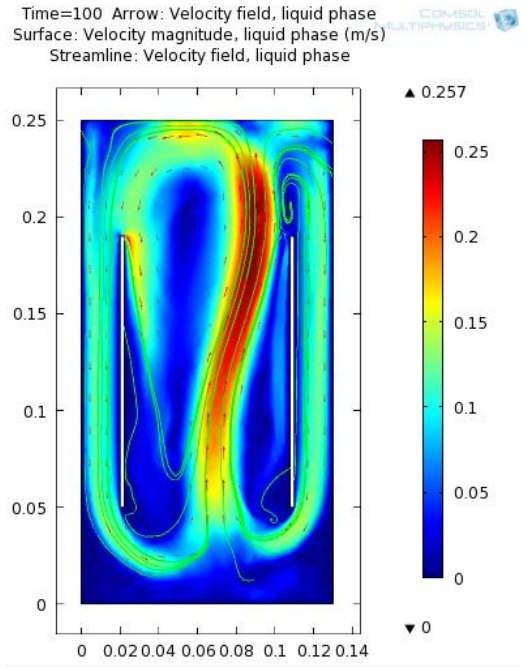
APPENDIX 1: Performance Study of the Screening System

COMSOL model simulation for the liquid circulation and mixing in the bioreactors

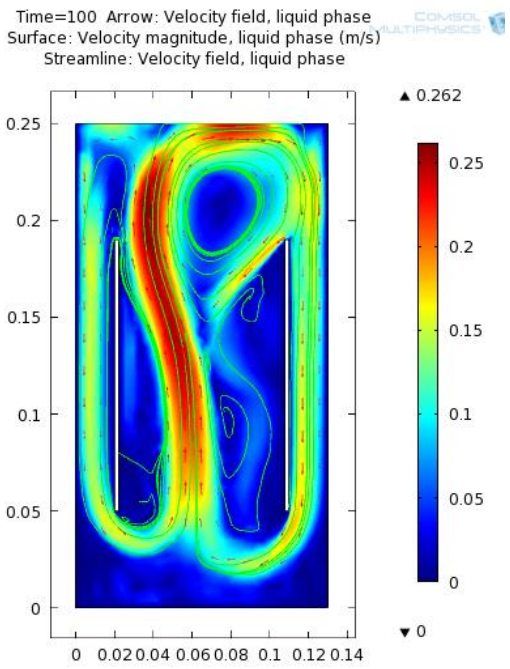
1. The simulation of liquid circulation in the bioreactor with oscillator



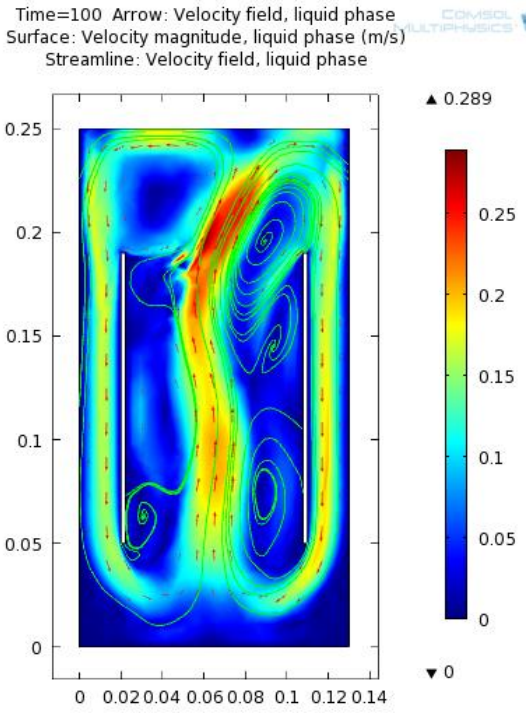
(a) Flow rate 0.1L/min (with oscillator) (b) Flow rate 0.3L/min (with oscillator)



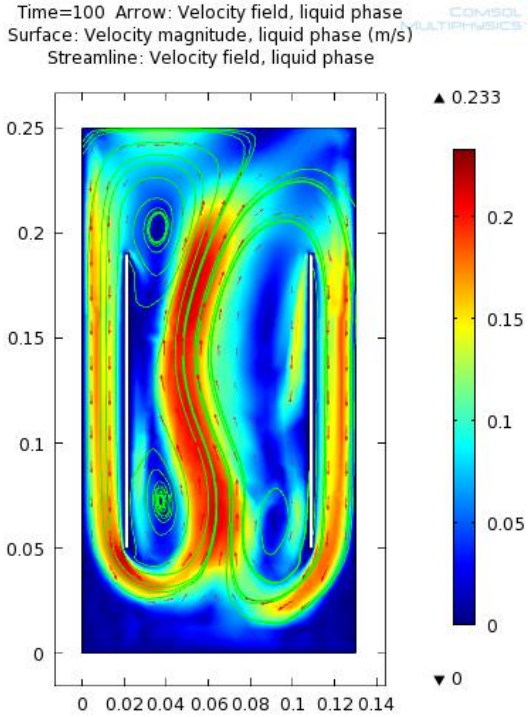
(c) Flow rate 0.5L/min (with oscillator)



(d) Flow rate 0.7L/min (with oscillator)

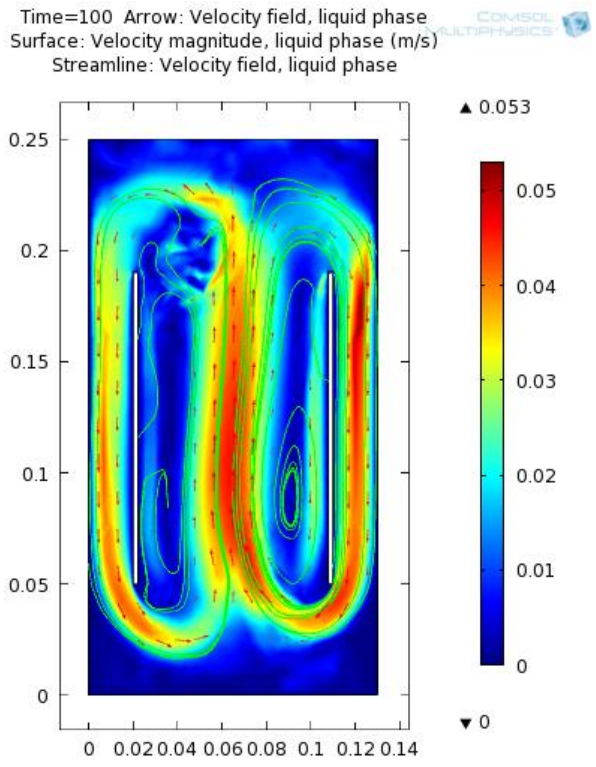


(e) Flow rate 0.9L/min (with oscillator)

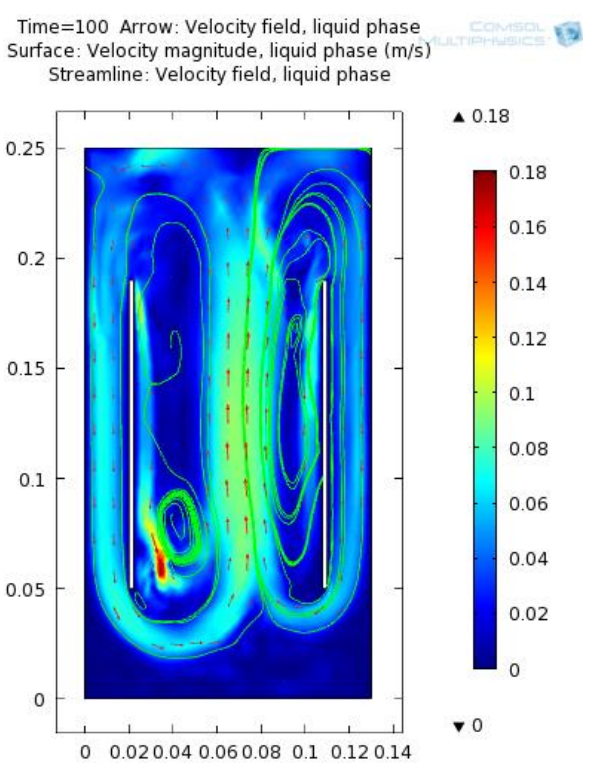


(f) Flow rate 1.1L/min (with oscillator)

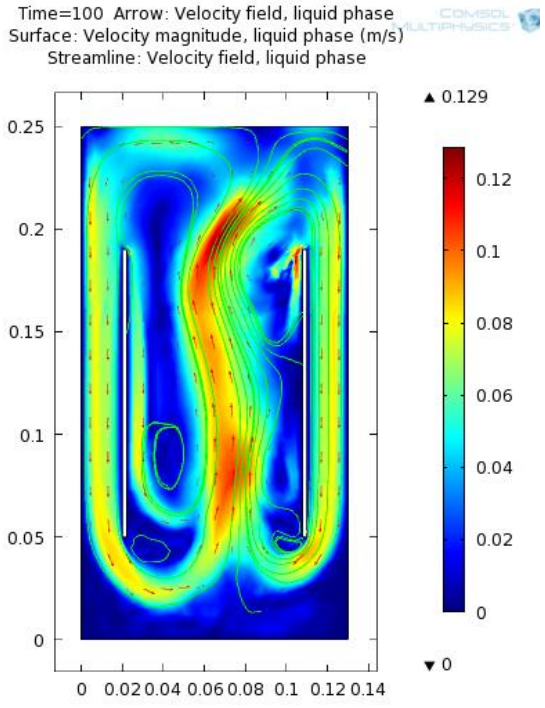
2. The simulation of liquid circulation in the bioreactor without oscillator



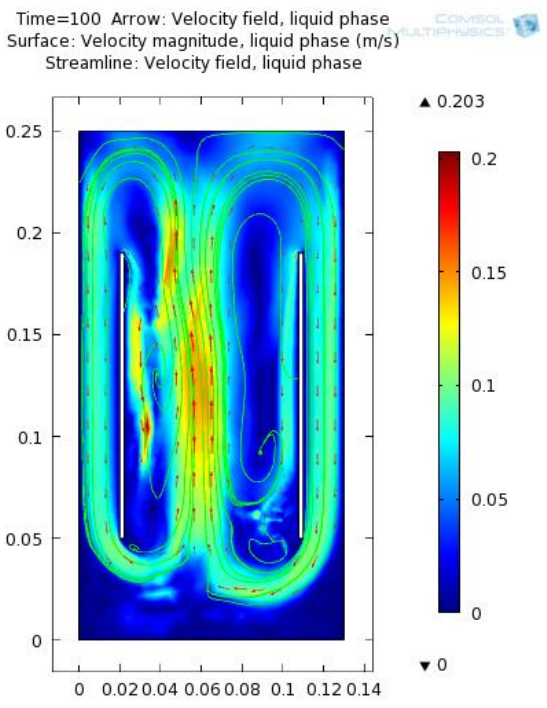
(g) Flow rate 0.1L/min (without oscillator)



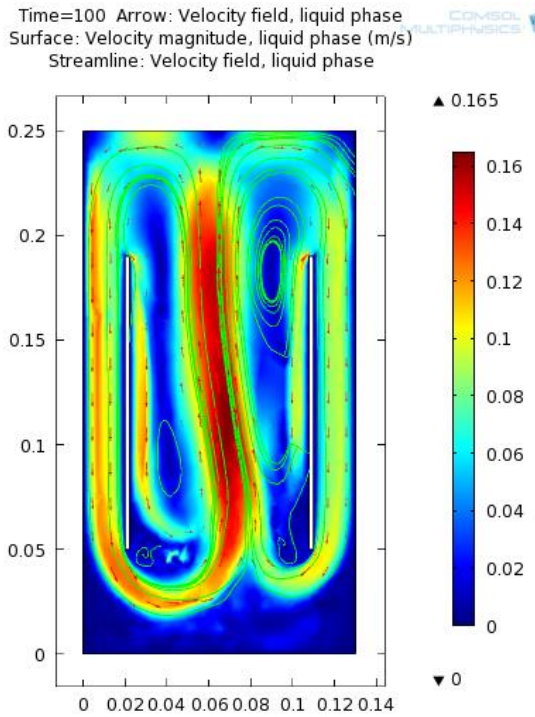
(h) Flow rate 0.3L/min (without oscillator)



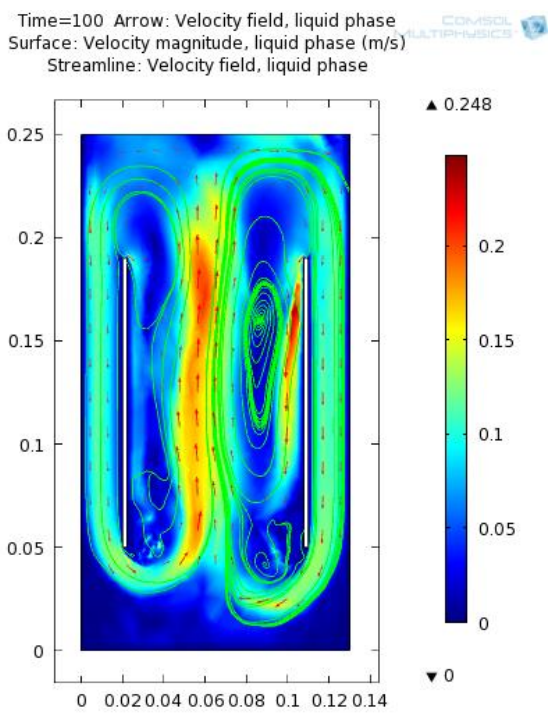
(i) Flow rate 0.5L/min (without oscillator)



(j) Flow rate 0.7L/min (without oscillator)



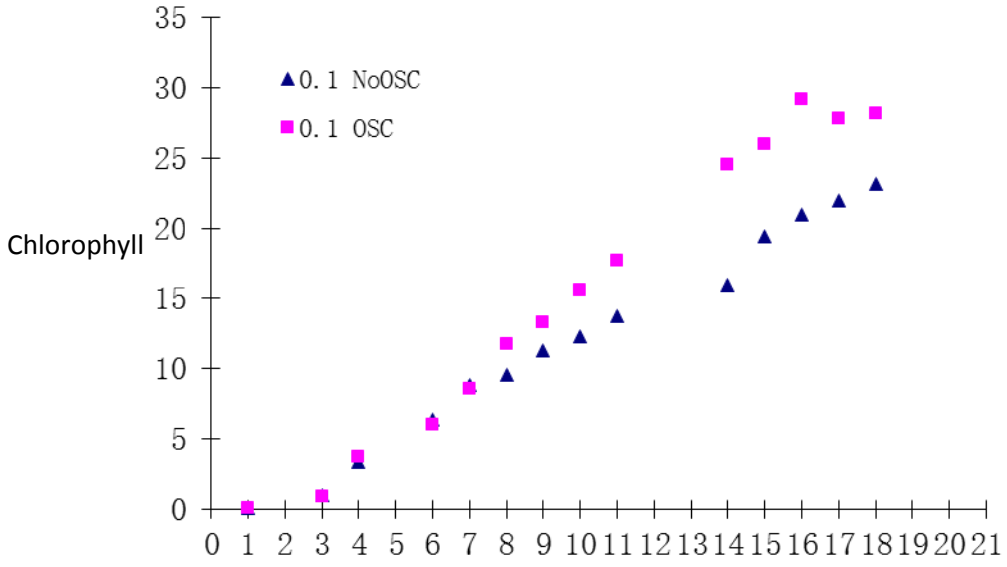
(k) Flow rate 0.9L/min (without oscillator)



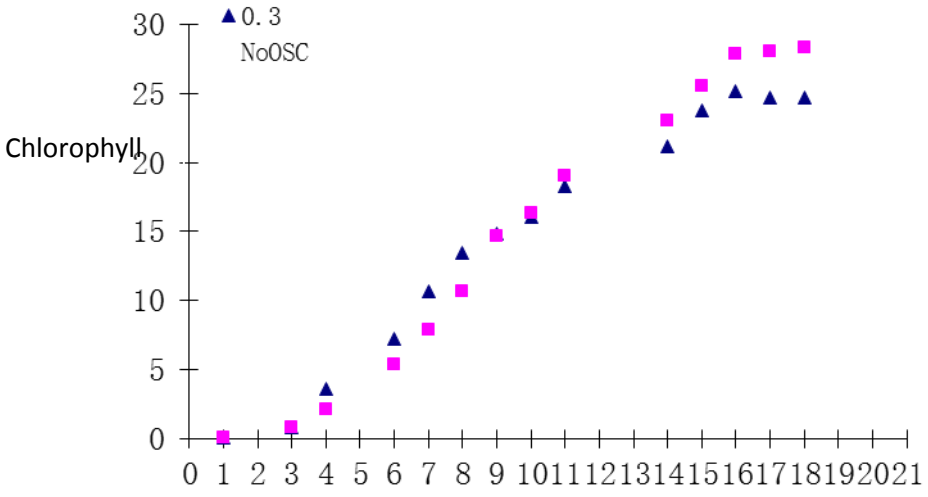
(l) Flow rate 1.1L/min (without oscillator)

APPENDIX 2

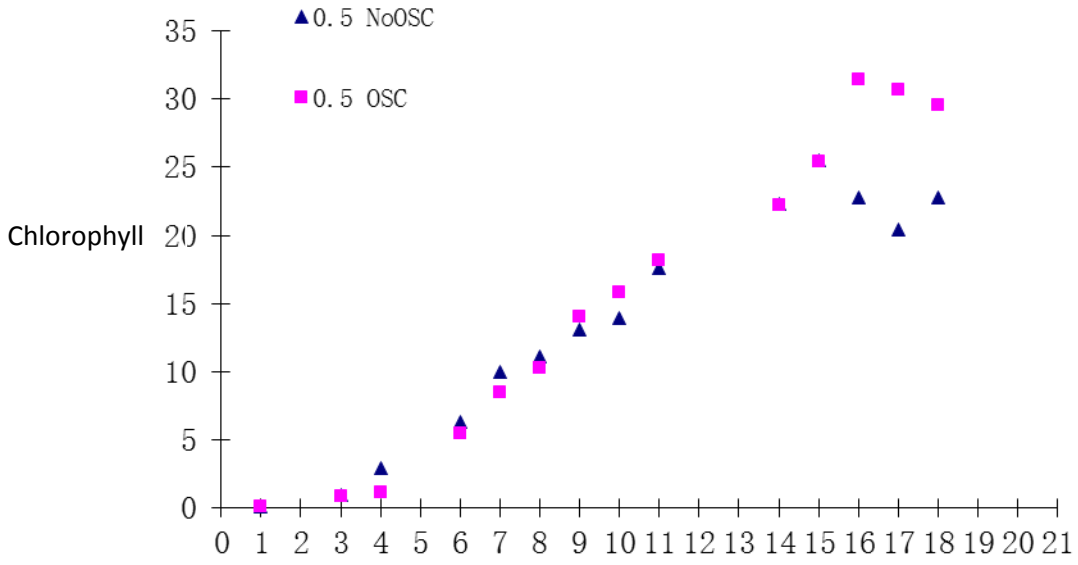
Algal growth in microbubble induced bioreactor and traditional bioreactor under different flow rate



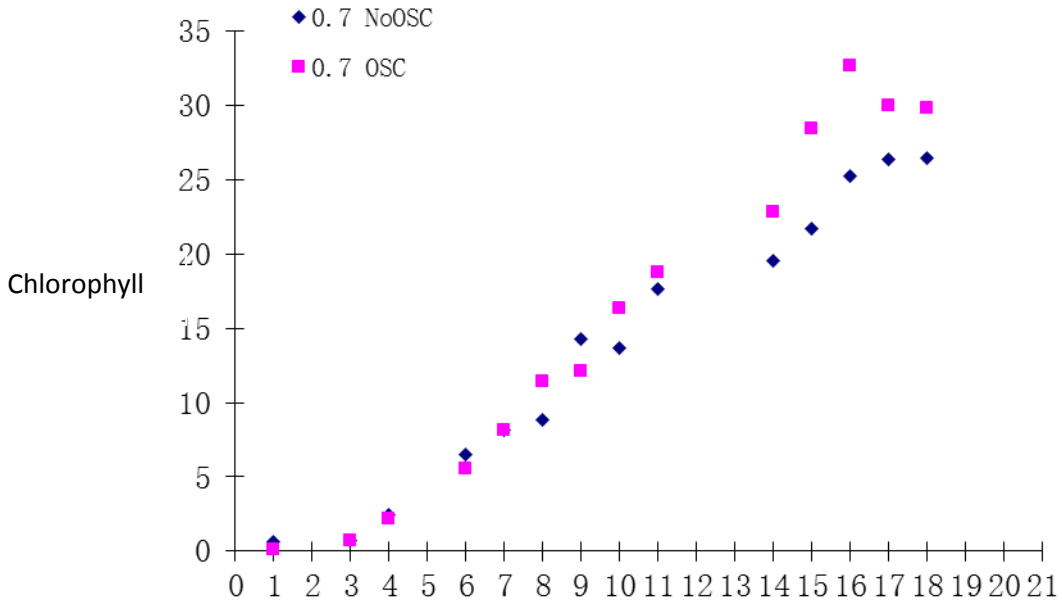
(a) The chlorophyll content increase under 0.1L/min flow rate (OSC-bioreactors with oscillator, NoOSC-bioreactors without oscillator)



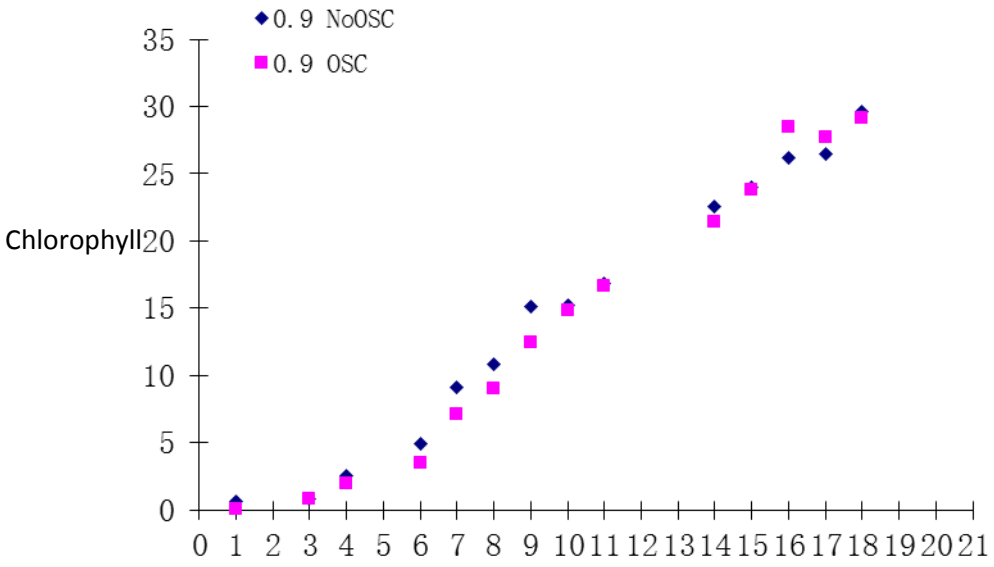
(b) The chlorophyll content increase under 0.3L/min flow rate (OSC-bioreactors with oscillator, NoOSC-bioreactors without oscillator)



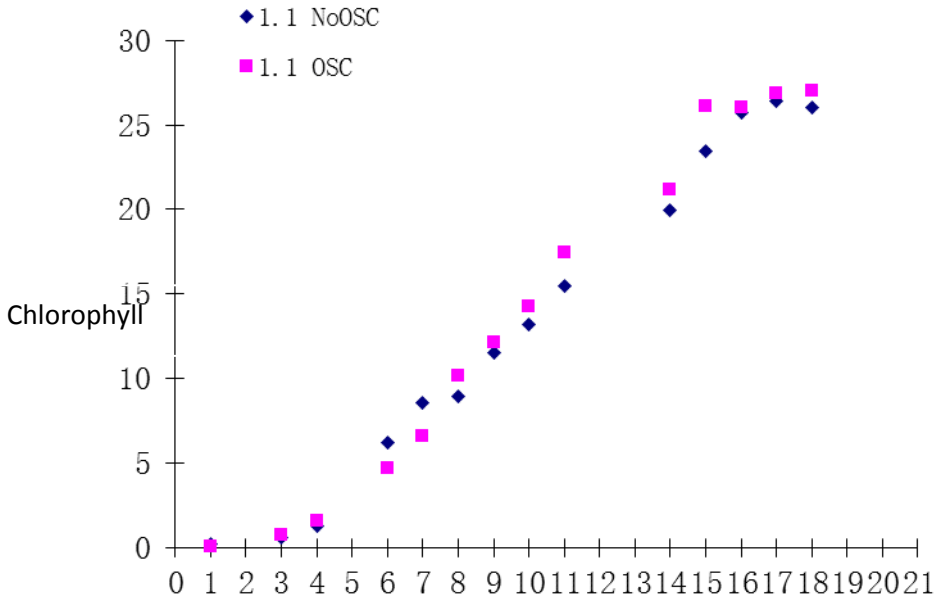
(c) The chlorophyll content increase under 0.5L/min flow rate (OSC-bioreactors with oscillator, NoOSC-bioreactors without oscillator)



(d) The chlorophyll content increase under 0.7L/min flow rate (OSC-bioreactors with oscillator, NoOSC-bioreactors without oscillator)



(e) The chlorophyll content increase under 0.9L/min flow rate (OSC-bioreactors with oscillator, NoOSC-bioreactors without oscillator)



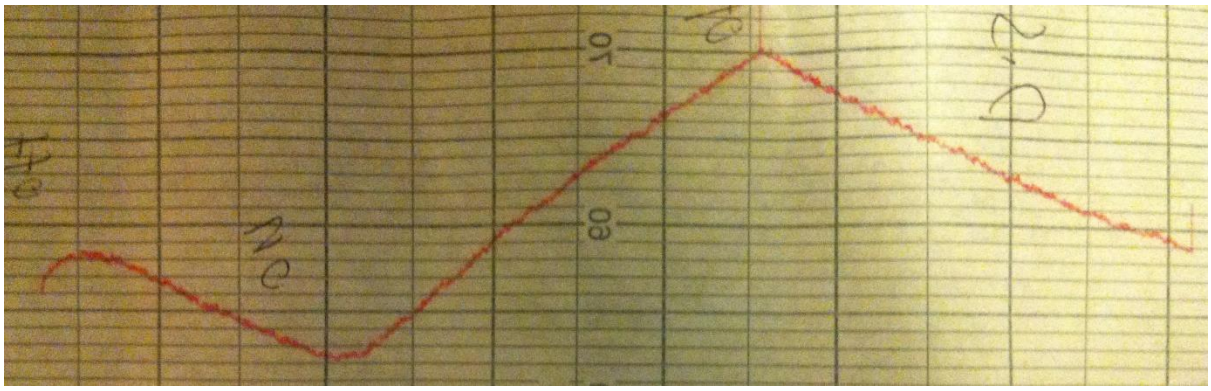
(f) The chlorophyll content increase under 1.1L/min flow rate (OSC-bioreactors with oscillator, NoOSC-bioreactors without oscillator)

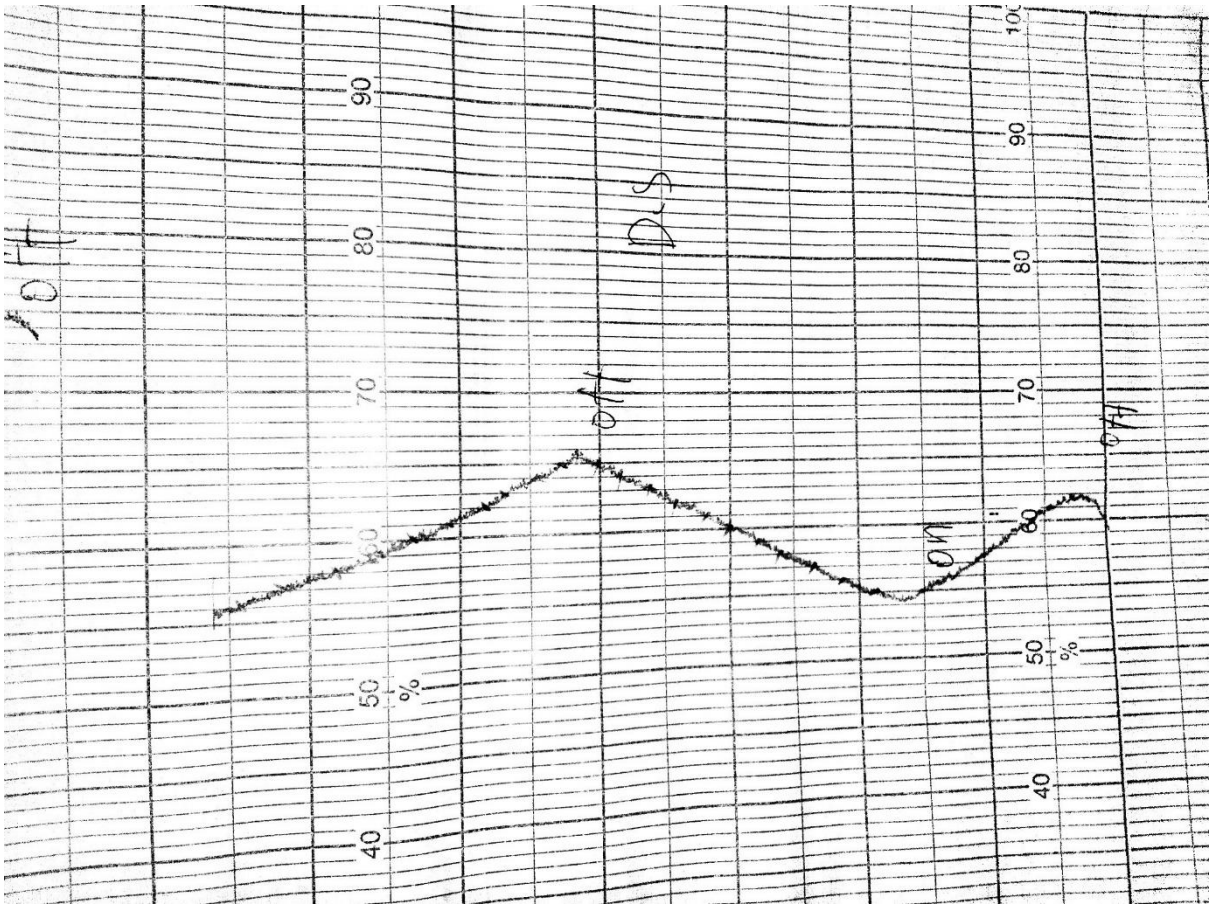
APPENDIX 3

The original chart of oxygen concentration changes due to photosynthesis and respiration

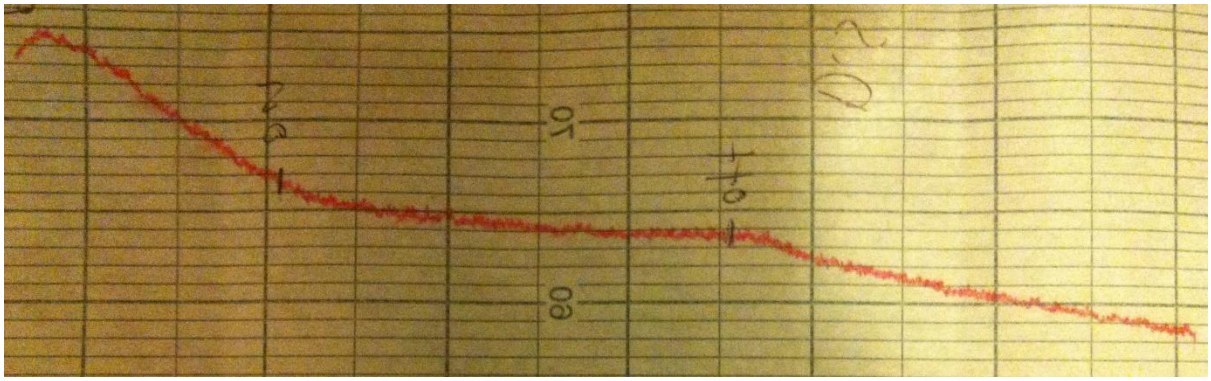
D salina

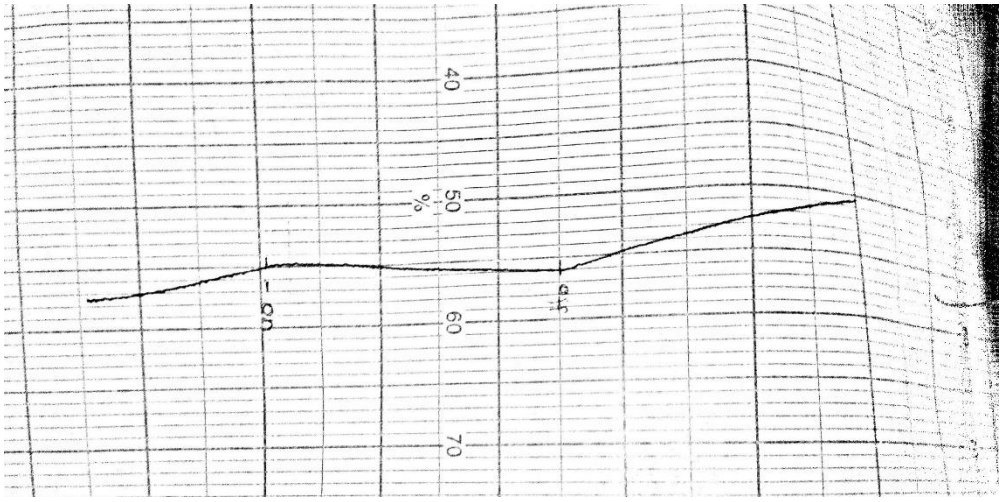
Control



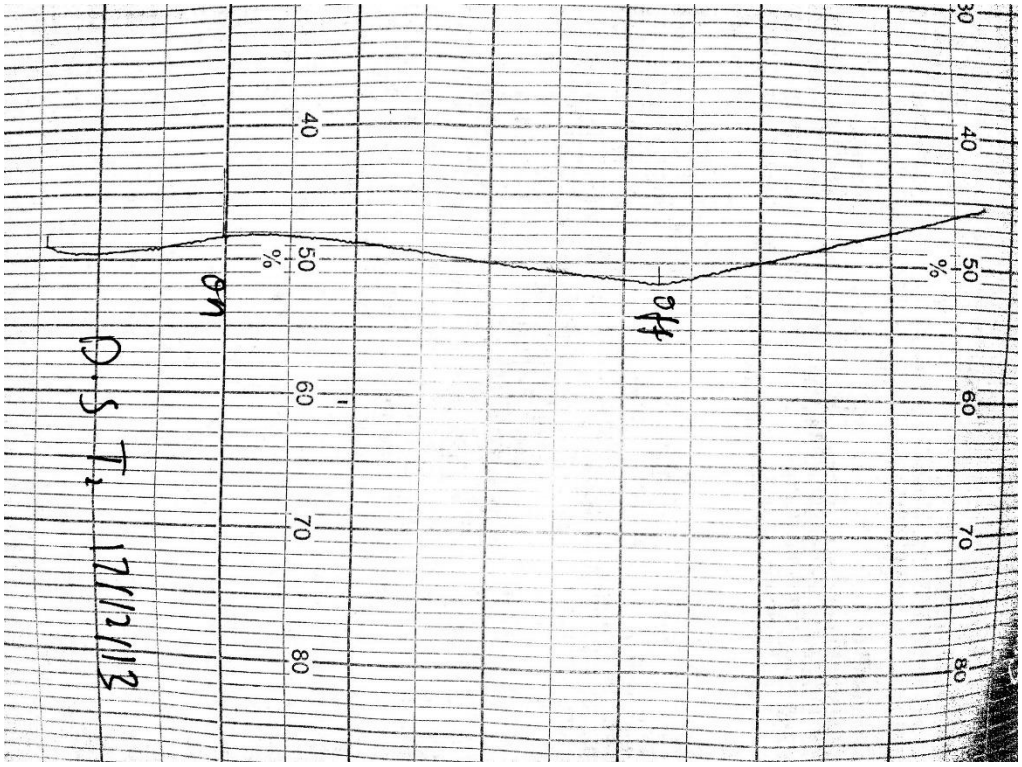


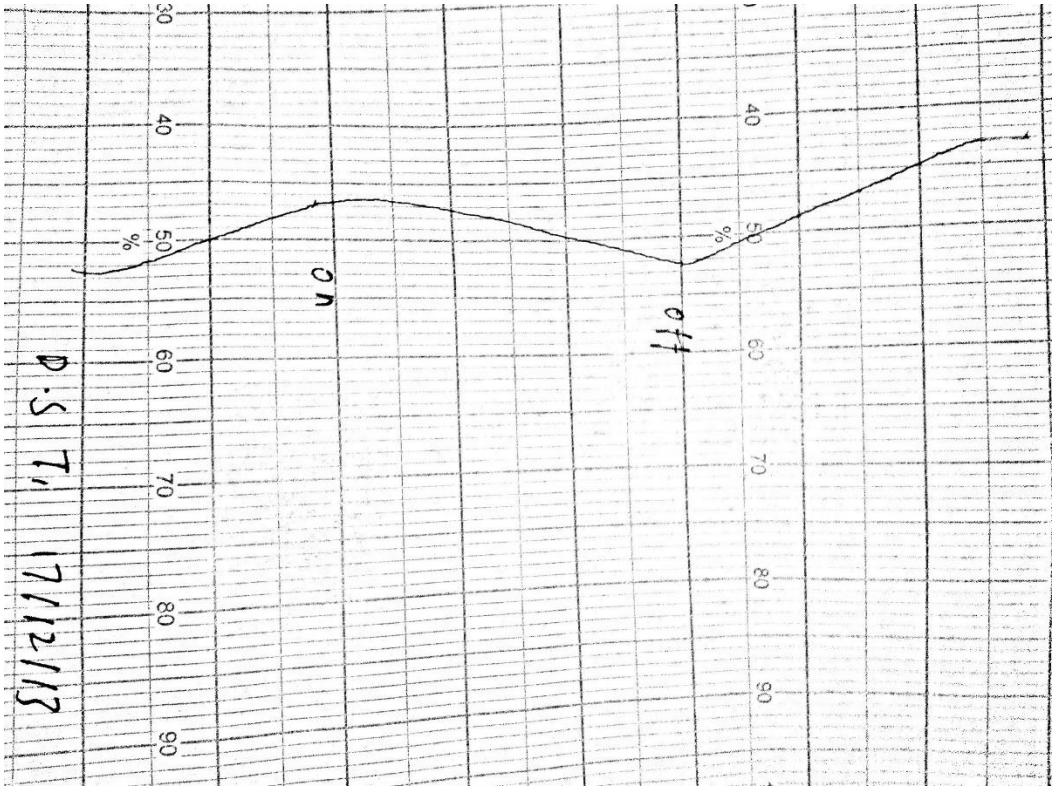
Control+acetaldehyde



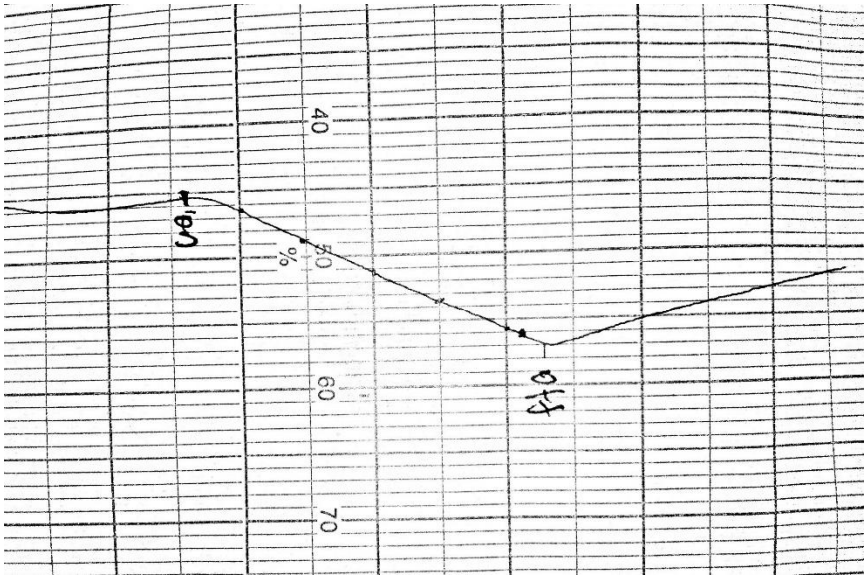


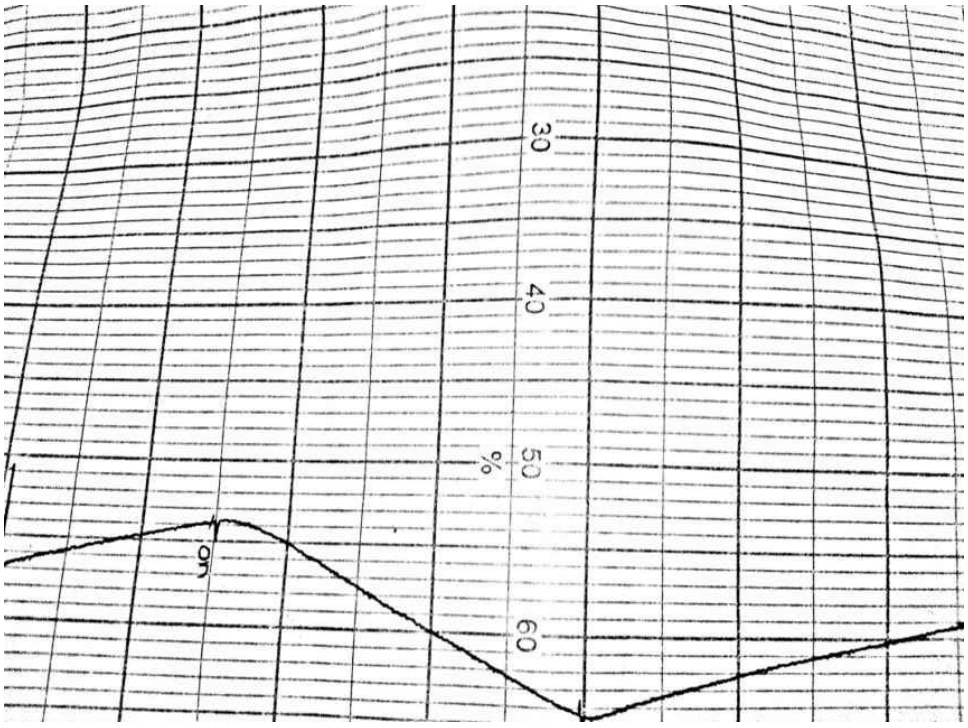
T1 non-accliamted



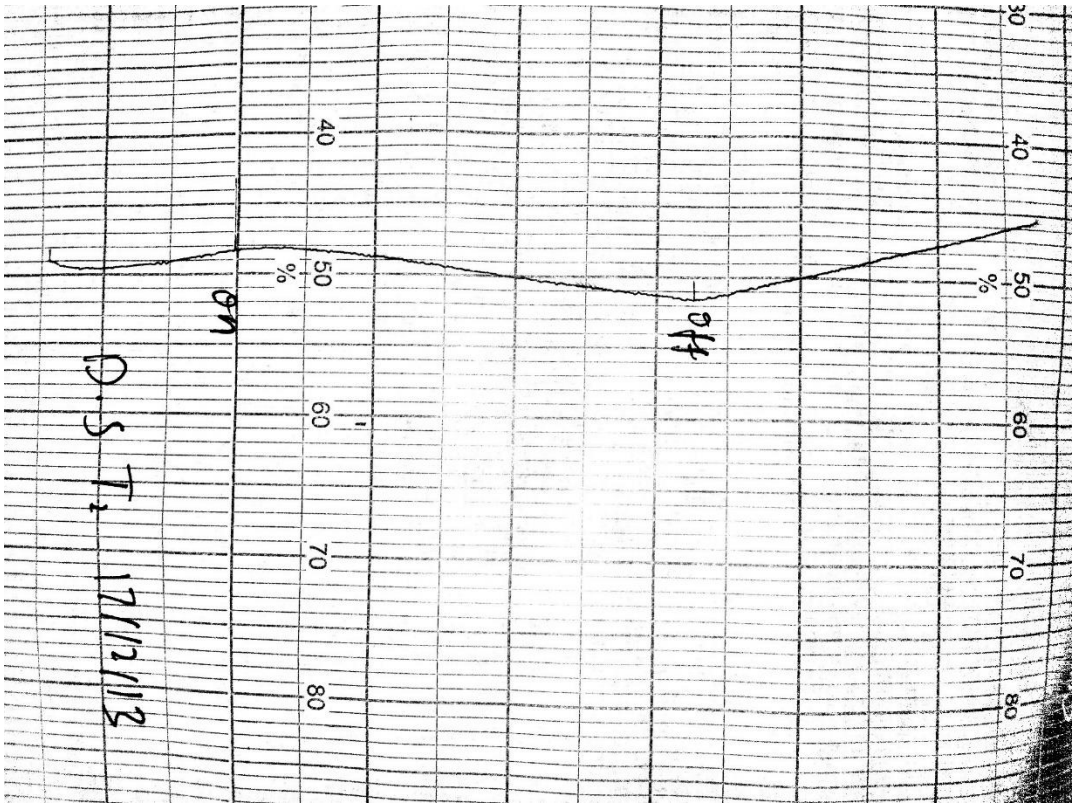


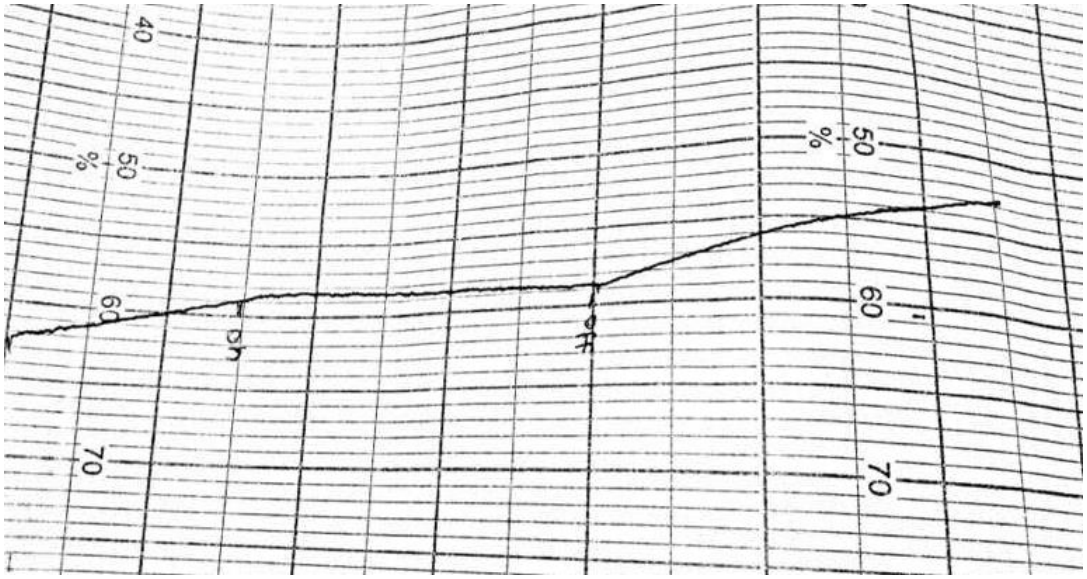
T1 acclimated



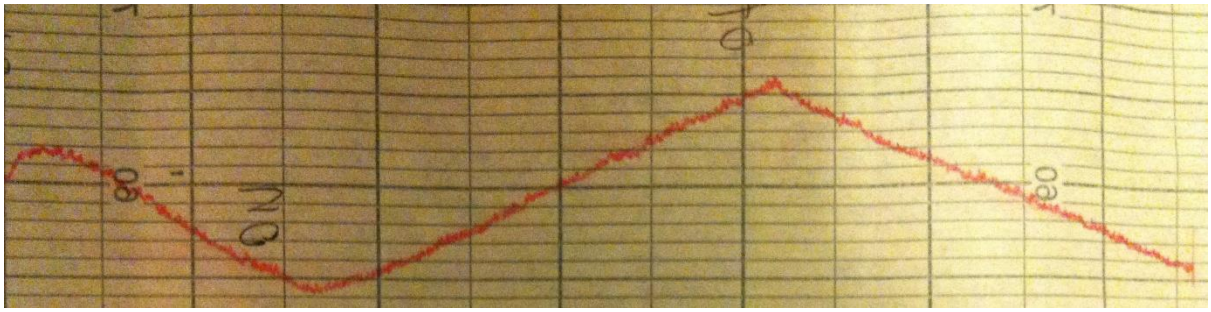


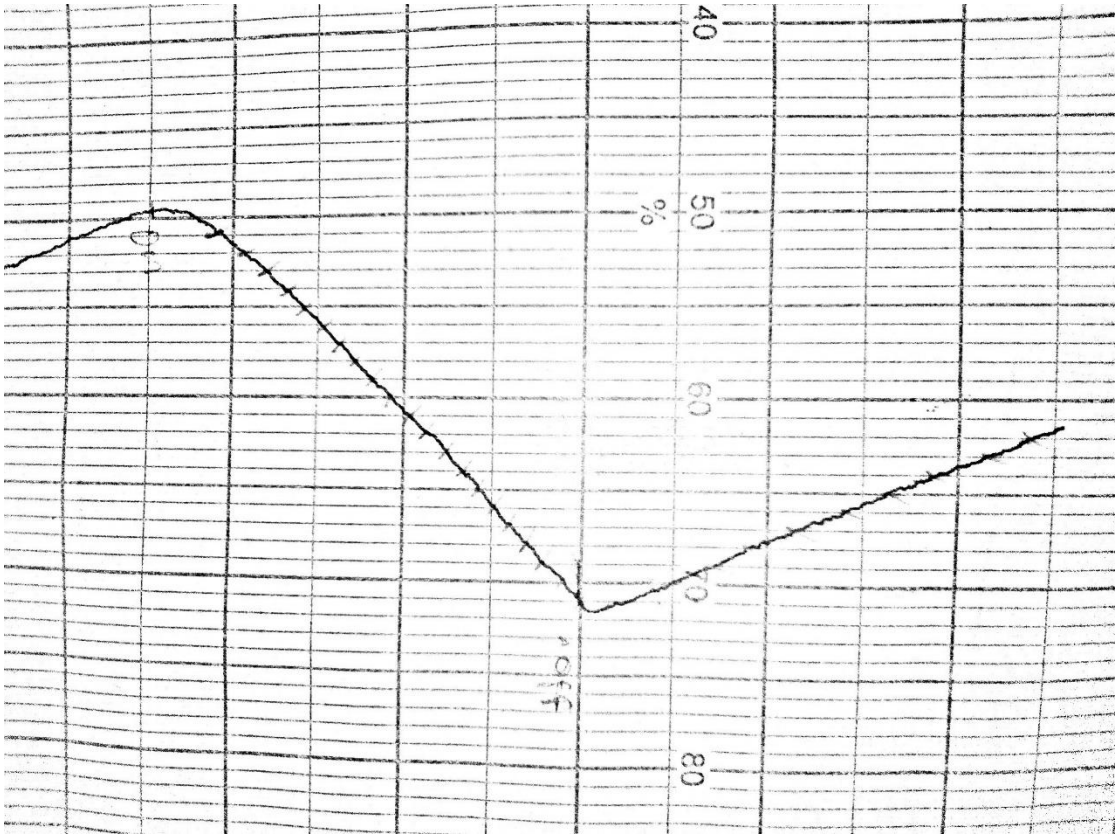
T2 non-acclimated





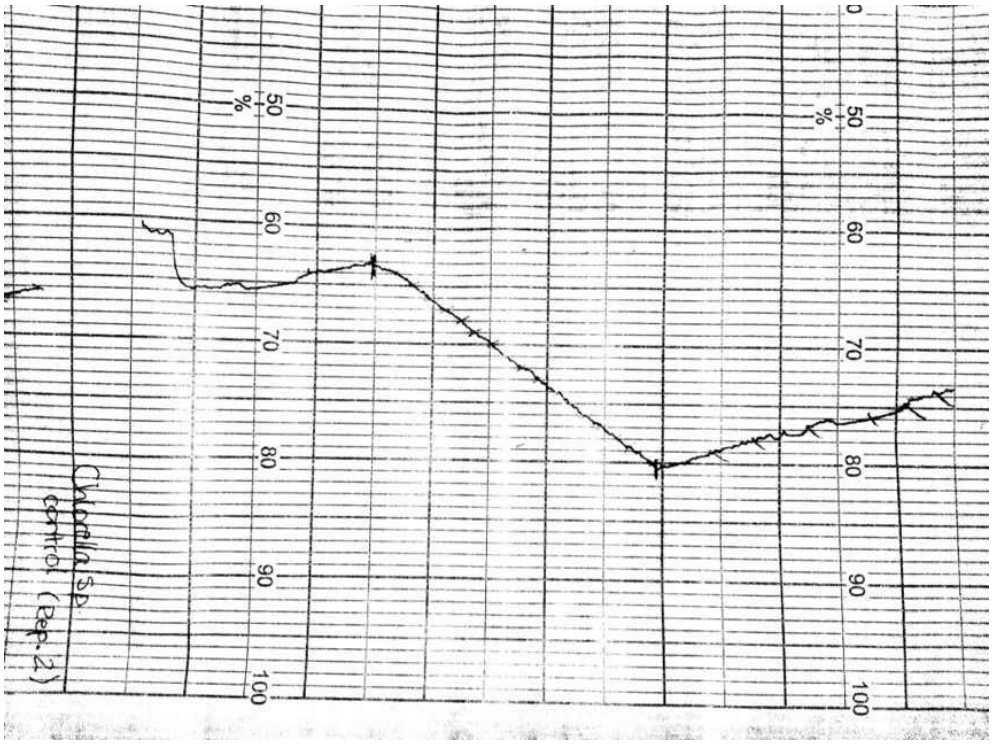
T2 acclimated



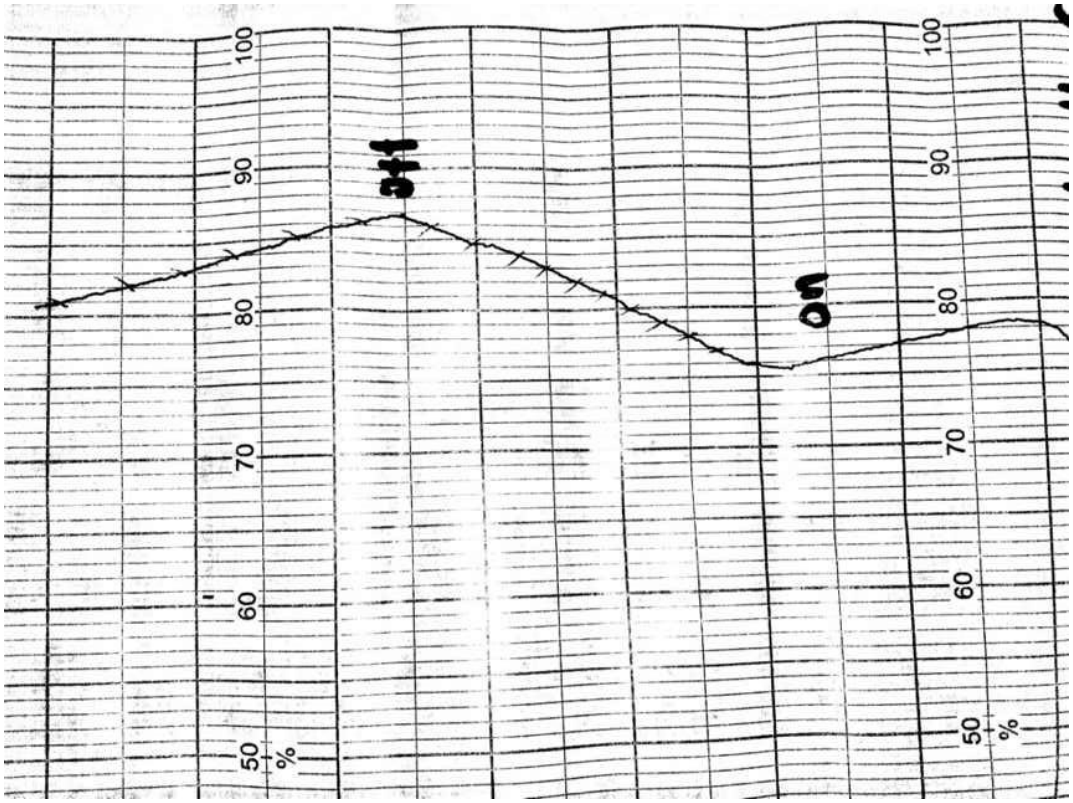


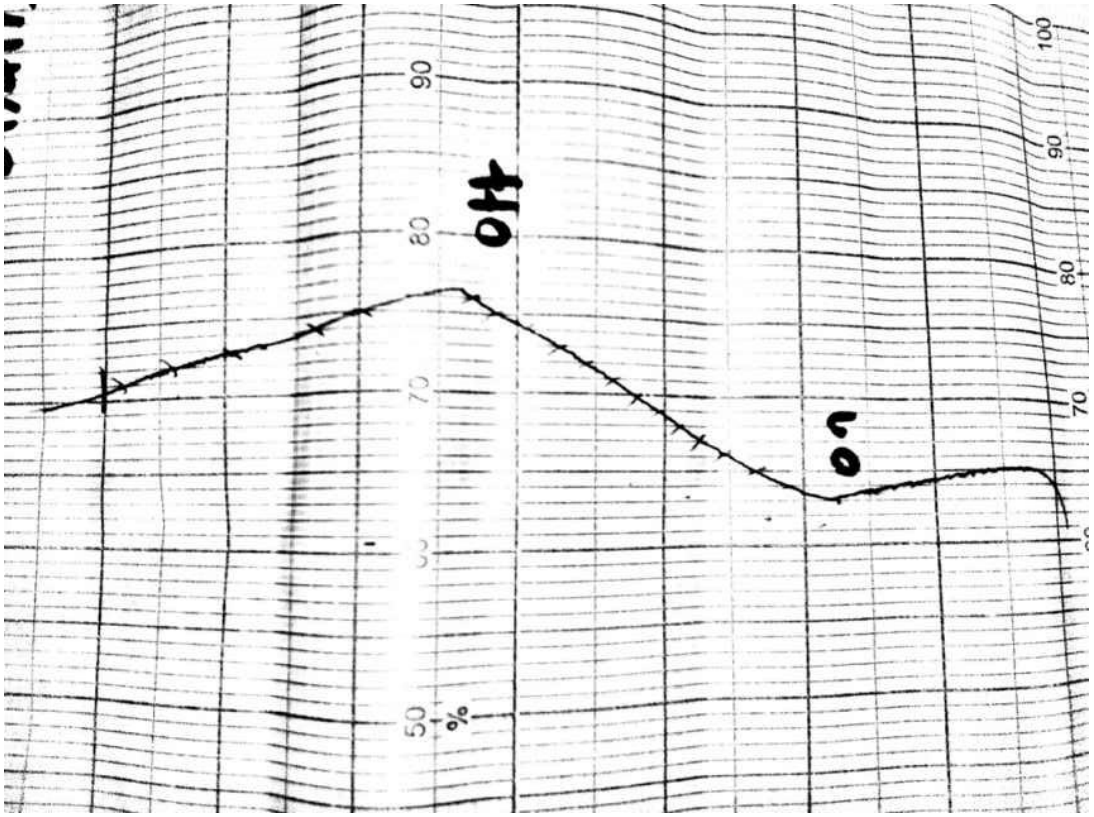
Chlorella

Control

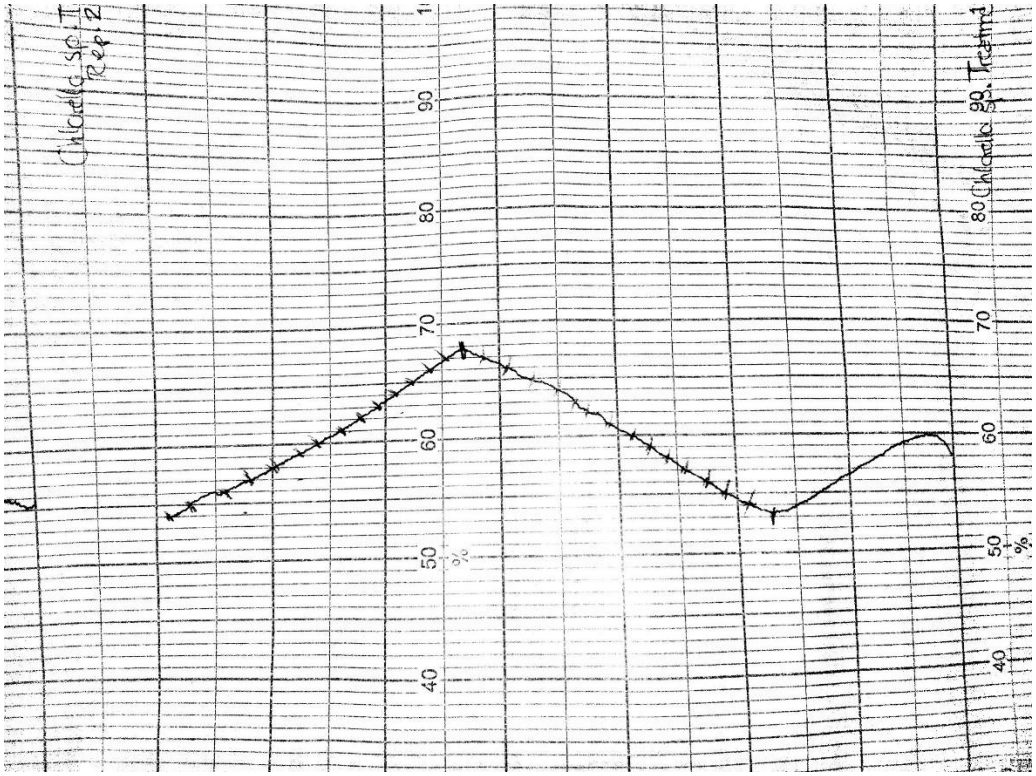


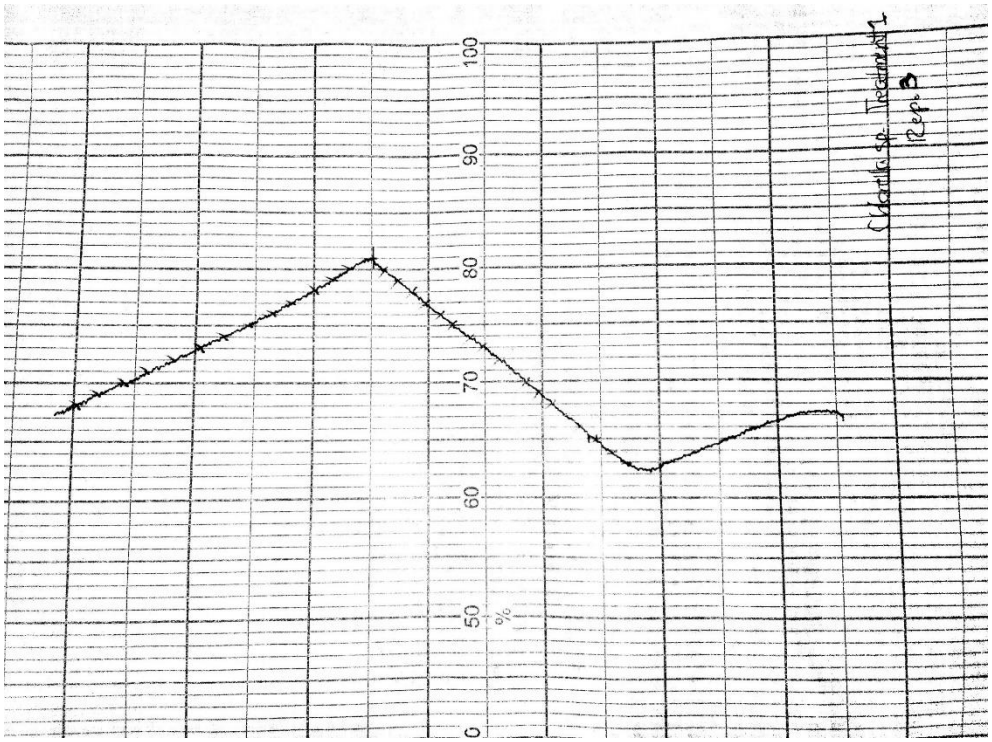
Control +acetaldehyde



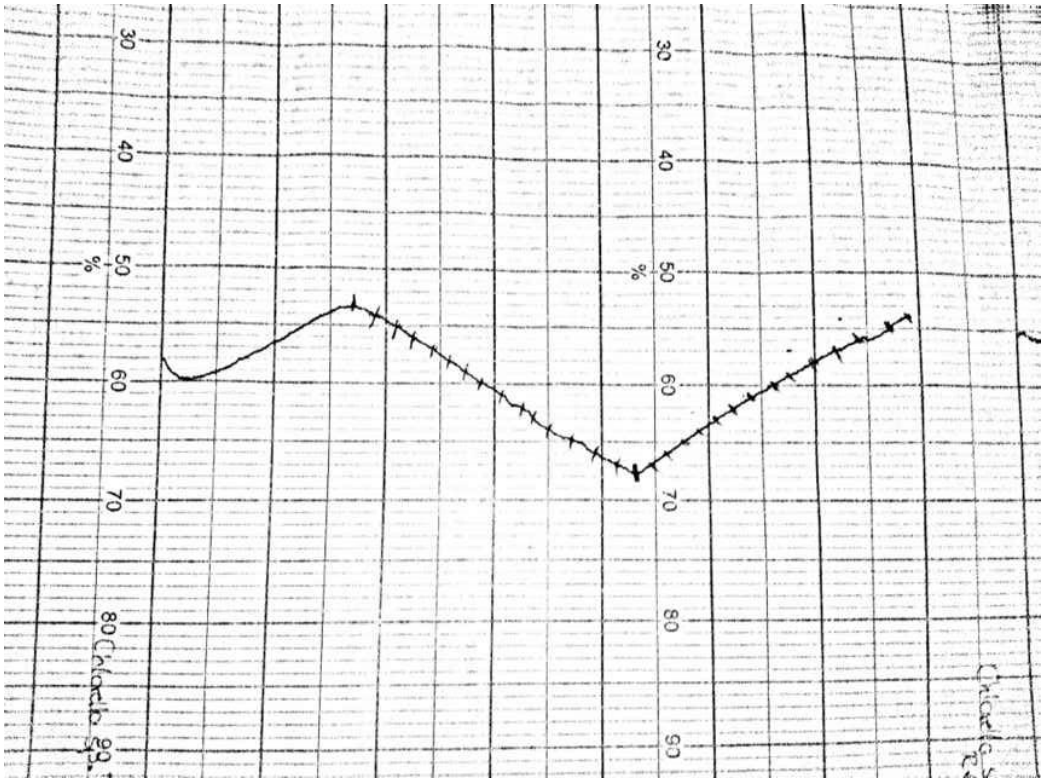


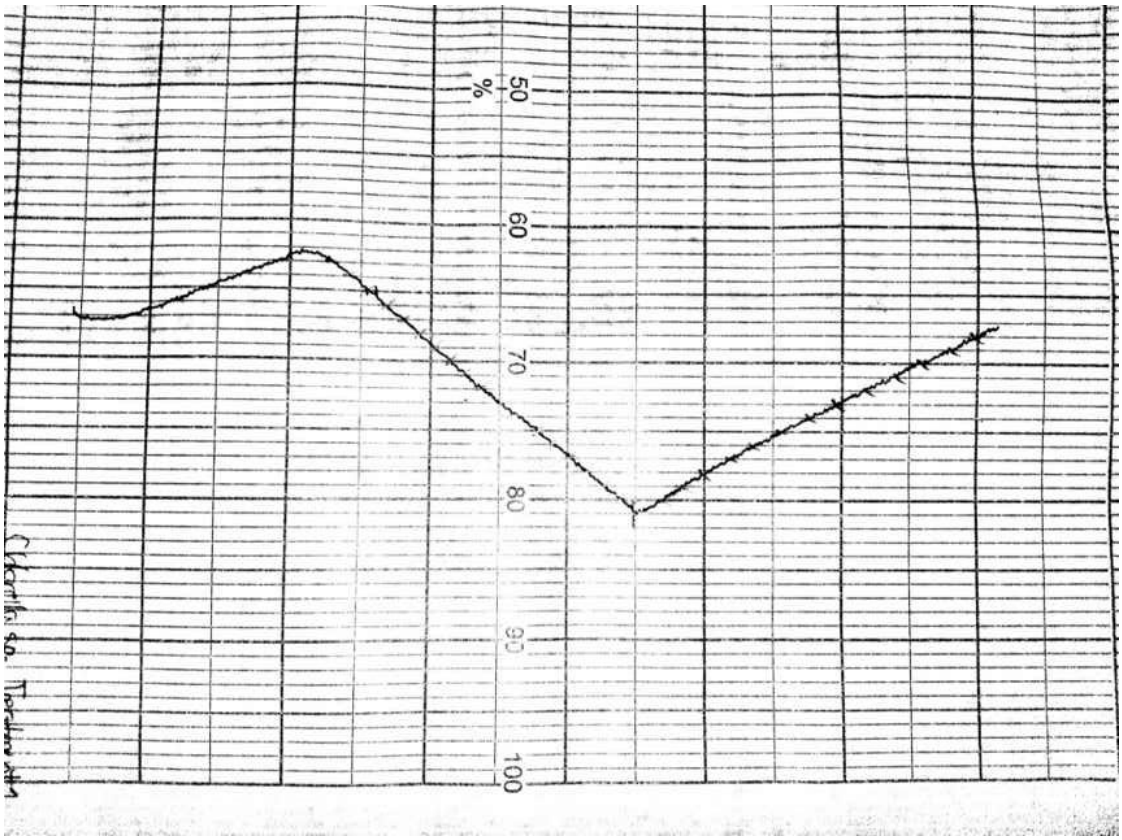
T1 non acclimated



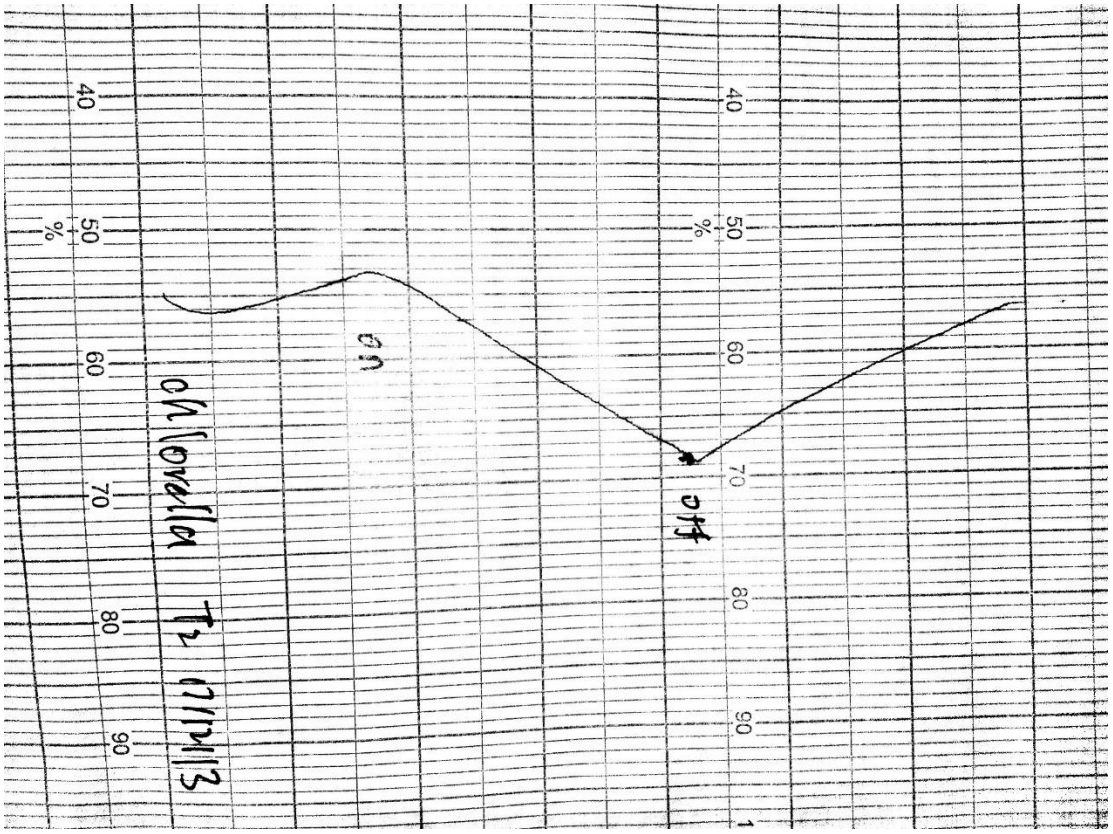


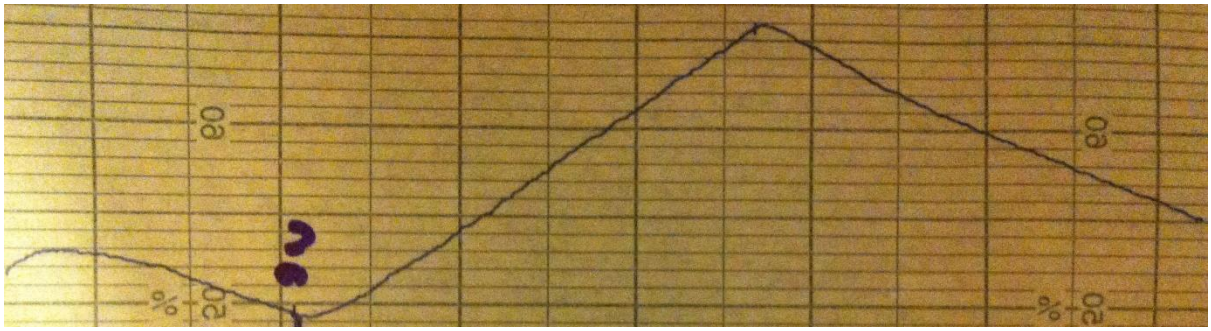
T1 acclimated



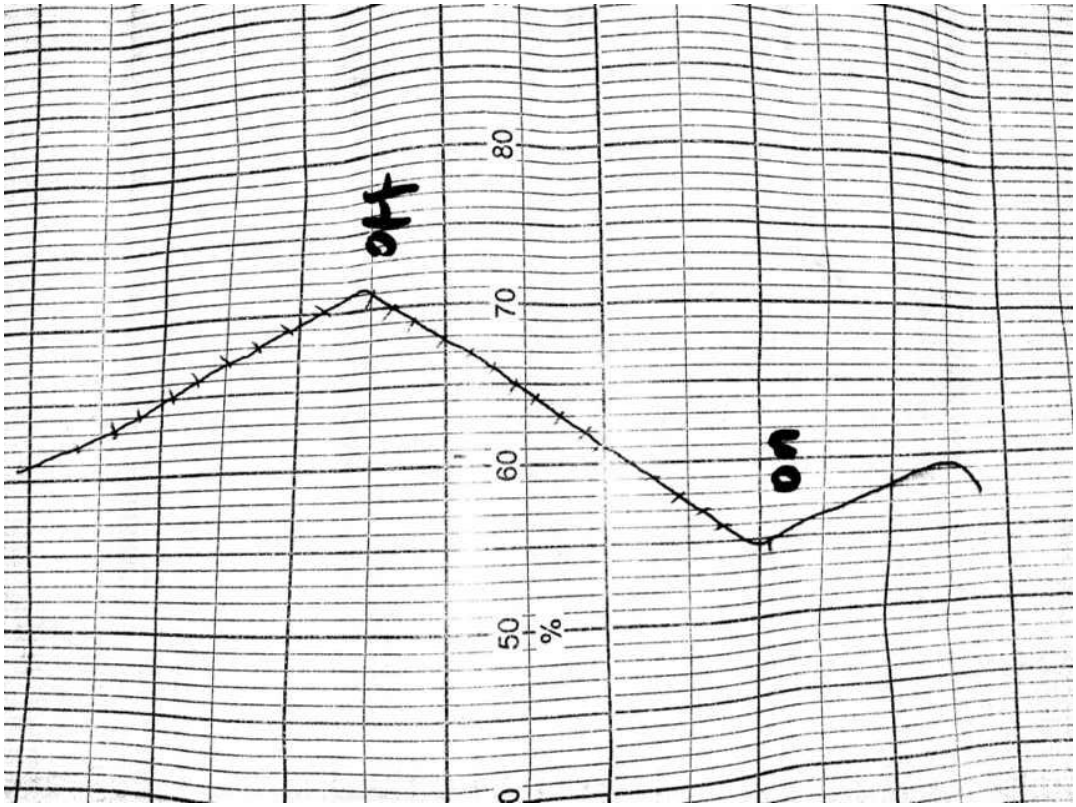


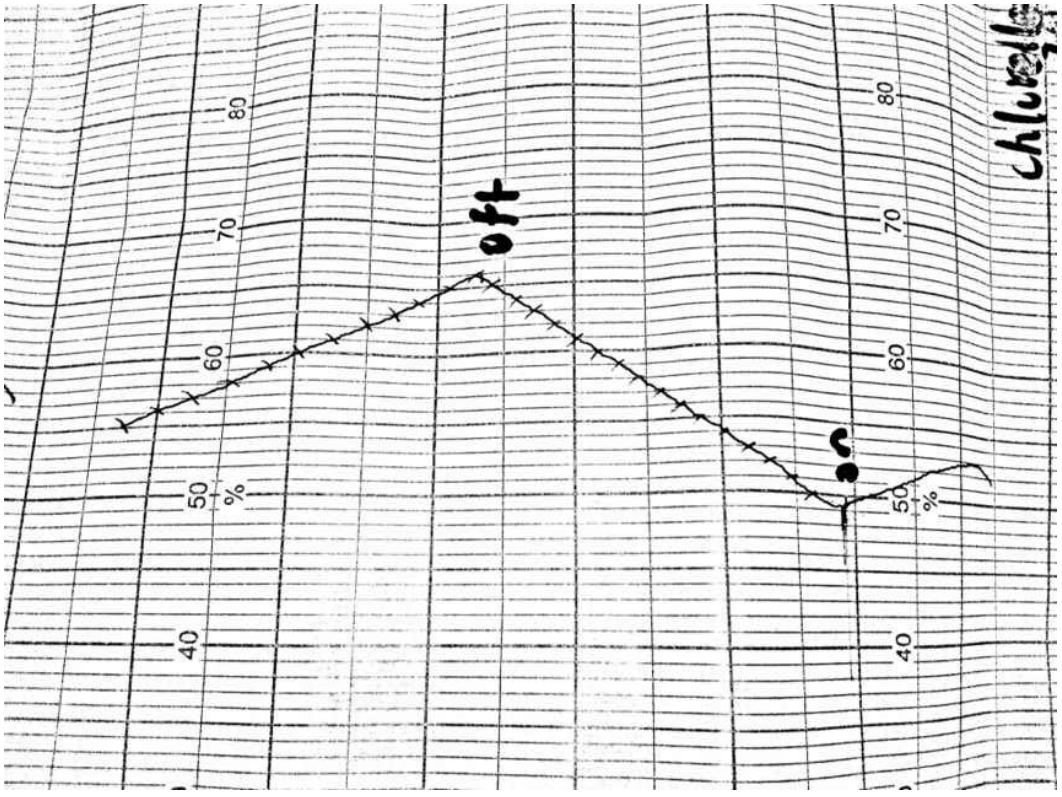
T2 non-acclimated





T2 acclimated

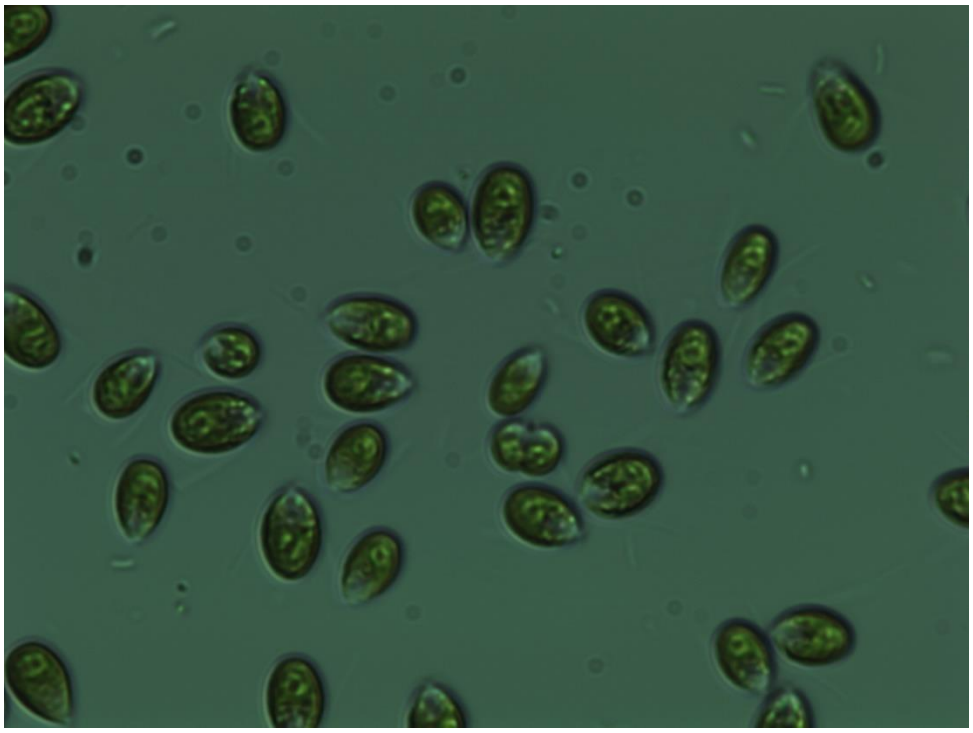


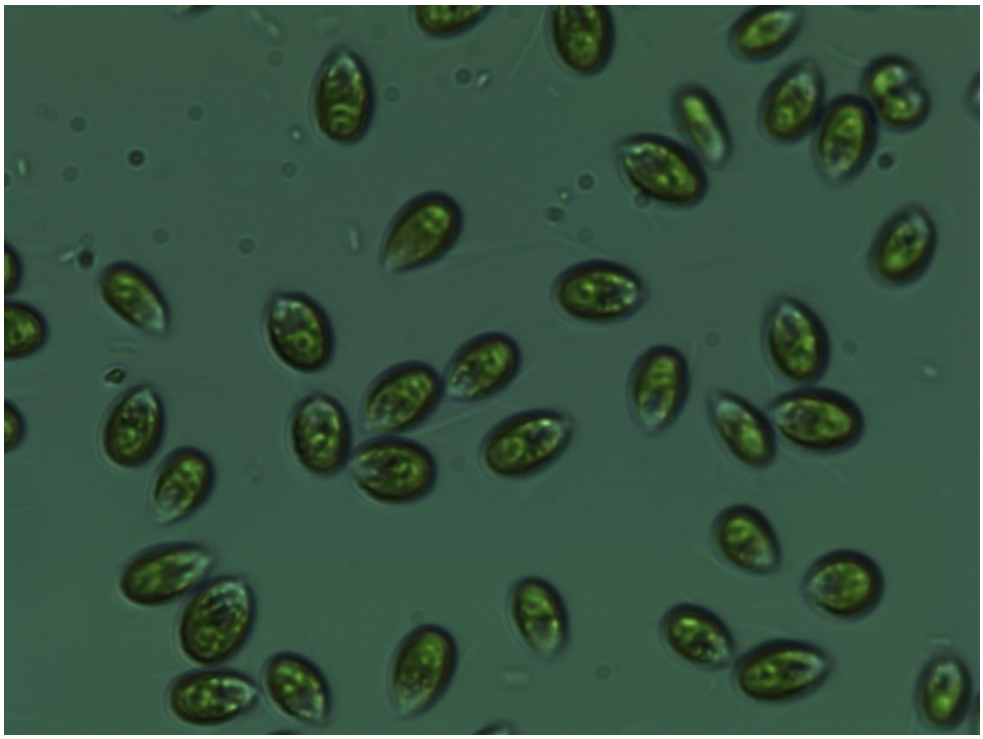


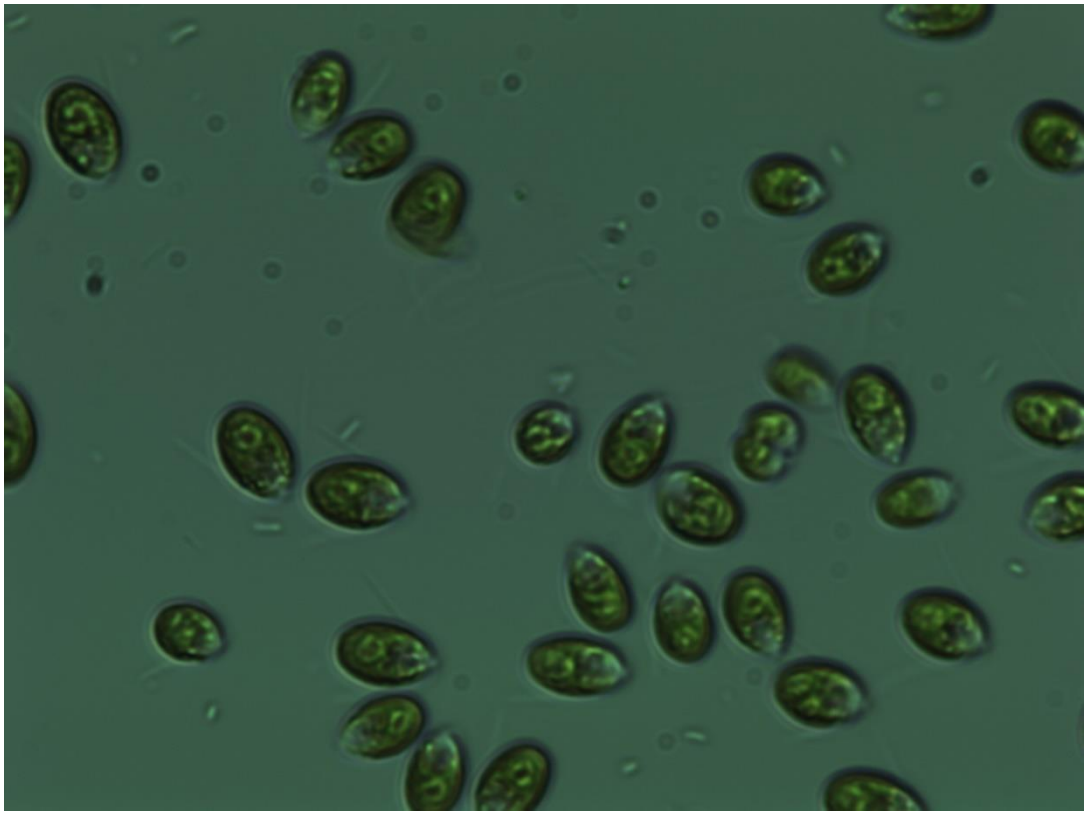
Morphology changes due to acetaldehyde

Dunaliella salina

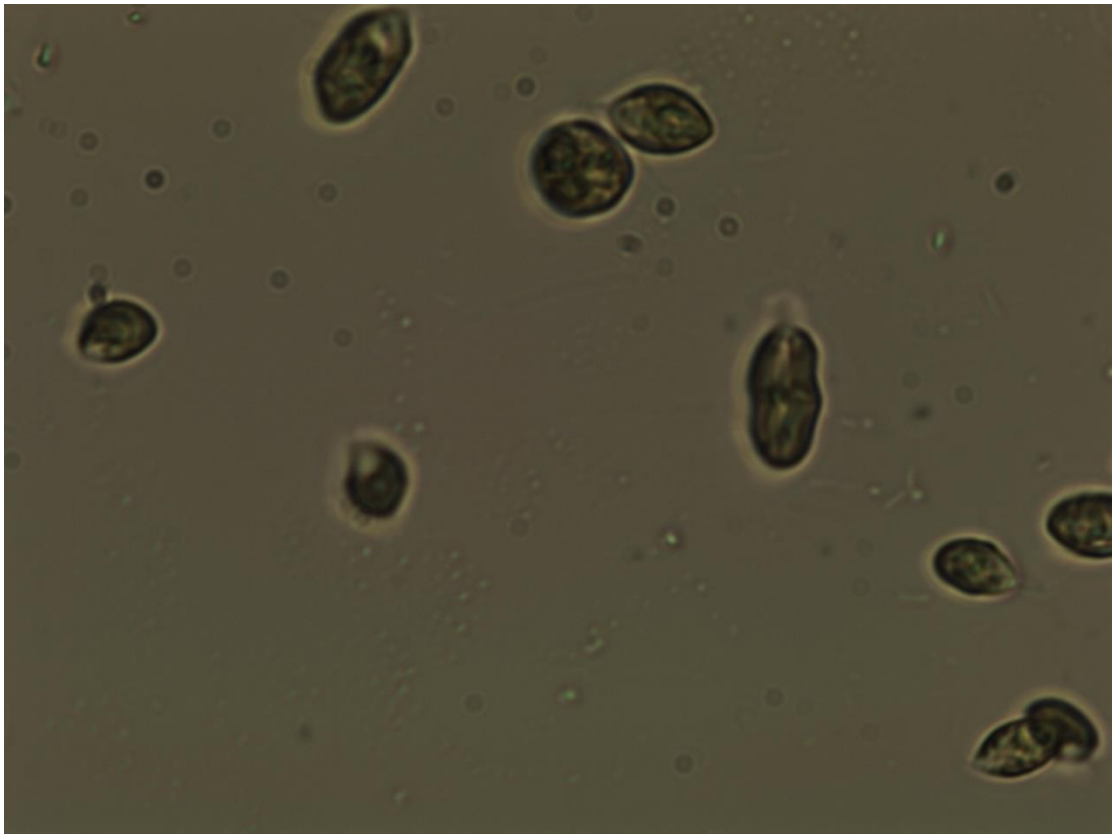
Control

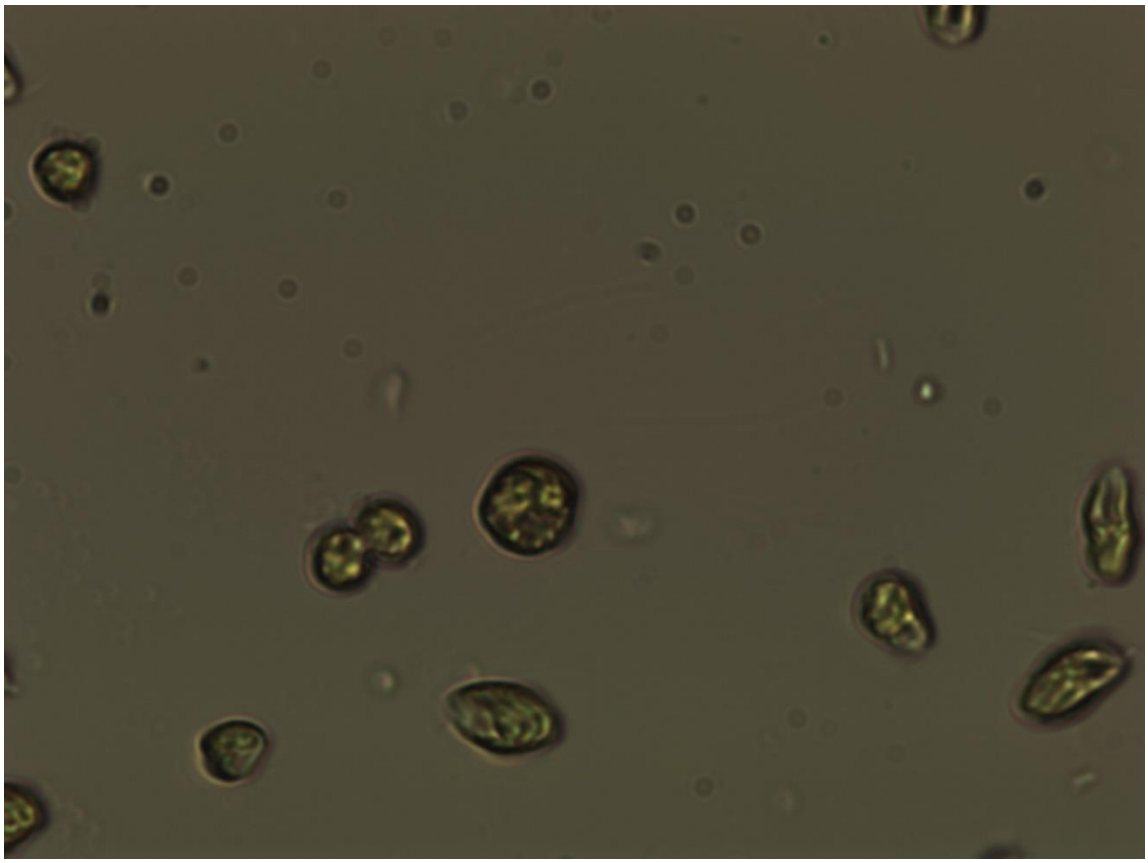


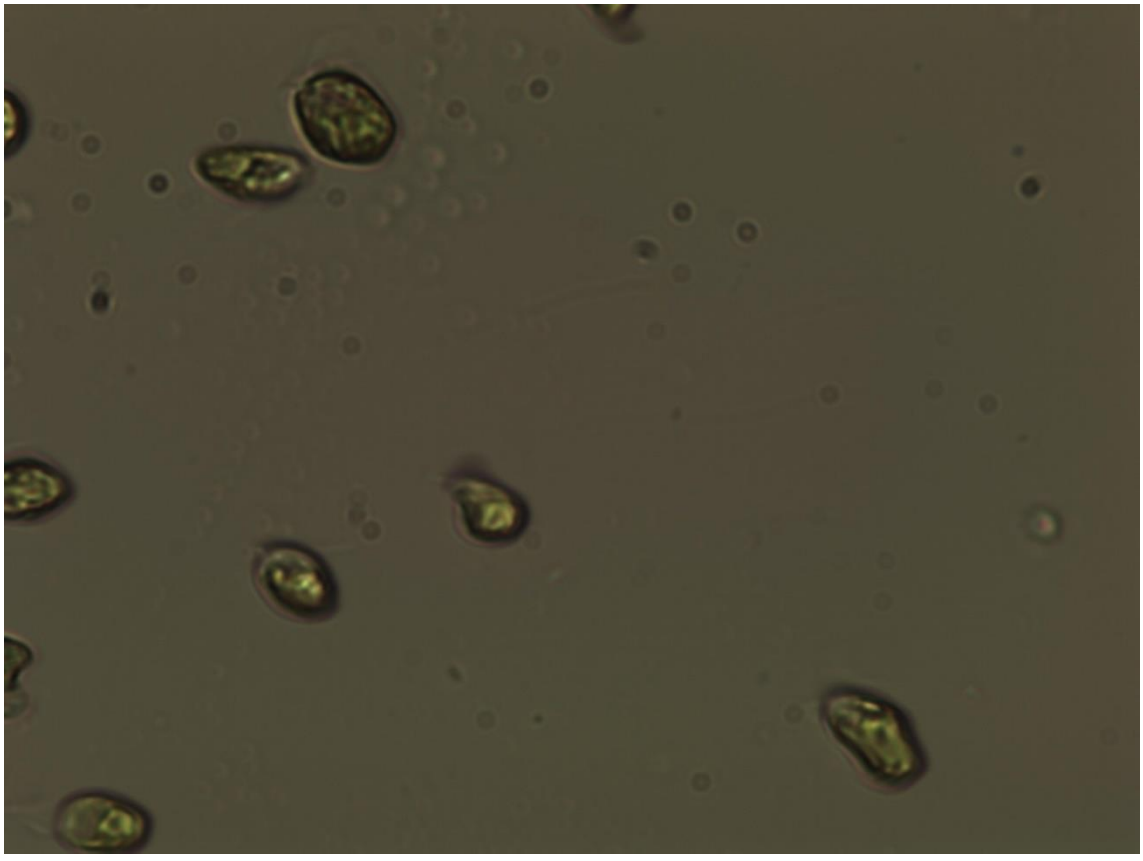




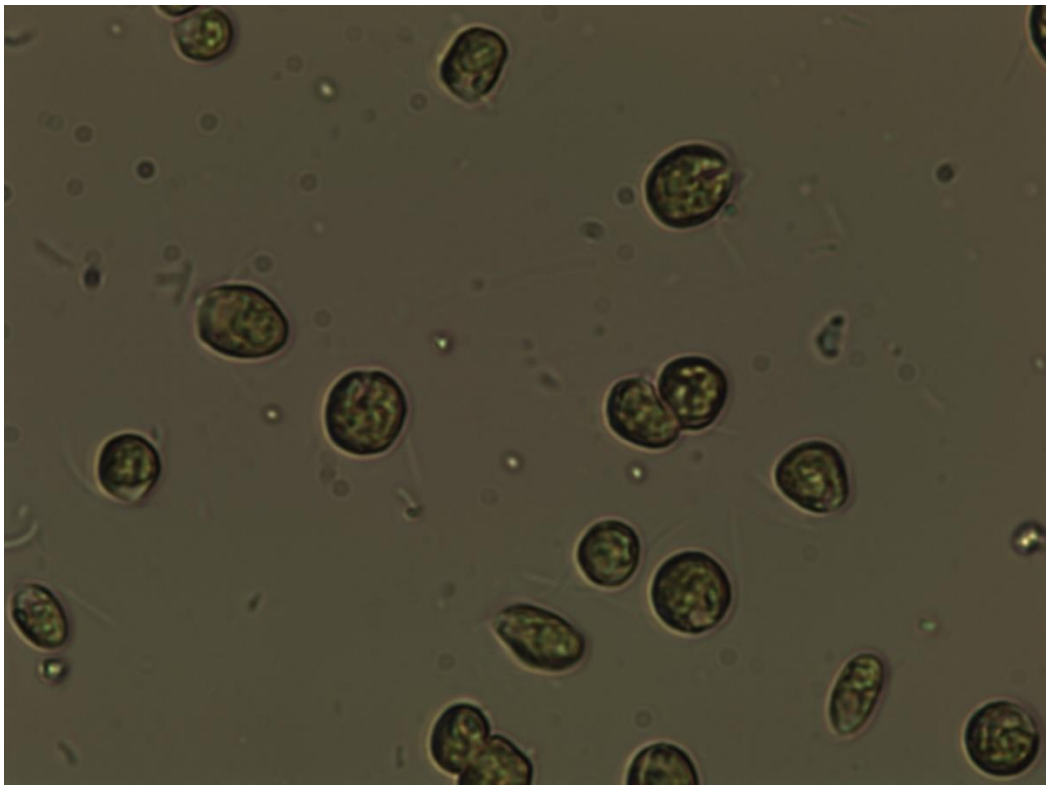
Treatment 1

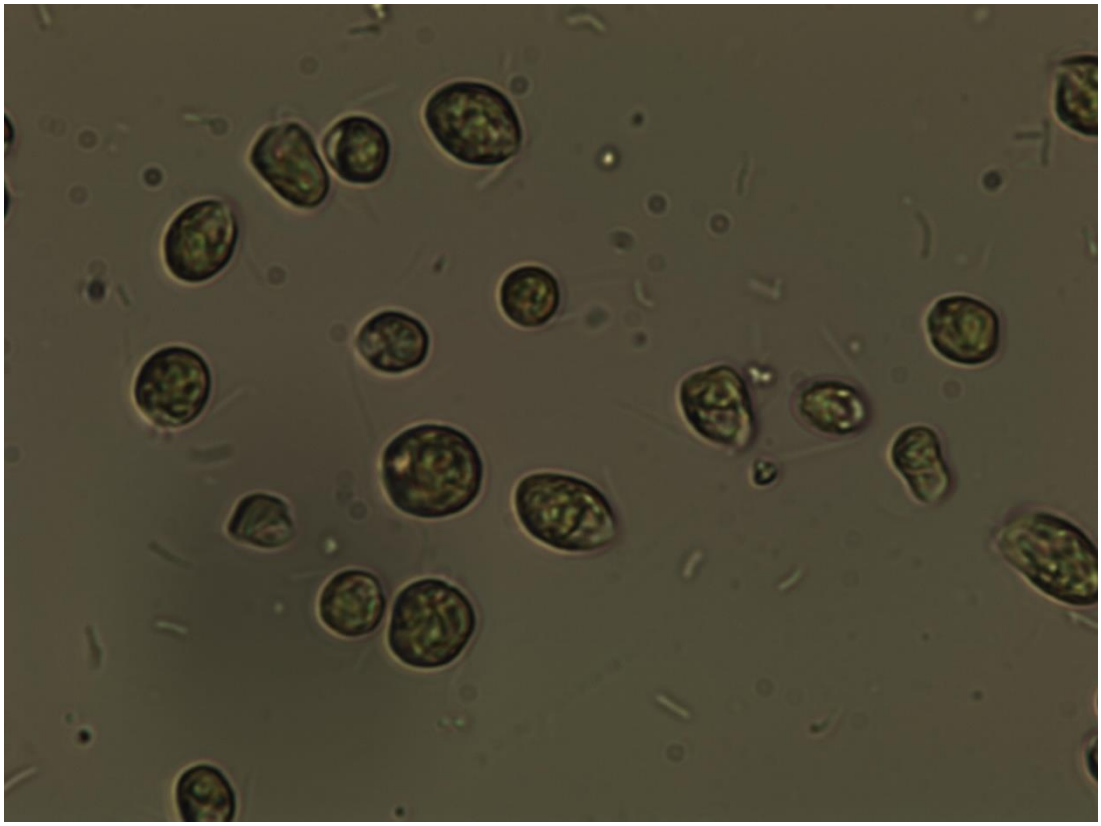


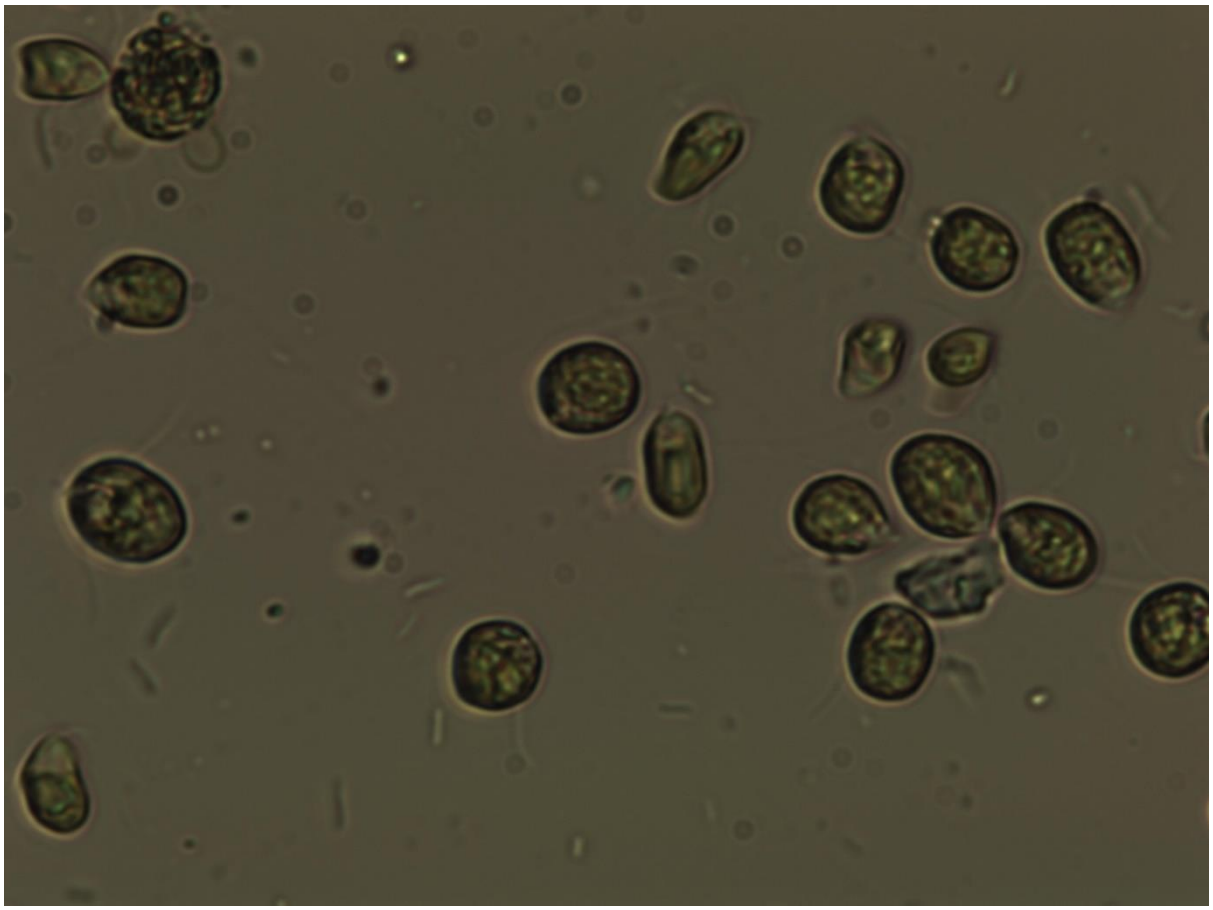




Treatment 2

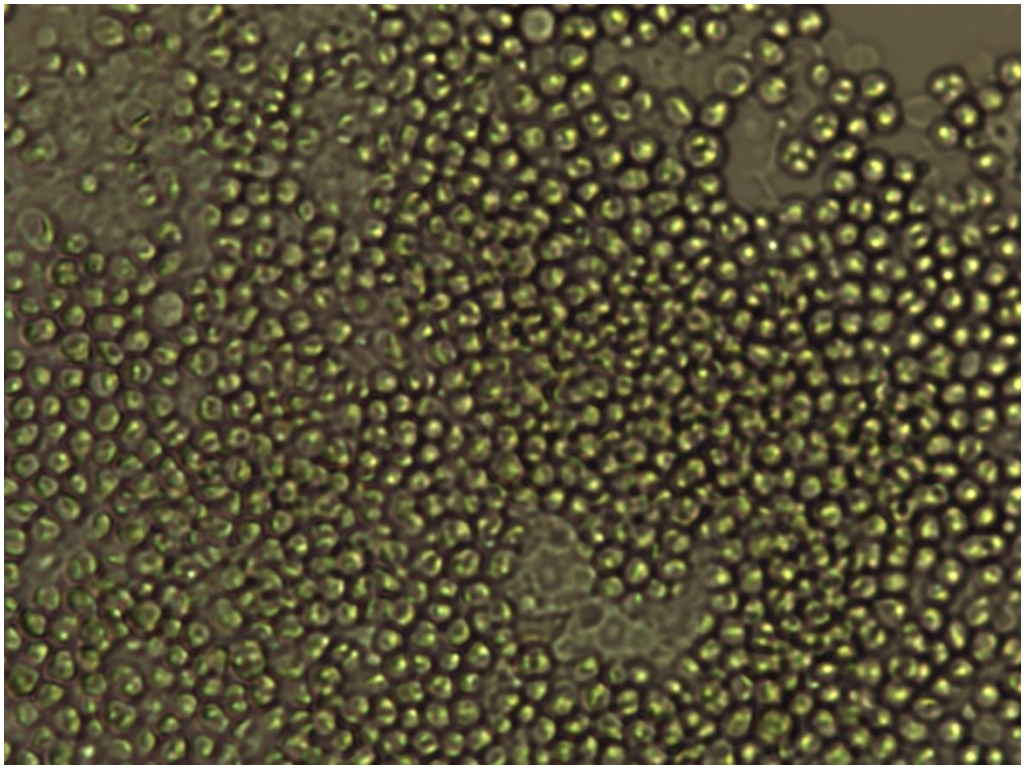


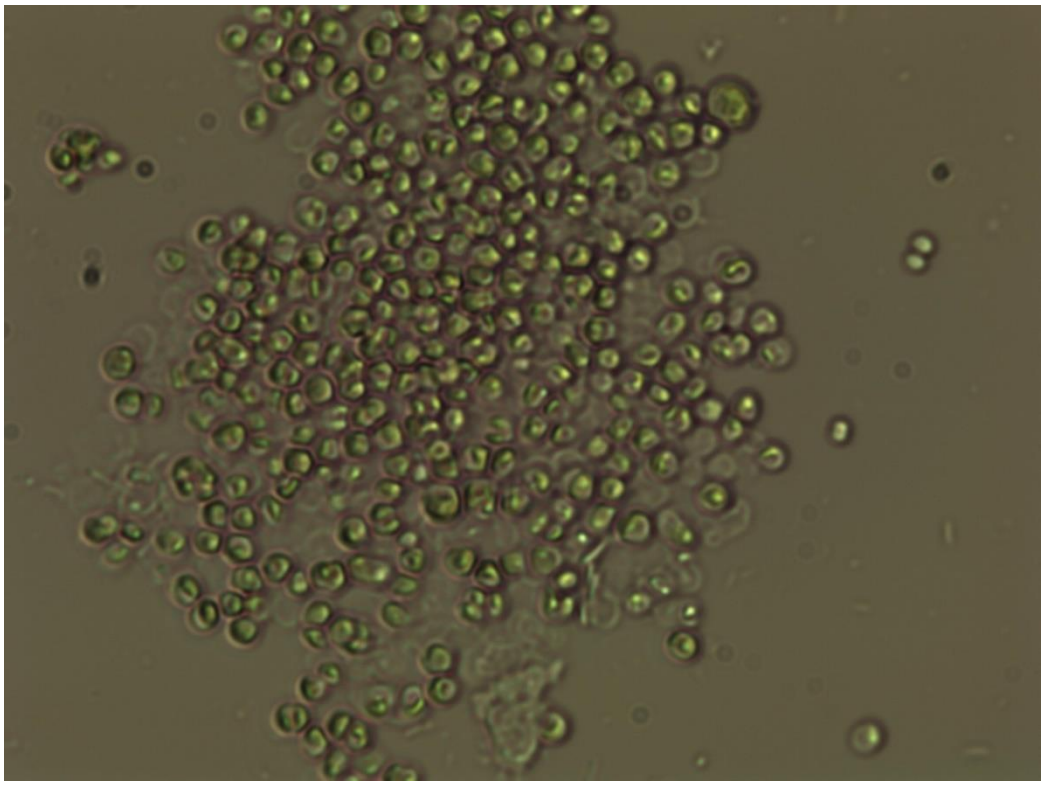


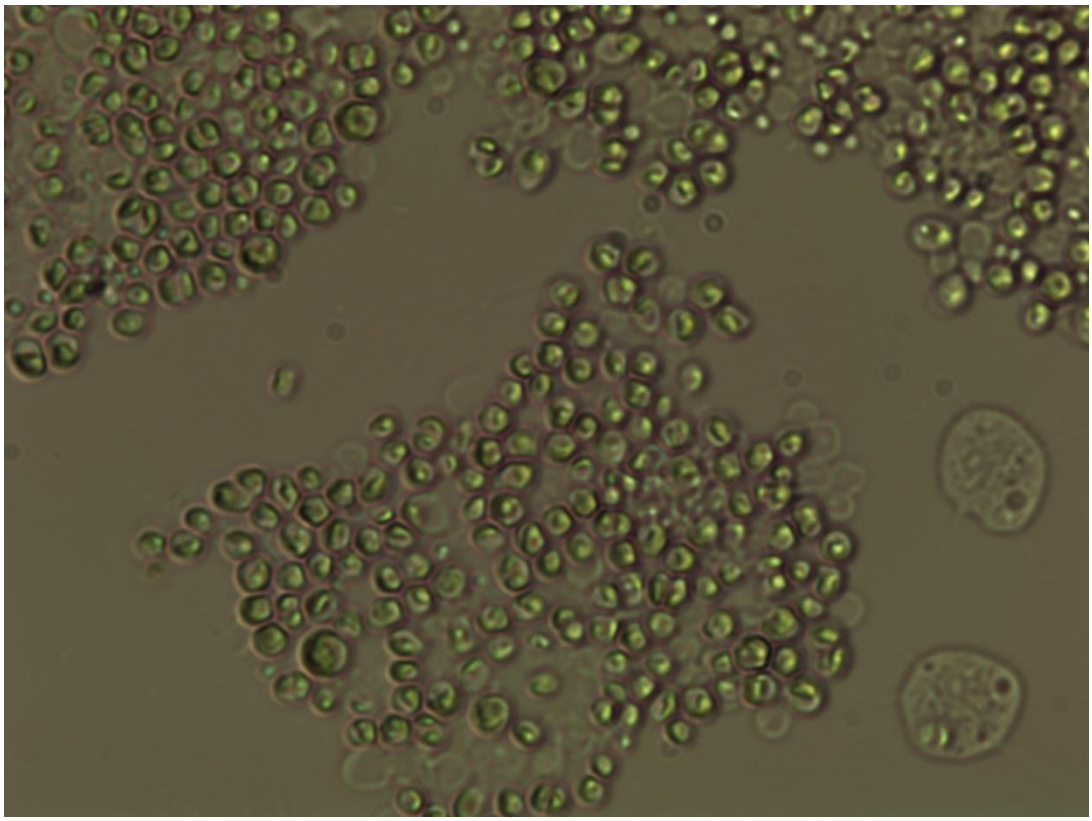


Chlorella

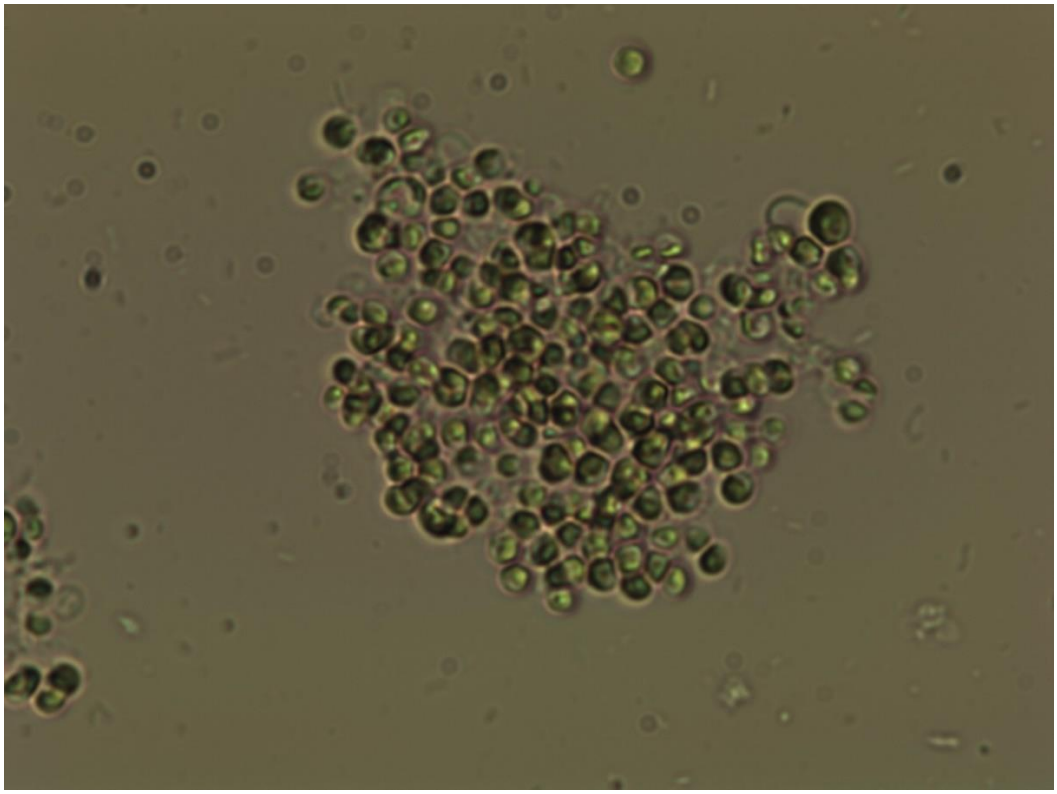
Control

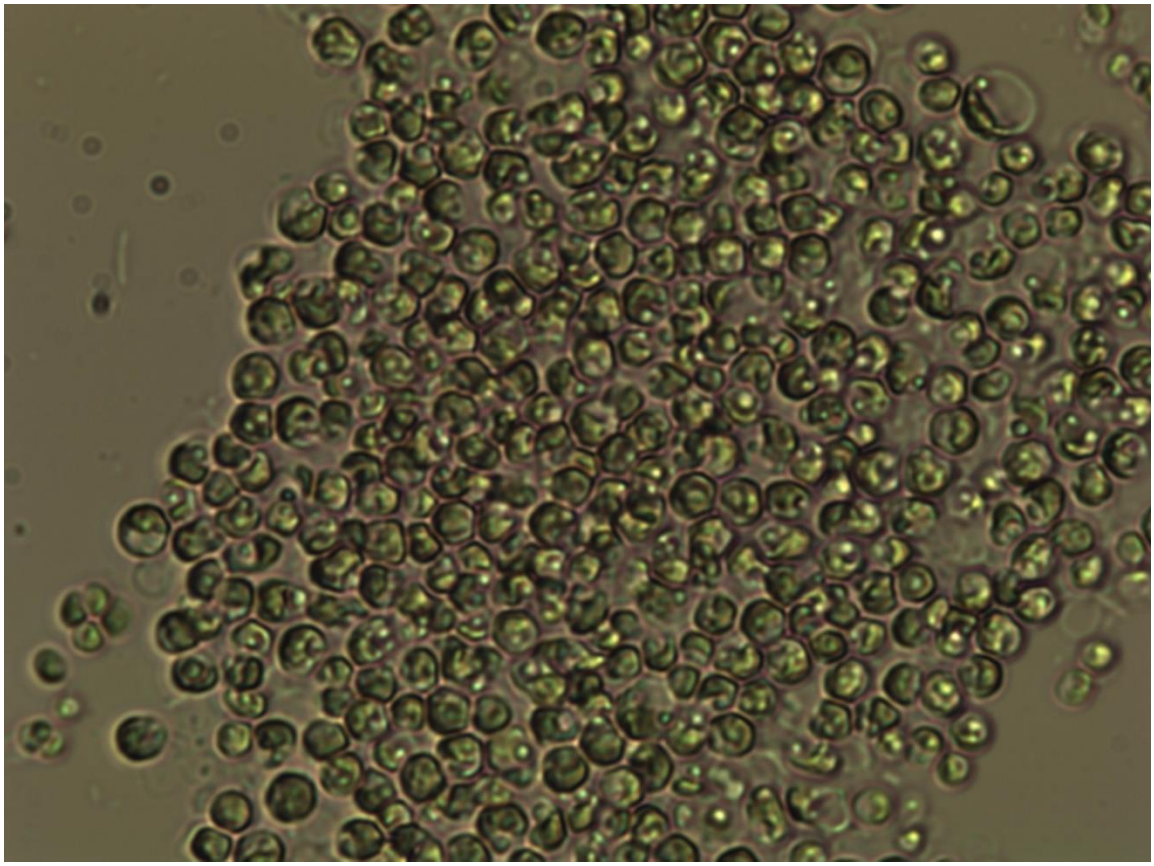


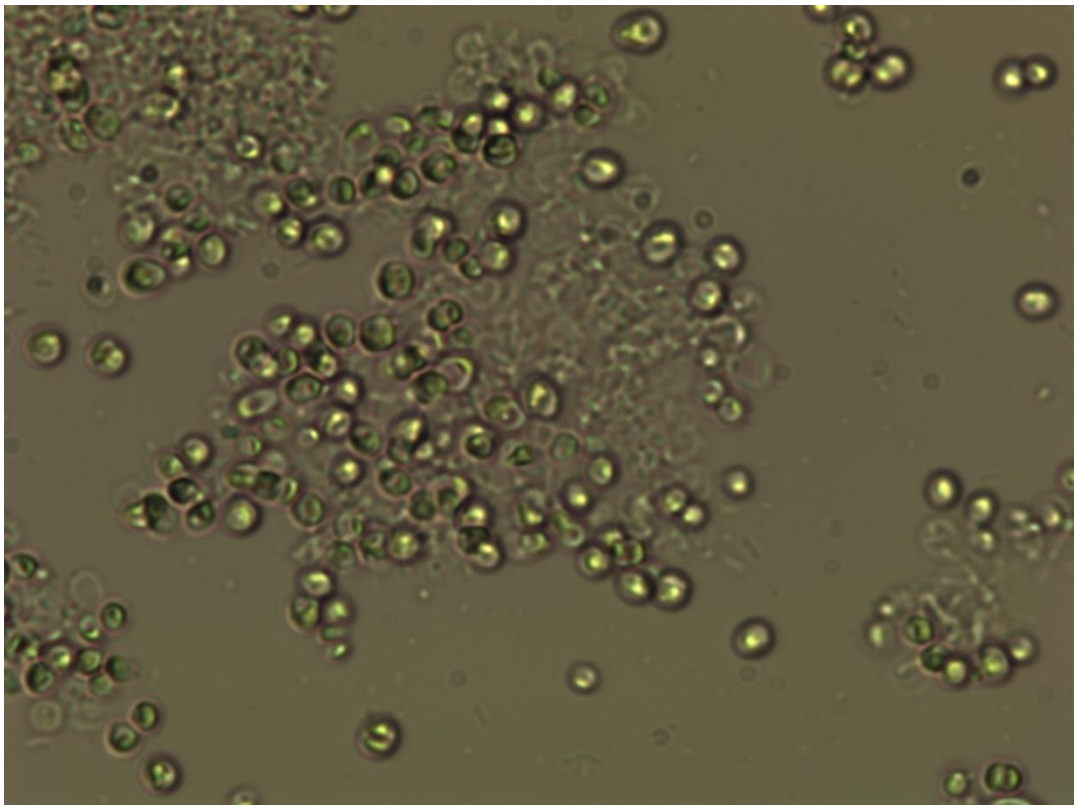




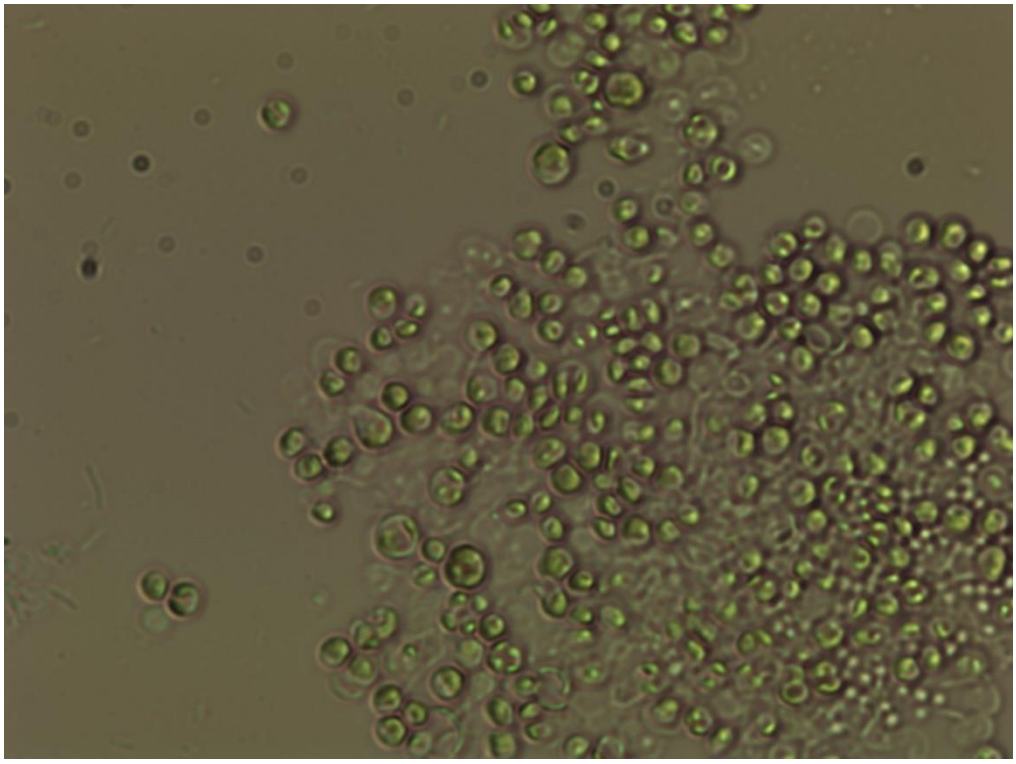
Treatment 1

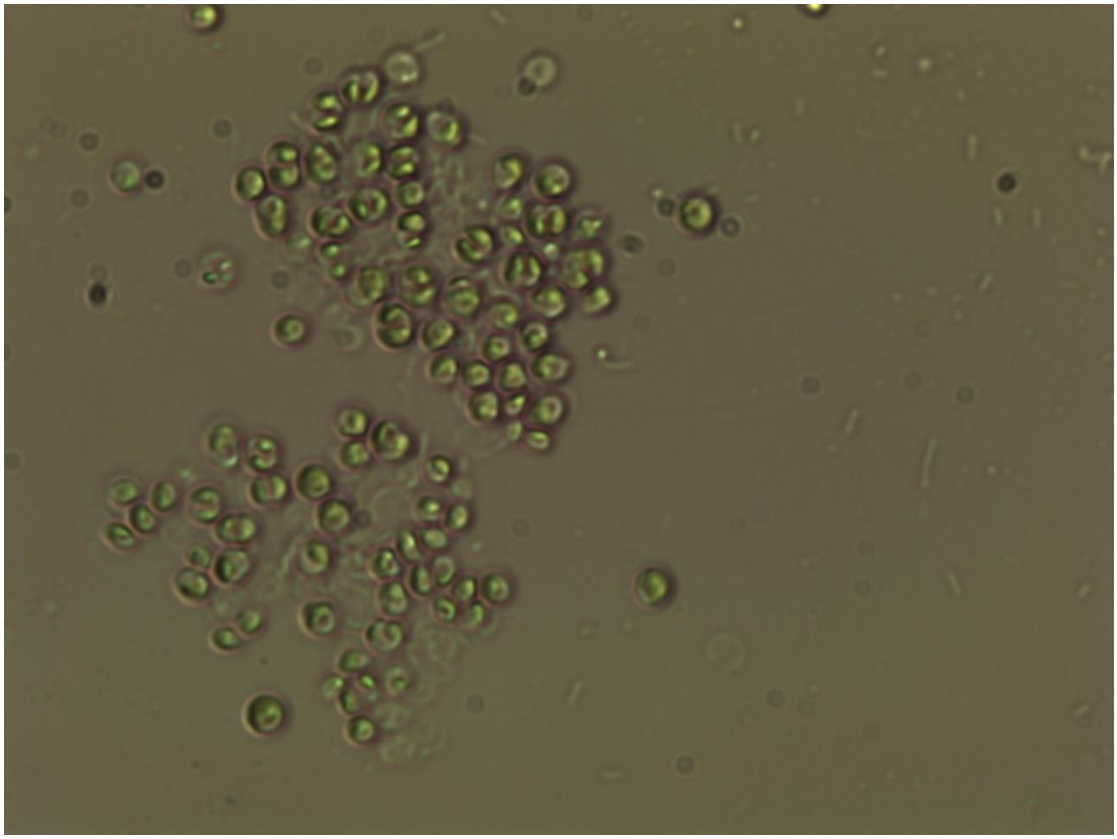


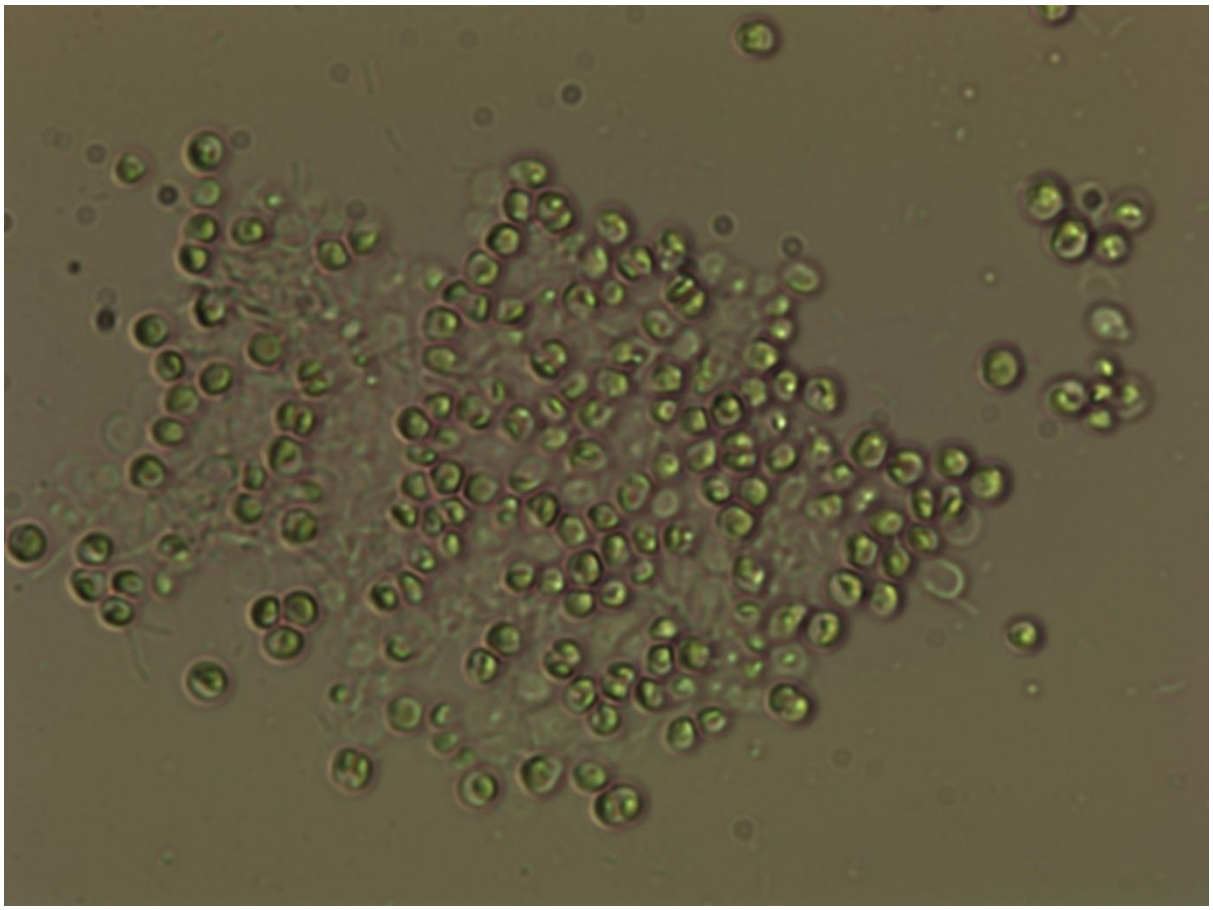




Treatment 2



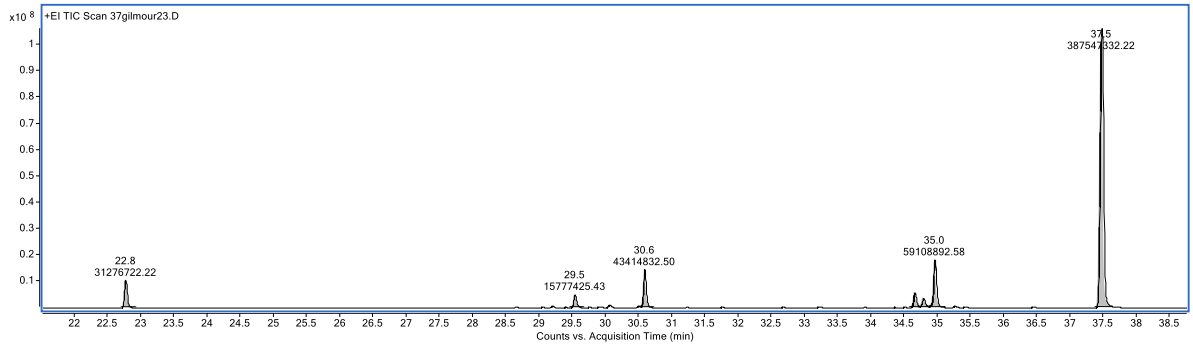




APPENDIX 4

The Chromatograph of Samples of *Dunaliella salina* and *Chlorella sp.*

D.salina Control group 1



1:Tridecanoic acid, Methyl ester (Methyl tridecanoate)

2: methyl 4, 7, 10, 13-hexadecateraenoate

3. Hexadecanoic acid, methyl ester (Palmitic acid, methyl ester)

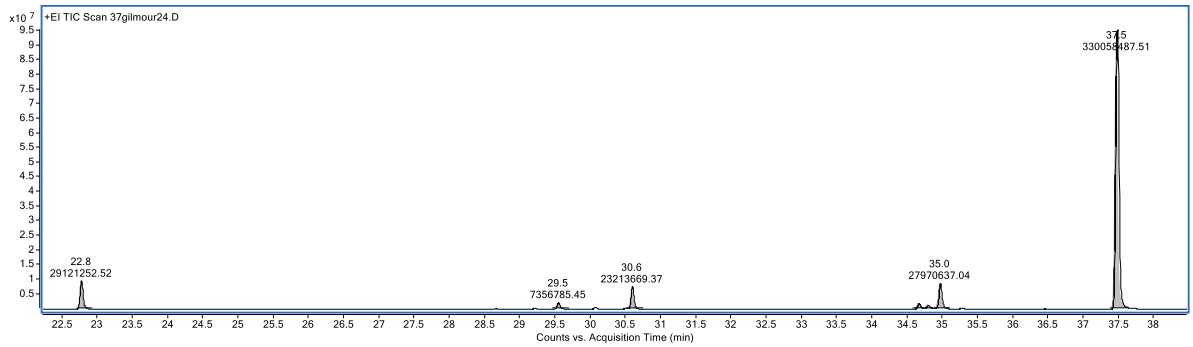
4. Linolenic acid, methyl ester (9,12,15- octadecadienoic acid (z,z)-, methyl ester) 34.7 17949926.81

5 trans-13-octadecenoic acid, methyl ester 34.8 12841189.99

6 Linoleic acid, methyl ester (9,12-octadecadienoic acid (z,z)-, methyl ester) 35.0 59108892.58

7 Methyl nonadecanoate 37.5 3875473322.22

D. salina Control group 2



1:Tridecanoic acid, Methyl ester (Methyl tridecanoate)

2: methyl 4, 7, 10, 13-hexadecateraenoate

3: Hexadecanoic acid, methyl ester (Palmitic acid, methyl ester)

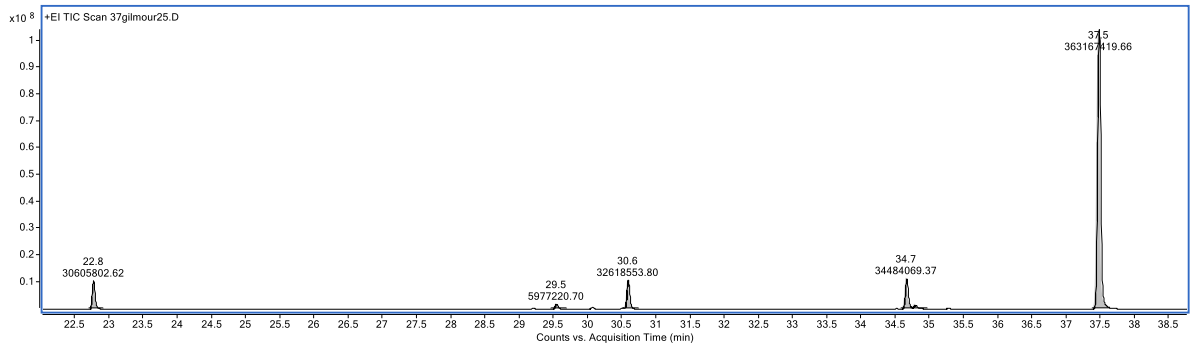
4. Linolenic acid, methyl ester (9,12,15- octadecadienoic acid (z,z)-, methyl ester) 34.7 5899032.35

5 trans-13-octadecenoic acid, methyl ester 34.8 5117102.32

6 Linoleic acid, methyl ester (9,12-octadecadienoic acid (z,z)-, methyl ester) 35.0 27970687.04

7 Methyl nonadecanoate 37.5 330058487.51

D.salina acclimated treatment 1



1:Tridecanoic acid, Methyl ester (Methyl tridecanoate)

2: methyl 4, 7, 10, 13-hexadecateraenoate

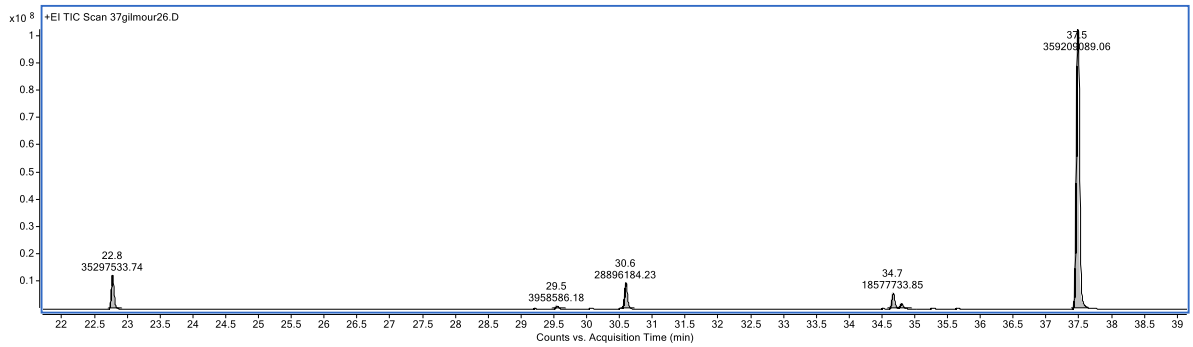
3: Hexadecanoic acid, methyl ester (Palmitic acid, methyl ester)

4. Linolenic acid, methyl ester (9,12,15- octadecadienoic acid (z,z)-, methyl ester) 34.7 34484069.37

5 trans-13-octadecenoic acid, methyl ester 34.8 6529057.44

7 Methyl nonadecanoate

D.salina acclimated Treatment 2



1:Tridecanoic acid, Methyl ester (Methyl tridecanoate)

2: methyl 4, 7, 10, 13-hexadecateraenoate

3: Hexadecanoic acid, methyl ester (Palmitic acid, methyl ester)

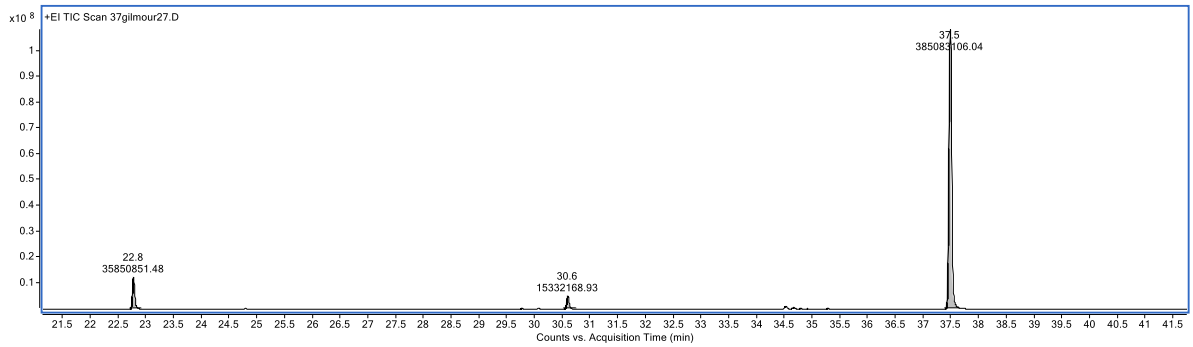
4. Linolenic acid, methyl ester (9,12,15- octadecadienoic acid (z,z)-, methyl ester) 34.7

18577733.85

5 trans-13-octadecenoic acid, methyl ester 34.8 7270049.78

6 Methyl nonadecanoate

Chlorella sp. Control group 1

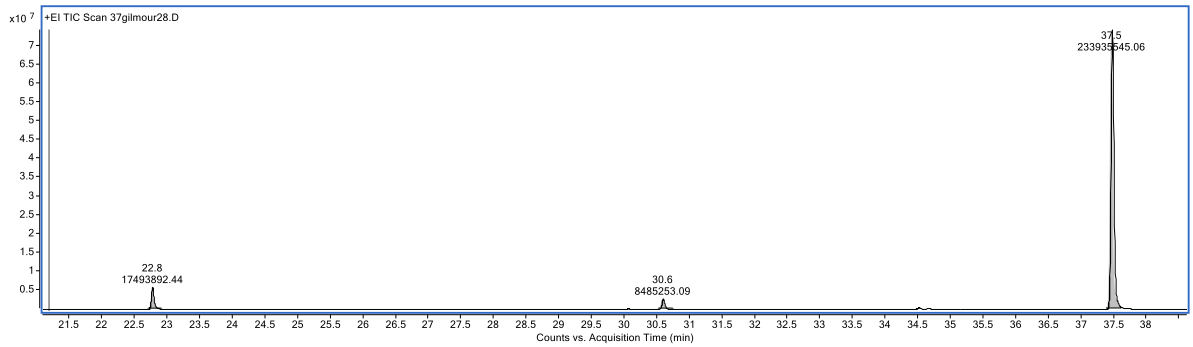


1: Tridecanoic acid, Methyl ester (Methyl tridecanoate)

2: Hexadecanoic acid, methyl ester

3: Methyl nonadecanoate

Chlorells sp. Control group2

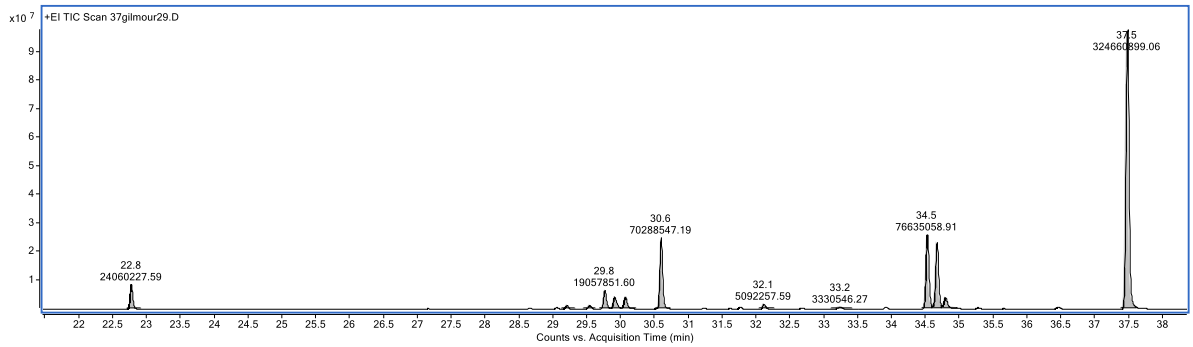


1: Tridecanoic acid, Methyl ester (Methyl tridecanoate)

2: Hexadecanoic acid, methyl ester

3: Methyl nonadecanoate

Chlorella sp acclimated treatment 1 sample 1



1: Tridecanoic acid, Methyl ester (Methyl tridecanoate)

2: 2,2-Dimethyl-3,4-octadienal 29.2 3539679.15

3: methyl 2-hydroxy-octadeca-9,12,15-trienoate; Archidonic acid; 5,8, 11-Hetadecatrien-1-ol;

3 Cyclohexene-1-carboxaldehyde 29.5 4078307.78

3 Methyl (7E,10E)-7,10-hexadecadienoate 29.8 19057851.60

4 Methyl (7E,10E, 13E)-7,10,13-hexadecadienoate 29.9 15605975.52

5 .(10Z,12Z)-10,12-Hexadecadienal; 10E,12E)-10,12-Hexadecadienal 30.1 14450386.17

6 hexadecanoic acid, methyl ester, Palmitic acid, methyl ester 30.6 70288547.19

7 Methyl 15-methylhexadecanoate 32.1 5092257.59

8 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl, 3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol 33.2

3330546.27

9 Methyl (9Z,12Z)-9,12-octadecadienoate 34.5 76635058.91

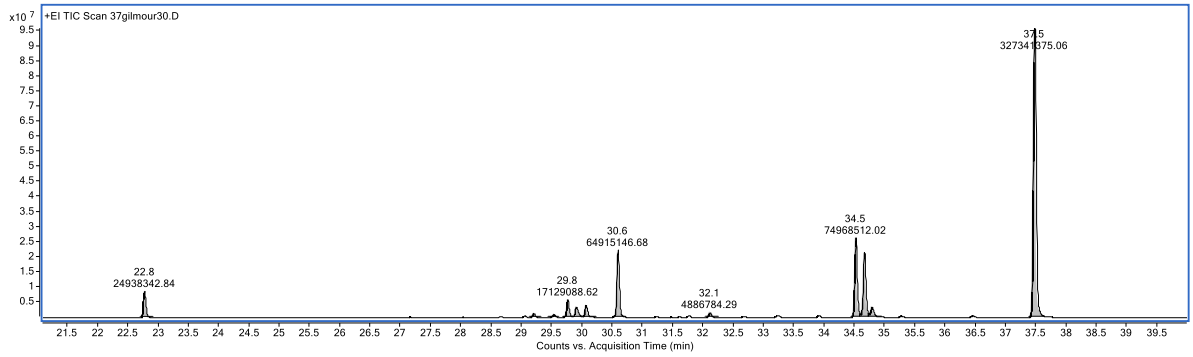
10 Linolenic acid, methyl ester (9,12,15- octadecadienoic acid (z,z)-, methyl ester) 34.7

71978531

11 trans-13-octadecenoic acid methyl ester 34.8 16660402.63

12 Nonadecanoic acid, methyl ester / methyl nonadecanoate 37.5 324660899.06

Chlorella sp acclimated treatment 1 sample 2



1: Tridecanoic acid, Methyl ester (Methyl tridecanoate)

2 2.2-Dimethylocta-3,4-dienal / 2,2-Dimethyl-3,4-octadienal 29.2 3790215.06

3 Methyl 2-hydroxy-octadeca-9,12,15-trienoate; 5,8,11-Heptadecatrien-1-ol; 29.5 3277483.01

4 Methyl (7E,10E, 13E)-7,10,13-hexadecadienoate 29.8 17129088.62

5 Methyl (7E,10E, 13E)-7,10,13-hexadecadienoate 29.9 13494060

6 Methyl (7E)-7-hexadecenoate; .(10Z,12Z)-10,12-Hexadecadienal; 10E,12E)-10,12-Hexadecadienal 30.1 13757563.25

7 hexadecanoic acid, methyl ester, Palmitic acid, methyl ester 30.6 64915146.68

8 Methyl 15-methylhexadecanoate 32.1 4886784.29

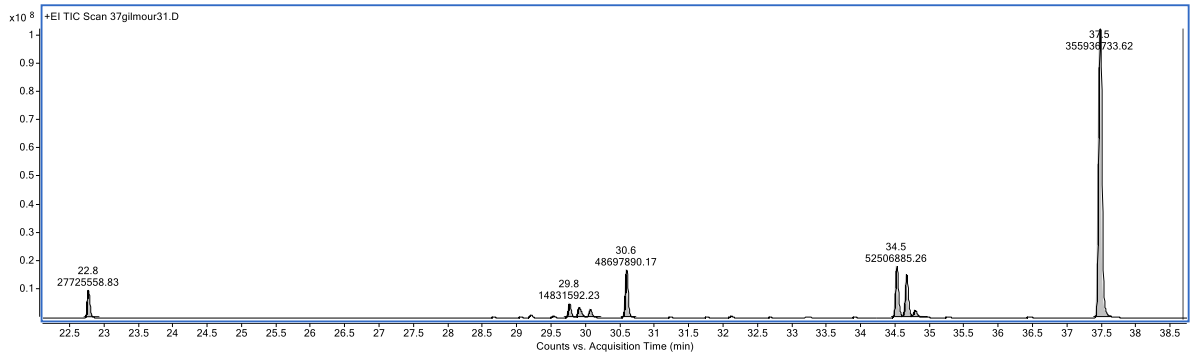
9 Methyl (9Z,12Z)-9,12-octadecadienoate 34.5 74968512.02

10 Linolenic acid, methyl ester (9,12,15- octadecadienoic acid (z,z)-, methyl ester) 34.7 66732655.98

11 trans-13-octadecenoic acid methyl ester 34.8 14107252.27

12 Nonadecanoic acid, methyl ester, methyl nonadecanoate 37.5 327341375.06

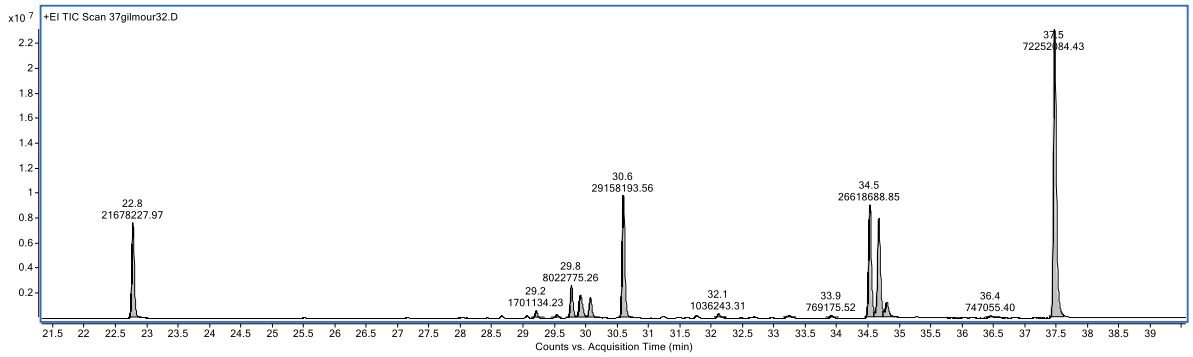
Chlorella sp acclimated treatment 2 sample 1



Butanedioic acid dimethyl ester 6.3 1102960.27

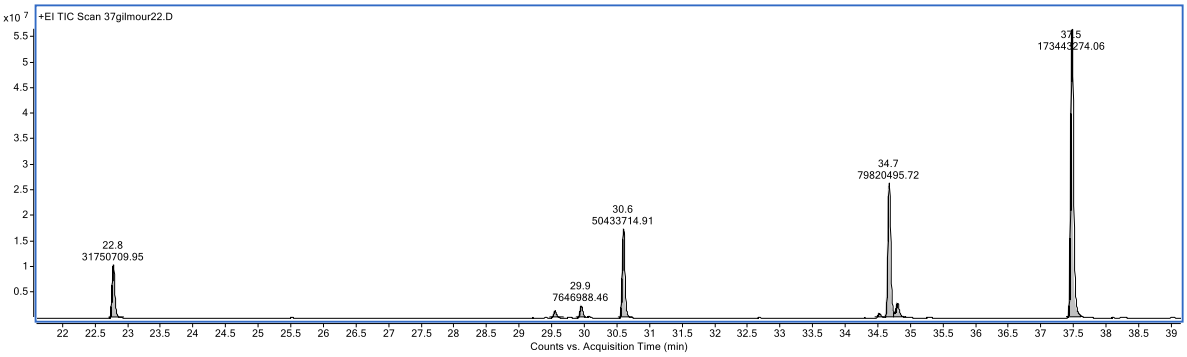
1. Tridecanoic acid methyl ester, methyl tridecanoate 22.8 27725558.83
2. Methyl (7E,10E)-7,10-hexadecadienoate 29.8 14831592.23
3. Methyl (7E,10E,13E)-7,10,13-hexadecatrienoate 29.9 13711355.07
4. (10Z,12Z)-10,12-Hexadecadienal 30.1 9776348.65
5. hexadecanoic acid, methyl ester, Palmitic acid, methyl ester 30.6 48697890.17
6. Methyl (9Z,12Z)-9,12-octadecadienoate 34.5 52506885.26
7. Linolenic acid, methyl ester (9,12,15- octadecadienoic acid (z,z)-, methyl ester) 34.7 47786781.80
8. trans-13-octadecenoic acid methyl ester 34.8 11478251.83
9. Nonadecanoic acid, methyl ester, methyl nonadecanoate 37.5 355936733.62

Chlorella sp acclimated treatment 2 sample 2



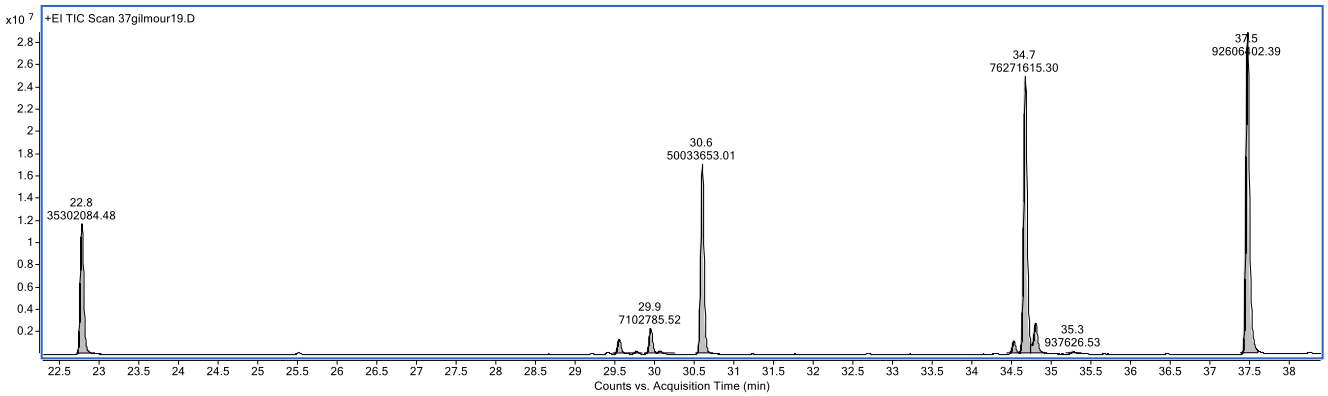
1. Tridecanoic acid methyl ester, methyl tridecanoate 22.8 21678227
2. 2.2-Dimethylocta-3,4-dienal / 2,2-Dimethyl-3,4-octadienal 29.2 1701134.23
3. Methyl 2-hydroxy-octadeca-9,12,15-trienoate; 5,8,11-Heptadecatrien-1-ol; 29.5 1012167.81
4. Methyl (7E,10E)-7,10-hexadecadienoate 29.8 8022775.26
5. Methyl (7E,10E,13E)-7,10,13-hexadecatrienoate 29.9 7483149.42
6. (10Z,12Z)-10,12-Hexadecadienal 30.1 5567924.37
7. hexadecanoic acid, methyl ester, Palmitic acid, methyl ester 30.6 29158193.56
8. Methyl 15-methylhexadecanoate 32.1 1036243.31
9. 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl 33.2 904093.87
10. 2-acetoxy-1,1,10-trimethyl-6,9-epidioxydecalin 33.9 769175.52
11. Methyl (9Z,12Z)-9,12-octadecadienoate 34.5 26618688.85
12. Linolenic acid, methyl ester (9,12,15- octadecadienoic acid (z,z)-, methyl ester) 34.7 26186294.27
13. trans-13-octadecenoic acid methyl ester 34.8 5763236.84
14. Nonadecanoic acid, methyl ester, methyl nonadecanoate 37.5 72252084.43

Tetraselmis Control group



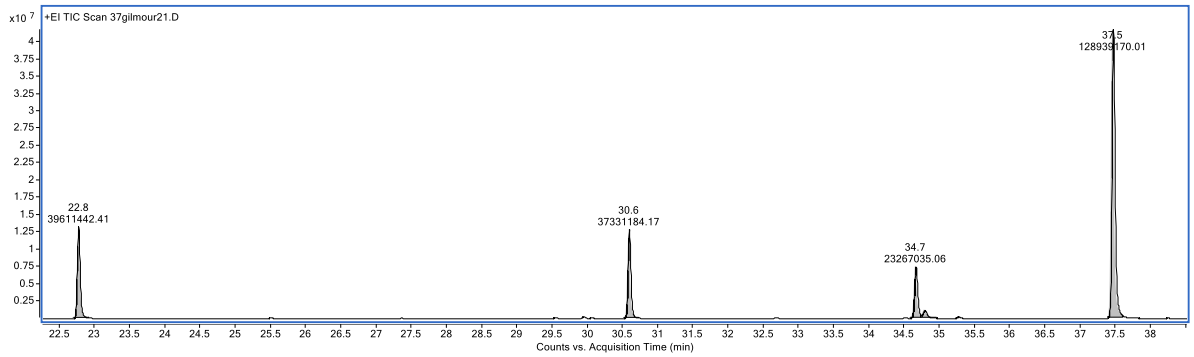
1. Tridecanoic acid methyl ester, methyl tridecanoate 22.8 31750709.95
2. Methyl 4,7,10,13-hexadecatetraenoate 29.5 4502551.98
3. Methyl (9Z)-9-hexadecenoate 29.9 7646988.46
4. hexadecanoic acid, methyl ester, Palmitic acid, methyl ester 30.6 50433714.91
5. Methyl (9Z,12Z)-9,12-octadecadienoate 34.5 2904525.91
6. trans-13-octadecenoic acid methyl ester, cis-13-octadecenoic acid methyl ester Methyl (9E)-9-octadecenoate, Methyl (11E)-11-octadecenoate, (Z)-9-Octadecenoic acid methyl ester 34.7 79820495.72
7. trans-13-octadecenoic acid methyl ester 34.8 11433424.90
8. Nonadecanoic acid, methyl ester, methyl nonadecanoate 37.5 173443274.06

Tetraselmis acclimated treatment 1 sample 1



1. Tridecanoic acid methyl ester, methyl tridecanoate 22.8 35302084.48
2. methyl 4, 7, 10, 13-hexadecateraenoate; Methyl (4Z,7Z,10Z,13Z,16Z,19Z)-4,7,10,13,16,19-docosahexaenoate 29.5 4396345.51
3. Methyl (7E,10E)-7,10-hexadecadienoate 29.8 954813.28
4. Methyl (9Z)-9-hexadecenoate 29.9 7102785.52
5. Methyl (7E)-7-hexadecenoate; .(10Z,12Z)-10,12-Hexadecadienal; 10E,12E)-10,12-Hexadecadienal 30.1 1240591.51
6. hexadecanoic acid, methyl ester, Palmitic acid, methyl ester 30.6 50033653.01
7. Methyl (9Z,12Z)-9,12-octadecadienoate 34.5 3741074.16
8. Cis-13-octadecenoic acid methyl ester; trans-13-octadecenoic acid ester; Methyl (9E)-9-octadecenoate, Methyl (9Z)-9-octadecenoate 34.7 76271615.30
9. Cis-13-octadecenoic acid methyl ester; trans-13-octadecenoic acid ester; Methyl (9E)-9-octadecenoate, Methyl (9Z)-9-octadecenoate 34.8 10525972.34
10. Methyl stearate 35.3 937626.53
11. Nonadecanoic acid, methyl ester, methyl nonadecanoate 37.5 92606402.39

Tetraselmis acclimated treatment 2 sample 1



1. Tridecanoic acid methyl ester, methyl tridecanoate 22.8 39611442.41
2. hexadecanoic acid, methyl ester, Palmitic acid, methyl ester 30.6 37331184.17
3. Cis-13-octadecenoic acid methyl ester; trans-13-octadecenoic acid ester; Methyl (9E)-9-octadecenoate, Methyl (9Z)-9-octadecenoate 34.7 23267035.06
4. trans-13-octadecenoic acid ester 34.8 4808788.11
5. Nonadecanoic acid, methyl ester, methyl nonadecanoate 37.5 128939170.01