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A thesis submitted in partial fulfillment of the requirements for the Master of Engineering Science degree in Chemical and Biochemical Engineering

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EFFECT OF PLANT HORMONES ON THE PRODUCTION OF BIOMASS AND
LIPID IN MICROALGAE

(Thesis format: Monograph)

by

Malihe Mehdizadeh Allaf

Graduate Program in Chemical and Biochemical Engineering

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Engineering Science

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Abstract

Limited fossil fuel reserves, increasing demand for energy in all parts of the world are some driving forces to look for new sources of transportation fuels. Among different options available, microalgae are currently attracting wide interests as an alternative and renewable fuel source.

Microalgae are single cell photosynthetic organisms that are known for rapid growth and high energy content and as a part of photosynthesis; they produce oil that can be used as a feedstock for biodiesel production. Some algae strains could contain lipid up to 80% of the dry biomass. The amount of lipid production is in direct relation with the medium composition and growth conditions of algae.

For biodiesel production from microalgae, increasing the growth rate and lipid content are the main goals. It has been suggested by some researchers that there are plant hormones capable of improving growth rate and biomass. Plant hormones are chemicals produced by plants and play a crucial role in controlling the way in which plants grow and develop.

In this research, the effect of different plant hormones from Brassinosteroids (BRs), Auxin and cytokinin families on biomass, growth kinetic and lipid content of *Chlorella vulgaris* was investigated, and it was found that of the tested hormones only Epibrassinolide has a positive effect on the growth of microalgae. At initial concentrations between 10^{-12} M and 10^{-10} M the total amount of biomass produced was doubled. The lipid content of the algae remained unchanged, resulting in an overall increase of lipid production.

Additionally an ionic liquid mediated process for the extraction of lipids was investigated and a one-pot process combining lipid extraction and trans-esterification was proposed.

Keywords

Microalgae, *Chlorella vulgaris*, Plant hormones, Brassinosteroids (BRs), Auxin, Cytokinin, Biomass, Growth kinetic, Lipid content, Ionic liquids

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Chapter 1

1 Introduction

1.1 Microalgae as Biofuel

Limited fossil fuel reserves, increasing demand for energy in all parts of the world and the likely link of increasing atmospheric carbon dioxide levels to global warming are some driving forces to investigate the production of renewable liquid transportation fuels. Globally 11,295 million tons of oil was used in 2008 and it is expected to increase by 60% in 2030. According to a recent study about the rate of energy consumption in the world, coal reserves are estimated to deplete in two or three centuries, while these periods are 40 years for oil and 63 years for natural gas (Gilbert & Perl, 2005; Gonçalves et al., 2013; S. Lee et al., 2007). However, there is no scientific consensus regarding the amount of recoverable fossil fuels (Shafiee & Topal, 2009). Estimates are constantly changing and new technologies such as hydraulic fracturing might completely change the global energy flow and will thereby influence the medium to long term interested in renewable fuels (King, 2012).

Main health concerns associated with burning fossil fuel are related to their complex composition and resulting incomplete combustion products such as carbon monoxide (CO), nitrogen oxides (NO_x), particle pollutants (acids, organic chemicals, metals), aldehydes, hydrocarbon and benzene derivatives (Hoffert et al., 2002). The increased concentration of atmospheric greenhouse gases is widely believed to result in extreme weather occurrences, rise in sea levels, extinction of species, retreat of glaciers and many other calamities (Gouveia & Oliveira, 2009; Hosikian et al., 2010).

Therefore finding alternative fuels which are clean, environmentally friendly, biodegradable, cheap and highly efficient, has become a challenge today. Political and economic factors have resulted largely in the search for alternatives to oil, rather than replacing the entire fossilize fuel sector with renewable source. This is largely due to current access capacity of coal and natural gas in North America, a trend likely to increase due to advancements in hydraulic fracturing (King, 2012). Some alternative

sources for intermediate and final products derived from petrochemicals are corn fermentation for ethanol, biodiesel from soybean or algae, BTX (benzene, toluene, and xylenes) from coal, biogas or bioliquid from agricultural wastes, hydrogen as transportation fuel, jet fuel from shale oil or crop oil, Fischer–Tropsch fuel from coal or biomass, bisphenols from agricultural sources, liquid transportation fuels from a natural gas source by ZSM-type catalysis, ethylene/propylene via conversion of synthesis gas, use of coal-derived acetylene for petroleum-derived ethylene as a building block chemical, and liquid fuels from spent tires or mixed wastes, etc.(Y. Gao et al., 2012; S. Lee et al., 2007).

Among all sources listed above, microalgae have attracted a great deal of interest as an alternative source for producing biodiesel. Microalgae are unicellular photosynthetic microorganisms, which alter sunlight, water and carbon dioxide to algal biomass. In large microalgae cultivation systems with high efficiency, the potential capture efficiency of CO₂ can be as high as 99% (Lim et al., 2012). They are also able to be grown on marginal land and be cultivated in fresh, waste or salt water. The growth yield of algae is higher compared to other feedstock being used at this time. The doubling time of algae can be changed between 4 to 24 hours. The oil content of some algae can be more than 80% while this number for most agricultural harvests which are being used for producing biodiesel is less than 5% of the biomass. Figure 1-1 compares the theoretical oil yield of different feedstocks per unit of growing area (Demirbas, 2010; Y. Gao et al., 2012; Lim et al., 2012; Radakovits et al., 2010; Wawrik & Harriman, 2010).

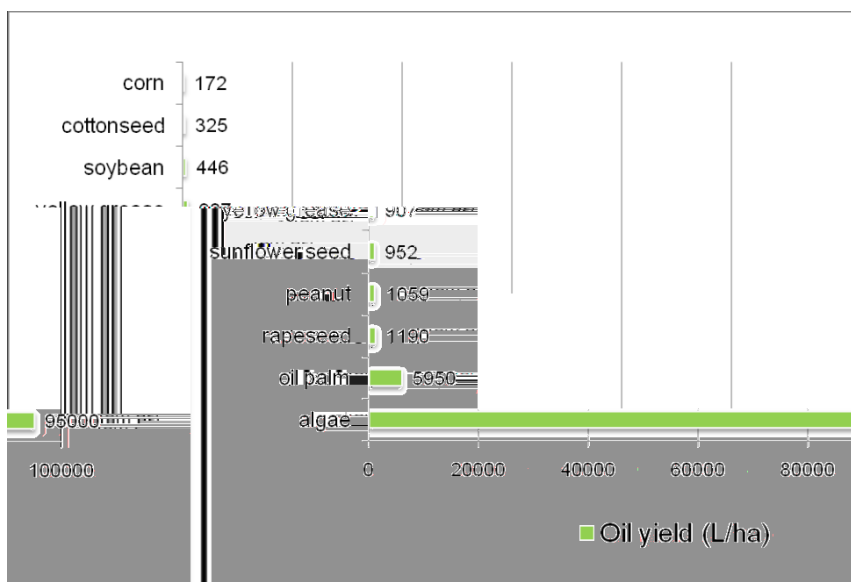


Figure 1-1: Oil Yields of Feedstocks for Biofuel from EarthTrends (2008). Reprinted with permission of Chemistry Central Journal (Y. Gao et al., 2012).

The amount of oil production in different species of algae is dissimilar and some strains could produce up to 80% oil by weight (Demirbas A. & Demirbas F., 2011). Table 1-1 shows the oil content of some microalgae.

Table 1-1: Lipid content of some microalgae (% dry matter). Reprinted with permission of Springer (Gouveia & Oliveira, 2009).

Species	Lipids (% dry matter)
<i>Scenedesmus obliquus</i>	11-22/33-55
<i>Scenedesmus dimorphus</i>	6-7/16-40
<i>Chlorella vulgaris</i>	14-40/56
<i>Chlorella emersonii</i>	63
<i>Chlorella protthecoides</i>	23/55
<i>Chlorella sorokiana</i>	22
<i>Chlorella minutissima</i>	57
<i>Dunaliella bioculata</i>	8
<i>Dunaliella salina</i>	14-20
<i>Neochloris oleoabundans</i>	35-65
<i>Spirulina maxima</i>	4-9

The main bottleneck of using microalgae for biofuel production is the high commercial cost of production. Sunlight, carbon dioxide, water and inorganic salts are the main factors for photosynthetic growth and the required temperature is within 293–303 K. Finding high lipid algae strains, maintaining selected species in outdoor culture, lack of commercial plants in operation, limited data on large scale cultivation of microalgae, high energy inputs for pumping water, CO₂ transfer and mixing the culture suspension, harvesting and dewatering the produced algal biomass are the main challenges, which increase the commercial price of the final product (Rodolfi et al., 2009). Therefore, free available sunlight and water containing essential salts and minerals could reduce the production cost (Demirbas, 2010; Hunt et al., 2011). These factors have stimulated the efforts to maximize the oil yield of these organisms and minimize the costs associated with growing the algae, extracting lipids, and converting these lipids into usable

biodiesel. Lipid extraction is a challenging procedure. Conventional methods in the presence of organic solvents have been used for a long time. Today a new class of solvent has emerged. Ionic liquids (ILs) are known as green solvents which can be used for extracting lipids from algal biomass (Earle & Seddon, 2000; Gonçalves et al., 2013; Kim et al., 2012).

In this study, *Chlorella vulgaris* was chosen because it grows fast and is easy to maintain (Andersen, 2005) and able to grow heterotrophic and mixotrophic (Liang, Sarkany, & Cui, 2009). It has been suggested by some researchers that there are plant hormones capable of improving growth rate and biomass of microalgae (A Bajguz & Czerpak, 1996; Andrzej Bajguz, 2010; Hunt et al., 2010).

Plant hormones or phytohormones are a class of chemical compounds that play a role in plant metabolism and work as signal molecules. Because of the close relationship between plants and algae, phytohormones are expected to play homologous roles in algae. These phytohormones are categorized in different groups, ranging from the growth-stimulating auxins to cell division-inducing cytokinins (Tsavkelova et al., 2006).

This research reports the lipid content of *Chlorella vulgaris* in the presence of different plant hormones in addition to biomass, which is typically reported by other researchers. Moreover, unlike previous studies where only shake flasks were used, in the current study measurements were made in microscale using a 96-well plate. In addition to plant hormones, ionic liquids were used for fractionating microalgae and extracting lipid.

1.2 Thesis Objective

The main objective of this thesis is to study the effect of different plant hormones on the growth rate, biomass concentration and lipid production of *Chlorella vulgaris*. In order to reach this goal, first a suitable assay to measure small changes had to be developed and validated. A secondary objective was to evaluate the use of ionic liquids to recover lipids.

Chapter 2

2 Literature Review

Modern societies have an ever increasing need for readily available energy. There are different available types of fuel around the world, for which the demand changes depending on their application, location, cost, environmental and socioeconomic factors and politics (S. Lee et al., 2007). Among all energy sources, fossil fuels like coal, petroleum and natural gas comprise more than 80% of the energy resources consumption (Scarsella et al., 2010). Limited reserves of fossil fuels, their negative impact on health and environment and unstable price are some driving forces for scientists to look for clean and renewable fuel replacements (S. Lee et al., 2007; Mulumba & Farag, 2012; Yu et al., 2011). From the other point of view, finding clean and renewable energy sources is one of the challenging problems for people in the medium to long term (Mata et al., 2010). Among different options, solar energy, either thermal or photovoltaic, hydroelectric, geothermal, wind, biofuels, and carbon sequestration are some sources, which have been studied and used. Depending on the area of application the better suited option can be chosen although, each selection has its own benefits and problems. Biofuels can be used as a substitute for energy production, which can help to reduce the adverse effects of the frequent oil supply crisis, decrease long-term replacement of fossil fuels, and help countries with no fossil fuel reserves to reduce their energy dependence (Gouveia & Oliveira, 2009; Mata et al., 2010).

2.1 Biofuels

Biofuels are renewable energy sources produced from living organisms or from metabolic by-products such as organic or food waste products. They can be liquid, solid or gaseous fuels (Yu et al., 2011; Horn, 2010). Lignified cellulose such as wood is the most common solid biofuel that has been burned for energy for a very long time. On the other hand, more refining is necessary for liquid and gaseous biofuels, which consist of bioethanol, biodiesel, and engine-combustible hydrocarbons as well as methane from anaerobic digestion (Yu et al., 2011). The first biofuel combustion engine was invented in

1929 by Rudolph Diesel when he fired his invented diesel engine with raw vegetable (peanut) oil (Gadonneix et al., 2010). Nowadays, there are many different forms of feedstocks for producing biofuels such as animal manures and municipal solid wastes, plant-derived starch and sugar feedstocks, animal fat and seed and algal oils (Drapcho, Nhuan, & Walker, 2008). Figure 2-1 illustrates the sources of energy used in the United States in 2007. The amount of biofuels consumption was about 53% of the total renewable energy.

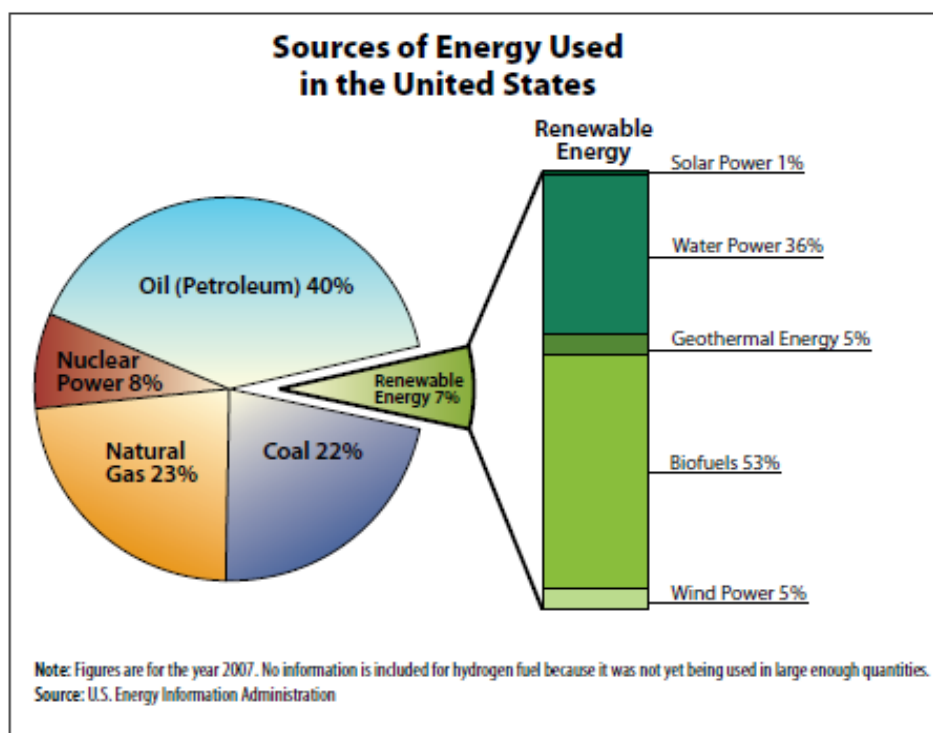


Figure 2-1: Sources of energy used in the United States in 2007. Reprinted with permission of Infobase Publishing (Horn, 2010).

2.2 Liquid Transportation Fuels

The first large scale, well-documented, modern attempt to convert biomass into a liquid transportation fuel was started in the United States during the energy crisis of October, 1973. Carbohydrate can be converted to alcohols (ethanol, butanol), lipids to fatty acid

esters (biodiesel), long chain and cyclic hydrocarbons (gasoline equivalents and jet fuels). Glucose from starch-containing crops such as corn; sucrose derived from sugar cane and sugar beets; palm and vegetable oils; and lignocellulosic biomass, including grasses such as *Miscanthus* and switchgrass, and woody biomass are some biomass sources for fuel production. (Blanch, 2012).

2.2.1 Ethanol

The first person envisioning to use ethanol as a transportation fuel was Henry Ford. In 1908 he proposed to use ethanol as the primary fuel for his Model T but then switched to less expensive gasoline (Mousdale, 2008). Sources which can be used for producing ethanol are starch containing feedstocks such as cereal grains, barley, sorghum, oat, and rice, sugar containing feedstocks such as sugarcane and sugar beet, Lignocellulosic feedstocks, microalgae and many more (Demirbas, 2010; Drapcho et al., 2008). Different feedstocks are used for the production of ethanol around the world. Brazil consumes sugarcane, United States corn, China corn and wheat, India sugarcane, and France utilizes sugar beets and wheat as feedstock for the production of ethanol (Drapcho et al., 2008). Conversion of biomass carbohydrates to ethanol is an anaerobic process. The yeast *Saccharomyces cerevisiae* is the universal organism for producing ethanol from starch and sugar feedstocks (Drapcho et al., 2008; Mielenz, 2009). The produced ethanol is blended into gasoline for use as transportation fuel (Gadonneix et al., 2010). Ethanol has a higher octane number, which leads to higher engine efficiencies and reduces carbon monoxide (CO) emissions from the engine (Mousdale, 2008).

2.2.2 Biodiesel

Biodiesel is another renewable biofuel which is derived from oil crops such as soybeans, canola oil, palm oil and corn oil, waste cooking oil, animal fat and microalgae (Chisti, 2007; Mielenz, 2009; Mousdale, 2008). The first idea of blending vegetable oils with conventional diesel fuels came from Rudolf Diesel and Henry Ford. In 2005, the amount of biodiesel produced around the world was equivalent to 2.91 million tonnes oil, of which 87% was manufactured in the European Union, 7.5% in the United States and 1.7% Brazil (Mousdale, 2008). Biodiesel is produced from transesterification of

triacylglycerides with monohydric alcohols (Mielenz, 2009). Figure 2-2 shows the procedure.

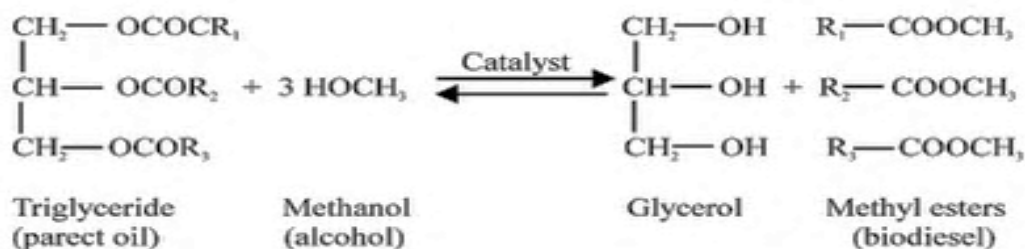


Figure 2-2: Transesterification of oil to biodiesel. R1–3 are hydrocarbon groups.

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Recently, microalgae have attracted lots of attention among these different feedstocks as a potential source of biodiesel. Some strains of microalgae are rich in oil which can be converted to biodiesel. In addition, they can grow rapidly and need a small area for producing algal biomass compared to other plants that are used for biodiesel production (Chisti, 2007; Demirbas, 2010; Gadonneix et al., 2010).

2.3 Algae

Algae are simple organisms with no vascular tissue except brown algae where they have a higher degree of organ differentiation. These organisms are photoautotrophic, which means they are able to produce organic carbon through photosynthesis by using sunlight, CO₂ and water. Some species of *Euglena* do not have chloroplasts; therefore they use other organisms as their food. Member of algae family vary in size, cellular structure and biology. Their size starts from 1µm in diameter for single cell microalgae to 50 m long multi-cellular forms such as kelp that belongs to macroalgae (Belcher & Swale, 1976; Frost et al., 2012; Serediak & Huynh, 2011). There are different methods for classification of algae. Except for Cyanobacteria, which are prokaryote the rest of them are eukaryote and belong to plant kingdom, phylum of *Cryptogamia* (Non flowering plants) and class of *Thallophyta*. Depending on their size algae are divided into two orders, *Microalgae* and *Macroalgae*. Microalgae contain four families while Macroalgae

contain three families. Figure 2-3 shows this classification (Smith et al., 1937; Transeau, 1952)

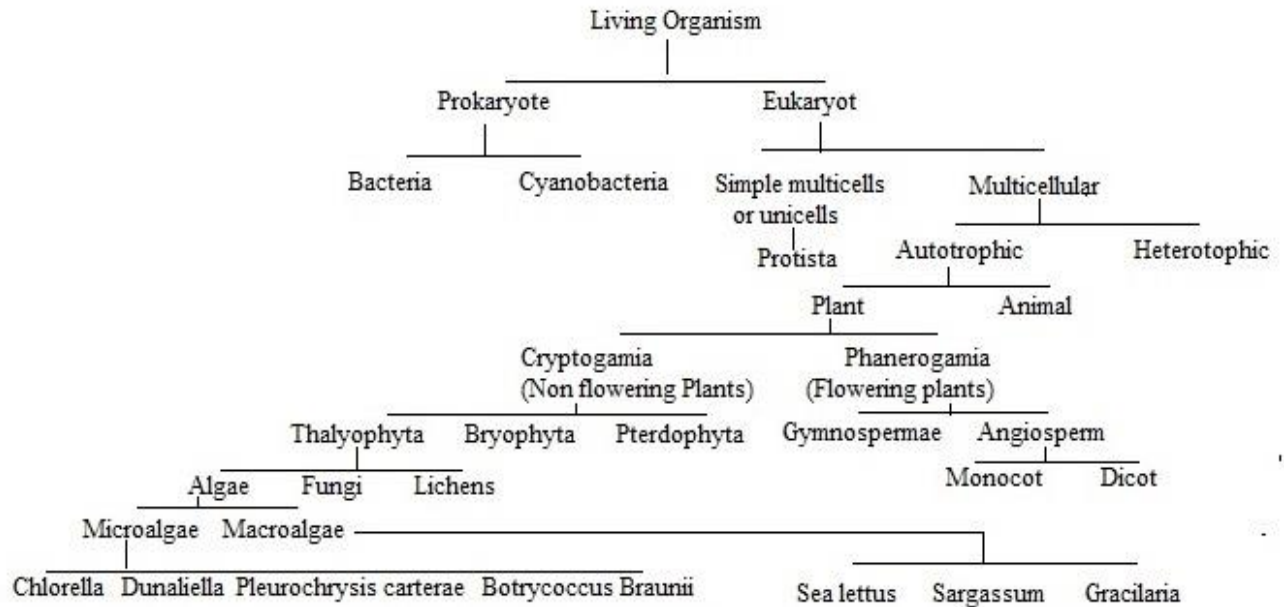


Figure 2-3: Algae classification

2.3.1 Algal Cultivation

Microalgae are able to grow in different types of metabolisms such as photrophic, heterotrophic, mixotrophic and photo-heterotrophic. In phototrophic system, light is the only source of energy for producing chemicals through photosynthetic reactions but in heterotrophic condition, only organic compounds are consumed as carbon and energy source. When these two conditions integrate and organisms need both light and organic compounds, mixotrophic condition results. Depending on the concentration of organic compounds and light intensity available, the organisms are able to live either autotrophically or heterotrophically. There is a small difference between, mixotrophic and photo-heterotrophic. In photo-heterotrophic, light is required to use organic

compounds as carbon source (Gouveia, 2011). Although different strains of microalgae contain different amount of biomass and lipid but it seems that heterotrophic condition results in better productivity and faster growth in comparison with other cultivation conditions. But this system is able to be contaminated very easily especially in open cultivation systems. On the other hand, the high cost of organic carbon is a limiting factor for this system (Y. Chen et al., 2011; Gouveia, 2011; Zheng et al., 2012).

Indoor and outdoor operated systems are used for microalgae cultivation, which can be closed (photobioreactor) or open (open ponds and raceway ponds) (Gouveia, 2011; Koller et al., 2012). The contamination such as unwanted algae, mould, yeast, fungi, protozoa and bacteria in closed systems is less than open systems (Y. Chen et al., 2011; Gouveia, 2011; Koller et al., 2012; Mata et al., 2010). Although productivity is higher in photobioreactors, operation and facilities cost is also higher than open ponds (Zheng et al., 2012).

2.3.2 Microalgae Growth Kinetics

Increase in the algal biomass or its growth takes place when the compounds from the surrounding are added to the organism and its size and mass increase. Increase in cell number can be followed by cell division, which can increase the number of cells in a population. In a simple batch culture, where algae growth is not restricted by external factors such as heat and mass-transfer, the algal growth can be divided into phases illustrated in Figure 2-4 (Andersen, 2005; Becker, 1994; Willey et al., 2009)

A. Adaptation (lag phase)

B. Exponential growth phase (log phase)

C. Stationary phase

D. Logarithmic death phase

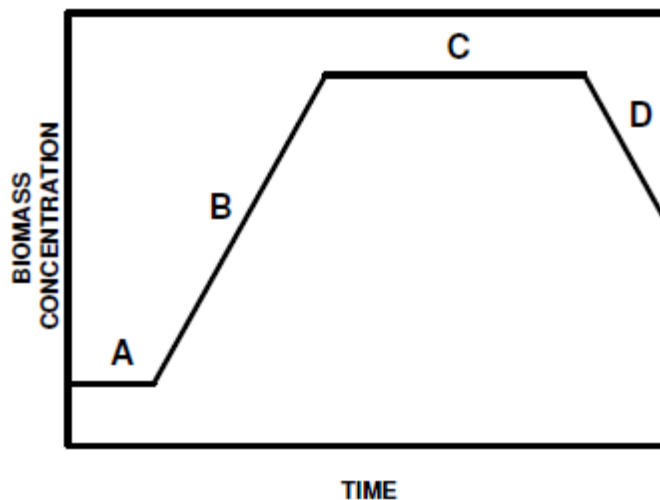


Figure 2-4: Unrestricted algae growth curve in a closed system (Willey et al., 2009).

At the beginning when algal cells are added to the new medium, they try to adapt themselves with the new condition. This stage is known as Lag phase. Then an accelerating growth starts and cells divide and grow at the maximal rate. This phase is named exponential (log) phase.

Limited nutrient supplies prevent microorganisms from growing and increasing their biomass when reaching the stationary phase. At this point an equilibrium between cell division and cell death occurs followed by a stage where cells stop dividing but remain metabolically active. This process continues till microorganisms run out of nutrients and die (Andersen, 2005; Becker, 1994; Willey et al., 2009).

2.3.3 Microalgae as Feedstock for Biofuel

Microalgae can be seen as a renewable energy source and a suitable alternative feedstock for producing biofuels. They are also used as water bioremediation agents, as feed for aquaculture, as food for humans and animals, in pigment production, in bioremoval of heavy metals, and in agriculture. Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms with a high growth rate and are able to consume carbon dioxide and convert it to potential biofuels, food, feed, and high-value products. They are able to grow on non-arable land and waste or salt water. They can be harvested daily and their

production is not seasonal. If inorganic elements can be reprocessed and saltwater-based cultivation systems are developed, there is no direct competition between agricultural food production resources and microalgae as biofuel feedstock. Some strains of microalgae contain high amounts of oil, which could be extracted and purified and converted to fuels. The amount of neutral lipid, which is stored in cytosolic lipid bodies as triacylglycerol, TAG, is about 20-50% of algae dry weight. Table 2-1 displays lipid content and productivities of different microalgae species (W. Chen et al., 2009; da Silva et al., 2009; Gonzalez & Bashan, 2000; Gouveia & Oliveira, 2009; Mata et al., 2010; Radakovits et al., 2010).

To improve biofuel production from microalgae, engineering solutions to optimize the productivity of any microalgae cultivation system and chose the suitable strains for biofuel production are important (Radakovits et al., 2010). There are major technological obstacles for microalgae production, harvesting and extraction of biofuel and co-products such as phytochemicals. Screening of wild-type microalgae species, possible production enhancement by genetic engineering, downstream processing requirements and biorefining are some limitations which need to be solved (Brennan et al., 2012). CO₂ fixations by microalgae has been proposed as a method for decreasing the CO₂ from the environment and therefore reduce emissions of greenhouse gases (Da Silva et al., 2009).

Table 2-1: Lipid content and productivities of different microalgae species.

Reprinted with permission of Elsevier (Mata et al., 2010).

Marine and fresh water microalgae species	Lipid content (% dry weight biomass)	Lipid productivity (mg/L/day)	Volumetric productivity of biomass (g/L/day)	Areal productivity of biomass (g/m ² /day)
<i>Ankistrodesmus sp.</i>	24.0-31.0	-	-	11.5-17.4
<i>Botryococcus braunii</i>	25.0-75.0	-	0.02	3
<i>Chaetoceros mulleri</i>	33.6	21.8	0.07	-
<i>Chaetoceros calcitrans</i>	14.6-16.4/39.8	17.6	0.04	-
<i>Chlorella emersonii</i>	25.0-63.0	10.3-50.0	0.036-0.041	0.91-0.97
<i>Chlorella protothecoides</i>	14.6-57.8	1214	2.0-7.70	-
<i>Chlorella sorokiniana</i>	19.0-22.0	44.7	0.23-1.47	-
<i>Chlorella vulgaris</i>	5.0-58	11.2-40.0	0.02-0.020	0.57-0.95
<i>Chlorella sp.</i>	10.0-48.0	42.1	0.02-2.5	1.61-16.47/25
<i>Chlorella pyrenoidosa</i>	2	-	2.90-3.64	72.5/130
<i>Chlorella</i>	18.0-57.0	18.7	-	3.50-13.90
<i>Chlorococcum sp.</i>	19.3	53.7	0.28	-
<i>Cryptocodinium cohnii</i>	20.0-51.1	-	10	-
<i>Dunaliella salina</i>	6.0-25.0	116	0.22-0.34	1.6-3.5/20-30
<i>Dunaliella primolecta</i>	23.1	-	0.09	14
<i>Dunaliella tertiolecta</i>	16.7-71.0	-	0.12	-
<i>Dunaliella sp.</i>	17.5-67.0	33.5	-	-
<i>Ellipsoidion sp.</i>	27.4	47.3	0.17	-
<i>Euglena gracillis</i>	14.0-20.0	-	7.7	-
<i>Haematococcus pluvialis</i>	25	-	0.05-0.06	10.2-36.4
<i>Isochrysis galbana</i>	7.0-40.0	-	0.32-1.60	-
<i>Isochrysis sp.</i>	7.1-33	37.8	0.08-0.17	-
<i>Mondou subterraneus</i>	16	30.4	0.19	-
<i>Montallanthus salina</i>	20.0-22.0	-	0.08	12
<i>Nannochloris sp.</i>	20.0-56.0	60.9-76.5	0.17-0.51	-
<i>Nannochloropsis oculata</i>	22.7-29.7	84.0-142.0	0.37-0.48	-
<i>Nannochloropsis sp.</i>	12.0-53.0	37.6-90.0	0.17-1.43	1.9-5.3
<i>Neochloris oleoabundans</i>	29.0-65.0	90.0-134.0	-	-
<i>Nitzschia sp.</i>	16.0-47.0	-	-	8.8-21.6
<i>Oocystis pusilla</i>	10.5	-	-	40.6-45.8
<i>Pavlova salina</i>	30.9	49.4	0.16	-
<i>Pavlova lutheri</i>	35.5	40.2	0.14	-
<i>Phaeodactylum tricorutum</i>	18.0-57.0	44.8	0.003-1.9	2.4-21
<i>Porphyridium cruentum</i>	9.0-18.8/60.7	34.8	0.36-1.50	25
<i>Scenedesmus obliquus</i>	11.0-55.0	-	0.004-0.74	-
<i>Scenedesmus quadricauda</i>	1.9-18.4	35.1	0.19	-
<i>Scenedesmus sp.</i>	19.6-21.1	40.8-53.9	0.03-0.26	2.43-13.52
<i>Skeletonema sp.</i>	13.3-31.8	27.3	0.09	-
<i>Skeletonema costatum</i>	13.5-51.3	17.4	0.08	-
<i>Spirulina platensis</i>	4.0-16.6	-	0.06-4.3	1.5-14.5/24-51
<i>Spirulina maxima</i>	4.0-9.0	-	0.21-0.25	25
<i>Thalassiosira pseudonana</i>	20.6	17.4	0.08	-
<i>Tetraselmis suecica</i>	8.5-23.0	27.0-36.4	0.12-0.32	19
<i>Tetraselmis sp.</i>	12.6-14.7	43.4	0.3	-

2.3.3.1 Biodiesel Production from Algae

For producing biodiesel from microalgae, the first bottleneck, as mentioned in previous sections, is choosing the right microalgae strain with high lipid content and productivity (Aguirre et al., 2012).

The next step is microalgal biomass production, the manipulation of which is the main goal in this research. In this stage, the biomass production and lipid content would be different depending on the cultivation mode such as photoautotrophic, heterotrophic and mixotrophic production, types of culture (open and closed systems), culture strategies (batch or continuous culture) and the essential nutrients in the growth medium (Chisti, 2007; Gallardo Rodríguez et al., 2010).

Pursuing this, harvesting and dewatering of microalgae biomass are followed. Finding a suitable method, which is technically appropriate and economically favorable for any species of algae is still an active research area (Mata et al., 2010). The biomass recovery process would be a challenge regarding the low cell densities and small size of cells. Flocculation, filtration, flotation, and centrifugation are some traditional methods which are used in this step (Aguirre et al., 2012; Mohan et al., 2009). The harvested biomass should be dry to prevent decomposition. Air drying, direct sun, use of rotating drums, spray dryers, freeze dryers or fluidized beds are some common used methods (Mata et al., 2010; Mielenz, 2009).

Disruption of cell wall and extracting lipids is the next step for biodiesel production from microalgae. This step is the most challenging section in the total process of biodiesel production. Finding an affordable and simple technique with high efficiency is still an area that needs more investigation. In order to disrupt cell wall, solvents, sonication, homogenization, bead-beating, lyophilization can be used (Ryckebosch et al., 2011). Solvent extraction is one the most common methods (Bligh & Dyer, 1959a; Floch et al., 1956; Long & Abdelkader, 2011). Ionic liquids have also been used as extraction solvents in biodiesel production which will be discussed in the next section (Kim et al., 2012; Zhao & Baker, 2013)

Converting the extracted lipid into biodiesel is the final step. Transesterification (alcoholysis) is the common synthetic route to biodiesel production. In transesterification process the triglycerides react with alcohol in the presence of catalyst and produce glycerol and methyl esters of fatty acids, which is known as biodiesel (Figure 2-2) (Chisti, 2007; Gonçalves et al., 2013; Pragma et al., 2013; Zhao & Baker, 2013)

2.3.3.2 Ionic Liquid-mediated Extraction of Lipids from Algal Biomass

Ionic liquids (ILs) often referred to as ‘green solvents’ are organic salts that usually melt below 100°C and consist of ions. They are able to dissolve polar and non-polar organic, inorganic, and polymeric compounds (Earle & Seddon, 2000; Gonçalves et al., 2013; S. H. Lee et al., 2009; Pragma et al., 2013). Selected ILs are also capable to dissolve cellulose, hemicelluloses and lignin (Blanch, 2012; Cruz et al., 2013; S. H. Lee et al., 2009). This extraction method for product recovery is very new and requires further investigations.

Kim and his team dissolved *Chlorella vulgaris* biomass with a mixture of ionic liquid and methanol which left lipids insoluble (Kim et al., 2012). They used commercial and cultivated *Chlorella vulgaris* and applied Bligh and Dyer gravimetric methods versus ionic liquid mixture for extracting the total lipid content. The final data showed that total contents of lipids extracted from commercial and cultivated *Chlorella vulgaris* were 10.6% and 11.1%, respectively, while with a mixture of ionic liquid these numbers increased to 12.5% and 19.0%, respectively. After analyzing fatty acids with GC they observed that C16:0, C16:1, C18:2, and C18:3 fatty acids were dominant, which can be used as a source of biodiesel production (Kim et al., 2012).

The low mass transfer rate caused by the high viscosity of ILs is the main problem of using this solvent. Microwave and ultrasound irradiation seem to be suitable options for overcoming this problem. Kim’s team reported that ultrasound irradiation could highly enhance the extraction rate and yield of the extraction system when using ILs. The total average extracted lipid from *Chlorella vulgaris* using four methods including soxhlet

method, the Bligh and Dyer's method, ILS, and ILS with ultrasonication was 21, 29, 47 and 75 mg/g dry cell weight, respectively (Kim et al., 2013).

2.3.3.3 *Chlorella vulgaris*

Chlorella vulgaris is a unicellular, spherical, fresh water green alga which belongs to phylum *Chlorophyta* and order *Chlorellales* (Gruneberg & Komor, 1976; Luz et al., 2002; Sharma et al., 2011). *Chlorella vulgaris* is a rich source of chlorophyll, which is used widely as a health food and feed supplement (Sharma et al., 2011), it is also used for tertiary wastewater treatment, and especially for nitrogen and phosphorus compounds and heavy metals removal (Gonzalez-Bashan et al., 2000; Luz et al., 2002). From the other point of view, *Chlorella vulgaris* is one of the best options for biodiesel production due to its high lipid production regarding quantity (combination of biomass productivity and lipid content) and quality (fatty acid composition) (Gouveia, 2011). This species of green algae is close to primitive land plants and is a suitable candidate for investigating the effect of complex plant chemicals such as plant hormones (Provasoli, 1958).

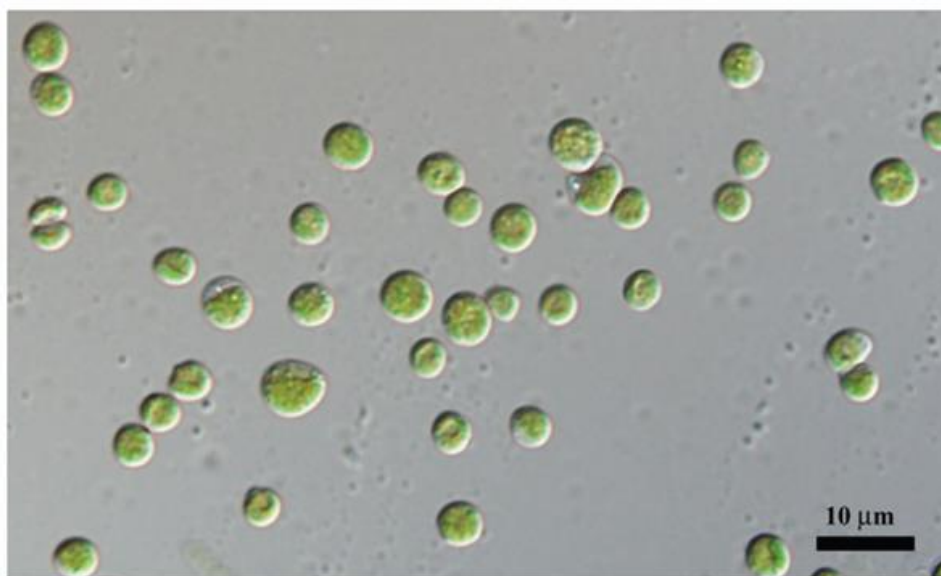


Figure 2-5: Light Microscopic observation of *Chlorella vulgaris* UTEX 2714.

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2.4 Plant Hormones

Plant hormones or phytohormones are signal molecules that are produced by plants and control a wide range of plant growth and developmental processes at low concentrations. Plant hormones are capable of adjusting the growth rate of the individual parts and producing the form that we recognize as a plant. They also regulate the processes of reproduction, stimulating defensive responses, differentiation, development, and formation of flowers, seeds and leaves, cell division or seed germination, inhibiting stem elongation and etc. Most plant hormones are able to tolerate charge, therefore they can be adsorbed to specific membrane lipid (Davies, 2004; Gzyl-Malchera et al., 2007).

These plant hormones are categorized in six different groups shown in Table 2-2.

Table 2-2: Hormones classification and function (Davies, 2004)

Hormones Name	Function in Plants	Anticipated effect on Microalgae
Brassinosteroids (BRs)	Control of division; Growth by elongation; differentiation of the vascular system; inhibiting root growth; BRs are needed for fertility; etc.	Increasing the growth rate
Cytokinin	Control of cell division; bud development; development of the leaf blade; senescence retardation; promote shoot initiation; etc.	Increasing the growth rate
Abscisic acid	Control of stomata apparatus function; growth inhibition; seed dormancy; inhibits shoot growth; induces storage protein synthesis in seed; etc.	Growth inhibition
Auxin	Induction of elongation growth and stem growth; stimulates cell division; differentiation of phloem elements; apical dominance; tropisms; initiation of root formation; etc.	Increasing the growth rate
Ethylene	Senescence induction; initiation of defensive responses; decrease elongation; Leaf and fruit abscission; etc.	accelerated ethylene synthesis underlies some auxin-depended responses

Gibberellins	Stem elongation; initiation of seed germination; cell division and elongation; Enzyme production during germination; etc.	Increasing the growth rate
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Some of these plant hormones have been biochemically detected in green algae, including *chlorophytes* and seem to have the same role as in land plants. On the other hand, green algae are unicellular and can be grown axenically in the laboratory, hence are appropriate models for investigating the effect of plant hormones on them (Blanc et al., 2010). Improving the biomass production of microalgae in the presence of plant hormones and micronutrients has been reported a few times since the 1930's (Hunt et al., 2010). In this research, different plant hormones from three different families were chosen as follows.

2.4.1 Brassinosteroids (BRs)

Brassinosteroids are steroidal compounds, which were first isolated from Brassica pollen. Today, over 40 brassinosteroids have been recognized. At low concentrations, they stimulate the growth and affect the development, cell division, cell elongation and vascular differentiation but have inhibitory effect on root growth. On the other hand, they are able to protect plants from various environmental stresses (Andrzej Bajguz & Asami, 2004; Davies, 2004; Fujioka & Sakurai, 1997). In green alga *Hydrodictyon reticulatum*, 24-epicastasterone and 28-homocastasterone were identified (Tarakhovskaya et al., 2007). The chemical structure of Epibrassinolide (EBL), the selected hormone from this family for this research is shown in Figure 2-6.

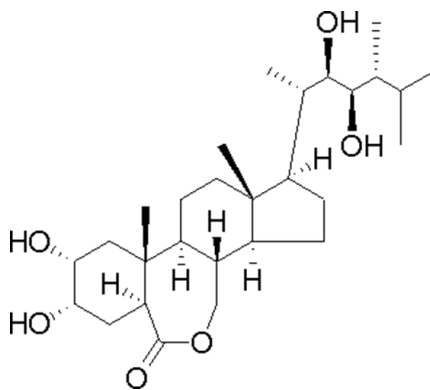


Figure 2-6: Structure of Epibrassinolide (EBL) (Fujioka & Sakurai, 1997).

2.4.2 Cytokinins (CKs)

Cytokinins are adenine derivatives; discovered in the 1950s, which have different effects on plant tissues such as, inducing cell division in the presence of Auxin, growth of lateral buds, leaf expansion and chloroplast development. Kinetin was the first element to be discovered as a cytokinin (Figure 2-7) (Clouse & Sasse, 1998; Davies, 2004). Some hormones from this family were found in the extract of furoid algae from the class of *Phaeophyceae*. Apparently algal cytokinins are generally produced at tRNA degradation (Tarakhovskaya et al., 2007).



Figure 2-7: Structure of Kinetin (Davies, 2004).

2.4.3 Auxins

According to studies in the 1960–1970s, Auxins and their inactive analogs were recognized in brown (*Macrocystis* and *Laminaria*), red (*Botryocladia*), and green (*Enteromorpha*, *Chlorella* , and *Cladophora*) algae and also in cyanobacteria (*Oscillatoria*) (Tarakhovskaya et al., 2007).

Cell enlargement, cell division, vascular tissue differentiation, root initiation, flowering, fruit setting and growth are some of Auxin roles in plants. It has been indicated that Auxin in the green alga *Bryopsis plumosa* stimulates rhizoid formation and activates growth in some cultured microalgae and cyanobacteria. The high concentration of Auxin has inhibitory effects (Davies, 2004; Tarakhovskaya et al., 2007). The main Auxin in most plants is Indole-3-acetic acid (IAA) (Figure 2-8A). Indole 3 butric acid (IBA) and 1-naphtalenacetic acid (NAA) are two different hormones from Auxin family with similar effects (Figure 2-8 B and C). Indole 3 butric acid (IBA) was introduced 50 years ago and has been studied widely for optimization of rooting in different plant species while 1-naphtalenacetic acid (NAA) is able to release the retarded gamete (Davies, 2004; Ludwig-müller, 2000; Tarakhovskaya et al., 2007)

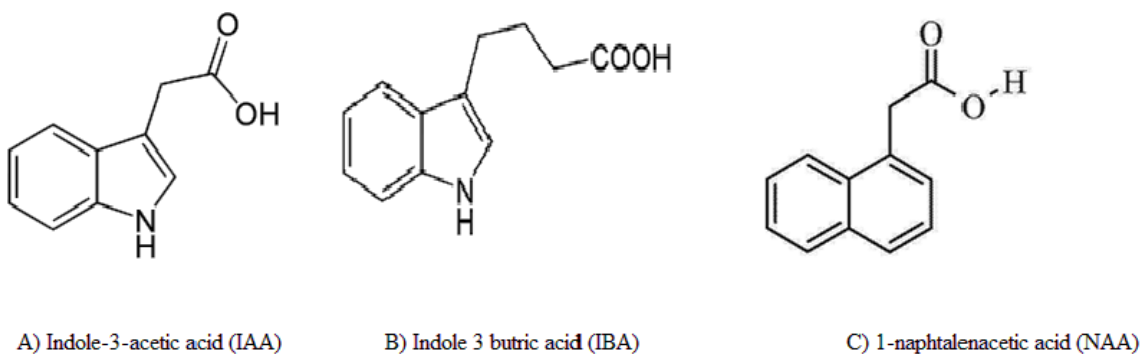


Figure 2-8: Structure of (A) Indole-3-acetic acid (IAA), (B) Indole 3 butric acid (IBA) and (C) 1-naphtalenacetic acid (NAA) (Davies, 2004).

2.5 Effect of Plant Hormones on the Growth and Biomass of Microalgae

2.5.1 Brassinosteroids (BRs)

As mentioned in section 1.3.1, Brassinosteroids are a group of plant hormones with stimulating effects on the growth of plants. Bajguz and Czerpak published some articles in 1996 and 1998 about the effects of this plant hormone on the growth of *Chlorella vulgaris*. Their results indicated that *Chlorella vulgaris* growth increased in the presence of concentrations of 10^{-15} to 10^{-8} M of BRs while its development cycle decreased. Furthermore, cell divisions increased and cell elongation improved with the growth of acid secretion. They deduced the growth stimulation to depend on pH reduction in the wall space and thus on acid-induced wall loosening (A Bajguz & Czerpak, 1996; A. Bajguz & Czerpak, 1998; Andrzej Bajguz, 2000).

Bajguz also investigated the effect of BRs on nucleic acids and protein content of *Chlorella vulgaris* and his results showed stimulating influence of this group of hormones on the DNA, RNA and protein content of microalgae (Andrzej Bajguz, 2000). His research also indicated that Brassinolide (BL) from Brassinosteroids improved the growth with increased cell number even under stress and restored the growth to the level of unstressed control (Andrzej Bajguz, 2010, 2011).

2.5.2 Cytokinins (CKs)

Piotrowska-Niczyporuk and her colleagues worked on *Chlorella vulgaris* under the influence of different phytohormones. Their results indicated that Cytokinins mixed with heavy metals persuaded the highest increase in the cell number of *Chlorella vulgaris* (Piotrowska-Niczyporuk et al., 2012).

Ryan W. Hunt and his group's work showed that in the presence of 0.002 ppm Zeatin and 0.22 ppm Thidiazuron from Cytokinin family the average productivity of *Chlorella vulgaris* increased 67% and 83% respectively over 10 days compared with control samples. Their results also indicated an approximately 160% increase in the chlorophyll productivity 10 days after adding Thidiazuron (Hunt et al., 2010).

The growth of *Dunaliella salina*, which is a green microalga from phylum of *Chlorophyta* was investigated by Raposo and Morais. Kinetins with concentrations of 0 to 2 mg/L were mixed with different concentrations of 2, 4-dichlorophenoxyacetic acid. They observed significant increase in algal growth in the presence of Kinetin (Filomena et al., 2013).

2.5.3 Auxins

Auxin is another group of phytohormones that has attracted the attention of researchers to study its effect on the growth and biomass production of microalgae. Hunt and his colleagues reported that the biomass productivity of *Chlorella sorokiniana* in the presence of 5 ppm 1-naphthaleneacetic acid (NAA) is about $0.042 \text{ g L}^{-1} \text{ day}^{-1}$ compared to $0.018 \text{ g L}^{-1} \text{ day}^{-1}$ in the control sample, which shows 133% biomass increase. This concentration of NAA showed the highest amount of biomass compared to other plant hormones from Auxin, Gibberellin and Cytokinin families. From the biomass data they also concluded that the Auxins such as 1-naphthaleneacetic acid (NAA), and indole-3-butyric acid (IBA) had the highest influence on improving the growth of microalgae (Hunt et al., 2010).

In another research, Hunt also figured out that mixing 1-naphthaleneacetic acid (NAA), with ethanol improved the biomass productivity in different strains of microalgae (Hunt et al., 2011).

Auxins such indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA), phenylacetic acid (PAA), and indole-3-butyric acid IBA) resulted in weaker biological activities compared to Cytokinins under stress conditions and in the presence of heavy metals in a research conducted by Piotrowska-Niczyporuk and her coworkers (Piotrowska-Niczyporuk et al., 2012).

Maria Filomena de Jesus Raposo and Rui Manuel Santos Costa de Morais observed that the cell number of *Haematococcus pluvialis*, a freshwater species of *Chlorophyta*, significantly increased after 13 days under Auxin stimulation. When a combination of Auxin and 1mg 2, 4-dichlorophenoxyacetic acid was used, the resulting cell number was

about 355% more than the control. In addition, the combination of these two chemicals significantly reduced the lag phase and therefore, increased the cell number three times on the 7th day of culture (Filomena et al., 2013).

2.6 Glucose Uptake by Microalgae

Organic carbon loading rate can influence the growth of algae and bacteria directly or indirectly. Algae get CO₂ from bacterial degradation of organic matter, bicarbonate alkalinity and dissolved atmospheric CO₂ in water (Mayo & Noike, 1994a). Martinez et al. reported that *Chlorella* species are able to grow well in the presence of glucose and it is the best substrate for algal population while they digest acetate and sucrose. Their results also showed that sugars stimulated the growth rate more than acids did and this growth stimulation is significant in the presence of glucose. Glucose uptake is faster in the light than in the dark (Martinez et al., 1987; Martinez & Orus, 1991).

Mayo and Noike investigated the effect of different concentrations of glucose (from 25 to 700 mg/l/d) on the growth behavior of *Chlorella vulgaris*. Their research indicated that algae growth was influenced by the concentration of added glucose. Algae growth rate during exponential phase was higher when increasing the glucose concentration. The highest concentration of glucose (700 mg/l/d) caused an inhibitory effect on the growth of microalgae, which happened because of insufficient dissolved oxygen and volatile fatty acids accumulation. On the other hand, bacterial population increased in the presence of glucose (Mayo & Noike, 1994a).

Glycerol is another option as a sole carbon source for microalgae growth. However, combination of this substrate with glucose gave the highest growth, biomass content, volumetric productivity, accumulation of lipids and soluble carbohydrates as the raw materials for biodiesel and bioethanol production in *Chlorella vulgaris* (Kong et al., 2013). Liang et al.'s studies also approved that the highest lipid productivity was achieved in the presence of glucose and light. Their studies demonstrated that both glucose and glycerol have inhibitory effects on *Chlorella vulgaris* at high concentrations. They also reported that a glucose concentration of 1% showed the fastest growth rate and with the addition of 2% glycerol, both the cellular lipid content and lipid productivity

increased (Liang et al., 2009). On the other hand, glucose increased the nitrate uptake in *Chlorella vulgaris*, which is the main source of nitrogen for producing and regulating the enzymes in plants (Schlee et al., 1985).

In mixotrophic condition in the presence of glucose and saturating light intensities, the *Chlorella vulgaris* cell size increased due to the higher metabolic activity. On the other hand, the volume density of chloroplasts was higher in mixotrophic situation, which was influenced by glucose and therefor increased the photosynthetic activity of cells (Griffiths, 1963; Martínez et al., 1991).

2.7 Glucose Analysis

Griffiths studied the effect of carbohydrate on the cell division of this microalga in 1963 by adding glucose to *Chlorella vulgaris* culture. In order to estimate the amount of glucose uptake, he used a colorimetric method. For this purpose, he added arsenomolybdate chromogenic reagent with Somogyi's 'Reagent 51' to dry the samples and measured the cell size on the haemocytometer slide under microscope (Griffiths, 1963).

In order to measure the soluble carbohydrates, Kong et al first extracted the carbohydrates by centrifuging, drying and homogenizing the samples and then by using Anthrone sulfuric acid method they determined the content of soluble carbohydrate and measured the color with a spectrophotometer (Kong et al., 2013).

Martinez and Orus used Somogyi method for carbohydrate determination in *Chlorella vulgaris*. In this method different Alkaline Copper and Arsenomolybdate color reagents were used and the color was measured by a photoelectric colorimeter (Martinez & Orus, 1991; Nelson, 1944)

Komor and Tanner invented a new colorimetric method, which used radioactive sugars to determine the amount of glucose uptake. They claimed that in this method no pigments were extracted and therefore sugars were directly measured (Gruneberg & Komor, 1976; Haass & Tanner, 1974; Komor & Tanner, 1971).

The techniques and methods, discussed in the previous sections are all colorimetric methods. Most of these methods are time consuming and there is a probability of sample loss during extraction. From another point of view, similar physical and chemical characteristics of carbohydrates cause difficulties in analyzing this group of chemicals. These similarities in chemical and physical properties result in improper chromophore for UV detection. Finding a technique with high sensitivity to distinguish these differences in a short time and with minimum error is a big concern. Consequently, several HPLC (high-pressure liquid chromatography) methods were developed to study carbohydrates (Andersen, 2005; Bailey et al., 2012).

In order to use HPLC, a calibration curve should be prepared with pure carbohydrates. The prepared samples for HPLC could be transferred to the instrument for separation and quantification of carbohydrates. This method shows high selectivity, accuracy, linearity, reliability and sensitivity (Parpinello & Versari, 2000; Wei et al., 2009).

2.8 Lipid Analysis

Today, biofuels production from plant oils, animal fats or microalgae is expanding quickly. Microalgae are an alternative substitute because of high biomass and high lipid content (W. Chen et al., 2011; Y. Feng, Li, & Zhang, 2011; Han et al., 2011).

The most important fact for biofuel production from microalgae is to choose the optimal strain with appropriate lipid yield. Consequently, finding the right and proper strain of microalgae and culture condition will require a suitable technique for monitoring the lipid content in microalgae. Microalgae contain different kinds of lipids, triglycerides and diglycerides, phospholipids and glycolipids, hydrocarbons and other kinds of lipids (Han et al., 2011). These lipids play different physiological roles in plants including energy storage, structural support as membranes, and intercellular signaling (Yu et al., 2011). The metabolic pathways of microalgae to produce a wide variety of biofuels are shown in Figure 2-9 (Radakovits et al., 2010).

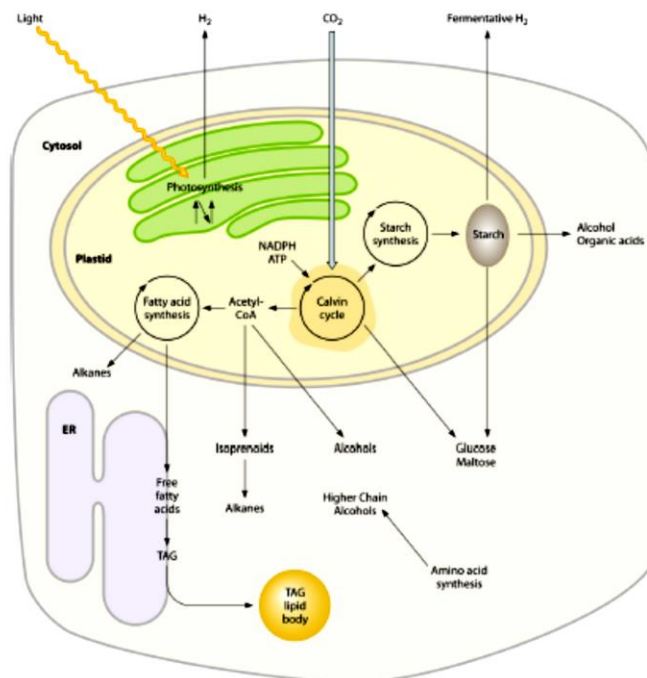


Figure 2-9: Microalgal metabolic pathways that can be leveraged for biofuel production. ER, endoplasmic reticulum. Reprinted with permission of ASM (Radakovits et al., 2010).

Species of microalgae used, culture nutrients, environmental and developmental conditions in which microalgae cells are cultured and harvested have a direct effect on the algal lipid composition (Han et al., 2011).

Different techniques are used for lipid analysis such as; gravimetric method, staining method, HPLC method, Near-infrared (NIR) and Fourier transform infrared (FTIR) and GC (Gas chromatography) technique which will be discussed in the following parts (Han et al., 2011).

2.8.1 Gravimetric Method

Jordi Folch and his colleagues established a standard gravimetric method for the isolation and purification of total lipids in tissues in 1956. In this method a chloroform-methanol mixture with a ratio of 2:1 (V/V) was used for extracting lipids from the homogenized tissue (Folch et al., 1956). This technique is one of the most reliable methods, which is still used by many researchers for extracting the total lipid. In 1959 Bligh and Dyer, two

Canadian scientists, tried to modify the Folch method and make it easier. Both fresh and frozen samples can be used in this method and this is what differentiates this method from Floch (Bligh & Dyer, 1959b). Iverson et al. compared these two methods with each other and found that both methods yield similar results in samples with low lipid contents but Folch method is more accurate for samples with high lipid contents (Iverson et al., 2001).

To increase the efficiency, Long and Abdelkader tried to use a mixture of hexane-isopropanol instead of chloroform-methanol for extracting lipid from *Nannochloropsis* microalgae. Their data indicated the extract yield is higher with chloroform-methanol compared to hexane-isopropanol. In the presence of chloroform-methanol the extract yield was about 25-27% while it decreased to 17% when hexane-isopropanol was used (Long & Abdelkader, 2011).

Although gravimetric methods are time consuming and labor-intensive, these methods are still used by many researchers especially to compare their results with other techniques (W. Chen et al., 2011, 2009; Da Silva et al., 2009; G.-D. Feng et al., 2012; C. Gao et al., 2008; Han et al., 2011; Huang et al., 2009; Kim et al., 2012; S. J. Lee et al., 1998; Mulbry et al., 2009; Salama et al., 2013; Scarsella et al., 2010; Soštarič et al., 2012).

2.8.2 Staining Method

Staining is a common and rapid screening method for lipid quantification, which is done in shorter times compared to gravimetric methods (Han et al., 2011). Microalgae, like higher plants, produce both neutral and polar lipids. Under unfavorable or restricted growth conditions microalgae produce neutral lipids such as triacylglycerol esters (TAG) and accumulate them in lipid droplets located in the cytoplasm. However, under favorable and unlimited growth conditions, the production of polar lipids such as glycolipids and phospholipids increases which are stored in chloroplast and cellular membranes (Held & Raymond, 2011).

Nile red (9-(Diethylamino)-5H benzo [∞] phenoxa- zin- 5-one) is a lipophilic fluorescent dye, which is used to evaluate the neutral lipids in animal cells and microorganisms, such as mammalian cells, bacteria, yeasts, zooplankton and microalgae (W. Chen et al., 2009; Held & Raymond, 2011). Figure 2-10 shows the chemical structure of Nile red.

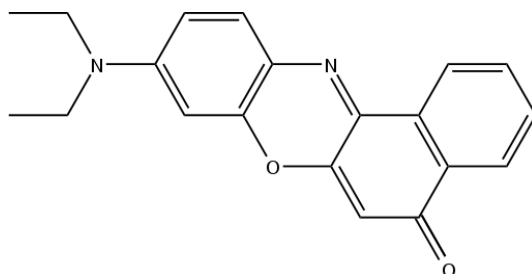


Figure 2-10: Chemical structure of Nile red

Researchers like Chen et al., Feng et al., Malapascua et al., Held and Raymond, Cooksey et al., Priscu et al. and many others have used Nile red for staining. They added the prepared dye to fresh or lyophilized samples and measured the fluorescence intensities with a fluorescence spectrophotometer when stable fluorescence readings were obtained for each sample (W. Chen et al., 2011, 2009; Cooksey et al., 1987; Da Silva et al., 2009; Elsey et al., 2007; G.-D. Feng et al., 2012; C. Gao et al., 2008; Held & Raymond, 2011; Priscu et al., 2004; Romel Malapascua et al., 2012)

In order to improve the results for staining and overcome the thick and rigid cell walls of some strains of microalgae such as *Pseudochlorococcum* species and *Scenedesmus dimorphus*, Chen and his colleagues used microwave to assist Nile red staining method. In this method, Nile red dye was added to the samples by using a microwave oven with 1200W power and maximum 2 minutes for the pretreatment process, and 2 minutes for the staining process. Then the fluorescence intensity was measured with a fluorescence spectrophotometer. Obtained results suggested microwave-assisted staining method can be used as a quantitative procedure to determine neutral lipids in algal cells at different stages of the life cycle. This method is rapid and smaller samples are required for detection and quantification of neutral lipids in microalgae (W. Chen et al., 2011).

After staining neutral lipids in the microalgae cells with the Nile red dye, a bright yellow fluorescence color was emitted under the fluorescence microscope which made it easy to distinguish the intracellular lipid droplets. The fluorescence wavelength which is used for neutral lipids is under 580 nm and can be distinguished from the fluorescence of chlorophyll which fluoresces at 680 nm (Cooksey et al., 1987; Elsey et al., 2007; Elumalai et al., 2011; G.-D. Feng et al., 2012; Huang et al., 2009; Lim et al., 2012; Priscu et al., 2004; Romel Malapascua et al., 2012).

BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene) is another lipophilic fluorescent dye which was reported as a vital stain for screening oil storage within live algal cells (Brennan et al., 2012; Cooper et al., 2010).

2.8.3 HPLC Method

HPLC (high-performance liquid chromatography) is a powerful technique for analyzing lipids. This chromatographic technique is suitable for separating, identifying, quantifying and purifying the individual components of the mixture (Han et al., 2011). However, a considerable sample preparation step is needed for isolating the lipid fractions before analyzing (Laurens & Wolfrum, 2011). Jones and her group published a paper in 2012 in which they used HPLC for analyzing lipid extraction. In their work, two different methods with two different mobile phases were used. Their results revealed that HPLC gives quantitative information about all extracted lipid classes (Jones et al., 2012).

2.8.4 NIR and FTIR Spectra

Near-infrared (NIR) and Fourier transform infrared (FTIR) are two new spectroscopy methods based on infrared for measuring the lipid content and composition in microalgae. In comparison with traditional methods, they are fast, accurate and non-destructive (Han et al., 2011; Laurens & Wolfrum, 2011).

2.8.5 GC (Gas Chromatography)

Another analyzing technique for lipid quantification is GC (Gas chromatography). The percentage of different fatty acids present in microalgae could be analyzed with GC. For this purpose, an internal standard for quantification of free and bounded fatty acids

converted into fatty acid methyl esters (FAMEs) is used (Elumalai et al., 2011; Samori et al., 2012). Gas chromatography was used with many researchers for analyzing fatty acid (Elumalai et al., 2011; Kim et al., 2012; Mallick et al., 2012; Mansson, 2012; Salama et al., 2013; Samori et al., 2012).

Chapter 3

3 Materials and Methods

3.1 Microalgae Strain

Chlorella vulgaris UTEX 2714 was used for this study. The strain was obtained through the Culture Collection of Algae from the University of Texas at Austin.

3.2 Growth Media

3.2.1 Bold's Basal Medium

For culturing the microalgae, a modified version of Bold's Basal (BBM) was used. This medium contains different micro and macro nutrients and is suitable for green microalgae such as *Chlorella vulgaris* (Andersen, 2005). Stock solutions were prepared according to Table 3-1 and kept in the fridge.

The media were prepared by dissolving the stock solution in distilled water. The pH of Bold's Basal media was adjusted at 6.6. with 1N NaOH. The prepared media were sterilized by AMSCO autoclave at a temperature of 121°C and a pressure of 15 psi above the atmospheric pressure for 15 minutes. The sterile media were cooled down to the room temperature and allowed to re-equilibrate of inorganic carbon species (Andersen, 2005).

Table 3-1: Modified Bold's Basal medium (Anderson, 2005)

Component	Stock solution g/L	Quantity used (ml)
NaNO ₃	25	10
CaCl ₂ ·2H ₂ O	2.5	10
MgSO ₄ ·7H ₂ O	7.5	10
K ₂ HPO ₄	7.5	10
KH ₂ PO ₄	17.5	10

NaCl	2.5	10
Alkaline EDTA		1
Na ₂ EDTA.2H ₂ O/EDTA	64	
KOH	31	
Acidified iron solution		1
FeSO ₄ .7H ₂ O	4.98	
H ₂ SO ₄		1
Boron solution		1
H ₃ BO ₃	11.42	
Trace metal Solution		1
ZnSO ₄ .7H ₂ O	8.82	
MnCl ₂ .4H ₂ O	1.44	
CuSO ₄ .5H ₂ O	1.57	

3.2.2 Mixotrophic Condition

Chlorella vulgaris is capable of growing mixotrophic. They can consume both inorganic (CO₂) and organic carbon substrates simultaneously and grow photoautotrophic and heterotrophic at the same time (Bhatnagar et al., 2011). In this study glucose with two different concentrations of 5 and 15 g/L was used as a carbon source for the media. Glucose, as an organic compound, has a positive effect on the growth rate and lipid content of microalgae (Feng et al., 2011; Pirson & Lorenzen, 1965).

3.3 Cultivation and Maintenance

The amount of algae inoculum was about 20% of the medium volume. 125 and 250 ml VWR flasks were used for culturing. The flasks were kept in Infors HT Multitron standard shaker-incubator at a temperature of $25\pm 1^\circ\text{C}$ with a speed of 120 RPM and a light intensity of $95\pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$.

3.3.1 Bioreactor

Fully controlled Infors HT bench-top bioreactor was used for cultivating large volumes of microalgae. The used vessel for this research had a volume of 7.5 liters. pH, dissolved oxygen (DO) and optical density (OD) were measured. The operation condition during cultivation was set as follows: temperature (T): $25\pm 1^\circ\text{C}$, air flow rate: 1.5 L/min, and the cultured was agitated at 250 RPM. The harvested algae were centrifuged at 4°C with a speed of 3500 RPM for 20 minutes. Then, the residue was washed 3 times with distilled water to eliminate the remaining salt of the medium from wet algal biomass. The wet biomass was transferred to the freezing dryer or lyophilization in order to dehydrate and dry under vacuum condition. The lyophilized powder was used for lipid extraction.



Figure 3-1: Labfors 5 bioreactor for large volume cultivation

3.4 Assay Development

The possible effect of plant hormones on the growth and lipid production rate of microalgae was expected to be of small magnitude. An assay had to be developed that could reproducibly detect differences in growth rates lower than 10%. Phototrophic and mixotrophic growth was investigated as follows:

In shake flask: Depending on the size of shake flask 100 to 200 ml media was added to each flask. Culturing and maintenance was done according to section 3.3. The optical density of each sample was measured once to twice a day.

In microtiter plate: In each well, 20 μl algae culture was added to 180 μl medium, and either kept in Infors- shaker incubator at $25\pm 1^\circ\text{C}$ with a speed of 120 RPM and a light intensity of $95\pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$ and OD was measured twice a day or incubated in Tecan plate reader and optical density was measured every hour.

3.4.1 Assay Verification: Measuring the Effect of Inhibitor Compounds on the Growth Kinetic of Microalgae

For establishing a technique to detect small differences in growth rate, compounds with known inhibiting effects were chosen. For this purpose, Ethanol and DMSO with concentrations of 1%, 2%, 3% and 4% (V/V) were added to the media.

3.5 Plant Hormones

For this study, five different plant hormones, Epibrassinolide (EBL) from Brassinosteroid family, 1-naphtalenacetic acid (NAA), Indole 3 acetic acid (IAA) and Indole 3 butric acid (IBA) from Auxin family and Kinetin from Cytokinin family were purchased from Sigma-Aldrich. For preparing hormone solutions they were dissolved in suitable solvents. Weaker solutions were prepared by serial dilution from stock solutions (Table 3-2).

Table 3-2: Family, solvent and working concentration of plant hormones

Family	Hormone	Working concentration (M)	Solvent
Cytokinin	Kinetin	10^{-5} to 10^{-9} M	1N NaOH
Auxin	Indole 3 acetic acid (IAA)	10^{-5} to 10^{-9} M	Water
Auxin	Indole 3 butric acid (IBA)	10^{-5} to 10^{-9} M	1N NaOH
Auxin	1-naphthalenacetic acid (NAA)	10^{-5} to 10^{-9} M	1N NaOH
Brassinosteroid	EpiBrassinolide (EBL)	10^{-6} to 10^{-12} M	Ethanol

For measuring the biomass concentration of microalgae in the presence of these plant hormones, a 96-well plate was used and 200 μ l of each sample was pipetted into each well. The optical density (OD) was measured with Tecan infinite M1000 plate reader.

3.6 Analysis of Samples

3.6.1 Cell Density

Growth and biomass concentration of *Chlorella vulgaris* were measured by a spectrophotometer. Optical density (OD) was recorded at 684 nm where it shows the highest peak for chlorophyll (Held, 2011). The samples were diluted whenever it was necessary to give an absorbance in the range 0.1–1.0 nm (Salama et al., 2013).

3.6.1.1 Spectrophotometer

Dual beam UV/VIS Spectrophotometer from Thermo Scientific is one of the devices that was used for measuring the cell density at 684 A. 1 cm disposable cuvettes were used for samples.

3.6.1.2 Plate Reader

Tecan infinite M1000 plate reader is another instrument used for measuring the optical density (OD) and fluorescent intensity in microscale. It is a multifunctional monochromator-based microplate reader with high performance and automatic control (Figure 3-2).



Figure 3-2: Infinite M1000 with injector box

3.6.1.3 Dry Weight and Biomass Concentration

Algal biomass concentration is one of the most critically needed measurements in this study. For this purpose a calibration curve was prepared as follows:

1. 15 ml sample was used and prepared twofold serial dilution.
2. The optical density (OD) was measured with two different devices.
3. A 4.7 cm glass microfiber filter was dried in the oven at 105 °C for 24 hours till its weight became constant.
4. The dry glass microfiber filter weight was measured (W_0).
5. The samples were filtered and dried in the oven for 24 hours.
6. The dry glass microfiber filter was weighed with the sample (W_s).
7. The dry cell weight was calculated using equation 3.1.

$$\text{Dry cell weight} = W_s - W_0 \quad (3.1)$$

The dry weight calibration curve, in Appendix 1, was created according to the above-mentioned procedure and used for measurements.

The relationship between the optical density and the dry cell weight of samples was obtained after an extensive data analysis and is given by equation 3.2 and 3.3 for *Chlorella vulgaris* cuvette (10mm path-length) and 96-well plate (200 μ L per well ~ 3mm path-length) respectively.

$$\text{Dry cell weight (g/L)} = \text{OD}_{684} / 3.2926 \quad (3.2)$$

$$\text{Dry cell weight (g/L)} = \text{OD}_{684} / 1.2047 \quad (3.3)$$

3.6.2 Growth Rate Measurement

The growth of algae during the exponential growth phase can be described by Equation (3.4) (Andersen, 2005; Kong et al., 2012; Martinez & Orus, 1991) where x is dry cell weight concentration (g/L) at time t and μ_{max} is the specific growth rate (μ , day⁻¹) of *chlorella vulgaris*. This equation can be integrated from t_0 to t with $x=x_0$ at $t=t_0$ (3.5) and linearized (3.6). The exponential range was determined for each growth experiment and the growth rate was calculated via linear regression of 3.6.

$$\frac{dx}{dt} = \mu_{max} x \quad (3.4)$$

$$X = X_0 e^{\mu_{max} t} \quad (3.5)$$

$$\mu_{max} t = \ln \frac{x}{x_0} \quad (3.6)$$

3.6.2.1 Doubling Time

The doubling time (t_d) is a period of time required for the algal biomass to double. This increase takes place in the exponential phase and is calculated according to the following equation (Andersen, 2005; Mulumba & Farag, 2012).

$$t_d = \frac{\ln 2}{\mu} \quad (3.7)$$

3.6.3 Glucose Detecting

Glucose consumption was measured using HPLC (Agilent 1260, Hi-Plex H column). At the beginning, the glucose calibration curve for HPLC was prepared (Appendix 2). Then, *Chlorella vulgaris* was cultivated in the presence of glucose. Different samples were taken during microalgae growth till it reached the death phase. The samples were prepared and filtered with 0.2 µm syringe filters to remove any remaining particles and then transferred to HPLC to measure the glucose consumption during microalgae growth.

3.6.3.1 High-performance Liquid Chromatography (HPLC)

High-performance liquid chromatography or HPLC is a chromatographic technique for identifying, quantifying or purifying the individual components of the mixture.

The setup in this study was as follows:

The filtered samples were transferred to the Agilent HPLC system. 10 µl of the prepared sample was injected into the HPLC system, which consisted of an Agilent 1260 Series Liquid Chromatograph, equipped with a quaternary pump (G1311B), auto sampler and column compartment, refractive index detector, and ChemStation software. The separation was done with Hi-Plex H column at a temperature of 65°C and a flow rate of 0.6 ml/min. The mobile phase used was milliQ water. The total run time under these conditions was 15 min.



Figure 3-3: Agilent 1260 HPLC

3.6.4 Lipid Analysis

In this research, two different methods were used for analyzing lipids in *Chlorella vulgaris*.

3.6.4.1 Folch Method

One of the most common gravimetric techniques is called Folch method, which was invented by Folch and his colleagues in 1953. In this classic method certain weighed samples were homogenized with a chloroform- methanol (2:1) mixture to final dilution 20 folds of the volume. Then the solution was mixed in a vortex and cells were disrupted for 15 minutes in an ultrasonic device (0.5 cycle, amplitude 60%). The samples were kept in ice to avoid heating. In the next step, the mixtures were kept in an Infors HT Shaker-incubator with a speed of 120 RPM and at a temperature of 25°C overnight. Then the samples were filtered and the extracted liquid was mixed with 20% water (v/v), centrifuged for 15 minutes at 4°C and 3500 RPM. In the last step, the lighter phase was removed, the solvent was evaporated and the lipid was recovered and weighed (Folch et al., 1956).

3.6.4.2 Nile-red Staining Method

In order to determine the amount of neutral lipid in the samples a 96-well plate was used. For this purpose, 100 μ l of aliquot sample was pipetted into a Corning 3603 black-sided clear bottomed plate and 100 μ l of the Nile Red dye (1 μ g/ml) solution, prepared in 50% DMSO, was added. Nile red (9-(Diethylamino)-5H benzo [a] phenoxazine-5-one) is a red phenoxazine and lipid soluble dye which can be used to detect neutral lipids in vivo. Although this dye is very poorly fluorescent in aqueous solutions but it is quite photostable and highly fluorescent in non-polar hydrophobic environments. Nile red is dissolved in Dimethyl sulfoxide (DMSO) so that it can pass through the thick and rigid cell walls in many green algae. To read the fluorescence intensity the plate was kept in the Tecan plate reader for 30 minutes. Fluorescence intensities were measured from the top using 530 nm excitation and 570 nm emission wavelengths with a Tecan M1000 plate reader and the readings were recorded (W. Chen et al., 2011, 2009; Held & Raymond, 2011). Nile red was purchased from Sigma- Aldrich.

3.6.4.3 Correlating Fluorescence Data with Gravimetric Measurements

Folch method is a classic gravimetric method that can be used for calculating the total lipid content of microalgae. Unfortunately, this method is time consuming and this is one of the weaknesses of the technique (Bligh & Dyer, 1959b). On the other hand, the amount of neutral lipids can be calculated with Nile-red method which uses Nile-red dye as a lipophilic stain. The fluorescence intensity corresponding to the sample and dye could be quantified with Tecan M1000 plate reader (Held & Raymond, 2011). One of the goals in this study was to find a fast and reliable technique for calculating the total lipid content by measuring the fluorescence intensity.

In the first step, a 4-liter medium with a concentration of 15 g/L glucose was prepared in a bioreactor. *Chlorella vulgaris* was cultivated inside the bioreactor. The cultivation process continued until the microalgae reached the stationary phase which contains the highest amount of lipid. 20 ml of culture was used for preparing twofold dilution series. Absorbance was measured and the neutral lipid was identified with Nile-red staining

method. The rest of the culture was centrifuged at 4°C with a speed of 3500 RPM for 20 minutes. The residue was washed 3 times with distilled water in order to remove the remaining salt of the medium from algae biomass. The wet biomass was transferred to the freezing dryer for dehydration and drying. In the next step, the total lipid content of lyophilized algae powder was calculated using the Folch method.

With the purpose of calculating the total lipid from neutral lipids by measuring the fluorescence intensity of microalgae samples, the obtained data from Folch and Nile-red method were correlated. The curve in Figure 3-4 was used as the calibration curve to estimate the lipid content in an algae culture using the fluorescence intensity.

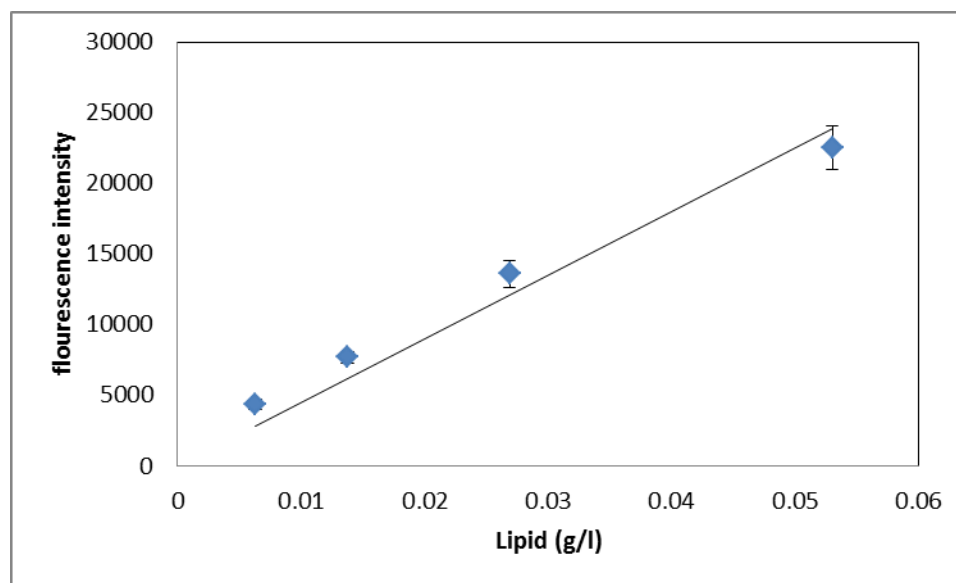


Figure 3-4: Amount of total lipid content in algae versus fluorescence intensity from Nile-red method (\pm SD).

3.7 Ionic Liquid Mediated Lipid Extraction

Most of the chemical reactions have been performed in molecular solvents. Ionic liquids (ILs) are a new class of solvents. They are organic salts that typically melt below 100°C and consist entirely of ionic species. They are suitable alternatives for volatile and unstable organic solvents due to their high thermal stability and nearly complete non-

volatility. They are also able to dissolve polar and non-polar organic, inorganic, and polymeric compounds and because of that have been given the title green solvents (Earle & Seddon, 2000; Kim et al., 2012; S. H. Lee et al., 2009)

In this stage, each sample vial was loaded with 1g ionic liquid and 0.2g lyophilized algae powder. As pretreatment, the mixtures in the vials were mixed in an ultrasonic device (0.5 cycle, amplitude 60%) for 30 seconds. The mixtures in vials were stirred at a speed of 250 RPM using magnetic bars at 95°C for 3 hours.

3.7.1 Fractionation

After cooling the extracted mixtures to room-temperature, each sample was washed three times with 1 ml n-Hexane. The n-Hexane extracts were combined, the solvent was evaporated and the lipids were weighed.

3.7.2 Fatty Acid Analysis

To determine the fatty acid composition of lipids, Gas Chromatography (GC) is used (Kim et al., 2012; Lepage & Roy, 1984). Transesterification process is required for preparing samples before analysis with GC.

3.7.2.1 Esterification and Transesterification

In 15 ml pressure tubes, 10 mg of the concentrated lipid solutions was dissolved in 1 ml chloroform, 1 ml methanol and 50 µl Methyl nonadecanoate. Methyl nonadecanoate (C19:0 FAME) played the internal standard role in the samples. The concentration of Methyl nonadecanoate (Internal standard) was 10 g/L which was dissolved in chloroform. Then, 16.5 µl sulfuric acid (98%) was added as a catalyst to each sample vial. Then vials were sealed properly and kept in a 100°C water bath for 3 hours. In the next step, 0.5 ml milliQ water was added to each cooled mixture. Two phases were formed after using a vortex equipment for 1 minute.

3.7.2.2 Fatty acids determination

The organic phase accumulated at the bottom and was filtered with a 0.45 µm PTFE filter and transferred into the GC vials for analysis.

3.7.2.3 Gas Chromatography (GC)

In this study, an Agilent 7890A gas chromatograph was used for identifying and quantifying the fatty acids in the organic phase (Figure 3-5). The Agilent 7890A GC-FID used was equipped with a J&W HP-5 column (weak polarity, length: 30 m, diameter: 0.320 mm, film: 0.25 μ m) and Helium was applied as the carrier gas. A 2 μ l portion of the organic phase was injected, and the inlet was maintained at 280°C. The oven was maintained at 80°C for 2 min, heated to 140°C at a rate of 20 °C/min, and from 140°C to 260 °C at a rate of 4 °C/min and then maintained at 260°C for 10 min. Peak detection was performed with a flame ionization detector, which was maintained at 280°C.



Figure 3-5: Agilent 7890A gas chromatograph

3.7.3 Analytical and Calculation Method

Qualitative and quantitative measurements were performed on fatty acids. The qualitative measurement was carried out via retention time by employing a FAMES mix standard as the reference (Appendix 3). For quantitative measurements Methyl nonadecanoate was used as the internal standard and the amounts of fatty acids were calculated via the following equation based on the internal standard principle (Appendix 4):

$$C_{\text{FAME}} = (A_{\text{FAME}} \times C_{\text{methyl nonadecanoate}} \times f_{\text{FAME}}) / A_{\text{methyl nonadecanoate}} \quad (3.6)$$

Where C_{FAME} , represents the concentration of the FAME in the sample. A_{FAME} , $C_{\text{methyl nonadecanoate}}$ and f_{FAME} are the FAME's peak area of FID signal, the concentration of the C19:0 FAME, and the coefficient, respectively. Each FAME has its unique f_{FAME} which can be calculated by using the peak area of the mixed FAMES standard and C19:0 FAME with known concentrations. $A_{\text{methyl nonadecanoate}}$ represents the C 19:0 FAME's peak area.

3.7.4 Experimental Set-up

In the first experiment, extraction efficiencies of Ionic liquids (ILs) were compared with organic electrolyte solution (OES). Because of that, two different ILs; AMIMCl (1-allyl-3-methylimidazolium chloride) and EMIMCl (1-Ethyl-3-methylimidazolium chloride); and one OES; Dimethyl sulfoxide (DMSO); were chosen. The chemicals were donated from Chinese Academy of Science.

Then, the extraction efficiencies of ILs were compared to the conventional gravimetric method. For this purpose, samples with two different ILs mentioned in the previous paragraph were compared with another sample prepared with the extracted lipid using Folch method. After sample preparation, the data were analyzed with GC.

In the final experiment, two different heterogeneous acidic catalysts were used in comparison with sulfuric acid during transesterification. The transesterification reaction can be catalyzed by acids or bases. In order to reduce the cost and recycle the catalyst, reduce alkaline waste-water treatment, and interference of free fatty acids and water, some new catalysts have been recommended such as heterogeneous catalysis (Zhao & Baker, 2013). Amberlyst 36 and 70 are strong acidic catalysts with high selectivity, long life time, resistance to superior resistance to thermal, mechanical, and osmotic shock and excellent stability, which are suitable for aqueous and non-aqueous media applications (Kunda et al., 2011).

3.8 Experimental Design and Statistical Analysis

All samples were prepared in triplicate and the mean values with their standard deviations are reported. Each experiment was repeated at least 4 times and the results of all repetitions were analyzed by Origin Pro 8.6 and Student T-test. In all cases, comparisons that showed a p value less than 0.05 were considered as significant.

Chapter 4

4 Results and Discussions

At the beginning of the project, before working with plant hormones, the sensitivity of the proposed methodology was tested and validated under different conditions. In the next step, the plant hormones were used to investigate their effect on biomass and lipid content of microalgae. Finally, the lipid content extracted from *Chlorella vulgaris* was analyzed.

4.1 Manipulating Algae Growth

4.1.1 Assay Development

4.1.1.1 Phototrophic Culture

In this experiment the prepared culture of *Chlorella vulgaris* was grown phototrophically and its growth rate was measured. According to Figure 4-1 data, there is no exponential growth phase, but linear growth instead. This is likely due to external limitations, either mass transfer or light. The system was therefore not biochemically limited; hence it would not be possible to detect any effects of hormones on growth rate. Therefore mixotrophic cultivation was investigated in subsequent experiments.

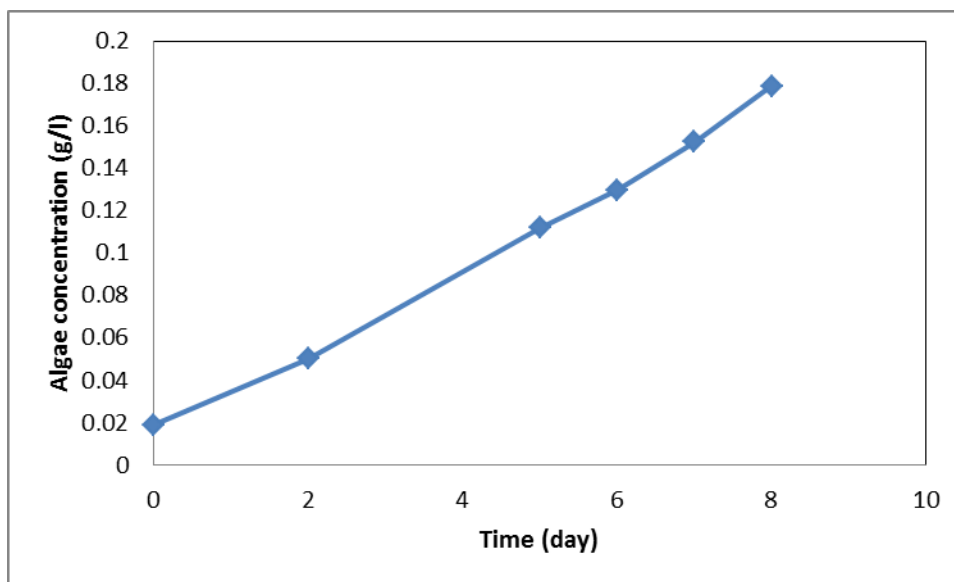


Figure 4-1: Growth curve for *Chlorella vulgaris* in phototrophic culture. The error bars represent standard deviations from the average biomass.

4.1.1.2 Mixotrophic Culture

In the first step, the growth rate of *Chlorella vulgaris* in the presence of different concentrations of glucose was investigated. The media were prepared in 125 ml flasks and glucose was added as a source of organic material. For this purpose, solution with glucose concentration of 5 g/L was added to Modified Bold's Basal medium. The optical density (OD) of microalgae biomass was measured at 684 nm everyday using a Tecan platereader and converted to dry biomass concentration g/L using the calibration curve (Appendix1). Results presented in Figure 4-2 demonstrate the effects of glucose on the growth curves, kinetics and biomass production of *Chlorella vulgaris* under mixotrophic cultivation. After the lag phase (24 hours), the algal cells entered their logarithmic growth phase in mixotrophic condition while phototrophic microalgae grew linearly. The maximum biomass content of 1 g/L was obtained in the mixotrophic culture in the presence of glucose. The results suggested that using glucose as an organic carbon source could increase the growth rate, cell density and dry weight of *chlorella vulgaris* for mixotrophic cultivation (Griffiths, 1963; Kong et al., 2013; Mayo & Noike, 1994b).

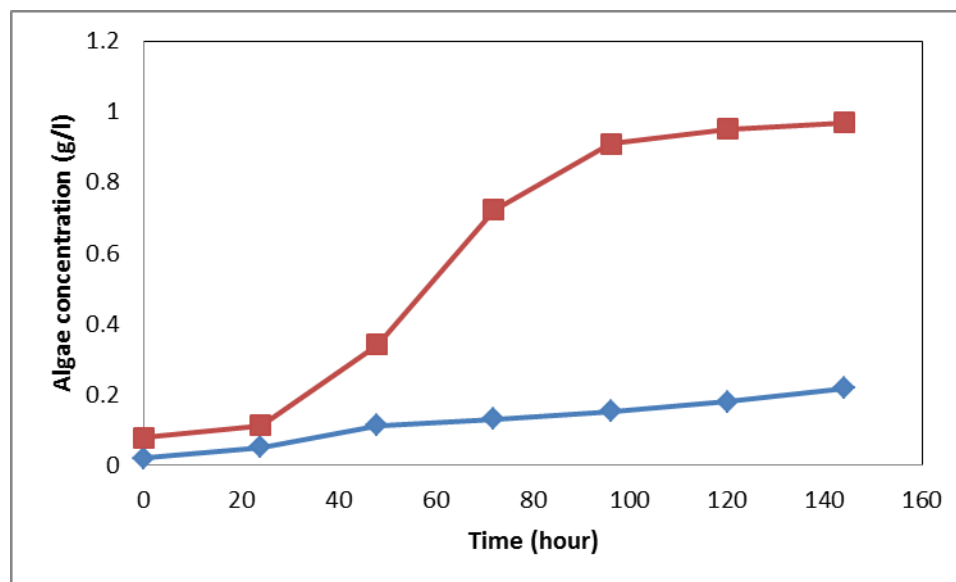


Figure 4-2: Growth curve for *Chlorella vulgaris* in the presence of 5 g/L glucose in the media. The error bars represent standard deviations from the average biomass.

Symbols: (◇) Control, (□) 5 g/L glucose

4.1.1.3 Glucose Detection

Subsequent experiments used chemotropically-growing algae to ensure that the metabolic rate was growth limiting, as it was expected this rate could be influenced by hormones. Monitoring the glucose uptake by *Chlorella vulgaris* is completed by high-pressure liquid chromatography (HPLC), which is a well-established tool for analyzing carbohydrates (Parpinello & Versari, 2000). As shown in Figure 4-3, the algae clearly grew exponentially. The maximum specific growth rate (μ_{\max}) is $0.072 \text{ h}^{-1} \pm 0.003$ and the doubling time is about 9.56 hours. At the end of the stationary phase, less than 1g/L glucose remained from the total initial amount. The ratio of the biomass produced to the amount of substrate consumed is defined as the biomass yield and was calculated according to the following equation:

$$\text{Biomass yield} = (X_{t1} - X_{t0}) / (S_{t0} - S_{t1}) \quad \text{Equation 4.1}$$

The amount of biomass yield at the end of the exponential phase was about 0.4 g/g. According to obtained data, at the end of the growth rate, still some glucose remained that showed that *Chlorella vulgaris* did not consume all the glucose. It can be concluded that after microalgae cells stopped growing but kept consuming glucose which indicated that they started making lipids, possibly ran out of nitrogen.

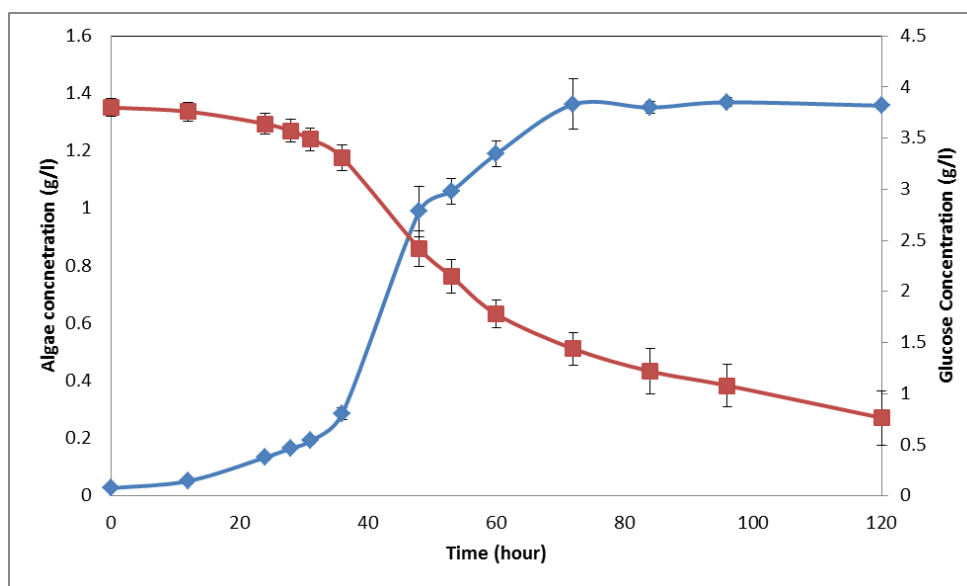


Figure 4-3: Kinetic growth and glucose consumption of *Chlorella vulgaris* in the presence of 5 g/L glucose. The (◇) is presenting the growth kinetic of *Chlorella vulgaris* and (□) is showing the glucose consumption by *Chlorella vulgaris*.

4.1.1.4 Comparing the Mixotrophic Growth of Microalgae in Shake-flask, 96-well Plate and Bioreactor

The next step after comparing mixotrophic with phototrophic culture growth was to find a suitable scale for culture growth for the rest of the study. Therefore, three different scales were used, which will be discussed in the following sections.

4.1.1.4.1 Mixotrophic Growth in Shake-flask

Growing *Chlorella vulgaris* in sake flask is one of the common options used by many researchers. In this experiment, a mixotrophic culture was prepared and the flasks were

kept in the Infors shaker incubator. The optical densities of samples were measured every 24 hours and the data were correlated to dry biomass. Figure 4-4 shows the obtained data for the growth rate of microalgae in shake flask.

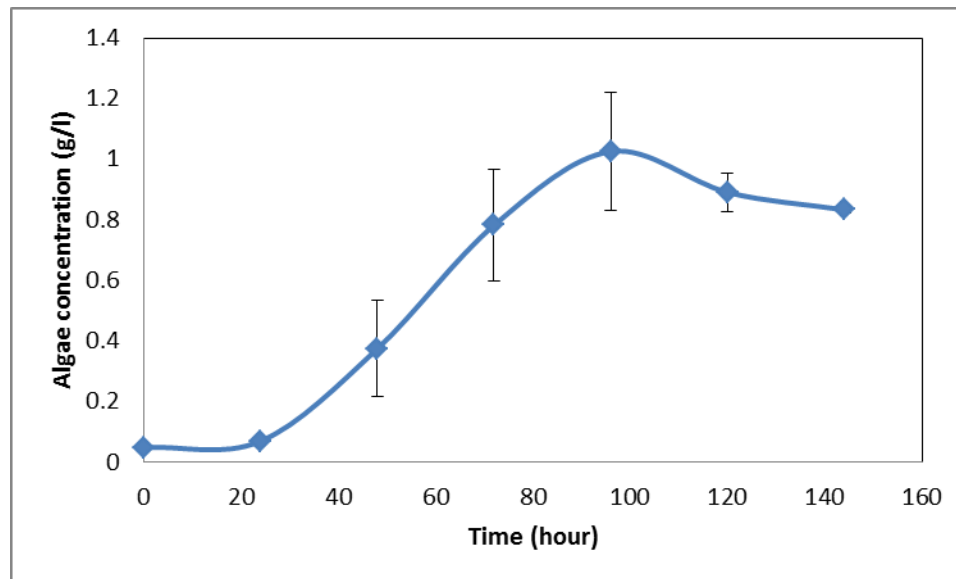


Figure 4-4: *Chlorella vulgaris* growth rate in shake flask

4.1.1.4.2 Mixotrophic Growth in 96-well Plate

Using a 96-well plate is another option for growing culture in microscale. The small volume of this type of plate, combination of more than one sample in a plate and the small space required for incubation are some of the advantages of this culture scale compared to other two methods. Figure 4-5 shows the growth of *chlorella vulgaris* in this scale. The standard deviations are small compared to shake flask and cannot be observed in the graph.

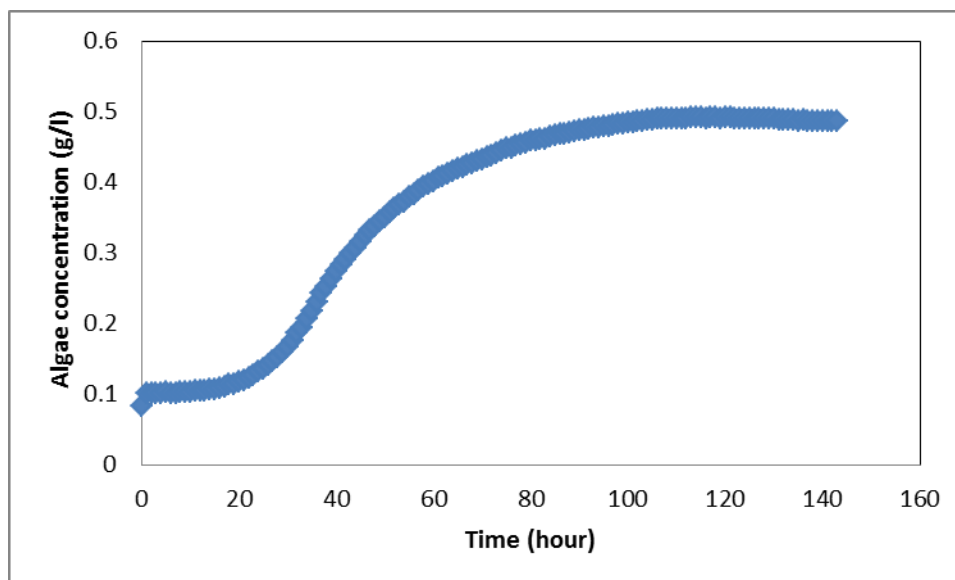


Figure 4-5: *Chlorella vulgaris* growth rate in 96-well plate

4.1.1.5 Mixotrophic Growth in Bioreactor

A fully controlled Infors HT bench-top bioreactor was used for mass cultivation of *Chlorella vulgaris* and to determine accurate growth kinetics as a benchmark. Infors HT bioreactors permit a better control of the cultivation conditions compared to flasks (Ugwu et al., 2008). A 4-liter Modified Bold's Basal medium was prepared and 5 g/L glucose was added to the medium. The bioreactor worked for almost 7 days and the biomass concentration of microalgae was measured with the spectrophotometer. It took 2.5 days till algae entered the logarithmic growth phase (Figure 4-6).

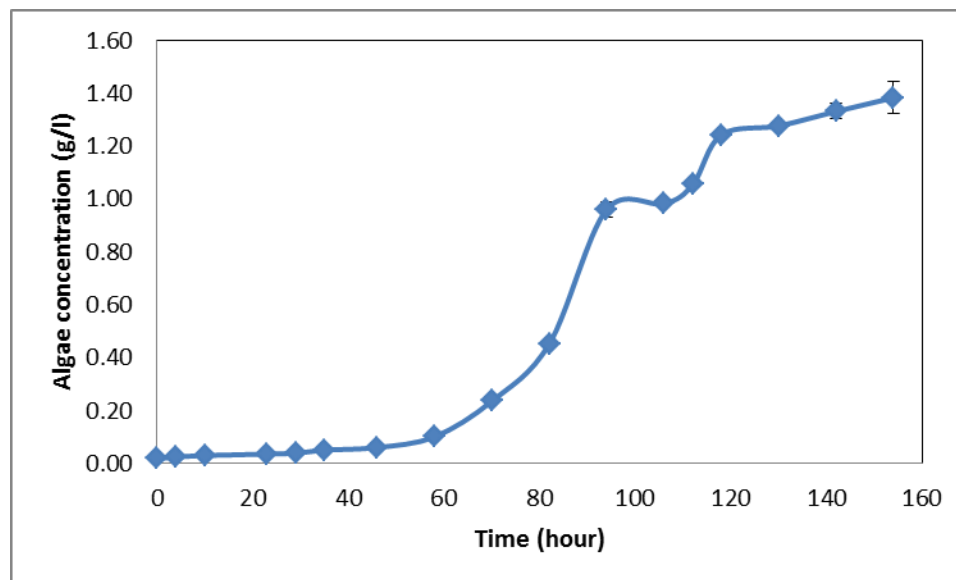


Figure 4-6: *Chlorella vulgaris* growth rate in Infors HT bioreactors

Comparing the microalgae growth results of the three different scales suggested that bioreactor could produce the highest amount of biomass among all but the maintenance and energy cost of bioreactor is higher compared to other scales. On the other hand, the standard deviation of the growth rate in plate reader is smaller and the amount of used media and culture is less than the other two.

4.1.2 Assay Validation

Before working with plant hormones the sensitivity of the proposed methodology was tested by investigating the effect of stress on growth rate and biomass concentration. Glucose was used as a carbon source and Ethanol and Dimethyl sulfoxide (DMSO) are two model compounds, which were used to disturb the system.

4.1.2.1 Ethanol

Street and Griffiths and their colleagues studied the effect of ethanol as a carbon source on the growth of microalgae. Their studies showed that ethanol as a carbon source had a positive effect on the growth of *chlorella vulgaris* below 0.1% but at 0.5% inhibited the cell division of *chlorella vulgaris* (El Jay, 1996a; Griffiths et al., 1960; Street, Griffiths et al., 1958). In this experiment, glucose was used as a carbon source and ethanol with

concentrations in the range 1-4% (v/v) was added. As can be seen in Figure 4-7 *Chlorella vulgaris* has the highest biomass production, the shortest lag phase and the longest exponential phase in the presence of glucose without any ethanol. With increasing the amount of Ethanol, the biomass production and exponential phase decreased while the lag phase increased. On the other hand, with increasing the ethanol concentration the specific growth rate (μ_{\max}) decreased and the doubling time increased (Table 4-1).

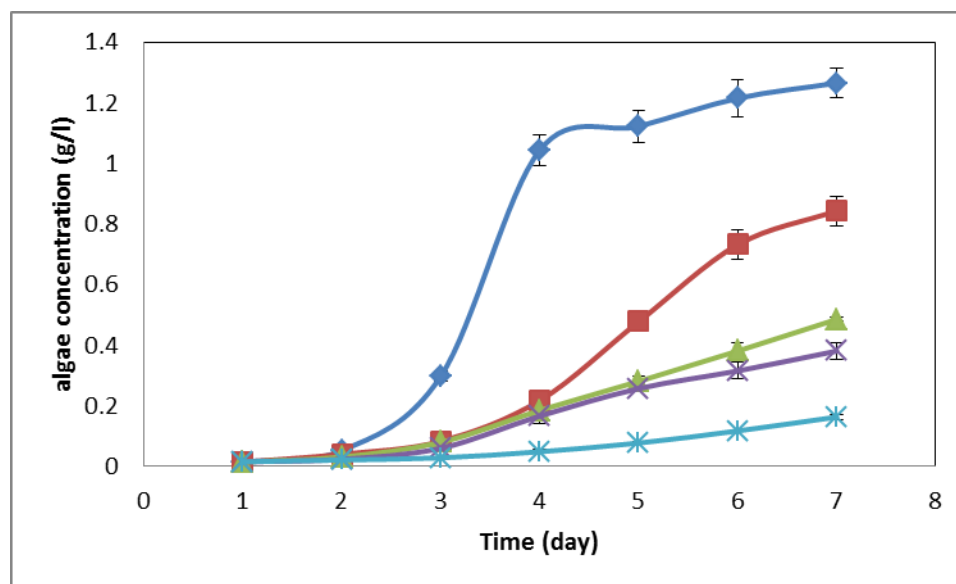


Figure 4-7: Growth kinetics of *Chlorella vulgaris* in the presence of 5 g/L glucose and different concentrations of ethanol (1, 2, 3 and 4% Ethanol (V/V)). Symbols: (◇), no Ethanol; (□) 1% Ethanol ;(Δ), 2% Ethanol; (X) 3% Ethanol; (*),4% Ethanol.

Table 4-1: The specific growth rate (μ_{\max}) and the doubling time of *Chlorella vulgaris* in the presence of 5 g/l glucose and different concentrations of ethanol (1, 2, 3 and 4% Ethanol (V/V)).

Ethanol Concentration (%)	μ_{\max} (d ⁻¹)±SD	Doubling time (d)±SD
No ethanol	1.50±0.14	0.46±0.06

1% ethanol	0.76±0.52	0.91±0.66
2% ethanol	0.63±0.08	1.10±0.2
3% ethanol	0.54±0.13	1.28±0.46
4% ethanol	0.43±0.02	1.61±0.11

4.1.2.2 Dimethyl Sulfoxide (DMSO)

Dimethyl sulfoxide (DMSO) is one of the organic solvents which is able to impose stress on test organisms (El Jay, 1996). For establishing a technique to detect the inhibitory effect of DMSO on the growth kinetics of *Chlorella vulgaris*, two different scale- size batch cultures were prepared. The screening test summarized in Figures 4-8 and 4-9 indicate the growth kinetics and biomass production of *Chlorella vulgaris* in the shake flask and 96-well plate, respectively. These two graphs illustrate the reverse relation between increasing DMSO and *Chlorella vulgaris* biomass. Using 4% DMSO (v/v) resulted in the lowest amount of algae biomass while 1% DMSO produced the highest biomass concentration and had the least effect on biomass production among all other concentrations. Instead, the doubling time and lag phase of microalgae augmented with increasing the DMSO concentration but the specific growth rate (μ_{\max}) declined (Table 4-2 and 4-3). As a result, it can be deduced that a DMSO concentration more than 1% causes growth inhibition and concentrations above 5% cause total inhibition (El Jay, 1996; Hess, 1980).

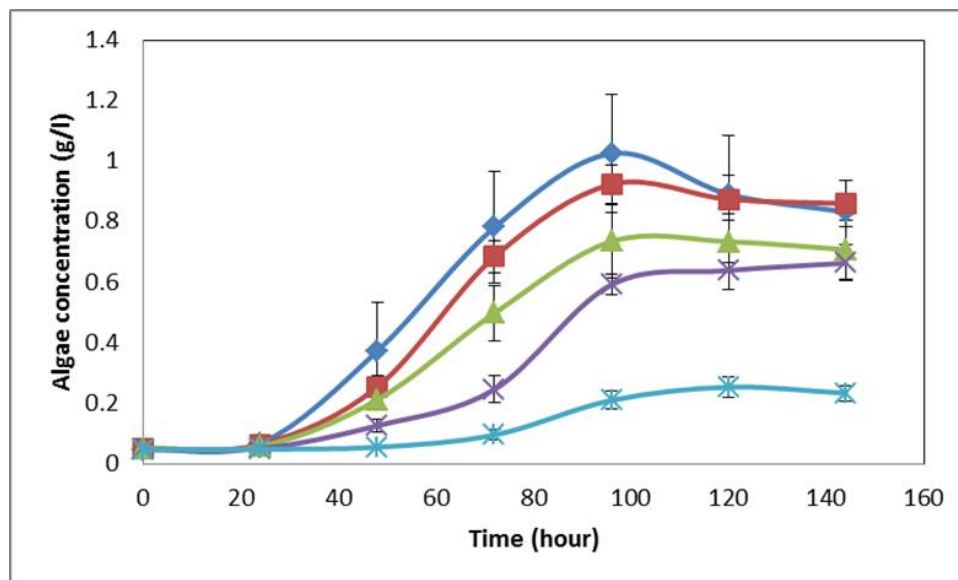


Figure 4-8: Effect of different concentrations of DMSO on the growth kinetics of *Chlorella vulgaris* in the presence of 15 g/L glucose in the shake flask. Symbols: (\diamond), no DMSO; (\square) 1% DMSO ;(Δ), 2% DMSO; (\times) 3% DMSO; (*),4% DMSO.

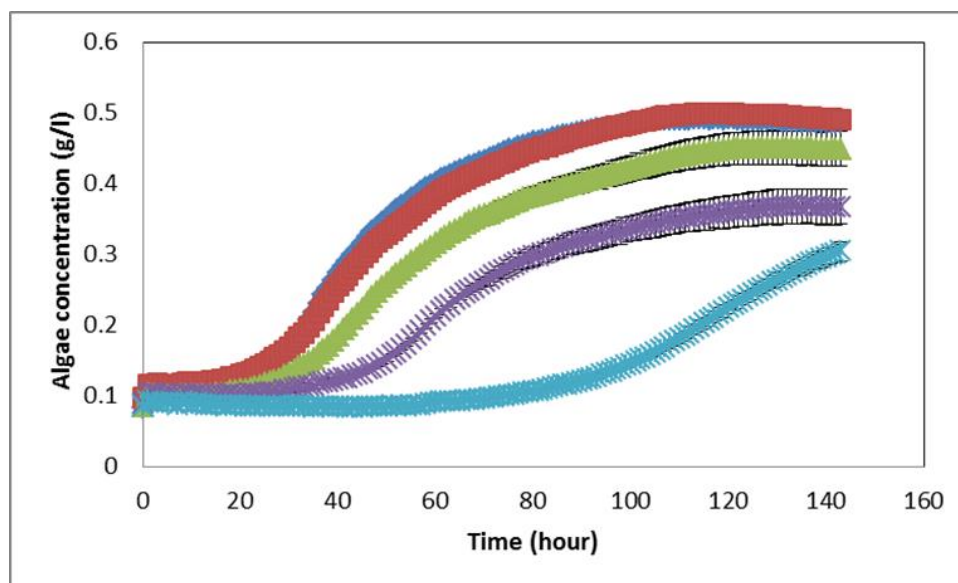


Figure 4-9: Effect of different concentrations of DMSO on the growth kinetics of *Chlorella vulgaris* in the presence of 15 g/L glucose in a 96-well plate. Symbols: (\diamond), no DMSO; (\square) 1% DMSO ;(Δ), 2% DMSO; (\times) 3% DMSO; (*),4% DMSO.

Table 4-2: Maximum specific growth rate (μ_{\max}) and doubling time of *Chlorella vulgaris* in the presence of 15 g/L glucose and 1 to 4% of DMSO.

DMSO concentration (%)	μ_{\max} (h^{-1}) \pm SD	Doubling time (h) \pm SD
No DMSO	0.042 \pm 0.007	16.64 \pm 4
DMSO 1%	0.037 \pm 0.007	18.71 \pm 5.2
DMSO 2%	0.035 \pm 0.006	19.86 \pm 4.95
DMSO 3%	0.033 \pm 0.001	20.95 \pm 0.90
DMSO 4%	0.022 \pm 0.003	31.48 \pm 6.19

Table 4-3: Maximum specific growth rate (μ_{\max}) and doubling time of *chlorella vulgaris* in the presence of 15 g/L glucose and 1 to 4% DMSO in the 96-well plate.

DMSO concentration (%)	μ_{\max} (h^{-1}) \pm SD	Doubling time (h)\pmSD
No DMSO	0.033 \pm 0.001	20.93 \pm 0.9
DMSO 1%	0.03 \pm 0.0006	23.51 \pm 0.65
DMSO 2%	0.028 \pm 0.0005	24.63 \pm 0.63
DMSO 3%	0.026 \pm 0.0005	27.11 \pm 0.73
DMSO 4%	0.016 \pm 0.0002	42.55 \pm 0.77

Preliminary conclusion:

Exponential algae growth can be detected in mixotrophic system. Influences of inhibitory compounds on the growth rate can be quantified. The standard deviation of the growth rate in shake flasks cultures was ~15%, rendering this method with the employed sampling rate not adequate to determine small differences. The standard deviation of the growth rate measured in microtiter plates was >3%. This method was therefore employed in future experiments.

4.1.3 Lipid Analysis

To analyze the lipid contents, two different methods were used. The classic Folch method is a gravimetric technique used for extracting the total lipid content of microalgae. Nile-red method can be used for measuring the neutral lipid in the living organism and Gas chromatography can be employed for the separation of lipids.

4.1.3.1 Folch Method

The results presented in Table 4-4 show the amount of lipid extracted from *Chlorella vulgaris* using Folch method. In this method more than 16% lipid was extracted from 1 gram of homogenized lyophilized microalgae biomass with a chloroform- methanol (2:1) mixture. The amount of extracted lipid grew linearly with increasing the biomass.

Table 4-4: Experimental results of lipid extraction with Folch method

Algae powder (g)	Lipid (g)±0.001
0.5	0.0737
1	0.1619
1.5	0.241
2	0.3249
3	0.4828

4.1.3.2 Nile-red Staining Method

Nile-red dye integration into *Chlorella vulgaris* cultures is fast and steady. DMSO affects the microalgae cell wall and the dye dissolved in DMSO partitions to the lipid beads contained in cells. A fluorescence peak was observed after the addition of dye (Held & Raymond, 2011). Comparing the culture with carbon source with the one without carbon source (Figure 4-10) shows that in the similar growing conditions and time, the microalgae without carbon source couldn't produce neutral lipids while the other series contained 5 g/L glucose as carbon source could produce neutral lipids and therefore showed fluorescence peaks after adding Nile-red dye, which could be measured with Tecan plate reader. On the other hand, measuring the biomass growth with the spectrophotometer and neutral lipid with Nile-red method showed that the total amount of neutral lipid increased with increasing the biomass concentration and the highest lipid content was obtained during the stationary phase while cells stopped growing and producing lipid (Figure 4-11). In this experiment the maximum lipid productivity was reached after 11 days.

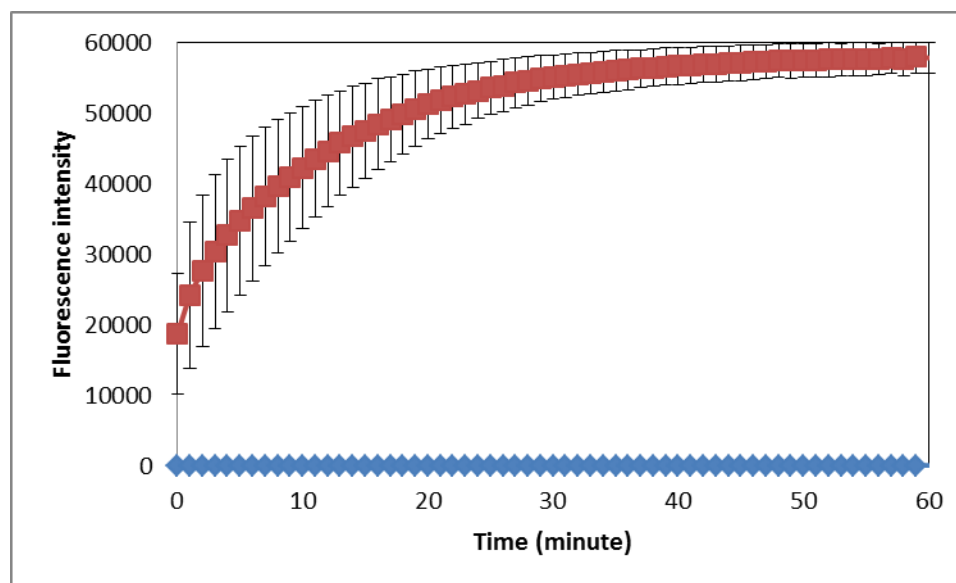


Figure 4-10: Lipid determination using Nile Red technique for *Chlorella vulgaris*. (□) sample with 5 g/L glucose as carbon source; (◇) sample without carbon source.

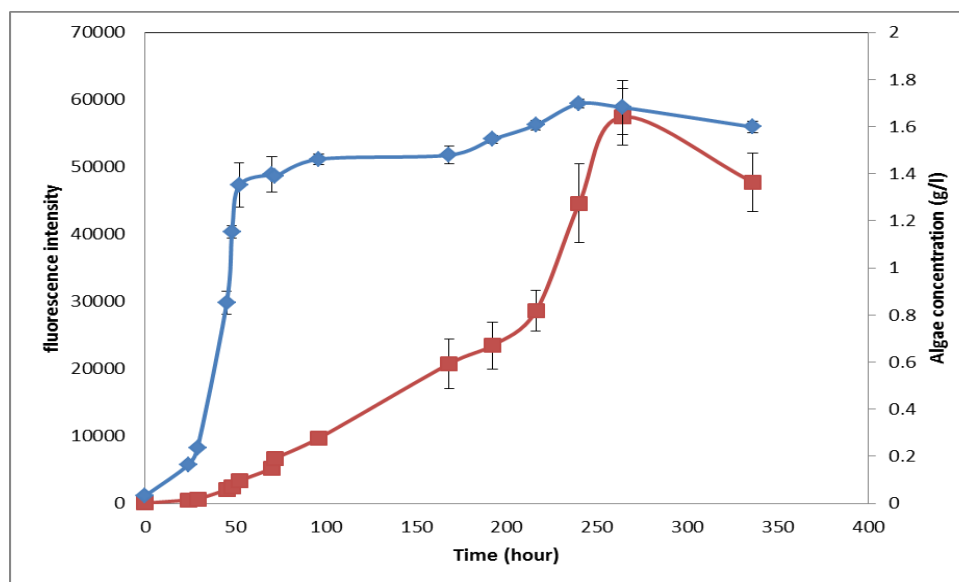


Figure 4-11: Growth kinetic and neutral lipid determination using Nile-red method in *Chlorella vulgaris* in the presence of 15 g/L glucose. (◇) Growth kinetic of *Chlorella vulgaris*; (□), amount of neutral lipid.

4.1.4 Effect of Plant Hormones on *Chlorella vulgaris*

Chlorella vulgaris was cultivated with different concentrations of five classes of plant hormones. The cell density was quantitatively measured by measuring the optical density (OD) of each sample and lipid content was estimated at the end of the stationary phase. The hormones were prepared according to Table 3-2. The concentration working range of Kinetin, Indole 3 acetic acid (IAA), Indole 3 butric acid (IBA), and 1-naphtalenacetic acid (NAA) were between 10^{-5} - 10^{-9} M. No significant effect on growth rate, yield and lipid content was found (detailed growth profiles are shown in Appendix 5). The normalized data based on the control for final biomass concentration, growth rate and lipid content of *Chlorella vulgaris* in the presence of these hormones is shown in Figure 4-12, 4-13 and 4-14, respectively. As seen in the Figures, small effects of the hormones might exist, however the data is at best inconclusive, as no clear trend with respect to hormone concentration can be seen. The applied assay could be refined further to detect

such small differences; however the differences are so small that there is no direct interest from an application point of view.

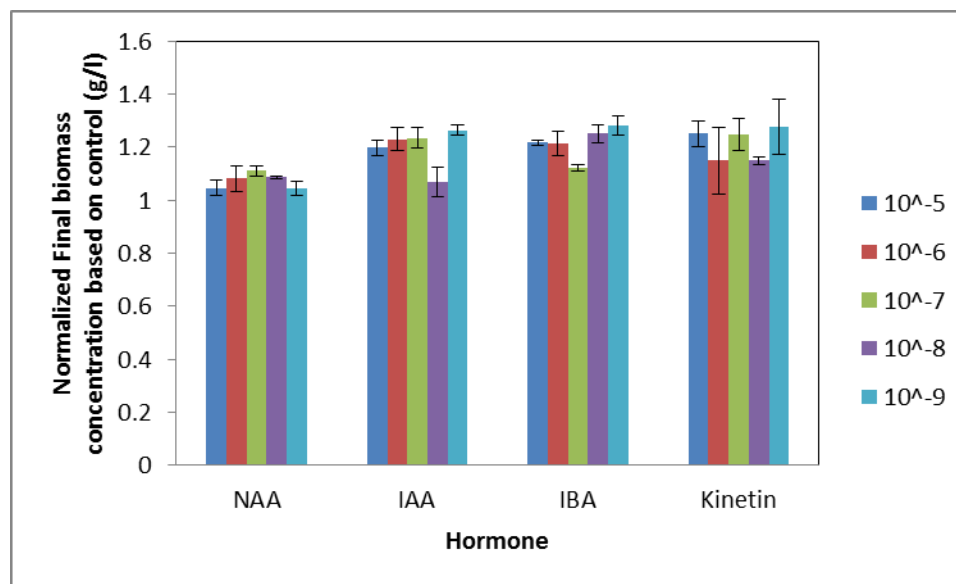


Figure 4-12: Normalized data for final biomass concentration of *Chlorella vulgaris* based on the control sample in the presence of 1-naphthalenacetic acid (NAA), Indole 3 acetic acid (IAA), Indole 3 butric acid (IBA), and Kinetin.

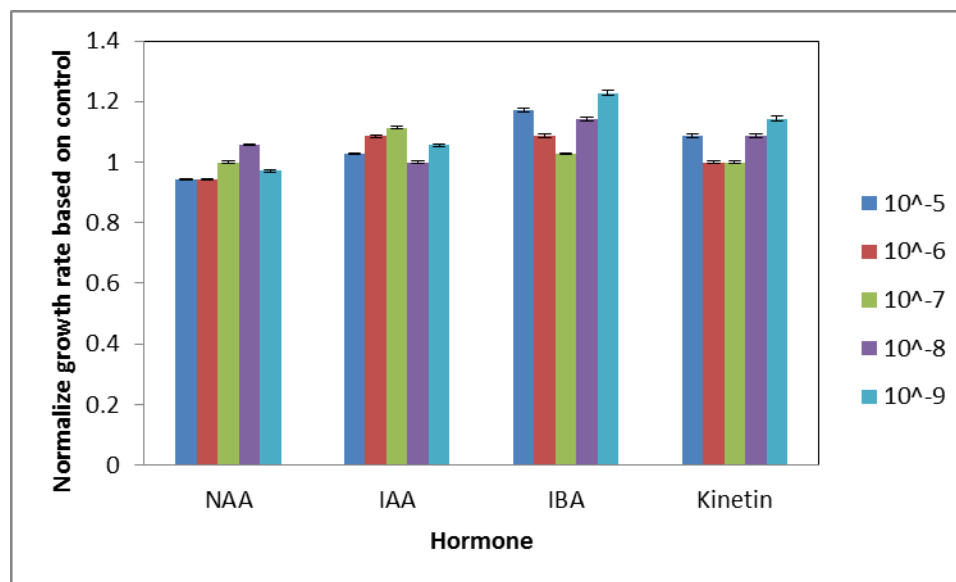


Figure 4-13: Normalized data for growth rate of *Chlorella vulgaris* based on control sample in the presence of 1-naphtalenacetic acid (NAA), Indole 3 acetic acid (IAA), Indole 3 butric acid (IBA), and Kinetin.

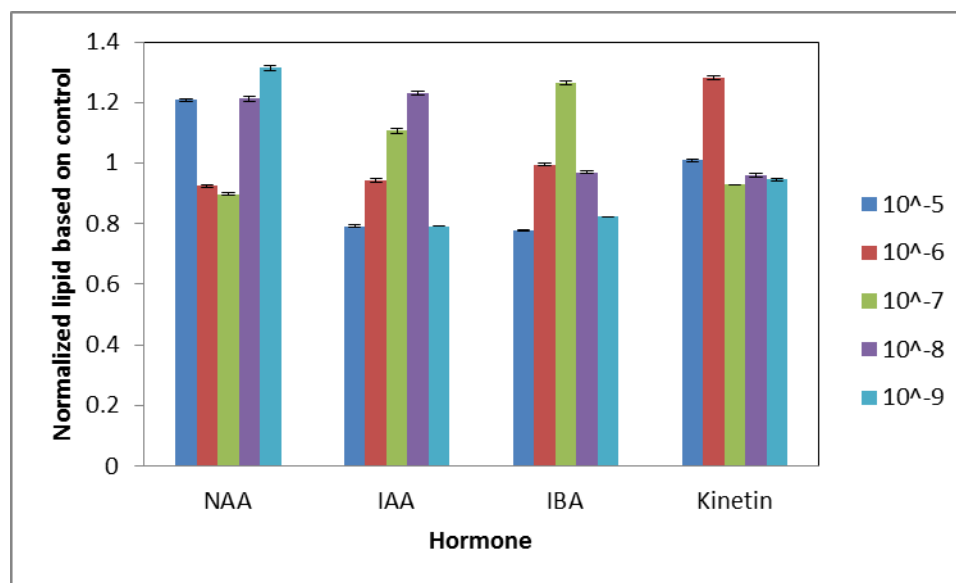


Figure 4-14: Normalized data for lipid content of *Chlorella vulgaris* based on control sample in the presence of 1-naphtalenacetic acid (NAA), Indole 3 acetic acid (IAA), Indole 3 butric acid (IBA), and Kinetin.

4.1.5 Effect of Epibrassinolide (EBL) on the Growth Kinetics and Lipid Content of *Chlorella vulgaris*

Epibrassinolide (EBL) was chosen from Brassinosteroid families which are steroidal compounds and stimulate growth and show different physiological responses at nanomolar to micromolar concentrations, (Andrzej Bajguz & Asami, 2004). The biomass concentration and growth kinetics data are presented in Figure 4-15. Among different concentrations, EBL with concentrations of 10^{-12} , 10^{-11} and 10^{-10} M produced more than 0.7 g/L dry biomass. This is statistically significant compared to the control sample which produced 0.34 g/L dry biomass. The lag phase was the same for all concentrations during the first 42 hours and after that different behaviors were observed in the exponential phase. These variations can be tracked in the specific growth rate (μ_{\max}) and doubling time of cultures too (Table 4-6). The lipid production of *Chlorella vulgaris* in the presence of this hormone significantly increased compared to the control sample without any Epibrassinolide. According to the obtained data, the lowest concentration hormone sample (10^{-12} M) which produced the highest final biomass concentration produced the most total amount of produced lipid among the other concentrations (Table 4-7).

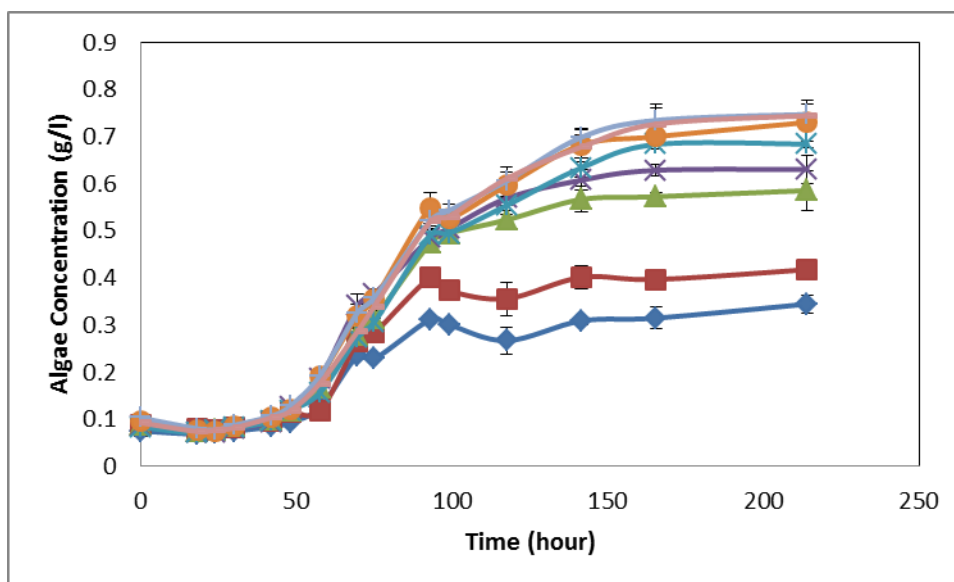


Figure 4-15: Effect of different concentrations of Epibrassinolide (EBL) on the growth kinetics of *Chlorella vulgaris* growing on glucose in the 96-well plate.

Symbols: (\diamond), Control; (\square), EBL 10^{-6} ; (Δ), EBL 10^{-7} ; (\times), EBL 10^{-8} ; (*), EBL 10^{-9} ; (\circ), EBL 10^{-10} ; (+), EBL 10^{-11} ; (-), EBL 10^{-12} .

Table 4-5: Maximum specific growth rate (μ_{\max}) and doubling time of *Chlorella vulgaris* in the presence of 15 g/l glucose and 10^{-6} - 10^{-12} M Epibrassinolide (EBL) in the 96-well plate.

Hormone concentration (M)	μ_{\max} (h^{-1}) \pm SD	Doubling time (h) \pm SD
EBL 10^{-6}	0.026 \pm 0.003	26.53 \pm 4.41
EBL 10^{-7}	0.027 \pm 0.002	25.32 \pm 2.70
EBL 10^{-8}	0.029 \pm 0.002	23.75 \pm 2.34
EBL 10^{-9}	0.031 \pm 0.002	22.45 \pm 2.05
EBL 10^{-10}	0.031 \pm 0.002	22.31 \pm 2.05
EBL 10^{-11}	0.031 \pm 0.002	22.17 \pm 2.05
EBL 10^{-12}	0.032 \pm 0.002	21.80 \pm 1.92
Control	0.023 \pm 0.002	30.74 \pm 3.73

Table 4-6: The amount of produced lipid (g/L) in *Chlorella vulgaris* in the presence of 10^{-6} - 10^{-12} M Epibrassinolide (EBL).

Hormone concentration (M)	Lipid content (%), \pm SD	Final biomass (g/L)	Total amount of lipid produced (mg/L) \pm SD
EBL 10^{-6}	26.37 \pm 0.002	0.42 \pm 0.01	111
EBL 10^{-7}	30.77 \pm 0.003	0.58 \pm 0.04	178

EBL 10 ⁻⁸	20.64±0.002	0.63±0.03	130
EBL 10 ⁻⁹	27.8±0.006	0.68±0.01	189
EBL 10 ⁻¹⁰	21.93±0.003	0.73±0.01	160
EBL 10 ⁻¹¹	24.11±0.003	0.75±0.03	181
EBL 10 ⁻¹²	26.9±0.006	0.74±0.03	199
Control	29.07±0.004	0.34±0.02	99

4.1.6 Preliminary Summary and Conclusion

An assay was developed to successfully measure small changes in growth-rate and lipid content of microalgae. The assay was verified for known growth inhibitors and subsequently used to evaluate the effect of plant hormone on the growth of *Chlorella vulgaris*. Five different hormones representing different hormonal categories were tested and only EBL showed significant effects on the tested microalgae. The algae growth rate was not significantly affected by EBL; however the right concentration (10⁻¹² M) had a positive effect on their overall production of algal biomass and therefore on the overall amounts of lipids produced. However, the presence of this hormone in the culture could increase the total lipid of microalgae. It was shown that not all glucose was consumed without the presence of the hormones (Figure 4-3); hence it was possible to achieve higher glucose conversion in the presence of EBL. Bajguz and Czerpak's work showed that, Brassinosteroids hormones are able to increase cell division (A Bajguz & Czerpak, 1996) and prevent chlorophyll, sugar, and protein loss (Andrzej Bajguz, 2011).

4.2 Ionic Liquid Mediated Lipid Extraction

Algae processing and lipid extraction is a very cost-intensive step. An innovative method using ionic liquids to fractionate algae biomass was investigated in this thesis.

In this section, the effects of different ionic-liquids and catalysts on the extraction efficiency were investigated. The goal of ionic liquid mediated lipid extraction is to use an ionic liquid that is capable of dissolving carbohydrates, but not able to dissolve lipids. Mixing microalgae with such liquids results in disintegration of the cell-wall as structural carbohydrates dissolve in the ionic liquid. The lipids form a distinct layer (the removal of which can be enhanced with a non-polar solvent such as hexane) that can be decanted. The protein fraction typically forms a non-soluble solid fraction.

4.2.1 Comparing Extraction Efficiencies of ILs and OESs

In this part two different ILs; AMIMCl (1-allyl-3-methylimidazolium chloride) and EMIMCl (1-Ethy-3-methylimidazolium chloride); were compared to IL/DMSO (Dimethyl sulfoxide) mixtures (organic electrolyte solution (OES)) (Figure 4-16). The experimental set-up was done based on Table 4-8. The rest of the experiments were done according to section 3.6.5.3. The amount of FAMEs derived from the extracted algae lipids is illustrated in Table 4-9.

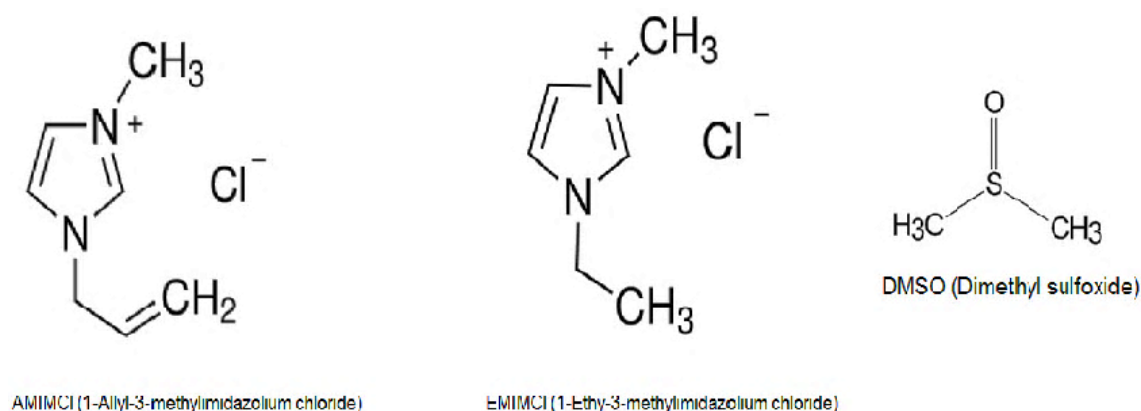


Figure 4-16: Chemical structure of AMIMCl (1-allyl-3-methylimidazolium chloride), EMIMCl (1-Ethy-3-methylimidazolium chloride); and DMSO (Dimethyl sulfoxide)

As can be seen in Table 4-9, the extraction efficiency of EMIMCl is higher compared to AMIMCl. On the other hand, mixing ILs with DMSO improved the extraction of total lipid from the samples. This increase is about 2 times when using an equal amount of

EMIMCl and DMSO in comparison with using EMIMCl alone. EMIMCl could extract C6:0 and C10:0 while AMIMCl could not extract these fatty acids, which shows the better performance of EMIMCl. DMSO has positive effects on the extraction yield of lipids from algae.

Table 4-7: Experimental set-up for comparing extraction efficiencies of ILs and OESs

Sample Name	Algae (g)	Extraction media (g)	
		IL	DMSO
RUN 1	0.2	2g AMIMCl	-
RUN 2	0.2	2g EMIMCl	-
RUN 3	0.2	1.4 g EMIMCl	0.6
RUN 4	0.2	1g EMIMCl	1

Table 4-8: Amount of FAMES derived from the extracted algae lipids

FAME	Amount of FAMES derived from the extracted algae fatty acids (mg/g D.W.)			
	RUN1	RUN2	RUN3	RUN4
C4:0	0.13	0.4	0.12	0.09
C6:0	0	0.09	0.01	0.02
C8:0	0.09	0.03	0	0
C10:0	0	0.34	0.17	0.31
C11:0	0.01	0	0.21	0
C12:0	0	0.01	0	0.02
C13:0	0	0.04	0.01	0
C14:0	0.03	0.01	0	0.02
C14:1	0.02	0.09	0.03	0.11
C15:1	0.02	0.03	0.02	0.14
C15:0	0.01	0.03	0.01	0.06
C16:1	0.15	0.05	0.23	0.1
C16:0	3.31	11.43	4.32	18.13
C17:1	0.04	0.07	0.03	0.06
C17:0	0.03	0.1	0.03	0.16
C18:3n6	0	0	0	0.03

C18:2n6c	2	2.91	1.63	8.32
C18:1n9/C18:2n6t/C18:3n3	2.97	4.59	2.56	12.66
C18:1n9t	0.84	2.52	1.4	5.19
C18:0	0.19	0.66	0.26	0.97
C20:5n3	0.02	0.01	0	0.06
C20:4n6	0	0.02	0	0.01
C20:3n6	0.01	0.01	0	0.04
C20:2	0.01	0.01	0	0.03
C20:3n3	0.01	0.01	0.02	0.05
C20:1n9	0	0.05	0	0.02
C20:0	0.03	0.1	0.04	0.15
C21:0	0.13	0.02	0.02	0.02
C22:6n3	0.28	0.2	0.15	0.11
C22:2	0	0	0	0
C22:1n9	0	0	0	0
C22:0	0.02	0.04	0.02	0.09
C23:0	0	0.01	0	0.01
C24:1n9	0	0	0	0.05
C24:0	0.02	0.07	0.03	0.11
Total	10.37	23.95	11.33	47.12

4.2.2 Comparison of Different Extractive Efficiencies of AMIMCl, EMIMCl and Folch Method

This section compares the extraction efficiency of two different ILs; AMIMCl (1-allyl-3-methylimidazolium chloride) and EMIMCl (1-Ethy-3-methylimidazolium chloride) with the extracted lipid using Folch method. The samples were prepared according to Table 4-10 and the results are shown in Table 4-11.

Table 4-9: Sample preparation procedure for comparing different extractive efficiencies of AMIMCl and EMIMCl

Sample Name	Algae (g)	Media
RUN 1	0.2	2g AMIMCl
RUN 2	0.2	2g EMIMCl
RUN 3	0.2	Folch method

Table 4-10: Amount of FAMES and their percentages derived from extracted algae lipids

Fatty acids	Amount of FAMES derived from extracted algae fatty acids (mg/g D.W.)					
	Folch		[AMIM]Cl		[EMIM]Cl	
C14:1	0.08±0.01	(0.12±0.01%)	0.01±0.01	(0.02±0.03%)	-	-
C14:0	0.12±0.01	(0.18±0%)	0.03±0.03	(0.18±0.08%)	0.03±0.01	(0.18±0.02%)
C15:1	0.02±0	(0.03±0%)	0.02±0.02	(0.09±0.08%)	0.03±0.01	(0.16±0.01%)
C15:0	0.11±0	(0.16±0.01%)	-	-	-	-
C16:1	0.22±0.01	(0.32±0.01%)	0.16±0.2	(1.63±2.43%)	0.04±0.02	(0.26±0.04%)
C16:0	20.55±1.12	(30.2±0.33%)	4.51±2.8	(28.84±0.72%)	4.92±1.73	(29.76±0.31%)
C17:1	0.16±0.01	(0.24±0.01%)	0.09±0.03	(0.79±0.57%)	0.04±0.02	(0.22±0.03%)
C17:0	0.19±0.01	(0.28±0.01%)	0.04±0.03	(0.24±0.08%)	0.05±0.02	(0.29±0%)
C18:3n6	0.05±0.01	(0.07±0%)	-	-	-	-
C18:2n6c	18.15±0.83	(26.69±0.51%)	4.19±2.59	(26.8±0.8%)	4.53±1.61	(27.36±0.22%)
C18:1n9/C18:2n6t/C18:3n3	25.11±1.27	(36.92±0.52%)	5.74±3.57	(36.7±1.12%)	6.2±2.19	(37.5±0.16%)
C18:1n9t	0.61±0.03	(0.9±0.02%)	0.15±0.09	(0.95±0.02%)	0.15±0.05	(0.91±0.01%)
C18:0	1.29±0.18	(1.89±0.15%)	0.27±0.19	(1.65±0.19%)	0.24±0.08	(1.48±0.04%)
C20:4n6	0.04±0.01	(0.05±0.02%)	-	-	-	-
C20:5n3	0.03±0	(0.05±0%)	-	-	-	-
C20:3n6	0.12±0.07	(0.17±0.09%)	-	-	0.01±0.02	(0.07±0.07%)
C20:1n9	0.11±0.03	(0.16±0.04%)	0.03±0.05	(0.13±0.16%)	0.02±0.01	(0.12±0.05%)
C20:2	0.12±0.02	(0.17±0.02%)	0.02±0.02	(0.15±0.06%)	0.03±0.01	(0.16±0.02%)
C20:3n3	0.03±0.05	(0.04±0.07%)	-	-	-	-
C20:0	0.17±0.01	(0.24±0%)	0.04±0.03	(0.25±0.01%)	0.04±0.01	(0.25±0.01%)
C21:0	0.03±0.01	(0.05±0.01%)	0.02±0.02	(0.12±0.04%)	0.01±0.01	(0.06±0.06%)
C22:6n3	0.44±0.52	(0.61±0.68%)	0.16±0.03	(1.17±0.44%)	0.13±0	(0.86±0.26%)
C22:1n9	0.04±0.06	(0.05±0.08%)	0.01±0.02	(0.05±0.08%)	-	-
C22:2	0.01±0.03	(0.02±0.03%)	-	-	-	-

C22:0	0.07±0.01	(0.09±0.01%)	-	(0.02±0.03%)	-	-
C23:0	0.03±0.05	(0.04±0.07%)	0.01±0.02	(0.05±0.08%)	-	-
C24:1n9	0.04±0.07	(0.06±0.1%)	-	-	-	-
C24:0	0.11±0.01	(0.17±0.01%)	-	-	0.01±0.02	(0.06±0.1%)
TOTAL	68.07±4.42	(100%)	15.55±9.48	(100%)	16.54±5.82	(100%)

Based on the obtained data, when using the extracted lipid from Folch method, the total amount of the extracted fatty acids is higher but there was no significant difference in extraction yield observed in the extracted fatty acids from samples with AMIMCl and EMIMCl.

4.2.3 Combining Lipid Extraction and Trans-esterification in a One-pot Process

The lipid recovery and trans-esterification are two separate processes in current methods for the production of biodiesel from microalgae. Process intensification has the potential to substantially reduce operating costs and therefore the possibility to combine ionic liquid mediated lipid extraction and trans-esterification into a single-pot process was investigated in this thesis.

Sulfuric acid is the most common used catalyst when converting lipids to actual biodiesel. Heterogeneous catalysts have the advantage that they can more easily be recycled, and that immersions can be generated in multiphase liquids, while homogenous catalysts would selectively partition between two immiscible liquid phases. The use of a heterogeneous catalyst might also allow for a simultaneous process combining ionic liquid based lipid extraction and trans-esterification in a one-pot process. Therefore two different heterogenous acidic catalysts; Amberlyst 36 and Amberlyst 70 (sulfuric acid as a control) were investigated and added during the extraction step.

The catalysts chosen for this work have high catalytic activity and long life time, they have high stability and also resistant to thermal and mechanical shocks (Kunda et al., 2011). The samples were prepared according to Table 4-12.

Table 4-11: Experimental set-up for comparing extraction efficiencies using different catalysts

Sample Name	Algae (g)	AMIMCl (g)	Methanol (g)	Catalyst
RUN A	0.2	2	0.5	0.02g H ₂ SO ₄
RUN B	0.2	2	0.5	0.02g Amberlyst 36
RUN C	0.2	2	0.5	0.02g Amberlyst 70

The amount of FAMES was directly analyzed after the extraction without a separate trans-esterification step. The results are presented in Table 4-13. When Amberlyst 70 was present in the sample more than 10 mg/g FAME was produced while the amount reduced to 0.76 mg/g when Amberlyst 36 was added and 1.45 mg/g when sulfuric acid was used as a catalyst. When separating extraction and esterification ~ 15 mg/g FAME could be recovered with the same ionic liquid (Table 4-10) under similar conditions. Sulfuric acid and Amberlyst 36 were not suitable for a combined extraction and trans-esterification process, however the initial results with Amberlyst 70 are highly promising and further optimization of the operating conditions and possibly catalyst composition might increase the overall yield of the process. These preliminary data clearly show that it is possible to combine lipid extraction and trans-esterification into a one-pot process, which might be able to reduce the overall cost of biodiesel production from micro-algae. Other challenges that still have to be overcome are suitable techniques to recycle the ionic liquid and the catalyst, which however extends the scope of this thesis.

Table 4-12: Amount of FAMES derived from extracted algae lipids

FAME	Amount of FAMES derived from extracted algae fatty acids (mg/g D.W.)		
	RUN A	RUN B	RUN C
C4:0	0.01	0.01	0.12
C6:0	0.06	0.02	0
C8:0	0	0	0.09
C10:0	0	0	0
C11:0	0	0	0.01
C12:0	0	0.01	0
C13:0	0	0	0

C14:0	0.02	0.01	0.03
C14:1	0.04	0.01	0.02
C15:1	0	0.04	0.01
C15:0	0.02	0.03	0.14
C16:1	0.01	0.04	0.13
C16:0	0.11	0.04	3.07
C17:1	0	0	0.03
C17:0	0	0	0.04
C18:3n6	0	0.02	0
C18:2n6c	0.04	0.01	1.84
C18:1n9/C18:2n6t/C18:3n3	0.07	0.01	2.73
C18:1n9t	0.31	0.09	0.79
C18:0	0.01	0.04	0.26
C20:5n3	0	0	0.02
C20:4n6	0	0	0
C20:3n6	0.01	0	0.07
C20:2	0.02	0	0.23
C20:3n3	0	0	0.01
C20:1n9	0	0	0
C20:0	0	0	0.04
C21:0	0.05	0.02	0.07
C22:6n3	0.39	0.17	0.29
C22:2	0.05	0.04	0
C22:1n9	0	0.08	0
C22:0	0.02	0.01	0.03
C23:0	0	0.01	0
C24:1n9	0	0.01	0
C24:0	0.2	0.04	0.04
Total	1.45	0.76	10.13

Chapter 5

5 Summary and Conclusions

5.1 Stimulatory or Inhibitory Effect on the Growth of Microalgae

The obtained data show that *Chlorella vulgaris* (UTEX 2714) can be manipulated with plant hormones when grown in mixotrophic condition with glucose as a carbon source. Comparing three different scales for growing microalgae showed that microplates were a suitable option for culturing microalgae in this project. The stress imposed on *Chlorella vulgaris* (UTEX 2714) by organic solvents such as Ethanol and Dimethyl sulfoxide (DMSO) was quantified in order to establish the sensitivity of the applied assay.

5.2 Effect of Plant Hormones on *Chlorella vulgaris*

Studying the effect of five different plant hormones from three families with dissimilar concentrations showed that some plant hormones had positive effects on the growth, biomass concentration and lipid content of *Chlorella vulgaris*; however, for the majority of investigated hormones no statistical difference was found. Among these five different plant hormones, Epibrassinolide (EBL) from the Brassinosteroid family was the only hormone that had a strong positive effect on the growth kinetics of *Chlorella vulgaris* compared to the control sample and the lowest concentration of this hormone yielded the maximum microalgae biomass concentration. The lipid content in the algae was not reduced; hence it also increased the total amount of lipid produced in the system. The four other plant hormones, which were used at similar working concentrations, did not show excessive changes in biomass concentration and growth kinetics of *Chlorella vulgaris*.

Due to the cost of plant hormones, their application to increase the biomass and lipid content of microalgae in large-scale is very expensive and therefore not likely economical.

5.3 Lipid Extraction and Analysis

Increasing the amount of lipid in microalgae is one of the major concerns for producing biodiesel. However, lipid extraction from microalgae is a technically challenging processing step. In this study, different techniques were used to extract and measure the lipid content.

Lipids from microalgae could successfully be extracted in an ionic liquid mediated process with high efficiency and the feasibility of a one-pot process was demonstrated, combining lipids extraction and trans-esterification via a heterogeneous catalyst.

5.4 Recommendations for Future Work

The work presented in this study was limited to one strain of microalgae and it is entirely possible that different species react more favorable to the hormones tested. Future studies could expand the number of strains. The genomes of multiple microalgae have recently become available and screening this data for possible hormone receptors might give additional clues, whether or not hormones might be used successfully.

The ability to recycle the ionic liquids of the proposed lipid extraction process has to be evaluated carefully. This line of work also requires to be extended beyond the strain used in this study to show its general applicability. The conditions for the simultaneous extraction and trans-esterification have to be optimized (e.g. residence time, catalyst loading, temperature) and the long term stability of the catalyst has to be evaluated.

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Appendices

Appendix 1: Dry Weight Calibration Curves

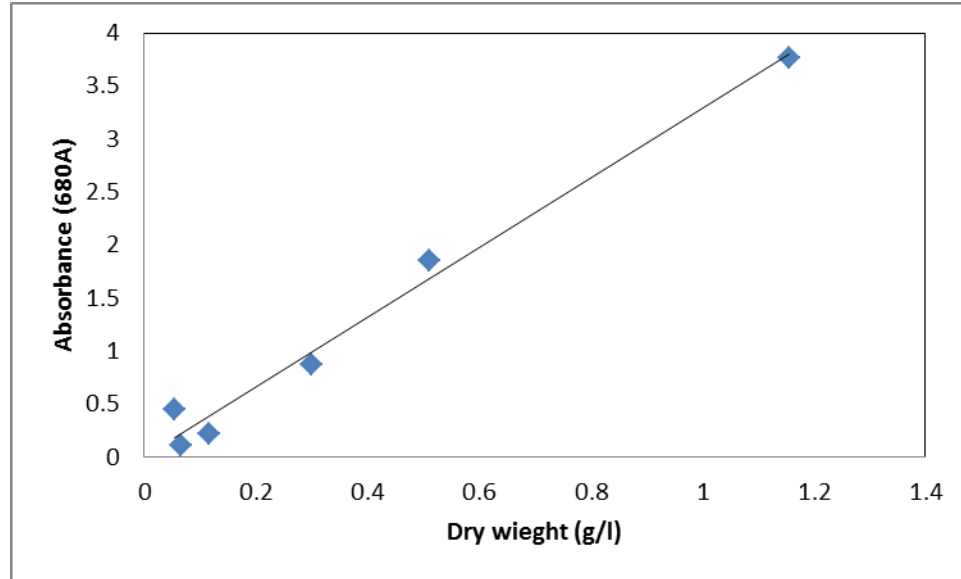


Figure A.1: Dry weight calibration curve measuring OD with spectrophotometer
(D.W=OD₆₈₄*3.2926)

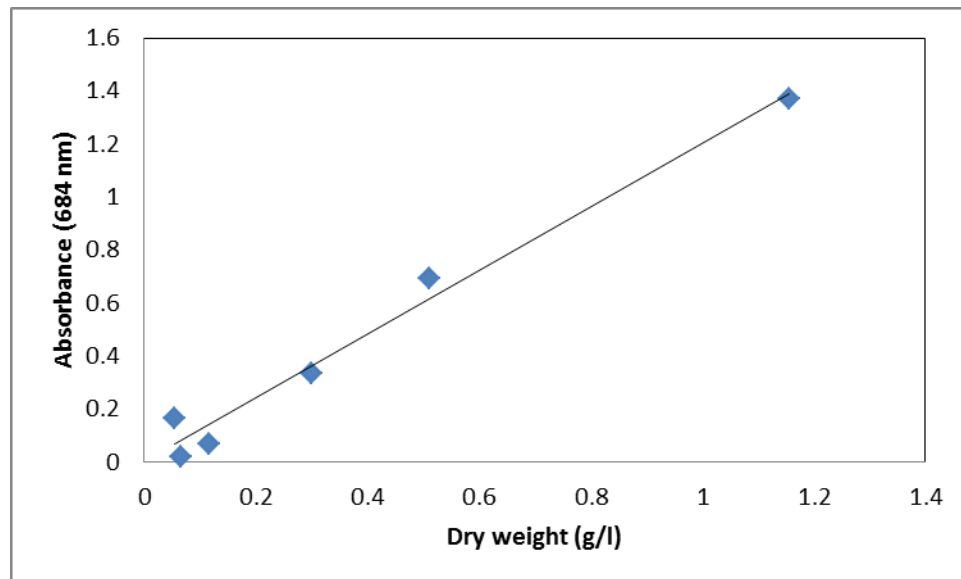


Figure A.2: Dry weight calibration curve measuring OD with Plate reader
(D.W=OD₆₈₄*1.2047)

Appendix 2: Glucose Calibration Curve with HPLC

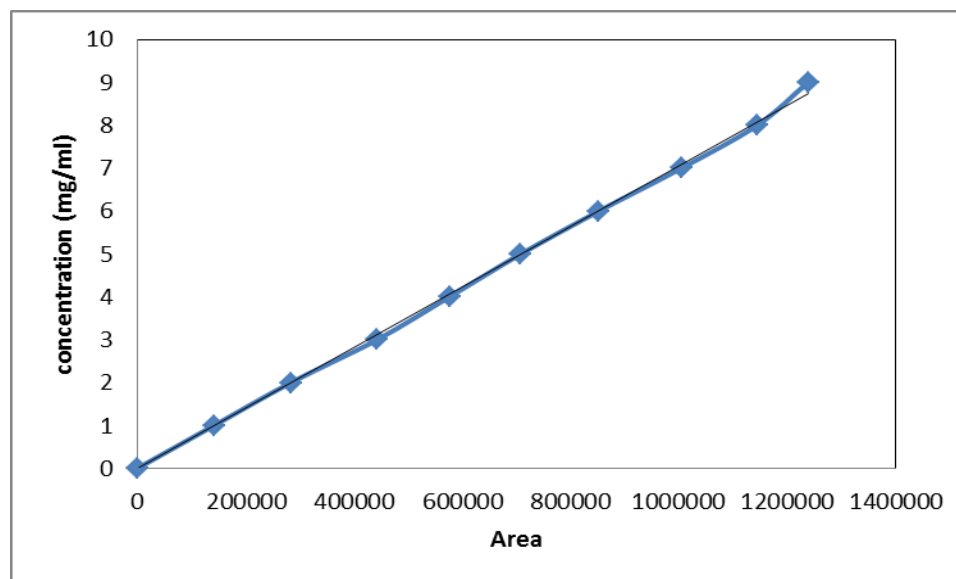


Figure A.3: Glucose calibration curve with HPLC ($C_g=7E-06 *A$)

Appendix 3: Chromatograph of 37 FAME Standards and C19:0 as Internal Standard

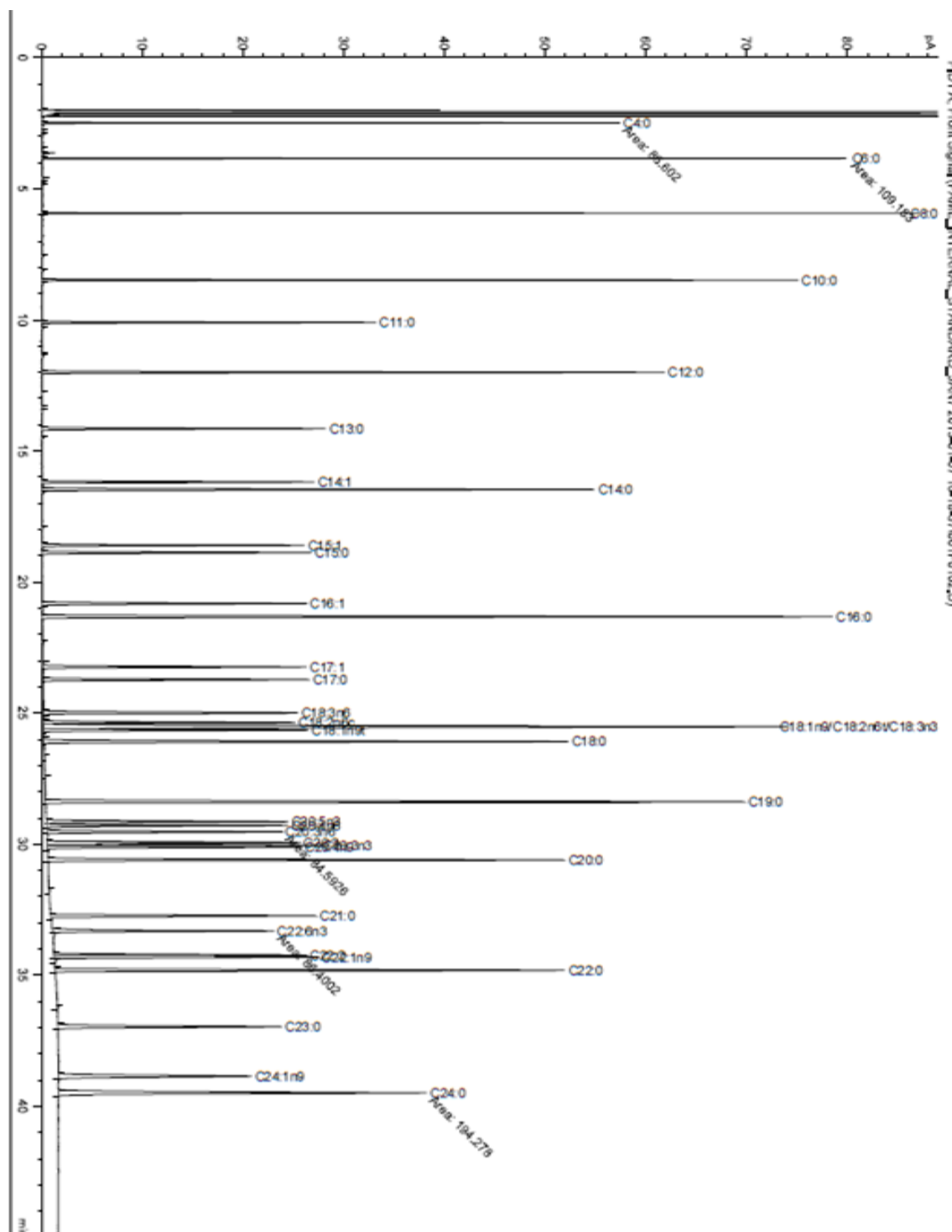


Figure A.4: Chromatograph of 37 FAME standards and C19:0 as internal standard

Appendix 4: Quantitative Analysis of 37 FAME Standards

Table A.1: Quantitative analysis of 37 FAME standards

Peak #	Retention time	FAME	Purification %	Actual concentration mg/ml	Average of areas	Stdev	f
1	2.48	C4:0 Butyric Acid Methyl Ester	99.9	0.3996	85.302	1.74	2.383488
2	3.84	C6:0 Caproic Acid Methyl Ester	99.9	0.3996	107.277	2.07	1.895246
3	5.93	C8:0 Caprylic Acid Methyl Ester	99.9	0.3996	127.507	2.16	1.59455
4	8.48	C10:0 Capric Acid Methyl Ester	99.9	0.3996	143.495	2.42	1.416888
5	10.1	C11:0 Undecanoic Acid Methyl Ester	99.5	0.199	74.003	1.24	1.368203
6	12	C12:0 Lauric Acid Methyl Ester	99.8	0.3992	155.339	2.56	1.307545
7	14.15	C13:0 Tridecanoic Acid Methyl Ester	99.9	0.1998	79.816	1.34	1.273656
8	16.19	C14:1 Myristoleic Acid Methyl Ester	99.9	0.1998	81.851	1.34	1.24199
9	16.47	C14:0 Myristic Acid Methyl Ester	99.9	0.3996	164.05	2.69	1.239356
10	18.6	C15:1 cis-10-Pentadecenoic Acid Methyl Ester	99	0.198	82.779	1.36	1.217003
11	18.88	C15:0 Pentadecanoic Acid Methyl Ester	99.9	0.1998	84.563	1.35	1.202159
12	20.82	C16:1 Palmitoleic Acid Methyl Ester	99.8	0.1996	86.255	1.41	1.177397
13	21.32	C16:0 Palmitic Acid Methyl Ester	99.7	0.5982	261.645	4.3	1.16327
14	23.24	C17:1 cis-10-Heptadecenoic Acid Methyl Ester	99.9	0.1998	88.217	1.42	1.152365
15	23.72	C17:0 Heptadecanoic Acid Methyl Ester	99.9	0.1998	88.633	1.42	1.146956
16	24.99	C18:3n6 γ -Linolenic Acid Methyl Ester	99.9	0.1998	87.632	1.42	1.160057

17	25.36	C18:2n 6c	Linoleic Acid Methy Ester	99.9	0.1998	88.766	1.41	1.145237
		C18:1n 9c	Oleic Acid Methy Ester	99.9	0.3996			
18	25.52	C18:2n 6t	Linolelaidic Acid Methy Ester	99.7	0.1994	355.678	5.67	1.142688
		C18:3n 3	a-Linolenic Acid Methy Ester	99.9	0.1998			
19	25.65	C18:1n 9t	Elaidic Acid Methy Ester	99.9	0.1998	89.464	1.41	1.136302
20	26.09	C18:0	Stearic Acid Methy Ester	99.7	0.3988	180.282	2.84	1.12551
21	28.39	C19:0	Nonadecanoatic Acid Methy Ester(Internal standard)	99.5	0.493719	251.204	3.97	1
22	29.14	C20:5n 3	cis-5,8,11,14,17-Eicosapentaenoic Acid Methy Ester	99.3	0.1986	86.837	1.36	1.163647
23	29.29	C20:4n 6	Arachidonic Acid Methy Ester	99.9	0.1998	85.332	1.36	1.191325
24	29.53	C20:3n 6	cis-8,11,14-Eicosatrienoic Acid Methy Ester	98.8	0.1976	83.448	1.57	1.204808
25	29.94	C20:2	cis-11,14-Eicosadienoic Acid Methy Ester	99.9	0.1998	90.38	1.43	1.124786
26	30.05	C20:3n 3	cis-11,14,17-Eicosatrienoic Acid Methy Ester	99.9	0.1998	97.936	1.58	1.038006
27	30.11	C20:1n 9	cis-11-Eicosenoic Acid Methy Ester	99.9	0.1998	79.116	2.19	1.284925
28	30.6	C20:0	Arachidic Acid Methy Ester	99.9	0.3996	186.203	2.97	1.091907
29	32.74	C21:0	Henicosanoic Acid Methy Ester	99.4	0.1988	93.418	1.43	1.082761
30	33.31	C22:6n 3	cis-4,7,10,13,16,19-Docosahexaenoic Acid Methy Ester	99.9	0.1998	86.441	2.88	1.176041
31	34.23	C22:2	cis-13,16-Docosadienoic Acid Methy Ester	99.9	0.1998	91.139	2.69	1.115419

32	34.31	C22:1n 9	Erucic Acid Methy Ester	99.9	0.1998	92.104	0.52	1.103732
33	34.81	C22:0	Behenic Acid Methy Ester	99.6	0.3984	188.465	2.93	1.075562
34	36.96	C23:0	Tricosanoic Acid Methy Ester	99.9	0.1998	95.036	1.42	1.06968
35	38.85	C24:1n 9	Nervonic Acid Methy Ester	97.4	0.1948	96.304	1.46	1.02918
36	39.49	C24:0	Lignoceric Acid Methy Ester	99.9	0.3996	191.348	3.15	1.062547

Appendix 5: Effect of Different Plant Hormones on the Growth, Biomass and Lipid Content of *Chlorella vulgaris*

Effect of 1-Naphtalenacetic Acid (NAA) on the Growth Kinetics and Lipid Content of *Chlorella vulgaris*

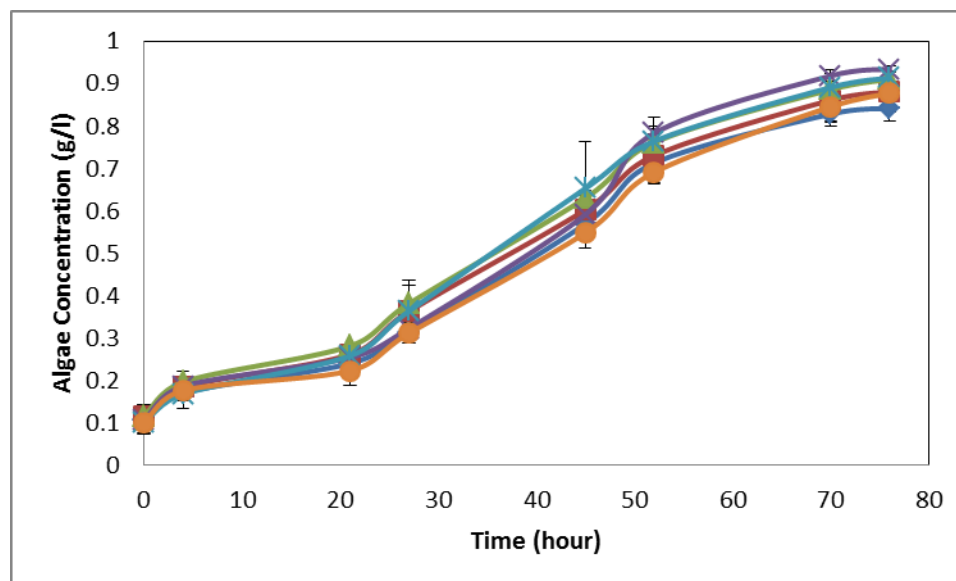


Figure A-5: Effect of different concentrations of 1-naphtalenacetic acid (NAA) on the growth kinetics of *Chlorella vulgaris* growing on glucose in the 96-well plate.

Symbols: (\diamond), Control; (\square), NAA 10^{-5} ; (Δ), NAA 10^{-6} ; (\times), NAA 10^{-7} ; (*), NAA 10^{-8} ; (\circ), NAA 10^{-9} .

Table A-2: Maximum specific growth rate (μ_{\max}) and doubling time of *chlorella vulgaris* in the presence of 15 g/L glucose and 10^{-5} - 10^{-9} M 1-naphtalenacetic acid (NAA) in the 96-well plate.

Hormone Concentration (M)	μ_{\max} (h^{-1}) \pm SD	Doubling time (h) \pm SD
NAA 10^{-5}	0.033 \pm 0.003	20.99 \pm 2.72
NAA 10^{-6}	0.033 \pm 0.003	20.83 \pm 2.72

NAA 10 ⁻⁷	0.035±0.003	20.09±2.42
NAA 10 ⁻⁸	0.037±0.003	18.89±2.16
NAA 10 ⁻⁹	0.034±0.004	20.62±3.44
Control	0.035±0.001	20.00±0.8

Table A-3: The amount of produced lipid (g/L) in *Chlorella vulgaris* in the presence of 10⁻⁵ -10⁻⁹ M 1-naphtalenacetic acid (NAA)

Hormone Concentration(M)	Lipid (% , g/g)±SD
NAA 10 ⁻⁵	2.73±0.004
NAA 10 ⁻⁶	2.09±0.004
NAA 10 ⁻⁷	2.03±0.004
NAA 10 ⁻⁸	2.74±0.009
NAA 10 ⁻⁹	2.97±0.007
Control	2.26±0.004

Effect of Indole 3 Acetic Acid (IAA) on the Growth Kinetics and Lipid Content of *Chlorella vulgaris*

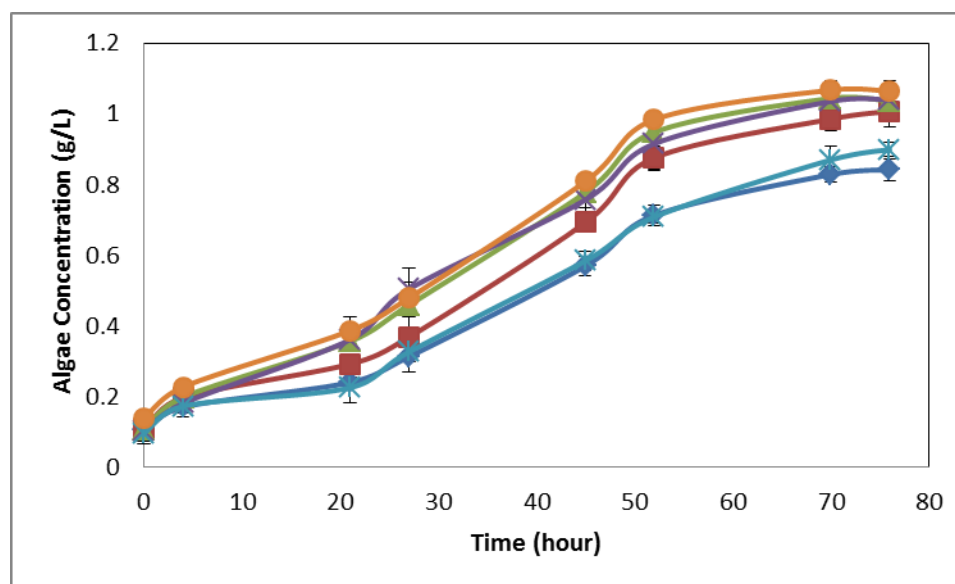


Figure A-6: Effect of different concentrations of Indole 3 acetic acid (IAA) on the growth kinetics of *Chlorella vulgaris* growing on glucose in the 96-well plate.
 Symbols: (◇), Control; (□), IAA 10⁻⁵; (Δ), IAA 10⁻⁶; (×), IAA 10⁻⁷; (*), IAA 10⁻⁸; (○), IAA 10⁻⁹.

Table A-4: Maximum specific growth rate (μ_{\max}) and doubling time of *chlorella vulgaris* in the presence of 15 g/l glucose and 10⁻⁵ -10⁻⁹ M Indole 3 acetic acid (IAA) in the 96-well plate.

Hormone concentration (M)	μ_{\max} (h ⁻¹)±SD	Doubling time (h)±SD
IAA 10 ⁻⁵	0.036±0.003	19.06±2.28
IAA 10 ⁻⁶	0.038±0.004	18.09±2.75
IAA 10 ⁻⁷	0.039±0.005	17.69±3.28

IAA 10 ⁻⁸	0.035±0.004	19.70±3.24
IAA 10 ⁻⁹	0.037±0.004	18.94±2.90
Control	0.035±0.001	20.00±0.80

Table A-5: The amount of produced lipid (g/L) in *Chlorella vulgaris* in the presence of 10⁻⁵ -10⁻⁹ M Indole 3 acetic acid (IAA)

Hormone concentration(M)	Lipid (% , g/g)±SD
IAA 10 ⁻⁵	1.79±0.004
IAA 10 ⁻⁶	2.13±0.005
IAA 10 ⁻⁷	2.50±0.008
IAA 10 ⁻⁸	2.78±0.007
IAA 10 ⁻⁹	1.79±0.001
Control	2.26±0.004

Effect of Indole 3 Butyric Acid (IBA) on the Growth Kinetics and Lipid Content of *Chlorella vulgaris*

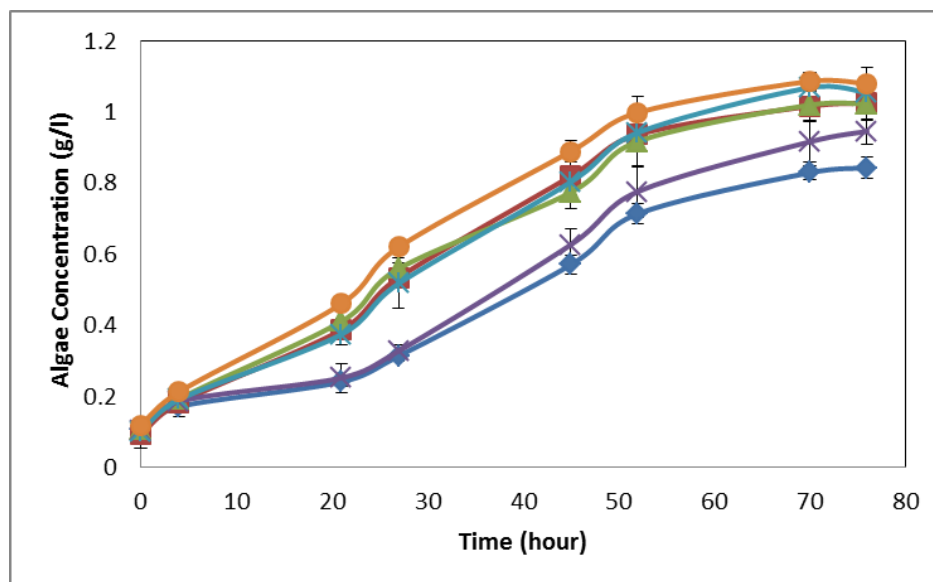


Figure 0-7: Effect of different concentrations of Indole 3 butyric acid (IBA) on the growth kinetics of *Chlorella vulgaris* growing on glucose in the 96-well plate. Symbols: (◇), Control; (□), IBA 10⁻⁵; (Δ), IBA 10⁻⁶; (×), IBA 10⁻⁷; (*), IBA 10⁻⁸; (○), IBA 10⁻⁹.

Table A-6: Maximum specific growth rate (μ_{\max}) and doubling time of *chlorella vulgaris* in the presence of 15 g/l glucose and 10⁻⁵ -10⁻⁹ M Indole 3 butyric acid (IBA) in the 96-well plate.

Hormone concentration (M)	μ_{\max} (h ⁻¹)±SD	Doubling time (h)±SD
IBA 10 ⁻⁵	0.041±0.006	16.77±3.58
IBA 10 ⁻⁶	0.038±0.006	18.04±4.18
IBA 10 ⁻⁷	0.036±0.001	19.13±0.76

IBA 10^{-8}	0.040±0.005	17.41±3.11
IBA 10^{-9}	0.043±0.007	16.04±3.81
Control	0.035±0.001	20.00±0.80

Table A-7: The amount of produced lipid (g/L) in *Chlorella vulgaris* in the presence of 10^{-5} - 10^{-9} M Indole 3 butric acid (IBA)

Hormone Concentration(M)	Lipid (% , g/g)±SD
IBA 10^{-5}	1.76±0.002
IBA 10^{-6}	2.25±0.003
IBA 10^{-7}	2.86±0.006
IBA 10^{-8}	2.19±0.004
IBA 10^{-9}	1.86±0.001
Control	2.26±0.004

Effect of Kinetin on the Growth Kinetics and Lipid Content of *Chlorella vulgaris*

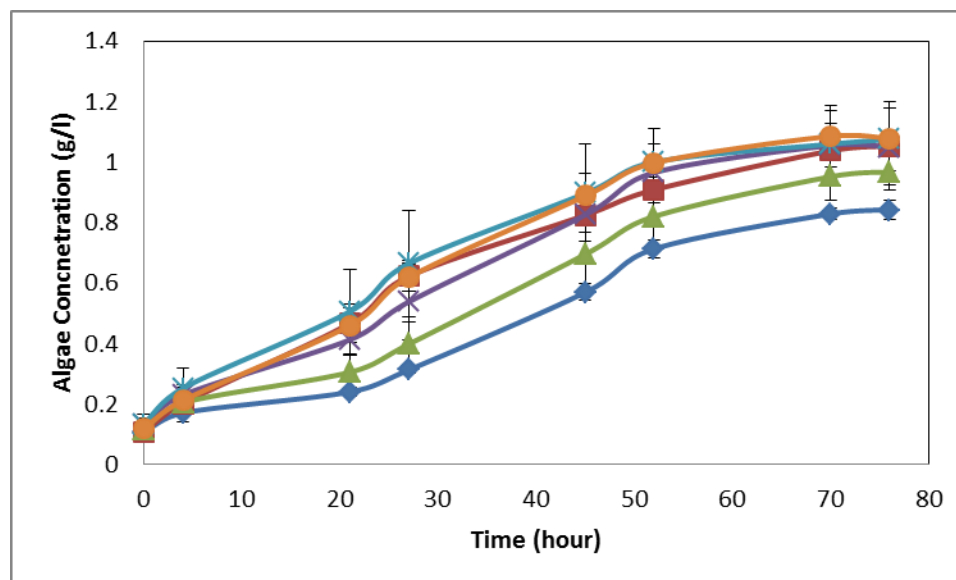


Figure 0-8: Effect of different concentrations of Kinetin on the growth kinetics of *Chlorella vulgaris* growing on glucose in the 96-well plate. Symbols: (◇), Control; (□), Kinetin 10^{-5} ; (△), Kinetin 10^{-6} ; (×), Kinetin 10^{-7} ; (*), Kinetin 10^{-8} ; (○), Kinetin 10^{-9}

Table 0-8: Maximum specific growth rate (μ_{\max}) and doubling time of *chlorella vulgaris* in the presence of 15 g/l glucose and 10^{-5} - 10^{-9} M Kinetin in the 96-well plate.

Hormone concentration (M)	μ_{\max} (h ⁻¹)±SD	Doubling time (h)±SD
Kinetin 10^{-5}	0.038±0.007	18.15±4.92
Kinetin 10^{-6}	0.035±0.004	19.99±3.24
Kinetin 10^{-7}	0.035±0.004	19.75±3.24
kinetin 10^{-8}	0.038±0.006	18.39±4.18

Kinetin 10^{-9}	0.04±0.009	17.29±5.81
Control	0.035±0.001	20.00±0.80

Table A-9: The amount of produced lipid (g/L) in *Chlorella vulgaris* in the presence of 10^{-5} - 10^{-9} M Kinetin.

Hormone concentration (M)	Lipid (% , g/g)±SD
kinetin 10^{-5}	2.28±0.006
Kinetin 10^{-6}	2.90±0.006
Kinetin 10^{-7}	2.10±0.001
Kinetin 10^{-8}	2.17±0.005
Kinetin 10^{-9}	2.14±0.004
Control	2.26±0.004

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