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BIODIESEL PRODUCTION FROM *CHLORELLA VULGARIS* ESP-6

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Selen ŞENAL

Signature

Dedicated to my family

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LIST OF SYMBOLS

Bbl	Barrel
C	Carbon
CO_2	Carbondioxide
Fe	Iron
Gal	Gallon
Ha	Hectar
Kj	Kilojoule
P_{Lipid}	Lipid Productivity
Mj	Megajoule
CH_4	Methane
N	Nitrogen
O_2	Oxygen
Rpm	Revolutions Per Minute
Sec	Second
NaCl	Sodium Chloride
$NaNO_3$	Sodium Nitrate
NaOH	Sodium Hydroxide
μ_{max}	Specific Growth Rate of Microalgae
H_2SO_4	Sulfiric Acid
X_{max}	The Highest Microorganism Concentration
°C	Temperature (Celcius)
P_x	Volumetric Biomass Productivity
Yr	Year

LIST OF ABBREVIATIONS

AG	Acyl Glycerides
ASTM	American Society for Testing and Materials
BBM	Bold Basal Medium
ADW	Digested Wastewater
DAG	Diglycerides
EN	European Standard
FAME	Fatty Acid Methyl Ester
FFA	Free Fatty Acid
GC	Gas Chromatography
GHG	Green House Gases
ILUC	Indirect Land Use Change
LCA	Life Cycle Assestment
MAG	Monoglycerides
MGDG	Mono Galactosyl Diacyl Glycerol
MPBR	Membrane Photobioreactor
MUFA	Mono Unsaturated Fatty Acids
MWI	Municipal Wastewater Influents
NREL	National Renewable Energy Laboratory
OD	Optical Density
PBRs	Photobioreactors
PUFA	Poly Unsaturated Fatty Acid
SAFA	Saturated Fatty Acids
SOR	Solvent to the Oil Ratio
SSR	Solvent to the Solid Ratio
TLC	Thin Layer Chromatography
ULSD	Ultra Low Sulfur Diesel
WTT	Well-to-Tank
WTW	Well-to-Wheel

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ABSTRACT

Biodiesel Production from *Chlorella vulgaris* ESP-6

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Department of Bioengineering

Master of Science Thesis

Advisor: Prof. Dr. Sevil YÜCEL

Microalgal biomass production is of great importance to different industries around the world. It can be used as a food supplement, biomass, feed, pigment, fertilizer and fuel while reducing CO_2 emissions during its production. The CO_2 level in the atmosphere has exceeded 413 ppm and this poses a serious threat to world as it exacerbates the effects of global warming. Therefore, emission-neutralizing biodiesel production from microalgal biomass has been accepted as a promising solution to combat this threat by all the countries in the world. Microalgae are living things that have the ability to absorb air pollutants (CO_2, NO_2, SO_2). Since microalgae contains fatty acids, it carries an important potential for biodiesel production. In this study, the growth conditions of *Chlorella vulgaris* ESP-6 in different environments were examined and biodiesel was produced from microalgae for the first time by *in-situ* esterification method. This study has two objectives; first one being the examination of the growth kinetics of *Chlorella vulgaris* ESP-6 with CO_2 supply, air supply and ambient conditions (without CO_2 and air supply), the other one is a combination of oil extraction and esterification in one step by optimizing various process parameters (catalyst quantity and type, alcohol amount) to produce biodiesel by *in-situ* esterification method. While the microalgae grown in CO_2 environment had the highest biomass yield (0.26 g / L), it has been found that m_{max}

value was 1.06 d^{-1}. Microalgal lipid content and lipid efficiency obtained from the culture grown under ambient conditions were determined as 24% and 3 mg / Lday, respectively, and the volume biomass productivity value was 13 mg / Lday. In *in-situ* esterification reaction used 100-150% by weight (oil-based) sulfuric acid as acid catalyst and 6.25-25% by weight NaOH as base catalyst. The reactions in both catalysts were carried out at 60˚C for 150 minutes. Methyl ester contents were determined by gas chromatography (GC) and qualitative results were determined by thin layer chromatography (TLC). Increasing the volume of reacted alcohol from 6:1 to 10:1 (v / w biomass) and increasing the catalyst rates, raised the oil extraction in the base catalyst, and enhanced the fatty acid methyl ester (FAME) of 29.1% and the methyl ester content of 7.16%. When using H_2SO_4, an acidic catalyst, the FAME efficiency (98%) was found to be much higher than the base catalyst's FAME efficiency (29.1%). When the amount of acidic catalyst was increased from 100% to 150% in the esterification reaction, it was observed that the FAME efficiency (98%) and the ester content (29.5%) were higher than 93.2% and 21.4%, respectively. Overall, it was deduced that the *Chlorella vulgaris* ESP-6 microalgae species can be used as an efficient potential source for reduction of CO_2 emissions and biodiesel production.

Keywords: Biodiesel, Carbondioxide, Microalgae, *Chlorella Vulgaris* ESP-6, Methyl Ester

ÖZET

Chlorella Vulgaris ESP-6 Mikroalg Türünden Biyodizel Üretimi

Selen ŞENAL

Biyomühendislik Bölümü

Yüksek Lisans Tezi

Danışman: Prof. Dr. Sevil YÜCEL

Mikroalgal biyokütle üretimi dünya çapında büyük bir öneme sahiptir. Bunun en büyük sebebi, mikroalglerin gıda takviyesi, biyokütle, yem, pigment, gübre ve yakıt olarak kullanılabilirken öte yandan üretimde CO_2 emisyonlarını azaltmasıdır. Atmosferdeki CO_2 seviyesi 413 ppm'i geçmiştir ve bu durum küresel ısınma açısından en büyük tehtidi oluşturmaktadır. Bu nedenle mikroalgal biyokütleden emisyon nötürleyici biyodizel üretimi tüm dünya ülkeleri tarafından desteklenmektedir. Mikroalgler hava kirleticilerini (CO_2, NO_2, SO_2) absorplama özelliğine sahip canlılardandır. İçerdikleri yağ asitleri sayesinde biyodizel üretimi için de önemli bir potansiyeldir. Bu çalışmada öncelikle *Chlorella vulgaris* ESP-6 mikroalg türünün farklı ortamlarda büyüme şartları incelenerek mikroalglerden ilk kez *in-situ* esterleşme yöntemi ile biyodizel üretimi gerçekleştirilmiştir. Bu çalışmanın 2 amacı bulunmaktadır; ilki *Chlorella vulgaris* ESP-6'nın CO_2 beslemeli, hava beslemeli ve ortam şartlarında (CO_2 ve hava beslemesiz) büyüme kinetiğini incelemek, diğeri ise çeşitli proses parametrelerini (katalizör miktrı ve cinsi, alkol miktarı) optimize ederek tek aşamada yağ ekstraksiyonu ve esterleşmenin bir arada yer aldığı in-situ esterleşme yöntemiyle biyodizel üretmektir. CO_2 ortamında büyütülen mikroalgler en yüksek biyokütle verimine

(0.26 g/L) sahip olurken, m_{max} değerinin 1.06 d^{-1} olduğu bulunmuştur. Ortam şartlarında büyütülen kültürden elde edilen mikroalgal lipit içeriği ve lipit verimliliği sırasıyla %24 ve 3 mg/Lgün olarak belirlenmiştir, hacimsel biyokütle üretkenlik değeri ise 13 mg/Lgün'dür. *Chlorella vulgaris* ESP-6 in-situ esterleşme reaksiyonunda asit katalizörü olarak ağırlıkça %100-150 (yağ bazlı) sülfürik asit ve baz katalizörü olarak ağırlıkça %6,25-25 NaOH kullanılmıştır. Her iki katalizörde de reaksiyonlar 60C'de ve 150 dakika süre ile gerçekleştirilmiştir. Metil ester içerikleri gaz kromotogrofisi (GC) ve kalitatif sonuçlar ince tabaka kromatografisi (TLC) ile belirlenmiştir. Reaksiyona giren alkol hacminin 6:1'den 10:1'e (v/w biyokütle) artırılması ve katalizörün oranlarının arttırılması, baz katalizörde yağ ekstraksiyonunu artırarak %29.1'luk yağ asidi metil esteri (FAME) verimi ve %7.16'lık metil ester içeriği sağlamıştır. Asidik bir katalizör olan H_2SO_4 kullanılığında FAME veriminin (%98), baz katalizörün FAME verimine (%29.1) kıyasla çok daha yüksek olduğu görülmüştür. Asidik katalizör miktarı esterleşme reksiyonunda %100'den %150'ye artırıldığında arttırıldığında, FAME veriminin (%98) ve ester içeriğinin (%29.5) sırasıyla %93.2'den ve %21.4'ten daha yüksek olduğu görülmüştür. Sonuç olarak *Chlorella vulgaris* ESP-6 mikroalg türünün CO_2 emisyonlarının azaltılmasında ve biyodizel üretiminde verimli bir potansiyel kaynak olarak kullanılabileceği uygun görülmüştür.

Anahtar Kelimeler: Biyodizel, Karbondioksit, Mikroalg, *Chlorella Vulgaris* ESP-6, Metil Ester

1
INTRODUCTION

1.1 Literature Review

All the time, fossil fuels are used as the world's energy source and scientists have cautioned about the exhaustion of this genuine source soon. In addition, increasing global warming effects have created restrictions on fuel exhaust gas emissions[2]. Nowadays the resources of fossil fuels available are steadily declining and the tought is that they will soon disappear. Sustainable energy and power sources should be used as an alternative in such conditions. Around the world, non-renewable energy sources are utilized for an enormous scope, but all of them are unsustainable because they have raised the level of CO_2 at 414 ppm and produce greenhouse gases which make the environment polluted. In order to keep the environment clean and sustainable. Consequently designing renewable and environmentally sustainable engineering solutions must be done [3]. Energy demand is the most important issue for the world that create unsafe conditions for all living life. Demand is growing day by day. The planet's condition is changed by global warming effects like greenhouse gases and it reached an alarming scale. Electricity generating companies contribute significantly to atmospheric CO_2 due to their increasing dependence on carbon resources such as coal, oil and natural gas to meet their power requirements. (U.S. GHG emissions and sinks inventory: 1990 2008) The alarming effects of CO_2 formation can be detected by Mauna Loa Observatory (Hawaii, USA), which recorded 414 ppm in 2020 equivalent to 280 ppm in 1958. The future interest for CO_2 sequestration from power plants is to limit the effect of CO_2 on worldwide environmental change. Microalgal growth for CO_2 sequestration is one of the interesting solution for CO_2 emissions.

The most important reasons for microalgal growth to be used for CO_2 fixation are: reducing CO_2 in the atmosphere which is the main cause of global warming, biofuel production and production of other important additional chemicals. One kilogram of dried algal cell mass uses around 1.83 kg of CO_2. Every year, approximately 54.9–67.7% of CO_2 is fixed in biomass with the growth of 30–37 tons of algal mass per hectare in open ponds [4]. Algae has already become the world's largest source of energy production. They can be used as cleaner types for biodiesel production, uncompetitive and rapidly developing species for land and food raw materials [3]. Algae biomass is used to produce biofuels (biodiesel, bioethanol, biohydrogen) and other commercial chemicals to create sustainable chemical solutions [1]. These have the potential to grow without much concern for waste nutrients and are regarded as the best source of biodiesel production because other resources can cause food problems, since they include certain plants which are used for biodiesel in crops that are less productive and less in quantity than algae. Algae contain about 80% of the energy content of petroleum [5]. Microalgae species have 40-70% hydrocarbon content, which can be easily extracted. In addition, algae can successfully remove hazardous nutrients from wastewater and therefore can play an important role in wastewater treatment. [3].

This thesis is mainly focused on biodiesel production from *Chlorella vulgaris* ESP-6 by sequestering of CO_2. It also gives a clear view of suitable biodiesel to be used in the future. The objective of this article is to critically describe various aspects of biodiesel production and examine different parameters in production like catalyst and alcohol ratios which can affect efficiency on industrial biodiesel production from *Chlorella vulgaris* ESP-6.

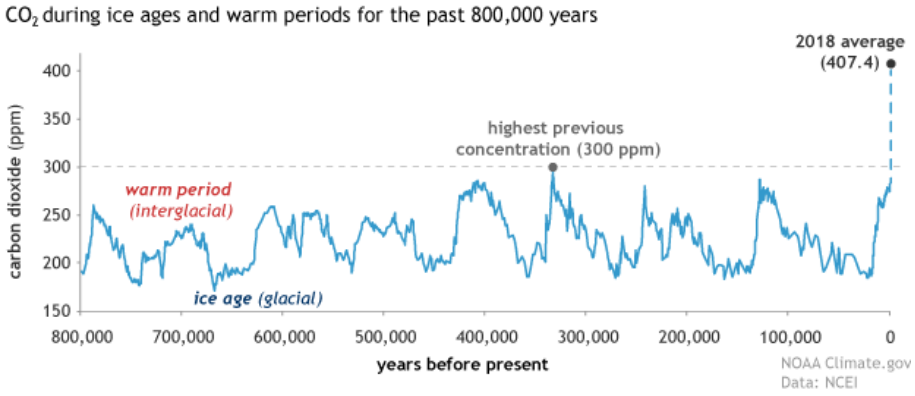


Figure 1.1 CO_2 ppm levels in atmosphere[15]

1.2 Objective of the Thesis

This study has two objectives; first one being the examination of the growth kinetics of *Chlorella vulgaris* ESP-6 with CO_2 supply, air supply and ambient conditions (without CO_2 and air supply), the other one is a combination of oil extraction and esterification in one step by optimizing various process parameters (catalyst quantity and type, alcohol amount) to produce biodiesel by *in-situ* esterification method.

1.3 Hypothesis

Microalgae are unicellular microns in size. The photosynthetic microalgae species convert CO_2 into biomass through photosynthesis just like large plants. The most important features that make microalgae superior to other plants in terms of CO_2 retention are that depending on the type, they can double their biomass in a short time like a day. Most strategies related to global warming involve the reduction of greenhouse gas emissions. This can be accomplished, among other things, by reducing fossil fuel use and carbon capture. Microalgae can play an important role in both strategies. As a result of all the examinations and calculations, it has been shown that a significant CO_2 reduction is possible and also economically advantageous method by means of an integrated microalgae growing facility for biomass extraction. Reducing greenhouse gas emissions that cause global warming

by producing biodiesel from microalgae grown in energy efficient environment conditions, has recently been considered as one of the most important developments.

Within the scope of this thesis, Chlorella vulgaris ESP-6 microalgae species is expected to be grown efficiently using carbon dioxide. There are studies to grow various microalgae species by feeding carbon dioxide abroad. However, as a result of the literature searches, no biodiesel production was found by in-situ transesterification method after growing *Chlorella vulgaris* ESP-6 species in different environments (carbon dioxide, air fed, ambient conditions).

2
BIODIESEL

2.1 Biodiesel Production

The cost of crude oil will be increasing for future decades because of increasing demand of users and sustainable energy sources of fuel must be made more feasible to supply oil requirement. Global warming will worsen ecology due to petroleum-derived fossil fuels that supply CO2 to the atmosphere. Biofuels (biodiesel and bioethanol) that can be used instead of oil can be produced using microalgae oil. For this reason, microalgae breeding farms have started to be established and technology development in order to make the microalgal biodiesel eco-manageable. Biodiesel is ester of triglycerides resulting from the transesterification process and can be used in all vehicles with diesel engines without modification.

It has been scientifically proven to emit less environmentally toxic particles than petro-diesel, when biodiesel is blended or used directly in diesel engine. Lapinskiene et al. proved that diesel fuel at exposure levels higher than 3% (w/w) is dangerous to soil organisms [6]. Since biodiesel is not environmentally toxic, increased environmental concerns are making biodiesel legally mandatory. Industrial biodiesel is generally produced from first and second generation oils (vegetable oils and waste oils from industries). However, the using vegetable oils as a raw stock of biofuel is too divisive and has a significant side effect on food safety. Agricultural lands used to obtain vegetable oils are a major obstacle to the production of vegetable biodiesel. 1% of land always accessible in rural areas was used for the first period of crop-bound biofuels. But this amount was enough to meet only 1% of the worldwide pre-demand. For this reason, microalgal biodiesel, which does not require any agricultural land or valuable vegetable oil in production, has bypassed an important industrial class as a 3rd generation biofuel. Studies on renewable energy production sources have concluded that microalgae biodiesel is the best alternative source of energy.

2.1.1 Crops vs. Algae for Biodiesel Production

Vegetable oils produced as edible oils have a worldwide use of 95% of raw materials for biodiesel production. However, this type of first-generation biodiesel fuels with raw materials have a significant disadvantage as they compete with our food sources. The first generation biodiesel production, which has the potential to increase the prices of food crops coming to our table, such as sunflower, can also cause extra costs within its own structure. In addition, second-generation biofuels such as industrially important oils such as Jatropha and waste cooking oils do not adversely affect food safety. However, second generation biofuels are problematic in terms of sustainability and their logistics costs are very high.

The second generation biofuels, which cause many problems in terms of engine performance and fuel characteristics, have been replaced by microalgae oil as an alternative. Algal biomass efficiency is several times higher than that of oilseed crops per hectare area. Microalgae have the ability to grow much faster than plants and can also grow in salt water. Using a critical degree of solar-powered life, they turn energy into efficient energy as solar converters. Microalgae show the best performance on photosynthetic efficiency than earthy plants and do not contain many essential supplements for development. While the lipid content in dry microalgae biomass regularly varies between 20-50%, some types of microalgae may have up to 85% high lipid content. Microalgae can produce average 20 times more noteworthy triglycerides than soil-bound plants. Microalgae oil is more suitable, especially compared to plant and seed biomass because transport costs are relatively low. Microalgae provides a significantly higher yield advantage compared to sunflower oil as a possible raw material for biodiesel production. In addition, the agricultural land used for the production of first and second generation biofuels decreases the carbon neutral balance it provides to the environment by causing more greenhouse gas emissions in production compared to microalgal biodiesel. Microalgal biodiesel, the third generation biofuel, provides 3.5 times less greenhouse gas emissions when used in diesel engines compared to petro-diesel. Algae are considered as the most suitable alternative fuel raw material for biofuel

production under favour of great oil efficiency and little land requirement compared to vegetableraw stocks. [11]. Researches about analysis of algal oils has previously been studied with recorded pH in the range 6–7, density between 0.85–0.89 g/cm^3, and viscosity between 3.8–4.4 mm^2/s [12].

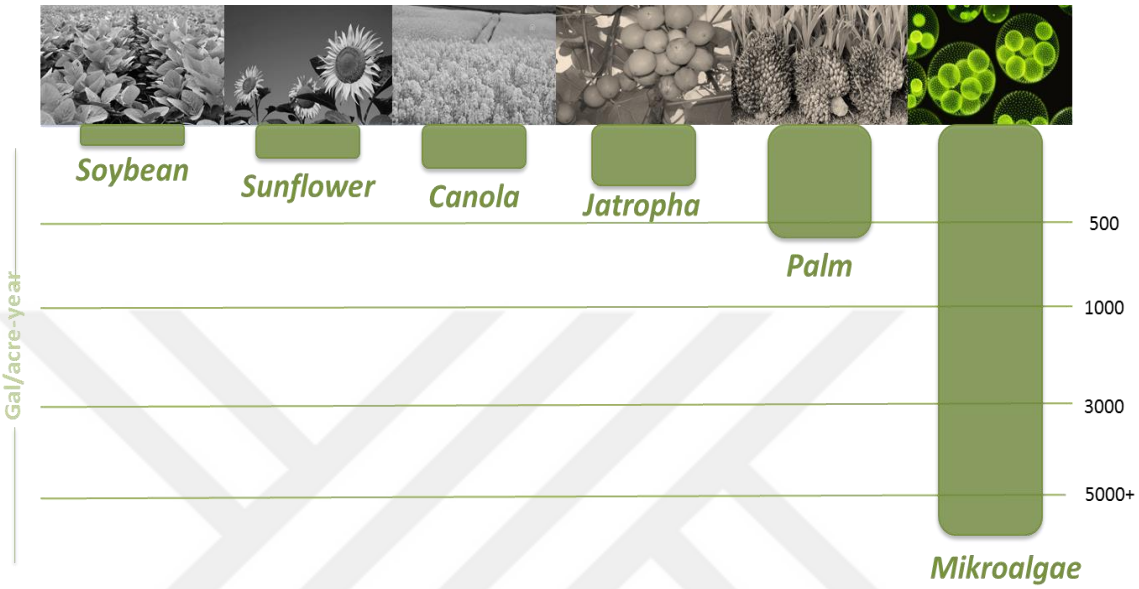


Figure 2.1 Comparison of productivity of vegetable biodiesel and algae biodiesel [49]

2.1.2 Advantages of Microalgae

In terms of harvesting time, microalgae have a much shorter life span compared to vegetable raw material. By providing higher solar-oriented energy yields, they provide higher lipid productivity. The generated lipids are typically neutral oils with a high saturation level which shows them ideal for biodiesel production. Microalgae provides economic profit in industrial production with its faster growing rates compared to the vegetables. Microalgal biomass weights within 24 hours.

Microalgae need less fresh water than soil-bound plants to grow. In non-arable lands, microalgal development may occur in salt or waste water. In microalgal growth some chemicals like herbicides are not needed to reduce costs and environmental effects, as in plants. Correspondingly, these microorganisms need less soil, about 2% of the soil is needed to obtain the same level of biodiesel from oil-containing plants. Rapid growth and high lipid production capacity in microalgae

performed much better than terrestrial products. Microalgal growth can adequately remove phosphates and nitrates which are located in water, fastly and this situation can be used as an economical model as a tertiary treatment system for wastewater. Microalgae are also rich in many basic raw materials lke high-value proteins, pigments, carbohydrates, etc. [7].

Manufacturing and transportation costs of microalgal biodiesel are generally lower than those of oily crops. Overabundance sub-extraction biomass develops a strategy for using the economy in addition or for combining other highly viable products [7].

Since microalgae reduces the average carbon dioxide in the air in culture environments, the biodiesel produced as a result of it is named carbon-neutral biofuel by neutralizing the emissions released when used in diesel engines [8]. The fuel properties of the microalgal biodiesel are very similar to that of the petroleum diesel, and are even more performance enhancing. These properties consist of density, viscosity, flash point, cold flow and heat value. None of the potential biodiesel raw materials are equivalent to algal biodiesel for sustainability. Because production in life cycle assessment they have a much higher environmental damage effect compared to algae at the stage [7]. Compared to lignocellulosic biomass, microalgae plays a more valuable role in the pyrolysis technique as an alternative approach to biofuel production. Since microalgae can store high levels of cellular lipids, polysaccharides and proteins, it is much more advantageous in pyrolysis techniques compared to vegetable raw materials [9] [10].

2.1.3 Cost of Biodiesel Production from Microalgae

A detailed cost-benefit analysis involving harvesting of biomass, centrifugation, extraction and transesterification process of microalgal biodiesel was conducted by the National Renewable Energy Laboratory (NREL), and the unit cost was calculated to be an average of $0.092/L (13). The outputs of this paper shows that the expense of biodiesel is diminished by 40.7–42.3% when the profitability of biomass is multiplied. The NREL report showed that when carbon dioxide in flue gas is used for

microalgal growth, production costs are reduced by almost 20%. In this production, 30 g of microalgae biomass per square meter per day was obtained.

This is becauseof precise estimate of transesterification costs, land costs and extr action efficiency [14].

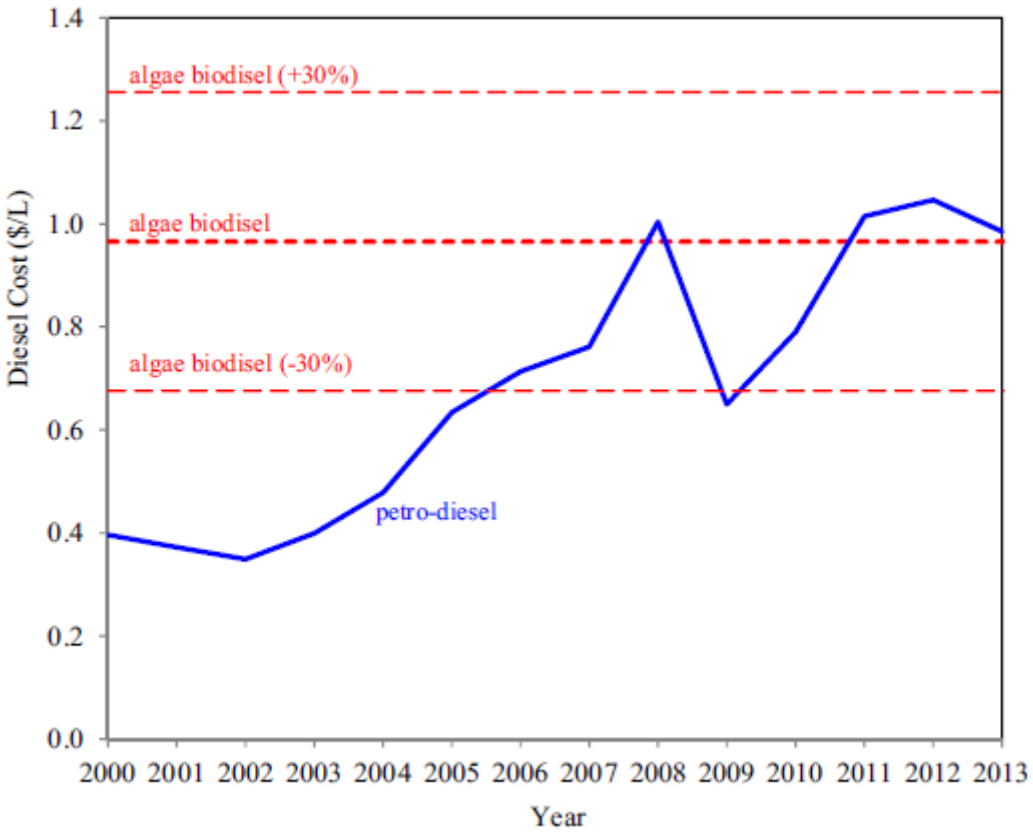


Figure 2.2 Cost comparison of petro-diesel vs. algae-diesel [14]

3
MICROALGAE

3.1 Microalgae

Algae; they are organisms that are divided into macroalgae or seaweed and microalgae and are not vascular (eg, without a specific nutrient delivery system). Microalgae can be of different structures, for example prokaryotic cyanobacteria and eukaryotes, diatomes, etc. Despite the fact that microalgae can take out oxygen by photosynthesis (for example split water to create O_2 and convert CO_2 into biomass by daylight), many can likewise utilize natural substrates (for example glucose, acidic corrosive, and so forth.) in obscurity and some may have returned to nonphotosynthetic, pigmentless species living forever in obscurity.

More than much of the atmospheric oxygen is generated thanks to microalgae. Unlike terrestrial oilseed plants, microalgae do not require soil requirements and fresh water sources. Since microalgae can grow in harsh environmental conditions, they can provide efficiency without competing with other terrestrial plants.

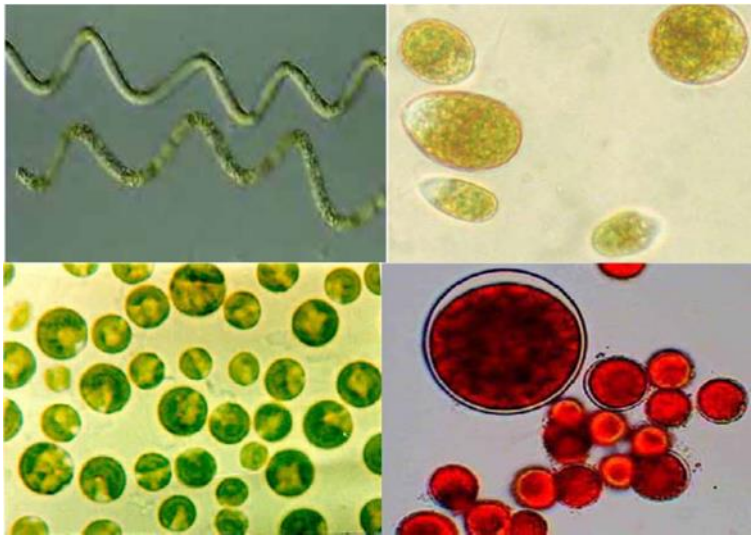


Figure 3.1 Microalgae species. *1. Spirulina (Arthrospira platensis) 2. Dunaliella salina 3. Chlorella vulgaris 4. Haematococcus pluvialis*

Since microalgae can grow in industrial wastewater environment, they can be used in wastewater treatment without forming active sludge by removing various chemicals such as nitrogen and phosphorus. Microalgal growth is much more resistant to contamination or environmental conditions, especially in closed photobioreactors, while plant growth and viability of soil-dependent oilseed crops rely on rare conditions, sunlight-based radiation, and all other climate-related changes. The phototransformation efficiency of terrestrial plants is typically below 1% in temperate climates against incoming solar radiation, while the efficiency of microalgae biomass is up to 5%.

The oil productivity efficiency of microalgae compared to vegetable oil raw materials is given by taking into account the land use in Table 3.1. Microalgae species, which are close to plant biomass and exhibit even more oil production, show that industrial production will have significant results in terms of profitability. For example, microalgae containing 30% dry biomass by weight can provide much more lipids than palm oil.

Microalgae efficiency is much higher compared to the amount of oil that can be obtained from jatropha or palm. It is seen that the use of micr In microalgae breeding, basically three steps are followed: (1) microalgal growth (in open ponds); (2) harvesting; and (3) extraction of lipids from algal biomass. Growing and harvesting processes constitute 40% and 20% cost of microalgal biodiesel manufacturing, respectively. Algae as biofuel raw material can have great financial and economic advantages.

3.2 Microalgae Celluar Structure

Microalgae are single-celled organisms. Green micro algae range from 1 to 50 microns in diameter, but grow in open water systems like ponds or oceans, and is the first step in the aquatic food chain. Microalgae, just like plants, perform photosynthesis using carbon dioxide and sunlight, and in the meantime, they accumulate nutrients such as lipids, carbohydrates and proteins in their structures and turn into nutrient-rich biomass.

Table 3.1 Microalgae oil potential vs. other biofuel feedstocks [36-50]

Plant	Gal Oil/Acre	Fat content
Oil Palm (*Elaeis guineensi*)	610	35,3
Macauba Palm(*Acrocomia aculeate*)	461	28,35
Pequi (*Caryocar brasiliense*)	383	45
Buriti Palm (*Mauritia flexuosa*)	335	19
Oiticica (*Licania rigida*)	307	80
Coconut (*Cocos nucifera*)	267	35,3
Avocado (*Persea Americana*)	270	20
Brazil Nut (Bertholletia excelsa)	245	66,9
Macadamia Nut (Macadamia terniflora)	230	71,6
Jatropha (Jatropha curcas)	194	28
Babassu Palm (Orbignya martiana)	188	60
Jojoba (Simmondsia chinensis)	186	50
Pecan (*Carya illinoensis*)	183	71,2
Bacuri (*Platonia insignis*)	146	13,5
Castor Bean (*Ricinus communis*)	145	48
Olive Tree (*Olea europaea*)	124	20
Rapeseed (*Brassica napus*)	122	30
Opium Poppy (*Papaver somniferum*)	119	48
Peanut (*Ariachis hypogaea*)	109	60
Sunflower (*Helianthus annuus*)	98	43,7
Tung Oil Tree (*Aleurites fordii*)	96	55
Rice (*Oriza sativa L.*)	85	10
Buffalo Gourd (*Cucurbita foetidissima*)	81	33
Safflower (*Carthamus tinctorius*)	80	35
Crambe (*Crambe abyssinica*)	72	30
Sesame (*Sesamum indicum*)	72	49,1
Camelina (*Camelina sativa*)	60	40
Mustard (*Brassica alba)*	59	40
Coriander (*Coriandrum sativum*)	55	15
Pumpkin Seed (*Cucurbita pepo*)	55	46,7
Euphorbia (*Euphorbia lagascae)*	54	50
Hazelnut (*Corylus avellana*)	49	60
Linseed (*Linum usitatissimum*)	49	34
Coffee (*Coffea arabica)*	47	15
Soybean (*Glycine max*)	46	17,7
Hemp (*Cannabis sativa)*	37	35
Cotton (*Gossypium hirsutum*)	33	40

Table 3.1 Microalgae oil potential vs. other biofuel feedstock (Cont.)

Calendula (*Calendula officinali*)	31	20
Kenaf (*Hibiscus cannabinus L.*)	28	20
Rubber Seed (*Hevea brasiliensis*)	26	24
Lupine (*Lupinus albus*)	24	7,5
Palm (*Erythea salvadorensis*)	23	30
Oat (*Avena sativa*)	22	8
Cashew Nut (*Anacardium occidentale*)	18	41,7
Corn (*Zea mays*)	18	4
Microalgae (low oil content)	6275	30
Microalgae (medium oil content)	10,455	50
Microalgae (high oil content)	14,635	70

More than 50,000 microalgae species have been identified to date. These species are classified separately as infrastructures, biochemical components, pigment composition and life cycles. Microplankton (20-1000 μm), nanoplankton (2–100 μm), ultraplankton (0.5-15 μm) and picoplankton (0.2-2 μm) are microalgae classification.

Microalgae include eukaryotic and prokaryotic cells, nuclear cells and organelles.. The DNA in eukaryotic microalgae is embedded in a finely perforated atomic film. In eukaryotic algae, an intracellular organelle called chloroplast performs the process of photosynthesis in microalgae. There are chloroplasts (which contain Chlorophyll A, A&B or A&C) containing different types of chlorophyll in different algae species. The chlorophyll type found in microalgae determines color. Microalgae, which have a simplified eukaryotic structure, play a role in effective carbon biophixation and energy production by providing lipid accumulation in their biomass with photosynthesis.

3.3 Microalgae Biochemical Composition

Microalgae contains four compounds which are proteins, carbohydrates, nucleic acids and lipids in different amounts.The energy-rich molecules are lipids (37.6 kJ g^{-1}) followed by protein (16.7 kJ g^{-1}) and carbohydrates (15.7 kJ g^{-1}). Microalgae consist of polar and non-polar lipids. Polar lipids are glycolipids-phospholipids.

The cell membrane, which fixes the cell and holds its structure, consists of glycolipids and phospholipids. The two-layer arrangement of phospholipids create cell membrane. Non-polar oils are triglycerides(TAGs), diglycerides(DAGs), monoglycerides(MAGs), free fatty acids (FFAs) and hydrocarbons.

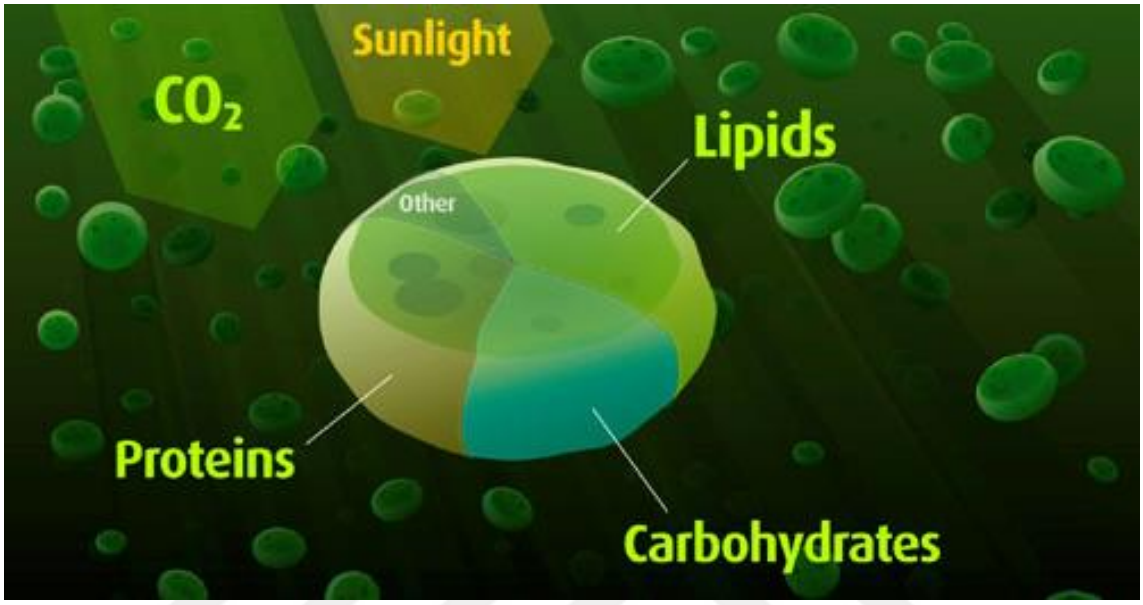


Figure 3.2 Biochemical composition of microalgae

Fatty acid compounds show various changes in different types of algae. Polyunsaturated fatty acids (PUFAs) constitute the structure oil frame, while monounsaturated fatty-acids (MUFAs), saturated fatty-acids (SAFAs) are storageable oil fractions [18,19,20].

Carbohydrates are another microalgae compound created in the dark phase of photosynthesis, unlike lipids. In the dark phase of photosynthesis, carbon dioxide is converted into carbohydrates in Calvin cycle [21]. Carbohydrates are sepearated as structural-storage molecules. Structural molecules are located in the cell walls of microalgae (eg pectin, cellulose and sulfate polysaccharides). All microalgae groups do not contain the same carbohydrate composition. Bioethanol can be produced by fermentation from carbohydrates in microalgae.

3.4 Microalgae Taxonomic Groups

More than 50,000 species of algae live in the world. Algae are usually classified according to distinctive nucleus, variable photosynthetic pigments, categories of nutrients,cell wall structure, structural flagella and type of reproduction and life background.

Table 3.2 Taxonomic groups of microalgae strains [36,97]

Taxonomy	Cellular Structure		Color
Bacillariophyceae	Eukaryotic	Marine & Freshwater	Golden-brown
Chlorophylceae	Eukaryotic	Mostly Freshwater	Green algae
Eustigmatophyceae	Eukaryotic	Mostly Freshwater	Pale-green or Yellow-green
Chrysophyceae	Eukaryotic	Freshwater & Marine	Golden-brown
Prymnesiophyceae	Eukaryotic	Mainly in Fresh Waters	Brown algae
Cyanophyceae	Prokaryotic	Freshwater	Blue-green

Not all taxonomic classes are suitable for the production of biodiesel from microalgae. Green microalgae species (Chlorophyceae) were determined to be the most productive species for biodiesel production.

They have a broad lipid efficiency fraction, such as microalgae, carotenoids such as chlorophylls, terpenes, tocopherols, hydrocarbons, sterols and prenil aids.Polar (auxiliary) lipids produce a critical amount of poly unsaturated fatty acids (PUFAs). Phospholipids and sterols are the basic polar materials of the cell layers that form a porous barrier for organelles and cells. Likewise, certain polar lipids work as basic intermediates in cell flagging pathways (e.g., sphingolipids, inositol lipids, oxidative

items). Storage lipids consist of triglycerides (TAGs) which have too much saturated and unsaturated fatty acid structures.

Microalgae synthesise TAG as they move to the stationary phase in their exponential growth phases. Therefore, the microalgae growth curve for biofuel production is harvested by stopping when it passes to the stationary phase.

Microalgae can catalyze TAGs too quickly in their bodies to provide structural energy. They are generally created in the presence of light source, aggregated in lipid structure, and afterward reused without light for polar lipid production. When sudden shift in the environmental condition to accelerate the creation of the adaptive membrane, PUFArich TAGs endue particular acyl groups to polar lipids[22].

3.5 Lipid Content of Microalgae

Different types of algae contain different amounts of lipids, so all of these species can contribute to high oil yield. The lipid composition of microalgae varies from 1 to 70%, but only some different situations a few microalgae species exceed 90% of dry weight [8,51,110,113].

Table 3.3 provides information about both lipid content and biomass efficiency in microalgae species growing in various seas and fresh waters.

It can be clearly seen in Table 3.3 that the fat content of microalgae can be 75% by weight in dry biomass, but in some cases low efficiency (eg *Botryococcus braunii*) can be achieved. But the most common algae species get an oil content approximately reached 50%.

As it is understood from the table, Chlorella species is a type that can be used for biodiesel production in terms of increasing oil content and productivity. In any case, as some different creatures are as effective and gainful as this one, the assessment of the most appropriate species needs to begin mulling over specific factors, the limit of microalgae to develop to use adequate assets or under explicit ecological conditions. When choosing the most suitable species to produce biodiesel, multiple environmental and chemical factors have to be considered.

The different saturated or unsaturated fatty acids found in microalgae has an huge effect in the efficiency and quality of the biodiesel produced in production. These are framed of immersed and unsaturated fats with 12–22 carbon iotas, some of them from the omega-3 and omega-6 gatherings. Thomas et al.[114] showed the unsaturated fatty acids of seven freshwater microalgae species demonstrating that they all created C14:0, C16:0, C18:1, C18:2, and C18:3 unsaturated fats. Different metabolic and ecological components, development environment and development stages can affect the content of unsaturated fats in microalgae.

Table 3.3 Lipid content and productivities of different microalgae species [51-71]

Microalgae species	Lipid content dry weight (%)	Lipid productivity (mg/L/day)	Volumetric productivity (g/L/day)
Ankistrodesmus sp.	24.0–31.0	–––	–––
Botryococcus braunii	25.0–75.0	–––	0,02
Chaetoceros muelleri	33,6	21,8	0,07
Chaetoceros calcitrans	14.6–16.4/39.8	17,6	0,04
Chlorella emersonii	25.0–63.0	10.3–50.0	0.036–0.041
Chlorella protothecoides	14.6–57.8	1214	2.00–7.70
Chlorella sorokiniana	19.0–22.0	44,7	0.23–1.47
Chlorella vulgaris	5.0–58.0	11.2–40.0	0.02–0.20
Chlorella sp.	10.0–48.0	42,1	0.02–2.5
Chlorella pyrenoidosa	2	–––	2.90–3.64
Chlorella	18.0–57.0	18,7	
Chlorococcum sp.	19,3	53,7	0,28
Crypthecodinium cohnii	20.0–51.1	–––	10
Dunaliella salina	6.0–25.0	116	0.22–0.34
Dunaliella primolecta	23,1	–––	0,09
Dunaliella tertiolecta	16.7–71.0	––	0,12
Dunaliella sp.	17.5–67.0	33,5	––
Ellipsoidion sp.	27,4	47,3	0,17
Euglena gracilis	14.0–20.0	––	7,7
Haematococcus pluvialis	25	––	0.05–0.06
Isochrysis galbana	7.0–40	––	0.32–1.60
Isochrysis sp.	7.1–33	37,8	0.08–0.17
Monodus subterraneus	16	30,4	0,19
Monallanthus salina	20.0–22.0	––	0,08

Table 3.3 Lipid content and productivities of different microalgae specie (Cont.)

Nannochloris sp.	20.0–56.0	60.9–76.5	0.17–0.51
Nannochloropsis oculata.	22.7–29.7	84.0–142.0	0.37–0.48
Nannochloropsis sp.	12.0–53.0	37.6–90.0	0.17–1.43
Neochloris oleoabundans	29.0–65.0	90.0–134.0	––
Nitzschia sp.	16.0–47.0	8.8–21.6	
Oocystis pusilla	10,5	––	––
Pavlova salina	30,9	49,4	0,16
Pavlova lutheri	35,5	40,2	0,14
Phaeodactylum tricornutum	18.0–57.0	44,8	0.003–1.9
Porphyridium cruentum	9.0–18.8/60.7	34,8	0.36–1.50
Scenedesmus obliquus	11.0–55.0	–	0.004–0.74
Scenedesmus quadricauda	1.9–18.4	35,1	0,19
Scenedesmus sp.	19.6–21.1	40.8–53.9	0.03–0.26
Skeletonema sp.	13.3–31.8	27,3	0,09
Skeletonema costatum	13.5–51.3	17,4	0,08
Spirulina platensis	4.0–16.6	–	0.06–4.3
Spirulina maxima	4.0–9.0	–	0.21–0.25
Thalassiosira pseudonana	20,6	17,4	0,08
Tetraselmis suecica	8.5–23.0	27.0–36.4	0.12–0.32
Tetraselmis sp.	12.6–14.7	43,4	0,3

Table 3.4 compares the oil contents between seed crops and microalgae and shows that there are large differences in the efficiency of the biodiesel produced from these two raw materials. According to land use, microalgal biodiesel are distinctly beneficial due to their higher production of biomass and oil yield when it compared with palm oil.

Table 3.4 Comparison of microalgae with other biodiesel feedstocks [8, 71-80]

Plant source	**Oil yield (L oil/ha year)**	**Land use (m^2 year/kg biodiesel)**	**Biodiesel productivity (kg biodiesel/ha year)**
Corn/Maize (*Zea mays L .*)	172	66	152
Hemp (*Cannabis sativa L.*)	363	31	321
Soybean (*Glycine max L.*)	636	18	562
Jatropha (Jatropha curcas L.)	741	15	656

Table 3.4 Comparison of microalgae with other biodiesel feedstocks (Cont.)

Camelina (Camelina sativa L.)	915	12	809
Canola/Rapeseed (Brassica napus L.)	974	12	862
Sunflower (Helianthus annuus L.)	1070	11	946
Castor (Ricinus communis)	1307	9	1156
Palm oil (Elaeis guineensis)	5366	2	4747
Microalgae (low oil content)	58,7	0.2	51,927
Microalgae (medium oil content)	97,8	0.1	86,515
Microalgae (high oil content)	136,9	0,1	121,104

In addition to all these benefits, microalgal biodiesel production brings together important difficulties in terms of technology development stage, but it is a more efficient raw material compared to the profitability analysis it will obtain.

The basic designs required for microalgal biodiesel production are based on the selection, harvesting and optimization of the transesterification process. As long as such technologies are developed in human life, the production of microalgal fuels will accelerate. In addition to all these, several different production techniques are being developed in place of the transesterification process for optimization in production including warm splitting (or pyrolysis) including warm decay or cleavage of triglycerides and other natural mixes found in the feedstock [100].

The fat content in plants is 30% on average dry mass, but microalgae generally have higher fat content than plants (Nannochloris sp. 56%, Chlorella sp. 53% and Neochloris oleoabundans 65%). Schizochytrium sp. Some types of algae can reach 80% lipid content in dry mass [23]. The quantity of saturated and unsaturated fatty acids is the way to evaluate the suitability of micro fuels as biofuel raw materials.

Unsaturated lipids, particularly palmitoleic (16:1), oleic (18:1), linoleic (18:2), linolenic (18:3) and saturated lipids as palmitic (16:0) acids are the significant segments of the oil produced by microalgae. The content of unsaturated lipids in the microalgae significantly affects the biodiesel specifications. Biodiesel which is generated from microalgae that contain low-chain saturated fatty acid lipids has more efficient cold temperature spesifications, because long-chain saturated fatty esters cause cloud point of biodiesel. Although, biodiesel which is produced from

high amounts of unsaturated compounds oxidizes faster than petro-diesel and causes insoluble particles which affect diesel engine. While efficient microalgae types are selected from the medium for biofuel production, it should be carefully examined how high the types of microalgae are capable of oil contet and the most desirible fatty acid compositions. Some environmental conditions (such as temperature or pH) can affect the oil efficiencies of microalgae, so considering these conditions on microlalgae efficiency gives a control units for a better quality biodiesel production. [24].

3.6 Selection for Improved Microalgae Strains

Algae farms usually choose *Spirulina* and *Dunaliella* species for large-scale production because their inoculum productions are very low. However, since these species grow in a specific environment (alkali or salinty), they can cause high production barriers. *Chlorella* and *Haematococcus* species microalgae can be grown without the need for specific media, but the first inoculum production rate is high. Species such as *Nanochloropsis* and *Cyclotella* (a diatom) have high productivity efficiency [17].

3.6.1 *Chlorella vulgaris* ESP-6

Choosing suitable algae types for an efficient CO_2 fixation and generating biodiesel must be critical since there are important differences in cell morphology, metabolic ability and genetics among microalgae species with morphological similarity.

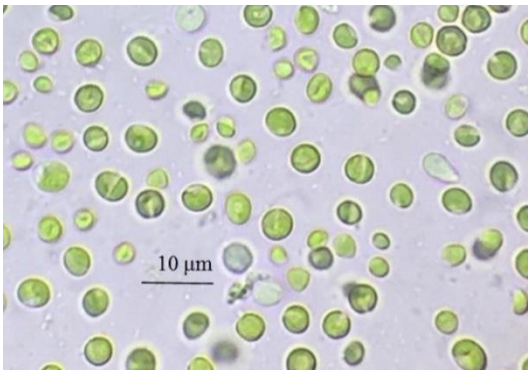


Figure 3.3 Microscopic image of *Chlorella vulgaris* [147]

Chen, X. et al. assessed that 10 speciesof *Chlorella* which were grown for industrial wastewater influents. The results proved, when microalgae growth systems are ventilated with the presence 10% CO_2, *Chlorella sp*. has the best CO_2 binding ability (35.51%) and *Chlorella vulgaris* has the highest lipid accumulation (58.48%). Scanning electron microscopy has proven that *Chlorella Vulgaris* maintains its normal morphology. These results are *Chlorella sp*. and *Chlorella vulgaris* emphasized that it has a very good tolerance for MWI and most importantly, *Chlorella vulgaris* is the most suitable species for biofuel generation due to its high lipid content. In the study, it was stated that FACHB729 and FACHB752 have not tolerating nitrogen deficiency, but ESP-6 and FACHB-484 proved to be a highly effective tolerance to nitrogen ratios. However, FACHB484 and ESP6 was decided to be most efficient species for CO_2 stabilization and production of biofuel. Moreover, they are proved to be suitable species in bioenergy production after industrial wastewater treatment, not only because of their high ability to fix CO_2 and lipid content, but also because of their high tolerance for nitrogen deficient MWI [25].

Xi Chen et al. proved that nutrients like itrogen, phosphorus were removed from anaerobically digested wastewater with four microalgae that are grew in a membrane-photobioreactor. It was performed with *S. dimorphus*, *S. quadricauda*, *C. sorokiniana* and *C. vulgaris* ESP-6 to evaluate the effectiveness of microalgae growth by the side. The biomass formed as a result of microalgal growth terminated in the membrane photobioreactor (MPBR) in 9 days. It has been observed that after four days of incubation in four algae species, they treated more than 75% of ammonia-nitrogen from MPBR. However, the quantity of ammonia-nutrient and phosphat-nutrient removed from the NPBR by the four species of algae was quite lowly that abrogated from the MPBR because of the contaminatings like suspende-solids in the digested wastewater that inhibited microalgal growth. *Chlorella vulgaris* ESP-6 had the second-best performance with a dry weight of 0.45 g / L in the MPBR [26].

Also, *Chlorella vulgaris* ESP-6, microalgae species was used to assimilate soluble metabolites in the hydrogen fermentation water of Clostridium butyricum CGS5 by Chien-Hung Liu et al.. The results obtained in the study prove that C. vulgaris ESP6

can perform microalgal growth in 4-fold diluted dark fermentation broth. Researchers also used acetate efficiently during algal production. *Chlorella vulgaris* ESP-6 algal growth was inhibited by lactate, butyrate and HCO_3 when concentrations were greater than 0.5, 0.1 and 2.72 g/L, respectively [27]. According to these studies *Chlorella Vulgaris* ESP-6 was selected for biodiesel production by in-situ transesterification that is deemed suitable to work for biodiesel production and CO_2 fixation.

3.7 Reactors for Microalgae Growing

Microalgae growth can be achieved by using both photobioreactors and open ponds. But in industrial scale open raceway ponds are generally more economic than the closed systems. New generation microalgal growth is achieved industrially with raceway pond systems and pond systems are generally operated at 20-40 cm height levels. About 0.5 hectares of land is needed for an effective microalgal production system. The most important parameter in microalgae production is mixing conditions. The channel stream speed should be in the range of 15 and 30 cm/sec. Higher rates can consume a lot of energy, for industrial production scale. A further consideration is the equilibrium of the production of O_2 and CO_2 in ponds, which includes depth optimisation, mixing rate, pH / alkali, pond scale and other parameters. There are lots of reactor and pond design for microalgae cultivation. Unidentified highlights include the impact of wind getting on headloss, waves, stream designs, and so on., yet it appears that multihectare pond scales ought to be possible without significant loss of command over principle parameters.



Figure 3.4 Microalgae production in open raceway paddle wheel mixed ponds and tubular photobioreactors. Upper Left, Earthrise Nutritionals, LLC, California. *Spirulina* production, Ponds ~ 1 acre. Upper Right, Haematococcus pluvialis production in tubular photobioreactors (Algatech Co., Israel) [17] Bottom Left, Seambiotic Nannochloropsis sp. culture ponds. Bottom Right, Tubular PBRs in operation, Varicon Aqua Solutions Ltd., UK [146]

Photobioreactors (PBRs) are normally the best solution technology for photosynthetic production which is enclosed. Numerous PBR structures have been created utilizing vessels, for example, tubes, plates, packs, vaults, and so on., and number of them was increased to a huge size (1ha). Tubular photobioreactors are suitableclean- tech solution in industrial scale. Less diameter (5-cm) and higher diameter (> 10 cm) tube reactors, several other models are head in pilot scale production, involving different types of flatplate reactors, hanging bag reactors, hemispheric reactors. Therefore, replacing the output of 4 hectare high rate pond will need many of such devices, each with its tubing, pump, carbonation-control system.Closed system photobioreactors can block to contamination andwater loss

from the culture but PBRs are seriously constrained in weight move. Also their capital cost for new technology pilot scales is too much. But open pond reactors are more scalable and easy-use for algal production. This is because they contain just paddle wheel and culture medium to growth large-scale algae species.

3.8 Supply of CO_2 and Other Nutrients

Carbon is the most important element for algal production besides sunlight. Microalgae can take its carbon in the form of CO_2 by photosynthesis, but the concentration of dissolved CO_2 in the growth medium can be reduced by alkali conditions. If carbondioxide amount is too much incultivation system, Ph will suddenly change and algal growth decrease. Also when CO_2 is produced in abundance, it will be transferred back to the environment from the top of the water. Because injecting CO_2 into cultivation systems would usually result in a detrimental energy loss, optimal use of CO_2 is required. CO_2 must, therefore, be applied regularly and in regulated quantities. The optimal availability of CO_2 is an issue in the construction of algae production. The microalgae medium must be carbonated each time intervals, with the pH decreased to 7.3 to guarantee that suitable CO_2 is adequate for ideal efficiency. pH should be fixed at 7-7.5 because alkali medium can cause microalgae to grow more slowly or die.

The numerous different supplements (P, K, Fe, Mn, Mg, and so on.) is likewise required. Food supplements like carbon, nitrogen and phosphorus are needed as nutrients for growing of microalgae. In addition, little quantities of nutrients like magnesium, potassium, iron, calcium, manganese, sulfur, zinc, copper, cobalt, etc. are needed. Since algae growth have high supplement content contrasted with most yields, reusing abundance biomass supplements after oil extraction is a significant issue. Microalgae generally use ammonium> urea> nitrate> nitrite in order of priority. When microalgae consume ammonium-source nutrient, the H + ion is rise and the pH reduces. Linear connection between the use of nitrate-nutrient and the pH of growth culture and microalgal development. Therefore, pH should be adjusted if ammonium and nitrate are used as nitrogen source.

More than one dietary supplement is needed for microalgal growth. These supplements can be provided with media such as Bold Basal Medium or BG-11, as well as nutrients such as nitrogen and phosphorus in various industrial-wastewater. It was proved that algae use or accumulate contaminants that are dangerous for the aquatic environment, such as nitrogen and phosphorus, in water and wastewater, such as nutrients, pesticides, organic and inorganic toxins, pathogenic organisms. There is also a symbiotic relationship between algae and bacteria in waters rich in organic matter, such as wastewater. In this relationship, while algae provide the aerobic bacterial oxidation of organic matter, carbondioxide and food substance released by oxidation are used by algae in the production of new biomass.

3.9 Temperature and Light Limitation on Productivity

One of the important factors affecting cell morphology and physiology is the temperature of the environment in which microalgae grow. Generally, high temperatures increase the metabolic rate, while low temperatures inhibit microalgal growth. Enzymes in microalgae cells have the highest activity at optimum temperature conditions. Microalgae can produce efficient production at wide temperatures such as 5-35 ° C, but the suitable culturing temperature is 25°C for most of the microalgae species selected for biofuel production. Temperature is also a physical stress factor for microalgae. The lipid composition of the cell, nutrient uptake, carbon fixation and growth rate are affected by temperature.The temperature required for the growth of microalgae must be provided by air conditioning and a special design must be developed for each region. [16,17].

Growth rate and lipid production of microalgae can be different depending on the level of illumination. Excessive light intensity can cause photooxidation and photoinhibition, while low light levels can be growth limiting. For this reason, in the microalgae development, at what intensity of the light reaches sufficient saturation determines the efficiency of light use. In general, the level of illumination at which many photosynthetic microorganisms reach saturation has been reported to be approximately 200 μmol/m^2 s^1.Wahidin et al.found that the effect of photoperiod time on microalgae development and lipid production, and found the best results for *Nannochloropsis sp.* (maximum cell concentration: 6.5x107 cells. ml^{-1}, lipid: 31.3% dry weight) in 18 hours light and 6 hours dark cycle [149].

4
IN-SITU TRANSESTERIFICATION PROCESS

4.1 *in-situ* Transesterification Process

Figure 4.1 shows comparison of traditional (trans)esterification techniques include an extraction of biomass lipids that will then be further used for the manufacturing of biodiesel and *in-situ* transesterification methods.

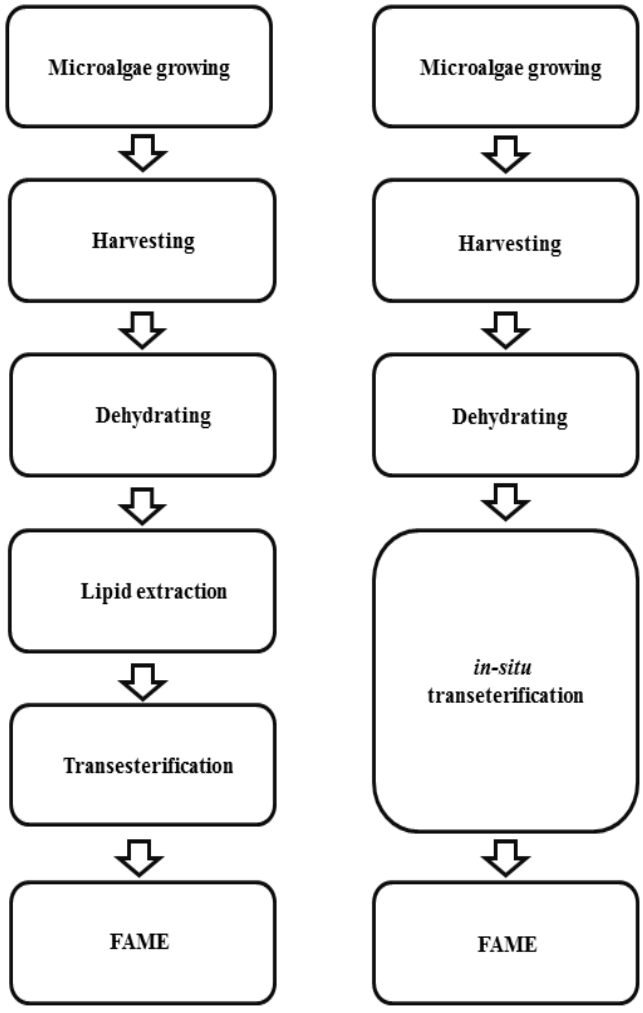


Figure 4.1 Comparison of traditional transesterification process and *in-situ* transesterification process

The transesterification process used for biodiesel production means converting an ester, that is, the lipid, into another ester by treating it with an alcohol accompanied by acid and base catalyst. In-situ transesterification is known as in situ esterification which is the transferation of lipids into biodiesel that do not need the lipid extraction from biomass [101-103]. Esters and by-products occur in the same reaction [104,105]. In some cases, extraction and (trans) esterification procedures may take place sequentially, in some reviews it is still described as *in-situ* (trans)esterification [106,107]. In-situ transesterification process, which is performed without oil extraction from biomass in industrial microalgal biodiesel productions, saves energy and time by reducing initial investment costs. Special presses and soluble extractors used in the oil extraction and purification process usually consist of costly devices, so removing lipid removal steps from the biomass not only eliminate process, but also helps to reduce initial investment costs and reduce operating costs. The quality of biodiesel derived from *in-situ* transesterification process was examined by Hass and Scott [108] and contrast to the ASTM D6751 standards. Kasim et al. [109] showed these findings were correlated to the EN 14214 standards, that the biodiesel generated from *in-situ* methods meeting all the analyses. In some cases, extra washing-step is needed to remove or reduce the FFA material.

Harrington and D'Arcy-Evan's works[101,103] are some of the oldest to study *in-situ* method or direct sunflower seed oils transesterification in 1985. This study could be traced back to 1966 when Dugan[102] contact direct acid catalyzed lipid methylation. Cost analysis of conventional biodiesel production is the main reason for the increase of in-situ process systems recently. In 2006, Haas and colleagues proved how the use of refined lipid as a biodiesel raw material in economic analysis made up about 70 percent of the total biodiesel production operating costs [116]. Lei et al. [138,139] used rice bran with too much FFA content to generate fatty acid ethyl ester (FAEE) by *in-situ* esterification process and proved that about 12% FAEE is taken if ethanol is consumed as solvent to remove rice bran fat. Efficiency of esterification-transesterification rate are reached 98%-83%, respectively.

4.1.1 Effect of Lipid Content of Microalgae on *in-situ* Transesterification

Differences in cultivation also clarify the variability of lipid content that found in microalgae, because of huge amount of lipid content (like 50 wt%), microorganisms are researched for energy production. If microalgae species contain high amount of lipids, in-situ transesterification process is more efficient, and biodiesel efficiency from biomass will be the highest.

4.1.2 Effect of Free Fatty Acid (FFA) Content of Microalgae on *in-situ* Process

FFA and moisture which founded in microalgae are two of the most important features that affect in the production of biodiesel from microalgal biomass and directly affect in-situ transesterification process yield. In order to prevent saponification, traditional base catalyzed technique requires FFA and moisture content under 0.5 and 0.05%, respectively [118,119]. Acid catalyzed esterification optimize superior levels of FFA and humidity (0.5%) [120], but this process was proven to have certain limitations [121]. Water which contained in reaction process adversely affects enzymatic activity for enzymatic processes [121]. Humidity and FFA included microalgae biomass can create saponification in the esterification proces. Therefore they must be reduced before the process. The storage process before the biomass production process affects the free fatty acid properties in the biomass and the humidity. In a study, the FFA level of the biomass increased significantly, even when stored at 4°C [122]. Microalgae generally consist of a higher FFA staple (42%) than agricultural biomass. Lipid biosynthetic pathway often begins by production of fatty acids which are then added to the backbone of glycerol phosphate before they are stored in the cell [9,124].

Inhibition of protein production occurs during the forced oil accumulation and thus disturbs this process causing un-reacted FFA in the organism. Another reason is triacylglycerol (TG) hydrolysis by cellular lipase catalysis reaction [124]. Park et al. conducted a detailed review of processes in the use of wet alga biomass at the time with extraction and esterification [125].

In *in-situ* transesterification process likewise the traditional one, methanol is commonly used as alcohol for distrupting cell Wall and catalyze the esterification

reaction. For fatty acid ethyl ester productioni ethanol is the second best option for biodiesel production. Experimentation of elective acyl acceptors is reliant on a few reasons which include: dissolvability between reactants[129], sustainability of acyl acceptor solvent[126], toxicity [130], reversibility of reactions[129], ecological and chemical conditions [130,126]. Likewise the traditional transesterification process, glycerol is obtained as a side product in in-situ transesterification process. The glycerol production in the market is supported by also side production of biodiesel [127,128].

4.2 Catalytic or Non-catalytic *in-situ* Transesterification Process

in-situ process will be further categorized as catalytic and non-catalytic or sub-and supercritical depending on the state of the solvent or reaction system.

4.2.1 Acid-catalyzed *in-situ* Transesterification Process

Acid catalysts are generally chosen for generating of biodiesel, especially sulfuric acid is widely used and preferred with an alkyl donor like methanol or ethanol [101,103]. Harrington et al. [103] have proven that *in-situ* is a more effective procedure than traditional procedures, with higher FAME yields. The most important reason for this was that it could be converted to FAME in non-extractable components.

Ozgul-Yucel and Turkay examined that optimization of *in-situ* transesterification of rice bran oil (RBO) consuming sulfuric acid as an catalyst. They found that rice bran include higher amounts of FFA that is effective for increasing methyl ester yield. When the FFA content of the oil increased from 16.6% to 84.5%, the ester content of the extract increased by about 67% [132,133]. Kalsum et al. showed that the efficiency of esterification of *Chlorella vulgaris* lipids which turned into FAME with acid-catalyst. According to the results of the experiment, the optimal efficiency of the reaction that took place in 70 minutes was determined as 31.56% [150].

Table 4.1 Acid catalyzed in-situ of microbial biomass [81,82, 86-93]

Feedstock	SSR (ml/g)	Temperature	Time (h)	%YS (%YP)
Slurry	30	75	24	3.93(82)
C. vulgaris	7	60	20	19.4(96.3)
C. Pyrenoidosa	3	60	4	(48.1)
C. Pyrenoidosa	10	90	2	(95-96)
C. Pyrenoidosa	10	120-150	2	(88-90)
Chlorella sp.	4	60	2	(86.1)
	2		8	
Chlorella sp.	4	60	0.5	
	2		2	(96.1)
Nanochloropsis sp.	15	95	2	(99.1)
R. Toruloides	20	70	20	48(94.16)
L. staryeki				48(96.02)
M. İsabellina				56.90(97.30)

Table 4.1 reported that summary of microalgae species studied in *in-situ* esterification method. All reactions were carried out with methanol as an alcohol and sulfiric acid as an catalyst for esterification processes.

4.2.2 Base catalyzed *in-situ* Transesterification Process

The use of the base catalyst in in situ transesterification is much newer than that of the acid catalyst. In 2004, Haas [134] tested the use of the base catalyst and found that 2–9% moisture content in soybean distillery grain[108] and bone meals[136] could be tolerated because of the excess methanol used in *in-situ* transesterification. The main obstacle for microbial biomass when using the base as a catalyst compared to agricultural biomass is its high FFA content.

Velasquez-Orta et al. [81] proved that the alkaline catalyst (sodium hydroxide) could increase amount of biodiesel produced in esterification with slightly greater efficiency than the acid catalyst (sulfuric acid). However, the use of an acid catalyst ratio of 0.35:1 for extended process times led to a higher efficiency 96.8 ± 6.3% by weight of algal biomass, which can be explained by more effective microalgae cell wall. As a result, esterification of microalgae in alkaline site reach higher

transformation in less time than an acid catalysed-esterification. But the most of time the base catalyst is not able to convert high FFA content directly to FAME. Also if the high amounts of alcohol used in *in-situ* transesterification, result will be in terms of saponification. As shown in Table 4.2., the base catalyst was used for algae feedstocks.

Table 4.2 Base catalyzed *in-situ* of microbial biomass [81-85]

Feedstock	SSR (ml/g)	Temperature	Time (h)	%YS (%YP)
C. vulgaris	7	60	20	19.6(96.9)
R. Toruloides	20	70	10	(96.93)
C. Vulgaris ESP-31	6	45	6	18.5(81.8)
Nanochloropsis sp.	45	65	4	26
Nanochloropsis sp.	9	Microwave	0	(80.1)

4.2.3 Two Step Acid-Base Catalyzed *in-situ* Transesterification Process

The *in-situ* transesterification method also has been developed with the two-stage acid base catalyst method for microalgal biomass with high moisture content and FFA. This method expects to esterify FFA with a corrosive impetus and afterward transesterify the abundance oil in the feedstock. This technique may be an expensive for industrial use because of the direct process of acid and base-catalyzed *in-situ* is bringing about high effectiveness attributable to the decreased response time required.

Dong et al.[35] proved biodiesel production with two-stage *in-situ* transesterification to reach high FAME yield. Heterogeneous acid catalyst was consumed to reduce the FFA content and was carried out by a pre-esterification process. As a result of the study, 94.87 ± 0.86% FAME recovery was obtained and the study proved to be much more efficient than a single-stage *in-situ* transesterification. Acid catalyst, Amberlyst-15, was consumed on 8 cycles without any a loss in reaction, which means a significant profit in the name of the catalyst in industrial production. This process is applicable to the industry as it reduces the

generation price of microalgae derivative FAME and achieves higher FAME efficiency with low catalyst consumption.

Table 4.3 Two-step acid –base catalyzed *in-situ* of microbial biomass [35,94,95]

Feedstock	SSR (ml/g)	Temperature	Time (h)	%YS (%YP)
Rice Bran	16	60	0.25 0.25	15.7(105.9)
Jatropha	15	110	0.58 0.22	38.3(97.2)
C. Sorokiniana	14	90	1 0.16	10.6(81.9)
	6	90	1 0.16	12.2(94.1)

4.3 Mechanism of *in-situ* Transesterification

in-situ is transfered as "in the spot" or "in location." It carries out (trans)esterification reactions making at the same time extracting the lipid and transfering it into FAME. Lipids are soluble in solvents like hexane or chloroform. Therefore in the reaction, firstly cell wall is disrupted by alcohol and then solvent solves the lipids and catalysts like sodium hydroxide or sulfiric acid act as a accelerator to convert lipids into biodiesel. Kildiran[140] studied the *in-situ* of soybean oil with specific alcohols and this study got a higher solubility of acyl glycerides, indicating the mechanism of progressive AG solution and alcoholysis-reaction. Haas et al.[134] and Qian et al.[141] stated that intracellular compartments were damaged by the existence of an alkaline catalyst, generating AG and allowing it to be solubilized and subsequently transected.

4.3.1 Solvent to Solid ratio

In *in-situ* transesterification processes, the solvent can be added according to the weight or fat ratio of the microalgal biomass. The solvent oil ratio (SOR) and the solvent solid ratio (SSR) refer to the amount of alkyl donors. Generally, the reaction is optimized according to the solvent alcohol ratio because the fat content in the weight of each algal biomass differs from each other. Similarly, the ideal SOR comparison for a high lipid content microalgal biomass and low content biomass is

difficult because the amount of solvent to be applied can vary depending on the accessible lipids. Although sufficient solvent injection is required for the reaction, it has been proved that excessive amount of solvent can cause slower reaction rate [142,143,144]. Considering SSR as the metric for contrast among different *in-situ*, SSR is strong impact on a process's efficiency and productivity. The SSR of an *in-situ* process depends primarily on the biomass's lipid efficiency, the biomass's ability to absorbing solvent, the biomass's density, and structure. Also reaction components can affect SSR, including the use of cosolvents, catalytic solvents and catalysts. The use of cosolvents is primarily to optimize the solubility between oil and alkyl donors, therefore increasing the rate of reaction in particular by alcohol. Co-solvents are changing as gas and liquid co-solvents. Their intended consumed is generally to increase the solubility of the lipids in alcohol, thereby increasing the reaction rate. Examples of carbondioxide, diethyl ether, hexane, pentane chloroform, acetic acid and acetone can be given for co-solvents. Co-solvents are usually added relative to a quantity of alkyl donors, which is observed with higher SSR. SSR using with gas cosolvent such as CO_2 rate does not change. Therefore the best co-solvent reaches the reducing of alkyl donor with decreasing the SSR [30].

El-Shimi, H. I. et al. Acid catalyzed in-situ transesterification from inedible Spirulina-Platensis microalgae lipids and proved that reaction parameters significantly affect the price of biodiesel production. The experiments were designed and optimized to show how the quantity of reacted methanol, quantity of the acid catalyst, time, temperature and mixing affect the quality of the product produced. The maximum oil amount of *Spirulina-Platensis* microalga was 0.1 g/g biomass. The highest (84.7%) fatty acid methyl ester (FAME) yield was observed when acid catalyst at the rate of 100% w / w lipid [31].

Viêgas, C. V. et al. expressed that the in-situ transesterification with acid catalyst for the *Chlorella* species was made using between 5%-20% by weight H_2SO_4 as catalyst. It was found to have a maximum ester yield of 96-98%. A high proportion of methanol was consumed for transesterification. The experiments proved that

biodiesel from *Chlorella* shows slight oxidative and thermal stability with contrast to vegetable-based biodiesel because of the polyunsaturated FAMEs. [32].

Ehimen et al. proved that the FAME balance transitions tend to be approaching challenges for reaction interval higher than 8 h for all temperatures under investigation. Mixing conditions are advantage for all forming of biodiesel. Rising the humidity content of the microalgae is a disadvantage on the FAME balance and inhibited in-situ esterification process [33].

Kim, G. V. et al. aimed to rise the quantity of the FAME using the type by optimizing various process parameters by in-situ transesterification with *Scenedesmus sp.*. The researches preferred the acidic catalyst analyzed the parameters affecting the reaction with the iortogonal matrix and found reaction temperature (47.5%)> amount of solvent (26.7%) > reaction time (17.5%) > quantity of catalyst (8.3%). However, in this study, biodiesel production achieved better FAME efficiency by using alkaline catalyst NaOH instead of using sulfiric acid as an acidic transesterification process at 55.07% (based on lipid weight) versus 48%. However, the acidic catalyst is average 2.5 times more effective than the NaOH with the following optimal conditions: 70 ° C temperature, 10 hours reaction time , catalyst amount 5% and biomass: solvent ratio 1: 15. Experiments showed that the acidic-solvent combines oil extraction with cell wall deformation in *in situ* transesterification for the production of qualified biodiesel from a Scenedesmus sp.[34].

5
LIFE CYCLE ASSESTMENT (LCA)

5.1 Life Cycle Assestment of Microalgal Biodiesel Production

Life cycle assessment (LCA) infers the environmental purchases of energy, feed stocks, wastes and emissions used for a production, process or service. Microalgal biodiesel production LCA shows an alteration according to many reasons. The main reasons why life cycle assessment is different in microalgal biodiesel production are due to discrepancy in microalgal growth systems, harvest systems which consumes electricity at high levels, logistic costs, transportation situations and land selection for building production plant. The existing LCA analysis for the production of algal oil hinges on point by point fabric structure and financial formation and designing huge acreage of paddle wheel ponds of different kind of photobioreactors. The key GHG emissions could be created from growing, drying, transporting, extracting process and refining algal oil that contains TAG, DAG and MAG into biodiesel. All algal biodiesel production plants cut down on greenhouse gas emissions that released to the environment by producing biodiesel when all of them recycle biomass, waste water or flue gas. Generating electricity from waste biomass and returning it to the facility, converting the heat in industrial process, transforming flue gases into a mechanical energy that can be operated in the facility, producing by-products such as carbohydrates, protein or pigment from the remaining biomass, are the positive effects which affect the life cycle assessment of the microalgal biodiesel. Anaerobic fermentation or biogas production from biomass are other results that positively affect the life cycle assessment in industrial processes.

Technologies needed for proficient production of microalgal biofuel are completely on the tenters, their particular specialized assumptions: (1) growing of algal species in photobioreactors or raceway ponds, (2)harvesting-procedures, (3) high productivity of algal biomass, (4) fertile recycling of clean water. All the above

processes are essential to generate algal biofuels economically viable and environmentally usable. The 400 ha farm is estimated to generate 22,600 L/ha.yr which i equalt to 2500 gallon and it was proved in the US DOE 2018 goal of 2,500 gal/a-yr for algal biofuels. In that study, well-to-wheel (WTW) procedure green house gases releases for biofuel production from microalgae are optimized and results show that when algal biodiesel is used in diesel engine, it will create 28.50 g CO_2e / MJ of biodiesel fuel. Results from the study demonstrate that algal biodiesel eliminates 70% ofgreen houses gases on the production, on the other hand petro-diesel releases 94.7g CO_2e/MJ measured for low sulfur diesel fuel (ULSD) on California EPA.

Even if the oil content in microalgal biomass is low, the same profitability model can be obtained by using it for biogas production in the production facility (sunlight based energy). These outcomes in much more desirable LCA results for biodiesel produced per unit of energy of 2.88 g of CO_2e/MJ at 10% of the tricacylglycerides recoverable in algal weight. Profit/price analysis of by-products is one of the most important challenges in LCA techniques that utilizing multiple allocation approaches, including strategies dependent on the energy output, quantity of the co-products. In general, when LCAs are examined, the benefits of greenhouse gas distribution per product are high as the main product and economic driving force of this process are biodiesel and by-products. Therefore, all increases in GHG resulting from the processing of biodiesel are highly profitable, as the product is reset when used in diesel engines and even lands in terms of carbon emissions.

Life cycle assessment studies of microalgae biofuels has shown that greenhouse gas emissions will be decreased by 70% ratio if biodiesel use in diesel engine. Because the calculated values for greenhouse gas emissions are 94.71 g CO_2e/MJ (CA-EPA) for refined ULSD in California, 85 g CO_2e/MJ for refined diesel-fuel, but microalga biodiesel creates just 28.5 g CO_2e/MJ reported as. Emissions of the soybean -based biodiesel are also low (21.25 g CO_2e / MJ), but this value can remain to 83.25 g CO_2e/MJ if parameters such as land used to grow vegetable biomass and required

logistics values are taken into consideration, it can be concluded that microalgal biodiesel is much more advantageous as a 3rd generation fuel. [28].

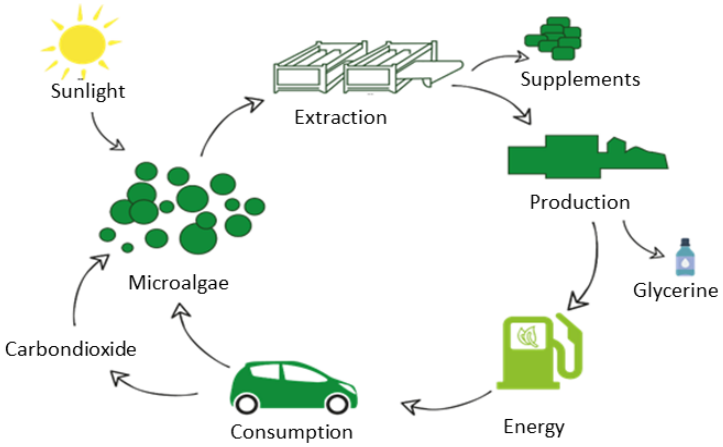


Figure 5.1 Life cycle assesment of microalgal biodiesel production

6
MATERIALS AND METHOD

6.1 Microalgae Species

The microalgae species used in this project, *Chlorella Vulgaris* ESP-6, was obtained from Kocaeli University Chemical Engineering Department.

6.2 Culture Medium

All microalgae cultures used in the study are stored in petri dishes at room temperature. Liquid stock cultures were made in Bold Basal Medium (BBM). The chemicals used in the BBM are given in Table 6.1. All chemicals were of analytical purity and were purchased from Sigma-Aldrich and Merck. The medium of stock cultures was prepared with distilled water and sterilized at 121 ° C for 15 minutes in an autoclave.

Table 6.1 Bold Basal Medium ingredients

	Stocks	**per 400 ml**
1	$NaNO_3$	10
2	$MgSO_4.7H_2O$	3
3	NaCl	1
4	K_2HPO_4	3
5	KH_2PO_4	7
6	$CaCl_2.2H_2O$	1
		per litre
7	Trace elements solution (autoclave to dissolve):	
	$ZnSO_4.7H_2O$	8,82
	$MnCl_2.4H_2O$	1,44
	MoO_3	0,71
	$CuSO_4.5H_2O$	1,57
	$Co(NO_3)2.6H_2O$	0,49
8	H_3BO_3	11,42
9	EDTA	50
	KOH	31
10	$FeSO_4.7H_2O$	4,98
	H_2SO_4 (conc)	1 ml
	Medium	**per litre**
	Stock solutions 1 - 6	10 ml each
	Stock solutions 7 - 10	1ml each

Preparation of 1 L medium; 10 ml of each stock solution was taken. 1 ml of the trace element solutions was added to the mixture and autoclaved to dissolve the compounds. Finally, it was completed to 1 L with distilled or deionized water.

6.3 Instruments

All devices used in the studies were located at Yıldız Technical University, Bioengineering Department Lipid Technology Laboratory. The ambient temperature and light suitable for microorganisms to grow were provided by Kerman incubator, optical density measurements were done by Scinco S-3150 UV-Vis Spectrophotometer, pH measurements were provided by Control 40 LZB-DK800-4 pH meter. Pure water used in the analysis and stock culture medium preparation was obtained from GFL Water Stills 2002 pure water device. Stanley Technical Silent brand pump was used to supply air to microalgae cultures and its flow rate was adjusted with Cole Parmer 1-800-323-4340 flowmeter. Seral Gaz branded CO2 cylinders were used to feed cultures. Precisa AB-0066-K was used for analytical balance weighing and mixing of cultures was provided by Heidolph Unimax 1010 shaker. In microorganism settling processes, the Nuve NF 1200R centrifuge device was used and the BINDER brand oven was used for harvesting of microalgae. Isopod brand soxhlet device was used with the Kerman brand back cooler to obtain oil from microorganisms. Solvents were obtained from the samples with Heidolph Laborota 4000 rotary evaporator. Heidolph Hei-Standard brand magnetic stirrer heater and back cooler were used for the biodiesel production reaction. Also, the Agilent 7820A model gas chromatography device was used to determine the fatty acid composition of oils obtained from microorganisms. Macherey Nagel brand chromatography paper and 2 tanks (iodized-iodine-free) were used for thin layer chromatography.

6.4 Chemicals

In transesterification reactions, 99.5% pure methyl alcohol (Merck), 96% pure ethyl alcohol (Merck) were used as alcohol, sodium hydroxide (Merck) was used as a catalyst. Benzene (Merck), chloroform (Merck), hexane (Merck), acetic acid (Merck) were used for Iatroscan analysis and hexane (Merck), diethyl ether (Merck), acetic acid (Merck), iodine (Advanced Diagnostic Research, ADR) were used for thin layer chromatography,

6.5 Experimental Methods

6.5.1 Production of Stock Cultures

The liquid medium was adjusted to pH 7.00 at the beginning. The temperature is constant at 25 ± 2 ˚C during culture production. Algae inoculation was made from an agar medium with a syringe into 250 ml of 3 erlens.

The cultures whose developments were monitored were transferred to 1L glass erlens by vaccinating approximately 20% algae. Carbondioxide was provided by CO_2 tube to the first one, air supply was provided to the second by a compressor, and no external factors were provided to the third. The flow rate of the air sent was adjusted with the help of a flowmeter. The liquid culture production mechanism is seen in Figure 3.3. The absorbances of liquid cultures at 600 nm were measured at certain time intervals with the help of a UV spectrophotometer. The cultures were renewed during the stopping phases.The inoculation rate was adjusted to be 20%. The following trials have been performed for *Chlorella vulgaris* ESP-6 cultures. Three working groups have been created:

Control group: Microalgae culture at atmospheric conditions

CO_2 group: Microalgae culture + CO_2

Ventilation group: Microalgae culture + air supply

The temperature of the cabin where the microalgae cultures were grown was adjusted to 25^0C and 40-60 watt daylight fluorescent lamps were used for lighting. Cultures grown in 250 ml flask were prevented from settling down by shaking on a

shaker at 150 rpm. Air supply was provided to the cultures placed in the air conditioner using a compressor. The flow rate of the air sent was adjusted with a flowmeter to be 1L/min. Cultivated *Chlorella vulgaris* ESP-6 was transferred to 1-liter glass bottles, modified with controlled carbon dioxide sequestration to keep the pH at the same level. CO_2 was provided at a flow rate of 0.2 vvm Agitation of cultures examined by air supply was achieved.



Figure 6.1. Growing of *Chlorella vulgaris* ESP-6 a) Cultivation of microalgae in incubator b) CO_2 tubes for CO_2 sequration c) Pump for O_2 mixing d) Flowmeter for pumping

Chlorella Vulgaris ESP-6 *Culture with Carbondioxide;* While airing to algae cultures with a volume of 1 liter, prepared for carbon dioxide experiments, air supply was provided from the 100% purity CO_2 cylinder with regulator through the flowmeter, with air. Since the volumes of the cultures were over 1 L, continuous air supply was applied during the experiments with the help of a compressor. The pH balance of the culture was controlled with a pH meter. The illumination was provided with daylight type fluorescent lamps. The trials lasted for 12 days. Culture parameters and optical densities were measured and recorded.

6.5.2 Harvesting Microalgae Cultures

The characteristic development stages of microalgae take place in five stages:

• Adaptation Phase (Lag Phase): It is the stage where the development is relatively slow since the cultivated algae cells are in the adaptation stage.

• Exponential Phase (Log Phase): The cells begin to divide regularly and continuously at a constant rate. At this stage, the rate of development is at the maximum level.

• Layover Phase (Stationary Phase): There is no change in the number of cells in this phase where the proliferation and extinction rates of the cells are equal.

• Collapse Phase (Death Phase): At this stage, the number of cells begins to decrease suddenly.

The end of the logarithmic phase and the stationary phase of the cultures were monitored according to the optical density values. The point where the microalgae pass from the log phase to the stationary phase is the point where the oil synthesis is maximal. *Chlorella Vulgaris* ESP-6 reached the stagnation phase in about 11-15 days. Algae, which reached the stationary phase, were harvested by centrifugation at 4800 rpm for 5 minutes at the end of the production period, and the remaining medium was removed. After this process, purified water was added to the algal biomass three times to clean the remaining traces of nutrient medium and centrifuged.



Figure 6.2 Harvesting of microalgae cultures a) Microalgae cultures in 50 ml centrifuge tubes for harvesting b) Centrifuge

6.5.3 Drying process of microalgae

Centrifuged wet microorganisms were dried in centrifuge tubes at 35 ºC for 1 day until reaching a fixed weight in BINDER brand oven.



Figure 6.3 Drying of microalgae for 1 day 35 ºC

6.5.4 Soxhlet extraction

After the cultures were harvested and dried by centrifugation, the dry biomass formed was pulverized using a grinder. 0.5 g of dried *Chlorella Vulgaris* ESP-6 sample was accurately weighed into the soxhlet glass flask with balance. The pre-weighed sample tube was placed in the soxhlet extraction section. Approximately 200 ml of hexane was transferred to the solvent container and put on the heating unit. The first siphon was taken together with the hexane, the flow of the hexane was

prevented in the soxhlet device operated with reflux at a maximum of 4 temperatures. Time-based extraction was carried out for 6 hours. The solvent was separated from microalgae oil using a rotary evaporator. The flask was then taken to an oven for 45 minutes at 100˚C and then to the desiccator for 20 minutes, processing was continued until constant weighing was achieved.

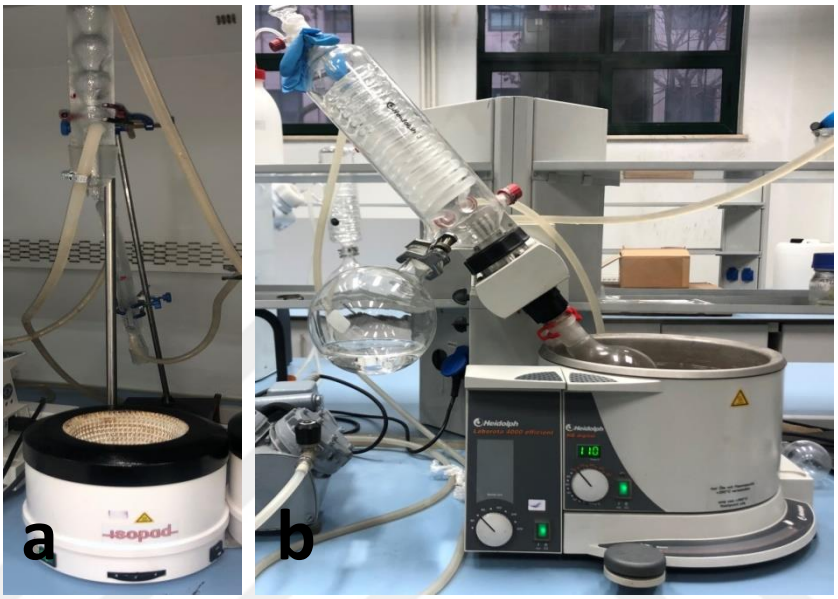


Figure 6.4 Lipid extraction from algal biomass a) Soxhlet extraction and condenser for lipid extraction b) Solvent recovery

6.5.5 *in-situ* Transesterification with Base Catalyst

The *in-situ* transesterification reaction and biodiesel purification steps are shown in Figure 1. 1 g dry microalgae mass was reacted with different concentrations of methanol which include different concentrations of transesterification reaction catalyst NaOH. The reactor system has a volume of 100 ml and was worked under reflux to prevent methanol from vaporazing. 1 g dry microalgae mass was pulverized with a grinder and mixed with methanol of different concentrations in which dissolved NaOH in different proportions beforehand. The reactions were carried out for 120 minutes at constant temperature at 64˚C.

Alkali process experiments were carried out as following;

Time 2 hours, reaction temperature 60˚C, amount of alcohol reacted (methanol, 99.5%) from 6ml to 10 ml, sodium hydroxide concentration 0.3-0.6% (g biomass-

based). The reaction system was integrated with the condenser to reduce solvent loss. NaOH and methanol were added to start the transesterification process. The entire reaction was proceed at a magnetic stirrer heater at a speed of 500 rpm.

After the reaction was completed, the solution was allowed to settle down and cool for 1 hour. Solution was filtered to remove excess biomass under suitable ambient conditions for later experiments. The reaction mixture was taken to the separatory funnel, 10 ml distilled water and 10 ml of hexane was put thereby separating the hydrophilic and hydrophobic components. After the extracted FAME (biodiesel) was washed 3 times with hexane, anhydrous sodium sulfate was put to seperate excess water. Before the hexane phase was blown, the sample tube was tared. Excess hexane was vaporized in the rotary evaporator to obtain the FAME mass.

6.5.6 *in-situ* Transesterification with Acid catalyst

Chlorella vulgaris ESP-6 powder (1 g) was mixed with 6ml and 10ml methanol containing different concentration of sulphuric acid (100% and 150% respectively, based on the lipid weight), these were proceed at 60°C for 150 minutes.

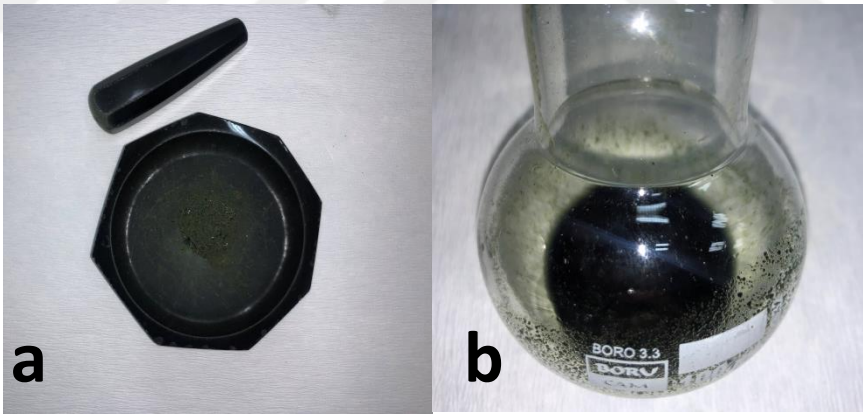




Figure 6.5 *in-situ* transesterification steps for biodiesel production a) Grinding of 1 g microalgae b) Mixing microalgae powder with methanol-NaOH solution d) Obtaining FAME products from reaction mixture by seperating funnel e) Recovery of excess solvent with condenser and pump

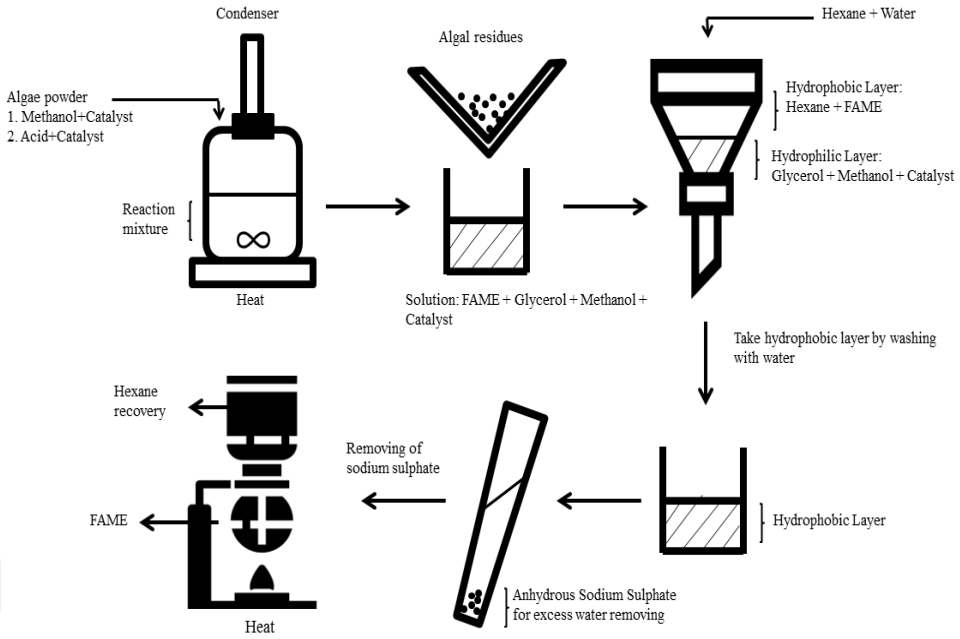


Figure 6.6 *in-situ* transesterification process diagram

6.6 Analysis Methods

6.6.1 Optical Density Measurement of Microalgae

Samples were taken from the culture medium with the help of sterile 1 ml micropipette. Samples were transferred to quartz cuvettes with volumes of 3 ml and their optical density (OD) resullts were measured in a 600 nm wavelength spectrophotometer. The media of the cultures were used as reference samples. When the optical density values exceed 1.00 absorbance value, the samples were diluted in certain ratios by volume and measurements were made.



Figure 6.7 Spectrophotometer for Optical Density (OD) measurements

6.6.2 pH and Temperature Measurement of Microalgae

Culture medium pH and temperature measurements were made with the help of Kontrol 40 pH meter. The probe of the device was immersed in the culture medium and readings were made.



Figure 6.8 pH meter for measurements

6.6.3 Cell Counting

The cell density of each culture batch should be measured daily to monitor growth and contamination in ideal conditions.

The cell count, which is viewed daily during the stock culture creation for large quantities in the party culture system, was not continued due to the high misleading share and difficult counting when large quantities were passed.

Cell counting of algae was performed under a biological microscope using a counting chamber (hemocytometer). It includes thick rectangles in the middle of the counting chamber or circular indentations in which algae cells to be counted as H are placed. Depending on the type of the cabin, there are one or two counting areas forming a chamber with a depth of 0.1 mm. Sample from stock culture are mixed thoroughly for counting purposes. Using a pasteur pipette, a drop from the sample is detected in the chamber in the counting chamber. For example, when placing in the counting chamber, the following points should be considered:

The counting chamber is cleaned and put into 90% alcohol. It must be absolutely free from dust and oil. It is placed on the dimensioned (ruler) part of the cover slip counting chamber.

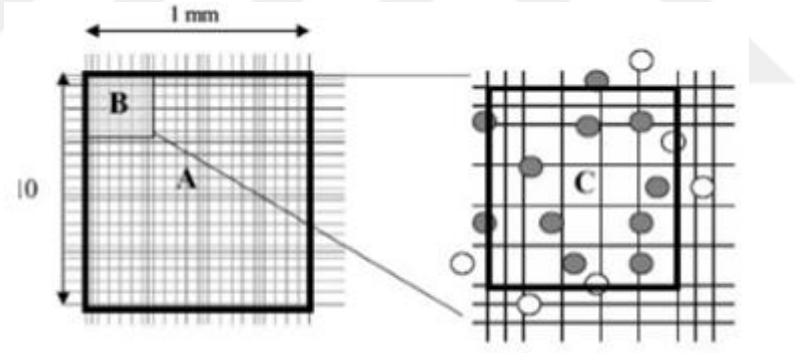


Figure 6.9 Cell counting by Thoma lamina A) Large square B) Medium square C) Small square

This counting chamber has large, medium and small squares. The area of the big square (A) is 1mm x 1mm. A large square usually has 16 or 25 medium-sized frames (B), while a medium-sized frame has 25 small squares (C). By selecting randomly 5 medium (B) sized frames, all the cells remaining in the area surrounded by thick lines in these frames are counted under the microscope 200 to 400 times (Figure) If the cell density of the culture is low, all cells in the large square (A) are counted. Cells that touch the top and left border line surrounding the counted square are counted,

while those touching the bottom and right border are not counted. The cell density of the culture is calculated as follows:

• Total number of cells counted in 5 medium sized squares: N1 + N2 + N3 + N4 + N5

• Average number of cells in the medium-sized square: (N1 +.... + N5) / 5

• If large squares (A) contains 16 medium squares (B), the total number of cells in the Census chamber; (N1 +.... + N5) / 5 x 16 Volume of the counting chamber;

Accordingly, microlgae cell density (cells / ml) can be calculated as follows.

(N1+....+N5)/5 x 16 x (1,000/0.1) = (N1+....+N5)/5 x 16 x 10,000

If the large square (A) contains 25 medium square (B), the total number of cells in the Counting chamber (cells / ml); (N1 +.... + N5) / 5 x 25 x 10,000

If all the big squares are counted (A); Algae density (cells / ml) = N x 10,000

6.6.4 Determination of Microalgae Growth Rate and Cell Retention Time

Specific growth rates of microalgae grown in different conditions were calculated with the excel chart according to Eq. 6.1.

$$\mu max = \frac{lnx2 - lnx1}{t2 - t1} \quad (6.1)$$

In the formula, X2 and X1 denote the biomass concentrations in the logarithmic (exponential) growth phase t 2 and t 1. The doubling time (T_d) of microalgae cells was calculated according to Eq. 6.2.

$$T_d = \frac{ln2}{\mu max} \quad (6.2)$$

μmax is the specific growth rate found with the help of Eq. 6.1.

6.6.5 Microorganism productivity calculation

Cell productivity is calculated by Eq. 6.3;

$$P_x = X_{max}/D \quad (6.3)$$

where P_x is the volumetric biomass productivity (g/L.day), X_{max} is the maximum cell dw concentration (g/L), and D is the cultivation time (day).

6.6.6 Lipit Productivity Calculation

When calculating the lipid mass in the microalgae biomass, the first weight and the subsequent weight of the sample tube are subtracted from each other before oil extraction. The resulting result should be calculated as a percentage value.The amount of lipid extract was calculated using the Eq. 6.4:

$$\text{Lipid content (\%)} = \frac{([\text{Sample tube tare (g)} + \text{Lipid (g)}] - [\text{Sample tube tare (g)}])}{(\text{Sample amount (g)})} \text{ x } 100 \qquad (6.4)$$

Lipid productivity of *Chlorella vulgaris* ESP-6 was calculated as in Eq. 6.5:
P_{lipid} is the volumetric lipid productivity (g/L.day), P_x is the volumetric biomass productivity (g/L.day), and $lipid\%$ is the final content of lipids and was given as percent dw.

$$P_{lipid} = P_x \, lipid\% \qquad (6.5)$$

6.6.7 Determination of Fatty Acid Composition

The contents and amount of the biodiesel obtained (FAME) was proved using GC (Agilent 7820A). The GC device consists of a FID detector and a 30 m long column with a film thickness of 0.25 µm and an inner diameter of 0.32 mm (CARBOWAX 20M). The detector temperature is 280ºC and the rate of separation is 1: 50. The column temperature was set at 50 ° C for 1 minute, then programmed at 25 ° C / min to 200 ° C, 3 ° C / min to 230 ° C, and kept constant at 230 ° C for 18 minutes. Following this, it was brought to 280ºC at 40ºC / minute and held again for 3 minutes. Helium was as the carrier gas of GC. FAMEs were primarily esterified to determine the fatty acid composition of the microalgal biodiesel. Then 0.1 g lipid the sample was adjusted and weighed into the sample tube. 10 ml of n-heptane was added to the sample and mixed until dissolved. Then, KOH dissolved in 0.2 ml 2 N methanol was added and vortexed. The supernatant containing the methyl ester was separated in the mixture, which was centrifuged at 5000 rpm for 10 minutes. Finally, 1 µl injection was given to the device [151].

The ester content, C, was calculated by using below equation and showed in terms of a mass fraction in percent.

$$C\% = \frac{(\sum A) - PAS}{PAS} X \frac{(CS) - VS}{m} X 100 \quad (6.6)$$

$\sum A$=total peak area from FAME

PAS=pek area of standart solution

CS=concentration, in mg/ml, of the standart

VS=volume, ,in ml, of the standart

m= mass, in mg, of the sample

6.6.8 *in-situ* Transesterification Efficiency

The resulting FAME mass is calculated using the formula:

$$\text{Biodiesel(FAME)(g)} = \frac{[Sample\ tube\ tare\ (g) + Biodiesel\ (FAME)\ (g)] - [Sample\ tube\ tare\ (g)]}{Algae\ amount\ (g)} \text{ x } 100 \quad (6.7)$$

Biodiesel efficiency of microalgae lipid was calculated using the formula:

$$\text{Biodiesel productivity (\%)} = \frac{Biodiesel\ (FAME)(g)}{Algae\ amount\ (g)\ x\ Lipid\ content\ (\%)} \quad (6.6)$$

6.6.9 Qualitative Analysis of Fat and Esters by TLC

TLC was performed for the qualitative analysis of FAMEs from microalgae. A mixture of hexane:diethyl ether:acetic acid (80:20:1 ml) was placed as a solvent for the layer prepared for thin layer chromatography. 1 µL of the samples previously dissolved in appropriate amounts of hexane was pulled with a micropipette and injected into a thin sheet paper at 1 cm intervals. Immediately after the injection, the thin layer was kept to the height set in the paper solvent tank, then kept at ambient temperature for 1 minute and taken into the iodine tank. Iodine vapors were given for 2-3 minutes to make the samples in thin layer paper visible.

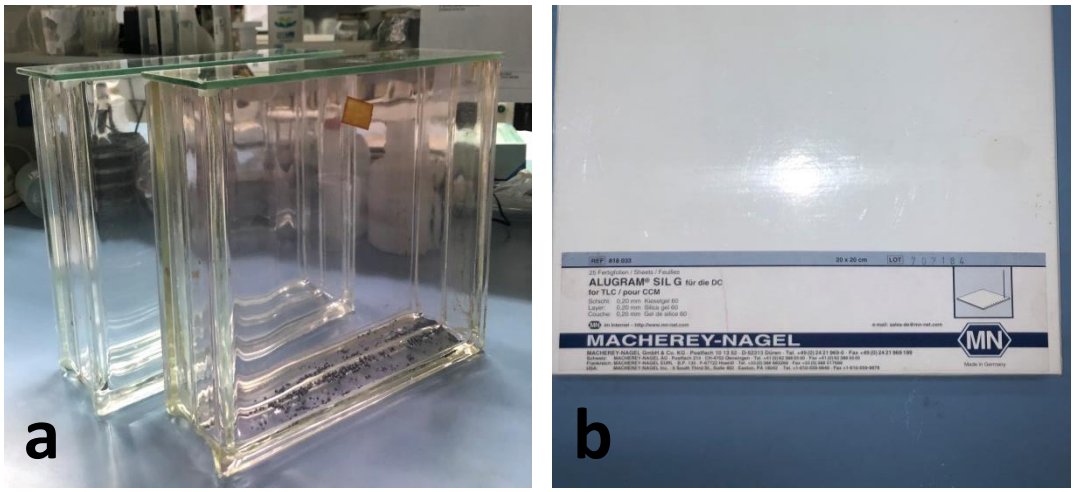


Figure 6.10 Thin layer chromatography (TLC) for methyl ester determination a) Tanks with iodine and without iodine b) Thin layer chromatography layers

7
RESULTS AND DISCUSSION

In this experimental study, *Chlorella Vulgaris* ESP-6 was cultered primarily. During this production, Bold's Basal Medium(BBM) was used as a medium. The effects of atmospheric conditions, air supply and CO_2 with different concentrations on the growth efficiency of *Chlorella Vulgaris* ESP-6 were investigated in constant temperature, light intensity and lighting times. Growth kinetics were detected. Oil was extracted from algae obtained at the end of the trials and biodiesel production was achived by different catalyts types and concentrations, different methanol quantities. FAME analyses were done by accurently.

7.1 Growth Conditions and Kinetics of *Chlorella vulgaris* ESP-6

7.1.1 *Chlorella Vulgaris* ESP-6 Used as Stock Culture in Production

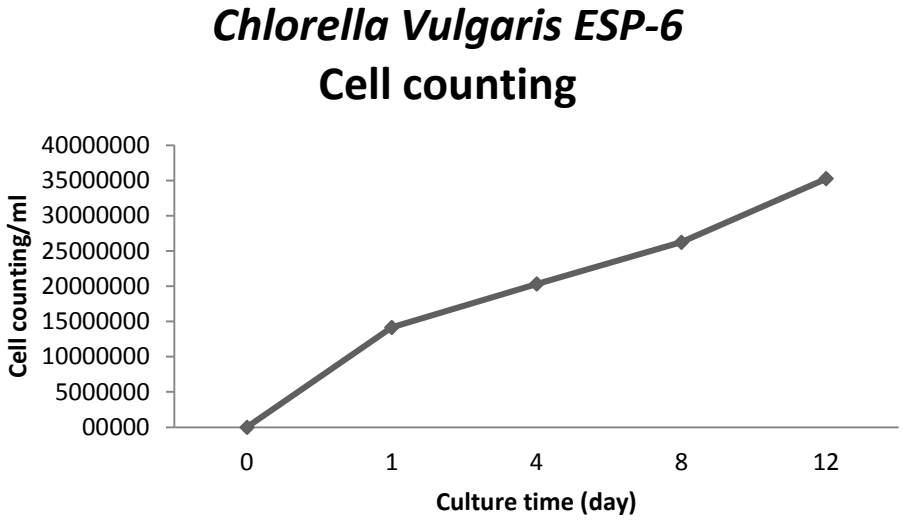


Figure 7.1 Cell number of *Chlorella vulgaris* ESP-6 in Bold Basal Medium

Cell counts of sample cultured in 50 ml falcon tube was given to start production in large quantities. Sample has reached the end of the lag phase on 12 days. With the highest cell density, 35248999 cells / ml.

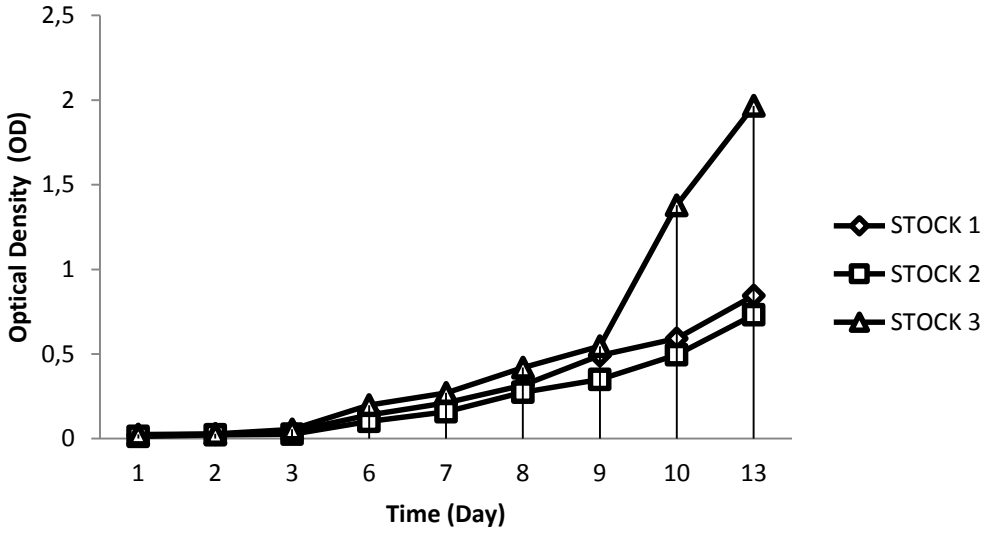


Figure 7.2 Optical density values of stock cultures at 600 nm

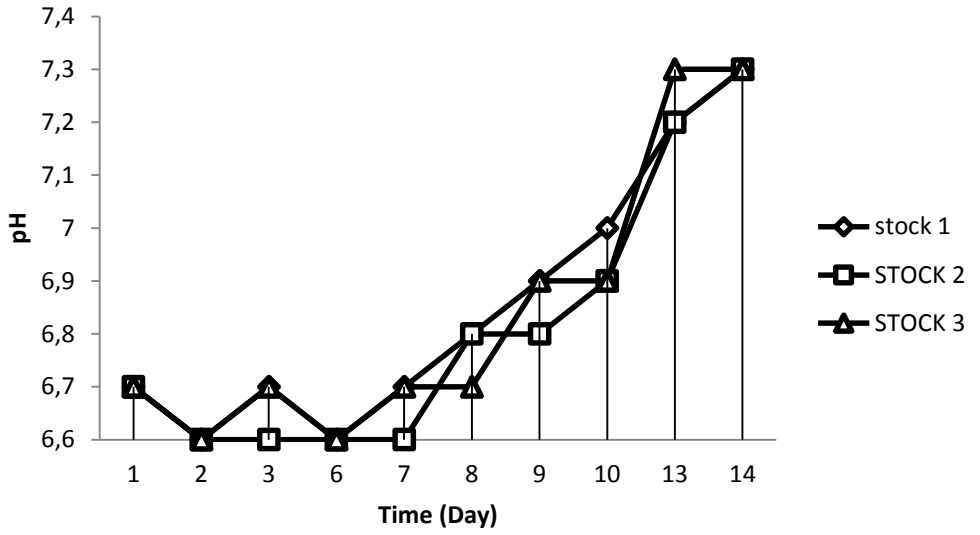


Figure 7.3 pH values of stock cultures

Stock cultures were inoculated from a 50 ml falcon tube and grown in suitable ambient conditions for further experiments. All of them reached their optimal growth at the end of 13th day. Their pH measurements changed between 6.6 to 7.3.3 different stock cultures were reproduced in 1 liter glass bottles for CO_2, atmospheric conditions and air supply.

7.1.2 Effect of CO_2 on Microorganism Growth

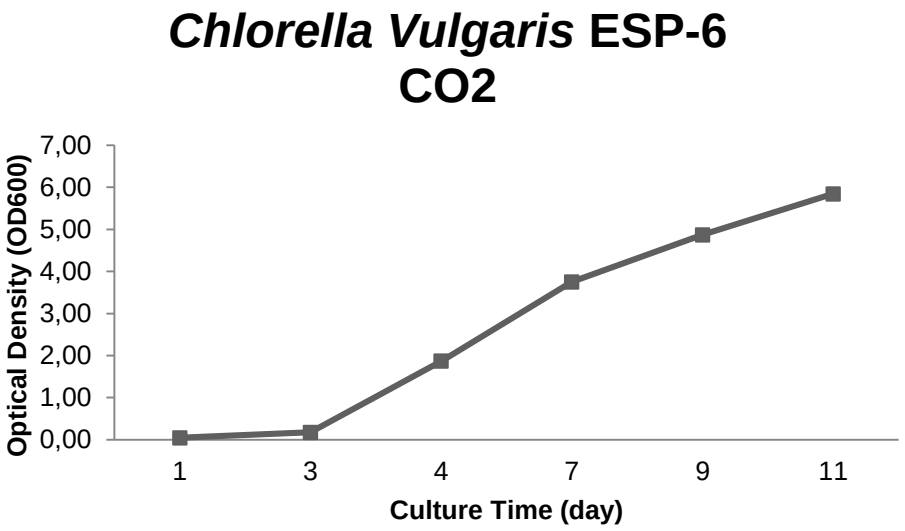


Figure 7.4 Optical density values of *Chlorella vulgaris* ESP-6 grown with CO_2 at 600 nm

100% pure CO_2 was used for maintain a stable level of pH in the cell culture. After 11 days *Chlorella vulgaris* ESP-6 was found to be reached at maximum level of its density. The effect of CO_2 on growth is obvious in the experiments. The highest cell density value was found to be 0.26 g/L at 100% pure CO_2-fed with 5.844 OD_{600} value. It was observed that the highest dry mass weight was reached with CO_2. As it is suitable in terms of production efficiency, it is at a level to be successful in large-scale productions. At high ratios of CO_2 tolerance varies depending on the species grown. Chiu proved that *Chlorella sp.* and *Nannochloropsis oculator* completed theirgrowing at %5 CO_2 concentration. In contrast, *Chlorella kessleri*, *Scenedesmus obliquus*, *Spirulina sp.*, *Chlorella vulgaris*, and *Chlorella sorokiniana* completed their best development under 6% CO_2. Hanagata reported that *Scenedesmus sp.* and *Chlorella sp.* stated that the efficiency rate for both organisms was 0.15 g/Lwith 10% CO_2 and 0.18 g/L with 40% CO_2. [148]. In this study, it was observed that *Chlorella vulgaris* ESP-6 has high tolerance to 100% purity CO_2 and showed high growing efficiency.

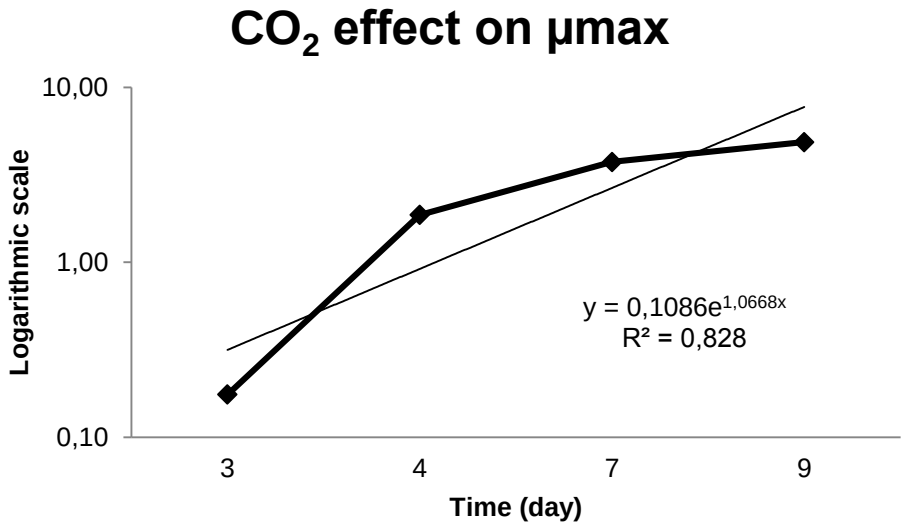


Figure 7.5 *Chlorella vulgaris* ESP-6 maximum specific growth rate under pure CO_2 supply

The highest microorganism growth rate (μ_{max}=1,06 d^{-1}) was achieved in the culture medium fed with CO_2. This has shown that *Chlorella vulgaris* ESP-6 can be used in an industrially sustainable way to reduce CO_2 emissions with its high CO_2 biofixation capacity as well as the potential to produce FAME (Biodiesel).

7.1.3. Effect of Atmospheric Conditions on Microorganism Growth

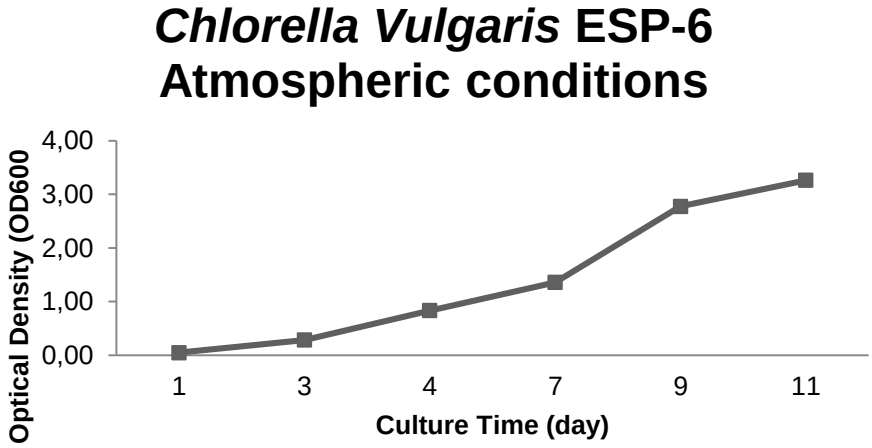


Figure 7.6 Optical density values of *Chlorella vulgaris* ESP-6 grown with atmospheric conditions at 600 nm

As can be seen from Figure 7.6, at the end of the 11th day, the optical density value of the culture, which was just grown with atmospheric conditions, reached 3.49. The most important reason for its low efficiency compared to other cultures is that sufficient mixing efficiency could not be achieved by only with atmospheric conditions.

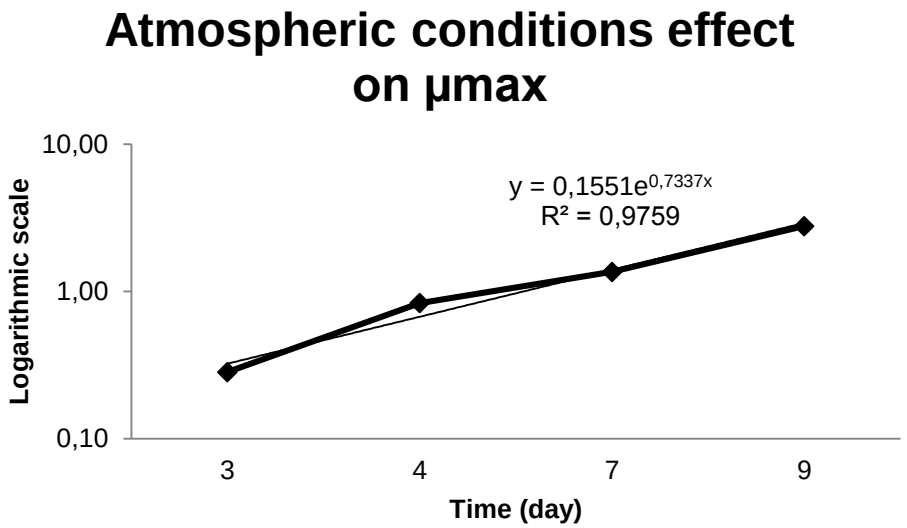


Figure 7.7 *Chlorella vulgaris* ESP-6 maximum specific growth rate under atmospheric conditions

The lowest microorganism concentration and growth rate (X_{max}= 0.15 g/L and μ_{max}=0.73 d^{-1}) were achieved in the culture medium fed with atmospheric conditions. Although the exponential phase in this cell culture was reached within the same time interval compared to other cultures, the mixing conditions reduced the specific growth rate compared to others, causing a slowing effect on growth kinetics. In industrial microorganism cultivation, it is foreseen that microalgae that will be grown only with atmospheric conditions will be insufficient in production.

7.1.4 Effect of O_2 on Microorganism Growth

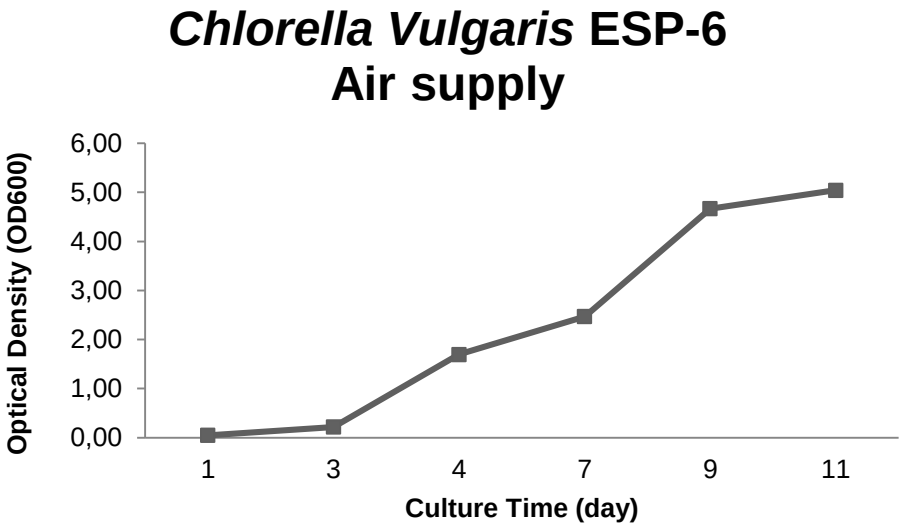


Figure 7.8 Optical density values of *Chlorella vulgaris* ESP-6 grown with air supply

At the end of the 11th day, the optical density value of the culture, which was fed by air, reached 5.042. Although the cell density of air supplied culture was lower than CO_2-fed culture, it showed the potential to achieve a faster saturation stage at the same time intervals

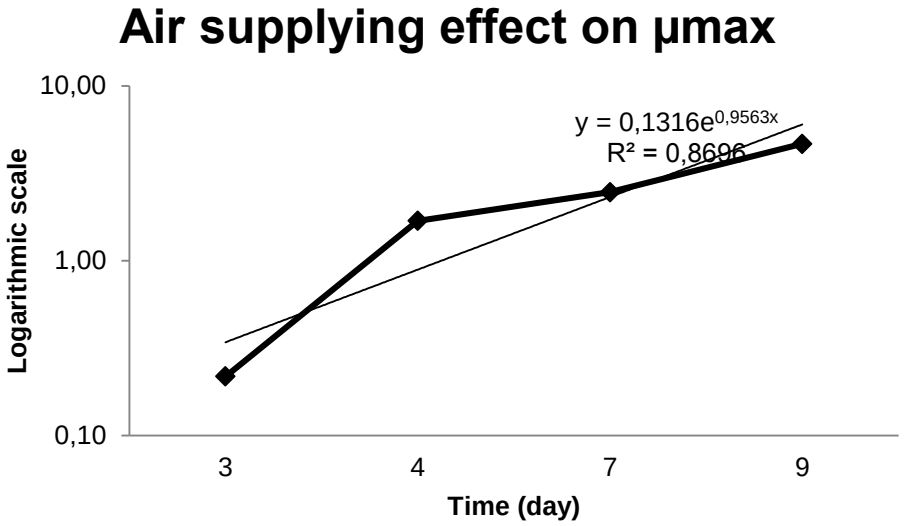


Figure 7.9 *Chlorella vulgaris* ESP-6 maximum specific growth rate under air supply

The efficient microorganism concentration and growth rate (X_{max}=0.22 g/L and μ_{max} = 0,95 d^{-1}) were achieved in the culture medium fed with air.

7.1.5 Effect of feeding source on microalgae cultures density

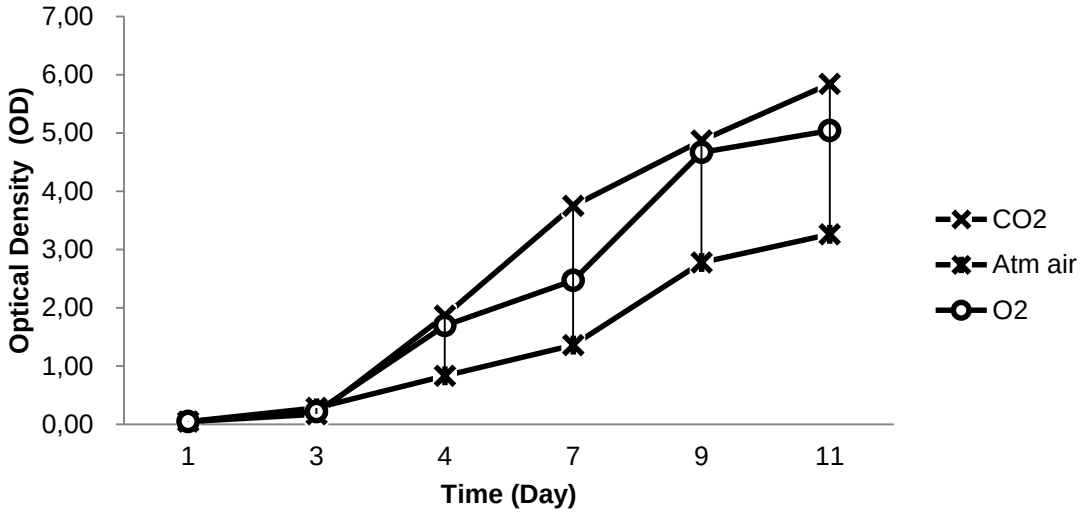


Figure 7.10 Effect of different feeding source on microalgae culture medium

In the Figure 7.10, a comparison of algae cultures fed with CO_2, atmospheric conditions and -air supply are given during the 11-day trial. It was observed that algae cultures fed with CO_2 and air supplied culture reached the highest cell density at the end of the 11th day with an optical density value of 5.84 and 5.04. The extra mixing effect of algae cultures fed with CO_2 and O_2 compared to the culture made only by atmospheric conditions had an impact on culture growth. The time interval to reach their exponential phase in three different cell cultures is close to each other. As it can be understood from the magnitude and duration of the growth in Figure 7.10, Chlorella vulgaris ESP-6 will show high efficiency in the environment fed by CO_2 for industrial microalgae production farm. Compared to the work done by Yin-Ru Chang a & Duu-Jong Lee, it was observed that the growth curve was showed at the same time intervals.

7.1.6 Effect of pH on Growing of Cultures

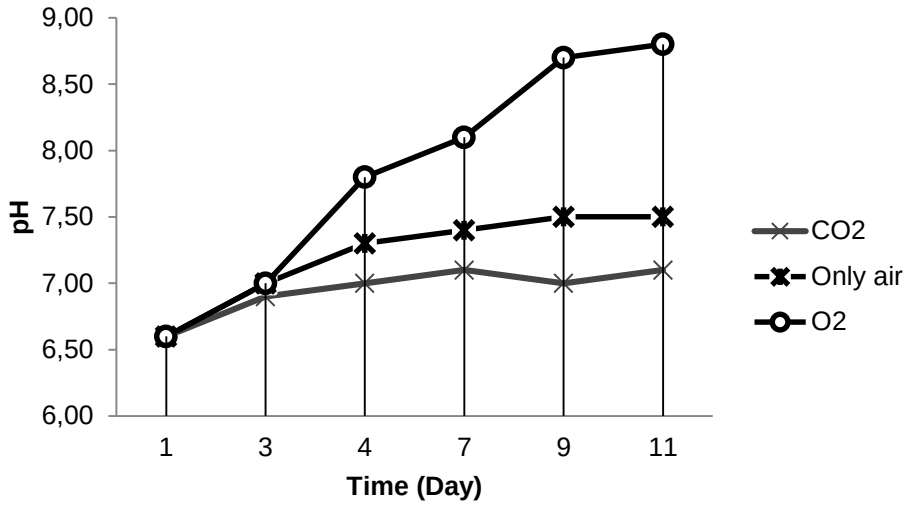


Figure 7.11 pH changes for all microalgae cultures

As seen in Figure 7.11, initially the pH of the environment varies between 6.5 and 8.7 for culture fed with O_2, 6.5 and 7.0 for cultures fed with CO_2 and atmospheric conditions. The initial temperatures of the cultures for *Chlorella vulgaris* ESP-6 were measured as 24.5 ± 0.2 ° C. During the culture, temperature values varied between 26 ° C and 24.5 ° C for each medium. Cultures are illuminated with LED bulbs 24 hours a day. Losses occur over time in the volume of cultures due to evaporation.

The ionic concentration of different species determines the pH of the medium. These are aqueous CO_2, carbonic acid (H_2CO3), bicarbonate (HCO^{-3}) and carbonate (CO_2^{-3}) formed when CO_2 dissolves in water. Depending on the temperature, pressure and concentration of the ions, cultures perform the following chemical equilibrium reaction and the addition of CO_2 lowers the pH of the medium.

$$CO_{2(aq)}+H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO^{-3} \leftrightarrow 2H^+ + CO^{-2} \quad (7.1)$$

As microalgae grow, they consume CO_2 in the environment and the equilibrium reaction shifts to the left and the pH rises. This was observed in culture fed with air. Between the 3rd and 11th days, pH increased faster than the others and reached 8.5.

The biggest reason for this is that no external CO_2 supply was made to the culture environment. So CO_2 inside was consumed and pH rised.

Similarly, the pH rises due to photosynthetic reactions. In autotrophic cultures, microalgae use inorganic carbon and give hydroxyl to the medium. Therefore, it is expected that the ambient pH will rise and be constant after a certain time. This has been observed only in air-fed culture. Ph increase due to photosynthetic reactions showed more in this culture. Since the CO_2 fed culture was adjusted to a stable 7 by pH meter, the change in pH from growth was not observed much. Therefore, growth is much more productive than other cultures.

7.1.7 Maximum Specific Growth Rates and Doubling Times

Table 7.1 gives the values of cell concentration, growth rate and replenishment time obtained in different feed sources for *Chlorella vulgaris* ESP-6 fed with CO_2, atmospheric conditions and air supply, respectively.

Table 7.1 Effect of different feeding source on the growth rate of *Chlorella vulgaris* ESP-6 species

Feeding Source	Xmax (g/L)	Px (g/L day)	μmax (d-1)	Doubling Time (Td) (h)
CO2	0,26	0,02	1,06	15,62264151
Atmospheric air	0,15	0,01	0,73	22,68493151
Air supply	0,22	0,02	0,95	17,43157895

Table 7.1 compares the maximum specific growth rates of cultures with different feed sources. While the maximum specific growth rate of the culture fed with CO_2 was calculated as 1.0668 day^{-1}, the culture fed with air was found to be 0.9563 day^{-1}. M_{max} rate of microalgae culture fed with just atmospheric conditions are the lowest one, 0.7337 day^{-1}.

For *Chlorella vulgaris* ESP-6, the highest microorganism concentration (X_{max} = 0.26 g/L) and growth rate (μmax = 1.0668 d^{-1}) were reached in CO_2-fed environment.

Doubling time of microalgae was reached almost 15 hours at CO2-fed culture. This means that it has little longer exponential phase but quicker because its growth faster.

Chlorella vulgaris ESP-6 grew high under pure CO_2 concentration compared to ventilated with air. Man Kee Lam and Keat Teong Lee reported tha same results as our study [115]. In literature studies, it was observed that CO_2 was used for microalgae cultivation as a source of carbon.
All of the above results suggested that providing micralgae setting with high CO_2 co ncentration could boost their photosynthesis efficiency, leading them to replicate a t a higher rate and acquire much more algae yield with less growing period. Providing the microalgae cultivation system with a high CO_2 concentration may increase the possibility of CO_2 dissolving in water, but may not mean that all CO_2 is dissolved and completely used by microalgae. By comparison, microalgae growing by ambient air (0.03% CO_2) reached higher CO_2 reduction efficiency while their biomass output was lower relative to the CO_2 supplement. From this, the real ability of high efficiency microalgal growth also depends on the nature and selection of photobioreactors

However, algal development in feeds with air continued with an increasing curve, it was slight similar to the culture fed with pure CO_2.
However, pH values that increase rapidly compared to other cultures will show growth inhibitory properties in industrial applications.

7.2 Determination of Lipid Content and Lipid Productivity of *Chlorella Vulgaris* ESP-6

Studies showed that the overall lipid quantity of *Chlorella vulgaris* approximately stayed at 18% and did not improve dramatically when the CO_2 concentration rise. The consequence was that a mere 1–6% rise in oil quantity in algae was detected, while 10–15% of CO_2 was supplied, in line with several recently published reports. The resulting carbon may be retained in protein form; sugars and pigments have been reported as a comparatively high composition opposed to lipid material

[123,131,137]. Nevertheless, utilizing high CO_2 concentrations to raise the lipid content of microalgae is not practical; nevertheless, this might be a technique to raise their growing conditions and the output of dry weight productivity. So lipid content was determined by using *Chlorella vulgaris* ESP-6 grown atmospheric conditions-fed. In this study, we provided a horizontal light source to minimize these changes in biomass. It was observed that the lipid production was 0.24 g/g biomass CO_2 feeding.

Table 7.2 Lipid content and lipid productivity of *Chlorella vulgaris* ESP-6

Feeding Source	X_{max} (g/L)	P_x (g/L day)	μ_{max} (d^{-1})	Doubling Time (Td) (h)	Lipid Content (%)	Px (g/L day)
Atmospheric air	0,15	0,01	0,73	22,68	24	0.0033

Since the feding sources are not enough for the other cultures, the lipid content could not be determined precisely. The microalgal lipid content and lipid productivity obtained from culture which was fed by atmospheric conditions was determined as 24%, and 3 mg/Lday, respectively, using the volumetric biomass productivity value (13 mg/Lday) calculated under these conditions.

Also some studies showed that algae can resist to adapt to changing conditions and stresses in their environment. While lipids act as structural components and organelles of the cell's plasma membranes, they act as signal components in the intracellular communication pathways. Lipids, which are part of the membrane, provide a barrier to separate compartments within the cell. Increased lipid concentration may increase of unsaturated fatty acid production at high CO_2 concentrations. Increased CO_2 concentration may affect enzymatic desaturation. On the other hand, increasing CO_2 concentration in some species may affect lipid accumulation since it will cause lower pH. High pH also contains higher amounts of bicarbonate available for carboxylation of lipid synthesis. High concentration of CO_2 reduces lipid accumulation in some species. In a Sheng study, Nannochloropsis

oculata-type microalgae reported that lipid content decreased with high rates of CO2 (5%, 10% and 15%) [87,92].

7.3 Effect of Base Catalyst on *in-situ* Transesterification Process

In general, methanol works the functions of *in situ* transesterification of both reactants and extractants, but it is weak [27]. This will definitely contribute to a poor transformation performance of *in situ* transesterification from microalgae to FAME. One potential solution to this issue is the introduction of a strong solvent for oil extraction, like hexane and chlorofor.

Table 7.3 Effect of base catalyst on in-situ transesterification FAME yield

Transesterification conditions					FAME Yield(%)	FAME content(%)
Exp	**Catalyst (NaOH, %)**	**MeOH:biomass (v/w)**	**Temperature (˚C)**	**Time (min)**		
1	6.25	6:1	60	150	8.3	3.16
2	12.5	6:1	60	150	16.6	4.43
3	12.5	10:1	60	150	29.1	4.45
4	25	10:1	60	150	33.3	7.16

The impact of the base catalyst quantity on the biodiesel yield is given in Table 7.3. (Exp.: 1–2). Base-catalyst quantity of 12.5% wt. (based on oil) was adequate to achieve a higher FAME yield with comparison of 6.25% wt. catalyst. The smaller catalyst dosage, 6.25% wt., did not successfully progress the reaction because the catalyst influence could be compromised by the existence of a number of organic compounds arising from micronutrients. When the same amount of alcohol was used compared to Experiment 3, the amount of catalyst increased from 12.5% to 25%, which did not significantly change the amount of FAME obtained, but increased the content of FAME to 7.16%. This clearly shows that as the amount of catalyst increases, the esterification rate increases. In addition, the reaction could

be constrained by the release rate of TAG and, thus, further NaOH catalyst may not improve the transesterification [135].

7.4 Effect of Acid Catalyst Concentration on *in-situ* Process

The *in situ* transesterification process was proceed with two acid-catalyst loadings (100% and 150% H_2SO_4 wt./wt. algae oil content) as illustrated in Table 7.4.
Increasing the acid catalyst content has a positive effect on the reaction process, resulting in higher FAME efficiency. Compared to the base catalyst, the acid catalyst provides much higher biodiesel yield. One of the main reasons for this is that microalgal oils have higher free fatty acid (FFA) and moisture content compared to vegetable oils, and thus the ending of base catalysts by saponification reactions.

Table 7.4 Effect of acif catalyst on in-situ transesterification FAME yield

Transesterification conditions					FAME Yield(%)	FAME Content (%)
Exp	**Catalyst (H_2SO_4,%)**	**MeOH:biomass (v/w)**	**Temperature (°C)**	**Time (min)**		
5	100	6:1	60	150	93.12	21.4
6	150	6:1	60	150	98	29.5

Biodiesel yield is significantly affected by the catalyst concentration used. In the in situ transesterification study using H_2SO_4 as an acid catalyst, 100% and 150% (based on algal oil) experiments were carried out at 60C temperature for 150 minutes at 6:1 methano to biomass ratio. The use of acid catalyst in the reaction significantly increased the lipid yield obtained by providing a faster and more efficient breakdown of the cell wall in situ esterification. As the amount of the acid catalyst was increased, the FAME yielding obtained increased by up to 98%, but an average of 29% of this oil content was esterified.

Obtained results showed similarities with H. I. El-Shimi et al. experiments that obtained from *Spirulina-Platensis* species *in situ* process using 100% and 200% acid catalyst [31]. A higher FAME yield was achieved at a rate of 98% compared to the 100% acid concentration test set (93.12% FAME) at a concentration of 150%

catalyst. These results are also assord with the methanolysis using 100% (wt./wt. of oil) H_2SO_4 as a catalyst resulted in the transformation of *Chlorella* lipid which providing the best yields of the methyl esters proved by E.A. Ehimen et al. [33].

7.5 Effect of Alcohol Ratio on *in-situ* Biodiesel Production

An excessive quantity of methanol is necessary for the development of *in-situ* process. Different amounts of methanol volumes (6 and 10 mL) were chosen to produce FAME with 1 g algae. NaOH was used in process (6.25%, 12.5% or 6 ml, 12.5%, 25% for 10 ml) at 60˚C with 150 minutes rection time. The amount of methanol from 6 ml to 10 ml measured a reacting alcohol-to-biomass weight ratio with acid and base catalyst.

The impact of concentration of alcohol is expressed in Table 7.3 (Exp.: 2 and 3). Tests showed that yields of up to 29.1% for the methanol-biomass ratio of 10 mL/g could be achieved, and more methanol showedd to produce 2 times more better results. When the ratio of alcohol in the reaction was increased from 6 ml to 10 ml compared to the biomass mass, it allowed the fat in the cell to dissolve more easily, thereby increasing the lipid content under the same catalyst amount conditions. But since the amount of catalyst did not change, the ester content of the lipid remained the same.

Dong et al., showed that higher alcohol ratio (4mL/g) gave the best yields (94.87±0.86%) with a comparison of 2 ml/g biomas [35]. In this study same results were obtained Since methanol is both solvent and reactant in reactions, using the required amount of alcohol is very important for biodiesel production [132]. The results obtained have actually proven that *in-situ* esterification can not only save time and process cost, but also achieve similar yields compared to the normal process. Ehimen et al. [33] produced 93% efficiency biodiesel from *Chlorella* type algae with *in situ* esterification method. The use of hexane has played a role in maintaining the amount of methanol as it provides extra solvent. The results showed that when using 4 mL of methanol in 1 g of algae powder, a suitable medium for *in*

situ transesterification was created, but in the study, they were warned that these rates are not constant for each species.

Effect of Solvent on dark color in-situ biodiesel production: It has been observed that the colors of biodiesel produced at different catalyst and alcohol ratios are different. As Kulkarni et al. [102] and Goff et al. [111] stated, oxidation of fatty acid and sulfone formation from sulfuric acid are factors that cause dark product outputs in biodiesel production. Dark colored FAME outputs can have a lower biodiesel fuel performance and have a lower cetane number. It can also lead to significant reductions in engine performance and life when used in diesel engines.It implied that nonpolarity solvents are better alternative than polarity ones *in situ* transesterification.

7.6 Gas Chromatography (GC) Analysis for FAME Products

The ester contents of FAMEs derived from base-catalysed in-situ transesterification by *Chlorella vulgaris* ESP-6 were low quantity (<10%) in contrast with FAMEs derived from acid-catalysed in-situ transesterification. This is because of base catalyst could cause saponification reaction instead of transesterification. Microalgae species contains high amounts of FFAs and moisture that can cause saponification.

The dominant acid methyl esters are palmitic acid methyl ester, heptadecanoic acid methyl ester, stearic acid methyl ester, and linoleic acid methyl ester, and the total content of these four FAMEs was about 30%. Reaction parameters can cause the efficiency of transesterification process and FAMEs quality.

The ester contents of each FAME can be seen in below figures. In addition of them, fatty acid composition was determined according to Experiment 6 which was resulted in the best FAME yield and content.

Table 7.5 Fatty acid composition of *Chlorella vulgaris* ESP-6 species

Fatty Acid	Content (%)
14:0	5
16:0	24
17:0	1.5
18:0	5.6
18:1	1.2
18:2	1.4
18:3	30
20:0	11
Unsaturated	32.6
Saturated	47.1

Fatty acids below 1% are not written on the table. It can be understood from here Chlorella vulgaris ESP-6 microalgae species contains palmitic acid, heptadecanoic acid, stearic acid, oleic acid, linoleic acid, linolenic acid and arachidic acid. This microalgae species contains both saturated and unsaturated lipids which can later determine biodiesel fuel species like cetane number.

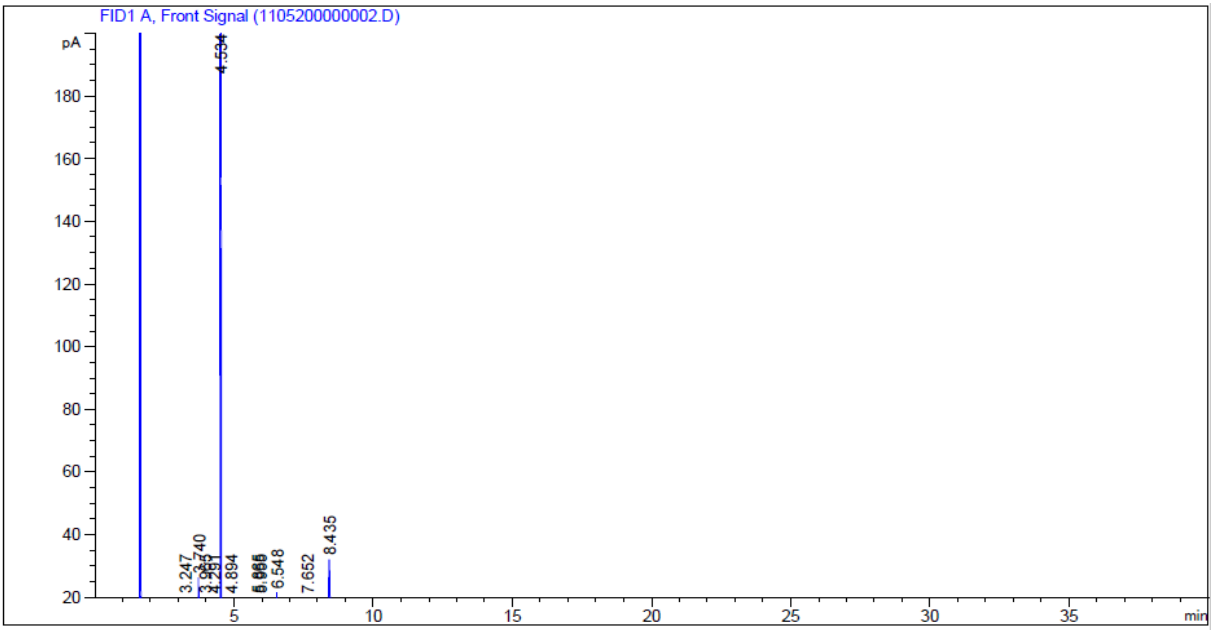


Figure 7.12 Chromatogram of FAME's ester content from base-catalyst in-situ transesterification of *Chlorella vulgaris* ESP-6 (6:1 methanol: biomass ratio (w / w), 6.25% catalyst ratio (based on oil content))

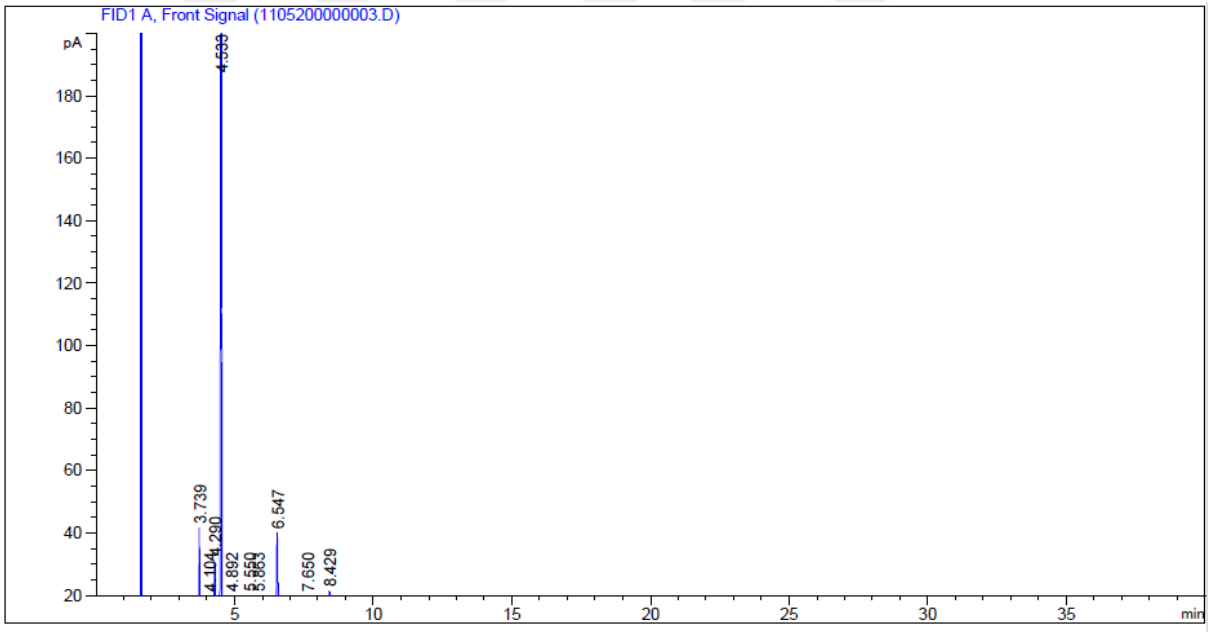


Figure 7.13 Chromatogram of FAME's ester content from base-catalyst in-situ transesterification of *Chlorella vulgaris* ESP-6 (6:1 methanol: biomass ratio (w / w), 12.5% catalyst ratio (based on oil content)

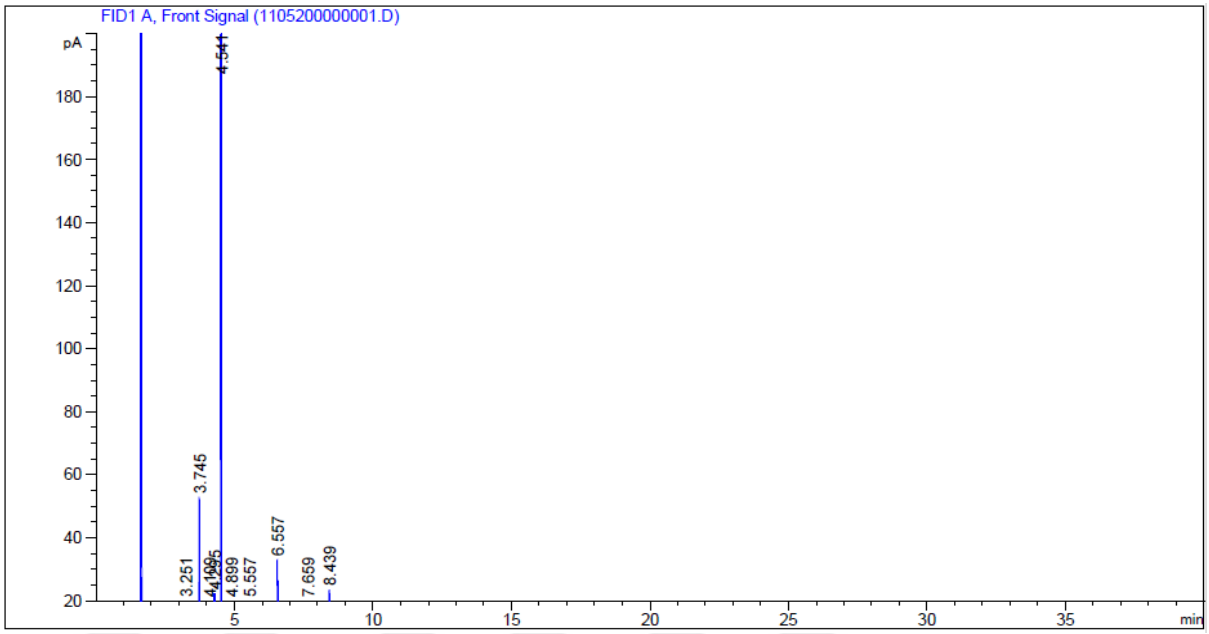


Figure 7.14 Chromatogram of FAME's ester content from base-catalyst in-situ transesterification of *Chlorella vulgaris* ESP-6 (10:1 methanol: biomass ratio (w / w), 12.5% catalyst ratio (based on oil content))

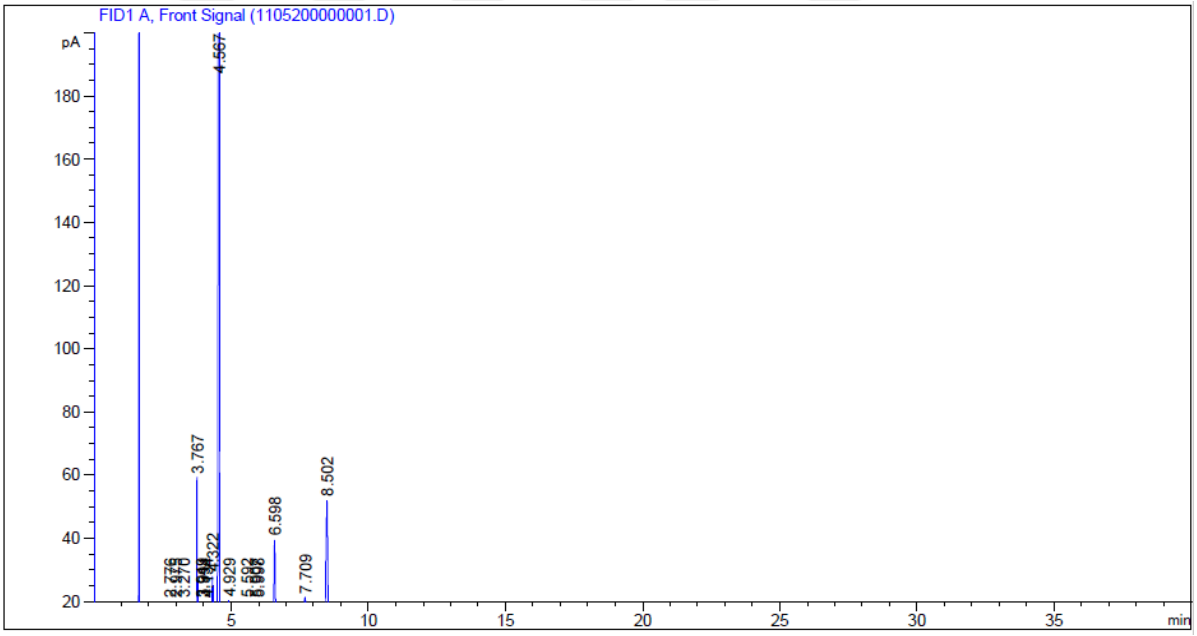


Figure 7.15 Chromatogram of FAME's ester content from base-catalyst in-situ transesterification of *Chlorella vulgaris* ESP-6 (10:1 methanol: biomass ratio (w / w), 25% catalyst ratio (based on oil content))

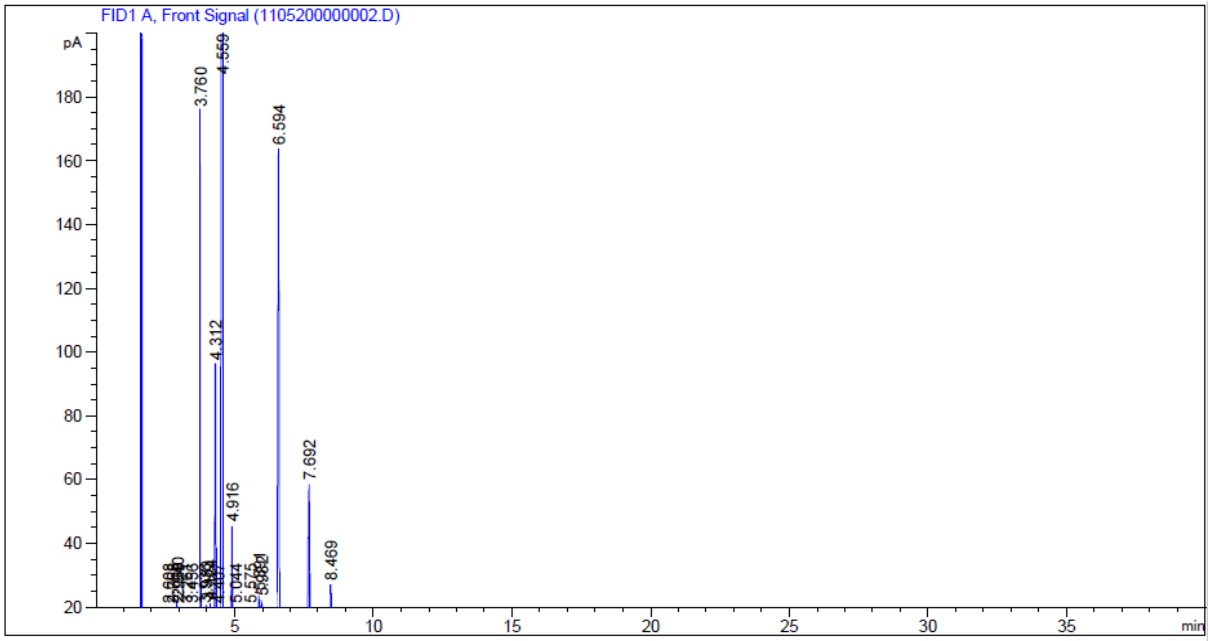


Figure 7.16 Chromatogram of FAME's ester content from acid-catalyst in-situ transesterification of *Chlorella vulgaris* ESP-6 (6:1 methanol:biomass ratio (w / w), 100% catalyst ratio (based on oil content))

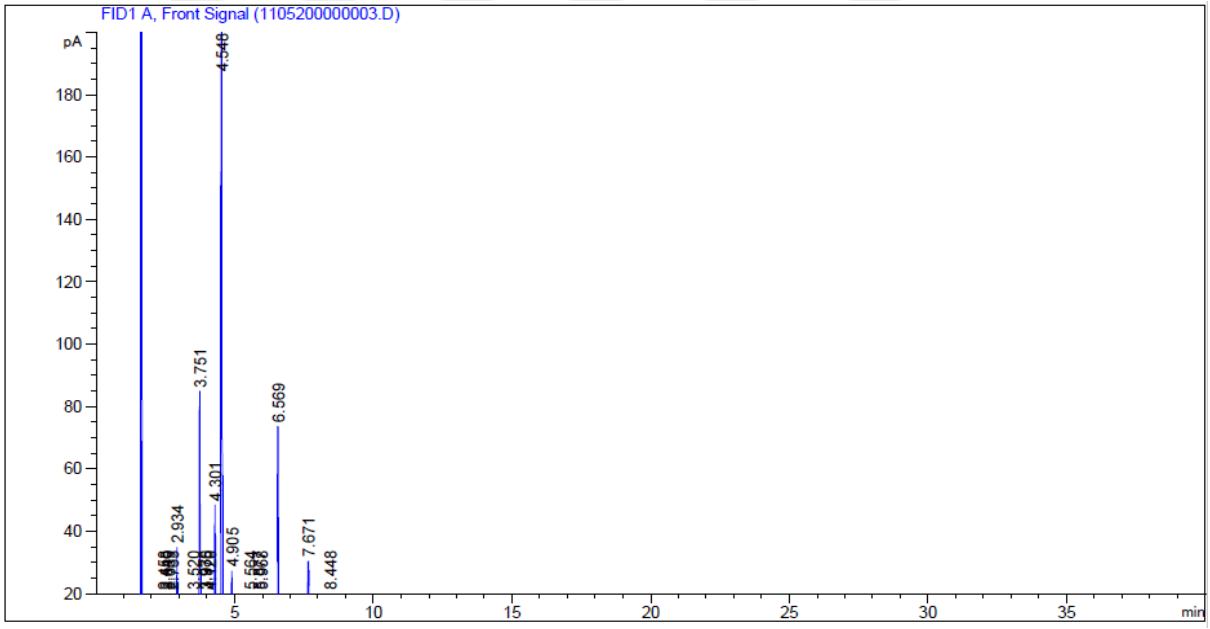


Figure 7.17 Chromatogram of FAME's ester content from acid-catalyst in-situ transesterification of *Chlorella vulgaris* ESP-6 (6:1 methanol:biomass ratio (w / w), 150% catalyst ratio (based on oil content))

7.7 Thin Layer Chromatography (TLC) Analysis

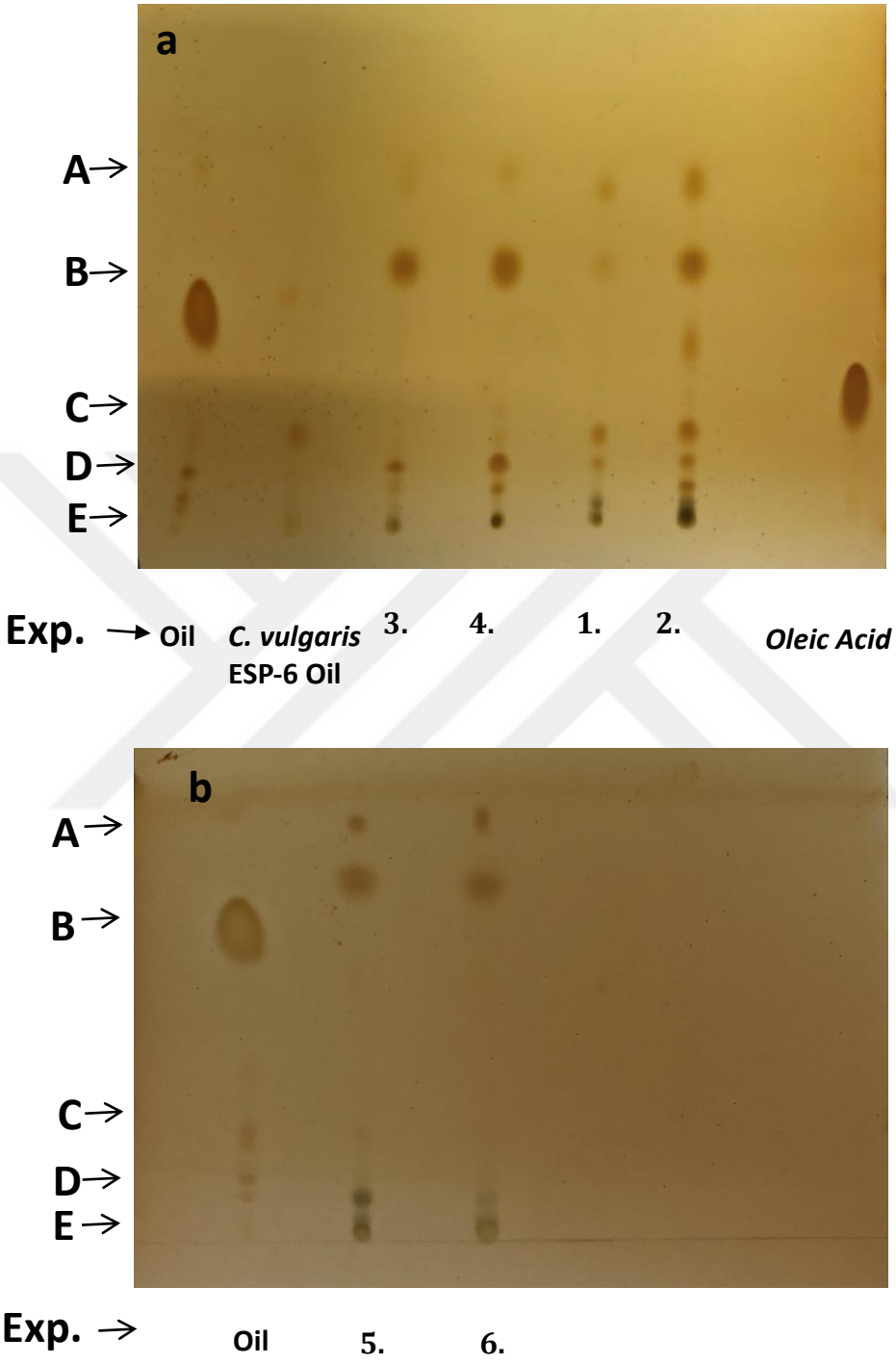


Figure 7.18 Thin layer chromatography view of the experiments performed in in-situ transesterification method a. base catalysed transesterification at autogenic temperature and b. acid catalysed transesterification at autogenic temperature (A → Fatty acid methyl ester, B → Triglyceride ,C → Free fatty acid, D → Diglyceride E → Monoglyceride)

Thin layer chromatography (TLC) is still one of the easiest and most widely used methods for analytical separation of mixtures. In this analysis, the processing of algal lipids to methyl esters of fatty acid (FAMEs) was used to monitor.

The TLC results showed FAME, TAG, and free fatty acids were present. The main TAG was a mixture of mono-, di-, and triglycerides and the main Popularity was commercial biodiesel (as a standard for comparison). The usage of n-hexane was eluted for each usual.

Esterification potential of biodiesel produced from *Chlorella vulgaris* ESP-6 was examined by TLC. The FAME outputs were diluted with the determined hexane ratios, and after dropping on a silica TLC plate, n-hexane / diethyl ether / acetic acid (80:20:1, v/v) was decomposed with the solvent system. Changes in product compositions were determined on the plate obtained after TLC analysis. The resulting methyl esters contain excess TAG and very little FFA. It has been observed in glycerides such as monoacylglycerol (MAG) and DAG in FAME products in TLC. The results show that esterification takes place during the reaction process, a certain percentage of TAG and most of the FFA can be converted to FAMEs. TLC results were also confirmed numerically with the results of the GC analysis. In Figure 7.18 it clearly showed that when the catalyst ratio was increased, the methyl ester yield increased.

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PUBLICATIONS FROM THESIS

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Conference Papers

1. Şenal S., Yücel S., (2019). Atmosferde Sera Etkisi Yaratan CO_2'in Fiksasyonu ile Mikroalglerden Biyodizel Eldesi, International Environmental Engineering Source Recovering Conference, 12-13 December 2019, Zeytinburnu, İstanbul, Türkiye, Sf. 479.

Awards

1. İlk İşim Girişim, Yıldız Technopark-Çalık Holding 2019 (Runner-up Prize)

2.Tusiad Bu Gençlikte İş Var, Tusiad-Güral Porselen 2020 (Finalist)