BIO-FUEL PRODUCTION FROM MICROALGAE

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BIO-FUEL PRODUCTION FROM MICROALGAE

submitted by **MELİH ONAY** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Biochemistry Department**, **Middle East Technical University** by,

Prof. Dr. Gülbin Dural Ünver
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Orhan Adalı
Head of Department, **Biochemistry** _____

Prof. Dr. A. Meral Yücel
Supervisor, **Biology Department, METU** _____

Prof. Dr. Hüseyin Avni Öktem
Co-Supervisor, **Biology Department, METU** _____

Examining Committee Members:

Prof. Dr. A. İnci Eroğlu
Chemical Engineering Dept., METU _____

Prof. Dr. A. Meral Yücel
Biology Department, METU _____

Prof. Dr. N. Tülin Güray
Biology Department, METU _____

Prof. Dr. I. Füsun Eyidoğan
Education Faculty, Baskent University _____

Assist. Prof. Dr. M. Cengiz Baloğlu
Genetics and Bioengineering Dept., Kastamonu University _____

Date:04.02.2015

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last Name: Melih Onay

Signature:

ABSTRACT

BIO-FUEL PRODUCTION FROM MICROALGAE

Onay, Melih

Ph.D., Department of Biochemistry

Supervisor: Prof. Dr. A. Meral Yücel

Co-supervisor: Prof. Dr. Hüseyin Avni Öktem

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Bio-fuel is a renewable fuel and it includes different biofuel energy sources such as methane, bioethanol and biodiesel. Generally, biodiesel is produced from agricultural waste, vegetable oils such as soybean and palm oil. Third generation biofuels called as microalgae have been appeared nowadays.

In the current study, biodiesel production from thermo-resistant green microalgae was focused on. For this aim, microalgae were sampled from a few hotspring points in Haymana and isolated. Identification and characterization of isolated microalgae were performed morphologically via scanning electron microscopy and molecularly via ITS2 region. They were named as *Scenedesmus sp.ME02, Hindakia tetrachotoma ME03 and Micractinium sp. ME05.*

Also, three different solvent extraction methods and five cell disruption techniques (Homogenization Assisted, Microwave Assisted, Ultrasonnication, Glass Bead and Lyophilization Assisted) were applied for effective lipid recovery from three green microalgae. The highest lipid content was found as 27.3 ± 2.6 % of dry weight for *Micractinium sp. ME05* at lyophilization and ultrasonnication assisted method with

Soxhlet method. Biodiesel productivity of *Micractinium sp. ME05* was 89 ± 3.1 %. Also, protein concentrations, carbohydrate percentages and chlorophyll contents of *Micractinium sp. ME05* were 56.48 ±2.99 %, 15.57 ± 0.79 %, and 114 ± 12.6 μg/mL, respectively.

The present work also comprised genetic manupilation of *Chlamydomonas reinhardtii* 137- by electroporation and the lipid contents of mutants were determined by using high throughput fluorescence technique.

As a conclusion, present study is the first report demonstrating biodiesel production from thermo-resistant green microalgae isolated from Central Anatolia, Haymana, Ankara.

Key words: Biodiesel, thermo-resistant microalgae, renewable energy, *Micractinium sp.*, *Scenedesmus sp.*, *Hindakia tetrachotoma*, transformation.

ÖZ

MİKROALGLERDEN BİYO-YAKIT ÜRETİMİ

Onay, Melih

Doktora, Biyokimya Bölümü

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Ortak Tez Yöneticisi: Prof. Dr. Hüseyin Avni Öktem

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Biyo-yakıt yenilenebilir bir enerji kaynağıdır ve metan, biyoetanol ve biyodizel gibi çeşitli enerji kaynaklarını içerir. Genellikle, biyodizel palmiye ve soya gibi bitkisel yağlar ile tarımsal atıkların yağlarından üretilir. Günümüzde mikroalgler üçüncü nesil biyo-yakıt olarak biyodiesel üretimi için kullanılmaya başlandı.

Bu çalışmada, sıcağa dayanıklı yeşil mikroalglerden biyodizel üretimine odaklanılmıştır. Bu amaç için, Mikroalgler Haymana termal sularından toplanarak izole edilmiştir. Izole edilen mikroalglerin belirlenmesi ve karakterizasyonu taramalı elekron mikroskopu ile morfolojik olarak ve ITS2 bölgesi belirlenmesiyle moleküler olarak yapılmıştır. Belirlenen bu mikroalgler *Scenedesmus sp.ME02, Hindakia tetrachotoma ME03 ve Micractinium sp. ME05* olarak adlandırılmışlardır.

Ayrıca, üç yeşil mikroalgden, etkili lipid eldesi için mikroalglere üç farklı özütleme metodu ve beş hücre parçalama tekniği (homojenizasyon, mikrodalga, ses ötesi, cam boncuk ve kurutarak dondurma) uygulanmıştır. En yüksek lipid içeriği, Soxhlet metod ile birlikte kurutarak dondurma ve ultrasonik yardımcı metodunun kombinasyonu sonucunda *Micractinium sp. ME05'*in lipid içeriği yüzde 27.3 ± 2.6 ve biyodizel verimi yüzde 89 ± 3.1 olarak bulunmuştur. *Micractinium sp. ME05*'in

protein, karbohidrat ve klorofil içeriği sırası ile % 56.48 ±2.99, % 15.57 ± 0.79, and 114 ± 12.6 µg/mL olarak saptanmıştır.

Bu çalışmanın içeriğinde, transformasyon tarafından elde edilen *Micractinium sp. ME05* mutantlarının yağ miktarlarını belirlemek için yüksek verimli ışınırlık metodu kullanıldı.

Bu çalışma aynı zamanda *Chlamydomonas reinhardtii 137*$^{-}$ nin elektroporasyon yöntemi ile genetik manupulasyonunu kapsamaktadır.

Sonuç olarak, bu çalışma Anadoludan izole edilen sıcağa dayanıklı yeşil mikroalglerden biyodizel üretimini gösteren ve yüksek verimli ışınırlık metodu aracılığıyla *Micractinium sp. ME05* mutantlarının yağ miktarlarını belirlemek için en uygun şartların belirlenmesini detaylı bir şekilde sağlayan ilk çalışmadır.

Anahtar kelimeler: Biyodizel, sıcaklığa dayanıklı mikroalg, yenilenebilir enerji, *Micractinium sp., Scenedesmus sp., Hindakia tetrachotoma*, transformasyon.

Dedicated to my sunshine,

Aytun & my son Rüzgar Çınar

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AFDW	: Ash free dry weight
ASTM	: American Society for Testing and Materials
BSA	: Bovine serum albumin
B-A	: Bead assisted
BD	: Bligh and Dyer
BG-11	: Allen and Stainer medium
BLAST	: Basic local alignment search tool
BODIPY	:4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3α,4adiaza-s-indacene
CBC	: Compensatory base change
CTAB	: Cetyltrimethyl-ammonium bromide
D	: Sheridan medium
DF	: Diluton factor
DMSO	: Dimethyl sulfoxide
DNA	: Deoxiribonucleic acid
EDTA	: Ethylenediamine tetra acetic acid
EIA	: Energy Information Administration
EN14214	: European Standard 14214
FAME	: Fatty acid methyl ester
FAO	: Food and Agriculture Organization
GPS	: Global Positioning System
GC	: Gas chromatography
H-A	: Homogenization assisted
ITS	: Internal Transcribed Sequences

L-A	: Lyophilization assisted
LB	: Luria Bertani
ME01-06	: METUNERGY1401-06
ME02	: *Scenedesmus sp.*
ME03	*: H. Tetrachotoma*
ME05	: *Micractinium sp.*
M-A	: Microwave assisted
MUFA	: Monounsaturated fatty acids
Nile red	: 9-diethylamino-5H-benzo [α] phenoxazine-5-one
OD	: Optical density
PCR	: Polymerase chain reaction
PUFA	: Polyunsaturated fatty acids
RNA	: Ribonucleic acid
SDS	: Sodium dodecyl sulfate
SEM	: Scanning electron microscopy
TAP	: Tris- Acetic acid-Phosphate
TD-NMR	: Time-domain nuclear magnetic resonance
TEM	: Transmission electron microscopy
TG	: Triglyceride
Tris	: Tris (hydroxymethyl) aminomethane
U-A	: Ultrasonnication assisted

CHAPTER 1

INTRODUCTION

1.1. Renewable Energy

Energy can be expressed as the ability to do work. Energy can be presented in two different patterns. These are kinetic and potential energies. Energy can be obtained in various forms such as thermal energy, chemical energy, electrical energy and mechanical energy. They are converted to one form to other forms. Large amount of energy are supplied by fossil fuels. Coil, petroleum fuels and natural gases are non-renewable energy (Azad et al., 2015). Production of petroleum-derived fuels and usage of them makes important effects on countries' economics and environments. Fossil fuels such as oil, coil and neutral gas cover 88 % of total energy consumption in the world (Tabatabaei et al., 2011).

Production of petroleum and other liquids was 34.1 in OPEC, 23.2 in Middle East and 9.9 million barrels per day in Russia in 2010. In addition to this, total production was 84.5 million barrels per day in the world in 2010. The United States Energy Information Administration (U.S. EIA) thinks that total production will have been reaching 119.4 million barrels per day in the world by the time 2040 (EIA, 2013). Moreover, in The United States of America, petroleum and other liquids consumption was 37.2 quadrillion Btu in 2010 and EIA estimates that consumption of them will be 35.4 quadrillion Btu in 2040. On the other hand, petroleum and other liquids consumption was 176 quadrillion Btu in 2010 and will be 243.1 quadrillion Btu in 2040. Moreover, crude oil price was 111.65 dollars per barrel in the world in 2012 (EAI, 2013). As a result of this, increasing of fossil fuel prices, depletion of fossil fuel and climate changes because of greenhouse effect increased renewable energy demand (Halim, 2012).

Types of renewable energy are hydroelectric, wood, biofuel, wind, waste, solar and geothermal energy. Energy information administration (EIA) estimates that 11 % of total energy consumption is supplied by renewable energy in the world and it will have reached to 15 % by the time 2015. Biofuel consumption is 2000 trillion btu per year (EIA, 2013). Renewable energy consumptions were given according to kinds of renewable energy in Figure 1.1.

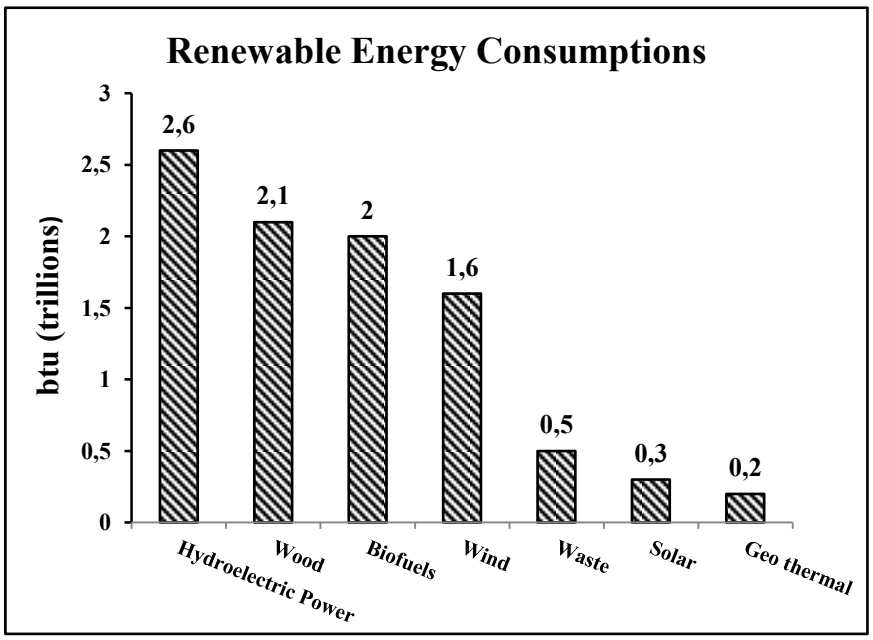

Figure 1.1. Amounts of renewable energy consumptions as trillions British thermal unit (btu) in 2013. (Adapted from EIA, 2013).

Earth's average temperature has risen by 0.74 ± 0.18 ^{0}C over the past century (1906–2005), and is projected to rise another 2–6 ^{0}C over the next hundred years (Trenberth et al., 2007). Carbon dioxide emission was twenty-nine billion tonnes in 2006 and this caused more acidic oceanic water and ecosystems surrounding environment were adversely affected in the ocean (Tabatabaei et al., 2011). Depletion of fossil fuels and energy demands of countries led to search new energy sources (Christiansen et al., 2011). Causes of these are anthropogenic activities such as usage of petroleum derived fuels and solution is actually simple, reducing of fossil fuels and producing biofuels (Rashid et al., 2014). Bio-fuel is a renewable fuel and there are many

different renewable energy sources such as methane, bioethanol and biodiesel. In addition, biofuel can be separated in three groups according to storage conditions such as solid, liquid and gaseous. Solids are materials made of straw and trees and plants. Liquids are alcohol fermentation products and transesterified oils such as bioethanol and biodiesel, respectively. The last is gaseous. It consists of fermentation of wastes of animals and plants such as methane. They are called as biogases (Frac et al., 2010). Biodiesel is liquid and one of the most important bio-fuels.

1.2. Biodiesel

Bio-diesel firstly was used by Rudolf Diesel. Rudolf Diesel produced biodiesel from peanut oil (Knothe et al., 2005). Amount of biodiesel production is 179 trillion btu per year (EIA, 2013). Biodiesel composed of fatty acid methyl ester (FAME). Generally, biodiesel is produced from agricultural waste, vegetable oils such as soybean and oil palm. First generation biodiesels have been produced by agricultural edible crop oils such as soy bean, rapeseed oil, sugarcane and sugar beet. However, these crops have different functions except for usage as biofuel. They can use food and they need vast areas for biodiesel production. This situation makes these crops much more expensive for biodiesel production (Demirbaş, 2011& Rawat et al., 2014).

The United States and Brazil are the most important biofuel producers from sugar crops such as sugar cane and sugar beet. But, sugar crops can produce only biofuel source. It is bioethanol. Bioethanol production was approximately 80 billion litres in the world in 2009. Bioethanol production increased nearly four-times when this result is compared with that of 2000's. On the other hand, FAO reports showed that bioethanol production except for sugar cane was not effective economically in Brazil (Tabatabaei et al., 2011).

1.3. Properties of Biodiesel

Biodiesel is nontoxic and includes lower carbon monoxide, carbon dioxide and hydrocarbon emissions when compared with petroleum derived diesel (Koberg et al., 2011). But, they have disadvantages because of high cost of biomass and their availability. This problem can be solved by using of available biomass. For example, palm oil in Malaysia, coconut oil in the Philippines, soybean in the USA and canola in Europa are found available. These oils can be used for biodiesel production (Yaakob, 2014). Another drawback of diesel obtained from plants has low stability point. They easily can oxidize with air during especially long term storage. This oxidation comes from fatty acid content of oil. Oil obtained from plant includes much more polyunsaturated fatty acids (PUFA) and it results in oxidation (Yaakob et al., 2014).

Saturated fatty acid methyl esters have higher cloud points and cetane numbers. These parameters are contributed to stabilization. But, PUFA's have lower clouds and cetane numbers. PUFA's are affected more easily than monounsaturated fatty acids (MUFA)'s to oxidation because of bis-allylic methylene structures (Das et al., 2009). Fatty acid contents of some oils for biodiesel production were given in Table 1.1.

There are many factors affecting biodiesel oxidation stability. Biodiesel has to show some properties for effective engine performances (Pruvost et al., 2009). These are iodine number, induction period, peroxide value, viscosity, structure indices, oxidisability, oil stability index, acid value and fatty acid methyl ester content (Yaakob et al., 2014).

Cetane number is measurement of ignition quality of biodiesel. Similarly, iodine number is an indicator of total unsaturation degree. It gives numbers of total double bonds in biodiesel and indicates any problems in engine. High iodine number value decreases the quality of lubrication in engine. Iodine number is expressed as the gram of iodine consumed per 100 g of the compound. Iodine number can be between 67 and 133 for biodiesel production. On the other hand, some standards such as

ASTM D975 don't determine any values for iodine number (Yaakob et al., 2014). Iodine numbers of various oils used for biodiesel production were given in Appendix A.

Induction period can be expressed as initial slow stage of a chemical reaction (Hydroperoxide formation). In this stage, hydroperoxide formation begins. In other words, oxidation starts. Induction periods for ASTM D6751 and EN14214 are 3 and 6 hours, respectively. Peroxide value can be determined as miliequivalents of peroxide units per kg of the biodiesel. High peroxide values result in engine operation problems. Viscosity can be defined as measurement of resistance to deformation (Yaakob et al., 2014).

Table 1.1. Fatty acid contents of some oils for biodiesel production (Adapted from Yaakob, 2014).

Fatty Acid	# of Carbon	Coconut	Palm	Sun Flower	Jatropha	Soy bean	Olive	Grape Seed	Chicken Fat	Beef Fat
Caprylic	8:0	7.0								
Capric	10:0	5.4								
Lauric	12:0	48.9	0.2							
Myristic	14:0	20.2	1.1		0.1				1.0	3.0
Palmitic	16:0	8.4	42.6	6.3	14.2	11.6	13.0	7.0	22.0	27.0
Stearic	18:0	2.5	3.8	3.0	7.0	4.0	3.7	4.0	6.0	7.0
Oleic	18:1	6.2	41.9	43.7	44.7	18.8	39.1	15.8	37.0	48.0
Rinsolenic	18:1									
Linoleic	18:2	1.4	10.4	47.0	32.8	56.1	35.4	69.6	20.0	2.0
Linolenic	18:3				0.2	8.5			1	
Arachidic	20:0				0.2		1.9			
Heneicosanoic	21:0									
Beheric	22:0				0.2		4.4			
Lignoceric	24:0						1.4			
Others					0.6	1.0	1.1	3.6	13.0	13.0

1.4. Biodiesel Production from Waste Cooking and Animal Oils

Second generation biodiesel composed of feedstock, especially lignocellulosic, wood, wood waste, waste cooking oils and animal oils. They can be used in diesel engines more effective than those of gasoline. Second generation biodiesels have low emission fuel and can emit lower carbonmonoxide, carbondioxide and hydrocarbon when compared with petroleum-derived fuels (Azad et al., 2015). Also, According to Azad, they have high cetane number and flash point (Azad et al., 2015). They are waste and don't use for food. But, these sources include many saturated fatty acid contents and this causes production difficulty and poor flow properties of fuel (Rawat, 2014). Also, these plants can mainly produce small quantity of oil when compared with microalgae according to oil/biomass ratio (Chisti, 2008).

1.5. Biodiesel Production from Microalgae

Microalgae, third generation biofuel, have appeared nowadays. Algae are similar to plant like organisms and separated to two groups (macroalgae and microalgae). Macroalgae consist of multiple cellular structures. They have root, stem and leave resembling structures (Chen et al., 2009). On the other hand, microalgae have unicellular structures and are subdivided into two groups as prokaryotic and eukaryotic. Prokaryotic microalgae don't have complex organelles such as chloroplast, mitochondria and nuclei but they present high amount of *chlorophyll a* in cells. Microalgae can be classified taxonomically as green, blue-green, yellow green, red, brown algae. Some microalgae are called autotrops and they can use carbon dioxide (inorganic carbon) as energy source. They can make photosynthesis and they convert sunlight to chemical energy such as organic energy. Other microalgae can use organic carbon such as glucose and they are called heterotrophic microalgae. The last group can utilise both organic also inorganic carbon sources and these microalgae are called mixotrops (Rashid et al., 2014). Microalgae can produce various types of bioenergy such as bio-methane, bio-hydrogen and bio-diesel (Moazami et al., 2011).

Microalgae can be habituated on different environments such as aqua, soil, fresh water, waste, brackish water and can be grown in extreme environmental conditions. There are approximately fifty thousand microalgae species in the world (Frac, 2010).

Microalgae have most important superior properties when they compared to plants for biodiesel production. They can grow fast (5-30 times more than oil crops) (Teo, 2014). In addition, they are non-food sources of biofuel, they have simple cell structures and can include high amount of lipid (up to 80% in dry weight). Microalgae can handle stress factors such as salty water and pH, and can emit carbon dioxide from coal-fired power plants.They don't include sulphur and easily biodegradable (Um, 2009).

Microalgae can keep potential energy in their cells. They can produce approximately 6x 10^{10} tons of biomass with photosynthesis per year. This value equals to 40 % of total photosynthesis biomass in the world (Cheng et al., 2013). This energy can be converted to biodiesel. A few plants and microalgae were compared according to their oil yields (L/ha) and land area needed (Mha) in Table 1.2. Many microalgae include high amount of macromolecules such as triacylglycerol and starch. These molecules can be used for bioethanol, biodiesel and biohydrogen production (Radakovits et al., 2010).

Table 1.2. Comparison of some plants and microalgae for biodiesel production (Adapted from Frac, 2010).

Crop	Oil (L/ha)	Land Area needed (M ha)
Corn	169	1545
Soybean	443	589
Coco Nut	2679	95
Palm Oil	5938	41
Microalgae- 70% oil/biomass	136	2
Microalgae- 30% oil/biomass	58	4.5

Biodiesel production from microalgae has been studying at CRTT-GEPEA Lab (Centre de Recherche et de Transfert de Technologies- Genie des Procedes Environnement et agroalimentaire) in France. In this international biodiesel production center from microalgae, there are many reactors and their types such as flat, tubuler closed photobioreactors, open photobioreactors and open ponds. Closed photobioreactors and open pond at the GEPEA lab were given in Figure 1.2.

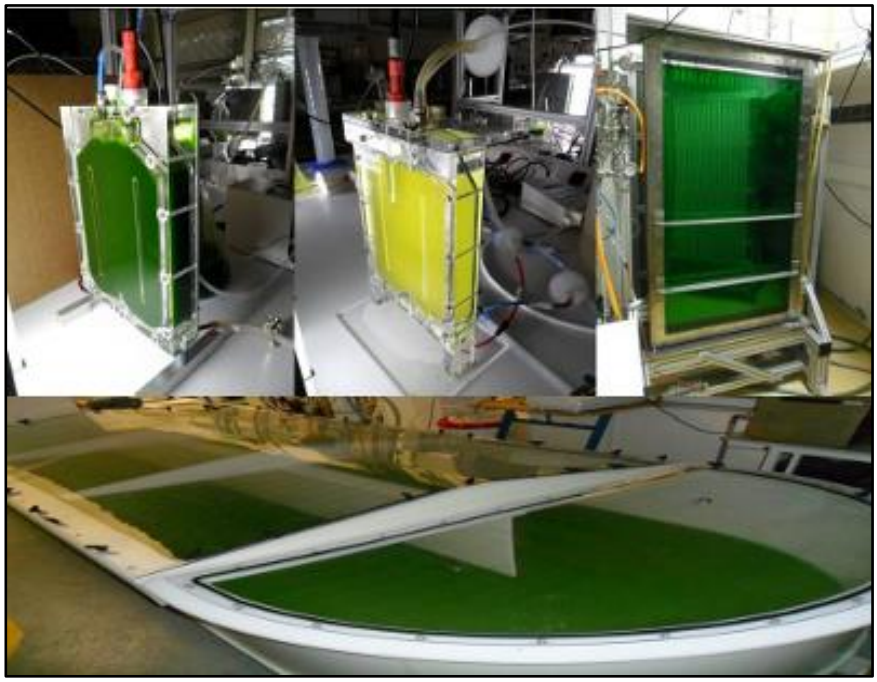

Figure 1.2. Closed photobioreactors and open pond at the GEPEA lab, France.

At this center, chemical engineer, chemist, biochemist, biologist and mechanical engineer have been studying for increasing of biodiesel quality and production. At CRTT-GEPEA Lab, Pruvost reported that *Neochloris oleoabundans,* fresh water microalgae, had high amount of lipid. They explored nitrogen limitation and deprivation experiments in continuous and batch culture and they tried to induce triacylglycerol (TAG) precursor of biodiesel (Pruvost et al., 2009). In other study, Pruvost et al. investigated continuous light effect and thickness of photobiorector on marine microalga species *Nannochloropsis oculata* for increasing of lipid and TAG content. In addition to this, they examined operating parameters such as incident photons flux density (PFD), initial and sudden starvation of microalgae (Vooren et

al., 2012). Also, Pruvost et al. (2014) investigated effect of light transfer in triglyceride fatty-acid cell content and productivity from microalgae during nitrogen starvation (Kandilian et al., 2014). Moreover, Pruvost et al. improved method for fast screening strains of *Nannochloropsis* and investigated this method at nine strains of *Nannochloropsis* (Talep et al., 2015).

Microalgae produce not only lipids but also proteins, enzymes, sugar, vitamins (niacin and riboflavin) and secondary metabolites such as β-carotene. So, they can be used for different industrial areas (Koller et al., 2014).

Biofuel is an alternative fuel to diesel fuel. It is obtained from oils via transesterification reaction. This chemical reaction is produced with acid or base catalysed transesterification of triglycerides. Also, there is enzyme catalyzed reaction. This reaction was enhanced by lipases which are biocatalysts and they work in mild environment conditions such as living cells. They can make esterification reaction directly without any pre-treatments and product, triglycerides, can be produced (Taher et al., 2014a). But, this procedure includes separation step and separation needs extra energy input and time consuming (McCurdy et al., 2014).

There are many steps for biodiesel production from microalgae. First of all, you have to make sampling for microalgae from environment. Secondly, it can be isolated with several methods such as single cell isolation and dilution method. Then, morphological and molecular analysis of microalgae can be examined. Next, microalgae cultivation and harvesting procedure are applied. Harvested samples are subjected to lipid extraction and transesterification. Schematic diagram of biodiesel production steps was summarized in Figure 1.3.

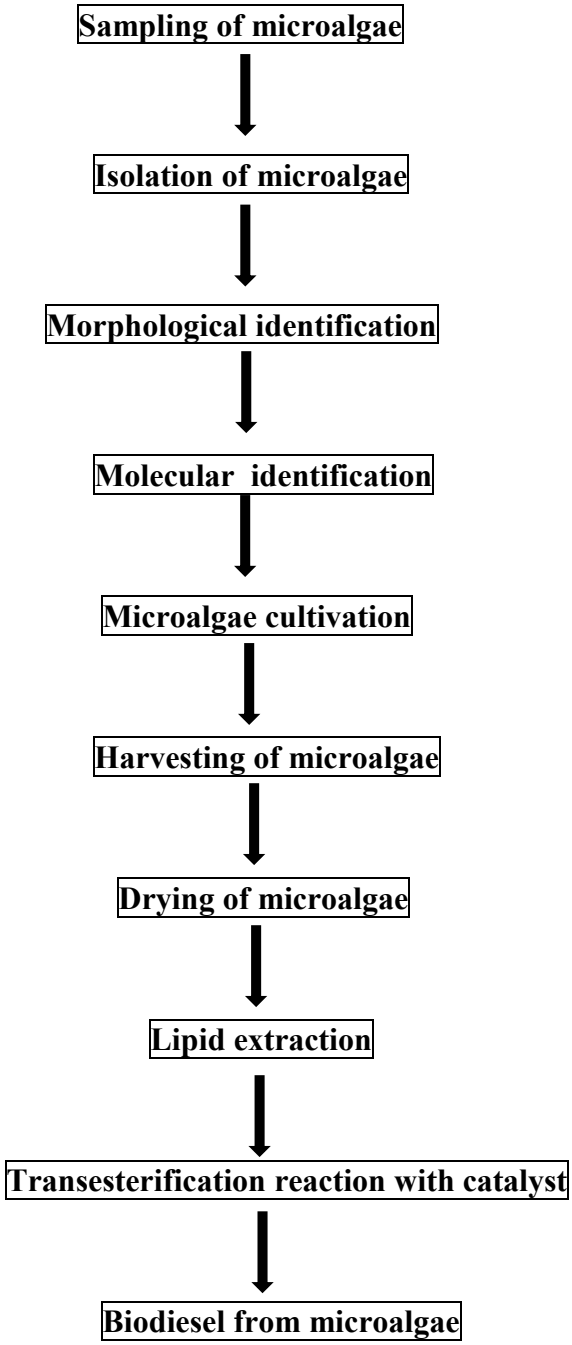

Figure 1.3. Schematic diagram of biodiesel production from microalgae

1.6. Steps from Strains to Biodiesel Production

1.6.1. Isolation of Microalgae

Isolation of microalgae needs an elaborate and careful study. Fresh water microalgae generally are isolated in each season except for winter because they are sensitive for temperature, pH and environmental conditions. (Andersen, 2005).

Nomenclatures of microalgae are important because some microalgae only grow in extreme conditions. Other crucial effect is contaminants. For successful isolation, contaminants should be eliminated (Andersen, 2005).

There are various methods for isolation of microalgae. These are enrichment cultures, single cell isolation by micropipette, streaking cells across agar plate, agar pour plate, atomized cell spray method and dilution technique. One of these can be chosen for isolation of microalgae. Sometimes, one technique can be combined with other ones. Success of isolation depends on microalgae species and isolated environment (Andersen, 2005).

Enrichment cultures can be prepared with adding of some nutrients such as nitrate, sulphate, phosphate or nitrate to natural water of microalgae. Single cell isolation method is difficult but effective if it can be applied properly. For single isolation method, Pasteur pipette is used and tried to get single cell. Streaking cells across agar plate, agar pour plate methods are common and deserved microalgae easily can be isolated on agar with suitable medium.

Atomized cell spray method can be applied to liquid medium. Suspension is atomized with sterile air to spread microalgae, then, appropriate microalgae are transferred on agar plate. Dilution method is a common method for isolation of microalgae. The aim is to collect dominant microalgae with suitable medium in test tube (Andersen, 2005).

1.6.2. Morphological and Molecular Identification of Microalgae

Identification of microalgae is a big problem for scientists. Convential light microscopy is not sufficient for identification because of small size of microalgae (generally 2-20 μm). Therefore, more complex microscopy methods are needed such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Sometimes, these methods can not reliable because cell sizes can change during life cycles. In addition, during fixation, they can lose some morphologic properties such as flagella. In these cases, it should be made molecular characterization (Godhe et al., 2002). At the molecular characterization studies, entire DNA sequence doesn't be required. Small conserved genes are examined in microalgae. These are ribosomal RNA genes (rRNA), mitochondria genes, plastid genes (rbcL), ITS (Internal Transcribed Sequences) and microsatellite DNA sequences. These regions help to identify microalgae (Mutanda et al., 2011). Tandem repeats can be located within ribosomal RNA operons (rRNA) of eukaryotic nuclear genome (Kocot, 2009).

There are three coding regions (18S, 5.8S and 28S-rDNA) and three non-coding spacers External transcribed spacer (ETS), Internal transcribed spacer 1 (ITS1) and Internal transcribed spacer 2 (ITS2). These are repeated in high copy number in the algal genome and these DNA sequences can be accepted for identification of microalgae (Kocot, 2009). The internal transcribed spacer consists of ITS1, 5.8S gene and ITS2. ITS2 regions are more confidential than those of ITS1 (Coleman, 2003). Target regions and primers used for molecular identification of microalgae and structure of nuclear ribosomal citrons in eukaryotes were shown in Table 1.3 and Figure 1.4, respectively.

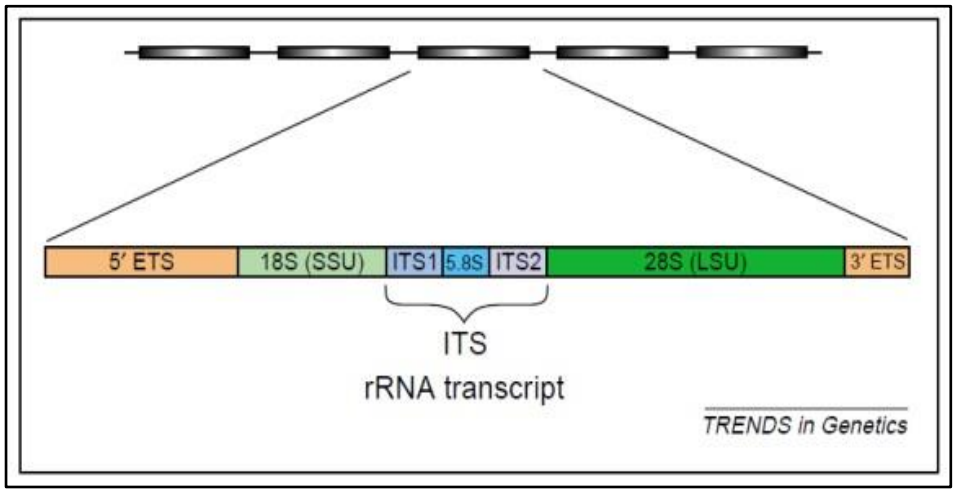

Figure 1.4. Structure of nuclear ribosomal citrons (grey boxes) in eukaryotes and their primary RNA transcript (Coleman, 2003).

Table 1.3. Target regions and primers used for molecular identification of microalgae (Adapted from Mutanda, 2011).

Target region	**Primers used**
18S rDNA	16S1N/16S2N
	ChloroF/ChloroR
	EK82F/Proto5R
	159F/1406R
Large subunit (LSU rDNA)	FD8/RB, D1/D2
Plastid rbcL	-------
Small subunit (SSU rDNA)	18comF1/Dino18SR1
	EK82F/Proto5R
Mitochondrial cytochrome c oxidase subunit	coxF/coxR
Internal transcript spacer (ITS)	ITS1/ITS2
Microsatellite locus	Kbr1/kbr10

1.6.3. Nomencluture of microalgae

1.6.3.1. Nomencluture of *Hindakia tetrachotoma*

Table 1.4. Systematic classification of *Hindakia tetrachotoma*

Kingdom: Plantae
Phylum: Chlorophyta
Class: Trebouxiophyceae
Order: Chlorellales
Family: Chlorellaceae
Genus: Hindakia
Species: *Hindakia tetrachotoma*

Hindakia was identified by Bock et al. (2010) within the *Chlorellaceae*. Morphologically, *Hindakia tetrachotoma* has oval cell shapes. *Hindakia tetrachotoma* was characterized from wastewater secondary treatment tanks by Osundeko and they applied oxidative stress tolerant test (Osundeko et al., 2013 and Osundeko et al., 2014).

1.6.3.2. Nomencluture of *Scenedesmus sp.*

Table 1.5. Systematic classification of *Scenedesmus sp.*

Kingdom: Plantae
Phylum: Chlorophyta
Class: Chlorophyceae
Order: Sphaeropleales
Family: Scenedesmaceae
Genus: Scenedesmus
Species: *Scenedesmus sp.*

Systematic classification of *Scenedesmus* was made by Komarek (1983) (Güçlü, 2009). *Scenedesmus* has *Scenedesmaceae* family. *Scenedesmus sp* has different cell wall. There are sporopollenine on its cell wall and this compound maintains rigidity to its cell wall (Mahdy et al., 2014). Also, cell wall of *Scenedesmus sp* includes galactose, mannose and glucose. Its cell wall properties prevent bacterial degredation (Mendez et al., 2014). It can easly grow in wastewater of plants and produce high lipid (Yuan et al., 2013). *Scenedesmus sp* includes high amount of protein. High level of protein results in high NH_4 content. This situation prevents anaerobic digestion and contributed to methane production (Fernandez et al., 2013). Species of *Scenedesmus* can show different morphological properties. For example, *Scenedesmus flavescens* grows together with other cells. Generally, four celled colonies can set up. Its outer cell is ellipsoidal and spindle. Inner cell is elongated oval and spindle. Its cell size is 8–10 µm wide and 18–30 µm long. On the other hand, *Scenedesmus multicauda* can be ellipsoidal or linear. They can be found as two celled colonies. Cell size is 3–10 µm wide and 7–13 µm long (Shin et al., 2013). They contain chlorophyll a and b also secondary metabolites such as β- carotene, γ-carotene, lycopene and luteine (Güçlü, 2009).

1.6.3.3. Nomencluture of *Micractinium sp.*

Table 1.6. Systematic classification of *Micractinium sp.*

Kingdom: Plantae
Phylum: Chlorophyta
Class: Trebouxiophyceae
Order: Chlorellales
Family: Chlorellaceae
Genus: Micractinium
Species: *Micractinium sp.*

Micractinium was identified by Fresenius (1858) (Luo, 2006). *Micractinium* has spherical and oval shape and they live in colonies. Cell walls consist of bristles and these bristles include protein and they don't have cellulosic membranes (Luo, 2006). These are appeared after cell wall formation. Species of *Micractinium* are similar every other and Length and number of bristles maintain distinguishment of species (Luo et al., 2006). *Micractinium pusillum* is similar to *Chlorella vulgaris* morphologically and both of them include spherical shapes without bristles. Also, they have similar cell wall structures and they don't consist of sporopollenin (Luo et al., 2006). On the other hand, there are diffferent properties between *Micractinium* and *Chlorella*. They have same family but different genera. Non bristed *Micractinium* cells produce bristles and these structures save them across rotifers. However, *Chlorella* doesn't contain bristles (Wang et al., 2014).

1.6.3.4. Nomencluture of Chlamydomonas reinhardtii

Table 1.7. Systematic classification of *Chlamydomonas reinhardtii*

Kingdom: Plantae
Phylum: Chlorophyta
Class: Chlorophyceae
Order: Chlamydomonadales
Family: Chlamydomonadaceae
Genus: Chlamydomonas
Species: *Chlamydomonas reinhardtii*

Chlamydomonas reinhardtii is unicellular green microalgae and has two flagella, 10μm long and multiple mitochondria. Eukaryotic photosynthesis has examined by means of *Chlamydomonas reinhardtii* because of its growth in dark with carbon sources (Merchant, 2007). It has haplontic life cycle, its reproduction can be induced by genetic manipulations and reproduces diploid zygotes. Also, it can be grown fast photoautrophy, heterotropy or mixotropy and reproduce as sexually or asexually (Aoyama et al., 2014 & Kumar et al., 2013). These properties make *Chlamydomonas reinhardtii* model organism and this species has been carried out in detail. In the future, probably, *Chlamydomonas reinhardtii* will be pioneer in a few industrial areas for production of biofuel, secondory metobolites, vacines and pharmacy (Kong et al., 2014). Main studies about *Chlamydomonas reinhardtii* are photosentesis, motility, reproduction and biofuel (Aoyama et al., 2014). *Chlamydomonas reinhardtii* has lipid bodies including triacylglycerols and these can be converted to biodiesel (Velmurugan et al., 2013). Nowadays, *Chlamydomonas reinhardtii* has been used for biohydrogen production. It can produce hydrogen in light condition because of iron hydrogenase activity. This enzyme functions reversible reduction of H^+ to H_2 via ferredoxin in anaerobic conditions (Mus et al., 2005).

1.6.4. Cultivation of Microalgae

Microalgae cultivation has been examined for long times. It was firstly thought microalgae might be energy supply in 1950s but this idea was postponed because of petroleum prices could be affordable (Farooq et al., 2014). Nowadays, crude oil prices have increased and microalgae cultivation studies have come into prominence (Farooq et al., 2014).

Microalgae can be grown in two different ways. First, an open pond and second is closed or open photobioreactor. Photobioreactors ensure huge amount of biomass production, light transfer and transmission (Junying et al., 2013). Open ponds are set up to outdoors and microalgae use CO_2 and sunlight from outside. Significant problem about open ponds is contamination and microalgae in this method aren't generally grown with controlled properties such as pH, light and temperature. On the other hand, open photobioreactors can be used as outdoor or indoor. When they are used in indoor, light can be maintained from lambs. In outdoor, they use sunlight (Bahadar, 2013).

Raceway ponds include a paddle wheel. This padel wheel can enable maximum light penetration and mixing of microalgae to prevent its precipitation in pond. They generally are set up as group of channels. Raceway ponds are cheaper because they don't need cooling systems and any energy inputs. But these systems produce lower biomass because of lower light intensity (Ugwu et al., 2008).

Closed photobioreactors have high biomass ratio, shorter harvesting time and efficiency. They also have reduced contamination risks. Light, flow rate and amount of carbondioxide are important in photobioreactors. Photobioreactors. High amount of light and carbondioxide increase photosynthesis and biomass. But, it results in high expense. Light between 10 and 30 m/s enables to high biomass concentration Junying et al., 2013). Advantages and disadvantages of open and closed photobioreactors were given in Table 1.8.

Table 1.8. Advantages and disadvantages of open and closed photobioreactors**.** (Adapted from Junying, 2013).

Parameters	**Open System**	**Closed System**
Biomass Concentration	low	high
Space required	low	high
Construction Cost	low	high
Contamination Risk	high	low
Water Losses	high	almost none
CO_2-Loses	high	low
Biomass Quality	Difficult to control	easy to control
Weather Dependence	high	low
Repeatability	low	high
Period of culture	long	relatively short

Closed photobioreactors are separated to a few groups such as tubular, flat, column and biofilm photobioreactors (Bahadar et al., 2013). Other novel method is non-suspended microalgae cultivation. At this method, microalgae are cultivated on attached surfaces. Microalgae have higher biomass yield , light can reach more easily in reactor and reactor can be controlled against contaminants. (Katarzyna et al., 2015). Advantages and disadvantages of various photobioreactors were given in Table 1.9.

Table 1.9. Advantages and disadvantages of various photobioreactors (Adapted from Bahadar, 2013).

Type of Photobioreactor	Advantages	Disadvantages
Tubular	Very effective light use; excellent temperature control, reasonable scale up	Fouling with some growth along walls
Vertical	Very high productivity and cell density	Scale up requires many compartments and support materials
Flat panel tubular with Fresnel coating	Absorbs more oblique light from light source	Difficulty controlling temperature, possible hydrodynamic stress to some algal stress
Helical tubular	High surface area	High heating and illumination costs
Air lift tubular	Easy CO_2 supply,	Complexity, difficult to scale up
Multiple airlifting membrane	Control over overall gas hold up and liquid circulation	High production cost
Cuboidal	High cell concentration	Can be dark regions in reactor
Stirred drought tube	Good light control	Higher cultivation cost
Stirred tank	Temperature control	Difficult to scale up
Air lift	Temperature control	Difficult to scale up
Flat plate	Excellent light use, temperature control	Difficult to scale up
Bubble column tubular	Effective light use	Low surface to volume ratio
Modified cascade	Effective light use	Limitation of biomass

1.6.5. Harvesting of Microalgae

Harvesting step is important for high biomass production. Sizes of microalgae can change between 1 μm and 30 μm. Low cell densities, tiny sizes and colloidal structures of microalgae led to difficult harvesting procedure (Misra et al., 2014). Choose of suitable harvesting method depends on morphological and anatomical properties of microalgae species. Harvesting of microalgae is expensive due to energy input for discarding of water. Near 30 % of total expense costs harvesting of microalgae (Molina et al., 2003).

Microalgae can be harvested with sedimentation, flocculation, filtration and centrifugation. Each stage can be used according to isolated microalgae. Sedimentation can not be used routinely for harvesting of microalgae. For flocculation, chemical agants can be used such as aluminium sulphate, ferric sulphate and ferric chloride. Polymer flocculants can be used for some microalgae such as *Chlorella*. Flocculation is a simple and fast method. This procedure is applied for concentration of microalgae. Massive biomass is used and microalgae biomass can be concentrated between twenty and a hundred times. This enables dewatering and reducing energy consumption. (Ndikubwimana et al., 2014 & Vandamme et al., 2013). On the other hand, chemical flocculants can be expensive and toxic.

Filitration needs pressure for filitrating of microalgae. The goal is to dewatering of microalgae. Generally, small scale microalgae samples are filitrated by this method (Barros et al., 2015). This method enables for high biomass concentrations. But, membranes are expensive and should be cleaned regularly.

Centrifugation procedure is fast but expensive because of energy input. Centrifugation can be applied to almost all microalgae. Gravitation and shear forces are important for centrifugation because these forces can distrupt cell membranes and damage product extracted. So, gravitation and shear forces should arrange among suitable ranges (Vandamme et al., 2013 & Barros et al., 2015). Filtration and centrifugation processes can be combined for harvesting of microalgae from enclosed photobioreactors (Gong, 2011). Advantages and disadvantages of harvesting methods for microalgae were given in Table 1.10.

Table. 1.10. Advantages and disadvantages of harvesting methods for microalgae (Adapted from Barros, 2015).

Harvesting Methods	**Advantages**	**Disadvantages**
Chemical Coagulation/Flocculation	Simple and fast method, no energy requirements	Can be expensive and toxic to microalgae biomass
Auto and bioflocculation	Inexpensive, allow culture medium recycling, non-toxic to microalgae biomass	Changes in cellular composition and contamination
Gravity sedimentation	Simple and inexpensive method	Time-consuming, possibility of biomass deterioration
Flotation	Low cost method, feasible for large scale applications	Requires the use of chemical flocculations
Electrical base process	Applicable to a wide variety of microalgal species	High energetic and equipment costs
Filtration	High recovery efficiency	Membrane should be regularly cleaned
Centrifugation	Fast, high recovery efficiency	Expensive and high energy requirements

1.6.6. Extraction of Microalgae Lipids

Microalgae can be extracted as wet and dried forms. Wet extraction procedure is fast and does not need energy input for drying of microalgae. But, this method isn't efficiency for lipid extraction. Microalgae include water and it is a polar molecule. This method needs to high pressure for decreasing of polarity. In addition to this, super critical carbondioxide is used for obtaining microalgae dewatered. But, lipid productivity decreases when super critical carbondioxide is used due to its ineffectiveness on polar lipid. On the other hand, drying procedure needs energy input but is efficient for lipid extraction (Mutanda et al., 2011 & Islam et al., 2014). These procedures can be applied when microalgae present in lipid soluble solvents such as chloroform, hexane and heptane. They are called as solvent extraction methods. Some assissted modifications such as microwave, bead, homogenization and ultrasound methods can be added to extraction procedure according to nature of microalgae species. It is more difficult when microalgae extraction compared with plant and animal fat extraction because of microalgae's small size and rigid cell walls (Islam et al., 2014).

1.6.7. Qualitative and Quantitative Determination of Microalgae Lipids

For determination of lipids, there are many different methods. These are organic solvent extraction, soxhlet extraction, high throughput Nile red (9-diethylamino-5H-benzo [α] phenoxazine-5-one) method, BODIPY 505/515 (4, 4-difluoro-1, 3, 5 ,7-tetramethyl-4-bora-3α,4adiaza-s-indacene) method, Time-domain nuclear magnetic resonance (TD-NMR) and Colorimetric quantification.

Conventional method for lipid extraction is organic solvent extraction. This method is labour intensive and time consuming also needs at least 15 mg of biomass but this method gives true results precisely (Akota et al., 2005). Bligh and Dyer (Bligh, 1959) and Folch methods (Folch et al., 1956) are organic solvent methods. These methods use chloroform and methonol and total lipids are measured gravimetrically. The other method is hexane extraction by soxhlet apparatus. Actually, hexane has a few advantages. It is less toxic than chloroform and neutral lipids can be extracted

selectively. Neutral lipids are extracted by hexane in the industries with current techniques (Shin et al., 2014).

Nile red is lipid soluable flourescence dye and can be used for in-situ lipid determination of microalgae (Bertozzini et al., 2011). This dye is photostable in hydrophilic and hydrophobic solutions and the maximum emmision peak is blue shifted when polarity of solvent decreases (Pick et al., 2012). This method is high throughput method for screening of microalgae strains (Chen et al., 2009).

BODIPY 505/515 (4, 4-difluoro-1, 3, 5, 7-tetramethyl-4-bora-3α,4adiaza-s-indacene) method is lipid soluable flourescence dye too. It dyes lipids to green color and this dye is non-destructive and can be used for other experiments after lipid determination (Govender et al., 2012). In calorimetric method, fatty acids react with copper and with additing of substrates, it occurs colored product and measured spectrophotometrically (Wawrik, 2010). Advantages and disadvantages of lipid determination methods for microalgae were given in Table 1.11.

Table 1.11. Advantages and disadvantages of lipid determination methods for microalgae (Adapted from Gong, 2011).

Methods	Advantages	Disadvantages
Solvent extraction and gravimetric method	High accuracy and reproducibility	Time consuming, labor intensive, large amount of biomass (15 mg)
Chromatographic method	Good reproducibility, single analysis generates data of both quantity and profile of fatty acids, small sample required	Requirement of cell disruption, requirement of expensive analytical equipment
Nile Red staining (NR)	In situ measurement, high throughput, simple, rapid and efficient	Variable efficiencies in some microalgae, accuracy can be affected by many factors
Time domain nuclear magnetic resonance (TD-NMR)	In situ measurement, rapid and less expensive	Accuracy is dependent on high lipid content
Colorimetric quantification	Rapid, simple, cheap	Not applicable to detect fatty acids with length chain of less than 12C atoms

1.6.8. Biodiesel Production from Microalgae via Transesterification Reaction

Extracted oils cannot be used for biodiesel. These oils have high viscosity. For decreasing of viscosity, obtained oils have to be converted to fatty acid methyl ester (FAME) via transesterification reaction (Yaakob et al., 2014). In transesterification reactions, triglycerides react with alcohols with suitable catalysts. Generally, methanol is used at these reactions because it is efficient and cheap. At the reaction, when three fatty acid molecules are combined with three methanol molecules via a suitable catalyst, one molecule biodiesel and one molecule glycerol is formed. Glycerol is waste product in here. But it can be used for pharmaceutical and cosmetics industries. The reaction is forwarded in three steps. At the first step, triglycerides are converted to diglycerides and next to monoglyceride. The last products are one mole of glycerol and three moles of fatty acid methyl ester. At the industry, one mole of triglyceride is reacted with six moles of methanol to increase biodiesel production. This results in above 98 % of biodiesel productivity (Frac et al., 2010).

This reaction is affected by a few parameters. These are temperature, time, catalyst and molar ratio between alcohol and triglyceride. There are three types of catalysts. These are i) base, ii) acid and iii) enzymatic catalysts. Base catalysts often are used in the industry. Base catalysed reactions are separated to two groups. The first is homogeneous base catalyst. These are KOH, NaOH and CH_3Na (Gemma et al., 2004). Second type is heterogeneous base catalyst such as MgO, CaO, Na and K. These are supported to metal support and the reaction forms heterogeneously. These have many advantages such as easy separation of catalyst, prevention of soap formation and reusing of catalyst (Yaakob et al., 2014). Base catalysts work fast and produce high amount of yield give little side product. But, they can produce saponification reaction with alcohol (Kulkarni et al., 2006). On the other hand, acid catalysed transesterification reactions are slow but don't interfere with saponification reactions. They are generally not preferred in the industry. Sulfuric acid can be used for acid catalysed reactions (Marchetti et al., 2007). The last is lipases. These are bio-enzymes and catalyse transesterification reactions. Lipases are effective but expensive. Application of lipases in transesterification reactions results in extra cost

in biodiesel production (Orcaire et al., 2006). Chemical schematic representation of biodiesel production from microalgae was given in Figure 1.5.

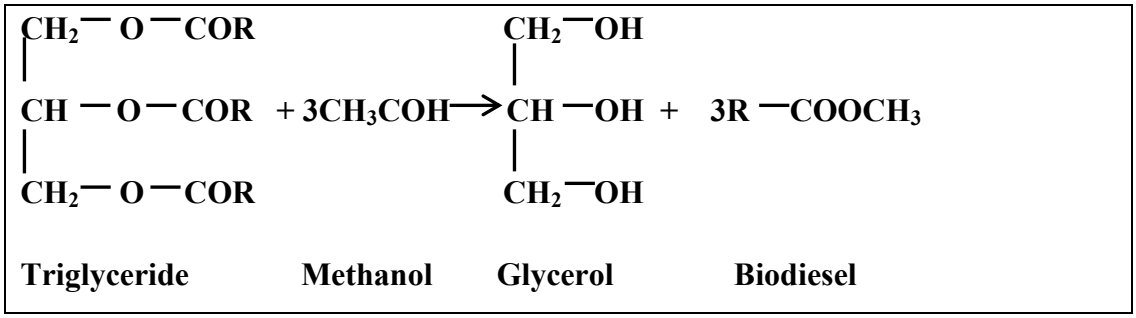

Figure 1.5. Chemical schematic presentation of biodiesel production

There are many processes for biodiesel production from microalgae. Main goal is to reduce expenses and find suitable microalgae strains including high lipid content and appropriate fatty acid compositions for biodiesel production. Integrated systems were given in Figure 1.6. One or a few of them can be optimized for obtaining of desired biodiesel production.

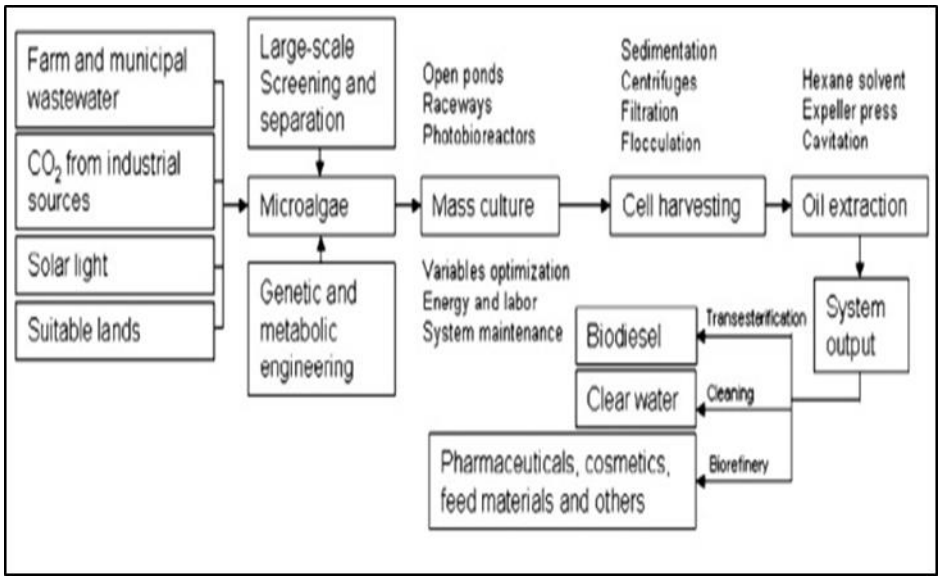

Figure 1.6. Integrated systems for deserved biodiesel production from microalgae (Gong, 2011).

Table 1.12. The standard values of some of the fuel parameters of biodiesel (Adapted from Yaakob, 2014).

Property	Biodiesel Test Method	Units	Biodiesel EN14214/ASTM D 6751	Diesel ASTM D 975
FAME Content	EN 1403	% m/m	96.5 min	-----
Mono-glyceride		% m/m	0.80	-----
Di-glyceride	EN 14105	% m/m	0.20	-----
Three-glyceride		% m/m	0.20	-----
Linolenic acid methyl ester	EN 1403	% m/m	12	-----
Free glycerine	D 6584	% m/m	0.020	-----
Total glycerine	D 6584	% m/m	0.240	-----
Phosphorus content	D 4951	% m/m	0.001 max	0.08 max
Flash point	D 93	^{0}C	93.0 min	60-80
Kinematic viscosity,40 ^{0}C	D 445	mm^2/s	1.9-6.0	1.3-4.1
Cetane number	D 613	-----	47 min	40-55
Cloud point	D 2500	^{0}C	-3 to 12	-15 to 5
Acid number	D 664	mg KOH/g	0.5 max	-----
Oxidation stability	EN 15751	hours	3 min	-----
Methanol content	EN 14110	vol %	0.2 max	-----
Lower heating value	-----	btu/gal	˜118,170	˜129,050
Specific gravity 60 ^{0}C	EN ISO 12185	kg/L	0.88	0.85
Iodine number	EN 14111	mg I/100g	120 max	-----
Pour point	ISO 3016	^{0}C	-15 to 10	-35 to -15

1.6.9. Determination of Biodiesel Quality

There are two standards known for determination of biodiesel quality. These are European standard EN 14214 and American standard ASTM D6751. These standards determine quality of biodiesel (Yaakob et al., 2014). The standard values of some of the fuel parameters of biodiesel were given in Table 1.12.

1.7. Transformation of DNA of Microalgae Nuclear Genome

There are several methods for transformation of DNA of *Chlamydomonas* nuclear genome. The first is to biolistic method. Firstly, genetic transformation of *Chlamydomonas* was tried in 1982. But, there was no successful result until 2000's. So, biolistic method has been generally used for transformation since 2000's (Ladygin et al., 2004). This method uses bombardment of these cells with coated tungsten particles on agar plate. This method was successful for nuclear and organelle transformation (Nickoloff et al., 1995). But, transformation efficiency remained low with biolistic method.

While genetic engineering was developing, other method (glass bead method) was found. This exhibited higher transformation efficiency (10^3 per μg DNA) than that of biolistic method. But, glass bead method failed in distruption of cell wall (Yamano et al., 2013).

The last method is electroporation method. Cell can be transformed by several copy of DNA without special cell strains or chemical treatment and efficiency of transformation (10^5 per μg DNA is high). Also this method doesn't permit production of harmful compounds (Nickoloff et al., 1995 & Yamano et al., 2013). On the other hand, this method has some backdraws such as removal of cell wall and gametolysin (Yamano et al., 2013). All methods excluded for biolistic method operate without cell walls. In other words, cell wall must remove from cell (Kumar et al., 2004).

1.8. Brief Information about Sampling Region (Haymana)

Haymana is located in the Central Anatolia region of the Turkey. It is the town of Ankara, the capital of Turkey. Haymana has distance of 74 km from Ankara. Population of Haymana is approximately 15000. The district covers area of near 3000 m^2 and its elevation is around 1260 (www.haymana.gov.tr). Haymana region is familiar with Turkish baths. Haymana is an old town. Its history reaches to antiquity. Tools from paleolithic, neolithic and metal age were found from ruins in Haymana. First inhabitants were Hittites in these areas. Then, The Phryges lived on this area around 1200 B.C and the Romans inhabited in Haymana and Anatolia near 25 B.C. They set up baths for remedy purposes. Arabian people invaded Haymana in 796.

When the Seljuks inhabited Anatolian lands, they captured this town in 1073. Next, the Ottomans settled Haymana. This town has been in the Republic of Turkey since 1923 (www.haymana.bel.tr). Nowadays, there have been three thermal baths and 5 spa hotels in 2014. Thermal spring water of Haymana has been used for rheumatism, brain, slipped disc, skin, hypertension, kidney and arteriosclerosis diseases (www.haymana.gov.tr). Properties and analysis results of thermal spring water were given in Table 1.13 and 1.14, respectively.

Table 1.13. Properties of Haymana thermal spring water (www.haymana.bel.tr).

Temperature: 41-44 ^oC
Radyoactivity: $0.155x10^{-9}$ g/L
Amount of salt: 1.221796 g/L
Density:1.00017 g/cm^3

Table.1.14. Analysis results of Haymana thermal spring water (www.haymana.bel.tr).

Chemicals	mg/L	mmol	mg-equivalent/L
Sodium (Na)	0.0814	3.5391	3.5391
Potassium (K)	0.00605	1.547	1.547
Calcium (Ca)	0.1140	2.8500	5.7000
Magnesium (Mg)	0.0240	0.9876	1.9752
Iron (Fe)	0.000308	0.00550	0.0110
Aluminium (Al)	0.000338	0.0125	0.0375
Clor (Cl)	0.024	0.6760	0.6760
Sulfate (S)	0.0094	0.09785	0.1957
Hydrocabonate (HCO_3)	0.6182	10.55372	10.55372
Silicic acid (H_2SiO_3)	0.0696	0.8910	
Free carbondioxide (CO_2)	0.947296	6.000	
Hydrogen sulfide (H_2S)	0.0005	0.0147	

1.9. Aim of the Study

The main goal of this study was to produce biodiesel from thermo-resistant green microalgae from Haymana. For this objective, firstly, micralgae were sampled from hot spring and applied isolation and characterization methods.

Next objective of this study was to characterize and identify the microalgae at morphologic and molecular level. For this purpose, Scanning electron microscopy (SEM) was used for morphological characterization. The molecular characterization was done by using PCR technique (ITS 2 region PCR).

Then, growth profiles, rates and dry cell weights of microalgae were determined.

The other purpose of the study was to optimize lipid extraction procedures for biodiesel production. Three techniques (Soxhlet, Bligh&Dyer and Folch) were tested. Also, protein concentrations, carbohydrate percentages and chlorophyll contents of microalgae were determined.

Next, lipid contents of Micractinium sp. mutants were measured by using Nile Red (Fluorescence technique).

The last, for improvement of biodiesel production capacity of microalgae by genomic approaches, the gene transformation studies were to achieve by using model microalgae *Chlamydomonas reinhardii.*

CHAPTER 2

MATERIALS AND METHODS

2.1. Microorganisms, Chemicals, Equipment and Glasswares

2.1.1. Microorganisms

In this study, *Scenedesmus sp.ME02, Hindakia tetrachotomaME03 and Micractinium sp.ME05* were used. These strains were sampled from a few hotspring points, Haymana, Ankara. *Chlamydomonas reinhardtii 137-* was obtained from Dr. Claire Remacle, Genetics of Microorganisms Laboratory, Department of Life Sciences, Université de Liège, B-4000 Liège, Belgium and used for genetic manipulation experiments.

2.1.2. Chemicals

Tris base, Ammonium chloride, Magnesium sulfate heptahydrate, Calcium chloride dihydrate, EDTA disodium dihydrate, Zinc sulfate heptahydrate, glacial acetic acid, Sodyum nitrat, Dipotassium phosphate, Citric acid monohydrate, Ferric Ammonium Citrate, Sodium carbonate, Nitrilotriacetic acid, Potassium nitrate, Sodyum nitrat, Disodium phosphate, Calcium sulfate dihydrate, Sodium chloride, Iron(III) chloride and Potassium hydroxide were purchased from Merck Company. Glutaraldehyde, sodium cacodylate trihydrate, osmium tetraoxide, acetone, ethanol, chloroform, methanol, phosphoric acid, sulfiric acid, hexane, bovine serum albumin and Nile red were purchased from Sigma Aldrich.

2.1.3. Equipment and Glasswares

In this study, we used some equipments for preparing of stock solutions and culture mediums. Glassware (e.g., pipets, bottles, erlenmayer flasks, test tubes, petri dishes) and plasticware were needed for preparation and storage of stock solution. pH meter and magnetic stirrer were used for pH arrangements of liquid mediums and stirring of chemicals in the solutions, respectively. Autoclave is necessary for sterilation of all equipments, glasswares and culture mediums. Filter sheets and filter syringes were essential for more sensitive sterilization. In addition to these, distelled water and ultrapure water were used for elimination of bacteria and viruses from medium cultures and in moleculer charecterization studies. After experiment steps, all glaswares were cleaned with deionized water and soaked in 1 M HCI.

2.2. Preparation of Stock Solutions and Storage of Materials

Preparation of stock solutions is important because amounts of macronutrients, trace elements and vitamins should be presented as correct precisely in the culture mediums. 1L of stock solutions was prepared during experiments. Also, trace solutions were prepared seperately and added in the final mediums after macronutrients were put. Materials were wrapped with aluminum foil before sterilization and autoclaved at 121 ^{o}C for 20 min. Then, they were kept in clean cabinets.

2.3. Sample Collection and Growth Conditions

Microalgae were collected from various hot spring locations defined as sources of water. GPS (Global Positioning System) coordinates of these locations were N: 39.793947, E: 32.80425; N: 39.79642, E: 32.801827; N: 39.794021, E: 32.802823 at Haymana, Ankara, Turkey. Sampling locations of microalgae were given in Figure 2.1 (A, B, C and D).

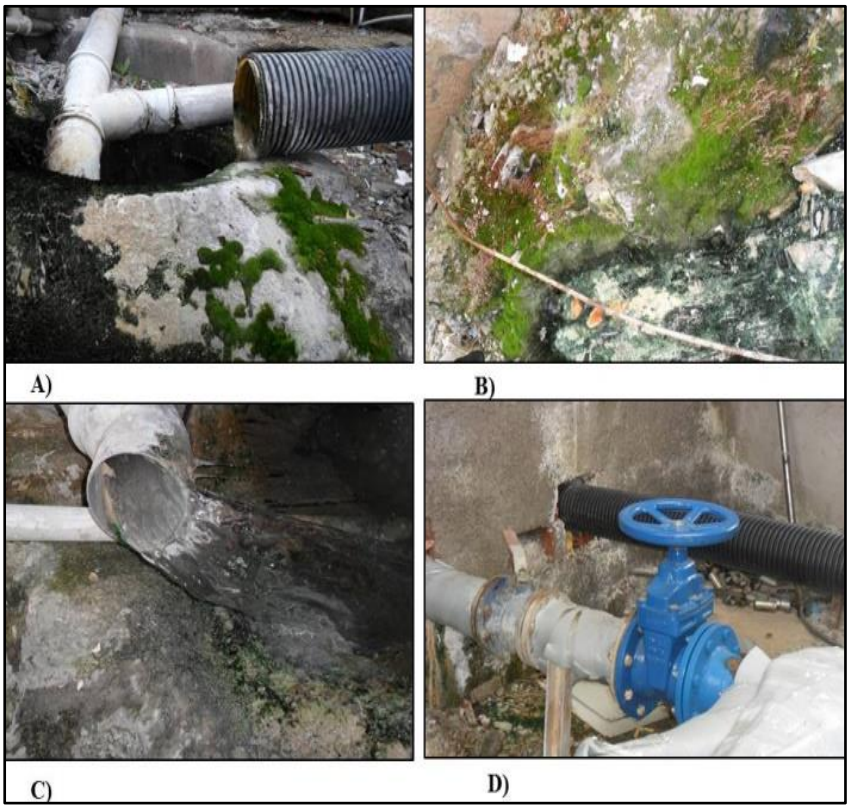

Figure 2.1. Sampling locations (A, B, C and D) of microalgae from various hot spring points defined as sources of water in Haymana.

Samples were placed in sterilized containers and brought directly to the lab. Water samples were filtered with double layered filter sheets to remove particle and debris. Then, 200 µL of filtrates were added on Tris- Acetic acid-Phosphate (TAP), BG-11 (Allen and Stainer medium) and D (Sheridan medium) agar at pH: 6.8. Compositions of mediums were given in Appendix B, C and D. Next, agar plates were left at the growth chamber to visible colonies. When colonies were visible, serial dilution and streaking cells techniques across agar plates were applied. After obtaining single colonies, microalgae were placed to be grown in TAP, BG-11 and D mediums. Microalgae were maintained at 4±1 ^{o}C, 24 ± 1 ^{o}C, 37±2 ^{o}C and 50±2 ^{o}C at diffterent pH values (5-8) and with a light intensity of 1 klux, 4 klux, 6 klux and 10 klux in TAP medium. All cultures were performed with 16:8 light: dark photoperiod cycle.

2.4. Morphological Characterization of Microalgae

2.4.1. Identification of Microalgae Samples by Light Microscopy

At the beginning of the study, morphological identifications of microalgae samples were carried by light microscopy. Microalgae samples were centrifuged at 3000g for 10 min at 4 ^{o}C. Supernatant was removed and pellet was resuspended in 4 % glutaraldehyde for 30 min at 4 ^{o}C. Then, 20 µL of microalgae sample was taken and observed under light microscopy. Photomicrographs were taken and compared with the micrograph catalogs of known genera.

2.4.2. Scanning Electron Microscopy (SEM) Analysis

For SEM analysis, isolated microalgae strains were centrifugated at 3000 *g* for 10 min at 4 ^{0}C. Supernatant was discarded and pellets were kept in 4 % glutaraldehyde for 3 hours at 4 ^{0}C to achieve primary fixation. Pellets were washed in 0.1 M sodium cacodylate buffer for three times to discard fixative agent. Washed pellets were subjected to 1 % osmium tetraoxide for an hour at room temperature to maintain secondary fixation. Then, pellet was washed in 0.1 M Sodium cacodylate buffer at pH: 7.4 and dehydration procedure was applied to isolated samples with different concentrations of aceton (30 %, 50 %, 70 %, 80 %, 90 %, 95 % and 100 %). Dehydrated samples were mounted on stubs and coated with gold and visualized with FEI Quanta 400 F scanning electron microscope according to Kalab (Kalab, 2008).

2.5. Molecular Characterization of Microalgae

2.5.1. PCR Amplification and Cloning of ITS2 Region

2.5.1.1. Isolation of DNA

Microalgal isolates were grown up to stationary phase in TAP medium. Grown cells were centrifuged for 4500 g at 10 minutes and cell pellets were lyophilized using Christ LGC Alpha 1-4 Loplus lyophilizer. Microalgae samples were examined for total genomic DNA extraction by CTAB method with a few modifications (Doyle and Doyle, 1987& Murray and Thompson, 1980). Preparation of CTAB extraction

buffer (Hexadecyl-trimethyl ammonium bromide) and the total genomic DNA isolation was given in Appendix E and Figure 2.1, respectively.

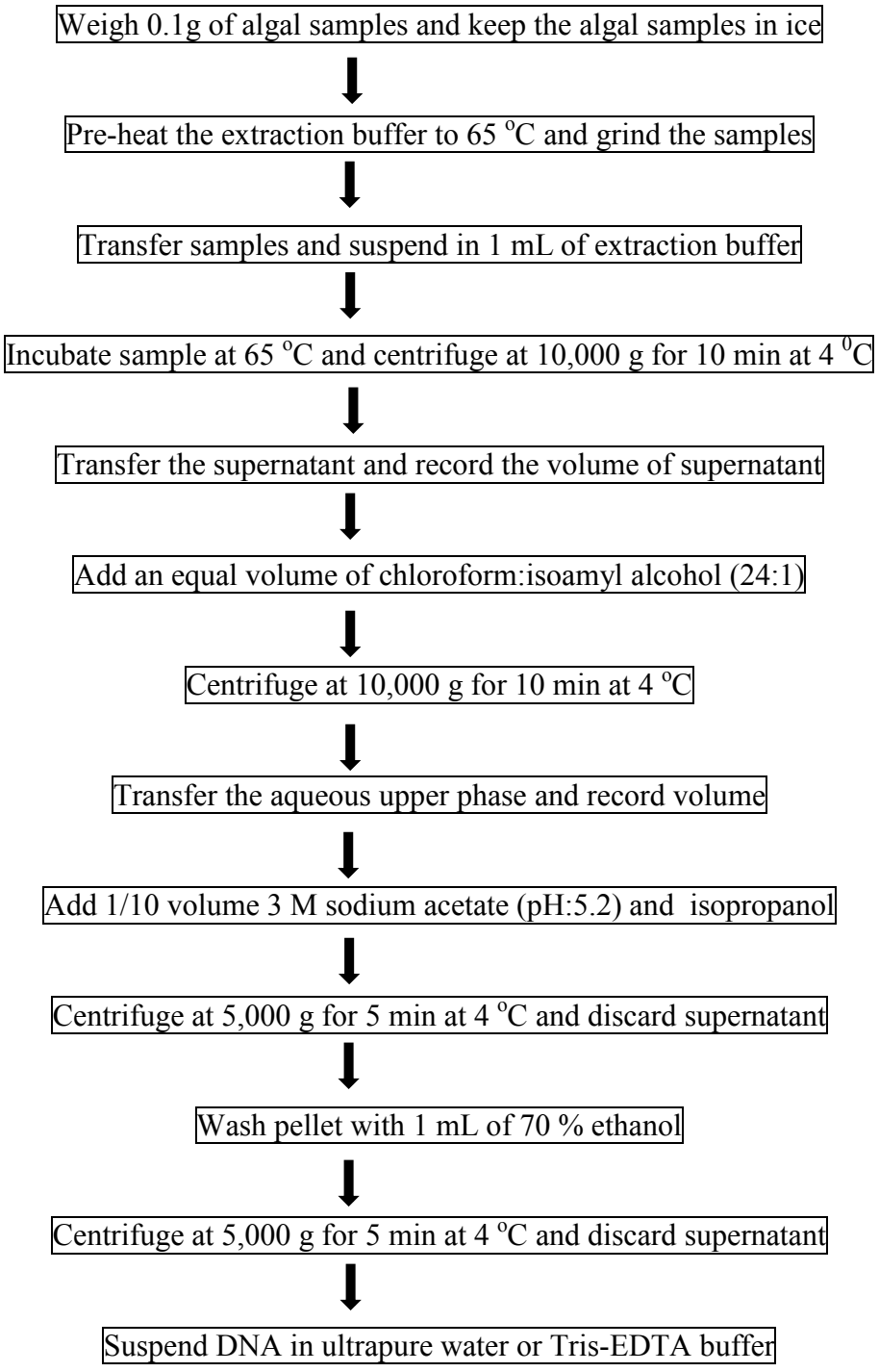

Figure 2.2. The total genomic DNA isolation procedure according to CTAB method (Doyle and Doyle, 1987 & Murray and Thompson, 1980).

2.5.1.2. Quantitation of DNA

Concentration of DNA was measured by NanoDrop 1000 Thermo scientific. To understand purify of DNA, the ratio of OD_{260}/OD_{280} was checked.

2.5.1.3. Amplification of the ITS2 rDNA Region

The ITS2 rDNA region was amplified via PCR containing stock 10X PCR buffer, 2 mM dNTP mix, 5 U Taq polymerase, 25 mM $MgCI_2$ and 10 µM concentration of reverse primer SQITS2 and forward primer. Composition of PCR was given in Table 2.1.

Table 2.1. Composition of PCR

Reagents	Stock Conc.	Final Conc.	Volume (µL)
Buffer	10X	1 X	5 µL
dNTPmix	2 mM	0.3 µM	7.5 µL
Forward Primer	10 µM	0.5 µM	2.5 µL
Reverse Primer	10 µM	0.5 µM	2.5 µL
$MgCI_2$	25 mM	2 µM	4 µL
Taq DNA polimerase	5U/ µL	0.06 U/ µL	0.6 µL
DNA (Template)	11ng/ µL	----------	1 µL
Ultrapure water			26.9 µL
Total			**50 µL**

The DNA region corresponding to the 18S ribosomal RNA gene internal transcribed sequence 2 (ITS2) was amplified as described by Kaur et al. (2012) using the following forward and reverse primers. Properties of forward and reverse primers were given in Table 2.2.

Table 2.2. Properties of forward and reverse primers

Primers	Sequence (5'→3)	A_{260}	M.W	Base number	Tm
SQITS1	GAGCATGTCTGCCTCAGC	0.21	5475	18	58.2
SQITS2	GGTAGCCTTGCCTGAGC	0.17	5202	17	57.6

PCR conditions were adjusted for 5 min at 95 ^{o}C for initial heating and followed by 30 s at 95 ^{o}C, 30 s at 55 C and 1 min 72 ^{o}C for 29 cycles and 5 min at 72 ^{o}C for the extension. Some modifications were applied for each strain (Thermal cycling conditions used in cycle sequencing was given in Table 2.3.

Table 2.3. Thermal cycling conditions used in cycle sequencing

	Duration	Temperature (^{0}C)	Number of Cycles
Initial Denaturation	5 min	95	1
Amplification			29
Denaturation	30 sec	95	
Annealing	30 sec	55	
Extension	1 min	72	
Final Extension	5 min	72	1

Finally, amplified DNAs were seperated on 1.5 % agarose gels including 1 X TAE buffer and bands were visualized with staining by ethidium bromide. The PCR gene

products were purified with the Nucleospin gel extraction. Procedure of PCR product purification was given in Appendix F.

2.5.1.4. Cloning of PCR Products

Then PCR products were cloned by TA cloning (Invitrogen) according to manufactures instructions and applied colony PCR by Qiagen PCR protocol using 10X PCR buffer, 10 mM dNTP mix, 5U Taq polymerase, reverse primer (M13) and forward primer (M13) and PCR optimum conditions were adjusted 15 min at 95 ^{o}C for Initial heating and followed 30 s at 95 ^{o}C, 30 s at 53 ^{o}C and 1 min 72 ^{o}C for 25 cycles and 5 min at 72 ^{o}C for the extension and sequenced using automated DNA sequencer by Ref-Gen.,Ankara, Turkey. Gel electrophoresis was carried out using systems manufactured by Bio-Rad Laboratories Inc (Hercules, CA, US) and C.B.S. Scientific Company Inc (Del Mar, CA, US). Gel documentation was achieved using GelDoc-ItImaging System (UVP Ltd; Cambridge, UK). Electrophoregrams were detected using Vector NTI (Invitrogen) and manually edited. We compared sequence similarities using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). GenBank accession numbers of microalgae isolates; *Hindakia tetrachotomaME03 Scenedesmus sp.ME02 and Micractinium sp.ME05* are KJ564283, KJ564284 and KJ564285, respectively.

2.5.2. Bioinformatic Analysis of the ITS2 Sequences and Construction of the Phylogenetic Tree

ITS2 sequences of microalgae isolates were carried out and analyzed using Contig Express software of Vector NTI (Invitrogen). ITS2 secondary structure prediction of sequences of microalge samples was examined using the prediction algorithm applied in the ITS2 database with the parameters of E<1e-16 and minimum helical transfer of 75% (Koetschan et al., 2012). The ITS2 sequence of each isolate and the predicted secondary structure was BLASTed against the ITS2 database with E<0.1. The BLAST results with minimum 40% total coverage were selected for CLUSTALW (Larkin et al., 2007) alignment within the 4SALE program (Seibel et al., 2008). The CBC matrix calculated by 4SALE was used for phylogenetic tree

generation in CBCanalyzer (Wolf et al., 2005). The phylogenetic tree was drawn by using TreeDyn (Chevenet et al., 2006).

2.6. Growth Evaluation and Harvesting of Microalgae

The optimum growth conditions of microalgal isolates were determined as 4 klux of light intensity, pH 7.4 and salt concentration of 1.5 x 10^{-2}M.

Growth of isolated microalgae was monitored at 530, 600, 680 and 750 nm using Shimadzu UV-1800 spectrophotometer. Microalgae dry weights were examined by filitration. Ash free dry weights (AFDW) were obtained for correlation between optical density and dry weight. The algal suspension was grown up to stationary phase. Then algae samples were harvested by centrifugation at 3000 *g* for 10 min at 4°C (Barsanti and Gualtieri, 2006). The pellet was washed three times with distilled water. Next, pellets of microalgae samples were filtered through glass fiber filters (1.2 μm GF/C; 47 mm, Whatman) and dried at 24h 105 °C and cooled in a dessicator for dry cell weight measurement. Next, microalgae samples were weighted again. In addition, the algal biomass was lyophilized and stored at -20 °C for next analysis. Results given were the average of three measurements.

2.7. Specific Growth Rate Calculation

The specific growth rates of microalgal isolates were calculated by the following equation (Clesceri et al., 1989): Specific Growth Rate: In (X_1- X_2) / (t_2 – t_1). Where, X_1: Biomass concentration at the end of the selected time interval; X_2: Biomass concentration at the beginning of the selected time interval; t_2 – t_1: Time elapsed between the selected time points.

2.8. Chlorophyll-a Content

Chlorophyll-a content was determined according to Arar method (1997). Firstly, microalgae were extracted by 90% acetone in cold. 1 mL of algae sample was taken and filtered. The filter was kept at dark and at -20 °C for 12 hours. Filter was put in tube and final volume of the sample was adjusted in 12 mL with 90% acetone. Then, the slurry was centrifuged at 1,000 rpm for 15 min at 4 °C. The supernatant was taken in a tube and the extract was filtered to decrease the turbidity with glass

syringe through Milipore membrane of 0.45 mm porosity. Finally, optical densities of the prepared samples were determined at 664, 647 and 630 nm wave length by Shimadzu UV-1800 spectrophotometer and subtracted the absorbance value at 750 nm from the absorbance values at 664, 647 and 630 nm.

Chlorophyll-a content of microalgae was calculated by tricromatic equations of Jeffrey and Humphrey (1975) according to the following equations;

Chlorophyll-a (µg/mL) = 11.85 (OD 664)-1.54 (OD 647)-0.08 (OD 630).

2.9. Determination of Carbohydrate Concentration of Microalgal Isolates

Total carbohydrates of microalgae were extracted with 80% H_2SO_4 according to Myklestad and Haug (1972). Carbohydrate concentration of species was carried out spectrophotometrically by anthrone-sulfuric acid method with modifications (Gerhardt et al., 1994 and Zahao et al., 2013). At the stationary phase, 0.5 mL of microalgae extract and 0.5 mL of distilled water were combined with 4 mL of anthrone solution (2g anthrone in 98 % H_2SO_4) and the mixture was placed in water bath at 100 ^oC for 10 min. The results were recorded with OD_{620} absorbance. In this study, glucose was used as a standard with different concentrations (10, 40, 60, 100, 150, 200 µg/ mL) and the standard curve was given in Appendix G. The equation was expressed as y= 0.0046x – 0.0135 (R^2 = 0.9986 P < 0.05). Carbohydrate concentration of extract was calculated according to the following equation:

Carboydrate concentration (w/w %) . (CV/M) * 100

where; C is carbohydrate concentration µg /ml)

Carboydrate concentration (µg /mL) = (Average OD 620 / slope) * DF

V is the volume (L) of the hydrolyis buffer used to resuspend the biomass and M is is the amount of extract (mg). OD: Optical density. DF: Dilution factor. slope: slope of the graph calculated for the standard.

2.10. Determination of Protein Concentration of Microalgae

Firstly, extraction procedure was applied to 0.1 g of microalgae according to Weis (2002). Then, cell pellets were resuspended in 10 mL of lysis buffer (50 mM Tris-HCI pH: 8, 10 mM EDTA, 1.5 % SDS). The resulting mixture was vortexed and centrifuged at 13000 g for 20 min at 4 ^{o}C. The supernatant was used for protein determination with Bradford method (Bradford, 1976).

0.1 ml of supernatant was taken and added to 0.9 mL of Bradford reagent (five times diluted). Preparation of Bradford reagent was given in Appendix H. Next, tubes were vortexed immediately and kept for 10 min at 25 ^{o}C. Bovine serum albumin (BSA) was used as standard and the tubes were vortexed and incubated for 10 min at RT. Then, the absorbances of the tubes were measured against at 595 nm. The equation of standart concentrations was shown as y=4.53x+0.0594. The protein content of the biomass was calculated using the following equation:

Protein content (w/w %): (CV/M) * 100

where; C is protein concentration (mg/ml)

Protein concentration (mg/mL) = (Average OD 595 / slope) * DF

V is the volume (L) of the lysis buffer used to resuspend the biomass and M is is the amount of biomass (mg). OD: Optical density. DF: Dilution factor., slope: slope of the graph calculated for the standard.

2.11. Lipid Content of Microalgae in Different Extraction Methods

0.1 g of microalgae strains were taken and the strains were extracted in 4 ml solvent via three different modified extraction methods: i) Bligh and Dyer method, ii) Folch method (Folch et al., 1956) and iii) Soxhlet extraction method with hexane (Koberg et al., 2011). For all three methods, initially two flasks of 100 ml of the same microalgal suspension were separated and centrifuged at 3000 *g* for 10 min at 4 $^{\circ}$C. Then, for dry weight calculation, one tube containing the pellet was taken and oven-dried. The second tube was used for lipid extraction according to the method of choice. All experiments were performed with three biological replicates.

2.11.1. Soxhlet Method

Soxhlet extraction method with hexane (GC grade) was applied according to Koberg and modified (Koberg et al., 2011). Firstly, 100 mL of 2 tubes of microalgal suspension were taken and centrifuged at 3000 g for 10 min at 4 $^{\circ}$C. Then, for dry weight calculation, one tube with pellet was taken and dried via Oven (NUVE ES 500). Another tube was used for Soxhlet method with n-hexane experiment. One part of pellet was mixed with forty parts of n- hexane in the Soxhlet apparatus. The solvent was heated to reflux. Oil of microalgae was dissolved in hexane. Pellet was left for 24 hours in Soxhlet apparatus and oil was taken from distillation flask. Remaining hexane was evaporated by rotary evaporator (BUCHI Rotavapor R-200). Microalgae oil was weighed gravimetrically and lipid productivity was calculated. Calculation of lipid content and productivity were given in Appendix I.

2.11.2. Bligh and Dyer Method

Bligh and Dyer method was carried out as described previously (Bligh, 1959). In this method, 100 mL of 2 tubes of microalgal suspension were taken and centrifuged at 3000 g for 10 min at 4 $^{\circ}$C. Then, for dry weight calculation, one tube with pellet was taken and dried via Oven (NUVE ES 500). Another tube was used for Bligh and Dyer method. Pellet was mixed with methanol and chloroform (2:1 v/v) as the extraction solvent and vortexed. Then, chloroform and water were added to the mixture. The mixture was shaken for 10 min in a seperating funnel and two phases formed subsequently.After adding water, two phases should be visible. If not, leave for 24 hours. Final ratio was adjusted to 1:1:0.9 v/v for methanol, chloroform, and water, respectively. Lower phase includes microalgae lipids. In this study, one part of pellet was mixed with forty parts of (methanol-chloroform-water). Lower phase was seperated from upper phase and evaporated by rotary evaporator (BUCHI Rotavapor R-200). Obtained lipids were weighed gravimetrically.

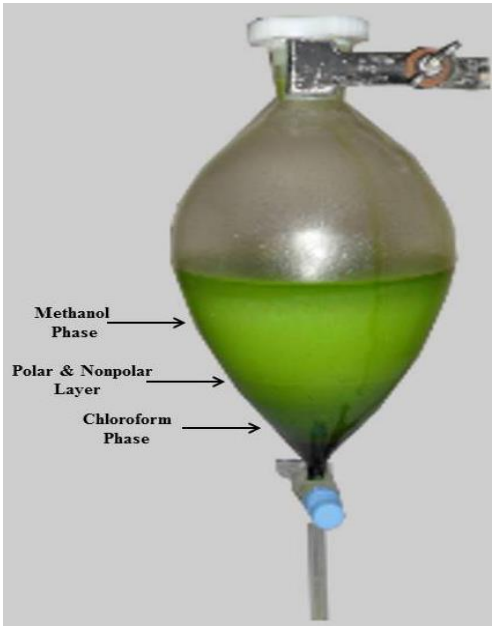

Figure 2.3. Solvent extraction by Bligh and Dyer for obtaining high amount of lipid.

2.11.3. Folch Method

This method was originally described by Folch (1956). 100 mL of 2 tubes of microalgal suspension were taken and centrifuged at 3000 *g* for 10 min at 4 °C. Then, for dry weight calculation, one tube with pellet was taken and dried via Oven (NUVE ES 500). Another tube was used for Folch method. Pellet was mixed with methanol and chloroform (1:2 v/v) as the extraction solvent and vortexed. Then, methanol and water were added to the mixture. After addition of water to the mixture, final ratio was 1:1:0.9 v/v for methanol, chloroform, and water, respectively. The mixture was shaken for 10 min in a seperating funnel and two phases formed subsequently. Next, lower phase including microalgae lipid was taken and evaporated by rotary evaporator (BUCHI Rotavapor R-200). After obtaining of lipid, microalgae lipids were weighed gravimetrically.

2.12. Cell Disruption Methods of Microalgal Strains for Assisted Lipid Extraction

We carried out five different cell disruption methods, namely; homogenization assisted (H-A), microwave assisted (MW-A), ultrasonnication assisted (US-A), bead assisted (B-A) and lyophilization assisted (L-A) and combinations of these together with the three solvent extraction procedures.

2.12.1. Homogenization Assisted (H-A) Method

100 mL of microalgal suspension was homogenized on ice by a homogenizer (Heideoph Diax-900) in tubes with 20 s homogenization time and 5 s interval. We tried three different time points; 5, 10 or 20 min for the total time of homogenization.

2.12.2. Microwave Assisted (MW-A) Method

100 mL of microalgal suspension was placed in a conventional microwave oven (Arcelik intellewave MD-599) and samples were disrupted at 900 W, 20 s microwave power and 5 s interval time for a total duration of 1, 5 or 10 min.

2.12.3. Ultrasonnication (US-A) Method

100 ml of microalgal suspension was extracted on ice via an ultrasonicator (Cole Palmer Ultrasonic Processor CPT) at 400 W, 20 s ultrasonication, 5 s interval time for a total duration of 5, 10 or 20 min.

2.12.4. Glass Bead (B-A) Method

100 ml of microalgal suspension was mixed with glass beads (0.4 mm diameter in size) and bead assisted extraction was subjected to distruption via shaking for 5,10 and 20 min at 1000 rpm rotation speed.

2.12.5. Lyophilization Assisted (L-A) Method

100 mL of microalgal suspension was centrifuged at 3000 g for 10 min at 4 $^{\circ}$C and pellet was lyophilized in a lyophilizator (Christ D-37520 Alpha 1-4 LD plus) for 24 hours. Then, 1 g lyophilized microalgae samples were mixed with 40 ml n-hexane in the Soxhlet apparatus. The solvent was heated to reflux. Oil of microalgae was dissolved in hexane. Pellet was left for 24 hours in Soxhlet apparatus and oil was taken from distillation flask. Remaining hexane was evaporated by rotary evaporator (BUCHI Rotavapor R-200). Microalgae oil was weighed gravimetrically and lipid yield was calculated.

On the other hand, 1 g lyophilized sample was mixed with 40 ml methanol and chloroform (2:1 v/v) as the extraction solvent for Bligh and Dyer method. Then, chloroform and water were added to the mixture. The mixture was shaken for 10 min in a seperating funnel and two phases formed subsequently. After adding water, two phases should be visible. If not, leave for 24 hours. Final ratio was adjusted to 1:1:0.9 v/v for methanol: chloroform: water, respectively. Lower phase includes microalgae lipids. In this study, one part of pellet was mixed with forty parts of (methanol-chloroform-water). Lower phase was seperated from upper phase and evaporated by rotary evaporator (BUCHI Rotavapor R-200). Obtained lipids were weighed gravimetrically.

1 g of lyophilized sample was mixed with 40 ml methanol and chloroform (1:2 v/v) as the extraction solvent for Folch method. Then, methanol and water were added to the mixture. After addition of water to the mixture, final ratio was 1:1:0.9 v/v for methanol, chloroform, and water, respectively. The mixture was shaken for 10 min in a seperating funnel and two phases formed subsequently. Next, lower phase including microalgae lipid was taken and evaporated by rotary evaporator (BUCHI Rotavapor R-200). After obtaining of lipid, microalgae lipids were weighed gravimetrically.

2.13. High Throughput Method for Determination of Microalgal Lipids (Nile Red Method)

Nile red method was used for *Micractinium sp* in acetone solutions according to Elsey method with some modifications (Elsey et al., 2007). Microalgae were examined at 5 different time intervals 5, 10, 15, 30 and 60 min and 6 various nile red concentrations as 0.1, 0.2, 0.5, 1, 2, and 5 µg/mL. Moreover, we studied at 5 different acetone concentrations as 0.1, 0.5, 1, 2, and 5 % and we used 5 various amounts of cells (1, 2, 4, 6, 8 x 10^6 cells/mL). Microalgae samples were taken at deserved cell, acetone, Nile red concentrations and final concentrations were adjusted to 50 µL. After microplates were completed to 50 µL, the 96-well plates were vortexed at 120 rpm and incubated at different time intervals for minute at 40 °C. Fluorescences were measured at excitation wavelength of 530 nm and emission wavelenght of 600 nm with Spectra Max Paradigm (Multi –Mode Detection Platform) and results were analyzed with Soft Max Pro 6.3.

2.14. Transformation of DNA in *Chlamydomonas* Nuclear Genome by Electroporation

2.14.1. Preparation of Luria Bertani (LB) Medium

10 g of triptone, 5 g yeast extract and 10 g sodium chloride were taken and dissolved in 800 mL of dH_2O. Then, volume was completed to 1 L with dH_2O and pH was adjusted to pH:7. For agar plate, added to 15 g of agar in the medium and autoclaved for 20 min at 121 °C.

2.14.2. Purification of pHyg3

Firstly, pHyg3 in *Escherichia coli* was taken from LB agar and grown in LB medium for 16 hours at 37 °C. Then, cells were centrifuged at 10000 rpm for 3 min at 4 °C. Pellet was taken and isolation of pHyg3 was carried out by plasmid DNA purification using the Qiaprep spin miniprep kit (Qiagen). Schematic presentation of pHyg3 was given in Appendix J.

2.14.3. *Hind III* Digestion of DNA

DNA was cutted with *Hind III* according to Fermentas *Hind III* digestion procedure and sample was runned on 1 % agarose gel to obtain deserved fragment with pHyg3.

2.14.4. Gel Extraction Procedure

Cleaning of DNA from agarose gel was applied by gel extraction procedure. We obtained fragment according to Nucleospin gel and PCR clean-up kit. Then, this DNA fragments was used for electroporation.

2.14.5. Transformation of *Chlamydomonas reinhardtii* 137^- by Electroporation

It was measured the optical density of *Chlamydomonas reinhardtii* 137^- at OD_{750} nm. Reaching absorbance to 0.5, it was centrifuged at 800 g for 5 min at 4 ^oC. Number of the cells was arranged to 3 x 10^7 cells / mL. Next, cells were resuspended in 500 µL of Tris-Acetate-Phosphate (TAP) buffer- 40 mM Sucrose solution at 4 ^oC and different concentrations (12, 72, 200, 660, 1200, 2000 ng) of pHyg3 DNA was added to mixture at 4 ^oC. Then, cells were electroporated in 4 cm of electroporation cuvette at various voltages (0.6, 1.8, 1.9 and 2.2 V) and capacities (10 and 50 µF). After electroporation, 1 mL of TAP medium was added to electroporated cells at at 4 ^oC and waited for 5 min and cells were incubated in water bath for 10 min at 25 ^oC. After incubation, cells were centrifuged at 800 g for 5 min at 4 ^oC. Supernatant was discarded and pellet was taken. Then, pellet was dissolved in 750 µL of Tris-Acetate-Phosphate (TAP) buffer. Finally, 100 µL and 300 µL of cell solutions were spread to prepared LB agar plates with hygromycin or not and agar plates were placed at growth chamber at 25 ^oC at 54 µE m^{-2} s^{-1}.

2.15. Transesterication and Characterization of Extracted Oils

Transesterication of extracted oils was carried out in methanol including 0.1 N KOH as catalyst and stirred for 3 hours at 60 ^oC. Mixture was kept for seperation of phases

at room temprature for 18 hours. The biodiesel was seperated by flask seperator. Then, biodiesel was washed by 5 % dH_2O. Fatty acid methyl esters of algae oils were detected with Gas chromatography (GC) after adding standarts including between 12 and 24 carbon atoms with double and tree bonds. Microalgae fatty acid methyl esters were analyzed by Agilent HP GC 6890 on a polar capillary column HP-88 (100 m, 0.25mm, 0.2 μm) using split sample injection mode (split ratio 1:100). The column was operated at 250 ^{o}C and 34.17 psi (split flow: 207.1 mL/min, total flow: 215.9 mL/min). Fatty acid methyl ester contents were detected by comparison of their peak areas with those of standards.

2.16. Statistical calculations

All of results are expressed as mean ± standard error (SE) and levels of significance were determined by regression analysis of variance using SPSS (IBM Corp.) and plotted. Multiple comparisons of concentration levels, time, techniques, cell numbers and their interactions were carried out General Linear Model, Univariate (Two-way Anova), Post-Hoc tests using Tukey, Bonferroni and Sidak. Statical tests were considered significantly at $p<0.05$. Univariate analyses of traits (concentration levels, time, techniques, cell numbers) were performed using General Linear Model in the program of SPSS (IBM Corp.).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Identification and Characterization of Microalgae

After processing of serial dilution techniques and streaking cells across agar plates to propagate homogenous clones genetically, six unialgal colonies were determined. Then, colonies were examined under the light microscope and named as METUNERGY1401 (*ME01*) to METUNERGY1406 (*ME6*). With light microscopy, microalgae isolates could not be characterized precisely. At the first characterization step, all of the isolates belonged to the division of Chlorophyta. Next, detailed characterization of these isolates was studied. Scanning electron microscopy **(**SEM) for further morphological characterization and PCR amplification of the gene internal transcribed sequence 2 (ITS2) region for molecular characterization of each algal species were performed.

3.2. Scanning Electron Microscopy (SEM) Analysis

Newly isolated microalgae were analyzed by SEM to characterize morphologically. (Kaur et al., 2012). We determined the key morphological characteristics of the microalgal isolates via SEM. Based on the general morphological features, similarities and differences between the six isolates, we divided them in three groups: METUNERGY1401, METUNERGY1403, METUNERGY1404 and METUNERGY1406 showed similar morphological features, thus comprised one group. METUNERGY1402 generated another group and METUNERGY110905 was classified in the other group because these isolates were different each other. Morphological identification of microalgae isolates was given in Figure 3.1. All three groups included unicellular alga without any flagella. Group one had a mixture of spherical and ellipsoid cells with a diameter of 2.5-3 μm. They were shown in Figure 3.1, A-D. The outer surface of group one had furrows and they are not smooth.

Another group, METUNERGY1402, had elliptical cells of approximately 3 μm in diameter. The cell surface was smoother compared to the first group. The most important distinctive property of METUNERGY1402 was the longitudinal rib-like structure on its outer surface. This was shown in Figure 3.1E. This structure can be presented at species of the *Scenedesmus* and *Desmedesmus* genus (An et al., 1999). *Desmedesmus* can be discriminated by submicroscopic properties on its cell wall outer membrane. *Scenedesmus* doesn't have this layer (An et al., 1999; Kaur et al., 2012). METUNERGY1405 doesn't have uniform structure. They were displayed in Figure 3. 1F. METUNERGY1405 had spherical and irregular shape. It consists of one or more dents on its surface area. The diameter of METUNERGY1405 cells was 2-3 μm (Onay et al., 2014).

3.3. Molecular Characterization of Isolated microalgae

Microalgae can not be distinguished correctly with just scanning electron microscopy (SEM). Internal properties of microalgae can not be observed with SEM. Therefore, microalgae have to be examined with molecular charecterization. We carried out molecular characterization of microalgae isolates by PCR-amplification and sequencing of the ITS2 rDNA region.

Detection of the ITS2 rDNA region of the isolates showed that METUNERGY1401, METUNERGY1403, METUNERGY1404, and METUNERGY1406 had the exact same bp length (265bp) for ITS2 sequence. These results were supported with the morphological similarities of these four isolates found by SEM (Figure 3.1) (Onay et al., 2014).

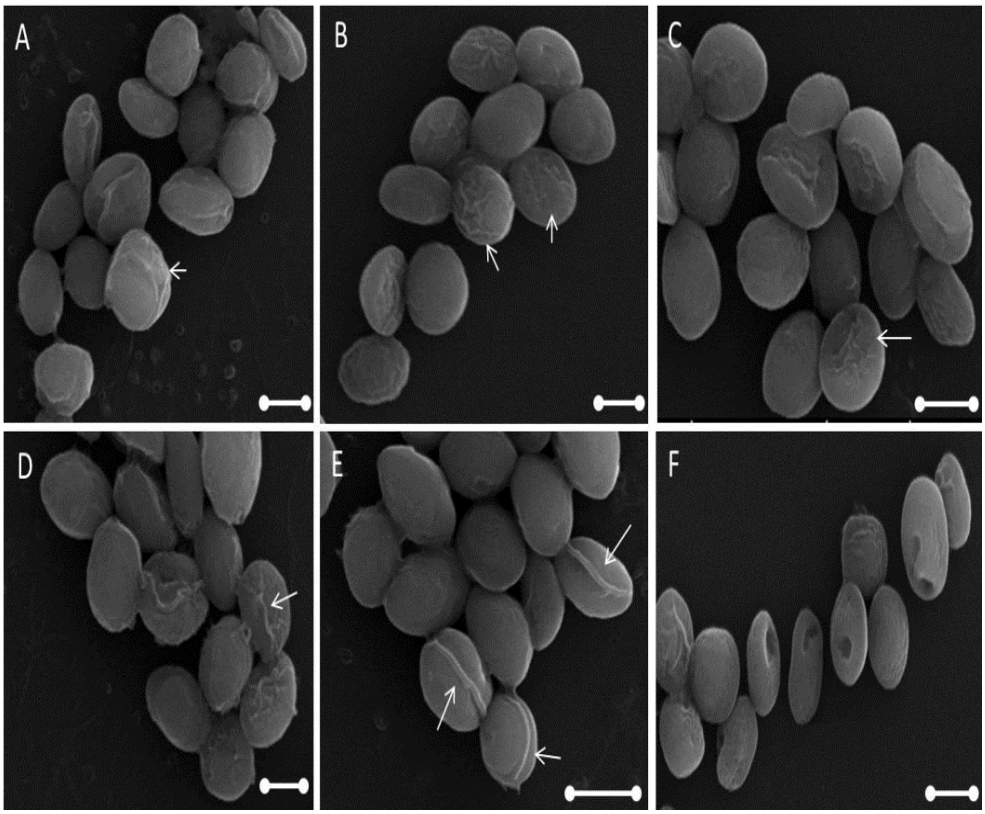

Figure 3.1. Scanning electron micrograph (SEM) images of A) METUNERGY1401, B) METUNERGY1403, C) METUNERGY1404, D) METUNERGY1406, E) METUNERGY1402 and F) METUNERGY1405. The scale bar is 2 μm. The arrows in A-D point to the furrows on the surface of the cells, and rib-like structures on the cell wall in E, (Onay et al., 2014).

ITS2 sequence lengths of METUNERGY1402 and METUNERGY1405 were found with 246 and 242 bp, respectively. Phylogenetic tree plots of isolated microalgae based on compensatory base change (CBC) matrices are shown in Figures 3.2-4. To the phylogenetic analysis, METUNERGY1401, METUNERGY1403, METUNERGY1404, and METUNERGY1406 belong to the *Hindakia tetrachotoma* species (Figure 3.2) (Onay et al., 2014). This species was originally described by Bock et al. (2010) as one of the most common species with the *Dictyosphaerium* morphotype within the *Chlorellaceae*. Morphologically, *Hindakia tetrachotoma* resembles the description of *Dictyosphaerium tetrachotomum*, characterized by its oval cells and connection of the young cells to the gelatinous stalks of the remnants

of the mother cell at their narrow ends. We performed our SEM experiments mainly with adult cells at stationary phase; therefore the branching morphology was not observed in our isolates. Additionally, the growth and culture conditions of the cells in this study and that of Bock et al.'s are quite different (i.e. growth temperature, medium and light/dark cycle). Hence, as previously reported, the typical morhology of cells in this study may show great diversity under different conditions (Komárek and Perman, 1978; Krienitz et al., 2010) (Onay et al., 2014).

Next, METUNERGY1402 was classified as a member of genus *Scenedesmus* (Figure 3.3). This genus usually contains 2, 4 or 8 elliptical to spindle-shaped cells in one or three rows, although unicellular strains have also been reported (An et al., 1999; Kaur et al., 2012). Although morphologically well defined, *Scenedesmus* can show great phenotypic plasticity depending on different growth and culture conditions and geographical origin (Trainor et al., 1998). Phylogenetic tree based on CBC matrix shows that METUNERGY1402 is closely related to a number of different *Scenedesmus* species such as *S. acutus*, *S. obliquus*, *S. deserticola*, *S. dimorphus* and *S. naegelii*. We found no CBCs between METUNERGY1402 and any of these species. Therefore, although METUNERGY1402 isolate was determined successfully at the genus level, it was reasoned as improper to assign any particular species name to this isolate (Onay et al., 2014).

Finally, phylogenetic analysis of METUNERGY1405 was performed via ITS2 structure and CBC matrix calculation. According to the phylogenetic tree, METUNERGY1405 is closely related to *Micractinium sp.* CCAP-211/92 and various *Chlorella species* (Figure 3.4). There were two CBCs between *Micractinium pusillum* ITS2 structure and sequences and METUNERGY1405 in Helices I and VI and no CBCs with *Chlorella vulgaris*, *Chlorella sorokiniana* and most *Micractinium sp.* structure and sequences. It is very difficult to morphologically distinguish *Micractinium* and *Chlorella* due to high phenotypic plasticity (Luo et al., 2006). Similar to other genera mentioned above, *Micractinium* can also be highly influenced by the environmental conditions (Luo et al., 2006). Therefore, phylogenetic analysis

based on ITS2 region is a more reliable method in differentiation of isolates at genus and species level (Onay et al., 2014).

Based on these results, our native isolates were named as follows: *Hindakia tetrachotoma* METUNERGY1401, *Scenedesmussp.* METUNERGY1402, *Hindakia tetrachotoma* METUNERGY1403, *Hindakia tetrachotoma* METUNERGY1404, *Micractinium sp.* METUNERGY1405, and *Hindakia tetrachotoma* METUNERGY1406. For ease of further analysis, we continued our experiments with the following isolates: *Scenedesmussp.* METUNERGY1402, *H. Tetrachotoma* METUNERGY1403 and *Micractinium sp.* METUNERGY1405 (Onay et al., 2014).

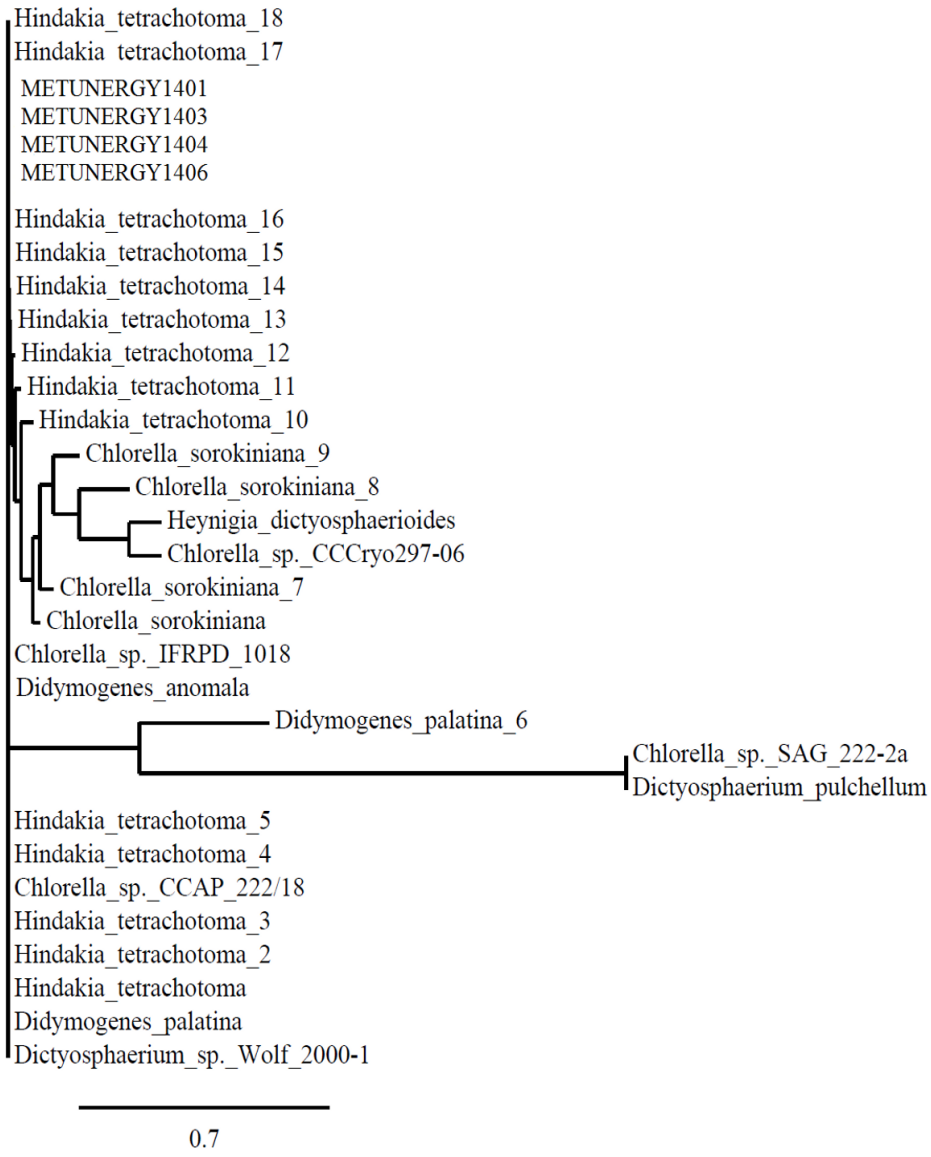

Figure 3.2. Phylogenetic tree plot of METUNERGY1401, METUNERGY1403, METUNERGY1404 and METUNERGY1406 based on CBC matrix, (Onay et al., 2014).

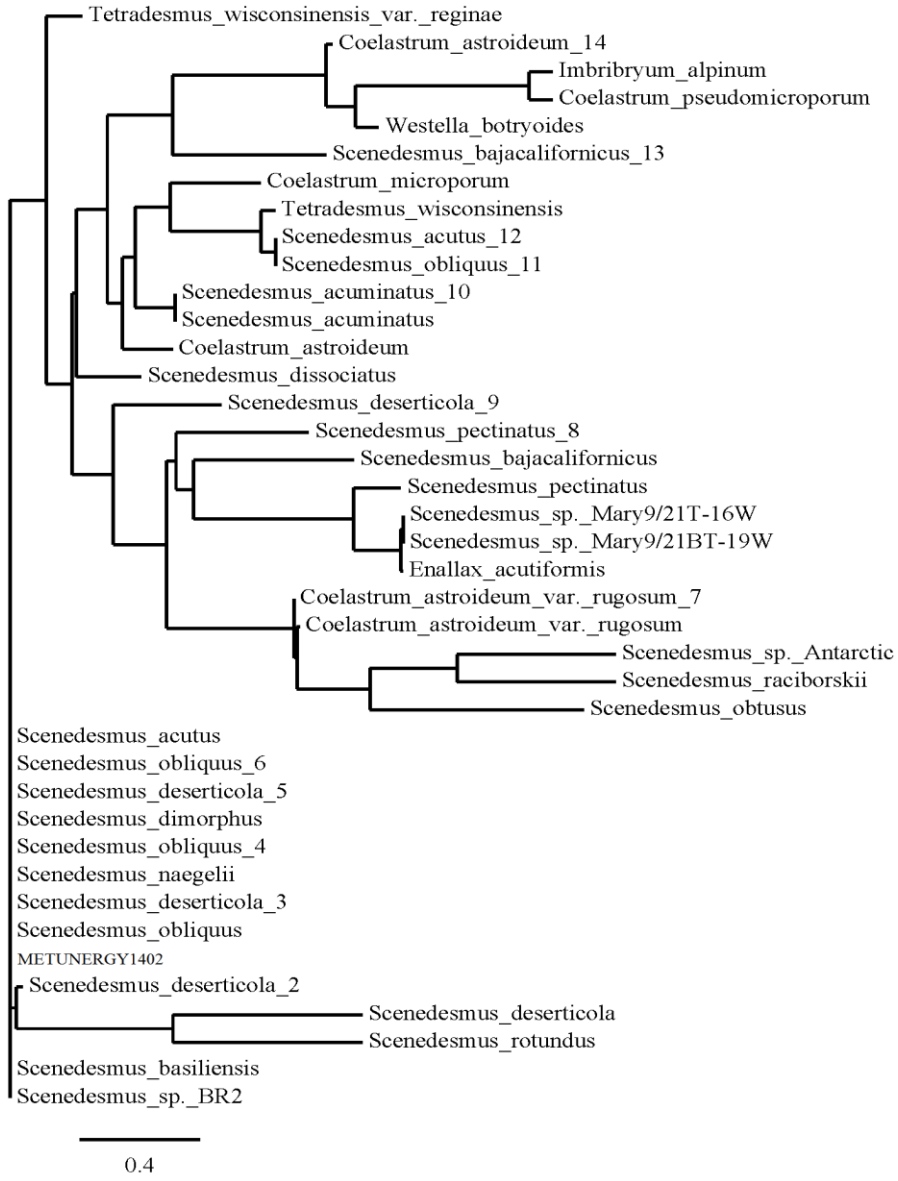

Figure 3.3. Phylogenetic tree plot of METUNERGY1402 based on CBC matrix, (Onay et al., 2014).

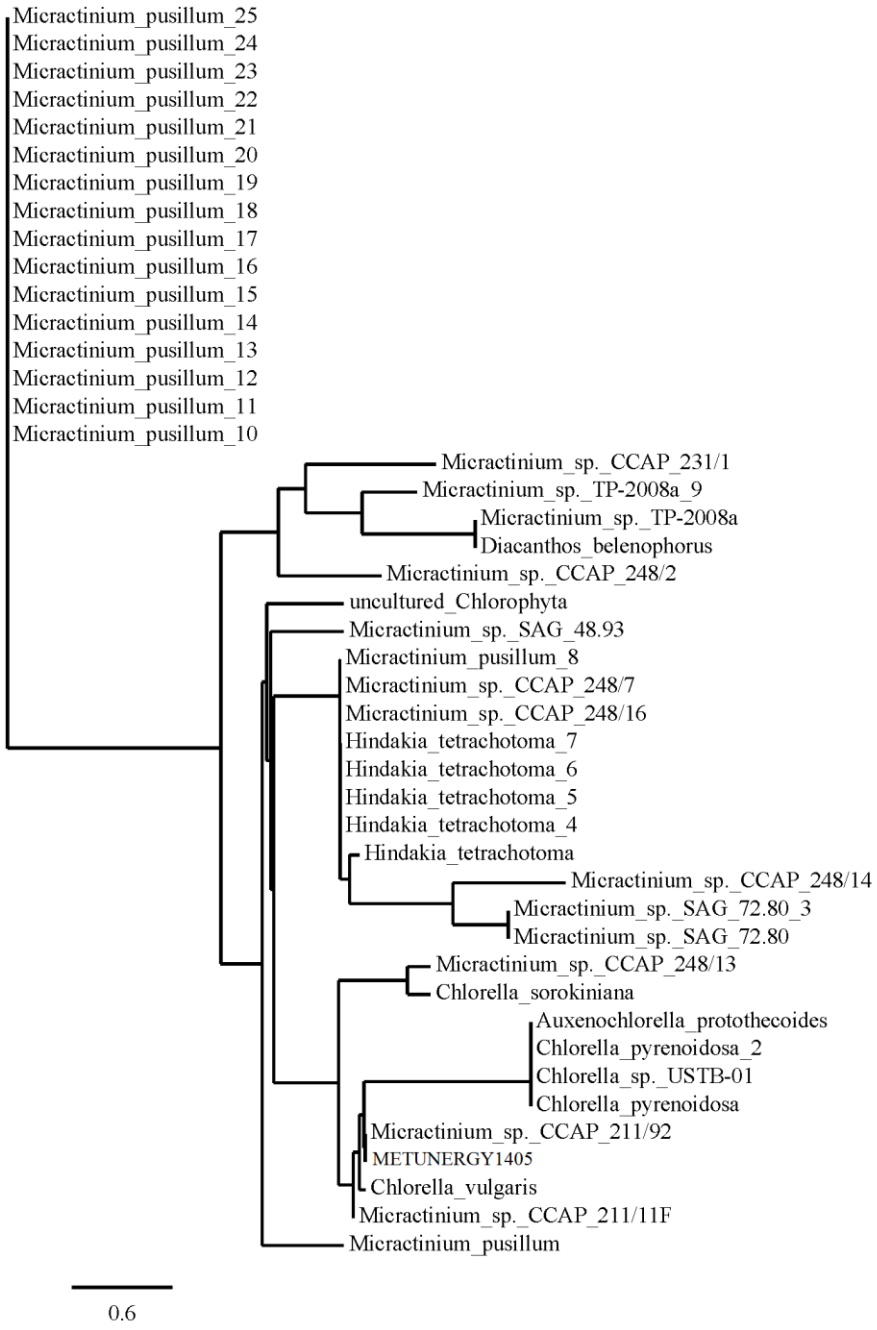

Figure 3.4. Phylogenetic tree plot of METUNERGY1405 based on CBC matrix, (Onay et al., 2014).

ITS2 secondary structure prediction of the consensus sequences of the isolates was carried out with the prediction algorithm implemented in the ITS2 database with the parameters of E < 1e -16 and minimum helical transfer of 75% and ITS2 secondary structure prediction of isolates (*ME01-ME06*) were shown in Figure 3.5, 3.6 and 3.7.

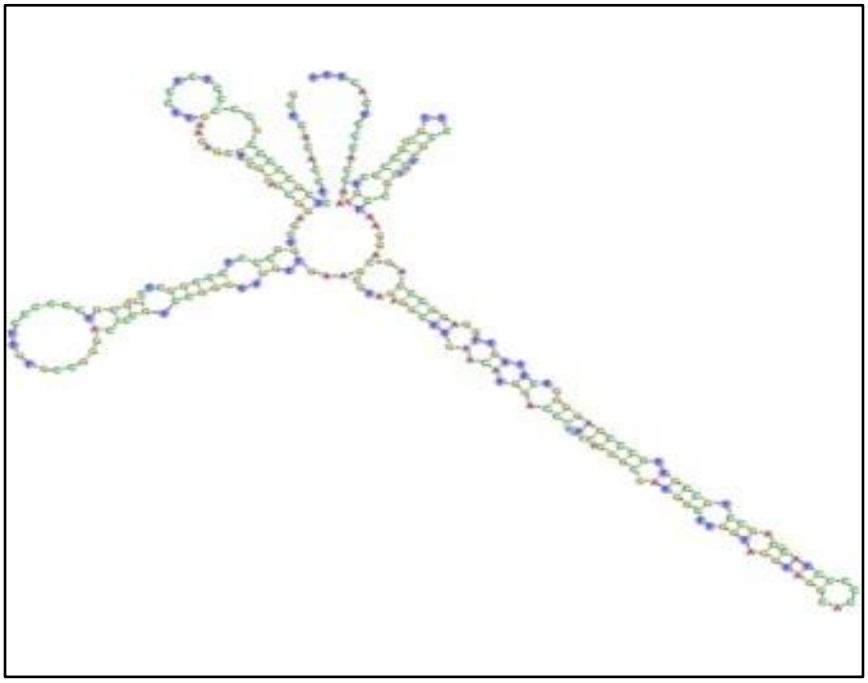

Figure 3.5. ITS2 secondary structure prediction of *ME01*. The green/grey structure shows the BLAST identified model for *ME01* where the green dots indicate homologue sequence and structure positions. *ME01* was homology modeled by Didymogenes palatina (GI: 37674531), (Onay et al., 2014).

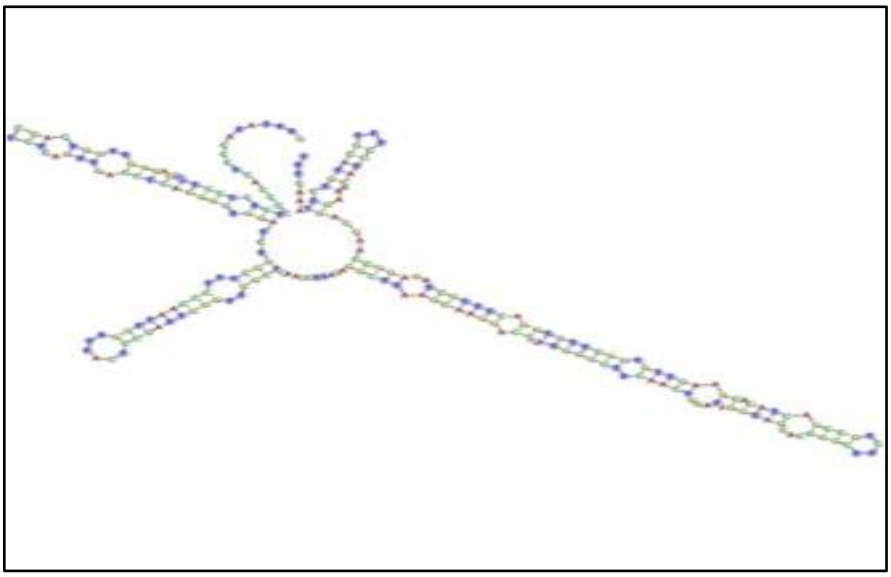

Figure 3.6. ITS2 secondary structure prediction of *ME02*. The green/grey structure shows the BLAST identified model for *ME02* where the green dots indicate homologue sequence and structure positions. *ME02* was homology modeled by Scenedesmus sp. BR2 (GI: 190151815), (Onay et al., 2014).

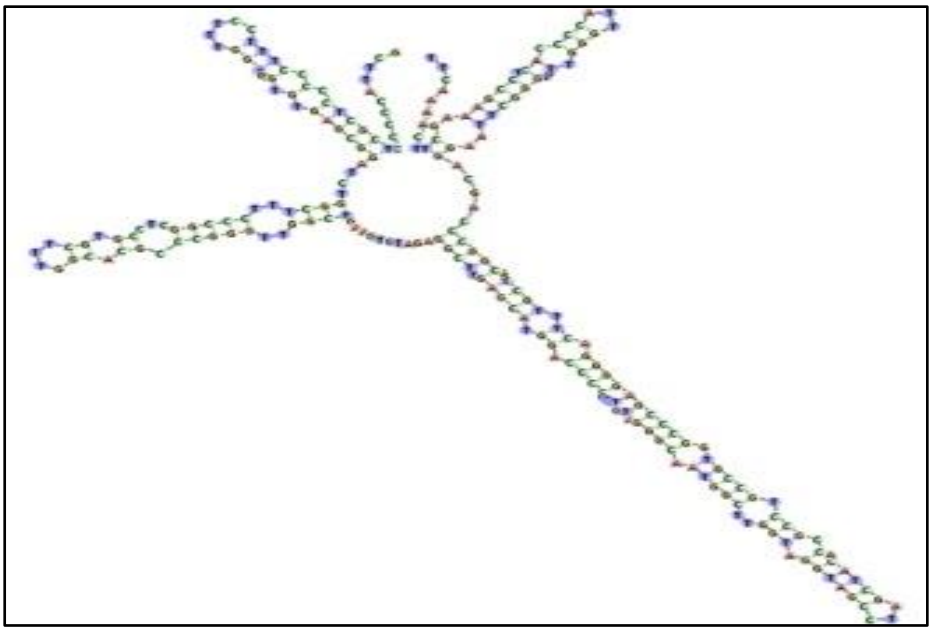

Figure 3.7. ITS2 secondary structure prediction of *ME05*. The green/grey structure shows the BLAST identified model for *ME05* where the green dots indicate homologue sequence and structure positions. *ME05* was homology modeled by Micractinium sp. CCAP-211/92(GI: 207366659), (Onay et al., 2014). Names and abbreviation of isolates was given in Table 3.1.

Table 3.1. Gene bank names, species and abbreviated names of microalgae isolates

Gene Bank Names	Species	Abbreviated Names
METUNERGY1401	*H. tetrachotoma*	*ME01*
METUNERGY1402	*Scenedesmus sp.*	*ME02*
METUNERGY1403	*H. tetrachotoma*	*ME03*
METUNERGY1404	*H. tetrachotoma*	*ME04*
METUNERGY1405	*Micractinium sp*.	*ME05*
METUNERGY1406	*H. tetrachotoma*	*ME06*

3.4. Growth Evaluation of Microalgae

Growth curves of *Scenedesmus sp*., *H. tetrachotoma* and *Micractinium sp.* were examined at different temperatures (4 ^{0}C, 25 ^{0}C, 37 ^{0}C and 50 ^{0}C) The optimum growth conditions of microalgal isolates were determined as 4 klux of light intensity, pH 7.4 and salt concentration of 1.5 x 10^{-2} M. Isolated microalgae were subcultivated on 1.5 % agar plates. *Scenedesmus sp*., *H. tetrachotoma* and *Micractinium sp*. on agar plates were shown in Figure 3.8.

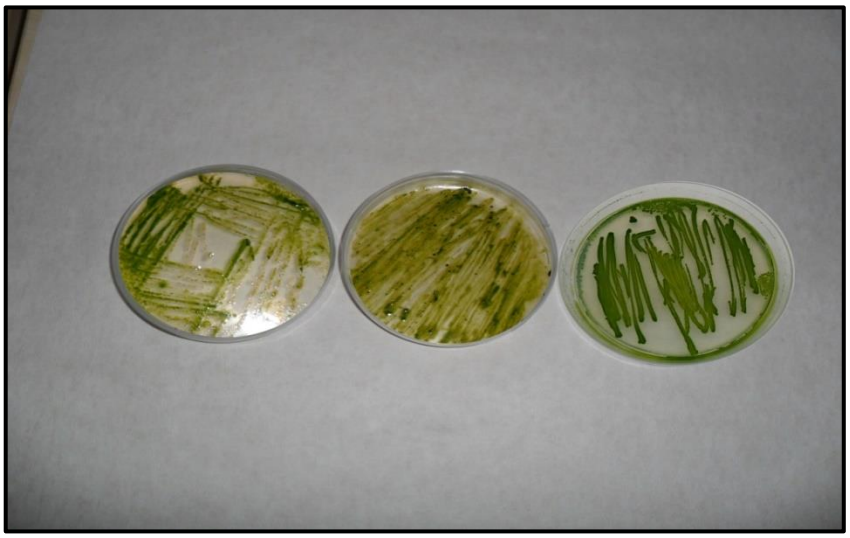

Figure 3.8. Subcultivated Scenedesmus sp., H. tetrachotoma and Micractinium sp. on agar plates.

3.5. Comparasion of Specific Growth Rates and Biomass Productivities of Microalgae

3.5.1. Growth Curves of *Scenedesmus sp. H. tetrachotoma* and *Micractinium sp.* at 4 ^{0}C

To observe growth curves of isolated microalgae at cold temparetures, microalgae were grown at 4°C. However, Scenedesmus sp., *H. Tetrachotoma* and *Micractinium sp.* did not show any meaningful data. They could not reach stationary phase and show specific growth curve. As a result, we found that *ME02*, *ME03*, and *ME05* were not suitable for growth at cold stress conditions at 4 °C.

3.5.2. Growth Curves of *Scenedesmus sp. ME02*, at 25 °C, 37 °Cand 50 °C

Growth curves of *Scenedesmus sp. ME02* were examined at 25 °C, 37 °C and 50 °C. At 25 °C and *Scenedesmus sp. ME02* was reached to statioanary phase for nearly 10 days. On the other hand, at the 37 °C and 50 °C, it showed slower growth than that of 25 °C although reaching to staionary phase at 4 days at 680 nm. Growth curves of *Scenedesmus sp. ME02* at 25 °C, 37 °C and 50 °C were given in Figure 3.9.

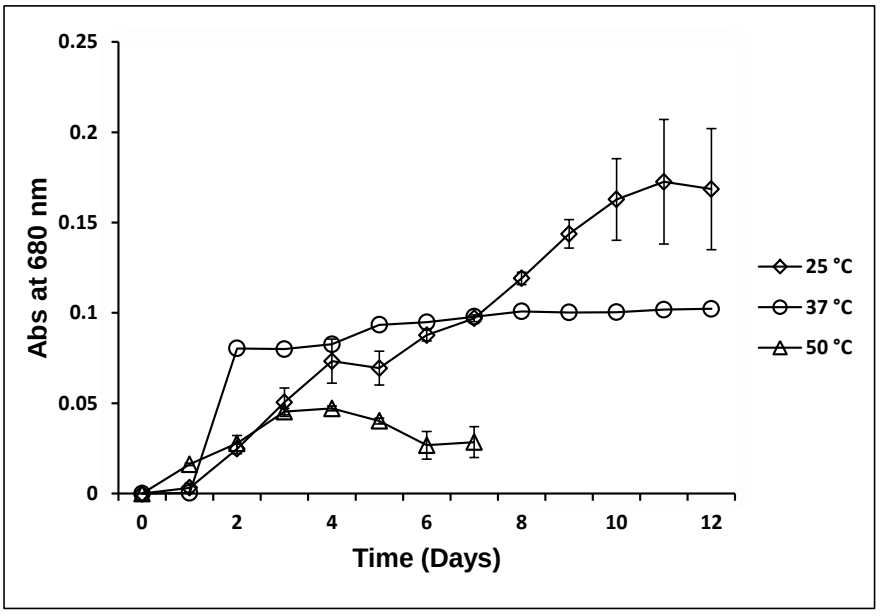

Figure 3.9. Growth curves of Scenedesmus sp. *ME02* at 25°C, 37°C and 50°C.

3.5.3. Growth Curves of *H. tetrachotoma ME03* at 25 °C, 37 °C and 50 °C.

Growth curves of *H. tetrachotoma ME03* were similar to Scenedesmus sp. *ME02*. It finalized growth at the end of the 8 days at 25 °C. At the other temperature values, it didnt show a remarkable growth and reached to stationary phase at the 7 days at 680 nm. Growth curves of H. tetrachotoma *ME03* at 25 °C, 37 °C and 50 °C were given in Figure 3.10.

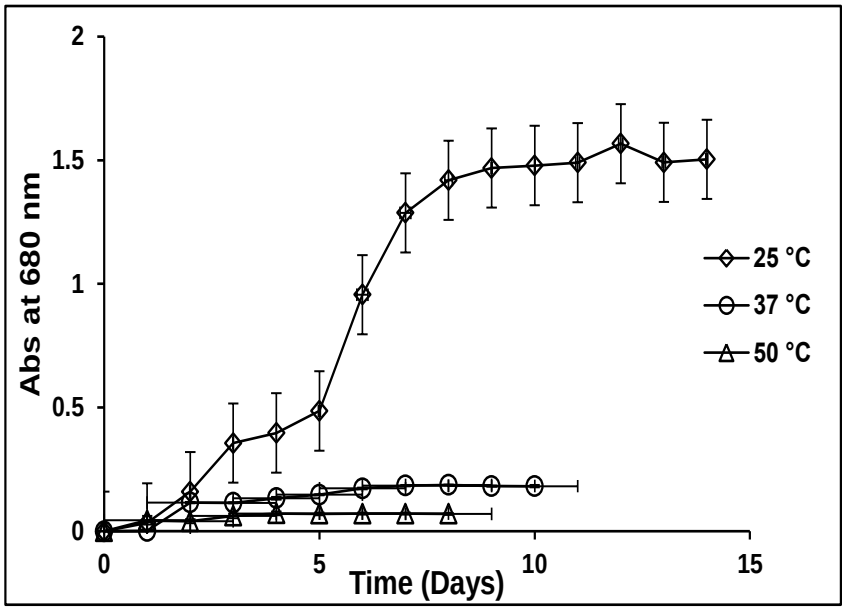

Figure 3.10. Growth curves of *H. tetrachotoma ME03* at 25 °C, 37 °C and 50 °C.

3.5.4. Growth Curves of *Micractinium sp. ME05* at 25 °C, 37 °C and 50 °C

On the other hand, *Micractinium sp. ME05* had different growth curves in the TAP medium. While it revealed the maximum growth rate at 2 days at 37 °C. At 25 °C, *Micractinium sp. ME05* showed high growth and reached to stationary phase at the 4.5 days and it had lower growth at athe 5 days at the 50 ° C at 680 nm. Growth curves of Micractinium sp. *ME05* at 25 °C, 37 °C and 50 °C were shown in Figure 3.11.

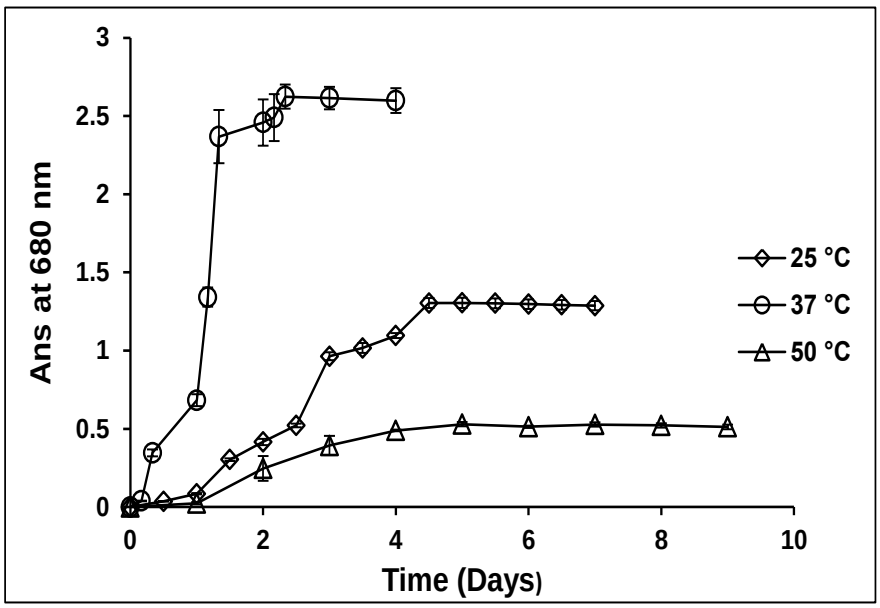

Figure 3.11. Growth curves of *Micractinium sp. ME05* at 25 °C, 37 °C and 50 °C.

3.5.5. Dry Cell Weights of *Scenedesmus sp. ME02* at 25 °C, 37 °C and 50 °C

Biomass concentration of *Scenedesmus sp. ME02* was found as 0.39±0.01 $g.L^{-1}$ at 25 °C while it showed dry weight with 0.12±0.01 $g.L^{-1}$ at the 37 °C and 0.11±0.01 $g.L^{-1}$ at 50 °C. These results displayed that Scenedesmus sp. *ME02* had much more biomass concentration at 25 °C than those at 37 °C and 50 °C. Dry weights of Scenedesmus sp. *ME02* at 25 °C, 37 °C and 50 °C were displayed in Figure 3.12.

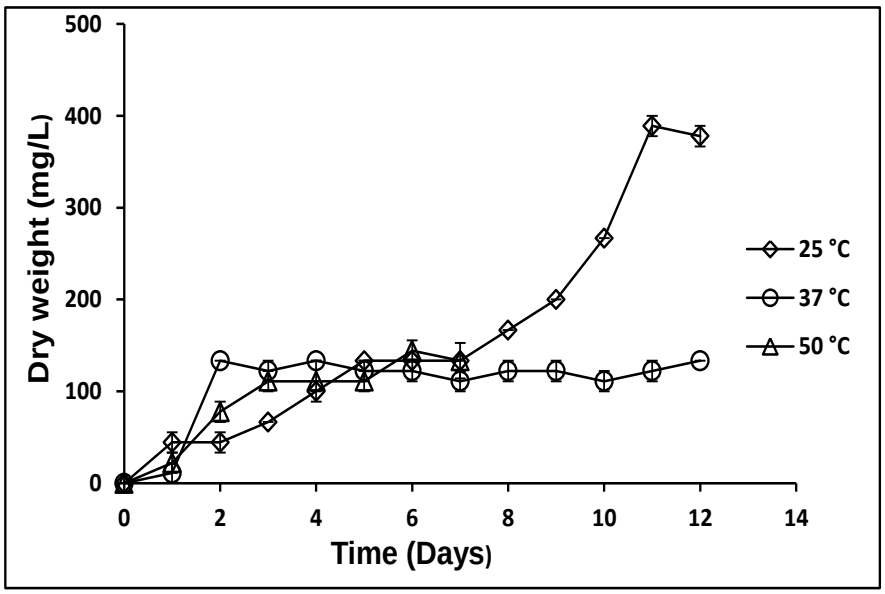

Figure 3.12. Dry cell weights of Scenedesmus sp. *ME02* at 25 °C, 37 °C and 50 °C.

3.5.6. Dry Cell Weights of *H. tetrachotoma ME03* at 25 °C, 37 °C and 50 °C

H. Tetrachotoma ME03 showed the highest biomass concentration with 0.94±0.05 $g.L^{-1}$ at 25 °C. It exhibited lower biomass concentration at 37 °C and 50 °C compared to its biomass concentration at 25°C. Dry cell weights of *H. tetrachotoma* ME03 at 25 °C, 37 °C and 50 °C were shown in Figure 3.13.

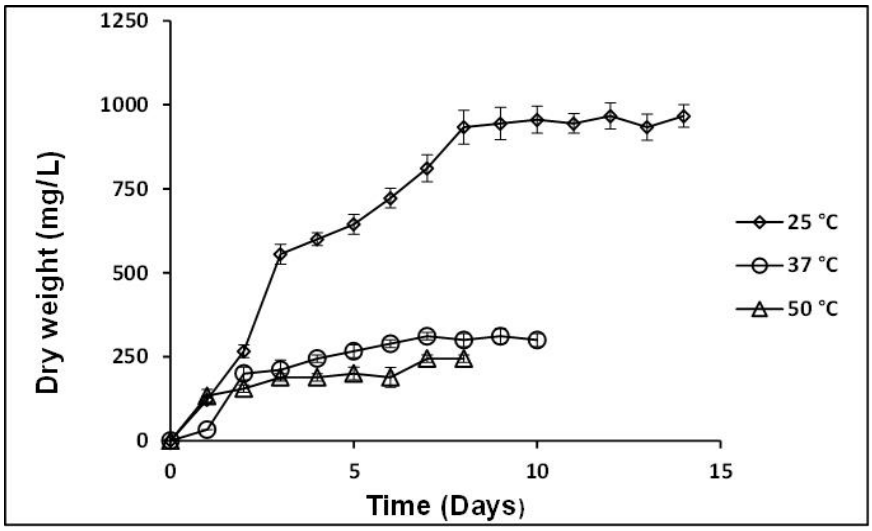

Figure 3.13. Dry cell weights of *H. tetrachotoma ME03* at 25 °C, 37 °C and 50 °C.

3.5.7. Dry Cell Weight Curves of *Micractinium sp. ME05* at 25 °C, 37 °C and 50 °C

Micractinium sp ME05 showed different biomass concentration than those of other. It had the highest biomass concentration at 37 °C. *Micractinium sp. ME05* had 0.76±0.03 $g.L^{-1}$, 0.93±0.04 $g.L^{-1}$ and , 0.14±0.01 $g.L^{-1}$ biomass concentration at 25 °C, 37 °C and 50 °C . Dry cell weights of Micractinium sp *ME05* at 25 °C, 37 °C and 50 °C were given in Figure 3.14.

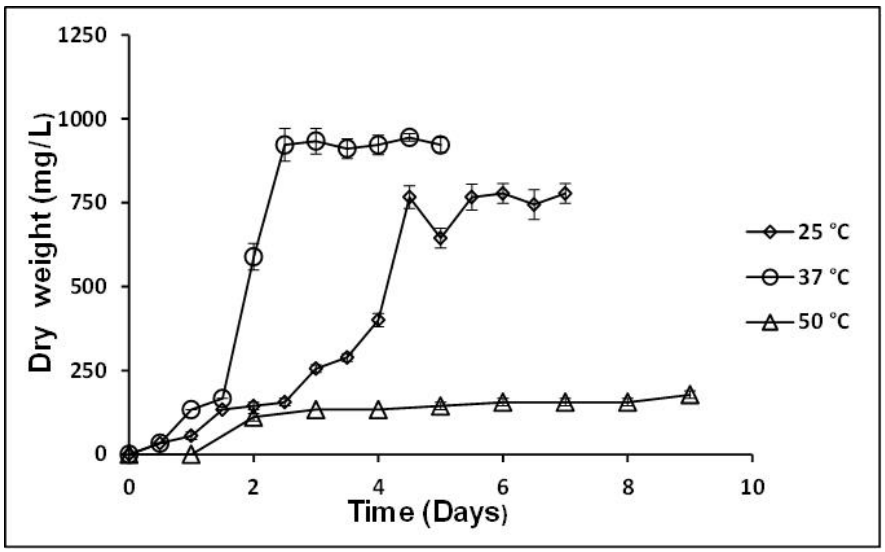

Figure 3.14. Dry cell weights of *Micractinium sp ME05* at 25 °C, 37 °C and 50 °C.

Microalgae have different behaviours under different cultures and growth conditions. We examined growth curves of *Scenedesmus sp. ME02*, *H. tetrachotoma ME03* and *Micractinium sp. ME05* at 25°C, 37°C and 50°C at 680 nm and dry cell weights were given as mg/L versus days of growth (Figure 3.15). In addition to this, growth rates, biomass concentrations and biomass productivities were determined at the 25°C, 37°C and 50°C. The results were shown in Table 3.2.

At three different temperatures, *Micractinium sp. ME05* had the highest growth, reaching stationary phase at four and a half days, two days and five days at 25°C, 37°C and 50°C, respectively (Figure 3.15). *H. tetrachotoma ME03* and *Scenedesmussp. ME02* grew more slowly compared to *Micractinium sp. ME05* reaching stationary phase at nine and eleven days, respectively at 25°C. Micractinium sp. *ME05* had the highest growth rate (5.2±0.04 at 25°C and 6.2±0.04at 37°C) among three isolated microalgae (Table 3.2).

Despite the slower growth rate (4.6±0.06), H. tetrachotoma *ME03* had higher biomass accumulation (0.94±0.05 g.L-1) than Micractinium sp. *ME05* (0.76±0.03 g.L-1) at 25°C.Scenedesmus sp. *ME02* had similar growth rates (3.5±0.03, 4.0±0.23 and 3.1±0.13) at 25°C, 37°C and 50°C, respectively. When the biomass productivities of each isolate are compared as a factor of gram dry weight per litre per day, Micractinium sp. *ME05* had the most the highest biomass productivity (0.47±0.02 g.L-1.day-1) at 37°C; nearly ten times higher than that of H. tetrachotoma *ME03* and Scenedesmussp. *ME02* at the same temperature.

Despite the higher biomass concentration at 25°C, H. tetrachotoma *ME03* still had lower biomass productivity (0.1±0.005g.L-1.day-1) compared to Micractinium sp. *ME05* (0.17±0.007g.L-1.day-1) at the same temperature. Taken together, in terms of growth rate, biomass accumulation and productivity, of the three different growth temperatures tested, the optimum growth temperature for Hindakia tetrachotoma *ME03* is 25°C whereas Micractinium sp. *ME05* exhibits highest growth rate and biomass productivity at 37°C.As for Scenedesmussp. *ME02*, the optimum growth temperature varies depending on the specific characteristic desired; this isolate

exhibits the highest growth rate at 37°C and the highest biomass concentration at 25°C (Onay et al., 2014).

Table: 3.2. Maximum growth rates, biomass concentrations and biomass productivities of *Micractinium sp. ME05 Scenedesmus sp. ME02 and Hindakia tetrachotoma ME03* at 25, 37 and 50°C, (Onay et al, 2014)

Temperature	Species	Growth Rate (μ) (d^{-1})	Biomass Concentration ($g.L^{-1}$)	Biomass productivity ($g.L^{-1}.day^{-1}$)
25 ^{0}C	*Micractinium sp.ME05*	5.2±0.04	0.76±0.03	0.17±0.007
	H. tetrachotoma ME03	4.6±0.06	0.94±0.05	0.1±0.005
	Scenedesmus sp. ME02	3.5±0.03	0.39±0.01	0.04±0.001
37 ^{0}C	*Micractinium sp ME05*	6.2±0.04	0.93±0.04	0.47±0.02
	H. tetrachotoma ME03	3.2±0.17	0.31±0.01	0.04±0.001
	Scenedesmus sp. ME02	4.0±0.23	0.12±0.01	0.06±0.003
50 ^{0}C	*Micractinium sp.ME05*	2.4±0.01	0.14±0.01	0.03±0.003
	H. tetrachotoma ME03	2.9±0.23	0.24±0.01	0.03±0.002
	Scenedesmus sp. ME02	3.1±0.13	0.11±0.01	0.02±0.002

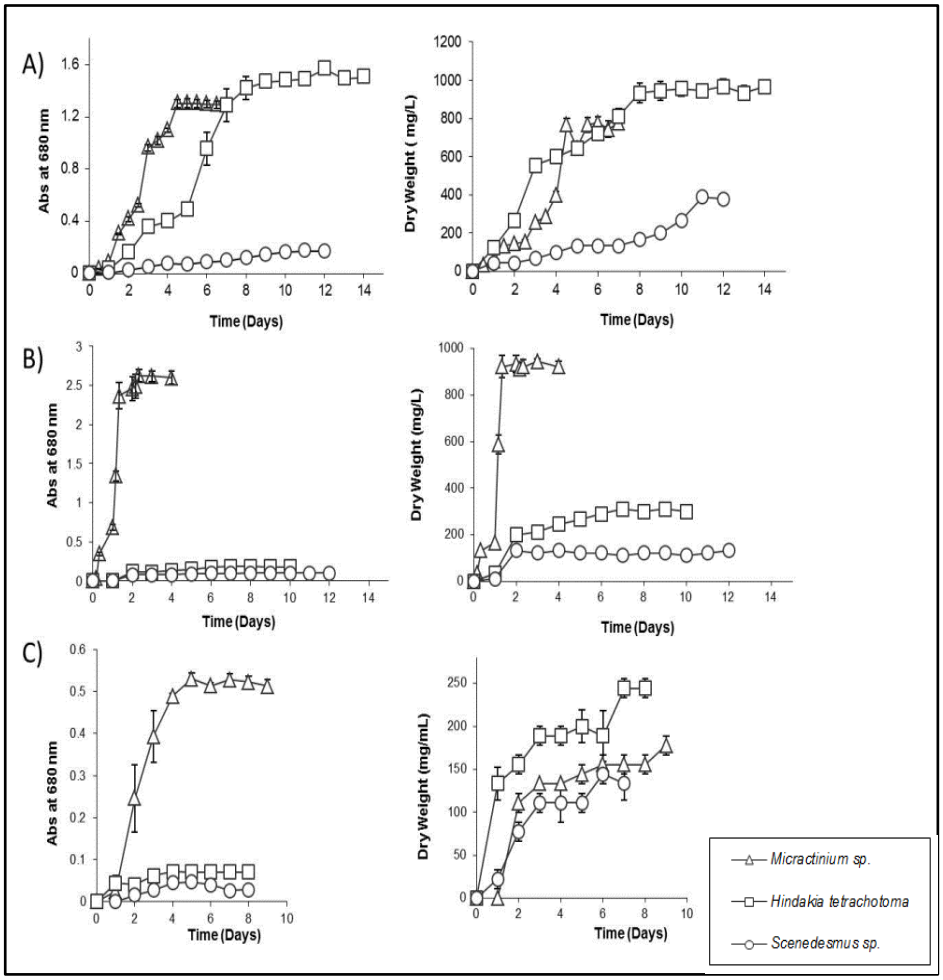

Figure 3.15. Growth curves of microalgal isolates as a function of either absorbance values at 680 nm or dry weight (mg/L) *vs* time (days) at A) 25°C, B) 37°C, C) 50°C. *Micractinium sp. ME05*, *H. tetrachotoma ME03*, *Scenedesmus sp. ME02* are represented by triangle, square and circle, respectively. Error bars are calculated by standard error, (Onay, 2014). In a recent study, Smith-Bädorf et al. (2013) tested the growth rates of geothermal strains of algae isolates from the Roman Baths at various temperatures (i.e. 20°C, 30°C and 40°C) and found that eukaryotic algae grew better lower temperatures at 20°C and didnt grow at 40°C. (Smith-Bädorf et al., 2013). In our study, although much slower, all of our isolates still showed growth at 50°C and similar to Smith-Bädorf et al.'s study, all microalgal isolates grew faster at temperatures lower than that of the hotspring water temperature (Onay et al., 2014).

3.6. Chlorophyll a content of microalgae

Chlorophyll contents of microalgae were calculated by tricromatic equations of Jeffrey and Humphrey (1975). *Micractinium sp. ME05* (37 ^{0}C) and (25 ^{0}C) revealed the highest chlorophyll content with 114±12.6 and 112 ± 8.2 µg/mL , respectively, among microalgae species. On the other hand, *Hindakia tetrachotoma ME03* and *Scenedesmus sp. ME02* represented amounts of lower chlorophyll a content with 77 ± 6.9 and 57 ± 8.7 µg/mL, respectively.

3.7. Carbohydrate concentration of microalgae

Carbohydrate concentrations of species were examined spectrophotometrically by anthrone method (Ludwig, 1956) with a few modifications (Gerhardt et al., 1994 and Zahao et al., 2013). Glucose was used as a standard with different concentrations (10, 40, 60, 100, 150, 200 µg/ mL). The equation was expressed as y= 0.0046x – 0.0135 (R^2 = 0.9986 P < 0.05). We found the highest carbohydrate concentration in *Scenedesmus sp. ME02* with 17.84 ± 1.6 % among isolated microalgae species. *Hindakia tetrachotoma ME03* and *Micractinium sp. ME05* (25 ^{0}C) and *Micractinium sp.* ME05 (37 ^{0}C) showed lower carbohydrate percentage amount with 14.5 ± 1.1, 11.11 ± 0.8 and 15.57 ± 1.9 % respectively.

3.8. Protein concentration of microalgae

Protein extraction procedure was applied according to Weis (Weis et al., 2002) and protein concentrations of microalgae were found by Bradford method (Bradford, 1976). Bovine serum albumin (BSA) was used as standard and equation was shown as y= 4.53x + 0.0594. In this study, *Hindakia tetrachotoma ME03* revealed the maximum protein concentration of 57.67 ± 2.81 % at 0.1 mg/mL. Similarly, *Scenedesmus sp. ME02* exhibited a second to the maximum protein concentration of 56.59 ± 2.3 %. On the other hand, while *Micractinium sp. ME05* grown at 37 ^{0}C showed protein concentration of 56.48 ± 7.33 %, *Micractinium sp. ME05* grown at 25 ^{0}C displayed the lowest protein concentration with 48.13 ± 2.23 % and the results were shown as percent concentration at Table 3.3.

Table 3.3: Chlorophyll-a and percent carbohydrate and protein amounts of *Micractinium sp ME05, Scenedesmus sp ME02 and Hindakia tetrachotoma ME03.*

Microalgae	Chlorophyll-a content (µg/mL)	Carbohydrate Content (%)	Protein Content (%)
Micractinium sp. ME05 (25°C)	112±8.2	11.11±0.34	48.13 ± 0.9
Micractinium sp. ME05 (37°C)	114±12.6	15.57±0.79	56.48±2.99
Hindakia tetrachotoma ME03	77±6.9	14.5±0.44	57.67 ± 1.15
Scenedesmus sp ME02	57±8.7	17.84±0.64	56.59 ± 0.94

3.9. Lipid extraction from microalgae

In this study, comparative analysis of three different solvent extraction methods were applied for lipids with or without assisted techniques. For this purpose, lipid extraction was performed from three different microalgae strains *Hindakia tetrachotoma*, *Scenedesmus sp.* and *Micractinium sp.* using Soxhlet method with n-hexane, Bligh and Dyer, and Folch methods initially with no additional assisted technique.

3.9.1. Solvent extraction methods with no assistance

3.9.1.1. Lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp.* by means of Soxhlet method

Lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. were carried out by means of Soxhlet method with no assistance. In this method, *Micractinium sp*. showed the highest lipid content (13.7 %) when compared to other microalgae. Lipid concents of *Scenedesmus sp.* *Hindakia tetrachotoma* were found as 9.3 % and 7 %, respectively. On the other hand, *Micractinium sp*. had more lower lipid content (8 %) at 37 °C. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp.* by Soxhlet method were given in Figure 3.16.

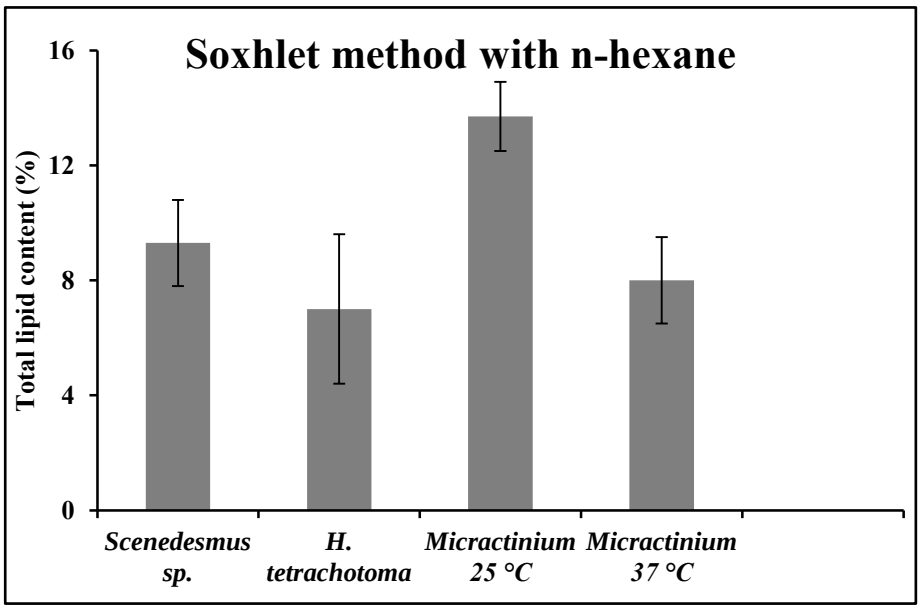

Figure 3.16. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. with Soxhlet method.

3.9.1.2. Lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of The Bligh and Dyer method

Lipid contents of microalgae were determined by another extraction method (the Bligh and Dyer method). In this method, microalgae, *Scenedesmus sp.* (9 %) *Hindakia tetrachotoma* (7 %), displayed total lipid content similar to Soxhlet method. Lipid content of *Micractinium sp*. was found as 13.3 % and 8.3 % at 25 °C and 37°C., respectively. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp.* with the Bligh and Dyer method were shown in Figure 3.17.

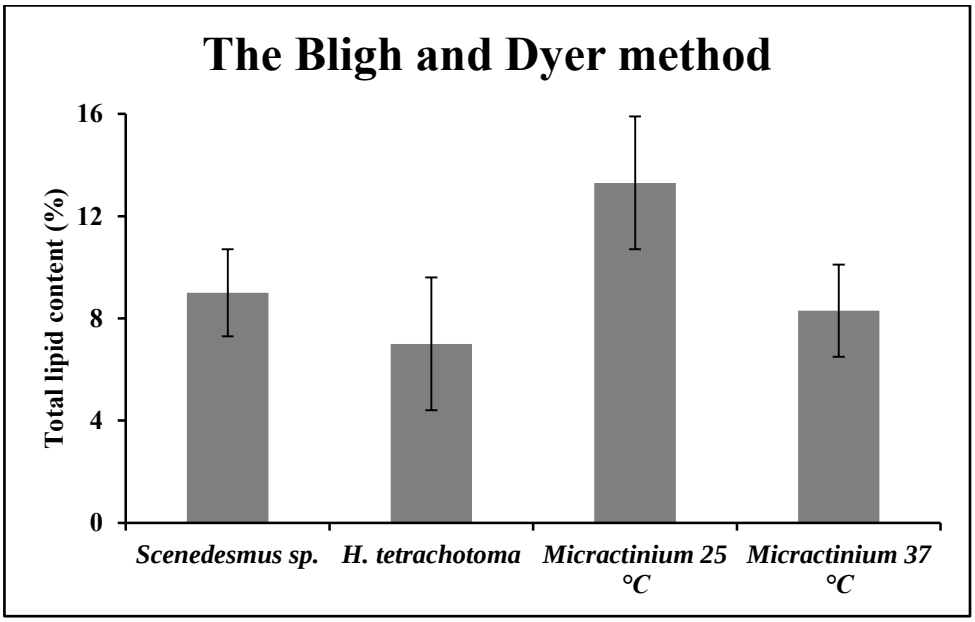

Figure 3.17. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. with the Bligh and Dyer method. There was no significant difference among species.

3.9.1.3. Lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Folch method

At the Folch method, *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. brought out a little difference results. *Hindakia tetrachotoma* (4.7 %) gave lower value compared to Soxhlet method with and the Bligh and Dyer method. Total lipid contents of *Scenedesmus sp.* *Micractinium sp*. were found as 10.3 % and 11.7 %, respectively. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. with Folch method were displayed in Figure in 3.18.

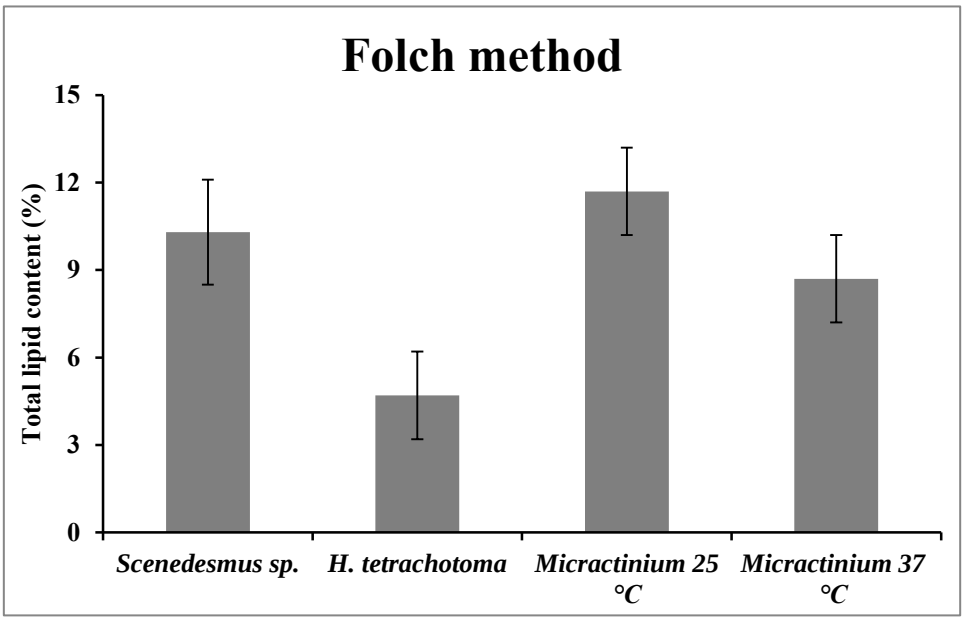

Figure 3.18. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. with Folch method. There was no significant difference among species.

3.9.2. Soxhlet method with assistance

3.9.2.1. Lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Soxhlet method with homogenization assisted (H-A) method

At the homogenization assisted method, three diffrent time periods (5, 10 and 20 min) were examined for homogenization method. All microalgae showed highest lipid contents for total time period of 10 min. *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. had 11 %, 8 % and 20.3 % of total lipid contents at 25 $^{\circ}$C, respectively. For 5 and 20 min points, *Scenedesmus sp* displayed same lipid contents with 10.7 %. Similarly, *Hindakia tetrachotoma* had nearly close total lipid contents (7.7 % and 8 %) for 5 and 20 min. *Micractinium sp*. had 16.7 % and 19 % of total lipid content for 5 and 20 min at 25 $^{\circ}$C , respectively. In addition, *Micractinium sp*. revealed 7.7 %, 9.7 % and 7.3 % of total lipid for 5, 10 and 20 min at 37 $^{\circ}$C, respectively. As a result, we can say that ten minutes of homogenization period is suitable from extraction of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Soxhlet method with homogenization assisted (H-A) method. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Soxhlet method with homogenization assisted (H-A) method were given in Figure 3.19.

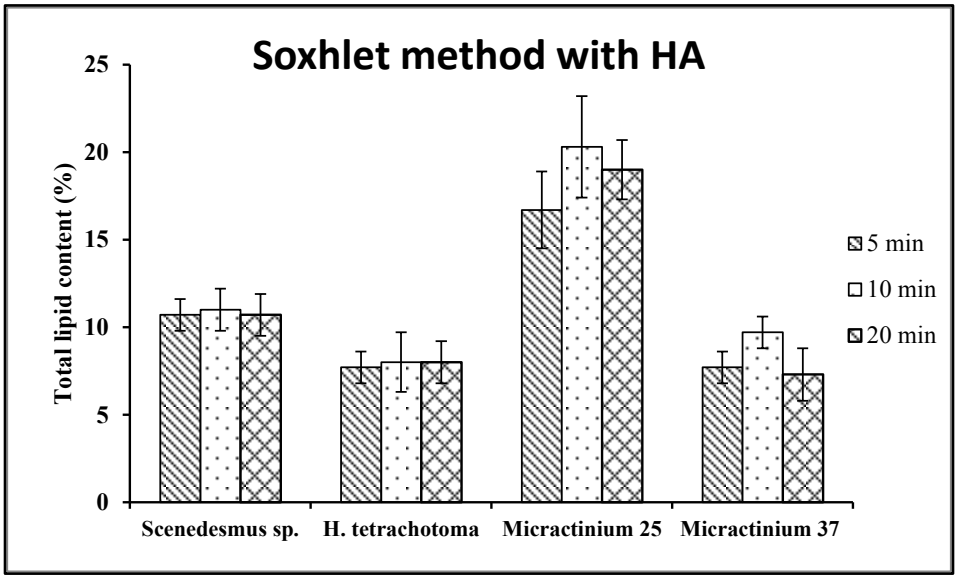

Figure 3.19. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Soxhlet method with homogenization assisted (H-A) method. *Micractinium sp.* had highly significant difference among other species at 25 ^{o}C ($p<0.001$). There was no effect of time, species and time interaction among other species.

3.9.2.2. Lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp.* by means of Soxhlet method with microwave assisted (M-A) method

Three different time points were carried out as 1, 5 and 10 min at the microwave assisted (M-A) method. The highest lipid contents of *Scenedesmus sp.*(11.3 %) *Hindakia tetrachotoma* (8.3 %) and *Micractinium sp*. (16.3 %) were found for 5 min. But, for 1 and 10 min, we encountered with similar results to 5 min results. For 1 min, *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. revealed 10.7 % and 7 % and 14 % of total lipid content, they displayed 9.3 %, 7.7 % and 16 % of total lipid in 10 min, respectively. Also, *Micractinium sp*. showed 7 %, 9.7 % and 8 % of total lipid for 1, 5 and 10 min at 37 $^{\circ}$C, respectively. At the microwave assisted (M-A) method, the maximum time was 5 min for extraction but we can use results for 1 min because of similar results for 1 and 5 min. Total lipid contents of

Scenedesmus sp. *Hindakia tetrachotoma* and *Micractinium sp*. by means of Soxhlet method with microwave assisted (M-A) method were displayed in Figure 3.20.

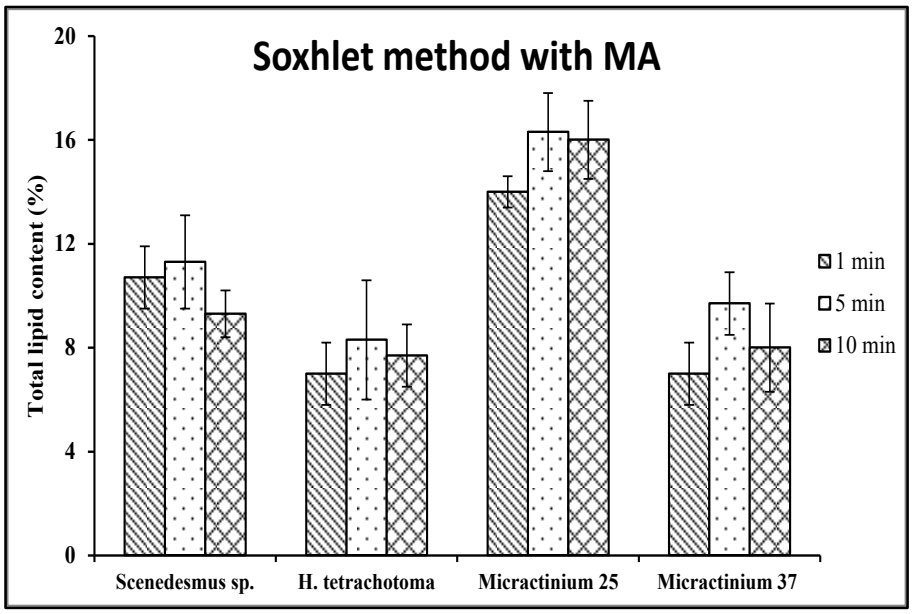

Figure 3.20. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Soxhlet method with microwave assisted (M-A) method. *Micractinium sp.* had highly significant difference among other species at 25 ^{o}C ($p<0.001$). There was no effect of time, species and time interaction among other species.

3.9.2.3. Lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp.* by means of Soxhlet method with ultrasonnication (U-A) method

Microalgae with ultrasonication method were examined for 5, 10 and 20 min. On ultrasonication of microalgae, *Scenedesmus sp.* (14 %) *Hindakia tetrachotoma* (8.7 %) and *Micractinium sp*.(18 %) gave the highest lipid contents for 20 min. For 5 min and 10 min, we determined lipid contents with 12 %, 11.3; 7 %, 8 %; and 17 %, 17 % for *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*., respectively. Moreover, *Micractinium sp* with ultrasonication revealed the highest lipid content (10.3 %) when compared to other methods at 37 $^{\circ}$C. Total lipid contents of

Scenedesmus sp. *Hindakia tetrachotoma* and *Micractinium sp*. by means of Soxhlet method with ultrasonnication (U-A) method were given in Figure 3.21.

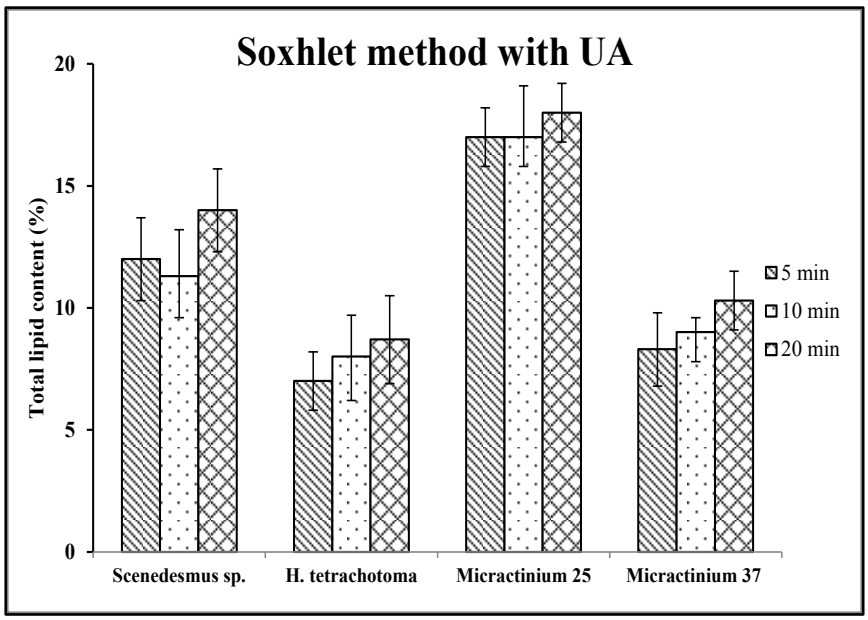

Figure 3.21. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Soxhlet method with ultrasonnication (U-A) method. *Micractinium sp.* had highly significant difference among other species at 25 ^{o}C ($p<0.001$). There was no effect of time, species and time interaction among other species.

3.9.2.4. Lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp.* by means of Soxhlet method with glass bead (B-A) method

On glass bead (B-A) method, *Hindakia tetrachotoma* (nearly 7 %) wasn't be affected much more by three time points (5, 10 and 20 min). While *Scenedesmus sp.* showed same results (9.7 %) for 5 and 10 min, it revealed the highest lipid content (11.3 %) for 20 min. *Micractinium sp*. increased lipid content with time (13.3 % for 5 min ; 14.7 % for 10 min and 15.7 % for 20 min. *Micractinium sp* had maximum lipid content (8.3 %) at 37 $^{\circ}$C for 20 min. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Soxhlet method with glass bead (B-A) method were shown in Figure 3.22.

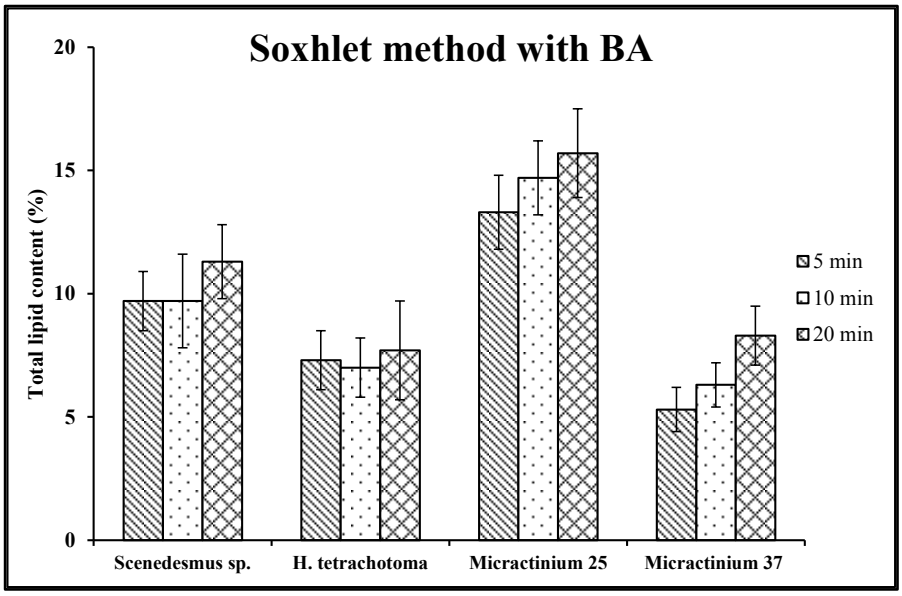

Figure 3.22. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Soxhlet method with glass bead (B-A) method. *Scenedesmus sp.* and *Micractinium sp*.at 25 ^{o}C had significantly difference p-values statistically 0.05 and 0.001, respectively.

3.9.2.5. Lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Soxhlet method with lyophilization assisted (L-A) and combination methods

lyophilization assisted (L-A) method and combinations methods including L-A, M-A and U-A were also anayzed. On lyophilization assisted method, microalgae displayed the highest total lipid contents compared to other extraction procedures. Total lipid content of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. were found with 12.3 %, 8.7 % and 22.7 %, respectively. When extraction methods were combined with each other, we observed that total lipid content increased at the some combinations. With combinaton of L-A&M-A, *Scenedesmus sp.* (13 %) *Hindakia tetrachotoma* (7.7 %) and *Micractinium sp*. (23.3 %) didn't show much more effect on lipid content.With L-A&B-A, *Scenedesmus sp.* (12.7 %) and *Hindakia tetrachotoma* (8.3 %) gave similar results to combinaton of LA-MA. In contrast, *Micractinium sp*. (24.7 %) continued to increase for extraction of total lipid content. The most striking results were found on combination of LA-UA. Total lipid contents

of three microalgae (*Scenedesmus sp.* 15.7 % *Hindakia tetrachotoma* 9.3 % and *Micractinium sp*. 27.3 %) increased significantly. *Micractinium sp*. didn't give an important effect with combination methods at 37 °C. Total lipid contents of *Scenedesmus sp. Hindakia tetrachotoma* and *Micractinium sp*. by means of Soxhlet method with lyophilization assisted (L-A) and combination methods were given in Figure 3.23.

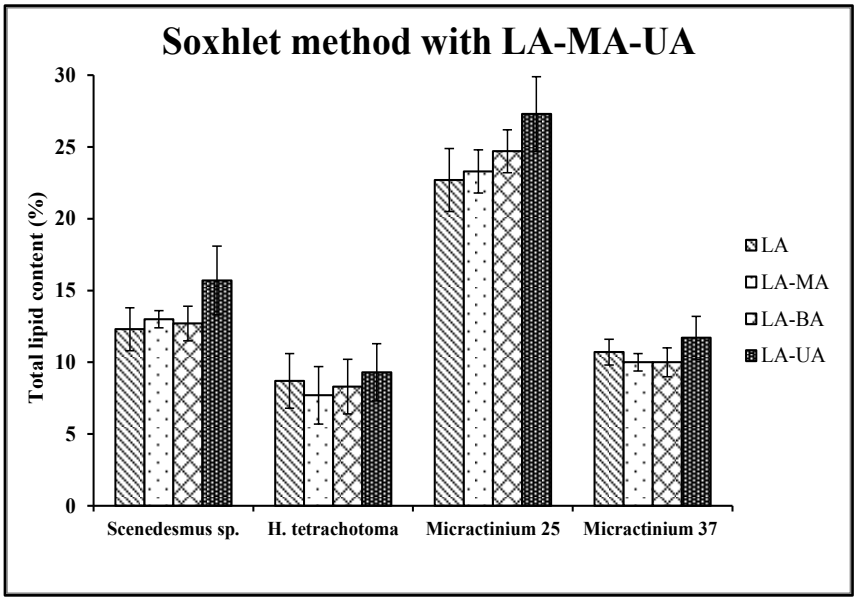

Figure 3.23. Total lipid contents of *Scenedesmus sp. Hindakia tetrachotoma* and *Micractinium sp*. by means of Soxhlet method with lyophilization assisted (L-A) and combination methods.In the same way, *Micractinium sp*. was distinguished from all other species with highly significant difference ($p<0.001$). Also, there was significant difference between *Scenedesmus sp.* and *Hindakia tetrachotoma* ($p<0.001$).

3.9.3. Bligh and Dyer method with assistance

3.9.3.1. Lipid contents of *Scenedesmus sp. Hindakia tetrachotoma* and *Micractinium sp.* by means of Bligh and Dyer method with homogenization assisted (H-A) method

Other extraction method (Bligh and Dyer) was applied to microalgae by homogenization for lipid extraction. Three different time points were used as 5, 10

and 20 min. At this experiment, maximum lipid contents were found for 10 min. At this time, while *Scenedesmus sp.* reached to 12 % of lipid content, *Micractinium sp*. showed 17.3 % of that. Although *Hindakia tetrachotoma* displayed maximum lipid content (7.7 %) for 10 min. it refleced similar values for 5 min (7.3 %) and 20 min (7 %). *Micractinium sp*. had 6.7 %, 9.7 % and 8.3 % lipid content at 37 °C.

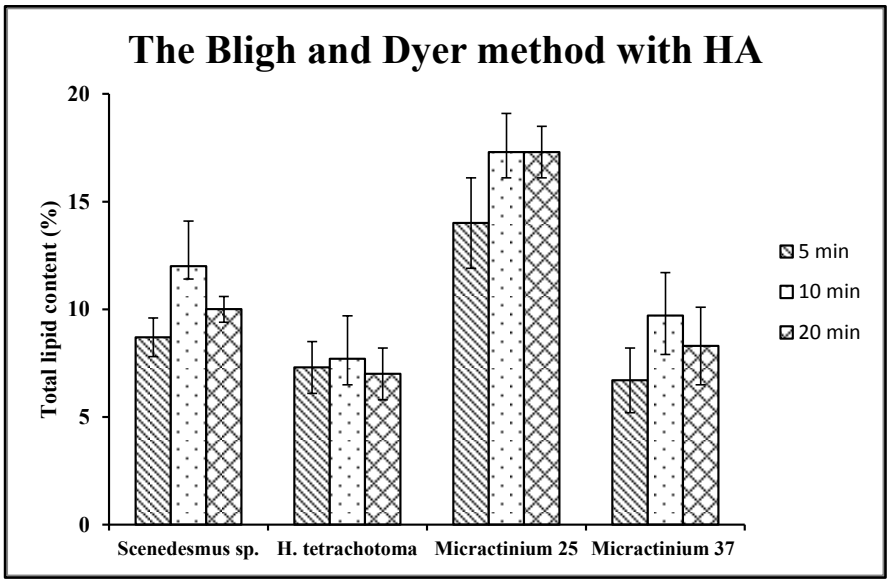

Figure 3.24. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Bligh and Dyer with homogenization assisted (H-A) method. *Micractinium sp.* had highly significant difference among other species at 25 °C ($p<0.001$). There was no effect of time, species and time interaction among other species.

3.9.3.2. Lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Bligh and Dyer method with microwave assisted (M-A) method

In this method, total lipid contents of three microalgae didnt change significantly when results were compared to no assistance method. Also, they didn't give distinctive results for 1, 5 and 10 min. Maximum values for *Scenedesmus sp.* and *Hindakia tetrachotoma* were 10.7 % and 6.3 % for 10 min. The highest lipid content of *Micractinium sp* was 13.7 % for 5 min. In addition, at 37 °C., *Micractinium sp* (8.3 %, 8.7 % and 8.3 %) had similar total lipid contents for 1 min, 5 min and 10 min,

respectively. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Bligh and Dyer with microwave assisted (M-A) method were given in Figure 3.25.

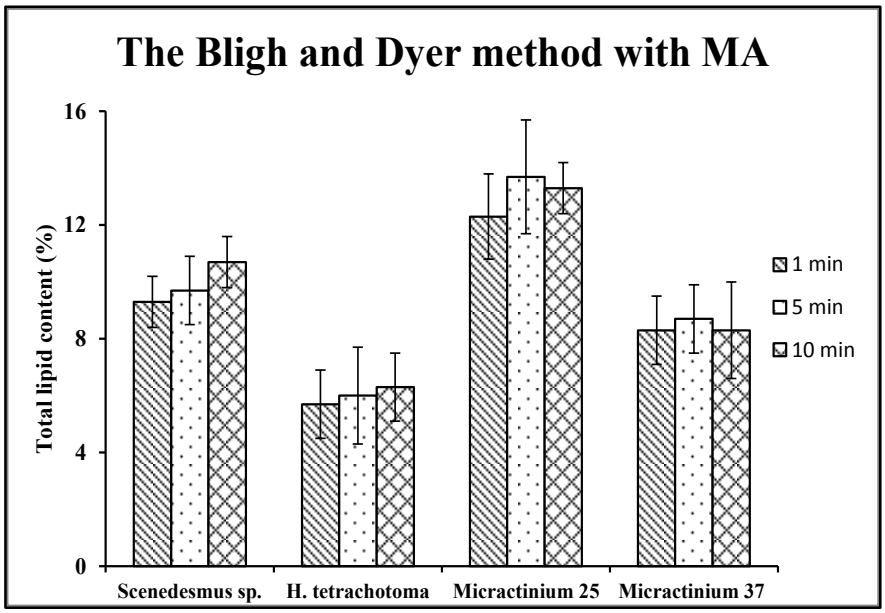

Figure 3.25. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Bligh and Dyer with microwave assisted (M-A) method. *Micractinium sp*. was distinguished from all other species with highly significant difference ($p<0.001$). Also, there was significant difference between *Scenedesmus sp.* and *Hindakia tetrachotoma* ($p<0.001$).

3.9.3.3. Lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Bligh and Dyer method with ultrasonnication (U-A) method

Three different time interveals were applied as 5, 10 and 20 min on microalgae. The most effective extraction time was found for 20 min. *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. revealed 12.3 %, 8.3 % and 15.7 %, respectively, for 20 min. Total lipid contents of *Micractinium sp*. were similar for 10 min (10.7 %) and 20 min (11 %). Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Bligh and Dyer with ultrasonnication (U-A) method were displayed in Figure 3.26.

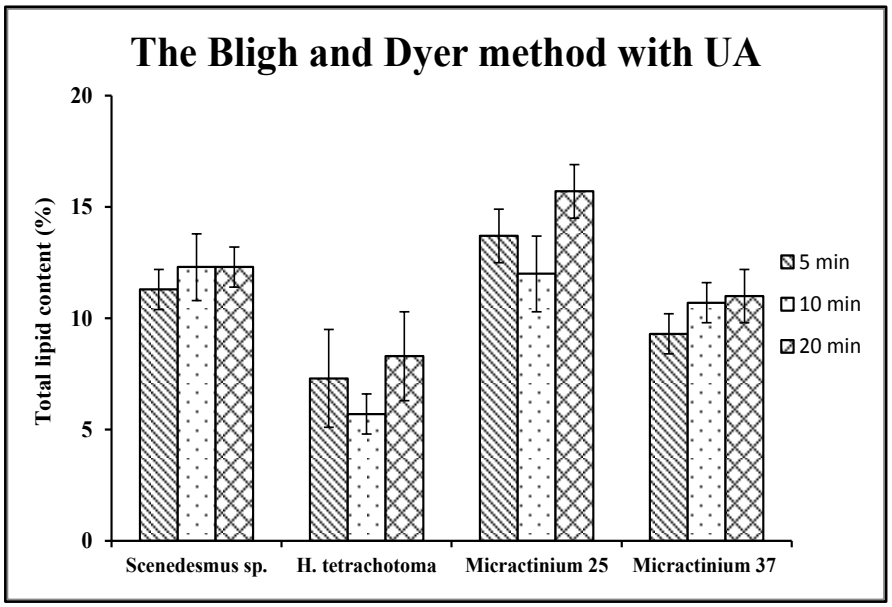

Figure 3.26. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Bligh and Dyer with ultrasonnication (U-A) method. *Scenedesmus sp.* and *Hindakia tetrachotoma* had significant difference ($p<0.001$). *Micractinium sp*. 25 ^{o}C was observed with significantly difference from *Hindakia tetrachotoma* and *Micractinium sp*. 37 ^{o}C ($p<0.001$). But, there was no significant difference between *Micractinium sp*. 25 ^{o}C and *Scenedesmus sp.*

3.9.3.4. Lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Bligh and Dyer method with glass bead (B-A) method

Scenedesmus sp. could not be affected by glass bead disruption depending on time remarkably. Its total lipid content percentages were 9.3 %, 9.3 % and 9 % for 5, 10 and 20 min, respectively. On the other hand, lipid content of *Hindakia tetrachotoma* increased during disruption time proportionally. Its values were 5.3 %, 6 % and 7.3 % for 5, 10, 20 min. *Micractinium sp*. showed the highest lipid content (17 %) for 20 min. Also, at 37 $^{\circ}$C, *Micractinium sp*. had 5.3 %, 6.3 % and 8.3 % for 5, 10 and 20 min. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Bligh and Dyer with glass bead (B-A) method were given in Figure 3.27.

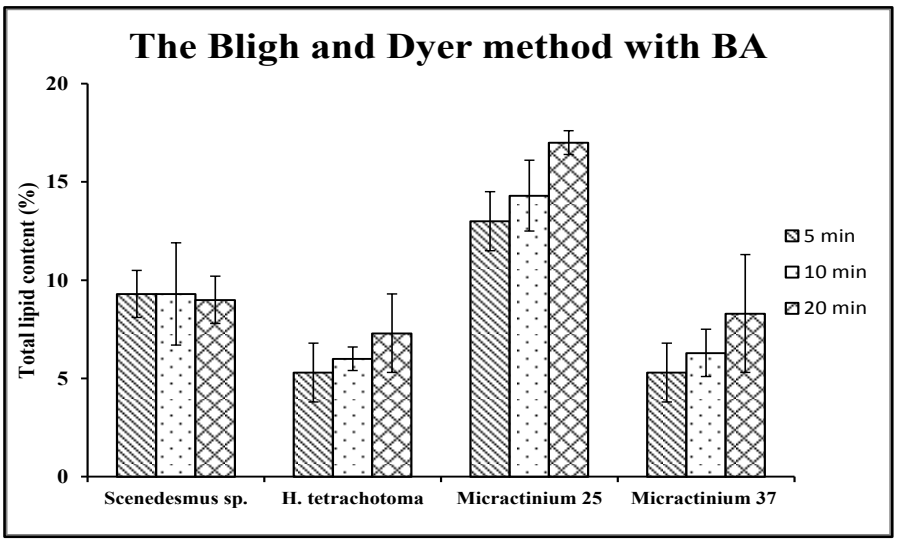

Figure 3.27. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Bligh and Dyer with glass bead (B-A) method. *Scenedesmus sp.* and *Hindakia tetrachotoma* had significant difference ($p<0.05$). Also, *Micractinium sp*. 25 ^{o}C was observed with significantly difference ($p<0.001$).

3.9.3.5. Lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Bligh and Dyer method with lyophilization assisted (L-A) and combination methods

Bligh and Dyer method was studied by combination methods and the most effective results were found for *Micractinium sp*. When we applied lyophilization assisted (L-A) method, total lipid content of *Micractinium sp*. raised to up to 20 %. Moreover, LA-UA combination method displayed the highest lipid content with 24 %. For Bligh and Dyer method. L-A&MA and L-A&B-A methods had 20.3 % and 21.7 % for *Micractinium sp*. In contrast, *Scenedesmus sp.* did not give distinctive results between methods. It showed nearly 12 % values for all combination methods. The other microalgae, *Hindakia tetrachotoma* had the highest lipid content (9.3 %) for L-A&U-A method. L-A, L-A&M-A and L-A&B-A methods reflected lipid contents as 7.7 %, 8 % and 8 % for *Hindakia tetrachotoma*, respectively. *Micractinium sp*.

represented the highest lipid content for L-A&B-A and L-A&U-A with 11.3 % at 37 °C. In addition to this, L-A and L-A&M-A had total lipid content with 10.7% and 10 %. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Bligh and Dyer with lyophilization assisted (L-A) and combination methods were shown in Figure 3.28.

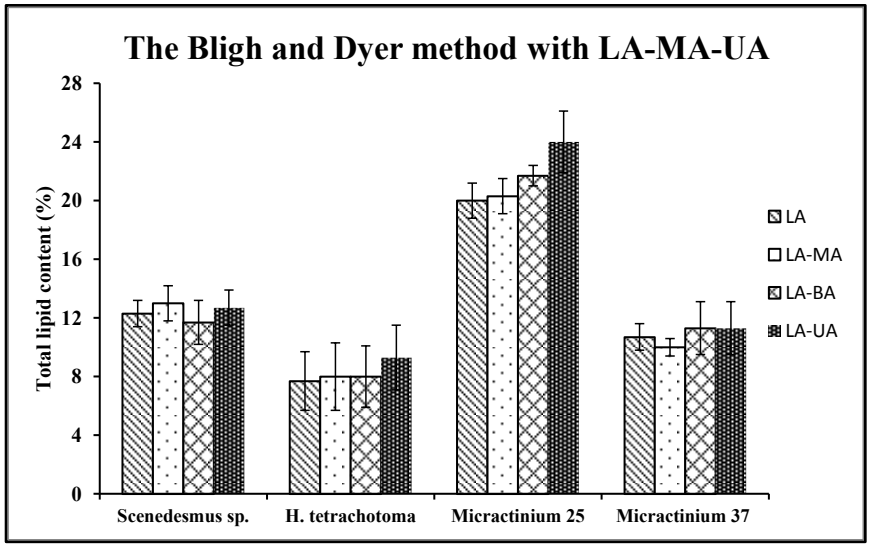

Figure 3.28. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Bligh and Dyer with lyophilization assisted (L-A) and combination methods. *Micractinium sp*. 25 °C showed with significantly difference ($p<0.001$). Also, *Scenedesmus sp.* and *Hindakia tetrachotoma* had significant difference ($p<0.001$).

3.9.4. Folch method with assistance

3.9.4.1. Lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Folch method with homogenization assisted (H-A) method

Microalgae were examined according to another extraction method, Folch method. Three microalgae didn't show meaningful results for different time points (5, 10, 20 min). *Scenedesmus sp*. had 10.3 %, 10.3 % and 7.3 % total lipid contents for 5, 10, 20 min, respectively. Total lipid content of *Hindakia tetrachotoma* was found

approximately 5 % for three different time interveals. *Micractinium sp*. showed nearly maximum lipid content with 16 %. *Micractinium sp*. had the highest lipid content (9 %) for 10 min at 37 °C. Generally, when this method is used for extraction with HA, it was enough time for 10 min.

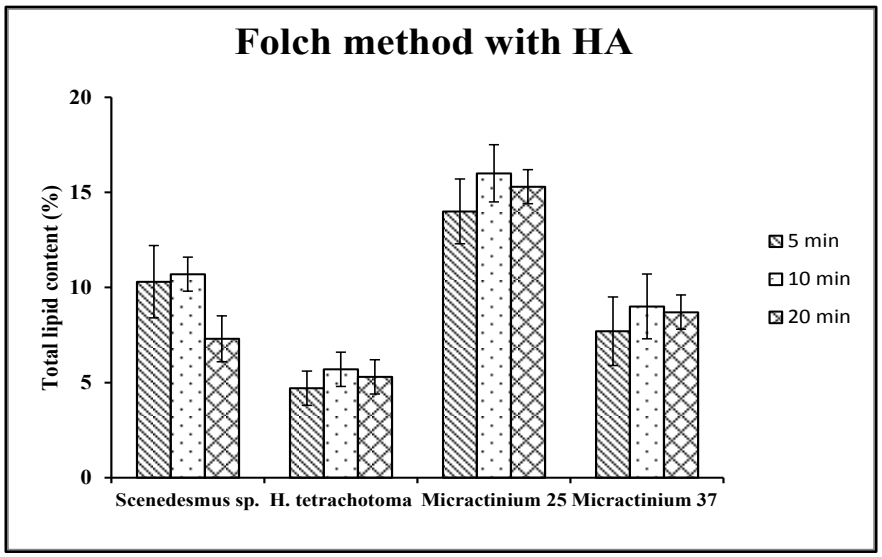

Figure 3.29. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Folch method with homogenization assisted (H-A) method. *Micractinium sp*. 25 °C showed with significantly difference ($p<0.001$). Also, *Scenedesmus sp.* and *Hindakia tetrachotoma* had significant difference ($p<0.001$).

3.9.4.2. Lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp.* by means of Folch method with microwave assisted (M-A) method

Scenedesmus sp. (9.7 %), *Hindakia tetrachotoma* (5 %) and *Micractinium sp*. (14.3 %) had maximum total lipid contents for 5 min. Total lipid contents of *Scenedesmus sp.* were 8.7 % for 1 min and 8.3 % for 10 min. For *Hindakia tetrachotoma*, lipid content was 4.7 % for 1 and 10 min. *Micractinium sp*. increased lipid content from 11.3 % to 14 % between 1 min and 10 min. *Micractinium sp*. showed the highest lipid content (8.7 %) for 5 min at 37 °C. Total lipid contents of *Scenedesmus sp.*

Hindakia tetrachotoma and *Micractinium sp*. by means of Folch method with microwave assisted (M-A) method were shown in Figure 3.30.

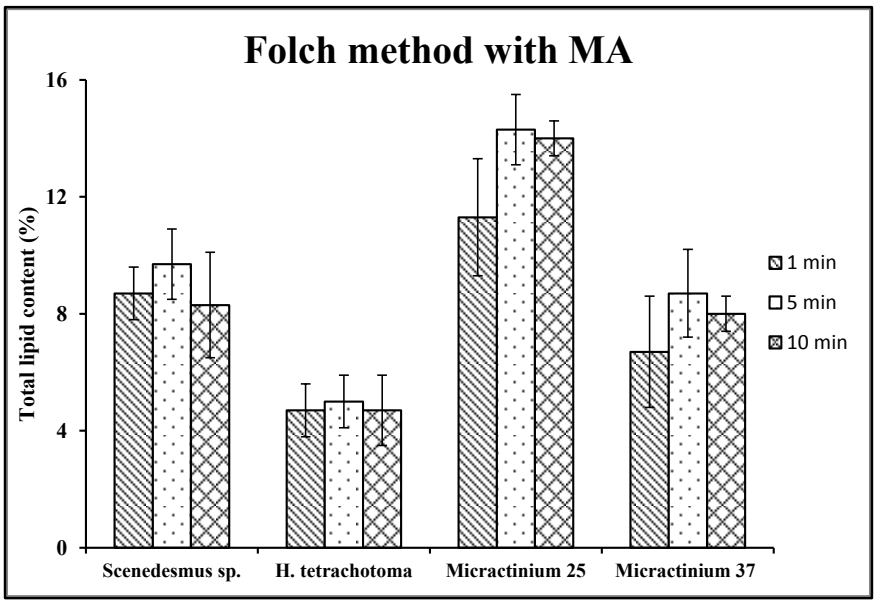

Figure 3.30. Total lipid contents of *Scenedesmus sp. Hindakia tetrachotoma* and *Micractinium sp*. by means of Folch method with microwave assisted (M-A) method. *Micractinium sp*. 25 ^{o}C showed with significantly difference ($p<0.001$). Also, *Scenedesmus sp.* and *Hindakia tetrachotoma* had significant difference ($p<0.001$).

3.9.4.3. Lipid contents of *Scenedesmus sp. Hindakia tetrachotoma* and *Micractinium sp*. by means of Folch method with ultrasonnication (U-A) method

On ultrasonication method, three microalgae displayed maximum effect on 20 min for lipid extraction. Lipid contents of *Scenedesmus sp. Hindakia tetrachotoma* and *Micractinium sp*.were found 11.7 %, 5 % and 15 % for 5, 10 and 20 min, respectively. *Scenedesmus sp.* increased lipid content from 8 % to 11 % in 10 min. *Micractinium sp*. had same value (13.7 %) for 5 and 10 min. For the highest lipid content with 9.7 %, *Micractinium sp*. showed for 20 min at 37 $^{\circ}$C. Total lipid contents of *Scenedesmus sp. Hindakia tetrachotoma* and *Micractinium sp*. by means of Folch method with ultrasonnication (U-A) method were given in Figure 3.31.

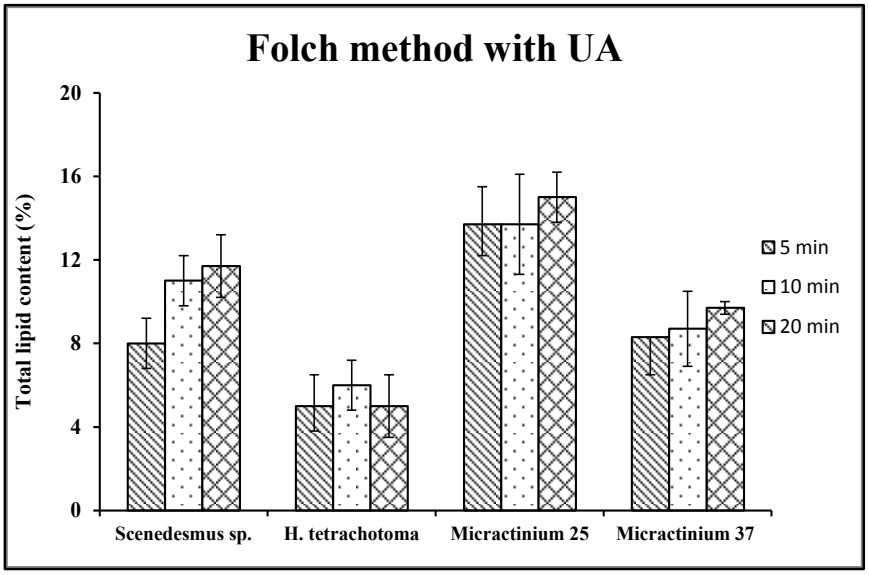

Figure 3.31. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Folch method with ultrasonnication (U-A) method. *Micractinium sp*. 25 ^{o}C showed with significantly difference ($p<0.001$). Also, *Scenedesmus sp.* and *Hindakia tetrachotoma* had significant difference ($p<0.001$).

3.9.4.4. Lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Folch method with glass bead (B-A) method

At the this method, lipid extraction was examined for 5, 10 and 20 min. *Scenedesmus sp.* raised lipid content from 8.3 % to 11.3 % in 20 min. On the other hand, *Hindakia tetrachotoma* and *Micractinium sp*. (37 $^{\circ}$C) didn't show any increase. Total lipid content of *Hindakia tetrachotoma* was nearly 6 % and that of *Micractinium sp*. (37 $^{\circ}$C) was 9 %. *Micractinium sp*. had the highest lipid content (13.3 %) for 20 min. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Folch method with glass bead (B-A) method were displayed in Figure 3.32.

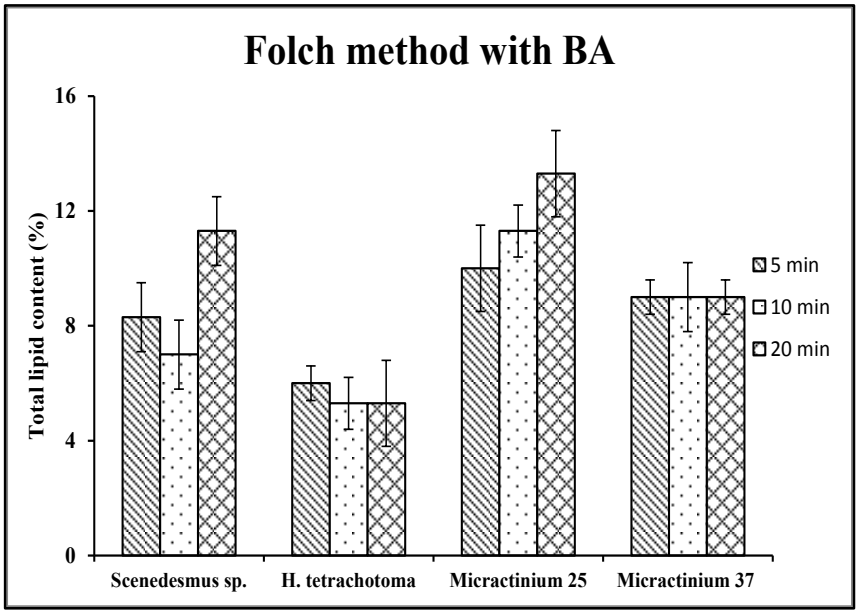

Figure 3.32. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Folch method with glass bead (B-A) method. *Micractinium sp*. 25 ^{o}C showed with significantly difference ($p<0.001$). Also, *Scenedesmus sp.* and *Hindakia tetrachotoma* had significant difference ($p<0.001$).

3.9.4.5. Lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Folch method with lyophilization assisted (L-A) and combination methods

Folch method with lyophilization assisted (L-A) and combination methods were carried out to understand whether lipid contents of microalgae increase or not. Maximum effects were observed for L-A&U-A combination method. In this method, total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. were found as 11.3 %, 7.3 %, and 22.3 %, respectively. *Scenedesmus sp.* had lipid contents as 11.7 %, 10.7 % and 10.7 % for L-A, L-A&M-A and L-A&B-A methods. *Hindakia tetrachotoma* gave similar results for L-A, L-A&M-A and L-A&B-A with nearly 6 %. In addition to these, *Micractinium sp*. displayed higher lipid contents for L-A (20 %) and L-A&B-A (20.7 %) than that of L-A&M-A (18.7 %). *Micractinium sp*. had the highest value for L-A&U-A with 9.7 % at 37 $^{\circ}$C. In this temperature, total lipid contents of *Micractinium sp* were found as 9 %, 8.3 % and 8.7 % for L-A, L-

A&M-A and L-A&B-A, respectively. Finally, L-A&U-A combination method should be prefered when Folch method with assistance is used for lipid extractions from *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Soxhlet method with lyophilization assisted (L-A) and combination methods were given in Figure 3.33.

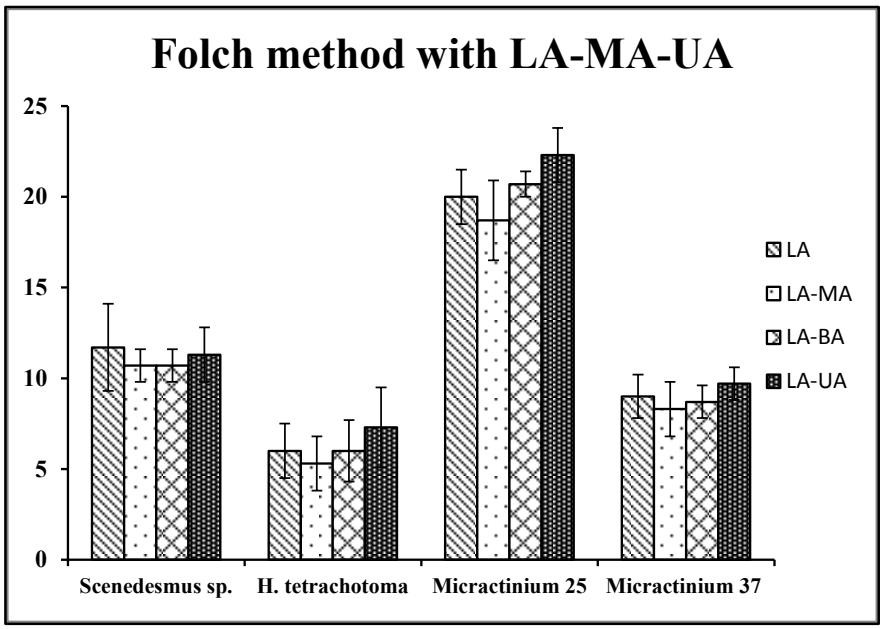

Figure 3.33. Total lipid contents of *Scenedesmus sp.* *Hindakia tetrachotoma* and *Micractinium sp*. by means of Soxhlet method with lyophilization assisted (L-A) and combination methods.

3.9.5. Evaluation of Maximum Lipid Contents of Microalgae

For *H. tetrachotoma*, final lipid content was calculated as 4.7% g of total lipid/ dryweight with the Folch method and 7.0% with Bligh and Dyer and Soxhlet methods. For *Scenedesmus sp.*, percent lipid contents were as follows: 9.0%, 9.3% and 10.3% with Bligh and Dyer, Soxhlet and Folch methods, respectively. Finally, *Micractinium sp.* had the highest lipid content among three species with all three methods tested. Percent lipid content for *Micractinium sp.* was 13.7%, 13.3%, and 11.7% with Soxhlet, Bligh and Dyer and Folch methods, respectively. Results are summarized in Figure 3.34.

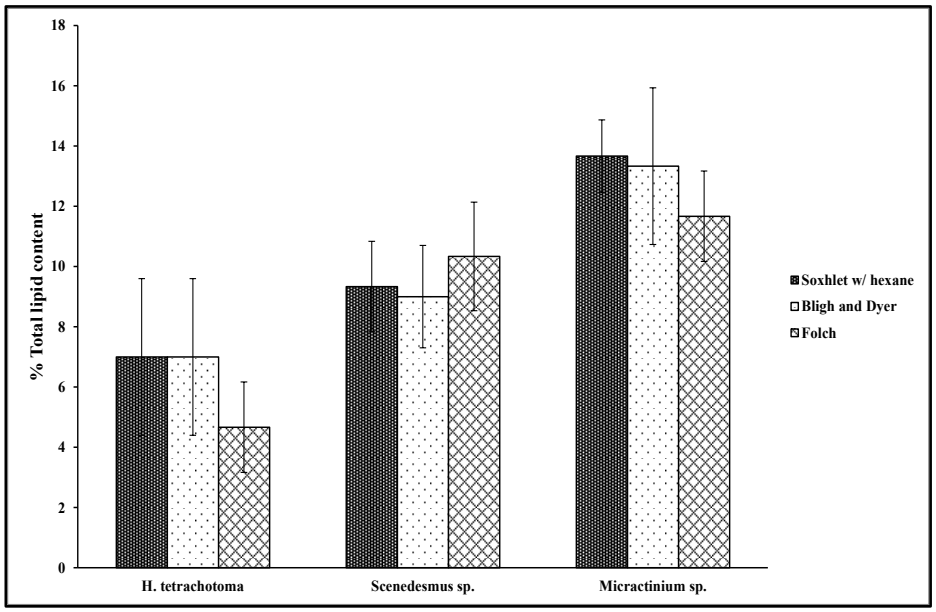

Figure 3.34: % lipid content of *H. tetrachotoma*, *Scenedesmus sp.* and *Micractinium sp.* with Soxhlet, Bligh and Dyer and Folch methods. Error bars show SEM. Species had nonsignificant statistically. Soxhlet and Folch methods showed highly significant difference ($p<0.001$). Bligh and Dyer method was distinguished from other techniques with $p<0.05$ value.

In addition to this, five various assisted extraction methods were carried out for obtaining of lipids from microalgal cells. These techniques were Homogenization assisted (H-A) method, microwave assisted (M-A) method, ultrasonication (U-A) method, glass bead (B-A) method, and lyophilization assisted (L-A) method. Moreover, we examined at least three different durations with assisted methods. For *H. Tetrachotoma*, U-A method (max. 20 min) and L-A method showed the highest lipid content with 8.7 %. Combination of L-A and U-A gave the maximum lipid content with 9.3 % with Bligh and Dyer and soxhlet method. For *Scenedesmus sp.*, U-A method (max. 20 min) gave the highest lipid content (14 %) with soxhlet. For combination of methods, LA and UA showed the maximum lipid content with 15.7 %. Last, L-A had the highest lipid content with 22.7 % for *Micractinium sp.* Combination of L-A and U-A showed 27.3 % lipid content with soxhlet.

Scenedesmus sp., H. Tetrachotoma and Micractinium sp. after lipid extraction procedure were given in Figure 3.35. Lipid contents of isolated microalgae via assisted method were given in Table 3.4, 3.5 and 3.6.

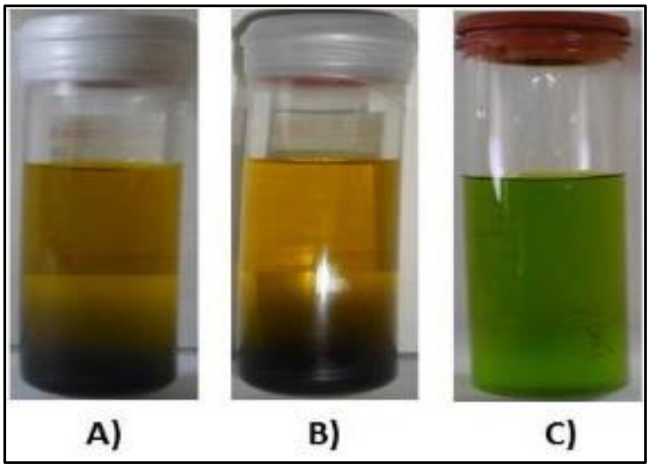

Figure 3.35. A) Scenedesmus sp., B) H. Tetrachotoma and C) Micractinium sp. in hexane after lipid extraction procedure.

The lipid productivity of each isolate was also check out as a factor of gram per litre dry weight per day at 25°C for *Scenedesmus sp.ME02* and *H. tetrachotoma ME03* and at both 25°C and 37°C for *Micractinium sp. ME05*. Although *Scenedesmus sp. ME02* had relatively high lipid content, it had the lowest lipid productivity (0.006±0.001) due to its slow growth rate. *H. tetrachotoma ME03*, on the other hand, showed lipid productivity of 0.01±0.002 $g.L^{-1}day^{-1}$. Interestingly, despite the dramatic difference between lipid contents of *Micractinium sp. ME05* at 25°C *vs.* 37°C, *Micractinium sp. ME05* had the same amount of lipid productivity (0.05±0.003 and 0.05±0.01), respectively, at either temperature due to changes in growth rate at these two different temperatures. Among all three isolates, *Micractinium sp. ME05* had the highest lipid productivity. The results were given in Table 3.7.

Studies related with *Micractinium sp.* on detailed lipid extraction haven't seen at the literature. However, Araujo studied different lipid extraction methods (Bligh and Dyer, Chen, Folch, Hara and Radin, and Soxhlet) with ultrasound on *Chlorella*

vulgaris and they found that Bligh and Dyer method gave the highest lipid content with 52.5 % (Araujo et al., 2013).

In other study, Santos (2015) carried out similar methods for *Chlorella vulgaris* and used as solvent such as ethanol, hexane and modified Bligh and Dyer and Folch (chloroform: methanol in ratios 1:2 and 2:1). Santos (2015) reported that mixture of chloroform: methanol (2:1) with ultrasound had the highest lipid content with 19 % (Santos, 2015). Microwave for 400, 800 and 1000 Watt and ultrasound assisted for 30 min combination extraction was studied by Dai. They selected various solvents such as methanol, n-hexane, iso-propanol, extraction time and energy power and n-hexane/iso-propanol (2:1) solvents showed the highest lipid content with 28 %. Also, they exhibited that the maximum productivity was 76.2% with a mixture of methanol-oil molar ratio (1:18) at 68 C for 4 h with Li_4SiO_4 (Dai, 2014).

In other study related with ultrasound and n-hexane extraction, *Chlorella minutissima, Thalassiosira fluviatilis and Thalassiosira pseudonana* were examined for lipid content. Lipid contents of *Chlorella minutissima, Thalassiosira fluviatilis and Thalassiosira pseudonana* were found with 15.5, 40.3 and 39.5 %, respectively (Neto et al., 2013). Moreover, asssisted methods were used for direct transesterification. For this goal, *Nannochloropsis* was converted to biodiesel directly and microwave asist method was found the most effective (Koberg et al., 2011).

Nannochloropsis sp. was transesterified with microwave method presence of methanol and NaOH and H_2SO_4 as wet microalgae and the highest biodiesel productivity was obtained with 43.7 % (Teo et al., 2014). In the other study, bath and microwave assisted method were carried out and microwave assisted method gave two fold of lipid content (38.31 %) compared to bath assisted method for *Nannochloropsis sp* (Wahidin et al., 2014).

In another recent study, Converti et al. (2009) tested the effect of temperature on the growth and lipid content of two microalgal species, *Chlorella vulgaris* and *Nannochloropsis oculata*. At *C.vulgaris*, a decrease in growth temperature from 30°C

to 25°C caused more than a two fold increase in lipid content from 5.9% to 14.7%. As the growth rate did not change, the overall lipid productivity increased from 8 to 20 mgL^{-1} day^{-1}. The lipid content of *N. oculata* was also variable at different growth temperatures (i.e. 14.9% at 15°C, 7.9% at 20°C and 13.9% at 25°C). However, as the growth rate also fluctuated at these temperatures, the lipid productivity remained unchanged. Smith-Bädorf et al. (2013), on the other hand, found that green algae used in their study produced less neutral lipid at higher temperatures (Smith-Bädorf et al., 2013).Hence, the effect of temperature on lipid content, growth rate and lipid productivity of microalgae is species-specific.

Table 3.4: Lipid contents of *Hindakia tetrachotoma ME03* by assited and non-assisted methods.

Species	Solvent extraction method	No assistance	Assisted techniques															
			H-A			M-A			U-A			B-A			L-A	LA&MA	LA&BA	LA&UA
			5’	**10’***	20’	1’	**5’**	10’*	5’	10’	**20’***	5’	10’	**20’***	**1 day**	**Maximum time**	**Maximum tme**	**Maximum time**
H. tetrachotoa	**Soxhlet w/ n-hexane**	7.0±2.6	7.7±0.9	**8.0±1.7**	8.0±1.2	7.0±1.2	**8.3±2.3**	7.7±1.2	7.0±1.2	8.0±1.7	**8.7±1.8**	7.3±1.2	7.0±1.2	**7.7±2.0**	8.7±1.9	7.7±**2**	8.3±**1.9**	9.3±**2**
	Bligh and Dyer	7.0±2.6	7.3±1.2	**7.7±2.0**	7.0±1.2	5.7±1.2	**6.0±1.7**	6.3±1.2	7.3±2.2	5.7±0.9	**8.3±2.0**	5.3±1.5	6.0±0.6	**7.3±2.0**	7.7±**2**	8±**2.3**	8±**2.1**	9.3±**2.2**
	Folch	4.7±1.5	4.7±0.9	**5.7±0.9**	5.3±0.9	4.7±0.9	**5.0±1.2**	4.7±1.2	5.0±1.2	6.0±1.2	**5.0±1.5**	6.0±0.6	5.3±0.9	**5.3±1.5**	6±1.5	5.3±1.5	6±**1.7**	7.3±**2.2**

Table 3.5: Lipid contents of *Scenedesmus sp.ME02* by assited and non-assisted methods

Species	Solvent extraction method	No assistance	Assisted Tecniques														
			H-A			M-A			U-A			B-A			L-A	LA&MA	LA&BA	LA&UA
			5’	**10’***	20’	1’	**5’**	10’*	5’	10’	**20’***	5’	10’	**20’***	**1 day**	**Maximum time**	**Maximum tme**	**Maximum time**
Scenedesmus sp.	**Soxhlet w/ n-hexane**	9.3±1.5	10.7±0.9	**11±1.2**	10.7±1.2	10.7±1.2	**11.3±1.8**	9.3±0.9	12±1.7	11.3±1.9	**14±1.7**	9.7±1.2	9.7±1.9	**11.3±1.5**	12.3±1.5	13±**0.6**	12.7±**1.2**	15.7±**2.4**
	Bligh and Dyer	9±1.7	8.7±0.9	**12±2.1**	10±0.6	9.3±0.9	**9.7±1.2**	10.7±0.9	11.3±0.9	12.3±1.5	**12.3±0.9**	9.3±1.2	9.3±2.6	**9±1.2**	12.3±**0.9**	13±**1.2**	11.7±**1.5**	12.7±**1.2**
	Folch	10.3±1.8	10.3±1.9	**10.7±0.9**	7.3±1.2	8.7±0.9	**9.7±1.2**	8.3±1.8	8±1.2	11 ±1.2	**11.7±1.5**	8.3±1.2	7±1.2	**11.3±1.2**	11.7±2.4	10.7±0.9	10.7±**0.9**	11.3±**1.5**

Table 3.6: Lipid contents of *Micractinium sp.ME05* by assited and non-assisted methods

Species	Solvent extraction method	No assistance	Assisted techniques														
			H-A			M-A			U-A			B-A			L-A	LA&MA	LA&BA	LA&UA
			5’	**10’***	20’	1’	**5’**	10’*	5’	10’	**20’***	5’	10’	**20’***	**1 day**	**Maximum time**	**Maximum tme**	**Maximum time**
Micractinium sp.	**Soxhlet w/ n-hexane**	13.7±1.2	16.7±2.2	**20.3±2.9**	19±1.7	14±0.6	**16.3±1.5**	16±1.5	17±1.2	17±2.1	**18±1.2**	13.3±1.5	14.7±1.5	**15.7±1.8**	22.7±2.2	23.3±**1.5**	24.7±**1.5**	27.3±**2.6**
	Bligh and Dyer	13.3±2.6	14±2.1	**17.3±1.8**	17.3±1.2	12.3±1.5	**13.7±2**	13.3±0.9	13.7±1.2	12±1.7	**15.7±1.2**	13±1.5	14.3±1.8	**17±0.6**	20±**1.2**	20.3±**1.2**	21.7±**0.7**	24±**2.1**
	Folch	11.7±1.5	14±1.7	**16±1.5**	15.3±0.9	11.3±2	**14.3±1.2**	14±0.6	13.7±1.5	13.7±2.4	**15±1.2**	10±1.5	11.3±0.9	**13.3±1.5**	20±1.5	18.7±2.2	20.7±**0.7**	22.3±**1.5**

3.10. High Throughput Method for Quantitative Lipid Measurement of *Micractinium sp.* Mutants

Neutral lipids of *Micractinium sp.* were carried out by Nile red method. For accurate and quick measurements of *Micractinium sp.* mutant samples, we needed a sensitive method. Therefore, we examined optimum conditions of mutant samples by Nile red method. Although conventional lipid extraction methods such as Soxhlet, the Bligh and Dyer methods and hexane extraction give accurate lipid content, they have drawbacks for microalgae mutants. These are labor-intensive, time consuming and expensive solvent inputs (Chen et al., 2009). This method includes a lipid-soluble fluorescent dye in hydrophobic solvents and soluble fluorescence of Nile red is related with triglycerides concentrations (Pick et al., 2012).

Nile red method can not be applied all microalgae because some microalgae have stick and rigid cell wall (Chen et al., 2009). Therefore, Nile red method needs optimization because this dye penetrates differently into cells. Also, choice of solvent, penetration time and solvent concentration, cell concentration affect accurate measurements of microalgae. Excitation and emmision wavelength can change because of microalgae nature. Generally, microalgae can be measured 480- 530 for excitation and 530- 600 for emmision (Ren et al., 2015).

In this study, we determined optimum conditions such as penetration time, cell concentration, Nile red concentration and amount of Nile red for exact lipid content determination of *Micractinium sp.* mutants. Samples were vortexed or sonicated for 5 min before experiment.

3.10.1. Determination of Nile Red Concentration and Time for Measurement of Lipid Content of *Micractinium sp.* Mutants.

5 different time intervals were carried out as 5, 10, 15, 30 and 60 min. In addition to this, we studied 6 various Nile red concentrations as 0.1, 0.2, 0.5, 1, 2, and 5 µg/mL. At 530 nm of excitation and 600 nm of emission, fluorescence intesities were found as 257 ± 9, 303 ± 12, 383 ± 23, 389 ±10 and 369 ± 24 a.u. in 5, 10, 15, 30 and 60 min. at 0.1 µg/mL of Nile red concentration, respectively. Fluorescence intensity in 0.2 µg/mL compared to that in 0.1 µg/mL, three times increased. At the 0.2 µg/mL, fluorescence intensities were calculated with 572 ± 41, 612 ± 34, 920 ± 29, 888 ± 23 and 880 ± 23 a.u. in 5, 10, 15, 30 and 60 min., respectively. Fluorescence intensity increased with 0.5 µg/mL of Nile red concentration. At this concentration, results were observed with 873 ± 20, 918 ± 24, 1128 ± 19, 1097 ± 15 and 1049 ± 29 a.u. in 5, 10, 15, 30 and 60 min., respectively. The highest fluorescence intensity with 1324 ± 24 a.u. was obtained at 15 min and 1 µg/mL of Nile red concentration in this experiment. At 1 µg/mL, fluorescence intensities were found with 1062 ± 42, 1108 ± 24, 1324 ± 24, 1296 ± 26 and 1160 ± 15 a.u. in 5, 10, 15, 30 and 60 min., respectively. After 1 µg/mL of Nile red concentration, fluorescence intensity decreases and as depending on time, it remains stable. At 2 µg/mL of Nile red concentration, the highest intisty was carried out with 1085 ± 30 a.u. in 30 min and the lowest intensity was determined with 965 ± 26 a.u. in 10 min. Fluorescence intensities were found with 791 ± 38, 868 ± 21, 826 ± 20, 873 ± 59 and 787 ± 16 a.u. in 5, 10, 15, 30 and 60 min at 2 µg/mL of Nile red concentration. In addition, in 5 min, the highest fluorescence intensity with 1062 ± 42 a.u. was exhibited at 1 µg/mL and the lowest intensity with 257 ± 9 was shown at 0.1mµg/mL., in other time intervals, 10, 15, 30 and 60 min, results supported us that the highest fluorescence intensities were found at 1 µg/mL. Results were given in Figure 3. 36.

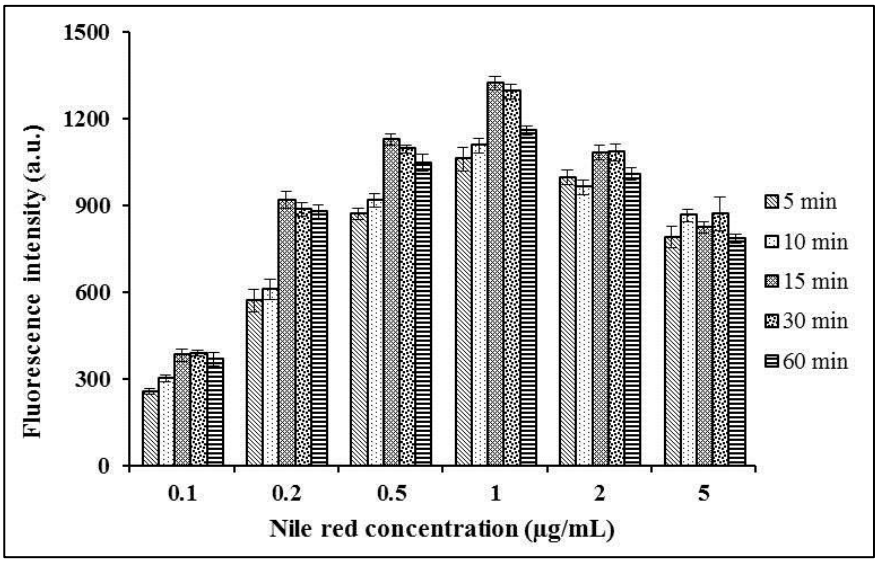

Figure 3. 36. Nile red concentrations and its time dependent graph of *Micractinium sp.*via Nile red method. Data are mean ± standard error from three replicates. There were statistically significant main effects among all Nile red concentrations ($p<0.001$). However, there were nonsignificant main effects between concentration levels of 0.5 and 2 µg/mL.

3.10.2. Determination of Amount of Cell for Measurement of Lipid Content of *Micractinium sp.* Mutants.

The assay was examined by using various Nile red concentrations combined with different cell numbers. We used 5 different amounts of cells (1, 2, 4, 6, 8 x 10^6 cells/mL) and 6 different Nile red concentrations (0.1, 0.2, 0.5, 1, 2, and 5 µg/mL). The highest fluorescence intensity was found with 1556 ± 23 (a.u.) at 8 x 10^6 cells/ mL and at 1 µg/mL of Nile red concentration. At the 1 x 10^6 cells/mL, relative fluorescence intensities were determined with 108 ± 19, 162 ± 11, 180 ± 10, 217 ± 13 and 161 ± 7 and 189 ± 22 a.u. at 0.1, 0.2, 0.5, 1, 2, and 5 µg/mL. in 15 min., respectively. At the 2 x 10^6 cells/mL, we examined that 1 µg/mL of Nile red concentration showed the highest intensity with 430 ± 12 a.u. The lowest intensity was determined at 0.1 µg/mL of Nile red concentration with 170 ± 15 a.u. at 530 nm of excitation and 600 nm of emission. While cell numbers and Nile red concentrations are increasing, fluorescence intensities continued to incerase and

showed linerity between Nile red concentration and cell number. At the 4 x 10^6 cells/mL, fluorescence intensities gained meaning much more and at the 6 x 10^6 cells/mL, Nile red concentrations correlated well with fluorescence intensities. Data were shown in Figure 3.37. At the 6 x 10^6 cells/mL, fluorescence intensities were 383 ± 23, 920 ± 29, 1194 ± 44, 1324 ± 24 and 1084 ± 25 and 826 ± 20 a.u. at 0.1, 0.2, 0.5, 1, 2, and 5 µg/mL. in 15 min. at 530 nm of excitation and 600 nm of emission, respectively. At these results, fluorescence intensities increased up to 1 µg/mL of Nile red concentration and above 1 µg/mL, intensities decreased gradually. We obtained similar results at the 8 x 10^6 cells/mL too. Fluorescence intensities were 490 ± 14, 1022 ± 8, 1381 ± 32, 1556 ± 23, 1370 ± 8 and 1256 ± 15 a.u. at 0.1, 0.2, 0.5, 1, 2, and 5 µg/mL. in 15 min., respectively. Results were given in Figure 3. 37.

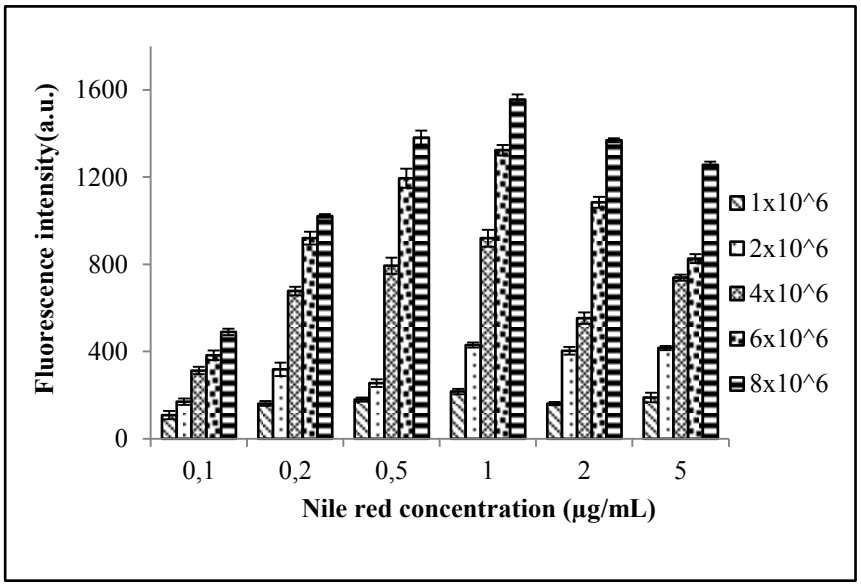

Figure 3. 37. Fluorescence intensities vs Nile red concentrations graph at different cell numbers of *Micractinium sp.*via Nile red method. Data are mean ± standard error from three replicates. Multiple comparisons of Tukey and Bonferroni tests showed that there were highly significant main effects among all Nile red concentrations except for one group ($p < 0.001$). There was no significant difference between 2 and 5 µg/mL of Nile red concentration. Moreover, cell number difference was monitored as highly significant and interaction of cell number and concentration of Nile red was observed ($p < 0.001$).

3.10.3. Correlation between Nile Red Concentration and Cell Numbers for Measurement of Lipid Content of *Micractinium sp.* Mutants.

To determine lipid content of *Micractinium sp.* mutants, we needed method including fast, easy and giving reproducible results. Nile red method was used and optimized for *Micractinium sp.* Suitable excitation and emmision value, Nile red concentration, duration time and cell number were examined.

We combined graphs and obtained correlation between Nile red concentrations and cell numbers. At 0.2 µg/mL of Nile red concentration, flurescence intensities were 162 ± 11, 318 ± 31, 678 ± 20, 920 ± 29 and 1022 ± 8 a.u. at 1, 2, 4, 6 and 8 x 10^6 cells /mL., respectively. When we graphed these results, R^2 value was found with 0.9608. In addition, at 0.5 µg/mL of Nile red concentration, R^2 value (0.9730) was better than 0.2 µg/mL. At this point, intensities were 180 ± 10, 255 ± 17, 793 ± 38, 1194 ± 44 and 1381 ± 32 a.u. at 1, 2, 4, 6 and 8 x 10^6 cells /mL., respectively.

The maximum value was found at 1 µg/mL of Nile red concentration. Its fluorescence intensities were 217 ± 13, 430 ± 12, 920 ± 39, 1324 ±24 and 1556 ± 23 a.u. at 1, 2, 4, 6 and 8 x 10^6 cells /mL., respectively. R^2 value was 0.9843. 1 µg/mL of Nile red concentration was suitable for cell number ranges between 1 and 8 x 10^6 cells /mL for measurement of lipid content of *Micractinium sp.* mutants.

Fluorescence intensities didnt show linearity well when Nile red concentration was 1 µg/mL below or above. In addition, fluorescence intensities were were 161 ± 7, 403 ± 17, 553 ± 27, 1084 ±25 and 1370 ± 8 a.u. at 1, 2, 4, 6 and 8 x 10^6 cells /mL., respectively. R^2 value was 0.9756 at 2 µg/mL. Results were given in Figure 3.38.

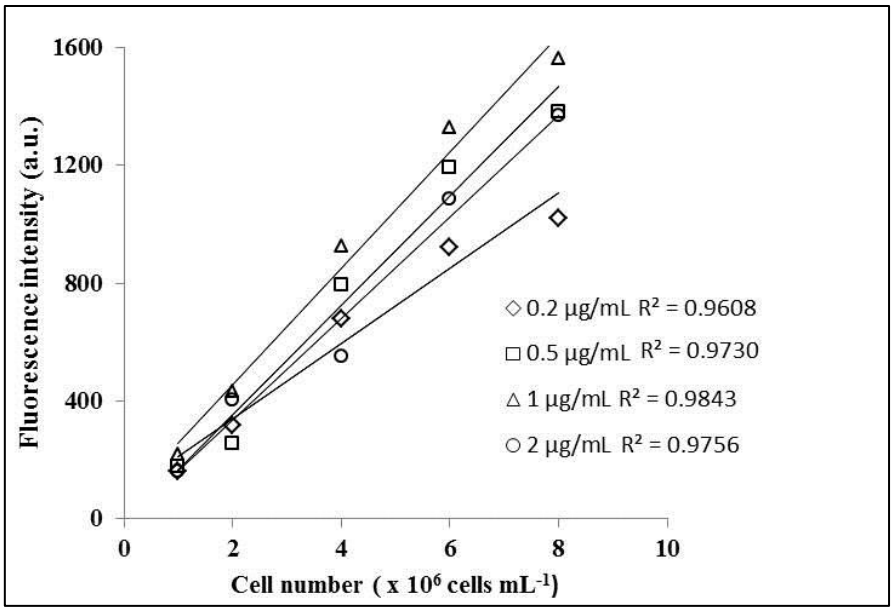

Figure 3. 38. Correlation graph between Nile red concentration and cell numbers for measurement of lipid content of Micractinium sp. mutants via Nile red method. Data are mean ± standard error from three replicates.

Detailed studies of *Micractinium sp.* haven't ever been presented for Nile Red. However, Chen (2009) studied development of conventional Nile red method. For microalgae with rigid cell walls, they used dimethyl sulfoxide (DMSO) and heating procedure and succeed to obtain reproducible results for several microalgae.

Doan et al., (2011) isolated 96 strains of microalgae from marine and screened them with Nile red method at excitation of 480 nm and emmision of 575 nm. They obtained approximately lipid content 45 % of dry weight biomass. Microwave assisted Nile red method was used for *Pseudochlorococcum sp* and *Scenedesmus dimorphus* and obtained higher fluroscence intensity (Chen et al., 2011).

Combination of DMSO and microwave pretreatment was used to pass cell walls of *Chlorella vulgaris* and *Pseudochlorococcum sp* but this method was found ineffective (Ren et al., 2015). Ultrasonic treatment was used without any chemicals and they found that ultrasonic treatment was effective for Nile red method (Ren,

2015). In other study, ethylenediaminetetraacetic acid (EDTA) was used as chemical agent and compared with glycerol and DMSO. EDTA showed higher florescence intensity for especially *Tetraselmis suecica* (Wong et al., 2014).

Pigment contamination sometimes can interfere with florescence intensity of Nile red so Higgins carried out 3 % hypochloride effect on Nile red method. They incubated suspension with 3 % hypochloride in 96 well microplates. They removed pigment and obtained more linear results (Higgins et al., 2014).

In addition to these, Nile red method was applied for wet microalgae (*Scenedesmus sp.*) and succeed with correlation between gravimetric method and Nile red method (Taher et al., 2014a). Teo (2014) used Nile red method for determination of lipid content of Tetraselmis sp. and compared with lipase enzyme and base catalyst (NaOH). Lipase enzyme had 9 folds higher transesterification capacity compared to NaOH (Teo et al., 2014).

3.11. Biodiesel Production and Characterization of Biodiesel

To produce biodiesel from microalgae oils, base-catalysis transesterification procedure was applied in this study. Therefore, It was used 0.1 N KOH as catalyst. Production of biodiesel from microalgae (*Scenedesmus sp.ME02* and *H. tetrachotoma ME03* and *Micractinium sp. ME05*) mentioned at material methods section.

Firstly, biodiesel productivity of the transesterified lipids of all three isolates was carried out. The highest biodiesel productivity with 89±3.1% was found for *Micractinium sp. ME05* grown at 25°C. *H. tetrachotoma ME03* showed biodiesel productivity with 83.7±3%. In addition, *Scenedesmus sp.ME02* had lower biodiesel productivity with 82±4.5% when it was compared with *Micractinium sp. ME05*. On the other hand, *Micractinium sp. ME05* grown at 37°C displayed biodiesel productivity with 87.3±3.3%. These results showed us that base catalysis transesterification reaction can be used for our isolates for biodiesel production.

At the transesterification of microalgae, acid catalyst (H_2S0_4) was used for direct transesterification of *I. zhangjiangensis Nannochloropsis, Tetraselmis subcordiformis, Spirulina platensis, Dunaliella salina, Chlamydomonas reinhartii, Synechocystis sp. 6803, and Chlorella pyrenoidosa*. 300 µg dry cell of microalgae was converted to biodiesel via H_2S0_4 directly (Liu et al., 2015).

Teo et al.,(2014) studied biodiesel production with alkali catalyzed transesterification (NaOH) by means of simultaneous cooling and microwave heating and obtained 83.3 % yield for *Nannochloropsis sp*. (Teo, 2014). Super critical transesterification in methanol and ethanol carried out for *Chlorella protothecoides and* optimum biodiesel yields were found with 90.8 % and 87.8 % in methanol and ethanol, respectively (Nan et al., 2014).
Enzyme catalyts (immobilized lipase) was used for transesterification of *C. Protothecoides* oil and 98 % yield obtained (Park et al., 2014). Teo reported transesterification of *Nannochloropsis oculata* with heterogenous catalyst $Ca(OCH_3)_2$ in methanol carried out and 92 % yield produced (Teo et al., 2014). Lee studied lipase catalyzed transesterification but they also added dimethyl carbonate and found with 90 % yield for *Chlorella sp. KR-1* (Lee et al., 2013).

Immobilized lipase effect in supercritical CO_2 examined for *Scenedesmus sp*. and the highest yield was found with 80 %. Convertion of oil occured at 200 bar, 9:1 methanol:oil molar ratio at 47 ◦C for 4 hours reaction time in the batch system (Taher et al., 2014b). Guerra studied transesterification of lipid for *Chlorella sp*. They used ethanol or hexane for solvent and catalyst (NaOH) with microwave and conventional Bligh and Dyer (BD) method. Maximum lipid yield was obtained with 20.1 % only microwave and microwave and hexane. On the other hand, with conventional Bligh and Dyer (BD) method, lipid yield was 13.9 %. Conversions of lipids were e 96.2%, 94.3%, and 78.1% for only microwave and microwave-hexane, conventional Bligh and Dyer (BD) method, respectively (Guerra et al., 2014).

After microalgae isolates were transesterified, fatty acid contents of *Scenedesmus sp.ME02*, *H. tetrachotoma ME03* and *Micractinium sp. ME05* were analyzed by gas

chromatography to understand biodiesel values of our samples. Results were expressed as relative percentage of the total peak area.

Most abundant fatty acids profiles are C14:0, C16:0, C18:1, C18:2, and C18:3 for freshwater microalgae and other types of fatty acid profiles of microalgae have different composition varierities according to species. (Ben-Amotzet et al.,1985). Actually, superior fatty acid compositions are not available for biodiesel production. However, we can use some guidelines and criteria for quality biodiesel production (Olmstead et al., 2013). Some monounsaturated fatty acids such as palmitoleic acid and oleic acid have advantageous over saturated and polyunsaturated fatty acids (PUFAs) for their desirable oxidative stability, cold flow and combustion properties (Knothe et al., 2009). But, some polyunsaturated fatty acids (PUFAs) are effective for biodiesel fuel on the flow properties under cold weather but can have adverse effects on oxidative stability (Knothe et al., 2008). On the other hand, saturated fatty acids such as palmitic acid and stearic acid have optimum combustion properties but can cause cold flow problems (Jeong et al., 2008).

Orta et al., (2013) studied for *Nannochloropsis oculata* and *Chlorella sp* with different catalysts such as acid catalyst H_2SO_4, base catalysts NaOH, sodium methoxide, and heterogeneous molecular sieve A on evaluation of FAME yields. Reaction with acid catalyst H_2SO_4 displayed the highest FAME yield with 73 ± 5 % for *Nannochloropsis oculata* (Orta et al., 2013).

In another study, FAME profile were affected by ozoflotation and obtained high biomass yield (79.6 %) of *Oscillatoria* and *Cyclotella* with ozone dose of 0.23 mg/mg of dried biomass. They also showed increase in FAME saturation contents (Orta et al., 2014). Daroch isolated and characterized 37 of microalgal strains and applied *in situ* transesterification with two various methods; nitrogen starvation and salt stress for inducing of FAME profile. They found that contents of fatty acids including carbon chains between C14 and C18 increased and amounts of polyunsaturated fatty acid decreased to 12 %. These results were suitable for EN14214 biodiesel standard (Daroch et al., 2013).

Park examined methods for increasing of FAME yield for *Chlorella vulgaris* OW-01. They used different medium including orange peel extract (OPE) for growth of microalgae and obtained 4.5-times more fatty acid methyl esters yields when comparing with conventional medium (Park et al., 2014).

In other study, microalgae were subjected to hydrolysis process, then, applied to esterification reaction and FAME yield was obtained. This result was compared to that of conventional direct transesterification. FAME yield increased 181.7 % by means of esterification of hydrolysates (Takisawa et al., 2013).

Ojeda et al., (2015) isolated and characterized five marine microalgae belonging to *Scenedesmaceae* family. A species, SCRE-2, met European biodiesel standard properties such as density, cetane number kinematic viscosity, iodine number, oxidative stability before N-limitation. In addition to this, two species, DSRE-1 and DSRE-2, were suitable for American biodiesel criteria except for their properties of oxidative stability but not European standard (Ojeda et al., 2015).

Ojo et al., (2014) carried out a shaken, single-use photobioreactor how to affect FAME profile for *Chlorella sorokiniana*. In early stage, microalgae were cultivated and conditions such as shaking frequency were examined. FAME profile changed when shaking increased. For example, while capric acid C(10:0) content was 8.5 %, 0.16 %, 1.62, 0.82% at 70 rpm, 180, 220 (V_f =0.25) and 180 (V_f =0.5), respectively. On the other hand, percentages of palmitic acid (16:0) were 27.18 %, 35.12 %, 33.8, 36.71 % at 70 rpm, 180, 220 (V_f =0.25) and 180 (V_f =0.5), respectively (Ojo, 2014).

Table 3.7: Lipid and biodiesel productivities of *Hindakia tetrachotoma, Scenedesmus sp., Micractinium sp.* based on lipid contents (%) obtained with Soxhlet method with hexane combined with lyophilization and ultrasonication.

Strains	Lipid content (%)	Lipid productivity ($g.L^{-1}. day^{-1}$)	Biodiesel Productivity (%)
Hindakia tetrachotoma	9.3±2.0	0.01±0.002	83.7±3.0
Scenedesmus sp.	15.7±2.4	0.006±0.003	82±4.5
Micractinium sp. (25 °C)	27.3± 2.6	0.05±0.003	89±3.1
Micractinium sp. (37 °C)	11.7± 1.5	0.05±0.001	87.3±3.3

According to our gas chromatography results, *Scenedesmus sp.* ME02 had approximately 25 % of linoleic acid methyl ester (18:2) and linolenic acid methyl ester (18:3). In addition, amounts of other two fatty acid methyl esters were found close to each other. These were palmitic acid methyl ester and oleic acid methyl ester. Palmitic acid methyl ester (16:0) was found with 17.9% and oleic acid methyl ester (18:1) with 16.8%. Detailed chromatogram of *Scenedesmus sp.* ME02 was given in Figure 3.39.

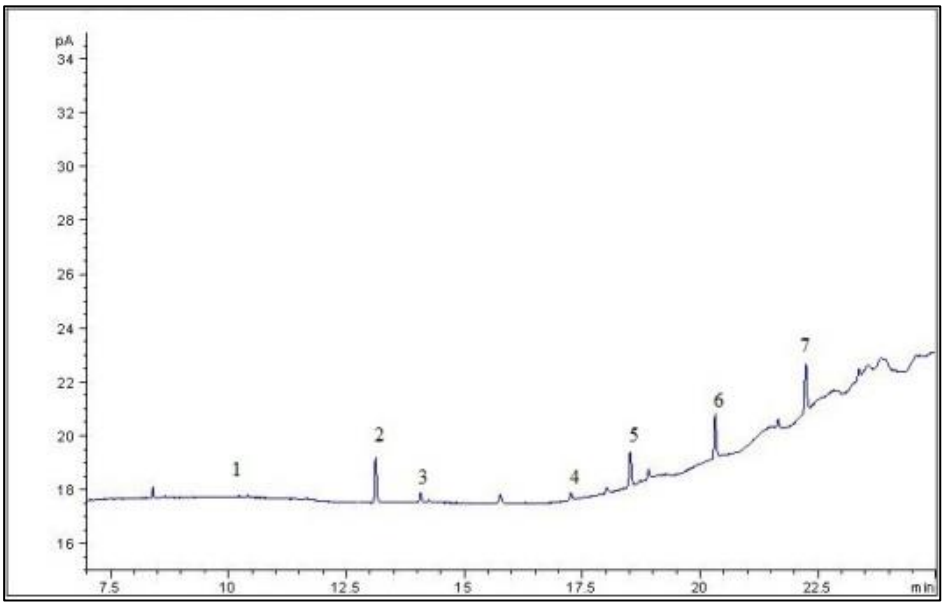

Figure 3. 39. Fatty acid methyl ester (FAME) profiles of *Scenedesmus sp. ME02* at 25°C. 1) Myristic acid methyl ester (14:0), 2) Palmitic acid methyl esters (16:0), 3) Palmitoleic acid methyl esters (16:1), 4) Stearic acid methyl esters (18:0), 5) Oleic acid methyl esters (18:1), 6) Linoleic acid methyl esters (18:2), 7) Linolenic acid methyl esters (18:3).

Another microalgae, *H. tetrachotoma ME03* was composed of 58.7% of eicosenoic acid methyl ester (20:1) and 11.2% of palmitic acid methyl ester (16:0). In addition, myristic acid methyl ester (14:0), palmitoleic acid methyl esters (16:1), heptadesenoic acid methyl esters (17:1), stearic acid methyl esters (18:0), oleic acid methyl esters (18:1), linoleic acid methyl esters (18:2), linolenic acid methyl esters (18:3) were tracely detected. Detailed chromatogram of *H. tetrachotoma ME03* was given in Figure 3.40.

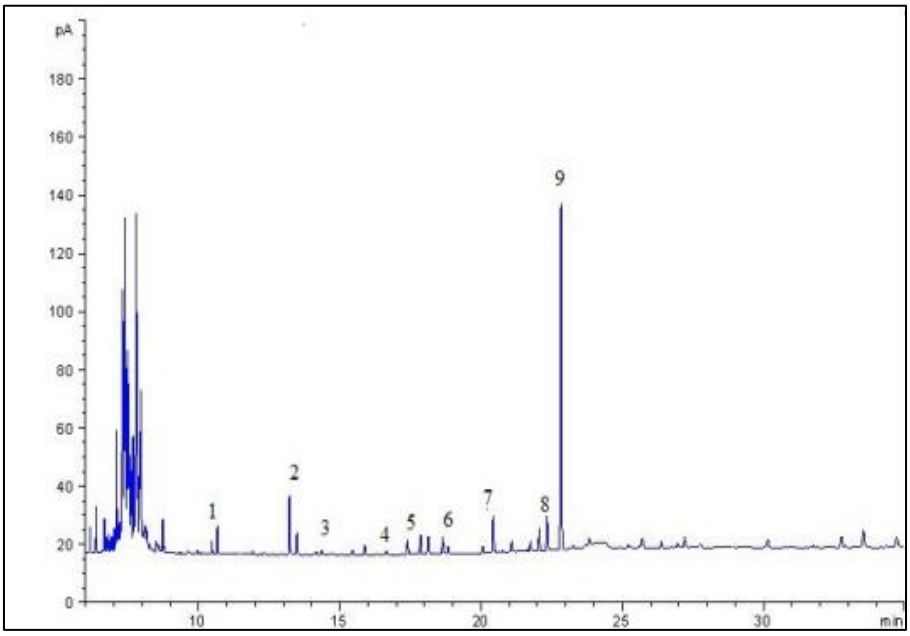

Figure 3. 40. Fatty acid methyl ester (FAME) profiles of *H. tetrachotoma ME03* at 25°C. 1) Myristic acid methyl ester (14:0), 2) Palmitic acid methyl esters (16:0), 3) Palmitoleic acid methyl esters (16:1), 4) Heptadesenoic acid methyl esters (17:1) 5) Stearic acid methyl esters (18:0), 6) Oleic acid methyl esters (18:1), 7) Linoleic acid methyl esters (18:2), 8) Linolenic acid methyl esters (18:3), 9) Eicosenoic acid methyl esters (20:1).

In our other study, the effect of temperature was carried out on fatty acid methyl ester composition of *Micractinium sp. ME05*. Thus, biodiesel content of this isolate grown at 25°C and 37°C was examined because it displayed the highest lipid content and growth rate, respectively. Strikingly, at two different temperatures, not only the lipid content but also the lipid composition of *Micractinium sp. ME05* changed (Figure 3.41).

Micractinium sp. ME05 grown at 25°C included 24.3% of oleic acid (18:1) methyl ester, 20.4% palmitic acid (16:0) methyl ester, 18.2% linolenic acid (18:3) methyl ester, 17.5% linoleic acid (18:2) methyl ester and 10% palmitoleic acid (16:1) methyl ester. In contrast, *Micractinium sp. ME05* grown at 37°C mainly consisted of the same fatty acid methyl esters but their percentages were different. The highest

component at 37°C was linoleic acid (18:2) methyl ester constituting 31.3% of the total amount of fatty acid methyl esters.The rest of the main fatty acid methyl esters was as follows: 17.4% palmitic acid (16:0) methyl ester, 10.2% each of oleic acid (18:1) methyl ester andlinolenic acid (18:3) methyl ester. Percent fatty acid methyl ester (FAME) amounts of *Micractinium sp. ME05*, *Scenedesmus sp. ME02* and *Hindakia tetrachotoma ME03* were given in Table 3.8.

Table 3.8. Percent fatty acid methyl ester (FAME) amounts of *Micractinium sp. ME05, Scenedesmus sp. ME02 and Hindakia tetrachotoma. ME03.* (Onay et al, 2014).

Fatty acid methyl esters	***Micractinium sp.* ME05 (25 ^{0}C) (%)**	***Micractinium sp.* ME05 (37 ^{0}C) (%)**	***Hindakia tetrachotoma* ME03 (%)**	***Scenedesmus sp.* ME02 (%)**
Myristic acid (14:0) methyl ester	1.54±0.22	0.77±0.08	1.3±0.12	0.56±0.06
Palmitic acid methyl esters (16:0)	20.42±1.62	17.44±1.12	11.21±1.78	17.88±2.26
Palmitoleic acid methyl esters (16:1)	9.96±0.7	3.09±0.28	1.49±0.08	5.65±0.77
Heptadecanoic acid methyl esters (17:0)	0.16±0.02	NI	0.18±0.02	NI
Heptadesenoic acid methyl esters (17:1)	0.14±0.02	NI	0.15±0.01	NI
Stearic acid methyl esters (18:0)	2.13±0.06	4.05±0.94	4.33±0.7	2.95±0.27
Oleic acid methyl esters (18:1)	24.33±1.93	10.19±1.7	5.81±0.45	16.78±1.63
Linoleic acid trans 1 methyl esters (18:2)	NI	NI	NI	NI
Linoleic acid trans 2 methyl esters (18:2)	1.41±0.19	NI	1.74±0.2	NI
Linoleic acid trans 3 methyl esters (18:2)	NI	NI	NI	NI
Linoleic acid (18:2)	17.49±1.06	31.28±2.13	1.45±0.12	24.6±1.08
γ - Linoleic acid methyl esters (18:2)	7.01±0.84	0.67±0.09	1.69±0.16	NI
Linolenic acid trans 1 methyl esters (18:3)	NI	NI	NI	NI
Linolenic acid trans 2 methyl esters (18:3)	NI	NI	NI	NI
Linolenic acid trans 3 methyl esters (18:3)	NI	NI	NI	NI
Linolenic acid methyl esters (18:3)	18.16±1.6	10.17±1.23	6.53±1.23	25.5±0.8
Arachidic acid methyl esters (20:0)	0.13±0.05	5.91±0.31	0.29±0.08	4.32±0.44
Eicosenoic acid methyl esters (20:1)	0.17±0.03	NI	58.67±3.16	NI
Behenic acid methyl esters (22:0)	0.07±0.02	NI	NI	NI
Licnoseric acid methyl esters (24:0)	0.27±0.1	NI	NI	NI
Nervonic acid methyl esters (24:1)	NI	NI	NI	NI

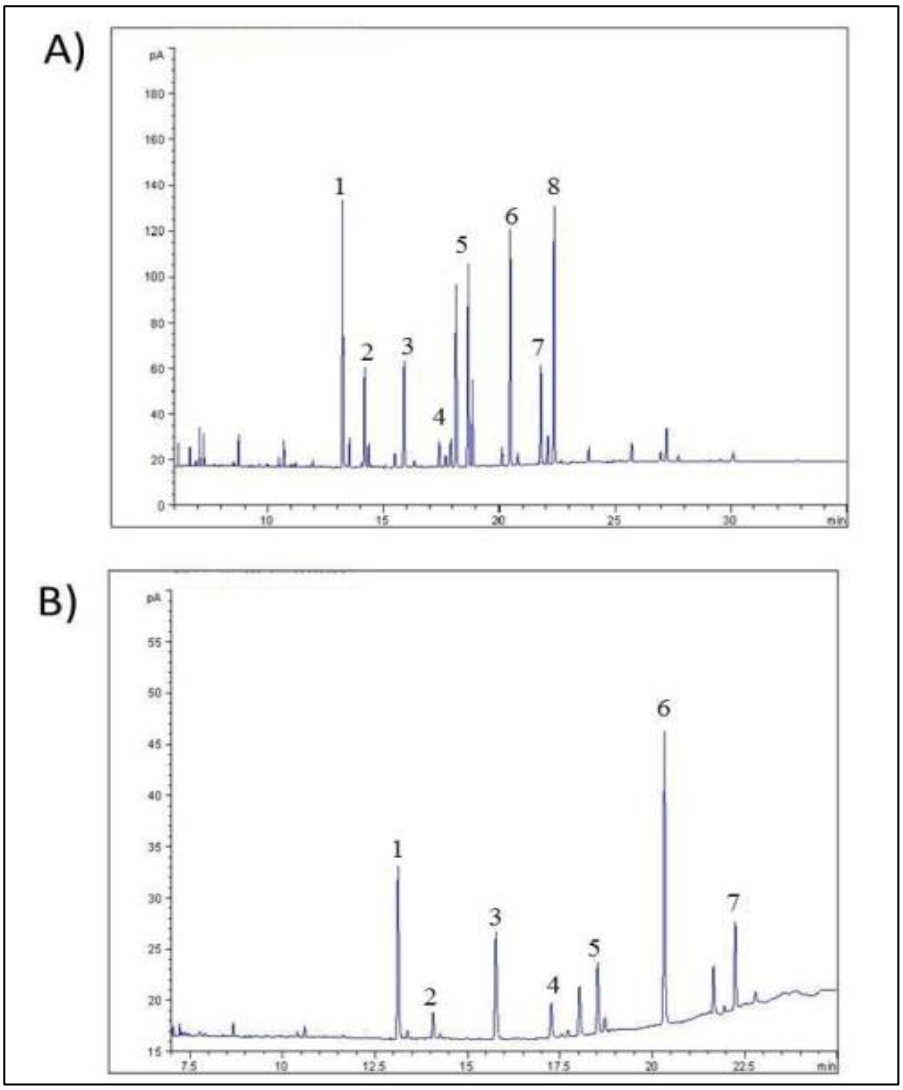

Figure 3. 41. Fatty acid methyl ester (FAME) profiles of *Micractinium sp ME05* at A) 25°C and B) 37°C. A-1) Palmitic acid methyl esters (16:0), 2) Palmitoleic acid methyl esters (16:1), 3) Heptadesenoic acid methyl esters (17:1), 4) Stearic acid methyl esters (18:0), 5) Oleic acid methyl esters (18:1), 6) Linoleic acid methyl esters (18:2), 7) Linolenic acid methyl esters (18:3). B-1) 1) Palmitic acid methyl esters (16:0), 2) Palmitoleic acid methyl esters (16:1), 3) Not identified, 4) Stearic acid methyl esters (18:0), 5) Oleic acid methyl esters (18:1), 6) Linoleic acid methyl esters (18:2), 7) Linolenic acid methyl esters (18:3).

3.12. Genetic Approaches for Improvement of biodiesel production capacity of *Chlamydomonas reinhardtii*

Chlamydomonas reinhardtii is unicellular green microalgae and has two flagella with 10µm long and multiple mitochondria. Scanning electron micrograph (SEM) image of *Chlamydomonas reinhardtii* was given in Figure 3.42. We transformed DNA in *Chlamydomonas reinhardtii* by electroporation.

Different concentrations (12, 72, 200, 660, 1200, 2000 ng) of pHyg3 DNA were used for electroporation. While DNA concentration was increasing, efficiency of electroporation was improved. In addition to this, various voltages (0.6, 1.8, 1,9, 2.2 kV) were applied on electroporation cuvettes and we obtained the highest electroporation efficiency with 0.6 kV. Next, we changed capacities between 10 and 50 µF. We found the highest electroporation efficiency with 2000 ng of pHyg3 DNA, 0.6 kV and 50 µF. Mutants of *Chlamydomonas reinhardtii 137*⁻ were shown in Figure 3. 43.

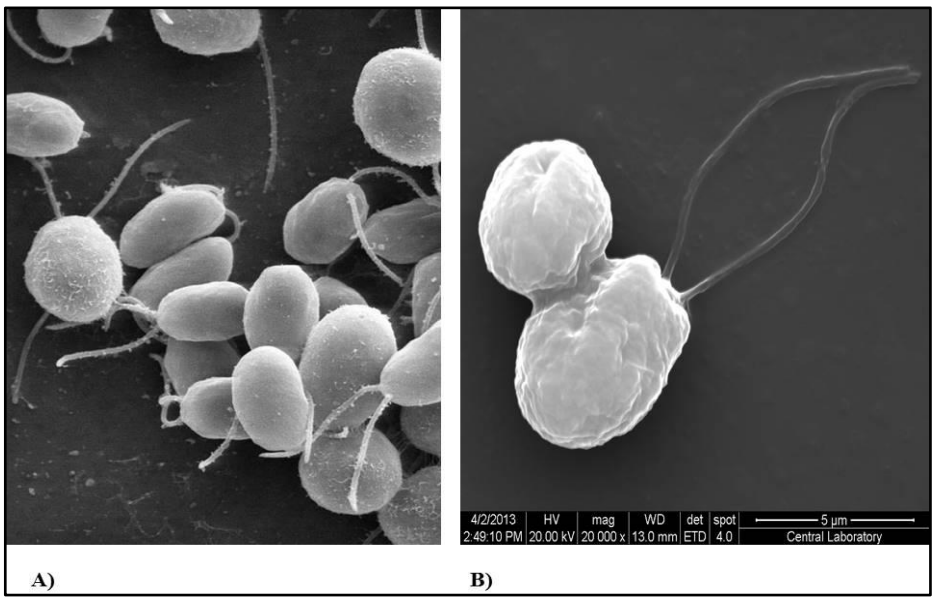

Figure 3.42. Scanning electron micrograph (SEM) images of *Chlamydomonas reinhardtii 137*⁻.

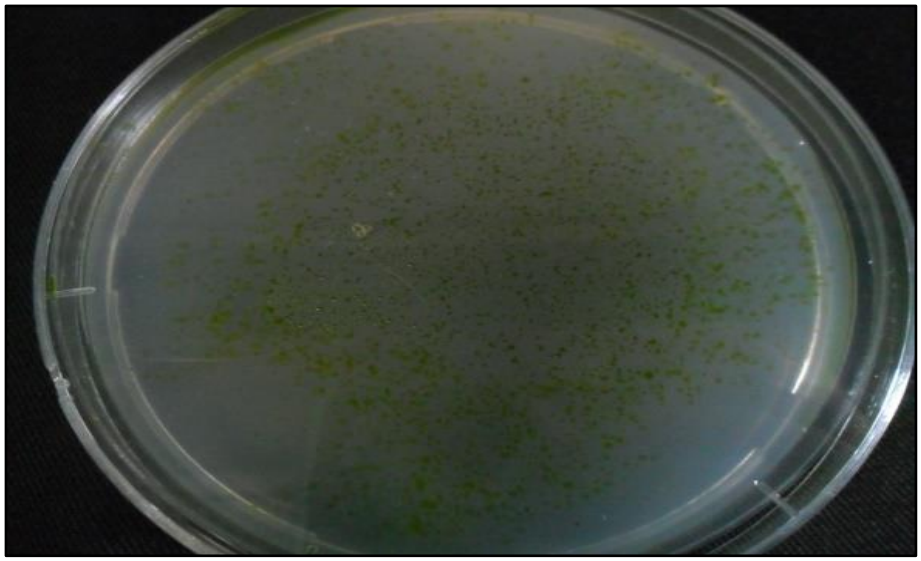

Figure 3.43. Transformation of DNA into *Chlamydomonas reinhardtii 137*⁻ in Tris-Acetate-Phosphate buffer with + Hygromycin at the 0.6 kV voltages, 50 µF capacity and 2000 ng of DNA.

In the current study, we used two different control groups. The first group is *Chlamydomonas reinhardtii 137*⁻ with - Hygromycin without selective marker. *Chlamydomonas reinhardtii 137*⁻ grown as expected and it was displayed in Figure 3. 44.

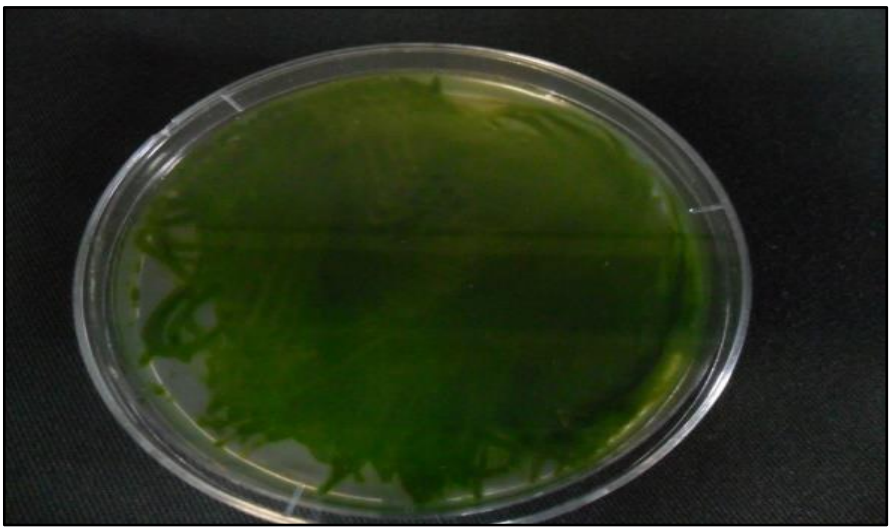

Figure 3. 44. Transformation of DNA into *Chlamydomonas reinhardtii 137*⁻ in Tris-Acetate-Phosphate buffer with - Hygromycin at the 0.6 kV voltages, 50 µF capacity without selective marker.

The second group is *Chlamydomonas reinhardtii 137*⁻ with + Hygromycin without selective marker. In this study, *Chlamydomonas reinhardtii 137*⁻ without selective marker didn't grow on agar plates because of its hygromycin sensitive. It was shown in Figure 3. 45.

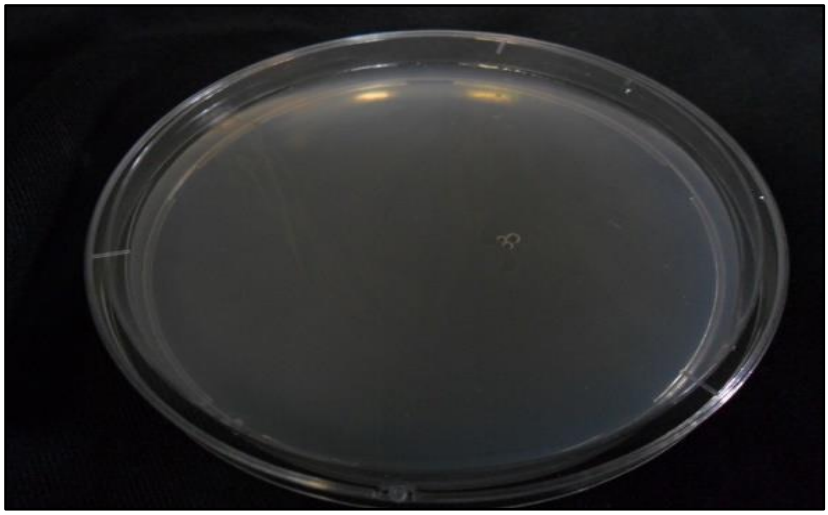

Figure 3. 45. Transformation of DNA into *Chlamydomonas reinhardtii 137*⁻ in Tris-Acetate-Phosphate buffer with + Hygromycin at the 0.6 kV voltages, 50 µF capacity without selective marker.

These mutants transformed by electroporation were grown at 25 ^{0}C, 16 h light-8 h dark photoperiod with a light intensity of 54 µmol m^{-2} s^{-1}. Mutants of *Chlamydomonas reinhardtii 137*⁻ were given in Figure 3. 46.

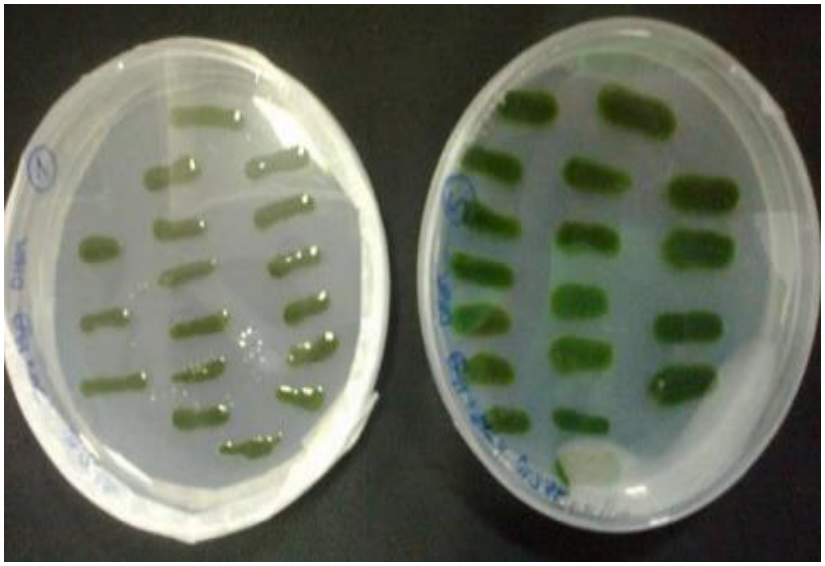

Figure 3.46. Mutants of cultivated *Chlamydomonas reinhardtii 137*- obtained by electroporation on agar plates.

In the literature, *Schizochytrium sp.* was studied for *Agrobacterium tumefaciens* mediated transformation. *Schizochytrium sp.* is classified into Stramenopiles which is kingdom and produces high amount of oil, especially, docosahexaenoic acid. pCAMBIA2301 containing the neomycin phosphotransferase II gene as the selectable marker,resistance to G418, was transfered to *Schizochytrium sp.* by means of *Agrobacterium tumefaciens* and transformants was determined on plates including G418 (Cheng et al., 2012).

Additonally, recombinant microalgae can be used in the industry. Xylitol has five carbon atoms and sugar alcohol. It is used for artificial sweetener. Xylitol genetically produced from *Chlamydomonas reinhardtii* by Pourmir with introduction of interested gene (xylose reductase gene) from fungi *Neurospora crassa* (Pourmir, 2013). Generally, transformation studies related with *Chlamydomonas reinhardtii* need microalgae without cell walls or zinc-containing metallo-protease gametolysin has to be used to remove cell wall. Yamano et al., (2013) succeed transformation of microalgae with its cell wall by means of a square electric pulses-generating electroporator (Yamano et al., 2013).

CHAPTER 4

CONCLUSION

The present study focuses on biodiesel production from thermo-resistant green microalgae isolated from Central Anatolia. Microalgae were sampled from a few hotspring points in Haymana and isolated. These microalgae were identified and characterized morphologically and molecularly. Then, they were named as *Scenedesmus sp.ME02*, *Hindakia tetrachotoma ME03* and *Micractinium sp. ME05.* All three isolates show thermotolerance and are also highly adaptible to a wide range of temperature for growth; an important advantage for cultivation in outdoor bioreactors. It has been shown that two of the isolates (*ME02* and *M05*) are promising candidates for biodiesel production with superior fatty acid methyl ester profiles. At 25 ^{0}C, *Micractinium sp. ME05* showed the highest growth rate and biomass productivity with 5.2 ± 0.04 μ d^{-1} and 0.17 ± 0.007 $g.L^{-1}.day^{-1}$, respectively. Similar to results in 25 ^{0}C, at 37 ^{0}C *Micractinium sp. ME05* had the highest growth rate and biomass productivity with 6.2 ± 0.04 μ d-1 and 0.47 ± 0.02 $g.L^{-1}.day^{-1}$, respectively. Moreover, *Micractinium sp. ME05* had the highest biodiesel productivity with 89 ± 3.1 %. Finally, one of the isolates (*ME05*) stands out with remarkable characteristics such as high growth rate, biomass and biodiesel productivities.

The comparative evaluations of three solvent extraction methods (Soxhlet extraction, Bligh and Dyer and Folch Method) and five cell disruption techniques (Homogenization Assisted, Microwave Assisted, Ultrasonnication, Glass Bead and Lyophilization Assisted) were achieved for effective lipid recovery from three green microalgae. Lipid contents of *Hindakia tetrachotoma ME03* at Soxhlet and Bligh and Dyer methods were near 9.3 % and 7.0 % of dry weight with assistance and no assistance, respectively. Lipid contents of *Hindakia tetrachotoma ME03* were not

affected too much from assistance methods. On the other hand, lipid contents of *Scenedesmus sp. ME02* were found as 15.7 % and 9.3 % of dry weight with assistance (L-A and U-A) and no assistance, respectively, at Soxhlet method. Lipid contents of *Scenedesmus sp. ME02* were not affected from assistance at Bligh and Dyer and Folch methods. Last, lipid contents of *Micractinium sp. ME05* increased two folds in assistance method compare to non-assistance methods at Soxhlet, Bligh and Dyer and Folch methods. Lipid contents of *Micractinium sp. ME05* were 27.3 % and 13.7 % of of dry weight with assistance and no assistance in Soxhlet method.

Moreover, in this study, high throughput fluorescence method was used for quantifying lipid content of *Micractinium sp. ME05* mutants

In the present work, the gene transformation technique (electroporation) was also optimized in model microalgae *Chlamydomonas reinhardii 137⁻ C* for improvement of biodiesel production capacity of microalgae by genomic approaches in the future.

To our knowledge, present study is the first report demonstrating biodiesel production from thermo-resistant green microalgae isolated from Central Anatolia, examining the effects of different lipid extraction and assisted methods in thermo-tolerant *Micractinium sp., Scenedesmus sp. and Hindakia tetrachotoma* strains in detail and demonstrating the optimum conditions of *Micractinium sp. ME05* via fluorescence Nile red method in detail.

Future studies can reveal how all three isolates perform in different and large reactor systems. Additionally, Lipid contens of all three isolates can be increased by genetic engineering methods for obtaining of huge amount of biodiesel production.

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APPENDIX

A. Iodine numbers of various oils used for biodiesel production (Adapted from Yaakob, 2014).

Fat or Oil	**Iodine Value (mg KOH/goil)**
Grape Seed Oil	124-143
Palm Oil	44-51
Olive Oil	80-88
Coconut Oil	07-10
Palm Kernel Oil	16-19
Cocoa butter	35-40
Jojoba Oil	~ 80
Cottonseed oil	100-117
Corn Oil	109-133
Wheat germ Oil	115-134
Sunflower oil	125-144
Linsee Oil	136-178
Soybean Oil	120-136
Peanut Oil	84-105
Ricebran Oil	99-1
Margarine light	37.3
Margarine	86.1
Lard	57.6
Rape oil	11.4
Crude fish Oil	108.5
Tung oil	163.1
Beef Fat	46.9

APPENDIX B

TAP (Tris-Acetate-Phosphate) –Medium (Adapted from Culture collection CCCryo of cryophilic algae).

Stock Solution (SL)	Volume	Component	Concentration in SL	Conc. in final Medium
Tris base	2.42 g	$H_2NC(CH_2OH)_3$ Tris(hydroxymethyl)-aminomethan		$2.00 \cdot 10^{-2}$ M
TAP-salts (Beijerinck salts)	25 mL	NH_4Cl	15 g · L^{-1}	$7.00 \cdot 10^{-3}$ M
		$MgSO_4 \cdot 7H_2O$	4 g · L^{-1}	$8.30 \cdot 10^{-4}$ M
		$CaCl_2 \cdot 2H_2O$	2 g · L^{-1}	$4.50 \cdot 10^{-4}$ M
Phophate solution	1 mL	K_2HPO_4	28.8 g · 100 mL^{-1}	$1.65 \cdot 10^{-3}$ M
		KH_2PO_4	14.4 g · 100 mL^{-1}	$1.05 \cdot 10^{-3}$ M
Trace elements solution (Hutner trace elements)	1 mL	$Na_2EDTA \cdot 2H_2O$	5.00 g · 100 mL^{-1}	$1.34 \cdot 10^{-4}$ M
		$ZnSO_4 \cdot 7H_2O$	2.20 g · 100 mL^{-1}	$1.36 \cdot 10^{-4}$ M
		H_3BO_3	1.14 g · 100 mL^{-1}	$1.84 \cdot 10^{-4}$ M
		$MnCl_2 \cdot 4H_2O$	0.50 g · 100 mL^{-1}	$4.00 \cdot 10^{-5}$ M
		$FeSO_4 \cdot 7H_2O$	0.50 g · 100 mL^{-1}	$3.29 \cdot 10^{-5}$ M
		$CoCl_2 \cdot 6H_2O$	0.16 g · 100 mL^{-1}	$1.23 \cdot 10^{-5}$ M
		$CuSO_4 \cdot 5H_2O$	0.16 g · 100 mL^{-1}	$1.00 \cdot 10^{-5}$ M
		$(NH_4)_6MoO_3$	0.11 g · 100 mL^{-1}	$4.44 \cdot 10^{-6}$ M
Acetic acid, conc.	1 mL	CH_3COOH		

APPENDIX C

BG-11 Medium (Adapted from Andersen, R.A., 2005, Algal culturing techniques).

Component	*Stock Solution* ($g \cdot L^{-1}$ dH_2O)	*Quantity Used*	*Concentration in Final Medium (M)*
Fe Citrate solution		1 mL	
Citric acid	6	1 mL	3.12×10^{-5}
Ferric ammonium citrate	6	1 mL	$\sim 3 \times 10^{-5}$
$NaNO_3$	—	1.5 g	1.76×10^{-2}
$K_2HPO_4 \cdot 3H_2O$	40	1 mL	1.75×10^{-4}
$MgSO_4 \cdot 7H_2O$	75	1 mL	3.04×10^{-4}
$CaCl_2 \cdot 2H_2O$	36	1 mL	2.45×10^{-4}
Na_2CO_3	20	1 mL	1.89×10^{-4}
$MgNa_2EDTA \cdot H_2O$	1.0	1 mL	2.79×10^{-6}
Trace metals solution	(See following recipe)	1 mL	—

Component	*1° Stock Solution* ($g \cdot L^{-1}$ dH_2O)	*Quantity Used*	*Concentration in Final Medium (M)*
H_3BO_3	—	2.860 g	4.63×10^{-5}
$MnCl_2 \cdot 4H_2O$	—	1.810 g	9.15×10^{-6}
$ZnSO_4 \cdot 7H_2O$	—	0.220 g	7.65×10^{-7}
$CuSO_4 \cdot 5H_2O$	79.0	1 mL	3.16×10^{-7}
$Na_2MoO_4 \cdot 2H_2O$	—	0.391 g	1.61×10^{-6}
$Co(NO_3)_2 \cdot 6H_2O$	49.4	1 mL	1.70×10^{-7}

APPENDIX D

D (Sheridan) Medium (Adapted from Andersen, R.A., 2005, Algal culturing techniques).

Component	*Stock Solution* ($g \cdot L^{-1}$ dH_2O)	*Quantity Used*	*Concentration in Final Medium (M)*
Nitrilotriacetic acid	—	0.100 g	5.23×10^{-4}
KNO_3	—	0.103 g	1.02×10^{-3}
$NaNO_3$	—	0.689 g	8.11×10^{-3}
Na_2HPO_4	—	0.111 g	7.82×10^{-4}
$MgSO_4 \cdot 7H_2O$	—	0.100 g	4.06×10^{-4}
$CaSO_4 \cdot 2H_2O$	60.00	1 mL	3.48×10^{-4}
NaCl	8.00	1 mL	1.37×10^{-4}
$FeCl_3$	0.29	1 mL	1.79×10^{-6}
Trace metals solution	—	0.5 mL	—

Trace Metals Solution

First, add 0.5 mL concentrated H_2SO_4 to 950 mL dH_2O, then dissolve or add the other components and bring final volume to 1 liter with dH_2O.

Component	*1° Stock Solution* ($g \cdot L^{-1}$ dH_2O)	*Quantity Used*	*Concentration in Final Medium (M)*
H_2SO_4 (concentrated)	—	0.5 mL	—
$MnSO_4 \cdot H_2O$	—	2.28 g	5.11×10^{-6}
$ZnSO_4 \cdot 7H_2O$	—	0.50 g	8.69×10^{-7}
H_3BO_3	—	0.50 g	4.04×10^{-6}
$CuSO_4 \cdot 5H_2O$	25	1 mL	5.00×10^{-8}
$Na_2MoO_4 \cdot 6H_2O$	25	1 mL	5.17×10^{-8}
$CoCl_2 \cdot 6H_2O$	45	1 mL	9.46×10^{-8}

APPENDIX E

Preparation of 2X CTAB Extraction Solution

Reagents	Amounts
CTAB	2g
1 M Tris. CI (pH:8.0)	10 mL
0.5 M EDTA (pH:8.0)	4 mL
5M NaCI	28 mL

All compounds were dissolved in dH_2O and volume was completed to 100 mL.

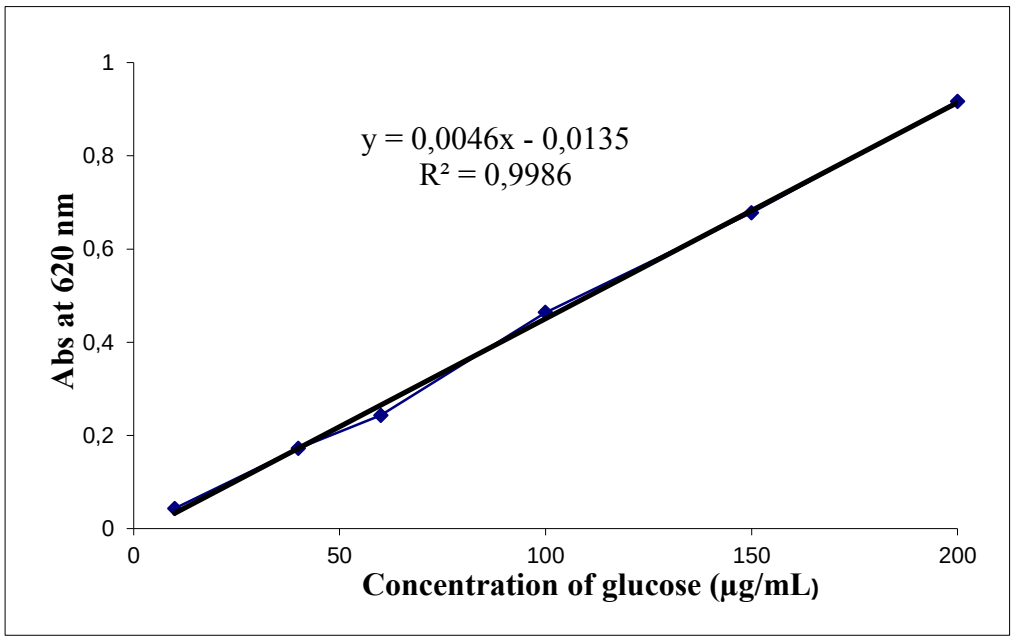
APPENDIX F

Procedure of Nucleospin PCR clean up and gel extraction (Nucleospin PCR clean up and gel extraction, Rev-03, July 2014, Macherey-Nagel).

	PCR clean-up	Gel extraction	DNA clean-up (with SDS)	Single stranded DNA clean-up
1 **PCR clean-up, DNA clean-up, or single stranded DNA clean-up:** Adjust binding condition **Gel extraction:** Excise DNA fragment / solubilize gel slice	 200 µL NTI/ 100 µL PCR	  200 µL NTI/ 100 mg gel 50 °C 5–10 min	 500 µL NTB/ 100 µL sample	 200 µL NTC/ 100 µL sample
2 **Bind DNA**	  11,000 x *g* 30 s			
3 **Wash silica membrane**	700 µL NT3 11,000 x *g* 30 s   ~~Recommended:~~ 2nd wash 700 µL NT3 11,000 x *g* 30 s			
4 **Dry silica membrane**	  11,000 x *g* 1 min			
5 **Elute DNA**	  15–30 µL NE RT 1 min 11,000 x *g* 1 min			

APPENDIX G

Standard curve of glucose for determination of carbohydrate concentration



APPENDIX H

5X Bradford Reagent

Bradford Reagent (5X concentrate)

100 mg	Coomassie Brilliant Blue G-250
47 ml	Ethanol (100%)
100 ml	Phosphoric Acid (85%)

Completed to 200 ml with H_2O.

APPENDIX I

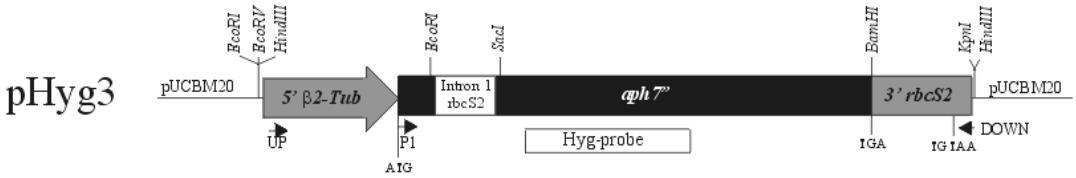
Calculation of lipid content and lipid productivity

$$\textbf{Lipid content (\%):} \quad \frac{\text{g of oil}}{\text{g of dry weight}} \times 100$$

$$\textbf{Lipid productivity (g } L^{-1}d^{-1}\textbf{):} \quad \frac{\text{g of oil}}{\text{(1 L of medium) x 1 day}}$$

APPENDIX J

Hygromycin Resistance Vector (pHyg3) for Chlamydomonas reinhardtii (Adapted from http://www.biologie.uni-regensburg.de/Genetik/Mages/)



Compensatory base change (CBC) table of *ME01, ME03, ME04 and ME06*

32	
Hindakia_tetrachotoma	0 0 0 0 1 0 3 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 3 0 0 0 0
Hindakia_tetrachotoma	0 0 0 0 1 0 2 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 2 0 0 0 0
Hindakia_tetrachotoma	0 0 0 0 1 0 3 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 3 0 0 0 0
Chlorella_sp._CCAP_222/18	0 0 0 0 2 0 2 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 2 1 0 0 0
Heynigia_dictyosphaerioides	1 1 1 2 0 0 2 1 0 1 1 1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 2 2 0 0 0
Chlorella_sorokiniana	0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 1 0 0 0 0
Chlorella_sp._SAG_222-2a	3 2 3 2 2 1 0 1 1 3 2 3 3 3 3 3 3 3 1 1 1 1 1 1 3 1 0 1 1 1 1
Didymogenes_palatina	0 0 0 0 1 1 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 2 1 1 0
Chlorella_sorokiniana	0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 1 0 0 0 0
Hindakia_tetrachotoma	0 0 0 0 1 0 3 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 3 0 0 0 0
Hindakia_tetrachotoma	0 0 0 0 1 0 2 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 2 0 0 0 0
Hindakia_tetrachotoma	0 0 0 0 1 0 3 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 3 0 0 0 0

Hindakia_tetrachotoma	0 0 0 0 0 0 3 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 3 0 0 0 0
Hindakia_tetrachotoma	0 0 0 0 0 0 3 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 3 0 0 0 0
Hindakia_tetrachotoma	0 0 0 0 0 0 3 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 3 0 0 0 0
Hindakia_tetrachotoma	0 0 0 0 0 0 3 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 3 0 0 0 0
Hindakia_tetrachotoma	0 0 0 0 0 0 3 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 3 0 0 0 0
Hindakia_tetrachotoma	0 0 0 0 0 0 3 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 3 0 0 0 0
Hindakia_tetrachotoma	0 0 0 0 0 0 3 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 3 0 0 0 0
METUNERGY1401	0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0
METUNERGY1403	0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0
Didymogenes_anomala	0 0 0 0 0 1 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 2 1 1 0
METUNERGY1404	0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0
METUNERGY1406	0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0
Didymogenes_palatina	0 0 0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0
Hindakia_tetrachotoma	0 0 0 0 0 0 3 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 3 0 0 0 0

Dictyosphaerium_sp.Wolf2000-1	0 0 0 0 2 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0*
Dictyosphaerium_pulchellum	3 2 3 2 2 1 0 1 1 3 2 3 3 3 3 3 3 3 3 1 1 1 1 1 1 3 1 0 1 1 1 1
Chlorella_sp._CCCryo297-06	0 0 0 1 0 0 1 2 0 0 0 0 0 0 0 0 0 0 0 0 2 0 0 1 0 1 1 0 0 0 0
Chlorella_sorokiniana	0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 1 0 0 0 0
Chlorella_sorokiniana	0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 1 0 0 0 0
Chlorella_sp._IFRPD_1018	0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0

Compensatory base change CBC table of *ME02*

40	
Scenedesmus_deserticola	0 1 0 2 0 0 0 0 1 2 1 1 0 0 0 0 0 0 0 0 0 1 0 0 0 1 0 0 1 0 1 0 1 0 0 0 0 0 0 0 1 2
Coelastrum_astroideum	1 0 1 1 1 1 1 1 1 4 1 0 2 1 2 1 1 1 1 3 3 2 1 2 2 1 1 1 1 3 1 0 1 1 1 1 0 2 1 2
Scenedesmus_bajacalifornicus	0 1 0 2 0 0 0 0 1 2 1 1 0 0 0 0 0 0 0 0 0 1 0 0 0 1 0 0 0 1 1 0 0 0 0 0 0 0 0 2
Imbribryum_alpinum	2 1 2 0 2 2 2 2 2 0 2 1 3 2 2 2 2 2 2 3 3 4 2 3 3 3 1 0 1 3 2 0 2 2 0 2 2 2 1 4
Scenedesmus_deserticola	0 1 0 2 0 0 0 0 1 2 1 1 1 0 0 0 0 0 0 1 1 2 0 1 1 1 0 0 1 1 1 0 0 0 0 0 0 0 1 2
Scenedesmus_obliquus	0 1 0 2 0 0 0 0 1 3 1 1 1 0 0 0 0 0 0 2 2 3 0 1 2 1 1 0 1 1 1 0 0 1 0 0 0 0 1 2
Tetradesmus_wisconsinensis_var._reginae	0 1 0 2 0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 1 1 2 0 1 1 1 0 0 1 2 0 0 0 0 0 0 0 0 1 2
Scenedesmus_deserticola	0 1 0 2 0 0 0 0 1 1 1 1 1 0 0 0 0 0 0 1 1 2 0 1 1 1 0 0 0 1 1 0 0 0 0 0 0 0 0 2
Tetradesmus_wisconsinensis	1 1 1 2 1 1 0 1 0 0 0 1 1 1 1 1 1 1 1 1 1 2 1 1 1 1 0 0 1 1 0 0 1 0 0 1 0 1 1 2
Coelastrum_pseudomicroporum	2 4 2 0 2 3 0 1 0 0 2 0 1 3 3 3 1 2 3 1 1 1 2 1 1 0 1 4 3 2 2 1 3 1 4 3 0 3 3 1

Scenedesmus_acutus	1 1 1 2 1 1 0 1 0 2 0 1 1 1 1 1 1 1 2 2 3 1 1 2 1 1 0 1 1 0 0 1 1 0 1 0 1 1 2
Westella_botryoides	1 0 1 1 1 1 1 1 1 0 1 0 4 1 1 1 1 1 1 3 3 4 1 3 2 2 1 0 1 2 1 0 1 1 0 1 1 1 1 3
Scenedesmus_pectinatus	0 2 0 3 1 1 1 1 1 1 1 4 0 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 3 1 0 1 1 1 1 1 1 1 2
Scenedesmus_dimorphus	0 1 0 2 0 0 0 0 1 3 1 1 1 0 0 0 0 0 0 2 2 3 0 1 2 1 1 0 1 1 1 0 0 1 0 0 0 0 1 2
Scenedesmus_obliquus	0 2 0 2 0 0 0 0 1 3 1 1 1 0 0 0 0 0 0 2 2 3 0 1 2 1 1 1 1 1 1 0 0 1 1 0 0 0 1 2
Scenedesmus_naegelii	0 1 0 2 0 0 0 0 1 3 1 1 1 0 0 0 0 0 0 2 2 3 0 1 2 1 1 0 1 1 1 0 0 1 0 0 0 0 1 2
Scenedesmus_deserticola	0 1 0 2 0 0 0 0 1 1 1 1 1 0 0 0 0 0 0 1 1 2 0 1 1 1 0 0 0 1 1 0 0 0 0 0 0 0 0 2
Scenedesmus_deserticola	0 1 0 2 0 0 0 0 1 2 1 1 0 0 0 0 0 0 0 0 0 1 0 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 2
Scenedesmus_obliquus	0 1 0 2 0 0 0 0 1 3 1 1 1 0 0 0 0 0 0 2 2 3 0 1 2 1 1 0 1 1 1 0 0 1 0 0 0 0 1 2
Scenedesmus_sp._Mary9/21T-16W	0 3 0 3 1 2 1 1 1 1 2 3 1 2 2 2 1 0 2 0 0 2 1 0 0 1 1 1 1 2 2 0 2 1 1 2 1 2 1 2
Scenedesmus_sp._Mary9/21BT-19W	0 3 0 3 1 2 1 1 1 1 2 3 1 2 2 2 1 0 2 0 0 2 1 0 0 1 1 1 1 2 2 0 2 1 1 2 1 2 1 2
Scenedesmus_sp._Antarctic	1 2 1 4 2 3 2 2 2 1 3 4 1 3 3 3 2 1 3 2 2 0 2 1 1 0 2 1 1 3 3 0 3 2 1 3 2 3 1 1
METUNERGY1402	0 1 0 2 0 0 0 0 1 2 1 1 1 0 0 0 0 0 0 1 1 2 0 1 1 1 0 0 1 1 1 0 0 0 0 0 0 0 1 2
Scenedesmus_pectinatus	0 2 0 3 1 1 1 1 1 1 1 3 1 1 1 1 1 0 1 0 0 1 1 0 0 1 1 1 1 3 1 0 1 1 1 1 1 1 1 2

Enallax_acutiformis	0 2 0 3 1 2 1 1 1 1 2 2 1 2 2 2 1 0 2 0 0 1 1 0 0 1 1 1 1 3 2 0 2 1 1 2 1 2 1 2
Scenedesmus_raciborskii	1 1 1 3 1 1 1 1 1 0 1 2 1 1 1 1 1 1 1 1 1 1 0 1 1 1 0 1 0 0 3 1 0 1 1 0 1 1 1 0 1
Scenedesmus_acuminatus	0 1 0 1 0 1 0 0 0 1 1 1 1 1 1 1 0 0 1 1 1 2 0 1 1 1 0 0 1 2 1 0 1 0 0 1 0 1 1 2
Coelastrum_microporum	0 1 0 0 0 0 0 0 0 4 0 0 1 0 1 0 0 0 0 1 1 1 0 1 1 0 0 0 2 2 0 0 0 0 0 0 0 1 2 1
Coelastrum_astroideum_var._rugosum	1 1 0 1 1 1 1 0 1 3 1 1 1 1 1 1 0 0 1 1 1 1 1 1 1 1 0 1 2 0 4 1 0 1 1 2 1 1 1 0 1
Scenedesmus_rotundus	0 3 1 3 1 1 2 1 1 2 1 2 3 1 1 1 1 0 1 2 2 3 1 3 3 3 2 2 4 0 1 1 1 2 2 1 1 1 4 4
Scenedesmus_obliquus	1 1 1 2 1 1 0 1 0 2 0 1 1 1 1 1 1 1 1 2 2 3 1 1 2 1 1 0 1 1 0 0 1 1 0 1 0 1 1 2
Scenedesmus_bajacalifornicus	0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0
Scenedesmus_basiliensis	0 1 0 2 0 0 0 0 1 3 1 1 1 0 0 0 0 0 0 2 2 3 0 1 2 1 1 0 1 1 1 0 0 1 0 0 0 0 1 2
Scenedesmus_acuminatus	0 1 0 2 0 1 0 0 0 1 1 1 1 1 1 1 1 0 0 1 1 1 2 0 1 1 1 0 0 1 2 1 0 1 0 0 1 0 1 1 2
Coelastrum_astroideum	0 1 0 0 0 0 0 0 0 4 0 0 1 0 1 0 0 0 0 1 1 1 0 1 1 0 0 0 2 2 0 0 0 0 0 0 0 1 2 1
Scenedesmus_sp._BR2	0 1 0 2 0 0 0 0 1 3 1 1 1 0 0 0 0 0 0 2 2 3 0 1 2 1 1 0 1 1 1 0 0 1 0 0 0 0 1 2
Scenedesmus_dissociatus	0 0 0 2 0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 1 1 2 0 1 1 1 0 0 1 1 0 0 0 0 0 0 0 0 1 2
Scenedesmus_acutus	0 2 0 2 0 0 0 0 1 3 1 1 1 0 0 0 0 0 0 2 2 3 0 1 2 1 1 1 1 1 1 0 0 1 1 0 0 0 1 2

Coelastrum_astroideum_var._rugosum	1 1 0 1 1 1 1 0 1 3 1 1 1 1 1 1 0 0 1 1 1 1 1 1 1 1 0 1 2 0 4 1 0 1 1 2 1 1 1 0 1
Scenedesmus_obtusus	2 2 2 4 2 2 2 2 2 1 2 3 2 2 2 2 2 2 2 2 2 2 1 2 2 2 1 2 1 1 4 2 0 2 2 1 2 2 2 1 0

Compensatory base change CBC table of *ME05*

45	
Micractinium_sp._CCAP_248/16	0 0 1 2 1 0 2 2 2 2 0 0 2 2 2 2 2 1 0 0 0 0 0 2 2 2 2 2 2 2 2 2 2 2 0 0 1 2 2 1 2 1 0 0 2 2
Micractinium_sp._SAG_48.93	0 0 1 1 0 0 1 1 1 1 0 0 1 1 1 1 1 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1 1 0 1 0 1 1 0 1 0 0 0 1 1
Micractinium_sp._TP-2008a	1 1 0 3 1 0 3 2 1 2 1 1 2 2 3 2 3 2 1 1 1 1 1 3 3 3 3 3 3 3 3 3 3 1 1 1 1 3 3 1 2 1 0 1 3 3
Micractinium_sp._CCAP_248/14	2 1 3 0 2 1 3 1 3 3 1 2 3 3 3 3 3 4 1 1 1 1 1 3 3 3 3 3 3 3 3 3 3 3 2 3 2 3 3 2 1 2 3 1 3 3
Micractinium_sp._CCAP_211/92	1 0 1 2 0 0 2 1 3 1 1 1 1 1 2 1 2 1 1 1 1 1 0 2 2 2 2 2 2 2 2 2 2 3 1 0 0 2 2 0 1 0 3 0 2 2
Micractinium_sp._CCAP_231/1	0 0 0 1 0 0 1 1 0 1 0 0 1 1 1 1 1 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1 0 0 1 0 1 1 0 1 0 0 0 1 1
Micractinium_pusillum	2 1 3 3 2 1 0 3 3 3 2 2 3 3 0 3 0 2 2 2 2 2 1 0 0 0 0 0 0 0 0 0 0 3 2 3 2 0 0 2 3 2 1 1 0 0
Micractinium_sp._SAG_72.80	2 1 2 1 1 1 3 0 2 2 1 2 2 2 3 2 3 2 1 1 1 1 1 3 3 3 3 3 3 3 3 3 3 2 2 1 1 3 3 1 0 1 1 1 3 3
Micractinium_sp._TP-2008a	2 1 1 3 3 0 3 2 0 4 1 2 4 4 3 4 3 3 1 1 1 1 1 3 3 3 3 3 3 3 3 3 3 0 2 1 3 3 3 2 2 2 1 1 3 3
Auxenochlorella_protothecoides	2 1 2 3 1 1 3 2 4 0 2 2 0 0 3 0 3 2 2 2 2 2 1 3 3 3 3 3 3 3 3 3 3 4 2 2 1 3 3 1 2 1 3 1 3 3

Hindakia_tetrachotoma	0 0 1 1 1 0 2 1 1 2 0 0 2 2 2 2 2 1 0 0 0 0 0 2 2 2 2 2 2 2 2 2 1 0 1 1 2 2 1 1 1 0 0 2 2
Micractinium_sp._CCAP_248/7	0 0 1 2 1 0 2 2 2 2 0 0 2 2 2 2 2 1 0 0 0 0 0 2 2 2 2 2 2 2 2 2 2 0 0 1 2 2 1 2 1 0 0 2 2
Chlorella_pyrenoidosa	2 1 2 3 1 1 3 2 4 0 2 2 0 0 3 0 3 2 2 2 2 2 1 3 3 3 3 3 3 3 3 3 4 2 2 1 3 3 1 2 1 3 1 3 3
Chlorella_sp._USTB-01	2 1 2 3 1 1 3 2 4 0 2 2 0 0 3 0 3 2 2 2 2 2 1 3 3 3 3 3 3 3 3 3 4 2 2 1 3 3 1 2 1 3 1 3 3
Micractinium_pusillum	2 1 3 3 2 1 0 3 3 3 2 2 3 3 0 3 0 2 2 2 2 2 1 0 0 0 0 0 0 0 0 0 3 2 3 2 0 0 2 3 2 1 1 0 0
Chlorella_pyrenoidosa	2 1 2 3 1 1 3 2 4 0 2 2 0 0 3 0 3 2 2 2 2 2 1 3 3 3 3 3 3 3 3 3 4 2 2 1 3 3 1 2 1 3 1 3 3
Micractinium_pusillum	2 1 3 3 2 1 0 3 3 3 2 2 3 3 0 3 0 2 2 2 2 2 1 0 0 0 0 0 0 0 0 0 3 2 3 2 0 0 2 3 2 1 1 0 0
Micractinium_sp._CCAP_248/13	1 0 2 4 1 0 2 2 3 2 1 1 2 2 2 2 2 0 1 1 1 1 0 2 2 2 2 2 2 2 2 2 3 1 0 1 2 2 1 2 1 1 0 2 2
Hindakia_tetrachotoma	0 0 1 1 1 0 2 1 1 2 0 0 2 2 2 2 2 1 0 0 0 0 0 2 2 2 2 2 2 2 2 2 1 0 1 1 2 2 1 1 1 0 0 2 2
Hindakia_tetrachotoma	0 0 1 1 1 0 2 1 1 2 0 0 2 2 2 2 2 1 0 0 0 0 0 2 2 2 2 2 2 2 2 2 1 0 1 1 2 2 1 1 1 0 0 2 2
Hindakia_tetrachotoma	0 0 1 1 1 0 2 1 1 2 0 0 2 2 2 2 2 1 0 0 0 0 0 2 2 2 2 2 2 2 2 2 1 0 1 1 2 2 1 1 1 0 0 2 2
Hindakia_tetrachotoma	0 0 1 1 1 0 2 1 1 2 0 0 2 2 2 2 2 1 0 0 0 0 0 2 2 2 2 2 2 2 2 2 1 0 1 1 2 2 1 1 1 0 0 2 2
uncultured_Chlorophyta	0 0 1 1 0 0 1 1 1 1 0 0 1 1 1 1 1 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1 0 1 0 1 1 0 1 0 0 0 1 1
Micractinium_pusillum	2 1 3 3 2 1 0 3 3 3 2 2 3 3 0 3 0 2 2 2 2 2 1 0 0 0 0 0 0 0 0 0 3 2 3 2 0 0 2 3 2 1 1 0 0

Micractinium_pusillum	2 1 3 3 2 1 0 3 3 3 2 2 3 3 0 3 0 2 2 2 2 2 1 0 0 0 0 0 0 0 0 0 3 2 3 2 0 0 2 3 2 1 1 0 0
Micractinium_pusillum	2 1 3 3 2 1 0 3 3 3 2 2 3 3 0 3 0 2 2 2 2 2 1 0 0 0 0 0 0 0 0 0 3 2 3 2 0 0 2 3 2 1 1 0 0
Micractinium_pusillum	2 1 3 3 2 1 0 3 3 3 2 2 3 3 0 3 0 2 2 2 2 2 1 0 0 0 0 0 0 0 0 0 3 2 3 2 0 0 2 3 2 1 1 0 0
Micractinium_pusillum	2 1 3 3 2 1 0 3 3 3 2 2 3 3 0 3 0 2 2 2 2 2 1 0 0 0 0 0 0 0 0 0 3 2 3 2 0 0 2 3 2 1 1 0 0
Micractinium_pusillum	2 1 3 3 2 1 0 3 3 3 2 2 3 3 0 3 0 2 2 2 2 2 1 0 0 0 0 0 0 0 0 0 3 2 3 2 0 0 2 3 2 1 1 0 0
Micractinium_pusillum	2 1 3 3 2 1 0 3 3 3 2 2 3 3 0 3 0 2 2 2 2 2 1 0 0 0 0 0 0 0 0 0 3 2 3 2 0 0 2 3 2 1 1 0 0
Micractinium_pusillum	2 1 3 3 2 1 0 3 3 3 2 2 3 3 0 3 0 2 2 2 2 2 1 0 0 0 0 0 0 0 0 0 3 2 3 2 0 0 2 3 2 1 1 0 0
Micractinium_pusillum	2 1 3 3 2 1 0 3 3 3 2 2 3 3 0 3 0 2 2 2 2 2 1 0 0 0 0 0 0 0 0 0 3 2 3 2 0 0 2 3 2 1 1 0 0
Diacanthos_belenophorus	2 1 1 3 3 0 3 2 0 4 1 2 4 4 3 4 3 3 1 1 1 1 1 3 3 3 3 3 3 3 3 0 2 1 3 3 3 2 2 2 1 1 3 3
Micractinium_pusillum	0 0 1 2 1 0 2 2 2 2 0 0 2 2 2 2 2 1 0 0 0 0 0 2 2 2 2 2 2 2 2 2 0 0 1 2 2 1 2 1 0 0 2 2
Chlorella_sorokiniana	0 1 1 3 0 1 3 1 1 2 1 0 2 2 3 2 3 0 1 1 1 1 1 3 3 3 3 3 3 3 3 1 0 0 0 3 3 0 1 0 2 1 3 3
METUNERGY1405	1 0 1 2 0 0 2 1 3 1 1 1 1 1 2 1 2 1 1 1 1 1 0 2 2 2 2 2 2 2 2 3 1 0 0 2 2 0 1 0 3 0 2 2
Micractinium_pusillum	2 1 3 3 2 1 0 3 3 3 2 2 3 3 0 3 0 2 2 2 2 2 1 0 0 0 0 0 0 0 0 0 3 2 3 2 0 0 2 3 2 1 1 0 0
Micractinium_pusillum	2 1 3 3 2 1 0 3 3 3 2 2 3 3 0 3 0 2 2 2 2 2 1 0 0 0 0 0 0 0 0 0 3 2 3 2 0 0 2 3 2 1 1 0 0

Chlorella_vulgaris	1 0 1 2 0 0 2 1 2 1 1 1 1 1 2 1 2 1 1 1 1 1 0 2 2 2 2 2 2 2 2 2 2 1 0 0 2 2 0 1 0 1 0 2 2
Micractinium_sp._SAG_72.80	2 1 2 1 1 1 3 0 2 2 1 2 2 2 3 2 3 2 1 1 1 1 1 3 3 3 3 3 3 3 3 3 3 2 2 1 1 3 3 1 0 1 1 1 3 3
Micractinium_sp._CCAP_211/11F	1 0 1 2 0 0 2 1 2 1 1 1 1 1 2 1 2 1 1 1 1 1 0 2 2 2 2 2 2 2 2 2 2 2 1 0 0 2 2 0 1 0 1 0 2 2
Micractinium_sp._CCAP_248/2	0 0 0 3 3 0 1 1 1 3 0 0 3 3 1 3 1 1 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1 1 0 2 3 1 1 1 1 1 0 0 1 1
Micractinium_pusillum	0 0 1 1 0 0 1 1 1 1 0 0 1 1 1 1 1 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1 1 0 1 0 1 1 0 1 0 0 0 1 1
Micractinium_pusillum	2 1 3 3 2 1 0 3 3 3 2 2 3 3 0 3 0 2 2 2 2 2 1 0 0 0 0 0 0 0 0 0 0 3 2 3 2 0 0 2 3 2 1 1 0 0
Micractinium_pusillum	2 1 3 3 2 1 0 3 3 3 2 2 3 3 0 3 0 2 2 2 2 2 1 0 0 0 0 0 0 0 0 0 0 3 2 3 2 0 0 2 3 2 1 1 0 0

CURRICULUM VITA

PERSONEL INFORMATION

Name & Surname:	Melih Onay
Nationality:	Turkish (T. C.)
Date and Place of Birth:	August 13, 1979, Gaziantep, Turkey
Adress:	Middle East Technical University, Dept. of Biological Sciences, 06800, Ankara, Turkey
Phone:	+ 90 312 210 51 56
Fax:	+90 312 210 79 76
e-mail:	monay@metu.edu.tr; momonay56@gmail.com

EDUCATION

Degree	Institution	Year of Graduation
Ph.D.	METU Biochemistry Dept.	2015
M.Sc.	METU Biochemistry Dept.	2008
B.S.	Ankara Unv. Biology Dept.	2003

ACADEMIC EXPERIENCE

Year	Place	Enrollment
2005- 2015	METU Department of Biochemistry	Research Assistant
2014-2015	GEPEA-CRTT,Universite de Nantes,France	Visiting Researcher

SCHOLARSHIPS AND AWARDS

- International Grant for PhD Students from TÜBİTAK (The Scientific and Technological Research Council of Turkey) (2014- 2015) (Department of Chemical Engineering, CRTT (Centre de Recherce et de Transfert de Technologies, University de Nantes)
- International Grant for PhD Students from TÜBİTAK (The Scientific and Technological Research Council of Turkey) (2013) Department of PMD, Biohydrogen production Center, University of California, Berkeley (UCB), USA
- METU (Middle East Technical University) Course Graduate Performance Award for PhD students. (First prize). (2012).
- National Scholarship Programme for PhD Students from TÜBİTAK (The Scientific and Technological Research Council of Turkey) (2008-2013).
- National Scholarship Programme for MSc Students from TÜBİTAK (The Scientific and Technological Research Council of Turkey) (2005-2008).

PUBLICATIONS / MEETING CONTRIBUTIONS / PUBLISHED ABSTRACTED

Onay, M., Sonmez, C., Oktem, H. A., Yucel, M. Thermo-resistant green microalgae for effective biodiesel production: Isolation and characterization of unialgal species from geothermal flora of Central Anatolia. **Bioresource Technology**. Volume ,169, 2014, pages: 62-71**.**

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Onay, M., Çoruh, N., Celep, F., Doğan, M. Antioxidant capacity of ethyl acetate extract of *Salvia aucheri*. International Food, Agriculture and Gastronomy Congress, February, 15-19, 2012, Belek, Antalya, Turkey.