

BIOCHEMICAL AND GENETIC ASSESSMENT OF
HIGH VALUE LIPID PRODUCTION FROM
LOCAL THERMO-RESISTANT GREEN MICROALGAE STRAIN

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOTECHNOLOGY

JANUARY 2017

Approval of the thesis:

**BIOCHEMICAL AND GENETIC ASSESSMENT OF HIGH VALUE LIPID
PRODUCTION FROM LOCAL THERMO-RESISTANT GREEN
MICROALGAE STRAIN**

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ABSTRACT

BIOCHEMICAL AND GENETIC ASSESSMENT OF HIGH VALUE LIPID PRODUCTION FROM LOCAL THERMO-RESISTANT GREEN MICROALGAE STRAIN

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January 2017, 110 pages

Green microalgae are a rich source of lipids available for animal and human consumption and for an important form of bioenergy; biodiesel. Selection and genetic engineering of a microalgal strain for sustainable and low-cost biotechnological processes is of utmost importance. In this study, a novel thermo-resistant microalgae strain; *Scenedesmus sp.* METUNERGY1402 (ME02) was assessed for high-value lipid production via biochemical and genetic approaches.

An optimized growth medium for efficient growth of *Scenedesmus sp.* ME02 has been developed. In terms of biochemical and physiological aspects of microalgae, temperature is critical parameter in cultivation of microalgae and temperature control

presents an additional cost in outdoor bioreactors. Previously, *Scenedesmus sp.* ME02 was reported to survive at 25-50°C. This study showed that this strain could also withstand temperatures as low as 10°C. The effects of diurnal temperature fluctuation on biomass and lipid productivities of *Scenedesmus sp.* ME02 were examined and compared to other temperature regimes. Fatty acid methyl ester (FAME) profiles of *Scenedesmus sp.* ME02 at different temperatures was also investigated in this study. Significant changes were observed particularly in economically important FAME profiles (palmitic, stearic, oleic, linoleic and linolenic acids). In order to dissect the genetic basis of the changes in fatty acid composition, fatty acid desaturase-2 (*fad2*) gene was identified and partially cloned from *Scenedesmus sp.* ME02.

Finally, *Scenedesmus sp.* ME02 was successfully and stably transformed via genetic engineering technology.

This is the first report of such a laboratory set-up implemented in batch cultures that can provide an insight for outdoor cultivation under extreme temperatures.

Keywords: Green Microalgae, *Scenedesmus sp.* METUNERGY1402, Biodiesel, Biochemical Assessment, Genetic Engineering

ÖZ

ISIL DİRENÇLİ YEREL YEŞİL MİKROALG SUŞUNDAN YÜKSEK DEĞERLİ LİPİT ÜRETİMİNİN BİYOKİMYASAL VE GENETİK AÇILARDAN DEĞERLENDİRMESİ

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Ocak 2017, 110 sayfa

Yeşil mikroalgler, hayvan ve insan tüketimine uygun yağlar ve ayrıca biyoenerjinin önemli bir formu olan biyodizel için zengin lipit kaynaklarıdır. Mikroalg suşunun seçimi ve mühendisliği, sürdürülebilir ve düşük maliyetli biyoteknolojik uygulamalar açısından son derece önemlidir. Bu çalışmada, özgün ve ısı dirençli bir mikroalg suşu olan *Scenedesmus sp.* METUNERGY1402 (ME02), biyokimyasal ve genetik yaklaşımlar aracılığıyla değerli yağ üretimi açısından değerlendirilmiştir.

Bu çalışmada, *Scenedesmus sp.* ME02 suşunun etkili bir şekilde büyümesi için optimize edilmiş besiyeri geliştirilmiştir. Biyokimyasal ve fizyolojik açıdan bakıldığında, mikroalg kültürünün yetiştirilmesinde, sıcaklık kritik bir parametredir ve dış mekân

biyoreaktörleri için sıcaklık kontrolü ek maliyete sebep olmaktadır. Daha önce *Scenedesmus sp.* ME02 suşunun 25-50°C dereceleri arasında yaşayabildiği rapor edilmiştir. Bu çalışmada buna ek olarak, bu türün 10°C kadar düşük bir sıcaklığa dahi dayanabildiği gösterilmiştir. Günlük sıcaklık değişiminin *Scenedesmus sp.* ME02 suşunun biyokütle ve yağ üretimine etkisi incelenmiş ve diğer sıcaklık rejimleriyle karşılaştırılmıştır.

Scenedesmus sp. ME02 suşuna ait farklı sıcaklıklardaki yağ asidi metil ester (FAME) profili bu çalışmada araştırılmıştır. Ekonomik olarak önemli olan yağ asidi metil esterlerinde (palmitik, stearik, oleik, linoleik ve linolenik asitler) anlamlı değişimler gözlemlenmiştir. Yağ asidi kompozisyonundaki değişimlerin genetik temelini incelemek amacıyla, yağ asidi desaturaz-2 (*fad2*) geni tespit edilmiş ve bir kısmı *Scenedesmus sp.* ME02 suşundan başarıyla klonlanmıştır.

Son olarak, *Scenedesmus sp.* ME02 suşuna genetik mühendisliği teknikleri kullanılarak başarılı ve kalıcı bir transformasyon gerçekleştirilmiştir.

Günlük sıcaklık değişimi olan laboratuvar ölçekli kesikli kültür ortamı düzeneği ilk kez bu çalışmada rapor edilmiş ve bu sayede, aşırı uç sıcaklıklarda dış mekân kültürasyon çalışmalarına ışık tutulmuştur.

Anahtar Kelimeler: Yeşil Mikroalgler, *Scenedesmus sp.* METUNERGY1402, Biyodizel, Biyokimyasal Değerlendirme, Genetik Mühendisliği

To my family...

ACKNOWLEDGEMENTS

I owe my deepest gratitude to my supervisor Prof. Dr. Meral Yücel, my co-supervisor Asst. Prof. Dr. Çağla Sönmez and Prof. Dr. Hüseyin Avni Öktem for their guidance, encouragements, patience, kindness and valuable advices.

I would like to thank to examining committee members; Prof. Dr. Füsün Eyidoğan and Assoc. Prof. Dr. Çağdaş Devrim Son for their contributions to my thesis.

I am truly grateful to my dear friends and colleagues Buse İşbilir, Işkın Engin, Evrim Aksu, Onur Bulut, M. Kaan Arıcı, Tuğçe Ceren Tuğrul and Evrim Elçin for their technical and moral supports, valuable comments and great friendships.

I would like to thank all members of Yücel & Öktem Lab family one by one for their great technical and moral supports, valuable comments and friendships.

My most sincere indebtedness belongs to my family; Sumru Akın, Arif Akın, Burak Akın and Tuğba Akın. I am really thankful to them for their constant support, helping me through the difficult times and for all the emotional reinforcements.

I would like to thank METU Central Laboratory Molecular Biology and Biotechnology R&D Center specialists, especially Tezcan Seymenler and Fatma Gül for their technical guidance, helpfulness and valuable comments.

This study was supported by Middle East Technical University-Scientific Research Projects Coordination Office (METU-BAP) Project No: BAP-07-02-2014-007-617 and by Scientific and Technical Research Council of Turkey (TÜBİTAK) 1001-Scientific and Technological Research Projects Funding Program; Project No: 114Z487.

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LIST OF ABBREVIATIONS

Acetyl-coA: Acetyl CoenzymeA

ACCase: Acetyl CoenzymeA Carboxylase

BBM: Bold's Basal Medium

BLAST: Basic Local Alignment Search Tool

bp: base pair

cDNA: Complementary Deoxyribonucleic Acid

CTAB: Cetyltrimethyl Ammonium Bromide

DAG: Diacyl Glycerol

DEPC: Diethylpyrocarbonate

DGAT: Diacylglycerol Acyltransferase

dH₂O: Distilled Water

DNA: Deoxyribonucleic Acid

DMSO: Dimethyl Sulfoxide

DRR: DNase Removal Reagent

μE: Micro Einstein

EtBr: Ethidium Bromide

FAD: Fatty Acid Desaturase

FAME: Fatty Acid Methyl Ester

G3PDH: Glycerol-3-Phosphate Dehydrogenase

GC: Guanine-Cytosine

GC: Gas Chromatography

GFP: Green Fluorescent Protein

ITS2: Internal Transcribed Sequence 2

IDT: Integrated DNA Technologies

KASIII: 3-Ketoacyl Carrier Protein Synthase III

kb: kilo base

kV: kilo Volt

LB Medium: Luria-Bertani Medium

log Phase: Logarithmic Phase

Malonyl-coA: Malonyl CoenzymeA

ME02: METUNERGY1402

μ F: micro Farad

mRNA: Messenger Ribonucleic Acid

NCBI: National Center of Biotechnology Information

nm: nanometer

OD: Optical Density

PCR: Polymerase Chain Reaction

PEG: Polyethylene Glycol

PUFA: Polyunsaturated Fatty Acid

RNA: Ribonucleic Acid

rpm: Revolution per Minute

rRNA: Ribosomal Ribonucleic Acid

RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

-RT-PCR: Negative Reverse Transcriptase Polymerase Chain Reaction

SS: Stock Solution

SOC: Super Optimal Broth with Catabolite Repression

TAE: Tris Acetate EDTA

TAG: Triacyl Glycerol

TAP: Tris Acetate Phosphate

T-DNA: Transferred Deoxyribonucleic Acid

Ti plasmid: Tumor Inducing Plasmid

UV: Ultraviolet

V: Volt

X-Gal: 5-bromo-4-chloro-3-indoyl- β -D-galactosylpyranoside

CHAPTER 1

INTRODUCTION

Green microalgae are important biological resources that have a wide range of biotechnological applications. They have potential uses which benefits environment and economy and rich in commercially and nutritionally valuable lipids also an important form of biofuel; biodiesel (Chu, 2012). Alternative fuels are receiving attention due to energy depletions and environmental pollution caused by fossil fuels. Biodiesel is considered as the main alternative to fossil fuels. Up to now, biodiesel has been obtained from oleaginous seed plants generally. However, plant-derived biodiesel has not been so much desired anymore due to competition with agricultural areas and low oil yield. At this stage, green microalgae become a strong candidate for biodiesel production because of several advantages over higher plants.

1.1. Green Microalgae

Green microalgae are small and unicellular microorganisms living individually, or in groups or in chain. They are classified under Kingdom Protista (Chu, 2012). Their sizes are about 1-50 μm in diameter. They are primitive plants, contain chlorophylls but do not have roots and leaves (Wolkers et al., 2011). They can produce biomass by converting CO_2 and use sunlight as a source of energy. Fixed CO_2 is converted to carbon including compounds like; polysaccharides, carbohydrates, proteins and lipids (Chisti, 2007). Oxygen is produced as a result of the photosynthetic process. During the

photosynthetic process, microalgae produce oxygen. Microalgae can live in freshwater rivers and lakes. Although, microalgae have a wide range of growth characteristics, generally they can double biomass in 24 hours periods. It is presumed that there are more than 50.000 different of microalgae species living in different environments; however only 30.000 of them have been studied (Mata et al., 2010).

1.1.1. Industrial Applications of Green Microalgae

Microalgae are an important bioresource particularly for applications of biotechnology. Since nutritional value of microalgae is very high, they can be used as pharmaceuticals, nutraceuticals, animal feeds and in healthy foods. Some strains are used for therapeutic purposes for example; in diabetes, heart diseases, diarrhea and hypertension. Also, valuable substances can be obtained from microalgae such as proteins, bioactive compounds, polyunsaturated fatty acids (PUFA), phycotoxins and antioxidants etc. Besides these, microalgae are also used for wastewater remediation and assessment of toxic substances in the environment (Chu, 2012). Most of all, green microalgae have great potential as a feedstock for biofuel, biohydrogen and bioethanol production; especially “biodiesel” due to their high lipid content (Huang et al., 2010).

1.1.2. Microalgal Biodiesel

Earth’s average temperature increases gradually due to global warming. Burning fossil fuels is the main contributor of this situation and due to these fuels greenhouse gases are emitted to environment. In this case, biodiesel is a conspicuous alternative to fossil fuels as biodiesel is renewable, nontoxic, biodegradable and environment friendly. It has an important role in decreasing of transportation derived CO₂ emissions (Zeng et al., 2011). Generally, biodiesel is produced from oil crops such as soy beans, canola, palm oil, sunflower and corn oil etc. However, green microalgae are more suitable alternative for biodiesel production (Onay et al., 2014).

1.1.3. Advantages of Microalgal Biodiesel

Producing biodiesel from green microalgae has many advantages compared to plant derived biodiesel. These are mainly;

- (i) The rate of biomass production is faster than that of plants.
- (ii) Photosynthetic efficiencies are higher than plants.
- (iii) Microalgae cells can live in a wide range of various environments.
- (iv) Numerous types of biofuel can be produced from microalgae.
- (v) They do not compete with food market and arable land for crops.
- (vi) Microalgal cells do not divide biomass for their auxiliary parts like plants, therefore harvest and biofuel production process is not difficult as in plants (Zeng et al., 2011).

1.1.4. Cultivation of Microalgae

Strict control and optimization of the culture conditions are necessary for cultivation of microalgae. For high biomass and lipid amount, some parameters such as pH, temperature, salinity, light cycle and intensity, water quality, mineral regulation and nutrient must be strictly monitored.

Growth medium is an important factor for microalgal growth. A suitable growth medium should include necessary nutrients for efficient microalgal growth. Carbon, nitrogen, sulphur and phosphorus are crucial elements for optimum growth and also some inorganic salts such as magnesium, iron and trace elements play an important role. The key point here is improving the balanced growth medium to obtain maximum yield and CO₂ fixation.

Neutral pH is preferred by most of the microalgae species but acidic or basic environments can also be favored by some species. Optimal cell growth can be observed at extreme pH levels as well. CO_2 and NH_4^+ have an important role on pH levels of growth environment.

The main energy source is sunlight for green microalgae, so light intensity is one of the important factors for microalgae growth. For most microalgae strains, metabolic activity accelerates when light intensity increases up to $400 \text{ mE m}^{-2} \text{ s}^{-1}$. Average light intensity is applied generally as $200 \text{ mE m}^{-2} \text{ s}^{-1}$. Light intensity adjustments for best cultivation can be possible for photobioreactors. In open pond cultivation, this strategy may not be valid.

Temperature is also one of the main parameters for effective growth of microalgae. In general, at elevated temperatures metabolic rates of microalgal cells become higher; on the other hand, at low temperatures the reverse situation is observed. In favorable temperature ranges, enzymes of microalgae cells show the maximum activity. Optimum temperature values vary according to species nature. For many microalgae species, optimum temperature range changes between 25°C to 35°C (Zeng et al., 2011).

Microalgae can be cultivated by either heterotrophic or autotrophic strategies. Even though green microalgae cells efficiently use sunlight as an energy source, growth is slower under autotrophic cultivation. Photoinhibition can be seen because of excess sunlight in summer days (Huang et al., 2010). In heterotrophic cultivation, biomass yield is generally higher than that of autotrophic one. Microalgal cells use carbon and nitrogen as an energy source and produce much more CO_2 , which is one of the main reasons of greenhouse gases (Zeng et al., 2011).

In autotrophic cultivation system, microalgae can produce several chemical energy forms including carbohydrates, proteins and lipids. Two main classes of photobioreactors are used in autotrophic microalgae cultivation such as open ponds and photobioreactors. Open pond system is generally used for large scale growth of microalgae (Huang et al., 2010). It is cheaper than photobioreactors and easy to operate however, it is prone to contamination and also stabilizing the cultivation conditions is

very difficult. Because of these disadvantages, photobioreactors are more suitable for biomass output (Zeng et al., 2011). Closed photobioreactors are more efficient for the production of valuable long chain fatty acids and proteins. Providing the aseptic conditions, contamination can be kept out. However, operation and investment is more costly than that of open ponds so it is not very economical in this respect (Huang et al., 2010).

Under laboratory conditions, small scale batch cultures are generally used. Batch cultures are closed systems and there is no input or output of materials during cultivation. This system is relatively simple and easier to operate compared to other cultivation types. Thus, starter experiments are initially conducted in batch cultures.

1.1.5. Growth Dynamics of Microalgae

In batch cultures, growth of microalgal culture is assessed under four phases namely; lag phase, exponential (logarithmic) phase, stationary phase and death phase (Figure 1.1).

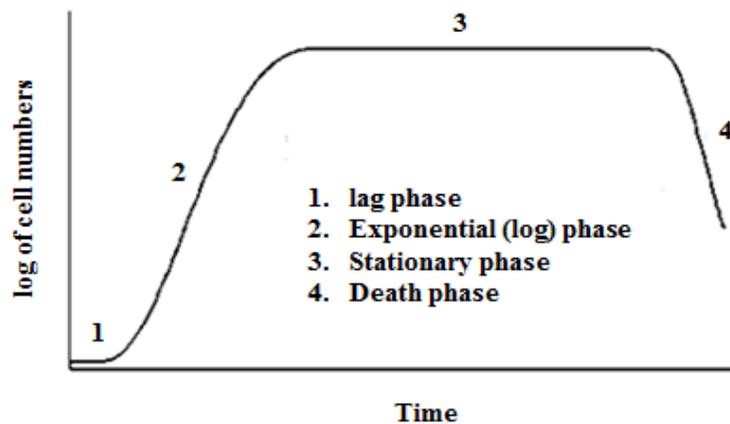


Figure 1.1 Growth dynamics in batch cultures

(Source; <http://www.fao.org/docrep/003/W3732E/w3732e06.htm#TopOfPage>)

During lag phase, only slight growth is observed. This phase is known as the physiological adaptation period to a new cultural environment. In exponential phase,

cell concentration rises as a logarithmic function. At stationary phase, cell density is constant and balanced growth rate is observed. Finally, at death phase, quality of water drops and nutrients are depleted so the number of cells suddenly decreases.

1.2. Biochemistry of Microalgae

1.2.1. Fatty Acid Biosynthesis in Microalgae

For lipid synthesis, microalgae cells utilize both organic (glucose, acetate etc.) and inorganic (CO₂) carbon sources. Lipids are mainly classified as neutral lipids (triacylglycerides) and polar lipids (glycerophosphatides). Triacylglycerides (TAGs) are main the constituents of biodiesel. Synthesis of triacylglycerides is composed of three steps namely; (1) acetyl coenzyme A (acetyl-coA) formation in the cytoplasm, (2) carbon chain elongation and (3) desaturation of fatty acids and biosynthesis of triacylglycerides. Overview of fatty acid biosynthesis pathway is represented in Figure 1.2.

Acetyl-coA carboxylase (ACCase) catalyzes the conversion reaction from acetyl-coA to malonyl-coA in the first step (Huang et al., 2010). ACCase is accepted as the building block of fatty acids and so it is a key enzyme in lipid metabolism (Dunahay et al., 1996). However, overexpression of ACCase studies to increase lipid production was not too successful in diatom and some plants. For example, in one of the studies by Dunahay et al. 1996, overexpression of ACCase was induced in diatom *Cyclotella cryptica* but almost no change in lipid production was observed. In addition, little increase in lipid content was obtained when ACCase was overexpressed in *Solanum tuberosum* (potato) and *Brassica napus* (canola) (Radakovits et al., 2010). Another trial was conducted with 3-ketoacyl-acyl carrier protein synthase III (KASIII) from *Spinacia oleracea* (spinach) and overexpressed in *Nicotiana tabacum* (tobacco). Expression of this protein was increased but lipid production rate did not improve. On the other hand, some achievements have been obtained via overexpressing other enzymes that take part in lipid biosynthesis. For instance, by increasing glycerol-3-phosphate dehydrogenase

(G3PDH) expression in *Brassica napus* and diacylglycerol acyltransferase (DGAT) expression, improvements in lipid production rate was observed (Radakovits et al., 2010). Acyl moiety of acetyl-coA is transferred to *sn*-1,2 diacylglycerol (DAG) via DGAT so TAG is synthesized. Model organism *Chlamydomonas reinhardtii* has 4 types of DGAT isoforms, which are named as DGTT (1-4) (Hung et al., 2013). DGAT manipulation is also a strong candidate for improvement of lipid production. For example, overexpression of DGAT resulted in 10-70% increase in lipid amount of *Arabidopsis thaliana* (Jako et al., 2001). In another study, DGTT2 gene isolated from *Chlamydomonas reinhardtii* and it was transferred to *Arabidopsis*. Approximately 10 fold increase was obtained in lipid amount of transgenic plants and also the newly synthesized lipids has very long chain fatty acids (Sanjaya et al., 2013). These kinds of overexpression studies in key enzymes of lipid biosynthesis pathway can be applied microalgae in order to achieve improvement in fatty acid content.

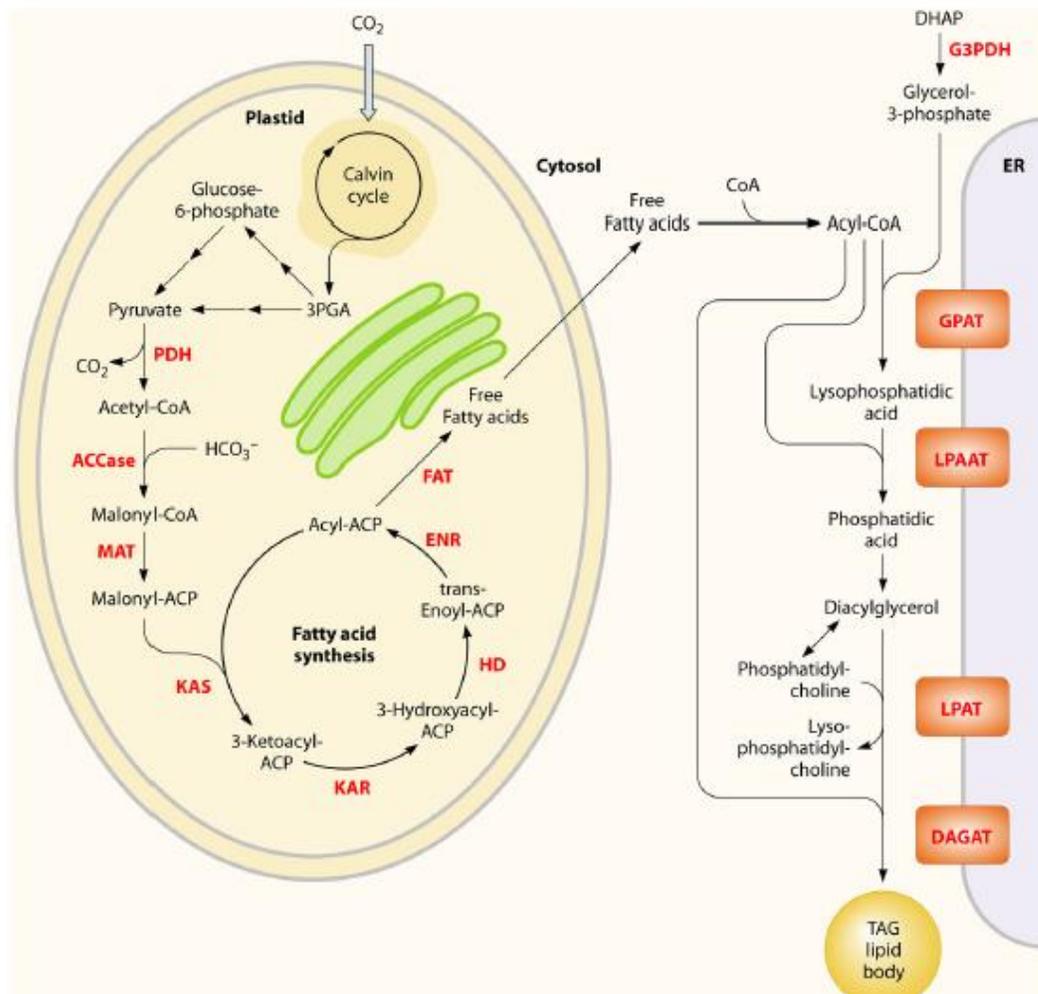


Figure 1.2 Fatty acid biosynthesis pathway in a microalgal cell (Radakovits et al., 2010)

1.2.2. Fatty Acid Desaturases

Fatty acid desaturase enzymes introduce double bond between two carbon atoms in fatty acyl chain (Los & Murata, 1998). There are different fatty acid desaturase enzymes that make fatty acids unsaturated. For instance; fatty acid desaturase-2 (FAD2) and fatty acid desaturase-6 (FAD6) and they are found in endoplasmic reticulum and plastids respectively. (Zhang et al., 2012) They convert monounsaturated oleic acid (18:1) to diunsaturated linoleic acid (18:2) by adding double bond between C12 and C13.

(Kargiotidou et al., 2008) Besides these, fatty acid desaturase-3 (FAD3), fatty acid desaturase-7 (FAD7) and fatty acid desaturase-8 (FAD8) add a double bond at ω 3 position of linoleic acid (18:2) and convert it to linolenic acid (18:3). Expression of genes that encode fatty acid desaturase enzymes are induced at stress conditions. For example, FAD2 and FAD6 increase in return for cold temperatures and drought in plants. According to Browse et al., elevation in unsaturated fatty acid composition of membrane lipids was observed in *fad6* mutant of *Arabidopsis thaliana*. (Zhang et al., 2012) FAD2 and FAD3 take part in PUFA production components of seed oils and these fatty acids are very important for nutrition and used in food applications. In general, all of these enzymes have crucial roles on PUFA production in plants. (Yurchenko et al., 2014) Since PUFAs are suitable for biodiesel production, using this gene in genetic engineering studies is useful for this purpose.

1.2.3. Biodiesel Production from Microalgae

Triacylglycerides (TAGs) are present for energy storage in microalgae and *in vitro* conditions they are converted to biodiesel via a reaction that is known as transesterification. Three long chain fatty acids are present in these neutral lipids (TAGs) and these fatty acids are esterified with glycerol. In transesterification reaction, a reaction occurs between TAGs and methanol and as a result, glycerol and fatty acid methyl esters (biodiesel) are produced (Chisti, 2007; Sharma et al., 2012) (Figure 1.3).

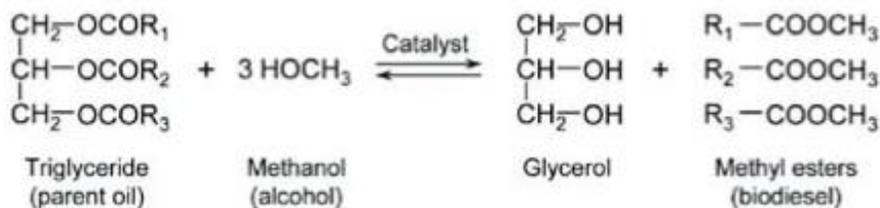


Figure 1.3 Transesterification reaction (Chisti, 2007)

For biodiesel production, neutral lipids are extracted from microalgae cells. According to Onay et al., when lyophilized and ultrasonication assisted Bligh and Dyer lipid extraction method is applied to *Scenedesmus sp.* METUNERGY1402 (ME02), neutral lipids can be extracted efficiently.

Acid or base catalysts are used in transesterification reaction. Base catalyzed reaction is faster than acid catalyzed one. Thus, generally NaOH and KOH base catalysts are used in transesterification. Base catalyzed transesterification reaction is conducted at 60°C and reaction lasts approximately 90 minutes (Chisti, 2007).

The aim of the transesterification is to decrease the viscosity of extracted oil. By transesterification process, large and branched structures of oils were converted to straight chained molecules which are suitable for diesel combustion engines. Fatty acid methyl esters for biodiesel are characterized for their viscosity, density, acid value, cetane number and flash point (Demirbas, 2008). Unsaturation level of fatty acids is significant for biodiesel quality because it affects cold flow property, oxidative stability and ignition quality of diesel which are important parameters for engine performance. Since cetane number of saturated fatty acids is high, they are more suitable for biodiesel production (Piloto-Rodríguez et al., 2017).

Higher lipid content, lipid productivity and biomass productivity rates of microalgal strain are advantageous factors for effective biodiesel production. Additionally, composition of fatty acids of microalgal species significantly affects characteristics of produced biodiesel (Mata et al., 2010).

1.3. Biochemical Assessment of Microalgae

Microalgae are potential source of many products such as biofuels and recombinant proteins, however; cultivation and other processing methods should be optimized for cost-effective large scale production. One way to achieve this is by means of biochemical assessment. Optimization and alterations of light intensity, nutrient supply,

temperature regimes and metabolism of species can create effective and economically valuable strains for biotechnological purposes (Gimpel et al., 2015).

The choice of a suitable growth medium is important for efficient microalgal growth. Essential nutrients such as carbon, nitrogen and phosphorus are supplied within the growth medium. The medium is also enriched with additional micronutrients. Using the best growth medium in microalgae cultivation is crucial for obtaining efficient results from subsequent studies. Previously, different types of media were tried for growth of *Scenedesmus sp.* ME02 namely, Tris-Acetate-Phosphate (TAP) medium, BG-11 (Allen and Stainer) medium and D (Sheridan) medium.

Temperature is a prominent factor for outdoor cultivation systems and often additional cost is required for temperature control. By using a thermo-resistant microalgae strain, this cost can be minimized.

Fatty acid methyl ester (FAME) profile is significant for biodiesel quality. In terms of combustion property, oxidative stability and cold flowing; monounsaturated fatty acids are more suitable rather than saturated and unsaturated fatty acids. On the other hand, polyunsaturated fatty acids make flowing easier especially in cold days but it negatively affects the oxidative stability. In other respects, some problems can be confronted about cold flow of saturated fatty acids. Microalgae synthesize mostly myristic acid (14:0), palmitic acid (16:0), stearic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3); and percentage varies from species to species (Onay et al., 2014).

Biomass productivity and lipid productivity are important parameters for biochemical assessment. Biomass productivity is defined as biomass concentration (dry cell weight per liter of culture) per day. Similarly, lipid productivity is lipid concentration (amount of extracted oil per liter of culture) per day. (Appendix C) When these parameters are measured at different environmental conditions; they are useful to determine suitable environmental conditions for organisms. Additionally, suitable microalgal strain can be assessed by comparing biomass and lipid productivity values.

1.4. Genetic Engineering of Microalgae

Genetic engineering enables introduction of foreign genes into host organisms (Leonor et al., 2012). *Scenedesmus* species has an environmental and economic importance however their genetic studies have not been sufficiently improved yet. Thus, genetic manipulation of *Scenedesmus sp.* needs to be developed. In comparison with bacteria, gene transformation process is slower in microalgae. Up to now, genetic transformation has been constituted only in some of the microalgal species For instance; Guo et al. successfully transformed a chloramphenicol resistance (*CAT*) gene as a marker and *gfp* gene as a reporter to *Scenedesmus obliquus* via electroporation (Guo et al., 2013).

Since *Scenedesmus sp.* ME02 is a novel strain, genetic information is not available in literature and any genetic study has not been conducted to *Scenedesmus sp.* ME02 so far.

1.4.1. Genetic Transformation Methods

Microalgal cell wall is a rigid barrier for microalgal gene transformation. Because of this reason, several different transformation methods have been applied to microalgae (Dunahay et al., 1996). Genetic transformation methods are separated into two branches as direct and indirect transformation. Indirect (biological) method uses bacteria as intermediary for transformation while in direct (physical) method cell wall is penetrated directly. Up to now, *Agrobacterium tumefaciens*-mediated gene transfer, agitation with glass beads, silicon carbide whiskers, microprojectile bombardment (biolistics) and electroporation methods were generally applied on microalgal cells (Leonor et al., 2012).

Agrobacterium tumefaciens-mediated gene transfer system is indirect method, a bacteria is used for transformation in this technique. *Agrobacterium* causes a tumor called as crown gall in plants (Nester, 2014). It bears tumor inducing (Ti) plasmid and plasmid contains T-region. Transferred DNA (T-DNA) is integrated into T-region of Ti plasmid.

When host cell is infected by *Agrobacterium*, T-region is integrated into host nuclear genome. Thereby, desired gene is transferred to host cell (Gelvin, 2003).

Agitation with glass beads, silicon carbide whiskers, biolistics and electroporation techniques are known as direct (physical) genetic transformation methods.

In agitation with glass beads method, membrane fusion is provided by polyethylene glycol (PEG). With glass beads, transient membrane pores are formed and foreign DNA is introduced into microalgae cells by this way (Coll, 2006).

Silicon carbide whiskers are coated with desired DNA molecule and they are added into cell suspension. Silicon carbide whiskers have a property to burst the cells without killing. After addition, mixture is mixed by vortex and DNA bearing whiskers puncture the cell membrane by collisions. As a result, desired DNA molecule enters to the cell by this way. This method can impair the cell and leads to irremediable damages. In addition, increased safety should be provided to avoid inhalation of silicon carbide whiskers since they can cause some hazards on respiratory tract.

In microprojectile bombardment or biolistic, also known as gene gun method, a heavy metal such as gold, tungsten and platinum nanoparticles, is coated with foreign DNA with CaCl_2 solution. These coated particles and cells bombard with each other. When microparticles hit the cells, DNA particles release and enter into cell. Then, foreign DNA integrates into chromosomal DNA. After bombardment, in order to visualize transformation efficiency, transformant cells are inoculated into separate media (Leonor et al., 2012).

Electroporation is most common method since it is rapid, simple and highly effective for genetic transformation. Short and high voltage is applied and it leads to formation of pores on cell wall because electrical field makes polarity change of cell membrane. As a result, foreign DNA enters into cell through these transient pores (Leonor et al., 2012). Despite high efficiency and simplicity of this method, optimization is highly required to deliver DNA molecules properly (Jeon et al., 2013).

In order to get efficient genetic transformation results, some subjects should be considered;

- Risky procedures should be avoided.
- Technique should not be too complex.

- High numbers of transformants should be obtained.
- Process should be easy and economical.
- Foreign DNA should be introduced stably.

When these factors are taken into consideration, the most suitable and efficient method seems as electroporation since it is simple, economical and effective method for microalgal transformation (Guo et al., 2013).

1.4.2. Marker Genes

1.4.2.1. Selectable Markers

Biotechnology in plants relies on delivery, integration and expression of desired gene into cell (Ziemienowicz, 2001). Genetic transformation efficiency is generally not so high and foreign DNA can integrate only small quantities of genome of the cells. Thus, using selectable marker genes become inevitable in order to distinguish transformant cells from non-transformants after plant transformation. Since first invention of transgenic plants, antibiotics, herbicide resistance genes or antimetabolites were used as selectable markers in genetic transformation studies (Sundar & Sakthivel, 2008). The selectable marker gene is transformed together with desired foreign gene. By means of selectable marker, transformed cells can survive on selective agent bearing growth medium (Miki & McHugh, 2004).

Selectable markers are classified as positive and negative, conditional and non-conditional selection systems. Positive selection allows the growth of transformant cells while negative selection eventuates with death of transformants. Positive selection system is divided as conditional and non-conditional positive selection system according to functionality of them. In conditional positive selection system, gene encodes a protein and it generally provides resistance to a certain substrate. Antibiotics, toxic and non-toxic drugs and herbicides are the examples of this system. In non-conditional positive selection system, there are no external substrates however selective growth is promoted (Sundar & Sakthivel, 2008).

In order to select microalgal transformants, different antibiotic resistance genes have been used until now such as, bleomycin, spectinomycin, streptomycin, paramomycin, G418, chloramphenicol, hygromycin and many others. Since many microalgae strains are resistant to wide variety of antibiotics, strain specific antibiotics are in limited number (Radakovits et al., 2010). Antibiotics affect metabolic processes of organism. For instance, aminoglycoside structure of hygromycin blocks the protein synthesis of organisms and ruins ribosomal A site therefore it results in aminoacyl tRNA misreading. Besides, hygromycin influences ribosomal translocation and leads to inaccurate translocation of mRNA (Borovinskaya et al., 2008).

1.4.2.2. Reporter Genes

On the contrary of selectable markers, reporter genes do not present resistance against selective agent. Visually detectable products are produced by these reporter genes. Assaying of reporter genes is easy and it is advantageous for selection. As an example; *lacZ*, *lux*, *luc* and *gfp* reporter genes are generally used in transformation experiments.

E.coli lacZ gene encodes the β -Galactosidase enzyme and it is so practical since it could be assayed easily. (Miki & McHugh, 2004) Enzyme is stable and under favour of this property LacZ is stabilized with glutaraldehyde and visualized by using 5-bromo-4-chloro-3-indoyl- β -D-galactosylpyranoside (X-Gal). However, in plants it is not preferable because detection is complicated than other organisms.

Bacterial or animal luciferases (LUC) are used for detection of gene expression. Bacterial luciferase enzyme is obtained from *Vibrio harveyi*. As a result of expression of *luxA* and *luxB* genes, bioluminescence is observed. Luciferase activity can be observed under 480 nm emission wavelengths. In addition, *luc* gene of firefly *Photinus pyralis* also encodes luciferase enzyme. In consequence of luciferin carboxylation by luciferase, oxyluciferin is formed and luminescence can be observed at 562 nm wavelength. Firefly luciferase has high efficiency and it is suitable for screening procedures such as

determination of transformants and induction kinetic experiments etc. (Ziemienowicz, 2001)

Green fluorescent protein (GFP) is a source of green bioluminescence of jellyfish *Aequorea victoria*. (Miki & McHugh, 2004) GFP fluorescence originates from a chromophore consists of an amino acid sequence; Ser-Tyr-Gly. When Ca^{2+} binds to aquorin molecules, GFP activation takes place. Excitation peak of GFP is about 480 nm while emission is at 509 nm. GFP is useful reporter gene due to a number of reasons. First of all, it is easy to detect by using standard ultraviolet (UV) light source and *in vivo* detection is feasible. Secondly, substrate is not necessary for GFP on the contrary of other reporter genes. Thirdly, due to its small protein size, fusion is possible. Because of these reasons, *gfp* is widely used reporter gene for detection of transformants, *in vitro* gene expression studies, localization of proteins, studying transmission of proteins, labeling cells and unicellular organisms. (Ziemienowicz, 2001) In this study, GFP marker was used for detection of transformed cells.

1.5. Green Microalgae Species Used in Study

Two different green microalgae species were used in this study namely; a local strain *Scenedesmus sp.* ME02 and model organism *Chlamydomonas reinhardtii*.

1.5.1. *Scenedesmus sp.* METUNERGY1402 (ME02)

The primary microalgal strain used in study is *Scenedesmus sp.* ME02. It was isolated from thermal springs of Haymana, Ankara by a previous PhD student in our laboratory (Onay, 2015). Despite of several advantages of salt water microalgae species compared to freshwater microalgae; in this study, freshwater microalgae were used since they have tolerance to wide temperature ranges. Detailed morphological and molecular characterization of this strain was done previous to this study and published (Onay et al., 2014). Amplification and bioinformatics analysis of the internal transcribed sequence 2

(ITS2) region of this strain was carried out and the strain was identified at the genus level. Phylogenetic tree is shown in Figure 1.4. Since it was isolated from thermal waters, this strain is resistant to high temperatures up to 50°C. This strain is very suitable for outdoor cultivation because of its thermo-tolerant nature. *Scenedesmus sp.* ME02 cells are small, unicellular, non-motile and are about 2-3µm in diameter. Scanning electron microscopy image of *Scenedesmus sp.* ME02 cells is seen in Figure 1.5. *Scenedesmus sp.* can be grown in commonly Tris- Acetate-Phosphate (TAP) and BG-11 growth media and optimum growth temperature is 25°C. It has richer FAME content therefore, so suitable for biodiesel production. Some biochemical and metabolic alterations of *Scenedesmus sp.* ME02 cells can be derived by manipulation of growth medium, nutrient content, growth conditions and genetic transformation (Kim et al., 2007).

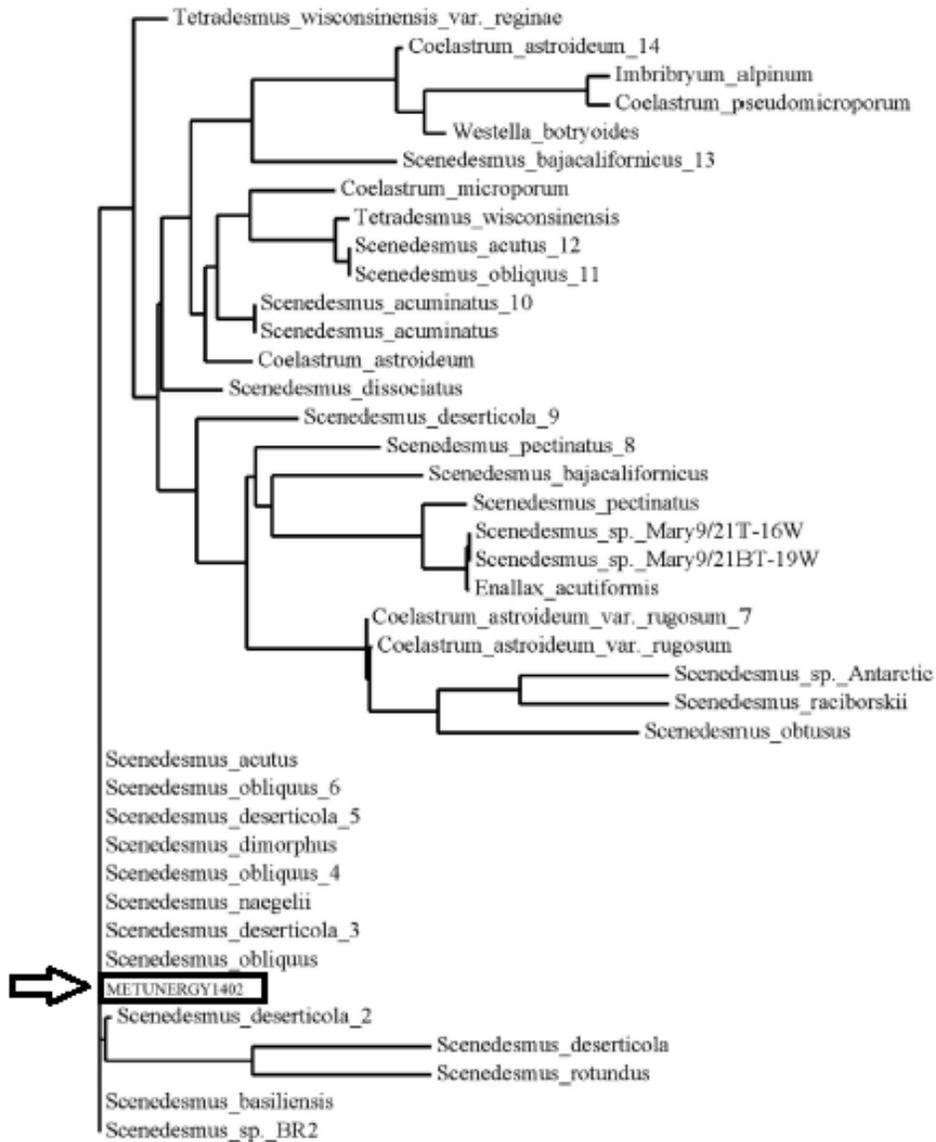


Figure 1.4 Phylogenetic tree of some microalgae species (Onay et al., 2014)

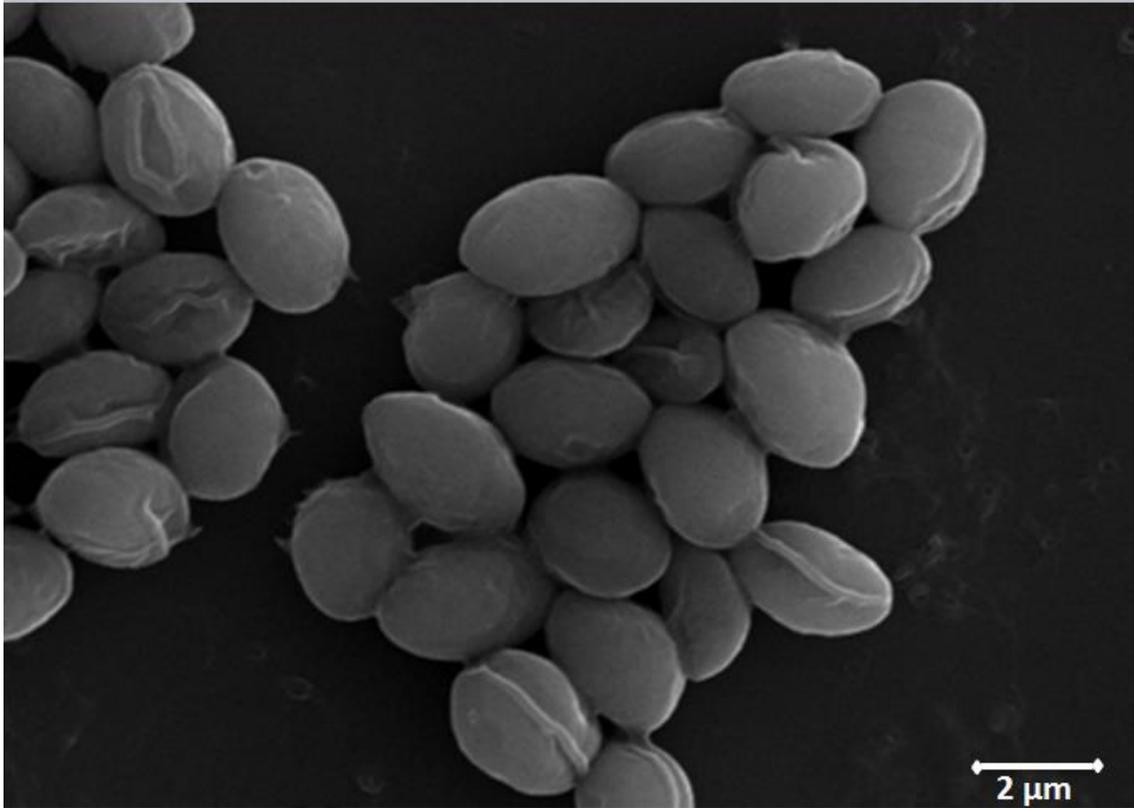


Figure 1.5 Scanning electron microscope image of *Scenedesmus sp.* ME02 cells (10000X magnification) (Onay et al., 2014) (METU Central Laboratory, SEM Facility)

1.5.2. *Chlamydomonas reinhardtii*

Chlamydomonas reinhardtii is small, single celled green microalgae with 5-8 μm diameters in size. It moves via its flagella (Figure 1.6). It is known as a model organism and the complete genome sequence is known and genetic engineering studies are available (Hung et al., 2013).

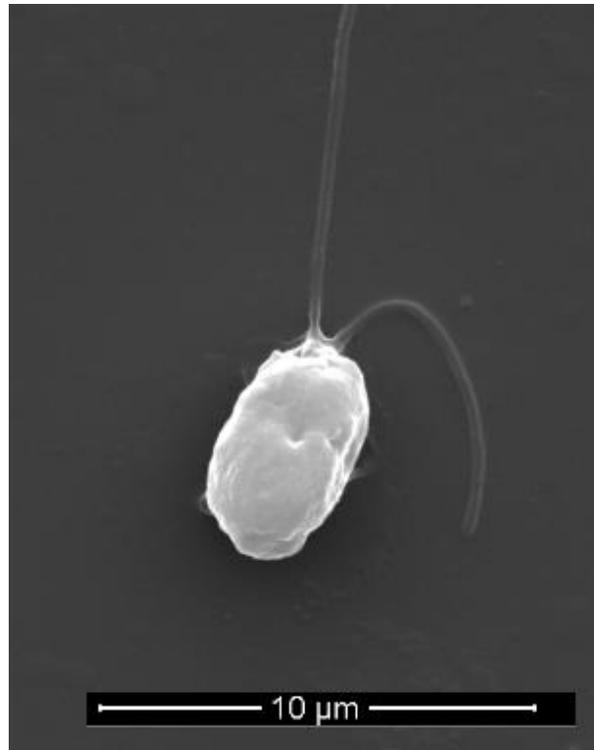


Figure 1.6 Scanning electron microscopy image of *Chlamydomonas reinhardtii* (10000X magnification) (Onay et al., 2014) (METU Central Laboratory, SEM Facility)

1.6. Aim of the Study

The main aim of this study was to assess the optimum growth conditions of *Scenedesmus sp.* ME02 for high lipid and biomass productivities by biochemical and genetic approaches under laboratory conditions. In order to reach this goal, *Scenedesmus sp.* ME02 were grown in different growth media and effect of different temperatures on biomass and lipid compositions were determined. This MSc thesis also aimed to set a stage for future genetic engineering studies of *Scenedesmus sp.* ME02 by cloning of *fad2* gene and optimization of stable genetic transformation into this microalgal strain. In this respect, this study will be a reference for future large scale bioreactor cultivation of *Scenedesmus sp.* ME02.

CHAPTER 2

MATERIALS & METHODS

2.1. MATERIALS

2.1.1. Chemicals

All of the chemicals that were used in this study were purchased from Merck, Sigma-Aldrich, AppliChem, Thermo Fisher Scientific, Qiagen, NanoBiz and Invitrogen companies.

2.1.2. Buffers and Solutions

All solutions were prepared by using distilled water. Ingredients and compositions of solutions and buffers were explained in Appendix A.

2.1.3. Microalgae and Bacteria Strains

In this study, *Chlamydomonas reinhardtii* and *Scenedesmus sp.* METUNERGY1402 (ME02) was used. *Chlamydomonas reinhardtii* strain was supplied by Dr. Claire Remacle's laboratory in Belgium. *Scenedesmus sp.* ME02 was isolated from geothermal waters of Haymana, Ankara and characterized in our laboratory (Onay et al., 2014).

One Shot Top 10 *E.coli* (Invitrogen) strain was used as competent bacterial cell. These cells were supplied commercially.

2.1.4. Primers

Primers that were used in this study were designed using Primer3 software. Five different designed primer sequences used in this study are listed in Appendix B. FAD2 primer set was used for amplifying *fad2* genes of *C. reinhardtii* and *Scenedesmus sp.* ME02. M13 primers were used as universal primers and they took part in colony PCR. Aph7 forward and reverse primer set was used to amplify hygromycin resistance sequence on pHyg3 plasmid via PCR. GFPqPCR forward and reverse primers were designed for conventional and RT-PCR amplification of GFP fragment on pChlamy_3-GFP construct. Internal transcribed sequence-2 (ITS2) primer set was used for amplification of internal control in RT-PCR experiments.

Stock solutions of PCR primers was prepared as 100 μ M with MilliQ water according to manufacturer's guide and stored at -20°C for long term. For PCR reaction, 10 μ M primer solutions were prepared from stock solution.

All designed primers were purchased from Integrated DNA Technologies (IDT), USA.

2.1.5. Plasmids

pHyg3 plasmid was supplied from Dr. Claire Remacle's laboratory in Belgium. This plasmid is 4376 base pair in length. It has *aph7* gene that provides Hygromycin resistance to host organism (Berthold et al., 2002) (Figure 2.1).

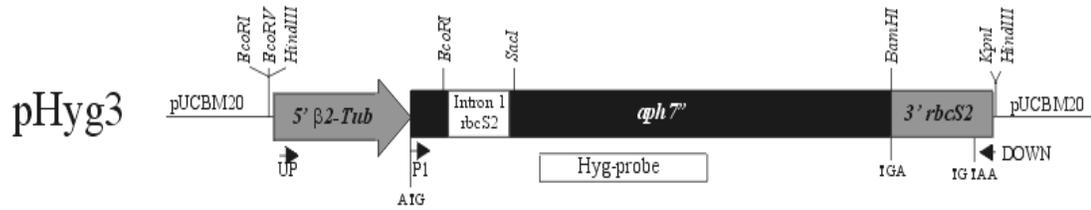


Figure 2.1 pHyg3 plasmid map

pChlamy_3 and pCR4 TOPO vectors were purchased from Invitrogen (Figure 2.2 and 2.3). Entire length of pChlamy_3 vector is 4517 base pair and pCR TOPO vector is 4 kilobase. These vectors contain multiple cloning site (MCS) with different restriction sites and these allow insertion of gene of interest properly. pChlamy_3 contains *aph7* gene for resistance to Hygromycin while pCR4 TOPO vector contains Ampicillin resistance gene fragment.

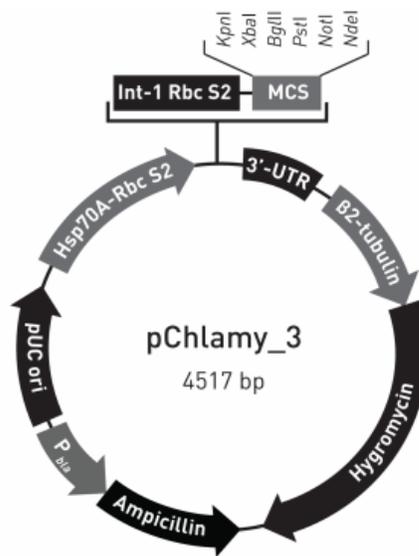


Figure 2.2 pChlamy_3 vector map

(Source; Invitrogen Gene Art *Chlamydomonas* Engineering Kit User Guide)

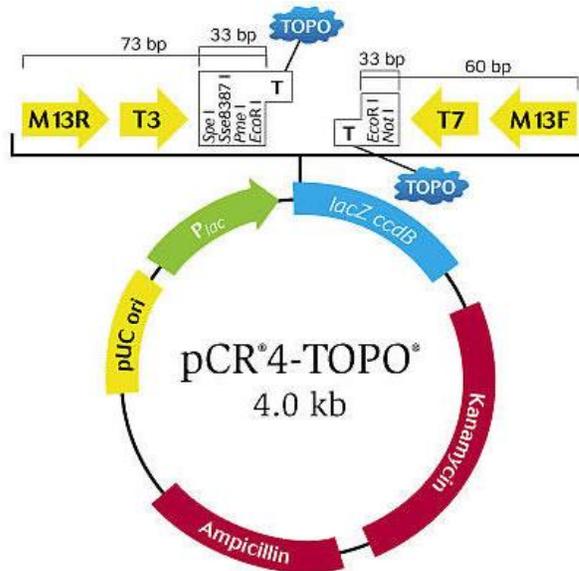


Figure 2.3 pCR4-TOPO vector map

(Source; Invitrogen TOPO TA Cloning Kit for Sequencing User Guide)

2.2. METHODS

2.2.1. Growth Conditions of Microalgae Strains Used in the Study

Daily maintenance of *Scenedesmus sp.* ME02 and *C. reinhardtii* were generally conducted in either petri plates with 1.5% agar or in flasks with liquid medium at 25°C and 16 hours light-8 hours dark photoperiod with constant shaking at 150 rpm in acclimation chamber (Nüve GC400). Lighting was supplied with fluorescent lamps with the intensity of $54 \mu\text{E m}^{-2} \text{s}^{-1}$ in laboratory conditions. Tris-Acetate-Phosphate (TAP) medium (pH:7) and BG-11 (pH:8) (Appendix A; Table A.1 and Table A.2) were the media for culturing *Scenedesmus sp.* ME02 under laboratory conditions. Only TAP medium was used to nourish *C. reinhardtii*. Stock cultures of microalgae cells were keep on the TAP agar plates and these plates were renewed monthly with streak plate technique (Figure 2.4 A and B).

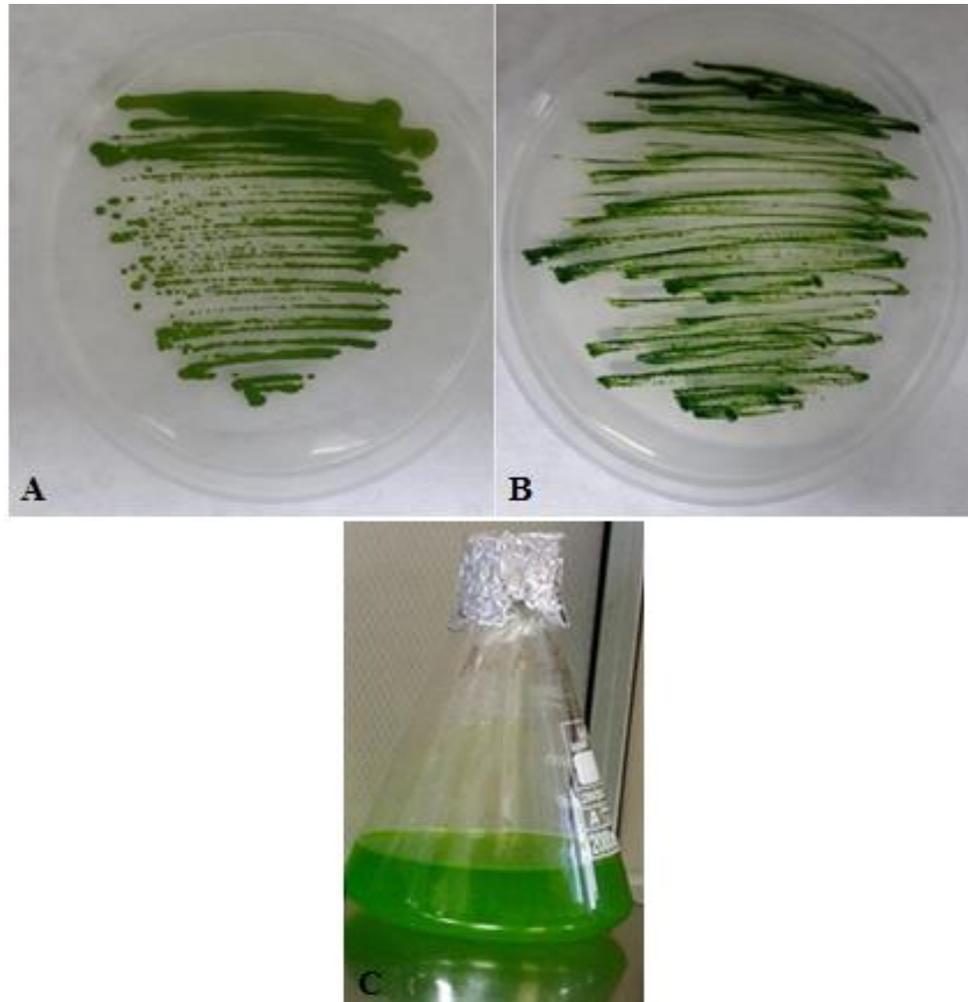


Figure 2.4 Microalgae cultures **A.** *Scenedesmus sp.* ME02 cells on solid agar plate **B.** *Chlamydomonas reinhardtii* cells on solid agar plate **C.** Liquid culture of microalgae cells

In order to prevent contamination, aseptic techniques were applied when microalgae were cultured. Cultures were handled in Type II laminar flow hood (Metisafe) in order to protect samples from contamination. Differently from other types of hoods, Type II laminar flow hood has HEPA filter so aseptic procedures can be conducted by preventing contamination of samples. Before working in the hood, it was sterilized with ultraviolet (UV) light and the base of the hood was wiped with 70% ethanol. The reason

of using 70% ethanol rather than absolute ethanol is contact coagulation of proteins of microorganisms in pure ethanol. If pure ethanol is used as antiseptic agent, cells become inactive but not dead. Bunsen burner was turned on in the hood and experiment was done near the flame. All of the equipment and liquids which were used in experiment were sterilized via autoclave before use.

Before starting the any experiment, starter culture was made. A loopful of microalgae cells from solid agar plate were taken via inoculation loop and inoculated into 50-60 liquid medium. Cells were grown at 25°C and 16 hours light-8 hours dark photoperiod with constant shaking at 150 rpm in acclimation chamber for 4-5 days and they were ready to be taken into fresh liquid media for subsequent experiments (Figure 2.4 C).

2.2.2. Cell Counting of *Scenedesmus sp.* ME02

Cell counting was done via hemocytometer. Microalgae culture was grown in 50 mL growth medium at 25°C for 5-6 days. Optical density (OD) was measured at 680 nm wavelength via spectrophotometer. Then, serial dilution was done namely; 1:2, 1:4, 1:8, and 1:16 (Figure 2.5).

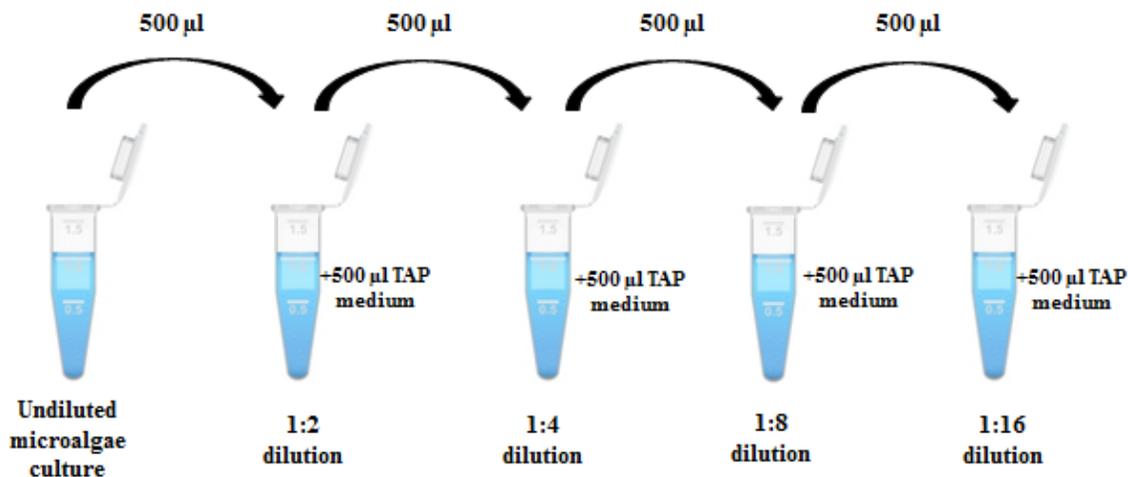


Figure 2.5 Serial dilution technique

About 5 μL formaldehyde was put into each tube in order to prevent movement of the microalgae cells. 10 μL cells were taken with pipette and injected between hemocytometer and coverslip carefully. After slide preparation was completed, it was observed and cells were counted under light microscope with 40X objective.

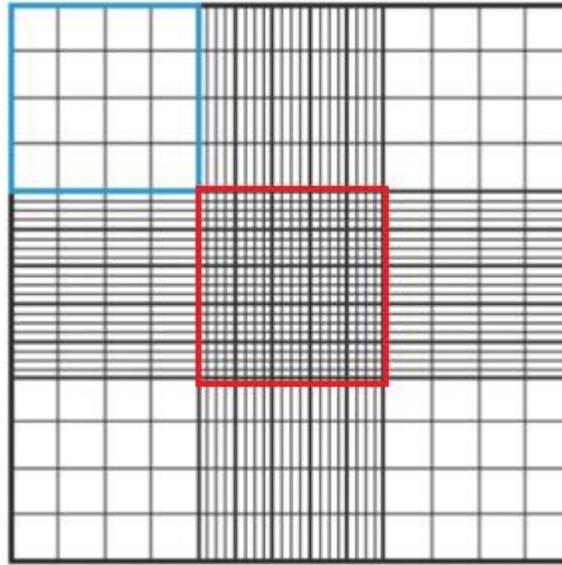


Figure 2.6 Hemocytometer (Thoma chamber) counting area

The cells on the large square in the middle of the chamber (red square) (Figure 2.6) were calculated and this calculation was done this way;

$$\text{Concentration}(\text{cell \# /mL}) = \text{Cell \#(in red square)} \times 10.000 \text{ mL}^{-1}$$

$$\text{width of small square} \times \text{height of small square} \times \text{depth of small square} = 1\text{mm} \times 1\text{mm} \times 0.1\text{mm} = 0.1 \text{ mm}^3 = 0.0001 \text{ mL} = 10000 \text{ mL}^{-1}$$

After calculations were completed, optical density at 680 nm (OD_{680}) versus cell number graph was sketched for *Scenedesmus sp.* ME02.

2.2.3. Growth Characteristics of *Scenedesmus sp.* ME02 in Different Growth Media

Scenedesmus sp. ME02 was grown in Bold's Basal Medium (BBM), 3N Basal Bold medium (3N-BB), BG-11, TAP and TAP:BG-11 (1:1) media (Sonmez et al., 2016) (Appendix A). In order to do this experiment initially starter culture was made with indicated media. Then, they were inoculated into 200 mL media in 500 mL Erlenmeyer flasks with 0.006 OD₆₈₀ and grown at 25°C and 16 hours light-8 hours dark photoperiod with constant shaking at 150 rpm. While growing, optical densities were measured daily (once every 24 hours) at 680 nm and recorded for 10 days. At the end of the 10th day growth curves were plotted for each medium as a factor of absorbance (OD₆₈₀) versus days.

Scenedesmus sp. ME02 cells, that were grown in TAP and TAP:BG-11 (1:1) media were investigated via light microscope. In order to examine the cells under the microscope, 1mL sample was taken into Eppendorf tube and 5µL formaldehyde was added to stop cell movement. Then, 10µL sample was loaded between microscope slide and coverslip. Cells were investigated under 100X magnification and photos were taken.

2.2.4. Effect of Different Temperature on Growth, Biomass and Lipid Composition

After determining the best growth medium for *Scenedesmus sp.* ME02, it was grown at different temperatures namely; 10°C, 16°C, 25°C and 30°C. As it was mentioned before, an initial starter culture was prepared and inoculation was done in 200 mL TAP:BG-11 (1:1) medium. Optical density was adjusted to 0.006 at 680 nm wavelength (Thermo Scientific Multiskan GO Spectrophotometer). Cells were left to grow for 16 hours light-8 hours dark photoperiod with constant shaking in acclimation chamber (Nüve GC400). Light intensity was 54 µE m⁻² s⁻¹ in the acclimation chamber. Optical densities were measured daily at 680 nm for 16°C, 25°C and 30°C experiment sets for 10 days. For the 10°C experiment, recording of optical density lasted for 12 days. In the end, growth curves were sketched for 4 different growth temperatures.

2.2.4.1. Dry Weight and Biomass Analysis at Different Temperature Regimes

As previously mentioned, a culture was prepared in 50-60 μL liquid medium. About 5-6 days later, this starter culture was inoculated into 800 mL TAP:BG-11 (1:1) medium and OD_{680} value was adjusted to 0.1. Inoculated cultures were grown at 10°C, 16°C, 25°C, and 30°C and 16 hours light-8 hours dark photoperiod with constant shaking for 7 days (until stationary phase). Additionally, 30°C-day, 16°C-night experiment was conducted with 16 hours light and 8 hours dark photoperiod respectively for 7 days. At the end of the 7th day, 5 mL culture was taken in 3 different glass test tubes from 10°C, 16°C, 25°C, and 30°C experiment sets. 5 mL samples of 30-16°C experiment were taken 7 hours into 16°C (at 11 am) and 3 hours into 30°C (at 3 pm) in same day. All of the samples were centrifuged at 3600 g for 10 minutes. After centrifugation, supernatant was discarded and 5 mL distilled water (dH_2O) was added into tubes for washing the pellet. Centrifugation was repeated one more time and remaining pellets were dried at 80°C oven for 24 hours. Next day, dried pellets were taken from oven and were weighed via precision balance for three times. According to the results, biomass concentration (g.L^{-1}), biomass productivity ($\text{g.L}^{-1}.\text{d}^{-1}$) and specific growth rates ($\mu.\text{d}^{-1}$) were calculated (Appendix C). All experiments were performed with 3 replicates.

2.2.4.2. Lipid Extraction and Fatty Acid Methyl Ester (FAME) Analysis at Different Temperature Regimes

Microalgae cultures were grown exactly like in biomass analysis experiments (section 2.2.4.1). 10°C, 16°C, 25°C, 30°C and 30-16°C experiment sets were conducted again. At the end of the 7th day of growth, cultures were centrifuged at 3600 g for 20 minutes. Most of supernatant was discarded and pellet was dissolved in remaining part of liquid supernatant. Dissolved cells were transferred to new falcon tubes and centrifuged again at 3600 g for 10 minutes. Supernatant were discarded and pellet was put at -80°C overnight. Next day, frozen cells were lyophilized (METU Central Laboratory Molecular Biology and Biotechnology R&D Center, Ankara) and after lyophilization

Bligh and Dyer lipid extraction protocol was applied (Onay et al., 2016). According to this protocol, approximately 0.120 g dried tissue was weighted and put into glass test tubes. Then 1.4 mL methanol and 0.7 mL chloroform was added. Methanol was used to get rid of impurities and the aim of using chloroform was to dissolve lipids. After addition of these chemicals 30 minutes incubation was done and solutions were mixed every 10 minutes via vortex for 1 minute. When incubation was completed, 15 minutes sonication was done and then 0.7 mL chloroform was added again and mixed with vortex. Then tubes were left to 3 hours incubation at room temperature. After incubation, 1.2 mL distilled water was added and centrifugation was done at 3600 g at room temperature for 5 minutes. When samples were taken from centrifuge, phase separation could easily be seen (Figure 2.7 A). It was desired to take between pellet and intermediate phase. In this phase, dissolved lipids in chloroform are present. Lipid phase was collected in to new clean test tube and upper phase was discarded. Remaining cell debris and pellet were dissolved again in 1.4 mL methanol and 0.7 mL chloroform and incubated 15 minutes with mixing via vortex. Then, 0.7 mL chloroform was added and left to overnight incubation. Next day, 1.2 mL distilled water was added and centrifuged 3600 g for 5 minutes. Same phase separations were formed and again dissolved lipid part was taken into collection tube. After all lipids were extracted and collected in one tube, this tube was centrifuged at 3600g at room temperature for 5 minutes. At the end of this centrifugation, 2 phases were formed as seen in Figure 2.7 B. Since protein and cell debris were unwanted parts, they were discarded and dissolved lipids were transferred into new clean test tube. This centrifugation and transferring to new tube were continued until all of the cell debris was cleaned. When clean lipid solution was obtained, lipid collection tube was left to air drying or rotary evaporator was used in order to evaporate all the chloroform. After entire chloroform was evaporated, tube was weighed and amount of lipid was calculated. In this way, lipid content and lipid productivity were measured gravimetrically (Appendix C). All of the lipid extraction experiments were conducted as three replicates.

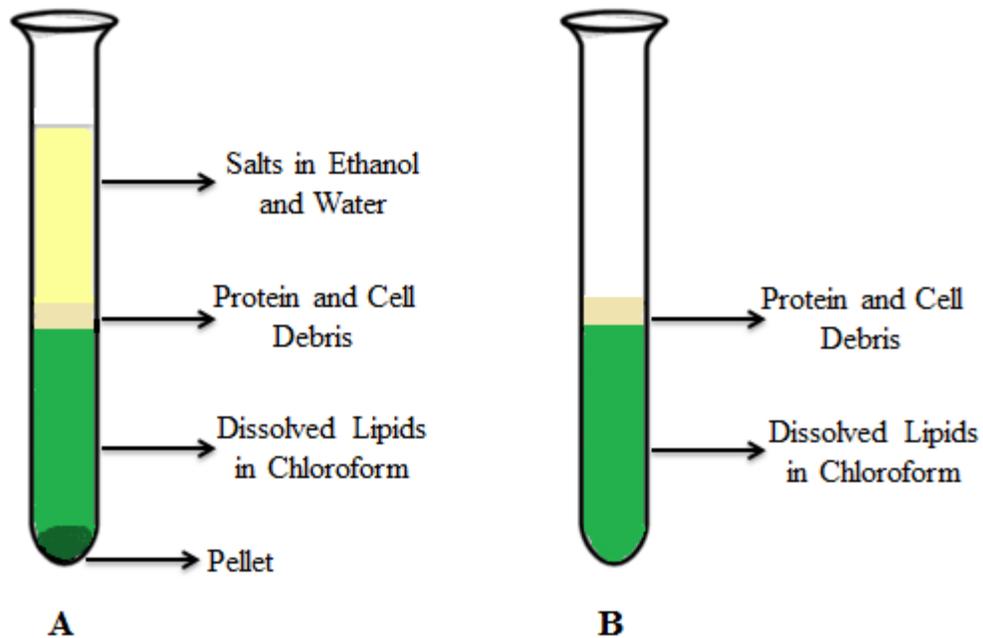


Figure 2.7 Phase separations during lipid extraction **A.** phase separation after phenol chloroform and water addition **B.** phase separation in second part of extraction

After lipid extraction was completed, oil contents were determined via Gas Chromatography (GC) (Agilent HP GC 6890) by Düzen-Norwest Laboratory, Ankara.

2.2.5. Genetic Engineering of *Scenedesmus sp.* ME02

2.2.5.1. Partial Cloning the Novel Fatty Acid Desaturase 2 (*fad2*) Gene from *Scenedesmus sp.* ME02

2.2.5.1.1. Design of PCR Primers for Cloning of *fad2* gene from *Scenedesmus sp.* ME02

Initially, *fad2* nucleotide sequence was determined in *Chlamydomonas reinhardtii*, *Chlorella vulgaris* and *Arabidopsis thaliana* genome. Then, these nucleotide sequences were translated to amino acid sequences. By using Clustal Omega web-based multiple sequence alignment tool, conserved areas were determined. Then, nucleotide sequences

were aligned via Basic Local Alignment Search Tool (BLAST) and appropriate primers were designed according to mostly conserved sequences between two species by using Primer3Plus web based primer designing tool.

2.2.5.1.2. Total RNA Isolation from Microalgae Strains

For total RNA isolation, *Chlamydomonas reinhardtii* and *Scenedesmus sp.* ME02 cells were cultured in 400 mL TAP and TAP:BG-11 (1:1) medium respectively at 25°C in acclimation chamber until stationary phase was reached (for 8 days). Then culture was centrifuged two times at 3600 g for 20 minutes in order to get rid of supernatant as much as possible. After this step, all of the equipment and solutions were treated with diethylpyrocarbonate (DEPC) or RNase Zap (RNase decontamination solution, Ambion) was used for wiping workbenches in order to expel RNase. For grinding, mortar and pestle were cooled with liquid nitrogen. The pellet was ground with liquid nitrogen and 3 mL Trizol was added on it. When powder became liquid, each 1 mL was taken to 2 mL Eppendorf tubes with pipette and vortexed for 2 minutes. Then, the mixture was centrifuged at 14000 rpm and room temperature for 5 minutes (MPW Med. Instruments, MPW-65R benchtop centrifuge). 900 µL supernatant was transferred to a new tube without touching the pellet. 900 µL phenol:chloroform (1:1) was added onto the supernatant and shaken vigorously for 15 seconds and incubated at room temperature for 5 minutes. Then, this mixture was centrifuged for 15 minutes at +4°C and 14000 rpm. After centrifugation, 450 µL upper phase was taken into new Eppendorf and 450 µL chloroform was added. Then, the mixture was shaken vigorously for 15 seconds and incubated at room temperature for 3 minutes. After incubation is completed, centrifuge was done for 15 minutes at room temperature and 14000 rpm. Next, 400-450 µL upper phase was taken carefully and transferred to a new 1.5 mL Eppendorf tube. Meanwhile, any contact with the interphase should be avoided. After that, 1 volume of isopropanol was added and RNA was precipitated via centrifugation for 20 minutes at +4°C and 14000 rpm. After centrifugation, supernatant was taken off and 1 mL 75% ethanol was added in order to wash the pellet. Tubes were left at room temperature for 3 minutes for

proper washing. Then, centrifugation was done for 5 minutes at +4°C and 14000 rpm and supernatant was poured off. Final centrifugation was done for 15 seconds to collect pellet at the bottom of the tube and to get rid of any visible remaining ethanol. After removal of ethanol, pellet was left to air dry for 10 minutes. When drying process was completed, 40 µL DEPC treated water was added and stored at +4°C overnight. For long term, RNA samples were stored at -80°C.

2.2.5.1.2.1. Characterization of the Nucleic Acid Samples

In order to check the integrity and quality of RNA, samples were loaded on agarose gel and electrophoresis was done at 70V. 1.5% agarose gel was prepared for this purpose and ethidium bromide (EtBr) was used as an indicator. Agarose and 1X Tris-Acetate-EDTA (TAE) Buffer (Appendix A; Table A.5) was mixed in Erlenmeyer flask and warmed until agarose dissolve completely in microwave oven. Then, the mixture was cooled under the tap water and 3 µL EtBr was added. EtBr was completely dissolved in agarose gel and then poured in gel tray and comb was placed at the top of tray. About 25-30 minutes later, gel became solid and comb was removed. Gel tray was placed in electrophoresis chamber and it was filled with 1X TAE buffer up to 5 mm above of the gel. 6 µL 1 kilobase (kb) gene ladder and 10 µL samples with 2 µL 6X loading dye mixtures were loaded into wells. Voltage was adjusted to 70 volt (V) for 45-50 minutes. After that, agarose gel was placed in gel imaging system and gel photo was taken.

2.2.5.1.3. DNase Treatment of Total RNA Samples

DNA contaminants in RNA were removed according to protocol of RapidOut DNA Removal Kit (Thermo Scientific).

Table 2.1 Composition of DNase treatment reaction

Ingredients	Final Concentration	Volume
RNA Sample	-	40 μ L
10X Reaction Buffer with MgCl ₂	1X	5 μ L
1U/ μ L DNaseI (RNase-free)	2.5 U	2.5 μ L
dH ₂ O	-	2.5 μ L
Total	-	50 μ L

Mixture was prepared as indicated in Table 2.1 and incubated at 37°C for 30 minutes. Meanwhile, DNase Removal Reagent (DRR) was vortexed well and 5 μ L DRR was added to mixture. In order to suspend DRR, mixture was incubated at room temperature for 2 minutes and mixed properly. Then, it was centrifuged at 1000 g for 1 minute to pellet DRR. Supernatant was transferred to new nuclease-free Eppendorf tube. Last centrifugation step was repeated in order to eliminate whole DRR completely. After DNase treatment, concentrations of RNA samples were measured via NanoDrop.

2.2.5.1.4. Complementary DNA (cDNA) Synthesis

cDNA synthesis was done according to the protocol of Reverse Aid First Strand cDNA Synthesis Kit (Thermo Scientific). cDNA was synthesized from *C. reinhardtii* and *Scenedesmus sp.* ME02 RNA samples. The protocol is as follows;

Table 2.2 First part of cDNA synthesis reaction composition

Ingredients	Volume
RNA (~5 μ g)	6 μ L
Oligo (dT) ₁₈ Primer	1 μ L
Water	5 μ L
Total	12 μ L

After this mixture was prepared (Table 2.2), they were mixed gently and incubated at 65°C for 5 minutes in thermal cycler. Then, vials were chilled on ice, spin down and placed back on ice. After incubation, following reagents were added in the indicated order (Table 2.3).

Table 2.3 Second part of cDNA synthesis reaction composition

Ingredients	Volume
5X Reaction Buffer	4 µL
Ribolock RNase Inhibitor	1 µL
10 mM dNTP Mix	2 µL
Revert Aid M-MuLV Reverse Transcriptase	1 µL
Grand Total	20 µL

All components were mixed gently and centrifuged briefly (Table 2.3). Then, they were incubated at 45°C for 1 hour. Reaction was terminated by heating at 70°C for 5 minutes.

2.2.5.1.5. Reverse Transcriptase PCR (RT-PCR) Optimization

Since *C. reinhardtii* and *Scenedesmus sp.* ME02 DNAs have high guanine-cytosine (GC) content, betaine or dimethyl sulfoxide (DMSO) was used in PCR. In order to determine the best DMSO or betaine concentration, different concentrations were tried and PCR was optimized (Table 2.4). In this reaction only *C.reinhardtii* was used because FAD2 primer was designed according to *C. reinhardtii fad2* DNA sequence.

Table 2.4 Composition of *fad2* RT-PCR with different DMSO and betaine concentrations

Ingredients	Final concentration	Volume per reaction (5% DMSO)	Volume per reaction (3% DMSO)	Volume per reaction (1M Betaine)	Volume per reaction (0.6M Betaine)
10X Taq Buffer	1X	2 μ L	2 μ L	2 μ L	2 μ L
25 mM MgCl ₂	2 mM	1.6 μ L	1.6 μ L	1.6 μ L	1.6 μ L
10 mM dNTP Mix	0.25 mM	0.5 μ L	0.5 μ L	0.5 μ L	0.5 μ L
DMSO	-	1 μL	0.6 μL	-	-
5M Betaine	-	-	-	4 μL	2.4 μL
10 μ M FAD2 Forward Primer	0.3 mM	0.6 μ L	0.6 μ L	0.6 μ L	0.6 μ L
10 μ M FAD2 Reverse Primer	0.3 mM	0.6 μ L	0.6 μ L	0.6 μ L	0.6 μ L
<i>C. reinhardtii</i> cDNA	-	2 μ L	2 μ L	2 μ L	2 μ L
5 U/ μ L Taq Polymerase	0.05 U/ μ L	0.2 μ L	0.2 μ L	0.2 μ L	0.2 μ L
dH ₂ O	-	11.5 μ L	11.9 μ L	8.5 μ L	10.1 μ L
Total	-	20 μ L	20 μ L	20 μ L	20 μ L

Table 2.5 Conditions of RT-PCR

Steps		Conditions
Initial denaturation		95°C, 5 minutes
36 Cycles	Denaturation	94°C, 30 seconds
	Annealing	58.5°C, 30 seconds
	Extension	72°C, 30 seconds
Final extension		72°C, 5 minutes

After PCR, products were run via agarose gel electrophoresis at 70V. Agarose gel was prepared in 1.5% concentration.

2.2.5.1.6. RT-PCR with *C. reinhardtii* and *Scenedesmus sp.* ME02 DNAs

According to optimized PCR results, RT-PCR was repeated with FAD2 primers and *C. reinhardtii* and *Scenedesmus sp.* ME02 cDNA in this time (Table 2.6).

Table 2.6 Optimized *fad2* RT-PCR composition

Ingredients	Final concentration	Volume per reaction
10X Taq Buffer	1X	2 µL
25 mM MgCl ₂	2 mM	1.6 µL
10 mM dNTP Mix	0.25 mM	0.5 µL
5 M Betaine	0.6 M	2.4 µL
10 µM FAD2 Forward Primer	0.4 µM	0.8 µL
10 µM FAD2 Reverse Primer	0.4 µM	0.8 µL
5 U/µL Taq Polymerase	0.05 U/µL	0.2 µL
<i>C. reinhardtii</i> and <i>Scenedesmus sp.</i> ME02 cDNA	-	2 µL
dH ₂ O	-	9.7 µL
Total	-	20 µL

Table 2.7 Conditions of RT-PCR

Steps		Conditions
Initial denaturation		95°C, 5 minutes
36 Cycles	Denaturation	94°C, 30 seconds
	Annealing	55°C, 30 seconds
	Extension	72°C, 30 seconds
Final extension		72°C, 5 minutes

In order to check the PCR results, agarose gel electrophoresis was done with 1.5% agarose.

2.2.5.1.7. Purification of PCR Products

Only *Scenedesmus sp.* ME02 RT-PCR products were purified with QIAquick PCR Purification Kit (Qiagen). According to the kit protocol; firstly 60 µL PCR products was taken into Eppendorf tubes, then 5 fold volume of PB buffer was added onto it. This mixture was loaded on the silica-gel membrane spin column. Then, tubes were centrifuged at 18000 g at room temperature for 1 minute. Flowthrough was discarded. Next, 750 µL buffer PE was added and centrifuged for 1 minute. Flowthrough was discarded from collection tube and again tubes were centrifuged for 2 minutes and collected fluid was poured. Then, spin column was placed on 1.5 mL Eppendorf tube and 50 µL buffer EB was added to the spin column to elute DNA. After that, centrifugation was done for 1 minute in order to collect the flowthrough. Collected fluid was loaded onto the spin column again and centrifugation was repeated. Eluted DNA concentration was measured with NanoDrop.

To check the quality of purified PCR product, agarose gel electrophoresis was conducted with 1.5% agarose gel.

2.2.5.1.8. Partial Cloning of *fad2* Gene Fragment from *Scenedesmus sp.* ME02 with TOPO TA Sequencing Kit

Purified *Scenedesmus sp.* ME02 PCR products were cloned in to pCR4-TOPO vector (Figure 2.3) by using TOPO TA Cloning Kit for Sequencing (Invitrogen).

Following reagents were used for TA cloning (Table 2.8).

Table 2.8 Composition of TOPO TA cloning reaction

Ingredients	Volume
Purified PCR Product	4 μ L
Salt Solution	1 μ L
TOPO Vector	1 μ L
Total	6 μ L

Mixture was prepared and incubated at 23°C for 30 minutes.

2.2.5.1.9. Chemical Transformation of Competent *E.coli* cells

TOPO TA cloned plasmids were chemically transformed to One Shot Chemically Competent *E.coli* (Invitrogen). Initially, competent *E.coli* cells were thawed on ice. Then, 2 μ L TOPO TA cloned plasmid was added onto 50 μ L *E.coli* cells and gently mixed. Mixture was incubated on ice for 30 minutes and at the end of this incubation immediately transferred to 42°C water bath for 30 seconds for heat shock. Next, tube was placed on ice again for 2 minutes. After incubation, 250 μ L super optimal broth with catabolite repression (SOC) medium (Invitrogen) was added. Then, incubation was done in incubator with orbital shaker (at 150 rpm) at 37°C for about 1 hour. Cells were inoculated on 75 mg/L ampicillin containing Luria-Bertani (LB) agar (Appendix A; Table A.4) plates in the volumes of 150 μ L, 100 μ L and 50 μ L. The reason of using

ampicillin was presence of ampicillin resistance marker on the TOPO TA vector. In this way positive colonies were selected on agar plate. Inoculated cells were incubated at 37°C overnight.

Next day, 8 randomly selected colonies were transferred to new LB agar plates with streak plate technique. They were incubated at 37°C overnight.

2.2.5.1.10. Colony PCR from Transformed *E.coli* Cells

In order to determine the positive colonies, colony PCR was conducted (Table 2.9).

Table 2.9 Composition of colony PCR

Ingredients	Final concentration	Volume per reaction
10X Taq Buffer	1X	2.5 µL
25 mM MgCl ₂	1.5 mM	1.5 µL
10 mM dNTP Mix	0.2 mM	0.5 µL
10 µM M13 Forward Primer	0.24 µM	0.6 µL
10 µM M13 Reverse Primer	0.24 µM	0.6 µL
DNA	-	pinch of colony
5 U/µL Taq Polymerase	0.05 U/µL	0.25 µL
dH ₂ O	-	19.05 µL
Total	-	25 µL+pinch of colony

Table 2.10 Conditions of colony PCR

Steps		Conditions
Initial denaturation		95°C, 5 minutes
22 Cycles	Denaturation	94°C, 30 seconds
	Annealing	53°C, 30 seconds
	Extension	72°C, 30 seconds
Final extension		72°C, 5 minutes

At the end of the PCR, positive colonies were visualized via agarose gel electrophoresis.

2.2.5.1.11. Plasmid Isolation from Transformed *E.coli* Cells

Presence of TOPO TA vector in competent *E.coli* cells was proven by colony PCR. Positive colonies were taken from LB agar plate and inoculated in 75mg/L ampicillin containing liquid LB medium. Incubation was done at 37°C overnight in orbital shaker (at 150 rpm).

Next day, plasmid isolation was performed from grown *E.coli* cells in liquid medium. Plasmids were isolated with Bacterial Plasmid Isolation Kit (Nano Biz). According to the kit protocol; 1.5 mL bacterial culture was centrifuged at 14000 rpm for 2 minutes (MPW Med. Instruments, MPW-65R benchtop centrifuge) and supernatant was discarded. Pellet was suspended in 200 µL Buffer-p1. Then, 200 µL Buffer-p2 and 200 µL Buffer-p3 were added and mixed with inversion. After suspension and mixing was completed, 5 minutes centrifugation was performed at 14000 rpm. 400 µL supernatant was transferred to new Eppendorf tube and 600 µL Buffer-p4 was added. 600 µL of the mixture was taken to spin column and centrifuged at 10000 rpm for 1 minute and flow through was discarded. Last step was repeated for remaining liquid in Eppendorf tube. 500 µL Buffer-p5 was put in spin column and then centrifugation was repeated at 10000 rpm for 1 minute. Again, flow through was discarded and centrifugation was done at 14000 rpm for 2 minutes for this time. Spin column was placed on sterile Eppendorf tube and 42 µL dH₂O was added. Tube was incubated for about 2 minutes at room temperature. After incubation, tube was centrifuged at 6000 rpm for 1 minute. At the end, elution contained the isolated plasmid. Concentration of the plasmid was measured via Nano Drop.

2.2.5.1.12. Sequencing the *fad2* Gene Insert in TOPO TA Vector

After plasmid isolation, plasmids were sequenced with T7 and M13 reverse primers (RefGen Gene Research and Biotechnology Company, Ankara).

Sequencing results were aligned and compared with *Chlamydomonas reinhardtii fad2* gene sequence and conserved areas were determined.

2.2.5.2. Transformation of *Scenedesmus sp.* ME02 Cells

2.2.5.2.1. Determination of Suitable Selectable Marker

Since pHyg3 and pChlamy_3 plasmids contain Hygromycin resistance gene region, Hygromycin sensitivity of *Scenedesmus sp.* ME02 was tested. Therefore, three different Hygromycin concentrations were tried namely; 25 mg/L, 50 mg/L and 75 mg/L. Molten TAP agar was mixed with Hygromycin antibiotic with specified concentrations and poured into sterile petri plates. Then, wild type *Scenedesmus sp.* ME02 was inoculated onto these plates via spread plate technique. About 10 days later, growth on the plates was observed and the most suitable concentration was determined before transformation.

2.2.5.2.2. Optimization of Transformation Conditions

2.2.5.2.2.1. Amplifying of Hygromycin Resistance Gene

Scenedesmus sp. ME02 was transformed with pHyg3 plasmid in order to optimize the transformation parameters for genetic transformation studies. pHyg3 plasmid was supplied by Dr. Claire Remacle's laboratory in Belgium. Hygromycin resistance marker cassette was contained within pHyg3. In order to have sufficient amount of DNA for

transformation, it needs to be amplified via Polymerase Chain Reaction (PCR). Firstly, PCR conditions were optimized with different amounts of DNA (Table 2.11).

Table 2.11 Composition of hygromycin resistance gene PCR

Ingredients	Final concentration	Volume per reaction (0.5 μ L DNA)	Volume per reaction (1 μ L DNA)	Volume per reaction (2 μ L DNA)
5X HF Phusion Buffer	1X	4 μ L	4 μ L	4 μ L
10 mM dNTP Mix	0.3 mM	0.6 μ L	0.6 μ L	0.6 μ L
DMSO	3%	0.6 μ L	0.6 μ L	0.6 μ L
10 μ M Aph7 Forward Primer	0.3 mM	0.6 μ L	0.6 μ L	0.6 μ L
10 μ M Aph7 Reverse Primer	0.3 mM	0.6 μ L	0.6 μ L	0.6 μ L
pHyg3 (1:20)	-	0.5 μL	1 μL	2 μL
2 U/ μ L Phusion Polymerase	0.02 U/ μ L	0.2 μ L	0.2 μ L	0.2 μ L
dH ₂ O	-	12.9 μ L	12.4 μ L	11.4 μ L
Total	-	20 μ L	20 μ L	20 μ L

Table 2.12 Hygromycin resistance gene PCR conditions

Steps		Conditions
Initial denaturation		98°C, 30 seconds
35 Cycles	Denaturation	98°C, 10 seconds
	Annealing	58°C, 30 seconds
	Extension	72°C, 30 seconds
Final extension		72°C, 5 minutes

PCR products needed to be purified before transformation via electroporation. In order to achieve this, PCR purification was done via QiaQuick PCR Purification Kit (Qiagen).

Purified PCR products were checked via Agarose Gel Electrophoresis.

2.2.5.2.2.2 pHyg3 Transformation of *Scenedesmus sp.* ME02

In order to optimize the transformation parameters (voltage and capacitance values in the course of electroporation) initially pHyg3 was transformed to *Scenedesmus sp.* ME02. For electroporation, 1 mL $\sim 10^8$ microalgae cell ($OD_{680}=0.4$) was taken into 1.5 mL Eppendorf tubes. Then, tubes were centrifuged at 800 g at $+4^\circ\text{C}$ for 5 minutes to collect the microalgae cells at the bottom of the tube and supernatant was discarded. 400 μL cold osmosis solution (Appendix A; Table A.6) was added onto pellet and incubated at 42°C water bath for 1 minute. After that, tubes were immediately put on ice and incubated for 5 minutes. After incubations were completed, 2 μg pHyg3 PCR product was added per 400 μL mixture and mixed gently. Again incubation on ice was done for 5 minutes. ~ 400 -450 μL solution was electroporated at different voltage and capacitance values via Bio Rad Gene Pulser II electroporation system. Different values were applied in order to optimize the conditions. Applied voltage and capacitance values are shown in Table 2.13 below;

Table 2.13 Voltage and capacitance values for pHyg3 transformation

Voltage	Capacitance
1 kV	25 μF
1.5 kV	10 μF
2 kV	10 μF
2 kV	25 μF

After electroporation was completed, electroporated cells were taken into 8 mL TAP:BG-11 (1:1) and incubated in dark at room temperature for 24 hours for recovery of the fragile cells. After 24 hours incubation, 10 mL electroporated culture was centrifuged at 800 g for 5 minutes and supernatant was discarded. Then, pellet was re-suspended in 350 μ L TAP:BG-11 (1:1) medium. 200 μ L suspended cells were spread onto hygromycin containing (final concentration: 75 mg/L) TAP agar plate by using sterile glass beads. As a control, the remaining 150 μ L cells were spread onto TAP agar plate without hygromycin. All of the agar plates were incubated at 25°C, 16 hours light and 8 hours dark photoperiod for 12-15 days. At the end of the incubation, newly formed colonies were transferred to new TAP agar plates.

2.2.5.2.3. Green Fluorescent Protein (GFP) Transformation via Electroporation

2.2.5.2.3.1. Vector Design and Cloning

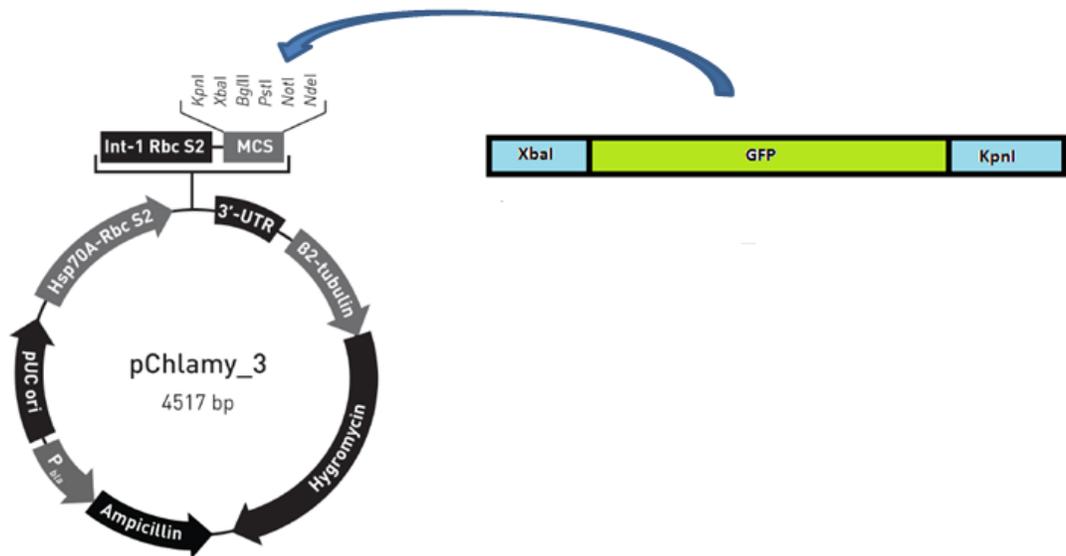


Figure 2.8 pChlamy_3 vector with GFP insert

GFP::pChlamy_3 construct was previously done by our laboratory members; Assist. Prof. Dr. Çağla Sönmez and Buse İşbilir (Figure 2.8). GFP was cut from pCAMBIA1302 with *XbaI* and *KpnI* restriction enzymes. Then, the fragment was ligated in multiple cloning site of pChlamy_3 vector with same restriction sites. This construct was optimized for plant transformation; therefore it has low GC content.

Constructed plasmid should be linearized before transformation to *Scenedesmus sp.* ME02 for proper integration of insert into genome of organism. In order to achieve this, *ScaI* digestion was done (Table 2.14).

Table 2.14 Composition of restriction enzyme digestion reaction

Ingredients	Final Concentration	Volume
10X Buffer <i>ScaI</i>	1X	2 μ L
DNA (plasmid)	2 μ g/ μ L	1 μ L
10 U/ μ L <i>ScaI</i>	0.75 U/ μ L	1.5 μ L
dH ₂ O	-	15.5 μ L
Total	-	20 μ L

After mixture was prepared, it was incubated at 37°C for 4 hours.

When restriction digestion was completed, mixture was purified with phenol-chloroform as described below:

Minimum 200 μ L restriction mixture was taken and equal volume of phenol:chloroform (1:1) was added. Mixture was vortexed briefly and incubated at 25°C for 5 minutes. After incubation, centrifugation was done at 14000 g for 5 minutes and phase separation was observed. Upper phase was taken into new Eppendorf tube and volume was measured. 1:10 (v/v) 3M sodium acetate (pH: 5.2) was added onto upper phase and also glycogen was added up to its final concentration becomes 0.5 μ g/ μ L in mixture. Then, 1:1 (v/v) isopropanol (100%) or 1:1 (v/v) ethanol (100%) was added onto mixture. After that, incubation was done -20°C for 1 hour or -80°C for 1 hour or -80°C overnight. After

incubation, tubes were centrifuged at 14000 g for 30 minutes at 4°C and supernatant was discarded with pipette and pellet was washed with 1 mL 70% ethanol. Centrifugation was repeated at 14000 g for 2 minutes. Supernatant was discarded and centrifuge was repeated one more time to get rid of remaining ethanol. Tubes were left to air dry for approximately 5 minutes until residual ethanol evaporate. After complete drying, pellet was dissolved in 15 µL 0.1 M Tris-HCl.

2.2.5.2.3.2. Transformation Conditions

The plasmid GFP::pChlamy_3 was transformed to *Scenedesmus sp.* ME02 via electroporation. Transformation procedure is the same as previous pHyg3 electrotransformation protocol. Since transformation parameters were optimized in previous pHyg3 transformation experiment, most efficient and suitable voltage and capacitance values were applied. These two voltage and capacitance values were 1kV 25µF and 2kV 10µF.

Since pChlamy_3 vector contains Hygromycin resistance gene fragment, positive transformants are expected to be resistant to hygromycin. Due to this reason, after transformation, transformant cells were spread onto 75 mg/L Hygromycin containing TAP agar plates and positive transformants were selected from plate.

2.2.5.2.3.3. Verification of Positive Clones

Three different GFP colonies were cultured in 1600 mL TAP:BG-11 (1:1) medium at 25°C until stationary phase was reached (for 8 days). At the end of the eighth day, culture was centrifuged at 3600g for 20 minutes and incubated at -80°C overnight. Next day, lyophilization was done in order to obtain dry tissues. 200 mg lyophilized tissue was weighed and 2X Cetyltrimethyl Ammonium Bromide (CTAB) extraction buffer (Appendix A, Table A.7) was pre-heated in 65°C water bath. Weighed sample was transferred into pre-cooled 2 mL Eppendorf tubes and suspended in 1 mL pre-heated

extraction buffer. Tubes were vortexed for complete suspension. Samples were transferred immediately to pre-cooled 2 mL Eppendorf tubes and suspended in 1 mL pre-heated extraction buffer. Vortex was done for complete suspension. Then, the mixture was incubated at 65°C for 45 minutes in a circulating water bath. After incubation, centrifugation was done at 10000 g for 10 minutes at 4°C. Then, supernatant was transferred to clean sterile 2 mL Eppendorf tube and volume of it was measured. Equal volume of chloroform:isoamylalcohol (24:1, v:v) was added on top of supernatant and mixed by inversion. Again, centrifugation was done at 10000 g for 10 minutes at 4°C. Upper aqueous phase was transferred to a clean sterile tube and volume of aqueous phase was recorded. Next, 1/10 volume of 3 M sodium acetate (pH=5.2) and equal volume of pre-chilled isopropanol were added in order to precipitate DNA. Tubes were gently inverted couple of times for complete suspension. Then, tubes were left at -80°C for 30 minutes or -20°C overnight. Extracted DNA was collected by centrifugation at 5000 g for 5 minutes at +4°C and supernatant was discarded. After that, pellet was washed with 1 mL of 70% ethanol and DNA was recovered by centrifugation at 5000 g for 5 minutes at +4°C. Supernatant was discarded after centrifugation and pellet was left to air drying at room temperature for 8-10 minutes in laminar flow hood. Dried DNA pellet was suspended in 40 µL 0.1 M Tris-HCl or ultrapure water. Finally, 1 µL RNase was added to degrade RNA. Isolated DNA was stored at -20°C for further use.

From isolated DNA samples, PCR was conducted. (Table 2.15) Concentrations of three different isolated DNA samples were measured via Nano Drop and they were between 2000-3000 ng/µL. For optimization, different DNA concentrations (1:2, 1:5, 1:10 and 1:20) were tried. In this PCR reaction, GFP gene fragment containing pChlamy_3 vector was used as a positive control.

After PCR reaction completed, samples were analyzed via agarose gel electrophoresis by using 1.5% agarose gel and at 70 V.

Table 2.15 Composition of GFP PCR

Ingredients	Final concentration	Volume per reaction
10X Taq Buffer	1X	2 μ L
25 mM MgCl ₂	1.75 mM	1.4 μ L
10 mM dNTP Mix	0.25 mM	0.5 μ L
10 μ M GFP qPCR Forward Primer	0.3 μ M	0.6 μ L
10 μ M GFP qPCR Reverse Primer	0.3 μ M	0.6 μ L
5 U/ μ L Taq Polymerase	0.05 U/ μ L	0.2 μ L
~2000-2500 ng/ μ L DNA sample	-	2 μ L
dH ₂ O	-	12.7 μ L
Total	-	20 μ L

Table 2.16 GFP PCR conditions

Steps		Conditions
Initial denaturation		95°C, 5 minutes
35 Cycles	Denaturation	94°C, 30 seconds
	Annealing	53°C, 30 seconds
	Extension	72°C, 30 seconds
Final extension		72°C, 5 minutes

2.2.5.2.3.4. Expression of GFP mRNA in Transformed Cells

For total RNA isolation, three different GFP colonies were cultured in 400 mL TAP:BG-11 (1:1) medium at 25°C until stationary phase was reached (for 8 days). Then, the total RNA isolation procedure was applied to GFP colonies as explained in part 2.2.4.1.2 previously.

After total RNA isolation was completed, DNase treatment and phenol-chloroform purification was done in order to remove DNA contamination. For this purpose, 5.5 μL DNase buffer (10X) was added onto 50 μL isolated RNA. Then, 1 μL DNase I was added onto the mixture and left to incubation in water bath at 37°C for 30 minutes.

Phenol-chloroform purification was done after DNase treatment to purify the RNA. In order to do this procedure, first of all mixture was completed to 200 μL with DEPC treated water. Then 200 μL phenol:chloroform (1:1) was added and shaken vigorously. In order to obtain phase separation, mixture was centrifuged for 15 minutes at +4°C and 14000 rpm (MPW Med. Instruments, MPW-65R benchtop centrifuge). After centrifugation, upper phase was taken into new Eppendorf tube and volume was measured. Equal volume of isopropanol was added and gently inverted. Tubes were left to incubate for 10 minutes at room temperature. After incubation, centrifugation was done for 20 minutes at +4°C and 14000 rpm. Supernatant was poured off and 1 mL 75% DEPC treated ethanol was added onto pellet and inverted for washing. Tubes were left at room temperature for 3 minutes for proper washing step. Then, tubes were centrifuged for 5 minutes at +4°C and 14000 rpm and supernatant was discarded. Next, in order to collect the pellet at the bottom of the tube and remove any visible liquid, additional centrifugation was done for 15 seconds and then left to air dry for 10 minutes. After drying process, pellet was dissolved in 40 μL DEPC treated water and mixed gently. For further use, isolated and purified RNA samples were stored at -80°C.

2.2.5.2.3.4.1. cDNA Synthesis from *Scenedesmus sp.* ME02 RNA Samples

cDNA synthesis was performed from RNA samples of GFP transformants. In order to do this, First Strand cDNA Synthesis Kit (Thermo Scientific) was used and kit protocol was applied as explained in section 2.2.5.1.4.

2.2.5.2.3.4.2. Negative Reverse Transcriptase PCR (-RT-PCR)

Negative control reaction was done with Reverse Aid First Strand cDNA Synthesis Kit (Thermo Scientific) (Table 2.17).

Table 2.17 First part of cDNA synthesis reaction composition

Ingredients	Volume
RNA (~1.1 µg)	3 µL
dH ₂ O	9 µL
Total	12 µL

Mixture was incubated at 65°C for 5 minutes and immediately put on ice. Then following reagents were added (Table 2.18).

Table 2.18 Second part of cDNA synthesis reaction composition

Ingredients	Volume
5X Reaction Buffer	6 µL
10 mM dNTP Mix	2 µL
Grand Total	20 µL

After all of reagents were mixed with each other, tubes were incubated at 45°C for 1 hour and reaction was terminated by heating at 70°C for 5 minutes.

2.2.5.2.3.4.3. Reverse Transcriptase PCR (RT-PCR)

Synthesized cDNA samples and negative RT controls were amplified by using internal control primer ITS2 and GFPqPCR primers. In this RT-PCR reaction pHyg3 cDNA was used as a positive control (Table 2.19 and Table 2.20).

After PCR reaction was complete, samples were analyzed via agarose gel electrophoresis by using 1.5% agarose gel and at 70 V.

Table 2.19 Composition of –RT-PCR with GFP primers

Ingredients	Final concentration	Volume per reaction
10X Taq Buffer	1X	2.5 μ L
25 mM MgCl ₂	1.75 mM	1.75 μ L
10 mM dNTP	0.25 mM	0.6 μ L
10 μ M GFP qPCR Forward Primer	0.25 μ M	0.75 μ L
10 μ M GFP qPCR Reverse Primer	0.25 μ M	0.75 μ L
5 U/ μ L Taq Polymerase	0.05 U/ μ L	0.25 μ L
cDNA sample	-	2.5 μ L
dH ₂ O	-	15.9 μ L
Total	-	25 μ L

Table 2.20 Composition of –RT-PCR with ITS2 primers

Ingredients	Final concentration	Volume per reaction
10X Taq Buffer	1X	2.5 µL
25 mM MgCl ₂	1.75 mM	1.75 µL
10 mM dNTP	0.25 mM	0.6 µL
10 µM ITS2 Forward Primer	0.25 µM	0.75 µL
10 µM ITS2 Reverse Primer	0.25 µM	0.75 µL
5 U/µL Taq Polymerase	0.05 U/µL	0.25 µL
cDNA sample	-	2.5 µL
dH ₂ O	-	15.9 µL
Total	-	25 µL

Table 2.21 Conditions of –RT-PCR

Steps		Conditions
Initial denaturation		95°C, 5 minutes
36 Cycles	Denaturation	94°C, 30 seconds
	Annealing	53°C, 30 seconds
	Extension	72°C, 30 seconds
Final extension		72°C, 5 minutes

2.2.5.2.3.5. Expression Analysis of GFP via Confocal Microscopy

A pinch of GFP transformant *Scenedesmus sp.* ME02 cells from solid media was inoculated into liquid 2 mL TAP:BG-11 (1:1) medium. They were left to proliferation for about 4-5 days. After growth, 10 µL formaldehyde was added to liquid culture and mixed well for fixation of cells. Then, 10 µL mixture was taken and put onto microscope slide and covered with coverslip without any air bubble. Finally, borders of coverslip were fixed to slide with transparent nail polish. When slide preparation was

completed, samples were observed under the laser scanning confocal microscope. Since we wanted to observe the presence of GFP, excitation and emission wavelengths were 488 nm and 505 nm respectively. Firstly, untransformed wild type cells were observed as negative control, after that other GFP transformant cells were visualized under the laser scanning confocal microscope. Cells were firstly observed under 10X magnification, then clear and desired image was obtained under 40X magnification. Laser scanning confocal microscope studies were done by METU Central Laboratory Molecular Biology and Biotechnology R&D Center, Ankara.

CHAPTER 3

RESULTS & DISCUSSION

3.1. Physiological Characterization of *Scenedesmus sp.* ME02

In this part, optimum growth conditions of *Scenedesmus sp.* ME02 for high lipid and biomass productivities were evaluated.

3.1.1. Growth Characteristics of *Scenedesmus sp.* ME02 in Different Growth Media

The growth of the *Scenedesmus sp.* ME02 was measured by counting the cells via hemocytometer after culturing in 50 mL TAP:BG-11 (1:1) medium at 25°C with constant shaking in acclimation chamber. When 0.8 OD₆₈₀ was reached, cells were counted under the light microscope. Concentrations at certain optical densities (at 680 nm) were calculated according to formula below.

$$\text{Concentration (cell number /ml)} = \text{Cell number} \times 10.000$$

Concentration versus OD₆₈₀ graph was sketched by using Excel software and data points and error bars were indicated on graph (Figure 3.1). In order to increase significance and confidence level, three replicates were used for cell counting. Error bars were calculated by standard error.

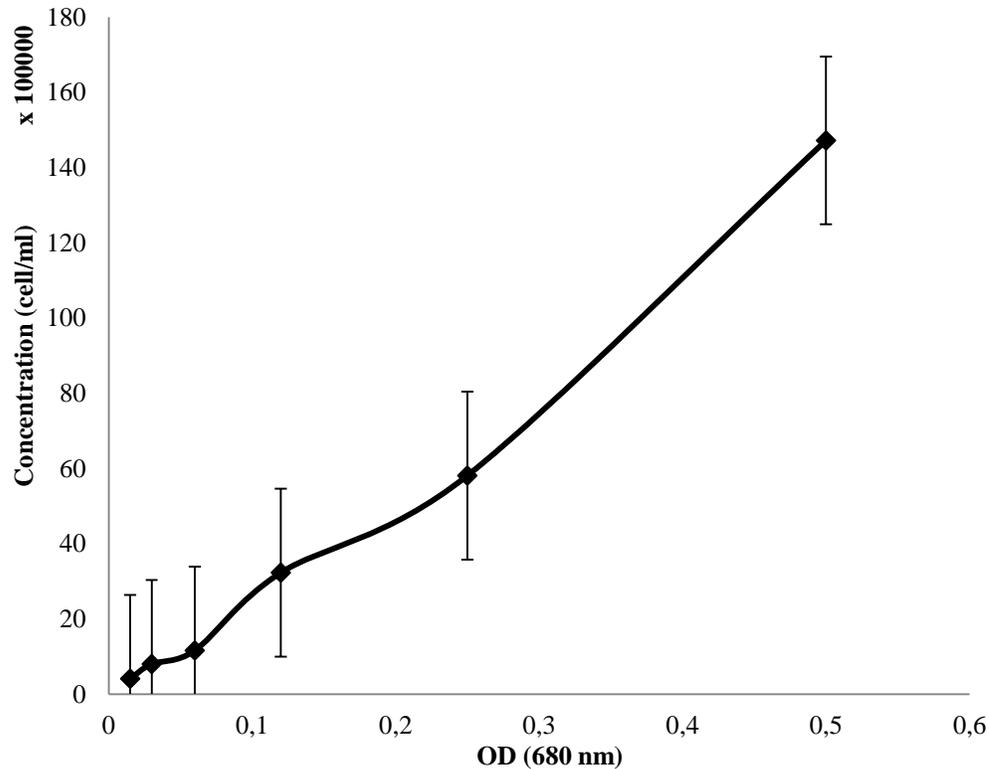


Figure 3.1 Optical density (OD) versus concentration graph for *Scenedesmus sp.* ME02. Error bars were calculated by standard error.

Cell number versus OD correlation required for subsequent transformation experiments and also some other studies like growth curves. By measuring OD value of microalgal culture, cell number could be predicted by means of this graph.

The graph shows linear trend since optical density and concentration of the cells are directly proportional to each other. However, some deviations were observed. The reason of this, at high concentration levels, cell number was too high and counting was hard due to intensity of cells. At low concentrations, due to low cell number, sample loading on slide was not too much uniform. Because of these reasons, some deviations from linear trend and wide error bars were observed.

In the previous study of Onay et al. *Scenedesmus sp.* ME02 cells were grown in BG-11 medium however very slow growth rate was observed. In order to improve growth rate

of *Scenedesmus sp.* ME02 cells, different growth media were tested in this study. These different growth media were Tris-Acetate-Phosphate (TAP), Bold's Basal Medium (BBM), 3N Bold's Basal medium (3N-BB) and BG-11. Growth curves of the *Scenedesmus sp.* ME02 cells that grown in different media were sketched till the end of stationary phase. All experiment sets were done with three replicates and error bars were calculated by standard error.

As it is seen in Figure 3.2, when BBM, 3N-BB and BG-11 media were used, *Scenedesmus sp.* ME02 growth was too slow and unhealthy. Also, logarithmic phase could not be reached even at the end of 10th day. On the other hand, in TAP and TAP:BG-11 (1:1) media, regular growth pattern could be observed from graph.

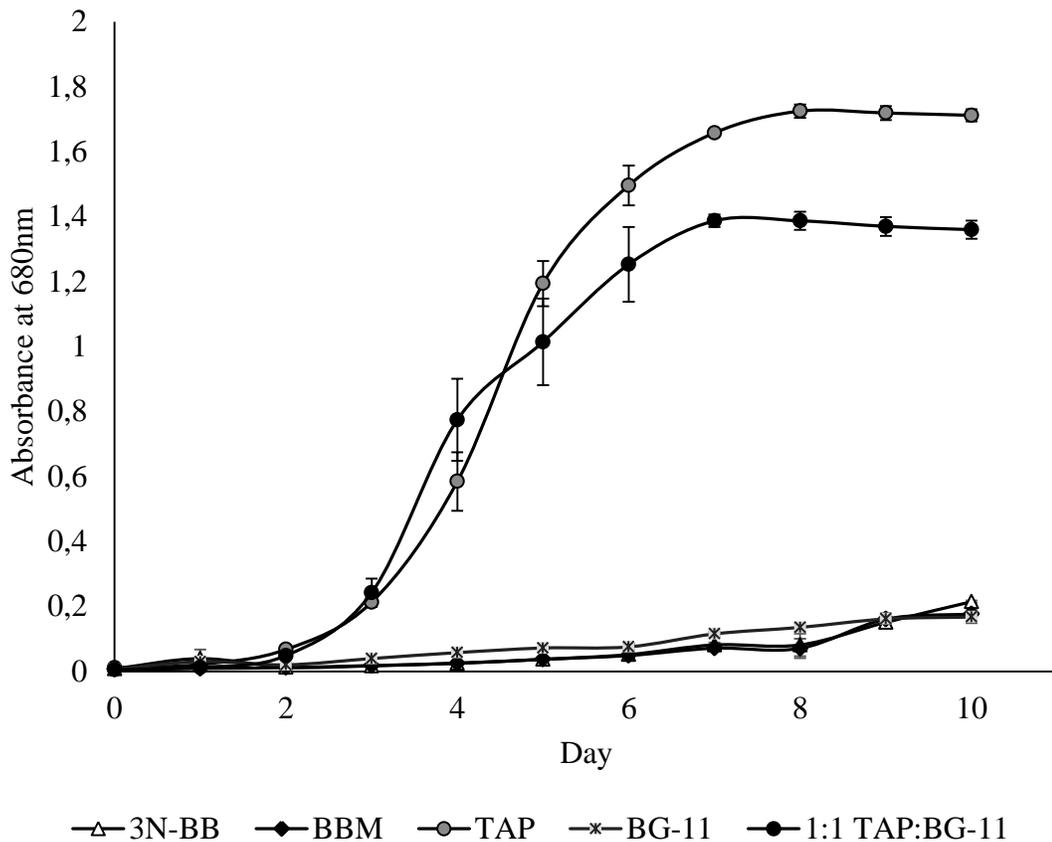


Figure 3.2 Growth curves of *Scenedesmus sp.* ME02 in different media (at 25°C, 16 h light/8 h dark photoperiod) Error bars were calculated by standard error.

Scenedesmus sp. ME02 cells grew more rapidly in TAP medium compared to TAP:BG-11 (1:1). It seemed that TAP medium was the most suitable growth medium for *Scenedesmus sp.* ME02. However, in TAP medium alone; cell clumps and secondary growth were observed (Figure 3.4). In order to eliminate these clumps, some modifications were tried in TAP medium. TAP and BG-11 media were mixed with equal volumes and *Scenedesmus sp.* ME02 cells were started to grow in it. In this case, healthy and uniform growth was observed and thus it was decided to use this medium mixture in subsequent experiments even growth was slower than that of in TAP medium.

3.1.1.1. Microscopic Characterization of *Scenedesmus sp.* ME02

Due to some aggregations of the cells in the TAP medium, closer examination was needed and *Scenedesmus sp.* ME02 cells were observed under the light microscope. Figure 3.3 shows light microscope image in TAP:BG-11 (1:1) medium. As it was mentioned above, uniform shaped and healthy growth was reached when TAP and BG-11 mediums were mixed with each other in one to one ratio. In Figure 3.4, cells were grown in only TAP medium and some aggregations were seen under microscope (Arrows show cell clumps). These cell clumps indicated the unhealthy growth of *Scenedesmus sp.* ME02 cells.

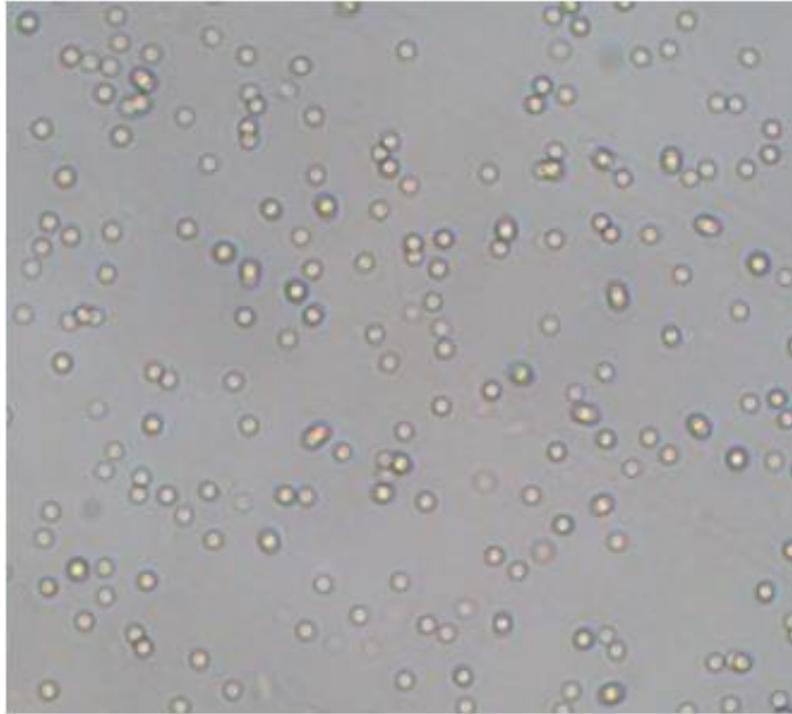


Figure 3.3 Light microscope image of *Scenedesmus sp.* ME02 grown in 1:1 TAP:BG-11 medium (100X magnification)

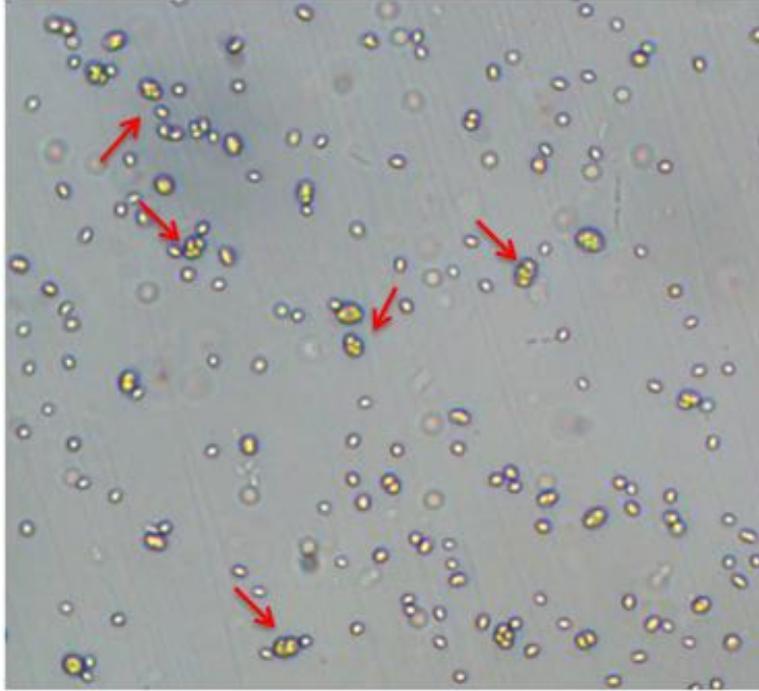


Figure 3.4 Light microscope image of *Scenedesmus sp.* ME02 grown in TAP medium (100X magnification)

Aggregation of the cells mainly results from high pH (>9) of the environment and also divalent cation concentrations such as Ca^{2+} ions (Powell & Hill, 2014). pH values of TAP and BG-11 media were adjusted at neutral pH however secondary metabolites that were produced during growth of the cells may cause pH increase and this condition may led to cell clumping. On the other side, TAP medium includes $8.30 \times 10^{-4} \text{M}$ CaCl_2 while BG-11 has $2.38 \times 10^{-4} \text{M}$ CaCl_2 as final concentration. Due to high Ca^{2+} concentration of TAP medium compared to BG-11, cells aggregated via hydrophobic or Van der Waals interactions (Powell & Hill, 2014). When TAP and BG-11 were mixed in 1:1 ratio, Ca^{2+} concentration dropped relatively and such condition was not observed.

3.1.2. Effect of Different Temperatures on Growth, Biomass and Lipid Compositions

After suitable growth medium was determined for *Scenedesmus sp.* ME02, growth curves were plotted at different temperatures (Figure 3.5). All experiment sets were done with three replicates and error bars were calculated by standard error.

This strain can be grown between 25-50°C and the optimum growth temperature is between 25°C-30°C as it is understood from graph in Figure 3.5 (Onay et al., 2014). In order to imitate the summer day in Central Anatolia, 16°C and 30°C were chosen as night and day temperatures because, in July and August day and night temperature changes between this range according to official statistics of Turkish State Meteorological Service. (Sonmez et al., 2016) The reason of imitating summer day temperatures in Central Anatolia was to provide an insight for outdoor cultivation at extreme temperatures without temperature control. Therefore, firstly growth curves were sketched at these defined temperatures. At 16°C, 25°C and 30°C, stationary phase was reached at 7th day. As it was expected, at 25°C *Scenedesmus sp.* ME02 showed optimum growth when compared with other temperature ranges. Besides, there was no significant difference between 25°C and 30°C growth curves, especially at stationary phase. When specific growth rate, biomass concentration and biomass productivity values were compared between 25°C and 30°C, the biomass values of 30°C was higher than that of 25°C (Table 3.1). Thus, it could be said that optimum temperature range is between 25°C and 30°C for *Scenedesmus sp.* ME02.

In addition, growth assessment was done at low temperature value (10°C). At 10°C, growth was very slow and even at the end of the 12th day, stationary phase was not reached. Despite this, *Scenedesmus sp.* ME02 cells could withstand this lower temperature. In future studies, cold tolerance of this strain can be improved by genetic engineering and normal growth manner can be achieved. Therefore, this situation shed light on outdoor cultivation in bioreactors at cold days without extensive temperature control.

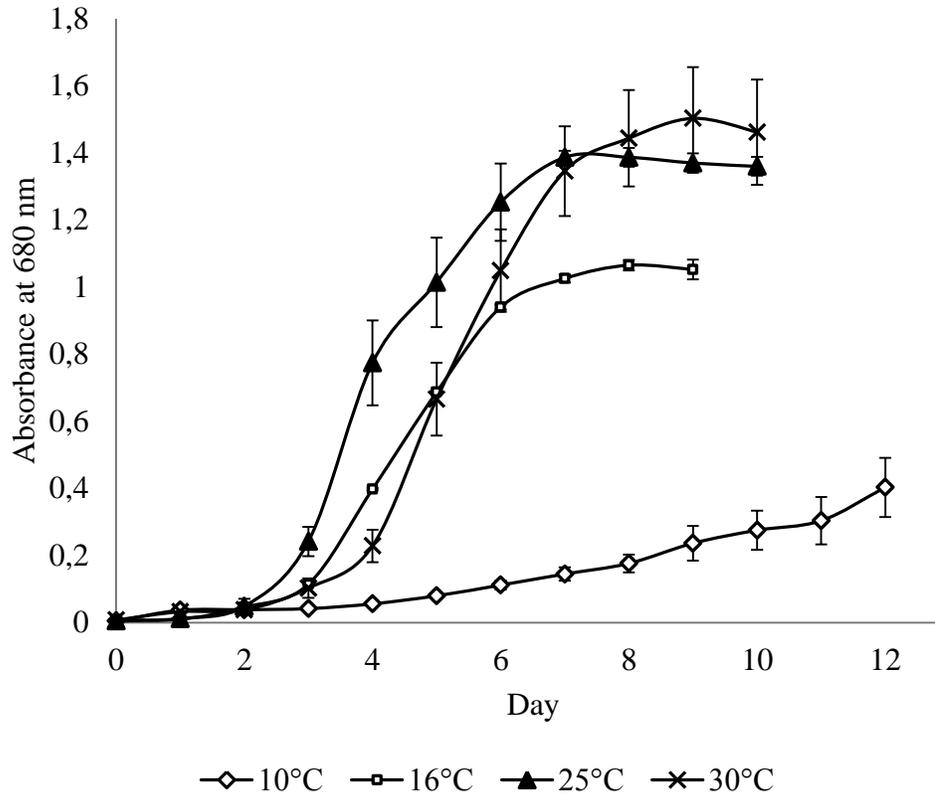


Figure 3.5 Growth curve of *Scenedesmus sp. ME02* at different temperatures (in TAP:BG-11 (1:1) medium, 16 h light/8 h dark photoperiod) Error bars were calculated by standard error.

3.1.2.1. Dry Weight and Biomass Analysis at Different Temperature Regimes

As it was mentioned before, in warm summer day in Central Anatolia, temperature fluctuates between 16-30°C daily. In laboratory conditions it was designed in acclimation chamber as 16°C night and 30°C day on 8 hours dark and 16 hours light photoperiod. These data were compared with daylong steady temperature at 16°C, 25°C and 30°C. Biomass measurements were taken at the end of logarithmic phase to ensure consistency of data. Specific growth rate, biomass concentration and biomass productivity formulas were given in Appendix C.

The highest values of specific growth rate, biomass concentration and biomass productivity were observed at 30°C. On the other hand, at 16-30°C (night/day) temperature cycle, the lowest values of specific growth rate, biomass concentration and biomass productivity were obtained compared to other temperatures apart from 10°C. Interestingly, biomass concentration at 16-30°C (night/day) was the same as 10°C. At 16-30°C (night/day) temperature cycle, a dramatic decrease was observed in biomass concentration and it was half of the 30°C (night/day) value. The lowness of 30-16°C biomass values at diurnal cycle may be due to unseasoned cells to temperature fluctuation. Strikingly, at 16-30°C (night/day), specific growth rate, biomass concentration and biomass productivity did not change through the cycle. The reason of this situation may be acclimation of the cells in this cycle (Table 3.1).

Table 3.1 Specific growth rate, biomass concentration and biomass productivity under different temperature regimes

	16 hours light/8 hours dark cycle					
	10°C	16°C	25°C	30°C	30-16°C	
					16°C (night)	30°C (day)
Specific Growth Rate (d ⁻¹)	0.47±0.020	0.75±0.005	0.76±0.006	0.78±0.040	0.71±0.008	0.71±0.004
Biomass Concentration (g.L ⁻¹)	0.3±0.05	0.4±0.02	0.4±0.02	0.6±0.16	0.3±0.02	0.3±0.01
Biomass Productivity (mg.L ⁻¹ .d ⁻¹)	23±4	51±2	54±3	73±2	36±2	36±1

In order to ignore the other parameters other than temperature, this study was conducted in batch cultures under laboratory conditions. It was shown that *Scenedesmus sp.* ME02 strain was adaptable to 30-16°C diurnal temperature cycle, so this strain can be cultured in outdoor photobioreactors in future studies.

3.1.2.2. Lipid and Fatty Acid Methyl Ester (FAME) Analysis at Different Temperature Regimes

Lipid content and lipid productivities were calculated at different temperature values (Formulas were given in Appendix C) (Table 3.2). These temperature regimes were the same as that of dry weight and biomass analysis studies. Lipid content of 16-30°C (night/day) temperature cycle was a little bit higher than that of 24 hours 16°C and 24 hours 30°C respectively. However, lipid productivity values were lower since biomass concentrations were also low at these temperature ranges. That is, low biomass concentration at 16-30°C (night/day) temperature cycle led to low lipid productivity because lipid productivity was calculated by using biomass concentration results. Despite the noticeable difference between lipid content of 25°C and 30°C, lipid productivities were nearly equal to each other. The reason was higher biomass concentration at 30°C with respect to 25°C. Highest lipid content and lipid productivity were 20.9±0.9 and 11.3±1 respectively when the algae were grown at 25°C in TAP:BG-11 (1:1) medium.

Table 3.2 Lipid content and lipid productivity under different temperature regimes

	16 hours light/8 hours dark cycle				
	16°C	25°C	30°C	30-16°C	
				16°C(night)	30°C(day)
Lipid Content (% w/w)	18.3±1.9	20.9±0.9	14.7±0.2	18.7±0.1	16.3±0.2
Lipid Productivity (mg.L ⁻¹ .d ⁻¹)	9.3±1	11.3±1	10.7±0.1	6.7±0.2	5.9±0.1

FAME profile of *Scenedesmus sp.* ME02 was measured at different temperatures. In general, palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) methyl esters were preferred for biodiesel production. (Onay et

al., 2014) These fatty acid types were present in abundance as indicated in Table 3.3. Unsaturation level of fatty acid influences the cold flow property, oxidative stability and combustion property of biodiesel. Polyunsaturated fatty acids are unstable because of their oxidative instability. On the other hand, saturated fatty acids have an important role in biodiesel properties since they have high cetane numbers (Piloto-Rodríguez et al., 2017).

Relative changes in FAME profile with temperature were searched in this study and noticeable results were obtained. For example, significant alterations observed for palmitic acid (16:0). When day and night temperatures were 16°C, 25°C and 30°C; palmitic acid percentage changed as 10%, 20% and 29% respectively. A contrary example was observed on γ -linolenic acid (18:3). While the percentage of γ -linolenic acid was 39.6% at 16°C day and night temperature; at other higher temperatures (25°C and 30°C) this value dropped to about 10-11%.

Saturated fatty acid composition dropped when temperature level decreased. This behavior was assessed as adaptation to colder temperature regimes. For instance, palmitic acid (16:0) level was 30% at 30°C; on the other hand this percentage dropped to 22% and 10% at 25°C and 16°C respectively. However, this was not the case at 16°C-30°C diurnal temperature cycle, because *Scenedesmus sp.* ME02 cells may have generated a memory about temperature stress and prepared for potential temperature change. (Sonmez et al., 2016)

Table 3.3 FAME profile of *Scenedesmus sp.* ME02 under different temperature regimes

FAME	16°C	25°C	30°C	30-16°C	
				16°C (night)	30°C (day)
Myristic Acid (14:0)	0.23±0.7	0.21±0.3	0.29±0.5	0.31±0.8	0.30±1.1
Palmitic Acid (16:0)	10.3±0.8	22.4±1.2	29.6±0.9	28.0±0.3	26.6±1.4
Palmitoleic Acid (16:1)	2.3±0.1	3.2±0.2	2.0±0.1	2.5±0.1	3.0±0.2
Heptadecanoic Acid (17:0)	0.06±0.2	0.09±0.5	0.16±0.9	0.10±0.2	0.12±0.3
Heptadecanoic Acid (17:1)	0.20±0.8	0.14±0.4	0.22±0.6	0.06±0.7	0.16±0.4
Stearic Acid (18:0)	0.36±0.1	0.42±0.4	0.29±0.2	0.31±0.1	0.30±0.2
Oleic Acid (18:1)	10.9±0.4	9.6±0.5	2.8±0.2	5.1±0.2	4.7±0.5
Linoleic Acid (18:2)	12.2±0.6	17.4±1.0	20.6±1.4	24.2±1.2	23.3±0.8
γ-Linolenic Acid (18:3)	39.6±2.1	10.0±0.8	11.0±0.6	10.3±0.7	10.6±1.1
Linolenic Acid (18:3)	23.6±1.4	36.0±1.8	33.0±1.0	29.0±2.0	30.7±1.5
Arachidic Acid (20:0)	0.29±0.4	0.19±0.4	0.09±0.2	0.12±0.1	0.11±0.3
Behenic Acid (22:0)	0.00	0.17±0.4	0.00	0.06±0.3	0.06±0.1
Lignoceric Acid (24:0)	0.00	0.17±0.5	0.08±0.1	0.11±0.6	0.10±0.1

3.2. Genetic Engineering of *Scenedesmus sp.* ME02

3.2.1. Partial Cloning the Novel Fatty Acid Desaturase 2 (*fad2*) Gene from *Scenedesmus sp.* ME02

Scenedesmus sp. ME02 could withstand a wide temperature range and its FAME profile changed depending upon different temperatures. There should be a genetic basis of this situation. Fatty acid desaturase 2 (*fad2*) gene provides cold and drought tolerance to the

plants and microalgae and the reason of temperature dependent FAME profile alterations can derive from *fad2* gene regulations. Thus, *fad2* gene may have a significant role at this point and as a first step, it was partially cloned from *Scenedesmus sp.* ME02.

In order to partially clone *fad2* gene from *Scenedesmus sp.* ME02, FAD2 amino acid sequences of *Chlorella vulgaris* (green microalgae), and model organisms *Chlamydomonas reinhardtii* and *Arabidopsis thaliana* were compared by Clustal Omega web based multiple sequence alignment tool. Highly conserved regions among them were determined and a primer set was designed for amplifying this region from *Scenedesmus sp.* ME02 (Figure 3.6).

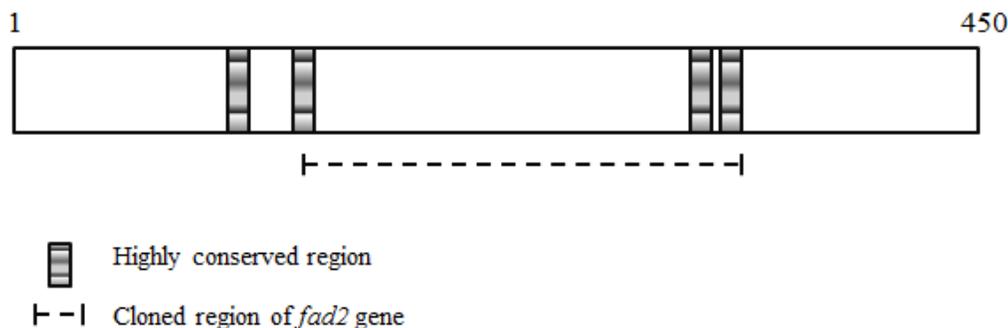


Figure 3.6 Highly conserved FAD2 amino acid regions of *Arabidopsis thaliana*, *Chlamydomonas reinhardtii* and *Chlorella vulgaris*

3.2.1.1. Total RNA Isolation from Microalgae Strains

Total RNA was isolated from *Chlamydomonas reinhardtii* and *Scenedesmus sp.* ME02 cells. Quality of RNA isolates were verified via agarose gel electrophoresis because RNA is a very fragile molecule and integrity should be checked before subsequent experiments (Figure 3.7). On gel image, 28S rRNA and 18S rRNA bands could be seen on labeled sites.

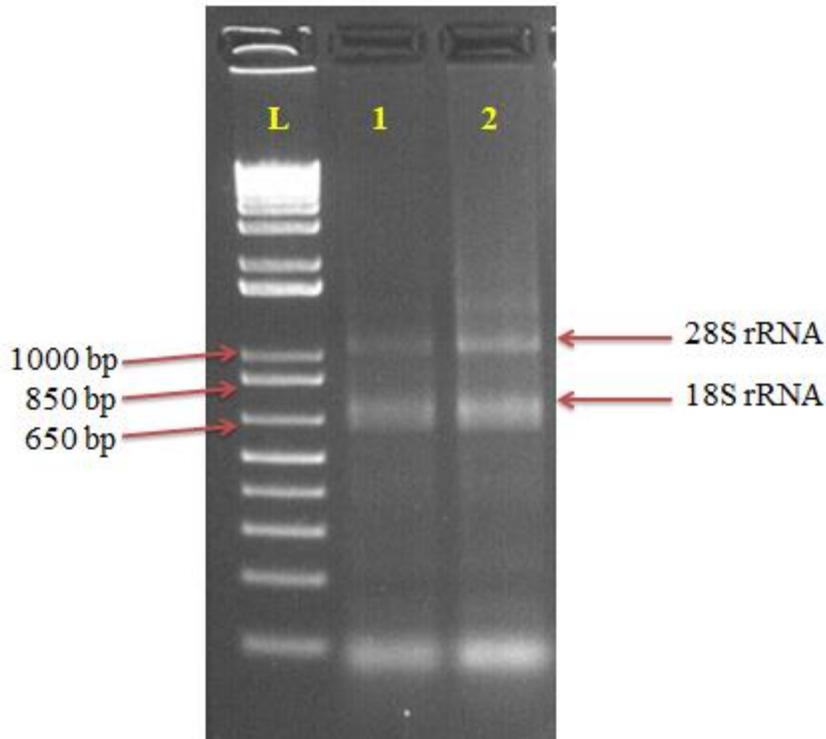


Figure 3.7 Agarose gel (1.5%) image of total RNA samples from microalgae strains. Lane L: 1kb plus ladder, Lane 1: *Chlamydomonas reinhardtii* total RNA, Lane 2: *Scenedesmus sp. ME02* total RNA

3.2.1.2. RT-PCR Optimization

Since GC content of *fad2* gene was high (about 50-60%) in *C. reinhardtii*, DMSO and betaine usage was beneficial in PCR studies. By using these kinds of agents, clearer and sharper bands were obtained on agarose gel. For optimization, two different DMSO (3% and 5%) and betaine concentrations (0.6M and 1M) were applied in RT-PCR.

PCR results were checked with agarose gel electrophoresis (Figure 3.8). There were nonspecific bands on lane 1 and 2. On the other hand, on lane 4; expected band was observed but it was blurred and weak. The clearest and sharpest band was seen on lane 3 (0.6 M betaine) when compared to others. Therefore, 0.6 M betaine was determined as most effective concentration for subsequent RT-PCR studies.

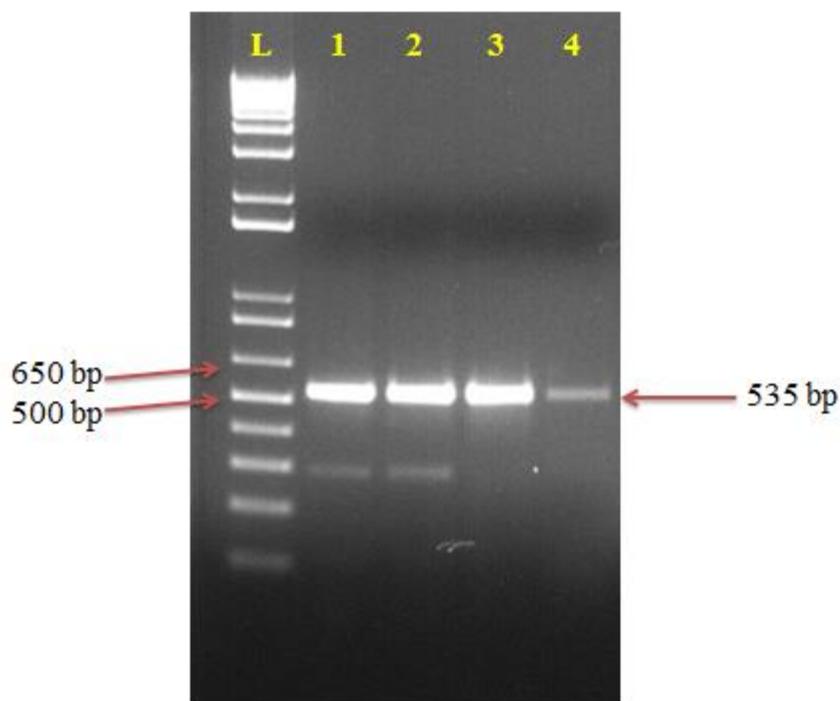


Figure 3.8 Agarose gel (1.5%) image of RT-PCR from *C. reinhardtii* cDNA. Lane L: 1kb plus ladder, Lane 1: 3% DMSO, Lane 2: 5% DMSO, Lane 3: 0.6M betaine, Lane 4: 1M betaine

3.2.1.3. RT-PCR with *C. reinhardtii* and *Scenedesmus sp.* ME02 cDNAs

After PCR optimization was completed with *C. reinhardtii* cDNA, the same RT-PCR conditions were applied for *Scenedesmus sp.* ME02 and *C. reinhardtii* cDNA.

C. reinhardtii was used as positive control in this experiment. Presence of the *fad2* gene was tried to be shown in *Scenedesmus sp.* ME02 gene. Although *Chlamydomonas reinhardtii* is model organism, *Scenedesmus sp.* ME02 was mainly used in these FAD2 studies. The reason was the adaptability of *Scenedesmus sp.* ME02 to a wide temperature range and also fatty acid content was richer than that of *C. reinhardtii*. These properties made *Scenedesmus sp.* ME02 more advantageous than *C. reinhardtii* for these studies. Genetic basis of resistivity to wide temperature range of *Scenedesmus*

sp. ME02 and alterations in FAME profile of this strain depending on temperature change were investigated in FAD2 studies.

As expected, 535 bp DNA band was seen for *C. reinhardtii* (lane 1). The same band was present on lane 2, too. However, this band was relatively indistinct than the band on lane 1. In this way, it was understood that *fad2* gene sequence was conserved in *Scenedesmus sp.* ME02. Lane 3 was made as negative control and any visible band was not seen on gel expectedly (Figure 3.9).

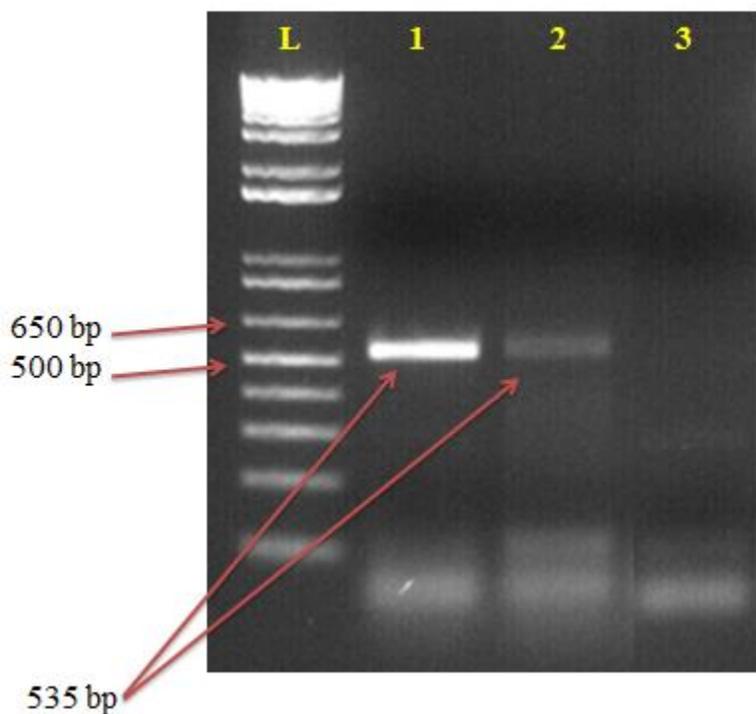


Figure 3.9 Agarose gel (1.5%) image of RT-PCR from microalgae cDNAs. Lane L: 1kb plus ladder, Lane 1: *fad2* of *Chlamydomonas reinhardtii*, Lane 2: *fad2* of *Scenedesmus sp.* ME02, Lane 3: negative control

3.2.1.4. Purification of PCR Products

PCR purification was conducted for effective cloning process of *Scenedesmus sp.* ME02 FAD2 gene fragment. PCR purification was done with QIAquick PCR Purification Kit

(Qiagen) and at the end of this process; concentration was measured as 37ng/μL via NanoDrop.

At the end of the purification, *Scenedesmus sp.* ME02 FAD2 PCR product was again visualized on agarose gel for double check. As expected, the same 535 bp band was seen on the gel (Figure 3.10).

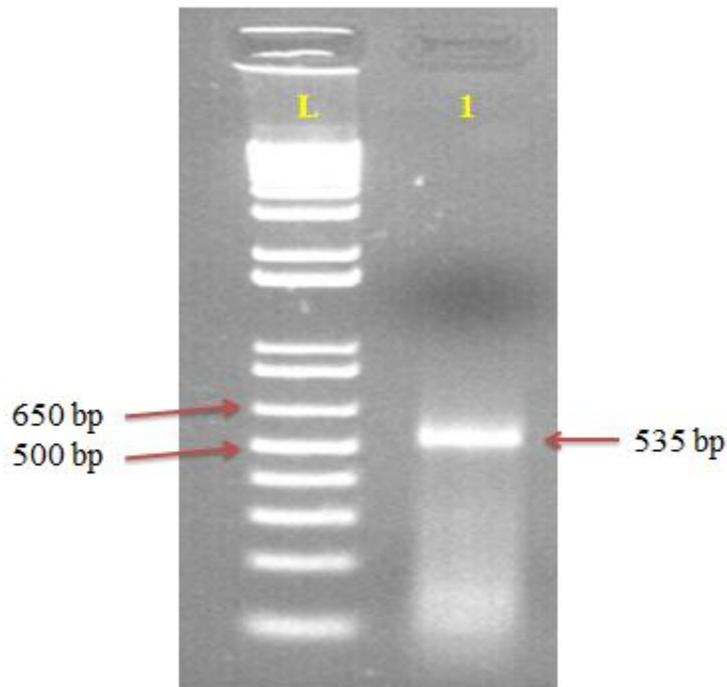


Figure 3.10 Agarose gel (1.5%) image of purified RT- PCR product. Lane L: 1kb plus ladder, Lane 1: purified *Scenedesmus sp.* ME02 *fad2* RT-PCR product

3.2.1.5. Chemical Transformation to Competent *E.coli*

TOPO TA cloned plasmids were transformed to competent *E.coli*. After chemical transformation to competent cells, colonies appeared on the 75 mg/L ampicillin containing LB agar plate (Figure 3.11 A). Relatively larger and single colonies were selected and again inoculated on agar plate with streak plate technique (Figure 3.11 B).

The purpose of second inoculation was increasing the cell number for subsequent experiments and testing the colonies against to selective marker for second time in order to eliminate escapes.

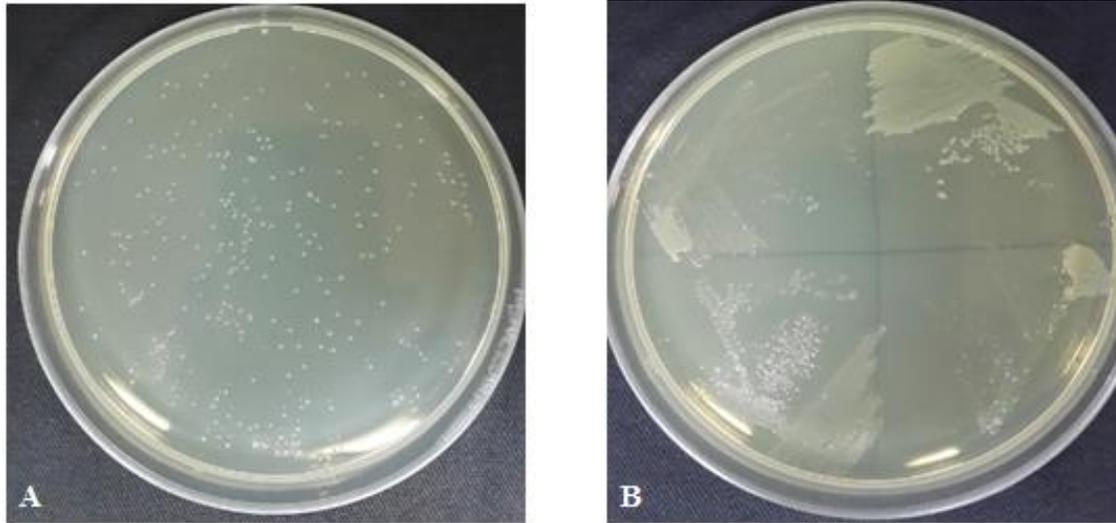


Figure 3.11 Chemically transformed *E.coli* cells on ampicillin containing LB agar plate. **A.** first appearing colonies after transformation **B.** randomly chosen transformant cells from plate A.

3.2.1.6. Colony PCR from Transformed *E.coli* Cells

Eight different colonies were chosen randomly on agar plate and colony PCR was done to verify the presence of FAD2 insert in the cell. Then, colony PCR results were analyzed on agarose gel (Figure 3.12). FAD2 primers amplified 535 bp gene region so expected band size was 535 bp on gel. Only on the lane 1, a band was observed around 500 bp. The other colonies did not give such bands.

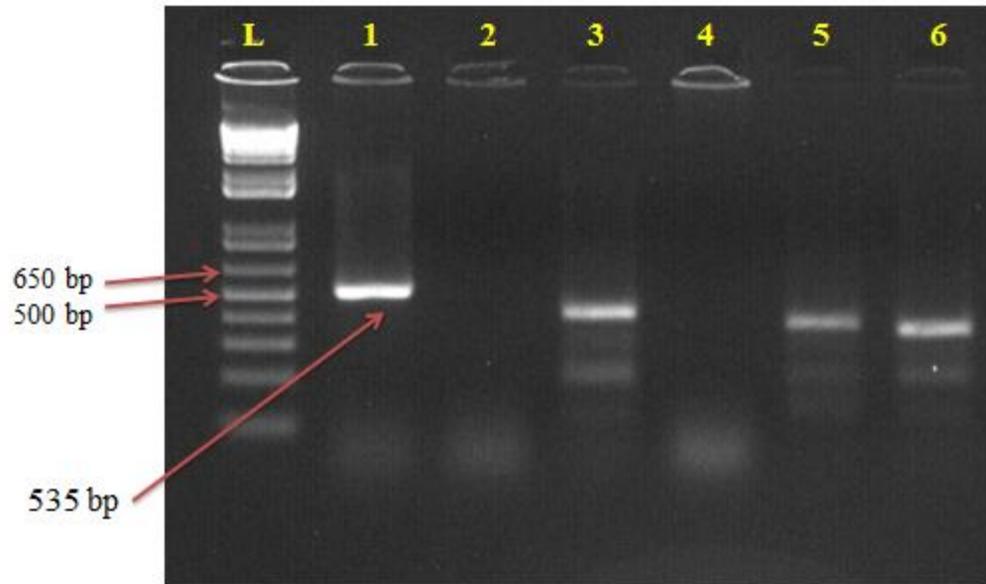


Figure 3.12 Agarose gel (1.5%) image of colony PCR. Lane L: 1kb plus ladder, Lane 1-6: different *E.coli* colonies

3.2.1.7. Plasmid Isolation from Transformed *E.coli* Cells

According to colony PCR results, plasmid was isolated from colony#1 since only it gave the positive result on colony PCR. Isolated plasmid concentration was measured as 1172 ng/ μ L.

3.2.1.8. Sequencing the *fad2* Gene Insert in TOPO TA Vector

TOPO TA cloned plasmid in colony#1 was sequenced from two directions with M13 reverse and T7 primers. Sequencing results were given below.

```
>HF1-M13R_D04 (1)
NNNNNNANCTGNNATNANCCCTCACTANNAGGNGTCCTGCAGGTTTAA
CGAATTCGCCCTTCGCGTGATAGTGCGGCATGTAGGTGACGGGGGGTCAC
GTGCGTGCGCGTAGGCGGCACCACAAACAATTGCGCAGACGGTGCAAACA
CAAACACCTCGTCCTTGGCCACAAACACCTCGTCCTTGGCCACAAACACC
TCGTCCTTGGCCACGTGATAGTGCGGCATGTAGGTGACGGGGGGTCACGT
GCGTGCGCGTAGGCGGCACCACAAACAATTGCGCAGACGGTGCAAACACA
AACACCTCGTCCTTGGCCACAAACACCTCGTCCTTGGCCACAAACACCTC
GTCCTTGGCCACAAACACCTCGTCCTTGGTTCGCGTGATAGTGCGGCATGT
ACAAACACCTCGTCCTTGGCCAAGGGCGAATTCGCGGCCGCTAAATTCAA
TTCGCCCTATAGTGAGTCGTATTACAATCACTGGCCGTCGTTTTACAAC
GTCGTGACTGGGAAAACCTGGCGTTACCCAACCTAATCGCCTTGCAGCA
CATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCG
CCCTTCCCAACAGTTGCGCAGCCTATACGTACGGCAGTTTAAGGTTTACA
CCTATAAAAGAGAGAGCCGTTATCGTCTGTTTGTGGATGTACAGAGTGAT
ATTATTGACACGCCGGGGGCGACGGATGGTGATCCCCCTGGCCAGTGAC
GTCTGCTGTCAGATAAAGTCTCCCGTGAACCTTACCCGGGGGGTGCATAT
CGGGGGATGAAAGCTGGGCGCATGATGACCACCCGATATGGGCCAGTGGG
NCCGGTCTCCCGTTATCCGGGGAAAAAAGTTGCCTGATCTCAGCCNCCCG
CGAAAATGACATCAAAAACCGCCATTAACCTGGATGTTCTGGGGGAATAT
AAATTGTCNNGGCATGAAAATTATCAAAAAGGATCTTCC
```

This sequence was obtained with M13 reverse primer. Bold and underlined area belongs to *fad2* gene region. The remaining sequence parts belong to TOPO TA vector.

```

>HF1-T7PROMOTER_H03 (1)
GNNNATNAATAANNGGCCGCGATTTCGCCCTTGGCCAGGACGAGGTGTTTGT
TACATGCCGCACTATCACGCGNCCAAGGACGAGGTGTTTGTGGCCAAGGA
CGAGGTGTTTGTGGCCAAGGACGAGGTGTTTGTGGCCAAGGACGAGGTGT
TTGTGTTTGCACCGTCTGCGCAATTGTTTGTGGTGCCGCCTACGCGCACG
CACGTGACCCCGTCACCTACATGCCGCACTATCACGTGGCCAAGGACG
AGGTGTTTGTGGCCAAGGACGAGGTGTTTGTGGCCAAGGACGAGGTGTTT
GTGTTTGCACCGTCTGCGCAATTGTTTGTGGTGCCGCCTACGCGCACGCA
CGTGACCCCGTCACCTACATGCCGCACTATCACGCGAAGGGCGAATTC
GTTTAAACCTGCANGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTG
GCGTAATCATGGTCATAGCTGTTTCTGTGTGAAATTGTTATCCGNTNAC
AATCCACACAACATAACNAGCCGGAAGCATAAAGTGTAAGCCTGGGGTG
CCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCT
TTCCAGTCGNAAACCTGTCNTGCNCTGCATTAATGAATCCGCCAACGCG
CGGGGAGAGGCGGTTGTCNTATTGGGCGCTNTTCCGNTTCTCNCCTACT
NACTNNTGCCTCGGNCNTCCGCTGCGCGANCGGATNNTCNTCAAAGGNNT
ATACNGTACCCAAATNNGGATAACCCAGGAAAACGTGTACAAAGGCNCCAA
GNCANAANNAANNCNCGTGCGGCGTTTCAAGNTNCCCCNTNAAGCTC
AAAANC

```

When sequencing was done from opposite direction with T7 primer, the sequence above was obtained.

```

GNTTTTGTAGCTTNANGGGGNANCTTGAAACGCCGCACGNGNNTTTTNNNT
TNTGNCTTGGNGCCTTTGTACAGTTTTTCTGGGTATCCCNATTTGGGTA
CNGTATANNCTTTGANGANNATCCGNTCGCGCAGCGGANGNCCGAGGCA
NNAGTNAGTGAGNGAGGAANCGGAANAGCGCCCAATANGCAAACCGCCTC
TCCCCGCGCGTTGGCGGATTCATTAATGCAGNGCANGACAGGTTTNCGA
CTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTC
ATTAGGCACCCAGGCTTTACACTTTATGCTTCCGGCTNGTATGTTGTGT
GGAATTGTNANCGGATAACAATTTACACAGGAAACAGCTATGACCATGA
TTACGCCAAGCTCAGAATTAACCCTCACTAAAGGGACTAGTCNTGCAGGT
TTAAACGAATTCGCCCTTTCGCGTGATAGTGCGGCATGTAGGTGACGGGGG
GTCACGTGCGTGCGCGTAGGCGGCACCACAAACAATTGCGCAGACGGTGC
AAACACAAACACCTCGTCCTTGGCCACAAACACCTCGTCCTTGGCCACAA
ACACCTCGTCCTTGGCCACGTGATAGTGCGGCATGTAGGTGACGGGGGGT
CACGTGCGTGCGCGTAGGCGGCACCACAAACAATTGCGCAGACGGTGCAA
ACACAAACACCTCGTCCTTGGCCACAAACACCTCGTCCTTGGCCACAAAC
ACCTCGTCCTTGGCCACAAACACCTCGTCCTTGGNCGCGTGATAGTGCGG
CATGTACAAACACCTCGTCCTGGCCAAGGGCGAATCGCGGCCNNTTATTN
ATNNNC

```

In order to align two sequences (M13 reverse and T7), reverse complement of T7 sequence was generated as shown above. Still, bold and underlined area belongs to *fad2* gene sequence.

Sequences producing significant alignments:

Select: [All](#) [None](#) Selected: 0

Alignments [Download](#) [GenBank](#) [Graphics](#) [Distance tree of results](#)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Chlamydomonas reinhardtii microsomal delta-12 fatty acid desaturase (FAD2) mRNA, complete cds	44.6	446	76%	4e-04	82%	EU596472.1

Figure 3.14 BLAST result between *Scenedesmus sp.* ME02 and *C.reinhardtii fad2* gene sequences.

In the future studies, full cloning of *fad2* gene can be achieved from *Scenedesmus sp.* ME02 and relationship between expression rate of *fad2* and temperature dependent FAME profile alterations can be investigated. Since *fad2* converts oleic acid to linoleic acid, it provides adaptability to low temperatures (An et al., 2013). If any significant changes are observed in *fad2* expression depending on temperature, overexpression of *fad2* gene can be attempted at the temperature that gives the richest FAME content. Another approach may be the transformation of *fad2* gene to different microalgal strains and improve the lipid content of them, after full cloning of *fad2* gene from *Scenedesmus sp.* ME02. Los et. al. reported that *Synechococcus sp.* PCC 7942 (cyanobacteria) was transformed with foreign fatty acid desaturase gene and excessive amount of unsaturated fatty acids were produced in its membrane lipids. This approach allowed understanding the role of desaturases in the adaptation of this microorganism to extensive temperatures.

3.2.2. Transformation of *Scenedesmus sp.* ME02 Cells

So far, metabolic improvement of *Scenedesmus sp.* ME02 has been provided by biochemical assessment. After this point, improvement was ensured by genetic engineering methods like transformation. Transgenic microalgae can be used as ‘green cell factory’ and high value molecules can be harmlessly produced such as valuable proteins and lipids. Additionally, healthy foods, food additives and pharmaceuticals can be manufactured from genetically engineered microalgae (Chu, 2012).

3.2.2.1. Determination of Selectable Marker

Before transformation, firstly selectable marker was determined. Since desired vector construct contained hygromycin resistance gene fragment and it was already known that *Scenedesmus sp.* ME02 was sensitive to hygromycin, it was chosen as a selectable marker. In order to determine the certain antibiotic concentration that *Scenedesmus sp.* ME02 was not alive, three different hygromycin concentrations were tried. These were namely, 25 mg/L, 50 mg/L and 75 mg/L. At 25 mg/L hygromycin concentration, *Scenedesmus sp.* ME02 cells were mostly alive on agar surface (Figure 3.14 A). At 50 mg/L concentration, most of the cells were dead but still live cells were present on plate (Figure 3.15 B). However, on 75 mg/L hygromycin containing agar plate (Figure 3.15 C), almost any cells were alive. Because of these reasons, 25 mg/L and 50 mg/L hygromycin concentrations were not chosen. Instead of these, 75 mg/L hygromycin concentration was determined to be used in subsequent transformation experiments.

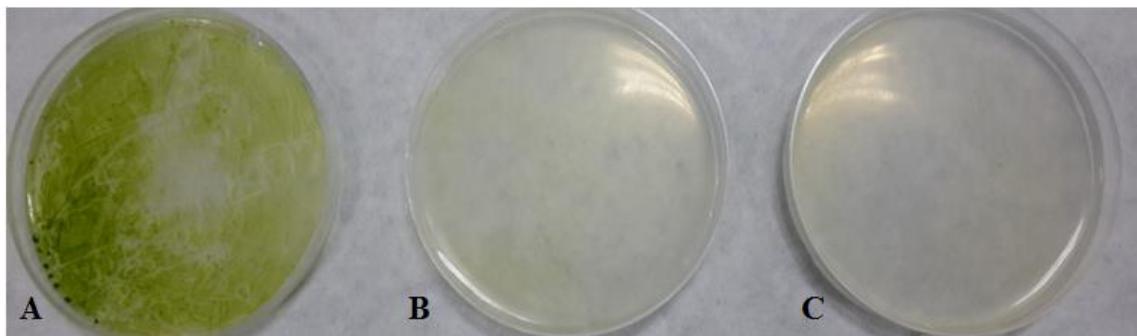


Figure 3.15 *Scenedesmus sp.* ME02 cells on different hygromycin concentrations. **A.** 25 mg/L hygromycin, **B.** 50 mg/L hygromycin, **C.** 75 mg/L hygromycin containing TAP agar plates.

3.2.2.2. Optimization of Transformation Conditions

Since transformation has not been applied to *Scenedesmus sp.* ME02 so far, initially conditions of electroporation were optimized. This optimization was conducted by electroporation of pHyg3 plasmid to *Scenedesmus sp.* ME02 cells.

3.2.2.2.1. Amplification of Hygromycin Resistance Gene

Before transformation of pHyg3 plasmid to *Scenedesmus sp.* ME02, hygromycin resistance gene (*aph7*) was amplified via PCR. Initially, DNA amount was optimized. For this purpose three different DNA concentrations were used in PCR namely; 0.5 μ L, 1 μ L and 2 μ L. Results of PCR was examined on agarose gel image. As it was seen on the Figure 3.16, in lane 1, DNA band was clearer than lane 2 and 3. At the bottom of the lane 1, there was no primer dimer band when compared to others. Due to these reasons, 0.5 μ L pHyg3 was suitable for this PCR. Therefore, required amount of hygromycin resistance gene for transformation was amplified via PCR by using 0.5 μ L DNA in following experiments.

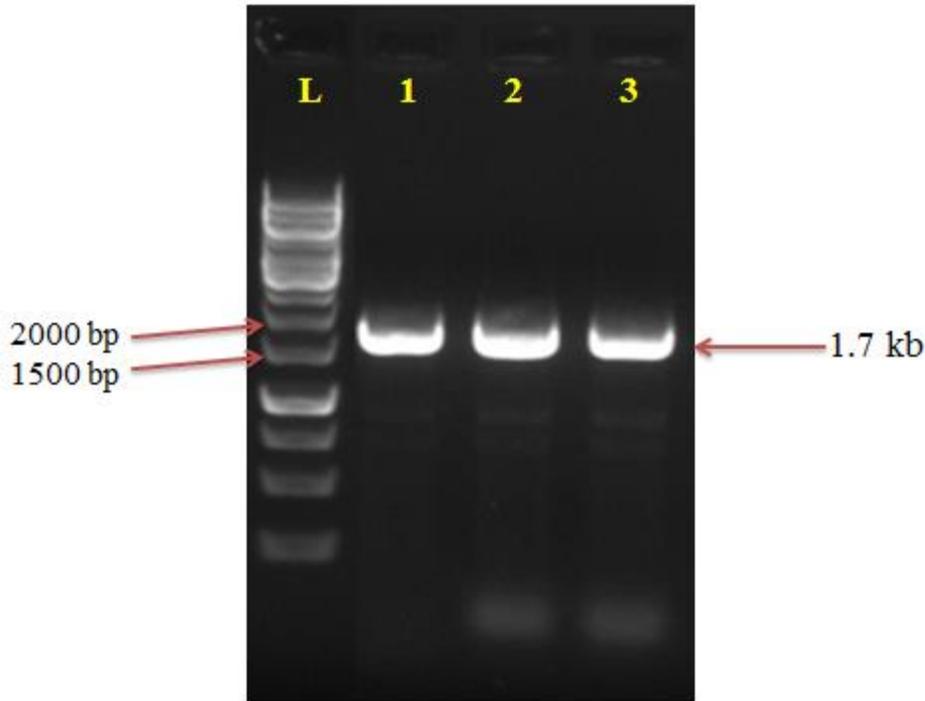


Figure 3.16 Agarose gel (1.5%) image of pHyg3 PCR. Lane L: 1 kb ladder, Lane 1: 0.5 μ L DNA, Lane 2: 1 μ L DNA, Lane 3: 2 μ L DNA.

3.2.2.2.2. pHyg3 Transformation of *Scenedesmus sp.* ME02

pHyg3 plasmid was transformed to *Scenedesmus sp.* ME02 in order to optimize the transformation parameters and verify the transformation efficiency. Four different voltage and capacitance values were applied to *Scenedesmus sp.* ME02 cells. These were 1.5kV-10 μ F, 2kV-25 μ F, 2kV-10 μ F and 1kV-25 μ F. Also, non-electroporated cells were used as control group.

In Figure 3.17 A, non-electroporated cells were inoculated on 75 mg/L hygromycin containing TAP agar plates. Since *Scenedesmus sp.* ME02 cells were sensitive to hygromycin, cells did not grow on the surface of the plate, as it was expected. On the other hand, electroporated cells were inoculated on TAP agar plate and many colonies

could grow (Figure 3.17 B). According to these results, it was inferred that there was no problem about electroporated cells.

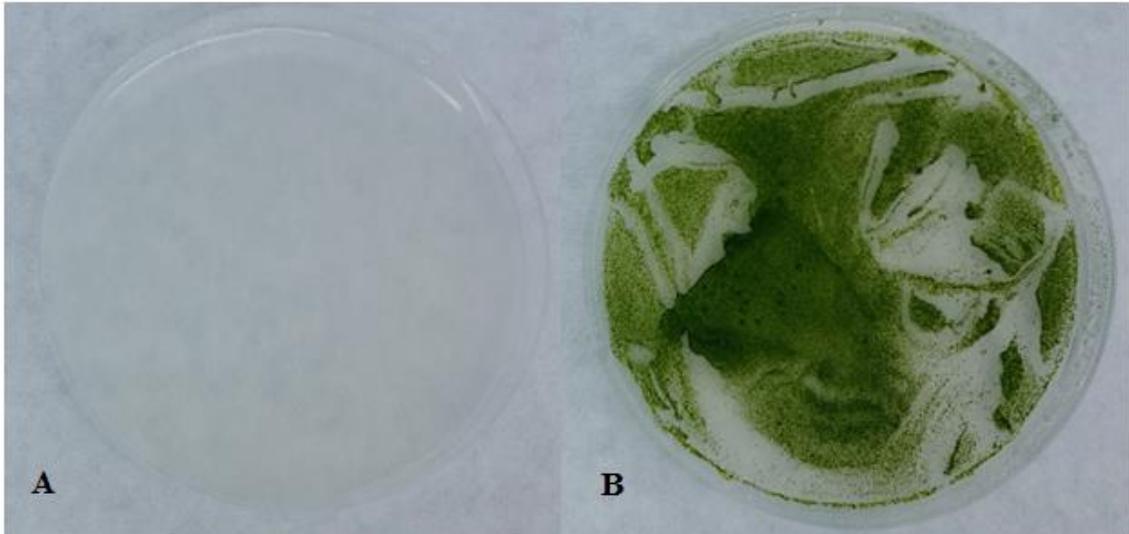


Figure 3.17 Control groups of pHyg3 transformation. **A.** non-electroporated cells on hygromycin containing agar plate. **B.** electroporated cells on agar plate

Transformed cells were inoculated on 75 mg/L hygromycin containing TAP agar plates. Since agar plates contained hygromycin antibiotics and pHyg3 carried hygromycin resistance gene fragment, colonies formed on the plate were called as positive transformants.

In Figure 3.18 A, *Scenedesmus sp.* ME02 was electroporated under 1.5kV voltage and 10 μ F capacitance values. Only 1 positive colony was observed on agar plate. Other green tiny colonies around the red arrow were satellite colonies. These colonies formed when antibiotics lost its effect. Thus, satellite colonies were not accepted as positive transformant.

When 2kV voltage and 25 μ F capacitance were applied on *Scenedesmus sp.* ME02 cells 6 positive transformants were observed on plate. Two of them were shown by red arrows (Figure 3.18 B).

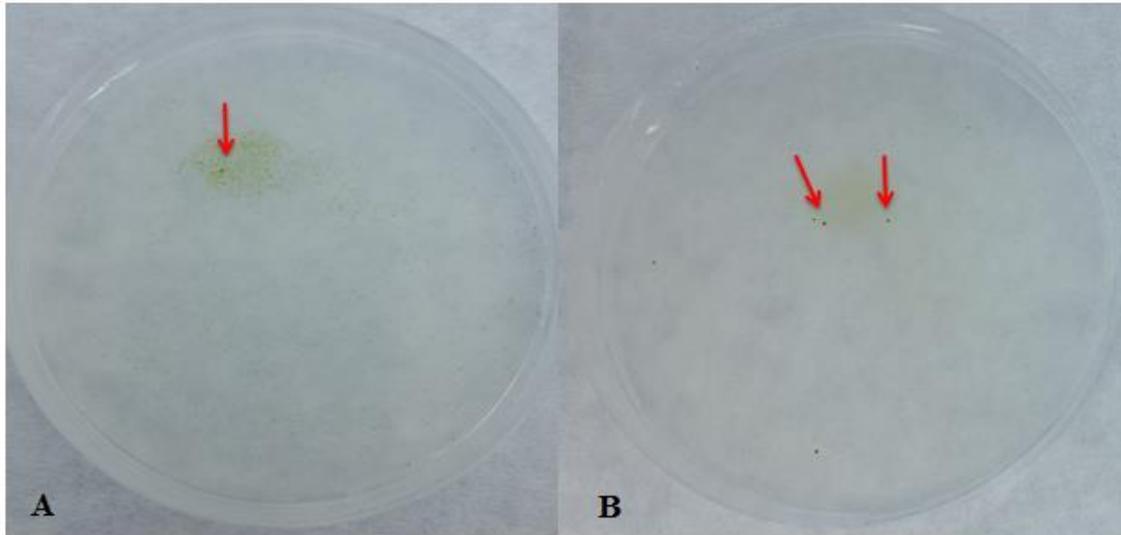


Figure 3.18 pHyg3 transformant cells on hygromycin containing agar plates. **A.** 1.5kV voltage and 10 μ F capacitance, **B.** 2 kV voltage and 25 μ F capacitance.

The most efficient results were obtained when transformation was done with 2kV-10 μ F and 1kV-25 μ F voltage and capacitance values. In Figure 3.19 A, 2kV-10 μ F transformation parameters were used and 21 colonies were observed. On the other hand, in Figure 3.19 B, 1kV voltage and 25 μ F capacitance was applied on the cells and 24 colonies were obtained on agar plate.

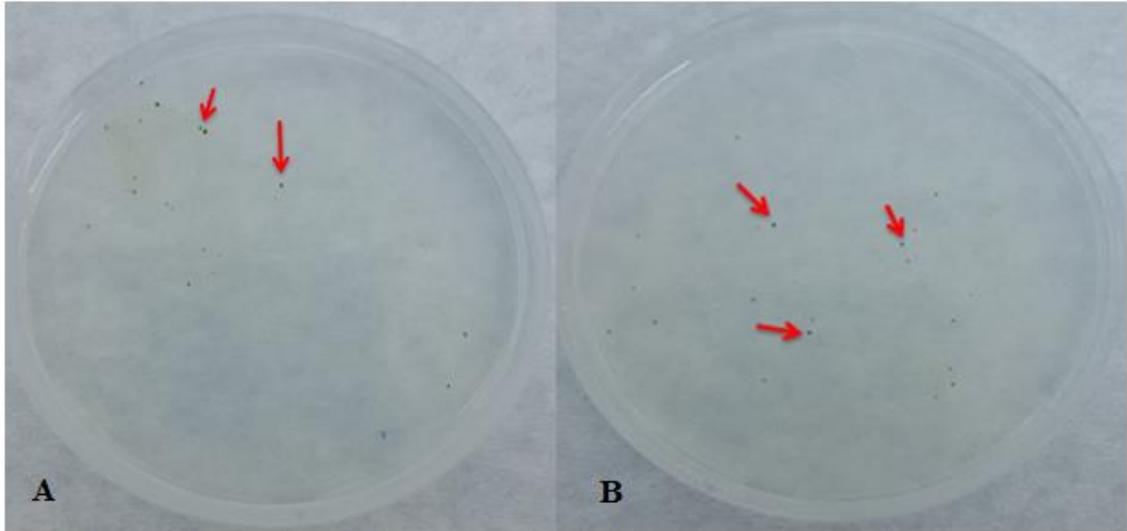


Figure 3.19 pHyg3 transformant cells on hygromycin containing agar plates. **A.** 2 kV voltage and 10 μ F capacitance, **B.** 1 kV voltage and 25 μ F capacitance.

As a result, considering the number of colony formation at 2kV-10 μ F (21 colonies) and 1kV-25 μ F (24 colonies); these electroporation parameters were determined as most productive and effective values for *Scenedesmus sp.* ME02 transformation. Following studies were conducted by using these best voltage and capacitance values.

3.2.2.3. GFP Transformation via Electroporation

3.2.2.3.1. Vector Design and Cloning

GFP gene fragment was previously constructed in pChlamy_3 vector from pCAMBIA1302 vector by our laboratory members. According to optimized parameters that were determined on pHyg3 transformation studies, GFP::pChlamy_3 transformation was conducted.

Before transformation, firstly plasmid was linearized to provide proper integration of the gene into genome of *Scenedesmus sp.* ME02 cells. GFP containing pChlamy_3 plasmid was digested with *ScaI* restriction enzyme. *ScaI* cut the plasmid at one site and it became linear. *ScaI* digested GFP::pChlamy_3 plasmid was checked via agarose gel electrophoresis. In order to verify the results, uncut GFP::pChlamy_3 was loaded on lane 1; digested plasmid was observed on the other site (lane 2) (Figure 3.20).

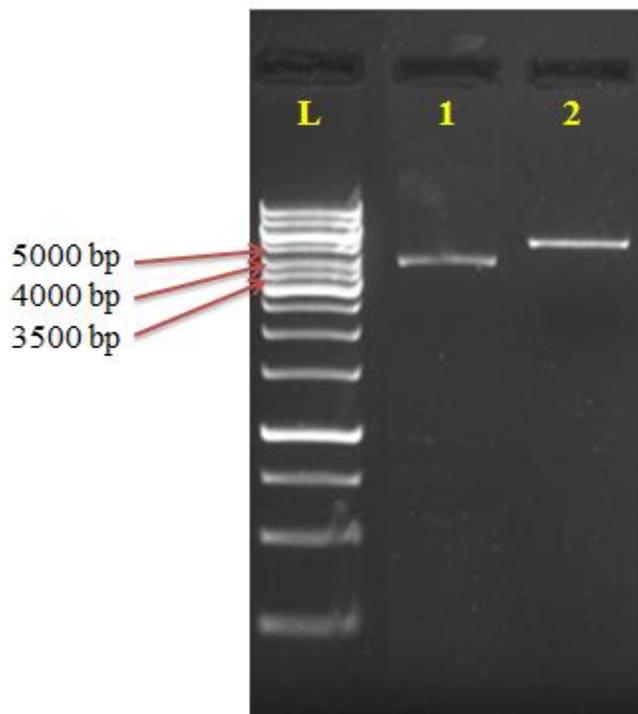


Figure 3.20 Agarose gel (1.5%) image after digestion of GFP::pChlamy_3 vector. Lane L: 1 kb ladder, Lane 1: undigested GFP::pChlamy_3, Lane 2: *ScaI* digested GFP::pChlamy_3

3.2.2.3.2. Transformation Conditions

For GFP transformation, two different voltage and capacitance values were applied to the *Scenedesmus sp.* ME02 cells. As previously determined in pHyg3 transformation

experiment, most effective parameters were 1kV-25 μ F and 2kV-10 μ F. Thus, these parameters were used in GFP transformation study. In addition, as a control group, non-electroporated cells were used.

Similar to pHyg3 transformation experiment, as a control group, non-electroporated cells were spread onto 75 mg/L hygromycin containing TAP agar plates. As it was expected, growth was not observed on the surface of the plate (Figure 3.21 A). If looked at the Figure 3.21 B, electroporation applied cells were observed on TAP agar. Since *Scenedesmus sp.* ME02 cells can grow on TAP agar in normal conditions, it was understood that electroporation process did not affect the cells vitality.

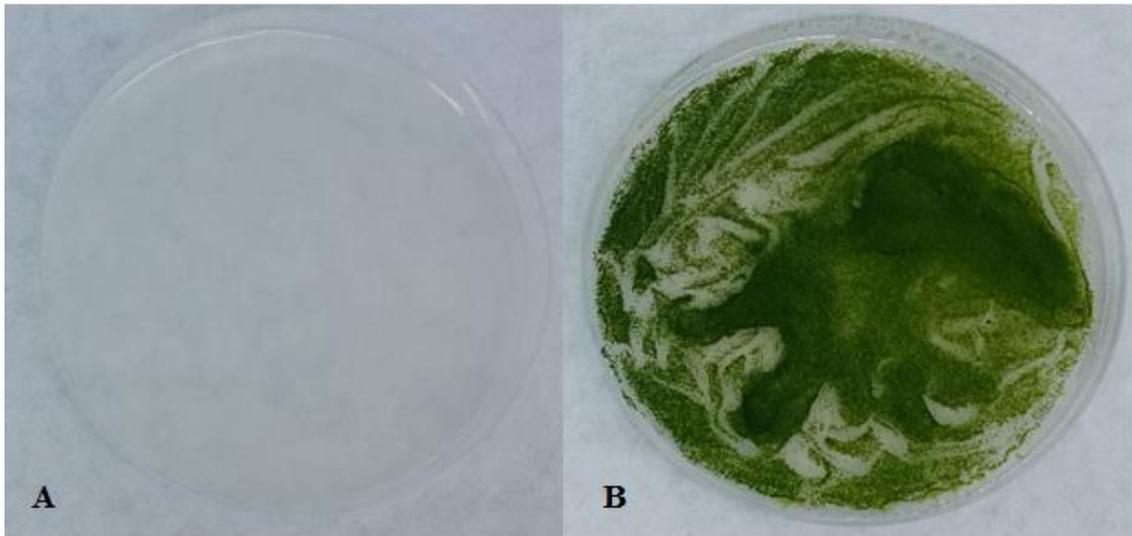


Figure 3.21 Control groups of GFP transformation. **A.** non-electroporated cells on hygromycin containing agar plate. **B.** electroporated cells on agar plate

In regard to pHyg3 transformation results, GFP transformation voltage and capacitance parameters were determined as 1kV-25 μ F and 2kV-10 μ F. These values were decided by looking at number of positive transformants at these particular conditions. Relatively larger colonies were selected and transformed to new 75 mg/L hygromycin containing

TAP agar plates (Figure 3.22). Small colonies around the large ones were not taken into consideration because they were accepted as satellite colonies.

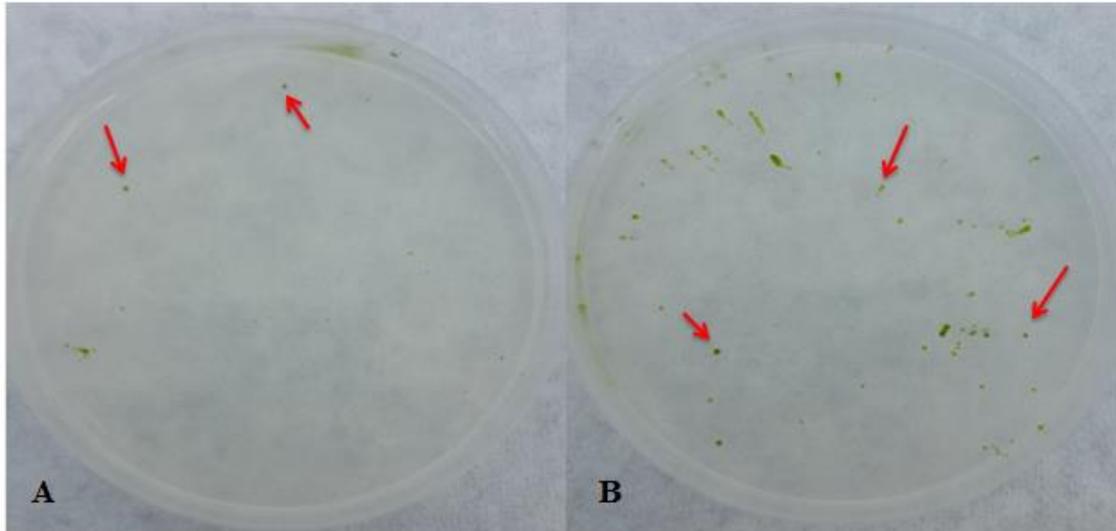


Figure 3.22 GFP transformant cells on hygromycin containing agar plates. **A.** 1 kV voltage and 25 μ F capacitance, **B.** 2 kV voltage and 10 μ F capacitance.

3.2.2.3.3. Verification of Positive Clones

After GFP transformation of *Scenedesmus sp.* ME02 was completed, it needed to be verified to understand whether transformation was successful or not. For this purpose, this verification was done firstly via selective marker and then at DNA level.

Hygromycin resistance was chosen as selective marker, because sensitivity of *Scenedesmus sp.* ME02 to certain amount of hygromycin was known previously (Guo et al., 2013). To this respect, hygromycin containing TAP agar plates were used in order to select positive transformants at first step of verification (Figure 3.21 & Figure 3.22).

In order to verify the presence of GFP insert into *Scenedesmus sp.* ME02 genome, first of all, DNA was isolated via CTAB DNA extraction method. In this part of experiment, three different colonies at their 5th generation were chosen randomly and these colonies were named as G12, G14 and G18. In addition, GFP::pChlamy_3 plasmid was used in PCR as positive control (Figure 3.23, lane 13 and 14). Then, isolated DNAs were amplified via conventional PCR by using GFPqPCR forward and reverse primers. DNAs that were used in this PCR, diluted in different ratios, namely; 1:2, 1:5, 1:10 and 1:20. On agarose gel electrophoresis image, all of the bands (lane 1-12) were at the same position with positive controls (lane 13 and 14). The expected size was 114 bp for this PCR and it was seen on the agarose gel.

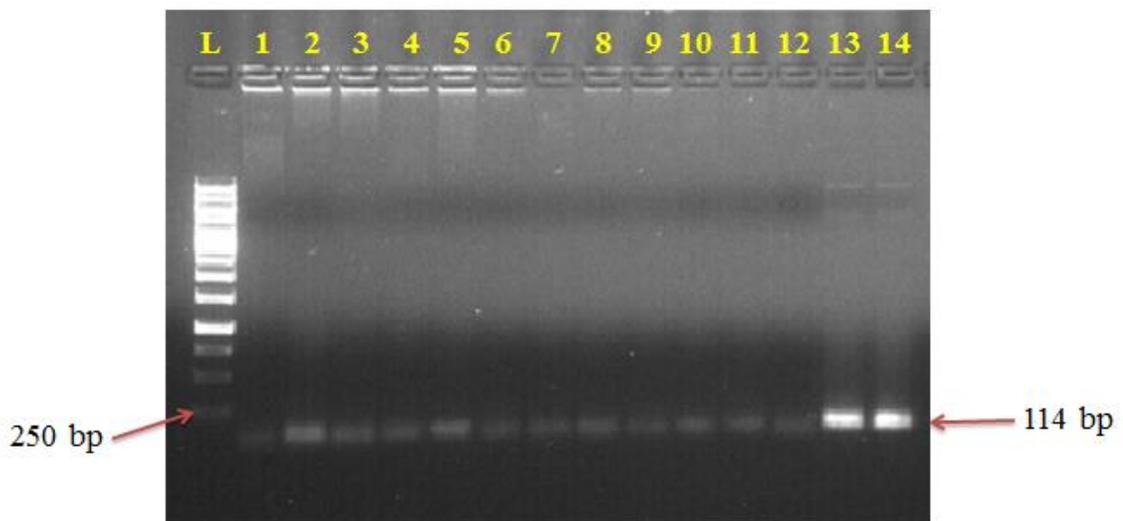


Figure 3.23 Agarose gel (1.5%) image of PCR with GFP primers. Lane L: 1 kb ladder, Lane (1-3): 1:2 diluted G12, G14, G18 respectively, Lane (4-6): 1:5 diluted G12, G14, G18 respectively, Lane (7-9): 1:10 diluted G12, G14, G18 respectively, Lane (10-12): 1:20 diluted G12, G14, G18 respectively, Lane 13: 1:10 diluted GFP::pChlamy3, Lane 14: 1:20 diluted GFP::pChlamy3

The most clear and sharp bands were chosen for each colony. For G12, 1:5 diluted DNA (lane 4) was clearer than other G12 PCR products (lane 1, 7, 10 and 13). For G14, 1:2 dilution (lane 2) was most suitable than others (lane 5, 8, 11 and 14). G18 has brighter band when 1:2 diluted DNA (lane 3) was used. Finally, in regards to the positive control, clearer band was seen at 1:10 dilution (lane 13) rather than 1:20 dilution (lane 14). As a result of these, PCR products that gave the best results in agarose gel electrophoresis, were chosen and run again on gel (Figure 3.24).

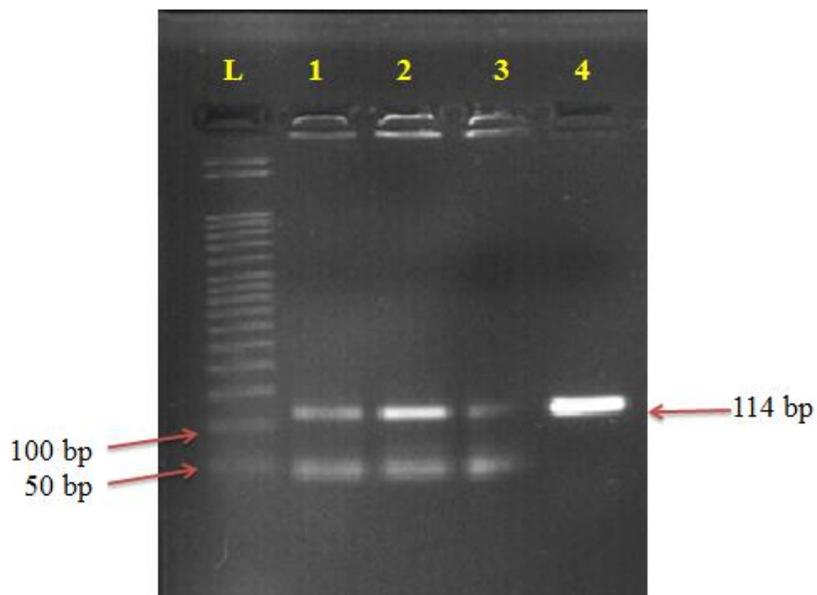


Figure 3.24 Agarose gel (1.5%) image of PCR with GFP primers. Lane L: 50 bp ladder, Lane 1: G12 (1:5 diluted DNA), G14 (1:2 diluted DNA), G18 (1:2 diluted DNA), GFP::pChlamy_3 (1:10 diluted)

In this agarose gel image, finest PCR products were loaded on agarose gel and electrophoresis was done one more time (Figure 3.24). Once again, the bands were at the same position with positive control (114bp). The sharpest and clearest band was seen on G14 (lane 2).

Eventually, according to DNA isolation and PCR results, presence of GFP in *Scenedesmus sp.* ME02 genome was proven at DNA level.

3.2.2.3.4. Expression of GFP mRNA in Transformed Cells

Total RNA isolation was done from G14, G18 and pHyg3 colonies. pHyg3 was used as negative control in RT-PCR studies.

RNA is very fragile against external factors and also RNase enzyme. Due to these reasons, quality and integrity of RNA was checked before next experiments. Thus, validation of RNA samples was done via agarose gel electrophoresis (Figure 3.25). On the gel photo, 28S rRNA and 18S rRNA bands could be seen clearly and smear between the bands was not too much. It showed that RNA integrity was provided and RNA samples were not degraded throughout the RNA isolation process.

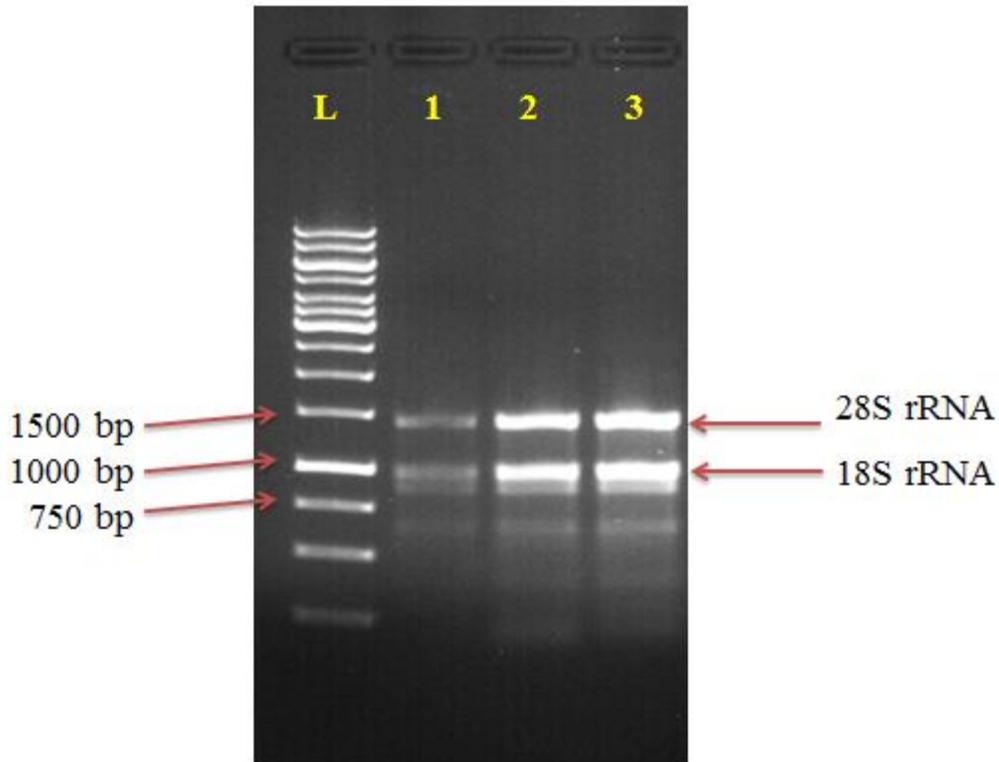


Figure 3.25 Agarose gel (1.5%) image of total RNA samples from GFP colonies. Lane L: 1 kb ladder, Lane 1: G14 RNA, Lane 2: G18 RNA, Lane 3: pHyg3 RNA

Then, DNase treatment was conducted in order to get rid of contaminant DNA pieces and cDNAs were synthesized from isolated and purified RNA samples.

3.2.2.3.4.1. Negative Reverse Transcriptase PCR (-RT-PCR)

-RT control PCR was performed to check DNA contamination in RNA samples. cDNA were made from G14, G18 and pHyg3 RNA. In this time, reverse transcriptase (RT) enzyme was not used for negative cDNA synthesis because main purpose was detecting the contaminant DNA, if any. After that, PCR was conducted with GFPqPCR and ITS2 primers and G14, G18 and pHyg3 cDNAs (Figure 3.26). On lane 1, 2 and 3; -RT-PCR products that were amplified with GFPqPCR primers were loaded. On the other hand,

7th, 8th and 9th lanes showed again –RT-PCR results but in this time negative cDNAs were amplified with ITS2 primers. As expected, there were no visible bands on gel image. It implied that there was no DNA contamination in RNA samples. In other respects, G14, G18 and pHyg3 cDNAs were also amplified with ITS2 primers via conventional PCR. (Lane 4, 5 and 6) ITS2 primers were used as internal control and they amplified a region of the 18S rRNA gene (Onay et al., 2014). The region was about 246 bp in length and Gen Bank number is KJ564284. Positive bands on lane 4, 5 and 6 showed that isolated RNA samples and synthesized cDNAs have good quality.

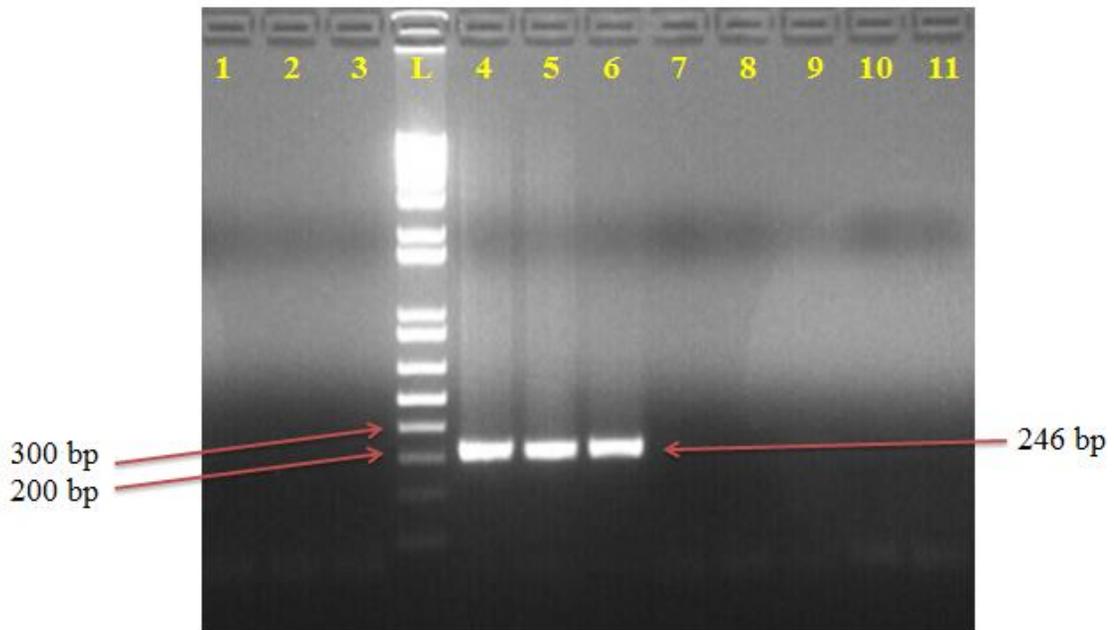


Figure 3.26 Agarose gel (1.5%) image of –RT-PCR. Lane L: 1 kb plus ladder, Lane 1, 2, 3: G14, G18 and pHyg3 –RT-PCR products respectively (with GFPqPCR primers), Lane 4, 5, 6: G14, G18 and pHyg3 RT-PCR products respectively (with ITS2 primers), Lane 7, 8, 9: G14, G18 and pHyg3 –RT-PCR products respectively (with ITS2 primers), Lane 10, 11: negative controls of PCR reactions with GFPqPCR and ITS2 primers respectively.

3.2.2.3.4.2. RT-PCR

RT-PCR was performed after cDNA synthesis from G14, G18 and pHyg3 RNAs. GFPqPCR primers were used and aim was verify the presence of GFP on RNA level. According to agarose gel electrophoresis results, only G18 gave the positive band on gel (Figure 3.27 A). Expected band size was 114 bp and it could be seen on the gel image. Unfortunately, G14 cDNA was not amplified with GFPqPCR primers. However, presence of GFP insert in *Scenedesmus sp.* ME02 genome was proven at least one colony which was G18 in here. pHyg3 cDNA was used as negative control in this experiment due to lack of GFP insert and so any visible bands could not be observed.

As a positive control, ITS2 primers were again used in this RT-PCR (Figure 3.27 B). RT-PCR was conducted with G14, G18 and pHyg3 cDNAs and ITS2 primers were used. 246 bp bands were anticipated on agarose gel and they were observed.

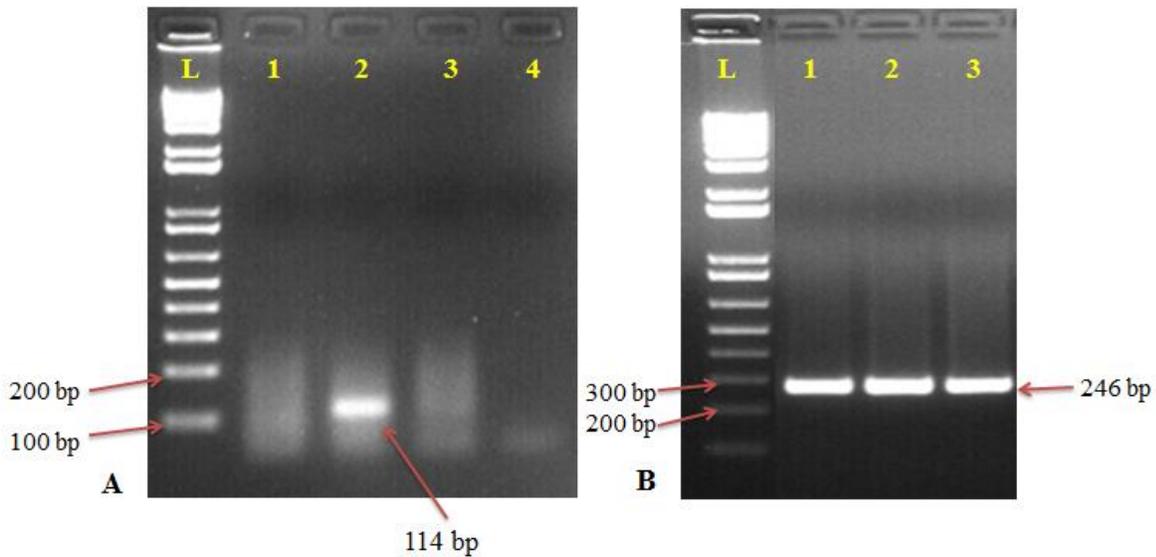


Figure 3.27 Agarose gel (1.5%) images of RT-PCR. **A.** RT-PCR with GFPqPCR primers. Lane L: 1kb plus ladder, Lane 1: G14, Lane 2: G18, Lane 3: pHyg3, Lane 4: negative control of reaction. **B.** RT-PCR with ITS2 primers. Lane L: 1kb plus ladder, Lane 1, 2, 3: G14, G18 and pHyg3 respectively.

As a result of this cDNA synthesis and RT-PCR experiments, the presence of GFP in *Scenedesmus sp.* ME02 genome was verified at RNA level.

3.2.2.3.5. Expression of GFP via Confocal Microscope

Lastly, GFP was displayed under laser scanning confocal microscope. This time, GFP was shown at the protein level. It means that, if GFP was translated into protein, it could be seen fluorescently under confocal microscope.

At Figure 3.28 A, green signals could be obtained from GFP bearing colonies. These colonies were shown with red arrows. Next to the confocal image, conventional light microscope image was observed (Figure 3.28 B). Red arrows on the Figure 3.28 A and B shows the corresponding cells. Since GFP::pChlamy_3 plasmid construct was plant optimized, GFP signal was not so much intense. However, GFP signal was obtained even if it was faint.

There were several reasons of faint signal of GFP in *Scenedesmus sp.* ME02. pChlamy_3 vector was privately constructed by Invitrogen company for genetic engineering of *Chlamydomonas reinhardtii* so it was not very suitable for *Scenedesmus sp.* transformation. Additionally, GFP insert was previously designed in compliance with plants and since *Scenedesmus sp.* ME02 has high GC content, plant optimized GFP did not suit so much for microalgal fluorescent protein expression. In order to overcome these problems, suitable vector for *Scenedesmus sp.* ME02 can be used and GFP codon optimization can be done in accordance with microalgae genome.

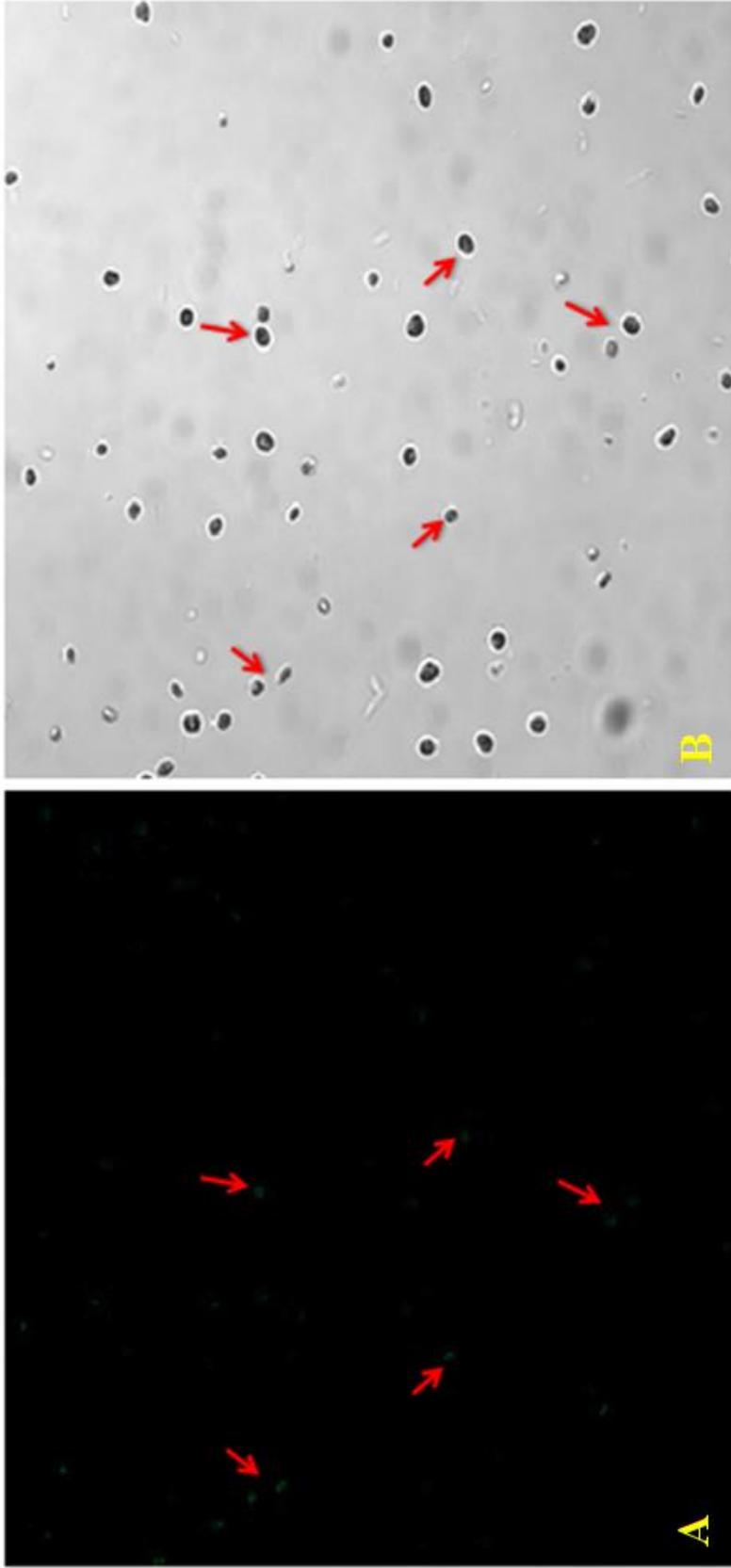


Figure 3.28 Laser scanning confocal microscope image of GFP colony (G18) **A.** confocal image **B.** light microscope image.
(Excitation: 488 nm. Emission: 505 nm. 40X magnification)

CHAPTER 4

CONCLUSION

Green microalgae are important sources for production of valuable proteins, carbohydrates and fatty acids. When environmental problems are taken into consideration, biodiesel seems as conspicuous solution instead of fossil fuels. Especially fatty acid production of microalgae was focused in this study. By using biochemical and genetic engineering methods, evaluation of novel thermo-resistant microalgae that is *Scenedesmus sp.* ME02 was performed. Most suitable growth medium and its composition were determined as TAP:BG-11 (1:1) according to most healthy growth pattern of microalgae cells. Besides, optimum temperature range was identified between 25°C to 30°C based on growth curves. Biomass and lipid productivities and also fatty acid profiles were examined at different growth temperatures (16°C, 25°C and 30°C). In addition, weather conditions of Central Anatolia in summer were mimicked at laboratory scale batch culture with 8 hours in dark at 16°C and 16 hours light at 30°C (16°C(night)/30°C(day)). Specific growth rate, biomass concentration and biomass productivity values were highest at 30°C. On the other hand, lipid content was slightly higher at 16°C(night)/30°C(day) than that of 24 hours (16 h light/8 h dark) 16°C and 24 hours (16 h light/8 h dark) 30°C respectively. However, lipid productivity was low at 16°C(night)/30°C(day) since biomass concentration values were lower at these temperature regimes. Fatty acid methyl ester (FAME) profiles of *Scenedesmus sp.* ME02 at different temperatures were also investigated. Significant changes in FAME profiles were observed particularly in palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) methyl esters, which are also

preferable for biodiesel production. Additionally, in order to investigate the genetic basis of the dramatic changes in temperature dependent fatty acid composition, fatty acid desaturase-2 (*fad2*) gene was identified and partially cloned from *Scenedesmus sp.* ME02 for the first time in this study. Fatty acid desaturase-2 (*fad2*) primers were designed according to *Chlamydomonas reinhardtii fad2* nucleotide sequence. By using these primers, *fad2* gene was partially cloned from *Scenedesmus sp.* ME02. And then, *Chlamydomonas* and *Scenedesmus fad2* gene sequences were aligned via BLAST and 82% similarity was obtained.

Besides these, transformation was applied to *Scenedesmus sp.* ME02 via electroporation. Since *Scenedesmus sp.* ME02 is novel strain and any genetic information has not been identified so far, initially transformation parameters were optimized by transforming pHyg3 plasmid into *Scenedesmus sp.* ME02 cells. After optimization, green fluorescent protein (GFP) gene was transformed to *Scenedesmus* cells with pChlamy_3 vector construct. Positive clones were proven by using hygromycin selective marker and GFP reporter gene. GFP bearing colonies was observed under the laser scanning confocal microscope and green fluorescent signal was obtained. Also, DNA was isolated and presence of GFP was verified at fifth generation via conventional PCR. After isolating total RNA of transformed cells and cDNA synthesis, GFP mRNA expression of transformed cells were proven via RT-PCR even at the twelfth generation. Therefore, stable transformation was achieved and proved with *Scenedesmus sp.* ME02.

In future, *fad2* gene can be fully cloned from *Scenedesmus sp.* ME02. After full cloning, relationship between expression rate of *fad2* and temperature dependent FAME profile alterations can be prospected. If any significant changes are observed in *fad2* expression depending on temperature, overexpression of *fad2* gene can be tested at the temperature that gives the richest FAME content. Another future prospect may be the transformation of *fad2* gene to different microalgal strains or other lipid productivity inducing genes such as DGTT2 to *Scenedesmus sp.* ME02 in order to improve the lipid content of microalgae.

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APPENDIX A

BUFFERS AND SOLUTIONS

Tris-Acetate-Phosphate (TAP) Medium

Table A.1 Ingredients of TAP medium (Adapted from Culture Collection of Cryophilic Algae)

Stock Solution (SS)	Volume	Component	Concentration in SS	Concentration in Final Medium
Tris Base	2.42 g	H ₂ NC(CH ₂ OH) ₃	-	2.00x10 ⁻² M
B-solution	50 mL	NH ₄ Cl	15.0 g/L	7.00x10 ⁻³ M
		CaCl ₂ .2H ₂ O	4.00 g/L	8.30x10 ⁻⁴ M
		MgSO ₄ .2H ₂ O	2.00 g/L	4.50x10 ⁻⁴ M
Phosphate Buffer	1 mL	K ₂ HPO ₄	288 g/L	1.65x10 ⁻³ M
		KH ₂ PO ₄	144 g/L	1.05x10 ⁻³ M
Trace Elements Solution	1 mL	Na ₂ EDTA.2H ₂ O	50.0 g/L	1.34x10 ⁻⁴ M
		ZnSO ₄ .7H ₂ O	22.0 g/L	1.36x10 ⁻⁴ M
		H ₃ BO ₃	11.4 g/L	1.84x10 ⁻⁴ M
		MnCl ₂ .4H ₂ O	5.00 g/L	4.00x10 ⁻⁵ M
		FeSO ₄ .7H ₂ O	5.00 g/L	3.29x10 ⁻⁵ M
		CoCl ₂ .6H ₂ O	1.60 g/L	1.23x10 ⁻⁵ M
		CuSO ₄ .5H ₂ O	1.60 g/L	1.00x10 ⁻⁵ M
		(NH ₄) ₆ MoO ₃	1.10 g/L	4.44x10 ⁻⁶ M
Acetic Acid	1 mL	CH ₃ COOH	-	-

Indicated quantities of medium components in Table A.1 are dissolved in approximately 850 mL distilled water and then completed to 1 L final volume. Final pH is adjusted to 6.5-7.0 and sterilized by autoclave at 121°C for 20 minutes.

For solid medium 1.5% Agar is added onto TAP medium before autoclaving.

BG-11 Medium

Table A.2 Ingredients of BG-11 medium (Adapted from Culture Collection of Cryophilic Algae)

Stock Solution (SS)	Volume	Component	Concentration in SS	Concentration in Final Medium
SS 1	60 mL	NaNO ₃	25.0 g/L	1.76x10 ⁻² M
SS 2	10 mL	MgSO ₄ .7H ₂ O	7.50 g/L	3.04x10 ⁻⁴ M
SS 3	20 mL	Na ₂ CO ₃	1.00 g/L	1.89x10 ⁻⁴ M
SS 4	5.3 mL	K ₂ HPO ₄	7.50 g/L	2.28x10 ⁻⁴ M
SS 5	14 mL	CaCl ₂ .2H ₂ O	2.50 g/L	2.38x10 ⁻⁴ M
SS 6	6 mL	Citric Acid	1.00 g/L	3.12x10 ⁻⁵ M
SS 7	10 mL	Ferric Ammonium Citrate	0.60 g/L	2.26x10 ⁻⁵ M
SS 8	10 mL	Na ₂ EDTA.2H ₂ O	0.10 g/L	2.69x10 ⁻⁶ M
Trace Elements Solution	1 mL	H ₃ BO ₃	61.0 mg/L	9.87x10 ⁻⁷ M
		MnSO ₄ .H ₂ O	169.0 mg/L	1.00x10 ⁻⁶ M
		ZnSO ₄ .7H ₂ O	287.0 mg/L	9.98x10 ⁻⁷ M
		CuSO ₄ .5H ₂ O	2.5 mg/L	1.00x10 ⁻⁸ M
		(NH ₄) ₆ Mo ₇ O ₂₄ .4H ₂ O	12.5 mg/L	1.01x10 ⁻⁸ M

Indicated quantities of medium components (except SS 6 and SS 7) in Table A.2 are dissolved in approximately 850 mL distilled water and then completed to 1 L final

volume. Final pH is adjusted to 8.0 and sterilized by autoclave at 121°C for 20 minutes. SS 6 and SS 7 stock solutions are filter-sterilized and added aseptically after autoclave.

Bold's Basal Medium (BBM)

Table A.3 Ingredients of BBM (Adapted from Culture Collection of Cryophilic Algae)

Stock Solution (SS)	Volume	Component	Concentration in SS	Concentration in Final Medium
SS 1	10 mL	NaNO ₃	25.0 g/L	2.98x10 ⁻³ M
SS 2	10 mL	MgSO ₄ .7H ₂ O	7.5 g/L	3.04x10 ⁻⁴ M
SS 3	10 mL	NaCl	2.5 g/L	4.28x10 ⁻⁴ M
SS 4	10 mL	K ₂ HPO ₄	7.5 g/L	4.31x10 ⁻⁴ M
SS 5	10 mL	KH ₂ PO ₄	17.5 g/L	1.29x10 ⁻³ M
SS 6	10 mL	CaCl ₂ .2H ₂ O	2.5 g/L	1.70x10 ⁻⁴ M
SS 7	1 mL	H ₃ BO ₃	11.4 g/L	1.85x10 ⁻⁴ M
EDTA-KOH Solution	1 mL	EDTA.Na ₂	50.0 g/L	1.71x10 ⁻⁴ M
		KOH	31.0 g/L	5.53x10 ⁻⁴ M
Ferric Solution	1 mL	FeSO ₄ .7H ₂ O	4.9 g/L	1.79x10 ⁻⁵ M
		H ₂ SO ₄	1 mL	-
Trace Elements Solution	1 mL	ZnSO ₄ .7H ₂ O	8.8 g/L	3.07x10 ⁻⁵ M
		MnCl ₂ .4H ₂ O	1.4 g/L	7.28x10 ⁻⁶ M
		MoO ₃	0.7 g/L	4.93x10 ⁻⁶ M
		CuSO ₄ .5H ₂ O	1.5 g/L	6.29x10 ⁻⁶ M
		Co(NO ₃) ₂ .6H ₂ O	0.5 g/L	1.68x10 ⁻⁶ M
Vitamin Mix	1 mL	Vitamin B ₁ (Thiamine HCl)	1.0 g/L	2.97x10 ⁻⁸ M
		Vitamin H (Biotin)	0.25 mg/L	1.02x10 ⁻¹⁰ M
		Vitamin B ₁₂	0.15 mg/L	1.11x10 ⁻¹¹ M

Indicated quantities of medium components in Table A.3 (except Vitamin Mix) are dissolved in approximately 850 mL distilled water and then completed to 1 L final volume. Final pH is adjusted to 5.5-6.5 and sterilized by autoclave at 121°C for 20 minutes. Vitamin mix is filter-sterilized and added aseptically after autoclave.

3N-BB (3N Bold's Basal) Medium

In order to prepare 3N-BB medium, BBM components shown in Table A.3 is mixed as explained above. Nevertheless, NaNO₃ is used in triple quantity (i.e. 30 mL for 1 L medium) in 3N-BB medium.

Luria-Bertani (LB) Broth

Table A.4 Composition of LB broth

Component	Quantity
Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g

Components that are shown in Table A.4 are mixed in distilled water. Total volume is completed to 1 L and pH is adjusted 7.0. Then, medium is sterilized by autoclave at 121°C for 20 minutes.

For solid medium 1.5% Agar is added onto LB broth before autoclaving.

50X Tris-Acetate-EDTA (TAE) Buffer

Table A.5 Composition of TAE buffer

Component	Quantity
Tris Base	242 g
Glacial Acetic Acid	57.1 mL
0.5 M EDTA (pH:8.0)	100 mL

Components that are shown in Table A.5 are mixed in about 600 mL distilled water. Then, total volume is completed to 1 L. In order to prepare 1X working solution, 50X stock is diluted in 1:4 ratio.

Osmosis Solution

Table A.6 Composition of osmosis solution

Component	Quantity	Concentration in Final Medium
Mannitol	0.36 g	0.2 M
Sorbitol	0.36 g	0.2 M
Glycerol	1 mL	10%

In order to prepare 0.2 M osmosis solution, components that are shown in Table A.6 are mixed in distilled water. Then, total volume is completed to 10 mL. Solution is stored at +4°C for cold utilization.

2X Cetyltrimethyl Ammonium Bromide (CTAB) Buffer

Table A.7 Composition of CTAB buffer

Component	Quantity	Concentration in Final Medium
CTAB	2 g	5.4×10^{-4} M
Tris.HCl	10 mL	1 M
EDTA	4 mL	0.5 M
NaCl	28 mL	5 M

In order to prepare 2X CTAB buffer, components that are shown in Table A.7 are mixed in distilled water. Then, total volume is completed to 100 ml.

APPENDIX B

SEQUENCES OF PCR PRIMERS

Table A.8 Sequences of PCR primers

Name of the Primer	Sequence (5'-3')
FAD2 Forward	GGCCAAGGACGAGGTGTTTGT
FAD2 Reverse	CGCGTGATAGTGCGGCATGTA
M13 Forward	TGTA AACGACGGCCAG
M13 Reverse	CAGGAAACAGCTATGAC
Aph7 Forward	TCGATATCAAGCTTCTTTCTTGC
Aph7 Reverse	AAGCTTCCATGGGATGACG
GFPqPCR Forward	TCAAGGAGGACGGAAACATC
GFPqPCR Reverse	GGGTCTTGAAGTTGGCTTTG
ITS2 Forward	GAGCATGTCTGCCTCAGC
ITS2 Reverse	GGTAGCCTTGCTGAGC

APPENDIX C

BIOMASS AND LIPID CALCULATIONS

Biomass Formulas

$$\text{Biomass concentration (g. L}^{-1}\text{)} = \frac{\text{dry weight (g)}}{\text{volume of culture (L)}}$$

$$\text{Biomass productivity (g.L}^{-1}\text{.d}^{-1}\text{)} = \frac{\text{dry weight (g)}}{\text{volume of culture (L) x time (day)}}$$

$$\text{Specific growth rate } (\mu) = \frac{\ln (X_2 - X_1)}{t_2 - t_1}$$

X_1 : biomass concentration at the beginning of time interval (mg/L)

X_2 : biomass concentration at the end of time interval (mg/L)

$t_2 - t_1$: time elapsed between selected interval (days)

Lipid Formulas

$$\text{Lipid content (\%)} = \frac{\text{weight of extracted oil (g)}}{\text{dry weight (g)}} \times 100$$

$$\text{Lipid productivity (g.L}^{-1}\text{.d}^{-1}\text{)} = \frac{\text{weight of extracted oil (g)}}{\text{volume of culture (L) x time (day)}}$$