



MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES
IN PURE AND APPLIED SCIENCES



**ASSESSMENT OF THE EFFECTS OF
GROWTH CONDITIONS ON STARCH AND
LIPID ACCUMULATION IN MICROALGAE**

ECEM KAPLAN OBAN

MASTER THESIS

Department of Bioengineering

Thesis Supervisor

Prof. Dr. Ahmet Alp SAYAR

ISTANBUL, 2018



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ÖZET

Tez Başlığı: Büyüme Koşullarının Mikroalglerde Nişasta ve Lipit Birikimine Etkisinin İncelenmesi

Mikroalglerin ürettiği lipitlerden elde edilen biyodizel umut vadeci bir enerji kaynağı olarak görülmektedir. Biyodizelin üretim maliyeti ve kalitesi ticarileşmesi açısından iki önemli parametredir. Sunulan çalışmada, potansiyel biyodizel kaynağı olarak bir tatlı su mikroalgi olan *Chlorella vulgaris* SAG 211-12, BG-11 besiyerinde büyütülmüştür. Farklı konsantrasyonlardaki NaCl, glukoz ve gliserol içeriğinin *Chlorella vulgaris*' in büyüme profili ile nişasta ve lipit üretimi üzerine etkisinin araştırılması hedeflenmiştir. NaCl, glukoz ve gliserol konsantrasyonları için deneysel aralık, merkezi karma dizayn (central composite design) yöntemi kullanılarak belirlenmiştir. En yüksek büyüme hızı olan 0.635 d^{-1} , 0.9% NaCl, 0.8% glukoz ve gliserol içeren BG-11 besiyerinde elde edilmiştir.

Palmitik asit (C16:0), stearik asit (C18:0), oleik asit (C18:1), linoleik asit (C18:2), linolenik asit (C18:3), miristik asit (C14:0) ve pentadekanoik asit (C15:0) *Chlorella vulgaris* SAG 211-12 hücreleri tarafından üretilen temel yağ asitleridir. Bu yağ asitlerinin, toplam kültür hacmi olan 150 mL' deki maksimum 2.571 mg değerine 0.9% NaCl, 0.8% glukoz ve gliserol varlığında ulaştığı görülmüştür. 0.9% NaCl, 0.3% glukoz ve gliserol içeren BG-11 besiyerinde en yüksek palmitik asit (C16:0) oranı (40.67%) elde edilmiştir. En yüksek stearik asit (C18:0) oranı ise (22.16%), 2.5% NaCl, 0.6% glukoz ve gliserol içeren BG-11 besiyerinde elde edilmiştir.

En yüksek toplam yağ asidi metil ester (mg) miktarı ve C16:0 oranına ulaşmak amacıyla besiyerine eklenmesi gereken NaCl, glukoz ve gliserol bileşenlerinin optimizasyonu gerçekleştirilmiş ve tahmin edilen maksimum toplam yağ asidi metil ester miktarına (2.6169 mg) 0.9% NaCl, 0.8% glukoz ve 0.9424% gliserol varlığında ulaşılabileceği belirlenmiştir. Optimizasyon sonuçlarına göre, en yüksek C16:0 oranını sağlayan optimum besiyerinin, 0.5% NaCl, 1.0% glukoz ve 1.0% gliserol içeren BG-11 besiyeri olduğu ve bu besiyerinde kütleye maksimum C16:0 yüzdesinin 39.76% olacağı bulunmuştur.

ABSTRACT

Thesis Title: Assessment of the Effects of Growth Conditions on Starch and Lipid Accumulation in Microalgae

Biodiesel obtained from microalgal lipids is considered as a promising energy source. Cost of production and quality of biodiesel are two important parameters for commercialization. In this study a freshwater microalgae *Chlorella vulgaris* SAG 211-12 as a potential biodiesel source was cultivated in BG-11 medium. Growth profile, carbohydrate and lipid accumulation of cells were investigated in the presence of various concentrations of NaCl, glucose and glycerol. Experimental range of NaCl, glucose and glycerol concentrations were determined using central composite design. Results showed that cells reached the highest growth rate of 0.635 d^{-1} in the BG-11 medium containing 0.9% NaCl, 0.8% glucose and glycerol.

Palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), myristic acid (C14:0) and pentadecanoic acid (C15:0) were the common fatty acids produced by *Chlorella vulgaris* SAG 211-12. Total amount of those fatty acids in 150 mL culture volume was maximum of 2.571 mg in the presence of 0.9% NaCl, 0.8% glucose and glycerol. In BG-11 medium including 0.9% NaCl, 0.3% glucose and glycerol, palmitic acid (C16:0) mass fraction was maximum of 40.67% of total fatty acids. The highest stearic acid (C18:0) mass fraction (22.16%) was obtained in the presence of 2.5% NaCl, 0.6% glucose and glycerol.

In order to reveal best medium for maximum total FAME (mg) accumulation and C16:0 mass fraction, optimization of NaCl, glucose and glycerol concentrations in growth medium was performed. Results demonstrated that maximum predicted value for total FAME amount was 2.6169 mg in the presence of 0.9 % NaCl, 0.8% glucose and 0.9424% glycerol. BG-11 medium containing 0.5% NaCl, 1.0% glucose and 1.0% glycerol was predicted to be the optimum medium for highest C16:0 mass fraction of 39.76%.

SYMBOLS

CO₂	: Carbon dioxide
CH₄	: Methane
C14:0	: Myristic acid
C15:0	: Pentadecanoic acid
C16:0	: Palmitic acid
C18:0	: Stearic acid
C18:1	: Oleic acid
C18:2	: Linoleic acid
C18:3	: Linolenic acid
d	: Day
g	: Grams
H₂O₂	: Hydrogen peroxide
KNO₃	: Potassium nitrate
L	: Liter
m	: Meter
mm	: Millimeter
mM	: Millimolar
mg	: Milligram
NaCl	: Sodium chloride
NaNO₃	: Sodium nitrate
nm	: Nanometer
N₂O	: Nitrous oxide
SF₆	: Sulphur hexafluoride
v	: Volume
w	: Weight
μ	: Specific growth rate
μm	: Micrometer

ABBREVIATIONS

BG-11	: Blue-Green 11
CCD	: Central composite design
DoE	: Design of experiments
DWC	: Dry cell weight
FAME	: Fatty acid methyl ester
GC-MS	: Gas Chromatography-Mass Spectrometry
GHG	: Greenhouse gas
GL	: Glycolipids
IEA	: International Energy Agency
OD	: Optical density
PL	: Phospholipids
PUFA	: Polyunsaturated fatty acid
RSM	: Response surface methodology
SD	: Standard deviation
TAG	: Triacylglycerol
UNFCCC	: The United Nations Framework Convention on Climate Change

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1. INTRODUCTION

Energy demand in World is increasing progressively due to the exponential growth of World population and industrialization. In order to meet escalating energy demand, fossil fuel consumption is increasing and this causes both environmental damage and depletion of crude oil reserves. Due to consumption of fossil fuel reserves, energy price is raised. In addition, greenhouse gas (GHG) emitted by fossil fuels to the atmosphere led to climate change (Makareviciene et al., 2013). Moreover, pH of oceans is altered when emitted carbon dioxide (CO₂) is solved in oceans. Eventually marine ecosystem is badly affected by pH drift (Mata et al., 2010).

Fossil fuels are utilized as the major energy sources, which meet almost 80% of energy demand in the world. It was reported that GHG that was emitted by transportation sector was increased by 20% between 1990 and 2001. Moreover, it is stated that limited oil reserves will be depleted in a short time, less than 10 decades (Atabani et al., 2012).

GHG emission is envisaged to be increased by more than 25% between 1995 and 2012 according the climate change and energy report prepared by International Energy Agency (IEA). Energy sector has the largest share with two-thirds of anthropogenically emitted GHG amount (International Energy Agency, 2015).

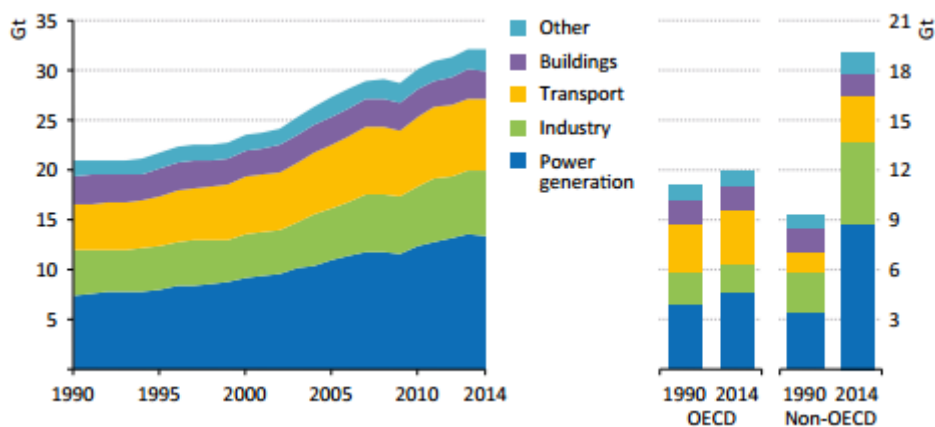


Figure 1.1. CO₂ emissions of different energy-related sectors and CO₂ amount emitted by OECD (The Organisation for Economic Co-operation and Development) and Non-OECD countries (International Energy Agency, 2015)

The term GHG includes CO₂, nitrous oxide (N₂O), methane (CH₄), sulphur hexafluoride (SF₆), hydrofluorocarbons (HFCs) and perfluorocarbons (PFCs) which are accumulated in the atmosphere cumulatively. CO₂ is the major GHG emitted by transportation sector (Atabani et al., 2012).

In 2030, all around the world total energy requirement is expected to be 50% more than today is energy demand, especially because of growing economies such as China and India (International Energy Agency, 2007 and Atabani et al., 2012).

In order to prevent the rise in GHG concentration in the atmosphere, appropriate policies should be implemented globally. Otherwise, adverse effect of climate change on all living organisms and physical environment will be irreversible. In 2010, within the context of The United Nations Framework Convention on Climate Change (UNFCCC) stabilization of global temperature increment below two-degree celsius was emphasized for long-term objective and parties to the convention committed taking action to reduce anthropogenically emitted GHG (International Energy Agency 2015, Cancun Agreements 2010). In addition, parties to the Kyoto Protocol agreed to lower GHG emission by 18% below of 1990s level in an eight-year period (2013-2020) (Kyoto Protocol 1997, Makareviciene, et al., 2013).

To overcome the globally important energy and environmental problem, renewable energy sources are emerged as clean alternative sources to meet world energy demand. Wind, biomass, geothermal, hydroelectric and solar energies are clean renewable energy forms that may be replenished (Mata et al., 2010, Lam and Lee, 2012).

Biomass is one of the most promising alternative energy sources, which are characterized with low CO₂ emission rate. Nowadays microalgal biomass as a third generation feedstock is widely used to produce biofuels. Microalgae biomass content can be converted to biogas, biodiesel, bioethanol, biomethane (Mata et al., 2010).

The aim of this study is cultivation of freshwater microalgae *Chlorella vulgaris* SAG 211-12 as a renewable energy feedstock in order to obtain high amount algal oil, which meets the required quality. For this reason, optimization of culture media of *C. vulgaris* SAG 211-12 is performed to manipulate the lipid production of freshwater microalgae and the fatty acid profile of algal oil obtained from SAG 211-12 cells.

1.1 Biodiesel

Chemically, biodiesel consists of fatty acid methyl esters (FAMEs) which are the product of transesterification reaction. Triacylglycerol (TAG) molecules are reacted with short chain alcohols in order to obtain biodiesel with the by-product glycerol (Chisti, 2007).

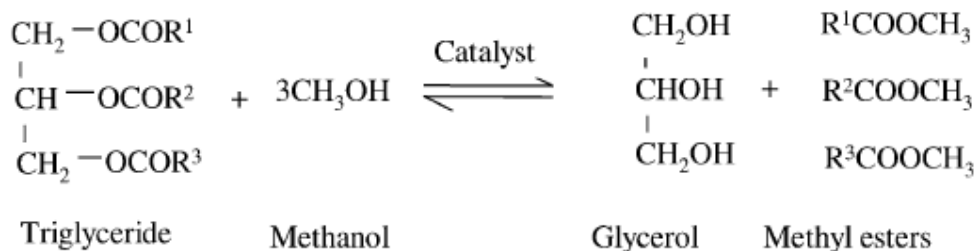


Figure 1.2. Reaction mechanism of transesterification (Meher et al., 2006).

The raw material, TAG molecules, affect both the cost and the quality of product. Chain length, degree of unsaturation, branching of fatty acids included in biodiesel have an impact on basic properties of biodiesel such as cetane number, viscosity, density, oxidative stability, lubricity and heat of combustion (Al-Iwayzy et al., 2014, Martinez et al., 2014). Due to the European Standard EN 14214 “Automotive fuels- Fatty acid methyl esters (FAMEs) for diesel engines- Requirements and test methods” linolenic acid methyl ester (C18:3) content should be maximum %12 (m/m) and polyunsaturated methyl ester (PUFA) with four and more than four double bonds should be maximum %1 (m/m) (Rutz and Janssen, 2006).

The first generation TAG sources are vegetable oils. It was the first time that vegetable oil was utilized to provide fuel when Rudolph Diesel used peanut oil as fuel. (Mandal and Mallick, 2014). In the United States soybean and in Europe rapeseed, in Indonesia and Malaysia palm oil are the most common crop plants consumed as a raw material for biodiesel production (Chisti, 2007, Makareviciene et al., 2013, Issariyakul and Dalai 2014). Sunflower, coconut, sesame, rice bran, palm oil, cottonseed oils are the other alternative oil producing feedstocks of biodiesel (Rashid et al., 2014). It was reported that depending on the fatty acid profile, vegetable oils have approximate quality with conventional diesel (Mandal and Mallick, 2014). In historic times, when vegetable oils were acknowledged as an energy source, the high viscosity of vegetable oils were caused some engine problems, then glycerol part of TAG molecule, which was found to be

responsible for high viscosity, was removed by transesterification reaction. The remaining part is called biodiesel (Issariyakul and Dalai, 2014).

On the other hand, biodiesel production from vegetable oils generates some social, economic and environmental drawbacks. Crop plants require huge amount of land for growth. In addition, these feedstocks are used to be food for human being and animals. A competition of food and energy occurs when the land is devoted to obtain energy instead producing food. Hence, the food price is increased (Chisti, 2007). Moreover, critical habitats may be used for cultivation of crop seeds in order to satisfy the biodiesel feedstock demand and that situation results in loss of biodiversity (Mata et al., 2010, Makareviciene et al., 2013).

Waste cooking oils and animal fats are defined as the second-generation biodiesel feedstocks. Considering that waste cooking oil cause environmental pollution, waste oils may be a promising alternative to vegetable oils (Doğan, 2016). However, limited stable supply of waste cooking oils like frying oil and animal fats may not fulfill the energy demand.

Microorganisms such as microalgae emerged as third generation biodiesel feedstock with high growth rate, high lipid content and its non-requirement of agricultural land for cultivation.

Table 1.1. Oil yield of several crop seeds and microalgae (Mata et al., 2010, Atabani et al., 2012, Trivedi et al., 2015).

Biodiesel Feedstock	Oil Yield (Loil/ha year)
Rubber seed	80-120
Corn	172
Cottonseed	325
Soybean	446
Sesame	696

Table 1.1 continued

Biodiesel Feedstock	Oil Yield (Loil/ha year)
Chinese tallow	907
Camelina	915
Sunflower	952
Peanut	1059
Canola	1190
Olive oil	1212
Castor	1413
Jatropha	1892
Karanj	2590
Coconut	2689
Palm oil	5950
Microalgae (30% oil by wt)	58,700
Microalgae (70% oil by wt)	136,900

1.2 Microalgae

Microalgae are unicellular or simple multicellular microorganisms, which are either prokaryotic or eukaryotic. Both prokaryotic and eukaryotic microalgae cells contain chlorophyll and thereby grow through photosynthesis. Although the mechanism of photosynthesis processes of microalgae and higher plants are similar, microalgae cells have 10-50 times higher solar conversion efficiency than that of terrestrial plants (Mandal and Mallick, 2014). They have ability to fix CO₂ released to atmosphere as a result of

industrial activities and aid reducing of GHG ratio in the atmosphere. It was reported that 1.8 ton of CO₂ is captured by each ton of microalgae (Chisti, 2007).

The term microalgae includes different sup-groups such as green algae, blue-green, red, golden and brown microalgae (Rashid et al. 2014). Although almost 30.000 different algae species are analyzed, it is expected that more than 50.000 algae species live in all earth ecosystems. They have advantage of adapting in diverse habitats on the earth including wastewater, fresh water, moist ground, wet stones, salt water and even habitats with extreme conditions (Mata et al., 2010, Makareviciene et al., 2013). University of Coimbra in Portugal has one of the largest microalgae cell collections. The other microalgae collection with 1273 different species and 2213 strains are in Germany in the Goettingen University (SAG) (Mata et al., 2010).

1.2.1 Utilization of microalgae

It was reported that about 2000 years ago the very first utilization of algal biomass was as food supply for human nutrition and animal feed (Harwood and Guschina 2009). Since microalgae contain eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), microalgae cells are considered a valuable omega-3 fatty acid source (Patil et al., 2006, Harun et al., 2010). Although these PUFAs have almost required quality, they are not economically feasible yet when compared to other commercial sources of that (Harwood and Guschina, 2009). It was stated that, generally marine microalgae accumulate high amount of ω 3-PUFAs while freshwater microalgae cells produce more saturated or monounsaturated fatty acids (Patil et al., 2006).

During reproduction, microalgae cells accumulate various compounds with high economic value. Chlorophyll a and chlorophyll b, carotenoids such as α carotene or β carotene and phycobilins are primary pigments, which absorb light energy and gives microalgae colorful appearance (Yen et al., 2013). In food and cosmetic industry, chlorophyll is utilized as natural pigment instead of synthetic additives (Harun et al., 2010).

Microalgal cells can be used for bioremediation process. Treatment of municipal and industrial wastewater is performed during reproduction of cells. Some pollutants such as heavy metals, xenobiotics, waste gases, nitrogen and phosphorus compounds in

wastewater are used by algae as nutrients. Oxygen gas generated by the process of photosynthesis is helpful for the oxidization and breakdown of pollutant molecules (Vidyashankar et al., 2016).

In the 1970s, after first oil crisis microalgae started to be studied as a feedstock for renewable energy. After that, several scientific research programmes have been supported in order to investigate raw materials for biofuel production.

1.2.2 Microalgae as a biofuel feedstock

Numerous types of energy produced by microalgal biomass have received considerable attention based upon renewable and sustainable properties of microalgae. Biogas, which is a mixture of CO₂ with the ratio of 25-45% and CH₄ range of 55-75%, is generated through anaerobic digestion of microalgal biomass. CH₄ also can be used to produce electricity and the biomass that remains after anaerobic degradation can be processed to obtain fertilizer in agriculture. Even though microalgae are suitable raw material to be converted to biogas, the product biogas is still not available commercially due to the process cost (Harun et al., 2010).

As an energy source, bio-hydrogen produced from microalgae is a promising alternative to the hydrogen formed by electrolysis of water. Bio-hydrogen is formed as a by-product of the process acidogenesis in which carbon substrates are converted into organic acid compounds (Koller et al., 2014).

As a commercially available biofuel source, bioethanol is the main product of anaerobic fermentation of carbohydrates and proteins. Some microorganisms such as fungi, bacteria and yeast have ability to carry out fermentation process under anaerobic conditions (Harun et al., 2010). Major feedstocks of bioethanol are food crops including starch. Taking into consideration that food crops are used as nutrient, lignocellulosic materials such as by-products of agriculture or forestry are encouraging raw materials for bioethanol production. On the other hand, lignocellulosic materials require a difficult pre-treatment process due to the high lignin content. Thus, they are considered not to be economically feasible (Ho et al., 2014). Microalgal biomass appears as an alternative source of fermentation process. Microalgae cells contain starch and cellulose (without

lignin) as carbohydrates, which are fermented to bioethanol. Much more efforts should be dedicated to improve bioethanol from microalgae (Harun et al. 2010).

Biodiesel is the other important biofuel. It is obtained from microalgal lipids. Two main objectives should be achieved in order to produce biodiesel from microalgae: determination of strains that have high lipid productivity and increase the productivity applying biochemical engineering and metabolic engineering methods (Mata et al., 2010).

It is stated that growth rate and lipid content are the most important properties of microalgae that are selected as a feedstock for biodiesel production. Microalgae with high growth rate require less time for cultivation thus operation cost is reduced. When species, which have high lipid content, are used as feedstock, biodiesel production yield is increased. Lipid profile of microalgae cells are also an important factor for biodiesel quality (Rashid et al., 2014). It was reported that fatty acids, which have 12-22 carbon atoms, are common in microalgae species. Cells accumulate both saturated and unsaturated fatty acids. It is possible to manipulate the fatty acid composition in microalgae by altering culture medium compositions, environmental conditions or cultivation period (Mata et al., 2010).

Microalgae cultivation may be performed by three different ways: phototrophic, heterotrophic and mixotrophic cultivation. Under phototrophic conditions, cells use light energy and CO₂ as inorganic carbon source. In the absence of light source, heterotrophic species can grow using organic carbon as energy and carbon source. Some microalgae species have ability to use both inorganic and organic carbon as carbon source and either light or organic carbon as energy source. Those organisms, which grow via both phototrophic and heterotrophic mechanisms, are called mixotrophs.

The most common cultivation system is autotrophic cultivation but lipid and biomass productivity of cultures can be improved by heterotrophic conditions. It was shown that under photoheterotrophic conditions, cells reached to higher biomass productivity than that was obtained in autotrophic and heterotrophic cultures (Isleten-Hosoglu et al., 2013).

Although microalgae are promising feedstock for biofuel production, microalgal oil has still higher cost than that of fossil fuels. It was reported that production cost per kg of algae oil in photobioreactors was \$24.60 whereas in fermenter it was \$ 1.54 (Rashid et

al., 2014). In order to compete with fossil fuels, microalgae based biodiesel should be economically feasible and in the same quality.

1.2.3 Microalgal lipids

The market price of biodiesel is directly related to the feedstock, in other words TAG ratio of the microalgal cells. The quality of biodiesel depends on the fatty acid profile. Microalgae cells accumulate two different types of lipids as non-polar storage lipids and structural lipids that are polar lipids. Structural lipids mainly consist of PUFAs (Sharma et al., 2012). Phospholipids (PL) and glycolipids (GL) are the major fractions of polar lipids, which are building blocks of cell and organelle membrane. The major forms of storage lipids are TAGs. Storage lipids are utilized to supply energy for metabolic reactions (Safi et al., 2014, D'Alessandro et al., 2016).

Microalgae tend to produce high amount of protein in exponential growth phase while in stationary phase microalgae cells accumulate high amount of lipids and carbohydrates instead of proteins (Rashid et al., 2014). Fatty acid molecules are synthesised by the chloroplast, TAG molecules are formed in endoplasmic reticulum and thylakoids of chloroplast. TAG synthesis is said to be occurred in the presence of light. The expression of enzymes responsible for lipid synthesis pathway is regulated depending on environmental conditions (Lv et al., 2010, Sharma et al., 2012, D'Alessandro et al., 2016).

Generally, there is a trade-off between growth and TAG synthesis. Under favorable conditions, microalgae cells tend to produce greater amount of biomass and biomass includes fatty acids, which are suitable for animal nutrition or polar lipids as constituents of intracellular membrane. On the other hand, under stress conditions microalgae cells switch their metabolic pathway to accumulate neutral lipids, generally in the form of TAG. Many processes including genetic engineering methods or biochemical engineering methods are applied to increase the oil content especially TAG content of microalgae cells (Fernandes et al., 2013).

1.3 Methods Applied to Increase TAG Content of Microalgae

Carbon, phosphorous and nitrogen are considered to the major elements to sustain growth of microalgae. In addition, trace amounts of cobalt, calcium, sulfur, iron, potassium,

sodium, manganese are required for cell growth. Nitrate, nitrite, ammonia, urea can be consumed by cells as nitrogen source which has a role in protein synthesis (Rashid et al., 2014). When microalgae cells are cultivated in a nitrogen poor medium, carbon in the cell is canalized to lipid biosynthesis instead of reproduction and starch formation. Concurrently, starch as an energy source in the cells will be depleted causing slow down of photosynthesis and growth (Lam and Lee, 2012).

Phosphorous and iron are the elements which have significant effects on growth and lipid accumulation of microalgae. Phosphorous is involved in energy transfer, signal transduction, respiration, photosynthesis and iron is an important element for enzymatic reaction of photosystem I and photosystem II (Singh et al., 2015).

Lv et al. (2010) utilized KNO_3 (Potassium nitrate) as nitrogen source for *C. vulgaris* cells and observed that when KNO_3 concentration in the cultivation medium is altered between 0.2 mM and 5 mM, cell growth increased continuously. At the same time lipid content is reduced from 22.5% to 15.9% and the highest lipid productivity as a result of both cell growth and lipid accumulation is found in the medium containing 1.0 mM KNO_3 (Lv et al., 2010).

Singh et al. (2015) examined the synergistic effect of nitrogen, phosphorous and iron on both growth and lipid accumulation of *Ankistrodesmus falcatus* KJ671624. It was observed that the maximum lipid productivity with higher saturation of fatty acids was obtained under nitrogen limited, phosphorous starved and sufficient iron conditions, although biomass productivity reduced under the same conditions. It was observed that in the absence of sufficient nitrogen for cell growth, iron supplement prevents sharp decrease of biomass productivity and increase the overall lipid productivity (Singh et al., 2015).

Converti et al. (2009) induced lipid productivity of *Chlorella vulgaris* and *Nannochloropsis oculata* by reducing NaNO_3 (sodium nitrate) concentration by 75%. In addition, lipid productivity of *C. vulgaris* was measured as 8.16 mg/L.d at a growth temperature of 30°C whereas lipid productivity was 20.22 mg/ L.d at a temperature of 25°C and growth rate remained constant as 0.14 d⁻¹. For *N. oculata* lipid productivity was unchanged with temperature while growth rate was affected by temperature. It was reported that lipid profile of the microalgae was also affected by temperature or nitrogen

concentration. Palmitic acid (C16:0) content of both microalgae was reduced with increasing temperature. Oleic acid (C18:1) formation was also significantly changed with temperature, C18:1 content of *C. vulgaris* and *N. oculata* were increased as a result of increasing temperature. On the other hand, NaNO₃ concentration did not have a significant effect on individual FAME concentrations of *C. vulgaris* (Converti et al., 2009).

Osmotic stress coupled with nitrogen starvation of *Neodesmus sp.* UTEX 2219-4 increased lipid content from 17% to 42% by DWC and starch also accumulated under stress conditions (Chang et al., 2016).

Iron deficiency influences growth and biosynthesis processes in algae, since iron is an important element for photosynthesis and electron transport chain. Che et al. (2015) investigated effect of iron on growth and lipid accumulation in *Monoraphidium sp.* FXY-10. It was observed that a certain concentration of iron enhanced both dry cell weight and lipid productivity. On the other hand, excessive iron inhibited both growth and lipid productivity (Che et al., 2015). It is consistent with the study presented by Ren et al. in which iron addition was stimulated cell reproduction and lipid accumulation, but further increase of iron ion concentration resulted in reduced cell concentration and lipid production (Ren et al., 2014).

Sulfur is an essential element for cell proliferation. As a result of a study in which four *Chlorellaceae* species were analyzed, in the absence of sulfur in the culture medium generally cell dry weight was increased. In addition, under sulfur deficient condition and 12h:12h light:dark cycle cells started to accumulate starch up to 50% and sulfur deficiency stimulates lipid accumulation (2.4-fold) in four *Chlorellaceae* species tested (Mizuno et al., 2013).

Isleten-Hosoglu et al. (2012) investigated growth and lipid accumulation of *Chlorella saccharophila* under heterotrophic conditions and analyzed the effect of different carbon sources as glucose, glycerol and sodium acetate. Glucose addition in the medium resulted in the highest cell dry weight, 1.39-fold and 3.74-fold of that was obtained with glycerol and sodium acetate supplementation, respectively (Isleten-Hosoglu et al., 2012).

Lipid productivity of *C. vulgaris* and *Scenedesmus sp.* cultivated in seawater was induced by Luangpipat and Chisti (2016). Whereas growth of *C. vulgaris*, *Neochloris sp.*, *C.*

minor, *Scenedesmus sp.* and *P. simplex* was reduced in seawater (Luangpipat and Chisti, 2016). Lipid production of marine microalgae *Dunaliella tertiolecta* was enhanced with the addition of NaCl in the growth medium until medium reached equal salinity to seawater (Takagi et al., 2006).

Hosoglu-Isleten et al. (2013) observed that photoheterotrophic cultivation mode induced the growth of microalgae *Ettlia texensis*. On the other hand, lipid content of cells grown under autotrophic and photoheterotrophic conditions were almost same and cell produced mainly C14:0, C16:0, C16:1, C18:0, C18:1, C18:2 and C18:3 fatty acids. Within the scope of same study, glucose was reported to be a better organic carbon source than glycerol, sodium acetate and ethanol (Isleten- Hosoglu et al., 2013).

1.4 Biorefinery Concept

Biorefinery concept is similar to petroleum refinery concept, which means fractionating petroleum in order to obtain various products. Biorefinery is the co-production of fuels, power, food, feed, chemicals from different components of biomass. In Bioenergy Task 42 prepared by IEA, biorefinery concept is defined as “Biorefinery is the sustainable processing of biomass into a spectrum of marketable products and energy” (Jong and Jungmeier, 2015, Safi et al., 2014).

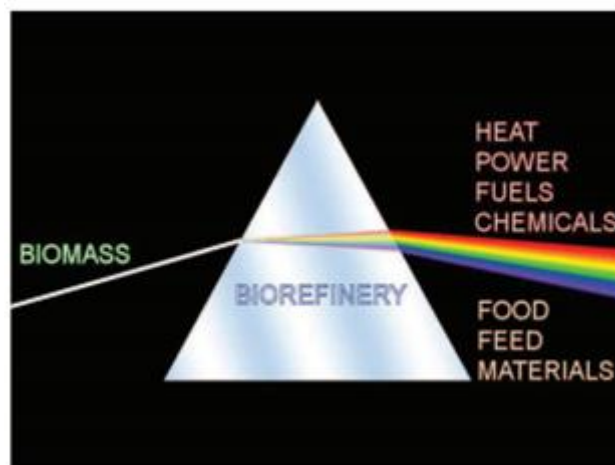


Figure 1.3. Biorefinery concept (Jong and Jungmeier, 2015)

Microalgae biomass with high carbohydrate, protein, lipid and pigments can be converted into various valuable products through algal refinery concept. TAG content of microalgal cells are used for biodiesel production and the remained biomass residue is still a source

of proteins, carbohydrates and PUFAs. Biofuel production may be integrated with wastewater treatment and CO₂ mitigation processes in order to maximize benefits and minimize the wastes of biomass.

1.5. Microalgae “*Chlorella vulgaris*”

Chlorella vulgaris is a unicellular, eukaryotic green microalga, discovered by Martinus Willem Beijerinck in 1890. It is a kind of freshwater microalgae and it is thought to be on earth for 2.5 billion years (Safi et al., 2014). It was examined that some strains of *C. vulgaris* are able to thrive in seawater (Luangpipat and Chisti 2016).

Due to its high protein content, it was used as a food source, in the beginning of 1900s. Nowadays *C. vulgaris* is consumed for human health due to protective properties for cardiovascular diseases, hypertension, Alzheimer, cancer. In addition, it is able to accumulate high amount of lipids and carbohydrates, which are converted to biofuel. *C. vulgaris* cells reproduce asexually by autospore and they have ability to grow rapidly with a short doubling time (Safi et al., 2014). Annual production of *C. vulgaris* is estimated to be 2000 t and Taiwan, Japan, China, Germany are the leader countries for its production (Trivedi et al., 2015).

Taxonomy of *C. vulgaris* (Safi et al., 2014):

Domain: Eukaryota

Kingdom: Protista

Division: Chlorophyta

Class: Trebouxiophyceae

Order: Chlorellales

Family: Chlorellaceae

Genus: *Chlorella*

Specie: *Chlorella vulgaris*

C. vulgaris cells can accumulate storage lipids in the cytoplasm and structural lipid components on the cell membrane and on the membranes of organelles. Total lipid content of cells is generally reported to be 5-40% of DCW (Marudhupandi et al., 2014, Safi et al., 2014). Lipid accumulation was increased up to almost 58% in the stationary phase (Pribyl et al., 2012).

It is stated that physicochemical properties such as pH, iodine value, density, viscosity, acid value of algal oil that is obtained from *C. vulgaris* meet the desired standards by European Standard (2003) for biodiesel (Marudhupandi et al., 2014). It is reported that calorific value (energy released with the combustion of a unit quantity of a fuel, J/kg) of biodiesel obtained from marine *C. vulgaris* is approximate to the conventional diesel (Mathimani et al., 2017).



2. MATERIALS AND METHOD

2.1. Materials

2.1.1. Algal strain and chemicals

Freshwater microalgae *Chlorella vulgaris* SAG 211-12 used in this study was kindly received from Prof. Dr. Murat Elibol (Ege University, Faculty of Engineering, Department of Bioengineering).

Chemicals used in the presented thesis were purchased from Merck (Germany), Sigma (USA). Total starch assay kit was supplied by Megazyme (Megazyme International Ireland).

2.1.2. Laboratory equipments

Laboratory equipments used in this study and their suppliers are listed in the Table 2.1.

Table 2.1. Laboratory Equipments

Equipment	Supplier
Balances	Mettler Tolerado/ Denver Instrument
Magnetic Stirrer	WiseStir MSH-20A/Heidolph
pH meter	inoLab
Autoclave	NUVE 032
Refrigerator	Arçelik
Deepfreezer (-20°C)	Arçelik
Biosafety Cabinet	ESCO
Micropipettes	Reinin
Spectrophotometer	PG Instruments Ltd
Incubator	BIOSAN
Centrifuge	Sigma
Vortex	ISOLAB
Rotary Evaporator	Heidolp
Ice Machine	Bar Line
Water Bath	Memmert/Heto

Table 2.1. Continued

Equipment	Supplier
Water Purification Systems	ELGA
GC-MS	Agilent

2.1.3. Cultivation media

In the presented work, BG-11 medium (Blue –Green 11 Medium) was used as basal medium in order to preserve and cultivate *C. vulgaris* SAG 211-12 cells. Composition of BG-11 is given in Table 2.2. Additionally, thiamine, biotin and B₁₂ vitamins were added to the BG-11 medium.

Table 2.2. BG-11 medium recipe

Component	Concentration	Trace metal mix	Concentration
NaNO ₃	1.5 g/L	H ₃ BO ₃	2,86 g/L
K ₂ HPO ₄	0.04 g/L	MnCl ₂ .4H ₂ O	1.81 g/L
MgSO ₄ .7H ₂ O	0.075 g/L	ZnSO ₄ .7H ₂ O	0.222 g/L
CaCl ₂ .2H ₂ O	0.036 g/L	Na ₂ MoO ₄ .2H ₂ O	0.39 g/L
Citric acid	0.006 g/L	CuSO ₄ .5H ₂ O	0.079 g/L
Ammonium ferric citrate	0.006 g/L		
EDTANa ₂	0.001 g/L		
Na ₂ CO ₃	0.02 g/L		
Trace metal mix	1 ml/L		

Stock solutions of all chemical components were prepared and sterilized in autoclave at 121°C for 15 minutes separately, to prevent precipitation of salts during autoclave. The initial pH values of the media were adjusted to 7.1 with HCl prior to sterilization. To prepare the agar medium of BG-11, 15 g/L of agar was added to BG-11 medium.

2.2. Experimental Methods

The main experimental steps used in the presented thesis are summarized in Figure 2.1.

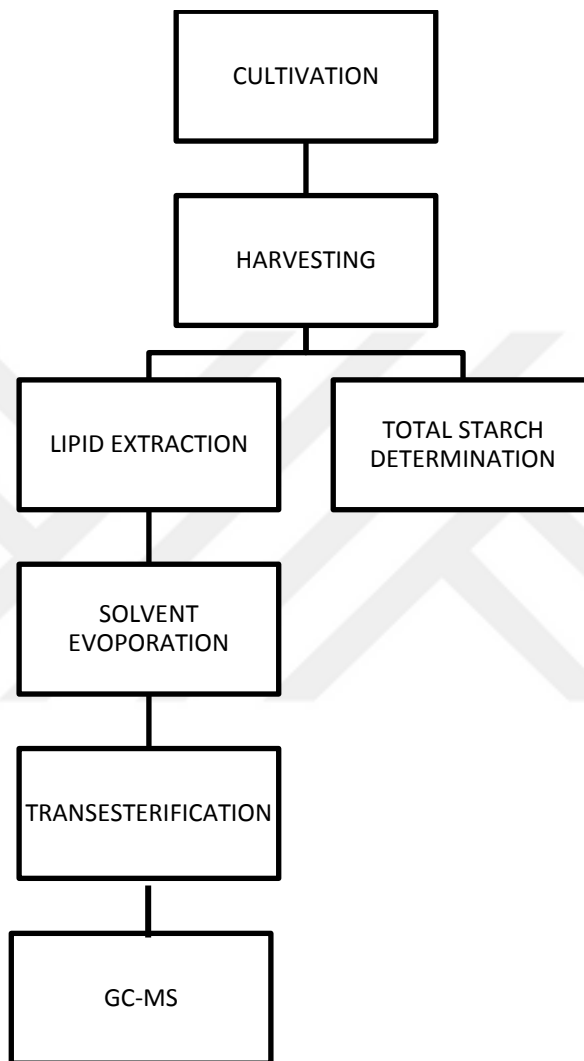


Figure 2.1. Main experimental steps

2.2.1. Optimization of glucose, glycerol and NaCl concentrations

Response surface methodology (RSM) is a technique that combines statistical and mathematical tools for empirical model development. It is used to describe the dependence of responses to several independent experimental variables. (Isleten-Hosoglu et al., 2013). First step of RSM is the experimental design for selection of independent variables (factors) which possibly affect the system and possible responses. Second step of the process is execution of experiments. Then a mathematical model is proposed in order to fit experimental results and finally optimum conditions are determined.

Design of experiments (DoE) is a process of determination of factor-level combinations and setting an experimental procedure in order to investigate the effect of selected factors on a system. In order to achieve this process, the first step is the identification of desired information. In the presented study, it was required to determine optimum concentration of NaCl, glucose and glycerol in basal medium in order to obtain maximum total FAME (mg) amount in 150 mL culture medium and maximum palmitic acid methyl ester (C16:0) ratio among all FAMES produced in microalgae cells.

Central composite design (CCD) which is a response surface design was applied to set factor-level combinations. In the present thesis, NaCl, glucose and glycerol concentrations were experimental variables (factors) and 5 levels for each of 3 factors were used in the design.

Table 2.3. Factors and levels of the experimental design

Factors	Level (-2)	Level (-)	Level (0)	Level (+)	Level (+2)
NaCl Concentration, X_1 (g/L)	0.5	0.9	1.5	2.0	2.5
Glucose Concentration, X_2 (g/L)	0.1	0.3	0.6	0.8	1.0
Glycerol Concentration, X_3 (g/L)	0.1	0.3	0.6	0.8	1.0

Table 2.4 shows the factor-level combinations ($k=3$, $n=5$). Total different fifteen combinations of factors were used and total fifteen experiments were performed. Medium 9 is central point in the design, which represents the middle levels of factors.

Table 2.4. Experimental design matrix

Medium Number	NaCl Concentration (% w/v)	Glucose Concentration (% w/v)	Glycerol Concentration (% w/v)
1	0.5	0.6	0.6
2	0.9	0.3	0.3
3	0.9	0.3	0.8
4	0.9	0.8	0.3
5	0.9	0.8	0.8
6	1.5	0.1	0.6
7	1.5	0.6	0.1
8	1.5	0.6	1.0
9	1.5	0.6	0.6
10	1.5	1.0	0.6
11	2.0	0.3	0.3
12	2.0	0.3	0.8
13	2.0	0.8	0.3
14	2.0	0.8	0.8
15	2.5	0.6	0.6

2.2.2. Cultivation and harvesting

Cultivation of *C. vulgaris* SAG 211-12 was maintained in 15 different media as shown in Table 2.3. Additionally, before application of experimental design *C. vulgaris* SAG 211-12 were grown in four different media, which were BG-11, BG-11 medium supplemented with 1% glucose, BG-11 medium supplemented with 1% glucose and 1% glycerol and BG-11 medium supplemented with 2.5% NaCl, in order to determine boundary concentrations of NaCl, glucose and glycerol.

Cultivation was performed in the 250 mL erlenmeyer flasks with the 150 mL working volume. The whole experimental studies were performed in an incubator illuminated with continuous white LED (light emitting diode) light. The cells were exposed to white LED light. To inoculate the broth media, same amount of cell was taken from agar plates and weighted. The initial cell concentration of each batch culture of *C. vulgaris* SAG 211-12 was maintained at 0.08g/L. Growth period was prolonged 15 and 27 days depending on the medium ingredients. Cultivation of cells were performed at 28±0.5 °C, with an agitation rate of 180 rpm. Experiments were triplicated.

The growth of *C. vulgaris* SAG 211-12 at different time intervals are shown in Figure 2.2-2.4. When cells reached to stationary phase, cells were harvested by centrifugation for 20 minutes at +4°C and 4000 rpm (Figure 2.5).



Figure 2.2. 1st day of cultivation



Figure 2.3. 4th day of cultivation



Figure 2.4. 14th day of cultivation

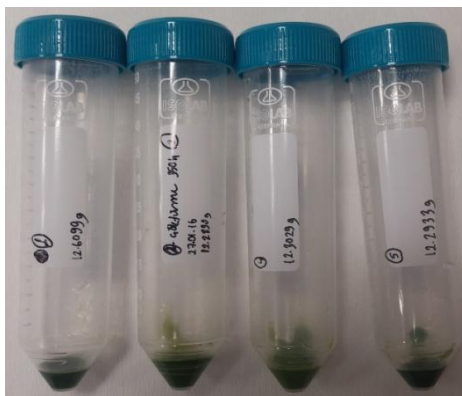


Figure 2.5. *C. vulgaris* SAG 211-12 cell pellet after centrifugation

Algal growth was measured by daily changes in optical density (OD) at 600 nm with a spectrophotometer (BIO-RAD). The maximum specific growth rate μ (d^{-1}) of *C. vulgaris* SAG 211-12 was calculated in exponential phase, according to the following equation:

$$\mu = \ln(N_2/N_1)/t_2 - t_1 \quad (2.1)$$

where, N_2 and N_1 are the final and initial cell concentrations at time t_2 and t_1 respectively.

2.2.3. Lipid extraction and solvent evaporation

After cell harvesting, lipids were extracted from wet cells with solvent extraction method using the procedure described by Yang et al. (2014). Harvested wet cells were frozen at -20°C before the extraction process and each sample was rinsed with 10 ml of 80% (v/v) hot ethanol since it is reported that ethanol has ability to penetrate through cell membrane and remove lipids (Yang et al., 2014). Then mixture was centrifuged at 4000 rpm, 20°C for 10 minutes. This step was applied for five times to all the samples and supernatants including algal lipids collected in each step (Figure 2.6).

After that, supernatant was transferred to a preweighed balloon and ethanol was evaporated from supernatants at 35°C and 210 rpm to obtain total lipids.



Figure 2.6. Ethanol phase including lipid molecules after lipid extraction

2.2.4. Determination of fatty acid composition of *C. vulgaris* SAG 211-12

In order to analyze fatty acid composition of the cells by Gas Chromatography- Mass Spectrometry (GC-MS), fatty acid molecules were converted to FAME molecules by alkali catalyzed transesterification reaction. In the first step, 1ml of NaOH-CH₃OH solution was added to each sample at 75°C and samples were incubated for 10 minutes. Then in the second step, 2ml of BF₃-CH₃OH was added and reaction had taken place for 10 minutes at 75°C. After the reaction, 3ml of hexane and 1 ml of deionized water was added to samples to form two phase including lipids and non-lipids separately. In the last step, the samples were centrifuged at 4000 rpm, 25 °C for 10 minutes in order to separate, the upper layer which was hexane phase including fatty acid methyl esters. The composition of upper layer was analysed by GC-MS.

Fatty acid composition was analyzed by GC-MS with a DB-Wax LTM module capillary column 30m x 0.25mm x 0.25µm (length x diameter x film thickness) using an Agilent 5973T LTM GC/MSD. Samples of 1 µl were injected into the capillary column with a split ratio of 50:1. Helium was employed as the carrier gas with flow rate of 1.0 mL/min. The temperatures of injector and detector both maintained at 250°C. The following temperature program was employed: 50°C for 1 minute, 25°C for 1 min, 200°C for 1 min, 3°C for a min and 230°C for 18 minutes (Zhao, Agilent Application Notes). Individual peaks were identified by comparison of their retention time with retention times of authentic standards (Supelco 37 Component FAME mix, Sigma-Aldrich).

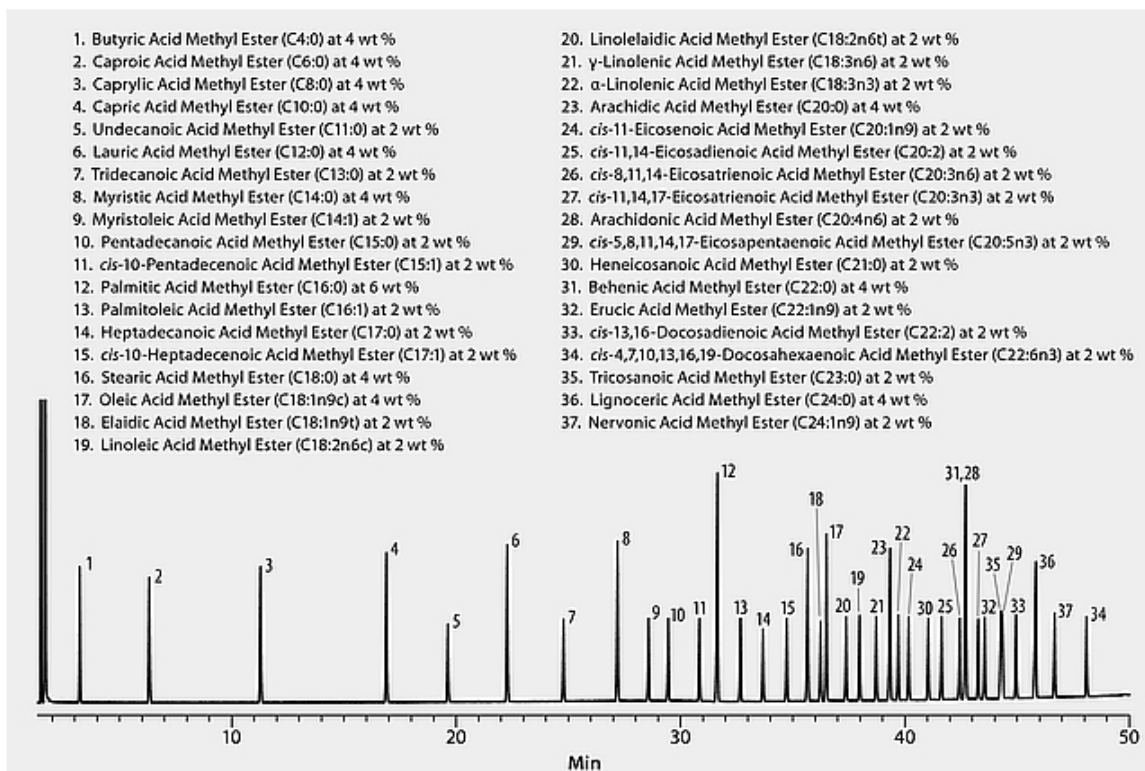


Figure 2.7. GC-MS analysis of Supelco 37 component FAME mix (Sigma-Aldrich)

2.2.5. Total starch determination

Besides fatty acid composition, starch content of *C. vulgaris* SAG 211-12 cells were also determined according to enzymatic procedure of Megazyme total starch assay kit. Thermostable α -amylase and amyloglucosidase were included in the kit in order to hydrolyze starch to maltodextrins and maltodextrins to D-glucose, respectively. Then in the presence of glucose oxidase, D-glucose was oxidised to D-gluconate with the formation of H_2O_2 . After reaction of H_2O_2 with p-hydroxybenzoic acid and 4-aminoantipyrine in the presence of peroxidase, a coloured product was formed. Starch content of samples were determined quantitatively with the aid of the absorbance of product.

3. RESULTS AND DISCUSSION

Biofuel was discovered even before the fossil fuels. However, the usage of it was not as effective as fossil fuels until the twentieth century. Since then, the increasing prices of fossil fuels, releasing huge amount of greenhouse gases and the presence of finite amount of oils shift the interests of people to biofuels again. Different attempts have been made to produce biofuels such as bioethanol and biodiesel from different types of biomass such as food crops, lignocellulosic materials on a commercial scale. Although food crops and lignocellulosic materials seemed to be convenient biomass for biofuel production, the debate between food and fuels, and limitations related to liquefaction of lignocellulose restrict the manufacturing of plant and lignocellulose based biofuels. Moreover, the demand for fresh water, the arable lands, and even effective saccharification methods force scientist and commercial companies to find suitable feedstocks for biofuels.

In recent years, much more attention has been paid to microalgae as a feedstock for biofuel production, because of the requirement of smaller land, faster growing cycle and containment of higher amount of oil. Due to the lipid and carbohydrate content of microalgal biomass, different type of biofuels such as biogas, biodiesel, bioethanol and even as biomethane could be produced from microalgal biomass. Among the other biofuels, biodiesel is one of the most important biofuel obtained from oils from algal biomass. Since better-quality and inexpensive diesel may be obtained by manipulating fatty acid composition of oil feedstock, and the quality of biodiesel depends on a balance between mono-unsaturated fatty acids and polyunsaturated fatty acids, different attempts have been made to determine and manipulate the fatty acid composition of algal oil (Yu et al., 2011, Juneja et al., 2013). However, the information about which factor is more crucial on fatty acid composition of algal biomass has not been clarified yet.

Therefore, preliminary evaluations based on an artificial neural network and feature selection have been carried out to find an approach to determine the degree of the effect of environmental stress. Based on the neural network calculations (data is not shown), it was revealed that salinity was the most important environmental stress on oil content of microalgae and biodiesel production. Additionally, it was reported that glucose and glycerol were the best carbon sources for enhancement of oil content of microalgae (Sharma et al., 2016).

In the light of these explanations, within the scope of this thesis, the effect of salt concentration together with glucose and glycerol on fatty acid composition of *Chlorella vulgaris* SAG 211-12 oil was investigated by using experimental design. Besides biodiesel production from microalgal oils, algal strains are also used to produce bioethanol because of the storage of starch within the cell. Thereby, the starch content of *C. vulgaris* SAG 211-12 biomass was also determined.

3.1. Determination of the Design Space Limits for the Concentrations of NaCl, Glucose and Glycerol

To explore the individual effect of NaCl, glucose and glycerol on growth, fatty acid and starch composition of *Chlorella vulgaris* SAG 211-12, *C. vulgaris* cells were cultivated in BG-11 medium supplemented with 0, 2.5, 5.0 and 10 % NaCl, 1% glucose and both 1% glucose and 1% glycerol.

Figure 3.1 shows how biomass concentration in terms of OD has changed in the absence and presence of NaCl, glucose and glycerol. According to the data, it seems likely that the highest biomass was obtained in the presence of 1% glucose (Figure 3.1 (c)) after 10 days of cultivation. In the presence of 1% glucose, cells reached the stationary phase within 8-10 days with the optical density of 14. Addition of 1% glycerol to BG-11 medium containing 1% glucose caused optical density to decrease from 14 to 10 at the end of the cultivation period (Figure 3.1 (d)).

It can be seen from Figure 3.1 (a) and (b) that the optical density of the strain SAG 211-12 was diminished in the absence of carbon sources and addition of NaCl. The biomass concentration in BG-11 and BG-11 containing 2.5% NaCl was closed to each other and almost 5 times lower than the biomass obtained using only glucose and both glucose and glycerol as carbon sources. The growth without carbon source was maintained at very long period and cells reached the stationary phase after 28-31 days of cultivation. Apart from 2.5% NaCl, growth ceased at 5 and 10% NaCl concentrations (data is not shown).

By using the growth curve, maximum specific growth rate of *Chlorella vulgaris* SAG 211-12 was also calculated and the results are illustrated in Table 3.1. Although addition of 1% glycerol caused to diminish the biomass content, maximum specific growth rate (Table 3.1) was slightly increased from 1.070 day⁻¹ to 1.077 day⁻¹. The lowest specific

growth rate of 0.098 day^{-1} was calculated in the BG-11 medium containing 2.5% NaCl. Removing NaCl from BG-11 medium did not change the specific growth rate significantly.

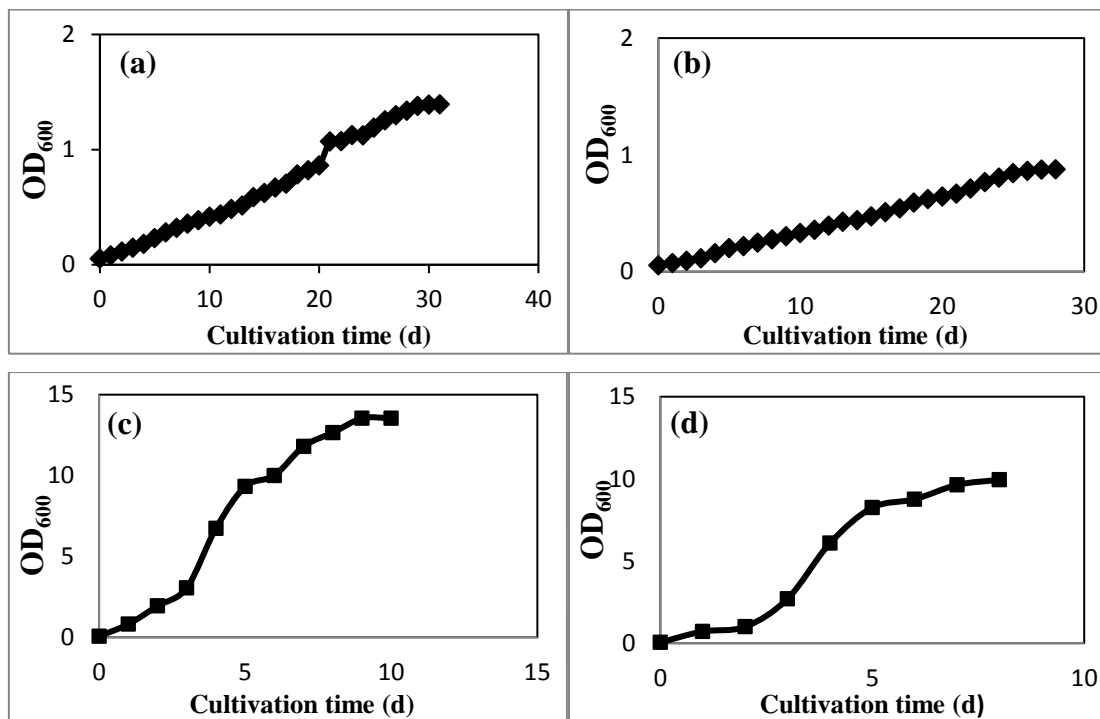


Figure 3.1. Growth profile of *C. vulgaris* SAG 211-12 in (a) BG-11 medium (b) BG-11 medium supplemented with 2.5 % (w/w) NaCl (c) BG-11 medium supplemented with 1% (w/w) glucose (d) BG-11 medium supplemented with 1% (w/w) glucose and 1% (w/w) glycerol

Table 3.1. Effect of NaCl, glucose and glycerol concentration on maximum specific growth rate (μ_{\max}) and dry cell weight (DCW) of *C. vulgaris* SAG 211-12 cells.

NaCl Concentration (% w/v)	Glucose Concentration (% w/v)	Glycerol Concentration (% w/v)	μ_{\max} (d^{-1})	DCW (g/L)
-	-	-	0.102	0.697
-	1	-	1.070	2.916
-	1	1	1.077	2.625
2.5	-	-	0.098	0.601

After harvesting microalgae cells, fatty acids were extracted directly by ethanol, and FAME composition was determined by GC-MS. Fatty acid composition of SAG 211-12 strain in the presence of glucose, glycerol and NaCl in mass fraction is given in Table 3.2.

Table 3.2. Effect of NaCl, glucose and glycerol concentration on fatty acid composition of *C. vulgaris* SAG 211-12.

NaCl (% w/v)	Glucose (% w/v)	Glycerol (% w/v)	Fatty acid composition (% w/w)				
			C16:0	C18:0	C18:1	C18:2	C18:3
-	-	-	26.82	30.10	-	11.84	31.24
2.5	-	-	62.39	37.55	-	-	-
-	1.0	-	50.34	-	17.61	16.36	15.68
-	1.0	1.0	53.01	-	26.37	20.63	-

Table 3.2 depicts that in modified BG-11 media, apart from poly-unsaturated alpha-linolenic acid (C18:3), mass fraction of saturated palmitic acid (C16:0) and stearic acid (C18:0), and mono-unsaturated oleic acid (C18:1), poly-unsaturated linoleic acid (LA) (C18:2) were increased significantly compared to fatty acids obtained in BG-11 medium. Although C18:3 was not synthesized in the presence of 2.5% NaCl and 1% glucose complemented with 1% glycerol, addition of 1% glucose as the sole carbon source dropped C18:3 ratio from 30.32% to 15.68%. According to the data C16:0 content was almost doubled and reached to 62.39%, 50.34% and 53.01% in the presence of 2.5% NaCl, 1% glucose, and glucose together with 1% glycerol, respectively. Considering mono-unsaturated oleic acid (C18:1) and poly-unsaturated linoleic acid (LA) (C18:2), C18:1 and C18:2 were not expressed in the presence of 2.5% NaCl, while removing NaCl, glucose and glycerol from BG-11 also stopped C18:1 synthesis. On the other hand, in the absence of glucose and glycerol and even NaCl, the fraction of C18:2 was almost 11.84%. In the presence of 1% glucose and glucose accompanied 1% glycerol, the percentage of C18:1 were almost 18% and 26%, respectively. The percentage of C18:2 was closed to C18:1 and equals to 16% and 21% in BG-11 supplemented with 1% glucose, and glucose together with 1% glycerol, respectively.

Besides fatty acids, starch content of *C. vulgaris* SAG 211-12 cells was also analyzed after lipid extraction and results are given in the Table 3.3. It can be seen from Table 3.3 that using 2.5% NaCl did not change starch content of *C. vulgaris* cells. In the presence and absence of NaCl, the starch content of cell was almost 6.5%. Although addition of

glucose enhanced starch content (8.37%), the highest value as 9.7% starch was observed in the medium containing both 1% glucose and 1% glycerol.

Table 3.3. Effect of NaCl, glucose and glycerol concentration on starch content of *C. vulgaris* SAG 211-12 cells.

NaCl (% w/v)	Glucose (% w/v)	Glycerol (% w/v)	Starch Content (%)
-	-	-	6.61
-	1	-	8.37
-	1	1	9.70
2.5	-	-	6.40

Overall, it is clear that NaCl, glucose and glucose complemented with glycerol manipulated fatty acid composition and starch content. Additionally, according to results, it seems that there is a synchronization between NaCl, glucose and glycerol for fatty acid synthesis. Therefore, CCD was applied to understand how NaCl, glucose and glycerol change fatty acid composition of *C. vulgaris* SAG 211-12.

In the previous studies, it was reported that glucose concentration up to 4% with 0.5% glycerol induced the cell growth. Additionally, combination of 2% glucose and 0.5% glycerol caused to increase the ratio between C16:0 and C18:0 (Hosoglu- Isleten et al., 2012). Additionally, Kong et al. (2013) reported that the growth of *C. vulgaris* was induced with the addition of 1% glycerol and 0.2% glucose into the growth medium. Therefore, in the presented thesis, 0.1 and 1% were chosen as lower and upper limit for glucose and glycerol, while NaCl concentration was changed between 0.5 and 2.5%, since above 2.5% NaCl growth was ceased.

3.2. Investigation of The Effect of NaCl, Glucose and Glycerol on Growth Profile of *C. vulgaris* SAG 211-12 by Using CCD

Cultivation of microalgae *C. vulgaris* SAG 211-12 cells were performed using fifteen different combinations of NaCl, glucose and glycerol concentration (% w/v). The raw data related to growth of strain SAG 211-12 is also given in figures between A.1 and A.15 in Appendix A. By using the growth profile of strain SAG 211-12, the maximum specific growth rates were calculated and results are tabulated in Table 3.4.

Table 3.4 compares the maximum specific growth rate of *C. vulgaris* SAG 211-12 at different concentrations of NaCl, glucose and glycerol. As an overall trend, the maximum specific growth rate decreased with an increase in NaCl concentration. The only exception is that, maximum specific growth rate of *C. vulgaris* SAG 211-12 was increased with augmentation of NaCl from 0.9 to 2.0% (at fixed glucose and glycerol concentrations of 0.3%).

When NaCl concentration was fixed as 2.0%, cell growth rate was dropped with an increase in glucose concentration. The lowest values were obtained at highest NaCl (2.0 and 2.5 %) and glucose concentrations at 0.8 and 0.6 %. At 2.0% NaCl concentration, the growth rate of strain SAG 211-12 doubled and reached to 0.439 by reducing both glucose and glycerol concentrations about three fold (from 0.8% to 0.3%). Although, at both glucose and glycerol concentration of 0.3%, specific growth rate declined almost two times by decreasing NaCl concentration from 2.0 % to 0.9 %, a surge took place slightly at 0.6% of glucose and glycerol concentration when NaCl concentration dropped down to 1.5 % from 2.5%.

Overall, the specific growth rate was varied considerably by changing the amount of NaCl, glucose and glycerol in growth medium. The medium in which the highest maximum specific growth rate obtained includes 0.9% NaCl, 0.8% of both glucose and glycerol, while the lowest one was obtained by keeping the glucose level at 0.8%, increasing NaCl fraction to 2.0% and decreasing glycerol to 0.3%.

Table 3.4. Effect of NaCl, glucose and glycerol concentration on maximum specific growth rates (μ_{\max}) of *C. vulgaris* SAG 211-12

Medium Number	NaCl Concentration (% w/v)	Glucose Concentration (% w/v)	Glycerol Concentration (% w/v)	μ_{\max} (d ⁻¹)
1	0.5	0.6	0.6	0.515±0.108
2	0.9	0.3	0.3	0.258±0.044
3	0.9	0.3	0.8	0.478±0.143
4	0.9	0.8	0.3	0.256±0.038
5	0.9	0.8	0.8	0.635±0.005
6	1.5	0.1	0.6	0.245±0.029
7	1.5	0.6	0.1	0.276±0.054
8	1.5	0.6	1.0	0.316±0.057
9	1.5	0.6	0.6	0.256±0.011

Table 3.4. Continued

10	1.5	1.0	0.6	0.392±0.016
11	2.0	0.3	0.3	0.439±0.091
12	2.0	0.3	0.8	0.376±0.014
13	2.0	0.8	0.3	0.156±0.005
14	2.0	0.8	0.8	0.188±0.001
15	2.5	0.6	0.6	0.182±0.052

Similar to presented results, in a previous study, NaCl and glucose were shown to be significant factors for cell growth as a result of Plackett-Burman design (Kong et al., 2012). *Chlorella vulgaris* is normally a type of freshwater microalgae. On the other hand, Luangpipat et al. (2016) confirmed that *C. vulgaris* could adapt brackish water (mixture of freshwater and seawater, 1:1 v/v) and even full strength seawater (including almost 4% NaCl). They also reported that the growth rate of *C. vulgaris* was slightly dropped in seawater (Luangpipat and Chisti 2016). However, the upper limit of NaCl that allow *C. vulgaris* SAG 211-12 cells to grow was 2.5%, lower than that reported by Luangpipat et al. (2016).

In order to understand the effect of NaCl on growth of microalgae, enzyme expressed by microalgae *C. protothecoides* were measured under salt stress, and it is reported that activity of isocitrate dehydrogenase (ICD) as an enzyme in TCA cycle was decreased with NaCl addition into the growth medium. Since TCA cycle provides carbon and energy for the cell growth, reduction of the activity of ICD caused the growth rate to decrease (Wang et al., 2016). That may be the one of the reasons behind inhibition of the growth of SAG 211-12 strain at 5 and 10% NaCl concentrations.

Recently, Church et al. (2017) investigated the effect of various salts at different concentration on growth and lipid accumulation of *C. vulgaris* cultivated in wastewater. The specific growth rate decreased from 0.27 d⁻¹ to 0.06 d⁻¹ with rising NaCl from 0 g/L to 45 g/L (4.5%). Similar to our data of preliminary experiments, time required to reach maximum cell concentration was prolonged at highest NaCl concentrations (Church et al., 2017).

Regarding glucose concentration, Cheirsilp et al. (2012) investigated the effect of glucose concentration on growth and lipid production of freshwater and marine *Chlorella sp.*, *Nannochloropsis sp.* and *Cheatocecos sp.* It was revealed that for marine *Chlorella sp.*

and *Nannochloropsis sp.*, dry cell weight was increased at a glucose concentration up to 10 g/L (1%); further addition of glucose up to 20 g/L did not increase cell growth.

In addition to glucose, effect of glycerol on growth of *C. vulgaris* was studied (Kong et al., 2013). It was reported that combination of glucose and glycerol was more effective on growth than using glycerol as sole carbon source. Kong et al. (2013) reported that maximum growth rate (0.99 d^{-1}) and biomass content of 2.62 g/L was obtained in the medium supplied with mixture of 2 g/L (0.2%) glucose and 5 g/L (0.5%) glycerol. However, glucose concentration at 2 g/L (0.2%) and glycerol concentration at 10 g/L (0.1%) gave the almost same results (Kong et al., 2013). As the opposite of these results, at fixed NaCl (0.9%) and glucose (0.8%) concentrations, decreasing glycerol concentration from 0.8 to 0.3% caused to decrease specific growth rate from 0.635 to 0.256 d^{-1} .

Overall, Figure 3.2. depicts that the maximum specific growth rate was decreased with increasing NaCl concentration at the same glucose and glycerol concentrations.

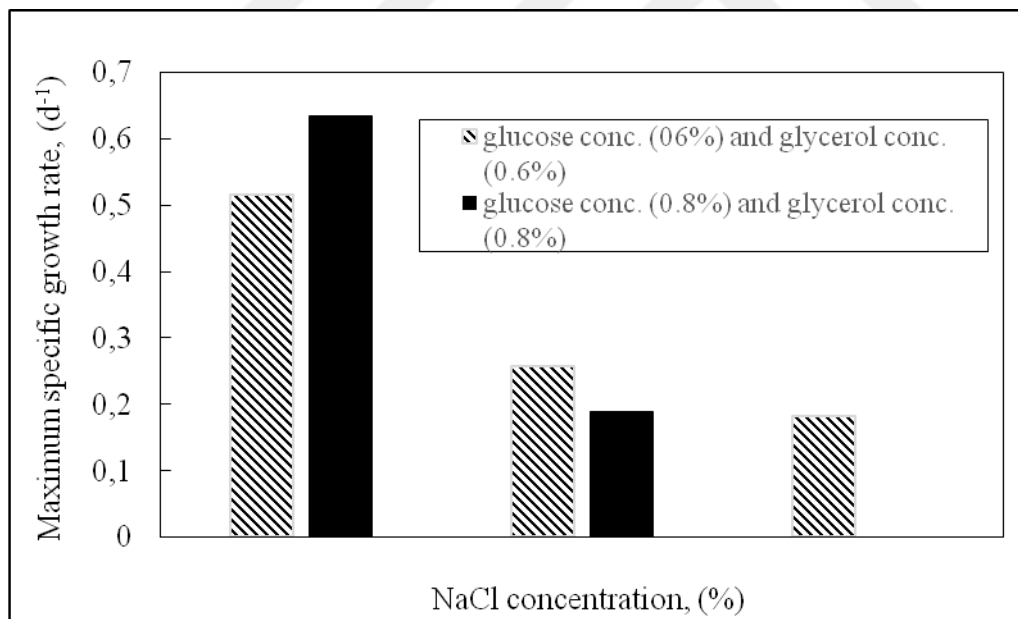


Figure 3.2. Effect of NaCl concentration on the maximum specific growth rate of strain SAG 211-12

On the other hand, depending on the NaCl concentration, glucose and glycerol showed opposite effects (Figure 3.3 and Figure 3.4). As seen in Figure 3.3., the maximum specific growth rate did not change with increasing glucose conc. from 0.3% to 0.8% at 0.9 NaCl

and 0.3% glycerol concentrations. However, at the same NaCl concentration (0.9%), when glycerol concentration was increased three fold, three-fold increase in glucose concentration caused to increase the specific growth rate. However, when the NaCl concentration was almost doubled, increasing glucose and glycerol concentration halved the maximum specific growth rate.

Opposite to glucose, glycerol had positive effect on the maximum specific growth rate of strain SAG 211-12 at low (0.9%) NaCl concentration. When glycerol concentration was almost tripled, the maximum specific growth rate was doubled at constant glucose concentrations. At higher NaCl concentration, changing glycerol concentration did not affect growth rate significantly at constant glucose concentrations.

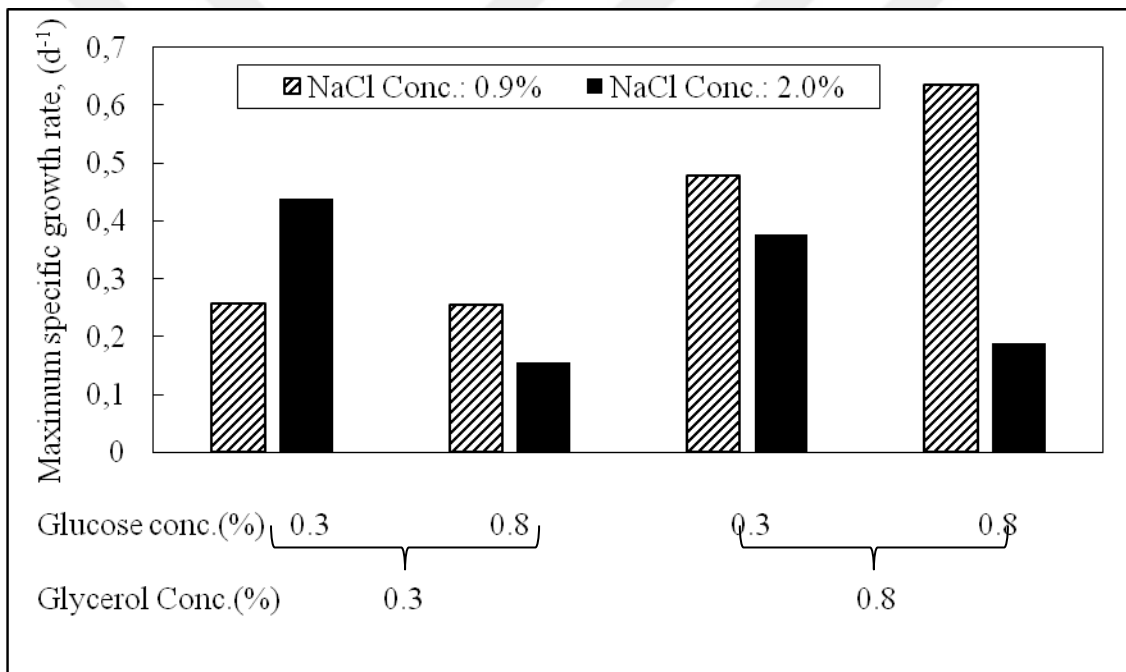


Figure 3.3. Effect of glucose concentration on the maximum specific growth rate of strain SAG 211-12

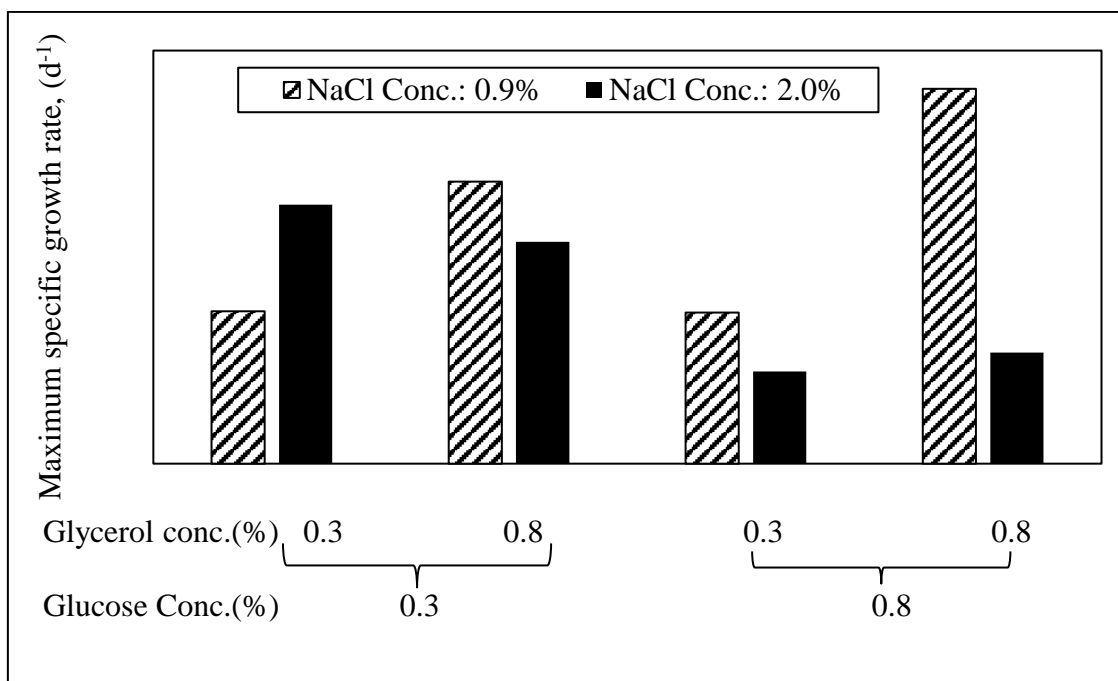


Figure 3.4. Effect of glycerol concentration on the maximum specific growth rate of strain SAG 211-12

3.3. Effect of NaCl, Glucose and Glycerol Concentrations on Fatty Acid Profile of *C. vulgaris* SAG 211-12

FAME molecules were analyzed with GC-MS in order to obtain fatty acid profile of *C. vulgaris* SAG 211-12 cells. Amount of FAMES (mg) accumulated in 150 mL culture medium, FAME yield based on biomass (mg/g DCW) and mass fraction of individual fatty acids in total fatty acids (% w/w) are illustrated in Appendix B and tabulated in Table 3.5, 3.6 and 3.7.

It can be seen from Figures B.1-B.15, fatty acids that were extracted from *C. vulgaris* SAG 211-12 were composed of saturated and unsaturated fatty acids with 16 carbon and 18 carbon chain length. C16:0, C18:0, C18:1, C18:2 and C18:3 were the common fatty acids for *C. vulgaris* SAG 211-12 cells in fifteen culture media. Although microalgae cells also accumulated myristic acid (C14:0) and pentadecanoic acid (C15:0), concentration of them was relatively low compared to other fatty acids.

According to data related to total amount of FAME of *C. vulgaris* SAG 211-12, the lowest amount of total fatty acid as 0.778 mg was obtained in medium 13 in which the lowest

specific growth rate was also obtained. Similarly, the highest amount of total fatty acid (mg) as 2.571 mg was obtained with medium 5, which resulted the highest specific growth rate. When glucose and glycerol concentrations were fixed at 0.8%, increasing NaCl from 0.9 to 2.0% caused the two times reduction of total FAME amount.

Accumulation of high amount of lipids is an important property for economics of biodiesel production from microalgae cells. In the study of Li et al. (2014), in which microalgae *Chodatella sp.* was cultivated in mixotrophically in piggery wastewater and also autotrophically and total FAME amount was increased under mixotrophic conditions (Li et al., 2014). It may be because of that in the presence of excess carbon, cells channel carbon for production of storage compounds especially lipids (Pribyl et al., 2012). It was seen from results of recent studies, mixotrophic growth induce growth and lipid accumulation in microalgae depending on the concentration of carbon source (Cheirsilp and Torpee, 2012). Furthermore, several researches reported the impact of NaCl and some other salt types on growth, lipid accumulation and lipid profile of various microalgae (Pandit et al., 2017, Church et al., 2017).

Excess salinity had led to environmental stress for microalgae cells and inhibited cell growth. However, under stress conditions cells switched the metabolic pathway and accumulate storage molecules especially triacylglycerol (TAG) instead of cell reproduction. Generally, between lipid production and cell growth, a trade-off was observed. Additionally, lipid profile of algal oil was influenced from salinity (An et al., 2013, Xia et al., 2014, Yang et al., 2015, Wang et al., 2016).

In the light of these explanations, we evaluated the effect of NaCl, glucose and glycerol on amount of total FAME of SAG 211-12 strain. The highest amount of total fatty acid (mg) was obtained at 0.9% NaCl concentration (Figure 3.5) and at constant NaCl concentration of 0.9 %, total FAME amount was slightly affected by glucose and glycerol concentration. Total FAME amount was increased from 2.037 to 2.571 mg with increasing glucose and glycerol concentration from 0.3% to 0.8% (Table 3.6). It can be seen also in Figure 3.6 and 3.7, the total amount of FAME did not change significantly with glucose concentration, (Figure 3.6), while increasing glycerol concentration at constant glucose concentration caused to tiny increase in total amount of FAME.

Considering the total FAME yield based on biomass (mg FAME/g DCW), similar to total amount of FAME, the highest FAME yield, which is higher than 7 mg/g DCW, was obtained at 0.9% NaCl concentrations (Table 3.6). The highest FAME yield of 8.521 mg/g DCW was obtained in the medium supplemented with 0.9% NaCl and 0.3% glucose and glycerol (medium 2).

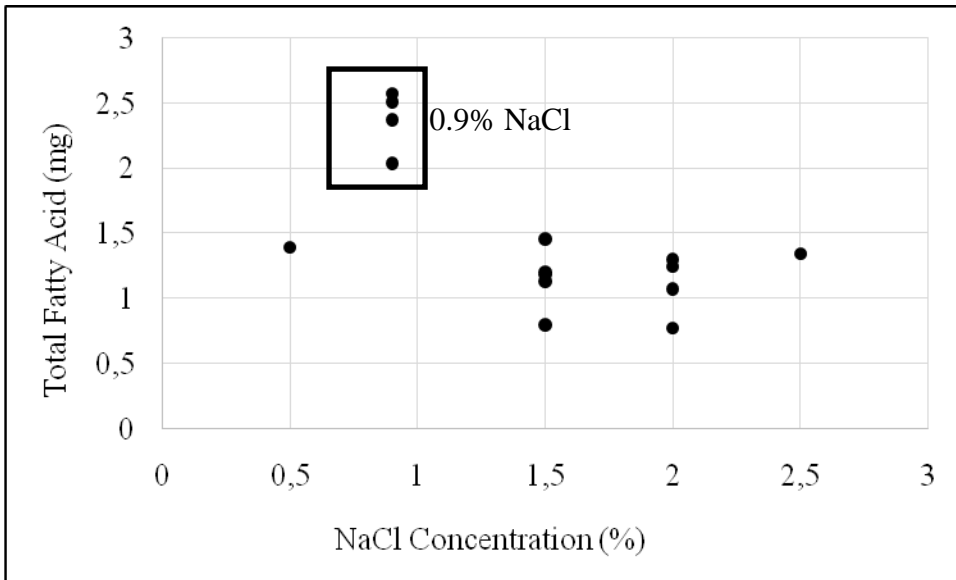


Figure 3.5. Effect of NaCl on amount of total FAME

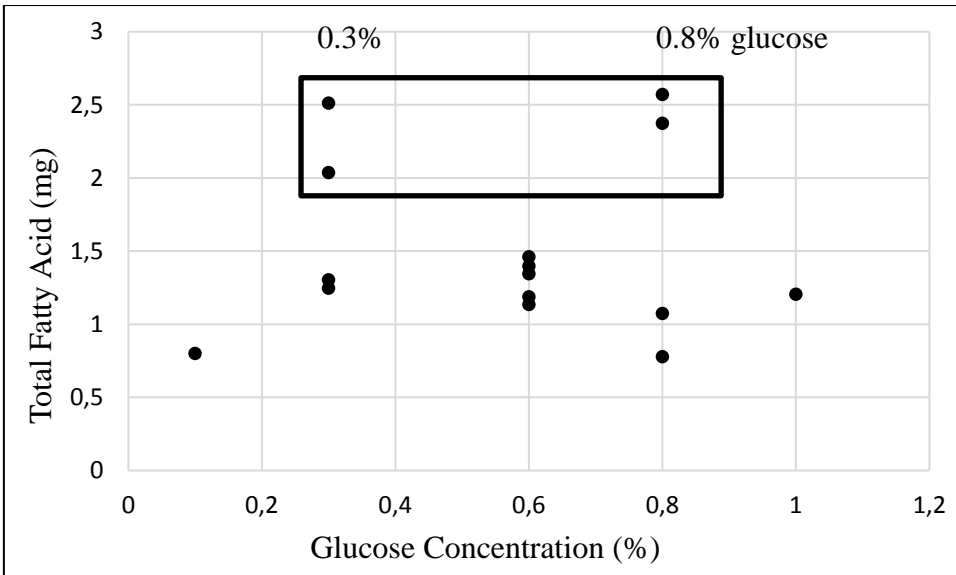


Figure 3.6. Effect of glucose on amount of total FAME

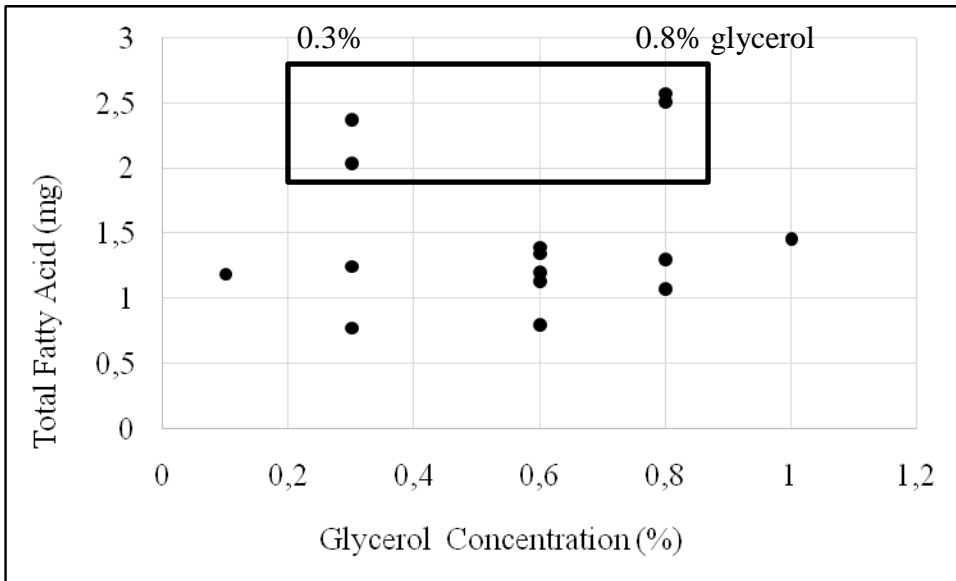


Figure 3.7. Effect of glycerol on amount of total FAME

Table 3.5. Amount of total FAMES (mg) and fatty acid composition of *C. vulgaris* SAG 211-12 cultured in various media containing glucose, glycerol and NaCl at different concentrations. Values were given as the means of FAME amount±standart deviation.

Culture Medium Number	NaCl (% w/v)	Glucose (% w/v)	Glycerol (% w/v)	Total FAME (mg)	mg						
					C14:0	C15:0	C16:0	C18:0	C18:1	C18:2	C18:3
1	0.5	0.6	0.6	1.397	-	-	0.491±0.251	-	0.215±0.119	0.292	0.399±0.300
2	0.9	0.3	0.3	2.037	-	-	0.829±0.478	-	0.302±0.122	0.25±0.111	0.657±0.306
3	0.9	0.3	0.8	2.510	0.045±0.009	0.022±0.019	0.919±0.589	0.148±0.018	0.294±0.136	0.344±0.198	0.737±0.449
4	0.9	0.8	0.3	2.372	0.037±0.006	-	0.715±0.186	0.212±0.070	0.439±0.221	0.503±0.353	0.466±0.126
5	0.9	0.8	0.8	2.571	0.055±0.055	0.010±0.010	0.915±0.346	0.158±0.072	0.469±0.322	0.300±0.114	0.664±0.062
6	1.5	0.1	0.6	0.799	0.027±0.022	0.068±0.022	0.292±0.098	-	0.097±0.035	0.108±0.035	0.208±0.054
7	1.5	0.6	0.1	1.186	0.045±0.006	-	0.421±0.113	-	0.174±0.049	0.161±0.044	0.387±0.114
8	1.5	0.6	1.0	1.460	0.045±0.007	0.029±0.008	0.525±0.069	-	0.173±0.013	0.190±0.040	0.498±0.139
9	1.5	0.6	0.6	1.134	-	-	0.380±0.087	-	0.225±0.086	0.157±0.095	0.374±0.256
10	1.5	1.0	0.6	1.205	0.032±0.027	-	0.421±0.055	-	0.181±0.040	0.165±0.028	0.406±0.093
11	2.0	0.3	0.3	1.247	0.046±0.003	0.030±0.005	0.379±0.043	0.188±0.132	0.136±0.005	0.173±0.034	0.295±0.052
12	2.0	0.3	0.8	1.302	0.022±0.018	0.042±0.072	0.379±0.044	0.140±0.054	0.166±0.059	0.147±0.031	0.407±0.013
13	2.0	0.8	0.3	0.778	-	-	0.238±0.058	0.152±0.050	0.182±0.040	0.086±0.005	0.130±0.052
14	2.0	0.8	0.8	1.074	-	-	0.362±0.094	0.183±0.071	0.275±0.076	0.095±0.036	0.159±0.072
15	2.5	0.6	0.6	1.345	-	-	0.345±0.070	0.298	0.258±0.117	0.116±0.050	0.33±0.184

Since the composition of FAME is also very important for biodiesel production, the fatty acid composition of *C. vulgaris* SAG 211-12's biomass obtained in fifteen different cultivation medium was investigated. As seen in Table 3.5, among all fatty acids determined in biomass, the highest amount was obtained for C16:0 in all media. The amount of C16:0 changed between 0.238 and 0.919 mg. The highest amount of C16:0 was determined at 0.9% NaCl, 0.3% glucose and 0.8% glycerol, while the lowest value was obtained at 2.0% NaCl, 0.8% glucose and 0.3% glycerol. Therefore, reducing NaCl concentration to 0.9% and increasing glycerol concentration from 0.3% to 0.8% caused to dramatic increase in C16:0 amounts. Additionally, C18:0 accumulation in mg and on dry cell weight basis was maximum in the medium 15, in the presence of 2.5% NaCl. When medium 1 (0.5% NaCl), medium 9 (1.5% NaCl) and medium 15 (2.5% NaCl) were compared, it can be seen that increased C18:1 amount was coupled with decreased C18:2 and C18:3 amounts. However, similar trend was not observed for other growth media.

Fatty acid compositions of cells were also determined on dry weight basis. Although FAME yield was almost doubled with augmentation of NaCl concentration from 0.5 to 2.5%, when NaCl content increased from 0.9 to 2.0%, total FAME yield was decreased significantly at any glucose and glycerol concentration (Table 3.6). It can be seen from Table 3.6 that in the presence of 0.5% NaCl and 0.6% glucose and glycerol, yield of C16:0 as 1.274 mg/g DCW was lower than C16:0 yield (1.656 mg/g DCW) in the presence of 2.5% NaCl and 0.6% glucose and glycerol. Nevertheless, regardless of glucose and glycerol concentration, reduction of C16:0 yield was observed when NaCl concentration increased from 0.9 to 2.0%, due to the reduction of total FAME yield. C18:0 production was not observed in medium 1 (0.5% NaCl), its production was enhanced up to 1.433 mg/g DCW in medium 15 (2.5% NaCl). Besides C18:0, C18:1 also showed similar alterations. C18:1 yield in medium 15 (1.238 mg/ g DCW) was more than two fold of the C18:1 yield in medium 1 (0.558 mg/ g DCW). In the presence of 2.0% NaCl, augmentation of both glucose and glycerol from 0.3% to 0.8% reduced total FAME yield whereas yield of C18:1 was increased two fold with the supplement of 0.8% glucose and glycerol. Under that conditions increasing C18:1 yield was coupled with reduction of C18:2 and C18:3 yields. In addition, increasing NaCl concentration from 0.9% to 2.0%, reduced C18:3 yield.

Table 3.6. Comparison of FAME yields (mg/g DCW) of microalgae cultured in various media. Values were given as the means of FAME yield±standart deviation.

Culture Medium Number	NaCl (% w/v)	Glucose (% w/v)	Glycerol (% w/v)	Total FAME (mg FAME/g DCW)	(mg/g DCW)						
					C14:0	C15:0	C16:0	C18:0	C18:1	C18:2	C18:3
1	0.5	0.6	0.6	3.626	-	-	1.274±0.512	-	0.558±0.554	0.758	1.036±0.752
2	0.9	0.3	0.3	8.521	-	-	3.467±0.614	-	1.262±0.455	1.046±0.492	2.747±0.511
3	0.9	0.3	0.8	8.098	0.146±0.208	0.072±0.829	2.966±0.644	0.476±0.134	0.950±0.465	1.111±0.578	2.377±0.612
4	0.9	0.8	0.3	7.320	0.113±0.234	-	2.205±0.313	0.655±0.372	1.355±0.533	1.552±0.724	1.439±0.320
5	0.9	0.8	0.8	7.161	0.152±1.017	0.029±1.031	2.548±0.421	0.441±0.490	1.306±0.711	0.836±0.423	1.850±0.206
6	1.5	0.1	0.6	3.717	0.124±0.817	0.314±0.325	1.357±0.336	-	0.452±0.362	0.502±0.330	0.968±0.261
7	1.5	0.6	0.1	4.283	0.161±0.141	-	1.519±0.270	-	0.627±0.283	0.580±0.278	1.396±0.297
8	1.5	0.6	1.0	3.936	0.121±0.198	0.079±0.296	1.414±0.170	-	0.466±0.132	0.513±0.236	1.343±0.300
9	1.5	0.6	0.6	4.021	-	-	1.346±0.229	-	0.796±0.381	0.555±0.604	1.324±0.684
10	1.5	1.0	0.6	3.221	0.087±0.847	-	1.125±0.156	-	0.484±0.235	0.442±0.192	1.084±0.245
11	2.0	0.3	0.3	5.197	0.190±0.180	0.124±0.230	1.579±0.206	0.783±0.725	0.567±0.175	0.723±0.261	1.230±0.246
12	2.0	0.3	0.8	4.535	0.078±0.864	0.145±0.736	1.319±0.299	0.486±0.476	0.577±0.451	0.511±0.348	1.419±0.277
13	2.0	0.8	0.3	3.682	-	-	1.113±0.294	0.711±0.368	0.852±0.274	0.401±0.172	0.605±0.435
14	2.0	0.8	0.8	4.817	-	-	1.623±0.267	0.821±0.396	1.234±0.283	0.424±0.388	0.715±0.460
15	2.5	0.6	0.6	6.466	-	-	1.656±0.205	1.433	1.238±0.453	0.555±0.429	1.584±0.557

Table 3.7. Comparison of mass fractions of individual fatty acids (% w/w) produced in various media.

Culture Medium Number	NaCl (% w/v)	Glucose (% w/v)	Glycerol (% w/v)	% w/w						
				C14:0	C15:0	C16:0	C18:0	C18:1	C18:2	C18:3
1	0.5	0.6	0.6	-	-	35.11	-	15.39	20.90	28.56
2	0.9	0.3	0.3	-	-	40.67	-	14.80	12.27	32.23
3	0.9	0.3	0.8	1.81	0.88	36.63	5.88	11.73	13.72	29.36
4	0.9	0.8	0.3	1.54	-	30.13	8.95	18.51	21.21	19.65
5	0.9	0.8	0.8	2.11	0.40	35.39	6.12	18.14	11.62	25.70
6	1.5	0.1	0.6	3.33	8.45	36.51	-	12.18	13.50	26.05
7	1.5	0.6	0.1	3.77	-	35.49	-	14.64	13.55	32.59
8	1.5	0.6	1.0	3.07	2.01	35.93	-	11.83	13.03	34.14
9	1.5	0.6	0.6	-	-	33.47	-	19.80	13.80	32.94
10	1.5	1.0	0.6	2.69	-	34.93	-	15.01	13.70	33.66
11	2.0	0.3	0.3	3.65	2.39	30.39	15.08	10.92	13.91	23.68
12	2.0	0.3	0.8	1.72	3.19	29.08	10.72	12.73	11.27	31.27
13	2.0	0.8	0.3	-	-	30.22	19.32	23.13	10.88	16.43
14	2.0	0.8	0.8	-	-	33.71	17.04	25.62	8.81	14.84
15	2.5	0.6	0.6	-	-	25.61	22.16	19.14	8.59	24.50

Quality of biodiesel is strictly dependent to the fatty acid profile of algal oil. Fatty acids with 16 and 18 carbon have been reported to be the most suitable fatty acids for biodiesel production (D'Alessandro and Antoniosi Filho, 2016). Chain length and unsaturation affect saponification and iodine value of biodiesel (Pandit et al., 2017). If average degree of unsaturation is high, then the cetane number of biodiesel is low and low cetane number cause low oxidation stability (Yang et al., 2015). In the literature (Fulekar et al., 2017, Mohan and Devi, 2014), raising saturated fatty acids in microalgal biomass is explained with a defense mechanism of microalgae cells against to high salinity. Saturated membrane fatty acids decreased membrane permeability to Na⁺ and Cl⁻ ions (Lu et al., 2012).

In this thesis, for all variations of experimental variables (NaCl, glucose and glycerol), C16:0 was the prominent fatty acid, while C14:0 and C15:0 percentages in terms of mass fraction were very low in all tested media. Total mass fraction of C14:0 and C15:0 were between 0-11.7%. In 2017, Pandit P.R. et al. investigated effect of different NaCl concentrations between 0-0.4 M on fatty acid profile of *C. vulgaris* cultured in BG-11 medium and reported the production of C15:0 as 0.24% (w/w) only in the presence of 0.1 M NaCl. In the same study, maximum C14:0 content was 0.6%. However, C15:0 has not been reported commonly for *C. vulgaris* (Pandit et al., 2017).

When C16:0 is examined, there were some quantitative differences of C16:0 content of *C. vulgaris* SAG 211-12 depending on the culture media. Its mass fraction was highest in medium 2 (40.67%) and lowest in medium 15 (25.61%) which includes 2.5% NaCl and 0.6% of both glucose and glycerol (Table 3.7). Mohan et al. (2014) showed that 1 g/L (0.1%) NaCl addition was resulted in 38.4% C16:0 accumulation in microalgae that was collected from domestic effluents. In a recent study performed by Church et al. (2017), *C. vulgaris* reached the maximum C16:0 content of 18% with the addition of 45 g/L (4.5%) NaCl or KCl in the synthetic wastewater. In the same study, the highest mass fraction of C18:0 (2.96%) was obtained in the presence of 45g/L NaCl (Church et al., 2017). In the presented work, in the presence of 0.9% NaCl C18:0 proportion was relatively low and increasing NaCl concentration to 1.5% ceased C18:0 accumulation. However, C18:0 mass fraction was increased with addition of 2.0% NaCl in the growth medium and peaked significantly in medium 15 in which NaCl concentration was 2.5%.

It seems like that at fixed NaCl concentration of 0.9%, increasing glucose and glycerol concentration affected C16:0 and C18:3 proportions negatively. C18:1 ratio was highest 23.13% and 25.62% in medium 13 (2% NaCl, 0.8% glucose and 0.3% glycerol) and medium 14 (2% NaCl, 0.8% glucose and 0.8 % glycerol), respectively. On the contrary, in the same media C18:3 mass fraction was observed to be lowest among fifteen media.

Kucharoenphaibul et al. (2014) also investigated the cultivation of freshwater *Chlorella sorokiniana* 1019 under mixotrophic conditions, using glucose, potassium acetate and glycerol as carbon source. They reported that while glycerol did not provide enhancement of growth and lipid production, glucose addition in the medium caused the reduction of C16:0, C18:0 and C18:1 ratio from 37.80, 4.30, 16.00 to 22.70, 2.10 and 9.20 respectively. On the other hand, C18:2 and C18:3 were increased from 28.40 and 6.30% to 32.60 and 14.40%, respectively (Kucharoenphaibul et al., 2014). In contrast, the optimization of growth and lipid production of *Ettlia texensis* under photoheterotrophic conditions was performed using 3 g/L glucose as an organic carbon source and C18:3 content of *Ettlia texensis* was reported to be slightly dropped under photoheterotrophic conditions in comparison with autotrophic and heterotrophic cultivation (Isleten-Hosoglu et al., 2013). In the presented study preliminary experiments showed a similar trend, in BG-11 medium mass fraction of C18:3 was 30.32% whereas it was dropped to 15.68% and 0% in the presence of 1% glucose and both 1% glucose and 1% glycerol, respectively. On the other hand, in general percentage of C18:3 was higher than 20% in mixotrophic cultures, that is higher than the limit of C18:3.

Generally, highest limit of C18:3 that determined by the European B100 biodiesel standard (EN 14214) is 12% (w/w) (Church et al., 2017). Comparing this value with C18:3 content of SAG 211-12, medium 14 (2% NaCl, and 0.8% of glucose and glycerol) and medium 13 (2.0 % NaCl, and 0.8% glucose and 0.3% glycerol) yielded C18:3 of 14.84% and 16.43%, respectively. These values are very close to the limit value of C18:3. It was noteworthy that when total mass fraction of C18:1 was significantly increased mass fraction of C18:3 reached the minimum values of 14.84%. In other words, at high salt concentration, it is required to use highest amount of both glucose and glycerol in order to reduce C18:3 fraction. However, at low NaCl concentration (0.9%), glucose concentration might be kept at much higher value than the glycerol concentrations.

3.4. Effects of NaCl and Carbon Source on Starch Content of *C. vulgaris* SAG 211-12

Starch content of residual biomass after lipid extraction was analyzed and starch accumulation in *C. vulgaris* SAG211-12 depending on the NaCl, glucose and glycerol content was determined. Results were calculated according to the Megazyme total starch assay kit and was demonstrated in Table 3.8.

Table 3.8. Effect of NaCl, glucose and glycerol on starch accumulation of *C. vulgaris* SAG 211-12

Culture Medium Number	NaCl (% w/v)	Glucose (% w/v)	Glycerol (% w/v)	Starch Content (%)
1	0.5	0.6	0.6	11.09
2	0.9	0.3	0.3	15.96
3	0.9	0.3	0.8	15.72
4	0.9	0.8	0.3	12.81
5	0.9	0.8	0.8	13.79
6	1.5	0.1	0.6	6.53
7	1.5	0.6	0.1	6.40
8	1.5	0.6	1.0	12.53
9	1.5	0.6	0.6	7.18
10	1.5	1.0	0.6	8.93
11	2.0	0.3	0.3	22.08
12	2.0	0.3	0.8	9.65
13	2.0	0.8	0.3	8.19
14	2.0	0.8	0.8	6.22
15	2.5	0.6	0.6	5.32

According to Table 3.8, the best medium composition for maximum starch accumulation (22.08%) was BG-11 medium supplemented with 2.0% NaCl, 0.3% glucose and glycerol. When glucose and glycerol concentrations were doubled (0.6%), increasing NaCl concentration caused drop of starch fraction. At 0.5% NaCl and 0.6% of both glucose and glycerol (medium 1), starch content was a small minority (11.9%), whereas when NaCl increased to 1.5% and 2.5%, starch content was a very small proportion (7.18% and 5.2%), respectively.

At 0.9% NaCl and 0.3% glycerol concentration, increasing glucose concentration in the medium from 0.3% to 0.8% resulted in decrease of starch content from 15.96% to 12.81%. Same trend was observed at 2.0% NaCl concentration. However, changing glucose concentration from 0.1% to 1.0% caused rise of starch content from 6.53 to 8.93%, when NaCl and glycerol concentration was 1.5% and 0.6%, respectively. When NaCl and glucose concentration was maintained as 1.5% and 0.6%, augmentation of glycerol concentration induced the starch accumulation. On the contrary, when 2.0% NaCl and 0.3% glucose was supplemented to the BG-11, starch content was decreased from 22.08 to 9.65% with the rising glycerol concentration from 0.3% to 0.8%.

Starch is the other important compound of microalgae cells and it can be used as raw material for bioethanol. Kong et al., in 2013 reported that mixotrophic growth induced the soluble carbohydrate accumulation up to 8.74% in the presence of 2 g/L glucose and 10 g/L glycerol. It was also stated that addition of 1g/L glycerol into autotrophic medium caused no significant change in carbohydrate content of *C. vulgaris*. But when glycerol concentration was increased to 5g/L, 10 g/L or glucose and glycerol were supplied together carbohydrate content was induced (Kong et al., 2013).

Rao et al. (2007) showed the increase of carbohydrate content of *Botryococcus braunii* as a result of high salinity up to 34 mM (Rao et al., 2007). Carbohydrate content of *Chlorella protothecoides* under heterotrophic culture conditions was reduced from 52% to 22.8% under NaCl stress whereas lipid and protein production was induced (Wang et al., 2016). Unlike that study, similar relation between total FAME amount and starch amount was not observed in the present study. In this study at a certain glucose and glycerol concentrations, increasing NaCl caused a decrease of starch content, except medium 11 in which maximum starch content was observed.

3.5. Optimization of NaCl, Glucose, Glycerol Concentrations in Growth Media

Optimization of NaCl, glucose and glycerol concentrations in the growth medium was studied for two major objectives:

- Maximization of total FAME amount (total TAG amount) produced in the 150 mL culture medium by *C. vulgaris* SAG 211-12

- Maximization of palmitic acid content (C16:0) as a most suitable fatty acid for biodiesel production.

CCD was applied for experimental design and NaCl with a concentration range of 0.5-2.5%, glucose and glycerol in the range of 0.1-1.0% were used as experimental variables. According to design, fifteen combinations of experimental variables were tested as medium components. Experimental and predicted results of responses as total fatty acid amount and palmitic acid content are demonstrated in table 3.9. According to the experimental results, medium 5 gave the maximum total FAME amount and medium 2 offered the highest palmitic acid (C16:0) fraction by mass among fifteen experiments.

Table 3.9. CCD matrix with three independent variables, experimental and predicted results

Runs	Experimental variables (factors) (% w/w)			Total FAME amount (mg)		C16:0 mass fraction (%w/w)	
	NaCl (X ₁)	Glucose (X ₂)	Glycerol (X ₃)	Experimental	Predicted	Experimental	Predicted
1	0.5	0.6	0.6	1.397	1.397	35.11	35.11
2	0.9	0.3	0.3	2.037	2.037	40.67	40.67
3	0.9	0.3	0.8	2.510	2.510	36.63	36.63
4	0.9	0.8	0.3	2.372	2.372	30.13	30.13
5	0.9	0.8	0.8	2.571	2.571	35.39	35.39
6	1.5	0.1	0.6	0.799	0.799	36.51	36.51
7	1.5	0.6	0.1	1.186	1.186	35.49	35.49
8	1.5	0.6	1.0	1.460	1.460	35.93	35.93
9	1.5	0.6	0.6	1.134	1.134	33.47	33.47
10	1.5	1.0	0.6	1.205	1.205	34.93	34.93
11	2.0	0.3	0.3	1.247	1.247	30.39	30.39
12	2.0	0.3	0.8	1.302	1.302	29.08	29.08
13	2.0	0.8	0.3	0.778	0.778	30.22	30.22
14	2.0	0.8	0.8	1.074	1.074	33.71	33.71
15	2.5	0.6	0.6	1.345	1.345	25.61	25.61

Full quadratic polynomial and Kriging models were tested to fit experimental data provided from CCD experiments. In a full quadratic polynomial model, relationship between experimental variables and response is expressed like in the following equation (Witek-Krowiak et al., 2014):

$$y = \beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_3x_3 + \beta_{12}x_1x_2 + \beta_{13}x_1x_3 + \beta_{23}x_2x_3 + \beta_{11}x_1^2 + \beta_{22}x_2^2 + \beta_{33}x_3^2 \quad (3.1)$$

where y is the response, x_1, x_2, x_3 are the coded levels of experimental variables, β_0 is model constant, the terms β_1x_1, β_2x_2 and β_3x_3 represent main effects of experimental variables, $\beta_{12}x_1x_2, \beta_{13}x_1x_3$ and $\beta_{23}x_2x_3$ are interaction terms and $\beta_{11}x_1^2, \beta_{22}x_2^2$ and $\beta_{33}x_3^2$ are quadratic terms. Polynomial models proposed in this study are shown below:

$$y = 0.965 - 0.398x_1 - 0.242x_2 + 0.219x_3 + 1.380x_1x_2 + 0.655x_1x_3 + 1.084x_2x_3 + 1.234x_1^2 + 2.512x_2^2 + 1.040x_3^2 \quad (3.2)$$

$$y = 1.255 - 0.345x_1 - 0.425x_2 + 0.038x_3 - 0.181x_1x_2 - 0.259x_1x_3 + 0.216x_2x_3 - 0.029x_1^2 - 0.336x_2^2 + 20.095x_3^2 \quad (3.3)$$

In equation 3.2, the response y represents the total FAME amount whereas in equation 3.3 y expresses the C16:0 mass fraction.

Statistical analysis of polynomial model resulted in low R^2 and adjusted R^2 values indicating inadequacy of model. Low R^2 value indicates that variance of response (dependent variable) poorly explained by the variance of independent variables. It can be increased by adding more terms in the mathematical model. On the other hand, adjusted R -squared value decreases if added term does not improve the model. Adjusted R^2 value is related with the number of independent variables in the model and it shows correlation of response with an independent variable (IBM Knowledge Center, 2018). In addition, p value was higher than 0.05, which meant that model was not significant. However, Kriging model resulted in R^2 value of 1. It indicated that Kriging model was suitable to express relationship between experimental variable and responses of the system. Comparison of R^2 and adj- R^2 obtained from two models are given in Table 3.10.

Table 3.10. Comparison of R^2 values for polynomial and Kriging models

	Polynomial Model	Kriging Model
R^2	0.497	1.000
Adj- R^2	-0.409	1.000

Kriging model cannot be expressed like a polynomial equation; it has more complex mathematical expression. It involves a deterministic part $\mu(x)$, which is a generalized linear model, and a stochastic part $Z(x)$ as shown below:

$$Y(x) = \mu(x) + Z(x) \quad (3.2)$$

Kriging model is considered as an exact predictor model for highly nonlinear systems. Nonlinearity of our system can be the reason of inadequacy of proposed polynomial model. In this case, responses were dependent to glucose, glycerol and NaCl concentration but they could also be affected by other environmental factors such as small deviations of temperature, light intensity, agitation rate and pH. A drawback of Kriging model is low accuracy of extrapolation out of experimental range. However, our goal is to find an optimum point within the experimental range, so the model provide exact prediction of unknown response.

Some optimization studies were performed in order to define the best medium composition or best environmental factors for maximization of microalgal cell growth and cellular components. Plackett-Burman design, Box-Behnken design and CCD of response surface methodology were reported to be common methods for experimental design. Although experimental variables and responses were different, polynomial models were proposed for similar studies. Generally, responses were selected as cell dry weight, biomass productivity, lipid content or starch content in those studies. On the other hand, optimization of total FAME amount as TAG source and fatty acid profile of algal oil should be investigated, too (Isleten-Hosoglu et al., 2012, Kong et al., 2012, Ho et al., 2013, Isleten-Hosoglu et al., 2013, Yang et al., 2014).

In the presented study total amount of FAMES (mg) and palmitic acid (C16:0) mass fraction were chosen of responses due to their importance for economics and quality of biodiesel, respectively. Glucose, glycerol and NaCl concentrations were experimental

variables because there were several reports about their ability to change metabolic pathways separately (Cheirsilp and Torpee, 2012, Isleten-Hosoglu et al., 2012, An et al., 2013,).

“Design and Analysis of Computer Experiments (DACE)” software package were used in order to fit Kriging model on Matlab. Global optimization process was applied to find global minima point. Interior point algorithm which is a widely preferred iterative method was used to solve optimization problem. Medium optimization process using interior point algorithm resulted in two different global optima points for two responses. Table 3.11 shows the optimum conditions for total FAME amount and C16:0 mass fraction. Model was predicted the highest total FAME amount of 2.6169 mg in the growth medium including 0.9% NaCl, 0.8% glucose and 0.9424% glycerol and maximum mass fraction of C16:0 was predicted to be 39.76 % in medium contains 0.5% NaCl, 1% glucose and 1 % glycerol.

In the presented study, optimum glucose and glycerol concentrations were close to maximum glucose and glycerol concentrations in the experimental domain. Kong et al. reported that 2 g/L (0.2%) glucose and 10 g/L (1%) glycerol supplement in the absence of NaCl provided the highest lipid content of *C. vulgaris* (Kong et al.2013). In a previous study, FAME yield of *C.vulgaris* OW-01 was increased from 0.03 g/L to 0.06 g/L with the addition of 0.5% glucose into BG-11 medium (Park et al., 2014). In the presented study, maximum total FAME amount was predicted to be 2.6169 mg in 150 mL growth medium, which was equal to 0.017 g/L.

Table 3.11. Optimum compositions of growth medium for maximization of total FAME (mg) and C16:0 mass fraction

X ₁ (% w/w)	X ₂ (% w/w)	X ₃ (% w/w)	Predicted Response	
			Total FAME amount (mg)	C16:0 mass fraction (% TFA)
0.9000	0.8000	0.9424	2.6169 mg	-
0.5000	1.0000	1.0000	-	39.76%

FAME production of *C. vulgaris* SAG 211-12 was highest at 0.9% NaCl concentration that was a moderate level in the range of 0.5%-2.5%. In previous report of Pandit P.R. et al. (2017), it is showed that when NaCl concentration was increased from 0.06 M to 0.4 M (equal to 2.338%) in BG-11 growth medium of *C. vulgaris*, enhancement of total lipid content was observed (Pandit et al., 2017).

According to the optimization results, it was predicted that when NaCl concentration is maintained at lowest concentration of experimental range (0.5%) and glucose and glycerol concentration increased up to 1.0%, C16:0 content reached the highest value of 39.76%. Table 3.12 represents the comparison of highest predicted value of C16:0 content observed in this study with results of previous studies. It is shown that, *C. vulgaris* SAG 211-12, which was grown with the synergistic effect of NaCl, glucose and glycerol, accumulated high amount of C16:0.

Table 3.12. Comparison of palmitic acid content (% TFA) in previous studies and this study

Microalgal Strain	Palmitic Acid Content (% TFA)	References
<i>C. vulgaris</i> SAG 211-12	39.76	In this study
<i>C. vulgaris</i> SAG 211-12	29.2	Sarayloo et al., 2018
<i>C. vulgaris</i>	32.30	Kong et al., 2012
<i>C. saccharophila</i>	24.20	Isleten-Hosoglu et al., 2012
<i>C. sorokiniana</i>	43.60	Ngangkham et al., 2012
<i>Ettlia texensis</i>	25.00	Isleten-Hosoglu et al., 2013
<i>Chlamydomonas</i> sp ICE-L	16.80	An et al., 2013
<i>Scenedesmus</i> sp.	30.77	Yang et al., 2014
<i>C. protothecoides</i>	20.90	Wang et al., 2016
<i>C. vulgaris</i>	37.00	Pandit et al., 2017
<i>C. vulgaris</i>	15.79	Heo et al., 2017
<i>A. obliquus</i>	54.00	Pandit et al., 2017

4. CONCLUSIONS

Biodiesel is regarded as a solution for increasing energy demand and environmental concerns. Several raw materials have been investigated in order to provide lipid, especially TAG for biodiesel production. Plant seeds are considered as first-generation feedstocks while animal fats and waste cooking oil are second-generation biodiesel feedstocks. Microalgal oil is emerged as third generation biodiesel feedstock. Microalgae have advantage of rapid growth and growth without land requirement.

Low production cost and required quality should be fulfilled for commercialization of biodiesel. Inducement of TAG accumulation of microalgae cells is a feasible way to reduce the production cost. Total TAG accumulation is related with both growth and lipid content of cells. Highest cell growth rate of 0.635 d^{-1} was obtained at 0.9% NaCl, 0.8% glucose and glycerol concentration.

Fatty acid composition of algal oil affects the quality of biodiesel. Many different ways have been applied to increase lipid production and manipulate the lipid profiles of microalgae cells. Studies generally have focused on the inducement of total lipid production. However, neutral lipids (TAGs) are preferred for biodiesel production. In the present study, instead of total lipid accumulation TAG accumulation of microalgae *C. vulgaris* was investigated. According to the presented study, addition of NaCl, glucose and glycerol into the growth medium can be used for the alteration of both total amount of microalgal fatty acids and profile of fatty acids. Total fatty acid accumulation reached to 2.571 mg in the medium in which highest growth rate was obtained. In addition, maximum total FAME yield of 8.521 mg/g DCW was observed in the medium including 0.9% NaCl, 0.3% glucose and glycerol.

The highest C16:0 ratio (40.67%) among total fatty acids was obtained with supplement of NaCl and carbon sources as 0.9, 0.8, 0.8%, respectively. On the other hand, for C18:0 maximum mass fraction was obtained at 0.6% glucose and glycerol and high concentration of NaCl of 2.5%. Optimization study results showed that low NaCl (0.5%), high glucose (1.0%) and glycerol (1.0%) supplement is required to obtain maximum C16:0 ratio.

In literature, there have been reports about two-stage cultivation method, which has been applied to induce growth in the first stage and induce lipid accumulation in the second stage. As a future work, the presented system can be designed as two staged cultivation system to induce growth and produce higher amount of TAG than that produced in the presented system. Glucose and glycerol together can be supplied for growth stage whereas NaCl stress can be applied in lipid production stage.

As a conclusion, *C. vulgaris* SAG 211-12 cultivated in the presence of NaCl, glucose and glycerol has a feasible fatty acid profile for biodiesel production with high saturated and monounsaturated fatty acid ratio. On the other hand, FAME yield of cells should be improved in the present circumstances. When total FAME yield of *C. vulgaris* SAG 211-12 is increased, it will be a promising feedstock for biodiesel production with high growth rate and suitable fatty acid profile. Furthermore, in the scope of biorefinery concept after lipids are extracted residual *C. vulgaris* SAG 211-12 biomass also can be used as a raw material for bioethanol production due to the starch content.

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APPENDIX A

Growth Profile of *C. vulgaris* SAG 211-12

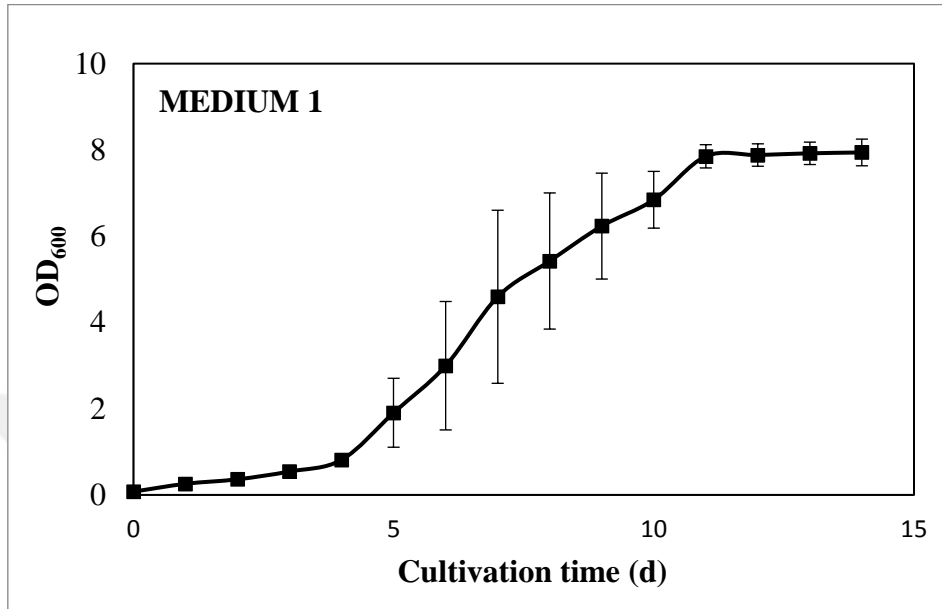


Figure A.1. Growth profile of *C. vulgaris* SAG 211-12 in M1 (BG-11 medium supplemented with a) 0.5 % NaCl, 0.6% glucose and 0.6% glycerol) Error bars, \pm SD.

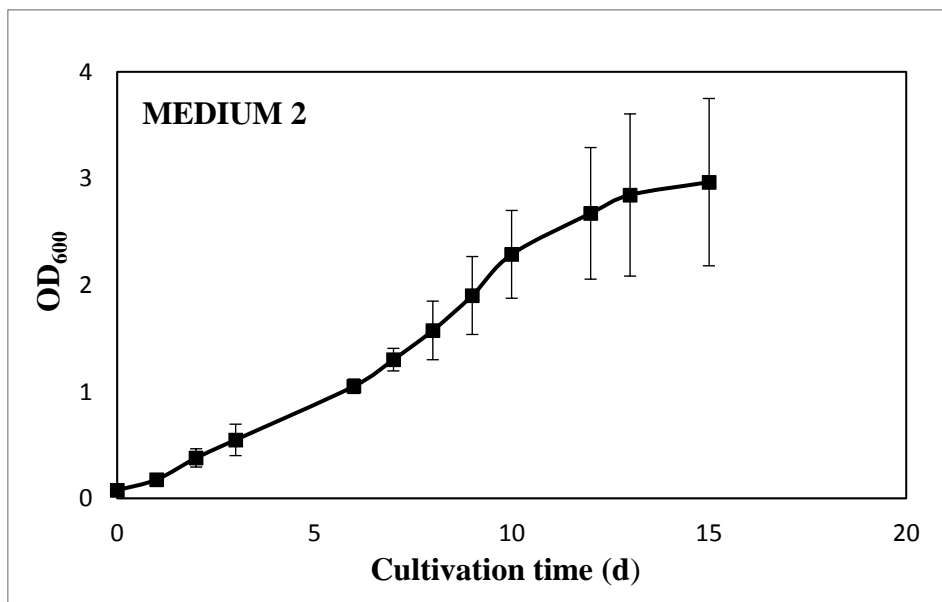


Figure A.2. Growth profile of *C. vulgaris* SAG 211-12 in M2 (BG-11 medium supplemented with 0.9 % NaCl, 0.3 % glucose and 0.3 % glycerol). Error bars, \pm SD.

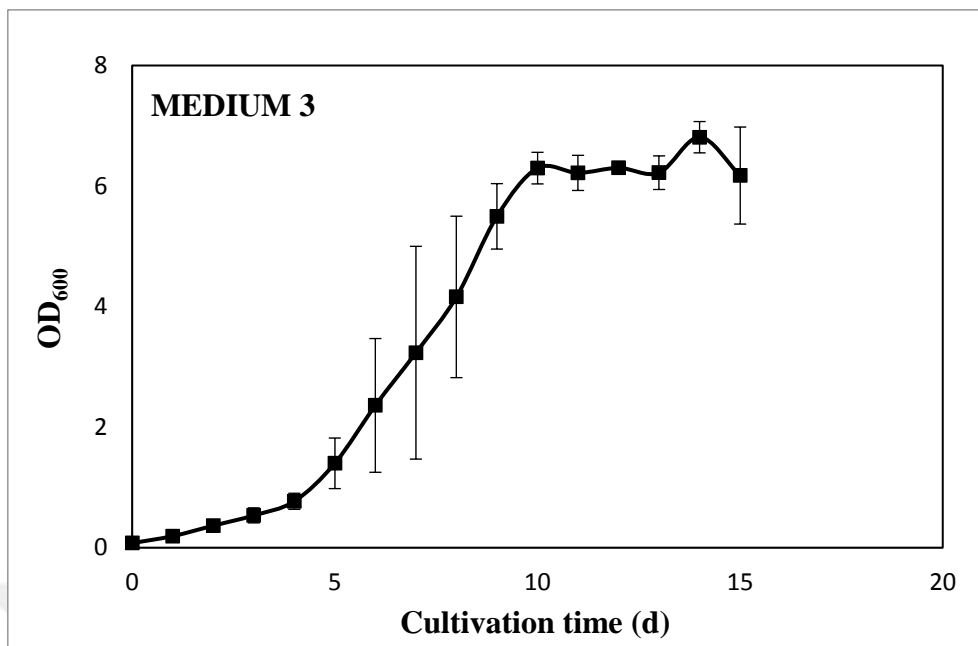


Figure A.3. Growth profile of *C. vulgaris* SAG 211-12 in M3 (BG-11 medium supplemented with 0.9 % NaCl, 0.3 % glucose and 0.8 % glycerol) Error bars, \pm SD.

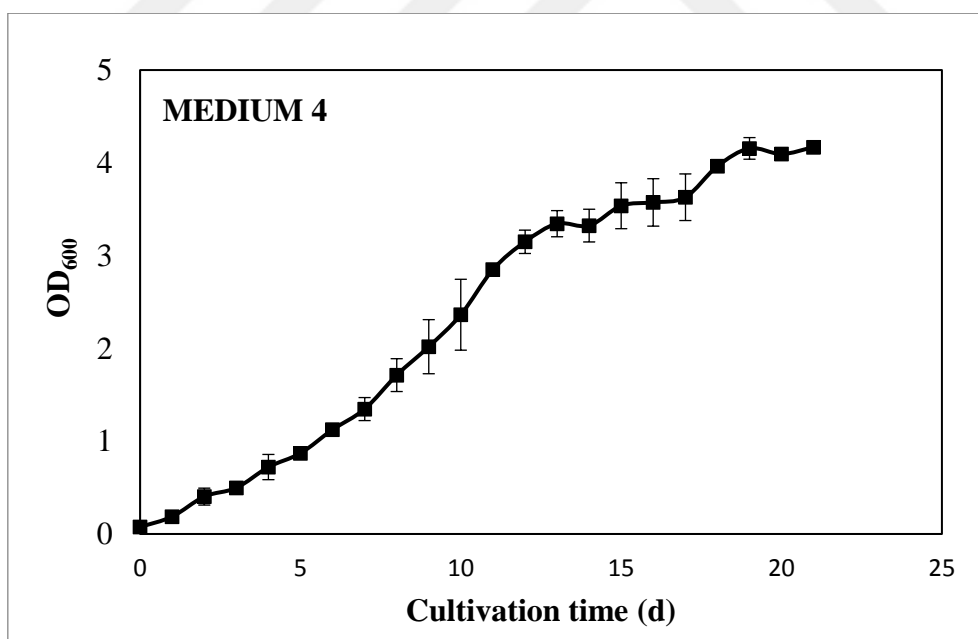


Figure A.4. Growth profile of *C. vulgaris* SAG 211-12 in M4 (BG-11 medium supplemented with 0.9 % NaCl, 0.8 % glucose and 0.3 % glycerol) Error bars, \pm SD.

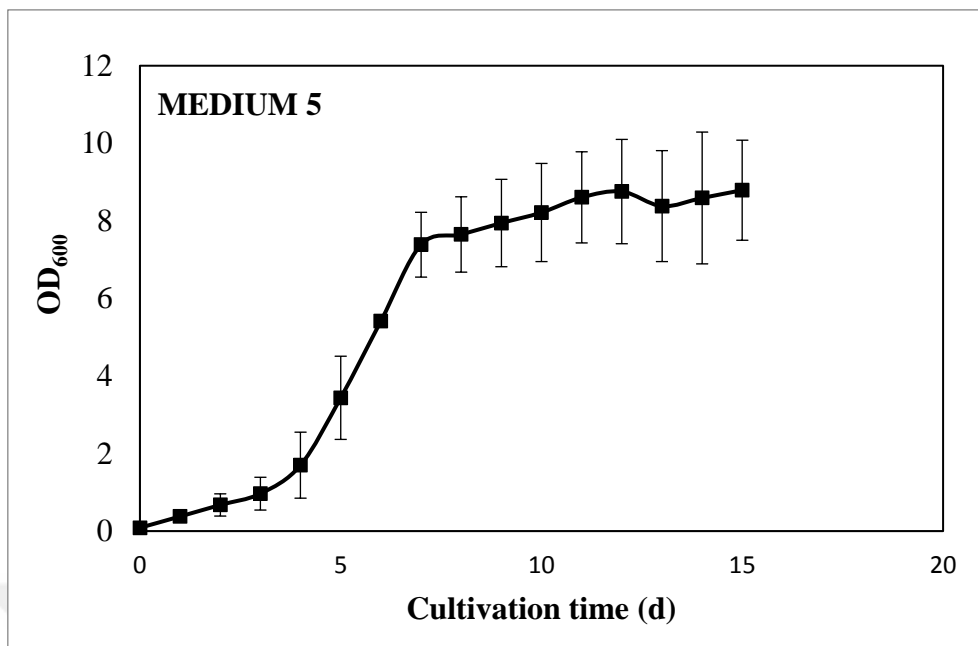


Figure A.5. Growth profile of *C. vulgaris* SAG 211-12 in M5 (BG-11 medium supplemented with 0.9 % NaCl, 0.8 % glucose and 0.8 % glycerol) Error bars, \pm SD.

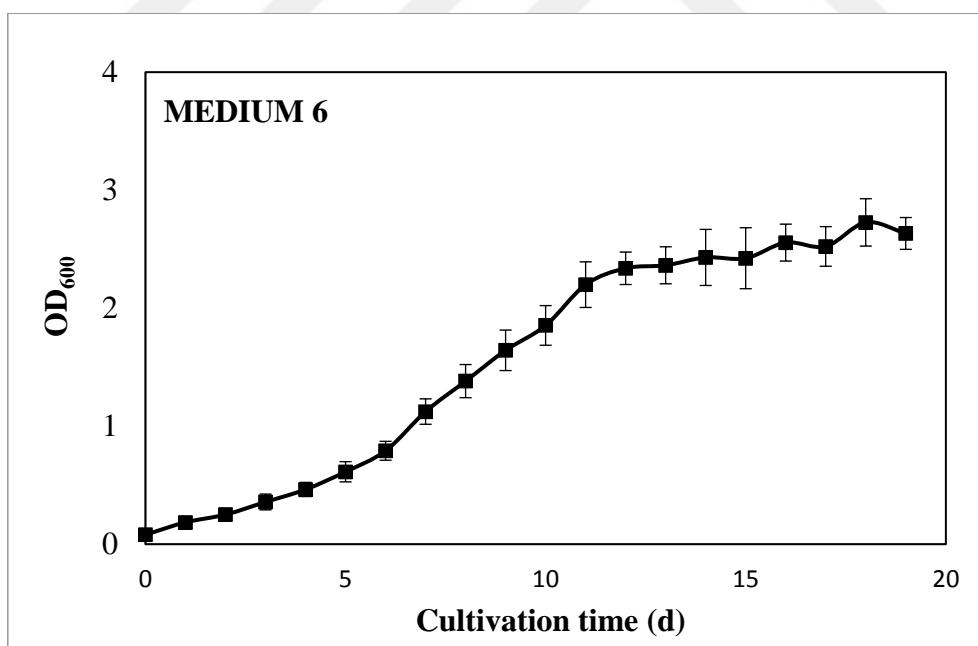


Figure A.6. Growth profile of *C. vulgaris* SAG 211-12 in M6 (BG-11 medium supplemented 1.5 % NaCl, 0.1 % glucose and 0.6 % glycerol) Error bars, \pm SD.

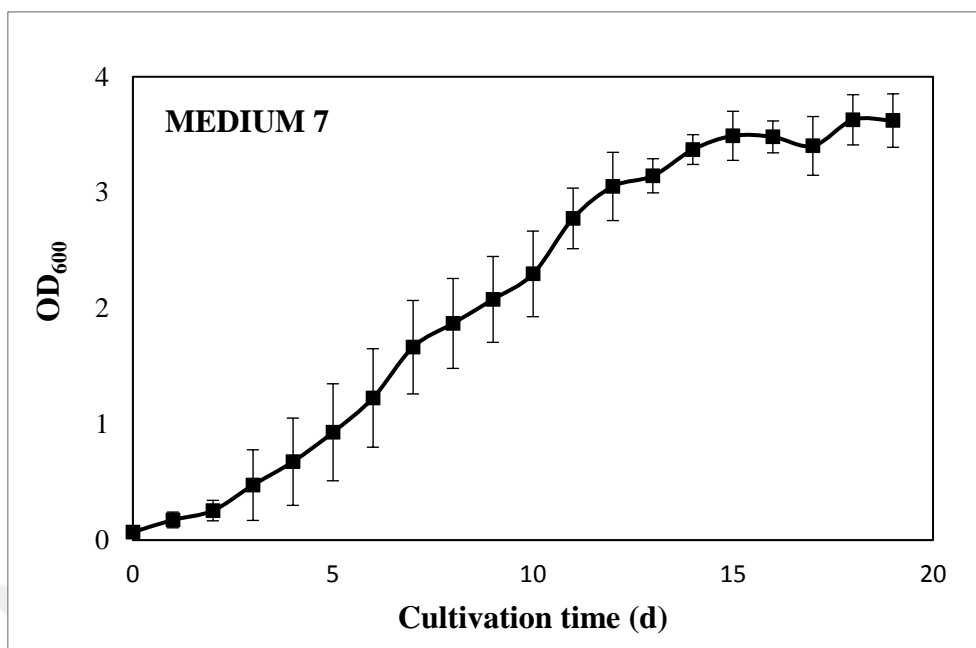


Figure A.7. Growth profile of *C. vulgaris* SAG 211-12 in M7 (BG-11 medium supplemented 1.5 % NaCl, 0.6 % glucose and 0.1 % glycerol) Error bars, \pm SD.

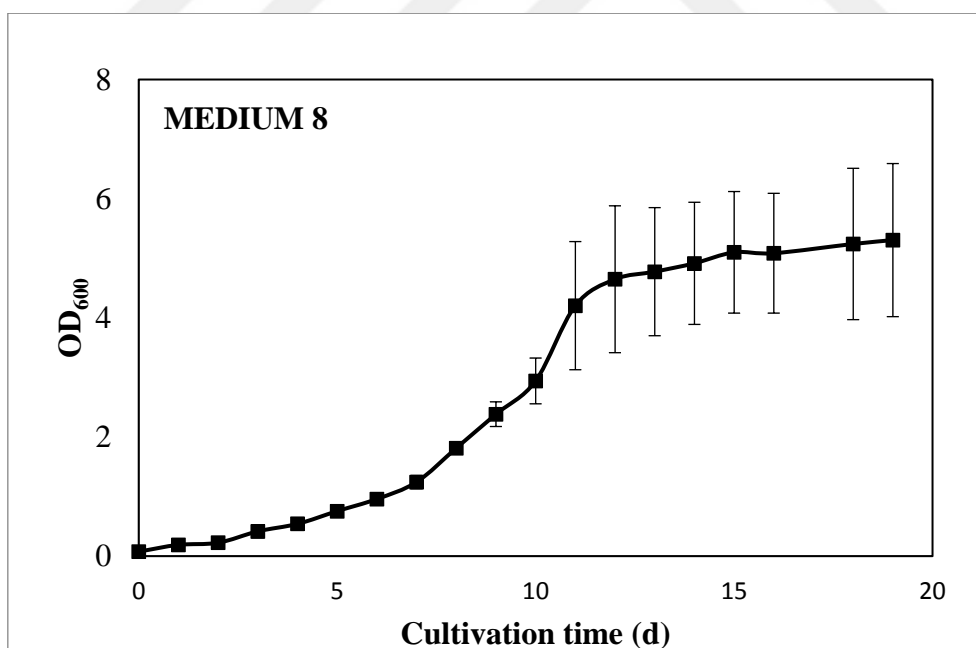


Figure A.8. Growth profile of *C. vulgaris* SAG 211-12 in M8 (BG-11 medium supplemented with 1.5 % NaCl, 0.6 % glucose and 1 % glycerol) Error bars, \pm SD.

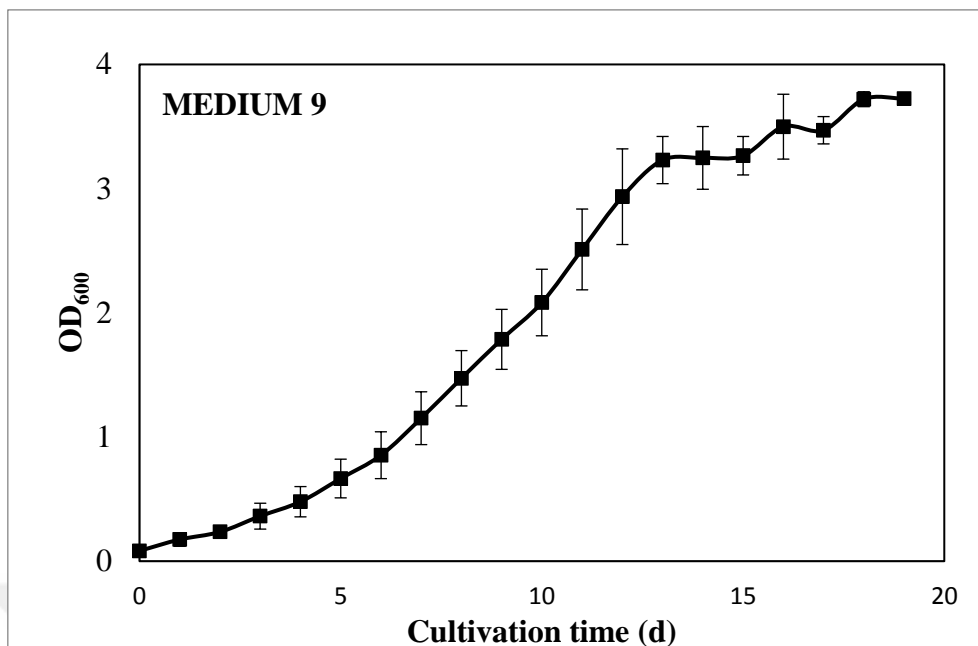


Figure A.9. Growth profile of *C. vulgaris* SAG 211-12 in M9 (BG-11 medium supplemented with 1.5 % NaCl, 0.6 % glucose and 0.6 % glycerol). Error bars, \pm SD.

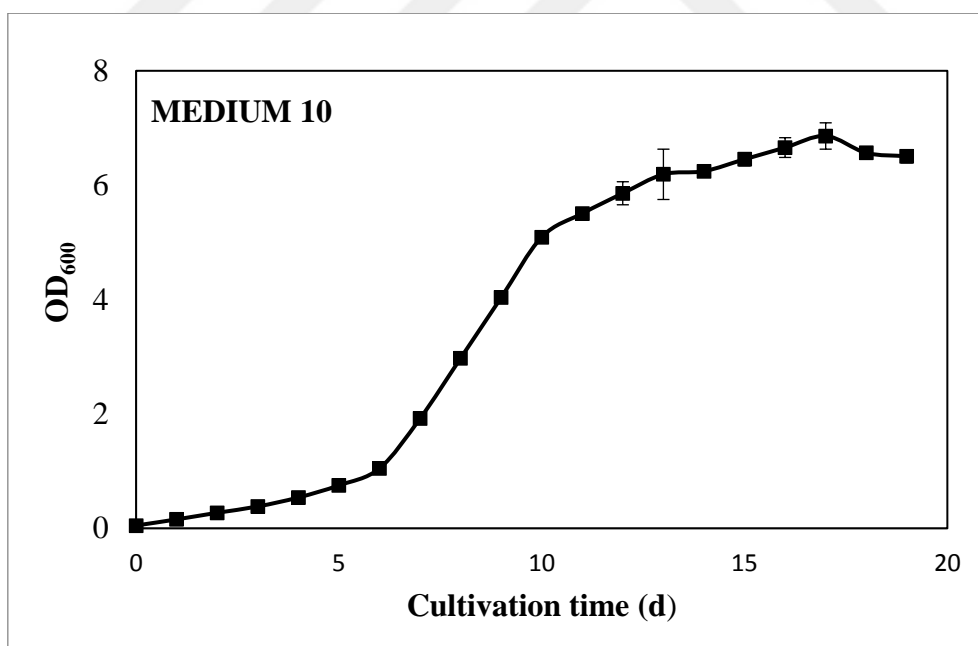


Figure A.10. Growth profile of *C. vulgaris* SAG 211-12 in M10 (BG-11 medium supplemented with 1.5 % NaCl, 1 % glucose and 0.6 % glycerol). Error bars, \pm SD.

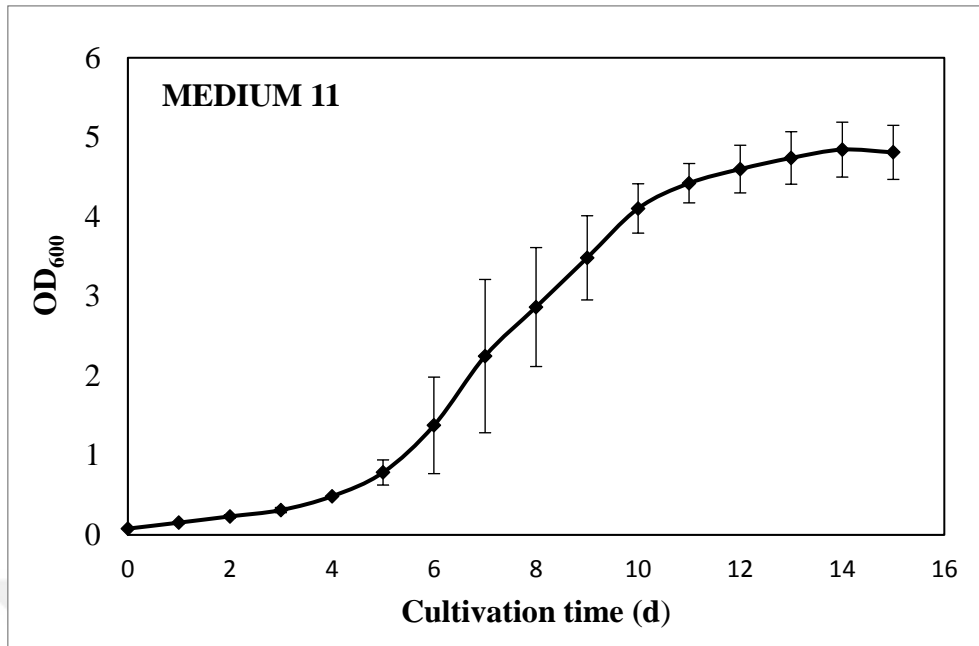


Figure A.11. Growth profile of *C. vulgaris* SAG 211-12 in M11 (BG-11 medium supplemented with 2 % NaCl, 0.3 % glucose and 0.3 % glycerol). Error bars, \pm SD.

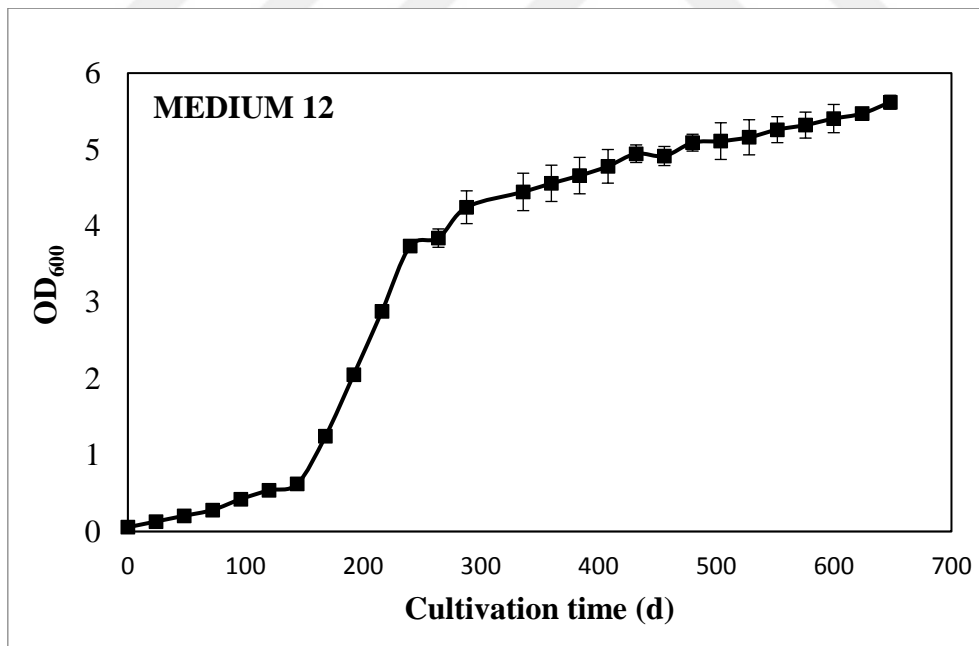


Figure A.12. Growth profile of *C. vulgaris* SAG 211-12 in M12 (BG-11 medium supplemented with 2 % NaCl, 0.3 % glucose and 0.8 % glycerol). Error bars, \pm SD.

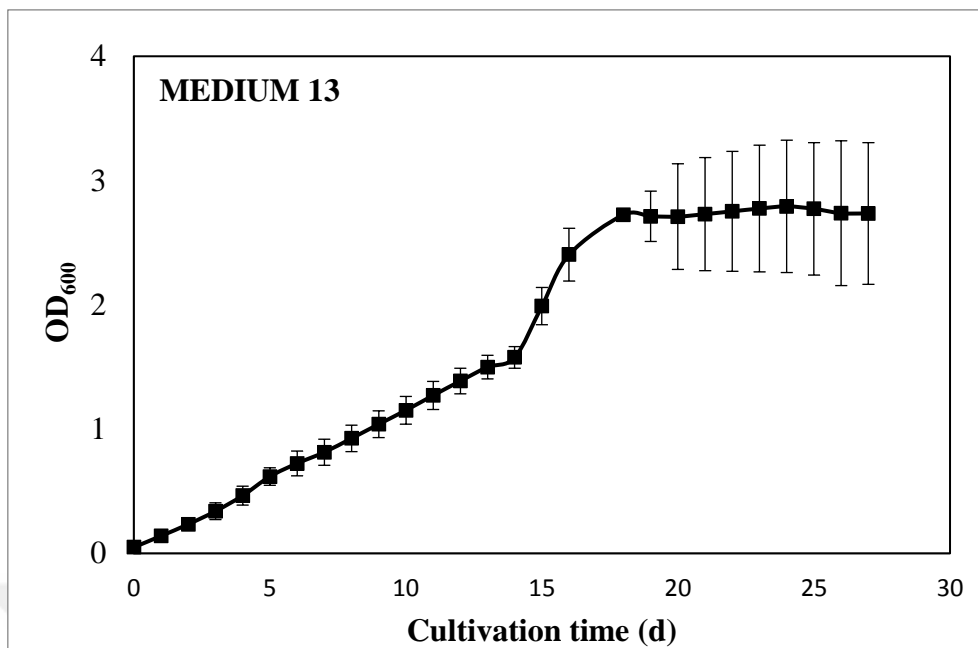


Figure A.13. Growth profile of *C. vulgaris* SAG 211-12 in M13 (BG-11 medium supplemented with 2 % NaCl, 0.8 % glucose and 0.3 % glycerol). Error bars, \pm SD.

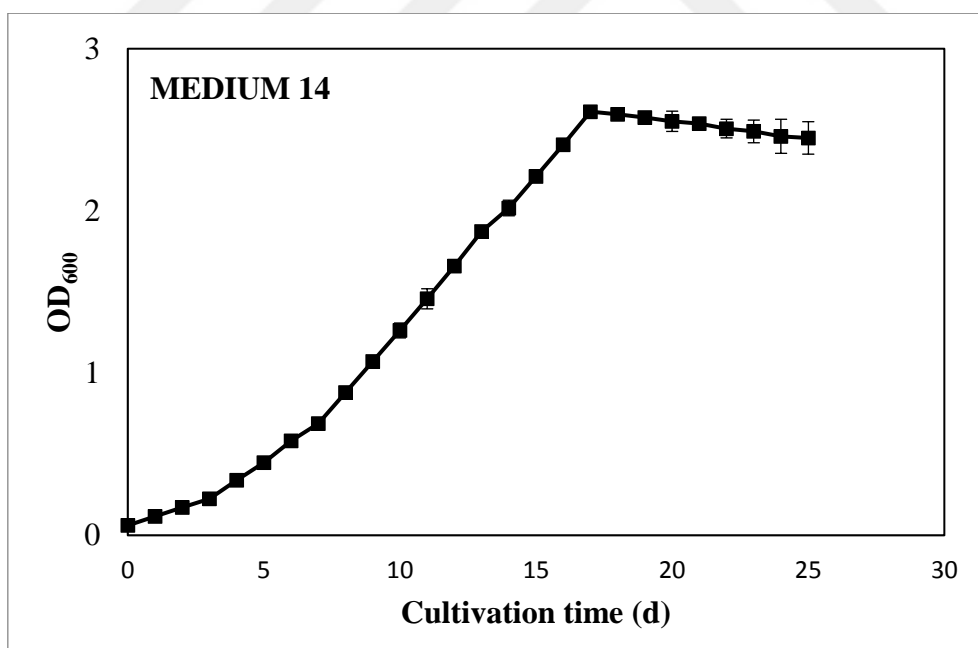


Figure A.14. Growth profile of *C. vulgaris* SAG 211-12 in M14 (BG-11 medium supplemented with 2 % NaCl, 0.8 % glucose and 0.8 % glycerol). Error bars, \pm SD.

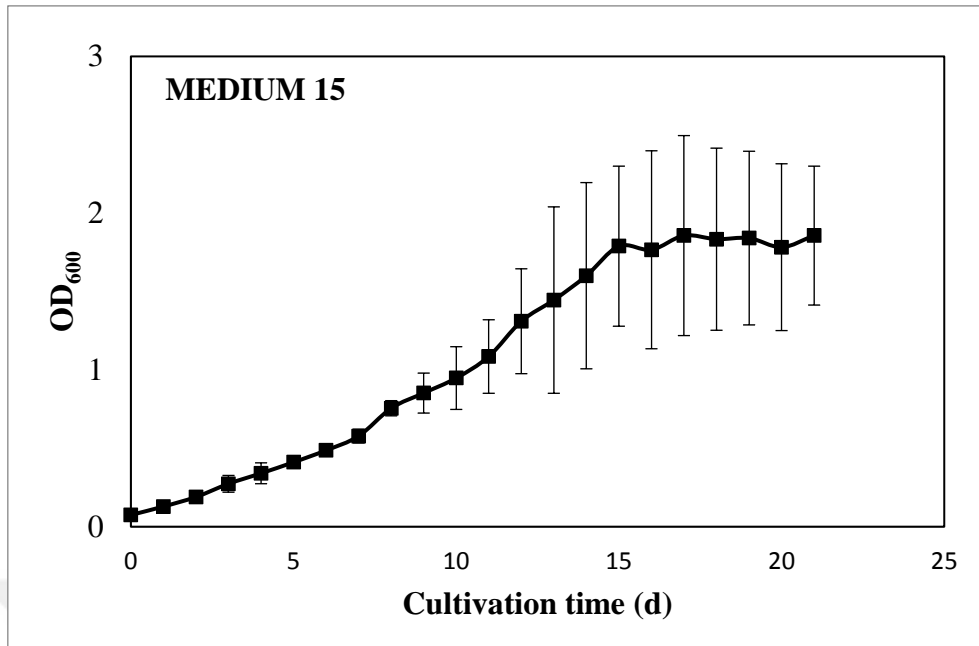


Figure A.15. Growth profile of *C. vulgaris* SAG 211-12 in M15 (BG-11 medium supplemented with 2.5 % NaCl, 0.6% glucose and 0.6% glycerol). Error bars, \pm SD.

APPENDIX B

Amount of FAMES (mg) and FAMES Yield (mg/g DCW) of *C. vulgaris* SAG 211-12 at Different NaCl, Glucose and Glycerol Concentrations

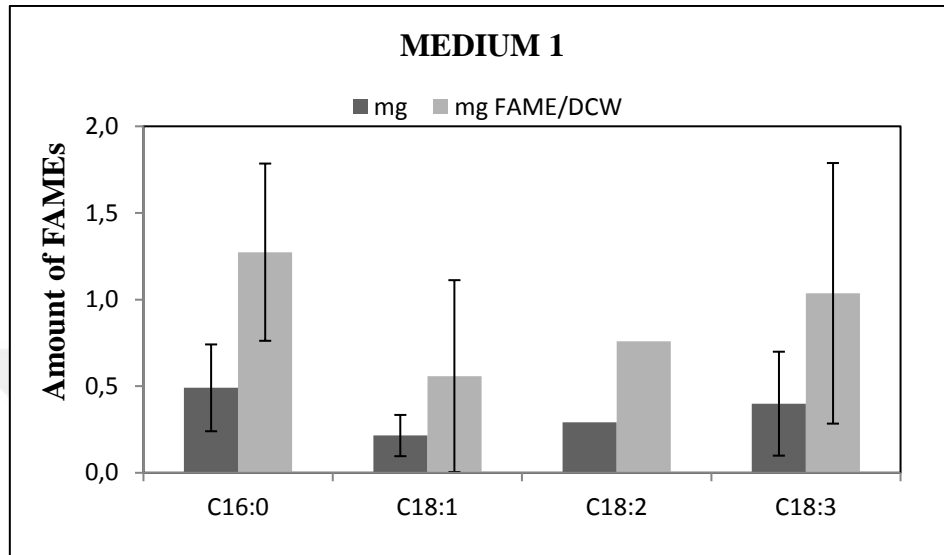


Figure B.1. Amount of fatty acid (mg) and fatty acid yield (mg/g DCW) of *C. vulgaris* SAG 211-12 in M1 (BG11 medium supplemented with 0.5% NaCl, 0.6% glucose and 0.6% glycerol). Error bars, \pm SD.

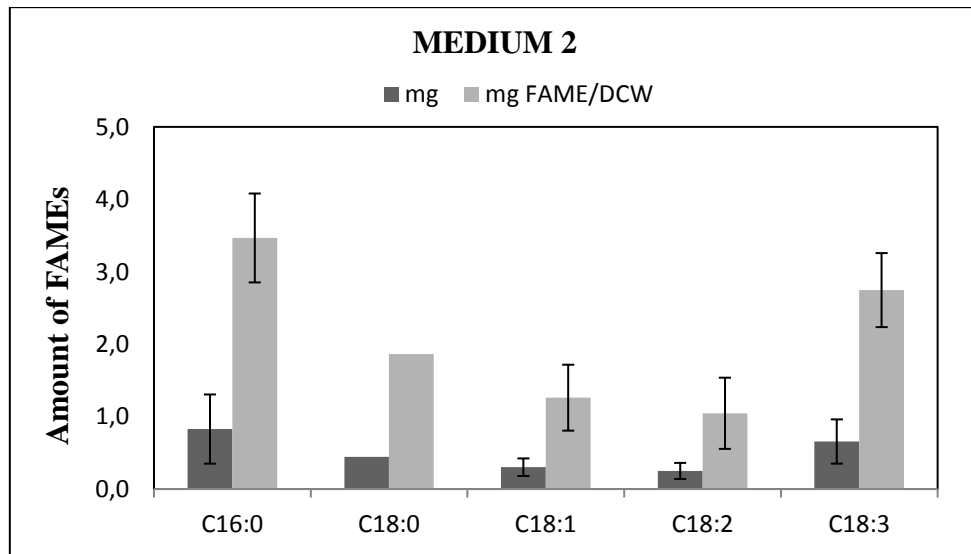


Figure B.2. Amount of fatty acid (mg) and fatty acid yield (mg/g DCW) of *C. vulgaris* SAG 211-12 in M2 (BG-11 medium supplemented with 0.9 % NaCl, 0.3 % glucose and 0.3 % glycerol) Error bars, \pm SD.

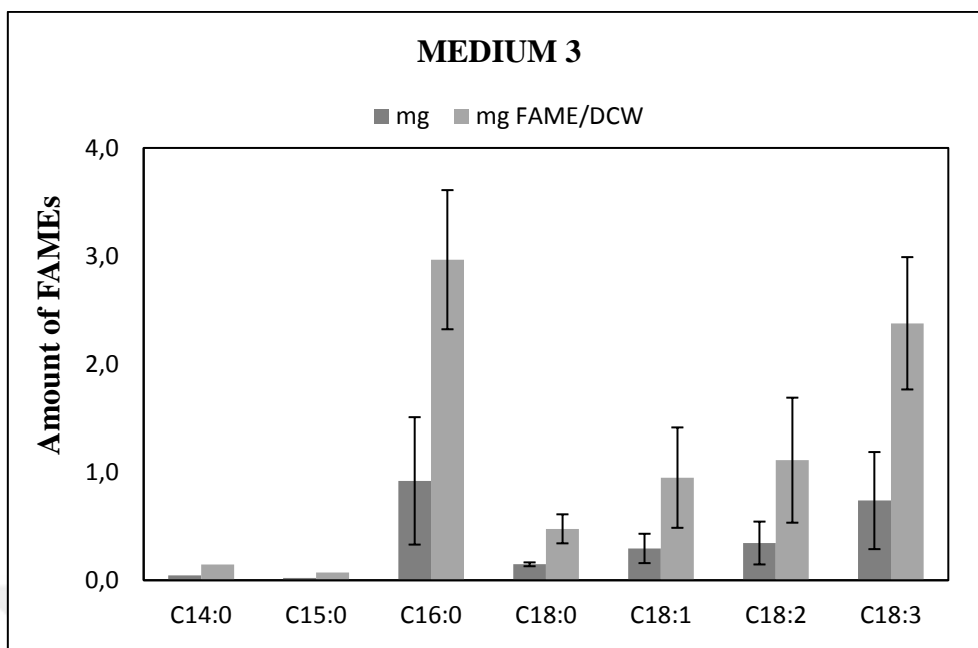


Figure B.3. Amount of fatty acid (mg) and fatty acid yield (mg/g DCW) of *C. vulgaris* SAG 211-12 in M3 (BG-11 medium supplemented with 0.9 % NaCl, 0.3 % glucose and 0.8 % glycerol). Error bars, \pm SD.

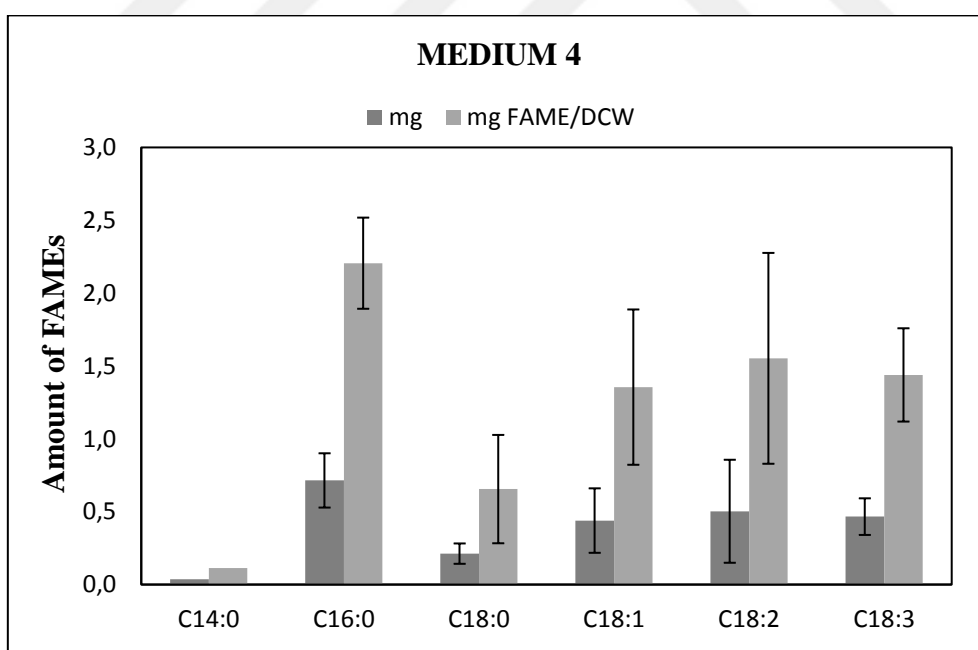


Figure B.4. Amount of fatty acid (mg) and fatty acid yield (mg/g DCW) of *C. vulgaris* SAG 211-12 in M4 (BG-11 medium supplemented with 0.9 % NaCl, 0.8 % glucose and 0.3 % glycerol). Error bars, \pm SD.

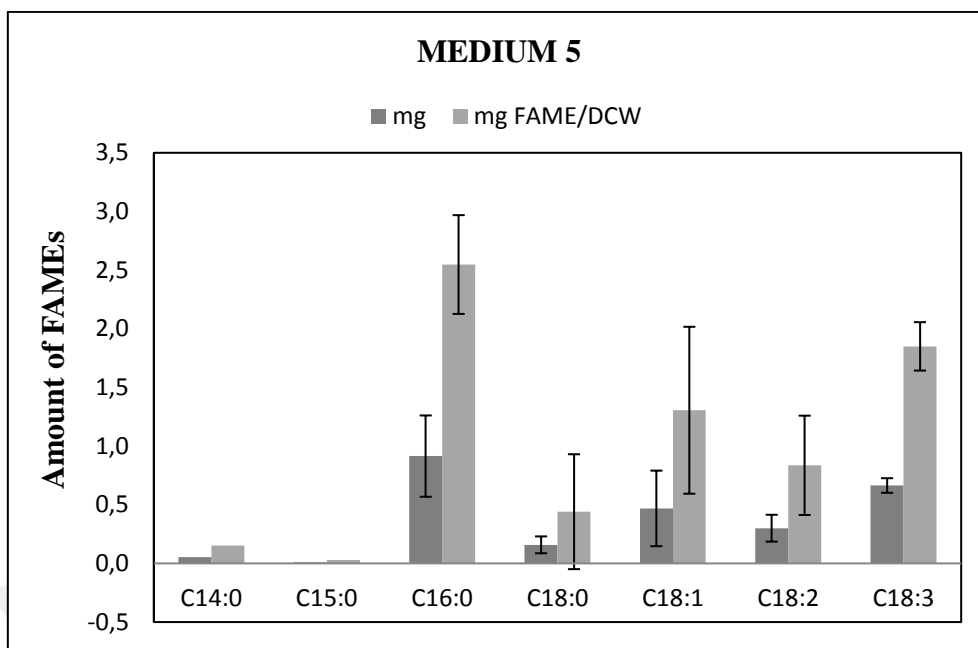


Figure B.5. Amount of fatty acid (mg) and fatty acid yield (mg/g DCW) of *C. vulgaris* SAG 211-12 in M5 (BG-11 medium supplemented with 0.9 % NaCl, 0.8 % glucose and 0.8 % glycerol) Error bars, \pm SD.

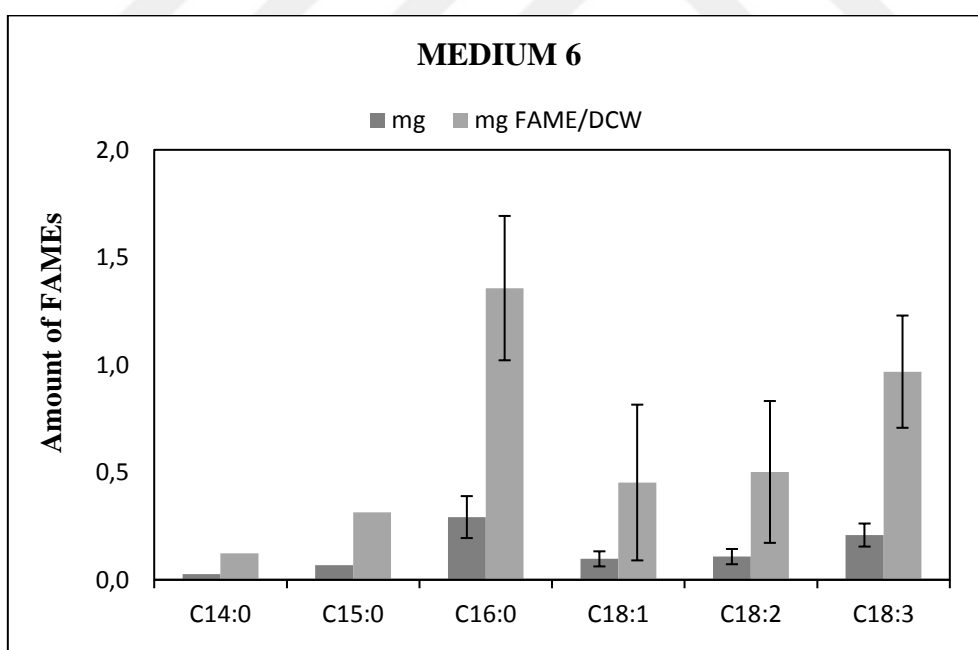


Figure B.6. Amount of fatty acid (mg) and fatty acid yield (mg/g DCW) of *C. vulgaris* SAG 211-12 in M6 (BG-11 medium supplemented 1.5 % NaCl, 0.1 % glucose and 0.6 % glycerol). Error bars, \pm SD.

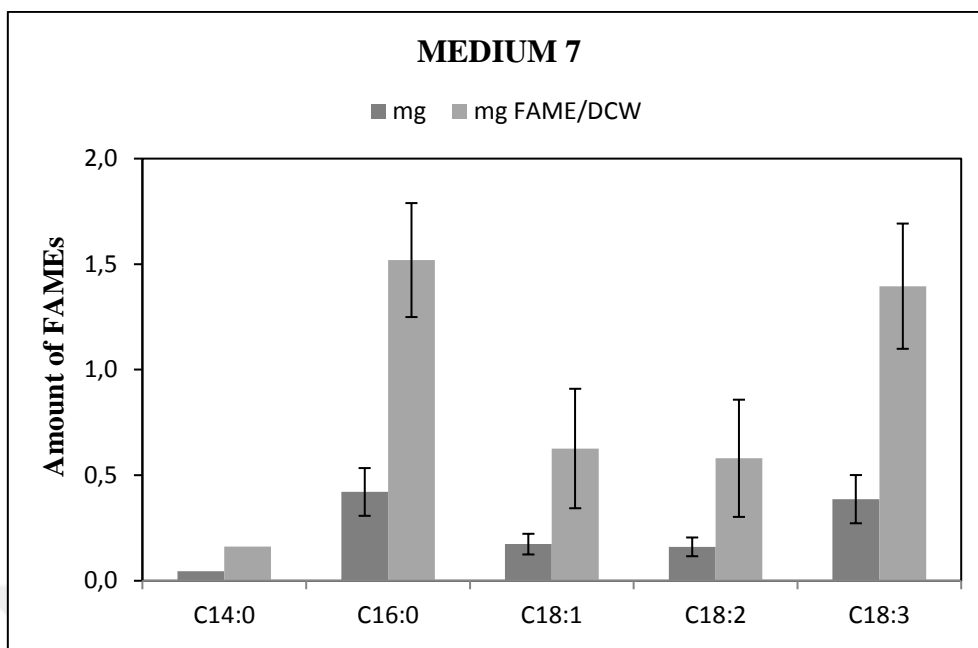


Figure B.7. Amount of fatty acid (mg) and fatty acid yield (mg/g DCW) of *C. vulgaris* SAG 211-12 in M7 (BG-11 medium supplemented 1.5 % NaCl, 0.6 % glucose and 0.1 % glycerol). Error bars, \pm SD.

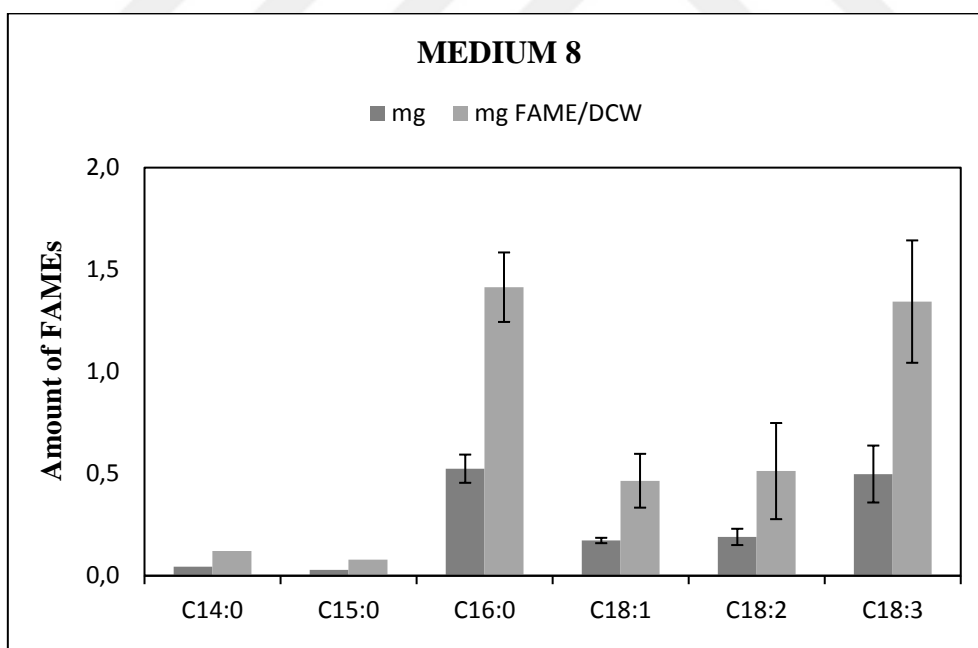


Figure B.8. Amount of fatty acid (mg) and fatty acid yield (mg/g DCW) of *C. vulgaris* SAG 211-12 in M8 (BG-11 medium supplemented with 1.5 % NaCl, 0.6 % glucose and 1 % glycerol). Error bars, \pm SD.

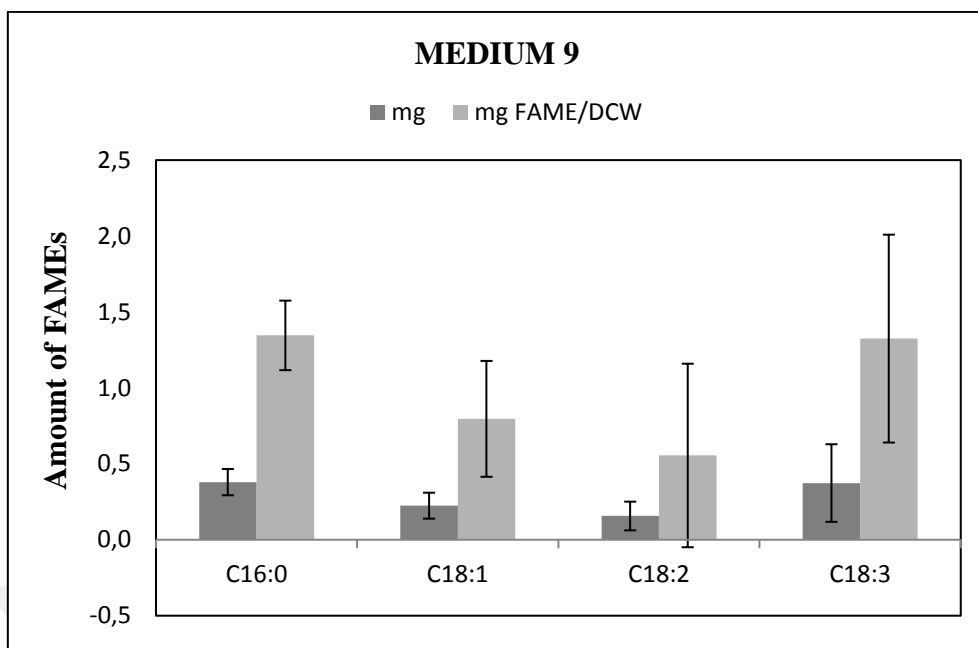


Figure B.9. Amount of fatty acid (mg) and fatty acid yield (mg/g DCW) of *C. vulgaris* SAG 211-12 in M9 (BG-11 medium supplemented with 1.5 % NaCl, 0.6 % glucose and 0.6 % glycerol). Error bars, \pm SD.

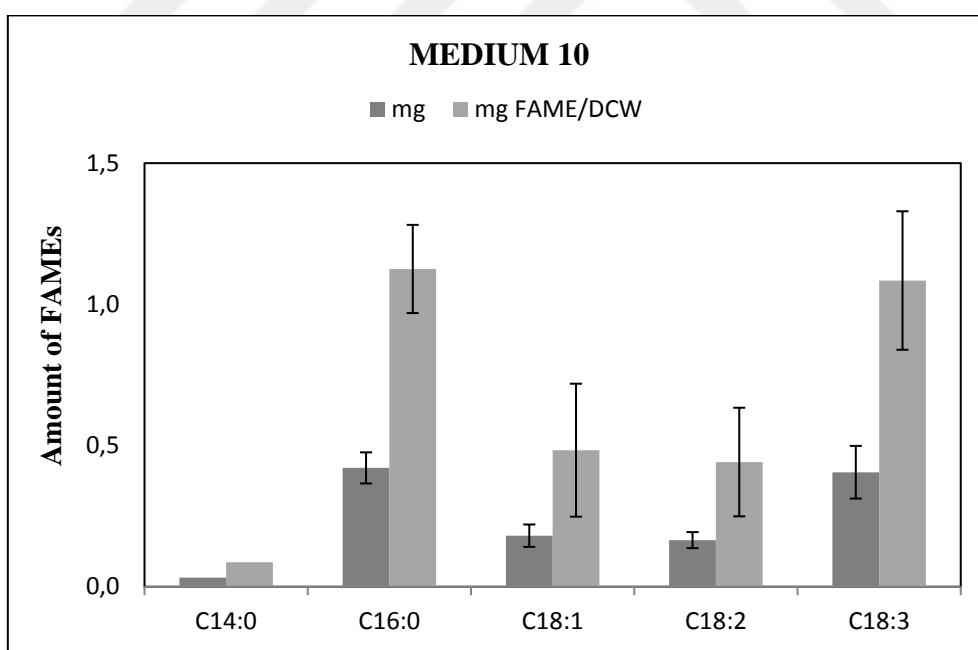


Figure B.10. Amount of fatty acid (mg) and fatty acid yield (mg/g DCW) of *C. vulgaris* SAG 211-12 in M10 (BG-11 medium supplemented with 1.5 % NaCl, 1 % glucose and 0.6 % glycerol). Error bars, \pm SD.

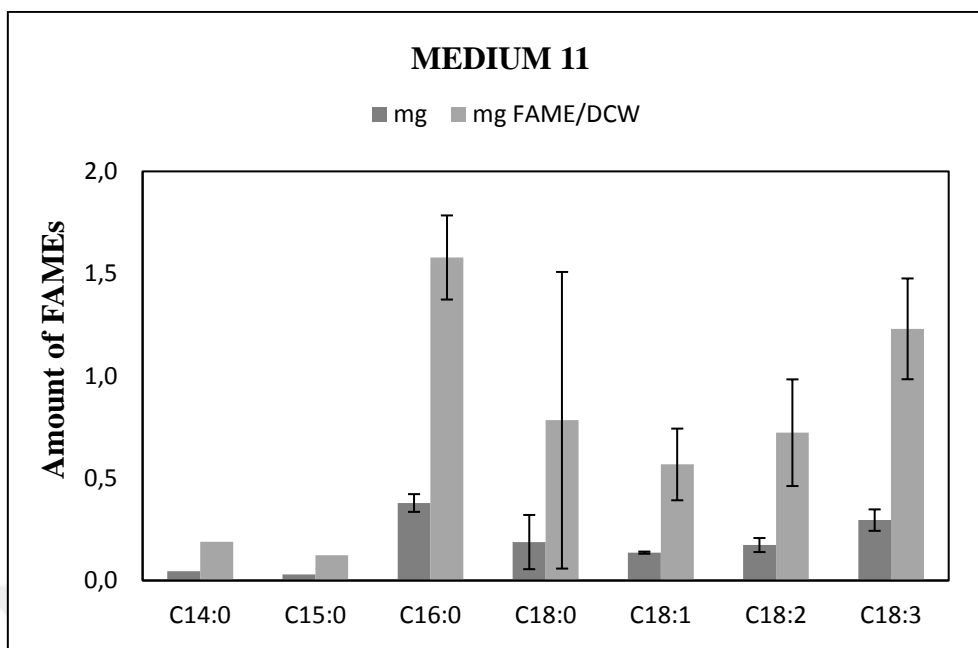


Figure B.11. Amount of fatty acid (mg) and fatty acid yield (mg/g DCW) of *C. vulgaris* SAG 211-12 in M11 (BG-11 medium supplemented with 2 % NaCl, 0.3 % glucose and 0.3 % glycerol). Error bars, \pm SD.

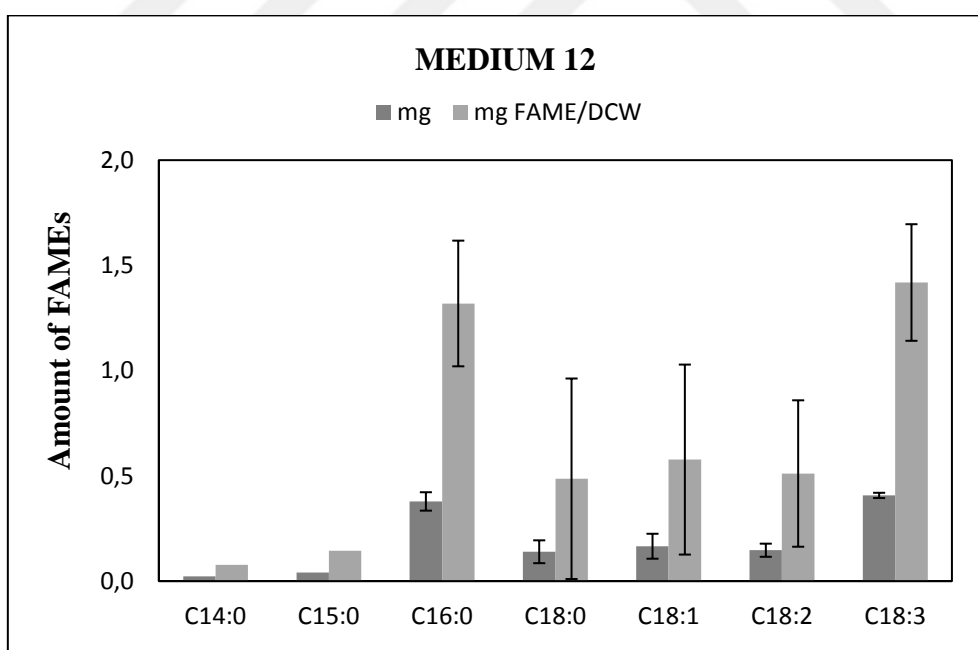


Figure B.12. Amount of fatty acid (mg) and fatty acid yield (mg/g DCW) of *C. vulgaris* SAG 211-12 in M12 (BG-11 medium supplemented with 2 % NaCl, 0.3 % glucose and 0.8 % glycerol). Error bars, \pm SD.

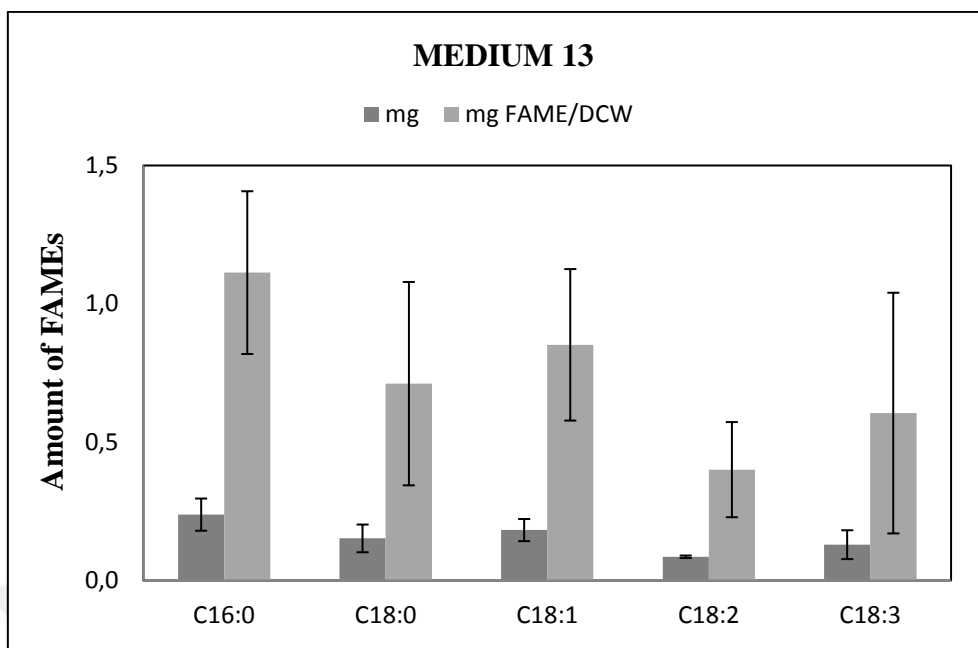


Figure B.13. Amount of fatty acid (mg) and fatty acid yield (mg/g DCW) of *C. vulgaris* SAG 211-12 in M13 (BG-11 medium supplemented with 2 % NaCl, 0.8 % glucose and 0.3 % glycerol). Error bars, \pm SD.

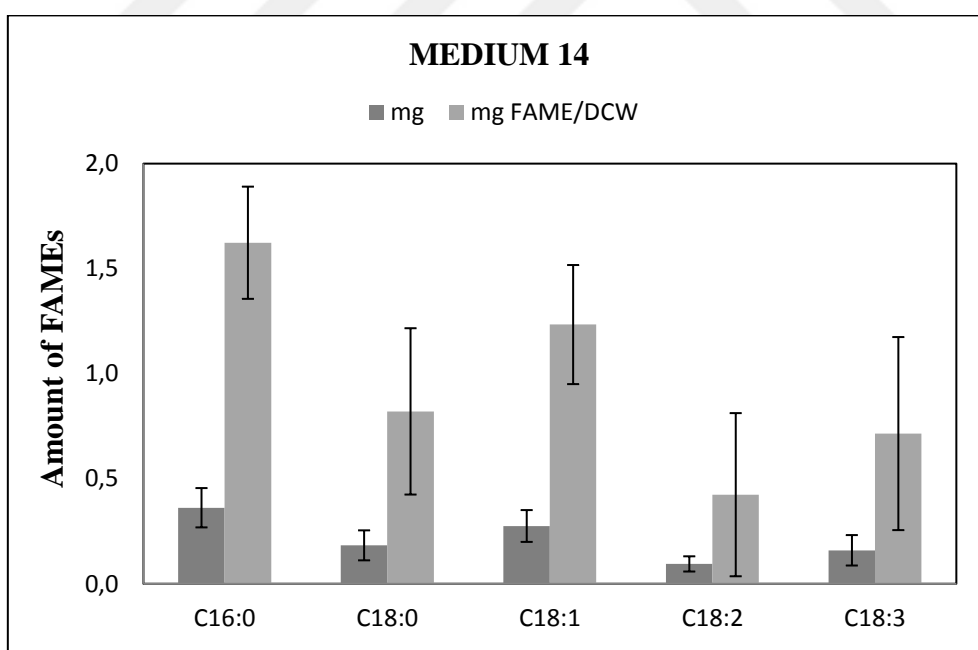


Figure B.14. Amount of fatty acid (mg) and fatty acid yield (mg/g DCW) of *C. vulgaris* SAG 211-12 in M14 (BG-11 medium supplemented with 2 % NaCl, 0.8 % glucose and 0.8 % glycerol). Error bars, \pm SD.

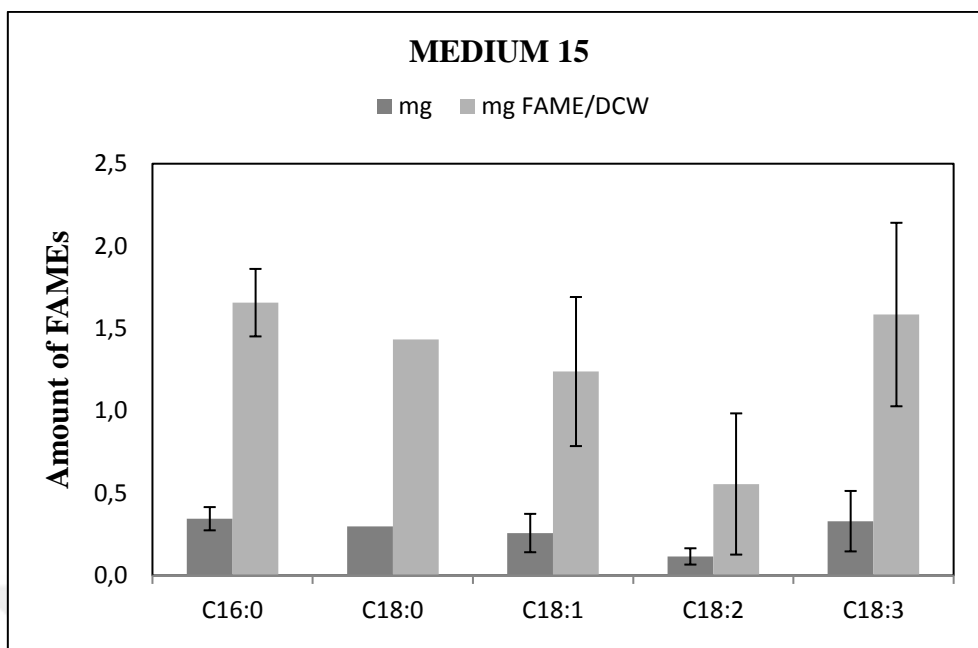


Figure B.15. Amount of fatty acid (mg) and fatty acid yield (mg/g DCW) of *C. vulgaris* SAG 211-12 in M15 (BG-11 medium supplemented with 2.5% NaCl, 0.6% glucose and 0.6% glycerol). Error bars, \pm SD.

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International Proceedings

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