Theoretical and Experimental Investigation of Supercritical Extraction of Triglycerides from Microalgae *Chlorella vulgaris* for Biodiesel Production

by

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Koç University

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ABSTRACT

Finite availability of fossil fuels and negative impacts of anthropogenic greenhouse gases emissions causing global warming have stimulated development of alternative resources for transportation fuels. Among alternatives, liquid biofuels such as biodiesel is attractive as they can be directly utilized in existing infrastructure. Recently, microalgae lipids has attracted increasing attention to be used for biodiesel resources thanks to superior properties of microalgae. They can be cultivated on non-arable land without competing with human food. Their lipid productivity is much higher than agricultural crops. Lipid extraction from microalgae is one of the steps affecting economic feasibility of biodiesel production from microalgae. Supercritical CO2 ($scCO_2$) extraction technology is considered as a promising alternative since it is environmentally friendly and prevents solvent contamination by enabling solvent free extract. In this study, $scCO_2$ extraction of high amount of lipid producing microalgae *Chlorella vulgaris* lipids was investigated in the pressure range from 200 to 400 bar and temperature range from 40 to 70 °C. Microalgae cultivation was performed with 27 liter flat panel type photobioreactor. Lipid productivity was tracked by Nile red method and it was found that highest lipid productivity was reached at early stationary phase. $ScCO_2$ extraction was compared with conventional Soxhlet extraction with n-hexane and comparable extraction yields were obtained. Moreover, it took far less time to reach same extraction yield with $scCO_2$. Increase in pressure at constant temperature and increase in temperature at constant pressure significantly improved $scCO_2$ extraction yield. Effluent lipid concentration curves showed a fast extraction period followed by a much slower extraction period. Sovova's model describing these periods as external mass transfer controlled and internal diffusion controlled was used to predict experimental data. Lipid solubility in $scCO_2$ at operating conditions was predicted from slope of the first part of the extraction curve. Predicted solubilities ranged between 2.7 and 9.0 mg lipid/g CO_2. Fluid and solid phase mass transfer coefficients regressed from experimental data were the order of 10^{-4} m/s and 10^{-8} m/s, respectively. Common correlations in mass transfer for forced convection

were used to predict fluid phase mass transfer coefficient. Best agreement was found with mass transfer correlation $Sh = 2 + 1.1 * Re^{0.6} * Sc^{1/3}$ which was developed for evaporation from drops. Fatty acid methyl esters (FAME) profiles obtained by transesterification were considerably affected by extraction method and operating conditions of $scCO_2$ extraction. No changes on FAME profiles were detected as a function of $scCO_2$ extraction time.

ÖZET

Fosil yakıtların tükenecek olması ve küresel ısınmaya sebep olan insan kaynaklı sera gazı emisyonlarının çevreye olumsuz etkileri ulaşım amaçlı kullanılan yakıtlara alternatif kaynaklar geliştirilmesini tetiklemiştir. Alternatifler arasında, mevcut altyapıda direkt olarak kullanılabilecek olan biyodizel gibi biyoyakıtlar ilgi çekmektedir. Son zamanlarda, biyodizel kaynağı olarak mikroalg lipitlerinin kullanılması, mikroalglerin üstün özellikleri sebebiyle büyük ilgi çekmektedir. Mikroalgler tarıma uygun olmayan arazilerde, insanların tükettikleri besinlerle rekabet etmeden yetiştirilebilirler. Mikroalglerin lipit verimleri tarımsal ürünlerden yüzlerce kat daha fazladır. Mikroalglerden lipit ekstraksiyonu, mikroalglerden biyodizel üretiminin ekonomik fizibilitesini etkileyen süreçlerden biridir. Çevre dostu olması ve solvent içermeyen ekstrakt elde edilmesiyle solvent kirliliğini engelleyen süperkritik karbondioksit ($scCO_2$) ekstraksiyon teknolojisi gelecek vaat eden bir alternatiftir. Bu çalışmada, yüksek miktarda lipit üreten mikroalg *Chlorella vulgaris*'ten $scCO_2$ ekstraksiyon yöntemiyle lipit ekstrakt edilmesi 200 ila 400 bar arası değişen basınçlarda ve 40 ila 70 °C arası değişen sıcaklıklarda incelenmiştir. Mikroalgler 27 litrelik düz panel fotobiyoreaktörlerde yetiştirilmiştir. Lipit verimi "Nile red" yöntemiyle takip edilmiştir ve en yüksek lipit veriminin durağan evrenin başında elde edildiği bulunmuştur. $ScCO_2$ ekstraksiyon yöntemi, geleneksel hekzan ile Soxhlet ekstraksiyonu yöntemiyle karşılaştırılmıştır ve benzer ekstraksiyon verimleri elde edilmiştir. Buna ek olarak, aynı ekstraksiyon verimine $scCO_2$ ekstraksiyon yöntemiyle çok daha hızlı ulaşıldığı gözlemlenmiştir. Sabit sıcaklıkta basınç artışı ve sabit basınçta sıcaklık artışı ekstraksiyon verimini önemli şekilde arttırmıştır. Lipit çıkış konsantrasyon eğrileri hızlı ekstraksiyon periyodunun ardından çok daha yavaş bir ekstraksiyon periyodu olduğunu göstermiştir. Deneysel sonuçlar, bu periyotları dış kütle transferi ve iç difüzyon tarafından kontrol edilen periyotlar olarak tanımlayan Sovova'nın modeli kullanılarak tahmin edilmiştir. Lipitlerin deney koşullarındaki çözünürlükleri ekstraksiyon eğrilerinin ilk kısmının eğimi kullanılarak bulunmuştur. Bulunan çözünürlükler 2,7 ve 9,0 mg lipit/g CO_2 arasında

değişmiştir. Deneysel sonuçlardan elde edilen akışkan faz kütle transfer katsayısı 10^{-4} m/s, katı faz kütle transfer katsayısı 10^{-8} m/s civarındadır. Kütle transferinde sıkça kullanılan korelasyonlar, deneysel olarak elde edilen akışkan faz kütle transfer katsayılarının tahmin edilmesinde kullanılmıştır. Damlaların buharlaşmasında kullanılmak üzere geliştirilen kütle transfer korelasyonu ($Sh = 2 + 1{,}1 * Re^{0,6} * Sc^{1/3}$) deneysel sonuçlara en iyi uyumu göstermiştir. Transesterifikasyon yöntemiyle elde edilen yağ asidi metil esterleri (FAME) profili ekstraksiyon yönteminden ve deneysel koşullardan önemli ölçüde etkilenmiştir. $ScCO_2$ ekstraksiyon süresinin FAME profiline herhangi bir etkisi gözlemlenmemiştir.

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NOMENCLATURE

a_0	*specific surface area per unit volume (m^2/m^3)*
e	*extraction yield (g/g algae)*
FAME	*fatty acid methyl esters*
GC-MS	*gas chromatography-mass spectrometry*
GRAS	*generally recognized as safe*
GHG	*greenhouse gases*
h	*bed height (m)*
k_f	*fluid phase mass transfer coefficient (m/s)*
k_s	*solid phase mass transfer coefficient (m/s)*
MUFA	*monounsaturated fatty acids*
q	*solvent to feed ratio (g/g)*
PUFA	*polyunsaturated fatty acids*
SFA	*saturated fatty acids*
Scco2	*supercritical carbon dioxide*
U	*superficial velocity of the solvent (m/s)*
x	*solid phase lipid fraction*
y	*fluid phase lipid fraction*
y_r	*lipid solubility in supercritical carbon dioxide (mg/g)*
ε	*bed porosity*
ρ	*solvent density (kg/m^3)*
ρ_s	*solid density (kg/m^3)*

Chapter 1

INTRODUCTION

Rapid depletion of crude oil reserves and global warming resulting from greenhouse gases emissions have triggered the search for alternative resources to produce transportation fuels. Immediate necessity to reduce carbon emissions and increasing demand for transportation fuels make biofuels produced from biomass attractive since production of biofuels sequester atmospheric CO_2 and biofuels can be directly blended with fossil fuels with a certain degree [1]. Contribution of bio-based resources into energy production is expected to increase and around one third of transportation fuels can be biofuels by 2050 [2]. Current regulations on biofuels have already introduced certain level of blending biofuels with fossil fuels and commercial biofuels such as bio-ethanol and bio-diesel are available in the market [3, 4]. Biofuels are classified according to feed stock type used during their production. First generation biofuels are derived from edible sources such as vegetable oil, sugar cane and corn [5, 6]. Usage of edible sources for biofuel production has brought about some concerns including food security and arable land availability [7, 8]. There has been intense debate about usage of these sources as fuel, known as food or fuel debate. Growing demand for edible feed stocks due to population growth and starvation problem all around the world make edible resources usage in biofuel production questionable [9]. Moreover, enormous land and fresh water requirement of edible sources plantation to meet global fuel demand doesn't seem to be sustainable due to low productivity of these sources [1]. Although second generation biofuels derived from non-edible sources such as Jatropha, switch grass and agricultural waste may eliminate concerns related with food security, they could not be enough to fulfill global

fuel demand in sustainable manner [10, 11]. Besides, studies on direct and indirect land use change show that utilization of first and second generation biofuels could increase greenhouse gases emissions due to elimination of positive contribution of forests and grass lands for carbon dioxide fixation [12]. Third generation biofuels obtained from microalgae, on the other hand, seem to overcome these concerns. Microalgae are unicellular and photosynthetic microorganisms that can store significant amount of protein, carbohydrates and lipids, which can be processed into biofuels, in a short period of time [13-15]. Non-arable land and saline or waste water can be used for microalgae cultivation. Microalgae doesn't compete with agricultural crops since they are not directly consumed by humans [16]. Microalgae-based biofuels may sustainably meet global fuel demand due to their faster growth rate, higher photosynthetic efficiency and biomass production when compared with land plants [1, 10, 17]. Among microalgae-based biofuels such as biomethane derived from anaerobic digestion of algal biomass and photobiologically derived biohydrogen, biodiesel obtained from algal lipids has attracted increasing attention due to higher amount of energy stored per carbon atoms in lipids than carbohydrates and proteins[5]. Moreover, aside from environmental and social advantages of using microalgae as a feed stock, lipid productivity of microalgae is significantly higher than terrestrial plants making them more efficient for biodiesel production [6, 13, 14].

Biodiesel production from microalgae requires several steps such as cultivation, harvesting, drying, pretreatment, lipid extraction and transesterification of lipids. Among these steps, effectiveness of lipid extraction is an important step for commercialization of microalgae-based biodiesel. Conventionally, organic solvent extraction techniques such as Bligh and Dyer, Soxhlet and Folch are applied to extract microalgae lipids by using chloroform, methanol, acetone and n-hexane [18]. There are some drawbacks of organic solvent usage including low selectivity, solvent contamination, high extraction temperatures and extraction times [18-20]. Extraction process requires selective extraction of neutral lipids since actual source of microalgae-based biodiesel is neutral lipids produced by microalgae. However, organic solvents also solubilize some polar components such as phospholipids, pigments etc. which are not utilized for biodiesel

production [20, 21]. Biorefinery concept exploiting biodiesel as well as high value algal products like proteins, pigments, omega-3 fatty acids have been introduced in order to improve economic of the process because current price of microalgae-based biodiesel is not competitive with conventional diesel [22]. Solvent contamination eliminating further usage of spent biomass and high extraction temperatures causing degradation of high value products are not desired when biorefinery concept is considered [23, 24]. Moreover, higher extraction times are not favorable for commercialization. Hence, new extraction methods should be introduced to overcome these issues. Recently, supercritical carbon dioxide ($scCO_2$) is considered as a promising candidate for extraction of neutral lipids from microalgae thanks to its superior properties eliminating drawbacks of organic solvents [18-20, 23-25]. $ScCO_2$ is a non-toxic, inert, inflammable solvent with a liquid-like density and gas like viscosity. Strong affinity of $scCO_2$ for non-polar molecules and not solubilizing phospholipids make $sccO_2$ selective solvent for neutral lipids such as triglycerides [26]. Extraction with $scCO_2$ eliminates solvent evaporation step as CO_2 is gas under ambient conditions yielding solvent free extract after depressurization. Mild critical point of CO_2 (31 °C and 73.8 bar) enables low temperature extraction without affecting heat sensitive biomolecules [27]. Besides, extraction time is significantly decreased with $scCO_2$ extraction when compared with organic solvent extraction [24].

Commercialization of $scCO_2$ extraction of algal lipids for biodiesel production necessitates investigation of operating parameters such as temperature, pressure on extraction yield and mathematical modeling studies to find mass transfer coefficients and identify applicable mass transfer correlations for $scCO_2$ extraction of microalgal lipids. Identification of effluent lipid profile may promote separation of high value lipids such as Eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) or lipids which are not favorable for biodiesel production [28].

Among a vast variety of microalgae species, fast growth rate and high lipid productivity of *Chlorella vulgaris* make it a very promising candidate for biodiesel production [29]. Moreover, commercial large scale cultivation of *Chlorella vulgaris* is performed in many countries in cost effective open ponds enabling high volume applications. In literature,

there is no study investigating applicable mass transfer correlations for $scCO_2$ extraction of *Chlorella vulgaris* lipids. Purpose of this thesis is to predict mass transfer coefficients from experimental data and compare them with mass transfer coefficients obtained from mass transfer correlations in $scCO_2$ environment and identify mass transfer correlations which can be used for $scCO_2$ extraction of *Chlorella vulgaris* lipids.

Within the scope of thesis fast growing, high amount of lipid producing microalgae *Chlorella vulgaris* were cultivated in flat panel type photobioreactor for biodiesel production. Harvesting was performed with centrifugation and oven drying. Prior to extraction, cells were ground in liquid nitrogen for cell disruption. Conventional and $scCO_2$ extraction methods were applied and compared in terms of lipid extraction efficiency. Effect of temperature and pressure on $scCO_2$ extraction yield were investigated. Mathematical model describing $scCO_2$ extraction process were introduced and mass transfer coefficients were regressed using extraction curves obtained from experimental data. Fatty acid methyl esters (FAME), namely biodiesel, produced after transesterification of lipids were analyzed by gas chromatography-mass spectrometry (GC-MS).

Chapter 2 includes literature review on world's energy demand, current regulations on biofuels, microalgae as a biofuel feedstock, lipid extraction methods from microalgae, supercritical extraction theory and mass transfer modeling of $scCO_2$ extraction process.

Chapter 3 describes experimental methods including microalgae cultivation and harvesting, lipid extraction, characterization as well as mathematical model used in this study.

Chapter 4 reports important results of this study and discuss reasoning behind these findings.

Finally, chapter 5 concludes the thesis by giving summary of the study.

Chapter 2

LITERATURE REVIEW

2.1 Current Status of World's Energy Demand and Concerns about Global Warming

Humanity needs energy sources due to several reasons such as electricity production, transportation, heating and cooking. Among these energy sources, fossil fuels are dominating and account for around 78 % world's total energy (**Fig. 1**). Source of fossil fuels is crude oil which is formed by heating and pressurization of organic material of dead animals and plants buried under the ocean million years ago. This formation process is very slow and consumption rate of crude oil is by far higher than its production rate. According to Organization of the Petroleum Exporting Countries (OPEC), proven crude oil reserves are about 1500 billion barrels and everyday 75 million barrels of crude oil are consumed [30]. Moreover, it is expected that energy demand of the world will go up by 50 % between 2020 and 2030 [31]. That's why crude oil is considered as a finite source and world will run out of crude oil in future with this huge consumption rate.

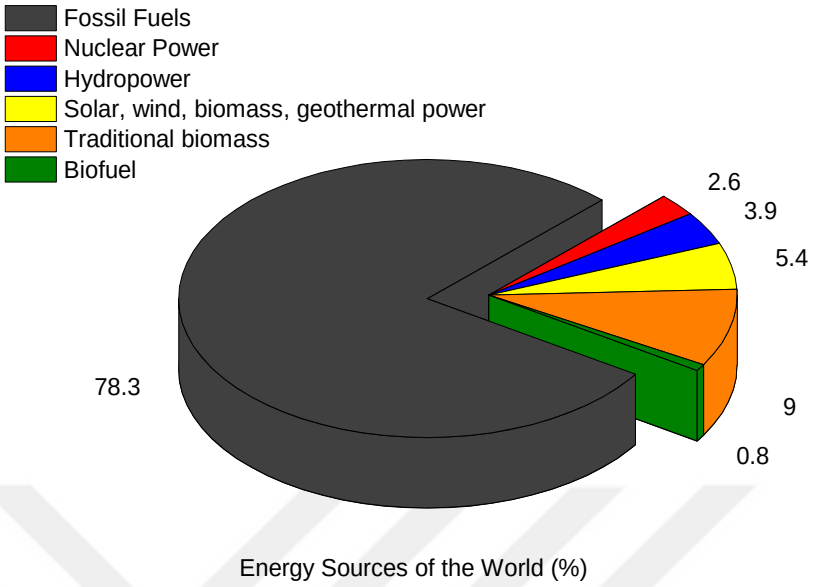

Figure 1: Total world energy consumption by source **[31]**

Aside from its possible scarcity in near future, greenhouse gases emissions (GHGs) resulting from fossil fuel usage are the main sources of global warming. Several evidences such as significant variation in temperature and sea level rise are considered as a strong indicator of global climate change. Surface temperature of the world have increased 0.8 °C and average 20 cm of sea level rise has been reported in 20th century [32]. Expected increase in GHGs will change climate more and affect ecosystem, agriculture and eventually society in a negative manner.

Transportation demand of the world is continuously increasing with urbanization, industrialization and irrepressible population growth. Great majority of transportation energy are provided by fossil fuels whereas natural gas and electricity contribute only 4 % of this energy [33]. Today, about 60 % of fossil fuels are used in transportation sector [34]. In spite of significant improvements in renewable energy sources such as solar and wind power integration to heat and electricity sector, fossil fuel usage in transportation sector is still increasing significantly [35]. This is mainly due to internal combustion engines which are commonly used in nearly all kind of transportation vehicles requiring

liquid fuels to operate. Finite availability of fossil fuels and negative effects of their usage on environment require replacement of these fuels with renewable, sustainable and environmentally friendly alternatives. Among these alternatives, biofuels derived from biomass are promising especially in short term since they have advantage of market maturity [36]. Moreover, biofuel production process sequester atmospheric carbon dioxide which is the second most important greenhouse gas after water vapor and responsible for 20 % of greenhouse effect [37].

Biofuels are categorized into generations by considering their biomass feedstock type. Edible, non-edible and microalgae feedstocks are used for production of first, second and third generation biofuels, respectively. All generations of liquid biofuels and their production routes can be seen in **Table 1**. First generation bioethanol form sugar cane and corn and biodiesel from vegetable oils are already available in the market [38]. Moreover, there are some regulations on biofuels requiring certain amount of blending into fossil fuels. For example, according to current regulations in Brazil, at least 27.5 % bioethanol and 7% biodiesel are needed to blend in gasoline and diesel, respectively [39].

Table 1: Classification of biofuels and their production routes [7, 36, 40]

Feedstock	Biofuel	Production Route
First Generation		
Edible plant oils (sunflower)	Biodiesel	Transesterification
Starch (wheat, barley, corn)	Bioethanol	Fermentation
Sugars (sugar cane)	Bioethanol	Fermentation
Second Generation		
Cellulosic Biomass	Biomethanol	Gasification to produce syngas (CO + H) and methanol production from syngas
Cellulosic Biomass	Biodiesel and Biogasoline	Pyrolysis or Liquefaction and upgrading of biooil
Cellulosic Biomass	Bioethanol	Hydrolysis to produce sugars and fermentation of sugars
Third Generation		
Microalgae	Biodiesel	Transesterification of microalgae lipids
Microalgae	Biomethanol	Gasification to produce syngas (CO + H) and methanol production from syngas
Microalgae	Biodiesel and Biogasoline	Pyrolysis or Liquefaction and upgrading of biooil

When global diesel demand is considered, it is important to replace diesel with environmentally friendly and sustainable alternative, namely biodiesel. Consumption of different fuel types in transportation sector can be seen in **Fig 2**. Diesel with a contribution of 38 % is the major transportation fuel together with gasoline.

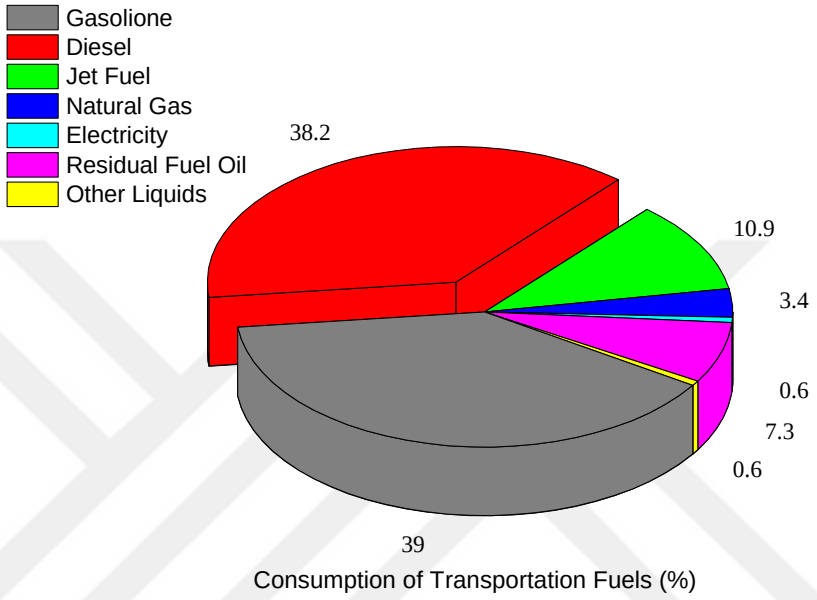

Figure 2: Total world transportation fuel consumption by source [33]

Aside from replacing high demanded transportation fuel, biodiesel has several advantages such as blending with conventional diesel in all blending ratios, low or no sulfur content, reduction of most regulated exhaust emissions, inherent lubricity, high flash point and no aromatics content [41, 42]. Global production of biodiesel increased 15 times from 2002 to 2012 [38]. It seems that global production of biodiesel will continue to increase as new regulations are on the way to reduce negative impacts of fossil fuels.

2.2 Biodiesel as an Alternative Fuel

Triglycerides molecules found in plants, animals and microorganisms are the actual sources of biodiesel. Triglycerides are class of neutral lipids and they are generally synthesized for energy storage in living things due to their higher energy density. They are composed of fatty acid molecules attached to glycerol (**Fig. 3**).

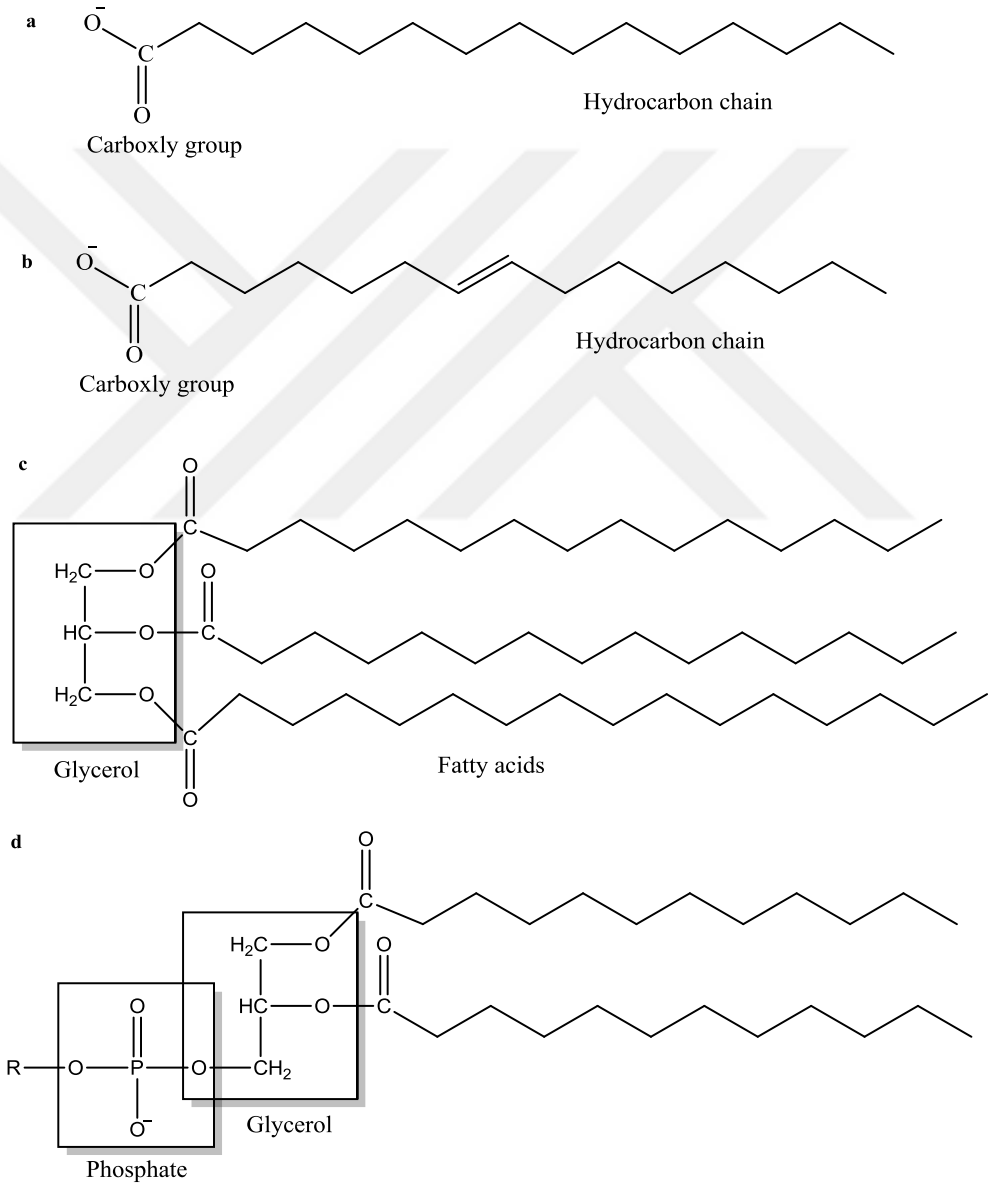

Figure 3: (a) Saturated fatty acid chain (C:15). (b) Unsaturated fatty acid chain with one double bond (C:15:1). (c) Triglyceride molecule (d) Phospholipid molecule **[43]**

Fatty acid molecules include highly non-polar hydrocarbon chain and polar carboxylate groups. These molecules are classified based on their two important properties, namely total number of carbon atoms and double bonds in hydrocarbon chain. Unsaturated fatty acids contain at least one double bond whereas saturated fatty acids have no double bonds. Fatty acid molecules are found in both neutral and polar lipids. There are some neutral lipids which don't contain fatty acids and cannot be converted to biodiesel such as pigments and hydrocarbons. Polarity of fatty acid containing lipids depends on bonding of carboxylate group in fatty acid molecules. If carboxylate group is associated with uncharged group neutral lipids are formed. Formation of polar lipids occurs when carboxylate group establishes a bond with charged group such as phosphate (**Fig. 3**). This bonding result in phospholipid formation. Phospholipids are not desired for biodiesel production since they form micelles in alcohols preventing contact of triglycerides with alcohol and catalyst [43, 44].

Production of biodiesel is not a recently developed process. When Rudolf Diesel (1858-1913) invented the diesel engine, he tried vegetable oil as a fuel for diesel engine. Although engine worked properly with this fuel, several reports emphasized that high viscosity of vegetable oil could bring operational problems including engine deposits. In 1937, transesterification of palm oil with ethanol to produce fatty acid ethyl esters which are considered as first report on today's biodiesel were introduced for viscosity reduction of palm oil. From beginning of World War II until the energy crises which occurred in 1970s, there were no significant reports on usage of vegetable oil as a fuel in diesel engine. After this period, rediscovery of vegetable oils led to new developments such as sun flower oil transesterification with methanol to produce fatty acid methyl esters for diesel engine. Usage of fatty acid methyl esters provided further viscosity reduction and eliminated operational problems. Today, fatty acid alkyl esters are considered as a biodiesel yet only FAME are commercially produced due to availability and price of methanol when compared with other alcohols [41].

FAME are produced by transesterification of triglycerides with methanol. Acid or base catalysts can be used in transesterification. Transesterification is a stepwise reaction. It includes three steps. At first step, triglycerides are converted to diglycerides. Then,

monoglycerides are obtained from diglycerides. Finally, conversion of monoglycerides give glycerol as a byproduct. In each step one mole of FAME is produced [45, 46]. Overall reaction can be seen in **Fig 4**.

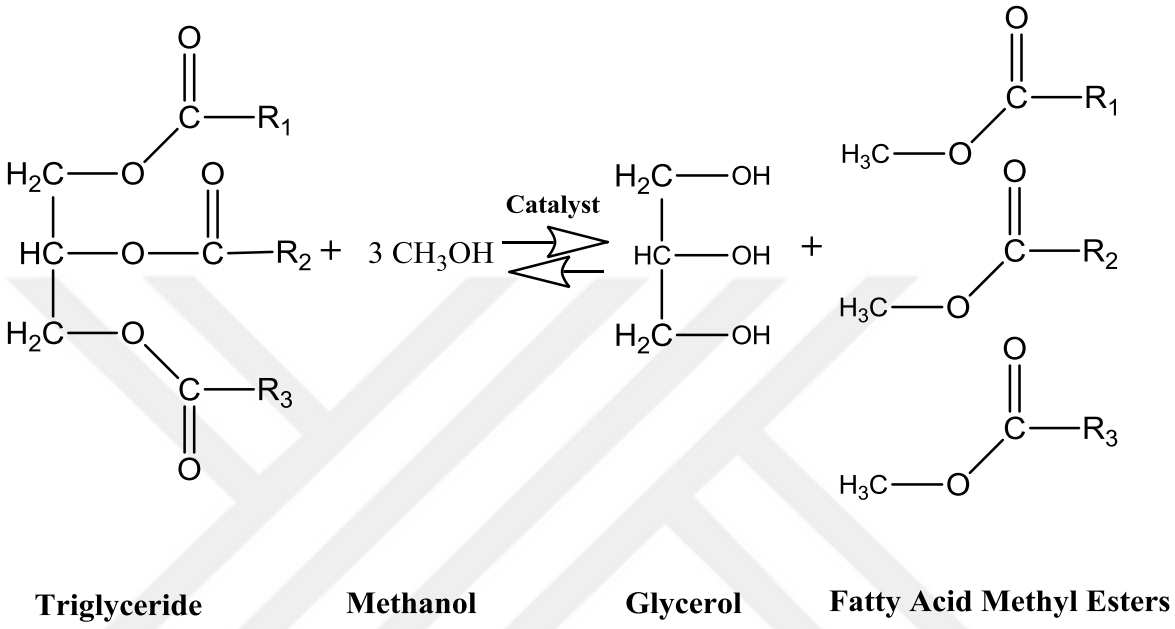

Figure 4: Transesterification reaction

Characteristics of biodiesel such as oxidation stability, heat of combustion, cold flow properties, lubricity and cetane number strongly depend on degree of unsaturation and chain length of the fatty acids found in the feedstock. Saturated and unsaturated fatty acids affect these properties oppositely. Saturated fatty acids are better for oxidation stability, heat of combustion and cetane number whereas unsaturated fatty acids improve cold flow properties and lubricity [47].

Triglycerides from edible, non-edible and microalgae feedstocks can be used for biodiesel production. However, increasing production of first generation biodiesel has brought new concerns such as food or fuel debate and land use changes. Edible feedstock usage in first generation biodiesel is likely to increase food and commodity prices which is not beneficial for society [48]. Besides, converting forests and grasslands or existing

croplands into biodiesel factories known as land use change will eliminate their positive contribution for carbon fixation and eventually increase GHGs [12]. Although second generation biofuels especially derived from agricultural waste seem to eliminate these concerns, they are far from enough to meet global demand and they require complex processing steps [11]. Biodiesel from microalgae seems only sustainable, renewable and environmentally friendly alternative to conventional diesel since it eliminates social, environmental and capacity related concern. They don't compete with human food and can be cultivated non-arable land without destroying existing forests and grasslands. Some strains with specific conditions produce enormous amount of lipids when compared with plant based alternatives as can be seen in **Table 2**.

Table 2: Lipid yield of some biodiesel feedstocks [8, 49].

Sources	Lipid Yield (L/ha)
Corn	172
Soybean	446
Canola	1190
Jatropa	1892
Coconut	2699
Palm oil	5950
Microalgae[a]	136900
Microalgae[b]	58700

[a] 70 % w/w lipid in biomass

[b] 30 % w/w lipid in biomass

Such a significant difference between microalgae and plant based materials in terms of lipid yield makes microalgae-based biodiesel only applicable alternative for meeting global diesel demand.

2.3 Microalgae as a Biodiesel Feedstock

Existence of life on earth was arisen thanks to cyanobacteria and microalgae performing photosynthesis and producing oxygen. Atmospheric CO_2 level which was 7000 ppm about 500 million years ago was decreased to suitable levels for existence of life by carbon fixation activity of these microorganisms [11]. Microalgae converts solar energy into chemical energy via photosynthesis and they produce macromolecules such as protein, carbohydrates and lipids to store chemical energy [9]. They can live almost in all ecosystems found on earth. Their simple unicellular structure facilitating high photosynthetic rates enables them to grow quickly and accumulate significant amount of lipids (**Table 2**) [43]. There are also microalgae strains which do not perform photosynthesis and grow heterotrophically; however, they are not in the scope of this study.

Microalgae cultivation require light, inorganic carbon source, nutrients, certain temperature and pH[8]:

- Light: Photosynthesis consist of two steps, light reactions and Calvin cycle. In light reactions, microalgae absorb light and split water to produce ATP. Sun light and artificial light can be used.
- Inorganic Carbon Source: This ATP is then utilized to produce glucose from inorganic carbon source in Calvin cycle. Microalgae use CO_2 as an inorganic carbon source.
- Nutrients: Aside from inorganic carbon source, microalgae need some nutrients such as N, P and K to produce essential macromolecules proteins, phospholipids etc.
- Temperature: Each strain has its own optimum growth temperature. Generally optimum growth temperature range is 20 and 24 °C. There are also some strains which can grow at very high and low temperatures.
- pH: Optimum pH range for microalgae growth is between 7 and 9.

Microalgae growth cycle includes lag phase, exponential growth phase, stationary phase and death phase as any other microbial growth cycles [50]. Lag phase happens at the very

beginning of the cultivation and cells do not perform cell division at this phase. It is considered as an adaptation period for new environmental conditions. After this adaptation phase, cell division starts and it happens really fast. Cell number increases exponentially until stationary phase where at least one stress condition such as nutrient depletion is present. In these two phases, cells follow two different strategy. In exponential growth phase, cells produce primary metabolites and perform only division whereas in stationary phase they produce secondary metabolites such as pigments, vitamins to sustain their growth rate or strengthen the possibility of their survival under stress conditions [51]. After depletion of nutrients and pollution resulting from secreted metabolites, death phase starts.

Microalgae generally do not synthesize large amount of lipids during the exponential growth phase. When an environmental stress such as nitrogen absence is introduced, they decelerate proliferation and start to synthesize lipids which is considered as an energy storage materials [52]. Nitrogen is essential for protein synthesis. Nitrogen starvation leads to alteration in the structure of RuBisCO enzyme which is also essential for protein synthesis. This alteration inhibits protein synthesis and let carbon resources flow for lipid production by increasing the activity of acetyl-coA carboxylase enzyme participating first step of lipid biosynthesis [11]. There are several strategies available to increase lipid production in microalgae. Different environmental stresses such as phosphorus starvation or insufficient/excess light can be introduced. Instead of promoting lipid synthesis, lipid catabolism can be inhibited by knocking out of some enzyme genes such as fatty acyl CoA dehydrogenase which is a key enzyme for oxidation of fatty acids [52].

Microalgae strain selection is a crucial step for biodiesel production. Each strain has its own characteristics. Growth rate, lipid content and lipid productivity of strain are the most important parameters to consider and these parameters change considerably from strain to strain. Aside from lipid production capacity related concerns, ease of cultivation and processing should be taken into account. Weak influence of environmental disturbances such as change in temperature, light availability and competition with other microorganisms on cultivation is desired for large scale operations. Moreover, ability of

microalgae to utilize cheap nutrients including CO_2 from flue gas or nitrogen and phosphorus from waste water would be beneficial for economics of overall process [1].

Microalgae cultivation can be performed with both open and closed systems. In open system operations generally raceway ponds are used. Tubular and flat panel type photobioreactors are commonly chosen for closed system cultivation. There is an ongoing debate about best production systems since both type of cultivation have their own pros and cons. Raceway ponds are easy to operate and require less initial investment yet they are less productive, they introduce greater volumes for processing due to more diluted cultures and they are more vulnerable to contamination. Closed systems have greater productivities; however, they require more investment and sophisticated control systems [53].

After cultivation either with open or closed system and using one of the lipid production strategies, water and biomass need to be separated for biodiesel production. This step is called harvesting. There are several methods for harvesting such as centrifugation, gravity sedimentation, flocculation and filtration [54]. Selection of harvesting methods depends on microalgae strain characteristics such as density and particle size since these characteristics strongly affect harvesting efficiency. Separation occurs in centrifugation and gravity sedimentation due to density difference of microalgae and water. Centrifugal force which is higher than gravity force is applied to accelerate separation in centrifugation whereas gravity force is used in sedimentation. Although centrifugation separates very fast and with high efficiency it consumes a lot of energy and negatively influence net energy balance of microalgae based biodiesel [55]. Sedimentation is simple and very effective process. However, sedimentation rate may be very low if the density of microalgae strain and water is close. To enhance sedimentation rate, flocculation method can be used. This method increases particle size of microalgae suspension by aggregation and expedites sedimentation [56]. In filtration method, microalgae suspension including water is passed through filters and cells are retained by filters.

Final step before lipid extraction is cell disruption and drying. Mechanical, physical, chemical and enzymatic methods can be applied for cell disruption [57]. Applicability of these methods depend on water content of biomass. For example, sonication can be

effectively applied to only wet biomass whereas grinding with liquid nitrogen is more suitable for dry biomass [55]. Drying can also be performed with several methods such as solar drying, oven drying, spray drying and freeze drying. Long drying times and large drying surface area requirement makes solar drying inefficient although it is cheapest method. Moreover, it may lead to biomass degradation. Spray and freeze drying are commonly used for drying high value microalgal products. Both of these methods preserve nutritional value of microalgae. Oven drying is also common method for drying. It may cause loss of some bioactive compounds such as phycocyanin but it doesn't affect fatty acid profile [58].

2.4 Lipid Extraction Methods

Physical state of microalgae prior to lipid extraction depends on pre-treatment and drying step. Lipid extraction can be applied to both wet and dry microalgal biomass. Wet microalgal biomass is in the form of either concentrate or disrupted concentrate whereas dry microalgal biomass is in the form of powder [43]. Although lipid extraction from wet microalgal biomass significantly reduces drying cost, low extraction yield due to immiscibility of water with non-polar solvents preventing dissolution of neutral lipids necessitates effective cell disruption methods which may be costly [55]. On the other hand, dry extraction route gives higher extraction yields with costly drying requirement. That's why lipid extraction from both wet and dry microalgal biomass are still being heavily studied.

2.4.1 Organic Solvent Extraction

Most common method for lipid extraction from microalgae is organic solvent extraction. Basic chemistry principle "like dissolves like" is the underlying mechanism of lipid extraction. When microalgal biomass is subjected to non-polar solvents, they can penetrate inside the cell and solubilize neutral lipids by forming wan der Waals interactions. However, some of the neutral lipids inside the cell together with polar lipids

form complex which is strongly bonded to cell membrane proteins by hydrogen bonding. Non-polar solvents are unable to disrupt these lipid-protein interactions whereas polar solvents can form hydrogen bonds with polar lipids in the complex resulting disruption of lipid-protein interactions. When non-polar and polar organic solvents are used together, non-polar solvents again form wan der Waals interaction with the neutral lipids in the complex and polar solvents associate with polar lipids in the complex via strong hydrogen bonding which lead to dissociation of complex including organic solvents and lipids. Collaboration of non-polar and polar solvents obviously lead to extraction of not only neutral lipids but also polar lipids which cannot be utilized for biodiesel production [10, 43, 55, 59].

There are three common methods available for lipid extraction. Folch [60] and Bligh and Dyer [61] methods target total lipid extraction including polar and neutral lipids whereas Soxhlet method which was invented by Franz von Soxhlet in 1879 [55] generally aims to extract only neutral lipids. In Folch and Bligh and Dyer methods, chloroform, methanol and water mixture is used with different ratios. Besides, initial sample to solvent ratios are different [62]. These methods are applied in batch mode. In Soxhlet extraction, n-hexane is the most commonly used non-polar solvent targeting only neutral lipid extraction. Sample is contained in extraction thimble where fresh solvent continuously provided with evaporation and condensation. This method provides high efficiency thanks to continuous supply of fresh solvent. Hence it is commonly used for lipid quantification, although it requires longer extraction times and consumes a lot of energy due to evaporation [55].

There are several problems associated with organic solvent usage in microalgal lipid extraction. Long extraction times are required in order to get a high extraction yield. After organic solvent extraction, an energy intensive separation unit is needed to separate lipids from organic solvents. High extraction temperature requirement of solvent extraction and toxicity of organic solvents prevents using remaining biomass containing high value products such as proteins, carbohydrates and pigments.

2.4.2 Supercritical CO_2 Extraction

When the pressure and the temperature of a fluid is increased above its critical point, a supercritical fluid (**Fig. 5**) is obtained. Supercritical state is identified as a state in which gas and fluid phase are indistinguishable. At this state, fluids have liquid-like densities and gas-like viscosities whereas their diffusivities are intermediate between gases and liquids. These different physicochemical properties of supercritical fluids makes them superior extraction solvent. Their relatively high diffusivity and low viscosity facilitate better transport properties when compared with organic solvents. They can easily penetrate into solid materials and provide faster extraction. Moreover, solvation power of the supercritical fluids can be modified by density change since solubility is a function of solvent density [63].

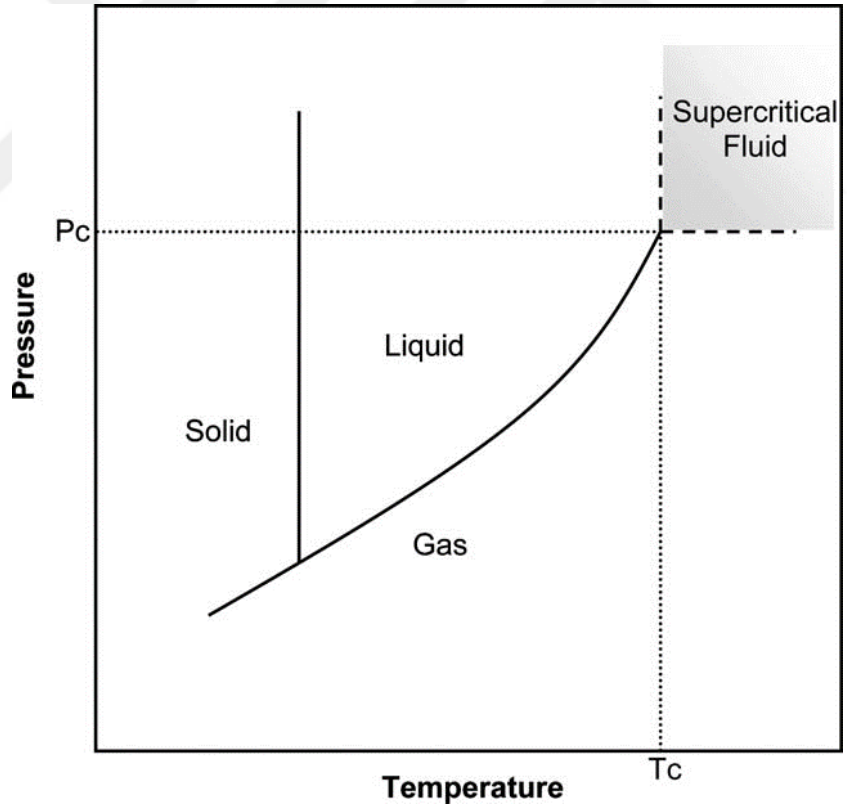

Figure 5: Typical phase diagram for a pure compound [63].

Among supercritical fluids, $scCO_2$ extraction has been extensively studied for neutral lipid extraction from microalgae due to its non-polar characteristic, moderate critical temperature (31 °C) and pressure (73.8 bar). It enables low temperature extraction protecting nutritional value of microalgal biomass. Besides, $scCO_2$ extraction is a GRAS solvent eliminating solvent contamination and it is a gas under ambient conditions resulting in solvent-free extract [20, 21, 25, 26, 64-66].

In order to investigate commercialization potential of the $scCO_2$ extraction of microalgal lipids for biodiesel production, understanding of effect of process conditions such as temperature, pressure on extraction mechanism and mathematical model describing overall process are needed.

2.4.2.1 Mass Transfer Modeling of Supercritical CO_2 Extraction

Mathematic modeling enables rational approach to design of extraction process, facilitate generalization of experimental results and gives opportunity to simulate different operating conditions without performing experiments. Three different approaches including empirical, based on heat and mass transfer analogy and differential mass balances integration are available in the literature [67]. Among these approaches, differential mass balances including time dependent concentration of both solid and fluid phase are more accurate and commonly used for the modeling of the $scCO_2$ extraction of the neutral lipids from microalgal biomass [18, 20, 68].

Sovova's model [69] for vegetable oil extraction from plant material with $scCO_2$ has been widely applied to extraction of neutral lipids from microalgae due to similar characteristics of plant material and microalgal biomass. This model is also used in this study with minor modifications.

Description and assumptions of the model are given below:

- $ScCO_2$ flows with superficial velocity U throughout the fixed-bed extractor containing grinded and dried microalgae powder.
- Solvent is solute-free at the extractor inlet.
- Temperature and pressure are constant during extraction.

- Particle size and initial lipid content are uniformly distributed.
- Bed porosity is constant.
- Physicochemical properties of $scCO_2$ doesn't change.
- Mass transfer by natural convection is neglected and only mass transfer by forced convection is considered.

By applying the differential mass balance approach, fluid (**Eq.1**) and solid (**Eq.2**) phase balance equations are obtained.

$$\rho * \varepsilon * \frac{\partial y}{\partial t} + \rho * U * \frac{\partial y}{\partial h} = J(x, y) \quad (1)$$

$$-\rho_s * (1 - \varepsilon) * \frac{\partial x}{\partial t} = J(x, y) \quad (2)$$

In these equations, ρ is the density of $scCO_2$, ε is the bed porosity, y is the fluid phase lipid concentration, ρ_s is the solid density, x is the solid phase lipid concentration and J is the mass transfer rate.

Experimentally obtained extraction curves (**Fig. 6**) show that there are two mass transfer periods in overall process. Extraction rate is very fast in the first period whereas it drops suddenly and occurs very slowly at second period. Broken and intact cells approach were introduced in order to represent sudden reduction in extraction rate [70]. According to this approach, lipid extraction from broken cells whose walls are damaged occurs in first period. Then, lipid extraction from intact cells which are located in the particle core starts. Mass transfer rates of these periods are significantly different.

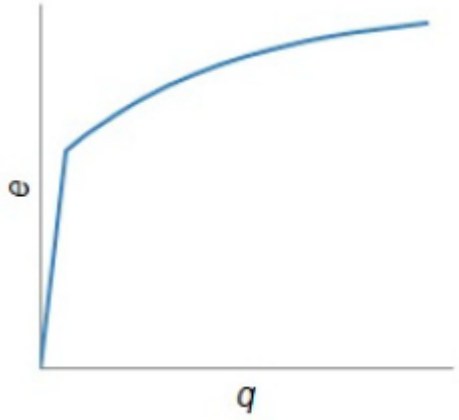

Figure 6: Typical extraction curve obtained by $scCO_2$ extraction of microalgal lipids [68]

First period of the extraction is controlled by phase equilibrium depending on extraction pressure and temperature as well as composition of lipids and $scCO_2$. It is considered that fluid phase at the extractor outlet is in equilibrium with solid phase. Fluid phase equilibrium concentration is assumed to be equal to solubility since lipid concentration in solid phase is high [69, 70]. It should be noted that here solubility term represents solubility of neutral lipid complex including different neutral lipid molecules in $scCO_2$.

Internal diffusion is rate-limiting step in the second period of the extraction. Lipid concentration in fluid phase is not equal to solubility anymore. Mass transfer resistance introduced by cell walls retards lipid extraction.

Mass transfer rate J in first period of the extraction is commonly defined as below (**Eq.3**).

$$J = k_f * a_0 * \rho * (y_r - y) \quad (3)$$

However, several mass transfer rates have been proposed to define mass transfer rate in second period as can be seen in **Table 3**.

Table 3: Mass transfer rates proposed for second period of extraction

Source	Second period
Lack[71]	$k_f * a_0 * \rho * (y_r - y) * x/x_k$
Pekhov and Goncharenko [69]	$k_s * a_0 * \rho_s * x$
Sovova [69]	$k_s * a_0 * \rho_s * x * (1 - y/y_r)$

2.4.2.2 Correlations for Mass Transfer Coefficients for Forced Convection

In order to predict mass transfer coefficients in different cases without performing tedious experiments all the time, mass transfer correlations have been developed [72]. These correlations were developed for specific applications and they have certain applicability range. They are expressed as dimensionless numbers. Sherwood number (**Eq.4**) is defined as ratio of mass transfer velocity to diffusion velocity and it includes mass transfer coefficient.

$$Sh = \frac{kl}{D} \tag{4}$$

Reynolds number (**Eq.5**) is defined as ratio of inertial forces to viscous forces for forced convection. It is related with flow characteristics.

$$Re = \frac{\rho * U * d_p}{\mu} \tag{5}$$

Schmidt number (**Eq.6**) is defined as ratio of diffusivity of momentum to diffusivity of mass.

$$Sc = \frac{\mu}{\rho D} \tag{6}$$

In these equations, k is the mass transfer coefficient, l is the characteristic length, D is the diffusion coefficient, ρ is fluid density, U is the fluid velocity, d_p is particle diameter and μ is the viscosity of the fluid.

In forced convection, Sherwood number is a function of Reynolds and Schmidt number. Mass transfer coefficients in forced convection can be predicted from Sherwood number. Previously, several authors have reported data including both experimental and modeling studies for $scCO_2$ extraction of microalgal lipids [18, 20, 21, 23, 25, 64]. Mendes et al. [64] studied $scCO_2$ extraction of freeze-dried microalgae *Chlorella vulgaris* at temperatures of 45 and 55 °C and pressures up to 350 bar. They reported positive effect of increasing pressure and cell disruption on extraction yield. Cheng et al. [25] compared organic solvent extraction and $scCO_2$ extraction of microalgae *Pavlova sp*. They showed that bead-beating pretreatment with $scCO_2$ extraction was both effective and selective method for triglycerides. According to their findings, 98.7 % of available FAME was extracted and 87 % of the extract was converted to FAME with this method. Baumgardt et al. [21] investigated effect of different organic solvent usage and co-solvent addition in $scCO_2$ on extraction yield of microalgae *Nannochloropsis occulata* lipids. They reported that Soxhlet extraction with ethanol gave highest yield among tested solvents and ethanol addition during $scCO_2$ extraction increased both extract and FAME yield. Aliev et al. [24] compared several extraction methods including $scCO_2$ extraction with and without acetone and Soxhlet extraction with n-hexane and chloroform for lipid extraction from microalgae *Nannochloropsis salina*. They claimed that extraction methods didn't affect total extract yield and fatty acid content although there are several literature findings opposing their arguments. Taher et al. [18] predicted extraction curves obtained from $scCO_2$ extraction of *Scenedesmus sp.* with different temperature, pressure and CO_2 flow rate by using Sovova's model [69]. They regressed fluid and solid phase mass transfer coefficients from their data as $6.7*10^{-6}$-$30.9*10^{-6}$ m/s and $2.08*10^{-10}$-$13.2*10^{-10}$ m/s, respectively. Mouahid et al. [20] studied $scCO_2$ extraction of freeze or oven dried microalgae with different pretreatments including *Nannochloropsis oculata*, *Cylindrotheca closterium*, *Chlorella vulgaris* and *Spirulina platensis*. They also used Sovova's model to fit experimental data and showed that all experimental cases could be represented by this model. Solana et al. [73] performed scCO2 extraction to obtain lipids of microalgae including *Scenedesmus obliquus*, *Chlorella protothecoides* and

Nannochloropsis salina. They found positive effect of pressure on extraction yield and identified a cross-over pressure as 250 bar. Moreover, they reported a good agreement between experimental and simulated extraction curves which were obtained by Sovova's model.

Chapter 3

MATERIALS AND METHODS

3.1 Materials

$NaNO_3$, $CaCl_2.2H_2O$, $MgSO_4.H_2O$, K_2HPO_4, KH_2PO_4, NaCl, FeSO4.H2O, H_3BO_3, EDTA, KOH, $ZnSO_4.7H_2O$, $MnCl_2.7H_2O$, $MoO_4Na_2.2H_2O$, $CuSO_4.5H_2O$, $CoCl_2$ and hexane with high purity (> 99.8%) were purchased from Sigma-Aldrich. CO_2 (>99.9) were obtained from Air Liquide.

3.2 Microalgae Cultivation

Microalgae *Chlorella vulgaris* (SAG 211-12) were provided from algae culture collection. Microalgae cultivation was performed under nitrogen starvation in a 27 liter flat panel type photobioreactor (PSI) available in our laboratory (**Fig. 7**) by using a slightly modified Bold's basal medium [74]. Composition of growth medium can be seen in **Table 4**. Aeration rate of 10 L/min (2 % CO_2) with a light intensity of 480 μE were supplied. Cell growth was monitored by cell density and nitrate concentration determined with absorbance measurement at 680 and 220 nm using spectrophotometer, respectively. Neutral lipid content of the cells were quantified by Nile red staining protocol using Triolein as a standard [75].

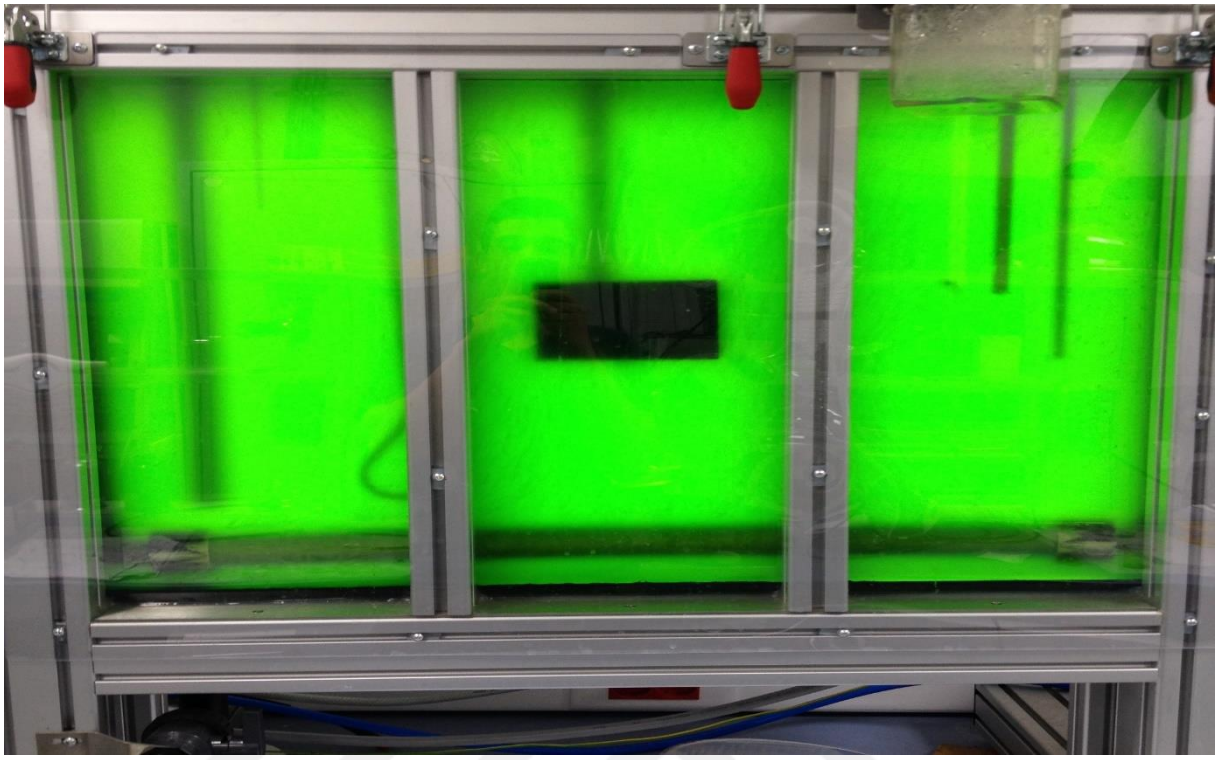

Figure 7: Flat panel type photobioreactor available in our laboratory.

Table 4: Growth medium ingredients and their concentrations

Ingredient	Concentration (g/L)
$NaNO_3$	0.278
$CaCl_2.2H_2O$	0.028
$MgSO4.H_2O$	0.083
K_2HPO_4	0.083
KH_2PO_4	0.194
NaCl	0.028
$FeSO_4.H_2O$	0.006
H_3BO_3	0.013
EDTA	0.056
KOH	0.034
$ZnSO_4.7H_2O$	0.010
$MnCl_2.7H_2O$	0.0005
$Na_2MoO_4H_2O$	0.0013
$CuSO_4.5H_2O$	0.0017
$CoCl_2$	0.0005

3.3 Harvesting

Cells were harvested at early stationary phase since higher lipid content which is favorable for lipid extraction was achieved during this phase. Harvesting was performed with centrifugation (Beckman Coulter). Algae paste obtained after centrifugation (**Fig. 8**) was put into oven for drying. Temperature was set to 50 °C and samples were kept in the oven for 24 hours.

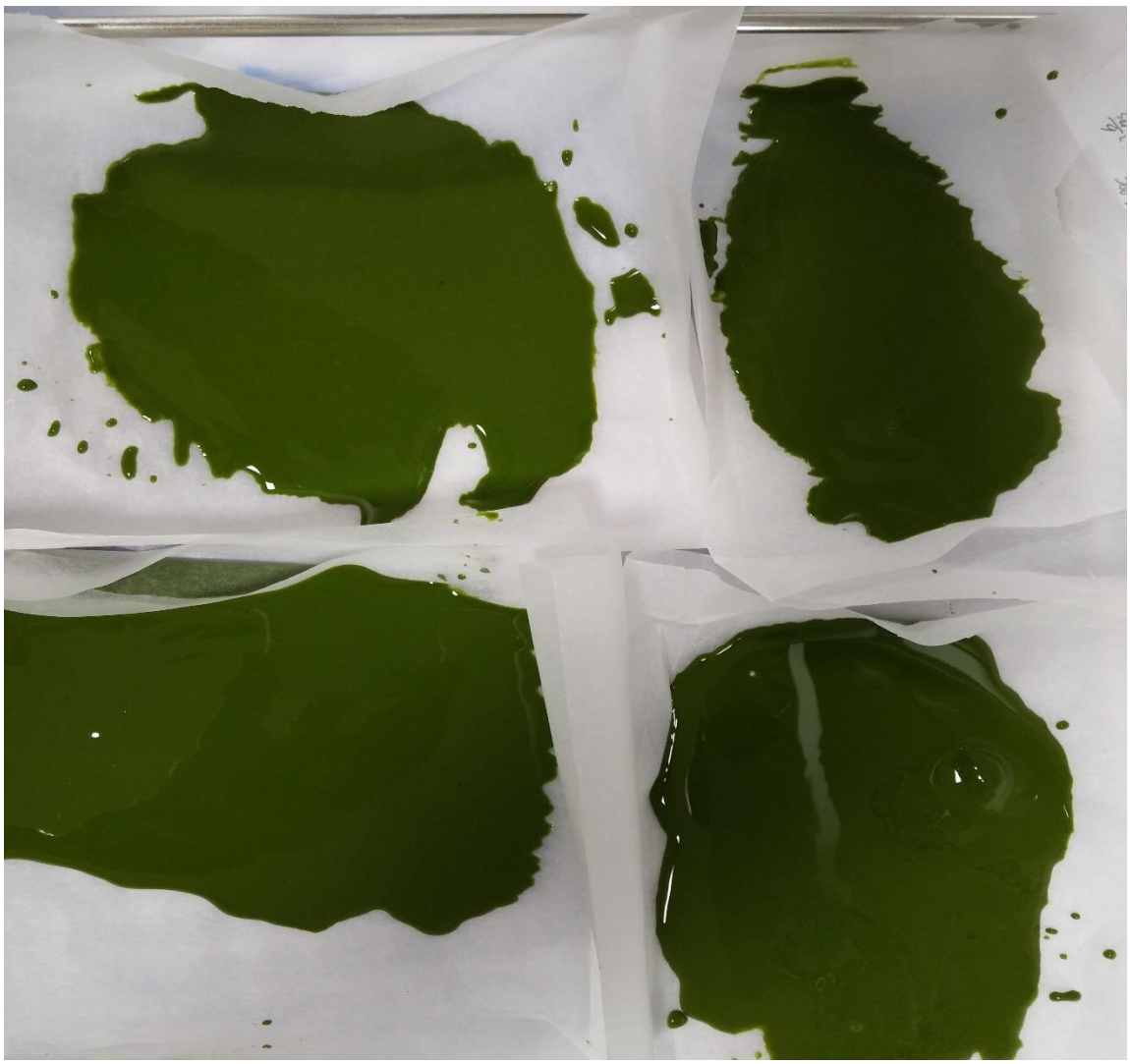

Figure 8: Microalgae paste obtained after centrifugation

3.4 Lipid Extraction

Prior to extraction, cells were subjected to liquid nitrogen and ground into powder (**Fig. 9**) with the help of mortar and pestle to enhance extraction yield.

Figure 9: Microalgae powder obtained after grinding with liquid nitrogen

3.4.1 Soxhlet Extraction

2 g of algae powder was put into cellulosic extraction thimble (Whatman GE). Then, thimble was placed into Soxhlet extractor. Round bottom flask containing 250 ml n-hexane and Soxhlet extractor were merged and placed on the heater. Glass condenser circulating cooling water was located at the top of the Soxhlet extractor. Experimental setup can be seen in **Fig.10**. Continuous heat was supplied to boil n-hexane and extraction

was performed for 24 hours. After extraction, solvent was evaporated by rotary evaporator and extract was quantified gravimetrically.

Figure 10: Soxhlet Extraction Experimental Setup (1.Condenser, 2.Extraction thimble, 3. Solvent, 4.Heater)

3.4.2 Supercritical CO_2 Extraction

Laboratory scale extraction equipment (Applied Separations) was used for supercritical extraction of microalgal lipids. Schematic representation of the process flow diagram can be seen in **Fig.11**.

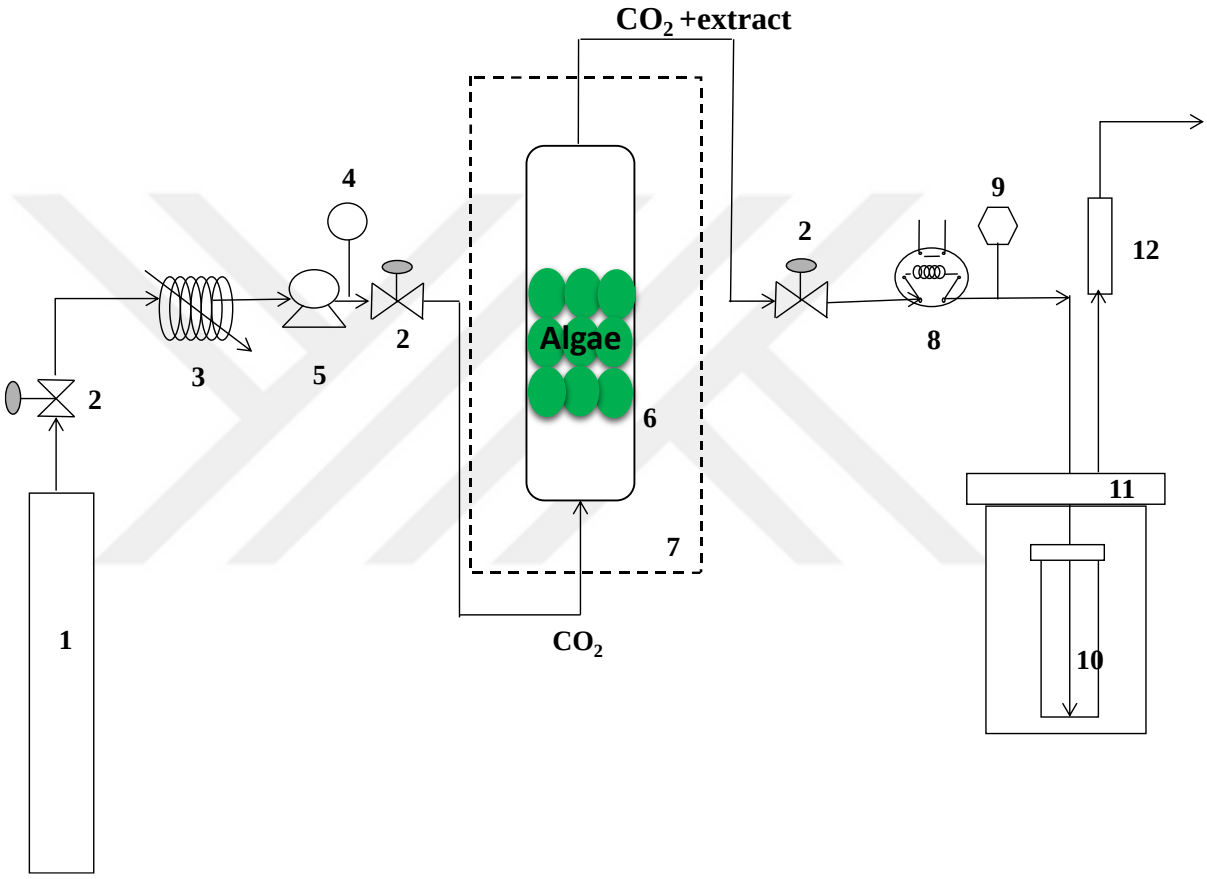

Figure 11: Schematic representation of $scCO_2$ extraction of microalgal lipids (1.CO2 tank, 2.Valve, 3.Cooler, 4.Pressure transducer, 5. CO2 pump, 6. High pressure extraction vessel, 7.Oven, 8.Micro-metering valve, 9.Thermocouple, 10.Sample collector, 11. Cooler, 12. Rotameter)

5 g of algae powder was put into 24 ml high pressure extraction vessel (Applied Separations). Glass wool was placed at both bottom and end of the vessel to prevent solid material leakage. Vessel was placed into pre-heated oven. After vessel reached to set temperature, it was pressurized to set pressure. Extraction was started by opening outlet

valve. Samples were collected with different time intervals and quantified gravimetrically. Extraction lasted 3 hours with a CO_2 flow rate of 2.5 L/min.

3.5 Transesterification

Quantified extracts either obtained with Soxhlet or $scCO_2$ extraction were transferred into round bottom flasks by using hexane and methanol. After solvent evaporation, 20 ml methanol containing 2 % HCl was added to the flasks and they were put on the heater. Transesterification reactions were performed at the boiling temperature of methanol 65 ℃ for 2 hours.

3.6 FAME Analysis

FAME analysis was performed by gas chromatography (Agilent 7890B) - mass spectrometry (Agilent 5977A) (GC-MS) equipped with highly polar capillary DB-23 column (60 m x 0.25 mm ID x 0.15 μm film thickness). Helium was used as a carrier gas and constant head pressure of 2.3 bar (33 cm/s at 50 ℃) was provided. Oven temperature was kept at 50 ℃ for 1 min, then it was increased to 175 ℃ with a heating rate of 25 ℃/min, after that it reached 230 ℃ with a heating rate of 4 ℃/min and it was kept constant at 230 ℃ for 5 min. 1 μL sample containing hexane and FAME was injected to GC-MS for analysis. FAME mixture (C4-C24 Supelco) was used as a FAME standard.
10 ml distilled water and 10 ml hexane were added to samples obtained by transesterification. After mixing, upper phase containing hexane and FAME was concentrated and injected to GC-MS.

3.7 Mathematical Model Used in the Study

Eq.3 was used to define mass transfer rate in the first part of the extraction. Mass transfer rate proposed by Pekhov and Goncharenko (**Table 3**) was used for representing mass transfer rate in the second part of extraction. According to this mass transfer rate, lipids

diffusing from inside of the cells are extracted as soon as they reach the surface where they contact with solvent. In other words, there is no accumulation of lipids at the surface. By combining **Eq.1** and **Eq.2** with mass transfer rates, model equations representing first (**Eqs. 7a**) and second (**Eqs.7b**) period of extraction were obtained.

$$\rho * \varepsilon * \frac{\partial y}{\partial t} + \rho * U * \frac{\partial y}{\partial h} = k_f * a_0 * \rho * (y_r - y) \tag{7a}$$

$$-\rho_s * (1 - \varepsilon) * \frac{\partial x}{\partial t} = k_f * a_0 * \rho * (y_r - y)$$

$$y(0, h) = 0$$

$$\rho * \varepsilon * \frac{\partial y}{\partial t} + \rho * U * \frac{\partial y}{\partial h} = k_s * a_0 * \rho_s * x \tag{7b}$$

$$-\rho_s * (1 - \varepsilon) * \frac{\partial x}{\partial t} = k_s * a_0 * \rho_s * x$$

$$x(0) = x_0$$

$$a_0 = \frac{6 * (1 - \varepsilon)}{D_p} \tag{8}$$

In **Eq.7b**, x_0 represents remaining solid phase concentration after first part of the extraction. Partial differential equation obtained in fluid phase mass balance was converted to set of ordinary differential equations by applying finite difference method to ∂y/∂h term. First part of the extraction curve was obtained by solving fluid phase mass balance (**Eqs. 7a**) using MATLAB® whereas second part of the extraction curve was predicted with solving solid phase mass balance (**Eqs. 7b**) analytically. Analytical solution of solid phase mass balance in **Eq.9**. The MATLAB program is given in Appendix A. Average particle diameter was found as 653.5 μm from SEM image of grounded microalgae powder. SEM image is given Appendix B.

$$x(t) = x_0 * e^{-\frac{k_S * a_o * t}{(1-\varepsilon)}} \quad (9)$$

3.8 Mass Transfer Correlations Used in the Study

Calculation of Sherwood, Reynolds and Schmidt numbers require knowledge of thermophysical properties of $scCO_2$ such as density and viscosity and diffusivity of lipids in $scCO_2$. Thermophysical properties of $scCO_2$ were obtained from NIST Standard Reference Simulation Website [76]. Lipid diffusivities in $scCO_2$ were estimated by using **Eq.10** [77].

$$D_{12} = \frac{4.505 * 10^{-13} * \mu^{-0.703} * T}{M^{0.5}} \quad (10)$$

In this equation, D_{12} is lipid diffusivity in $scCO_2$, μ is viscosity of the $scCO_2$, T is the temperature and M is the molecular weight of the lipid. Triolein was used as a standard lipid molecule representing complex lipid mixture in microalgae for molecular weight calculation.

To compare mass transfer coefficients regressed from experimental data, following common correlations in mass transfer for forced convection (**Table 5**) were used.

Table 5: Common correlations in mass transfer for forced convection

Correlation	Applicability	Application	Reference
$Sh = 2 + 1.1Re^{0.6}Sc^{1/3}$	General	Evaporation from drops	Ranz and Marshall [78]
$k_f = U(1.1086Re^{-0.72}Sc^{-2/3})$	Re<10	Particle-fluid mass transfer in fixed beds	Dwivedi and Uphadhyay [79]
$k_f = U(0.765Re^{-0.82}+0.365Re^{-0.82})Sc^{-2/3}$	General	Particle-fluid mass transfer in fixed beds	Dwivedi and Uphadhyay [79]
$k_f = U(1.1Re^{-0.42}Sc^{-2/3})$	General	Particle-fluid mass transfer in fixed beds	Cussler [72]
$Sh = 0.380Re^{0.83}Sc^{1/3}$	General	$ScCO_2$ extraction of woody material in fixed bed	Lin et al. [80]

Chapter 4

RESULTS and DISCUSSION

4.1 Determination of Harvesting Point

Fig. 12 shows microalgae growth curve and nitrate concentration as a function of time which was the only nitrogen source in the growth medium. When nitrogen source was available, cells performed proliferation and a significant increase in cell concentration was detected which is the characteristic of exponential growth phase. Growth rate of the cells started to decrease as nitrate was depleted and eventually cell concentration remained almost constant indicating beginning of stationary phase where protein synthesis is inhibited and cell division almost stops.

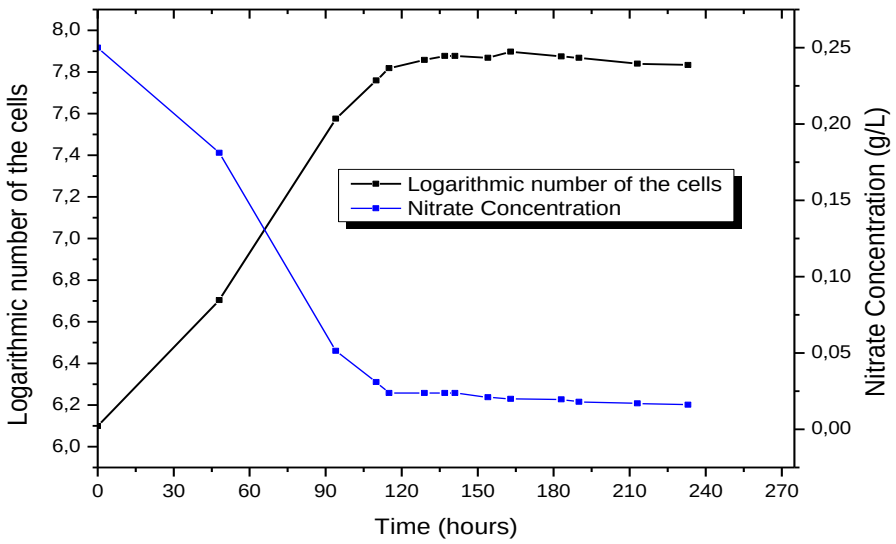

Figure 12: Variation of cell and nitrate concentration during growth

Lipid productivity of the cells determined by Nile red method is presented in **Fig.13**. With the beginning of stationary phase (~120 hours), lipid productivity started to increase and reached maximum value at early stationary phase around 140 hours. It can be seen that lipid production started with nitrogen starvation (**Fig.12**) inhibiting protein synthesis and letting carbon resources be used up for lipid production [11]. Then, a considerable decrease was seen in lipid productivity most likely due to decrease in cell viability and catabolism of lipid molecules. Significant variations in lipid productivity as a function of time shows importance of harvesting point. In order to maximize biodiesel productivity, it is crucial to harvest the cells at the right point where maximum lipid productivity is reached. It seems that early stationary phase is more appropriate for harvesting as it gives higher lipid productivity.

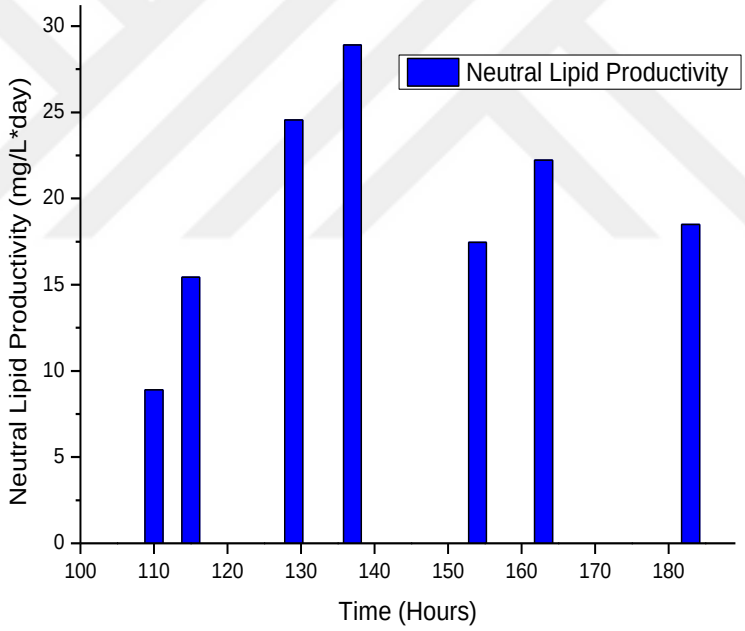

Figure 13: Variation of lipid productivity during growth

4.2 Comparison of Soxhlet Extraction with n-Hexane and $ScCO_2$ Extraction Yield

To investigate applicability of $scCO_2$ extraction for neutral lipid extraction, it was compared with most efficient and widely used conventional lipid extraction method which is Soxhlet extraction with n-hexane in terms of extraction yield. **Table 6** shows

extraction yields obtained with both Soxhlet extraction with n-hexane and $scCO_2$ extraction with different operating conditions. $ScCO_2$ extractions were performed with operating pressures of 200, 300 and 400 bar and temperatures of 40, 50 and 70 °C. Although all available neutral lipids couldn't be extracted with $scCO_2$, significant amount of them were extracted within a very short period of time. For example, it took 12 hours to reach extraction yield of 112 mg/g with Soxhlet extraction whereas it took only 3 hours with $scCO_2$ extraction performed at 400 bar and 50 °C. These results show that $scCO_2$ extraction is both efficient and fast method for extraction of neutral lipids.

Table 6: Extraction yield obtained with different operating conditions

Method	**Pressure (bar)**	**Temperature (°C)**	**Duration (hours)**	**% extract in 1 hour**	**% extract in 12 hours**	**Total Extraction yield (mg/g)**
Soxhlet	1	68	24	n.a*	81	139
$scCO_2$	200	50	3	73	n.a*	24
$scCO_2$	300	50	3	74	n.a*	65
$scCO_2$	400	50	3	76	n.a*	90
$scCO_2$	400	40	3	74	n.a*	55
$scCO_2$	400	70	3	71	n.a*	112

*not available

4.3 Effect of Operating Conditions on $ScCO_2$ Extraction Yield

In order to investigate effect of pressure on extraction yield, $scCO_2$ extraction was performed at constant temperature 50 °C and at different pressures which were 200, 300 and 400 bar. **Fig.14** shows $scCO_2$ extraction yield at different pressures. Increase in pressure at constant temperature significantly increased extraction yield. This result was expected as solvent density determines solvation power as well as solubility increases with increasing pressure [18, 20, 23].

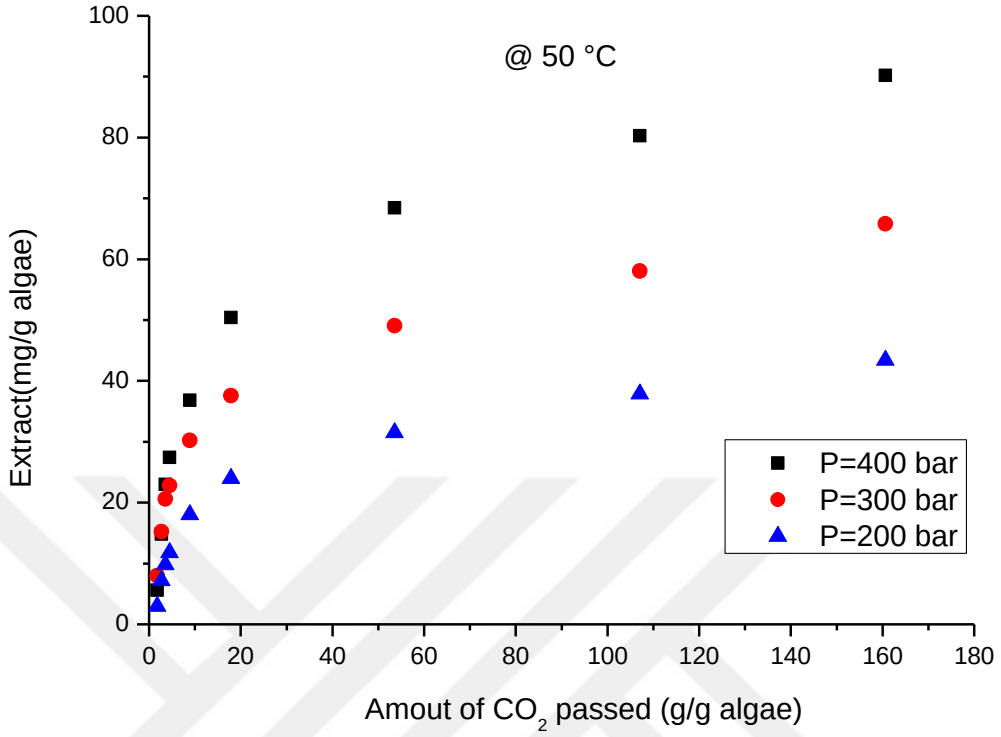

Figure 14: Extraction yields obtained at different pressures with CO_2 flow rate of 2.5 L/min

Effect of temperature on extraction yield is more complex due to competing effects of solvent density and lipid volatility. At constant pressure, increase in temperature results in higher lipid volatility which enhances solubility whereas higher operating temperatures decrease solvent density reducing solvation power and solubility. **Fig.15** shows effect of temperature on extraction yield at constant pressure. Operating pressure of 400 bar was selected for investigating effect of temperature since it gave higher extraction yield. Increasing temperature improved extraction yield due to dominating effect of lipid volatility.

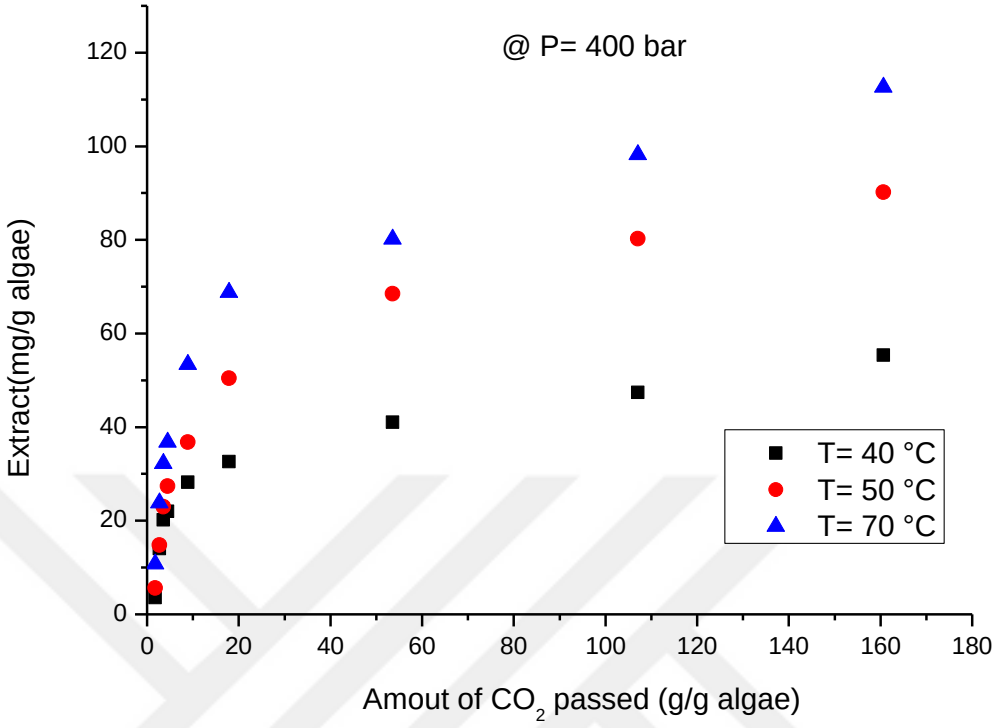

Figure 15: Extraction yields obtained at different temperatures with CO_2 flow rate of 2.5 L/min

4.4 Effect of Extraction Method and Operating Conditions on FAME Profile

Properties of biodiesel depend on degree of unsaturation and chain length of fatty acids found in FAME. Previous studies suggested that biodiesel with high MUFA content is better in terms of ignition quality, cold flow properties and iodine number whereas biodiesel with high SFA is better for oxidation stability and has higher heat of combustion and cetane number [47, 81]. Kinematic viscosity and cetane number of biodiesel increase with increasing chain length. Increase in degree of unsaturation and decrease in chain length of fatty acids increase NO_x emissions [81]. According to these findings, it is hard to define optimum FAME composition. Instead, desired characteristics should be identified and blending with conventional diesel should be done accordingly by considering conventional biodiesel properties. For example, in winter biodiesel with more MUFA content may be blended into conventional biodiesel so that fuel has better cold

flow properties. That's why it would be beneficial if FAME profile obtained from microalgal lipids can be controlled with extraction methods. In order to see effect of extraction methods and operating conditions on extraction yield, FAME profile obtained with Soxhlet extraction with n-hexane and $scCO_2$ with different operating conditions were compared. **Fig.16** shows fatty acid profile obtained with Soxhlet extraction with n-hexane and $scCO_2$ extraction. With all extraction methods, FAME mainly consisted of three major fatty acid chains C16:0, C18:1 and C18:2. However, their composition changed with extraction method and operating conditions. Similar changes on FAME profile with different extraction method and operating conditions were reported by Mendes et al. [66] whereas some other studies like Aliev et al. [24] reported that FAME profiles were not affected by extraction method and operating conditions.

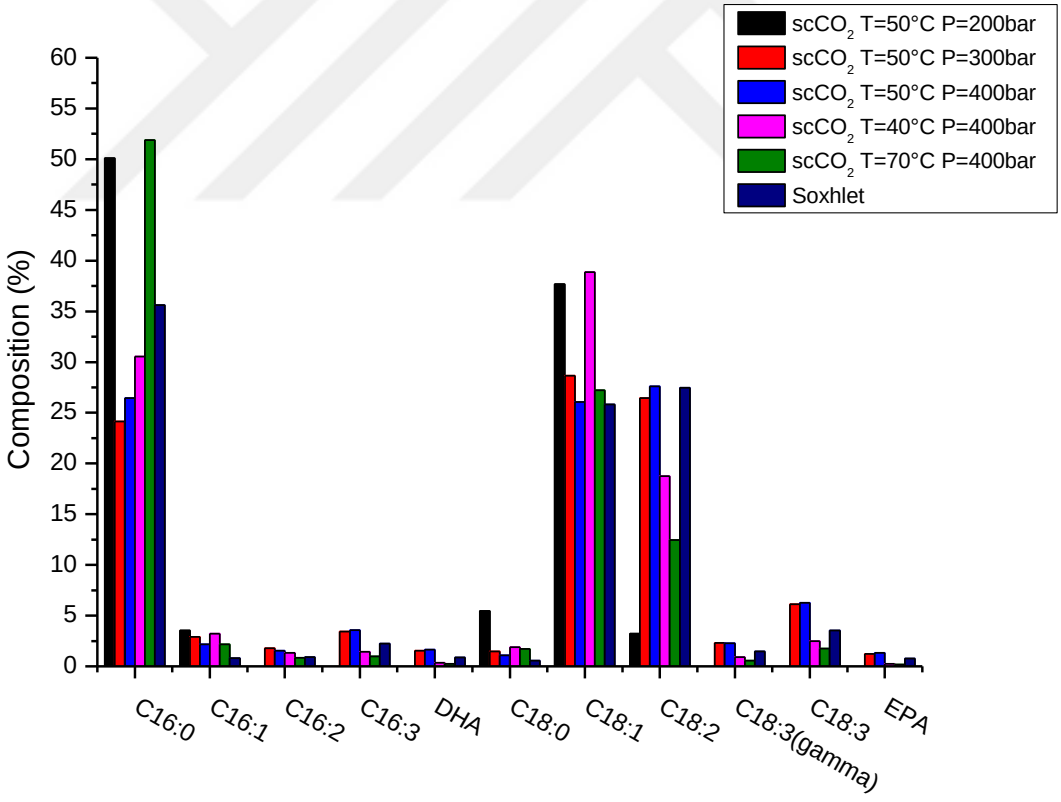

Figure 16: FAME profiles obtained with different operating conditions

Fig. 17 shows FAME composition as a function of $scCO_2$ extraction time. Extracts obtained at different time intervals were converted to FAME by transesterification. It was found that FAME profile didn't change with different time intervals.

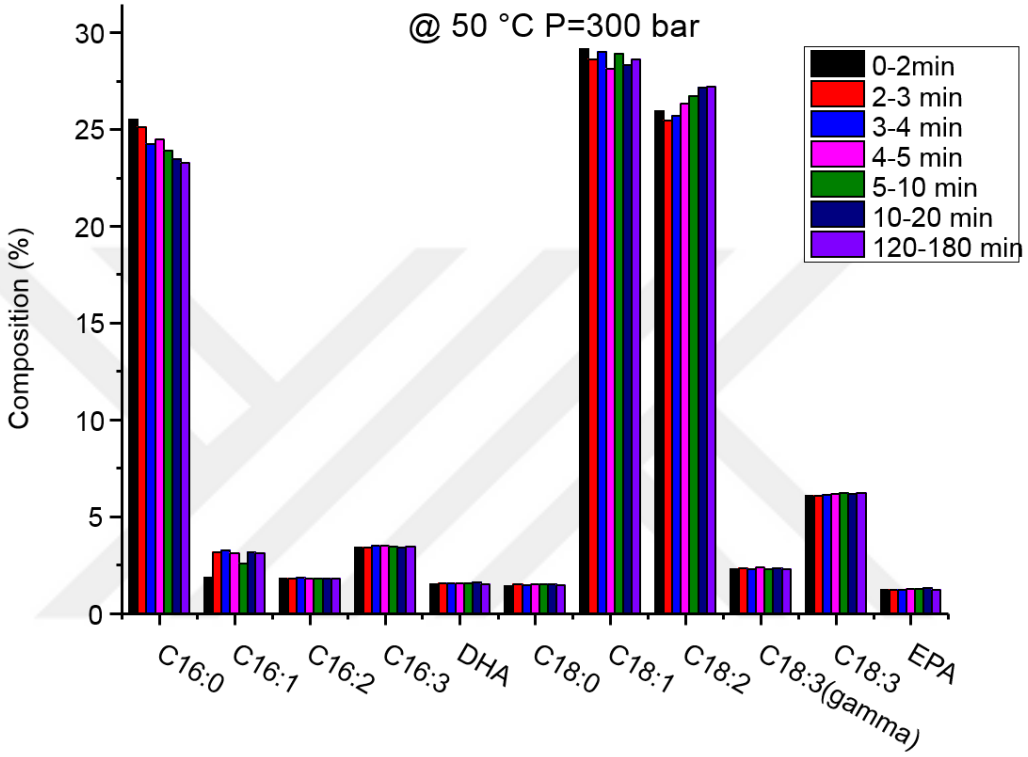

Figure 17: FAME profile obtained at 50 °C and 300 bar with CO_2 flow rate of 2.5 L/min as a function of time

Table 7 shows SFA, MUFA and PUFA contents of FAME obtained with Soxhlet extraction with n-hexane and $scCO_2$ extraction at different operating conditions. Change in extraction method and operating conditions significantly changed SFA, MUFA and PUFA content of the FAME. Increase in pressure at constant temperature significantly increased PUFA content and decreased SFA content of the extract. No trend was identified for MUFA content of the FAME.

Table 7: SFA, MUFA and PUFA content of FAME obtained with Soxhlet extraction with n-hexane and $scCO_2$ extraction with different operating conditions with CO_2 flow rate of 2.5 L/min

Method	Pressure (bar)	Temperature (°C)	ΣSFA (%)	ΣMUFA (%)	ΣPUFA (%)
Soxhlet	1	68	36	27	37
$scCO_2$	200	50	56	41	3
$scCO_2$	300	50	32	42	26
$scCO_2$	400	50	28	28	44
$scCO_2$	400	40	32	26	42
$scCO_2$	400	70	54	29	17

4.5 Extraction Curve Modeling

Experimental extraction curves were used to fit model equations given in **Eqs. 7a** and **Eqs. 7b**. In order to solve these equations, solubility of neutral lipids in $scCO_2$ at operating conditions were required. Extract obtained from microalgae may contain both neutral lipids and other complex molecules and solubility measurement can be tedious and uncertain. Thermodynamic and empirical approaches have been proposed for determining solubility [18]. Thermodynamic approach requires complex modeling due to complex nature of extract. That's why, solubility of neutral lipids in $scCO_2$ is generally approximated with slope of the first part of the experimental extraction curve known as empirical approach as it is assumed that CO_2 leaving the extractor is saturated with neutral lipids at the first part of the extraction [20, 23, 68]. Same approach was used in this study. **Table 8** shows predicted solubility of neutral lipids in $scCO_2$ with different operating conditions. Positive effect of increase in both pressure and temperature on solubility was observed. Fatty acid profile of *Chlorella vulgaris* is similar to vegetable oil. When solubility values obtained in this study are compared with vegetable oil solubility in $scCO_2$, it can be seen that they are comparable indicating similar chemical composition of vegetable oil and *Chlorella vulgaris* lipids [68].

Table 8: Predicted solubility of neutral lipids in $scCO_2$ as a function operating pressure and temperature

Temperature (°C)	**Pressure (bar)**	**CO_2 Density (kg/m^3)**	**Predicted Solubility (mg/gCO_2)**
40	400	956.1	5.7
50	200	784.3	2.7
50	300	870.4	5.8
50	400	923.3	7.6
70	400	856.7	9.0

Experimental and model extraction curves obtained with different operating temperatures are presented in **Figs. 18-22**. Fluid and solid phase mass transfer coefficients were also included. Very fast first extraction period followed by slower second extraction period can be observed at all operating conditions. Deviation from this behavior indicating beginning of second extraction period started after 5 g CO_2 was passed per g of algae (5 min of extraction time). Combination of model extraction curves successfully predicted experimental extraction curves with appropriate fluid and solid mass transfer coefficients which were on the order of 10^{-4} m/s and 10^{-8} m/s, respectively.

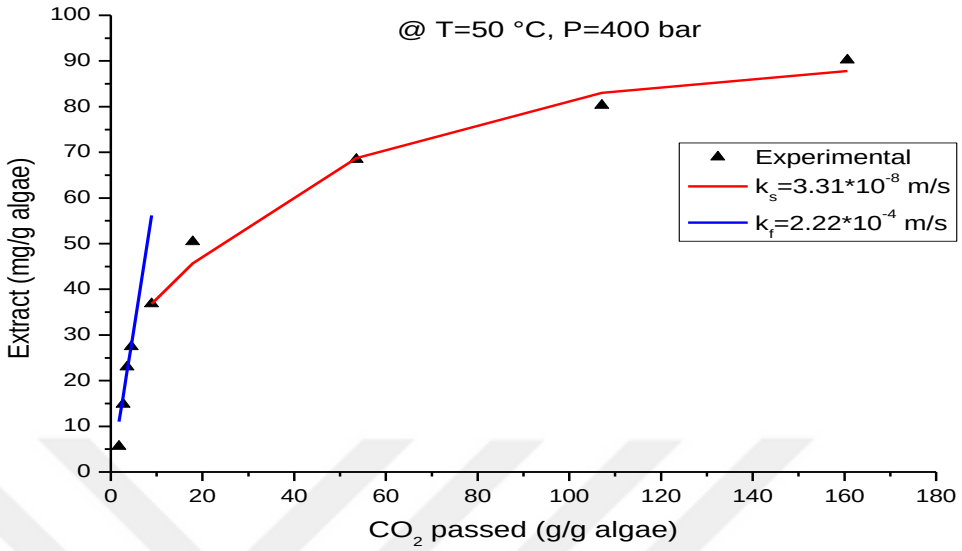

Figure 18: Experimental and model extraction curves obtained at 50 °C 400 bar with CO_2 flow rate of 2.5 L/min

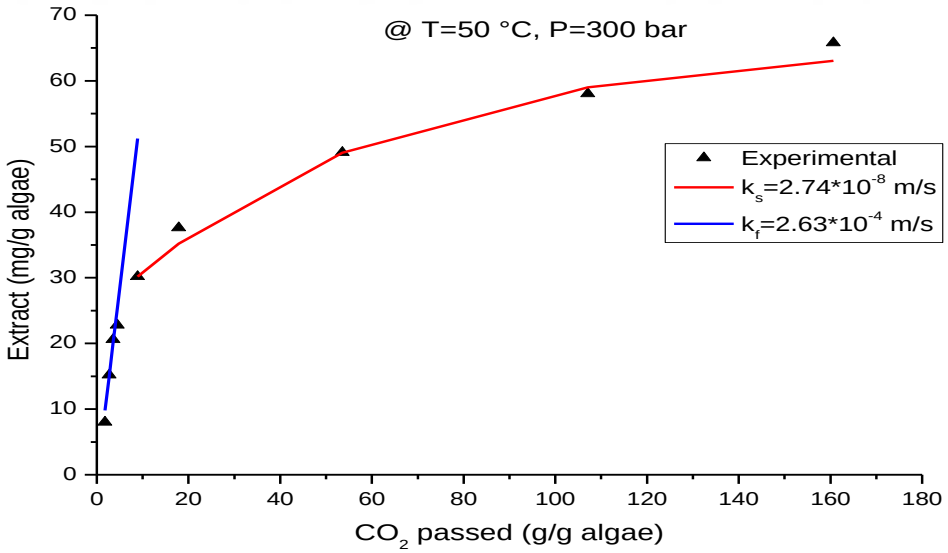

Figure 19: Experimental and model extraction curves obtained at 50 °C 300 bar with CO_2 flow rate of 2.5 L/min

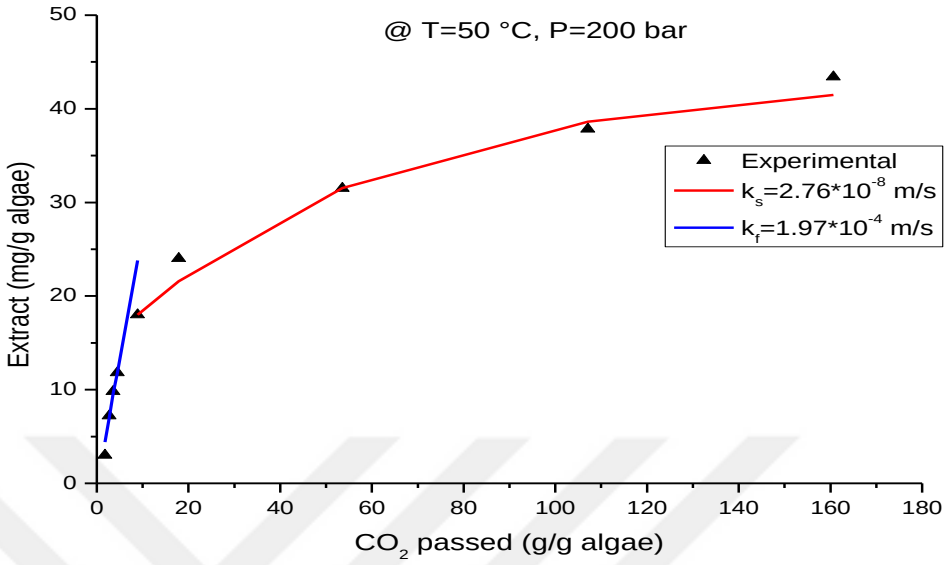

Figure 20: Experimental and model extraction curves obtained at 50 °C 200 bar with CO_2 flow rate of 2.5 L/min

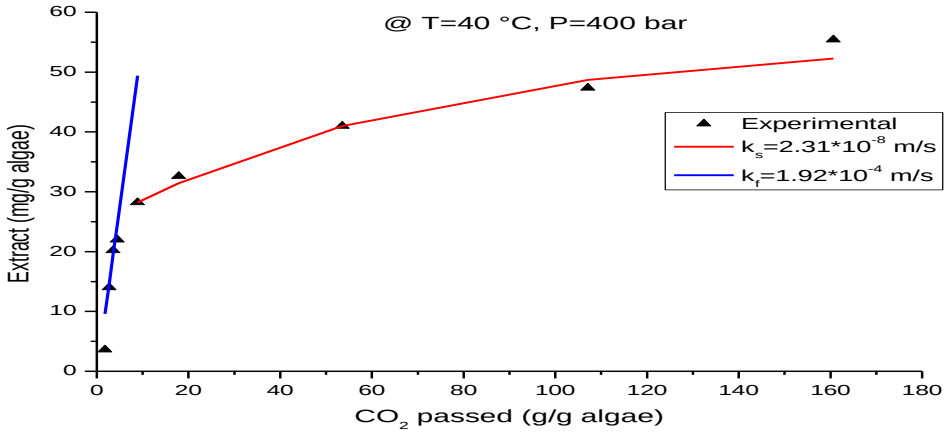

Figure 21: Experimental and model extraction curves obtained at 40 °C 400 bar with CO_2 flow rate of 2.5 L/min

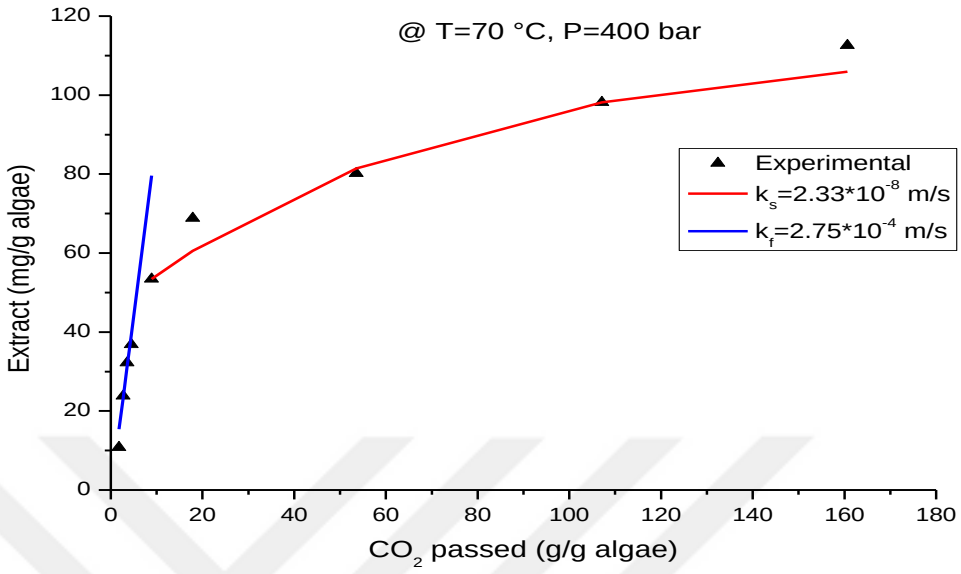

Figure 22: Experimental and model extraction curves obtained at 70 °C 400 bar with CO_2 flow rate of 2.5 L/min

Fluid and solid phase mass transfer coefficients obtained for different operating conditions are presented in **Table 9**. Since controlling mechanism is different in first and second extraction period, fluid and solid phase mass transfer coefficients should be analyzed separately. In first period of the extraction, convective mass transfer is dominant. Increase in pressure at constant temperature decreased fluid phase mass transfer coefficient. Fluid mass transfer coefficient is directly proportional to diffusivity (**Eq.4**). Since an increase in pressure results in an increase in viscosity and thus a decrease in diffusivity (**Eq.8**), negative effect of pressure on fluid phase mass transfer coefficient is expected. Second extraction period is controlled by internal diffusion. Although diffusivity is also affected by temperature and pressure, main factor which controls internal diffusion is the morphology of the solid microalgae matrix which was almost identical in all experiments. That's why comparable solid phase mass transfer coefficients were obtained at different operating conditions.

Table 9: Fluid and solid phase mass transfer coefficients obtained by fitting experimental data to a mathematical model at different operating conditions with CO_2 flow rate of 2.5 L/min

Temperature(°C)	Pressure(bar)	k_f (m/s)	k_s (m/s)
40	400	$1.92*10^{-4}$	$2.31*10^{-8}$
50	200	$3.0*10^{-4}$	$2.76*10^{-8}$
50	300	$2.63*10^{-4}$	$2.74*10^{-8}$
50	400	$2.22*10^{-4}$	$3.31*10^{-8}$
70	400	$2.75*10^{-4}$	$2.33*10^{-8}$

4.6 Mass Transfer Correlations

In order to predict fluid phase mass transfer coefficients from correlations for forced convection, physicochemical properties of $scCO_2$, diffusivity of lipids in $scCO_2$, Reynolds and Schmidt number which are presented in **Table 10** were needed.

Table 10: Physicochemical properties of $scCO_2$ and calculated dimensionless numbers with different operating conditions

Temperature (°C)	Pressure (bar)	Density (kg/m^3)	Viscosity (Pa.s)	Diffusivity (m^2/s)	Re	Sc
40	400	956.1	0.00011	$2.9*10^{-9}$	6.5	37.9
50	200	784.3	0.00007	$4.1*10^{-9}$	9.7	21.2
50	300	870.4	0.00008	$3.6*10^{-9}$	8.1	27.3
50	400	923.3	0.00010	$3.2*10^{-9}$	6.8	32.6
70	400	856.7	0.00008	$3.9*10^{-9}$	8.2	24.9

Fluid phase mass transfer coefficients obtained from both correlations and experimental data are presented in **Table 11**. Best agreement was found with correlation $Sh = 2 + 1.1Re^{0.6}Sc^{1/3}$ which was developed for evaporation from drops.

Table 11: Comparison of fluid phase mass transfer coefficients obtained from correlations and experimental data

10	T (°C)	P (bar)	k_f*10^5 (correlation)	$kf*10^5$ (Exp.)	Percent Error (%)
$Sh = 2 + 1.1Re^{0.6}Sc^{1/3}$	40	400	6.0	19.2	221
	50	200	8.8	30.0	241
	50	300	7.4	26.3	255
	50	400	6.5	22.2	242
	70	400	7.9	27.5	250
$k_f = U(1.1086Re^{-0.72}Sc^{-2/3})$	40	400	2.8	19.2	581
	50	200	3.7	30.0	720
	50	300	3.3	26.3	707
	50	400	3.0	22.2	641
	70	400	3.4	27.5	700
$k_f = U(0.765Re^{-0.82}+0.365Re^{-0.386})Sc^{-2/3}$	40	400	3.3	19.2	473
	50	200	4.6	30.0	554
	50	300	4.0	26.3	560
	50	400	3.6	22.2	520
	70	400	4.2	27.5	553
$k_f = U(1.17Re^{-0.42}Sc^{-2/3})$	40	400	2.0	19.2	838
	50	200	3.0	30.0	903
	50	300	2.5	26.3	943
	50	400	2.2	22.2	905
	70	400	2.7	27.5	930
$Sh = 0.380Re^{0.83}Sc^{1/3}$	40	400	2.7	19.2	611
	50	200	4.4	30.0	584
	50	300	3.5	26.3	646
	50	400	3.0	22.2	651
	70	400	3.7	27.5	634

In all operating conditions, fluid phase mass transfer coefficients obtained from experimental data are higher than fluid phase mass transfer coefficients obtained from correlations. This indicates that extraction occurs faster than predicted and common correlations bring significant errors while predicting mass transfer coefficients. Possible

reason of errors is particle size used in this study. During development of the mathematical model, it was assumed that at first part of the extraction some of the lipids are directly exposed to $scCO_2$. That's why, particle size should be defined as the size of lipid droplets available in microalgae powder not microalgae particle size. However, it is hard to measure and it may not be uniform. That's why, more detailed study is required for determination of the particle size. After this step, more appropriate mass transfer correlations can be identified or developed.

Chapter 5

CONCLUSIONS

Microalgae *Chlorella vulgaris* were cultivated in flat panel type photobioreactor for biodiesel production. Early stationary phase was found to be best harvesting point for biodiesel production as it gave highest lipid productivity. Similar extraction yields were obtained faster with $scCO_2$ extraction especially when compared with Soxhlet extraction with n-hexane. Lipid solubility in $scCO_2$ and $scCO_2$ extraction yield significantly increased with increasing pressure at constant temperature and increasing temperature at constant pressure. Sovova's model predicted effluent lipid concentration as a function of time indicating fast extraction period followed by slow extraction period fairly well. Fluid and solid phase mass transfer coefficients were regressed from experimental data and they were varied in the range between $1.92*10^{-4}$ and $3.0*10^{-4}$ m/s and $2.31*10^{-8}$ and $3.31*10^{-8}$ m/s, respectively. Mass transfer correlation $Sh = 2 + 1.1 * Re^{0.6} * Sc^{1/3}$ was found to predict fluid phase mass transfer coefficients better than other mass transfer correlations used in this study. Extraction method and operating conditions changed FAME profiles significantly.

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APPENDIX A

MATLAB Code Used for Solution of Mass Transfer Modeling Equations

```
function y=salim(t,yo);
global n height kf ye p eparticle a0 dext As QCO2 UCO2 eCO2 mextract
time mCO2 yexp eCO2vessel

kf=35e-5;  %liquid phase mass transfer coefficient (m/s)
p=0.4;  % porosity
d0=653.5e-6; % microalgae particle diameter (m)
a0=6*(1-p)/d0; %spesific surface area (1/m)
dext=0.015; % Extractor bed diameter(m)
As=(pi*dext^2)/4; % Flow surface area (m^2)
QCO2=(2.5*10^-3)/60; %Volumetric flow rate of CO2 (m^3/s)
eCO2=1.7842*10^3; % g/m^3 @1 bar 25 °C
eCO2vessel=784.3*10^3;% g/m^3 @400 bar 50 °C
eparticle=1.1*10^6; %g/m^3
UCO2=(QCO2*eCO2/(As*eCO2vessel*p)); %interstital velocity of CO2 (m/s)

ye=2.75e-3; %solubility of lipids in scCO2 (mg/g)
height=0.025; %m
delz=height/n; %m
yo(1)=0.;
y(1)=0;
for i=2:n+1
    y(i)=((kf*a0*(ye-yo(i)))/p)-UCO2*((yo(i)-yo(i-1))/delz)/p;
end
%pause
y=y';

tspan=[0:10:1800];
n=49;
N0=zeros(n+1,1);

[t,N]=ode45('salim',tspan,N0);
```

APPENDIX B

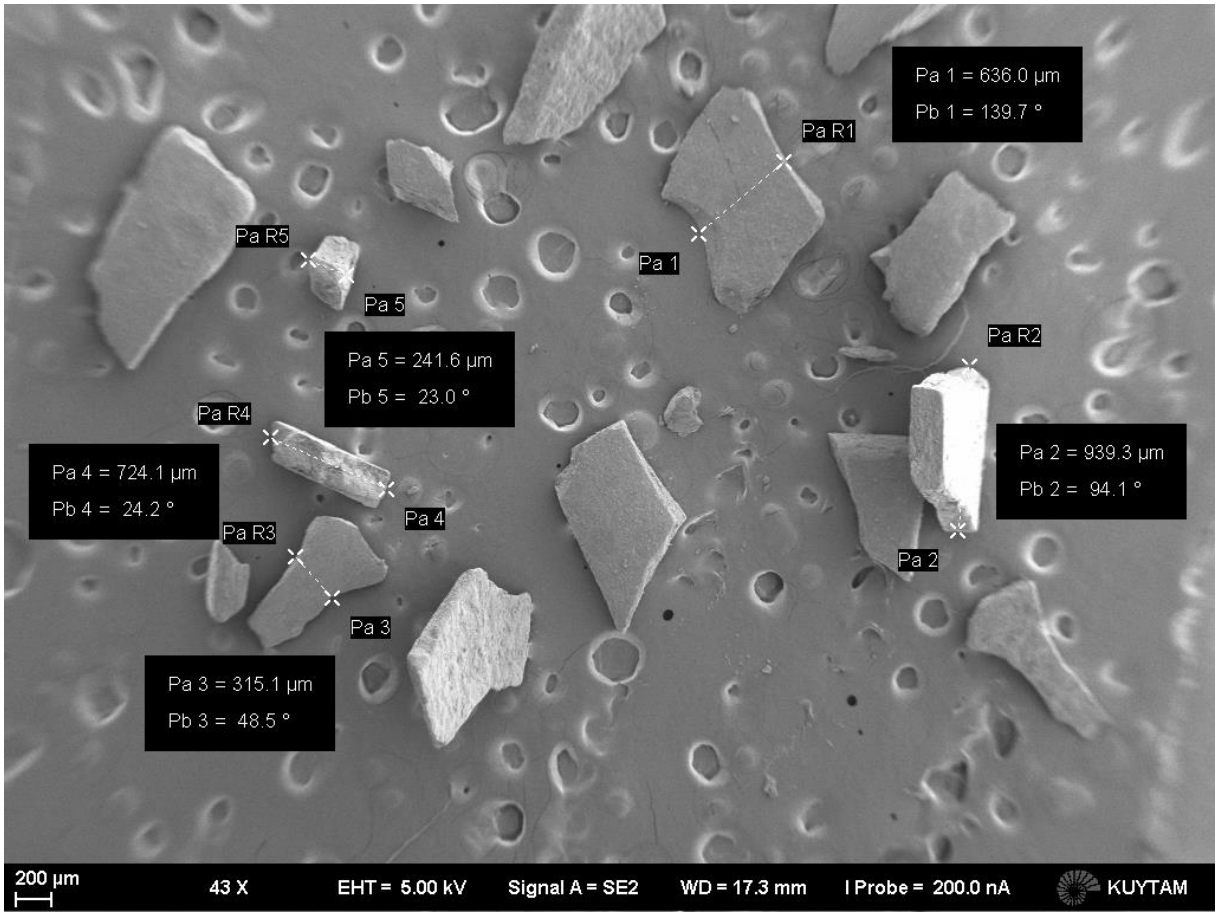

Figure 23: Dry microalgae powder SEM image