BIODIESEL PRODUCTION FROM MUNICIPAL WASTEWATER TREATMENT PLANT SLUDGE BY DIRECT LIQUID-LIQUID LIPID EXTRACTION METHOD

by

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ABSTRACT

BIODIESEL PRODUCTION FROM MUNICIPAL WASTEWATER TREATMENT PLANT SLUDGE BY DIRECT LIQUID-LIQUID LIPID EXTRACTION METHOD

Energy plays a significant role in sustaining the modern society. Currently, most of the energy demand is satisfied from fossil sources which are non-renewable and cause environmental problems. In order to eliminate these problems, biodiesel is accepted to be the best alternative energy source as it can be used directly without requiring any kind of modification and it is harmless to the environment. Biodiesel production is not expanded because of high raw material costs. Thus, it is essential to find a cheap alternative feedstock, uncompetitive with food market and easily available in large quantities. Municipal wastewater treatment plant (WWTP) sludges have high lipid content and can be used as feedstock for biodiesel production. The aim of the study was explore the lipid extraction efficiency for biodiesel production from municipal WWTP sludges by using the novel direct liquid-liquid lipid extraction method eliminating the expensive dewatering and drying steps and compare it with standard lipid extraction method. The study also comparatively investigated the effects of acid, ultrasonic and microwave pretreatments on the lipid and biodiesel yields. The results of the study showed that the primary sludge is better lipid feedstock for biodiesel production compared to secondary sludge. Although higher lipid yields were attained from primary sludge using the standard method compared to the direct liquid-liquid lipid extraction method, almost the same biodiesel yields were achieved by both of the extraction methods. Acid pre-treatment increased the lipid and biodiesel yields. The highest increasing impact was obtained by the application of combined acidification/ultrasonication pretreatment.

ÖZET

DİREKT SIVI-SIVI LİPİT EKSTRAKSİYON YÖNTEMİ İLE BELEDİYE ATIKSU ARITMA TESİSİ ÇAMURUNDAN BİYODİZEL ÜRETİMİ

Enerji, modern toplumun sürdürülebilmesinde önemli bir rol oynamaktadır. Günümüzde, enerji ihtiyacının çoğu yenilenemeyen ve çevresel sorunlara neden olan fosil kaynaklardan karşılanmaktadır. Fosil kaynakların sebep olduğu bu problemlerden kurtulmak için biyodizel, herhangi bir modifikasyon gerektirmeden doğrudan kullanılabileceği ve çevre dostu olduğundan dolayı en iyi alternatif enerji kaynağı olarak kabul edilmektedir. Hammadde maliyetinin yüksek olması nedeniyle biyodizel üretimi yaygınlaşamamaktadır. Bu nedenle ucuz, gıda pazarı ile rekabette olmayan ve büyük miktarlarda kolayca ulaşılabilir alternatif bir hammadde bulmak gerekir. Belediye atıksu arıtma tesisi (AAT) çamurları yüksek oranda lipit içerdiğinden dolayı biyodizel üretimi için hammadde olarak kullanılabilir. Bu çalışmanın amacı çamur susuzlaştırma ve kurutma aşamalarını gerektirmeyen direkt sıvı-sıvı lipit ekstraksiyon yöntemini kullanarak atıksu arıtma çamurlarından elde edilen lipit ekstrasyon verimini araştırmak ve bu yöntemi standart lipit ekstraksiyon yöntemiyle karşılaştırmaktır. Çalışma ayrıca asit, ultrasonik ve mikrodalga ön işlemlerinin lipit ve biyodizel verimleri üzerindeki etkilerini karşılaştırmalı olarak incelemiştir. Çalışmanın sonuçları birincil çamurun ikincil çamura kıyasla biyodizel üretimi için daha iyi bir lipid hammaddesi olduğunu gösterdi. Birincil çamurun, direkt sıvı-sıvı lipit ekstraksiyon yöntemine kıyasla standart yöntem kullanılarak daha yüksek lipit verimleri vermesine rağmen, her iki ekstraksiyon yöntemi kullanılarak elde edilen biyodizel verimlerinin hemen hemen aynı miktarda olduğu tespit edilmiştir. Lipit ekstraksiyonu öncesinde asit ilavesi lipit ve biyodizel verimlerini arttırdı. En yüksek arttırıcı etki, asitlendirme/ultrasonikasyon ön işlemlerinin kombine olarak uygulanmasıyla elde edildi.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol/Abbreviation	**Explanation**	**Unit**
$AgSO_4$	Silver Sulfate	
CO	Carbon Monoxide	
CO_2	Carbon Dioxide	
COD	Chemical Oxygen Demand	mg/L
EU	European Union	
FAME	Fatty Acid Methyl Esters	%
FFA	Free Fatty Acids	%
FID	Flame Ionization Detector	
GC	Gas Chromatograph	
GHG	Greenhouse Gas	
HC	Hydrocarbon	
HCl	Hydrogen Chloride	
Hg	Mercury	
$HgSO_4$	Mercury Sulfate	
H_2O_3S	Sulphonic Acid	
H_2SO_4	Sulfuric Acid	
K_2CrO_7	Potassium Dichromate	
KHP	Potassium Hydrogen Phthalate	
KOH	Potassium Hydroxide	
$MgSO_4.H_2O$	Magnesium Sulfate Monohydrate	
MLSS	Mixed Liquor Suspended Solids	mg/L
MW	Microwave	
MLVSS	Mixed Liquor Volatile Suspended Solids	mg/L
NaOH	Sodium Hydroxide	
$NaHCO_3$	Sodium Bicarbonate	
NO_x	Nitrogen Oxides	
ORP	Oxidation Reduction Potential	mV
sCOD	Soluble Chemical Oxygen Demand	mg/L
SO_x	Sulfur Oxides	
SO_2	Sulfur Dioxide	
TS	Total Solids	mg/L

US	Ultrasonication	
VS	Volatile Solids	mg/L
WWTP	Wastewater Treatment Plant	

1. INTRODUCTION

Energy is one of the indispensable factors for humanity and constitutes the basis of economic development. Increase in urbanization and industrialization with development of the society, population growth and rising the standard of living increase the energy demand in the world. Total world energy consumption will be 37 % more in 2040 compared to the world's current situation, according to the International Energy Agency. Nowadays, 85 % of all energy consumed comes from fossil fuel sources of which worldwide reserve distributions are 33 % coal, 39 % petroleum and 28 % natural gas. The alternatives to these resources, such as nuclear power, hydropower, and biomass costitute only 15 % (British Petroleum, 2017). Even though the use of non-fossil fuels is anticipated to increase faster than fossil fuels, fossil fuels will still constitute of 77 % of total energy consumption in 2040 (International Energy Agency, 2017). The fossil fuel sources are non-renewable and will be exhausted in the near future. There is a prediction is that coal reserves will be consumed by 2112 while oil and gas will be available up to 2040 and 2042, respectively (International Energy Agency, 2014).

The world oil (petroleum) demand will increase by 9,5 % in 2030 and 19 % in 2040. The majority of increase in oil consumption is in the transportation and industrial sectors. In the transportation sector which is the second largest energy consuming sector, oil maintains to meet most of the energy demand. As number of cars increases around the world, transportation sector will be become strongest growing energy consumption sector. Oil consumed by transportation will rise by 0,7 % per year from 2015 to 2040. At present, the transportation sector is responsible for 60 % of world oil demand (Atabani et al., 2012; U.S. Energy Information Administration, 2017). Extreme usage of fossil fuels leads to exhaustion of fossil fuel reserve and some problems such as greenhouse gas (GHG) emissions, air pollution and global warming. According to the studies, the global energy gap and pollution will increase by 50 % until 2030 if the use of energy sources is not changed (Alptekin, 2016). In addition to effect of GHG on global warming, it has also impacts on the environment and human life. Human societies and industries produce huge amount of the wastes. Accumulation of extremely polluted wastes threatens soil and fresh water resources because of high nutrient, toxic matters, and organic substances containing oils and fats (Willson et al., 2010).

Taking account energy profile of Turkey into consideration, economic development of Turkey has been growing rapidly due to the increase in its own energy consumption. Turkey has become the second country after China in terms of increase in need for electricity and natural gas. Due to the fact that Turkey can satisfy only 26 % of its total energy need by using its own finite domestic energy, it depends on energy imports, primarily of oil and natural gas. Approximately 90 % of oil supply and 98 % of natural gas supply are imported. According to the Ministry of Energy and Natural Resources (MENR) statistics, petroleum products, natural gas and coal compose of the primary energy consumption in Turkey. It is expected that the total primary energy demand will be 218 Mtoe by 2023 which means nearly 75 % increase from the current level of 125 Mtoe. Recently, primary energy consumption is based on coal (28,5 %), natural gas (35 %), oil (27 %), hydro (17 %) and other renewables (2,5 %) in Turkey (MENR, 2018). Energy production and consumption are mainly composed of fossil fuel producing carbon dioxide (CO_2) emissions that result in many environmental problems. Owing to many factors such as the dependence of Turkey on import energy supplies, the depletion of petroleum derived fuels and increasing prices of petroleum products, domestic renewable energy sources such as hydro, wind, biofuels, and biodiesel have to be utilized (Boluk et al., 2013).

In order to overcome the problems related with non-renewable sources, the use of renewable energy resources having little or no environmentally impact is the best solution. Among the alternative fuel sources such as alcohol, biomass, biogas, biodiesel and synthetic fuels, biodiesel is the best alternative to traditional diesel (Srivastava et al., 2000). The reasons behind choosing biodiesel as the best candidate for diesel fuels are sevaral. Biodiesel is gaining traction as a renewable trasportation fuel in the United States after obtaining a remarkable success in Europe. Its primary advantage is that it does not require new refuelling station. Biodiesel can be used efficiently as both, blend with conventional diesel fuel and in a pure form. In addition, biodiesel is environmentally friendly, less toxic, renewable and biodegredable (four times faster than conventional diesel), safer for storage and handling. Furthermore, it has perfect lubricity and can provide energy density similar to diesel. It has low emission profiles compared to petroleum diesel as it includes oxygen and decreases most exhaust emissions (CO_2, carbon monoxide (CO), particulate, hydrocarbons, except nitrogen oxides (NO_x)) and decreases emissions of sulfur oxides (SO_x) and sulfates (Siddiquee et al., 2011; Olkiewicz et al., 2015). The use of biodiesel as an alternative energy source would decrease 30 % of discharge of sulfure content and 10 % of carbon monoxide. In addition, biodiesel does not include any aromatic compounds and other chemical components which are destructive to the environment. Recent studies have demonstrated that air toxicity and cancers can be cut down by 90 % and 95 % respectively, compared to conventional diesel source, by using biodiesel as energy source (Sharp, 1996).

Turkey has various feedstocks for biodiesel production; however, utilization of the biodiesel production capacity is not been entirely provided. Turkey has a capacity of 235 thousand tons for biodiesel production, annually, but only 70 thousand tons biodiesel was produced in 2017. Although there were more than 100 small scale biodiesel production plants in 2011, most of them are repealled. In Turkey, the progress of biodiesel industry is prevented due to the improper changes in regulative environment and relevant tax laws. Many licensed biorafineries have stopped production because of bureaucratic procedures and high tax rates. Recently, 23 biodiesel plants have operating licenses and 15 facilities have distribution licenses (Ar et al., 2010; EMRA, 2016). In order to increase the use of renewable enegy applications supporting procedures should be implemented.

Chemically, biodiesel which is the known as fatty acid methyl ester (FAME), can be defined as the mono alkyl esters of long chain fatty acids obtained from lipid sources by transesterification of pre-extracted oils with alcohol by using a catalyst (Siddique et al., 2011). Currently, biodiesel is produced from a variety of feedstocks such as soybean, rapeseed, palm, sunflower and coconut oils, waste cooking oils and animal fat. The overall biodiesel production cost is high due to fact that pure vegetable oil and seed oils constitute between 70 % and 85 % of the total cost (Haas et al., 2005). Because of the limited supply of these feedstocks and high cost of them, the further expansion of biodiesel production is blocked. Therefore there is a need for alternative feedstocks that are readily available and cheap to produce biodiesel. Among the alternative biodiesel feedstocks, municipal wastewater treatment plant (WWTP) sludge can be considered to be a good lipid feedstock because it is plentiful and includes significant amount of lipid (Mondala et al., 2009; Kargbo, 2010).

Previous investigations have demonstrated that municipal sludges from WWTPs are hopeful lipid feedstocks for biodiesel production due to their high lipid content. In most studies, dry sludge was used as a raw material to produce biodiesel from municipal primary and secondary sludges. As municipal wastewater sludges have high water content of 95-98 % by weight, the energy cost of sludge dewatering and drying processes almost 50 % of the total biodiesel production cost making the whole process very expensive and difficult the scale up (Dufreche et al., 2007, Olkiewicz et al., 2014). In order to eliminate the expensive preliminary step of sludge drying, some researchers used dewatered sludge without drying for biodiesel production. However, the energy necessary for water elimination still constitutes 14 % of the total biodiesel production cost (Dufreche et al., 2007). Moreover, the use of conventional thermal drying or freezee-drying to remove water from sludge causes to the loss of valuable organic compounds leading to loss of lipids and decrease of biodiesel production yield (Blight et al, 1959). The proposed alternative, direct liquid-liquid lipid extraction method has rather limited information on the biodiesel production from municipal WWTP (sewage) sludges. The novel direct liquid-liquid lipid extraction tecnique was investigated by Olkiewicz et al, (2014), but the effects of pretreatment methods on biodiesel production by using this method has not been studied up to now.

The aim of this study was to investigate the efficiency of direct liquid-liquid extraction method to obtain lipid from municipal WWTP sludges for biodiesel production without applying any expensive sludge dewatering or drying steps. The study also investigates the application of pre-acidification, ultrasonication and microwave pretreatments to the sludge samples to show the effects of pretreatments on the lipid and biodiesel yields. The direct liquid-liquid extraction of lipid from sludge samples was compared with the conventional reference drying method 5520E. Two different types of municipal WWTP sludges "primary sludge" and "secondary sludge" were used for biodiesel production by using both of the lipid extraction techniques. Optimization of the direct liquid-liquid lipid extraction was studied by changing the organic solvents (chloroform, hexane, petroleum ether and toluene), contact times of organic solvent with sludge samples, the sludge to solvent ratios (by volume) and the number of sequential lipid extraction stages in order to acquire the most appropriate process resulting in the maximum lipid yield.

2. LITERATURE REVIEW

2.1. Biodiesel

Rapidly growing world population, inattentive and unbalanced use of fossil resources, increasing industrial production and unlimited human needs have been rapidly increasing demand for energy in the world. Increasing energy demand leads to overuse and consumption of fossil resources and more importantly environmental damage that is not reversed. As the increasing environmental problems over time are not limited to the regional dimension, become major problems in the global dimension. Researchers have searched for alternative energy sources. The production and use of alternative fuels such as biofuels, which are supported by developed countries, have gained importance. Among the alternative fuels, biodiesel is preferred because it is renewable and contains less hydrocarbon (HC), carbon monoxide (CO), sulfur dioxide (SO_2), mercury (Hg) and particulate in exhaust emissions than fossil fuels (Yasar, 2009; Szulczyk et al., 2010). In addition, if biodiesel is produced on a large scale it reduces demand for fossil fuels and creates a new market for agricultural raw materials. Biodiesel attracts worldwide attention leading an increase in the amount of biodiesel production. Biodiesel, an alternative renewable fuel, is monoalkyl esters of long chain fatty acids derived from a variety of feedstock.

2.1.1. Biodiesel Feedstocks

The feedstock selection for biodiesel production depends on the oil content of feedstock, process chemistry and the economy of the whole biodiesel production process. The cost of raw material constitutes 80 % of the total production cost. Therefore, the choice of the cheapest feedstock is very significant to reduce the cost of the process (Karmakar et al., 2010). Mainly used feedstocks for biodiesel production can be categorized as; edible and non-edible plant oils, algae, animal fats, waste cooking oil and municipal WWTP sludges.

Primarily feedstocks used for biodiesel production are edible plant oils that costitute more than 95 % of total biodiesel production in the world such as rapeseed (84 %), sunflower oil (13 %), palm oil (1 %), soybean oil and others (2 %). Increase in the use of edible plant oils to satisfy rising demand for biodiesel production has resulted to increase in the worldwide plant oil market. Their fields have been founded in many countries in the world such as USA, Germany and Malaysia (Atabani et al., 2012; Khan et al., 2014). Cultivation of biodiesel feedstocks has been limited due to the lack of agricultural lands reducing the amount of biodiesel production. Due to fact that edible plant oils are basically prefered as food materials, there is a competition between food sector and fuel production making more expensive the use of these edible oils as a feedstock to produce biodiesel (Olkiewicz et al., 2012; Monisha et al., 2013). It is unavoidable that there is a high cost to process pure vegetable or seed oils which limits the development and commercialization of biodiesel production. This process composes of 70-80 % of whole biodiesel production cost (Haas et al., 2005; Revellame et al., 2010; Olkiewicz et al., 2015). Additionally, the overuse of edible plant oil seeds causes many concerns such as food and fuel crisis and also significant environmental problems such as forest destruction, damage of vital soil resources and use of many existing cultivable lands (Atabani et al., 2012; Thompson, 2012).

Dependency on edible plant oils can be reduced by using non-edible plant oils, such as jatropha, neem, castor, karanja, pongomia, and sea mango for biodiesel production (Gui et al., 2008). Non-edible plant oils gather more interest around the world as a biodiesel feedstock because they are grown wastelands that are not convient for planting of food crops eliminating land use competing with food production. In addition, they are more economical and more environmentally friendly compared to the edible plant oils. However, their extreme cultivation may result in destruction of the ecosystem and deforestation (Atabani et al., 2012; Khan et al., 2014).

Algae can be also used as a significant feedstock for biodiesel production. It has been considered as a very promising alternative to plant oils due to its higher growth ratios, high photosynthetic activity to generate biomass and higher lipid content compared to edible and non-edible feedstocks. Algae has the potential to achieve biodiesel yield that is up to 200 times higher than the yield from that of vegetable oils as algae can be grown naturally all over the world and throughout the year (Sheehan et al., 1998; Atabani et al., 2012). However, the commercialization of biodiesel production from algae is limited because of the need for high-oil-yielding algae species and the need for efficient large-scale bioreactors (Vasudevan et al., 2008).

Animal fats such as poultry fat and beef tallow also can be used as feedstock to produce biodiesel eliminating the requirement to dispose them. Animal fats are easily available, but those obtained from the contaminated animals have biosafety problems (Janaun and Ellis, 2010). Additionally waste cooking oil is considered to be a good feedstock as it does not require the production stage. In this case, the use of these feedstocks will be more cost-effective. Discharge of waste cooking oils sourced from kitchens, restaurants, hospitals, institutions brings extra pollution load into wastewater and causes to adhesion of wastes in the sewage pipe leading to narrowing of the pipeline. Therefore, the use of waste cooking oils in biodiesel production does not only reduces the total production cost, but also reduces the negative impact of the waste cooking oils on the environment (Alptekin et al., 2006). However, there is a great problem related with collection substructure and logistics in order to produce enough amount, as the sources are usually dispersed (Atabani et al., 2012).

Biodiesel production is an expensive process due to the high cost of the raw materials. The raw materials are responsible for 80 % of the total cost which prevent expansion of biodiesel production process (Haas et al., 2005; Mondala et al., 2009; Revellama et al., 2010). Therefore, there is an urgent demand for a low cost, easily accessible, and plentiful lipid sources. Municipal WWTP sludge is a resource that can be used a lipid feedstock for biodiesel production providing these features.

2.1.2. Municipal WWTP Sludge as a Feedstock

Municipal sludge is produced in WWTP facilities after primary and secondary processes and it has a solid, semisolid or liquid muddy looking. Municipal wastewater sludge includes a sort of organic materials suh as fats, oil and grease, detergents, plant residues, pesticidies, and solvents and inorganic matters (Bharathiraja et al., 2014). Two main types of sludge stream, primary sludge and secondary sludge (also known as activated sludge) are generated from a wastewater treatment process having an activated sludge facility. The municipal primary sludge is composed of floating grease and solids while the municipal secondary sludge is comprised primarily of microbial cells and suspended solids generated during the aerobic biological treatment. That’s why primary sludge has a different composition from secondary sludge. The primary sludge is taken from the bottom of the primary clarifier after screening and grit removal and the activated sludge is taken from the secondary clarifier. A part of collected activated sludge is recycled back to aeration basins to provide an adequate concentration of microorganisms. The rest of the sludge requires further treatment and stabilization by aerobic and anaerobic digestion to handle and dispose it (Mondala et al., 2009; Pokoo-Aikins et al., 2010).

Nowadays, municipal wastewater sludge has been considered as a hopeful lipid feedstock for biodiesel production. It has been attracting attention as an alternative biodiesel feedstock around the world. It is assumed that the amount of municipal wastewater sludge will raise in the future because of the increase in urbanised and industrial areas (Mondala et al., 2009).

Municipal WWTP sludge includes remarkable amount of lipids, up to 30 wt. % based on dry sludge, that is a combined of organic matrix resulting from the direct adsorbtion of lipids from domestic wastes in the sludge. These lipids are composed of free fatty acids, monoglycerides, diglycerides, and triglycerides. Additionally, the phospholipids in the cell membranes of microorganisms used in the wastewater treatmet process, their metabolites and by-product of cell lysis are essential costituents of municipal wstewater sludge (Rittmann et al., 2001; Mondala et al., 2009).

Huge quantities of municipal sludge that is readily attainable is produced in plentiful in wastewater treatment plants. Municipal sludge is an indispensable waste produced in WWTPs that's why it is considered as a non-cost feedstock (Olkiewicz et al., 2015).

Municipal sludge management (handling, treatment, and disposal) causes a complicated challenge for any WWTP and contstitutes of 20-60 % of the total cost (Andreoli et al., 2007). Municipal wastewater sludge includes lots of dangerous matters such as heavy metals, pathogens, and dioxins that may result in disposal problem related with environmental issues and human health. Sludge incineration causes emissions including heavy metals and dioxins and the use of it as fertilizer in land application is limited in many countries because of bad odour and the existence of heavy metals and toxic organic matters. The ocean dumping of sludge has the potential of releasing dangerous substances that may harm to the environment (Shen et al., 2003; Angerbauer et al., 2008; Kargbo, 2010; Siddiquee et al., 2011; Zhu et al., 2012).

2.2. Pretreatment Applications

As municipal WWTP sludges include complex components, extraction of lipids from these sludges becomes difficult. Pretreatments are able to improve the lipid extraction efficiency from biologic samples. Pretreatments have the ability to expose the lipids present in the sludge bonded to macromolecules which can not be reached by the solvent. Furthermore, application of pretreatment methods to the biologic samples disrupt cell wall releasing phospholipids and makes them easily accessible by the solvent (Olkiewicz et al., 2015). In this section, three pretreatment methods including acid, ultrasonic and microwave pretreatments are briefly described. Combined sludge pre-treatment methods such as acidic/ultrasonic or acidic/microwave pretreatments can also be applied. For example, the combination of the ultrasonic and acid pretreatment achieves better treatment efficiency, because they have different mechanisms of sludge dissolution. (Olkiewicz et al., 2015).

2.2.1. Pre-acidification (Acid Pretreatment)

Acid pretreatment has the ability to increase the lipid extraction efficiency from biological sources. Lipids present to be bonded to carbonhyrates, proteins, and minerals in municipal wastewater sludge as it is a processed sample. Acid can hydrolyze the sludge samples and release the lipids from the macromolecules which can not be reached by the solvent (McNichol et al., 2012; Olkiewicz et al., 2015).

2.2.2. Ultrasonic Pretreatment

Ultrasonic pretreatment is the most widely used method for sludge disintegration. Although it has the ability to improve the efficiency of lipid extraction from biological materials, its application to enhance yield of lipid extracted from municipal WWTP sludges using the direct liquid-liquid lipid extraction method has not been investigated. In the ultrasonication process, sound is used at frequencies between 20 kHz and 10 MHz, which are not heard by humans. The bottom of this voice range produces ultrasonic waves on a fluid which results in periodic compression and dilution of the medium. Ultrasonication achievement primarily depends on the cavitation mechanism. There are various factors that affect cavitation; viscosity of liquid, the temperature of the fluid, ultrasonic intensity, the period of the ultrasonication and the vibration frequency (Bourgrier et al., 2006; Roxburgh et al., 2006 Pilli et al., 2011). Cavitation begins above a spesific density threshold, when gas bubbles are formed, they grow in size before precipitating severely in a few microseconds. The temperature and pressure within the precipitating cavitation bubbles increase to approximately 5000 Kelvin (K) and several hundred atmospheres. This operation causes to disintegration of sludge flocks, disruption large organic substances and the destroying of the bacterial cell walls in sludge (Tiehm et al., 2001; Olkiewicz et al., 2015). The formation of the cavitation mechanism is shown in Figure 2.2.

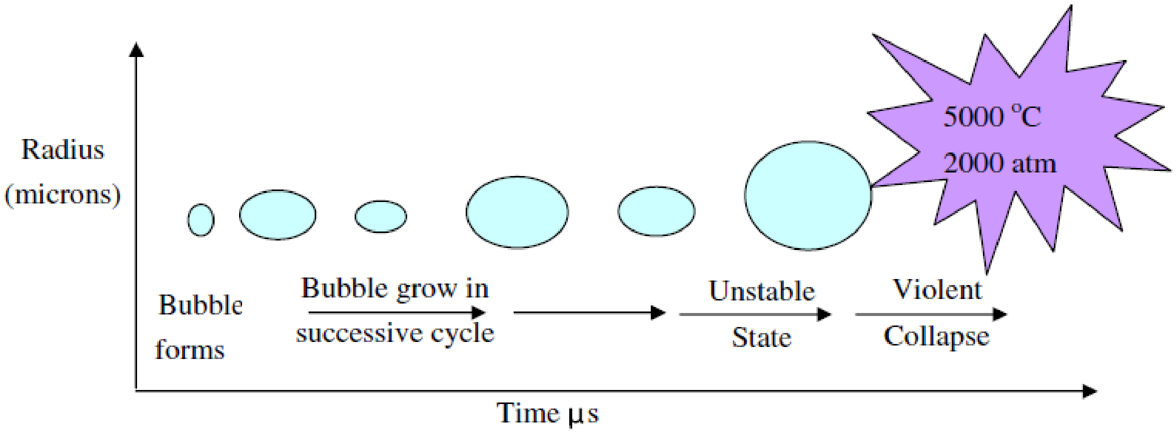

Figure 2.1. Development and collapse of the cavitation bubble (Pilli et al., 2011).

2.2.3. Microwave Pretreatment

Microwaves have been widely applied in environmental engineering. The reasons behind the utilization of microwave are its ability to complete reactions in very short time, rapid heating and cooling, ease of the application, operational cost saving, high efficiencies, and the compactness of the system (Gude et al., 2013). Microwave energy can be used in two main stages in the production of biodiesel; lipid extraction and transesterification. However, it has not been applied to municipal wastewater sludges either in dry nor wet form to investigate the efficiency on biodiesel production process as a pretreatment method.

Microwaves, as an energy source, generate heat by interacting with substances at the molecular scale without changing the molecular structure. The microwave area corresponds to a wavelength of 1 mm to 1 m at frequencies of 300 GHz to 300 MHz, respectively. Domestic and industrial microwaves commonly operate at a wavelength of 12.2 cm which corresponds to 2.45 GHz and energy of 1.02×10^{-5} eV (Jacob et al., 1995).

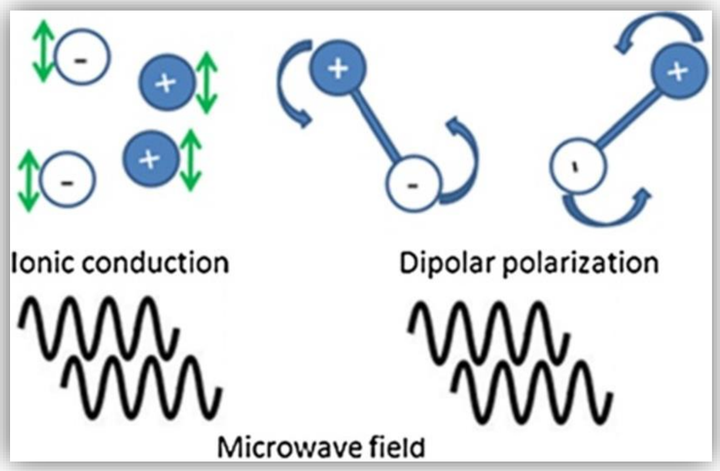

Figure 2.2. Ionic conduction and dipolar polarization under microwave conditions (Gude et al., 2013).

Energy transfer is the basic feature of microwave heating. It takes place through dipolar polarization and ionic conduction via the reversals of the dipoles and displacement of the charged ions existing in the solvent and solute (Figure 2.3). In many applications, these two mechanisms occur at the same time. When a molecule containing a dipole moment is subjected to the microwave irradiation, the dipole attemps to align with the applied electric field. As the electric field is oscillating, the dipoles frequently make an effort to realign to maintain this motion. This permanent reorientation of the molecules leads to friction and thus heat. This mechanism is called as dipolar polarization (Gude et al., 2013). Ionic conduction is defined as the electrophoretic migration of ions when they are exposed to an electromagnetic field and the resistance of the solution to this flow of ions (Eskilsson et al., 2000).

2.3. Lipid Extraction

Lipid extraction from municipal wastewater sludges is the first and most significant stage of the biodiesel production process. The chemical complication and variety of the sludges generated in WWTPs makes the lipid extraction process more difficult (Bharathiraja et al., 2014). Extraction of lipids from municipal wastewater sludges to produce biodiesel has many economic and environmental advantages (Kwon et al., 2012). Lipid extraction can be affected by many factors such as extraction time, type of sludge, temperature, type and amount of organic solvent, mixing rate, etc (Ibrahim, 2017). In literature, many studies have been done to extract the lipids from municipal wastewater sludge for biodiesel production. The majority of the lipid extraction methods necessitate dewatering and drying processes.

2.3.1. Conventional Organic Solvent Extraction Methods

Currently, there are primarily three methods to extract lipids from biological sources, namely, acid hydrolysis extraction, the water bath shaking extraction, and Soxhlet extraction methods. Some researchers have also investigated supercritical lipid extraction method. However, these methods are more economical and application of them easier compared to supercritical extraction method. In these three methods, organic solvents are used for extraction of lipids and aso called ‘organic solvent extraction’. Many investigations have been done in order to find the most appropriate solvent system. For this purpose, many different organic solvents were used such as toluene, methanol, ethanol, chloroform and hexane, alone or in the mixture (Boocock et al., 1992; Dufreche et al., 2007; Pokoo-Aikins et al., 2010; Siddiquee et al., 2011).

In the acid hydrolysis extraction method, after sludge acidification of the sludge samples the mixtures are heated at a temperature for a while. After cooling, ethanol is added to the solutions and the mixtures are filtered. Then, extraction solvent is added to the mixtures and lipids are extracted.

In water bath shaking method, extraction solvent is added to the sludge samples and they are placed in a water shaking bath. Afterward, the mixtures are shaken and heated. Finally, extraction procedure is completed (Zhu et al., 2014).

Soxhlet extraction is carried out on a special device. This method is suitable for solid or semi-solid samples. The Soxhlet extractor is one of the oldest extraction systems and is still widely used. The Soxhlet extractor consists of a solvent bottle, a liquid flow pipe in the middle circle (siphon), a cooled condenser and heating system. The sludge sample is placed inside the extraction chamber in the middle circle and the solvent is added into the solvent bottle. The solvent is heated above the boiling temperature and the vapor from the boiling solvent moves to the condenser. Then, the vapor drips towards the sample with condensation. The solvent soaks the sample, discharges the entire sample chamber as soon as the solvent level reaches the top of the siphon, and starts dripping back into the solvent bottle. Thus, the hot solvent is circulated in the sample several times. When the extracted analytes remain in the solvent bottle, only fresh solvent evaporates, so fresh solvent is used in each circulation. Modern versions of Soxhlet extractors based on the same basic principle have been developed including pressurized Soxhlet extraction, automated Soxhlet extraction, sonication-assisted Soxhlet extraction, microwave-assisted Soxhlet extraction, and supercritical fluid extraction. The main advantages of several extractors used in automated Soxhlet extraction are to reduce extraction time, reduce extractant volume and allow simultaneous extraction of several samples. In this study, semi-automated Soxhlet extraction was used. The most important disadvantage of this Soxhlet extraction is that it requires drying of the sludge samples prior to use. Sludge dewatering and drying processes constitute an important part of total biodiesel production cost. Another disadvantage is that the samples are usually extracted for a long time at a temperature higher than the boiling point of the solvent, resulting in contamination of the lipids to be extracted (Buyuktuncel, 2012; Olkiewicz et al., 2015).

2.3.2. Direct Liquid-Liquid Lipid Extraction Method

Conventional lipid extraction methods commonly include dewatering and drying processes prior to lipid extraction from municipal wastewater sludges. As municipal wastewater sludges have higher water content of 95-98 % by weight, the energy cost of sludge dewatering and drying processes constitute almost 50 % of the total biodiesel production cost making the process very expensive and difficult the scale up (Mondala et al., 2009; Olkiewicz et al., 2012). A new method called direct liquid-liquid lipid extraction has been developed by Olkiewicz and co-workers to eliminate these drawbacks.

The sludge samples were used in liquid form without dewatering and drying in this method and lipid extraction was performed in a batch mixer settler reactor at room temperature.

2.4. Lipids

Biodiesel is comprised of fatty acid methyl esters which can be generated from a variety of lipid feedstocks generally by transesterification or esterification. The municipal wastewater sludges include high quantity of lipidic compounds resulted from adsorption of lipids from domestic wastes into the sludge, directly. In addition, the phospholipids consisting in the cell membranes of microorganisms, their metabolites and cell disruption products contributes to the lipidic fraction in the sludge (Mondala et al., 2009). Lipids are made of the elements carbon, hydrogen, oxygen, nitrogen and consist of both polar and nonpolar groups. Lipids dissolve very poorly in water while they are very soluble in nonpolar solvents like chloroform, hexane and diethyl ether. The lipids consisting in municipal wastewater sludge are mainly composed of fatty acids, triglycerides, diglycerides, monoglycerides, and phospholipids (Campell, 1991; Smesdes et al., 1999).

2.4.1. Fatty acids

Fatty acids present in great amount in biological systems and they are composed of a long hydrocarbon chain and a terminal carboxyl group. The hydrocarbon chain may be saturated or unsaturated. Most natural fatty acids have unbranched chains with an equal number of carbon atoms (usually 14 to 24) and they are produced from phospholipids or triglycerides (Garett et al., 1995). Fatty acids composed of saturated carbon chains have higher melting points than unbranched ones and they are called fats. A single fatty acid is called "free" fatty acid if it is not bound to other molecules (Bozaghian, 2014).

2.4.2. Triglycerides

Triglycerides constitute 95 % of common fats existing in the municipal primary wastewater sludge and the remaining 5 % contains monoglycerides, diglycerides, free fatty acids, phosphatides, streols, fatty alcohols, vitamins soluble in fats (Aksoy, 2010). Triglycerides are formed of a glycerol backbone esterified with three fatty acid chains**.** If all three fatty acids linked to glycerol are similar, the molecule is called a simple triglyceride. On the contrary, if triglycerides contain different fatty acid chains, they are named mixed triglycerides (Garett et al., 1995). Solid and liquid fats are a mixture of triglycerides and they can be liquid or solid depending on the fatty acids they contain.

Triglycerides, mostly containing saturated fatty acids, are solid at room temperature (20°C) and are known as solid fats. Conversely, those consisting mainly of unsaturated fatty acids are liquid and are commonly known as oils. Because of Van der Waals' reduced attractiveness between the molecules by the double bonds, triglyceride fats have higher melting points than oils (Clayden et al., 2012).

2.4.3. Phospholipids

Phospholipid is the most common lipid group found in the structure of microorganisms consisting in municipal activated sludge. Phospholipids contain a glycerol linked to two fatty acid chains and a phosphate group. Entire components of the phospholipids contain a polar head and a nonpolar tail. Phospholipids are generally obtained by the esterification of alcohol groups of a glycerol with a phosphoric acid (Campell, 1991).

2.5. Biodiesel Production Methods

In order to use lipids extracted from the biological samples as fuel, it is first necessary to subject them to processes that reduce their viscosities. For this purpose thermal and chemical methods can be used. In the thermal method, it is aimed to reduce the viscosity of the oils by preheating. The chemical method is being used more frequently because of the problems that can occur during the application of the thermal method in a moving vehicle motor. The chemical method is examined in four parts; Blending with petro-diesel, microemulsions, pyrolysis (thermal cracking) and transesterification (Ulusoy, 1999).

2.5.1. Blending with Petro-diesel

It is a method for lowering the viscosities of oils by mixing with diesel fuel at certain ratios. In the fuels expressed as B20, B30, B40; 20 %, 30 %, 40 % of the oils are contained in the diesel fuels respectively. The cost of fuels obtained by using this method is lower than that of diesel fuels. When studies on mixtures made from diesel fuel and vegetable oil were examined, it was seen that the properties of the mixture and the diesel fuel were not very different from each other. The oils most commonly used for biodiesel production by blending; waste cooking oils and vegetable oils such as peanuts, sunflowers, rapeseed (Srivastava et al., 2000; Oguz, 2004).

2.5.2. Microemulsions

The microemulsion is defined as a stable colloidal dispersion of 1-150 nm in size, formed by spontaneous mixing of two liquids with ionic or nonionic organic mixtures. The microemulsion is formed by the combination of two normally miscible liquids and one or more amphiphiles. It is possible to produce alternative diesel fuels out of petroleum with this method. In the microemulsion process, short-chain aliphatic alcohols such as methanol and ethanol are used (Schwab et al., 1987).

2.5.3. Pyrolysis (Thermal cracking)

Pyrolysis is the conversion of high-molecular compounds into lower-molecular compounds at high temperatures. Pyrolysis of vegetable oils takes place in two forms. In the first, vegetable oils are disintegrated by heat effect in a closed container. In the second, vegetable oil is thermally decomposed by distillation using standard substances. The fuel properties obtained by applying the second process are closer to the diesel (Srivastava et al., 2000). In pyrolysis, the compound is transformed into another compound in the airless environment by using catalyst as well as heat. It is very difficult to understand the mechanism of the pyrolysis process because it consists of many different reactions occurring at the same time and in succession (Formo et al., 1979). Defining pyrolytic chemistry is difficult because of the diversity of reaction paths and the variety of reaction products obtained from the reactions. Pyrolyzed substances may be vegetable oils, animal oils, natural fatty acids and methyl esters of fatty acids. Generally, thermal degradation proceeds by forming free radicals or carbonium ions. Pyrolysis is difficult to produce and costs are high. In addition, the products obtained are chemically similar to petroleum products, which are gasoline and diesel fuel. Removal of oxygen during pyrolysis also eliminates some environmental benefits of the use of an oxygenated fuel (Zhenyi et al., 2004).

2.5.4. Transesterification

Transesterification (also called alcoholysis) is the reaction of the monoglycerides, diglycerides, triglycerides, and phospholipids in fats and oils with an alcohol to form esters and glycerol. Transesterification reaction is mostly used method for biodiesel production. Biodiesel can be also produced from free fatty acids (FFAs) by esterification reaction with methanol. The biodiesel formation reaction is shown in Figure 2.4. The cell membrane of microorganisms, the primary material of municipal wastewater sludges, commonly comprised of phospholipids also contributes to biodiesel production. Stoichiometrically a 3:1 molar ratio of alcohol to triglyceride is required to complete a transesterification reaction. Due to the fact that the reaction is comprised of a series of consecutive and reversible reactions, excess alcohol have to be added to change the equilibrium to the product side (Ma et al., 1999; Rittmann et al., 2001). With the effect of primary alcohol the triglycerides is converted to diglyceride, monoglyceride, and finally glycerol step by step. Glycerol is a by-product resulted from these reactions can be utilized by industry for manufactoring cosmetics and phamaceutical formulations contibuting to biodiesel process as an economically positive element (Dufreche et al., 2007, Siddiquee et al., 2011).

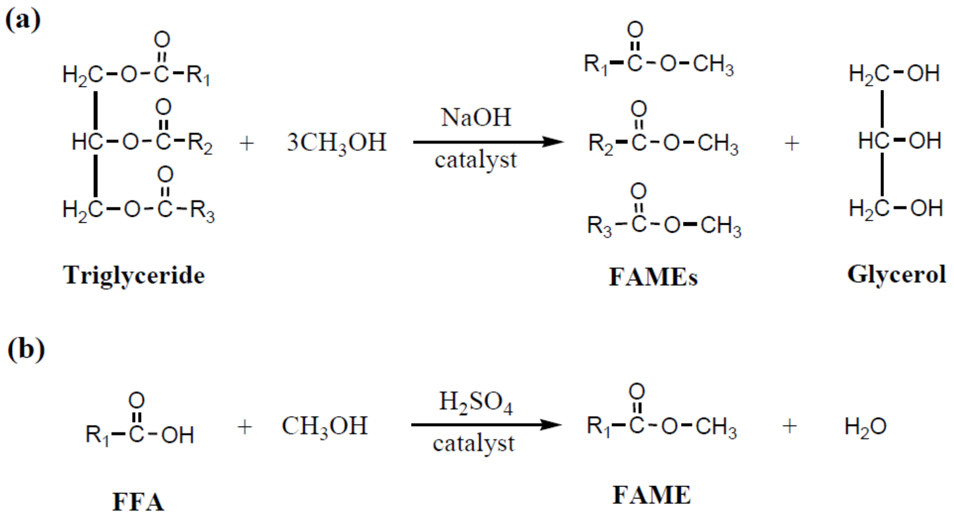

Figure 2.3. Transesterification and esterification processes for lipids (a) Transesterification of triglyceride and (b) esterification of FFA (Mondala et al., 2009).

Methanol, ethanol, propanol, and butanol can be used in transesterification process as the primary alcohol. Methanol and ethanol are used widely, particularly methanol due to its physical and chemical features and low cost. In addition, it has high reactivity, it does not absorb water during transesterificacion reaction, it prevents soap formation and also it can be recovered easily (Ma et al., 1999; Demirbas, 2008; Chongkhong et al., 2009). The basic parameters affecting transesterification yield are water content of fats or oils, reaction temperature, molar ratio of alcohol/oil, reaction time, the content of fatty acid, and catalyst type (base, acid, or enzyme) (Siddiquee et al., 2011).

In transesterification, there are two methods, catalyzed and non-catalyzed transesterification reactions. Catalytic transesterification reactions are mostly carried out using alkaline (base), acidic and enzymatic catalysts. Recent studies have begun to use heterogeneous catalysts such as microporous zirconium, sulphated zirconium and titanium-based zeolite. Supercritical processes or co-solvent systems are used for non-catalyzed transesterification reactions (Prakash et al., 2006; Karmakar et al., 2010).

2.5.4.1. Base/Alkali catalyzed transesterification. Sodium hydroxide (NaOH), potassium hydroxide (KOH), carbonates and alkoxides (sodium methoxide, sodium ethoxide, sodium butoxide etc.) are used in the production of biodiesel as alkaline catalysts. Base/alkali catalyzed transesterification is commonly used commercially because it has the fastest reaction rate among other catalyzed reactions. However, for a base catalyzed transesterification the glicerides and alcohol must be anhydrous due to the fact that water causes the catalyst consumption and saponification. Also, high free fatty acids contained in lipid sources result in catalyst consumption and soap formation that obstructs glycerol seperation and decreases the biodiesel production yield. Low free fatty acid content is necessary for base catalyzed transesterification. If triglycerides contains more water and free fatty acids acid catalyzed transesterification must be used (Ma et al., 1999; Siddiquee et al., 2011).

2.5.4.2. Acid catalyzed transesterification. Acid catalyzed transesterification is 4000 times slower than the based catalyzed one and needs high alcohol to lipid ratio. However, acid catalysts have the ability to convert free fatty acids and triglycerides to fatty acid methyl esters since they catalyze both of esterification and transesterification reactions simultaneously resulting more biodiesel production yield (Demirbas, 2005). Bronsted acids such as hydrogen chloride (HCl), sulfuric acid (H_2SO_4), sulphonic acid (H_2O_3S) are used as acidic catalysts in transesterification reactions. Lipid feedstocks containing low FFA is required base catalyzed transesterification. If lipid feedstocks include greater than 1 % FFAs and more water such as in municipal wastewater sludge, the use of acid catalyzed transesterification is recommended (Kargbo, 2010; Siddiquee et al., 2011).

2.5.4.3. Enzymatic transesterification. Enzymatic transesterification can be used to overcome the problems related with product separation, glycerol recovery, and side reaction (Raman et al., 2008). In enzyme catalyzed transesterification, lipase that breaks down fats into fatty acids is used as enzyme. Enzyme catalyzed reactions occur at lower temperatures (30-40 ºC) so that they can act without degrading the enzyme structure (Yuji et al., 2002). The enzyme has to be isolated from microorganisms. Nowadays, this method is not chosen due to having some disadvantages such as high cost of enzym, slow reaction rate, strenght of extraction and contamination of the product with remnants (Korbitz, 1999).

2.5.4.4. In situ transesterification. In situ transesterification is dissimilar with conventional transesterification because the lipid containing material comes into contact with directly alcohol rahter than reaction of alcohol with extracted lipid. Lipid extraction and transesterification occur in the same process, organic solvent is added to system for extraction of biodiesel instead of lipids. Therefore, the process eliminates the requirement for extraction and separation of lipids. The in situ process decreases the reaction time, the quantity of organic solvent and sludge samples used in the system reducing the total cost of biodiesel production compared to conventional transesterification process. On the other hand, the application of this process on an industrial scale is unfavourable because of the difficulties of sustaining the process design and parameters (Mondala et al., 2009; Bharathiraja et al., 2014).

2.5.4.5. Non-catalytic transesterification. Non-catalytic transesterification does not need a further purification because of not containing any catalyst. Apart from triglycerides, free fatty acids can be also converted into fatty acid methyl ester (FAME) by using this method. Non-catalytic transesterification process consists of BIOX process and supercritical process (Monisha et al., 2013).

BIOX process. BIOX process utilizing of a co-solvent that assists to cope with the problem of slow reaction time was found out by Professor David Boocock. Glycerol and ester phase do not include any catalyst remnants. This process requires an operating temperature of 30°C. The final product generated by using BIOX process does not contain water and catalyst, also recovery and recycling of co-solvent can be done easily (Freedman et al., 1984).

Supercritical process. Conventional transesterification process has some negative aspects, for instance it is time consuming and purification of products is difficult. To overcome these problems Kusdiana and Saka have found out a supercritical tecnique. Biodiesel can be generated by supercritical process without a catalyst using supercritical methanol. Even thought lipid feedstocks contain high amount of FFA, biodiesel can be also produced simultaneously by the esterification reaction of FFAs. The high yields of biodiesel can be produced without soap formation. However, in order to complete this process at a very short period of time, oil has to react with excess methanol at extreme temperature and pressure conditions of 350°C and 43 MPa, respectively (Kusdiana et al., 2001; Imahara et al., 2008; Fjerbaek et al., 2009).

2.6. Applications of Lipid Extraction and Biodiesel Production from Municipal WWTP Sludges

In literature, there are several types of lipid extraction procedure and biodiesel production method using the municipal wastewater sludges as feedstock. Municipal primary sludge and secondary sludge include 4-5 % (w/v) and 1-2 % (w/v) solid matters, respectively (Mondala et al., 2009). Due to the high water content of these sludges, most researchers have applied dewatering and drying processes in order to remove the water and increase the yield of biodiesel production.

Boocock et al. (1992), utilized the Soxhlet and boiling solvent lipid extraction methods in their study. They used the sludge samples in dry form. They employed a sludge to solvent ratio of 1 to 6 and used chloroform and toluene as solvents. They achieved 12 % and 17-18 % (based on dry weight of sludge) of lipid yields using both of the solvents by Soxhlet and boiling solvent lipid extraction methods, respectively. They also reported that toulene is preferable solvent due to economic and environmental concerns although similar lipid extraction yields were attained by both solvents.

Dufreche et al. (2007), demonstrated that lipids can be extracted from municipal activated sludge through use of pure or mixture of hexane, methanol and acetone as solvents. They pretreated the activated sludge (2 % solid) by gravity settling followed by centrifugation or pressure filtration and got 7-14 % (w\w) solids containing sludge. Then, they obtained the maximum biodiesel yield, 4,41 % (based on dry weight of sludge) by using acid catalyzed esterification-transesterification method and the mixture of 60 % hexane/20 % methanol/20 % acetone as organic solvent. They also used supercritical-CO_2 tecnique and supercritical-CO_2 with methanol co-solvent tecnique for lipid extraction to produce biodiesel and obtained 0,28 % and 2,31 % (based on dry weight of sludge) biodiesel, respectively. Additionally, they achieved the biodiesel yield of 6,23 % (based on dry weight of sludge) by using in-situ transesterification. They claimed that lipid extraction using solvent was more effective than expensive supercritical-CO_2 tecnique. They also concluded that biodiesel production by using in-situ transesterification resulted with the highest biodiesel yield compared with other methods.

In the study by Mondala and co-workers (2009), it has been indicated that municipal primary and secondary sludges can be used to generate biodiesel through acid catalyzed in-situ transterification. They used the raw municipal wastewater sludges which were concentrated by gravity settling, dewatered by centrifugation and dried by freze-drying. They used methanol as solvent and determined the impacts of three process parameters (temperature, catalyst concentration and solvent to sludge ratio) on the biodiesel production yield. Results indicated that the yield of biodiesel from primary sludge was remarkably affected by the interactive impacts of temperature, acid catalyst concentration and methanol to sludge mass ratio, while the biodiesel from secondary sludge was affected by the independed impacts of the three investigated process parameters. They also achieved highest yields of biodiesel for primary 14,5 % and secondary sludge 2,5 % (based on dry weight of sludge) at 75°C, 5 % (v/v) H_2SO_4, and 12:1 methanol to sludge mass ratio.

Revellame et al. (2009), reported that lipidic compounds in municipal activated sludge can be used as a biodiesel feedstock. They optimized in-situ transesterification process in order to attain the best performance of operational circumstances and highest biodiesel yield. 4,88 % (based on dry weight of sludge) biodiesel yield was achieved at 55°C, 25 methanol to sludge ratio and 4 % volume of sulfuric acid. Also, they demonstrated that biodiesel yield at temperature above 60°C reduces because of acid-catalyzed polymerization of unsaturated fatty acids or their esters.

Pokoo-Aikins et al. (2010), extracted lipid and produced biodiesel from municipal wastewater sludge however, they did not advert the type of sludge they used primary or secondary. They preferred toluene, hexane, ethanol, and methanol as solvents to extract lipids. They used a sludge to solvent ratio 1:5 and separated the free fatty acids, triglycerides and the solvent. The results indicated that the yield of FFA was 24,8 %, 24,9 %, 25,5 %, and 25,5 % (based on dry weight of sludge) for toluene, hexane, methanol, and ethanol, repectively and the highest yield of 13,4 w % (on the basis of dry weight of sludge) was taken from the triglyceride.

Siddiquee et al. (2011), investigated the effect of different factors on the biodiesel production from dried municipal primary and secondary sludges and found that temperature is the most important parameter affecting lipid extraction by using methanol and hexane as solvents. They conducted that lipid extraction using methanol resulted with a higher lipid yield, 14,46 % and 10,04 % (based on dry weight of sludge), from the primary and secondary sludge sources, respectively, as compared to 11,6 % and 3,04 % (based on dry weight of sludge) from primary and secondary sludges, respectively, using hexane as a solvent. Additionally, the results of the study showed that biodiesel conversion yields of hexane and methanol extracted lipids were 41.25 w % and 38.94 w % (on the basis of lipid) for primary sludge, and 26.89 w % and 30.28 w % (on the basis of lipid) for the secondary sludge. Also they reported that the use of natural zeolite as a desiccant raised the biodiesel yield by nearly 18 %.

A research by Kwon et al. (2012), has indicated that biodiesel can be produced from municipal wastewater sludge economically as it includes high lipid content and has low cost as a feedstock. They used a noncatalytic biodiesel conversion mechanism (a thermochemical process) under ambient pressure in a continuous flow system and achieved to convert FFAs and triglycerides to FAMEs. Hexane was used as a solvent in the study. They concluded that the lipid yield of municipal wastewater sludge is 88 and 2200 times higher than microalgae and soybean oil, respectively. This study also confirmed that municipal wastewater sludge can be the most economical feedstock for biodiesel production among the common various feedstocks.

Zhu and colleagues (2012), utilized different organic solvents and their mixtures to find the most efficient solvent in lipid extraction from the municipal wastewater sludge. They showed that mixed solvent has better performance compared to the single solvent.

In the study by Olkiewicz et al. (2012), the compatibility of four different types of municipal WWTP sludge (primary, secondary, blended and stabilized) for biodiesel production were investigated. They extracted lipid from sludges via Soxhlet extractor, using hexane as a solvent and then converted the extracted lipids to biodiesel by acid catalysis transesterification. Lipid extraction yields of 25,3 %, 21,9 %, 10,1 % and 9,1 % (based on dry weight of sludge) was obtained from primary, blended, stabilized and secondary sludge samples, respectively. In terms of biodiesel yield primary, blended, secondary, and stabilized sludges achieved 13,9 %, 10,9 %, 2,9 %, and 1 % (based on dry weight of sludge), in the same order. The results of the study demonstrated that the greatest lipid and biodiesel yields were attained from the primary sludge samples. They also compared primary sludge with common biodiesel feedstocks in terms of fatty acid profile and demonstrated their compatibility for the biodiesel production.

Another study by Olkiewicz et al. (2015), indicated the effects of acid, ultrasonic and mechanical pretreatments on the lipid extraction and biodiesel production yields. They used primary, secondary, blended and stabilized sludges as lipid feedstocks. The lipids consisting in the municipal WWTP sludge samples were extracted by through Soxhlet apparatus, using hexane as a solvent. Then they converted the obtained lipids to biodiesel by acid catalysis transesterification. The results showed that the pretreatment methods did not increase remarkably the lipid extraction and biodiesel production efficiencies.

Bharathi and co-workers (2014), reported that municipal secondary sludge has biodiesel production capacity as a lipid feedstock. They acquired the maximum lipid extraction yield using hexane and methanol as solvents from dried secondary sludge. Then they converted extracted lipids to FAMEs by acid cataysed transesterification using sulfuric acid.

Zhu et al. (2014), comparatively investigated the lipid extraction efficiency attained from municipal wastewater sludge with three different methods (acid hydrolysis extraction, soxhlet extraction, and the water bath shaking extraction) by using various organic solvents. They concluded that the highest lipid yield (10.3 %, based on dry weight of sludge) and biodiesel yield (6.35 %, based on dry weight of sludge) were achieved by using Soxhlet extraction compared to other methods.

In aforementioned investigations, dry sludge was used directly as a raw material for biodiesel production from municipal WWTP sludges. As municipal wastewater sludges have higher water content of 95-98 % by weight, the energy cost of sludge dewatering and drying processes constitute almost 50 % of the total biodiesel production cost making the process very expensive and difficult the scale up (Mondala et al., 2009; Olkiewicz et al., 2012). In order to eliminate the expensive preliminary step of sludge drying Pastore and co-workers (2013), used dewatered sludge as raw material for extraction of lipidic compounds. However, the energy necessary for water elimination still constitues 14 % of the total biodiesel production cost (Dufreche et al., 2007; Pastore et al., 2013). They achieved the yield of 18 % biodiesel (based on dry weight of sludge) with the lowest energy need by using a new two-step method consisting of lipid extraction directly from dewatered acidified primary sludge using hexane as solvent and methanolysis of extracted lipids. They also demonstrated that sulphuric acid has an important role in biodiesel production process. It has also an impact on the transterification of glycerides and generation of new FFAs from soaps and their esterification with methanol.

Demirbas et al. (2017), used municipal wastewater sludge samples in wet form eliminating the high energy required for removing its high water content that is a significant drawback of the process for scaling up. Firstly, they extracted the lipids from sludge samples with a Soxhlet apparatus by using hexane as solvent and then produced biodiesel via supercritical methanol transesterification (SCMT) process. The study demonstrated that biodiesel production performance in SCMT did not affected by high water and FFA contents in the sludge.

Olkiewicz et al. (2014), developed a new method for extraction of lipids from municipal wastewater sludges different from the methods used by other researchers. They used the sludge samples directly in liquid form without dewatering and drying. They explored the novel direct liquid-liquid lipid extraction method and compared that method with standard reference method in terms of lipid extraction and biodiesel production efficiencies. In this new method, hexane was used as an organic solvent and extraction was performed in batch mixer settler reactor at room temperature. The results of this study demonstrated that higher lipid yield (27 %, based on dry weight of sludge) and higher biodiesel yield (19.2 %, based on dry weight of sludge) were obtained from primary sludge samples via direct liquid-liquid lipid extraction technique, whereas 25 % of lipid and 17,6 % of biodiesel yields (based on dry weight of sludge) were produced by using the standard method. They concluded that direct liquid-liquid lipid extraction method can be used effectively for primary sludges; however it is not efficient for secondary sludges.

Kech et al. (2018), optimized the direct liquid-liquid lipid extraction method proposed by Olkiewicz and co-workers. In the study, municipal primary and secondary sludge samples were initially pretreated by ultrasonication application and centrifuged for an efficient separation for phases. Then, lipids were extracted from sludge samples by organic solvent extraction using different solvents. They did not convert directly extracted lipids to biodiesel instead of that analysed the saponification number to determine the biodiesel conversion capacity of the obtained lipids. They obtained higher lipid yield of 34.5 % (based on dry weight of sludge) by optimization using Smedes mixture (cyclohexane/isopropyl/alcohol/water) as solvent compared hexane extraction (32.8 %, based on dry weight of sludge) from wet primary sludge samples.

Literature data based on the lipid extraction and biodiesel production from municipal WWTP sludges summarized in the Table 2.1.

Table 2.1. Literature data related with the biodiesel production from municipal WWTP sludges.

References	**Used sludge type**	**Methodology**	**Resullts**[a]
Boocock et al. (1992)	Dried municipal WWTP sludge	Boiling solvent extraction	17-18 % lipid
		Soxhlet extraction	12 % lipid
Dufreche et al. (2007)	Dried activated sludge	Supercritical-CO_2	3.5 % lipid
		Supercritical-CO_2 with methanol co-solvent	13.5 % lipid
		Organic solvent extraction using the mixture of hexane, methanol, and acetone	27.4 % lipid
		In-situ transesterification	6.2 % FAME
Mondala et al. (2009)	Dried primary sludge	In-situ transesterification	14.5 % FAME
	Dried secondary sludge		2.5 % FAME
Revellame et al. (2010)	Dried secondary sludge	In-situ transesterification	4.8 % FAME
Pokoo-Aikins et al. (2010)	Dried municipal WWTP sludge	Organic solvent extraction with toluene	24.8 % FFA

		Organic solvent extraction with hexane	24.9 % FFA
		Organic solvent extraction with methanol	25.5 % FFA
		Organic solvent extraction with ethanol	25.5 % FFA
Siddiquee et al. (2011)	Dried primary sludge	In-situ transesterification using methanol	5.6 % FAME
		In-situ transesterification using hexane	4.6 % FAME
	Dried secondary sludge	In-situ transesterification using methanol	3 % FAME
		In-situ transesterification using hexane	0.8 % FAME
Kwon et al. (2012)	Dried municipal sewage sludge	Noncatalytic esterification/transesterification	18-20 % lipid
Zhu et al. (2012)	Dried municipal WWTP sludge	Organic solvent extraction with the mixture of hexane-ethanol	15 % lipid
		Organic solvent extraction with the mixture of ether-petroleum ether	12.5 % lipid
		Organic solvent extraction with hexane	9 % lipid
		Organic solvent extraction with petroleum ether	7 % lipid
Olkiewicz et al. (2012)	Dried primary sludge	Acid catalyzed esterification/transesterifacion	13.9 % FAME
	Dried blended sludge		10.9 % FAME
	Dried stabilized sludge		1 % FAME
	Dried secondary sludge		2.9 % FAME
Pastore et al. (2013)	Dewatered primary sludge	Noncatalytic esterification/transesterifacion	9 % FAME
		Acid catalyzed esterification/transesterifacion	17.9 % FAME
Bharathi et al. (2014)	Dried secondary sludge	Acid catalyzed esterification/transesterifacion	Not given
Zhu et al. (2014)	Dried secondary sludge	Acid hydrolysis extraction	4.8 % lipid
		Soxhlet extraction	6 % lipid
		Water bath shaking extraction	5.1 % lipid
Olkiewicz et al. (2014)	Dried primary sludge	Soxhlet extraction	26.3 % lipid
	Wet primary sludge	Direct liquid-liquid extraction	13 % lipid
	Wet acidified primary sludge		26.6 % lipid
	Wet secondary sludge		6.3 % lipid
	Wet acidified secondary sludge		5.1 % lipid
	Wet blended sludge		10.7 % lipid

	Wet acidified blended sludge		19.1 % lipid
Olkiewicz et al. (2015)	Dried primary sludge	Acid catalyzed esterification/transesterifacion	19 % FAME
	Dried blended sludge		15 % FAME
	Dried stabilized sludge		2 % FAME
	Dried secondary sludge		4 % FAME
Ayhan et al. (2017)	Wet municipal WWTP sludge	Soxhlet extraction	24 % lipid
Kech et al. (2018)	Acidified dried primary sludge	Organic solvent extraction with Smedes mixture	37.2 % lipid
	Wet primary sludge	Direct liquid-liquid extraction with Smedes mixture	31.8 % lipid
	Wet acidified primary sludge		33.5 % lipid
	Wet acidified primary sludge	Direct liquid-liquid extraction with hexane	32.8 % lipid
	Dried secondary sludge	Organic solvent extraction	28.6 % lipid

[a] Lipid and biodiesel (FAME) yields (%) based on dry weight of sludge

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Feedstock: Municipal Wastewater Sludge

The sludge samples used in this study were supplied from a municipal wastewater treatment plant located in İstanbul with a capacity to process near 400.000 m^3 of wastewater per day. Primary sludge samples were collected at the bottom of the primary clarifier and secondary sludge samples were taken from the recycling line of the biological treatment. The collected sludge samples were stored at 4°C.

3.1.2. Chemicals

For the lipid extraction experiments, hexane, petroleum ether, toluene, chloroform and magnesium sulphate monohydrate were supplied from Sigma-Aldrich. Sulphuric acid and anhydrous methanol used in the esterification/transesterification experiments were purchased from Merck. Hyrochloric acid was also supplied from Merck for sludge acidification step. For the biodiesel production process, anhydrous sodium sulphate, sodium chloride and sodium bicarbonate were purchased from Sigma–Aldrich. 37 component FAMEs mix standard used for quantification FAMEs was provided by Supelco.

3.1.3. Instrumental Equipments

The equipments used in this study for analyses are given in Table 3.1.

Table 3.1. Instrumental equipment list and methodology.

Analysis	**Instrumental Equipment**	**Method**
TS (mg\L)	Nüve FN 500 Oven (105°C)	Gravimetric Method
VS (mg\L)	Protherm Furnance (550°C)	Gravimetric Method
MLSS (mg\L)	Schott Duran Filtration Apparatus	Gravimetric Method
MLVSS (mg\L)	Protherm Furnance (550°C), Schott Duran Filtration Apparatus	Gravimetric Method
COD (mg\L)	HACH COD Digester HACH DR/2010 Spectrophotometer	Dichromate Closed Reflux Method, Spectrometric Method
sCOD (mg\L)	Hettich Universal 16A Centrifuge, HACH OD Digester HACH DR/2010 Spectrophotometer	Centrifugation and Spectrometric Method
pH, ORP (mV) and temperature	WTW 3110 Inolab pH meter	Electrometric Method
Viscosity (cP)	Brookfield DV-I Prime Viscometer	Instrumental Method
Alkalinity (mg\L)	Magnetic Stirrer	Titrimetric Method
Disintegration	Bandelin Sonopuls HD 3400 Ultrasonic Homogenizer	Instrumental Method
Disintegration	Berghof Speedwave MWS+3 Microwave Digestion System	Instrumental Method
Lipid extraction (%)	Velp Scientifica SER 148 Solvent Extractor	Gravimetric Method
Lipid extraction (%)	Jar Test F.6/S	Gravimetric Method
FAME (%)	Agilent Gas Chromatograph 6890GC with a flame-ionization detector (FID)	Instrumental Method

3.2. Methods

3.2.1. Pretreatment Applications

In this study, acid, ultrasonication and microwave pretreatment methods were applied to the sludge samples due to their ability to disintegrate the sludges improving homogenisation of the samples and penetration of the solvent into the samples. Moreover, the methods have the ability to disrupt cell wall, disintegtare sludge flocks, and expose the lipids present in the sludge bonded to macromolecules (Olkiewicz et al., 2015). In order to compare the effects of pretreatment methods on the lipid and biodiesel yields obtained from municipal primary wastewater sludge samples, the lipid extraction experiments were conducted by using untreated, acid, ultrasonication, microwave, combined acid-ultrasonication and combined acid-microwave pretreated sludge samples.

3.2.1.1. Pre-acidification (Acid pretreatment). Acid pretreatment was applied to the sludge samples before extraction process. 0.1 N concentrated hydrochloric acid (HCl) was added into the samples to decrease the pH to about 2. Addition of almost 0.6 ml concentrated HCl to sludge samples of 40 ml was sufficient to attain this pH.

3.2.1.2. Ultrasonication pretreatment. Ultrasonication (US) pretreatment was applied to both untreated sludge and acidified sludge samples to investigate the effect of ultrasonication alone and combination with acidification process. 200 mL of the sludge sample was placed in a vessel and the sample was ultrasonically pretreated using an ultrasonic homogenizer (Bandelin-Sonopuls HD 3400) shown in Figure 3.1. The ultrasonic homogenizer used in the study is equipped with a booster horn (SH 3425), an ultrasonic converter (UW 3400), a probe (VS 200 T) and a generator (GM 3400). The generator transforms the received power (50 or 60 Hz) into high-frequency power at a frequency of 20 kHz (Bandelin, 2009).

Figure 3.1. The ultrasonic homogenizer (Bandelin-Sonopuls HD 3400).

Ultrasonic experiments were conducted at 20 kHz of working frequency, 70 % amplitude and 200 W of the input of energy at room temperature for 13 min to get specific energy of 15,000 kJ/kg TS. Ice packs were placed around the sample to prevent it warming. The whole properties of the ultrasonic equipment used in the study are listed in Table 3.2.

Table 3.2. Characteristics of the ultrasonic homogenizer.

GM 3400 Generator	
Power Supply	230V~50/60Hz (alternatively115V~50/60Hz)
Ultrasonic frequency	20 kHz
Maximum Power	400 W
Power setting range	60 – 300 W
Weight	3.1 kg
Dimentions (l× w × h)	324 × 230 × 131 mm
Time setting range	0:00:01-9:59:59 (h:mm:ss) or continuous operation
Amplitude setting range	10 – 100 % , 1 % increments
UW 3400 Ultrasonic Converter	
Frequency	20 kHz
Weight	2.2 kg
Dimensions	Ø 90 ×180 mm
Degree of protection	IP 20
VS 200 T Probe	
Diameter	Ø 25 mm
Connection to standard horn	SH 3425
Volume range	100 – 2500 mL
Maximum admissible amplitude setting	100 %
Immersion depth (recommended)	10 – 20 mm

Spesific Energy

The specific energy can be described as the energy input per unit of sludge (as TS) to attain a certain degree of disintegration (Khanal et al., 2007). Specific Energy (E_s) is a function of ultrasonic period, ultrasonic power, volume of sonicated sludge and TS concentration, and can be computed by using the following Equation 3.1 (Bougrier et al., 2006);

$$E_s = \frac{(P\times t)}{(v\times TS)} \tag{3.1}$$

where E_s: the Specific Energy input in kW s/kg TS (kJ/kg TS); P: the ultrasonic power in kW; t: the ultrasonic period in seconds; V: the volume of sonicated sludge in liters; TS: the total solids concentration in kg/L.

3.2.1.3. Microwave pretreatment. Microwave (MW) pretreatment of the sludge samples was performed by using a Microwave Digestion Unit (Berghoff Speedwave MWS-3+) shown in Figure 3.2. Microwave irradiation was applied to both untreated sludge and acidified sludge samples to determine the effect of MW pretreatment alone and combination with acidification process. The sludge samples in the volume of 50 ml were put into MW vessels and placed in the MW device.

Figure 3.2. The microwave equipment used in the study (Berghof Speedway MWS+3).

In literature, the optimal microwave temperature differs from 160°C to 180°C while duration of the application is in the range of 30-60 min. In order to attain these temperature values the pressures in the range of 600–2500 kPa is performed (Weemaes et al., 1998). Considering this information, the MW pretreatment was conducted to the sludge samples at 175ºC and 2000 kP during 30 minutes. The whole characteristics of the microwave equipment used in the study are listed in Table 3.3.

Table 3.3. Specifications of the microwave equipment.

Power Supply	230 V/50 Hz/ 1,350 W
Microwave Output	1000 W
Frequency	2450 Hz
Weight/dimensions (W x D x H)	Standard device: approx. 14 kg/ 520 x 460 x 330 mm Control unit: approx. 0.5 kg/ 188 x 35 x 114 mm
Oven chamber	Approx. 27 Liter/ 350x x340 x 215 mm (W x D x H)
Noise Level	<60 dB
Ambient Conditions	15- 35 ºC / 85 % relative air humidity
Temperature measurements	Measurement range 50-260 ºC, accuracy 1ºC at 200 ºC

3.2.2. Lipid extraction

Lipid extraction is the first step of biodiesel production process. In this study, two different lipid extraction methods; Standard lipid extraction method (Soxhlet extraction method) and direct liquid-liquid lipid extraction method were used to make a comparison in terms of lipid and biodiesel yields obtained from municipal WWTP sludges. In the standard extraction method, sludge samples were processed after drying of them. The reason of choosing this method to extract of lipids for comparison is that magnesium sulfate monohydrate ($MgSO_4.H_2O$) is used to dry the sludge samples. The water content of the sludge samples is a significant factor that could block the extraction of lipid by solvent. Using $MgSO_4.H_2O$, the sludge samples were assumed entirely dried. Olkiewicz at al. (2014) investigated the comparison of different drying methods and they indicated that common sludge drying methods resulted with the lower lipid and biodiesel yields compared to drying with $MgSO_4.H_2O$.

3.2.2.1. Standard Lipid Extraction Method (Soxhlet Extraction Method). In this method, the lipid extraction procedure was applied by making minor modification according to standard method 5520E (Rice et al., 2012). In the standard method, a traditional Soxhlet apparatus has been used for the lipid extraction. Differently a semi-automatic Soxhlet extractor operating according to the Randall technique consisting of immersion, washing and solvent recovery steps was used in this study.

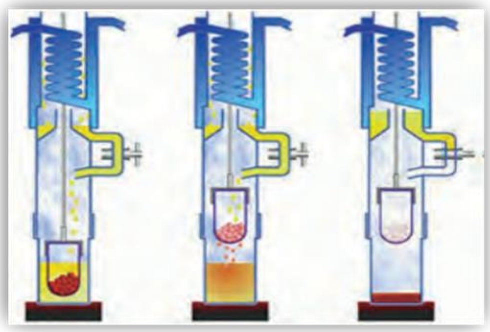

Figure 3.3. Randall tecnique used in the Soxhlet extraction method (Velp Scientifica, 2014).

Sludge samples of 20 grams were taken. Some part of the samples were used without acidification and the other part was acidified with 0.3 mL of fuming hydrochloric acid to determine the acid pretreatment effect on the yields. 25 g of magnesium sulfate monohydrate was added into the sludge samples for drying. For the Soxhlet extraction, the mixture was stirred to obtain a smooth appearance. The homogeneous sample was put in a cellulose thimble and glass wool was placed to the the top of the thimble to eliminate the spilling of the solid. 70 ml of solvent was added into a vessel. The timble and vessel were placed the Soxhlet apparatus. After settling a proper programme (20 min for immersion, 50 min for washing and 15 min recovery of solvent) for lipid extraction the Soxhlet was started to operate. As the boiling point of each solvent is different, the temperature of the heating plate in the Soxhlet was also adjusted to the solvents. The temperature values were set to 210 ℃, 160 ℃, 240 ℃ and 180 ℃ for chloroform, petroleum ether, toluene and hexane, respectively. In the first phase of extraction, the sample containing thimble was immersed in boiling solvent for an effective defatting action. After 20 min the thimble was lifted up. In the washing step, the condensed solvent flowed over the sample and though the thimble for 50 min to complete the extraction process. The final step of the is that solvent consisting in the vessel was removed and recovered for the purpose of using it the further stages. By using Soxhlet apparatus, up to % 70 of the solvent can be recovered. After completion of lipid extraction process, the glass cups containing extracted lipids were cooled then put in a desiccator, stored overnight and weighed the next day to calculate the lipid yield. The lipid yield was determined gravimetrically and calculated as in the Equation (3.2),

$$Lipid\ as\ \%\ of\ dry\ solids = \frac{\text{gain in weight of vessel, g} \times 100}{\text{weight of wet solids, g dry solids fraction}} \tag{3.2}$$

Following the determination of the yield, the lipids were dissolved in the solvent and kept frozen until next analysis.

(a)

(b)

Figure 3.4. Two different devices for lipid extraction; (a) Traditional Soxhlet apparatus, (b) Semi-automatic Soxhlet extractor.

3.2.2.2. Direct Liquid-Liquid Lipid Extraction Method. Initially, direct liquid-liquid lipid extraction method was applied to municipal WWTP sludge samples to extract lipids by using the different solvents like hexane, petroleum ether, chloroform, and toluene. The results of the study indicated that the hexane was the most proper solvent, resulting with the highest lipid yields. Then, the optimisation of the extraction process was done by using different sludge to hexane (S/H) volume ratios (4/1, 2/1, 1/1 and 1/2), different contact times (10, 20, 40 and 60 minutes) in each extraction stage and the number of the extraction stages (up to nine stages). The lipid extraction experiments were performed by using the optimum process conditions throughout the study.

In this study, the direct liquid-liquid lipid extraction was performed consecutively by processing the 40 ml of sludge samples with organic solvent, in a six-paddle mixer-settler batch reactor set an ambient temperature as described by Olkiewicz and colleagues (2014) with little modification. Firstly, a spesific volume of hexane was added to each sludge sample. The sludge samples were stirred with hexane at 200 rpm for 20 minutes and settled at 60 rpm for 15 minutes. After each consecutive extraction stage, samples were extracted again with extra solvent. Later, the hexane phase was filtered using a 4 μm filter paper in order to prevent the residual solids and dried by using anhyrous sodium sulfate. Then, the hexane was recovered by using a Soxhlet apparatus and reused in the sequential stages. After the completion of lipid extracion procedure, extracted lipids were put in a desiccator, stored overnight and weighed the next day to determine the extraction yield. Lipid yield was calculated as in the Equation (3.2). Then, the lipids were dissolved in the solvent and kept frozen until next analysis.

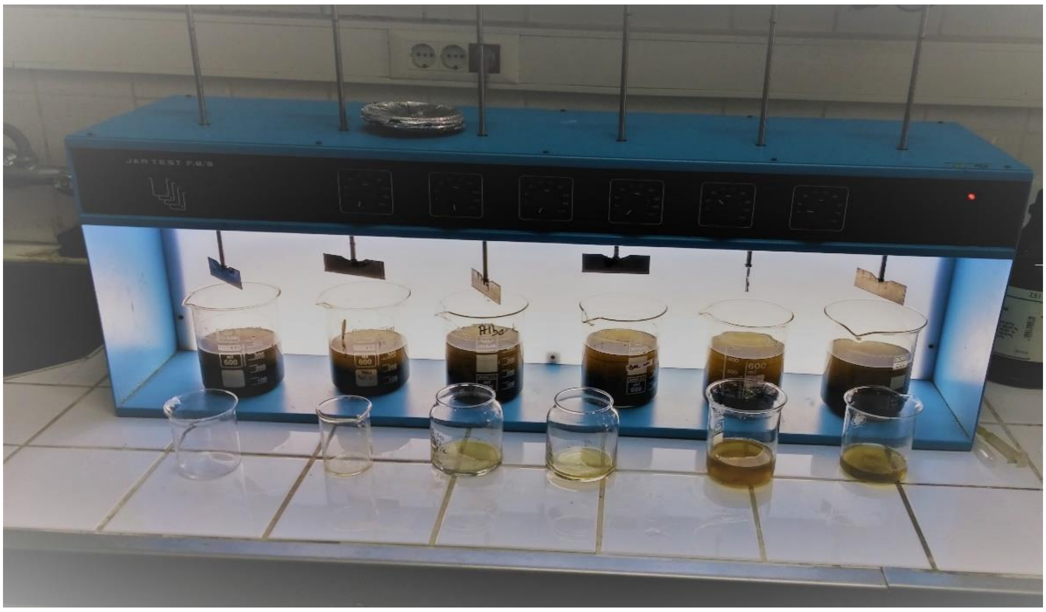

Figure 3.5. The six-paddle mixer-settler batch reactor.

3.2.3. Biodiesel Production

The extracted lipids from the municipal WWTP sludge samples were converted into fatty acid methyl esters (FAMEs – biodiesel) through acid catalyzed transesterification by using the modified Christie's method (Christie at al., 2010). In addition to transesterification of triglycerides, free fatty acids (FFA) existing in the extracted lipids were also esterified in acidic media and produced FAMEs.

Biodiesel was produced following the steps below;

1. The extracted lipids were dissolved in 10 ml hexane in a flask and 20 ml of 2 % H_2SO_4 in methanol was added into the mixture.
2. The flask was capped and put on a magnetic stirrer to mix and heat the mixture overnight at 50°C.
3. The mixture was cooled, transferred to a separatory funnel and 10 ml of 5 % sodium chloride (NaCl) in water was added into the mixture.
4. The FAMEs were extracted three times with 20 mL of hexane by shaking for three minutes to recover the FAMEs.
5. The hexane phase was washed with 10 mL of 2 % sodium bicarbonate ($NaHCO_3$) in water followed by 10 ml of warm water and dried over anhydrous sodium sulfate.
6. The samples were filtered from a filter with 0.45µm pore size and kept frozen with the addition hexane until further analysis.

Figure 3.6. Biodiesel separation after transesterification reaction.

Figure 3.7. Biodiesel produced by transesterificaton.

3.2.4. Biodiesel Analysis

The FAMEs obtained by transesterification were analyzed by an Agilent gas chromatograph-flame ionization detector (GC-FID) and for the calibration of the method, a 37 component FAMEs standard mixture (SUPELCO 47885-U) was used. The separation was provided in the HP-INNOWax column 30 m × 0.32 mm × 0.25 µm with helium as carrier gas and with a stable injector temperature 260ºC. The injection volume of sample was set to 1.5 ml with a split ratio 20/1. The FID was at 260ºC for the period of the analysis. The column oven temperature programme was start from 150 ºC, holding for 1 min and then increased by 2.9 ºC/min to 230 ºC. The results of the GC-FID were used to determine the amount of saponifiable matters in extracted lipids and hence the mass of biodiesel produced from the sludge.

$$\frac{Saponifiable}{\%} = \frac{\left(\frac{FAMEs}{g}\right)}{\left(\frac{Lipid}{g}\right)} \times 100 \qquad (3.3)$$

where FAMEs is the total FAMEs produced after transesterification, determined by the GC-FID run, and Lipid is amount of lipid used for transesterification.

$$\frac{FAMEs}{\%} = \frac{Lipid}{\%} \times \frac{Saponifiable}{\%} \qquad (3.4)$$

where Lipid is the lipid content in sludge samples, Equation (3.3), and Saponifiable is the FAMEs content in the lipid, Equation (3.4).

3.2.5. Analytical Methods

3.2.5.1. Total solids and volatile solids (TS & VS). TS and VS analyses were performed in accordance with the Standard Methods for the Examination of Water and Wastewater (APHA, 2012) using method 2540B and 2540C, respectively. In order to get accurate results, evaporating dishes were brought to a constant weight by using a muffle furnance. For TS measurement, 10 ml of well mixed sludge samples were put in evaporating dishes and placed the steam bath to evaporate moisture in the sludges and then dried for one hour at 103°C in oven. Subsequently, they were cooled in desiccator for 30 minutes and were weighed. For VS measurement, ignition of the sludge samples were performed in the muffle furnace at 550ºC for 30 minutes, then the samples were cooled in the desiccator and weighed. TS and VS contents of the sludge samples were determined by calculations.

3.2.5.2. Mixed liquor suspended solids and mixed liquor volatile suspended solids (MLSS & MLVSS). MLSS and MLVSS determination were conducted according to the Standard Methods (APHA, 2012) using the method 2540D and 2540E, respectively. Firstly, filter papers and crucibles used in these analyses were brought to a constant weight. For MLSS analysis, sludge samples were filtered through a pre-weighed glass fiber filter (Whatman GF/C) and dried in oven for 1 hour at 105ºC. The filter papers were cooled in the desiccator for 30 minutes and weighed. For MLVSS determination, the remnants on the filter papers used in MLSS analysis were ignited in the muffle furnace at 550ºC for 30 minutes. The filter papers with inorganic residual were cooled in the desiccator and weighed. Measurement of MLSS and MLVSS concentrations of the sludge samples were determined by calculations.

3.2.5.3. Chemical oxygen demand and soluble chemical oxygen demand (COD & sCOD). COD determination of the sludge samples was performed by using dichromate closed reflux method in accordance with the Standard Methods for the Examination of Water and Wastewater (APHA, 2012), method 5220D. Initiallly, sludge samples were diluted in some ratios and then they were refluxed with potassium dichromate (K_2CrO_7) and sulfuric acid (H_2SO_4) for two hours at 150°C in HACH COD digester. Mercury sulfate ($HgSO_4$) was added into the samples to block the inhibition of chloride and silver sulfate (Ag_2SO_4) was added as catalyst. For sCOD determination, supernatant portions were obtained by centrifugation of 40 ml of sludge samples at 2850 rpm for 15 minutes as described by Bougrier et al. (2006). The supernatant parts were removed using a syringe and conducted by using dichromate closed reflux method. After the samples were cooled, absorbance values of them were measured at 600 nm by using HACH DR/2010 Portable Data Logging Spectrophotometer. Potassium hydrogen phthalate (KHP) solutions were used to prepare the calibration curve.

3.2.5.4. pH, oxidation reduction potential (ORP) and temperature. All pH, ORP, and temperature determinations were performed by using WTW Inolab pH meter.

3.2.5.5. Viscosity. Viscosity analysis were performed by using Brookfield DV-I Prime digital viscometer. For accurate measurement, the viscometer was switched on and adjusted using the apparatus under the three feet of the device controlling the bubble on the top of the head. The setting was checked before each measurement. Viscosity of the 500ml sludge samples was measured by using Spindle No:2. All viscosity experiments were performed at ambient temperature of 25º C and operating speed was set to 100 rpm.

3.2.5.6. Alkalinity. Alkalinity analyses were carried out in accordance with the Standard Methods for the Examination of Water and Wastewater (APHA,2012). The sludge samples were adjusted to room temperature because they had been cooled for stored. 10 ml of the samples were taken and the initial pH of them was measured. 5 drops of phenolphthalein indicator solution were added and any color change was not observed. Then 5 drops of methyl orange indicator solution was added. The initial pH values of the sludge samples were measured and 0,02 N sulfuric acid (H_2SO_4) was added into the samples slowly for titration. After each addition, the samples were mixed thoroughly. The pH values were recorded to reach a constant reading. Adding titrant was continued until the samples' pH reached 3,7. Alkalinity measurements of the sludge samples were found by calculations.

4. RESULTS AND DISCUSSIONS

4.1. Sludge characterization

The results of characterization of municipal primary and secondary sludge samples used in the study are given in Table 4.1. All of the measurements were performed in triplicate according to the Standard Methods of the Examination of Water and Wastewaters. Primary and secondary sludge samples were collected from a municipal wastewater treatment plant located in İstanbul. Primary sludge samples were collected at the bottom of the primary clarifier and secondary sludge samples produced by an activated sludge process were taken from the secondary clarifier. The sludge composition depends on the sludge type and varies during the wastewater treatment. The samples were sent to the laboratory with an urgent need to store at 4ºC before using. The sludge samples were characterized by measuring their total solid (TS), volatile solid (VS), mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), chemical oxygen demand (COD), soluble chemical oxygen demand (sCOD) concentrations, viscosity, pH, oxidation reduction potential (ORP) and alkalinity.

Table 4.1. Characteristics of sludges used in experiments.

Parameter	**Unit**	**Primary Sludge**	**Secondary Sludge**
TS	(mg/L)	42100	13108
VS	(mg/L)	24430	7790
MLSS	(mg/L)	38600	12100
MLVSS	(mg/L)	21960	7050
COD	(mg/L)	40280	8780
sCOD	(mg/L)	5580	2240
pH	-	6.2	6.1
ORP	(mV)	45	41.6
Viscosity	(mPa.s)	8	6.4
Alkalinity	(mg/L)	4800	1600

4.2. Effect of the sludge and solvent types on the lipid yield

Some properties should be considered when selecting the proper solvent for extraction of lipids such as boiling point, polarity, immiscibility with water, cost and environmental issues (Siddiquee et al., 2011). The selected solvents should be nonpolar, water immiscible, volatile and have low viscosity because a high viscosity adversely affect recovery of solvent and extraction efficiency (Dong et al., 2016). In the lipid extraction step, hexane, toluene, petroleum ether and chloroform were used to determine the which solvent has the highest extraction efficiency. The physical features in respect of lipid extraction of the solvents used in the study are shown in Table 4.2. Mixed solvents were not utilized in this study. They can extract high amounts of lipids. However, the use of them is not profitable as the separation of solvent from mixture is hard and results more energy consumption making the total biodiesel production process costly. In addition, polar solvents extract larger amount of extractable fraction, resulting a prominent increase in lipid yield, because they have the ability to extract both polar lipid and non-lipid portion. This is accompanied by a rising in the nonsaponifiable fraction resulting higher biodiesel contamination. Moreover, the use of polar solvents is not economical compared the nonpolar solvents as an effective separation of them from post-extracted sludge is impossible. (Pokoo-Aikins et al., 2010; Siddiquee et al., 2011). In addition to all these reasons, as the sludge samples were used in wet form in direct liquid-liquid lipid extraction method polar solvents such as methanol and ethanol which can miscible with water can not be used as organic solvent in the experiments.

Table 4.2. Physical features of some common organic solvents (Reichardt, 2003; Dong et al., 2016).

Solvent	**Formula**	**Boiling point (°C)**	**Viscosity (cP,25°C)**	**Solubility in water (g/100g)[a]**	**Relative polarity**
Methanol	CH_4O	64.6	0.54	M	0.762
Ethanol	C_2H_6O	78.5	1.07	M	0.654
Hexane	C_6H_{14}	69	0.31	0.0014	0.009
Chloroform	$CHCl_3$	61.2	0.54	0.8	0.259
Toluene	C_7H_8	110.6	0.56	0.05	0.099

[a] M: completely miscible

In the liquid–liquid lipid extraction process the solvent and sludge samples were mixed to sludge to solvent (S/H) ratio of 1:2 at 200 rpm for 20 min and settled at 60 rpm for 15 min (three extraction steps) at the ambient temperature.

The results showing the effects of different solvents on the lipid yields of the municipal primary and secondary WWTP sludge samples for both of standard drying and liquid–liquid lipid extraction methods are given in Table 4.3. In the liquid-liquid lipid extraction method, utilization of hexane as a solvent resulted with higher lipid yields for the municipal primary sludges compared to other solvents. This can be explained related with polarity. Based on the ‘‘like dissolve like” principle, nonpolar solvents are the most favorable for extraction of lipids from primary sludge that is composed of mostly nonpolar lipids due to deriving from glyceride in wastewater (Pokoo-Aikins et al., 2010). As the hexane has the lowest polarity compared to other solvents, it achieved to extract the highest lipid from primary sludge samples.

The highest lipid yields were obtained from primary sludge samples by standard dry lipid extraction method. The lipid yields were close to each other for all of the solvents. In this method, Soxhlet extraction led to extraction of the lipids in primary sludge samples near to maximum levels. In lipid extraction process, the performance of solvents having different polarities may change according to applied extraction method (Zhu et al., 2014).

Table 4.3. Lipid yields obtained from acidified municipal WWTP sludges by using different solvents.

	Lipid Yield (%)[a]			
Solvent Type	**Liquid-liquid extraction method**[b]		**Standard dry extraction method**[c]	
	Primary sludge	**Secondary sludge**	**Primary sludge**	**Secondary sludge**
Hexane	19.35±1.45	2.72±0.16	24.83±0.6	7.31±0.17
Petroleum ether	13.43±0.43	2.27±0.13	23.8±1.3	11.9±0.7
Chloroform	12.33±0.67	1.27±0.07	23.1±1.43	10.08± 0.95
Toluene	11.31±1.11	1.32±0.08	22.93±1.85	10.33±0.62

[a] Lipid yield (%), on the basis of dry sludge

[b] Extraction conditions: Sludge/Solvent 1:2, 20 min, ambient temperature, three extraction stages, 200 rpm of mixing speed

[c] Extraction according to standard $MgSO_4 \cdot H_2O$ method

Considering the lipid efficiency of the secondary sludge samples, lipid extraction performance of all solvents were very low and similar with each other in the liquid-liquid lipid extraction method. According to these results it can be concluded that the liquid-liquid lipid extraction method was not efficient for the secondary sludges. By using standard dry extraction method, lipid extraction yields of solvents except for hexane were almost the same. Secondary sludge has lots of microorganisms whose cell wall includes polar phospholipids. Hexane has not ability to extract these polar compounds because of its nonpolar structure.

The higher lipid yields were acquired from the primary sludge samples compared to the secondary sludge samples. This is because the combination of primary sludge primarily includes organic material coming from raw wastewater which contains a mixture of floating grease and solids. Otherwise, secondary sludge is a composition of suspended solids and microorganisms originated from aerobic biological treatment of the primary treated wastewater (Olkiewicz et al., 2014).

As the primary sludge samples resulted with the higher lipid yields than the secondary sludge samples, optimization studies of the liquid-liquid lipid extraction were conducted with the primary sludge samples. In these optimization studies, hexane was used as organic solvent because it achieved the highest lipid yield from primary sludges. In order to make comparison between two sludge types in further steps under same conditions, hexane was also used as organic solvent in experiments of secondary sludges. Additionally, hexane has several advantages over other solvents. While chloroform and toluene have harmful effects on environment, hexane is a environmentally friendly solvent. Recovery of toluene consumes more energy because of its higher boiling point. However, hexane has high volatility and also it has higher immiscibility with water (Siddiquee et al., 2011).

4.3. Effect of lipid extraction methods on yields

The high water content of municipal WWTP sludges is an inconvient factor that prevents the widespread of biodiesel production process. In order to eliminate this situation, the direct liquid-liquid lipid extraction method was used in this study. In addition, for determining the efficiency of this method it was compared with standard dry extraction method in terms of lipid and biodiesel yields.

The results of the study as shown in Table 4.4 demonstrated that the standard drying method resulted with the higher lipid yields from the primary sludge samples compared to the liquid-liquid lipid extraction method. These outcomes are consistent with the results of the study by Kech and colleques (2018). They also found that the lipid fraction in the dry sludge is higher than that in the wet sludge. Obtaining higher lipid yields with the standard drying method was possibly because the dry sludge was crushed prior to lipid extraction, thereby increasing the accessibility of the lipids for solvent. Additionally, water present in the wet sludges tends to retain lipids from the extraction by solvent and it can wrap the sludge particles causing to prevent hexane from penetrating well into the solid particles. Moreover, temperature has an important effect on the lipid extraction process increasing the lipid yield. While the liquid-liquid lipid extraction was performed at ambient temperature, lipid extraction of dry sludges was carried out in a Soxhlet apparatus at a temperature as high as 180°C. In the study by Olkiewicz and co-workers (2015), it was indicated that the lipid extraction at ambient temperature (25°C) resulted in a reduction of the lipid yield by approximately 20 % compared to higher temperatures.

Table 4.4. Lipid extraction and transesterification yields obtained from acidified municipal WWTP sludges by using two different lipid extraction methods.

Sludge type	**Liquid-liquid extraction method**[a]			**Standard dry extraction method**[b]		
	Lipid (%)[c]	**Saponifiable (%)**[d]	**Biodiesel (%)**[c]	**Lipid (%)**[c]	**Saponifiable (%)**[d]	**Biodiesel (%)**[c]
Primary sludge	19.35±1.4	74±6	14.7±1.6	24.83±0.6	60±5	14.5±1.3
Secondary sludge	2.72±0.1	39±5	1.05±0.07	7.31±0.17	32±4	2.35±0.4

[a] Extraction conditions: S/H 1:2, 20 min, ambient temperature, three extraction stages, 200 rpm of mixing speed

[b] Extraction according to standard $MgSO_4 \cdot H_2O$ method

[c] Lipid and biodiesel yields (%), on the basis of dry sludge

[d] Saponifiable yield (%), on the basis of lipid

Although 28 % higher lipid yield was achieved from the primary sludge samples by using the standard reference method, almost the same biodiesel yields were attained by both of the extraction methods. Total amount of lipid fraction extracted from sludge is not the actual value that could be convertible biodiesel (FAMEs). The lipid fraction extracted from sludge includes not just as acyglycerols and free fatty acids (saponifiable lipids). It also includes pigments, linear alkyl benzenes, trepans, hydrocarbons, polycyclic aromatic hydrocarbons, sterols and some waxes (Jarde et al., 2005; Pastore et al., 2013). Just acyglycerols and free fatty acids (FFAs) constituting the saponifiable part of lipids can be converted into biodiesel (transesterifiable/esterifiable to FAMEs). Hydrocarbons, other non-polar substances (non-lipids) and some parts of lipids such as waxes and sterol which can be co-extracted with hexane are considered as non-saponifiable lipids. Non-saponifiable lipids can not be converted into biodiesel and represent lipid contaminants. In a study by Olkiewicz et al. (2015), the decrease in temperature caused to increase the quantity of saponifiable lipids, indicating that the lipid extraction at higher temperature attained more lipid contamination with non-saponifiable substance not converting into biodiesel. Regardless of variations in the amount of extracted lipid and saponifiable portion, the biodiesel yields remained almost unchanged (Olkiewicz et al., 2015).

Lipid and biodiesel yields obtained from the secondary sludge samples by liquid–liquid lipid extraction method were 62.6 % and 55.3 % lower than those achieved by standard drying method. Therefore, the liquid–liquid lipid extraction method from the secondary sludge is not efficient for extraction of lipids consisting in this sludge and conversion of them to biodiesel. Due to the liquid–liquid lipid extraction from acidified primary sludge is more convenient than secondary sludge, the optimization of this method was carried out using the primary sludge.

4.4. Optimization of the direct liquid-liquid lipid extraction from primary sludge

The optimization of the lipid extraction from acidified municipal primary sludge samples was conducted for nine stages at different contact times of 10, 20, 40 and 60 minutes (min) and different sludge to hexane volume ratios (S/H) of 4:1, 2:1, 1:1 and 1:2. The experiments were carried out at ambient temperature by using 40 mL of sludge samples at 200 rpm mixing speed. These operating conditions were kept constant throughout the experiments. Figures 4.1 and 4.2 show the optimization of the direct liquid–liquid lipid extraction process for acidifed primary sludge samples.

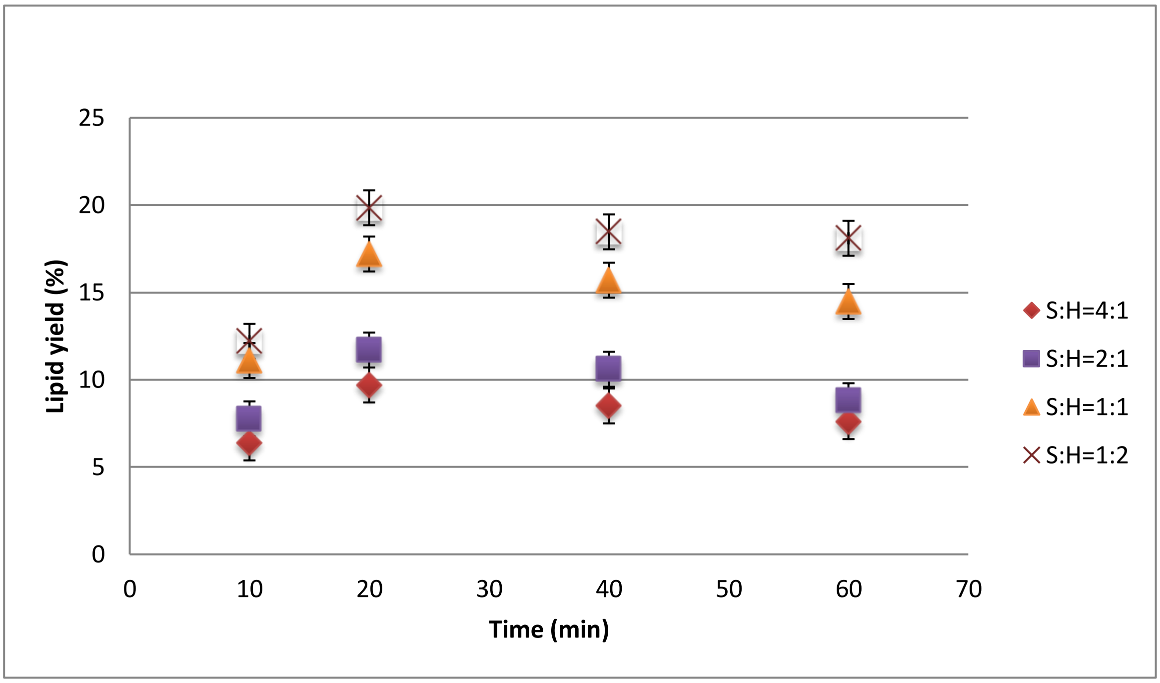

Figure 4.1. Optimization of extraction time and sludge to hexane volume ratios for acidified municipal primary WWTP sludge (Extraction conditions: three extraction stages, ambient temperature, 200 rpm of mixing speed).

As it can be seen in Figure 4.1, the lowest lipid extraction yields were obtained for 10 min of extraction time giving 6.38 %, 7.76 %, 11.1 % and 12.2 % of lipids for sludge to hexane volume ratios 4:1, 2:1, 1:1 and 1:2, respectively. This is because 10 minutes contact time of sludge with solvent was not sufficient for an effective lipid extraction. Extraction of sludge samples with hexane for 20 min resulted with the best values achieving the yields of 9.7 %, 11.7 %, 17.2 % and 19.85 % lipid for sludge to hexane volume ratios 4:1, 2:1, 1:1 and 1:2, respectively. Generally lipid extraction efficiency is expected to rise with the increasing contact time (Olkiewicz et al., 2014). However, after 20 min the lipid yield decreased as the contact time increased for the all of the sludge to hexane ratios. Because hexane is highly volatile organic solvent and used extraction process in the study is an open system, as the mixing time increased the evaporation of the hexane from the system also increased, decreasing the lipid extracton yield. An evaporation test was conducted to determine the degree of vaporization of the hexane. At the end of 60-min stirring period the volume of hexane drastically reduced from 80 mL to 10 mL. The highest lipid yield was achieved at a sludge to hexane volume ratio of 1:2 for the 20 min mixing time. According to the results of optimization studies, the more solvent was added, the more lipid yield was achieved.

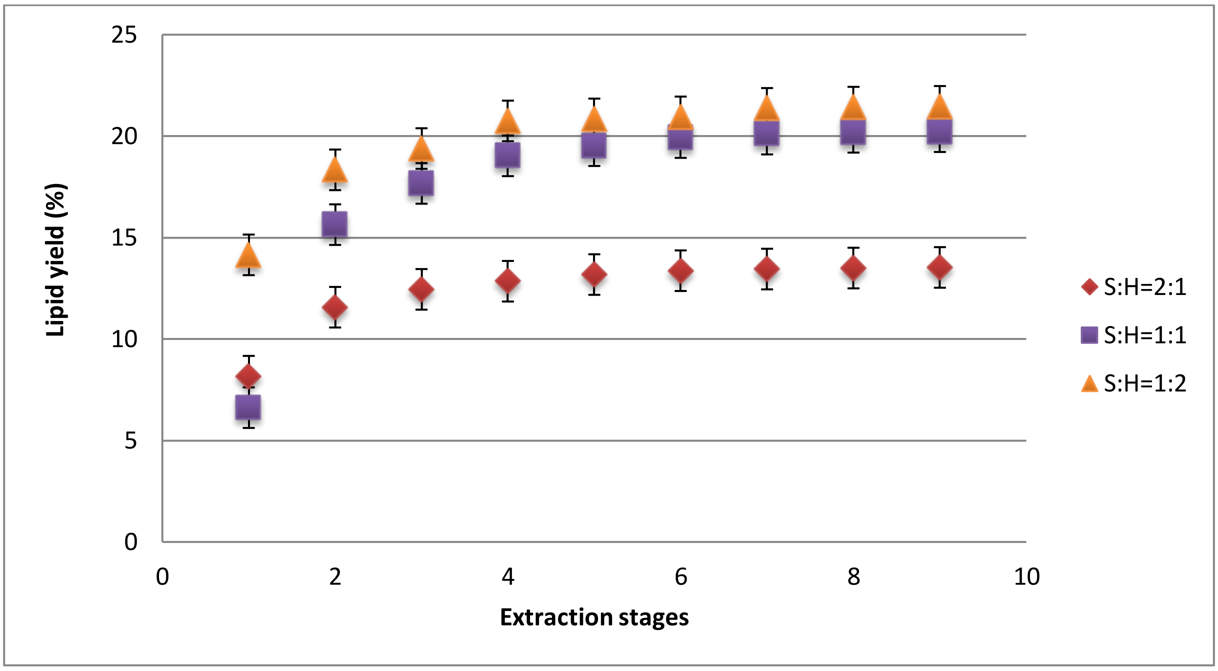

Figure 4.2. Optimization of sludge to hexane volume ratios for acidified municipal primary WWTP sludge (Extraction conditions: ambient temperature, 20 min, 200 rpm of mixing speed).

The outcomes of the lipid extraction studies demonstrated that the amount of cumulative lipid yield increased with sequential extraction steps, attaining a stable value at the end of the nine extraction steps. As the rate of increase in lipid yield was very little, lipid extraction was terminated at the end of the ninth step. The obtained lipid yield at the end of the three steps constitutes approximately 91 % of the accessible lipid yield. According to Olkiewicz et al. (2014), three sequential extraction steps are enough to achieve 91 % of lipids existing in the municipal primary WWTP sludge. In order to attain this lipid extraction efficiency there are two situations. If extraction time is to be minimized, the ideal process conditions are 3 steps using a 1:2 sludge to hexane volume ratio for 20 min in accordance with the results of the study by Olkiewicz et al. (2014). Conversely, if the amount of solvent used is to be minimized, the ideal process conditions are 5 steps using a 1:1 sludge to hexane volume ratio. Olkiewicz et al. (2014), determined the best extraction conditions for municipal primary WWTP sludges. Considering the minimization of solvent used, S/H 2:1 for 60 min extraction period and for minimizing of extraction time, S/H 1:2 for 20 min extraction period were found as ideal process conditions. Kech et al. (2018) also performed the liquid–liquid lipid extraction method by using municipal WWTP sludges. They extracted lipids at three sequential extraction steps in a closed system. They used Smedes mixture (isopropyl alcohol/cyclohexane/water) as solvent and found the best operation conditions for lipid extraction were determined to be 60 min sludge to solvent ratio of 1:1.6:2.

The further experimental studies were performed under optimum conditions of sludge to hexane volume ratio of 1:2, 20 minutes mixing period and the extraction stage numbers to be three.

4.5. Effect of sludge pretreatments

Acidification, ultrasonication and microwave techniques were applied to municipal primary sludge samples as pretreatment prior to lipid extraction step. These pretreatment methods were prefered because of the their ability to expose the lipids present in the sludge bonded to macromolecules which can not be reached by the solvent (Olkiewicz et al., 2015). Each method was first applied alone and then ultrasonication and microwave applications were performed in combined with acid pretreatment to determine their efficiency by themselves and in combination.

Table 4.5. Lipid and biodiesel yields obtained from municipal primary WWTP sludge pretreated with different methods.

Acidification	Pre-treatment	Extraction type					
		Standard dry method[a]			Liquid-liquid method[b]		
		Lipid (%)[c]	Saponifiable (%)[d]	Biodiesel (%)[c]	Lipid (%)[c]	Saponifiable (%)[d]	Biodiesel (%)[c]
Acidified	Untreated	24.8±0.6	60±5	14.5±1.3	19.35±1.4	72±6	14.7±1.6
	Ultrasonic	25.8±0.43	62±6	15.7±1.8	21.8±0.11	73±5	15.8±1.1
	Microwaved	25.1±0.3	61±4	15.1±0.5	20.6±0.02	72±2	15.3±0.4
Non-acidified	Untreated	22.6±0.25	55±3	12.5±0.5	10.5±0.28	54±5	5.6±0.4
	Ultrasonic	20.7±0.35	55±5	11.5±1.2	10.2±0.6	54±6	5.5±0.5
	Microwaved	19.8±0.19	52±3	1.2±0.7	8.1±0.8	53±3	4.29±0.2

[a] Extraction according to standard $MgSO_4 \cdot H_2O$ method

[b] Extraction conditions: S/H 1:2, 20 min, ambient temperature, three extraction stages, 200 rpm of mixing speed

[c] Lipid and biodiesel yields (%), on the basis of dry sludge

[d] Saponifiable yield (%), on the basis of lipid

4.5.1. Effect of pre-acidification (acid pretreatment)

The results of the study demostrated that, in both of the lipid extraction methods, acid pretreatment (pre-acidification) improved the lipid and biodiesel yields obtained from the municipal primary sludge samples. In the direct liquid-liquid lipid extraction method, acid pretreatment increased the lipid yield by 84 % and biodiesel yield by 162 %. Olkiewicz et al. (2014) carried out a study to determine the efficiency of acid pretreatment application to wet sludge using the direct liquid-

liquid lipid extraction method with hexane. They found that acidification increased the lipid and biodiesel yields at high levels as in this study. Pastore et al. (2013) conducted out a study aiming to analyse the exact characterization of the lipidic compounds extracted from the primary sludge samples with hexane. They discovered the basic constituents of the sludge to be soap. Therefore, the big difference between the yields attained with and without acidification can be explained with the presence of fatty acids from commercial soaps, potassium and sodium from household cleaning products, cosmetics, lubricant and coatings in primary sludge. The physico-chemical process causes to generate insoluble calcium and magnesium salts settling during the primary wastewater treatment, that present in the primary sludge at the end of the process. Therefore, acidification contributes to the conversion of insoluble soaps to FFA, which can be solubilized by the solvent, enhancing lipid yield and saponifiable substances (Pastore et al., 2013).

On the other hand, the improvements in lipid and biodiesel yields remained in a range of 10–16 % in the standard drying method. There is a minor difference between the yields obtained with and without acidification. After acidification process the hydrolysis could exposure the lipids bonded to proteins and/or carbohydrates achieving more esterifiable (saponifiable) lipids and leaving the polar materials unextracted. Moreover, the triglycerides, phospholipids, wax esters and sterol esters present in the sludge may have been also hydrolysed into FFAs leaving the polar portion (glycerol, alcohol and phosphate group) unextracted, therefore there is a little improvement in the yields gravimetrically (Olkiewicz et al., 2015).

4.5.2. Effect of ultrasonication and microwave pretreatments

The lipid and biodiesel yields obtained from the municipal primary sludge samples using the standard drying and direct liquid-liquid lipid extraction methods are presented in Table 4.5. The values represent the average of at least three parallels of the samples collected in WWTP.

The application of ultrasonication and microwave pretreatments did not improve the lipid and biodiesel yields obtained from the non-acidified primary sludge samples by both of the drying and liquid-liquid lipid extraction methods. Ultrasound is a well-known technique that helps to create emulsions when the cavitation threshold is obtained, even without emulsifiers (Gaikwad et al., 2008). The formation of stabilized emulsions and spread of lipid droplets during the ultrasonication pretreatment may cause a decrease in the lipid extraction efficiency. Microwave treatment also results in the formation of emulsion inhibiting lipid extraction performance (Dong et al., 2016). Moreover, after a while these pretreatments cause to an increase in the size particle progressively because of reflocculation of the particles by the formation of new connections of the organic substances from cellular materials that were released before (Pilli et al., 2011). On the other hand, when compared to the untreated sludge samples, the combined acidification/ultrasonication and acidification/microwave pretreatments were very effective on improving the lipid and biodiesel yields obtained by the direct liquid-liquid lipid extraction method. The pretreatment applications increased the lipid yield by approximately 2 folds and biodiesel yield by 2.5-2.8 folds for the primary sludge samples. This may be resulted from hydrolysis of the emulsions and re-floccculated large size particles formed during ultrasonicaton and microwave processes by the effect of the acidification, thus achieving more efficient lipid extraction (Dong et al., 2016).

However, the effect of combined acidification/ultrasonication and acidification/microwave pretreatments were much smaller when the standard drying method was used for lipid extraction. The combined acidification/ultrasonication and acidification/microwave pretreatments increased the lipid yields by 14.2 % and 11 % and biodiesel yields by 25.6 % and 20.85 % obtained from the non-acidified primary sludge samples, respectively.

The application of ultrasonication and microwave pretreatments slightly enhanced the lipid and biodiesel yields obtained from the acidified primary sludge samples by both of lipid extraction methods. Only minor increases of 6-12 % and 4-7 % were observed in the lipid and biodiesel yields, respectively, attained from the primary sludge samples by using liquid-liquid lipid extraction method and 1-4 % and 4-8 % were obtained in the lipid and biodiesel yields, respectively, through standard dry method.

It seems that in lipid extraction and biodiesel production, acidification itself is enough for the pretreatment of municipal primary WWTP sludges. Application of the combined acidification/ultrasonication and acidification/microwave pretreatments to the primary sludge samples is not beneficial due to the high cost for intense energy consumption, low efficiency and difficult to scale up.

4.6. Biodiesel production

The yields obtained from the liquid-liquid lipid extraction were compared with those obtained from standard dry method to determine the effects of the former method on biodiesel yield and FAME composition. According to the results it was confirmed that there was not significant differences between two methods in terms of FAME composition and biodiesel production yield.

The total lipidic compounds extracted from the municipal WWTP sludge samples do not represent the real value. Only some part of them can be converted to the biodiesel. This is the reason for that while higher lipid yield is obtained by using an extraction method lower saponifiable matters can be obtained by the same method.

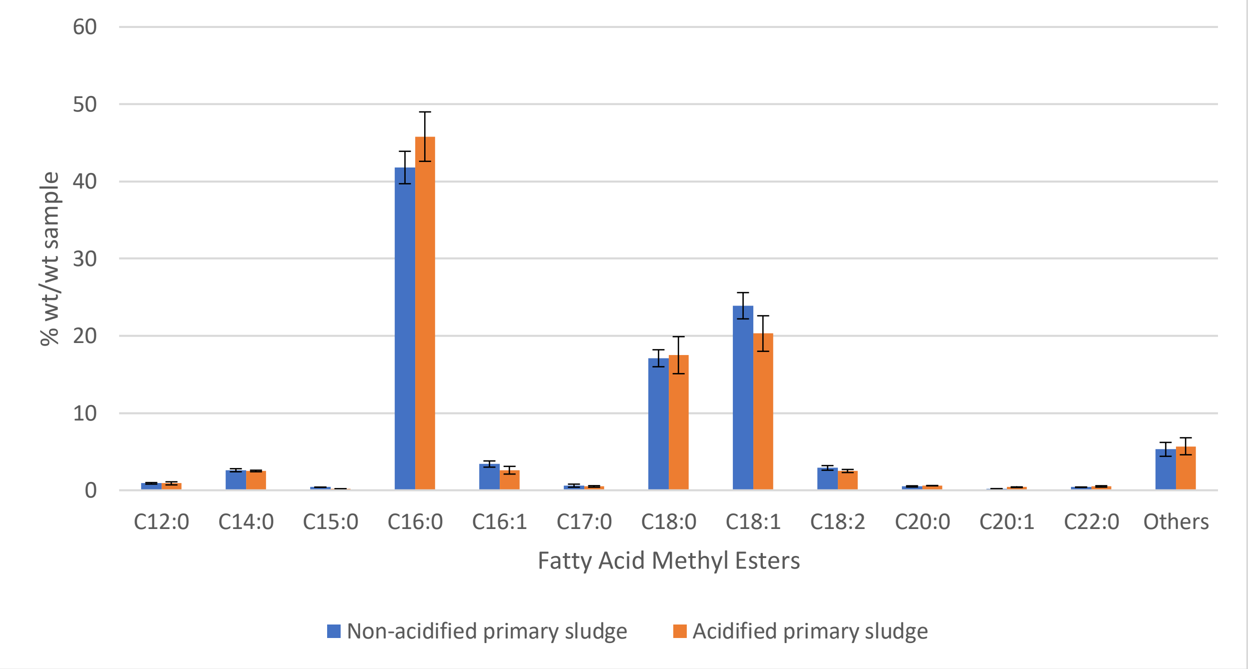

Figure 4.3. FAME composition of biodiesel obtained from standard dry lipid extraction method.

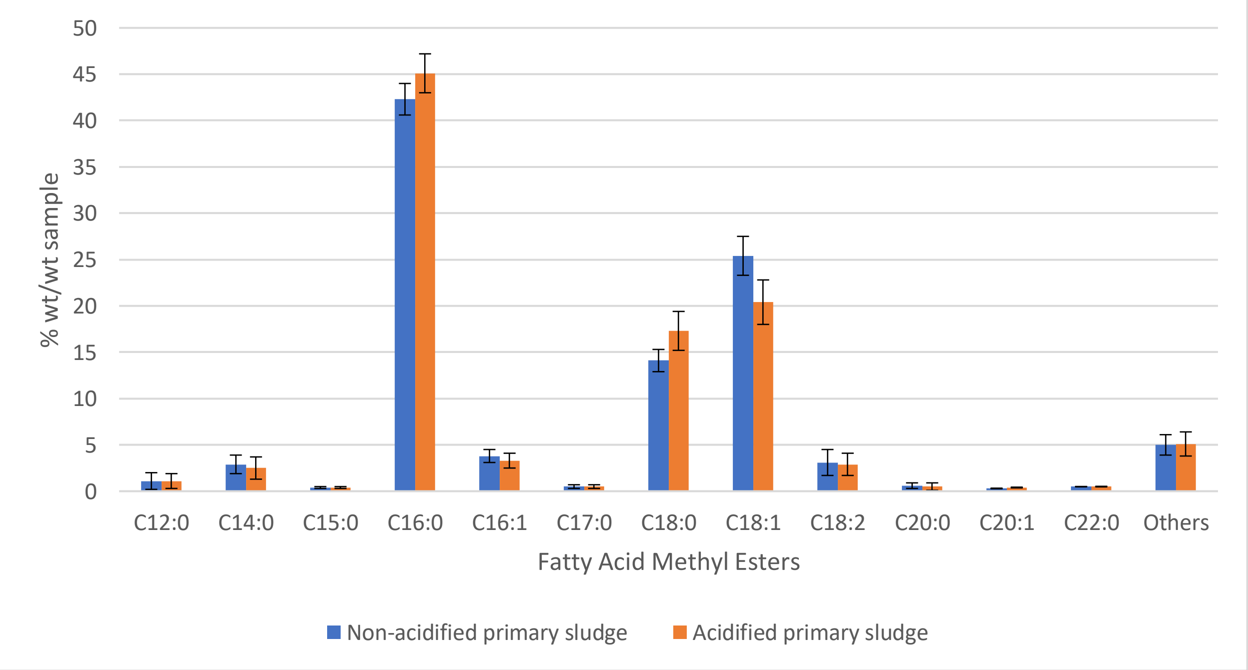

Figure 4.4. FAME composition of biodiesel obtained from direct liquid-liquid lipid extraction method.

The optimized liquid-liquid lipid extraction from the acidified primary sludge samples achieved 72 % of saponifiable matter (on the basis of lipid) and 14,7 % of biodiesel (on the basis of dry sludge). These yields are higher that those attained by standard dry extraction method (60 % of saponifiable matter and 14,5 % of biodiesel).

The characteristics of fuel generated mainly rely on fatty acid composition of biodiesel. Therefore, determination of FAME composition of biodiesel produced is important when examining compatibility of potential biodiesel feedstock (Knothe, 2005). As the primary sludge samples resulted with the higher lipid and biodiesel yields compared to the secondary sludge samples samples, the fatty acid composition of biodiesel produced from the primary sludge by using both of extraction methods was analyzed and the results were shown in the Figure 4.3 and Figure 4.4. The analyses of the biodiesel samples demonstrated that biodiesel produced from the primary sludge samples mainly includes methyl esters of palmitic acid (C16:0), oleic acid (C18:1) and stearic acid (C18:0). Additionally, acidification of sludge samples had no essential impact on fatty acid profile of the primary sludge samples. These outcomes showed compatibility with the findings of Olkiewicz and co-workers and it can be deduced that the primary sludge is an proper raw material for biodiesel production (Olkiewicz et al., 2015; Kech et al., 2018). As the FAME compositions of biodiesel produced by two extraction methods are the similar, the observed distinctions are not important. This case is crucial since the direct liquid-liquid lipid extraction method had no effect on the properties of biodiesel produced which makes that process convenient.

5. CONCLUSIONS

In this study, some experiments were conducted to explore the lipid extraction efficiency for biodiesel production from municipal WWTP sludges by using the novel direct liquid-liquid lipid extraction method without applying expensive sludge dewatering and drying processes. This extraction method was compared with the conventional reference drying method 5520E. Two different types of municipal WWTP sludges "primary sludge" and "secondary sludge" were used for biodiesel production in both lipid extraction techniques. Effects of different reaction conditions of the direct liquid-liquid lipid extraction method on lipid yields were investigated. This research also contains application of acid, ultrasonic and microwave pretreatments to the primary sludge samples to demonstrate the impacts of pretreatments on the lipid and biodiesel yields.

Based on the experimental results of the study the following conclusions were attained;

- The results indicated that municipal primary WWTP sludges having high lipid content can be used as efficient feedstocks for biodiesel production. However, municipal secondary WWTP sludges were found to be inefficient to be used as lipid feedstocks for biodiesel production due to their low lipid contents.

- Although, the standard drying method was found to be more effective in the lipid extraction from the primary sludge samples, the similar biodiesel yields were achieved by both of the standard drying and liquid–liquid lipid extraction methods. The lower lipid and biodiesel yields were obtained from the secondary sludge samples applying the liquid–liquid lipid extraction method compared to the standard drying method. Therefore, that can be concluded that the liquid-liquid lipid extraction from the secondary sludge is not effective for the extraction of lipids formed in this sludge and their conversion to biodiesel.

- Acid pretreatment (pre-acidification) enhanced the lipid and biodiesel yields obtained from the primary sludge samples in both of the lipid extraction methods. The application of ultrasonication and microwave pretreatments did not improve the lipid and biodiesel yields obtained from the non-acidified primary sludge samples by both of the drying and liquid-liquid lipid extraction methods. On the other hand, when compared to untreated sludge sample, the combined acidification/ultrasonication and acidification/microwave pretreatments were very effective on improving the lipid and biodiesel yields obtained by the direct liquid-liquid lipid extraction method. The highest biodiesel yields were attained by the application of combined acidification/ultrasonication pretreatment to the primary sludge samples by using the direct liquid-liquid lipid extraction method.

- The FAME analyses of biodiesel obtained from the standard dry extraction and liquid-liquid lipid extraction methods indicated that methyl esters of palmitic acid (C16:0), oleic acid (C18:1) and stearic acid (C18:0) are the main costituents of biodiesel obtained from the primary sludge samples. Different lipid extraction methods did not have a remarkable impact on the FAME composition. This case is crucial since the liquid-liquid lipid extraction method had no effect on the properties of biodiesel produced which makes that process convenient

- Even though lipid extraction from primary dry sludge is more efficient, biodiesel production from dry and wet sludges is equally effective. Moreover, drying of sludge samples before the standard drying extraction method is economically disadvantageous for large-scale utilization because of enormous energy need. It was apparent that the new method was simple and energy saving method due to the elimination of drying and dewatering steps. In both of the methods, hexane recovery also brings some economy. The direct liquid-liquid lipid extraction method is an alternative reuse/disposal method, decreasing the amount of municipal WWTP sludges, requiring high treatment and handling cost. Therefore, it has a potential to replace this conventional method.

REFERENCES

Aksoy, L., 2010. Alternatif Enerji Kaynağı Olarak Biyodizel ve Üretim Prosesleri. Electronic Journal of Vehicle Technologies, 2, 45-52.

Alptekin, E., Canakci, M., 2006. Biyodizel ve Türkiye'deki durumu. Mühendis ve Makine, 47, 57-64.

Andreoli, C.V., Speling, M.V., Fernandes, F., 2007. Sludge treatment and disposal. Biological wastewater treatment series 6, IWA Publishing, London.

Angerbauer, C., Siebenhofer, M., Mittelbach, M., Guebitz, G.M., 2008. Conversion of sewage sludge into lipids by Lipomyces starkeyi for biodiesel production. Biosource Technology, 99, 3051-3059.

APHA/AWWA/WPCF, 2012. Standard Methods for the examination of Water and Wastewater. 22nd ed. Washington, DC: American Public Health Association/American Water Works Association/Water Pollution Control Federation, USA.

Ar, F., Karaosmanoglu, F., Koc, A.A., Acaroglu, M., Sarsu, F., Ozsoyler, Y., Boluk, G., Isler, A., Aygun, O.M., 2010. Biofuels Report. World Energy Council, Turkish National Committee Publication, Ankara.

Atabani, A.E., Silitonga, A.S., Badruddin, I.A., Mahlia, T.M.I., Masjuki, H.H., Mekhilef, S., 2012. A comprehensive review on biodiesel as an alternative energy resource and its characteristics. Renewable and Sustainable Energy Reviews, 16, 2070–2093.

Bandelin, 2009. Sonopuls Ultrasonic Homogenizers Operating Instructions. Bandelin Electronic GmbH & Co. KG.

Bharathi, P., Karthiga, R., Shamli, M., Vivetha, K., Subathra, M., 2014. Extraction of lipids from municipal sewage sludge for production of biodiesel via transesterification process. International Journal of Pharma and Bio Sciences, 5, 132–141.

Bharathiraja, B., Yogendran, D., Kumar, R.R., Chakravarthy, M., Palani, S., 2014. Biofuels from sewage sludge- A review. International Journal of Chemical Techonology Research, 6, 4417-4427.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology, 37, 911-917.

Boluk, G., Koc, A., 2013. The implications of biofuel policy in Turkey. International Journal of Energy Economics and Policy, 3, 14-22.

Bougrier, C., Albasi, C., Delgenes, J.P., Carrere, H., 2006. Effect of ultrasonic, thermal and ozone pretreatments on waste activated sludge solubilization and anaerobic biodegradability. Chemical Engineering and Processing, 45, 711-718.

Boocock, D.G.B., Konar, S.K., Leung, A., Ly, L.D., 1992. Fuels and chemicals from sewage sludge: 1. The solvent extraction and composition of a lipid from raw sewage sludge. Fuel, 71, 1283–1289.

Bozaghian, M., 2014. Characterization and synthesis of biodiesel from sludge available in the Umeå region. Degree Project in Engineering Chemistry, Umeå University.

British Petroleum, BP Statistical Review of World Energy 2017. https://www.bp.com/content/dam/bp-country/de_ch/PDF/bp-statistical-review-of-world-energy-2017-full-report.pdf. Date accessed October 2018.

Buyuktuncel, S.E., 2012. Gelişmiş Ekstraksiyon Teknikleri I. Hacettepe University Faculty of Pharmacy Journal, 32, 209-242.

Campell, M.K., 1991. Biochemistry, 2nd edition, Orlando, Florida. Saunders College Publishing.

Chongkhong, S., Tongurai, C., Chetpattananondh, P., 2009. Continuous esterification for biodiesel production from palm fatty acid distillate using economical process. Renewable Energy, 34, 1059-1063.

Christie, W.W., Han, X., 2010. Lipid Analysis: Isolation, Separation, Identification and Lipidomic Analysis, Fourth Ed., The Oily Press, Bridgwater.

Clayden, J., Geeves, N., Warren, S., 2012. Organic Chemistry, 2nd edition, Oxford. Oxford University Press.

Demirbas, A., 2008. Biofuels sources, biofuel policy, biofuel economy and global biofuel projections. Energy Conversion and Management, 49, 2106–2116.

Demirbas, A., 2005. Biodiesel production from vegetable oils via catalytic and non-catalytic supercritical methanol transesterification methods. Progress in Energy and Combustion Science, 31, 466-487.

Demirbas, A., Bamufleh, H.S., Edris, G., Al-Sasi, B.O., 2017. Biodiesel production from lipids of municipal sewage sludge by direct methanol transesterification. Energy Sources, Part A: Recovery, Utilization, and Environmental Effects, 39, 800-805.

Dong, T., Knoshaug, E.P., Pienkos, P.T., Laurens, L.M.L., 2016. Lipid recovery from wet oleaginous microbial biomass for biofuel production: A critical review. Applied Energy, 177, 879-895.

Dufreche, S., Hernandez, R., French, T., Sparks, D., Zappi, M., Alley, E., 2007. Extraction of lipids from municipal wastewater plant microorganisms for production of biodiesel. Journal of the American Oil Chemists' Society, 84, 181–187.

Energy Market Regulatory Authority (EMRA), 2016. http://www.emra.org.tr/. Date accessed October 2018.

Eskilsson, C.S., Björklund, E., 2000. Analytical-scale microwave-assisted extraction. Journal of Chromatography A, 902, 227–250.

Fjerbaek, L., Christensen, K.V., Norddahl, B., 2009. A review of the current state of biodiesel production using enzymatic transesterification. Biotechnology and Bioengineering, 10, 1298-1315.

Formo, W.M., Jungermann, E., Norris, F.A., Sonntag, N.O.V., 1979. Bailey's Industrial Oil and Fat Products, 99 -159, John Wiley and Sons, A.B.D.

Freedman, B., Pryde, E.H., Mounts, T.L., 1984. Variables affecting the yields of fatty esters from transesterified vegetable oils. Journal of the American Oil Chemists Society, 61, 1638–1643.

Gaikwad, S.G., Pandit, A.B., 2008. Ultrasound emulsification: Effect of ultrasonic and physicochemical properties on dispersed phase volume and droplet size. Ultrasonics Sonochemistry, 15, 5545–63.

Garett, R.H., Grisham, C.M., 1995. Biochemistry, 1st edition, Orlando, Florida. Saunders College Publishing.

Gui, M.M., Lee, K.T., Bhatia, S., 2008. Feasibility of edible oil vs. non-edible oil vs, waste edible oil as biodiesel feedstocks. Energy, 33, 1646-1653.

Gude, V.G., Patil, P., Martinez-Guerra, E., Deng, S., Nirmalakhandan, N., 2013. Microwave energy potential for biodiesel production. Sustainable Chemical Processes, 1-5.

Haas, M. J., Foglia, T. A., 2005. Alternate feedstocks and technologies for biodiesel production. In Biodiesel Handbook 2005; Knothe, G., Krahl, J., Gerpen, J. V., Eds.; AOCS Press: Urbana, IL, 2005; pp 42-61.

Ibrahim, S.N.H., 2017. Modeling and optimization of lipid extraction process from municipal secondary sludge for biodiesel production. Journal of Bioresources and Bioproducts, 2, 123-131.

Imahara, H., Minami, E., Saka, S., 2008. Thermal stability of biodiesel in supercritical methanol. Fuel, 87, 1-6.

International Energy Agency. World Energy Outlook 2012. http://www.iea.org/publications/freepublications/publication/WEO2012_free.pdf. Date accessed October 2018.

International Energy Agency, World Energy Outlook 2014 executive summary. https://www.iea.org/Textbase/npsum/WEO2014SUM.pdf. Date accessed October 2018.

International Energy Agency. Key World Energy Statistics 2017. https://www.iea.org/publications/freepublications/publication/KeyWorld2017.pdf. Date accessed October 2018.

Jacob, J., Chia, L.H.L., Boey, F.Y.C., 1995. Review: Thermal and non-thermal interactions of microwave radiation with materials. Journal of Materials Science, 30, 5321-5327.

Janaun, J., Ellis, N., 2010. Perspectives on biodiesel as a sustainable fuel. Renewable and Sustainable Energy Reviews, 14, 1312-1320.

Jarde, E., Mansuy, L., Faure, P., 2005. Organic markers in the lipidic fraction of sewage sludge. Water Research, 39, 1215–1232.

Kargbo, D.M., 2010. Biodiesel production from municipal sewage sludge. Energy Fuels, 24, 2791-2794.

Karmakar, A., Karmakar, S., Mukher jee, S., 2010. Properties of various plants and feedstocks for biodiesel production. Biosource Technology, 101, 7201-7210.

Kech, C., Galloy, A., Frippiat, C., Piel, A., Garot, D., 2018. Optimization of direct liquid-liquid extraction of lipids from wet urban sewage sludge for biodiesel production. Fuel, 212, 132–139.

Khan, T.M.Y., Atabani A.E., Badruddin, I.A., Badarudin, A., Khayoon, M.S., Triwahyono, S., 2014. Recent scenario and technologies to utilize non-edible oils for biodiesel production. Renewable and Sustainable Energy Reviews, 37, 840–851.

Khanal, S.K., Grewell, D., Sung, S., Leeuwen, J.H.V., 2007. Ultrasound Applications in Wastewater Sludge Pretreatment: A Review. Critical Reviews in Environmental Science and Technology, 37, 277-313.

Knothe, G., 2005. Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. Fuel Processing Technology, 86, 1059–1070.

Korbitz, W., 1999. Biodiesel production in Europe and North America, an encouraging prospect. Renewable Energy, 16, 1078-1083.

Kumar, A., Sharma, S., 2011. Potential non-edible oil resources as biodiesel feedstock: an Indian perspective. Renewable and Sustainable Energy Reviews. 15, 791–800.

Kusdiana, D., Saka, S., 2001. Kinetics of transesterification in rapeseed oil to biodiesel fuels as treated in supercritical methanol. Fuel, 80, 693–698.

Kwon, E.E., Kim, S., Jeon, Y.J., Yi, H., 2012. Biodiesel production from sewage sludge: new paradigm for mining energy from municipal hazardous material. Environmental Science and Technology, 46, 10222-10228.

Ma, F., Hanna, M.A., 1999. Biodiesel production: a review. Biosource Technology, 70, 1-15.

McNichol, J., MacDougall, K.M., Melanson, J.E., McGinn, P.J., 2012. Suitability of soxhlet extraction to quantify microalgal fatty acids as determined by comparison with in situ transesterification. Lipids, 47, 195–207.

Mondala, A., Liang, K., Toghiani, H., Hernandez, R., French, T., 2009. Biodiesel production by in situ transesterification of municipal primary and secondary sludge. Bioresource Technology, 100, 1203–1210.

Monisha, J., Harish, A., Sushma, R., Murthy, T.P.K., Mathew, B.B., Ananda, S., 2013. Biodiesel: A review. Journal of Engineering Research and Applications, 3, 902-912.

Oguz, H., 2004. Tarım Kesiminde Yaygın Olarak Kullanılan Dizel Motorlarında Fındık Yağı Biyodizelinin Yakıt Olarak Kullanım İmkanlarının İncelenmesi, Ph.D. Thesis, Institute of Science and Technology, Selçuk University, Turkey.

Olkiewicz, M., Fortuny, A., Stüber, F., Fabregat, A., Font, J., Bengoa, C., 2012. Evaluation of different sludges from WWTP as a potential source for biodiesel production. Procedia Engineering, 42, 695–706.

Olkiewicz, M., Caporgno, M.P., Fortuny, A., Stüber, F., Fabregat, A., Font, J., Bengoa, C., 2014. Direct liquid–liquid extraction of lipid from municipal sewage sludge for biodiesel production. Fuel Processing Technology 128, 331–338.

Olkiewicz, M., Fortuny, A., Stüber, F., Fabregat, A., Font, J., Bengoa, C., 2015. Effects of pre-treatments on the lipid extraction and biodiesel production from municipal WWTP sludge. Fuel the science and technology of Fuel and Energy, 141, 250–257.

Olkiewicz, M., Plechkova, N.V., Fabregat, A., Stüber, F., Fortuny, A., Font, J., Bengoa, C., 2015. Efficient extraction of lipids from primary sewage sludge using ionic liquids for biodiesel production. Separation and Purification Technology, 153, 118-125.

Pastore, C., Lopez, A., Lotito, V., Mascolo, G., 2013. Biodiesel from dewatered wastewater sludge: A two-step process for a more advantageous production. Chemosphere, 92, 667–673.

Pilli, S., Bhunia, P., Yan, S., LeBlanc, R.J., Tyagi, R.D., Surampalli, R.Y., 2011. Ultrasonic pretreatment of sludge: A review. Ultrasonics Sonochemistry, 18, 1-18.

Pokoo-Aikins, G., Heath, A., Mentzer, R.A., Mannan, M.S., Rogers, W.J., El-Halwagi, M.M., 2010. A multicriteria approach to screening alternatives for converting sewage sludge to biodiesel. Journal of Loss Prevention in the Process Industries, 23, 412–420.

Prakash, N., Arul, J.A., Devanesan, M.G.T., 2006. Optimization of Karanja oil transesterification. Indian Journal of Chemical Technology, 13, 505-509.

Raman, J.K., Sariah, A., Denis, P., Seng, C.E., Pogaku, R., 2008. Production of biodiesel using immobilized Lipase. Critical Reviews in Biotechnology, 28, 253–264.

Reichardt, C., 2003. Solvents and solvent effects in organic chemistry, Third Ed., John Wiley and Sons, VCH.

Republic of Turkey Ministry of Energy and Natural Resources, Turkey's energy profile and strategy. http://www.mfa.gov.tr/turkeys-energy-strategy.en.mfa. Date accessed October 2018.

Republic of Turkey Ministry of Energy and Natural Resources, Turkey's energy profile and strategy. Electricity. http://www.enerji.gov.tr/en-US/Pages/Electricity. Date accessed October 2018.

Revellame, E., Hernandez, R., French, W., Holmes, W., Alley, E., 2010. Biodiesel from activated sludge through in situ transesterification. Journal of Chemical Technology and Biotechnologly, 85, 614–620.

Rice, E.W., Baird, R.B., Eaton, A.D., Clesceri, L.S., 2012. Standard Methods for the Examination of Water and Wastewater. 22nd, APHA AWWA WEF, Washington, 2012.

Rittmann, B.; McCarty, P., 2001. Environmental Biotechnology: Principles and Applications, McGraw-Hill, New York.

Roxburgh, R., Sieger, R., Johnson, B., Rabinowit, B., Goodwin, S., Crawford, G., Daigger, G., 2006. Sludge minimization technologies – doing more to get less. Proceedings of the Water Environment Federation, WEFTEC 2006: Session 1- 10, 506-525.

Schwab, A.W., Bagby, M.O., Freedman, B., 1987. Preparation and properties of diesel fuels from vegetables oils. Fuel, 66, 1372–1378.

Sharp, C.A., 1996. Emissions and lubricity evaluation of rapeseed derived biodiesel fuels. Final Report for Montana Department of Environmental Quality. Southwest Research Institute.

Sheehan, J., Dunahay, T., Benemann, J., Roessler, P., 1998. Energy’s aquatic species program-biodiesel from algae. National renewable energy laboratory (NREL). Golden, CO.

Shen, L., Zhang, D.K., 2003. An experimental study of oil recovery from sewage sludge by low-temperature pyrolysis in a fluidised-bed. Fuel, 82, 465-72.

Siddiquee, M.N., Rohani, S., 2011. Experimental analysis of lipid extraction and biodiesel production from wastewater sludge. Fuel Processing Technology, 92, 2241–2251.

Siddiquee, M.N., Rohani, S., 2011. Lipid extraction and biodiesel production from municipal sewage sludge: A review. Renewable and Sustainable Energy Reviews, 15, 1067-1072.

Smesdes, F., Askaland, T.K., 1999. Revisiting the development of the Bligh Dyer total lipid determination method. Marine Pollution Bulletin, 38, 193-201.

Srivastava, A., Prasad, R., 2000. Triglycerides-based diesel fuels. Renewable and Sustainable Energy Reviews, 4, 111–133.

Szulczyk, K.R., McCarl, B.A., 2010. Market penetration of biodiesel. Renewable and Sustainable Energy Reviews, 14, 2426–2433.

Thompson, P.B., 2012. The Agricultural Ethics of Biofuels: The Food vs. Fuel Debate. Agriculture, 2, 339–358.

Tiehm, A., Nickel, K., Zellhorn, M., Neis, U., 2001. Ultrasonic waste activated sludge disintegration for improving anaerobic stabilization. Water Research, 35, 2003-2009.

Ulusoy, Y., 1999. Ayçiçeği, Kolza, Pamuk ve Soya Yağlarının Dizel Motorlarında Yakıt Olarak Kullanım Olanaklarının belirlenmesi Üzerine Karşılaştırmalı Bir Araştırma, Ph.D. Thesis, Institute of Science and Technology, Uludağ University, Turkey.

U.S. Energy Information Administration. International Energy outlook, 2017. https://www.eia.gov/outlooks/ieo/pdf/0484(2017).pdf. Date accessed October 2018.

Vasudevan, P.T., Briggs, M., 2008. Biodiesel production-current state of the art and challenges. Journal of Industrial Microbiology and Biotechnology, 35, 421-30.

Velp Scientifica, 2014. FOOD&FEED LINE, SER 148 Solvent Extractor brochure.

Weemaes, M., Verstraete, W.H., 1998. Evaluation of current wet sludge disintegration techniques. Journal of Chemical Technology and Biotechnology, 73, 83–92.

Willson, R.M., Wiesman, Z., Brenner, A., 2010. Analyzing alternative bio-waste feedstocks for potential biodiesel production using time domain (TD)-NMR. Waste Management, 30, 1881-1888.

Yasar, B., 2009. Evaluation of Biodiesel Production and Utilization of Biodiesel As An Alternative Source of Energy from The Point of View of Turkish Agriculture and EU Adaptation Process, Ph.D. Thesis, Institute of Science and Technology, Çukurova University, Turkey.

Yuji, S., Yomi, W., Akio, S., Yoshio, T., 2002. Enzymatic alcoholysis for biodiesel fuel production and application of the reaction to oil processing. Journal of Molecular Catalysis B: Enzymatic, 17, 133-142.

Zhenyi, C., Xing, J., Shuyuan, L., Li, L., 2004. Thermodynamics Calculation of the Pyrolysis of Vegetable Oils. Energy Sources, 26, 849-856.

Zhu, F., Zhao, L., Zhang, Z., Jiang, H., 2012. Preliminary study at lipids extraction technology from municipal sludge by organic solvent. Procedia Environmental Sciences, 16, 352-356.

Zhu, F., Zhao, L., Jiang, H., Zhang, Z., Xiong, Y., Qi, J., Wang, J., 2014. Comparison of the lipid content and biodiesel production from municipal sludge using three extraction methods. Energy Fuels, 28, 5277–5283.