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Activated Sludge as Renewable Fuels and Oleochemicals Feedstock

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ACTIVATED SLUDGE AS RENEWABLE FUELS AND OLEOCHEMICALS FEEDSTOCK

By

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FEEDSTOCK

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The utilization of activated sludge as feedstock for biofuel and oleochemical production was investigated. Initial studies included optimization of biodiesel production from this feedstock through in situ transesterification. Results of these studies indicated that activated sludge biodiesel is not economically viable. This was primarily due to relatively low yields and the high economics of feedstock dewatering.

Strategies to increase biofuel yield from activated sludge were then evaluated. Bacterial species present in activated sludge are known to produce a wide variety of lipidic compounds as carbon and energy storage material and as components of their cellular structures. In addition to lipidic compounds, activated sludge bacteria might also contain other compounds depending on wastewater characteristics.

Among these bacterial compounds, only the saponifiable ones can be converted to biodiesel. The unsaponifiable compounds present in the activated sludge are also important, not only for biofuel production, but also for a wide variety of applications. Characterization of lipids in activated sludge revealed that it contains significant amount of polyhydroxyalkanoates, wax esters, acylglycerides and fatty acids. It also contains

sterols, steryl esters and phospholipids as well as small but detectable amounts of hydrocarbons. This indicated that activated sludge could be also an inexpensive source of oleochemicals.

Another strategy that was evaluated was lipid-enhancement by fermentation of activated sludge. Since the majority of products from petroleum oil are used as transportation fuel, the aim here was to increase the saponifiable lipids in activated sludge bacteria by applying a biochemical stimulus (i.e. high C:N ratio). Results showed that application of this stimulus increased the amount of saponifiable lipids, particularly triacyglycerides, in the activated sludge. Furthermore, fermentation homogenized the lipids in the sludge regardless of its source. This solidified the concept of utilizing wastewater treatment facilities as biorefineries.

To support the utilization of other compounds in raw activated sludge for biofuel production, a model compound was chosen for catalytic cracking experiments. Results indicated that catalytic cracking of 1-octadecanol over H^{+}ZSM5 proceeds via dehydration, producing octadecene. The octadecene then undergoes a series of reactions including β-C─C bond scission, alkylation, oligomerization, dehydrocyclization and aromatization producing aromatics, paraffins and olefins suitable for fuel applications.

DEDICATION

This work is dedicated to my beloved wife – Miriam, my precious son – Erastus, and to my parents – Vevencio and Nenita Revellame.

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I am sincerely grateful to my major professor, Dr. Rafael Hernandez, for all the guidance, help and patience. He is an inspiration for being a great teacher, researcher and mentor. Thank you for pushing me to go beyond my limits. Equally, I would like to thank Dr. W. Todd French. Thank you also for always reminding me that there is always something to learn and for allowing me to teach Mass and Energy Balances. I also want to thank my committee members, Dr. L. Antonio Estévez, Dr. Benjamin Magbanua, Jr. and Dr. Priscilla Hill for all the suggestions that improved this dissertation.

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CHAPTER I

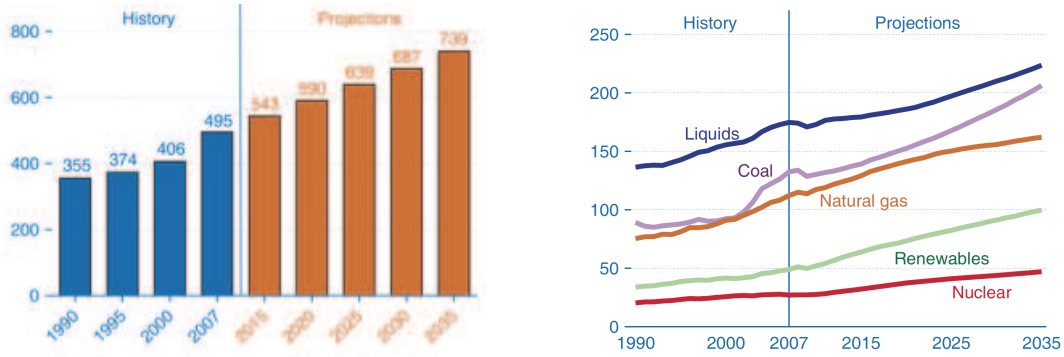
INTRODUCTION

"Crude oil is being consumed a million times faster than it was made".

– Armstrong et al. (2007) in *Energy... beyond oil* [1]

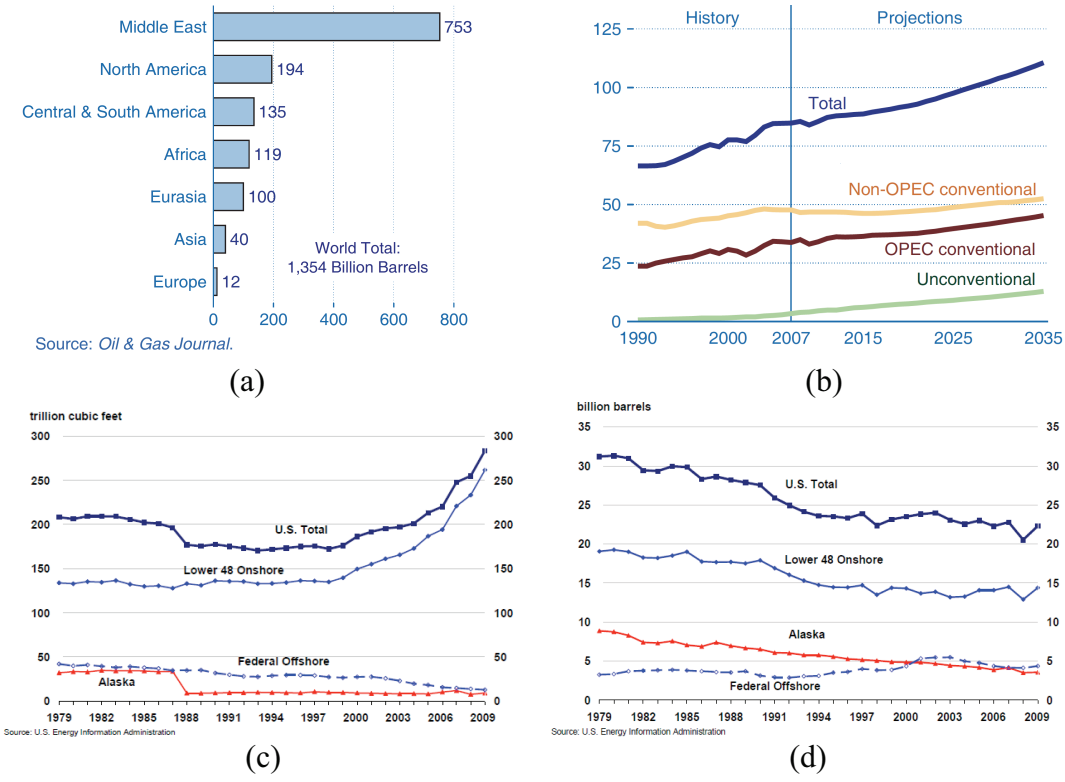
1.1 Energy Outlook: Facts and Figures

The above quotation could be true not only for crude oil, but for all fossil energy reserves such as coal and natural gas. According to United States Department of Energy (US DOE), the worldwide energy consumption will increase from 495 quadrillion Btu (Quads) in 2007 to 739 Quads in 2035 (an increase of 49.30%) (Figure 1a). Leading the sources of the world's energy demand in 2035 are crude oil, coal and natural gas, all of which are fossil energy (non-renewable) resources (Figure 1b) [2]. Crude oil and coal have fueled the world's industries and transport systems since the Industrial Revolution, a period of about two centuries [1]. As of January 01, 2010, the world's proved crude oil reserves were about 1,354 billion barrels (Figure 1.2a) [2]. These reserves might change in the future as new fossil deposits could possibly be discovered. For example, the United States natural gas reserves continuously increased from year 2000 to 2009 and the crude oil reserves increased from year 2008 to 2009 (Figure 1.2c, d) [3]. Despite this, with the continuously increasing consumption of liquid fuels at an average rate of 1.3 percent per year (Figure 1.2b), it has been estimated that the extraction of crude oil will require great effort after year 2025 [4].



(a) (b)

Figure 1.1 (a) World marketed energy consumption; (b) World marketed energy use by fuel type, 1990-2035 (quadrillion Btu) [2].

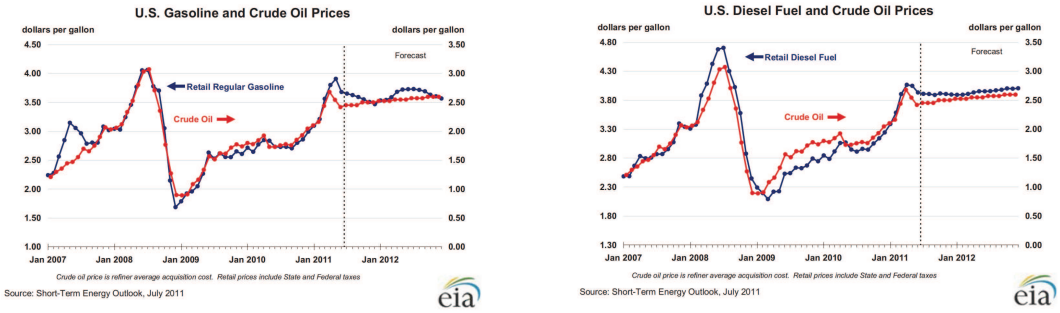


(a) (b)

(c) (d)

Figure 1.2 (a) World proved oil reserves by geographic region as of January 1, 2010 (billion barrels) [2]; (b) World liquid fuels production, 1990-2035 (million barrels per day)[2]; (c) United States proved wet natural gas reserves (1979 – 2009)[3]; (d) United States proved crude oil plus condensate reserves (1979 – 2009) [3].

The projected price of crude oil is very stable from the third quarter of 2011 throughout year 2012 (Fig 1.3) [5]. However, it is expected to increase by almost 100% from 2012 to 2035 (Figure 1.4) [6]. This expected increase is probably due to depletion of easily extractable crude oil deposits because of continuously increasing consumption of liquid fuels. Consequently, this has triggered the search for sustainable and renewable energy resources across the globe. These renewable energy resources include solar, wind, water biomass, geothermal, and hydrogen and fuel cells [7]. They are considered clean or environmentally-friendly with little to no net CO_2 (or greenhouse gas) generation even for geothermal resources [8]. Furthermore, they are available domestically and thus, will eliminate dependence in foreign countries in terms of energy. For example, from the period of 1980 – 2035, the United States' total energy production is less than its energy consumption/demand (Figure 1.5a). In terms of petroleum oil, the country's importation will decrease from 52% (of total liquid fuel consumption) in 2009 to 41% in 2035 [9]. This will be primarily due to an expected increase in the production of biofuels from 4% in 2009 to 11% in 2035 (Figure 1.5b) [10]. Tapping locally available resources for the production of inexpensive, clean, sustainable and renewable energy will not only reduce green house gas emissions but could also generate new jobs, increase farm incomes, contribute to rural development and increase or improve the United States' energy security in the long term [11].



(a) (b)

Figure 1.3 (a) United States gasoline and crude oil prices; (b) United States diesel fuel and crude oil prices (Jan. 2007 – January 2012) [5].

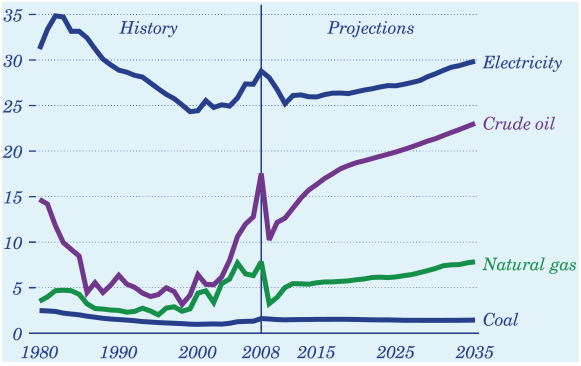
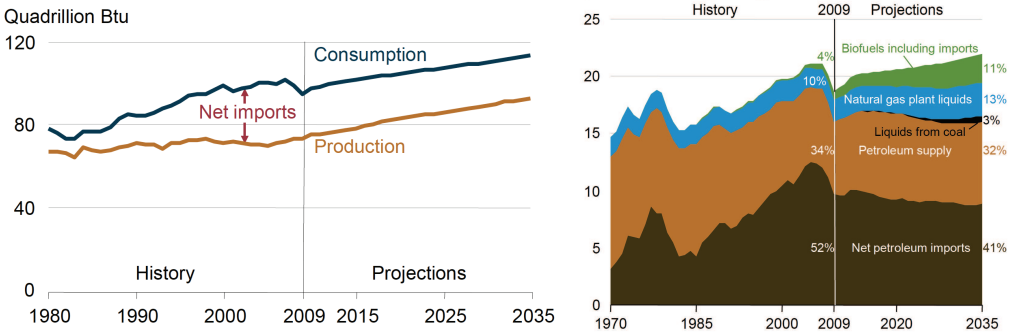


Figure 1.4 Energy prices, 1980 – 2035 (2008 dollars per million Btu) [6].



(a) (b)

Figure 1.5 (a) United States total energy production and consumption [9];(b) United States liquid fuels consumption, 1970-2035 (million barrels per day) [10].

Among the renewable energy resources mentioned earlier, biofuels from biomass alone can be used as an alternative to petroleum-based transportation fuels with little to no modifications in the existing infrastructures for fuel production, distribution and utilization [12]. In addition to fuel, biomass in all its form has been and will be the most important source of humans' basic needs which is usually summarized as six *fs*: food, feed, fuel, feedstock, fiber and fertilizer. These six *fs* are associated and commonly lead to the seventh *f* – finance. Biomass has also the potential of being an infinite, largest and sustainable energy source with an annual global production of 220 billion oven dry tons or about 4500 exajoules (10^{18} joules) [13].

Biomass resources include forest and mill residues, agricultural crops and wastes, wood and wood wastes, animal wastes, livestock operation residues, aquatic plants, fast-growing trees and plants, and municipal and industrial wastes. Any fuel derived from any of these biomass resources is termed "biofuel" and is considered to be a clean, renewable and sustainable fuel. The majority of compounds present in biomass that may be used for biofuel production include cellulose, hemicelluloses, lignin, lipids, proteins, simple sugars, starches and hydrocarbons [14].

1.2 Biofuels or Renewable Fuels

Biofuels has been defined as any fuel with at least 80% content (by volume) derived from living organisms harvested within the 10 years preceding its manufacture. With respect to composition, biomass has some advantages over conventional fossil fuels including low sulfur content and highly reactive char. In addition, catalyst poisons are not present in biomass in significant concentrations which is advantageous for its initial thermal processing as well as for subsequent upgrading operations [14].

Nowadays, biofuels are usually classified as 1st, 2nd or 3rd generation biofuels [15]. This classification leads to the development of the biofuel ladder or roadmap presented in Figure 1.6. Regardless of their classification, biofuels are produced either through chemical (i.e. acid-/base-catalyzed transesterification), biochemical (i.e. enzyme-catalyzed transesterification), thermochemical (i.e. pyrolysis followed by catalytic synthesis), biological conversion (i.e. fermentation) or their combination(s) [11, 16]. First generation biofuels are those that are manufactured from readily available biomass such as crops rich in sugar, starch and oil/lipids. Among the 1st generation biofuels, biodiesel and bioethanol are the most common ones. Also included in this classification are biofuels from catalytic cracking and biobutanol [11].

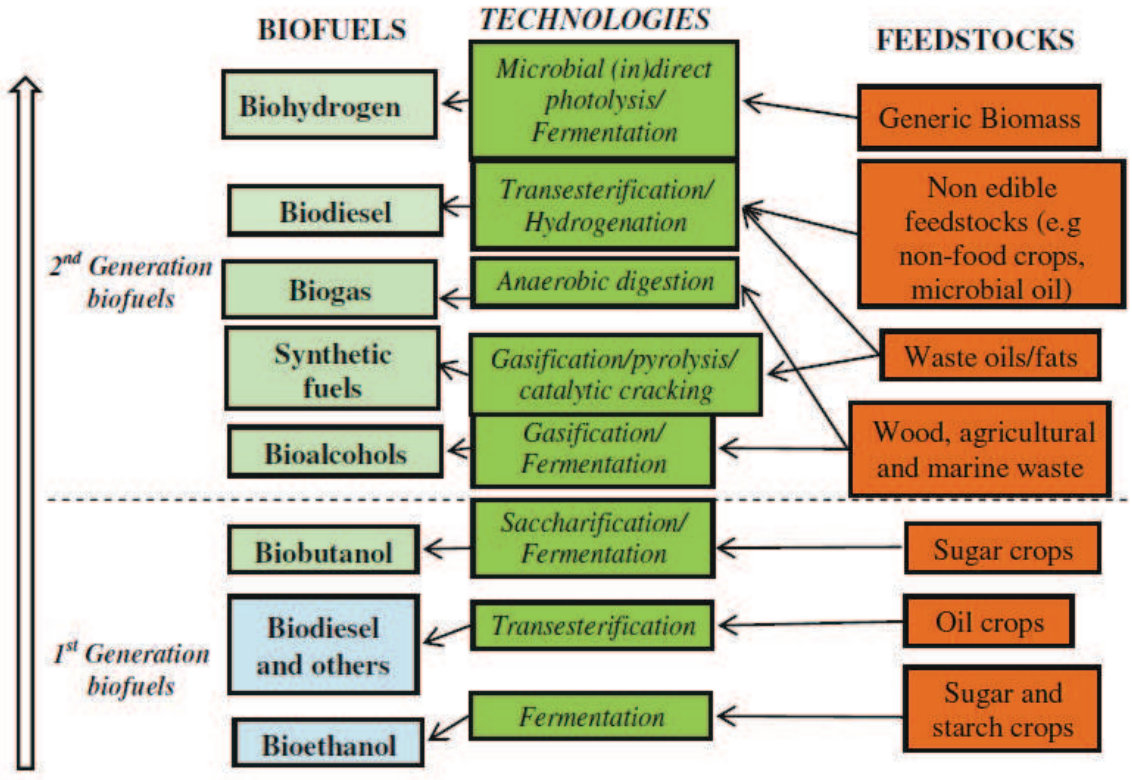


Figure 1.6 The biofuels ladder. Roadmap of biofuels production from feedstocks and technologies [11].

The pros and cons of 1st generation biofuels are presented in Table 1.1. The main issue associated with the 1st generation biofuels is the utilization of food crops. With a limited arable land and grain reserves, the utilization of this food crops for biofuel production could cause food prices to skyrocket. In addition, production of 1st generation biofuels could cause deforestation and could threaten natural biodiversity. These issues had lead to the search for alternative feedstocks and technologies and gave rise to the 2nd generation biofuels [16].

Table 1.1 First generation biofuels [16].

Pros	Cons
1. Simple and well known production methods	1. Feedstocks compete with crops grown for food
2. Familiar feedstocks	2. Production by-products need markets
3. Scalable to smaller production capacities	3. High-cost of feedstocks (except for Brazilian sugar cane)
4. Fungibility with existing petroleum-derived fuels	4. Low land-use efficiency
5. Experience in commercial production and use in several countries	5. Modest net reductions in fossil fuel use and greenhouse gas emissions with current processing methods (except for Brazilian sugar cane)

Second generation biofuels share the same feature as the 1st generation in that they are also from renewable resources. However, consideration was given to alternative feedstocks, which are in general non-edible. These feedstocks include waste vegetable oils and fats, non-food crops and biomass sources. Technologies were also implemented/developed (i.e. green diesel production technology) in an attempt to

overcome the major shortcomings of the production of first generation biofuels. The utilization of these alternative feedstocks could not only solve the problems associated with the 1st generation biofuels, but also can potentially supply a larger proportion of fuel in a more sustainable and reasonable price with greater environmental benefits. In addition to biodiesel and bioalcohols, 2nd generation biofuels include refined Fischer-Tropsch liquids (FTL), dimethyl ether (DME), biogas and biohydrogen [11, 16].

The utilization of various alternative feedstocks for production of 2nd generation biofuels poses several issues. For the case of non-food crops, their cultivation patterns are still not fully understood. For waste oils and fats, the variability of their properties (i.e. water and free fatty acids content) could result to a more expensive processing cost and could potentially jeopardize the quality of the final product. Furthermore, technologies available for the majority of the second generation biofuels are still premature for a large scale biofuel production [11].

Several strategies to produce economical biofuels include incorporation of several processing steps into a single step. These strategies include in situ transesterification for the case of biodiesel and simultaneous saccharification and fermentation or simultaneous saccharification and co-fermentation as for the case of bioethanol [17]. Despite these strategies, the successful production and utilization of 2nd generation biofuels still needs major development. Take cellulosic ethanol for example; while cellulosic ethanol can be produced today, producing it competitively (without subsidies) from lignocellulosic biomass still requires significant research on:

1. Developing biomass feedstocks with physical and chemical structures that facilitate processing to ethanol, e.g. lower lignin content, higher cellulose content, etc.;

2. Improving enzymes (i.e. cellulase) to achieve higher activities, higher substrate specificities, reduced inhibitor production and other features to facilitate hydrolysis;
3. Developing new microorganisms that are high-temperature tolerant, ethanol-tolerant, and able to ferment multiple types of sugars (6-carbon and 5-carbon).

As a result of intensive experimentation both in the academe and industry, researchers concluded that these objectives may be achieved by application of genetic engineering and thus, the 3rd generation biofuels was introduced [16].

The literature definition of 3rd generation biofuels is vague. Bessou et al. (2011) defined them as an extension or follow-up of the 2nd generation biofuels with the inclusion of biohydrogen [15]. According to Demirbas (2009) and Ngô and Natowitz (2009), algae as feedstock for biofuel production are the only difference between 2nd and 3rd generations biofuel. Accordingly, *oilgea* or the oil from algae is a unique feedstock for the production of 3rd generation biofuels [18, 19]. However, based on the definition of 2nd generation biofuels, the most logical definition of 3rd generation biofuels is close to the one given by Maxwell (2009). His implicit definition suggests that 3rd generation biofuels involves utilization of synthetic biocatalysts designed to efficiently convert carbon dioxide and sunlight into a high-octane hydrocarbon. These biocatalysts could be genetically modified or engineered algae, bacteria and any other organisms including higher plants [20]. Furthermore, carbon source for these biocatalysts is not limited to carbon dioxide. It may include any of the alternative feedstocks considered for 2nd generation biofuels. The main objectives of genetically modifying these organisms are to enhance their feedstock producing capacity, to obtain a more homogeneous and desirable feedstock and to reduce feedstock recovery cost (i.e. microbes designed to excrete

metabolites). Some recent developments associated with 3rd generation biofuel include consolidated bioprocessing and engineered cyanobacteria that secrete fungible hydrocarbon products in a continuous process (also known as Joule's solar-to-fuel) [17, 21]. Consolidated bioprocessing is an alternative processing strategy wherein cellulose production, substrate hydrolysis, and fermentation are accomplished in a single process step by microorganisms that express cellulolytic and hemicellulolytic enzymes [17].

Biofuels that are either created using petroleum-like hydroprocessing, advanced biochemistry, or revolutionary processes like the Joule's solar-to-fuel are also sometimes referred as 4th generation biofuels [18, 22]. However, 4th generation biofuels are also sometimes referred to as carbon-negative fuels. The idea is basically to engineer microorganisms that can absorb more CO_2 than would be released during combustion [23, 24]. Regardless of its suitable definition, the idea of a 4th generation biofuels suggests that intensive studies are being conducted to solve the current energy issues the world is facing.

1.3 Lipids as Biofuels Feedstock

Among the major compounds directly extracted from biomass resources, lipids, were the only ones that had been used as transportation fuel [25]. Vegetable oils were also the primary lubricants for machinery and transportation vehicles for thousands of years until the discovery of petroleum [26]. About a hundred years ago, Rudolf Diesel tested vegetable oil as fuel for diesel engines. They were also used during the 1930s and World War II as fuel in critical situations. Perhaps the most important advantage of using vegetable oils as fuel is that its properties are close to that of diesel fuel, except for viscosity and volatility. Vegetable oils have relatively high viscosity and low volatility,

which can reduce the fuel atomization and increased fuel spray penetration [27]. These could cause several problems, which include coking and trumpet formation on the injectors; carbon deposits; oil ring sticking; thickening or gelling of the lubricating oil as a result of contamination by vegetable oils, and lubricating problems. As a result, vegetable oils for biofuel application were modified using processes such as pyrolysis, alcoholysis, hydroprocessing, dilution with hydrocarbons and emulsification [28].

1.3.1 Biodiesel

Lipid feedstocks could come from sources such as soybean, rapeseed, canola, corn, coconut, etc. [29]. However, as mentioned earlier, these feedstocks are also used in the edible oil industry and thus being used to produce 1st generation biofuels. The 2nd generation biofuels considered non-food crops such as castor and jatropha, waste oils and microbial sources. The most frequently used method of modifying lipid feedstocks to fuel is alcoholysis to produce biodiesel. Alcoholysis reaction produces alkyl esters, commonly known as biodiesel (Figure 1.7). Depending on the nature of the lipid feedstock a glycerol by-product may or may not be produced. Alcoholysis is the reaction of an ester and an alcohol. In general however, biodiesel can be produced by interaction of a carboxylic acid (such as fatty acid) or an acyl derivative with an alcohol or its equivalent (esterification) or another ester (ester–ester interchange, interesterification, transesterification). In the biofuel technology, these terms are being used interchangeably, such that all of them mean biodiesel production [30].

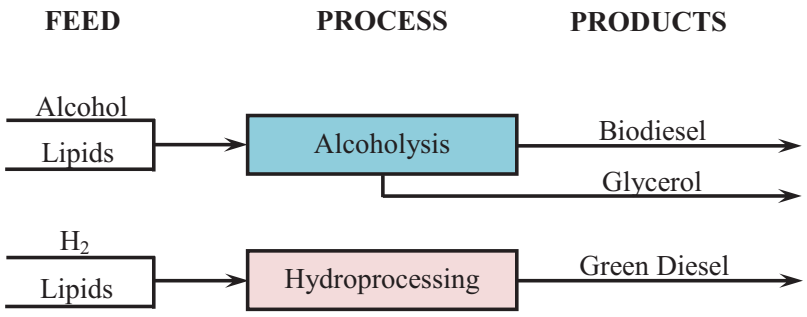


Figure 1.7 Production of biofuels from lipid feedstocks (Redrawn and modified from reference [31]).

Biodiesel, particularly (m)ethyl esters have properties, which are similar to petroleum-derived diesel making them suitable as petroleum diesel substitute. Additionally, their production process is relatively simple [32]. Despite the many advantages of biodiesel; there are issues associated with its distribution, storage and utilization, such as poor stability, poor cold flow properties and high solvency, leading to problems with filter plugging [31, 33]. The latter hindered the usage of pure biodiesel in existing diesel engines.

1.3.2 Green Diesel

A different processing route to convert lipid feedstock into a high-quality diesel fuel was sought to avoid biodiesel performance issues. The route, called hydroprocessing or hydrotreatment, is widely used in petroleum refineries and thus existing infrastructures can be utilized for its manufacture and distribution [31, 34]. Oxygenated compounds, such as lipids, are deoxygenated by a series of cracking and hydrogenation reactions to form mostly linear aliphatic hydrocarbons within the range of petroleum-derived diesel fuel compounds [35]. As such, green diesel is fully compatible with existing diesel engines and can be used without modifications [34].

Some properties of green diesel as compared to biodiesel are listed in Table 1.2. The important differences between the two fuels are oxygen content, cloud point and cetane number. The oxygen content of green diesel is negligible, making it a very stable fuel. As mentioned, this is advantageous for its transportation, storage and utilization. The cloud point, which is the temperature where the fuel begins to crystallize, is lower for green diesel. This makes green fuel a suitable fuel even for countries with very cold climates [36]. The cetane number of an ultra-low sulfur diesel is about 40 while that of green diesel ranges 70-90 [31]. This high cetane number of green diesel makes it a premium diesel-blending component. This property can potentially be used to optimize the amount of lower-value refinery streams that can be introduced into the refinery diesel pool without sacrificing the quality of the final product [37].

Table 1.2 Comparison of properties of biodiesel and green diesel [31, 38].

Property	**Biodiesel**	**Green diesel**
Oxygen, %	11	0
Specific gravity	0.883	0.78
Sulfur content, ppm	<1	<1
Heating value, MJ/kg	38	44
Cloud point, ºC	-5 to +15	-30 to -10
Distillation, ºC	340 – 355	265 – 320
Cetane number	50 – 65	70 – 90
Stability	Marginal	Good

In the search for a feedstock for production of 2nd generation biofuels, one that is available in large quantities all year round is preferable. Furthermore, feedstocks which do not require acreage and energy (i.e. crops) would be beneficial. These are the primary reasons why microorganisms (i.e. microalgae, yeasts, bacteria, fungi) have gained

attention in recent years. Microalgae utilize carbon dioxide as carbon source and sunlight as energy for lipid accumulation and thus can also help in greenhouse gas mitigation [39]. On the other hand, bacteria, yeasts and fungi can utilize a wide range of carbon sources and thus can also help in waste minimization. It is in this regard that bacteria, yeast and fungi are preferable over microalgae as source of biofuel feedstock. Microalgae will require construction of large infrastructures to be able to significantly displace petroleum fuel demands. Construction of these infrastructures requires large land usage, which could also affect food supply. Bacteria, on the other hand, are the ones responsible for the biological treatment of wastewaters [40]. Since wastewater treatment facilities are already in place, bacteria will require little to no additional infrastructures. These bacteria are utilizing an abundant supply of inexpensive carbon and nutrient source(s) – wastewater. In the United States alone, there are about 16,583 wastewater treatment facilities treating more than 32 billion gallons of municipal wastewater daily [41, 42]. These numbers are expected to increase due to the anticipated increase in United States' population (from 282 million in 2000 to 364 million in 2030) and urbanization (from 81% in 2000 to 85% in 2030) [43, 44]. This is an attractive feature of feedstock from wastewater bacteria. As population, urbanization and industrialization increases, fuel demand will increase and so is feedstock from wastewater bacteria.

1.4 Wastewater Treatment

Industrialization has brought with it a level of pollution never before seen in the United States. In the 1960s, environmental problem(s) was one of the critical issues faced by the government. In response, the President of the United States created the U.S. Environmental Protection Agency (EPA) in December of 1970 and the Congress passed

the Federal Water Pollution Control Act (Clean Water Act-CWA) in 1972 to restore and maintain the integrity of the nation's waters. The CWA established a regulatory program, through the National Pretreatment Program, that requires direct (domestic) and indirect (non-domestic) wastewaters to be discharged to publicly owned treatment works (POTWs) or municipal wastewater treatment plants (MWWTPs) [41].

Domestic wastewater contains all the materials added to the water during its use such as human body wastes (feces and urine) together with the water used for flushing toilets, and wastewater resulting from personal washing, laundry, food preparation and the cleaning of kitchen utensils [45]. Wastewaters may also come from other sources such as industrial, institutional and recreational facilities, storm water (runoff) and groundwater (infiltration) [46]. MWWTPs are designed for treatment of domestic wastewaters, but they are also treating wastewaters from these other sources. As for institutional and industrial wastewaters, their discharge to MWWTPs may be permitted depending on the nature and level of pollutant(s) present [41].

A schematic diagram of a typical MWWTP is shown in Figure 1.8. Collected wastewater or influent undergoes preliminary treatment wherein the large solid particles (e.g. rags, cans, rocks, leaves, etc.) are either shredded into smaller particles or removed by screening. Preliminary treatment may also include removal of grit (e.g. sand, gravel, egg shells, etc.) from the wastewater stream. The wastewater then goes through primary treatment wherein settleable organics and floatable solids are removed by sedimentation. The effluent from the primary treatment is then subjected to secondary or biological treatment to remove biodegradable organics. The most commonly used biological treatment technologies include activated sludge, trickling filters, and rotating biological contactors [41, 46]. Figure 1.8 shows a conventional activated sludge process and is

given emphasis in this discussion. Modifications of the activated sludge process will be discussed below.

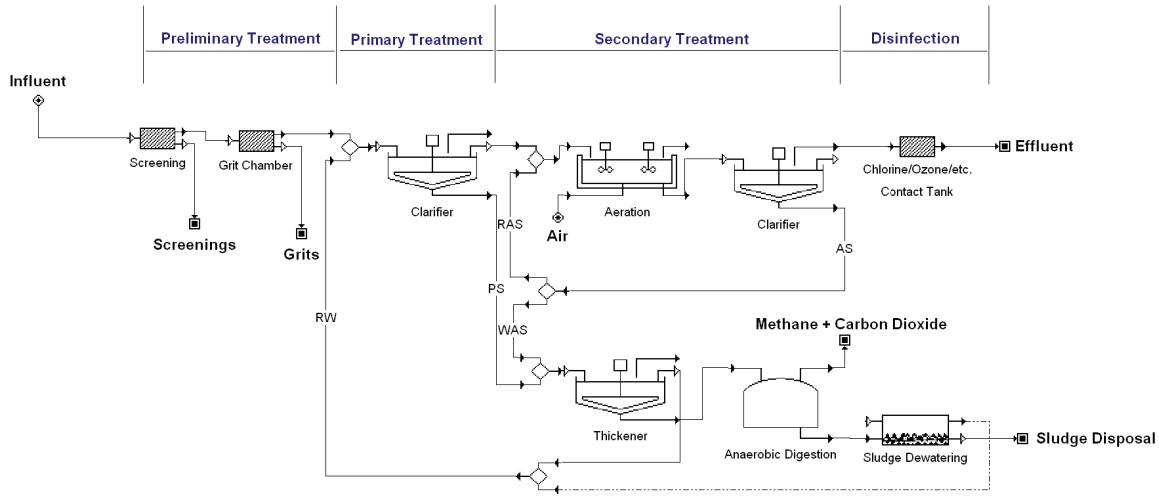


Figure 1.8 Schematic diagram of a typical municipal wastewater treatment plant.*

*AS, Activated Sludge; RAS, Return Activated Sludge; WAS, Waste Activated Sludge; PS, Primary Sludge; RW, Recycled Water. (Redrawn and modified from reference [46]).

The most common components of this process are aeration tank and settling basin/clarifier. Aerobic oxidation of organic matter is carried out in the aeration tank where the microorganisms metabolize and biologically flocculate the organics in the wastewater [46, 47]. To maintain a certain food to microorganism ratio in the aeration tank, the effluent from the primary treatment is mixed with a portion of solids from the clarifier as it enters the aeration tank. This is also called the return activated sludge (RAS) which contains microorganisms that have been in a food-depleted environment for some time, thus they are *hungry* or *activated* [48]. The supernatant from the clarifier is disinfected before discharge as plant effluent. The microbial floc (sludge) produced during oxidation is recovered using the settling basin/clarifier. Part of this recovered

sludge is the RAS and the remaining portion is wasted (WAS). MWWTPs can also perform advanced treatment operations which might include nitrification (to convert ammonia and nitrite to the less-toxic nitrate form of nitrogen), denitrification (to convert nitrate to molecular nitrogen) and physical-chemical treatment (to remove dissolved metals and organics) [41].

The amount of sludge generated during oxidation can be as high as 2% of the original volume of wastewater with ~97% water content. This sludge also contains organic matters and microorganisms. For these reasons, volume reduction, stabilization of organics and elimination of microorganisms is necessary prior to reuse or disposal. These are usually done using a combination of thickener (sedimentation tank) and digester (aerobic or anaerobic). Another process that is also being used is thermal incineration [46].

1.5 Activated Sludge

In a conventional activated sludge system (Figure 1.9), a large portion of the biomass is recycled. This important characteristic makes the mean cell residence time (also called sludge age) much greater than the hydraulic retention time (average time spent by the influent liquid in the aeration tank). This practice helps maintain a large number of microorganisms that effectively oxidize organic compounds in a relatively short time. In a conventional activated sludge system, hydraulic retention time (detention time) in the aeration basin usually varies between 4 and 8 hours while solids (cells) retention time may vary from 5 to 15 days [47].

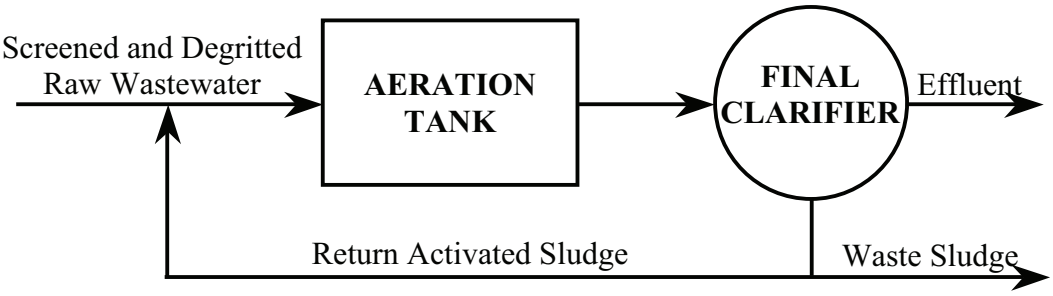


Figure 1.9 Conventional activated sludge system (Redrawn from reference [47]).

The conventional activated sludge process provides excellent treatment. However, it requires primary treatment, large aeration tank capacity, and high initial oxygen demand and is very sensitive to operational problems. Variability in wastewater characteristics and operational sensitivity have created the need to modify the process over the years to provide better performance and to tailor specific operating conditions. Modifications include step aeration, complete mix, pure oxygen, contact stabilization, extended aeration and oxidation ditch (Figure 1.10) activated sludge processes. The characteristics of these modifications are presented in Table 1.3. Among the modifications of activated sludge process, the most commonly used are contact stabilization, extended aeration and oxidation ditch [46].

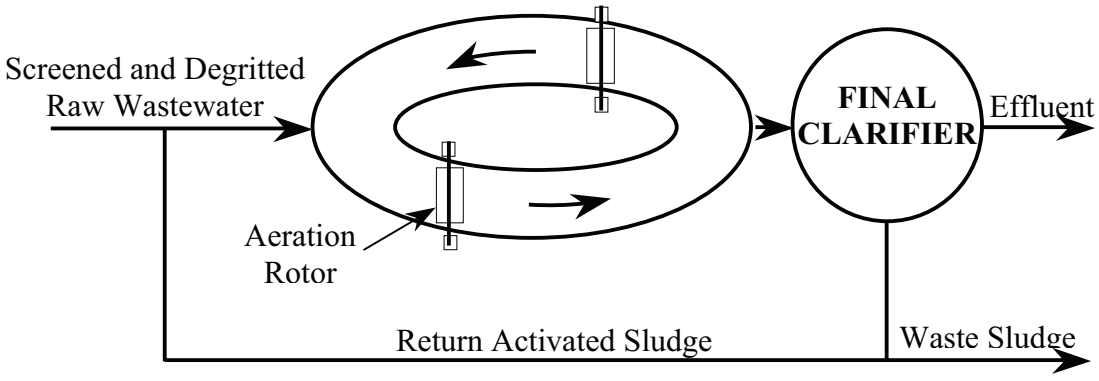


Figure 1.10 Oxidation ditch activated sludge process (Redrawn from reference [47]).

Table 1.3 Modifications of the conventional activated sludge process [46, 47].

PROCESS	CHARACTERISTICS
Step Aeration	• Requires primary treatment • Provides excellent treatment • Operation characteristics are similar to conventional • Distributes organic loading by splitting influent flow • Reduces oxygen demand at the head of the system • Reduces solids loading on the settling tank
Completely Mixed Aerated System	• May or may not include primary treatment • Distributes waste and oxygen evenly throughout the tank • Aeration may be more efficient • Maximizes tank use • Permits higher organic loading • Can sustain shock and toxic loads.
Pure Oxygen	• Requires primary treatment • Permits higher organic loading • Uses higher solids levels • Improves treatment and reduces production of sludge • Operates at higher food to microorganism ratios • Uses covered tanks • Poses a potential safety hazard • Oxygen production is expensive
Contact Stabilization	• Does not require primary treatment • During operation, organisms collect organic matter (during contact) • Solids and activated sludge are separated from flow via settling • Activated sludge and solids are aerated for 3 to 6 hours (stabilization) • Return sludge is aerated before it is mixed within effluent flow • The activated sludge oxidizes available organic matter • Requires less tank volume than other modifications and can be prefabricated as a package unit for flows of 0.05 to 1.0 million gallons per day • A disadvantage is that common process control calculations do not provide usable information

Table 1.3 (Continued).

Extended Aeration	• Does not require primary treatment • Frequently used for small flows such as for schools and subdivisions • Uses 24 to 30 hours aeration • Sludge age can be extended to >15 days • Produces low BOD effluent • Produces the least amount of waste activated sludge • Capable of achieving 95% or greater removal of biological oxygen demand • Can produce effluent low in organic and ammonia nitrogen
Oxidation Ditch (Figure 1.10)	• Does not require primary treatment • Consists of an oval channel with one or more rotating rotors for wastewater aeration • Hydraulic retention time is approximately 24 hours. • Similar to the extended aeration process

1.5.1 Current Uses and Disposal Practices

Waste or excess activated sludge (WAS) is an unwanted by-product of MWWTPs and its treatment and disposal represents a major bottleneck of treatment facilities all over the world. In the United States, current production of WAS is about 7.6 – 8.2 million U.S. dry tons annually [49]. WAS needs to be processed prior to disposal and the cost of these processes could account to 30% to more than 50% of WWTPs' operating cost [50]. WAS is a Class B biosolid which contains detectable level of pathogenic microorganisms. Although Class B biosolid can be directly applied as land fertilizer, its application has site restrictions to prevent or minimize human exposure. Treatment such as composting or lime/heat treatment is necessary for Class B biosolid to be a Class A biosolid, which does not have site restrictions and can be bagged and sold to the public for use as fertilizer [51]. Heat treatments include incineration, pyrolysis, gasification, wet air oxidation and supercritical water oxidation. The treated solids are then disposed through

landfilling and land application [42, 50, 52]. However, with increasing strict regulations governing disposal and decreasing availability of disposal sites, most WWTPs conduct in-pipe sludge minimization techniques. These techniques are mainly focused on the long retention times within the activated sludge process. The main idea is to reduce sludge production by using processes with either reduced or low cell yield coefficient. Reductions of yield coefficient are usually accomplished by cell lysis using ozone, chlorine, ultrasound, mechanical shear forces, etc. It can also be done in a two-stage system that promotes predation in bacteria. Some of the available technologies in the market that uses this technique include Biolysis® 'O', Cannibal™ and Microsludge™ process. Sludge reduction can also be accomplished by using processes with intrinsically low yield coefficient (i.e. anaerobic – aerobic process) [50, 53].

Sewage contains approximately 10 times the energy needed for its treatment. And thus, it is feasible to recover some energy from sludge, which can be used within the WWTP. Some technologies for energy extraction from sludge are as follows:

1. Use of restaurant greases to increase biogas production in the digester by more than 50% (Watsonville, CA);
2. Substitution of 5 – 10% thermally treated biosolids for coal to fuel cement kiln (Maryland);
3. Energy from waste (including sewer sludge) combustion and biogas production accounted for 10.8% and 4.2%, respectively of all United Kingdom's renewable energy (2005);
4. Gas engines produced 113% of electricity used by a German plant (2005);
5. Use of biogas from a treatment plant to fuel at least 30 buses (Sweden);

6. Use of heat recovery pumps to extract heat from treated sewage that provides hot water and heating to 80,000 apartments (Stockholm, Sweden); and
7. Use of dewatered sludge as fuel charcoal for thermal power generation (Tokyo, Japan) [42].

1.5.2 Microbial Community

Activated sludge flocs contain a wide range of prokaryotic and eukaryotic organisms, which include bacteria, fungi, protozoa, rotifers and nematodes. Among these microorganisms, bacteria, particularly eubacteria and archaebacteria, are the most important biological wastewater treatment. Thus, they constitute the majority of the microorganisms in the activated sludge. Studies indicated that the major genera in the activated sludge flocs are *Zooglea*, *Pseudomonas*, *Flavobacterium*, *Alcaligenes*, *Achromobacter*, *Corynebacterium*, *Comomonas*, *Brevibacterium*, *Acinetobacter*, *Bacillus* sp., as well as filamentous microorganisms such as *Sphaerotilus*, *Beggiatoa* and *Vitreoscilla* [40, 47]. Table 1.4 shows a typical distribution of heterotrophic bacteria in activated sludge.

1.5.2.1 Bacterial Lipids

Any discussion of lipids in bacteria is complicated by the wide variety of such compounds that are found. In general, bacterial lipids predominantly contain fatty acids in the C_{12} to C_{20} chain-length range, which are usually saturated or monounsaturated. These fatty acids, however, are usually associated with a variety of lipidic compounds such as acylglycerides, wax esters, phospholipids, etc. [54]. There is no clear classification of lipidic compounds present in bacteria. Thus, in this document, they were

classified according to function: as storage compounds and as component of cellular structure.

Table 1.4 Distribution of aerobic heterotrophic bacteria in a standard activated sludge process [47].

Genus or Group	% Total Isolates
Comamonas-Pseudomonas	50.0
Alcaligenes	5.8
Pseudomonas (fluorescent group)	1.9
Paracoccus	11.5
Unidentified (gram-negative rods)	1.9
Aeromonas	1.9
Flavobacterium-Cytophaga	13.5
Bacillus	1.9
Micrococcus	1.9
Coryneform	5.8
Arthrobacter	1.9
Aureobacterium-Microbacterium	1.9

1.5.2.1.1 Lipid Storage Compounds

Some bacteria are known to produce lipidic compounds as energy and/or carbon storage materials. These include polyhydroxyalkanoates (PHAs), triacylglycerides (TGs) and wax esters (WEs). Bacterial syntheses of these compounds are usually in response to environmental stresses such as nitrogen, oxygen and nutrient (i.e. phosphorus, magnesium, manganese, iron, potassium and sodium) limitations [55-58]. A complete discussion on functions, specific bacterial species and possible industrial applications is given in Chapter V. Furthermore, chemical structures of these compounds are given in Appendix A.

1.5.2.1.2 Bacterial Cellular Structure Composition

All bacteria, except Mycoplasma, have cell walls. Cell walls are composed of a mucopolysaccharide called peptidoglycan or murein (glycan strands cross-linked by peptide chains). Peptidoglycan is composed of *N*-acetylglucosamine and *N*-acetylmuramic acid and amino acids. A cell wall stain, called the Gram stain separates bacteria into gram-negative and gram-positive species. Peptidoglycan layers in gram-positive bacteria are thicker than that of gram-negative bacteria (see Figures 1.11 and 1.12). Peptidoglycan makes up about 10% of the dry weight of the cell wall in gram-negative bacteria and as much as 20 – 25% of the dry weight in gram-positive bacteria. In addition to peptidoglycan, gram-positive bacteria contain teichoic acids which are complex polymers consisting of either phosphoglycerol- or phosphoribitol-modified carbohydrates or amino acids [59, 60].

The cell wall of gram-negative bacteria, such as *Escherichia coli*, also contains outer membrane (Figure 1.11). In between the inner and the outer membranes is the periplasm. In addition to peptidoglycans, the periplasm can also contain β-glucans, which is a polysaccharide of D-glucose. The outer membrane of gram-negative bacteria predominantly contains lipopolysaccharide (LPS). In general however, it contains 30-40% proteins, 35-45% LPS and 25% lipids [59, 61]. In general, cell walls of gram-negative bacteria contain 20-30% lipids while those of gram-positive bacteria contain 2-4% lipids. For both gram-negative and positive bacteria, most of the lipids are phospholipids [62].

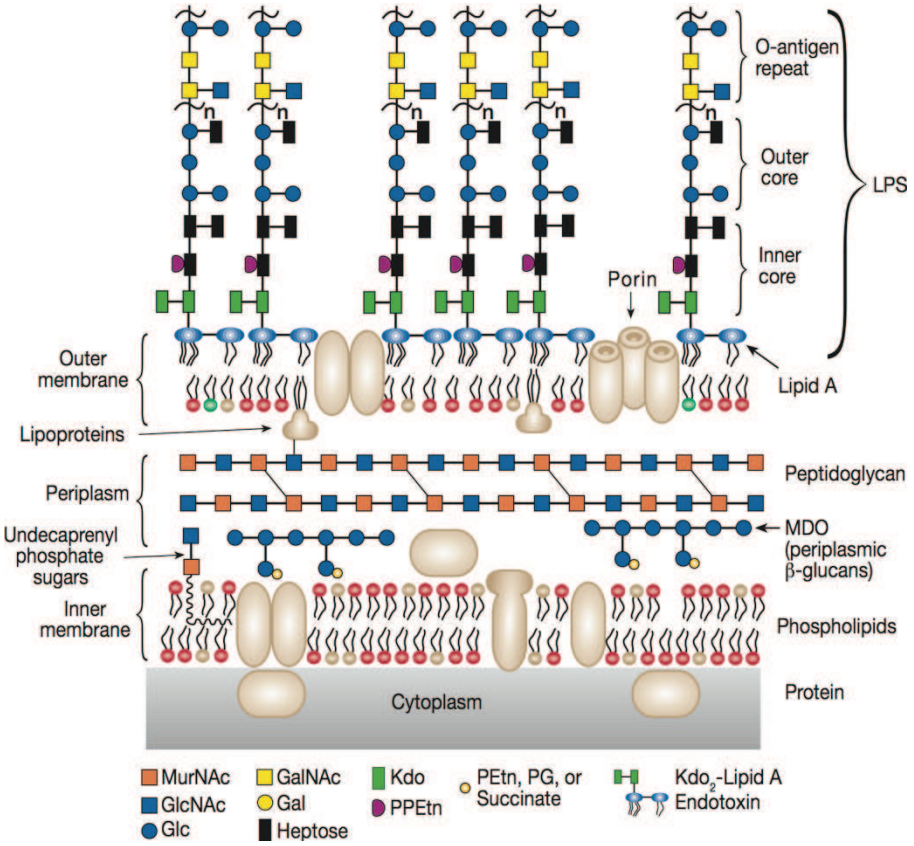


Figure 1.11 Schematic representation of the cell wall of gram-negative bacteria.*

* The periplasm contains peptidoglycan, which is a copolymer of *N*-acetylglucosamine and *N*-acetylmuramic acid with peptide cross-links, and a class of *β*-glucans known as membrane-derived oligosaccharides (MDO). The outer leaflet of the outer membrane is rich in lipopolysaccharide (LPS). (Red-lipids) Phosphatidylethanolamine; (yellow-lipids) phosphatidylglycerol; (Kdo) 3-deoxy-D-*manno*-octulosonic acid; (heptose) L-glycero-D-manno-heptose; (n) variable number of O-antigen repeats; (PPEtn) pyrophosphoethanolamine [59].

Immediately below the peptidoglycan layers is the cytoplasmic membrane (inner membrane for gram-negative bacteria). This membrane, which is semi-permeable and controls the passage of nutrients and metabolites into and out of the cell, contains about 75% protein, 20-30% lipids (predominantly phospholipids) and 2% carbohydrate [63]. In addition to phospholipids, some bacteria, such as Mycoplasma, require sterols (i.e.

cholesterol and cholesteryl ester) for growth. The cell membrane's lipids of these bacteria could contain up to 35% sterols [64].

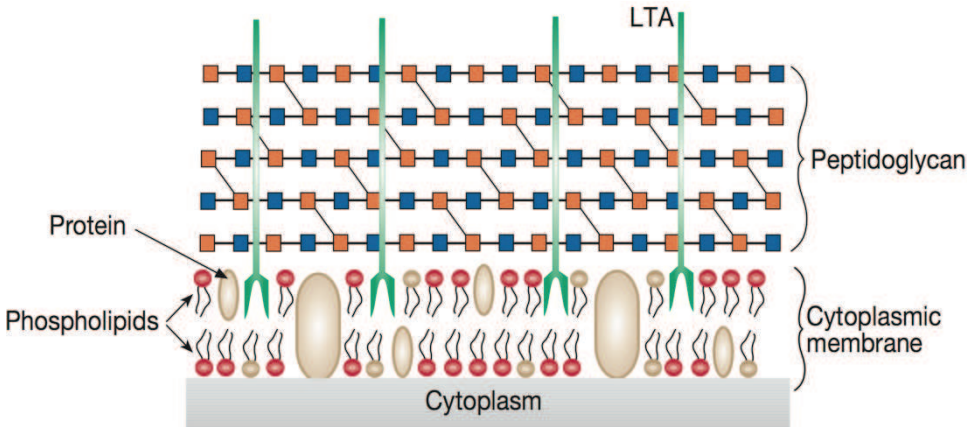


Figure 1.12 Cell wall of gram-positive bacteria.*

*LTA - Lipoteichoic acid; (Red-lipids) Phosphatidylethanolamine; (yellow-lipids) phosphatidylglycerol [59].

1.5.2.2 Other Organic Compounds

Bacterial cells also produce a type of compound called glycocalyx. Glycocalyx is made up of extracellular polymeric substances (EPS), which are composed mainly of polysaccharides. Glycocalyx is either organized as a capsule or loose polymeric materials dispersed in the growth medium. In activated sludge process, EPS are produced during the endogenous phase of growth and help bridge the microbial cells to form a three-dimensional matrix.

Some species of bacteria can also synthesize straight chain hydrocarbons and trace amounts of isoprenoid hydrocarbons such as prispane, phytane and squalene [65]. In addition to storage and structural compounds, activated sludge bacteria can also contain other organic compounds such as polycyclic aromatic hydrocarbons, linear alkyl benzenes and pentacyclic triterpanes [66]. The concentration and type of compound

classes in activated sludge bacteria are dictated by process configurations but more importantly, by the type of the influent wastewater (i.e. municipal, food, etc.).

1.6 Enhanced Activated Sludge

As mentioned earlier, bacteria synthesize lipid storage compounds under stressful conditions (i.e. nitrogen, oxygen and nutrient limitation) provided that there is an excessive supply of carbon source. Commonly, nitrogen limitation is the one that is being used to induce lipid production in bacteria.

On the average, heterotrophic bacterial biomass has a carbon to nitrogen (C:N) mass ratio of 12:1. Typically, domestic wastewaters shift this ratio towards a higher N content [67]. For example, the C:N ratio of human feces and urine are about 6-10:1 and 1:1, respectively [68]. To increase the production of lipids in activated sludge operations, it has been suggested that a C:N ratio of around 40-50:1 must be employed [69, 70]. At this C:N ratio, lipid accumulation is triggered producing sludge with high lipid content. Depending on the microbial species present, accumulated lipids can be any or all of the storage compounds discussed in section 1.5.2.2.1 (i.e. PHAs, TGs, WEs). Accumulation of lipids in high concentration is highest in group of microorganisms called oleaginous microorganisms. These microorganisms, which can accumulate more than 20% (cell dry weight) of lipids, include species of yeasts, fungi and some bacteria.

An idealized representation of the lipid accumulation process in oleaginous microorganisms is presented in Figure 1.13. It can be seen from the figure that the exhaustion of nitrogen in the medium in the onset of lipid accumulation. Once nitrogen is depleted, the cells stop to multiply and begin to consume the carbon source for the synthesis of storage compounds (i.e. PHAs, TGs, WEs). Depending on microbial species

and nature of substrate, lipid accumulation could vary from 20% to 70% of cell dry weight [70].

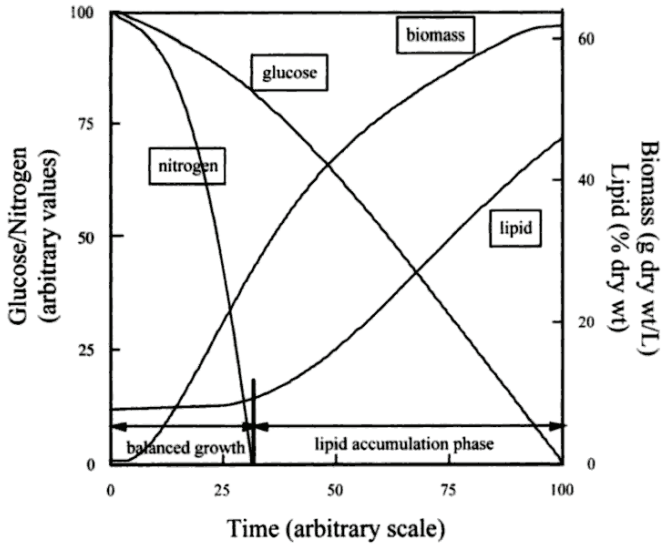


Figure 1.13 Idealized representation of the process of lipid accumulation in an oleaginous microorganism [70].

Nutrient limitation is a physiological stress that is commonly used as a strategy to channel metabolic fluxes to lipid accumulation, even for microalgae. This strategy is a biochemical engineering approach well studied in yeasts. Here, it is believed that:

1. Upon nutrient (i.e. nitrogen) exhaustion, the growth rate of oleaginous species is much lower than the intrinsic rate of lipid biosynthesis;
2. The acetyl acid-CoA carboxylase, which is the regulatory enzyme for fatty acid biosynthesis is either hyper-active or not repressed or not subjected to feedback inhibition during lipid accumulation in oleaginous species;
3. ATP:citrate lyase, an enzyme that is not present in non-oleaginous species is responsible for lipid accumulation;

4. In non-oleaginous species, there may also be a lipid production cycle, but it is accompanied by simultaneous lipid oxidation resulting to a negligible net lipid production;
5. There might be some intermediary metabolism differences between oleaginous and non-oleaginous species (i.e. increase of carbon flux into acetyl-CoA for oleaginous species when subjected to physiological stress [71-74].

Although these postulates were made from studies involving oleaginous yeasts and fungi, these might be applicable for all oleaginous species, including bacteria. In addition to biochemical engineering, other approaches that are recently being applied include genetic and transcription factor engineering. Both approaches exploit advance understanding of metabolic pathways in oleaginous species. In general, synthesis of target metabolites (i.e. lipids) can be enhanced by overexpression of key enzyme(s) or transcription factor(s) to the lipid metabolic pathway. The use of transcription factor engineering is a promising technique that utilizes transcription factor(s) (i.e. proteins) to regulate activity of multiple enzymes relevant to biosynthesis of target metabolite(s) [72].

Throughout this document, the terms enhanced sludge, enhanced activated sludge and lipid-enhanced sludge are used interchangeably to mean sludge(s) produce from fermentation of raw activated sludge under high C:N ratios. On the other hand, activated sludge and raw activated sludge refer to sludge(s) obtained directly from a wastewater treatment facility.

1.7 Oleochemicals

Petroleum refinery separates crude oil into different fractions, each of which goes to different applications. As shown in Figure 1.14, the majority of these products are

consumed as fuel. However, some products (about 10%) from refineries are consumed as petrochemical feedstocks. These are being used to manufacture products such as ink, crayons, dishwashing liquids, deodorants, eyeglasses, CDs and DVDs, tires, ammonia, heart valves, etc. [75].

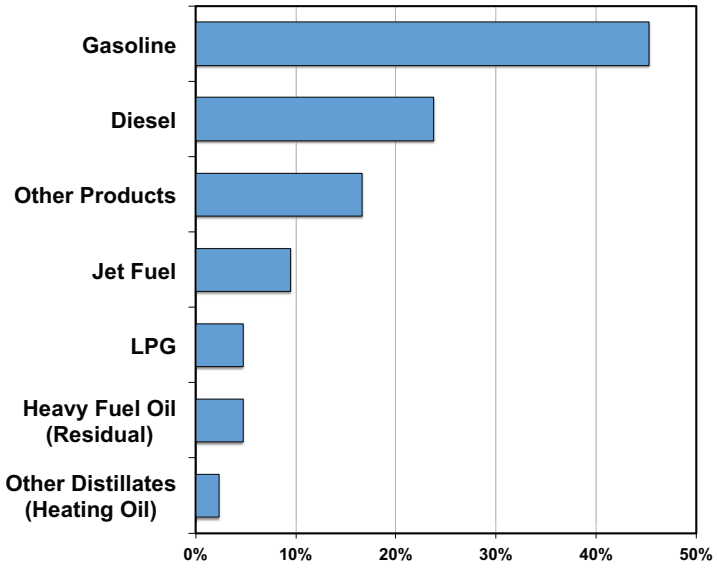


Figure 1.14 Profile of products from petroleum oil refining [75].

Oleochemicals are the renewable equivalent of petrochemical feedstocks. As such, vegetable oils and animal fats are their major source. Some of their sources include soybean, cottonseed, groundnut, sunflower, rapeseed, canola, sesame, corn, olive, coconut, palm, butterfat, lard, castor, linseed, tallow and fish oils [76, 77]. Another important source of oleochemicals is tall oil, which is one of the by-products of wood pulping industry. This oil is an important and cheap source of oleic-linoleic acid mixture in a suitable ratio for the synthesis of dimer acids for production of polyamides with excellent adhesive properties [76, 78].

There are four basic oleochemicals; fatty acids, fatty acid methyl (alkyl) esters, fatty alcohols and glycerol (Figure 1.15). These basic oleochemicals then undergo several operations (reactions) including amidation, chlorination, dimerization, epoxidation, ethoxylation, quaternization, sulfation, sulfonation, transesterification, saponification, etc., to produce derivatives that eventually end up as marketable products [76, 79].

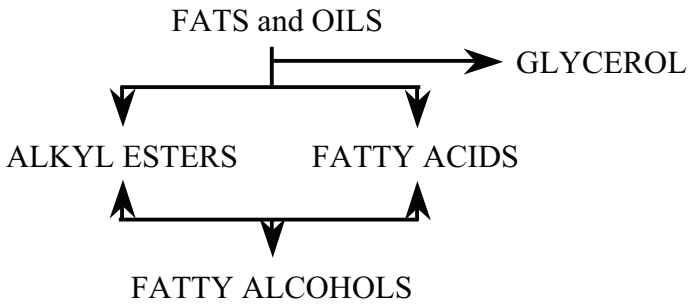


Figure 1.15 The basic oleochemicals. Redrawn from reference [79].

1.7.1 Alkyl Esters

Alkyl esters can be made by several routes using different alcohols. However, like the biodiesel industry, methanol is the commonly used alcohol in the oleochemical industry, thus producing methyl esters. Historically, methyl esters had only limited use as intermediates for the production of fatty alcohols and specialty surfactants. But, with the development of biodiesel in recent years, methyl esters have become the fastest growing oleochemicals [76, 80].

1.7.2 Fatty acids

Fatty acids can also be produced by the petrochemical industry. However, even during the times when petroleum was a lot cheaper, they only played a minor part in the oleochemical industry. Presently, only acids with alkyl branching in the carbon chain,

and acids with odd-number carbon chains, which are not produced by nature in large quantities, are manufactured from petrochemical raw materials. Fatty acids are mainly used for the production of soaps, esters, amines, but they have a lot of other minor applications (see Figure 1.16) [76, 80].

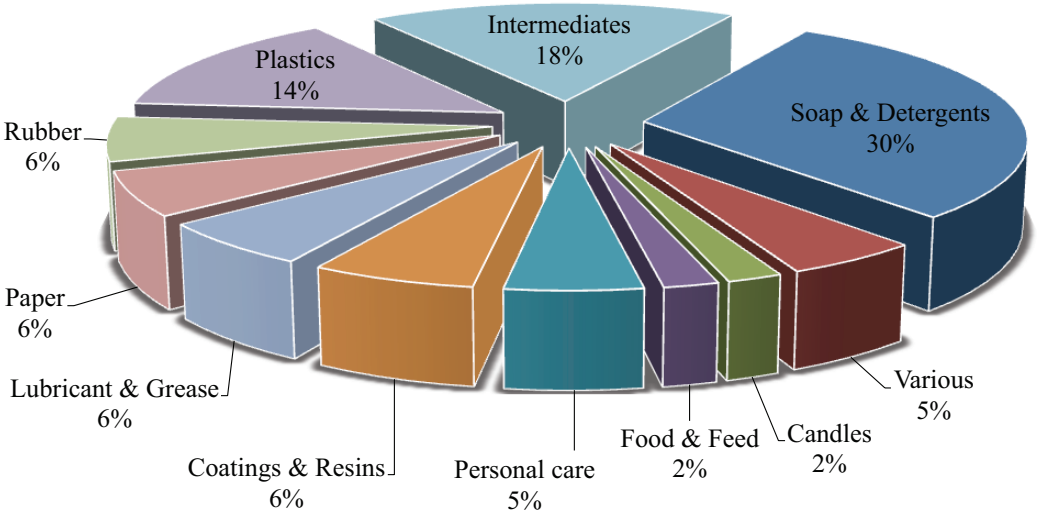


Figure 1.16 Market for oleochemical fatty acids. (Redrawn from reference [80]).

1.7.3 Fatty Alcohols

Oleochemical fatty alcohols are produced through hydrogenation of fatty acids or fatty acid methyl ester. In the petroleum industry, fatty alcohols are commonly produced from the kerosene and gas oil fraction of crude oil [77]. Due to increasing price of petroleum, the production of oleochemical fatty alcohols has become more economical than petrochemical fatty alcohols resulting to decreasing production of the latter (Table 1.5) [80].

Fatty alcohols in the range $C_6 – C_{22}$ are mainly used (~50%) in the manufacture of ionic and anionic surfactants. They can be found in many industrial products including paints, lubricants, emulsifiers, plastics, paper, leather, etc. (see Figure 1.17) [77].

Table 1.5 Global origin of fatty alcohols [80].

Year	Petrochemical	Oleochemical
	Percentage	
1980	64	36
1990	52	48
2000	40	60
2010	>35	<65

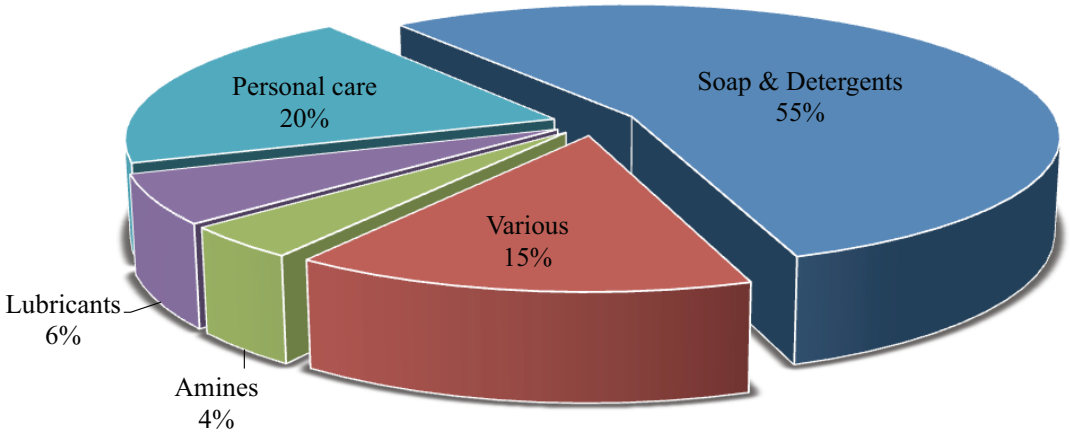


Figure 1.17 Market for oleochemical fatty alcohols. (Redrawn from reference [80]).

1.7.4 Glycerol

Glycerol is mainly obtained from the manufacture of soaps, free fatty acids and fatty acid alkyl ester. The glycerol industry was quite in order until recently due to the growth of the biodiesel industry. This has resulted to continuous decline in prices of refined and crude glycerol during the past years. The cost of glycerol purification is around $300 per metric ton. Therefore, glycerols from the biodiesel industry are sometimes used as feed additive or as raw material for production of biogas [76, 79].

Presently, there is a significant amount of research being carried out by different government and private organizations to find new uses for glycerol. Utilization of glycerol for production of high value product(s) will not only help the glycerol industry,

but the biodiesel industry as well. Figure 1.18 shows several markets for glycerol [79, 80].

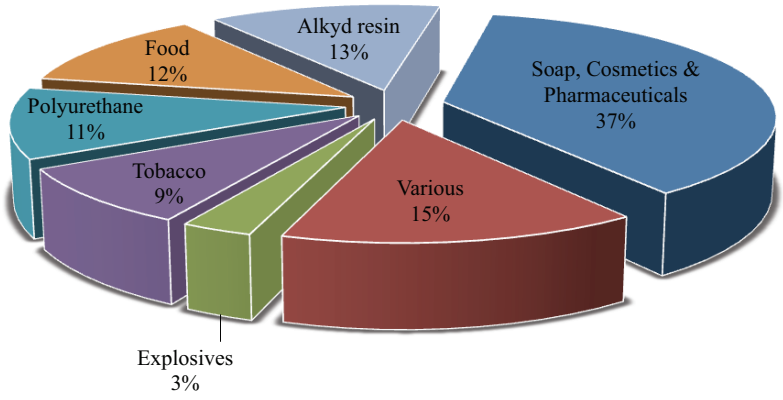


Figure 1.18 Market for oleochemical fatty alcohols. (Redrawn from reference [80]).

1.8 Remarks

The world has been and is still is very much dependent on fossil resources for its energy needs. In recent years, the declining levels of these fossil resources and the growing environmental concerns triggered the search for alternative renewable resources. Vegetable oil is one of the attractive alternative resources. However, it is also an important food commodity. With the growing malnourished population of the world (~3.7 billion), the question of whether it is moral or ethical to use vegetable oils to run transportation vehicles and industries is a major concern [81].

Fossil resources are mainly used for fuel. Nevertheless, they are also the source of other products that are necessary for everyday life. Thus, in the search for fossil substitute, these other products should also be considered. This will bring back the stability of vegetable oil usage as food and oleochemical feedstock. It is for these reasons that an abundant, non-food and unwanted alternative feedstock was considered in this work ─ activated sludge. Consistency with respect to the amounts and classes of lipidic

materials that may be obtained from this feedstock could be problematic due to differences in wastewater characteristics and treatment technologies currently in place. However, with current advances in engineering, this concern could be easily addressed.

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CHAPTER II

RESEARCH HYPOTHESIS AND OBJECTIVES

2.1 Research Hypothesis

Previous studies indicated that reasonable yields of biodiesel could be obtained from activated sludge. However, the economics of this feedstock was not fully understood due to lack of information with regards to optimum biodiesel yield and availability of other compounds that can be used either for production of biofuel through other route or as precursor for production of high value chemicals.

The guiding hypothesis of this work was that activated sludge could be made an economically viable source of lipids for the biofuel and oleochemical industries by applying different engineering strategies. Activated sludge contains microorganisms, which are predominantly heterotrophic bacteria. Species of bacteria are known to produce a wide variety of lipidic compounds. Some of these compounds serve as carbon and energy storage material when they under stressful environment (i.e. nutrient limited medium). Compounds in this category include polyhydroxyalkanoates, triacylglycerides and wax esters. Other lipidic compounds are associated with cellular structures (i.e. cell wall) of bacteria. These include phospholipids, lipopolysaccharides and in some cases sterols and steryl esters. In addition to lipidic compounds, activated sludge bacteria might also contain glycocalyx or exopolysaccharides, straight chain hydrocarbons and isoprenoid hydrocarbons such as prispane, phytane and squalene. Among these compounds, only the saponifiable ones (those that contain fatty acid moiety) can be

converted to fuel via the biodiesel pathway (i.e. transesterification). The unsaponifiable compounds present in the activated sludge are also important, not only for biofuel production, but also for a wide variety of applications.

2.2 Objectives

The main objective of this work was to evaluate different strategies to fill in research gaps with regards to a cost-effective utilization of activated sludge as biofuel and oleochemical feedstock. And for its accomplishment, it was divided into three primary objectives.

1. Optimization of biodiesel production through in situ transesterification.
2. Identification and quantitation of lipidic compounds present in raw and enhanced activated sludges.
3. Develop reaction mechanism for catalytic cracking of a model compound present in activated sludge.

2.2.1 Primary Objective 1

As previously mentioned, there are available literatures with regards to the utilization of activated sludge for biodiesel production. Most of the previous studies utilized the in situ transesterification process for the evaluation of its economics. Their economic evaluations were based on reasonably assumed biodiesel yield. Nevertheless, this presents some inaccuracies. For a more accurate economic evaluation of the process and the feedstock, optimization is of utmost importance. The optimization of biodiesel production from activated sludge using the in situ process is the topic of Chapter III. The in situ process was also applied on partially dewatered sludge for the purpose of reducing

the cost associated with feedstock drying. The results of economic analysis for these two processes can also be found in Chapter III.

2.2.2 Primary Objective 2

The biodiesel industry is mainly concern with the fatty acid component of any lipidic materials. This is the main cause of the recent disturbances in the oleochemical glycerol industry. In the search for alternative biofuel feedstock, it is important to know what by-products could be formed to strategize their possible utilization. This might even improve the economics of any feedstock as what was anticipated for activated sludge. Therefore, knowledge of the nature and relative concentrations of major lipidic compounds present in activated sludge would help researchers in designing unit operations for subsequent processes.

Extraction experiments were conducted to efficiently extract different compounds from activated sludge without affecting their quality. This was necessary for a more accurate compound characterization. Chapter IV presents the results of extraction comparison using different techniques. Subsequently, a solid phase extraction technique was developed for the characterization of lipidic compounds in activated sludge extracts. This is the subject of Chapter V.

A biochemical stimulus (high C:N ratio) was also used to induce accumulation of lipids (particularly the saponifiable ones) in activated sludge. The main aim was to increase biofuel yield from this feedstock. However, this was also done to solidify the concept of using existing WWTPs as source of lipids for biofuel production. Existing WWTPs are configured tailored specific conditions. The class(es) of lipidic materials present in sludges from different facilities could be entirely different from each other and

their combined utilization for biofuel production might not be attractive. It is envisioned that, regardless of the source of activated sludge, they will accumulate lipids with uniform (homogenous) characteristics. The result of this study is presented in Chapter VI.

2.2.3 Primary Objective 3

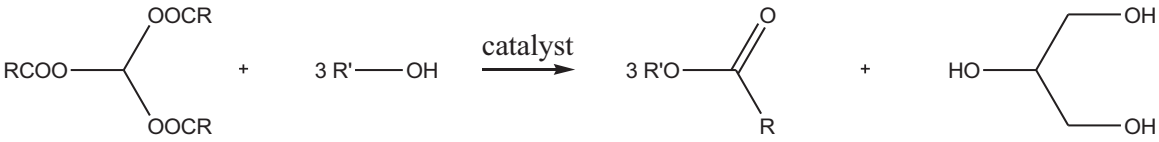
Other compounds present in activated sludge include wax esters. Wax esters are esters of fatty acids and a long chain alcohol. If activated sludge lipids are to be used for the production of biofuel alone, a different process routes must be used. One such route is catalytic cracking to produce green fuels. Catalytic cracking of fatty acids have been and still is the subject of significant researches and thus the availability of literature data is enormous. For this reason a model fatty alcohol (1-octadecanol) was chosen for catalytic cracking studies presented in Chapter VII. A mechanistic approach was undertaken to better understand the chemistry involve in cracking of this compound. The data obtained would help researchers to design catalysts process conditions for the conversion of this compound into biofuel.

CHAPTER III

PRODUCTION OF BIODIESEL FROM ACTIVATED SLUDGE: OPTIMIZATION STUDIES (Revellame et al., 2010, Revellame et al., 2011) [1, 2]

3.1 Introduction

Biodiesel, also known as fatty acid alkyl ester, is an alternative renewable fuel that may be derived from a variety of feedstock (i.e. vegetable oils, animal fats, used frying oils, microbial oils) [3, 4]. It is commonly produced by the reaction of pre-extracted refined oils (mostly triacylglycerides) and an alcohol in the presence of a catalyst to generate fatty acid alkyl esters (Figures 3.1 and 3.2) [5]. In addition to being renewable and biodegradable, biodiesel could provide displacement of imported petroleum-based diesel, it has similar energy density as petroleum diesel, higher flash point, inherent lubricity, and it could reduce most exhaust emissions (except NO_x) [3, 5, 6]. These advantages make biodiesel a promising alternative energy carrier.



Triacylglycerol Alcohol Fatty Acid Alkyl Ester Glycerol

Figure 3.1 The transesterification reaction. R is a mixture of various fatty acid chains. Alcohol is usually methanol ($R' = CH_3$) [3, 7].

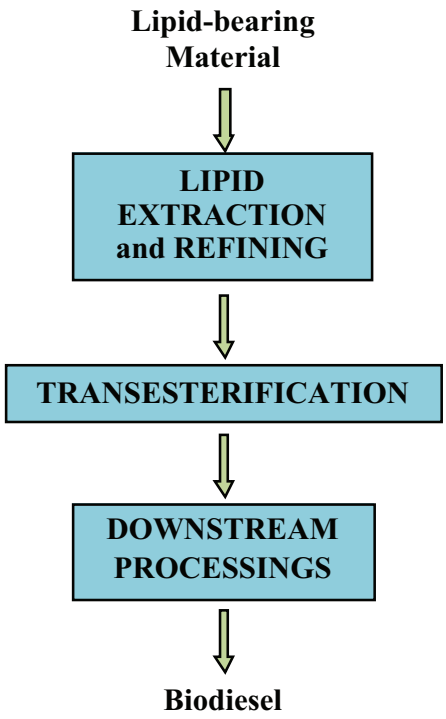
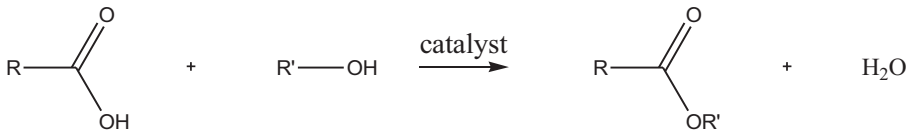


Figure 3.2 The general steps for biodiesel production.

The most commonly used alcohol for biodiesel production is methanol, thus producing Fatty Acid Methyl Esters (FAMEs). Methanol, in general is the cheapest alcohol except in some countries such as Brazil, where ethanol is the least expensive one [8]. The use of longer-chain (straight or branched) alcohols have also been used and reported to produce fatty esters with lower freezing points than FAMEs [9-11]. For refined feedstocks, alkalis such as sodium hydroxide, potassium hydroxide and their alkoxides are the most commonly used catalysts. It has been reported that for the transesterification reaction, alkali catalysis is a much more rapid process than acid catalysis [12]. Alkali catalysis, however, cannot be applied on lower quality feedstocks (i.e. with high free fatty acid content) due to soap formation. Utilization of these feedstocks requires either one-step acid catalysis or a two-step acid-alkali catalysis

depending on its free fatty acid level. For feedstocks with very high free fatty acid level, such as trap grease (as high as 100% free fatty acids), one-step acid catalysis is more applicable while for feedstocks such as animal fats (5 – 30% free fatty acids) the two-step process is more suitable. According to Ramadhas (2009), the two-step process is preferred for feedstocks containing 20 – 50% free fatty acids [13]. For this two-step process, the acid-catalyzed esterification (Figure 3.2), which converts free fatty acids to alkyl esters, serves as a pretreatment step prior to the alkali-catalyzed transesterification of the acylglycerides in the feedstock [9, 14]. Other catalysts that have been tested for biodiesel production include enzymes (lipases), calcium methoxide and BaOH, CaO, K_2CO_3, Na_2CO_3, Fe_2O_3, $NaAlO_2$, Zn, Cu, Sn, Pb, ZnO, anion exchange resins, zeolites, Cs-MCM-41, Cs-sepiolite, hydrotalcites and even ashes from the combustion of fibers, shell and husk of coconut and palm seeds [9, 12].



Free Fatty Acid Alcohol Fatty Acid Alkyl Ester Water

Figure 3.3 Esterification of free fatty acids. R = fatty acid chain. Alcohol is usually methanol (R′ = CH_3) [15, 16].

Biodiesel's main economic challenge is the high feedstock/raw material cost, which for refined vegetable oil, accounts for 70-85% of the total biodiesel production cost [9, 14, 17]. The growth of the biodiesel industry is limited by the availability of farmland and vegetable oil inventories, which could result in high sensitivity of prices to oil demand from the industry. Majority of the 100+ million tons of oils/fats generated

annually are consumed as human food (80-81%). The remaining portion enters livestock rations (5-6%) to produce more human food and the remainder goes to the oleochemical industry [18]. These create a competition between the biofuel and food industries that require urgent consideration of non-food related feedstock [19-23]. This competition could result in unattractive increases in both fuel and food costs [24].

In the United States, the major biodiesel feedstocks are soybean oil and animal fats. Rapeseed and sunflower oils are predominant in the European Union [9]. Among possible alternative biodiesel feedstock are oils of non-edible crops like jatropha, castor, neem, and karanja [17], used frying oils [21], microalgae [19], soapstocks [9] and microbial biomass [5]. Although these alternative feedstocks might be cheaper than soybean or rapeseed, crops require energy and acreage to grow, used frying oils have broad properties that may affect the consistency of biodiesel production [9], and microorganisms require energy and acreage for sufficient oil production. Excessive acreage requirement for planting crops or generate microbial oils could also displace lands necessary to grow crops for food in the future.

Efforts to reduce biodiesel cost include utilization of cheap, non-food sources of oil (i.e. from non-food sources mentioned above), application of low cost, highly active catalyst and process modifications. Such modifications include process flexibility to accommodate wide range of feedstock (i.e. the two-step acid-alkali catalysis [9]) and elimination of some conventional processing steps (i.e. in situ transesterification [9, 12]). Other processes that have been tested for biodiesel production include supercritical alcohol, ultrasonic and monophasic (co-solvent) transesterification and application of sub-critical water pre-treatment [9, 13, 25-30].

3.1.1 The In situ Process

The in situ transesterification process was developed by Michael Haas, a biochemist with the USDA Agricultural Research Service (ARS). In situ is the Latin for "in place" which indicates that the transesterification reaction happens in the place of origin of the oil (triacylglyceride), which is the oil-bearing material [31]. This process eliminates the expensive extraction step or rather it combines the lipid extraction and fuel conversion steps into a single step, thereby reducing the cost of the process (see Figure 3.2) [8, 12].

3.1.2 Biodiesel from Activated Sludge

Previous studies have demonstrated the potential of activated sludge from MWWTPs as biodiesel feedstock. Dufreche et al. (2007) compared biodiesel yield from activated sludge using different extraction procedures. They tested accelerated solvent extraction (ASE®) using different organic solvents, supercritical CO_2 extraction, and in situ transesterification. They concluded that the in situ transesterification extraction procedure gave the highest yield of biodiesel (6.23% of dry sludge) since the reagents have access to all lipids in the feedstock. They estimated that for a biodiesel yield of 7% weight, the cost of biodiesel from in situ transesterification of activated sludge is around $3.11 per gallon [32]. In a related study, Mondala et al. (2009) determined the effect of three process parameters (reaction temperature, methanol loading, and catalyst concentration) on the yield of biodiesel from primary and secondary sludges obtained from a MWWTP. They utilized the in situ procedure with *n*-hexane as co-solvent. Two levels of temperature (50 and 75°C), two levels of methanol to sludge ratio (8:1 and 12:1 weight/weight which correspond to 10:1 and 15:1 volume/weight, respectively), and two levels of sulfuric acid concentration (1 and 5% volume/volume) were studied by these

investigators. They concluded that for the secondary or activated sludge, the biodiesel yield is affected by independent effects of the three investigated process parameters. Also, a maximum yield of 2.5% was obtained at 75°C, 5% (v/v) sulfuric acid, and 12:1 methanol to sludge ratio. Results of their kinetic experiments showed that for the secondary sludge, reaction completion was achieved after 24 hours reaction time. Their economic analysis indicated that at a biodiesel yield of 10% (weight), the break-even price of biodiesel from sludges (primary and secondary/activated) is $3.23 per gallon [14].

Although the effect of different process parameters on the biodiesel yield using in situ transesterification procedure has been demonstrated, optimization of the process is necessary to predict performance of operating conditions and determine processing costs more accurately. Thus, the optimization of in situ transesterification of dried activated sludge obtained from a MWWTP in Tuscaloosa, AL was conducted. Process parameters (temperature, methanol to sludge ratio, and catalyst concentration) were varied to determine the combination resulting in the maximum yield of FAMEs.

All the previous studies on the in situ transesterification of activated sludge were conducted using nearly dried sludges (~5% weight moisture). The reduction of water content of the activated sludge from 98% to 5% (weight) could add up to 55% of the biodiesel cost [32]. Using a feedstock for in situ transesterification with as near as its natural moisture content could reduce the drying cost but may require relatively large amount of methanol [24, 33]. On a study conducted by Haas and co-workers (2007) on the in situ transesterification of distillers dried grains with solubles (DDGS), they found that the removal of 20% weight moisture from the sample has no effect on the methanol requirement of the reaction. They further concluded that more complete drying (2.62%

weight) reduces the methanol requirement of the process for high reaction conversion [24].

For the case of substrate with relatively low moisture, like DDGS (8.7% weight natural moisture), reduction of the moisture content to reduce the methanol requirement may be the best option to reduce the overall cost of the process, hence the product. However, for substrates with high moisture content, like activated sludge (98% weight) [32], increasing the methanol loading might be more economical than the reduction of water to a very low level so as to obtain high reaction conversion. For substrates with this high moisture level, reduction of water to a certain level that will result to an acceptable yield might also be necessary. This might jeopardize the yield of biodiesel but could result to a remarkable reduction of production cost and hence the cost of biodiesel.

The in situ transesterification process was also applied to partially dewatered activated sludge (84.50% weight moisture). The optimization of the process was conducted by varying process parameters (temperature, methanol to sludge ratio, and catalyst concentration) to determine the combination that will give the maximum/optimum yield of biodiesel based on FAMEs.

The in situ transesterification process utilizes either acid or base liquid catalyst depending on the nature of the lipids present in the substrate [14, 24, 33, 34]. Owing to the possible high level of free fatty acids in the activated sludge, an acid catalyst specifically sulfuric acid was chosen. This was to maximize biodiesel yield and avoid soap formation as for the case of base catalysts. Among possible acids (sulfuric, hydrochloric, formic, acetic, and nitric acids) that can be used as catalyst for transesterification process, previous study showed that sulfuric acid had significantly higher activity as compared to the others [35]. Furthermore, sulfuric acid has been shown

to be an effective catalyst for the in situ esterification of rice bran oil even in the presence of significant amount of moisture (13.40% weight) [34].

The results of optimizations were then used to calculate the economics of the two processes and the possible cost reduction associated with the utilization of activated sludge with high water content for biodiesel production was estimated.

3.2 Materials and Methods

3.2.1 Chemicals and Gases

All Chemicals (methanol, sulfuric acid, *n*-hexane, toluene, anhydrous sodium sulfate, 1,3-dichlorobenzene (1,3-DCB), and butylated hydroxytoluene (BHT) were purchased from Fisher Scientific (Pittsburgh, U.S.A.). The 14-component FAMEs standard with saturated, mono-unsaturated and poly-unsaturated fatty acids was purchased from Supelco (Bellefonte, PA, U.S.A.) and all the gases used (He, H_2, and air) for gas chromatography were of high purity grade and distributed by nexAir (Columbus, MS, U.S.A.). All chemicals, standard, and gases were used as received.

3.2.2 Sample Collection and Preparation

All activated sludge samples used in this chapter were obtained from a MWWTP in Tuscaloosa, AL, U.S.A. [36]. Samples were collected from the return activated sludge line into 4-gallon plastic buckets and were transported in ice chests to the Renewable Fuels and Chemicals Laboratory at Dave C. Swalm School of Chemical Engineering, Mississippi State University.

3.2.2.1 Partially Dewatered Sludge

Samples were concentrated by gravity settling in ice-bath overnight. The supernatant was discarded and the settled solids were centrifuged using an IEC Centra GP6 centrifuge (Thermo Electron Corp., Milford, MA, U.S.A.) operated at 3000 rpm for 20 minutes. The solid content of the concentrated sludge was determined using an Ohaus MB45 infrared heater (Ohaus, Pine Brook, NJ, U.S.A.) and it was found to contain an average of 15.50% (weight) solid. The concentrated sludge sample was stored below 0°C until further use.

3.2.2.2 Freeze-dried Sludge

The concentrated (partially dewatered) sludge was spread into 150 x 15 mm standard polystyrene Petri dishes (Fisher Scientific, Pittsburgh, U.S.A.), frozen at -18°C using a ColdTech freezer (Jimex Corp., Hayward, CA, U.S.A.) and freeze-dried using Freezone 2.5 freeze dry system (Labconco, Kansas City, MO, U.S.A.) for 5 days. The freeze-dried sludge, which contains an average of 95% (weight) solid, was then pulverized using mortar and pestle, homogenized, and stored in the freezer until further use.

3.2.3 Experimental Design

3.2.3.1 Freeze-dried Sludge

The optimization of biodiesel production from freeze-dried activated sludge was conducted using four levels of temperature (45, 55, 65, and 75°C), six levels of methanol to sludge ratio (5, 10, 15, 20, 25, and 30 mL/g) and five levels of catalyst (H_2SO_4) concentration (0.5, 1, 2, 4, and 6% volume/methanol volume). A full factorial design (4 x

6 x 5) was utilized giving a total of 120 treatment combinations. Three replicates were done for all treatment combinations.

3.2.3.2 Partially Dewatered Sludge

Temperatures from 45 to 75°C, methanol to sludge (solids) ratios from 5 to 30 mL/g and H_2SO_4 concentrations from 1 to 10% volume/methanol volume were considered for optimization. A wider range of catalyst loading was considered to include higher loading to compensate for the dilution effect due to the presence of high amount of water. An orthogonal central composite response surface design with 9 center points was used as experimental design giving a total of 23 treatment combinations. The treatment combinations are presented in Table 3.1. Triplicate runs were conducted for all treatment combinations.

Table 3.1 Orthogonal central composite response surface design for the in situ transesterification of partially dewatered activated sludge.

Experimental run	Factors		
	Temperature (°C)	Methanol to sludge ratio (mL/g solid)	Sulfuric acid (% vol./methanol vol.)
1	51	10.07	2.82
2	51	10.07	8.18
3	51	24.93	2.82
4	51	24.93	8.18
5	69	10.07	2.82
6	69	10.07	8.18
7	69	24.93	2.82
8	69	24.93	8.18
9	45	17.50	5.50
10	75	17.50	5.50
11	60	5.00	5.50
12	60	30.00	5.50

Table 3.1 (Continued).

13	60	17.50	1.00
14	60	17.50	10.00
15 – 23	60	17.50	5.50

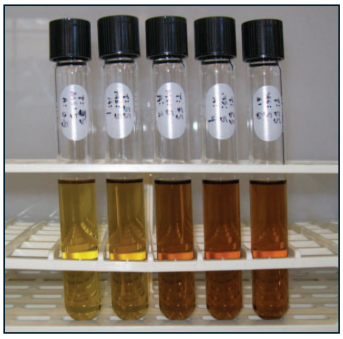
3.2.4 In situ Transesterification

Reactions were carried out using Instatherm® block system (Ace Glass Inc., Vineland, NJ, U.S.A.) for 24 hours. One gram of freeze-dried activated sludge (one gram equivalent solid which is equal to 6.45 g for partially dewatered sludge) was weighed into screw-capped (PTFE-lined) reaction vials with a capacity of at least two times the reaction volume. Treatments were then randomly assigned to each of the vials. Then, assigned volume of methanolic sulfuric acid was added and the mixture was heated to the desired temperature at ambient pressure. The solids were kept suspended in solution by using a magnetic stirring bar.

3.2.4.1 Freeze-dried Sludge

After 24 hours, the mixture was allowed to cool down to room temperature. The mixture was then centrifuged at 3000 rpm for 5 minutes and the supernatant was transferred into a 60-mL vial. To recover any FAMEs adhered to the solid residue, it was washed two times with 5 mL of methanol, vortex-mixed for 2 minutes, and centrifuged at 3000 rpm for 5 minutes. The supernatants were pooled, after which the methanol was removed at 45°C under 15 psi stream of N_2 using TurboVap LV (Caliper Life Sciences, Hopkinton, MA, U.S.A.). Then, the residue was re-dissolved in 15 mL of *n*-hexane and washed three times with 5 mL distilled water. Vigorous mixing was done during water washing and any emulsions formed were broken by centrifugation at 1500 rpm for 5 minutes. The hexane layer was then transferred to a 20-mL screw-capped test tube

passing through ~2 grams of anhydrous sodium sulfate to remove traces of water (Figure 3.4). Then, the hexane was removed using TurboVap LV (45°C, 15 psi of N_2) and the residue was re-constituted in 10 mL of GC-diluent (toluene with 200 ppm 1, 3-DCB and 100 ppm BHT).



a b c d e

Figure 3.4 Biodiesel dissolved in *n*-hexane from the in situ transesterification of activated sludge; 55ºC, 5 mL methanol/g sludge, a: 0.5%, b: 1.0%, c: 2.0%, d: 4.0% and e: 6.0% H_2SO_4.

3.2.4.2 Partially Dewatered Sludge

After reaction completion, the mixture was allowed to cool to room temperature. The supernatant was recovered into 60-mL glass vial by centrifugation at 3000 rpm for 5 minutes. The solid residue was washed twice with 5 mL methanol, vortex-mixed for 2 minutes and centrifuged at 3000 rpm for 5 minutes. The supernatants were pooled and the volume was reduced to ~6 mL using a TurboVap at 45°C under 15 psi stream of N_2. The FAMEs were then extracted four times with *n*-hexane (20 mL total) and the extract was washed three times with 5mL distilled water. During water washings, emulsions formed were broken down by centrifugation at 1500 rpm for 5 minutes. The residual water in the extract was removed by passing it through ~2 grams of anhydrous sodium sulfate. The hexane was removed using a TurboVap LV as described above and the FAMEs were re-

dissolved in 5 mL of GC-diluent. The 1,3-DCB was used as internal standard for FAMEs analysis, while BHT was added primarily because of its antioxidant property.

3.2.5 Methanol Loss

Experiments similar to the in situ transesterification experiments were done but without freeze-dried sludge to determine methanol loss due to evaporation. Different initial weights of the methanolic sulfuric acid (corresponding to 5 – 30 mL) were recorded and heated to a given temperature (45 to 75°C) for 24 hours. Final weights were determined and the percentage methanol losses were calculated by difference.

3.2.6 FAME Analysis

The FAMEs obtained from in situ transesterification of both freeze-dried and partially dewatered sludges were analyzed in the same way. FAMEs samples were diluted (1:1) with the GC-diluent prior to analysis, which was carried out using an Agilent 6890N gas chromatograph equipped with flame ionization detector (GC-FID) (Agilent, Santa Clara, CA, U.S.A.). The column used was a Restek Stabilwax-DA capillary column (Restek, Bellefonte, PA, U.S.A.) with dimensions of 30 m x 0.25 mm I.D. and 0.25 μm film thickness. Analyses were conducted using helium as carrier gas with a constant injector temperature of 260°C in splitless mode. The GC oven temperature was programmed at an initial temperature of 50°C, held for 2 minutes; then ramp to 250°C at 10°C/minute, and held for 18 minutes. The FID was held at 260°C for the duration of analysis. A 14-component FAMEs standard mixture containing C_8-C_{24} fatty acids was used for instrument calibration. The percent yields of FAME/biodiesel based on dry weight of sludge using the in situ transesterification procedure were

calculated based on the data obtained from GC-FID runs, neglecting compounds with concentration of less than 1% weight/weight.

3.3 Results and Discussion

3.3.1 Methanol Loss

Methanol loading is one of the critical factors for overall energy efficiency of biodiesel. This is not only because high methanol loading will increase raw material costs but also because removal of methanol after transesterification can be energy intensive [5]. For all the temperature investigated, no significant methanol loss due to evaporation (1.15 ± 0.46 % weight) was observed. This suggests that the set-up used is suitable for studying effects of different factors for the purpose of optimizing biodiesel production from activated sludge using an in situ transesterification process.

3.3.2 Statistical Analyses and Regressions

All statistical analyses were done using SAS® software*, a statistical analysis software package [37]. The software's ADX interface was utilized in numerical optimization, and surface plot for data analyses and presentation. Regression analyses were done at a significance level of 0.05.

For all the treatment combinations, the highest coefficient of variation (relative standard deviation) obtained were 7.37% and 8.20% for freeze-dried sludge and partially dewatered sludge, respectively indicating satisfactory data agreement between replicate runs. Combined with partial least square regression method, the SAS software's ADX

* SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc. in the U.S.A. and other countries. ® indicates U.S.A. registration.

interface was used to determine main and interactive effects of factors on the response. The most commonly used form of regression relation between experimental response, Y, and factors is the quadratic response surface model which is given by:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j} \sum \beta_{ij} x_i x_j + \varepsilon \qquad (3.1)$$

Here, k is the number of factors, β_0 is the constant term, β_i is the linear coefficient of factor i, β_{ii} is the quadratic coefficient of factor i, β_{ij} is the interactive effect coefficient for factor i and factor j, and ε is the random error [38]. Although higher order model can be used, difficulties in result interpretation may arise. Thus, the quadratic response surface model was tested for adequacy.

Expansion of the quadratic response surface model for $k = 3$ for temperature, t, methanol to sludge ratio, m, and sulfuric acid concentration, a results in;

$$Y = \beta_0 + \left(\beta_1 t + \beta_2 m + \beta_3 a\right) + \left(\beta_{11} t^2 + \beta_{22} m^2 + \beta_{33} a^2\right) + \left(\beta_{12} tm + \beta_{13} ta + \beta_{23} ma\right) + \varepsilon \qquad (3.2)$$

3.3.2.1 Freeze-dried Sludge

For the freeze-dried sludge (at $\alpha = 0.05$), all the factors and factor interactions were found to be statistically significant ($p<0.05$) and the uncoded model was found to be:

$$\begin{aligned} Y = & -1.77x10^{1} + \left(5.29x10^{-1} t + 2.97x10^{-1} m + 2.05a\right) \\ & - \left(4.27x10^{-3} t^2 + 3.25x10^{-3} m^2 + 1.55x10^{-1} a^2\right) \\ & - \left(1.13x10^{-3} tm + 5.34x10^{-3} ta + 1.98x10^{-2} ma\right) \end{aligned} \qquad (3.3)$$

with R^2 of 0.843 and statistically not significant lack of fit ($p=0.152$) indicating that the model is adequate and no additional term is necessary. The three-way interaction effect of

the factors was tested and the result showed that it has insignificant effect on the response ($p=0.525$) confirming the sufficiency of the quadratic response surface model. The model presented in Equation 3.3 corresponds to the uncoded model of the data, which can be used to generate predicted values of biodiesel yield. This model is dependent on the unit of measure, and thus cannot be used to interpret how the response is being influenced by the different effects [39].

To determine the factors and factor interactions with the greatest influence on the response, the model was coded using a standard coding scale of -1 to +1 for the low versus high end, respectively, of the factorial ranges: 45 to 75°C of temperature, 5 to 30 mL/g of methanol to sludge ratio, and 0.5 to 6% volume of sulfuric acid concentration. Factor coding is a linear transformation of the factor space coordinates. It removes the unit of measure, and thus, can be used to determine how the response changes relative to a representative center of design, the intercept [40, 41]. Coded coefficients are synonymous to standardized regression coefficients which represent the change in standard deviation units of the dependent variable per one standard deviation change in the independent variable with all other variables held constant [42]. The coded model is given by;

$$\begin{aligned} Y = {} & 4.53 + \left(-3.11x10^{-1}t + 6.40x10^{-1}m + 1.04a\right) \\ & - \left(9.61x10^{-1}t^2 + 5.08x10^{-1}m^2 + 1.18a^2\right) \\ & - \left(2.13x10^{-1}tm + 2.20x10^{-1}ta + 6.80x10^{-1}ma\right) \end{aligned} \tag{3.4}$$

with the coded coefficients plotted in Figure 3.5. As mentioned, the intercept of 4.53 was used as the center of design. Results indicated that methanol to sludge ratio and sulfuric acid concentration have positive linear influence on the response while temperature has a negative linear influence on the response. The catalyst concentration was the most

effective factor that influenced the yield of biodiesel followed by methanol to sludge ratio. All the quadratic elements and interactions have negative influence on the response.

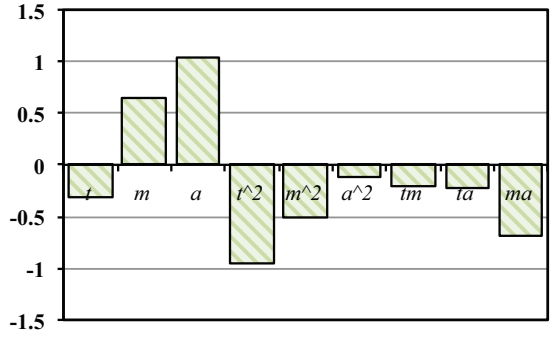


Figure 3.5 Coded coefficients or standardized regression coefficients of different factors and factor combinations for biodiesel yield: *t*, temperature; *m*, methanol to sludge ratio; *a*, sulfuric acid concentration.

The negative coded coefficients on main effect of *t* indicated that both the low (-1) and high (+1) levels have negative influence on the response at constant *m* and *a*. Furthermore, the low level of *t* has a lesser negative influence on the response than the high one. The quadratic effect of *t*, indicates that there exists a value of *t* within the experimental design, which will give a maximum positive influence on the response. This can be seen also on the main factor effect of *t* on the biodiesel yield presented in Figure 3.6a indicating that the response can be maximized at a temperature region from 55 to 65°C.

The influence of *t* can be explained by the fact that unsaturated fatty acids, their glycerides, and their esters can undergo polymerization at high temperature. Methyl oleate for example has been known to undergo polymerization at 300°C [43]. Although the maximum temperature included in the design was 75°C, sulfuric acid is a known catalyst for polymerization of unsaturated fatty acids. The acid-catalyzed production of

estolides, an oligomeric fatty acid esters, can proceed at a slow reaction rate at 50°C. At 75°C, the reaction rate is faster even at a reduced concentration of acid catalyst [44, 45]. The study conducted by Isbell et al. (1997) showed that commercial oleic acid forms estolide when reacted at 55°C with 5% (by volume) concentrated sulfuric acid for 24 hours under vacuum [46]. As indicated in section 3.3.1, no significant losses of methanol were observed for the temperature range studied. Thus, it is safe to assume that losses of products (i.e. biodiesel) due to evaporation were also not significant. And thus, polymerization of unsaturated fatty acids and their derivatives might have caused the decline in biodiesel yield at temperature above 60°C.

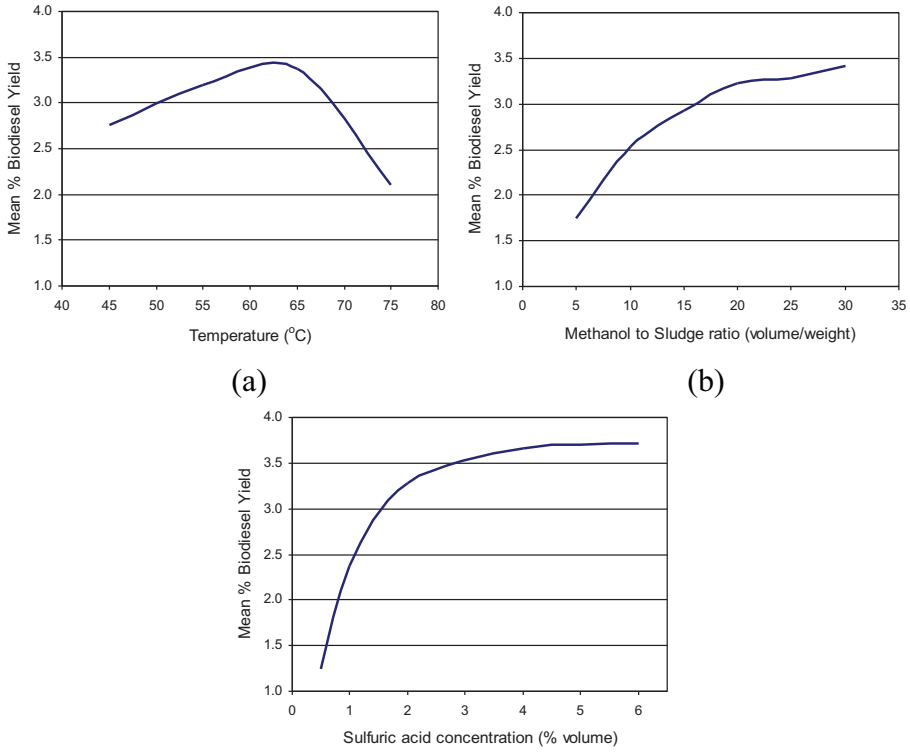


Figure 3.6 Main effects of process parameters on the yield of biodiesel for in situ transesterification of activated sludge; (a) temperature, *t*, (b) methanol to sludge ratio, *m* and (c) sulfuric acid concentration, *a*.

The main effect of methanol to sludge ratio, *m* on the response indicated that there is a positive increasing influence on biodiesel yield from low to high level. However, because of the quadratic main effect term, the increase in the response gradually decreases from low to high level. This is also evident on the main factor effect of *m* on the percent biodiesel yield shown in Figure 3.6b. The same is true for the main effect of acid concentration, *a* on the biodiesel yield (Figure 3.6c). There is an increasing influence on biodiesel yield, which progressively decreases from low to high level. For both *m* and *a*, these suggested that there is a value within the experimental design wherein above that value, the increase in response will be insignificant. Referring to Figures 3.7b and 3.7c, these values seem to be 20 and 4 for methanol to sludge ratio and sulfuric acid concentration, respectively.

As for the interaction effects; *tm*, *ta*, and *ma*, the negative coded regression coefficients indicate that for them to have a positive effect on the response low and high levels should be combined. For example, high level of *t* should be combined with low level of *m* for the *tm* interaction to have a positive effect on the biodiesel yield. The same is true for *ta* and *ma* interactive effects. These might be due to the acid-promoted polymerization of unsaturated fatty acids and their derivatives. For the interactive factor *tm*, for example, high level of *m* should result to high biodiesel yield based on the main effect of *m*. However, if this high level of *m* is combined with high level of *t*, low biodiesel yield will be obtained since at temperature above 60°C polymerization of unsaturated fatty acids and their derivatives might be significant.

3.3.2.2 Partially Dewatered Sludge

The same statistical analyses as with the freeze-dried sludge were applied on the in situ transesterification of partially dewatered sludge. The significant effects (main effects and interaction effects) of the factors investigated (temperature, methanol to solid ratio and catalyst concentration) on the biodiesel yield were determined utilizing the quadratic response surface model given by Equation 3.1. Data regression showed that the uncoded model showing only the significant effects ($p< 0.05$) is represented by:

$$Y = 4.63 + (-2.44x10^{-1}t + 1.94x10^{-1}m + 8.78x10^{-2}a) + 2.69x10^{-3}t^2 - 1.96x10^{-3}tm \quad (3.5)$$

This model gave a R^2 of 0.836 and a not significant lack of fit ($p = 0.254$). This indicates good agreement between the model and the data. Furthermore, this implies that the reliability of the model is reasonably high [24].

The factors were then coded with a standard coding scale of -1 to +1 for the low versus high level, respectively. By doing so, resulting coefficients of the coded model can then be compared with one another with respect to the model's intercept which is the representative center of experimental design [40, 41]. The coded model was found to be;

$$Y = 1.51 + (6.78x10^{-1}t + 9.54x10^{-1}m + 3.95x10^{-1}a) + 6.06x10^{-1}t^2 - 3.67x10^{-1}tm \quad (3.6)$$

with the coded coefficients plotted in Figure 3.7. Results showed that all the significant effects except for the interaction between temperature and methanol to sludge ratio, have positive influence on biodiesel yield. Moreover, the methanol to sludge ratio or methanol loading has the highest influence on the response.

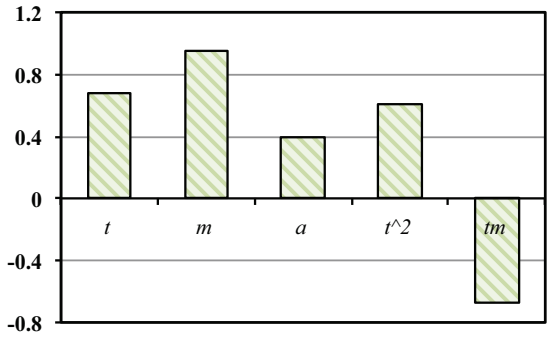


Figure 3.7 Coefficients for the coded model: *t*, temperature; *m*, methanol to sludge ratio; *a*, sulfuric acid concentration.

To better understand the effect of each of the factors on biodiesel yield, main effect plots were generated as shown in Figure 3.8. The positive linear influence of temperature indicates that as the temperature was increased, biodiesel yield increased. Moreover, the positive quadratic effect of temperature indicated that higher temperatures will result to a greater increase in biodiesel yield. It can be seen from Figure 3.8 that the temperature where the biodiesel yield started to increase drastically is around 60°C. This behavior is the exact opposite of what was observed on the effect of temperature on the in situ transesterification of freeze-dried sludge. This might be due to the presence of high concentration of water in partially dewatered sludge. A study conducted by Isbell et al. (1994) on the acid-catalyzed condensation of oleic acid indicated that addition of water impedes the formation of estolides and polyestolides [47]. This explains the differences in the effects of the factors investigated on the yield of biodiesel from freeze-dried and partially dewatered sludges.

The positive linear influence for both methanol to sludge ratio, *m*, and acid concentration, *a*, indicated that biodiesel yield increases as the level of both factors increases. But since the coded coefficient of methanol to sludge ratio is higher than that of acid concentration (Figure 3.7), it is expected that the increase in biodiesel yield would

be greater for methanol to sludge ratio than for acid concentration. This is also evident on the main effect plots showing steeper slope for methanol to sludge ratio than for acid concentration (Figure 3.8).

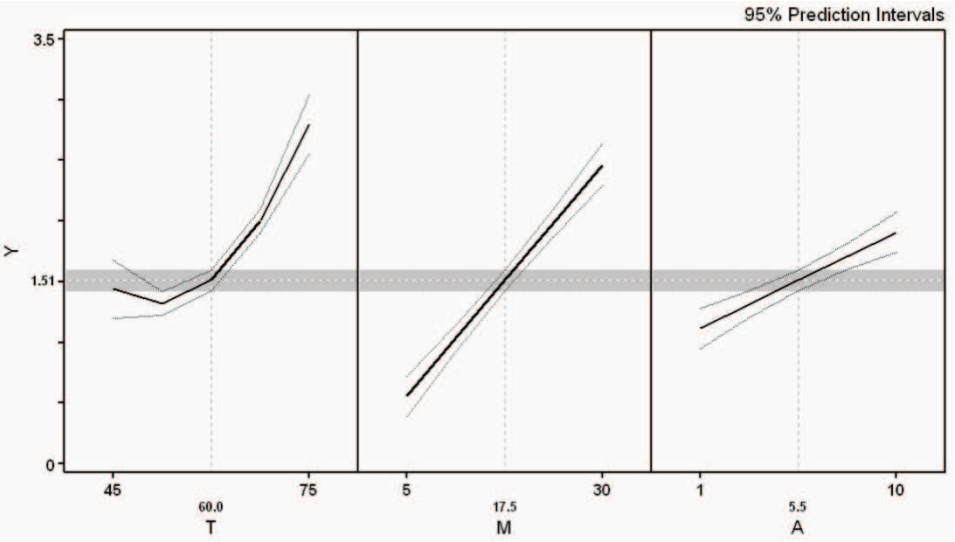


Figure 3.8 Main effect plots of the factors investigated (T = temperature; M = methanol to sludge ratio; A = acid concentration), showing how the biodiesel yield, Y, changes with the change in the level of the factors relative to the center of design (β_0 = 1.51).

3.3.3 Optimization

Based on the obtained model for the yield of biodiesel, surface plots were generated. Since three factors were investigated, surface plots were generated at fixed values of one of the factors.

3.3.3.1 Freeze-dried Sludge

The effect of methanol to sludge ratio and sulfuric acid concentration can be clearly seen on Figure 3.9 confirming the analyses in the previous section (section 3.3.2.1). It can be concluded by comparing the y-axis of the plots that the highest percent biodiesel yield was obtained at 55°C. However, these plots do not provide exact values of

methanol to sludge ratio and sulfuric acid concentration that will give maximum biodiesel yield. Thus, numerical optimizations using discrete and continuous values of the factors used in the design were conducted.

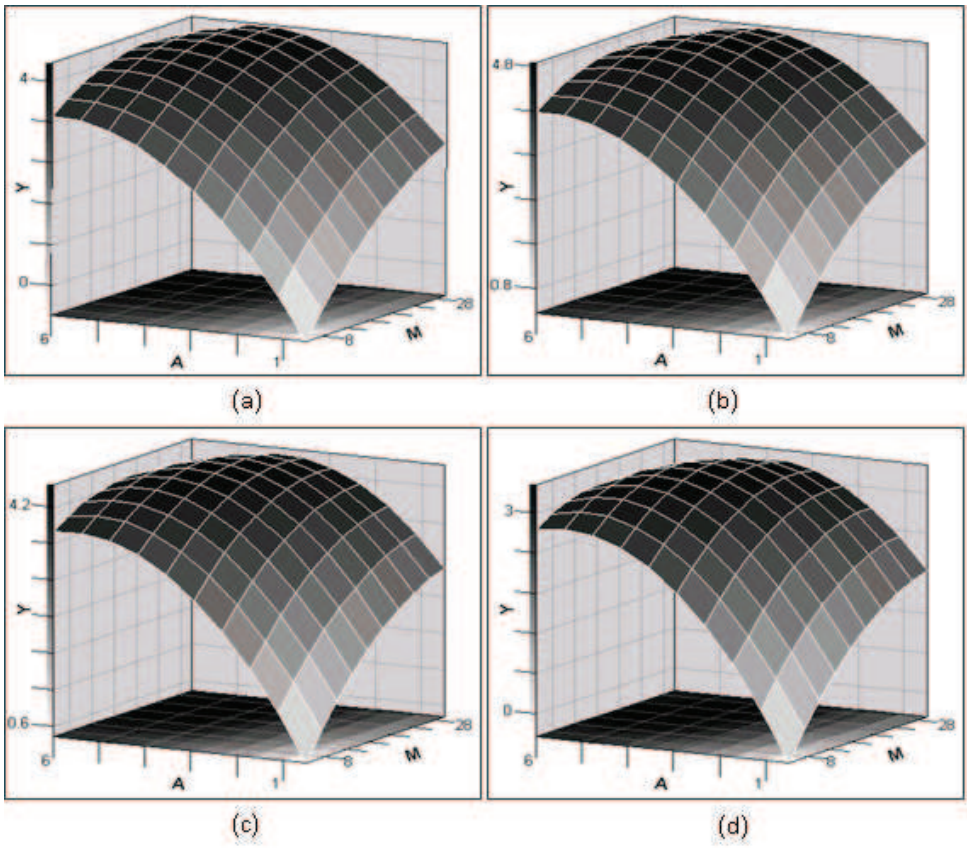


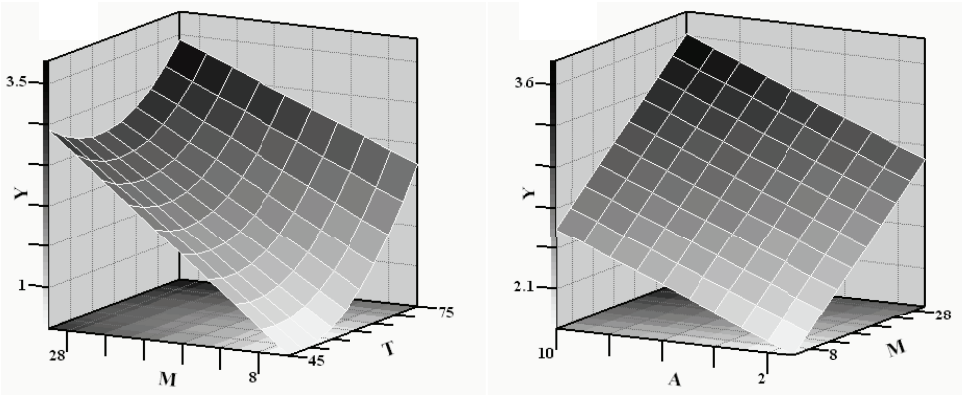
Figure 3.9 Predicted percent biodiesel yield (Y) at different temperatures as a function of methanol to sludge ratio (M) and sulfuric acid concentration (A): a) 45°C b) 55°C c) 65°C d) 75°C.

Optimization using discrete values of factors showed that an optimum biodiesel yield of 4.88% can be obtained at 55°C with methanol to sludge ratio of 25 mL/g and sulfuric acid concentration of 4% H_2SO_4 volume /methanol volume. These values are roughly the same as those obtained by optimization using continuous values; a yield of 4.89% at 56.2°C, 23.4 mL methanol/g sludge and 4% H_2SO_4 volume/methanol volume.

This indicated that the experimental design captured the necessary points for optimization. The experimental yield of biodiesel at 55°C, 25 methanol to sludge ratio, and 4% sulfuric acid was 4.79 ± 0.02% which was the maximum obtained for all the treatment tested. At this optimum condition, the quadratic response surface model gave a maximum percent error of 2.30% indicating satisfactory agreement between the model and the experimental data.

3.3.3.2 Partially Dewatered Sludge

The analyses done on the in situ transesterification of partially dewatered activated sludge (section 3.3.2.2) implied that no optimum condition could be attained for the process. To see this more directly, surface plots were generated and are presented in Figure 3.10. As can be verified from the figure, there is no optimum condition present within the experimental design. However, results indicated that there is a process condition that will give maximum yield of biodiesel. It is apparent from Figures 3.9 and 3.11 that this condition is at the high level of all the factors studied; specifically, at temperature of 75°C, methanol to sludge ratio of 30 mL/g and sulfuric acid concentration of 10% H_2SO_4 volume/methanol volume. This specific combination of the factors was not included in the design. Thus, to verify these findings and to further asses the reliability of the model obtained, the in situ transesterification experiment of partially dewatered sludge was conducted at this condition. A biodiesel yield of 3.93 ± 0.15% (weight) was obtained at this condition.



(a) (b)

Figure 3.10 Predicted percent biodiesel yield, Y: (a) as a function of methanol to sludge ratio, M and temperature, T at fixed level of acid concentration, A = 10% volume; (b) as a function of acid concentration, A and methanol to sludge ratio, M at fixed level of temperature, T = 75°C.

Numerical optimization was conducted to determine the predicted yield of the model at the condition that gave maximum biodiesel yield. Since this condition was already established, optimization was conducted using discrete values of factors used in the experimental design. Result showed that a maximum biodiesel yield of 3.78% (weight) can be obtained at this condition, giving a model error of at most 7.35%. This indicates high reliability of the model obtained.

3.3.4 FAME Analysis

A sample chromatogram of the FAMEs analysis of biodiesel produced by in situ transesterification of activated sludge is shown in Figure 3.11. Calculations of percent biodiesel yields for all treatments were based on FAME analysis, which was used to infer the biodiesel composition obtained from activated sludge. A gravimetric yield as high as 13.30% and 15.00% (weight/sludge dry weight) were obtained for freeze-dried and partially dewatered sludges, respectively, using an in situ transesterification process. Aside from fatty acids, glycerides and phospholipids, bacterial lipids may also contain

wax esters, steroids, terpenoids, polyhydroxyalkanoates and hydrocarbons [48, 49]. Activated sludge is also known to contain linear alkyl benzenes and polycyclic aromatic hydrocarbons [50]. These compounds might also have been extracted during the in situ transesterification of activated sludge and contributed to the high gravimetric yields.

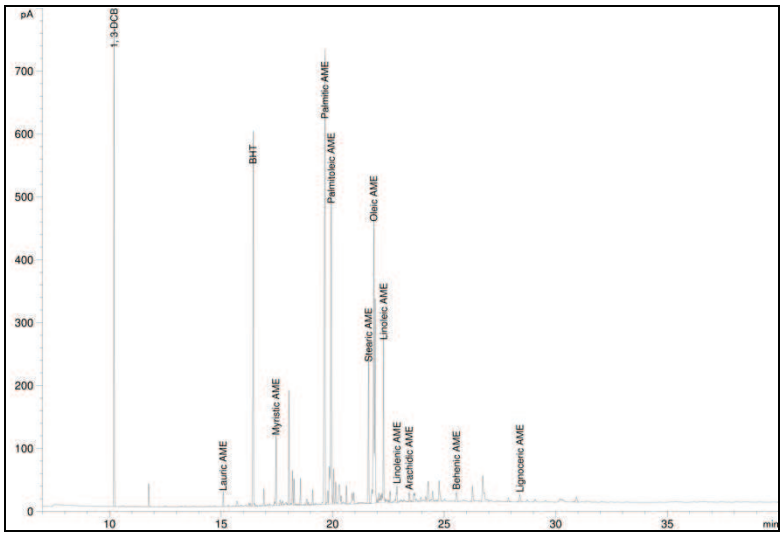


Figure 3.11 Sample chromatogram from the GC-FID analysis of FAMEs from activated sludge through in situ transesterification.

Due to the limited number of standards, some components were not identified and were presented as total unknowns in Figure 3.12. Results indicated that methyl esters of palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2) are the major components of the biodiesel from activated sludge. A target biodiesel with improved properties (i.e., cold flow, cetane number and oxidative stability) mainly contains oleic acid (71.3%) and linoleic acid (21.4%) [51]. However, according to Knothe (2008), palmitoleic acid methyl ester is more suitable in low temperature applications than oleic acid methyl ester because of its advantages in terms of kinematic viscosity. This is primarily due to low melting point of methyl

palmitoleate (-33.9°C), which is 14°C below that of methyl oleate [6]. This indicates that activated sludge is a suitable biodiesel feedstock. The obtained fatty acid profile is in agreement with the results obtained by Mondala et al. (2009) and Dufreche et al (2007) on the composition of biodiesel produced by in situ transesterification of activated sludge from the same MWWTP as this study [14, 32].

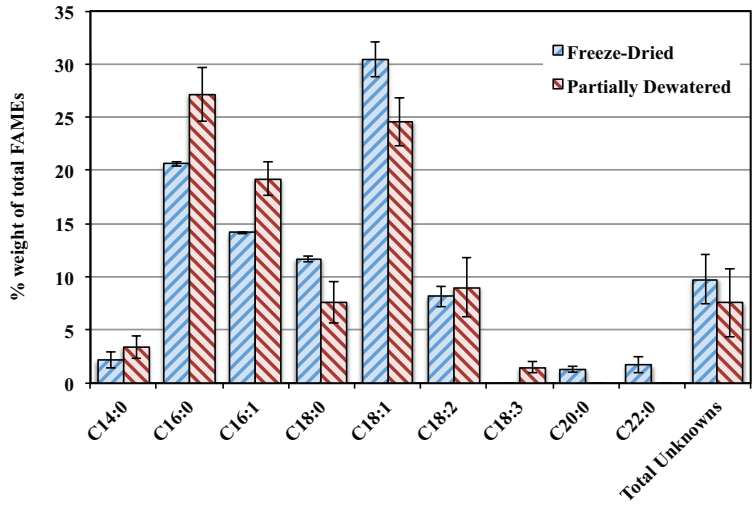


Figure 3.12 Fatty acid profile of biodiesel from in situ transesterification of activated sludge.

3.3.5 Economic Analysis

Dufreche et al. (2007) estimated the cost of biodiesel from in situ transesterification of activated sludge at $3.11 per gallon. Their calculations were based on an assumed biodiesel yield of 7% (weight) [32]. In a related study, Mondala and co-workers (2009) estimated that at a yield of 10% (weight), the break-even price of biodiesel from in situ transesterification of sludge (primary and activated) was around $3.23 per gallon. Furthermore, they estimated the annual production costs to be $992,327.00 for a biodiesel plant with an annual production of 3.05 x 10^5 gal [14]. The optimization of in situ transesterification of freeze-dried sludge indicated that an

optimum yield of 4.79 ± 0.02% (dry sludge weight) could be obtained at reaction temperature of 55°C, methanol to sludge ratio of 25 (mL/g) and sulfuric acid concentration of 4% (volume). The optimum biodiesel yield obtained was far below the value that Dufreche et al. (2007) and Mondala et al. (2009) had assumed. This could result in a dramatic increase of the break-even price of biodiesel from in situ transesterification of activated sludge. Break-even price is the price for which the revenue is the same as total manufacturing cost of a plant [52]. Based on the calculations conducted by Mondala et al. (2009), the economics of biodiesel production from in situ transesterification of dried activated sludge was re-calculated to reflect the optimum yield and condition obtained in this study. The results showed that at an annual production capacity of 1.47×10^5 gallons biodiesel, the break-even cost of biodiesel is about $7.42 per gallon (Table 3.2). This cost is more than twice as much as the cost obtained by Mondala et al. (2009) due to lower biodiesel yield (10% versus 4.79% weight) and higher methanol loading (15 versus 25 mL per gram dry sludge weight).

The study conducted by Dufreche et al (2007) on the economics of in situ transesterification of dried activated sludge showed that the drying step (from 98% to 5% weight moisture) could add up to 55% of the biodiesel cost [32]. Thus, the in situ transesterification of activated sludge with high level of water was optimized. Assuming that the optimum biodiesel yield obtained from the freeze-dried sludge is the highest biodiesel yield obtainable; the maximum yield obtained from partially dewatered sludge was ~17.95% lower.

Using the results of the optimization of the in situ transesterification of activated sludge, the economics of the two processes were estimated. Results of the analysis showed that the in situ transesterification of dried sludge (5% moisture) is still more

economical than that of the partially dewatered sludge (84.5% moisture). Although the in situ transesterification of wet sludge eliminates the drying cost, the high methanol and catalyst requirements and large equipment sizes for the process resulted in higher break-even price of biodiesel (Table 3.2).

Table 3.2 Cost estimates for in situ transesterification dried and partially dewatered activated sludges.

		Dried Sludge [a] (5% weight moisture)	**Partially Dewatered Sludge**[b] (84.5% weight moisture)
Annual Biodiesel Production, gal		1.47×10^5	1.21×10^5
A. Feedstock Preparation			
1. Centrifugation	\$0.43/gal[c]	\$63,232.79	\$51,879.93
2. Drying	\$1.29/gal[c]	\$189,698.37	\$0.00
B. Methanol	\$0.08/gal[d]	\$169,254.24	\$203,105.09
C. Catalyst	\$0.15/gal[d]	\$77,721.60	\$233,164.80
D. Equipment Cost		\$276,728.92[d]	\$1,660,373.5
Total annual production cost[d]		\$1,091,546.19	\$1,747,470.13
Biodiesel Price (break-even), gal^{-1}		\$7.42	\$14.48

[a] methanol requirement = 25 mL per gram dried sludge, catalyst requirement = 4% (H_2SO_4 volume/methanol volume).
[b] methanol requirement = 30 mL per gram dried sludge, catalyst requirement = 10% (H_2SO_4 volume/methanol volume).
[c] Dufreche et al. (2007) [32].
[d] Mondala et al. (2009) [14].

3.3.6 Sensitivity Analyses

Once some factors (e.g. yield, process technology, operating parameters, raw material costs and plant capacity) are identified, the economic performance of a plant such as fixed capital cost, total manufacturing cost and break-even price can be estimated. This was done in the previous section. The economic viability of a plant is

affected by the variability of these factors and thus, it is important to measure the relative magnitude of their effects.

The results of the economic analyses indicated that the removal of ~14% of the water initially present in the activated sludge is not enough to reduce the cost of biodiesel. Thus, a sensitivity analysis was conducted to determine how the break-even biodiesel price changes with the moisture content of this feedstock. For this analysis a linear relationship among biodiesel yield, moisture content, and methanol and catalyst requirements was assumed. As shown in Figure 3.13, a lowest biodiesel break-even price of ~$7.00 per gallon can be obtained at 50% moisture content. However, this price is still not economically competitive at current petroleum-based diesel (around $2.95 per gallon) [53]. Thus, another sensitivity analysis was conducted to determine how the break-even price changes with the biodiesel yield. Based on this analysis, it was estimated that a biodiesel yield of more than 10% (weight), which corresponds to at least 3 x 10^5 gal/year biodiesel production capacity (Figure 3.14), would make the fuel from activated sludge cheaper compared to petroleum-based diesel.

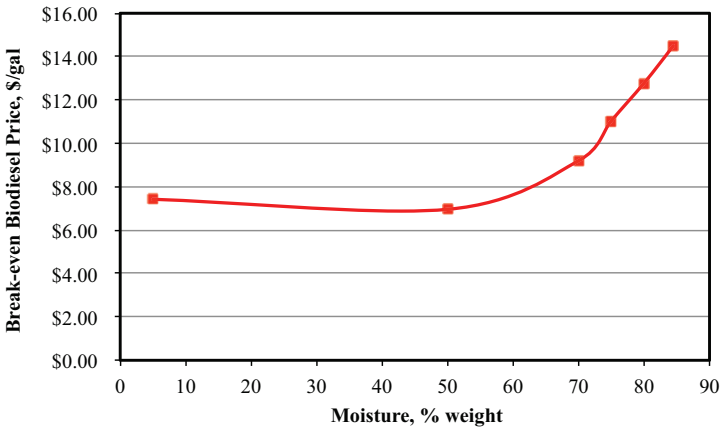


Figure 3.13 The impact of moisture content of activated sludge on the break-even price of biodiesel.

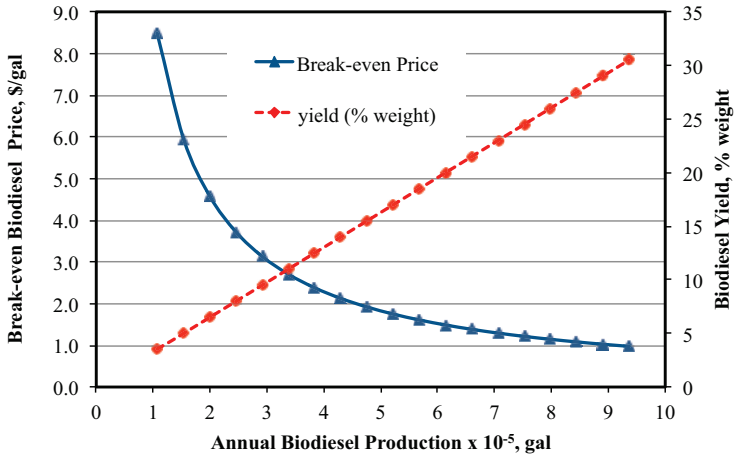


Figure 3.14 The influence of yield on the break-even price of biodiesel from activated sludge.

3.3.7 Possible Strategies to Improve the Economics of Biofuel from Activated Sludge

The in situ transesterification of wet activated sludge was conducted for 24 hours. This was based on the study conducted by Mondala et al. (2009) on the kinetics of in situ transesterification of activated sludge. It is well established that in biodiesel production, acid-catalysis is slower than base-catalysis [16]. Additionally, the long reaction time might be due to mass transfer resistance of methanol and oil during the in situ transesterification process [54]. Increasing the agitation speed might shorten the reaction time by minimizing the mass transfer limitations. This has been proven true even for transesterification of pre-extracted oils [55]. Agitation speed could be another potential cost saving strategy for the in situ transesterification of partially dewatered activated sludge and might reduce the break-even price of biodiesel from this process.

Yield of fuel that can be obtained from activated sludge can be increase significantly by conversion of other compounds that might have also been extracted during the in situ transesterification reaction into fuel. The extract may contain other compounds such as sterols, fatty alcohols, alkyl benzenes, hydrocarbons, polycyclic

aromatic hydrocarbons, etc. resulting to a gravimetric extract yield of as high as 13 – 15% (sludge dry weight) [50]. Analysis of relative concentrations of these compounds in the activated sludge is necessary in order to determine suitable processes for fuel conversion (e.g. hydrocracking, hydrotreating) [56, 57]. The extraction, identification and quantitation of these compounds are the subjects of the next two chapters.

Among the compounds present in raw activated sludge, only the saponifiable lipids were converted to biodiesel during the in situ transesterification reaction. Thus, increasing the amount of saponifiable lipids will increase biodiesel yield from this feedstock. This can be accomplished by subjecting activated sludge microorganisms to a biochemical stimulus (i.e. high C:N ratio). This strategy is the subject of Chapter VI.

3.3.8 Biodiesel Quality

Biodiesel properties (i.e. low-temperature operability, oxidative and storage stability, viscosity, cetane number, exhaust emissions, and energy content) are highly dictated by the presence of contaminants and other minor components [58]. In addition to the components mentioned above, the biodiesel from activated sludge may also contain contaminants such as metals, free fatty acids, triacylglycerides, diacylglycerides, monoacylglycerides, methanol, sulfuric acid (catalyst), and water. These compounds need to be minimized or removed from the activated sludge biodiesel for it to meet the ASTM D6751 or EN 14214 specifications. It was expected that additional processes might be necessary for the biodiesel from wet activated sludge to pass the ASTM or EN specifications. Therefore, the processing cost allotted in the economic analysis was at least twice the processing cost of biodiesel from soybean oil, which is approximately $0.30 per gallon [32, 59].

3.4 Conclusions

The production of biodiesel from dried and partially dewatered activated sludge using in situ transesterification was optimized. The quadratic response surface model is adequate enough to describe both processes at 0.05 significance level. For the in situ transesterification of dried activated sludge, statistical analyses showed that within the experimental design there is a value of temperature, which will give a maximum biodiesel yield. This was possibly caused by acid-catalyzed polymerization of unsaturated fatty acids or their esters, which significantly affected the biodiesel yield above 60°C. As for the methanol to sludge ratio and sulfuric acid concentration, the coded regression coefficients indicated direct relationship with biodiesel yield. However, the relationships weaken at high levels of these factors. The two-way interactions of the three factors investigated showed that combination of the low and high levels of the factors would have a positive impact on biodiesel yield. Numerical optimization showed that an optimum yield of 4.89% can be obtained at 56.2°C, 23.4 mL methanol per g sludge ratio and 4% (volume) sulfuric acid. This optimum value is roughly similar to that obtained by discrete numerical optimization, which was 4.88% at 55°C, 25 mL methanol per g sludge ratio and 4% (volume) sulfuric acid. At this optimum condition, a maximum percent error of 2.30% was obtained indicating satisfactory agreement between the model and the experimental data.

For the in situ transesterification of partially dewatered activated sludge, the statistical analyses showed that within the experimental design there exists a condition where the yield of biodiesel is highest. This condition was at temperature of 75°C, methanol to sludge ratio of 30 mL/g and catalyst concentration of 10% (volume). The

model predicted a biodiesel yield of 3.78% (weight) at this condition. Experimental verification gave a yield of 3.93 ± 0.15% (weight) giving a model error of 7.35%.

For both processes, FAME analysis of the biodiesel produced showed significant amount of methyl esters of palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2). These results are in agreement with the results obtained by previous researchers who worked on activated sludge obtained from the same MWWTP as this study [14, 32].

Results of the economic analyses indicated that in situ transesterification of dry activated sludge is more economical than that of partially dewatered activated sludge ($7.42 versus $14.42 per gallon). This was primarily due to high methanol and catalyst requirements, and high equipment costs associated with the latter.

The sensitivity analysis indicated that a moisture content of around 50% (weight) and a biodiesel yield of greater than 10% (weight) will make the biodiesel obtained from in situ transesterification of activated sludge less expensive than petroleum-based diesel. A fuel yield of greater than 10% (weight) might be attainable by identifying other compounds present in the extract that can be converted into fuel.

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CHAPTER IV

SOLVENT EXTRACTION OF ORGANIC COMPOUNDS FROM ACTIVATED SLUDGE: A COMPARATIVE STUDY

4.1 Introduction

Trade in oil and oilseeds depends on the ability of the purchaser to determine the yield and consequently the price of value-added products [1]. Thus, extraction of solid and semisolid samples using liquid solvents is a very routine practice in many laboratories. Solubility of the target compound is the main criterion in choosing suitable extraction solvent. For example, solvents for extraction of lipids from source material depend heavily on the type of lipid present and the proportion of nonpolar (principally triacyglycerides) and polar (i.e. phospholipids and glycolipids) components [2]. The determination of oil content of solid samples using nonpolar solvents such as petroleum ether can be accomplished either directly or indirectly as shown in Figure 4.1 [3]. Prior to extraction, samples are usually subjected to various pre-treatment such as drying, size reduction and if necessary acid hydrolysis [2].

Solvent types and pre-treatment methods are chosen based on their ability to break the analyte-matrix binding which could be Van der Waals attractions, hydrophobic and electrostatic interactions, and hydrogen and covalent bonding [4]. For example, extraction of lipids from starchy materials such as wheat, rice, corn, fababean, lentil, potato and cassava include acid hydrolysis followed by selective solvent extraction with 2:1 (volume ratio) of chloroform:methanol at ambient temperature and then by 3:1

(volume ratio) of *n*-propanol:water at 90 – 100°C [5]. In lipid extraction from milk, it is a common practice to add sodium hydroxide prior to extraction to dissolve casein and eventually release the lipids from its surrounding matrix [2]. Another extraction technique that involves hydrolysis prior to extraction is the Roese-Gottlieb extraction procedure. In this method, samples are pre-treated with boiling water and then with 25% (weight/volume) ammonia solution followed by repeated extraction with ethanol, diethyl ether and hexane/petroleum ether [2, 6]. In some cases, treatment such as acid hydrolysis is conducted after a prior extraction procedure (i.e. Soxhlet extraction) [7].

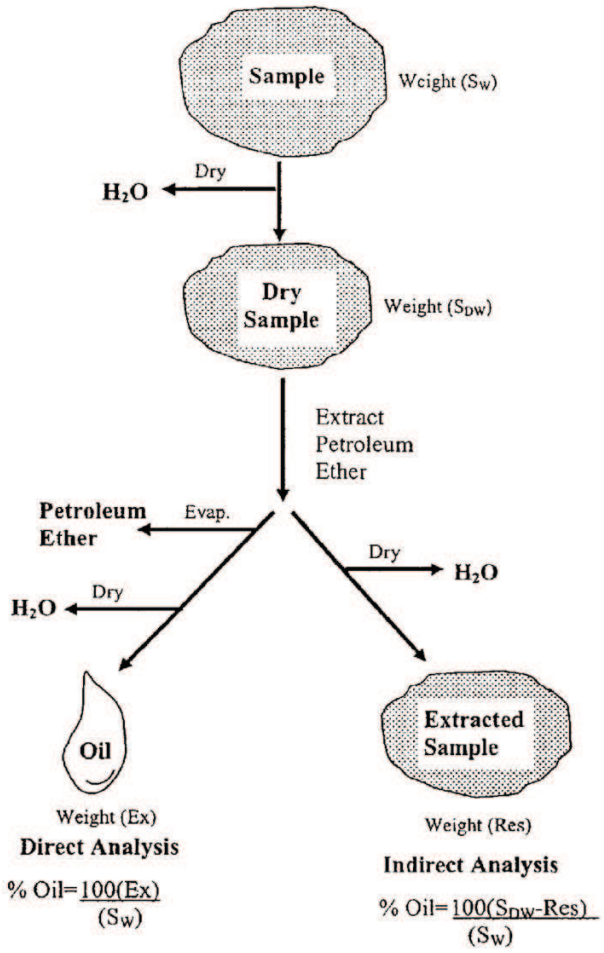

Figure 4.1 A diagrammatic representation of the analysis of fat/oil by solvent extraction [3].

4.1.1 Soxhlet Extraction

The most classical and exhaustive solvent extraction technique, which is still very widely used, is the Soxhlet extraction method. This method, which was invented by a Franz Von Soxhlet in 1879, involves placing a sample-loaded thimble over a boiling solvent. Condensed solvent would be in contact with the sample, solubilizing extractable materials and would be siphoned back into the boiling solvent (Figure 4.2) [8, 9]. This cycle is repeated many times (usually for a period of 6 – 48 hours). To recover extractable materials, the solvent is evaporated off leaving the residue for further analysis.

The main disadvantage of the Soxhlet extraction is the long extraction time. Thus, in early 1970s, Edward Randall modified the Soxhlet extraction to cut the extraction time to as short as 30 minutes (Figure 4.2). In this method, which is also known as the Randall method, the sample to be extracted is totally immersed in the boiling solvent. The principle behind this method is that the solubility of most materials increases with temperature. Complete immersion of the sample into the boiling solvent decreases the extraction time. In Soxhlet extraction, the solvent that comes in contact with the sample passed through a condenser, and thus the extraction temperature is lower than that of Randall method [8].

Another modification of the Soxhlet extraction involved pre-treatment of samples by boiling in 3M HCl followed by filtration and drying. The Soxhlet extraction is then applied to the dried sample using petrol ether as solvent. This method, which is called the Stoldt fat method, is well known for extraction of fats in foods and feeds [10, 11].

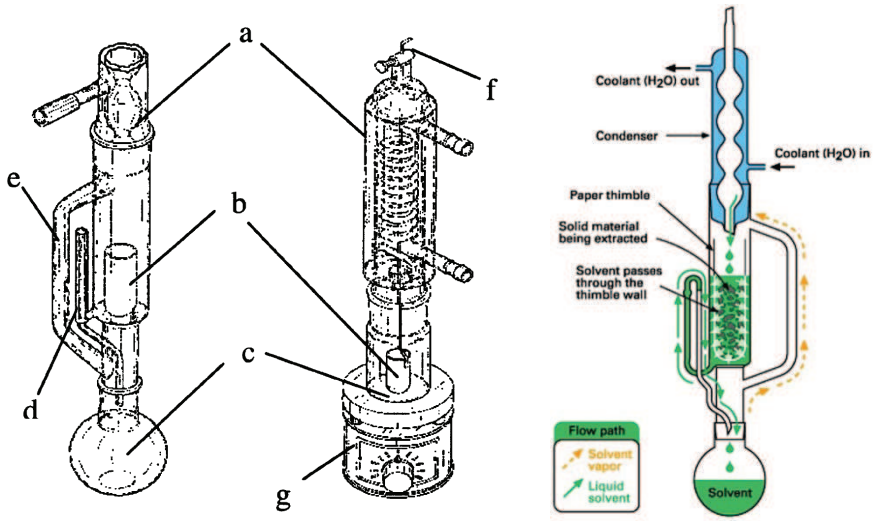

Figure 4.2 Original Soxhlet (left) and Randall extraction apparatus (middle). Solvent flow paths during Soxhlet extraction (right).*

*(a) condenser (b) sample thimble (c) solvent flask (d) siphon tube (e) solvent vapor tube (f) thimble positioning mechanism – slide rod (g) heater (not shown on the Soxhlet) [8, 12].

Most automated solvent extraction systems such as the Soxtec™ Avanti 2050 extraction system shown in Figure 4.3 are based on the Randall method [8]. However, this method is limited by the boiling point of the extraction solvent. Extraction efficiency increases with temperature due to decrease in viscosity, allowing enhanced solvent penetration and analyte diffusion. But solvent loss is high at temperatures higher than the boiling point of the solvent because this system operates at atmospheric pressure. Another disadvantage of most systems is the utilization of large volumes of extraction solvent, which increases purchase and disposal costs along with health and environmental concerns. In 1995, Dionex Corporation (Salt Lake City, UT) introduced the accelerated solvent extraction (ASE®) in response to these concerns [9].

Figure 4.3 The Soxtec™ Avanti 2050 automated extraction system [8].

4.1.2 Accelerated Solvent Extraction (ASE®)

Accelerated solvent extraction is also referred to as pressurized fluid extraction (PFE) and pressurized liquid extraction (PLE). It is a liquid extraction technique, which utilizes organic and/or aqueous solvent at higher temperature (100 – 180ºC) and pressure (1500 – 2000 psi). Higher temperatures can disrupt the strong solute-matrix interactions caused by van der Waals forces, hydrogen bonding, and dipole attractions of the solute molecules and active sites on the matrix. Thermal energy can overcome cohesive (solute-solute) and adhesive (solute-matrix) interactions thereby facilitating the desorption process. In addition, hydrogen bonding is weakened with increased temperature [13]. By operating at high pressure, the solvent(s) can be maintained in liquid state and thus eliminates temperature limitation (boiling point of the extraction solvent). ASE® can accomplish extraction in as short as 12 minutes using as low as 1.2 mL extraction solvent for every gram of sample [9]. Due to these advantages, ASE® has been approved as EPA

standard method for extraction of organics from a variety of biological samples including municipal sewage sludge, beginning in 1996 [14].

A general schematic of an ASE® system is shown in Figure 4.4. Extractions are accomplished by a combination of static and dynamic flow of the solvent through a heated extraction cell loaded with the sample. The sample cell is initially filled with solvent. Once filled, it is then heated by direct contact with a heat source. Pressure is applied to maintain the solvent in liquid state and to be able to move the solvent through the sample cell in a reasonable amount of time. The sample cell is usually maintained at the set extraction temperature for a period of 5 – 10 minutes. This period is called the static phase where analyte diffusion is believed to take place. After the static hold-up, fresh solvent is allowed to flush over the sample pushing the previous solvent volume out of the sample cell into collection vial. The last step is by using nitrogen gas to collect the remaining solvent from the sample cell and lines [9].

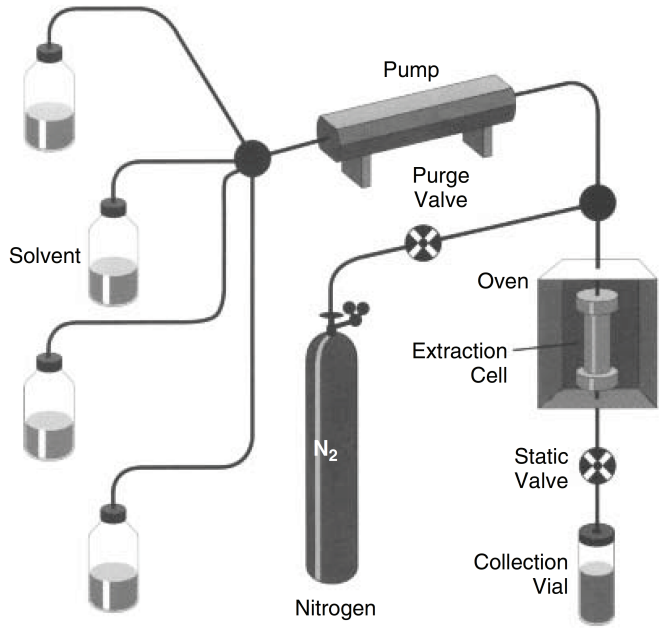

Figure 4.4 Schematic diagram of an ASE® system [14].

ASE® can extract various analytes including PAHs, PCBs, hydrocarbons, chlorobenzenes, phenols, fatty acids and lipids from different matrices such as soil, chicken meat, medicinal tablets and plants [14, 15]. Pinto and Lanças (2009) compared pressurized solvent extraction (PSE) and Soxhlet extraction of soybean oil using pentane as extraction solvent. They obtained a yield of 21.25 ± 0.36% (weight) and 21.55 ± 0.65% (weight) for PSE and Soxhlet extraction, respectively. They concluded that PSE was more efficient and faster extraction technique with reduced solvent consumption. Furthermore, extraction in an inert atmosphere (nitrogen) prevents sample and analyte oxidative decomposition [16]. This technique have also been used for the determination of lipid biomarkers from vegetative and/or sporulated biomasses of *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium fortuitum*, *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Aspergillus niger* as well as from environmental samples collected from water, soil and air. Results indicated that ASE® is a rapid and efficient technique that can speed-up data collection for microbial community analysis [17].

On the extraction of lipids from activated sludge, Dufreche et al. (2007) tested the effectiveness of different extraction solvents including hexane, methanol and a mixture of hexane/methanol/acetone (60/20/20 by volume). The ASE® was conducted at 100ºC and 10.3 MPa for an hour. Among these solvents, the highest yield of extractable materials was obtained using the hexane/methanol/acetone mixture with a value of 27.43 ± 0.98% (weight) [18].

ASE® evolved into supercritical fluid extraction (SFE) as a consequence of the need for higher working temperature. In many cases, however, extraction is much faster and efficient with liquid solvents at elevated temperature and pressure than with SFE [14]. In addition to SFE, other common techniques for extraction of semi-volatile

compounds from solid matrices include ultrasonic extraction [14] and microwave-assisted extraction (MAE) [19, 20].

4.1.3 Bligh & Dyer Extraction (BDE)

Extraction at elevated temperature is not applicable for heat sensitive analytes and for analyses that require negligible transformation (degradation) of the analyte(s). Thus, ASE® and SFE cannot be used for such cases. Microwave can also cause degradation and chemical reaction during extraction while ultrasonic extraction is not as efficient as the others [14]. Most of these extraction techniques also require sample drying prior to extraction, which might also alter the nature of the analyte(s). It is for these reasons that for extractions involving biological materials, the labor-intensive Bligh & Dyer extraction technique is still widely used.

BDE was originally developed for the extraction of lipids from fish tissues [21]. However, because of its effectiveness it is also being used for extraction of lipids from a wide range of matrices including soil, cattle manure, pig slurry and microbial biomass [20, 22-24]. BDE utilizes a ternary solvent mixture of chloroform, methanol and water as extraction solvent. Since water is one of the extraction solvents, sample drying prior to extraction is not required for this technique. The initial step for this extraction technique is to use the solvent system in 1:2:0.8 (chloroform:methanol:water) volume ratio with a 3:1 initial solvent to sample ratio. This step puts the extraction system in the monophasic region (point C) of the phase diagram shown in Figure 4.5. This step is necessary to break the association between lipids and cell membranes and lipoproteins. After homogenization, chloroform and water are added to obtain a biphasic mixture containing 2:2:1.8 (chloroform:methanol:water) volume ratio (point C' in Figure 4.5). This step

leads to extraction of lipids, which can be recovered from the organic (chloroform) layer [21, 25, 26]. According to Bligh and Dyer (1959), optimum lipid yield can be obtained around points C, D and E in Figure 4.5. However, the region around point C is more economical in terms of solvent consumption. Furthermore, the final biphasic system should fall on or below the maximum chloroform tie-line shown in Figure 4.5. This is to make sure that the organic layer contains 100% chloroform as solvent and thus, preventing recovery of non-lipid materials [21].

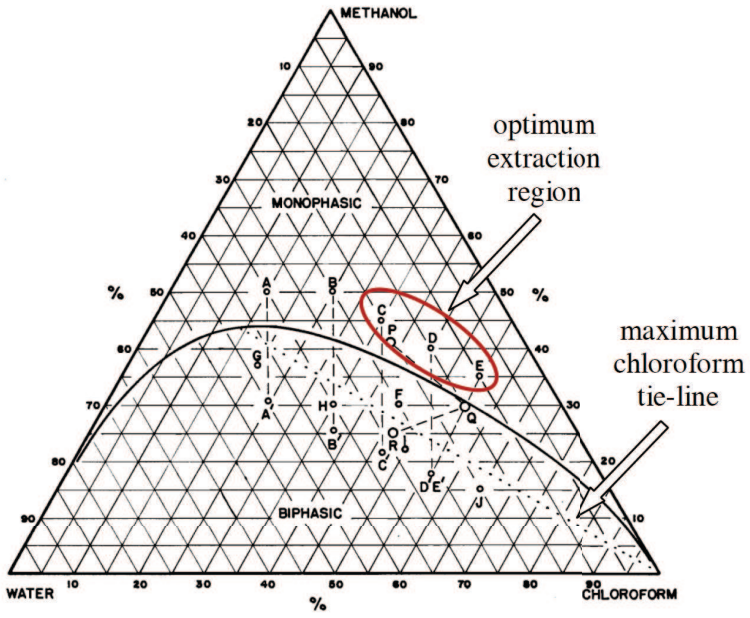

Figure 4.5 Chloroform-methanol-water phase diagram, % weight at 20ºC showing the points considered in the method development and maximum chloroform tie-line [21].

The BDE has been modified in various ways to tailor extraction of lipids from various matrices. One such modification is the HCl-BDE method, which was applied for the extraction of fats from pig feces rich in calcium soaps and herring/mackerel scrap rich in polyunsaturated fatty acids. This method was based on the Stoldt fat method and thus

includes acid pre-treatment (1 mL of 3M HCl) of the samples (0.6 g) followed by heating at 80ºC for an hour. Samples were then subjected to BDE procedure [11]. The hazardous nature of chloroform and toxicity of methanol had also led to modification of the BDE procedure. This concern was first addressed by Hara and Radin in 1978, where they replaced chloroform with hexane and methanol with isopropanol. Results of their study indicated several advantages over the original BDE protocol. These advantages include, in addition to less solvent toxicity, easier phase separation and cheaper solvents. However, this solvent combination is inefficient for extraction of gangliosides [4, 27]. In a similar study, Smedes (1999) used a mixture of isopropanol and cyclohexane for the extraction of lipid from plaice, mussel and herring samples. They concluded that extraction efficiency is similar to the original BDE protocol for a solvent volume ratio of 8:10:11 isopropanol:cyclohexane:water [28]. This procedure was successfully applied by Manirakiza et al. (2001) for extraction of lipid from food samples including margarine, eggs, milks, chicken feed and fish flour [6]. The suitability of using methyl-*tert*-butyl ether (MTBE) as chloroform replacement in the original BDE has also been studied. Matyash et al. (2008) applied MTBE for extraction of lipids from *Escherichia coli*, *Caenorhabditis elegans*, mouse brain and human blood plasma. They demonstrated that the MTBE protocol delivers similar or better recoveries of species of most all major lipid classes compared with the BDE and its forerunner Folch method [29].

This chapter evaluated the suitability of ASE® and BDE for the extraction of lipids and other compounds from activated sludge. The evaluation was mainly based on gravimetric and FAMEs yields. Results of this evaluation were used in the succeeding two chapters.

4.2 Materials and Methods

4.2.1 Sample Collection and Preparation

All activated sludge samples used in this study were collected from a MWWTP in Tuscaloosa, AL, U.S.A. Samples were collected from the return activated sludge line into 4-gallon plastic buckets and were transported in ice chests to the Renewable Fuels and Chemicals Laboratory at Dave C. Swalm School of Chemical Engineering, Mississippi State University. The solids were concentrated by gravity-settling overnight, followed by either centrifugation at 3000 rpm for 20 minutes or vacuum filtration using a P8-creped cellulose fiber filter (Fisher Scientific, Pittsburg, U.S.A.). A portion of the concentrated sludge was frozen at -18°C and was freeze-dried for 5 days. The centrifugation/filtration of the sludge gave a concentrated sludge containing 8-16% weight solids. Freeze-drying of the concentrated sludge resulted in sludge with an average solids content of 95.74% weight.

4.2.2 BDE Experiments

The lipids and other compounds present in the partially dewatered sludge (8-16% weight solid) and the freeze-dried sludge were extracted using the Bligh & Dyer extraction procedure [21]. Initial experiments were conducted to optimize some extraction parameters, particularly the solids to solvent ratio and number of extraction stages. The appropriate values for these parameters were then used for the extraction of partially dewatered and freeze-dried sludges. All extractions were conducted at ambient temperature.

Preliminary experiments were conducted to determine the suitable solids to solvent ratio and number of extraction stages for BDE. For these experiments, the

partially dewatered sludge was used. This was also done to determine if the water content (84-92% weight) of the partially dewatered sludge is enough for the initial steps of the BDE. Samples with solid content ranges from 0 – 16% (with corresponding weight of solids from 0 – 0.57 grams) were prepared (Table 4.1). Samples were then extracted with the same volume of solvent as discussed below.

4.2.2.1 Single Extraction

To each of the samples, 7.5 mL of methanol and 3.75 mL of chloroform were added. Samples were then vortex-mixed for 30 minutes for homogenization. Then, 3.75 mL of water and 3.75 mL of chloroform were added and the mixtures were again vortex-mixed for 2 minutes. Phases were separated by centrifugation at 3000 rpm for 10 minutes after which, the organic (chloroform) layer was recovered by using Pasteur pipettes passing through another Pasteur pipette packed with glass wool. This was done to remove traces of cell debris from the extract. The extractable materials were recovered by removal of the solvent at 45°C under 15 psi stream of N_2 using a TurboVap LV.

4.2.2.2 Double Extraction

Another set of samples (as in the previous section) was subjected to double extraction. After the removal of the organic layer, 9.50 mL of methanol and 4.75 mL of chloroform were added to the raffinate (aqueous layer). The mixture was vortex-mixed for 30 minutes and then 8.5 mL of water and 8.5 mL of chloroform were added. The mixture was homogenized and centrifuged to separate the phases. The lower extract-rich layer was combined with the first one and the solvent was removed by using a TurboVap LV. Separation of the extract and solvent removal were conducted as described in the previous section (single extraction).

Table 4.1 Experimental design for the Bligh & Dyer extraction.

Experimental Run	Amount of Solids(g)	% Solids (weight)[b]	Solids to Solvent Ratio(g/L)[c]	Solids to Solvent Ratio(g/L)[d]
1[a]	0.00	0.00	0.00	0.00
2	0.10	3.23	5.33	2.00
3	0.20	6.25	10.67	4.00
4	0.30	9.09	16.00	6.00
5	0.40	11.76	21.33	8.00
6	0.57	15.97	30.40	11.40

[a]Blank/Control run.
[b]Water content of all samples = 3.0 mL (3.0 grams).
[c]Single extraction. Total volume of solvent used = 18.75 mL (water content of samples not included).
[d]Double extraction. Total volume of solvent used = 50.00 mL (water content of samples not included).

4.2.3 ASE® Experiments

Accelerated solvent extraction was applied to the freeze-dried sludge using a modified procedure of Dufreche et al. (2007) [18]. Prior to extraction, the sample (4.0 g) was mixed with 1.0 g diatomaceous earth (Dionex, Sunnyvale, CA, U.S.A.), homogenized and then loaded into 22-mL stainless steel extraction cell. After sample loading, void spaces in the extraction cells were filled up with enough diatomaceous earth. An ASE 200 system equipped with a multi-solvent control system (Dionex, Sunnyvale, CA, U.S.A.) was used for extraction. The extraction was conducted at 100°C and 10.34 MPa using a solvent system containing 60/20/20 volume ratio of *n*-hexane/methanol/acetone. Three extractions were done per cell for 1-hour total extraction time. Each extraction was followed by a solvent flush equivalent to 75% of the extraction cell's volume. The solvent was removed using a TurboVap LV as was done with BDE experiments. The recovered extract was stored below 0°C until further analysis.

4.2.4 Analysis of Extraction Yields

The gravimetric yields of the two extraction procedures were compared. In addition, the yields of FAMEs were also determined. The extracts from the two extraction procedures were subjected to methanolysis using 5 mL of 14% BF_3 – methanol solution (Fisher Scientific, Pittsburg, U.S.A.) added to 100 mg of sample. The mixture was vortex-mixed and reacted at 65°C for 30 minutes. After reaction, the mixture was allowed to cool to room temperature and 10 mL of saturated NaCl in water was added. FAMEs were then extracted three times with 5 mL *n*-hexane. The pooled organic layers were dried using a TurboVap LV as described in BDE experiments. The recovered FAMEs were re-constituted in toluene containing 100 ppm BHT and 200 ppm 1,3-DCB.

Quantitation of FAMEs was conducted using an Agilent 6890N gas chromatograph equipped with flame ionization detector (GC-FID). The GC-FID was equipped with a Restek Stabilwax-DA capillary column (Restek, Bellefonte, PA, U.S.A.) having dimensions of 30 m x 0.25 mm I.D. and 0.25 μm film thickness. Samples were introduced to the injector set at 260°C in splitless mode. The GC oven was programmed at an initial temperature of 50°C for 2 minutes, ramped to 250°C at 10°C/minute, and was held at 250°C for 18 minutes. The FID was at 260°C for the duration of the analysis. The calibration of the GC-FID was conducted using a 14-component FAMEs standard mixture containing saturated, mono-unsaturated and poly-unsaturated C_8 – C_{24} fatty acids (Sigma-Aldrich, St. Louis, MO, U.S.A.).

4.3 Results and Discussion

All extraction experiments were conducted using a single batch of sludge. The main purpose of evaluating different extraction procedures is to maximize extract yield from activated sludge, and thus, obtain more complete characterization results. However,

since the major products from petroleum oil are fuels, the extraction procedure that maximized the yield of FAMEs/biodiesel was considered more intensively. Focus was given on those extraction procedures, which are known for effectiveness. The BDE procedure was applied on both partially dried and freeze-dried sludges. The BDE procedure is the most well known method for determination of total lipid content in biological samples [21, 30]. The study conducted by Dufreche et al. (2007) on the ex situ biodiesel production from activated sludge showed that the highest yield could be obtained using a 60/20/20 volume ratio of hexane/methanol/acetone as solvent for ASE [18]. Thus, this procedure was also employed on the extraction of freeze-dried activated sludge without prior optimization.

4.3.1 BDE: A Pseudo Ternary Extraction Technique

One of the common classifications of lipids is based on polarity. Neutral or non-polar lipids include triacylglycerides, diacylglycerides, monoacylglycerides, sterols and waxes, while polar lipids include free fatty acids, phospholipids, sphingolipids, etc. [25]. Activated sludge microorganisms are mostly heterotrophic bacteria and bacterial lipids contain all the classes of lipids mentioned above and possibly others [31]. Thus, in the extraction of bacterial lipids, the solvent or solvent system selected should be polar enough to dissociate the polar lipids from cell membranes and lipoproteins but adequately non-polar to dissolve neutral lipids. This need was first recognized by Folch et al. (1957) who devised an extraction procedure using chloroform and methanol in 2:1 ratio followed by washing with water or salt solution [32]. The Bligh & Dyer extraction procedure was a modification of the Folch method with the intention of: (1) reducing the volume of

extraction solvent, and (2) shortening the extraction time making the procedure applicable for routine works [21, 25].

One of the advantages of BDE is that the samples can be extracted without prior drying because water is one of the extraction solvents. Furthermore, extractions are accomplished at ambient temperature and thus, BDE is considered to be a mild extraction technique. Figure 4.6 shows the BDE path on the chloroform-methanol-water phase diagram. This path is specific for the procedure described in the BDE experiments (section 4.2.2). The sample (containing 3 mL water) to be extracted can be located on the apex representing 100% water. For dried samples, the same is true after addition of sufficient water. After addition of 11.25 mL of 2:1 (volume) methanol:chloroform solution (point S_1), the resulting system is represented by point $M_{1(1)}$. With respect to the three solvents (chloroform, methanol and water), this point is in the monophasic region and is a necessary step to remove the association of the lipids (especially phospholipids) from cellular membrane and lipoproteins. Samples, especially biological materials, contain enzymes that degrade lipids during extraction. In addition to disruption of lipid-protein association, this step also inactivates lipid-degrading phosphatidases and lipases [33]. The next step, which is the addition of 7.5 mL of 1:1 (volume) chloroform:water solution (point S_2), brings the mixture to the biphasic region (point $M_{2(1)}$). According to Bligh and Dyer (1959), this point must be on or below the maximum chloroform tie-line to ensure that the extract layer ($E_{1-\infty}$) contains only chloroform as solvent [21]. To determine the location and solvent composition of the raffinate (aqueous layer), a tie-line from the chloroform apex (100% chloroform, point $E_{1-\infty}$) passing through $M_{2(1)}$ can be drawn. The intersection of the tie-line and the phase envelope is the raffinate ($R_{1-\infty}$). This

covers the single BDE, which is represented by red pathway in Figure 4.6. For illustrative purposes, 100% recovery of $E_{1-\infty}$ was assumed.

In the original BDE procedure, the solids were separated from the liquid phase (solvents) after homogenization of the mixture at point $M_{1(1)}$. The solids were washed once with chloroform and the washing was combined with the separated liquid phase from $M_{1(1)}$. Thus, in the original BDE, point $M_{2(1)}$ was a solid-free mixture. It was noted that for samples with high concentration of triacylglycerides, re-extraction of the solids with chloroform alone is recommended. The extracts from the first extraction and re-extraction should be combined. According to Christie (2003), this is much misunderstood and thus, the BDE is commonly misused [34]. Alteration of the original BDE can lead to erroneous results. Thus, in 1994, a lipid intercomparison exercise using the BDE method was conducted within the QUASIMEME, a quality assurance project of the European community. The results of the exercise indicated differences, although not significant, could be attributed to the method alterations. Furthermore, re-extraction resulted in higher results compared to the original single extraction of Bligh & Dyer [7, 35].

Removal of the solids from mixture $M_{1(1)}$ might be beneficial during the phase separation of the mixture at $M_{2(1)}$. However, it might result in sample losses during filtration. And thus, for this study, the solids were kept in the mixture throughout the BDE. In this manner, re-extractions (multi-stage extraction) of the solids were easily done as discussed below.

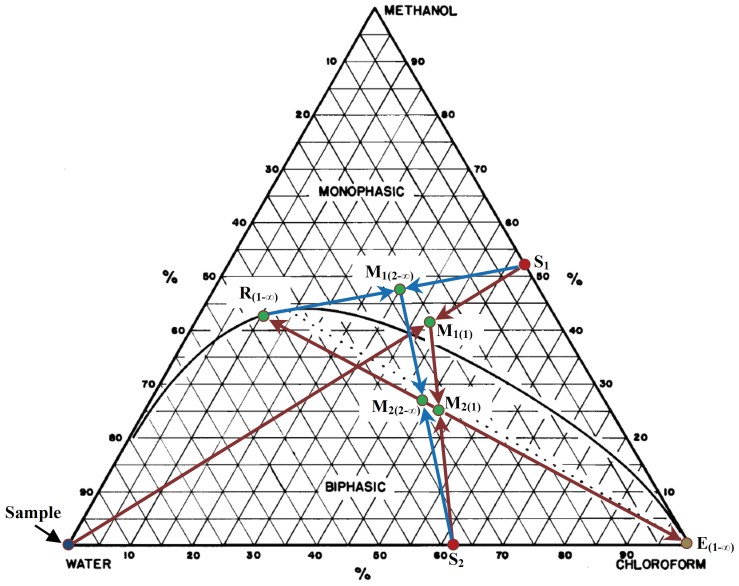

Figure 4.6 The Bligh & Dyer extraction pathway.*

*—single extraction —double extraction

The second extraction can be accomplished in two ways. The first one is by adding equal amount of pure chloroform to $R_{1-\infty}$. The resulting mixture will be on the midpoint of $R_{1-\infty}$ - $E_{1-\infty}$ tie-line. Separation of the phases can then be accomplished and extraction can be done as many times as necessary. However, doing multiple stage extraction in this manner will only recover free (dissociated) lipids from the first monophasic extraction step. Thus, the second extraction was conducted in a different approach. To the raffinate from the first extraction, 14.25 mL of S_1 was added to bring the mixture around the monophasic optimum extraction region again (point $M_{1(2-\infty)}$) (see Figure 4.5). The mixture was then homogenized to maximize the dissociation of bound lipids. Addition of 17 mL S_2 brings the mixture to the biphasic region again (point $M_{2(2-\infty)}$). Just like the first extraction, a tie-line passing through $M_{1(2-\infty)}$ can be drawn to determine the location and composition of the raffinate from the second extraction. This concludes second extraction (represented by blue pathway in Figure 4.6) and this can be

done as many times as necessary. The third, fourth and fifth extractions were conducted by addition of 20 mL chloroform to the raffinate from the previous extraction stage followed by phase separation and extract recovery.

The % solid content of sludge from centrifugation or filtration ranges from 8 – 16% (weight). Thus, experiments were conducted to determine if the water content for this range of solid content is enough for the DBE, especially for the high end. This is because the water content of the sample was the basis for calculating the volumes of the other solvents. The original BDE method utilized fish tissues containing 80% (weight) water. This water content was also the basis of volumes of the other solvents. Considering that the lowest water content of the partially dewatered sludge was about 84% (weight), the range of solid content of samples should be adequate for BDE (solids to solvent ratio decreases as water content increases). However, due to the differences in samples (fish tissues versus activated sludge), verification was considered necessary. Results indicated that for the range of solid content studied (3 – 16% weight) and for the same number of extraction stages, the FAMEs yields were not significantly different from each other. The results suggested that the range of water content of the samples is sufficient for BDE.

Multi-stage extraction is necessary for efficient extraction of any target compound. A study on the comparison between BDE and Folch extraction methods showed that BDE is applicable only for the extraction of samples containing <2% lipids [26]. This is probably due to large difference in volume of extraction solvents that the two methods require. The BDE requires a solvent:sample ratio of (3+1):1 while the Folch method requires 20:1. The in situ transesterification of activated sludge yielded 4 – 5% (weight) biodiesel (Chapter III), which in terms of lipids is equivalent to about the same

range as triacyglycerides. This means that the original BDE, which is a single-stage extraction procedure, is not applicable for extraction of lipids from activated sludge. This is evident from Figure 4.7. At least two extraction stages are necessary for complete extraction of lipidic materials from partially dewatered activated sludge. Any additional extraction stages will result to a not significant yield of FAMEs. Thus for the remainder of the study, two-stage extractions were conducted for both partially dewatered and freeze-dried sludges. For the freeze-dried sludge, 3.00 mL of water was added to 0.50 g samples (equivalent to 14.00% solids), and the BDE was conducted in the same manner as the partially dewatered sludge.

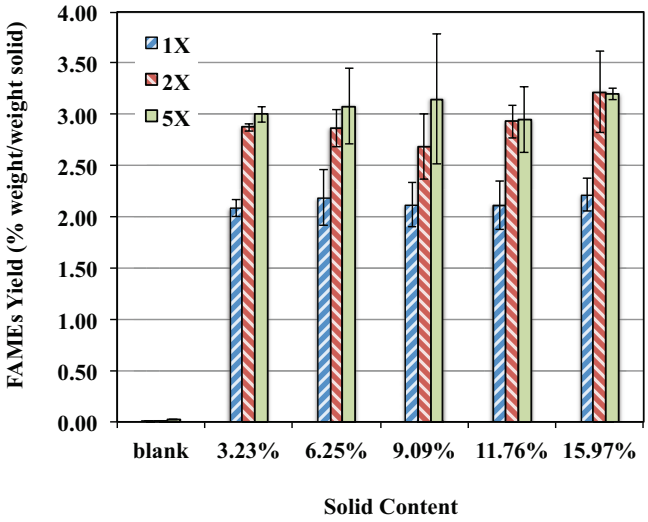

Figure 4.7 FAMEs yield as a function of solid content.*

*Single Extraction (1X): 18.75 mL Extraction solvent. Double Extraction (2X): 50 mL Extraction solvent. Five-stage extraction (5X): 110.00 mL Extraction solvent. Water content of samples: 3.00 mL. Extraction Temperature: ambient.

4.3.2 ASE®

Accelerated extraction was conducted for freeze-dried sludge only. This was due to the high water content of partially dewatered sludge, which will require large amount

of diatomaceous earth for drying and dispersing. Studies on the recovery of analytes (i.e. hydrocarbons) from dry and wet sample using ASE® showed that higher yields can be obtained for dry samples using the same extraction solvent(s) [36]. Furthermore, ASE® was conducted at elevated temperature (100ºC), and in the presence of as low as 1% (weight) moisture, the rate of hydrolysis of lipids is known to be significant [37]. Thus, on the freeze-dried samples were subjected to ASE®.

The two extraction procedures tested might not be economically feasible in an industrial scale. However, as previously mentioned, the main objective of the study is to evaluate these extraction techniques in terms of extraction yields (gravimetric and FAMEs). Maximization of extraction yield is necessary for a more complete characterization result, which was the subject of the next two chapters.

The results of extraction experiments are shown in Figure 4.8. On the average, the gravimetric yield of the BDE using partially dewatered sludge was the highest among the three extraction techniques tested. However, it was not significantly different with the yields of the other two extraction procedures. This also applies to the biodiesel yields. Regardless of the extraction technique used, the fatty acid profiles of the biodiesel obtained are similar (Figure 4.9). This profile is also similar to those obtained by previous researchers who worked on activated sludge from the same wastewater treatment facility [18, 38]. The dominant fatty acids present in the sludge ranges from C_{14} – C_{18}, which has been suggested to reflect bacterial contribution [39].

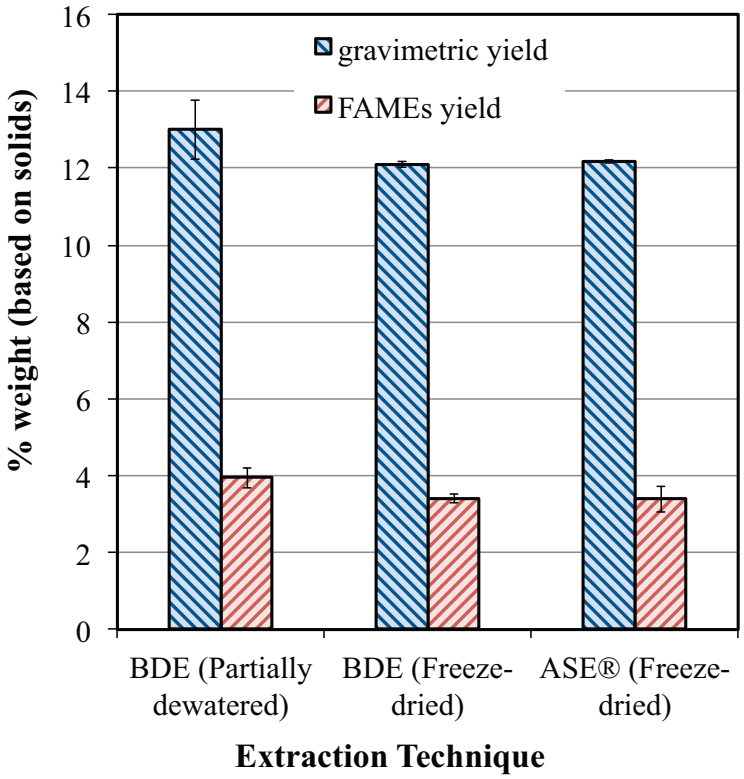

Figure 4.8 Yield comparison for the extraction of activated sludge.

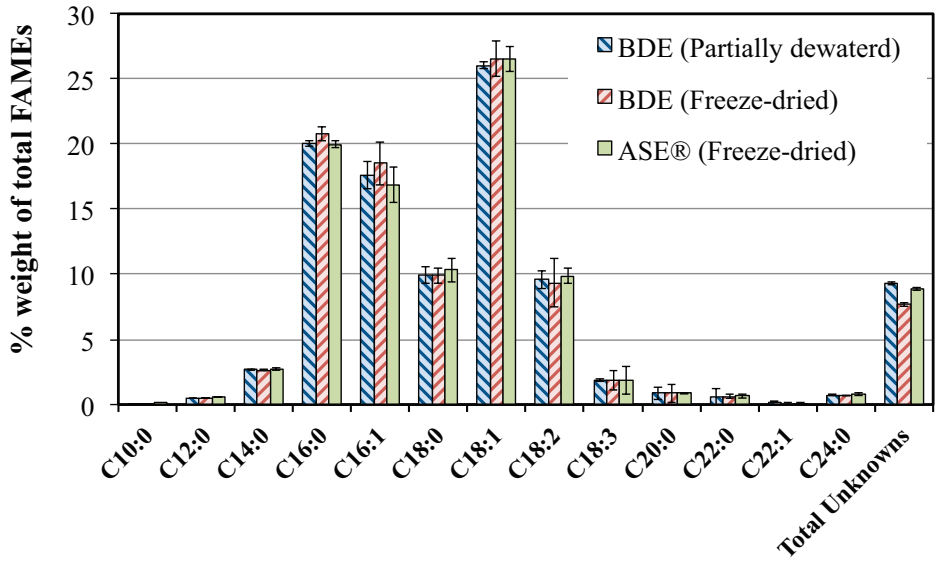

Figure 4.9 Fatty acid profiles of biodiesel from activated sludge using different extraction techniques.

In selecting the suitable extraction procedure for extract characterization, the one that minimizes the changes or transformations of extractable compounds is preferable. Freezing has been known to trigger microbial cell damages resulting to a considerable breakdown of cellular organization. It can cause alterations in phospholipid composition of the cells due to either peroxidation or phospholipase activity [40]. Thus, the samples that underwent freeze-drying were not preferable. Furthermore, the ASE® was conducted at 100°C using methanol as one of the extraction solvents. At this condition, significant production of FAMEs was observed. To verify this observation, activated sludge samples were spiked with 200 mg soybean oil and were subjected to ASE®. On the average, a conversion of about 80% was obtained on these experiments. For these reasons, the BDE of partially dewatered sludge seemed to be the most desirable one among the evaluated extraction techniques.

4.4 Conclusions

The extraction of lipidic materials and other compounds from activated was evaluated. Two extraction procedures were considered based on their effectiveness. The BDE was applied on both partially dewatered and freeze-dried activated sludges. Results indicated that the water content of the partially dewatered sludge was sufficient for the initial BDE steps. On the other hand, ASE® was only applied on freeze-dried samples.

The gravimetric and FAMEs yields of all the techniques tested were statistically similar. However, the BDE of partially dewatered sludge minimizes alteration(s) of the compounds present in the samples. Thus, this combination of sample and extraction technique was considered to be the most suitable one for the extraction of lipidic and other organic materials from activated sludge.

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CHAPTER V

LIPID STORAGE COMPOUNDS IN ACTIVATED SLUDGE MICROORGANISMS: METHOD DEVELOPMENT, QUANTITATION AND ASSOCIATED PRODUCTS (Revellame et al., 2011) [1]

5.1 Introduction

In response to stressful conditions (e.g. nitrogen, phosphorus or oxygen limitation), bacteria produce compounds such as triacylglycerides, wax esters and polyhydroxyalkanoates as carbon or energy storage materials [2]. These compounds are important raw materials or intermediates for a variety of applications. For example, wax esters and fatty alcohols with C_{12} and higher are important basic material for the production of fragrances, detergents, toothpastes, shampoos and lubricants [3-5].

Since activated sludge contains a mix microbial community, it may also contain other organic compounds such as alkanes, polycyclic aromatic hydrocarbons, polychlorinated biphenyl, linear alkyl benzenes, sterols and pentacyclic triterpanes. The relative amount of these compounds varies depending on microbial strains comprising the activated sludge, type of wastewater being treated and treatment process configurations [6-8].

Aside from transportation fuels, the world is also very much dependent on other products (i.e. crayons, eyeglasses, tires, heart valves, etc.) derived from petroleum oil [9]. Thus, in the search for an alternative to petroleum oil, the importance of these other products must also be considered. In this context, activated sludge, as feedstock for fuels

and oleochemicals production, might have an advantage compared to other conventional feedstocks because of the variety of compounds that activated sludge can potentially offer.

Previous studies on activated sludge showed relatively low yields (3 – 6% weight of dry solid) of biodiesel. Raw activated sludge, applied just for biodiesel production is not economically competitive at current petroleum prices [10, 11]. In Chapter III, a gravimetric yield as high as 13 – 15% (dry sludge weight) was obtained on the in situ transesterification of activated sludge because of the extraction of other compounds aside from biodiesel. These compounds could be any or all of the compound classes mentioned above. The sensitivity analysis indicated that a yield of at least 10% (dry sludge weight) biodiesel yield must be attained for activated sludge to be economically competitive at current petroleum prices. If the other unidentified compounds (the difference between 3 – 6% and 13 – 15%) can be converted to biofuel, or other useful chemical reaction precursors, the economics of this feedstock may improve dramatically. However, identification and quantitation of these compounds are necessary for their strategic separation and utilization. Once identified, reaction pathways to fuel or oleochemical conversion can then be established.

5.1.1 Solid Phase Extraction (SPE)

Solid phase extraction is in a sense another form of adsorption by which a solid surface or a "meta-surface" (i.e. organic layer of a C18 bonded silica) is being used for extraction. Scientists claim that the first literature reference about the use SPE can be found in the Bible [Exodus, Chapter 15, verses 24 – 25]. The first modern use of SPE employed charcoal, diatomaceous earth and zeolites as sorbents to remove pigments from

chemical reactions. SPE was considered a scientific technique in the 1970s and in 1977, the first disposable, pre-packaged cartridges/columns were introduced (Figure 5.1) [12].

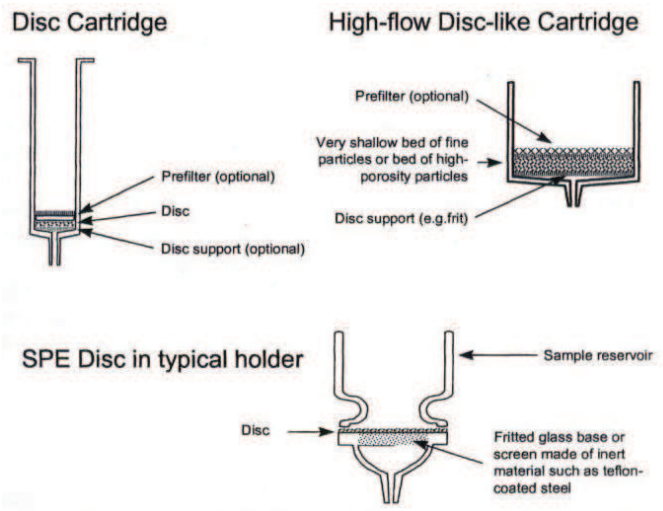


Figure 5.1 Examples of pre-packed SPE cartridges [13].

The basic steps of SPE are shown in Figure 5.2, where the compound of interest (analyte) is represented by black circles. Just like any other separation process, such as liquid-liquid extraction (LLE), a distribution coefficient, K_D as function of analyte concentration can be defined for SPE and is given in Equation 5.1. Since the analyte distributes on the surface of the sorbent, SPE closely resembles distillation [12].

$$K_D = [analyte]_{solid} / [analyte]_{sample} \tag{5.1}$$

The main requirement of SPE is that the analyte must have very high distribution coefficient such that it is almost completely adsorbed on the sorbent's surface. This process/step is called retention. However, during this step, co-retention of other unwanted compounds might occur. These unwanted compounds are removed by using an appropriate wash solvent during the rinsing step. The last step is elution using a solvent or solvent mixture that can cause desorption of the analyte from the sorbent. For each

step, care should be taken in the selection of wash and elution solvents, and sample loading conditions. In most cases, the elution solvent must be chosen not just due to its desirability to a given analyte, but also for convenience in subsequent handling and analyses [12].

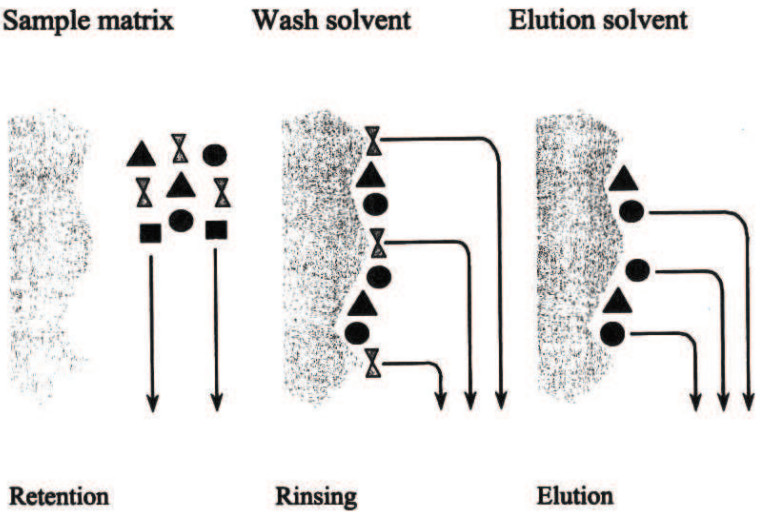


Figure 5.2 The three steps of a solid phase extraction of a compound represented by •after conditioning of the sorbent [12].

Silica, which is an inorganic polymer with a general formula of $(SiO_2)_x$, is the most widely used sorbent or stationary phase in SPE procedures. The main advantages of silica are its availability in a wide range of surface areas (50 – 500 m^2/g) and pore sizes (50 – 500 Å) at a relatively low cost. Its surface is dominated by the presence of hydroxide groups called silanols. These hydroxide groups normally exist as single silanol (Figure 5.3a), but in few cases, two hydroxyl groups are attached to a silicon atom. These sites are called germinal silanols (Figure 5.3b). Furthermore, associated silanols can also be formed when two hydroxyl groups from adjacent silicon atoms are arranged in a way that facilitates hydrogen bonding (Figure 5.3c) [13].

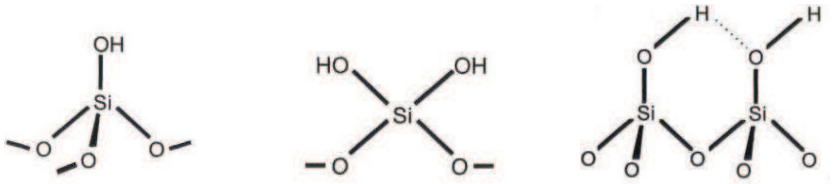


Figure 5.3 Functional groups at the surface of silica. (a) Single silanol (b) Geminal silanol (c) Associated silanol [13].

Chemically bonded stationary phases can be produced by reacting the silanol groups of silica with various organic reagents. These bonded phases have greater bonding potential for specific analytes and thus the choice of reagent will depend on the functional group(s) present on these analytes [14]. The nature of the bonded phases can be hydrophobic (R is an alkyl such as C18), hydrophilic (R has polar functional groups such as hydroxyl, cyano and amine) or ionic (R = sulfonic acid, carboxylic acid or amine) (Figure 5.4) [13].

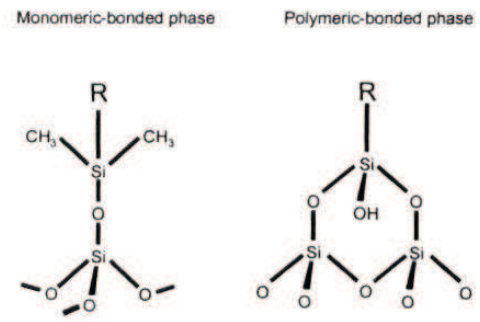


Figure 5.4 Representation of two approaches to bonding of silica surfaces [13].

5.1.2 SPE for Lipid Classes Separation

Separation of lipid classes by SPE is mostly accomplished by using aminopropyl-bonded silica stationary phase, which are usually used in-tandem with other analytical techniques such as liquid chromatography-mass spectrometry (LCMS) and gas chromatography-mass spectrometry (GC-MS).

The procedure reported by Kaluzny et al. (1985) has been the basis of most studies on the separation of lipid classes. They utilized two aminopropyl-bonded silica columns to separate a lipid mixture from fatty adipose tissue into seven fractions. The first column was used to separate the mixture into neutral lipids, free fatty acids and phospholipids. The second column was then used to separate the neutral lipids fraction into cholesteryl esters, triacylglycerides, diacylglycerides, monoacylglycerides and cholesterol [15]. The procedure was modified by several authors to separate lipid classes from rat plasma [16], Iberian pig muscle [17], mix microbial cultures [18] and to separate free fatty acids in shellfish [19].

In 1998, Pinkart and co-workers developed a SPE method to rapidly separate lipid classes commonly found in microorganisms. They utilized an aminopropyl-bonded silica column to initially separate a lipid extract from *Spirula plantesis* into neutral lipids, polyhydroxyalkanoates and polar lipids. The neutral lipids fraction was then separated into steryl esters, triacylglycerides, diacylglycerides, monoacylglycerides and sterols by using another aminopropyl-bonded silica column [20]. This was the first and only detailed procedure designed for separation of microbial lipids using SPE. In this method however, the recovery of polyhydroxyalkanoates was relatively low at 69% and free fatty acids were not separated as one fraction. Thus, Ruiz et al. (2004) modified the procedure to separate free fatty acids instead of polyhydroxyalkanoates [17]. They used the modified procedure to separate lipid classes from Iberian pig muscle and was later used by Dybvik et al. (2008) for separation of lipids from cod roe [21].

The procedure described by Hamilton and Comai (1988) was the only study about lipid separation that used unmodified silica columns. They used a single silica column to separate triacylglycerides, cholesteryl esters, fatty acids, cholesterol and phospholipids

from human serum [22]. However, the application of this method is limited by the narrow lipid classes considered and thus cannot be applied to separate activated sludge lipids, which can contain compounds from hydrocarbons to polyhydroxyalkanoates.

The presence of polyhydroxalkanoates in the lipid of activated sludge increases its complexity. The most commonly used method for isolation of this polymer from different biological materials is solvent extraction with acetone, chloroform, dichloromethane, dichloroethane and propylene carbonate. Other extraction procedure that had been tested include digestion using enzymes, sodium hypochlorite and surfactants, treatment with ammonia, supercritical fluid disruption, dissolved-air flotation and selective dissolution of cell mass [23, 24]. After isolation, the purification is normally conducted by precipitation in chilled methanol followed by either hydrolysis or alcoholysis for identification of monomers present in the polymer [2, 25].

This chapter deals with characterization of Bligh & Dyer extract from raw activated sludge to support the evaluation of activated sludge as a feedstock for renewable fuels and oleochemicals production. Characterization was accomplished by a combination of a method available in the literature and a SPE technique, which was designed for activated microbial lipids. Furthermore, an extensive literature survey was conducted to identify potential products that can be obtained from different compound classes present in raw activated sludge.

5.2 Materials and Methods

5.2.1 Sample Collection and Preparation

All activated sludge samples used in this Chapter were obtained from a MWWTP in Tuscaloosa, AL, U.S.A. [26]. Samples were collected from the return activated sludge

line into 4-gallon plastic buckets and were transported in ice chests to the Renewable Fuels and Chemicals Laboratory at Dave C. Swalm School of Chemical Engineering, Mississippi State University. Samples were concentrated by gravity-settling in ice-bath overnight, followed by either centrifugation at 3000 rpm for 20 minutes or vacuum filtration using a P8-creped cellulose fiber filter.

5.2.2 BDE

The lipids and other compounds present in the partially dewatered activated sludge were extracted by the Bligh & Dyer extraction method as was discussed in the preceding chapter [27]. However, the BDE was conducted in 1-L stirred vessels to obtain more samples. The volume of solvents (methanol, chloroform and water) added were calculated based on the average solid content (8 – 16% weight) of the samples. After extraction, the extract-rich layer was filtered through a funnel packed with glass wool to remove traces of cell debris. Most of the solvent from the extract-rich layer was removed using a Büchi R-205 rotary evaporator (Brinkmann Instruments, Inc., Westbury, NY, U.S.A.) at 40°C under 300 mbar of vacuum. The remaining solvent was removed at 45°C under 15 psi stream of N_2 using a TurboVap LV. The recovered extract was stored below 0°C until further analysis.

5.2.3 Analysis of Storage Compounds

5.2.3.1 Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates in the extract were isolated and analyzed using a modified procedure presented by Kathiraser et al. (2007) [25]. The BD extract (~ 250 mg) dissolved in 1 mL chloroform was added drop-wise to 10 mL chilled methanol to precipitate the PHAs. The precipitate was then recovered by centrifugation at 3000 rpm

for 5 minutes and by filtration of the supernatant using a 0.45 μm PTFE filter. The precipitate was re-dissolved in 1 mL chloroform and re-precipitation was done twice more. The isolated PHAs were washed 3 times with 1 mL of *n*-hexane. The isolated polymer was air-dried overnight and re-dissolved in 1 mL of chloroform for further analysis. The supernatants from PHA precipitations and hexane washings were pooled and dried using a TurboVap LV as described in BDE experiments. The dried extract (free of PHAs) was analyzed for other compound classes (see SPE experiments).

The isolated PHAs dissolved in chloroform were de-polymerized/derivatized by addition of 1 mL of 2.0 N HCl in methanol (Sigma-Aldrich, St. Louis, MO, U.S.A.). The mixture was refluxed using an Instatherm® heating block system for 16 hours at 80°C in a screw-capped (PTFE-lined) vial. After the reaction, the mixture was allowed to cool to room temperature after which, 2.5 mL of distilled water containing 5% NaCl and 2% $NaHCO_3$ was added. After addition of 1 mL chloroform, the mixture was vortex-mixed and set aside for phase separation. The organic (chloroform) layer was withdrawn and extraction using 1 mL chloroform was repeated twice more. The organic layers were pooled and dried using a TurboVap LV as in BDE experiments. The solid residue was re-dissolved in chloroform and was analyzed using a Varian 3400 GC equipped with a Saturn 2000 ion-trap mass spectrometer (GC-MS) (Varian Inc., Palo Alto, CA, U.S.A.). Electron impact (EI) and chemical ionization (CI) using acetonitrile as CI gas were utilized for peak identification.

Quantitation of hydroxy acid methyl esters was conducted using an Agilent 6890N gas chromatograph equipped with flame ionization detector (GC-FID). The GC-FID was equipped with a Restek Stabilwax-DA capillary column (Restek, Bellefonte, PA, U.S.A.) having dimensions of 30 m x 0.25 mm I.D. and 0.25 μm film thickness.

Samples were introduced to the injector set at 260°C in splitless mode. The GC oven was programmed at an initial temperature of 50°C for 2 minutes, ramped to 250°C at 10°C/minute, and was held at 250°C for 18 minutes. The FID was at 260°C for the duration of analysis. The GC-FID and GC-MS were running at the same condition and were equipped with the same column. The calibration of the GC-FID was conducted using methyl3-hydroxybutyrate and methyl 3-hydroxyvalerate standards (Sigma-Aldrich, St. Louis, MO, U.S.A.).

5.2.3.2 SPE

A method for the separation of compound classes present in the BD extract (free of PHAs) from activated sludge was developed using a solid phase extraction technique. Elution of different compound classes was conducted on a 1000-mg Extra-clean™ SPE silica column [average particle size: 50 μm, pore size: 60Å, surface area: 479 m^2/g] (Grace Davison Discovery Sciences, Deerfield, IL, U.S.A.) utilizing three solvent systems [94/6 (by volume) *n*-hexane/diethyl ether, 85/15/2 (by volume) *n*-hexane/diethyl ether/acetic acid, and pure methanol]. Optimization of the separation was conducted with thin layer chromatography (TLC) and high temperature gas chromatograph (HT-GC) monitoring. The PHA-free extract (20-30mg) in minimal volume of chloroform was loaded into the silica column which was pre-conditioned with 2 × 5 mL *n*-hexane. Elution of different compound classes was conducted following the scheme presented in Figure 5.5 with the solvent volumes at the best separation obtained shown in Table 5.2.

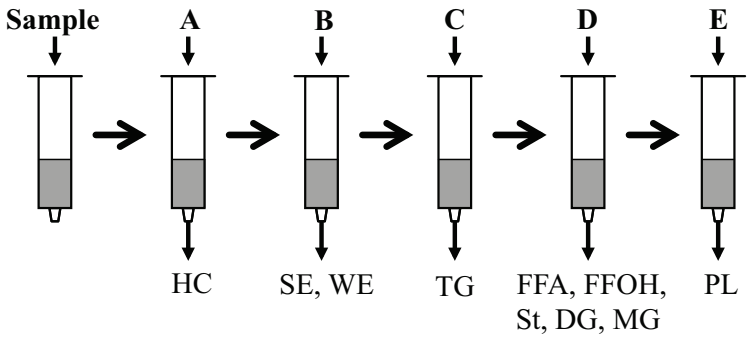


Figure 5.5 Sequential elution scheme for the separation of PHA-free activated sludge extract using 1000-mg Extra-clean™ silica solid phase extraction column.*

*HC = Hydrocarbon, SE = Steryl ester, WE = Wax ester, TG = Triacylglyceride, FFA = Free fatty acid, FFOH = Free fatty alcohol, St = Sterol, DG = Diacylglyceride, MG = Monoacylglyceride, PL = Phospholipid. The composition and volumes of solvents A-E are presented in Table 5.1.

Table 5.1 Composition and volumes of solvents used in the elution scheme presented in Figure 5.5 with the best compound class separation.*

Name	Solvent components	Composition (by vol.)	Vol. (mL)	Compound class eluted
A	*n*-Hexane/Diethyl ether	94/6	3.70	Hydrocarbons
B	*n*-Hexane/Diethyl ether	94/6	4.50	Steryl esters and Wax esters
C	*n*-Hexane/Diethyl ether	94/6	10.00	Triacylglycerides
D	*n*-Hexane/Diethyl ether:Acetic acid	85/15/2	15.00	Free fatty acids, Free fatty alcohols, Sterols, Diacylglycerides and Monoacylglycerides
E	Methanol	pure	5.00	Phospholipids

*Elution volumes are highly dependent on sample load and lipid class concentration.

The fractions obtained from the SPE were dried using a TurboVap LV as described BDE experiments. The dried fractions were re-constituted in 100 µL of chloroform and were subjected to TLC based on the method by Hwang et al. (2002). Fractions (5-10 µL) were spotted on 20 × 20 cm glass-backed Analtech Uniplates™ pre-coated with 250 µm silica gel-G (Sigma-Aldrich, St. Louis, MO, U.S.A.). Sample

applications were conducted using Drummond microcaps® disposable pipets (Fisher Scientific, Pittsburg, U.S.A.). Representative standards (20-30 μg) for each compound class were also spotted on the plates. Plates were developed either in 94/6 (v/v) *n*-hexane/diethyl ether or 85/15/2 (v/v/v) *n*-hexane/diethyl ether/acetic acid. Bands were visualized by spraying the plates with a solution of 10% (w/v) cupric sulfate in 8% phosphoric acid aqueous solution. The plates were then allowed to dry for 5 minutes and the developed bands were charred in an oven at 150°C for visualization [28].

The re-constituted fractions (in chloroform) were also analyzed on a HT-GC using a Varian 3600 GC (Varian Inc., Palo Alto, CA, U.S.A.) equipped with a flame ionization detector (FID). The GC column was an Rtx®-Biodiesel TG (15m × 0.32mm I.D., with a 0.10 μm film thickness) and utilized a 2m × 0.53mm Rxi® guard column (Restek, Bellefonte, PA, U.S.A.). Samples were analyzed using cool-on-column injection with an initial injector temperature of 50°C and a final injector temperature of 380°C, at a ramp rate of 180°C/min. The GC oven temperature was programmed at an initial temperature of 50°C, held for 1 min, then ramped to 180°C at 15°C/min, then ramped to 230°C at 7°C/min, then ramped to 370 at 20°C/min, and finally held for 11.20 minutes. The FID was held constant at 380°C for the duration of the analysis.

5.2.3.2.1 Fraction 1: Hydrocarbons

The hydrocarbon fraction was analyzed on a Varian 3400 GC equipped with a Saturn 2000 ion-trap mass spectrometer. Both EI and CI were used for compound identification. Quantitation of the peaks was conducted using an Agilent 6890N GC-FID equipped with a Restek Stabilwax-DA capillary column with dimensions of 30 m x 0.25 mm I.D. and 0.25 μm film thickness. Samples were introduced to the injector which was

at 260°C in splitless mode. The GC oven was programmed at an initial temperature of 50°C for 2 minutes, ramped to 250°C at 2°C/minute, and was held at 250°C for 18 minutes. The FID was at 260°C for the duration of analysis. The calibration of the GC-FID was done using *n*-octacosane (Sigma-Aldrich, St. Louis, MO, U.S.A.) and all responses were calculated based on this compound.

5.2.3.2.2 Fraction 2: Wax Esters and Steryl Esters

The wax and steryl esters fraction was subjected to methanolysis using a modified procedure by Bernasconi et al. (2007) [29]. The fraction from SPE was dried under N_2 using the procedure described in BDE experiments. After addition of 1 mL 14% BF_3-methanol solution, the mixture was vortex-mixed and the methanolysis was carried out at 60°C for 30 minutes. The mixture was then allowed to cool to room temperature. Products from methanolysis were extracted using 3 × 2 mL of chloroform. The chloroform extracts were pooled and dried using a TurboVap LV as in BDE experiments. The dried extract was dissolved in chloroform and was subjected to TLC as described in SPE experiments to determine if the methanolysis reaction achieved completion. The FAMEs were then separated from sterols and fatty alcohols using another 1000-mg SPE silica column. The methanolysis products, dissolved in minimal volume of chloroform, was loaded into a pre-conditioned column (2 × 5 mL *n*-hexane). FAMEs were eluted using 17 mL of 94/6 (v/v) *n*-hexane/diethyl ether solvent mixture and the sterols and fatty alcohols were eluted using 85/15/2 (v/v/v) *n*-hexane/diethyl ether/acetic acid solvent mixture. These two fractions were subjected to TLC as in SPE experiments to verify the separation of the methanolysis products.

Quantitation of FAMEs was conducted using an Agilent 6890N GC-FID using the procedure described earlier (see PHAs), while the sterols and fatty alcohols were analyzed using an Agilent 6890N gas chromatograph equipped with a 5975 inert Mass Selective Detector. The column was a Restek Rxi®-1MS having dimensions of 10 m × 0.10 mm I.D. with 0.10 μm film thickness. Samples were introduced to the injector which was held constant at 280°C for the duration of the analysis. The GC oven was programmed at an initial temperature of 50°C for 1.50 minutes, then ramped to 100°C at 35°C/minute, then ramped to 310°C at 20°C/minute and was held at 310°C for 5 minutes. Calibration of the instrument was accomplished using standards of primary fatty alcohols (tetradecanol, tetradecenol, pentadecanol, hexadecanol, hexadecenol, heptadecanol, octadecanol, octadecenol, nonadecanol, eicosanol and heneicosanol) and sterols (coprostanol, cholesterol, campesterol, stigmastanol, stigmasterol and β-sitosterol) (Sigma-Aldrich, St. Louis, MO, U.S.A.).

5.2.3.2.3 Fraction 3: Triacylglycerides

This fraction was subjected to methanolysis as was done with Fraction 2: Wax Esters and Steryl Esters, and quantitation of FAMEs was conducted using an Agilent 6890N GC-FID using the procedure described previously (see PHAs).

5.2.3.2.4 Fraction 4: Free Fatty Acids, Free Fatty Alcohols, Sterols, Diacylglycerides and Monoacylglycerides

The free fatty acids, diacylglycerides and monoacylglycerides were converted to FAMEs by methanolysis using 14% BF_3-methanol solution followed by SPE to separate the FAMEs from free fatty alcohols and free sterols. The procedure described earlier was employed (see Fraction 2: Wax Esters and Steryl Esters).

5.2.3.2.5 Fraction 5: Phospholipids

This fraction was subjected to methanolysis and analyzed by GC-FID as was done with Fraction 3: Triacylglycerides.

5.3 Results and Discussion

The availability of a wide range of compounds that can be obtained from activated sludge is advantageous for its potential use as an alternative to petroleum oil. These compounds are either intermediates or products of microbial degradation of organic and inorganic present in the wastewater. Some microorganisms that are usually involve in the activated sludge process include *Xanthomonas, Vibrio, Sphingomonas, Achromobacter*, *Aerobacter*, *Alcaligenes*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Comamonas*, *Flavobacterium*, *Micrococcus*, *Pseudomonas*, *Spirillum*, *Zooglea*, and *E. coli* [30-33]. All activated sludge samples used in this study came from a MWWTP in Tuscaloosa, AL, U.S.A., which utilizes a conventional aerobic treatment configuration. Based on the study conducted by Mondala et al. (2011), the activated sludge from this facility contains bacteria in phyla Proteobacteria [α-/β-/γ-/δ-/ε-Proteobacteria (i.e. Rhodobacterales and Xanthomonadales)], Verrucomicrobia (class Verrucomicrobiae), Bacteriodetes (class Flavobacteria and Sphingobacteria), Firmicutes (class Clostridia) and Actinobacteria [34].

Three batches of activated sludge were collected in the months of April, June and October (coded A, J and O, respectively) during the plant's normal operation. The yield of extract ranges 6 – 16% (weight) based on the dry solids. This wide range of extractables represents the inherent variability of activated sludge within a treatment plant. The range obtained corresponds to approximately 1.20 – 3.50 % (weight) FAMEs yield based on dried activated sludge (see Table 5.2). This range is in agreement with the

results obtained by Dufreche et al. (2007) on the ex situ biodiesel production from activated sludge obtained from the same waste treatment facility as this study. However, the range of biodiesel yield obtained is lower than the yields obtained from Chapter III and by previous workers using in situ biodiesel production from activated sludge [10]. This could be due to inherent variability of sludge sample with time or due to differences between the processes used. According to Dufreche and co-workers (2007), all saponifiable lipids are in contact with the reagents during the in situ process resulting to a higher biodiesel yield [10]. The yields of FAMEs were calculated from the results of the SPE experiments. The FAMEs yield shown in Table 5.2 is the sum of FAMEs obtained from all SPE fractions.

5.3.1 PHAs

PHAs are polyesters of hydroxyalkanoic acids and are well-known as biodegradable alternative to petroleum plastics [35, 36]. PHA is the main storage compound in most bacteria with poly(3-hydroxybutyrate) as the most abundant one [37, 38]. Numerous microorganisms are known to accumulate PHAs. Some microorganisms such as *Cupriavidus necator, Rhodopseudomonas palustris, Methylobacterium organophilum* and *Alcaligenes euthropus* (also known as *Ralstonia eutropha*), require limitation of an essential nutrient (i.e. nitrogen, phosphorus, magnesium, manganese, iron, potassium, sodium and oxygen) with an excess of carbon source. On the other hand, some microorganisms are known to accumulate PHAs during the growth phase. Examples of these microorganisms include *Alcaligenes latus* and *Azotobacter vinelandii*. Efforts to increase the yield of PHAs by microbial fermentation include cloning and expression of genes involved in PHA biosynthesis in *Escherichia coli*. Recombinant *E.*

coli can accumulate PHAs up to 80 – 90% of cell dry weight. Other microorganisms that can produce PHAs include *Protomonas extorquens, Bacillus subtilis, Bacillus thuringiensis, Bacillus megatarium, Rhodococcus ruber, Rhodococcus opacus, Rhodococcus jostii* RHAI, *Syntrophomonas wolfei, Rhodospirillum rubrum, Rhizobium japonicum, Halobacterium mediterranei, Azotobacter beijerinckii, Zoogloea ramigera, Methylobacterium rhodesianum, Methylocystis pervus, Methylosinus trichosporium, Rhizobium meliloti, Thiocapsa pfennigii, Sphaerotilus natans, Streptomyces lividans, Protomonas aeruginosa, Protomonas mendocina, Pseudomonas flourescens, Pseudomonas testosterone, Pseudomonas denitrificans, Rickettsia prowazekii* and *Pseudomonas oleovorans* [24, 36, 39-47]. Nowadays, approximately 300 microbial species are known to produce PHAs [48]. The properties of microbial PHAs such as molecular weight, polydispersity and hydroxyacid monomer length are highly dependent on the microorganism(s) involved, fermentation conditions and method of isolation [36, 39]. PHAs in microorganisms, particularly in bacteria, serve as carbon and energy reserve (storage) and/or as sink for redundant reducing power or electrons under stressful conditions [24, 39].

Microorganisms in activated sludges are known to accumulate PHAs ranging from 0.30 to 22.70 mg polymer per gram of sludge [7]. Reddy et al. in 2008 isolated PHA producing bacteria from activated sludge obtained from a MWWTP. Out of 480 bacterial isolates that they screened, 21.87% are PHA-accumulators. Furthermore, they identified seven *Bacillus* species, two *Alcaligenes* species, two *Aeromonas* species and one *Chromobacterium* species as PHA-accumulators [49]. In a related study, Law et al. (2001) isolated *Bacillus* species from municipal activated sludge. They found that the species is closely similar to *Brevibacillus laterosporus* and *Bacillus megaterium* [50].

The study conducted by Jiang et al. (2009) on PHA production from waste activated sludge showed that *γ-Proteobacteria, β-Proteobacteria* and *α-Proteobacteria* were the major PHA-producing microorganisms [51].

The PHAs content of the three batches analyzed is shown in Table 5.2 and a representative total ion chromatogram from GC-MS analysis is presented in Figure 5.6. The activated sludge extracts contains about 2 – 4% PHAs based on the weight of extract, which corresponds to ~1.83 – 4.69 mg PHAs per gram of dried solids. The isolated PHAs have purities of 80 – 90% by weight. Furthermore, only two hydroxyacid monomers were detected which are hydroxybutyric (HB) and hydroxyvaleric (HV) acids. These findings on the yields and monomers present are in agreement with the results obtained by other researchers on the isolation of PHAs from municipal activated sludges [7, 48, 52-54]. On the average, the ratio of HB to HV of the isolated PHAs was 1.20 by weight. This result is lower than that obtained by Hesselmann et al. (1999), which was ~2.91 by weight [53]. The difference is potentially due to differences in influent wastewater characteristics, specifically the volatile fatty acids (VFA) content. According to Yan et al. (2006), aside from environmental stress (i.e. high C:N ratio), there is a direct correlation between VFA content of the wastewater and PHA production in activated sludge. The type of VFA (i.e. acetic, propiopic, butyric, valeric) and the presence of other carbon sources in the wastewater also affects the ratio of different PHA monomers in the activated sludge [52]. For example, Alvarez et al. (1997) obtained PHAs consisting mostly of 3-hydroxyoctanoic acids from *Pseudomonas* species (Isolate 319) when either octanoate or octanol was used as carbon source [55]. Takabatake et al. (2002) studied PHA production using 18 activated sludges from MWWTPs with excess acetate as carbon source. They concluded that PHA production is more affected by influent characteristics than activated

sludge operating conditions [56]. In a similar study, Takabatake et al. (2000) concluded that regulating the composition of VFA such as acetate and propionate in the wastewater influent could control the monomer units of PHAs from activated sludge [57].

Table 5.2 Composition of Bligh and Dyer extract from activated sludge.

	A	**J**	**O**
Sludge Collection Date	7-Apr-2010	30-Jun-2010	13-Oct-2009
Aeration Basin Temperature, °C	24.80	20.10	26.00
Bligh & Dyer extract yield, % weight of dry solid	9.41 ± 0.21	5.88 ± 0.43	16.30 ± 1.28
Total FAMEs yield, % weight of extract	19.42 ± 0.33	20.68 ± 0.05	21.53 ± 0.32
Total FAMEs yield, % weight of solid	1.83 ± 0.05	1.22 ± 0.09	3.51 ± 0.28
PHAs, % weight of extract	1.95 ± 0.14	3.70 ± 0.72	2.88 ± 0.12
FRACTION 1			
Hydrocarbons, ppm (based on extract weight)	2.14 ± 0.18	1.39 ± 0.15	0.95 ± 0.20
FRACTION 2			
Fatty Alcohol (from WEs), % weight of extract	1.79 ± 0.22	5.24 ± 0.38	5.55 ± 0.36
Sterols (from SEs), % weight of extract	1.54 ± 0.09	2.83 ± 0.13	2.58 ± 0.27
FAMEs yield, % weight of extract	5.24	6.66	6.27
FRACTION 3			
Triacylglycerides, % weight of extract	2.82 ± 0.09	2.01 ± 0.00	2.08 ± 0.02
FAMEs yield, % weight of extract	2.84	2.02	2.09
FRACTION 4			
Free Sterols, % weight of extract	10.75 ± 0.01	18.42 ± 0.23	12.13 ± 3.55

Table 5.2 (Continued).

FAMEs yield, % weight of extract	11.04	11.31	12.73
FRACTION 5			
FAMEs yield, % weight of extract	0.31	0.70	0.44

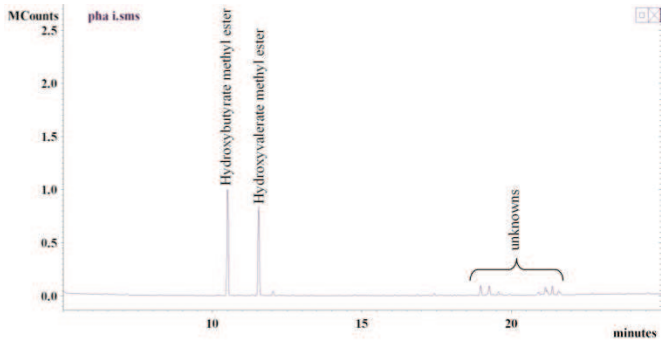


Figure 5.6 GC-MS analysis of PHAs isolated from activated sludge.

Presently, there are several PHA products such as Biopol, Mirel and Nodax (U.S.A.), Biomer (Germany), Biocyle (Brazil), DegraPol (Italy) and Tianan PHBV and PHB (China) that are available commercially [24, 58]. Companies that manufacture microbial PHAs include ZENECA Bio-products (UK), Biotechnolgische Froschungs gessellschaft mbH (Austria), Petrochemia Danubai, Bio Ventures Alberta Inc. (Canada), Biocorp (U.S.A.), Metabolix (U.S.A.), Procter and Gamble (U.S.A.) and Asahi Chemicals and Institute of Physical and Chemical Research (Japan) [43, 59]. The current production cost of microbial PHAs is about $4 – 6 per kilogram, which is approximately 10 times higher than petroleum plastic [36, 48]. The cost of carbon source has caused the slow growth experienced by the PHA industry. For example, the cost of substrate or carbon source accounts for about 50% of the microbial PHA cost [50]. Even with

genetically engineered *E. coli*, the carbon source is still about 31% of PHAs production cost [60].

Efforts to reduce microbial PHAs cost include searching for inexpensive carbon sources or substrates, advancement of fermentation, extraction and purification strategies and development of genetically engineered microorganisms [36, 39, 42, 61]. Carbon sources such as whey, wheat and rice brans, starch, molasses, waste vegetable and plant oils, CO_2 and H_2, methanol, industrial and biological wastes and wastewater are some of alternative substrates that have been considered to produce less expensive microbial PHAs [36, 43, 58, 61-64]. In the past several years, researchers all over the world have been looking at PHAs production co-current with wastewater treatment facilities particularly by utilizing the biological or activated sludge treatment of wastewater. Aside from the fact that this configuration might not need additional infrastructure, this has the potential of reducing the amount of waste sludge to about 20% after PHA extraction [8, 48-52, 54, 56, 57, 60, 65-72].

PHAs are attractive as packaging films and disposable commodity plastics (i.e. razor, utensils, diapers, cosmetic containers, bottles and cups, etc.) due to their complete microbial biodegradability [43, 73]. In medicine, PHAs can be used as functionalized nano/micro beads for diagnostic and therapeutic applications, as devices for sutures and wound dressings, as conduits and carrier scaffolds for nerve repairs, as drug delivery systems, as drug eluting stents for cardiovascular applications, for soft and hard-tissue repairs and regenerations and as heart valve in heart tissue engineering [41, 59, 74-78].

PHAs from activated sludges might not be applicable for use as everyday commodities and medical devices. One possible application is to convert the PHAs to hydroxyacid alkyl esters by acid-/enzyme-/alkaline-catalyzed alcoholysis to produce

biodiesel (see Table A.1). It was estimated that the production cost of PHA-based biodiesel is about $1,200 per ton, which is clearly not practical and economical [79]. However, according to Thomson et al. (2009), all known chiral PHAs are purely composed of (*R*)-hydroxyalkanoate monomers and thus can be used as a good raw material for production of enantiomerically pure drugs and specialty chemicals [35].

5.3.2 SPE: Method Development

Due to low solubility of PHAs in most organic solvents, they were removed prior to SPE to prevent their possible effect on the elution flow rate. The elution solvents were chosen on the basis of separation of lipid classes on silica-coated TLC plates. Based on observation, the resolution of hydrocarbons, wax esters and triacyglycerides was higher with 94/6 hexane/diethyl ether solution as developing solution than with 85/15/2 hexane/diethyl ether/acetic acid solution. For the rest of the compound classes, the resolution was reversed for the two solvents (see Figure 5.7-lanes S1 and S2). Thus, these two solvent systems were used as elution solvents. The polar lipids, phospholipids in particular, were eluted using methanol.

One disadvantage of using the elution scheme shown in Figure 5.5 is its inability to separate wax esters and steryl esters as individual fractions. Thus, they were collected as single fraction (Fraction 2). To analyze for fatty alcohols and sterols in this fraction, a derivatization procedure followed by another SPE method was conducted as described in the methods section. Another disadvantage was that free fatty acids, diacyglycerides, monoacylglycerides, fatty alcohols and sterols were collected also as single fraction (Fraction 4). It was decided not to separate these compounds into different fractions since the lipid extract from activated sludge contains negligible amount of diacylglycerides and

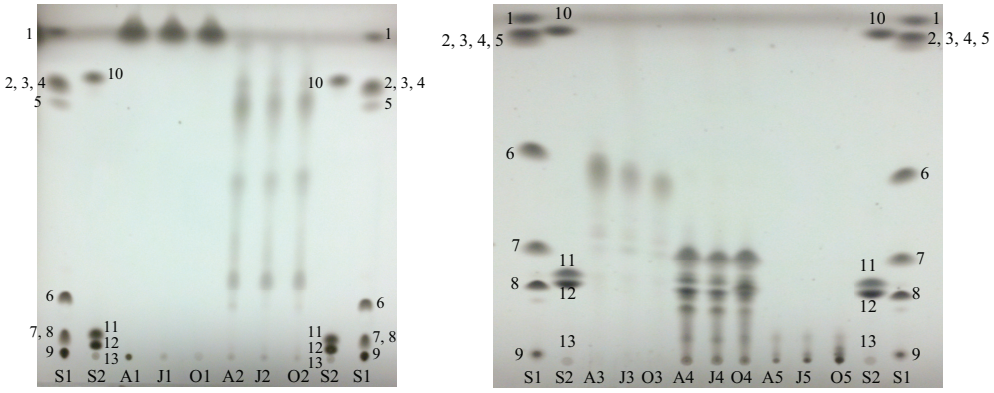
monoacylglycerides (see Figure 5.8d). As for the fatty alcohols and sterols present in this fraction, they were easily analyzed using the same procedure described above for Fraction 2. For sample containing high amount of diacylglycerides and monoacylglycerides, the method needs to be extended to separate them into individual fractions.

The SPE procedure that was developed utilizes just three elution solvents, two of which are exactly the same as the TLC developing solution. This minimizes possible analyte transformations by using several elution solvents especially if they contain salts or acids. Most of the SPE procedures available in the literature were conducted under vacuum, which requires careful timing as to when to add the succeeding elution solvent. Drying of the SPE column is critical for separation and thus, an automated SPE apparatus is probably the best option for systems under vacuum. Since the SPE protocol presented herein was conducted at ambient pressure, this concern was eliminated.

The results of TLC analysis of SPE fractions are shown in Figure 5.7. To increase the resolution of the chromatograms (as described earlier), the TLC plates for Fractions 1 and 2 were developed in 94/6 hexane/diethyl ether solution while that of Fractions 3 – 5 were developed in 85/15/2 hexane/diethyl ether/acetic acid solution. Fraction 1 showed only one band corresponding to hydrocarbon (Figure 5.7a-lanes A1, J1 and O1) while Fraction 2 showed several bands that correspond to steryl ester, wax ester and probably fatty acid alkyl esters (Figure 5.7a-lanes A2, J2 and O2). As shown in Figure 5b, Fraction 3 is composed mainly of triacylglycerides (lanes A3, J3 and O3), Fraction 4 predominantly contains free fatty acids, free fatty alcohol, sterols, diacylglycerides and monoacylglycerides (lanes A4, J4 and O4) and Fraction 5 contains mainly phospholipids (lanes A5, J5 and O5). To verify these findings, the fractions (except Fraction 5) were

injected to a HT-GC. The results are shown in Figure 5.8 with the range of retention times for specific group of compounds. The retention time ranges were identified by analyzing standard mixtures of different compound groups. As can be seen in Figure 5.8a, the hydrocarbon fraction (Fraction 1) is characterized by the presence of an unresolved complex mixture (UCM). This will be discussed in the next section.

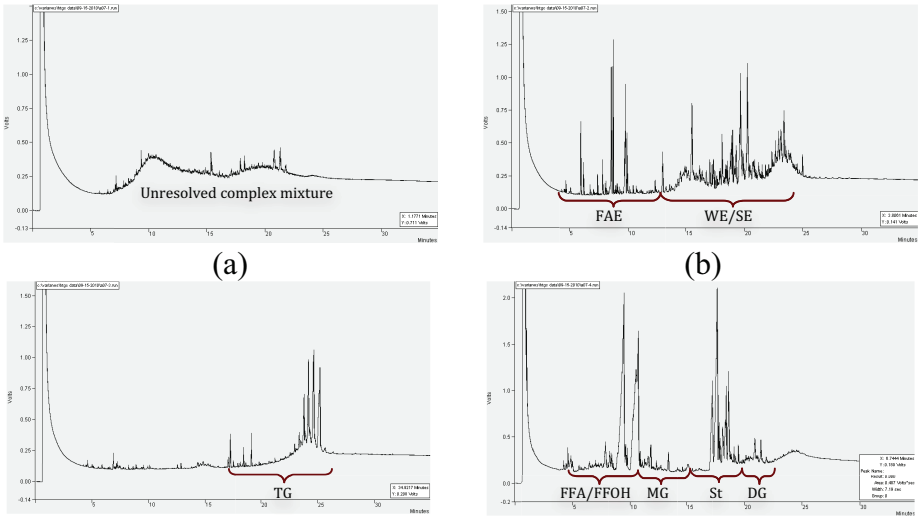
As can be seen in Figures 5.4b – 5.4d, there were co-elutions of different compounds, which makes quantitation of peaks or responses difficult. Thus, all the fractions were subjected to methanolysis. Although Fraction 1 appeared to contain only unsaponifiable hydrocarbons, it was also subjected to methanolysis for further verification. After methanolysis, the products were subjected to TLC and the results are shown in Figure 5.9. The TLC result for Fraction 1 showed that it indeed contained unsaponifiable materials as indicated by the absence of methyl ester products (Figure 5.9a-lanes A1, J1 and O1). Fraction 3 (Figure 5.9b-lanes A3, J3 and O3) and Fraction 5 (Figure 5.9b-lanes A5, J5 and O5) showed only one distinct band, which corresponded to methyl oleate. For Fraction 2 (Figure 5.9a-lanes A2, J2 and O2) and Fraction 4 (Figure 5.9b-lanes A4, J4 and O4), bands corresponding to fatty alcohol and sterol were also developed in addition to the methyl ester band. This result was expected since Fraction 2 contained wax esters and steryl esters while Fraction 4 contained free fatty alcohols and sterols as indicated by TLC and HT-GC of the original fractions (Figure 5.7a-lanes A2, J2 and O2, Figure 5.7b-lanes A4, J4 and O4, Figure 5.8b and Figure 5.8d).



(a) (b)

Figure 5.7 Thin layer chromatography of fractions from solid phase extraction.*

*(a) Fractions 1 and 2, developed in hexane/diethyl ether (94/6). (b) Fractions 3 – 5, developed in hexane/diethyl ether/acetic acid (85/15/2). S1 and S2 are standard mixtures: (1) *n*-octacosane, (2) behenyl oleate, (3) behenyl stearate, (4) palmityl palmitate, (5) lauryl palmitate, (6) triolein, (7) palmitoleic acid, (8) 1, 2-diolein, (9) monoolein, (10) cholesteryl myristate, (11) 1-hexadecanol, (12) cholesterol and (13) phospholipid standard mixture.



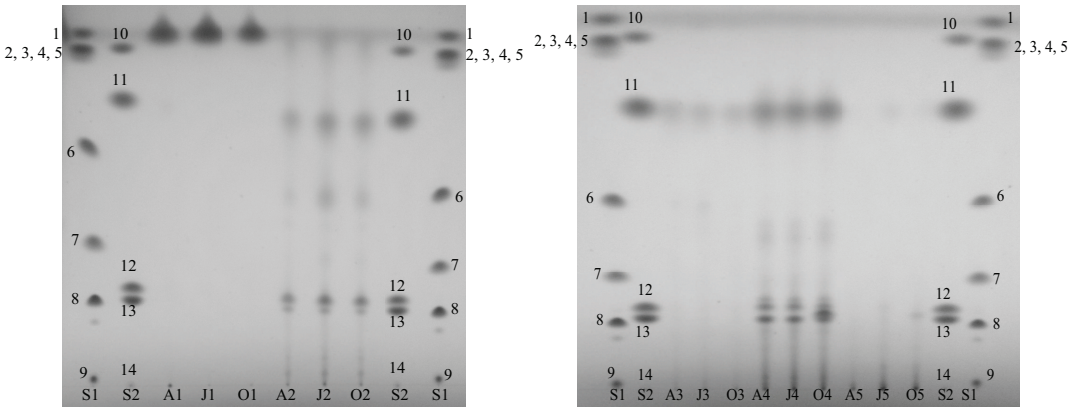
(a) (b)

(c) (d)

Figure 5.8 Representative high temperature gas chromatographs of fractions from solid phase extraction.*

*a – d: Fractions 1 – 4. FAE – Fatty acid alkyl ester, WE – wax ester, SE – Steryl ester, TG – Triacylglyceride, DG – Diacylglyceride, MG – Monoacylglyceride, FFA – free fatty acid, FFOH – free fatty alcohol, St – Sterol.

The FAMEs analysis showed that Fractions 2 – 5 are dominated by saturated and unsaturated C_{16} and C_{18} fatty acids (Figure 5.10). This result indicated microbial activity and is in agreement with the result obtained from Chapters III and IV and with other workers [10, 11]. The result of fatty alcohols and sterols analyses are shown in Figure 5.11 and Table 5.2. As can be seen in Figure 5.11, there were free sterols but no free fatty alcohols detected on the samples. Furthermore, results indicated that there were wax esters and steryl esters present in the samples (Figure 5.11a). For more detailed discussions on these, see **Wax Esters and Free Fatty Alcohols** and **Steryl Esters and Free Sterols** sections.



(a) (b)

Figure 5.9 Thin layer chromatography of fractions after methanolysis.*

*Developing solution: hexane/diethyl ether/acetic acid (85/15/2) (a) Fractions 1 and 2. (b) Fractions 3 – 5. S1 and S2 are standard mixtures: (1) *n*-octacosane, (2) behenyl oleate, (3) behenyl stearate, (4) palmityl palmitate, (5) lauryl palmitate, (6) triolein, (7) palmitoleic acid, (8) 1, 2-diolein, (9) monoolein, (10) cholesteryl myristate, (11) methyl oleate, (12) 1-hexadecanol, (13) cholesterol and (14) phospholipid standard mixture.

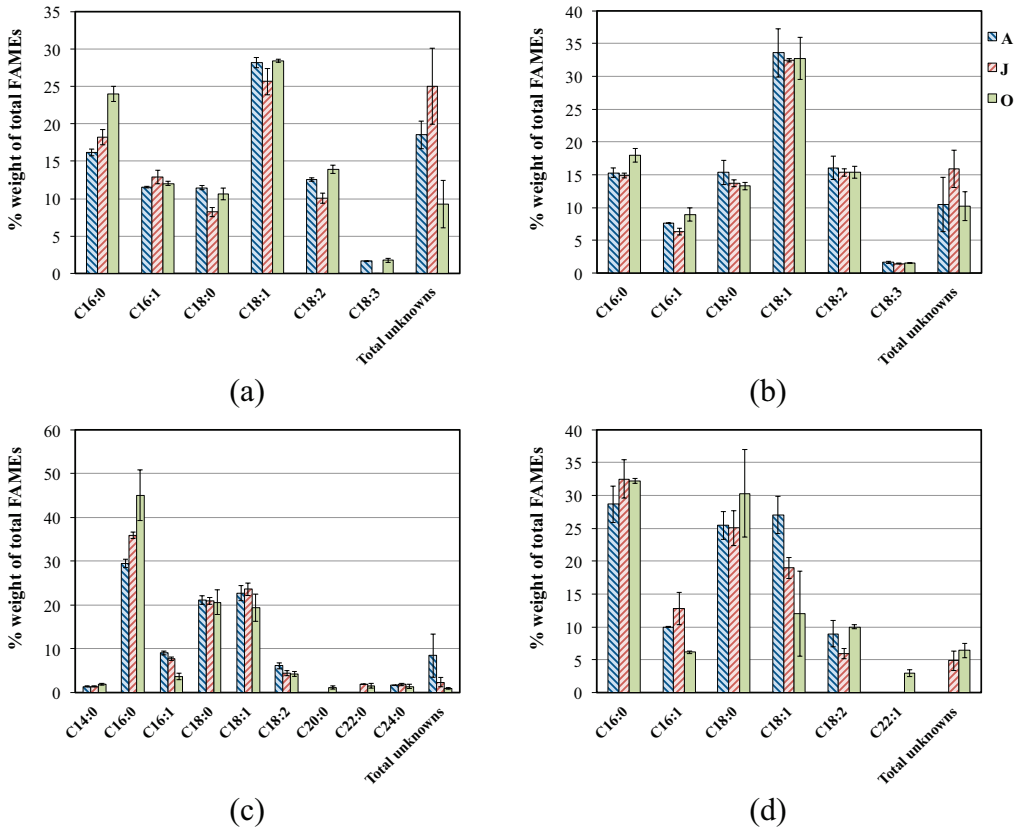


Figure 5.10 Fatty acid profiles of different fractions from SPE. (a-d: Fractions 2-5).

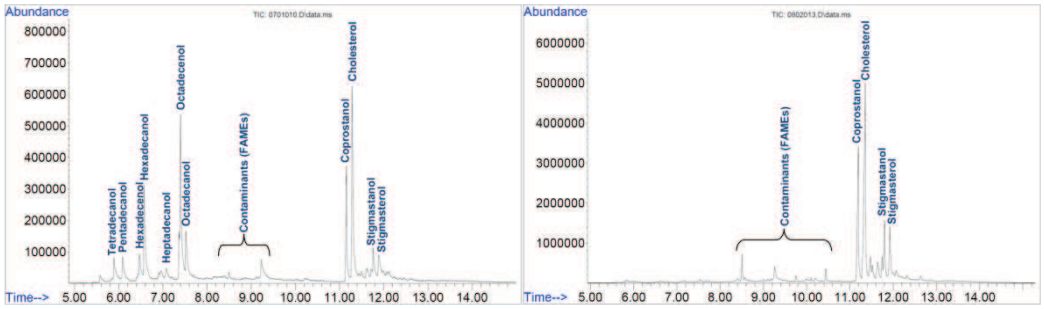


Figure 5.11 Representative total ion chromatograms from GC-MS analysis of fatty alcohols and sterols from activated sludge.*

*(a) Separated from Fraction 2. (b) Separated from Fraction 4.

5.3.3 Hydrocarbons

The result of the hydrocarbon analysis is shown in Table 5.2. As mentioned earlier, the hydrocarbon fractions were characterized by the occurrence of UCM [Figures 5.8a and 5.12]. However, some major peaks were identified, quantified and presented as total hydrocarbons in Table 5.2. Identified peaks include hydrocarbons from $C_{16} - C_{20}$ and some linear alkyl benzene (LABs) particularly 1-pentyloctyl benzene and 1-butylnonyl benzene. According to Jardé et al. (2005), LABs are found as unsulphonated detergent residue and are characteristics of domestic sludges. Due to their resistance to microbial attack, LABS are recognized as molecular markers for domestic waste contribution. On the other hand, low molecular weight *n*-alkanes (from C_{15} to C_{22}) are characteristics of petroleum products or fossil organic matter. This is supported by the presence of the UCM, which can be attributed to microbially degraded petroleum residue and are characteristics of petroleum-polluted sediments [6].

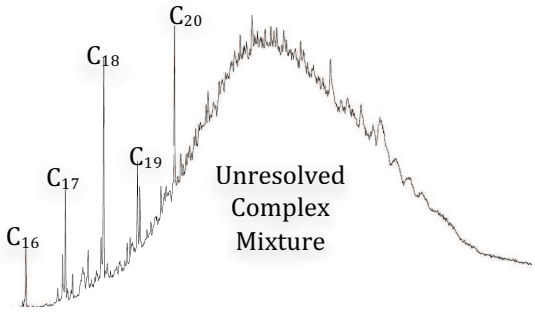


Figure 5.12 A typical total ion chromatogram of the hydrocarbon fraction from SPE of activated sludge extract showing the presence of alkanes and unresolved complex mixture.

Although the results of the hydrocarbon analysis showed contribution from petroleum products, it is still possible that some of these compounds were synthesized by activated sludge microorganisms. In a study by Moreda et al. (1998), aliphatic

hydrocarbons on domestic sludges ranging from 230 to 1420 mg/kg of dry matter were detected [80]. Some species of bacteria are known to produce small amounts of hydrocarbons (<1.0%). For example, 25% of the 5.9% cellular lipids of *Desulfovibrio* are straight chain hydrocarbons ranging from C_{15} to C_{31} while 17.4% of the 7.4% total lipids of *Pseudomonas maltophilia* are from C_{22} to C_{32} hydrocarbons. In addition to straight chain hydrocarbons, most bacteria produce trace amounts of isoprenoid hydrocarbons such as prispane, phytane and squalene [81]. Other bacteria that produce hydrocarbons include *Desulfovibrio desulfuricans, Pseudomonas flourecens, Clostridium pasteurianum, Clostridium tetanomorphum, Synechococcus elongatus, Anabaena variabilis, Micrococcus luteus, Micrococcus lysodeikiticus, Bacillus* sp., *E. coli, Mycobacterium* sp. and *Arthrobacter* sp. [82-87]. Some microorganisms from the phyla *Verucomicrobia*, *Planctomyces*, *Chloroflexi*, *Proteobacteria* and *Actinobacteria* can also produce hydrocarbons [88]. The hydrocarbons produced by these microorganisms can be either intracellular or extracellular. Furthermore, yeasts, fungal spores, fungal mycelia and algae were also reported to produce hydrocarbons [20, 81, 85, 89-91]. A broad list of microorganisms that produce intracellular and extracellular hydrocarbons is given by Ladygina et al. (2006) [85].

The formation of intracellular hydrocarbons in microorganisms is essential for the regulation of the cellular fatty acid pool [81]. Furthermore, intracellular hydrocarbons might have protective functions (i.e. promote resistance to desiccation) and control some physicochemical properties of the cytoplasmic membrane [85]. On the other hand, the extracellular hydrocarbons synthesized by microorganisms promote cell wall hydrophobicity, protecting them from extreme condition changes (i.e. high concentration of excreted acids). In most bacteria, extracellular hydrocarbons decrease glass adhesion

of the cells and promote cell aggregations [82, 85]. Bagaeva and Zinurova (2004) obtained 3.7% and 6.9% (weight of biomass) intracellular and extracellular hydrocarbons, respectively from a culture of *Clostridium pasteurianum* grown in a 10% CO_2 – 90% H_2 atmosphere [82]. In other microorganisms, hydrocarbons aid in cell development and interspecies interactions [85].

Hydrocarbons are considered to be the most stable group of compounds and are the main component of petroleum-based fuels and thus, a very advantageous target for the biofuel industry. They can be used in existing engines, refineries and distribution systems without modifications [84, 85]. Since the role of hydrocarbons in microorganisms is not fully understood, genetic engineering seems to be the only way to increase microbial production of hydrocarbons [83, 84, 92]. To date, there have been no reports of hydrocarbon production co-current with wastewater treatment plants. However, in 2001, Park and co-workers isolated a halotolerant bacterial strain (close in characteristics to *Vibrio furnissii*) from sewage, which can accumulate large amount of extracellular lipids and hydrocarbons (120% of cell dry weight). The accumulated hydrocarbons included C_{15}, C_{18}, C_{21}, C_{22} and C_{24} alkanes totaling to 50% of cell dry weight [93]. In terms of industrial production, Robertson et al. (2011) claimed to develop a genetically engineered cyanobacteria capable of producing alkanes and ethanol on a commercial scale [94].

5.3.4 Wax Esters and Free Fatty alcohols

Wax esters (WEs), waxes or cerides are another class of storage compounds that microorganisms can synthesize under stressful environment. In particular, some prokaryotes can accumulate large amount of WEs under nitrogen-limited condition when

there is an excess of carbon [95-99]. WEs contain fatty acids, which are ester-linked to long chain alcohols or fatty alcohols that can have chain length up to C_{64} [5, 100]. Accumulation of WEs have been reported involving microorganisms in the genus *Acinetobacter*, *Moraxella*, *Micrococcus*, *Fundibacter*, *Neisseria*, *Pseudomonas*, *Marinobacter*, *Corynebacterium*, *Nocardia*, *Mycobacterium* and *Rhodococcus* [96, 101]. Microbial species that are known to be WE-producers include *Aeromonas hydrophila*, *Fundibacter jadensis*, *Micrococcus cryophilus* ATCC15174, *Acinetobacter calcoaceticus*, *Rhodococcus opacus* PD630, *Rhodococcus jostii* RHAI, *Marinobacter hydrocarbonoclasticus* [ATCC 49840], *Marinobacter aquaeolei* VT8, *Pseudomonas nautical* [IP85/617], *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Mycobacterium bovis*, *Mycobacterium smegmatis*, *Streptomyces coelicolor*, *Alcanivorax borkumensis* and *Euglena gracilis* [ATCC 12716] [5, 47, 95, 96, 100-108]. For a more complete listing of microbial species, see Kalscheuer (2009). The list includes gram – negative bacteria (α-, β-, γ-, δ-Proteobacteria) and gram – positive bacteria (Actinobacteria, Bacteriodetes/Chlorobi) [109]. In the environment, waxes can be produced not only by microorganisms, but also by marine and terrestrial plants, marine animals, insects and birds [100].

Fatty alcohols (a.k.a. alkanols) normally exist in the environment as wax esters [100]. In microbial cultivation, fatty alcohols serve as intermediates during aerobic catabolism of long chain *n*-alkanes for WE biosynthesis [109, 110]. However, in microbial catabolism of detergent fatty alcohols as polyethoxylates, free fatty alcohols can potentially be found as one of the cultivation products [111].

WEs in microorganisms serve mainly as energy and carbon storage reserves during starvation. In addition, WEs also act as metabolic water reserves, buoyancy

generators, thermal insulators and as sinks for toxic or useless fatty acids during growth on recalcitrant hydrocarbons [95, 96, 100, 101]. In some microorganisms such as *Fundibacter jadensis* and some strains of *Acinetobacter* sp., production of extracellular WEs has also been reported but their functions in living microbial cells are yet to be determined [101, 112]. Microorganisms can produce WEs from a variety of carbon sources including hydrocarbons, alkanols, fatty acids, triacylglycerides and phytol [95, 96, 103, 110, 113, 114].

Analyses showed that WEs were present in the samples (Figures 5.3 and 5.5, Table 5.2). The complexity of the samples, however, made it impossible to analyze the WEs without derivatization. The methanolysis of WEs separated the fatty acid and fatty alcohol components of the molecule and independent analyses of the components were made without much interference. The fatty alcohol associated with WEs in the samples ranges from about 1.80 – 5.55 % (weight) of extract which correspond to ~0.17 – 0.90 % (weight) based on dried sludge. According to Mudge et al. (2008), due to the synthetic pathway for fatty alcohols, fatty acids should act as indicator of the likely fatty alcohols that can be found in bacteria [100]. The fatty acids present in the samples have $C_{14} – C_{24}$, peaking at $C_{14} – C_{18}$ (Figure 5.10). By looking at the fatty acid profile of the samples, it was expected that fatty alcohols from $C_{14} – C_{18}$ should be present in the samples. And as shown in Figure 5.13, this was indeed the case. Saturated $C_{14} – C_{18}$ and monounsaturated C_{16} and C_{18} fatty alcohols were found in all the samples. Furthermore, odd numbered fatty alcohols were detected (C_{15} and C_{17}), which are mainly produced by bacteria [115]. Although, this is a good indication of bacterial activity, exogenous contributions cannot be neglected since fatty alcohols may come from other sources. For example, fatty alcohols from *Ascophyllum nodosum* and *Fucus spiralis* (brown algae) contain $C_{12} – C_{28}$

range peaking at C_{14} – C_{18} [100]. The fatty alcohols of the samples may also have anthropogenic contribution. The sludge samples were obtained from a MWWTP and C_{12} – C_{18} fatty alcohols are usually used in detergent applications [3, 116]. Fatty alcohols are not completely degraded in wastewater treatment facilities, with degradation fraction ranging from 0.993 for C_6 to 0.159 for C_{22}. The remaining fraction goes to air (0.004 for C_6 to 0.000094 for C_{22}), water (0.001 for C_6 to 0.045 for C_{22}) and sludge (0.470 for C_{12} – 0.729 for C_{18}) [111]. Thus, the total fatty alcohols detected in the samples might be a sum of contributions from all these sources.

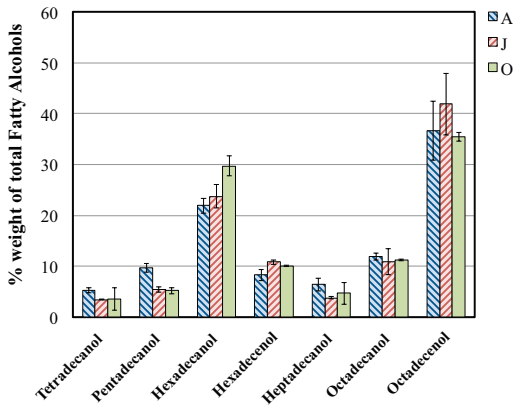


Figure 5.13 Fatty alcohol profile of wax esters isolated from activated sludge extract.

Although, as mentioned earlier, in the presence of detergent fatty alcohols, free fatty alcohols are possible to be found in microbial extracts, they were not detected on any of the samples (see Fig 5.7b). Nagao et al. (2009) studied the conversion of vegetable oils to rare fatty acids and fatty alcohols using *Aeromonas hydrophila*. They detected wax esters but not free fatty alcohols in their samples [103]. In their case, the microorganisms most likely synthesized the free fatty alcohols as precursors for WEs biosynthesis since they used vegetable oil as carbon source. As for the case of activated sludge, the same might be true. Detergent fatty alcohols especially in the form of polyethoxylates are

considered to be bioavailable [111]. Thus, the activated sludge microorganism might have used them as intermediates for WEs production, which is the main function of free fatty alcohols in living microbial cells.

WEs and fatty alcohols are important raw materials for a variety of surfactant, polymer, leather, solid coating, lubricants, toiletry, cosmetic, food and pharmaceutical products [4, 95, 110, 112, 117-119]. The major sources of natural WEs are jojoba and carnauba oils. However, due to high price of jojoba oil (~7,000 USD per ton), most commercial WEs available nowadays are of synthetic origin, which are mainly consumed by cosmetic and pharmaceutical industries [120, 121]. In the past several years, researchers have been considering other sources of WEs such as microbial (including genetically engineered microbes), and crambe and rice bran oils [5, 119, 122, 123]. Free fatty alcohols that are commercially available (i.e. Lurgi manufacturing company) are usually produced by catalytic hydrogenation of fatty acids or FAMEs at high temperature (523 – 573 K) and under high hydrogen pressure (25 – 35 MPa) [124]. They are also produced from ethylene via the Ziegler Alfol process and by hydroformylation of olefins [125].

There are no reports regarding production of WEs co-current with wastewater treatment facilities. Aside from possible applications of activated sludge WEs in different industries mentioned earlier, they could also be used as feedstock for the renewable fuel industry. The fatty acid component of the WE can be converted to biodiesel (by methanolysis) or green fuel (via catalytic cracking) [126]. The fatty alcohol component can be converted to its alkyl acetate derivative (by transesterification/transacetylation), which has been recently considered as a new class of biofuel, or to green fuel (via catalytic cracking) (see Table A.1) [127-129].

5.3.5 Steryl Esters and Free sterols

Steryl esters (SEs) and sterols are usually associated with lipids found in animals, plants, yeasts and fungi [101, 130-132]. Only a few species of bacteria are known to produce sterols. These include *Flovobacterium dehydrogenes*, *Methylcoccus capsulatus, Methylosphaera hansonii, Nannocystis exedens, Rhodococcus rhodochrous, Bacillus* sp., *Cellulomonas dehydrogenans* and *Mycobacterium smegmitis* [130, 132-137]. Most bacteria belonging to the genus *Mycoplasma* (i.e. *M. salivarium* PG-20, *M. fermentans* PG-18, and *M. canis* PG-14), are known to require sterol for growth [138]. Some bacteria such as *Streptobacillus moniliformis, Proteus mirabilis, Streptococcus pyogenes,* and *Staphylococcus aureus* can incorporate cholesterol into their cell membranes [139]. Moreover, some bacteria such as *Staphylococcus epidermis, Propionibacterium acnes* and *Propionibacterium granulosum* can esterify cholesterol if it is present in the growth medium [136]. In *Mycoplasma* species and other sterol-requiring species (i.e. *Borrelia afzelii* and *Helicobacter pyroli*), the presence of steryl glycosides (sterol with an attached sugar moiety) has also been reported [140].

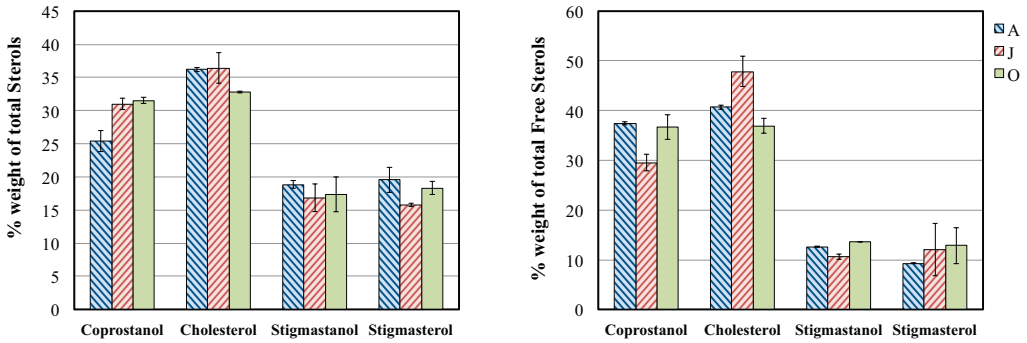


Figure 5.14 Profiles of sterols isolated from activated sludge extract. (A) Associated with steryl esters. (B) Free sterols.

Other than structural functions in sterol-requiring microorganisms, the role of sterols and SEs in living bacterial cells is not clearly understood. It has been suggested that the SEs might be involved in the transport of sterols to different parts of plants [141]. Sterols can also eliminate the thermotrophic transition (lamellar gel phase to liquid-crystalline phase) of phosphoglycerolipid bilayers. This will result in constant membrane properties such as membrane fluidity for wide temperature ranges [140]. Aside from membrane fluidity, free sterols were also suggested to have important functions in the sensitivity of yeasts to the action of polyene antibiotics [142]. In a study conducted by Grunwald (1971) on the effects of sterols, SEs and steryl glycoside on membrane permeability of barley roots, they found that free sterols particularly cholesterol and campesterol can greatly stimulate or inhibit (depending on the concentration) the permeability of the phospholipid layer of the barley root membrane. However, cholesteryl palmitate and cholesteryl glucoside did not have any effect on the membrane permeability [141]. This study, as indicated by Grille et al. (2010), suggested that free sterols might have similar effect on permeability of most biomembranes [140]. These functions of free sterols and SEs on plants, yeasts and biomembranes might be also true for bacteria.

There are four major sterols (as SEs and free sterols) present in the activated sludge samples (Figure 5.14). In both cases, coprostanol and cholesterol are the most dominant ones. Cholesterol has a variety of possible sources including animals and microalgae in addition to sewage while coprostanol is considered to be the principal indicator of mammalian sewage [115, 130]. Coprostanol is produced in the digestive track by anaerobic microbial hydrogenation of cholesterol and can comprise 24 – 89% of total sterols in human feces [6, 143]. Moreover, coprostanol might be a product of

reduction of cholesterol during the activated sludge treatment process [6, 144]. Stigmasterol together with β-sitosterol is known to be a higher plant sterol and usually associated with herbivore fecal contamination [6, 130]. As was for the case of coprostanol, the stigmastanol detected in the samples could have been produced by hydrogenation of stigmasterol during the treatment process [144].

Fecal sterols are known to be excreted in esterified form [143, 145]. Thus, the SEs in the samples could have been from the influent wastewater or due to microbial activity. On the average, the percentages of esterified sterols in the samples were 12.53%, 13.31% and 17.54% (weight of total sterols) for batch A, J and O, respectively. Based on these results, the SEs and free sterols in the samples could be a sum of contributions from two main sources, which are human and animal feces and treatment due to microbial activity.

Possible industrial application of SEs and free sterols from microorganisms could be the same as plant sterols. They can be used as starting material for steroids synthesis and steroid-based drug production, as bioactive pharmaceutical compounds, as food and nutraceutical additives and as surfactants [130]. In addition to these possible applications, SEs and free sterols from activated sludge can also be used as feedstock for renewable fuel production. This might be possible via either catalytic cracking (hydroprocessing) or pyrolysis (thermal cracking) as indicated by several studies [144, 146-150].

5.3.6 Glycerides and Free fatty acids

Triacylglycerides (TGs) are triesters of glycerol with fatty acids. They are commonly present in most eukaryotic organisms such as animals, plants, yeasts and fungi [151, 152]. In prokaryotic microorganisms, accumulation of TGs have been reported in some bacteria belonging to the genera *Mycobacterium, Rhodococcus, Micromonospora,*

Dietzia, Gordonia, Nocardia (*Streptomyces*) and *Acinetobacter* [55, 101, 151, 152]. Species that are known to accumulate TGs include *Rhodococcus opacus* PD630, *Rhodococcus opacus* DSM1069, *Rhodococcus jostii* RHAI, *Rhodococcus aetherivorans* IAR1, *Rhodococcus fascins, Rhodococcus erythropolis, Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Nocardia corallina, Nocardia globerula* 432, *Streptomyces coelicolor*, *Streptomyces lividans*, *Mycobacterium smegmatis*, *Rhodococcus ruber NCIMB 40126*, *Alcanivorax borkumensis*, *Mycobacterium tuberculosis, Dietzia maris, Gordonia amarae, Acinetobacter lwoffi* and *Acinetobacter calcoaceticus* [see Alvarez (2006) and Alvarez and Steinbüchel (2002) for more complete listings] [38, 44, 47, 101, 103-106, 151-158]. These microorganisms can use a wide range of carbon sources such as wastewaters, sugars, vegetable oils, food wastes, hydrocarbons and halogenated aliphatics and aromatics [32, 154, 155, 158, 159].

The accumulation of TGs is usually triggered when a carbon source is available in excess in a nitrogen-limited environment. However, it has been also suggested that TG-accumulations in bacteria can be accomplished under limited aeration conditions [151]. Similar to PHAs and WEs, the main function of TGs is as carbon and energy reserve compound. In addition to this, TG serve as a sink for reducing equivalents, as a reservoir of metabolic water, as a means of adjusting membrane fluidity by regulating the fatty acid pool of the membrane lipids, as a raw material for phospholipids biosynthesis, as acceptor for toxic or unwanted fatty acids, as a means of balancing cell metabolism depending on environmental conditions by reducing pyridine nucleotides in the cells and as precursor for antibiotics and mycolic acids biosynthesis [151, 152].

Diacylglycerides (DGs) and monoacylglycerides (MGs) serve mainly as intermediates for synthesis of TGs and phospholipids [142, 151]. They are usually

present in minute but detectable concentrations [160]. In a study conducted by Wältermann et al. (2000) on lipid accumulation of *Rhodococcus opacus* PD630, they detected DGs and MGs along with TGs and free fatty acids. However, the concentrations of DGs, MGs and free fatty acids were almost negligible relative to the TGs [105]. According to Alvarez (2006), free fatty acids are biologically toxic and hence they do not occur in living cells in high quantity [151]. DGs and MGs being intermediate compounds and free fatty acids being toxic are probably the reason why they occur in very small quantities in living cells. Furthermore, DGs, MGs and free fatty acids could be products of TGs and phospholipids degradations possibly during sample extraction, preparation and storage.

The result of the analysis of extract from activated sludge showed the presence of free fatty acids, MGs, DGs and TGs (Figures5.3b and 5.4c-d). As mentioned, the presence of DGs and MGs might have been due to sample extraction, preparation and storage. The same might be true about the presence of high proportion of free fatty acids in the samples. The extract from activated sludge contains 2 – 3% (weight of extract) TGs (Fraction 3) which yielded FAMEs in the same range. On the other hand, the FAMEs obtained from Fraction 4 (Free Fatty Acids, DGs and MGs) were 11 – 13% (weight of extract), which constitute to more than 50% of total FAMEs obtained (Table 5.2). The BDE uses chloroform, methanol and water as solvents. In the presence of water and methanol, TGs, DGs and MGs (even WEs, SEs and phospholipids) can undergo hydrolysis and methanolysis, respectively. Aside from the occurrence of high proportion of free fatty acids, this is also evident by the presence of fatty acid alkyl esters in the samples [Figure 5.7a (lanes A2, J2 and O2), Figure 5.7b (lanes A4, J4 and O4) and Figure 5.8b].

Glycerides are probably the most important basic oleochemicals including free fatty acids, fatty acid alkyl esters, fatty alcohols and fatty amines [125]. Generally, possible applications of bacterial glycerides and fatty acids may be the same as that of vegetable sources, which include soaps, detergents, plastics, personal care products, resins and lubricants. Among these possible applications, glycerides and fatty acids from activated sludge might be well suited as renewable fuel feedstock either via alcoholysis or catalytic cracking.

5.3.7 Phospholipids

Like most biological membranes, bacterial membranes consist of a lipid bilayer. For gram-negative bacteria, in general, their outer membrane contains 25% phospholipids with 75% phosphatidylethanolamine, 20% phosphatidylglycerols and 5% cardiolipin [161]. These phospholipids are also present in gram-positive bacteria but in different proportions. For example, phospholipids from *Bacillus megaterium* contains 16% phophatidylethanolamine, 40% phosphatidylglycerols, 40% cardiolipin and 4% other [162]. However, the compositions and even the amount of phospholipids present in microorganisms are dictated by environmental conditions such as nutrient deficiency (C, N, Na and Mn) and temperature. Mn deficiency has been reported to reduce phospholipid content of *Brevibacterium ammoniagenes*. N-/C-/Na-limitation affects the composition of phospholipids in *Rhodotorula glutinis* and *Staphylococcus aureus*. As for temperature, unsaturation of phospholipid fatty acids increases with decreasing temperature as has been reported for *Neurospora crassa* and *Paecilomyces persicinus* [163].

In most cells, phospholipids play a vital role in cellular structure and functions. They also have a function in transport of important cellular material such as protein and

they regulate materials coming in and out of the cell [164, 165]. The yields of FAMEs from the phospholipid fraction of the sludge extract ranges 0.30 – 0.70% (weight extract) (Table 5.2). This range corresponds to 0.03 – 0.07% (weight dry sludge). These results are within the range obtained by Forney et al. (2001) on their study about fatty acids associated with activated sludge phospholipids obtained from different wastewater treatment facilities in the United States. They obtained a range of 0.40 – 15.3 nmol fatty acids/mg dry biomass which is equivalent to (as stearic acid) 0.01 – 0.44% (weight dry biomass) [166].

Phospholipids can be used as a source of oleochemical fatty acids, which can be utilized for the production of fatty alcohols, biofuels and other useful products. They can be utilized for production of polymerizable phospholipids, which can be used in biomedical and microelectronic applications [167]. Individually, phospholipids are nutritious, biodegradable, biocompatible and a good source of organic phosphate and choline. As a group, phospholipids can form supramolecular structures that self-assemble. Furthermore, phospholipids can spontaneously self-associate into bilayer membranes that can separate compartments of the same aqueous phase from each other geographically. The resulting structures from this self-association have predictable properties. Due to these properties of phospholipids, they are widely used in different industrial applications (i.e. paints, magnetic recording media, controlled microparticle crystallization), molecular biology (as genetic material carrier) and food technology (i.e. accelerated cheese ripening, reduction of bacterial spoilage and encapsulation of antioxidants) [168].

5.4 Conclusions

The Bligh & Dyer extracts of activated sludge obtained from Tuscaloosa, AL, U.S.A. were analyzed for major bacterial storage compounds. Due to the diversity of microbial community present in the sludge, all types of storage compounds were detected including PHAs, WEs, SEs and TGs. The PHAs were isolated and analyzed using a precipitation technique. A SPE technique utilizing silica column was then developed to separate different compound classes from the PHA-free extract. As far as the author know, this SPE technique cannot be found anywhere in the literature.

The input of PHAs in the activated sludge process is highly likely to be negligible and thus all the PHAs present in the sludge are due to microbial activity. Although there is a very high possibility that the WEs and TGs present were produced by activated sludge microorganisms, the probability of exogenous contributions may not be neglected. As for SEs, their occurrence in the sludge can be accounted mainly from anthropogenic contributions. Regardless of the source of these compounds, their availability in the sludge offers a wide range of applications in the renewable fuel and oleochemical industries. The results also explain the high gravimetric yield (~13 – 15%weight) obtained from Chapter III on the in situ transesterification of activated sludge. Other compounds, particularly fatty alcohols and sterols, were also extracted during the process resulting in a high gravimetric yield.

5.5 References

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CHAPTER VI

ANALYSIS OF LIPID STORAGE COMPOUNDS IN ENHANCED ACTIVATED SLUDGE MICROORGANISMS

6.1 Introduction

The utilization of activated sludge from different wastewater treatment facilities across the United States may be solidified and can be made economically feasible by addressing several issues. These issues are mainly due to the different factors listed below. These factors could result in wide variations in types and amounts of compound classes that may be obtained from activated sludges.

1. Low lipid yields.
2. Existing wastewater treatment facilities are treating specific type of wastewaters (i.e. domestic, food, agricultural, clinical and industrial).
3. Due to differences in type of wastewaters, facilities were configured in different ways (i.e. conventional activated sludge, oxidation ditch, trickling filter, rotating biological contactors, etc.).
4. Differences in microorganisms present in the biological treatment unit.

Mondala et al. (2011) proposed a modification of existing wastewater treatment facilities that can possibly address these issues. The proposed concept, which is shown in Figure 6.1, involved an additional lipid-accumulation unit where the waste activated sludge from a plant, is subjected to environmental condition (stressed condition) that facilitates lipid production. Results of their batch fermentation experiments using glucose

and ammonium sulfate as carbon and nitrogen sources, respectively, showed that maximum lipid yield of 17.5 ± 3.9% (cell dry weight) can be obtained at a glucose loading of 60 g/L with a corresponding carbon:nitrogen mass ratio of 70:1. At this fermentation condition, they obtained a biodiesel yield of 10.2 ± 2.0% (cell dry weight) [1]. The activated sludge that they used as seed for fermentation was obtained from Tuscaloosa, AL, U.S.A., which is the same plant where the samples used for Chapters III-V were taken. The results obtained in Chapter III and by other workers on the biodiesel production from activated sludge obtained from this facility showed a yield from 3-6% (dry sludge weight) [2, 3]. Based on the results of economic analysis conducted in Chapter III, a yield of at least 10% (sludge dry weight) is necessary for this feedstock to be economically viable. Lipid enhancement shown in Figure 6.1 can be one strategy to achieve the required biodiesel yield. A portion of the wastewater input to the plant may be used as carbon and nutrient source. However, to induce lipid-accumulation, additional carbon and nutrient sources might be needed. This might affect the economics of this feedstock negatively. This negative effect might be compensated by using relatively inexpensive carbon sources (i.e. lignocellulosic materials). In the United States alone, approximately 1.3 billion tons per year of lignocellulosic biomass could be used sustainably for biofuel production [4].

Oil accumulation is highest for a group of microorganisms called “oleaginous” species. These species are capable of accumulating oil more than 20% of their biomass weight and are mainly species of yeast, fungi and a few bacteria [5]. Standard activated sludge contains mostly heterotrophic bacteria totaling to about 10^8 colony-forming unit (CFU) per milligram. Commonly, these bacteria belong to phyla Proteobacteria, Bacteriodetes and Actinobacteria [6]. As discussed in Chapter V, bacteria can accumulate

a wide range of compounds depending on strain, carbon source and environmental condition. However, for most application, both in biofuel and oleochemical industries, triacylglycerides are the most favorable target group of compounds. Only bacteria belonging to the actinomycetes group including *Mycobacterium*, *Rhodococcus*, *Nocardia* and *Streptomyces* are known to accumulate large amounts of triacylglycerides, which serve as storage reservoirs for energy and carbon [7-9] (see Chapter V, Section 5.3.6 for specific bacterial species).

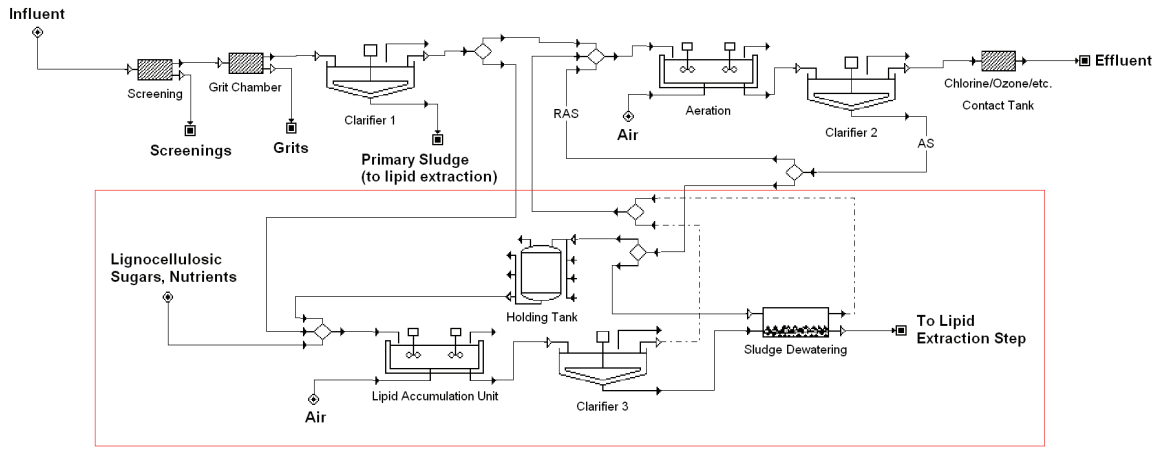


Figure 6.1 Proposed modification (red rectangle) of wastewater treatment facilities for lipid enhancement of activated sludge. Redrawn from reference [1].

In this chapter, the modification of existing wastewater treatment infrastructures (Figure 6.1) was evaluated. Lipid enhancements were applied on two activated sludges from conventional and oxidation ditch treatment configurations. For evaluation purposes, glucose was used as sole carbon source. Different compounds present in the sludge before and after lipid enhancement were analyzed using the method developed in Chapter V. This was conducted to determine the effect of enhancement on the quality of lipids that can be obtained.

6.2 Materials and Methods

6.2.1 Activated Sludge Collection and Preparation

Samples were collected from MWWTPs in Tuscaloosa, AL, U.S.A. (capacity: 30 million gallons per day) and Tupelo, MS, U.S.A. (capacity: 10.50 million gallons per day) during normal plant operations. The Tuscaloosa plant utilizes a conventional activated sludge treatment configuration (Chapter I, Figure 1.9), while the one in Tupelo utilizes an oxidation ditch (Chapter I, Figure 1.10). Samples were collected in 1-L plastic containers from the return activated sludge line of the Tuscaloosa plant and from the effluent of the oxidation ditch unit of the Tupelo plant and were transported in ice-bath. The collected samples from a plant were mixed and homogenized after which a portion was transferred in 1-L Thermo Scientific Nalgene Culture Vessel (Fisher Scientific, Pittsburgh, U.S.A.) maintained with agitation and aeration at ambient temperature.

6.2.2 Fermentation

The fermentation was conducted for seven (7) days using a synthetic wastewater as cultivation medium. Glucose and ammonium sulfate were used as carbon and nitrogen sources, respectively and the complete composition of the medium is presented in Tables 6.1 and 6.2. Fermentation parameters and conditions were based on the procedure by Mondala (2010) [10]. A carbon loading of 60 g/L (as glucose) and a nitrogen loading of 1.62 g/L (as ammonium sulfate) were used giving a C:N mass ratio of 70:1. Prior to fermentation, the medium was autoclaved at 121ºC and 240kPa for 20 minutes. As suggested by Mondala et al. (2011), glucose solution was autoclaved separately to prevent caramelization and was then combined with the rest of the medium components [1].

Table 6.1 Formulation of synthetic wastewater.*

Component	Concentration (g/L de-ionized H_2O)
Glucose	60
$(NH_4)_2SO_4$	1.62
Gelatin	0.15
Starch	0.07
Yeast extract	0.07
Casamino acids	0.01
KH_2PO_4	1.50
NaH_2PO_4	1.00
Trace Mineral Supplement	5 (mL/L)

*Mondala, 2010 [10]: based on formulation of Ghosh and LaPara, 2004 [11].

Table 6.2 Trace mineral supplement formulation.*

Component	Concentration (g/L de-ionized H_2O)
EDTA	0.50
$MgSO_4 \cdot 7H_2O$	3.00
$MnSO_4 \cdot H_2O$	0.50
NaCl	1.00
$FeSO_4 \cdot 7H_2O$	0.10
$CaCl_2$ (anhydrous)	0.10
$ZnSO_4 \cdot 7H_2O$	0.10
$CuSO_4 \cdot 5H_2O$	0.01

*Mondala, 2010 [10]: based on Wolfe's trace mineral supplement formulation [12].

Fermentation experiments were conducted using two 5-L BIOFLO 310 Bioreactors (New Brunswick Scientific, Edison, NJ, U.S.A.). Six hundred milliliters (600 mL) of activated sludge was inoculated to 2.4 L of sterile medium giving a total cultivation volume of 3 L. No initial pH adjustments were done and throughout the experiments, only the cultivation temperature was monitored and controlled (25 ± 1ºC). The bioreactors were equipped with a platinum resistance temperature detector (RTD) for

temperature monitoring and with a water jacket for temperature control. Foaming was minimized by using diluted (1:10) nonoil, polypropylene-based Antifoam 204 concentrate (Sigma–Aldrich, St. Louis, MO, U.S.A.). An aeration rate of 1 vvm (volume of air per volume of media per minute) was applied. The supplied air was pre-treated by filtration using a 0.45-µm HEPA vent filter (Whatman, Kent, U.K.). Agitation rate was at 300 rpm for the first 24 hours and was then increased to 400 rpm for 24 hours and was set at 500 rpm for the rest of the fermentation experiment. This was done to maintain a minimum dissolved oxygen level of 20% saturation throughout the experiment [1].

6.2.3 Biomass Recovery, Extraction and Analysis

Samples (~35 mL) were taken at the start and conclusion of the fermentation experiments. These samples were used for the determination of biomass and lipid concentrations. The samples were centrifuged at 3000 rpm for 20 minutes and the supernatants were discarded. The concentrated solids were frozen at -18 ºC using a ColdTech freezer and freeze-dried in a Freezone 6 Bulk Tray freeze dry system (Labconco, Kansas City, MO, U.S.A.). The weights of the solids were recorded and were used to calculate biomass concentration. The dried solids were then subjected to BDE to determine the gravimetric lipid yields following the protocol discussed in Chapter IV.

The remainder of the fermentation broth was recovered and was also subjected to centrifugation and freeze-drying as was mentioned above. The BDE was then conducted in 1-L stirred glass reactor as was done in Chapter V. Analysis of different lipidic material present in the extract was conducted utilizing the protocol developed in the same chapter.

6.3 Results and Discussion

The evaluation of using wastewater treatment facilities as source of feedstock for biofuels and oleochemicals production was conducted in Chapter V. However, the profiles of compounds that may be obtained from activated sludge may vary with respect to wastewater type and process configurations. This might affect the consistency of resulting products (i.e. biodiesel) and might require different downstream processing strategies.

Three batches of activated sludges were collected from each of the Tuscaloosa and Tupelo wastewater treatment facilities. They were collected in the months of October, November and February (coded O, N and F, respectively) during the plants' normal operations. The enhancements of the sludges were conducted and all the sludges were analyzed using the protocol developed in Chapter V. The PHAs analysis of the sludges showed significant reduction after enhancement. Furthermore, for both raw and enhanced sludges, only two hydroxyacid monomers were detected which are hydroxybutyric (HB) and hydroxyvaleric (HV) acids. Regardless of the source of the raw sludges, the PHAs content were statistically similar (Tables 6.3 and 6.4). Although the two plants utilize different treatment configurations, their biological treatment units are both aerated. According to Takabatake et al.(2002) PHA production in activated sludge is more affected by influent characteristics than activated sludge operating conditions [13]. It is also a well established fact that microorganisms produce PHAs under anaerobic condition [14]. Furthermore, production of large quantities of PHAs require high concentration of phosphates in the influent wastewater and thus usually happens during biological phosphorus removal [15]. For the raw sludge from Tuscaloosa, the ratio of HB:HV was 1.20 while for raw sludge from Tupelo, a ratio of about 2.00 was obtained.

The difference could be due to the influent wastewater characteristics. Unlike the Tuscaloosa plant, the Tupelo plant does not have primary treatment unit(s). As was discussed in Chapter V, the ratio of PHA monomers are greatly affected by concentrations of and types of volatile fatty acids as well as other carbon sources present in the influent wastewater [16]. Although both plants are treating domestic wastewaters, the absence of primary treatment as for the case of Tupelo plant could have caused the differences on the ratio of PHA monomers.

The solid phase extractions were conducted following the protocol developed in the preceding Chapter. However, due to high concentration of triacylglycerides in the enhanced sludges, the amount of samples loaded on silica columns were reduced to about 10 – 15 mgs. If one looks at Figures 6.2 and 6.3, the types of compounds present in raw sludges were similar regardless of the source. However, the concentrations of these compounds were different for the two plants (Tables 6.3 and 6.4). Again these could be accounted for the differences in configurations of the two treatment plants.

Table 6.3 Composition of lipid extract from Tuscaloosa, AL, U.S.A. raw and enhanced activated sludge.

	N		**O**		**F**	
Sludge Collection Date	6-Oct-10	14-Oct-10	29-Nov-10	7-Dec-10	15-Feb-11	23-Feb-11
Aeration Basin Temperature, °C	24.50	25 ± 1	20.80	25 ± 1	15.60	25 ± 1
ΔBiomass Concentration, mg/mL	5.73 ± 0.63		8.61 ± 0.28		5.32 ± 0.22	
Bligh & Dyer extract yield, % dry sludge weight	8.27 ± 0.99	15.57 ± 0.40	9.07 ± 0.24	19.68 ± 0.34	9.60 ± 0.81	15.11 ± 0.33
Total FAMEs yield, % weight of extract	20.47 ± 1.04	17.08 ± 1.20	19.72 ± 1.06	29.37 ± 1.76	21.05 ± 0.39	31.93 ± 1.22
Total FAMEs yield, % weight of solid	1.69 ± 0.22	2.66 ± 0.20	1.79 ± 0.11	5.78 ± 0.36	2.02 ± 0.17	4.82 ± 0.21
PHAs, % weight of extract	2.64 ± 0.76	1.99 ± 0.13	1.81 ± 0.73	1.24± 0.28	3.01 ± 1.07	0.68 ± 0.24

Table 6.3 (Continued)

FRACTION 1						
Hydrocarbons, ppm (based on weight extract)	3.25 ± 1.88	-	5.26 ± 2.63	-	2.39 ± 0.96	-
FRACTION 2						
Fatty Alcohol (from WEs), % weight of extract	0.51 ± 0.10	-	0.35 ± 0.05	-	1.20 ± 0.12	-
Sterols (from SEs), % weight of extract	0.18 ± 0.06	-	0.14 ± 0.01	-	0.26 ± 0.03	-
FAMEs yield, % weight of extract	1.78	1.00	1.94	0.50	2.95	1.12
FRACTION 3						
Triacylglycerides, % weight of extract	1.76 ± 0.02	11.37 ± 0.95	1.21 ± 0.20	17.82 ± 0.84	2.10 ± 0.55	18.20 ± 0.21
FAMEs yield, % weight of extract	1.77	11.42	1.22	17.90	2.11	18.34
FRACTION 4						
Free Sterols, % weight of extract	1.35 ± 0.08	-	1.38 ± 0.96	-	2.01 ± 0.14	-
FAMEs yield, % weight of extract	5.29	3.70	6.90	8.99	8.30	10.60
FRACTION 5						
FAMEs yield, % weight of extract	11.62	0.97	9.66	1.98	7.69	1.87

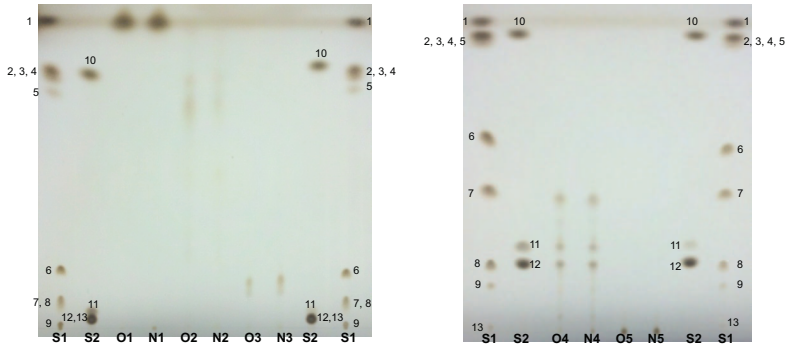
Table 6.4 Composition of lipid extract from Tupelo, MS, U.S.A. raw and enhanced activated sludge.

	N		**O**		**F**	
Sludge Collection Date	6-Oct-10	14-Oct-10	29-Nov-10	7-Dec-10	15-Feb-11	23-Feb-11
Aeration Basin Temperature, °C	26.00	25 ± 1	20.00	25 ± 1	15.00	25 ± 1
ΔBiomass Concentration, mg/mL	9.90 ± 0.14		10.37 ± 0.58		10.07 ± 0.29	
Bligh & Dyer extract yield, % dry sludge weight	6.40 ± 1.25	15.69 ± 0.45	8.05 ± 0.21	18.20 ± 0.74	7.25 ± 0.21	16.42 ± 0.22
Total FAMEs yield, % weight of extract	27.82 ± 1.08	40.02 ± 1.13	27.29 ± 0.29	30.20 ± 1.16	25.78 ± 0.96	37.08 ± 2.15
Total FAMEs yield, % weight of solid	1.78 ± 0.35	6.27 ± 0.25	2.19 ± 0.06	5.94 ± 0.31	1.87 ± 0.09	6.09 ± 0.36
PHAs, % weight of extract	4.29 ± 1.37	3.00 ± 1.52	1.87 ± 0.41	0.52 ± 0.04	3.10 ± 0.52	1.95 ± 0.88

Table 6.4 (Continued)

FRACTION 1						
Hydrocarbons, ppm (based on weight extract)	1.25 ± 0.21	-	2.67 ± 1.11	-	4.23 ± 2.00	-
FRACTION 2						
Fatty Alcohol (from WEs), % weight of extract	0.84 ± 0.01	-	1.08 ± 0.06	-	2.25 ± 0.98	-
Sterols (from SEs), % weight of extract	0.19 ± 0.05	-	0.27 ± 0.04	-	0.32 ± 0.10	-
FAMEs yield, % weight of extract	2.07	0.68	2.13	0.82	3.21	0.34
FRACTION 3						
Triacylglycerides, % weight of extract	1.00 ± 0.01	23.83 ± 0.81	1.72 ± 0.22	21.83 ± 0.15	1.97 ± 0.16	20.09 ± 1.01
FAMEs yield, % weight of extract	1.01	23.93	1.72	21.93	1.98	20.19
FRACTION 4						
Free Sterols, % weight of extract	1.02 ± 0.05	-	1.11 ± 0.18	-	1.52 ± 0.25	-
FAMEs yield, % weight of extract	17.70	12.10	18.58	2.78	15.55	14.24
FRACTION 5						
FAMEs yield, % weight of extract	7.04	3.30	4.86	4.67	5.04	2.31

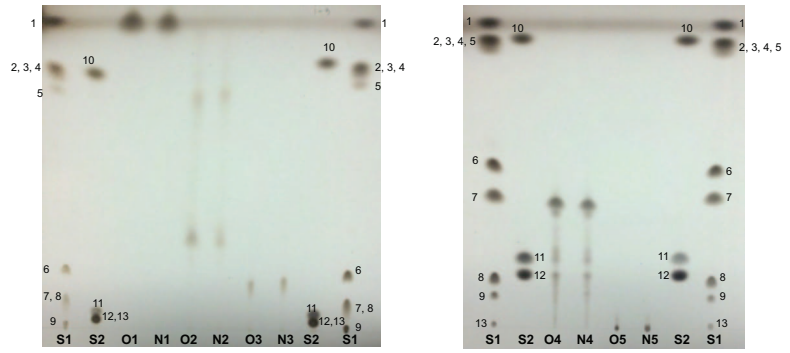
Figures 6.4 and 6.5 show the thin layer chromatography analysis on the lipid-enhanced sludges from the two plants. It can be seen that after enhancement hydrocarbons were not detected in any of the samples (Figures 6.4 and 6.5: lanes O1 and N1). The same is true for wax esters and steryl esters (Figures 6.4 and 6.5: lanes O2 and N2). However, analysis indicated that there were FAMEs present on Fraction 2 (wax esters and steryl esters). This could be accounted from the BDE of the lipid-enhanced activated sludges. As mentioned before, methanol was one of the extraction solvents, which could have resulted in methanolysis of lipids present in the samples. This was also observed in the analyses conducted in Chapter V.



(a) (b)

Figure 6.2 Thin layer chromatography of fractions from solid phase extraction of PHA-free extract from raw activated sludge obtained from Tuscaloosa, AL, U.S.A.*

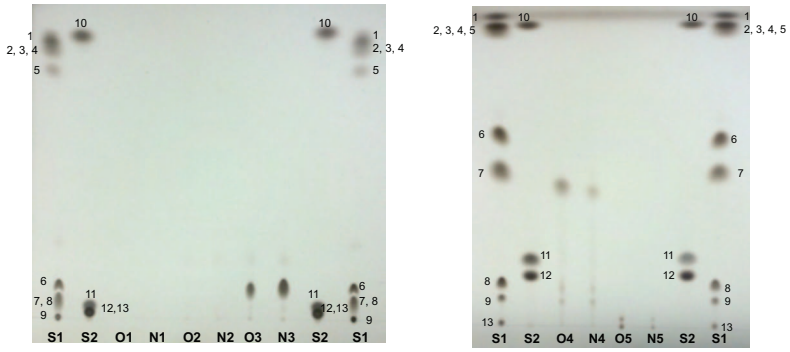
*(a) Fractions 1, 2and 3, developed in hexane/diethyl ether (94/6). (b) Fractions 4 and 5, developed in hexane/diethyl ether/acetic acid (85/15/2). S1 and S2 are standard mixtures: (1) *n*-octacosane, (2) behenyl oleate, (3) behenyl stearate, (4) palmityl palmitate, (5) lauryl palmitate, (6) triolein, (7) palmitoleic acid, (8) 1, 2-diolein, (9) monoolein, (10) cholesteryl myristate, (11) 1-hexadecanol, (12) cholesterol and (13) phospholipid standard mixture.



(a) (b)

Figure 6.3 Thin layer chromatography of fractions from solid phase extraction of PHA-free extract from raw activated sludge obtained from Tupelo, MS, U.S.A.*

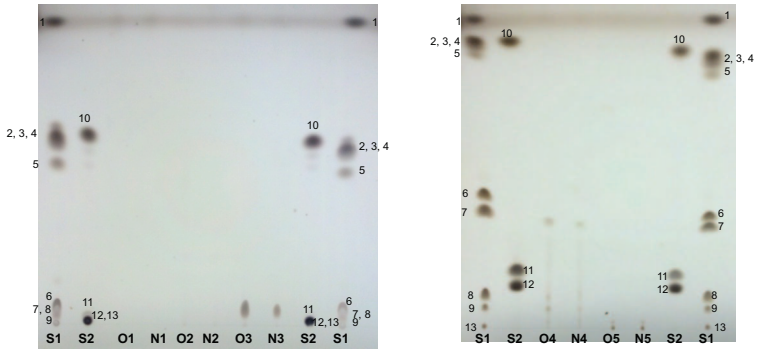
*(a) Fractions 1, 2and 3, developed in hexane/diethyl ether (94/6). (b) Fractions 4 and 5, developed in hexane/diethyl ether/acetic acid (85/15/2). S1 and S2 are standard mixtures: (1) *n*-octacosane, (2) behenyl oleate, (3) behenyl stearate, (4) palmityl palmitate, (5) lauryl palmitate, (6) triolein, (7) palmitoleic acid, (8) 1, 2-diolein, (9) monoolein, (10) cholesteryl myristate, (11) 1-hexadecanol, (12) cholesterol and (13) phospholipid standard mixture.



(a) (b)

Figure 6.4 Thin layer chromatography of fractions from solid phase extraction of PHA-free extract from lipid-enhanced Tuscaloosa activated sludge.*

*(a) Fractions 1, 2and 3, developed in hexane/diethyl ether (94/6). (b) Fractions 4 and 5, developed in hexane/diethyl ether/acetic acid (85/15/2). S1 and S2 are standard mixtures: (1) *n*-octacosane, (2) behenyl oleate, (3) behenyl stearate, (4) palmityl palmitate, (5) lauryl palmitate, (6) triolein, (7) palmitoleic acid, (8) 1, 2-diolein, (9) monoolein, (10) cholesteryl myristate, (11) 1-hexadecanol, (12) cholesterol and (13) phospholipid standard mixture.



(a) (b)

Figure 6.5 Thin layer chromatography of fractions from solid phase extraction of PHA-free extract from lipid-enhanced Tupelo activated sludge.*

*(a) Fractions 1, 2and 3, developed in hexane/diethyl ether (94/6). (b) Fractions 4 and 5, developed in hexane/diethyl ether/acetic acid (85/15/2). S1 and S2 are standard mixtures: (1) *n*-octacosane, (2) behenyl oleate, (3) behenyl stearate, (4) palmityl palmitate, (5) lauryl palmitate, (6) triolein, (7) palmitoleic acid, (8) 1, 2-diolein, (9) monoolein, (10) cholesteryl myristate, (11) 1-hexadecanol, (12) cholesterol and (13) phospholipid standard mixture.

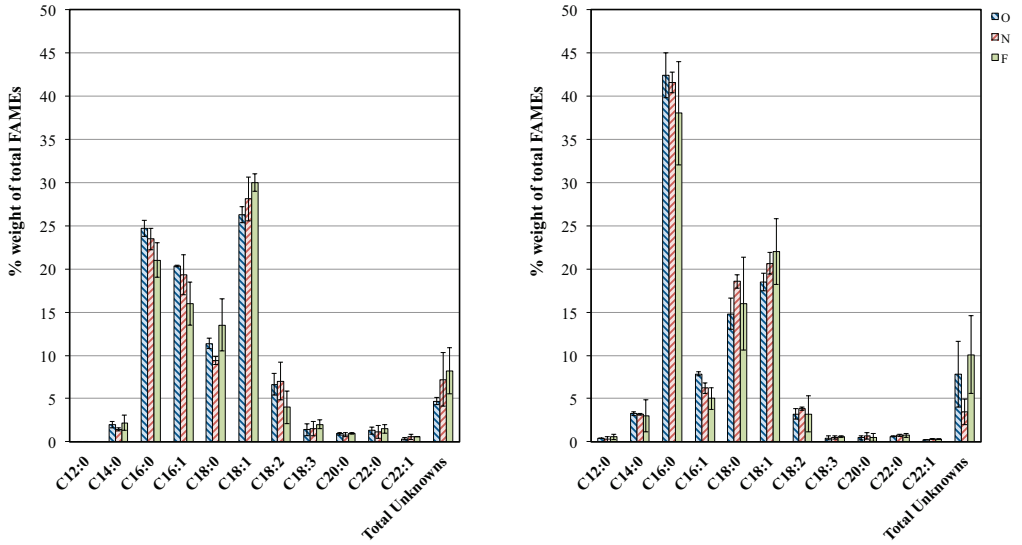
The undetected levels of hydrocarbons, wax esters, steryl esters and free sterols in lipid-enhanced activated sludges could be accounted to several factors. The most obvious one is the switch in carbon sources. As discussed in Chapter V, the presence of unresolved complex mixture in the hydrocarbon fraction of the raw sludges can be attributed to microbially degraded petroleum residue and are characteristics of petroleum-polluted sediments [17]. Since the enhancement of the sludges used glucose as sole carbon source, the input of petroleum products residue was eliminated and thus hydrocarbons were not detected in the samples. The same is true for wax esters, steryl esters and free sterols. The wax esters in the raw sludges could be due to possible presence of detergent fatty alcohols in the influent wastewaters of the two plants. Detergent fatty alcohols are considered bioavailable and might have been used by raw activated sludge microorganisms for wax ester synthesis [18]. The input of these detergent fatty alcohols was eliminated and thus, waxes were not detected in the lipid-enhanced activated sludges. As for the case of steryl esters and free sterols, their presence in the raw sludges can be accounted mainly due to anthropogenic contributions, particularly human feces. Cessation of such contributions resulted to undetected level of steryl esters and free sterols on the resulting sludge. However, one might ask what happened to these compounds (hydrocarbons, wax esters, steryl esters and free sterols) initially present in the raw sludges, which were used as fermentation seeds. It is highly unlikely that the microorganisms consumed these compounds for growth instead of glucose. After the 7-day fermentation, the glucose concentration in the broth was still above 20 g/L, which suggest that these compounds would still be present in the lipid-enhanced activated sludges. The raw sludge seed was only 20% (volume) of the total fermentation volume. This resulted to dilution of these compounds to a level that cannot

be detected. Another way to look at this dilution effect is by considering the change in biomass concentration as a result of enhancement. On the average, the biomass of the Tuscaloosa sludges increased by 6.55 mg/mL while that of the Tupelo increased by 10.11 mg/mL (Tables 6.3 and 6.4). This indicates that an inert material initially present in the raw sludges will be diluted by 1:7.55 and 1:11.11 for Tuscaloosa and Tupelo sludges, respectively.

As was discussed in Chapter V, the ability of raw activated sludge microorganisms to synthesize hydrocarbons, wax esters, steryl esters and free sterols cannot be neglected. Thus, their absence in the lipid-enhanced activated sludges may be due to microbial population shift brought about by the changes in carbon source and/or cultivation condition. Recent study on the lipid-enhancement of activated sludge from municipal wastewater treatment facility indicated significant changes in microbial population. At the end of the 7-day fermentation period, the pH of the broth decreased from 6.50 to 2.00 and 99.5% of bacterial population shifted to α-Proteobacteria [1]. Sequencing showed that these bacteria are similar in characteristics to *Acidomonas methanolica*, an acidotolerant bacteria [19]. The decrease in pH could have caused the shift in bacterial population, favoring the ones that can survive under acidic environment.

Figure 6.6 shows the fatty acid profile of the lipids in the raw sludges. It can be seen that within a plant, the profiles are significantly similar. Also, between the two plants considered, the fatty acids present were similar, ranging from $C_{12:0}$ to $C_{22:1}$. However, between the two plants the concentrations of fatty acids were significantly different particularly those of C_{16}s and C_{18}s fatty acids. The main purpose of primary treatment (clarifier) in a wastewater treatment facility is for removal of settleable and floatable solids. It is also in this section where oil and grease are skimmed along with

other floatable materials [20]. The amount of lipids (oil, grease, fats and fatty acids) in most municipal wastewater amounts to about 30 – 40% of its total chemical oxygen demand. Studies on their fate in biological waste treatment indicated that in addition to biodegradation, they are also adsorbed by the biomass [21]. Thus, the differences in concentrations of compounds present (i.e. fatty acids) in the raw activated sludges could be attributed to the absence of primary treatment unit(s) of the Tupelo plant.

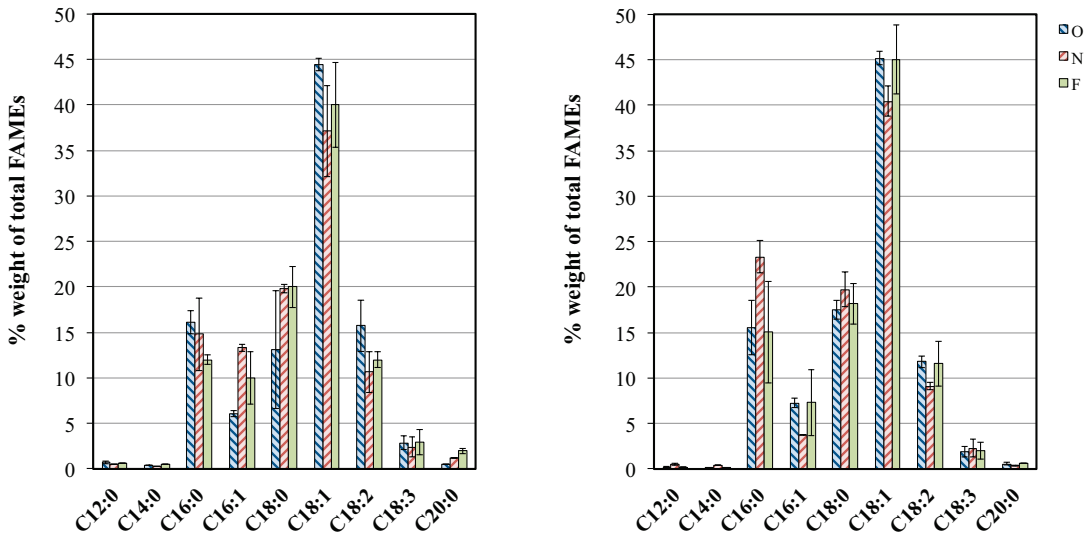


(a) (b)

Figure 6.6 Fatty acid profile of lipids extracted from raw activated sludge. (a) Tuscaloosa, AL, U.S.A. (b) Tupelo, MS, U.S.A.

Enhancement of the sludges resulted to homogenization of the lipidic compounds associated with microbial biomass. This is evident in the fatty acid profiles of the lipids in enhanced sludges (Figure 6.7). Regardless of the source of the raw sludges, the lipid-enhancement resulted to an almost similar fatty acid profile. Furthermore, analysis of the lipid-enhanced sludges indicated that 57 – 67% and 60 – 73% of the FAMEs for Tuscaloosa and Tupelo, respectively, were coming from triacylglycerides. This was a

very significant improvement considering that the FAMEs from triacylglycerides fractions of raw activated sludges ranges about 6 – 10% and 4 – 8% for Tuscaloosa and Tupelo, respectively (Tables 6.3 and 6.4). However, the yield of total FAMEs (2.5 – 6.0% and 6.0 – 6.5% dry sludge weight for enhanced Tuscaloosa and Tupelo sludges, respectively) were significantly lower than what previous workers obtained (10.2 ± 2.0% dry sludge weight) at the same cultivation condition (Tables 6.3 and 6.4, [10]) . This could be due to losses during sample preparation and handling or to inherent variability of raw activated sludge samples within a plant. Nevertheless, the total FAMEs of the enhanced activated sludges are significantly higher than that of the raw activated sludges (1.5 – 2.0% and 1.8 – 2.2% dry sludge weight for Tuscaloosa and Tupelo sludges, respectively (Tables 6.3 and 6.4).



(a) (b)

Figure 6.7 Fatty acid profile of lipids extracted from enhanced activated sludge. (a) Tuscaloosa, AL, U.S.A. (b) Tupelo, MS, U.S.A.

6.4 Conclusions

Lipid-enhancement of activated sludges from two WWTPs via fermentation was conducted for the purpose of increasing the amount of saponifiable lipids. Fermentation experiments were conducted at conditions previously identified to trigger accumulation of lipids. This was also conducted to determine if activated sludge fermentation could be a possible strategy for successful utilization of existing WWTPs as biorefineries.

Two WWTPs were considered for the study, one utilizes a conventional activated sludge process while the other one uses an oxidation ditch configuration. Results indicated that fermentation increases the amount of saponifiable lipids in the activated sludges irrespective of their WWTP source. Most of these saponifiable lipids are associated with triacylglycerides, which is the ideal lipid compound class for biofuel and oleochemical production. Furthermore, the fatty acid profiles of the enhanced sludges were similar indicating that this strategy can homogenize activated sludges from different WWTPs. This solidifies the concept of converting existing WWTPs into biorefineries that can provide significant amount of high quality lipids for various applications.

6.5 References

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CHAPTER VII

ELUCIDATION OF REACTION PATHWAY FOR THE HETEROGENEOUS CRACKING OF A SATURATED FATTY ALCOHOL OVER H^+ZSM5 CATALYST

7.1 Introduction

Hydrocarbon cracking on acid catalysts is a well-established and critically important industrial process. Since its development in 1930s, it has undergone many improvements both in reactor configurations and catalyst formulations. Natural clays were the catalyst initially used for hydrocarbon cracking. Even then, it was well understood that cracking happens on acidic surfaces [1].

Catalytic cracking plays a vital role in petroleum refinery processes. It is the most extensively used process for conversion of heavy oils into valuable products like gasoline and other lighter products. Typically, catalytic cracking reactions are carried out at 290-400°C and 1200-2000 psig [2]. For this process, zeolite catalysts are known to have high selectivity and activity, which translate to profitable liquid product yields and high cracking capacity. However, modifications of the catalyst's surface (i.e. removal of some aluminum atoms from the framework) are sometimes necessary to meet industrial demands and specifications (i.e. higher octane rating) [3].

Environmental concerns regarding the limits of sulfur and aromatic compounds in motor fuels had triggered the utilization of pretreatment processes in oil refineries. These processes include catalytic hydrotreating, which involves the use of hydrogen gas in the

presence of a catalyst (usually mixed sulfides of CoMo, NiMo or NiW supported on γ-Al_2O_3) to convert organic sulfur and nitrogen compounds to hydrogen sulfides, ammonia and hydrocarbons [4, 5]. The hydrocarbons will then undergo the usual catalytic cracking processes. The terms *hydrotreating*, *hydrocracking*, *hydroprocessing*, and *hydrodesulfurization* are being used rather loosely in the industry because they occur simultaneously. However, *hydrotreating* is a more suitable term for catalytic stabilization of petroleum products and feedstocks by removing unwanted elements like sulfur, nitrogen, oxygen, halides, and trace metals [2].

Not until recently that the investigation on catalytic cracking of oxygenated compounds has gained much attention. Most research had focused on compounds present in vegetable oils and bio-oil, a product of wood pyrolysis. Reports indicated that vegetable oil cracking is a promising alternative route for the preparation of 1st generation biofuels that are within the boiling range of gasoline compounds [6-10].

Lipids are oxygenated organic compounds that may also contain nitrogen and phosphorus. In addition to these, lipidic material considered for production of 2nd generation biofuels might also contain considerable amount of metals (i.e. calcium, potassium, magnesium) that may poison cracking catalysts. Aside from the removal of oxygen, another major challenge would be the purification of these feedstocks with variable compositions prior to catalytic cracking [11].

Several studies about catalytic cracking of lipidic materials are reported in the literature. Bhatia et al. (2007) studied the modeling and simulation of the catalytic cracking palm oil using rare earth-Y as catalyst in a transport riser reactor. They obtained good agreement between the experimental and predicted yield of gasoline fraction, kerosene fraction, and diesel fraction, and gaseous product. Several workers also

investigated the hydro-deoxygenation of esters of fatty acids. Şenol et al. (2007) used NiMo/γ-Al_2O_3 and CoMo/γ-Al_2O_3. Hydrocarbons (C_6 and C_7) were produced together with some intermediates which include alcohols, aldehydes, carboxylic acids and ethers [12]. Danuthai et al. (2009) on the other hand used H^+ZSM5 for conversion of methyl octanoate into hydrocarbons. The reaction produced different hydrocarbons (C_1-C_7) as well as significant amount of aromatics [13]. Benson et al. (2009) investigated the catalytic pathway for the heterogeneous cracking of unsaturated acylglycerides with oleic acid as substituent. Cracking reactions were conducted at 400°C using H^+ZSM5, faujasite, or Si-Al as catalyst. They concluded that heteroatom removal was accomplished by the formation of CO and CO_2. Furthermore, they concluded that the H^+ZSM5 and faujasite catalysts promoted aromatic compound formation while the Si-Al catalyst supported the formation of dienes [5].

In the cracking of lipidic materials, microporous catalysts are advantageous due to their high selectivity for production of products within the gasoline and diesel fractions [14]. For this reason, zeolites are widely employed in lipid cracking. Zeolites, which are hydrated crystalline microporous aluminosilicates with open regular frameworks, have high thermal stability and excellent selectivity for gasoline production. The zeolite micropores are of molecular size, which give them adsorption, catalytic and ion-exchange properties of paramount importance in the chemical industrial field. Interest is growing on the study of their applications related to process intensification, green chemistry, hybrid materials, medicine, animal food uses, optical- and electrical-based applications, multifunctional fabrics and nanotechnology [14-16].

Zeolites are used in the refinery as strong and thermally stable solid acid catalysts for cracking alkanes, alkenes, and alkylaromatics, isomerization (in almost all processes),

and their oligomerization (see Figure 7.1). In all uses, shape selectivity has some role to play: in some processes it is essential, whereas in other processes, it is perhaps a restriction that has to be accepted. Zeolites have replaced silica alumina catalysts in hydrocracking and fluid catalytic cracking due to their better stability and regenerability [17].

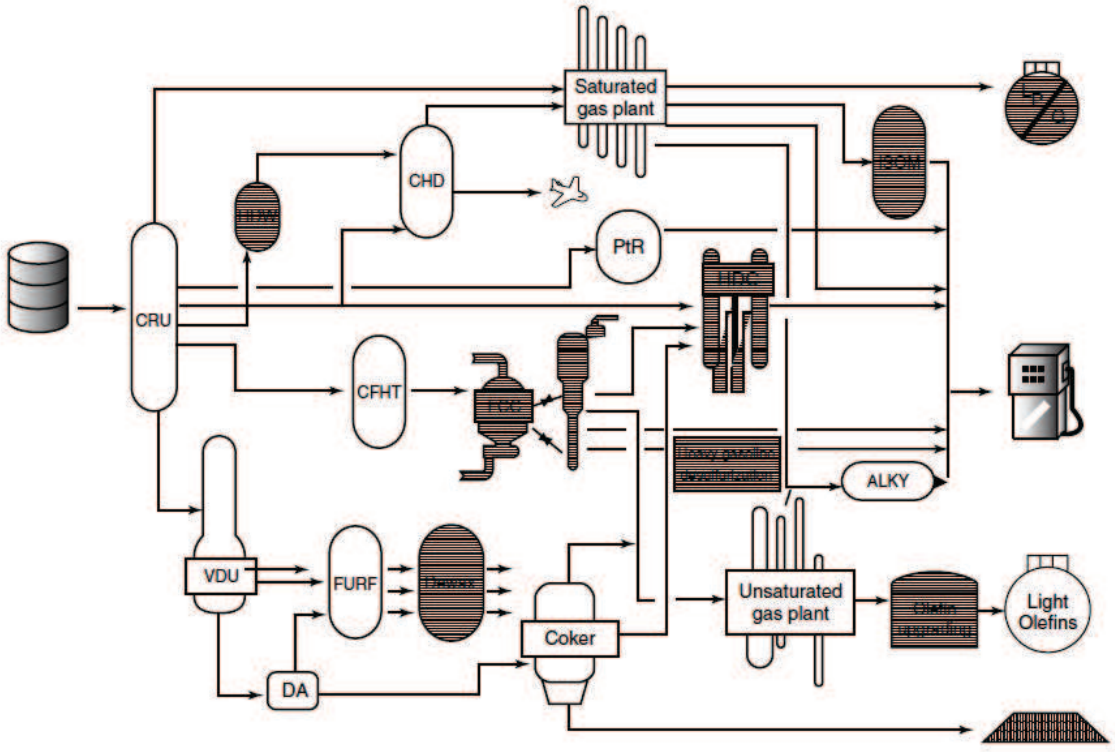

Figure 7.1 A hypothetical complex refinery showing processes where zeolite(s) are utilized*.

*Zeolite-catalyzed processes are shaded. CRU, crude distillation unit; HDW, hydrodewaxing; CHD, catalytic hydrodesulfurization; PtR, reforming; ISOM, isomerization; CFHT, catalytic feed hydrotreating; FCC , fluid catalytic cracking; HDC, hydrocracking; ALKY, alkylation; VDU, vacuum distillation unit; FURF, furfural extraction; DEWAX, lube hydrodewaxing; and DA, deasphalting [17].

In catalytic cracking of lipidic compounds, ZSM5, particularly H^{+}ZSM5 (Figure 7.2) is one the most commonly used zeolites [6, 7, 18-29]. ZSM5 is a shape-selective

catalyst developed by Mobil Research and Development Corp. in the mid-1970s. Due to its shape selective characteristics, ZSM5 are being used for materials that require minor cracking which would result to higher proportion of liquid relative to gaseous products [15]. The role of H^+ZSM5 in a catalytic cracking unit is normally as an octane-boosting additive due to its higher selectivity towards aromatic hydrocarbons [14].

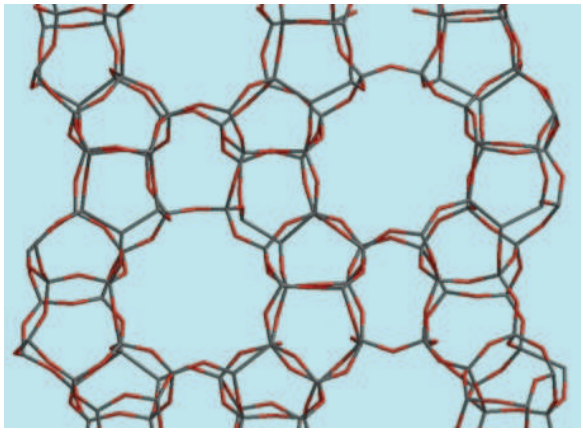

Figure 7.2 Structure of alominusilicate H^+ZSM5 (Gray, Silicon/Aluminum; Red, Oxygen).

Most studies on the conversion of lipidic materials into renewable fuel by catalysis were focused on vegetable oils (which are mostly triacyglycerides), fatty acids (including their esters) and bio-oil (a by-product of wood pyrolysis). However, for an effective utilization of 2nd generation biofuel feedstocks (i.e. waste oils, microbial oils), the cracking of other compounds present must also be understood. The results of the study conducted in Chapters V and VI showed that fatty alcohols (associated with wax esters) were consistently present in raw activated sludges. These compounds were not converted to biodiesel during the in situ transesterification studies conducted in Chapter III.

Fatty alcohols are important oleochemicals for manufacture of variety of products and thus, their occurrence in activated sludge makes it a very attractive petroleum alternative. However, their utilization as raw material for production of everyday commodities might be subjected to public scrutiny. The production of 1st generation biofuels, particularly biodiesel, had caused disturbances in the oleochemical industry. The utilization of all compounds present in 2nd generation feedstock (i.e. activated sludge) for fuel production could avoid disturbances of the oleochemical industry supply and demand structure. Thus, it is important to study possible routes for their conversion to fuel.

This chapter deals with catalytic cracking of a model compound that is present in activated sludge, particularly 1-octadecanol. This fatty alcohol is a saturated 18-carbon compound, which is solid at ambient condition. Activated sludge contains $C_{14} - C_{18}$ fatty alcohols with octadecenol as the dominant one. Nevertheless, octadecanol was chosen as the model compounds due to its possible unreactivity. For example, the study conducted by Benson (2008) on the super acid trifluoromethanesulfonic acid (triflic acid) cracking of fatty acids, showed a highly unreactive behavior of palmitic acid, a 16-carbon saturated fatty acid. Furthermore, it is long established that alkane cracking, although similar to alkene cracking, proceeds with slow reaction rates [15]. It was anticipated that catalytic cracking of octadecanol would be difficult as compared to octadecenol and thus, the former was chosen as model compound.

Cracking mechanism for the conversion of 1-octadecanol to fuel using H^+ZSM5 was developed to support its utilization as fuel. Although H^+ZSM5 is not an industrially used catalyst due to its instability at high temperature, its intrinsically high acidity (mostly Brønsted) made it suitable for development of catalytic cracking mechanisms

[15]. Zeolitic Brønsted acid sites are the active species in different hydrocarbon transformations including cracking, isomerization, and alkylation [30].

7.2 Materials and Methods

7.2.1 Chemicals, Gases and Catalyst

All analytical standards, silica gel, silanized glass wool and 1-octadecanol (properties are given in Table 7.1) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Acetone and chloroform were procured from Fisher Scientific (Pittsburgh, U.S.A.) and He and liquid CO_2 were distributed by nexAir (Columbus, MS, U.S.A.). Liquid CO_2 was used for cool-on-column analysis. All chemicals, standards and gases were used as received.

The H^+ZSM5 (Si/Al = 23) was obtained from Zeolyst International (Valley Forge, PA, USA) in the ammonium form $[((C_3H_7)_4NOH)_4][Si_{95.7}Al_{0.3}O_{192}]$. Prior to use, it was calcined at 550°C for 12 hours in air using a muffle furnace to produce the acidic form $[H_{0.32}][Si_{95.68}\ Al_{0.32}\ O_{192}]$. According to the manufacture, the catalyst has a particle diameter of ~1 μm, surface area of 425 m^2/g, and pore diameter of 5.5 Å.

7.2.2 Quatra C

Reactions and analyses were performed using Quatra C (Cryogenic Capillary Catalytic Cracker), which is an in-house built reactor/analyzer (see Figure 7.3). This system is a modified Varian 3600 (Walnut Creek, CA, U.S.A.) gas chromatograph with front and rear injectors being used for reactant injector and reactor (catalyst bed), respectively. This system can take samples in any form: solid, liquid or gas. Liquid and gas reactants are injected using syringes while solid reactants are loaded in a glass crucible and are directly injected using a Varian ChromatoProbe (see Figure 7.4).

Table 7.1 Properties of 1-octadecanol.*

Formula	$C_{18}H_{38}O$
CAS #	112-92-5
Structure	
Molecular Weight[a]	270.49
Synonyms	*n*-Octadecanol, *n*-Octadecyl alcohol, Octadecan-1-ol, Octadecyl alcohol, Stearol, Stearyl alcohol, Stenol, Steraffine, Decyl octyl alcohol, Adol 68, Dytol e-46, Aldol 62, Alfol 18, Atalco S, Cachalot S-43, Crodacol-S, Lanol S, Lorol 28, Sipol S, Siponol S, Polaax, CO-1895, CO-1897, Follestrine, Kalcohl 80, Conol 30F, 1-Hydroxyoctadecane, C18 Linear alcohol, Cachalot S-56, Ceteareth-20, CO 1895F, Conol 1675, Crodacol S70, Crodacol S95NF, Dehydag Wax 18, Emery 3343, Epal 18NF, Fancol SA, Lanette 18 DEO, Lipocol S, Lorol C18, Loxiol VPG 1354, Philcohol 1800, Rita SA, Stearal, Varonic BG
Melting point (°C)[a]	56 to 59°C
Normal Boiling point (°C)[b]	335°C

*Properties were obtained from National Institute of Standards and Technology (NIST) unless noted.
[a]Sigma-Aldrich [31].
[b]Cheméo [32].

The catalyst bed (reactor) is normally operated up to 400°C. This is a dual-column system equipped with a Saturn 3 mass spectrometer (Varian, Walnut Creek, CA, U.S.A.) and a thermal conductivity detector (TCD). This feature of the system allows automatic analyses of reaction products including fixed gases. To maintain constant flow to the mass spectrometer, an open-split interface, manufactured by SGE Analytical Science (Victoria, Australia) was utilized. The open-split interface allows only 1 mL/min of flow to the mass spectrometer and thus offers flexibility of the system to higher gas flowrates across the catalyst bed.

For this study, an Rxi®-1ms (30m x 0.53mm, with a 1.50 µm film thickness) (Restek, Bellefonte, PA, U.S.A.) was use as chromatographic column for the mass spectrometer. This is an ultra-low bleed column with a working temperature range of -60°C to 350°C. On the other hand, an Rt®-Q-Bond column (Restek, Bellefonte, PA, U.S.A.) with dimensions of 30 m x 0.53 mm and a film thickness of 20 µm was chosen for the TCD. This column is suitable for analysis of fixed gases, C_1 – C_3 isomers and up to C_{12} alkanes and can withstand temperatures up to 300°C. The wide temperature span for both columns allows separation of a wide range of compounds.

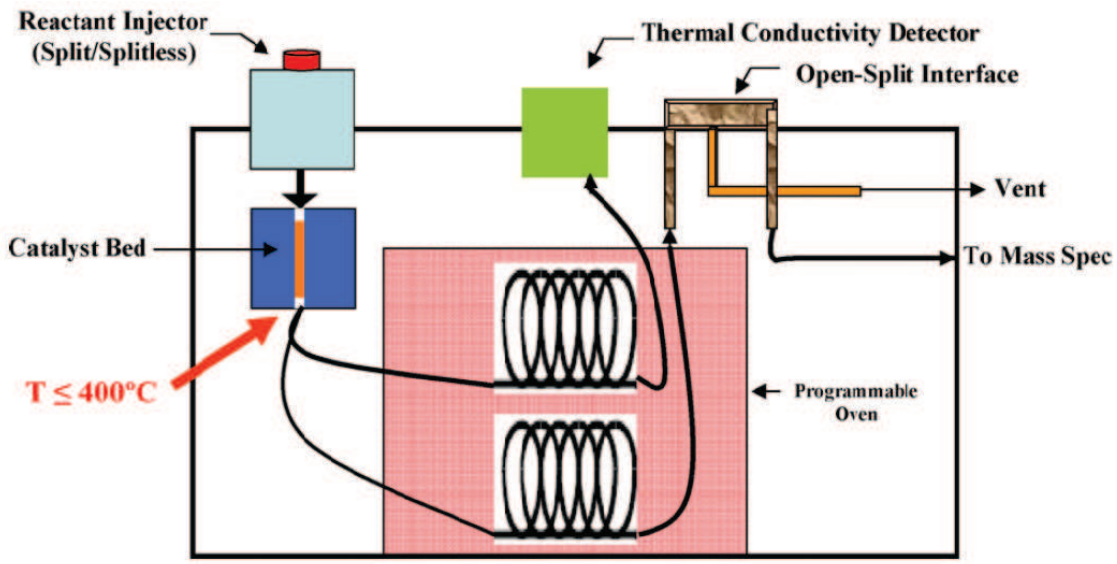

Figure 7.3 Diagram of the Quatra C illustrating the use of dual chromatographic columns [5].

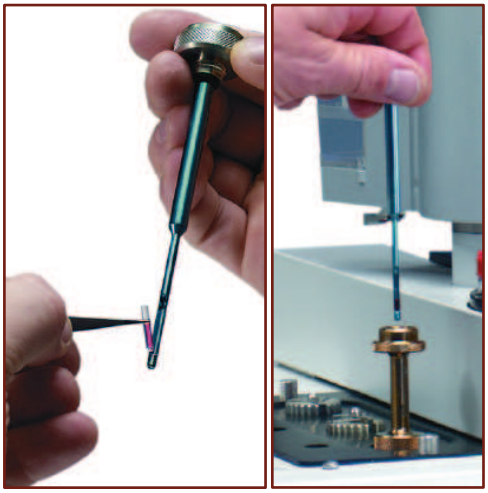

Figure 7.4 Sample introduction using ChromatoProbe [33].

7.2.3 Catalytic Cracking of 1-Octadecanol

Cracking reactions were performed by first adding the catalyst to the reaction tube. The reaction tube was a ¼ in. O.D. glass tubing (7.2 cm length x 3.9mm I.D.) that was subjected to sonication with acetone and chloroform (1 hour each) and then heated in a furnace at 500°C. Catalyst amounts were varied from 0 – 20 mg. Silanized glass wool was used to hold the catalyst in place and to reduce channeling through the catalyst. For catalyst loading less than 20 mg, silica gel (200/400 mesh) was added in appropriate amount to keep the amount of solids inside the tube constant. This was done to maintain constant hydrodynamics inside the reactor.

The catalyst-loaded reaction tube was then placed inside the reaction zone of the Quatra C. The desired temperature was set, and the catalyst was given time for off gassing of air and adsorbed water vapor. The air/H_2O indicator of the mass spectrometer was monitored, and once within acceptable levels ($\leq$ 0.5 amu for air and $\leq$ 5% H_2O^+/H_2O for water), the reaction/analysis was initiated. High levels of air and water can burnout the filament in the mass spectrometer.

The reactant (1-octadecanol) is a solid at ambient conditions. Initial reaction runs indicated that the lowest temperature at which the GC oven can be programmed was 50°C. This was due to high melting point (56 to 59°C) of the reactant. Below 50°C, the reactant condenses along the transfer line (the line connecting the reactant injector and the catalyst bed) (see Figure 7.5). The transfer line was ⅛ in. stainless steel tubing heated isothermally by heating tape that was controlled by a rheostat placed externally to the machine. However, unlike what is depicted in Figure 7.3, the transfer line is just above the GC oven and so changes in the GC oven temperature causes fluctuations on transfer line temperatures. For example, if the initial GC oven temperature 20°C, the reactant condenses along the transfer line for some time until the GC oven reaches a certain temperature higher than the reactant's melting point. At this temperature, the reactant starts to vaporize, passes through the catalyst bed and the products formed through the chromatographic columns. However, by the time this happens, the temperature of the GC oven is high and separation of products is difficult.

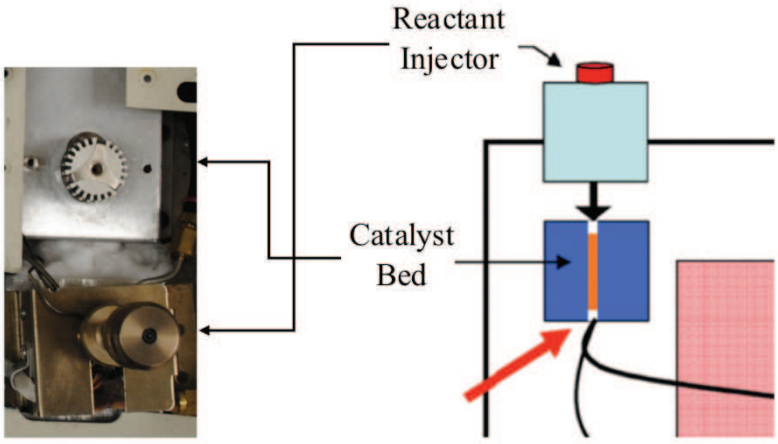

Figure 7.5 Reactant transfer line of the Quatra C.

Due to this limitation of the Quatra C, the injection procedure was modified to inject the reactant directly to the catalyst bed. It was necessary to keep the reactant in liquid form. This was accomplished by mildly heating the reactant in a hot plate until it melted. Direct reactant injections were accomplished by using a syringe heated isothermally by a heating tape that was controlled by a rheostat (see Figure 7.6). Air bubbles inside the syringe were eliminated, after which, the syringe was cooled down and weighed. Prior to injection, the syringe was mildly heated again to melt the reactant. The amount of reactant injected was determined by getting the difference of the weights before and after injection. This modification allowed GC oven programming down to -40°C.

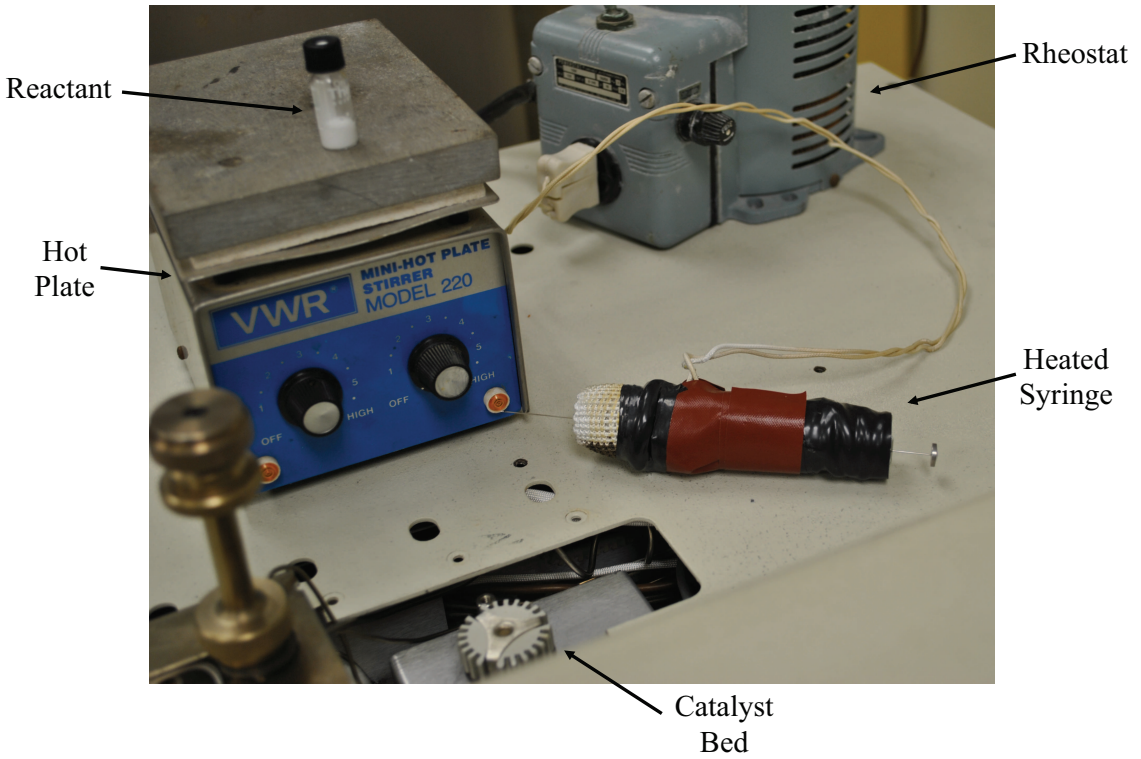

Figure 7.6 Direct injection of 1-octadecanol to the catalyst bed using a heated syringe.

Chromatographic analysis was conducted using helium as carrier gas. The flow of helium through the catalyst bed and through the chromatographic columns was adjusted to ~15 mL/min using an Intelligent Digital Flowmeter (Varian, Walnut Creek, CA, U.S.A.). The GC oven was programmed with an initial temperature of 0°C for 10 minutes and was then ramped to 50°C at 5°C/min, then to 100°C at 3°C/min, and finally to 300°C at 10°C/min and was held at 300°C for 3.34 minutes.

Quantitation was performed using calibration curves from standard compounds. For all the experiments, the mass spectrometer was configured to operate only in electron impact (EI) mode. Reaction runs were segmented to analyze for different mass-charge (m/z) ranges. Low molecular weight compounds eluting during the first 10 minutes were scanned from 10 – 80 m/z and for the rest of the chromatographic run, the mass range was 40 – 350 m/z to analyze high molecular weight compounds.

7.3 Results and Discussion

Catalytic cracking processes are applied to convert high molecular weight gas oil into valuable gasoline and olefins at petroleum refineries. In the same way, catalytic cracking can be used for the conversion of vegetable oils, animal fats and waste lipidic materials into biofuels that contain linear and cyclic paraffins, olefins, aldehydes, ketones and carboxylic acids. Several researchers proposed a mechanism for the conversion of vegetable oils to hydrocarbon products by cracking over H^+ZSM5 catalyst (Figure 7.7) [14].

The first step involves thermal decomposition of triacylglyceride molecules to form heavy oxygenated hydrocarbons such as fatty acids, aldehydes, ketones and esters. The next step involves secondary cracking of oxygenates to form gaseous products such

as paraffins, olefins, CO, CO_2, H_2O and alcohols. Secondary cracking involves breaking of C-O and C-C bonds through decarboxylation (CO_2) and decarbonylation (CO). The products from secondary cracking can undergo oligomerization to form olefins and paraffins within the range of gasoline, diesel and kerosene compounds. These compounds can undergo aromatization, isomerization and alkylation to form aromatic hydrocarbons, which can serve as coke precursors [14].

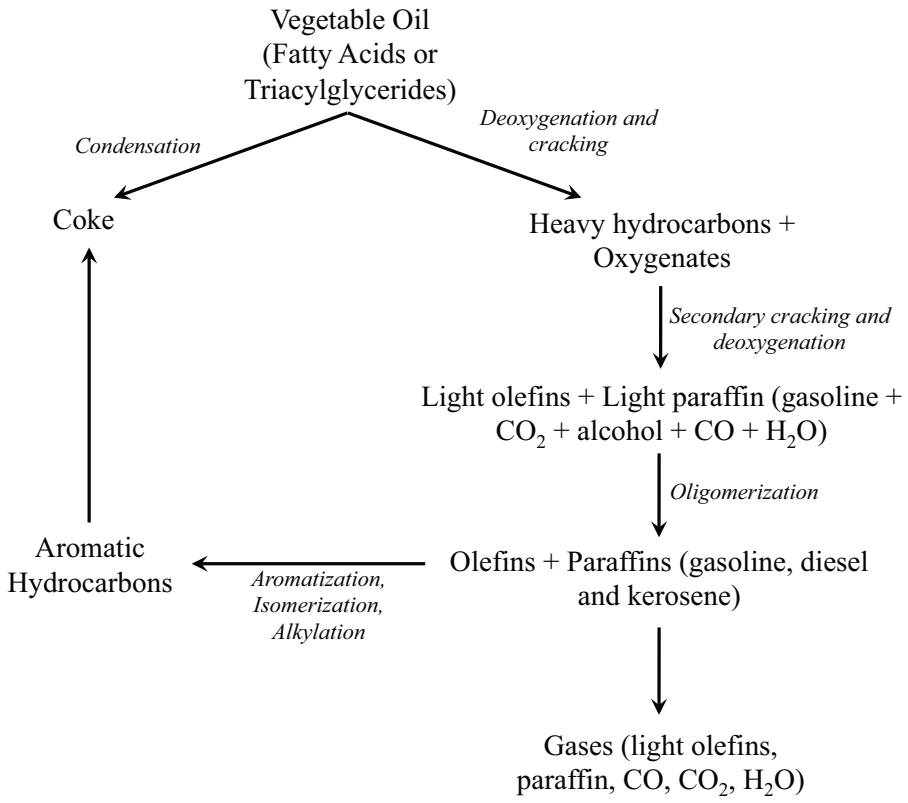

Figure 7.7 Proposed reaction pathway for the conversion of vegetable oils over an H^+ZSM5 catalyst to hydrocarbon products [14].

7.3.1 Thermal Cracking

Pyrolysis, or burning in the absence oxygen, is an important step for catalytic cracking of vegetable oils. Thermal decomposition (cracking) of triacylglycerides starts

at temperatures above 200°C and produces mostly hydrocarbons, carboxylic acids, a few carbonyl compounds and possibly some alcohols [34]. Pyrolysis is considered a non-catalytic homogenous reaction, which rapidly occurs due to thermal unstability of vegetable oils. Catalytic cracking, on the other hand, normally occurs at a much lower temperature than thermal cracking [14]. Knowing this, in mechanistic studies, it is important to determine the temperature at which thermal cracking is negligible.

Experiments were conducted to determine the range of temperatures suitable for the study of catalytic cracking mechanism of 1-octadecanol. For these experiments, ~ 1 mg of 1-octadecanol was injected to the catalyst bed containing 20 mg silica gel (no H^+ZSM5). The temperature of the catalyst bed was varied from 325 to 400°C.

It has long been known that in thermal decomposition of alcohols, two reactions predominate; dehydration and dehydrogenation (Figure 7.8) Studies indicated that these two reactions can be greatly enhanced by catalysts. These catalysts were subdivided into three categories, namely; dehydrating, dehydrogenating and mixed. Some of the dehydrating catalysts include thorium, tungsten and aluminum. Ethanol pyrolysis over reduced copper leads to the formation of acetaldehyde as dominant product. Silica, on the other hand, is considered to be a mixed catalyst that enhances both dehydration and dehydrogenation of alcohols [35, 36].

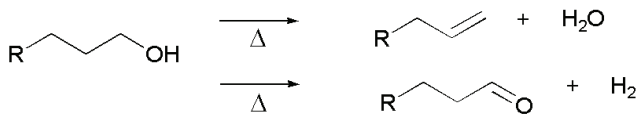

Figure 7.8 Thermal decomposition (pyrolysis or thermal cracking) of alcohols [36].

The results of the thermal cracking experiments are shown in Figure 7.9 (see also Figure 7.10). It can be seen that at 400°C, around 90% of the initial reactant was converted to two other compounds (labeled Unknown 1 and Unknown 2 in Figure 7.9). Since silica gel was used for these experiments, it was suspected that Unknown 1 and Unknown 2 were octadecene and octadecanal, respectively. Octadecene will be the product of octadecanol dehydration, while octadecanal will be produced by octadecanol dehydrogenation. Nevertheless, further analysis of these two unknown peaks was not conducted at this part of the study. As also shown in Figure 7.9, the extent of octadecanol conversion decreases with temperature and is almost negligible at 325ºC. Thus, for the catalytic cracking experiments, temperatures from 325 to 375°C were initially considered.

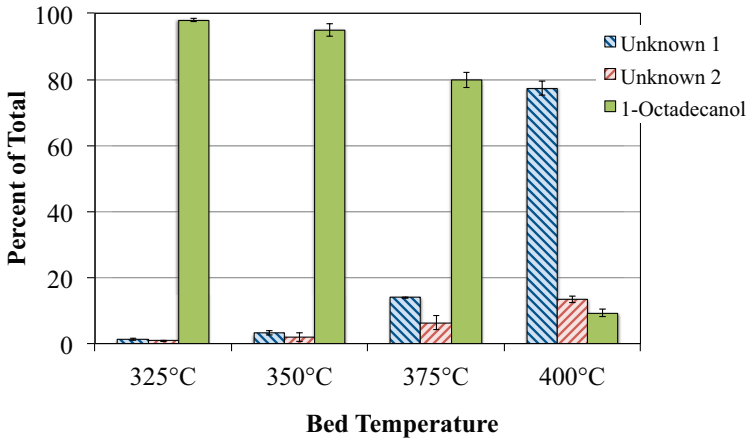

Figure 7.9 Thermal cracking of 1-octadecanol.

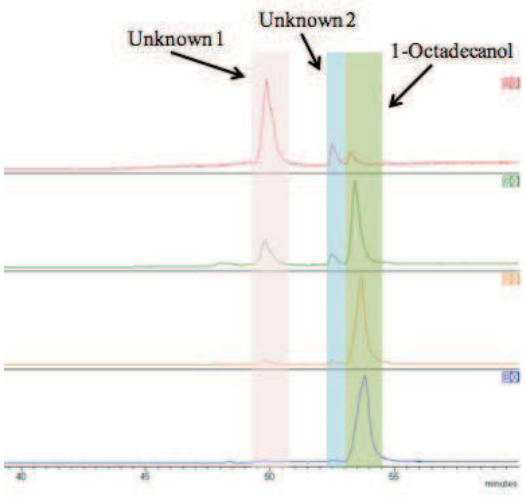

Figure 7.10 Effect of temperature on the thermal cracking of 1-octadecanol.

7.3.2 Catalytic Cracking

Catalytic cracking of any compound using a shape selective catalyst, like H^+ZSM5, is greatly affected by the compounds geometry. For example, dehydration of 1-butanol using zeolite-A catalyst is easier compared to 2-butanol. The linear geometry of 1-butanol allows it to enter the catalyst pores, access the active sites and undergo dehydration more easily [37].

The catalyst used in this study had a pore diameter of 5.5 Å. As evident by the AM-1 molecular geometry calculations, which were conducted using Spartan software (Wavefunction, Irvine, CA, USA), the 1-octadecanol is small enough to enter the pores of the H^+ZSM5 catalyst (Figure 7.11). However, there might still be some site restrictions due to the length of the molecule, which is about 25 Å. Thus, initial cracking of this molecule could be the sum of contributions from cracking inside and outside the pores of the H^+ZSM5 catalyst.

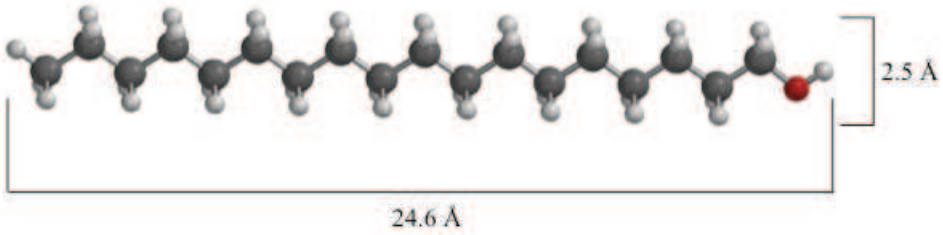

Figure 7.11 Equilibrium geometry of 1-octadecanol (calculated from semi-empirical AM-1 calculations using Spartan '06 [38].

7.3.2.1 Effect of Temperature

As mentioned earlier, temperatures from 325 to 375ºC were considered for catalytic cracking of 1-octadecanol over H^+ZSM5. However, no significant conversion was observed at 325ºC. Thus, experiments were conducted between 350 and 375°C using 2.5 mg H^+ZSM5. The main aim of this set of experiments was to determine the temperature at which cracking intermediates would be observed. It was anticipated that catalytic cracking of 1-octadecanol would be faster than that of vegetable oils (i.e. triacylglycerides) due to high molecular weight and molecular geometry of the latter. Thus, a low catalyst loading (2.5 mg) was used for this set of experiments.

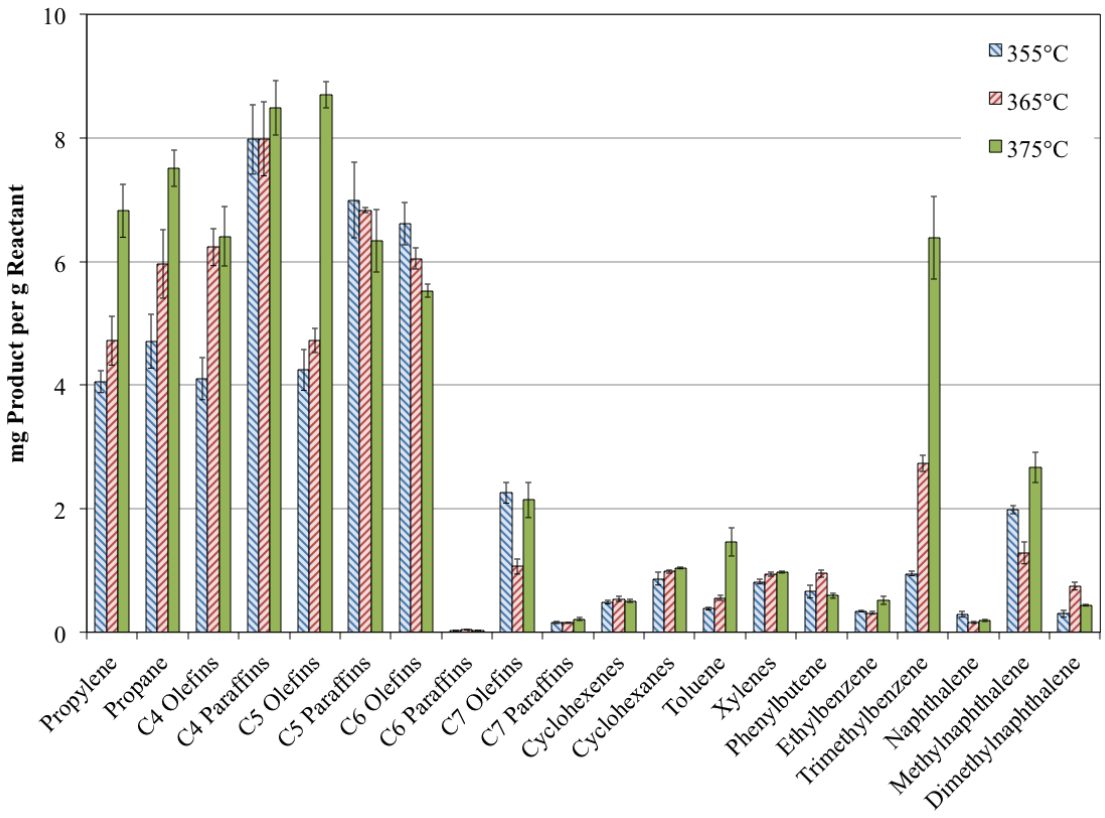

Figure 7.12 Effect of temperature on the catalytic cracking of 1-octadecanol using 2.5 mg H^+ZSM5.

The effect of temperature on the catalytic cracking of 1-octadecanol is shown on Figure 7.12. It can be seen that, except for C_5 paraffins and C_6 olefins, all the cracking products increased with temperature. This increase in product formation could be due to increase in kinetic energy of the 1-octadecanol at higher temperatures. Increase in kinetic energy means increase collision between the reactant (and/or initial cracking products) and the active sites of the catalyst resulting in a higher reactant conversion. The decrease in the formation C_5 paraffins and C_6 olefins at higher temperature suggests that they serve as intermediates for the production of other products (i.e. cyclic hydrocarbons and eventually to aromatic hydrocarbons). However, this could not be confirmed since this set

of experiments was conducted with a constant amount of catalyst. This translates to constant retention time of the reactant in the catalyst bed.

7.3.2.2 Effect of Catalyst Loading

To better understand the cracking mechanism of 1-octadecanol, the catalyst loading was varied from 2.5 to 20 mg. As was shown in the previous section, the qualitative profile of cracking products were the same for the three temperatures investigated. However, as shown in Figure 7.9, thermal cracking is still significant at 375°C. Thus, 365ºC was chosen for this set of experiments to minimize the effect of thermal cracking while ensuring high reactant conversion. Varying the amount of catalyst does not provide kinetic data. Nonetheless, at constant bed diameter, increasing the catalyst loading means increase in reactant retention time.

The results of this set of experiments are shown in Figure 7.13. Generally, all the cracking products increased with catalyst loading, except for C_6 olefins. Unlike what was observed in the previous section, the C_5 paraffins increased with catalyst loading which indicates that this group of compounds is not the major intermediate(s) for the catalytic cracking of 1-octadecanol over H^+ZSM5. This suggests that C_6 olefins serve as main intermediates for catalytic cracking of 1-octadecanol.

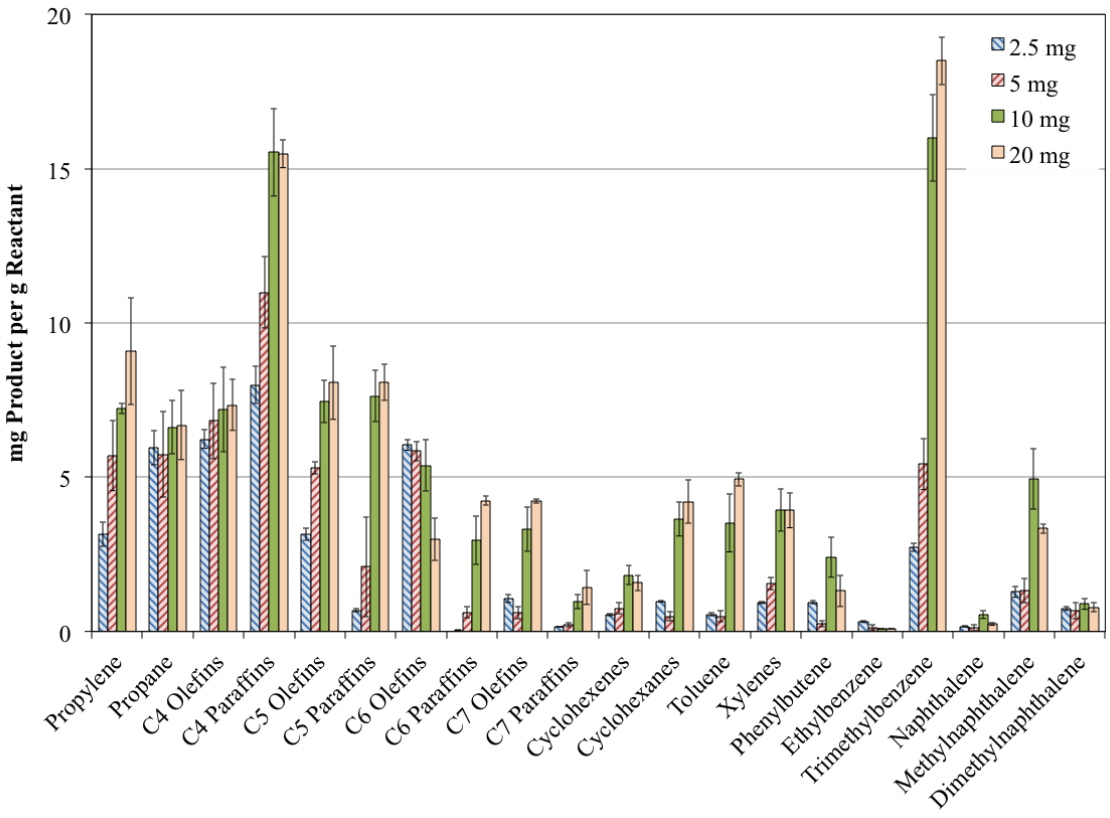

Figure 7.13 Effect of catalyst loading on the catalytic cracking of 1-octadecanol (Temperature: 365ºC).

7.3.2.3 Proposed Catalytic Cracking Pathway

It was discussed earlier in this chapter that zeolite catalysts are also being used for alcohol dehydration. To determine if the H^+ZSM5 catalyst used in this study favored dehydration over dehydrogenation, identification of unknown peaks seen during the thermal cracking experiments was necessary. A typical gas total ion chromatogram from gas chromatography-mass spectrometric (GC-MS) runs is shown in Figure 7.14. It is apparent that at 365°C, the conversion of 1-octadecanol to Unknown 1 was favored over Unknown 2. Thus, it was safe to assume that primary cracking by H^+ZSM5 primarily produced Unknown 1.

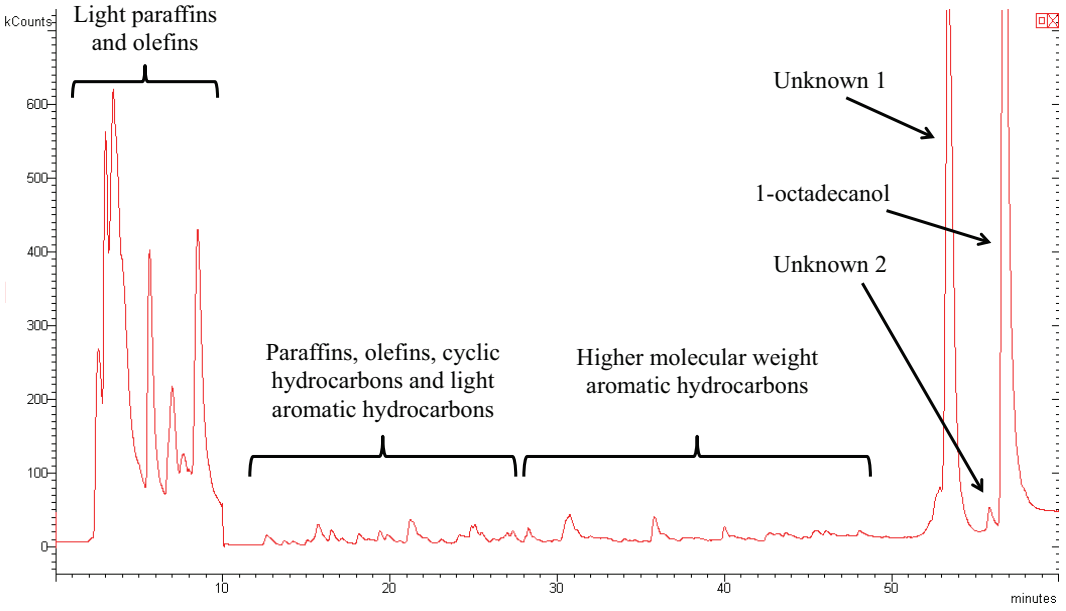

Figure 7.14 Typical GC/MS total ion chromatogram for cracking reaction of 1-octadecanolon H^{+}ZSM5 at 365ºC.*

*Scanning segmentation change at 10 min (m/z = 10 – 100 for first 10 min and then m/z = 40 – 350 for remainder of chromatographic run).

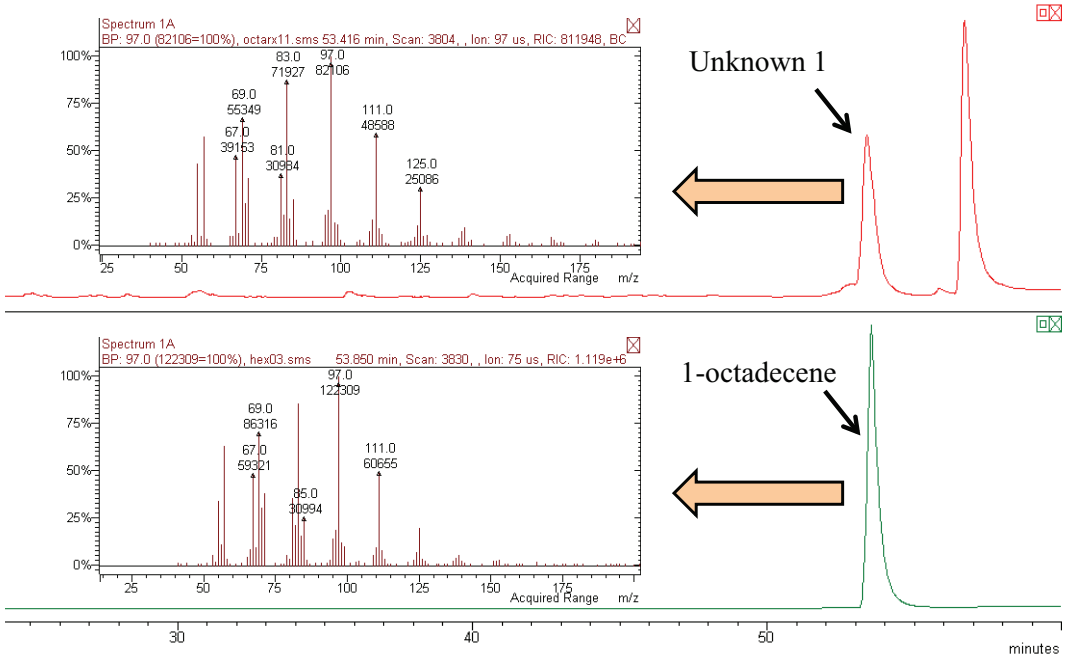

Figure 7.15 Comparison of unknown peak 1 and 1-octadecene.

To better understand the mechanism of primary steps in catalytic cracking of 1-octadecanol, identification of unknown 1 was necessary. As was mentioned earlier in this chapter, pyrolysis of 1-octadecanol will produce mainly octadecene and octadecanal. If one looks at Figures 7.12 and 7.13, it can be seen that CO and CO_2 were not detected in the cracking products. If initial cracking of 1-octadecanol produces significant amount of 1-octadecanal, CO and CO_2 should be present in the reaction products due to decarbonylation and decarboxylation reactions. Thus, it was presumed that Unknown 1 was octadecene and as shown in Figure 7.15, this was indeed the case.

Knowing that octadecene is the favored primary cracking product, a mechanism was developed to describe the conversion of 1-octadecanol to fuel related compounds over H^+ZSM5. The proposed mechanism is presented in Figures 7.17 and 7.18. Figure 7.17 covers primary cracking reactions, which starts by dehydration of 1-octadecanol catalyzed by H^+ZSM5 and heat. Figure 7.18 covers secondary and tertiary cracking of 1-octadecanol. For this discussion 1-octadecene was considered, although in reality it can isomerize by double bond shift. Nevertheless, the mechanism would be the same and throughout this discussion, alkene will be used in place of 1-octadecene.

Initial stages of alkene cracking were proposed to be the formation of a complex, which involves the double bond in the molecule. These complex results to the formation of carbenium ions (see Figure 7.16). Primary carbenium ions are the least stable among these type of compounds, followed by secondary and then tertiary carbeniums. Thus, if a primary carbenium is produced during cracking, it rapidly decomposes or isomerizes to secondary carbenium ion. From the carbenium ions, primary cracking of alkene molecules starts [39].

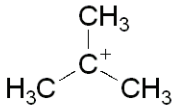

Figure 7.16 An example of tertiary carbenium ion [15].

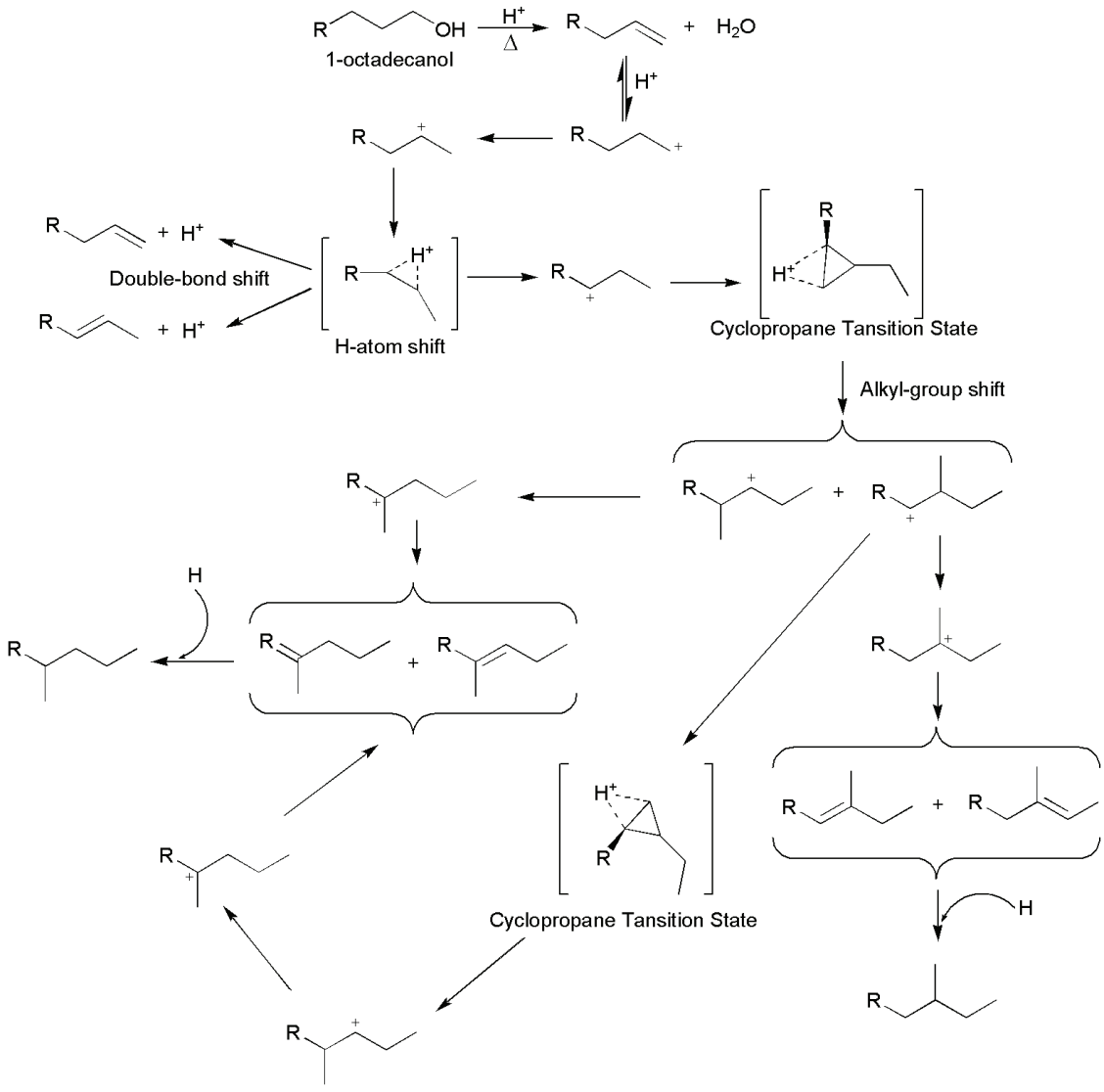

Figure 7.17 Primary cracking of 1-octadecanol over H^+ZSM5.

The carbenium ions can undergo H-atom shift, which could regenerate the original alkene or produce an isomer of it via double-bond shift. Branching of molecules during cracking can be made possible by an alkyl-group shift through the cyclopropane transition state. This reaction is commonly referred to as type-B skeleton rearrangement. This alkyl-group shift is followed by an H-atom shift, which generates ternary carbenium ions, which increases the profiles of products from primary cracking of 1-octadecanol. Further reaction includes methyl-group shift involving branched tertiary carbenium ions, which also involves a cyclopropane transition state. This is also called the type-A skeleton rearrangement reactions. Cracking of alkenes over acidic catalysts are usually accompanied by formation of alkanes with the same skeleton. Thus a hydrogenation reaction step occurs during catalytic cracking of these molecules. The source of hydrogen for these hydrogenation reactions is discussed below [39].

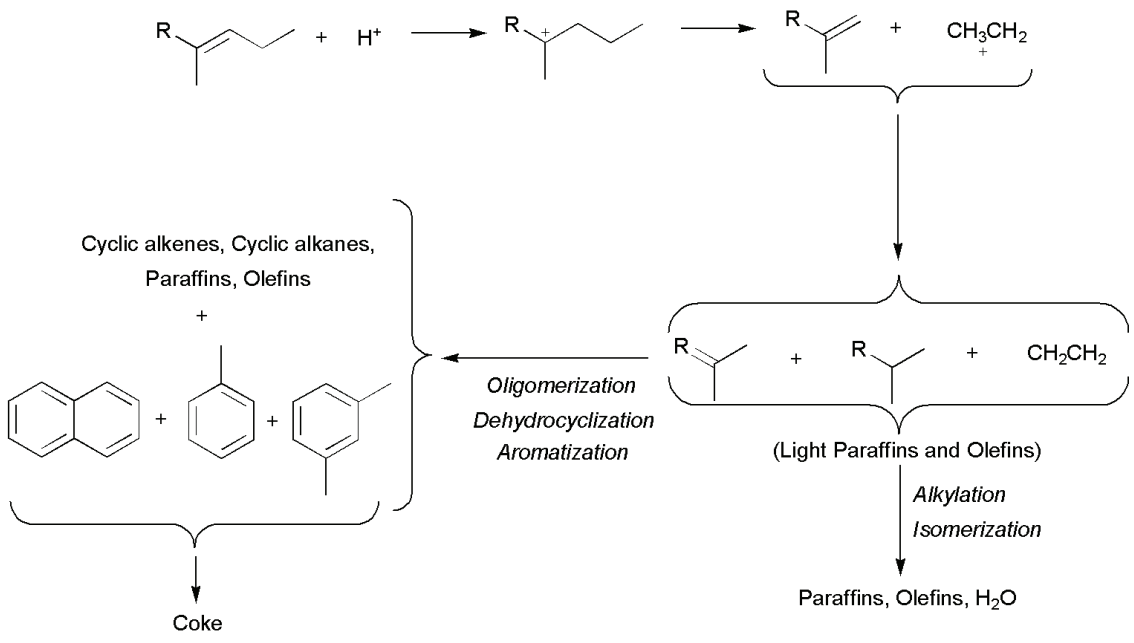

Figure 7.18 Secondary and tertiary cracking of 1-octadecanol over H^{+}ZSM5.

Primary products from 1-octadecanol cracking such as a branched alkene can react with the acidic sites on the H^+ZSM5 producing more carbenium ions. These carbenium ions can undergo C─C bond (i.e. β-C─C bond) scission reducing the size of molecules from primary cracking steps. The products from C─C scission can also undergo cracking similar to primary cracking mechanism. These C─C scission reactions are the ones responsible for the production of light paraffins and olefins. Light paraffins and olefins can undergo alkylation and isomerization producing paraffins and olefins within the range of gasoline and diesel compounds. Light paraffins and olefins can also undergo oligomerization, cyclization and aromatization. Oligomerization of secondary products could lead to the formation of long chain hydrocarbons, which could then undergo β-C─C bond scission producing smaller molecules. These molecules are usually referred to as tertiary cracking products. The oligomerization, cyclization and aromatization steps together with the coke formation reactions are believed to be the source of hydrogen for hydrogenation reactions mentioned earlier. These steps are also referred as hydrogen redistribution reactions. Thus, production of alkanes during catalytic cracking of 1-octadecanol is accompanied by the generation of hydrogen-deficient compounds such as cyclic and aromatic hydrocarbons. The production of hydrogen from oligomerization reactions usually starts with the formation of cycloalkenes. This validates the detection of cyclohexenes in the reaction products [39]. These cyclohexenes, together with cyclohexanes could be also formed from cyclization of paraffins and olefins. Due to the decreasing amount of C_6 olefins in the products as the catalyst loading was increased, they were thought to be the main intermediate for the formation of cyclic hydrocarbons. This is possible through direct cyclization or through C_6 olefin cracking followed by oligomerization reactions.

Benzene, which is commonly produced during catalytic cracking of vegetable oils, was not detected in any reaction runs. This suggests the validity of the proposed reaction pathway for 1-octadecanol cracking. According to Kissin (2001), cracking of 1-hexene over ZSM5 produces light aromatics such as toluene, ethylbenzene, xylenes, and other disubstituted benzenes while 1-octene generates mostly C_8 aromatic hydrocarbons [39]. Moreover, methane and C_2 hydrocarbons were not detected in the cracking products of 1-octadecanol. According to Kissin (2001), methane, C_2 and C_3 are not usually observed in catalytic cracking of alkenes under mild conditions (i.e. 150°C). The observed formation of C_3 hydrocarbons could be attributed to more severe cracking conditions (365ºC) employed in this study [39]. These literature observations, and the fact that CO and CO_2 where not produced during the catalytic cracking of 1-octadecanol over H^+ZSM5 supports the validity of the proposed cracking mechanisms.

7.4 Conclusions

The catalytic cracking of 1-octadecanol over H^+ZSM5 was conducted for mechanistic studies. Octadecan-1-ol is one of the many compounds present in activated sludges. Thus, this study was conducted to support the utilization of activated sludge as feedstock for the production of 2nd generation biofuel. For this study, an in-house built GC-MS unit capable of on-line automatic product analyses – Quatra C was utilized.

Due to the high melting point of 1-octadecanol and the configurations of reactant injection on the Quatra C, a modified injection procedure was developed – a heated syringe injector system. This allowed chromatographic analyses at low temperatures.

It was believed that catalytic cracking of 1-octadecanol over H^+ZSM5 starts with the alcohol dehydration producing octadecene. The octadecene then undergoes a series of

reaction involving carbenium ions. These reactions include H-atom shift, double-bond shift and alkyl-group shift. These reactions, which also involve the cyclopropane transition state, produced primary cracking products from octadecene.

Secondary cracking produces smaller molecule hydrocarbons via C─C scission. These light hydrocarbons undergo several reactions such as alkylation and isomerization producing paraffins and olefins suitable as fuel. Cyclic hydrocarbons and aromatic hydrocarbons are produced through oligomerization, dehyrocyclization and aromatization of the light hydrocarbons. The proposed cracking mechanism is supported by collected data as well as facts available in the literature. These include zero (undetected levels) CO and CO_2 in the cracking products as well as negligible net production of methane and C_2 hydrocarbons.

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CHAPTER VIII

CONCLUSIONS

The utilization of bacterial lipids and other single cell oils as feedstock for different applications can be made economically feasible by addressing three main conditions: (1) sustainable utilization of inexpensive bio-based carbon source, (2) high lipid yield, and (3) reproducibility and quality of lipids produced. Activated sludges generated by wastewater treatment operations have the potential of meeting these three conditions. Wastewater as carbon source is essentially free thus activated sludge lipids meets condition 1. This work sought to provide strategies for activated sludge lipids to meet conditions 2 and 3.

Initial work began with the optimization of biodiesel production from raw activated sludge. Results of this study indicated that activated sludge biodiesel is not economically attractive due to the following reasons:

1. Low saponifiable lipid present in raw activated sludge, and
2. High processing cost particularly the feedstock dewatering steps.

Nevertheless, this study provided information on the difference between activated sludge biodiesel and petroleum diesel.

Activated sludge lipids are mostly coming from bacteria, which are the ones responsible for biological wastewater treatment. Bacteria are known to produce different types of lipidic materials, both saponifiable and unsaponifiable ones. Biodiesel can be produce only from fatty acid (or saponifiable) component of any lipidic materials.

Unsaponifiable lipids are unwanted in biodiesel processes. However, in significant quantities, they can be used for a wide variety of other applications. They can also be converted to biofuel through catalytic cracking or they can be a good source of compounds for the oleochemical industry.

The activated sludge lipids were then characterized to determine the profiles of saponifiable and unsaponifiable lipids. Results indicated that activated sludge lipids contain significant amount of unsaponifiable lipids including sterols and fatty alcohols. In addition, polyhydroxyalkanoates were also a significant component of activated sludge lipids. These compounds can be used for different applications, which could improve the economics of activated sludge lipids. For example, fatty alcohols are important oleochemicals for the production of products such as lubricants, soaps and detergents. Although petroleum oil is mostly consumed for fuel production, it is also the source of feedstocks, which are being used as raw materials for the synthesis of different products for everyday life. Thus, the consistent presence of these compounds in activated sludge is advantageous both in biofuel and oleochemical industries.

The main issue with the use of activated sludge as biofuel feedstock is consistency. It was shown that activated sludge contains different classes of lipidic compounds including saponifiable and unsaponifiable ones. However, if all sludges from different wastewater treatment facilities will be used as lipid source, the question of whether they will all have similar lipid profiles is a big concern. Thus, lipid enhancement by activated sludge fermentation was conducted to address this issue. Fermentations were conducted using conditions previously identified to induce lipid accumulation in activated sludge bacteria. Results showed that fermentation increased the saponifiable lipids in activated sludge. Also, there were no unsaponifiable lipids detected, which

makes the lipid produced of higher quality. Moreover, enhancement of activated sludge obtained from two MWWTPs utilizing different treatment configurations, produced lipids with similar characteristics as indicated by their fatty acid profiles. Thus, not only that enhancement increased the amount of saponifiable lipids, it caused homogenization of lipids produced.

To further support the utilization of raw activated sludge for biofuel production, catalytic study of a model compound was conducted using H^{+}ZSM5 as catalyst. The main aim was to determine cracking chemistry of 1-octadecanol, a fatty alcohol present in activated sludges. Results indicated that catalytic cracking of this compound starts by dehydration reaction producing an alkene. The alkene molecules then undergo a series of reactions including C─C bond scission, alkylation, isomerization, oligomerization, dehydrocyclization and aromatization. These reactions produced paraffins, olefins and aromatic hydrocarbons, which are suitable for fuel consumption.

8.1 Significance of the Study

The results of this research provided significant contributions in the fields of engineering and analytical chemistry. These contributions are outlined below.

1. In the production of biodiesel, the results of the process optimization provided a more accurate economics of the activated sludge biodiesel. This gave a general idea of how far the economics was, as compared to petroleum-based diesel.
2. The characterization results provided information regarding different compounds in activated sludge. This information will be useful in designing unit operations to separate the activated sludge lipids in different fractions.

Based on the results, each fraction will be useful either as biofuel or as oleochemical feedstock. The results also provided an idea of the range of products that can be obtained from activated sludge lipids. These products are shown in Figure 8.1

3. Another significant contribution comes from the development of the solid phase extraction protocol for the analysis of lipidic compounds from activated sludge. Although the protocol was developed for activated sludge lipids, it can be applied to any lipidic material with little to no modifications.

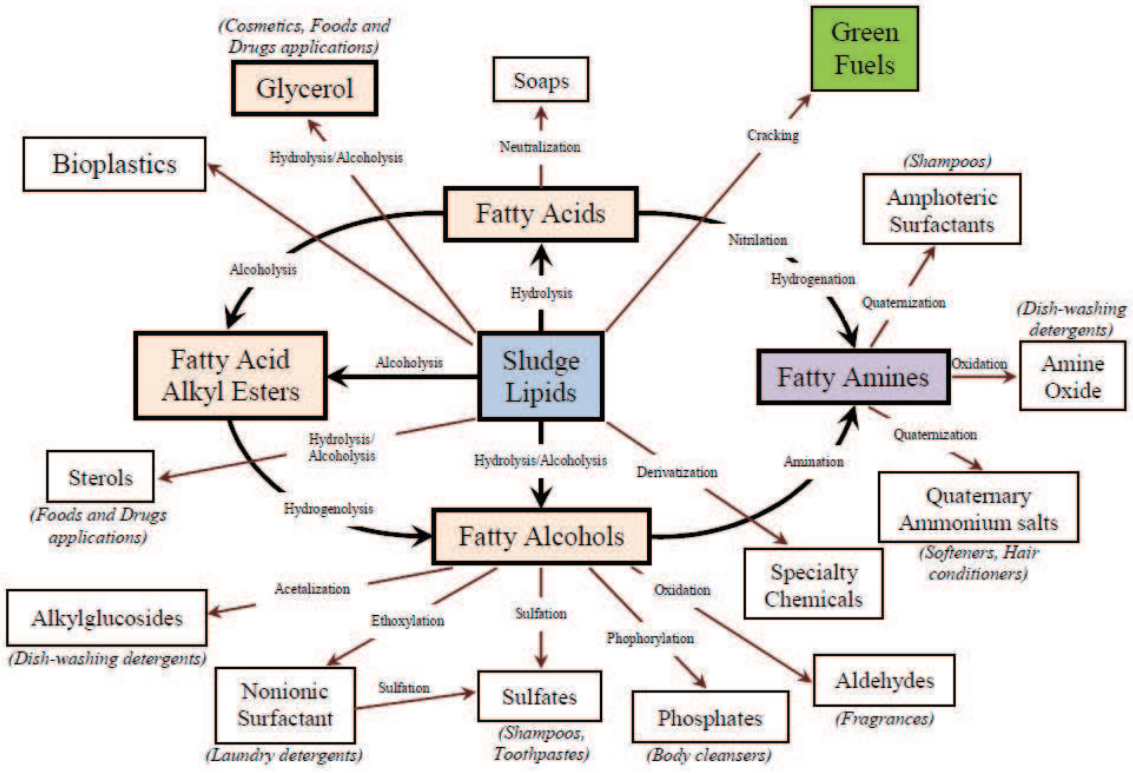


Figure 8.1 Range of products that can be obtained from activated sludge.

4. The lipid enhancement study showed that metabolic products from activated sludge bacteria can be controlled by application of biochemical stimulus. The

stimulus used in this study allowed them to accumulate triacylglycerides. It is well established that accumulation of PHAs in bacteria is enhanced in anaerobic treatment processes. By applying this stimulus, WWTPs can also be used as source of polymers that will displace petroleum-based plastics (see Figure 8.2). In addition, activated sludge microorganisms can also be directed to produce wax esters. Wax esters can be an important source of biofuel and/or oleochemical fatty alcohol. It is possible to include all these enhancement units in all wastewater treatment facilities. However, it is more logical to distribute this in different waste treatment facilities. For example, TG-enhancement unit(s) could be integrated in MWWTPs, PHA-accumulation unit(s) in wastewaters high in phosphorus, and WE-accumulation unit(s) in wastewaters with high concentration of detergent fatty alcohols.

5. The catalytic cracking study provided sufficient information regarding cracking mechanism of saturated fatty alcohols. If activated sludge lipids will be used for biofuel production alone, this will be useful for designing of catalyst and process.

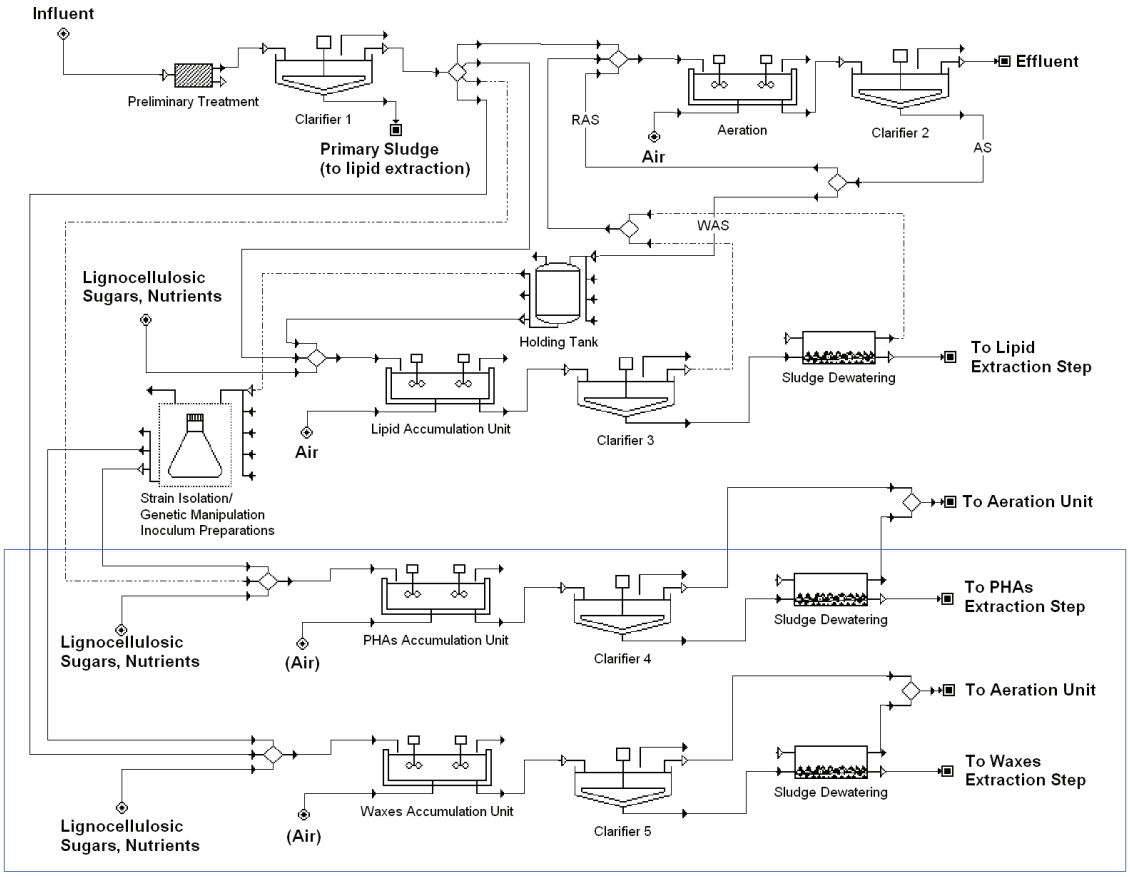


Figure 8.2 Modification of existing wastewater treatment facilities to accommodate production of lipids for different applications.

8.2 Research Needs

This work provided enough information that solidified the concept of utilizing existing WWTPs as biorefineries. However, there are still works to be done which are listed below:

1. This study covered only two types of wastewater treatment configurations both of which utilizes aerobic activated sludge treatment. Realistically, there are other biological treatment configurations or processes being utilized especially for industrial wastewaters. The biorefinery concept can be extended to include such processes.

2. Estimation of overall economics of the overall biorefinery concept.
3. The catalytic study only covered one of the unsaponifiable lipids in activated sludge. If activated sludge lipids are to be used for biofuel conversion alone, it is necessary to study the other ones too (i.e. sterols). Furthermore, evaluation of commercially used catalyst would be beneficial and would provide information on yield of biofuel from the unsaponifiable lipids.

APPENDIX A

CHEMICAL STRUCTURES

A.1 Structures of different compounds present in Bligh & Dyer extract from raw activated sludge.

Table A.1 Chemical structures and example(s).

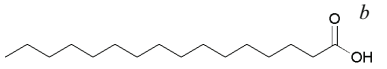
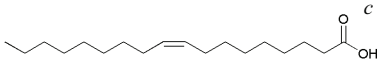
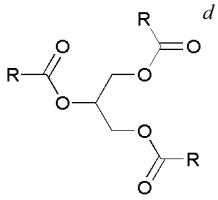
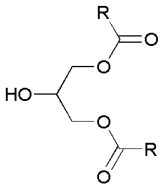
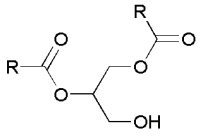
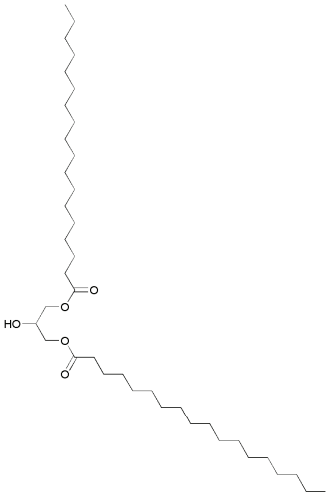
Compound Class	General Structure	Example	Structure
Free fatty acids	*a*	Palmitic acid	*b* 
		Oleic acid	*c* 
Triacyl-glycerides	*d*  R = mixture of various fatty acid chain	Triolein	
Diacyl-glycerides	 1,3 - Diacylglycerides  2,3 - Diacylglycerides R = mixture of various fatty acid chain	1, 3-Distearin	

Table A.1 (Continued)

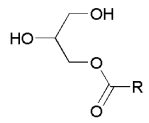
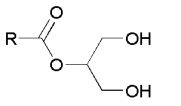
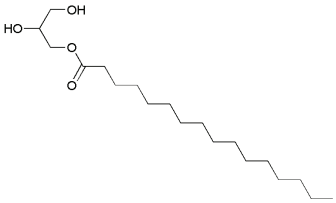
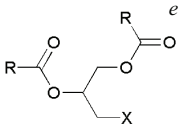
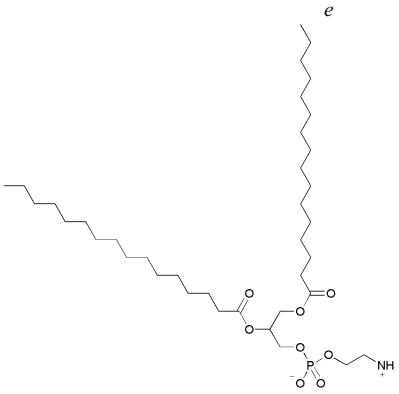
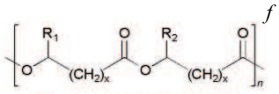
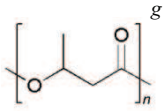
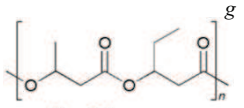
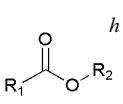
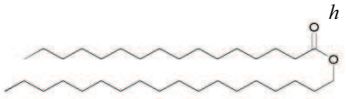
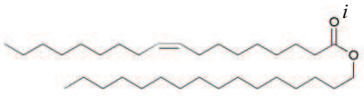
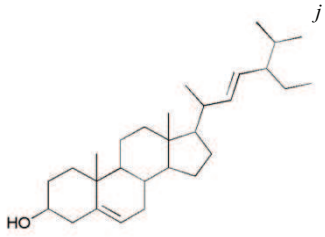
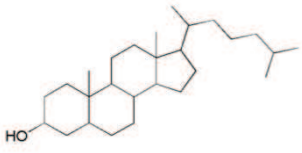
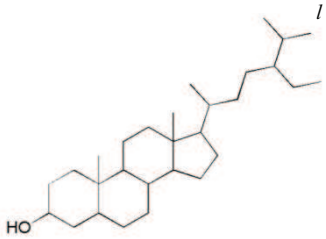
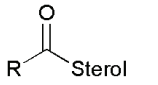
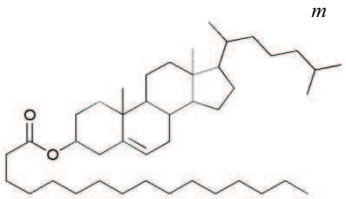
Monoacyl-glycerides	 1 - Monoacylglycerides  2 - Monoacylglycerides R = fatty acid chain	1-Mono-palmitin	
Phospholipids	 X = Polar head group	Phosphatidyl-ethanolamine	
Polyhydroxy-alkanoates	 R_1/R_2 = alkyl groups from C_1 to C_{13} x = 1 - 4 n = 100 - 30000	Polyhydroxy-butyrate	 $R_1 = R_2 = CH_3$ x = 1
		Poly(hydroxyl-butyrate-co-hydroxy-valerate)	 $R_1 = CH_3$ $R_2 = CH_2CH_3$ x = 1
Wax Esters (Waxes)	 R_1/R_2 = aliphatic (acyclic) groups	Stearyl Palmitate	
		Palmityl Oleate	

Table A.1 (Continued)

Sterols	-	Stigmasterol	 *j*
		Coprostanol	 *k*
		Stigmastanol	 *l*
Steryl Esters	 R = Fatty acid chain	Cholesteryl Palmitate	 *m*

[a]Van Gerpen et al., 2006 [1].
[b]About.com, 2011 [2].
[c]About.com, 2011 [3].
[d]Knothe and Dunn, 2005 [4].
[e]Hills, 1988 [5].
[f]Akaraonye et al., 2010 [6].
[g]Verlindenet al., 2007 [7].
[h]Garrett and Grisham, 2010 [8].
[i]Kalscheuer et al., 2006 [9].
[j]Zoebelein, 2001 [10].
[k]Ehlerset al., 2007 [11].
[l]Coleet al., 2003 [12].
[m]Hajjar, 1994 [13].

A.2 References

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[11] MM Ehlers, A Sundram, MM Kock, N Potgieter, Microbial and Chemical Assays to Determine the Origin of Faecal Pollution and the Presence of Waterborne Pathogens in *Environmental microbiology research trends*, ed by GV Kurladze. Nova Science Publishers, Inc., New York, Ch. 4, pp. 137-166 (2007).

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APPENDIX B

EXPERIMENTAL TOOLS, TECHNIQUES AND EQUIPMENTS

Figure B.1 Ohaus MB45 infrared heater (Ohaus, Pine Brook, NJ, U.S.A.).

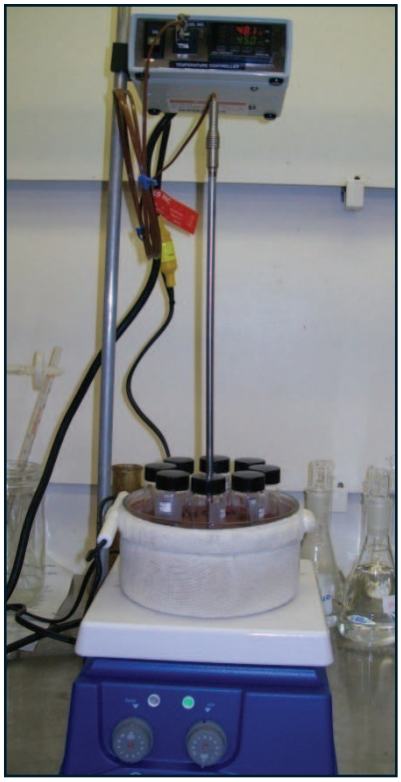

Figure B.2 Instatherm® block system (Ace Glass Inc., Vineland, NJ, U.S.A.).

Figure B.3 IEC Centra GP6 centrifuge (Thermo Electron Corp., Milford, MA, U.S.A.).

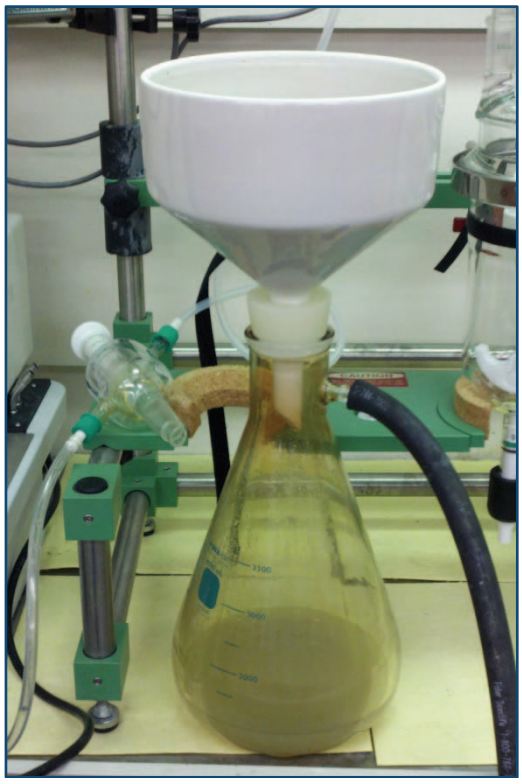

Figure B.4 Vacuum filtration set-up.

Figure B.5 ColdTech freezer (Jimex Corp., Hayward, CA, U.S.A.).

Figure B.6 Freezone 6 Bulk Tray Freeze dry system (Labconco, Kansas City, MO, U.S.A.).

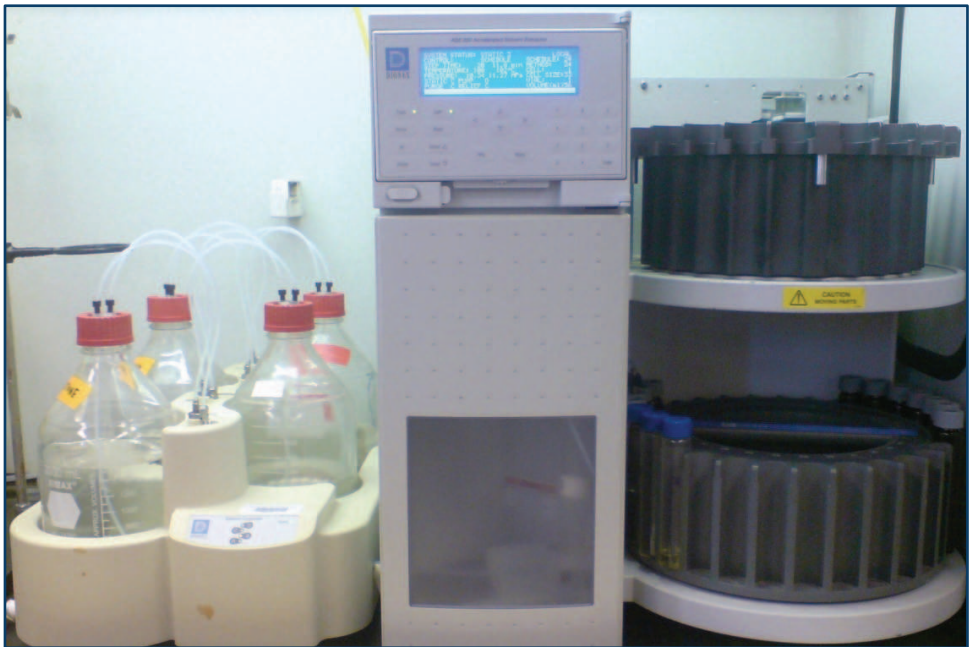

Figure B.7 ASE® 200 system equipped with a multi-solvent control system (Dionex, Sunnyvale, CA, U.S.A.).

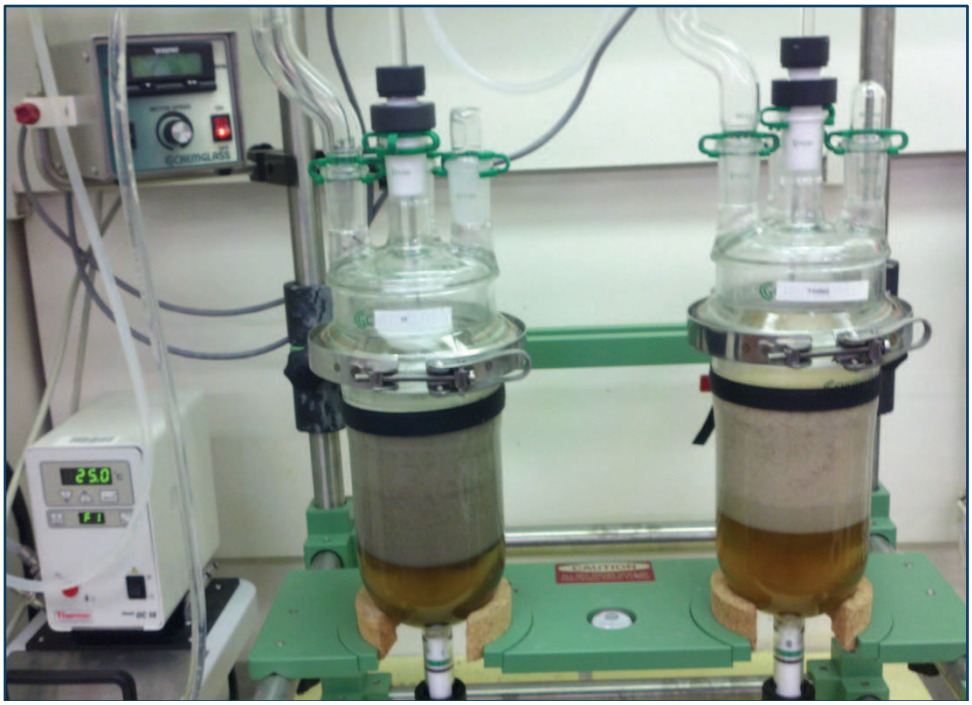

Figure B.8 Bligh & Dyer extraction (1-L reactors).

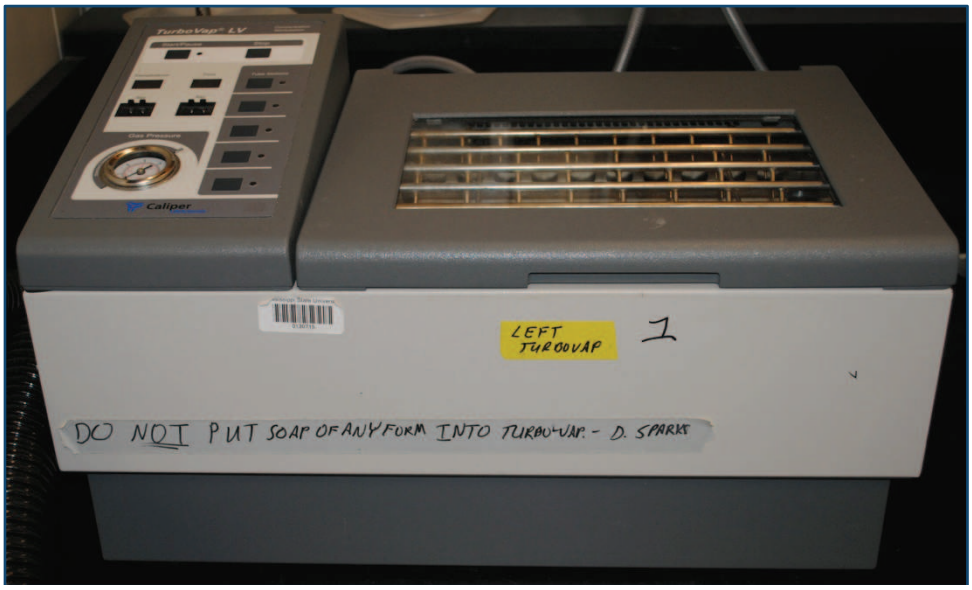

Figure B.9 TurboVap LV (Caliper Life Sciences, Hopkinton, MA, U.S.A.).

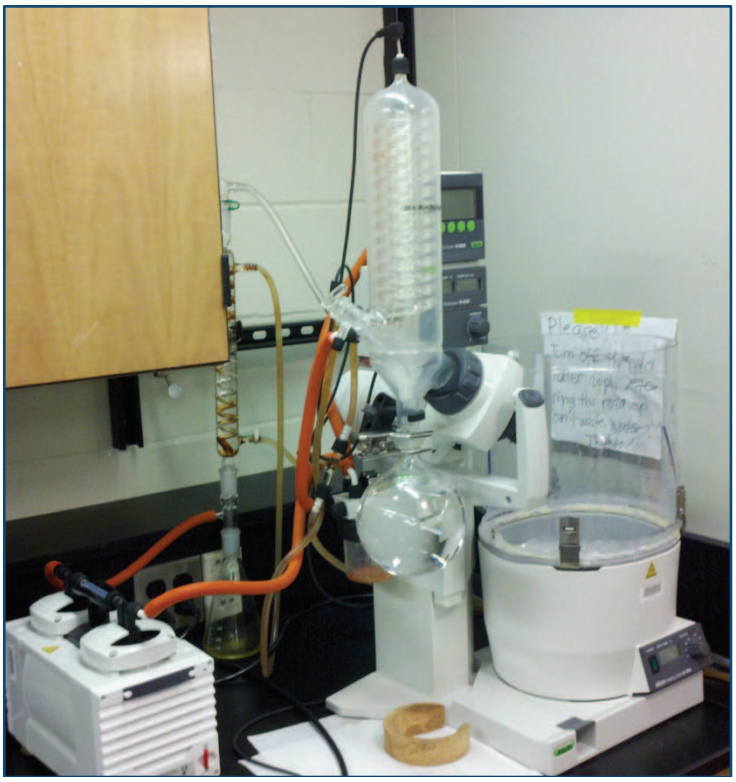

Figure B.10 Büchi R-205 rotary evaporator (Brinkmann Instruments, Inc., Westbury, NY, U.S.A.).

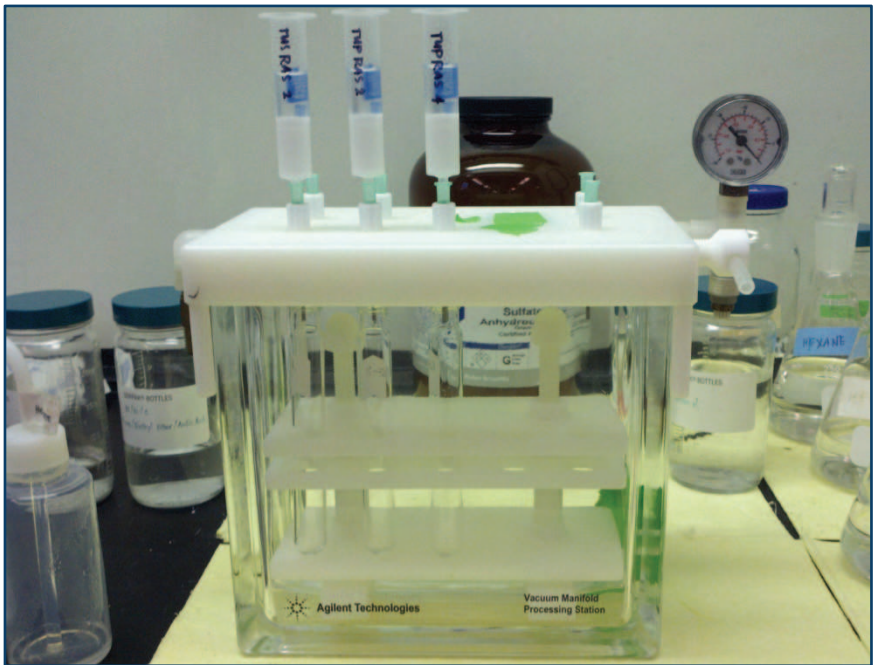

Figure B.11 Solid Phase Extraction set-up.

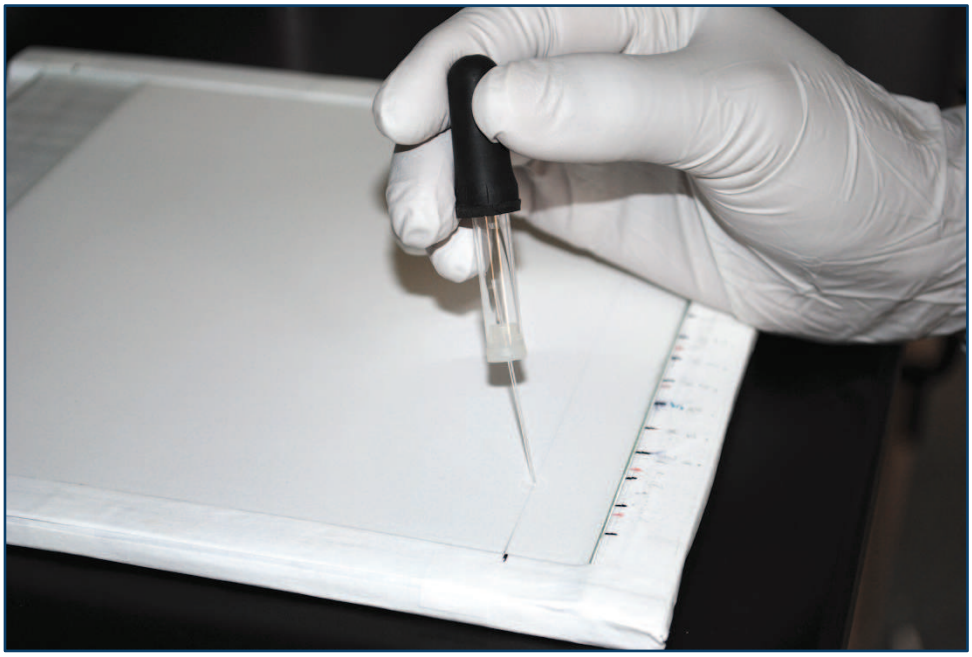

Figure B.12 Thin Layer Chromatography sample application.

Figure B.13 Thin Layer Chromatography developing chamber.

Figure B.14 Activated sludge samples for fermentation.

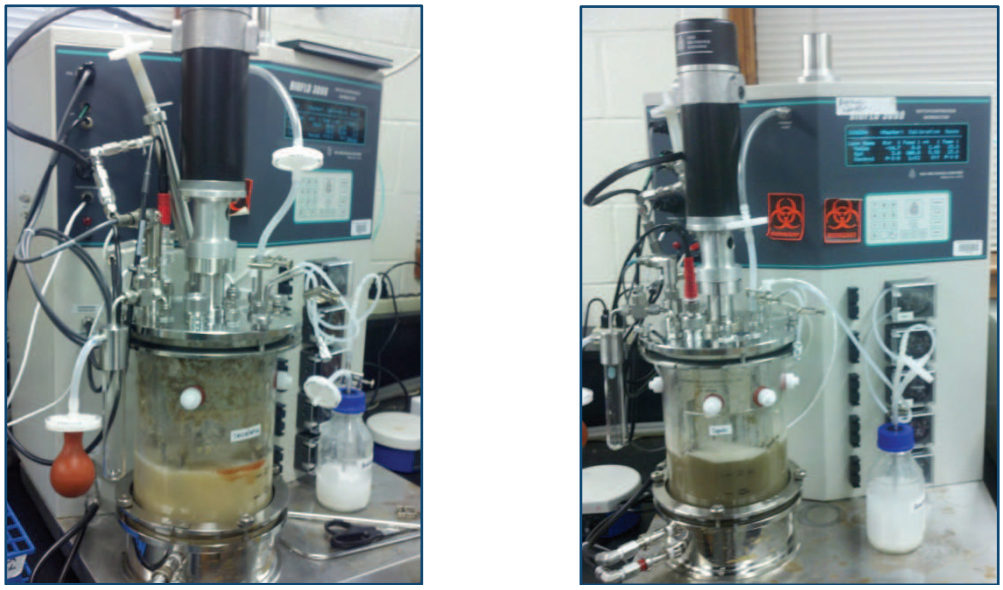

Figure B.15 BIOFLO 310 Bioreactors (New Brunswick Scientific, Edison, NJ, U.S.A.).

Figure B.16 Agilent 6890N gas chromatograph equipped with flame ionization detector (Agilent, Santa Clara, CA, U.S.A.).

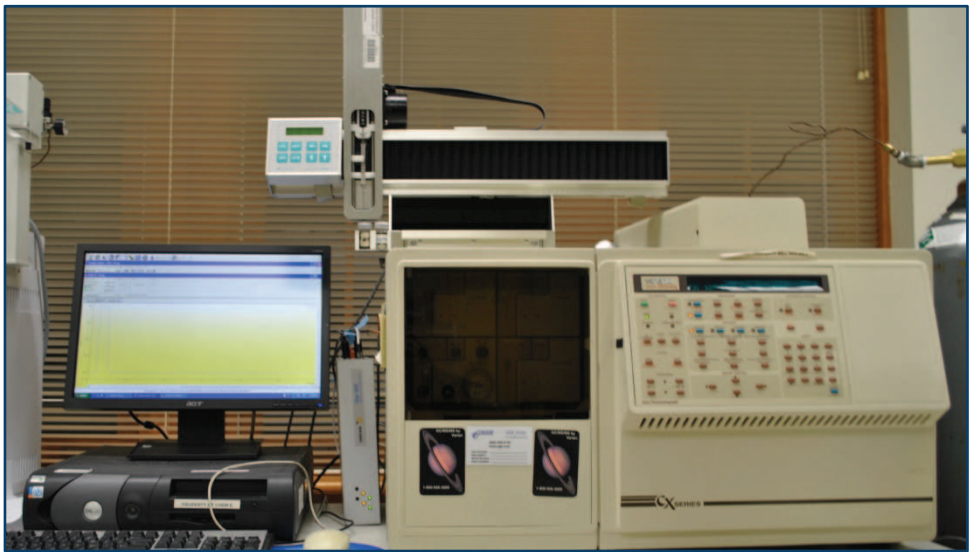

Figure B.17 Varian 3600 GC equipped with flame ionization detector (Varian Inc., Palo Alto, CA, U.S.A.).

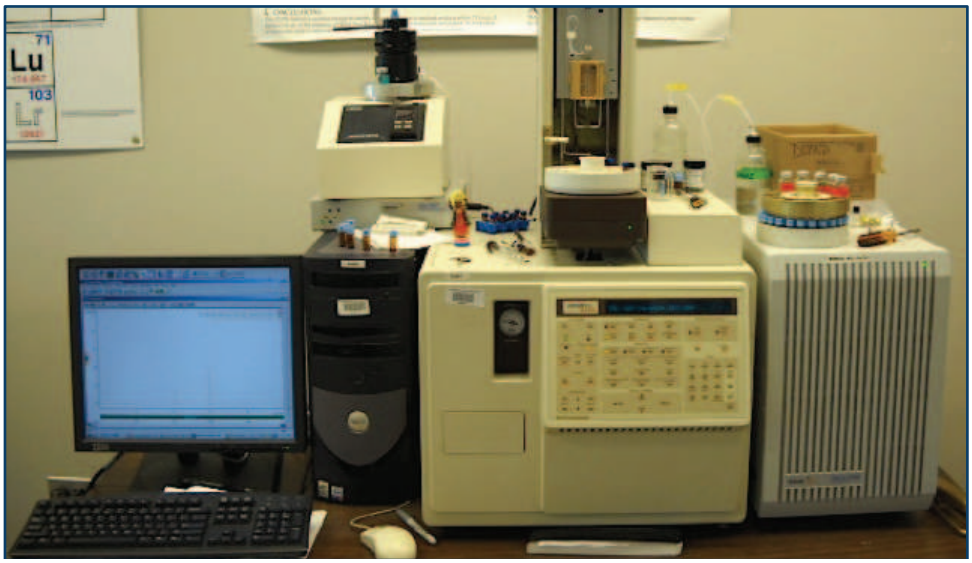

Figure B.18 Varian 3400 GC equipped with a Saturn 2000 ion-trap mass spectrometer (GC-MS) (Varian Inc., Palo Alto, CA, U.S.A.).

Figure B.19 Agilent 6890N gas chromatograph equipped with a 5975 inert Mass Selective Detector (Agilent, Santa Clara, CA, U.S.A.).

Figure B.20 Quatra C.