CONVERTING WASTEWATER TREATMENT FACILITIES INTO BIOREFINERIES: BIODIESEL FROM WASTEWATER MICROORGANISMS

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

In a world with growing energy demands, increasing dependence on fossil fuels has become the norm, but given the finite supply of these fuels, this is no longer a sustainable approach. Biodiesel is one alternative renewable fuel source with great promise. Unlike other forms of renewable energy, biodiesel is directly usable in existing forms of technology, such as the diesel engine, and does not require significant retrofitting. Biodiesel is composed of fatty acid esters, like fatty acid methyl ester (FAME), which are made via the transesterification reaction, wherein fatty acids are esterified with alcohol in the presence of a catalyst. Traditionally biodiesel has been produced from vegetable oils, however, recently many alternative feedstocks, such as used cooking oil, have been gaining significant attention. One interesting alternative feedstock with very limited research is wastewater sludge, a major by product of the wastewater treatment process. Rich in fatty acids that are ripe for transesterification, wastewater sludge offers significant potential as a biodiesel feedstock.

Currently, treatment and disposal of sludge have significant economic and energy costs for wastewater treatment plants. Some of these costs have been mitigated through the use of anaerobic digesters, but often significant pretreatment is required for these systems suggesting that alternative cost-saving technologies are desirable. Biodiesel production from sludge is one such technology. Furthermore, the sludge-derived biodiesel could be used to power equipment at wastewater treatment plants making the energy intensive treatment process itself more sustainable. A final

possibility posed by sludge-derived biodiesel is the potential for anaerobic digester pretreatment via the transesterification reaction used in this process. In this scenario producing biodiesel from wastewater sludge would have the dual effect of both producing a useable fuel product and improving downstream sludge management.

In this study the suitability of wastewater sludge as feedstock for biodiesel was evaluated by several different approaches, including extraction yields via *in situ* transesterification, FAME analysis via gas chromatography, fuel property analysis, and varying microbial populations. Additional tests were performed on the portion of sludge remaining after the biodiesel extraction process, termed extracted sludge (ES), to determine whether or not the biodiesel production process could simultaneously serve as a type of anaerobic digester pretreatment. ES and untreated control sample of waste activated sludge (CWAS) were subjected to dewaterability tests and soluble chemical oxygen demand (COD) analysis in order to assess the pretreatment potential of transesterification.

This research has shown that it is possible to produce biodiesel possessing good heating values (39-44 MJ/kg), densities (0.84-0.86 kg/L), and FAME profiles (12 to 20 carbon chains), from wastewater sludge with a relatively high yield (9-13%). This study has also shown that ES has good dewaterability and soluble COD in comparison to CWAS, confirming the pretreatment potential of transesterification. Further research is required before this technology can be applied at full-scale. In particular, sludge-derived biodiesel should be subjected to a more rigorous battery of fuel quality tests to determine whether or not the fuel meets all of the specifications for biodiesel required by the ASTM and the EN.

Chapter 1

INTRODUCTION

1.1 Biodiesel Overview

With the predicted depletion of conventional petroleum resources and the negative externalities associated with fossil fuel use, such as global climate change and environmental pollution, renewable fuels like biodiesel have been getting considerable attention. Biodiesel has several advantages over petroleum diesel including lower harmful emissions, lower carcinogen production potential, and biodegradability (Krawcyzk, 1996; Ma and Hanna, 1999; Knothe et al., 2005). Biodiesel is comprised of esterified fatty acids and is produced through a process known as transesterification, wherein lipids such as triglycerides, phospholipids, or free fatty acids, are reacted with an alcohol, in either an acid or base catalyzed reaction (Ma and Hanna, 1999; Carrapiso and Garcia, 2000).

Biodiesel is typically made from vegetable oils taken from agricultural crops, including soybeans, cottonseed, and canola oil (NREL, 2009). While vegetable oils are the most popular feedstock used for biodiesel production, other possible feedstocks that have been investigated include wasted cooking oil, animal fats, algae, and bacteria (Ma and Hanna, 1999; Encinar et al., 2005). The use of waste products as feedstocks for biodiesel is ideal because they tend to be of low cost and can contribute to making the production process more sustainable. Recently, wastewater sludge has been proposed as a potential feedstock for biodiesel and although some preliminary

investigations have been performed using primary and secondary sludge, further research is needed (Dufreche et al. 2007; Mondala et al., 2009).

1.2 Waste Activated Sludge Overview

Of the many different wastewater treatment processes available the most commonly used treatment technique is the activated sludge process (Metcalf & Eddy, 1991; Rittman and McCarty, 2001). In this process, bacteria suspended in an aeration basin are used to remove organic materials in the wastewater stream. The bacteria in the aeration basin effluent are then removed from the water phase via sedimentation and either wasted or recycled back into the aeration basin (Tchobanoglous and Schroeder, 1985). This wasted bacteria, called waste activated sludge (WAS), is critical for maintaining proper microbial density in the aeration basin and is an inevitable byproduct of the activated treatment process. Management of WAS is one of the major challenges for a wastewater treatment plant and contributes to the high costs and energy demands of the activated sludge process (Metcalf & Eddy, 1991; Appels et al., 2008). Extracting biodiesel from WAS prior to disposal is one method of adding value to what would otherwise be considered a limited use waste product.

1.3 Anaerobic Digestion Overview

Anaerobic digestion is a process wherein anaerobic methanogenic bacteria are used to convert organic materials into methane gas, which in turn can be recovered as a potential fuel source (Tchobanoglous and Schroeder, 1985). The anaerobic digestion process is most commonly used at municipal wastewater treatment plants to further treat sludge to both reduce its solid content and render it less active, a process known as waste stabilization (Metcalf & Eddy, 1991). Anaerobic digestion is ideal for

waste stabilization because high loading rates can be used, cell growth is low, the nutrient requirements are low, and methane gas is produced (Rittman and McCarty, 2001). Because anaerobic digestion of WAS is limited by the hydrolysis of organic material, techniques for pretreating WAS, such as thermal and chemical pretreatment, in order to solubilize organic matter and improve sludge digestibility have been investigated (Li and Noike, 1992; Lin et al., 1997; Kim et al., 2003; Neyens et al., 2003; Appels et al., 2008; Bougrier et al., 2008). Another potential benefit of extracting biodiesel from wastewater sludge is that the high temperature and caustic catalyst required for the transesterification reaction may also function as thermochemical pretreatment to improve the digestibility of the remaining sludge.

1.4 Research Objectives

The primary objective of this research is comprised of two parts. First, to demonstrate the potential for extracting biodiesel from WAS and second, to demonstrate preliminary techniques to manage the fraction of sludge remaining after the biodiesel extraction process. These broad objectives can be broken down into several specific tasks as follows:

1) To evaluate the potential of WAS as a biodiesel feedstock and the effect of different microbial populations in WAS on the biodiesel extraction yield

2) To determine some of the fuel properties of sludge-derived biodiesel

3) To demonstrate the potential of *in situ* transesterification as an anaerobic digestion pretreatment technique

Chapter 2

LITERATURE REVIEW

2.1 Biodiesel Sources and Use

Despite being invented over 100 years ago, the eponymous diesel engine still remains one of the most popular types of engines in use today. While the majority of diesel engines currently utilize petroleum-derived diesel as their primary fuel source, Rudolf Diesel himself also tested his engines with renewable plant based fuel sources, demonstrating successful operation of the engine using peanut oil (Knothe et al., 2005). However, with the rise of inexpensive oil sources, the use of vegetable oils was quickly supplanted by petroleum diesel (Ma and Hanna, 1999). The growing concern over global warming due to rising carbon dioxide emission along with depleting oil supplies has renewed interest in alternative fuel sources, looking beyond merely vegetables oils into the realm of biodiesel.

Conventionally, biodiesel has been made primarily from vegetable oils, but there are several major barriers to widespread adoption of vegetable oil-derived biodiesel. First, because the vegetables oils used for biodiesel are highly refined virgin oils, the cost of biodiesel is much higher than petroleum diesel (Encinar et al., 2005). Second, as conventional biodiesel is derived from agricultural crops that are primarily used as food crops, diverting portions of this crop can affect food availability. Some studies have even reported that producing fuel from food crops has contributed to the global rise in food prices (Mitchell, 2008). Finally, land use changes need to be

carefully considered when assessing the impact of biodiesel production. In some circumstances, the drive to produce more crops for fuel production can result in the destruction of forested regions for new farmland, resulting in a net increase in carbon dioxide emissions (Fargione et al., 2008).

Given the problems associated with using agricultural crops as a source for biodiesel, many alternative biodiesel feedstocks have been investigated including non-food sources such as algae, bacteria, and non-edible oil crops as well as waste products such as used cooking oil and animal fats (Ma and Hanna, 1999, Knothe et al., 2005). It is important to note that while many of these alternative feedstocks have numerous advantages, there is no one single feedstock that can ultimately provide for the current diesel demand. Therefore, a variety of sources should be explored.

Currently in the U.S. biodiesel has limited use and availability. The total production of biodiesel in the U.S. was estimated to be 600 million gallons in 2008, but this amount only represents 2% of the total diesel fuel usage for that year (EIA, 2007; NREL, 2009). One way of expanding the usage of biodiesel is to blend it in a certain percentage with petroleum diesel. For example, B20, a blend consisting of 20% biodiesel and 80% petroleum diesel, is popular because it balances out the considerations of economic cost, engine performance, and environmental cost while maintaining similar properties to pure petroleum diesel (NREL, 2009).

2.2 Biodiesel Properties and Specifications

In addition to being a renewable fuel source, biodiesel has several other important advantages over petroleum diesel. First, biodiesel has the potential to be carbon neutral (i.e. it will not contribute to further increasing green house gas emissions in the form of carbon dioxide) (Chisti, 2008). Second, biodiesel is both

non-toxic and biodegradable, making accidental spills less of a concern (Ma and Hanna, 1999; Knothe et al, 2005; Lotero et al., 2005; Meher et al., 2006). Third, biodiesel burns more cleanly than diesel with lower carbon dioxide and particulate matter emissions (Canakci and Van Gerpen, 2001; NREL, 2009). However, biodiesel has not been shown to reduce NO_x emissions, sometimes even showing an increase in NO_x emissions (Knothe et al, 2005; NREL, 2009).

There are a variety of standards available for biodiesel in both the U.S. and the European Union (E.U.). The U.S. standard is provided by the American Society for Testing Materials (ASTM) and the E.U. standard is provided by the European Committee for Standardization (EN) (EN, 2008; ASTM, 2010). These specifications are benchmarks for the necessary properties biodiesel fuel must possess in order to be acceptable for use in motor vehicles and are displayed in Table 2.1. Many of the standards govern the physical property requirements such as density, viscosity, cetane number, heating value, flash point, cloud point, and pour point, which critically affect diesel engine performance. While most of the specifications for these parameters are similar to the petroleum diesel specifications, two notable exceptions are the pour point and flash point. The higher flash point of biodiesel reduces the risk of accidental combustion making biodiesel easier to transport (Meher et al., 2006). The lower cloud point and pour point of biodiesel suggests that there is a risk of biodiesel gelling under cold weather conditions if this specification is not met (Knothe et al., 2005; Meher et al., 2006; NREL, 2009). The other parameters included in the specification are chemical composition issues that are consequences of the production process, such as residual water, methanol, glycerin, and residual catalyst, but as these parameters are feedstock specific, they have not been included in Table 2.1 (Meher et al., 2006).

Finally, the elemental composition of biodiesel is also listed in Table 2.1 and is noteworthy for the high oxygen content, an additional benefit of biodiesel because it allows for better combustion and lower emissions in comparison to petroleum diesel (Canakci and Van Gerpen, 2001; NREL, 2009),

Table 2.1 Table showing the various properties of diesel and biodiesel according to both the ASTM and EN standards (Knothe et. al., 2005; Lotero et al., 2005; EN, 2008; NREL, 2009; ASTM, 2010).

Property	Units	Diesel	Biodiesel - USA	Biodiesel - EU
Standard		ASTM D975	ASTM D6751	EN 14214
Composition		Hydrocarbon (C10 -C21)	FAME (C12 -C22)	96.5% FAME
Kin. Viscosity at 40°C	mm^2/s	1.9 to 4.1	1.9 to 6	3.5 to 5
Density	g/mL	0.85	0.88	0.86 to 0.90
Flash Point	°C	60 to 80	100 to 170	120
Cloud Point	°C	-15 to 5	-3 to 12	-
Pour Point	°C	-35 to -15	-15 to 16	-
Water	vol %	0.05	0.05	-
Oxygen	wt %	0	11	-
Cetane Number	#	40 to 55	48 to 60	51
Heating Value	MJ/kg	42 to 45	39 to 42	35[a] [a]EN Biodiesel as Heating Oil Standard

2.3 Biodiesel Production

Lipids are a diverse group of molecules used for both energy storage and structure and are made up of fatty acids, which are a class of carboxylic acids possessing hydrocarbon chains that vary from four to thirty six carbons long (Nelson and Cox, 2005). Biodiesel is produced through the transesterification of lipids and fatty acids, wherein the lipids or fatty acids are reacted with an alcohol to create an ester bond. While a variety of alcohols can be used in the transesterification reaction, methanol is by far the most common (Freedman et al., 1984; Ma and Hanna, 1999). When using methanol as the alcohol, the major fatty acid ester created is fatty acid methyl ester (FAME). Transesterification can be broadly defined as a reaction in which FAME is created from a mixture of fatty acids and lipids. In i*n situ* transesterification, fatty acids and lipids are simultaneously extracted from the source material and then transesterified in one step rather than as two separate steps (Carrapiso and Garcia, 2000). This reaction can be catalyzed using a variety of different catalysts (Meher et al., 2006).

The most frequently considered form of transesterification is when a molecule of triglyceride is reacted with three molecules of alcohol to form three ester molecules and one molecule of glycerin (Figure 2.1). As major energy storing compounds, triglycerides are one of the simplest lipids, consisting of a glycerin group ($C_3H_5(OH)_3$) plus three fatty acids chains (Figure 2.1), and are the primary constituent of many plant oils and animal fats (Nelson and Cox, 2005). Given that plant oils are the most commonly used biodiesel feedstock, few lipids, beyond triglycerides, were initially considered in the transesterification process. However with the advent of waste feedstocks, the range of lipid consideration has been re-evaluated.

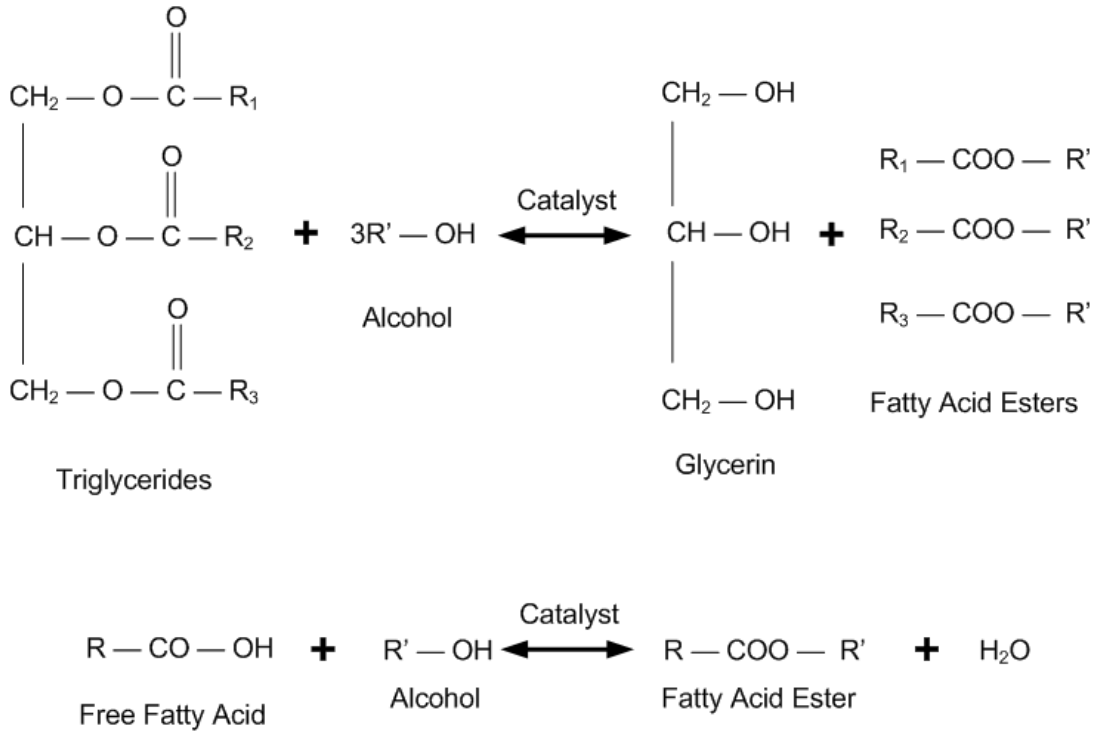


Figure 2.1 The transesterification reaction. Triglycerides (top) and free fatty acids (bottom) are being converted to fatty acid esters. Adapted from Carrapiso and Garcia (2000) and Marchetti et al. (2006).

Unlike plant oils, many alternative feedstocks do not contain a uniform distribution of lipids in one particular form but rather a complex mixture of lipids and fatty acids. Most notably, many waste product feedstocks possess a high quantity of free fatty acids (FFA) with some waste grease possessing between 10-25% FFAs (Canakci and Van Gerpen, 2001). Some other lipids present in alternative feedstocks include diglycerides and monoglycerides, which are similar in structure to triglycerides but lacking in one or two fatty acid chains. As they are also intermediates of triglyceride transesterification, mono- and di- glycerides should be readily accessible for transesterification. Finally, phospholipids (Figure 2.4(a)) in the form of glycerophospholipids are a common component of cell membranes and also possess a similar structure to triglyceride, with a phosphate group substituted for one of the fatty acid chains, and given their structural similarity to triglycerides are also readily available for transesterification (Figure 2.1) (Carrapiso and Garcia, 2000; Nelson and Cox, 2005). Transesterification of these other types of lipids is possible, but their effect on the transesterification reaction, particularly in the case of FFA, is highly dependent on the choice of catalyst and the moisture content of the feedstock, catalyst, and alcohol.

2.3.1 Catalyst Selection

Transesterification is typically acid or base catalyzed. Recently, enzyme catalyzed reactions using lipases have also been investigated, but currently their usage is limited by the high catalyst cost (Fukuda et al., 2001; Meher et al., 2006). Currently alkaline catalysts in the form of sodium hydroxide or potassium hydroxide are the most commonly used catalysts for biodiesel production from plant oils (NREL, 2009). Base catalysts are significantly faster than acid catalysts and base catalyzed

transesterification can be conducted at room temperature (Freedman et al., 1984; Ma and Hanna, 1999; Carrapiso and Garcia, 2000). However, there are some significant interference considerations for base catalysts, most notably the effect of free fatty acids (FFA) and water. It has been demonstrated that base catalyzed transesterification yields will be significantly reduced unless the feedstocks contain less than 0.5% FFA and anhydrous alcohol and catalyst are used (Bradshaw and Meuly, 1944; Feuge and Grose, 1949; Freedman et al., 1984; Ma and Hanna, 1998; Lotero et al., 2005). Ma et al. (1998) have shown that, for base catalyzed transesterification of beef tallow, the water content should be kept below 0.06% and the FFA content below 0.5%, both on a weight/weight basis, in order to ensure an efficient reaction (Ma and Hanna, 1998). The FFA and sodium hydroxide can also form soap (Figure 2.2(a)) which can lead to major separation issues, particularly in the case of glycerin, as well as significant emulsion problems (Canakci and Van Gerpen, 2001; Lotero et al., 2005). Likewise the presence of water can lead to the hydrolysis of the newly formed FAMEs back into FFA that can in turn lead to more soap formation, as shown in Figure 2.2(b) (Lotero et al., 2005).

Acid catalysts, while slower than base catalysts and requiring higher temperatures, have a significant advantage over base catalysts in that they are not only uninhibited by the presence of FFA, but they can actually transesterify FFA into FAMEs (Figure 2.1) (Carrapiso and Garcia, 2000). One drawback of the transesterification of FFA, however, is that water, a byproduct of the reaction, can have inhibitory effects and will hydrolyze FAME as shown in the reaction in Figure 2.2 (b). It has been suggested that feedstock water content should be kept below 0.5 %

when using acid catalysts in order to reduce these inhibition effects (Canakci and Van Gerpen, 2001; Lotero et al., 2005).

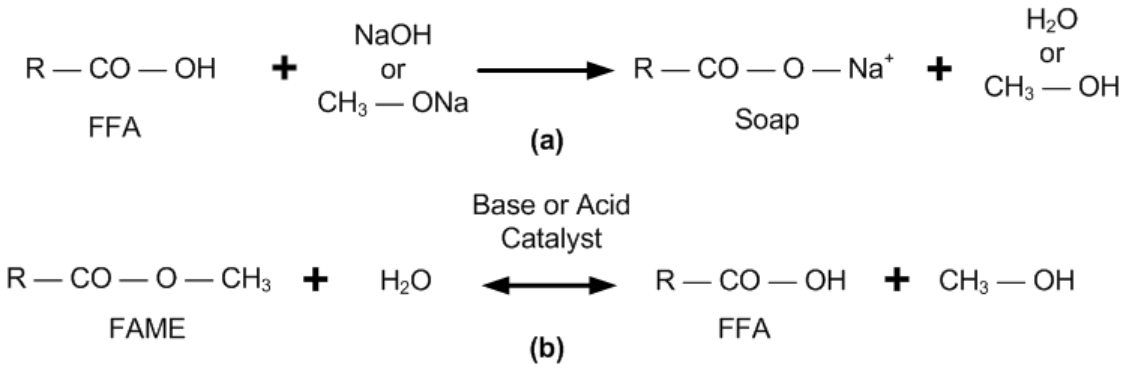


Figure 2.2 Formation of soap due to the interaction of FFA and sodium hydroxide (a) and hydrolysis of FAMEs by water (b). Adapted from Lotero et al. (2005).

While acid and base catalysts have been described separately thus far, it is possible to use the two catalysts in tandem. Canakci and Van Gerpen (2001) have reported on the feasibility of base catalyzed transesterification using a two-step acid catalyzed pretreatment procedure. In this study, waste grease of up to 33% FFA were first reduced to 1% FFA using an acid catalyzed pretreatment with water removal. The remaining lipid fraction was then transesterified using a base catalyst. This combined process attempts to maximize the strengths of both catalysts in order to derive the optimal amount of biodiesel from any feedstock regardless of the fatty acid

composition. Encinar et al. (2005) have also improved biodiesel yields from used frying oil by using two-stage transesterification.

In review, under optimal conditions using uniform feedstocks, base catalysts are significantly faster than acid catalyst. However, base catalysts are much more process sensitive and certain impurities can lead to significant biodiesel yield reductions. Acid catalysts, while slower, are a more robust catalyst and should be used for feedstocks with high FFA, such as many waste materials.

2.3.2 Separation Considerations

Following the transesterification process, there are a variety of residual materials which can remain in the biodiesel including catalyst, alcohol, un-reacted lipids, and glycerin. While it is not possible to remove all of these materials completely, the presence of some of these residuals can affect fuel quality. Glycerin is relatively insoluble in biodiesel and thus can be easily separated from the biodiesel phase via simple techniques such as gravity settling (Van Gerpen, 2005). Glycerin is then recoverable as a useful byproduct of the biodiesel production process. However, the glycerin levels depend on the level of triglycerides present in the initial feedstock. Since transesterified FFA do not produce glycerin, a feedstock high in FFA would have relatively less glycerin. Excess alcohol can be removed from the biodiesel via vacuum flash distillation (Van Gerpen, 2005). Finally, residual catalyst can then be removed by the addition of acid or base depending on the type of catalyst chosen and any other residual materials, such as salt and un-reacted lipids, can be removed by washing the biodiesel with water (Van Gerpen, 2005).

2.4 Wastewater Sludge

Within the wastewater treatment process there are two main sources of sludge, first from primary treatment (i.e. sedimentation processes) and second from secondary treatment (i.e. biological processes such as activated sludge). The sludge from primary treatment is called primary sludge and is composed of settlable solids from raw wastewater and tends to be small, simple organic molecules that are readily digestible (Tchobanoglous and Schroeder, 1985). The activated sludge process is the most commonly used type of secondary treatment, and sludge from this process is termed secondary sludge. Secondary sludge can be further subdivided into two sludge streams: return activated sludge (RAS) and waste activated sludge (WAS) (See Figure 2.3). Secondary sludge is primarily composed of microbial cells produced during the activated sludge process (Tchobanoglous and Schroeder, 1985). Primary sludge and WAS, the wasted portion of secondary sludge, are typically combined and pumped to an anaerobic digester for waste stabilization, resulting in digested sludge or biosolids (Metcalf & Eddy, 1991).

Wastewater sludge is the major waste product of municipal wastewater treatment. In the U.S., annual sludge production for 2010 is expected to increase to 8.2 million dry tons and between 1972 and 1998, sludge production increased by 50%, although the U.S. population increased by only 29% (WEF, 2008). Management of sludge is estimated to be about 50% of the working costs for municipal wastewater treatment plants (Appels et al., 2008). By 2010 it is estimated that the main management techniques for all of this sludge after stabilization will be land application (48%), disposal in landfills (10%) or incineration (19%) (WEF, 2008). Despite being the principal sludge disposal techniques, all of these management strategies are under increasing pressure because of the declining land availability for land application or

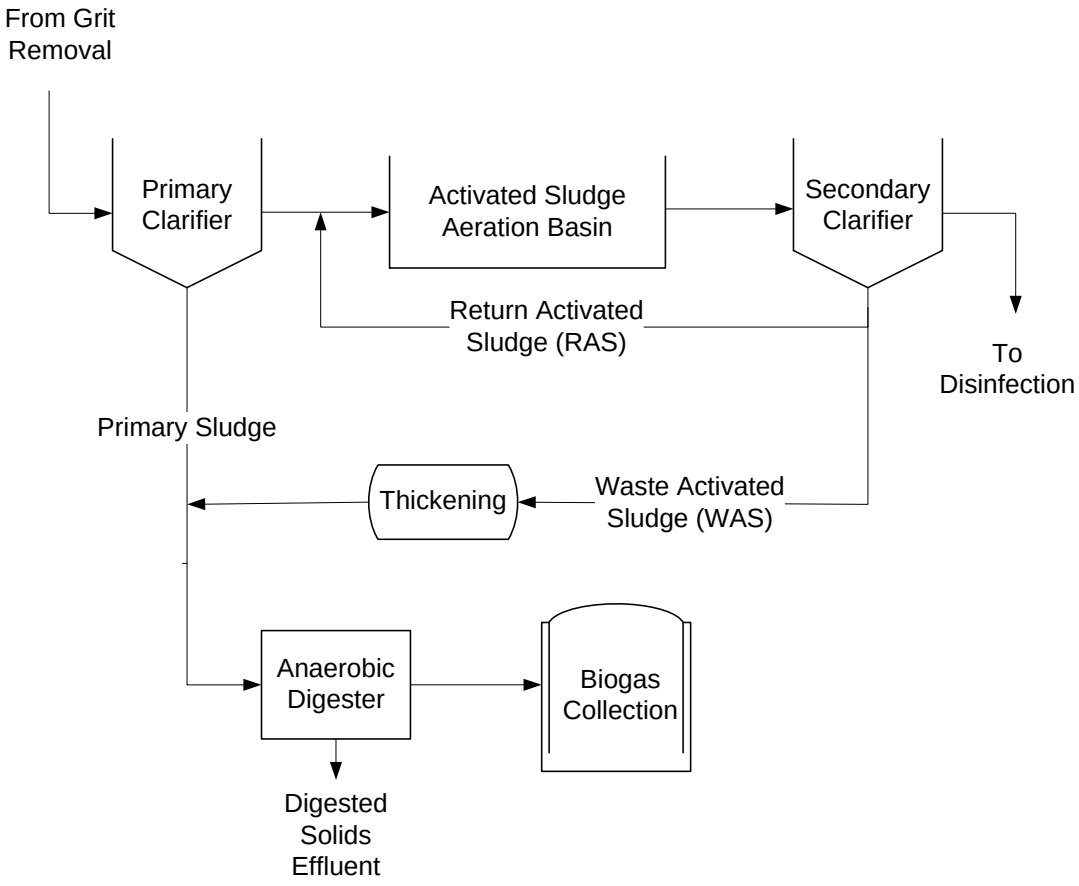
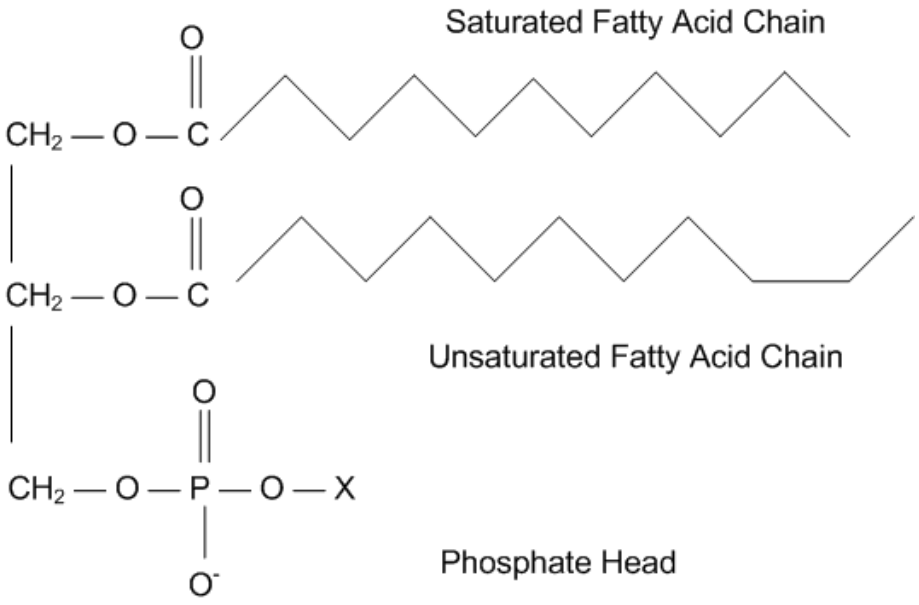


Figure 2.3 Primary and secondary treatment in a municipal wastewater treatment plant. Adapted from Tchobanoglous and Schroeder (1985) and Metcalf & Eddy (1991).

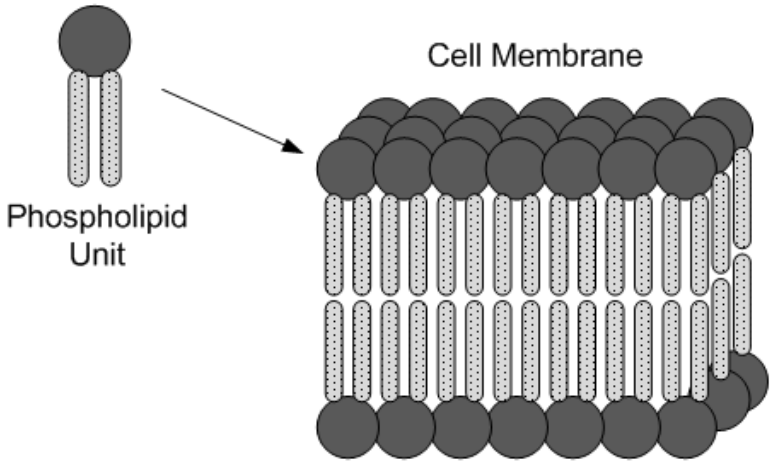
landfill use and the strict regulations on incineration emissions (Metcalf & Eddy, 1991). Land application in particular has come under scrutiny for uncertainty about the potential health risks, although no proven risks have been found at this time (NRC, 2002; WEF, 2008). Given these problems, finding alternative uses for this excess of sludge offers significant environmental and economic benefits for wastewater treatment plants and the surrounding communities.

2.4.1 Sludge as Biodiesel Feedstock

Primary sludge has been estimated to contain 20-30% grease and fat on a dry weight basis, whereas activated sludge has been reported to contain 5-12% grease and fat (Metcalf & Eddy, 1991). It is important to note that these grease and fat values do not necessarily reflect the total lipid content, especially in regards to microbial lipids. For example, WAS is comprised of bacteria with cell membranes containing glycerophospholipids, as shown in Figure 2.4, which are readily transesterified (Carrapiso and Garcia, 2000). It has been estimated that cell membranes of microorganisms in sludge may contain up to 24% phospholipids on a dry weight basis (Dufreche et al., 2007) (Figure 2.4). Activated sludge has been reported to contain 5-10% FFAs as a percentage of total fat (Fransen et al., 1995). Lipid values of 17-18% on a dry weight basis have been reported for primary sludge, with 65% of these lipids being FFA and 7% being glycerides (Boocock et al., 1992). Thus both primary sludge and WAS have been shown to contain a mixture of lipids and fatty acids, such as phospholipids, FFA, and glycerides. Primary sludge tends to have a higher FFA and lipid content than WAS. This difference is because primary sludge, being mostly composed of raw putrescible matter, usually has more free organic material and less complex organic structures than WAS (Appels et al., 2008).



(a) Phospholipid Molecule



(b) Phospholipid Bi-layer

Figure 2.4 Phospholipid structure (a) and phospholipid bi-layer structure (b). Adapted from Nelson and Cox (2005).

Some preliminary investigations using primary and secondary sludge as a biodiesel feedstock have been performed. Dufreche et al. (2007) reported a FAME yield of 6.23% for dry secondary sludge using *in situ* transesterification at 50°C with 1% (v/v) sulfuric acid in methanol and a 40:1 methanol to sludge mass ratio. Mondala et al. (2009) found in their studies of the *in situ* transesterification of dry wastewater sludge, a maximum FAME yield of 14.5% for primary sludge and 2.5% for secondary sludge in a reaction at 75°C using 5% (v/v) sulfuric acid in methanol and a 12:1 methanol to sludge mass ratio.

2.4.2 Enhancing Sludge Potential with *Gordona amarae*

Activated sludge is comprised of an assortment of different bacteria with different properties. Filamentous bacteria are typically present in activated sludge in small numbers, but certain environmental conditions can trigger them to grow in excess leading to a problem known as sludge bulking or foaming (Tchobanoglous and Schroeder, 1985). Sludge foaming is characterized by thick brown foam on the surface of the sludge aeration basins and can seriously disrupt operations at a wastewater treatment plant (Richards et al., 1990). This problem has been well studied over the years and a variety of management techniques, such as controlling the mean cell residence time or using an anaerobic selector, have been explored and implemented (Pitt and Jenkins, 1990; Cha et al., 1992). Separation of the foam layer by air stripping the filamentous species has also shown to be possible, driving up to 90% of the bacteria, based on filament length, into the foam layer (Richards et al., 1990; Jenkins et al., 2004). One species that has been identified as a major contributor to sludge foaming situations is the gram positive bacteria *Gordona amarae* (formerly classified as *Nocardia amarae*) (Lechevalier and Lechevalier, 1974; Goodfellow et al.,

1994). It has been determined from survey data that *G. amarae* is the most frequently occurring filamentous bacteria in wastewater treatment plants in the United States (Jenkins et al., 2004).

In addition to the inherent potential offered by sludge as a feedstock for biodiesel, *G. amarae* features some additional characteristics that may make it especially well suited for biodiesel extraction. This species has a high surface area to volume ratio in comparison to other bacteria present in wastewater sludge. (Kim et al., 2002). This high surface area to volume ratio indicates that the species would have relatively larger amount of phospholipids available for biodiesel conversion (Figure 2.4). *G. amarae* has also been shown to contain a special class of fatty acids known as mycolic acids, which are long chain fatty acids, up to 54 carbons long in the case of *G. amarae* (Goodfellow et al., 1982). Despite the long chain length, techniques have been developed for transesterifying mycolic acids, although it is important to note that these long chain fatty acids are not detectable via conventional FAME gas chromatography analysis and special pyrolytic cleaving chromatography techniques must be utilized to detect them (Minnikin et al., 1975; Guerrant et al., 1981). In addition to mycolic acids, FAME profiles of the other fatty acids present in *G. amarae* have been investigated as well. These profiles have demonstrated that the fatty acids in *G. amara*e are primary composed of myristic, either palmitoleic or sapienic acid, oleic, stearic acid, as well as the uncommon tuberculostearic acids, with the highest percentage by far (up to 44.2%) being palmitic acid (Goodfellow et al., 1982). All of these fatty acids, except tuberculostearic acids, fall in the specified range for biodiesel chain lengths of 12 to 20 carbons (ASTM, 2010).

Finally, *G. amarae* has been shown to be capable of storing and accumulating energy in the form of triglycerides, an unusual characteristic of prokaryotic organisms, and it has been further shown that *G. amarae* can accumulate as much as 6.1% triglyceride on a dry weight basis when grown with gluconoate or hexadecane (Alvarez and Steinbüchel, 2002; Alvarez, 2003). Given this ability for triglyceride accumulation, it is possible that *G. amarae* from a typical activated sludge process will have relatively higher amount of triglyceride in comparison to other bacteria present in the sludge. Thus, it has been demonstrated that *G. amarae* contains a variety of fatty acids and lipids which are suitable for biodiesel conversion in potentially higher quantities than other types of activated sludge bacteria.

2.5 Anaerobic Digestion

One of the oldest wastewater treatment techniques in use today, anaerobic digestion, is a biological process involving anaerobic microorganisms primarily used for sludge stabilization processes (Metcalf & Eddy, 1991). Anaerobic digestion can be operated in either mesophilic (37°C) or thermophilic (55°C) conditions. Primary and thickened secondary sludges are fed at either a high rate or standard rate depending on digester design (Figure 2.3). Anaerobic digestion treats the sludge by reducing the total solids content of the sludge, reducing the strong odors associated with sludge, and producing methane gas. The microbial population in a digester is a complex mixture of anaerobes that break down the organic material in the feed sludge first to organic acids and hydrogen via hydrolysis and fermentation and second to methane and carbon dioxide via methanogenic bacteria (Rittman and McCarthy, 2001). The hydrolysis step has been identified as the limiting step in anaerobic digestion and research into improving anaerobic digestion has focused on increasing this rate of hydrolysis (Li and

Noike, 1992; Kim et al., 2003; Appels et al., 2008). While primary sludge tends to be readily digestible, the complex structure of the microbial cells in WAS are more difficult to hydrolyze (Tchobanoglous and Schroeder, 1985). Mechanical, thermal, chemical, and biological pretreatment techniques have been used to solubilize the organic material in WAS in order to improve the rate of hydrolysis (Appels et al., 2008). The key parameter investigated in pretreatment studies is soluble COD, a measurement of the soluble organic matter readily available for anaerobic digestion.

Two heavily studied pretreatment techniques are thermal and chemical pretreatment. Various thermal pretreatment conditions have been reported, with a pretreatment of 60 minutes at 170°C, being reported as the most favorable conditions for COD solubilization (Li and Noike, 1992). However, a thermal pretreatment of 30 minutes at a temperature of 120°C has been shown to cause a COD solubilization of 17.6% (Kim et al., 2003). Bougrier et al. (2008) have shown that thermal pretreatment at temperatures above 150°C can also lead to improvements in dewaterability and a reduction in sludge volume index and sludge viscosity. Chemical treatment techniques have also been investigated such as alkali and acid pretreatment (Neyens et al., 2003; Appels et al., 2008). Alkali pretreatment has shown a COD solubilization between 36 and 40% when using sodium hydroxide at ambient temperatures (Lin et al., 1997; Kim et al., 2003). Finally, thermochemical pretreatment, in which both alkali and thermal treatment techniques are combined, has been shown to have COD solubilization of 51.8% (Kim et al., 2003).

Given the use of either alkaline or acidic catalysts, as well as the heating required for rapid transesterification, the transesterification conditions used in biodiesel production resemble the thermochemical pretreatment techniques outlined

above. Thus it is possible that the *in situ* transesterification of sludge could function concurrently as an anaerobic digestion pretreatment process.

2.6 Summary

Biodiesel is produced from a variety of lipids and fatty acids via transesterification using acid or base catalysts, has numerous advantages over conventional diesel, and has strict set of property standard requirements. Catalyst and feedstock selection have a strong influence on the transesterification reaction and must be chosen carefully in order to optimize reaction efficiency. Waste feedstocks offer significant potential for biodiesel production. Wastewater sludge, as a waste material from the wastewater treatment process, could be a feedstock for biodiesel. Although some research using both primary and secondary sludge as a feedstock for biodiesel production has been performed, there is room for further research. In particular, the use of WAS as a biodiesel feedstock should be explored further. Additionally, the yields of biodiesel from WAS may be enhanced by promoting the growth of certain organisms in WAS. Finally, the parameters used in the transesterification reaction simulate the conditions used in the pretreatment of sludge for an anaerobic digester, and therefore may also be able to function as an anaerobic digester pretreatment for wastewater sludge.

Chapter 3

MATERIALS AND METHODS

3.1 Wastewater Sludge Samples

Samples of waste activated sludge (WAS) were acquired from both Wilmington Wastewater Treatment Plant (WL Sludge; Wilmington, DE) and the San Francisco Water Pollution Control Plant (SF Sludge; San Francisco, CA). The samples had an initial total suspended solids concentration (TSS) of ~5000 mg/L and ~10000 mg/L, respectively. In order to concentrate the sludge, the samples were allowed to gravity-thicken overnight and the supernatant was poured off.

SF Sludge was only used in the preliminary fatty acid methyl ester (FAME) comparison and biodiesel experiments. WL Sludge was used in these experiments as well as the later anaerobic digester experiments. Although most of these samples would be dried for the biodiesel experiments, an additional sample of WL Sludge was also prepared without drying, labeled as Wet WL Sludge. To determine the effect of different microbial populations on biodiesel yields, one species of interest, *G. amarae*, was selected, and samples containing different amounts of this species were compared.

3.1.1 Detection of G. amarae

Microscope slide smears of the WAS from both treatment plants were prepared according to the gram stain procedure outlined by Pitt and Jenkins (1990). A

Microphot-FX microscope (Nikon, Tokyo, Japan) was used to confirm the presence of *G. amarae,* which appeared as purple rod-shaped bacteria expressing a branch like growth pattern. Only the SF Sludge was found to contain significant levels of *G. amarae*.

3.1.2 Air-Stripping Procedure

To separate *G. amarae* from the rest of the WAS sample, an air-stripping technique was used. SF Sludge was placed into a large cylindrical glass column with a tapered end and an opening at the bottom (Figure 3.1). A piece of size 17 Tygon Masterflex tubing (Cole Parmer, Vernon Hills, IL) was attached to the bottom of the column. The open tube was sealed with a clamp. A Deep Water DW 24-2 air pump (Tetratec, Pasadena, California) attached to an air stone was used to provide aeration. The air stone was placed towards the bottom of the glass cylinder as shown in Figure 3.1. After an aeration period of 5 minutes, the air pump was stopped and the air stone was removed. The bottom portion of the sludge was withdrawn to a glass beaker for storage, and the foam layer was collected in another beaker. SF Sludge supernatant was used to wash any foam bubbles stuck on the glass surface into the collection beaker. In order to ensure that the sludge was fully stripped of *G. amarae,* the sludge sample was stripped in twice in. The resulting *G. amarae* foam sample from this air-stripping process was designated as GA Foam and the biodiesel yields of this sample would be compared along with WL Sludge, Wet WL Sludge, and SF Sludge samples.

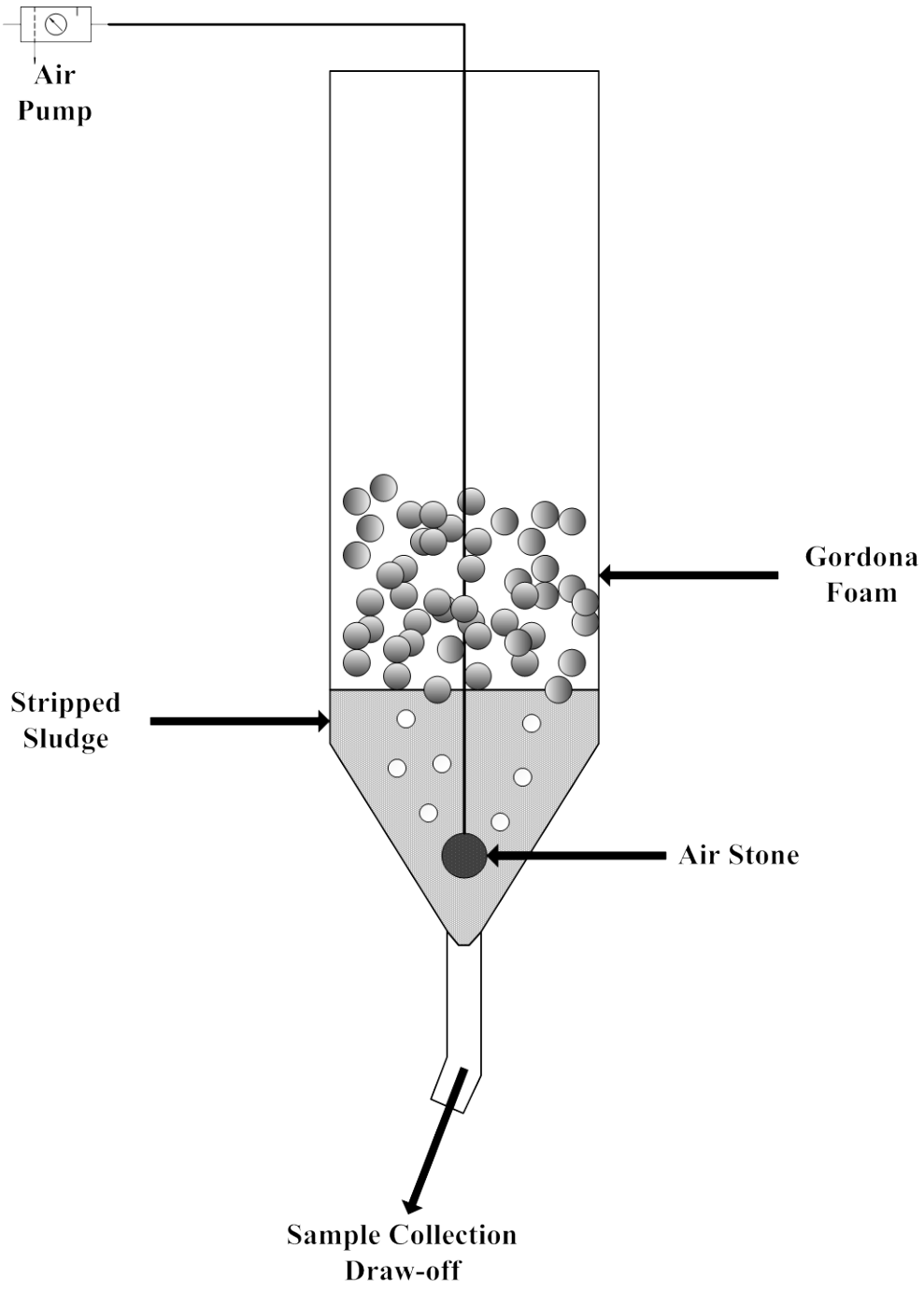


Figure 3.1 Diagram of air stripping procedure

3.2 Preliminary Fatty Acid Comparison

Prior to performing biodiesel experiments, a preliminary study was conducted to assess the relative amounts of fatty acids present in different sludge samples. The three sludge samples investigated were WL Sludge, with a TSS of 5438 ± 125 mg/L, SF Sludge, with a TSS of 10910 ± 122 mg/L, and GA Foam, with a TSS of 1461 ± 14 mg/L. Fatty acids from these samples were extracted and analyzed as fatty acid methyl esters (FAMEs) using the Sherlock Instant FAME™ technique (MIDI, Inc., 2009). The reagents used in this procedure were potassium hydroxide in methanol (Reagent #1), hexane (Reagent #2), and acid in water dyed red (Reagent #3). The following extraction procedure from MIDI, Inc. (2009) was used:

4) Samples of a known volume were centrifuged in gas chromatography vials with a microcentrifuge to achieve a known cell mass of between 2 or 3 mg.

5) 250 µL of Reagent 1 was added to extract and methylate the fatty acids, and the vial was then vortex for 10 seconds.

6) 250 µL of Reagent 2 was added to transfer the fatty acids to an organic phase, and the vial was then vortex for 3 seconds.

7) 250 µL of Reagent 3 to induce phase separation. The resulting phases are a clear top layer and a red bottom layer.

8) 70 µL of the top phase was removed to gas chromatography vial with a glass insert. The samples were then analyzed using a gas chromatograph equipped with a flame ionization detector.

Six runs were performed for each sample. The peaks were identified, and the area was calculated using the Sherlock™ software system (MIDI, Inc., Newark, DE). The total peak response of the different samples were compared and normalized by the cell mass used.

3.3 Biodiesel Experiments

3.3.1 Sludge Preparation

Prior to *in situ* transesterification, sludge samples needed to be dewatered and then dried. The gravity-thickened sludge was dewatered by centrifuging at 4000 rpm for 15 minutes in a Marathon 22K centrifuge (Fisher Scientific, Pittsburgh, PA). After centrifuging, the centrate was decanted off and the dewatered sludge was collected in a glass container and placed in either a Precision Scientific Oven (Fisher Scientific) at 104 ± 1°C for 3-4 hours or a Precision Economy Incubator at 60°C overnight (Fisher Scientific). Once dried, sludge samples were pulverized with a mortar and pestle and stored at -15°C.

In preparing the Wet WL Sludge and GA Foam, there were some deviations from the above procedure. The GA Foam was not centrifuged. Instead the separated foam was transferred directly to a glass container and oven-dried. The Wet WL Sludge preparation will be discussed in detail below.

3.3.2 *In situ* Transesterification

In situ transesterification was used simultaneously to transesterify and extract lipids and fatty acids from sludge samples. *In situ* transesterification converts the fatty acids in phospholipids into FAMEs (i.e. biodiesel). The *in situ* transesterification method used in this study followed a modified procedure from Mondala et al. (2009). The reagents used are found in Table 3.1 and were prepared using ACS reagent grade chemicals from Fisher Scientific.

Table 3.1 Reagents used for *in situ* transesterification

Reagent #	Composition
#1	Sulfuric Acid in Methanol (5% v/v, 25 mL sulfuric acid in 475 mL of methanol)
#2	Hexane
#3	Saturated Sodium Chloride Solution (36 g in 100 mL of DI Water)
#4	Potassium Carbonate Solution (2% w/w, 4 g of potassium carbonate in 200 g of DI Water)

For the *in situ* transesterification experiments, a known mass of dried and crushed sludge (about 3.5 g) was measured into a 250 mL fluted Erlenmeyer flask containing a magnetic stir bar. Reagent #1 was then added in a 12:1 methanol to sludge mass ratio. Then 25 mL of Reagent #2 was added to the flask. The reaction flask was then attached to a condenser and placed inside of an improvised water bath consisting of 1 L beaker filled with 400 mL of water on top of an Isotemp heated magnetic stirrer (Fisher Scientific). The water bath was maintained at a temperature between 70-75°C and the magnetic stirrer was adjusted to a setting of approximately 200 rpm to ensure complete mixing. The reaction flask was kept at these conditions for 2 hours to allow for sufficient conversion of the sludge lipids and fatty acids to FAMEs. After 2 hours, the reaction flask was removed from the water bath and allowed to cool for 10 minutes.

Next, the FAMEs had to be separated from the resulting aqueous phase. This separation was achieved by means of a hexane extraction. The steps were as follows:

1) The contents of the reaction flask were divided equally among three 50 mL centrifuge tubes. 2 mL of Reagent #3 were added to each tube to prevent emulsion formation.

2) 15 mL of Reagent #2 (pure hexane) was added to each centrifuge tube to extract the FAMEs from the aqueous phase.

3) The centrifuge tubes were then shaken at 300 rpm on an Innova 2000 platform shaker (New Brunswick Scientific, Edison, NJ) for 3 minutes.

4) The centrifuge tubes were then centrifuged in a Marathon 22K centrifuge at 3000 rpm for 3 minutes in order to separate the aqueous phase from the hexane phase.

5) The hexane phase was then removed with a pipette and collected in a 250 mL separatory flask.

6) Steps 2-5 were repeated two more times.

The FAME-bearing hexane, now collected in a separatory flask, was then washed with 10 mL of Reagent #4, and the precipitates and water subsequently formed were allowed to settle for 10 minutes. The water and precipitates were then removed and the hexane phase was collected in a 250 mL ball flask of known mass. The contents of the flask were then evaporated for 20 minutes using a rotary evaporator set at 320 mbar with a bath temperature of 60°C and a rotation of 30 rpm. After evaporation, the ball flask was purged with nitrogen gas for 1 minute. The mass of the residue remaining was determined by subtracting the mass of the empty flask from the mass of the residue containing flask. This residue was assumed to be 100% FAMEs. The biodiesel yield was then calculated by dividing the FAME mass by the initial sludge mass.

3.3.3 *In situ* Transesterification, Wet Sludge Modification

In situ transesterification of the Wet WL Sludge was performed using the same procedure as above with some minor modifications. To keep the wet sludge

experiment consistent with the dry sludge experiment, a relationship between wet and dry sludge had to be determined. To do this, sludge samples of a known volume and a known TSS were placed into a container of predetermined mass. The mass of sludge in the container on a dry weight basis could be calculated from the volume and TSS values. The Wet WL Sludge samples were then centrifuged at 3000 rpm for 30 minutes in a Marathon 3000 centrifuge (Fisher Scientific). After centrifuging, the centrate was decanted off. The mass of the dewatered wet sludge plus the container was then measured, and the mass of the wet sludge was calculated by subtraction. From these values a ratio between the dry and wet sludge was calculated and used as a conversion factor. With this conversion factor the equivalent amount of wet sludge on a dry weight basis sludge could then be calculated. For example, given that 5 g of samples on a dry weight was desired and that this conversion factor was typically around 0.1 g dry sludge per gram, the wet sludge would then come out to a mass of 50 g. Finally, the Wet WL Sludge was collected in a glass container and stored at 4°C without drying.

Due to the increase in sludge volume, the total volume also increased requiring the use of a 250 mL glass bottle with Teflon-lined silicone septa instead of the centrifuge tubes used previously. The change in extraction vessel necessitated some other volume changes specifically, in step 1, 5 mL of Reagent #3 were added, and in step 2, 50 mL of Reagent #2 were added instead of the original volumes. Also, a larger Marathon 3000 centrifuge was then required for step 4. Otherwise, the *in situ* transesterification procedure remained unchanged.

3.3.4 Fuel Property Analysis

The ASTM D6751 standard specifies a number of specific test methods for different properties of biodiesel (ASTM, 2010). However, since these test methods were designed for large quantities of biodiesel derived from highly refined vegetable oils, it was not feasible at this time to use the ASTM methods to analyze the sludge-derived biodiesel. Furthermore, a detailed property analysis of the sludge-derived biodiesel was beyond the scope of this study. Instead simpler methods were used to estimate values for heating value and density to provide a general indication of the properties of sludge-derived biodiesel.

Density was estimated by taking the mass of a known volume of a particular biodiesel sample. An aliquot of biodiesel was pipetted using an Eppendorf micropipette (Eppendorf AG, Hamburg, Germany) onto a weight dish. The mass of the sample was then determined using an A-160 Balance (Denver Instruments, Bohemia, NY). Heating values were estimated from the chemical oxygen demand (COD) of a biodiesel sample. The COD measurements were done in duplicate using HACH high range COD digestion vials (Loveland, CO, USA) with a spectrophotometer.

3.3.5 FAME Analysis

FAME analysis was performed using a gas chromatograph equipped with a flame ionization detector. The retention times of the unknown samples were compared to the retention times of a known calibration standard provided by MIDI, Inc. Peak identification and peak area calculation were performed using the Sherlock^{TM} software system. The samples tested consisted of 10 mL aliquots taken from the hexane layer collected during the *in situ* transesterification procedure.

Following the gas chromatography analysis, it was determined that there were several unknown compounds present in the sample and that more refined analytical techniques were required. To this end, a gas chromatography-mass spectrophotometer device (GC-MS) was employed to provide greater resolution and insight into the FAME composition of the sludge-derived biodiesel samples.

3.4 Anaerobic Digester Pretreatment Experiments

While the primary focus during the biodiesel experiments was on the FAMEs laden hexane phase, for the anaerobic digester pretreatment experiments, the focus was shifted to the residual sludge matter in the aqueous phase. Tests were performed on this residual sludge to assess the potential for *in situ* transesterification as a digester pretreatment technique. Two types of samples were used. The first sample, termed control WAS (CWAS), consisted of concentrated WAS prepared by combining centrifuged WAS with gravity thickened WAS. The second sample, termed extracted sludge (ES), was prepared using a modified version of the wet sludge *in situ* transesterification step outlined earlier (described in detail below). Both CWAS and ES samples were adjusted to a TSS concentration of 31-34 g/L. The similar TSS range allowed for a basis of comparison between the two samples. The level of pretreatment achieved by *in situ* transesterification was assessed by measuring the capillary suction time and soluble chemical oxygen demand (SCOD) of the CWAS and ES samples.

3.4.1 Preparation of Extracted Sludge

ES consisted of the residual sludge remaining after transesterification. However, in order to remove some potential interference in the digester pretreatment

performance analyses, the *in situ* transesterification step for preparing ES had to be modified. To avoid an excess of sodium or chloride in the extracted sludge samples, the volume of Reagent #3 (Table 3.1) used in step 1 was reduced from 2 mL per centrifuge tube to 0.25 mL per bottle. Chloride is known to cause significant interference in COD analysis, and a high chloride concentration would have had a substantial effect on the SCOD analysis. Except for this modification, the rest of the *in situ* transesterification procedure remained unchanged for ES.

Some additional preparation was required for ES before the pretreatment tests could be performed. Since the large amount of excess methanol remaining in the aqueous phase could have interfered in the SCOD measurement, the liquid had to be removed by evaporation. While water and methanol do not form an azeotropic mixture, methanol is still soluble in water. As a result methanol evaporation conditions alone would not have been sufficient to remove the majority of the methanol (Methanex Corporation, 2006). To this end, a two-stage evaporation procedure was developed using a rotary evaporator. First, following the removal of hexane phase after transesterification, the remaining aqueous phase transferred to a 500 mL ball flask. Second the sample was evaporated under normal methanol evaporation conditions (settings of 337 mbar, 60°C, and 125 rpm) for 20 minutes (Büchi Labortechnik AG, 2001). Finally third, a higher setting (settings of 200 mbar, 80°C, and 125 rpm) was used for additional 20 minutes to evaporate most of the methanol-water mixture remaining.

As the original dry weight used for the wet sludge *in situ* transesterification was ~5 g, by transferring this evaporated sludge residue to a graduated cylinder and diluting it to 300 mL, a TSS in the range of 31-34 g/L was

achieved. The sample was then transferred to a beaker. Since the sulfuric acid catalyst was still remaining in the ES and required neutralization, the pH was adjusted with 6 M sodium hydroxide. The TSS of the resulting ES was then determined and further adjusted if necessary.

3.4.2 Capillary Suction Time

Dewaterability is a measurement of the capacity of a particular wastewater sludge sample to discharge water (APHA et al., 1992). Capillary suction time (CST) is a test for determining the relative dewaterability of different sludges by measuring the time it takes for water to leave a sample of sludge. CST tests were performed using a Venture Innovations CST Instrument (Venture Innovations, Inc., Lafayette, LA) with chromatography paper (Fisher Scientific). The test procedure was performed according to Standard Methods (APHA et al., 1992). Samples were performed in triplicate. Because the WAS samples were relatively slow to dewater, the wide opening of the sludge reservoir was used consistently for all samples. The CST values for the ES and CWAS samples were then normalized by the TSS.

3.4.3 Soluble Chemical Oxygen Demand

For the purpose of this study a simple SCOD determination technique was modified and adapted from several other techniques (Mamais et al., 1993; Nah et al., 2000; Kim et al., 2003). First, a sludge sample was centrifuged for 15 minutes at 1000 rpm to partially pelletize the cell matter without rupturing any of the cells. The centrate was then filtered twice in succession, first with a Whatman 934-H glass fiber filters (Fisher Scientific) with a 1.2 µm particle retention capacity filter and then second, with a Whatman cellulose nitrate filter (Fisher Scientific) with a pore size of

0.45 μm. The COD of this filtrate was then measured in duplicate using HACH high range COD digestion vials. The total COD (TCOD) of the samples was also determined. TCOD was determined by measuring the COD of an unfiltered and unadjusted sample. SCOD values were reported as solubilization percentages, which were calculated according to the following formula from Kim et al. (2003):

$$COD\ Solubilization\ (\%) = \frac{SCOD}{TCOD} \times 100$$

3.5 Analytical Procedures and Instrumentation

Total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to standard methods (APHA et al., 1992). An aliquot of sludge of known volume was filtered with a filter of known mass using a vacuum pump. Filters were then dried at 104 ± 1°C in an oven overnight. Following the determination of TSS, VSS were also determined by placing the dried filters in an Isotemp Muffle Furnace Model 186 A (Fisher Scientific) at 550 ± 50°C for 15 minutes. Whatman 934-H glass microfiber filters were used for all TSS and VSS determinations. A Mettler H54AR Balance (Mettler-Toledo, Columbus, OH), Mettler PE II Balance (Mettler-Toledo), or an A-160 Balance were used for the various mass determinations. All FAME analyses were performed using an Agilent 6840 gas chromatograph equipped with a flame ionization detector and an Agilent gas chromatography mass spectrophotometer (Santa Clara, CA). The FAME equipment was provided by MIDI, Inc. All evaporations were accomplished using a Büchi R-205 Rotovap (Büchi Scientific, Flawil, Switzerland). All COD measurements were performed using a HACH DR 5000 spectrophotometer set to a wavelength of 620 nm. The pH of different samples was measured using a pH meter (Cole Parmer).

Chapter 4

RESULTS

4.1 Fatty Acid Comparison

Three activated sludge samples from two wastewater treatment plants were compared for the preliminary fatty acid analysis: WL Sludge, SF Sludge, and GA Foams. Six trials of each sample were run and the results are shown in Figure 4.1. The total peak area for each sample was averaged and then normalized by the cell mass used in the analysis. All peak areas were measurements of fatty acids in the form of FAMEs.

SF Sludge and GA Foam samples have relatively higher fatty acid amounts (323 ± 13 and 286 ± 13 respectively) in comparison to the WL Sludge sample (185 ± 18). While it was expected that GA Foam samples, the sample with the largest amount of *G. amarae*, would also have the highest relative amount of fatty acid, this was not the case. However, because mycolic acids are not detectable using the Sherlock Instant FAME^{TM} technique, the contribution of mycolic acid to the total peak area is not being reflected thus lowering the relative amount of fatty acids in the GA Foam sample. Nevertheless, since both SF Sludge and GA Foam were known to have substantial levels of *G. amarae* and WL Sludge was known to contain negligible levels of *G. amarae*, this analysis confirms the hypothesis that samples containing *G. amarae* should have relatively higher amounts of fatty acids than samples without *G. amarae*. This confirmation further implies that since samples containing *G. amarae*

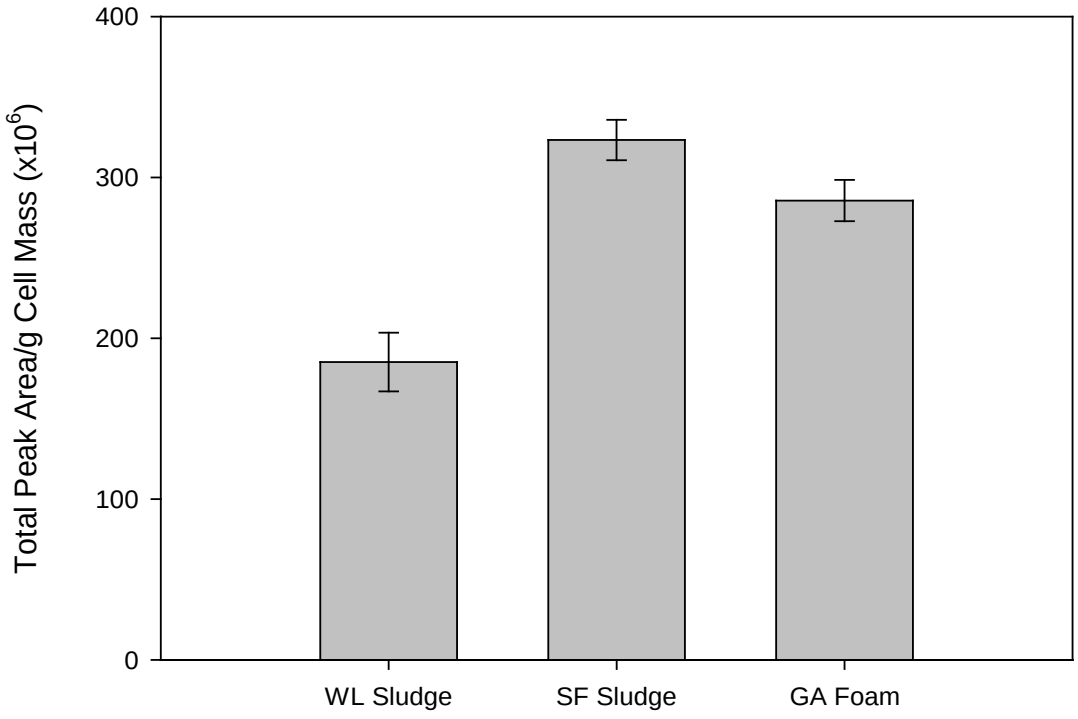


Figure 4.1 **Relative amounts of fatty acids for different sludge samples normalized by the cell mass. The bar data signify the average of duplicate samples and the error bars correspond to the standard deviation.**

have relatively larger amounts of fatty acids, they should also have higher biodiesel yields.

4.2 Biodiesel

4.2.1 Extraction Yields

In situ transesterification and extraction experiments were performed on four samples: WL Sludge, SF Sludge, GA Foam, and Wet WL Sludge. Each sample was tested in duplicate and the results were calculated as percent yields on a weight/weight basis. The average of these duplicate analyses is shown in Figure 4.2 along with a reference value for soybean oil yield. Although the soybean oil value actually refers to the yield of soybean oil (not biodiesel) from the soybean plant, it can be reasonably assumed that virtually all of the oil would be converted to biodiesel because the conversion efficiency of soybean oil to biodiesel is very high (>99%) (Freedman et al., 1984).

Similar to the fatty acid analysis earlier, these biodiesel yield results show that the samples containing *G. amarae,* with yields of 12.60 ± 0.06% (SF Sludge) and 13.02 ± 0.36% (GA Foam), have a substantially higher yield than samples low in *G. amarae,* with a yield of only 11.12 ± 0.29% (WL Sludge). To further compare these samples, a Two-Sample t-Test, assuming equal variances with a 95% confidence interval, was performed using the Microsoft Excel Analysis ToolPak. The t-Test was used to determine whether or not the average values were statistically different from each other with the null hypothesis being that the two values are statistically identical. The summary of these results can be found in Table 4.1. A value of “Yes” means the

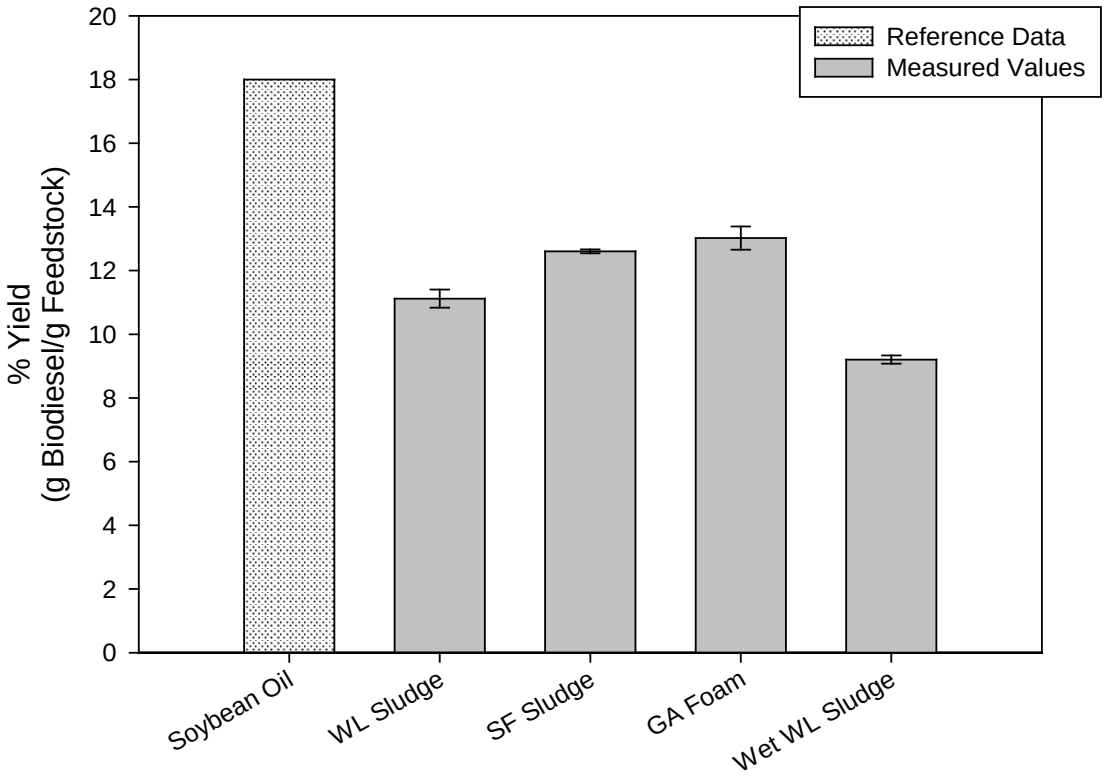


Figure 4.2 **Biodiesel yields for different sludges as compared to yields for soybean oil (Freedman et al., 1984; USDA, 2008). The bar data signify the average of duplicate samples and the error bars correspond to the standard deviation.**

two values are statistically different and a value of "No" means the values are not statistically different.

Table 4.1 Statistical significance comparison between different biodiesel yields for different sludge samples using a t-Test

Sample	WL Sludge	SF Sludge	GA Foam
WL Sludge	X	Yes	Yes
SF Sludge	Yes	X	No
GA Foam	Yes	No	X

The t-Tests confirm that there is a statistically significant difference between the biodiesel yields for both SF Sludge and GA Foam and the biodiesel yield for WL Sludge. Thus, it can be concluded that sludge containing *G. amarae* will result in better biodiesel yields. However, these t-Tests also show that the averages for SF Sludge and GA Foam are not statistically different. The expectation was that GA Foam, being predominately composed of *G. amarae*, would have a higher yield than SF Sludge. Like the preliminary fatty acid analysis, however, this was not the case. The explanation for this result is again most likely due to unique chemistry of mycolic acids. Given the significant chain length of these fatty acids, they are probably not being extracted successfully and their potential contribution to the biodiesel yield is not being reflected.

Finally, it is valuable to compare the Wet WL Sludge sample biodiesel yield of 9.20 ± 0.13% to the WL Sludge sample yield of 11.12 ± 0.29%. As previously mentioned, the presence of water can negatively impact acid-catalyzed transesterification because water can hydrolyze FAMEs back into FFA causing a reduction in conversion efficiency (Canakci and Van Gerpen, 2001; Lotero et al.;

2005). Despite the known negative impacts of water, the reduction in biodiesel yield for Wet WL sludge is only 1.91%. This data suggests that it may be possible to still get adequate biodiesel yields using wet sludge without the energy-intensive drying step. This in turn could improve the overall economics of using sludge as a feedstock for biodiesel. Furthermore, Mondala et al. (2009) estimated that sludge-derived biodiesel could be produced for $3.23/gallon assuming a yield of 10%, which is quite close to the wet sludge yield. However, it is important that a balance be established between moisture level and conversion efficiency as exceedingly watery sludges would most likely see an uneconomical reduction in biodiesel yield.

The biodiesel yields for all the sludge samples were lower than the yield reported for soybean oil. However, the difference was rather small, between 5-7%, showing that sludge is a viable alternative biodiesel feedstock. All of the sludge-derived biodiesel yields in this study exceed the maximum values previously reported in the literature for biodiesel derived from secondary sludge (6.23%; 2.5%) but are lower than the reported yields for biodiesel from primary sludge (14.5%) (Dufreche et al. 2007; Mondala et al., 2009). This shows that even higher biodiesel yields from WAS are possible than were originally measured. Thus, these *in situ* transesterification experiments have shown it is possible to produce biodiesel from WAS sludge with yields higher than previously measured and that the presence of filamentous bacteria like *G. amarae* appears to have a positive effect on the total yield.

4.2.2 Fuel Property Analysis

The resulting biodiesel from the extractions for WL Sludge, SF Sludge, and GA Foam were tested for heating value and density. The biodiesel yield for Wet

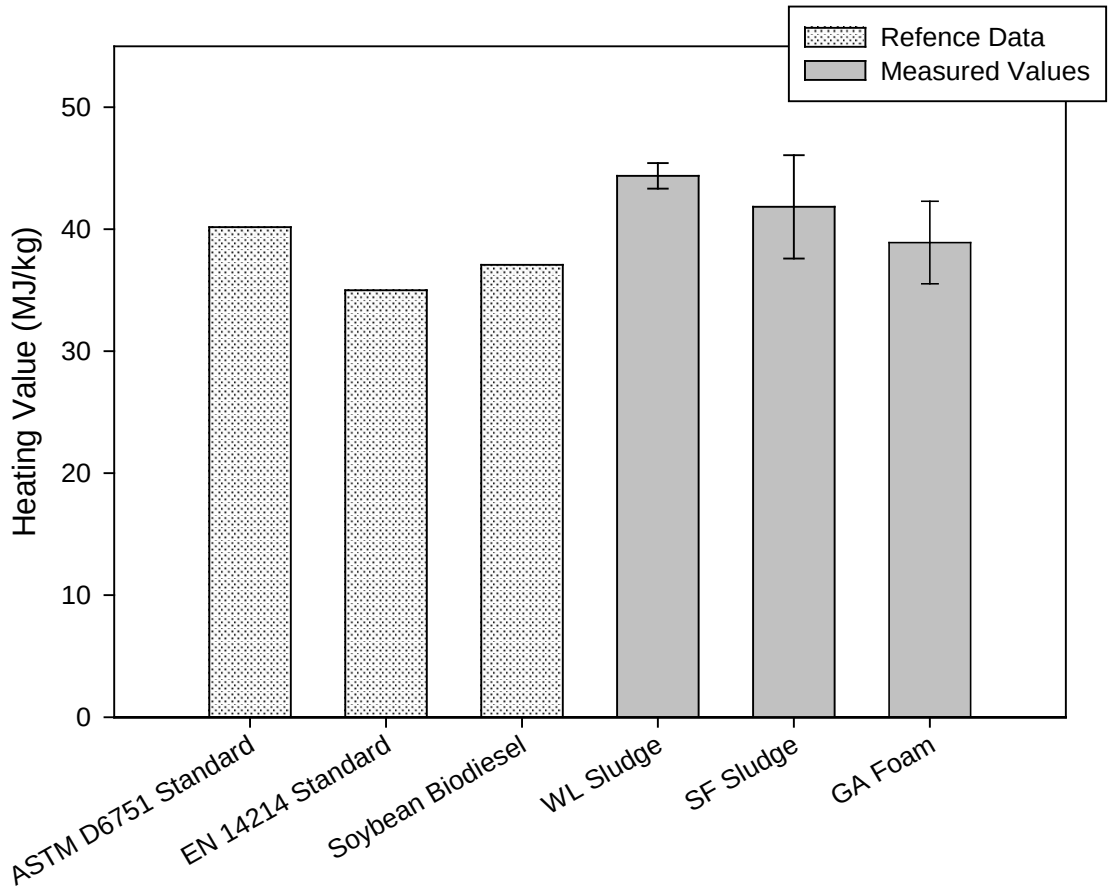


Figure 4.3 **Biodiesel heating values for different sludge biodiesel samples as compared to soybean diesel and biodiesel standard values (Knothe et al., 2005; EN, 2008; NREL, 2009; ASTM, 2010). The bar data signify the average of duplicate samples and the error bars correspond to the standard deviation.**

WL Sludge samples was too low to provide a sufficient quantity of sample for testing, thus no density or heating values were reported for these samples. Only one density test was performed. Heating value estimates were performed in duplicate. Heating values were calculated from the COD using the conversion factors adapted from Niessen (2002) and the density measurement, as shown in the following equation:

$$COD,\ \frac{kg\ O_2}{L} \times 4.32 \frac{kg\ Air}{kg\ O_2} \times Density, \frac{L}{kg\ Fuel} \times 3.08 \frac{MJ}{kg\ Air} = Heating\ Value, \frac{MJ}{kg\ Fuel}$$

The heating value results are shown in Figure 4.3 and the density results are shown in Figure 4.4. For reference purposes, the specifications for heating value and density for biodiesel from the ASTM and EN standards have been reported as well as the heating values and density for a typical soybean biodiesel.

These results show that for all categories of sludge-derived biodiesel, the heating values compare favorably to the ASTM and EN specification in some cases even exceeding the standard value. Every sample of biodiesel exceeded the soybean biodiesel heating value indicating that sludge-derived biodiesel may be more energy-rich and will likely burn better than soybean biodiesel (Knothe et al., 2005). Like the heating values, the density values for all of the biodiesel samples show favorable comparison to the ASTM and EN specifications. The soybean biodiesel density actually exceeds all of the sludge-derived biodiesel densities, but only slightly.

Overall, these preliminary fuel quality results indicate that sludge-derived biodiesel, regardless of sludge source and composition, show compliance with the ASTM and EN fuel standards for density, and favorably exceed the standards for heating values.

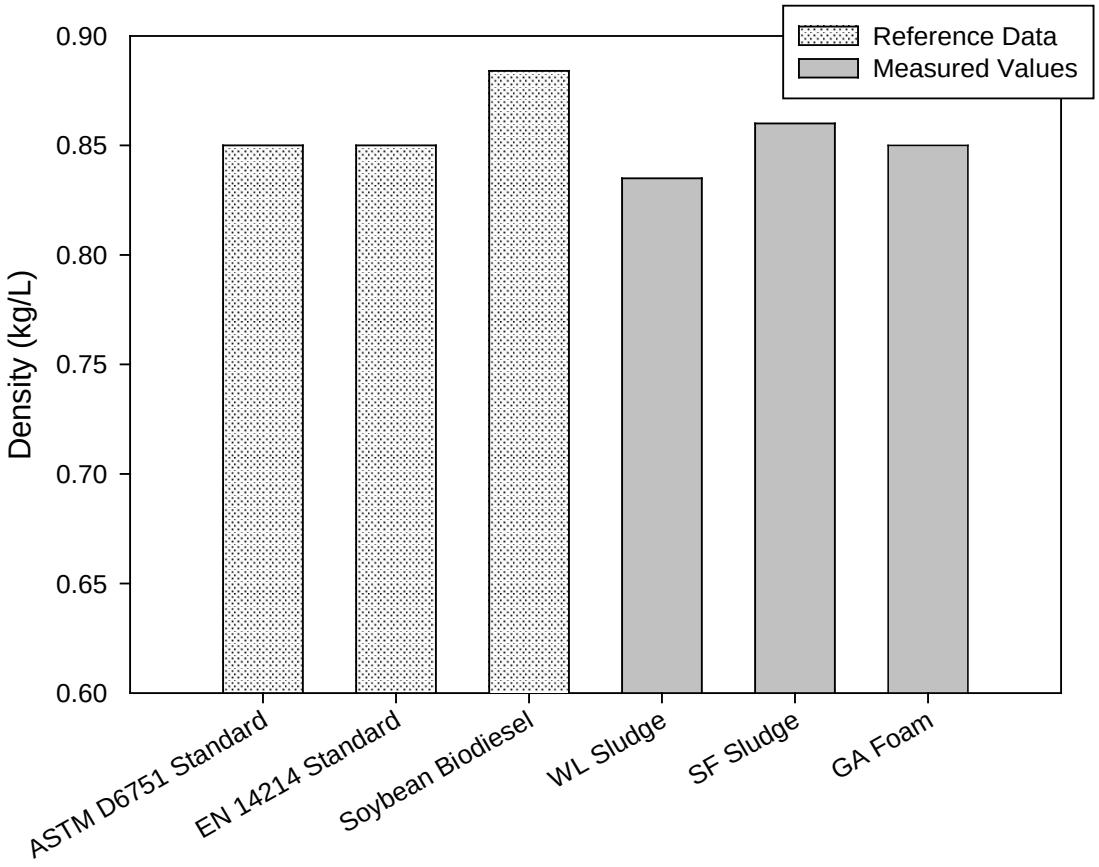


Figure 4.4 Biodiesel density values for different sludge biodiesel samples as compared to soybean diesel and biodiesel standard values (Knothe et al., 2005; EN, 2008; NREL, 2009; ASTM, 2010).

4.2.3 FAME Analysis

Two samples of hexane containing FAME from the *in situ* transesterification procedure were analyzed using gas chromatography. An example of one of the gas chromatographs with FAME peaks is shown in Figure 4.5. The full FAME profile is shown in Figure 4.6. The FAME profile for soybean oil biodiesel has also been included for reference purposes.

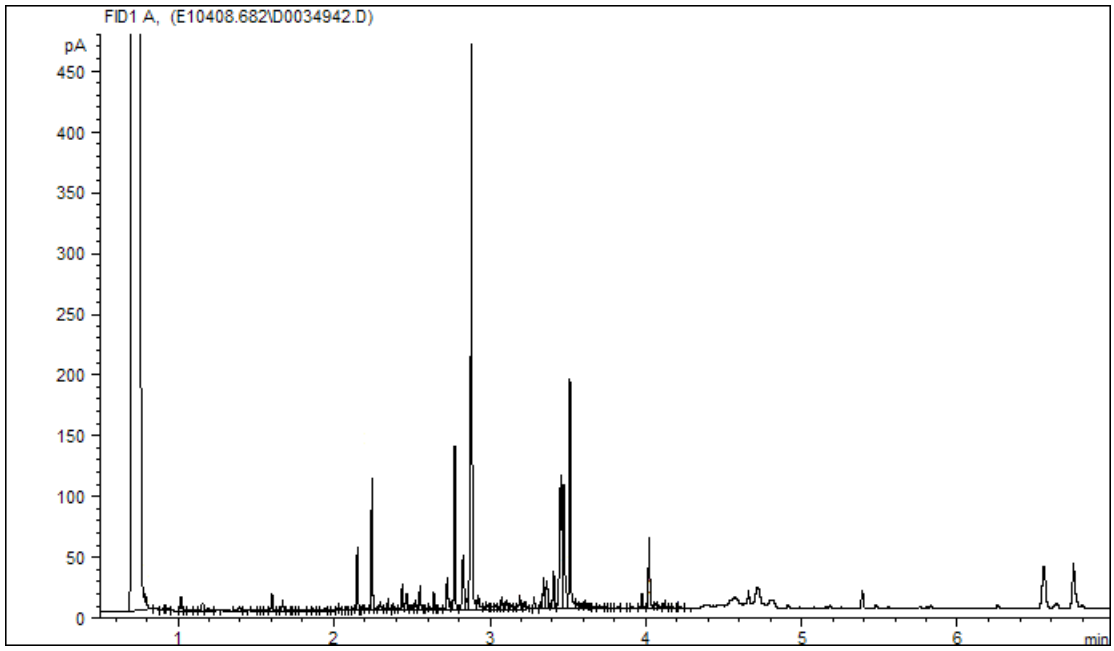


Figure 4.5 Gas chromatograph of a sludge biodiesel sample.

The distribution of saturated versus unsaturated fatty acid content is very important for determining several physical properties of biodiesel. In particular, the fatty acid content can affect the cloud point, oxidative stability, and cetane number

(Knothe et al., 1997; Knothe et al., 2005). Generally, saturated FAMEs burn better than unsaturated ones, but solidify at low temperatures more easily, causing an increase in cloud point and pour point (Dunn and Bagby, 1995; Lee et al., 1995; Ritz and Croudace, 2003; Knothe et al., 2005; Dufreche et al., 2007). On the other hand, biodiesel with a high percentage of unsaturated FAMEs, while possessing good cold weather performance, has a lower oxidative stability which affects the storage capacity (Knothe et al., 2005; Meher et al., 2006).

For the sludge-derived biodiesel samples, all of the FAMEs are within the range of 12 – 20 carbon chains, which meets the ASTM specification (ASTM, 2010). From the references values, soybean oil is shown to consist primarily of unsaturated fatty acids (83%) as well as some saturated fatty acids (15.5%). Given the complex nature of bacterial cell walls, the distribution of different fatty acids in the sludge-derived biodiesel is very different from the soybean distribution, with many more odd numbered chain length fatty acids and substituted functional groups (Christie, 2010). For the sludge-derived biodiesel data, several of the peaks are unidentified, however of the known peaks it can be shown that 23.26% are saturated FAMEs and 14.70% are unsaturated FAMEs. Thus, the saturated FAME content of sludge-derived biodiesel is higher than soybean biodiesel, while the unsaturated content is lower.

While this saturated FAME content value is lower than the 60% reported by Dufreche et al. (2007) for sludge-derived biodiesel, it is likely some of the unknown peaks are saturated FAMEs and this percentage of saturated FAMEs is being underreported. Thus these data show that the saturated FAME content of sludge-derived biodiesel is higher than the unsaturated FAME content, which indicates that biodiesel will burn better and have a better oxidative stability but may be prone to

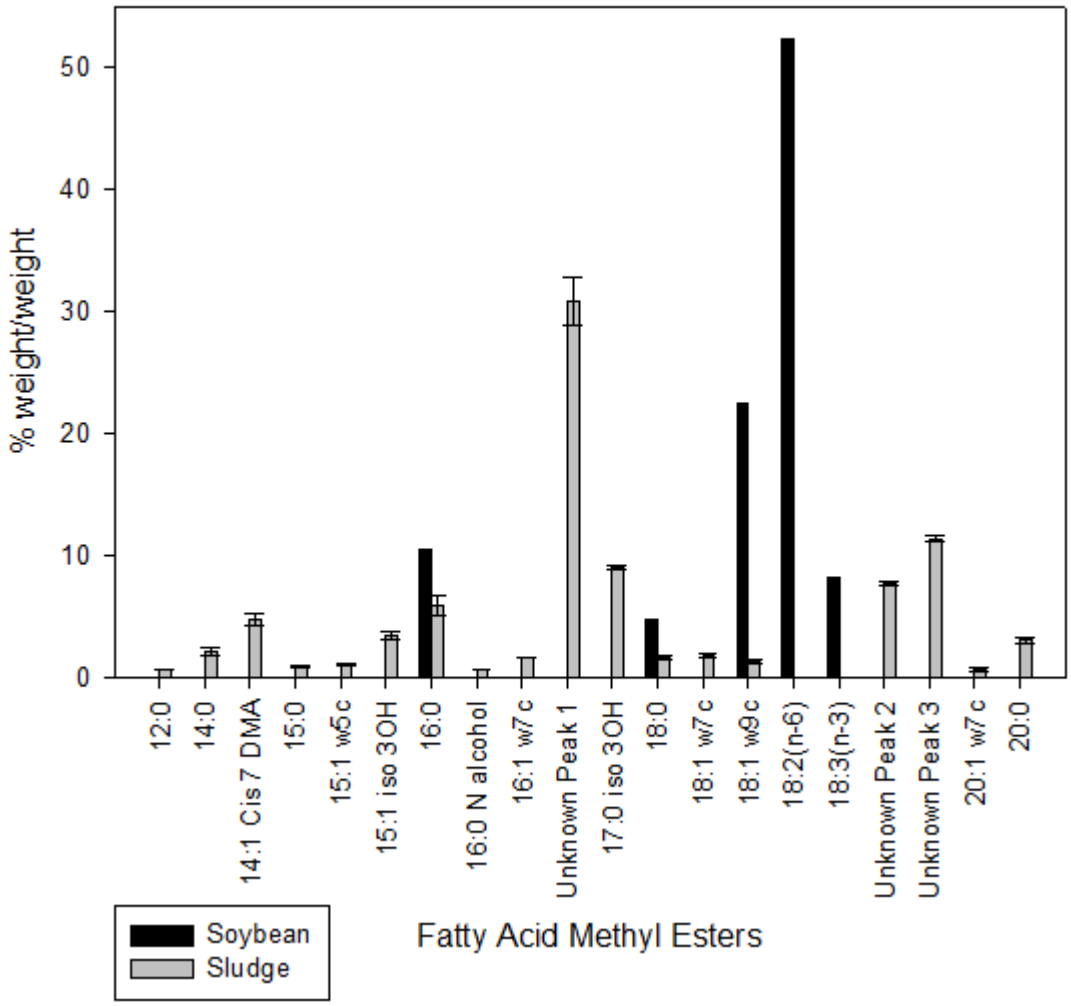


Figure 4.6 FAME profiles for sludge biodiesel samples as compared to soybean biodiesel samples (Canakci and Van Gerpen, 2003; Lotero et al., 2005).

solidification problems during cold weather conditions (Knothe et al., 2005; NREL, 2009). Thus, sludge-derived biodiesel has been shown to contain FAMEs in the ideal range and composition.

Because some of the peaks were not identified during the gas chromatography analysis, a GC-MS was used to further the illuminate the composition of the biodiesel sample. The GC-MS data indentified the methylated forms the saturated fatty acids lauric acid (12:0), myristic acid (14:0), palmitic acid, and stearic acid, as well as the methylated forms the unsaturated fatty acids palmitoleic acid (16:1) and several linoleic acid (18:1) varieties. Interestingly, the GC-MS data also show the presence of un-methylated palmitic acids. This indicates that not all of the fatty acids and lipids present in the sludge were converted to FAMEs, which means that higher yields are possible. In previous studies, the maximum FAME yields for wastewater sludge samples using acid catalyzed transesterification were achieved after a 24 hour period (Mondala et al., 2009). As this was not a kinetic analysis study, the reaction time was kept at a fixed time of 2 hours, so all samples received a consistent treatment. However, this reduced reaction time may have resulted in this incomplete conversion of fatty acids. Given that not all of the fatty acids were converted, in the future further refinement of the necessary reaction time will be required.

4.3 Anaerobic Digester Pretreatment

Control waste activated sludge (CWAS) and extracted sludge (ES), two samples of concentrated WAS receiving different treatments, were compared via capillary suction time (CST) and percent COD solubilization analysis. In addition to these parameters, the samples were also measured for TSS, VSS, and TCOD. An overview of these parameters can be found in Table 4.2.

Table 4.2 Characteristics of sludges used in pretreatment experiments

Parameter	CWAS	ES
TSS (mg/L)	33065 ± 742	31608 ± 374
VSS (mg/L)	24698 ± 364	20508 ± 1064
TCOD (mg/L)	44225 ± 742	41400 ± 1273
SCOD (mg/L)	4225 ± 35	19000 ± 354
pH	6.03	6.03

The CST test results, normalized by the TSS, were run in duplicate and are shown in Figure 4.7. Likewise the percent COD solubilization tests were also run in duplicate and these results are shown in Figure 4.8.

The dewaterability of WAS was found to improve substantially after *in situ* transesterification with a value of 3.21 ± 0.20 seconds per g/L TSS for ES. By comparison CWAS sludge, with a value of 16.93 ± 0.37 seconds per g/L TSS, was far less easily dewatered. Previous research by Bougrier et al. (2008) has found that thermal treatment is only effective for reducing CST at temperatures of 150°C or greater, but Neyens et al. (2003) have shown that the addition of sulfuric acid to achieve pH values of 3 or less can significantly improve dewaterability. Given that the temperature of 70-75°C used for *in situ* transesterification is much lower than the 150°C threshold from the literature, it likely that the reduction in CST value for the ES sample is attributable to the addition of sulfuric acid catalyst.

The percent COD solubilization data shows that using *in situ* transesterification as a pretreatment technique can substantially improve the level of soluble material available in the sludge. After transesterification, the ES was found to have percent solubilization of 45.93 ± 2.26%. In comparison, the CWAS was found to have a percent COD solubilization of only 9.56 ± 0.24%. It was previously reported by Kim et al. (2003) that thermochemical pretreatment of activated sludge at 121°C

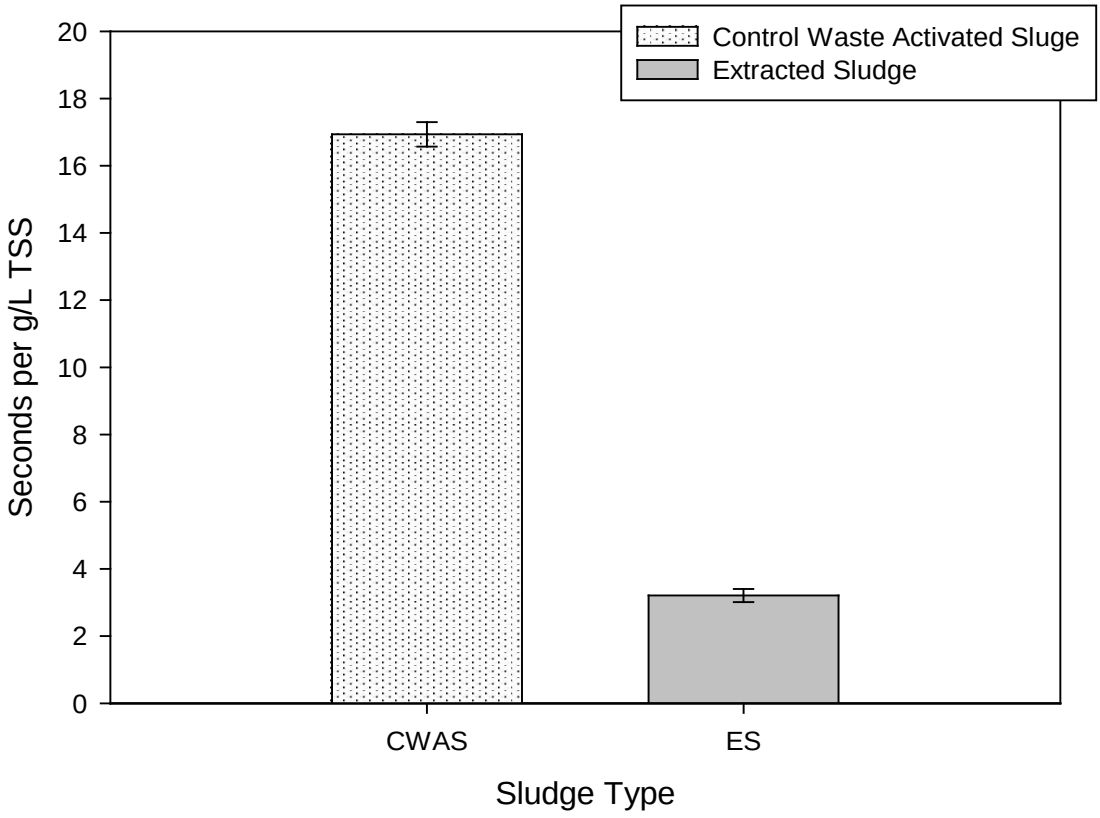


Figure 4.7 Dewaterability of sludges with and without pretreatment. The bar data signify the average of duplicate samples and the error bars correspond to the standard deviation

for 30 minutes at pH 12 (adjusted with sodium hydroxide) would achieve a percent COD solubilization of 51.8%. The value from this study of 45.93% compares favorably to this data, having the added benefit of requiring a much lower temperature.

Neyens et al. (2003) previously reported that the optimal conditions for thermochemical treatment using sulfuric acid were a temperature of 120°C, a pH of 3, and a reaction time of 60 minutes. Although the transesterification conditions of 70-75°C, a pH of less than 2, and a reaction time of 120 minutes were not optimized for pretreatment, these conditions have still demonstrated good pretreatment capability. These results successfully confirm the hypothesis that *in situ* transesterification can function simultaneously as a type of sludge anaerobic digester pretreatment. Thus, these experiments indicate that rate-limiting effects of the hydrolysis step in the anaerobic digestion process will be substantially reduced and improve the overall digestibility of the sludge. Furthermore, this analysis suggests that there is significant potential for using transesterification as thermochemical pretreatment concurrently with full scale biodiesel production, refuting the assertion of Appels et al. (2008) that thermochemical pretreatment is limited in application.

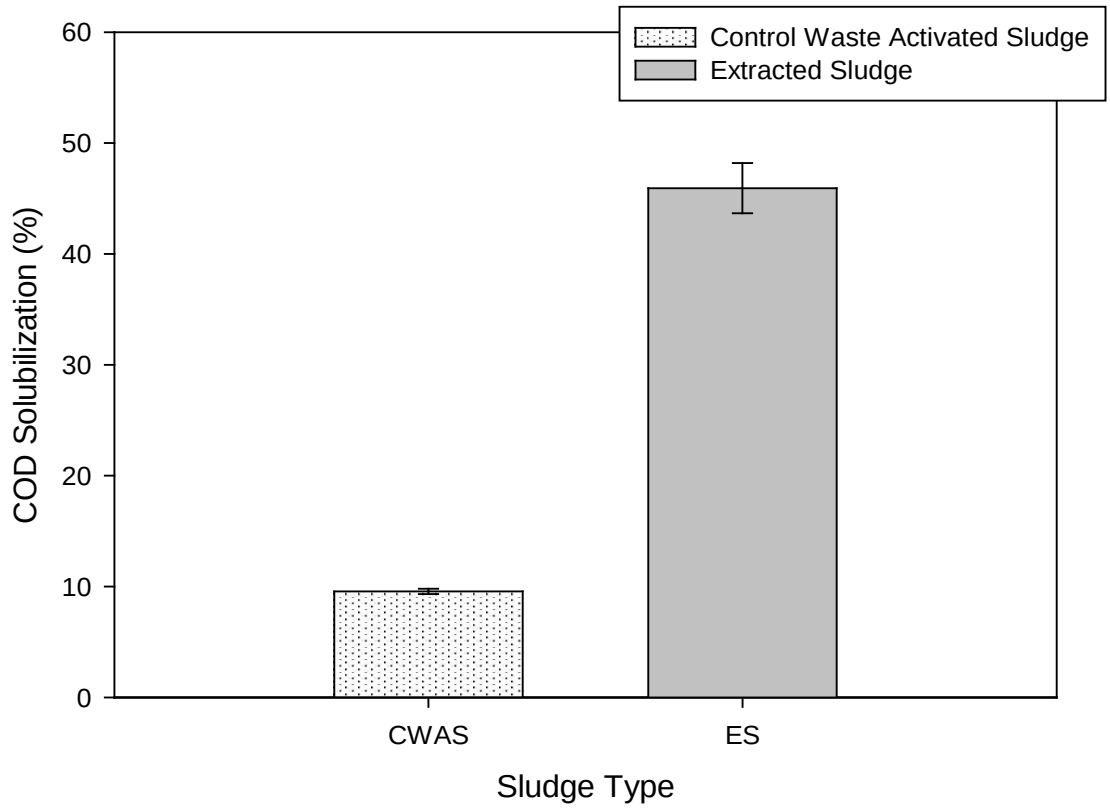


Figure 4.8 Percent COD solubilization in different sludges. The bar data signify the average of duplicate samples and the error bars correspond to the standard deviation.

Chapter 5

CONCLUSIONS AND FUTURE RESEARCH

5.1 Conclusions

The primary objective of this research was to evaluate WAS as a feedstock for biodiesel production. Biodiesel extraction via *in situ* transesterification was performed on WAS samples from two different wastewater treatment plants. This research has successfully demonstrated that biodiesel can be produced from WAS with high yields of up to 12.60 ± 0.06%. It has also been shown that the presence of *G. amarae* in sludge feedstocks causes a statistically significant increase in biodiesel yield from 11.12 ± 0.29% to 12.60 ± 0.06%. Using wet sludge as feedstock, in spite of the known inhibitory effects of water on *in situ* transesterification, was found to only slightly reduce the biodiesel yield from 11.12 ± 0.29% to 9.20 ± 0.13%.

Sludge-derived biodiesel was also subjected to some preliminary fuel property analysis. The estimated heating values and density values for sludge-derived biodiesel were shown to meet or exceed both the ASTM and EN specifications. The FAME profile of the biodiesel sample has shown a complex mixture of FAMEs present, with the majority in the preferred range for biodiesel. The level of saturated FAMEs present in sludge-derived biodiesel exceed the level of unsaturated FAMEs, suggesting that this biodiesel will burn better and have a superior storage capacity. The presence of unconverted fatty acids, as shown by the GC-MS data, indicate that

the efficiency of the transesterification procedure used in not yet optimized and that further optimization is required.

Finally, anaerobic digester pretreatment potential was also investigated. The usefulness of *in situ* transesterification as an anaerobic digester pretreatment technique has been demonstrated from the dewaterability values of 3.21 ± 0.20 seconds per g/L TSS and percent COD solubilization values of 45.93 ± 2.26%, both of which were significantly improved over the control values. This data indicates a high degree of pretreatment is possible with *in situ* transesterification.

5.2 Integrated Treatment Process

Based on the results of this study a novel wastewater treatment plant that can simultaneously produce biodiesel and treat wastewater was proposed. Figure 5.1 shows a conventional wastewater treatment facility that has been re-envisioned as a biorefinery. In a biorefinery setup, many of the electrical requirements within the wastewater treatment plant could be fulfilled with either the methane gas production or the biodiesel derived from the wastewater sludge. If integrated systems like this were implemented at all the municipal treatment plants in the U.S., assuming a biodiesel yield of 10% and a fuel density of 0.88 kg/L, 223 million gallons of biodiesel could be produced in the year 2010 from the projected sludge production of 8.2 million dry tons (WEF, 2008).

5.2 Future Research

While this research has successfully demonstrated the feasibility of using wastewater sludge as a feedstock for biodiesel, more research is required before this technology can be implemented at a full scale. The currently employed acid catalyzed

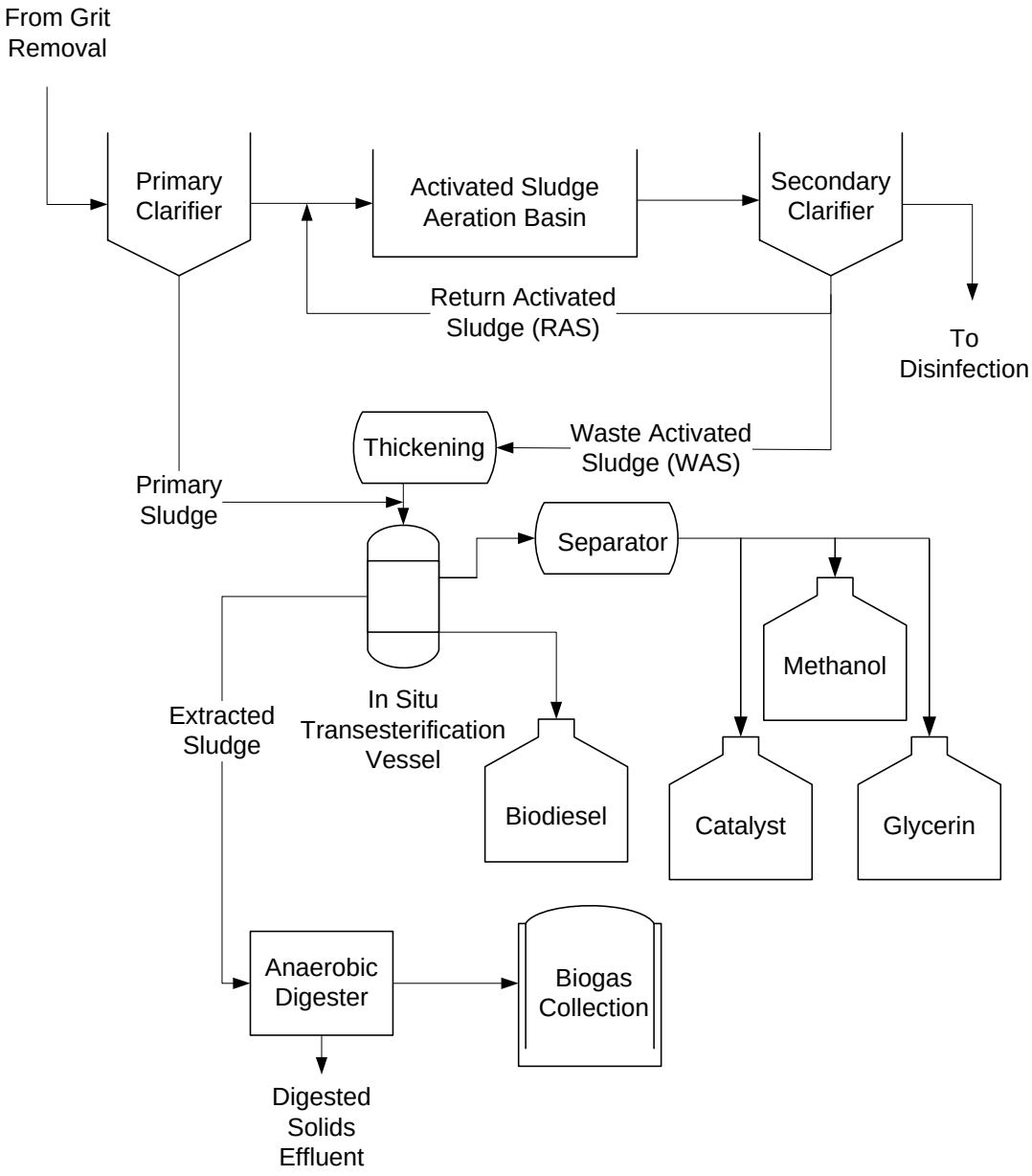


Figure 5.1 Integrated biodiesel and wastewater treatment process

transesterification technique needs further refinement. Examining the biodiesel yields of sludge using two-step transesterification, a well studied transesterification technique for feedstocks with high FFA, is one experiment with a great deal of potential for both improving biodiesel yields and improving reaction efficiency. Further testing of the quality and performance characteristics of sludge-derived biodiesel is also required. This study only examined two parameters, density and heating value, but as shown in Table 2.1, there are a variety of other specifications for biodiesel that must be met before the fuel can be used in general applications. Fuel specification testing would also necessitate scaling up production to produce sufficient quantities of biodiesel for all of the required tests. Thus a larger lab scale *in situ* transesterification procedure should also be investigated.

Finally, although *in situ* transesterification has been effectively demonstrated as an anaerobic digestion pretreatment technique, further testing is required to assess the use of transesterification pretreated sludge as an anaerobic digester feed. Two important parameters need to be assessed before the extracted sludge can be used successfully in long term anaerobic digester operations. First, since the sludge extraction process removes the natural buffering capacity of the WAS, the buffer and pH requirements of the digesters will need to be carefully considered. Second, since a significant quantity of sodium is added both in the form of sodium chloride solution and sodium hydroxide, the sodium concentration of the extracted sludge needs to be carefully monitored as excess concentrations of sodium can affect digester performance. Developing techniques for removing excess sodium from the extracted sludge prior to feeding is also recommended.

REFERENCES

Alvarez, H. M. (2003). Relationship between β-oxidation pathway and the hydrocarbon-degrading profile in actinomycetes bacteria. *International Biodeterioration & Biodegradation, 52*(1),35-42. doi:10.1016/S0964-8305(02)00120-8

Alvarez, H. M., and Steinbüchel, A. (2002). Triacylglycerols in prokaryotic microorganisms. *Applied Microbiology and Biotechnology, 60*(4), 367–376. doi:10.1007/s00253-002-1135-0

American Public Health Association (APHA), American Water Works Association (AWWA), and Water Environment Federation (WEF). (1992). *Standard methods for the examination of water and wastewater* (18th Ed.) Greenberg, A. E., Clesceri, L. S., and Eaton, A. D. (Eds.). Washington, DC: APHA, AWWA, and WEF.

American Society for Testing and Material International (ASTM). (2010). ASTM D6751 - 09a Standard Specification for Biodiesel Fuel Blend Stock (B100) for Middle Distillate Fuels. In *ASTM Book of Standards, Volume 05.04 Petroleum Products and Lubricants (IV).* West Conshohocken, PA: American Society for Testing and Material International. doi:10.1520/D6751-09A

Appels, L., Baeyens, J., Degrève, J., and Dewil, R. (2008). Principles and potential of the anaerobic digestion of waste-activated sludge. *Progress in Energy and Combustion Science, 34*(6), 755-781. doi:10.1016/j.pecs.2008.06.002

Boocock, D. G. B., Konar, S. K., Leung, A., and Ly, L. D. (1992). Fuels and chemicals from sewage sludge: 1. The solvent extraction and composition of a lipid from a raw sewage sludge. *Fuel, 71*(11), 1283-1289. doi:10.1016/0016-2361(92)90055-S

Bougrier, C., Delgenès, J. P., and Carrère, H. (2008). Effects of thermal treatments on five different waste activated sludge samples solubilisation, physical properties and anaerobic digestion. *Chemical Engineering Journal, 139*(2), 236-244. doi:10.1016/j.cej.2007.07.099

Bradshaw, G. B., Meuly, W. C. (1944). Preparation of detergents. *US Patent No. 2, 360-844*. Washington, DC: U.S. Patent and Trademark Office.

Büchi Labortechnik AG. (2001). *BÜCHI Rotavapor R-200/205 Manual*. Flawil, Switzerland: Büchi Labortechnik AG.

Canakci, M., and Van Gerpen, J. (2001). Biodiesel production from oils and fats with high free fatty acids. *Transactions of the American Society of Agricultural Engineers, 44*(6), 1429-1436.

Carrapiso, A. I., and García, C. (2000). Development in lipid analysis: Some new extraction techniques and *in situ* transesterification. *Lipids, 35*(11), 1167-1177. doi:10.1007/s11745-000-0633-8

Cha, D. K., Jenkins, D., Lewis, W. P., and Kido, W. H. (1992). Process Control Factors Influencing *Nocardia* Populations in Activated Sludge. *Water Environment Research, 64*(1), 37-43.

Chisti, Y. (2008). Biodiesel from Microalgae beats Bioethanol. *Trends in Biotechnology, 26*(3), 126–131.

Christie, W. W. (2010, March 12). *Fatty acids: straight-chain saturated, structure, occurrence and biosynthesis*. Retrieved April 10, 2010 from The AOCS Lipid Library: http://lipidlibrary.aocs.org/Lipids/fa_sat/index.htm

Dufreche, S., Hernandez, R., French, T., Sparks, D., Zappi, M., and Alley, E. (2007). Extraction of Lipids from Municipal Wastewater Plant Microorganisms for Production of Biodiesel. *Journal of the American Oil Chemists' Society, 84*(2), 181-187. doi:10.1007/s11746-006-1022-4

Dunn, R.O., and Bagby, M. O. (1995). Low-Temperature Properties of Triglyceride-Based Diesel Fuels: Transesterified Methyl Esters and Petroleum Middle Distillate/Ester Blends. *Journal of the American Oil Chemists' Society, 72*, 895–904.

Encinar, J. M., González, J. F., and Rodríguez-Reinares, A. (2005). Biodiesel from Used Frying Oil. Variables Affecting the Yields and Characteristics of the Biodiesel. *Industrial & Engineering Chemistry Research, 44*(15), 5491–5499. doi:10.1021/ie040214f

Energy Information Administration (EIA). (2007). *Annual Energy Outlook 2007: With Projections to 2030* (DOE/EIA-0383). Washington, DC: U.S. Government Printing Office.

European Committee for Standardization (EN). (2008). EN 14214 - Automotive fuels - Fatty acid methyl esters (FAME) for diesel engines - Requirements and test methods. In *CEN/TC 19 - Gaseous and liquid fuels, lubricants and related products of petroleum, synthetic and biological origin*. Brussels, Belgium: European Committee for Standardization.

Fargione, J., Hill, J., Tilman, D., Polasky, S., and Hawthorne, P. (2008). Land Clearing and the Biofuel Carbon Debt. *Science, 319,*1235-1237

Feuge, R.O. and Grose, T. (1949). Modification of vegetable oils. VII. Alkali catalyzed interesterification of peanut oil with ethanol. *Journal of the American Oil Chemists' Society, 26*, 97–102.

Fransen, G., Urlings, B. A. P., Kessels, B. G. F., and Bijker, P. G. H. (1995). Chemical and Biochemical Aspects of Slaughterhouse Sludge Intended for Feed Purposes. *Journal of Agricultural and Food Chemistry. 43* (8), 2071–2076. doi:10.1021/jf00056a022

Freedman, B., Pryde, E.H., and Mounts, T.L. (1984) Variables affecting the yields of fatty esters from transesterified vegetable oils. *Journal of the American Oil Chemists' Society, 61*(10), 1638–1643.

Fukuda, H., Kondo, A., and Noda, H. (2001). Biodiesel fuel production by transesterification of oils. *Journal of Bioscience and Bioengineering, 92*(5), 405-416. doi:10.1016/S1389-1723(01)80288-7

Goodfellow, M., Minnikin, D. E., Todd, C., Alderson, G., Minnikin, S. M., and Collins, M. D. (1982). Numerical and Chemical Classification of *Nocardia amarae*. *Journal of General Microbiology, 128*, 1283-1297. doi:10.1099/00221287-128-6-1283

Goodfellow, M., Chun, J., Stubbs, S., and Tobili, A. S. (1994). Transfer of *Nocardia amarae* Lechevalier and Lechevalier 1974 to the genus *Gordona* as *Gordona amarae* comb. nov. *Letters in Applied Microbiology, 19*, 401-405.

Guerrant, G. O., Lambert, M. A., and Moss, C. W. (1981). Gas-Chromatographic Analysis of Mycolic Acid Cleavage Products in Mycobacteria. *Journal of Clinical Microbiology, 13*(5), 899-907.

Jenkins, D., Richard, M. G., and Daigger, G. T. (2004). *Manual on the Causes and Control of Activated Sludge Bulking and Foaming and Other Solids Separation Problems* (3rd ed.). Boca Raton, FL: CRC Press.

Kim, D. W., Cha, D. K., Wang, J., and Huang, C. P. (2002). Heavy metal removal by activated sludge: influence of Nocardia amarae. *Chemosphere, 46*(1), 137-142. doi:10.1016/S0045-6535(00)00598-1

Kim, J., Park, C., Kim, T. H., Lee, M., Kim, S., Kim, S. W., and Lee, J. (2003). Effects of various pretreatments for enhanced anaerobic digestion with waste activated sludge. *Journal of Bioscience and Biotechnology, 95*(3), 271-275.

Knothe, G. H., Bagby, M. O., and Ryan, T.W. (1997). Cetane Numbers of Fatty Compounds: Influence of Compound Structure and of Various Potential Cetane Improvers, *Society of Auto Engineers (SAE) Paper 971681*, (SP-1274).

Knothe, G., Van Gerpen, J., and Krahl, J. (Eds.). (2005) *The Biodiesel Handbook.* Champaign, IL: AOCS Press.

Krawczyk, T. (1996). Biodiesel - Alternative fuel makes inroads but hurdles remain. *INFORM. 7*, 801-829.

Lechevalier, M. P., and Lechevalier, H. A. (1974). Nocardia amarae sp. nov., an Actinomycete Common in Foaming Activated Sludge. *International Journal of Systematic Bacteriology, 24*(2), 278-288.

Lee, I., Johnson, L. A., and Hammond, E.G. (1995). Use of Branched-Chain Esters to Reduce the Crystallization Temperature of Biodiesel. *Journal of the American Oil Chemists' Society, 72*, 1155–1160.

Li, Y. Y., and Noike, T. (1992). Upgrading of anaerobic digestion of waste activated sludge by thermal pretreatment. *Water Science and Technology 26*(3–4), 857–866.

Lin J.-G., Chang C.-N., and Chang, S.-C. (1997). Enhancement of anaerobic digestion of waste activated sludge by alkaline solubilization. *Bioresource Technology, 62*(3), 85-90. doi:10.1016/S0960-8524(97)00121-1

Lotero, E., Liu, Y., Lopez, D., Suwannakarn, K., Bruce, D. A., and Goodwin, J. G., Jr. (2005). Synthesis of Biodiesel via Acid Catalysis. *Industrial & Engineering Chemistry Research, 44*(14), 5353-5363.

Ma, F., and Hanna, M. A. (1999). Biodiesel production: a review. *Bioresource Technology, 70*(1), 1-15.

Mamais, D., Jenkins, D., and Pitt, P. (1993). A rapid physical-chemical method for the determination of readily biodegradable soluble COD in municipal wastewater. *Water Research (27)*1, 195-197. doi:10.1016/0043-1354(93)90211-Y

Marchetti, J. M., Miguel, V. U., and Errazu, A. F. (2007). Heterogeneous esterification of oil with high amount of free fatty acids. *Fuel, 86*(5-6), 906-910. doi:10.1016/j.fuel.2006.09.006

Meher, L. C., Sagar, D. V., and Naik, S.N. (2006).Technical aspects of biodiesel production by transesterification—a review. *Renewable and Sustainable Energy Reviews, 10*(3), 248-268. doi:10.1016/j.rser.2004.09.002

Metcalf & Eddy, Inc. (1991). *Wastewater Engineering: Treatment, Disposal, and Reuse* (4th ed.). New York, NY: McGraw-Hill, Inc.

Methanex Corporation. (2006). *Technical Information & Safe Handling Guide for Methanol, Version 3.0*. Vancouver, Canada: Methanex Corporation.

MIDI, Inc. (2009). *Sherlock Instant FAME™ User Manual* (4th Ed.). Newark, DE: MIDI, Inc.

Minnikin, D. E., Alshamaony, L., and Goodfellow, M. (1975). Differentiation of *Mycobacterium*, *Nocardia*, and Related Taxa by Thin-layer Chromatographic Analysis of Whole-organism Methanolysates. *Journal of General Microbiology, 88*, 200-204.

Mitchell, D. (2008). *A Note on Rising Food Prices (POLICY RESEARCH WORKING PAPER 4682)*. Retrieved April 15, 2010 from The World Bank Development Prospects Group: http://go.worldbank.org/31PG0020G0

Mondala, A., Liang, K., Toghiani, H., Hernandez, R., and French, T. (2009). Biodiesel production by *in situ* transesterification of municipal primary and secondary sludges. *Bioresource Technology, 100*(3), 1203-1210. doi:10.1016/j.biortech.2008.08.020

Nah, I. W., Kang, Y. W., Hwang, K.-Y., and Song, W.-K. (2000). Mechanical pretreatment of waste activated sludge for anaerobic digestion process. *Water Research (34)*8, 2362-2368. doi:10.1016/S0043-1354(99)00361-9

National Renewable Energy Laboratory (NREL). (2009). *Biodiesel Handling and Use Guide, Fourth Edition* (NREL/TP-540-43672). Washington, DC: U.S. Government Printing Office.

National Research Council (NRC) (U.S.). Committee on Toxicants and Pathogens in Biosolids Applied to Land. (2002). *Biosolids applied to land: Advancing standards and practices*. Washington, DC: The National Academies Press.

Nelson, D. L., and Cox, M. M. (2005). *Lehninger Principles of Biochemistry* (4th Ed.). New York, NY: W. H. Freeman and Company.

Neyens, E., Baeyens, J., Weemaes, M., and De heyder, B. (2003). Hot acid hydrolysis as a potential treatment of thickened sewage sludge. *Journal of Hazardous Materials*, 98(1-3), 275-293. doi:10.1016/S0304-3894(03)00002-5

Niessen, W. R. (2002). *Combustion and incineration processes* (3rd Ed.). New York, NY: Marcel Dekker, Inc.

Pitt, P., and Jenkins, D. (1990). Causes and Control of *Nocardia* in Activated Sludge. *Research Journal of the Water Pollution Control Federation, 62*(2), 143-150.

Richards, T., Nungesser, P., and Jones, C. (1990). Solution of *Nocardia* Foaming Problems. *Research Journal of the Water Pollution Control Federation, 62*(7), 915-919.

Ritz, G. P. and Croudace, M. C. (2003). Biodiesel/FAME analysis. *Hydrocarbon Engineering, 8*(7), 83-84.

Rittmann, B. E., and McCarty, P. E. (2001) *Environmental Biotechnology: Principles and Applications*. New York, NY: McGraw-Hill.

Tchobanoglous, G., and Schroeder, E. D. (1985) *Water Quality: Characteristics, Modeling, Modification*. Reading, MA: Addison, Wesley, Longman.

United States Department of Agriculture (USDA). (2010, May 7). *Soybeans and Oil Crops: Background*. Retrieved April 10, 2010 from United States Department of Agriculture Economic Research Service: http://www.ers.usda.gov/Briefing/SoybeansOilcrops/background.htm

Van Gerpen, J. (2005). Biodiesel processing and production. *Fuel Processing Technology, 86*(10), 1097-1107. doi:10.1016/j.fuproc.2004.11.005

Water Environment Federation (WEF). (2008). *Operation of Municipal Wastewater Treatment Plants: Manual of Practice No. 11, Volume I: Management and Support Systems* (6th Ed.). New York, NY: McGraw Hill.