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Cultivation of Oleaginous Microorganism Consortium on Municipal Wastewater for the Production of Lipids

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CULTIVATION OF OLEAGINOUS MICROORGANISM CONSORTIUM ON MUNICIPAL WASTEWATER FOR THE PRODUCTION OF LIPIDS

By

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Alternative fuels are necessary to meet the increasing demands for fuels. Alternative fuels such as biodiesel are produced using vegetable oils, which are prominentt in the food industry. An alternate feedstock could be oil-producing microorganisms. These oleaginous microorganisms are defined as accumulating more than 20% of their weight in oil as lipids. Cultivating these microorganisms for oil production is not economical due to the high production costs from the sugars in the culture medium. Municipal wastewater could be a potential growth medium that has not previously been considered for cultivating oleaginous microorganisms. However, municipal wastewater contains a low concentration of carbon, which does not promote oil accumulation in the oleaginous microorganisms. To increase the carbon concentration in the wastewater, lignocellulosic sugars could be added to the municipal wastewater. These sugars are a potential alternative to sugars that are in the food industry.

The goal of this research is to determine the efficacy of using municipal wastewater to cultivate a consortium of oleaginous microorganisms, thus, producing oil for biodiesel production. First, a consortium of oleaginous microorganisms was cultivated on autoclaved wastewater to determine if the wastewater contains any inhibiting substances for the microorganisms. In addition to the substances in the wastewater, indigenous microorganisms are possible inhibitors to the consortium. Therefore, to determine the effect these indigenous microorganisms have on the oleaginous microorganisms, the consortium was cultivated on raw municipal wastewater amended with varying amounts of sugar. Since the municipal wastewater can be used as a cultivation medium, the effect of the addition of lignocellulosic sugars was determined. During the production of lignocellulosic sugars, furfural and acetic acid, known microbial inhibitors, are formed. The effect of these inhibitors on the consortium's growth and oil accumulation ability was ascertained, and inhibition models were developed to describe their impact. With these results, SuperPro Designer v6.0 was used to perform simulations and economic analyses to determine the efficacy of incorporating an oleaginous microorganism consortium in a wastewater treatment facility.

Keywords: Oleaginous microorganism, municipal wastewater, primary effluent, biofuel

DEDICATION

This dissertation is dedicated to my husband, Christopher Hall, my parents, James and Judith Isonhood, and my family. Thank you for your unconditional love and support.

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CHAPTER I

INTRODUCTION

The United States currently depends on foreign countries to meet the country's growing need for fuel. In the United States, the primary energy consumption has increased from 78 quadrillion Btu in 1980 to 99 quadrillion Btu in 2008. This consumption is projected to increase at an average annual growth rate of 0.5% from 2008 to 2035 (Energy Information System 2010b). In 2008, the United States imported a total of 10.98 million barrels of oil per day (Energy Information System 2010a). Dependence on foreign oil can lead to further increase in oil prices as well as straining diplomatic relationships with those countries. Eliminating this dependence could enable stability in the fuels market. To reduce this dependence and avoid the increasing fuel prices, alternative fuels to petroleum diesel and gasoline must be investigated. Alternative fuels such as ethanol and biodiesel are viable options that are more environmentally friendly than the petroleum fuels (You et al., 2008). These alternative fuels are mostly produced using corn and soybeans in the United States, which are also dominant in the food industry (Antoni et al., 2007). The dependence on alternative fuel feedstocks that are also in the food industry limits the alternative fuel production. Using feedstocks produced from waste and wastewater could increase the overall alternative fuel production.

Alternative Fuels

Biodiesel and Ethanol

Two common alternative fuels are ethanol and biodiesel. These fuels are produced from renewable feedstocks such as corn, rapeseed, soybeans, and other vegetable sources. Ethanol as an alternative is produced by two methods. One of the most common methods is the conversion of cornstarch by fermentation (State Energy Conservation Office, 2010). The United States has over 147 operational ethanol plants. Ethanol can be blended with petroleum to produce an E85, which consists of 85% ethanol and 15% gasoline. This E85 has been shown to burn cleaner than conventional fuels, but the vehicle consumes more fuel since ethanol is less efficient (Texas Comptroller of Public Accounts, 2008).

Biodiesel is an alternative to petroleum diesel. Biodiesel is produced by transesterification of triglycerides from various oils. The transesterification reaction consists of combining a triglyceride with an acid or base to yield fatty acid methyl esters (FAMEs), which is biodiesel, and glycerol (Lui and Zhao, 2007). The advantages of using biodiesel versus petroleum diesel include the fact that biodiesel uses renewable raw materials, is more environmentally friendly, has economic benefits, and provides energy security for the United States (Pioneer: DuPont, 2007). The triglyceride feedstocks include rapeseed, soybean, flax, waste vegetable oil, animal fats, algae, and microorganisms (Lui and Zhao, 2007). Soybeans account for approximately 90% of all fuel stocks for the U.S (Pioneer: DuPont, 2007). However, soybeans are also very prominent in the food industry, thus increasing the market price. The feedstock is

responsible for approximately 70-75% of the total biodiesel production cost (Xue et al., 2006). Producing these oil-filled plants requires an entire season as well as a large amount of land. In January 2008, the United States had a production capacity for more than 2.2 billion gallons of biodiesel but only produced 683 million gallons (Energy Information System 2010c; United States Department of Energy, 2008). There exists an obvious shortage in biodiesel feedstocks compared to the biodiesel refining capacity. Given this shortage of traditional feedstocks, economical alternatives are desperately needed. Cultivating oleaginous microorganisms could be an alternative biodiesel feedstock that increases overall biodiesel production that does not require a large amount of land or a large amount of time due to their cultivating methods.

Oleaginous Microorganisms

One alternative to using oil from crops is using oil produced from oleaginous microorganisms. These microorganisms are defined as microorganisms that produce more than 20% of their body weight in oil as lipids. These microorganisms have been shown to produce oil from 20% up to 80% of their cell dry mass (Alvarez and Steinbuchel, 2002; Wynn and Ratledge, 2005). Preliminary results from Hall et al. have shown that these microorganisms reach their stationary phase within 48 hours of cultivation (Hall et al., 2011). Moreover, these microorganisms are able to utilize a multitude of various carbon sources. The most common carbon sources utilized to cultivate these oleaginous microorganisms are various sugars such as glucose and xylose. They have also been shown to utilize xylose, sucrose, glycerol, and waste whey from the

cheese manufacturing process (Easterling et al., 2009; Ratledge, 1994). These microorganisms have been shown to thrive on wastewater streams from a soybean and olive oil manufacturing plant (Chigusa et al., 1996; D'Annibale et al., 2005).

To accumulate a large amount of oil as lipids, these microorganisms require a high carbon to nitrogen ratio (Ratledge, 1994). The carbon source most readily assimilated is glucose; however, industrial glucose is often produced by the hydrolysis of cornstarch (Karkalas, 1985). Because cornstarch can be used to produce food products and ethanol, using glucose as a substrate to produce oil for biodiesel via oleaginous microorganisms increases competition in the market for this raw material. This market competition can increase the cost, thus increasing the cost for producing biodiesel. Therefore, an inexpensive, abundant source of carbon would benefit the cultivation of the oleaginous microorganisms. One source that has not been researched is municipal wastewater.

Wastewater Production

Municipal wastewater is an inexpensive source of carbon, nitrogen, phosphorous, and other trace minerals. These components are necessary for microorganisms to grow. Wastewater is also found in abundance; in the United States, more than 40 billion gallons of municipal wastewater is treated daily (USEPA, 2003). By implementing oleaginous microorganisms into the wastewater treatment process, the microorganisms can treat the water as well as accumulate oil.

The wastewater treatment process consists of primary, secondary, and tertiary treatment (Metcalf & Eddy, 2003). The wastewater enters the plant by directly flowing to the preliminary treatment. Preliminary treatment consists of coarse screening, medium screening, grit removal, and occasionally pre-aeration. These units are arranged according to the wastewater influent characteristics. Furthermore, after the grit is removed, it continues to the primary treatment unit, where the solids are allowed to settle via gravity. The water from the primary settling tank continues to the biological treatment and begins secondary treatment (Viessman and Hammer, 2005).

The biological treatment consists of a consortium of microorganisms that further metabolize the organic and inorganic material in the wastewater to reduce the chemical oxygen demand (COD) and the biochemical oxygen demand (BOD). COD and BOD, among other parameters, are used to determine water quality (Metcalf & Eddy, 2003). The wastewater treatment facility in Tuscaloosa, AL reduces COD, on average, to below 5 mg/L, resulting in a 97% reduction in COD (Heany, 2008). Then, from the aeration tank, the water and biosolids continue to the secondary settling tank (Metcalf & Eddy, 2003).

The secondary settling tank allows the waste biosolids to settle out of the wastewater. The waste biosolids are, then, thickened and sent to the biosolids processing facilities. The biosolids processing facility includes an anaerobic digester and/or mechanical dewatering unit. If an anaerobic digester is used, the treated biosolids continue to liquid land application or mechanically dewatered. After dewatering, it can be applied as a fertilizer or soil conditioner. The dewatered, treated biosolids can also be incinerated or taken to a designated landfill. Incineration is mostly used in large urban

areas due to land limitations. In determining the best method to dispose of these treated solids, cost and environmental conditions must be considered. A portion of wastewater from the secondary settling tank will be recycled to the influent into the primary settling tank (Viessman and Hammer, 2005). Next, the water flows from the secondary settling tank to the tertiary treatment. The tertiary treatment step is the final unit treatment process. The tertiary treatment consists of coagulation, flocculation, clarification, direct or contact filtration, and disinfection. The arrangement of these units depends highly on the wastewater constituents (Metcalf & Eddy, 2003).

Using a portion of the primary settling effluent, a second aeration tank parallel to the biological treatment aeration tank in the secondary treatment stage will contain oleaginous microorganism consortium. By cultivating the oleaginous microorganism consortium in the aeration tank, the consortium could produce oil, thereby, increasing biodiesel production as well as not disrupt the wastewater treatment process. Utilizing the existing wastewater treatment facility minimizes the overall costs to produce oil from these microorganisms. However, the wastewater is low in carbon concentration. Since oleaginous microorganisms require a high concentration of carbon to accumulate oil, the carbon concentration in the wastewater needs to be increased. Utilizing lignocellulosic sugars to increase the carbon concentration could be a less expensive alternative to sugars typically used in the food industry.

Lignocellulosic Sugars

Lignocellulosic sugars are an available, renewable carbon supplement since they are produced from plants such as switchgrass, miscanthus, wood chips, corn stover, etc. In the United States, approximately 368 million tons per acre of forest-derived biomass and 194 million tons per acre of agricultural residue are available as a bioenergy feedstock (Frederick et al., 2008). Lignocellulosic sugars are produced from hydrolysis of lignocellulose, which is mostly composed of cellulose, hemicellulose, and lignin. Hydrolysis can be carried out using acid hydrolysis or enzymatic hydrolysis. This hydrolysate is often composed of five and six carbon simple sugars. These sugars could be utilized to boost oil accumulation in the oleaginous consortium by increasing the available carbon. If these agricultural residues could be used as a carbon source for oleaginous microorganisms, biodiesel production rates could be increased without the limitation of the feedstock (Dai et al., 2007).

Furthermore, with the diminishing crude oil supplies and the increase in fuel consumption, alternative fuels such as biodiesel are required to meet these growing demands. Currently, biodiesel is produced from feedstocks that are dominant in the food industry. To increase biodiesel production in the United States, oleaginous microorganisms are a potential alternative to the feedstocks in the food industry. By cultivating these oleaginous microorganisms on municipal wastewater, the overall production costs decrease while increasing biodiesel production. Municipal wastewater contains all components that a microorganism requires and is found in abundance. To promote oil accumulation in the oleaginous microorganisms, lignocellulosic sugars are proposed to increase the carbon concentration in the municipal wastewater. This research

is directed to determine the effect that municipal wastewater constituents, indigenous microorganisms, and lignocellulosic sugars have on the consortium's growth.

CHAPTER II

RESEARCH HYPOTHESIS

The objective of this work is to incorporate oleaginous microorganisms into a wastewater treatment facility that could increase availability of feedstocks to produce alternative fuels. To meet this objective the following hypothesis must be proven or disproven, a consortium of oleaginous microorganisms originally comprised of *Rhodotorula glutinis* (ATCC 15125) (Wynn and Ratledge, 2006), *Cryptococcus curvatus* (ATCC 20509) (Daniel et al., 1999), *Cryptococcus albidus* (ATCC 32040) (Wynn and Ratledge, 2006), *Candida valida* (ATCC 22687) (Nakahara, 2005), *Candida utilis* (ATCC 22023) (Nakahara, 2005), *Codermyces poitrasii* (ATCC 13844) (Nakahara, 2005), *Rhodosporidium toruloides* (ATCC 10788) (Wynn and Ratledge, 2006), *Lipomyces starkeyi* (ATCC 64135) (Fall et al., 1984), and *Pichia angusta* (ATCC 34438) (Van der Heijden et al., 1999) can thrive in unmodified municipal wastewaters. If disproven, the alternative hypothesis would be modifying the municipal wastewater treatment so that the oleaginous microbial consortium thrives. Chosen for their ability to accumulate an abundant amount of oil as lipids, oleaginous microorganisms are the main agent for producing triacylglycerides for the eventual production of biofuels. In cultivating these microorganisms, a carbon source plays a large role in the accumulation of the oil as lipids. Due to the price and availability of the common carbon sources such

as sugars, the use of an inexpensive and abundant growth medium is required. Since these microorganisms can utilize a wide range of carbon sources, wastewater, which contains a multitude of carbon compounds, is a potentially inexpensive, abundant growth medium. With these wastewaters containing a variety of carbon, nitrogen, and micronutrient sources, it is doubtful that a single oleaginous microorganism would possess the catalytic capacity to maximize the utilization of all of these components. Therefore, another strategy would be to utilize a consortium of oleaginous microorganisms that could utilize the various compounds further treating the wastewater as well as using the nutrients to accumulate oil. By utilizing a wastewater treatment facility to cultivate a consortium of oleaginous microorganisms and lignocellulosic sugars for the oleaginous consortium to convert into lipids, the production of triacylglycerides for biofuel production could be increased in addition to treating the wastewater. This research will generate various growth kinetic data for this consortium to be available for future comparisons.

Research Goal

The goal of this research was to determine if the consortium of oleaginous microorganisms could be cultivated on municipal wastewater to accumulate oil as lipids as well as treat the wastewater. The goal can be divided into two primary objectives:

1. Evaluating the feasibility of cultivating a consortium of oleaginous microorganisms on municipal wastewater

2. Determining the effect of the indigenous microorganisms on the oleaginous microorganism consortium growth.

Primary Objective 1: Evaluate the Growth of Oleaginous Microorganism Consortium on Municipal Wastewater

The first primary objective required a thorough search of the various oleaginous microorganisms available to form a consortium. These oleaginous microorganisms were chosen for their ability to accumulate a large amount of oil as lipids as well as to utilize a wide range of carbon sources. With this ability, a consortium of oleaginous microorganisms could be cultivated on municipal wastewater, which contains a multitude of various carbon compounds. However, since the carbon to nitrogen ratio dictates the amount of oil accumulated within these microorganisms, the carbon content in the municipal wastewater must be increased. Amending the wastewater with glucose and synthetic acid hydrolysate increased the carbon to nitrogen ratio. Cultivating this consortium on autoclaved wastewater and synthetic wastewater amended with these compounds can be found in Chapter VI and Chapter VIII.

After cultivating this consortium on the amended wastewater, the growth of the consortium as found to be inhibited by furfural and acetic acid, which are two compounds that are commonly found in lignocellulosic sugars. Based on the experimental data, models were developed to describe the inhibition of furfural and acetic acid on the growth of the consortium by modifying the Monod model and the Contois model. The experimental data is shown and discussed in Chapter IX while the model development is

shown in Chapter X. The background information for the kinetic models is discussed in Chapter V. In addition, the simulation and economic analysis for modifying the wastewater treatment facility to cultivate the consortium on wastewater without disrupting the wastewater treatment facility is developed and discussed in Chapter XI.

Primary Objective 2: Effect of Indigenous Microorganisms on the Growth of the Oleaginous Microorganism Consortium

The second primary research objective was to determine if the presence of indigenous microorganisms in the wastewater would inhibit the growth and oil accumulation of the oleaginous microorganism consortium. In addition to determining the effect on growth and oil accumulation, the effect on fatty acid methyl ester (FAME) production and microbial populations were also factors to consider. The indigenous microorganisms and the oleaginous microorganism consortium were inoculated into the wastewater supplemented with a carbon to nitrogen ratio of 1:1 and 60:1. The study for the indigenous microorganisms are inoculated with the consortium into the autoclaved wastewater amended with glucose is discussed in Chapter VII.

CHAPTER III

LITERATURE REVIEW

Alternative Fuels and Feedstocks

Petroleum based fuels are currently used to power cars, trucks, airplanes, and other modes of transportation. Petroleum or fossil fuels naturally occur in the environment as a flammable liquid consisting of a mixture of hydrocarbons with varying molecular weights. Through refining and separation processes, the fossil fuels are converted into usable products such as gasoline and diesel. Gasoline and diesel are the most common fuels used. The issues associated with continuing to rely on gasoline and diesel consists of the following: limited supply and environmental impact on harvesting and utilizing energy. In order to reduce the reliance on these fuels, alternative fuels have been developed.

Alternative fuels to gasoline and diesel include biofuels such as ethanol and biodiesel, respectfully. The criteria for a comparable alternative fuel to petroleum-based fuels consists of remaining a liquid, being pumpable for all possible temperatures, and having a high heat of combustion value for reducing energy losses as well as the transportation cost. In addition, it is required to remain stable for storage purposes to ensure the fuel remains safe during storage and environmentally friendly (Antoni et al.,

2007). To be able to use the existing engines, refineries, and various modes of transportation, the alternative fuels must be designed to contain the important features of the petroleum-based fuels such as flash point and oxidation stability (Antoni et al., 2007; International, 1996-2011). The alternative fuel could also be blended with the petroleum-based fuel to account for any differences (Alptekin and Canakci, 2008).

Ethanol is an alternative fuel for gasoline. It is most readily produced by the fermentation of lignocellulosic biomass, including starch from corn and grains (Prasad et al., 2007; Rooney et al., 2007). Alcohols have been used as biofuels since the early nineteenth century (Solomon et al., 2007). In the 1860's, Nikolaus August Otto used ethanol to develop a spark ignition engine. Moreover, in 1902, one third of the Duetz Gas Engine Work's locomotives were run on pure ethanol. Ethanol was soon recognized for its allowance of higher piston compression, which increased engine efficiency, thus being added to the gasoline from 1925 to 1945 (Antoni et al., 2007). In the United States, Henry Ford's Model T ran on pure ethanol (Solomon et al., 2007). During the 1920's, Standard Oil sold a 25% blend of ethanol in gasoline in the Midwest (Antoni et al., 2007). However, low gasoline prices caused the use of ethanol and ethanol blends to be uneconomical in the 1940's. Since gasoline dominated the market with its low prices, ethanol was obsolete until the mid-1980's when ethanol oxygenates were added to reduce carbon monoxide emissions in vehicles (Solomon et al., 2007). Currently, a blend of 10% ethanol and gasoline is sold on the market to promote usage of alternative fuels (Prasad et al., 2007).

The production of ethanol consists of the fermentation of a carbon source by yeast that are able to utilize pentose and hexose sugars as well as tolerate inhibitory substances

produced from the hydrolyzed biomass (Tian et al., 2009). The carbon sources range from starch-derived glucose to cellulose-containing waste material (Antoni et al., 2007). In the United States, over 95% of the ethanol produced is from corn (Solomon et al., 2007). However, the concerns with ethanol production include low energy density of ethanol, low percentage (20% v/v) of production on an industrial scale, and the economic cost of overall production (Antoni et al., 2007).

The concept of utilizing vegetable oil as a fuel has been around since the 1890's. In 1895, Rudolf Diesel developed the diesel engine to be fueled by peanut oil (Akbas and Ozgur, 2008). By 1900, peanut oil was used as fuel in the internal combustion engine at the Paris Expedition (Akbas and Ozgur, 2008; Pousa et al., 2007). During the Great Depression and World War II, vegetable oils were used in the place of petroleum-diesel (Akbas and Ozgur, 2008). From 1970's to 1990's, a new enthusiasm for utilizing renewable fuels such as biodiesel was ignited by the increase in petroleum prices, the concern for the supply of fossil fuels, and the increase in environmental awareness (Pousa et al., 2007). Due to the vegetable oil's high viscosity and low volatility, engines were quickly damaged from the deposits formed from incomplete burning, thus resulting in the need to convert the vegetable oil into biodiesel (Schuchardt et al., 1998).

Biodiesel is defined as a monoalkyl fatty acid ester, more specifically fatty acid methyl ester (Antoni et al., 2007). It is produced by the equilibrium reaction, transesterification, shown in Figure 3.1 (Abdullah et al., 2007). Transesterification is defined as a class of organic reactions that involves an ester transforming into another ester by interchanging the alkoxy moiety. Since the transesterification in producing biodiesel involves the use of an alcohol, this specific reaction is known as alcoholysis of

carboxylic esters (Schuchardt et al., 1998). The catalyst is typically a strong acid or base but can also be an enzyme (Sun et al., 2010). Transesterification for biodiesel production includes a triglyceride reacting with an alcohol and a strong acid or base as the catalyst to produce the fatty acid methyl esters (FAMEs) and glycerol (Kildiran et al., 1996). For the overall process, three consecutive and reversible reactions occur and form two intermediates as diglycerides and monoglycerides (Behzadi and Farid, 2009). In order to produce a high yield of FAMEs, 1 mol of triglycerides is reacted with 3 mol of alcohol as the optimum stoichiometric ratio. Acid-catalyzed transesterification reaction is a common reaction to produce biodiesel because it produces very high alkyl ester yields (Schuchardt et al., 1998). The most common acid and alcohol are sulfuric acid and methanol, respectively (Behzadi and Farid, 2009).

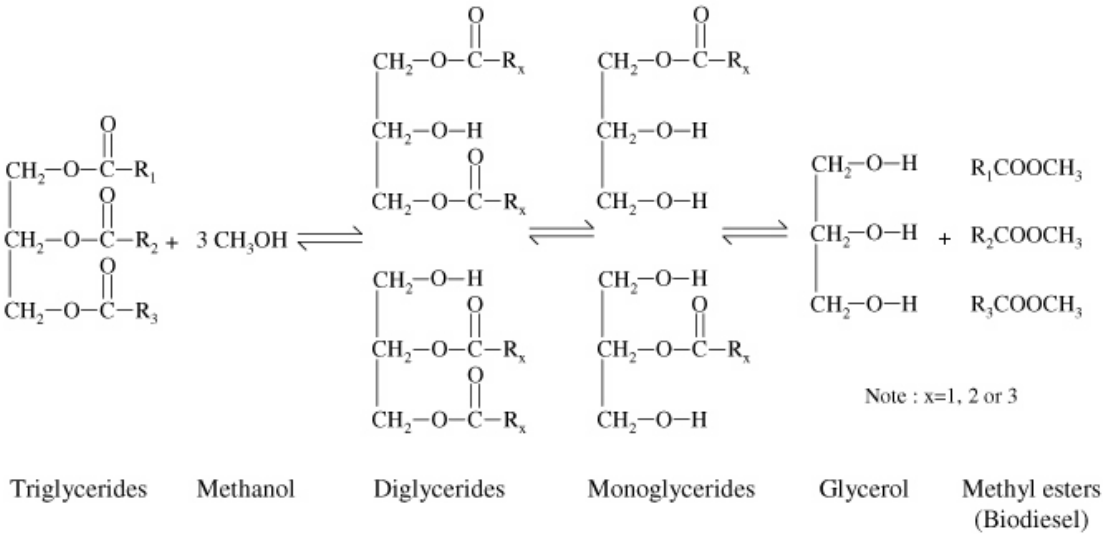

Figure 3.1 Basic transesterification reaction for biodiesel production (Abdullah et al., 2007).

The acid-catalyzed transesterification reaction mechanism is shown in Figure 3.2 (Christie, 1993). This mechanism consists of the carbonyl group from the ester being protonated and producing the carbocation. When the alcohol reacts with a nucleophilic attraction, the tetrahedral intermediate is formed, eliminating glycerol. This glycerol elimination allowed the formation of the new ester as well as generating the catalyst H+ (Schuchardt et al., 1998; Sun et al., 2010).

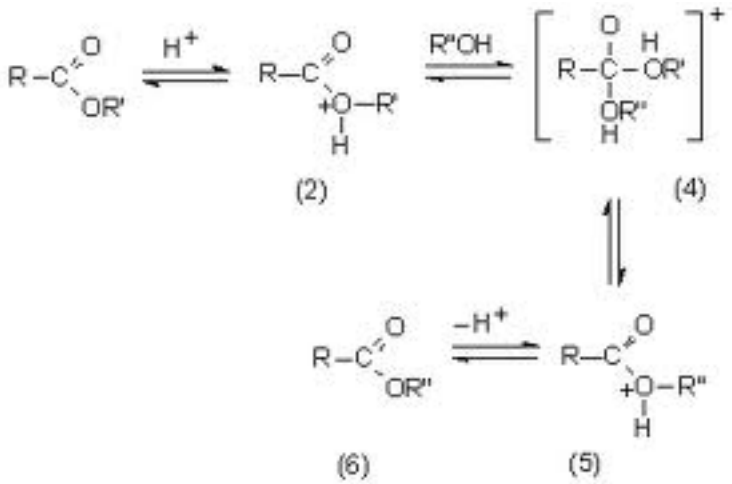

Figure 3.2 Mechanism of the acid-catalyzed transesterification for biodiesel production (Christie, 1993).

Common biodiesel feedstocks mainly include plant oils such as soybean oil, rapeseed oil, canola oil, sunflower oil, and tallow oil (Nelson et al., 1994). In addition to plant oils, biodiesel is also produced from used frying oil, yellow grease, and microbial oil (Oner and Altun, 2009). Table 3.1 compares the fatty acid compositions to common oil feedstocks (Rickdatech, 2010). Soybean oil is the most common biodiesel feedstock in the United States. According to the Energy Information Administration (EIA), the price of soybean oil is expected to reach $2.67 per gallon in 2010/2011 with yellow grease

$1.47 per gallon. In addition, the prices of both soybean oil and yellow grease are predicted to increase up to $2.80 per gallon for soybean oil and $1.55 per gallon for yellow grease (Radich). Meanwhile, crude oil prices are $2.56 per gallon on average in 2010 (Administration, 2011). With the increasing prices of soybean oil and biodiesel prices, the need for a feedstock alternative to plant-based oils is required. Oleaginous microorganisms are a potential oil source for biodiesel production.

Table 3.1 Fatty acid compositions of common biodiesel feedstocks (Rickdatech, 2010).

	Fatty Acid Compositions (%)									
Fat or Oil	**12:0**	**14:0**	**16:0**	**18:0**	**18:1**	**18:2**	**18:3**	**20:0**	**20:1**	**22:1**
Soybean	----	----	6-10	2-5	20-30	50-60	5-11	----	----	----
Corn	----	1-2	8-12	2-5	19-49	34-62	----	----	----	----
Peanut	----	----	8-9	2-3	50-65	20-30	----	----	----	----
Olive	----	----	9-10	2-3	73-84	10-12	----	----	----	----
Cotton-seed	----	0-2	20-25	1-2	23-35	40-50	----	----	----	----
Butter	----	7-10	24-26	10-13	28-31	1-2.5	0.2-0.5	----	----	----
Lard	----	1-2	28-30	12-18	40-50	7-13	0-1	----	----	----
Tallow	----	3-6	24-32	20-25	37-43	2-3	----	----	----	----
Linseed	----	----	4-7	2-4	25-40	35-40	25-60	----	----	----
Yellow Grease	----	2	23	13	44	7	1	----	----	----
Coconut	45-53	17-21	7-10	2-4	5-10	1-3	----	----	----	----
Palm	----	----	44	5	39	10	----	----	----	----
Palm kernel	48	16	8	----	15	3	----	----	----	----
Pongamia pinnata	----	----	4-8	3-9	45-71	11-18	----	2-5	10-12	4-5

Oleaginous Microorganisms

With their ability to produce oil, oleaginous microorganisms are a possible alternative biofuel feedstock to plant oils. These microorganisms are found most commonly in natural environments with high carbon concentrations. The benefits of using microorganisms over plant-based oils include the fact that microorganisms can utilize a wide range of carbon sources, grow relatively quickly, and adapt to varying environments (Wynn and Ratledge, 2005). In addition to the benefits, the production of oil from microorganisms that are equivalent in composition to plants or animals is defined as single cell oils (SCO) in commercial production (Ratledge, 1994). The oil-producing or oleaginous microorganisms are defined as producing more than 20% of their weight in oil as lipids (Ratledge, 2005b). These microorganisms are mostly composed of yeast, fungi, and algae with a very small amount of oleaginous bacteria (Wynn and Ratledge, 2006).

Oleaginous microorganisms mainly produce triacylglycerols and phospholipids (Wynn and Ratledge, 2005). The fatty acid profiles of these oils consist of Palmitic, Palmitoleic, Stearic, Oleic, Linoleic, and g-Linoleic acid methyl esters (Ratledge, 1994; Ratledge, 2005a; Wynn and Ratledge, 2006). Table 3.2 shows a typical fatty acid profile for these microorganisms (Ratledge, 1994). These fatty acid profiles are very specific to oleaginous microorganisms. This range of fatty acids is also found in plant and animal fats and oils (Wynn and Ratledge, 2006). Oil accumulation within the microbial cell occurs when either nitrogen or phosphorous is exhausted in the medium. When nitrogen or phosphorous are not present, the microorganisms cannot replicate or function, thus causing the storage of oil as lipids. Oleaginous microorganisms require a large amount of

carbon to a low amount of nitrogen. This ratio is required for the microbes to encourage oil accumulation. The microbes accumulate oil as food reserves during possible periods of starvation (Wynn and Ratledge, 2005).

Table 3.2 Fatty acid concentration of the oleaginous microorganisms (Ratledge, 1994).

Oleaginous Microorganisms	Max. Lipid %	Fatty Acid Compositions (%)						
		16:0	16:1	18:0	18:1	18:2	18:3	Others
Candida curvata	58	32		15	44	8		
Cryptococcus albidus	65	12	1	3	73	12		
Lipomyces lipofer	64	37	4	7	48	3		
Lipomyces starkeyi	63	34	6	5	51	3		
Rhodosporidium toruloides	66	18	3	3	66			22:0 (3%)
Rhodotorula glutinis	72	37	1	3	47	8		
Yarrowia lipolytica	36	11	6	1	28	51	1	

The mechanism for oil accumulation begins with the nitrogen-limited environment. When nitrogen is exhausted in the environment, the cells cease to produce ATP, which is the generation of energy. Without ATP, the cells are unable to grow and divide since nitrogen is required for protein biosynthesis. Immediately after the nitrogen is depleted, the main enzyme in the citric acid cycle, Krebs' cycle, is inactivated. This inactivation causes the rapid accumulation of isocitrate and citric acid within the mitochondria. The citrate is immediately transported into the cytoplasm of the cell and cleaved by the enzyme ATP: citrate lyase. This reaction generates acetyl-CoA, which is the major C2 building unit for fatty acid biosynthesis. This enzyme is specific to

oleaginous microorganisms. The cleavage of the first main reaction generates oxaloacetate and produces malate with the malate dehydrogenase. The malate is then converted to pyruvate by the malic enzyme and subsequently produces NADPH, reducing power equivalent (Wynn and Ratledge, 2005). The malic enzyme simultaneously produces NADPH as well as reduces the long acyl chain produced from the acetyl-coenzyme into the long-chain fatty acid (Ratledge, 2002). The NADPH and the acetyl-CoA are both continuously required for fatty acid biosynthesis, thus oil accumulation (Wynn and Ratledge, 2005).

The accumulation of oil relies on the activity of the malic enzyme instead of the ATP: citrate lyase (Ratledge, 2002). The activity of this enzyme is based on the genetic makeup of the cell (Wynn and Ratledge, 2005). For example, the cells that accumulate a large amount of lipid have the gene responsible for malic enzyme synthesis is continuous (Ratledge, 2002). Cells with low amounts of lipids turn off the enzyme synthesis directly after nitrogen exhaustion (Wynn and Ratledge, 2005).

In the early twentieth century, plant oils were in short supply due to the two world wars, especially in Germany. Thus, microbial oil or Single Cell Oil (SCO) was considered as a possible substitute for the plant oil. Research on isolating the strains of microorganisms that accumulated the highest lipids was conducted in the first half of the twentieth century (Wynn and Ratledge, 2005). By late 1950's, a knowledge of how to cultivate these microorganisms to produce the highest amount of oil was determined as well as the range of oils produced by these microorganisms (Ratledge, 2005b). During the 1960's, agriculture production increased, thus reducing the prices on plant oil. With

the plant oil cheaper and more plentiful, the microbial oil became somewhat not economical to produce (Wynn and Ratledge, 2005).

However, four microbial oils were in full-scale production in the first decade in the twenty-first century. These microbial oils are specific polyunsaturated fatty acids (PUFAs) (Wynn and Ratledge, 2005). These PUFAs are included in people's diets, especially for infants, babies, and the elderly (Nakahara, 2005). Only certain plants are able to produce these PUFAs but not easily produced. Some of these PUFAs are obtained from animals and fish; fish oil has the potential to be contaminated by pollutants such as organo-mercury compounds and dioxanes (Wynn and Ratledge, 2005). In addition to PUFAs, oleaginous microorganisms were considered being utilized as a cocoa butter equivalent in the late 1970's. By the early 1980's, cocoa butter prices were exceedingly high, thus, resulting in the search for a less expensive alternative such as microbial oils (Wynn and Ratledge, 2006). The microbial oil was not publicly accepted as an alternative to oil produced from plants or animals, which caused this alternative to cocoa butter extract to not be marketable (Ratledge, 2005b). Currently, oleaginous microorganisms are being used to produce fatty acids used in dietary supplements and infant formula with their ability to produce PUFAs such as DHAs (Wynn and Ratledge, 2005).

In addition to PUFAs, these oleaginous microorganisms have a multitude of uses. A common oleaginous microorganism, Rhodotorula glutinis, has been shown to produce microbial oil from agricultural and forestry residues. This oleaginous microorganism was chosen for the study by Dai et al because of its ability to utilize a wide range of sugars present in the hydrolyzed residues (Dai et al., 2007). Daniel et al showed that oleaginous

microorganisms, Cryptococcus curvatus and Candida bombicola, can produce sophorolipids, biosurfactant, from the deproteinized whey concentrates in the dairy industry. Since disposing of the cheese whey has become an increasing problem in the dairy industry, these microorganisms are able to produce a profitable product (Daniel et al., 1999). Another usage of oleaginous microorganisms includes the treatment of olive-mill wastewaters, showing that these microorganisms can remove oil from this type of wastewater (D'Annibale et al., 2005). Davis et al showed that lipids are accumulated by Nocardia sp. when grown the Propane and n-Butane as the sole carbon source (Davis, 1964). Moreover, research has been conducted showing the production of lipids with the Acinetobacter sp. when grown on hexadecane (Makula et al., 1975). Raza et al shows that a biosurfactant was produced from Pseudomonas aerouginosa, an oleaginous microorganism, when cultivated on vegetable oil refinery waste (Raza et al., 2007). Easterling et al shows that Rhodotorula glutinis is able to grow when cultivated on glycerol, a by-product of biodiesel production (Easterling et al., 2009). Angerbauer et al shows that an oleaginous microorganism, Lipomyces starkeyi, produced lipids when grown on sewage sludge (Angerbauer et al., 2008). With the ability to produce lipids with a variety of carbon sources, these oleaginous microorganisms are a potential source of oil when cultivated on municipal wastewater.

Municipal Wastewater Treatment

As a potential medium, municipal wastewater contains multiple nutrients that a microorganism can utilize. These nutrients include carbon, nitrogen, phosphorous, and

biodegradable organic and inorganic material. In addition to the nutrients, wastewater is found in abundance. The water consumption for developing countries such as China is estimated to be 21 gal d^{-1} with the world average ranging from 9 to 24 gal d^{-1} per capita (Metcalf & Eddy, 2003).

In order to fully treat the wastewater for reuse, a typical wastewater treatment in the United States consists of multiple treatment levels. The preliminary treatment level consists of removing large objects such as rags, sticks, and grit that could potentially interfere with the processes downstream. Primary treatment level is required to remove organic matter and suspended solids; an advanced primary treatment level is also utilized to enhance the removal of suspended solids using filtration or chemical addition. Secondary treatment level uses biological and chemical processes to remove biodegradable organic matter as well as suspended solids. This level can include a disinfection step to potentially remove pathogens from the effluent. The disinfection includes chlorine, ozone, or ultraviolet light to pre-treat wastewater before reaching the biological processes. Tertiary treatment level consists of disinfection, nutrient removal, and removal of residual suspended solids by microscreens or granular medium filtration. The advanced treatment level is the final level that removes the suspended and dissolved materials when it is required for water reuse applications (Metcalf & Eddy, 2003).

Figure 3.3 shows the flow scheme of a typical United States municipal wastewater treatment facility (Viessman and Hammer, 2005). In the flow scheme, the secondary treatment level consists of biological processes removing biodegradable particulate and soluble organic matter in an aeration tank. This aeration tank promotes aerobic digestion through a consortium of aerobic microorganisms. The biodegradable

particulate organic matter goes through hydrolysis and is reduced to biodegradable soluble organic matter. In this reduction, nutrients such as ammonia and phosphate are released. The biodegradable soluble organic matter is further broken down to carbon dioxide and water as well as active biomass by means of heterotrophic bacteria. Further decay of the active biomass releases carbon dioxide, water, and inactive biomass. The nonbiodegradable particulate organic matter contained in the influent becomes a part of the digested solids since it is not affected by digestion (Grady et al., 1999).

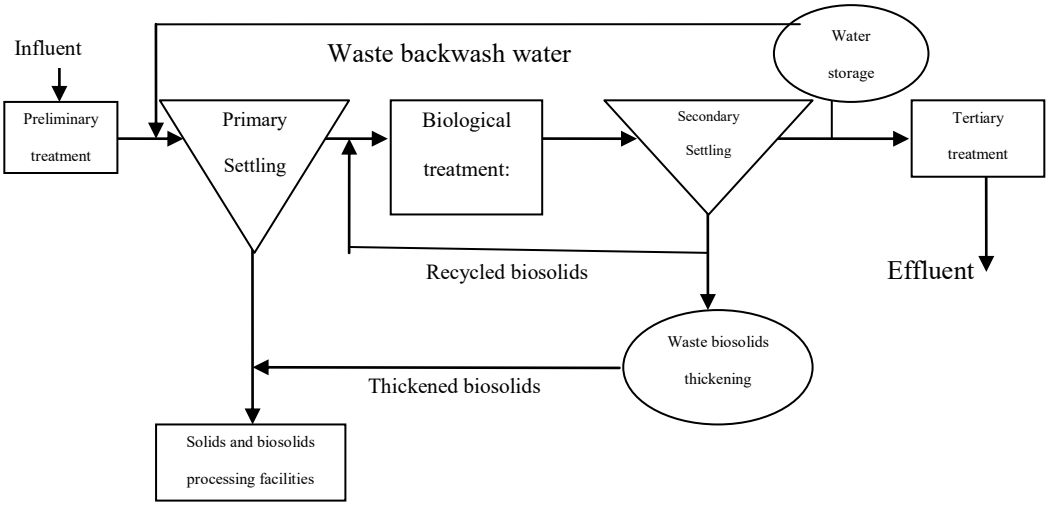

Figure 3.3 Flowchart of a conventional wastewater treatment facility (Viessman and Hammer, 2005).

The biomass produced in the aeration tank is termed activated sludge. The activated sludge is composed of various microorganisms mainly belonging to the domains Bacteria and Archaea. A small portion of the activated sludge is composed of protozoa and other Eucarya. In the domain Bacteria, heterotrophic bacteria are the dominant microbes in the activated sludge. Heterotrophic bacteria are defined by their ability to use organic compounds as a carbon source for cell synthesis. The other type of

bacteria that utilize the inorganic compounds is defined as chemoautotrophic bacteria. These bacteria utilize ammonia and nitrite and are responsible for nitrification. Most of these bacteria are either obligately or facultatively aerobic, which discerns that these microbes utilize oxygen as an electron acceptor. In contrast, the Arhaea are typically useful in anaerobic operations, where they are more prone to produce methane. The Eucarya that can be found in the activated sludge consists of fungi, yeast, protozoa, rotifers, and nematodes (Grady et al., 1999). Each microorganism makes up the consortium that further treats the wastewater, reducing the organic and inorganic material in the wastewater and producing carbon dioxide and water in its place.

The activated sludge can be processed, reused, or disposed, depending on the regulations and its components. Land application is one way to reuse the biosolids. This application involves using bulk or bagged biosolids to land in order to provide the soil with nitrogen for crops or for vegetation as well as decreasing the amount of nutrients lost below the root zone. Surface disposal consists of dedicating surface disposal sites, solids-only landfills, lagoons, and piles. These surface disposal sites do not require a protective liner or leachate collection system. Incineration is also a method of disposal for biosolids. Each disposal or reuse option requires regulations on the amount of heavy metals, fecal coliform, and other possible pathogens. These methods for reuse and disposal show the potential for oil extraction in order to increase reuse as well as profitability (Metcalf & Eddy, 2003).

The oil extracted from a typical activated sludge is approximately 1.5-7.5% of oil on a dry mass basis (Dufreche et al., 2007). Oil can already be extracted with the current activated sludge microorganisms. The oils extracted from these microorganisms are not

as high in percent yield as the oil that can be extracted from oleaginous microorganisms. By incorporating oleaginous microorganisms, the amount of oil extracted from the activated sludge should increase. However, for a medium-strength wastewater in the United States, the chemical oxygen demand (COD) is 430 mgL^{-1}, nitrogen is 25 mgL^{-1}, and phosphorous is 7 mgL^{-1} (Metcalf & Eddy, 2003). With 430 mgL^{-1}, which is approximately 0.4 mgL^{-1} of sugar, the municipal wastewater carbon concentration needs to be increased. Since oleaginous microorganisms require a high carbon concentration to low nitrogen concentration, this increase in carbon concentration will create an environment conducive for oil accumulation. In order to minimize production costs, the carbon source supplement could be lignocellulosic sugars.

Lignocellulosic Sugar Production

Lignocellulosic sugars are produced from hydrolyzing lignocellulosic biomass. Lignocellulosic biomass includes forestry residue, herbaceous energy crops, agricultural residues, and woody biomass (Keshwani and Cheng, 2009). The benefits of using biomass to increase the carbon concentration in wastewater include that they are renewable, not used in food industry, and abundant source of carbon. This biomass is mostly composed of lignin, cellulose, and hemicellulose (Agbogbo and Wenger, 2007). Cellulose is a polysaccharide, linear chain of linked glucose units; the linearality of this compound makes it easy to breakdown into glucose (Palmqvist and Hahn-Hagerdal, 2000b). Hemicellulose consists of heterogeneous polymers. These polymers are mostly composed of pentoses, hexoses, and sugar acids (Saha, 2003). Lignin is a large aromatic

chemical compounds; it cross-links many plant polysaccharides, which provides structure to the cell wall in plants. Since lignin is such a large aromatic compound, it is difficult to breakdown into simple sugars (Whetten and Sederoff, 1995).

In order to obtain simple sugars that are biologically available, the biomass must be broken down either chemically and/or using enzymes. A common chemical method is using an acid to break down the components in biomass into lignocellulosic sugars that can be utilized to cultivate microorganisms. The benefits of using acid instead of enzymes include the acid can break down the lignin without the need of a pretreatment step and the hydrolysis rate is faster than using enzymes. Common acids that are used in catalyzing the reaction include sulphuric, hydrochloric, and phosphoric acids (Lenihan et al., 2010). Another method to break down the components to form simple sugars is to utilize a combination of dilute acid and enzymes. The dilute acid is used as a pretreatment step that opens the structures of the biomass compounds to allow the enzymes to break down the structures further into glucose and pentoses. The most commonly used and studied treatment is the dilute acid hydrolysis. The dilute acid hydrolysis is beneficial to make sure to minimize xylose degradation (Jensen et al., 2008).

These components vary in composition depending on the source of the biomass. Switchgrass contains a low concentration of lignin but high concentration of cellulose. In contrast, woody biomass contains a higher concentration of lignin and lower concentrations of cellulose. Overall, each biomass produces glucose, xylose, mannose, galactose, and arabinose in varying concentrations (Jensen et al., 2008). For example, Table 3.3 shows the varying percentages of cellulose, lignin, and hemicelluloses for

biomass feedstocks (Olsson and Hahn-Hagerdal, 1996). With the varying raw materials, the amounts of sugars produced are just as varied. The higher concentration of hemicellulose, the higher concentration of xylose produced (Aguilar et al., 2002). Table 3.4 shows the varying concentrations of the sugars from the different biomass feedstocks (Olsson and Hahn-Hagerdal, 1996).

Table 3.4 Sugar concentrations (gL^{-1}) from different biomass feedstocks.

Materials	Glucose	Xylose	Galactose	Arabinose	Mannose	Reference
Wood chips	57.5	11.2	9.3	----	15.1	(Katahira et al., 2006)
Forest residue	4	77	20	30	13	(Nilsson, 2005)
Spruce	21.9	8.1	6.7	----	16.9	(Nilsson et al., 2005)
Vineshoot trimmings	11.8	17.9	----	5.3	-----	(Bustos et al., 2004)
Hardwoods	3.2	-----	1.7	----	6.8	(Keating et al., 2006)
Corn stover	8.19	33.54	----	----	----	(Agbogbo and Wenger, 2007)

In addition to producing sugars, the hydrolysis of biomass also produces growth inhibitors. The growth inhibitors include acetic acid, furfural, hydroxymethylfurfural (HMF), and phenolic compounds. The acetic acid is produced from the acetyl group in hemicelluloses (Agbogbo and Wenger, 2007). With the degradation of xylose, furfural is produced (Vazquez et al., 2007). HMF is formed with the dehydration of hexose (Sanchez, 1988). When the lignin is degraded, phenolic compounds are formed (Palmqvist and Hahn-Hagerdal, 2000b). Each one of these inhibitors is produced in

varying concentrations, depending on the hydrolysis process as well as the type of biomass (Olsson and Hahn-Hagerdal, 1996). Table 3.5 shows the concentrations of the inhibitors with the different biomass.

Table 3.5 Inhibitor concentrations (gL^{-1}) from different biomass feedstocks.

Materials	Furfural	Acetic Acid	Reference
Wood chips	Not reported	Not reported	(Katahira et al., 2006)
Forest residue	0.2	1.5	(Nilsson et al., 2005)
Spruce	0.4	1.6	(Nilsson, 2005)
Vineshoot trimmings	3.1	5.4	(Bustos et al., 2004)
Hardwoods	0.18	15	(Keating et al., 2006)
Corn stover	0.73	7.93	(Agbogbo and Wenger, 2007)

The main compounds that are inhibitory are considered furfural and acetic acid. Furfural is known to decrease the specific growth rate of yeast (Horvath et al., 2003). Furfural has also been known to inhibit protein and RNA synthesis, and other biological activities (Horvath et al., 2003; Lui et al., 2005; Sanchez, 1988). For example, furfural is known to cause cell replication to be inactivated (Palmqvist and Hahn-Hagerdal, 2000b). For microorganisms, the NADH produced is needed for furfural reduction, thus not enough for terminal respiration. Since ATP is required for microorganisms to sustain cell growth, the insufficient production of ATP explains furfural inhibition on cell growth and the direct inhibition to enzymes (Keating et al., 2006; Taherzadeh et al., 2000).

Moreover, acetic acid is known to also decrease cell growth as well as the functionality of glycolysis enzymes and catabolic activity, resulting in inhibition of cell growth despite buffering of pH (Zhao et al., 2008). The concentration and pH at which the acetic acid is inhibitory is different for each microorganism. Acetic acid inhibition on microbial growth is most often explained by anion accumulation. This anion accumulation theory is based on the acetic acid anionic form accumulating within the cell while simultaneously the undissociated acetic acid diffuses into the cell reaching an equilibrium concentration. pH is a function of this equilibrium concentration. Therefore, the concentration of anions accumulated within the cell is a function of the pH gradient that occurs across the cell. With acetic acid present, the pH of the media is a low extracellular pH. This low pH is what causes the accumulation of anions while the cell tries to maintain a neutral intracellular pH (Palmqvist and Hahn-Hagerdal, 2000b). All the energy of the cell is directed toward maintaining pH instead of maintaining cell growth, thus causing the inhibition.

Currently, these sugars that are hydrolyzed from biomass are utilized for ethanol production. The ethanol is produced using microorganisms to ferment the lignocellulosic sugars. Since the microorganisms can use the sugars to produce ethanol through fermentation, oleaginous microorganisms could potentially utilize these sugars to accumulate oil.

CHAPTER IV

MATERIALS AND METHODS

Introduction

Chapter IV discusses the details for this study of the experimental materials, experimental methods, and analytical methods. Moreover, the individual specificities are included in the chapters in which the results are shown.

For this research, municipal wastewater was collected to evaluate how the microorganisms will perform with the components typically found in wastewater. Using municipal wastewater from an actual treatment plant will demonstrate whether the wastewater contains major inhibitory compounds that could potentially inhibit the growth of the microorganisms. Since municipal wastewater components are widely varied in concentration, a synthetic wastewater was used in subsequent experiments to control the variability of the wastewater. By controlling the variability of the wastewater growth medium, the consortium's growth and response to the environment can be fully understood.

Wastewater Collection and Synthetic Wastewater Medium

Municipal wastewater was obtained from Tuscaloosa Wastewater Treatment Facility in Tuscaloosa, Alabama (4010 Kauloosa Avenue, Tuscaloosa, AL 35401). From the primary effluent clarifier, the wastewater was collected and transported in 1 L, clear Nalgene bottles. After collection, the bottles were immediately placed into an ice chest with ice to stabilize the microbial populations and transported to the laboratory at Mississippi State University.

Synthetic wastewater medium from Ghosh et al. was utilized to eliminate the variability in municipal wastewater nutrients and concentration (Ghosh and LaPara, 2004). Table 4.1 shows the chemical species and concentrations that form the synthetic wastewater medium. The pH was adjusted to 7 using NaOH. This synthetic medium was developed for treating wastewater with membrane-coupled reactors that separate the biomass from the wastewater effluent.

Table 4.1 Chemical compounds and concentrations in the synthetic wastewater (Ghosh and LaPara, 2004).

Ingredients	Concentration (gL^{-1})
Gelatin	0.15
Starch	0.07
Yeast extract	0.07
Casamino acids	0.01
Ammonium sulfate	0.05
Sodium phosphate	0.025
Potassium phosphate	0.03
Calcium chloride	0.0006
SL7 trace mineral solution (mL)	0.0001

Consortium Formation and Maintenance

Consortium Formation

The consortium was developed utilizing nine known oleaginous yeast and one oleaginous bacterium. The oleaginous bacterium *Rhodococcus opacus* (DSM 44193; Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH German Collection, DSMZ, Braunschweig, Germany) has shown to accumulate up to 70% of its weight in oil as lipids (Alvarez and Steinbuchel, 2002). This bacterium was cultivated on nutrient broth (Fisher Scientific, Pittsburgh, Pennsylvania) prior to formation of this consortium. The nine oleaginous yeast consisted of *Rhodotorula glutinis* (ATCC 15125) (Wynn and Ratledge, 2006), *Cryptococcus curvatus* (ATCC 20509) (Daniel et al., 1999), *Cryptococcus albidus* (ATCC 32040) (Wynn and Ratledge, 2006), *Candida valida* (ATCC 22687) (Nakahara, 2005), *Candida utilis* (ATCC 22023) (Nakahara, 2005), *Codermyces poitrasii* (ATCC 13844) (Nakahara, 2005), *Rhodosporidium toruloides* (ATCC 10788) (Wynn and Ratledge, 2006), *Lipomyces starkeyi* (ATCC 64135) (Fall et al., 1984), and *Pichia angusta* (ATCC 34438) (Van der Heijden et al., 1999). Prior to consortium formation, these yeast were cultivated on yeast mold (YM) broth and yeast extract, peptone, and dextrose (YPD) broth. The broths were made with deionized water and YM and YPD powder from Fisher Scientific. Then, the broths were autoclaved in an Amsco Steris steam sterilizer (Westbury, New York) for 15 minutes at 121^{o}C and 15 atm. In addition, these microorganisms were chosen for their ability to accumulate oil as well as their commonality.

The consortium was formed by inoculating 30mL from each culture after three days of cultivation into 3L of autoclaved synthetic wastewater medium (Ghosh and LaPara, 2004). It was contained in a Corning bioreactor with working volume of 3L (Fisher Scientific, Pittsburgh, Pennsylvania). The synthetic wastewater medium utilizes soluble starch from Fisher Scientific (Pittsburgh, Pennsylvania) as the main carbon source. Since these oleaginous microorganisms are copiotrophic, the carbon source concentration shown in Table 4.1 was increased to 3g to boost the cell concentration in the consortium (Wynn and Ratledge, 2006).

Consortium Maintenance

The consortium of oleaginous microorganisms was developed in May 2007 and sustained weekly. To maintain the consortium, 1 L was removed from the consortium bioreactor followed by the addition of 1 L of autoclaved synthetic wastewater medium via aseptic technique.

Analytical Methods

Chemical Oxygen Demand (COD)

For each treatment, a 50 mL aliquot was taken and placed in a 50 mL Corning centrifuge tube (Fisher Scientific, Pittsburgh, Pennsylvania). The centrifuge tubes were centrifuged at 3200 rpm for 20 minutes using a Sorvall ST 40 (Thermo Scientific, Asheville, North Carolina). Using the supernatant for the COD analysis, 2 mL of the

supernatant was added to 0-15,000 mgL^{-1} COD reagent vials (Fisher Scientific, Pittsburgh, Pennsylvania). After mixing, the COD reagent vials were digested in a COD digester for 2 hr at 150°C (EPA). Once the reagent vials cool, the COD values were measured using a Genesys 20 spectrophotometer (Thermo Fisher Scientific, West Palm Beach, Florida) at an absorbance wavelength of 620nm.

Sugar sample and cell mass preparation

For sugar analysis, 1 mL of the supernatant was stored in a 1.5 mL GC vial in the freezer until ready for analysis. The remainder of the supernatant as well as the remaining cell pellet was stored in 15 mL centrifuge tubes in a Revco -80°C freezer (Thermo Scientific, Asheville, North Carolina). After thoroughly frozen, the cell pellets were freeze-dried using the Labconco Freezone 2.5 freeze drier (Labconco, Kansas City, Missouri). Once the pellets were completely dry, the pellets were weighed in 50 mL glass, round-bottom centrifuge tubes using an analytical balance (Model XS204; Mettler-Toledo, Incorporated; Columbus, Ohio).

Lipid extraction

Furthermore, to extract the lipids from the freeze-dried cell pellets, the solvent extraction method using chloroform, methanol, and water as described by Bligh et al was used (Bligh and Dyer, 1959). The freeze-dried pellets were first re-suspended in 5 mL of deionized water by vortexing. Next, 12.5 mL of methanol was added to the mixture. To complete the monophase mixture, 6.25 mL of chloroform was also added. The mixture

was then vortexed and vigorously shaken for 10 minutes either by hand or by Innova 2000 platform shaker (New Brunswick Scientific; Edison, New Jersey). After shaking, 6.25 mL of chloroform was added to form two phases and 12.5 mL of deionized water with 0.5 % NaCl to enhance the phase separation. Then, the mixture was vortexed, shaken for 15 minutes, and centrifuged at 1800 rpm for 20 minutes using a Sorvall ST 40 (Thermo Scientific, Asheville, North Carolina). After centrifuging, the bottom layer (lipid dissolved chloroform layer) was extracted using a pasteur pipet and filtered through fiberglass wool into a 60 mL previously weighed, amber vial. The chloroform was evaporated off using a TurboVap LV (Caliper Life Sciences, Hopkinton, Massachusetts) and TuboVap LV Evaporator (GenTech, Arcade, New York). The lipids were weighed using an analytical balance after the chloroform was completely evaporated.

Analysis of Sugars with High-Performance Liquid Chromotography

Glucose and xylose concentrations were measured using an Agilent 1100 High Performance Liquid Chromatography (HPLC; GMI Inc., Ramsey, Minnesota) system coupled with a Varian 385-LC evaporative light scattering detector (ELSD; Varian Inc., Palo Alto, California). The ELSD used nitrogen as the nebulizer gas with a temperature set to 60^{o}C. The column was Restek Pinnacle II Amino (5µm, 150×4.6mm). The mobile phase consisted of Acetonitrile and water in a ratio of 83:17. The injection volume was 2 micro-liters with a flow rate of 1 mL per minute.

Conversion of Lipids to Fatty Acid Methyl Esters

Transesterification reaction was utilized to convert the lipids into fatty acid methyl esters (FAME) using sulfuric acid as the catalyst and methanol as the reactant. The reaction takes place in 60 mL amber glass vial and heated for 2 hours in a hot water bath set to 60^{o}C once the methanol with 2% sulfuric acid was added. After the sample cools down, 5 mL of a mixture of 2% KHCO3 and 5% NaCl in water was added to the FAMEs and glycerol to quench the reaction. In order to separate the FAMEs from the glycerol, 2 mL of toluene with an internal standard of 200 ppm 1,3 dichlorobenzene and 100 ppm of BHT to inhibit oxidation was added to the sample and vortexed. This step was repeated twice to increase the amount of fatty acids that are dissolved in the toluene. After allowing the mixture to separate, 1 mL of the top layer that consists of the toluene with the fatty acids was dispensed into gc vials for analysis.

Analysis of Fatty Acid Methyl Esters with Gas Chromatography

Gas chromatography was utilized to identify and quantify the various FAMEs produced from transesterification of the microbial lipids. The concentrations of these FAMEs were determined with Agilent 6890N gas chromatogram with a flame-ionization detector (GC-FID; Agilent Technologies Inc., Wilmington, Delaware) using a fused silica column Stabilwax-DA (30 m × 0.25mm, film thickness 0.25µm). The operating conditions were as follows: oven temperature 50-250 ^{o}C with a rate of 10 ^{o}C per minute increase; carrier gas helium; 1.5mLmin-1 flow; 260^{o}C detector temperature. Comparing

the retention times of FAMEs contained in standard mixtures identified FAMEs in the samples.

Analysis of Furfural and Acetic Acid with Gas Chromotography

Furfural and acetic acid sample preparation began by dispensing 0.5mL of the supernatant from the experiments into 2 mL microcentrifuge tubes. Then, 0.2g of sodium chloride, 0.1 mL of the 50% v/v sulfuric acid solution, and 1 mL of chloroform with 1 mg mL^{-1} of hexanoic acid were added to the microcentrifuge tubes. Each microcentrifuge tube was inverted 18 times to completely mix the sample. The tubes were centrifuged in a microcentrifuge (Model 5415D; Eppendorf North America, Hauppauge, New York) at 12,000 rpm for 30 seconds. After centrifuging, the organic layer was removed using Pasteur pipets and dispensed into 1.8 mL autosampler vials with a 300 L insert. Calibration standards were developed using synthetic wastewater medium with $5gL^{-1}$ of furfural and serial dilutions to a concentration of $0.31gL^{-1}$ of furfural. Acetic acid calibration standards were developed with the exact same concentrations and follow the same methods. Each calibration standard and dilution was prepared the same as the samples.

To analyze these samples for furfural and acetic acid, an Agilent 6890N gas chromatogram with a flame-ionization detector (GC-FID; Agilent Technologies Inc., Wilmington, Delaware) that features a CombiPAL autosampling system that was manufactured by LEAP Technologies (Carrboro, North Carolina) was used. This GC-FID used a Stabilwax-DA (30m, 0.25mmID, 0.25 m df). The flow through the column

was 1.2 mL min^{-1} with an injection volume of 2 L. The detector temperature was 300^{o}C with hydrogen flow of 40 mL min^{-1}, air flow of 400 mL min^{-1}, and a helium makeup of 33.8 mL min^{-1}. The oven temperature increased from 50 ^{o}C to 230 ^{o}C at a rate of 10 ^{o}C min^{-1}.

When analyzing the furfural and acetic acid samples, each sample produced an unknown peak. In order to identify this peak as a by-product of the furfural and acetic acid, a Varian 3400 gas chromatogram coupled to Varian Saturn was used. This GC was operating in electron impact mode, using a scan from 40 to 110 amu. The column for this GC is a Stabilwax-DA (30m, 0.25mmID, 0.25 m df).

CHAPTER V

CELL GROWTH KINETIC MODELING

Introduction

The purpose of Chapter V is to provide the background for the growth kinetics utilized to describe the consortium's growth in the subsequent chapters. The three equations discussed in this chapter include the Monod equation model, Contois model, and the yield coefficients. The Monod equation model is used to describe the consortium's growth on the wastewater. The Contois equation model describes the inhibited growth of the consortium on wastewater with furfural and acetic acid as the inhibitory substance. The yield coefficients are used to quantify the effects the various sugars as well as the inhibitory substances have in relation to the consortium's growth, sugar consumption, and lipid accumulation.

Monod Equation Model

The growth of a microorganism is described in four main phases. Phase 1 is known as the lag phase, where the microorganisms become acclimated to the environment. This phase results in no growth or replication of cells. The lag phase also results in the synthesis of protein transport, enzymes for new substrate and for replicating

cells. Phase 2 shows the exponential growth phase. In this phase, the cells efficiently utilize the nutrients and divide at a maximum rate. The cell mass concentration is directly proportional to the rate of the cell's growth. In phase 3, the cells have reached the stationary phase, where the cells have exhausted one or more nutrients necessary for growth. This phase is where the net growth rate is zero. Many products are produced during this phase such as antibiotics and lipids. Phase 4 is known as the death phase, where the live cell concentrations are decreased. This decrease is due to the lack of nutrients (Fogler, 2006).

At first, the exponential growth phase, phase 2, was thought to only be possible when nutrients were present in high concentrations. However, in the 1940's, it was determined that microorganisms show an exponential growth rate when one nutrient is limiting. The basic concept of microbial growth is represented by the specific growth rate coefficient, μ, as a function of the limiting nutrient concentration. The limiting nutrient can be the electron donor, electron acceptor, nitrogen, carbon source, or other nutrients needed for growth. Figure 5.1 shows the typical growth of microorganisms (Toprak, 2000). This figure shows the specific growth rate versus the substrate concentration. The growth rate typically shows a sharp increase initially and then asymptotically approaches the maximum specific growth rate, μ_{max} (Grady et al., 1999).

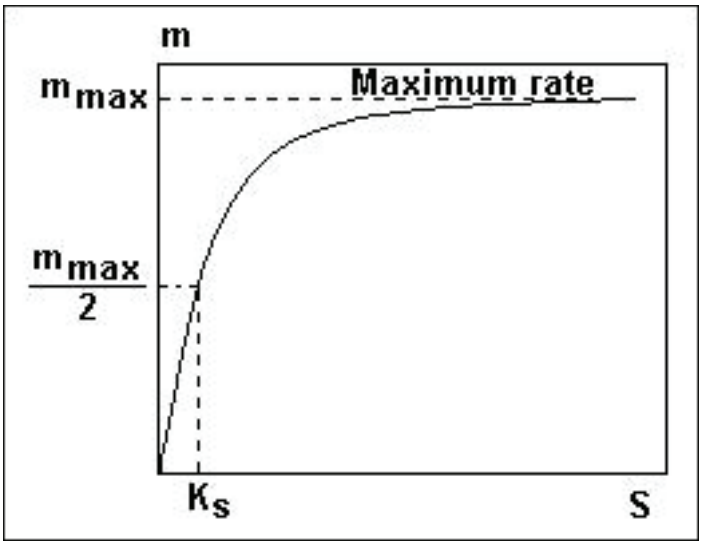

Figure 5.1 Typical growth rate of microorganisms with one limiting substrate (Toprak, 2000).

The overall cell growth rate of new cells is described by Equation 5.1 (Fogler, 2006).

$$Cells + Substrate \rightarrow Cells + \text{Pr}oduct \qquad [5.1]$$

The exact mechanism of this overall reaction equation for cell growth is not known. Therefore, to develop a mathematical expression, empirical models have been fitted to experimental data based on goodness of fit, broad acceptance, and easy to use. Monod proposed an equation that related specific growth rate, μ, to substrate concentration, CA, with coefficients of maximum specific growth rate, μ_{max}, and the Monod constant (half-saturation constant), K_S, as shown by Equation 5.2 (Grady et al., 1999).

$$\mu = \mu_{\max} \frac{C_A}{K_S + C_A} \qquad [5.2]$$

K_S shows how quickly the specific growth rate approaches the maximum specific growth rate. It is also measured as the substrate concentration where specific growth rate is half of the maximum specific growth rate. This equation is strictly empirical even though it is similar to the Michaelis-Menten equation that is based on reaction mechanisms of enzyme kinetics (Grady et al., 1999). To determine μ_{max} and K_S, the inverse of specific growth rate was plotted against the inverse of substrate concentration, using Equation 5.3. The y-intercept from the plot is the inverse of the maximum of specific growth rate. Using the maximum specific growth rate and the slope, K_S was then calculated.

$$\frac{1}{\mu} = \frac{K_S}{\mu_{\max} C_A} + \frac{1}{\mu_{\max}} \qquad [5.3]$$

The Monod equation is the most widely used equation to describe microbial growth. This equation has been used to describe pure cultures that are cultivated on single substrates and used as a basis for developing models to best describe continuous cultures (Grady et al., 1999). Moreover, the Monod equation shows a satisfactory fit when applied to a wide range of data (Shuler and Kargi, 2002). The Monod equation has been used to describe the kinetics of biodegradation of substrates or nutrients (Nakhla et al., 2005). The Monod equation assumptions include that the vessel is well-mixed, uniform conditions throughout the container, and agitation speed provided adequate mass transfer and uniform substrate availability (Govindaswamy and Vane, 2007). In addition, this model was used to describe the growth rate of activated sludge in municipal wastewater treatment (Grady et al., 1999). Many modifications to the Monod model have been developed to describe growth inhibition and mixed microbial cultures such as the

Luong equation, Han-Levenspiel equation, and Haldane equation (Grady et al., 1999; Han and Levenspiel, 1988; Luong, 1986).

Contois Equation Model

The Contois equation model is similar to the Monod model in that it also describes the growth rate of microorganisms. The Contois equation is based on the assumption that the specific growth rates are functions of substrate or nutrient concentration as well as population density. Equation 5.4 is the Contois model, where B is a growth parameter similar to the Monod constant, K_S, and P represents the population density.

$$\mu = \mu_{\max} \frac{S_S}{BP + S_S} \qquad [5.4]$$

The difference between the Contois model and the Monod model is that the data that Monod used to develop his model consisted of different concentrations of limiting nutrients and the same initial population densities from culture to culture (Contois, 1959). The specific growth rate is inversely proportional to population density or cell concentration (Baei et al., 2011). The relationship of the population density to the limiting nutrient concentration is possibly due to environmental condition changes with increasing population density or oxygen may be limiting (Contois, 1959). This relationship depicts substrate utilization at high cell densities (Ahmad et al., 2011). According to Gerber et al, the Contois model describes microbial growth for discontinuous and continuous processes but is limited in describing dynamic processes (Gerber and Span, 2008). Cell concentration or biomass has also been used

interchangeably with the population density when applying this model to experimental data such as fermentation process of gluconic acid production by *Aspergillus niger* (Znad et al., 2004).

The Contois model has been used to describe the growth of microorganisms in many different industries such as wastewater treatment, biodegradation, and fermentation. In particular, this model has been known to describe the aerobic biodegradation of solid municipal organic waste well (Ajbar et al., 2011). The Contois model also fit experimental data on the decomposition of grey waste significantly better than the traditional first-order kinetics (Vavilin et al., 2004), the growth of *Peniscillium brevicompactum* in a submerged batch bioreactor culture (Ardestani, 2012), and the hydrolysis step of insoluble fraction in a batch anaerobic digestion of municipal solid waste (Nopharatana et al., 2007).

Yield Coefficients

Methods to quantify the consortium's growth, utilize sugars, and accumulate oil are yield coefficients. The purpose of the yield coefficient is to relate the cell mass production, lipid mass production, and sugar consumption. Equation 5.4 shows how the yield coefficient, $Y_{C/S}$, for cell mass production, C_C, relates to sugar consumption, C_S (Fogler, 2006).

$$Y_{C/S} = -\frac{\Delta C_C}{\Delta C_S} \qquad [5.4]$$

The yield coefficient, $Y_{C/P}$, that relates the oil accumulation, C_P, to the cell mass production, C_C, is shown in Equation 5.5 (Fogler, 2006).

$$Y_{C/P} = -\frac{\Delta C_C}{\Delta C_P} \qquad [5.5]$$

Moreover, the relationship of the oil accumulation, C_P, to the substrate consumption, C_S, is shown in Equation 5.6 (Fogler, 2006).

$$Y_{P/S} = -\frac{\Delta C_P}{\Delta C_S} \qquad [5.6]$$

CHAPTER VI

MUNICIPAL WASTEWATER AS A MEDIUM TO CULTIVATE OLEAGINOUS MICROORGANISMS

Introduction

Municipal wastewater contains a multitude of nutrients for microorganisms. In addition to these nutrients, wastewater also contains many other compounds. These compounds can include pharmaceuticals, phenols, organic solvents, and other possible hazardous chemicals (Grady et al., 1999). This experiment is focused on evaluating the ability of oleaginous microorganisms to use municipal wastewater as a medium. The first objective involves the cultivation of two well-known oleaginous microorganisms, *Rhodotorula glutinis* and *Cryptococcus curvatus*. This objective will determine whether the wastewater contains any major inhibitory constituents that prevent the growth of these microorganisms.

In addition to pure cultures, a consortium of oleaginous microorganisms is proposed to be grown on the wastewater based on the pure culture growth results. Currently, wastewater treatment facilities utilize a consortium of microorganisms to treat the wastewater. Therefore, a consortium of oleaginous microorganisms could utilize the nutrients and compete with indigenous microorganisms more efficiently than a pure culture. The second objective involves cultivating a consortium of oleaginous

microorganism on primary effluent wastewater. This objective will determine whether a consortium of oleaginous microorganisms can utilize the nutrients in the wastewater. However, the wastewater constituents vary hourly. In addition to the component variability, wastewater nutrients are low in carbon, whereas oleaginous microorganisms require a high carbon concentration to a low nitrogen concentration. Therefore, to increase the carbon concentration, sugar could be added to the wastewater. In order to determine how the consortium is affected by the sugar concentrations, a synthetic wastewater is utilized. The third objective involves cultivating the consortium of oleaginous microorganism on a synthetic wastewater to reduce the variability in the wastewater nutrients.

Methodology

Objective 1: Cultivation of *R. glutinis* and *C. curvatus* on Autoclaved Wastewater

The cultures *R. glutinis* and *C. curvatus* were cultivated on autoclaved primary effluent wastewater. The wastewater was autoclaved (described in detail in Chapter IV) in order to eliminate indigenous microorganisms in the wastewater that could potentially inhibit the growth of the pure cultures. This experiment was conducted in 250 mL Nalgene bottles with 100 mL of autoclaved wastewater. The wastewater was inoculated with 10 mL of the pure culture grown on yeast extract, peptone, and dextrose (YPD) broth. The treatments consisted of autoclaved wastewater (negative control), autoclaved

wastewater with *R. glutinis*, and autoclaved wastewater with *C. curvatus*. To eliminate the lack of carbon as a factor for non-growth of the cultures, 0.1 gL^{-1} and 1 gL^{-1} of glucose was added to wastewater. These treatments included autoclaved wastewater with each concentration of glucose (negative control), autoclaved wastewater with each glucose concentration inoculated with *R. glutinis*, and autoclaved wastewater with each glucose concentrations, inoculated with *C. curvatus*. The positive controls consisted of the pure cultures inoculated into YPD broth. Each treatment was conducted in triplicate and cultivated in a New Brunswick Incubator at 28^{o}C and 110 rpm. The samples were taken every 24 hours for a 72-hour period. Lipid mass concentrations were measured for the 0-hour time point and the 72-hour time point. The optical density measurements and lipid concentrations are shown below, and these samples were taken as described in Chapter IV: Materials and Methods.

Objective 2: Cultivation of the Oleaginous Microorganism Consortium on Autoclaved Wastewater

The second objective involved cultivating the developed consortium, as described in Chapter IV, on autoclaved, primary effluent wastewater. This experiment was conducted in 1 L, baffled flasks with 400 mL of autoclaved wastewater. The autoclaved wastewater was inoculated with 25 mL of the consortium. The treatments included autoclaved wastewater inoculated with the consortium without the addition of glucose and autoclaved wastewater with consortium amended with 1 gL^{-1}. Each treatment was conducted in triplicate. The flasks were incubated using the same incubator and settings

as the first approach. Samples were taken every 24 hours for a 96-hour period as described in Chapter IV. The cell mass concentrations and fatty acid profiles were determined using the methods described in Chapter IV.

Objective 3: Cultivation of Oleaginous Microorganism Consortium on Synthetic Wastewater

The third objective included cultivating the oleaginous microbial consortium on a synthetic wastewater medium developed by Ghosh et al (Ghosh and LaPara, 2004). The concentrations of the chemicals that compose the synthetic wastewater are shown in Table 4.1 (Chapter IV). This experiment was conducted in 1 L, baffled flasks with 25 mL of the consortium inoculated into 400 mL of the synthetic wastewater. The flasks were incubated at the same parameters as those used to address Objective 1. The treatments for this experiment include increasing the starch concentration, the main carbon source in the synthetic wastewater, to 1-4 gL^{-1} of starch. Increasing the starch concentration also resulted in the increase in the other components in the same ratio. The samples were collected every 24 hours for a 96-hour cultivation period as described in Chapter IV. Cell mass concentrations and chemical oxygen demand (COD) samples were measured in the methods described in Chapter IV.

Results

Objective 1: Cultivation of *R. glutinis* and *C. curvatus* on Autoclaved Wastewater

The results to this experiment show that autoclave municipal wastewater does not contain any major inhibitory compounds on the growth of *Rhodotorula glutinis* and *Cryptococcus curvatus*. Figure 6.1 shows the optical density of the treatments in the experiment compared to the positive controls and the negative control. This figure shows that *R. glutinis* and *C. curvatus* do not grow on unamended autoclaved wastewater when comparing the optical density measurements to the controls. The positive controls show that both yeast are viable at inoculation as shown by the increase in optical density measurements for both positive controls. Since the treatments do not show any difference in optical density over the 72-hour cultivation period, the autoclaved municipal wastewater possibly does not provide enough nutrients for oleaginous yeast or inhibitors are present.

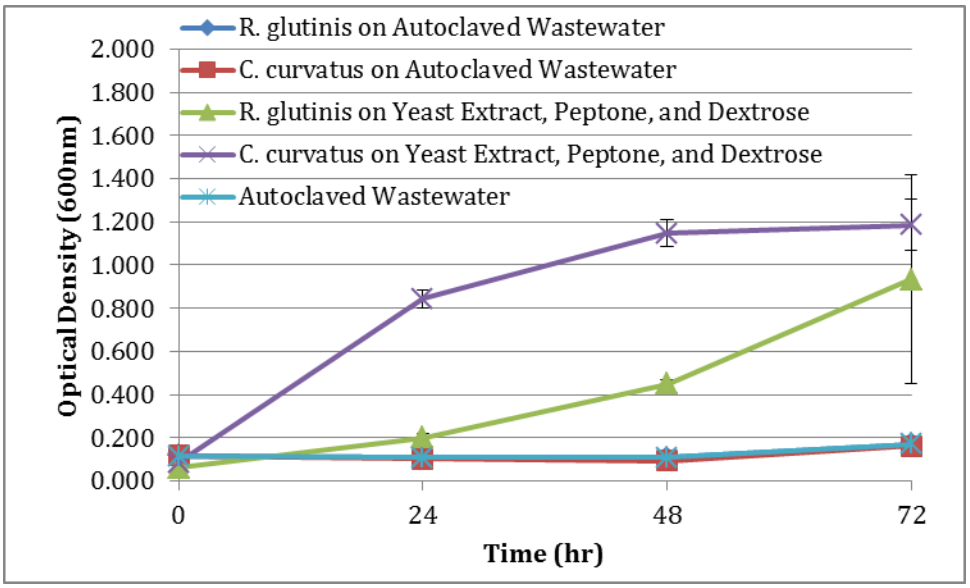

Figure 6.1 Optical density for the growth of *R. glutinis* on autoclaved wastewater, *C.curvatus* on autoclaved wastewater, *R. glutinis* on YPD broth, *C. curvatus* on YPD broth, and autoclaved wastewater.

Oleaginous microorganisms are typically copiotrophic in nature, meaning that they require an environment with a high concentration of nutrients (Sylvia et al., 2005). Municipal wastewater typically has an average COD between 250 mgL^{-1} and 800 mgL^{-1} (Metcalf & Eddy, 2003). These COD amounts are low values compared to the high nutrient concentration environments from which they are isolated. For example, *C. curvatus* was isolated from waste whey from the dairy industry, which has a high COD value of 4, 400 mgL^{-1} (Porges and Jasewicz, 1959).

Figure 6.2 shows the optical density measurements for the cultures that are cultivated on autoclaved municipal wastewater amended with 1 gL^{-1} of glucose. This figure shows that with the addition of a small amount of glucose results in substantial growth within the 72 hours of cultivation. *R. glutinis* and *C. curvatus* grown on autoclaved wastewater with 1 gL^{-1} of glucose also shows a 24 hour lag phase, during

which time the microbes began acclimating to the autoclaved wastewater and developing the enzymes to utilize the nutrients in the wastewater. The negative control did not show any changes in optical density and was interpreted as an indication of no growth, thus demonstrating that autoclaving eliminated indigenous microorganisms in the wastewater. The positive controls did show growth. However, the treatments grew at a higher rate than the positive controls. This result could potentially show that municipal wastewater is a more complete medium than the YPD broth. Municipal wastewater does contain a multitude of nutrients while the YPD contains only 3 components.

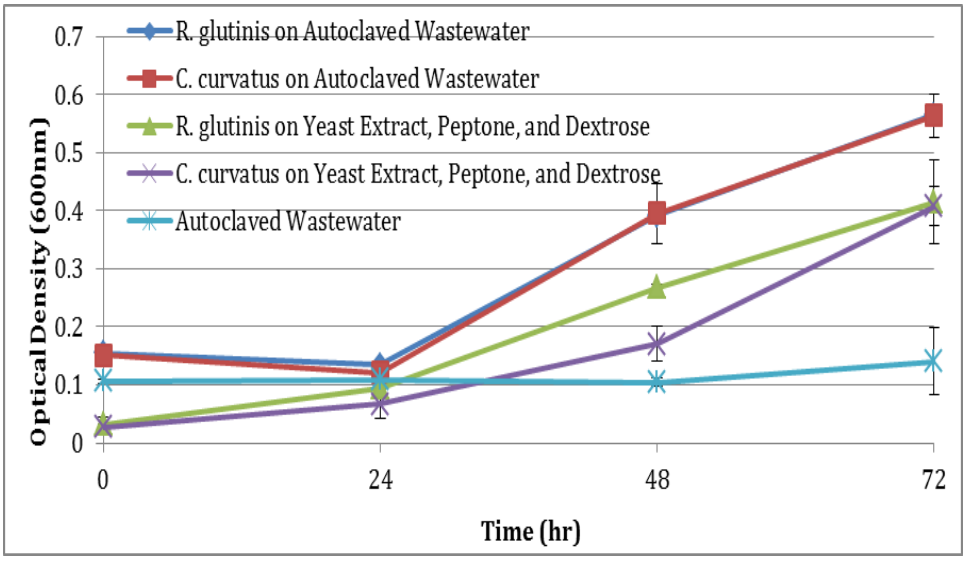

Figure 6.2 Growth of *R. glutinis* on autoclaved wastewater, *C.curvatus* on autoclaved wastewater, *R. glutinis* on YPD broth, *C. curvatus* on YPD broth, and autoclaved wastewater amended with 1 gL^{-1} of glucose.

In addition to growth, lipid mass concentrations were also measured for the 0-hour and the 72-hour time points. Figure 6.3 shows the lipid mass concentration results comparing *R. glutinis* and *C. curvatus* grown on autoclaved wastewater with and without

1 gL^{-1} of glucose. Without glucose, the both cultures show a decrease in lipid mass concentrations. This result could show that these microorganisms are utilizing their oil stored in their lipids to cope with the low concentrations of carbon source in the wastewater. When 1gL^{-1} of glucose was added to autoclaved wastewater, the lipid mass concentrations show an increase over the 72-hour cultivation period. Therefore, a small increase in carbon concentration can activate the oil accumulation.

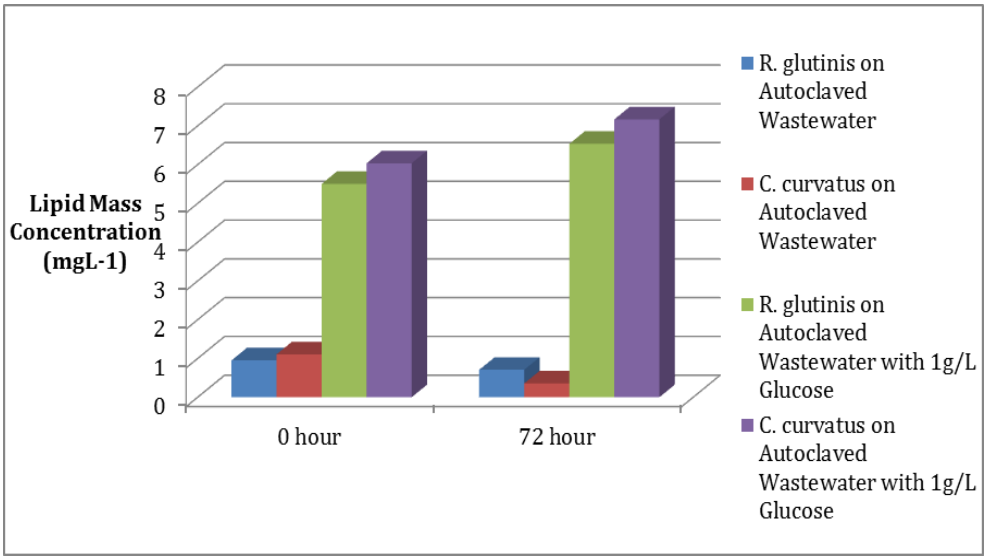

Figure 6.3 Lipid mass concentration of the growth of *R. glutinis* on autoclaved wastewater, *C.curvatus* on autoclaved wastewater, *R. glutinis* on autoclaved wastewater with 1 gL^{-1} of glucose, and *C. curvatus* autoclaved wastewater with 1 gL^{-1} of glucose.

Objective 2: Cultivation of the Oleaginous Microorganism Consortium on Autoclaved Wastewater

Oleaginous microorganisms, *R. glutinis* and *C. curvatus*, were successfully cultivated on autoclaved municipal wastewater. Since the wastewater treatment process

currently utilizes a consortium of microorganisms to utilize the vast organic and inorganic materials in the wastewater, it therefore stands to reason that a consortium of oleaginous microorganisms could also be cultivated on autoclaved wastewater. Figure 6.4 shows the cell mass concentrations of the consortium grown on autoclaved wastewater with and without 1 gL^{-1} of glucose. These results are similar to the results with the pure cultures. The consortium shows very little growth within the first 24 hours and then decreasing steadily until 72 hours. At the 72-hour time point, the net cell mass concentration is zero. Thus, when 1 gL^{-1} of glucose is added to the autoclaved wastewater, the consortium shows an 80% increase in cell mass concentration with the maximum at 72 hours. The increase shown within the first 24 hours could be the consortium utilizing the remaining carbon from the inoculum. The stationary phase between the 24-hour and the 48-hour could be the acclimation to the autoclaved wastewater nutrients, resulting in the increase to the 72-hr time point. The cell mass concentration decreased from 72 hours until 96 hours for the consortium cultivated on autoclaved wastewater.

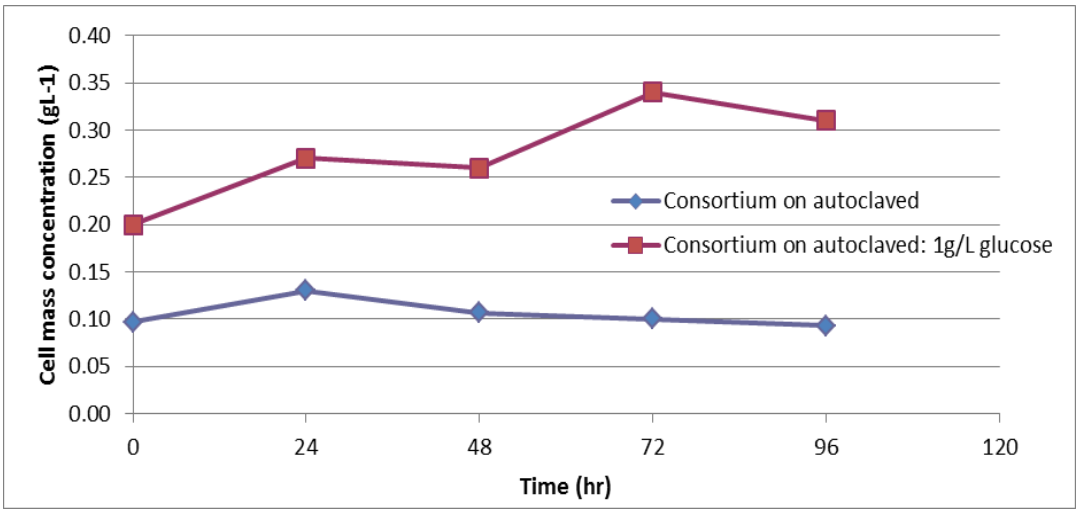

Figure 6.4 Cell mass concentration of the consortium grown on autoclaved wastewater and autoclaved wastewater with 1 gL^{-1} of glucose added.

In addition to cell mass concentrations, the fatty acid profile was also measured to determine if the addition of glucose changes the fatty acid profile of the consortium. Fatty acid profiles are used to characterize various microorganisms. Therefore, by measuring the fatty acid profiles, whether the addition of glucose will cause a major shift in microbial population within the consortium will be determined. Table 6.1 shows the fatty acid profiles for the consortium grown on autoclaved wastewater with and without glucose after 96 hours of cultivation. The consortium grown on autoclaved wastewater shows the majority of the fatty acid profile consisting of palmitic acid and stearic acid. These fatty acids are the most common present in fatty acid profiles for oleaginous microorganisms (Ratledge, 2005b). With the addition of 1 gL^{-1} of glucose, the consortium fatty acid profile shows the majority of palmitic, palmitoleic, and oleic acids. By adding the glucose, the consortium's profile increased the production of palmitoleic acid as well as showing a small percentage of myristic and linoleic, which was not present in the consortium grown on autoclaved wastewater without glucose added.

Overall, the addition of glucose did not show a major shift in the fatty acid profiles, but increased the different fatty acids produced.

Table 6.1 Fatty acid profile for the consortium on autoclaved wastewater and on autoclaved wastewater amended with 1 gL^{-1} of glucose.

Fatty Acid Profile	Consortium Autoclaved	Consortium Autoclaved with 1gL^{-1} glucose
Lauric ACM (C12:0) %	0	0
Myristic ACM (C14:0) %	0	0.35
Palmitic ACM (C16:0) %	48.46	44.11
Palmitoleic ACM (C16:1) %	8.19	19.18
Stearic ACM (C18:0) %	31.82	27.57
Oleic ACM (C18:1) %	2.49	0.00
Linoleic ACM (C18:2) %	0	0.60
Linolenic ACM (C18:3) %	0	0
Arachidic ACM (C20:0) %	0	0
Lignoceric ACM (C24:0) %	0	0

Objective 3: Cultivation of Oleaginous Microorganism Consortium on Synthetic Wastewater

Following confirmation of the oleaginous consortium ability to grow in amended and autoclaved primary wastewater, more data was needed to begin describing this growth mathematically. Since actual wastewater constantly changes, the need for a surrogate wastewater that was consistent was obvious. Figure 6.5 shows the cell mass concentration of the consortium grown on the synthetic wastewater medium described in the Methodology section with the starch concentrations ranging from 1 to 4 gL^{-1}. When comparing the growth of the consortium on the different starch concentrations, overall the consortium shows an increase in cell mass concentrations with increasing starch

concentrations. The consortium grown on 1 gL^{-1} of starch synthetic wastewater showed an increase for the first 24 hours before reaching stationary phase. The cell mass concentration remained constant during the stationary phase from 24 hours to 96 hours. By increasing the concentration of the starch to 2 gL^{-1}, the cell mass increased steadily throughout the 96 hours of cultivation. When cultivated on 2 gL^{-1} of starch, the consortium produces more cell mass concentration when compared to the growth on the 1 gL^{-1}. The growth of the consortium on 3 gL^{-1} starch shows the same trend as the consortium grown on 2 gL^{-1} of starch. This growth comparison from 2 to 3 gL^{-1} of starch does not show the same increase as seen from 1 to 2 gL^{-1}. Moreover, the consortium's growth on 4 gL^{-1} of starch shows the exponential growth phase through the first 48 hours. From 48 hours to 72 hours, the growth does not change, therefore, resulting in the stationary phase. The increase in cell mass concentration produced on 4 gL^{-1} is a substantial increase from the consortium grown on the other concentrations.

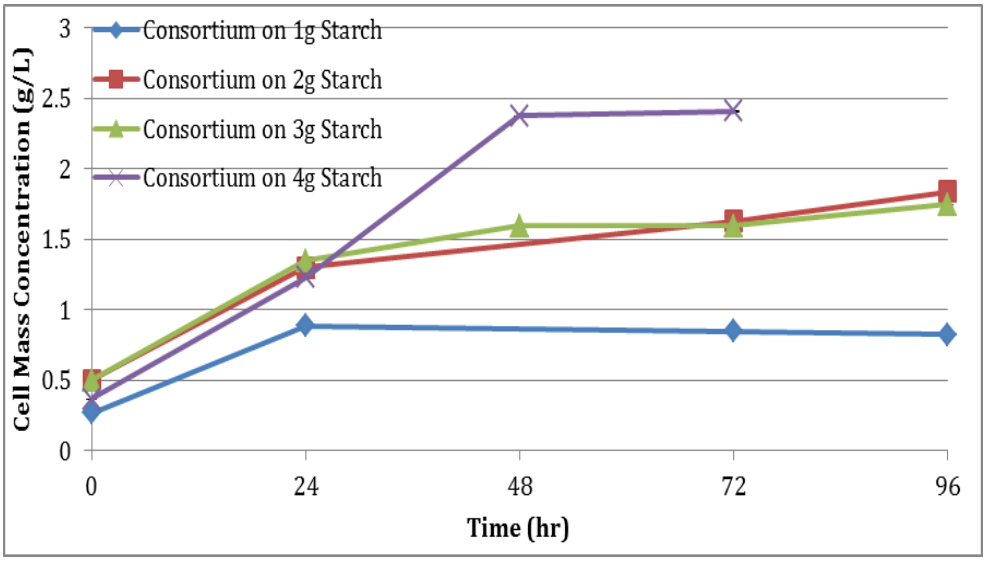

Figure 6.5 Cell mass concentrations of the consortium grown on synthetic wastewater with starch concentrations of 1 to 4 gL^{-1}.

The treatment of municipal wastewater is measured by multiple components. One such component is chemical oxygen demand (COD). In order to determine how well the consortium is treating the wastewater, the COD values are measured for the consortium grown on starch concentrations from 1 to 4 gL^{-1}, shown in Figure 6.6.

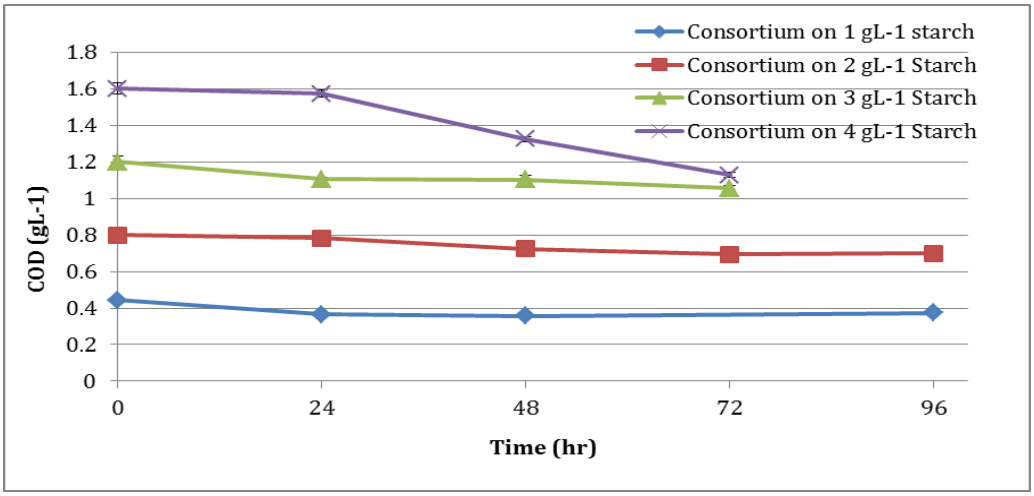

Figure 6.6 Chemical Oxygen Demand (COD) of the consortium grown on synthetic wastewater with starch concentrations of 1 to 4 gL^{-1}.

For the consortium grown on 1 gL^{-1} starch concentration, the reduction is approximately 18.3%. This small reduction in COD corresponds with the cell mass concentration data. The decrease in COD is only for the first 24 hours, which is when the consortium showed growth. When the consortium was grown on 2 gL^{-1} and 3 gL^{-1} of starch, the COD reduction for both concentrations was 14.3% and 13.8%, respectively. The COD results for the 2 gL^{-1} and 3 gL^{-1} of starch concentrations show a steady reduction throughout the 96 hours of cultivation. These COD results show a steady decrease in COD, corresponding to the steady increase in cell mass concentration. The highest reduction is 29.5% for the consortium grown on synthetic wastewater with 4 gL^{-1} of starch. This result also relates to the cell mass concentration in which the highest cell mass concentration was achieved at the same time point as the lowest COD value. However, the amount of starch consumed does not correlate to the large increase in cell mass. This result could be due to the fact that the cell mass samples were not washed prior to drying and weighing, so the cell mass includes starch as well as cell mass and

lipid mass. As the starch concentration increased, the COD reduction also increased. This reduction in COD could have been improved through aeration of the system.

Conclusion

In the investigation of using municipal wastewater as a culture medium, the results show that the oleaginous yeast, *R. glutinis* and *C. curvatus*, can be cultivated on municipal wastewater when the carbon concentration is increased. In addition, the municipal wastewater does not contain growth-inhibiting components, thus the ability to use the wastewater as a culture medium. The pure cultures also showed an increase in lipid production when glucose was added. In addition to the cultures, the consortium could also be grown on the municipal wastewater, especially when sugar is added. The fatty acid profile does not change significantly by adding glucose. Moreover, the consortium when grown on synthetic wastewater shows an increase in cell mass concentration and a decrease in COD. The highest cell mass concentration and highest reduction in COD was shown with the consortium grown on synthetic wastewater with 4 gL^{-1} of starch, proving the need for additional carbon for oleaginous and copiotropic microorganisms.

CHAPTER VII

OLEAGINOUS MICROORGANISM CONSORTIUM GROWN WITH INDIGENOUS MICROORGANISMS PRESENT AND ON OZONATED WASTEWATER

Introduction

Wastewater contains not only nutrients but also a variety of indigenous microorganisms. These indigenous microorganisms can include any bacteria, viruses, fungi, and yeasts (Grady et al., 1999). The current consortium of microorganisms in the return activated sludge that is used in the wastewater treatment process is stable since they are not completely inhibited by the microorganisms indigenous to the influent wastewaters. However, these indigenous microorganisms could have a negative effect on the growth and activity of a consortium of oleaginous microorganisms if introduced into the wastewater treatment process. This chapter focuses on determining the effect of microorganisms indigenous to municipal wastewater influent on the growth of a consortium of oleaginous microorganism.

The first step is to determine effect of the consortium grown on raw wastewater with 1 gL^{-1} of glucose as set forth in Objective 1. Cultivating the consortium on raw wastewater with a small amount of glucose added shows how the consortium microorganisms can compete with the indigenous microorganisms. However, if

pretreatment is required to increase the survivability of the oleaginous consortium, there are two common pre-treatments that could be employed such as chlorination or ozonation. If a pre-treatment is necessary, ozonation would be the most viable because it increases the dissolved oxygen in the wastewater once the ozone decomposes, which can benefit the growth of the aerobic microorganisms in the consortium. The goal of Objective 2 is to determine whether the consortium can be grown on ozonated wastewater. In order to increase the survivability for the consortium, Objective 3 was used to cultivate the consortium on raw wastewater amended with 60 gL^{-1} of glucose. Increasing the amount of glucose added to the wastewater converts the wastewater into a copiotrophic medium, thereby, increasing the coptiotrophic consortium's survivability. If the consortium can compete well with indigenous microorganisms, then no pre-treatment of the wastewater is necessary, thus reducing production costs.

Methodology

Objective 1: Survivability of Oleaginous Microorganism Consortium on Raw Wastewater with 1 gL^{-1} of Glucose (Hall et al., 2011)

Objective 1 included collecting the primary effluent wastewater as described in Chapter IV. The primary effluent wastewater (400 mL) was added to nine one-liter, baffled flasks. Six of the flasks were autoclaved at 121^{o}C for 15 min, and the remaining three flasks were utilized as negative controls. After autoclaving the six flasks were allowed to cool to room temperature. All nine flasks were then kept overnight in the

refrigerator. Before inoculation, glucose was added to each of the nine flasks to yield a concentration of 1 gL^{-1} of glucose to increase the carbon in the wastewater to enhance consortium cell concentration. After the contents of each flask were mixed by swirling each flask ten times, the six flasks that were autoclaved were inoculated with 30 mL of the oleaginous consortium seed. The consortium seed was not washed prior to inoculation since the washing would not be an actual processing step when implemented into a wastewater treatment facility. Three of the six flasks with autoclaved wastewater that was inoculated with only the oleaginous consortium were used as the positive control. The remaining three of six flasks were inoculated with 30 mL of raw wastewater, which served as the treatment to determine the effect of indigenous microorganisms from a municipal wastewater on the growth of a consortium of oleaginous microorganism. Each flask was incubated at 30oC in a shaking incubator (New Brunswick Scientific Model I26, Edison, New Jersey) at 110 rpm for 48 hr.

Samples were collected at 0, 6, 12, 24 and 48 hr in triplicate from the raw wastewater, raw wastewater with the consortium added, and the autoclaved wastewater with the consortium added. Cell mass concentration, COD, glucose concentration, lipid mass concentration, and FAME samples were processed using methods described in Chapter IV. DNA was isolated using the QiaAMP DNA Stool Mini Kit as described by the manufacturer (Qiagen, Valencia, California). The concentration and purity of DNA isolated was determined spectrophotometrically (Nanodrop 1000, Thermo Fisher Scientific, Waltham, Massachusetts).

The primers selected for bacteria detection were p201f/p1370r (Tseng et al., 2003)and primers selected for yeast detection were NS5f/NS6r (White et al., 1990). To

verify that these primers were specific to bacteria and yeast present in the consortium, DNA from *Rhodococcus opacus* and *Rhodotorula glutinis* was isolated and subjected to polymerase chain reaction (PCR). PCR was performed under standard conditions in 50 µl volumes using a MJ Mini Gradient Thermocycler (Bio-Rad Laboratories Inc,. Hercules, California). Products were analyzed on a 0.8% agarose gel (0.5X TAE). Primers were found to be suitable for amplifying bacterial and yeast populations in the consortium (data not shown).

Quantitative Polymerase Chain Reaction (qPCR) amplification was performed in 25µl final volumes containing 5µl of DNA, 1mM of each respective primer, and 12.5µl of SybrGreen Master Mix (Applied Biosystems, Foster City, California). All amplifications were performed in optical-grade 48-well plates on an ABI StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, California) with an initial step at 95°C for 10 min., followed by 40 cycles of 95°C for 15 sec., 60°C for 30 sec., and 72°C for 30 sec. The CT values obtained from associated software were averaged and normalized against standards generated for known bacteria (R. opacus) and yeast (R. glutinis). Standards were performed in 1:2 dilutions, with a starting concentration of 100ng. Curves were obtained by plotting the mean values of C_T over time. All samples were analyzed in triplicate. For the bacteria standard curve, y = (-0.8833x) + 12.527 with R2 = 0.992. For the yeast standard curve, y = (-0.6615x) + 23.598 with R2 = 0.9785. The standard deviation was calculated for each set of samples collected.

Objective 2: Effect of Ozonated Wastewater on the Growth of the Oleaginous Microorganism Consortium

Objective 2 was conducted using primary effluent wastewater (27 L) as described in Chapter IV. For the negative control, 9 L of raw wastewater was mixed in a large bucket, dispensing 800 mL into 9, 1 L baffled flasks. For the positive control, 800 mL was dispensed into 9 flasks and autoclaved as described in Chapter IV. The wastewater for the treatment was prepared by ozonating 9 L. The ozonation process consisted of using 2 mgL^{-1} of ozone for 1 hour per 3 L. After ozonating, 800 mL was dispensed into 9 flasks. Before inoculation, 1 gL^{-1} of glucose was added to 3 raw wastewater flasks, 3 autoclaved wastewater flasks, and 3 ozonated wastewater flasks. Also, 10 gL^{-1} of glucose was added to 3 flasks from each control and treatment. The positive control and the treatment flasks were inoculated with 30 mL of consortium. Cell mass concentration, COD, and FAME samples were processed as described in Chapter IV.

Objective 3: Effect of the Growth of the Oleaginous Microorganism Consortium on Raw Wastewater Amended with 60 gL^{-1} of Glucose

For Objective 3, the primary effluent wastewater was obtained using the same method as described in Chapter IV. Before inoculation, 450mL of the wastewater was dispensed into 3, 1 L, baffled flasks and autoclaved at 121^{o}C for 15 min to be used as the positive control. For the treatment, 450 mL of wastewater was dispensed into 3, 1 L, baffled flasks. The negative control consisted of dispensing 480 mL of wastewater into 3, 1 L, baffled flasks. Each flask contained a stir bar and air dispersing rod in order to

allow agitation as well as aeration throughout the duration of the experiment. The constant agitation and aeration will simulate the growth in a typical wastewater treatment facility.

To increase the carbon concentration in the wastewater, 30 g of glucose was dissolved in 50 mL of distilled water in separate 50 mL Nalgene bottles and autoclaved. The 50 mL glucose mixture was added to the wastewater dispensed into the flasks to achieve an overall concentration of 60 gL^{-1} in the flasks. Once the glucose was added, the positive control and the treatment were inoculated with 30 mL of the developed consortium as described in (Hall et al., 2011). Each flask has an overall liquid working volume of 530 mL. For the duration of the experiment, each flask was placed on a stir plate with a low agitation speed while aerating the flasks with a low pressure of air to keep the cultures fully aerated. With a sterile syringe, 50 mL of a 50% antifoam and water mixture was added to the cultures prior to being aerated to reduce foaming. Samples were collected at 0, 12, 24, 36, 48, 72, 96, 120 hours. Cell mass concentration, lipid mass concentration, FAMEs, pH, and glucose concentrations were processed as described by Chapter IV.

To further determine the effect the indigenous microorganisms have on the consortium, DNA was extracted and quantitative polymeric chain reaction (qPCR) was performed. The samples for this analysis were collected by centrifuging 1mL of the sample at 12,000 rpm for 1.5 min. After centrifuging, each sample was kept at -80^{o}C (Revco -80 freezer). PowerSoil DNA Isolation Kit (MO BIO laboratories, Inc.; Carlsbad, CA) was used to isolate the DNA from the cell mass samples for particular sample points. Then, qPCR was conducted on the extracted DNA using a 7900HT Fast Real-Time PCR

system (Applied Biosystems by Life Technologies Corp; Carlsbad, CA). Four primers were used; two selective primers for *Rhodotorula glutinis* and two primers more broad for yeast. *R. glutinis* is an oleaginous yeast found in a multitude of environments.

Results

Objective 1: Survivability of Oleaginous Microorganism Consortium on Raw Wastewater with 1 gL^{-1} of Glucose (Hall et al., 2011)

The oleaginous microorganism consortium was grown on wastewater supplemented with 1 gL^{-1} of glucose and inoculated with indigenous microorganisms. The growth of the consortium with indigenous microorganisms was compared to the growth of the consortium on autoclaved wastewater (positive control) and raw wastewater (negative control) both supplemented with 1 gL^{-1} of glucose. Figure 7.1 shows the cell mass of the consortium grown on wastewater. The positive control shows an increase in cell mass until 24 hr and then remains steady until the 48-hr point. The consortium grown on autoclaved wastewater inoculated with indigenous microorganisms follows a similar trend to the positive control, except the death phase from the 24 to 48 hr point. The negative control of raw wastewater shows an increase until the 12-hr point during the exponential growth phase when the death phase takes place from 12 to 48 hr points. All three treatments respond very similarly to the treatments through the first 12 hr of incubation, indicating that the oleaginous consortium members and those microbes

indigenous to the wastewater respond similarly to the addition of sugar. Xue et al showed a similar result when comparing the growth of three different yeast, including C. utilis, when cultivated on monosodium glutamate wastewater from the food industry (Xue et al., 2006). The 48 hr sampling is interesting in the fact that the cell concentrations for those samples that contain raw wastewater were similar but different from the pure oleaginous consortium. This indicates that there is a difference in the make-up of the microorganisms found in these samples.

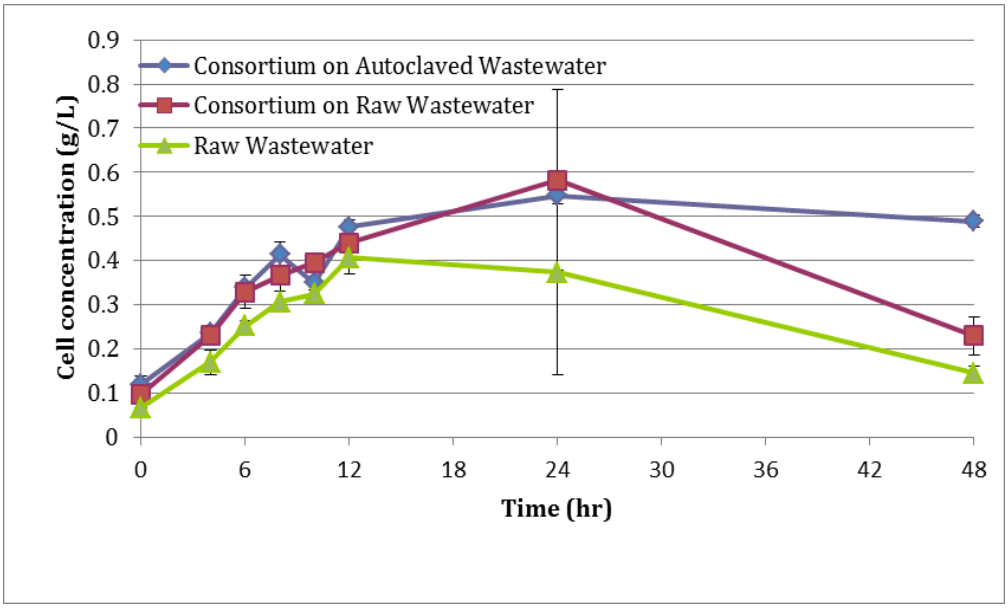

Figure 7.1 Cell mass concentration of the consortium grown on autoclaved wastewater, consortium grown on raw wastewater, and raw wastewater with 1 gL^{-1} of glucose.

In addition to measuring growth, water quality was determined by measuring chemical oxygen demand (COD). Figure 7.2 shows the trend of chemical oxygen demand for the different treatments. The consortium grown on autoclaved wastewater shows a steady decrease until the 24-hr point and then remains steady until the 48-hr

point. The consortium grown on autoclaved wastewater inoculated with indigenous microorganisms follows the same trend as the positive control with a decrease until the 24-hour point. The raw wastewater decreased until the 18-hr point. The difference between the initial COD values for the raw wastewater and the consortium trends is due to the growth of the consortium on a high carbon source medium that is transferred when inoculated, thus resulting in the increase in COD initially at inoculation of the wastewater. The raw wastewater reached a minimum COD in a shorter amount of time because the microorganisms in the raw wastewater are acclimated to the wastewater.

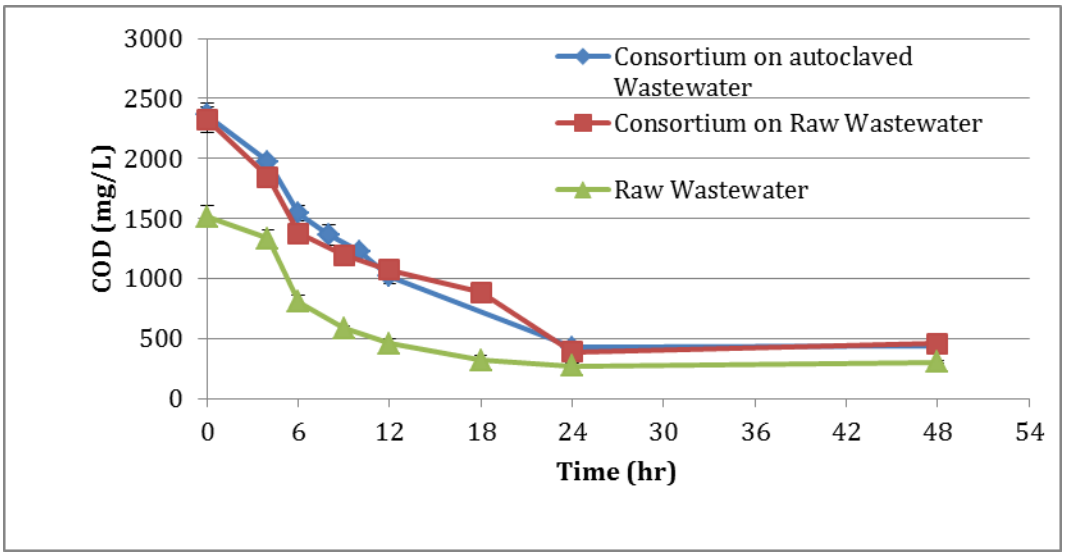

Figure 7.2 COD of the consortium grown on autoclaved wastewater, consortium grown on raw wastewater, and raw wastewater with 1 gL^{-1} of glucose.

The percent reductions in COD's were calculated, resulting in an 81.4% reduction for the positive control, 79.9% for the negative control, and 80.4% for the consortium inoculated with indigenous microorganisms. It should be noted that this reduction observed for all of these samples is most likely the result of the consumption of the

additional sugar that was added to achieve 1 gL^{-1}. The basis for this observation is that the COD of the wastewater used in this experiment was 0.389 gL^{-1} prior to the addition of any exogenous sugar (e.g. 1 gL^{-1} glucose) and the final COD of the wastewater following treatment was approximately the same. These results do demonstrate that the consortium can remove the additional COD and make use of the inorganic nutrients present therein. With a COD of 2.45 gL^{-1}, the cell concentration reached a maximum of 0.6 gL^{-1}. This is a relatively low COD compared to the COD of 40 gL^{-1} for glutamate wastewater from the food industry (Zheng et al., 2005), and 43 gL^{-1} for olive-mill wastewater (D'Annibale et al., 2005). With a high COD, the consortium of oleaginous microorganisms could produce a higher cell mass concentration and accumulate more oil (Xue et al., 2006; Zheng et al., 2005).

Furthermore, the COD removal could be improved through the addition of air sparging. Sparging air into the flasks will increase the available oxygen to these aerobic microorganisms, allowing the metabolizing of all the carbon nutrients in the wastewater. The wastewater treatment facilities utilize an aeration tank, sparging air into the tank, to increase the degradation of the carbon content by the activated sludge (Grady et al., 1999). Hall et al. performed a study testing the oil accumulation of oleaginous microorganism *Candida* 107 with varying aeration conditions (Hall and Ratledge, 1977). As the aeration rate increased from 0.05 to 1.0 vol of air per vol of medium per min, the cell mass concentration and the total lipid increased from 5.3 to 10.5 gL^{-1}, and 0.55 to 2.26 gL^{-1}, respectively (Hall and Ratledge, 1977). The consortium utilization of the COD correlates with the data in the cell mass concentration graph (Fig. 7.1). The COD reached a minimum at the point when the cell mass showed a maximum and either plateaued or

declined with additional cultivation time. The majority of the reductions are seen within the first 24 hr of cultivation, which also provides evidence for the relatively quick growth and utilization of the nutrients in the wastewater.

Since the wastewater was supplemented with glucose, glucose concentrations were measured to determine the extent of consumption. Figure 7.3 shows the consumption of the glucose for the consortium grown on wastewater. The consortium grown on autoclaved wastewater consumes all the glucose within the first 8 hr of cultivation. The oleaginous microbial consortium inoculated with indigenous microorganisms showed similar trends to the positive control in that it consumed all the glucose within 8 hr. The two consortium trends showed a linear decrease in glucose concentration from the time of inoculation until six hours after inoculation. R^2 value of the consortium and the consortium with indigenous microorganisms based on a linear trendline fit was 0.9909 and 0.9962, respectively. However, the raw wastewater showed a slight decrease for the first four hours and then a linear decrease until the glucose was consumed at the 8-hr time point. The raw wastewater also showed a similar decrease in sugar concentrations when compared to the other treatments. This acclimation time for the raw wastewater was also seen in the COD trend results for the first four hours.

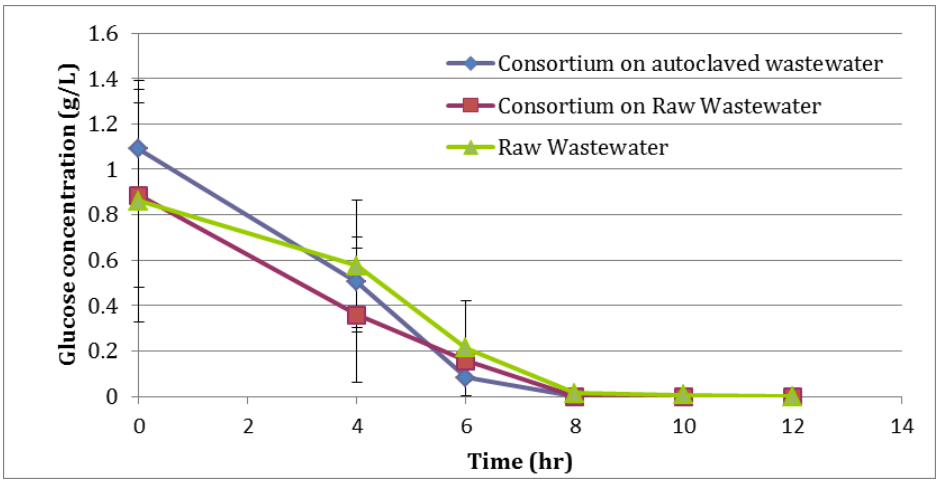

Figure 7.3 Glucose concentrations of the consortium grown on autoclaved wastewater, consortium grown on raw wastewater, and raw wastewater with 1 gL^{-1} of glucose.

Since the additional glucose was consumed within 8 hours and the cell concentration maximum was seen at 12-hr and 24-hr point, the glucose was utilized for cell mass production instead of oil accumulation. In order for oil accumulation to take place, the carbon concentration must be high enough to be in excess when the microorganisms reach stationary phase (Wynn and Ratledge, 2006). When comparing Figure 7.1 and Figure 7.3, the carbon concentration was utilized before the microorganisms could complete their exponential growth phase. These microorganisms ability to utilize glucose quickly could be an asset to the wastewater treatment facilities.

Since there were so many similarities with the treatments, there did not appear to be any substantial proof that the indigenous microorganisms negatively impacted the oleaginous microorganisms. In order to gain a better understanding of the interactions with the oleaginous microorganisms in the consortium with the indigenous microbes of the wastewater, the fatty acid profiles were evaluated. After cultivating these microorganisms for 48 hr, the oil was extracted from the cells using the solvent extraction

method previously described in Materials and Methods section. The percentage of solvent extractables was 10% for the positive control, 21% for the consortium inoculated with indigenous microorganisms, and 30% for the negative control. These solvent extractables include not only triacylglycerides and phospholipids but could also contain other nonsaponifiable lipids such as cholesterols (Lessellier, 2001). In order to quantify the amount that can be converted to biodiesel, these extractables are transesterified to FAMEs.

Table 7.1 compares the various FAMEs from each sample. The positive control is mainly composed of FAMEs in the lower range of the methyl esters with 35% palmitic acid methyl ester. In a study performed by Daniel et al, *C. curvatus* showed a similar distribution of FAMEs when grown on whey wastewater from the dairy industry (Daniel et al., 1999). In addition, Dufreche et al also reported similar FAME distribution for waste activated sludge with mostly composed of palmitic acid methyl ester (Dufreche et al., 2007). The negative control and the consortium inoculated with indigenous microorganisms show an even distribution throughout the range of FAMEs. The consortium inoculated with indigenous microorganisms consists mainly of oleic acid methyl ester with 25% of the total FAMEs. Similarly, the negative control had 21% of the total FAMEs as oleic acid methyl ester. Common FAME distribution for oleaginous microorganisms consists of mostly palmitic acid methyl ester, stearic acid methyl ester, and oleic acid methyl ester. These FAMES were produced from activated sludge shown by Dufreche et al. *R. glutinis* grown on lignocellulosic material from agricultural and forestry residues also resulted in this FAME distribution (Dai et al., 2007). In addition, *L. starkeyi* when cultivated on sewage sludge showed similar FAME distribution

(Angerbauer et al., 2008). Comparison of the percentages indicates that the consortium inoculated with indigenous microorganisms is in between the two controls, potentially denoting the combination of activity among the oleaginous microorganism consortium and the indigenous microorganisms. This similarity in FAME distribution supports the possibility that the indigenous microorganisms effect the lipid production in the microorganisms in the consortium. Also, only the positive control showed any Heptadecenoic acid methyl ester with a 12% of the total FAMEs. This particular fatty acid has been shown to be produced by R. opacus, which was one of the oleaginous microorganisms initially added to the consortium (Waltermann et al., 2000). Since it is only shown in the positive control, this denotes that the R. opacus or another oleaginous microorganism that produces that FAME could not compete with the indigenous microorganisms for 48 hr cultivation period.

Table 7.1 Fatty Acid Methyl Ester (FAME) percentages and totals for consortium grown on primary effluent wastewater with 1 gL^{-1} of glucose compared to the FAME produced by *R. opacus*.

FAME	Consortium Autoclaved	Consortium + Raw	Raw Wastewater	*R. opacus* (Mol%) (Waltermann et al., 2000)
Myristic AME (%) C14:0	1.65	3.23	2.18	5.2
Palmitic AME (%) C16:0	35.39	16.55	14.71	25.9
Palmitoleic AME (%) C16:1	19.51	10.80	11.14	9.5
Heptadecenoic AME (%) C17:1	12.25	------	-------	15.4
Stearic AME (%) C18:0	2.45	5.91	9.57	3.1
Oleic AME (%) C18:1	18.56	25.02	21.69	22.0
Linoleic AME (%) C18:2	3.07	13.95	8.56	Not Reported
Linolenic AME (%) C18:3	2.02	3.92	4.54	Not Reported
Arachidic AME (%) C20:0	1.19	5.95	6.05	Not Reported
Behenic AME (%) C22:0	1.94	6.30	10.41	Not Reported
Erucic AME (%) C22:1	0.76	3.93	5.60	Not Reported
Lignoceric AME (%) C24:0	1.21	4.44	6.03	Not Reported
Total (mgL^{-1})	465.52	122.17	106.40	
Solvent Extractables (%)	9.77	20.57	30.10	

The positive control showed the lowest percentage of solvent extractables but the highest total FAMEs. This result shows that most of the lipids extracted could be converted to FAMEs, while the raw wastewater had the highest percentage of solvent extractables and the lowest total FAMEs, meaning that only a portion of the lipids could be converted to FAMEs. The total FAME concentrations were 466 mgL^{-1} for the positive control, 122 mgL^{-1} for the consortium with indigenous microorganisms, and 106mgL^{-1} for the raw wastewater. The similarities in the FAME concentrations for the raw wastewater and the consortium with the indigenous microorganisms suggest that the members of the oleaginous consortium were not able to successfully compete for food

and nutrients. The consortium alone produces four times the amount of FAMEs than the raw wastewater or the wastewater with the consortium and the indigenous microorganisms with the experimental conditions. Using a statistical t-test with a 5% significance level, the total FAME ratio for the consortium with indigenous microorganisms is not significantly different when compared to the total FAME ratio for the raw wastewater. However, for oil accumulation to occur, the carbon to nitrogen ratio should be above 20:1 (Ratledge, 2005b). This experiment used a carbon to nitrogen ratio of 1:1. Papanikolaou et al adjusted the carbon to nitrogen ratio from 150:1 to 340:1 of the medium to increase lipid production from 8.2 gL^{-1} at 150:1 to 18.1 gL^{-1} at 340:1 when cultivating *Mortierella isabellina* on various glucose concentrations (Papankikolaou et al., 2004). Therefore, by increasing the carbon to nitrogen ratio, the amount of oil produced should increase and potentially favor oleaginous microorganisms over those that are non-oleaginous and indigenous to the influent wastewater.

Table 7.2 shows the net specific growth rate as well as the doubling times for each sample. The doubling time for the negative control was approximately 4.65 hr, which was expected to be faster due to the fact that the microbes in wastewater were acclimated to the composition of the wastewater. The positive control also showed a relatively quick doubling time of approximately 4.93 hr. The slowest doubling time of 5.88 hr occurred with the consortium inoculated with indigenous microorganisms. This slow doubling time is believed to be the result of competition for nutrients among the bacteria from the consortium and in the raw wastewater. However, the doubling time of the consortium inoculated with indigenous microorganisms is not significantly different when compared to the doubling time of the raw wastewater based on the t-test with a 5% significance

level. The doubling times of the controls are not significantly different from each other, supporting the qPCR results that the population of each treatment is predominantly bacteria.

Table 7.2 The consortium doubling time with 1 gL^{-1} of glucose

Doubling Time			
Sample	**u_{net}**	**R^2**	**t_d (hr)**
Consortium Autoclaved	0.1406	0.9742	4.93
Consortium Raw	0.1178	0.9119	5.884
Raw Wastewater	0.1491	0.965	4.649

The doubling rate provides residence time predictions when sizing the aeration tank. The raw wastewater and the consortium both have doubling times faster than the consortium with indigenous microorganisms. This would require a longer residence time for the consortium with indigenous microorganisms as compared to the other samples.

The level of glucose tested in this study was relatively low compared to the levels typically used in pure culture investigations with oleaginous microorganisms (Shuler and Kargi, 2002). The survivability of these oleaginous microorganisms could be enhanced via the supplementation of additional sugars that are higher than 20 gL^{-1}.

In addition to the doubling time, Monod constants were determined to compare the growth of the consortium on raw wastewater to the negative and positive controls. Table 7.3 shows the Monod constants for each treatment. The maximum specific growth rate, μ_{max}, shows an increase from 0.18 to 0.6 hr^{-1} for the consortium grown on raw wastewater when compared to the positive control, showing a quick growth rate. When

compared to the negative control, the consortium grown on raw wastewater shows a decrease in the maximum specific growth rate, resulting in the quick biomass production by the microorganisms indigenous to wastewater. Since the maximum specific growth rate is in between the two controls, the consortium and the indigenous microorganisms could potentially form a symbiosis, supporting the cell mass production growth and the total FAMEs produced. The Monod constant, K_S, shows similar results in that the consortium on raw wastewater is in between the two controls. This constant measures how well the microorganisms utilize the substrate, thus showing that the consortium on autoclaved wastewater utilizes the glucose quickly. The K_S values support the results shown in the glucose consumption (Figure 7.3).

Table 7.3 Monod constants for the consortium grown on autoclaved and raw wastewater compared to raw wastewater with 1 gL^{-1} of glucose.

Monod Constants			
Constants	Consortium on Autoclaved	Raw Wastewater	Consortium on Raw wastewater
μ_{max} (hr^{-1})	0.18	0.80	0.60
K_S (gL^{-1})	0.14	1.94	0.95
R^2	0.82	0.87	0.94

Prior to extracting the oils, an aliquot was removed from each sample to enumerate the microorganisms in the sample via Real-Time quantitative polymerase chain reaction (qPCR). Figure 7.4 shows the graphs of each treatment comparing bacteria and yeast populations over time for each treatment. The C_T values represent the inverse of the populations; the lower the C_T values, the higher the relative concentrations

of the populations. Figure 4A shows the bacteria and yeast population for raw wastewater over a period of 48 hr. The microbial population before the sugars were added was undetectable. When the 1 gL^{-1} of glucose was added at the zero hour, the bacteria population showed an increase until the 24-hr time point. The maximum bacteria population was seen at the 24-hr time point, which is also when the maximum cell concentration occurs. The yeast population remains constant around a C_T value of 32. This increase in bacteria population shows that the indigenous microorganism population in raw wastewater is predominantly bacteria. Bacteria have an average doubling time between 45 min to 1 hr, and yeast have a doubling time of approximately 1.5 to 2 hr (Fogler, 2006). This drastic increase in bacteria population compared to the raw wastewater shows that the consortium is surviving in the presence of indigenous microorganisms.

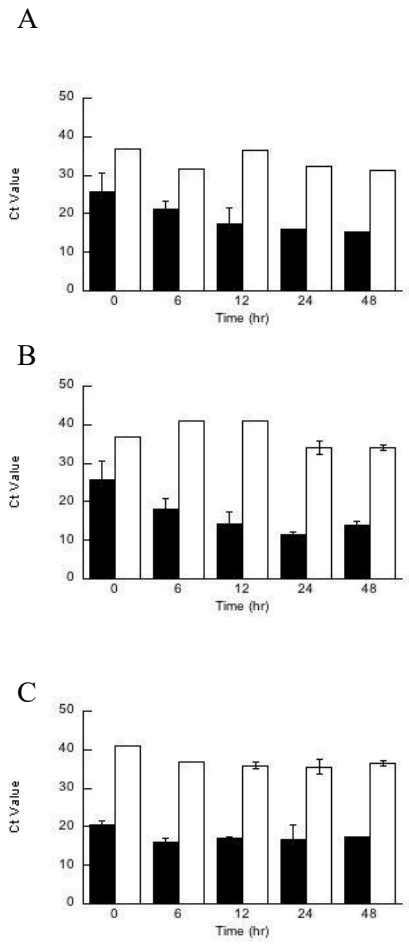

Figure 7.4 Relative amounts of bacterial (black bar) and yeast (white bar) species present in wastewater with 1 gL^{-1} of glucose. A) C_T values of bacteria and yeast are plotted from raw wastewater. B) C_T values of bacteria and yeast are plotted from raw wastewater with consortium added. C) C_T values of bacteria and yeast are plotted from autoclaved raw wastewater with consortium added. Error bars represent standard deviation.

Figure 7.4B shows the bacteria and yeast populations in the raw wastewater inoculated with the oleaginous microorganism consortium. Initially, the consortium population showed a C_T value of 7.24 for bacteria and 30.87 for yeast. From this initial analysis of the species, the consortium is composed of approximately 80% bacteria and 20% yeast. This graph shows a larger increase in bacteria population than in the raw

wastewater, which is due to the introduction of bacteria contained in the oleaginous consortium or the introduction of micronutrients also contained in the aqueous phase of oleaginous inoculum. Also, the maximum bacterial population was shown at the same point that the cell mass concentration was a maximum. The yeast population shown is inconclusive since the C_T values are so high. This population shift shows the bacteria out-competing the yeast for nutrients. Bacteria essentially metabolize and multiply faster than yeast. Which is the expected result given the doubling time of bacteria is on average significantly faster than other microorganisms such as yeast and fungi.

Figure 7.4C shows the bacteria and yeast populations for the consortium grown on autoclaved raw wastewater. These data do not show a population shift with the bacteria and the yeast population remaining constant throughout the 48 hours, denoting a negligible microbial population shift. Autoclaving the wastewater removed the indigenous microorganisms prior to the introduction of the consortium. This figure shows that bacteria and potentially yeast can be maintained without the indigenous microbes, yet are more efficient with this community present. When compared to the cell mass concentration (Fig. 1), the ordinary growth curve supports the lack of population change throughout the cultivation period.

The microorganisms indigenous to the influent wastewater had a negative impact on some of the members of the oleaginous consortium when low concentrations of glucose were utilized. The data show that *R. opacus* of the oleaginous consortium is out competed for nutrients by those microorganisms present in the wastewater. The FAME analysis and qPCR population enumeration support the observations on the impact of the indigenous microorganisms on the oleaginous consortium. The fact that the oleaginous

consortium was capable of growing and producing more transesterifiable lipids than the indigenous microbes is encouraging. However the data clearly demonstrates the need to either reduce the number of indigenous microorganisms or modify the conditions (e.g. sugar supplementation) to increase the survivability of the oleaginous microorganisms.

With 1 gL^{-1} of glucose added, the consortium with indigenous microorganisms showed a 13% increase in FAMEs produced. Although not statistically significant it does suggest that with supplemental sugars and/or oleaginous microorganisms, additional increases in lipids could be achieved. By increasing the amount of sugar added to a known optimum carbon to nitrogen ratio of 40:1, the amount of oil accumulated could also increase (Ratledge, 2005b).

Objective 2: Effect of Ozonated Wastewater on the Growth of the Oleaginous Microorganism Consortium

Even though the consortium grown on raw wastewater showed positive results, an experiment was conducted to determine the effect of a common pre-treatment, ozone, on the growth of the consortium. Figure 7.5 illustrates the change over time in the cell mass concentration of the consortium grown on autoclaved and ozonated wastewater as well as raw wastewater with 1 gL^{-1} of glucose added. This figure shows that the consortium on the ozonated wastewater resulted in the largest cell mass concentration at 72 hours of cultivation when compared to the positive and negative controls. The consortium on the ozonated wastewater also demonstrated a steady increase from inoculation at 0 hour to 72 hours. From 72 hour to 96 hours, the cell mass concentration shows a large decrease.

The growth of the consortium on the autoclaved wastewater shows a steady increase until 72 hours with a slight decrease to 96 hours. The raw wastewater cell mass concentration shows a similar trend to the consortium on ozonated wastewater, except the treatment shows a higher increase. This result shows that the consortium can be cultivated on ozonated wastewater as well as show an increase in overall cell mass production.

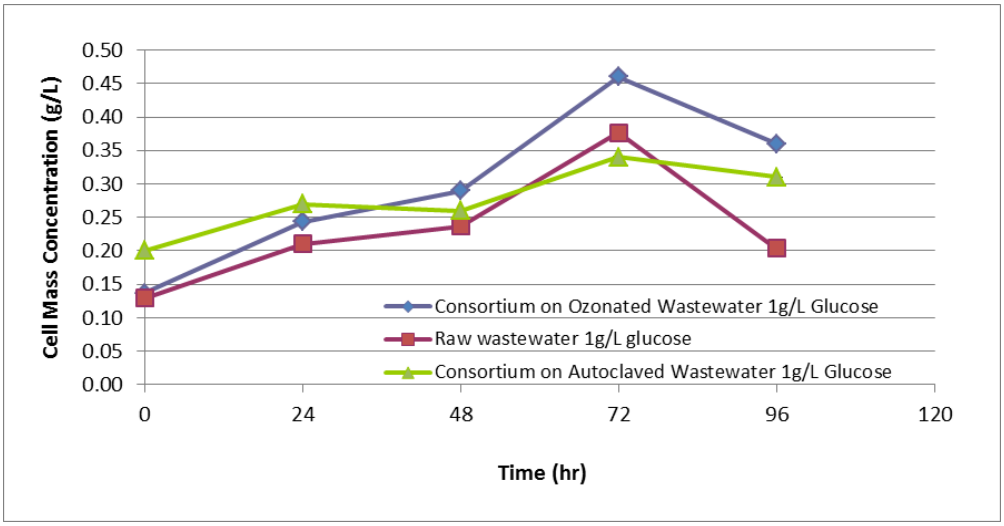

Figure 7.5 Cell mass concentration of the consortium grown on autoclaved wastewater and ozonated wastewater with 1 gL^{-1} of glucose compared to raw wastewater with 1 gL^{-1} of glucose.

When cultivating the consortium on ozonated wastewater with 10 gL^{-1} of glucose, the results are presented in Figure 7.6. By increasing the glucose concentration, the treatment results show an increase from 0.45 to 0.6 gL^{-1} in cell mass concentration. The treatment shows a lag phase for the first 24 hours, exponential growth phase from 24 hours to 48 hours, and death phase from 48 hours to 96 hours. The positive control of the autoclaved wastewater shows the same trend as the treatment except not as large as the treatment. The negative control shows an initial increase from 0 to 24 hours, stationary

phase from 24 to 72 hours, and a death phase from 72 to 96 hours. This trend is very different from the consortium's growth on autoclaved and ozonated wastewater.

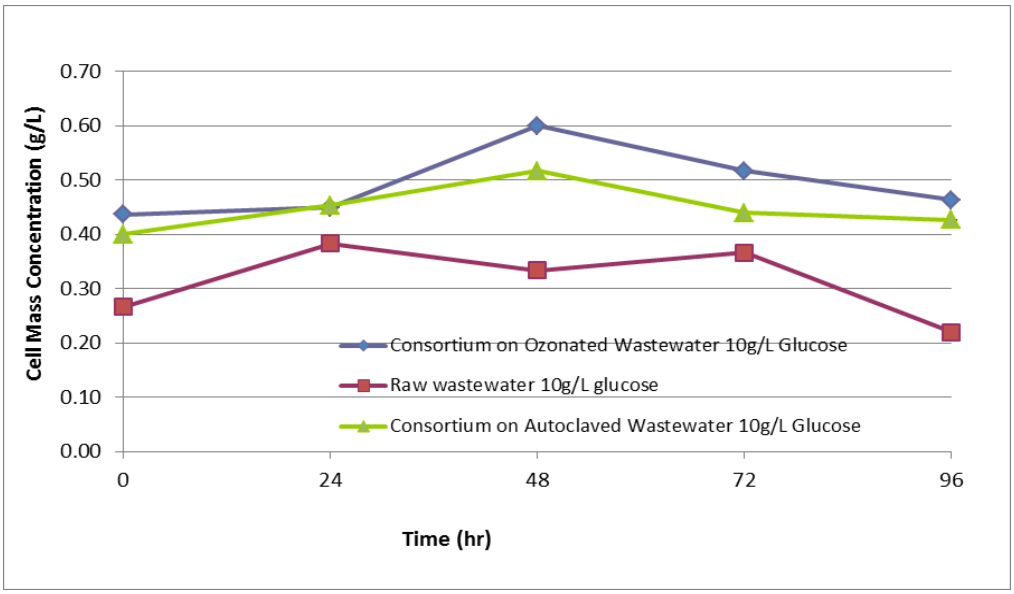

Figure 7.6 Cell mass concentration of the consortium grown on autoclaved wastewater and ozonated wastewater with 10 gL^{-1} of glucose compared to raw wastewater with 10 gL^{-1} of glucose.

In addition to the cell mass concentrations, chemical oxygen demand (COD) is also measured to determine how well the microorganisms utilize the nutrients and treat the wastewater. Figure 7.7 shows the COD results for the consortium grown on autoclaved and ozonated wastewater as well as raw wastewater with 1 gL^{-1} of glucose. Both the positive control and the treatment show a steady decrease throughout 72 hours, which corresponds to the steady increase seen in the cell mass concentrations (Figure 7.5). The negative control shows the maximum reduction shown within the first 48 hours and remaining constant until the 96 hours since the indigenous microorganisms are already acclimated to the wastewater and can efficiently utilize these nutrients.

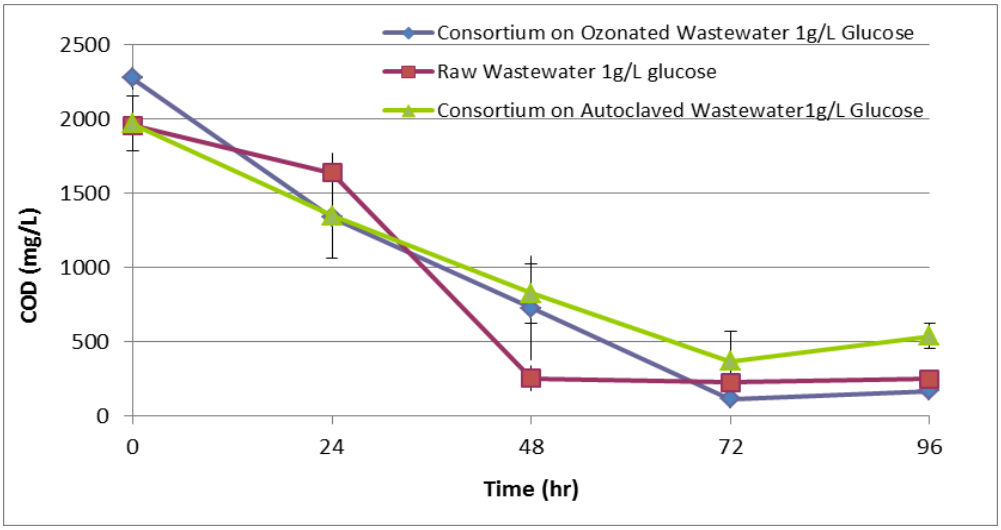

Figure 7.7 COD of the consortium grown on autoclaved wastewater and ozonated wastewater with 1 gL^{-1} of glucose compared to raw wastewater with 1 gL^{-1} of glucose.

In contrast to the COD results shown with the growth on 1 gL^{-1}, the COD reduction on the growth with 10 gL^{-1} of glucose is shown in Figure 7.8. This figure shows the consortium grown on autoclaved wastewater and the raw wastewater trends are the same. They both decrease for the first 48 hours. After 48 hours, they increase to a maximum, which could be due to the death of the cells as shown in the cell mass concentration data (Figure 7.5). The positive control shows a constant decrease within the first 48 hours, with a slight increase to 72 hours where it remains constant. The COD results show that the microorganisms do not reduce the COD as well when cultivated with 10 gL^{-1} of glucose added as well as when only 1 gL^{-1} of glucose was added. This result could possibly be due to the limited amount of oxygen since these samples were not aerated throughout the experiment.

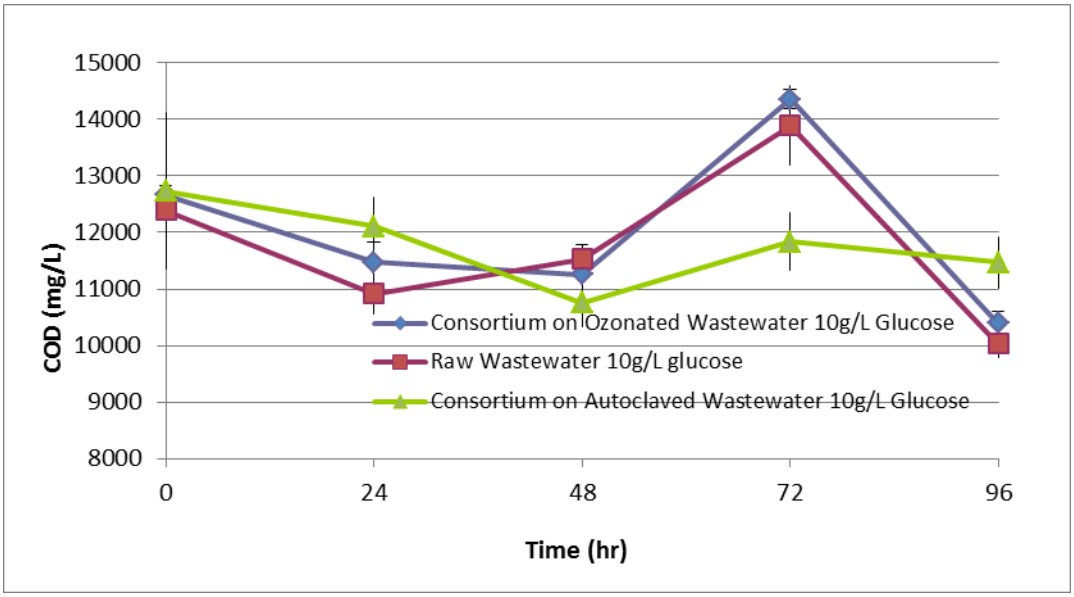

Figure 7.8 COD of the consortium grown on autoclaved wastewater and ozonated wastewater with 10 gL^{-1} of glucose compared to raw wastewater with 10 gL^{-1} of glucose.

Furthermore, the fatty acid profiles were also measured. Table 7.4 shows the fatty acid profiles comparing the growth of the consortium on autoclaved and ozonated wastewater as well as raw wastewater with 1 gL^{-1} and 10 gL^{-1} of glucose. The fatty acid profiles for the consortium grown on autoclaved wastewater show a high percentage of palmitic, palmitoleic, and stearic acid for the 1 gL^{-1} of glucose. When cultivated with 10 gL^{-1} of glucose, the fatty acid profile is similar to 1 gL^{-1} of glucose but an increase in oleic acid and a decrease in palmitoleic acid. These fatty acids are commonly found in oleaginous microorganisms. The treatment fatty acid profile shows high percentages of palmitic, palmitoleic, and oleic acids. These fatty acid percentages do not show a large change when increasing glucose concentrations from 1 gL^{-1} to 10 gL^{-1}. The main difference includes decreases in linoleic acid and palmitoleic acid, increases in myristic acid, palmitic acid, and stearic acid. The raw wastewater profiles show a wider range of

fatty acids than the positive control or treatment with the majority consisting of palmitic, palmitoleic, stearic, and oleic acids. Increasing the glucose concentration, the myrisitic, linoleic, and linolenic acid increased while the palmitic, stearic, and oleic acids decrease. The consortium grown on the autoclaved wastewater and ozonated wastewater show no major shift in fatty acid profile and are very similar in fatty acid compositions. The treatment fatty acid profiles when compared to raw wastewater are not similar.

Table 7.4 Fatty acid profile comparing the consortium grown on autoclaved and ozonated wastewater to raw wastewater with 1 gL^{-1} and 10 gL^{-1} of glucose.

Fatty Acid Profile	Consortium on Autoclaved with 1g/L glucose	Consortium on Autoclaved with 10g/L glucose	Consortium on Ozonated with 1g/L glucose	Consortium on Ozonated with 10g/L glucose	Raw Wastewater with 1g/L glucose	Raw Wastewater with 10g/L glucose
Lauric ACM (C12:0) %	0	0	0	0.05	0	0.02
Myristic ACM (C14:0) %	0.35	0	1.59	4.05	2.84	17.68
Palmitic ACM (C16:0) %	44.11	61.96	33.49	45.17	36.46	19.13
Palmitoleic ACM (C16:1) %	19.18	4.29	20.11	13.00	11.60	12.82
Stearic ACM (C18:0) %	27.57	29.47	8.30	12.12	21.43	14.42
Oleic ACM (C18:1) %	0.00	4.27	30.16	22.56	16.32	10.16
Linoleic ACM (C18:2) %	0.60	0	3.17	0.47	8.59	22.32
Linolenic ACM (C18:3) %	0	0	3.18	2.58	1.71	2.86
Arachidic ACM (C20:0) %	0	0	0	0	0.14	0.36
Lignoceric ACM (C24:0) %	0	0	0	0	0.90	0.22

Objective 3: Effect of the Growth of the Oleaginous Microorganism Consortium on Raw Wastewater Amended with 60 gL^{-1} of Glucose

When 1 gL^{-1} of glucose was added, the indigenous microorganisms did show a negative impact on the microbial populations of the consortium. To increase the chances of the consortium thriving and accumulating oil in raw wastewater, it was hypothesized that oleaginous consortium could compete with the indigenous microbial community if the concentration of sugar was significantly increased. A study conducted by Modala et al showed that oleaginous microorganisms contained in the return activated sludge began to proliferate when the sugar concentrations were raised to 60 gL^{-1} (Mondala, 2010). Therefore this experiment focuses on the growth of the consortium on raw wastewater amended with 60 gL^{-1} of glucose to increase the survivability of the members of the oleaginous consortium.

The results of this research showed that the consortium with indigenous microorganisms improved overall cell mass concentration compared to the positive control and negative control. Figure 7.9 shows the cell mass production throughout the 120 hr cultivation period. The first 24 hr for both the treatments and the controls shows the lag phase or acclimation period where the microorganisms typically develop the enzymes to utilize the nutrients in the wastewater while competing with the indigenous microorganisms. The exponential growth phase for the treatment is from the 24-hr point to the 72-hr point. At 72 hr, stationary phase is apparent until the end of the experiment. The positive control shows a steady increase in cell mass concentration from 24 hours until the completion of the experiment. The negative control also shows a similar result to the positive control. The maximum cell mass concentration of the treatment is 5.3gL^{-1},

whereas the maximum for the positive control is 1.7gL^{-1} and for the negative control is 0.96gL^{-1}. The cell mass produced by the consortium and indigenous microorganisms is approximately five times the amount that is produced by either of the controls. Thus, the combination of the consortium microorganisms with the indigenous microorganisms results in a significant amount of cell mass concentration produced.

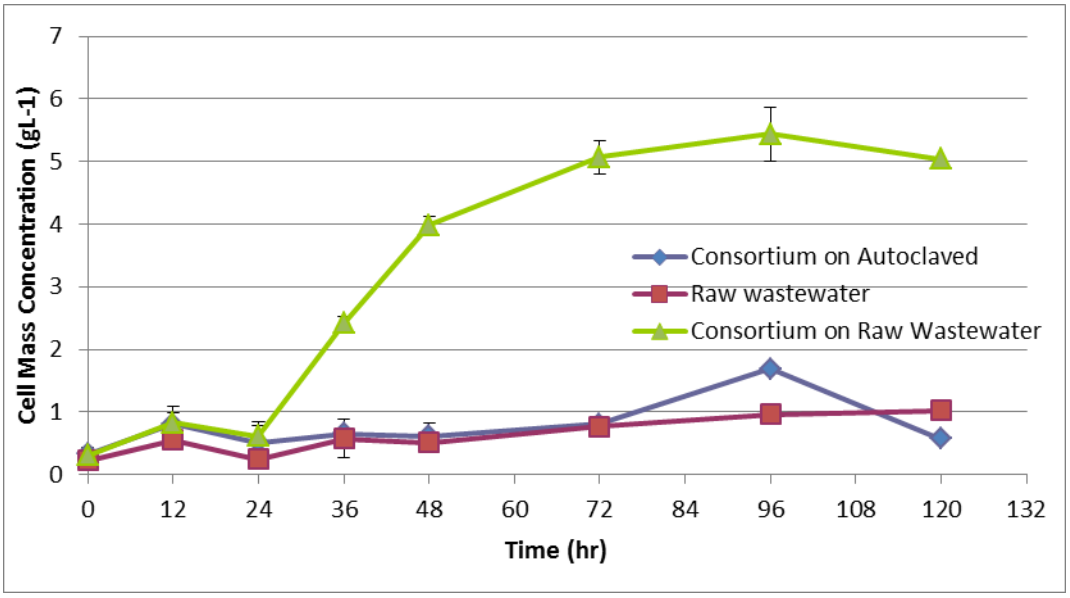

Figure 7.9 Cell mass production of the consortium on raw wastewater, consortium on autoclaved wastewater, and raw wastewater with 60 gL^{-1} of glucose.

In addition to cell mass concentration, glucose concentration was also measured throughout the experiment to determine if the indigenous microorganisms have an effect on the consortium's ability to consume glucose. Figure 7.10 shows the consumption of the supplemented glucose in the varying treatments. The sugar consumption shows that the consortium with indigenous microorganisms consumes the additional 60 gL^{-1} of glucose within 72 hours of cultivation. This consumption of glucose supports the

significant amount of cell mass produced (Figure 7.9). The positive control shows a steady decrease until the 36-hr point where it shows a slowing of glucose being consumed. From 36 hours to 120 hours, the positive control shows an increased consumption rate. The positive control also showed 33 gL^{-1} of glucose remaining in the wastewater at the end of the experiment. The negative control shows a similar trend to the positive control for the first 36 hours. After 36 hours, the negative control shows a slower decrease in glucose concentrations until the 96 hours, where it remains constant at 20 gL^{-1} at 120 hours. This steady decrease supports the slight increase in cell mass concentration (Figure 7.9).

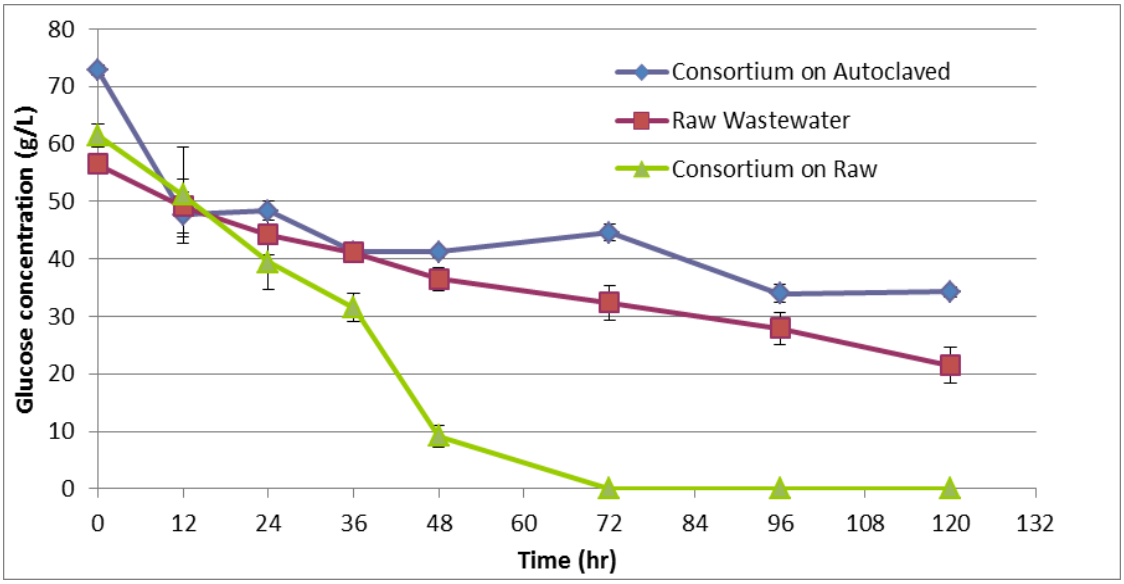

Figure 7.10 Glucose concentration of the consortium on raw wastewater, consortium on autoclaved wastewater, and raw wastewater with 60 gL^{-1} of glucose.

In order to determine the effect indigenous microorganisms have on the consortium's ability to accumulate oil, lipid mass concentrations were measured. Figure 7.11 shows the production of lipids over time for each treatment. The consortium with

indigenous microorganisms shows the largest amount of lipid production of 0.25gL^{-1} after only 36 hours of cultivation. The amount of lipid remained steady from 12 hr to 120hr. This result shows that the microorganisms are not using the metabolic pathway for lipid production but instead are using the pathway for replication/cell mass production, supporting the cell mass concentration (Figure 7.9). This result could be due to the low pH since the samples were not buffered. The positive control shows lipid production within the first 12 hours, then showing a steady decrease until 96 hr. The maximum lipid produced by the positive control is 0.15 gL^{-1}. The negative control shows a sharp increase from 0 hour to 12 hour. At the 12 hour, it shows a constant decrease until 72 hour. The negative control reaches a maximum of 0.14 gL^{-1} at the 12-hour point. The negative control then shows a stationary lipid production from 72-hr to 96-hr point. The varying trends show that the combination of the microorganisms in the consortium and in the raw wastewater produces the largest amount of lipids within the first 12 hours.

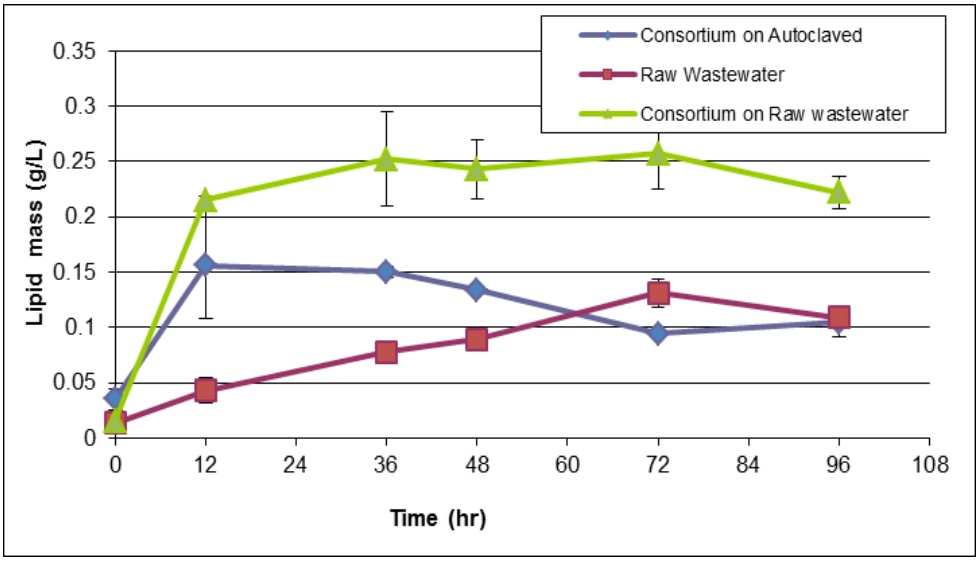

Figure 7.11 Lipid concentration of the consortium on raw wastewater, consortium on autoclaved wastewater, and raw wastewater with 60 gL^{-1} of glucose.

When comparing the treatment to the negative control, the trends are similar in the fact that they produce a large amount of lipid within the first 12 hours of cultivation. This result could potentially show that the indigenous microorganisms outcompete some of the microorganisms within the consortium. However, since the consortium on raw wastewater shows a larger increase in cell mass production and lipid production than the negative control, the microorganisms are obviously forming a symbiosis relationship within the first 12 hours to promote growth, glucose consumption, and lipid production.

The percentage of lipids on a dry mass basis in this experiment is shown in Figure 7.12. For the consortium grown on raw wastewater, the percentage of lipids shows a high peak at 20% at 12 hours of cultivation and then decreasing to 5%. The lipid percentage remained at 5% from 48 hours to 120 hours. This result is consistent with the cell mass data and the lipid mass data, where the percentage does not change from 48 hours to 120 hours. The low percentage shows that the microorganisms produced more biomass than

lipids from 48 hours to 120 hours, which is consistent with quick growing microorganisms. The positive control shows a steady increase to 20% for the first 36 hours and then decreasing to 10% from 36 hours to 96 hours. The negative control shows a steady increase to 17% within 48 hours and a slight decrease to 10% at 120 hours. The control results for lipid percentage support the cell mass growth and the lipid mass growth.

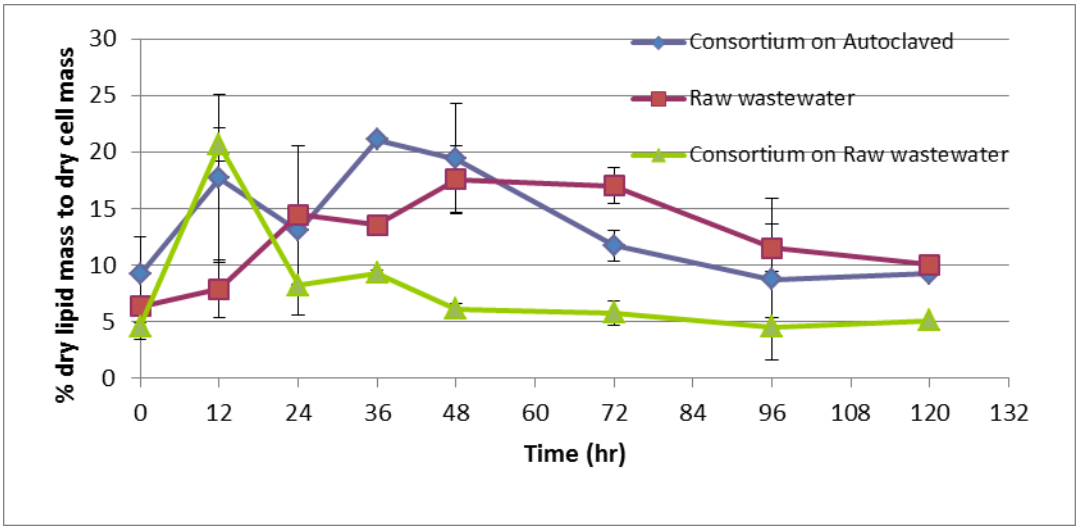

Figure 7.12 Percentage of lipids over time on a dry mass basis with 60 gL^{-1} of glucose.

After lipid was extracted from the cell mass, the lipids were transesterified into FAMEs. Figure 7.13 shows the total FAMEs produced by each treatment over the duration of the experiment. The total FAMEs for the consortium grown on raw wastewater shows a steady increase from 0.17 gL^{-1} at 0 hr to 1.8 gL^{-1} at 120 hr. The amount produced for the treatment is six times larger than the total FAMEs produced compared to both controls. The combination of consortium microorganisms and

indigenous microorganisms produced the largest amount of total FAMEs when compared to the controls, which supports the lipid mass concentration data (Figure 7.11). The positive control shows a steady increase until the 48 hr but decreased at 120 hr. The negative control showed a steady increase until 120 hr where it produced 1gL^{-1}.

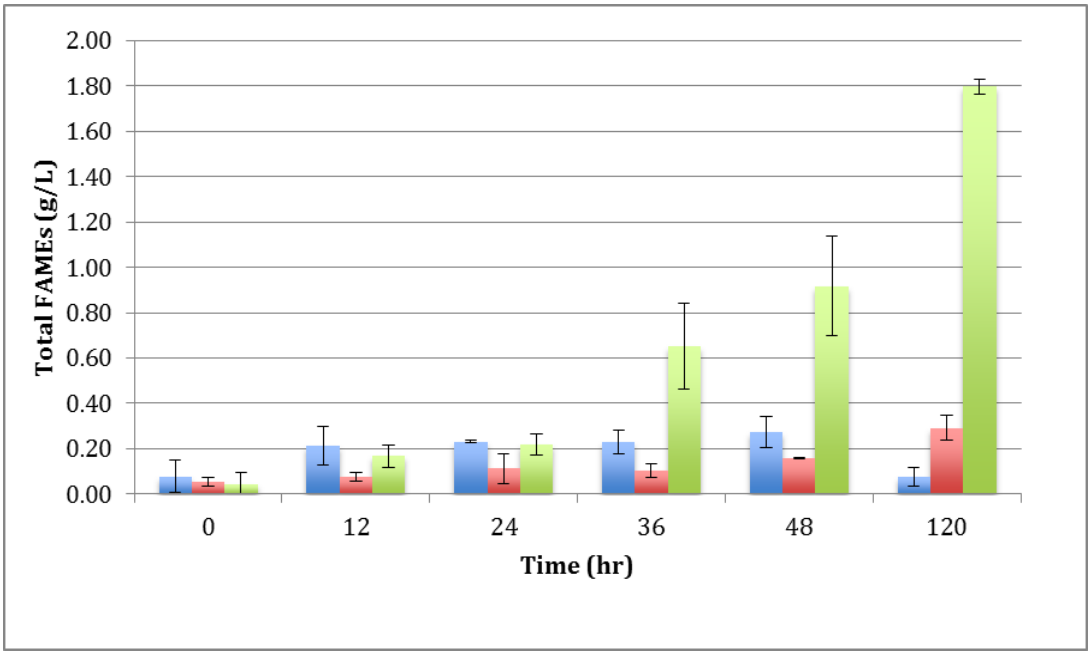

Figure 7.13 Total FAMEs from the consortium on raw wastewater (green), consortium on autoclaved wastewater (blue), and raw wastewater (red) with 60 gL^{-1} of glucose.

Along with the total FAMEs, the fatty acid profiles for the controls and the treatment were measured. Table 7.5 shows the fatty acid profile results for the consortium grown on autoclaved and raw wastewater compared to raw wastewater. The profile from the consortium grown on raw wastewater shows the majority consisting of palmitic, oleic, and linoleic acid. The positive control profile includes high percentages of palmitic, heptadecanoic, and oleic acid. The negative control shows a majority of

palmitic, stearic, oleic, and linoleic acid. Each sample showed a large percentage of palmitic and oleic acid, which is a fatty acid commonly found in oleaginous microorganisms. The palmitoleic, stearic, and linolenic acids are similar values for both the consortium on raw wastewater and on autoclaved wastewater, thus potentially showing survivability of the microorganisms in the consortium. In addition, each sample also showed heptadecanoic acid, which can be related to a common oleaginous bacterium, R. opacus. Overall, these profiles show that the raw wastewater potentially contains oleaginous microorganisms since the fatty acids found in the raw wastewater are similar to oleaginous microorganisms.

Table 7.5 Fatty acid profile of the consortium grown on autoclaved and raw wastewater as well as raw wastewater with 60 gL^{-1} of glucose after 120 hours of cultivation

FAME	Consortium on Autoclaved	Raw Wastewater	Consortium on Raw Wastewater
Octanoic AME %	6.08	2.53	0.15
Decanoic AME %	2.08	0.46	0.03
Lauric AME %	0.24	0.16	0.02
Myristic AME %	3.94	1.07	0.70
Pamitic AME %	37.73	24.37	20.51
Palmitoleic AME %	9.79	5.07	9.07
Heptadecanoic AME %	10.22	3.10	2.63
Stearic AME %	6.70	24.98	7.20
Oleic AME %	14.30	20.89	39.72
Linoleic AME %	2.08	14.02	16.54
Linolenic AME %	3.39	1.77	3.11
Arachidic AME %	1.24	0.78	0.22
Behenic AME %	0.14	0.15	0.04
Erucic AME %	0.71	0.20	0.06
Lignoceric AME %	1.36	0.45	0.00
Total (gL^{-1})	0.30	1.16	7.19

When comparing the total FAMEs produced after 120 hours of cultivation, the highest amount was produced from the consortium grown on raw wastewater and the lowest amount shown with the consortium grown on autoclaved wastewater. For raw wastewater, the total FAMEs produced is approximately 1.16 gL^{-1}. By adding the consortium to the wastewater, the amount of FAMEs increased by a factor of seven. This result shows that the microorganisms in both the raw wastewater and the consortium work together to utilize the nutrients as well as produce oil. The FAME results support the results for the cell mass concentrations (Figure 7.9), lipid concentrations (Figure 7.11), and glucose consumption (Figure 7.10).

In addition to the fatty acid profiles, DNA isolations and qPCR were conducted to further identify the change in microbial population of the consortium when grown on raw wastewater. Figure 7.14 shows the qPCR results used to quantify the shifts in microbial population. The primers were used to identify *R. glutinis* and other yeast with less specificity. These results show that the yeast population for the consortium with indigenous microorganisms and for the raw wastewater steadily increased through the first 24 hours, which corresponds to the lag phase in Figure 7.11. After 48 hours, the microbial population stabilizes, showing a large amount of yeast in the consortium with indigenous microorganisms and raw wastewater. The amount of yeast in the consortium with indigenous microorganisms is very similar to the amount of yeast in the raw wastewater, thus showing that the raw wastewater indigenous microorganisms could have dominated over the consortium microorganisms. The positive control does show the same amount of yeast at the beginning of the experiment as the treatment and negative control. After inoculation, the consortium shows a decrease in yeast for the first 24

hours, increasing until 48 hours. At 48 hours, the yeast population stabilized to the same level as at the 0 hour and remaining stable throughout 120 hours. This result for the consortium supports the lag phase shown in the cell mass concentration (Figure 7.9).

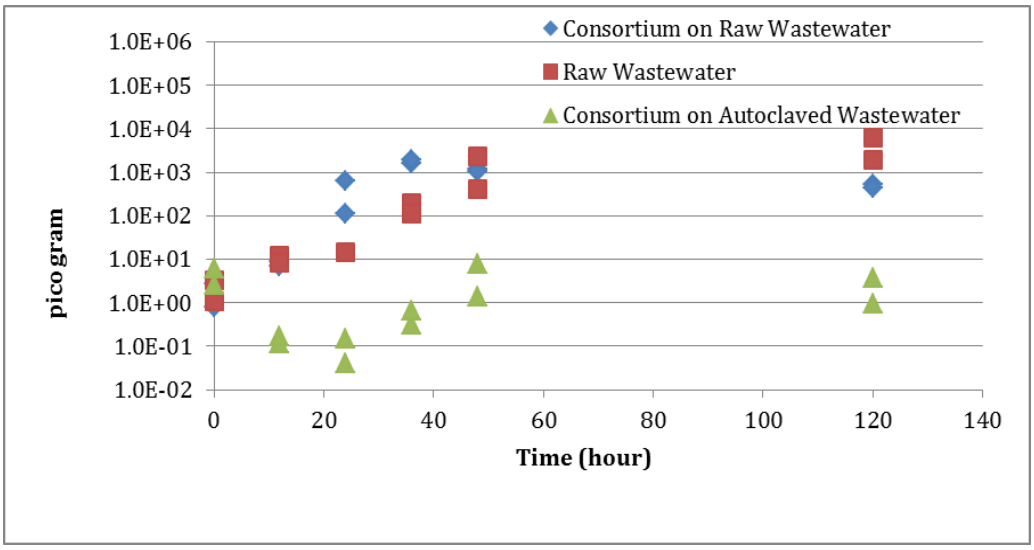

Figure 7.14 qPCR results of the consortium on raw wastewater, consortium on autoclaved wastewater, and raw wastewater with 60 gL^{-1} of glucose using yeast specific primers.

From measuring the pH over time as shown in Figure 7.15, the pH drops from 7 to 2 within 24 hours. This drop in pH could allow for the selectiveness for the yeast since yeast grow well at low pH while bacteria mainly grow best at a neutral or high pH. In addition to the pH change, the large amount of glucose and aeration also aid in the selection for yeast. Oleaginous microorganisms are more likely to be yeast than bacteria based on literature (Ratledge, 2005b). Each control and treatment shows a similar decreasing trend even though each treatment received the same amount of aeration and agitation. The consortium grown on raw wastewater decreased to the lowest pH of 2 at 36 hours, then remaining stable until 120 hours. Once at a pH of 2, the cell mass

concentration and lipid mass concentration increased drastically, which supports the qPCR results (Figure 7.14) of the yeast dominating.

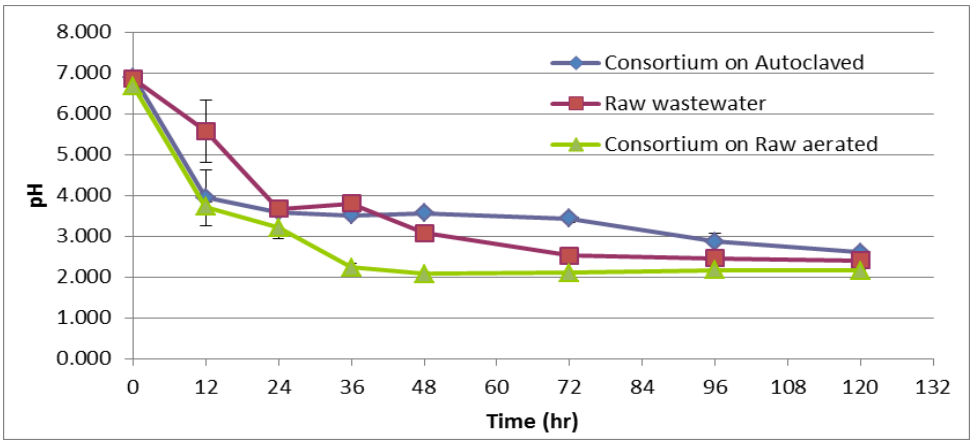

Figure 7.15 pH results of the consortium on raw wastewater, consortium on autoclaved wastewater, and raw wastewater with 60 gL^{-1} of glucose.

Table 7.6 and Table 7.7 show the results of the kinetic analysis. Table 7.6 consists of the calculated constants from applying Monod kinetic model to the data. The μ_{max} represents the maximum specific growth rate, thus it relates the growth of the microorganisms to substrate consumption. In comparing the μ_{max} for each of the treatments, the highest μmax is seen with the consortium with indigenous microorganisms. The lowest μmax was shown in the negative control. These results reflect the cell mass growth in Figure 7.9 that shows the highest μ_{max} with the highest production of cell mass by the consortium with indigenous microorganisms. The lowest μmax was seen with the lowest cell mass produced by the negative control. When compared to the consortium grown on raw wastewater amended with 1 gL^{-1} of glucose, the μ_{max} is drastically lower from 0.6 hr^{-1} to 0.086 hr^{-1}. This result is due to the fact that the increase in sugar concentration decreases the rate of the microbes' growth, supporting

the differences in the glucose consumption with 1 gL^{-1} and 60 gL^{-1}. The Monod constant K_S shows no significant difference between the consortium with indigenous microorganisms and the positive control, thus showing similarity in microbial growth and potentially population. The Ks for the negative control is significantly lower than the values for either the treatment or the positive control. From 1 to 60 gL^{-1} of glucose, the consortium grown on raw wastewater shows a large increase in the Monod constant from 0.95 to 78.9 gL^{-1}, coinciding with the μ_{max} results as well as the glucose consumption results.

Table 7.6 Monod constants, μ_{max} and K_S, for each treatment with 60 gL^{-1} of glucose.

Monod constants			
Constants	**Consortium on Autoclaved**	**Raw Wastewater**	**Consortium on Raw**
μ_{max} (hr^{-1})	0.013	0.001	0.086
K_s (gL^{-1})	77.9	37.0	78.9
R^2	0.9635	0.925	0.9997

Table 7.7 shows the yield coefficients for the different treatments. The $Y_{c/s}$ shows the change in cell mass over the change in glucose concentration. This yield shows the highest amount for the positive control, the lowest amount for the negative control, and the consortium with indigenous microorganisms in between. The yield $Y_{c/p}$ shows the change in cell mass over the change in lipid production. This yield shows the highest amount for the consortium with indigenous microorganisms while the negative control shows the smallest amount. However, these amounts are not statistically significant when comparing the two controls using a t-test. The $Y_{p/s}$ shows the change in

lipid production over the change in glucose consumption. The largest amount is shown with the positive control and the smallest amount is seen with the negative control. Since the consortium with indigenous microorganisms is in between both of the controls, some of the microorganisms in the consortium have competed with the indigenous microorganisms to survive and possibly forming a symbiosis with the indigenous microorganisms.

Table 7.7 Yield coefficients for each treatment with 60 gL^{-1} of glucose.

Yield Coefficients			
Constants	Consortium on Autoclaved	Raw Wastewater	Consortium on Raw
$Y_{c/s}$	0.73	0.05	0.14
$Y_{c/p}$	19.37	17.48	22.29
$Y_{p/s}$	0.015	0.004	0.008

The addition of indigenous microorganisms and the consortium showed a significant increase in production of cell mass, lipid mass, and FAMEs with 60 gL^{-1} of glucose added. When compared to the raw wastewater and consortium grown on autoclaved wastewater, the amount of FAMEs increased by 85% and 96%, respectively. According to Ratledge et al, the optimal carbon to nitrogen ratio to achieve a high amount of oil accumulated was 40:1, thus supporting the large increase in oil accumulated with the addition of 60 gL^{-1} of glucose. With 1 gL^{-1} of glucose added, the indigenous microorganisms negatively impacted the growth of the consortium when grown on raw wastewater. Therefore, the addition of the high concentration of glucose did have a positive impact on the survivability of the consortium and the overall oil accumulation.

Conclusion

These results show that the consortium grown on raw wastewater amended with 1 gL^{-1} of glucose was negatively impacted by the presence of the indigenous microorganisms. The total FAMEs produced are increased by 13% by adding the consortium into the wastewater. To overcome the negative impact of the indigenous microorganisms, the wastewater can be ozonated or the sugar concentration could be increased. When the consortium is grown on ozonated wastewater, the amount of cell mass concentration is increased, thus showing that ozonation possibly increases the amount of available oxygen within the wastewater, thereby, increasing the cell mass produced. By increasing the amount of glucose added to 60 gL^{-1}, the consortium increased the total FAMEs by 85% when added to the raw wastewater. Therefore, the high concentration of glucose shows that the consortium can accumulate a large amount of lipids for oil production when grown on raw wastewater.

CHAPTER VIII

EFFECT OF CULTIVATING OLEAGINOUS MICROORGANISMS ON SYNTHETIC WASTEWATER AMENDED WITH LIGNOCELLULOSIC SUGARS

Introduction

Municipal wastewater contains many nutrients that microorganisms need to grow. However, this wastewater typically has a low concentration of these nutrients (Grady et al., 1999). Since the consortium is composed of oleaginous microorganisms that require a medium with high nutrient concentrations, the wastewater needs to be supplemented with a carbon source to ensure growth. In addition to growth, these microorganisms require a high concentration of carbon and a low concentration of nitrogen to accumulate oil (Ratledge, 2005b). Therefore, the supplemented carbon will also encourage oil accumulation for the oleaginous microorganisms in the wastewater. An alternative carbon source to sugars from food sources could be sugars derived from lignocellulosic biomass.

Lignocellulosic sugars are produced from hydrolyzing biomass from agricultural residues and other related sources. These sugars are commonly composed mostly of glucose, xylose, galactose, arabinose, and mannose. In addition to these sugars, the

hydrolysis process produces furfural and acetic acid. The concentrations of these compounds vary according to the source of the biomass as well as the hydrolysis process (Olsson and Hahn-Hagerdal, 1996). The purpose of this chapter is to determine the effect of cultivating the oleaginous microorganism consortium on synthetic wastewater with varying concentrations of lignocellulosic sugars.

Methodology

This experiment was conducted using autoclaved primary effluent wastewater. The wastewater was collected and transported to Mississippi State University laboratory as described in Chapter IV. The concentrations of the compounds in the synthetic lignocellulosic sugar hydrolysate are shown in Table 8.1.

Table 8.1 Lignocellulosic sugar compounds and concentrations.

Composite of Sugars	Concentration (gL^{-1})
Glucose (Bakker et al., 2004; Cara et al., 2008)	10.5
Xylose (Bakker et al., 2004; Cara et al., 2008)	8.0
Galactose (Cara et al., 2008)	0.3
Arabinose (Cara et al., 2008)	1.0
Mannose (Cara et al., 2008)	0.1
Acetic acid (Bakker et al., 2004; Bustos et al., 2004; Diaz et al., 2009; Lima et al., 2004)	5.2
Furfural (Bustos et al., 2004)	5

The sugar concentrations are based on literature from the hydrolysis of switchgrass, willow, and vineshoot trimmings (Bakker et al., 2004; Cara et al., 2008). The acetic acid concentration is uniformly found in hydrolysates of biomass from

switchgrass, willow, olive tree trimmings, vineshoot trimmings, and sugarcane bagasse (Bakker et al., 2004; Bustos et al., 2004; Diaz et al., 2009; Lima et al., 2004). Because furfural is known to be highly inhibitive to microbial growth, the highest furfural concentration found in literature was used, which is from hydrolyzing biomass of vineshoot trimmings (Bustos et al., 2004). The justification for using this high furfural concentration was to ensure the consortium's ability to utilize and grow on any lignocellulosic sugars from any biomass source when added to the wastewater.

The concentration of sugars was decreased from a total of 20 gL^{-1} to 3, 5, 7, and 9 gL^{-1} of sugars to increase the growth rate. The carbon to nitrogen ratios for each sugar concentration are 3:2 for 3 gL^{-1} of sugars, 5:3 for 5 gL^{-1} of sugars, 7:5 for 7 gL^{-1} of sugars, and 9:7 for 9 gL^{-1} of sugars. The treatments involve reducing the furfural and acetic acid concentrations shown in Table 8.1 in half for each total sugar of 3, 5, 7, and 9 gL^{-1}, determining if the furfural and acetic acid concentrations have an effect on the growth of the consortium. These furfural and acetic acid concentrations were reduced by the same ratios as the sugar concentrations. The positive controls included only the sugars added to the autoclaved wastewater, excluding the acetic acid and furfural. For each treatment and positive control, 400 mL of primary effluent wastewater was dispensed into 1 L, baffled flasks and autoclaved as described in Chapter IV. Each flask was inoculated with 30 mL of the consortium and grown in the New Brunswick Scientific incubator at 110 rpm and 28^{o}C for 52 hours. Cell mass, lipid mass, glucose and xylose concentrations were measured as described in Chapter IV. The Monod kinetics and yield coefficients were determined by the methods described in Chapter V.

Results

The results of cultivating oleaginous microorganism consortium on autoclaved wastewater amended with a model lignocellulosic sugars have shown that the presence of furfural and acetic acid have a growth inhibitory effect on the consortium. The data shown in Figure 8.1 demonstrates the growth of the consortium on autoclaved wastewater amended with 3 gL^{-1} of sugar with the furfural and acetic acid concentrations of 0.4 and 0.8 gL^{-1}. This figure shows that as the furfural and acetic acid concentrations increase from 0.4 to 0.8 gL^{-1}, the cell mass concentration decreases when compared to the positive control. The maximum cell mass for the positive control was approximately 0.66 gL^{-1} after 30 hours of cultivation. With the addition of 0.4 gL^{-1} of acetic acid and furfural, the cell mass increases steadily for the first 12 hours and then reaches stationary phase between 12 and 52 hours. The maximum cell mass is approximately 0.35 gL^{-1} at 30 hours, which is a significantly smaller amount compared to the positive control. With the addition of 0.8 gL^{-1} of furfural and acetic acid, the cell mass concentration shows a similar trend to the treatment with 0.4 gL^{-1} of acetic acid and furfural with a maximum of 0.18 gL^{-1} at 30 hours, which is also significantly lower than the positive control. With 3 gL^{-1} of sugars, the presence of acetic acid and furfural inhibited the cell mass growth.

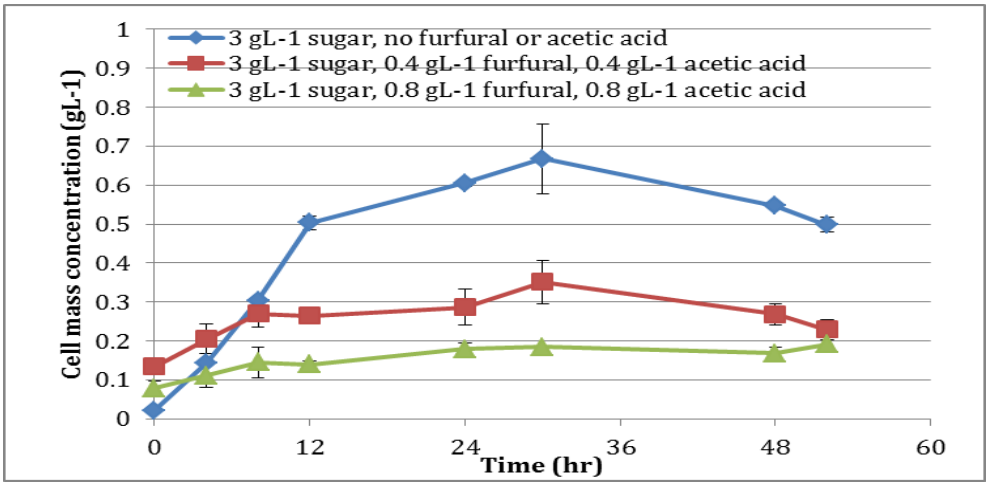

Figure 8.1 Growth of the consortium on autoclaved wastewater amended with 3 gL-1 of sugars, 0.4 gL^{-1} and 0.8 gL^{-1} acetic acid, and 0.4 gL^{-1} and 0.8 gL^{-1} of furfural.

By increasing the sugar concentration to 5 gL^{-1} and subsequently the acetic acid and furfural concentrations, Figure 8.2 shows the effects of these compounds on the production of cell mass. The positive control containing the consortium with only sugars added shows the exponential growth rate occurred from 0 hours to 30 hours of cultivation, at which the stationary phase begins and continues until 52 hours. The cell mass concentration shows a maximum of 0.72 gL-1 at 30 hours. With the addition of 0.6 gL^{-1} of furfural and acetic acid, the cell mass concentration increased steadily throughout the first 12 hours and then reaching the stationary phase at 24 hours. After 24 hours, the cell mass decreases from 24 hours to 52 hours as the death phase. Once the furfural and acetic acid was increased from 0.6 to 1.3 gL^{-1}, there was no measureable growth observed for the consortium. Even with the increase in sugars to 5gL^{-1}, the cell mass of the consortium was still inhibited by the presence of furfural and acetic acid.

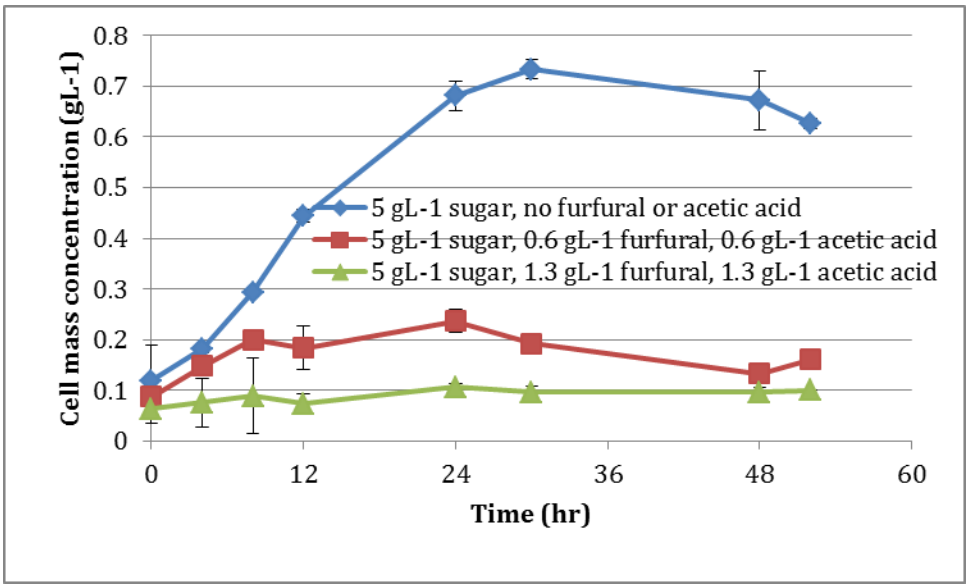

Figure 8.2 Growth of the consortium on autoclaved wastewater amended with 5 gL^{-1} of sugars, 0.6 gL^{-1} and 1.3 gL^{-1} of acetic acid, and 0.6 gL^{-1} and 1.3 gL^{-1} of furfural.

As the sugar concentration was increased to 7 gL^{-1}, the furfural and acetic acid increased to 0.9 and 1.8 gL^{-1}. Figure 8.3 shows that the effect of cultivating the consortium on autoclaved wastewater with 7 gL^{-1} of lignocellulosic sugars. The positive control with 7 gL^{-1} of sugars without acetic acid and furfural shows a steady increase as the exponential growth rate for the first 30 hours of cultivation. The stationary phase was observed to have occurred from 30 hours until 52 hours for the positive control. With the addition of 0.9 gL^{-1} of furfural and acetic acid, a small amount of growth of 0.05 gL^{-1} increase for the first 12 hours were shown with the stationary phase occurring from 12 hours to 30 hours. The furfural and acetic acid concentration of 1.8 gL^{-1} resulted in relatively no growth.

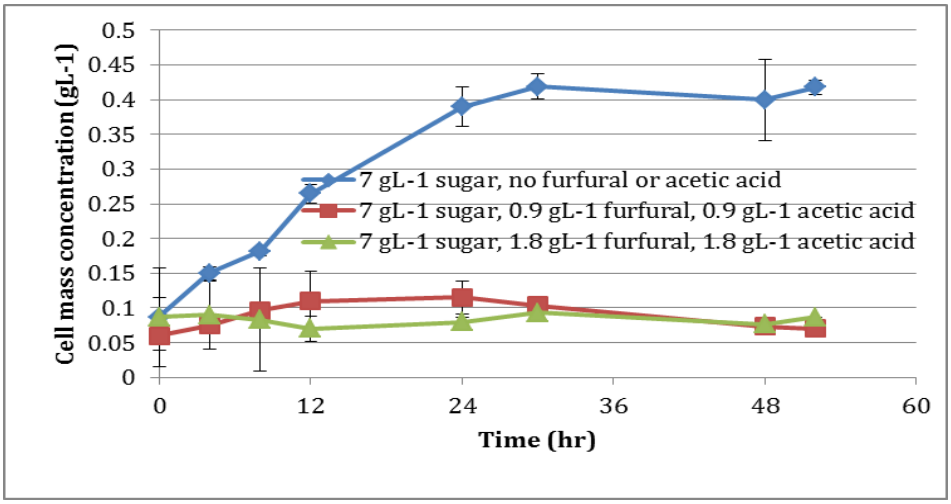

Figure 8.3 Growth of the consortium on autoclaved wastewater amended with 7 gL^{-1} of sugars, 0.9 gL^{-1} and 1.8 gL^{-1} of acetic acid, and 0.9 gL^{-1} and 1.8 gL^{-1} of furfural.

Therefore, as shown in Figure 8.4, the addition of 9 gL^{-1} of sugar with 1.1 gL^{-1} and 2.3 gL^{-1} of furfural and acetic acid on autoclaved wastewater resulted in no growth. The positive control for the 9 gL^{-1} of sugar shows the increase for the first 24 hours as the exponential growth phase. The stationary phase was noticed to have occurred from 24 hours to 52 hours. The growth of the consortium with 7 gL^{-1} and 9 gL^{-1} of sugar with no furfural and acetic acid added show similar trends.

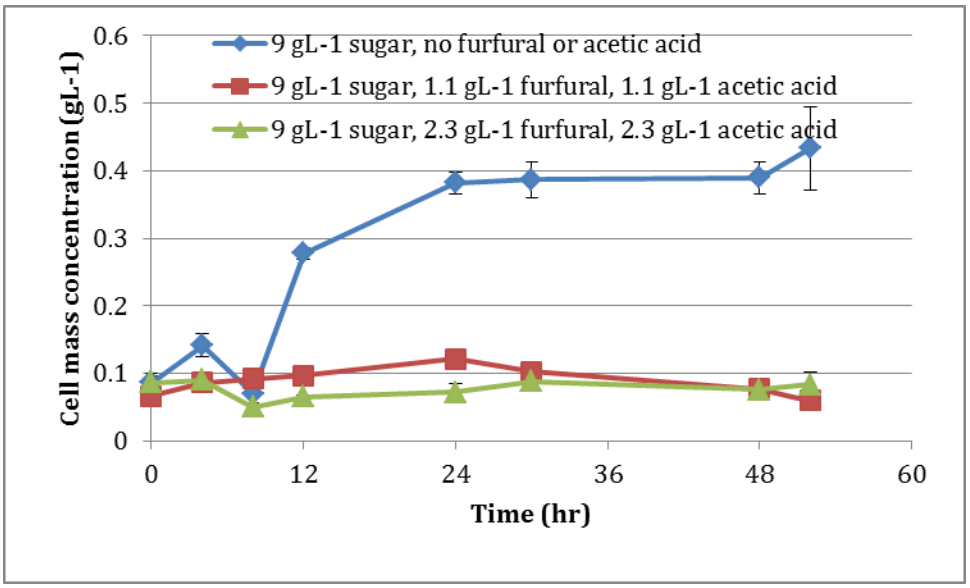

Figure 8.4 Growth of the consortium on autoclaved wastewater amended with 9 gL^{-1} of sugars, 1.1 gL^{-1} and 2.3 gL^{-1} of acetic acid, and 1.1 gL^{-1} and 2.3 gL^{-1} of furfural.

The furfural and acetic acid have a substantial effect on the growth of the consortium. Furfural and acetic acid have been known to decrease the effectiveness of microorganism's ability to thrive in their environment. When acetic acid and furfural reached a concentration above 0.6 gL^{-1}, the cell mass concentration showed a negligible amount of growth. The increase in sugar did not show any benefit to overcome the presence of furfural and acetic acid.

In addition to cell mass concentrations, the lipid mass concentrations were measured to determine the effect of lignocellulosic sugars with furfural and acetic acid concentrations on the consortium's ability to accumulate oil on autoclaved wastewater. Figure 8.5 shows the lipid mass production of the consortium grown on autoclaved wastewater amended with 3 gL^{-1} of sugar with furfural and acetic acid concentrations of 0.4 gL^{-1} to 0.8 gL^{-1}. This figure shows that only a little lipid mass was produced with 3

gL^{-1} of sugar with and without furfural and acetic acid. The positive control increased approximately 0.07 gL^{-1} of lipids in 30 hours of cultivation. This increase is a very small amount when compared to the amount of cell mass produced within the same time period. The positive control also produced 10.5 % of lipids on a dry cell mass basis. This percentage of lipids is small when compared to the definition of oleaginous microorganisms that is defined to produce over 20% of its weight in lipids. This effect is most likely due to the small amount of sugars added to the autoclaved wastewater. A significant accumulation of lipids was not expected with a carbon to nitrogen ratio of 3:2 since oleaginous microorganisms require a carbon to nitrogen ratio of 40:1 to accumulate oil (Ratledge, 2005b). However, with the addition of furfural and acetic acid, the lipids produced showed similar results to the positive control, thus showing that possibly furfural and acetic acid with concentrations of 0.4 and 0.8 gL^{-1} did not affect lipid mass production. Lipid accumulation in these organisms is a response to stress, and the presence of furfural and acetic acid is obviously a stressor. The consortium produced 25% and 30% (dry weight basis) of lipids due to the low production of cell mass when 0.4 gL^{-1} and 0.8 gL^{-1} of furfural and acetic acid.

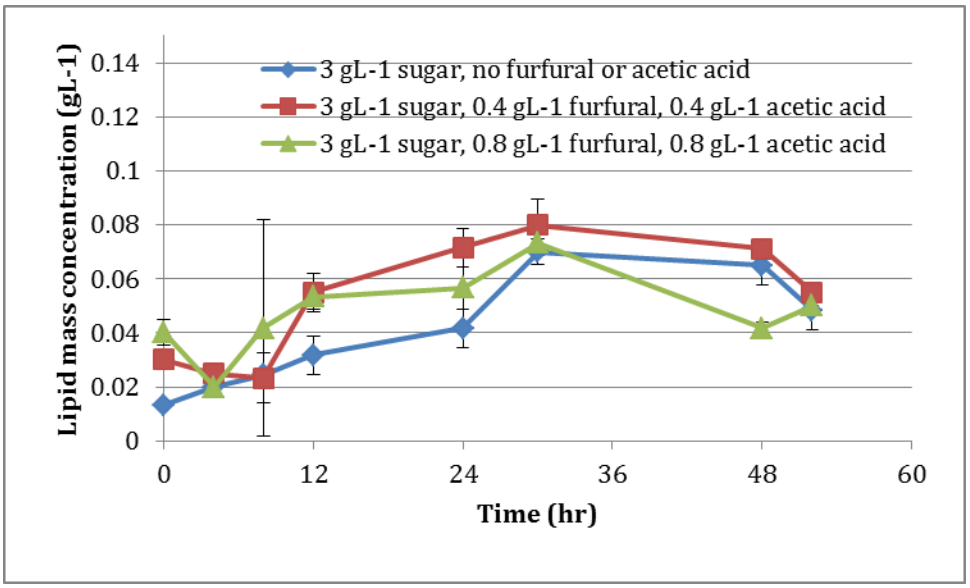

Figure 8.5 Lipid mass production of the consortium on autoclaved wastewater amended with 3 gL^{-1} of sugars, 0.4 gL^{-1} and 0.8 gL^{-1} of acetic acid, and 0.4 gL^{-1} and 0.8 gL^{-1} of furfural.

When comparing the positive controls, the effect of increasing sugars from 3 to 5 gL^{-1} is shown in Figure 8.6. This result shows that with the addition of furfural and acetic acid, the overall lipid production decreases when compared to the positive control. This result supports the growth curve shown in Figure 8.2. The positive control accumulated 14.3% of lipids within 52 hours. Only a small amount of lipids were accumulated in experiments containing 0.6 gL^{-1} of furfural and acetic acid, also coinciding with the small amount of cell growth shown in Figure 8.2. The treatment with 1.3 gL^{-1} of furfural and acetic acid showed no accumulation of oil throughout the 52-hour cultivation period. Thus, the furfural and acetic acid concentration of 0.6 gL^{-1} of furfural inhibited the consortium's ability to accumulate oil when grown on autoclaved wastewater amended with 5 gL^{-1} of sugar.

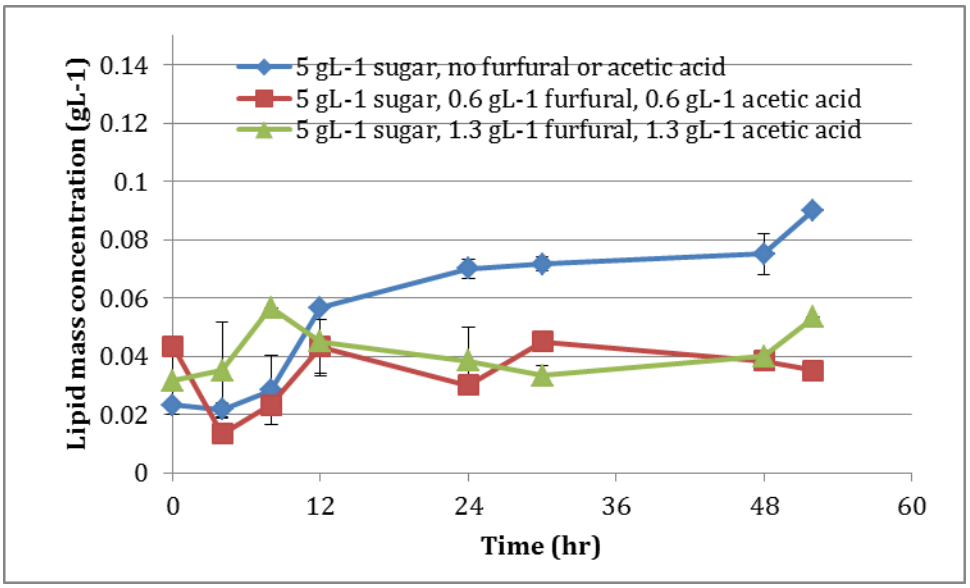

Figure 8.6 Lipid mass concentration of the consortium on autoclaved wastewater amended with 5 gL^{-1} of sugars, 0.6 gL^{-1} and 1.3 gL^{-1} of acetic acid, and 0.6 gL^{-1} and 1.3 gL^{-1} of furfural.

Figure 8.7 shows the consortium grown on autoclaved wastewater with 7 gL^{-1} of sugars and 0.9 gL^{-1} and 1.8 gL^{-1} of furfural and acetic acid. This result shows that without furfural and acetic acid the consortium shows a consistent accumulation of oil throughout the 52 hours of cultivation. The maximum lipid accumulated was 23.9% at the 24 hour point, which corresponds to the highest cell mass produced shown in Figure 8.3. By adding 0.9 gL^{-1} of furfural and acetic acid, the amount lipids extracted from the cell mass remained constant throughout the first 24 hours at approximately 0.03 gL^{-1}. After 24 hours, the lipid mass concentration decreases steadily. When 1.8 gL^{-1} of acetic acid and furfural, the lipid mass shows a similar trend to when 0.9 gL^{-1} furfural and acetic acid, resulting in the decrease in lipid mass after 30 hours. This decrease could be due to the microorgnaisms in the consortium consuming the lipid stores to detoxify inhibitors, furfural and acetic acid, present in the system.

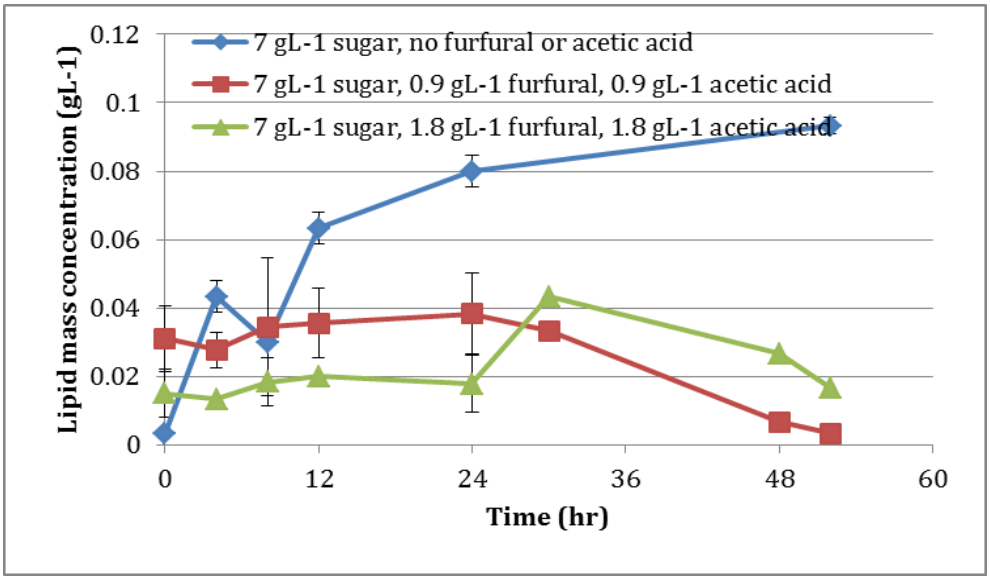

Figure 8.7 Lipid mass concentration of the consortium on autoclaved wastewater amended with 7 gL^{-1} of sugars, 0.9 and 1.8 gL^{-1} of acetic acid, and 0.9 and 1.8 gL^{-1} of furfural.

The increase to 9 gL^{-1} of sugars with 1.1 gL^{-1} and 2.3 gL^{-1} of furfural and acetic acid is shown in Figure 8.8. The positive control for the 9 gL^{-1} of sugars shows a lipid accumulation of approximately 16.9 % after 52 hours of cultivation. The result of the addition of 1.1 gL^{-1} of furfural and acetic acid with 9 gL^{-1} shows that the lipid mass remains constant until the 24 hour point. From 24 hours to 52 hours, the lipid mass shows a slight decrease. The overall trend of the treatment with 2.3 gL^{-1} acetic acid and furfural shows similar results to when 1.1 gL^{-1} of furfural and acetic acid is added. The constant lipid mass over the duration of the experiment is consistent with the lack of cell mass production as shown in Figure 8.4. This data demonstrates the inactivity of the microorganisms in the autoclave wastewater in the presences of furfural and acetic acid.

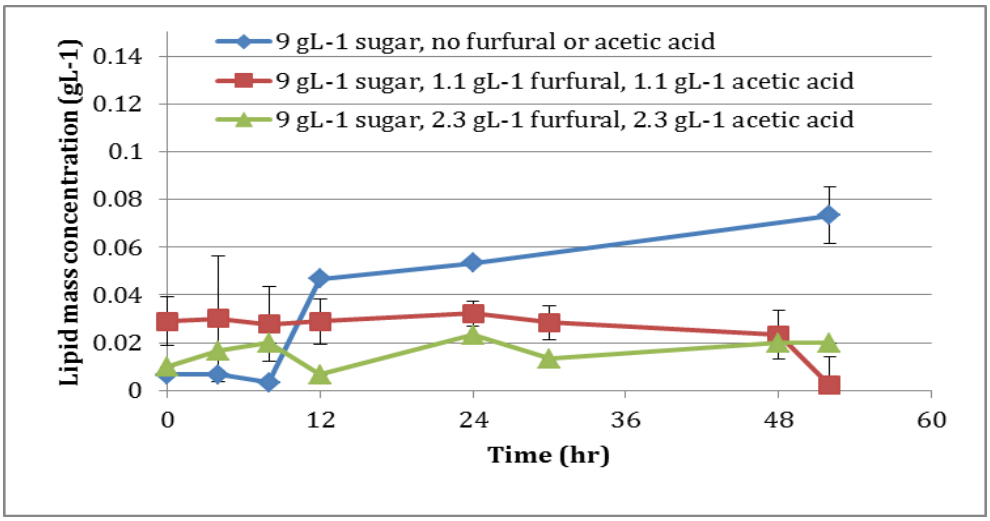

Figure 8.8 Lipid mass concentration of the consortium on autoclaved wastewater amended with 9 gL^{-1} of sugars, 1.1 and 2.3 gL^{-1} of acetic acid, and 1.1 and 2.3 gL^{-1} of furfural.

The increase in sugars from 3 gL^{-1} to 9 gL^{-1} shows an increase in lipid percentage accumulated on the positive controls. However, the increase in sugars did not assist in overcoming the inhibitory effects of the furfural and acetic acid. With the increase in sugars, furfural, and acetic acid, the consortium decreases in overall cell mass production as well as to the point of no additional lipid production. Lipid accumulation does not occur at concentrations above 0.6 gL^{-1} for furfural and acetic acid.

Since glucose and xylose were the two primary compounds in the synthetic lignocellulosic sugar, glucose and xylose concentrations were monitored over the duration of the experiment. The highest consumption of sugars occurs during the exponential growth phase. The glucose and xylose concentrations for the consortium grown on autoclaved wastewater with 3 gL^{-1} of sugars and 0.2 and 0.4 gL^{-1} furfural and acetic acid are shown in Figure 8.9 and Figure 8.10. These figure shows that the without furfural and acetic acid, the glucose and xylose sugars are consumed within the first 8

hours of cultivation. By adding 0.2 gL^{-1} of furfural and acetic acid, the glucose is consumed within 12 hours and the xylose shows a steady consumption through 24 hours of cultivation but tapers off at 24 hours without disappearing completely. Increasing the furfural and acetic acid to 0.8 gL^{-1} results in the consumption of glucose from 1.5 to 0.2 gL^{-1} within 12 hours while the xylose concentration shows a decrease from 1.24 gL^{-1} to 0.8 gL^{-1} within 24 hours. When comparing the treatments to the positive control with 3 gL^{-1} of total sugars, the glucose and xylose consumption are inhibited with the furfural and acetic acid present in 0.4 gL^{-1} and 0.8 gL^{-1}.

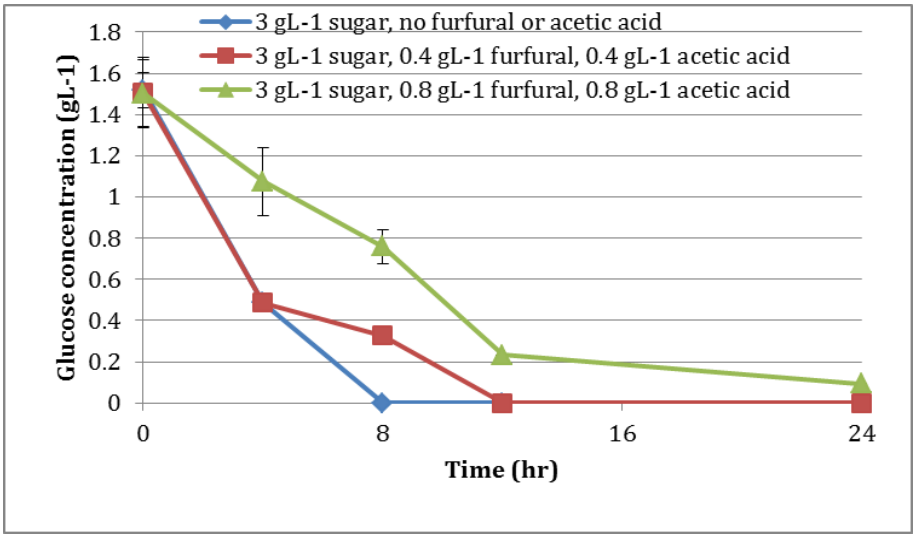

Figure 8.9 Glucose concentrations of the consortium grown on autoclaved wastewater amended with 3 gL^{-1} of sugars, 0.2 and 0.4 gL^{-1} of furfural, and 0.2 and 0.4 gL^{-1} of acetic acid.

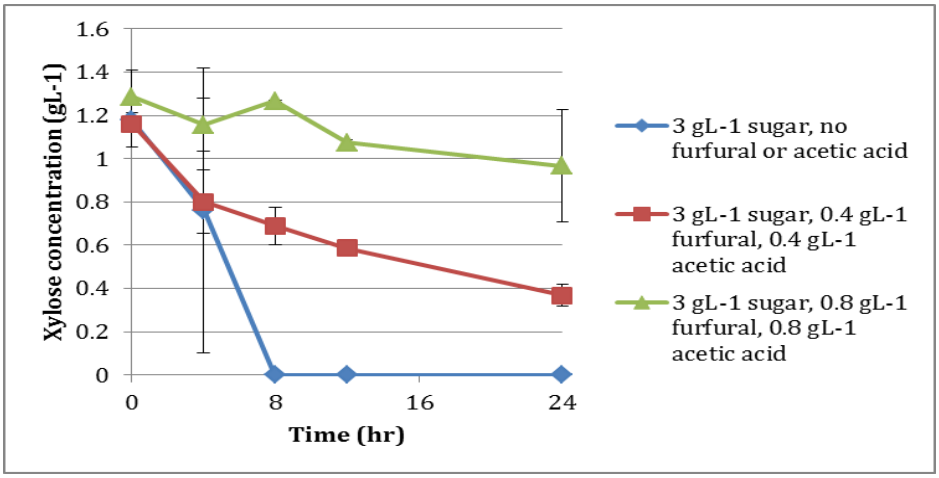

Figure 8.10 Xylose concentration of the consortium grown on autoclaved wastewater amended with 3 gL^{-1} of sugars, 0.2 and 0.4 gL^{-1} of furfural, and 0.2 nd 0.4 gL^{-1} of acetic acid.

With the increase to 5 gL^{-1} of sugars, the consortium shows similar results to when grown on 3 gL^{-1} of sugars. Figure 8.11 and 8.12 show the glucose and xylose concentrations of the consortium grown on 5 gL^{-1} of sugars, 0.6 and 1.3 gL^{-1} of furfural and acetic acid. The positive control shows the complete consumption of glucose within 8 hours of cultivation and xylose within 24 hours. The addition of 0.6 gL^{-1} of furfural and acetic acid slows the consumption of glucose and inhibits the consumption of xylose. The glucose is consumed steadily through the first 12 hours of cultivation while the xylose shows a maximum reduction within the first 4 hours of 0.5 gL^{-1} and an overall reduction of 0.1 gL^{-1} by 24 hours. A further increase of furfural and acetic acid of 1.3 gL^{-1} results in the complete glucose consumption within 24 hours and an overall consumption of 0.2 gL^{-1} of xylose within 24 hours. The increase in time it takes to consume the glucose is attributed to the inhibition by the furfural and acetic acid. At these concentrations, the furfural and acetic has not completely inhibited the consumption of glucose but merely increased the time it takes for the microorganisms to consume it.

The small consumption of xylose within 24 hours could be due to the fact that glucose is metabolized before xylose (Easterling et al., 2009).

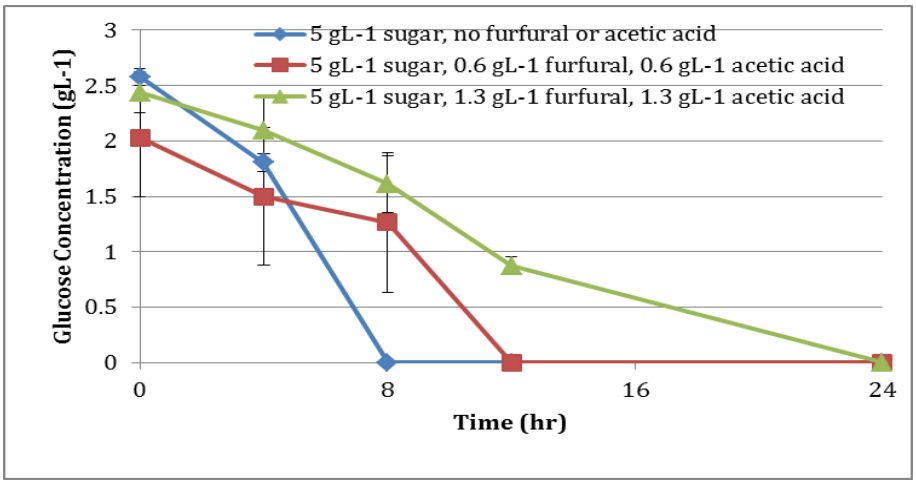

Figure 8.11 Glucose concentrations of the consortium grown on autoclaved wastewater amended with 5 gL^{-1} of sugars, 0.6 gL^{-1} and 1.3 gL^{-1} of furfural, and 0.6 gL^{-1} and 1.3 gL^{-1} of acetic acid.

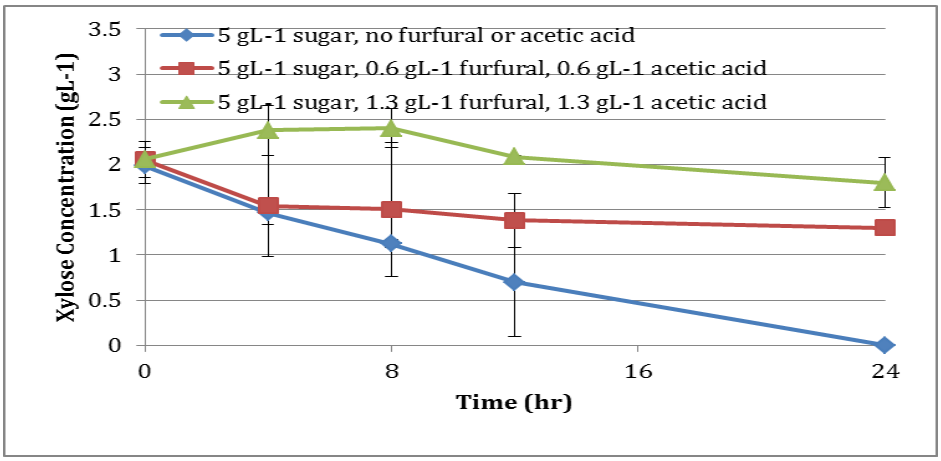

Figure 8.12 Xylose concentrations of the consortium grown on autoclaved wastewater amended with 5 gL^{-1} of sugars, 0.6 gL^{-1} and 1.3 gL^{-1} of furfural, and 0.6 gL^{-1} and 1.3 gL^{-1} of acetic acid.

The consumption of glucose and xylose for the consortium grown on autoclaved wastewater with 7 gL^{-1} of sugars and 0.9 and 1.8 gL^{-1} of furfural and acetic acid is shown in Figure 8.13 and 8.14. This figure shows similar glucose consumption to the consortium grown on 3 and 5 gL^{-1} of sugars, except this positive control takes 24 hours to completely consume the increased amount of glucose. The treatment at this sugar concentration shows similar trends for glucose consumption when compared to the other sugar concentrations, whereas the glucose was consumed in the first 24 hours for each furfural and acetic acid concentration. Thus, the furfural and acetic acid at concentrations above 0.6 gL^{-1} do not seem to further inhibit the consumption of glucose. However, the xylose consumption shows inhibition due to the utilization of only 0.1 gL^{-1} of xylose within 24 hours when 1.8 gL^{-1} of furfural and acetic acid is included. With 0.9 gL^{-1} of furfural and acetic acid added, the xylose consumption does not show much difference in the trend than with 0.6 or 0.8 gL^{-1} of furfural and acetic acid added, despite the increase in xylose concentration.

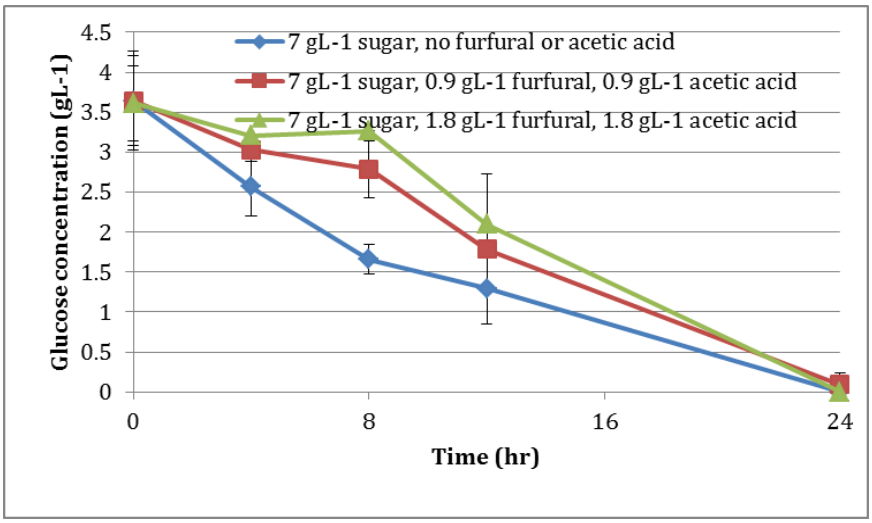

Figure 8.13 Glucose concentrations of the consortium grown on autoclaved wastewater amended with 7 gL^{-1} of sugars, 0.9 and 1.8 gL^{-1} of furfural, and 0.9 and 1.8 gL^{-1} of acetic acid.

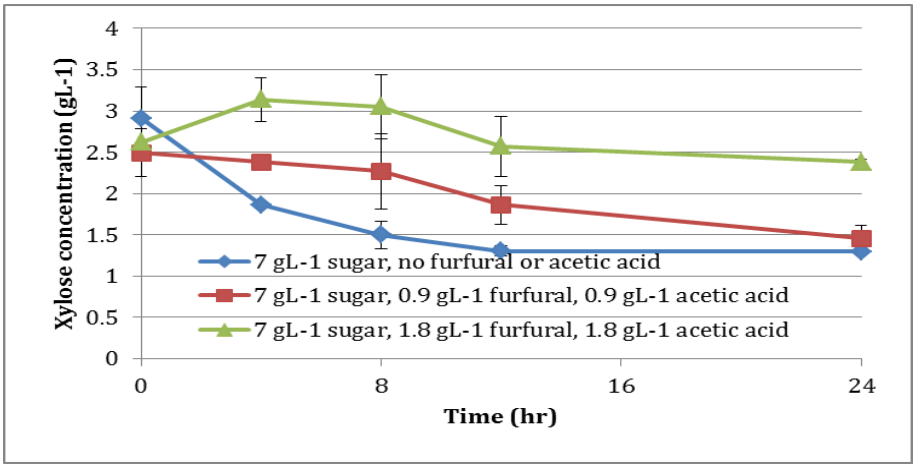

Figure 8.14 Xylose concentrations of the consortium grown on autoclaved wastewater amended with 7 gL^{-1} of sugars, 0.9 and 1.8 gL^{-1} of furfural, and 0.9 and 1.8 gL^{-1} of acetic acid.

The effect of these inhibitors on the consortium's ability to consume glucose and xylose is shown in Figure 8.15 and 8.16. This result shows that increasing the sugar concentration to 9 gL^{-1} without furfural and acetic acid, the consortium does not consume all the glucose or xylose within 24 hours, which is the exponential growth phase shown in

Figure 8.4. In addition, the addition of 1.1 gL^{-1} of furfural and acetic acid does not show an increased inhibition effect. However, with the same sugar concentration, the increase in furfural from 1.1 to 2.3 gL^{-1} results in the minimal glucose consumption. In addition, the xylose consumption is completely inhibited with 2.3 gL^{-1} of furfural and acetic acid with the negligible amount of xylose consumed. However, the furfural and acetic acid concentration of 1.1 gL^{-1} does show some consumption with the increased amount of sugars.

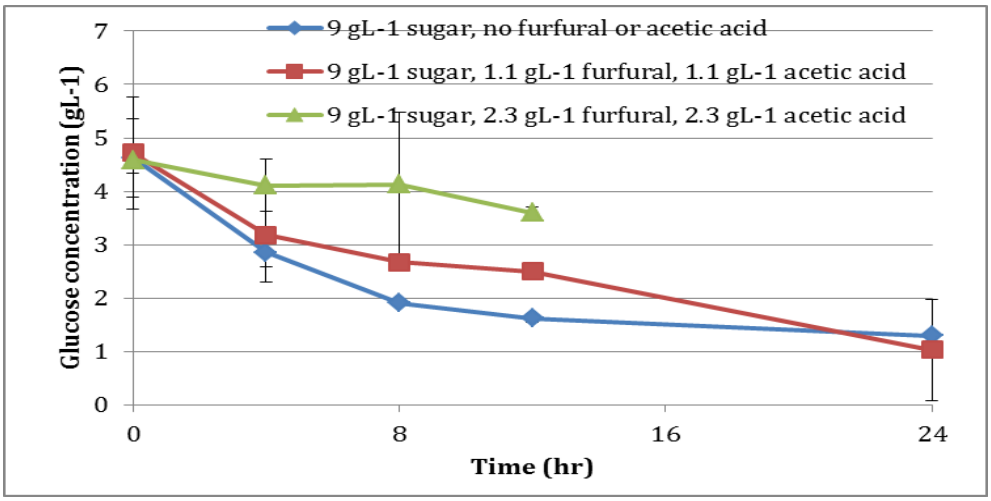

Figure 8.15 Glucose concentrations of the consortium grown on autoclaved wastewater amended with 9 gL^{-1} of sugars, 1.1 and 2.3 gL^{-1} of furfural, and 1.1 and 2.3 gL^{-1} of acetic acid.

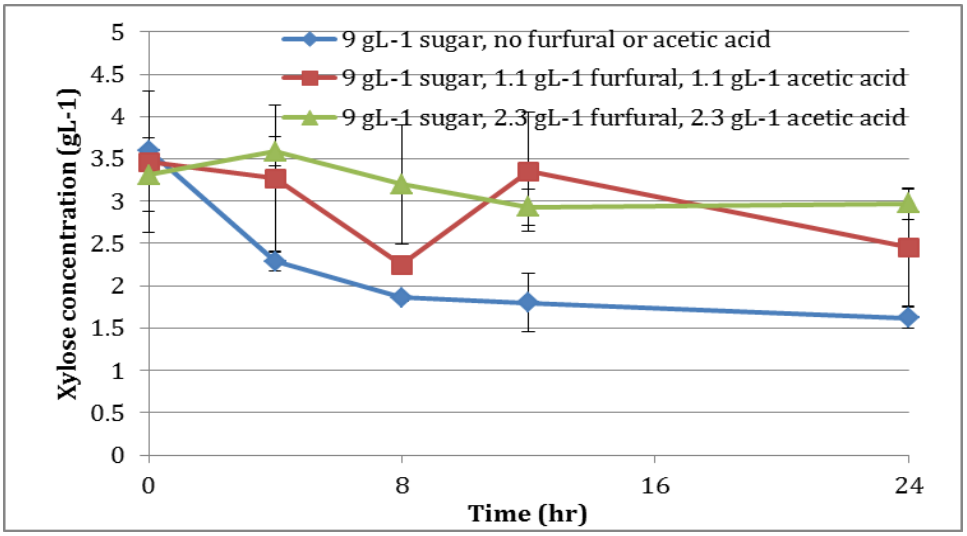

Figure 8.16 Xylose concentrations of the consortium grown on autoclaved wastewater amended with 9 gL^{-1} of sugars, 1.1 nd 2.3 gL^{-1} of furfural, and 1.1 and 2.3 gL^{-1} of acetic acid.

The glucose and xylose concentration data does not correlate with the growth of the consortium because the glucose and xylose are still being consumed at 9gL^{-1} of sugars and 1.1 gL^{-1} of furfural and acetic acid while the cell mass concentrations are not increasing. This effect is attributed to the microorganisms consuming the sugars as energy to convert the furfural and/or acetic acid to a compound less inhibitory.

Furthermore, the yield coefficients for the different treatments and sugar concentrations were determined to quantify the effect of varying concentrations of lignocellulosic sugars has on the consortium growing on autoclaved wastewater. Table 8.2 compares the results for each treatment and positive control for the overall yield ratio of cell mass to lipid mass ($Y_{c/p}$), yield ratio of cell mass to sugar consumption for both glucose and xylose ($Y_{c/s}$), and the yield ratio of lipid mass to sugar consumption for both glucose and xylose ($Y_{p/s}$). These values describe the overall yield through the 52 hours of cultivation. No yields could be calculated for the autoclaved wastewater with 5 gL^{-1} of

sugars and 1.3 gL^{-1} of furfural and acetic acid because no growth was observed in the cell mass and no lipid production was seen in Figures 8.2 and 8.6. In addition, no yields could be calculated for 7 gL^{-1} of sugars with 0.9 and 1.8 gL^{-1} of furfural and acetic acid as well as 9 gL^{-1} of sugars with 1.1 and 2.3 gL^{-1} of furfural and acetic acid since no growth or lipid mass production occurred.

Table 8.2 Yield coefficients for the consortium grown on autoclaved wastewater with lignocellulosic sugars.

Sugar (gL^{-1})	Sample	$Y_{c/p}$	$Y_{c/s}$		$Y_{p/s}$	
			Glucose	Xylose	Glucose	Xylose
3	no furfural or acetic acid	17.33	0.22	0.25	0.01	0.01
	0.4 gL^{-1} furfural 0.4 gL^{-1} acetic acid	5.26	0.15	0.20	0.05	0.08
	0.8 gL^{-1} furfural 0.8 gL^{-1} acetic acid	2.36	0.12	0.06	0.04	0.04
5	no furfural or acetic acid	8.29	0.07	0.29	0.004	0.03
	0.6 gL^{-1} furfural 0.6 gL^{-1} acetic acid	2.56	0.11	0.37	0.001	0.04
	1.3 gL^{-1} furfural 1.3 gL^{-1} acetic acid	------	------	------	------	------
7	no furfural or acetic acid	3.42	0.10	0.19	0.03	0.06
	0.9 gL^{-1} furfural 0.9 gL^{-1} acetic acid	-----	-----	-----	-----	-----
	1.8 gL^{-1} furfural 1.8 gL^{-1} acetic acid	-----	-----	-----	-----	-----
9	no furfural or acetic acid	5.98	0.20	0.10	0.03	0.13
	1.1 gL^{-1} furfural 1.1 gL^{-1} acetic acid	-----	-----	-----	-----	-----
	2.3 gL^{-1} furfural 2.3 gL^{-1} acetic acid	-----	-----	-----	-----	-----

With 3 gL^{-1} of sugars, the yield coefficient ($Y_{c/p}$) shows a large decrease from 17.33 to 5.26 when 0.4 gL^{-1} of furfural and acetic acid is added. This yield shows even more of a decrease to 2.36 with 0.8 gL^{-1} of furfural added. These yield values support the cell mass and lipid mass concentrations shown in Figure 8.1 and 8.5 in showing that more cell mass is produced than lipid mass throughout the experiment. A similar result is observed when cultivated with 5 gL^{-1} of sugars, where a decrease from 8.29 to 2.56 is shown when 0.6 gL^{-1} of furfural and acetic acid. Thus, the positive controls for cell mass to lipid mass yield shows a decrease with increasing sugar concentrations, except for with 7 gL^{-1} of sugars with 3.42 cell mass to lipid mass yield. This decrease shows the lipid mass increases with increasing sugar concentrations as shown in Figures 8.5-8.

The cell mass to sugar consumption yield shows similar results as seen with the cell mass and lipid mass yield for the 3 gL^{-1} of sugars. These yields show a decrease when adding 0.4 and 0.8 gL^{-1} of furfural and acetic acid when considering the glucose and xylose consumptions without inhibitors present. The decrease in yields shows that the cell mass production decreases in the presence of furfural and acetic acid, supporting the results shown in Figures 8.1 and 8.5. However, when grown on 5 gL^{-1} of sugars, the cell mass to sugar consumption yield shows an increase when 0.6 gL^{-1} of furfural and acetic acid are added. This increase is due to the increase in the rate of cell mass production before the glucose and xylose is completely consumed. For the positive control, the sugar concentration increases from 5 to 7 gL^{-1} shows a 0.1 increase in the cell mass and glucose consumption yield. For the positive control from 7 to 9 gL^{-1}, the cell mass to glucose consumption yield increases by 0.2. For the increase from 5 to 7 and 9 gL^{-1} of sugars, the cell mass to xylose consumption yield results in a decrease to 0.19 and

0.1, respectively. This decrease is due to the decrease in the change in cell mass with respect to the xylose consumption, which coincides with the cell mass and xylose consumption figures shown.

The yield for the lipid mass to sugar consumption shows a slight increase for the 3 gL^{-1} of sugars when adding 0.4 and 0.8 gL^{-1} of furfural and acetic acid. This increase is due to the small change in lipids with respect to the change in sugar consumption, which coincides with the lipid mass concentration and sugar consumption shown in Figures 8.5 and 8.9. With 5 gL^{-1} of sugars, the addition of 0.6 gL^{-1} of furfural and acetic acid results in a decrease when only including the glucose consumption while an increase in this yield is shown when considering the xylose consumption. When increasing the sugar concentrations, the yield of lipid mass and xylose consumption shows an increase from 0.01 to 0.13 for 3 to 9 gL^{-1}. The yield for lipid mass and glucose consumption shows a decrease from 3 to 5 gL^{-1} but continues to increase with 7 and 9 gL^{-1} from 0.01 to 0.03. This overall increase is due to the increase in lipids produced with the increasing sugar concentration.

The Monod kinetic model was applied to the data to quantify how the consortium grows on autoclaved wastewater with lignocellulosic sugars. Table 8.3 shows the Monod constants for each sugar concentration and furfural and acetic acid concentrations. The Monod constants for the growth on 5 gL^{-1} with 1.3 gL^{-1} of furfural and acetic acid, 7 gL^{-1} of sugars with 0.9 and 1.8 gL^{-1} of furfural and acetic acid, and 9 gL^{-1} of sugars with 1.1 gL^{-1} and 2.3 gL^{-1} of furfural and acetic acid could not be determined since no growth was shown throughout the experiment.

Table 8.3 Monod constants for the consortium grown on autoclaved wastewater amended with lignocellulosic sugars.

Sugar Concentration (gL^{-1})	Sample	Glucose			Xylose		
		μ_{max} (hr^{-1})	K_S (gL^{-1})	R^2	μ_{max} (hr^{-1})	K_S (gL^{-1})	R^2
3	no furfural or acetic acid	0.99	2.22	1	0.19	1.28	1
	0.4 gL^{-1} furfural 0.4 gL^{-1} acetic acid	0.20	1.56	0.97	0.02	1.16	0.95
	0.8 gL^{-1} furfural 0.8 gL^{-1} acetic acid	0.01	1.73	0.91	0.02	0.81	0.55
5	no furfural or acetic acid	0.49	7.39	1	0.25	3.95	0.99
	0.6 gL^{-1} furfural 0.6 gL^{-1} acetic acid	0.04	2.54	0.96	0.02	2.27	0.82
	1.3 gL^{-1} furfural 1.3 gL^{-1} acetic acid	-----	-----	-----	-----	-----	-----
7	no furfural or acetic acid	0.74	19.27	0.89	0.17	5.21	0.85
	0.9 gL^{-1} furfural 0.9 gL^{-1} acetic acid	-----	-----	-----	-----	-----	-----
	1.8 gL^{-1} furfural 1.8 gL^{-1} acetic acid	-----	-----	-----	-----	-----	-----
9	no furfural or acetic acid	0.16	6.78	0.97	0.07	4.18	0.82
	1.1 gL^{-1} furfural 1.1 gL^{-1} acetic acid	-----	-----	-----	-----	-----	-----
	2.3 gL^{-1} furfural 2.3 gL^{-1} acetic acid	-----	-----	-----	-----	-----	-----

Similarly to the yields, the consortium grown on 3 gL^{-1} of sugars shows a decrease in the maximum specific growth rate, μmax, as 0.4 and 0.8 gL^{-1} of furfural and acetic acid was added when considering both glucose and xylose consumption. When 0.4 gL^{-1} of furfural and acetic acid is present, the μmax decreases from 0.99 to 0.2 hr^{-1} with glucose consumption and from 0.19 to 0.02 hr^{-1} with xylose consumption. The half-saturation constant, K_S, also was decreased with the addition of 0.4 and 0.8 gL^{-1} of furfural and acetic acid. This result is supported by the decrease in yield coefficients as well as the cell mass concentration shown in Table 8.2 and Figure 8.1. A typical value for μmax is 1.3 hr^{-1} for *E. coli* grown on glucose (Fogler, 2006). Comparatively, the consortium grown on 3 gL^{-1} of sugars without furfural and acetic acid is fairly close with

a value of 0.99 hr^{-1} when utilizing glucose consumption. The calculations involve the cell mass growth as well as the sugar consumption. Since the positive control amended with 3 gL^{-1} of sugar utilized all the glucose and xylose within the first 8 hours, only two points were utilized to calculate these constants, thus resulting in the R^2 value of 1. In order to achieve a more representative value, more samples needed to be taken between 0 hours and 8 hours. The increase in sugar to 5 gL^{-1} results in a decrease from 0.99 to 0.49 hr^{-1} with glucose consumption, but showed an increase from 0.19 to 0.25 hr^{-1} with xylose consumption. By including 0.6 gL^{-1} of furfural and acetic acid with 5 gL^{-1} of sugars, the μ_{max} decreased by a factor of 10 for both glucose and xylose consumption. In addition, the K_S constant also decreased with the addition of furfural and acetic acid from 7.39 gL^{-1} and 3.95 gL^{-1} to 2.54 gL^{-1} and 2.27 gL^{-1} for glucose and xylose consumption, respectively. With 7 gL^{-1} of sugars, the μmax increased to 0.74 hr^{-1} for glucose consumption and decreased to 0.17 hr^{-1} for xylose consumption. The K_S constant increased to 19.27 gL^{-1} for glucose consumption and 5.21 gL^{-1} for xylose consumption when the consortium was grown with 7 gL^{-1} of sugars. For the growth with 9 gL^{-1} of sugars, the μmax decreased to 0.16 hr^{-1} and 0.07 hr^{-1} for glucose and xylose consumption, which coincides with the yield coefficients, cell mass production, and the sugar consumptions. The K_S constant shows a decrease to 6.78 gL^{-1} and 4.18 gL^{-1} of glucose and xylose consumption, respectively for 9 gL^{-1} of sugars.

Conclusion

The cell mass, lipid mass, sugar consumption, maximum specific growth rate, and overall yield coefficients are inhibited in the presence of furfural and acetic acid. Increasing the total sugar concentrations did not decrease the inhibitory effects of the furfural and acetic acid. At a low lignocellulosic sugar concentration of 3 gL^{-1}, the consortium showed the lease amount of inhibition on the cell mass production with 0.4 and 0.8 gL^{-1} of furfural and acetic acid. The maximum specific growth rate and the Monod constant were affected by the presence of furfural and acetic acid, showing a decrease in both constants as the furfural and acetic acid concentrations increase. However, it is not known how furfural or acetic acid individually inhibits the growth of the consortium. In addition, developing models to describe the inhibition of the consortium by furfural and acetic acid could allow a more accurate prediction of how the consortium will grow when incorporated in the wastewater treatment plant.

CHAPTER IX

PARAMETRIC STUDY OF THE GROWTH OF CONSORTIUM ON GLUCOSE AND XYLOSE WITH FURFURAL AND ACETIC ACID

Introduction

Oleaginous microorganisms are defined as microorganisms that have the ability to accumulate at least 20% of their body weight as lipids (Ratledge, 1994). The lipids that these microorganisms accumulate are mostly composed of triacylglycerides (Alvarez and Steinbuchel, 2002). These triacylglycerides can be converted to fatty acid methyl esters (FAMEs) and glycerol by reacting with an alcohol, such as methanol, and using an acid or base as the catalyst (Jain and Sharma, 2010). Mixtures of FAMEs are commonly known as biodiesel. The FAME profiles of triacylglycerides from oleaginous microorganisms are similar to the profiles produced from common vegetable oils such as soy and rapeseed (Hall et al., 2011). Therefore, oleaginous microorganisms could be a potential lipid feedstock to produce biodiesel, resulting in an increase in feedstock diversity and supply, and consequently reduce cost. An inexpensive and abundant source of nutrients is required to cultivate these microorganisms.

Municipal wastewaters are a potential source of nutrients, such as carbon, nitrogen, and phosphorus to cultivate microorganisms (Hall et al., 2011). Wastewater

contains all nutrients that microorganisms require to thrive (Metcalf & Eddy, 2003). In addition to the nutrients available in wastewater, oleaginous microorganisms require a high carbon to nitrogen ratio to activate the metabolic processes for oil accumulation (Ratledge, 1994). Since the carbon to nitrogen ratio of a typical municipal wastewater is relatively low (Metcalf & Eddy, 2003), an abundant, and preferably a non-food source of carbon, is required to activate oil accumulation pathways. To increase the carbon to nitrogen ratio of municipal wastewater, hydrolyzed biomass could be added to the wastewater. Zhang et al showed substantial growth from a common oleaginous microorganism, Rhodotorula glutinis, when cultivated on a model acid hydrolysate from switchgrass (Zhang et al., 2010). Hydrolysis of biomass results in various sugars, such as glucose and xylose (Petersson and Liden, 2007). Other byproducts generated include furfural and acetic acid (Horvath et al., 2003). These compounds are known inhibitors to microbial growth (Horvath et al., 2003). Furfural has been known to inhibit cell growth, protein and RNA synthesis, and other biological activities (Horvath et al., 2003; Lui et al., 2005; Sanchez, 1988). Acetic acid has been shown to decrease functionality of glycolysis enzymes and block catabolic activity, resulting in inhibition of cell growth (Zhao et al., 2008). The purpose of this chapter is to determine how furfural and acetic acid affect the growth of the oleaginous consortium.

Methodology

Objective 1: Effect of furfural and acetic acid on the consortium when cultivated on glucose amended synthetic wastewater

In Objective 1, the oleaginous microbial consortium was developed using nine oleaginous yeasts and one oleaginous bacterium. The consortium was formed and maintained as described in Hall et al 2010 (Hall et al., 2011). Since municipal wastewater varies frequently in nutrients and composition, a synthetic wastewater was utilized to decrease variability. A synthetic wastewater developed by Ghosh et al was used in this investigation (Ghosh and LaPara, 2004). The concentrations were adjusted to 1 gL^{-1} of ammonium nitrate. Since the main sugar produced in hydrolyzing biomass is glucose, 5gL^{-1} of glucose was the only carbon source used in the experiment. The experiment was designed to determine the effects on cell mass, lipid mass, and glucose consumption when furfural was present in concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 gL^{-1} and the effects on glucose consumption when acetic acid was present in concentrations of 0.5, 0.6, 0.7, 0.8, and 1.5 gL^{-1}. These concentrations are based on screening studies using varying amounts of furfural or acetic acid to determine the optimum range of inhibition. Each concentration was evaluated in triplicate, using 500 mL of the synthetic wastewater with glucose and acetic acid or furfural in 1 L, baffled flasks. The pH of the synthetic wastewater with the additional acetic acid or furfural was adjusted to a pH of 6 before being autoclaved as described in Chapter IV. Since the optimum pH for the members of the oleaginous consortium are between 5 and 7, a pH of 6 was selected to decrease the impact pH would have on the experimental results. Each flask was

inoculated with 30 mL of the oleaginous consortium and grown in a New Brunswick incubator, shaking at 110 rpm and 28°C. Cell mass, lipid mass, glucose concentrations, FAMEs, pH, furfural concentration, and acetic acid concentration were measured as described in Chapter IV.

Objective 2: Effect of furfural and acetic acid on the consortium when cultivated on xylose amended synthetic wastewater

Objective 2 is similar to Objective 1 in the setup. These experiments were conducted using synthetic wastewater with 5 gL^{-1} of xylose to determine what effect furfural or acetic acid had on cell mass, lipid mass, and xylose consumption. The concentration of the chemical components in synthetic wastewater by Ghosh et al was adjusted to 1 gL^{-1} of ammonium nitrate (Ghosh and LaPara, 2004). The furfural concentrations used in this experiment include 0.1, 0.5, 1, 1.5, and 2 gL^{-1}. The acetic acid concentrations tested are 0.75, 1, 1.25, 1.5, and 1.75 gL^{-1}. For each concentration, 500 mL of the synthetic wastewater with xylose and furfural or acetic acid was dispensed into 1 L, baffled flasks and autoclaved. The pH was adjusted to a pH of 6 so that the oleaginous microorganisms would not be inhibited by the low pH that the acetic acid or furfural causes. These flasks were incubated using the same conditions described for Objective 1. Cell mass, xylose concentrations, lipid mass, FAMEs, pH, furfural concentration, and acetic acid concentration was measured using the methods described in Chapter IV.

Results

Objective 1: Effect of furfural and acetic acid on the consortium when cultivated on glucose amended synthetic wastewater

In experiment for Objective 1, the oleaginous microorganism consortium was inoculated into a synthetic wastewater medium supplemented with 5 gL^{-1} of glucose and various furfural and acetic acid concentrations. Figures 9.1and 9.2 show the cell mass concentrations of the consortium grown for 96 hours. Figure 9.1 shows the cell mass production for the consortium with the varying amounts of furfural. These results show that as furfural increases from 0.1 to 0.5 gL^{-1} the cell mass production over the duration of the experiment decreases. From these results, furfural inhibits the consortium's growth through the first 24 hours of incubation for samples containing less than 0.2 gL^{-1} furfural. For those samples containing less than 0.4 gL^{-1} furfural but greater than 0.2 gL^{-1} inhibition of growth was observed for the first 48 hours. When compared to the control, the biomass reduction ranges from 20% with 0.1 gL^{-1} furfural and 80% with 0.5 gL^{-1} of furfural, which is supported by the significant decrease in specific growth rates (Palmqvist and Hahn-Hagerdal, 2000b). Olsson et al showed a 47% growth inhibition of Pichia stipitis with 1 gL^{-1} of furfural present (Olsson and Hahn-Hagerdal, 1996). Furfural is known to cause inactivation of cell replication (Palmqvist and Hahn-Hagerdal, 2000b). In the presence of furfural, NADH is required to reduce the furfural, thus leaving an insufficient amount for terminal respiration (Keating et al., 2006; Taherzadeh et al., 2000). The furfural inhibition can be explained by this insufficient ATP generation that

is required to sustain cell growth, and inhibition on the enzymes directly (Keating et al., 2006; Taherzadeh et al., 2000).

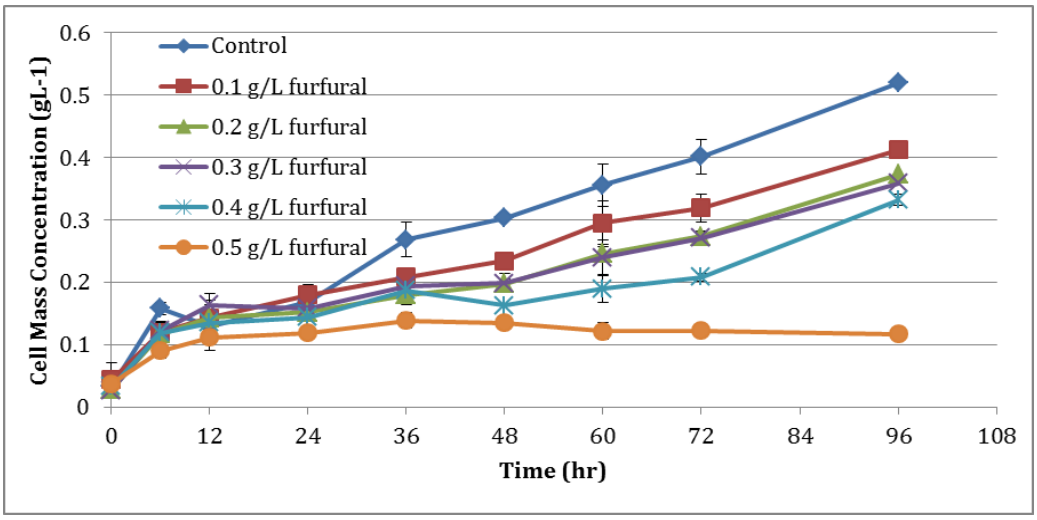

Figure 9.1 Consortium grown on glucose with varying amounts of furfural concentration 0.1 to 0.5 gL^{-1}.

Figure 9.2 shows the cell mass production over the 96-hour sample period for the consortium growth with acetic acid present. These results show that the presence of acetic acid does inhibit the growth of the consortium. With an initial 5 gL^{-1} of acetic acid, the cell mass production is not inhibited when compared to the control with no acetic acid added. However, with the acetic acid concentration increasing from 0.5 to 1.5 gL^{-1}, the inhibition of cell mass production for the 4 concentrations tested does not vary significantly. These results showed an average of 30% reduction in biomass production, which is supported by Phowchinda et al that showed a 20% decrease in biomass production when cultivating Saccharomyces cerevisiae with 1 gL^{-1} acetic acid (Phowchinda et al., 1995). One mechanism of acetic acid inhibition on microbial growth was explained by anion accumulation as shown in Figure 9.3(Palmqvist and Hahn-

Hagerdal, 2000b). The anion accumulation theory is based on the fact that the anionic form of acetic acid is being accumulated within the cell from the undissociated acetic acid diffusing into the cell (Palmqvist and Hahn-Hagerdal, 2000b). The proton released from the dissociation of the acetic acid inside the cell causes the intracellular pH to decrease. To maintain a neutral intracellular pH, the cell expends energy in transporting the proton out of the cell. Because the cell's energy is used to balance the pH, the cell functions are disrupted, thereby, resulting in an inhibition (Palmqvist and Hahn-Hagerdal, 2000b).

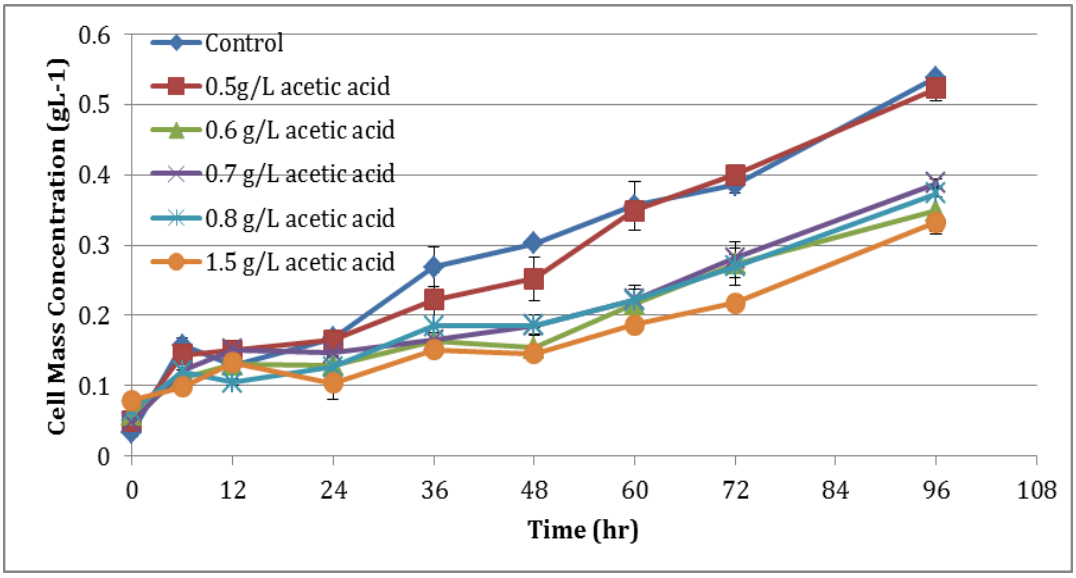

Figure 9.2 Consortium grown on glucose with varying amounts of acetic acid concentration 0.5 to 1.5 gL^{-1}.

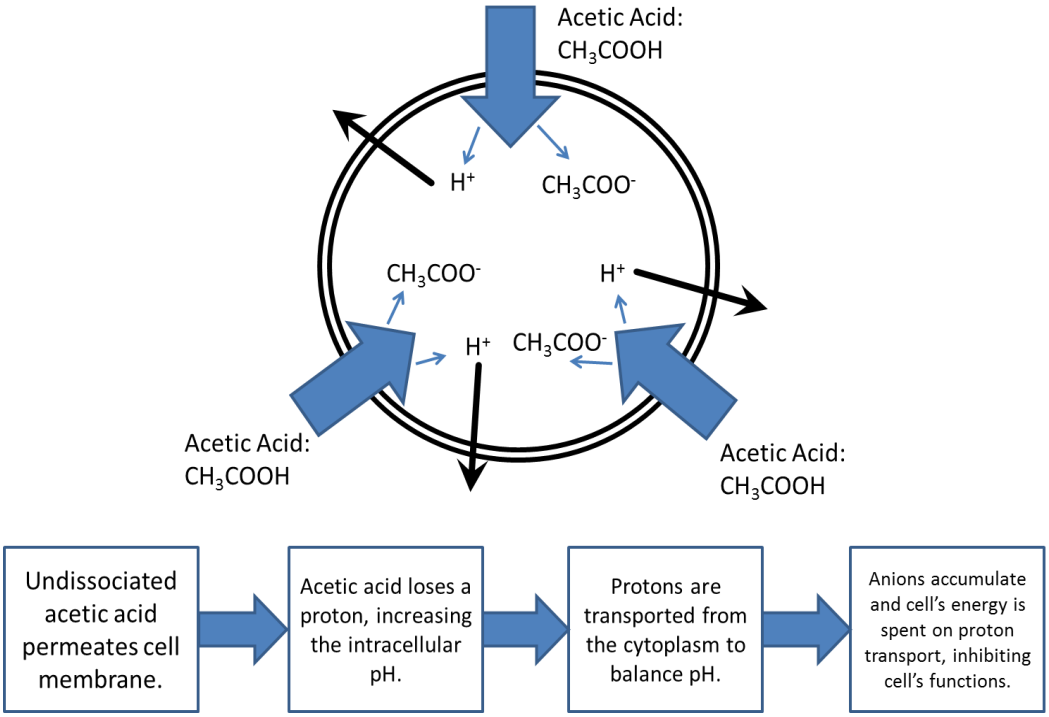

Figure 9.3 Anion accumulation theory for acetic acid inhibiting microorganisms.

Figure 9.4 shows the glucose consumption of the consortium in the presence of various furfural concentrations. The rate of glucose consumption does not seem to be effected with the increasing amounts of furfural compared to the control. All the glucose is consumed within the 72 hours of cultivation for all furfural concentrations tested. Palmqvist et al shows that furfural is often biologically converted into a by-product of furfuryl alcohol and furoic acid (Palmqvist and Hahn-Hagerdal, 2000a). The furfural concentration was determined at each time point and it was observed to be decreasing within the first 6 hours of cultivation for each furfural concentration. This decrease was attributed to the consortium members metabolizing the furfural as evidenced by a new peak after 6 hours of incubation. At the 6-hr point, a separate peak was identified as furfuryl alcohol. It is believed that the cells utilized the glucose to convert the furfural

into furfuryl alcohol instead of producing more cell mass. This further supported by the fact that the glucose consumption did not vary among samples with and without furfural additions. Since the furfural was utilized quickly, the enzymes that metabolize furfural must be expressed by consortium members and are in fact found within the microorganisms of the consortium as reported by Sanchez et al (Sanchez, 1988). This result shows that the consortium produced furfuryl alcohol as a means of detoxifying furfural. Figures 9.5 and 9.6 show the identification in the unknown peak produced during the experiment. With the usage of the identification library, the unknown compound is determined to be 2-Furanmethanol, which is a known by-product of microbial growth with furfural present (Palmqvist and Hahn-Hagerdal, 2000b; Taherzadeh et al., 2000).

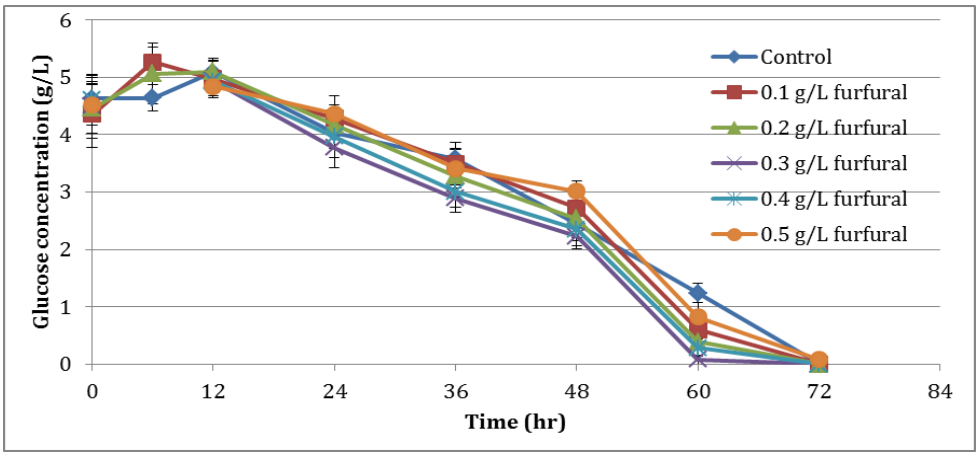

Figure 9.4 Glucose concentrations for the consortium grown on glucose with furfural concentration 0.1 to 0.5 gL^{-1}.

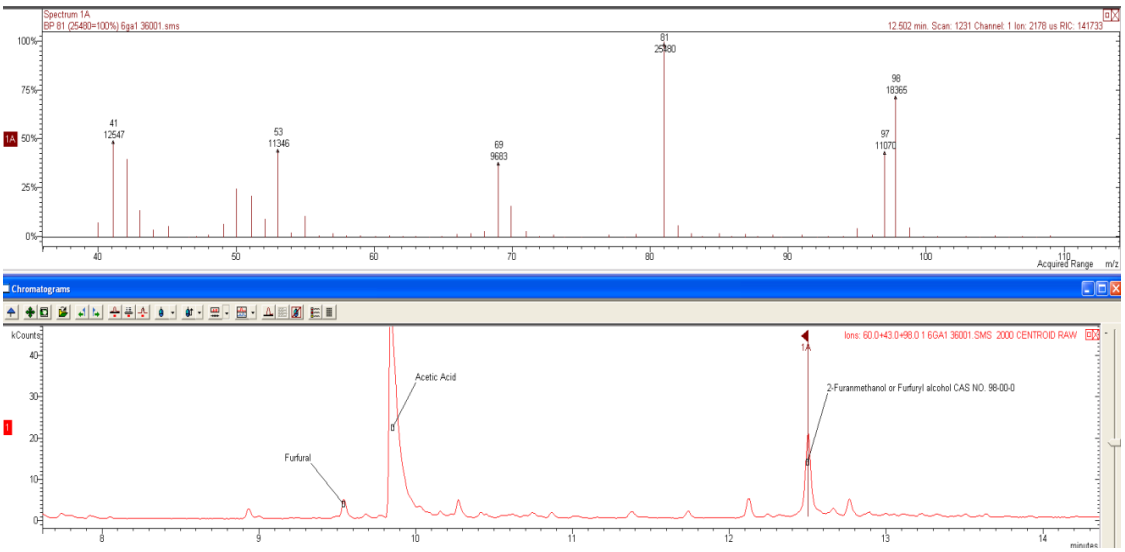

Figure 9.5 On the bottom an extracted ion chromatogram of the sample using a sum of m/z 43, 60, and 98 is shown and labeled. The unknown peak at retention time 12.5 minutes produced an electron impact spectrum shown on top.

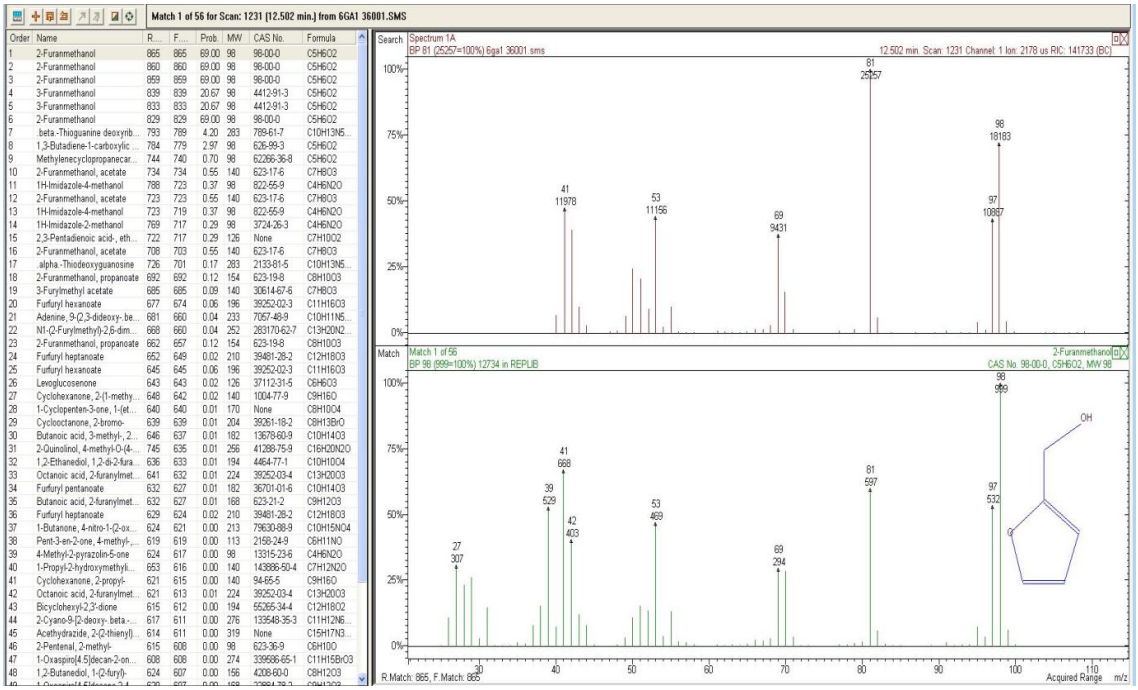

Figure 9.6 The chromatogram of the matches of spectra in the 2005 NIST library to the unknown spectrum producing 2-Furanmethanol as the top 6 to confirm the identification.

Figure 9.7 also shows the glucose consumption of the consortium with acetic acid. The glucose consumption with the presence of furfural is similar to the consumption in the presence of acetic acid. However, the consumption with 0.5 gL^{-1} of acetic acid showed consumption at a quicker rate than even the positive control, and the glucose was completely consumed within 60 hours, which corresponds to the cell mass production. The similarity is also due to the fact that acetic acid does not inhibit the uptake of glucose but does inhibit the cell mass production. In addition to the glucose consumption, acetic acid concentration decreased steadily over the 96-hr cultivation period. The % reduction for the acetic acid consisted of 39% for 0.5 gL^{-1}, 49% for 0.6 gL^{-1}, 38% for 0.7 gL^{-1}, 50% for 0.8 gL^{-1}, and 26% for 1.5 gL^{-1}.

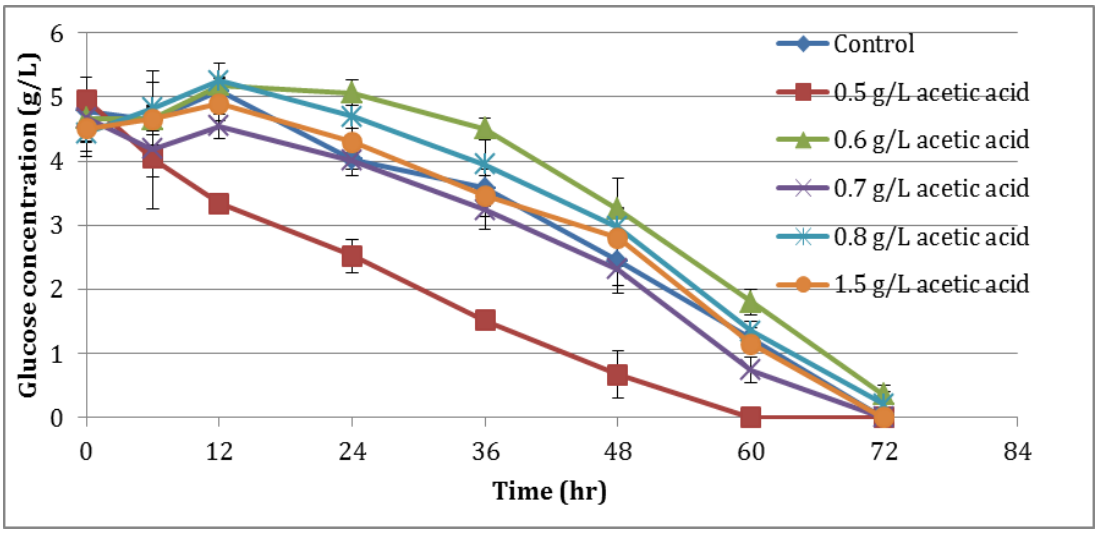

Figure 9.7 Glucose concentrations for the consortium grown on glucose with acetic acid concentration from 0.5 to 1.5 gL^{-1}.

Figure 9.8 shows the lipid production with the addition of furfural. This result is similar to the cell mass production. Lipid production decreased with increasing furfural concentration; at 0.5 gL^{-1} of furfural, no lipid was produced. Oleaginous microorganisms do not accumulate oil until the nitrogen source is eliminated (Certik et al., 1999); therefore, oil accumulation for the consortium does not occur until after approximately 36 hours of cultivation and continues throughout the duration of the experiment. Moreover, since the consortium with 0.5 gL^{-1} of furfural showed minimal growth, the cells did not utilize all the nitrogen. Because nitrogen remains in the media, the cells do not accumulate lipids.

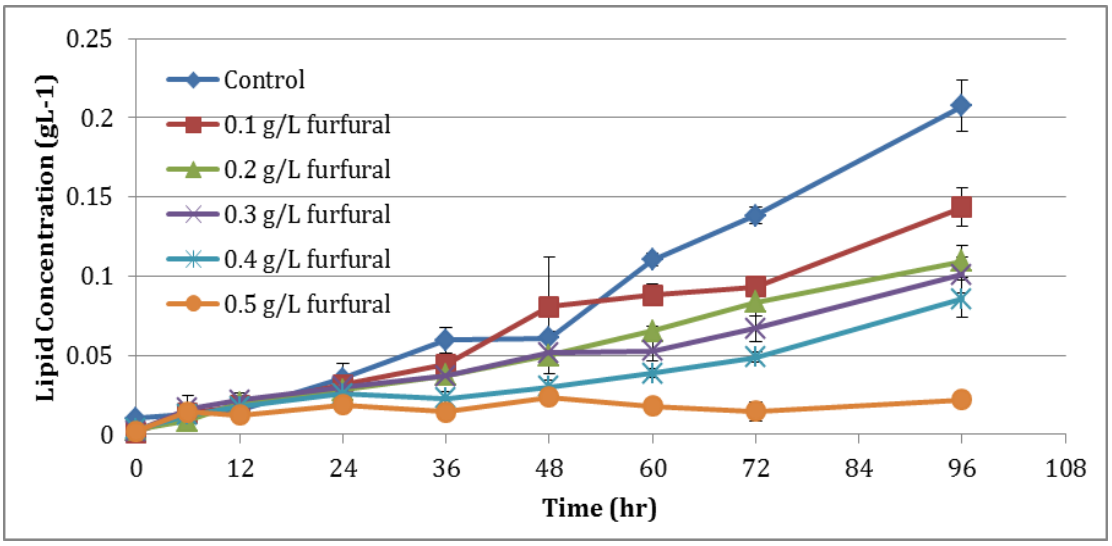

Figure 9.8 Lipid mass concentrations for the consortium grown on glucose with furfural concentration 0.1 to 0.5 gL^{-1}.

Figure 9.9 shows the consortium's lipid production in the presence of acetic acid. The lipid production follows similar trends as the growth trends. Acetic acid does not appear to have an effect on lipid production until it is above 0.5 gL^{-1}. Once the acetic acid concentration reaches 0.6 gL^{-1}, the consortium does not accumulate as much lipids as the control. The lipids produced from the consortium do not change with acetic acid concentrations from 0.6 to 1.5 gL^{-1}. When acetic acid concentration increases, the cells develop the ability to consume the glucose at a higher rate without increasing biomass or products as supported by Pampulha et al (Pampulha and Loureiro-Dias, 2000). Pampulha et al also showed an increase in intracellular pH as well as an elongation of the lag phase (Pampulha and Loureiro-Dias, 2000). Since the cells must maintain a neutral intracellular pH for viability, the cells ability to replicate decreases as well as oil accumulation (Palmqvist and Hahn-Hagerdal, 2000b). This effect could show that the

acetic acid crosses the cell membrane and blocks the enzyme or mechanism to accumulate oil and increase biomass.

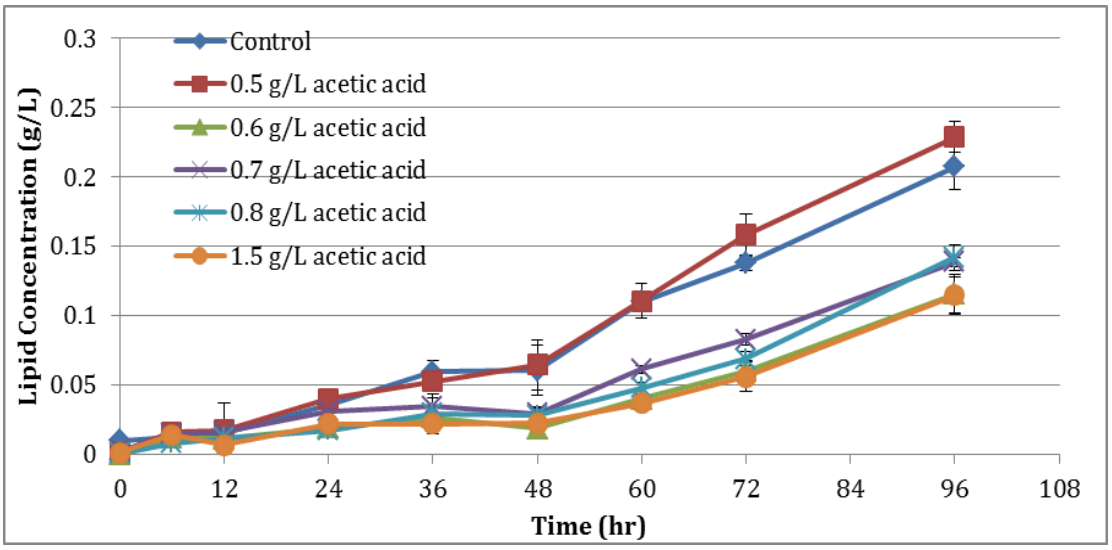

Figure 9.9 Lipid mass concentrations for the consortium grown on glucose with acetic acid concentration from 0.5 to 1.5 gL^{-1}.

Table 9.1 shows the fatty acid profiles, % lipids produced, and total FAMEs produced for the consortium with furfural present after 96 hours of cultivation. FAME profiles are commonly used to determine types of microorganisms (Welch, 1991). These profiles could denote whether the consortium shows a microbial population shift with the inhibitory effects of either furfural or acetic acid. These profiles do not show a population shift with increasing furfural concentrations when compared to the control. The FAMEs produced mostly consist of palmitic and heptadecanoic acid with an increase in oleic acid with increasing furfural concentrations. Gill et al showed that microorganisms FAMEs consisted of palmitic acid when grown on glucose for lipid production (Gill et al., 1977). Hall et al showed this consortium with similar fatty acid

profiles thus showing consistent microbial population (Hall et al., 2011). Thus, a shift in microbial population was not seen in the presence of furfural. However, the increase in oleic acid production has been shown to be a result of stress on microorganisms, especially yeast (You et al., 2003). The % lipid accumulated from the consortium decreases with increasing furfural concentrations, where 0.5 gL^{-1} furfural shows the lowest percentage with 19%. Also, the total FAMEs produced decrease with furfural present when compared to the control. The decrease in total FAMEs is statistically significant relative to the positive control using a t-test with a 0.05 alpha level for all concentrations of furfural. Furfural does inhibit the oil accumulation and overall FAME production of this oleaginous consortium.

Table 9.1 Percentage of lipids accumulated and the total FAMEs produced after 96 hours of cultivation for each furfural concentration.

Compound	Glucose	0.1 gL^{-1} Furfural	0.2 gL^{-1} Furfural	0.3 gL^{-1} Furfural	0.4 gL^{-1} Furfural	0.5 gL^{-1} Furfural
Octanoic (C8:0) %	0.00	0.00	0.00	0.00	0.00	0.00
Decanoic (C10:0) %	0.00	0.00	0.00	0.00	0.00	0.00
Lauric (C12:0) %	0.72	0.76	0.17	0.24	0.27	0.20
Myristic (C14:0) %	2.24	2.61	3.08	3.32	3.73	3.14
Palmitic (C16:0) %	39.58	38.67	39.89	40.72	40.34	39.84
Palmitoleic (C16:1) %	6.69	7.07	7.88	7.22	9.35	8.14
Heptadecanoic (C17) %	30.59	30.50	29.48	29.66	26.43	26.95
Stearic (C18:0) %	9.53	5.73	5.16	5.02	5.67	5.15
Oleic (C18:1n9c) %	0.46	6.26	6.79	6.84	7.91	10.62
Linoleic (C18:2n6c) %	0.50	0.47	0.54	0.41	0.46	1.03
g-Linoleic (C18:3n6) %	7.38	7.41	6.74	6.01	5.22	3.56
Arachidic (C20:0) %	0.41	0.24	0.27	0.27	0.37	0.63
Behenic (C22:0) %	0.94	0.27	0.00	0.00	0.00	0.00
Erucic (C22:1n9) %	0.96	0.00	0.00	0.29	0.24	0.74
Lignoceric (C24:0) %	0.00	0.00	0.00	0.00	0.00	0.00
% Lipid	40	35	30	28	27	19
FAME total (gL-1)	0.133	0.106	0.095	0.099	0.0965	0.039

Table 9.2 shows the fatty acid profiles, % lipids produced, and total FAMEs produced for the consortium with acetic acid present after 96 hours of cultivation. These FAME profiles with acetic acid present show similar results to when furfural is added, where palmitic and heptadecanoic acid are the dominant fatty acids that are present. In addition the microorganisms also show an increase in oleic acid when acetic acid is present. Moreover, the inhibitory effects of acetic acid do not cause a shift in the microbial population of the consortium. The percentage of lipids accumulated decreases with acetic acid present except an increase with 0.5 gL^{-1} of acetic acid when compared to the control. In addition, the total FAMEs show similar results to the % lipids with a decrease when acetic acid is present. The decrease in total FAMEs is statistically significant relative to the positive control using a t-test with a 0.05 alpha level for 0.6 up to 1.5 gL^{-1} of acetic acid. At 0.5 gL^{-1} of acetic acid, the decrease in total FAMEs is not statistically significant when compared to the positive control. Therefore, acetic acid has an inhibitory effect on lipid accumulation and on the total FAMEs produced.

Table 9.2 Percentage of lipids accumulated and the total FAMEs produced after 96 hours of cultivation for each acetic acid concentration.

Compound	Glucose	0.5 gL^{-1} Acetic Acid	0.6 gL^{-1} Acetic Acid	0.7 gL^{-1} Acetic Acid	0.8 gL^{-1} Acetic Acid	1.5 gL^{-1} Acetic Acid
Octanoic (C8:0) %	0	0	0	0	0	0
Decanoic (C10:0) %	0	0	0	0	0	0
Lauric (C12:0) %	0.73	0.98	0.74	0.56	0.44	0.11
Myristic (C14:0) %	2.14	2.41	1.97	2.49	2.13	2.15
Palmitic (C16:0) %	39.06	38.10	39.83	40.85	40.65	41.20
Palmitoleic (C16:1) %	6.71	8.31	11.98	8.49	11.99	9.93
Heptadecanoic (C17) %	30.87	29.91	28.80	31.13	29.15	30.99
Stearic (C18:0) %	9.53	8.10	6.36	6.75	6.87	6.92
Oleic (C18:1n9c) %	0.55	4.66	4.80	4.64	2.65	4.38
Linoleic (C18:2n6c) %	0.33	0.36	0.31	0.45	0.33	0.45
g-Linoleic (C18:3n6) %	7.71	5.05	4.02	3.21	4.04	3.10
Arachidic (C20:0) %	0.32	0.29	0.29	0.38	0.30	0.36
Behenic (C22:0) %	0.63	0.83	0.91	0.61	0.87	0.00
Erucic (C22:1n9) %	1.44	1.00	0.00	0.43	0.56	0.42
Lignoceric (C24:0) %	0	0	0	0	0	0
% Lipid	40	44	33	36	37	32
FAME total (gL^{-1})	0.133	0.128	0.105	0.101	0.108	0.097

Objective 2: Effect of furfural and acetic acid on the consortium grown on xylose amended synthetic wastewater

In this experiment for Objective 2, the consortium is cultivated on synthetic wastewater amended with 5 gL^{-1} of xylose. By adding varying concentrations of furfural or acetic acid, the effect of these compounds on the consortium's ability to growth and utilize nutrients can be determined. Figure 9.10 and 9.11 show the cell mass concentration produced throughout the 96 hours of cultivation.

Figure 9.10 shows the cell mass concentration produced when grown on synthetic wastewater amended with xylose and furfural concentrations ranging from 0.1 to 2 gL^{-1}. This data demonstrates that as furfural concentration increases, the cell mass concentration decreases. However, 0.5 gL^{-1} of furfural resulted in the lowest cell mass

concentration. With the addition of 0.1 gL^{-1} of furfural, the cell mass concentration exhibited an 18.6% reduction when compared to the positive control. The highest reduction of 69.8% is reported with 0.5 gL^{-1} of furfural. A reduction of 37.2% in cell mass production is shown when 1 and 1.5 gL^{-1} of furfural is added. With 2 gL^{-1} of furfural, the cell mass is reduced by 30.2%. These results reveal that concentrations above 0.1 gL^{-1} of furfural inhibit cell growth. When the consortium was cultivated on synthetic wastewater with glucose, the consortium's ability to grow was more sensitive to furfural concentration, showing a range of only 0.1 to 0.5 gL^{-1} of furfural inhibiting consortium growth.

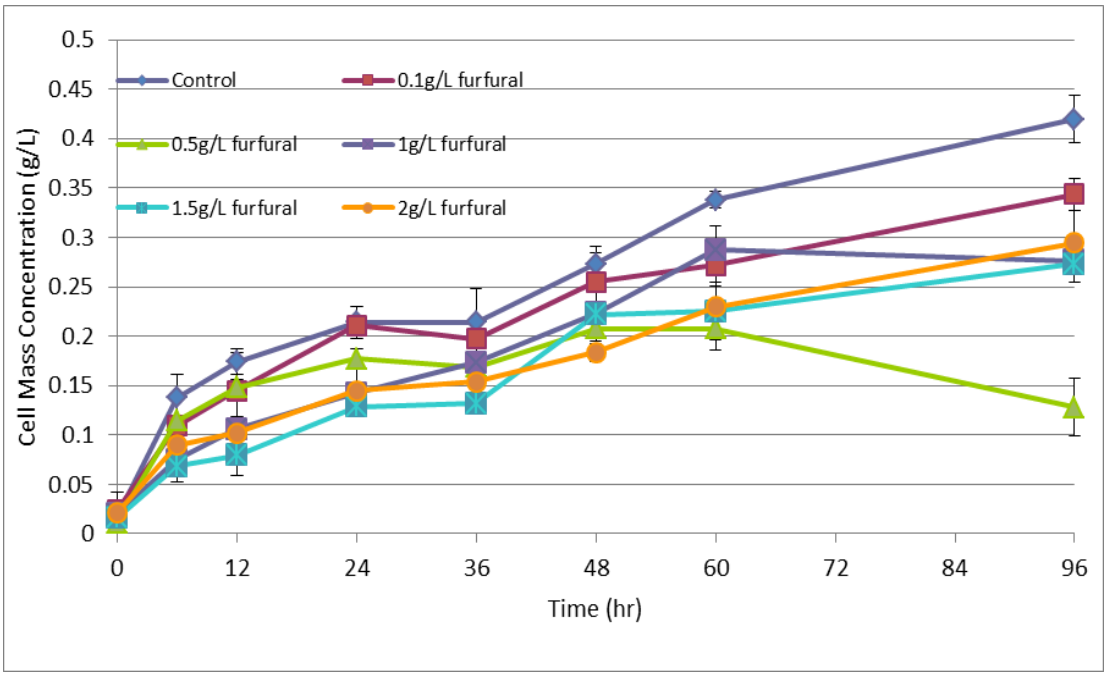

Figure 9.10 Growth of the consortium on synthetic wastewater with 5 gL^{-1} of xylose and furfural concentrations ranging from 0.1 to 2 gL^{-1}.

Figure 9.11 shows the cell mass concentration produced from the consortium with acetic acid concentrations ranging from 0.75 to 1.75 gL^{-1}. This result shows that the addition of acetic acid inhibits the cell mass growth of the consortium on xylose. When 0.75 gL^{-1} of acetic acid is added, the reduction in cell mass is approximately16.7%. With 1 gL^{-1} of acetic acid, the cell mass production shows a 33.3% reduction. Acetic acid concentrations of 1.25 and 1.5 gL^{-1} results in approximately 25% reduction in cell mass. The highest reduction of 50% in cell mass concentration is shown with 1.75 gL^{-1} of acetic acid. These reductions coincide with the reductions seen when the consortium is grown on glucose in the presence of acetic acid.

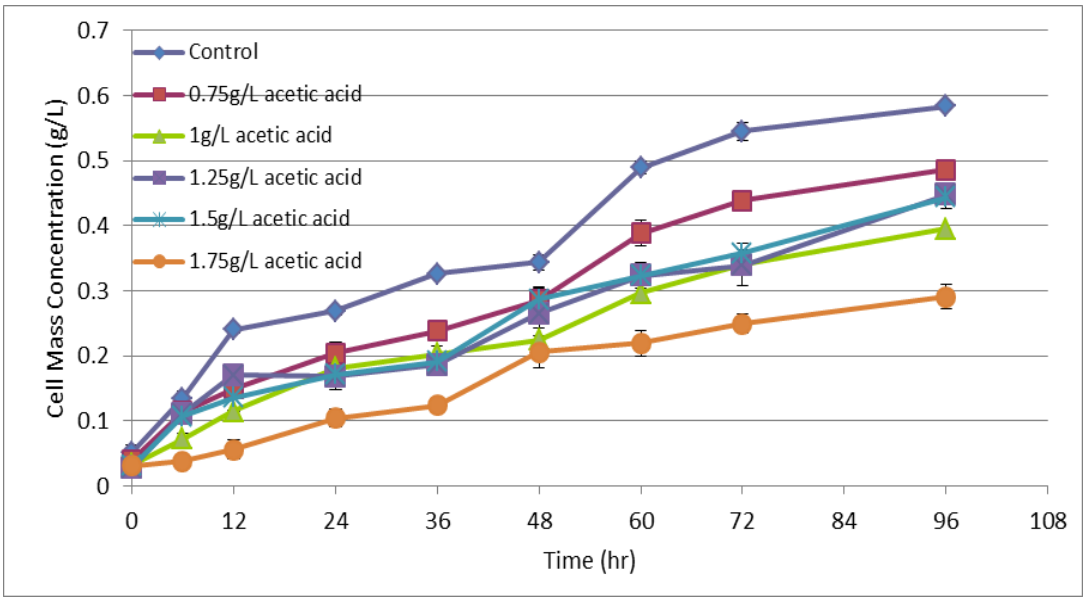

Figure 9.11 Growth of the consortium on synthetic wastewater with 5 gL^{-1} of xylose and acetic acid concentrations ranging from 0.75 to 1.75 gL^{-1}.

In addition to cell mass concentration, the xylose concentration was also measured over the 96-hour cultivation. The xylose concentrations for each concentration

of furfural are shown in Figure 9.12. For each concentration of furfural, the xylose concentration shows a steady decrease, except for 0.5 gL^{-1} of furfural. This finding is supported by the cell mass concentration data shown in Figure 9.10 where the cell mass decrease beginning at the 60 hr reading and continuing until the 96 hr measurement. This result is similar to the glucose consumption in that the consumption of the main carbon source is not negatively affected by the furfural. Since this is the same consortium used in the glucose experiments a similar detoxification process would be expected with xylose as the substrate. In other words, the consortium could be consuming the xylose to convert the furfural into a less-inhibiting substance.

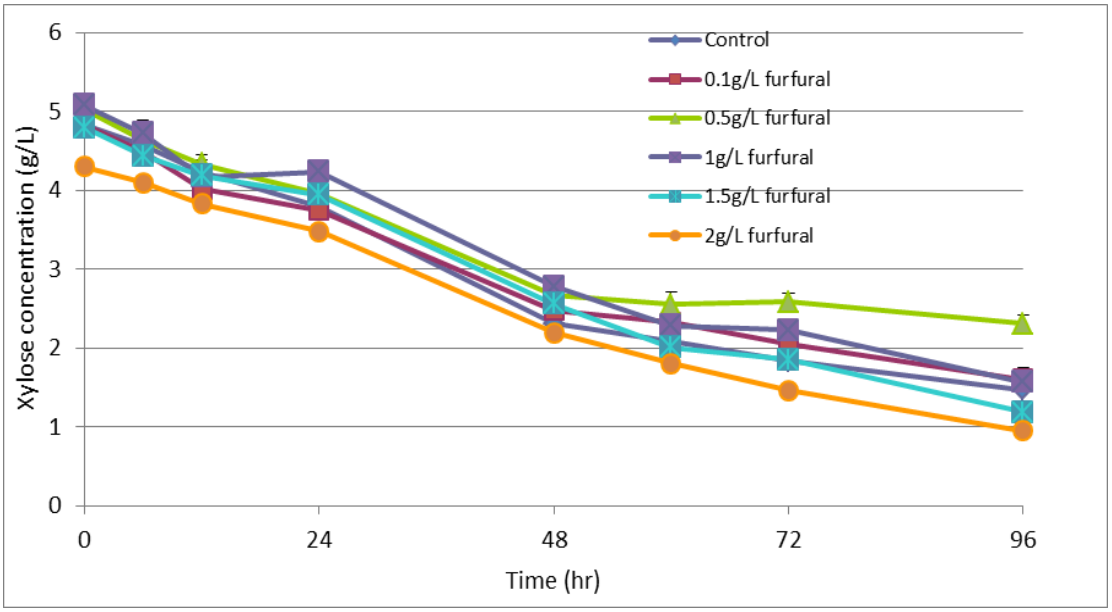

Figure 9.12 Xylose concentrations for the growth of the consortium on synthetic wastewater with furfural concentrations 0.1 to 2 gL^{-1}.

For the acetic acid, Figure 9.13 exhibits the results for the xylose consumption through 96-hours of cultivation. The data in this figure demonstrates that the xylose

consumption does not change with respect to acetic acid concentration when compared to the positive control. The xylose concentration only showed an average reduction of 1 gL^{-1} for the first 48 hours of cultivation. From 48 to 72 hours, the xylose concentration indicates a steady decrease to approximately 2 gL^{-1} of xylose. Overall, 3 gL^{-1} xylose was consumed within 72 hours. Based on this data, inhibition is not as clear as it is with the cell mass over time data. Therefore, the acetic acid also does not inhibit xylose consumption. When compared to the glucose consumption, the acetic acid has more effect on the glucose consumption than on the xylose consumption.

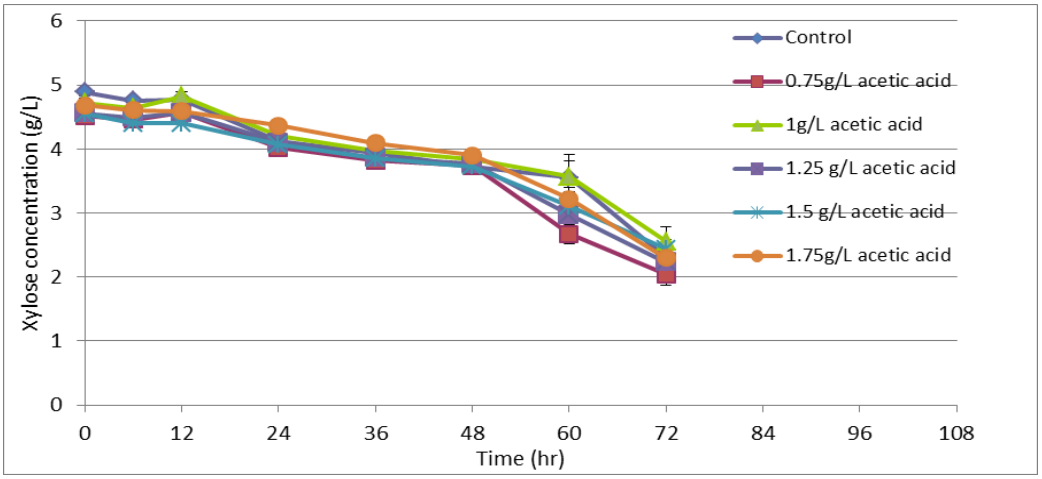

Figure 9.13 Xylose concentrations for the growth of the consortium on synthetic wastewater with acetic acid concentrations 0.75 to 1.75 gL^{-1}.

The purpose of cultivating this consortium on synthetic wastewater is to accumulate lipids in a wastewater treatment facility. Therefore, the lipid mass concentration is shown in Figures 9.14 and 9.15. Figure 9.14 presents the lipid mass-produced by the consortium growing on synthetic wastewater amended with xylose and furfural of varying concentrations. The result indicate that the lipid mass is highly

inhibited by the increasing furfural concentrations with the positive control, demonstrating a steady increase to a maximum lipid mass of 0.14 gL^{-1}. For furfural concentrations of 0.5 to 2 gL^{-1}, the lipid mass increases for the first 12 hours and then exhibits a steady decrease at the 48 hours. From the 48-hour measurement, the lipid mass for concentrations 1, 1.5, and 2 gL^{-1} of furfural shows an increase from 0.02 gL^{-1} to approximately 0.06 gL^{-1}. However, the 0.5 gL^{-1} of furfural shows approximately no change in lipid mass from 48 to 96 hours, which coincides with the cell mass production shown in Figure 9.10. This result shows that in addition to cell mass inhibition, furfural also inhibits the overall increase in lipid mass accumulated. Similar results were observed when the consortium is cultivated with glucose and furfural. Since the cells are dependent upon the generation of NADH/NADPH for the production of lipids and these compounds are needed to detoxify the furfural, the results agree with the theory of inhibition and past research (Palmqvist and Hahn-Hagerdal, 2000b).

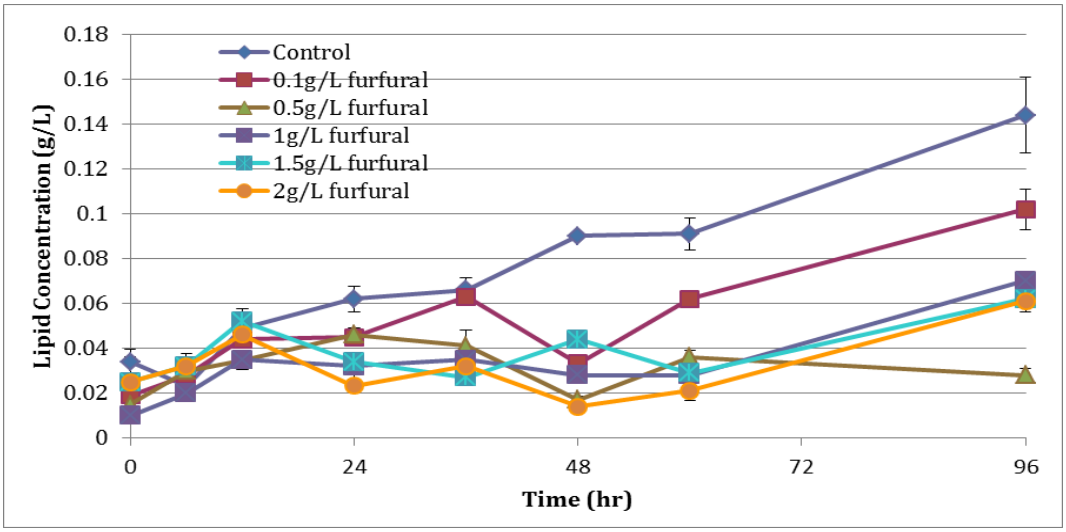

Figure 9.14 Lipid mass concentration of the consortium grown on synthetic wastewater with 0.1 to 2 gL^{-1} of furfural.

Figure 9.15 presents the data for the lipid mass concentration for the consortium grown with xylose and acetic acid concentrations from 0.75 to 1.75 gL^{-1}. The lipid mass illustrates an overall decrease as the acetic acid concentration increases. The decrease in lipid mass produced is statistically significant using a t-test with an alpha level of 0.05 in comparison to the positive control. The consortium shows a increase from 12 hours to 96 hours with 0.2 gL^{-1} of lipids accumulated at 96 hours in comparison to the positive control. With acetic acid present, the lipid mass shows a delay in lipid mass production with a small increase for the first 48 hours. After 48 hours, the lipid mass shows a greater increase, resulting in the maximum lipid mass ranging from 0.12 to 0.15 gL^{-1}. There is no statistically significant difference among the lipid mass produced for 1, 1.25, and 1.5 gL^{-1} of acetic acid, using a t-test with a 0.05 alpha level. The least amount of lipids produced was demonstrated with 1.75 gL^{-1} of acetic acid as 0.075 gL^{-1}. This result supports the cell mass data shown in Figure 9.11. This result also coincides with the consortium grown with glucose and acetic acid.

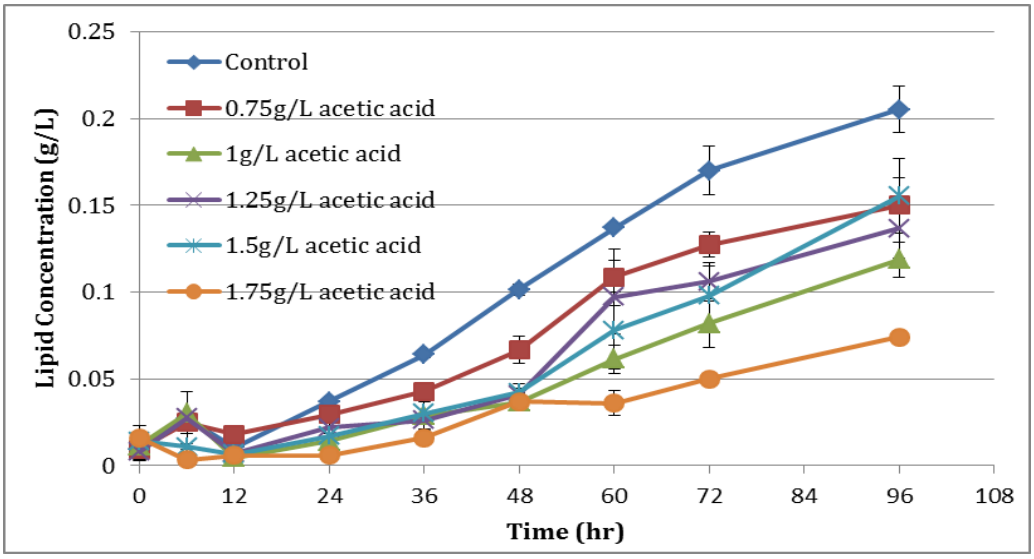

Figure 9.15 Lipid mass concentration of the consortium grown on synthetic wastewater with 0.75 to 1.75 gL^{-1} of acetic acid.

In addition to cell mass, lipid mass, and xylose consumption, fatty acid profiles were used to determine inhibitory effects of the furfural and acetic acid on the consortium. Table 9.3 and 9.4 show the fatty acid profile, percent lipids, and total FAMEs for each concentration of furfural and acetic acid. Table 9.3 presents the fatty acid profiles for the consortium grown with xylose and furfural. For each furfural concentration and positive control, the main fatty acids produced consist of palmitic and heptadecanoic acid. As the furfural concentration increases, the fatty acid profile demonstrates a slight decrease in lauric acid, palmitoleic acid, stearic acid, and oleic acid. An increase in myrisitc acid, palmitic acid, and heptadecanoic acid is shown in the fatty acid composition as the furfural concentration increases while relatively no change in fatty acids larger than linoleic acid. *R. opacus* is an oleaginous bacteria which commonly is known to produce heptadecanoic acid in its fatty acid profile (Waltermann et al., 2000). The increase in heptadecanoic acid could possibly show that *R. opacus* is not as inhibited

by the presence of furfural as the other microorganisms in the consortium. The lipid percentage is shown in Table 9.3 and shows a decrease from 1 to 2 gL^{-1} of furfural concentration when compared to the positive control. For concentrations of 0.1 and 0.5 gL^{-1} of furfural, the lipid percentage is not statistically significantly different from the positive control when using a t-test with an alpha level of 0.05. The total FAMEs produced show an overall reduction as the increase in furfural concentration. When compared to the positive control, the total FAMEs demonstrate a statistically significant decrease using the t-test with a 0.05 alpha level when the furfural concentrations are 0.5 gL^{-1} and above.

Table 9.3 Fatty acid profiles for the consortium grown on synthetic wastewater with xylose and 0.1 to 2 gL^{-1} of furfural.

Compound	Xylose	0.1 gL^{-1} furfural	0.5 gL^{-1} furfural	1 gL^{-1} furfural	1.5 gL^{-1} furfural	2 gL^{-1} furfural
Octanoic (C8:0) %	0	0	0	0	0	0
Decanoic (C10:0) %	0	0	0	0	0	0
Lauric (C12:0) %	0.09	0.04	0.14	0.39	0.08	0.09
Myristic (C14:0) %	2.62	2.70	3.88	3.11	2.97	2.69
Palmitic (C16:0) %	38.99	39.93	38.79	43.75	44.81	44.78
Palmitoleic (C16:1) %	10.90	11.98	14.11	6.68	3.82	4.19
Heptadecanoic (C17) %	27.09	25.73	18.94	30.78	35.29	34.02
Stearic (C18:0) %	6.83	5.38	4.16	4.69	4.34	4.75
Oleic (C18:1n9c) %	10.09	10.92	13.95	6.09	5.12	5.66
Linoleic (C18:2n6c) %	0.67	0.74	1.80	0.73	0.71	0.70
g-Linoleic (C18:3n6) %	0.19	0.23	0.53	0.24	0.24	0.23
Arachidic (C20:0) %	0.36	0.26	0.82	0.35	0.35	0.36
Behenic (C22:0) %	0.97	0.94	0.57	1.05	1.08	0.91
Eruic (C22:1n9) %	1.18	0.87	2.31	2.14	1.21	1.61
Lignoceric (C24:0) %	0	0.28	0	0	0	0
% Lipid	34.3	29.7	19.6	25.2	22.5	20
FAME total (gL^{-1})	0.129	0.117	0.044	0.099	0.083	0.098

Table 9.4 presents the fatty acid profiles for the consortium grown on xylose with acetic acid concentrations. The fatty acid profiles mainly consist of palmitic acid and heptadecanoic acid. With increasing acetic acid concentrations, the myristic acid, stearic acid, oleic acid, g-linoleic acid, and erucic acids decrease. Palmitic acid and heptadecanoic acid show an increase as the acetic acid increases. This result in fatty acid profiles is similar to the results when grown with furfural. The percent lipids show a decrease when acetic acid is added. The lipid percentage is statistically significantly decreased with 1.75 gL^{-1} of acetic acid. Furthermore, the total FAMEs produced show a statistically significant decrease using a t-test with an alpha level of 0.05 as the acetic acid increases when compared to the positive control.

Table 9.4 Fatty acid profiles for the consortium grown on synthetic wastewater with xylose and 0.75 to 1.75 gL^{-1} of acetic acid.

Compound	Xylose	0.75 gL^{-1} acetic acid	1 gL^{-1} acetic acid	1.25 gL^{-1} acetic acid	1.5 gL^{-1} acetic acid	1.75 gL^{-1} acetic acid
Octanoic (C8:0) %	0	0	0	0	0	0
Decanoic (C10:0) %	0	0	0	0	0	0
Lauric (C12:0) %	0.25	0.29	0.16	0.30	0.27	0.05
Myristic (C14:0) %	1.88	1.85	1.41	1.43	1.32	1.49
Palmitic (C16:0) %	37.03	27.07	37.68	38.52	38.61	38.18
Palmitoleic (C16:1) %	8.28	7.59	8.79	7.72	7.03	9.24
Heptadecanoic (C17) %	29.29	40.17	31.22	34.34	34.93	29.65
Stearic (C18:0) %	8.14	7.89	8.53	6.07	6.54	7.38
Oleic (C18:1n9c) %	8.47	8.19	7.70	6.82	6.40	9.04
Linoleic (C18:2n6c) %	0.22	0.33	0.29	0.29	0.29	0.32
g-Linoleic (C18:3n6) %	4.95	4.25	2.91	3.09	3.14	3.50
Arachidic (C20:0) %	0.31	0.56	0.43	0.47	0.49	0.43
Behenic (C22:0) %	0.94	1.22	0.90	0.97	0.97	0.71
Eruic (C22:1n9) %	0.25	0.59	0	0	0	0
Lignoceric (C24:0) %	0	0	0	0	0	0
% Lipid	35.2	30.8	30	30.5	31.9	24
FAME total (gL^{-1})	0.151	0.126	0.125	0.129	0.128	0.106

Conclusion

The consortium grown on synthetic wastewater amended with glucose shows an inhibition when furfural or acetic acid was present. Furfural and acetic acid have shown inhibition on cell growth and lipid accumulation of the consortium. The presence of these inhibitors did not have a large impact on glucose consumption. The FAME analysis has shown that the microbial population of the consortium does not shift with these inhibitors present.

When the consortium was cultivated on synthetic wastewater with xylose, the furfural and acetic acid inhibited the cell mass and lipid mass production. However, the presence of these two compounds did not affect the consortium's ability to consume the xylose. The FAME analysis did show a potential microbial population shift due to the increase in Heptadecanoic acid. The total FAMEs produced did show a decrease for each concentration of furfural and acetic acid.

CHAPTER X

DEVELOPMENT OF MODEL TO DESCRIBE GROWTH INHIBITION OF CONSORTIUM ON GLUCOSE AND XYLOSE WITH FURFURAL AND ACETIC ACID

Introduction

Biodiesel is produced using oil from various sources, including plants such as soybeans. To aid in the growing demand for biodiesel, oleaginous microorganism have shown to be a viable alternative in oil production for biodiesel. Utilizing wastewater as a growth medium for these microorganisms provides water and nutrients that these microorganisms need to thrive. By using lignocellulosic sugars that are produced from hydrolyzing biomass, the carbon to nitrogen ratio is increased in wastewater. This increase in carbon allows for an environment conducive for oil accumulation. Along with increasing the carbon, the lignocellulosic sugars contain two main inhibiting compounds, acetic acid and furfural.

Chapter IX discussed the extent to which furfural and acetic acid inhibited the growth of the oleaginous microorganism consortium. This chapter is focused on developing a model that describes the growth inhibition of furfural and acetic acid on the oleaginous consortium cultivated on synthetic wastewater, amended with glucose or

xylose. Although both of the substrates are sugars, they are transported into the cells by different mechanisms and metabolized by different pathways and therefore are not readily described with a single model. The Monod and Contois equations were modified to model inhibition using glucose or xylose as the main carbon sources, respectively.

Methodology

Objective 1: Effect of furfural or acetic acid on the consortium when cultivated on glucose amended synthetic wastewater

In order to describe the growth of the consortium, the Monod model was initially applied to the data. Equation 9.1 shows the Monod equation with net specific growth rate, μ, reaction rate constant, k, substrate concentration, C_A, and Monod constant, C_M. For each inhibitor concentration, the reaction rate constant k and Monod constant (C_M) were determined using Polymath 5.1 non-linear regression. The Monod equation was developed to describe typical microbial growth (Shuler and Kargi, 2002). Because the Monod equation does not describe all phases of microbial growth, initial and final points (acclimation and stationary phases) were eliminated in the model development.

$$\mu = k\frac{C_A}{C_M + C_A} \qquad [10.1]$$

Since furfural and acetic acid have been shown to inhibit the growth of the consortium, an inhibition model must be evaluated to fully describe this effect on the microorganisms in the oleaginous consortium. Three different inhibition models Andrews, Equation 10.2, (Andrews, 1968; Grady et al., 1999); Aiba, Equation 10.3,

(Aiba et al., 1968; Han and Levenspiel, 1988); and a Tessier-type inhibition model, Equation 10.4, (Han and Levenspiel, 1988) were chosen to describe the data. Each inhibition equation consists of specific growth rate, μ, reaction rate constant, k, substrate concentration, C_A, inhibition constant, K_I, and Monod constant, C_M. To fit the data to the equation, Polymath 5.1 non-linear regression was used to determine the constants k and K_I in each inhibition model while using the Monod constant, C_M, from the Monod equation. These constants were determined in the same manner for each inhibitor concentration of furfural and acetic acid.

$$\mu = k\frac{C_A}{(C_A + C_M)(1 + {}^{C_A}\!/_{K_I})} \qquad [10.2]$$

$$\mu = k\frac{C_A \exp(-C_A/K_I)}{C_A + C_M} \qquad [10.3]$$

$$\mu = k\left[\exp(-\frac{C_A}{K_I}) - \exp(-\frac{C_A}{C_M})\right] \qquad [10.4]$$

The Andrews model (Equation 10.2) has been shown to describe inhibition effects in wastewater (Grady et al., 1999; Nakhla et al., 2005). This equation is based on the Haldane model, describing enzyme inhibition kinetics (Andrews, 1968). The governing assumption involves the formation of an inactive enzyme-substrate complex with two substrate molecules per enzyme (Tan et al., 1996). When this model was applied to the experimental data, the model did not fit the data potentially due to the fact that this equation is based on the inhibition of substrate consumption. Since the presence of furfural or acetic acid did not result in inhibition of substrate consumption as shown in Chapter IX, this equation did not describe the growth inhibition. Table 10.1 shows a

Polymath 5.1 output as an example of how the Andrews model does not fully describe the growth inhibition.

Table 10.1 Polymath 5.1 output for the fit of Andrews model to experimental data from glucose with acetic acid.

Nonlinear regression (L-M)

Model: u = k*CA/((CA+CM)*(1+CA/KI))

Variable	Ini guess	Value	95% confidence
k	0.1	0.0285356	3.027E-05
KI	10	1010.9896	250.1774

Nonlinear regression settings
Max # iterations = 64

Precision

R^2	= 0.2591935
R^2adj	= 0.1357257
Rmsd	= 0.0021489
Variance	= 4.925E-05

General

Sample size	= 8
# Model vars	= 2
# Indep vars	= 2
# Iterations	= 32

The Aiba model, Equation 10.3, is a modification of the Monod equation that was originally developed to describe product inhibition, specifically ethanol inhibition, with glucose as the carbon source (Aiba et al., 1968). The modification was determined to be an exponential function of product or inhibitor concentration by plotting semi-logarithmic specific growth rate versus product concentration. Similarly to the Andrews model, the Aiba model did not fit the data because this model is dependent on the inhibition of substrate consumption and substrate consumption was not affected with furfural or acetic acid present. Table 10.2 shows a Polymath 5.1 output example of fitting the Aiba equation to the data and the lack of fit as shown by the low R^2 values.

Table 10.2 Polymath 5.1 output for the fit of Aiba model to experimental data.

Nonlinear regression (L-M)

Model: u = k*CA*exp(-CA/KI)/(CA+CM)

Variable	Ini guess	Value	95% confidence
k	0.1	0.0283654	3.029E-06
KI	10	1010.9985	25.069033

Nonlinear regression settings

Max # iterations = 64

Precision

R^2 = 0.2583258

R^2adj = 0.1347134

Rmsd = 0.0021501

Variance = 4.931E-05

General

Sample size = 8

Model vars = 2

Indep vars = 2

Iterations = 24

The Tessier-type inhibition model is based on the Tessier model shown in Equation 10.5, which is based on assuming diffusion-controlled substrate [Luong et al., 1986]. The Tessier model includes net specific growth rate, μ, reaction rate constant, k, substrate concentration, C_A, and Monod constant, C_M (Shuler and Kargi, 2002).

$$\mu = k(1 - \exp(-\frac{C_A}{C_M})) \qquad [10.5]$$

The inhibition model, Equation 10.4, was based on the assumption of combining the mechanism for the Tessier model, Equation 10.5, with a protective diffusional limitation for high concentrations of inhibitory compounds (Luong, 1986). When this model was applied to the data, the model did not fit the data as observed by the low R^2 value. Table 10.3 is a Polymath 5.1 output example of how the Tessier-type inhibition model fit the experimental data.

Table 10.3 Polymath 5.1 output example of fitting Tessier-type inhibition model to experimental data.

Nonlinear regression (L-M)

Model: u = k*(exp(-CA/KI)-exp(-CA/CM))

Variable	Ini guess	Value	95% confidence
k	0.1	0.0225535	7.671E-05
KI	5	505.97163	183.74796

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.231723
R^2adj	= 0.1036768
Rmsd	= 0.0021883
Variance	= 5.108E-05

General

Sample size	= 8
# Model vars	= 2
# Indep vars	= 2
# Iterations	= 28

Based on the results of the Polymath analysis, these models did not describe the data adequately as shown by the low R^2 values. A different model needs to be developed to describe the results of the inhibition experiments. Since the Monod model fit the data with a higher R^2 value than the previous models tested, then a modification to this model to incorporate the presence of inhibitors might improve the fit to the experimental data. This strategy is similar to that applied by Andrews, Equation 10.2. Since the constants k and CM from the Monod equation resulted in different values for each inhibitor concentration, a relationship could be established to describe the inhibition of the consortium's growth by plotting the constants versus the inhibitor concentration. A mathematical relationship was determined by applying a trend line. The non-linear trend lines, exponential and power functions, showed an R2 fit greater than 0.90. An example of the trend line for the relationship of furfural concentration and reaction rate constant, k, is shown in Figure 10.1.

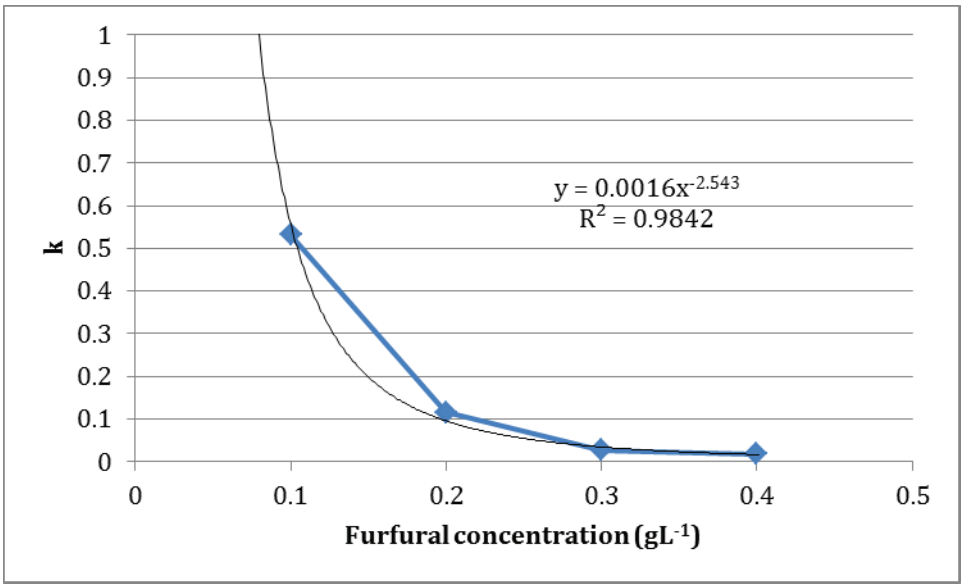

Figure 10.1 Reaction rate constant k versus furfural concentration with a power law trend line applied.

The relationship shown in Figure 10.1 and the relationship of furfural concentration to Monod constant, C_M, was incorporated into the Monod model. This modification is used to describe the effect of furfural on the consortium's growth with glucose as the main source of carbon. Equation 10.6 shows an example of incorporating these mathematical relationships for furfural inhibition into the Monod equation. This method of equation development also applies to acetic acid inhibition with glucose.

$$\mu = 0.0016 C_I^a \frac{C_A}{102 C_I^b + C_A} \qquad [10.6]$$

Polymath 5.1 non-linear regression to determine constants a and b for different equation variations were used to fit the data for all inhibitor concentrations. The best fit was with the modified equation that describes the inhibited growth of the consortium. The Polymath output is shown in Appendix A.

Objective 2: Effect of furfural or acetic acid on the consortium grown on xylose amended synthetic wastewater

Developing a model to describe the furfural and acetic acid inhibition on the consortium amended with xylose followed a similar method. However, the Monod model did not fit the individual inhibitor concentrations for either acetic acid or furfural. Therefore, other models such as Moser, Equation 10.7; Tessier, Equation 10.8; and Contois, Equation 10.9, were explored (Aguiar et al., 2002; Shuler and Kargi, 2002). Each inhibition equation consists of specific growth rate, μ, reaction rate constant, k, substrate concentration, C_A, and Monod constant, C_M.

$$\mu = k(1 + C_M C_A^{-\lambda})^{-1} \quad [10.7]$$

$$\mu = k\left[1 - \exp(-\frac{C_A}{C_M})\right] \quad [10.8]$$

$$\mu = k\frac{C_A}{A * C_C + C_A} \quad [10.9]$$

The Moser equation involves an empirical constant, λ, and the Contois equation involves the apparent saturation constant, A, with cell mass concentration, C_C. The Moser equation is a model that is a modified Monod equation that is also commonly used to describe cell growth (Shuler and Kargi, 2002). The Tessier model is based on the diffusion-controlled substrate assumption to describe microbial growth (Luong, 1986). The Contois model is typically used to describe substrate-limited growth with high cell densities; this model also is structured similarly to Monod model with the exception of the Monod constant as a function of cell mass concentration (Contois, 1959; Shuler and

Kargi, 2002). Table 10.4 shows how each equation fit to the experimental data using non-linear regression in Polymath.

Table 10.4 R^2 values comparing the equation fit to experimental data from Polymath

Acetic Acid (gL^{-1})	Tessier	Moser	Contois
	R^2	R^2	R^2
0.75	0.68	0.88	0.94
1	0.44	0.74	0.97
1.25	0.6	0.81	0.99
1.5	0.46	0.73	0.99
1.75	0.45	0.68	0.99

Based on these results, the Contois model fit the data the best with R^2 values above 0.8. The Polymath output can be seen in Appendix B for the different variations of the Contois model. The remaining steps in the model development are the same as the methods for developing a model to describe furfural and acetic acid inhibition on the growth of the consortium on synthetic wastewater amended with glucose.

Results

Objective 1: Effect of furfural and acetic acid on the consortium when cultivated on glucose amended synthetic wastewater

The Monod model was modified to describe the inhibition of furfural on the growth of the consortium in synthetic wastewater with glucose as the primary carbon source. The first step in modifying the Monod model was to determine the Monod constants. Table 10.5 shows the results and goodness of fit for each furfural concentration. Since the Monod results showed higher values than 0.3 and 0.4 gL^{-1} of furfural for reaction rate constant k and higher values than 0.2, 0.3, and 0.4 gL^{-1} for Monod constants, the 0.5 gL^{-1} furfural concentration was eliminated from the model development. At furfural concentrations higher than 0.1 gL^{-1}, the Monod constant shows a steep decrease in value with increasing furfural concentration.

Table 10.5 Monod model constants determined for each furfural concentration.

Furfural concentration (gL^{-1})	k	C_M	R^2
0	0.718	102	0.8
0.1	0.531	102	0.94
0.2	0.116	11.1	0.99
0.3	0.0265	2.01	0.83
0.4	0.0181	1.695	0.69
0.5	0.0712	100	0.23

The next step involved plotting furfural concentration versus each constant and applying trend lines that fit the data best. Figure 10.2 shows the reaction rate constant k as a function of furfural concentration. The two trend lines that fit best consisted of the power and exponential function with R^2 of 0.98 and 0.95, respectively. Figure 10.3 shows the Monod constant as a function of furfural concentration. Similarly to the reaction rate constant k, the best-fit trend lines consisted of power and exponential function with R^2 of 0.97 and 0.90, respectively.

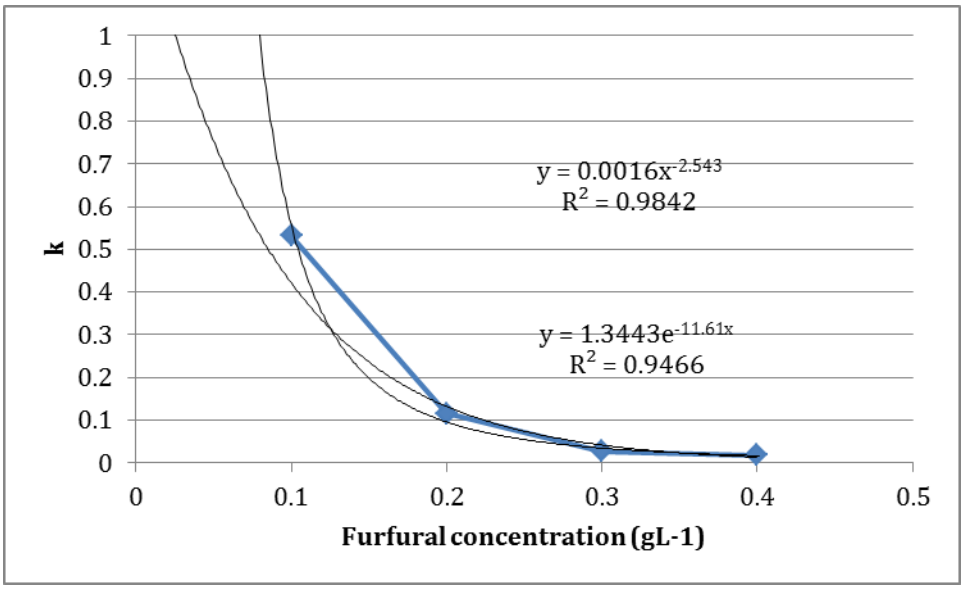

Figure 10.2 Reaction rate constant k versus furfural concentration.

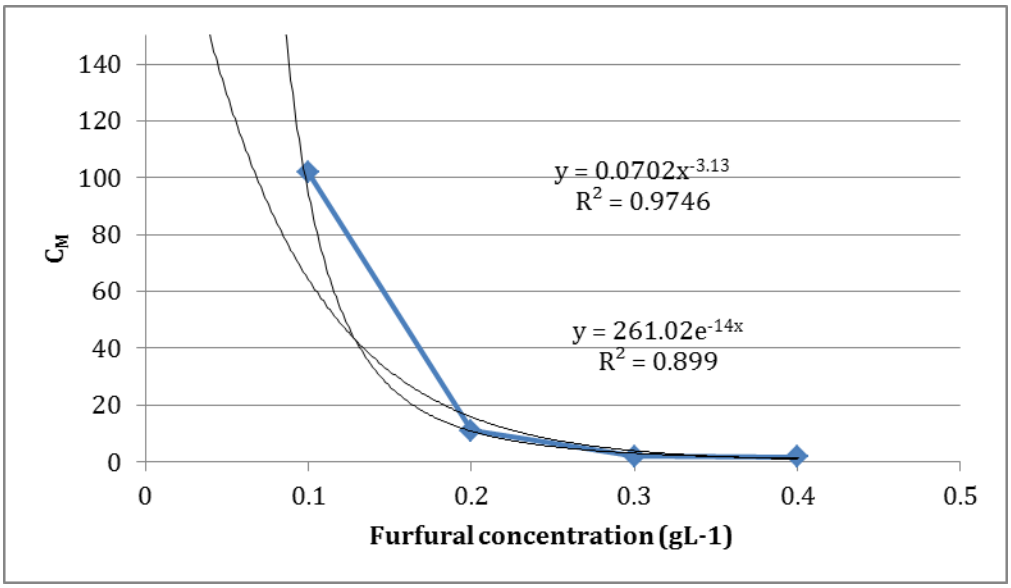

Figure 10.3 Monod constant as a function of furfural concentration

These functions that describe the reaction rate constant k and Monod constant were implemented into the Monod model in different variations. To incorporate the positive control, the reaction constant k 0.718 was used as the coefficient in the function of furfural concentration. Table 10.6 shows the summary of the equation variations from Polymath.

Table 10.6 Summary of equation variations to describe furfural inhibition with glucose.

Equation	a	b	R^2
u=0.718*CI^a*CA/((102*CI^b)+CA)	0.222	0.083	-0.03
u=0.718*exp(CI*a)*CA/((102*CI^b)+CA)	-1.25	0.004	0.395
u=0.718exp(CI*a)*CA/((102exp(CI*b))+CA)	-8.94	-9.4	0.57
u=0.718exp(CI*a)*CA/((261exp(CI*b))+CA)	-11.1	-18.3	0.82
u=0.718exp(CI*a)*CA/((0.0702*CI^b)+CA)	-10.75	-2.83	0.87

The modified Monod that best fits the data with an R^2 value of 0.87 is shown in Equation 10.10.

$$\mu = 0.718\exp(-10.8C_I)\frac{C_A}{0.0702C_I^{-2.8} + C_A} \qquad [10.10]$$

This equation shows that the maximum specific growth rate best described as an exponential function while the Monod constant is best described by a power function. The exponential function shows that the maximum specific growth rate decreases exponentially as inhibitor concentration increases. The coefficient of the exponential function is the maximum specific growth rate when inhibitor concentration is zero since it showed the best fit. Aiba et al developed a similar equation to describe product inhibition (Aiba et al., 1968). The difference between the Aiba equation and Equation 10.7 is that the Monod constant is a power function of inhibitor concentration. The exponent of the inhibitor concentration is -2.8, which shows that the Monod constant is inversely proportional to inhibitor concentration raised to the 2.8. The Monod constant is relatively small when compared to the substrate concentration, and as the furfural

concentration increases, the Monod constant gets even smaller. Equation 10.10 also shows that the consortium is highly sensitive to the amount of furfural in the media. The fact that experiments with furfural concentrations, ranging from 0.1 to 0.5 gL^{-1}, showed growth of the consortium is highly inhibited by the presence of furfural supports the previous statement.

In the presence of furfural, the microorganisms reduce the furfural to furfuryl alcohol. This reduction to furfuryl alcohol is typically due to the NADH-dependent alcohol dehydrogenase (ADH) (Palmqvist and Hahn-Hagerdal, 2000b). Typically, glycerol is produced to regenerate excess NADH in biosynthesis to NAD^+. However, research has shown that glycerol production is decreased in the presence of furfural, thus concluding that furfural reduction regenerates NAD^+ (Palmqvist and Hahn-Hagerdal, 2000b). Furthermore, glycolytic enzymes and ADH could have been inhibited by furfural, contributing to the excretion of acetaldehyde. Accumulation of acetaldehyde within the cell has also been suggested to be responsible for growth inhibition (Palmqvist and Hahn-Hagerdal, 2000b). Therefore, furfural concentration has a larger effect on growth inhibition than the substrate concentration.

Using the same method, a model was developed to describe the inhibition of acetic acid with glucose on the consortium's growth. Table 10.7 shows the results of determining the Monod model constants. Based on these results, acetic acid concentration 0.7 gL^{-1} was eliminated as an outlier due to the constants being a lower value than 0.8 gL^{-1} of acetic acid. At acetic acid concentrations higher than 0.6 gL^{-1}, the Monod constant showed a drastic decrease in value as the acetic acid concentrations increased.

Table 10.7 Summary of the Monod model constants fit to inhibition of acetic acid with glucose

Acetic Acid Concentration (gL^{-1})	k	C_M	R^2
0.5	0.402	102	0.926
0.6	0.141	102	0.916
0.7	0.0195	0.879	0.875
0.8	0.044	6.05	0.986
1.5	0.0145	0.254	0.958

Figure 10.4 shows the reaction rate constant k as a function of acetic acid concentration. This plot shows that the power and exponential function fit the trend best with an R2 of 0.93 and 0.84, respectively. Figure 10.5 is the plot of the Monod constant as a function of acetic acid concentration for the consortium grown on glucose. The trend lines that best describe this plot are similar to the reaction rate constant k.

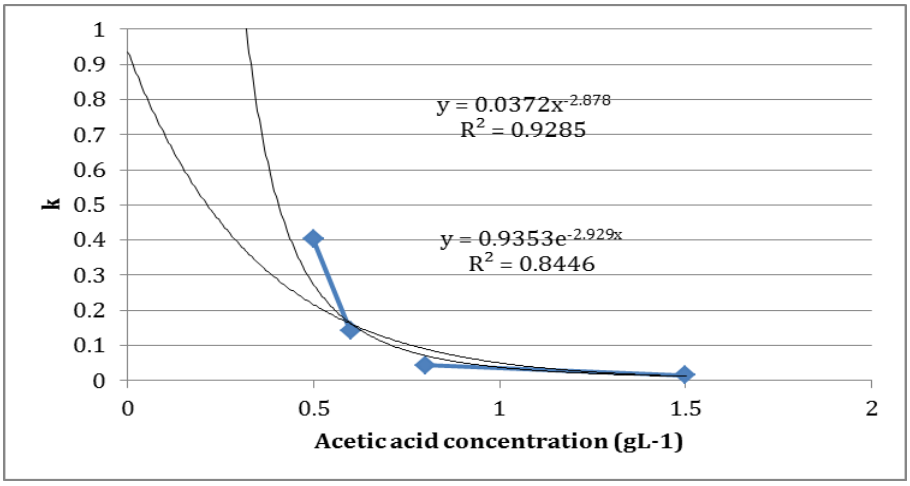

Figure 10.4 Reaction rate constant k versus acetic acid concentration

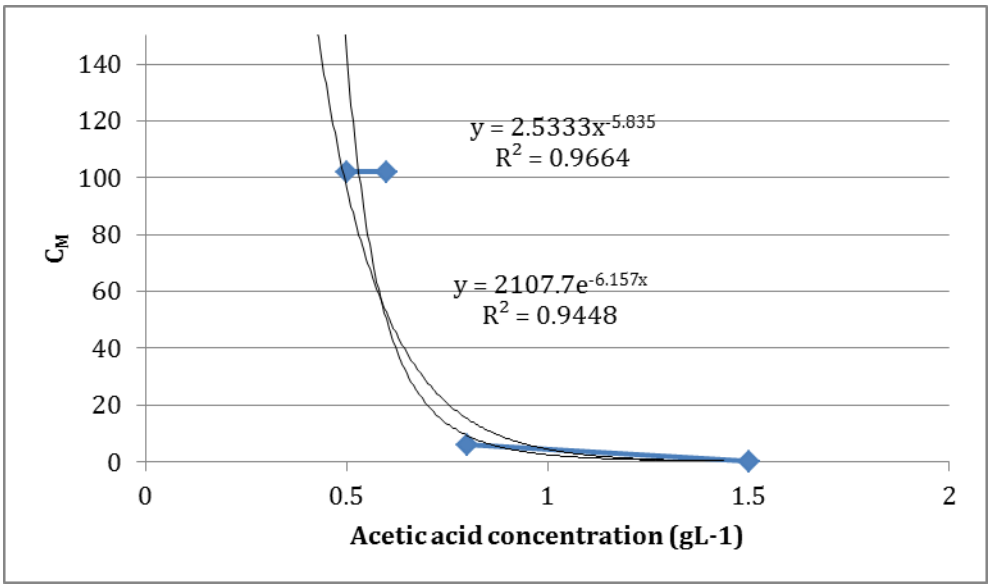

Figure 10.5 Monod constant as a function of acetic acid concentration with power and exponential trend line.

Using these trend lines, the Monod model is modified to include acetic acid inhibitor concentration. Table 10.8 summarizes the variations of the inhibitor functions to fully describe the inhibition of acetic acid on the growth of consortium. As seen in the equation development, the Monod constant was best described by the power function as well as the reaction rate constant k. After the 4th variation, the data for the 0.6 gL^{-1} of acetic acid was eliminated as an outlier because the calculated specific growth rate was unusually high compared to the specific growth rate for 0.5 gL^{-1} of acetic acid. With the elimination of 0.6 and 0.7 gL^{-1}, the equation fit increased to an R^2 of 0.9 and above.

Table 10.8 Summary of the equation variations for acetic acid inhibition with glucose.

Equation	A	B	C	R^2
u=0.037CI^A*CA/(2107exp(B*CI)+CA)	-2.5	-6.43	-----	0.595
u=0.935exp(A*CI)*CA/(2107exp(B*CI)+CA)	1.82	0.179	-----	-0.538
u=0.935exp(A*CI)*CA/(2.53*CI^(B)+CA)	-3.08	-6.75	-----	0.079
u=0.037CI^A*CA/(2.53*CI^(B)+CA)	2.62	5.26	-----	0.227
u=0.037CI^A*CA/(Cexp(B*CI)+CA)	-1.5	-3.07	102	0.903
u=0.037CI^A*CA/(C*CI^(B)+CA)	-1.5	-2.61	4.17	0.922

The results for the model development to describe the acetic acid inhibition effect on the consortium cultivated on synthetic wastewater with glucose are shown in Equation 10.11 with an R^2 fit of 0.92.

$$\mu = 0.037 C_I^{-1.5} \frac{C_A}{4.2 C_I^{-2.6} + C_A} \qquad [10.11]$$

Equation 10.11 shows that the maximum specific growth rate for this system is described by a function of inhibitor concentration raised to the -1.5. This function shows that the maximum specific growth rate is an inverse relationship to inhibitor concentration, showing a decrease in maximum specific growth rate as inhibitor concentration increases. This inverse effect could possibly show that as the acetic acid concentration increases, the uncoupling and intracellular anion accumulation increases, thus resulting in a decrease in specific growth rate. The inhibitory effect of acetic acid on the cells is based on the dissociated form of the acetic acid diffusing across the cell's plasma membrane and dissociating because of the higher intracellular pH. This increase

in intracellular pH decreases the cytosolic pH. Along with this pH decrease, the anionic accumulation within the cell is also a factor in acetic acid inhibition (Hasunuma et al., 2011). As the acetic acid concentration increases, the anionic accumulation increases within the cell, thus reduces the cell's ability to grow and accumulate lipids.

To achieve the best fit to the data, the Monod constant is described as a function of inhibitor concentration raised to the -2.6, which is similar to the Monod constant function for the furfural inhibition on glucose. The overall equation shows that the consortium's growth is sensitive to the presence of acetic acid. However, the sensitivity to the acetic acid is much less than the sensitivity to the furfural since growth was shown up to an acetic acid concentration of 1.5 gL^{-1}.

Equation 10.11 shows similarity in the basic structure as mixed inhibition with simplifying the equation further as shown in Equation 10.12.

$$\mu = \frac{kC_A}{(C_M C_I^{-1.1} + C_A C_I^{1.5})} \qquad [10.12]$$

The mixed inhibition equation is shown in Equation 10.13. The similarity stems from the k and C_M having a function of C_I.

$$r_P = \frac{kC_A}{(C_M(1+\frac{C_I}{K_i}) + C_A(1+\frac{C_I}{K_j}))} \qquad [10.13]$$

Since these two equations are similar, the proposed mechanism should be similar to the reaction mechanism for mixed inhibition. Mixed inhibition is defined as the combination of competitive and noncompetitive inhibition (Kalra et al., 2007). In this type of inhibition, the inhibitor shows an affinity only for the free enzyme (E) or the enzyme-substrate complex (ES) (Converti et al., 2000).

The following set of reactions is the governing reactions for mixed inhibition with the substrate as (S), enzyme-substrate complex as (ES), association rate constant as k, inhibitor concentration as (I), enzyme-inhibitor complex (EI), enzyme-substrate-inhibitor complex as (ESI), and product as (P). Coefficients of n and m were implemented in the formation of (EI) and (ESI) equations, respectively. These coefficients describe the interactions of the inhibitor with more than one enzyme and enzyme-substrate complex. Since all microorganisms in the consortium were oleaginous microorganisms, the enzymes could have similar active sites based on the fact that the same substrate is binding to the enzyme.

$$E + S \underset{k2}{\overset{k1}{\leftrightarrow}} E \bullet S$$

$$nE + I \underset{k4}{\overset{k3}{\leftrightarrow}} nE \bullet I$$

$$mE \bullet S + I \underset{k6}{\overset{k5}{\leftrightarrow}} mE \bullet S \bullet I$$

$$E \bullet S \overset{k7}{\rightarrow} P + E$$

From these reaction steps, the rate laws were developed assuming elementary reactions. Equation 10.14 shows the rate of product formation also known as substrate uptake rate that is assumed to be irreversible. For this derivation, Equation 10.14 is assumed to be the rate-limiting step. The remaining rate laws, Equations 10.15 through 10.17, were developed and set equal to zero.

$$r_P = -r_S = k_7(E \bullet S) \quad [10.14]$$

$$r_{E \cdot S} = 0 = k_1(E)(S) - k_2(E \bullet S) - k_9(E \bullet S) \quad [10.15]$$

$$r_{E \cdot I} = 0 = k_3(E)^n(I) - k_4(E \bullet I)^n \quad [10.16]$$

$$r_{E \cdot S \cdot I} = 0 = k_5(E \bullet S)^m(I) - k_6(E \bullet S \bullet I)^m \quad [10.17]$$

Equation 10.15 was solved for (E) in terms of the (ES) as shown in the following equation.

$$k_1(E)(S) = (k_2 + k_9)(E \bullet S) \qquad [10.18]$$

$$(E) = \frac{(k_2 + k_9)(E \bullet S)}{k_1(S)} \qquad [10.19]$$

When $C_M=(k_2+k_9)/k_1$,

$$(E) = \frac{C_M(E \bullet S)}{(S)} \qquad [10.20]$$

Equation 10.16 was set equal to zero and solved in terms of (E).

$$k_3(E)^n(I) = k_4(E \bullet I)^n \qquad [10.21]$$

$$\left[k_3(E)^n(I)\right]^{1/n} = \left[k_4(E \bullet I)^n\right]^{1/n} \qquad [10.22]$$

$$(E \bullet I) = \frac{k_3}{k_4}(E)(I)^{1/n} \qquad [10.23]$$

By substituting Equation 10.20 into 10.23, the following equation was developed.

$$(E \bullet I) = \frac{k_3 C_M(E \bullet S)(I)^{1/n}}{k_4(S)} \qquad [10.24]$$

Equation 10.17 was solved for (ESI).

$$k_5(E \bullet S)^m(I) = k_6(E \bullet S \bullet I)^m \qquad [10.25]$$

$$\left[k_5(E \bullet S)^m(I)\right]^{1/m} = \left[k_6(E \bullet S \bullet I)^m\right]^{1/m} \qquad [10.26]$$

$$(E \bullet S \bullet I) = \frac{k_5}{k_6}(E \bullet S)(I)^{1/m} \qquad [10.27]$$

The total Enzyme equation is the following with the assumption that all the reversible reactions are quick (Converti et al., 2000).

$$E_t = (E) + (E \bullet S) + (E \bullet I) + (E \bullet S \bullet I) \quad [10.28]$$

By substituting Equations 10.19, 10.21, and 10.22 into Equation 10.23,

$$E_t = \frac{C_M (E \bullet S)}{(S)} + (E \bullet S) + \frac{k_3 C_M (E \bullet S)(I)^{1/n}}{k_4 (S)} + \frac{k_5 (E \bullet S)(I)^{1/m}}{k_6} \quad [10.29]$$

Multiplying both sides by (S) to Equation 10.24,

$$E_t (S) = C_M (E \bullet S) + (S)(E \bullet S) + \frac{k_3 C_M (E \bullet S)(I)^{1/n}}{k_4} + \frac{k_5 (E \bullet S)(S)(I)^{1/m}}{k_6} \quad [10.30]$$

Taking the common factor (ES) out of Equation 10.30,

$$E_t (S) = (C_M + (S) + \frac{k_3 C_M (I)^{1/n}}{k_4} + \frac{k_5 (S)(I)^{1/m}}{k_6})(E \bullet S) \quad [10.31]$$

Combining terms on the right side of Equation 10.31 is shown in the following.

$$E_t (S) = (C_M (1 + \frac{k_3 (I)^{1/n}}{k_4}) + (S)(1 + \frac{k_5 (I)^{1/m}}{k_6}))(E \bullet S) \quad [10.32]$$

Solving Equation 10.32 for (ES),

$$(E \bullet S) = \frac{E_t (S)}{C_M (1 + \frac{k_3}{k_4}(I)^{1/n}) + (S)(1 + \frac{k_5}{k_6}(I)^{1/m})} \quad [10.33]$$

Substituting Equation 10.33 into the rate-limiting step, Equation 10.14, shows

$$r_P = -r_S = \frac{k_7 E_t (S)}{C_M (1 + \frac{k_3}{k_4}(I)^{1/n}) + (S)(1 + \frac{k_5}{k_6}(I)^{1/m})} \quad [10.34]$$

Assuming that k_3 and k_5 are very large, Equation 10.34 further reduces to the following,

$$r_P = -r_S = \frac{k_7 E_t (S)}{C_M (\frac{k_3}{k_4}(I)^{1/n}) + (S)(\frac{k_5}{k_6}(I)^{1/m})} \quad [10.35]$$

With the assumption that k equals k7 times E_t, Equation 10.35 results in the following.

$$r_P = -r_S = \frac{k(S)}{C_M (\frac{k_3}{k_4}(I)^{1/n}) + (S)(\frac{k_5}{k_6}(I)^{1/m})} \quad [10.36]$$

This proposed mechanism shows that mixed inhibition best describes the consortium's growth inhibited by acetic acid with glucose as the substrate. According to Converti et al, growth inhibition by the presence of acetic acid has been described by mixed inhibition on xylose fermentation by *C. guilliermondii* (Converti et al., 2000). Equation 10.37 is the equation that best described acetic inhibition on xylose fermentation.

$$r_S = \frac{k(S)}{C_M (1 + \frac{(I)}{K_3}) + (S)(1 + \frac{(I)}{K_4})} \quad [10.37]$$

Equation 10.37 contains K_3 and K_4 that are reaction equilibrium constants for formation of (E) and (ES) from (EI) and (ESI). *C. guilliermondii* is a yeast strain that is known to produce xylitol, a polyol that contains a sweetness similar to sucrose. This research article was a kinetic study of determining the inhibition of methanol, furfural, and acetic acid on xylose fermentation by *C. guilliermondii*. A batch experiment set up to test four different concentrations of each inhibitor concentration was conducted. The acetic acid concentrations that were used are similar to the acetic acid range of concentrations used in this dissertation research. The results in Converti et al showed that furfural was the most inhibitory substance followed by acetic acid and then methanol. In addition, the results also showed a decrease in both k and C_M values for

each inhibitor, which corresponds with the decrease in k and C_M value in this dissertation research (Converti et al., 2000).

In addition, mixed inhibition described the 2-amino-6-hydroxyl-8-mercaptopurine (AHMP) inhibition of xanthine oxidase during xanthine metabolism and the anticancer drug 6-mercaptopurine (6MP) transformation (Kalra et al., 2007). This experiment was geared to finding an inhibitor of 6MP transformation while leaving xanthine metabolism unaffected to eliminate the accumulation of xanthine in the body that causes xanthine nephropathy during chemotherapy for leukemia. The experiment used different concentrations for three different inhibitors. The results showed that the xanthine oxidase activity decreased with increasing inhibitor concentration similarly to the results in this dissertation research (Kalra et al., 2007).

Kim et al showed that mixed inhibition is the best type that describes butane inhibition of 1,1,1-trichloroethane (TCA) transformation by a butane-grown mixed culture (Kim et al., 2002). This experiment focused on bioremediation of 1,1,1-TCA that is a common groundwater contaminant. The experiment consisted of measuring the growth of the butane-grown mixed culture on utilizing 1,1,1-TCA with varying concentrations of butane. The results were similar to this dissertation research in that as the butane concentration increased, the growth of the microorganisms decreased at a similar rate (Kim et al., 2002).

The fact that this mechanism and rate expression fits the data indicates what is physically happening in the system. The acetic acid affecting the growth and lipid production of the oleaginous microorganism consortium can be described by mixed inhibition.

Objective 2: Effect of furfural and acetic acid on the consortium grown on xylose amended synthetic wastewater

Since both furfural and acetic acid inhibit the growth and lipid production of the consortium when cultivated on synthetic wastewater amended with xylose, models were developed to fully describe the inhibitory effect on consortium's growth. The Monod model did not fit the experimental data. This is believed to be due to the fact that glucose and xylose are consumed in different metabolic pathways and are transported into the cell with different mechanisms. After applying multiple models discussed in the methods section, the Contois model was the model that fit the experimental data the best. The reason that the Monod fit the data with the glucose and not with the xylose is because glucose is metabolized in a different pathway than xylose. Figure 10.6 shows the different glucose and xylose metabolic pathways (Zhang, 2003).

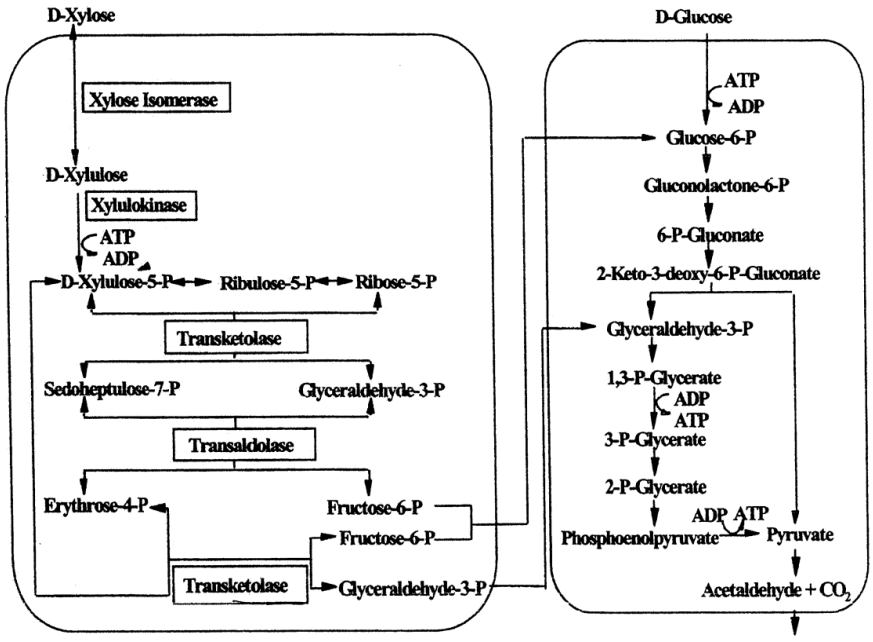

Figure 10.6 Metabolic pathways of glucose and xylose (Zhang, 2003).

The Contois growth model was applied to the data as describe in the methods model development section. Table 10.9 shows the Contois constants for the different furfural concentrations. This table shows both the reaction rate constant k and the Contois apparent saturation constant. Since the Contois apparent saturation constant was the same for all concentrations, there is no need to plot this constant versus furfural concentration. When looking at the reaction rate constant k, the value for 0.5 gL^{-1} of furfural concentration is extremely low when compared to the higher furfural concentrations, thus, eliminating this concentration as an outlier. Figure 10.7 shows the reaction rate constant k as a function of furfural concentration. This trend is best described by the power and exponential function with an R^2 fit of 0.95 and 0.88, respectively.

Table 10.9 Contois constants for the furfural concentrations.

Furfural concentration (gL^{-1})	k	A	R^2
0	0.156	92	0.964
0.1	0.161	92	0.781
0.5	0.128	92	0.616
1	0.135	92	0.948
1.5	0.137	92	0.977
2	0.128	92	0.976

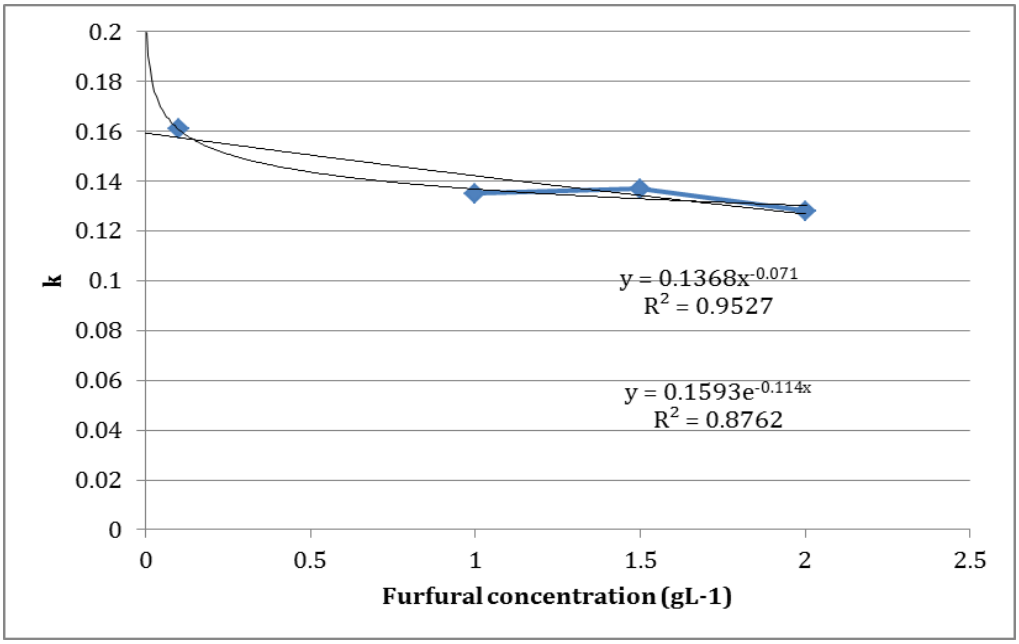

Figure 10.7 Plot of reaction rate constant k versus furfural concentration

To determine an equation that describes furfural inhibition with xylose, variations of the power and exponential functions were applied to the Contois model. Table 10.10 shows the variations of incorporating furfural concentration. The equation that best fit the furfural inhibition of the consortium grown with xylose is the power function incorporated into the Contois model with an R^2 of 0.86. Equation 10.38 shows the model developed by modifying the Contois model to fully describe furfural inhibition on the consortium's growth.

Table 10.10 Summary of the equation variations of furfural concentration functions

	Equation	A	B	R^2
1	u=0.16*exp(A*CI)*CA/(92*CC+CA)	-0.117	----	0.83
2	u=0.16*exp(A*CI)*CA/(B*CC+CA)	-0.173	81.2	0.84
3	u=0.14*CI^A*CA/(92*CC+CA)	-0.13	----	0.863
4	u=0.14*CI^A*CA/(B*CC+CA)	-0.13	92	0.863
5	u=0.16*CI^A*CA/(B*CC+CA)	-0.124	102	0.854

$$\mu = 0.14 C_I^{-0.13} \frac{C_A}{92 C_C + C_A} \qquad [10.38]$$

The modification to the Contois model consists of describing the maximum specific growth rate as a function of inhibitor concentration raised to the -0.13. This result shows that maximum specific growth rate is inversely related to the furfural concentration. Furfural has shown a small impact on the overall specific growth rate when compared to the sugar concentration, C_A, and the cell mass concentration, C_C, since furfural, C_I, is raised to a power less than one. The effect of furfural on microbial growth with xylose is not as large of an inhibition when compared to the growth with glucose, supporting results from Zhang et al (Zhang et al., 2010). This effect is also shown with growth shown in the presence of furfural in higher concentrations.

The intracellular effect of furfural is similar despite the carbon source, so the difference in growth models must be due to how the cell is able to metabolize the carbon source in the presence of furfural. Based on the model results, the cell functionality is not as inhibited with furfural when the main carbon source is xylose when compared to cell functionality on glucose. This result shows that furfural does not inhibit metabolizing xylose as much as it does glucose.

Similarly to the furfural concentration, the acetic acid inhibition on the growth of the consortium can be described by modifying the Contois model. The experimental data was applied to the Contois model, determining the constants for each acetic acid concentration. Table 10.11 shows the Contois model constants determined for each acetic acid concentration. This table shows both the reaction rate constant k and the Contois apparent saturation constant for each acetic acid concentration. The reaction rate

constant k for 1.5 gL^{-1} of acetic acid was eliminated as an outlier because it is high compared to the other acetic acid concentration values. The Contois apparent saturation constant did not change with acetic acid concentration, so there is no need to plot this constant versus the acetic acid concentration. Figure 10.8 shows the plot of reaction rate constant k and the acetic acid concentration.

Table 10.11 Contois model constants for the acetic acid concentrations.

Acetic acid Concentration (gL^{-1})	k	A	R^2
0	0.182	102	0.987
0.75	0.1545	102	0.944
1	0.1298	102	0.973
1.25	0.1294	102	0.987
1.5	0.143	102	0.986
1.75	0.1087	102	0.988

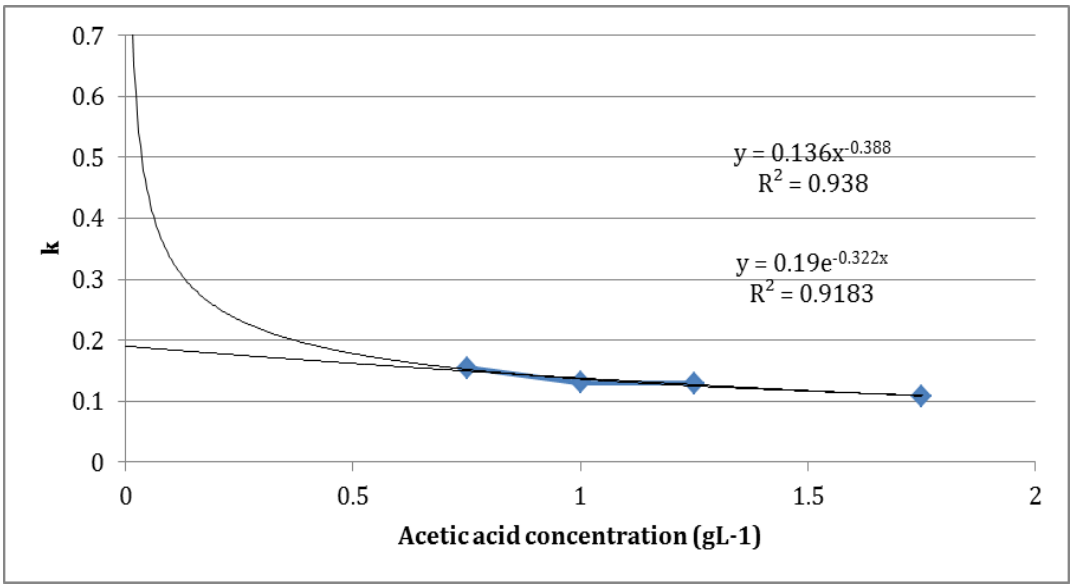

Figure 10.8 Graph of reaction rate constant k and the acetic acid concentration.

The function that describes the reaction rate constant k and acetic acid concentration was a power and exponential function with an R^2 of 0.94 and 0.92, respectively. Variations of these functions were implemented into the Contois model. The results of these equations are shown in Table 10.12.

Table 10.12 Summary of the equation variations for the acetic acid inhibition with xylose.

Equation	A	B	R^2
u=0.136CI^A*CA/(102*CC+CA)	-0.39	-----	0.963
u=0.19exp(A*CI)*CA/(102*CC+CA)	-0.319	-----	0.96
u=0.19exp(A*CI)*CA/(B*CC+CA)	-0.328	101	0.96

Each variation resulted in a relatively the same R^2 value. However, the equation chosen also resembled the modified equation for furfural inhibition with xylose. Equation 10.39 shows the model that describes the effect of acetic acid on the growth of the consortium.

$$\mu = 0.14 C_I^{-0.4} \frac{C_A}{102 C_C + C_A} \quad [10.39]$$

This model is similar to the model that describes the furfural inhibition, including similar power functions for reaction rate constant k with same coefficient. The effect of acetic acid is higher than the effect of furfural based on the comparison of the power functions.

Conclusion

The consortium grown on synthetic wastewater amended with glucose shows an inhibition when furfural or acetic acid was present. The model developed is a modification of the Monod model. This model describes the inhibition of the consortium's growth for glucose with furfural as an exponential function of furfural concentration for the reaction rate constant k. To describe the acetic acid inhibition with glucose, the model developed was a power function, showing that acetic acid concentration is inversely proportional to specific growth rate. The inhibition of acetic acid on the consortium grown on glucose can best be described by mixed inhibition mechanism. The inhibition of furfural and acetic acid is best described by the inverse relationship of inhibitor concentration and specific growth rate to modify the Contois

equation. Overall, furfural inhibition with glucose as the carbon source was observed to have the highest inhibitory effect on the growth of the consortium.

CHAPTER XI

ENGINEERING SIGNIFICANCE OF CULTIVATING OLEAGINOUS MICROORGANISM CONSORTIUM ON MUNICIPAL WASTEWATER

Introduction

Oleaginous microorganisms are potential source of oil for biodiesel. These microorganisms produce oil similar to plant oils such as soybeans, canola, and rapeseed (Ratledge, 2005b). These microorganisms have been shown in the previous chapters to be cultivated on municipal wastewater. A consortium of oleaginous microorganisms was developed based on literature showing their ability to accumulate oil. Since the wastewater constituents vary hourly, a consortium of oleaginous microorganisms is more beneficial to production over a pure culture. A consortium allows for growth and accumulation of lipids despite the varying nutrient concentrations. One microorganism in the consortium could be inhibited by a substance in the influent wastewater that another microorganism utilizes efficiently. In using a pure culture, the inhibitory substance could drastically reduce the microorganism's productivity in the aeration tank, where a consortium of microorganisms provides the ability to adapt to the ever-changing wastewater.

Using an existing municipal wastewater treatment facility has the potential to reduce the overall production costs of the oil. Figure 11.1 shows the typical wastewater treatment process. Wastewater enters the treatment facility and immediately the large items are screened out and removed. The wastewater continues to the primary clarifier, where additional solids are removed. After removal of the suspended solids, the wastewater is treated in the aeration tank. This aeration tank is where a consortium of microorganisms utilize the dissolved carbon, nitrogen, and phosphorous for growth. The activated sludge in the aeration tank has been shown to reduce 95% of the biochemical oxygen demand in the wastewater (Grady et al., 1999). The secondary clarifier is used to remove the activated sludge from the wastewater. The water from the secondary clarifier continues to the final treatment stage where ultraviolet light and or chlorine are used to eliminate any contaminants. A portion of the activated sludge from the secondary clarifier is recycled to the aeration tank while the remaining continues to the anaerobic digestion tank. After the digestion tank, the sludge is usually disposed of by incineration, sent to landfills, or used in agriculture (Grady et al., 1999; Metcalf & Eddy, 2003).

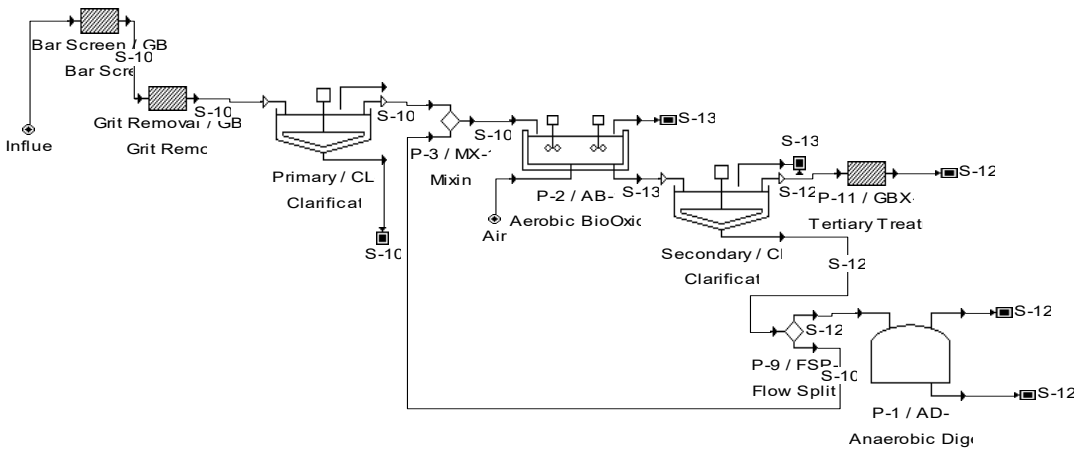

Figure 11.1 Municipal wastewater treatment process flow diagram.

Municipal wastewater typically contains a low concentration of carbon, whereas these microorganisms require a high concentration of carbon to accumulate oil (Ratledge, 2002). Therefore, lignocellulosic sugars produced from hydrolyzed biomass could be used to increase the carbon concentration, thus, inducing oil accumulation in the consortium of oleaginous microorganisms. However, lignocellulosic sugars contain inhibitory substances such as furfural and acetic acid. Advances in research associated with the hydrolysis of lignocellulose have resulted in technologies that could yield sugars with small concentrations of inhibitors at cost, competitive with sugar cane, reducing the overall production cost of biofuels.

To incorporate the oleaginous microorganisms, modifications should be made to the existing wastewater treatment facilities. Figure 11.2 shows the modified wastewater treatment flow diagram. This figure shows the aeration tank that will contain the oleaginous microorganism consortium tied into the treatment system as a subsection of the overall treatment process. This subsection should not interfere with the wastewater

treatment plant's treating the water for public use. The oleaginous microorganism consortium will be grown in an aeration tank that utilizes a portion of treated wastewater from the primary clarifier. Prior to entering the aeration tank with the oleaginous microorganism consortium, lignocellulosic sugars and the recycle stream will be mixed with the influent. After the aeration tank, the wastewater continues into a clarifier to separate the biomass from the water. The clarifier effluent will then be recycled to the main influent of the wastewater treatment plant for further treatment to meet EPA specifications. The oleaginous biomass from the clarifier will be dewatered using a centrifuge or a similar unit. The water removed from the dewatering unit will be joined with the recycle stream to the aeration tank. After dewatering, the biomass will be dried using a rotary dryer or some comparable unit. The dried biomass will then be sent to the biodiesel plant for production of biodiesel.

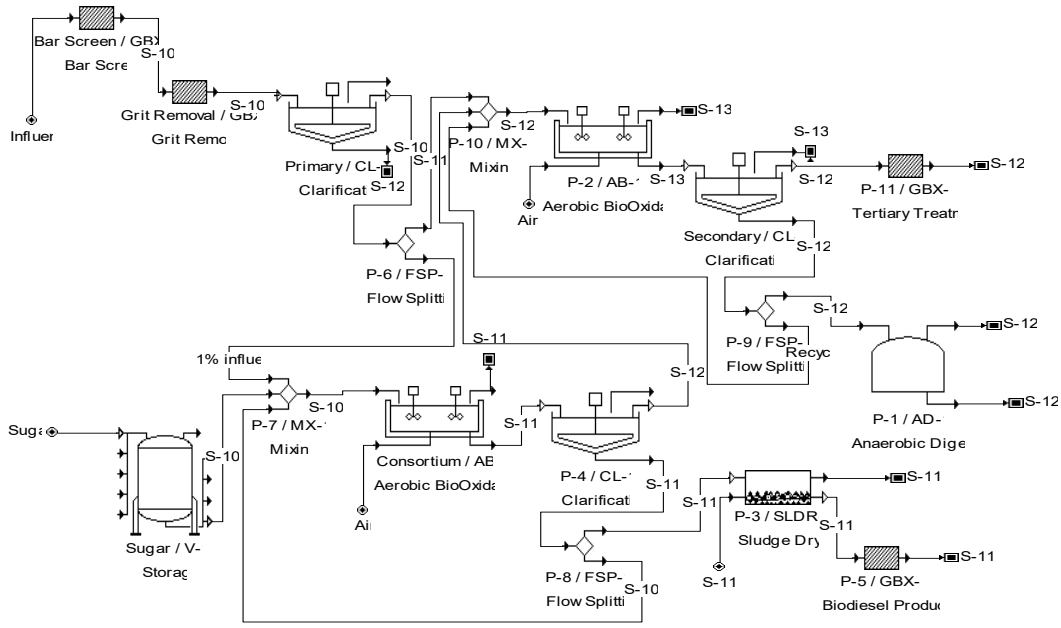

Figure 11.2 Modification of the wastewater treatment process.

The purpose of this chapter is to simulate the growth of the oleaginous microorganism consortium in the modified wastewater treatment plant and the production of biodiesel from the biomass, using SuperPro Designer v6.0. The simulation includes the incorporation of the lignocellulosic sugars, biomass production, biomass drying, and biodiesel production. An economic analysis is completed using SuperPro Designer v6.0 for the oleaginous microorganism consortium cultivated on wastewater and for the production of biodiesel from the biomass.

Modification of the Wastewater Treatment Process Simulation

The modified wastewater treatment facility was simulated using SuperPro Designer v6.0. The flow diagram of the process is shown in Figure 11.3. The wastewater influent was assumed to be 10% of the total daily influent to the wastewater treatment facility in Tuscaloosa, AL, which is 30 million gallons per day. This influent was assumed to be a fraction of the primary clarifier effluent. To increase the carbon concentration prior to entering the aeration tank, the wastewater influent, lignocellulosic sugar stream, and recycle were mixed upstream of the aeration tank. A holding tank for the lignocellulosic sugars (V-108) was included in the basic flow diagram to store lignocellulosic sugars. Lignocellulosic sugar concentrations and inhibitor concentrations were chosen based on hydrolyzed forest residue biomass presented by Nilsson et al (Nilsson et al., 2005), as shown in Table 11.1.

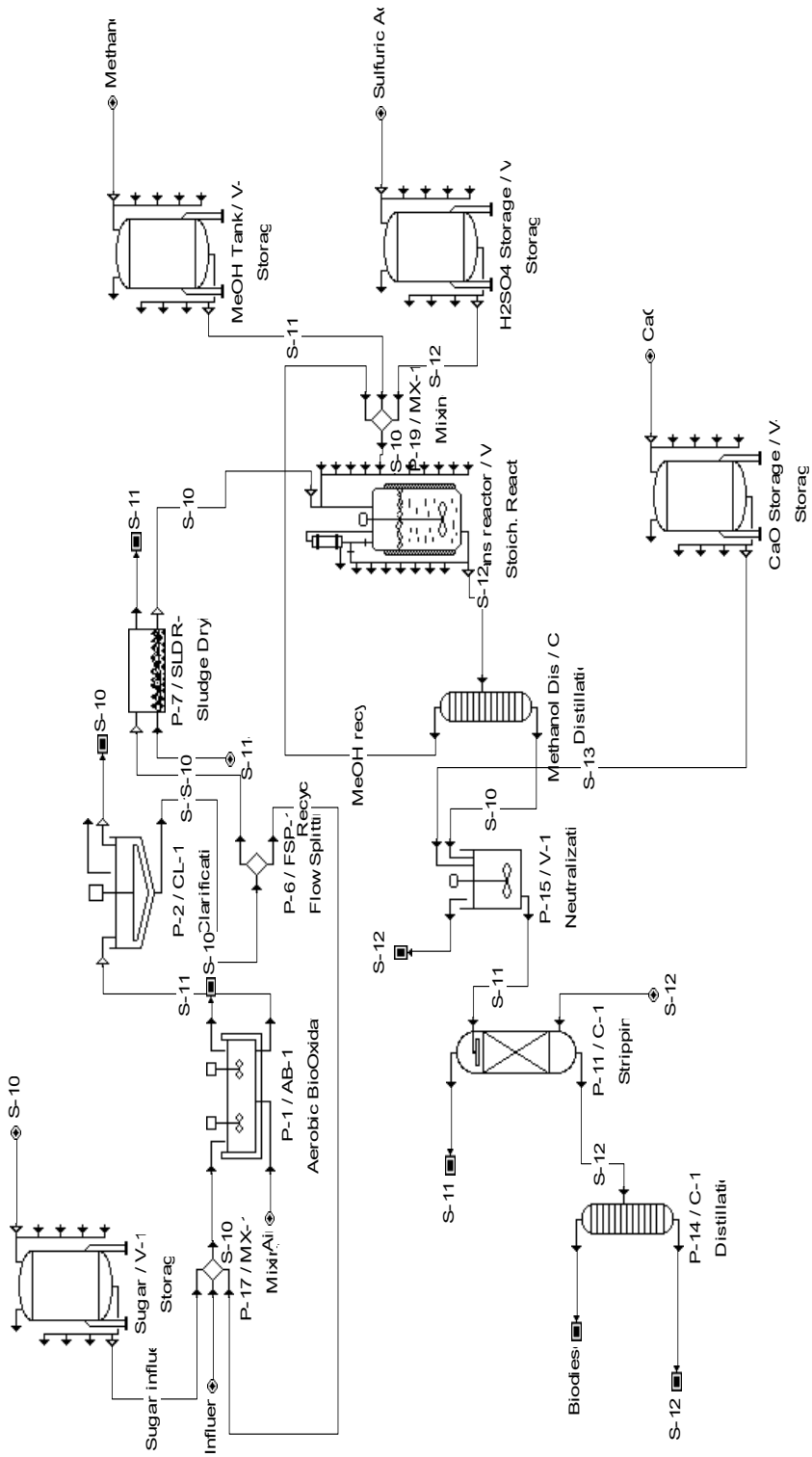

Figure 11.3 Schematic for the wastewater treatment modification for the oleaginous microorganisms and the biodiesel production plant.

Table 11.1 Sugar and inhibitor concentration of hydrolyzed forest residue (Nilsson et al., 2005).

Sugar	Concentration (gL^{-1})
Glucose	16
Xylose	6.1
Acetic Acid	1.5
Furfural	0.2

With these inhibitors, the experimental data and the developed inhibition models from Chapters IX and X were used in the simulation. A sensitivity analysis was performed to determine which inhibitor produced the highest amount of inhibition to cultivate the consortium in an existing wastewater treatment facility. Figure 11.4 and Figure 11.5 show the results of the sensitivity analysis for determining the inhibitory concentrations. Based on a sensitivity analysis comparing the four inhibition models, furfural appears to be the most inhibitory substance when glucose is present.

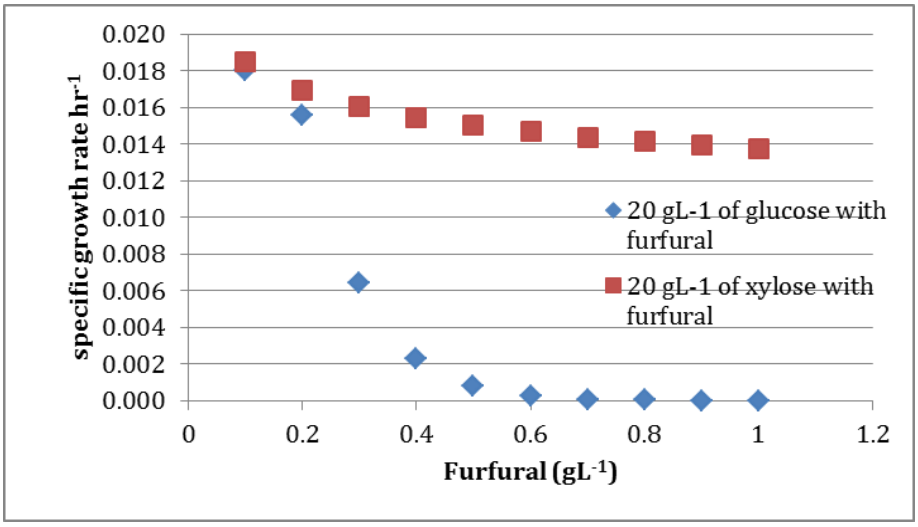

Figure 11.4 Consortium's specific growth rate with 20 gL^{-1} of glucose or xylose with varying amounts of furfural using the models developed in Chapter X.

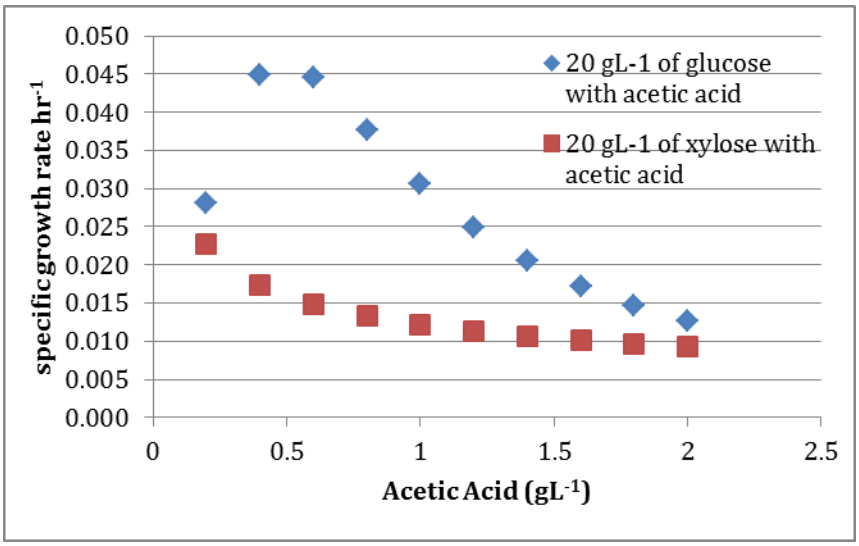

Figure 11.5 Consortium's specific growth rate with 20 gL^{-1} of glucose or xylose with varying amounts of furfural using the models developed in Chapter X.

A sensitivity analysis comparing the developed models from Chapter X was also performed to determine the specific growth rate changing with respect to glucose or xylose concentrations. Figure 11.6 and 11.7 show the results of the substrate sensitivity analysis.

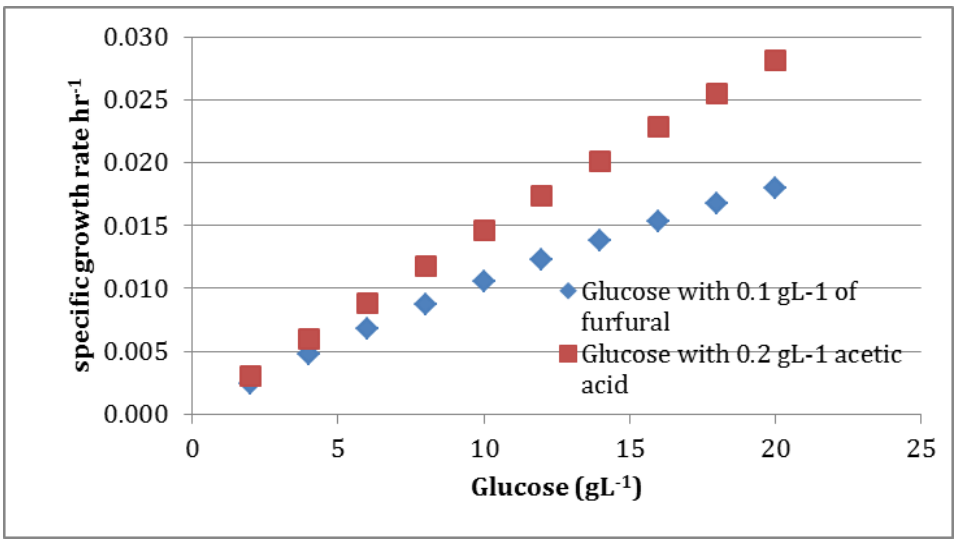

Figure 11.6 Consortium's specific growth rate with 0.1 gL^{-1} of furfural and 0.2 gL^{-1} of acetic acid with varying amounts of glucose using the models developed in Chapter X for glucose with furfural and glucose with acetic acid.

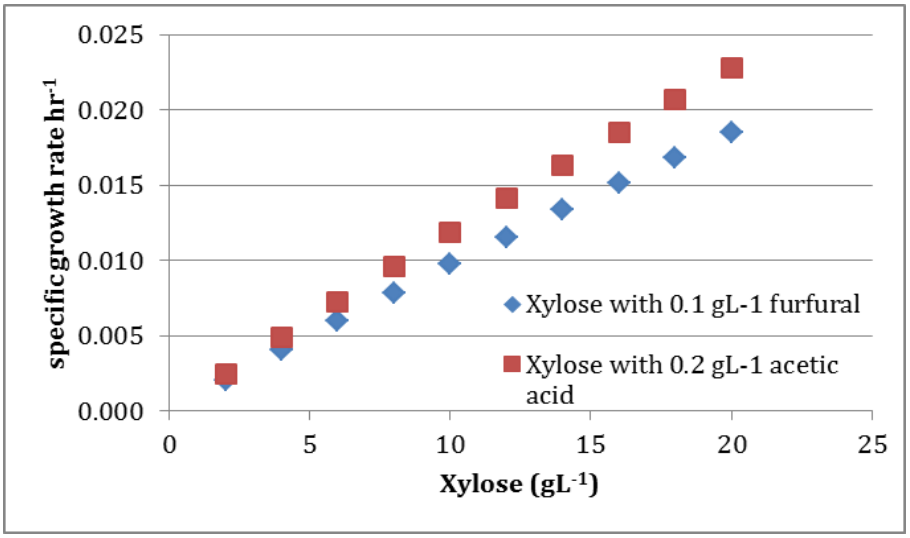

Figure 11.7 Consortium's specific growth rate with 0.1 gL^{-1} of furfural and 0.2 gL^{-1} of acetic acid with varying amounts of xylose using the models developed in Chapter X for xylose with furfural and glucose with acetic acid.

Based on these results, the furfural shows the most inhibition when both glucose and xylose are present. Therefore, the rate expression for the consortium grown in the

aeration tank is best described by the glucose with furfural model and the xylose with furfural model. Furthermore, since microorganisms utilize glucose first and xylose second (Easterling et al., 2009), the two rate expressions are sequential. In specifying the reaction expressions, Monod kinetics was chosen in SuperPro aeration tank operations. Since models were developed based on Monod, the Monod option was used, adjusting the constants k and K_s for the furfural concentration of the lignocellulosic sugars. The influent sugar concentration was 4 gL^{-1} for the aeration tank influent, which is the optimum amount of sugar to reduce the cost of raw materials. The design specifications for the aeration tank are shown in Table 11.2.

Table 11.2 Design specifications for the aeration tank with inhibitors present.

Aeration Tank Parameters		Inhibitors
Glucose:	k (1/hr)	0.244
	KS (g/L)	44.3
	Yc/s (g cell/g sugar)	0.07
Xylose:	k (1/hr)	0.189
	KS (g/L)	27.6
	Yc/s (g cell/g sugar)	0.06
SRT (hours)		6.8
HRT (hours)		6
Working Volume (L)		2,860,617

In addition to using these models, stoichiometric reactions to describe the substrate utilization were also required as input into the simulation. The reactions were mass based using experimental yields in Chapter IX. Equation 11.1 shows the reaction

for glucose conversion to biomass with the corresponding mass values, using the yield of 0.07 g of biomass per g of glucose consumed.

$$Glucose \rightarrow Biomass + CO_2 + H_2O$$
$$1g. \quad 0.07g. \quad 0.46g. \quad 0.47g. \qquad [11.1]$$

Equation 11.2 shows the utilization of xylose for biomass production, using the yield of 0.06 g of biomass per g of xylose consumed.

$$Xylose \rightarrow Biomass + CO_2 + H_2O$$
$$1g. \quad 0.06g. \quad 0.47g. \quad 0.47g. \qquad [11.2]$$

In addition to the reaction kinetics, the design of a wastewater treatment facility is based on the determined hydraulic retention time (HRT) and the solids residence time (SRT) as shown in Table 11.2. The HRT for this system was assumed to be 6 hours, which is a common HRT for a typical wastewater treatment plant (Metcalf & Eddy, 2003; NRC-CNRC, 2004). Based on the HRT and the wastewater influent, the volume of the aeration tank was calculated to be 2,860,617 L (0.75 million gallons). SuperPro Designer calculates the SRT based on the ratio of biomass concentration in the reactor to the biomass concentration sent to processing, which in this case is the influent to the sludge drier. The SRT was calculated to be 6.78 hours. After the Aeration tank, the biomass and wastewater continue to the clarification unit (CL-10) for concentrating the biomass. The overflow water from the clarifier was sent to the beginning of the wastewater treatment plant for further treatment. The concentrated biomass from the clarifier was split into two streams, where 75% of the stream continues to a sludge drying

unit (SLDR-101) and 25% was recycled to be mixed with the wastewater influent and lignocellulosic sugars. The sludge dryer removed 95% of the water using air.

The biodiesel production plant portion of this simulation was modeled using acid-catalyzed transesterification of waste cooking oil by Zhang et al and soybean oil by Haas et al (Haas et al., 2006; Zhang et al., 2003). The dried biomass continues to the transesterification reactor (V-101). The in situ transesterification reactor includes the addition of methanol and sulfuric acid, which was mixed prior to entering the reactor. The methanol and sulfuric acid influents were calculated based on 12:1 ratio of methanol to biomass and 5 % (v/v) sulfuric acid to biomass (Mondala et al., 2009). The extent of reaction was determined to be 6.4% based on the assumption that 10% of the biomass was oil and 64% of the oil is converted to FAMEs.

Since the effluent from the transesterification reactor contains a large amount of methanol, the effluent continues to the distillation column, where the methanol is recovered and recycled to the mixing point prior to entering the reactor. The bottoms product from the distillation column continues to a neutralization reactor, where the unreacted sulfuric acid was neutralized by calcium oxide (CaO) to form calcium sulfate (CaSO4) (Zhang et al., 2003). A wash column was used to remove the calcium sulfate, biomass, and other solids. To separate the biodiesel from the waste stream, a distillation column was used, with the distillate containing 99% of biodiesel. Table 11.3 shows a summary of the simulation parameters for this described process.

Table 11.3 Simulation parameters for the production of biodiesel from oleaginous microorganism consortium grown on amended wastewater.

Simulation Parameters	Consortium Grown on Amended Wastewater with Inhibitors	
Wastewater	473,176	L/h
Lignocellulosic sugars	2,000	kg/h
Sugar Effluent	1,935	kg/h
Methanol	14.4	L/h
Sulfuric Acid	1.7	L/h
Calcium Oxide	1.06	L/h
Biomass production	1.5	kg/h
Biomass recycle	0.38	kg/h
Oil Extracted	----	----
Biodiesel production	0.07	kg/h

To prevent the oleaginous microorganisms from consuming their oil storage, the sugar effluent maintains a flow rate 1,935 kgh^{-1}. The amount of biomass produced is small compared to the typical wastewater treatment facility activated sludge production. This value is attributed to the inhibition kinetic parameters that were developed to describe furfural inhibition for the oleaginous microorganism consortium. The consortium grown on amended wastewater with inhibitors present produced 176 gal per year, which is also a low value considering the increasing fuel demands. To increase the amount of biodiesel produced, the inhibitors could be removed using a simple wash column prior to mixing with the wastewater influent. Figure 11.8 shows the schematic for producing biodiesel from cultivating an oleaginous microorganism consortium on amended wastewater with the wash column to remove inhibitors. With the removal of acetic acid and furfural from the lignocellulosic sugar influent, the aeration tank

parameters change to incorporate no inhibited growth of the biomass. Table 11.4 shows the parameters used for the aeration tank in the simulation with no inhibited growth.

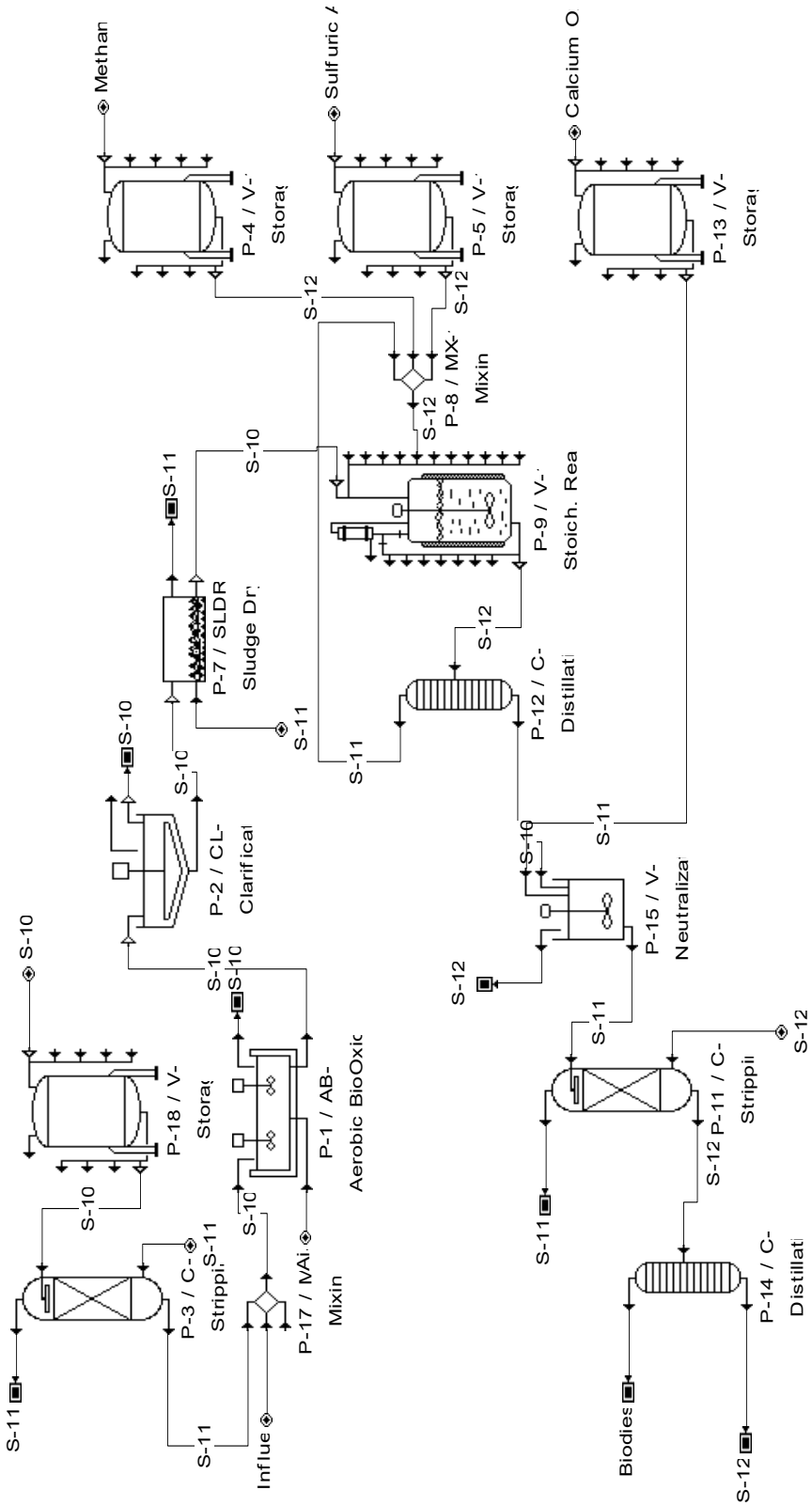

Figure 11.8 Schematic for the wastewater treatment modification for the oleaginous microorganisms with inhibitor removal and the biodiesel production plan.

Table 11.4 Design specifications for the aeration tank when no inhibitors are present.

Aeration Tank Parameters		Value for no inhibition
Glucose:	k (1/hr)	0.718
	KS (g/L)	102
	Yc/s (g cell/g sugar)	0.11
Xylose:	k (1/hr)	0.156
	KS (g/L)	36.8
	Yc/s (g cell/g sugar)	0.12
SRT (hours)		29.4
HRT (hours)		6
Working Volume (L)		2,851,402

This simulation was conducted without a biomass recycle stream due to computation errors on the biomass production in the aeration tank. The amount of biomass produced with the recycle was magnitudes lower than without the recycle stream. After simulating various configurations of equalization tanks, storage tanks, and mixing points for the influent and recycle streams, the simulation showed no increase in biomass production. These kinetic parameters were taken from Chapters IX and X, using the glucose and xylose with no inhibitors present. These parameters increase when compared to the aeration tank parameters for the simulation conducted with inhibitors present. The SRT was calculated to be 29.4 hours, using the inverse of net specific growth rate since there is no biomass in the influent (Metcalf & Eddy, 2003; NRC-CNRC, 2004). With an HRT of 6 hours, the working volume was calculated for the aeration tank with no inhibition was 2,851,402 L (0.75 million gallons).

As shown by Figure 11.8, the simulation process did not change with the exception of the furfural and acetic acid. However, the methanol, sulfuric acid, and

calcium oxide influents did change to compensate for the increase in biomass produced. Table 11.5 shows the simulation parameters used for the production of biodiesel with no inhibition. With keeping the wastewater influent and the lignocellulosic sugar the same, the amount of biomass produced is 96.3 kgh^{-1} compared to 1.5 kgh^{-1} with the inhibition. From previous studies, 20% of the biomass is oil with 64% conversion of oil to biodiesel, thus resulting in 12.8% conversion from biomass to biodiesel. Without inhibition, the biodiesel production is 12.3 kgh^{-1} (29,382 gal per yr). This biodiesel production is still small, but quite larger than the production with the inhibition. In addition to the biomass and biodiesel production increasing, the methanol increased substantially from 14.4 kgh^{-1} to 1,152 kgh^{-1}, using the same 12:1 methanol to biomass ratio. Since such a large amount of methanol is required and could increase the cost of the raw materials, the oil could be extracted prior to the transesterification reactor.

Table 11.5 Simulation parameters for the production of biodiesel from oleaginous microorganism consortium grown on wastewater without inhibitors.

Simulation Parameters	**Consortium Grown on Amended Wastewater without Inhibitors**	
Wastewater	473,176	L/h
Lignocellulosic sugars	2,416	kg/h
Sugar Effluent	1,926	kg/h
Methanol	1,152	kg/h
Sulfuric Acid	4.8	L/h
Calcium Oxide	3	L/h
Biomass production	96.3	kg/h
Biomass recycle	----	----
Oil Extracted	----	----
Biodiesel production	12.3	kg/h

The process schematic that includes the extraction of oil is shown in Figure 11.9. The oil extraction unit in Figure 11.9 consists of a storage tank, to control the flow into the high pressure homogenizer. There are many ways to disrupt cell membranes and extract intercellular products such as solvent extraction. However, solvent extraction on a large scale can be very expensive due to the cost of chemicals needed for the extraction. Therefore, high pressure homogenizers are the best option for industrial use. The high pressure homogenizer has been used in multiple bioprocesses to disrupt the cell membranes to extrude inclusion bodies within the microorganisms. For instance, the pharmaceutical industry used the high pressure homogenizers for extraction of enzymes and proteins within the microorganisms on an industrial scale. One such process includes the production of proinsulin in inclusion bodies of E. coli (Petrides, 2000). A high pressure homogenizer contains a positive displacement piston pump with one or more plungers. The biomass enters the check valve and into the pump cylinder. With the pressure stroke, the biomass is pushed through a discharge valve and restricted orifice (Chisti and Moo-Young, 1986). This pressure change causes the disruption of the cell membrane. Homogenizers have also been used to disrupt cell membranes of yeast, such as Candida utilis, which is one of the yeast in the oleaginous microorganism consortium, in addition to algae, bacteria, and fungi (Nasseri et al., 2011).

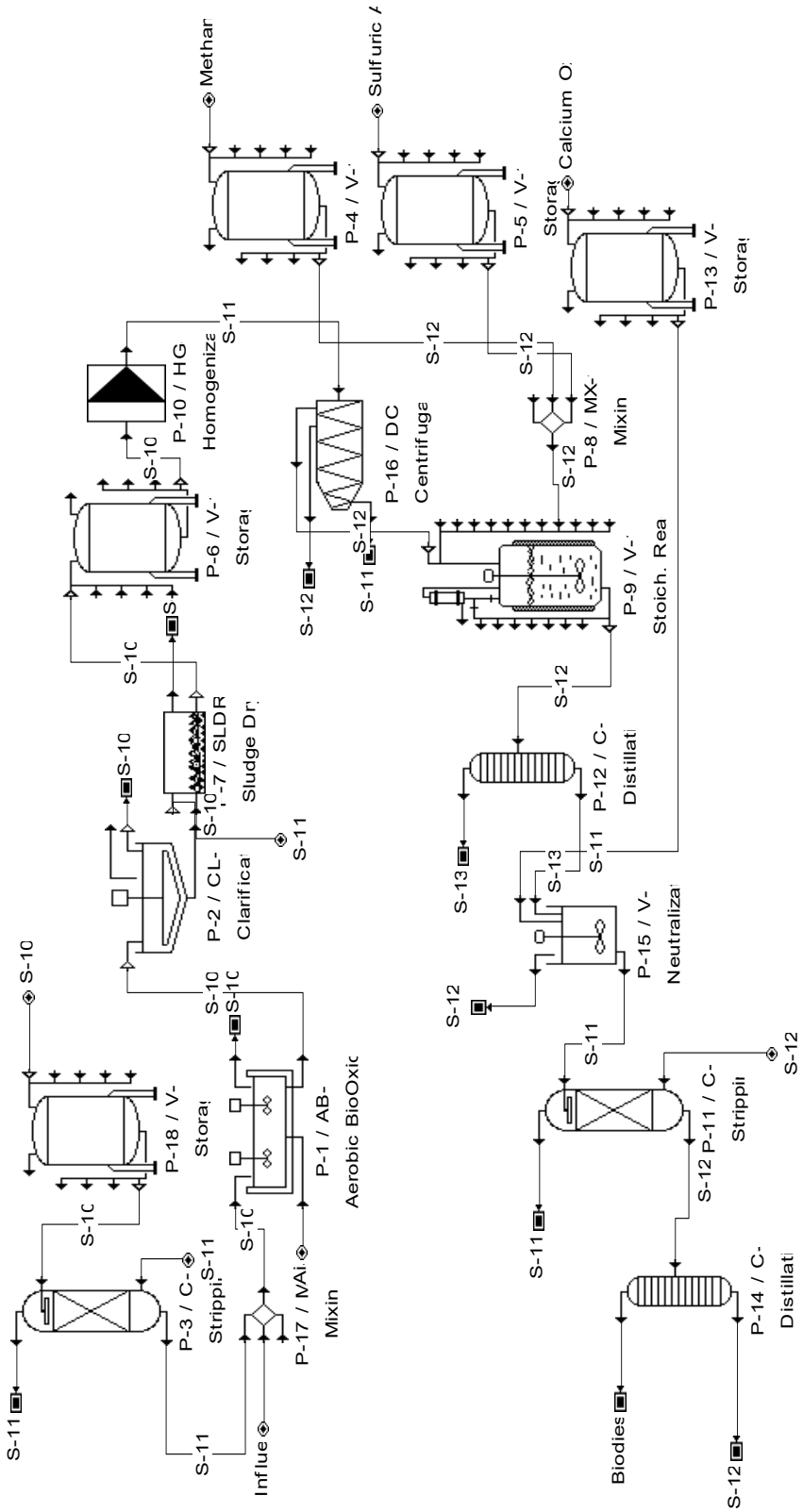

Figure 11.9 Schematic for the wastewater treatment modification for the oleaginous microorganisms with inhibitor removal and the biodiesel production plant.

For the process in Figure 11.9, the biomass was dried to maintain moisture content of 15% instead of 5% to decrease the load on the high pressure homogenizer. Once the oil is removed from within the microorganisms, the oil is separated from the water and the lysed cells, using a centrifuge. The centrifuge uses densities to separate the oil from the lysed cells. The extracted oil was then pumped into the transesterification reactor. The amount of methanol utilized was calculated based on stoichiometric coefficients of the acid-catalyzed transesterification reaction. The calculated methanol recycle was approximately 11,300 kg/h, which was a magnitude larger than the required methanol for the transesterification reaction. In addition, the large amount of methanol recycled required 3 distillation columns with large amounts of high pressure steam and cooling water to operate all 3 distillation columns. Therefore, the methanol was not recycled for this simulation. Table 11.6 shows the simulation parameters for the biodiesel production process with no inhibitors and the oil extracted.

Table 11.6 Simulation parameters for the process of biodiesel production from consortium of oleaginous microorganisms grown on amended wastewater with no inhibitors and oil extracted.

Simulation Parameters	Consortium Grown on Amended Wastewater without Inhibitors and Oil extracted	
Wastewater	473,176	L/h
Lignocellulosic sugars	2,415	kg/h
Sugar Effluent	1,926	g/h
Methanol	51	L/h
Sulfuric Acid	0.85	L/h
Calcium Oxide	0.51	L/h
Biomass production	96.3	kg/h
Biomass recycle	----	kg/h
Oil Extracted	17.4	kg/h
Biodiesel production	10.97	kg/h

The wastewater and lignocellulosic influents are kept the same for this simulation. The biomass production also remains the same since no kinetic parameters are changed in this simulation. However, the methanol influent is drastically decreased as well as the sulfuric acid and calcium oxide. Based on previous results, the percent of oil in biomass is 20%, with a 64% conversion from oil to biodiesel. These parameters result in an annual biodiesel production of 26,292 gal per year. This value is lower than the in situ transesterification without inhibitors but is still substantially greater than the biodiesel production with inhibitors present.

Economic analysis

With these design specifications, the economic analysis can be determined for cultivating the oleaginous microorganism consortium on wastewater amended with lignocellulosic sugar, using a portion of the treatment facility. This economic analysis includes the modifications to the wastewater treatment facility as well as biodiesel production. This economic analysis will compare the three simulations previously discussed and determine the most economical configuration, using SuperPro Designer v6.0.

Table 11.7 compares the total capital cost investment for each simulation. The total plant direct cost (TPDC) was calculated as the sum of the equipment purchase price, installation of equipment, process piping, instrumentation, insulation, electrical, buildings, yard improvement, and auxiliary facilities. The total plan indirect cost (TPIC) is the addition of engineering and construction. The total plant cost (TPC) is the total of TPDC and TPIC. The Direct Fixed Capital Cost (DFC) is the combination of TPC and the contractor's fee and contingency (CFC).

Table 11.7 Fixed capital estimate summary for each simulation.

Fixed Capital Estimate Summary	Consortium Grown on Amended Wastewater with Inhibitors	Consortium Grown on Amended Wastewater without Inhibitors	Consortium Grown on Amended Wastewater without Inhibitors and Oil extracted
1.Total Plant Direct Cost (TPDC)	$20,842,000.00	$43,425,000.00	$43,462,000.00
1.1 Equipment Purchase Price	$6,869,000.00	$13,553,000.00	$13,556,000.00
1.2 Installation	$1,059,000.00	$4,392,000.00	$4,422,000.00
1.3 Process piping	$2,404,000.00	$4,744,000.00	$4,744,000.00
1.4 Instrumentation	$2,748,000.00	$5,421,000.00	$5,422,000.00
1.5 Insulation	$206,000.00	$407,000.00	$407,000.00
1.6 Electrical	$687,000.00	$1,355,000.00	$1,356,000.00
1.7 Buildings	$3,091,000.00	$6,099,000.00	$6,100,000.00
1.8 Yard Improvement	$1,030,000.00	$2,033,000.00	$2,033,000.00
1.9 Auxiliary Facilities	$2,748,000.00	$5,421,000.00	$5,422,000.00
2. Total Plant Indirect Cost (TPIC)	$12,506,000.00	$26,055,000.00	$26,078,000.00
2.1 Engineering	$5,211,000.00	$10,856,000.00	$10,866,000.00
2.2 Construction	$7,295,000.00	$15,199,000.00	$15,212,000.00
3. Total Plant Cost (TPC=TPDC+TPIC)	$33,348,000.00	$69,480,000.00	$69,540,000.00
4. Contractor's Fee and Contingency (CFC)	$3,335,000.00	$10,422,000.00	$9,923,000.00
5. Direct Fixed Capital Cost (DFC=TPC+CFC)	$36,683,000.00	$79,902,000.00	$79,463,000.00

The TPDC for the simulation with the inhibitors is approximately $20.8 million. When removing the inhibitors, the TPDC increases to $43.4 million, due to the 6

methanol storage tanks, methanol recovery distillation column, and the additional wash column to remove the inhibitors. By extracting the oil, the TPDC is slightly higher at $43.5 million due to the addition of the oil extraction equipment even though the methanol requirement decreased. The TPIC also increased by removing the inhibitors. The TPIC for the removal of inhibitors is twice as much as with the inhibitors. The TPIC with the oil extraction is similar to the TPIC of the removed inhibitors. The total capital investment increases with the removal of inhibitors, beginning with $36.7 million for inhibitors, $79.9 million for inhibitor removal, and $79.5 million for inhibitor removal with oil extraction.

Table 11.8 compares the equipment costs, size or capacity, and number of units for each of the simulations. The first simulation includes inhibition model, the second simulation is with inhibitors removed, and the third is with inhibitors removed and oil extracted. The simulation with the inhibition models contains the least number of equipment for biodiesel production when compared to the other two simulations. With removal of the inhibitors, the amount of methanol increased substantially enough that 6 storage tanks are now required instead of 2. In addition, the methanol recovery distillation column is required to recover and recycle the methanol. The lignocellulosic sugar amount did not change with inhibitor removal, so 2 storage tanks are still required. The washing column that removes the furfural and acetic acid is an additional $5.1 million. With extracting the oil, the washing column to remove the furfural and acetic acid, biomass storage, high pressure homogenizer, and decanter centrifuge are all additional pieces of equipment that are required to produce biodiesel. The cost of the oil

extraction equipment is an additional $310,000, which is much smaller than the additional wash column.

Table 11.8 Comparison of the number of units, sizes or capacities, and equipment costs for the different simulations.

	With Inhibitor			Without Inhibitor			Without Inhibitor with Oil Extraction		
Equipment	# of units	Size/Capacity	Cost ($)	# of units	Size/Capacity	Cost ($)	# of units	Size/Capacity	Cost ($)
Aeration Basin	1	3,181,700 L	$5,655,000.00	1	3,168,224 L	$3,982,000.00	1	3,168,224 L	$3,982,000.00
Clarifier	1	1989.5 m2	$279,000.00	1	2,273 m2	$824,000.00	1	2,273 m2	$824,000.00
Wash column to remove CaSO4 and Biomass	1	84.1 L	$6,000.00	1	46.81 L	$3,000.00	1	308 L	$7,000.00
Methanol Recovery Distillation Column (C-102)	1	0.01 L	$8,000.00	1	0.02 L	$8,000.00	1	.01 L	$8,000.00
Transesterification Reactor (V-101)	1	28.56 L	$399,000.00	1	141 L	$399,000.00	1	80.35 L	$399,000.00
Lignocellulosic sugar storage tank (V-108)	1	39,941 L	$86,000.00	2	24,963 L	$94,000.00	2	24,963 L	$94,000.00
Methanol Storage Tank (V-105)	1	2,688 L	$120,000.00	6	35,840 L	$348,000.00	1	9,520 L	$34,000.00
Sulfuric Acid Storage Tank (V-103)	1	6.12 L	$18,000.00	1	896 L	$18,000.00	1	158 L	$18,000.00
Calcium Oxide Storage Tank (V-104)	1	3.82 L	$18,000.00	1	896 L	$18,000.00	1	95 L	$18,000.00
Biodiesel distillation column (C-103)	1	0.15 L	$9,000.00	1	0.59 L	$11,000.00	1	0.46 L	$12,000.00
Neutralization reactor (V-106)	1	12.52 L	$31,000.00	1	70.39 L	$31,000.00	1	8.15 L	$31,000.00
Sludge Dryer (SLDR-101)	1	5,390 kg/h	$31,000.00	1	6,582 kg/h	$31,000.00	1	6,566 kg/h	$31,000.00
Lignocellulosic sugar wash column (C-104)	----	----	----	1	324,670 L	$5,077,000.00	1	324,670 L	$5,077,000.00
Storage of dried biomass (V-102)	----	----	----	----	----	----	1	153.6 L	$18,000.00
High Pressure Homogenizer (HG-101)	----	----	----	----	----	----	1	138 L/h	$22,000.00
Decanter Centrifuge (DC-101)	----	----	----	----	----	----	1	140.5 L/h	$270,000.00

Table 11.9 shows the annual utilities for each simulation. The total annual utilities cost for the simulation with inhibition consists of 25% cooling water, 53% steam and 21% high pressure steam with a total of $490,921 per year. With the removal of inhibitors, 65% of the annual utilities cost consists of cooling water and 33% as high pressure steam. The annual utilities with inhibitor removal are $15 million, which are much higher than either simulation and contributed to the methanol recovery distillation column. The utilities could be reduced by using a different heat exchanger or by routing a cold stream that needs to be heated through the heat exchanger. However, SuperPro simulation has limitations on distillation column operations, thus the calculated costs are general estimations.

Table 11.9 Utilities for each simulation with inhibition and with inhibition removal.

Utilities	Consortium Grown on Amended Wastewater with Inhibitors	Consortium Grown on Amended Wastewater without Inhibitors	Consortium Grown on Amended Wastewater without Inhibitors and Oil extracted
Electricity	$6,422.00	$5,611.00	$17,881.00
Steam	$257,768.00	$314,799.00	$314,022.00
High Pressure Steam	$103,184.00	$4,998,173.00	$7,424.00
Cooling Water	$123,547.00	$9,894,134.00	$4,368.00
Chilled Water	----	----	$5,274.00
Total Annual Utilities Cost	**$490,921.00**	**$15,212,717.00**	**$348,969.00**

With oil extracted and inhibitor removed, the annual utilities of $348,969 are smaller than either of the two simulations. This decrease could be due to the fact that methanol is distilled but not recycled. When the recycle was implemented, the amount of

cooling water and high pressure steam to recycle the large amount of methanol was closer to the utilities values of the simulation without inhibitors. With the oil extraction, the utilities mainly consist of 90% of steam.

The annual raw materials costs for each of the simulations are shown in Table 11.10. The price for lignocellulosic sugars of $0.10 per lb ($0.22 per kg) was estimated using Department of Energy (DOE) target lignocellulosic sugar price. However, according to Israel-based HCL Clean Tech, the lignocellulosic sugar cost is approximately $0.20 per lb ($0.44 per kg), which doubles the annual cost of sugar from $3.4 million to $7 million. Lignocellulosic sugars represent 97% to 99% of the total raw materials cost for the simulations. The cost of lignocellulosic sugars does not change for each simulation since the amount of lignocellulosic sugar used did not change with each simulation. The cost of methanol was $0.02 per kg based on Mondala et al price of $0.08 per gal (Mondala et al., 2009). The lowest annual cost for methanol is the simulation with inhibitors $2,000. The highest at $144,000 was with the removal of inhibitors in order to conduct in situ transesterification without extracting the oil. The sulfuric acid annual cost also increased due to the in situ transesterification. Since the sulfuric acid usage increased, the calcium oxide also increased and thus the increase in annual calcium oxide cost.

Table 11.10 Annual Operating Costs for the simulations using the minimum lignocellulosic price of $0.10 per lb.

Raw Materials	Consortium Grown on Amended Wastewater with Inhibitors	Consortium Grown on Amended Wastewater without Inhibitors	Consortium Grown on Amended Wastewater without Inhibitors and Oil extracted
Lignocellulosic Sugars ($0.22/kg)	$3,366,000.00	$4,208,000.00	$4,208,000.00
Methanol ($0.02/kg)	$2,000.00	$144,000.00	$6,000.00
Sulfuric Acid ($0.070/kg)	$33.25	$5,000.00	$1,000.00
Calcium Oxide ($0.15/kg)	$70.50	$10,000.00	$2,000.00
Total Annual Raw Materials Cost	**$3,368,103.75**	**$4,367,000.00**	**$4,217,000.00**

Using the total annual raw materials cost, the annual operating costs for each simulation are shown in Table 11.11. The total annual raw materials can increase from $3.8 mil up to $7 mil, depending on the price of sugar. Raw materials cost accounts for 10% up to 17% of the annual operating costs for the simulations with inhibitors present and the simulation with inhibitors removed with oil extracted. The labor dependent annual cost increased with inhibitor removal and oil extraction. This increase is due to the increase in equipment required to remove inhibitors and extract the oil. The facility dependent annual cost was the highest with the inhibitor removal and oil extraction amounting to $15 million per year. The laboratory annual costs for each simulation remained around $1 million for each simulation. The annual utilities as shown in Table 11.9 contributed to 35% of the annual operating costs for the simulation with inhibitors removed, 2.6% for the simulation with inhibition, and 1.2% for the simulation with inhibitors removed and oil extracted. By reducing the methanol requirement, recovery,

and recycle, the utilities and thus the overall annual operating costs would decrease. Since SuperPro provides an estimated cost for each equipment and utilities, the utilities and process could be improved to lower the annual operating costs. Using these annual operating costs and the annual biodiesel production, the break-even price for biodiesel would be $107,051 per gal for the simulation without inhibitor removal, $1,449 per gal for removing the inhibitor, and $1,135 per gal for removing inhibitor and extracting oil. The break-even prices calculated use the minimum price of lignocellulosic sugars as a target price set by DOE. Therefore, the break-even prices could increase, depending on the lignocellulosic sugar price. The break-even prices are not competitive when compared to the cost of petroleum diesel at $4.12 per gal (Energy Information System 2012b). However, in this comparison, the most economical choice would be to remove the inhibitor and extract the oil.

Table 11.11 Annual operating costs for each simulation.

Annual Operating Costs	**Consortium Grown on Amended Wastewater with Inhibitors**	**Consortium Grown on Amended Wastewater without Inhibitors**	**Consortium Grown on Amended Wastewater without Inhibitors and Oil extracted**
Raw Materials	$3,368,104	$4,367,000	$4,217,000
Labor-Dependent	$6,675,000	$6,870,000	$8,822,000
Facility-Dependent	$7,306,000	$15,108,000	$15,121,000
Laboratory/QC/QA	$1,001,000	$1,031,000	$1,323,000
Utilities	$490,921	$15,212,717	$348,969
Total Annual Operating Cost	$18,841,025	$42,588,717	$29,831,969
Total Annual Biodiesel Production (gal/yr)	176	29,382	26,292.00
Breakeven price for biodiesel: ($/gal)	**$107,051**	**$1,449**	**$1,135**

To improve the economics of producing oil on wastewater, a simulation and economic analysis was performed using the same design parameters as the simulation with inhibitor removal and extracting the oil from the biomass. The assumption in this economic analysis is that the lignocellulosic sugar price of $0.44 per kg ($0.20 per lb) includes the removal of furfural and acetic acid. In addition, the extracted oil would be sold for $0.76 per kg ($106.55 per barrel), which is based on the price of crude oil as of February 28, 2012 (Energy Information System 2012a).

Table 11.12 shows the overall economic analysis summary that includes the capital cost, annual operating cost, production rate, and total revenue. The capital cost uses the same equipment shown in Table 11.8 for the simulation with inhibitor removed and oil extraction, excluding the pieces of equipment required for biodiesel production and inhibitor removal. The annual operating cost is also similar to the simulation with inhibitor removal and oil extraction, involving the production of biomass and extraction of oil. The annual production rate accounts for the total flow of stream influent per year, and the unit production cost is the cost per total flow of stream influent per year. The total revenue is based on how much oil is produced per year and sold for the price of crude oil.

Table 11.12 Economic analysis summary for the simulation with inhibitor removal and oil extraction without biodiesel production units and inhibitor removal units.

Economic Analysis Summary	Consortium Grown on Amended Wastewater without Inhibitors and Oil extracted
Direct Fixed Capital Cost (DFC=TPC+CFC)	**$76,076,000.00**
1. Total Plant Direct Cost (TPDC)	$41,345,000.00
1.1 Equipment Purchase Price	$12,898,000.00
1.2 Installation	$4,199,000.00
1.3 Process piping	$4,514,000.00
1.4 Instrumentation	$5,159,000.00
1.5 Insulation	$387,000.00
1.6 Electrical	$1,290,000.00
1.7 Buildings	$5,804,000.00
1.8 Yard Improvement	$1,935,000.00
1.9 Auxiliary Facilities	$5,159,000.00
2. Total Plant Indirect Cost (TPIC)	$24,807,000.00
3. Total Plant Cost (TPC=TPDC+TPIC)	$66,153,000.00
4. Contractor's Fee and Contingency (CFC)	$9,923,000.00
Annual Operating Costs	**$23,016,000.00**
Raw Materials	$4,208,000.00
Labor-Dependent	$3,552,000.00
Facility-Dependent	$14,386,000.00
Laboratory/QC/QA	$533,000.00
Utilities	$337,000.00
Annual Oil Production Rate (gal Oil/yr)	189,283
Unit Production Cost ($/gal Oil)	$121.60
Total Annual Revenue	**$109,000.00**

The direct fixed capital cost (DFC) was calculated to be $76 million with an equipment cost of $12.9 million. Compared to each of the simulations of $36.7 million without inhibitor, $79.9 million with inhibitor removal, and $79.5 million with inhibitor removal and oil extraction, the capital costs are much reduced. The annual operating costs were determined to be $23 million with a raw materials cost of $4.2 million and

utilities of $337,000 per year. The raw materials cost is made up of lignocellulosic sugars for a cost of $0.20 per lb. The utilities cost consists of 93% of steam, 5% of electricity, and 2% of chilled water. When compared to the other simulations, the raw materials cost does not change since the same amount of sugars were used in this simulation. The utilities cost of $337,000 for this simulation is much smaller than the $490,921 without inhibitor removal, $15 million with inhibitor removal. However, the utilities are similar to the simulation with inhibitor removal and oil extraction with $348,969 per year. The total annual revenue is $109,000 with an oil production rate of 189,283 gal of oil per year and a unit production cost of $121 per gal of oil. The unit production price is similar to the break-even price calculated for the previously discussed simulations. When comparing these simulations, the most economical process appears to be the production of biomass and selling the extracted oil to a biodiesel refinery. Therefore, the wastewater treatment process modification has shown to produce oil from biomass as a biodiesel feedstock.

Conclusion

By modifying the wastewater treatment process and adding a biodiesel production plant, biodiesel can be produced using wastewater with an oleaginous microorganism consortium. Simulations were performed to compare 3 variations of the modified wastewater treatment plant with the addition of lignocellulosic sugars. The first simulation modeled the biomass and biodiesel production in the presence of inhibitors from lignocellulosic sugars. With the inhibition results from Chapter IX and X, 1.5 kgh^{-1} of biomass was produced along with 0.07 kgh^{-1} of biodiesel, with an SRT of 6.78 hours.

When the inhibitors were removed, the biomass and biodiesel produced were 96.3 kgh^{-1} and 12.3 kgh^{-1}, respectively. Without the inhibition, the aeration tank was modeled using SRT of 29.4 hours. In order to reduce the amount of methanol required, the oil was extracted using high pressure homogenizer to produce 10.97 kgh^{-1} of biodiesel.

The economic analysis consisted of comparing direct fixed capital cost for each simulation. With the inhibitors, the capital investment consists of $36.7. By removing the inhibitors, the capital investment cost is $79.9 million, including the 2 lignocellulosic sugar storage tanks and 6 storage tanks for methanol. The capital investment cost with the simulation for the removal of inhibitors and oil extracted was $79.5 million. The annual operating costs were $18.8 million, $42.6 million, and $29.8 million for simulation with inhibitors, with inhibitor removal, and oil extraction, respectively. The break-even price for biodiesel comes to $107,051 per gal for the simulation with inhibitors, $1,449 per gallon for simulation with inhibitors removed, and $1,135 per gallon for oil extraction. To further reduce capital costs and annual operating costs, the oil was sold to a biodiesel refinery at the price of crude oil as well as the purchase price for lignocellulosic sugars includes the removal of inhibitors. Selling the oil reduces the capital costs to $66 million. With an annual operating cost of $23 million, the break-even or unit production cost is $121 per gal of oil. Thus, the most economical option is to sell the oil to a biodiesel refinery and purchase lignocellulosic sugars with inhibitor removed.

To further reduce the break-even cost for this process to become economical, this process should be optimized. Optimization of this process should include including a recycle stream, recycling the biomass. The addition of nutrient reactions, including

nitrogen and phosphorus, should improve the design. For this simulation, using a dry hydrolysate could possibly have improved the process instead of a liquid hydrolysate.

CHAPTER XII

CONCLUSIONS

Cultivating the oleaginous microorganism consortium on municipal wastewater to produce oil for biofuels was evaluated. Based on the research results, municipal wastewater can be used as a cultivation medium for oleaginous microorganism consortium when amended with lignocellulosic sugars for oil production. Incorporating these microorganisms into the wastewater treatment facility can cause an increase in the total biodiesel production. Also, producing oil from the wastewater treatment facility can reduce the dependence on oil from foods prominent in the food industry. Below are the individual conclusions.

- The result of cultivating Rhodotorula glutinis and Cryptococcus curvatus on autoclaved primary effluent wastewater determined that oleaginous microorganisms could be cultivated on wastewater with the addition of sugars. In addition, these results showed that municipal wastewater does not contain growth-inhibiting components. Therefore, the wastewater can be utilized as a growth medium. The lipid production for these pure cultures resulted in an increase in glucose concentration. Since wastewater constituents vary, a consortium of oleaginous microorganisms is a better fit than a pure culture. The result for the consortium grown on

autoclaved wastewater showed no major inhibitory substances. Increasing the sugar concentration in the wastewater did not show a significant change in the fatty acid profile of the consortium. In investigating the effect carbon concentration has on the consortium's growth, a synthetic wastewater was utilized and resulted in an increase in cell mass production and reduction in COD.

- In investigating the effect indigenous microorganisms have on the consortium, the results show that the indigenous microorganisms have a negative impact on the presence of the microorganisms in the consortium with the addition of 1 gL^{-1} of glucose. When considering the total FAMEs produced, the incorporation of the consortium into raw wastewater showed a 13% increase. To overcome this negative effect, a pre-treatment of ozonation can be utilized. The consortium grown on ozonated wastewater results in an increase in cell mass production, proving that ozonating wastewater could increase the available oxygen. With the increase of glucose to 60 gL^{-1}, the addition of the consortium into the raw wastewater showed a large increase in cell mass production as well as an 85% increase in total FAMEs. Therefore, by incorporating the consortium into the wastewater treatment facility, the total production of oil is increased, especially compared to raw wastewater with the high concentration of glucose.
- To increase the carbon concentration in wastewater, lignocellulosic sugars are an alternative source of sugars to sugars found in the food industry.

However, in the production of lignocellulosic sugars, two major growth inhibitory substances, furfural and acetic acid, are by-products. In determining the consortium's ability to withstand these inhibitory compounds, the results showed that overall the cell mass, lipid production, sugar consumption, and, inherently, kinetic parameters are inhibited by the presence of furfural and acetic acid. The increase in sugar concentration did not decrease the inhibitory effects of the furfural and acetic acid. Since the least inhibition was shown with the lowest concentration of furfural and acetic acid, the presence of these compounds is inhibitory to the consortium. Moreover, since furfural and acetic acid are inhibitory to the consortium, the next phase is to determine the individual effects of furfural and acetic acid as well as the type of inhibition. The type of inhibition allows the consortium's behavior to be predicted and thus overcome using substrate concentration or initial cell concentration.

- With the cultivation of the consortium on synthetic wastewater amended with glucose and varying amounts of furfural and acetic acid, cell mass and lipid production were inhibited. When glucose was used as the primary carbon source, the glucose consumption showed inhibition with acetic acid concentration increasing but slight inhibition with furfural present. The FAME analysis did not show a microbial population shift with furfural and acetic acid when glucose is used as the carbon source. With xylose, the FAME analysis resulted in a possible microbial shift due

to the increase in Heptadecanoic acid with increasing furfural and acetic acid concentration.

- Models were developed to describe the inhibition of furfural and acetic acid when cultivated on synthetic wastewater amended with glucose and xylose. The models for acetic acid and furfural with glucose as the main carbon source were developed by modifying the constants in the Monod model to incorporate inhibitor concentration. The modification for the furfural model with glucose consisted of an exponential function for the maximum specific growth rate and a power function for the Monod constant. The model to describe acetic acid inhibition with glucose is best described by power functions for both constants in the Monod model, which is proposed to follow mixed inhibition. The model to describe the inhibition of acetic acid and furfural with xylose as the main carbon source is developed by modifying the Contois model maximum specific growth rate. For the both furfural and acetic acid inhibition, the power function of the inhibitors described the inhibition. Overall, from the model development, the furfural with glucose as the carbon source has the highest inhibitor effect on the consortium.
- Incorporating the oleaginous microorganism consortium into the wastewater treatment facility requires modification to the current wastewater treatment facilities to avoid interfering with the wastewater treatment process. To simulate the modification, SuperPro Designer v6.0 was used. One simulation consisted of incorporating the inhibition

kinetics developed in Chapter X to account for the inhibitors in the lignocellulosic sugars. The solids residence time (SRT) was calculated to be 6.78 hours with an annual biodiesel production rate of 0.07 kgh^{-1}. This simulation resulted in a capital cost of $36.7 million with an annual operating cost of $24.2 million. The break-even price for biodiesel production with inhibitors was calculated to be $137,255 per gal. To improve the productivity, a simulation was performed to remove the inhibitors, which included the un-inhibited kinetics shown in Chapter X. The SRT was determined to be 29.4 hours without the inhibitors. This simulation increased the production of biodiesel. However, the methanol required to perform in situ transesterification added to the capital cost and operating cost due to the 6 methanol storage tanks as well as the methanol recovery system. The capital cost is $79.9 million with an annual operating cost of $42.6 million and a break-even price of $1,449 per gal, so this simulation is more economical than with the inhibitor. To reduce the methanol requirement, a simulation and analysis was performed to extract the oil prior to the transesterification reactor, which decreased the capital cost to $79.5 million and the annual operating cost to $30.6 million. The break-even cost was reduced to $1,057 per gal. If the oil was sold to a biodiesel refinery and the lignocellulosic sugars purchase cost included removal of inhibitors, the capital cost is calculated to be $76 million with an annual operating cost of $23 million. The break-even cost was determined to be $121 per gal of oil. With these results, the most

economical option is selling the oil at crude oil prices to a biodiesel refinery.

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APPENDIX A

COMPLETE POLYMATH MODELING RESULTS FOR FITTING MONOD MODEL IN CHAPTER IX

Table A.1 Polymath output for fitting the Monod model to the data of the consortium grown on glucose in the presence of 0.1 gL^{-1} of furfural.

Nonlinear regression (L-M)

Model: u = smax*CA/(CM1+CA)

Variable	Ini guess	Value	95% confidence
smax	0.01	0.5312023	0.0698566
CM1	1	101.99958	13.964579

Nonlinear regression settings
Max # iterations = 64
Precision

R^2	= 0.9400156
R^2adj	= 0.9100234
Rmsd	= 5.241E-04
Variance	= 2.197E-06

General

Sample size	= 4
# Model vars	= 2
# Indep vars	= 1
# Iterations	= 20

Table A.2 Polymath output for fitting the Monod model to the data of the consortium grown on glucose in the presence of 0.2 gL^{-1} of furfural.

Nonlinear regression (L-M)

Model: u = smax*CA/(CM1+CA)

Variable	Ini guess	Value	95% confidence
smax	1	0.3863774	0.1421853
CM1	1	46.544401	18.665759

Nonlinear regression settings
Max # iterations = 64
Precision

R^2	= 0.9944506
R^2adj	= 0.9916759
Rmsd	= 2.548E-04
Variance	= 5.193E-07

General

Sample size	= 4
# Model vars	= 2
# Indep vars	= 1
# Iterations	= 11

Table A.3 Polymath output for fitting the Monod model to the data of the consortium grown on glucose in the presence of 0.3 gL^{-1} of furfural.

Nonlinear regression (L-M)

Model: u = smax*CA/(CM1+CA)

Variable	Ini guess	Value	95% confidence
smax	1	0.0244659	0.0184901
CM1	2	1.8173207	3.927867

Nonlinear regression settings
Max # iterations = 64
Precision
R^2 = 0.8273104
R^2adj = 0.7409656
Rmsd = 3.784E-04
Variance = 1.146E-06
General
Sample size = 4
Model vars = 2
Indep vars = 1
Iterations = 7

Table A.4 Polymath output for fitting the Monod model to the data of the consortium grown on glucose in the presence of 0.4 gL^{-1} of furfural.

Nonlinear regression (L-M)

Model: u = smax*CA/(CM1+CA)

Variable	Ini guess	Value	95% confidence
smax	0.1	0.0181043	0.0700648
CM1	1	1.694801	21.664717

Nonlinear regression settings
Max # iterations = 64
Precision
R^2 = 0.6916994
R^2adj = 0.3833988
Rmsd = 3.097E-04
Variance = 8.634E-07
General
Sample size = 3
Model vars = 2
Indep vars = 1
Iterations = 7

Table A.5 Polymath output for fitting the Monod model to the data of the consortium grown on glucose in the presence of 0.5 gL^{-1} of furfural.

Nonlinear regression (L-M)

Model: u = smax*CA/(CM1+CA)

Variable	Ini guess	Value	95% confidence
smax	0.5	0.0711675	0.0239359
CM1	-1	99.999911	35.096016

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.2336914
R^2adj	= -0.5326171
Rmsd	= 0.0014009
Variance	= 1.766E-05

General

Sample size	= 3
# Model vars	= 2
# Indep vars	= 1
# Iterations	= 28

Table A.6 Polymath output for fitting the Monod model to the data of the consortium grown on glucose in the presence of 0.5 gL^{-1} of acetic acid.

Nonlinear regression (L-M)

Model: u = k*CA/(B+CA)

Variable	Ini guess	Value	95% confidence
k	1	0.401872	0.031933
B	1	101.99767	8.40299

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.9264264
R^2adj	= 0.9019019
Rmsd	= 6.961E-04
Variance	= 4.038E-06

General

Sample size	= 5
# Model vars	= 2
# Indep vars	= 1
# Iterations	= 27

Table A.7 Polymath output for fitting the Monod model to the data of the consortium grown on glucose in the presence of 0.6 gL^{-1} of acetic acid.

Nonlinear regression (L-M)

Model: u = k*CA/(B+CA)

Variable	Ini guess	Value	95% confidence
k	1	0.1410023	0.0150093
B	1	101.99942	11.31951

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.9160376
R^2adj	= 0.8740564
Rmsd	= 2.96E-04
Variance	= 7.01E-07

General

Sample size	= 4
# Model vars	= 2
# Indep vars	= 1
# Iterations	= 33

Table A.8 Polymath output for fitting the Monod model to the data of the consortium grown on glucose in the presence of 0.7 gL^{-1} of acetic acid.

Nonlinear regression (L-M)

Model: u = k*CA/(B+CA)

Variable	Ini guess	Value	95% confidence
k	1	0.0194986	0.0061603
B	1	0.8787291	1.3218027

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.8747766
R^2adj	= 0.8121649
Rmsd	= 1.469E-04
Variance	= 1.726E-07

General

Sample size	= 4
# Model vars	= 2
# Indep vars	= 1
# Iterations	= 7

Table A.9 Polymath output for fitting the Monod model to the data of the consortium grown on glucose in the presence of 0.8 gL^{-1} of acetic acid.

Nonlinear regression (L-M)

Model: u = k*CA/(B+CA)

Variable	Ini guess	Value	95% confidence
k	1	0.0441504	1.644E-04
B	0.05	6.0499854	0.037941

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.985847
R^2adj	= 0.9787704
Rmsd	= 3.165E-04
Variance	= 8.012E-07

General

Sample size	= 4
# Model vars	= 2
# Indep vars	= 1
# Iterations	= 19

Table A.10 Polymath output for fitting the Monod model to the data of the consortium grown on glucose in the presence of 1.5 gL^{-1} of acetic acid.

Nonlinear regression (L-M)

Model: u = k*CA/(B+CA)

Variable	Ini guess	Value	95% confidence
k	1	0.0144801	0.0051254
B	0.9	0.2542621	0.7969041

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.9576039
R^2adj	= 0.9152078
Rmsd	= 9.527E-05
Variance	= 8.168E-08

General

Sample size	= 3
# Model vars	= 2
# Indep vars	= 1
# Iterations	= 8

APPENDIX B

COMPLETE POLYMATH MODELING RESULTS FOR MODIFYING THE MONOD MODEL IN CHAPTER IX FOR CONSORTIUM GROWN ON GLUCOSE WITH FURFURAL AND ACETIC ACID

Table B.1 Polymath output for the modifying the Monod model for the consortium grown on glucose with furfural.

Nonlinear regression (L-M)

Model: u = .718*Cl^m*CA/((102*Cl^n)+CA)

Variable	Ini guess	Value	95% confidence
m	0.1	0.2217276	0.966058
n	-1	0.0830227	1.0121171

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= -0.0304527
R^2adj	= -0.1097183
Rmsd	= 0.0019434
Variance	= 6.537E-05

General

Sample size	= 15
# Model vars	= 2
# Indep vars	= 2
# Iterations	= 11

Table B.2 Polymath output for the modifying the Monod model for the consortium grown on glucose with furfural.

Nonlinear regression (L-M)

Model: u = .718*exp(Cl*A)*CA/((102*(Cl^B))+CA)

Variable	Ini guess	Value	95% confidence
A	1	-1.2485408	0.0736724
B	1	0.0042637	0.0101003

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.3951317
R^2adj	= 0.3486034
Rmsd	= 0.0014889
Variance	= 3.837E-05

General

Sample size	= 15
# Model vars	= 2
# Indep vars	= 2
# Iterations	= 11

Table B.3 Polymath output for the modifying the Monod model for the consortium grown on glucose with furfural.

Nonlinear regression (L-M)

Model: u = .718*exp(CI*A)*CA/((102*exp(CI*B))+CA)

Variable	Ini guess	Value	95% confidence
A	0.1	-8.9396864	2.3335054
B	-0.1	-9.4049118	3.5311004

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.570145
R^2adj	= 0.5370793
Rmsd	= 0.0012552
Variance	= 2.727E-05

General

Sample size	= 15
# Model vars	= 2
# Indep vars	= 2
# Iterations	= 11

Table B.4 Polymath output for the modifying the Monod model for the consortium grown on glucose with furfural.

Nonlinear regression (L-M)

Model: u = .718*exp(CI*A)*CA/((261*exp(CI*B))+CA)

Variable	Ini guess	Value	95% confidence
A	-1	-11.121258	0.8200914
B	1	-18.288313	1.6970482

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.8187476
R^2adj	= 0.8048051
Rmsd	= 8.151E-04
Variance	= 1.15E-05

General

Sample size	= 15
# Model vars	= 2
# Indep vars	= 2
# Iterations	= 16

Table B.5 Polymath output for the modifying the Monod model for the consortium grown on glucose with furfural.

Nonlinear regression (L-M)

Model: u = .718*exp(CI*A)*CA/((.0702*CI^n)+CA)

Variable	Ini guess	Value	95% confidence
A	1	-10.748692	0.4434634
n	-3	-2.8272558	0.0753769

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.866619
R^2adj	= 0.856359
Rmsd	= 6.992E-04
Variance	= 8.461E-06

General

Sample size	= 15
# Model vars	= 2
# Indep vars	= 2
# Iterations	= 10

Table B.6 Polymath output for the modifying the Monod model for the consortium grown on glucose with acetic acid.

Nonlinear regression (L-M)

Model: u = .037*CI^(A)*CA/(2107.7*exp(B*CI)+CA)

Variable	Ini guess	Value	95% confidence
A	1	-2.4898961	0.6597196
B	1	-6.425825	0.5505082

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.5950556
R^2adj	= 0.566131
Rmsd	= 9.352E-04
Variance	= 1.599E-05

General

Sample size	= 16
# Model vars	= 2
# Indep vars	= 2
# Iterations	= 11

Table B.7 Polymath output for the modifying the Monod model for the consortium grown on glucose with acetic acid.

Nonlinear regression (L-M)

Model: u = .935*exp(A*CI)*CA/(2107.7*exp(B*CI)+CA)

Variable	Ini guess	Value	95% confidence
A	1	1.8206367	0.0012363
B	1	0.1785118	0.0012382

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= -0.5375897
R^2adj	= -0.6474175
Rmsd	= 0.0018223
Variance	= 6.072E-05

General

Sample size	= 16
# Model vars	= 2
# Indep vars	= 2
# Iterations	= 7

Table B.8 Polymath output for the modifying the Monod model for the consortium grown on glucose with acetic acid.

Nonlinear regression (L-M)

Model: u = .935*exp(A*CI)*CA/(2.53*CI^(B)+CA)

Variable	Ini guess	Value	95% confidence
A	1	-3.0807068	0.4365309
B	1	-6.7482946	1.770572

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.0786062
R^2adj	= 0.0127923
Rmsd	= 0.0014106
Variance	= 3.639E-05

General

Sample size	= 16
# Model vars	= 2
# Indep vars	= 2
# Iterations	= 28

Table B.9 Polymath output for the modifying the Monod model for the consortium grown on glucose with acetic acid.

Nonlinear regression (L-M)

Model: u = .037*CI^(A)*CA/(2.53*CI^(B)+CA)

Variable	Ini guess	Value	95% confidence
A	1	2.6243127	0.8788218
B	1	5.2576597	1.9213584

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.2271106
R^2adj	= 0.1719043
Rmsd	= 0.001292
Variance	= 3.052E-05

General

Sample size	= 16
# Model vars	= 2
# Indep vars	= 2
# Iterations	= 8

Table B.10 Polymath output for the modifying the Monod model for the consortium grown on glucose with acetic acid.

Nonlinear regression (L-M)

Model: u = .037*CI^(A)*CA/(B*exp(C*CI)+CA)

Variable	Ini guess	Value	95% confidence
A	1	-1.5061578	0.0204329
B	1	101.99453	1.4538741
C	1	-3.0712422	0.0197697

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.903259
R^2adj	= 0.881761
Rmsd	= 4.944E-04
Variance	= 3.911E-06

General

Sample size	= 12
# Model vars	= 3
# Indep vars	= 2
# Iterations	= 37

Table B.11 Polymath output for the modifying the Monod model for the consortium grown on glucose with acetic acid.

Nonlinear regression (L-M)

Model: u = .037*CI^(A)*CA/(B*CI^(C)+CA)

Variable	Ini guess	Value	95% confidence
A	1	-1.5317436	6.348E-05
B	1	4.1684352	1.717E-04
C	1	-2.6095708	8.266E-05

Nonlinear regression settings
Max # iterations = 64

Precision

R^2	= 0.9220165
R^2adj	= 0.9046868
Rmsd	= 4.439E-04
Variance	= 3.153E-06

General

Sample size	= 12
# Model vars	= 3
# Indep vars	= 2
# Iterations	= 8

APPENDIX C

COMPLETE POLYMATH RESULTS FOR FITTING DATA FROM THE CONSORTIUM GROWN ON XYLOSE WITH FURFURAL AND ACETIC ACID TO THE CONTOIS MODEL IN CHAPTER IX

Table C.1 Polymath output for fitting the Contois model to the data of the consortium grown on xylose in the presence of 0.1 gL^{-1} of furfural.

Nonlinear regression (L-M)

Model: u = k*CA/(B*CC+CA)

Variable	Ini guess	Value	95% confidence
k	0.01	0.1606812	0.0032981
B	0.9	91.898462	2.4999574

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.7814753
R^2adj	= 0.7086338
Rmsd	= 0.0049794
Variance	= 2.066E-04

General

Sample size	= 5
# Model vars	= 2
# Indep vars	= 2
# Iterations	= 26

Table C.2 Polymath output for fitting the Contois model to the data of the consortium grown on xylose in the presence of 0.5 gL^{-1} of furfural.

Nonlinear regression (L-M)

Model: u = k*CA/(B*CC+CA)

Variable	Ini guess	Value	95% confidence
k	0.01	0.1258065	0.0038227
B	0.9	91.899442	3.6864276

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.6144374
R^2adj	= 0.4859165
Rmsd	= 0.0061687
Variance	= 3.171E-04

General

Sample size	= 5
# Model vars	= 2
# Indep vars	= 2
# Iterations	= 15

Table C.3 Polymath output for fitting the Contois model to the data of the consortium grown on xylose in the presence of 1 gL^{-1} of furfural.

Nonlinear regression (L-M)

Model: u = k*CA/(B*CC+CA)

Variable	Ini guess	Value	95% confidence
k	1	0.1346872	0.0073945
B	0.9	91.899219	7.4820789

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.9478512
R^2adj	= 0.9304683
Rmsd	= 0.0015421
Variance	= 1.982E-05

General

Sample size	= 5
# Model vars	= 2
# Indep vars	= 2
# Iterations	= 18

Table C.4 Polymath output for fitting the Contois model to the data of the consortium grown on xylose in the presence of 1.5 gL^{-1} of furfural.

Nonlinear regression (L-M)

Model: u = k*CA/(B*CC+CA)

Variable	Ini guess	Value	95% confidence
k	1	0.1365648	0.0018792
B	0.9	91.897862	1.9441255

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.9765744
R^2adj	= 0.9687658
Rmsd	= 0.0012804
Variance	= 1.366E-05

General

Sample size	= 5
# Model vars	= 2
# Indep vars	= 2
# Iterations	= 12

Table C.5 Polymath output for fitting the Contois model to the data of the consortium grown on xylose in the presence of 2 gL^{-1} of furfural.

Nonlinear regression (L-M)

Model: u = k*CA/(B*CC+CA)

Variable	Ini guess	Value	95% confidence
k	1	0.1281904	0.0017146
B	0.9	91.89761	1.6982854

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.9755297
R^2adj	= 0.9673729
Rmsd	= 9.486E-04
Variance	= 7.499E-06

General

Sample size	= 5
# Model vars	= 2
# Indep vars	= 2
# Iterations	= 17

Table C.6 Polymath output for fitting the Contois model to the data of the consortium grown on xylose in the presence of 0.75 gL^{-1} of acetic acid.

Nonlinear regression (L-M)

Model: u = k*CA/(102*CC+CA)

Variable	Ini guess	Value	95% confidence
k	1	0.1544501	0.0154242

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.94403
R^2adj	= 0.94403
Rmsd	= 7.536E-04
Variance	= 3.55E-06

General

Sample size	= 5
# Model vars	= 1
# Indep vars	= 2
# Iterations	= 4

Table C.7 Polymath output for fitting the Contois model to the data of the consortium grown on xylose in the presence of 1 gL^{-1} of acetic acid.

Nonlinear regression (L-M)

Model: u = k*CA/(B*CC+CA)

Variable	Ini guess	Value	95% confidence
k	1	0.1297848	0.0043877
B	1	101.99523	4.0998649

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.9733331
R^2adj	= 0.9599996
Rmsd	= 3.612E-04
Variance	= 1.044E-06

General

Sample size	= 4
# Model vars	= 2
# Indep vars	= 2
# Iterations	= 27

Table C.8 Polymath output for fitting the Contois model to the data of the consortium grown on xylose in the presence of 1.25 gL^{-1} of acetic acid.

Nonlinear regression (L-M)

Model: u = k*CA/(B*CC+CA)

Variable	Ini guess	Value	95% confidence
k	1	0.1293699	0.0024391
B	1	101.99771	2.340205

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.9868274
R^2adj	= 0.9824365
Rmsd	= 3.175E-04
Variance	= 8.401E-07

General

Sample size	= 5
# Model vars	= 2
# Indep vars	= 2
# Iterations	= 15

Table C.9 Polymath output for fitting the Contois model to the data of the consortium grown on xylose in the presence of 1.5 gL^{-1} of acetic acid.

Nonlinear regression (L-M)

Model: u = k*CA/(B*CC+CA)

Variable	Ini guess	Value	95% confidence
k	1	0.1426608	0.0011237
B	1	101.99908	0.9943847

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.9855826
R^2adj	= 0.9807768
Rmsd	= 4.622E-04
Variance	= 1.78E-06

General

Sample size	= 5
# Model vars	= 2
# Indep vars	= 2
# Iterations	= 18

Table C.10 Polymath output for fitting the Contois model to the data of the consortium grown on xylose in the presence of 1.75 gL^{-1} of acetic acid.

Nonlinear regression (L-M)

Model: u = k*CA/(B*CC+CA)

Variable	Ini guess	Value	95% confidence
k	1	0.1087018	0.0011765
B	1	101.99948	1.4565113

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.9876216
R^2adj	= 0.9814325
Rmsd	= 4.179E-04
Variance	= 1.397E-06

General

Sample size	= 4
# Model vars	= 2
# Indep vars	= 2
# Iterations	= 25

APPENDIX D

COMPLETE POLYMATH RESULTS FOR FITTING DATA FROM THE CONSORTIUM GROWN ON XYLOSE WITH FURFURAL AND ACETIC ACID TO THE MODIFIED CONTOIS MODEL IN CHAPTER IX

Table D.1 Polymath output for the modifying the Contois model for the consortium grown on xylose with furfural.

Nonlinear regression (L-M)

Model: u = .16*exp(A*CI)*CA/(92*CC+CA)

Variable	Ini guess	Value	95% confidence
A	0.1	-0.1170584	0.089331

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.8298181
R^2adj	= 0.8298181
Rmsd	= 0.0019797
Variance	= 6.689E-05

General

Sample size	= 16
# Model vars	= 1
# Indep vars	= 3
# Iterations	= 5

Table D.2 Polymath output for the modifying the Contois model for the consortium grown on xylose with furfural.

Nonlinear regression (L-M)

Model: u = .16*exp(A*CI)*CA/(B*CC+CA)

Variable	Ini guess	Value	95% confidence
A	0.1	-0.1729874	0.1666715
B	0.9	81.20453	25.938393

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.8378366
R^2adj	= 0.8262535
Rmsd	= 0.0019325
Variance	= 6.829E-05

General

Sample size	= 16
# Model vars	= 2
# Indep vars	= 3
# Iterations	= 8

Table D.3 Polymath output for the modifying the Contois model for the consortium grown on xylose with furfural.

Nonlinear regression (L-M)

Model: u = .14*Cl^A*CA/(92*CC+CA)

Variable	Ini guess	Value	95% confidence
A	0.1	-0.1299236	0.0802615

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.8631675
R^2adj	= 0.8631675
Rmsd	= 0.0017751
Variance	= 5.378E-05

General

Sample size	= 16
# Model vars	= 1
# Indep vars	= 3
# Iterations	= 5

Table D.4 Polymath output for the modifying the Contois model for the consortium grown on xylose with furfural.

Nonlinear regression (L-M)

Model: u = .14*Cl^A*CA/(B*CC+CA)

Variable	Ini guess	Value	95% confidence
A	0.1	-0.1313785	0.002634
B	0.9	91.898554	0.441673

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.8630645
R^2adj	= 0.8532834
Rmsd	= 0.0017758
Variance	= 5.766E-05

General

Sample size	= 16
# Model vars	= 2
# Indep vars	= 3
# Iterations	= 17

Table D.5 Polymath output for the modifying the Contois model for the consortium grown on xylose with furfural.

Nonlinear regression (L-M)

Model: u = .16*CI^A*CA/(B*CC+CA)

Variable	Ini guess	Value	95% confidence
A	1	-0.1239024	8.279E-04
B	1	101.99991	0.1473702

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.8535945
R^2adj	= 0.843137
Rmsd	= 0.0018362
Variance	= 6.165E-05

General

Sample size	= 16
# Model vars	= 2
# Indep vars	= 3
# Iterations	= 13

Table D.6 Polymath output for the modifying the Contois model for the consortium grown on xylose with acetic acid.

Nonlinear regression (L-M)

Model: u = .136*CI^(A)*CA/(102*CC+CA)

Variable	Ini guess	Value	95% confidence
A	1	-0.3902606	0.0861185

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.9633512
R^2adj	= 0.9633512
Rmsd	= 2.977E-04
Variance	= 1.689E-06

General

Sample size	= 18
# Model vars	= 1
# Indep vars	= 3
# Iterations	= 5

Table D.7 Polymath output for the modifying the Contois model for the consortium grown on xylose with acetic acid.

Nonlinear regression (L-M)

Model: u = .19*exp(A*CI)*CA/(102*CC+CA)

Variable	Ini guess	Value	95% confidence
A	1	-0.3193147	0.0248735

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.9601444
R^2adj	= 0.9601444
Rmsd	= 3.105E-04
Variance	= 1.837E-06

General

Sample size	= 18
# Model vars	= 1
# Indep vars	= 3
# Iterations	= 7

Table D.8 Polymath output for the modifying the Contois model for the consortium grown on xylose with acetic acid.

Nonlinear regression (L-M)

Model: u = .19*exp(A*CI)*CA/(B*CC+CA)

Variable	Ini guess	Value	95% confidence
A	1	-0.3279632	0.0750011
B	10	100.57695	11.521159

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.9603093
R^2adj	= 0.9578286
Rmsd	= 3.098E-04
Variance	= 1.944E-06

General

Sample size	= 18
# Model vars	= 2
# Indep vars	= 3
# Iterations	= 9