THE INTEGRATION OF AUTOTROPHIC AND HETEROTROPHIC MICROBIAL PROCESSES FOR BIOFUEL PRODUCTION

By

Yingkui Zhong

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ABSTRACT

THE INTEGRATION OF AUTOTROPHIC AND HETEROTROPHIC MICROBIAL PROCESSES FOR BIOFUEL PRODUCTION

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Biofuel is an alternative energy source that has drawn much attention. Microbes that can utilize various types of carbon sources are considered as potential biofuel producers. *Umbelopsis isabellina*, a fungal strain that consumes sugars in a corn hydrolysate in the presence of inhibitors, showed excellent potential for biofuel production in our previous study. However, some disadvantages in this energy production process still exist, such as costly nitrogen sources for fungal culture and the lack of an efficient lipid extraction method. To address these problems, this study has developed two processes that could make large scale production using this strain more feasible. First, in order to further reduce the substrate cost, algal biomass was added to a corn stover hydrolysis process to eliminate the cost of the yeast extract used for fungal culture. The addition of the algal biomass had the same effect as the addition of yeast extract on biomass accumulation and lipid yield after hydrolysis. The algal hydrolysate could also be prepared separately and used for fungal culture. In addition to reducing the substrate cost of the fungal culture, this process also provides another use for the algal biomass, which could help to further shrink the cost of the algae-based biofuel. After fungal biomass accumulation, lipid extraction is another critical step in biofuel production. The traditional Bligh & Dyer method, which uses a highly toxic and dangerous solvent system (methanol and chloroform) was considered the best method for fungal lipid extraction. Instead of using that solvent system, the less toxic solvent hexane was selected in this study as an alternative, and a three-stage lipid extraction method for the fungus *Umbelopsis isabellina* was developed. When applied to 1 g of biomass, this method extracted the same amount of lipids as the Bligh & Dyer method, and the lipid profile was similar.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1. Literature Review

1.1 Introduction

As societies around the world continue to develop, alternative energy sources are needed to replace limited fossil fuel resources and reduce greenhouse gas emissions. In addition to wind, hydropower, solar energy and other types of renewable energy sources, biodiesel has drawn much attention. Common raw materials for biodiesel production include soybean, sunflower, coconut, peanut, and rapeseed oils (Shay 1993). To convert those oils into biodiesel, vegetable oil or animal fat is transesterified to convert it into alkyl esters, which make up biodiesel. The biodiesel fuel B20, which is a blend of 6-20% biodiesel and petroleum diesel, is currently in wide use all over the world (Knothe et al. 2005). The benefits of using biodiesel are lower SOx and NOx emissions, the conversion of CO_2 into biomass, and a reduced reliance on fossil fuels (Shay 1993).

Although the use of biodiesel is environmentally beneficial, biodiesel production might cause a rise in greenhouse gas emissions. When land is cleared during the conversion process of a carbon-rich habitat into a bioenergy cropland, the habitat is usually burned to prepare it for the bioenergy species and an enormous amount of greenhouse gases are emitted. The greenhouse gases produced from burning and the degradation of the underground roots is huge, and it can take up to hundreds of years for a habitat to recover from biodiesel production (Joseph Fargione 2008). Additionally, biodiesel production from vegetable oil has also posed a threat to food production. The use of edible oil for biodiesel production might create an imbalance between food and energy. The use of land for energy crop growth might limit the land area available for food cultivation (Yusuf, Kamarudin, and Yaakub 2011).

Such concerns have resulted in more attention to a different type of biodiesel - microbial biodiesel. The most common microbes used for biodiesel production are microalgae, bacteria, yeast, and fungi (Meng et al. 2009). The advantages of microbial biodiesel include a shorter life cycle, less land usage,

less impact due to environmental changes, and ease of maintenance without intensive labor (Q. Li, Du, and Liu 2008).

1.2 The use of microbes for energy production

Microbes that can accumulate lipids and contain more than 20-25% lipid in their biomass are considered oleaginous species (C. Ratledge 1991). The major oils from microbes are triglycerides, which is the same as the vegetable oil that is commonly used for biodiesel production. In biodiesel production, this kind of microbial oil can be used to produce fatty acid methyl esters that are known as FAMEs (Vicente, Martínez, and Aracil 2004).

Many microbial lipids have been recently studied. Microbial lipids can be used to produce cocoa butter substitutes (Papanikolaou et al. 2001; Davies, Holdsworth, and Reader 1990), γ-linolenic acid (Fakas et al. 2007; Papanikolaou, Komaitis, and Aggelis 2004; Dyal, Bouzidi, and Narine 2005; Jang, Lin, and Yang 2005), docosahexaenoic acid (Wu, Yu, and Lin 2005; Chi et al. 2007; Bigogno et al. 2002), eicosapentaenoic acid (Tsunehiro Aki et al. 1998; Guo et al. 1999), and biodiesel (Easterling et al. 2009; Yonghong Li, Zhao, and Bai 2007; L. Y. Zhu, Zong, and Wu 2008; Chiu et al. 2009; Angerbauer et al. 2008). Polyunsaturated fatty acids (PUFAs) are types of fatty acids that exist in humans and other higher organisms. This type of fatty acid has been demonstrated to be beneficial for several health issues, such as cardiovascular disease, brain disorders, cancer, inflammatory disease, obesity, autoimmune disease, and diabetes. The primary sources of PUFA production are fungi and algae. Yeast is preferred in industry, but it cannot synthesize PUFAs unless the strains are genetically modified. Bacterial fatty acids are not exploitable (Bellou et al. 2016; Simopoulos 2006; Abedi and Sahari 2014).

1.2.1 Bacteria and bacterial lipids

Because they reduce land use and have high productivity, bacteria have been considered as a potential biodiesel source. The average lipid content of bacterial dry biomass is approximately 30%, for example,

the oil content of *Arthobacter sp*. can achieve 40%. Compared with other microbes, bacteria that have a rapid growth rate are easy to cultivate. The bacterial strain *Rhodococcus opaacus* PD630 can even accumulate fatty acids in acylglycerols up to 87% of its dry weight. High lipid yields seem to be a characteristic of only certain bacterial groups - most bacteria do not produce high lipid yields. Since most lipids exist in the bacterial outer membrane, they are difficult to extract, which in turn makes it difficult to commercially produce biodiesel from bacteria (Meng et al. 2009; Alvarez and Steinbüchel 2003).

1.2.2 Yeast

The major biofuel product from yeast cultures is ethanol. Metabolically engineered yeast strains are currently used for ethanol production. Via fermentation, yeast can convert sugar from a substrate into a dilute ethanol solution, which can then be concentrated via distillation. Genetically modified strains can even produce bio-butanol, which has a higher energy content and is more similar to gasoline than ethanol (Coyle 2010). The use of yeast to produce lipids has been studied for a long time. The advantages of oleaginous yeast strains are a high lipid content, rapid growth, a high cell concentration, the utilization of all carbohydrates in the hydrolysate, and a high hydrolysate inhibitor tolerance (Yu et al. 2011).

In addition to bioenergy production, yeast has been widely cultured by humans to produce alcoholic beverages, bread dough, and food ingredients (Boulton 2001; Tanguler and Erten 2008; Peppler 1982; Walker 1998). Yeast extract, collected from autolyzed yeast, is often used as a nitrogen source in a microbial culture for the growth of the microbe. It contains peptides, amino acids, carbohydrates, and soluble vitamins. It is used in many types of media for various microbial cultures; such media include Sigma LB medium, Sigma Minimal salts (M9) medium, Terrific Broth, SOB medium, SOC medium, and 2X YT medium (Sigma-Aldrich 2016). Degradative enzymes in the yeast itself can be activated via autolysis, and can solubilize different cellular components. During this process, insoluble molecules such as nucleic acids and proteins are converted to nucleotides and amino acids, which are essential for microbial growth (Sommer 1998; Reed, Gerald 1991).

1.2.3 Algae and algal lipid

In addition to yeast, algae also have excellent potential as an alternative energy source because they can fix carbon dioxide using sunlight as an energy source. Algae also exhibit a higher oil content and higher production rates than traditional energy crops (Chisti 2007a; Q. Li, Du, and Liu 2008). Several types of biofuel can be produced from microalgal cultures, including methane (Chisti 2007a; Spolaore et al. 2006), biodiesel (Chisti 2007a; Gavrilescu and Chisti 2005; Sawayama et al. 1995; Roessler et al. 1994), and bio-hydrogen (Akkerman, Janssen, Rocha, & Wijffels, 2002; Chisti, 2007; Ghirardi et al., 2000; Melis, 2002). Much less land use is necessary for algal growth than for any vegetable, and several types of algae have excellent potential for algal oil production due to their high oil content. The oil content of certain algae can achieve 80%, and their biomass accumulation time is short. It usually takes less than one day for an algae to double its biomass (Metting 1996; Spolaore et al. 2006). In addition to producing biofuel, algae can also be used in human nutrition due to their high protein content and nutritional value (Spolaore et al. 2006; Soletto et al. 2005; Radmer 1996; Desmorieux and Decaen 2005), as a feed additive for many types of animals, which is an industry that comprises 30% of global algal production (Spolaore et al. 2006; Richmond 2004), and in the production of cosmetic products, high-value molecules, fatty acids, and pigments (Spolaore et al. 2006).

Several disadvantages of algal cultures for biodiesel production exist. An algal culture usually occupies much space, because when the concentration of algae is high, light cannot penetrate the algal solution, which slows down the growth rate. Reliance on light results in shorter growth periods per day compared to other microbes. Even though an external light source can be used for culture, additional energy is required (Chisti 2007a). Additionally, high water usage and a low cell concentration after harvest are also problems that must be solved to economically to use algae as an alternative biodiesel source. (Q. Li, Du, and Liu 2008).

1.2.4 Fungus and fungal lipids

Fungi have recently been drawing much attention as a potential biodiesel source. Their lipid content can achieve up to 80% under certain conditions (Murphy 1991; Subramaniam et al. 2010). Fungal lipid content is affected by several factors - the C/N ratio, the carbon source, the nitrogen source, mixing, the pH, and the temperature. Fungal lipids contain high concentrations of PUFAs, such as arachidonic acid (AA) and γ-linolenic acid (GLA), which are fatty acids also used to produce high-value products other than biofuels. *Mortierella alpine* can generate 4.5 g/L of AA, which is a significant portion (18%) of its dry biomass (Eroshin et al. 2000). *Mortierella alliance* also produced 7.1 g/L of AA when cultured in a 50 L jar (T. Aki et al. 2001). The capacity to produce such high-value fatty acids does increase the value of fungal biomass, but the lipid production rate of a fungus is usually lower than that of a yeast. It usually takes fungus longer to produce the same amount of lipids as yeast. For example, *Mortierella ramanniana*, MM15-1 can accumulate 68% lipid in its biomass in 9 days with a glucose-only medium in a Maxblend Fermentor (Hiruta et al. 1997). Fakas et al. (Fakas et al. 2009) cultured two fungal strains, *Cunninghamella echinulate* and *Umbelopsis isabellina* (previously known as *Mortierella isabellina*) in glucose, xylose, and glycerol media. Both strains showed similar lipid yields in the glucose medium (g lipid/g sugar consumed) and *Umbelopsis isabellina* had a very high biomass yield (27 g/L). *Cunninghamella echinulate* demonstrated a better lipid yield in the xylose medium and *Umbelopsis isabellina* had a better lipid yield in glycerol medium. *Umbelopsis isabellina* ATHUM 2935 produced 44-56% oleic acid and 10-16% linoleic acid, which qualifies it as a potential biodiesel production strain.

The production cost must be reduced to use microbial biomass to produce commercial biodiesel instead of vegetable oil and animal fat. Two major components must be improved – the culturing cost and the transesterification efficiency. To reduce the cost of culturing microbes, the primary cost, which is the substrate cost, must be decreased. Instead of pure sugar as a carbon source, an alternative substrate is essential for the development of microbial lipids. Studies have explored the viability of several types of alternative substrates, including a whey substrate for the growth of *Cryptococcus curvatus* ATCC 20509

and *Candida bombicola* ATCC 22214 cultures (Daniel et al. 1999), *Umbelopsis isabellina* with glycerol (Fakas et al. 2009), *Cunninghamella echinulata* and *Umbelopsis isabellina* with molasses (Chatzifragkou et al. 2010), *Lipomyces starkeyi* with sewage sludge (Angerbauer et al. 2008), *Lipomyces starkeyi* with olive oil mill wastewaters (Yousuf et al. 2010), *Mortierella alpina* with maize starch hydrolysate (M. Zhu, Yu, and Wu 2003), and *Cryptococcus curvatus* with N-acetylglucosamine (Zhang et al. 2011). Although a potential for lignocellulosic material to be utilized by different types of the fungus has been indicated, a limited supply and a high transportation cost are still problems (C. Huang, Chen, Xiong, Chen, et al. 2013). The cost of raw materials for biodiesel production is approximately 75% of the total production cost (Srinivasan 2009). Therefore, a cheap and abundant raw material is important. Lignocellulose is considered a potential substrate, because it is a more available biofuel source and has been studied by many researchers (C. Huang, Chen, Xiong, Chen, et al. 2013), which includes fungal cultured with corn stover hydrolysate (Ruan et al. 2012).

1.3 Biofuel from lignocellulosic material

1.3.1 Lignocellulosic material

Lignocellulosic material is the most abundant resource for microbial lipid production. The low cost and high availability of this material make it an ideal substrate for energy production (Srinivasan 2009). Over 1.3 billion tons of dry lignocellulosic material that can be used for biofuel production are produced in the US every year (Robert D. Perlack, Lynn L. Wright, Anthony F. Turhollow, Robin L. Graham, Bryce J. Stokes 2005). If this much material were transformed into energy, the energy produced would be equivalent to 3.8 billion barrels of oil (Kumar, Jones, and Hanna 2009). Therefore, it is very important to study the best way to use this energy source for optimized energy production.

Lignocellulosic biomass usually has three main parts: cellulose, lignin, and hemicellulose. The concentration of those three components varies depending on the biomass (Reddy and Yang 2005). Since

lignocellulosic biomass cannot be directly used by microbes, the material must be degraded before use in bioconversion and fermentation (Rubin 2008). Acid is usually used to degrade the biomass during the breakdown process. Long-chain polysaccharides are generated during this process, and then converted to the corresponding monosaccharides (F. LaForge 1918). During the breakdown of lignocellulosic material, inhibitors such as weak acids, aromatic compounds, and aldehydes are produced. Those compounds are usually toxic to microbes. Most microbes cannot grow in the presences of such inhibitors (Palmqvist and Hahn-Hägerdal 2000). Hence, the discovery of microbes that can tolerate these inhibitors while producing energy sources is critical for renewable energy development. Lignocellulosic biomass-degrading microbes are being considered as a novel way to breakdown a lignocellulosic biomass, and the enzymes extracted from those microbes can be further applied in industrial production (Gilbert 2007). Due to the difficulty of culturing the microbes that can produce these enzymes, the cost of this process is still currently higher than acid treatment (Hugenholtz 2002).

Various fermentable sugars are produced by the hydrolysis of a lignocellulosic biomass. The primary sugars are hexoses and pentoses and the hexose concentration is usually 1.5 to 3 times higher than that of pentoses (Balan et al. 2009; Lau and Dale 2009; Sassner, Galbe, and Zacchi 2005; Rahman, Choudhury, and Ahmad 2006). Because both sugars are present in the hydrolysate, the microbes selected for biofuel production should have the ability to use those sugars efficiently and to grow well in the presence of inhibitors. Screening or mutagenesis are strategies that scientists can employ to find the best strain for culturing with a lignocellulosic hydrolysate. Therefore, the ability to accumulate lipids or other types of high-value chemical products from xylose is critical for the discovery of suitable microbes since most of them cannot use xylose as an energy source (C. Huang, Chen, Xiong, Chen, et al. 2013).

1.3.2 Yeast culture with lignocellulosic material

Yeasts are currently the primary microbes studied for use with lignocellulose material for biofuel production. Several yeast strains have been studied. *Trichosporon dermatitis CH007* and *Trichosporon*

corymbiform were cultured with corncobs, and their lipid concentrations achieved 9.8 and 7.7 g/L, respectively (C. Huang, Chen, et al. 2012; C. Huang, Chen, Xiong, Yang, et al. 2013). In addition to corncobs, several types of yeast have been cultured with corn stover, and *Rhodotorula garminis* showed excellent utilization of sugar with 14.1 g/L of lipid produced (Hu et al. 2011; X. Huang et al. 2011; Gong et al. 2013; Galafassi et al. 2012). Rice straw has also been used with *Hosporon fermentans CICC 1368* and 12.5/L of lipid was accumulated (C. Huang et al. 2009). *Trichosporon fermentans* and *Yarrowia lipolytica* have been cultured on sugarcane bagasse and accumulated 15.8 and 6.7 g/L of lipids respectively (C. Huang, Wu, et al. 2012; Tsigie et al. 2011). *E. veralis* (*G.pullulans*) achieved a 45% lipid content when cultured with waste sulfite liquor (Lindner 1922). *R.gracilis* (*R. toruloides*) achieved a 64% lipid content when cultured with wood or molasses hydrolysates (Lundin 1950; Tornqvist 1951). For most yeast fermentations with lignocellulose material, the primary substrates have usually been detoxified due to the sensitivity of the yeast to the inhibitors in the substrate, such as furan derivatives, weak acids, and phenolic compounds. Not many yeasts that can be cultured in non-detoxified substrates have been reported. Yu et al. succeeded in culturing *Cryptococcus curvatus*, *Rhodosporidium toruloides*, *Rhodotorula glutinis*, *Yarrowia lipolytica*, and *Lipomyces starkeyi* in a non-detoxified hydrolysate. *Cryptococcus curvatus* accumulated 5.8 g/L of lipids. The primary inhibitor of growth was HMF, while 1 g/L furfural reduced the lipid content by 62% (Yu et al. 2011). Inhibitors produced from the degradation of lignin and carbohydrates are the major inhibitors of yeast lipid accumulation. Those inhibitors are generated by pretreatment methods and the lignocellulosic biomass cell wall composition (Helene B. Klinke et al. 2002; H. B. Klinke, Thomsen, and Ahring 2004). Since most oleaginous yeast cannot accumulate lipids in xylose media, the development of oleaginous microbes that can utilize sugar produced from lignocellulosic material that have high inhibitor resistance are necessary for biodiesel production from lignocellulosic material.

1.3.3 Fungal culture with lignocellulosic material

Yeasts are not the only microbe that can utilize lignocellulosic material for biofuel production - some other fungi are also considered potential "lignocellulosic utilizers". The filamentous fungus *Mucor hiemalis* can produce over 50 g/L of ethanol in a high glucose concentration (up to 190 g/L) wheat hydrolysate. The high value byproduct chitosan has also been produced in this fermentation (Heidary Vinche et al. 2013). *Hohenbuehelia* sp. ZW-16 has been cultured in a corn straw hydrolysate and a corncob hydrolysate. The highest yield was achieved in the corn straw hydrolysate, which produced 4.6 g/L of bioethanol in 8 days (Liang et al. 2013). It has been reported that 11 fungal strains were cultured in synthetic media containing glucose and xylose and 6 fungal strains were selected for culture in detoxified and non-detoxified acid-pretreated wheat straw media. The fungal strain *Umbelopsis isabellina* showed the highest lipid yield (39.4%) in a non-detoxified liquid hydrolysate (Zheng et al. 2012). *Umbelopsis isabellina* has also been grown in a rice hull hydrolysate and has accumulated oil comprising up to 64.3% of the biomass with a C/N ratio of 57 (Economou et al. 2011). The same strain was studied by Zhenhua Ruan, who combined acid and alkali pretreated corn stover and treated it with enzymes to generate a hydrolysate without the need for pH adjustment. In this case, the fungus could accumulate biomass and lipids in the presence of inhibitors (Ruan et al. 2014).

1.4 Algal hydrolysate

Carbon sources and nitrogen sources are critical for fungal cultures. Many studies have explored the use of different lignocellulosic materials as carbon sources for fungal culture. However, few studies have focused on alternative nitrogen sources for fungal culture. Yeast extract is still the major nitrogen source currently used in microbial cultures, and it is quite expensive.

In a study by Maurya, a harmful bloom-forming algal biomass *(Lyngbya majuscule)* was hydrolyzed and used for oleaginous microalgal culture. The use of the hydrolysate significantly increased the dry cell weight and the productivity of the algae *C. vulgaris* during the culture (Maurya et al. 2016). In a study by

Kightlinger, an autolyzed algae (*Chlamydomonas reinhardtii*) biomass showed similar or even better effects than yeast extract for the culturing of common lavatory strains such as *E. coli* and *S. cerevisiae*. If algal extract could bring the same price as yeast extract, algae-based biofuel products would be more compatible (Kightlinger et al. 2014). These studies showed the potential of using algae hydrolysate as a substitute for yeast extract. More ways to utilize an algal biomass would make the large-scale production of algae-based bioenergy more feasible.

1.5 Lipid extraction

Extraction is one of the most energy-intensive stages of the microbial lipid production process (de Boer et al. 2012). Therefore, the discovery of a stable, economical, environmentally friendly, and easy extraction method is critical for the development of microbial lipids. Organic solvent extraction and SCCO2 extraction are two current, popular extraction methods. The advantages of the SCCO2 extraction method are neutral lipid selectivity, the total lipid yield, the extraction time, hazard and toxicity, and reactivity with lipids. The advantages of the organic solvent extraction method are the energy requirements, the installation cost, and the operation cost. Organic solvent extraction is more commonly used in industry due to the high installation cost for high pressure vessels for SCCO2 extraction (Halim, Danquah, and Webley 2012).

Various other extraction methods are used in the laboratory and in industry. The Bligh & Dyer method (E. G. Bligh 1959) is one of the most popular extraction methods for all living tissue lipids. Two organic solvents, chloroform and methanol, are used in this method. By using polar and non-polar solvents, this extraction method can completely extract both types of lipids. First, chloroform, methanol, and water in a ratio of 1:2:0.8 (v/v/v) is used to extract the lipid from the tissue. Then, additional chloroform and water is added to a total ratio of 2:2:1.8 (v/v/v) to form a biphasic structure. The bottom layer contains all the lipids while the top layer contains all the non-lipids. Homogenization may be used depending on the biomass extracted. The advantage of this method is that both polar and non-polar lipids

are recovered. The disadvantage is that the solvents are extremely toxic and flammable (Medina et al. 1998). Hexane-isopropanol at a ratio of 3:2 (v/v) can also be used for lipid extraction. The solvent in this method is less toxic compared to those in the Bligh & Dyer method. After extraction, the extract is washed in sodium sulfate to further remove the non-lipid contaminants. Since the density of the solvent is low, a centrifuge can be used to separate the solids and liquids instead of filtration as in the Bligh & Dyer method. This method has been reported to have a good lipid extraction rate for rat or mouse brain lipids. However, when applied to various algae and fungal strains, the yields were not as high as those of the Bligh & Dyer method (Grima et al. 1994; Halim, Danquah, and Webley 2012; Hussain et al. 2014). Extraction with hexane alone has been used for the high oil content algae *Chlorella protothecoides* (Miao and Wu 2006). Hexane has a low affinity for non-lipid materials, and it is cheaper and much less toxic than the solvents used in the Bligh & Dyer method (Halim et al. 2011).

Due to different cell structures, lipid compositions, strain physiologies, and other uncertain variables, the best extraction method varies by strain. For example, for the algae *Botryococcus braunii*, the chloroform/methanol method has resulted in a higher lipid yield compared to four other organic solvent extraction methods, including a dichloroethane-based organic solvent mixture, which is an extraction method recommended for the algae *Cladofora* (Lee et al. 2010; Halim, Danquah, and Webley 2012). Therefore, selecting the appropriate solvent and procedure for the target strain is very important. Javid Hussain studied four solvent-based extraction methods for the fungus *Umbelopsis isabellina*, including a modified Bligh & Dyer method, hexane and isopropanol, dichloromethane & methanol, and hexane extraction. The Bligh & Dyer method gave the best lipid yield for this fungus.

Chapter 2. The integration of autotrophic and heterotrophic microbial processing for biofuel production

2.1 Abstract

With an increasing energy demand, an alternative energy source is needed for the sustainability of this world. Biofuel produced from microbes has excellent potential because it produces less pollution, is a renewable energy source, and has a smaller carbon footprint. The filamentous, oleaginous fungus *Umbelopsis isabellina* was studied for its ability to utilize carbon sources in a corn stover hydrolysate in the presence of inhibitors. To further reduce the substrate cost, this study investigated the possibility of adding an algal biomass to the corn stover hydrolysis process to reduce the subsequent addition of yeast extract. The fungus was successfully cultured on the hydrolysate containing an algal biomass and corn stover. The lipid yield was comparable to the corn stover hydrolysate medium with yeast extract.

2.2 Introduction

Biodiesel is attracting more interest recently as a renewable energy source. The conventional sources for biodiesel production are peanut oil, soybean oil, coconut oil and rapeseed oil. Vegetable oil-based biodiesel has environmental and social advantages over petroleum diesels such as low sulfur oxides (SOx) emissions, low nitrogen oxide (NOx) emissions, CO_2 sequestration from crop growth, and the production of valuable byproducts from the biomass. Despite its benefits, biofuel has several drawbacks including low fuel production yields, performance disadvantages compared to fossil-based diesel, and a high production cost (Shay 1993). Additionally, increasing concern about crop land use and emissions produced from cultivation raise questions as to whether plant-based biodiesel is truly environmentally beneficial (Joseph Fargione 2008). Microbial biodiesel, which is produced from microalgae, fungi, and yeast, is becoming a more environmentally friendly alternative for biodiesel production. For example, Yanqun Li (Yanqun Li et al. 2008) claimed that with a high-value co-product strategy, an advanced

reactor design, and cost-effective harvesting and drying technology, algal biofuel production could fulfill the increasing energy demand. Yeast and fungi are also considered as potential microorganisms for fuel production. Some yeasts can accumulate up to 80% of their biomass as lipids, and the fatty acids produced from fungi and yeast are similar to those produced from fish oil and other animal sources (C 1993)

Microalgae are considered an ideal candidate for microbial biodiesel generation due to their ability to utilize CO_2 to generate lipids and their high photosynthetic efficiency (Chisti 2007b; Yanqun Li et al. 2008). The rapid growth of algae requires a significant amount of CO_2. Since power plants are one of the largest CO_2 contributors, the abundant CO_2 from power plant flue gas could potentially be used as a carbon source for algal growth. Vunjak-Novakovic used an air-lift reactor to culture microalgae. Flue gas was pumped into the reactors to provide the carbon source for algal growth and the biomass was harvested during the growth phase. Light intensity played a major role in the CO_2 reduction rate (Vunjak-Novakovic et al. 2005). The system could reduce the CO_2 concentration of the incoming flue gas by 82.3% on a sunny day and the CO_2 reduction rate dropped to 50.1% on a cloudy day. In addition to CO_2 reduction, microalgae can also be used as a biofuel source. Depending on the culture medium composition, the algal protein, carbohydrate, and lipid composition can vary significantly. The algal biomass produced from continuous culturing usually has a lower lipid content because algae accumulate more lipids or carbohydrates in an environment that has a low nitrogen concentration and a high protein content (Wang et al. 2015). On the other hand, algae grow rapidly on a nitrogen-rich medium, and a significant amount of protein is naturally present in most algae species that can be used for human consumption and animal feed, making algae a more desirable biofuel (Becker 2007). Although several benefits and uses for culturing microalgae exist, commercialization of algal cultivation is still not feasible due to the high cost of operation, nutrients, and extraction (Wijffel, Barbosa, and Eppink 2010). To make algal production more cost efficient, several studies have been performed to identify more uses for algal biomass. Algal hydrolysate was used as a medium to improve the hydrolysis processes for other

biomasses such as anaerobic digestion fiber and poplar for glucan conversion (Chen et al. 2014). An autolyzed algal biomass (*Chlamydomonas reinhardtii*) was demonstrated to provide effects similar to yeast extract for general laboratory microorganism culturing except for ethanol production from yeast (Kightlinger et al. 2014).

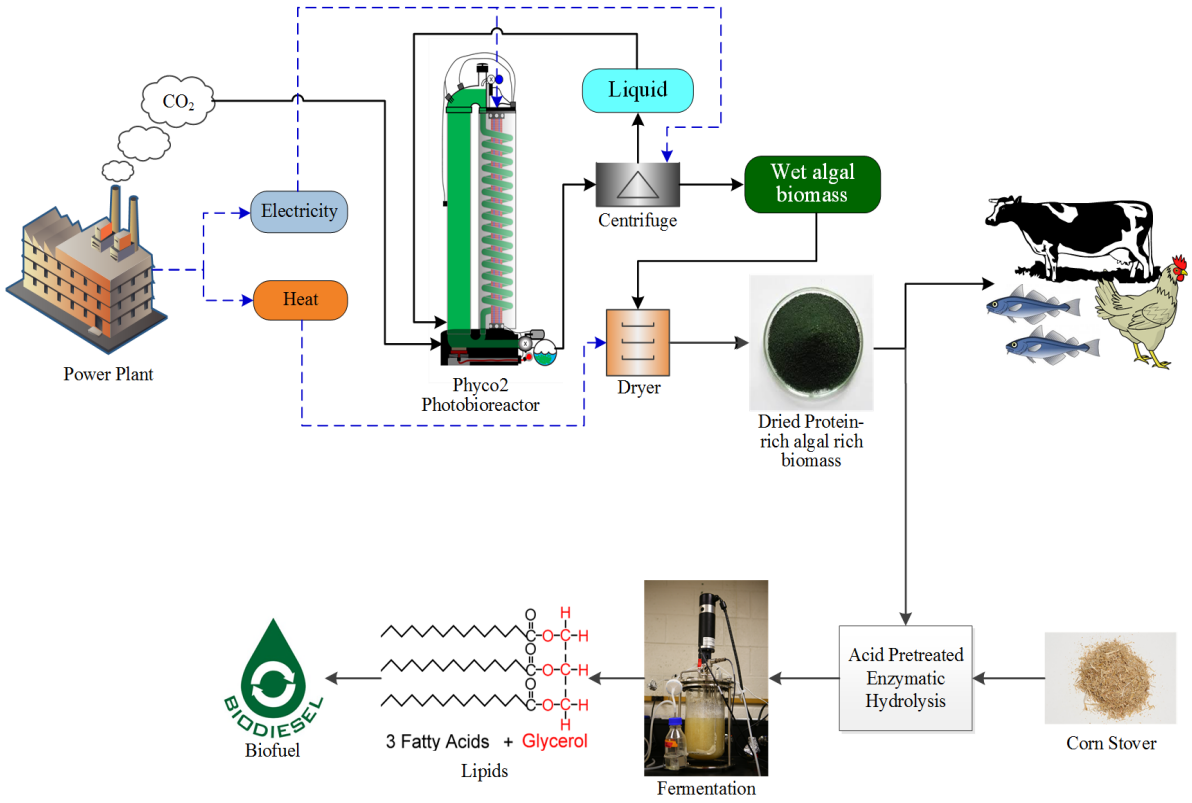
Oleaginous fungi have also been studied as an alternative energy source. Ostensibly, fungi have some clear advantages - their growth does not require light, they have high lipid yields (C. Ratledge 1991), and can utilize different types of sugar (Fakas et al. 2009). However, one of the greatest problems with fungal culture is the medium cost. To make it more affordable, pure substrates must be replaced by an alternative source such as a lignocellulosic biomass (Ruan et al. 2013). In a previous study, *Umbelopsis isabellina* (previously known as *Mortierella isabellina*) tolerated inhibitors such as furfural and hydroxymethylfurfural (HMF), while consuming carbon sources in an acid-pretreated corn stover hydrolysate. This study indicated a further improved possibility of using fungal biomass as a potential biodiesel feedstock, because it replaced an expensive culture media with abundant corn stover. It is remarkable that this strain can tolerate inhibitors while accumulating lipids. In these fungal culture experiments, yeast extract was used as the nitrogen source for the fungal cultivation, which would increase the production cost. Other cheap organic nitrogen sources could be used to replace yeast extract to make fungal lipid production more economically feasible (Ruan et al. 2012; Ruan et al. 2013; Ruan et al. 2014; Ruan et al. 2015).

With the previous research in mind, the objectives of this study are to evaluate the hydrolysis of corn stover and an algal biomass and to investigate fungal ability to utilize nitrogen from an algal extract as a nitrogen source. The improved process demonstrates an alternative strategy for nitrogen source production, and a feasible way to combine nitrogen and carbon source production for a fungal culture.

2.3 Materials and methods

2.3.1 Algal and fungal strains and seed culture medium

Figure 1. Integration of autotrophic and heterotrophic microbial processing for biofuel production



The microalga *Chlorella vulgaris* 395 was purchased from the UTEX Culture Collection of Algae (Austin, TX) and stored on Tris-Acetate-Phosphate (TAP) agar medium (Wang et al. 2015) at room temperature under constant fluorescent light. Liquid TAP medium (without agar) was used for the seed cultures. Modified TAP medium was used as the photoautotrophic culture medium and contained the following substances: 7.5 mmol L^{-1} of NH_4Cl, 0.34 mmol L^{-1} of $CaCl_2 \cdot 2H_2O$, 0.4 mmol L^{-1} of $MgSO_4 \cdot 7H_2O$, 0.68 mmol L^{-1} of K_2HPO_4 (anhydrous), 0.45 mmol L^{-1} of KH_2PO_4 (anhydrous), and 1 mL of TAP trace elements solution.

The fungal strain *U. isabellina* (ATCC 42613) was used for this experiment. First, the spores of this strain were cultured on potato dextrose agar for 14 days at room temperature. After good growth had occurred the plate, the spores were transferred into a sealed bottle with autoclaved DI Water and refrigerated at 4°C. To prepare the seed for this experiment, 1 mL of spore solution (4.2×10^7 spore·mL^{-1}) was added to 100 mL seed medium with 8 g/L yeast extract and 24 g/L potato dextrose broth for two days (Ruan et al. 2012).

2.3.2 Pilot-scale algae photobioreactors to accumulate biomass

An algal culture system (algae photobioreactors APB), which could convert the carbon dioxide in the power plant flue gas to a carbon source for algal growth, was designed by PHYCO2 and installed in the Michigan State University T. B. Simon Power Plant. The PHYCO2 APB unit (US Patent #8,476,067 B2, Canada Patent #2,712,862) has a total volume of 118 L with a working volume of 100 L. The goal of this reactor is to decrease the carbon dioxide emissions from the power plant and accumulate algal biomass for biofuel and animal feed production. A portion of flue gases produced from the plant was pumped from the exhaust into the reactor via a vacuum pump to provide a carbon source for algal growth. The reactor was filled with the algal culture medium including approximately 100 mg/L nitrogen, 20 mg/L phosphorus, and 1.7 mg/L iron during the experiments. The pH of the culture was kept at 6.5±0.3. The flue gas and the design of the reactor created a flow in the reactor without the use of a pump, which helped the recirculation and mixing of the algae without any energy use. Since the system was located inside of the plant, high-intensity LEDs lights were used to provide a constant light source for the reactor. The LED panel included 20% blue and 80% red light, which was designed by PHYCO2 to provide enough light energy for photosynthesis. The Chlorella strain was stored in a solid Tris-Acetate-Phosphate (TAP) medium. Liquid TAP medium without acetate was used for seed culture under an LED light panel, which was set up with the same light penetration and design as the one in the power plant. The LED lights were the only light source and were turned on 24 hours a day. The seed was then inoculated into the reactor, which was filled with the same TAP medium without acetate. With 24 hours of LED lighting and

a continuous gas input (7% CO_2), the concentration of the algae could achieve higher than 2.5 g/L. 20% of the volume, and it was harvested every day for the different harvesting tests until the algal concentration achieved stationary phase. Each time after harvesting, the same amount of fresh medium was added to replace the volume removed. The total iron and nitrogen content of the medium were monitored daily and maintained at their original level. The harvested algal solution was centrifuged in a disc centrifuge (Alfa Laval MAB 104B) at 7350 rpm to collect the biomass. After drying at 105°C overnight, the biomass was cooled to room temperature, ground in a coffee grinder, and stored in a sealed container for future experiments.

2.3.3 Dilute acid pretreatment

The corn stover used for this hydrolysis experiment was collected from the Michigan State University Crop and Soil Science Teaching and Research Field Facility. The samples were collected after harvesting in 2015, air dried, and ground in a mill (Willey Mill, Standard Model No. 3, Arthur H. Thomas, Philadelphia, PA). The average particle size of the sample was 2 mm.

Diluted acid pretreatment was used to extract the sugar from the biomass[3]. DI water, sulfuric acid, and biomass were added to a 2 L beaker and mixed well. The total weight of the mixture was 700 g. Three types of biomass were designed for this experiment. The first was a mixture, which included 10% (w/w) corn stover and 1% (w/w) algal biomass. The second was 10% (w/w) corn stover, and the third was 10% (w/w) algal biomass. The pretreatment solution was prepared from 98% sulfuric acid, and the final acid concentration was 2% (w/w). The remaining mass comprised DI water. Three replicates were prepared for each treatment. After mixing thoroughly, the beakers were covered with aluminum foil and autoclaved at 120ºC for 1 hour. Afterwards, the beakers were cooled to room temperature, and the pH of each beaker was adjusted to 6 using a 30% (w/w) Sodium Hydroxide solution.

2.3.4 Enzymatic Hydrolysis

After acid pretreatment, the mixtures were transferred into a 1 L sealed bottle for enzyme hydrolysis. Cellulase from *Trichoderma reesei* ATCC 26921 was used for the hydrolysis experiment, and the enzyme activity was 120FPU/ml enzyme. The dosage used for the hydrolysis was 30 FPU cellulose per gram of corn stover. The same amount of enzyme was added to the pretreated algal biomass and the mixed biomass. After the addition of enzyme, the bottles were sealed and mixed on a temperature controlled shaker (Thermal Scientific) for three days. The temperature was 50ºC, and the shaking speed was 150 rpm. After enzyme hydrolysis, the samples were separated with a centrifuge, and the liquid components were collected and stored at -18C.

2.3.5 Fungal culture to accumulate lipids

After analyzing the sugar concentration and total nitrogen of the hydrolysate of the various treatments, media were prepared as follows:

Medium 1, 100 mL corn stover enzymatic hydrolysate only;

Medium 2, 100 mL corn stover enzymatic hydrolysate and 0.489 g yeast extract;

Medium 3, 88.5 mL corn stover enzymatic hydrolysate and 11.5 mL algal biomass enzymatic hydrolysate;

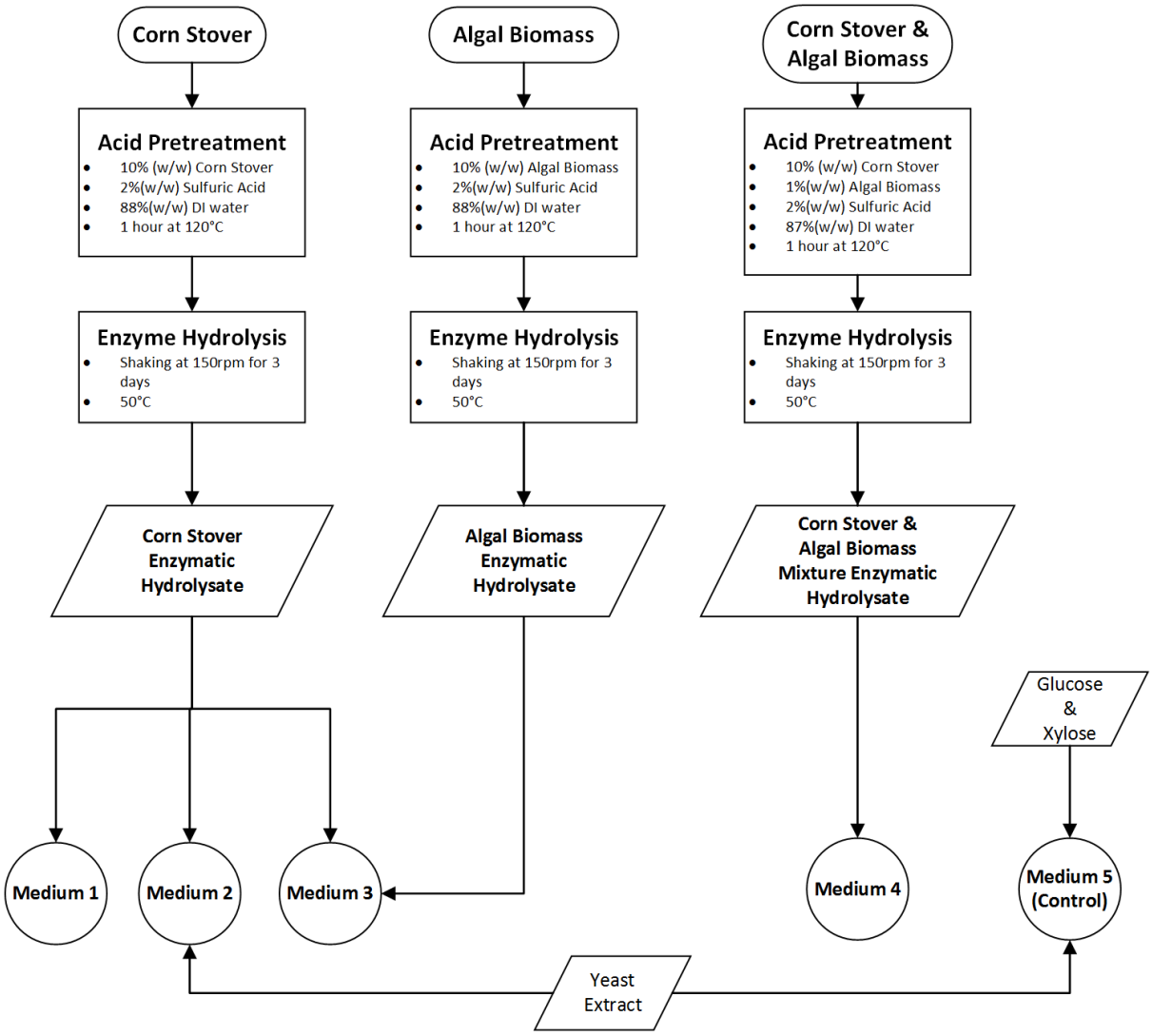
Medium 4, 100 mL corn stover and algal biomass mixture enzymatic hydrolysate;

Medium 5, 100 mL control medium with 25.5 g/L glucose, 16.6 g/L xylose, 0.93 g/L sodium acetate, and 4.98 g/L yeast extract.

The detailed medium preparation steps are shown in Figure 2. Each medium was autoclaved for 15 minutes at 121ºC and cooled to room temperature before seed inoculation. Each flask was then inoculated with 10% (v/v) inoculum, and cultured on a temperature controlled shaker (Thermal Scientific). Samples

were taken each day for HPLC sugar analysis. When the total sugar concentration achieved approximately 0 g/L, the biomass was collected by filtration and dried overnight, then the biomass concentration of each treatment was determined.

Figure 2. Fungal cultivation medium preparation



2.3.6 Analysis methods

Medium samples were taken each day from each treatment and filtered through a 0.22 µm syringe filter. Glucose, xylose, acetate, furfural, and hydroxymethylfurfural (HMF) were determined via HPLC (Agilent 1100). A refractive index detector and a Biorad Aminex HPX-87H analytical column were used for the determination. A mobile phase of 0.005 M sulfuric acid with a 0.6 mL/min flow rate was used for the column, and the column temperature was at 65°C. Glucose, xylose, sodium acetate, furfural, and hydroxymethylfurfural (HMF) from Sigma were diluted with DI water and used as standards. The fungal biomass was collected by filtration. DI water was used to wash off the residual sugars attached to the biomass by rinsing the biomass twice. The biomass collected from the filtration was dried at 105C overnight. The biomass was then ground with a mortar for lipid extraction. The lipid extraction method was the Bligh and Dyer method (E. G. Bligh 1959). The nitrogen content was analyzed using the HACH High Range Nitrogen (Total) TNTplus Vial Test.

2.3.7 Statistical analysis

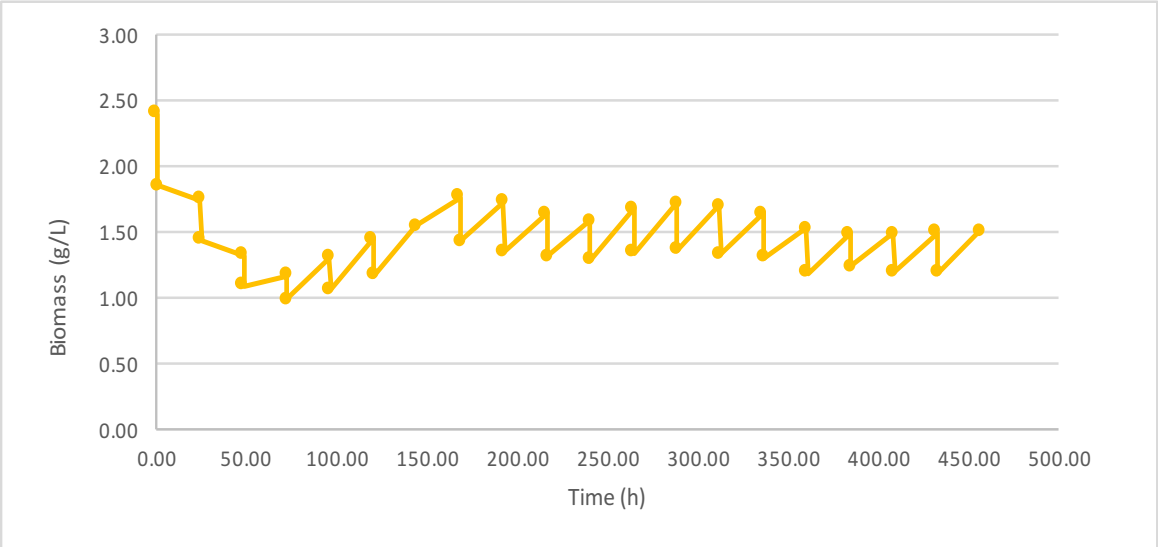
Statistical analysis was performed by using Minitab Express 2016. A two sample t test and a one-way ANOVA test was chosen for the treatment comparisons for each dataset. The confidence level was 95%.

2.4 Results and discussion

2.4.1 Algal growth and biomass details

Daily harvesting was done after the algal concentration had achieved 2.4 g/L to accumulate biomass. Twenty percent of the total reactor volume was harvested every day. In the beginning, the biomass concentration decreased until it achieved 1.2 g/L. After the decline, the concentration increased until it achieved 1.8 g/L. After 20 days of harvesting, the biomass concentration achieved equilibrium at approximately 1.5 g/L

Figure 3. Biomass concentration with 20% harvesting everyday



As the harvest amount increased, the algal concentration dropped due to the growth rate of the algae. In future experiments or production, it is important to discover the balance point between the amount harvested and growth rate, which can help optimize the algal biomass production. After harvesting the biomass, it was dried, ground, and ready for the hydrolysis process.

2.4.2 Hydrolysis of the algal biomass, corn stover, and a mixture of both

Table 1. Sugar and nitrogen concentration of the algal biomass hydrolysate

Treatment	Experiment	Glucose	Xylose	Acetate	Total Sugar	Total Nitrogen
Acid Pretreatment	10% algal biomass	3.51±0.05	4.47±0.04	0.15±0.01	8.13±0.01	3.98±0.29

The sugar concentration of the algal biomass hydrolysate was 3.51 g/L glucose, 4.47 g/L xylose, and 0.15 g/L acetate. The nitrogen concentration achieved 3.98 g/L after hydrolysis. Compared to the corn stover hydrolysate, the sugar concentration of the algal biomass was very low, but the nitrogen

concentration was much higher. This result showed the potential for using this hydrolysate as a nitrogen source for fungal culture.

The glucose, xylose, acetate, and total sugar concentrations were not significantly different between the pretreated mixed hydrolysate and pretreated corn stover hydrolysate. The glucose, xylose and acetate concentrations of the mixed hydrolysate were 25.47 g/L, 16.56 g/L, 0.93 g/L, respectively. The sugar concentrations of the corn stover hydrolysate were 26.46 g/L, 17.44 g/L, and 0.95 g/L, respectively. The results showed that no significant differences existed for glucose (P-value=0.5011, for a for a detailed statistical analysis, see Appendix B.1), xylose (P-value=0.0950, for a detailed statistical analysis, see Appendix B.1), acetate (P-value=0.4512, for a detailed statistical analysis, see Appendix B.1), and the total sugar concentrations (P-value=0.2714, for a detailed statistical analysis, see Appendix B.1) when 1% (w/w) algal biomass was added to a 10% (w/w) corn stover pretreatment.

Table 2. Sugar and nitrogen concentration of the corn stover hydrolysate and the mixed corn stover and algal biomass hydrolysate

Treatment	Experiment	Glucose	Xylose	Acetate	Total Sugar	Total Nitrogen
Enzyme Hydrolysis	10% Corn Stover + 1% Algal Biomass	25.47±1.05	16.56±0.48	0.93±0.03	42.97±0.62	1.2±0.24
	10% Corn Stover	26.46±1.99	17.44±0.15	0.95±0.00	44.86±2.09	0.66±0.06

The total nitrogen contents of the mixed enzymatic hydrolysate and corn stover enzymatic hydrolysate were 1.2 g/L and 0.66 g/L, respectively. An extra 0.54 g/L of nitrogen was extracted from the 10%(w/w) corn stover hydrolysis with 1%(w/w) algal biomass added, which was higher than the nitrogen concentration of the 10%(w/w) algal hydrolysate. The nitrogen in the corn stover could be the reason for

the higher nitrogen concentration. This experiment showed that it is beneficial to hydrolyze a mixture of corn stover and algal biomass. The addition of the algal biomass did not show significant inhibition of the sugar production due to hydrolysis. The extra nitrogen showed that the mixed hydrolysate could be potentially used for fungal culture without an extra nitrogen source such as yeast extract.

2.4.3 Fungal culture in different hydrolysates

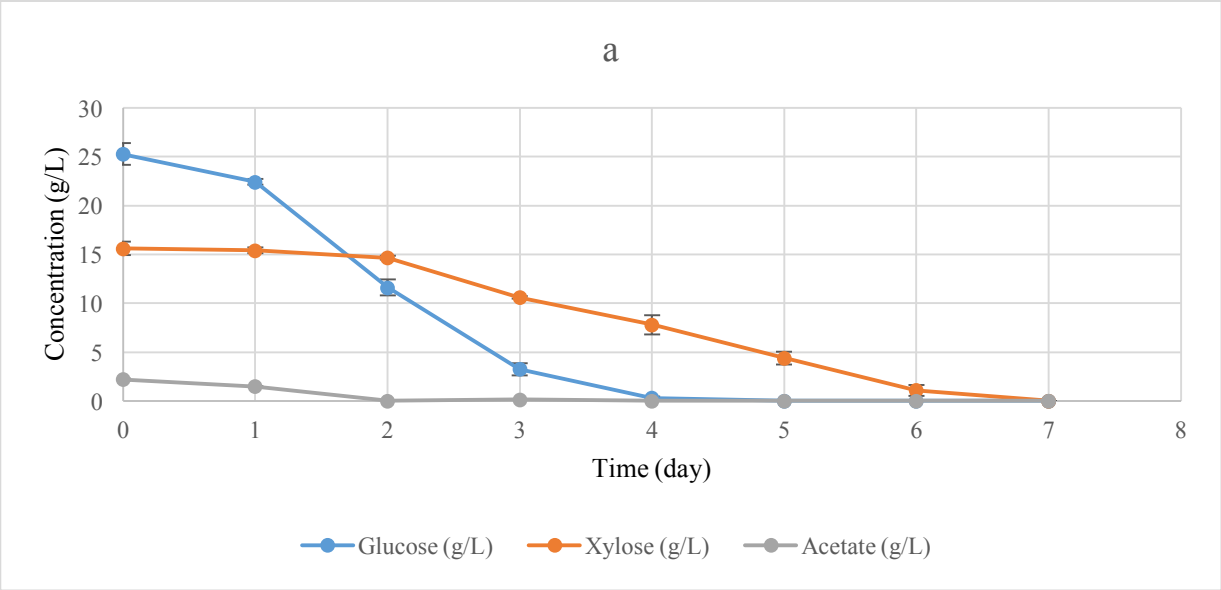
Table 3. Sugar consumption rate for different media

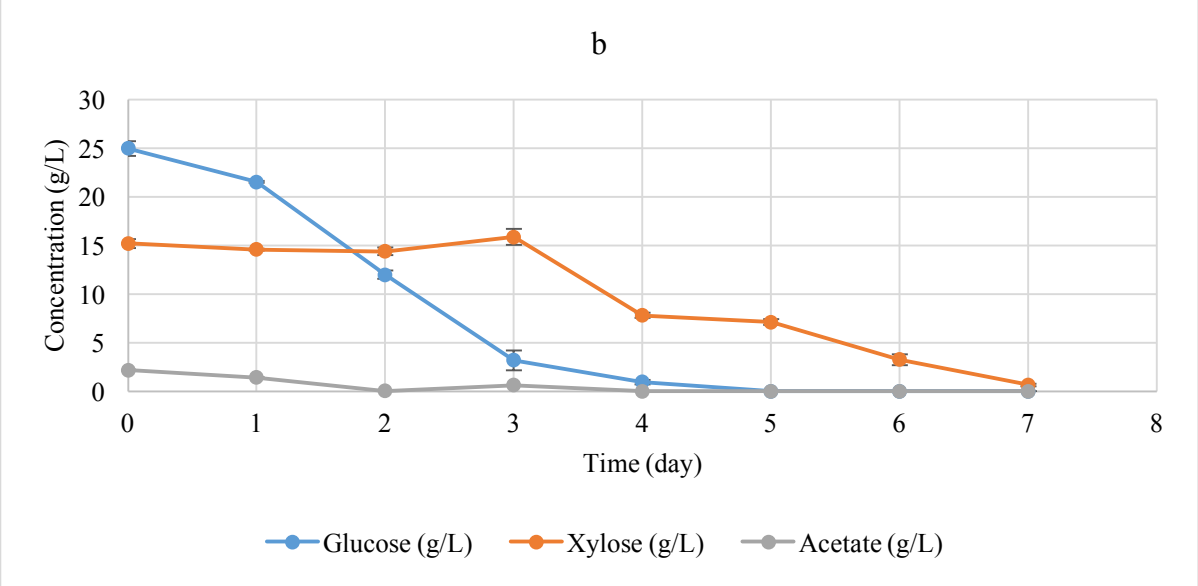
Medium number (#)	Detatil	Total sugar at beginning (g/L)	Time when total sugar is lower than 5g/L (day)	Sugar consumption rate (g/L*day-1)
1	corn stover hydrolysate	43.08	5	7.74
2	corn stover hydrolysate with yeast extract	42.33	6	6.51
3	corn stover hydrolysate with algal biomass hydrolysate	39.18	5	7.14
4	corn stover and algal biomass mixture hydrolysate	39.76	6	6.16
5	control with pure sugar	35.12	3	10.84

Table 3 shows the sugar consumption rate for different media. The control medium had the best sugar consumption rate due to the absence of inhibitors generated during hydrolysis.

With all of the media, glucose was consumed first. On the first day, which comprised the lag phase of the fungal culture, both glucose and xylose consumption were low. On the second day, the glucose consumption rate peaked, and the peak xylose consumption rate occurred on the third day when glucose became limited. Acetate was consumed simultaneously with glucose. At the end of the fermentation, all of the sugars in each medium were consumed by the fungus. The sugar utilization trend was similar with all media. Since control medium contained no inhibitors, it had the highest sugar consumption rate and was the first in which all of the available sugar was consumed.

Figure 4. Carbon source consumption by the fungal culture on (a) corn stover hydrolysate, (b) corn stover hydrolysate with yeast extract, (c) corn stover hydrolysate with algal biomass hydrolysate, (d) corn stover and algal biomass mixed hydrolysate, (e) control with pure sugar





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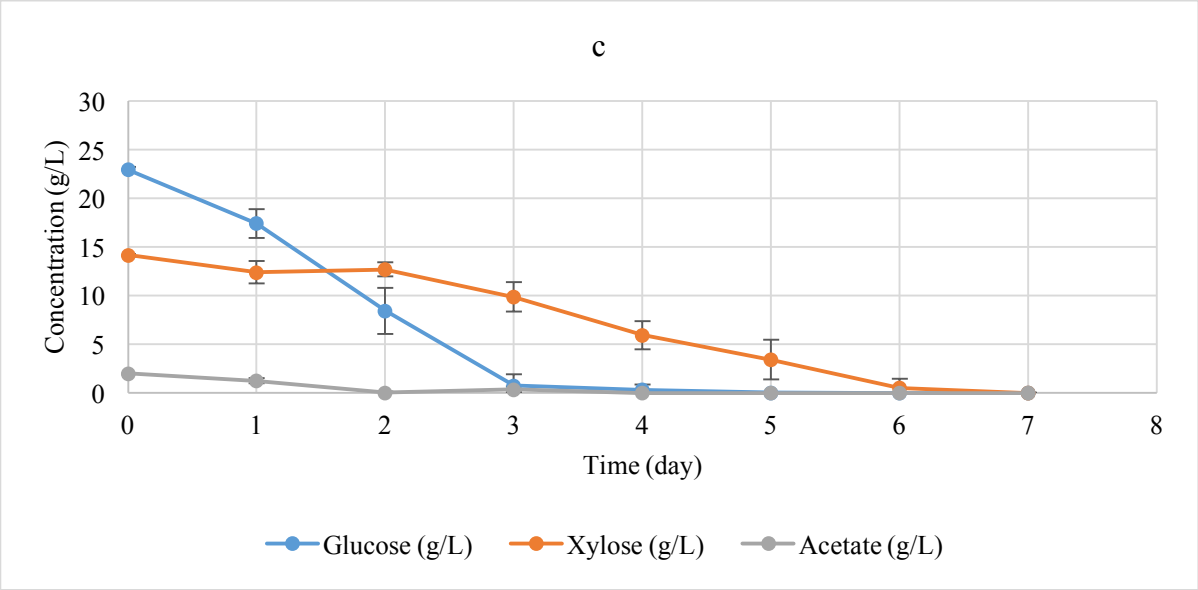

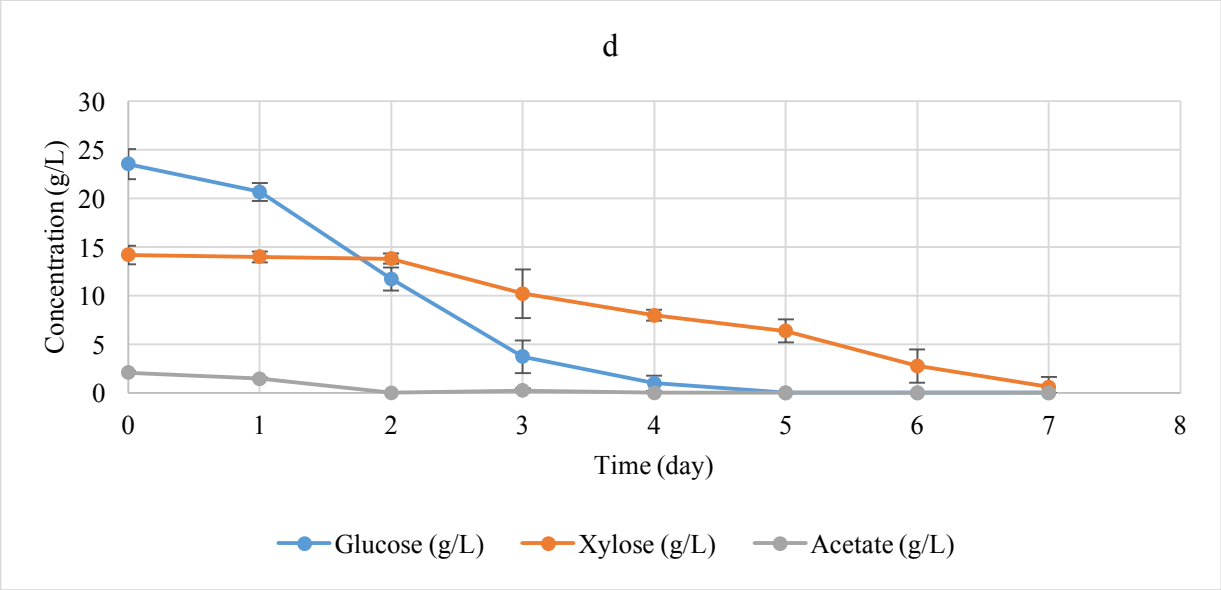

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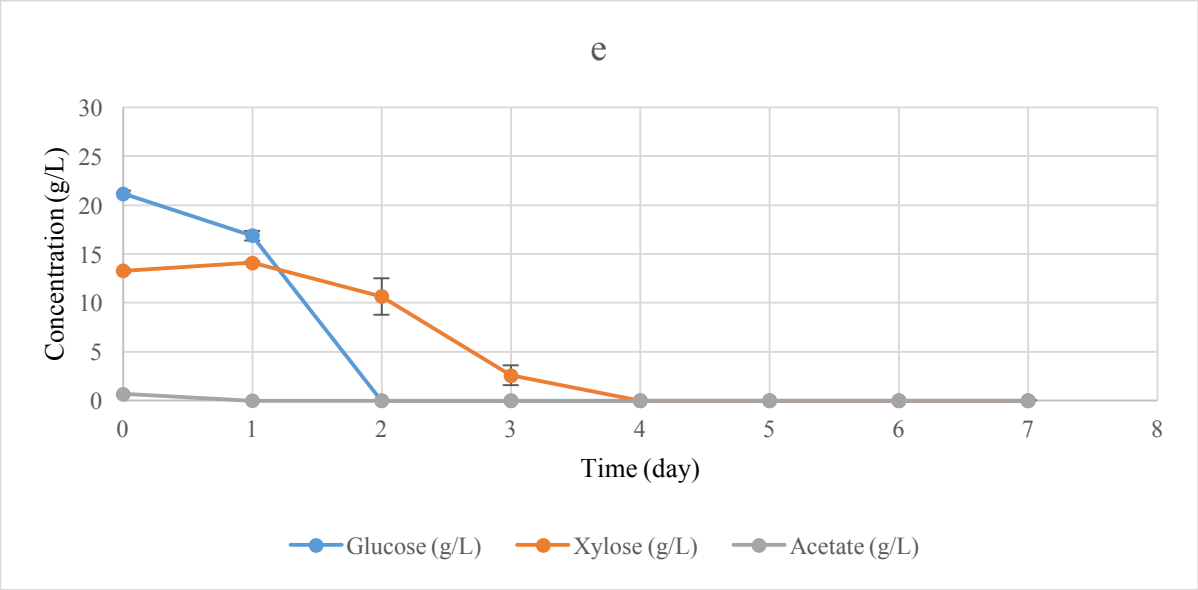
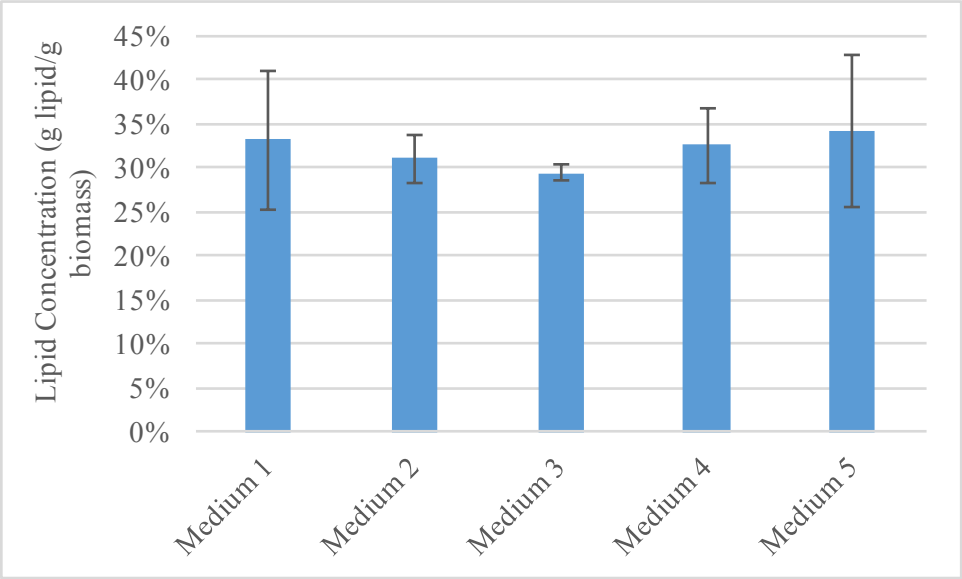


The lipid content of the biomass is shown in Figure 5. No significant difference (P-value = 0.8518, for a detailed statistical analysis, see Appendix B.2) of the lipid concentration occurred in any of the treatments. The highest lipid concentration was found in medium 5 (34.2%), and the lowest was found in medium 4 (29.5%).

Figure 5. Lipid concentration (g lipid/g biomass) in biomass using different culture media

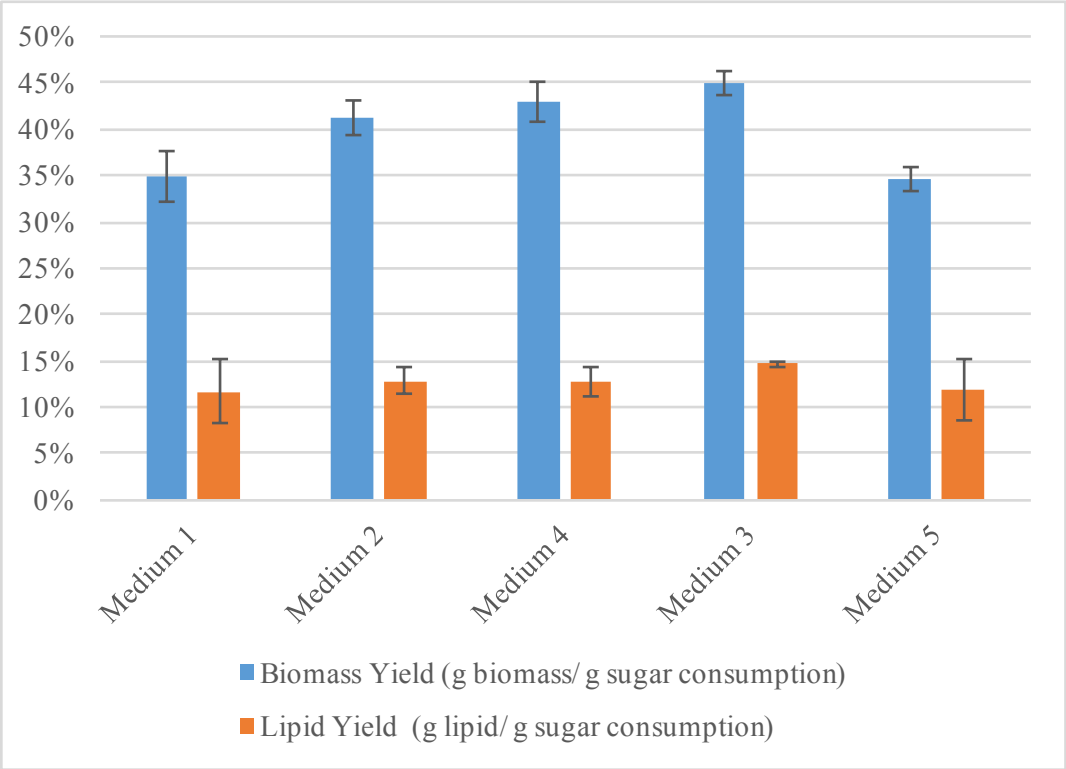


The biomass yield (g biomass produced/ g sugar consumed) and the lipid yield (g lipid produced/ g sugar consumed) were calculated after the experiment based on biomass/lipid production and sugar consumption.

Medium 3 (corn stover hydrolysate and algal biomass hydrolysate) had the highest biomass yield (0.45 g biomass/ g sugar), which was significantly higher than in medium 1 (corn stover hydrolysate alone) (0.35 g biomass/ g sugar) and the control (0.35 g biomass/g sugar). A comparison of medium 2 to medium 3 indicated no significant differences in biomass yield if an algal biomass hydrolysate or yeast extract was used as the nitrogen source for fermentation. The addition of an algal biomass to corn stover and hydrolyzing the mixture did not significantly affect the biomass yield. This could be a new approach for preparing a fungal medium in the future since it simplified the process.

None of the media showed a significant difference in lipid yield (P-value= 0.5767, for a detailed statistical analysis, see Appendix B.2). The algal enzymatic hydrolysate could be a replacement for yeast extract because the same lipid production was achieved. Furthermore, mixing corn stover and algal hydrolysate did not affect the fungal lipid yield.

Figure 6. Biomass yield and lipid yield (g/g total sugar consumed) on different medium



2.5 Conclusion

This study demonstrated that *U. isabellina* ATCC 42613 can utilize an acid pre-treated algal hydrolysate as a nitrogen source, and it can replace the yeast extract in a cell culture. The use of the algal biomass has no negative influence on the lipid concentration. In addition, no inhibition was detected in a culture that had algal hydrolysate as the nitrogen source. By hydrolyzing a mixture of algal biomass and corn stover, the hydrolysate can be directly used for cell culture without an external nitrogen source. The fungi cultured on the mixed hydrolysate showed a similar sugar utilization trend to the corn hydrolysate medium with yeast extract. In the future, algal hydrolysis using different hydrolysis methods should be tested to archive a higher nitrogen recovery rate. Additionally, the addition of algal hydrolysate to different microbial cultures should be tested. Other sources such as other algae strains, a mixed culture of algae, and the biomass collected from an algal bloom should be used for algal hydrolysate production.

Chapter 3. A high-efficiency extraction approach to recover lipids from the oleaginous fungus Umbelopsis isabellina

3.1 Abstract

An increasing demand for alternative energy sources has brought much current attention to biofuel. *Umbelopsis isabellina*, an oleaginous fungal strain, was considered as a potential biofuel producer due to its ability to use sugars in a non-detoxified, acid pretreated corn stover hydrolysate. The Bligh & Dyer lipid extraction method that uses highly toxic and dangerous solvents has been suggested for use as the extraction method. This method is difficult to scale up due to the solvent properties. In this study, a method using the less toxic solvent hexane was studied as a replacement for the Bligh & Dyer method. By shaking the ground biomass with hexane in three stages, this method could achieve same lipid content as the Bligh & Dyer method. The lipid profile of the extract is also comparable with that from the Bligh & Dyer method. This method could be used as an extraction method for the fungus *Umbelopsis isabellina*.

3.2 Introduction

An increasing demand for alternative energy sources has brought much current attention to biofuel because it is carbon neutral and can be produced from a renewable feedstock. However, biodiesel production from vegetables and other plant-based sources potentially has negative environmental impacts for various reasons, including excessive land use for large scale production and massive greenhouse gas emission during land conversion (Shay 1993; Joseph Fargione 2008). Biodiesel derived from lipids from microorganisms such as fungi, algae and bacteria can be sustainably produced because they require less land use, do not cause food source competition, and have a high lipid content (Meng et al. 2009). In addition to biodiesel production, other value-added products such as PUFAs, and essential fatty acids for humans and animals can be generated from these microorganisms. Fungi are the predominant microorganisms for PUFA production compared to algae, bacteria, and yeasts (C 1993). Furthermore,

fungi also has the most rapid biomass accumulation compared to algae and bacteria (Colin Ratledge and Wynn 2002). Fungi have the potential for high-value lipid production, and our previous studies showed that the fungal strain *Umbelopsis isabellina* (previously known as *Mortierella isabellina*) can utilize lignocellulosic material and convert the carbon source in corn stover hydrolysate to lipids in the presence of inhibitors such as furfural and hydroxymethylfurfural (HMF). Demonstrating that this fungal strain can produce lipids from a renewable carbon source caused the use of fungal lipids for biodiesel and other high-value fatty acids productions to become more realistic (Ruan et al. 2012). Since different extraction methods performs differently for different microorganisms, the discovery of a highly efficient, economically feasible, and environmentally friendly extraction method is essential for microbial biodiesel production and necessary for the further development of this particular fungal strain (Medina et al. 1998; Forfang et al. 2017).

Lipids can be extracted from biomass in several different ways - organic solvent extraction, supercritical fluid extraction, organic solvent extraction with a Soxhlet apparatus (Soxhlet 1879), ultrasound-assisted organic solvent extraction, and microwave-assisted organic solvent extraction. None of these methods are very scalable due to the complicated extraction setup or the large amount of organic solvents required. Organic solvent extraction is the traditional extraction method that is widely used in many laboratories (Halim, Danquah, and Webley 2012). Several organic solvent methods are generally used for the extraction of microbial lipids: The Bligh & Dyer method uses methanol and chloroform (E. G. Bligh 1959), the Atsushi method uses hexane & isopropanol (Hara and Radin 1978), the Soxhlet method uses hexane (Soxhlet 1879), and the Jordi method uses methanol and chloroform (Folch, Lees, and Sloane Stanley 1957). The extraction methods vary for different biomasses. For example, dichloroethane-based organic solvent mixtures show excellent potential for the algae Cladofora. However, limitations were found when this method was applied to the algae *B. braunii* (Halim, Danquah, and Webley 2012). Therefore, the discovery of the appropriate extraction method for a specific microorganism for biodiesel production is essential. For the lipid extraction of *Umbelopsis isabellina*

biomass, our previous study suggested that the Bligh & Dyer method was better than the hexane & isopropanol method, the CH2Cl2 & methanol method, and the hexane method (Hussain et al. 2014). However, the solvents used in this method are highly toxic and a large quantity of solvents is required (Halim, Danquah, and Webley 2012). Furthermore, this method showed high variation when conducted in different labs (Manirakiza, Covaci, and Schepens 2001). Therefore, the discovery of an alternative lipid extraction method which uses a less toxic solvent, and has an easier and more stable procedure, is essential for the further development of fungal lipid production. Hexane is the most widely used solvent for oil extraction in industry for primary oil seeds such as soybeans and canola seeds. Compared to the chloroform and methanol used in the Bligh & Dyer method, hexane is less toxic and more environmentally friendly (Capello, Fischer, and Hungerbuhler 2007). Furthermore, hexane can be evaporated and condensed after extraction, then reused in the next round of extraction. In the economic feasibility report of Delmer L. Helgeson, large-sized extraction equipment from 500 tons to 2000 tons per day was surveyed, and hexane was used as the primary extraction solvent. (Helgeson, D., D. Cobia, R. Coon, W. Hardie, L. Schaffner 1977).

Hexane therefore plays a major role as a solvent in industrial extraction methods. The development of an efficient, and stable extraction method for fungal biomass could contribute to the development of biodiesel production from oleaginous fungi.

3.3 Material and methods

3.3.1 Fungal culture

The fungal strain used for this experiment was *U. isabellina* ATCC 42613. *U. isabellina* spore*s* were cultured on potato dextrose agar (Sigma, USA) at 30°C for 14 days, washed with sterile distilled water, and stored at 4°C. To generate the seed for the culture, 1 mL of spore solution (4.2×10^7 spore·mL^{-1}) was first cultured for two days in 100 mL medium with 24 g/L potato dextrose broth and 8 g/L yeast extract as a seed for fermentation. Seed inoculum was added at 10% to a 7.5 L New Brunswick Bioflo

115 fermenter with 4.0 L of fermentation medium and cultured at 1 vvm and 25°C for 4 days. The fermentation medium included 20 g/L glucose, 20 g/L xylose, 4 g/L acetate, 1 g/L furfural and 1 g/L HMF to mimic a pretreated corn stover hydrolysate. The biomass was collected After each batch was cultured using a centrifuge and washed three times with DI water (Beckman, Allegra X-12R). The washed biomass was then oven-dried for 24 hours at 100°C. Several batches of culture were harvested to accumulate enough biomass for the experiment. The biomass from each batch was mixed and ground with a Waring Blender and then stored at room temperature in a sealed bottle for future use.

3.3.2 Lipid extraction

The biomass and hexane were mixed in different gram to milliliter ratios in a 2 mL Denvile microtube. Three ratios were tested in this study (1:4, 1:8, and 1:12). The tubes were shaken on an Innova 2000 platform shaker for various amounts of time (1 minute, 3 minutes, 5 minutes, 10 minutes, 20 minutes, 60 minutes, and 1800 minutes) at room temperature. The shaking speed used for this experiment was 400rpm. After shaking, the samples were centrifuged at 13000 rpm for 5 minutes using an Eppendorf Refrigerated Microcentrifuge (5417R). After centrifugation, the supernatant, which contained hexane and extracted lipid, was poured onto pre-weighed aluminum trays and dried in a fume hood overnight. The weight of the aluminum trays and lipid was measured after the hexane was evaporated to determine the mass of the lipid extracted.

The process was also studied for multiple extraction stages. To perform the second and third stage, the same amount of hexane was added to the biomass left in the microtube after it was centrifuged and the supernatant was removed, and same methods were repeated. After each stage, the hexane layer was poured into the same aluminum tray so that all of the lipid extracted from one sample could be accumulated and recorded.

To test on a larger scale with a larger amount of biomass, 1 g of biomass and 4 mL hexane were mixed in a 50 mL centrifuge tube and shaken on the shaker. Two layers were formed After shaking, the

hexane layer and a solid layer. The hexane layer was transferred to several Denvile microtubes using a 1 mL pipette, and the settled solids were left in the tube and dried. The microtubes were centrifuged for five minutes in the microcentrifuge to separate the liquid and solid. The supernatants from all of the tubes that contained the same sample were poured into the same aluminum tray, and the solid was dried and transferred back to the original 50 mL centrifuge tube for the next stage of extraction. The same amount of hexane was added for the second and third stages, and the same protocols for mixing and separation were employed. The liquid fraction, containing lipid and solvent in the aluminum trays, was dried in the fume hood overnight and then weighed to determine the total lipid concentration. The Bligh & Dyer method with a modified methanol: chloroform: water ratio of 2:1:0.8 (v/v/v) was conducted as a positive control and the total lipid content of the same biomass was determined (Bligh and Dyer 1959).

3.3.3 Lipid profile

Fatty acid methyl ester (FAME) synthesis was used to analyze the fatty acid profile of the lipids extracted by the various methods. The lipids were first treated with a conventional transesterification procedure to form FAMEs. The lipids were treated with methanol, sulfuric acid, and chloroform. The chloroform layer, which included the FAMEs, was collected and stored at -18°C for GC–MS analysis (Indarti et al. 2005). A Thermo GC-MS equipped with an Agilent DB-23 column (30 m, 0.25 mm, 0.25 μm, 7-inch cage) was used to analyze the fatty acids. The standard used for this analysis was a Supelco 37 Component FAME Mix.

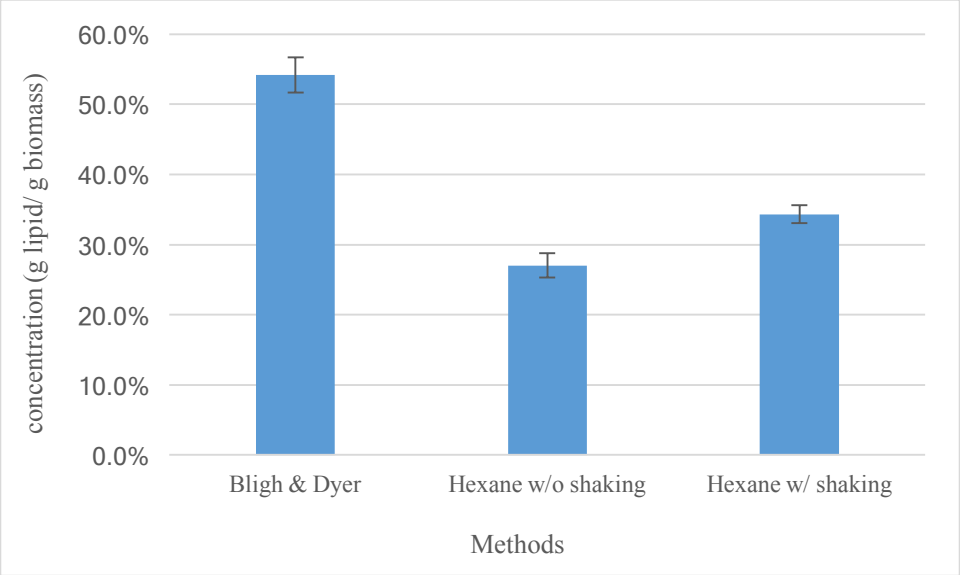
3.3.4 Statistical analysis

Statistical analysis was performed by using the Minitab Express 2016. A two sample t test and a one-way ANOVA was chosen for the treatment comparisons for each dataset. The confidence level was 95%.

3.4 Results and discussion

3.4.1 Comparison of lipid extraction using different extraction methods

Figure 7. Lipid concentration of same fungal biomass using different extraction methods



The Bligh & Dyer method was conducted to determine the total extractable lipids in the biomass. The lipid yield was 54.2±3%. Two experiments using hexane as the solvent were performed and compared with the Bligh & Dyer method, one with and one without shaking using the same hexane ratio (1:4). Shaking the mixture significantly increased the lipid yield from 27.0±1.7% to 34.3±1.3%. This experiment showed that hexane extraction has the potential to extract part of the lipids but not 100% of the total extractable lipids. It did however demonstrate that hexane had the potential to extract some of the lipids and that mixing is critical for lipid extraction with hexane. The shaking time, the extraction protocol, and the biomass to hexane ratio were optimized in future experiments.

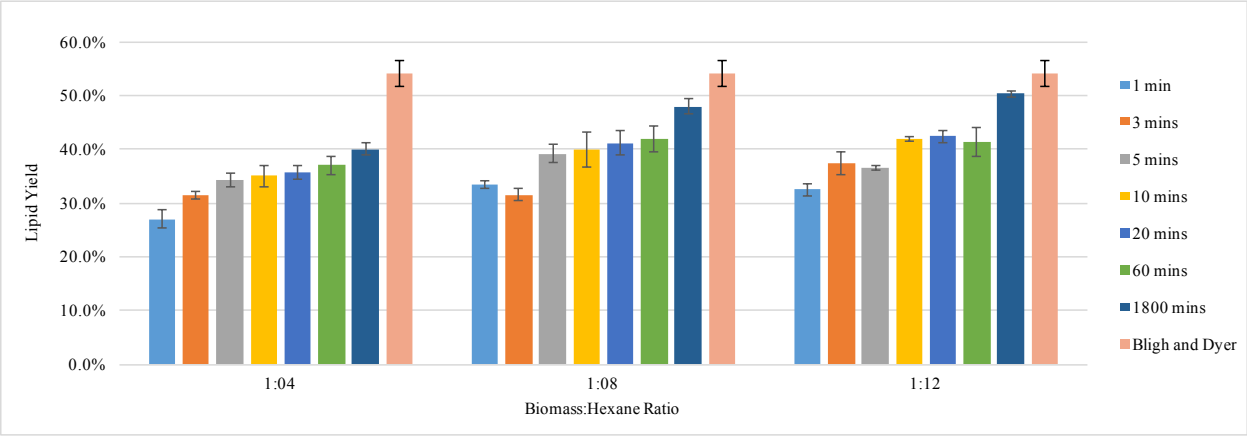
3.4.2 Optimization of the hexane extraction method

The biomass and hexane were mixed in three ratios (1:4, 1:8 and 1:12) and shaken at 400 rpm for various amounts of time (1 minute, 3 minutes, 5 minutes, 10 minutes, 20 minutes, 60 minutes and 1800

minutes). As the shaking time increased, the lipid yield increased from 27.1% to 40.0% for the 1:4 ratio, 33.4% to 48.1% for the 1:8 ratio, and 32.5% to 50.3% for the 1:12 ratio. The standard fungal lipid extraction method (i.e., the Bligh and Dyer method) was conducted on the same biomass and the lipid yield was 54%±2.5%. The lipid ratio increased rapidly during the first five minutes of shaking time. After the first five minutes, the lipid yield exhibited a lag phase. From five minutes to sixty minutes, the lipid content remained steady for the 1:4 and 1:8 ratios. As the mixing time increased, the extracted lipid yield increased. For all of the ratios, 1800 minutes of shaking was best. A larger amount of lipids were extracted with a higher amount of solvent. The 1:8 biomass: hexane ratio had the highest lipid yield at five minutes, No significant differences were seen between the 1:8 and the 1:12 ratios. No significant difference was found between the 1:4 and 1:12 ratios (for a detailed statistical analysis, see Appendix B.3). The 1:4 ratio was chosen for future experiments because it had a good lipid extraction yield and required the least amount of hexane, and all three ratios extracted more than half of the lipids extracted by the Bligh & Dyer method.

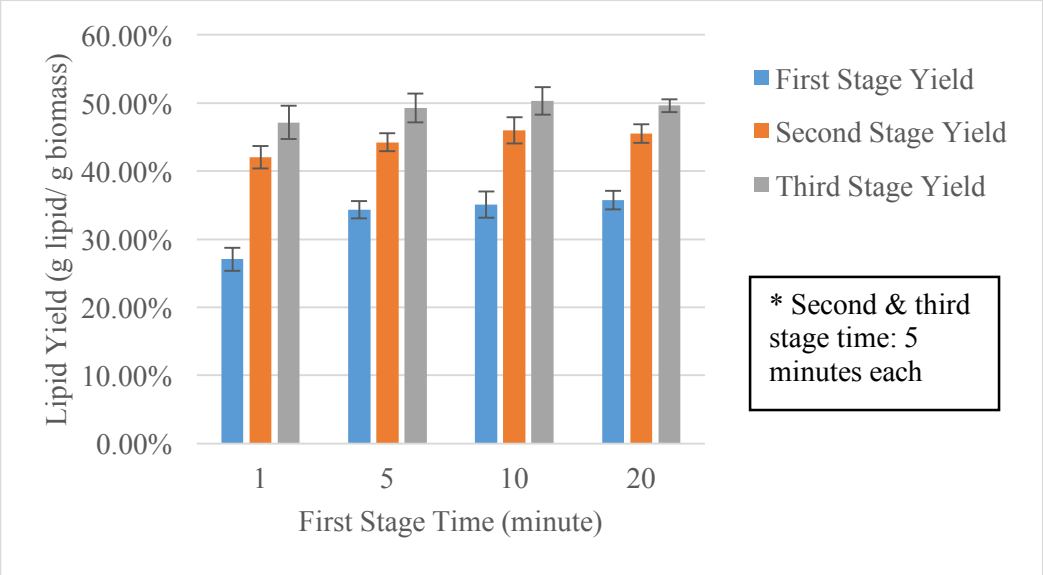
Although the 1:12 biomass: hexane ratio showed a comparable result after 1800 minutes, the long extraction time, and the energy consumed by the shaking was not ideal. Therefore, hexane cannot extract as much lipid as the Bligh & Dyer method in a one stage extraction. However, the lipid yield showed that a hexane extraction solvent could eventually achieve a yield comparable to the Bligh & Dyer method. To shorten the extraction time and achieve a similar or even higher yield than the Bligh & Dyer method, multiple stage extraction was considered as the next step. The inspiration for multiple stage extraction came from Soxhlet method, in which hexane is evaporated and then condensed to continuously wash the biomass so that during the extraction period, the biomass contacts the hexane, but not the lipid.

Figure 8. The effects of different biomass: hexane ratios and extraction time on lipid yield



To further increase the yield, the extraction processes was performed in three stages. Since the first stage could extract more than half of the lipid; it was important to ensure that the highest yield was achieved in a short time. Four first stage mixing times (1 minute, 5 minutes, 10 minutes, and 20 minutes) were tested, followed by two stages of extraction for 5 minutes. The biomass: hexane ratio was 1:4 for all three of these stages. Figure 9 shows the lipid yield from the first stage, which was processed for 1 minute and was significantly lower than the yield after 5, 10, or 20 minutes of extraction. No significant difference was found for 5, 10, and 20 minutes for the first stage extraction (for a detailed statistical analysis, see Appendix B.3), and no significant difference was found for 1, 5, 10, 20 minutes for second stage lipid yields (P-value=0.0936, for a detailed statistical analysis, see Appendix B.3). After the second and third stages, the final lipid yield for those four experiments was respectively 47%, 49%, 50%, and 50% and t no significant difference was found for any treatments (P-value=0.2944, for a detailed statistical analysis, see Appendix B.3).

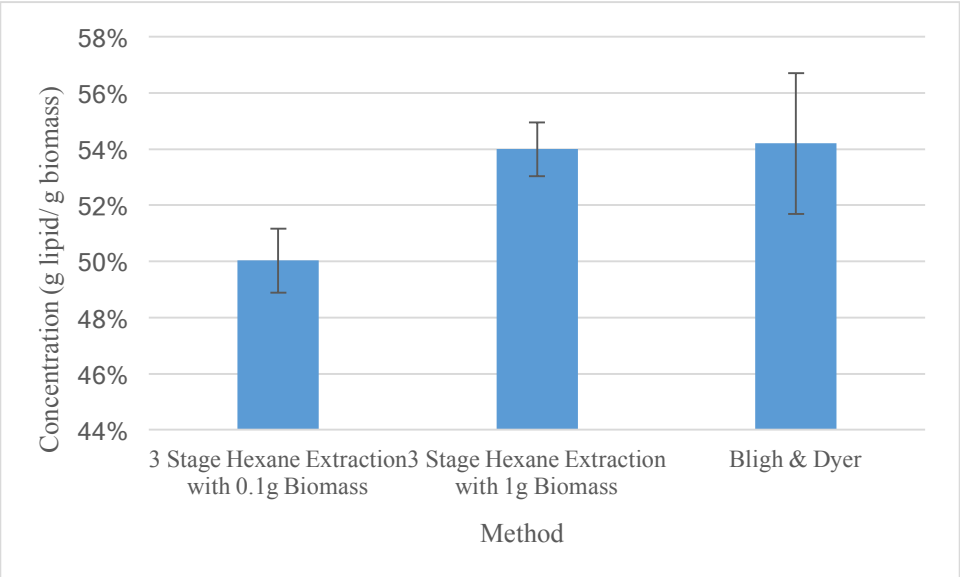
Figure 9. Staged extraction of different first stage extraction times



Since there was no significant difference in the final lipid yield for 1, 5, 10, and 20 minutes, 5 minutes was selected for the first stage. The reason 5 minutes was chosen instead of 1 minute was that more lipids were extracted after 5 minutes than 1 minute. This could help when applied to a biomass with a higher lipid content. In addition, because all three stages of extraction repeated the same protocol for the same time, it was easier to conduct the experiment. After three extraction stages of 5 minutes each, this multiple stages extraction method extracted over 90% of the lipids extracted by the Bligh & Dyer method.

3.4.3 Three stage extraction with a larger amount of biomass

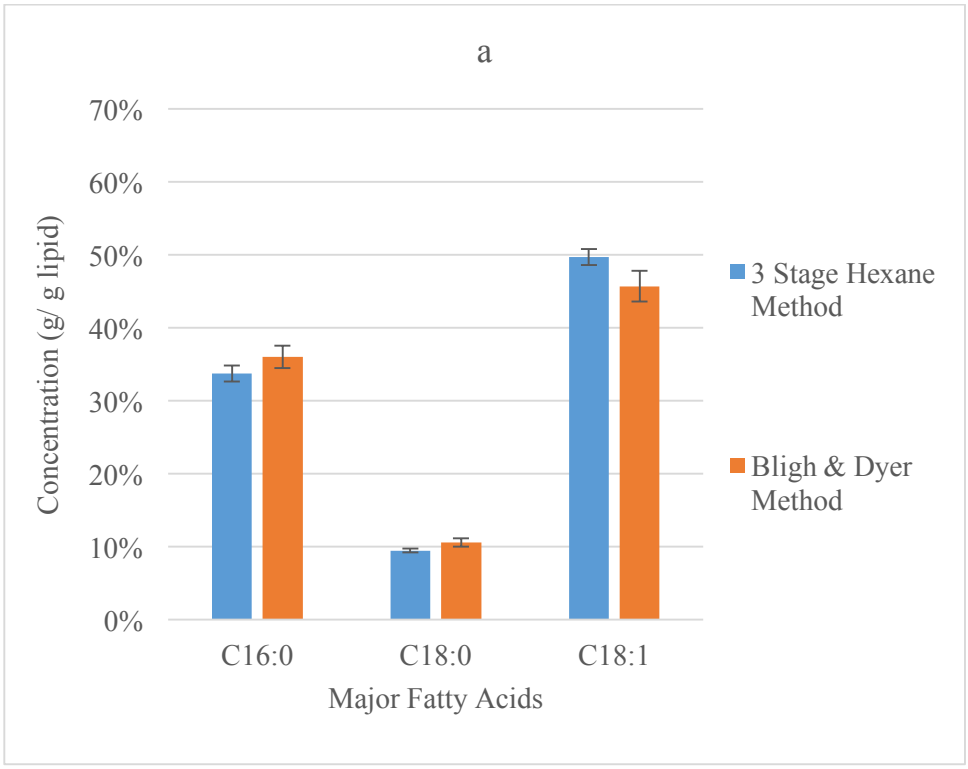
Figure 10. Scaling up the three stage hexane extraction



After extracting 0.1 g biomass, a larger amount of biomass (1 g) was tested for verification, because 0.5 g biomass was used for the Bligh & Dyer method since it is difficult to perform accurately on a smaller scale. The lipid yield after three stages of extraction increased as the biomass amount increased. The final yield of hexane method showed no significant differences from the Bligh & Dyer yield (P-value=0.9099, for a detailed statistical analysis, see Appendix B.4). The method also showed less variation between the three replicates. A possible explanation for the increase in the lipids extracted from 50% to 54% is that the mixing was likely more effective in the 1 g experiment because the sample was in a 50 mL centrifuge tube. It appeared that a larger tube size and a larger head space for the mixture allowed the biomass and hexane to mix better. Therefore, the three-stage extraction method can extract the same amount of lipid as the Bligh & Dyer method for the fungus *U. isabellina* biomass.

3.4.4 Fatty acid profile of the extracted lipids

Figure 11. Fatty acid profiles for different extraction methods: (a) major fatty acids; (b) C16 and C18; (c) Saturated fatty acids and unsaturated acids



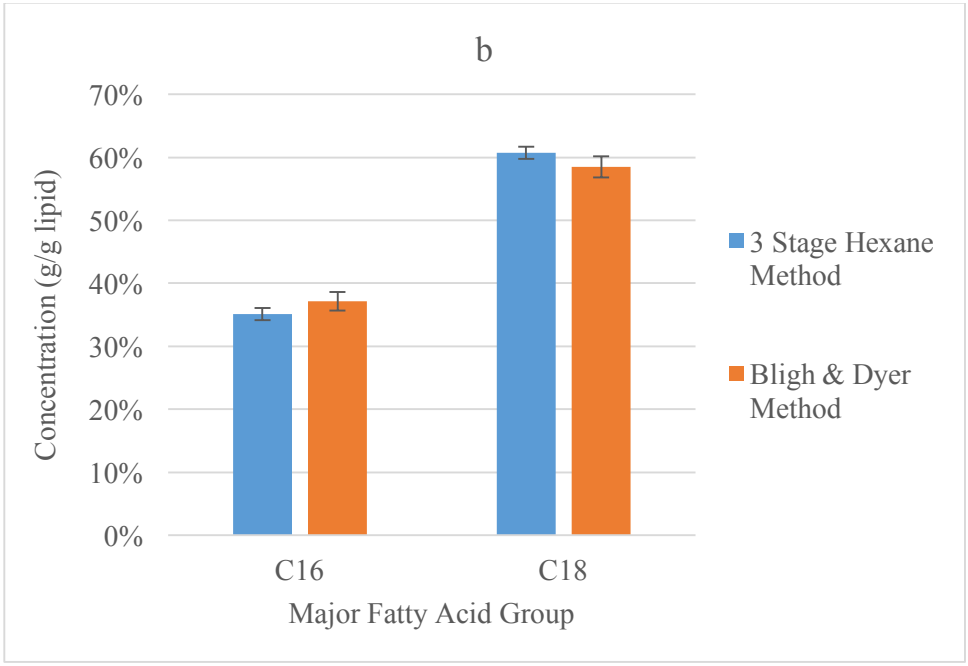
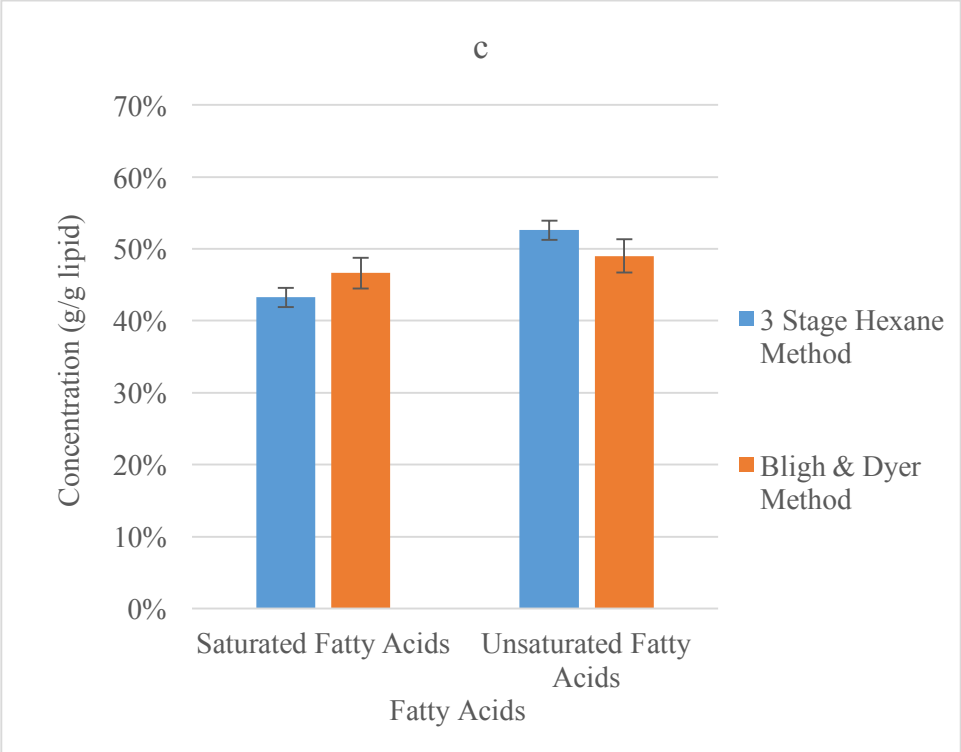


Figure 11 (cont'd)



Different fatty acids were analyzed using the FAME method to determine differences between solvents for fatty acid extraction. In Figure 11, different fatty acid profiles were analyzed for these two methods. Compared with Bligh & Dyer, no significant differences were found for the two methods for the extraction of the major fatty acids: C16:0 (P-value=0.1287, for a detailed statistical analysis, see Appendix B.5), C18:0 (P-value=0.0990, for a detailed statistical analysis, see Appendix B.5), and C18:1 (P-value=0.0635, for a detailed statistical analysis, see Appendix B.5). Oleic acid (C18:1) was the most abundant fatty acid, followed by Palmitic acid (C16:0) and stearic acid (C18:0). The lipid profile was comparable with our previous study (Ruan et al. 2012). Additionally, as seen in Fig. 11b, no significant differences were seen for C16 (P-value=0.1432, for a detailed statistical analysis, see Appendix B.5) and C18 (P-value=0.1425, for a detailed statistical analysis, see Appendix B.5), which are the major carbon chains for biodiesel production. Additionally, no significant differences were seen for saturated (P-

value=0.1009, for a detailed statistical analysis, see Appendix B.5) and unsaturated fatty acids (P-value=0.1028, for a detailed statistical analysis, see Appendix B.5) (Fig. 11c).

3.5 Conclusion

This study demonstrated that three-stage extraction process with hexane can be to extract lipids the fungus *U. isabellina*. This method is easier to conduct and more environmentally friendly compared to the standard fungal lipid extraction method of Bligh & Dyer. This study only tested 0.1 g and 1 g of biomass. When this method was applied to 0.1 g of biomass, over 90% of lipid was extracted compared to the Bligh & Dyer method. This approach could be helpful when not much biomass is available and only a rough estimate of the lipid yield is required. When applied to 1 g of biomass, the lipid yield achieved the same level as the Bligh & Dyer method with a lower standard deviation. This study demonstrated that hexane could be used to extract lipids from *U. isabellina*. In the future, larger amounts of biomass should be tested, as well as the reuse of hexane, and eventually an industry level reactor could be used with *U. isabellina* biomass. This could potentially make biofuel products extracted from *U. isabellina* biomass more feasible because hexane extraction is currently a mature industrial technology.

Chapter 4. Conclusion and future work

4.1 Conclusion.

U. isabellina, a filamentous oleaginous fungus, can utilize non-detoxified corn stover hydrolysate for lipid production. To further promote this strain as feasible for the production of high-value biofuel products, an economical and abundant substrate, and a highly efficient, environmentally friendly extraction method are required

A low lipid content algal biomass was mixed with corn stover, pre-treated with acid, and enzymatically hydrolyzed. The hydrolysate could provide a nitrogen source for fungal growth and the addition of algal biomass did not inhibit growth. Since no significant differences in lipid production were found for the algal hydrolysate and yeast extract as a nitrogen source, this approach could save the expense of an external nitrogen source for fungal culture. The substrate cost was also reduced. Additionally, the results of this study suggest that the algal hydrolysate can be sold as a nitrogen supplement for microbial culture, which would make algal biofuel products more economically practical.

Lipid extraction was another critical process in fungal lipid production that was studied in this research. The most popular laboratory method is the Bligh & Dyer method, which is a complex method using two solvents. To simplify the process, this study revealed that a three-stage extraction method that used hexane alone can replace of the Bligh & Dyer method for the lipid extraction of *U. isabellina* biomass. This method can extract the same amount of lipid as the Bligh & Dyer method for 1 g of biomass. Using hexane as the only solvent is desirable due to less environmental impact and a less potential for human error compared to the Bligh & Dyer method. Furthermore, pilot scale extraction systems for major oil seed extraction using hexane already exist in industry (Ayorinde et al. 1990).

4.2 Future work

The following future studies may be useful for microbial lipid production with fungal fermentation.

1. Optimization of algal hydrolysis with different hydrolysis methods to achieve a high nitrogen content and the application of the hydrolysate to different microbes.
2. Investigation of different algae strains, mixed algal cultures, or biomass collected from an algal bloom for algal hydrolysate production.
3. A scale-up of the fungal extraction to an industrial level reactor and an energy balance analysis for multi-stage hexane extraction.

APPENDICES

APPENDIX A: DATA

Chapter 2 Data

Table 4. Algae concentration for figure 3

Time (h)	Dry Biomass (g/L)
0.0	2.39
0.4	1.85
24.2	1.74
24.8	1.43
48.0	1.32
48.5	1.08
72.0	1.16
72.5	0.98
96.0	1.30
96.4	1.06
120.0	1.44
120.4	1.16
144.0	1.53
168.0	1.76
168.4	1.42
192.0	1.72
192.5	1.35
216.0	1.63
216.5	1.31
240.0	1.57
240.4	1.28
264.0	1.67
264.4	1.34
288.0	1.71
288.4	1.35
312.0	1.69
312.4	1.33
336.0	1.64
336.4	1.29
360.0	1.52
360.4	1.18
384.0	1.48
384.5	1.23
408.0	1.48
408.4	1.19
432.0	1.49
432.4	1.18

Table 5. Sugar concentration data for figure 4

	Time (Day)	0	1	2	3	4	5	6	7
Medium		Sugar Concentration (g/L)							
Corn Stover Hydrolysate	Glucose (g/L)	25.28	22.41	11.62	3.24	0.30	0.00	0.00	0.00
	Xylose (g/L)	15.61	15.42	14.67	10.60	7.81	4.41	1.09	0.00
	Acetate (g/L)	2.20	1.48	0.00	0.14	0.00	0.00	0.00	0.00
	Total	43.08	39.31	26.29	13.98	8.10	4.41	1.09	0.00
Corn Stover Hydrolysate with Yeast Extract	Glucose (g/L)	24.97	21.52	11.98	3.19	0.94	0.00	0.00	0.00
	Xylose (g/L)	15.19	14.59	14.40	15.87	7.80	7.10	3.26	0.66
	Acetate (g/L)	2.17	1.42	0.05	0.63	0.00	0.00	0.00	0.00
	Total	42.33	37.54	26.43	19.69	8.74	7.10	3.26	0.66
Corn Stover Hydrolysate with Algal Biomass Hydrolysate	Glucose (g/L)	22.98	17.44	8.46	0.76	0.33	0.04	0.00	0.00
	Xylose (g/L)	14.18	12.41	12.69	9.86	5.94	3.43	0.53	0.00
	Acetate (g/L)	2.02	1.26	0.05	0.37	0.00	0.00	0.00	0.00
	Total	39.18	31.11	21.20	11.00	6.27	3.46	0.53	0.00
Corn Stover And Algal Biomass Mixture Hydrolysate	Glucose (g/L)	23.51	20.68	11.71	3.71	0.98	0.00	0.00	0.00
	Xylose (g/L)	14.17	13.98	13.78	10.19	7.97	6.36	2.75	0.59
	Acetate (g/L)	2.08	1.46	0.00	0.22	0.00	0.00	0.00	0.00
	Total	39.76	36.12	25.48	14.12	8.95	6.36	2.75	0.59
Control with Pure Sugar	Glucose (g/L)	21.17	16.88	0.00	0.00	0.00	0.00	0.00	0.00
	Xylose (g/L)	13.27	14.09	10.66	2.59	0.00	0.00	0.00	0.00
	Acetate (g/L)	0.69	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Total	35.12	30.97	10.66	2.59	0.00	0.00	0.00	0.00

Table 6. Lipid concentrations and standard deviations for figure 5

	Lipid Concentration (g lipid/g biomass)	Standard Deviation
Medium 1	33.24%	7.92%
Medium 2	31.13%	2.71%
Medium 3	32.60%	0.95%
Medium 4	30.84%	4.18%
Medium 5	34.22%	8.61%

Table 7. Biomass yield and lipid data for figure 6

	Biomass Yield (g biomass/ g sugar consumption)	Lipid Yield (g lipid/ g sugar consumption)	Biomass Yield Standard Deviation	Lipid Yield Standard Deviation
Medium 1	34.90%	11.71%	2.80%	3.42%
Medium 2	41.24%	12.85%	1.80%	1.51%
Medium 4	42.99%	12.69%	2.16%	1.51%
Medium 3	44.98%	14.66%	1.33%	0.34%
Medium 5	34.64%	11.93%	1.39%	3.38%

Chapter 3 Data

Table 8. Lipid yield and standard deviation data for figure 7

	Lipid Yield (g lipid/ g biomass)	Lipid Yield Standard Deviation
0.1g Biomass Extraction	50.25%	1.14%
1g Biomass Extraction	54.41%	0.95%
Bligh & Dyer	53.64%	2.51%

Table 9. Lipid yield and standard deviation for figure 8

Ratio	1:04	1:08	1:12	1:04	1:08	1:12
Time	Average Lipid Yield (g lipid/ g biomass)			Standard Deviation		
1 min	27.06%	33.45%	32.52%	1.68%	0.68%	1.04%
3 mins	31.44%	31.55%	37.45%	0.70%	1.12%	2.09%
5 mins	34.31%	39.25%	36.62%	1.29%	1.73%	0.33%
10 mins	35.10%	39.97%	41.91%	1.93%	3.23%	0.36%
20 mins	35.76%	41.25%	42.44%	1.38%	2.39%	1.19%
60 mins	37.10%	41.92%	41.32%	1.71%	2.41%	2.74%
1800 mins	40.03%	48.06%	50.35%	1.10%	1.36%	0.53%
Bligh and Dyer	54.20%	54.20%	54.20%	2.51%	2.51%	2.51%

Table 10. 3 stage lipid yields for different first stage reaction time (figure 9)

First Stage Time (minute)	1	5	10	20
	Lipid Yield (g lipid/ g biomass)			
First Stage Yield	27.06%	34.31%	35.10%	35.76%
Second Stage Yield	42.04%	44.25%	45.97%	45.49%
Third Stage Yield	47.15%	49.26%	50.34%	49.62%

Table 11. Scale up lipid yield data for figure 10

	3 Stage Hexane Extraction with 0.1g Biomass	3 Stage Hexane Extraction with 1g Biomass	Bligh & Dyer
Lipid Yield (g lipid/ g biomass)	50.03%	53.99%	54.20%
Standard Deviation	1.14%	0.95%	2.51%

Table 12. Fatty acid profile data for figure 11

Graph	a			b		c	
	C16:0	C18:0	C18:1	C16	C18	Saturated Fatty Acids	Unsaturated Fatty Acids
3 Stage Hexane Method	33.74%	9.50%	49.69%	35.10%	60.72%	43.24%	52.58%
Bligh & Dyer Method	36.02%	10.60%	45.69%	37.15%	58.49%	46.62%	49.01%
Hexane Method Standard Deviation	1.09%	0.24%	1.12%	1.00%	1.00%	1.33%	1.33%
Bligh & Dyer Method Standard Deviation	1.55%	0.60%	2.13%	1.50%	1.68%	2.12%	2.30%

APPENDIX B: STATISTICAL ANALYSIS

B1. Statistical analysis for different sugar concentration (glucose, xylose, acetate, and total sugar) of hydrolysate in chapter 2 section 2.4.2

Table 13. Data for appendix B1

Hydrolysate	Glucose (g/L)	Xylose (g/L)	Acetate (g/L)	Total Sugar (g/L)
Corn Stover Hydrolysate	25.28	16.4	0.93	42.61
Corn Stover Hydrolysate	26.6	16.19	0.91	43.69
Corn Stover Hydrolysate	24.53	17.11	0.97	42.6
Corn Stover + Algal Biomass Hydrolysate	28.32	17.61	0.95	46.88
Corn Stover + Algal Biomass Hydrolysate	26.72	17.32	0.95	44.99
Corn Stover + Algal Biomass Hydrolysate	24.35	17.4	0.96	42.71

2-Sample t: Glucose by Hydrolysate

Method

μ_1: mean of Glucose when Hydrolysate = Corn Stover + Algal Biomass Hydrolysate

μ_2: mean of Glucose when Hydrolysate = Corn Stover Hydrolysate

Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics: Glucose

Hydrolysate	N	Mean	StDev	SE Mean
Corn Stover + Algal Biomass Hydrolysate	3	26.463	1.997	1.153
Corn Stover Hydrolysate	3	25.4700	1.0480	0.6051

Estimation for Difference

Difference	95% CI for Difference
0.993	(-3.151, 5.138)

Test

Null hypothesis	H_0: $\mu_1 - \mu_2 = 0$
Alternative hypothesis	H_1: $\mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
0.76	3	0.5011

2-Sample t: Xylose by Hydrolysate

Method

μ_1: mean of Xylose when Hydrolysate = Corn Stover + Algal Biomass Hydrolysate

μ_2: mean of Xylose when Hydrolysate = Corn Stover Hydrolysate

Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics: Xylose

Hydrolysate	N	Mean	StDev	SE Mean
Corn Stover + Algal Biomass Hydrolysate	3	17.4433	0.14978	0.08647
Corn Stover Hydrolysate	3	16.5667	0.4821	0.2783

Estimation for Difference

Difference	95% CI for Difference
0.8767	(-0.3774, 2.1308)

Test

Null hypothesis	H_0: $\mu_1 - \mu_2 = 0$
Alternative hypothesis	H_1: $\mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
3.01	2	0.0950

2-Sample t: Xylose by Hydrolysate

Method

μ_1: mean of Xylose when Hydrolysate = Corn Stover + Algal Biomass Hydrolysate

μ_2: mean of Xylose when Hydrolysate = Corn Stover Hydrolysate

Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics: Xylose

Hydrolysate	N	Mean	StDev	SE Mean
Corn Stover + Algal Biomass Hydrolysate	3	17.4433	0.14978	0.08647
Corn Stover Hydrolysate	3	16.5667	0.4821	0.2783

Estimation for Difference

Difference	95% CI for Difference
0.8767	(-0.3774, 2.1308)

Test

Null hypothesis	H_0: $\mu_1 - \mu_2 = 0$
Alternative hypothesis	H_1: $\mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
3.01	2	0.0950

2-Sample t: Total Sugar by Hydrolysate

Method

μ_1: mean of Total Sugar when Hydrolysate = Corn Stover + Algal Biomass Hydrolysate
μ_2: mean of Total Sugar when Hydrolysate = Corn Stover Hydrolysate
Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics: Total Sugar

Hydrolysate	N	Mean	StDev	SE Mean
Corn Stover + Algal Biomass Hydrolysate	3	44.860	2.088	1.206
Corn Stover Hydrolysate	3	42.9667	0.6264	0.3617

Estimation for Difference

Difference	95% CI for Difference
1.893	(-3.522, 7.309)

Test

Null hypothesis	H_0: $\mu_1 - \mu_2 = 0$
Alternative hypothesis	H_1: $\mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
1.50	2	0.2714

B2. Statistical analysis for lipid concentration, biomass yield, and lipid yield of different hydrolysate mediums (with control) in chapter 2 section 2.4.3

One-Way ANOVA: Lipid Con (g lipid/g biomass) versus Medium

Method

Null hypothesis	H_o: All means are equal
Alternative hypothesis	H_1: At least one mean is different

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Medium	5	control with pure sugar, corn stover and algal biomass mixture hydrolysate, corn stover hydrolysate, corn stover hydrolysate with algal biomass hydrolysate, corn stover hydrolysate with yeast extract

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Medium	4	0.0041067	0.00102667	0.33	0.8518
Error	10	0.0311333	0.00311333		
Total	14	0.0352400			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0557973	11.65%	0.00%	0.00%

Means

Medium	N	Mean	StDev	95% CI
control with pure sugar	3	0.34333	0.08963	(0.27155, 0.41511)
corn stover and algal biomass mixture hydrolysate	3	0.29667	0.02517	(0.22489, 0.36845)
corn stover hydrolysate	3	0.33000	0.07810	(0.25822, 0.40178)

corn stover hydrolysate with algal biomass hydrolysate	3	0.330000	0.010000	(0.258221, 0.401779)
corn stover hydrolysate with yeast extract	3	0.31000	0.02646	(0.23822, 0.38178)

Pooled StDev = 0.0557973

One-Way ANOVA: Biomass Yield versus Medium

Method

Null hypothesis	H_0: All means are equal
Alternative hypothesis	H_1: At least one mean is different

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Medium	5	control with pure sugar, corn stover and algal biomass mixture hydrolysate, corn stover hydrolysate, corn stover hydrolysate with algal biomass hydrolysate, corn stover hydrolysate with yeast extract

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Medium	4	0.02676	0.00669	16.73	0.0002
Error	10	0.00400	0.00040		
Total	14	0.03076			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.02	87.00%	81.79%	70.74%

Means

Medium	N	Mean	StDev	95% CI
control with pure sugar	3	0.346667	0.015275	(0.320938, 0.372395)
corn stover and algal biomass mixture hydrolysate	3	0.42667	0.02082	(0.40094, 0.45239)
corn stover hydrolysate	3	0.34667	0.03055	(0.32094, 0.37239)
corn stover hydrolysate with algal biomass hydrolysate	3	0.450000	0.010000	(0.424272, 0.475728)

corn stover hydrolysate with yeast extract	3	0.410000	0.017321	(0.384272, 0.435728)

Pooled StDev = 0.02

Grouping Information Using the Tukey Method and 95% Confidence

Medium	N	Mean	Grouping	
corn stover hydrolysate with algal biomass hydrolysate	3	0.450000	A	
corn stover and algal biomass mixture hydrolysate	3	0.42667	A	
corn stover hydrolysate with yeast extract	3	0.410000	A	
corn stover hydrolysate	3	0.34667		B
control with pure sugar	3	0.346667		B

Means that do not share a letter are significantly different.

Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
corn stover and algal biomass mixture hydrolysate-control with pure sugar	0.08000	0.01633	(0.02631, 0.13369)	4.90	0.0044
corn stover hydrolysate-control with pure sugar	0.00000	0.01633	(-0.05369, 0.05369)	0.00	1.0000
corn stover hydrolysate with algal biomass hydrolysate-control with pure sugar	0.10333	0.01633	(0.04964, 0.15703)	6.33	0.0006
corn stover hydrolysate with yeast extract-control with pure sugar	0.06333	0.01633	(0.00964, 0.11703)	3.88	0.0201
corn stover hydrolysate-corn stover and algal biomass mixture hydrolysate	-0.08000	0.01633	(-0.13369, -0.02631)	-4.90	0.0044

corn stover hydrolysate with algal biomass hydrolysate-corn stover and algal biomass mixture hydrolysate	0.02333	0.01633	(-0.03036, 0.07703)	1.43	0.6248
corn stover hydrolysate with yeast extract-corn stover and algal biomass mixture hydrolysate	-0.01667	0.01633	(-0.07036, 0.03703)	-1.02	0.8405
corn stover hydrolysate with algal biomass hydrolysate-corn stover hydrolysate	0.10333	0.01633	(0.04964, 0.15703)	6.33	0.0006
corn stover hydrolysate with yeast extract-corn stover hydrolysate	0.06333	0.01633	(0.00964, 0.11703)	3.88	0.0201
corn stover hydrolysate with yeast extract-corn stover hydrolysate with algal biomass hydrolysate	-0.04000	0.01633	(-0.09369, 0.01369)	-2.45	0.1791

Individual confidence level = 99.18%

One-Way ANOVA: lipid yield versus Medium

Method

Null hypothesis	H_0: All means are equal
Alternative hypothesis	H_1: At least one mean is different

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Medium	5	control with pure sugar, corn stover and algal biomass mixture hydrolysate, corn stover hydrolysate, corn stover hydrolysate with algal biomass hydrolysate, corn stover hydrolysate with yeast extract

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Medium	4	0.00169333	0.00042333	0.76	0.5767
Error	10	0.00560000	0.00056000		
Total	14	0.00729333			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0236643	23.22%	0.00%	0.00%

Means

Medium	N	Mean	StDev	95% CI
control with pure sugar	3	0.12000	0.03464	(0.08956, 0.15044)
corn stover and algal biomass mixture hydrolysate	3	0.123333	0.015275	(0.092891, 0.153776)

corn stover hydrolysate	3	0.11667	0.03215	(0.08622, 0.14711)
corn stover hydrolysate with algal biomass hydrolysate	3	0.146667	0.005774	(0.116224, 0.177109)
corn stover hydrolysate with yeast extract	3	0.130000	0.017321	(0.099558, 0.160442)

Pooled StDev = 0.0236643

B3. Statistical analysis for lipid yield of different hexane ratio at 5 minutes in chapter 3 section 3.4.2

One-Way ANOVA: Lipid Yield versus Hexane ratio

Method

Null hypothesis	H_o: All means are equal
Alternative hypothesis	H_1: At least one mean is different

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Hexane ratio	3	4, 8, 12

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Hexane ratio	2	0.00650771	0.00325386	18.11	0.0003
Error	11	0.00197626	0.00017966		
Total	13	0.00848397			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0134037	76.71%	72.47%	64.26%

Means

Hexane ratio	N	Mean	StDev	95% CI
4	6	0.343167	0.012881	(0.331123, 0.355211)
8	5	0.392000	0.016432	(0.378807, 0.405193)
12	3	0.366667	0.005774	(0.349634, 0.383699)

Pooled StDev = 0.0134037

Grouping Information Using the Tukey Method and 95% Confidence

Hexane ratio	N	Mean	Grouping

8	5	0.392000	A	
12	3	0.366667	A	B
4	6	0.343167		B

Means that do not share a letter are significantly different.

Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
8-4	0.048833	0.008116	(0.026910, 0.070757)	6.02	0.0002
12-4	0.023500	0.009478	(-0.002101, 0.049101)	2.48	0.0727
12-8	-0.025333	0.009789	(-0.051774, 0.001107)	-2.59	0.0605

Individual confidence level = 97.94%

One-Way ANOVA: First Stage (1/5/10/20minute) versus First Stage Time

Method

Null hypothesis	H_0: All means are equal
Alternative hypothesis	H_1: At least one mean is different

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
First Stage Time	4	1, 5, 10, 20

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
First Stage Time	3	0.0150754	0.00502513	21.97	<0.0001
Error	11	0.0025162	0.00022875		
Total	14	0.0175916			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0151245	85.70%	81.80%	71.64%

Means

First Stage Time	N	Mean	StDev	95% CI
1	3	0.270600	0.016802	(0.251381, 0.289819)
5	6	0.343167	0.012881	(0.329577, 0.356757)
10	3	0.35097	0.01928	(0.33175, 0.37019)
20	3	0.357533	0.013761	(0.338314, 0.376753)

Pooled StDev = 0.0151245

Grouping Information Using the Tukey Method and 95% Confidence

First Stage Time	N	Mean	Grouping

20	3	0.357533	A	
10	3	0.35097	A	
5	6	0.343167	A	
1	3	0.270600		B

Means that do not share a letter are significantly different.

Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
5-1	0.07257	0.01069	(0.04035, 0.10478)	6.79	0.0002
10-1	0.08037	0.01235	(0.04317, 0.11757)	6.51	0.0002
20-1	0.08693	0.01235	(0.04973, 0.12413)	7.04	0.0001
10-5	0.00780	0.01069	(-0.02442, 0.04002)	0.73	0.8833
20-5	0.01437	0.01069	(-0.01785, 0.04658)	1.34	0.5566
20-10	0.00657	0.01235	(-0.03063, 0.04377)	0.53	0.9495

Individual confidence level = 98.82%

One-Way ANOVA: Second Stage (5 minutes) versus First Stage Time

Method

Null hypothesis	H_o: All means are equal
Alternative hypothesis	H_1: At least one mean is different

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
First Stage Time	4	1, 5, 10, 20

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
First Stage Time	3	0.00278307	0.00092769	2.74	0.0936
Error	11	0.00371934	0.00033812		
Total	14	0.00650241			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0183881	42.80%	27.20%	0.00%

Means

First Stage Time	N	Mean	StDev	95% CI
1	3	0.42040	0.02219	(0.39703, 0.44377)
5	6	0.442467	0.017232	(0.425944, 0.458989)
10	3	0.45970	0.02233	(0.43633, 0.48307)
20	3	0.454933	0.011235	(0.431567, 0.478300)

Pooled StDev = 0.0183881

One-Way ANOVA: Third Stage (5 minutes) versus First Stage Time

Method

Null hypothesis	H_o: All means are equal
Alternative hypothesis	H_1: At least one mean is different

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
First Stage Time	4	1, 5, 10, 20

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
First Stage Time	3	0.00169894	0.00056631	1.40	0.2944
Error	11	0.00444621	0.00040420		
Total	14	0.00614515			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0201047	27.65%	7.91%	0.00%

Means

First Stage Time	N	Mean	StDev	95% CI
1	3	0.47153	0.02425	(0.44599, 0.49708)
5	6	0.492633	0.021365	(0.474568, 0.510698)
10	3	0.50347	0.02014	(0.47792, 0.52901)
20	3	0.496200	0.009412	(0.470652, 0.521748)

Pooled StDev = 0.0201047

B4. Statistical analysis for lipid yield of 1g hexane extraction and Bligh & Dyer in chapter 3 section 3.4.3

2-Sample t: Lipid Yield by Method

Method

μ_1: mean of Lipid Yield when Method = Bligh & Dyer

μ_2: mean of Lipid Yield when Method = Hexane Method (1g)

Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics: Lipid Yield

Method	N	Mean	StDev	SE Mean
Bligh & Dyer	3	0.54199	0.02506	0.01447
Hexane Method (1g)	3	0.540000	0.010000	0.005774

Estimation for Difference

Difference	95% CI for Difference
0.00199	(-0.06502, 0.06901)

Test

Null hypothesis $H_0: \mu_1 - \mu_2 = 0$

Alternative hypothesis $H_1: \mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
0.13	2	0.9099

B.5. Statistical analysis for lipid profile of hexane extraction and Bligh & Dyer in chapter 3 section 3.4.4

2-Sample t: C16:0 by Method

Method

μ_1: mean of C16:0 when Method = 3 Stage Hexane

μ_2: mean of C16:0 when Method = Bligh & Dyer

Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics: C16:0

Method	N	Mean	StDev	SE Mean
3 Stage Hexane	3	0.337393	0.010935	0.006313
Bligh & Dyer	3	0.360207	0.015509	0.008954

Estimation for Difference

Difference	95% CI for Difference
-0.02281	(-0.05768, 0.01205)

Test

Null hypothesis	H_0: $\mu_1 - \mu_2 = 0$
Alternative hypothesis	H_1: $\mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
-2.08	3	0.1287

2-Sample t: C18:0 by Method

Method

μ_1: mean of C18:0 when Method = 3 Stage Hexane

μ_2: mean of C18:0 when Method = Bligh & Dyer

Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics: C18:0

Method	N	Mean	StDev	SE Mean
3 Stage Hexane	3	0.095030	0.002399	0.001385
Bligh & Dyer	3	0.106013	0.006017	0.003474

Estimation for Difference

Difference	95% CI for Difference
-0.010983	(-0.027075, 0.005108)

Test

Null hypothesis	H_0: $\mu_1 - \mu_2 = 0$
Alternative hypothesis	H_1: $\mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
-2.94	2	0.0990

2-Sample t: C18:1 by Method

Method

μ_1: mean of C18:1 when Method = 3 Stage Hexane

μ_2: mean of C18:1 when Method = Bligh & Dyer

Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics: C18:1

Method	N	Mean	StDev	SE Mean
3 Stage Hexane	3	0.496887	0.011187	0.006459
Bligh & Dyer	3	0.45695	0.02125	0.01227

Estimation for Difference

Difference	95% CI for Difference
0.03994	(-0.00418, 0.08406)

Test

Null hypothesis	H_0: $\mu_1 - \mu_2 = 0$
Alternative hypothesis	H_1: $\mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
2.88	3	0.0635

2-Sample t: C16 by Method

Method

μ_1: mean of C16 when Method = 3 Stage Hexane

μ_2: mean of C16 when Method = Bligh & Dyer

Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics: C16

Method	N	Mean	StDev	SE Mean
3 Stage Hexane	3	0.351000	0.009987	0.005766
Bligh & Dyer	3	0.371500	0.014981	0.008649

Estimation for Difference

Difference	95% CI for Difference
-0.02050	(-0.05358, 0.01258)

Test

Null hypothesis	H_0: $\mu_1 - \mu_2 = 0$
Alternative hypothesis	H_1: $\mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
-1.97	3	0.1432

2-Sample t: C18 by Method

Method

μ_1: mean of C18 when Method = 3 Stage Hexane

μ_2: mean of C18 when Method = Bligh & Dyer

Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics: C18

Method	N	Mean	StDev	SE Mean
3 Stage Hexane	3	0.607200	0.009974	0.005759
Bligh & Dyer	3	0.584867	0.016836	0.009720

Estimation for Difference

Difference	95% CI for Difference
0.02233	(-0.01362, 0.05829)

Test

Null hypothesis	H_0: $\mu_1 - \mu_2 = 0$
Alternative hypothesis	H_1: $\mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
1.98	3	0.1425

2-Sample t: saturated fatty acids by Method

Method

μ_1: mean of saturated fatty acids when Method = 3 Stage Hexane

μ_2: mean of saturated fatty acids when Method = Bligh & Dyer

Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics: saturated fatty acids

Method	N	Mean	StDev	SE Mean
3 Stage Hexane	3	0.432433	0.013282	0.007669
Bligh & Dyer	3	0.46623	0.02115	0.01221

Estimation for Difference

Difference	95% CI for Difference
-0.03380	(-0.07969, 0.01209)

Test

Null hypothesis	H_0: $\mu_1 - \mu_2 = 0$
Alternative hypothesis	H_1: $\mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
-2.34	3	0.1009

2-Sample t: unsaturated fatty acids by Method

Method

μ_1: mean of unsaturated fatty acids when Method = 3 Stage Hexane

μ_2: mean of unsaturated fatty acids when Method = Bligh & Dyer

Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics: unsaturated fatty acids

Method	N	Mean	StDev	SE Mean
3 Stage Hexane	3	0.525800	0.013251	0.007650
Bligh & Dyer	3	0.49013	0.02306	0.01331

Estimation for Difference

Difference	95% CI for Difference
0.03567	(-0.01320, 0.08453)

Test

Null hypothesis	H_0: $\mu_1 - \mu_2 = 0$
Alternative hypothesis	H_1: $\mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
2.32	3	0.1028

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