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Pilot-scale continuous-flow hydrothermal liquefaction of filamentous fungi cultivated in thin stillage

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ABSTRACT

With an ever increasing demand for energy and a better awareness of its environmental consequences, renewable fuels have become a desirable solution. The growing industry of second generation biofuels, such as renewable diesel, can further supplement energy needs and decrease reliance on fossil fuels. Lipid-rich biomass is a prime candidate for new drop-in biofuel feedstocks. While it has received only minor attention from researchers, fungi has the potential to become an effective source of biofuel. Due to high moisture content, fungal biomass is well-suited for the process of hydrothermal liquefaction. This thermochemical process uses water under near-supercritical conditions to convert biomass into biocrude oil. The use of water eliminates the need for energy-intensive drying processes needed in pyrolysis or gasification. A 1.5-L pilot-scale continuous-flow hydrothermal liquefaction process was optimized for the conversion of filamentous fungi *Rhizopus oligosporus* to biocrude.

To increase efficiency of a pilot-scale fungi-to-fuel process, improvements to fungal cultivation methods were studied. Large variation in growth yields have been noted for fungi cultivated in thin stillage, and were presumed to be the result of bacterial contamination. However, it was unknown if variations in growth were due to the quality of the thin stillage, contamination during collection, or contamination during lab procedures. Therefore, a lab-scale study was conducted to determine the source of diminished fungal growth yields, and possible methods to overcome these challenges were studied. Specifically, hydrogen peroxide was employed as a disinfectant for thin stillage, and its effect on fungal yields were observed. Results from lab-scale tests helped develop methods used during pilot-scale cultivation of fungi in a 1600-L bioreactor.

CHAPTER 1

INTRODUCTION

Since 2007, the corn ethanol industry in the U.S. has seen rapid growth, resulting in the production of 15 billion gallons of ethanol from over 200 plants across the country (Nebraska Energy Office, 2016). This rate of production will be sustained through the year 2022 as mandated by the Renewable Fuel Standard – Energy Independence and Security Act of 2007 (USDOE, 2014). The growth of the corn ethanol industry has reduced reliance on foreign fossil fuels while providing employment for nearly 86,000 people and contributing $44 billion to the GDP last year alone (RFA, 2016a). However, corn ethanol has been the target of much criticism, and the sustainability of the process has been questioned (Dias de Oliveira et al., 2005; Bhat, 2008). Improvements to the energy efficiency of the process can enhance sustainability and further limit environmental impact of corn ethanol.

A potential method of reducing energy consumption at corn ethanol plants is by eliminating an energy intensive flash evaporation process, in which the co-product thin stillage is converted to a syrup. This syrup is used as an additive for animal feed, however the value of the product is low and demand is limited (Mitra et al., 2012; RFA, 2016b). Instead of consuming large amounts of energy to produce a product with negligible value, thin stillage can be used as a medium for cultivation of fungi. Previous studies have demonstrated the advantages of fungal cultivation on thin stillage. Research performed by Rasmussen et al. observed *Rhizopus oligosporus* could provide wastewater treatment for the thin stillage by removing 80% COD, 98% suspended solids, and 100% glycerol and organic acids (2014). This treatment would allow for water reuse in other processes, resulting in a potential water demand reduction of 75%

(Jessen, 2010). Subsequently, the harvested fungi can be used as an animal feed. Mitra et al. determined that fungi cultivated in thin stillage contain protein and essential amino acids levels suitable for use as a feed for non-ruminant animals (2012).

In addition to use as an animal feed, fungi can also be used as a feedstock for biofuels. It has been documented that the lipid content of the fungal biomass increased when cultivated in thin stillage, (Mitra et al., 2012), resulting in an attractive feedstock for biofuel production. The focus of this thesis is the pilot-scale cultivation and conversion of fungal biomass to biofuel. The process selected for biofuel processing was hydrothermal liquefaction (HTL). HTL is a thermochemical process which mimics Earth's natural conversion of biomass to fossil fuels. In HTL, biomass is heated under pressure in the presence of water to produce a liquid fuel known as biocrude. The biocrude can then be upgraded to renewable diesel, and subsequently be used as a drop-in biofuel. Production of biocrude with the use of a corn ethanol byproduct and fungi can potentially improve the sustainability of the corn ethanol industry. Energy once used to generate a low value syrup, can instead be used to create a second-generation biofuel.

Research for this thesis is divided into two parts. The Chapter 3 focuses on methods used to efficiently cultivate *R. oligosporus* in thin stillage. Lab-scale testing was used to investigate the cause of high variability of fungal biomass yields previously seen (Erickson, 2010; McMahon, 2015). Collection, storage, and disinfection methods were studied to find a means of cultivating consistent growth yields. Methods determined to be produce consistent maximal growth were implemented on pilot-scale. A 1600-L bioreactor was operated to determine the potential for large-scale fungal biomass growth to be used as a biofuel feedstock. Biomass harvested from the pilot-scale bioreactor was used in HTL processing.

Chapter 4 of this thesis focuses on optimization of a pilot-scale continuous-flow HTL system. Reaction temperatures and residence times were investigated to determine the optimal conditions for biocrude production. Analyses were conducted to determine the yield of biocrude, as well as elemental and biochemical composition. Since little research has been performed using fungi as a HTL feedstock, results were compared to other continuous-flow systems using microalgal biomass. In addition to providing a higher value co-product from corn ethanol plants, this research was also performed to establish fungi as a potential biofuel feedstock.

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CHAPTER 2

HYDROTHERMAL LIQUEFACTION: A LITERATURE REVIEW

Abstract

This literature review provided an investigation of the thermochemical process of hydrothermal liquefaction (HTL) to produce biofuel. An overview of the process and its background, different types of feedstocks used, and possible future implementations of HTL was discussed. Through discussions of these topics, advantages and limitations of this biofuel production process as compared to other biofuel processes will be highlighted. The majority of previously published material focuses on lab scale batch reactors, as well as using microalgae as a feedstock. While these developments will be discussed, investigation of other feedstock potential and observation of current trends in the upgrades and improvements of HTL technology will be addressed.

2.1 Introduction

As the world population ever increases, the unique challenge of providing resources to meet the exponentially growing demand arises. No concern over resource consumption has gained more interest and controversy than the topic of fossil fuels. Since Marion King Hubbert's peak oil theory in the 1950's, the concept of the finite quantity of fossil fuels has become a part of the public's consciousness. The theory predicts that peak oil production follows a bell-shaped trend whose maximum occurs when half of the non-renewable resources have been consumed, with many scientists believing that this point will be reached in the first half of the 21st century, if not already (Bardi, 2009). Besides the fact that fossil fuels will eventually run out, a reliance

on petroleum raises environmental concerns such as climate change, and can be an issue of national security (Guo et al., 2015).

For these reasons, and more, a growing interest in biorenewable energy has occurred over the past few decades. In the United States, this was especially prompted by the Oil Crisis of the 1970's (Lifset, 2014). One process that has gained interest since this event is hydrothermal liquefaction (HTL). This thermochemical process can convert biomass into a liquid fuel, which can be further upgraded to a renewable drop-in fuel. Elevated pressure and temperature subject the biomass to near-supercritical conditions, resulting in the creation of biocrude. HTL is unique to other thermochemical processes in that the conversion process is carried out in an aqueous solution. This allows for a wide array of wet biomass to be employed, ranging from waste feedstocks such as manure (Yin et al., 2010) to dedicated feedstocks like microalgae (Biller and Ross, 2011).

While this technology has been studied since the 1970's, it has not been until recently that this area has begun to expand (Zhang, 2010). Currently, there has been an evolution from lab-scale batch reactors to demonstrations on continuous-flow pilot-scale reactors (Valdez et al., 2012; Jazrawi et al., 2013). This transition is an indication that HTL technologies are still in developing stages, and there is much room to grow. Maturation of this technology may lead to exploration of different feedstocks, process optimization and enhancements, reduction of carbon and waste footprints, and implementation of larger scale equipment. Once these have been achieved, biocrude obtained from HTL can contend as a desirable second generation biofuel with the potential to reduce greenhouse gas emissions and provide energy security. This review will investigate the process of HTL, and identify possible improvements it will need in order to be adopted on a larger scale for economic and environmental benefits.

2.2 Background on HTL

As previously mentioned, HTL came out of the 1970's boom of renewable energy research. While technically the process of creating liquid fuel from biomass in hot water was first discovered in the 1920's, it was not until fifty years later with the Pittsburgh Energy Research Center (PERC) that there was a renewed interest in HTL (Zhu et al., 2014). At this time PERC was experimenting with using Douglas fir as a biomass feedstock, with their research culminating in a 100 kg/h capacity pilot-plant (Appell et al., 1971). Concurrently, Lawrence Berkeley Laboratory (LBL) began similar work with Douglas fir on a pilot-plant of a comparable scale while introducing a pretreatment process of the woody biomass (Zhu et al., 2014). Research at both of these facilities continued through the 1970's and 1980's. Recently, HTL research has found a renewed interest sparked by experiments conducted by the Pacific Northwest National Laboratory (PNNL, 2016) and by the Savage Research Group at the University of Michigan (Savage, 2012), where each group has primarily focused on the prospect of microalgae as a feedstock.

2.2.1 How HTL Works

HTL can simply be described as pyrolysis in water. It is a thermochemical process in which biomass, in the presence of water, undergoes elevated temperatures and pressures (creating a near-supercritical environment). These conditions break the long-chain organic compounds of the biomass, forming short-chain hydrocarbons which result in the production of a liquid biofuel referred to as biocrude. HTL follows the same general concept of Earth's natural formation of fossil fuels. Plants and other biomass trapped under the Earth's surface for millions of years lead to the creation of coal or petroleum. With HTL this process is mimicked, but on a much shorter timeline.

Within this process, there are two unique approaches: direct conversion and pretreatment before conversion (Zhang, 2010). The latter method is used for lignocellulosic feedstocks in order to break the polysaccharides into monosaccharides, so that they are more easily converted to biocrude. Pretreatment typically done using acid hydrolysis, but can lead to the disadvantage of having to dispose of the acid waste stream (Briones et al., 2011). Direct conversion is simply subjecting the biomass to heat and pressure without extensive pretreatment, other than size reduction to improve pumping capabilities. Once the biomass has undergone pretreatment (if this method is selected), the conversion process is then carried out. A water environment inside of a reactor reaches temperatures of 250 - 400°C and pressures of 4-22 MPa (Elliott et al., 2015; Yoo et al., 2015; and Brown et al., 2010). At these conditions, water is at near-supercritical conditions. The elevated pressure keeps the water in liquid form, despite reaching temperatures that would generate steam at atmospheric conditions. Aside from keeping water in the liquid phase, the effect of pressure on biocrude quantity or quality is low to negligible (Akhtar and Amin, 2010).

When these conditions are reached, water takes on physical properties unseen at standard conditions. Most notably, water begins to act like an organic solvent during HTL. At 300°C water has solvent-like properties similar to that of acetone at 25°C (Zhang, 2010). Biomass can be readily suspended into the water, without the need for other chemical solvents which could be costly or require disposal. The near-supercritical conditions alter the electronegative of the water molecule, actually making the water molecule less polar. When water loses its polarity it is better able to react with the organic hydrocarbons, and thus assist in the formation of biocrude. Additionally, higher temperatures aid in the dissociation of the water molecule resulting in hydrogen and hydroxide ions. Figure 2.1 demonstrates the effect temperature has on the

dissociation constant. At 25°C the constant is 10^{-14}, and is almost 500 times lower when compared to constants in the temperature range where HTL is conducted (IAPWS, 2004).

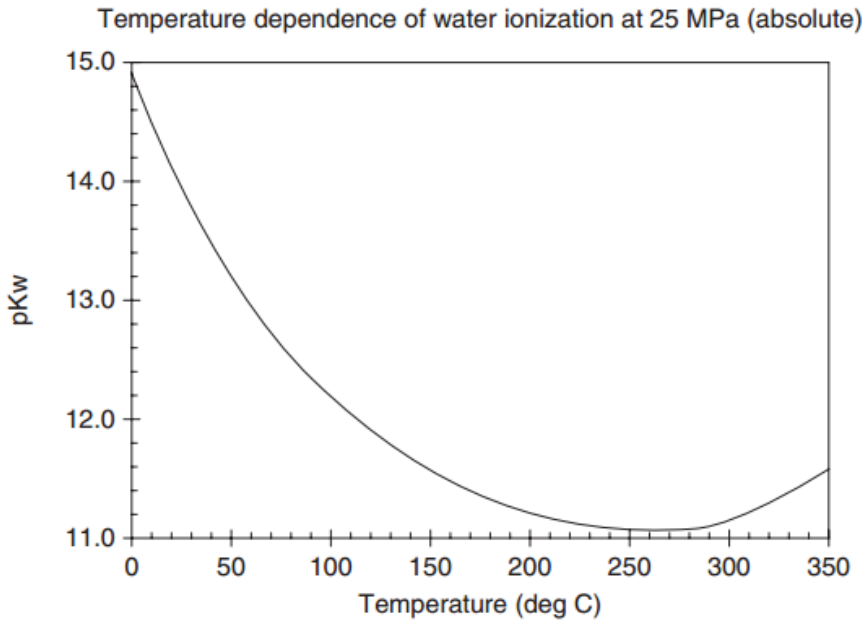

Figure 2.1. Effect of temperature on water dissociation constant (IAPWS, 2004).

The dissociation allows water to act as both an acid as well as a base. This duality is advantageous in the formation of biocrude. First, water as a base begins to break the organic bonds of the biomass. Then, water acting as an acid accelerates the reactions. The physical and chemical nature of water when heated can provide condensation, cleavage, and hydrolysis reactions, making water a key factor in the viability of HTL as a pathway to biofuel (Siskin and Katritzky, 1991; Zhang, 2010).

2.2.2 Operating Conditions

Temperature is regarded as being the most important factor in HTL (Akhtar and Amin, 2011). The temperatures mentioned above are of particular importance because they dictate the

yield of biocrude obtained as compared to other products formed. At temperatures below 250°C, a process known as hydrothermal carbonization occurs. Reactions at these temperatures favor the formation of biochar, composed mostly of proteins and carbohydrates. Because the char is composed of proteins and carbohydrates, it is possible to extract lipids prior to hydrothermal carbonization if it is desired to obtain char as well as lipids for possible biodiesel production (Heilmann et al., 2011). At temperatures above 400°C (entering into the range of supercritical conditions for water), the process of hydrothermal gasification occurs. The production of synthetic fuel gas is favored at these conditions (Elliot et al., 2015). HTL happens in the temperature range of 250 – 400°C. This process is defined by the production of a liquid fuel. While biocrude is produced at the extremes of this temperature range (250°C and 400°C), it is often at considerably lower yields (Valdez et al., 2012). The low yields are due to the fact that some hydrothermal carbonization and hydrothermal gasification is happening simultaneously with HTL at these overlapping temperature ranges. Thus, some of the biomass is converted to synthetic gas or char instead of predominately to biocrude. To ensure sufficient biomass conversion is occurring, approximate temperatures need to be above 280°C and lower than 374°C (the supercritical temperature of water) (Akhtar and Amin, 2011). Therefore, the optimum temperatures generally regarded for biocrude production fall between 300 – 350°C (Garcia et al., 2012; Yoo et al., 2015; Tian et al., 2014; and Brown et al., 2010).

Retention time varies greatly depending on scale, process system set-up, feedstock used, and methodology. For lab-scale batch reactors, recorded results for time held in the reactor range from anywhere between 0 – 120 minutes (Chow et al., 2013). These times are reported for the use of microalgae as a feedstock, however studies using other varieties of biomass can be assumed to fall in this range as well. While the research of continuous-flow pilot-plant systems is

still relatively limited, retention times have ranged from 3 – 40 minutes (Jazarawi et al., 2013; and Elliott et al., 2013). Overall, it is believed that retention time has a moderate to low impact on biocrude production, and only becomes a significant consideration if low temperatures are used (Akhtar and Amin, 2011). If lower temperatures are used, retention times should be longer to aid in a more complete conversion of the biomass.

Likewise, concentration of biomass in the feedstock slurry depends largely on the aforementioned methodological parameters. This is especially true for continuous-flow pilot-plant studies where the ability to pump the biomass slurry becomes a key factor. During scale-up of the HTL process to commercial-scale it is not anticipated that solids concentrations can reach much higher than 30% dry weight (Wender et al., 1975). To date, pilot-scale tests have remained in the range of 1 – 35% (Jazarawi et al., 2013; and Elliott et al., 2013). Increased solids loading would result in an increased overall efficiency in the system. However, biomass loading does not appear to have a significant impact on the quality of biocrude produced from biomass. This has been demonstrated on both lab-scale batch tests (Jena et al., 2011) and continuous-flow pilot-scale (Jazarawi et al., 2013).

2.2.3 Benefits of HTL

What sets HTL apart from other bioenergy production methods is its use of water as a reaction medium. Other thermochemical processes such as pyrolysis or gasification require that the feedstock be completely dried before undergoing conversion. Drying feedstock requires large quantities of heat. This becomes an energy intensive procedure and accounts for high energy costs (Onarheim et al., 2015). Since HTL uses water to its advantage, there is no need for extensive dewatering or drying. Biomass can be left as is and can simply be blended into a biomass-water slurry. An added benefit of using water is that other types of solvents are not

required. To extract lipids for biodiesel production from feedstocks like microalgae, an acid solution is needed as a catalyst, followed by a solvent such as methanol or hexane to obtain the lipid for fuel (Sathish and Sims, 2012). In addition to material costs, disposal or recovery can become expensive as well. On a commercial scale, costs for these chemicals become a significant factor. Whereas water, for use in the HTL process, is an abundant resource and does not carry the same cost as more commonly used solvents.

Allowing for the presence of water in biomass also opens up the potential for a larger variety of feedstock to be considered. Biomass that is cultivated in a liquid environment has greater appeal when energy costs due to drying are not an issue. Microalgae, for instance, has been receiving great amounts of interests due to its high oil content compared to that of terrestrial oilseed crops, such as soy (Sheehan, 1998). However, microalgae is cultivated in water and contains high moisture concentrations, making drying and dewatering difficult. HTL becomes more economical and sustainable for wet biomass when compared to pyrolysis or gasification (Zhu et al., 2013).

Additionally, many of the feedstocks that are compatible with HTL also exclude it from the food versus fuel debate that plagues biodiesel and ethanol production. First generation biofuels that use traditional food crops like soybeans, corn, or palm oil have come under scrutiny for straining food resources. Some claim that by dedicating more of these crops to energy instead of food, the price of food will begin to rise. Also, less food will be exported to other countries. This is thought to encourage countries to deforest land for agricultural purposes to adjust for lower food imports (Laursen, 2007). With regards to HTL, food versus fuel does not apply to many of the biomass sources research has been focused on. Feedstocks that have been used in HTL studies such as microalgae (Biller and Ross, 2011), manure (Vardon et al., 2011), corn

stover (Wang et al., 2011), sewage sludge (Fonts et al., 2012), forest residue and fungi (Jena et al., 2015) are not traditionally used as a primary food source. Therefore, the potential conflict between food and fuel with regards to HTL is not applicable.

2.3 Thermochemical Reaction

An understanding of the general concept of HTL and what sets it apart from other biofuel pathways, provides a base understanding for the thermochemical reactions taking place. This section will focus on what is being converted during this process. Biochemical and elemental analysis can demonstrate the reactions taking place and help with selection of the optimum operating conditions.

2.3.1 Conversion of Lipids, Carbohydrates, and Proteins

During biodiesel production, transesterification requires the extraction of lipids from biomass. To obtain the most efficient process, therefore, it is imperative to use feedstocks with high lipid content. This is evidenced by the growing interest in microalgae, a lipid-rich biomass. However, lipids alone are not processed in HTL. Instead, proteins and carbohydrates are reacted simultaneously with lipids. In order to determine the effect that a biomass' biochemical composition has on biocrude yields, Biller and Ross (2011) conducted a study observing the yields of microalgae compared with feedstocks high in specific macromolecules. Four strains of microalgae were selected with ranging levels of lipid (5 – 32%), carbohydrate (9 – 40%), and protein (43 – 65%). *Nannochloropsis oculata* contained the highest levels of lipid, *Spirulina* had the highest level of protein, and *Porphyridium cruentum* contained the highest level of carbohydrate. To highlight the impact of each biochemical make-up, seven other representative samples were analyzed. Notably, albumin and soya protein were used to investigate protein,

glucose and starch represented carbohydrates, and sunflower oil demonstrated the lipid impact. Results from this study are summarized in Figure 2.2.

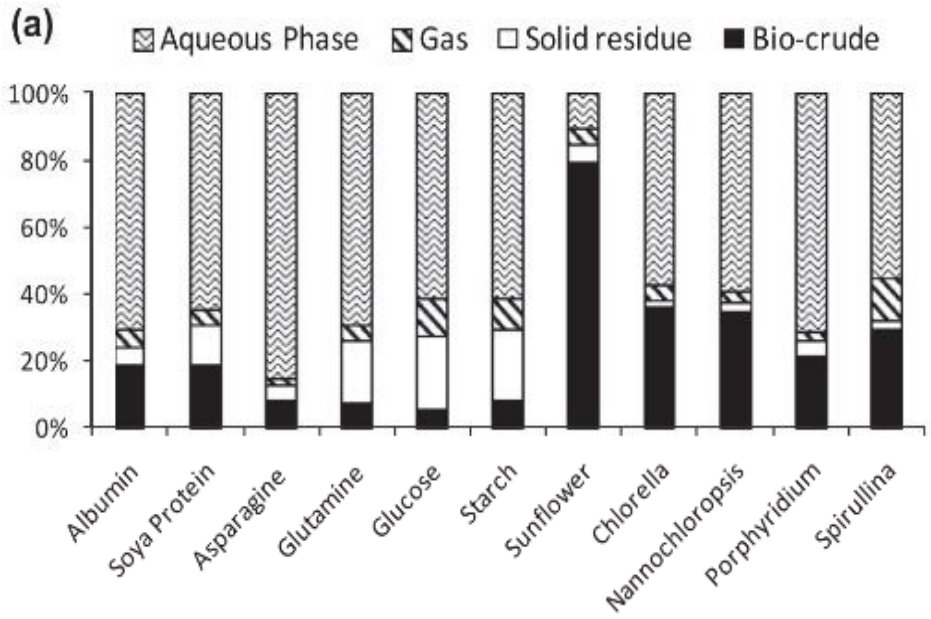

Figure 2.2. Dry ash-free (daf) yields of products from HTL (Biller and Ross, 2011)

Biocrude was obtained in the highest levels when using sunflower oil. Approximately 80% of the mass of collected product was composed of biocrude, with the remainder going mostly into aqueous phase. While it can be assumed that the lipid composition of the sunflower oil will differ than that of the microalgae feedstocks, Figure 2.2 demonstrates that it offers a good approximation of what may happen in biomass containing high lipid levels. *Chlorella vulgaris* and *Nannochloropsis oculata* contained the top two highest amounts of lipids, and likewise produced the most amount of biocrude for the microalgae samples. Conversely, carbohydrates appeared to result in the lowest amount of biocrude created. Biocrude from glucose and starch models accounted for less than 10% of the products collected. Again, this is paralleled in the

microalgae feedstock. As the carbohydrate representative, *Porphyridium cruentum* saw the lowest biocrude yields. During HTL, carbohydrates are not broken down into hydrocarbons, but rather polar water-soluble products like formic acid, acetic acid, lactic acid, and acrylic acid (Srokol et al., 2004; Biller and Ross, 2011). Therefore feedstocks containing higher levels of lipid are more favorable in HTL than biomass primarily composed of starch or glucose. A study conducted by Yoo et al. found that the energy return on investment (EROI) and biocrude quality were directly related to the lipid content of the feedstock, more so than carbohydrate or protein content (2015). However, it should be noted that although biocrude from carbohydrates (and to a lesser extent protein) is minimal, biocrude is nonetheless being produced. Production from protein, lipid, or carbohydrate gives HTL an advantage over other biofuel pathways that simply utilize lipids.

2.3.2 Elemental Analysis of Feedstock and Biocrude

Elemental analysis of biocrude can provide a look into the efficiency of the HTL process. Specific attention should be paid to the oxygen and nitrogen content of both the feedstock and biocrude product. Oxygen is directly related to quality of the biocrude. More oxygen in the product lowers the energy efficiency of the fuel. While some oxygen is needed to aid in combustion and reduction of certain byproducts, generally lower oxygen level are desired for biofuels in order to be comparable to petroleum. Oxygen content of the feedstock depends on the biomass used, but typically ranges between 25-45% for some of the commonly used HTL feedstock (Elliott et al., 2015). When a continuous-flow system is employed, significant reduction of oxygen from biomass to biocrude has been observed. A study conducted by Jazrawi et al. (2013) observed oxygen levels of 12 – 21% in the biocrude, compared to the microalgae biomass with oxygen levels near 30%. Reduction in oxygen has been found to occur in the

greatest amount when more severe reaction conditions are used (Jazrawi et al., 2013). Higher temperatures and longer retention times produce biocrude with less oxygen. Additionally, this range of oxygen is significantly lower than what is seen in pyrolysis bio-oil, making it a more attractive product with regards to upgrading (Zhu et al., 2014).

Nitrogen is also an important element to monitor through the HTL process. A low concentration of nitrogen is typically needed for a sustainable system. Nitrogen present in biocrude can directly form NO_x compounds, having serious environmental implications (Biller and Ross, 2011). Nitrogen is seen at highest levels when reaction conditions are more severe. This is due to an increase in biocrude formation from the protein fraction at higher temperatures (Jazrawi et al., 2013). A unique problem is presented if both low oxygen and low nitrogen are desired, since the two are inversely related to each other with respect to operating temperatures. An optimum reaction condition needs to be implemented to have a balance acceptable nitrogen and oxygen content.

2.3.3 HHV

As discussed, lower oxygen levels yields are seen at more severe conditions. Subsequently, heating values are generally higher when more severe conditions are used (Elliott et al., 2015; Jazrawi et al., 2013). Typical HHVs for a lipid-rich feedstock like microalgae range from 21.8 – 39.6 MJ/kg, with the low end of the range occurring at 200°C and the upper end occurring at 350°C (Yoo et al., 2015). An average heating value for HTL can be estimated to be about 35 MJ/kg (Zhu et al., 2014). When compared to other fuels, HTL biocrude outperforms pyrolysis bio-oil (which falls in the range of 16 – 19 MJ/kg), and is slightly less than conventional petroleum fuels (40-45 MJ/kg) (Demirbas, 2011; Zhu et al, 2014). In a transition to

biofuels, a replacement fuel should be selected that most resembles petroleum products. This will reduce the amount of alteration needed to existing infrastructure.

2.4 Feedstocks

A variety of biomass feedstocks can be considered good candidates for HTL due to the compatibility with wet biomass. This following section will observe some of the feedstocks drawing the most attention from researchers, and will explore why they are favorable. A summary of the characteristics of commonly studied HTL feedstocks are presented below in Table 2.1.

Table 2.1. Common HTL feedstocks' characteristics (Elliott et al., 2015)

Summary of HTL feedstock and continuous-flow reactor results.

Feedstock (dry basis)	Lignocellulosics	Macroalgae	Microalgae	Manures
Ash	3–8	15–35	7–26	10–20
H/C	1.2	1.2	1.6	1.5
O%	35–45	25–40	25–30	35–45
N%	0.5–3	3–7	5–9	3–6
HHV, MJ/kg	12–20	10–20	25–30	10–20
Size	1–100,000 mm	1–10,000 mm	1–100 μm	1–10,000 μm
Feed formatting required	Yes	Not all strains	No	No
Reference	Umeki et al. (2010), Wang et al. (2011)	Ross et al. (2008)	Biller and Ross (2011)	Vardon et al. (2011), Wang et al. (2011)
Biocrude			Continuous HTL results	
Yield, % daf	35	27	38–64	–
Energy Recovery %	64	52	60–78	–
N%	0.3	3–4	4–8	–
O%	12	6–8	5–18	–
Reference	Tews et al. (2014), NABC (2014)	Elliott et al. (2013a)	Jazrawi et al. (2013), Elliott et al. (2013b)	NA

2.4.1 Lignocellulosic

HTL work first began using woody biomass, rich in lignocellulose, for many early studies. Since the primary work of PERC using Douglas fir as a feedstock, other varieties of lignocellulosic material have been evaluated for potential with HTL (Appell et al., 1971). Woody biomass such as beech, Ailanthus, spruce, Jack pine, and Chinese fir have reportedly been used

(Demirbas, 2000; Qu et al., 2003; and Xu et al., 2008). Biomass is typically ground into a sawdust like material, then mixed with water. Lignocellulosic biomass for HTL is appealing for biofuels because it can be derived from waste products. Forest debris, building materials, sawdust, among other wood waste streams are abundant in some areas, and can be used as a source of fuel (Qu et al., 2003). Compared to other HTL feedstocks, however, woody biomass does not perform as well.

Because a large portion of woody biomass is composed of lignin, biocrude produced from this feedstock is of a poorer quality. Hardwood and lignin are not easily converted to hydrocarbons, and need temperatures higher than average to hydrolyze. Also, biomass high in lignin results in the formation of biochar (Akhtar and Amin, 2011). Levels of oxygen in the biomass are typically only reduced to a range of 19 – 32 % in the biocrude (Qu et al., 2003). However some report a 12% content is achievable (Wang et al., 2011). High oxygen results in a lower heating value of the biocrude, generally around 22 – 30 MJ/kg (Qu et al., 2003). Lignocellulosic feedstocks are also higher in carbohydrates than others, and therefore lower overall biocrude yields are seen. Total yields fall in the range of 25 – 35% daf (Demirbas, 2000; Qu et al., 2003; and Xu et al., 2008).

2.4.2 Manure

The use of manure from agricultural livestock has received attention as an HTL feedstock. What makes this an attractive biomass source is simply the amount of manure that is produced. Over 250 million tons on a dry basis of manure is produced each year in the United States (Xiu et al., 2010). A portion of this amount is land applied as a fertilizer, however there remains an abundance. In addition to volume produced, manure also has moderate lipid levels. A study using swine manure found that the feedstock was composed of 22% lipid, although a

large portion was composed of carbohydrate as well (37%) (Vardon et al., 2011). Oxygen levels in manure are also significant, with chicken and swine manure demonstrating a composition of 31 – 32% (Ekpo et al., 2016). Significant reduction in oxygen has been recorded, making up only 6.5 – 15% when HTL was conducted at 300 - 350°C (Ekpo et al., 2016; Vardon et al., 2011). A biochemical composition of moderate lipid, high carbohydrate, and low protein results in modest biocrude yields. Results for swine manure have been approximately 30% daf.

A unique benefit of HTL with respect to manure feedstocks is the potential for bioactive contaminant removal. Due to the pervasiveness of antibiotic and hormone use in the agricultural livestock industry these compounds often appear in manure. If manure is used in land application, there is a potential for the spread of bioactive contaminants (Cantrell et al., 2007). A study conducted by Pham et al. observed the fate of two kinds of contaminants present in swine manure after HTL (2013). Three antibiotics (florfenicol, ceftiofur and carbenicillin) and a hormone (estrone) were subjected to an HTL process consisting of either 250°C for 60 minutes or 300°C for 30 minutes. It was found that these HTL conditions contributed to the reduction of 98 – 99.5% of all the bioactive contaminants. These results indicate that HTL can provide contaminant removal as well as produce a renewable fuel from a waste stream.

2.4.3 Microalgae

Perhaps the feedstock that has received the most attention for HTL work is microalgae. This coincides with increased attention of microalgae for other renewable fuel processes, such as biodiesel and thermochemical processes like pyrolysis (Mata et al., 2010; Li et al., 2008). Algae are a desirable feedstock for a few reasons. First, their biochemical composition lends itself to high oil production. Lipid content in algae is considerably higher than other traditional oilseed crops, like soybeans. For comparison, if all the U.S. soybean fields were replaced with algae they

could replace 61% of petroleum diesel consumed annually (compared to soybean's 4.5%) (Pienkos and Darzins, 2009). Second, algae have a better photosynthetic efficiency than traditional crops and therefore has higher growth rates (Elliott et al., 2015; Brennan and Owede, 2009). In addition, they can be cultivated in non-arable landscapes (Liu et al., 2013), are not traditionally a source of food (Zhu et al., 2013), and can be cultivated in fresh, brackish, or wastewater (reducing the impact on freshwater resources) (Savage, 2012).These attributes make algae appealing for any biofuel pathways. However, what makes algae particularly suited for HTL is that no drying is needed, which is especially important for a feedstock that is cultivated in water (Yoo et al., 2015).

These advantages have resulted in extensive studies in HTL, in both batch and continuous-flow systems. There has been specific interest in the microalgae strains of *Chlorella, Spirulina,* and *Nannochloropsis* due to the aforementioned advantages (Elliott et al., 2015; Biller and Ross, 2011). Lipid content can vary drastically from species to species, and will have an impact on the overall biocrude yields. However, biocrude yields have been demonstrated to range from 38 – 64% daf on a pilot-scale continuous-flow system (Elliott et al., 2015; Elliott et al., 2013; Jazrawi et al., 2013). These yields exceed the other common HTL feedstocks used. HTL of microalgae has also shown to greatly reduce oxygen and nitrogen from the biomass to the biocrude, with approximately 8 – 10% oxygen and 3 – 5% nitrogen left remaining in extracted biocrude (Savage, 2012).

2.4.4 Fungi

Fungi have many of the advantages that algae have, but there have been very few HTL studies in this area. To the author's knowledge, there have been four studies using a fungus as a feedstock and have been limited to yeasts *Cryptococcus curvatus* or *Saccharomyces cerevisiae*,

all of which were conducted on a lab batch-scale (Jena et al., 2015; Miao et al., 2014; Valdez et al., 2013; Hammerschmidt et al., 2011). Similar to algae, fungi can be cultivated in an aqueous environment and can have high moisture contents, making them more adaptable to HTL as compared to pyrolysis or gasification. There is also no food versus fuel debate, and fungi have quick cultivation periods. Additionally, fungi have similar lipid content to microalgae. Oleaginous fungi species are typically composed of 25 – 50% lipid, with some species such as *Trichosporon fermentans* as high as 62.4% (Thevenieau and Nicaud, 2013).

In the few studies using yeast as a feedstock, biocrude yield and quality have been promising. Jena et al. found that *Cryptococcus curvatus* produced yields higher than most other biomass types, and were comparable to microalgae (2015). HTL at 300°C for a 30 minute retention time resulted in a yield of 49% daf with a heating value of 36.55 MJ/kg. Other studies using *Saccharomyces cerevisiae* have also seen high yields around 40% daf (Hammerschmidt et al., 2011; Valdez et al., 2013). Based on these findings, and similarities in advantages to microalgae, other species of fungi aside from yeast should be researched. Oleaginous multi-cellular fungi could be well suited for HTL.

2.5 Optimization and Potential Upgrades

HTL has been demonstrated to produce a biofuel of near-fossil fuel quality from an assortment of feedstocks in both lab-scale batch reactors and pilot-scale continuous-flow reactors. The evolution of research in this area has, of recently, been drawn to the optimization of the process. Improvements to the process in order to reduce energy consumption and cost have been studied via life-cycle analyses (LCA) and techno-economic analyses (TEA).

2.5.1 Optimization of Reaction Conditions

In order to be viable on commercial scale, HTL processes need to have an efficient use of energy. The main source of energy consumption is the result of reactor heating. More severe reaction conditions are generally considered to produce a higher quality biocrude, and have been shown to have higher overall yields due to increased hydrocarbon production from protein and carbohydrate fractions at higher temperatures (Jazrawi et al., 2013; Elliott et al., 2015; Valdez et al., 2012). However, operating at severe conditions to increase biocrude yields and HHV may not necessarily be efficient. Yoo et al. observed the relationship of EROI and reaction temperature for a high-lipid (*Nannochloropsis oceanica*) and a moderate-lipid (*Golenkinia* sp.) microalgae (2015). Results are presented in Figure 2.3 below.

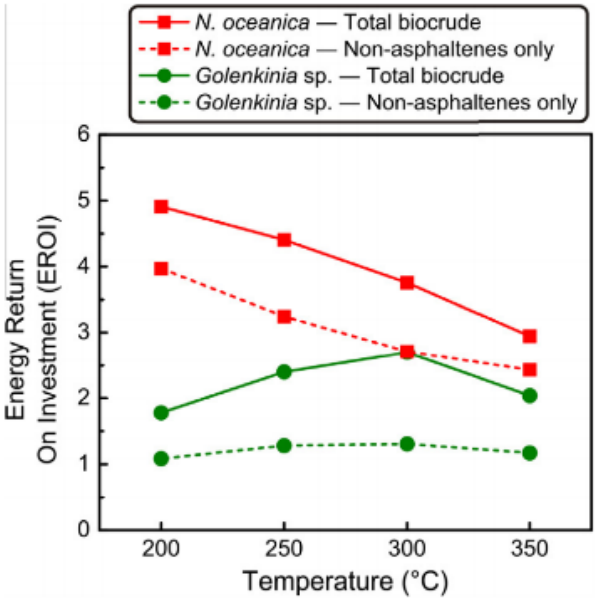

Figure 2.3. Relationship between EROI and operating temperature (Yoo et al., 2015)

Results were obtained from 42 mL batch reactors at a constant retention time of 60 minutes. EROI was determined by calculating the HHV of the biocrude sample divided by the

amount of energy needed to heat the microalgae and water. It was found that although higher yields and larger HHVs were seen at 350°C, this did not directly translate to a more efficient process. The increase in biocrude at high temperatures provided a lower return on energy than when operating at 200°C when using the high lipid feedstock lipid *N. oceanica*. Since lipid is converted to biocrude at lower temperatures, severe reaction conditions are not needed to obtain the majority of readily available biocrude. For high lipid feedstocks, there is no payoff for converting the smaller amounts of protein and carbohydrates at higher temperatures when compared to the energy required. This however, is not necessarily true for biomass with a lower lipid content. *Golenkinia* sp. saw the best EROI at 300°C. Biocrude yields benefitted from the conversion of proteins at higher temperatures, with regards to the energy consumed. This indicates that optimum reaction temperature will vary with feedstock used, and is dependent on the biochemical composition of the biomass.

Similarly, optimization of residence time can impact energy efficiency in HTL. Recent studies point to the use of shorter residence to generate better energy recovery. Faeth et al. demonstrated that 91% energy recovery was possible in a batch reactor with heating rates much higher than typically used (set point of 600°C) and a short residence time of 1 minute (2013). These conditions were able to produce a large biocrude yield of 66% daf. Similar success with shorter residence times have been found on a continuous-flow reactor. Significant yields of greater than 40% daf have been recorded using a 3 min residence time and temperature of 350°C at pilot-scale (Jazrawi et al., 2013). These studies provide evidence that acceptable biocrude yields can be obtained without extended residence times, possibly improving EROI and reducing capital costs.

2.5.2 HTL Process Design

In addition to optimizing reaction conditions, studies have begun looking at altering HTL systems to be more efficient environmentally and economically. A key factor in enhancing efficiency is the recovery of resources. Zhu et al. investigated improving the economics of HTL of woody biomass through process design (2014). It was found that co-products generated during HTL could be recovered and used elsewhere in the biofuel production process. The aqueous product left over after the separation of biocrude can be recycled back into the feedstock slurry preparation, instead of using clean water to mix with the biomass. Excess wastewater not used can undergo anaerobic digestion to produce methane and carbon dioxide. These gases can in turn be used for heat generation at the plant. Similarly, the offgas created from HTL can be captured, compressed, and used to generate hydrogen. This hydrogen can then be used in the hydrotreating process of the biocrude to form a renewable fuel (Zhu et al., 2014). Interest in recovery of solid co-products has also been demonstrated. Studies have shown that a large portion of phosphorus is found in solids collected from HTL (Elliott et al., 2013). P is an essential element for biomass growth, and can be recycled for cultivation purposes (Liu et al., 2013). Recovery of these co-products can help reduce the nutrient, water, and energy consumption, and will be necessary if HTL is implemented on a large-scale.

2.5.3 Techno-economic Analysis (TEA)

With the evolution of lab to pilot-scale, research has looked at the viability of further increasing the size of production to commercial-scale. Major costs of the process have been identified. Primarily, feedstock cultivation, HTL reaction, and hydrotreating the biocrude account for the highest costs (Jones et al., 2014; Zhu et al., 2013). Feedstock cultivation will vary with each biomass used, especially between waste stream sources and dedicated crops. HTL

reaction, however, needs consistent improvement for any methodology to reduce costs. In addition to optimizing time and temperature for energy consumption benefits as previously stated, better recovery of biocrude is needed. Losses of organics to the aqueous product reduces the biocrude yield and leads to higher wastewater treatment costs. Reduction in this loss could contribute to a 27% decrease of the minimum fuel selling price (MFSP) (Zhu et al., 2014). To a lesser extent, reducing the pressure used in the HTL reaction could also improve the MFSP by 4% (Zhu et al., 2014).

HTL has been compared to other fuel processes in terms of life-cycle and techno-economics at assumed large scale performance. Greenhouse gas emissions are currently lower for pilot-scale HTL than petroleum fuel and corn ethanol production, and will remain so if scaled-up (Liu et al., 2013). When EROI is considered, HTL is comparable to other biofuel pathways (Liu et al., 2013). However, when compared to the other biofuel technologies, HTL has higher capital costs. This is a direct result of using high pressure, and the equipment needed to facilitate these conditions (Zhu et al., 2014). Overall, TEA of HTL is promising. Using current technologies the cost of renewable fuel generated from HTL is considered to be around $4.45 per gallon of gasoline equivalent (GGE) (Jones et al., 2014; Zhu et al., 2014). With significant improvements to biocrude recovery, feedstock cultivation, and upgrades to the hydrotreating process, MFSP is hypothesized to be as low as $2.07 – $2.52/GGE (Zhu et al., 2013; Zhu et al., 2014).

2.6 Conclusion

HTL is a promising pathway to second generation biofuels. The aqueous environment used to carry out the reaction allows for a greater potential to use wet biomass feedstocks. Feedstocks analyzed thus far have produced renewable fuels of quality equivalent to more traditional

technologies. However, lack of extensive research at the pilot-scale and limited investigation of other oleaginous biomass highlights the need for increased analysis before HTL can be implemented on a large scale. Selection of appropriate feedstock and process design developments can lead to a renewable fuel that will reduce the reliance on fossil fuels and limit impact to the environment.

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CHAPTER 3

FUNGAL CULTIVATION IN THIN STILLAGE: LAB TO PILOT-SCALE DEVELOPMENT

Abstract

This study examined the variability in fungal biomass yields previously seen during the cultivation of *Rhizopus oligosporus* on thin stillage (TS). Fungi cultivated on this corn ethanol co-product can provide higher value products such as animal feed, nutrient supplements, or biofuel feedstock while potentially reducing energy demand in the process. However, bacterial contamination present in the TS was found to be inhibitory to fungal growth, resulting in variable yields. A 100 mL lab-scale study determined the major cause of diminished biomass yields was extended storage, and not collection methods. Fungal growth began to decline after 2 weeks of storage, resulting in yields consistently <15 g/L. Maximal yields (>30 g/L) were seen on TS less than 2 weeks old, and could be achieved without hydrogen peroxide as a disinfectant. The finding that recently collected TS was the biggest factor for high yields was applied to the 1600-L pilot-scale bioreactor. A yield of 9 g/L was obtained at pilot-scale, and provided a sufficient amount of biomass for the HTL processing in Chapter 4.

1. Introduction

As mandated by the Energy Independence and Security Act of 2007, the volume of biofuels produced in the U.S. has experienced extensive growth, with projections of 15 billion gallons of ethanol to be produced each year between 2015 - 2022 (USDOE, 2014). In 2015

alone, the corn ethanol industry directly employed 85,967 people, and contributed $44 billion to the GDP (RFA, 2016a). However, modifications to the corn ethanol processes are needed to improve energy return on investment and enhance sustainability of the industry. This can be accomplished by providing additional uses for the centrate collected from the suspended solids remaining after fermentation in dry-grind plants. The centrate, referred to as thin stillage (TS), is currently converted to a syrup to be used as an animal feed additive. The process to obtain this syrup is energy intensive and results in a low-value co-product (Mitra et al., 2012; Rasmussen et al., 2014).

Alternative uses of TS have consisted of cultivating fungi in the liquid medium. Non-aseptic growth of fungi in similar waste streams has been demonstrated to provide treatment for the waste as well as generate a biomass product (Jin et al., 1998). When applied to TS, previous studies have shown that fungi can remove 80% COD, 98% suspended solids, and 100% glycerol and organic acids (Rasmussen et al., 2014). This treatment allows for water reuse at corn ethanol plants, ultimately reducing the water demand by up to 75% (Jessen, 2010). In addition to providing water treatment, the harvested fungal biomass itself has been shown to be a high value product. The protein-rich fungi can serve as an animal feed or nutrient supplement for humans (Mitra et al., 2012). Also, lipid accumulation of species *Rhizopus oligosporus* cultivated in TS can potentially serve as an effective feedstock for biofuel processes. The use of fungi can reduce the energy costs needed to transform TS into a syrup while simultaneously aiding in waste treatment and generating a more desirable product.

While previous studies have demonstrated the advantages of cultivating *R. oligosporus* on TS, consistent fungal biomass yields have been difficult to obtain. High variability of growth has been documented at a range of scales (Erickson, 2012; McMahon, 2015). Presumably, the TS

has contained bacterial contamination that was outcompeting the fungal inoculum. However, it was not understood where this contamination was coming from; whether it was present in TS at the corn ethanol plant, or if laboratory use had exposed TS to ubiquitous bacteria. Therefore, this study was conducted to determine the source of contamination. One test studied the impact the TS collection method had on the capability to support growth. Another test demonstrated the benefit of limiting air exposure. The consequence of the age of TS, as well as the result of adding a disinfectant was also investigated in this test.

Hydrogen peroxide was used for this test based on research showing its usefulness in aquatic settings. In treating for cyanobacteria in lakes, it was found that the bacteria were impacted at doses 10 times lower than eukaryotic organisms (Drabkova et al., 2007). At an appropriate dose, cyanobacteria populations could be removed by 99% without major impact to eukaryotic phytoplankton, zooplankton, and macrofauna (Matthijs et al., 2012). Also, hydrogen peroxide byproducts are non-toxic, resulting in the formation of water and oxygen (Linley et al., 2012). This could allow for reuse of the TS without forming a hazardous waste. In applications similar to this current research, a waste stream from a wet-grind corn ethanol plant was disinfected with hydrogen peroxide to achieve a bacterial reduction of over 50% (Miao, 2002).

This research followed the progression from lab-scale results to pilot-scale. Lab-scale findings determining the source of contamination, the impact of the age of TS, and benefit of hydrogen peroxide helped dictate methods implemented on the 1600-L pilot-scale bioreactor. It was necessary to generate maximal and consistent biomass yields in order to make this process viable at commercial-scale. The purpose of this study was to determine the most efficient methods to achieve consistent biomass yields to be used as a feedstock for biofuel. The biomass cultivated at pilot-scale was used for hydrothermal liquefaction processes studied in Chapter 4.

2. Methods

2.1. Growth Medium

Fungal sp. *R. oligosporus* was cultivated in thin stillage (TS) obtained from Golden Grain Energy LLC (Mason City, IA), a dry-grind corn ethanol plant. For lab studies, two batches of TS obtained on different days were used. One batch was used for the contamination source tests, while the other was used for hydrogen peroxide tests Transfer hoses used for TS collection were soaked in 3% (v/v) sodium hypochlorite prior to use. All hose and sampling port connections were doused with ethanol during collection. TS was collected in an autoclaved 20-L polypropylene carboy for hydrogen peroxide disinfection tests. Similarly, sterile 500 mL polypropylene sampling bottles were used for contamination source tests. TS was stored in a 10 °C cooler when not in use. For the pilot-scale test, 1400 L of thin stillage was collected for bioreactor operations. During collection, this volume was split between two 1000-L chemical totes, disinfected with 3% (v/v) sodium hypochlorite. TS for the pilot-scale bioreactor was used within 24 proceeding collection. Freshly collected TS had an average pH of 5.0, and was not adjusted for these tests as this fell within tolerable conditions for fungal growth (Mitra et al., 2012). Typical thin stillage characteristics are listed in Table 1, although exact concentrations will vary from batch to batch.

Table 1. Typical Thin Stillage Characteristics (Rasmussen et al., 2014)

Typical Thin Stillage	**Concentration**
COD	90 g/L
Total Sugar	17 g/L
Reducing Sugar	6 g/L
Suspended Solids	20-30 g/L
Nitrogen	6 wt.%

2.2. Fungal inoculum preparation

The culture of fungus, *R. oligosporus* was obtained from the American Type Culture Collection (ATCC# 22959, Rockville, MD). Cultivation, collection and storage of spores were conducted according to methods used by Ozsoy et al. (2008), and the same procedures were used as outlined in Chapter 4, Section 2.1.1 of this thesis.

2.3. Cultivation

Prior to transferring the TS to the growth vessels, the carboy or bottles were thoroughly shaken to ensure homogeneity, due to solids settling to the bottom when the TS was stored for an extended period of time. For lab scale-tests, cultivation of fungal biomass was conducted on a 250 mL scale for each test. A total of 100 mL of TS was transferred to heat sterilized (121 °C, 15

min) 250 mL Erlenmeyer flasks. Sterilization wrap covered each flask, and was only removed during TS transfer and inoculation. The mycelium inoculum was added to each flask at a concentration of 5% (v/v). Flasks were immediately covered following this inoculation in order to limit contact with potential air particulates. Flasks were then placed in a shaker incubator at 200 rpm and 37 °C for 48 h (Mitra et al., 2012). After the cultivation period was completed, fungi were harvested via fine mesh colander. Each flask was individually poured through the colander to remove excess TS, and then transferred to a glass Petri dish to be dried. Growth yields were reported as dried fungal mass per L of TS, and were determined by drying the harvested wet fungi at 70 °C for 48 h and recording the dried mass. This drying temperature was determined to achieve adequate drying without volatilizing lipids or inducing a Maillard reaction. Each test was performed in triplicate, and all data points are reported as averages.

At pilot-scale, procedures were followed as outlined in Chapter 3, Section 2.1.2. The 1600-L bioreactor was cleaned and disinfected with a 3% (v/v) sodium hypochlorite solution, followed by a sodium hydroxide solution (pH ~10.5) 24 h prior to thin stillage transfer. Since the temperature of thin stillage can reach 80 °C at the time of collection, it was allowed to cool to 35°C over the course of 15 h before inoculation to prevent heat damage to the fungal inoculum. Following the cooling period, thin stillage was transferred to the bioreactor, and 8 L of fungal inoculum were added to the thin stillage (Erickson, 2012). Cultivation was allowed to proceed over the following 48 h (Mitra et al., 2012). During cultivation, temperature in the bioreactor was held at 37±3 °C (Mitra et al., 2012), and four ceramic diffusors provided air at a flow rate of 300 L/min. In order to control excessive foaming, 450 mL of a liquid anti-foaming agent (Sigma-Aldrich, Antifoam 204) was added to the bioreactor. Upon completion of fermentation, fungal biomass was harvested by filtering over a fine mesh screen to remove excess thin stillage.

2.3.1. Contamination source identification

To determine whether contamination was a result of TS collected at the corn ethanol plant or from laboratory conditions, 500 mL sampling bottles were filled during a typical TS collection process. Standard operating procedure dictated that the sampling line was flushed to remove any old TS that may be remaining before carboys were filled. However, to determine if contamination was a result of collection at the plant, one bottle was filled before the sampling line had been flushed. After this initial pre-flush sample was collected, approximately 2 L of TS were discarded from the sampling port. A second 500 mL bottle was then collected, followed by a 20 L carboy, then another sampling bottle, and so forth until six sampling bottles had been collected between carboy fillings. Bottles remained closed until they had cooled to room temperature. At this point, the bottles were opened in a UV light sanitized biosafety hood, where TS was transferred to sanitized 250 mL flasks. Cultivation procedures as described in Section 2.3 above were then employed.

2.3.2. Hydrogen peroxide and storage tests

TS for hydrogen peroxide and storage tests were collected in a sterilized 20 L carboy with a spigot. The spigot was used in order to avoid having to pour TS directly from the mouth of the carboy, which was thought to draw potentially contaminated air into the carboy. To limit this exposure of air, sterilization wrap was used to cover the mouth of the carboy while 100 mL of TS was dispensed into a sterilized flask from the spigot below. Hydrogen peroxide (Fisher Science, Lab Grade S25359) was next added to the flask at a one-time dose of 250 mg/L, determined to be optimal. Previous studies observing growth of fungi in TS had used 60 mg/L (Miao, 2005), however fungal growth was determined to benefit from higher doses. This

hydrogen peroxide dosed TS was compared with a control flask, which did not receive any disinfectant. TS used for both the control and dosed flasks came from the same carboy and were prepared at the same time. All tests were conducted in triplicate. Cultivation then took place as described above in Section 2.3. This test was conducted on a weekly basis for a total of 8 consecutive weeks following collection, and using TS from the same carboy. Average growth yield was reported for each week.

2.4. Analyses

2.4.1. Thioglycollate Analysis

A thioglycollate test was conducted as a way of quickly determining possible contamination, and identifying the general type of bacteria present (i.e. aerobic, anaerobic, facultative anaerobic, etc.). This analysis was performed for each week of the hydrogen peroxide and storage test to have a better understanding of the quality of TS used that week. The medium for the thioglycollate test was prepared according to the manufacturer's instructions (Hardy Diagnostics, 2016). A total of 29.75 g/L of thioglycollate broth with resazurin (Fluka Analytical, 90404-500G) was dissolved in distilled water. Approximately 10 mL of the prepared broth were transferred to 16-mL test tubes. Test tubes were then loosely capped, and autoclaved for at least 20 min. Subsequently, the test tubes were then capped tightly and allowed to cool to room temperature. The resazurin in the broth indicated oxidation by the presence of a pink color. Less than the top third of the test tube turned pink due to the interface of broth and air, while the remaining broth was yellow. Careful consideration was taken to avoid excessive agitation to the test tubes, as this could aerate the broth causing a pink color throughout.

Once the broth had cooled, the test tube was then inoculated with a sample of TS. The TS sample was collected in a sterilized 250-mL flask from the spigot of the carboy as described above, and was then immediately covered with sterilization wrap. A small cut was made in the top of the sterilization wrap, and a sterile pipette was inserted to extract approximately 1 mL of TS. Subsequently, the TS was transferred to the test tube, creating a thin vertical line of sample starting from the bottom of the test tube. Again, care was taken to not shake the test tube. Test tubes were then incubated at 38 °C for 48 h. All samples were conducted in triplicate. Observations were made 24 and 48 h following the start of incubation. Determination of contamination was based on visual observation of the test tubes. Bacterial presence was confirmed by visible growth of a culture and/or an absence of pink coloration at the top of the flask, indicating oxidation/reduction reactions had occurred with aerobic organisms. Location and concentration of the growth indicated whether the bacteria were anaerobic, aerobic, or facultative.

2.4.2. Microbiological Analysis of Fresh Thin Stillage

Exact Scientific Services, Inc. (Ferndale, WA) performed microbiology and organic chemistry analyses on a sample of TS from the batch collected for the hydrogen peroxide tests. Two separate sets of analyses were conducted: one for the TS when it had been recently collected (less than one week old), and a second for when the TS was demonstrating significantly decreased yields (approximately 4 months old). Analyses consisted of counts for anaerobic bacteria, aerobic bacteria, lactic acid bacteria, thermophilic spores, *Bacillus* spp., *lactobacillus* spp., as well as lactic and acetic acid concentrations.

2.5. Statistical analysis

A statistical analysis was performed to determine the significance of biomass yields and impact of age and hydrogen peroxide. Software JMP (SAS Institute, Cary, NC) was used to perform a Tukey's honest significant difference (HSD) test. P values greater than 0.05 were determined to show significance.

3. Results and discussion

3.1. Variability in fungal growth

In previous studies exploring fungal cultivation in TS, a variation in biomass yields was noted (Erickson, 2012; McMahon, 2015). The difference in biomass yields occurred despite the fact that the same TS was used for each test, reducing the uncertainty that the composition of the TS itself was the cause. Before testing to determine the source of variation began, a preliminary test to determine the extent of biomass yield variability was performed. This test was conducted according to methods outlined in Section 2.3. The results for this test are summarized in Fig. 1.

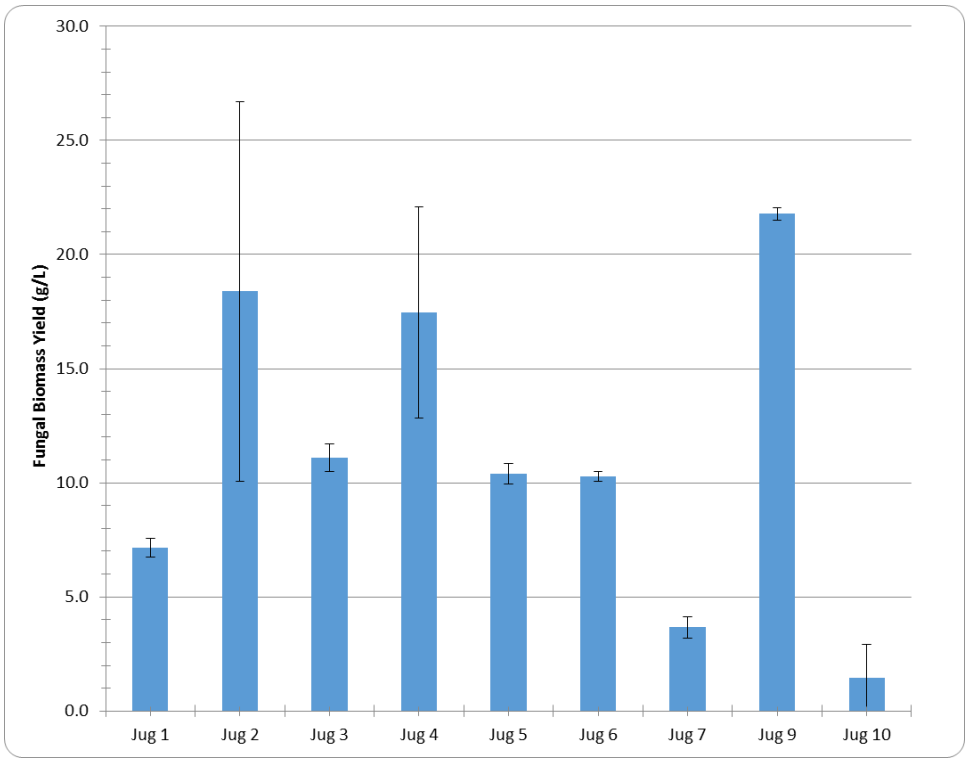

Fig. 1. Variability in fungal biomass yields using TS collected on the same day. Error bars represent one standard deviation.

Jugs 1 – 10 were filled with TS on the same day, and came from the same corn ethanol plant. Labels 1 – 10 were given arbitrarily, and did not reflect the order in which they were filled or subsequently used for testing. All jugs were kept in cold storage for 8 weeks before this test was conducted. Samples were taken periodically for other non-related tests, so it was unknown how many times each jug had been opened.

A wide range of biomass yields were recorded for this comparison study, with TS from some jugs supporting growth of over 20 g/L and others with less than 5 g/L. Since the TS was collected on the same day, each jug was presumed to have a similar composition. Thus, contamination was believed to be the cause of the high variability. Although previous studies

have reported that bacterial contamination was a concern with fungal cultivation in TS (Erickson, 2012; Mitra et al., 2012; McMahon; 2015), storage of TS in autoclaved jugs at 10 °C for multiple weeks was assumed to provide acceptable control of contamination. As seen in Fig. 1, however, this was not found to be true for all jugs of TS. Growth of fungi appeared to be inhibited by bacteria in some TS samples, but not in others.

Yet, the source of contamination was not known. Because the order in which the jugs had been filled was not recorded, one hypothesis was that the TS sampling line at the corn ethanol plant had not been properly flushed prior to collection. Samples collected earlier in the order were posited to be exposed to stagnant and potentially contaminated TS, while jugs towards the end of the order were unaffected due to sufficient flushing provided during filling. Another proposed explanation for the variation was the handling of the TS during lab tests. As noted, the number of times each jug had been opened was unknown. While methods to transfer TS in an aseptic manner were employed, exposure to air was believed to bring the growth medium in contact with bacteria. Jugs opened more frequently than others could have had greater exposure to bacteria, and thus supported less biomass growth. Both of these hypotheses were tested to determine the effect of sample line flushing and interaction with air. Since exposure to air is inevitable at pilot-scale, the use of a chemical additive was also investigated to see if this could aid in the suppression of bacterial population.

3.2. Contamination due to sample line flushing

Sample bottles were collected sequentially to determine the effect of filling order on the ability of TS to support fungal biomass growth. The results are presented in Fig. 2.

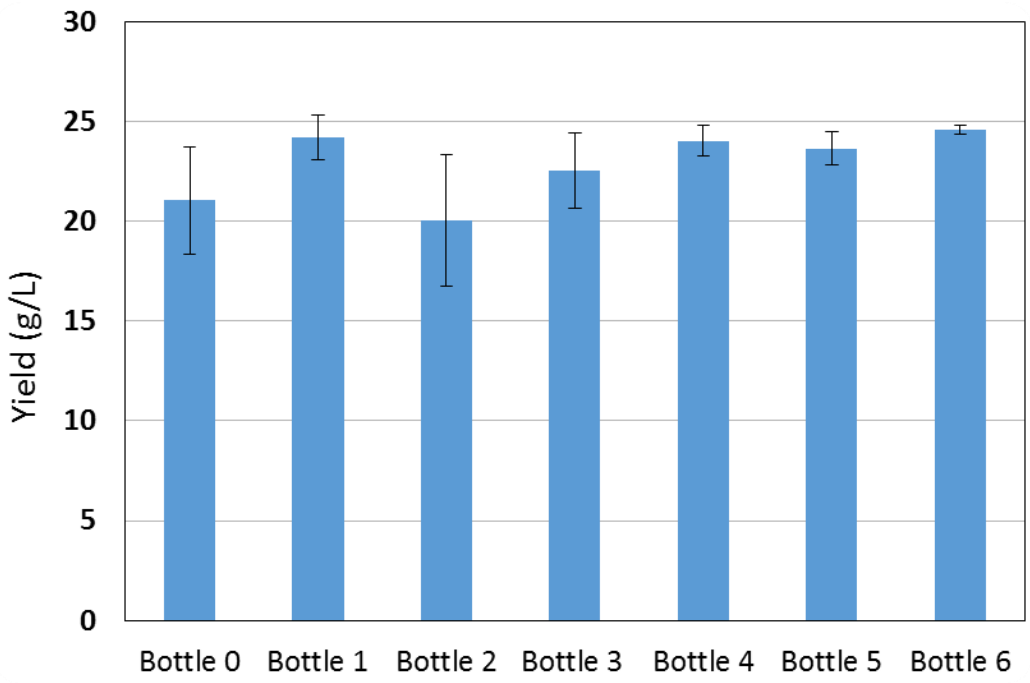

Fig. 2. Effect TS sample line flushing has on fungal biomass yields. Error bars represent one standard deviation

As evidenced by Fig. 2, neither the flushing of the sampling line nor the order in which the bottles were filled affected the growth of fungi. Even the sample bottle collected before the line was flushed at all (Bottle 0) supported a high level of biomass growth (>20 g/L). Therefore, flushing of the sampling line was not a major contributor to contamination or variability seen in previous tests. Likewise, the order in which the samples were collected did not play a role.

3.3. Contamination present in TS

Findings in the previous section were supported by an analysis of the microbiology and organic chemistry of the TS. The sample of TS used for the analysis had been recently collected from the corn ethanol plant (< 1 week), and the results from the analyses are presented below in Table 2.

Table 2. Microbiological Analyses of TS

	Concentration	Units
Microbiology		
Anaerobic plate count	<10	cfu/ml
Lactic acid bacteria	<10	cfu/g
Thermophilic spores	<10	cfu/g
Aerobic plate count	<10	cfu/g
Bacillus spp.	Negative	-
Lactobacillus	<10	cfu/g
Organic Chemistry		
Acetic acid	910	ppm
Lactic acid	<250	ppm

There were no bacteria found over the detection limit for any of the species tested. Interest in the concentration of *Lactobacillus* spp. and thermophilic spores was taken due to the temperature of TS during collection. Because TS can reach 80 °C, microorganisms capable of withstanding high temperatures were hypothesized to survive in the medium and decrease fungal growth (Erickson, 2012). Analyses of microbiology indicated that no such contaminants were identified. Data found in Sections 3.2 and 3.3 indicate that contamination did not originate from

the TS itself or during collection, and these findings show that transportation of TS to the bioreactor would not pose an obvious source of contamination at commercial-scale.

3.4. Storage and hydrogen peroxide study

Since collection and fresh TS were not the source of contamination, storage and exposure to air was believed to be the most likely source. Therefore, a study spanning 8 weeks using the same TS was conducted to confirm this assumption. Since storage of TS at commercial-scale may need to take place, this test was performed to determine the maximum length of time that TS can be stored before it is no longer able to support a sufficient biomass yield. As previously described, a carboy with a spigot and filter paper limiting direct exposure to air was used. While these preventative measures can be implemented at lab-scales, TS will inevitably come into contact with air at commercial-scale. Thus, the use of hydrogen peroxide as a chemical disinfectant was also studied to combat bacterial contamination. Hydrogen peroxide had been previously used to improve fungal biomass growth in related experiments using wastewater from a wet-grind corn ethanol plant. A dose of 60 mg/L was used, and found to reduce bacterial population by 50% (Miao, 2002). To determine the optimum dose on TS, a sample that was only able to support minimal biomass yields (<5 g/L) was used. A dose of 60 mg/L was initially tested, but it was discovered that TS benefits from higher concentrations of hydrogen peroxide. Results from doses are presented in Fig. 3.

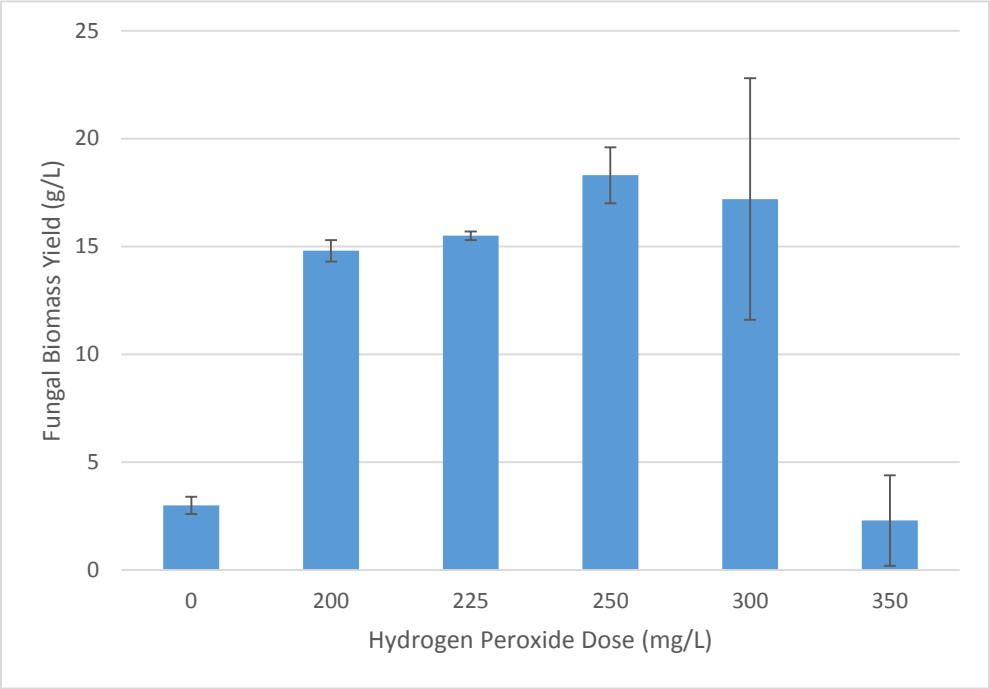

Fig. 3. Optimum dosage of hydrogen peroxide to improve fungal biomass growth. Error bars represent one standard deviation.

The optimum dose of hydrogen peroxide was found to be 250 mg/L. This dose provided a biomass yield more than 6 times greater that of the control. This was the hydrogen peroxide concentration at which bacteria were most inhibited, but could still be tolerated by the fungi. Yields greater than 15 g/L were also seen when using a dose of 300 mg/L. At this concentration, however, greater variability in growth between the flasks was noted (yields ranged between 9.8 g/L and 23.5 g/L). Additionally, the morphology of the fungi at this dose was a mixture between pelleted and dispersed growth, which is not typically seen at lab-scale. Due to the variability and atypical morphology, this dose was not considered to be optimal. Likewise, concentrations above

this (≥350 mg/L) resulted in poor growth. This was found to be the hydrogen peroxide concentration at which the tolerance of the fungi is exceeded.

Once the optimum dose had been selected, the weekly test observing the effect of hydrogen peroxide and age of TS was performed. Results are reported in Fig. 4.

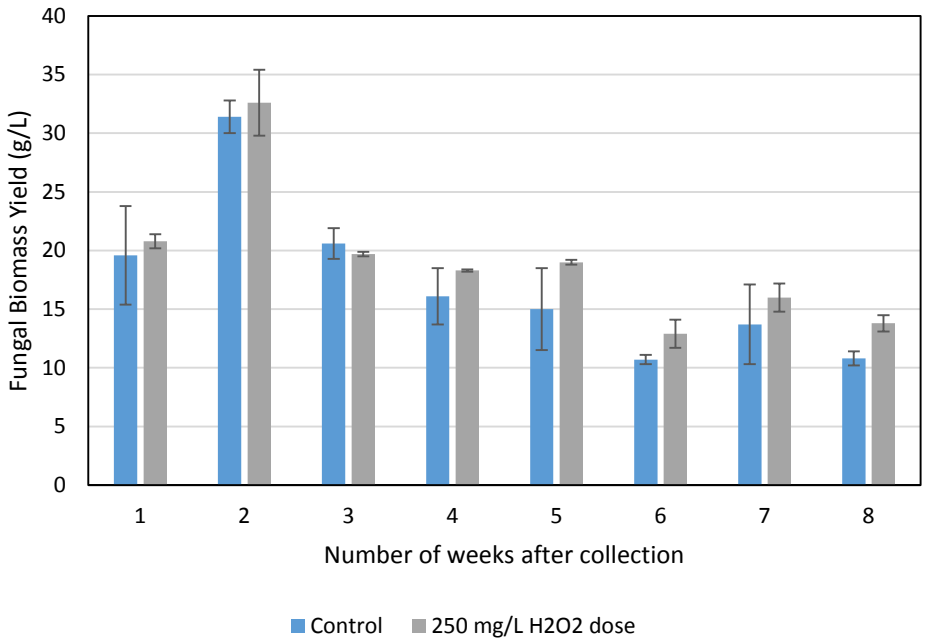

Fig. 4. Effect of age and a weekly one time dose of hydrogen peroxide on biomass yields using stored TS. Error bars represent one standard deviation.

With regards to the age of TS, a trend in fungal biomass yields was observed. Maximum growth on the control TS was seen within the first two weeks (31.4 g/L), followed by a gradual decrease in biomass yields each subsequent week. Diminished growth in Week 1 was likely due to improper disinfection of the carboy spigot. In following weeks, the spigot was swabbed with ethanol to remove any particulates or residual TS prior to assembling the experiment. Despite the use of filter paper over the mouth of the carboy and the use of a spigot, decreasing growth was

still observed. TS began losing its capability to support fungal cultivation after two weeks even though contact with air was restricted. Therefore, TS should not be stored for more than 2 weeks when considering commercial-scale operations. It can be presumed that this time may even be shorter for scaled-up processes, due to the precautions taken at lab-scale that cannot be feasibly implemented.

Results using a chemical disinfectant provided a similar decreasing trend in growth. Hydrogen peroxide did not demonstrate to significantly improve fungal yields. While it has been shown to decrease cyanobacteria levels in aquatic environments without detriment to other microorganisms (Drabkova et al., 2007; Matthijs et al., 2012), it provided little benefit to the cultivation of fungi in TS. Yet it did not suppress fungal growth either, as nearly each week saw higher average yields using hydrogen peroxide. While these increases were not found to be statistically significant ($P<0.05$), less variation of growth between flasks was observed with the chemical addition, and may be a result of bacterial suppression. Dosing with hydrogen peroxide was most beneficial when using contaminated TS where minimal fungal growth (<5 g/L) was supported, as seen in the optimum dose determination (Fig. 3). Fungal growth could be increased by a multiple of 6 on this contaminated TS, compared to only a maximum of 1.3 when the TS was 5 weeks old in Fig 4. Larger increases in growth compared to the control may have been seen if this test had been carried out longer. However, it is unlikely that this extended length of storage time would be used in commercial-scale operations, or would result in biomass yields significantly greater than what was seen in the final weeks in Fig. 4.

While the initial purpose of using hydrogen peroxide was to inhibit contaminants, the increase in fungal biomass may not be completely due to the suppression of bacteria. Instead, it is likely the result of the byproducts formed from hydrogen peroxide (i.e. oxygen and water).

The decomposition into oxygen and water aids in COD removal (Linley et al., 2012; Bauza et al., 2014), and likely occurred in the TS based on similar studies using this disinfectant (Miao, 2002). Reduction of COD could account for the increase each week between the dosed and control flasks. Since oxidation improved yields, other methods to increase dissolved oxygen should be studied. Additionally, hydrogen peroxide may have oxidized the complex organic structures of the TS. Thus, more labile compounds could have been available to the fungi.

3.5. Thioglycollate Analysis Results

At the start of each week's test, a thioglycollate analysis was conducted on the carboy of TS to determine if any microbial contamination was evident. It was hypothesized that a decrease in fungal yields would begin when a presence of contamination was noted in the thioglycollate test. This, however, was not seen. The first recorded appearance of bacterial contamination occurred during Week 5 of the test. The thioglycollate analysis indicated the presence of aerobic bacteria in the TS, and was observed for each of the following weeks. As seen in Fig. 4, biomass yields had already began to decline prior to Week 5. Therefore, contamination in the TS had been inhibiting fungal growth even before it was detected in the thioglycollate analysis. This demonstrates that the thioglycollate analysis is not sensitive enough to identify concentrations of bacteria inhibitory to growth of *R. oligosporus.*

3.6. Pilot-scale cultivation

Findings in Sections 3.1 – 3.5 indicate that the most significant cause of variability in fungal yields was due to storage of TS for longer than 2 weeks. Neither limited contact to air nor hydrogen peroxide improved the ability of TS to support growth. Based on these findings, maximal biomass yields are expected to occur using freshly collected TS, and a chemical disinfectant is not needed. This conclusion is supported by subsequent testing that has routinely resulted in decreased fungal growth after 2 – 3 weeks from the same sample of TS.

Therefore, it was decided that the 1600-L pilot-scale bioreactor could be operated successfully without a chemical disinfectant, provided newly collected TS would be used. This hypothesis was tested according to pilot-scale procedures outlined in Section 2.3. While the bioreactor itself was cleaned and disinfected, no addition to the TS was made aside from an anti-foaming agent. Following 48 h of cultivation, a biomass yield of 9 g/L dried fungi was harvested. This yield is lower than what is typically seen at lab-scale, but is consistent with what has been previously reported during scale-up (approximately 10 g/L) (Erickson, 2012). In addition to a lower yield, a different morphology of fungi was also seen at pilot-scale. Fungal biomass in pellet morphology was harvested, and is believed to be the result of a different aeration method than the one performed at lab-scale. At pilot-scale the vessel is aerated via ceramic diffusers, compared to aeration due to mixing at lab-scale. Regardless of the differences in morphology and yield, it was found that sufficient biomass yields for hydrothermal liquefaction processing in Chapter 4 could be obtained at pilot-scale if recently collected TS is used.

4. Conclusion

The cause of high variability between batches of TS was identified. Studies observing collection methods, microbial content, and lab handling of TS determined that extended storage resulted in diminished fungal growth yields. The addition of hydrogen peroxide and restricted contact with air did not significantly improve the biomass yields. It was found that the most effective method to obtain high yields (>20 g/L at lab-scale) was to use newly collected TS. This was confirmed by the use of fresh TS without a disinfectant at pilot-scale, where a dry yield of 9 g/L was reported. At commercial-scale, efficient growth can be expected using a disinfected bioreactor and TS less than 2 weeks old.

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Chapter 4

PILOT-SCALE CONTINUOUS-FLOW HYDROTHERMAL LIQUEFACTION OF FILAMENTOUS FUNGI

Abstract

This study examined the potential of the filamentous fungus *Rhizopus oligosporus* as a feedstock for hydrothermal liquefaction (HTL). The fungal biomass, cultivated in a byproduct of corn ethanol, was processed at pilot-scale using a 1.5-L capacity continuous-flow HTL system. HTL operating conditions of 300 – 400 °C at 27 MPa for 12 – 30 min were tested. Yields ranging from 48.2 – 60.9% were obtained. At low reaction temperatures (300 °C), yields as high as 59.9% could still be achieved. Neither yield nor elemental composition of biocrude was significantly impacted by residence time or temperature, as is typically seen with batch reactors, aside from the least severe reaction condition studied (300 °C, 12 min). Similarities in biochemical and elemental composition between *R. oligosporus* and microalgae resulted in comparable biocrude yields previously recorded for continuous-flow systems. These findings demonstrate the viability of fungal biomass as a feedstock, and that lower temperatures can be used at pilot-scale while still achieving maximal yields.

1. Introduction

The U.S. corn ethanol industry has seen a considerable amount of growth over the past decade, resulting in the production of 15 billion gallons of ethanol per year from over 200 plants nationwide (Nebraska Energy Office, 2016). Biofuels provide a renewable source of energy and

reduce dependency on fossil fuels. However, some remain skeptical of the sustainability of corn ethanol (Dias de Oliveira et al., 2005; Bhat, 2008), and issues regarding energy efficiency and handling of byproducts remain. Specifically, the liquid centrate produced from centrifugation of suspended solids following fermentation is a resource that can be better utilized to improve plant operations. This centrate, referred to as thin stillage, is currently being evaporated in an energy-intensive process to produce a syrup containing 30% solids. The syrup is subsequently mixed with the dried suspended solids to form an animal feed called dried distillers grains with solubles (DDGS). (RFA, 2016). There is, however, limited demand for the syrup and it is often sold at low prices. The added nutritional benefit of the syrup is also questionable (Rasmussen et al., 2014).

In order to avoid high energy requirements to provide a low-value coproduct, alternative uses of thin stillage are being considered. Exploiting a high organic content (90 g/L COD) and acidic pH range ideal for fungal growth (4 – 5), previous studies have investigated the cultivation of the filamentous fungi *Rhizopus oligosporus* and *Mucor circinelloides* in thin stillage (Mitra et al., 2012; Rasmussen et al., 2014). Fungi were found to provide effective water treatment of the thin stillage by reducing COD, suspended solids, and organic acids. This could result in reuse opportunities at the plant possible, and potentially reduce water needs by 75% (Jessen, 2010; Rasmussen et al., 2014). Additionally, the harvested fungi could be used as an animal feed due to their high protein and essential amino acid content.

Fungal biomass cultivated in thin stillage was also found to be a possible feedstock for biofuels, due to a short cultivation period of 2 – 3 days and increased lipid content when compared to fungi cultivated in a yeast malt broth (Mitra et al., 2012). Production of a renewable diesel fuel from filamentous fungi *Rhizopus oligosporus* cultivated on thin stillage could

potentially add a high value coproduct from the corn ethanol process, and improve energy return on investment. Hydrothermal liquefaction (HTL) was selected in this present study as the pathway for fungal biocrude production. This thermochemical process uses water at high temperature and pressure to break down the long-chain organics of the biomass and re-polymerize them into short-chain hydrocarbons to form a liquid fuel referred to as biocrude. Unlike other thermochemical processes like pyrolysis or gasification, no drying of the biomass is required as water is required for HTL. Thus, energy consumption due to drying is eliminated (Biller and Ross, 2011). Therefore, HTL is an optimal process for use with fungal biomass cultivated in thin stillage due to high moisture content.

To date, the majority of studies using HTL as a biofuel production process have used microalgal or lignocellulosic biomass (Qu et al., 2003; Valdez et al., 2012; Tian et al., 2014; Elliott et al., 2015). Limited research has been conducted using fungi as a feedstock, and are limited to the yeasts *Cryptococcus curvatus* or *Saccharomyces cerevisiae* (Hammerschmidt et al., 2011; Valdez et al., 2013; Miao et al., 2014; Jena et al., 2015). Additionally, continuous-flow HTL processes have exclusively investigated use of microalgae and lignocellulosics (Elliott et al., 2013; Jazrawi et al., 2014; Elliott et al., 2015). However, continuous-flow HTL for fungi has not been thoroughly investigated. This study aimed to determine the feasibility of fungal biomass as an HTL feedstock. Our results will be compared to other feedstocks that have received more attention. Performance at pilot-scale in a continuous-flow reactor was studied, and optimization of reaction conditions was performed. Feasibility and optimization were evaluated based on quantity and composition of biocrude created at reaction conditions previously determined to be optimal for biocrude production (300 – 400 °C) (Akhtar and Amin, 2011; Valdez et al., 2012).

Specifically, the oxygen and nitrogen content of the biocrude was studied. Low oxygen levels were desired to produce a biofuel comparable to fossil fuels. Similar composition would allow for the fungal biofuel to be more readily utilized in existing infrastructure. Nitrogen content was also of concern. Increased nitrogen in the biocrude demonstrates improved protein conversion during the HTL process. However, excessive amounts would potentially increase NO_X emissions when combusted (Biller and Ross, 2011). Therefore, it was important to determine reaction conditions which produced high yields of biocrude with a composition similar to petroleum crude oil.

2. Methods

2.1. Feedstock cultivation

2.1.1. Fungal inoculum preparation

The culture of the fungus, *R. oligosporus* was obtained from the American Type Culture Collection (ATCC# 22959, Rockville, MD). Cultivation, collection and storage of spores were conducted according to methods used by Ozsoy et al. (2008). The spores were aseptically cultured using HIMEDIA™ RM301 agar for 36-48 h at 30 °C. Spores were then collected using a deionized dilution water containing 0.85% (w/v) sodium chloride and 0.05% polysorbate 80. The collected spores and solution were passed through a 50 mL syringe containing glass wool to remove any mycelia. Subsequently, yeast malt (YM) broth (Difco Laboratories, Detroit, MI) was mixed with the filtrate at a 1:1 ratio, and glycerin was added to a final concentration of 20% (v/v). This spore stock solution was collected in sterile 2 mL cryo-vials and stored at -80 °C.

A fungal inoculum was prepared by transferring one 2 mL cryo-vial of spore suspension to 1-L of autoclaved YM broth. The inoculum flask was incubated at 37°C on an orbital shaker at 200 rpm for 24 h prior to inoculating the thin stillage (Mitra et al., 2012). A total of 8 L of fungal inoculum was prepared for the pilot-scale bioreactor.

2.1.2. Pilot-scale bioreactor and fungal biomass cultivation

R. oligosporus was cultivated in thin stillage obtained from Golden Grain Energy LLC (Mason City, IA), a dry-grind corn ethanol plant. 1400 L of thin stillage was collected for the pilot-scale bioreactor operations. Since the temperature of thin stillage can reach 80 °C at the time of collection, it was allowed to cool to 35°C over the course of 15 h before inoculation to prevent heat damage to the fungal inoculum. Prior to fungal cultivation, the average pH was 5.0.

The 1600-L pilot-scale bioreactor was cleaned and disinfected with a 3% (v/v) sodium hypochlorite solution, followed by a sodium hydroxide solution (pH ~10.5) 24 h prior to thin stillage transfer. Following the cooling period, thin stillage was transferred to the bioreactor, and 8 L of fungal inoculum was added to the thin stillage. Cultivation was allowed to proceed over the following 48 h (Mitra et al., 2012). During cultivation, temperature in the bioreactor was maintained at 37±3 °C (Mitra et al., 2012), and four ceramic diffusors provided air at a flow rate of 300 L/min. In order to control excessive foaming, 450 mL of a liquid anti-foaming agent (Antifoam 204, Sigma-Aldrich, St. Louis, MO) was added to the bioreactor. Upon completion of fermentation, fungal biomass was harvested by filtering over a fine mesh screen to remove excess thin stillage, and achieve a solids content of approximately 25 wt.%. The solids content was confirmed by drying a known weight of wet fungal biomass (approximately 5 -10 g) at 70 °C for 48 h. This drying temperature was determined to achieve adequate drying without

volatilizing lipids or inducing a Maillard reaction. After harvesting, the fungi were stored at 10 °C for up to 4 months until further use.

Analysis of fungal biomass from the pilot-scale bioreactor to determine the elemental compositions of C, H, N, O, S and heating values were conducted by Keystone Materials Testing, Inc. (Newton, IA) using ASTM methods D5291 and E711, respectively. Exact Scientific Services, Inc. (Ferndale, WA) performed proximate analysis to determine carbohydrate, lipid, and protein content of the biomass.

2.2. Feedstock slurry

Prior to feedstock slurry preparation, the stored fungi were mixed until uniform consistency was achieved to ensure a homogeneous moisture content. Fungal slurries containing 4 wt.% solids were prepared 1 – 7 days prior to each HTL test and stored at 10 °C until needed. Solid content was limited by the supercritical reactor equipment due to difficulties in pumping, and risk of plugging when slurries containing greater than 5 wt.% solids were used. All of the biomass used in the slurries came from one batch of fungi grown together on the same thin stillage. The same batch of fungi was used in order to eliminate the potential risk of biochemical variations between batches, which could influence biocrude yields (Biller and Ross, 2011).

Fungi and water were blended into a slurry in a two-step method. First, the biomass and water were mixed in a commercial-grade food blender. Approximately 3000 g of wet fungal biomass was blended with an equal amount of distilled water for about 1 min. Additional

distilled water was subsequently added to the blend, and mixed to a 4 wt.% solids content in a high-shear inline mixer until a uniform slurry was obtained.

A proximate analysis was performed to confirm a 4 wt.% solids loading and determine the amount of ash present in each slurry sample. An amount of fungal slurry (50 -75 g) was added to a ceramic crucible and dried in an oven at 105 °C for at least 24 h. Moisture content was calculated by the mass difference of the wet and dried fungal biomass. Ash determination was conducted in a similar manner by heating the dried biomass in a muffle furnace for a minimum of 3h at 550 °C (Jazrawi et al., 2013). Analyses of fungal slurry were performed in triplicate with the average values reported.

2.3. HTL

2.3.1. Equipment

HTL was performed in a 1.5-L capacity pilot-scale supercritical flow reactor (SCFR) located at the Iowa Energy Center's Biomass Energy Conversion (BECON) Facility in Nevada, IA. The SCFR was designed by Supercritical Fluid Technologies Inc. (Newark, DE), with maximum operating conditions of 450 °C and 69 MPa. A 10-L tank equipped with a paddle mixer was used to contain the prepared slurry. The paddle mixer provided continuous agitation, so the slurry would remain homogeneous. A plunger pump was used to transport slurry from the tank through a preheater, consisting of stainless steel piping coiled around a cylindrical heating unit, before entering the SCFR. Effluent was discharged from the SCFR via pneumatic valve when pressure inside the reactor reached the set point. A liquid-to-liquid heat exchanger cooled effluent to approximately 25 °C.

2.3.2. Operation

HTL of fungi was studied at 12 different operating conditions. Four retention times ranging from 12 – 30 min for temperatures 300, 350, and 400 °C were studied using a constant pressure of 27 MPa for each test. Attempts to keep retention times constant across the temperature range proved to be difficult based on the SCFR set-up. Since effluent flow rate was dictated by the reactor reaching the set-point pressure, retention times would vary at different temperatures despite operating the pump at a constant speed. This variation was most noticeable when comparing the longest retention time setting at 300 and 400 °C, where a 5 min difference was noted. All other retention times varied by less than 1.5 min for the same pump rate across the temperature range. Thus, rounded averages of 12, 16, 19, and 30 min were reported for the retention times. Although previous continuous-flow reactor studies have used shorter residence times (Elliott et al., 2013; Jazrawi et al., 2013), these times were selected based on the operating capacity of the slurry pump and the rate at which the heat exchanger could effectively cool the effluent.

Prior to each HTL test, distilled water was pumped through the SCFR for a minimum of 4 h to achieve the desired operating conditions. The system was switched over to fungal slurry once a steady state of temperature and retention time had been reached. The preheater was set at 133 °C, as temperatures above this could potentially bake the slurry and cause plugging issues (Elliott et al., 2013). After switching to the slurry, the initial 2-L of collected effluent was discarded. This effluent was found to be predominately composed of the distilled water remaining in the SCFR prior to switching. Due to the aforementioned issues with retention time consistency, samples of aqueous effluent were collected on a volumetric basis as opposed to time

increments. Samples of 500 mL were collected in polypropylene bottles and processed individually. A minimum of 12 samples of effluent were collected, since bio-crude production was found to achieve steady state at this point.

2.4. Bio-crude

Determination of bio-crude yield consisted of the separation of oil from solid and aqueous phase fractions. Dichloromethane (DCM) (Fisher Scientific, Waltham, MA) was used as a solvent to extract bio-crude from the collected effluent. 100 – 200 mL of DCM was added to each 500 mL sample and shaken at 30 rpm in a rotator for 60 min. Subsequently, solids from the DCM and effluent mixture were vacuum-filtered and removed via 1.6 µm pore sized glass fiber filter. After filtration was complete, the filter paper was placed in an oven at 105 °C for 24 h, and then weighed to determine the mass of solids present in the aqueous sample. The filtrate was next collected in a separatory funnel, allowing the denser DCM and oil to separate from the lighter aqueous fraction. The oil and DCM fraction was transferred to a crystallizing dish, where the DCM was allowed to evaporate at room temperature for 3 – 5 days. The remaining bio-crude oil was weighed and recorded, and a dry, ash-free (daf) oil yield was determined using Eq. 1 (Biller and Ross, 2011; Jazrawi et al., 2013). Results of bio-crude yields are reported as an average of the samples determined to be at steady state. Analyses for each of the 12 HTL tests were conducted by Keystone Material Testing, Inc. (Newton, IA) to determine heating values and elemental composition (C, H, N, O, and S) using ASTM methods D240-09 and D5291, respectively.

$$yield\ (daf\ \%) = \frac{Oil\ Mass}{Effluent\ Sample\ Mass \times (Slurry\ \%\ solids - \%\ ash)} \times 100 \quad (1)$$

2.5. Statistical analysis

A statistical analysis was performed to determine the significance of biocrude and biochar yields. Software JMP (SAS Institute, Cary, NC) was used to perform a Tukey's honest significant difference (HSD) test. P values greater than 0.05 were determined to show significance.

3. Results and discussion

3.1. Fungal feedstock analysis

R. oligosporus was cultivated on thin stillage, and the fungal feedstock composition is shown in Table 1. The biomass was predominately composed of carbohydrates (34.8%) and proteins (34.2%). The crude lipid content accounted for 22.4% of the biochemical composition. Fungi benefit from being cultivated in thin stillage due to their ability to accumulate 20% more lipids compared to the same fungi cultivated in YM broth (Mitra et al., 2011). Increased lipid content is desirable to have since biocrude production has been demonstrated to come primarily from lipids, followed by proteins, and to a lesser extent carbohydrates (Biller and Ross, 2011). Compared to other fungal species used in HTL studies, *R. oligosporus* contained a significantly higher lipid content than yeast *Saccharomyces cerevisiae* (2.7%) (Valdez et al., 2013), and less

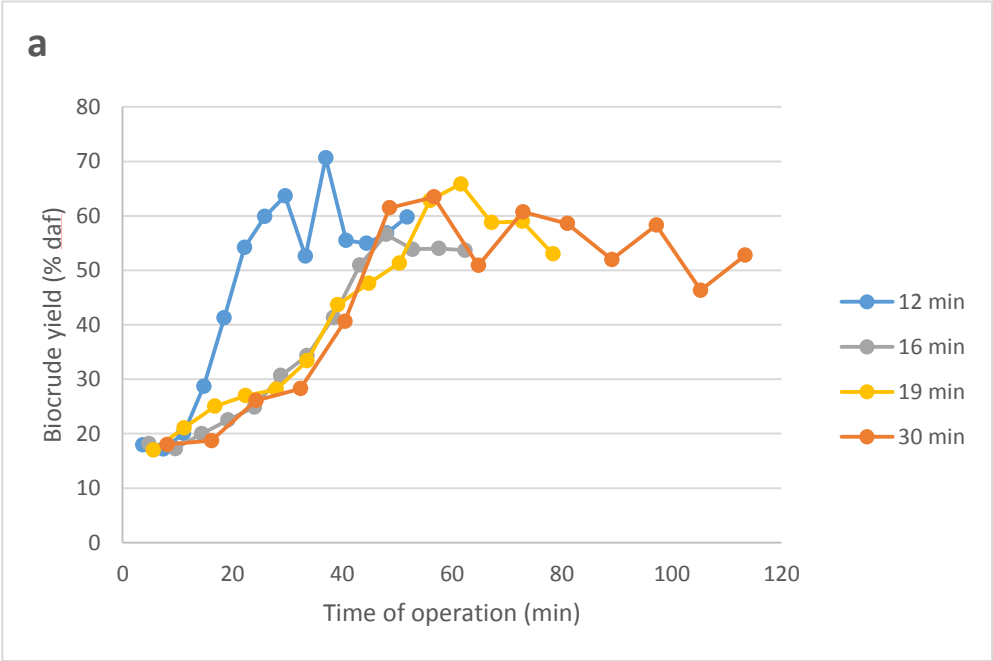
than oleaginous yeast *Cryptococcus curvatus* (32.8%) (Jena et al., 2015). With regards to other feedstocks used in continuous-flow HTL systems, *R. oligosporus* lipid content was comparable to the biomass of microalgae (Elliott et al., 2015); specifically, *Chlorella* (25%) (Biller and Ross, 2011) and *Nannochloropsis* (28%) (Brown et al,. 2010). The fungal biomass feedstock was found to have a similar elemental composition and higher heating value (HHV) to these microalgae as well. Biochemical and elemental similarities to microalgae confirm the fungal biomass' potential as a feedstock, as biocrude yields of up to 64% daf and oxygen content as low as 5% have been recorded for microalgae in continuous-flow systems (Elliott et al., 2013).

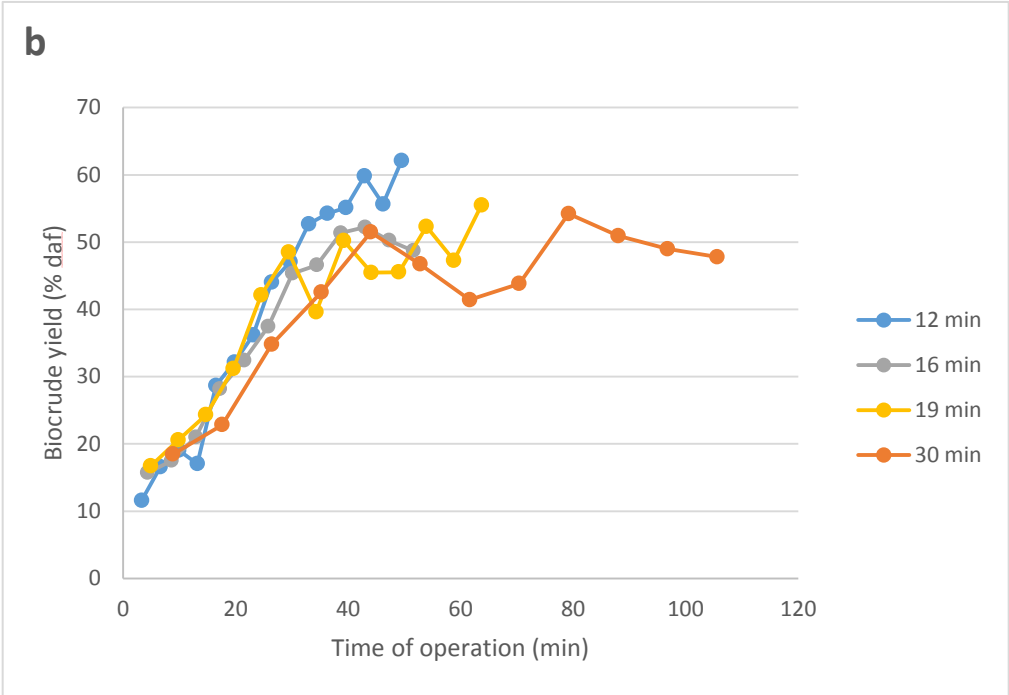
Table 1. Analysis of *R. oligosporus* feedstock.

Proximate Analysis (wt.% as received)	
Ash	7.13
Moisture	1.44
Elemental composition (wt.% daf)	
C	59.4
H	8.7
N	5.5
O (by diff.)	19.9
S	0.4
Biochemical content (wt.%)	
Carbohydrates	34.8
Proteins	34.2
Lipids	22.4
HHV (MJ/kg)	28.3

3.2. Reactor performance

Biocrude production in relation to a continuous-flow process was recorded in order to study the performance of the SCFR (Fig. 1). Biocrude yield results were reported on a dry, ash-free (% daf) basis for each 500 mL sample that was collected.





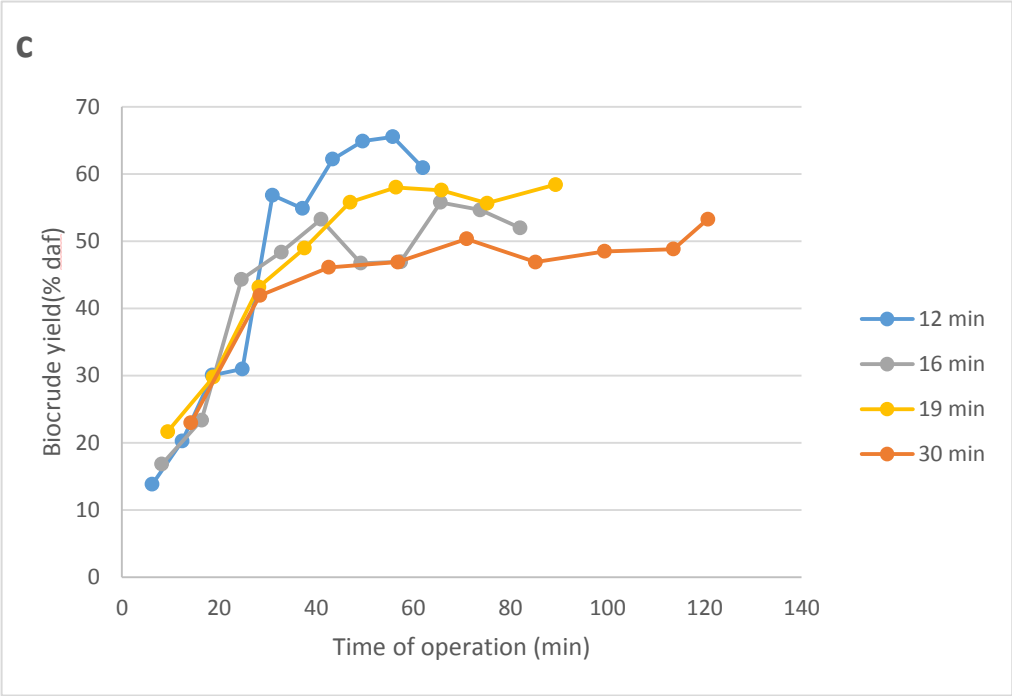


Fig. 1. Biocrude production as a function of SCFR operation time for given residence times and temperatures (a) 300 °C, (b) 350 °C, and (c) 400 °C.

Despite being held at constant temperatures, biocrude production was shown to require a start-up period between 30 – 60 min before a near-steady state was reached. This lag time indicates that the water used for heating was not completely removed from the reactor during this initial start-up time, despite the first 2 L of effluent being discarded. Water mixed with the slurry, reducing the actual solids content present at the time of reaction. The assumption that the SCFR behaved as a plug-flow reactor, and that the solids content remained at a constant 4 wt.%, resulted in decreased recorded biocrude production. The up-flow design of the SCFR may have contributed to additional mixing due to settling by gravity of the water and slurry.

Once water had been sufficiently purged from the reactor, after approximately 3 L of effluent samples had been collected, a near steady state production of biocrude was achieved.

Residence time and temperature appeared to influence biocrude production trends at steady state. The more severe the reaction conditions were, the more stable the biocrude yields became. This is most evident during the 30 min residence time at 400 °C, where the yields at steady state ranged by ±2.4% compared to ±5.76% seen at the same residence time at 300 °C. These variations occurred despite the fact that the same number of samples had been collected and the same amount of effluent slurry had been pumped through the reactor. Even greater variation was found to occur at the least severe condition (300 °C, 12 min), when steady state yields differed by as much as ±6.22%. Stability of reaction conditions at higher temperatures and longer retention times at pilot-scale have been noted in other studies using continuous-flow systems (Jazrawi et al., 2013).

3.3. Biocrude oil yield

The average biocrude yield (% daf) obtained after the reactor reached steady state is reported in Fig. 2 for all temperatures and residence times. As noted in Section 3.2, longer times of operation could have produced more well-defined trends, specifically at lower temperatures and residence times where more variation was noted to occur. However, 3 – 8 samples at steady state were averaged from each condition tested, and were found to provide representative biocrude yields for the test parameters. Reliability of these results was determined by conducting a repeat HTL test for one of the given operating conditions. Only a 0.52% difference in biocrude yield was seen between the repeat tests.

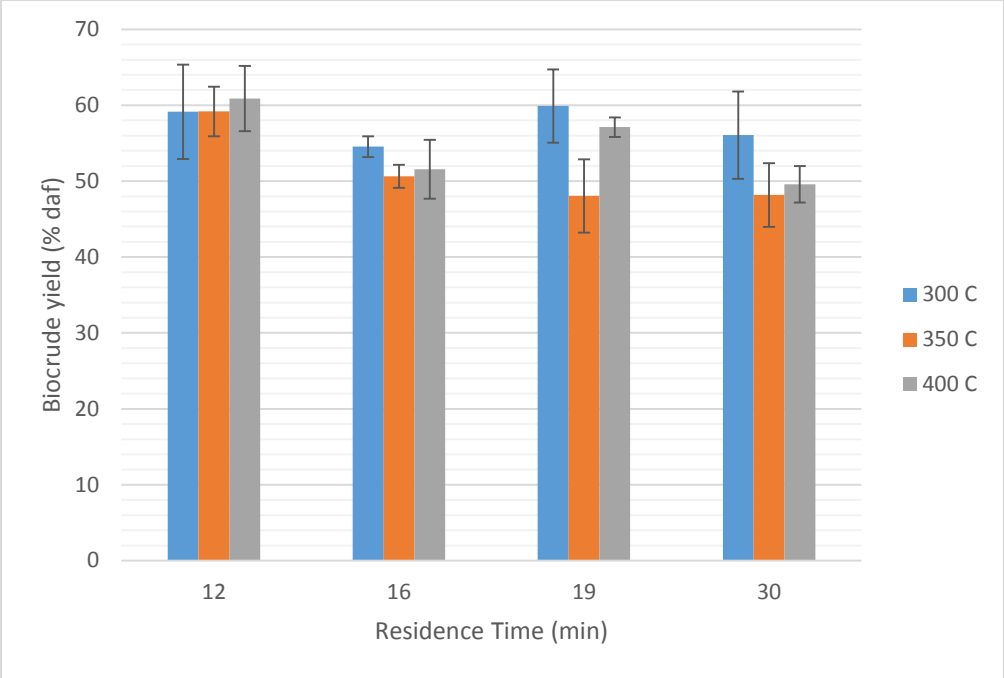


Fig. 2. Biocrude yields (% daf) from HTL testing. Results are averages of at least three samples collected at steady state. Error bars represent one standard deviation.

The optimum temperature range for biocrude production during HTL, as opposed to biochar and biogas formation, is generally reported to be 300 - 350 °C (Akhtar and Amin, 2011; Garcia et al., 2012; Tian et al., 2014; Yoo et al., 2015). However, more severe temperatures are believed to increase biocrude formation from the protein fraction, as evidenced by higher nitrogen content in biocrude samples created at higher temperatures (Elliott et al., 2015). Therefore, the reaction temperature of 400 °C was also studied as fungal slurry contains a high level of proteins. From the HTL test data, it appears that higher temperatures did result in increased protein conversion, since the maximum yield (60.9% daf) was obtained at 400 °C for 12 min. This finding is consistent with studies both in batch reactors, as well as continuous-flow systems, in which the highest biocrude production is observed at the most severe reaction

temperature due to improved conversion of proteins and carbohydrates to biocrude (Valdez et al., 2012; Jazrawi et al., 2013; Elliott et al., 2015).

While the maximum yield was obtained at the most severe reaction temperature (400 °C), it was not significantly higher than the other temperatures tested ($P<0.05$). A range of yields between 48.2 – 60.9% daf was found to be attainable using *R. oligosporus* as a HTL feedstock, but did not appear to be dependent on temperature or residence time. The fungal biomass used for this study produced higher yields than the oleaginous yeasts used in other previous batch reactor tests, which ranged between 40 – 49% daf (Hammerschmidt et al., 2011; Valdez et al., 2013; Jena et al., 2015). When compared to other continuous-flow systems, the observed biocrude range for *R. oligosporus* is similar to systems using microalgae as a feedstock, in which biocrude yields of 38 – 64% daf have been recorded (Elliott et al., 2013; Jazrawi et al., 2013; Elliott et al., 2015). It should be noted that in a previous continuous-flow study conducted by Elliott et al., temperatures of 350 °C were required to produce yields near 60% (2013). Conversely, the HTL system used herein for these fungal biomass tests achieved yields as high as 59.9% daf even when the lowest reaction temperature of 300 °C was employed. Therefore, these observations suggest that pilot-scale continuous-flow processes are highly system specific, and the significance of temperature will vary depending on the equipment and parameters used.

For this system, acceptable biocrude yields were obtained when the least severe reaction conditions (300 °C) were used. This has implications for commercial-scale applications, where energy consumption of the HTL process could be reduced by lowering reaction temperatures to 300 °C. Improved energy return on investment can occur when lower HTL temperatures are employed with lipid-rich feedstocks (Yoo et al., 2015). As previous studies have shown that temperatures below 300 °C result in decreased yields (Valdez et al., 2012), this fungal HTL

process should be further investigated using lower temperatures to determine when temperature starts to become a significant factor.

As determined by Anastasakis and Ross (2011) and Jena et al. (2011) residence time significantly impacts biocrude yields in batch reactors. However, residence time does not play a large role in biocrude production during pilot-scale continuous-flow HTL tests. This was observed in our results, and has been reported by Jazrawi et al. (2013) using microalgae as well. Extending residence time from 12 to 30 min did not improve yields in this present study. Maximal biocrude production (60.9%) was found to occur even at the shortest residence time of 12 min. Shorter residence times allow for higher biocrude output at commercial-scale, and can help improve the economic viability of the technology.

3.4. Solids content analysis of HTL effluent

While it was found that the reaction conditions do not impact the yield of the biocrude, the same cannot be said about the quality of the product. This section will observe the composition of biocrude with respect to the operating conditions. Solids were collected from the effluent samples and were recorded as %daf mass of sample. The results for each reaction condition are presented in Fig. 3. Solids collected in the effluent were typically low, ranging from 0.13 – 0.71% when the reactor was determined to be at steady state. A considerably higher solids content (2.53%) was found to occur at 300 °C for 12 min during an initial test, and is likely the result of improper reactor cleaning. This value was not consistent with the rest of the collected data, and upon a repeated test at this condition a more consistent value of 0.48% was seen. This value is therefore reported in Fig. 3.

A greater amount of solids was expected to occur at lower temperatures due to this reaction condition favoring biochar formation via hydrothermal carbonization (Heilmann et al., 2011). Overall, a trend of decreasing biochar formation with increasing temperatures was observed at each residence time. Deviation from this trend occurred at the 12 min residence time, and may be an indication that this length of time is not sufficient for maximal biochar formation. Due to the relatively low solids content, biocrude yields were not impacted by solids formation as seen in Fig. 2. While the overall yield was not affected, solids do raise a concern with regards to further processing of the biocrude. Excessive solids proved to make filtration and separation of biocrude from the aqueous fraction difficult, and samples collected from tests at 300 °C took longer to process.

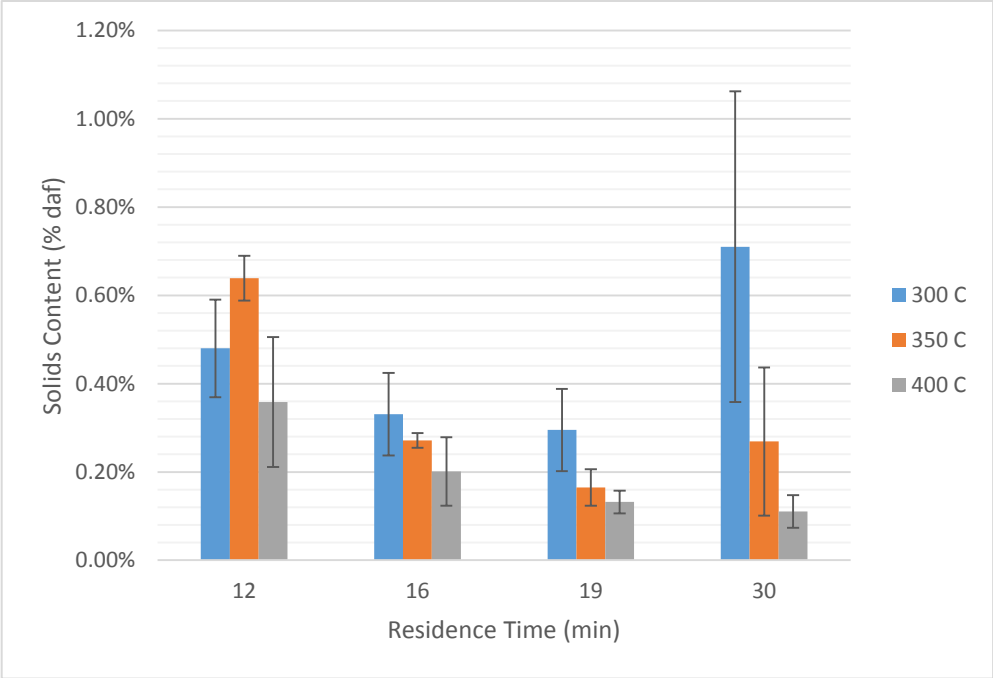


Fig. 3. Solids content present in aqueous sample collected for each given reaction condition. Error bars represent one standard deviation.

3.5. Elemental analysis of biocrude

The fate of C, H, N, O, and S from the fungal biomass to the biocrude was determined. A summary of biocrude composition at each reaction condition is presented in Table 2. HTL provided oxygen and nitrogen removal from the fungal biomass. Over 30% of the oxygen present in the feedstock was removed during the process, and an average of 14% oxygen was obtained in the biocrude. Previous continuous-flow reactor results show a broad range of oxygen levels achievable, with approximately 5 – 18% seen when using microalgae (Elliott et al., 2013; Jazrawi et al., 2013). Batch reactor studies previously performed by Miao et al. (2014) and Jena et al. (2015) using yeast have demonstrated higher oxygen removal, with over 63% removal from the starting feedstock. Differences in biochemical composition and a larger scale may have contributed to less efficient oxygen removal when using this fungal feedstock. With regards to operating conditions, the oxygen value in the biocrude produced in 12 min at 300 °C was most notable. A higher amount of oxygen (16.2%) was found at this condition compared to the rest of the samples. All other reaction conditions produced biocrude with lower oxygen content (13.7% average), and demonstrated little deviation between each condition tested (±0.95%). Higher levels of oxygen represent an insufficient amount of residence time, resulting in decreased quality of biocrude (Biller and Ross, 2011). Longer residence times at each temperature generally resulted in greater oxygen removal, and are consistent with previous work (Jazrawi et al., 2013).

Table 2. Elemental composition of biocrude as percent of total sample.

Temp. (°C)	Residence Time (min)	Carbon	Hydrogen	Nitrogen	Oxygen	Sulfur
300	12	70.50	10.30	2.51	16.20	0.47
	16	72.70	10.30	3.19	13.20	0.57
	19	72.10	10.30	3.05	14.00	0.56
	30	72.40	10.40	2.92	13.70	0.53
350	12	73.40	10.70	2.87	12.50	0.52
	16	71.50	10.30	3.05	14.60	0.55
	19	71.30	10.30	3.02	14.80	0.57
	30	73.60	10.80	2.81	12.20	0.56
400	12	72.30	10.40	2.85	13.90	0.52
	16	71.60	10.40	2.76	14.60	0.57
	19	71.50	10.30	2.83	14.70	0.64
	30	73.40	10.60	2.79	12.60	0.61

The lowest nitrogen content was seen at the least severe condition tested (300 °C, 12 min), with the remaining reaction conditions showing only minor variations. Nitrogen is often found in lowest amounts when reaction severity is minimal, and is a result of fewer proteins being converted to biocrude (Valdez et al., 2012). Similar to oxygen, our results suggest that 12 min at 300 °C is not sufficient for complete reactions to occur. Conversely, all other reaction conditions demonstrated an improved conversion of proteins. While higher nitrogen content in biocrude is an indication of increased biomass conversion, elevated levels of nitrogen can become a concern due to the potential formation of NO_x compounds (Biller and Ross, 2011). Therefore, a balance between optimal conversion and low nitrogen levels needs to be met. While more nitrogen present in biocrude is potentially detrimental to the environment, the nitrogen levels found in this biocrude were lower than what has typically been seen in continuous-flow systems (4 – 8%) (Elliott et al., 2015). Further treatment of biocrude will be needed to remove nitrogen to obtain petroleum-like quality (<2%), if NO_x production is a concern.

3.6. Higher heating value of biocrude

Due to the majority of reaction conditions producing a biocrude of similar composition, the estimated HHV varied only slightly (±0.7 MJ/kg) for the parameters tested. However, the minor variations in oxygen and nitrogen content did provide an overall trend for the impact of temperature and residence time on HHV (Fig. 4).

Higher HHVs were obtained at 400 °C (37.2 MJ/kg)**,** while lower values were obtained at 300 °C (35.8 MJ/kg). This trend resulted from slightly improved oxygen removal at higher temperatures and longer residence times. However, the improvement in biocrude quality seen at more severe reaction conditions was minimal, and it is unlikely that an energy return on investment analysis would show that increased temperature provides a significant benefit. This range of HHVs demonstrates the potential for HTL biocrude derived from fungi. These values are larger than what has been seen in other continuous-flow HTL systems using microalgae (Elliott et al., 2013; Jazrawi et al., 2013) as well as lignocellulosic biomass (Qu et al., 2003). Additionally, the fungal biocrude HHVs greatly exceed pyrolysis bio-oil (16 – 19 MJ/kg) and reach values near those typically observed in petroleum fossil fuels (40 – 45 MJ/kg) (Zhu et al., 2014). Additional upgrading would be needed to further remove oxygen and nitrogen to produce a biofuel compatible with existing infrastructure.

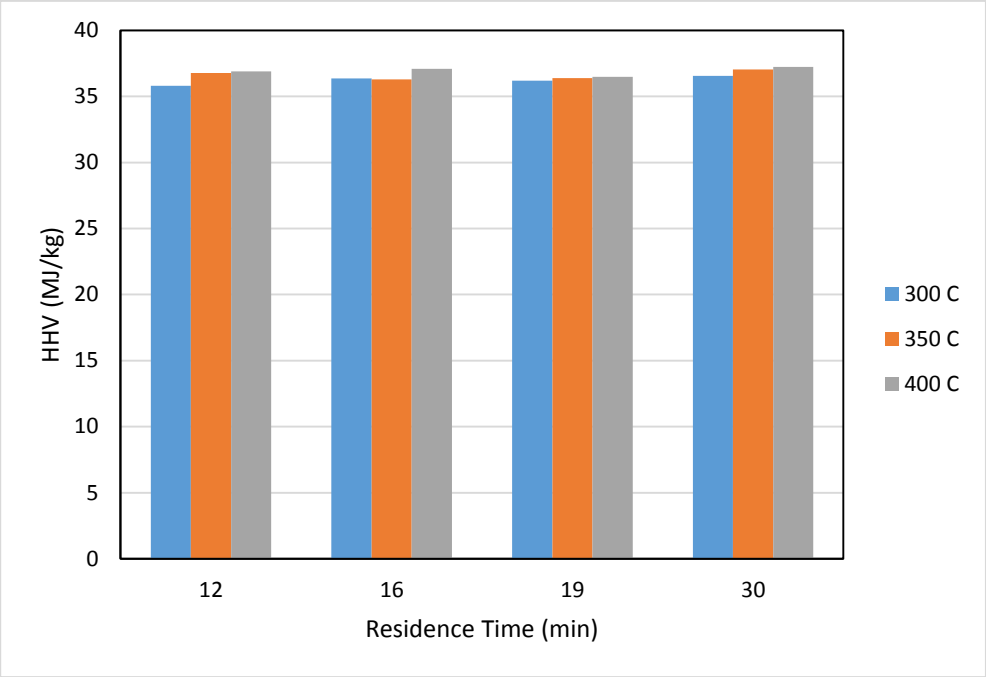


Fig. 4. Higher heating values of biocrude.

4. Conclusion

Biocrude derived from fungi *R. oligosporus* was successfully produced using a pilot-scale continuous-flow HTL reactor. At pilot-scale, higher temperatures did not prove to significantly enhance biocrude production, and 300 °C was found to generate yields equal to those seen at 400 °C. Reaction conditions had less of an impact than with what has been previously reported in batch reactors, and only became a significant factor at the least severe condition of 300 °C for 12 min. Biocrude from fungi had similar yields and quality to that of microalgae at temperatures of 300 °C for residence times of 16 min or longer, making fungi a viable feedstock.

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Chapter 5

Conclusions

Pilot-scale cultivation and continuous-flow HTL was successfully completed to generate a biocrude oil derived from *R. oligosporus* grown in thin stillage. In order for this process to be feasible at commercial-scale it is imperative to produce consistent and maximal fungal biomass yields. Controlling bacterial contamination is a key factor in doing so. At lab-scale, bacterial contamination became a significant factor in biomass yields when thin stillage stored for an extended period was used. The addition of hydrogen peroxide was not an effective method in addressing these contamination issues, and it was determined that recently collected thin stillage could achieve high biomass yields (>30 g/L) without the use of a chemical disinfectant. Since it is unlikely that thin stillage would be stored for longer than 2 weeks in commercial applications, thin stillage itself would not pose an obvious source of contamination. Rather, future research at pilot and commercial-scale should focus on disinfection of the bioreactor. Ensuring a controlled environment, as performed at lab-scale using autoclaved equipment, would improve biomass yield consistency. Since autoclaving equipment at commercial-scale is not practical, use of other disinfection methods should be studied.

Fungal biomass harvested from the pilot-scale bioreactor was successfully converted to biocrude using a continuous-flow HTL system. Biocrude yields of approximately 60% daf were attained even when reaction temperatures were as low as 300 °C. The biocrude was also determined to be similar in composition to biocrude seen in other studies using microalgae (approximately 13% oxygen, 3% nitrogen), and have high HHVs close to that of petroleum fossil fuel values (37 MJ/kg). The overall pilot-scale process produced approximately 5.5 g of biocrude

for every L of thin stillage. At a mid-sized corn ethanol plant (50 MGY), these observations could result in over 4,000 tons of biocrude per year. This biofuel process has the potential to generate larger profits at plants, improve energy return on investment, and reduce environmental impact.