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Improvements in non-aseptic methods for fungal cultivation on corn-ethanol thin stillage and continuous hydrothermal liquefaction of fungal biomass feedstock

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Improvements in non-aseptic methods for fungal cultivation on corn-ethanol thin stillage and continuous hydrothermal liquefaction of fungal biomass feedstock

by

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ABSTRACT

Cultivation of *Rhizopus oligosporus* and *Mucor circinelloides* on corn-ethanol thin stillage has been reported to significantly remove COD, TSS, glycerol and organic acids; making in-plant water reuse much more viable while producing a protein-rich animal feed biomass with a high oil content and valuable fatty acids. As fungal biomass cultivation is scaled up from lab to bench and pilot scale, aseptic methods become difficult and cost prohibitive. Non-aseptic fungal cultivation on thin stillage has been reported to have fungal and bacterial contaminants resulting in reduced biomass yields. Chemical oxidizers sodium hypochlorite, chlorine dioxide, iodophor and ozone along with antibiotics penicillin-streptomycin were tested as potential selective disinfectants to enhance fungal biomass yields.

Fungal biomass grown on thin stillage also has the potential to be a promising feedstock for advanced biofuels due to its short 2-3 day fermentation period and high oil content. Drop-in biofuel consumption is projected to more than double from 2014-2019. Fungal biomass feedstock for thermochemical processing to produce bio-crude oil that is upgradable to drop-in renewable diesel could help to meet this increasing demand. Suspended growth fungal biomass cultivated in thin stillage has a high moisture content making aqueous-phase processing through hydrothermal liquefaction (HTL) a favorable thermochemical process for generating biofuels compared to dry feedstock required for traditional gasification and pyrolysis. Continuous HTL of fungal biomass using a 1.5L supercritical flow reactor to produce bio-crude oil was accomplished. The fungal bio-crude oil was then analyzed and compared with microalgae bio-crude oil produced by like methods.

CHAPTER I

INTRODUCTION

1.1 Introduction

Total biofuel consumption is expected to grow from 14 billion gallons to 16 billion gallons from 2012 to 2022, respectively (US EIA, 2014). With the sustained projected ethanol production, the volume of ethanol by-products like thin stillage and dried distillers grains are also projected to be stable. 5-6 gallons of stillage per gallon of ethanol are produced at a conventional dry-grind ethanol plant, approximately half of which is recycled directly as backset (Ethanol Producer Magazine, 2006).

Innovations in corn ethanol efficiencies and higher value byproducts stand to improve the ethanol biofuel industry. *Rhizopus oligosporus* and *Mucor circinelloides* fungal growth has been reported to have numerous benefits for thin stillage treatment and produce valuable products. *Rhizopus oligosporus* cultivation on thin stillage has been reported to remove 80% COD, 98% suspended solids, 100% glycerol, and 100% organic acids making in-plant water reuse much more viable (Rasmussen et al., 2014).

Fungal biomass (*Mucor circinelloides* and *Pythium irregulare*) cultivated corn ethanol thin stillage have been shown to produce oil containing eicosapentaenoic acid (EPA) polyunsaturated fatty acids (Ravi, 2014, Liang et al., 2012). EPA is a high-value omega-3-polyunsaturated fatty acid that has important human health properties and is predominantly obtained from fish oil (Simopoulus, 1999). *Rhizopus oligosporus* cultivated on thin stillage could also be a viable protein rich animal feed (Rasmussen et al., 2014; Van Sambeek et al., 2012).

However, non-aseptic *Rhizopus oligosporus* and *Mucor circinelloides* fungal cultivation on thin stillage has had issues with competing bacterial and fungal contaminants (Erickson, 2012). Thin stillage that has not been heat sterilized especially when stored for periods of 15 days or more, have reported to contain bacterial contamination resulting in decreased fungal biomass yields (Mitra et al., 2012). Using selective disinfection to improve biomass yields and reduce bacterial counts for *Rhizopus oligosporus* non-aseptic fungal cultivation has been reported to be effective (Miao, 2005; Sankaran et al., 2008). With the high value potential of fungal biomass cultivated on ethanol thin stillage but with the challenges of microorganism contamination, attempts to improve mono-cultivation of *Rhizopus oligosporus* and *Mucor circinelloides* biomass yield through a variety of oxidizing disinfectants and antibiotics is described in Chapter 3.

US consumption of "drop-in" biofuels at 135 million ethanol gallons equivalent in 2014 is expected to grow by a factor of 2.4 by 2019 (US EIA, 2014). "Drop-in" biofuels differ from renewable biofuels in that they do not need to blended with petroleum products; but rather, can be drop in replacement for diesel or gasoline. The potential for fungal biomass as a potential feedstock for biofuels has been reported, *Mucor circinelloides* fungal biomass has a suitable fatty acid profile for biodiesel production (Vicente et al., 2009). The opportunity for an ethanol plant to turn a low-value thin stillage byproduct into high-value bio-crude oil which could be upgraded to drop-in renewable diesel has the potential help meet this increasing drop-in fuel demand. Readily harvestable co-cultures of algae and filamentous fungal have been reported to produce wet slurries suitable for hydrothermal gasification (Mackay et al., 2015). Chapter 4 describes continuous hydrothermal liquefaction performed using fungal biomass feedstock to produce bio-

crude oil. The fungal bio-crude oil was then analyzed and compared with microalgae bio-crude oil produced by like methods.

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CHAPTER 2

NON-ASEPTIC FUNGAL CULTIVATION

A Literature Review

Abstract

This literature review focuses on fungi competition for limited resources with bacteria and other fungi species. Fungi compete with bacteria in the rhizosphere for plant exudates and decomposing lignocellulosic material. Bacteria competing with fungi produce antifungal enzymes chitinase and siderophore iron chelating enzyme to starve fungi of available iron. Lactic and acetic acid producing bacteria have been reported to inhibit ethanol producing fungi in the biofuel fermentation industry. Fungi defenses and beneficial bacterial relationships with fungi are also discussed. Chitinase and β-1, 3 glucanase are important enzymes in mycoparasitic fungi activity. Enzyme activity has been shown to be affected by metal concentrations. A study on fungal competitive growth and preferences for food sources precolonized with other fungal species reported by Boddy and Abdalla (1998) is discussed in detail.

2.1 Introduction

Achieving consistent yields of *Rhizopus oligosporus* and *Mucor circinelloides* has proved challenging as discussed in detail in Chapter 3. Biomass has been the measurement made on each fungal cultivation batch; however, fungi can use the same amount of biomass to conduct different types of search patterns (Boddy, 2000). Therefore, although fungal biomass production can be measured, little of the fungal biological activities are known. Previous *Mucor circinelloides* fungal cultivation of thin stillage exceeding 15 days of storage at 5°C was observed to have 75% reduction in biomass yields attributed likely to psychrophilic bacteria

(Mitra, 2012). *Bacillus spp.* bacteria and fungi have been identified as bioreactor contaminants during *Rhizopus oligosporus* fungal monoculture on thin stillage (Erickson, 2012). Competition between fungi and bacteria are well known in the rhizosphere and have also been reported in biofuel ethanol fermentation. One fungus can replace another as the dominant species for an available nutrient source through competitive interactions through antagonistic volatile organic compound production (Boddy, 2000). Mycoparasitic and antagonistic fungi such as *Trichoderma spp*. and *T. harzianum* are known antifungal biocontrol fungi (Lorito et al., 2008). Aside from microorganism competition, soil oxygen content, organic matter, pH and composition have been reported as important factors in natural flora selection (Ross et al., 2000).

Chitinases and β-1, 3 glucanase are enzymes known for fungi cell wall degrading and/or preventing hyphal growth (Gohel et al., 2006, 2004,; Vaidya et al., 2001; Cruz et al.1992, 1995; Shen et al., 1991; de Boer et al., 2008). Bacteria utilize chitinase for parasitism of fungi, nutrition and mineralization of chitin (Gohel et al., 2006). However, the role of chitinase depends on the organism it is found in. For plants chitinase is used as a defense against fungal and bacterial phytopathogens. Chitinase in fungi are used in cell division, differentiation and mycoparasitic nutrition (Gohel et al., 2006). B-1, 3 glucanase has been determined in plants to have antifungal role (Grenier et al., 1993; Mauch et al.; 1988); however, bacteria usually lack β-1, 3 glucanase and a nutritional role in bacteria for β-1, 3 glucanase has been established (Watanabe et al., 1992). β-1, 3 glucanase in fungi have a physiological role during fungal development (Rapp, 1992; Stahman et al., 1992), mobilization of β-1, 3 glucans and a nutritional role (Chet, 1987; Lorito et al., 1994; Sivan and Chet, 1989).

Siderophore and cyanide are antifungal enzymes produced by bacteria during competitive growth (Ellis et al., 2000; Cornelis and Matthijs, 2002). Siderophore is an iron-chelating

compound that acts deprives the fungus of iron nutrition, thus inhibiting the fungal growth (Scher and Baker, 1982). The siderophore acts to inhibition the conidial germination or early germ tube elongation (Sulochana et al., 2013).

2.2 Review and Discussion

2.2.1 Bacterial Competition

A. General

Bacteria are the by far the prevailing decomposers in aquatic systems (del Giorgio et al., 1998). However, in terrestrial microbial systems bacteria have not had such a dominant role but have competed more evenly with fungi. Cellulose degradation in anoxic conditions is nearly wholly performed by anaerobic bacteria (Leschine et al., 1995; Lynd et al., 2002). Aerobic decomposition of cellulose, on the other hand, is performed by fungi, filamentous bacteria and non-filamentous bacteria (Lynd et al., 2002). However, in general it is considered that by far most aerobic degradation of cellulose is accomplished by fungi (de Boer et al., 2005). Bacteria isolated from fungal rich rhizosphere soils have a higher occurrence of antifungal properties, supporting that a selection pressure naturally occurs in fungal rich environments (de Boer et al., 2008). Recalcitrant organic matter such as lignocellulosic material has primarily been decomposed by fungi (de Boer et al., 2005). In land systems, bacteria were thought to nearly exclusively degrade simple substrates such as plant exudates (de Boer et al., 2005); however, recent publications show that fungi may have an important role in degrading root exudates (Butler et al., 2003; Treonis et al., 2004). Selective respiration studies have shown the potential

for fungi to compete for simple substrates; however, there have been no studies showing the competitive strength fungi have compared to bacteria (de Boer et al., 2005).

B. Chitinase and siderophore producing bacteria

Bacteria isolated from fungi rich soils showed antifungal properties in in vitro tests and production of siderophores, cyanide and lytic antifungal enzymes as shown in Figure 2.1 (de Boer et al., 2008). *Fluorescent pseudomonads* are one of the most widely studied bacteria found in the rhizosphere (Gohel et al., 2006). In a study by Verma et al. (2007) soil reactions were assessed the antifungal activity of *fluorescent Pseudomonads* found in the soil rhizosphere. Figure 2.2 (Verma et al., 2007) shows a pH of 5.2 had higher antifungal affects than at 5.8 or 6.4. Chitinase enzyme production at pH 5.2 was observed to be greatly reduced when the pH was increased to 5.8 and 6.4 as shown in Figure 2.3 (Verma et al., 2007). Siderophore and chitinase production was reported to decrease corresponding to a decreased inhibition of mycelial growth (Verma et al., 2007). This is logical since chitinase is a known antifungal enzyme (Gohel et al., 2006) as well as siderophores (Sulochana et al., 2013). Siderophore from *P. aeruginosa* antifungal activity have been tested by well-plate assay and reported zones of inhibition for *Fusarium oxysporium f.* sp. *Cicero, Fusarium udum* and *Aspergillus niger* (Sulochana et al., 2013). Siderophore at a concentration of 1 µg/L from *P. aeruginosa* was reported to be antifungal (Siddiqui and Shaukat, 2003). Verma et al. (2007) reported that although all of the *fluorescent Pseudomonads* produced varying levels of siderophore, only the isolates that produced chitinases showed fungal inhibition. Suggesting that chitinases is a much more deleterious enzyme to fungi.

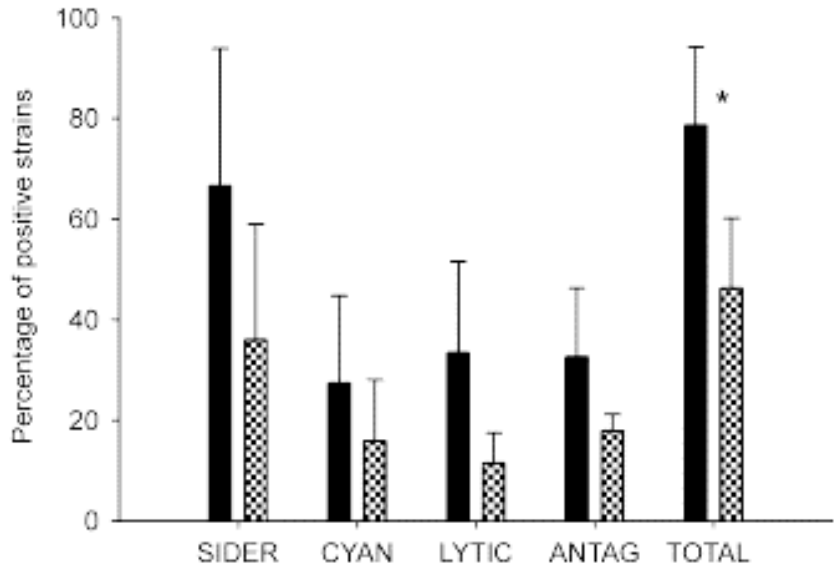

Figure 2.1. Data represent the mean and standard deviation of bacteria isolated from fungal-rich soils (black bars) and fungal-poor soils (checkered bars). SIDER: siderophore producing strains; CYAN: cyanide producing strains; LYTIC: strains producing potential fungal cell wall lytic enzymes (chitinase and/or β-1, 3-glucanase); ANTAG: strains showing in vitro antagonism (de Boer et al., 2008)

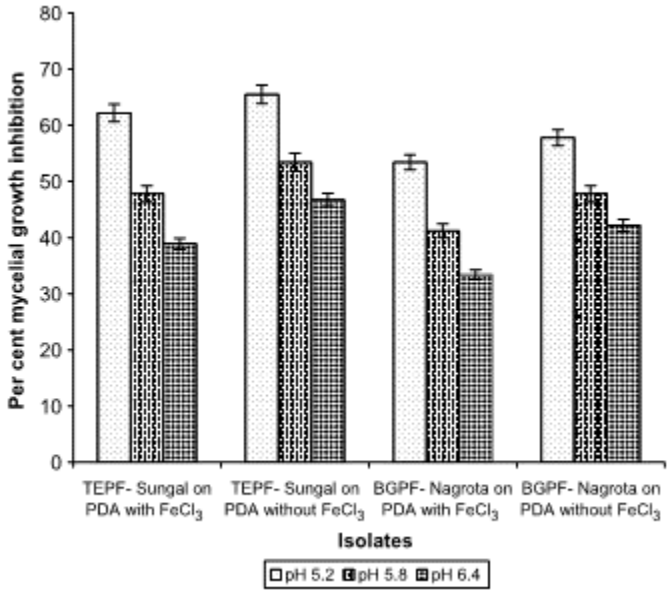

Figure 2.2. Antagonism of *fluorescent Pseudomonads* in presence and absence of $FeCl_3$ at different pH levels (Verma et al., 2007)

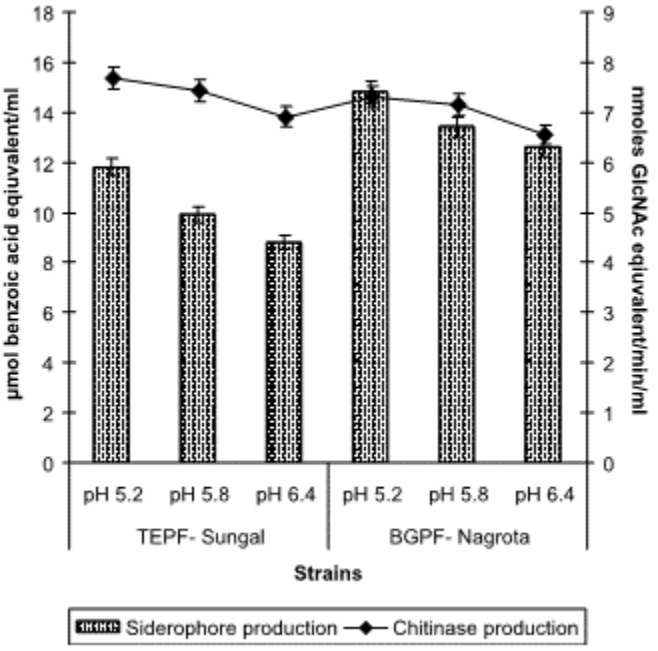

Figure 2.3. Siderophore and chitinase production by *fluorescent Pseudomonads* at different pH (Verma et al., 2007)

Frankowski et al. (2001) studied chitinolytic enzymes CHIT60 and CHIT100 isolated from *Serratia plymuthica* bacteria and were reported to inhibit to spore germination and germ tube elongation. CHIT 60 had peak enzyme activity observed at 55°C and pH 5.4. Ca^{2+}, Co^{2+} or Mn^{2+} at 10mM concentration showed a 20% increase in activity. A 10mM of Cu^{2+} inhibited the activity of CHIT60. CHIT 100 was reported an optimal activity at a temperature of 43°C and pH 6.6. A 90% decrease the enzyme activity for CHIT reported with a 10mM Co^{2+} or Cu^{2+}. The inhibition of CHIT 60 and CHIT 100 on fungi *Botrytis cinerea* was approximately 30% and 70%, respectively.

C. Lactic and acetic acid producing bacteria

Bacteria have been known to compete with yeast for nutrient sugar substrates in fuel ethanol fermentation process (Skinner and Leathers, 2004). Increased lactic acid concentrations during contamination indicate that lactic acid producing bacteria are the likely biological contaminants (Skinner and Leathers, 2004). Lactic acid producing bacteria are thought to be the dominant bacterial contaminant and have been species of *Bacillus subtilis* and *Bacillus cereaus* have been shown in lab tests to be inhibitory to ethanol producing yeasts (Manitchotpisit, 2013) Ethanol production facilities commonly monitor lactic and acetic acid level as a means to determine bacterial contamination (Skinner and Leathers, 2004). Numerous artificial infections studies have been done to show that various species of *Lactobacillus* bacteria increase lactic acid concentrations and reduce ethanol production, (Makanjuol et al., 1992; Essia Ngang et al., 1990; Oliva-Neto and Yokoya, 1994; Narendranath et al., 1997). Samples from two dry-grind fuel ethanol facilities over a nine month period often reached 10^8 bacteria/ml with *Lactobacillus* species being the most predominant (Skinner and Leathers, 2004). Dosages of antibiotics at various levels at the dry-grind ethanol facilities reliably reduced overall contaminations (Skinner and Leathers, 2004).

D. Fungi antibacterial defense

Although bacteria produce a number of inhibitory and nutrient sequestering enzymes, fungi have produced compounds with antibacterial activity to combat bacterial antagonism. Fungi employ detoxification, effluxion for the flushing of antibiotics and modification of bacterial gene expression to combat bacterial assaults (Duffy et al., 2003). Fungal exudation of

strong organic acids have been known to acidify the substrate area and inhibit bacterial strains (Goodell, 2003).

E. Beneficial bacteria for fungi

In the case where available carbohydrates are not the limiting growth factor for fungi, bacteria have been in some cases shown to be beneficial (de Boer et al., 2005). Bacteria and white-rot fungi co-inoculation of wood blocks showed consistently higher decomposition of the wood than monocultures of white-rot fungi tests even though the bacteria could not be directly accredited for any degradation of the wood (Murray and Wooward, 2003). The bacteria may have produced fungi essential vitamins or increased fungal enzyme activity through consumption of a fraction of the breakdown products (de Boer et al., 2005). Bacteria can also be beneficial through increasing available nitrogen via nitrogen fixation or through degrading fungi toxic solutes (Greaves, 1971; Jurgensen et al., 1989; Hendrickson, 1991).

2.2.2 Fungal Competition

Chitinase and β-1, 3 glucanase important enzymes in in cell wall lysis in mycoparasitic activity (Cruz et al.1992, 1995; Shen et al., 1991). Chitinase is an important enzyme in bacterial competition; however, bacteria usually lack β-1, 3 glucanase (Watanabe et al., 1992). El-Katatny et al. (2001) isolated and purified chitinase and β-1, 3 glucanase from *T. harzianum* fungi were tested for the effectiveness and stability. The half-life of *T. harzianum* chitinase increased from 5 hours to 20 hours with a corresponding pH of 3 to 6 (El-Katatny et al. 2001). β-1, 3 glucanase from *T. harzianum* stability also increase with pH. The half-life of β-1, 3 glucanase increased from 6 hours to 30 hours with a pH of 3 compared to 7 (El-Katatny et al. 2001). Various

compounds affect the activity of chitinase and β-1, 3 glucanase. Table 2.1 shows the effect of different chemical additives on the activity chitinase and β-1, 3 glucanase from *T. harzianum* (El-Katatny et al. 2001). Mercury and to a lesser extent iron proved to greatly reduce and chitinase and β-1, 3 glucanase activity (El-Katatny et al. 2001). Chitinase and β-1, 3 glucanase have a synergistic effect of against certain fungi (El-Katatny et al. 2001).

Table 2.1. Effect of various compounds on chitinase and β-1, 3-glucanase from *T. harianum* (El-Katatny et al., 2001)
[a] 100% activity corresponds to 39.1 pkat ml^{-1} (chitinase) and 8.5 nkat ml^{-1} (β-1, 3-glucanase)

Items	Concentration	Relative activity(%)[a]	
		Chitinase	β-1,3-Glucanase
None	–	100.0	100.0
$CaCl_2.2H_2O$	5 mM	100.0	97.3
$CuCl_2.2H_2O$	5 mM	123.8	100.4
$FeSO_4.7H_2O$	5 mM	52.0	79.0
KCl	5 mM	88.0	100.0
$MnCl_2.4H_2O$	5 mM	133.3	132.0
$NiCl_2.6H_2O$	5 mM	104.9	98.7
NaN_3 (sodium azide)	5 mM	114.0	97.2
$ZnSO_4.7H_2O$	5 mM	98.0	98.7
EDTA	5 mM	88.8	91.7
EDTA	10 mM	82.0	91.7
$HgCl_2$	5 mM	10.0	6.6
SDS	5 mM	76.0	86.2
$HgSO_4$	5 mM	15.0	7.3
Urea	5 mM	133.0	86.2
Cycloheximide	5 mM	113.0	97.2
Hydrazinum sulphate	5 mM	105.0	106.4
LiCl	5 mM	79.0	95.4
Glucono-δ-lactone	5 mM	100.0	100.0
Glucono-δ-lactone	10 mM	100.0	97.2

A study performed by Boddy and Abdalla (1998) compared *Phan. veluntina* fungi competition with *B. adusta* precolonized and *X. polymorpha* precolonized on agar plates with woodblock food sources. Boddy and Abdalla (1998) reported their findings on fungi competitive growth for food source that had been precolonized with a competing species. As shown in Figure 2.4 (Boddy and Abdalla, 1998)

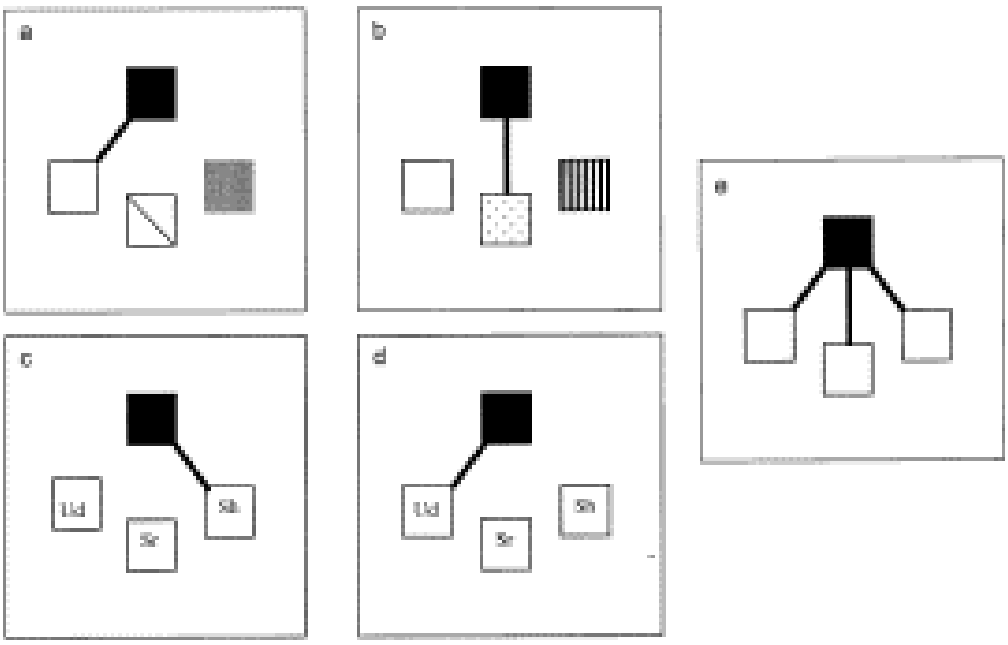

Figure 2.4. Schematic diagram illustrating the general patterns of colonization of three resource baits (4 cm3) by *Phanerochaete velutina* after 60 days. Lines connecting inocula and baits represent persistent cords. In all treatments: ■,inoculum; □, uncolonized baits; Sh, *Stereum hirsutum*; Ud, *Ustulina deusta*; and Sr, *Stereum rugosum*. (a) baits from left to right are uncolonized, precolonized for 6 weeks and precolonized for 2 years; (b) baits from left to right are uncolonized, precolonized for 6 weeks then autoclaved and precolonized for 2 years then autoclaved; (c) baits precolonized for 6 weeks; (d) baits precolonized for 2 years; (e) control. (Boddy and Abdalla, 1998)

P. velutina preferred uncolonized food sources over food sources that had been precolonized for six weeks or two years (Fig. 2.4, a). Interestingly, the *P. velutina* preference order changed when the precolonized food sources had been autoclaved after being precolonized. *P. velutina* preferred the 6 week precolonized then autoclaved food source over the uncolonized and the 2 year precolonized then autoclaved food source (Figure 2.4, b). *P. velutina* also showed a preference depending on the type of fungus and how long the food source had been precolonized. When the food source had been precolonized for six weeks *P. velutina* preferred *Stereum hirsutum* precolonized food sources and did not colonize the other two precolonized

food source (Figure 2.4, c). However, when the food source had been precolonized for 2 years, *P. velutina* showed only a slight preference for food sources that had been precolonized with *Ustulina deusta* (Figure 2.4, d) by either having more connective mycelia or by being more colonized with *P. veluntina*. The preference for either the uncolonized or 6-week precolonized food sources could be due to the 2-year precolonized food source being better established by the competing in fungi and *P. veluntina* must use antagonistic mechanisms to overtake the food source or it is possible that *P. veluntina* may be able to sense that the lower carbon content of the food source that has been precolonized longer.

2.3 Conclusion

Terrestrial vs. aquatic systems, recalcitrant vs. simple substrates and anaerobic vs. aerobic environments play an important role determining whether fungi vs bacteria are likely to be predominant decomposers of organic matter. Chitinase is a highly inhibitory antifungal enzyme produced by both bacteria and competing fungi. Siderophore an iron chelating compound and cyanide are antifungal enzymes produced by bacteria. β-1, 3 glucanase is an enzyme for cell wall lysis produced by mycoparasitic fungi. Increased lactic acid concentrations are a good indicator of lactic and acetic acid producing bacteria contamination of fungal monocultures. In certain nutrient deficient situations, or when breakdown products become inhibitory, bacteria may benefit fungal growth. Fungi defend against bacteria through acidification, flushing of antibiotics for detoxification and producing antibacterial compounds. In fungi-to-fungi competition, the species of competing fungi, how long the competing fungi has inhabited a food source, if the food source has been autoclaved after preinoculation with the

competing fungi are all important factors in determining if the fungi will overtake its competitors and which food source the fungi will prefer.

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CHAPTER 3

SELECTIVE DISINFECTION FOR NON-ASEPTIC FUNGAL CULTIVATION ON CORN-ETHANOL THIN STILLAGE

Abstract

Mucor circinelloides and *Rhizopus oligosporus* fungal biomass non-aseptic corn-ethanol cultivation on thin stillage substrate was attempted to be improved using selective disinfects in lieu of heat sterilization pretreatment of the thin stillage. The chemical disinfectants attempted were: sodium hypochlorite, chlorine dioxide, iodophor, penicillin-streptomycin (p-s) and ozone. The chemical disinfectants tests were done at the 250mL and 2L-scale. 2L-scale test were observed to have approximately one third the average fungal biomass yields (3.4-5.8 g/L (dry)) compared to the 250mL-scale (9-20.4 g/L (dry)). Aerating the 2L-scale using pure oxygen instead of air was found to greatly increase yields and bringing them to within the range observed at the 250mL-scale. No discernable optimal or inhibitory dosage was observed for sodium hypochlorite dosage range of 24-216 mg/L. *Mucor circinelloides* with chlorine dioxide dosages of 9-90 ppm showed increased fungal biomass yields ranging from 15-25 g/L (dry) compared to control flasks averaging 10 g/L (dry). Penicillin-streptomycin at the 250mL-scale with a dosage of 25-100 units/mL of penicillin and 25-100 µg/mL of streptomycin increased yields over heat sterilized thin stillage by a factor of 1.0-1.4 and non-autoclaved control by a factor of and 1.3-2.4, respectively. The 400 ppm dosage of iodine was observed to be optimal and produced yields 13% higher than the autoclaved control flasks and 60% higher than non-autoclaved control flasks. However, iodine dosages of 600 ppm and greater proved to be toxic to

fungal growth. The 10 mg/L/h ozone dosage had an average biomass yield of only 10% lower than autoclaved control flasks.

3.1 Introduction

At a dry-grind corn-ethanol plant, ethanol is separated and recovered through the process of distillation. Ethanol exits the top of the column and whole stillage containing less than 0.1% ethanol exits the bottom. The whole stillage solids are removed through centrifugation. 5-6 gallons of the liquid centrate, known as thin stillage, per gallon of ethanol are produced at a conventional dry-grind ethanol plant (Ethanol Producer Magazine, 2006). The total US ethanol production of 13 billion gallons in 2012 (Rasmussen, 2014), also produces approximately 72 billion gallons of thin stillage byproduct. Approximately half of the thin stillage is recycled as backset (Ethanol Producer Magazine, 2006); therefore, a remaining 36 billion gallons per year of thin stillage must be condensed to a syrup. The syrup (containing 30% solids) is typically added to the wet distillers' grains and co-dried to produce dried distillers' grains with solubles (a.k.a. DDGS) (RFA, 2013). However, demand for syrup addition to distillers' grains has declined due to its low nutritional value, causing a very low market price and at times is even given away (Johnson, 2013).

Rhizopus oligosporus and *Mucor circinelloides* fungal biomass cultivated on low-value corn-ethanol byproduct thin stillage substrate has the potential to produce a variety of high-value products while reclaiming the thin stillage by removing COD, suspended solids, glycerol and organic acids to obtain a recyclable effluent (Rasmussen et al., 2014). The *Mucor circinelloides* can produce bio-oil containing eicosapentaenoic acid (EPA) polyunsaturated fatty acids (Ravi, 2014). EPA is a high-value omega-3-polyunsaturated fatty acid that has important human health

properties and is predominantly obtained from fish oil (Simopoulus, 1999). *Rhizopus oligosporus* has been demonstrated to be potential protein rich animal feed (Rasmussen et al., 2014; Van Sambeek et al., 2012).

Previous research cultivating *Rhizopus oligosporus* and *Mucor circinelloides* fungus on ethanol plant byproduct thin stillage that has not been heat sterilized prior to inoculation have had bacterial contamination causing reduced fungal biomass yields. Thin stillage stored at 4 °C for >15 days has been reported to produce 75% lower yields at 5 g/L (dry); which, has been attributed to psychrophilic bacteria propagated during cold storage (Mitra et al., 2012). Devastating effects *Bacillus* bacteria contamination at the 1600L pilot-scale caused fungal biomass deterioration from 7 g/L (dry) to 0 g/L (dry) in 24 hours (Erickson, 2012). Gram positive bacteria *Bacillus cereus* and *Bacillus megaterium* and yeasts *Rhodotorula mucilaginosa* and *Pichia kudriavzevii* were identified as bioreactor contaminants during *Rhizopus oligosporus* fungal cultivation on thin stillage (Erickson, 2012).

Disinfecting large volumes of thin stillage substrate by autoclaving at 121°C for a process scaled-up to the 1600L pilot-scale or larger is energy intensive and costly. The thin stillage after being autoclaved must be cooled to <40 °C prior to inoculation which again would be energy intensive and costly. The aim of this research was to test various chemical disinfectants as a selective disinfectant to determine their effectiveness at maximizing fungal biomass yields for potential scale up.

The chemical disinfectants attempted for selective disinfection were: sodium hypochlorite, chlorine dioxide, iodophor, penicillin-streptomycin and ozone. The chemical disinfectants attempted were then compared with thin stillage sterilized by autoclaving at 121°C and non-sterilized control flasks. Sodium hypochlorite and ozone have been successfully

demonstrated as selective disinfectant for non-aseptic *Rhizopus oligosporus* fungal production on corn processing wastewater substrate (Miao, 2005; Sankaran et al., 2008). At a 10 mg/L sodium hypochlorite concentration bacterial populations were observed to be reduced by 50% and fungal biomass yields increased by 39% (Miao, 2005). Ozonation at a dosage of 57 mg/L of wet corn-milling wastewater for selective disinfection against bacteria for non-aseptic *Rhizopus oligosporus* fungal production reduced bacterial population optimized fungal biomass causing a 20% increase in yields (Sankaran et al., 2008). Lactic and acetic acid producing bacteria have been identified as primary bacteria contaminants in corn grain fuel ethanol production (Skinner and Leathers, 2004). Penicillin and virginiamycin are commonly used commercially available antibiotics for ethanol production sold to treat bacterial contaminants (Hynes et al., 1997; Bayrock et. al., 2003). Chemical solutions containing chlorine dioxide precursors are commercially available and are marketed to attack lactic and acetic acid producing bacteria (Sumner, 2013). Therefore, antibiotics penicillin-streptomycin and chlorine dioxide selective disinfectants were attempted to improve fungal biomass yields. Iodophors have been reported to be bactericidal, virucidal and mycobactericidal, but may require longer contact times to kill bacterial spores and certain fungi (Rutala, 1996). Therefore iodophor may be an affective selective disinfectant against bacteria.

3.2 Methods and Materials

3.2.1 Thin Stillage

Thin stillage was obtained from Lincolnway Energy LLC dry-grind ethanol plant located seven miles east of the Iowa State University campus. The Lincolnway Energy performs process control HPLC analysis on the thin stillage. The general range of the constituents values from the

test reports obtained (n=5) is shown in Table 3.1. Since fresh thin stillage is obtained for each test run the constituents of the thin stillage growth substrate would vary from batch to batch.

Table 3.1. Thin Stillage HPLC Analysis (from five samples obtained from Lincolnway Energy 5/31/2014 – 2/10/15)

Constituent	Concentration (wt. %)
DP4+	0.746 – 0.863
DP3	0.107 – 0.158
Maltose	0.523 – 0.669
Glucose	0.019 – 0.238
Lactic Acid	0.155 – 0.424
Glycerol	1.742 – 1.795
Acetic Acid	0.079 – 4.689
Ethanol	0.043 – 0.076

Thin stillage was transported from the ethanol plant to the laboratory using 8 L, 10 L and 20 L polypropylene Nalgene™ carboy jugs. The carboy jugs were washed using soap and water, after each use. Within 24 hours prior to transporting the thin stillage the carboy jugs were sterilized by steam autoclave for a minimum of 15 minutes at 121°C.

Thin stillage was transferred to the carboy jugs from the thin stillage storage tank. The storage tank circulation pump effluent piping has a sampling valve that can be opened to divert thin stillage into the carboy jugs. The thin stillage temperature was 50-60 °C at the time it was obtained. The thin stillage was then placed in 10 °C cold storage with the lids left partially open to vent during cooling. The thin stillage was cooled to a temperature from 10 °C to 40 °C.

3.2.2 Fungal Spore Description, Cultivation and Storage

Rhizopus oligosporus and *Mucor circinelloides* were obtained from the American Type Culture Collection (Rockville, MD). Fungal spores were stored in 2 mL vials kept in an ultra-

low freezer set at -80°C. The ultra-low freezer was a So-Low™ model number U85-18. Fungal spores were grown on agar solution and the collected into vials. The fungal spore cultivation procedure follows the procedure described by Ozsoy et al. (2008) under aseptic conditions and have been reported to produce a spore count of 10^6-10^7 spores per mL as determined by haemocytometer counts. The agar powder was HIMEDIA™ RM301, CAS. No. 9002-18-0 41g/L (dry). The agar powder chemical analysis performed by the manufacturer is shown in Table 3.2. The agar solution was prepared by adding 41 g/L (dry) of agar powder to deionized water and homogenized on a hot plate. The agar solution was autoclaved and allowed to cool to near room temperature. Then 15-20 mL of agar solution was added to each agar plate. The agar plates were then left at room temperature for 24 h right-side up in a fume hood. In a HEPA laminar flow hood the agar plates were inoculated by adding 100 µL of frozen spores to each plate. The spores were then spread evenly across the agar plate using a glass hockey stick. The inoculated spore plates were then incubated for 3-4 days at 30 °C. For the first 24 hours the agar plates were right-side up, for the remaining incubation period the agar plates were orientated upside down. 10mL of autoclaved deionized dilution water was added to each agar plate. The dilution water contains 0.85% (w/v) sodium chloride, 0.05% (v/v) Tween 80®. Tween 80® is a nonionic surfactant and emulsifier manufactured my Sigma-Aldrich. The dilution water was mixed with the spores grown on each of the agar plates and transferred to a 100mL beaker. The collected spore solution is then poured into a 50-60 cc syringe loosely packed with approximately 30cc of glass wool. The glass wool retains the mycelia and passes the fungal spores. The approximately 100mL spore containing filtrate was collected into a 200 mL beaker. The spore containing filtrate is then diluted with YM broth at 1:1 ratio. Glycerin was added to the

spore containing filtrate at a ratio of 20% of the undiluted filtrate volume. The spore solution was continually magnetically stirred while 2 mL of spore solution were withdrawn using a micropipette and transferred to 2mL Nalgene™ Cryoware vials (Thermo Scientific, #5000-0020). The 2 mL vials were than labeled and placed and stored at -80 °C in a So-Low™ model number U85-18 ultra-low freezer.

Table 3.2. Agar Powder (HIMEDIA™ RM301) Chemical Analysis

Description	Value Range
Gelling Temperature	34-36°C
Melting Range	≥85°C
Water (KF)	≤20%
Calcium	≤0.1%
Heavy Metals (as Pb)	≤40ppm
Lead	≤10ppm
Arsenic	≤3ppm
Total Ash	≤6.5%
Acid insoluble matter (on dry basis)	≤0.5%
Foreign organic matter	≤1.0%
Foreign insoluble matter	≤15 mg in 7.5 gm of Agar

3.2.3 Fungal Spore Germination on YM Broth

Fungal spore germination followed the general inoculum preparation procedures described by Mitra et al. (2012). Prior to inoculating the thin stillage the fungal spores were germinated by inoculating yeast malt (YM) broth solution followed by incubation. YM broth powder was manufactured by Alpha Biosciences Inc. catalog number Y25-108, lot number H08-37. The YM broth powder has been microbiologically tested by the manufacturer for quality control to support growth for the following microorganisms: *Aspergillus niger, Candida albicans, Escherichia coli, Lactobacillus casei, Saccharomyces cerevisiae*. The YM broth

solution was prepared within 24 hours of being inoculated with fungal spores. The YM broth solution was prepared by adding 21g/L (dry) of YM broth powder to deionized water in a flask. The YM broth powder was stirred and heated using magnetically stirred hot plate to dissolve the powder in the deionized water. The flask was then covered with Kimberly-Clark™ KC200 Kimguard™ sterilization wrap. The sterilization wrap was secured to the flask with a rubber band. Then the covered flask containing the YM broth solution was steam autoclaved for 45 minutes at 121°C. The YM broth was then allowed to cool for 24 hours to a temperature of 10-40°C.

The YM broth was inoculated with fungal spores in a HEPA filter laminar flow hood. One 2mL vial of frozen fungal spores was removed from the -80°C freezer storage and thawed at room temperature. The laminar flow hood surfaces were sterilized using a UV lamp prior to each use. The YM broth flask was placed in the laminar flow hood. The sterilization wrap cover was briefly removed and one 2 mL vial of fungal spores was added to 1L (or less) of YM broth solution. The sterilization wrap was then immediately replaced on the flask. The covered flask of inoculated YM broth solution was then removed from the laminar flow hood and placed in incubator shaker. The incubator shaker was maintained at a temperature of 34±3°C and an orbital shaking speed of 200 revolutions per minute (rpm). The incubator shaker is a floor model Lab Companion™ model IS-971 with a 270 L internal working volume. The inoculated YM broth was shaken and incubated for 24-48 hours to allow for fungal spore germination.

3.2.4 Thin Stillage Fungal Inoculation and Fermentation

A. General

Once the frozen fungal spores have germinated on YM broth they are then ready for inoculating the thin stillage sample. The spores in the YM broth grew out into pellets or filamentous flocs. The fungal cultivation on thin stillage substrate was performed at the 250mL-scale and 2L-scale. Optimal biomass yields of *Mucor circinelloides* grown on thin stillage have been reported at a pH of 4-7 (Mitra et al., 2012). The initial pH of the thin stillage ranged from 4-6 and was therefore not adjusted prior to inoculation. Mitra et al. 2012 reported *Mucor circinelloides* fungal biomass yields of 19.2 g/L (dry) at 30°C grown for five days and 20.0 g/L (dry) at 37 °C grown for two days. Although Mitra et al. (2008) found that 37°C was the optimal temperature for optimizing fungal biomass yields, 30°C only had reduction in yield of 0.8 g/L (dry). The fungal growth tests performed in this study were conducted at 34 °C ± 3 °C. The method of inoculation, heating and aeration were different for the 250mL-scale and 2L-scale.

At the 250mL-scale the reactors consisted of glass 250 mL Erlenmeyer flasks. The flasks were cleaned with soap and water after each use and autoclaved within twenty four hours prior to thin stillage addition. Each 250mL-scale reactor was filled with 100 mL of thin stillage. The 250mL-scale reactors were inoculated by transferring 5mL of the YM broth fungal flocculent solution to the 250mL-scale reactor using a micropipette. The YM broth fungal flocculent solution was continuously mixed during the transfer. Each of the 250mL-scale reactors were then covered with Kimberly-Clark™ KC200 sterilization wrap. The inoculated 250mL-scale reactors were heated and shake aerated during fermentation by a floor model Lab Companion™ model IS-971 shaker incubator. The 250 mL-scale reactors are shown along with a 2L flask of YM

broth inside the shaker incubator in Figure 3.1. The 270 L internal compartment of the shaker incubator was maintained at a constant air temperature using an integral digitally controlled electric heater. The flasks were mounted to a shaker plate and aerated by orbital motion shaking action at 200 rpm. The fermentation period was one to seven days, depending on the test.

Figure 3.1. 250mL-scale reactors and 2 L flask of YM broth in shaker incubator

The 2L-scale tests reactor vessels were glass 2 L Erlenmeyer flasks. Each flask was filled with 1400-1800 mL of thin stillage substrate. The 2L-scale reactors were inoculated by transferring 100 mL of the YM broth fungal flocculent solution to each flask. The inoculated 2L-scale reactors were then placed in a heated water bath to maintain a fluid temperature of 34±3°C. The water bath was heated using an isotemp immersion circulator manufactured by Fisher

Scientific, model 70. The reactors were aerated by bubbling air through a submersible diffuser. Compressed air was pressure regulated to 20 psi. The air flow rate was measured and controlled using an air rotameter. Flexible fish tank air tubing along with a check valve to prevent backflow routed air from the rotameters to each flask. Figure 3.2 shows the 2L-scale reactors in the warm water bath with the airlines connected.

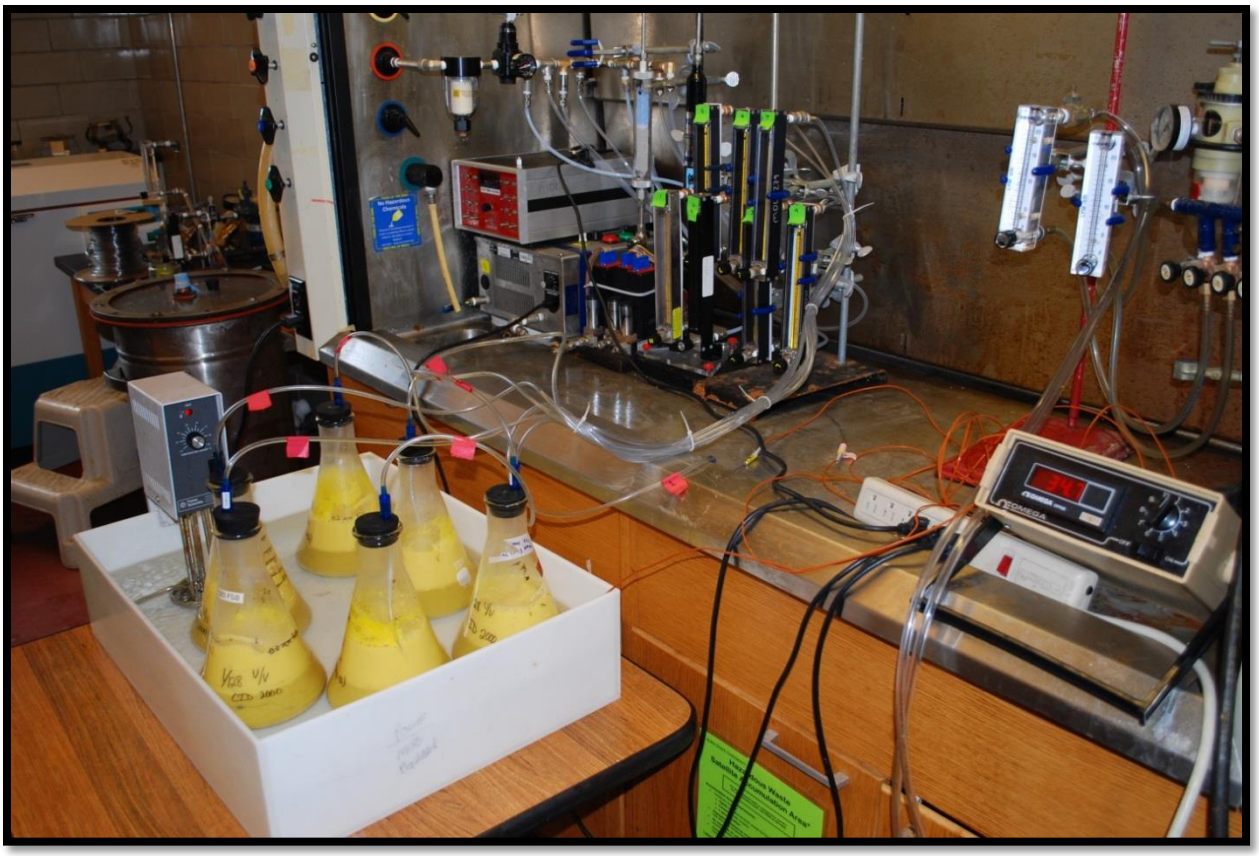

Figure 3.2. 2L reactors set up

A number 10 rubber stopper was inserted in the top of each flask. The rubber stopper had two, one for air tubing to pass through and second hole for venting. An approximately 12 inch length of air tubing was cut and inserted through the rubber stopper into the flask. A disposable aquarium 1 inch porous stone was attached to the end of the air tubing as the submersible air

diffusers. Air flow rate was set at 2.0 liters per minute which equates to an air/L working volume/min (vvm) of 1.1-1.4 vvm. The fermentation period at the 2L-scale was two days unless otherwise stated.

B. Sodium Hypochlorite Tests

The fungal fermentation period was for seven (7) days. The 250mL-scale reactors were dosed with the 6% sodium hypochlorite solution once during inoculation of the thin stillage and once per day throughout the fermentation period. The thin stillage substrate disinfected with sodium hypochlorite solution was not autoclaved. One control flask inoculated with *Mucor circinelloides* and one control flask inoculated with *Rhizopus oligosporus* was prepared with each test. All of the flask with a sodium hypochlorite dosages >48 mg/L were performed in duplicate. Various dosages of bleach were attempted with each test. Later tests attempted higher dosages of 180 mg/L and 216 mg/L. The purchased household bleach had an 8.25% sodium hypochlorite dosage; however, it was diluted in the lab using deionized water to a dosage of 6% sodium hypochlorite. The volume of the bleach solution dosage was up to 216 mg/L. For example at a 216 mg/L sodium hypochlorite dosage the 100mL of thin stillage was dosed with 360 µL of the 6% sodium hypochlorite solution daily.

C. Chlorine Dioxide Tests

Dupont™ Fermasure® XL was the chemical used for the chlorine dioxide tests. Fermasure® XL contains of 15-25% sodium chlorite (Fermasure XL® MSDS). Sodium chlorite is a chlorite containing compound that is a stabilized precursor to chlorine dioxide as shown in the equation below:

$$5NaClO_2 + 4H^+ \rightarrow 4ClO_2(g) + 2H_2O + Cl^- + 5Na^+$$

1 g of sodium chlorite produced 0.597g of available chlorine dioxide (Patent EP2480088A1) Therefore 15-25% sodium chlorite produces 9-15% available chlorine dioxide. The thin stillage substrate disinfected with chlorine dioxide was not autoclaved. A series of six tests were performed from September 2013 through December 2013. Tests were performed in a similar manner to the sodium hypochlorite tests. Tests were done at the 250mL flask scale, fermentation period was seven days, and the disinfectant was dosed daily. The control along with all of the chlorine dioxide dosages flasks were performed in duplicate for each test, accept for the test performed the week of 9-13-13 which was performed in triplicate.

D. Penicillin-streptomycin Tests

A combination of penicillin and streptomycin (p-s) antibiotics were attempted as a selective disinfectant to prevent bacterial contamination. The P-S Test 1, P-S Test 2 and P-S Test 3 were performed at the 250mL-scale inoculated with *Rhizopus oligosporus*. The thin stillage substrate disinfected with p-s was not autoclaved. The penicillin-streptomycin (p-s) was manufactured by Life Technologies™ catalog number 15140-122. Penicillin-streptomycin was a 100mL unit size containing frozen antibiotic stored at -5°C to -20 °C. According to the manufacturer, the penicillin and streptomycin were originally purified from the fungi *Penicillium* and *Streptomyces griseus*, respectively. The manufacturer listed antibiotic solution concentration was 10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin. The penicillin-streptomycin was dosed once to the thin stillage during the inoculation. The fermentation period was 40-48 hours. One

set of three control flasks of thin stillage was autoclaved with each p-s test. The penicillin-streptomycin dosage tests were each done in triplicate. When harvesting the triplicate flasks of the same antibiotic dosage, they were combined together onto the same petri dish for drying and weighing. The combined dry mass (grams) was then divided by 0.3 L to obtain and average yield (g/L (dry)) for the three flasks.

P-S Test 4 and P-S Test 5 were performed at the 2L-scale and were also inoculated with *Rhizopus oligosporus*. These two tests were not identical. They were each done using different samples of thin stillage and the P-S Test 4 control flasks air was filtered using 0.45 micron filters. In P-S Test 5 consisted of two flasks and P-S Test 4 consisted of three flasks.

E. Iodophor Tests

Iodophor was tested as a potential oxidant selective disinfectant using BTF® Iodophor. Two tests were performed at the 250mL reactor scale. BTF® Iodophor contains butoxy polypropoxy polyethoxy ethanol-iodine complex that is water soluble which releases 1.6% free iodine when in solution. The thin stillage substrate disinfected with iodophor was not autoclaved. The thin stillage was inoculated with *Rhizopus oligosporus* fungus and dosed once with iodophor at the time of inoculation. I-Test 1 was performed in duplicate with iodine dosages of 25 and 50 ppm. I-Test 2 was done in triplicate with iodine dosages of 200, 400, 600 and 800 ppm. Both tests had an autoclaved and non-autoclaved control.

F. Ozone Tests

A series of seven (7) tests were performed where ozone gas was diffused into the thin stillage as an oxidizing disinfectant. These tests were performed at the 2L-scale. Ozone gas was continuously diffused into the thin stillage during the fermentation period. Ozone gas flow rates 5 mL/min, 10 mL/min, 15 mL/min, 80 mL/min and 100 mL/min were attempted. An ozone dosage determination was performed for each ozone gas flow rate to determine the mass of dissolved ozone per min dosage. It was found the 5-15 mL/min all had approximately the same dissolved ozone dosage rate of 10 mg/L/h). 80 and 100 mL/min delivered 24 mg/L/h ozone dosage. Each flask was inoculated with 100mL of *Mucor circinelloides* fungal biomass. Air flow rate to each flask was 2 L/min. Each flask was filled with 1800 mL of thin stillage.

G. Aeration and Oxygenation Tests

The aeration and oxygenation tests were performed at the 2L-scale. Two tests were performed comparing glass verses disposable diffusers and various air flow rates. Three test were performed comparing oxygenation to aeration. All of the thin stillage was autoclaved prior to inoculation. Air flow rates of 1 L/min and 2L/min used one diffuser. Air flow rates of 4 L/min used two diffusers. Glass diffusers were Pyrex® (12C) gas dispersion with 12 mm O.D. fritted cylinder, 250 mm L × 8 mm diam. stem. The pores size of the fritted glass was 40-60 µm. Disposable diffusers were 1 inch aquarium porous stone diffusers.

3.2.5 Fungal Harvesting

The contents of the 2L or 250mL flasks were poured into a wire mesh kitchen food strainer as shown in Figure 3.3. Water was separated from the fungal biomass by an orbital wrist action

of the operator. Figure 3.4 shows the dewatered fungal biomass placed in the glass petri dishes for further drying. The petri dish with the wet fungal biomass was then placed in an oven at 60°C for 1 to 3 days. The dry fungal biomass and petri dish were then removed and weighed to determine the dry grams of fungal biomass yield. Figure 3.4 shows the harvested fungal biomass placed in petri dishes after being dewatered using the strainer.

Figure 3.3. Harvesting of fungal biomass. Straining fungal biomass from thin stillage (left).
Figure 3.4. Dewatered fungal biomass placed in petri dishes (right).

3.3 Results and Discussion

3.3.1 Autoclaved

Autoclaving the thin stillage has been a standard form of sterilization done for the control flasks in the selective disinfection studies. Many of the tests had a set of control flasks that were filled with autoclaved thin stillage, non-autoclaved thin stillage or a set of both. All of the yield data for the autoclaved and non-autoclaved control flasks are shown in Table 3.3. The largest number of test have been done with non-autoclaved thin stillage at a total count of 53. There have been a total of 123 reactor fermentations at the 250mL-scale, which is 2.6 times more than the total 47 reached with flask fermentations at the 2L-scale. 250mL-scale testing was easier to

perform and served to test a wider range of variable per batch. Control flasks at the 250mL-scale were generally done in triplicate. Control flasks at the 2L-scale were generally done in singles or duplicates due to space limitations.

Figure 3.5 shows the average dry fungal biomass yields and one standard deviation error bar. *Mucor circinelloides* grown on autoclaved thin stillage at the 250mL-scale had biomass yields of 14.5 g/L (dry) which was 4.5 g/L (dry) less than 20 g/L (dry) reported by Mitra et al. (2012) using a 6-L airlift bioreactor fermented for 2 days. Non-autoclaved fresh thin stillage was observed to produce a *Mucor circinelloides* biomass yield of 9.0 g/L (dry). This was 38% lower biomass yield than was observed using autoclaved thin stillage and half the biomass yield reported by Mitra et al. (2012). Mitra et al. (2012) reported non-autoclaved fresh thin stillage produced an average *Mucor circinelloides* biomass yield of 18 g/L (dry), which was 10% less than the heat sterilized thin stillage produced. *Rhizopus oligosporus* grown on thin stillage in a 5L bench-top bioreactor aerated at 0.4-1.0 vvm aeration rate was reported to yield 27-36 g/L (dry) of fungal biomass (Rasmussen, 2014). At the 250mL-scale *Rhizopus oligosporus* fungal biomass yields averaged 12.5 -20g/L (dry), which was only approximately half the yield reported by Rasmussen et al. (2014). At the 2L-scale *Rhizopus oligosporus* fungal biomass yields averaged 4.1-5.1 g/L (dry) at an aeration rate of 1.1-1.4 vvm, which is only 11-19% of the yield reported by Rasmussen et al. (2014) even though a higher aeration rate was used. At the 250mL-scale autoclaving was observed to increase yields over non-autoclaving by 5.5 g/L (dry) for *Mucor circinelloides* and 7.9 g/L (dry) for *Rhizopus oligosporus*. However, the biggest difference autoclaving the thin stillage substrate, was in the reduced variability of the fungal biomass yields. At the 250mL-scale autoclaved thin stillage produced a fungal biomass yield with a standard deviation of approximately half of the non-autoclaved thin stillage.

The 2L-scale produced fungal biomass yields of approximately one third the cultivations done at 250mL-scale. Decreased dissolved oxygen transfer to the thin stillage liquid at the 2L-scale was suspected as the likely cause of the decreased yields. Refer to Section 2.3.7 Aeration and Oxygenation for more detailed information.

Table 3.3. Fungal biomass yields for autoclaved and non-autoclaved control flask.

Test	250mL-scale				2L-scale			
	Non-autoclaved		Autoclaved		Non-autoclaved		Autoclaved	
	Mucor	***Rhizopus***	***Mucor***	***Rhizopus***	***Mucor***	***Rhizopus***	***Mucor***	***Rhizopus***
Count	20	53	35	15	6	14	23	4
Max	33.1	32.8	23.4	27.2	7.2	8.4	15.5	13.6
Min	0.0	0.0	0.0	12.4	2.2	0.0	2.5	2.3
Std. Dev.	9.4	8.2	4.4	5.4	1.8	3.1	3.1	4.9
Avg. Yield (g/L (dry))	9.0	12.5	14.5	20.4	3.4	4.1	5.8	5.1

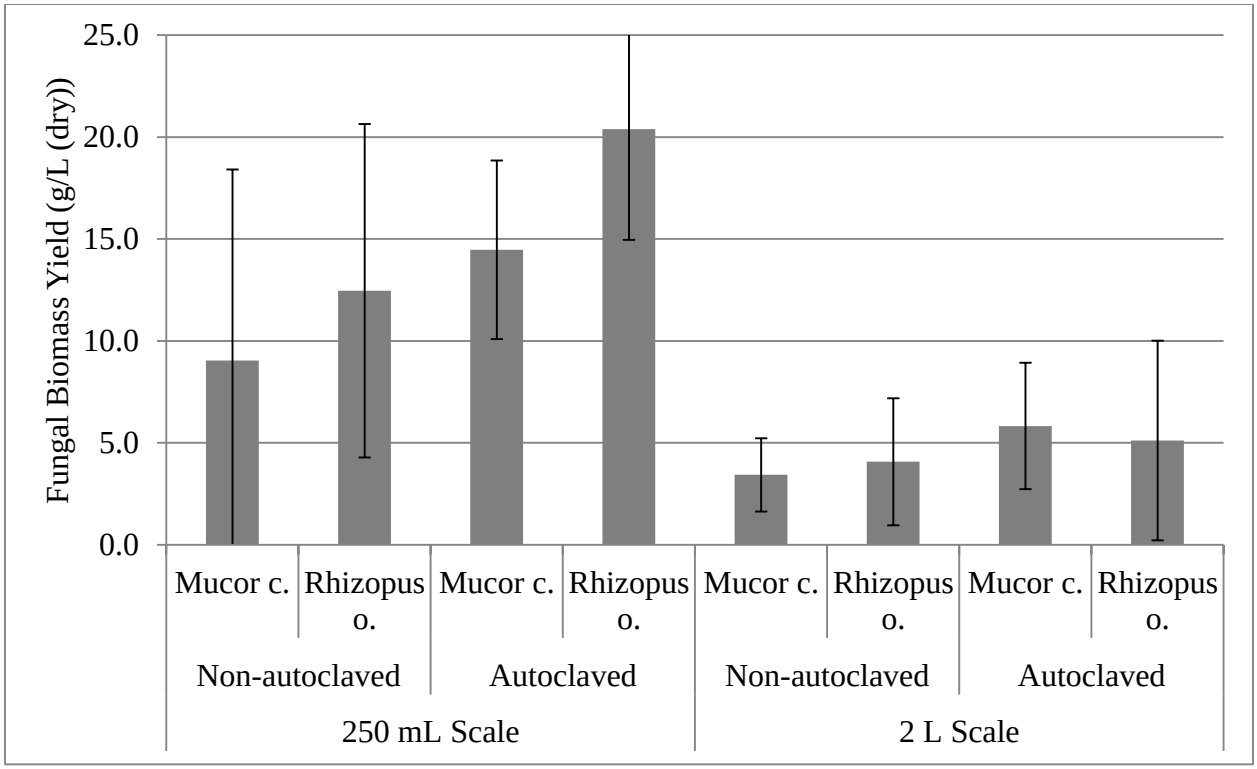

Figure 3.5. Autoclaved and non-autoclaved control fungal biomass yields

3.3.2 Sodium Hypochlorite

Table 3.4 shows the total number of 250mL-scale reactor that were performed at each dosage of sodium hypochlorite (bleach) solution during the series of eight tests. Figure 3.6 shows the average fungal biomass yields and one standard deviation error bar for the flasks at that dosage of disinfectant. The control flasks had no sodium hypochlorite addition for *Mucor circinelloides* and *Rhizopus oligosporus* and an average fungal biomass yield of 10 g/L (dry) was observed. Many of the flasks dosed with sodium hypochlorite solution produced average yields at or below control flask yield of 10 g/L (dry).

Table 3.4. The total count of flasks tested each sodium hypochlorite dosage during the series of tests performed.

* Fungal biomass yields are not shown for this dosage in Figure 3.4 since during the series of tests less than three flasks inoculated with *Mucor c.* and no flasks inoculated with *Rhizopus o.*

NaClO Solution Dosage	Total count of flasks tested	
(mg/L)	*Mucor c.*	*Rhizopus o.*
0.00	8	7
*12	1	---
24	6	5
36	5	3
48	10	8
*60	2	---
72	16	14
96	8	8
108	6	6
120	8	8
144	14	14
180	6	6
216	6	6

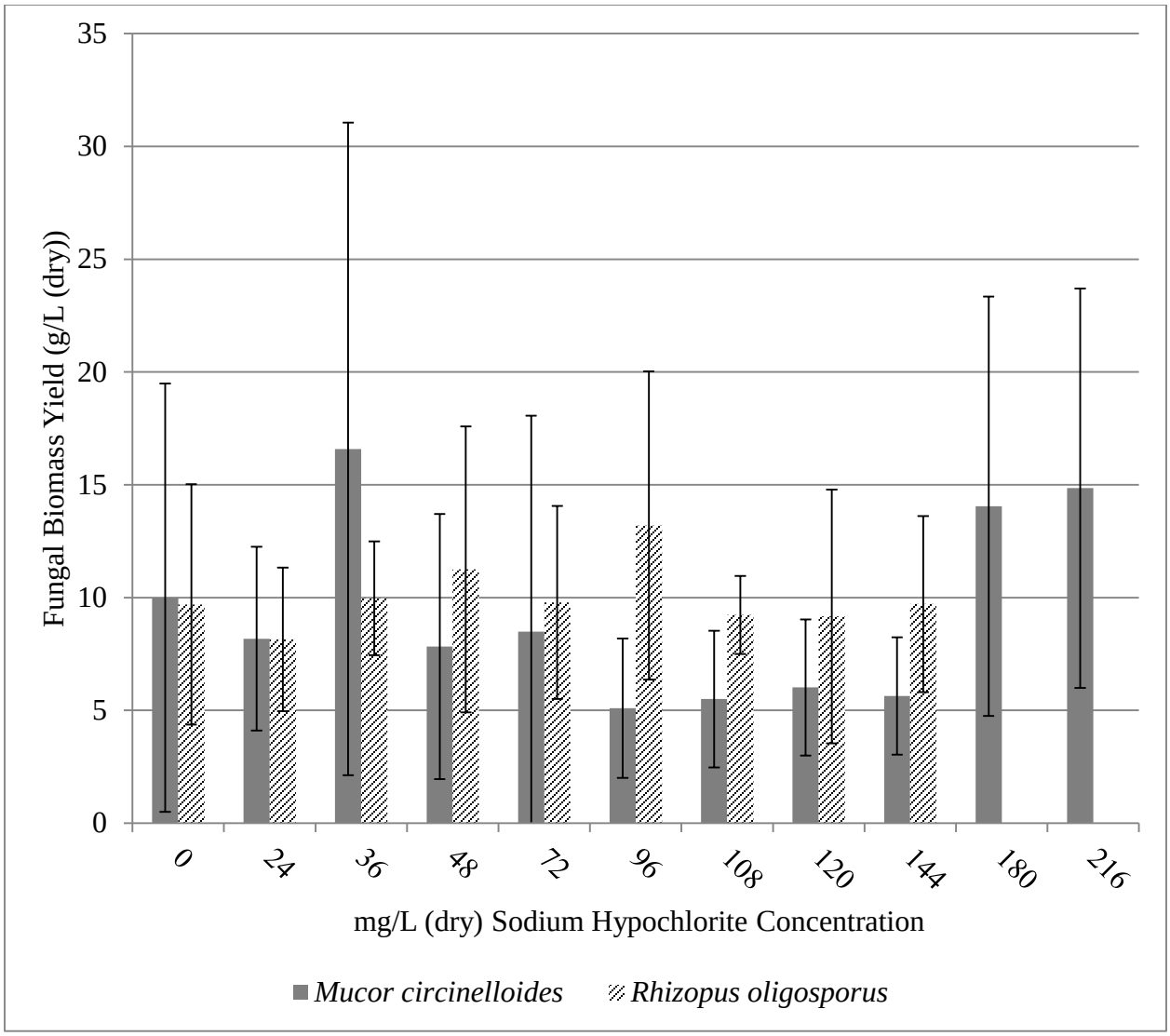

Figure 3.6. Sodium hypochlorite selective disinfection test results with average fungal biomass and standard deviation shown.

The flasks that did result in an increased yield were highly variable as shown by the single standard deviation error bar shown in Figure 3.6. The *Mucor circinelloides* 36 mg/L dosage of sodium hypochlorite had the highest average yield of 16.6 g/L (dry). However, there were only five *Mucor circinelloides* flasks that performed at 36 mg/L dosage. There were ten *Mucor circinelloides* flasks performed at the 48 mg/L dosage, and a much lower average yield of

7.8 g/L (dry) was observed. The highest yield for *Rhizopus oligosporus* of 13.2 g/L (dry) was observed at a 96 mg/L sodium hypochlorite dosage. There were eight *Rhizopus oligosporus* flasks performed at a 96 mg/L dosage. At the 72 mg/L dosage 14 *Rhizopus oligosporus* flasks were performed; however, a much lower yield approximately equal to the average control flasks of 9.8 g/L (dry) was observed. Miao (2005) reported an optimal sodium hypochlorite concentration of 10 mg/L for non-aseptic *Rhizopus oligosporus* cultivation on corn processing wastewater resulting in a 39% biomass yield increase. Higher sodium hypochlorite concentrations were reported to be inhibitory resulting in a 50% fungal biomass decrease at a 50mg/L concentration (Miao, 2005). The much higher sodium hypochlorite dosage range of 24-216 mg/L was tested in this study; however, no discernable optimal or inhibitory dosage was observed.

3.3.3 Chlorine Dioxide

Stabilized chlorine dioxide is specifically designed for the ethanol industry to attack organic acid producing bacteria, namely lactic and acetic acid (Sumner, 2013). Since the Fermasure® XL dosing chemical contained of 15-25% sodium chlorite (Fermasure XL® MSDS), the available chlorine dioxide concentration was 9-15%. Because the available chlorine dioxide concentration in the dosing chemical ranged from 9-15%, the ppm dosages in Table 3.5 and Figure 3.7 show a range of values for each dosage. The 9-150 ppm chlorine dioxide dosage range tested was based on guidance given from the ethanol plant that supplied the disinfectant. The last test of the series attempted higher 72-120 ppm and 90-150 ppm dosage. Table 3.5 shows the total number of 250mL-scale reactors that were dosed at each dosage during the entire test series.

Table 3.5. Total count of flasks tested during the series of tests performed at each Fermasure® XL solution dosage.

Chlorine Dioxide Dosage	Total count of flasks tested	
ppm	*Mucor c.*	*Rhizopus o.*
Control, 0	12	13
9-15	3	3
18-30	11	8
27-35	11	8
65-60	10	8
45-75	8	6
54-90	10	8
72-120	2	2
90-150	2	2

Figure 3.7 shows the average fungal biomass yields and a one standard deviation error bar. Similar to the tests performed using sodium hypochlorite disinfectant the control flasks had an average yield of approximately 10 g/L (dry). In this series of tests, the highest yields observed were approximately 25 g/L (dry) for both *Mucor circinelloides* and *Rhizopus oligosporus* fungal cultivations at a 9-15 ppm chlorine dioxide dosage. However, there were only three flasks performed for both *Mucor circinelloides* and *Rhizopus oligosporus* at the of 9-15 ppm chlorine dioxide dosage. There were eleven *Mucor circinelloides* flasks dosed at 18-30 ppm chlorine dioxide dosage and the average yield was 19.9 g/L (dry). A specific optimal dosage of chlorine dioxide for *Mucor circinelloides* was unable to be determined. However, *Mucor circinelloides* with chlorine dioxide dosages of 9-90 ppm showed increased biomass yields over the control flasks with fungal biomass yield ranging from 15-25 g/L (dry). At dosages of 72-120 ppm and 90-150 ppm *Mucor circinelloides* fungal biomass average yields were approximately equal to the control flasks 10 g/L (dry) ± 1 g/L (dry). *Rhizopus oligosporus* fungal cultivation results using chlorine dioxide were mixed. 9-15 ppm, 27-45 ppm, and 45-75 ppm showed fungal biomass

yields higher than the control flasks with yield of 12.5-25 g/L (dry). Chlorine dioxide dosage of 18-30 ppm, 36-60 ppm, 54-90 ppm, 72-120 ppm and 90-150 ppm had fungal biomass yield below the control flask average of 10 g/L (dry). It was odd chlorine dioxide dosages of 18-30 ppm and 36-60 ppm were below the average control flask yields when the 9-15 ppm, 27-45 ppm, and 45-75 ppm showed above average yields.

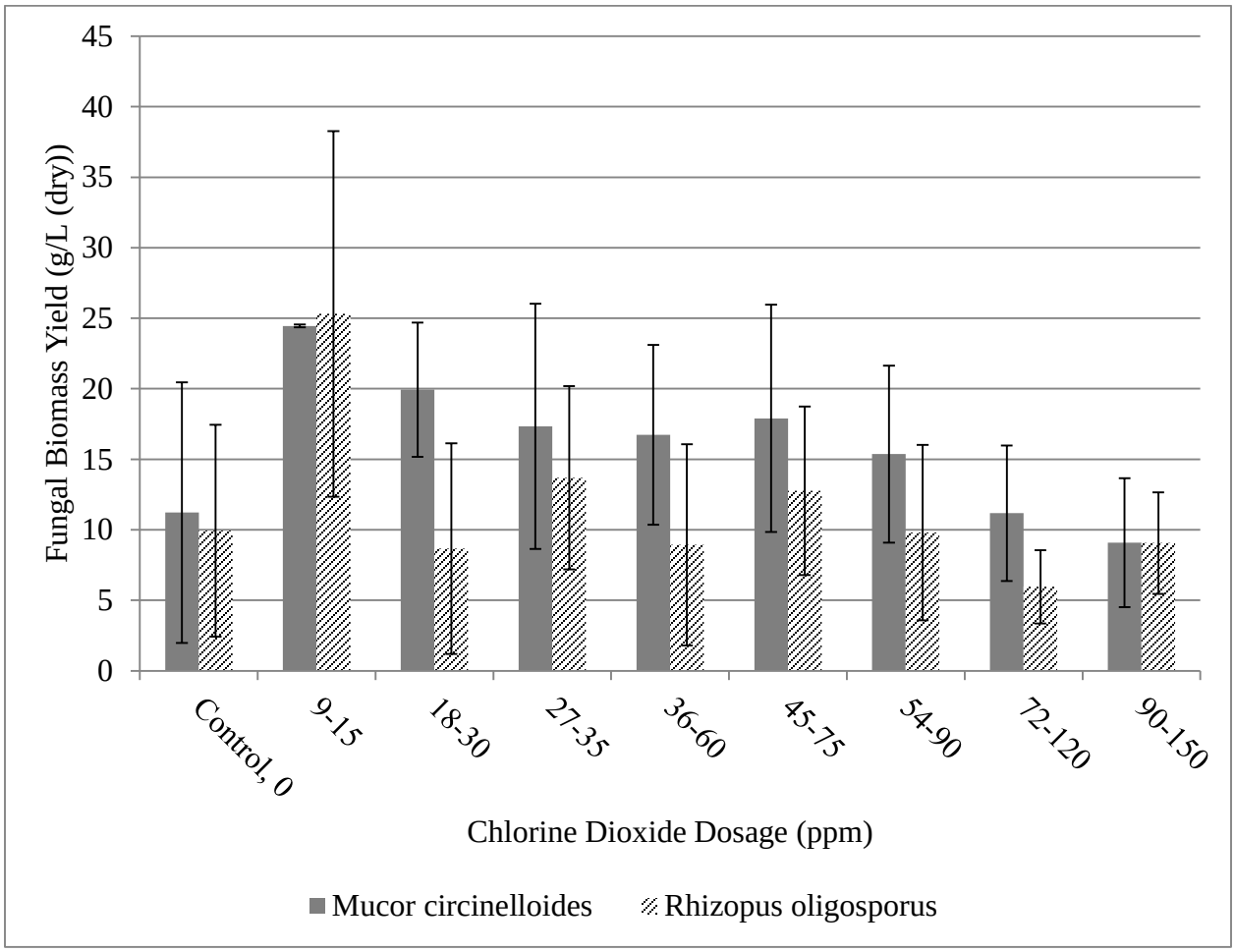

Figure 3.7. Chlorine dioxide selective disinfection test results with average fungal biomass and standard deviation shown.

3.3.4 Penicillin and streptomycin

A combination of penicillin and streptomycin (p-s) antibiotics were attempted as a selective disinfectant to prevent bacterial contamination. Penicillin-streptomycin was tested at different dosages following the procedures described in Section 3.2.4 Thin Stillage Fungal Inoculation and Fermentation. The average fungal biomass yields are shown in Table 3.6. In P-S Test 2 at all of the p-s dosages performed significantly better than the control with a combined average yield of 29.4 g/L (dry) and small standard deviation of 1.1 g/L (dry). The combined average yield for P-S Test 2 was higher than the P-S test 1 autoclaved and non-autoclaved control flasks by a factor of 1.65 and 2.07, respectively.

Autoclaved and non-autoclaved thin stillage substrate control flasks at the 250mL-scale inoculated with *Rhizopus oligosporus* was part of most tests as described in Section 3.3.1 Autoclaved vs. Non-autoclaved. The average yield for all of the autoclaved and non-autoclaved 250mL-scale *Rhizopus oligosporus* control flasks throughout the two-year study period was 20.4 g/L (dry) and 12.46 g/L (dry), respectively. The combined average yield for P-S Test 2 flasks dosed with 25-100 (units/mL of penicillin and mg/L of streptomycin) was 29.4 g/L (dry). This was higher than the overall autoclaved and non-autoclaved control by a factor of 1.44 and 2.36, respectively.

P-S Test 1 and P-S Test 3 both had biomass yields approximately equal to the autoclaved control flasks. P-S Test 1dosed with 100 (units/mL of penicillin and mg/L of streptomycin) performed equivalent the control autoclaved flasks at approximately 18 g/L (dry). P-S Test 3 25 (units/mL of penicillin and mg/L of streptomycin) had a yield of 16.4 g/L (dry).

In P-S Test 1 a set of triplicate 250mL flasks were dosed with 100 (units/mL of penicillin and mg/L of streptomycin) and 50 ppm iodine was added to the thin stillage at the time of

inoculation. The combined average yield of the triplicate flasks was 19.6 g/L (dry), which was 8% higher than the flasks dosed with 100 (units/mL of penicillin and mg/L of streptomycin) alone. Both performed better than the non-autoclaved control by only 26-40%.

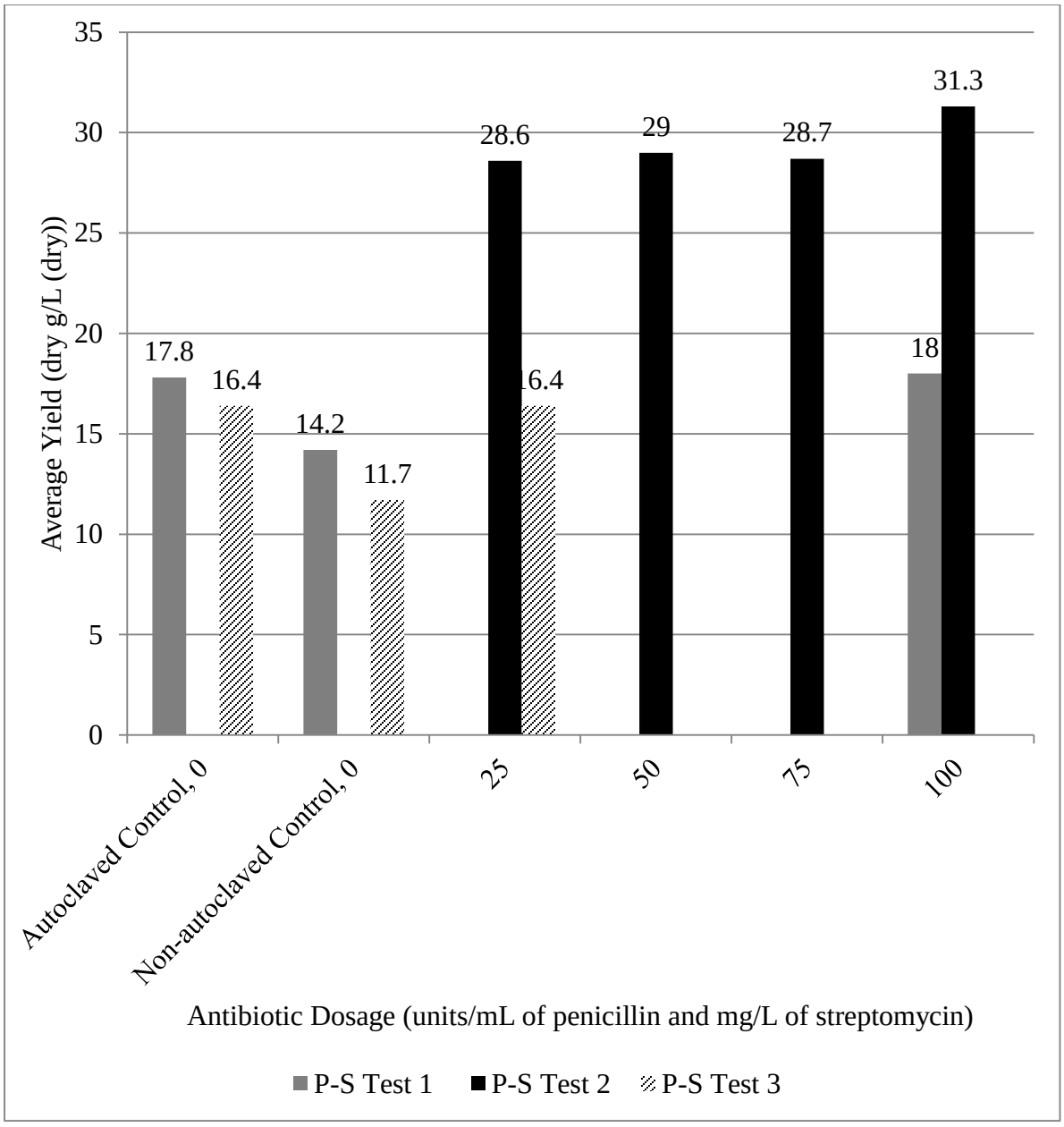

Figure 3.8. Penicillin-streptomycin tests. The control and flasks dosed with penicillin-streptomycin (P-S) used non-autoclaved thin stillage substrate.

P-S Test 4 and P-S Test 5 were performed at the 2L reactor scale. In P-S Test 4 the 0.25% (v/v) addition of penicillin-streptomycin did not increase the yields or the consistency compared to the air filtered control fungal biomass yield of approximately 6 g/L (dry). P-S Test 4 control flasks had a standard deviation of 1.7 and the 0.25% (v/v) P-S flasks had a standard deviation of 2.0. The addition of penicillin-streptomycin in P-S Test 5 improved fungal biomass yields much more significantly than in P-S Test 4. The average yields in P-S Test 5 were 9.06 g/L (dry) for the 0.25% (v/v) P-S addition and only 2.55 g/L (dry) for the control flasks. P-S Test 5 control flasks were less than half the yield of the P-S Test 4 control flask. The lower yield of the control flasks in P-S Test 5 could be due to that different batches of thin stillage used as the substrate or possibly due to the air supply was not filtered for the control flasks. The 2L-scale penicillin-streptomycin tests (P-S Test 4 and 5) overall had much lower yields than the test performed at 250mL-scale (P-S Test 1-3). The autoclaved and non-autoclaved control flasks at the 250mL-scale had an average yield of 15.0 g/L (dry). At the 2L-scale the control flasks had an average yield of 4.7 g/L (dry). The 2L-scale control flasks had a yield of approximately a third of the 250 mL with a similar standard deviation. All of the flasks with penicillin-streptomycin addition at the 250mL-scale had a combined average yield of 25.3 g/L (dry) with a standard deviation of 5.8. At the 2L-scale the flasks dosed with penicillin-streptomycin had an average yield of only 7.4 g/L (dry) and a standard deviation of 2.8. Similar to the control flasks, the 2L flasks dosed with penicillin-streptomycin had approximately a third the yield of the 250mL-scale flasks. However, the 250mL-scale flasks did have a significantly higher standard deviation.

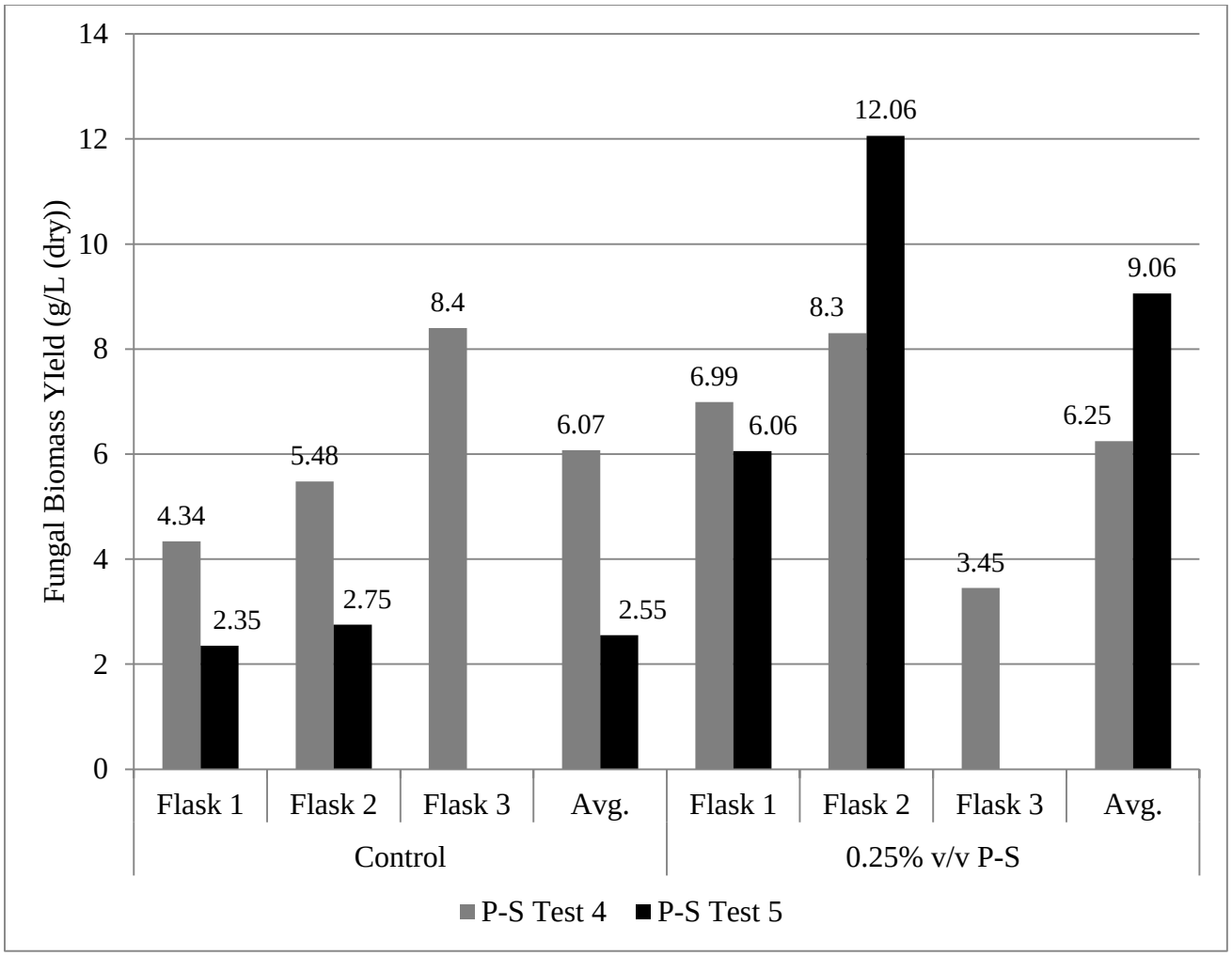

Figure 3.9. 2L-scale penicillin-streptomycin tests. The air supply for the P-S Test 4 control flasks were filtered, all other air supplies were unfiltered.

The promising results of the initial tests using penicillin-streptomycin were followed by later tests where little no improvement over the control flasks was observed. Two subsequent tests were done in two successive weeks at the 250mL reactor scale using p-s where no biomass growth occurred.

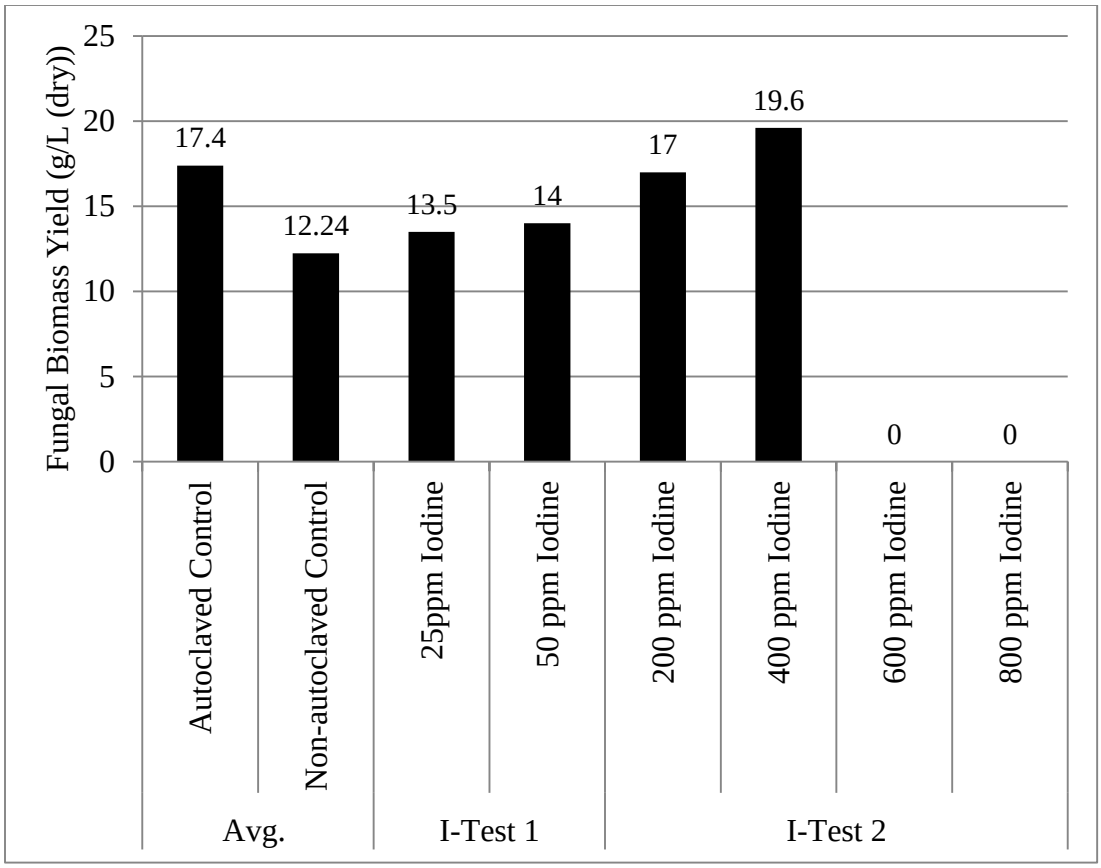

Figure 3.10. Iodophor oxidant selective disinfectant tests. Autoclaved and Non-autoclaved control for I-Test 1 and I-Test 2 were averaged.

3.3.5 Iodophor

The combined average yield for the autoclaved and non-autoclaved controls from I-Test 1 and I-Test 2 are shown in Figure 3.10. The 25 and 50 ppm dosage of iodine only performed 10-14% better than the non-autoclaved control. The 200 ppm dosage of iodine had approximately the same yield as the autoclaved control. The 400 ppm dosage had a yield 13% higher than the autoclaved control and 60% higher than non-autoclaved control. Iodine dosages of 600 ppm and higher proved to be toxic to fungal growth, since no growth was observed.

3.3.6 Ozone

In the series of seven ozone tests, an ozone dosage of 24 mg/L/h had approximately the same yield as the average autoclaved control. An ozone dosage of 10 mg/L/h ozone dosage achieved biomass yield of 4.9 g/L (dry) which was almost as high as 5.4 g/L (dry) autoclaved control. 24 mg/L/h ozone dosage had a much lower average yield at 3.7 g/L (dry), which was significantly lower than the autoclaved control and only slightly high than 3.5 g/L (dry) observed for non-autoclaved control. 10 mg/L/h dosage of ozone could potentially be good selective disinfectant for fungi; however, yields were still quite variable.

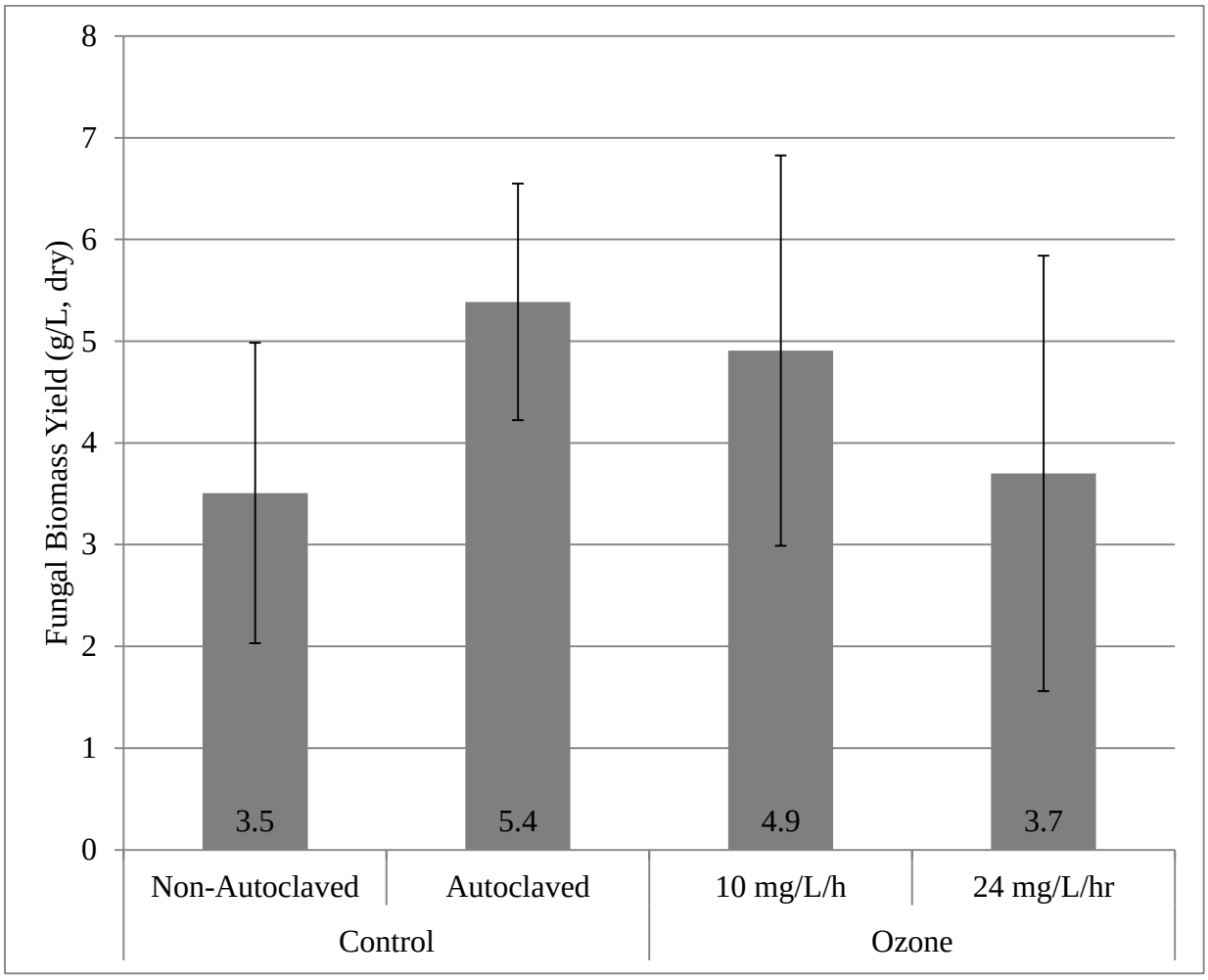

Figure 3.11. Ozone oxidant selective disinfectant tests fungal biomass yields.

3.3.7 Aeration and Oxygenation

The 2L-scale had significantly lower yields with an average yield of 4.9 g/L (dry) compared to an average yield of 13.45 g/L (dry) observed at the 250mL-scale. The 2L-scale on average had almost one third the biomass yield of the 250 mL flasks. This could be attributed to the different method of mixing and aerating in the 2L-scale. 2L-scale are mixed and aerated by bubbling compressed air through submerged diffuser. 250mL-scale reactors are aerated and mixed through orbital motion shaking action. It is possible that the compressed air rate is insufficient to provide proper aeration and mixing to achieve optimal growth yields. At an air flow rate of 2 L/min (1.1 vvm) the glass diffuser had a higher yield than the disposable diffuser by 38-39%. However, at an air flow rate of 4 L/min (2.2 vvm), the glass diffuser had yield of 9% higher on the 3/24/14 test, but was 29% lower than the disposable diffuser on the 3/27/14 test. The test indicates that there is likely no significant fungal biomass yield improvement with the glass diffuser compared to the disposable diffusers. Air flow rates seemed to make a much more significant difference in the fungal biomass yield. In the 3/24/24 test an air flow rate of 4 L/min (2.2 vvm) had a higher yield than the 2 L/min (1.1 vvm) air flow rate by a factor of 2.57. However, the 3/27/14 results were mixed. In the case of the glass diffuser the yield at 4 L/min (2.2 vvm) was actually lower than at 2 L/min (1.1 vvm). It is possible in the 3/27/14 test that even at 4L/min (2.2 vvm) the air flow rate was insufficient to satisfy the oxygen demand.

Further tests were done using compressed oxygen cylinders to oxygenate the thin stillage. The relatively low oxygen transfer efficiency of sparged air can be greatly increase by bubbling pure oxygen. The 3/27/14 test shown in Figure 3.13 indicates that an air flow rate of 4 L/min (2.2 vvm) still had suppressed biomass yield of only 4 g/L (dry) compared to an average of 13.5 g/L (dry) at the 250mL-scale. For the oxygenation test both oxygen and air flow rates were 4 L/min

(2.2 vvm). Similar to the air flow rate tests, the thin stillage substrate was steam autoclaved prior to inoculation. In all three tests the oxygenated thin stillage yield significantly higher fungal biomass than the aerated flasks. The oxygenated flasks had twice the average yield of the aerated flasks. The oxygenated thin stillage had an average fungal biomass yield of 18.3 g/L (dry). The aerated flasks had fungal biomass yield of 9.1 g/L (dry). The aerated flasks showed higher variability in the series of tests with standard deviation of 3.9 compared to standard deviation of only 1.5 with the oxygenated flasks. Every oxygenated flask had a higher yield that the 12.5 g/L (dry) average of the 250mL-scale.

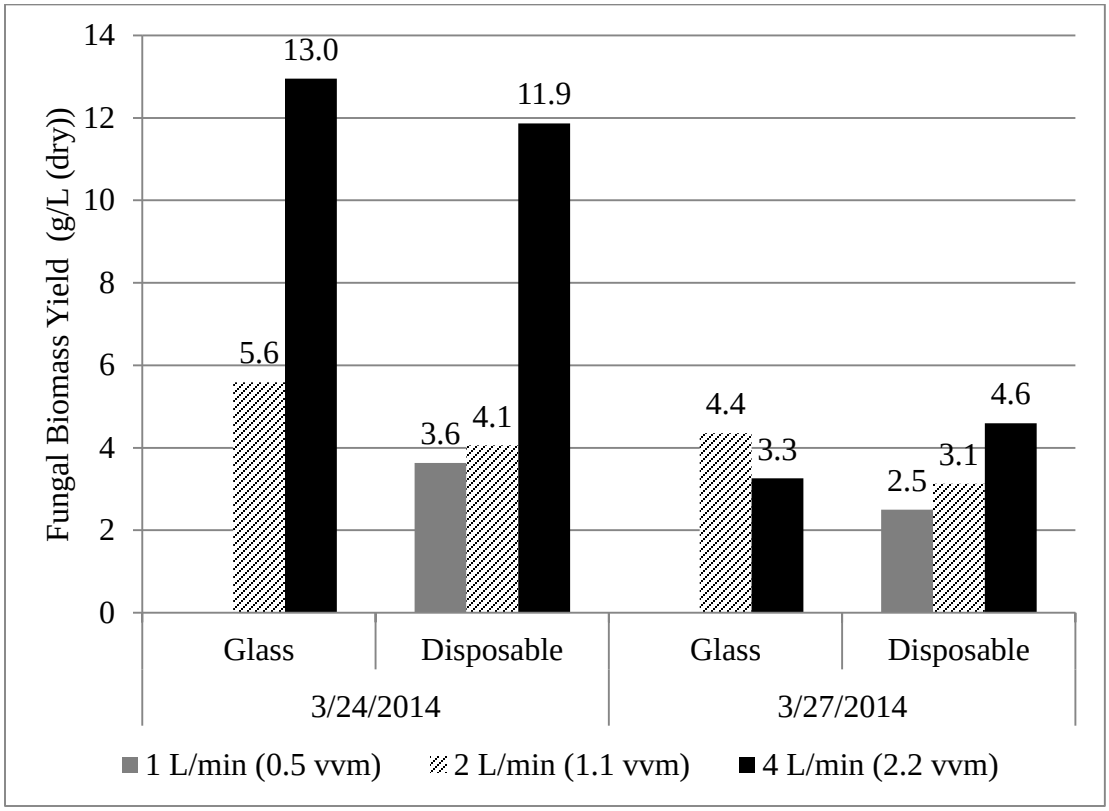

Figure 3.12. Aeration comparison test with glass and disposable diffusers.

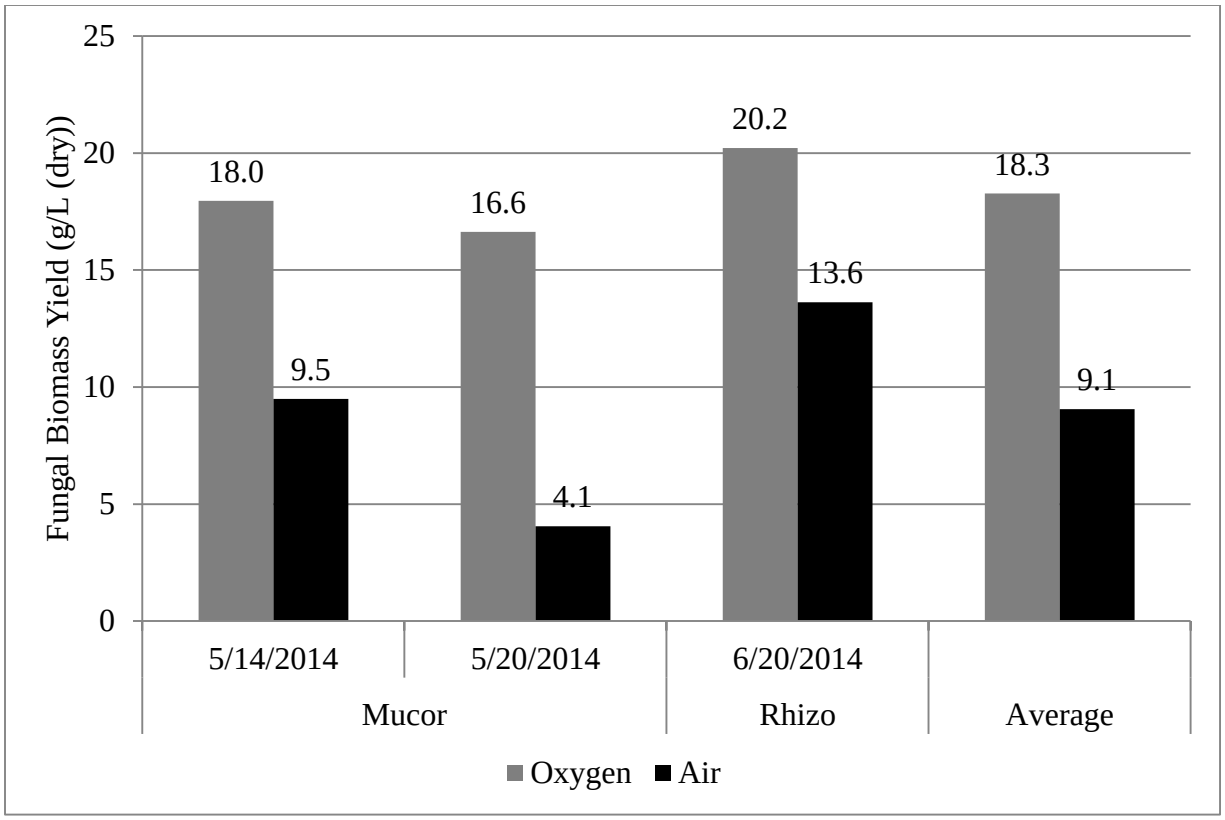

Figure 3.13. Oxygenation Test

3.4 Conclusion

High standard deviations and repeatability issues observed throughout the selective disinfectant study shows that *Mucor circinelloides* and *Rhizopus oligosporus* fungal biomass cultivation is highly variable indicating that non-aseptic methods and/or batch-to-batch variation of the fresh thin stillage substrate greatly affect biomass yields. Heat sterilization pretreatment of the thin stillage at the 250mL-scale prior to inoculation more significantly decreased the variance of the biomass yields compared to increasing the average yields. Penicillin-streptomycin antibiotics showed the greatest potential as a selective disinfectant with potentially producing yields significantly higher than even heat sterilized thin stillage. Iodine at a 400 ppm dosage was

as affective as heat sterilization at increasing fungal biomass yields. Chlorine dioxide at 9-90 ppm dosage proved to be affective at increasing fungal biomass yields for *Mucor circinelloides* equally as well as heat sterilized pretreated thin stillage; however, little to no improvement was observed for *Rhizopus oligosporus*. Chlorine dioxide showed no noticeable effect on the variance of the fungal biomass yields for either species. Sodium hypochlorite tested at a range of 24-216 mg/L dosage does not seem to have any discernable positive or inhibitory effect of fungal biomass yields or variability. Air sparging at the 2L-scale provided insufficient available dissolved oxygen to achieve fungal biomass yields of that observed by at the 250mL-scale by shake aerating. Tests performed at the 2L-scale produced highly variable yields of approximately one third the 250mL-scale regardless of the disinfectant added or if thin stillage was heat sterilized prior to inoculation. When the 2L-scale reactors were oxygenated instead of aerated, yields increased comparable to that observed at the 250mL-scale. Continuous ozonation of the thin stillage during fungal cultivation was also observed to increase yields approximately equal to autoclave pretreated thin stillage. However, since ozonation tests were done only at the 2L-scale using air yields were still very low and more difficult to interpret.

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CHAPTER 4

CONTINUOUS-FLOW HYDROTHERMAL LIQUEFACTION OF FUNGAL BIOMASS

Abstract

Rhizopus oligosporus and *Mucor circinelloides* fungal biomass slurries cultivated on thin stillage corn-ethanol byproduct was processed by hydrothermal liquefaction (HTL) thermochemical reforming process to produce bio-crude oil using a continuous supercritical flow reactor operated at subcritical conditions. Fungal slurry with 5-12% solids content went through HTL operated at 350-392 °C, 2900-4000 psi, and 15-36 minute residence time. Bio-crude oil yields of 36-54 wt. %, dry ash free (daf.) were obtained. Carbon, Hydrogen, Nitrogen, Sulfur and Oxygen (by difference) assay showed that the largest elemental change was increased carbon and a decreased oxygen content. Fatty acid fraction analysis revealed the total fatty acid content of the fungal biomass decrease by 70-80% after undergoing the HTL process and C18:2 fatty acids were likely 95% converted to C14:0 – C18:1 fatty acids. The higher heating value of the bio-crude oil was 33.5-37.5 MJ/kg (dried basis).

4.1 Introduction

Rhizopus oligosporus and *Mucor circinelloides* fungal biomass cultivated on low-value corn-ethanol byproduct thin stillage substrate has the potential to produce a variety of high value byproducts while reclaiming thin stillage to obtain a recyclable effluent by removing COD, suspended solids, glycerol and organic acids (Rasmussen et al., 2014; Ravi, 2014; van Sambeek et al., 2012). Fungal biomass grown on thin stillage also has the potential to be a promising

feedstock for advanced biofuels due to its short 2-3 day fermentation period and high oil content reported for *Mucor circinelloides* 46%±2% (Mitra et al., 2012) , 39% *Pythiem irregulare* (Liang et al., 2012) and *Rhizopus oligosporus* 20-25% (Rasmussen et al., 2014).

Fossil fuels account for 82% of the total energy consumption in the United States; however, it is expected to decline to 80% by 2040 (US EAI, 2014). The U.S. liquid biofuels production is projected to grow 22% from 0.9 MMbbl/d in 2012 to 1.1 MMbbl/d in 2040. A 92% growth from 1.3 MMbbl/d in 2012 to 2.5 MMbbl/d in 2040 of world liquid biofuel production is predicted (US EAI, 2014). With the expected continued growth of the liquid biofuel market and the continued food versus fuel debate of first-generation biofuels, second-generation and third-generation biofuels become more appealing to meet liquid biofuel demands.

A dry feedstock is required for traditional gasification and pyrolysis thermochemical processes for producing biofuels. Energy costs needed for vaporizing moisture content in the feedstock becomes substantial if the feedstock has a significant water percentage (Duan and Savage, 2011). Fungal biomass cultivated on thin stillage substrate similar to most microalgae is grown in a liquid suspension. Therefore, fungal biomass and most microalgae have very high moisture contents making aqueous-phase processing through hydrothermal liquefaction (HTL) a more promising thermochemical process for generating biofuels (Duan and Savage, 2011). Heat can be recovered from the HTL process for energy savings to improve efficiency (Jazrawi et al., 2013).

Hydrothermal liquefaction is thought to imitate the geological processes considered to naturally produce fossil fuels. Long-chain organic compounds (biomass) break into short chain hydrocarbons through a chemical reforming process (HTL) in a heated and pressurized oxygen deprived vessel. Water is considered to play an important role in HTL carrying out condensation,

cleavage and hydrolysis reactions and affecting selective ionic chemistry. Water likely causes organic material to break down and rebuild in shorter hydrocarbon chains by adding H^+ to open carbon bonds (Zhang, 2010).

In order to make HTL chemically controllable and economically feasible, HTL must be a continuous process (Jazrawi et al., 2013). Batch HTL of aqueous-phase algal biomass for conversion into biofuels has been researched extensively (Chow et al., 2013). However, very little research has been done on continuous HTL. Simple water soluble monosaccharide model compounds have been studied using semi-continuous or continuous HTL (Promedej et al., 2011; Srokol et al., 2004) and on food industry waste sludge and machinery works wastewater treatment sludge (Hammerschmidt et al., 2011). Only two published articles could be found that used a continuous flow reactor for HTL of aquatic biomass both using microalgae biomass feedstock (Elliot et al., 2013; Ross et al., 2013). No research has been published on HTL of fungal biomass neither in batch, nor in continuous. The aim of this research is to perform HTL on fungal biomass feedstock slurry using a continuous supercritical flow reactor (SCFR) operated at subcritical conditions and compare bio-crude oil yields with microalgae feedstocks processed through a similar continuous HTL process.

4.2 Methods and Materials

4.2.1 Fungal Slurry

A. Fungal Cultivation and Harvesting

Rhizopus oligosporus and *Mucor circinelloides* fungus was grown on thin stillage substrate (refer to Section 3.2.1 Thin Stillage and Section 3.2.2 Fungal Spore Description, Cultivation and

Storage for a detailed description of the thin stillage substrate and the fungal spores). The fungal spores were first germinated on YM broth prior to inoculation of the thin stillage as described in Section 3.2.3 Fungal Spore Germination on YM Broth.

Two (2) twin stainless steel fungal bioreactors with an internal volume of 50L were used for growing the fungal biomass. 1L of YM broth fungal flocculent solution was added to each 50L reactor for inoculation. The twin 50 L reactors have a diameter of 36 cm and a depth of 64 cm. One ceramic dome fine bubble diffuser with a 304 stainless steel base was installed in each 50L reactor. The diffuser is 19 cm in diameter and has a height of 9 cm. The diffuser was purchase from Cole-Parmer® item #EW-70025-22. Compressed air supply was pressure regulated to 15-20 psi and was routed to the submerged diffusers at an airflow rate of 40 L/min which equates 1.0 l air/L working volume/min (vvm). The 50L reactor was maintained at 3443±3 °C using a 200 W electric AquaTop™ submersible fish tank heater.

Prior to each reactor start-up the 50L reactor and diffuser were autoclaved at 121°C for a minimum of 30 min. The tank heater and the reactor plastic lid were chemically sterilized using bleach and 70% ethanol. 40L of thin stillage, 1L of YM broth fungal flocculent solution, and 13.2 mL of Sigma-Adlrich® Antifoam 204 (0.33 mL antifoam/L thin stillage) were added to each 50 L reactor. The initial pH of the thin stillage was recorded. 48-72 h was allowed for fungal fermentation.

The fungal biomass was harvested from the 50 L reactors as described in Section 2.2.5 Fungal Harvesting. The harvested fungal biomass was further dewatered placing it in a woven polypropylene bag and hand squeezing to remove excess water. The fungal biomass was then transferred into freezer storage bags and placed in a 10°C cooler. The percent dry-solids of each batch was determined by placing 5-10 g sample of wet fungal biomass on a petri dish and oven

drying it at 60 °C for 24-48 h. The dewatered fungal biomass had a dry-solids concentration ranging from 10% to 38%.

B. Fungal Slurry Preparation

Multiple harvested fungal biomass batches were combined, hydrated to a desired percent solids concentration and mechanically mixed. Based on the percent dry-solids and total mass of each batch combined the total water content and total dry-solids content of the combined batches was determined. Water was then added to the fungal biomass to achieve the desired percent solids of the fungal slurry.

The fungal slurry was mixed in two stages, a course mix and a high-shear mix. The course mix was done using a commercial grade food blender. The fungal biomass and dilution water were combined in the blender and pureed for approximately one minute. The second stage mix was done using a DR module IKA Works Inc. Labor-Pilot UTL-2000/4 model number P01191 high shear mixer. A high-speed rotor with narrow slot spins inside a stator. The rotor-stator dispersion action produces high shearing energy between the rotor and stator. The high shear mixer is of modular design and can be equipped with multiple stages of rotor-stator couplings. The high shear mixer was equipped with three fine toothed (6F) generator stages. The mixer is equipped with a 3450 rpm motor connected to a variable frequency drive which has a range of 24– 60 Hz. The transmission ratio of the belt drive increases the speed of the high shear mixer rotor shaft to 3,028 –7,570 rpm. The circumferential speed at the periphery of the high shear mixer rotor given this speed range is approximately 8.2– 22 m/s.

An 18 gallon (68L) cone bottomed hopper was used to feed the coarse mixed fungal slurry in the high shear mixer. The hopper was attached to the inlet flange of the high shear

mixer. The hopper was mixed using two (2) 3.5 cm three blade propellers mounted to a shaft. The hopper mixer motor has speed of 2700 rpm at 60 Hz. However, the hopper mixer shaft would vibrate excessively at higher speeds therefore the hopper mixer was limited to a max speed of 675 rpm at 15 Hz (25% of full speed). A flexible hose was attached to the outlet of the high shear mixer and recirculated to the inlet hopper. The entire fungal slurry was high shear mixed until a uniform consistent mixture was produced. A sample of the mixed and sheared fungal slurry was taken from each batch for a percent solids and ash determination. The fungal slurry was then stored at 10 °C for a later supercritical reaction test or used directly after high shear mixing.

Figure 4.1. 5% fungal slurry course mixed (left), high shear mixed (right). Fungal slurry prepared 7/2/14.

The manufacturer's operating manual for the supercritical flow reactor slurry pump stated that the pump had a solids handling of 10-15% (by weight). In two tests 8% solids fungal slurries were used in the HTL reactor. However, in subsequent tests it was found that fungal slurries with 8% solids or higher caused the influent double ball check valve on the slurry pump to stick open.

A solids content of 4-6% was found to be ideal. It was determined that rehydrating pre-dried fungi will allow fungal slurries with up to 13% solids to be successfully passed through the HTL reactor. However, because of the drying costs, this was not considered an economical approach for potential scale up.

4.2.2 Supercritical Flow Reaction

A. Equipment

The fungal biomass slurry underwent hydrothermal liquefaction (HTL) thermochemical process using a supercritical flow reactor (SCFR) located at the Biomass Energy Conversion (BECON) Facility located in Nevada, IA. The supercritical flow reactor was designed by Supercritical Fluid Technologies Inc. located in Newark, DE. The maximum operating temperature and pressure of the SCFR is 450 °C and 10,000 psi.

Figure 4.2 shows the flow diagram of the primary components of the supercritical flow reactor. There is also solvent pump, not shown, that could be used for feedstock additive and cleaning the reactor. However, it is not shown in the process flow diagram because it was not used in the HTL process of the fungal slurry. A 50L Nalgene® carboy jug was used for holding the deionized water feed water to the supercritical flow reactor. The 10L-column for holding the fungal slurry had a diameter of 18 cm and height of 52 cm. The fungal slurry column was mixed using a two-blade paddle mixer. The paddle mixer was rotated using an IKA® RW 16 basic overhead stirrer, 40-1200 rpm. The slurry pump was a Milroyal® C packed plunger liquid end pump.

The slurry pump had a maximum flow capacity of 250 mL/min, and maximum discharge pressure of 10,000 psi. The preheater consisted of 20 coil wraps of tubing around a 19 cm

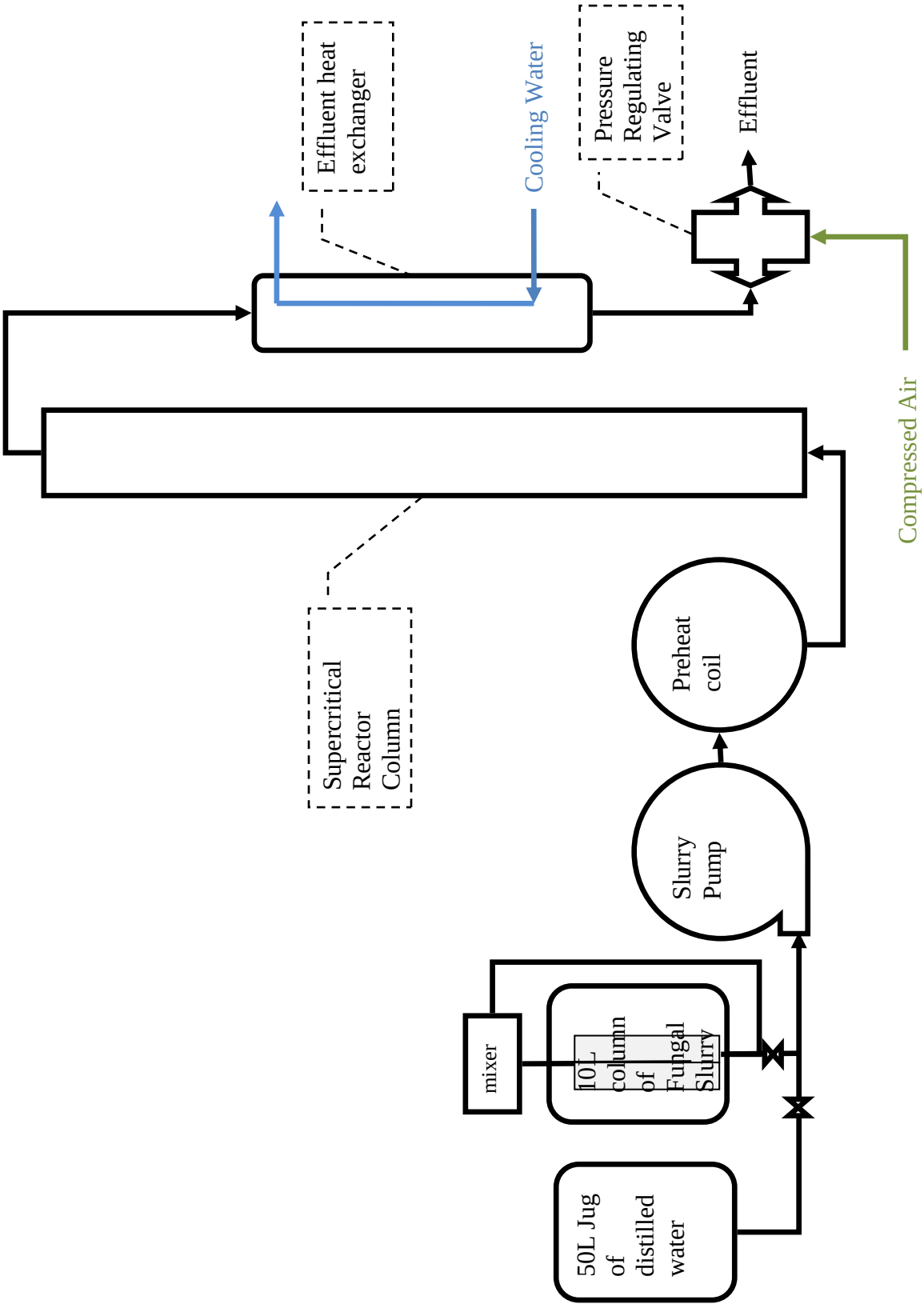

Figure 4.2 Supercritical Flow Reactor Diagram

diameter column. The coil length was approximately 12 m. The reactor column was 212 cm in height and had an inside diameter of 3.2 cm. The reactor volume was measured by filling with water from a graduated cylinder and was found to be 1.5 L. The reactor column effluent was cooled using a liquid-to-liquid heat exchanger. The heat exchanger consists of a Swagelok® 304L SS Double-Ended DOT-Compliant Sample Cylinder, part number 304L-HDF4-2250 with a domestic cold water connected to the cold water inlet for cooling the hot effluent of the reactor. The preheat coil and the reactor column were insulated and electrically heated.

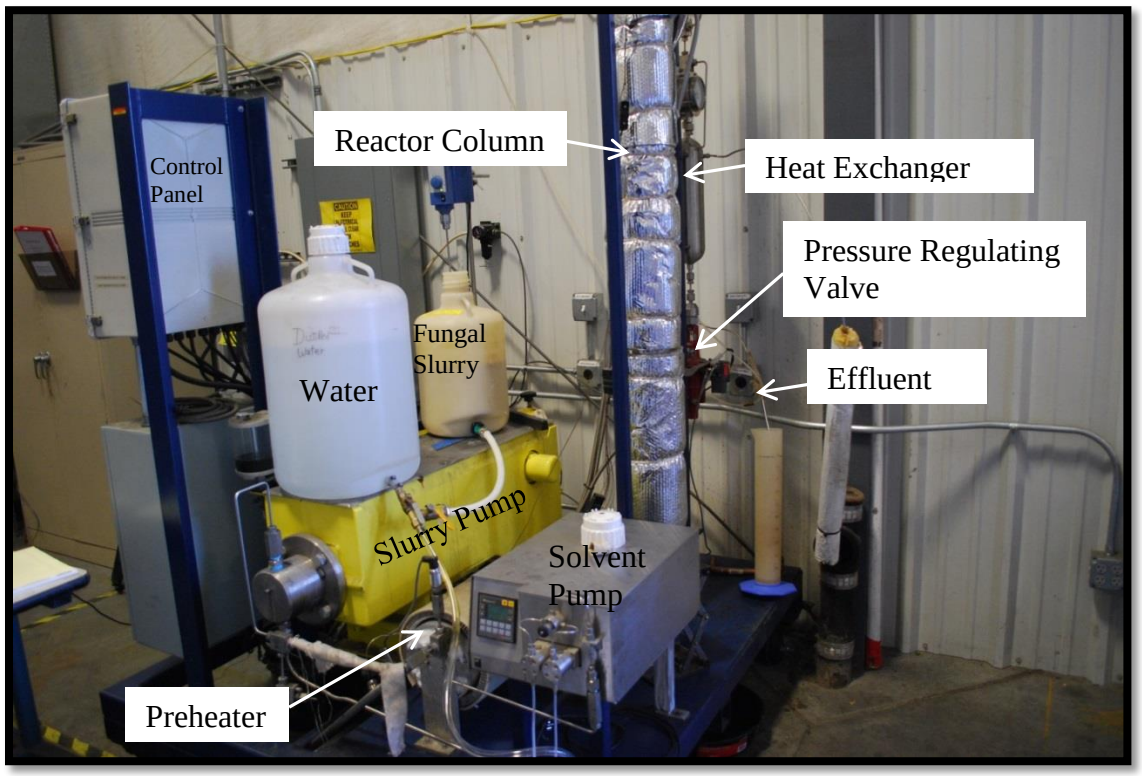

Figure 4.3. Supercritical flow reactor

The digital control system allowed for control of the reactor column pressure, reactor column temperature and preheater coil temperature. The reactor column consisted of three temperature control zones. The bottom, middle and top temperature zones could each have a different target

temperature. The system pressure was automatic controlled by the pneumatic pressure regulating valve near the system discharge point. Two rupture discs located between the prior to the preheat coil and the effluent of the reactor column protected the system from accidental over pressure of 10,000 psi. A dial pressure gauge installed near the heat exchange was available for confirmation of system pressure. Figure 4.3 shows a labeled picture of the supercritical flow reactor at BECON used to perform the HTL of the fungal slurries.

B. Operation

To start-up the supercritical flow reactor first air supply was provided to the pressure regulating valve, water was supplied to the effluent heat exchanger and the control panel was powered on. The reactor was prefilled with deionized water by either removing the reactor column top cap and pouring the water in, or by pumping it in using the slurry pump. The valve to 50 L jug of deionized water was opened to supply deionized water while the SCFR was brought up to the set point temperatures and pressure. The preheat coil temperature was set at 133 °C. Preheat temperatures greater than 133 °C were not recommended by Elliot et al. (2013) to avoid baking fungal slurry onto the side walls of the preheat coil potential causing the narrow diameter tubing to plug . The slurry pump continued to pump deionized water into the reactor column for 2-3 h until the supercritical reactor column was up to the set point temperatures. While the SCFR was warming up, the fungal slurry that had previously been course mixed was high shear mixed as described in Section 4.2.1 Fungal slurry. The high shear mixed fungal slurry was then poured into SCFR 10L column. The paddle mixer was turned on to continuously mix the slurry. Once the reactor column was fully up to the set point temperatures, the valve to the 50 L jug of deionized water was closed and the valve to the 10 L column of fungal slurry was opened. Once

the fungal slurry started flowing to the slurry pump, the first 2 L of liquid was collected and wasted. The reactor column volume was filled with 1.5 L of deionized water, so the first 2 L of liquid was wasted because it was likely diluted since the plug of deionized water in the column had not yet been displaced with fungal slurry.

Samples were collected using 500 mL Nalgene® high density polyethylene bottles. The hydraulic residence time in the reactor column was varied by changing the flow rate of the slurry pump for different test runs. Effluent 500mL samples were collected every 4-22.5 min depending on the selected slurry pump flow rate. The sample bottles were weighed empty and again after they were filled. The sample volume was estimated as 1g = 1mL since the sample contents were greater than 95% water. Direct volumetric determination of the sample contents would have been difficult due to that the 5% or less oil content of the sample tended to adhere to the side walls of the sample bottle and must be removed using a solvent.

The reactor column was cleaned using superheated deionized water after each supercritical flow reactor run. The upper and lower column end caps were removed and cleaned in the lab using a brass brush and lacquer thinner. The end caps were then reinstalled on the reactor column. The reactor column was filled with deionized water and heated to an internal temperature of 385 °C. The total time to heat up the reactor column was approximately 4 h. Water was added to the column for 1-2 min every hour to make up for any water loss and ensure the reactor was completely full during the cleaning process. The column pressure was regulated at 4000 psi. Once the column was fully up to temperature, the preheat temperature was set at 133°C and the slurry pump was turned on at 38 to 50 ml/min. A minimum 2 L of deionized water was pumped through the reactor column to displace the 1.5 L volume that was in contact

with the reactor column side walls. The pump remained on until the discharge effluent contained no visible discoloration from char or oil.

4.2.3 Percent Solid and Ash Determination

Percent solids and ash determination on the fungal slurry was done on each fungal slurry that was prepared. The fungal slurry was hydrated to the desired percent solids, blended and high shear mixed as described in Section 4.2.1 Fungal Slurry. After the fungal slurry was high shear mixed a 500-2,000 mL sample was collected. The sample was transported to the Metals Development building on the Iowa State University campus for percent solids and ash determination. The solids and ash determination test was done in triplicate. The average percent solids and ash was used for determining the percent dry ash free oil content.

The method for percent solids and ash determination generally followed the methods described by Jazrawi et al. (2013) using a Coors™ 150 mL (84 x 52 mm) porcelain crucible and 50-100 g of wet fungal slurry. The wet fungal slurry was dried for a minimum of 24 h at 105 °C. The percent solids content of the fungal slurry was determined using Equation 4.1.

$$Fungal\ Slurry\ \%\ Solids = \frac{(crucible + dry\ fungal\ mass) - (empty\ crucible\ weight)}{(crucible + wet\ fungal\ mass) - (empty\ crucible\ weight)}$$

Equation 4.1. Solids content of fungal slurry

The crucible containing the dried fungal mass was then placed in a Fischer-Scientific Isotemp® programmable muffle furnace set at 550 °C for 3 h. Later ash determination tests were done using a three staged heating at 200 °C, 250 °C and 550 °C for 3 h. It was found that having

only a single stage cause the dry fungal mass sample to ignite possibly causing ash particle to be lost to the atmosphere. The ash content was calculated by Equation 4.2.

$$Fungal\ Slurry\ \%\ Ash = \frac{(crucible + ash) - (empty\ crucible\ weight)}{(crucible + dry\ fungal\ mass) - (empty\ crucible\ weight)}$$

Equation 4.2. Ash content of solids in the fungal slurry

4.2.4 Bio-Crude Oil Yield Determination

The HTL samples of the fungal slurry after undergoing the hydrothermal liquefaction process were collected and taken to Metals Development Laboratory at the Iowa State University campus for oil yield determination. An HTL effluent liquid sample poured into a glass jar for demonstration purposes is shown in Figure 4.4. The HTL effluent sample contains a visible aqueous and oil fraction. The much smaller solids (char) content of the sample is not readily visible aqueous HTL effluent solution but was observed during filtration.

Figure 4.4. HTL effluent sample (left)
Figure 4.5. Bio-crude oil sample from HTL 1 test (right)

The oil yield determination test used methylene chloride as the solvent and generally followed the procedures of Ross et al. (2011). Bio-crude oil was defined as the methylene chloride soluble fraction. Figure 4.5 shows the bio-crude oil sample from HTL 1 being poured into a beaker. Methylene chloride is a probable human carcinogen and is ranked in EPA's Group B2 (US EPA). Due to the carcinogenic risk, all work done with methylene chloride was performed in a fume hood wearing 5-layer ethylene vinyl alcohol copolymer Sliver Shield® chemical resistant gloves. Other standard personal protective equipment included safety glasses, laboratory coat and closed toed shoes. The 500 mL HTL sample was transferred into a 1 L polypropylene (PP) bottle. 150-250 mL of methylene chloride solvent was added to the 500 mL HTL sample. The 1L bottle containing the methylene chloride and the HTL sample was placed in a rotator and rotated for 60 min at 30 rpm to assure the bio-crude oil present was completely dissolved in the methylene chloride.

Figure 4.4. HTL samples with methylene chloride solvent being rotated

The sample was then filtered using Whatman®, 934-AH™, 110mm glass microfiber filter paper, porcelain Buchner filter funnel, 2-L vacuum flask, and a vacuum pump as shown in Figure 4.6. The filter paper was stored in a desiccator and weighed prior to filtration. The filter paper was then placed on a petri dish and dried overnight in an oven set at 105 °C. The filter paper was then cooled in a desiccator and weighed to determine the percent solids in the HTL sample. The char content was only the fluidized portion that discharged from the SCFR. A significant amount of solids settled in the up flow column due to the low up flow velocity. Figure 4.5 shows the solids that had settled in the SCFR during an HTL run. The picture was taken during the disassembly and cleaning process performed between HTL runs. Equation 4.3 and 4.4 were used to calculate the dry ash free (daf.) fungal biomass of the HTL sample, and the bio-crude oil percent solids.

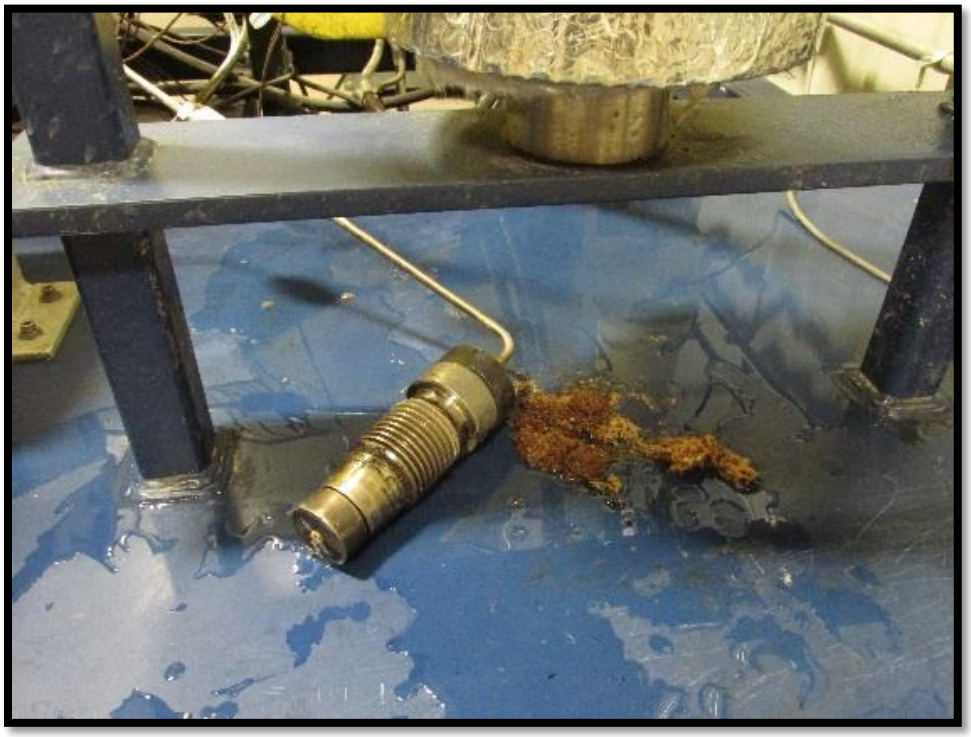

Figure 4.5. Solids that settled out of the SCFR HTL run

$$Fungal\ Biomass\ Content\ of\ HTL\ Sample\ (g, daf.)$$
$$= HTL\ sample\ mass * (fungal\ slurry\ \%\ solids - fungal\ slurry\ \%\ solids * fungal\ slurry\ \%\ ash)$$

Equation 4.3. Mass of dry ash free fungal biomass present in the HTL sample

$$Bio - crude\ oil\ \%\ Char = \frac{(Filter\ Paper + Solids) - (dry\ filter\ paper\ wt.)}{Fungal\ Biomass\ content\ of\ HTL\ sample\ (g, daf.)}$$

Equation 4.4. % Solids of the bio-crude oil

The filtered HTL sample was then poured into a 1-L glass separatory funnel as shown in Figure 4.7. The separatory funnel glass stopper was put in place while the water and methylene chloride layers were allowed to separate for 15 min. Methylene chloride is denser then water at a density of 1.3266 g/cm^{3}and settled to the bottom of the separatory funnel as shown in Figure 4.8. An empty 325 mL (100x50mm) crystalizing dish was weighed and placed under the separatory funnel. The methylene chloride layer was drained into the crystalizing dish as shown in Figure 4.9. The crystalizing dish was then kept in a fume hood for a minimum of 3 days to allow the methylene chloride solvent to evaporate. The crystalizing dish containing the remaining methylene chloride soluble fraction of oil (bio-crude oil) and was weighed and recorded. The bio-crude oil yield was calculated using Equation 4.5.

$$\%\ yield = \frac{oil\ mass}{Fungal\ Biomass\ Content\ of\ HTL\ Sample\ (g, daf.)}$$

$$oil\ mass = (crystalizing\ dish + oil) - crystaling\ dish\ wt.$$

Equation 4.5. % Yield of bio-crude oil relative to the mass of dry ash free fungal biomass present in the HTL sample

Figure 4.6. HTL sample with methylene chloride being poured into a Buchner funnel for % char determination (left)
Figure 4.7. HTL sample with methylene chloride settling in separatory funnel (right)

Figure 4.8. Stratification in the separatory funnel of HTL sample aqueous fraction (top layer) and methylene chloride soluble fraction (bottom layer) (left)
Figure 4.9. (Right) Draining methylene chloride soluble fraction from the HTL sample into a crystalizing dish

4.2.5 Analytical Laboratory Tests

All of the analytical laboratory tests listed in Table 4.1 were completed by the Agricultural Utilization Research Institute on September 23rd, 2014. The test results of the analytical laboratory are shown and discussed in Section 4.3. Fungal biomass samples were dried at 80°C and ground using an analytical mill and a porcelain mortar and pestle. Ground fungal biomass samples were ground until it completely passed through a -40 mesh sieve.

Table 4.1 Analytical Laboratory Testing Methods

Assay	Method#
Loss on Drying	AOAC 950.46b
Karl Fisher Moisture	AOCS Ca 2e-84
Ash	AOAC 920.153
Lipid	AOAC 991.36
Protein	AOAC 981.10
Fatty Acid Profile	AOCS Ce 2-66, Ce 1j-07
Carbohydrates	Calculated
Heating Value	ASTM E711
Sulfur	ASTM D4239
Carbon, Hydrogen, Nitrogen	ASTM D5373

4.3 Results and Discussion

A. Oil Yields

Oil yields of 36-54% dry, ash-free basis (daf.) were obtained with fungal slurries in the HTL reactor. Table 4.2 shows the reaction, fungal biomass slurry and % yield details. Figure 4.2 shows a typical oil yield profile throughout an HTL test run, with sampling being performed every 6-8 min. The oil yield was low for the first few samples because water was being purged from the reactor and being replaced with the fungal slurry. Likely the up-flow through the reactor column was not a true plug flow and some mixing took place causing dilution of the first few

samples taken. It must be noted that the majority of the oil obtained from the HTL reactor is due to the inherent oil content of the raw fungus. Figure 4.3 shows the oil yields obtained from four of the tests done with fungal biomass. Test HTL 1 was performed on a mixture of 45% *Mucor circinelloides* and 55% *Rhizopus oligosporus*. The other three tests were performed using 100% *Rhizopus oligosporus* fungal slurry.

Table 4.2 Hydrothermal liquefaction test description and details

HTL Reaction	**HTL 1**	**HTL 2**	**HTL 3**	**HTL 4**
HTL Reaction Date	6/6/2014	7/25/2014	8/4/2014	9/5/2013
Fungus Species	45% *Mucor c.*, 55% *Rhizopus o.*	*Rhizopus o.*	*Rhizopus o.*	*Rhizopus o.*
Fungal Slurry Date	6/1/2014	7/17/2014	7/31/2014	8/26/2014
Fungal Production Date Range	2/15/14 – 5/30/14	Unknown	6/24/14 – 7/24/14	8/8/14 – 8/22/14
Dried and Rehydrated	No	Yes	No	No
Temperature				
Top	354	368	351	325
Middle	365	392	377	350
Bottom	173	186	194	174
Pressure	2,900	4,000	4,000	4,000
Residence Time (min.)	36.1	32.1	20.6	15.4
Fungal Slurry % Solids	4.9%	12.6%	7.2%	7.3%
Fungal Slurry % Ash (dry)	13.5%	7.7%	8.1%	13.0%
Raw Fungus Lipid (wt.% dry)	---	36%	40%	---
Raw Fungus Lipid (wt.% daf.)	---	39%	43%	---
Bio-Crude Oil Yield (wt.% daf.)	36%	50%	54%	52%
Standard Deviation	1.3%	1.7%	2.9%	2.5%

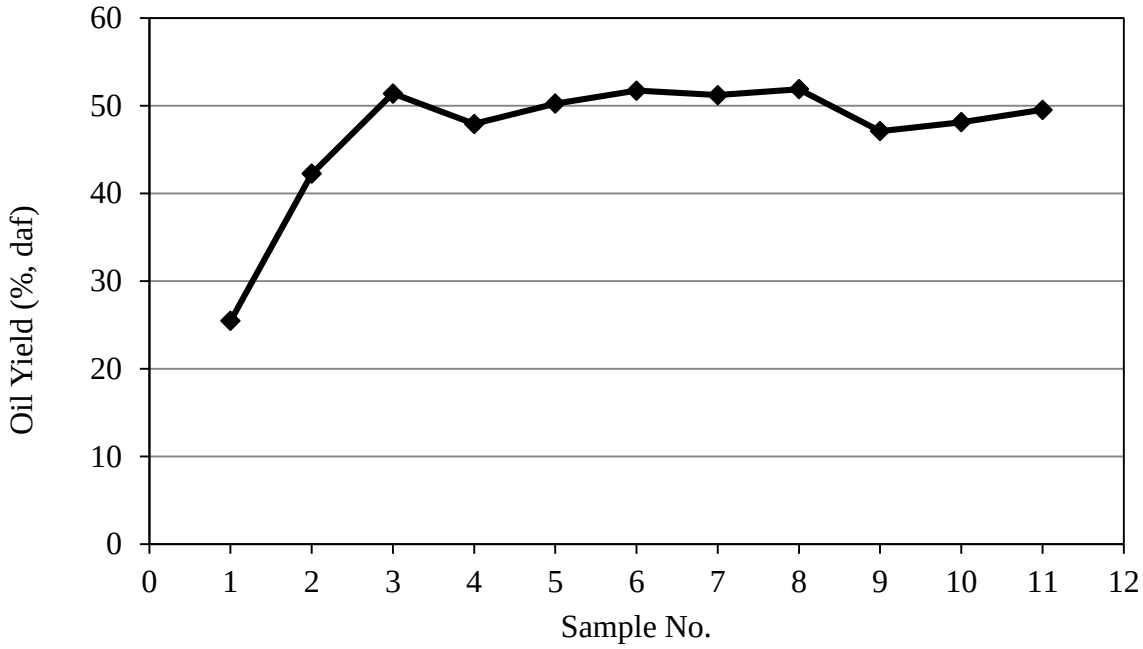

Figure 4.2. Typical oil yields throughout an HTL test using fungal biomass. Test HTL 1 shown.

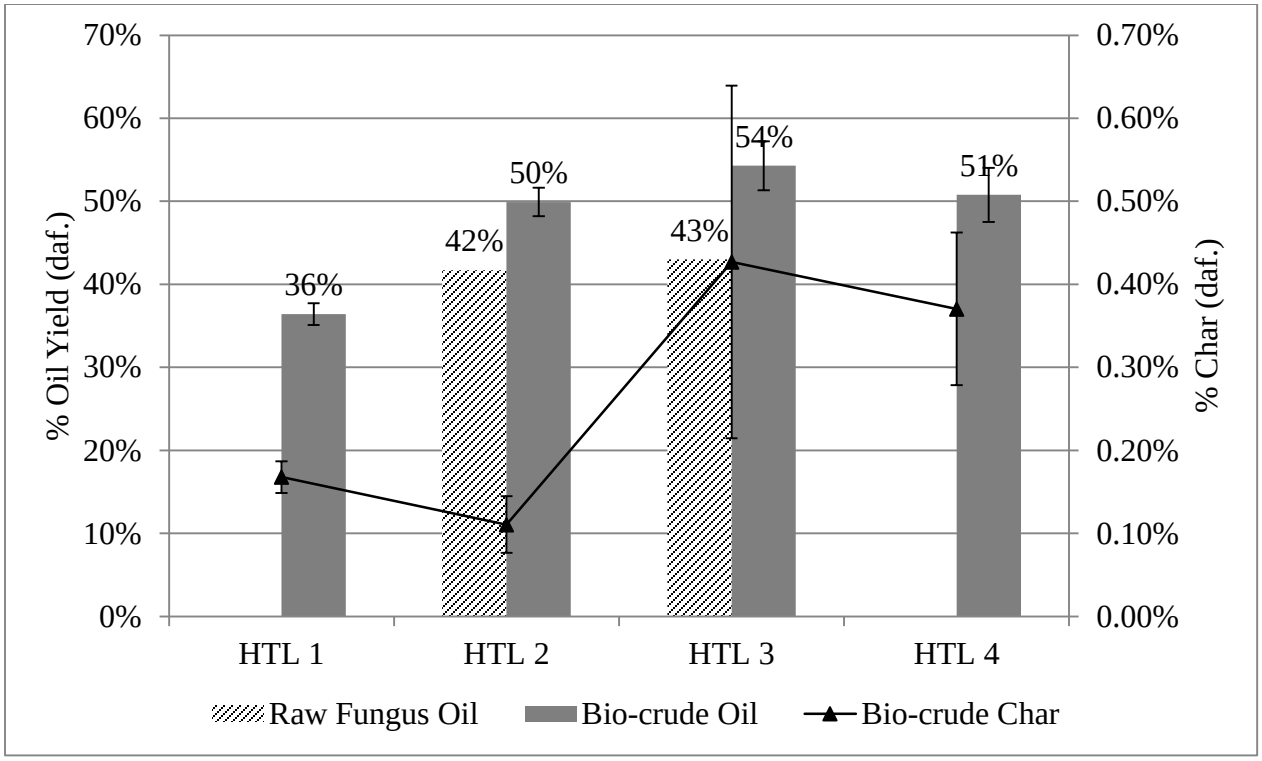

Figure 4.3 Oil yields from HTL processing of fungal biomass

A literature search yielded no previous research done on hydrothermal liquefaction of fungal biomass. Chow et al. (2013) reported the results of nineteen research articles on hydrothermal liquefaction of algae. Of the nineteen algal HTL articles reviewed, yields of up to 70-80% bio-oil (%) to feed stock dried wt. basis were reported (Chow, 2013). However, all of the algae HTL reviewed by Chow et al. (2013) were done using batch reactors. Elliot et al. (2013) and Jazrawi et al. (2013) performed hydrothermal liquefaction using a continuous flow reactor of wet algal slurries. Since the HTL research done by Elliot et al. (2013) and Jazrawi et al. (2013) were performed using a similar continuous flow reactor as was used in the HTL of the fungal biomass, a more extensive comparison of the Elliot et al. (2013) and Jazrawi et al. (2013) results were done throughout this chapter. Four algal slurry feed stocks were tested by Elliot et al. (2013) at temperatures ranging from 344-362°C, pressures of 2966-3020 psig, and residence time of 1.5-2.2 h. Jazrawi et al. (2013) had a solids loading rate of 1-10 wt. %, a temperature of 250-350°C, a residence time of 3-5 min, and a pressure was held constant at 2,175-2,900 psi. The bio-crude oil yield from HTL of the algal slurry reported by Elliot et al. (2012) ranged from 38 – 63.6 wt. % daf, and 10-42 wt. % daf by Jazrawi et al. (2013). This compared closely with the 36-54% bio-crude oil yields observed from HTL of the fungal biomass. Jazrawi et al. (2013) reported that the wt. % daf. of bio-crude oil was 1.1-10 times the oil original oil present in the algae. Tests HTL 2 and HTL 3 yielded 1.28 and 1.26 bio-crude oil, respectively, compared to the original oil in the fungus. This shows that the continuous flow HTL of fungi falls within the expected range reported for algae. However, the bio-crude oil yield compared to the original biomass oil content is low end of the range of what is likely achievable. It must be noted that the majority of the oil obtained from the HTL reactor is due to the inherent oil content of the raw

fungus. Other sources of oil come from the conversion of protein and carbohydrates to bio-crude oil.

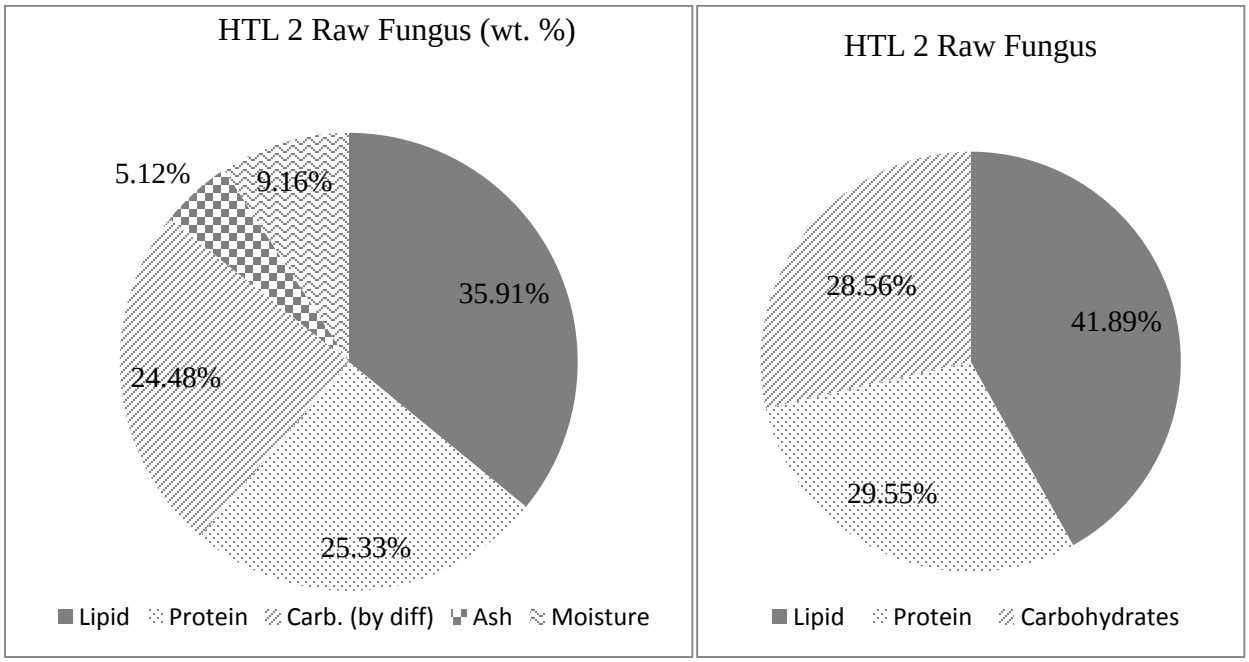

Figure 4.4 HTL 2 raw fungus lipid, protein and carbohydrate content

B. Protein, Carbohydrate and Lipid Content

The lipid, protein, ash and Karl Fisher moisture content of the raw fungal sample for the HTL 2 reaction were as shown in Figure 4.4. The lipid content of HTL 2 and HTL 3*Rhizopus oligosporus* raw fungus was 42 and 43 wt. % daf., respectively. The lipid content of the *Rhizopus oligosporus* raw fungus for HTL 2 and HTL 3 was approximately double the reported a 20-25% lipid content for *Rhizopus oligosporus* grown on corn-ethanol thin stillage by Rasmussen et al. (2014). *Mucor circinelloides* fungus grown on thin stillage substrate has been reported to contain 46 ±2 wt. %, dry (Mitra et al., 2012) which is only 4-12% higher lipid content of the *Rhizopus oligosporus* fungal biomass used in HTL 2 and 3.

Biller and Ross (2011) tested different proteins, amino acids, carbohydrates, oil and algae with various carbohydrate, protein an oil make ups. Biller and Ross (2011) found that lipids form hydrothermal liquefaction bio-crude oil yields of 55-80%, protein 11-18% and carbohydrates 6-15%.

$$bio - crude\ oil\ yield\ \% = (protein\ yield\ 11 - 18\% * protein\ content\ \%) + (carbohydrate\ yield\ 6 - 15\% * carbohydrate\ content\ \%) + (lipid\ yield\ 55 - 80\% * lipid\ content\ \%)$$

Equation 4.6. Predicted bio-crude yield % based on protein, carbohydrate, lipid content (Biller & Ross, 2011)

Based on the dry ash free weight % (daf. wt.%) of the lipid, protein and carbohydrate of HTL 2 raw fungus the bio-crude oil yield predicted to be the range of 28-43 wt. % daf. using Equation 4.6 as shown in Table 4.3. The actual bio-crude oil yield of the HTL 2 sample was 50%, exceeding the predicted range using Equation 4.6. Applying Equation 4.6 to the protein, carbohydrate, lipid content of the *Chlorella* and *Spirulina* algae that underwent continuous HTL by Jazrawi et al. (2013) projected bio-crude oil yields of 12-20% for *Chlorella* and 14-23% for Spirulina. The actual bio-crude yield of continuous HTL of *Chlorella* and *Spirulina* was 10-45% and 10-18%, respectively (Jazrawi, 2013). In the case of *Spirulina* the actual bio-crude oil yield fell within the range predicted by Equation 4.6. However, the actual maximum bio-crude yield reported for *Chlorella* (Jazrawi, 2013) was over double the projected high yield. This shows that the HTL2 yield of 16% greater than the projected high yield is reasonable and potentially could still be increased. Since both the *Chlorella* algae bio-crude yields (Jazrawi, 2013) and the fungi

HTL 2 yield exceed the predicted yield, the yield % factors proposed by Biller and Ross (2011) may be too low.

Table 4.3 Predicted bio-crude oil yield % (daf.) based on the oil, protein and carbohydrate content using Equation 4.6.

Biomass Slurry Description	Compound	Biomass Assay wt.% daf.	Bio-Crude Yield %			
			% of compound wt.		% of total wt.	
			Low	High	Low	High
Rhizopus oligosporus fungus, HTL 2	Lipid	41.89%	55%	80%	23%	34%
	Protein	29.55%	11%	18%	3%	5%
	Carbohydrates	28.56%	6%	15%	2%	4%
	Predicted Total	---	---	---	28%	43%
	Actual Total	---	---	---	50%	
Chlorella Algae (Jazrawi et al., 2013)	Lipid	5%	55%	80%	2%	4%
	Protein	68%	11%	18%	7%	12%
	Carbohydrates	28%	6%	15%	2%	4%
	Predicted Total	---	---	---	12%	20%
	Actual Total	---	---	---	10%	45%
Spirulina Algae (Jazrawi et al., 2013)	Lipid	9%	55%	80%	5%	7%
	Protein	78%	11%	18%	9%	14%
	Carbohydrates	13%	6%	15%	1%	2%
	Predicted Total	---	---	---	14%	23%
	Actual Total	---	---	---	10%	18%

C. Carbon, Hydrogen, Nitrogen, Sulfur and Oxygen Assay

Carbon, hydrogen, nitrogen, sulfur and oxygen (by difference) wt. % assay analysis was performed on seven different raw fungal slurries. Only two of the fungal slurries were used in an HTL reaction. Figure 4.4 shows how the two fungal slurries used in HTL 2 and HTL 3 compare with the other five fungal slurries. The average wt.% along with one standard deviation error bar is shown for each element. As can be seen by the low standard deviation, all of the raw fungal slurry samples had little variation in their elemental make up. HTL 2 and HTL 3 CHNSO wt. %

assay is shown alongside the average for comparison. The rank of HTL 2 and HTL3 in the data set of seven raw fungal slurry samples as a percentage of the data set is shown in Figure 4.4 (secondary axis). A 50% rank would be an average wt.%. A 90% rank would mean that 90% of the samples had lower wt. % of that element. From Figure 4.4 it can be seen that HTL 2 raw fungal slurry elemental constituents are closer to the 50% percent rank than the HTL 3 raw fungal slurry for all elements and therefore HTL 2 raw fungal slurry more closely represents the average raw fungal slurry elemental make-up.

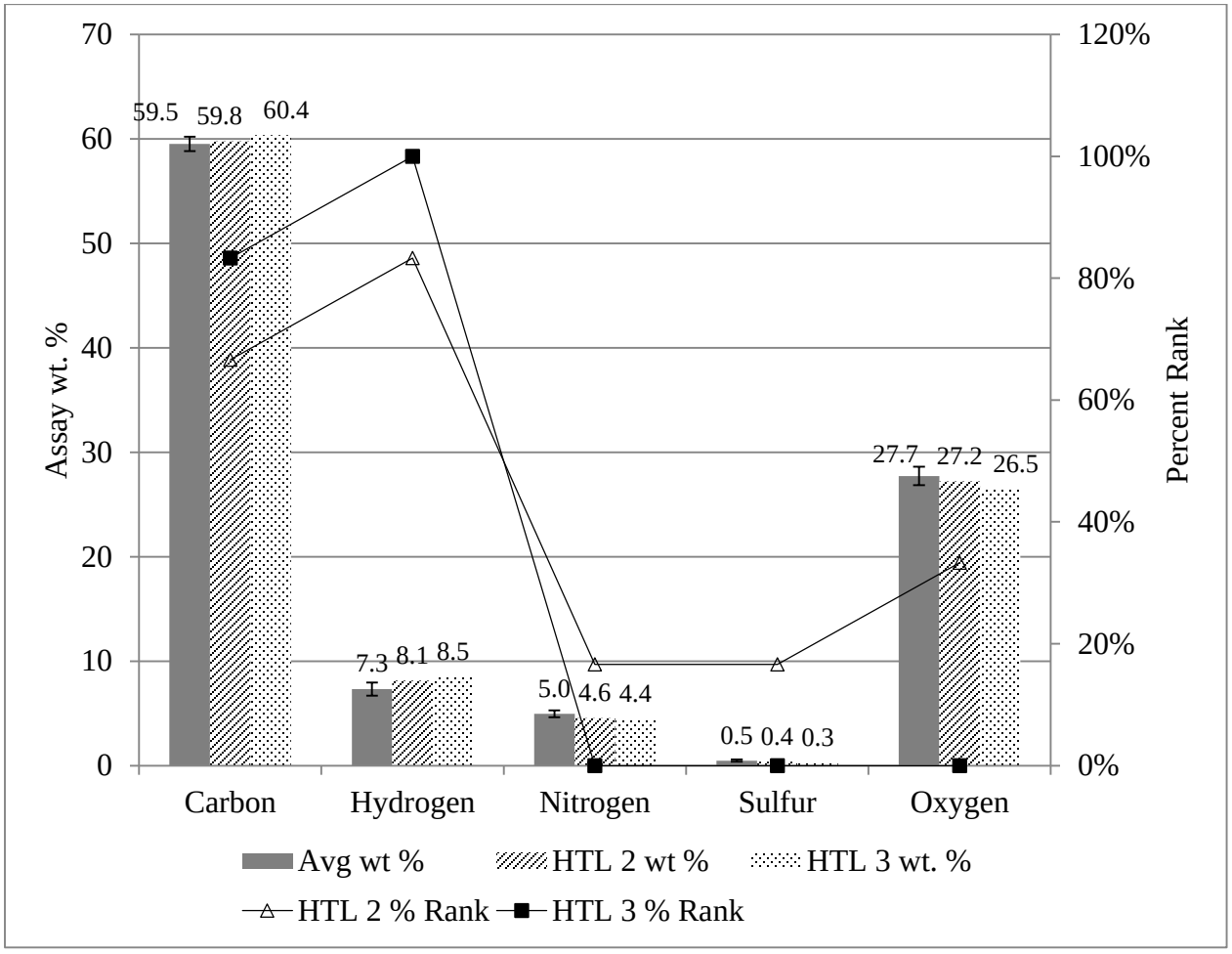

Figure 4.4. C, H, N, S, and O (by diff.) assay wt. % for the seven raw fungal slurries

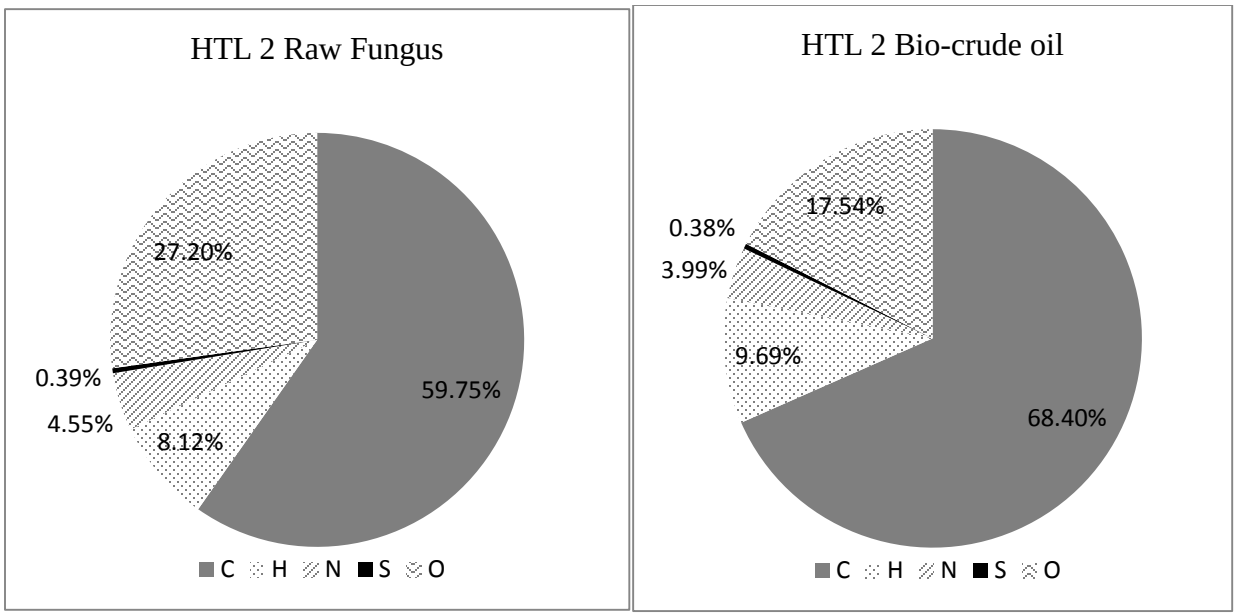

Figure 4.5. C, H, N, S, and O (by diff.) assay for the fungal slurry and Bio-crude oil from HTL 2

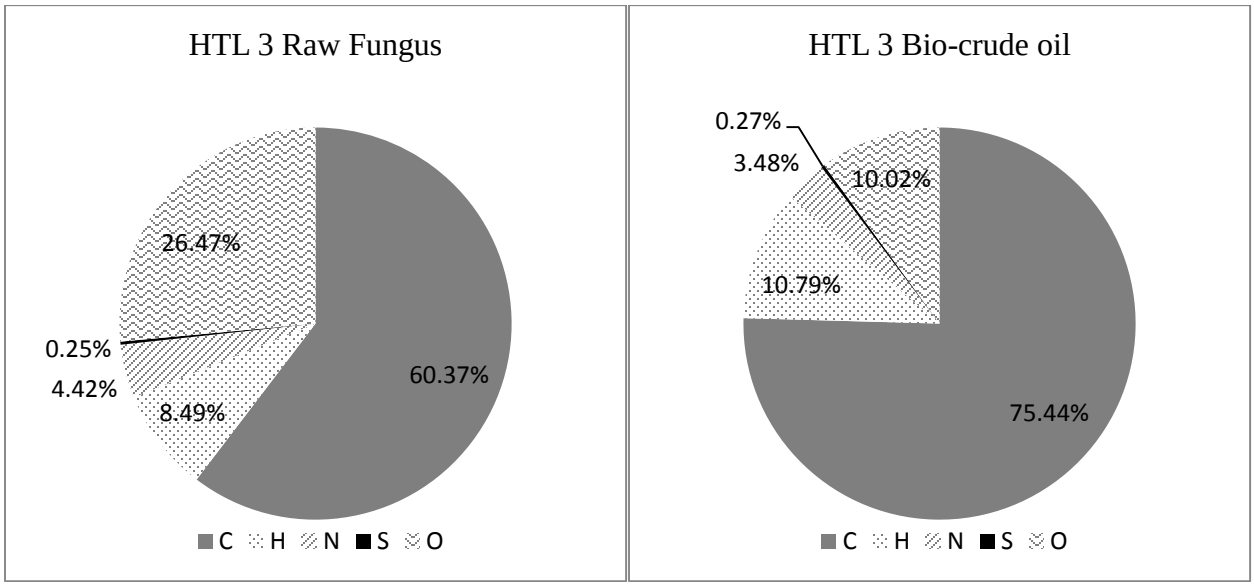

Figure 4.6. C, H, N, S, and O (by diff.) assay wt. % for the raw fungal slurry and Bio-crude oil from HTL 3 reaction

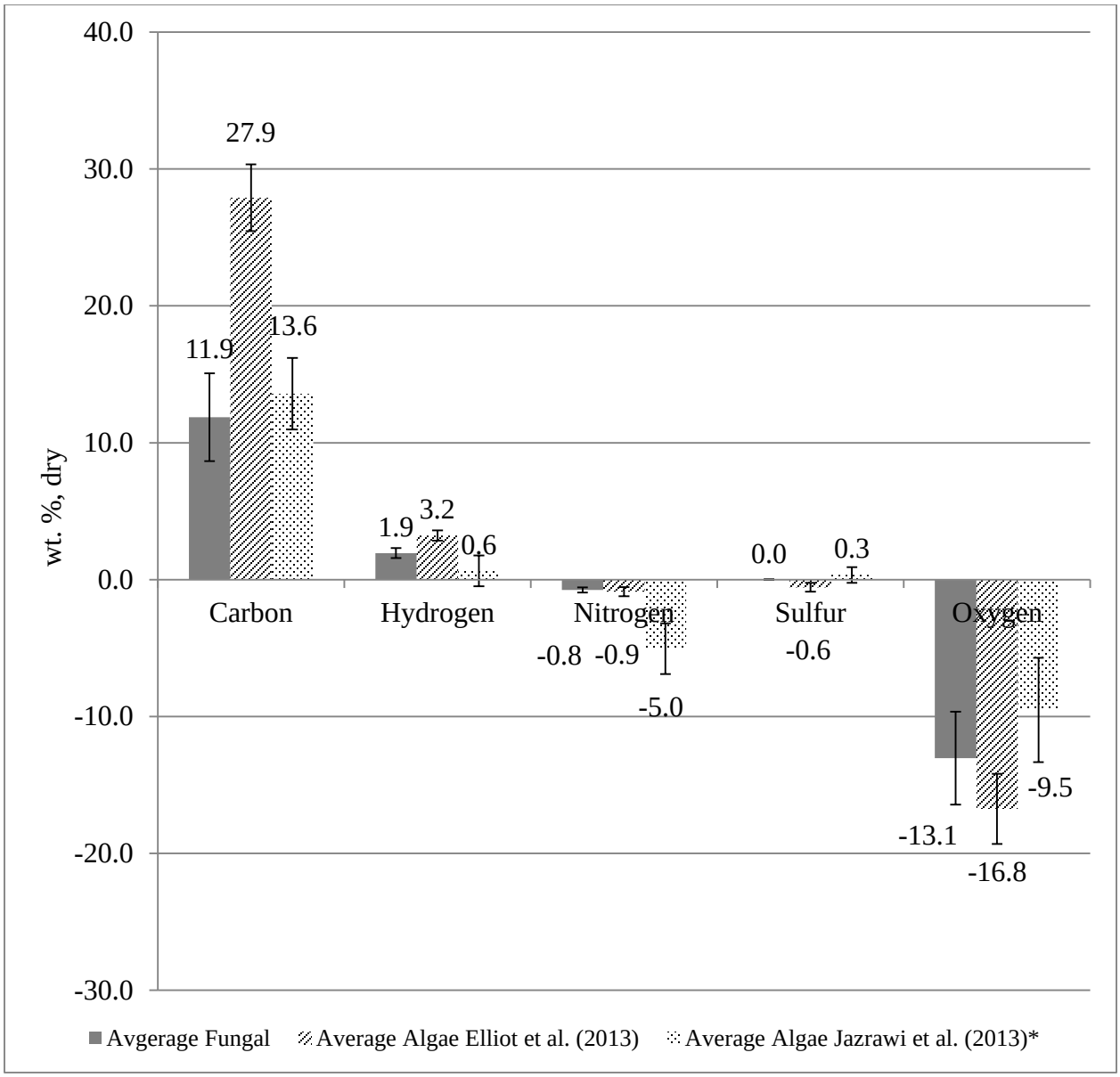

Figure 4.7 Change in C, H, N, S, and O (by diff.) assay wt. % from the raw biomass slurry to bio-crude oil. Avg. fungi values came from HTL2 and HTL3 shown in Figures 4.5 and 4.6. Avg. Algae values came from Elliot et al. (2013) and Jazrawi et al. (2013)
* Jazrawi et al. (2013) values are reported as wt. %, daf.

The raw fungal slurry underwent changes in its elemental constituency during the HTL reaction as shown in Figure 4.5 and 4.6. Figure 4.7 shows the percent change in the C, H, N, S, and O (by diff.) content. The fungal biomass percentage was subtracted from the bio-crude oil

percentage of C, H, N, S, and O (by diff.). A positive value indicates that the elemental wt.% increased after undergoing the HTL process. A negative value indicates that the elemental wt.% decreased after undergoing the HTL process. The carbon and oxygen wt. % changed the largest amount in the assay of the HTL 2 and HTL 3 fungal slurries. On average the fungi carbon weight content went up by 11.9% and the oxygen content went down by 13.1%. The hydrogen content weight percent change was much smaller at only 1.94% wt. on average. Nitrogen and Sulfur content change by less than 1%. Since HTL of fungal biomass has not previously been reported the closest comparison as shown in Figure 4.7 is with continuous-flow reactor HTL of algae research by Elliot et al. (2013) and Jazrawi et al. (2013). The same trend was reported both in the continuous flow HTL of the fungal biomass as was observed by HTL of algal biomass. Carbon and hydrogen wt. % increased. Oxygen and nitrogen content decreased. However, there was virtually no change in the sulfur content of the fungal biomass after undergoing the HTL process. The change in sulfur content of the algal biomass was mixed. A sulfur content decrease of 0.6 wt. % dry (Elliot, 2013) and increased of 0.3 wt. % daf. (Jazrawi, 2013). Although the same trend was observed between algal biomass and the fungal biomass, the algal biomass showed greater wt. % change in C, H, N, S, and O (by diff.) than reported by Elliot et al. (2013). Algal biomass wt. % change of carbon, hydrogen, sulfur and oxygen reported by Jazrawi et al. (2013) was less than reported by Elliot et al. (2013). In general the C, H, N, S, and O (by diff.) changes observed in the fungal biomass fell within the range reported for continuous flow HTL of algal biomass.

D. Fatty Acids Analysis

Rhizopus oligosporus fungal biomass underwent hydrothermal liquefaction and bio-crude oil was determined as described in Section 4.2.2 Super Critical Reaction and 4.2.4 Bio-Crude Oil Yield. The bio-crude oil fatty acid profile shown in Figure 4.9 was from HTL 2 and HTL 3 reactions detailed in Table 4.2. The *Rhizopus oligosporus* fungal biomass average fatty acid profile shown in Figure 4.9 is from seven samples obtained from 2012 through 2013. Even though the *Rhizopus oligosporus* fungal biomass samples that were analyzed for fatty acids were from previous *Rhizopus oligosporus* harvests, the standard deviation of the fatty acid content was low showing that the fatty acid content fungal biomass was consistent.

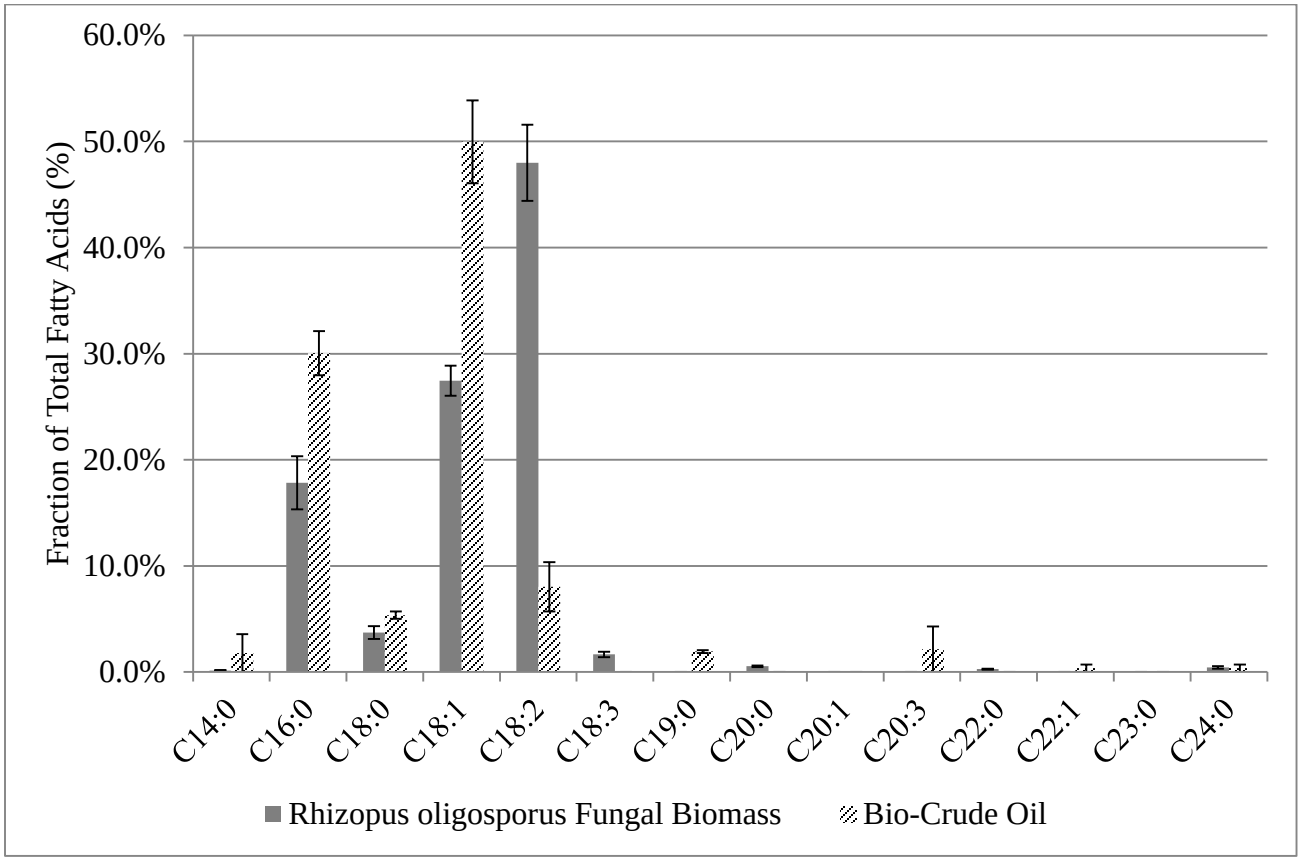

Figure 4.9 Fraction of total fatty acid profile of *Rhizopus oligosporus* fungal biomass and bio-crude oil product from HTL reaction of *Rhizopus oligosporus* fungal biomass

The total content and distribution of the C16:0, C18:0, C18:1, C18:2 and C18:3 fatty acids are important since it affects fuel stability and cold flow performance. The fatty acid content and distribution is also an important factor affecting the ability to further refine the bio-crude oil. The total percent fatty acid content fungal biomass was 98.5% with standard deviation of 0.3%. The total percent fatty acid content bio-crude oil was 23.7% with standard deviation of 4.4%. The total fatty acid content of the fungal biomass after undergoing the HTL process decreased by 79.5% – 70.1%. This indicates that 79.5% – 70.1% of the fatty acids present in the fungal biomass were converted to hydrocarbons.

Figure 4.10 shows the percent change in the fraction of total fatty acid. The fungal biomass percentage was subtracted from the bio-crude oil percentage of fraction of total fatty acid. A positive value indicates that the fraction of total fatty acid increased after undergoing the HTL process. A negative value indicates that the fraction of total fatty acid decreased after undergoing the HTL process. The largest change in the fraction of the total fatty acid content was the C18:2. The C18:2 fatty acid fraction of the total content was reduced by 40% after the fungal biomass underwent the HTL process. C14:0 – C18:1fraction of the total fatty acid % all increased. The sum total of the C14:0-C18:1 was 38.1%. The C18:1 fatty acid chain went up the greatest at 22.5%, followed by C16:0 which went up by 12.2%. This may indicate that the 40% C18:2 fatty acids chains were 95% converted to C14:0 – C18:1 fatty acids, since the 38.1% increase is 95% of the 40% C18:2 fatty acids chain reduction. The 22.5% increase of the C18:1 fatty acid is 56% of the C18:2 40% decrease. This indicates that likely approximately half of the C18:2 fatty acids had a double bond broken converting them from C18:2 to C18:1 fatty acids. Approximately one third of the C18:2 fatty acids were therefore likely converted to C16:0.

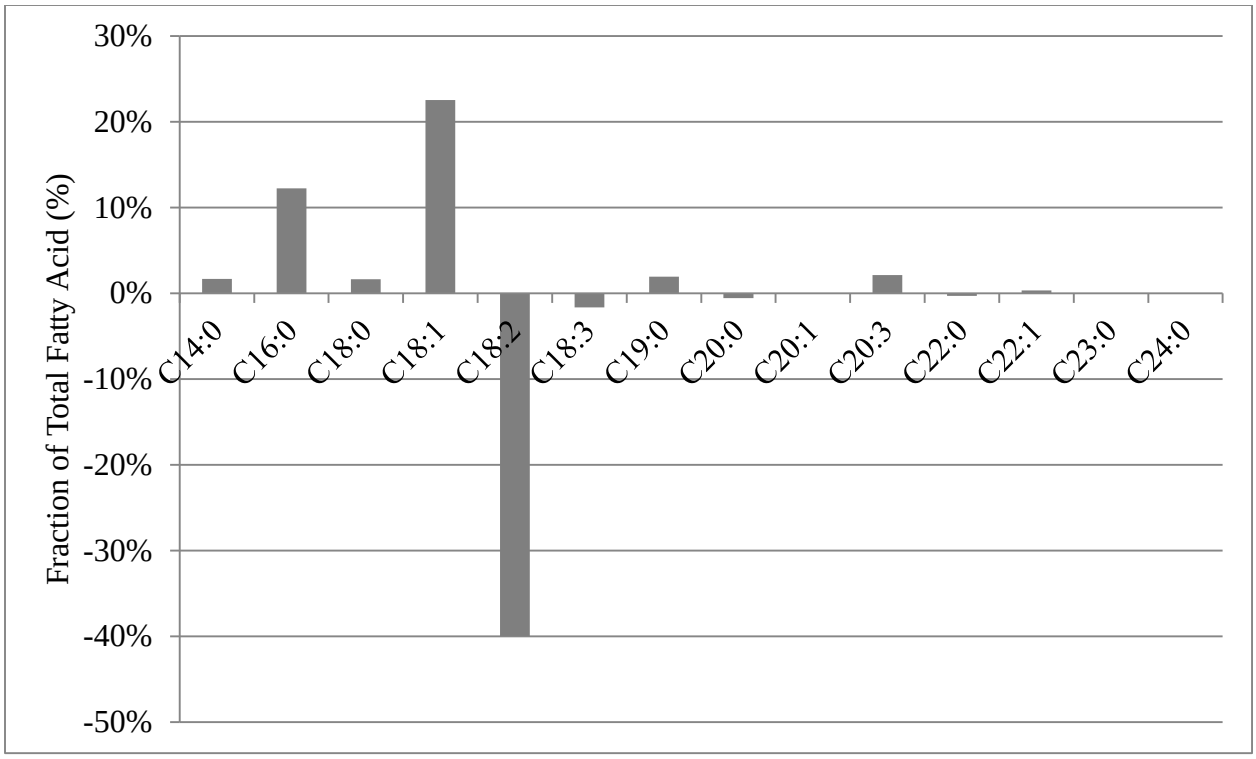

Figure 4.10. Change in fraction of total fatty acid profile of fungal biomass after HTL reaction to production bio-crude oil.

E. Higher Heating Value

The higher heating value (HHV) of HTL2 and HTL 3 bio-crude oils were 33.51 and 37.47 MJ/kg (dried basis) respectively. Continuous HTL of *Chlorella* and *Spirulina* have produced bio-crude oil with an average HHV of 31.7 MJ/kg and 30.3 MJ/kg (Jazrawi, 2013). HHV of the fungal biomass exceeded the algae bio-crude produced using a similar process by 5-24%. The fungal bio-crude oil is 73.5 – 82.3% of the HHV of petroleum crude oil is 45.54 (GREET, 2010).

4.4 Conclusion

Continuous HTL of fungal biomass grown on corn-ethanol thin stillage byproduct has been proven to produce comparable quantities of bio-crude oil % yields with similar C, H, N, S,

and O (by diff.) elemental content to microalgae slurries under like continuous HTL thermochemical treatment process conditions. Bio-crude oil derived from fungal biomass can have higher heating values greater than reported for bio-crude oil derived from algae biomass. The total fatty acid content of the bio-crude oil product was much less than the original fatty acid content of the fungal biomass. The fraction of long chained fatty acids present in the bio-crude oil after the biomass underwent HTL was greatly reduced to shorter chained fatty acids with fewer double bonds. Less fatty acid content combined with shorter chained fatty acid profile could greatly improve the bio-crude oil stability and cold flow properties as biofuel.

4.5 References

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CHAPTER 5

CONCLUSIONS

Selective disinfection during nonaseptic cultivation of *Rhizopus oligosporus* and *Mucor circinelloides* fungus on thin stillage substrate using chlorine dioxide, iodophor, penicillin-streptomycin ozone showed modest improvements over non-autoclaved thin stillage with fungal biomass yields comparable to heat sterilized thin stillage. However, results were highly variable making drawing conclusions from the data more challenging. Sodium hypochlorite addition was not observed to have any discernable positive or inhibitory effect on fungal biomass yields. Scale up from 250mL lab scale to 2L bench scale resulted in significant decline in fungal biomass yields. All of the selective disinfects attempted did little to improve biomass yields at the 2L bench scale. Oxygenation at the 2L scale greatly improved biomass yields to the point of being in range of that achieved at the 250mL scale suggesting that oxygen mass transfer through air sparing was insufficient.

Continuous hydrothermal liquefaction (HTL) was successful using fungal biomass feedstock. Bio-crude oil percent yield and C, H, N, S and O (by diff.) elemental content to microalgae slurries processed by continuous HTL. Higher heating values of bio-crude oil from fungal biomass were observed to be greater for bio-crude oil derived from microalgae biomass processed by continuous HTL. Long chain fatty acid content was reduced through HTL of the fungal biomass and the fatty acid chains remaining were shorter with fewer double bonds.