Study of Oleaginous Fungi Screened from Oil-Rich Plants for Improved Lipid Production

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Yan Yang

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Dedication

This work is dedicated to my dear daughter Julia Yan and husband Mi Yan, and my loved farther Yuanding Yang and mother Bilan Feng.

Abstract

Biodiesel generated from lipid transesterification can be used as a replacement or blended with for petroleum diesel in any proportion. Current processes to produce biodiesel mostly use plant oil as feedstocks, which keep the costs at high levels and result in a shortage of edible oil in food market. Lipids created via microbial biosynthesis are a potential raw material to replace plant-based oil for biodiesel production. The production of biodiesel from lignocellulosic biomass and other waste materials would have both economic and environmental benefits. This research focused on the screening and identifying novel oleaginous fungal strains with both high lipid content and capability of cellulase enzyme production, the study of the characteristics of selected strain in lipid accumulation process, and the utilization of selected strain in converting lignocellulosic biomass to fungal lipids for biodiesel production.

The first step of this research is the screening and identifying fungi from soybean and soil surrounding to soybean plants, and analyzing fungal community of the screened isolates. From two sets of screenings, 33 fungal isolates were obtained from soybean samples collected in August and 17 fungal isolates were obtained from soybean samples collected in October. Meanwhile, the screening from soil surrounding to soybean plant roots obtained 38 fungal isolates. Soybean samples and soil samples showed a great difference in the isolated fungal community, and difference of fungal diversity was also detected from soybean samples collected in August and samples collected in October. Also, different sampling locations had differences in fungal community. These results

demonstrated an impact of environment on fungal community, and also indicated a change of plant-associated fungal community through time in soybean samples. For soybean samples, *Fusarium* and *Alternaria* were the dominant genera, and other frequently detected genera included *Penicillium, Nigrospora, Cercospora* and *Epicoccum*. For soil samples, *Trichoderma, Fusarium, Mucor* and *Talaromyces* were most frequently isolated genera.

The second step of this research is the bioprospecting of fungal strains isolated from soybean and soil for lipid accumulation to identify strains with high lipid content and cellulase production. Among 33 fungal isolates screened from soybean plant, 13 were oleaginous fungi (lipid content>20% dry biomass weight); among 38 fungal isolates screened from the surrounding soil, 14 were oleaginous fungi. A considerable amount of fungi were identified as oleaginous fungi, and fungi with highest lipid content (>40%) belong to *Fusarium* genus. One of the strains was selected as the most promising strain was *Fusarium equiseti* UMN-1 strain. This strain has high lipid content (>56%) and high fatty acid methyl ester (FAME) content (98% in total lipid), also produces cellulase. In addition, it can utilize a wide range of substrates and has promising oil composition for biodiesel production. The *F. equiseti* UMN-1 strain offers great potential for biodiesel production by directly utilizing lignocellulosic biomass as feedstocks, and was used in the following studies.

The third step of this research is the investigation on the characteristic of *F. equiseti* UMN-1 strain and optimization of cultivation conditions for higher lipid production. Characteristic study of this strain determined the optimal temperature as 27 ℃ and agitation speed as 150 rpm. The best C:N ratio for lipid accumulation was 80, strong light during cultivation was not suggested, and 6 to 8 day's culture was sufficient for this strain to reach a high level of lipid production. This fungal strain obtained higher lipid production when using fructose and mannose as carbon source, but it also can grow well on a variety of carbon sources. Most suitable nitrogen source for lipid production was the combination of $(NH_4)_2SO_4$ and yeast extract. According to the response surface analyses results of the central composite design (CCD), the optimal growth condition for flask culture was 23.7 ℃, 37.39 g/L glucose and 0.236 g/L nitrogen (N). The maximum lipid production was predicted as 3.91 g/L, and 3.89 g/L lipid production was verified from flask culture under the optimized conditions.

The fourth step of research is to explore the application of *F. equiseti* UMN-1 strain in lignocellulosic lipid production and study different fermentation process to improve lipid accumulation. When directly using lignocellulosic biomass for lipid production, this strain achieved a lipid yield of 59.1±2.7 mg/g from soybean hulls and 61.1±2.6 mg/g from corn stover through solid state fermentation with 90% moisture content. Application of pretreatment and cellulase hydrolysis further increased the lipid yield to 69.2±5.0 mg/g from corn stover in integrated fermentation. *F. equiseti* UMN-1 strain was shown to have

the capability of lipid accumulation from a variety of materials, and it could be a potential candidate as a lipid source in the production of lignocellulosic biodiesel.

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CHAPTER 1 INTRODUCTION

1.1 Background and significance of the research

Global demand for petroleum is predicted to increase up to 40% by 2025 (Hirsch R.L., 2005). Fossil fuel consumption is not a sustainable solution in the long term due to its rapid depletion and associated environmental problems. Research has increasingly addressed alternative energy with a focus on reducing greenhouse gas emissions and enhancing a sustainable economy (Zinoviev S. et al., 2010). In various types of alternative energy, "drop-in" biofuel attracts great attention to serve as alternative liquid fuel that can be directly utilized in current engine system and compatible with current petroleum-based fuels for transportation. Bioethanol and biodiesel are currently the primary choices of alternative fuel. While bioethanol is widely used in ethanol-blended gasoline to drive gasoline-engine vehicles, biodiesel is used for diesel engine vehicles and provides great environmental benefits, such as serving as high-energy fuel, reducing CO_2 emission, and reducing emission of air pollutants such as carbon monoxide and sulfur oxides.

Plant oil is used as the main feedstock for biodiesel production, which has limited availability due to the competition with human consumption. In order to find cheaper feedstock to replace plant-based oil for biodiesel production, other oil sources needs to be explored. Recently, microbial lipids, also referred to as single cell oils (SCO) from oleaginous microorganisms via microbial biosynthesis have been an attractive alternative raw material for biofuel production, including biodiesel, hydrocarbon compounds and

other complex oils. These organisms accumulate lipids in the form of triacylglycerols (TAG), which are also the main component in vegetable oils and animal fats (Vicente G. et al., 2009). The occurrence of TAG as energy storage compounds have been widely found in eukaryotic organisms such as plants, animals, algae, fungi and yeasts, but was rarely reported in bacteria. Most bacteria are not oil producers and bacteria generally accumulate polyhydroxyalkanotes as storage material (Meng X. et al., 2009). Among these oleaginous microorganisms, cultivation of oleaginous fungi (filamentous molds) and yeasts has some favorable properties for industrial biofuel production compared to algae. Fungi and yeast have a short life cycle, high growth rate and high biomass density, and can be grown in conventional bioreactors to maximize yield and profile. In addition, filamentous fungal cells in general are more easily harvested compared to algae and yeasts, especially when they grow in the form of pellets or mycelium (Xia C. et al., 2011). These characteristics make oleaginous fungi a good candidate for industrial-scale lipid production.

The oleaginous microbial species currently available are capable of accumulating high content of lipids in their cell biomass, but the high cost of substrate, for instance, glucose, greatly increases the price of biodiesel produced from microbial lipids, which makes this source economically impracticable. Alternative cheap and sustainable substrates have been explored, such as non-grain plants, agricultural and forest residues, lignocellulosic biomass, food wastes and other waste materials. Cellulose and hemicelluloses containing fermentable sugars in lignocellulosic biomass can be potential substrates; however,

oleaginous microbial species rarely can directly utilize lignocellulosic biomass (Zhang J. G., & Hu B., 2012). This research focuses on the screening and selection of oleaginous fungal strains that generate both high lipid content and hydrolytic enzymes. Successful screening and cultivation will provide a promising strain that can use lignocellulosic biomass and accumulate a high amount of lipids, thus offering a practical method to create low-cost biodiesel.

1.2 Objectives

The goal of this research is to screen and identify novel oleaginous fungal strains with desirable characteristics, including high lipid content and hydrolytic enzymes, to achieve high lipid production that can be utilized in converting lignocellulosic biomass to fungal lipids for biodiesel production. The specific objectives of the research are:

1) Isolating and analyzing the fungal community associated within soybean plants and from surrounding soil;

2) Bioprospecting of fungi isolated from soybean and soil for lipid production to screen out fungal isolates with high biomass, high lipid content, promising oil composition, a variety of substrate utilization, and cellulase production;

3) Investigating the cultivation conditions of selected fungal strains for enhanced lipid production, optimizing cultivation condition with central composite design (CCD). The study on cultivation includes the effect of temperature, agitation, light, C:N ratio, medium composition, and different carbon sources and nitrogen sources;

4) Studying the lipid production of selected fungal strains utilizing lignocellulosic biomass and evaluating lipid production with different fermentation process, such as solid-state fermentation and submerged fermentation. In addition, investigate the effect of pretreatment and cellulase enzyme with the integrated process to achieve higher efficiency of converting lignocellulosic biomass to lipids.

CHAPTER 2 LITERATURE REVIEW

2.1 Potential of microbial lipids as biofuel feedstock

Current raw materials to produce biodiesel are primarily plant-based, for example, soybean and vegetable oil (Bunyakiat K. et al., 2006), palm oil (Al-Widyan M.I., & Al-Shyoukh A.O., 2002), sunflower oil (Antolin G. et al., 2002), and restaurant waste oil (Demirbas A.H., 2009). These feedstock sources contribute to more than 75% of the total production costs, which makes biodiesel still expensive compared with conventional fuels (Durrett T.P. et al., 2008). In economic terms, microbial sources become a feasible alternative for lipid production to create fuel.

Microorganisms containing more than 20%–25% lipids in their cell biomass (on dry basis) are defined as oleaginous microorganisms (Ratledge C., 1991). Microbial lipids are accumulated as energy storage when cell growth becomes limited due to the shortage of nitrogen or other nutrients, while an excessive carbon source is still available (Ratledge C., 1991). Several species of microalgae, yeasts and filamentous fungi have the capability to accumulate as high as 70% of lipids based on dry cell weight (Meng X. et al., 2009). Microorganisms have many advantages over plants for the production of lipids, such as short life cycles, ease of scale up, less demand on space, and will not compete with food sources.

These organisms accumulate lipids, mostly in the form of TAG, with some in the form of free fatty acids (FA). Microbial lipids have similar composition and energy value to plant

and animal oils, and they can be used for economical biofuel production especially if it is based on inexpensive carbon source, such as waste materials, byproducts and lignocellulosic substrates. The most common biofuels produced from lipids are biodiesel via transesterification of TAG. Various oleochemical products can also be generated from the microbial lipids. The major process for transforming lipids into oleochemicals is the hydrolysis of TAG into glycerol and FA, and more products can be formed from FA, such as fatty alcohols and fatty amines (Biermann U. et al., 2000; Corma A. et al., 2007). Some microbial lipids contain a special group of fatty acids called essential fatty acids (EFA), which are valuable as food supplements. Important EFA include gamma linoleic acid (GLA), arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA).

2.2 Microbial lipid production from lignocellulosic biomass

Lignocellulosic biomass is a biological material that is composed mainly of cellulose (35%–50%, dry weight basis), hemicelluloses (20%–35%), and lignin (10%–25%) (Sun Y., & Cheng J., 2002). As one of the most abundant materials in the world, it serves as a feedstock for the traditional paper industry and is used for production of chemicals and biomaterials/biopolymers. In recent years, more studies have recognized the potential to use lignocellulosic biomass as a sustainable feedstock for the biofuel production. The fermentable sugars in cellulose and hemicellulose components can be suitable carbon source for microbial growth. However, these sugars are not easily accessible. Together with lignin, cellulose and hemicellulose comprise a complex structure with cellulose

fibers embedded in a hemicellulose and lignin matrix (Figure 2.1). Cellulose crystallinity, accessible surface area, lignin seal, and the heterogeneous character of biomass particles all contribute to the recalcitrance of lignocellulosic biomass and prohibit the utilization of sugars (Chang V. S., & Holtzapple M. T., 2000). In this case, either pretreatment of lignocellulosic biomass or a high activity of cellulase is needed to break the structure and release the polysaccharides for its utilization.

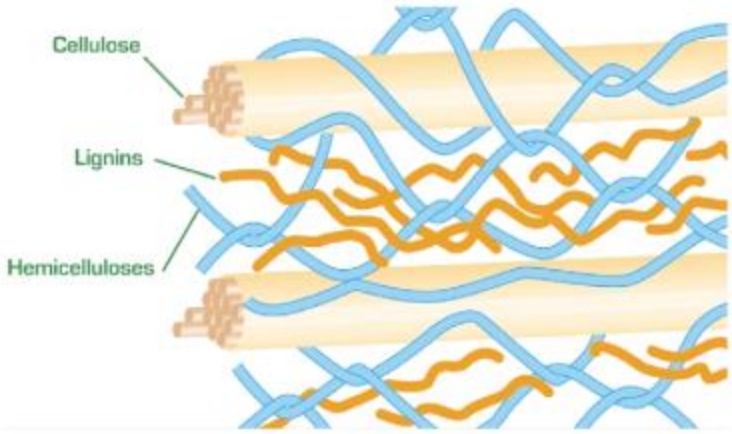

Figure 2.1. Schematic representation of the main biopolymers in plant cell wall (Boudet A.M. et al., 2003).

The bioconversion of lignocellulosic biomass into lipids by oleaginous microorganisms generally involves biomass pretreatment, enzymatic hydrolysis and fermentation of sugars into lipids. This oleaginous cell cultivation on lignocelluloses shares many similarities with lignocellulosic ethanol production. Pretreatment is the key step to disrupt the complex lignocellulose structure and make cellulose and hemicellulose more accessible to cellulase enzymes for hydrolysis. Acid, alkaline, or ammonia fiber explosion (AFEX) pretreatment methods have been successfully applied in industrial

conditions. The major sugars hydrolyzed from lignocellulosic biomass are glucose and xylose, with some amount of minor sugars such as arabinose, mannose, or galactose. Thus the oleaginous strains that can utilize both glucose and xylose are preferred, and the ability to utilize those minor sugars is also desirable (Huang C. et al., 2013). Separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) are the two common fermentation strategies in lignocellulose-based lipid conversion. In SHF, the pretreated lignocellulosic biomass is enzymatically hydrolyzed first, and the hydrolysate is used as the substrate for microbial cultivation. SSF combines cellulose hydrolysis with microbial lipid fermentation. SSF can prevent the inhibition of enzymes by sugars and improve the yield of enzymatic hydrolysis, thus improve lipid yield. It also renders some difficulties and challenges due to the higher working temperature of cellulase, compared to the lower temperatures where most fungi and algae strains grow.

Lignocellulosic biomass represents one of the most important potential feedstock to produce biodiesel. Recent studies confirmed the conversion of cellulose and hemicellulose hydrolysate into lipids by oleaginous fungi, yeast or algae strains (Hu C. et al., 2009; Li P. et al., 2011; Ruan Z. et al., 2012). Both C5 and C6 monomeric sugars become available after hydrolysis, as well as numerous byproducts that may have inhibitive effects on cell growth, such as acetate, formic acid, furfural, 5-hydroxymethyl-2-furaldehyde (HMF), and hydroxybenzaldehyde (Aden A. et al., 2002; Yang B., & Wyman C.E., 2004). Selecting the strains that can utilize both C5 and C6 sugars and also

high tolerance to lignocellulose degradation compounds (inhibitors) will be an important factor to the success of lignocellulosic based biofuel production.

2.3 Studies on oleaginous fungi and yeasts

2.3.1 Oleaginous fungi and yeast strains for lipid production

Oleaginous fungi and yeasts have been known as favorable oil-producing organisms since 1980s (Ratledge C., 1993). Some oleaginous fungal species such as *Mortierella* can store up to 80% lipids based on dry weight (Meng X. et al., 2009; Xia C. et al., 2011). Some oleaginous yeasts, for example, *Rhodotorula* species and *Lipomyces* species, can accumulate lipids at the level of 40% to 70% under nutrient limiting conditions (Beopoulos A. et al., 2009). Table 2.1 lists the oil content of several oleaginous fungi and yeast species.

Table 2.1. Oil content of some oleaginous fungi and yeast species (Li Y. et al., 2007; Meng X. et al., 2009; Beopoulos A. et al., 2009; Vicente G. et al., 2010; Lin H. et al., 2010).

Fungi	**Oil Content (% dry weight)**	**Yeast**	**Oil Content (% dry weight)**
Aspergillus niger	23-57	*Candida curvata*	58
Aspergillus oryzae	15-57	*Cryptococcus albidus*	65
Cunninghamella echinulata	40-57	*Lipomyces starkeyi*	64
Humicola lanuginosa	75	*Rhodotorula glutinis*	72
Mortierella alliacea	42	*Rhodotorula graminis*	36
Mortierella alpina	31	*Rhodosporidium toruloides*	76
Mortierella isabellina	60-86	*Rhizopus arrhizus*	57
Mortierella vinacea	66	*Trichosporon pullulans*	65
Mucor circinelloides	20	*Trichosporon fermentans*	62
Mucor rouxii	32	*Yarrowia lipolytica*	36

Lipid accumulation via oleaginous fungal strains has been extensively studied and some commercial successes have been generated, especially for the production of nutraceuticals such as polyunsaturated fatty acids (PUFA). Some *Mortierella* and *Mucor* species fungi produce a high concentration of PUFA such as GLA and AA (Mamatha S.S. et al., 2009; Eroshin V.K. et al., 2000). Somashekar D. et al. (2002) reported *M. rouxii* and *Mucor* sp. resulted in a production of 30% lipids and up to 17% GLA in total lipids. *M. alpina* was considered as a suitable source for the production of AA, and AA was more than 18% of dry cell mass and over 60% of the total lipids reported by Eroshin V.K. et al. (2000). However, research on the exploitation of SCO for biofuel production was just emerged in the last few years. Recent studies have primarily focused on lipid production through different carbon sources, especially lignocellulosic biomass and waste materials for low-cost biofuel production (Papanikolaou S. et al., 2003; Zhu L.Y., et al., 2008; Hu C. et al. 2009; Lin H. et al., 2010; Ruan Z. et al., 2012). Oleaginous filamentous fungi *M. circinelloides* and *M. isabellina* have been heavily studied for this purpose using various substrates (Ratledge, C., 2004; Ruan Z. et al., 2012; Zeng J. et al., 2013; Wei H. et al., 2013).

The most widely investigated oleaginous yeasts belong to the genera *Candida*, *Cryptococcus*, *Rhodotorula*, *Rhodosporodium*, *Lypomyces*, *Yarrowia*, and *Trichosporon* (Ageitos J.M. et al., 2011; Li Q. et al., 2008; Meng X. et al., 2009). *Cryptococcus curvatus* has a good industrial potential since it requires minimal nutrients for growth, accumulates up to 60% lipids, and can grow on a very broad range of substrates

(Meesters P. et al., 1996; Zhang J. et al., 2011). The red yeasts *R. glutinis* and *R. toruloides* are able to synthesize the natural pigments carotenoids in addition to lipids (Wang S.L. et al., 2007; de Miguel T. et al., 1997). Being considered as a potential pigment source, their biotechnological application is also associated with their capability to utilize glycerol and lignocellulosic materials for biofuel production (Easterling E.R., 2009; Hu C. et al., 2009; Yu X. et al., 2011).

2.3.2 Improvement of lipid production

Strain development

The extent of lipid accumulation for oleaginous microorganisms is determined by the genetics and the growth conditions (Meng X. et al., 2009). The isolation, identification and selection of appropriate oleaginous fungi and yeast strains with high growth rates and productivities are crucial steps for the overall success of biofuel production. The current studied oleaginous strains are not as efficient or robust as would be preferred for industrial applications (Jin M. et al., 2015). The use of strain development techniques such as screening, directed evolution, and metabolic pathway engineering can lead to better oleaginous strains with higher biomass and lipid production than wild-type strains.

Generally, there are three different strategies available for screening and selection. The first strategy is isolation from local environments where they are supposed to be grown on a large scale. Local species have competitive advantages under the local geographical, climatic and ecological conditions. The second strategy is acclimatization under an

environment in which the microorganisms do not normally grow well. The studies of microbial species under some extreme conditions may result in some special strains with desired properties. The third strategy is to use genetic and metabolic engineering to modify the regulation of metabolic pathways. The genetic engineering method can improve the intrinsic properties of strains and make significant improvement.

Several studies have already been conducted for the screening and selection of oleaginous fungi and yeasts. Dey P. et al. (2011) screened two endophytic oleaginous fungi *Colletotrichum* sp. and *Alternaria* sp. with lipid content 30% and 58% respectively, and studied their growth under optimum and nutrient-stress conditions. The endophytic oleaginous fungus *Microsphaeropsis* sp. was isolated from oily wood-stem and used for lipid production from wheat straw hemicellulose hydrolysate (Peng X. W., & Chen H. Z., 2007; Peng X.W., Chen H.Z., 2012). Other studies reported oleaginous fungus *Aspergillus* sp. from soil samples and accumulated 22% lipid using corncob waste liquor as a substrate, which was used for biodiesel production (Subhash G.V., & Mohan S.V., 2011). Kitchaa S. & Cheirsilp B. (2011) screened multiple oleaginous yeast strains such as *Trichosporonoides spathulata* with more than 40% lipid from soils and palm oil mill wastes, and optimized lipid production with crude glycerol. Tanimura A. et al. (2014) selected *Cryptococcus* sp. with high lipid content (>60% lipid) and productivity from hundreds of yeast isolates, and studied their properties for biodiesel production.

Considering using the lignocellulosic biomass such as wheat straw or corn stover as feedstock for microbial lipid production, the currently available oleaginous fungi and yeast strains still have some disadvantages. They have either low tolerance to lignocellulose degradation compounds (inhibitors) or insufficient degrading enzymes to utilize lignocellulosic biomass. If a suitable fungal strain were screened and developed with the capability to produce both lipids and hydrolytic enzymes, it can directly utilize lignocelluloses to generate lipids for biodiesel production to reduce the costs remarkably.

Cultivation conditions

The theoretical yield for converting sugars to lipids is 32% from glucose and 34% from xylose (Papanikolaou, S., & Aggelis, G., 2011), and most of oleaginous cell cultures can reach the lipid yield of 20-25% (Weete J.D., & Weber D.J., 1980; Li Q. et al., 2008) while some sugars have to be diverted to support the cell growth and metabolism. This conversion efficiency relies on the genetics of microorganism, but the cultivation conditions also greatly affect microbial lipid content and lipid composition accumulated in fungi and yeasts (Somashekar D. et al., 2002; Beopoulos A. et al., 2009; Sitepu I.R. et al., 2013). Lipid accumulation by oleaginous fungi and yeasts mostly happens when a nutrient in the medium (e.g. nitrogen or phosphorus source) becomes limited and excessive carbon source is present; and nitrogen limitation is the most effective condition for most oleaginous fungi and yeast species in lipid accumulation (Rossi M. et al., 2011). An optimally high C:N molar ratio for lipid accumulation is usually at the range of 65 to near 100 (Weete J.D., & Weber D.J., 1980; Ageitos J.M. et al., 2011; Jin M. et al., 2015).

Besides the most important factor C:N ratio, many nutritional and environmental factors control the cell growth and lipid content of fungi and yeasts. Nutritional factors include carbon sources, nitrogen sources, and other essential macro- and micronutrients in the growth medium; environmental factors include temperature, pH level, and dissolved oxygen. Somashekar D. et al. (2002) studied the medium composition for the lipid accumulation of *Mucor* species and found that cultivation using different carbon sources lead to different lipid production, and lactose was a poor promoter for biomass and lipid production. Nitrogen sources were also found to affect the lipid production and lipid composition, and the selectivity of nitrogen sources varied among different fungi and yeast species (Somashekar D. et al., 2002). As an example, switch of nitrogen source from nitrate to urea diminished the accumulation of unsaturated fatty acids in *Ustilago maydis* (Zavala-Moreno A. et al., 2014).

Growth temperature of oleaginous microorganisms also influenced the lipid production and fatty acid composition (Ageitos J.M. et al., 2011). Culture of *Sporobolomyces roseus* at different temperatures obtained the lowest lipid production of 0.96 g/L at 30 ^{o}C and the highest lipid production of 2.35 g/L at 14 ^{o}C. Meanwhile, the C18:1, C18:2 and C18:3 fatty acids represented 60, 10 and 1%, respectively, of the total fatty acids at 30 ^{o}C. These percentages switched to 49.2, 15 and 9%, respectively, at a lower growth temperature (between 6 and 22 ^{o}C). Similar result was obtained on the study of lipid accumulation by *Metschnikowia pulcherrima*, in which lower temperature triggered high levels of oil

production (Santamauro F. et al., 2014). Initial pH is another critical parameter in lipid accumulation. Angerbauer C. et al. (2008) studied the influence of the pH value (from 5 to 7) on the lipid production of *L. starkeyi* in a basal medium (C/N ratio of 100 g/g). The highest lipid production (56% biomass DW; 7.5 g/L) was obtained at pH 5, while the highest biomass was obtained at pH 6.5 (15.12 g/L). The lipid accumulation was not significantly affected by the pH values that ranged from 5.5 to 6.5 but decreased dramatically (7% biomass DW, 1.1 g/L) at pH 7. It is considered that optimal growth temperature for oleaginous fungi is generally 20-28 ^{o}C, and 25-30 ^{o}C for yeasts, and optimal growth pH ranges are typically 5-7 for fungi and yeasts (Ageitos J.M. et al., 2011; Papanikolaou S., & Aggelis G., 2011).

Metabolic engineering

Metabolic engineering is another method that can efficiently improve the overall lipid production besides the management of cultivation conditions. Extensive studies have been carried out on this aspect, and the development of genome data and genetic tools provide the possibility to increase the yield of stored lipids by metabolic engineering. The filamentous fungus *M. circinelloides* represents an excellent model within *Zygomycota* phylum since the whole genome sequence and an efficient transformation procedure are readily available (Gutiérrez A. et al., 2011; Rossi M. et al., 2011).

Microbial lipid biosynthesis process includes fatty acid (FA) synthesis approach and TAG synthesis approach (Figure 2.2), and the biochemical pathway of lipid biosynthesis

is not very different among eukaryotic microorganisms or between oleaginous and non-oleaginous strains. In the fatty acid synthesis approach, various carbon sources are firstly converted to pyruvate via glycolysis in cytoplasm. For example, glucose is firstly converted to fructose-1,6-bisphosphate (F-1,6-BP), and converted to glyceraldehyde 3-phosphate (G-A-P). G-A-P is converted to phosphoenolpyruvate (PEP) and then to pyruvate. Pyruvate will enter the mitochondria and be converted to acetyl-CoA, and further to citrate to enter tricarboxylic acid (TCA) cycle. When citrate level in mitochondria is sufficiently high, excessive citrate will enter into cytoplasm and be cleaved to form acetyl-CoA. Acetyl-CoA will be converted to malonyl-CoA by acetyl-CoA carboxylic enzyme (ACC). Once malonyl-CoA is synthesized, fatty acid synthase multi-enzymatic complex (FAS) can transfer it into acyl-carrier protein (ACP) and use the fatty acyl-ACP as carbon source for the synthesis of long chain fatty acids, mainly C16 and C18 (Liang M., & Jiang J., 2013).

TAG formation happens in endoplasmic reticulum (ER). In the TAG synthesis approach, carbon sources like glucose are converted to glycerol-3-phosphate (G3P) first, and then catalyzed by glycerol-sn-3-phosphate acyl-transferase (GPAT) to react with acyl-CoA and form lysophosphatidate (LPA). LPA is catalyzed by lysophosphatidate acyl-transferase (LPAT) to react with another acyl-CoA and produce phosphatidate (PA). Then PA can be further dephosphorylated to produce diacylglycerol (DAG) and TAG by phosphatidic acid phosphatase (PAP) and diacylglycerol acyl-transferase (DGAT). Meanwhile, FAS can convert Acetyl-CoA and malonyl-CoA into acyl-CoA, transport

into endoplasmic reticulum (ER) and enter this TAG synthesis pathway. Besides this G-3-P pathway, a new dihydroxyacetone phosphate (DHAP) pathway in yeast was also reported. In this pathway, DHAP is acylated at the sn-1 position by DHAP acyltransferase (DHAPAT), and the product 1-acyl-DHAP is reduced by 1-acyl-DHAP reductase to yield LPA, which is further acylated to PA by LPAT (Coleman R., & Lee D.P., 2004; Liang M., & Jiang J., 2013).

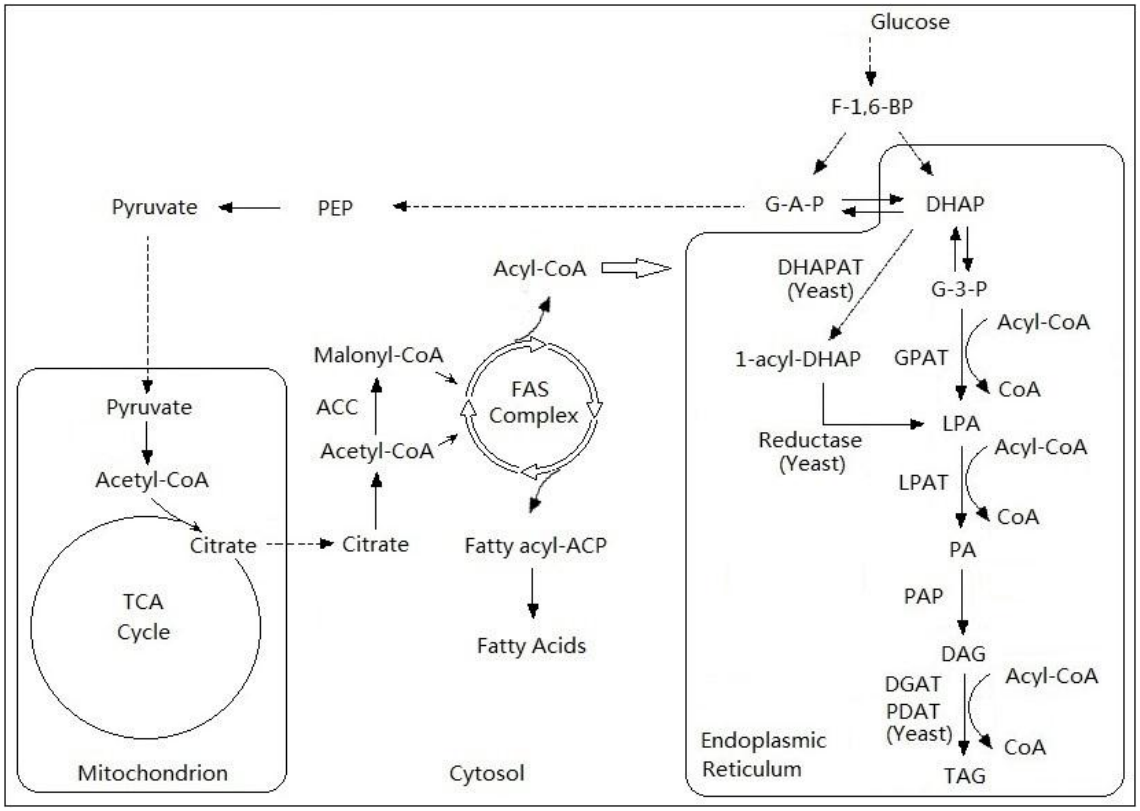

Figure 2.2. Microbial lipid biosynthesis pathway. Figure adapted from Liang M., & Jiang J., 2013; Jin M. et al., 2015; Zhu Z. et al., 2012.

The ability of oleaginous microorganisms to accumulate high amounts of lipids depends mostly on the regulation of biosynthesis pathway and the supply of the precursors (e.g. acetyl-CoA, malonyl-CoA, and glycerol-3-phosphate) and the cofactor NADPH. Regulation methods can be classified into five approaches: (1) overexpressing enzymes

of FA biosynthesis pathway; (2) overexpressing enzymes of TAG biosynthesis pathway; (3) regulation of related TAG biosynthesis bypasses; (4) blocking competing pathways; and (5) multi-gene transgenic methods (Liang M., & Jiang J., 2013).

Overexpressing enzymes is one of the most common strategies, which aims to increase the expression of rate limiting enzyme and increase lipid production. It was reported that overexpression of diacylglycerol acyltransferase (DGA1) in *Y. lipolytica* received a 4-fold increase in lipid production over control, and the overexpression of acetyl-CoA carboxylase (ACC1) in *Y. lipolytica* also received a 2-fold increase over control (Tai M., & Stephanopoulos G., 2013). Regulation of related TAG biosynthesis bypasses focuses on some enzymes that are not directly involved but influence the rate of lipid production. Regulation of these enzymes will mainly affect the content of essential metabolites for lipid synthesis. For example, the overexpression of malic enzyme in *M. circinelloides*, which is involved in NADPH formation and pyruvate generation from malate, lead to a 2.5-fold increase of lipid production over control (Zhang Y. et al., 2007). Blocking competing pathways are aimed to direct the metabolic flux to TAG biosynthesis rather than other metabolic pathways. Deletion of GUT2 gene, which codes for glycerol-3-phosphate dehydrogenase isomer in *Y. lipolytica*, was also demonstrated to have a 3-fold increase in lipid production compared to the wild-type strain (Beopoulos A. et al., 2008). Multi-gene transgenic methods are mostly related to the manipulation of more than one key enzyme in lipid synthesis pathway. Besides the lipid production increased by the overexpression of DGA1 and ACC1 described above, the co-overexpression of these two

genes resulted in an almost 5-fold increase than control (Tai M., & Stephanopoulos G., 2013). There are also successful examples in recombination oleaginous microorganisms, like FAME production by heterologous expression of bacterial acyltransferase in *Saccharomyces cerevisiae* (Kalscheuer R. et al., 2004), and fatty acid ethyl ester (FAEE) production by heterologous expression of *Zymomonas mobilis* pyruvate decarboxylase, alcohol dehydrogenase and unspecific acyltransferase in *E. coli* (Kalscheuer R. et al., 2006).

Fermentation processes

In order to obtain a higher microbial lipid production rate, various cultivation modes have been developed in lab to culture oleaginous microorganisms. Mostly studied cultivation modes include batch, fed-batch, and continuous mode. Besides these widely studies methods, solid state fermentation and pelletized submerged fermentation have also been investigated.

Batch cultivation is a partially closed system in which most of the materials required are loaded into the reactor in the first place. In this mode of operation, conditions are continuously changing with time, and the reactor is an unsteady-state system. Culture medium in batch cultivation always has a high initial C:N ratio, and the change of nitrogen concentration determines the passage from a balanced growth phase to a lipid accumulation phase. During the growth phase, the carbon source is mainly contributed to satisfy the growth need and low lipid content biomass is mostly produced. As nitrogen

concentration becomes limited, biomass generation decreases while the microbial metabolism shifts into lipid accumulation (Duhalt R.V., & Greppin H., 1987). Therefore, the initial C:N ratio of growth medium in batch cultures is vital in determining the bioprocess performance, affecting both biomass and lipid content within cells. It was reported that batch culture of *M. isabellina* using corn stover hydrolysate can achieve a lipid production of 6.9 g/L in a 7.5 L fermenter (Ruan Z. et al., 2014), culture of *L. starkeyi* using glucose and xylose achieved a lipid production of 12.6 g/L in flasks (Zhao X. et al., 2008), and the culture of *C. echinulate* using starch obtained a lipid production of 7.65 g/L in flasks (Chen H.C., & Liu T.M., 1997).

Fed-batch cultivation has been proved to effectively increase both the cell density and lipid content of oleaginous microorganisms. In fed-batch processes, cells are grown under a batch mode for some time, usually until close to the end of the exponential growth phase, and then fed with a solution of substrates without the removal of culture fluid. The control of nutrient concentrations in reactor can help to monitor and control the specific growth rate and carbon utilization, and also maintain the oleaginous microorganisms at a desired specific growth phase. Cultivation of oleaginous yeast *R. toruloides* using flask fed-batch cultivation has reached a 151.5 g/L dry cell concentration with a lipid content of 48.0% (w/w) in 25 days, a pilot-scale fed-batch cultures in 15 L stirred-tank fermenter also reached a 106.5 g/L dry cell concentration with a lipid content of 67.5% (w/w) in 5.5 days (Li Y. et al., 2007). Similarly, cultivation of oleaginous yeast *C. curvatus* in 30 L

stirred-tank fermenter has reached a 104.1 g/L dry cell concentration with a lipid content of 82.7% (w/w) in 8 days (Zhang J. et al., 2011).

Unlike batch culture, continuous culture feeds the microorganisms with fresh nutrients, and at the same time removing spent medium plus cells from the system. At the steady state when high cell yield is reached, the microbial growth, assimilation of C and N sources, and withdrawal of nutrients are maintained at constant rates. Continuous culture usually results in a high biomass and end-product productivity in the fermentation process. The constant C:N ratio in the reactor will help to reduce the loss of cell viability and acid production (Beopoulos A. et al., 2009). Besides the C:N ratio in growth medium, microbial growth and nutrient assimilation both largely depend on the dilution rate. It was reported that as dilution rate increased in continuous cultivation of *C. curvata*, specific lipid accumulation rate increased and non-lipid cell accumulation decreased (Brown B.D. et al., 1989). This result indicates that the microbial metabolism is partially controlled by dilution rate. Another report mentioned that lipid production of *Y. lipolytica* was favored at low specific dilution rates and highly aerated condition, and a high lipid production (3.5 g/L) with high lipid content (43% w/w) can be achieved (Papanikolaou S., & Aggelis G., 2002).

Submerged culture is the major cultivation process used for lipid production from lignocellulosic biomass, but researchers also explore solid state fermentation with oleaginous fungi. This is a method to directly use lignocellulosic material as feedstock,

aimed to reduce the pretreatment cost in lipid production. Meanwhile, solid state fermentation has drawbacks in areas such as heat and mass transfer, scale-up and cell and lipid harvest. Zhang J.G. and Hu B. (2012) conducted such fermentation using *M. isabellina* on soybean hulls at around 40-90% moisture content, but an overall low efficiency was obtained due to the lack of cellulase for degrading cellulose to provide sugar. Other attempts to carry out lipid production in solid state fermentation were also found: Lin H. et al. (2010) performed solid state fermentation using a cellulolytic strain of *A. oryzae* on wheat straw. This strain can directly convert cellulose into lipids in a low cost fermentation system. Economou C.N. et al. (2010) also performed semi-solid state fermentation using *M. isabellina* on sweet sorghum, and the highest oil yield of 11 g/100 g dry weight of substrate was obtained at 92% moisture content.

Another novel method is pelletized submerged fermentation, in which filamentous microorganisms aggregate in medium and grow as pellets/granules. As a commercial fungal strain to produce cellulase, *A. oryzae* is easily to form homogenous, stable and relatively large cell pellets during the cell growth without any inducing approach. The major benefit for pelletized submerged fermentation is easy harvest of biomass after lipid production, which reduces the cost in biomass harvest and finally decreases the cost of biodiesel. One approach for pelletized submerged fermentation is to use $CaCO_3$ powder to induce the fungal pelletization (Liao W. et al., 2007; Liu Y. et al., 2008), and another approach is to conduct pH adjustment during cell growth (Xia C. et al., 2011).

There are two schemes of fermentation process, SHF and SSF, which are based on lignocellulosic biomass derived lipid production. SSF combines enzymatic hydrolysis and fermentation steps and improves the yield of hydrolysis. SSF also reduces the contamination risk and capital cost of lipid production. However, SSF is usually set at temperature and pH suitable for fermentation (around 30^{o}C, pH >5), which is normally lower than the temperature required for hydrolysis. This will affect the release of sugar from lignocellulosic biomass, decrease lipid production, and leave more unhydrolyzed solids to reduce lipid recovery efficiency (Jin M. et al., 2015). Currently, most studies still focuses on SHF, in which both hydrolysis and fermentation can be controlled and optimized. Culture of *M. isabellina* using SHF in glucose medium obtained a lipid production of 10.2 g/L in flask (Ruan Z. et al., 2012). A few studies use SSF process, for example, Liu W. et al. (2012) cultured *T. cutaneum* using pretreated corn stover and achieved a lipid production of 3.23 g/L in 50 L bioreactor.

2.3.3 Utilization of different substrates for lipid production

Oleaginous fungi and yeasts are able to utilize a variety of carbon sources. Glucose is most commonly used in the research of fungal growth and lipid production, and other carbon sources such as fructose, xylose (Heredia L., & Ratledge C., 1988), lactose (Daniel H.J. et al., 1999), arabinose, mannose (Hansson L., & Dostalek M., 1986) and ethanol (Christopher T. et al., 1983) have also been used. In order to reduce the cost of microbial lipids, abundant and inexpensive carbon source including lignocellulosic biomass, agricultural/forest residues and waste materials have been explored for their

potential in lipid production. Cellulose and hemicellulose are the main compounds in these materials to release sugars for the growth of oleaginous microorganisms, while pretreatment and hydrolysis processes are usually required before fermentation. Besides the relatively high cost of the pretreatment and hydrolysis processes, the difficulty to use these materials for lipid production is the inhibitory compounds released during these steps such as acetic acid, furfural, 5-hydroxymethylfurfural, and water soluble lignin (Huang C. et al., 2009). Detoxification is an essential step on efficient utilization of these hydrolysates. Many oleaginous fungi and yeasts have been studied for lipid accumulation on different kinds of substrates, such as sweet sorghum juice (Economou C.N. et al., 2010), potato starch (Wild R. et al., 2010), tomato waste (Fakas S. et al., 2007), sugar cane molasses (Zhu L.Y. et al., 2008), whey permeate (Ykema A. et al., 1988 ; Akhtar P. et al., 1998), crude glycerol (Athalye S.K. et al., 2009; Andréa A. et al., 2010; Makri A. et al., 2010; Kitchaa S., & Cheirsilp B., 2011), corncob hydrolysate (Chang Y.H. et al., 2015), sewage sludge (Angerbauer C. et al., 2008), starch wastewater (Xue F. et al., 2010), wheat straw (Hui L. et al., 2010), and rice straw hydrolysate (Huang C. et al., 2009).

Table 2.2. Examples of fungi and yeast cultivation for oil accumulation on various substrates.

Fungi/Yeast species	Substrates	Biomass (g/L)	Biomass productivity (g/L/d)	Lipid content (% dry weight)	Lipid (g/L)	Lipid productivity (g/L/d)	Lipid yield (g/g substrate)	Culture mode	Reference
Mortierella isabellina	Glucose	11.4-22.3	1.32-4.80	40.0-45.0	5.0-10.2	0.52-2.11	0.12	Batch	Ruan Z. et al., 2012
Mortierella isabellina	Xylose	9.8-21.6	1.11-3.36	37.0-43.0	3.8-8.8	0.38-1.27	0.10	Batch	Ruan Z. et al., 2012
Mortierella isabellina	Corn stover hydrolysate	10.9-14.1	2.84-4.5	29.5-38.4	2.48-4.82	0.65-1.61	-	Batch	Ruan Z. et al., 2012

Mortierella isabellina	Enzymatic hydrolysate slurry	-	-	24.8	3.2	0.87	0.07	Batch	Ruan Z. et al., 2013
Trichosporon cutaneum	Glucose	22.9	4.58	52.4	12.0	2.4	0.20	Batch	Hu C. et al., 2011
Trichosporon cutaneum	Xylose	21.2	4.24	46.5	9.9	1.98	0.16	Batch	Hu C. et al., 2011
Trichosporon fermentans	Glucose	28.1	4.0	62.4	17.5	2.50	0.18	Batch	Zhu L.Y. et al., 2008
Trichosporon fermentans	Molasses + glucose	27.9	3.99	52.7	14.7	2.09	0.13	Batch	Zhu L.Y. et al., 2008
Trichosporon fermentans	rice straw hydrolysate	28.6	3.58	40.1	11.5	1.44	-	Batch	Huang C. et al., 2009
Yarrowia lipolytica	Glucose	9.3	0.30	24.7	2.3	1.78	0.12	Continuous	Aggelis G., & Komaitis M., 1999.
Yarrowia lipolytica	Rice bran hydrolysate	10.8	-	48.0	5.16	-	0.17	Batch	Tsigie Y.A. et al., 2012
Yarrowia lipolytica	Sugarcane bagasse hydrolysate	11.4	3.01	58.5	6.68	1.76	0.33	Batch	Tsigie Y.A., et al., 2011
Yarrowia lipolytica	Glycerol	4.7	2.26	22.30	1.05	0.50	0.04	Repeated-batch	Makri A. et al., 2010
Rhodosporidium toruloides	Glucose	106.5	19.07	67.5	71.89	12.96	-	Fed-batch	Li Y. et al., 2007
Cryptococcus curvatus	Glucose	104.1	20.82	82.7	16.54	11.28	-	Fed-batch	Zhang J. et al., 2011
Apiotrichum curvatum	Whey	85.0	29.1	35.0	29.75	9.60	-	Fed-batch	Ykema A. et al., 1988
Rhodotorula glutinis	Starch wastewater	60.0	24.0	30.0	18.0	7.20	-	Batch	Xue F. et al., 2010
Lipomyces starkeyi	Cellobiose	28.0	6.43	50.0	14.0	3.22	0.20	Batch	Gong Z. et al., 2012
Lipomyces starkeyi	Cellobiose + xylose	31.5	5.24	55.0	17.3	2.88	0.19	Batch	Gong Z. et al., 2012
Mucor circinelloides	Stillage + glycerol	20.0	10.0	46.0	9.2	4.60	-	Batch	Mitra D. et al., 2012

2.4 Technical challenges and future development

Oleaginous microorganisms have become promising candidates for biofuel production. They do not compete with food crops for arable lands, and can reach a very high production rate compared to crops. In general, high growth rates, reasonable growth densities and high lipid contents are the main reasons for microbial lipid to be attractive as biodiesel feedstocks. Correspondingly, the main technical challenges for the feasible

biofuel include: biomass productivity, cellular lipid content, overall lipid productivity and yield, and substrate untilization including carbon sources and other nutrients.

In early explorations, expensive carbon sources such as starch are the substrates of interest, but the microbial production of TAGs from starch is not profitable compared to low-value plant oils and fats. For profitability, the relatively low-value biodiesel end-product will require low-cost substrates, highly efficient conversion system and the capability to yield valuable products (Jin M. et al., 2015). Fermentation using lignocellulosic sources of carbohydrates may best fit these requirements. Current cultures of oleaginous strains on lignocellulosic hydrolysates are facing several challenges such as low lipid content, low lipid productivity and low lipid yield, which are caused by several technical problems like limited sugar concentrations, low control on C:N ratio and low tolerance of inhibitor generated from pretreatment (Jin M. et al., 2015). Besides carbon source, the major nutrients required for biomass growth like nitrogen, phosphorous, iron and sulfur are also crucial. Stable supply and management of nutrients are also important in the consistent accumulation of intercellular lipids. Once the culture of oleaginous microorganisms finished, there is also technical requirements on low water use, high efficiency harvest, high efficiency oil extraction and downstream processing. The capital cost of required equipment and energy required to extract the oil are still relatively high. Obviously, it is needed to overcome these technical hurdles and make the microbial-based biodiesel economical feasible.

The crude microbial oil is chemically similar to crude fossil fuel oil, and the engineering challenges on oil conversion are mostly well managed by current petroleum companies (Maher K.D., & Bressler D.C., 2007). It inspires researchers to build collaborations between microbial lipid production companies and major oil companies for maximizing downstream processing efficiencies. In addition, the potential to yield separable, valued co-products would be a bonus for the profitability of microbial-based biodiesel. A recent popular concept is SCO biorefinery, which integrates the conversion process of biomass into biofuels with production of co-products like chemicals and power (Figure 2.3). Furthermore, biorefinery process can be more economical and efficient when integrated with industries dealing with large amounts of biomass, such as pulp and paper mill, agricultural residues, and municipal solid wastes treatment. It has been considered as a way to maximize the biofuel production and co-products while reducing industrial waste disposal.

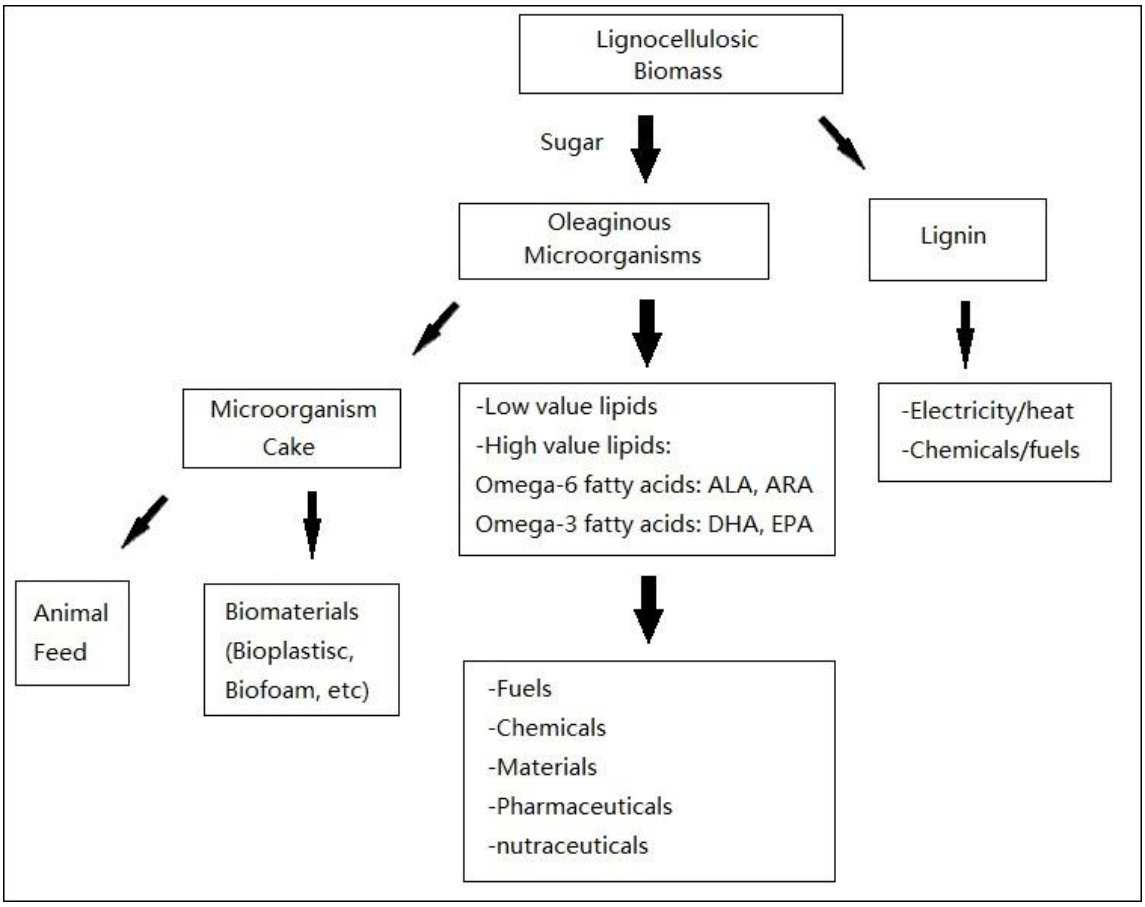

Figure 2.3. Products that could be generated from lipid biorefinery. Adapted from Jin M. et al., 2015.

CHAPTER 3 ANALYZING FUNGAL COMMUNITY ISOLATED FROM SOYBEAN

Outline

Soybean was used as the host plant in this research to explore the fungal community associated within soybean grains and husks and from soil, and also search for potential oleaginous fungal species. The species composition and diversity of associated fungi that inhabit in soybean samples were studied from different locations and time periods (August and October). Endophytic fungi were screened from soybean collected from five sites of croplands in Rosemount and Hastings, MN. After pure isolation was obtained, sequencing of fungal ITS regions and morphology characterization were used to identify the isolated fungal strains. 33 fungal isolates were obtained from soybean samples collected in August, 17 fungal isolates were obtained from soybean samples collected in October, and 38 fungal isolates were obtained from soil samples collected in August. For soybean samples, *Fusarium* and *Alternaria* were the two dominant genera detected, and other frequently detected genera included *Penicillium*, *Nigrospora*, *Cercospora* and *Epicoccum*. From soil samples, *Trichoderma*, *Fusarium*, *Mucor* and *Talaromyces* were the most frequently isolated genera. The results suggested a significant difference of fungal diversity between soybean samples collected in August and October, indicating a change of fungal community through time. There was also difference in fungal community from different locations. Oleaginous fungi were also discovered in the screened fungal isolates, with *Fusarium* species as the most promising ones, representing oil-rich plants a good reservoir of oleaginous fungi.

3.1 Introduction

Endophytic fungi are fungi that inhabit plant tissues and cause no apparent or immediate negative effect to plants (Petrini O., 1991; Bacon C.W., & White J.F., 2000). Endophytic fungi are extensively found in the 300,000 existing plant species and a lot of them have not been discovered or fully studied. It is now commonly accepted that each plant species host at least one or even several hundred strains of endophytes (Strobel G., & Daisy B., 2003). Of the current 500,000 known fungal species, endophytes only represent a small fraction, but probably contribute greatly to the estimated 1.5 million fungal species (Hawksworth D.L., 2001; Stadler M., & Schulz B., 2009; Strobel G., 2014). Endophytic fungi can be incorporated into different stages of growth during plant life cycle (Petrini O. et al., 1992). They grow within roots, stems, leaves, flowers, fruits and/or seeds of plants (Rodriguez R.J. et al., 2009; Strobel G., 2014). The tremendous biodiversity and taxonomic variety of endophytic fungi make them an important part of the overall fungal diversity on earth. Many studies have started to address their ecological function and evolution significance, especially the interaction with host plants, but our understanding of these questions are still limited (Schulz B., & Boyle C., 2005; Arnold A.E., 2007; Rodriguez R.J. et al., 2009; Aly A.H. et al., 2011).

Besides the fundamental studies on the ecological roles, researchers also look for the biotechnological potential for utilizing this wide range resource of fungi in the last few years. Although the utilization of these poorly understood endophytic microorganisms

has just begun, it has already showed promising potential and exciting future prospects to discover novel microorganisms and products (Strobel G., & Daisy B., 2003; Aly A.H. et al., 2011; Strobel G., 2014). Endophytic microorganisms provide a great diversity of novel secondary metabolites or other unique and important characteristics for agricultural, medical, and industrial purposes (Strobel G., & Daisy B., 2003; Stadler M., & Schulz B., 2009; Santos-Fo F.C. et al., 2011). They can be used as biological control agents to suppress plant disease (insects and pathogens) (Bacon C.W. et al., 2001; Schena L. et al., 2003; Zhang Q. et al., 2014; Backman P.A. & Sikora R.A., 2008), provide bioactive metabolites for pharmaceuticals (Schulz B. et al., 2002; Strobel G., & Daisy B., 2003; Strobel G., 2014) and other unique metabolites for chemicals and biofuels (Strobel G. et al., 2008; Gonz âez M.C. et al., 2009; Stadler M., & Schulz B., 2009). Recently, it has been discovered that some of the endophytic fungi are capable of producing fuel-related compounds, like oil and other hydrocarbon compounds. This discovery has brought in new interests and development for the biofuel and bioenergy application since these new sources of fuel-like compounds are renewable and compatible with current engine systems, and can be processed as “drop-in” fuels.

During the search for endophytes, it is relevant to consider that some endophytic microbes can produce the same metabolite as their plant host (Strobel G., & Daisy B., 2003; Stadler M., & Schulz B., 2009). Such consideration makes oil-rich plants a potential reservoir of promising fungal strains for fuel production. This research focused on the biodiversity of endophytic or plant-associated fungal species in soybean, and also

investigated their lipid accumulating capability. It was expected to isolate promising oleaginous fungi strains from the plants, which will be suitable for the production of biofuel. This study represents an important initial step in understanding the role of fungal endophytes to soybean and the presence of oleaginous fungi in soybean.

3.2 Materials and Methods

3.2.1 Isolation of fungi

Soybean grains and husks samples were collected from five sites of croplands in Rosemount and Hastings, MN, August 2011. In order to make a comparison, soil samples from sampling sites were also collected. Another set of samples was collected from the same sites in October 2011 and only soybean samples were obtained. Soybeans without symptoms of disease were randomly chosen from each site, detached from plants by scissor/tweezer, put into sterilized glass container immediately, sealed and stored at 4℃ (no more than 48 h) until processed. Soybean samples were dipped into 70% (v/v) ethanol for 10 sec to sterilize the surface, and then soybean grains were separated from soybean husks by sterilized tweezer and scissor. Both soybean grains and soybean husks were cut into small pieces (about 2~3mm^3) by sterilized scissor, and incubated at 27 ^oC on Potato Dextrose Agar (PDA) -Ampicillin plates. Soil samples were added into autoclaved distilled water, vertexed to mix well, and then 20 ul of wash-out was inoculated into PDA - Ampicillin plates (50ug/ml Ampicillin) and incubated at 27 ^oC. Individual fungal hyphae isolate generated from the plates was transferred to another

fresh PDA plate and continued incubation at 27 ^{o}C. This transfer was repeated until pure isolates were obtained with microscope examination.

3.2.2 Genetic identification with sequencing of ITS regions

Fungal isolates were identified by genetic identification of fungal internal transcribed spacer (ITS) regions. The hyphae of pure fungal isolates were inoculated into 100 mL potato dextrose (PD) broth medium in 250 mL flasks. After 6 days of cultivation at 27 ℃, 150 rpm, 1 mL fungal culture was collected to extract genomic DNA by a Promega extraction kit (Promega, USA. Cat No. A1120). Universal primer ITS1: TCCGTAGGTGAACCTGCGG ITS4: TCCTCCGCTTATTGATATGC were used to amplify DNA in ITS1, 5.8s ribosomal RNA and ITS2 regions. PCR reactions were performed under the following condition: initial denaturation at 95 ℃ for 5 min, 35 cycles of 95 ℃ for 45 s, 55 ℃ for 1 min, and 72 ℃ for 1 min, and final elongation at 72 ℃ for 10 min. Amplified PCR products were purified by Promega PCR clean-up kit (Promega, USA. Cat No.A9281) and sequenced by Sanger sequencing at University of Minnesota Genomic Center (UMGC). Results were analyzed by comparing with sequences in NCBI nucleotide collection database with BLASTn program.

3.2.3 Flasks cultivation and determination of lipid content

The hyphae of pure fungal isolates were inoculated into a 100 mL modified PD medium in 250 ml flasks. The medium contains 9.6 g/L PD broth and 12 g/L glucose with a C:N molar ratio of 100. After 6 days of cultivation at 27 ^{o}C, 150 rpm, the culture broth was

centrifuged at 9000 rpm for 5 min to obtain fungal biomass. The biomass was then oven dried overnight at 105 ^{o}C and ground into powders. The weight of dry biomass was biomass content. Lipids were extracted from the dry fungal biomass by using mixed solvents of chloroform and methanol with the following procedure (Folch J. et al., 1957): approximately 0.1 g dry fungal biomass was added into a 10 mL mixture of chloroform and methanol (2:1 ratio of chloroform/methanol) and shaken at 150 rpm for 16 h. Then, 2.5 mL of water was added into the mixture and vortexed for 1 min, which was then centrifuged at 7,000 rpm for 7 min. After centrifugation, the lower layer was filtered through a 0.45 μm filter, and then the solvent was evaporated to obtain the total lipids. Lipid content was determined by calculating the weight percentage of lipid in dry biomass.

3.3 Results

The screening of fungi generated 33 fungal isolates from August soybean samples, 17 fungal isolates from October soybean samples and 38 fungal isolates from soil samples. 18 different species were screened from August soybean, 8 different species were screened from October soybean, and 25 different species were screened from soil samples.

In the 33 fungal isolates from August soybean samples, 10 different genera were identified and the most abundant genera were *Fusarium, Alternaria, Penicillium* and *Nigrospora*. These 33 isolates encompass total 18 different species, in which 6 of them

belong to the genus of *Fusarium*. *Fusarium* and *Alternaria* are the two predominant genera in total endophytes that have been collected. All screened species have been detected from previous research work about endophytic fungi in soybean (Dalal J.M. & Kulkarni N.S., 2014; Impullitti A.E., & Malvick D.K., 2013; Muhammad H. et al., 2009; Pimentel I.C., et al., 2006), and a similar result of the predominance of *Fusarium* and *Alternaria* has been reported in some studies (Dalal J.M., & Kulkarni N.S., 2014). Table 3.1 listed all the fungal species obtained from August soybean samples.

Table 3.1. Fungi isolated from August soybean samples (Soy1 A).

Soy1 A-1*	*Fusarium graminearum*	Soy1 A-18	*Leptosphaerulina chartarum*
Soy1 A-2*	*Fusarium sporotrichioides*	Soy1 A-19	*Curvularia lunata*
Soy1 A-3*	*Fusarium sporotrichioides*	Soy1 A-20*	*Alternaria alternata*
Soy1 A-4*	*Fusarium acuminatum*	Soy1 A-21	*Alternaria alternata*
Soy1 A-5*	*Fusarium acuminatum*	Soy1 A-22	*Cladosporium cladosporioides*
Soy1 A-6*	*Fusarium acuminatum*	Soy1 A-23	*Alternaria alternata*
Soy1 A-7*	*Fusarium sporotrichioides*	Soy1 A-24	*Alternaria alternata*
Soy1 A-8	*Aspergillus flavus*	Soy1 A-25	*Alternaria alternata*
Soy1 A-9	*Penicillium aculeatum*	Soy1 A-26	*Alternaria alternata*
Soy1 A-10	*Penicillium aculeatum*	Soy1 A-27	*Alternaria alternata*
Soy1 A-11*	*Fusarium equiseti*	Soy1 A-28	*Alternaria* sp.
Soy1 A-12	*Nigrospora oryzae*	Soy1 A-29	Yeast 1
Soy1 A-13*	*Fusarium lacertarum*	Soy1 A-30	Yeast 2
Soy1 A-14	*Myrothecium verrucaria*	Soy1 A-31	*Alternaria alternata*
Soy1 A-15*	*Fusarium moniliforme*	Soy1 A-32*	*Fusarium equiseti*
Soy1 A-16	*Nigrospora oryzae*	Soy1 A-33	*Sistotrema brinkmannii*
Soy1 A-17*	*Fusarium equiseti*		

*: isolates which have lipid content of more than 20% of dry biomass.

In the 38 fungal isolates screened from soil samples, 15 different genera were identified and the most frequent isolated genera were *Trichoderma*, *Fusarium*, *Mucor* and *Talaromyces*. These genera were also reported by other researchers from soil (Manter

D.K., & Vivanco J.M., 2007; Manici L.M., & Caputo F., 2010; Vujanovic V. et al., 2012). Most of the genera from August soybean samples, such as *Fusarium, Alternaria*, *Aspergillus* and *Penicillium* have also been detected from soil samples, but they may not be the same species in these two types of samples. In the fungal strains that were isolated from soil samples, *Talaromyces*, *Mortierella*, *Aspergillus*, *Trichoderma*, *Mucor* are the genera that haven't been found from soybean samples. In contrast to soybean samples that several genera dominate the acquired fungal isolates, there is only one genus (*Trichoderma*) with higher abundance in soil samples. The detail isolated fungal species were listed in Table 3.2.

Table 3.2. Fungi isolated from August soil samples (Soy1 B).

Soy1 B-1	*Aureobasidium pullulans*	Soy1 B-20*	*Trichoderma* sp.
Soy1 B-2	*Sordaria tomento-alba*	Soy1 B-21	*Talaromyces purpurogenus*
Soy1 B-3*	*Fusarium equiseti*	Soy1 B-22*	*Mucor hiemalis*
Soy1 B-4*	*Fusarium oxysporum*	Soy1 B-23*	*Trichoderma koningiopsis*
Soy1 B-5*	*Fusarium equiseti*	Soy1 B-24*	*Trichoderma koningiopsis*
Soy1 B-6	*Trametes pubescens*	Soy1 B-25	*Cladosporium cladosporioides*
Soy1 B-7*	*Mucor hiemalis*	Soy1 B-26	*Talaromyces purpurogenus*
Soy1 B-8	*Neosartorya fischeri*	Soy1 B-27	*Epicoccum nigrum*
Soy1 B-9	*Trichoderma gamsii*	Soy1 B-28*	*Fusarium graminearum*
Soy1 B-10	*Trichoderma gamsii*	Soy1 B-29*	*Mortierella alpina*
Soy1 B-11	*Trichoderma harzianum*	Soy1 B-30	*Trichoderma* sp.
Soy1 B-12	*Trichoderma harzianum*	Soy1 B-31	*Sordaria fimicola*
Soy1 B-13*	*Trichoderma koningiopsis*	Soy1 B-32	*Aspergillus flavus*
Soy1 B-14*	*Trichoderma koningiopsis*	Soy1 B-33	*Alternaria alternata*
Soy1 B-15	*Penicillium pinophilum*	Soy1 B-34*	*Mucor circinelloides*
Soy1 B-16	*Aspergillus niger*	Soy1 B-35	*Talaromyces flavus*
Soy1 B-17	*Lichtheimia ramosa*	Soy1 B-36	*Alternaria alternata*
Soy1 B-18	*Trichoderma* sp.	Soy1 B-37	*Cladosporium cladosporioides*
Soy1 B-19*	*Penicillium verruculosum*	Soy1 B-38	*Neosartorya fischeri*

*: isolates which have lipid content of more than 20% of dry biomass.

In the 17 fungal isolates screened from October soybean samples, 7 different genera were identified and the most abundant fungi genera are *Alternaria*, *Cercospora*, and *Epicoccum*. All fungal species isolated from October soybean have also been detected from previous research work (Dalal J.M., & Kulkarni N.S., 2014; Impullitti A.E., & Malvick D.K., 2013; Muhammad H. et al., 2009; Pimentel I.C. et al., 2006). Compared to fungi isolated from August soybean samples, fewer genera were obtained. *Alternaria* remained its dominant position in the isolated strains, but the frequency of isolated *Fusarium* has been largely declined. In addition, the screening of October soybean generated some fungal genera that haven't been detected in August soybean samples, like *Cercospora* and *Epicoccum*. The detail isolated fungal species were listed in Table 3.3.

Table 3.3. Fungi isolated from October soybean samples (Soy2 A).

Soy2 A-1	*Alternaria alternata*	Soy2 A-10	*Epicoccum nigrum*
Soy2 A-2*	*Alternaria* sp.	Soy2 A-11	*Alternaria alternata*
Soy2 A-3	*Cercospora* sp.	Soy2 A-12	*Alternaria alternata*
Soy2 A-4	*Diaporthe longicolla*	Soy2 A-13*	*Cladosporium cladosporioides*
Soy2 A-5*	*Scopulariopsis brevicaulis*	Soy2 A-14*	*Fusarium equiseti*
Soy2 A-6*	*Alternaria alternata*	Soy2 A-15	*Alternaria alternata*
Soy2 A-7*	*Alternaria alternata*	Soy2 A-16	*Alternaria alternata*
Soy2 A-8*	*Alternaria alternata*	Soy2 A-17	*Cercospora* sp.
Soy2 A-9	*Epicoccum nigrum*		

*: isolates which have lipid content of more than 20% of dry biomass.

August soybean samples and October soybean samples were both collected from five different sites of farmlands inRosemont and Hastings, MN. From the list of fungal species that obtained from each site (Table 3.4 & Table 3.5), it is obvious that most species were screened out from one or two sites. The only species that isolated from

nearly all sites for both August and October is *A. alternate*. This may be related to the fast growth rate of *A. alternata*, but it also showed that *A. alternate* is widely distributed in the sampled farmlands across the time. For August soybean samples (Table 3.4), *A. alternate* and *F. acuminatum* are most frequently detected species. Location 2, 3, 4, and 5 tends to share several same species from the screening results, while Location 1 appeared to have more distinct species. But all five locations screened out *Fusarium* genus fungi, showing *Fusarium* as the most prevalent and active fungi in August season. For October soybean samples (Table 3.5), all locations showed less diversity than August samples, and Location 2 appeared to have distinct species than other four locations. *A. alternate* is the most frequently detected species in October samples, and there is only one location obtained *Fusarium* genus fungi compared to August samples, suggesting the spatial and temporal change in fungal community.

Table 3.4. Fungal species detected from August soybean sample at different sites.

August soybean	Location 1	Location 2	Location 3	Location 4	Location 5
Alternaria alternata		x	x	x	x
Alternaria sp.					x
Aspergillus flavus			x		
Cladosporium cladosporioides			x		
Curvularia lunata		x			
Fusarium acuminatum		x		x	x
Fusarium equiseti		x			x
Fusarium graminearum	x				
Fusarium lacertarum			x		
Fusarium moniliforme				x	
Fusarium sporotrichioides	x			x	
Leptosphaerulina chartarum	x				
Myrothecium verrucaria			x		
Nigrospora oryzae			x	x	
Penicillium aculeatum	x				
Sistotrema brinkmannii					x

Table 3.5. Fungal species detected from October soybean sample at different sites.

October Soybean	Location 1	Location 2	Location 3	Location 4	Location 5
Alternaria alternata	x		x	x	x
Alternaria sp.	x				
Cercospora sp.	x			x	
Cladosporium cladosporioides		x			
Diaporthe longicolla	x				
Epicoccum nigrum					x
Fusarium equiseti				x	
Scopulariopsis brevicaulis			x		

3.4 Discussion

The plant-associated fungal species of soybean in this research have been widely isolated from environments and are primarily recognized as plant pathogen in other fungal community studies. But when isolated with no adverse effects on plants, these fungi are not considered to be pathogenic to host plants at the stage when isolated. They might play an important role in the physiology of the plant growth by delivering the resistance to herbivory and insects, drought tolerance, and protection against pathogens (Higgins K.L. et al., 2007; Senthilkumar M. et al., 2007). There is also possibility that the fungal endophytes will not show any observable symptoms of their presence within their host. This status was described by Schulz B. & Boyle C. (2005) that endophytes maintained a finely balanced interaction with their host. As long as fungal virulence and the host defense reaction are balanced, disease does not develop and the endophytes do not show their pathogenicity.

The major research methods on fungal communities can be categorized as culture-dependent method and culture-independent methods. Culture-dependent methods use

prepared growth medium to isolate and grow fungi for identification. It is effective for rapid recovery of a large number of endophytic or plant-associated fungal species from plant tissues and isolate specific group of fungi (Orphan V.J. et al., 2000). Undoubtedly, culture-dependent screening process only detects species that grow under specific culture conditions, and there are likely more species of fungi that live within soybean grain. Culture-independent methods have generally been considered to be more reliable, fast, and economical in profiling complex community structure and evolution dynamics (Cocolin L. et al., 2007; Jany J.L., & Barbier G., 2008). It can detect unculturable, low abundance or slow-growth microorganisms, provide a less biased picture of the richness of microbial communities than culture-dependent methods (Gherbawy Y., & Voigt K., 2010). However, the culture-independent method is also interfered by the primer selection, unequal amplification, taq polymerase error and the formation of chimerical molecules or heteroduplex molecules (Acinas S.G. et al., 2005). In this research, the culture-dependent method is chosen based on the purpose to both study soybean fungal community and isolate suitable fungal strains for biofuel production. Without the acquisition of pure strains, culture-independent method is not sufficient for subsequent research on selection, characterization and improvement of potential oleaginous fungal strains. This research focuses on the fungal species that can be detected by this traditional method.

Among all the fungal isolates obtained in this research, *Fusarium*, *Alternaria*, *Penicillium* and *Nigrospora* were the most abundant genera screened from August soybean samples.

From October soybean samples, *Cercospora* and *Epicoccum* were also found to be frequently present in addition to *Alternaria*. *Fusarium* and *Alternaria* fungi were usually the dominant species that can be isolated from plants. Many *Fusarium* species are commonly found as plant pathogens and they produce a range of phytotoxic compounds, including fusaric acid, fumonisins, moniliformin and trichothecenes, causing a variety of morphological, physiological and metabolic effects including necrosis, chlorosis, growth inhibition, wilting, and inhibition of seed germination (Van Asch M.A.J. et al., 1992; McLean M., 1996). Some *Fusarium* fungi are also found to be beneficial to plants. For example, *F. equiseti* and *F. solani* strains that grow endophytically in crops can control plant disease and promote plant growth (Motlagh M.R.S., 2001; Saldajeno M.G.B., & Hyakumachi M., 2011; Karpouzas D.G. et al., 2011). *Alternaria* species are considered as plant-pathogens and saprophytes, causing common diseases including leaf blight and leaf spot diseases on various types of crops. Various *Alternaria* species are known to produce approximately 30 metabolites with possible toxicity (Robiglio A.L., & López S.E., 1995). They have also been closely related to postharvest diseases, and it was reported that at least 20% of agricultural spoilage was caused by *Alternaria* species (Nowicki M., et al., 2012). In addition, *A. alternata* has been related to food poisoning, and produces several different mycotoxins include alternariol, altenuene, alternariol monomethyl ether, altertoxins and L-tenuazonic acid (Scott P.M., 2001). The soybeans selected for sampling in this research have no symptoms of disease, and these endophytes are more likely to provide beneficial metabolites for plant growth and biocontrol of plant disease in their symbiotic relationship with their host plant.

Similar to *Fusarium* and *Alternaria*, *Penicillium* is also a common mycotoxin producer. *Penicillium* is a genus of fungi important in food and drug production. Some members of this genus like *P. chrysogenum* are used in the production of antibiotic penicillin, and some are used in cheese making including *P. camemberti* and *P. candidum*. Like *Alternaria*, some species in *Penicillium* can cause postharvest diseases, affecting the quality of the product and generating large economic loss. The Penicillium mycotoxins (PMs), such as citrinin, ochratoxin A, patulin, mycophenolic acid and penicillic acids, can be frequently found in animal feeds (Mansfield M.A. et al., 2008; Reyes-Vel ázquez W.P. et al., 2008). *P. aculeatum* isolated in this research are found to be capable of producing chitinases and dextranase (Binod P. et al., 2005; Shukla G.L. et al., 1989). *Nigrospora*, *Cercospora*, and *Epicoccum* are the other three genera that have relative higher abundance in isolates from soybean samples. Similar to the previous discussed endophytes, pathogenic species in these genera have been reported to cause losses in agriculture by reducing crop yield in the field and through spoilage during storage (Ayob F.W., & Simarani K., 2016; Shane W.W., & Teng P.S., 1992; Sorensen J.L. et al., 2009). Their pathogenicity are mainly related to mycotoxins (Dong J. et al., 2014), and cellulase production from these fungal strains (Ayob F.W., & Simarani K., 2016; Brown A.E., 1984). Meanwhile, the mycotoxin production also makes these species gain the capability to be used as biocontrol agents in management of specific plant diseases (Larena I. et al., 2005; Mari M. et al., 2007; Groenewald J.Z. et al., 2013),

Trichoderma, *Fusarium*, *Mucor* and *Talaromyces* are the genera that are relatively abundant in fungal isolates screened from soil samples. *Trichoderma* fungi are discovered in many types of soil, and have been screened as endophytic fungi to form mutualistic endophytic relationships with host plants (Bae H. et al., 2011). They are found to serve as biocontrol agents against fungal diseases of plants (Harman G.E., 2006), and one *T. asperellum* strain was reported to have high disease reduction of 71.7% (Saravanakumar K. et al., 2016). Certain *Trichoderma* sp. is also applied in the production of commercial cellulase. *Mucor* is another fungal genus commonly found in soil. They are normally encountered as contaminants of meat, fruits, vegetables or processed products, but certain *Mucor* sp. is also involved in food production (Karimi K., & Zamani A., 2013). *Mucor* species have also been reported to enhance plant uptake of mineral nutrients and can accumulate phosphorus from the surrounding as the phosphorus storage to improve plant growth (Wang G.H. et al., 2007). Furthermore, *M. circinelloides* is a well-known lipid producer capable of accumulating a high level of TAG (Xia C. et al., 2011). *Talaromyces* is also reported to be screened as endophytes (Ding H. et al., 2015; Kaur A. et al., 2016). Current research is mainly focused on the utilization of *Talaromyces* metabolites, like lipase (Romdhane I.B. et al., 2012) and asparaginase (Krishnapura P.R., & Belur P.D., 2016).

As expected, high lipid content fungi were screened out from the fungal isolates. There were 13 oleaginous fungal strains screened out of 33 strains isolated from August soybean samples, lipid content ranges from 20.6% to 47.6%. Among the 13 oleaginous

strains, 5 have especially high lipid content (>40%), which belong to 3 species *F. sporotrichioides*, *F. acuminatum*, *F. equiseti*. And *F. equiseti* has the highest lipid content. From October soybean samples, 7 oleaginous fungal strains were screened out of 17 isolated strains. Their lipid content ranges from 20.1% to 46.4%, and only one strain (*F. equiseti*) has lipid content higher than 40%. The abundance of oleaginous strains out of total strains screened from these two types of samples are very close, but oleaginous fungi strains isolated from August soybean generally have higher lipid content. In fungal isolates obtained from soil samples, oleaginous fungi were also discovered. 4 strains have lipid content higher than 40%, and they belong to 3 species *F. equiseti*, *M. hiemalis* and *M. circinelloides*. Screening of these 3 types of sample provided *F. equiseti* as the most promising oleaginous fungal species, and the application of it on lipid production merit future investigation. Figure 3.1 shows the number of isolates in each lipid content range.

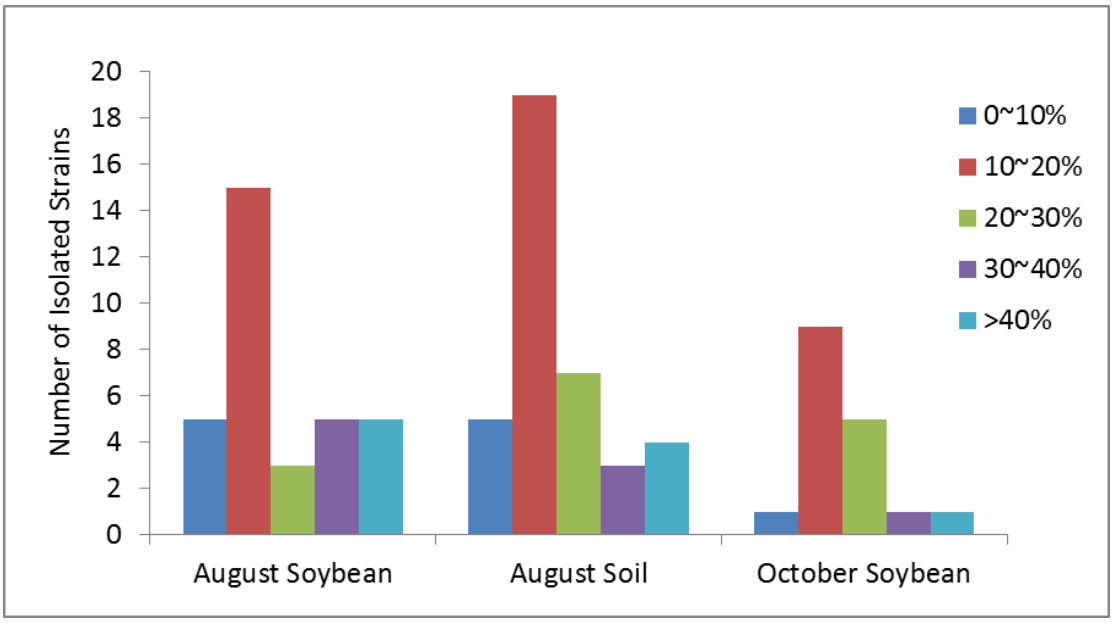

Figure 3.1. Number of fungal isolates in each lipid content range.

All *Fusarium* strains isolated in this research were identified as oleaginous fungi, and *F. equiseti* reached the highest lipid content in obtained strains. Previous research on *Fusarium* focused more on its toxicity and its role as a plant pathogen. Only in recent years, there are reports of *Fusarium* species as promising candidates for lipid production (Li S. et al., 2012; El-haj M. et al., 2015). Besides *Fusarium* strains, *Alternaria*, *Scopulariopsis* and *Cladosporium* species screened out from soybean, *Mucor*, *Mortierella*, *Penicillium* and *Trichoderma* species screened out from soil are also identified as oleaginous fungi in this research. Some of these genera have been identified as oleaginous fungi in previous reports. For example, *Mucor* and *Mortierella* strains have long been studied as important model organisms for lipid accumulation (Papanikolaou S. et al., 2004; Ratledge C., 2005). *Penicillium* and *Trichoderma* have also been reported as oleaginous fungi (Li S. et al., 2011; Serrano-Carreon L. et al., 1992). *Alternaria* is considered as a plant pathogen, and one report claimed *Alternaria* sp. as oleaginous fungi (Dey P. et al., 2011). *Scopulariopsis* and *Cladosporium* were demonstrated to function as plant pathogens (Rivas S., & Thomas C.M., 2005; Lavin P. et al., 2016). To our knowledge, this is the first time that species in *Scopulariopsis* and *Cladosporium* are considered as oleaginous fungi.

Oleaginous fungi isolated from this research are located in four classes: Sordariomycetes, Dothideomycetes, Eurotiomycetes, and Mucormycotina (Table 3.6). Among all the isolated oleaginous genera, *Fusarium*, *Scopulariopsis* and *Trichoderma* belong to the class of Sordariomycetes. *Alternaria* and *Cladosporium* belong to the class of

Dothideomycetes. *Mucor* and *Mortierella* belong to the class of Mucormycotina. The class of Eurotiomycetes have only one oleaginous fungi genus in this research, but there are reports identified *Aspergillus* sp. in Eurotiomycetes as oleaginous fungi (Khot M. et al., 2012). The common habitats of investigated oleaginous fungi are oil-polluted or oil-rich environments, in which relatively excessive carbon source is existed. Researches on lipid accumulation of multiple oleaginous fungi strains revealed that nitrogen limited condition is required for them to accumulate high intracellular lipid content. And it is possible for oleaginous fungal species to share some specific metabolic pathways to enrich their lipid content under certain living environments. According to the fungal genetic tree that has been built (James T.Y. et al., 2006), these oleaginous fungal genera represent a minor proportion of the total fungal population and locate in some specific classes. These specific classes may serve as oleaginous fungi reservoir to screen out promising strains when needed.

Table 3.6. Scientific classification of obtained oleaginous fungal genera.

Kingdom	Phylum	Class	Order	Family	Genus
Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Alternaria*
Fungi	Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	*Cladosporium*
Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*
Fungi	Zygomycota	Mucormycotina	Mortierellales	Mortierellaceae	*Mortierella*
Fungi	Zygomycota	Mucormycotina	Mucorales	Mucoraceae	*Mucor*
Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Penicillium*
Fungi	Ascomycota	Sordariomycetes	Microascales	Microascaceae	*Scopulariopsis*
Fungi	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	*Trichoderma*

Compared to fungal strains isolated from soil, fewer fungi have been isolated from soybean. The environment could be the main factor to limit the community size. Soil environment has various nutrients to be utilized by different types of microorganisms, but

the internal environment of plants is more unique. Less variety of carbon source and other nutrients are provided inside plants, which limits the types of endophytic fungi. For endophytes within plants, plant materials and nutrients are their only nutrient source. Although they do not cause immediate negative effect, endophytes may turn pathogenic during host senescence (Rodriguez R., & Redman R., 2008). The capability of plant structure degradation may provide extra benefits when producing lignocellulose-based lipids by endophytic fungi. *Fusarium* and *Alternaria* species are screened out as endophytic fungi in this research. They may have better utilization of cellulose-based material (eg. corn stover, wheat straw) to produce lipids from agricultural wastes.

Fungal species isolates in this study are relatively less than other studies on soybean endophytes. One possible reason is the sampling location. Compared to soybean plants grown in tropical environment like in Brazil or India (Dalal J.M., & Kulkarni N.S., 2014; Pimentel I.C. et al., 2006), the cold temperature in Minnesota may lower the diversity of endophytes. Another important possibility is that fungal strains in this study are isolated only from soybean grains and husks. It has been pointed out that greater numbers of endophytes near the soil line in stems were observed for fungi in soybean (Pimentel I.C. et al., 2006; Impullitti A.E., & Malvick D.K., 2013), while different organs and parts of soybean plants may also be preferentially occupied by certain groups of fungal isolates.

Another finding is that from August to October, the fungal community in soybean had a change. In August soybean samples, *Fusarium* and *Alternaria* are the two predominant

genera. In October soybean samples, *Alternaria* remained its dominant position but the abundance of *Fusarium* had been largely declined. The most likely reason is the change of soybean grain environment, such as moisture content and nutrients. Soybean in August was still in the process of fruit bearing, the samples collected were small size grain with healthy green color. While in October, soybean sample collected were dry, bigger size grain with yellow color. It was found that some essential nutrients needed for endophytic microorganisms were unavailable during the maturation and senescence of plants (Pimentel I.C. et al., 2006), which could be the main reason for the less detection of endophytes and lower appearance of *Fusarium* strains in October sample. For the screening process with the purpose to obtain promising strains, it may be better to choose samples before its maturation.

3.5 Conclusions

This research emphasized fungi isolated from soybean grains, and compared the fungal isolates obtained from two time periods (August and October) and from two types of samples (soybean and soil). Difference in fungal diversity was detected from soybean samples collected in August and samples collected in October. Soybean samples and soil samples also showed a difference in the isolated fungal community, and different sampling locations lead to differences in fungal community. These results demonstrated an impact of environment on fungal community. And the change of acquired fungal isolates through time was discovered, indicating that plant-associated fungal community was in a dynamic status. From all the fungal isolates, *F. equiseti* was identified as the

most promising oleaginous fungi in this research. Besides the discovery of *F. equiseti*, this research also observed and explored the correlation between the plant-associated fungi and oleaginous fungi, exploring a new method in the search of oleaginous fungi for prospective function as biotechnological applications.

CHAPTER 4 FUNGAL STRAINS OF SOYBEAN FOR LIPID PRODUCTION

Outline

Lipids created via microbial biosynthesis are a potential raw material to replace plant-based oil for biodiesel production. Oleaginous microbial species currently available are capable of accumulating high amount of lipids in their cell biomass, but rarely can directly utilize lignocellulosic biomass as substrates. Thus this research focused on the screening and selection of new fungal strains that generate both lipids and hydrolytic enzymes. To search for oleaginous fungal strains in the soybean plant, endophytic fungi and fungi close to the plant roots were studied as a microbial source. Among 33 endophytic fungal isolates screened from soybean plant collected in August, 13 have high lipid content (>20 % dry biomass weight). Among 38 fungal isolates screened from the soil surrounding the soybean roots, 14 have high lipid content. Among 17 fungal isolates screened from soybean plant collected in October, 7 have high lipid content. Also, five fungal isolates with both high lipid content and promising biomass production were selected for further studies on their cell growth, oil accumulation, lipid content and profile, utilization of various carbon sources, and cellulase production. The results indicate that most strains could utilize different types of carbon sources and some strains accumulated >40 % of the lipids based on the dry cell biomass weight. Among these promising strains, some Fusarium strains specifically showed considerable production of cellulase, which offers great potential for biodiesel production by directly utilizing inexpensive lignocellulosic material as feedstock.

4.1 Introduction

With increasing environmental concerns over fossil fuels and demands for renewable energy products, microbial lipids synthesized by oleaginous microorganisms are potentially an important and attractive feedstock for biodiesel production. Current raw materials to produce biodiesel, primarily plant-based (e.g., soybean and vegetable oil), contribute to >75 % of the total production cost, which signifies that biodiesel still more expensive compared with conventional fuels (Durrett T.P. et al., 2008). Meanwhile, the biodiesel industry is competing primarily with the food industry for oil crops and available arable land (Vicente G. et al., 2009). As a potential solution to address these issues, microbial lipids, referred to as single cell oils (SCO), accumulated during cultivations of microalgae (Cheng Y. et al., 2009), oleaginous yeast, and fungi (Papanikolaou S. et al., 2004; Ratledge C., & Hopkins S., 2006), can replace vegetable oils to produce biodiesel. The theoretical yield for converting sugars to SCO is 32 %; however, most oleaginous microorganisms can reach the yield of 20 %–25 % because a portion of the sugars must be diverted to support the cell growth and metabolism. Therefore, the use of non-starch biomass such as lignocellulose for the organic carbon supply is critical.

Microorganisms containing more than 20%–25% lipids in their cell biomass are usually considered to be oleaginous microorganisms (Ratledge C., 1991). It is generally accepted that microbial lipids are accumulated as energy storage when cell growth becomes limited due to the shortage of nitrogen or other nutrients, while excessive carbon source

is still available (Ratledge C., 1991). In most cases, lipids from these microorganisms are in the form of triglycerides, which are also the main component in vegetable oils and animal fats (Vicente G. et al., 2009). Among these different oleaginous microorganisms, filamentous fungi have some favorable properties. Filamentous fungal cells, in general, are more easily harvested, especially when they grow in the form of pellets or mycelium (Xia C. et al., 2011). Some fungal species, such as *Mortierella* sp., can accumulate the intracellular lipids as high as 80 % based on dry weight (Meng X. et al., 2009); however, they cannot generate cellulase to directly grow on cellulose ((Zhang J.G., & Hu B., 2012). While some fungal species can grow on inexpensive substrates such as lignocellulosic biomass ((Zhang J.G., & Hu B., 2012), none can accumulate large amounts of oil and thus be considered as oleaginous strains (Ahamed A., & Ahring B.K., 2011). Recent studies confirmed the conversion of hemicellulose hydrolysate into lipids by oleaginous yeast strains (Huang C. et al., 2009; Hu C. et al., 2009; Chen X. et al., 2009). However, these yeast strains have low tolerance to lignocellulose degradation compounds and were unable to efficiently produce lipids in the presence of inhibitors in the hydrolysate. If a suitable fungal strain were developed with the capability to produce both lipids and hydrolytic enzymes, it would then be possible to develop consolidated bioprocesses to directly convert lignocellulose to microbial lipids for biodiesel production.

Endophytic fungi are the fungal species that colonize inside plants, while no immediate negative effect to plants is observed (Bacon C.W., & White J.F., 2000). Recently, endophytic microbes have been extensively studied for their wide range of beneficial

applications (Strobel G.A., & Daisy B., 2003), including their production of bioactive metabolites such as nutraceuticals and drugs, their agricultural use for crop performance improvement, and their oil accumulation for biofuels (Stierle A. et al., 1993; Strobel G.A. et al., 2008; Schulz B. et al., 2002; Zhang H.W. et al., 2006; Aly A.H. et al., 2010). This research illustrates that plants may serve as a reservoir of untold numbers of endophytes (Bacon C.W., & White J.F., 2000) and that endophytic microorganisms can provide a great diversity of secondary metabolites or other unique and important characteristics for agricultural, medical, and industrial purposes (Stadler M., & Schulz B., 2009; Santos-Fo F.C. et al., 2011). During the search for endophytes, it is relevant to consider that the endophytic microbes can also produce the same metabolite as their plant host (Strobel G.A., & Daisy B., 2003; Stadler M., & Schulz B., 2009). For example, the anticancer drug taxol, originally derived from the inner bark of yew tree, was also produced by the endophytic fungi of the tree (Stierle A. et al., 1993; Strobel G.A. et al., 1996; Strobel G.A., 2003). With this consideration, endophytic fungi of oil-seed plants can be expected to have lipid-accumulating capability; thus oil-rich plants become a potential reservoir of oleaginous fungi. It is hypothesized that endophytic fungi in oil-rich plants had a strong possibility to obtain the ability of lipid accumulation and that they also had the capability to generate cellulase in order to obtain the organic carbon source for their growth. Researchers have isolated lipid-accumulating fungi from oily wood-stem (Peng X.W., & Chen H.Z., 2007) and oil-seed crops (Dey P. et al., 2011; Venkatesagowda B. et al., 2012). Minnesota and the Midwest United States have traditionally been an agricultural region to mainly produce corn and oilseed crops, such as soybeans, sunflowers, and

canola. If oleaginous strains can be screened from these locally grown oil crops, then they might be a more suitable source for microbial oil accumulation by utilizing agricultural residue, which is considered to be readily available, low-value feedstock. In this research, the soybean plant was used as the oil-rich plant source for fungal strain screening, and fungal strains with high lipid content and with cellulase production were isolated and studied. Sunflower and canola samples from local farms were also used in the screening process; however, they offered a very limited number of oleaginous fungal strains with lower oil content (20%–30%), thus those data are not discussed in this research.

4.2 Materials and Methods

4.2.1 Isolation of fungi

Soybean grains and husks, and soil samples surrounding to the soybean roots were collected from croplands in Rosemount and Hastings, MN. Please refer to 3.2.1 for the detailed method of fungal isolation.

4.2.2 Flask cultivations to evaluate the lipid production

Please refer to 3.2.3 for the detailed methods of biomass content and lipid content determination.

4.2.3 Genetic identification with sequencing of ITS regions

With the flask cultures to evaluate the lipid production of all the fungal isolates obtained from the field samples, only the fungal isolates with high lipid content (>20 % of dry

biomass weight) were selected for genetic identification of ITS regions. Please refer to 3.2.2 for the detailed sequencing method. In addition, phylogenetic trees were constructed by the neighbor-joining method using software MEGA (Molecular Evolutionary Genetics Analysis; version 5.05). The nucleotide sequences of reference strains used in tree construction were obtained from the NCBI nucleotide collection database. Nearly complete ITS1, 5.8 s rRNA, and ITS2 region sequences of both reference strains and isolated fungal strains with high-lipid content were covered for the tree construction.

4.2.4 Petri dish cultivation with different carbon sources

Only five fungal isolates were tested for their carbon utilization, fatty acid pofile and cellulase production. To determine if fungal isolates have the capability to utilize different carbon sources, solid growth medium with various carbon sources were prepared. The medium contained: 2 g/L KH_2PO_4, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 3 g/L $(NH_4)_2SO_4$, 0.1 mg/L $CaCl_2 \cdot H_2O$, 0.1 mg/L $CoCl_2$, 0.1 mg/L $MnSO_4 \cdot 5H_2O$, and 20 g/L agar. In addition, different carbon sources were added at the same concentration of 20 g/L. Carbon sources used in this test included glucose, fructose, galactose, glycerol, lactose, starch, mannose, xylose, xylan, cellulose, sucrose, and cellobiose. All chemicals are ordered from Fisher Scientific or Sigma-Aldrich. Fungal spores were inoculated into each medium, and then cultivated at 27 ℃ for 4 days. The cell growth on the Petri dish was evaluated based on the visual appearance of the fungal hyphae. The growth performance

was categorized into three levels: (1) –, no growth, no hyphae seen; (2) +, limited growth, few hyphae seen; or (3) ++, active growth, massive spread of hyphae seen.

4.2.5 Analysis of fatty acid profile and cellulase production

FAME was prepared by extraction–transesterification method (Indarti E. et al., 2005). Approximately 0.1 g dry fungal biomass was placed in a 25 mL screw-top glass tube, and a 10 mL extraction mixture of methanol, concentrated sulfuric acid, and chloroform (4.25:0.75:5) was added into the glass tube for the extraction and transesterification reaction in a 90 ℃ water bath for 90 min. When the reaction finished, tubes were cooled to room temperature and the lower layer, which contains FAME, was collected. The FAME profile was then determined with gas chromatography (GC) (Agilent 6890, Santa Clara, CA, USA), equipped with flame ionization detector (FID) and DB-FFAP capillary column. The oven temperature was 140 ℃, held for 5 min, raised to 240 ℃ at a rate of 4 ℃/min, and held at 240 ℃ for 10 min, while the injector and detector temperature were both set at 250 ℃. Hydrogen was used as carrier gas at a flow rate of 0.6 ml/min. FAME in the sample was identified and quantified by comparing the peak area with the standards (FAME Mix C8-C22 standard, 18920-1AMP; Sigma-Aldrich, St. Louis, MO, USA) using octanol as the internal standard.

Cellulase activity of fungal isolates was tested by filter paper assay (Ghose T.K., 1987). Fungi were cultivated in 100 mL growth medium at 27 ℃ for 8 days; the culture medium contained 2% cellulose as well as the other nutrients described in 4.2.4. The cultural

broth was centrifuged at 9,000 rpm for 5 min and the supernatants were tested for the cellulase activity. Cellulase activity results were represented as filter paper unit per milliliter (FPU/mL).

4.3 Results and Discussion

4.3.1 Lipid content of oleaginous fungi isolates and their identification

A total of 33 fungal isolates were obtained from soybeans and 38 fungal isolates from surrounding soil samples. For the 33 isolates from August soybean grains and husks, 13 have lipid content >20% (Table 4.1). For the 38 isolates from surrounding soil, 14 have lipid content >20 % (Table 4.1). For the 17 isolates from October soybean grains and husks, 7 have lipid content >20 % (Table 4.1). The high percentage of oleaginous fungi suggests that the soybean plant could be a "hot spot" in the isolation of oleaginous fungi.

Table 4.1. Biomass production and lipid content of fungal isolates from soybean and surrounding soil.

Fungal isolates from August soybean			Fungal isolates from soybean surrounding soil		
Isolate	Biomass (g/100ml)	Oil Content (%)	Isolate	Biomass (g/100ml)	Oil Content (%)
Soy1 A-1	0.700 ±0.080	25.11 ±0.77	Soy1 B-3	0.475 ±0.033	44.55 ±1.16
Soy1 A-2	0.312 ±0.016	36.43 ±0.46	Soy1 B-4	0.363 ±0.027	29.57 ±1.00
Soy1 A-3	0.340 ±0.015	44.95 ±0.22	Soy1 B-5	0.516 ±0.007	53.83 ±2.56
Soy1 A-4	0.547 ±0.025	40.61 ±0.03	Soy1 B-7	0.570 ±0.011	42.11 ±3.95
Soy1 A-5	0.685 ±0.004	33.13 ±2.20	Soy1 B-13	0.342 ±0.019	26.19 ±1.28
Soy1 A-6	0.626 ±0.037	30.56 ±0.30	Soy1 B-14	0.513 ±0.010	27.34 ±1.05
Soy1 A-7	0.572 ±0.033	23.90 ±3.05	Soy1 B-19	0.400 ±0.029	24.57 ±1.05
Soy1 A-11	0.505 ±0.015	42.82 ±0.10	Soy1 B-20	0.406 ±0.051	26.78 ±0.72
Soy1 A-13	0.460 ±0.010	37.44 ±0.42	Soy1 B-22	0.541 ±0.022	30.50 ±0.50
Soy1 A-15	0.503 ±0.025	30.07 ±0.13	Soy1 B-23	0.480 ±0.067	24.42 ±0.93
Soy1 A-17	0.478 ±0.021	47.56 ±0.20	Soy1 B-24	0.484 ±0.059	24.33 ±0.92
Soy1 A-20	0.777 ±0.063	20.64 ±0.68	Soy1 B-28	0.619 ±0.029	34.80 ±0.41

Soy1 A-32	0.592 ±0.013	43.17 ±0.72	Soy1 B-29	0.300 ±0.030	30.24 ±0.81
			Soy1 B-34	0.415 ±0.013	45.38 ±1.98
Fungal isolates from October soybean					
Isolate	Biomass (g/100ml)	Oil Content (%)			
Soy2 A-2	0.618 ±0.079	20.09 ±0.17			
Soy2 A-5	0.237 ±0.035	30.04 ±4.00			
Soy2 A-6	0.444 ±0.019	23.18 ±1.52			
Soy2 A-7	0.522 ±0.034	23.86 ±1.91			
Soy2 A-8	0.550 ±0.015	21.52 ±0.87			
Soy2 A-13	0.466 ±0.026	23.50 ±0.58			
Soy2 A-14	0.516 ±0.007	46.43 ±2.75			

Based on the sequencing results, a phylogenetic tree was built to describe the similarity of fungal isolates with high lipid content. A key observation is that almost all of the oleaginous fungi obtained from August soybean belong to *Fusarium* genus except one *A. alternata* strian (Figure 4.1 a), and many species of the *Fusarium* fungi such as *F. graminearum*, *F. oxysporum*, *F. moniliforme*, and *F. acuminatum*, are commonly found as plant pathogens in crops (Goswami R.S., & Kistler H.C., 2004; Bienapfl J.C. et al., 2009). Some *Fusarium* fungi are also found to be beneficial for plant growth. For example, *F. equiseti* (teleomorph: *Gibberella intricans*), a typical endophytic fungus living in many cereal crops, has been described in research as a plant growth-promoting fungus and a disease-control agent in order to increase yields of agricultural crops (Saldajeno M.G.B., & Hyakumachi M., 2011; Motlagh M.R.S., 2011). *Fusarium* strains from these isolates can generate tremendously high amount of lipids in their cell biomass. The endophytic living environment inside the soybean may enable these strains to possess the capability to accumulate large amount of lipids. Almost all of the oleaginous

fungi obtained from October soybean (Figure 4.1 c) belong to *Alternaria* genus. Many species of *Alternaria* such as *A. alternata* are common plant pathogens (Peever T.L. et al., 2004; Pryor B.M., & Michailides T.J., 2002). In previous research about endophytic fungi, *Fusarium* and *Alternaria* were reported to be isolated from soybean seeds, leaves, and stems (Pimentel I.C. et al., 2006; Miller W.A., & Roy K.W., 1982), and these two genera are the most commonly isolated fungi from crops (Vujanovic V., et al., 2012). Besides *Fusarium and Alternaria, Scopulariopsis* and *Cladosporium* species screened from October soybean were also identified as oleaginous fungi in this research. *Scopulariopsis* and *Cladosporium* were also demonstrated to function as plant pathogens (Rivas S., & Thomas C.M., 2005; Lavin P. et al., 2016).

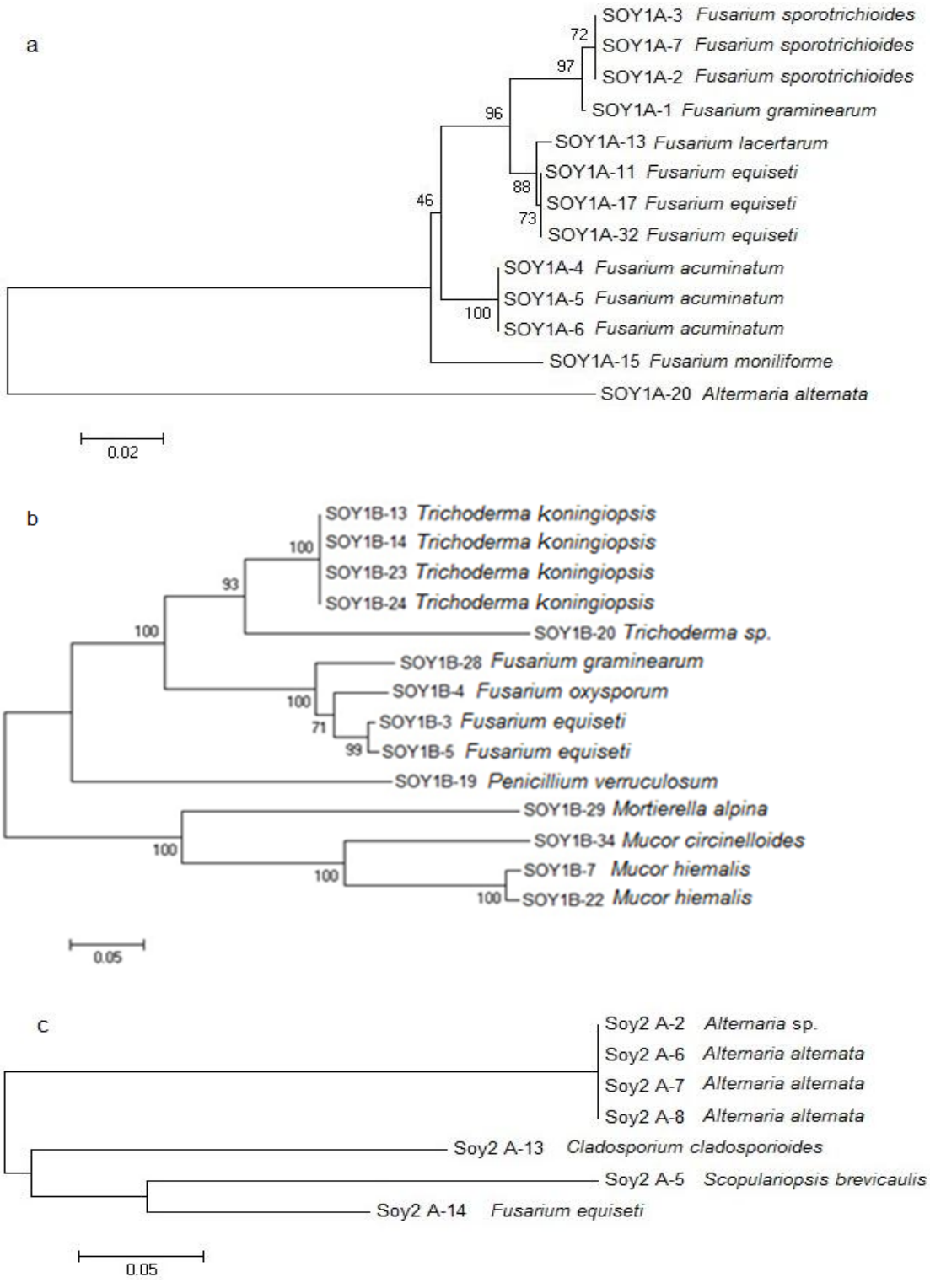

Figure 4.1. Phylogenetic tree of oleaginous fungal isolates from soybean and surrounding soil. a: August soybean samples; b: soil samples; c: October soybean samples.

For soil samples, screened fungal isolates are more diverse: the 14 fungal isolates with high lipid content belong to five different genera (Figure 4.1 b). In addition to the *Fusarium* genus, four oleaginous fungal isolates belong to the *Mucor* genus and *Mortierella* genus, including one *M. circinelloides* isolate, one *M. alpina* isolate, and two *M. hiemalis* isolates. In these two genera, many fungal species have already been recognized and studied as oleaginous microorganisms, such as *M. circinelloides*, *M. isabellina*, and *M. alpine* (Vicente G. et al., 2009; Zhang J.G., & Hu B., 2012; Mitra D. et al., 2012; Fakas S. et al., 2009; Takeno S. et al., 2005). For *M. circinelloides*, a dimorphic fungus, the U.S. Department of Energy (U.S. DOE) has decided to sequence its whole genome through the bioenergy program at the Joint Genome Institute due to its many excellent features for biodiesel production. *M. circinelloides* has a high level of lipids in the mycelium, good biomass production using a wide range of carbon sources, and a proven capacity to grow in large fermenters (Fakas S. et al., 2009; Aggelis G., 1996; Du Preez J.C. et al., 1995; Wynn J.P. et al., 2001). *M. isabellina* and *M. alpina* are two other promising fungal strains to produce lipids (especially GLA) in submerged fermentation using various agricultural waste feedstocks, for example, sweet sorghum and cheese whey (Fakas S. et al., 2009; Papanikolaou S. et al., 2004). *M. hiemalis* is also a dimorphic plant pathogen, and has been studied to generate ethanol from sweet sorghum bagasse, as well as generating polyunsaturated fatty acid (PUFA; GLA, in this case). Four isolates from soil samples were identified as *T. koningiopsis*, and these four isolates are not significant oil generators compared with other species in the screening.

Screening results for this current study supported the research work in the area of mycorrhiza, where *Fusarium, Trichoderma, Penicillium, Mucor* and *Mortierella* were also isolated from soil samples (Vujanovic V. et al., 2012; Manter D.K., & Vivanco J.M., 2007; Manici L.M., & Caputo F., 2010). Many plant-associated fungi have been found to enhance plant uptake of mineral nutrients such as phosphorus and to promote plant growth (Franken P. et al., 2007; Trillas M.I., & Segarra G., 2009). For example, *Mucor* species have been reported to accumulate phosphorus from the surrounding as the phosphorus storage and *Penicillium* strains are typical phosphatesolubilizing fungi (Wang G.H. et al., 2007). *Trichoderma* can colonize the plant root system and protect from soil-borne pathogens, decompose organic material in which the plant grows, attack other fungi in the plant's root system, and release compounds that can activate the plant's defense mechanism (Carvalho D.D.C. et al., 2011).

Among the 13 oleaginous fungal isolates from August soybean, Soy1 A-11, A-17, and A-32 were selected for further studies since they have both high biomass production and high lipid content. These three isolates belong to the same species of *F. equiseti*, but were screened from soybean samples collected in different locations. Among the 14 fungal isolates from surrounding soil, Soy1 B-7 and B-34 were selected for further studies. Soy1 B-7 was identified as *M. hiemalis* and Soy1 B-34 was identified as *M. circinelloides*. Soy1 B-3 and B-5 screened from soil and Soy2 A-14 screened from October soybean also showed good oil accumulation, but were not included in the further study because they

are the same species (*F. equiseti*) as the selected fungal isolates obtained from August soybean. Figure 4.2 shows the Sudan IV staining of lipids stored inside Soy1 A-32 fungal cells.

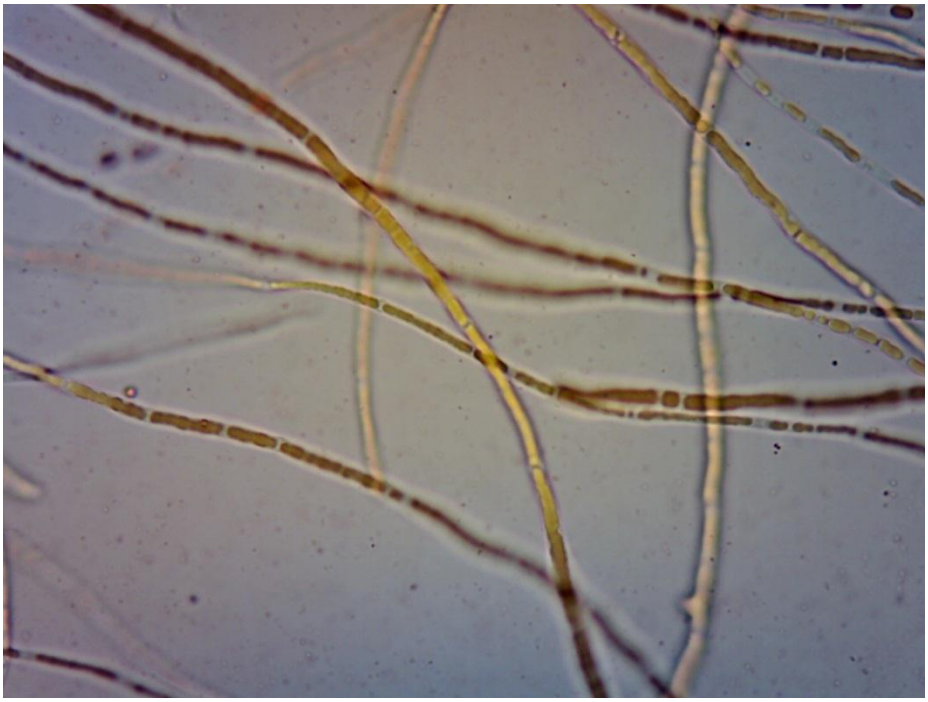

Figure 4.2. Sudan staining of fungal isolate Soy1 A-32 (Sudan IV, 1000x magnitude).

4.3.2 FAME analysis for fungal lipids

The fatty acid compositions of the three Fusarium fungal isolates screened from soybeans are quite similar, and the two *Mucor* fungal isolates screened from soils surrounding soybean roots also have similar fatty acid profile (Table 4.2). All these fungal isolates are rich in C16 and C18, and the most abundant fatty acids are palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2), which account for >90% of the total fatty acid production. From the preliminary screening results, the yield of fungal isolates to accumulate lipids is approximately 0.1 g of oil from 1 g of sugar, which

prevents its possible industrial commercialization, considering the relatively high cost of pure sugar and the low price for the lipids. To commercially utilize the fungal lipids in biodiesel production, an inexpensive source of feedstock is required for lipid accumulation, such as lignocellulosic materials from various agricultural residues, and byproducts or waste materials such as glycerol, corn steep liquor, or cheese whey. Also, if the process of fungal oil accumulation can be scaled up with bioreactors/fermenters to enhance the production and to improve the utilization of feedstock (especially waste materials), then fungal oil accumulation can potentially be a vital supplement to current biodiesel production by converting those waste materials into desirable liquid fuels.

Table 4.2. Fatty acid profile (%) in lipid produced from selected fungal isolates.

Fatty acid	Soy1 A-11	Soy1 A-17	Soy1 A-32	Soy1 B-7	Soy1 B-34
Myristic acid, C14:0	0.57	0.55	0.52	4.04	4.31
Pentadecanoic acid, C15:0	0.14	NA	NA	NA	NA
Palmitic acid, C16:0	26.06	25.11	25.77	33.83	29.69
Palmitoleic acid, C16:1	1.77	1.53	2.01	3.34	6.43
Stearic acid, C18:0	9.66	9.57	8.10	10.52	13.82
Oleic acid, C18:1	28.97	28.64	30.90	40.13	40.37
Linoleic acid, C18:2	31.12	32.81	30.96	1.95	2.22
Linolenic acid, C18:3	0.73	0.84	0.90	NA	NA
Arachidic acid, C20:0	0.57	0.58	0.52	0.81	0.53
cis-11-Eicosenoic acid, C20:1	NA	NA	NA	4.19	2.17
Behenic acid, C22:0	0.42	0.38	0.31	1.19	0.47

4.3.3 Utilization of different carbon sources

The five fungal isolates that selected for further studies showed different trends in the utilization of carbon sources. Three isolates from soybean grain (Soy1 A-11, A-17, and A-32) have a high utilization rate of various carbon sources and a good utilization rate of

cellulose. The reason for this utilization is likely that *Fusarium* species are screened out as endophytic fungi, which requires the production of cellulase to break down plant structure for their own growth (Carapito R. et al., 2008; King B.C. et al., 2011). The other two fungal isolates from soybean surrounding soil (Soy1 B-7 and B-34) did not have the ability to hydrolyze cellulose, while they can still utilize other carbon sources very well for good growth in this experiment (Table 4.3). This wide adaptation of carbon sources shows the ability of the screened fungi to utilize a variety of materials as feedstock.

Table 4.3. Carbon source utilization after 4 days culture on solid plates.

Carbon Source	Soy1 A-11	Soy1 A-17	Soy1 A-32	Soy1 B-7	Soy1 B-34
Glucose	++	++	++	++	++
Fructose	++	++	++	++	++
Galactose	++	++	++	++	++
Glycerol	++	++	++	++	++
Lactose	++	++	++	++	++
Starch	++	++	++	++	++
Mannose	++	++	++	++	++
Xylose	++	++	++	++	++
Xylan	++	++	++	++	++
Cellulose	+	+	+	-	-
Sucrose	++	++	++	++	++
Cellobiose	++	++	++	++	++

4.3.4 Cellulase enzyme activity analysis

Analysis of cellulase enzyme activity of some selected fungal isolates showed that cellulase can be secreted by these three *Fusarium* fungal isolates (Soy1 A-11, A-17, and A-32) (Table 4.4). Cellulase is one of the most important hydrolytic enzymes involved in the degradation of lignocelluloses, and the capability of cellulose degradation by these

three isolates also makes lignocellulosic material a potential carbon and nutrient sources for lipid accumulation. Unlike the current biodiesel production using plant based oil or animal oil as feedstock, the utilization of negatively valued materials will highly reduce the cost of the raw materials and helps to lower the price of biodiesel from microorganisms. Meanwhile, the two fungal isolates screened from surrounding soil (Soy1 B-7 and B-34) did not show any cellulase activity. For current industrial cellulase-producing fungi such as *Trichoderma reesei* or *Aspergillus niger*, the general cellulase activity by standard assay is approximately 1 FPU/g (Deshpande S.K. et al., 2008). Compared with those fungi, *Fusarium* fungi isolated in this research have a quite low cellulase production. However, simple prediction of the cellulase efficiency in biomass conversion based on standard cellulase enzyme activity test is inadequate (Zhang Y.H.P. et al., 2006). A better method to estimate the enzyme efficiency is to also consider the particular biomass characteristics and conduct specific assays (Kabel M.A. et al., 2006). The efficiency of cellulase must be further evaluated by feedstock, such as lignocellulosic biomass, and cellulase activity can be further increased by modification of fermentation conditions in future research to increase the overall conversion efficiency. Endophytic fungi in oilseed crops can be a rich resource to screen oleaginous strains, and these strains may primarily live on lignocelluloses. Compared to well-researched strains (*M. circinelloides* and *M. isabellina*) and other yeast and fungal strains (Table 4.5), the screened *F. equiseti* strains not only produce similar amount of lipids but also a significant amount of cellulase. Current research on *Fusarium* focuses on their pathogenesis (Di Pietro A. et al., 2003; Ortoneda M. et al., 2004; Voigt C.A. et al., 2005),

plant protection, and plant–fungi interaction (Hano C. et al., 2008; Christian D.A., & Hadwiger L.A., 1989; L'Haridon F. et al., 2011); no discussion about their lipid accumulating capability was found. This research is the first show that native strain can both generate cellulase and a high lipid content, potentially suitable for consolidated bioprocessing (CBP) as one of the promising processes for scaling-up application and commercialization.

Table 4.4. Cellulase activity of selected screened fungal isolates.

Isolate	Celllulase activity (FPU/mL)
Soy1 A-11	0.040±0.004
Soy1 A-17	0.041±0.003
Soy1 A-32	0.045±0.003
Soy1 B-7	0
Soy1 B-34	0

Table 4.5. Lipid production by oleaginous yeasts and fungi on different substrates

Species	Substrate	Lipid content (%)	Reference
Yeast			
Y. lipolytica	Industrial glycerol	42.0	Papanikolaou S., & Aggelis G., 2002
C. curvatus	Glycerol	25.0	Meesters P.A.E.P. et al., 1996
A. cuvatum	Glucose	45.6	Hassan M. et al., 1993
R. glutinis	Sugar cane molasses	39.2	Alvarez R.M. et al., 1992
R. glutinis	Glucose	66.0	Johnson V. et al., 1992
R. toruloides & *S. fibuliger*	Starch	36.5	Dostalek M., 1986
T. fermentans	Galactose	59.0	Huang C. et al., 2009
T. fermentans	Dilute sulfuric acid pretreated rice straw hydrolysate	40.1	Huang C. et al., 2009
Fungi			
A. niger	Glycerol	41.0	Andre A. et al., 2010

M. isabellina	Glucose	50.0-55.0	Papanikolaou S. et al., 2004
M. circinelloides	Acetic acid	34.4	Du Preez J.C. et al., 1995
F.equiseti	Glucose	42.8-47.6	This study

CBP is a new concept, in which biological conversion is consolidated into a single-process step that comprises cellulase production, cellulose hydrolysis, and fermentation. CBP originated from the lignocellulosic ethanol fermentation process, in which multiple foreign genes are introduced to the ethanol-producing strains so that the yeast can utilize all types of polysaccharides available in lignocellulosic biomass. Although no such strains are available yet due to the inherent technical difficulties in developing such strains with many foreign genes, CBP shows great potential. The native strains of F. equiseti screened in this research, which can both generate lipids and cellulase, are therefore promising for developing CBP. This choice of strain will be superior to the genetically modified CBP strains that are currently under development because *F. equiseti* is a wild-type strain with natural genetic stability.

4.4 Conclusions

This research was conducted to verify the hypothesis that an oil-rich plant may contain some endophytic fungi that have the ability to accumulate high oil content and that isolation of oleaginous fungi from oil-rich plants is relatively easy compared with such isolation in other circumstances. As detailed in the research approach and methodology, five fungal isolates with high-oil accumulation were screened from the soybean plant and surrounding soil. Among the 33 fungal isolates screened from August soybean plant, 13 have high lipid content (>20 % dry biomass weight). Among 38 fungal isolates screened

from surrounding soil, 14 have lipid content (>20 % dry biomass weight). Among the 17 fungal isolates screened from October soybean plant, 7 have high lipid content (>20 % dry biomass weight). From the total 34 oleaginous fungal isolates, 10 were able to accumulate content >40 % lipid. These findings suggest the strong possibility of an oil-rich plant as a good reservoir of oleaginous fungi. In addition to their lipid-accumulating capability, some oleaginous fungi also show the ability to utilize cellulose as carbon source, which provides benefits to reduce the expense of biodiesel production by using inexpensive lignocellulosic material as feedstock. For future applications, it is expected that the new promising oleaginous fungal strains isolated in this study will be applied in processes for accumulation of lignocellulosic microbial oil.

CHAPTER 5 INVESTIGATION ON THE CULTIVATION CONDITIONS OF A NEWLY ISOLATED *FUSARIUM* FUNGAL STRAIN FOR ENHANCED LIPID PRODUCTION

Outline

Fusarium equiseti UMN-1 fungal strain isolated from soybean is selected as a potential oleaginous fungal strain for biodiesel generation as in our previous studies. It has high lipid content (up to 56%) and high fatty acid methyl esters (FAME) content (more than 98%) in total lipids, and also has the capability to produce cellulase. This research focused on the investigation of the characteristics of this strain and optimization of culture conditions to enhance lipid production. Impact of temperature, agitation, light, C:N ratio, medium composition, and carbon and nitrogen sources have been investigated, and central composite design (CCD) have been applied to improve the lipid accumulation. The optimum range for temperature, agitation, C:N ratio, and carbon and nitrogen concentrations have been discovered, and the CCD model provided optimized growth medium to achieve a maximum lipid production of 3.885 g/L. The research on *F. equiseti* UMN-1 fungal strain is expected to improve the feasibility of microbial lipids as biofuels.

5.1 Introduction

Biodiesel is a renewable, clean-burning diesel replacement. It is compatible with diesel engines when mixed with petroleum diesel, and have a wide application in Europe and the US. Biodiesel is produced by the transesterification of triglycerides, which converts triglycerides into long-chain alkyl esters as biodiesel. It can be made from a diverse mix of feedstocks including recycled cooking oil, plant oil, and animal fats. But from the economic terms, these feedstocks account for more than 75% of the total production costs, making biodiesel more expensive than conventional fuels (Durrett Y.P. et al., 2008). Meanwhile, the use of plant oil in biodiesel production also leads to food vs. fuel controversy (Miao X., & Wu Q., 2006). The high costs of lipid feedstocks make research groups looking for alternative lipid source for biodiesel production, and single cell oils (SCO) have emerged as potential candidates.

SCO are the edible oils extracted from microorganisms. They are lipophilic compounds in microbial cells, produced for their cell metabolism and survival. While excessive carbon source is available due to the shortage of nitrogen or other nutrients, the cell growth becomes limited, and microbial lipids are accumulated as energy storage (Ratledge C., 1991). SCO can be the source of oils & fats that serve as raw materials for oleochemicals industries, or source of poly unsaturated fatty acids (PUFA) and other essential fatty acids (EFA). Their compositions are similar to traditional vegetable oils (Li Q. et al., 2008), and the production of SCO requires lower land resources compared with plant oil. However, SCO production largely depends on the microbial strain used for

oil accumulation. Only a small group of microorganisms were identified as oleaginous microorganism, which can accumulate high intracellular lipid content (>20% of dry cell biomass weight). And it is also revealed that biodiesel quality depends upon the fatty acid composition of the oil feedstock (Ramos M.J. et al., 2009). Both the lipid content and the types of their intracellular lipids are important criteria for microorganisms to serve as a suitable feedstock for biodiesel production.

Besides lipid content and lipid composition, fermentation substrate is also an important factor in the development of SCO. Screening of oleaginous microorganisms normally uses sugars as carbon source, but the high price of pure sugar limits its use in the industrial production of SCO. To lower the cost of substrates, the use of inexpensive waste materials and agricultural residue for SCO production has been studied (Angerbauer C. et al., 2008; Fakas S. et al., 2008; Huang C. et al., 2009; Chen X. et al., 2009). This requires oleaginous microorganisms to maintain a high lipid content during growth when utilizing these wastes or lignocellulosic materials as carbon source.

From the search of oleaginous fungi from oil-rich plants, one specific *F. equiseti* UMN-1 strain (Soy1 A-11 strain) was screened from soybean (Yang Y. et al., 2014). It is able to accumulate around 56% of lipid content when using glucose as carbon source in optimized medium, and nearly all lipids extracted from cells can be converted to FAME. This strain was shown to utilize various types of carbon sources, and showed the capability to degrade plant tissue with considerable production of cellulase. These

characteristics provide possibility for this strain to accumulate high amount of SCO on inexpensive lignocellulosic material.

Besides the selection of strains, accumulation of lipids by oleaginous fungi can also be impacted by many factors, such as the nutrient level and culture conditions (e.g. temperature, pH, carbon and nitrogen source, and agitation) (Athenstaedt K. et al., 2006; Somashekar D. et al., 2002; Jang H.D. et al., 2005). Usually under stressed conditions such as the limitation of nitrogen will lead to an enhancement of lipid production by fungi, and the excess of carbon is assimilated by the cells and converted into TAG (Ratledge C., 1991; Ratledge C., &Wynn J.P., 2002). C:N ratio is one of the most commonly studied stress factors to stimulate the oil accumulation for the oleaginous species (Sharma K.K. et al., 2012). Thus the search for suitable range of C:N molar ratio is also important to improve microbial lipid production. Cultivation conditions also greatly affect microbial lipid content and lipid composition accumulated in fungi. This research aimed to investigate the characteristics of this strain under pure culture and optimize cultivation conditions, and explore the possibility of this strain for industrial application.

5.2 Materials and Methods

5.2.1 Effect of temperature and agitation speed

F. equiseti UMN-1 strain used in this study was isolated from healthy soybean samples collected from farmland in Minnesota, USA (Yang Y. et al., 2014). In the investigation of

temperature effect, fungal hyphae were inoculated into 100 mL modified PD medium (please refer to 3.2.3) in 250 mL flasks. Flasks were cultivated at 150 rpm for 6 days at different temperatures (20, 27, 35, 42 ℃). In the investigation of agitation effect, fungal hyphae were inoculated into the same medium. Flasks were cultivated at 27 ℃ for 6 days at different agitation speeds (100, 150, 200 rpm). The biomass, lipid content, FAME content, and fatty acid profile were tested.

5.2.2 Effect of light condition

Fungal hyphae were inoculated into 100 mL modified PD medium in 250 mL flasks. Fungal cultivation was set up under three light conditions. In no light condition, flasks were fully covered with aluminum foil to prevent any light. In moderate light condition, flasks were put in a shaker incubator with transparent lid, and room light was used as the only light source. In strong light condition, flasks were put in another shaker incubator, which use fluorescent lamps to provide intense continuous light. All flasks were cultivated at 27 ℃ and 150 rpm for 6 days. The biomass, lipid content, FAME content, and fatty acid profile were tested.

5.2.3 Effect of C:N ratio

The effect of different C:N molar ratios was observed for *F. equiseti* UMN-1 strain to detect if there was any difference in lipid production. Medium used in this test contained: 0.75 g/L $(NH_4)_2SO_4$, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 2 g/L KH_2PO_4, 0.1 mg/L $CaCl_2 \cdot H_2O$, 0.1 mg/L $CoCl_2$, 0.1 mg/L $MnSO_4 \cdot H_2O$, 0.75 g/L yeast extract, and 20 g/L glucose (Zhang

J.G., & Hu B., 2012). Different C:N molar ratios were based on the change of nitrogen source concentration: 6 g/L $(NH_4)_2SO_4$+6 g/L yeast extract (C:N=5), 1.5 g/L $(NH_4)_2SO_4$+1.5 g/L yeast extract (C:N=20), 0.75 g/L $(NH_4)_2SO_4$+0.75 g/L yeast extract (C:N=40), 0.375 g/L $(NH_4)_2SO_4$+0.375 g/L yeast extract (C:N=80), 0.3 g/L $(NH_4)_2SO_4$+0.3 g/L yeast extract (C:N=100). The fungal hyphae were inoculated into 100 mL medium in 250 mL flasks and cutivated at 27 ℃ and 150 rpm for 6 days. The biomass and lipid content were tested.

5.2.4 Effect of growth medium

The optimization of growth medium for *F. equiseti* UMN-1 strain was conducted to improve lipid production. The design of different media was based on a growth medium (M3 medium) used for *Fusarium* fungi (Carapito R. et al., 2008), and compared with medium for oleaginous fungi (Zhang J.G., & Hu B., 2012). The M3 medium contained: 0.667 g/L $NaNO_3$, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 1.4 g/L KH_2PO_4, 0.68 g/L K_2HPO_4, 0.1 g/L KCl, 0.8 mg/L $MnSO_4 \cdot H_2O$, 0.4 mg/L $CuSO_4$, 0.8 mg/L $FeSO_4 \cdot 7H_2O$, 0.8 mg/L $Na_2MoO_4 \cdot 2H_2O$, 8 mg/L $ZnSO_4 \cdot 7H_2O$, 0.04 mg/L $Na_2B_4O_7 \cdot 10H_2O$, 1 g/L yeast extract, and 20 g/L glucose. Each type of medium had only one component changed, and C:N molar ratio was 40 for all media (Table 5.1). The fungal hyphae were inoculted into 100 mL medium in 250 mL falsks, and cultivated at 27 ℃ and 150 rpm for 6 days. The biomass and lipid content were tested.

Table 5.1. Different types of medium for lipid production.

Components	Type 1	Type 2	Type 3	Type 4	Type 5	Type 6
$NaNO_3$ (g/L)	**NA**	0.667	0.667	0.667	0.667	0.667
$(NH_4)_2SO_4$ (g/L)	**0.75**	NA	NA	NA	NA	NA
$MgSO_4 \cdot 7H_2O$ (g/L)	0.1	**1.5**	0.1	0.1	0.1	0.1
KH_2PO_4 (g/L)	1.4	1.4	1.4	1.4	1.4	1.4
K_2HPO_4 (g/L)	0.68	0.68	0.68	0.68	0.68	0.68
KCl (g/L)	0.1	0.1	**NA**	0.1	0.1	0.1
$CaCl_2 \cdot H_2O$ (mg/L)	NA	NA	NA	**0.1**	NA	NA
$CoCl_2$ (mg/L)	NA	NA	NA	NA	**0.1**	NA
$MnSO_4 \cdot H_2O$ (mg/L)	0.8	0.8	0.8	0.8	0.8	0.8
$CuSO_4$ (mg/L)	0.4	0.4	0.4	0.4	0.4	0.4
$FeSO_4 \cdot 7H_2O$ (mg/L)	0.8	0.8	0.8	0.8	0.8	0.8
$Na_2MoO_4 \cdot 2H_2O$(mg/L)	0.8	0.8	0.8	0.8	0.8	0.8
$ZnSO_4 \cdot 7H_2O$ (mg/L)	8	8	8	8	8	8
$Na_2B_4O_7 \cdot 10H_2O$ (mg/L)	0.04	0.04	0.04	0.04	0.04	0.04
Yeast extract (g/L)	**0.75**	1	1	1	1	1
Glucose (g/L)	20	20	20	20	20	20

Note: NA, not applicable. Type 6 was the original M3 medium.

5.2.5 Time-course growth of *F. equiseti* UMN-1 strain

The time-course growth experiment studied the physiological characteristics of the strain growth. Fungal hyphae were inoculated into 100 mL of optimized M3 medium (the Type 1 medium in 5.2.4) in 250 mL flasks, and cultivated at 27 ℃ and 150 rpm for different days (2, 4, 6, 8, 10, 12, 14 days). The biomass, lipid content, and residual sugar were tested.

5.2.6 Effect of different carbon sources and nitrogen sources

This test was to observe the effect of different carbon sources and nitrogen sources on the fungal growth and lipid production. The medium used was optimized M3 medium *F*.

equiseti UMN-1 strain was inoculated into 100 mL medium containing different carbon and nitrogen sources in 250 mL flasks, cultivated at 27 ℃ and 150 rpm for 6 days. The C:N molar ratio was approximately 40 for all medium. The biomass and lipid content were tested.

For carbon sources, medium contained 0.75 g/L $(NH_4)_2SO_4$ and 0.75 g/L yeast extract and included: 20 g/L glucose, xylose, galactose, mannose, fructose, sucrose, lactose, starch, cellulose, cellobiose, xylan, and glycerol (total C was approximately 0.667 mol/L). For nitrogen sources, medium contained 20 g/L glucose and included: 1.172 g/L $(NH_4)_2SO_4$, 1.509 g/L $NaNO_3$, 1.225 g/L $NaNO_2$, 0.71 g/L NH_4NO_3, 2.048 g/L yeast extract, 1.775 g/L proteose peptone, 0.533 g/L urea, 0.956 g/L $NaNO_3$+0.75 g/L yeast extract, 0.776 g/L $NaNO_2$+0.75 g/L yeast extract, and 0.45 g/L NH_4NO_3+0.75 g/L yeast extract (total N was approximately 0.01775 mol/L).

5.2.7 Effect of initial carbon and nitrogen concentrations

F. equiseti UMN-1 fungal hyphae were inoculated into 100 mL optimized M3 medium containing different concentrations of carbon (glucose) and nitrogen ($(NH_4)_2SO_4$ and yeast extract) compounds. Initial carbon included: 10, 20, 40, 60, 80, 100, 120, and 140 g/L glucose. Initial N included: 0.1, 0.25, 0.5, 0.75, 1, 1.25 g/L N, corresponding to 0.3 g/L $(NH_4)_2SO_4$+0.3 g/L yeast extract, 0.75 g/L $(NH_4)_2SO_4$+0.75 g/L yeast extract, 1.5 g/L $(NH_4)_2SO_4$+1.5 g/L yeast extract, 2.25 g/L $(NH_4)_2SO_4$+2.25 g/L yeast extract, 3 g/L $(NH_4)_2SO_4$+3 g/L yeast extract, and 3.75 g/L $(NH_4)_2SO_4$+3.75g/L yeast extract. Flasks

were cultivated at 27 ℃ and 150 rpm for 6 days. The biomass and lipid content were tested.

5.2.8 Central composite design (CCD) for optimized cultivation condition

The central composite design (CCD) was used to determine the optimum value of each variable that significantly affects the *F. equiseti* UMN-1 strain fungal growth and lipid production. It is one of the response surface methodologies to build a second order (quadratic) model for the response variables. Each factor would be designed with 5 coded levels ($-\alpha$, -1, 0, +1, $+\alpha$), in which α is a function of the numbers of factors. The general equation for α value is $\alpha= (2^k)^{1/4}$. In this research, the independent factors included temperature, carbon source concentration and nitrogen concentration, thus k=3 and α value in this design was 1.682. Coded levels and the actual values for the independent variables were listed in Table 5.2. The lipid production was the dependent variable (the response for CCD).

Table 5.2. Coded levels and true values of independent variables

Parameter	Variables	$-\alpha$ level	-1 level	0 level	+1 level	$+\alpha$ level
Temperature	X1	18.6 ℃	22 ℃	27 ℃	32 ℃	35.4 ℃
Glucose	X2	4.77 g/L	15 g/L	30 g/L	45 g/L	55.23 g/L
Nitrogen	X3	0.032 g/L	0.1 g/L	0.2 g/L	0.3 g/L	0.368 g/L

In Table 5.2, carbon source was glucose and nitrogen was the combination of $(NH_4)_2SO_4$ and yeast extract. 0.2 g/L N in medium required 0.6 g/L $(NH_4)_2SO_4$ and 0.6 g/L yeast extract, and other concentrations followed the same ratio. All media were prepared based

on the optimized M3 medium as above. The design of the experiment matrix followed Design Expert 8.0 (Static Made Easy, Minneapolis, MN) to acquire a random order. After the designed experiment matrix was performed, biomass and lipid content were tested to obtain the value of lipid production. Linear regression was used to obtain the predicted optimum values of each variable. Then the predicted model was validated with fungal cultivation. All fungal cultivation was carried out in 100 mL optimized M3 medium in 250 mL flasks at 150 rpm for 6 days.

5.2.9 Analytical methods

After cultivation, the culture broth was centrifuged at 9000 rpm for 5 min to obtain fungal biomass. Please refer to 3.2.3 for the detailed methods of biomass content and lipid content determination. FAME was prepared by extraction–transesterification procedure (Indarti E. et al., 2005) to analyze the fatty acid profile of lipids accumulated in fungal cells. Please refer to 4.2.5 for the detailed methods of fatty acid profile anaylsis.

The fungal culture broth after cultivation was centrifuged at 9,000 rpm for 5 min to collect supernatants for cellulase activity analysis. Cellulase activity was tested by filter paper assay (Ghose T. K., 1987), and was represented as filter paper unit per milliliter (FPU/mL). For residual sugar, the fungal culture broth was centrifuged at 9000 rpm for 5 min to collect supernatants. The residual sugar content in supernatants was analyzed by dinitrosalicylic acid (DNS) method (Miller G.L., 1959).

5.3 Results and Discussion

5.3.1 Effect of temperature and agitation speed

Results represented that the optimum temperature for *F. equiseti* UMN-1 strain was around 27 ℃. The highest lipid production was obtained at 27 ℃, with 0.22±0.02 g lipid/100mL (Figure 5.1). The highest lipid content of 49.74±0.35% was obtained at 35 ℃, but the biomass growth had a significant drop that reduced the total lipid production. No fungal growth was detected at 42 ℃, which suggests that 42 ℃ is beyond the suitable temperature range of *F. equiseti* UMN-1 strain. This is similar to a report on the impact of temperature on *F. oxysporium*, from which 30 ℃ was found as the optimum temperature and fungal growth drastically reduced below 15 ℃ and above 35 ℃ (Farooq S. et al., 2005). The FAME content in fungal lipids reached the highest ratio of 99.76% at 27 ℃, indicating that nearly all lipids produced by *F. equiseti* UMN-1 strain at this temperature can be converted to FAME. It was significantly higher than the value achieved at 20 ℃ (65.27%) and 35 ℃ (43.47%) (Table 5.3). Meanwhile, the detail FAME profile also changed with temperature. From 20 ℃ to 35 ℃, the percentage of palmitic acid (C16:0) increased from 22.86% to 38.65%, and the percentage of stearic acid (C18:0) increased from 10.09% to 22.15%. Correspondingly, the percentage of linoleic acid (C18:2) received an obvious decrease. Generally, as growth temperature increases, fungi tend to produce more saturated fatty acids in lipids while reduces the proportion of unsaturated fatty acids. The degree of fatty acids unsaturation has been found to decrease with higher temperatures in many oleaginous microorganisms like fungi, yeasts, and bacteria (Suutari M. et al., 1990; Gounot A.M., 1991; Beales N., 2004).

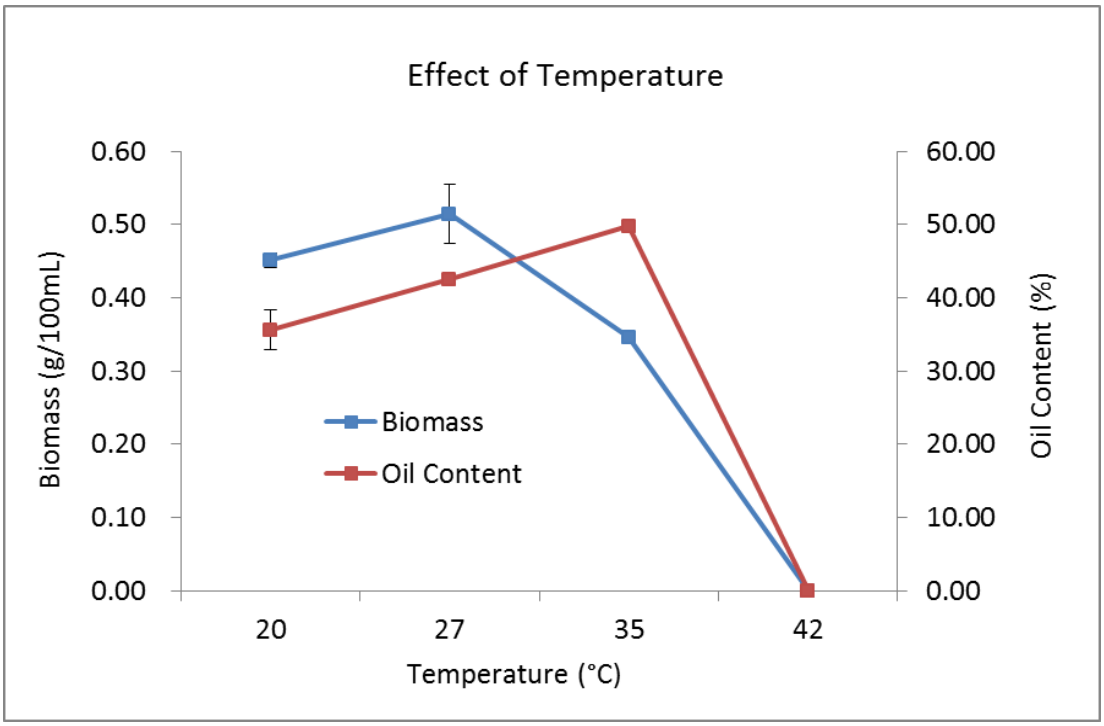

Figure 5.1. Effect of temperature on biomass and lipid content.

Table 5.3. Effect of temperature on fatty acid profile.

Fatty Acids (%)	**20 ℃**	**27 ℃**	**35 ℃**
Myristic acid C14:0	NA	0.57	0.9
Pentadecanoic acid C15:0	NA	0.14	0.36
Palmitic acid C16:0	22.86	26.06	38.65
Palmitoleic acid C16:1	1.38	1.77	0.72
Stearic acid C18:0	10.09	9.66	22.15
Oleic acid C18:1	32.41	28.97	29.48
Linoleic acid C18:2	26.11	31.12	4.98
GLA (r-Linolenic acid, C18:3)	NA	0.12	NA
Linolenic acid C18:3	6.06	0.73	NA
Arachidic acid C20:0	0.64	0.57	1.22
cis-11- Eicosenoic Acid C20:1	NA	NA	0.76
Behenic acid C22:0	0.44	0.42	0.78
FAME Content in lipids (%)	**65.72**	**99.76**	**43.47**

Temperature is one of the most influencing factors for fungal growth. Impact of temperature on lipid accumulation has also been discovered, for example, lower temperature was discovered to trigger high levels of oil production on *M. pulcherrima* (Santamauro F. et al., 2014), and the presence of stearidonic acid (C18:4) in *Mortierella elongate* at low temperature (Weinstein R.N. et al., 2000). Fungi can live in a relatively large range of temperatures, but their growth rate and metabolism are different at different temperatures, which mostly relates to chemical reactions within fungal cells. The temperature at which fungi has highest biomass growth rate is normally accepted as the optimum temperature (Carlile M.J. et al. 2001). It is considered that optimal growth temperature for oleaginous fungi is generally 20-28 ℃ (Ageitos J.M. et al., 2011; Papanikolaou S., & Aggelis G., 2011). Such optimum temperature should allow the most efficient progression of chemical reactions necessary for growth. However, in this test, the biomass growth and lipid accumulation in cells has different optimum temperature. Similar phenomenon has been reported for *Penicillium roqueforti*, that the best temperature for cell growth and substrate carbon conversion efficiency were different (Li Y. et al., 2009). This is probably due to different metabolic reactions required for cell growth and generation of secondary metabolism products. In this test, the highest lipid production for *F. equiseti* UMN-1 strain was obtained at 27 ℃ and lipid content was 42.54±0.46%. When temperature increased to 35 ℃, lipid content can increase to 49.74±0.35%. This higher lipid content could be interesting for oil production industry to consider higher culture temperature during continuous culture.

Additionally, three agitation speeds were tested to explore the impact of agitation on lipid production. Results revealed that 150 rpm agitation speed was the best on both biomass production and oil accumulation, while either higher or lower speed had negative impact compared to 150 rpm (Figure 5.2). The FAME composition was not significantly affected by the change of agitation speed, and relative percentages of each major component remained at a stable level (Table 5.4).

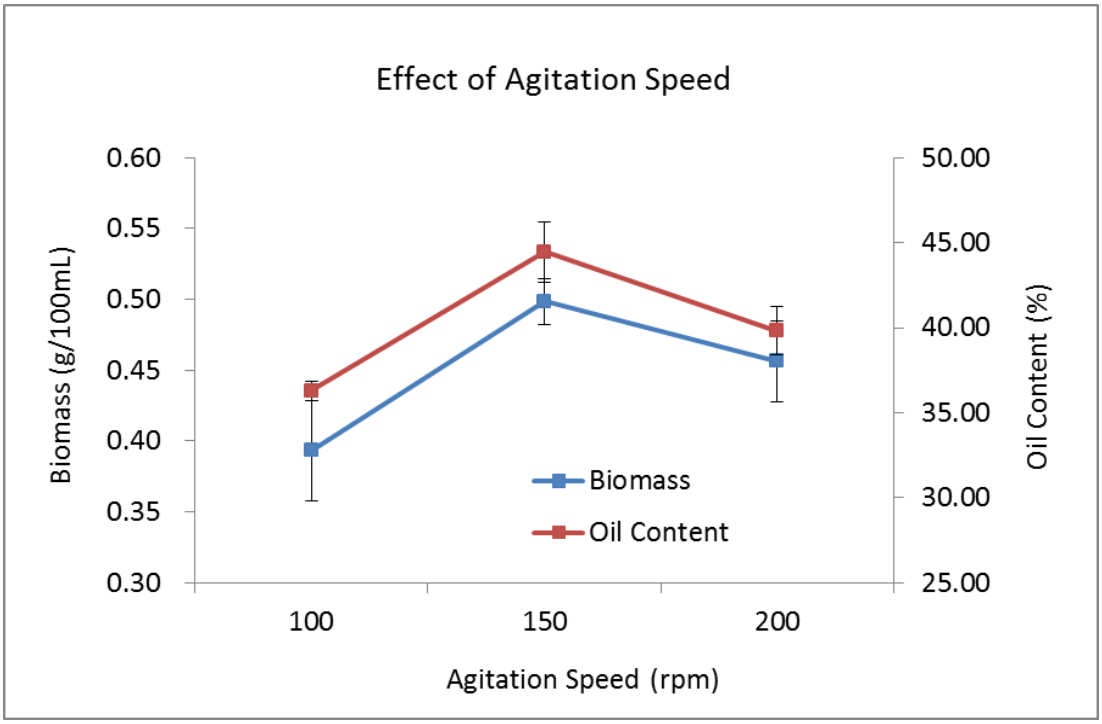

Figure 5.2. Effect of agitation speed on biomass and lipid content.

Table 5.4. Effect of agitation speed on fatty acid profile.

Fatty Acids (%)	**100 rpm**	**150 rpm**	**200 rpm**
Myristic acid C14:0	0.83	0.64	0.60
Pentadecanoic acid C15:0	0.56	0.39	0.40
Palmitic acid C16:0	38.21	34.11	36.00
Palmitoleic acid C16:1	0.97	0.97	0.97
Stearic acid C18:0	23.81	23.59	21.56

Oleic acid C18:1	22.07	22.75	24.05
Linoleic acid C18:2	8.51	13.41	11.91
GLA (r-Linolenic acid, C18:3)	NA	NA	NA
Linolenic acid C18:3	NA	0.25	NA
Arachidic acid C20:0	1.31	1.32	1.30
cis-11- Eicosenoic Acid C20:1	2.69	1.56	2.24
Behenic acid C22:0	1.04	1.00	.097
FAME Content in lipids (%)	**97.62**	**98.92**	**97.71**

The oxygen in liquid substrate affects cell growth and metabolite biosynthesis. And usually oxygen level in cuture broth is related to the cutivation modes and agitation speed. During aerobic fermentation process in bioreactor, the generation of fermentation products is limited by oxygen availability. From an investigation on L-lysine fermentation in a continuous culture, it was found that L-lysine production was strongly influenced by the dissolved oxygen level, and 50% or above of dissolved oxygen was suggested to maximize the production of lysine (Ensari S., & Lim H.C., 2003). Unlike bioreactor fermentation, external aeration is generally not applicable in flask cultivation. Due to the small surface area of growth medium in flasks, the oxygen transfer rate could be highly limited; this makes agitation a very important method to increase the dissolved oxygen in growth medium. Results in this test showed that from 100 rpm to 150 rpm, both biomass growth and oil accumulation had a significant increase, which was probably due to higher dissolved oxygen content at 150 rpm speed.

Besides increasing the oxygen content in liquid, agitation also helps to form uniform suspension of microbial cells in homogeneous medium and increase mass transfer rate. It was found that the mycelial morphology was significantly affected by agitation intensity

(Amanullah A. et al., 2002). However, higher agitation speed also leads to higher shear stress, which could be detrimental to mycelial growth (Yang F.C., & Liau C.B., 1998). During the cultivation of *F. equiseti* UMN-1 strain, both the biomass growth and oil accumulation decreased when agitation speed raised from 150 rpm to 200 rpm. Thus agitation speed around 150 rpm was most suitable for *F. equiseti* UMN-1 strain lipid production during flask cultivation.

5.3.2 Effect of light condition

Unlike the temperature and agitation that had clear impacts on intracellular oil accumulation, the effect of light did not have a clear trend. The highest biomass was achieved under moderate light condition, and the highest oil content was obtained under no light condition (Figure 5.3). For lipid production, both no light condition and moderate condition achieved around 0.22 g lipid/100mL, which was significantly higher than the value achieved at strong light condition. The FAME contents in fungal lipids were relatively constant in these three light conditions, but the detail FAME profile was different. Palmitic acid (C16:0) content at moderate light condition was 36.70%, which was higher than the content at no light condition (28.34%) and strong light condition (25.67%). Stearic acid (C18:0) content at moderate light condition (21.10%) was also higher than those at no light (16.43%) and strong light conditions (12.64%). In contrast, linoleic acid (C18:2) content (10.92%) was lower compared to those at no light (23.94%) and strong light condition (32.73%) (Table 5.5). Compared to algae using light as their energy source, fungi use light as a source of information to influence their metabolic

processes (Tisch D., & Schmoll M., 2010). There were reports claimed higher mycelia grown of *Neurospora crassa* and *Alternaria alternate* in light as compared to cultivation in darkness (Ram S. et al., 1984; Haggblom P., & Unestam T., 1979), and cultivation of *Monascus purpureus, Isaria farinosa, Emericella nidulans, Fusarium verticillioides* and *Penicillium purpurogenum* reported higher biomass, extracellular and intracellular pigment production when incubated in total darkness (Velmurugan P. et al., 2010). Meanwhile, the *velvet* gene that involved in the regulation of diverse cellular processes had been described to mediate development in response to light in *Aspergillus nidulans* (Calvo A.M., 2008), indicating that light might influence the metabolic process through the regulation of gene expression. Also studies showed that light regulated the production of some *Fusarium* secondary metabolites, such as the carotenoids, and in many species it influenced fungal morphology like the production of asexual spores and sexual fruiting bodies (Rau W., 1980; Avalos J., & Estrada A.F., 2010). But there were rare studies on the light regulation of oil accumulation by *Fusarium* fungi, and it is worth further investigation on the effect of light on *F. equiseti* UMN-1 strain lipid production.

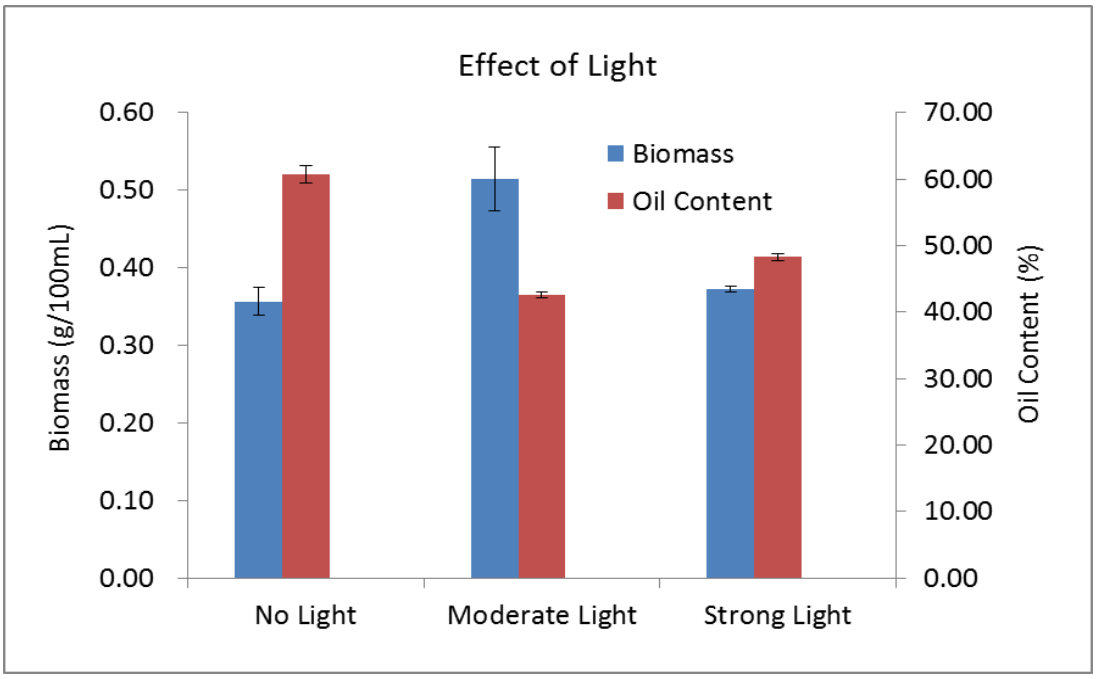

Figure 5.3. Effect of light condition on biomass and lipid content.

Table 5.5. Effect of light condition on fatty acid profile.

Fatty Acids (%)	**No Light**	**Moderate Light**	**Strong Light**
Myristic acid C14:0	0.53	0.59	0.52
Pentadecanoic acid C15:0	0.17	NA	0.19
Palmitic acid C16:0	28.34	36.70	25.67
Palmitoleic acid C16:1	1.07	0.83	1.17
Stearic acid C18:0	16.43	21.10	12.64
Oleic acid C18:1	27.41	26.72	25.22
Linoleic acid C18:2	23.94	10.92	32.73
GLA (r-Linolenic acid, C18:3)	0.07	NA	0.12
Linolenic acid C18:3	0.29	NA	0.46
Arachidic acid C20:0	0.99	1.19	0.72
cis-11- Eicosenoic Acid C20:1	NA	1.08	NA
Behenic acid C22:0	0.74	0.88	0.56
FAME Content in lipids (%)	**93.53**	**98.75**	**96.15**

5.3.3 Effect of C:N ratio

C:N molar ratio showed a siginficant impact on fungal lipid accumulation (Figure 5.4). Fungal biomass production was pretty high (1.14±0.10 g/100mL) when C:N ratio was at 5. As C:N ratio increased to 20, the biomass production decreased to 0.68±0.01 g/100mL. Further increase of C:N ratio did not reduce biomass production a lot, and in general biomass production kept at a stable level (0.62-0.69 g/100mL) from the C:N ratio of 20 to 100. Meanwhile, as C:N ratio increased from 5 to 80, lipid content increased from 12.36±1.91% to 36.45±1.51%. Beyond the C:N ratio of 80, both biomass and lipid content began to decline as at the C:N ratio of 100. Thus the optimal C:N molar ratio for *F. equiseti* UMN-1 strain was 80, at which the highest lipid production was obtained.

Since carbon (glucose) concentration was fixed at 20 g/L for all growth media, the C:N ratio actually reflected nitrogen concentration in medium. When C:N ratio was at 5, nitrogen content in medium was 6 g/L $(NH_4)_2SO_4$+6 g/L yeast extract. When increasing C:N ratio to 20, the nitrogen content in medium decreased to 1.5 g/L$(NH_4)_2SO_4$+1.5 g/L yeast extract, and such big decrease of nitrogen content lead to the siginifcant reduction of biomass production. In contrast, the nitrogen reduction when C:N ratio increased from 20 to 100 was much smaller, and correspondently a smaller reduction of biomass was received.

Lipid accumulation by oleaginous fungi mostly happens when a nutrient in the medium becomes limited and excessive carbon source is present. Under this circumstance fungal

growth is inhibited and the synthesis of protein and nucleic acid tend to cease. The excessive carbon is preferentially channeled toward lipid synthesis, leading to the accumulation of TAG within intracellular lipid bodies (Ratledge C., &Wynn J.P., 2002). Nitrogen limitation is considered as the most effective condition for most oleaginous fungal species in lipid accumulation (Rossi M. et al., 2011), and an optimally high C:N molar ratio for lipid accumulation by fungi and yeasts was reported at the range of 65 to near 100 (Weete J.D., & Weber D.J., 1980; Ageitos J.M. et al., 2011; Jin M. et al., 2015).

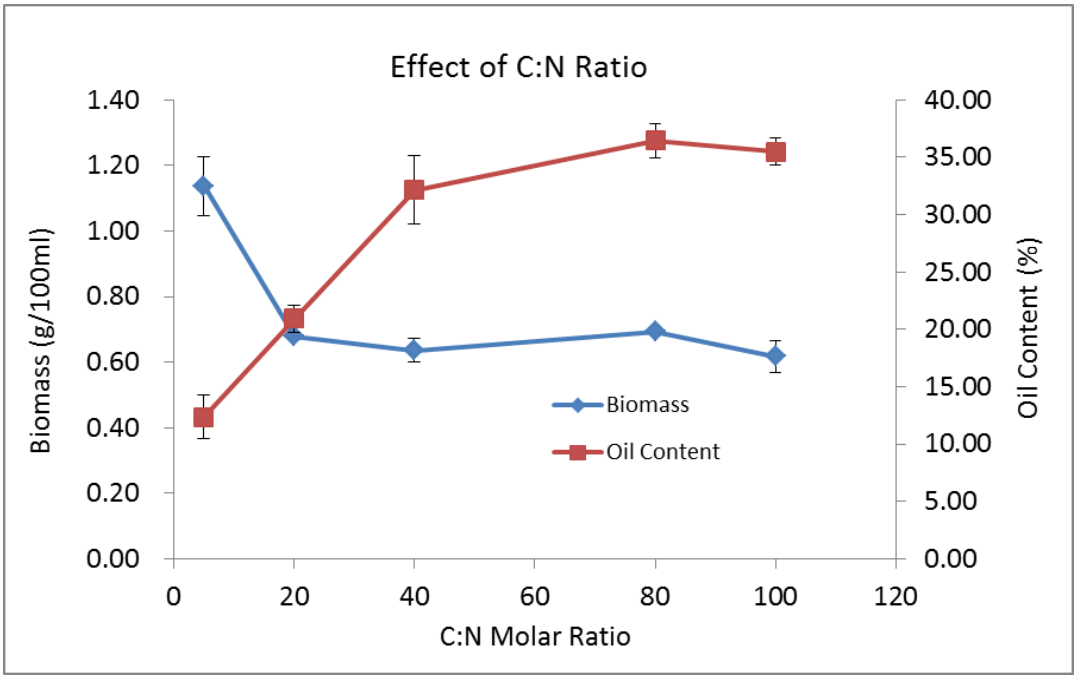

Figure 5.4. Effect of C:N ratio on biomass and lipid content.

5.3.4 Effect of growth medium

In the medium optimization, M3 medium was used as the base for improvement. M3 medium was firstly designed for cultivation of actinomycetes (Rowbotham T.J., & Cross T., 1977), but it was also found to be suitable for growth of *Fusarium* and other fungal

species (Mitchell D.B. et al., 1997; Carapito R. et al., 2008). To improve the original M3 medium for lipid production from *F. equiseti* UMN-1 strain, another growth medium for oleaginous fungi (Zhang J.G., & Hu B., 2012) was used as reference. Result showed that the best improvement of growth medium on lipid accumulation was the change of nitrogen source. While the total nitrogen content stayed at the same level, switching the inorganic nitrogen source from $NaNO_3$ to $(NH_4)_2SO_4$ (Type 1 medium) lead to a dramatic increase of both biomass and lipid content compared to original M3 medium (Type 6). The original M3 medium with $NaNO_3$ was generally good for the growth of *Fusarium* sp. fungi, but in this investigation the *F. equiseti* UMN-1 strain had better utilization of $(NH_4)_2SO_4$ than $NaNO_3$ to accumulate lipids. Increasing the concentration of $MgSO_4$ did not have an obvious impact (Type 2). And the removal of Cl^- significantly reduced both the biomass production and lipid content (Type 3), which means Cl^- is vital for this strain's growth and oil accumulation. The addition of Ca^{2+} (Type 4) or Co^{2+} (Type 5) increased the biomass production and slightly reduced lipid content, but the lipid production was about the same as the amount produced by original M3 medium. The highest lipid production was obtained with Type 1 medium, with high biomass (0.64 ± 0.02 g/100mL), high lipid content (56.24 ± 0.24%), and a lipid production of 0.360 ± 0.006 g lipid/100mL (Figure 5.5). Compared to the lipid yield of 0.182 g/g glucose from *M. isabellina* (Papanikolaou, S., et al., 2004) and 0.093 g/g glucose from *M. circinelloides* (Carvalho A.K.F. et al., 2015), the lipid yield from *F. equiseti* UMN-1 strain can reach 0.180 g/g glucose, which is no less than these two widely studied

oleaginous strains. Also, the high FAME content in intracellular further made this strain an ideal candidates for biodiesel production.

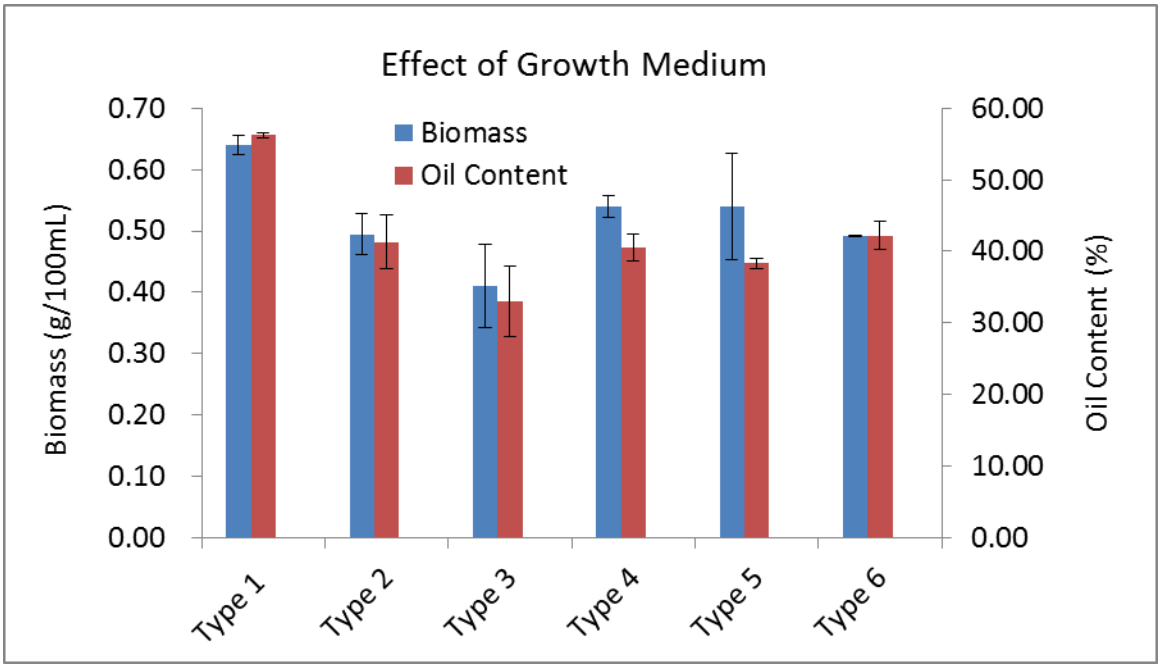

Figure 5.5. Effect of growth medium on biomass and lipid content. Note: Type 6 was the original M3 medium

5.3.5 Time-course growth of *F. equiseti* UMN-1 strain

With the improved M3 medium, the growth and lipid accumulation process of *F. equiseti* UMN-1 strain was also observed. From the growth curve (Figure 5.6), Day 4 can be considered as the turning point for fungal growth. Sugar in medium was consumed at a high rate before Day 4 and biomass production had reached a high level on Day 4. After that point, the utilization of sugar decreased to a low rate, and biomass in medium slowly increased to its peak value on Day 8. Unlike biomass content, the intracellular lipid accumulation continued to increase steadily after Day 4, and reached its maximum level of around 50% on Day 10.

It was normally recognized that lipid accumulation was triggered when cell growth was inhibited by nutrient limitation like nitrogen depletion (Ratledge C., 2002), and *F. equiseti* UMN-1 strain followed a similar pattern in this growth curve. The lipid content reached 24.94 ±0.96% on Day 2, and at that time biomass production remained a high rate. There is high possibility that nitrogen in medium was nearly depleted before Day 2, and the increase of fungal biomass after Day 2 was mainly the result of lipid accumulation in cells, but not the result of cell proliferation. The high concentration of residual carbon source on Day 2 drove the lipid synthesis at a high rate. As the carbon concentration decreases, the lipid accumulation gets more slowly. More than 80% of sugar had been consumed on Day 6, and from Day 6 to Day 10, both biomass amount and lipid content stayed at a relatively constant level. Instead of longer culture time like 2 weeks, a short cultivation time (like 6 to 8 days) would be considered to increase the lipid yield per unit of time in lipid production process. The overall growth curve of *F. equiseti* UMN-1 strain indicates this strain's good capability to utilize simple sugar and rapidly converted that to intracellular lipids, which is a favorable characteristic for oleaginous fungal oil accumulation.

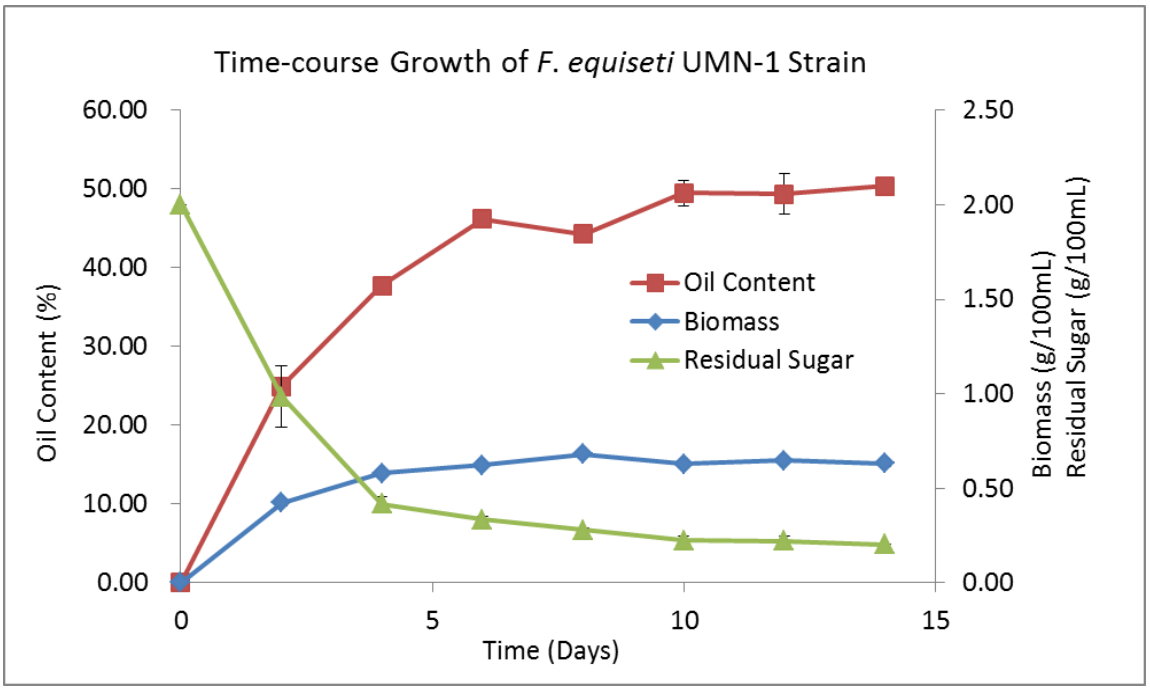

Figure 5.6. Time-course growth of *F. equiseti* UMN-1 strain in optimized M3 medium.

5.3.6 Effect of different carbon sources and nitrogen sources

The source of carbon can influence the fungal growth and lipid synthesis process; hence influence the efficiency of lipid accumulation. The results showed that *F. equiseti* UMN-1 strain can grow well on most of the carbon sources such as fructose, mannose, glucose and xylose, etc. (Figure 7). But this *Fusarium* strain had much less biomass growth when using lactose as carbon source. Some other oleaginous fungi such as *M. rouxii* and *C. echinulata* have also been described to have a poor growth on lactose (Somashekar D. et al., 2002; Chen H.C., & Chang C.C., 1996). Meanwhile, this strain was also able to synthesize a significant amount of lipids on most of the carbon sources explored in this research. Fructose and mannose lead to the highest lipid production, 0.39 ±0.00 g lipid/100mL and 0.37 ±0.01 g lipid/100mL respectively. Glucose also gave high lipid

production of 0.34±0.03 g lipid/100mL, and glucose is a more commonly used carbon source in fungal cultivation.

Since this strain was found to have the capability to produce cellulase, the cellulase activity with different carbon substrates was also tested. Xylose, galactose, starch, and cellobiose can all significantly increase cellulase activity, and xylose obtained the best lipid production (0.26±0.03 g lipid/100mL) as well as the stimulation of cellulase production. They can be added as additional nutrients in fungal culture to enhance cellulase production when lignocellulosic biomass is adapted as carbon source. On the other hand, some carbon sources such as glucose, mannose, fructose, sucrose and glycerol inhibited the cellulase production and nearly no cellulase activity was detected when *F. equiseti* UMN-1 strain grew on these substrates. The production of cellulase can be affected by the carbon source in medium and some simple carbon sources (e.g. glucose) will repress cellulase in some fungi (Niranjane A.P. et al., 2007).

Oleaginous fungi also showed their preference on the utilization of nitrogen source for growth and lipid accumulation (Figure 5.7). In the nitrogen source test, $(NH_4)_2SO_4$ was the best inorganic nitrogen source for total lipid production. Fungal growth in medium with $(NH_4)_2SO_4$ achieved higher biomass and lipid content than $NaNO_3$, $NaNO_2$ and NH_4NO_3, and obtained a lipid production of 0.27±0.00 g lipid/100mL. Urea was the best organic nitrogen source among yeast extract, peptone and urea for total lipid production, which obtained 0.25±0.03 g lipid/100mL. Moreover, the combination of inorganic

nitrogen and organic nitrogen achieved higher lipid production, and the highest lipid production was 0.34 ± 0.03 g lipid/100mL by using $(NH_4)_2SO_4$ and yeast extract. This combination was also set as nitrogen source in the optimized M3 medium for *F. equiseti* UMN-1 strain. Different fungal species may have different preference on using nitrogen compounds for growth and lipid accumulation. For example, *Mucor rouxii* had higher biomass and lipid production in medium containing KNO_3 (Somashekar D. et al., 2002), and *C. echinulata* had better growth with NH_4NO_3 and urea while KNO_3 was best for its lipid production (Chen H.C., & Chang C.C., 1996).

The source of carbon and nitrogen may have an influence on fungal growth, lipid accumulation, and also the type of fatty acids synthesized (Somashekar D. et al., 2002). In this study, *F. equiseti* UMN-1 strain showed the capability to utilize various monosaccharides, disaccharides, polysaccharides, and glycerol. The wide range of carbon source utilization is a key feature for the oleaginous microbial species. And it can use both hexose (6-C sugars) and pentose (5-C sugars). These characteristic indicated the strain's good potential to use all available sugars in lignocellulosic biomass.

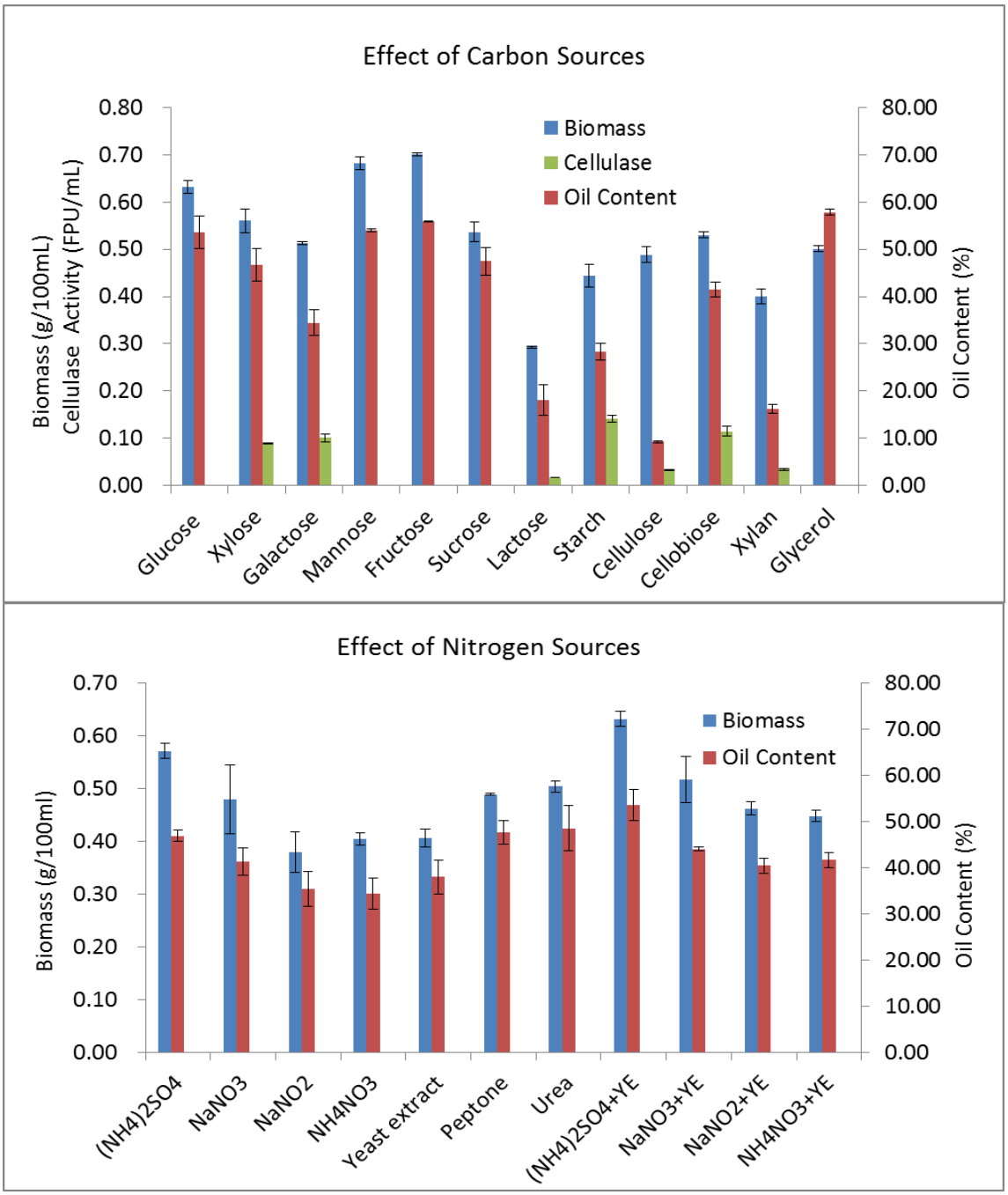

Figure 5.7. Effect of different carbon sources and nitrogen sources on biomass and lipid content.

5.3.7 Effect of initial carbon and nitrogen concentrations

The concentration of carbon/nitrogen source is also important for fungal growth and lipid accumulation. Different initial carbon and nitrogen concentrations were tested separately to explore their suitable ranges for lipid production. Glucose is the most commonly used carbon source for culturing oleaginous fungi in lab or industry. For the test of carbon concentration, nitrogen concentration was fixed with 0.75 g/L $(NH_4)_2SO_4$+0.75 g/L yeast extract and glucose concentration changed in the optimized M3 medium. When carbon source (glucose) increased from 10 g/L to 20 g/L, both fungal biomass and lipid content reached a higher level. When glucose increased beyond 20 g/L, biomass production raised steadily. On the contrary, lipid content didn't get any improvement but gradually decreased to a low level (Figure 5.8). This was probably correlated with the increasing C:N ratio as glucose increased. Lipids are generally accumulated under condition of N starvation, and higher concentrations of sugar should be easier to create such circumstance. However, there is an optimum C:N ratio range for fungal lipid generation; extremely high values of C:N ratio will not promote lipid accumulation but decline lipid content.

For the condition with 10 g/L glucose, the total lipid production was the lowest among all sugar concentrations tested. 20 g/L, 40 g/L, 60 g/L, 80 g/L and 100 g/L glucose produced around 0.20 g/100mL lipids, and 120 g/L and 140 g/L glucose produced 0.22 ± 0.02 g lipid/100mL and 0.26 ± 0.03 g lipid/100mL respectively. Although the 140 g/L glucose obtained highest lipid production, the high cost and low lipid yield of high sugar

concentration made it unsuitable for flask cultivation. The possible reason for the low lipid yield is the limitation of oxygen during batch culture in flasks. Since a large amount of carbon source was provided, significant amount of sugar were still existed in medium when nitrogen in medium was depleted. These sugars can be utilized for lipid accumulation, but oxygen is also required in the synthesis of saturated fatty acid and unsaturated fatty acids (Egner R. et al., 1993; Martin C.E. et al., 2007; Mansilla M.C., & de Mendoza D., 2005). The flask culture method had poor gas transfer efficiency and the dissolved oxygen in medium was easy to be exhausted. Bioreactor is helpful to avoid this oxygen limitation by continuous supply of air, and the utilization of bioreactor was shown to achieve high lipid content and lipid yield (Wiebe M.G. et al., 2012). When using flask culture method, a high sugar concentration will not be recommended. Results showed that a glucose concentration of around 20 g/L would be more suitable for flask culture of *F. equiseti* UMN-1 strain.

The impact of nitrogen concentration showed a similar trend. Carbon concentration was fixed with 20 g/L glucose and the increase of nitrogen source ($(NH_4)_2SO_4$+ yeast extract) beyond 0.25 g/L N lead to a steady increase of biomass and a decrease of lipid content (Figure 8). This means high levels of nitrogen will inhibit fungal lipid accumulation in cells. And more nitrogen existed in medium resulted in lower C:N ratio; extreme low C:N ratio will also reduce lipid content. 0.1 g/L N and 0.25 g/L N provided the highest lipid production of 0.26 ± 0.01 g lipid/100mL and 0.25 ± 0.01 g lipid/100mL respectively. The suitable range of nitrogen concentration should be 0.1~0.25 g/L N for this strain. In the

oleaginous fungal lipid production process, low level of glucose and/or nitrogen can become a limiting factor since there is not enough carbon and nitrogen source in the medium, and high levels of glucose and/or nitrogen will have negative effects. To guarantee carbon and nitrogen supply for a high cell-density growth in batch culture and also avoid the inhibitory effects, the suitable range of carbon and nitrogen are important parameters for *F. equiseti* UMN-1 strain lipid production.

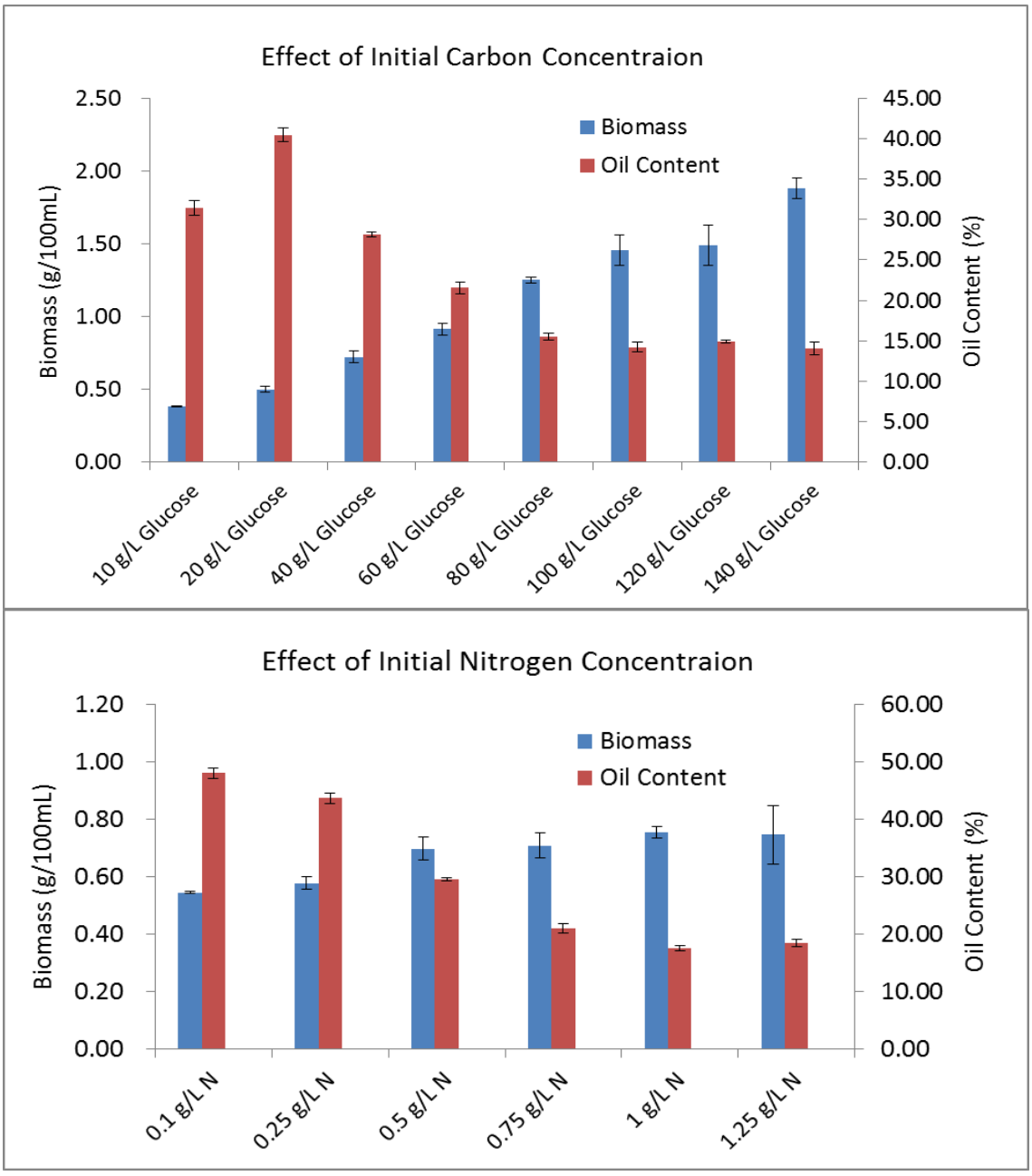

Figure 5.8. Effect of initial carbon and nitrogen concentrations on biomass and lipid content.

5.3.8 Central composite design (CCD) for optimized cultivation condition

Based on the studies of factors discussed in this experiment, a central composite design (CCD) was developed to determine the optimum values of variables that significantly affect lipid production in *F. equiseti* UMN-1 strain. The design of three independent variables (temperature, glucose concentration, and nitrogen concentration) in actual values and the response (lipid production) were presented in Table 5.6. CCD experiment results of lipid production at various conditions ranged from 0.57 g/L to 3.80 g/L. A quadratic model was proposed to calculate the optimum levels of temperature, glucose and nitrogen concentration to determine the maximum lipid production corresponding to these factors. Table 5.7 showed ANOVA results of the quadratic model. The F-test value for the overall model is 6.40, which implies the model is significant. And there is only a 0.38% chance that an F-value this large could occur due to noise. In the quadratic model, A, B, A^2, B^2, C^2 are significant model terms. The fitness of the model was checked by coefficient (R^2 = 0.8520), suggested that about 14.80% of the total variance could not be explained by this model. Multiple regression analysis on the experiment data provided the following second-order equation to explain the relationship between response variable and the tested variables:

Lipid Production = -22.35 + 1.45*Temperature + 0.23*Glucose + 39.77*Nitrogen - 0.0018*Temperature*Glucose - 0.72* Temperature*Nitrogen + 0.015*Glucose*Nitrogen - 0.026*$Temperature^2$ - 0.0026*$Glucose^2$ - 49.19*$Nitrogen^2$

Table 5.6. Central composition design (CCD) of three variables with lipid production as response value.

Std	Run	Factor 1	Factor 2	Factor 3	Response
		Temperature (X_1) (℃)	Glucose (X2) (g/L)	Nitrogen (X_3) (g/L)	Lipid Production (g/L)
1	8	22	15	0.1	2.11
2	4	32	15	0.1	1.72
3	17	22	45	0.1	2.54
4	5	32	45	0.1	1.92
5	12	22	15	0.3	2.21
6	9	32	15	0.3	0.69
7	16	22	45	0.3	3.04
8	7	32	45	0.3	0.66
9	13	18.6	30	0.2	2.88
10	2	35.4	30	0.2	0.57
11	15	27	4.77	0.2	0.40
12	19	27	55.23	0.2	3.40
13	1	27	30	0.032	1.23
14	18	27	30	0.368	3.07
15	10	27	30	0.2	3.41
16	14	27	30	0.2	3.62
17	20	27	30	0.2	3.29
18	6	27	30	0.2	3.80
19	3	27	30	0.2	3.57
20	11	27	30	0.2	3.67

Table 5.7. ANOVA for Response Surface Quadratic model for optimization of lipid production.

Source	**Sum of Squares**	**df**	**Mean Square**	**F value**	**P value Prob > F**
Model	22.03	9	2.45	6.40	0.0038
A-Temperature	5.69	1	5.69	14.88	0.0032
B-Glucose	3.07	1	3.07	8.03	0.0177
C-Nitrogen	0.14	1	0.14	0.37	0.5561
AB	0.15	1	0.15	0.38	0.5497
AC	1.04	1	1.04	2.73	0.1294
BC	3.828E-003	1	3.828E-003	0.010	0.9223
A^2	5.92	1	5.92	15.47	0.0028
B^2	4.84	1	4.84	12.66	0.0052
C^2	3.49	1	3.49	9.12	0.0129

Residual	3.82	10	0.38		
Lack of Fit	3.66	5	0.73	21.65	0.0021
Pure Error	0.17	5	0.034		
Cor Total	25.85	19			

R^2 = 0.8520; Adj-R^2 = 0.7189; Pred-R^2 = -0.9867

Besides the regression equation, the two-dimensional (2D) contour plots and three-dimensional (3D) response surface curves provided a graphical solution to identify the types of interactions between every two test variables, located the optimum ranges of variables and predicted the response value (Figure 5.9). In the present study, each figure presented the effect of two test variables on lipid production, while the third variable was fixed at the 0 level. It is obvious from the Figures 9a, 9c and 9e that the 3D response surface curves are convex in nature. The optimum values for glucose, nitrogen and temperature are all located within the ±1 level range, implying that values for temperature, glucose concentration, and nitrogen concentration are well-defined and fitted in this model.

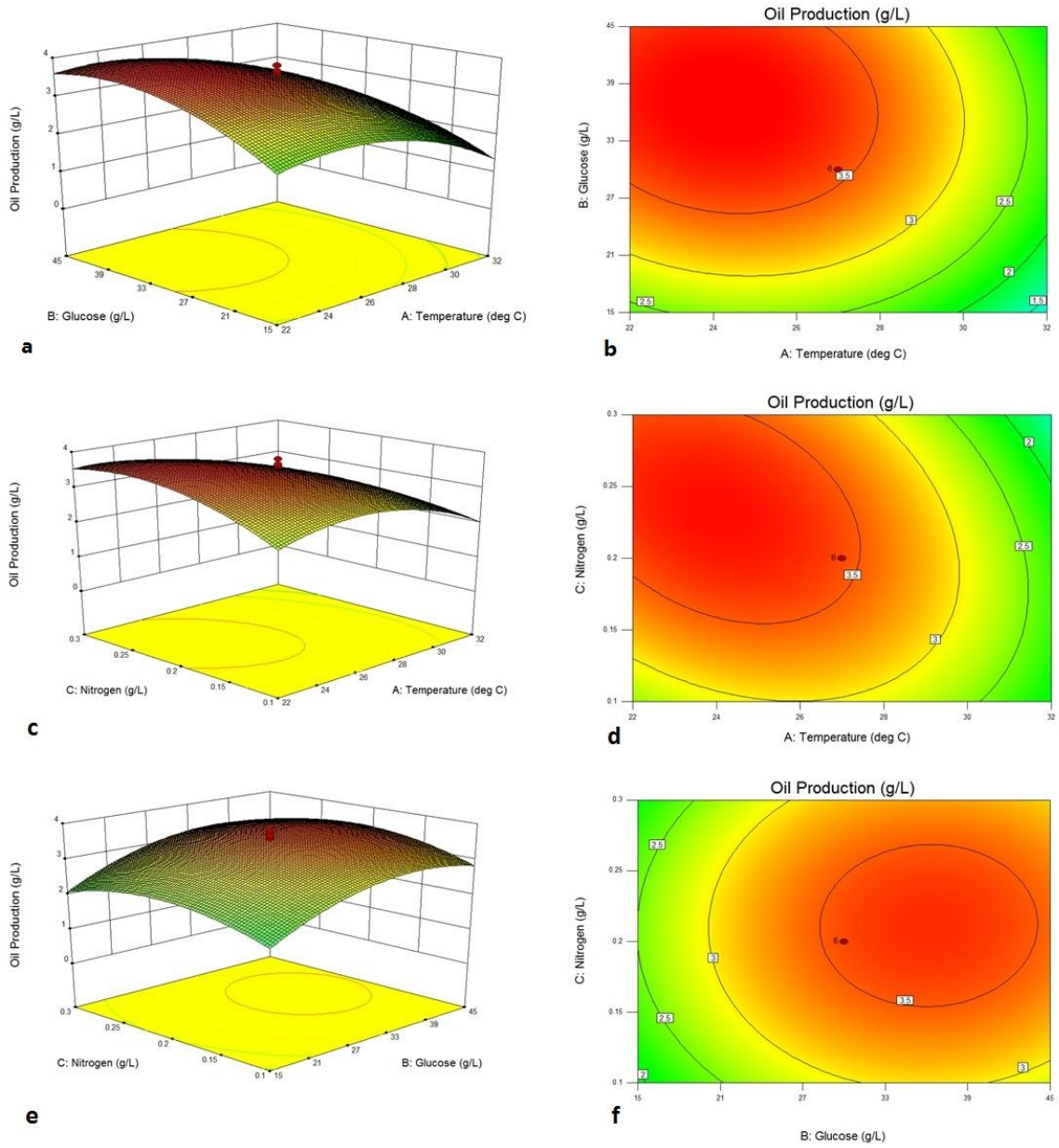

Figure 5.9. 3D-response surface curves (a, c and e) and 2D-contour plots (b, d and f) of oil production (g/L) versus the test variables (glucose, nitrogen at g/L in medium and temperature at ℃).

From the optimization result of CCD model, the optimal values of the tested variables were predicted as follows: 23.7 ℃, 37.39 g/L glucose and 0.236 g/L nitrogen. The maximum lipid production was predicted as 3.91 g/L. Validation of the experimental

design was performed by cultivation of *F. equiseti* UMN-1 strain under the above optimal condition, and an average lipid production of 3.89 g/L with 3 replicates was obtained. This validation result was in close agreement with the model-predicted response of 3.91 g/L, therefore the validation experiments confirmed the predicted values and the accuracy of the model equation.

5.4 Conclusions

This experiment explored the growth characteristic of *F. equiseti* UMN-1 strain, focused on the improvement of growth condition for production of intracellular lipids. With the high FAME percentage in fungal lipids, this strain has high potential to contribute in the renewable biodiesel production. Various factors such as temperature, agitation, light condition, carbon and nitrogen sources and growth medium were observed to improve lipid production. This strain is able to achieve a high efficiency of 0.180 g/g sugar under the optimized M3 growth medium. Then temperature, carbon concentration and nitrogen concentration were identified as the most important factors in lipid accumulation process, and a relative high lipid production of 3.89 g/L was achieved under the optimized growth medium and growth condition using CCD model. Furthermore, this strain has the capability to utilize a variety of carbon sources and generate extracellular cellulase, showed a great potential to produce economically feasible lipid using lignocellulosic biomass. As a conclusion, this *F. equiseti* UMN-1 strain is a good candidate of microbial lipid production to be used in biodiesel industry.

CHAPTER 6 MICROBIAL LIPID PRODUCTION FROM LIGNOCELLULOSIC BIOMASS BY A NEWLY ISOLATED *FUSARIUM* STRAIN

Outline

Microbial lipids can be an attractive feedstock for biodiesel production to replace the current plant-oil based biodiesel. To increase the economic feasibility of biodiesel, abundant and renewable lignocellulosic biomass has been studies to serve as the substrate for the accumulation of microbial lipids. In the biodiesel generation, oleaginous fungi have some significant advantages such as high growth rate and high lipid production. A newly isolated *Fusarium equiseti* UMN-1 strain was discovered to generate high content of lipids and also a significant amount of cellulase enzyme. In an attempt to directly utilize lignocellulosic materials by this strain for lipid accumulation, the solid state fermentation and integrated fermentation with pretreatment and cellulase hydrolysis were explored. A lipid yield of 59.1 mg/g soybean hulls and a lipid yield of 61.1 mg/g corn stover were achieved from solid state fermentation with 90% moisture content. When dilute acid pretreatment and cellulase hydrolysis were applied, the integrated fermentation of corn stover achieved a lipid yield of 69.2 mg/g corn stover. For soybean hulls, such treatment didn't improve lipid accumulation efficiency, and highest lipid yield of 54.4 mg/g soybean hulls was achieved without pretreatment or cellulase hydrolysis. This study proved the feasibility of using *F. equiseti* UMN-1 strain in lignocellulosic biodiesel production, and proper treatment of feedstocks was evaluated to enhance its lipid production.

6.1 Introduction

Biodiesel is one of the various alternative energies that attract great attention. Current biodiesel production heavily relies on plant oil thus the production cost is not competitive with fossil diesel, and there is also competition with food consumption. As a potential alternative lipid source, microbial lipids or single cell oils (SCO) from oleaginous microorganisms have been extensively studies for its application in biodiesel production. The rapid growth and high oil production of oleaginous microorganisms also provide some advantages compared to conventional oilseed crops. Numerous oleaginous fungi, yeasts, and microalgae have been reported to accumulate significant amounts of lipids similar to vegetable oils (Aggelis G., & Sourdis J., 1997; Papanikolaou S. et al., 2004; Ratlegde C., & Hopkins S., 2006; Cheng Y. et al., 2009) and methyl-esters (Metzger P., & Largeau C., 2005). The use of lignocellulosic agricultural residues to cultivate oleaginous microorganisms has been explored to lower the cost of substrates in lipid production (Huang C. et al., 2009; Galafassi S. et al., 2012; Patel A. et al., 2015; Zhong Y. et al., 2015; Cheirsilpa B., & Kitcha S., 2015).

Lignocellulosic materials are renewable and abundant biomass. The typical chemical composition of lignocellulosic materials is 48% C, 6% H, 45% O by dry weight, with the inorganic matter being a minor component (Molina S.M., & Rodr ǵuez R.F., 2004). Cellulose, hemicellulose and lignin are the three most important components in lignocellulosic materials, in which cellulose and hemicellulose can be degraded into single sugars and utilized for microbial growth. However, these sugars are not easily

accessible. Cellulose is a long polymeric chain composed of β 1→4 linked D-glucose units. In its native state, cellulose chains are held together laterally by intermolecular hydrogen bonds, and intramolecular hydrogen bonds are also formed between glucose units of the same chain (Fengel D., & Wegener G., 1984). These crystalline aggregates (microfibrils) are combined to form larger fibrils, which are the main structure of plant cell wall (Glazer A.N., & Nikaido H., 1995). Furthermore, cellulose, hemicellulose and lignin comprise a complex structure with cellulose fibers embedded in a hemicellulose and lignin polysaccharide matrix, while lignin is a phenolic polymer and cannot be hydrolyzed by microorganisms. In this case, either pretreatment of lignocellulosic biomass or a high activity of cellulase is needed to break the structure and release the polysaccharide for its utilization.

In the lignocellulosic biodiesel production, the process generally involves pretreating the biomass to loosen plant cell walls, saccharifying pretreated biomass with enzymes, fermentation of sugars into lipids by oleaginous microorganisms, and generation of methyl/ethyl esters from lipids. Two common fermentation strategies in lignocellulose-based lipid conversion are separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). Compare to SHF that separates the enzymatic hydrolysis and fermentation of sugars, SSF combines these two steps into an integrated process. For the two SHF studies using wheat straw hydrolysates as feedstock, *C. curvatus* achieved a lipid production yield of 47 mg/g wheat straw (Yu X. et al., 2011) and *M. isabellina* achieved a lipid production yield of 44 mg/g wheat straw (Zeng J. et al.,

2013). When using both liquid and solid portions from hydrolysis, *M. isabellina* was able to achieve a lipid yield of 44 mg/g from switchgrass in the SSF process (Ruan Z. et al., 2013). For a SSF study using corn stover as feedstock, a lipid yield of 81 mg/g corn stover was achieved by *C. curvatus* (Gong Z. et al., 2013). Although the production efficiency was expected to be further increased, these studies proved the feasibility of lignocellulosic lipid production.

Several oleaginous yeast and fungal strains are capable of accumulating high amount of lipids inside cells, but rare species can directly utilize lignocellulosic biomass as the substrate since the lack of cellulase producing capability. From the search of oleaginous fungi from oil-rich plants, one specific *F. equiseti* UMN-1 strain was screened from soybean (Yang Y. et al., 2014). It is able to accumulate up to 56% of oil content when using glucose as carbon source in optimized medium, and can utilize various types of carbon sources. A considerable production of cellulase was also discovered when using cellulose as carbon source in cultivation (Yang Y. et al., 2014), indicating this fungal strain has the capability to degrade lignocellulosic biomass and use the various degraded sugars for fungal growth and oil accumulation. The purpose of this study is to explore the feasibility of producing lipids from lignocellulosic biomass by this isolated fungal strain. Different fermentation processes were explored to improve lipid production. In the integrated process, the additional treatments on biomass (e.g. pretreatment and/or enzymatic hydrolysis) was also investigated to test if they were required in the utilization of lignocellulosic biomass by *F. equiseti* UMN-1 strain.

6.2 Materials and Methods

6.2.1 Microorganisms and lignocellulosic biomass

F. equiseti UMN-1 strain used in this study was isolated from healthy soybean samples collected from farmland in Minnesota, USA (Yang Y. et al., 2014). Soybean hulls samples were obtained from Minnesota Soybean Processor at Brewster, MN, and corn stover samples were collected from University of Minnesota Southern Research and Outreach Center (SROC) at Waseca, MN. These two lignocellulosic materials were ground separately into particles by an electric laboratory mill through a 30 mesh sieve, and were oven-dried at 105 ℃ for 24 hours before used in fermentation.

6.2.2 Impact of inoculum type on lipid production

Submerged fermentation was carried out with soybean hulls and corn stover by *F. equiseti* UMN-1 strain to observe the impact of two types of inocula on lipid production. 100 g mixture containing 5% (w/w) soybean hulls or corn stover was added into 250 mL flasks and autoclaved at 121 ℃ for 20 min. Then fungal hyphae of *F. equiseti* UMN-1 strain were inoculated and flasks were cultivated at 27 ℃, 150 rpm for 8 days or 12 days. Two kinds of inoculum were used: one was from the hyphae on PDA plate; the other was from the hyphae in PD medium. The eight fermentation conditions were shown in Table 6.1. When fermentation was completed, the cultivation mixture was centrifuged at 9000 rpm for 5 min and the solid was collected to test solid and lipid content.

Table 6.1. Fermentation conditions for impact of inoculum type.

Soybean hulls	Corn stover
Inoculated from plate, Day 8	Inoculated from plate, Day 8
Inoculated from liquid culture, Day 8	Inoculated from liquid culture, Day 8
Inoculated from plate, Day 12	Inoculated from plate, Day 12
Inoculated from liquid culture, Day 12	Inoculated from liquid culture, Day 12

6.2.3 Solid state fermentation of lignocellulosic biomass

To explore the feasibility of solid state fermentation on the two types of lignocellulosic biomass, a fermentation process with different moisture contents was conducted. Four initial moisture levels (75%, 85%, 90%, and 95%) were tested to explore the impact of moisture level on lipid production. Fermentation was carried out in 250 mL flasks and each flask contained 100 g mixture. Samples with 75% moisture contained 25 g oven-dried biomass and 75 mL distilled water, samples with 85% moisture contained 15 g oven-dried biomass and 85 mL distilled water, 90% and 95% moisture used 10 g dry biomass and 5 g dry biomass respectively. All flasks were autoclaved at 121 °C for 20 min before inoculation. Fungal hyphae of *F. equiseti* UMN-1 strain were inoculated and flasks were kept at room temperature (around 25 ℃) for 28 days. Samples were taken on Day 4, 8, 12, 16, 21, and 28. On each sampling date, 5 g of mixture was collected and weighted immediately as fresh sample weight, and then samples were dried to test solid and lipid content. Moisture content (%) was calculated as follows:

$$\text{Moisture content (\%)}_i = (FW_i - DW_i)/FW_i * 100\%$$

FW_i: Fresh sample weight at sampling i, i=1, 2, 3, 4, 5, 6

DW_i: Oven-dried sample weight at sampling i

The cumulative solid weight loss was measured according to the following method: Before the fermentation started, the initial weight of fermentation mixture was recorded as W_0. On the first sampling date, the weight of fermentation mixture was measured before and after sampling, recorded as $W1\alpha$ and $W_{1\beta}$ respectively. Same measurement was applied on each sampling, and the weight data $W_{2\alpha}$, $W_{2\beta}$, $W_{3\alpha}$, $W_{3\beta}$…...were obtained. The weight loss (%) from the starting point to the first sampling point was calculated using the following equation:

$$\text{Weight loss (\%)}_1 = (W_0 - W_{1\alpha})*100\%/W_0$$

And the weight loss percentage from the first sampling point to the second sampling point was calculated using the following equation:

$$\text{Weight loss (\%)}_2 = (W_{1\beta} - W_{2\alpha})*100\%/W_{1\beta}$$

Using the same method, weight loss (%) between two sampling points can be calculated. Cumulative weight loss is the sum of weight loss (%) on each specific sampling point, shown as the following equation:

$$\text{Cumulative weight loss (\%)}_i = \sum_{k=1}^{i} [\text{Weight loss (\%)}\ i]$$

The remaining weight of mixture (RW_i) was calculated by the following equation:

$$RW_i = W_0 * (1 - \text{Cumulative weight loss (\%)}_i/100)$$

And the remaining solid in flasks on each specific sampling point (SW_i) was calculated by the following equation:

$$SW_i = RW_i * (1 - M_i/100)$$

M_i: Moisture content at sampling i

And the cumulative solid weight loss (%) on each specific sampling point was calculated by the following equation:

$$\text{Cumulative solid weight loss (\%)}_i = (SW_0 - SW_i) *100\%/SW_0$$

SW_0: Initial dry weight of solid.

SWi: Solid weight at sampling i

In addition, the composition of soybean hulls and corn stover including cellulose, hemicellulose, lignin, protein, lipid, ash, C, H, and N content was analyzed (NREL 2005; NREL 2008; Folch J. et al., 1957). Scanning electron microscope (SEM) was also used to observe soybean hulls and corn stover samples before and after solid state fermentation to evaluate whether the fermentation affects lignocellulosic material structure. SEM observation was carried out as the following procedure: A layer of sample material was mounted on individual aluminum stubs with double-sided carbon adhesive tabs. The specimen stubs were sputter-coated with gold-palladium and observed in a Hitachi S3500N scanning electron microscope at an accelerating voltage of 10 kV.

6.2.4 Integrated fermentation with pretreatment and cellulase enzyme

Integrated fermentation process was conducted with some treatments (pretreatment and/or cellulase enzyme addition) for the hydrolysis of lignocellulosic materials. Four experiment conditions were applied on soybean hulls and corn stover samples as shown in Table 6.2. To supply sufficient nutrients and provide similar initial C:N molar ratio in mixture, 2 g/L KH_2PO_4 and 1 g/L $MgSO_4 \cdot 7H_2O$ were added for soybean hulls for all four conditions, and 2 g/L KH_2PO_4, 1 g/L $MgSO_4 \cdot 7H_2O$ and 1.5 g/L yeast extract were added for corn stover for all four conditions.

For pretreatment, dilute acid pretreatment was applied: 5% (w/w) soybean hulls or corn stover was mixed with 2% (v/v) sulfuric acid and soaked at room temperature for 16 h. Then the mixture was autoclaved at 121 ^{o}C for 1 h, and pH was adjusted to 5. After pH adjustment, nutrients were added. For cellulase enzyme addition, 20 U/g cellulase from *T. reesei* ATTC 26921 and 20 g/U β-glucosidase (cellobiase) from *A. niger* (Sigma-Aldrich, St. Louis, MO, USA) were added on Day 2 of fermentation.

All fermentation conditions started with 100 g mixture containing 5 g soybean hulls or corn stover, and fermentation was carried out in 250 mL flasks. After nutrient addition, flasks were autoclaved at 121 ℃ for 20 min. Then fungal hyphae of *F. equiseti* UMN-1 strain were inoculated and flasks were cultivated at 27 ℃, 150 rpm for 14 days. 6 mL of mixture was taken on Day 2, 4, 6, 8, 10, 12 and 14. Samples were filtered by Whatman 934 glass microfiber filter (1.5 μm). Liquid portion was collected for residual sugar content and the solid portion was dried to test solid and lipid content. In addition, the composition (cellulose, hemicellulose, and lignin) of solid samples after 14 days'

fermentation was tested (NREL 2005; NREL 2008). Fungal biomass in the solid samples after 14 days' fermentation was tested by glucosamine analysis method modified from Roopesh K. et al., 2006.

Table 6.2. Treatments of soybean hulls and corn stover sample

Soybean hulls	Corn stover
No pretreatment and no enzyme addition	No pretreatment and no enzyme addition
Only pretreatment	Only pretreatment
Only enzyme addition	Only enzyme addition
Both pretreatment and enzyme addition	Both pretreatment and enzyme addition

6.2.5 Analytical methods

For solid content, samples were oven-dried overnight at 105 ℃ till constant weight can be obtained. The weight of oven-dried solid (a mixture of fungal biomass and lignocellulosic material) in whole collected sample was the solid content (g/L). For lipid content test, oven-dried solid was ground into powder for lipid extraction. Around 0.3 g dry solid was used for lipid content test. Please refer to 3.2.3 for lipid content test method. The residual sugar content (g/L) in supernatant was analyzed by DNS method (Miller G.L., 1959).

6.3 Results

6.3.1 Impact of inoculum type on lipid production

To decide the inoculation type, two types of inocula (fungal hyphae on PDA plate and from liquid medium) were tested in cultivation with 100 g of 5% w/w soybean hulls or corn stover. Results indicated that inoculum of fungal hyphae from PDA plate was better

for oil accumulation (Figure 6.1). Compared to the fermentation inoculated with hyphae from liquid medium, hyphae from PDA plate achieved higher lipid content and lipid production for both soybean hulls and corn stover. Thus the fungal hyphae from PDA plate were selected as the inoculum for other experiments in this study.

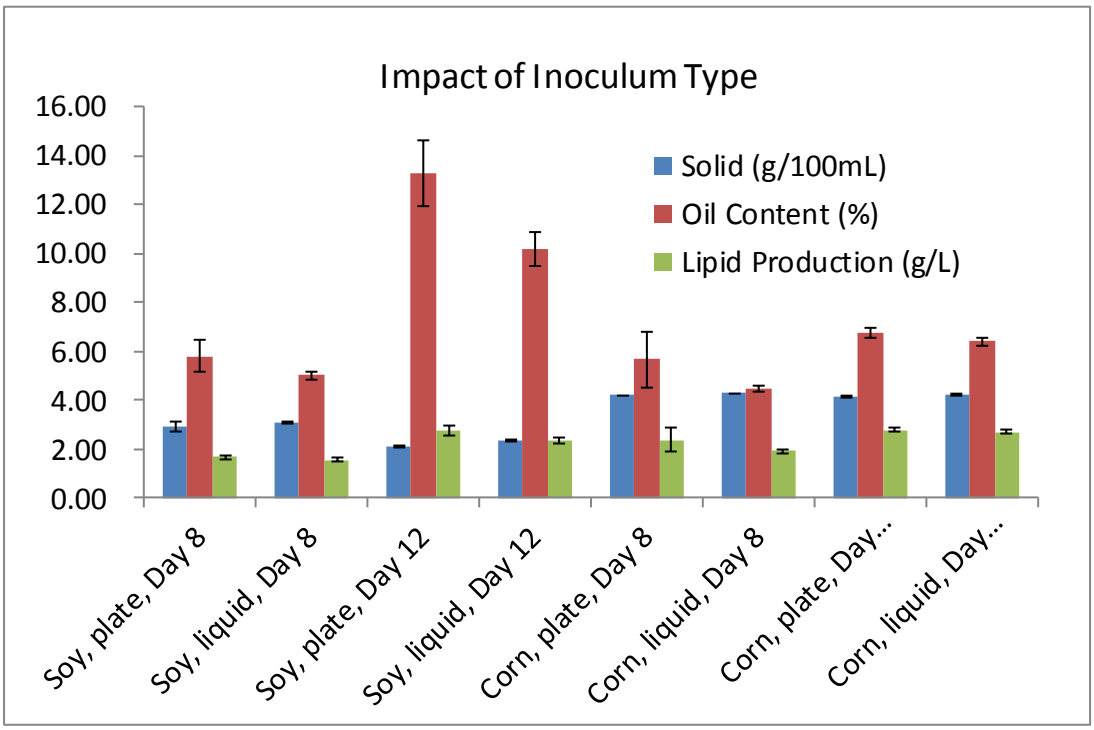

Figure 6.1. Impact of inoculum type on lipid production.

6.3.2 Solid state fermentation of lignocellulosic biomass

Evaluation of these two lignocellulosic materials was conducted by compositional analysis before fermentation (Table 6.3). Cellulose and hemicellulose content in these two materials is very similar, which accounts for more than 50% of the total dry weight. Soybean hulls samples were discovered to have higher protein content and much lower

lignin content compared with corn stover. As a by-product from soybean oil and soybean meal production, soybean hulls have been successfully utilized as an economic substitute in animal diets (Chee K.M. et al., 2005; Costa S.B.M. et al., 2012). The low lignin content also makes it an attractive source of fermentable sugars for cellulosic ethanol production (Mielenz J.R. et al., 2009). Corn stover is mainly comprised of the stalks and leaves. It has been evaluated by DOE that have great potential to produce biofuel (DOE-EERE, 2009), but the high lignin content creates a sturdy structure and increases the difficulty of its utilization.

Table 6.3. Composition of soybean hulls and corn stover raw materials.

	Soybean hulls (Raw material)	Corn stover (Raw material)
Protein%	12.54±0.28	2.34±0.07
C%	42.85±0.17	41.24±0.36
H%	5.98±0.01	5.37±0.08
N%	2.01±0.05	0.37±0.01
Lipid%	3.39±0.16	3.10±0.20
Ash%	6.31±0.11	8.20±0.44
Cellulose%	31.66±0.40	32.45±0.14
Hemicellulose%	21.08±0.68	22.72±0.49
Lignin%	6.03±0.49	21.35±0.27

The solid state fermentation showed steady weight loss with various initial moisture contents on both soybean hulls and corn stover within 28 days. Cumulative weight loss of soybean hulls on Day 28 was 27.01±1.52 ~ 33.69±2.29% for the four moisture contents, meanwhile the result on corn stover was 9.74±0.29 ~ 14.30±0.44% (Figure 6.2). The most possible reason for weight loss is the continuous degradation of these two

lignocellulosic materials by fungal strain during fermentation process to provide nutrient for fungal growth. A further evaluation of samples' ash content showed that ash content of soybean hulls with 90% moisture content increased from 6.31±0.11% to 8.66±0.24%. Under the same condition, ash content of corn stover increased from 8.20±0.44% to 10.31±0.28%. The increase of ash content indicated that the total weight of biomass was significantly reduced, which further confirmed the degradation of lignocellulosic materials during fermentation. In addition, soybean hulls had a much higher degradation rate. From the compositional analysis, cellulose and hemicellulose percentage in these two feedstocks were very close, and the most major difference was the lignin content. In soybean hulls the lignin content was 6.03±0.49%, while in corn stover the lignin content was 21.35±0.27%. Such difference could be the main reason to make soybean hulls easier to be degraded than corn stover.

During the fermentation process, lipid content increased to a relatively high level in 8 to 12 days, then remained stable or dropped to lower value. The lipid content of soybean hulls culture reached the highest value of 6.88±0.20% on Day 12 with 90% moisture content, and the lipid content of corn stover culture reached the highest value of 6.61±0.18% on Day 12 with 90 % moisture content. Similar to lipid content, the total lipid production reached a high point and then fell back to lower values. All of these 8 cultures obtained their highest lipid production before Day 12, indicating that consumption of lipids was faster than assimilation after Day 12. From the results, it was found that 90% moisture content worked best for lipid production. Under this moisture

content, soybean hulls sample reached its highest lipid yield of 59.1 mg/g biomass on Day 8, and corn stover samples reached its highest lipid yield of 61.1 mg/g biomass on Day 12.

In all four moisture contents, steady weight loss was observed in both feedstocks within 28 days. Weight loss of soybean hulls samples was much faster than corn stover samples, and difference of lignin content between these two materials was considered as the major contributing factor. Meanwhile, the weight loss percentage also varied among the four moisture contents. The highest weight loss in soybean hulls samples reached 33.69±2.29% with 95% moisture content on Day 28 while the weight loss with 75% moisture content also reached 32.90±1.70% on Day 28; and the difference between these two moisture levels was statistically insignificant. The highest weight loss in corn stover reached 14.30±0.44% with75% moisture content on Day 28, and the weight loss in corn stover steadily decreased with the increasing moisture content.

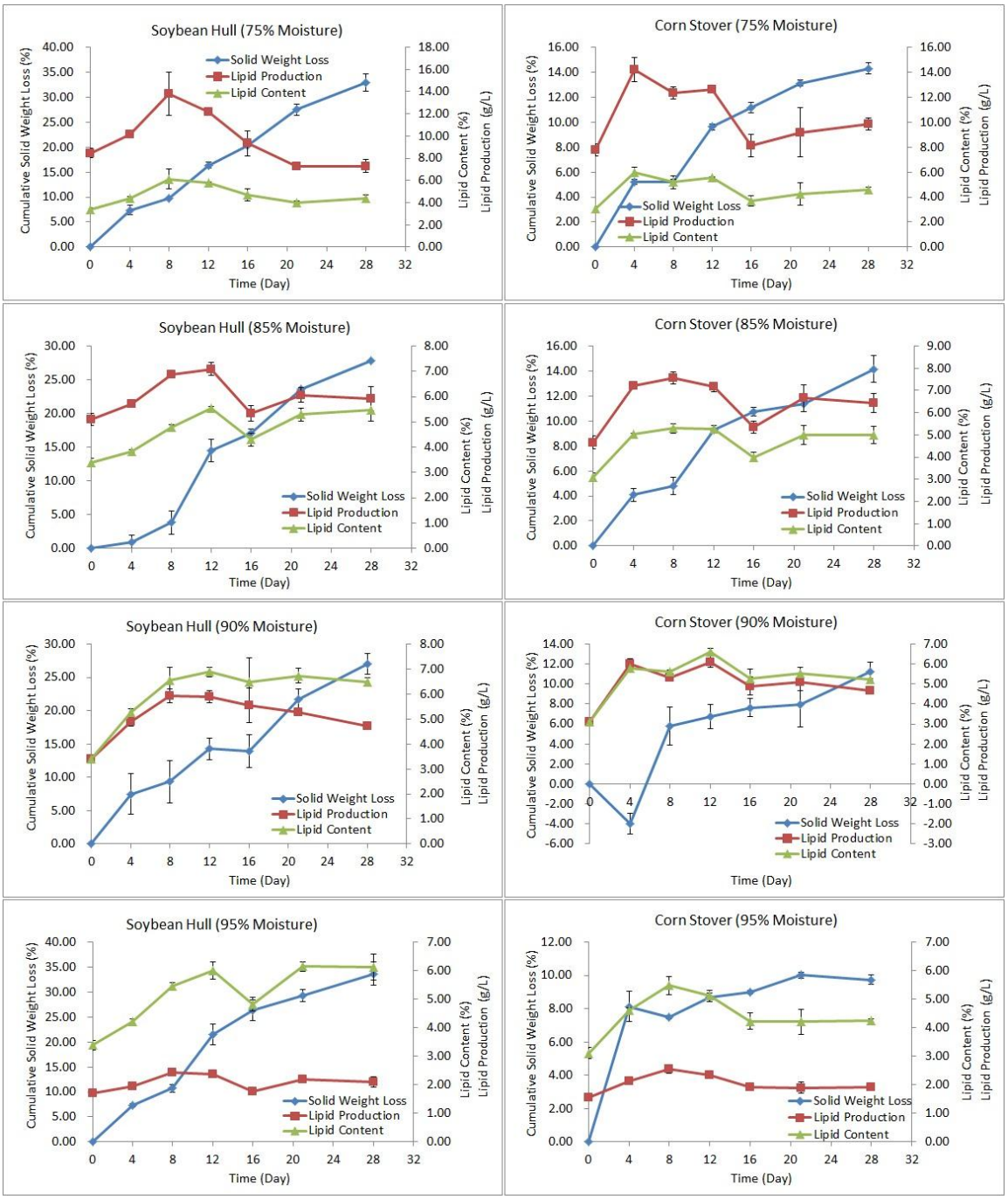

Figure 6.2. Solid state fermentation of soybean hulls and corn stover under different moisture contents.

SEM photo of soybean hulls and corn stover before and after degradation showed the lignocellulosic biomass structure. Compared figure 6.3(c) and 6.3(d) to 6.3(a) and 6.3(b), biomass was observed to have a slight degradation after autoclave. Some cracks and holes appeared on biomass surface. Compared figure 6.3(e) and 6.3(f) to 6.3(c) and 6.3(d), more degradation was observed after 28 days solid state fermentation. The overall structure was loose, and more holes were discovered on biomass surface. Before taking SEM photo, samples were oven dried at 105 ℃ overnight for preparation, so fungal hyphae growing on lignocellulosic biomass have been dried and they are probably difficult to be detected. It proved that *F. equiseti* UMN-1 strain was able to degrade these two lignocellulosic feedstocks by its own cellulase production.

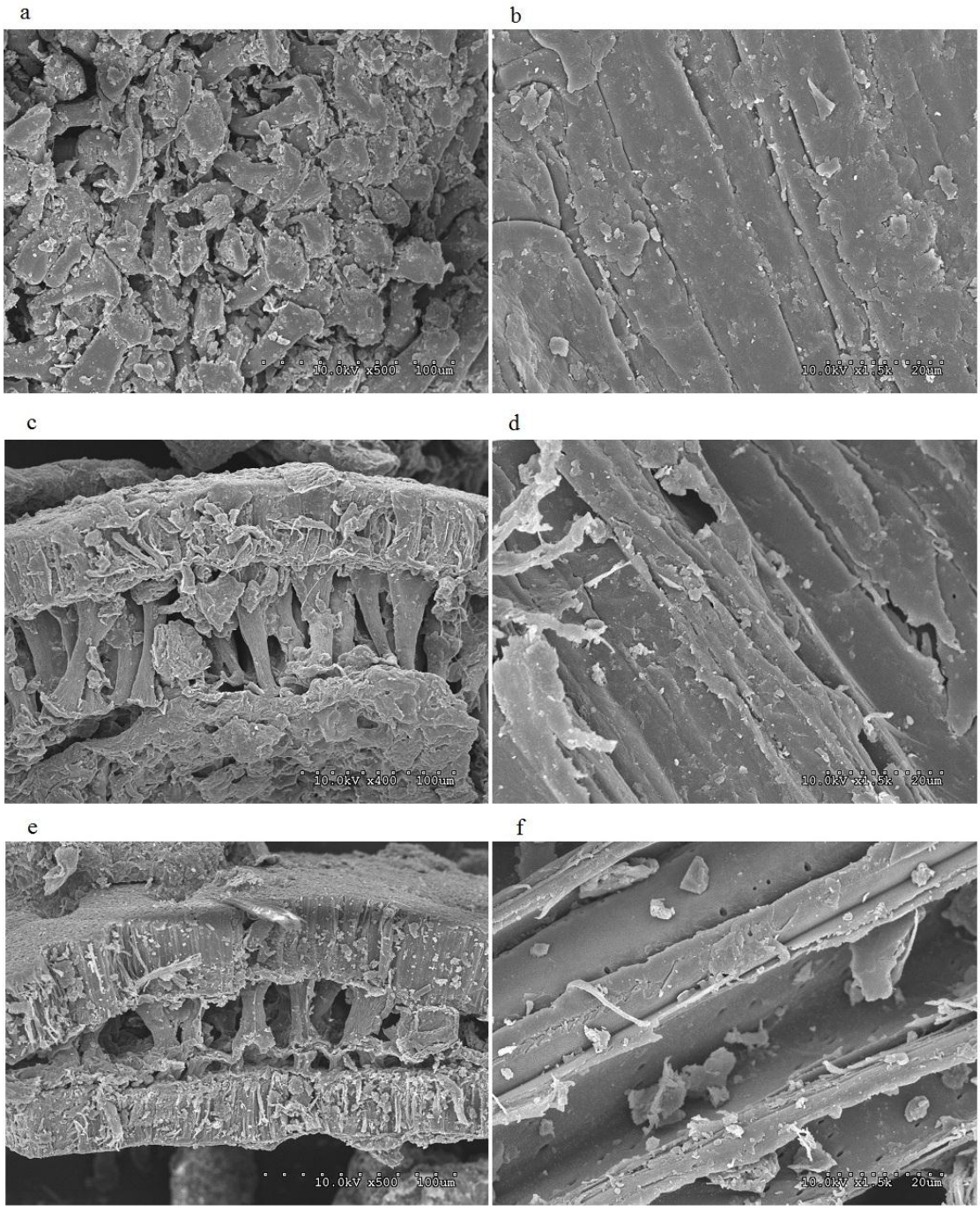

Figure 6.3. SEM photo of raw, autoclaved and fungi degraded samples. Fungi degraded samples were samples after 28 days solid state fermentation under 90% moisture content. a: soybean hulls; b: corn stover; c: autoclaved soybean hulls; d: autoclaved corn stover; e: fungi degraded soybean hulls; f: fungi degraded corn stover.

6.3.3 Integrated fermentation with pretreatment and cellulase enzyme

To test if the application of pretreatment and cellulase hydrolysis will help to improve lipid accumulation, an integrated fermentation process using 5% w/w ratio was conducted. Unlike the solid state fermentation without agitation, this fermentation process used 150 rpm agitation to accelerate mass transfer and raise dissolve oxygen content in medium. It obtained higher lipid content and lipid production than solid state fermentation with 95% moisture content. In control condition that without pretreatment or cellulase addition, both soybean hulls and corn stover received a steady reduction of solid content. Lipid content and lipid production in these two feedstocks also increased to their highest value near the end of fermentation (on Day12). The addition of cellulase enzyme (on Day 2) showed its effect to enhance lignocellulosic biomass degradation, and obtained a higher reduction of solid content and higher lipid content on Day 4 and Day 6. But after that, this enhancement became insignificant, which probably due to the gradual loss of enzyme activity. For soybean hulls and corn stover samples with cellulase enzyme addition, highest lipid production (on Day 14) was close to the highest lipid production of control samples. The addition of cellulase boosted the oil accumulation during a short time, but in long term the effect was not obvious.

When dilute acid pretreatment was applied, both these two feedstocks were partially digested. The solid content was reduced to a lower level and some sugars were already released into growth medium by dilute acid pretreatment, so the fermentation started with lower solid content, higher lipid content and higher residual sugar. In soybean hulls

samples the residual sugar was continuously consumed, solid content slightly decreased till Day 8 and then started to rise up till the end of fermentation. Lipid content gradually increased in the first 4 days, but started to decrease after that point. Although it started to increase again after Day 8, the final lipid content was still lower than control samples. However, the highest lipid production (on Day 14) was close to control samples. In corn stover samples, sugar concentration in medium slightly decreased in the first 2 days, then stopped decreasing and kept at a relatively stable level. Solid content did not have a significant change, thus the corn stover biomass was probably not largely degraded for fungal growth. Lipid content slightly increased in the first 6 days, and then dropped to a low level. The final lipid content and lipid production were close to the starting value, and highest lipid content and lipid production were achieved on Day 6. Also, the highest lipid production was close to control samples. Inhibitors generated through dilute acid pretreatment process might be the main factor that affected fungal growth and lipid accumulation.

One more tested fermentation condition was the application of both dilute acid pretreatment and enzymatic hydrolysis. In soybean hulls samples, concentration of residual sugars started to decrease from Day 4 and decreased to a low level at the end of fermentation. Solid content gradually declined till Day 12, obviously a large portion of pretreated soybean hulls samples had been hydrolyzed. Lipid content increased gradually till the end of fermentation, but the final lipid content was very close to the value obtained in control condition. Also, highest lipid production (on Day 14) was close to

control samples. In corn stover samples, concentration of residual sugars started to decrease from Day 6 and decreased to a low level at the end of fermentation. Solid content decreased in the first few days, and started to rise again from Day 8 till the end of fermentation. Lipid content generally increased from beginning to the end, and on Day 14 the lipid content was higher than the values obtained from other conditions. Highest lipid production (on Day 14) was close to control samples based on statistical analysis. The application of dilute acid pretreatment plus enzymatic hydrolysis didn't show an obvious effect on the improvement of lipid production.

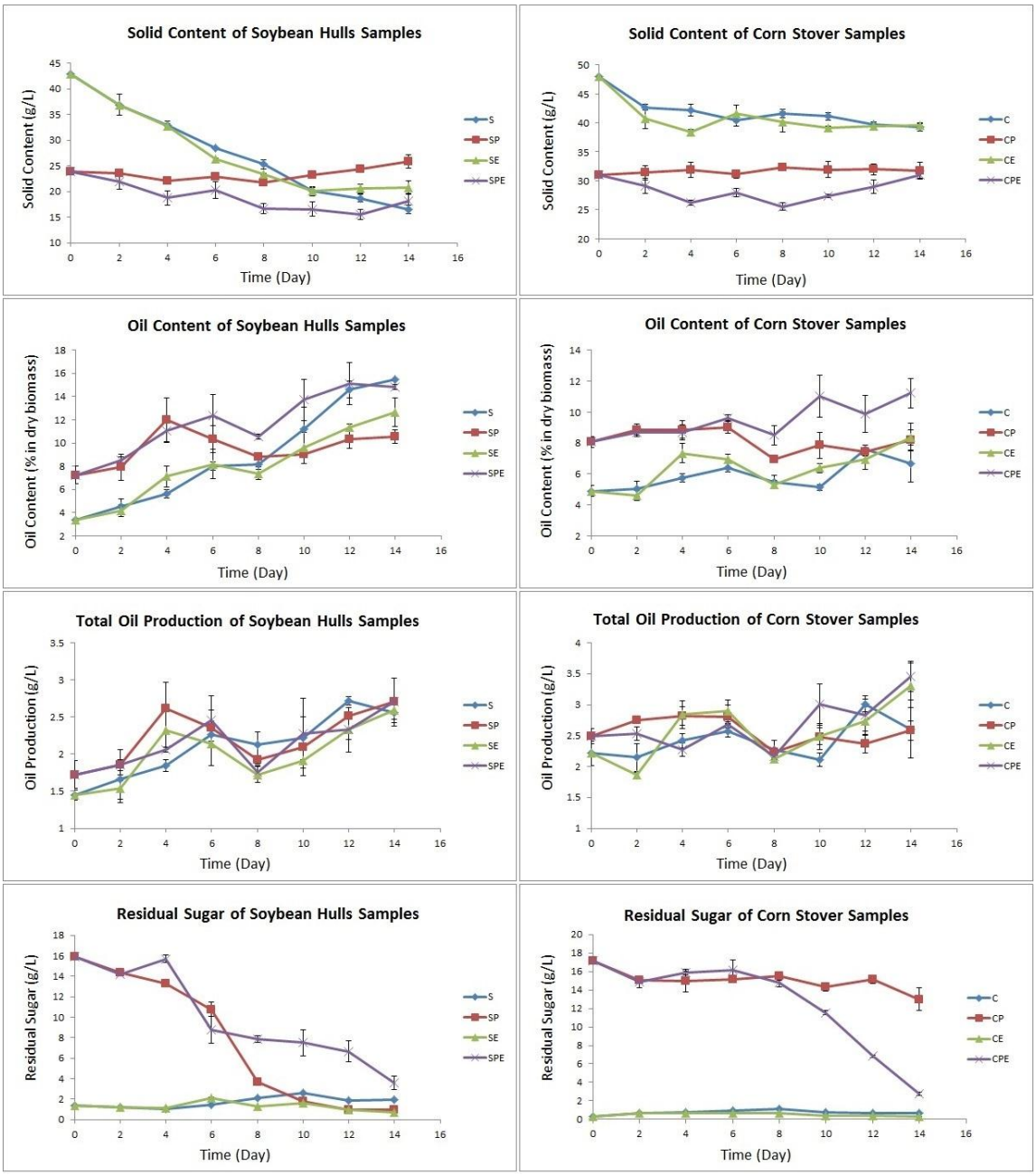

Figure 6.4. Integrated fermentation of soybean hulls and corn stover with different treatments. S: soybean hulls with no treatment (control); SP: soybean hulls with pretreatment; SE: soybean hulls with enzyme addition; SPE: soybean hulls with both pretreatment and enzyme. C: corn stover with no treatment (control); CP: corn stover with pretreatment; CE: corn stover with enzyme addition; CPE: corn stover with both pretreatment and enzyme.

Cellulose, hemicellulose, and lignin content in solid portion after 14 days fermentation have been measured to evaluate the utilization of feedstocks. In general, soybean hulls samples had higher cellulose and hemicellulose degradation. The highest cellulose degradation ratio was found in soybean hulls samples with both dilute acid pretreatment and cellulase addition, in which cellulose percentage in dry biomass dropped from $47.50 \pm 0.27\%$ to $19.16 \pm 2.41\%$ (Figure 6.5). In soybean hulls samples with no pretreatment, cellulose percentage also dropped from $36.08 \pm 0.56\%$ to $15.17 \pm 0.15\%$. Considering the rapid decline of solid content, actual cellulose degradation was close to 80% under these two conditions. In contrast, corn stover samples had much less cellulose degradation, and the highest cellulose degradation ratio was also detected when both dilute acid pretreatment and cellulase hydrolysis were applied. Hemicellulose had a similar trend that higher degradation was found in soybean hulls samples without dilute acid pretreatment. When dilute acid pretreatment was applied to soybean hulls and corn stover samples, hemicellulose was reduced to a low level during pretreatment and a minor degradation was detected during fungal fermentation. No significant lignin degradation was found in any samples, which indicated neither the *F. equiseti* UMN-1 fungus nor dilute acid pretreatment will significantly degrade lignin. In addition, the strain had better fungal growth in samples without dilute acid pretreatment for both soybean hulls and corn stover. And more fungal biomass generated from soybean hulls feedstock. Since the *Fusarium equiseti* UMN-1 strain was isolated from soybean plants, it should have better degradation capability on the original habitat that it resides.

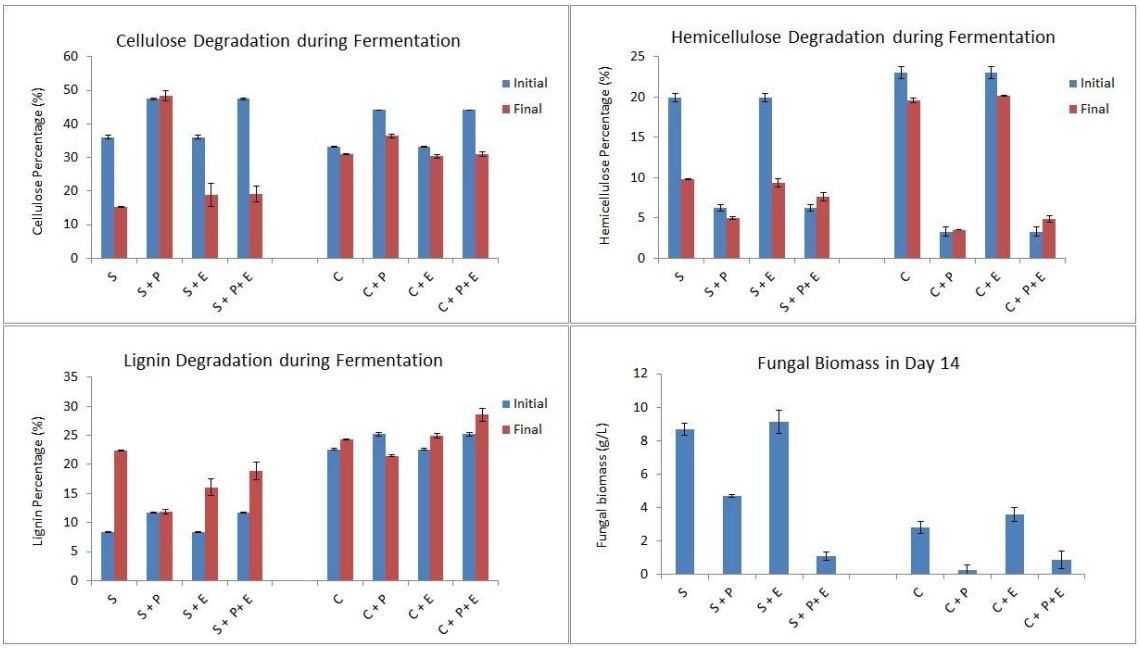

Figure 6.5. Composition and fungal biomass of samples after fermentation.

6.4 Discussion

6.4.1 Solid state fermentation of lignocellulosic biomass

Breaking the structure to release sugars is the first step in the utilization of lignocellulosic biomass. Lignin seal, accessible surface area and cellulose crystallinity are the major factors that inhibit the release of sugar in cellulosic material (Chang V. S., & Holtzapple M. T., 2000). In industrial scale, dilute acid hydrolysis is by far the most convenient method to release sugars from cellulosic material. Acid can penetrate lignin without any preliminary pretreatment of biomass to break down cellulose and hemicellulose polymers, while sulphuric and hydrochloric acids are the most commonly used catalysts for hydrolysis of lignocellulosic biomass (Lenihan P. et al., 2010). However, dilute acid hydrolysis need to be carried out at high temperatures to achieve acceptable rate of

cellulose conversion. Besides equipment corrosion, it also leads to higher rate of hemicellulose decomposition and formation of fermentation inhibitors such as furfural and 5-hydroxymethyl-furfural (HMF) (Kootstra A.M.J. et al., 2009). Compared to acid hydrolysis, enzymatic hydrolysis requires less energy and milder environment conditions (Banerjee S., et al., 2010), but it requires a pretreatment step to deconstruct lignocellulosic material and make cellulose more accessible to hydrolytic enzymes (Hendriks A., & Zeeman G., 2009). Both these steps are major economic costs of lignocellulosic biofuel (Luo L. et al., 2009; Marcuschamer D.K. et al., 2012), and it is expected to search for more cost-effective method to hydrolyze sugar from lignocellulosic materials.

With the purpose to save costs from pretreatment and hydrolysis steps, a solid state fermentation was firstly explored in this study to evaluate the efficiency of cellulose degradation and lipid production. This fermentation method provides the microorganisms an environment close to natural environment where they are isolated, and microbes normally perform well and give higher products yields compared with the liquid fermentation (Thomasa L. et al., 2013). Moisture content is one of the most important control factors in solid state fermentation. It is recognized that solute diffusion in the substrate must occur in the liquid phase, and the growth and metabolism of microorganisms in solid state fermentation always or almost occur in the liquid phase (Gervais P., & Molin P., 2003). Humid environment is preferred by most fungi, and low moisture content limits the activity of fungal inoculation. On the other side, there is a

close relationship between moisture content and oxygen availability. Like many wood degrading fungi, the *F.equiseti* UMN-1 strain is an aerobic fungus that needs oxygen in degradation process. Increase of moisture content in substrate tends to decrease oxygen availability as the inter-particular spaces become filled with water and air is forced out (Pham T.A. et al., 2010; Mekala N.K. et al., 2008; Misra A. K. et al., 2007). To optimize oxygen requirements without limiting the liquid diffusion of nutrients, it is generally beneficial to keep the level of water content just below the water holding capacity of the solid substrate (Gervais P., & Molin P., 2003). From the results of this solid state fermentation, 75% moisture content was the best condition for cellulosic material degradation, and 90% moisture content was the best condition for lipid production. This study used 250 mL Erlenmeyer flasks, where the gas exchange between flasks and outer environment was limited. If switched to better designed reactor, additional functions like mixing and oxygen supply could be helpful to further increase the lipid yield (Tengerdy R.P., & Szakacs G., 2003).

Lipid production reached its peak value in the early stage of solid state fermentation. In every moisture content of both these two feedstocks, lipid content reached a relatively high level in 8 to 12 days, and then remained stable or drop to lower value. This was an implication that cellulase produced by *F. equiseti* UMN-1 strain may not be sufficient to support a long term lipid accumulation. Intracellular lipid accumulation mostly happens when nitrogen becomes limited and excessive carbon source is present (Rossi M. et al., 2011), and enzymatic hydrolysis with low cellulase activity will be difficult to create a

carbon-rich environment for lipid accumulation. Compared to white-rot fungus *Irpex lacteus* that reached a weight loss of 21.1±1.5% on corn stover within 21 days (García-Torreiro M. et al., 2016), the highest weight loss reached by *F. equiseti* UMN-1 strain on Day 21 was significantly lower (13.13±0.27%). There is high possibility that released sugars from enzymatic hydrolysis stayed at a low concentration in the later stage of fermentation, and these sugars were majorly used in the growth and multiplying of oleaginous fungi instead of lipid accumulation.

6.4.2 Integrated fermentation with pretreatment and cellulase enzyme

Pretreatment and enzymatic hydrolysis are necessary steps in the production of lignocellulosic biodiesel. Pretreatment mainly reduces the crystallinity of lignocellulosic biomass and remove lignin to make the cellulose accessible, and enzymatic hydrolysis is aimed to break down lignocellulosic biomass and release sugars for fermentation. Compared to enzymatic hydrolysis which considers the enzyme loading as the major criterion, pretreatment methods and conditions are more complicated and significantly depend on the type of lignocelluloses that make up the lignocellulosic biomass (Taherzadeh M.J., & Karimi K., 2008). Various types of pretreatment methods are developed based on the properties of different substrates. In this research, two types of lignocellulosic biomass were tested. For soybean hulls, the differences of the highest lipid production among the four experiment conditions were statistically insignificant (5% level). The pretreatment and enzymatic hydrolysis applied in this study didn't show strong promotion on lipid production for the two feedstocks.

The optimum condition for dilute acid pretreatment was not evaluated in this study. This pretreatment method leads to a low solid content at the starting point of fermentation, because it breaks down cellulose and hemicellulose polymers and releases a large amount of sugar in medium. The high initial concentration of sugar can promote fungal growth and subsequent lipid accumulation. However, the degradation of hemicellulose also generates fermentation inhibitors (mainly acetic acid, furfural and HMF) which severely affects microbial metabolism (Huang C. et al., 2009; Ask M. et al., 2013). With inhibitors present in the fermentation broth, the *F. equiseti* UMN-1 strain fungal growth was affected for both feedstocks and the lipid production was not improved. Unlike fungal cultivation in pure growth medium that consumes most sugar rapidly in the first several days, the high concentration of released sugar in fermentation broth was slowly decreased during the fermentation process. In some extreme conditions like pretreated corn stover without enzyme addition, sugar content in fermentation broth remained at a high level till near the end of fermentation and fungal growth was inhibited. This slow utilization efficiency is not preferred for lipid accumulation. To improve the fermentation efficiency of this strain, we need to reduce the inhibitory effects during fungal fermentation. One option is to select less recalcitrant feedstocks so that mild pretreatment conditions (e.g. lower concentration of acid) can be applied and less inhibitors will be generated (Larsson S. et al., 1999). Another option is to improve pretreatment methods for less inhibitor generation. Detoxification process can be added on the existing pretreatment for efficient utilization of hydrolysates.

For both soybean hulls and corn stover samples, pretreatment enhanced lignocellulose degradation. More biomass degradation was discovered in samples with both pretreatment and enzyme compared to samples with only pretreatment. And similarly, more biomass degradation was discovered in samples with enzyme compared to samples without any treatment (control samples). The increase of degradation was higher in the groups when pretreatment was applied. This means the pretreatment can help to reduce the crystallinity of lignocellulosic biomass. It might be difficult for cellulase to degrade lignocellulose without breaking the lignin and hemicellulose structures by pretreatment, and the exploration of pretreatment on specific biomass is important to improve fermentation efficiency.

On the other side, 20 U/g cellulase and 20 U/g β-glucosidase was added in fermentation broth on Day 2. After the enzyme addition, a corresponding reduction of solid content and increase of lipid content were detected in these two feedstocks. This result indicated that enzyme can help with lignocellulose degradation in the early stage of fermentation (first 4 or 6 days). But in the long term, lipid production was not significantly improved. This may relates to the gradual loss of enzyme activity after several days of hydrolysis. In the later stage of fermentation the cellulase produced by the strain itself could be the major source of enzyme to further degrade materials for growth and lipid accumulation.

6.4.3 Prospects of lipid production by *F. equiseti* UMN-1 strain

In the generation of biofuel from lignocellulosic material, bioethanol and biodiesel are the two most attractive options. The bioethanol production briefly involves pretreatment of biomass, enzymatic hydrolysis, fermentation of sugar, and distillation of ethanol. In this complex process, several challenges exist for a cost-effective bioethanol production, including rigorous pretreatment, reduction of cellulase costs, development of more robust industrial strains, approaches for enzymatic hydrolysis and fermentation, and high capital costs associated with complex processes (Mood S.H. et al., 2013; Aditiya H.B. et al., 2016). Pretreatment and saccharification are the main costs and significantly increase the price of final product. Current microbial strains used in ethanol fermentation are majorly industrial strains of *S. cerevisiae* (Wang Y. et al., 2004; Olofsson K. et al., 2010; Jin M.J. et al., 2012), which do not have any cellulose degradation capability and make pretreatment and saccharification inevitable. Lignocellulosic biodiesel also aims to utilize sugars that released from cellulose and hemicellulose. The main process includes release of sugar from lignocellulosic material, fermentation of sugar for lipid production and transesterification of lipids for methyl/ethyl esters generation. Similar to lignocellulosic bioethanol, the major obstacle for widespread of lignocellulosic biodiesel is the lack of economically feasible technology to convert lignocellulosic material into useful lipid products. When using oleaginous fungi as lipid-producting microorganisms, one potential advantage is the secretion of cellulase from these fungal strains (Peng X., & Chen H., 2008; Hui L. et al., 2010; Roche C.M. et al., 2014). This characteristic will reduce the

requirements in pretreatment and hydrolysis steps to simplify the process and lower the price of final product.

This study explored the production of lignocellulosic biodiesel by a newly isolated strain, and it was found oleaginous fungi *F. equiseti* UMN-1 strain could degrade lignocellulosic materials and accumulate lipids by its own cellulase. From the solid state fermentation results, soybean hulls samples achieved a lipid yield of 59.1 ±2.7 mg/g feedstock with 90% moisture content in 8 days, while corn stover samples achieved 61.1 ±2.6 mg/g feedstock with 90% moisture content in 12 days (Table 6.4). In similar studies on lipid production directly using lignocellulosic feedstocks with solid state fermentation, *Microsphaeropsis* sp. was able to produce 42 mg lipid from 1g steam-exploded wheat straw and wheat bran (Peng X., & Chen H., 2008), *M. isabellina* was able to achieve a lipid yield of 47.9 mg/g soybean hulls (Zhang J.G., & Hu B., 2012), and *A. oryzae* strain achieved a lipid yield of 36.6 mg/g dry substrate of wheat straw and bran mixture (Hui L. et al., 2010). The high lipid yield of *F. equiseti* UMN-1 makes it a promising oleaginous strain than other fungal species.

From the integrated fermentation with pretreatment and cellulase enzyme results, this strain achieved a lipid yield production yield of 69.2 ±5.0 mg/g corn stover and 54.4 ±1.0 mg/g soybean hulls (Table 6.4). This integrated process received a slight increase of lipid yield than solid state fermentation with corn stover, and received a similar lipid yield to that of solid state fermentation with soybean hulls. It is believed that the inhibitors

generated from dilute acid pretreatment inhibit the metabolic activity of *F. equiseti* UMN-1 strain, thus limit its growth and lipid accumulation. In similar studies using both liquid and solid portions from hydrolysis, oleaginous yeast *T. cutaneum* was able to produce 32.3 mg lipid from 1g of corn stover (Liu W. et al., 2012); *M. isabellina* was able to achieve a lipid yield of 31.8 mg/g from corn stover and 44 mg/g from switchgrass (Ruan Z. et al., 2013). With dilution method to reduce the impact of inhibitor, a higher lipid yield of 85.8 mg/g corn stover was achieved by *M. isabellina* (Zhang J.G., & Hu B., 2014). Improvement of pretreatment method especially reducing the inhibitors is expected to enhance the fungal growth and lipid production efficiency of *F. equiseti* UMN-1 strain, making it a good candidate in lignocellulosic biodiesel production.

Table 6.4. Lipid yields of solid state fermentation and integrated fermentation from soybean hulls and corn stover.

Lipid yield (mg/g feedstock)	**Soybean hulls**	**Corn stover**
Solid state fermentation		
75% Moisture	55.2±7.8 (on Day 8)	56.8±3.8 (on Day 4)
85% Moisture	47.3±1.7 (on Day 12)	50.4±1.9 (on Day 8)
90% Moisture	59.1±2.7 (on Day 8)	61.1±2.6 (on Day 12)
95% Moisture	48.6±1.0 (on Day 8)	50.8±2.8 (on Day 8)
Integrated fermentation		
No Treatment (Control)	54.4±1.0 (on Day12)	60.2±2.6 (on Day12)
Pretreatment	54.0±0.4 (on Day14)	56.2±3.8 (on Day 6)
Enzyme Addition	52.0±2.6 (on Day14)	66.2±7.2 (on Day14)
Pretreatment + Enzyme	54.2±6.4 (on Day14)	69.2±5.0 (on Day14)

6.5 Conclusions

F. equiseti UMN-1 strain was investigated in this study for its potential application in lignocellulosic biodiesel production. In solid state fermentation, a lipid yield of 59.1±2.7

mg/g soybean hulls was obtained with 90% moisture content in 8 days and 61.1 ±2.6 mg/g corn stover was obtained with 90% moisture content in 12 days. Application of pretreatment and cellulase hydrolysis didn't achieve an improvement of lipid production from soybean hulls, but increased the lipid yield to 69.2 ±5.0 mg/g from corn stover. This result showed that *F. equiseti* UMN-1 strain was feasible to accumulate lipids from various lignocellulosic materials, and solid state fermentation could be applied to reduce the production costs. Better process and reactor design are expected to improve the final lipid yield.

CHAPTER 7 CONCLUSIONS

7.1 Summary of this research

With a long term environmental concern and economic consideration, a large amount of work has been devoted to renewable liquid fuels to partially replace the consumption of fossil fuels. Biodiesel is a potential alternative to diesel fuels, compatible with diesel engines and have a wide application in Europe and the US. This research focused on the production of microbial lipid from oleaginous fungi; explore the possibility of low-cost biodiesel from lignocellulosic feedstocks.

With this purpose, a screening of oleaginous fungi from soybean was conducted to search for promising strain. 33 isolates from August soybean sample and 17 isolates from October soybean samples were obtained, and different fungal diversity from collected August soybean samples and October soybean samples was detected. *Fusarium* and *Alternaria* were the dominant genera, and other frequently detected genera included *Penicillium*, *Nigrospora*, *Cercospora* and *Epicoccum*. Result of fungi screening suggests a change of plant associated fungi community through time, and from these isolates, several fungi (especially *Fusarium* sp.) were identified as oleaginous fungi. This screening suggests oil-rich plants are a good reservoir of oleaginous fungi.

Among the oleaginous fungi obtained from the fungi screening, one strain was named as *F. equiseti* UMN-1 strain and selected for the further study of its characteristic and potential of using lignocelluosic biomass for biodiesel generation. It has high lipid

content and high FAME content, promising oil composition, and produces cellulase. Study of this strain revealed its optimum temperature, agitation speed, light, C: N ratio, medium composition, and carbon and nitrogen sources. Based on these investigations, a CCD model was applied to optimize growth medium and cultivation conditions, and was validated to achieve a maximum lipid production of 3.89 g/L through flask cultivation. In another experiment that directly used lignocellulosic material as feedstock, this strain achieved a lipid yield of 59.1 ±2.7 mg/g from soybean hulls and 61.1 ±2.6 mg/g from corn stover through solid state fermentation (90% moisture content), and application of pretreatment and cellulase hydrolysis further increase the lipid yield to 69.2 ±5.0 mg/g from corn stover in integrated fermentation. This result showed that *F. equiseti* UMN-1 was feasible to accumulate lipids from various lignocellulosic materials. The application of sole pretreatment or sole enzyme didn't improve the lipid yield, and improvement of lipid yield from both pretreatment and addition of cellulase was statistically insignificant. More suitable pretreatment and improved design of reactor and production processes are expected to enhance the final lipid yield.

7.2 Future study

This study explored the possibility of lignocellulosic biodiesel, and isolated a new promising strain from soybean for the microbial lipids production. Microbial lipids offer a great potential for sufficient production of renewable fuels, the cultivation of fungi also can accumulate a significant amount of microbial lipid for downstream process. However, to make biofuel economically competitive with petroleum fuels, low cost substrates are

required in lipid production process to provide inexpensive carbon sources. Current solution to the substrate is lignocellulosic materials, but there are still several technical obstacles that hinder the efficient utilization of these materials. In the future study of economically feasible biodiesel, there are some areas that worth further investigation:

1. Improvement of the isolated oleaginous fungal strain

   Besides screening of oleaginous fungi from various sources, genetic modification of oleaginous microorganisms is another method to improve its lipid accumulation capability. Depending on the choice of process design, various genetic modification methods can be used. Researchers can regulate the lipid synthesis pathway to increase its lipid accumulation capability, or enhance its cellulase generation for higher cellulose degradation capability. It has been reported that UV mutated *Fusarium* sp. had an increased cellulose degradation (Makeshkumar V., & Mahalingam P.U., 2011), and similar method can also applied on the *F. equiseti* UMN-1 strain.

2. Further improvement of pretreatment technologies

   Besides strain improvement, improvement of pretreatment is another direction to increase the production rate. There is considerable economic interest in the development of processes that can pretreat inexpensive cellulosic wastes, which can provide large amount of low-cost substrate for microbial growth. Dilute acid pretreatment used in this research had a good effect on releasing of sugar from lignocellulosic materials, but generated inhibitor through the treatment process and seriously reduced the microbial lipid accumulation. The optimization of dilute acid

pretreatment and decrease of inhibitor concentration is highly expected to get better result. Besides dilute acid pretreatment, there are also various methods that haven't been used in this research including alkaline pretreatment, liquid hot water, stem explosion, AFEX, etc. Exploration of suitable pretreatment methods for specific feedstock is also essential to increase the production rate.

3. Development of downstream processes

   Lipid accumulated in microbial cells cannot be directly used as biofuels. They need further steps such as extraction and transesterification to be converted to biodiesel. Extraction process is followed after lipid production with the purpose to separate lipids from other constituents, and it is one of the most costly steps determining the economic sustainability of whole biofuel production process. After extraction, lipids can go through transesterification process to produce biodiesel, or transfer into value-added products through other process like thermal-chemical conversion. Besides lipids, oleaginous microorganism production can also provide many valuable byproducts like lubricants, emulsifier, biopolymer, plasticizers, nutraceuticals, pharmaceuticals, animal feed, and fertilizer (Dahiya A., 2015; Jin M. et al., 2015). Efficient extraction and biofuel production systems are essential components, meanwhile utilization of co-products is also an effective component to reduce the cost for better economic feasibility. This research didn't discuss the downstream processes, but these have been recognized and studied by other researchers. SCO biorefinery is a popular concept that integrates the conversion of biofuels with production of co-products to reduce costs. Further research on downstream process and collaboration

with industries are expected to further optimize the production of biofuel and co-products in a cost-effective way.

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