Sustainable production of microbial lipids from renewable biomass:
Evaluation of oleaginous yeast cultures for high yield and productivity

by

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# Abstract

Microbial lipids derived from oleaginous yeasts are a promising alternative source of edible oils due to the following advantages: no requirement of broad lands; availability of year-round production; and no food versus fuels controversy. Oleaginous yeast has an inherent ability to accumulate lipids inside cells and their lipids are preferable as starting materials in oleo-chemical industries because of their distinct fatty acid composition. Lignocellulosic biomass is a promising substrate to supply carbon sources for oleaginous yeast to produce lipids due to the high content of polysaccharides and their abundancy. Lignocellulosic-based sugar streams, which can be generated *via* pretreatment and enzymatic hydrolysis, contained diverse monosaccharides and inhibitors. The major objectives of this study were: 1) to develop a novel purification method to generate clean sugar stream using sorghum stalks after acid pretreatment; 2) to optimize fermentation conditions for *Trichosporon oleaginosus* to achieve high yields and productivity of microbial lipids using lignocellulosic hydrolysates; 3) to investigate the potentials of sorghum stalks and switchgrass as feedstocks for microbial lipid production using oleaginous yeast strains, such as *T. oleaginosus, Lipomyces starkeyi, and Cryptococcus albidus*; 4) to develop an integrated process of corn bran based-microbial lipids production using *T. oleaginosus*; and 5) to develop bioconversion process for high yields of lipids from switchgrass using engineered *Escherichia coli*.

In our investigation, major inhibitory compounds of lignocellulosic hydrolysates induced by pretreatment were acetic acid, formic acid, hydroxymethyl furfural (HMF) and furfural. The activated charcoal was effective in removing hydrophobic compounds from sorghum stalk hydrolysates. Resin mixtures containing cationic exchangers and anionic exchangers in 7:3 ratio at pH 2.7 completely removed HMF, acetic acid, and formic acid from sorghum stalk hydrolysates.

*T. oleaginosus* was a robust yeast strain for lipid production. In the nitrogen-limited synthetic media, total 22 g/L of lipid titers were achieved by *T. oleaginosus* with a lipid content of 76% (w/w). In addition, *T. oleaginosus* efficiently produced microbial lipids from lignocellulosic biomass hydrolysates. The highest lipid titers of 13 g/L lipids were achieved by *T. oleaginosus* using sorghum stalk hydrolysates with a lipid content of 60% (w/w). *L. starkeyi and C. albidus* also successfully produced microbial lipids using lignocellulosic hydrolysate with a lipid content of 40% (w/w). Furthermore, corn bran was a promising feedstock for microbial lipid production. The highest sugar yields of 0.53 g/g were achieved from corn bran at the pretreatment condition of 1% acid and 5% solid loading. Microbial lipids were successfully produced from corn bran hydrolysates by *T. oleaginosus* with lipid yields of 216 mg/g. Engineered *E. coli* also effectively produced lipids using switchgrass as feedstocks. *E. coli* ML103 *pXZ18Z* produced a total of 3.3 g/L free fatty acids with a yield of 0.23 g/g. The overall yield of free fatty acids was 0.12 g/g of raw switchgrass and it was 51 % of the maximum theoretical yield. This study provided useful strategies for the development of sustainable bioconversion processes for microbial lipids from renewable biomass and demonstrated the economic viability of a lignocellulosic based-biorefinery.

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# List of Abbreviations

ACC: acetyl-CoA carboxylase

ACL: ATP-citrate lyase

ARA: arachidonic acid

DAG: diacylglycerol

DGAT: diacylglycerol acyltransferase

DHA: docosahexanoic acid

EPA: eicosapentaenoic acid

FAS: fatty acid synthetase

FFAs: free fatty acids

FAME: fatty acid methyl ester

G3P: glycerol-3-phosphate

GLA: γ-linolinic acid

GPAT: glycerol-3-phosphate acyltransferase

HMF: 5-hydroxymethylfurfural

LCAs: life cycle assessments

LPAAT: lysophosphatidic acid acyltransferase

MFS: metabolic flux analysis

TAG: triacylglycerides

TE: acyl-ACP thioesterase

TY: theoretical yields

PA: phosphatidic acid

PUFAs: polyunsaturated fatty acids

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# Dedication

To my parents, Lee Soon-ki and Oh Jung-hee, my sister, Lee Jae-eun
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# Chapter 1 - Sustainable production of lignocellulose-based microbial lipids

## *Microbial lipids*

Bio-based lipids produced by oleaginous microorganisms are promising feedstocks for use in the oleochemical and biodiesel industry. Microbial lipids are primarily composed of triacylglycerides (TAG), which can be easily converted to biodiesel fatty acid methyl esters (FAME) *via* a transesterification process (Figure 1.1). Also, phospholipids, sphingolipids, sterols, and free fatty acids can be produced but the amount is insubstantial (Probst et al., 2015).

Microbial lipids were known to have a similar composition of fatty acids with plant oils containing high oleic acid (Table 1.1). Normally, plant oil is composed of 55% oleic acid and 20% linoleic acid (Sitepu et al., 2014). Microbial lipid composition varies depending on the species, but oleic acid is the most predominant fatty acid species produced by oleaginous microorganisms. Oleic acid is preferred in the biodiesel industry because of its improvement of cold-flow properties, ignition quality, ideal melting point, kinematic viscosity, and oxidative stability (Matsakas et al., 2014; Zhou et al., 2016; Steen et al., 2010; Knothe, 2008). Other than utilization in the biodiesel industry, microbial lipids can be alternative starting materials in the oleochemical industry, used to convert invaluable oleochemicals *via* chemical or biological synthesis. In addition, a few studies attempted to develop oleaginous strains to produce microbial lipids for food additives, since specific polyunsaturated fatty acids (PUFAs) can be produced *via* metabolic engineering. Homologous or heterologous expression of specific enlongases or desaturases in oleaginous microorganisms are capable of synthesizing essential fatty acids such as γ-linolenic acid (GLA), arachidonic acid (ARA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA), which

are in high demand in the food industry (Huang et al., 2013) (Table 1.2). The attention of PUFA — usually provided from seafood oil and vegetable oil — is an essential oil in human metabolism to prevent cardiovascular and inflammatory diseases, brain disorders, and diabetes (Béligon et al., 2016). Also, such PUFAs are known to contribute to the brain development of infants, proper functioning of the eye, synthesis of hormones, and signaling of molecules (SanGiovanni and Chew, 2005). As their nutritional importance has led to increased demand, PUFAs synthetized by oleaginous microorganisms are anticipated to meet the growing PUFA market in the food industry.

### *Oleaginous microorganisms for lipid production*

Microbial lipids can be produced by oleaginous microorganisms such as yeast, fungi, and microalgae. Oleaginous microorganisms have an inherent ability to accumulate lipids, mainly in the form of triacylglycerides (TAG), with more than 20% (dry-mass basis) using carbon sources (Ageitos et al., 2011). Table 1.3 shows lipid production of oleaginous microorganisms from different families. Numerous yeasts and fungi accumulate higher percentages of lipids compared with other oleaginous microorganisms. Each oleaginous microorganism has its own properties, which affects cost of lipid production during fermentation (Table 1.4). Microalgae are photosynthesizing organisms requiring sunlight, water, and carbon dioxide for their growth and lipid accumulation. Many species of microalgae, such as, *Chlorella sp* or *Schizochytirum sp*, grow rapidly and accumulate more than 60% (w/w) lipids. Their lipid accumulation properties are dependent on temperature, nitrogen concentration, or $CO_2$ enrichment of the environment (Calvey et al., 2016). Despite their ability of higher lipid accumulation, a larger acreage and longer fermentation period are required to grow microalgae, compared to other bacteria and yeast cells (Meng et al., 2009). Some species of bacteria also are capable of accumulating lipids, such as *Acinetobacter calcoaceticus* and *Rhodococcus opacus*. Their rapid growth and easy cultivation

methods are the biggest advantages. However, lipid contents are substantially lower than other oleaginous species, and bacterial lipids exist in the outer membrane, which makes extracting lipids from the cell wall challenging. Therefore, many limitations remain for developing lipid production using oleaginous bacteria in a biorefinery. Many studies successfully developed non-oleaginous bacterial strains — typical industrial bacteria with fully identified genetic information such as *Escherichia coli* and *Corynebacterium glutamicum* — for lipid production, mainly free fatty acids or unusual fatty acids synthesis (Haushalter et al., 2014; Bule et al., 2016; Yuan et al., 2016). With use of synthetic biology, tailored fatty acid species can be produced such as odd-number fatty acids and unusual fatty acids, which are insubstantial chemicals in oleochemistry. Still, challenges remain to achieve high titers of fatty acids using metabolically engineered bacteria species, due to antibacterial properties of free fatty acids (Cray et al., 2015; Desbois and Smith, 2010; Sherkhanov, Korman and Bowie, 2014). Some filamentous fungi are classified as oleaginous species, including *Aspergillus oryzae, Mortierella isabellina,* and *Mucor circinelloides*. While other oleaginous microorganisms accumulate lipids mainly in TAG, lipids produced by oleaginous fungus are composed of a high content of polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA), γ-linolenic acid (GLA), eicosapentaenoic acid (EPA), and arachidonic acid (ARA) (Pinzi et al., 2014). *Mortierella,* especially, are regarded as the most efficient fungus species for PUFA synthesis with the capacity of 20-70% of total lipids containing mostly PUFAs (Coradini et al., 2015). However, filamentous fungus preferably grows under solid-state fermentation, where it is not easy to control fermentation conditions, and requires precise moisture control. There have been many attempts to grow them in a traditional bioreactor to reduce production cost and wastes. Only a few studies successfully cultivated them in a liquid environment, with low lipid content and fungal oil production still at laboratory scale (Pinzi et al., 2014). Another interesting microbial

system for lipid production is oleaginous yeast. Compared to microalgae and fungus, oleaginous yeast grows fast and accumulates lipids of high content in submerged cultivation. Oil accumulation varies depending on the yeast species and cultivation conditions. Among a variety of oleaginous species, *Rhodosporidium toruloides, Lipomyces starkeyi* and *Trichosporon oleaginosus* have been found to accumulate lipids up to 60 or 70% in dry-cell mass (Görner et al., 2016). Since high titers and productivity can be achieved, compared to other oleaginous microorganisms, microbial lipid production using oleaginous yeast cultures has gained substantial attention in biorefineries.

## *Metabolic pathway for TAG synthesis from diverse sugar sources in oleaginous yeast cultures*

### *Importance of acetyl-CoA for a lipid synthesis*

Figure 1.2 shows the metabolic pathway of lipid synthesis in oleaginous yeast. Acetyl-CoA represents a key node in metabolism due to its intersection with many metabolic pathways (Shi and Tu, 2015). Also, acetyl-CoA is an important intermediate metabolite for acetylation of several enzymes involved in lipid synthesis (Galdieri and Vancura, 2012; Baumann, 2015; Cai et al., 2011). Carbon sources are metabolized to cytosolic pyruvate *via* glycolysis and then enter the mitochondria. The mitochondrial pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase and utilized for ATP synthesis *via* Kreb cycle and oxidative phosphorylation at the electron transport chain.

Oleaginous microorganisms have interesting metabolic properties with which to synthesize TAG as a form of a lipid body. In the nitrogen-starvation condition, mitochondrial cAMP levels are decreased *via* increased AMP-deaminase activity (Calvey et al., 2016). It causes a decrease of isocitrate dehydrogenase activity, and subsequently, citrate — an intermediate of the Kreb cycle

— is accumulated in the mitochondria. Finally, it leads to the export of mitochondrial citrate to the cytosol through citrate/malate translocase (Kosa and Ragauskas, 2011). The citrate is converted to oxaloacetate and acetyl-CoA by ATP-cytrate synthetase (*ACL*), and acetyl-CoA carboxylase (*ACC*) converts it to malonyl CoA as a starting step of FA synthesis (*FAS*) (Kosa and Ragauskas, 2011). Therefore, the *ACL* enzyme provides an important function for carbon flow to synthesize TAG. Oxaloacetate produced from citrate is converted to malate and joins again to the Kreb cycle. Fatty acyl-CoA is synthesized by *FAS* and undergoes elongation and desaturation (Probst et al., 2015).

### *De novo lipid synthesis*

*De novo* lipid synthesis begins from glycerol-3-phosphate (G3P), which is the glycolysis intermediate. G3P provides glycerol backbone to synthesize TAG. G3P is converted to lysophosphatidic acid (LPA) by glycerol-3-phosphate acyltransferase (*GPAT*), followed by the action of lysoPA-acyltransferase (*LPAAT*) to form phosphatidic acid (PA). PA is an another key intermediate for synthesizing storage lipids (TAG). The cellular level of PA regulates the transcription of several genes, including *ACC* and *FAS*, which play a crucial role in lipid synthesis (Kosa and Ragauskas, 2011). PA is further dephosphorylated to DAG by phosphatidic acid phosphatase (*PAP*). The final step in the biosynthesis of storage lipids, TAG, is catalyzed by diacylglycerol acyltransferase (*DGAT*). The de novo lipid synthesis is an acyl-CoA-dependent reaction required for three acyl-CoAs during TAG synthesis.

## Production of lignocellulose-based microbial lipids

### Cost effective sugar sources for microbial lipid production: lignocellulosic biomass

Lignocellulosic biomass is a promising new sugar supplier to replace expensive carbon sources such as starch and refined sugars. Lignocellulosic biomass, which is known widely as an existing renewable carbohydrate source, generally consists of ~75% carbohydrate polymers (cellulose and hemicellulose) and ~25% lignin in a dry-mass basis (Lopes, 2015). Table 1.5 shows the composition of the most commonly utilized lignocellulosic biomass, which varies among species. Cellulose has a highly crystalline structure consisting of β-1,4-linked glucose polysaccharides, and is tightly linked with hemicellulose and lignin in the plant structure (Limayem and Ricke, 2012; Guerriero et al., 2016). Hemicellulose is an amorphous and branched single-chain polysaccharides mainly composed of glucuronoxylan, glucomannan, and other polysaccharides (Anwar, Gulfraz and Irshad, 2014). Among the hemicelluloses, xylan is the most abundant polymer and is characterized by a linear backbone of β-1,4-linked xylosyl residues substituted with acetyl, glucuronic acid, and arabinose(Guerriero et al., 2016). Hemicellulose interacts with more than one cellulose polymer with non-covalent cross-links between cellulose bundles (Yang et al., 2011). Lignin, the smallest fraction of the lignocellulosic biomass, fills the gap between and around the cellulose and hemicellulose complexion with a long-chain polymer composed of phenyl-propane units normally linked by either bond (Anwar, Gulfraz and Irshad, 2014).

Cellulose and hemicellulose can be separated *via* pretreatment, and depolymerized to monosaccharides — mainly glucose, xylose, and arabinose — *via* enzymatic hydrolysis. Pretreatment is an energy-intensive process to efficiently recover hemicellulose and cellulose fractions without substantial loss due to breaking the lignin structure. Therefore, harsh conditions, such as high temperature and pressure with chemical reagents, are required for successful

pretreatment. Acid- and alkali-based pretreatments are the most commonly utilized methods. The exposed cellulose and hemicellulose portions were depolymerized to a fermentable sugar stream *via* hydrolysis process of synergic action of several enzymes. Cellulases, including endoglucanases, exoglucanases (cellobiohydrolases), and β – glucosidases, catalyze the hydrolysis reaction of cellulose to hexose sugars (Ding et al., 2016). Since hemicellulose is composed of more varied compounds, a mixture of more diverse enzymes — endo-xylanase, acetyl xylan esterase, β-mannosidase, α-L-arabinofuranosidase, α-glucoronidase, and ferulic acid esterase — is required for the effective hydrolysis (Dashtban et al., 2010).

### *Promising lignocellulosic feedstocks for bioconversion*

One key factor to improve the viability of lignocellulose-based biorefinery is the economics of bioenergy crop production. The most important consideration in the production of lignocellulosic crops are (1) yield, (2) land costs, and (3) the price of other feedstocks for biofuel production (Rinehart, 2006). High-biomass-yielding energy crops will reduce the total acreage required, minimizing potential competition for land utilization (Olson et al., 2012). The promising bioenergy crops would have high resistance to pest and disease, tolerance of poor soils, high yields of cellulose, drought and flood tolerance, efficient water use and low fertility requirement to achieve high yield crop productions (Rinehart, 2006). Most of the perennial C4 grasses being developed for bioenergy purposes are due to low input requirements on land, its tolerance to water limited environments and C4 photosynthesis which contributes to improved nitrogen use efficiency (Mullet et al., 2014). Sorghum and switchgrass are the typical C4 perennial bioenergy crops that have been identified as the most promising renewable feedstocks among a variety species of crops (Fu et al., 2016). Table 1.5 shows that both sorghum stalks and switchgrass contain high contents of polysaccharides (cellulose and hemicellulose), which is a key requirement for bioenergy crops.

*Switchgrass*

Switchgrass - a perennial C4 grass and warm seasons species - evolved across Central and North America and has recently gained huge attention as energy crops because of its low-cost investment and abundance. A study demonstrated that switchgrass can be grown on the soils of moderate fertility without fertilizing, or with limited additions of fertilizer, and still maintain productivity (Parrish and Fike, 2005). Higher net energy efficiency was obtained from switchgrass among energy crops because of moderate inputs including N fertilizer and facile management (Schmer et al., 2008). In addition, many studies reported high yield of switchgrass compared with other energy crops. In a recent study, more than 20 Mg/ha per year was observed in systems managed for maximum sustained biomass (Parrish and Fike, 2005). The productivity of switchgrass (13.4 – 22.3 t/ha) exceeds the yield range of corn (6.3 – 8.7 t/ha) (McLaughlin and Kszos, 2005).

*Sorghum*

Sorghum is a fast growing C4 plant native to tropical zones, but with a wide adaptability to different environmental conditions due to its relative lower agronomic requirements compared to other crops (Zegada-Lizarazu and Monti, 2012). Sorghum was not initially targeted for energy crop development, but was principally grown as an annual grain and forage crop. The studies demonstrated the potential of sorghum as a bioenergy crop, specifically due to its high drought tolerance (Monk, Miller and McBee, 1984; Borrell et al., 2006; Rosenow and Clark, 1995). Sorghum has an extensive root system, which enables it to penetrate 1.5 to 2.5 meters into the soil and extend one meter away from the stem. Its unique stomata in their leaves makes them tolerate water deficit conditions (Mullet et al., 2014). The biomass yield of energy sorghum grown in small

irrigated plots was ~45-50 dry Mg/ha compared with ~15-25 dry Mg/ha under non-irrigated conditions when grown in larger plots (Olson et al., 2012).

### *Sustainable production of lignocelluose-based microbial lipids*

The platform of lignocellulose-based microbial lipids was proposed to identify and evaluate environmental impacts associated with process operations (Figure 1.3). The proposed platform utilized alkaline pretreatment was utilized to effectively remove lignin compounds with minimal sugar loss. For the lipid extraction produced by oleaginous yeast, the traditional method developed by (Bligh and Dyer, 1959) was utilized. Also, the lignin fraction obtained after enzymatic hydrolysis was collected and utilized for energy generation *via* combustion or a source of high-value aromatic monomers. One of the merits of biorefineries is their environmental friendly process compared with chemical reactions. Many life-cycle assessments (LCAs) showed a reduction of $CO_2$ emissions in biofuel production (Jin et al., 2015). However, the generation of a waste stream and usage of hazardous organic solvent during the process is inevitable. Possible waste streams are identified in Table 1.6. The major waste stream is effluent. A large quantity of water is required during the overall process, such as for pretreatment, enzymatic hydrolysis, and fermentation. After these processes, huge amounts of liquid effluents are generated, which are normally contained organic compounds. The pretreatment process generates diluted alkaline or acidic effluents, containing various phenolic compounds and aliphatic acids. Even if they are considered as wastes in the proposed biorefinery, this waste stream contains a variety of invaluable products to the industry. Development of a novel separation method to harvest valuable industrial compounds will increase the substrate utilization and sustainability of the proposed biorefinery. The fermentation process also causes huge amounts of waste water streams, which normally contain spent broth, buffer solution, and secondary metabolites of microorganisms. Extraction

methods developed by (Bligh and Dyer, 1959) are known as the most efficient lipid extraction methods, with 95% efficiency. However, this method requires hazardous chemicals such as chloroform and hexane. It can be the drawback of this proposed platform for the sustainable production of microbial lipids.

Suggestions for improving sustainability of the proposed platform for lignocellulosic-based microbial lipid production are efficient procurement of raw feedstocks which is one of the challenges. To increase competitiveness and sustainability, the efficient procurement of raw material is required. In 2030, 10% of global residues could yield around 4.1 % of the projected transport fuel demand (Eisentraut, 2010). Although more than one billion tons of biomass per year would be potentially available to meet the 30% replacement of petroleum-derived gasoline in 2030, the high cost of biomass could be a serious hindrance if potential lands and feedstocks are not managed and utilized efficiently (Perlack et al., 2005). Therefore, it is important to develop efficient management strategies for energy crops. It will help alleviate land competition with food crops and reduce costs for feedstock production. Another important factor to make bio-refineries sustainable and economically viable is optimization and achievement of high process efficiency (Cherubini and Strømman, 2011). Pretreatment is an energy-intensive process directly affecting product yield. Therefore, optimized and effective pretreatment conditions will reduce production costs and environmental concerns. Many attempts have been explored to efficiently recover the portion of polysaccharides from lignocellulosic biomass. However, the pretreatment process remains one of the challenges for the sustainability of biorefineries.

Table 1-1 Fatty acid composition of oleaginous microorganisms

| Organisms | Relative average fatty acids (%, w/w) | | | | | | | References |
|---|---|---|---|---|---|---|---|---|
| | C14:0 | C16:0 | C16:1 | C18:0 | C18:1 | C18:2 | C18:3 | |
| Plant | | | | | | | | |
| Palm oil | NA | 33 | 0.1 | 4.7 | 55.1 | 1.6 | NA | (Coradini et al., 2015) |
| Sunflower oil | NA | 6.9 | NA | 6 | 26.5 | 66.5 | NA | (Pinzi et al., 2014) |
| Rapeseed oil | 0.9 | 40 | NA | 4.1 | 43 | 11 | 0.2 | (Pinzi et al., 2014) |
| Microalgae | | | | | | | | |
| *Psedokirchneriella subcaptiata* | 0.1 | 16.2 | 1.0 | 1.3 | 31.1 | 5.1 | 11.4 | (Patil et al., 2007) |
| *Chlorella vulgaris* | NA | 16 | 11.9 | 2 | 58 | 9 | 14 | (Wynn and Ratledge, 2005) |
| *Nanochloropsis oceanica* | 16.9 | 17.2 | 18.2 | 1.8 | 4.1 | 9.7 | 0.5 | (Patil et al., 2007) |
| Oleaginous yeast | | | | | | | | |
| *Cryptococcus albidus* | NA | 19.4 | NA | 4.9 | 10.9 | 27.3 | 7.4 | This study |
| *Lipomyces starkeyi* | NA | 22.3 | 8.9 | 2.7 | 62.9 | 2.7 | 0.5 | (Probst and Vadlani, 2015) |
| *Trichosporon oleaginosus* | 0.8 | 25.4 | 0.47 | 10.8 | 40.5 | 20.1 | 2.0 | This study |
| *Yarrowia lipolytica* | NA | 11 | 6 | 1 | 28 | 51 | 1 | (Li, Du and Liu, 2008) |
| Oleaginous bacteria | | | | | | | | |
| *Rhodococci sp.* | 2.3 | 7.0 | 27 | 13.9 | 14.5 | 11.4 | NA | (Wei et al., 2015) |
| *Rhodococcus opacus* | 2 | 27.3 | 9.3 | 3.3 | 14.0 | NA | NA | (Kurosawa et al., 2015) |
| Oleaginous fungi | | | | | | | | |
| *Mucor circinelloides* | NA | 20 | 2.3 | 2 | 37 | 14.3 | 18.5 | (Vicente et al., 2009) |
| Fugal strain IBB G4 | 1.1 | 22.9 | NA | 23.7 | 26.9 | 17.8 | NA | (Kakkad et al., 2015) |

NA = Not Available

Table 1-2 Production of polyunsaturated fatty acids using oleaginous microorganisms

| Polyunsaturated fatty acids | Microorganisms | | References |
|---|---|---|---|
| γ-Linolenic acid (GLA) | *Gunninghamella echinulate* | Oleaginous fungi | (Fakas et al., 2007) |
| | *Mortierella ramanniana* | Oleaginous fungi | (Dyal and Narine, 2005) |
| | *Mucor rouxii* | Oleaginous fungi | (Muniraj et al., 2015) |
| Deocosahexaenoic acid (DHA) | *Candida guilliermondii* | Oleaginous fungi | (Guo and Ota, 2000) |
| | *Chlorella sp.* | Microalgae | (Lee et al., 2013) |
| | *Aurantiochytirum sp.* | Microalgae | (Choi et al., 2014) |
| Arachidonic acid (ARA) | *Sirodotia Kylin* | Microalgae | (Bhosale, Velankar and Chaugule, 2009) |
| | *Mortierella alpine* | Oleaginous fungi | (Kikukawa et al., 2013) |
| Eicosapentaenoic acid (EPA) | *Schizochytrium limacinum* | Microalgae | (Chi et al., 2007) |
| | *Mortierella alpina* | Oleaginous fungi | (Okuda et al., 2015) |
| | *Vischeria stella* | Microalgae | (Gao et al., 2016) |
| | *Nannochloropsis oceanica* | Microalgae | (Meng et al., 2015) |

Table 1-3 Lipid accumulation by oleaginous microorganisms

| Organisms | Nutrient | Lipid content (%, w/w) | References |
|---|---|---|---|
| **Microalgae** | | | |
| *Chlorella sp.* | - | 45-47 | (Pinzi et al., 2014) |
| *Schizochytrium sp.* | - | 50-77 | (Pinzi et al., 2014) |
| *Nitzschia sp.* | - | 45-47 | (Pinzi et al., 2014) |
| **Yeast** | | | |
| *Trichosporon oleaginosus* | Woody biomass | 61 | This study |
| *Lipomyces starkeyi* | Sorghum stalk | 44 | This study |
| *Cryptococcus albidus* | Woody biomass | 50 | This study |
| *Yarrowia lipolytica* | Sugarcane baggase | 58.5 | (Tsigie et al., 2011) |
| *Trichosporon fermentans* | Rice straw | 40.1 | (Huang et al., 2009) |
| **Bacteria** | | | |
| *Acinetobacter sp.* | - | 27-38 | (Pinzi et al., 2014) |
| *Bacillus alcalophilus* | - | 18-24 | (Pinzi et al., 2014) |
| *Rhodococcus opacus* | Glucose | 38 | (Kurosawa et al., 2010) |
| **Fungi** | | | |
| *Mortierella alpina* | Glucose | 64 | (Nie et al., 2014) |
| *Mucor circinelloides* | Corn stillage | 61 | (Mitra et al., 2012) |
| *Mortierella isabellina* | Rice hull | 64 | (Economou et al., 2011) |
| *Aspergillus oryzae* | Potato waste | 40 | (Muniraj et al., 2013) |

Table 1-4 Properties of oleaginous microorganisms (Cray et al., 2015; Desbois and Smith, 2010; Sherkhanov, Korman and Bowie, 2014)

| Advantages | Drawbacks |
|---|---|
| *Microalgae* | |
| ▪ High content of lipids<br>▪ Submerge fermentation | ▪ High energy requirement to provide $CO_2$ and light<br>▪ Intracellular product<br>▪ Requirement of large acreages<br>▪ Long fermentation period |
| *Oleaginous yeast* | |
| ▪ High content of lipids<br>▪ Fast growth rate<br>▪ High-density growth<br>▪ High intolerance to toxic compounds and high concentrations of sugars<br>▪ Submerge fermentation | ▪ Intracellular product |
| *Oleaginous bacteria* | |
| ▪ Submerge fermentation<br>▪ Fast growth rate | ▪ Low content of lipids<br>▪ Vulnerable to toxic compounds<br>▪ Vulnerable to high concentrations of substrate and product<br>▪ Intracellular product |
| *Fungi* | |
| ▪ High content of lipids<br>▪ High content of unsaturated fatty acids | ▪ Solid-state fermentations<br>▪ Intracellular product |

Table 1-5 Composition of lignocellulosic biomass (% dry mass)

| Biomass species | Hemicellulose | Cellulose | Lignin | References |
|---|---|---|---|---|
| Switchgrass | 29 | 35 | 17 | This study |
| Sorghum stalks | 28 | 21 | 20 | This study |
| Waste papers | 25-40 | 40-55 | 6-10 | (Limayem and Ricke, 2012) |
| Nut shell | 20-30 | 30-40 | 25-30 | (Anwar, Gulfraz and Irshad, 2014) |
| Corn stover | 26 | 38 | 19 | (Zhu, Lee and Elander, 2005) |
| Rice straw | 24 | 32.1 | 18 | (Prasad, Singh and Joshi, 2007) |
| Soybean hulls | 33 | 17 | 10 | (Brijwani, Oberoi and Vadlani, 2010) |
| Poplar | 40 | 20 | 21 | (Guragain, Wang and Vadlani, 2016) |
| Douglas fir | 37 | 20 | 29 | (Guragain, Wang and Vadlani, 2016) |
| Sugarcane bagasse | 25 | 42 | 20 | (Anwar, Gulfraz and Irshad, 2014) |
| Wheat straw | 16-21 | 26-32 | 29-35 | (Anwar, Gulfraz and Irshad, 2014) |
| Banana waste | 14.8 | 13.2 | 14 | (Anwar, Gulfraz and Irshad, 2014) |

Table 1-6 Identified waste stream in proposed platform

| Process | Waste |
|---------|-------|
| Transport | $CO_2$ emission |
| Pretreatment | Liquid effluent- phenolic, aliphatic |
|  | Gas emission – volatile compounds |
| Enzymatic hydrolysis | Lignin residue |
| Fermentation | Solid effluent – cell biomass |
|  | Liquid effluent – nutrient residue, by-product |
|  | $CO_2$ emission, volatile metabolites |
| Downstream – lipid extraction | Liquid effluent: solvents – chloroform, hexane |
|  | Gas emission |
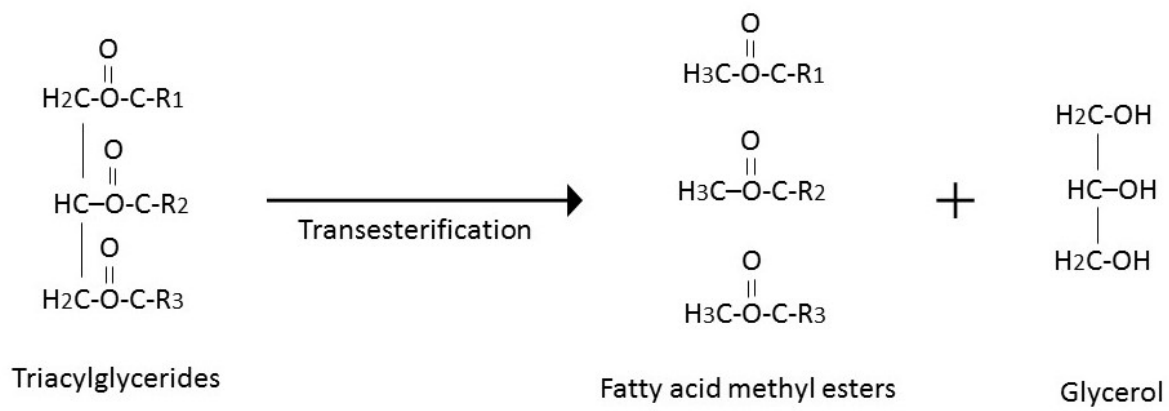
Figure 1-1 Conversion of triacyglycerides to fatty acid methyl esters *via* transesterification
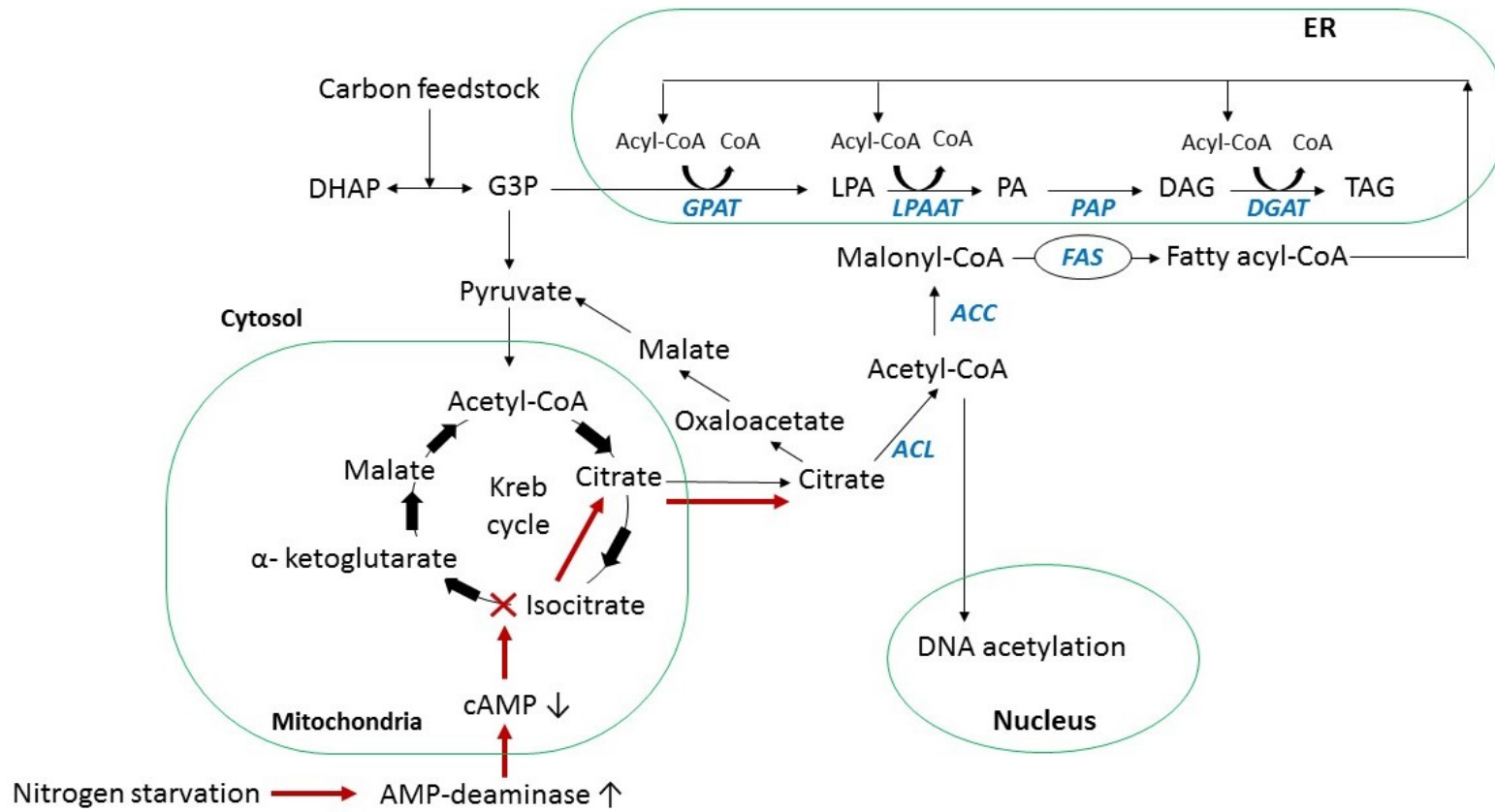
Figure 1-2 Metabolic pathway for lipid accumulation in oleaginous yeast

Figure 1-3 Lignocellulosic biomass (A) switchgrass; (B) sorghum (Teel, 2003; Assefa et al.,)
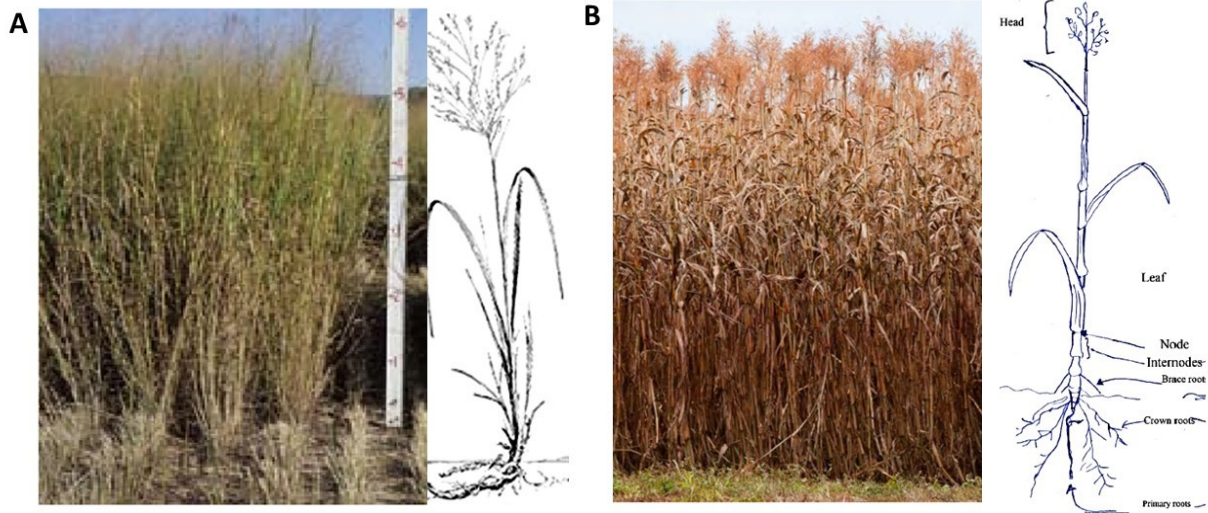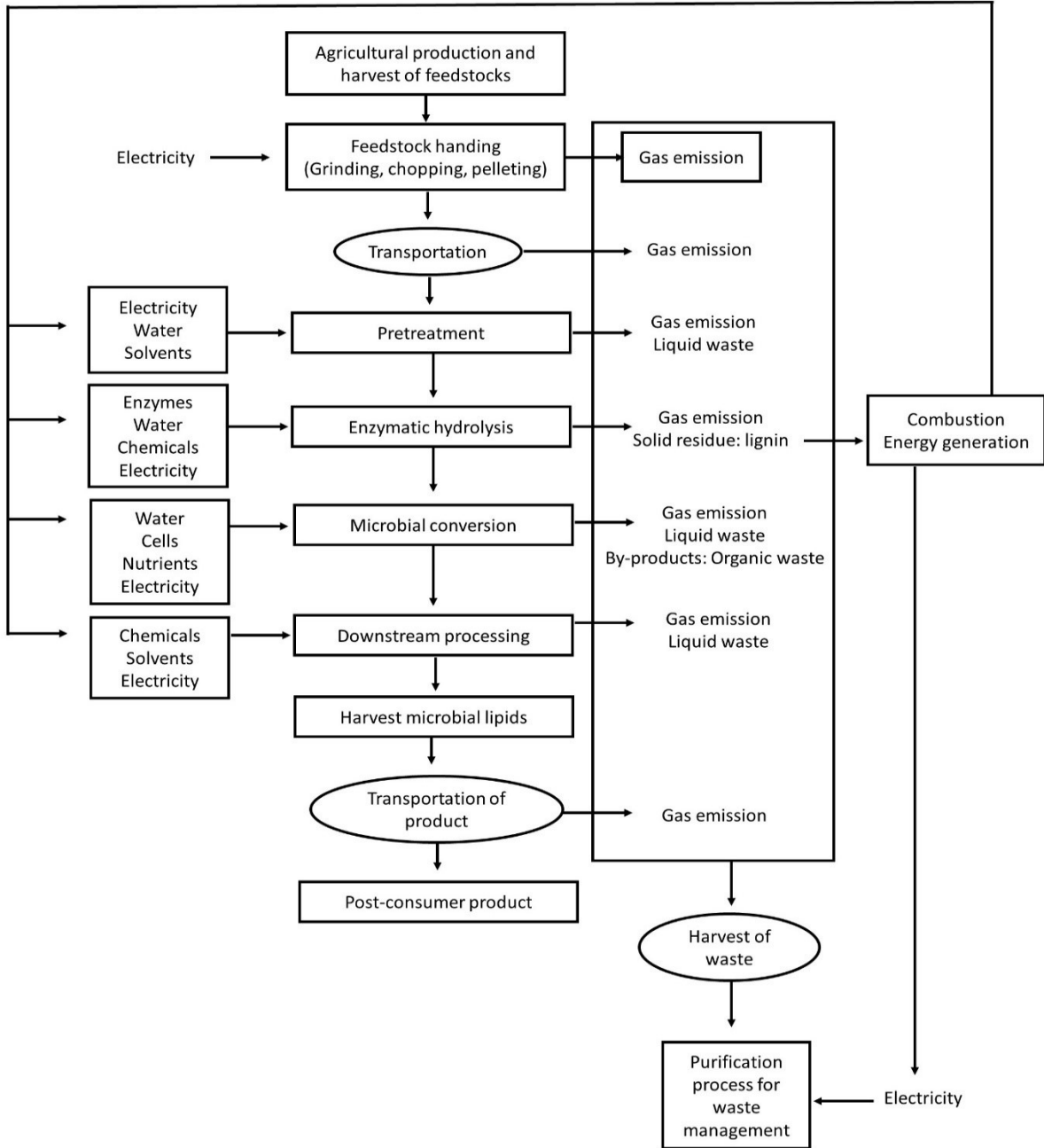
Figure 1-4 Diagram of proposed process for lignocellulose-based lipid production

# Chapter 2 - Research Objectives

Lignocellulose-based microbial lipids are attractive substrates to replace edible oils. However, several challenges remain for economically feasible production of microbial lipids. This study explored how to improve the potential of an industrially viable bioconversion platform of microbial lipids through utilization of non-edible agricultural wastes. Therefore, the purpose of this study was to develop and evaluate an efficient bioconversion process, to achieve high yields and productivity of microbial lipids from inexpensive sugar sources by oleaginous yeast cultures and engineered *E. coli*. Specific objectives for this dissertation are listed below:

- Develop a novel purification method to generate a clean sugar stream using sorghum stalks after acid pretreatment. (Chapter-3)

- Optimize fermentation conditions for *T. oleaginosus* to achieve high yields and productivity of microbial lipids using lignocellulosic hydrolysates. (Chapter-4)

- Develop a bioconversion process for high yields of microbial lipids from switchgrass using engineered *E. coli*. (Chapter-5)

- Investigate the potential of sorghum stalk and switchgrass as feedstock for microbial lipid production, using oleaginous yeast strains such as *T. oleaginosus, L. starkeyi, and C. albidus*. (Chapter-6)

- Develop an integrated process of corn-bran-based microbial lipid production using *T. oleaginosus*. (Chapter-7)

# Chapter 3 - Innovative methods to generate clean sugar stream from biomass feedstocks for efficient fermentation[1]

## Abstract

The objective of this study was development of a novel purification method using activated charcoal and ionic exchangers to utilize xylose-rich stream of sorghum stalk hydrolysates after acid pretreatment. Sorghum stalks were evaluated to generate clean sugars; enzymatic hydrolysis efficiency of acid pretreated biomass was low (12.3 g sugar/g pretreated biomass) compared to alkali method (49.1 g sugar/g pretreated biomass). Inhibitory compounds derived from acid pretreatment, such as, hydroxymethyl furfural (HMF) and furfural, were effectively removed using an appropriate detoxification method using 1% (w/v) activated charcoal. The resin mixture containing cationic exchangers (DOWEX 50WX8) and anionic exchangers (IRA 743) in 7:3 ratio at pH 2.7 completely removed HMF, acetic acid, and formic acid with less than 2% (w/w) sugar loss, leading to efficient detoxification of inhibitory compounds. The information is pertinent to develop a detoxification method based on the inhibitory compounds present in the biomass hydrolyzate and the relative adverse effect of these compounds to the targeted culture used in the subsequent fermentation process.

## Introduction

Lignocellulosic biomass, including forest and agricultural residues, represent widespread and cost effective resources for the production of specialty chemicals and advanced biofuels (Chandra, Takeuchi and Hasegawa, 2012).  Lignocellulosic biomass is composed of three types of bio-polymers: cellulose, hemicellulose, and lignin. Cellulose is a structural linear component of a

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plant's cell wall consisting of a long-chain of glucose monomers linked by β (1→4) glycosidic bonds that reach to several thousand glucose units in length (Limayem and Ricke, 2012). Hemicelluloses are repeated polymers of both hexoses (D-glucose, D-galactose and D-mannose) and pentoses (D-xylose and L-arabinose) (Anwar, Gulfraz and Irshad, 2014). Lignin is an aromatic and rigid biopolymer, and acts as glue by filling the gap between and around the cellulose and hemicellulose complexes. It is considered as a byproduct in the bioconversion of lignocellulosic feedstocks *via* the sugar platform route (Anwar, Gulfraz and Irshad, 2014). Monomer sugars are mainly obtained *via* hydrolysis of cellulose and hemicellulose which are utilized as carbon sources during fermentation to produce targeted fuels and chemicals.

Lignocellulosic biomass is recalcitrant to the action of cellulolytic enzymes to depolymerize carbohydrate polymers to monomer sugars because of the strong biomass structure due to lignin-hemicellulose-cellulose complex (Anwar, Gulfraz and Irshad, 2014). Harsh conditions, such as, high pressure and temperature and/or high chemical concentrations, should be employed during pretreatment process to deconstruct the complex biomass structures and to recover cellulose and hemicellulose. Pretreatment processes mainly focus on disrupting and removing the cross-linked matrix of lignin and hemicelluloses to increase porosity and surface area of cellulose for subsequent enzymatic hydrolysis (Li et al., 2010). There are several physical, chemical, and biological techniques for biomass pretreatment, such as ball milling, steam explosion, acid, alkali, and enzymes (Guragain et al., 2011). Among those pretreatment techniques, a dilute sulfuric acid pretreatment has been shown as a leading pretreatment process that is currently under commercial development (Li et al., 2010). In order to develop an economically feasible biofuel production process, several factors should be considered, such as labor requirement and cost, sample size, and cost of process to overcome biomass recalcitrance (Wyman, 1999). Recently, many studies tried

to integrate or combine the pretreatment and enzymatic hydrolysis process because of a number of attributes of such combined process, such as requirement of smaller quantity of biomass, highly automated process, reduced labor, and production time (Schell et al., 2016; Banerjee et al., 2012).

The pretreatment process inevitably generates several compounds from lignocellulosic biomass depending upon pretreatment methods and severity of the pretreatment processes (Guragain et al., 2016). Some of the pretreatment-induced compounds inhibit microbial growth and product formation during the fermentation process. Such inhibitory compounds can be divided into three groups, as shown in Figure 3.1.: (1) Monomer sugar derivatives, such as, 5-hydroxymethylfurfural (HMF) and furfural, (2) lignin degradation products, such as phenolics, and (3) compounds derived from lignocellulosic structure, such as acetic acid released from acetyl groups on hemicellulose and lignin. HMF and furfural are generated due to the degradation of hexose and pentose sugars, respectively (Palmqvist and Hahn-Hagerdal, 2000). Many studies reported that these furans (HMF and furfural) have negative influence on the enzymatic hydrolysis, as well as fermentation process. The study by Rajan and Carrier (Rajan and Carrier, 2016) showed that furans and organic acid reduced the specific activity of cellulytic enzymes. Also, furans inhibit the microbial growth and output. The microorganism converts HMF and furfural to their respective alcohols by oxidizing NADPH to $NADP^+$ or NADH to $NAD^+$ (Almeida et al., 2007). Furans, at a concentration of 10 g/L or greater, affect yeast metabolism and cellular growth by inhibiting glycolytic enzymes, such as alcohol dehydrogenase (Palmqvist et al., 1999). Lignin degradation products generated during the pretreatment process include a variety of compounds, such as phenolics and aldehydes. Phenolics are known as the strongest inhibitors for the action of cellulytic and hemicellulytic enzymes (Ximenes et al., 2010). In addition, phenols inhibit microbial fermentation by reducing integrity of biological membranes (Almeida et al., 2007). The major

compound derived from lignocellulosic structure is acetic acid. It is mainly released from acetyl groups of hemicellulose during enzymatic hydrolysis. Acetylated lignin also generates acetic acid during the pretreatment process (Guragain et al., 2015). Acetic acid leads to the depletion of the reducing agent, such as NADH or NADPH and ATP, and thereby adversely effects the microbial metabolism. It also has an adverse impact on the function of membrane embedded protein (Almeida et al., 2007). Therefore, the biomass-derived inhibitory compounds produced during pretreatment and hydrolysis retard microbial growth and product formation. In addition to these biomass-derived compounds, a number of heavy metal ions, such as iron, chromium, nickel, and copper, can be leached from processing equipment, and act as inhibitors for microbial fermentation (Mussatto and Roberto, 2004). Therefore, detoxification is an essential process to eliminate pretreatment- and hydrolysis-induced inhibitory compounds in lignocellulosic biomass hydrolysates, and to generate a clean sugar stream for efficient fermentation.

A number of detoxification methods are available to remove inhibitory compounds in biomass hydrolyzate depending on the types of inhibitors and scale of operation. These methods can be physical (such as evaporation and membrane filtration), physicochemical (such as activated charcoal, ion exchange, neutralization, over-liming, and extraction with organic solvents) or biological (such as use of specific enzymes or microorganisms) (Mussatto and Roberto, 2004). Among these detoxification methods, adsorption is a convenient and effective technique to remove low concentrations of chemicals from the aqueous phase because of simple operation, easy recovery, and reuse of adsorbent (Jeong et al., 2014). Activated charcoal was utilized for several years to eliminate pollutants due to its high adsorption capacity (Kim et al., 2013). Adsorption using activated carbon shows high effectiveness in the removal of hydrophobic compounds (Ra et al., 2015). However, the high cost to regenerate the exhausted charcoal, due to the energy-intensive

regeneration process, is the greatest challenge for economic viability of the charcoal detoxification process. The most common exhausted charcoal regeneration process is a thermal method using more than 600 °C (Cazetta et al., 2013). Use of ion exchangers is another effective adsorption method for the detoxification of biomass hydrolyzates. This method encomoasses on easy regeneration process using dilute chemicals, such as sodium hydroxide or hydrochloric acid (Chandrasekara and Pashley, 2015). The ion-exchange resins are normally used to remove low or medium levels of undesirable ions (Rudnicki, Hubicki and Kolodynska, 2014). The binding efficiency of ionic exchangers are affected by a number of factors, such as size exclusion, Donnan exclusion by functional group of resins and hydrophobic interactions with matrix of resins (Valentin et al., 2014). Cationic exchange resins effectively remove hydrophobic compounds, such as HMF and furfural, without substantial sugar loss, but they cannot remove acetic acid. While anionic exchange resins effectively remove acetic acid, the monomer sugar loss is the biggest challenge in this resin (Fernandes et al., 2012). Therefore, an optimum mixture of cationic and anionic resins could be an alternative approach for the detoxification of biomass hydrolyzates. Since commercial mixed resins were developed to purify water using a higher level of anionic exchangers, they may lead to substantial sugar loss and hence may not be appropriate for the detoxification of biomass hydrolyzates. To the best of our knowledge, no attempt has been made thus far to detoxify biomass hydrolysates using a mixture of cationic and anionic resins.

In this study, we evaluated detoxification methods using activated charcoal and three types of commercial resins: cationic exchangers (DOWEX-50WX), anionic exchangers (IRA-743), and mixed resins (MB-20). We also evaluated the mixture of cation exchange (DOWEX-50WX) and anion exchange resins (IRA-743) at a specific ratio. The objective of this study was to optimize

the detoxification process for biomass hydrolyzates in order to maximize toxin removal with minimum sugar loss.

## Materials and Methods

### *Materials*

Sorghum stalks were obtained from Texas A&M University, College Station, TX. The hydrolytic enzymes, Cellic C-Tec2 and Cellic H-Tec2, were provided by Novozymes Inc., Franklinton, NC, USA. Activated carbon (50-200 Mesh) was purchased from Fisher Scientific, USA. DOWEX 50WX8, Amberlite IRA743 and MB-20 were purchased supplied from Sigma Aldrich, USA. DOWEX 50WX8 is a gel type cationic resin with a sulfonic acid functional group. Amberlite IRA is a weakly basic resin with N-methylglucaomine (free base form) functionality. MB-20 resin is a mixed resin with 60% anionic exchangers and 40% cationic exchangers containing sulphonic acid and trimethylammonium as functional groups.

### *Pretreatment and hydrolysis stream preparation*

Sorghum stalks were ground with a Thomas-Whiley Laboratory Mill (Model 4) using a 1 mm sieve, and the biomass hydrolyzate samples were prepared as shown in Figure 3.2. Biomass pretreatment was conducted by mixing ground sorghum stalks with 1% (w/v) sodium hydroxide or 1% (v/v) sulfuric acid solution at the rate of 10% (w/v) solids loading, that is, 20 g biomass in 200 mL alkali or acid solution in a 500-mL flask. The biomass slurry was autoclaved at 121 °C for 30 min. Acid pretreatment was also conducted at 140 °C for 30 min using a 1% (v/v) sulfuric acid solution and 10% solids loading in a Parr Reactor (4520 Bench Top Reactors, Parr Instrument Company, Moline, Illinois). The pretreated biomass slurry was filtered, and the filtrate (called "pretreated stream") was collected for the detoxification study. The solid residues of the biomass were washed using distilled water until the filtrate became clear and neutral to litmus paper; around

5 L of water was used to wash 20 g biomass. The pretreated biomass was dried at room temperature for 48 h so that the moisture content was decreased to less than 10% (w/v). The pretreated sorghum stalks were mixed with a citrate buffer (0.05 M and 4.8 pH) at the rate of 5% (w/v) solids loading, that is, 2 g of pretreated biomass in 40 ml of buffer solution in a 125 ml screw cap flask. The Cellic C-Tec2 and Cellic H-Tec2 enzymes were added in each flask at the rate of 5.4% and 0.6% (w/v) of biomass, respectively, and incubated in a temperature-controlled shaker (Innova 4300, New Brunswick Scientific, NJ, USA) at 50 °C for 48 h. The supernatants for the hydrolysate (called "hydrolysis stream") were separated by centrifugation (Sorvall Super T21, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 13,000 rpm for 15 min and used for detoxification study.

### *Detoxification using activated charcoal*

Activated charcoal detoxification was carried out by mixing using 1% or 2% (w/v) charcoal in 30 mL of sample solution in 250 mL flasks. The flasks were incubated in a temperature-controlled shaker at 50 °C and 150 rpm for 1h. The exhausted charcoal was separated by filtration using 90 mm pore size of filter papers (Whatman, Fisher Scientific, Hamton, NH). The filtrate was analyzed for sugar and inhibitors.

### *Detoxification using resins*

Four types of resins were evaluated in this study to remove inhibitors from sample solutions: cationic resin (DOWEX 50WX8), anionic resin (IRA743), commercial mixed resin (M-20), and mixture of DOWEX and IRA743 (in 7:3 ratio). The glass column (Pyrex, ID 1.5 cm, height 16-20 cm, bed volume 30 mL) was packed with each type of resins as shown in Figure 3.3. The flow rate of outlet was controlled to one drop per 2 to 3 sec by adjusting a valve at the bottom of column, and the experiment was carried out at room temperature. A 10 mL aliquot of each sample solution

was poured from top of the column, and 40 mL of distilled water was added as an eluent. The eluted samples were analyzed for sugars and inhibitors.

### *Analytical methods*

The monomer sugars and inhibitors were measured using High Performance Liquid Chromatography (HPLC, Prominence LC20AB, Shimadzu Scientific Instruments, Columbia, MD, USA). All samples were diluted ten times and filtered using a 0.45 μm membrane filter (Phenomenex, Torrance, CA). The organic acid column (Rezex$^{TM}$ ROA-Organic Acid 150 x 7.8mm, Phenomenex), and both refractive index detector (RID) and Photodiode Array detector (PDA) were used. The mobile phase (0.005 N sulfuric acid) was pumped at a rate of 1.0 mL/min at a column temperature of 80 °C.

### *Statistical analysis*

All experiments were conducted in triplicate, and data were statistically analyzed using JMP software (SAS Institute Inc., Cary, NC, USA). The least significant difference (LSD) test was carried out with 95% confidence level ($P < 0.05$).

## Results and Discussion

### *The mass balance study from sorghum stalks to released sugars*

The dilute acid and alkali pretreatment processes were evaluated and compared for mass balance from ground sorghum stalks to sugar released from enzymatic hydrolysis of the pretreated biomass. Figure 3.4 shows that using equal acid and alkali concentrations, and the same processing conditions for time, temperature and solids loading, acid pretreatment led to 10% higher mass recovery after pretreatment compared with alkali pretreatment. The pretreatment was carried out at 121 °C for 30 min with 10% solids loading using 1% acid (v/v) or alkali (w/v) solutions. On the other hand, because of the lower saccharification efficiency in acid pretreated biomass, less sugar

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was released from acid pretreated biomass compared with alkali pretreated biomass. Starting from 100 g of raw biomass in each pretreatment method, acid pretreatment process released 12.28 g total monomer sugars during enzymatic hydrolysis of pretreated biomass whereas alkali pretreatment process released 49.14 g total monomers sugars. These data shows that, if similar pretreatment conditions, including acid/alkali concentration, time, temperature and solids loading, were used, the alkali pretreatment process was four times more effective than acid pretreatment at improving the enzymatic hydrolysis efficiency of pretreated biomass. However, further research is necessary in order to optimize pretreatment conditions for each acid and alkali process using a three-factor ($3^k$) factorial design for acid/alkali concentration, time and temperature. Taking conversion factors of 0.90 from glucose to glucan, and 0.88 from xylose (or arabinose) to xylan (or arabinan) (Guragain et al., 2014), the monomer sugar recoveries were 88%, 81%, and 86% of theoretical maximum value for glucose, xylose and arabinose, respectively, for the alkali pretreatment process. Higher glucose recovery compared with xylose could be because of higher amount of hemicellulose loss during the pretreatment process. In the case of acid pretreatment, glucose and xylose recoveries were 32% and 9% of the theoretical maximum value, respectively, and no arabinose was recovered at all. The very low monomer sugar recovery from the acid pretreatment process was because of inefficient saccharification during the enzymatic hydrolysis of acid pretreated biomass, as well as hemicellulose loss during pretreatment (Guragain, Wang and Vadlani, 2016). The above results show that, compared to the alkali pretreatment process, the acid process required harsher pretreatment conditions, such as higher acid concentration, higher processing temperature and a longer time processing to make the process more effective at improving the hydrolysis efficiency of pretreated biomass. Therefore, we increased the

pretreatment temperature to 140 °C for the acid pretreatment process, keeping all other conditions (time, acid concentration and solids loading) same, in subsequent experiments of our study.

### *Sugars and inhibitors released during acid pretreatment and enzymatic hydrolysis*

Sorghum stalks were pretreated using 1% (v/v) sulfuric acid at 140 °C for 30 min, and the pretreated biomass was hydrolyzed using an enzyme cocktail, as shown Figure 3.2. The pretreatment stream and hydrolysis streams were analyzed to identify and quantify compounds released from sorghum stalk during acid pretreatment and hydrolysis, respectively. Table 3.1 shows that a total 23 g/L of sugars were detected in the pretreatment stream, which was three times higher than sugars in the hydrolysis stream. The Table 3.1 and Figure 3.4 show that increasing temperature from 121 °C to 140 °C for acid pretreatment did not improve saccharification efficiency of pretreated biomass, indicating even harsher pretreatment conditions are necessary for efficient saccharification. Xylose was the major sugar in the pretreatment stream, whereas the glucose was the main sugar in hydrolysis stream. This was because most hemicellulose was solubilized during pretreatment in the diluted acidic condition, and remaining hemicellulose and cellulose were hydrolyzed during enzymatic hydrolysis (Guragain, Wang and Vadlani, 2016). Besides monomer sugars, acetic acid, formic acid, 5-hydroxymethylfurfural (HMF), and furfural were identified in the pretreatment stream, whereas the hydrolysis stream contained less than 0.01 g/L of these inhibitory compounds. Djioleu and Carrier (Djioleu and Carrier, 2016) reported that severe pretreatment conditions, especially temperature, was an important factor for the generation of inhibitors and directly affected the saccharification efficiency. Therefore, detoxification of pretreated biomass slurry is critical for efficient hydrolysis, especially for the acid pretreatment process because of the requirement of higher temperature compared to the alkali pretreatment process. The results also showed that the acid pretreatment stream can be utilized as a xylose-rich

medium for fermentation with an appropriate detoxification method to eliminate inhibitory compounds, and the hydrolysis stream can be utilized as a glucose rich medium; however, further research is needed to optimize pretreatment conditions in order to improve enzymatic hydrolysis efficiency.

### *Detoxification of sorghum hydrolysate using activated charcoal*

The sorghum hydrolysate samples were detoxified using two concentrations (1% or 2%, w/v) of activated charcoals. Monomer sugars and inhibitors were measured in the hydrolyzate before and after detoxification. Table 3.2 shows that 1% (w/v) activated charcoal effectively removed both HMF and furfural from sample solutions with minimal (around 7%) sugar loss, but acetic acid was not removed at all. By increasing activated charcoal concentration from 1% to 2%, acetic acid removal was slightly improved; however, detoxification efficiency of acetic acid was still very low (less than 4% removal).

The adsorption behavior of any compound to a specific activated charcoal depends on the micropore diameter distribution of the granular activated charcoal, and the molecular weight of the compound (Hidaka, Kohno and Eida, 1981). In addition, the activated charcoal has hydrophobic surface, and hence more hydrophobic compounds are absorbed on the charcoal (Nobre, Teixeira and Rodrigues, 2012). Therefore, the differences in absorption of the sugars, acetic acid, HMF, and furfural on activated charcoal can be explained based on the hydrophobic property (Log P value) and molecular weight of these compounds. Table 3.3 shows that HMF and furfural had a substantially higher Log P value compared with sugars and acetic acid and, hence, effectively absorbed on the activated charcoal surface. Though, Log P value of acetic acid is higher than sugars, but it has a lower molecular weight, which might be the reason for the substantially higher sugar loss in activated charcoal detoxification compared with acetic acid removal. The

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results also indicated that the effect of hydrophobic property is more profound compared with molecular weight.

### *Selection of an appropriate resin for detoxification*

Experiments were conducted to select an appropriate resin for the detoxification of biomass hydrolyzates using a synthetic sample solution containing sugars and inhibitors of the following concentrations: 40 g/L glucose, 20 g/L xylose, 1.3 g/L acetic acid, 0.5 g/L formic acid, 0.5 g/L HMF, and 0.1 g/L furfural. Three types of commercial resins, with the properties as shown in Table 3.4, were evaluated for these experiments. Monomer sugars and inhibitors of synthetic sample solutions were measured before and after each type of resin detoxification. Figure 3.5 shows that the cationic exchanger (DOWEX-50WX) led to a minimum sugar loss during the detoxification process, but was not effective at removing aliphatic acids (acetic acid and formic acid). On the other hand, the anionic exchanger (IRA 743) effectively removed all inhibitors; however, all glucose and a substantial amount of xylose were also lost during the detoxification process. Mixed resin (MB-20) removed all of the compounds, including sugars and inhibitors.

Monosaccharides have several hydroxyl groups in their structures and they could be deprotonated in the aqueous phase (Lopes and Gaspar, 2008). Therefore, cationic exchangers, which are in hydrogen ion form in the aqueous phase, did not interact with the deprotonated sugar; therefore, it was not absorbed in the cationic exchangers. Similarly, the negatively charged aliphatic acids were not absorbed by cationic exchangers. Though, HMF has a structure and pKa value similar to sugars, but cationic exchangers effectively removed HMF without sugar loss. The removal of HMF could be due to the hydrophobic interaction between HMF and resin matrix. It was reported that HMF has hydrophobic interactions with the polystyrene-divinylbenzene copolymer, a matrix of cationic exchanger (Valentin et al., 2014). Mixed resins and anion

exchangers removed all inhibitory compounds, including aliphatic acids, because cations (free base in IRA 743 and hydrogen group in mixed resins) in the resins effectively bind with the anions of dissociated acids, which are negatively charged in the aqueous medium. The sugar loss in anionic and mixed resins is probably due to the formation of covalent bonds between hydroxyl groups of sugars and resins' functional groups. Based on these results, it was proposed that mixtures of cationic exchangers (DOWEX 50WX) and anionic exchangers (IRA743) with the ratio of 7 to 3 could be an appropriate resin for effective detoxification of biomass hydrolyzate with minimum sugar loss.

### *Detoxification of sorghum hydrolysate using resin mixture*

The cationic exchangers (DOWEX 50WX) and anionic exchangers (IRA743) were mixed in 7:3 ratios to prepare a resin mixture, and preliminary experiments were carried out to optimize the initial pH of the hydrolyzate to minimize sugar loss during the detoxification process. The data show that the initial pH of 2.7 led to the minimum sugar loss (data not shown here), and hence, further experiments were carried out using hydrolyzate with pH 2.7. Equal volumes of sorghum pretreatment stream and hydrolysis stream (Figure 3.2) were mixed, and pH of the mixture was adjusted to 2.7 to prepare hydrolyzate samples for these experiments. Table 3.5 shows that all types of monomer sugars in the sample solution were completely recovered without being absorbed in the resin mixture. It is possible that most sugars in the sample solutions were not dissociated at pH 2.7; so, net charge may be close to zero. In addition, monomer sugars have higher molecular weight compared to other compounds of sample solutions so they may migrate faster according to the principle of size-exclusion (Valentin et al., 2014). The aliphatic acids, including acetic acid and formic acid, were completely removed by the resin mixture. This was probably because the two aliphatic acids, based on their pKa values, are present in the anionic form, and they easily bind

with the functional groups of anionic exchangers. HMF was also completely removed in the detoxification process, and the removal could be due to the hydrophobic interaction with the matrix of DOWEX-50WX (polystyrene-divinylbenzene copolymer) based on the observation made by Valentin and coworkers (Valentin et al., 2014).

Furfural was not removed during the detoxification process. The Log P value of furfural indicates that it has the highest hydrophobicity compared to other compounds of the hydrolyzate sample, but furfural was not retained in the resin matrix by hydrophobic interaction. Hydrophobic interaction with the resin matrix is not only dependent on the hydrophobicity, but also on the molecular size of the compound (Kim, Snoeyink and Saunders, 1976). Furfural has a lower molar mass than HMF, and therefore, a weak hydrophobic interaction was expected between the resin matrix and the furfural, despite its high hydrophobicity. Our results showed that the 7 to 3 ratio of DOWEX 50WX8 and IRA743 efficiently removed most inhibitory compounds, including aliphatic acids, without substantial sugar loss. The above results indicate that an appropriate detoxification strategy can be made depending on the type of inhibitory compounds in biomass hydrolyzates and relative adverse effect of these compounds to the targeted culture used in the subsequent fermentation process. However, the economics of the detoxification process is equally critical for large-scale commercial use. Therefore, the economic analysis of the detoxification process must be simultaneously evaluated, in addition to technical efficiency of the process.

## Conclusions

Acid pretreatment process possessed the possibility to generate separate glucose-rich and xylose-rich streams from biomass hydrolyzates. However, harsher acid pretreatment conditions were required in order to make the process effective at improving enzymatic hydrolysis efficiency

of pretreatment biomass to generate sugar. This led to the requirement of developing appropriate detoxification methods to remove pretreatment-induced inhibitors, such as HMF, furfural, and acetic acid. A 1% (w/v) activated charcoal was effective to remove HMF and furfural with 7% sugar loss, but acetic acid was not removed in this method. The mixture of cationic exchangers (DOWEX 50WX8) and anionic exchangers (IRA 743) in 7:3 ratio at pH 2.7 was effective at removing HMF, acetic acid and formic acid with less than 2% sugar loss, but the furfural was not removed in this method. These results indicated that the appropriate detoxification method depends on the type of inhibitors present in the biomass hydrolyzate and the relative adverse effect of these compounds to the targeted culture used in the subsequent fermentation process.

Table 3-1 Sugar and inhibitors released during acid pretreatment and enzymatic hydrolysis

| Sample stream | Glucose (g/L) | Xylose (g/L) | Arabinose (g/L) | Acetic acid (g/L) | HMF (g/L) | Furfural (g/L) |
|---|---|---|---|---|---|---|
| Pretreatment stream | $3.2\pm0.2^A$ | $17.2\pm0.7^A$ | $2.1\pm0.2^A$ | $0.9\pm0.1^A$ | $0.06\pm0.01^A$ | $1.5\pm0.1^A$ |
| Hydrolysis stream | $6.2\pm0.2^B$ | $0.9\pm0.1^B$ | $\leq0.01^B$ | $\leq0.01^B$ | $\leq0.01^B$ | $\leq0.01^B$ |

Acid pretreatment was carried out at 140 °C for 30 min using 1% (v/v) sulfuric acid with 10% (w/v) solids loading. Enzymatic hydrolysis of pretreated biomass was carried out at 50 °C for 48 h using C-Tec2 and H-Tec2 enzymes with 5% (w/v) solids loading. Data represent average value of triplicate experiments ± sample standard deviation. The values with the same letters, in superscripts, within the same column are not significantly different from each other at the $p<0.05$.

Table 3-2 Sugar and inhibitors removal during activated charcoal detoxification

| Charcoal (%) | Total sugars (%) | Acetic acid (%) | HMF (%) | Furfural (%) |
|---|---|---|---|---|
| 1 | 6.8±0.1[A] | 0.9±0.9[A] | 100±0.0[A] | 100±0.0[A] |
| 2 | 7.5±0.3[B] | 3.6±1.3[B] | 100±0.0[A] | 100±0.0[A] |

Activated charcoal detoxification was carried out at 50 °C and 150 rpm for 1 h, and the removal percentage (w/w) was calculated for each compound based on the concentration of the compound before and after detoxification. Data represent average value of triplicate experiments ± sample standard deviation. The values with the same letters, in superscripts, within the same column are not significantly different from each other at the $p < 0.05$

Table 3-3 Properties of monomer sugars and inhibitory compounds

| Compounds | Molecular formula | Molar mass g/mol | Log P[a] | pKa at 25 °C | Precursor |
|---|---|---|---|---|---|
| Glucose | $C_6H_{12}O_6$ | 180.16 | -3.1 | 12.28 | Cellulose |
| Xylose | $C_5H_{10}O_5$ | 150.13 | -3.4 | 12.15 | Hemicellulose |
| Arabinose | $C_5H_{10}O_5$ | 150.13 | -3.02 | 12.43 | Hemicellulose |
| Acetic acid | $C_2H_4O_2$ | 60.05 | -0.17 | 4.76 | Hemicellulose |
| Formic acid | $CH_2O_2$ | 46.03 | -0.54 | 3.75 | Furfural, HMF |
| 5-Hydromethyl furfural | $C_6H_6O_3$ | 126.11 | -0.09 | 12.82 | Hexose |
| Furfural | $C_5H_4O_2$ | 96.08 | 0.41 | Not available | Pentose |

[a] $Log P = log \frac{[\text{ concentrations in } 1-\text{octanol}]}{[\text{concentration in water}]}$

Table 3-4 Ionic exchange resins used in this study and their properties

| Resins | Type | Structure | Mesh size |
| --- | --- | --- | --- |
| DOWEX 50WX8-200 | Strongly acidic cation exchangers | Gel | 100-200 |
| Amberlite IRA743 | Weakly basic anion exchangers | Macroporous | 20-35 |
| Amberlite MB-20 | Mixed bed ion exchangers | Macroporous | 20-50 |
| Resin mixture | Mixture of 50WX8-200 and IRA743 with the ratio of 7 to 3 | Gel/Macroporous | 20-200 |

Table 3-5 Sugar and inhibitors recovery during resin mixture detoxification

| Compounds | Recovery (%) |
|---|---|
| Glucose | $99.5 \pm 0.4$ |
| Xylose | $98.5 \pm 0.4$ |
| Arabinose | $100 \pm 0.0$ |
| Acetic acid | $\leq 0.01$ |
| Formic acid | $\leq 0.01$ |
| HMF | $\leq 0.01$ |
| Furfural | $95.3 \pm 0.2$ |

Resin detoxification was carried out by passing the sample through the column packed with resin mixture, and the recovery percentage (w/w) was calculated for each compound based on the concentration of the compound before and after detoxification. Data represent average value of triplicate experiments ± sample standard deviation.
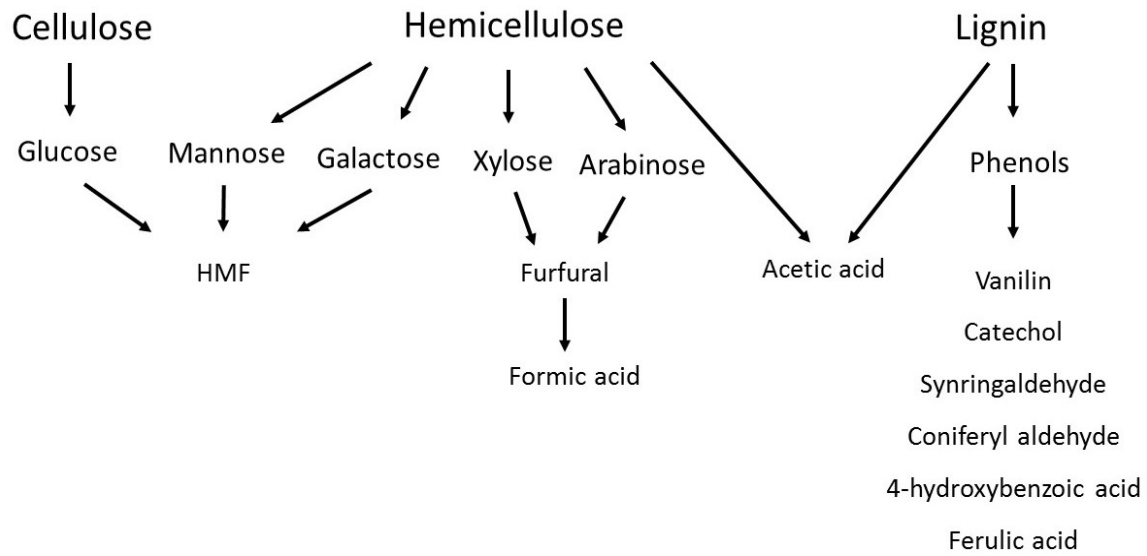
Figure 3-1 Sugar and inhibitors generated during pretreatment and hydrolysis. HMF = Hydroxymethyl fufural
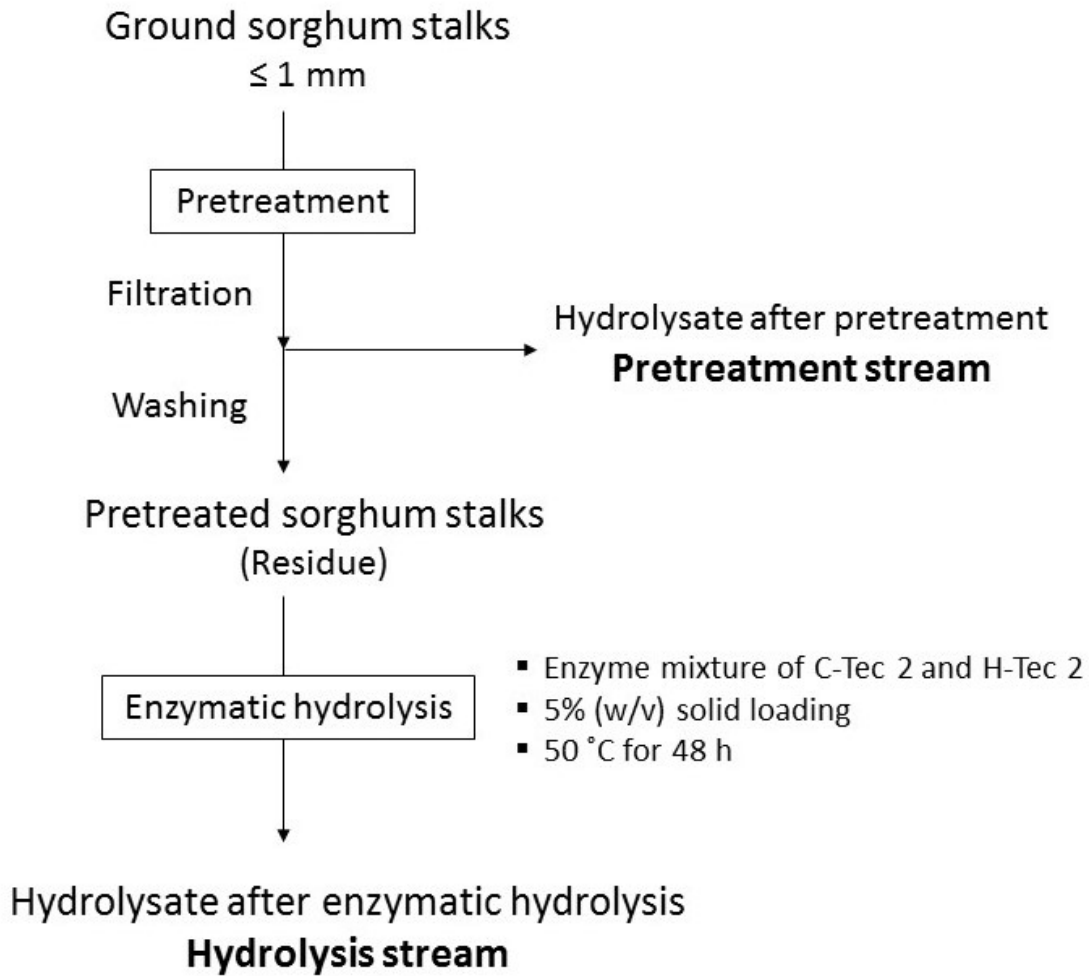
Figure 3-2 Schematic diagram for sample preparation procedure. Pretreatment was carried out using either 1% (v/v) sulfuric acid or 1% (w/v) sodium hydroxide at different time and temperature combinations
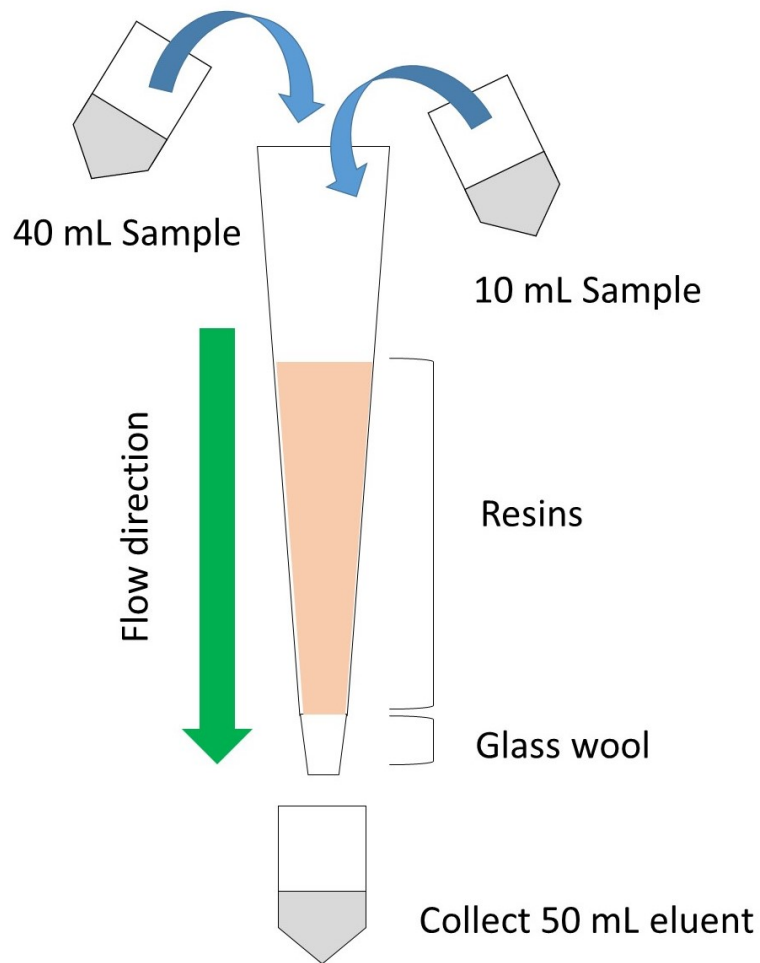
Figure 3-3 Schematic diagram for the detoxification column used in ionic exchangers. A 10 mL sample followed by 40 mL water (eluent) was passed though the column packed with ionic exchange resins, and the throughput was analyzed for sugar and inhibitors
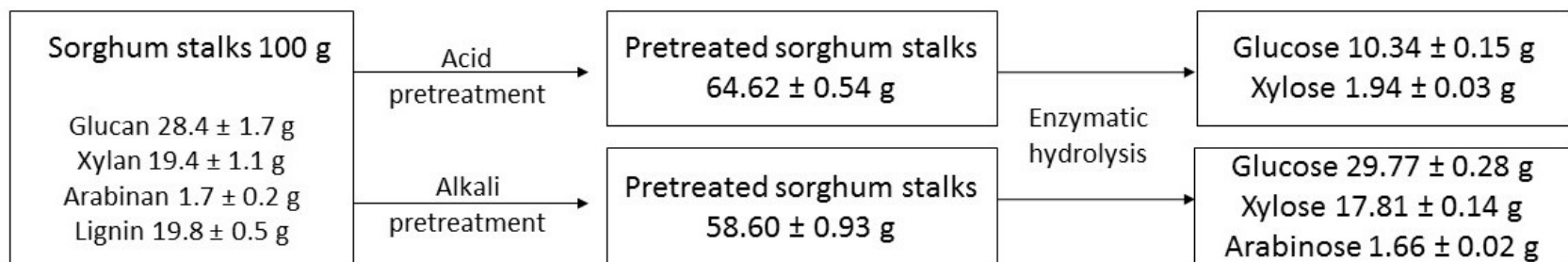
Figure 3-4 Mass balance of monomer sugar released from ground sorghum stalk. Pretreatment was conducted at 121 °C for 30 min using 1% (v/v) sulfuric acid and 1% (w/v) sodium hydroxide for acid and alkali pretreatment, respectively. All the experiments were carried out in triplicate, and the data are the mean value ± sample standard deviation.
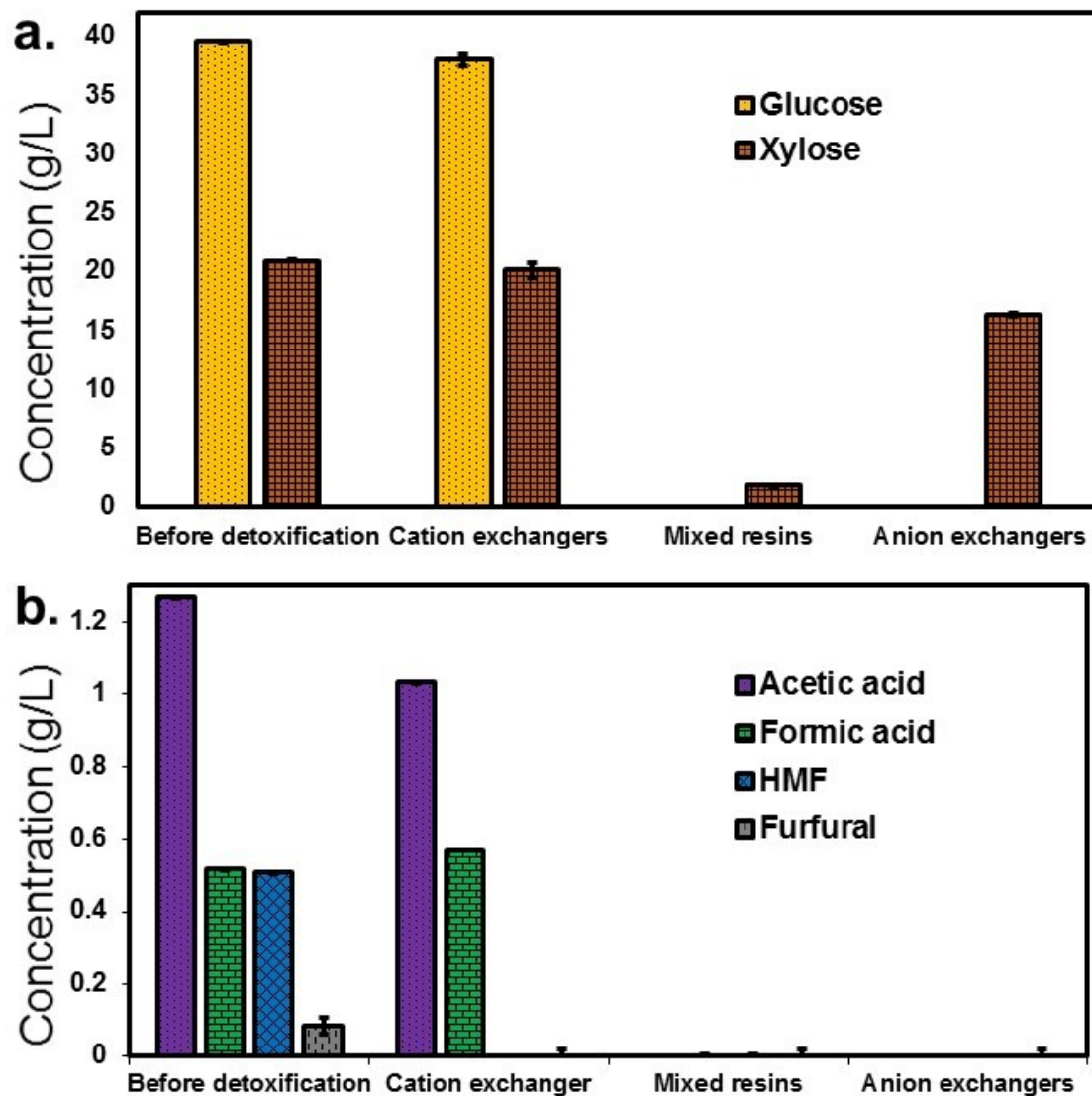
Figure 3-5 Detoxification of sorghum hydrolysate using different resin; A. Sugar concentration before and after detoxification with different types of resins; B. Inhibitors concentration before and after detoxification with different types of resins. The data are average values of triplicate experiments, and the error bars represent sample standard deviation of the data.

# Chapter 4 - Optimization of microbial lipids production from lignocellulosic hydrolysates using *Trichosporon oleaginosus*

## Abstract

For industrial feasibility of lipids production, achieving a high titer of lipids on a large scale is still challenging due to complex factors affecting the lipids production by oleaginous yeasts. The objective of this study was optimization of fermentation conditions for *T. oleaginosus* ATCC20509 to obtain high concentrations and content of microbial lipids. In this study, the molar ratio of carbon and nitrogen sources (C:N ratio) for *Trichosporon oleaginosus* was optimized using response surface methodology (RSM). In fed-batch fermentation using optimized media, a total 9 g/L lipids concentration was produced from the initial 50 g/L of glucose. A total of 22 g/L lipids were achieved with a 76 % (w/w) lipids content by supplementation of additional glucose. Also, the optimized C:N ratio was tested using different types of lignocellulosic hydrolysates such as switchgrass, sorghum stalks and woody biomass. *T. oleaginosus* showed the best performance in the sorghum stalk hydrolysates achieving 13g/L lipids concentrations with 60% (w/w) lipid accumulation. In the switchgrass and woody biomass hydrolysates, total 12 g/L and 9 g/L lipids were produced, respectively. Our investigation showed the potentiality of *T. oleaginosus* as an industrial strain for microbial lipid production from lignocellulosic biomass in biorefineries.

## Introduction

Recently, industrial production of microbial lipids from agricultural biomass has been gaining substantial attention. Microbial lipids are a third-generation biofuel source for biodiesel production via transesterification process (Sitepu et al., 2014). Life cycle assessments (LCAs) showed great reduction of $CO_2$ emission in biofuel production (Jin et al., 2015). The main reason is $CO_2$

produced by fuel combustion is offset by $CO_2$ captured from the growing biomass, which is later utilized as feedstock for biofuel fermentation (d'Espaux et al., 2015). Microbial lipids contain high portions of oleic acid (more than 40%), which is preferred for the biodiesel industry (Firestone, 1999). Also, biofuel production using microorganisms can avoid the public debate of "food vs fuel", and year-round production is available (Sitepu et al., 2014). Conventional biofuel fermentation utilizes naturally occurring organisms that are highly competitive in specific operating environments (Shaw et al., 2016). There have been many attempts at producing microbial lipids using bacteria, yeasts and filamentous fungi. Among these microorganisms, yeasts are typical industrial microbes that have been utilized for the production of bio-based chemicals and fuels. The advantages of yeasts in biotechnology applications are faster growth, less susceptibility to infection, long history of safe use, and the ability to control bacterial contamination using low pH growth (Sitepu et al., 2014). Special types of yeasts are classified as "oleaginous" yeasts, which can accumulate at least 20% oils as a percentage of dry cell weight (Ratledge, 1979). Various species of lipids could be generated by oleaginous yeasts including triacylglycerols, diacylglycerols, monoacylglycerols, fatty acids, steryl esters, free sterols, glycerophospholipids, etc (Schweizer, 2004). However, oleaginous yeasts store lipids mainly in the form of triacylglycerides (TAG), and fatty acids are the primary components in TAG. (Sitepu et al., 2014).

Oleaginous yeasts are known to accumulate higher levels of lipids in the nitrogen limited condition (Ratledge and Wynn, 2002). Nitrogen deficient conditions cause the reduced flux of citric acid cycle, and mitochondrial citrate are pumped into the cytosol inducing flux through lipid synthesis by increasing the cytosolic Acetyl-CoA pool (Calvey et al., 2016). For obtaining a high content of lipids within cells, molar ratio of carbon and nitrogen sources (C:N ratio) in the media

should be optimized. However, higher lipids content of oleaginous yeasts does not guarantee a high titer of lipids production because they are intracellular products. Cell density is also a critical factor for achieving high concentration of lipids using oleaginous yeasts. To the best of our knowledge, most research for optimizing C:N ratio have been focused on improving lipid content in oleaginous yeasts.

Previous studies have reported a high level of lipid accumulation (30 to 70%) in *Trichosoporon oleaginosus ATCC20509* using lignocellulosic hydrolysates, such as sweet sorghum bagasse, wheat straw, and corn stover (Liang et al., 2014; Gong et al., 2014; Yu et al., 2011). *T. oleaginosus* ATCC20509 was previously referred to as *Cryptococcus curvatus, Candida curvata* or *Apiotrichum curvatum*, and recently was assigned to the basidomycetous genus *Trichosporon* (Görner et al., 2016). *T. oleaginosus* ATCC20509 is considered an ideal oleaginous strain for producing microbial lipids from lignocellulosic hydrolysates because their lipid composition resembles seed oils, which are preferred in biodiesel industry (Slininger et al., 2016). Also, *T. oleaginosus* ATCC20509 consumes a diverse variety of weak acids as nutrients, including acetic acid, levulinic acid, and formic acid, which are inevitable inhibitory compounds generated during processing for hydrolysates production (Liang et al., 2014). Therefore, the additional process for purifying lignocellulosic hydrolysates is not required for lipid production using *T. oleaginosus*.

The purpose of this study is to optimize fermentation conditions for *T. oleaginosus* to achieve a higher titer of lipids from lignocellulosic hydrolysates. First, the C:N ratio of the fermentation medium was optimized using the response surface method (RSM) for obtaining a high concentration of lipids. In addition, lipid production was scaled up on the fermenter level to test the optimized C:N ratio using synthetic sugars. The optimized C:N ratio was applied for lipid

production from lignocellulosic hydrolysates, including switchgrass, sorghum stalks and woody biomass.

## Materials and Methods

### *Microorganism and culture condition*

*Trichosporon oleaginosus* ATCC 20509 was obtained from the American Type Culture Collection (ATCC, Manassa, VA, USA) for this study. Inoculum of *T. oleaginosus* was prepared by growing them into 50mL of liquid YM media (YM broth, Difco Laboratories, Detroit, MI, USA). A 10% (v/v) solution of inoculum was transferred into the fermentation media. Cells were incubated at 25 °C and 200 rpm in the shakers (Innova 4300, New Brunswick Scientific, NJ, USA) for 120h. The initial pH was adjusted at 5 using 3M hydrochloric acid or 10N sodium hydroxide.

### *Lignocellulosic hydrolysates preparation*

Woody biomass hydrolysates containing 403 g/L of glucose and 140 g/L of xylose were obtained from Technology Holding LLC, Salt Lake City, Utha, USA. Sorghum stalks were obtained from Texas A&M University, College station, Texas, USA, and switchgrass was obtained from the Kansas State University Agronomy Farm, Manhattan, Kansas, USA. These were all utilized as feedstock for producing hydrolysates *via* the developed process in our lab. Sorghum stalks and switchgrass were pretreated with 1.25 % (w/v) sodium hydroxides at 121 °C for 30 min. and 1 h, respectively. The pretreated biomass was washed with water until sodium hydroxide residue was removed, and completely dried at room temperature for 72 h.  The pretreated biomass was mixed with 50 mM citrate buffer solution, and Cellic C-Tec2 and Cellic H-Tec2 enzymes (Novozyme Inc, Franklinton, NC) were added with a dosage of 8 FPU per gram of biomass. Enzymatic hydrolysis was carried out at 50 °C for 48 h and harvested *via* centrifugation at 8500 rpm for 20 min (Sorvall Super T21, Thermo Fisher Scientific Inc., Waltham, MA, USA). Sorghum

stalks were composed of 49.9 g/L of glucose, 22.2 g/L of xylose, 2.8 g/L of arabinose, and switchgrass hydrolysates were composed of 44.3 g/L of glucose, 19.7 g/L of xylose, and 2.8 g/L of arabinose.

### *Fermentation media*

Shake flask fermentation was performed in 50 mL of synthetic minimal media or lignocellulosic hydrolysates (sorghum stalks, switchgrass and woody biomass hydrolysates). Minimal media contained 50 g/L of glucose, 0.65 g/L of urea, 2.7 g/L of $KH_2PO_4$, 0.95 g/L of $NaHPO_4$, 0.2 g/L of $MgSO_4 \cdot 7H_2O$, 0.1 g/L of yeast extract, 0.1 g/L of ethylene- diamine-tetra-aceticacid, 0.04 g/L of $CaCl_2 \cdot 2H_2O$, 0.0055 g/L of $FeSO_4 \cdot 7H_2O$, 0.0052 g/L of citric acid$\cdot H_2O$, 0.001 g/L of $ZnSO_4 \cdot 7H_2O$, 0.00076 g/L of $MnSO_4 \cdot H_2O$. Each lignocellulosic hydrolysate (sorghum stalks, switchgrass and woody biomass hydrolysates) was diluted using water to adjust the total sugar concentrations to 50 g/L. The ratio of carbon to nitrogen was adjusted to 76. The pH of the media was adjusted to 5.0 using 3 M hydrochloric acid and 10 N sodium hydroxide.

Fed-batch fermentation was carried out in a 7 L fermenter with a working volume of 5 L (Bioflo 110, New Brunswick Scientific Inc., Enfield, CT, USA). The fermentation broth was minimal media containing 50 g/L of glucose, 0.65 g/L of urea, 2.7 g/L of $KH_2PO_4$, 0.95 g/L of $NaHPO_4$, 0.2 g/L of $MgSO_4 \cdot 7H_2O$, 0.1 g/L of yeast extract, 0.1 g/L of ethylene- diamine-tetra-aceticacid, 0.04 g/L of $CaCl_2 \cdot 2H_2O$, 0.0055 g/L of $FeSO_4 \cdot 7H_2O$, 0.0052 g/L of citric acid$\cdot H_2O$, 0.001 g/L of $ZnSO_4 \cdot 7H_2O$, 0.00076 g/L of $MnSO_4 \cdot H_2O$. Agitation speed was maintained at 300

rpm and the temperature was kept at 25 ˚C. The pH was maintained at 5 using 10 N sodium hydroxide and air was supplied at 1 vvm during fermentation.

## *Analytical methods*

Cell counting was conducted using a hemacytometer (Hausser Scientific, Horsham, PA, USA). Dry-cell weight (DCW) was determined by drying cells at 80 ˚C for 14h and then utilized for the calculation of lipid yields. Glucose, xylose, arabinose, acetic acid, hydroxymethylfurfural (HMF), and furfural were quantified using HPLC (Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a refractive index detector (RID-10A) and a UV/VIS detector (SPD-M20A). The mobile phase was 0.005 N sulfuric acid and went through the column with a flow rate of 0.6 mL /min. Oven temperature was maintained at 80 ˚C during analysis.

## *Lipid extraction and fatty acid analysis*

Cells were harvested *via* centrifugation at 8500 rpm for 20 min and washed using $dH_2O$ two times to remove residues from the culture media. Yeast cells were concentrated to $\leq 10^9$ cells m/L. The concentrated cells were transferred into micro tubes for short-term storage at -80 ˚C. Dry weight of the concentrated cells was measured to calculate the lipid content within yeast cells by drying cells at 80 °C for 14 h. Lipid content was calculated by dividing the weight of extracted lipids by the amount of dry cell mass.

For lipid extraction, a 0.5 mL aliquot of yeast cells was centrifuged and the supernatant was discarded. To break the cells, 1mL of silica beads (0.55 mm cubic zirconia beads, BioSpec Products, Bartlesville, OK, USA) was added followed by the addition of 0.5 mL of chloroform and 0.5 mL of methanol. Cells were lysed by beads beating (Mini-Beadbeater-24, BioSpec Products, Bartlesville, OK, USA) for a total of six cycles of 45 sec intervals and cooling on ice for 10 min. The cell lysates were transferred into a 16x150 mm kimax glass tube and additional

chloroform, methanol, and water were added with the ratio of 1:2:0.8 (chloroform:methanol:water). The cell lysate mixtures were vortexed and centrifuged at 4000 rpm for 15 min. After centrifugation, two separated liquid layers were obtained. The bottom layer of chloroform containing lipids was collected using Pasteur pipette from the mixture and transferred into a clean Kimax tube. This step was repeated three times to completely take the chloroform layer to minimize lipid loss. The combined chloroform layer was filtered using a synringe filter (Whatman, Fisher Scientific., 0.22 μm of pore size) and completely dried out under nitrogen gas at 40 ˚C. The weight of residual lipids were measured to calculate the oil content in yeast cells. Lipids were re-dissolved in the chloroform for analyzing fatty acid composition.

Transesterification of fatty acids was conducted for the analyzing the profile of fatty acids. Fatty acids were methylated using 3 M methanolic hydrochloric acid at 78 ˚C for 30 min. Hexane was added and vortexed to extract the fatty acid methyl esters (FAME). Pentadecanoic acid (C15:0) was added as an internal standard. Samples were analyzed using a gas chromatograph (GC-2014, Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a capillary column (Zebron ZB-Waxplus 30 m x 0.25 mm x 0.25 μm, Phenomenex, Torrance, CA, USA) and a flame ionization detector (FID). Samples were injected to 220 ˚C and the column temperature was increased to 160 ˚C with a flow rate of 3mL/min. The temperature of FID was set at 250 ˚C. An external standard (Supleco 37 Component FAME Mix) was utilized to confirm the retention time of each FAME compound.

## *Statistical analysis*

All experiments were conducted in triplicate, and data were statistically analyzed using JMP software (SAS Institute Inc., Cary, NC, USA). The least significant difference (LSD) test was carried out with 95% confidence level ($P < 0.05$).

# Results and discussion

## *Lipid production by T. oleaginosus in batch fermentation*

Batch fermentation was performed in a fermenter scale to understand the fermentation properties of *T. oleaginosus* during lipids production. General YM broth containing 20 g/L of glucose was utilized as the fermentation medium without adjusting the C:N ratio. Figure 4.1 shows the fermentation profile of *T. oleaginosus*. A total of 30 % lipids per dry cell mass were accumulated within yeast cells and 8 g/L dry cell weight were sustained for 40h. Therefore, a total of 2 g/L lipids were produced using 20 g/L glucose by *T. oleaginosus*. The composition of fatty acids in lipids produced by *T. oleaginosus* was investigated (Figure 4.2). The major fatty acids were oleic acid (C18:1), palmitic acid (C16:0), and linoleic acid (C18:2) consisting of about 40 % (w/w), 28 % (w/w), and 20 % (w/w) respectively. The remaining fatty acids in the lipids were composed of stearic acid (C18:0), linolenic acid (C18:3), and palmitoleic acid (C16:1) which correlate with other studies. Gong and coworkers (2014) also reported that the major fatty acid produced by *T. oleaginosus* ATCC20509 was oleic acid, which was almost 50 % (w/w) of fatty acids in lipids, whereas palmitic acid was about 28 % (w/w).

## *Optimization of initial sugar concentrations and C:N ratio using RSM*

To improve lipid concentrations by *T. oleaginosus*, initial glucose concentrations and C:N ratio were optimized using response surface methodology (RSM). Glucose concentrations and C:N ratio were selected as input factors, while DCW and lipid content were chosen as responses. Previous results in our lab showed that glucose inhibition was observed (not included in this study) when initial concentrations of glucose were more than 50 g/L. Due to this, the range of glucose concentrations were adjusted between 20 to 50 g/L. Table 4.1.B shows thirteen types of experimental runs with results for central composite design. The experimental design showed the

best desirability when glucose concentrations were 50 g/L and C:N ratio was 76 (Table 4.1.C). Figure 4.3 shows 3D surface plots to optimize C:N ratio and initial glucose concentrations. For high levels of lipids content, C:N ratio was a more critical factor than glucose concentrations (Figure 4.3.A). It seems that the C:N ratio is more related with lipids content rather than glucose concentrations. The experimental design expected that the highest content of lipids would be obtained when C:N ratio was 76 regardless of glucose concentrations. Figure 4.3.B shows that both glucose concentrations and C:N ratio were crucial for obtaining high concentrations of DCW. As glucose concentration was increased, cell density was also increased. The surface plot for lipid concentrations had similar patterns with DCW; both glucose concentrations and C:N ratio affected the final lipid concentrations (Figure 4.3.C).

### *Lipid production by T. oleaginosus in the optimized fermentation conditions*

Fed-batch fermentation was performed based on optimized conditions using RSM. Figure 4.4 shows the fermentation profile of lipid production in nitrogen-limited medium. The initial glucose concentration was 50 g/L and the C:N ratio was adjusted to 76. Additional 50 g/L of glucose was fed into the fermenter at 40 h and all of the glucose was consumed at 76 h. The dry cell weight did not reflect an increase of cell numbers. Cell counting results showed that cell growth was stopped, and the stationary phase was started at 24 h. However, DCW was continuously increased during fermentation because the yeast cells kept accumulating lipid. It was assumed that the lipid accumulation mode was started at 24 h by increasing the flux of acetyl CoA toward lipid instead of the citric acid cycle. The initial 50 g/L of glucose was completely consumed at 40 h, and a total of 9 g/L of lipids were produced by accumulating 45% (v/v) lipids within 20 g/L of yeast cells (dry weight basis). Additional 50 g/L of glucose was supplemented at 40 h to induce more accumulations of lipids in yeast. Fermentation was continued until all of the glucose was

consumed. Finally, a total of 22 g/L of lipids were produced by 32 g/L of yeast cells (dry weight basis) with an accumulation of 67% (w/w) lipids (Table 4.2). Improved fermentation performance was observed for the 76 h of fermentation process compared with 40 h. Higher levels of productivity, oil content and product concentrations were achieved. This is because lipid accumulation of oleaginous yeast has mainly occurred during the stationary phase due to secondary metabolites (Calvey et al., 2016).

Figure 4.5 shows the observed yields during the fed-batch fermentation of *T. oleaginosus*. As fermentation continued, Yps and Ypx were continuously increased with a rate of 0.02 g/g/h and 0.01 g/g/h, respectively. However, Yxs was almost the same during fermentation. These results indicate that substrate utilization for biomass production was constant whereas the lipid accumulation rate rapidly increased as fermentation went on. This is probably because cells accumulated lipid using the remaining carbon sources when all of the nitrogen sources were depleted.

Figure 4.6 shows the profile of fatty acids in lipid from *T. oleaginosus* produced during fed-batch fermentation in nitrogen limited media. Major fatty acids are palmitic acid and oleic acid. Besides those, myristic acid, palmitoleic acid, stearic acid, linoleic acid and linolenic acid were produced. The composition of fatty acids produced in nitrogen limited media were similar with fatty acids in YM broth (Figure 4.2). The percentage of palmitic acid and oleic acid were increased, and linoleic acid and linolenic acid were decreased as fermentation proceeded in the nitrogen limited media. A similar trend was reported by other studie; the major fatty acids were palmitic acid and oleic acid, and increased oleic acid and decreased linoleic acid percentages were observed as fermentation progressed in the nitrogen limited media (Tchakouteu et al., 2015).

### *Lipid production from lignocellulosic hydrolysates using T. oleaginosus*

Switchgrass, sorghum stalks and woody biomass hydrolysates were utilized as a feedstock for lipid production using *T. oleaginosus*. Switchgrass and sorghum stalk hydrolysates were prepared *via* the alkali pretreatment and enzymatic hydrolysis processes, and woody biomass hydrolysates were obtained from its manufacturer. Each of the biomass hydrolysates were diluted to make the concentration of initial sugars to around 50 g/L, and the molar ratio of nitrogen and carbon sources was adjusted to 76. Figure 4.7 shows the composition of hydrolysates utilized for lipid production. During the production process of hydrolysates, citric and acetic acid were produced and were included in the fermentation media. All biomass hydrolysates contained a similar amount of glucose (30 g/L) and xylose (15 g/L), and arabinose was only in the switchgrass and sorghum stalk hydrolysates. In the switchgrass and sorghum stalk hydrolysates, about 10 g/L of citrate and less than 1 g/L of acetic acid were included and woody biomass hydrolysates had 2 g/L of citrate.

Lipid production was carried out in the shake flask containing 50 mL of each hydrolysates media at 25 ˚C for 120 h and cells were harvested *via* centrifugation to extract lipids from the yeast. *T. oleaginosus* consumed all of the carbon sources and also citrate, acetate, and monomer sugars for 120 h. Consumption of organic acids by oleaginous yeasts were also reported by other studies (Slininger et al., 2016; Li et al., 2015). Normally, acid compounds were known to be inhibitory compounds for yeast growth by inducing a decrease of intracellular pH (Almeida et al., 2007). However, the consumption of acid compounds by *T. oleaginosus* was desirable for lipid production from lignocellulosic hydrolysates. Table 4.3 shows the fermentation performances from lignocellulosic hydrolysates. In the sorghum stalk hydrolysates, it was observed that *T. oleaginosus* had the best performance of lipid production. The highest lipid concentration of (13 g/L) was achieved using sorghum stalk hydrolysates. Yxs and Yps showed that the substrate

utilization for product and cell biomass was also the most efficient in the sorghum stalk hydrolysate. The highest lipid content was observed in the woody biomass hydrolysate, but the lowest lipid concentration of (15 g/L) was obtained from woody biomass hydrolysates because the lowest level of dry cell weight was achieved. This result showed that both lipid content and dry cell weight are crucial factors for getting high titers of lipids because lipids are intracellular products. A higher level of lipid concentration from switchgrass and sorghum stalk hydrolysates might be since they contained a higher level of citrate compared with woody biomass hydrolysates. Higher levels of cytosolic citrate increased the cytosolic acetyl-CoA pool, which is an important substrate for both lipid synthesis and the acetylation of enzymes participating lipid synthesis (Shi and Tu, 2015). Therefore, citrate included during the process of hydrolysate production was a positive effect on the lipid production of *T. oleaginosus*. The fatty acid profile of lipids produced from different hydrolysate media is shown in Figure 4.8. The main fatty acids in *T. oleaginosus* were palmitic and oleic acid, regardless of the hydrolysate type. This result showed similar patterns with lipid production in YM broth and nitrogen limited media. Same species of fatty acids were produced by *T. oleaginosus* regardless of hydrolysate media types. However, a slightly higher level of oleic acid and a lower level of linoleic acid was obtained from woody biomass hydrolysates compared with switchgrass and sorghum stalks hydrolysates. Other studies also reported that slightly different relative fatty acid content was observed in the different media (Gong et al., 2014; Thiru, Sankh and Rangaswamy, 2011), thus, fermentation media has an effect on fatty acid composition.

## Conclusions

This study shows that *T. oleaginous* is an ideal cell factory for lipid production from lignocellulosic biomass. The optimized C:N ratio of both lipid content and cell density for *T.*

*oleaginosus* provided high levels of lipid accumulation and lipid concentrations in the fermenter level using synthetic medium. Also, this investigation revealed that microbial lipids can be successfully produced by lignocellulosic biomass such as switchgrass, sorghum stalks and woody biomass by applying the optimized C:N ratio for high titers of lipid production. Future work should be directed towards increasing the lipid production process using lignocellulosic hydrolysates.

Table 4-1 Central composite experimental design (A) Factors, inputs and responses utilized for central composite design; (B) Experimental design matrix suggested C:N ratio and initial glucose concentrations; (C) Optimized initial glucose concentrations and C:N ratio for maximizing lipid concentrations.

**(A)**

| | Goal | Lower limit | Upper limit |
|---|---|---|---|
| (A) Glucose | In range | 20 | 50 |
| (B) C:N ratio[a] | In range | 20 | 100 |
| DCW[b] | Maximize | 0.27 | 10 |
| Lipid content | Maximize | 0 | 81 |

**(B)**

| Run | Suggested conditions | | Experimental results | | |
|---|---|---|---|---|---|
| | Glucose | C:N Ratio[a] | DCW[b] | Lipid titers | Lipid content |
| 1 | 35 | 60 | 9.40 | 6.70 | 71 |
| 2 | 20 | 100 | 8.27 | 4.83 | 58 |
| 3 | 20 | 20 | 8.87 | 5.76 | 65 |
| 4 | 14 | 60 | 7.47 | 4.58 | 61 |
| 5 | 35 | 60 | 9.27 | 7.51 | 81 |
| 6 | 35 | 60 | 9.47 | 5.94 | 63 |
| 7 | 35 | 117 | 8.40 | 5.17 | 62 |
| 8 | 56 | 60 | 10.00 | 6.05 | 61 |
| 9 | 50 | 100 | 9.87 | 5.58 | 57 |
| 10 | 35 | 60 | 9.27 | 5.69 | 61 |
| 11 | 50 | 20 | 7.60 | 4.66 | 61 |
| 12 | 35 | 3 | 0.27 | 0.00 | 0 |
| 13 | 35 | 60 | 9.80 | 4.25 | 43 |

**(C)**

| Rank | Glucose | C:N ratio[a] | DCW[b] | Lipid content | Desirability |
|---|---|---|---|---|---|
| 1 | 50 | 76.14 | 10.7 | 67 | 0.91 |
| 2 | 20 | 71.29 | 9.2 | 68 | 0.88 |
| 3 | 20 | 70.42 | 9.2 | 68 | 0.88 |

[a]C:N ratio = the molar ratio of carbon to nitrogen sources, [b]DCW = dry cell weight

Table 4-2 Fermentation performance of *T. oleaginosus* using nitrogen limited media

| Fermentation time | Productivity[a] (g/L/h) | Lipid concentrations (g/L) | Lipid content[b] (%) | DCW (g/L) |
|---|---|---|---|---|
| 40h | $0.2 \pm 0.0^A$ | $9 \pm 0.4^A$ | $45 \pm 1^A$ | $20 \pm 0.9^A$ |
| 76h | $0.3 \pm 0.0^B$ | $22 \pm 1.9^B$ | $67 \pm 2^B$ | $21 \pm 2^B$ |

This data represents the average value of triplicate experiments ± sample standard deviation. The values with the same letters, in superscripts, within the same column are not significantly different from each other at the $p<0.05$.
[a]Productivity was defined as the amount of lipids produced per liter per hour.
[b]Lipid content was calculated by dividing the weight of extracted lipids by the amount of dry cell mass.

Table 4-3 Fermentation performance during lipid production from different types of lignocellulosic hydrolysates

| Lignocellulosic hydrolysates type | $Yxs^a$ (g/g) | $Yps^b$ (g/g) | $Ypx^c$ (g/g) | Lipid content$^d$ (%) | DCW (g/L) | Lipid concentrations (g/L) | Productivity$^e$ (g/L/h) |
|---|---|---|---|---|---|---|---|
| Switchgrass | $0.46 \pm 0.0^A$ | $0.27 \pm 0.01^A$ | $0.58 \pm 0.03^A$ | $58 \pm 2.6^A$ | $21 \pm 0.6^A$ | $12 \pm 0.2^A$ | $0.1 \pm 0.0^A$ |
| Sorghum stalks | $0.48 \pm 0.0^A$ | $0.29 \pm 0.01^B$ | $0.60 \pm 0.03^A$ | $60 \pm 2.5^A$ | $22 \pm 0.3^A$ | $13 \pm 0.7^A$ | $0.1 \pm 0.0^A$ |
| Woody biomass | $0.3 \pm 0.0^B$ | $0.2 \pm 0.01^C$ | $0.60 \pm 0.02^A$ | $62 \pm 2.0^A$ | $15 \pm 0.3^B$ | $9 \pm 0.3^B$ | $0.1 \pm 0.0^B$ |

This data represents the average value of triplicate experiments ± sample standard deviation. The values with the same letters, in superscripts, within the same column are not significantly different from each other at the $p<0.05$.
[a]Ypx was calculated by dividing the amount of lipids by the amount of dry cell mass
[b]Yps was calculated by dividing the amount of lipids by the amount of consumed glucose
[c]Yxs was calculated by dividing the amount of dry cell mass by the amount of consumed glucose
[d]Lipid content was calculated dividing the weight of extracted lipids by the amount of dry cell mass.
[e]Productivity was defined as the amount of lipids produced per liter per hour.
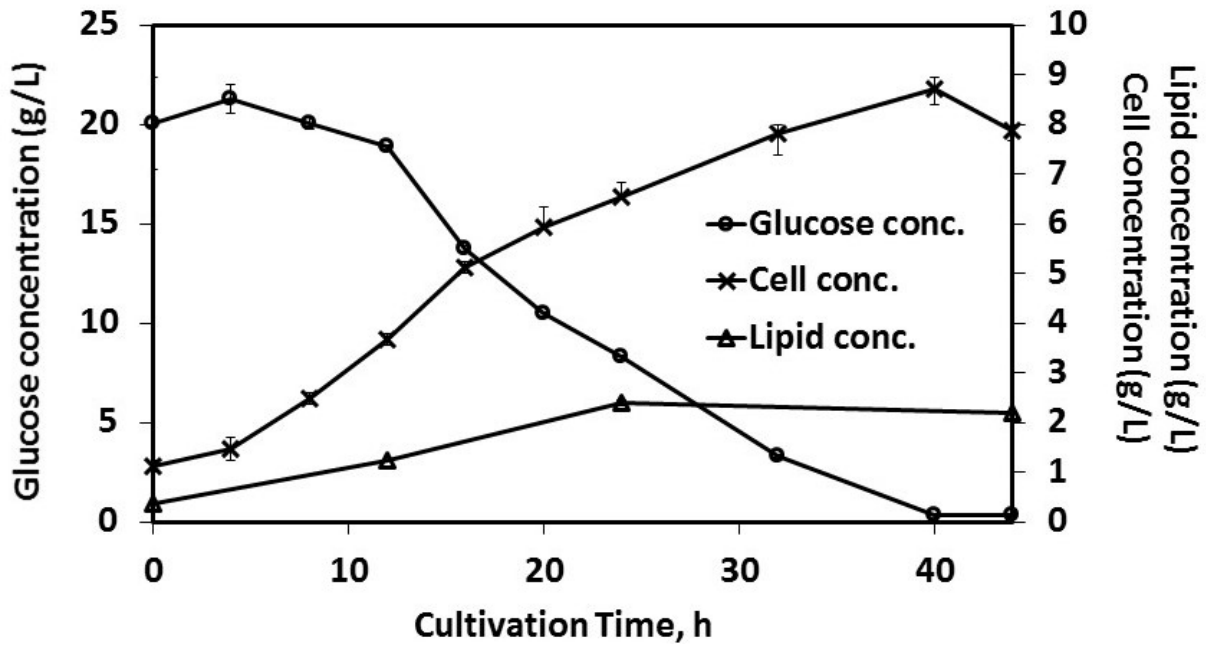
Figure 4-1 Batch fermentation of lipid production by *T. oleaginosus* in a YM broth. Fermentation was carried out at 25 ˚C and 300 rpm in the fermenter level with a 5 L working volume. Data shows the average value of triplicate experiments and error bars representing sample standard deviation.
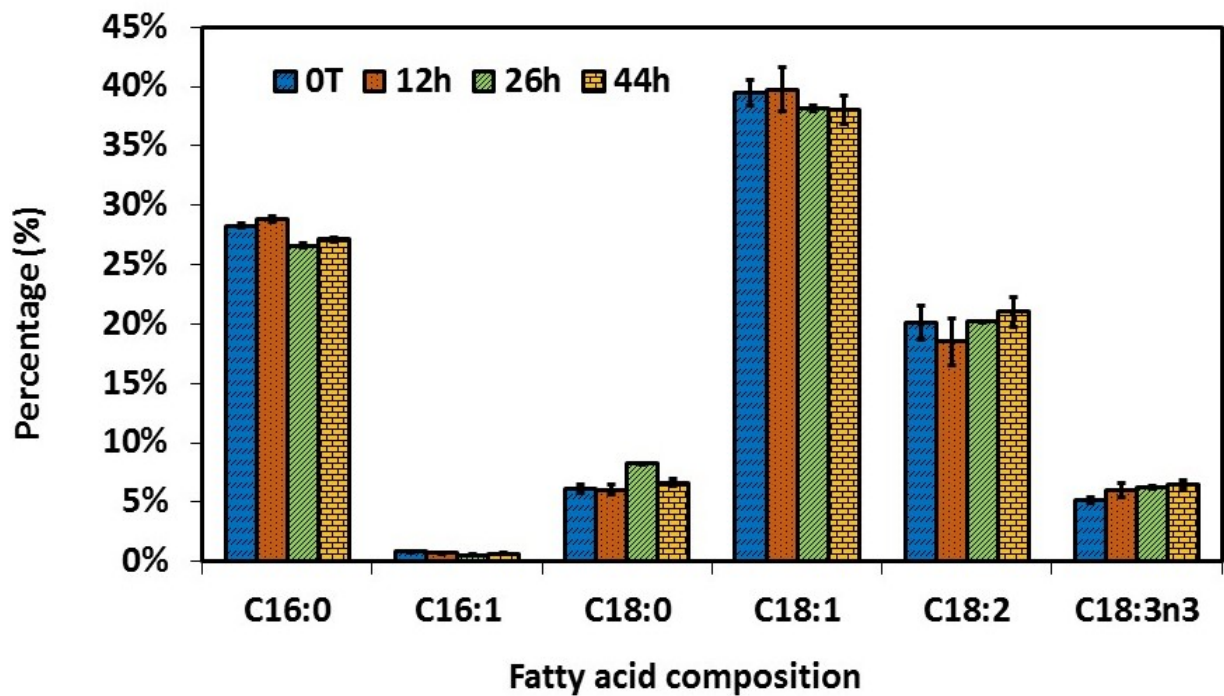
Figure 4-2 Fatty acid profile of lipids accumulated in *T. oleaginosus* from YM broth. The data shows average value of triplicate experiments and error bars representing sample standard deviation.
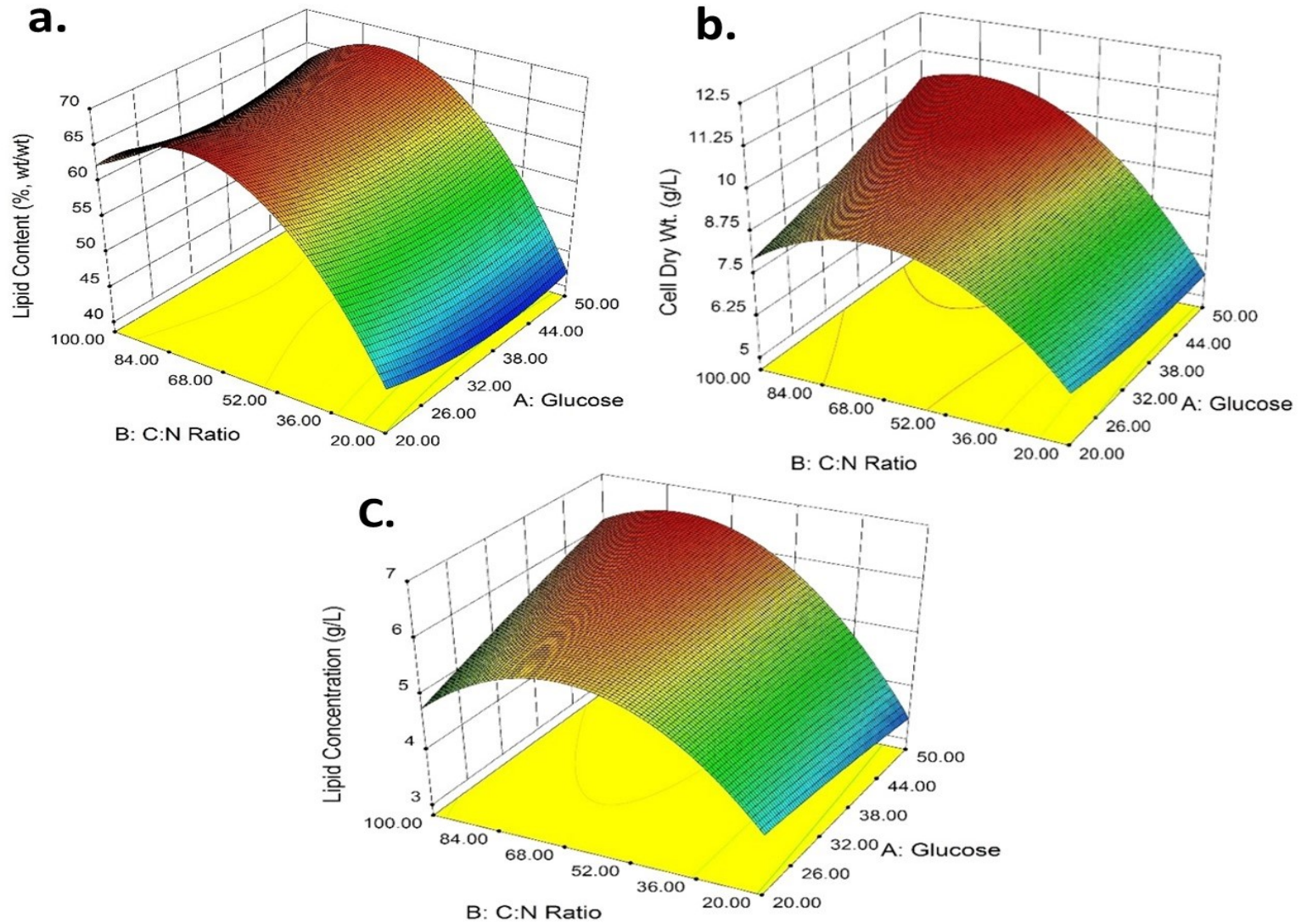
Figure 4-3 Surface plot for optimizing C:N ratio and initial glucose concentrations. (A) for maximizing lipid content; (B) for maximizing dry cell weight; (C) for maximizing lipid concentrations.
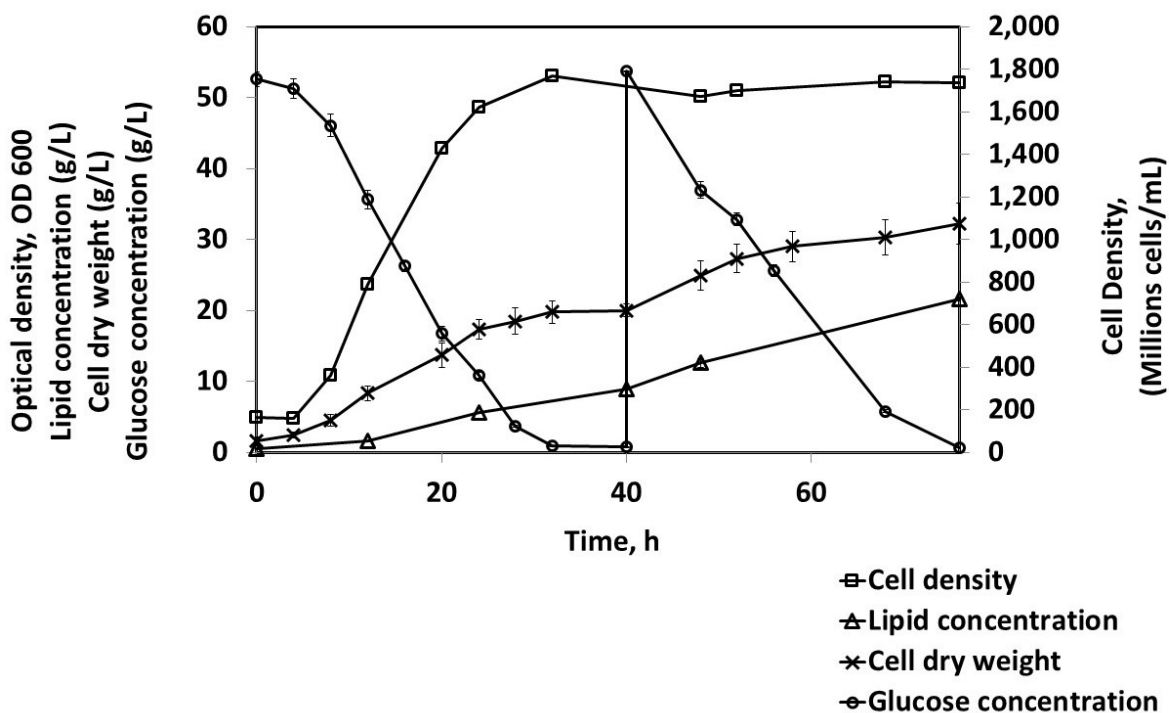
Figure 4-4 Fed-batch fermentation of lipid production by *T. oleaginosus* in N-limited media. Fermentation was carried out at 25˚C and 300 rpm in the fermenter level with a 5 L working volume and additional glucose was fed at 40 h. The data shows average value of triplicate experiments and error bars representing sample standard deviation.
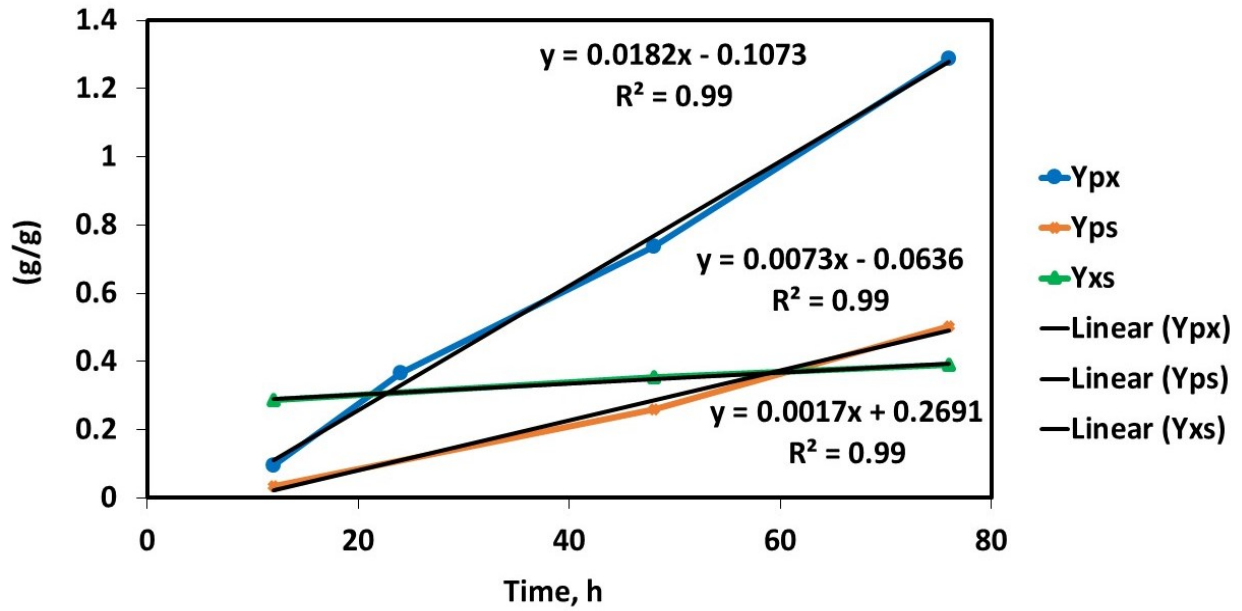
Figure 4-5 Time courses of observed yields during lipid production by *T. oleaginosus.*
Ypx was calculated by dividing the amount of lipids to the amount of dry cell mass; Yps was calculated by dividing the amount of lipids to the amount of consumed glucose; Yxs was calculated by dividing the amount of dry cell mass to the amount of consumed glucose.
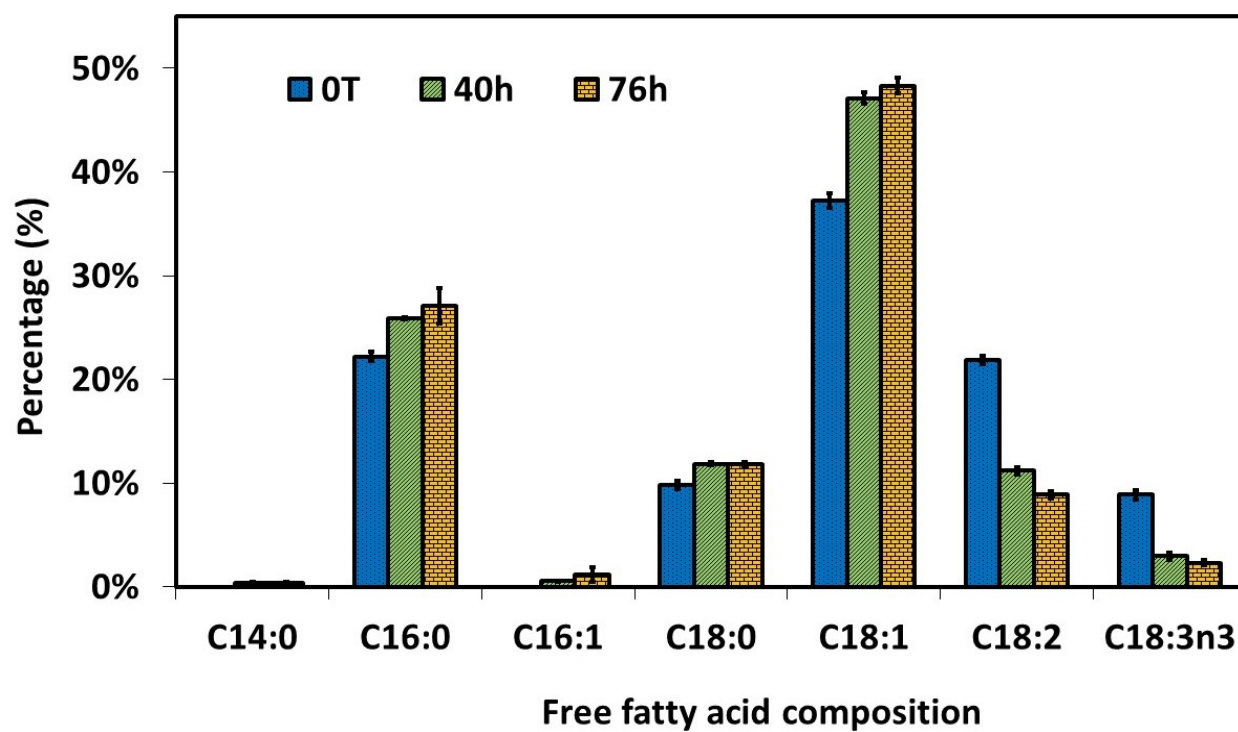
Figure 4-6 Fatty acid profile of lipids accumulated by *T. oleaginosus* in N-limited media.

The data shows average value of triplicate experiments and error bars representing sample standard deviation.
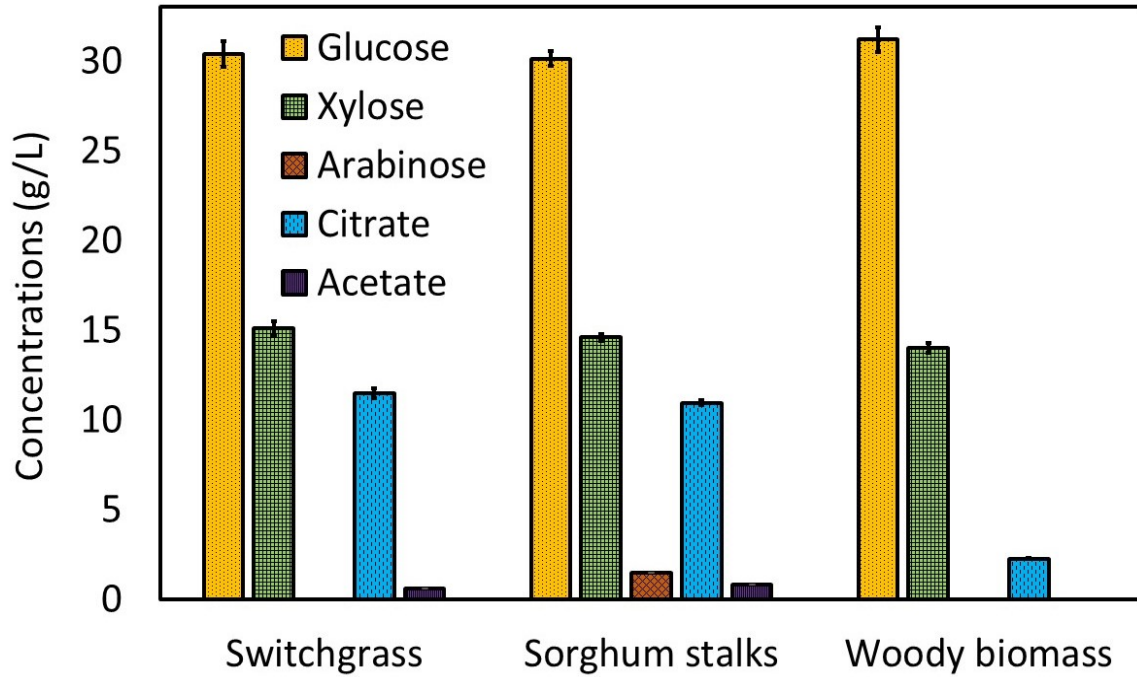
Figure 4-7 Composition of lignocellulosic hydrolysates utilized for fermentation.

The data shows average value of triplicate experiments and error bars representing sample standard deviation.
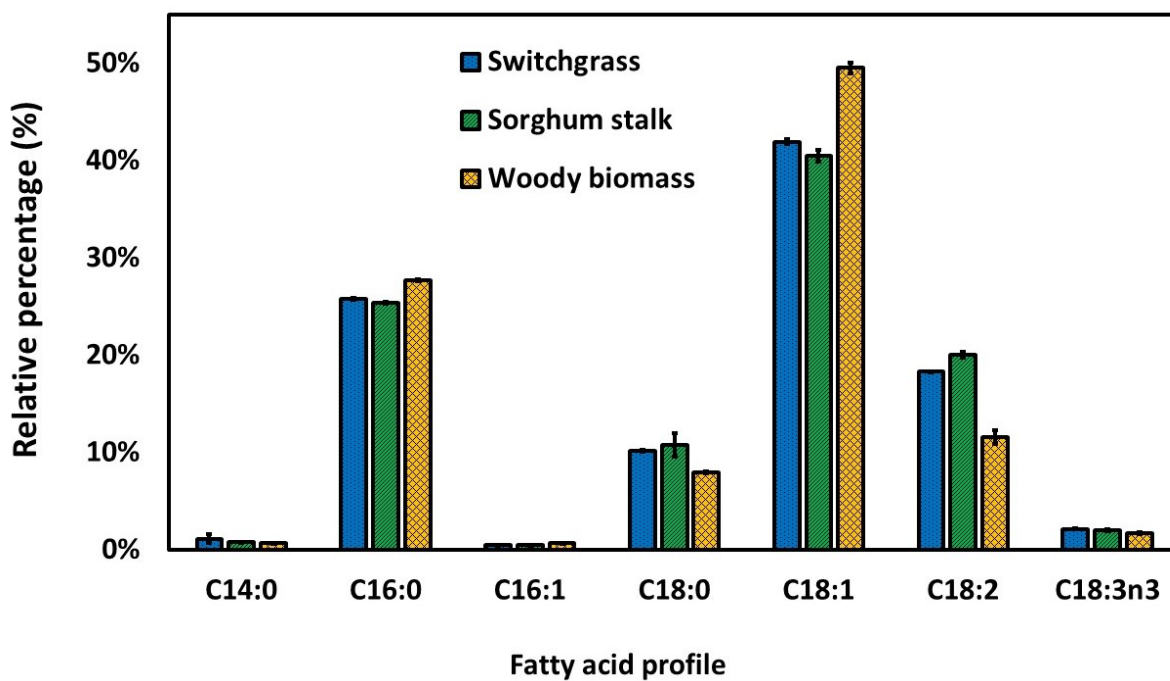
Figure 4-8 Fatty acid profile of lipid accumulated in *T. oleaginosus* from lignocellulosic hydrolysates. The data shows average value of triplicate experiments and error bars representing sample standard deviation.

# Chapter 5 - Production of free fatty acids from switchgrass using recombinant *Escherichia coli*

## Abstract

Switchgrass is a promising feedstock to generate fermentable sugars required for the sustainable operation of biorefineries because of their abundant availability, easy cropping system, and high cellulosic content. The objective of this study was to investigate the potentiality of switchgrass as an alternative sugar supplier for free fatty acid (FFA) production using recombinant *Escherichia coli* strains, ML103 *pXZ18* and ML103 *pXZ18Z*. Both recombinant *E. coli* strains successfully produced FFA using switchgrass hydrolysates attaining substantially higher concentrations of FFA compared with synthetic sugars. Total of 2.8 and 3.3 g/L FFA were attained from switchgrass hydrolysates by ML103 *pXZ18* and ML103 *pXZ18Z* strains, respectively, which were 8% and 14% higher amount of FFA, respectively than that of using synthetic sugars. Further, overall yield assessment of our bioconversion process showed that 88% and 46% of the theoretical maximal yields of glucose and xylose were attained from raw switchgrass during sugar generation. Additionally, 72% of theoretical maximum yield of FFA were achieved from switchgrass hydrolysates by ML103 *pXZ18Z* strain during fermentation. These shake-flask results were successfully scaled up to a laboratory scale bioreactor with 4 L working volume, in which the FFA productivity was doubled without affecting product yields. This study demonstrated an efficient bioconversion process of switchgrass-based FFAs using engineered microbial system for targeted fatty acids production that are secreted into the fermentation broth with associated lower downstream processing cost, which is pertinent to develop an integrated bioconversion process using lignocellulosic biomass.

# Introduction

Bio-based fatty acids (FAs) produced by microbes are promising chemical feedstocks for replacing plant oils. Microbial fatty acids can be effectively utilized for oleochemical industries such as lubricants, surfactants, soaps, and polymer additives (Lennen and Pfleger, 2012). The genetically modified *Escherichia coli* is an excellent cell factory for fatty acid production due to its abundance of genetic information and well-studied fatty acid metabolism (Tee et al., 2014). Fatty acids are inherent components in *E. coli* metabolism for energy storage, membrane integrity, and signaling metabolism (Jin et al., 2015). Despite the intrinsic ability for FAs synthesis, wild type *E. coli* normally does not secrete free fatty acids as intermediates in the fermentation medium (Tee et al., 2014). However, FA production in engineered *E. coli* is typically achieved by an expression of thioesterase with deletion of the ß-oxidation gene (*FadE*) to make *E. coli* secrete the FA in the medium (Torella et al., 2013). While most of the oleaginous microorganism accumulates lipids within their body, the recombinant *E. coli* strains secret the FA in the media, and thereby make easy to harvest final product from medium for the down-stream process without an extra process or using hazardous solvents during product extraction.

Figure 5.1 shows the metabolic pathway for FFA production in the recombinant *E. coli* strains used in this study. FA synthesis is initiated by the carboxylation of acetyl-CoA to malonyl CoA, followed by condensation to a linear acyl chain, normally $C_{12}$ to $C_{22}$ carbons. The FAs are released by thioesterase and secreted into the medium (d'Espaux et al., 2015). Wu and coworkers expressed acyl-ACP thioesterase derived from *Ricinus communis* in the recombinant *E. coli* strains (ML103 *pXZ18* and ML103 *pXZ18Z*) and provided them for this study. Therefore, the recombinant *E. coli* strains (ML103 *pXZ18* and ML103 *pXZ18Z*) used in this study have an ability to secrete FFA into media. The biggest difference between *E. coli* ML103 *pXZ18* and *pXZ18Z* is the expression of β-

hydroxyacyl-ACP dehydratase; plasmid *pXZ18* is *pTrc99a* carries an acyl-ACP thioesterase, whereas plasmid *pXZ18Z* is *pTrc99a* carries an acyl-ACP thioesterase and β-hydroxyacyl-ACP dehydratase (Wu et al., 2014; Wu et al., 2015).

Microbial lipid production from expensive sugar sources such as starch has been explored extensively, but it is yet to be economically viable as an alternative of plant oils and fats markets (Jin et al., 2015). For economic feasibility of microbial lipid production, lignocellulosic biomass has received extensive attention as a low-cost substrate in a biorefinery (Gong et al., 2016). Lignocellulosic biomass can be supplied on a large-scale from different low-cost materials such as industrial wastes, wood, and agricultural residues (Limayem and Ricke, 2012). Lignocellulosic biomass varies among species but generally consists of ~25% lignin and ~75% carbohydrate polymers in dry weight (cellulose and hemicellulose), and is the largest known renewable carbohydrate source (Zhang et al., 2016). Switchgrass (*Panicum virgatum, L*) is a warm season herbaceous crop, and one of the most promising bioenergy crops among lignocellulosic biomass due to its high cellulose (40-45%) and hemicellulose (30-35%) content, low-cost investment, facile management, and abundance in the U.S. (Limayem and Ricke, 2012; Liu et al., 2015). The productivity of switchgrass is 13.4 – 22.3 t/ha, which is much higher than the yield range of corn (6.3 – 8.7 t/ha) (McLaughlin and Kszos, 2005). A recent energy model analyses estimated that switchgrass could produce >700% more output than input energy, whereas an estimated average greenhouse gas (GHG) has been estimated to be slightly positive for ethanol derived from switchgrass (Farrell et al., 2006). The switchgrass was chosen as an important herbaceous plant at the Bioenergy Feedstock Development Program (BFDP) in the Department of Energy (DOE) and Biofuels Feedstock Program in the Oak Ridge National Laboratory (Min et al., 2017). For these

reasons, switchgrass has been intensively studied as a new substrate for bio-based chemical production.

Previous studies have confirmed that engineered *E. coli* successfully produced free fatty acids using renewable biomass hydrolysates such as woody hydrolysates, sorghum extract sugars (Wu et al., 2015; Bule et al., 2016). To the best of our knowledge, switchgrass has not previously been studied as a feedstock for free fatty acid production.

The purpose of this study was to develop the overall process of FFA production from switchgrass using engineered *E. coli*. First, sugar stream was generated using switchgrass via pretreatment and fermentation. The switchgrass hydrolysates were, then, tested as feedstocks for FFA production using two types of engineered *E. coli* in flask fermentation level. The developed bioconversion process for switchgrass based-FFAs was evaluated for an overall yield assessment. Finally, the fermentation process for FFA production was evaluated using a bioreactor.

## Materials and methods

### *Composition of switchgrass*

Switchgrass was obtained from the Kansas State University Agronomy Farm, Manhattan, Kansas, and grounded using a Tomas-Wiley laboratory mill (model 4) fitted with a 1 mm sieve. The composition of ground switchgrass was determined by following protocol NREL/TP-510-42618 (Sluiter et al., 2008).

### *Pretreatment of sorghum stalks and switchgrass*

Switchgrass was obtained from the Kansas State University agronomy farm, Manhattan, Kansas, and grounded using a Tomas-Wiley laboratory mill (model 4) fitted with a 1 mm sieve. Ground switchgrass was pretreated with 1.25% (w/v) sodium hydroxide at 121 ˚C for 1 h.

Pretreated biomass was washed with about 5 L of water to completely remove the sodium hydroxide residue, and dried at room temperature for 72 h.

### *Enzymatic hydrolysis of sorghum stalks and switchgrass*

C-Tec2 and H-Tec2 enzymes for producing sugar solutions were obtained from Novozymes Inc., Franklinton, North Carolina. Ten percent (w/v) of the pretreated biomass was suspended with 50 mM of a citrate buffer solution (pH 4.8), and enzyme mixtures were added at a dosage of 8 FPU per gram of pretreated switchgrass. Enzymatic hydrolysis was conducted at 50 ˚C and 140 rpm for 72 h. Switchgrass hydrolysates, which were a supernatant after centrifugation, were harvested *via* centrifugation at 8500 rpm for 20 min (Sorvall Super T21, Thermo Fisher Scientific Inc., Waltham, MA, USA).

### *E. coli strain and plasmids*

Recombinant *E. coli* ML103 *pXZ18* and *E. coli* ML103 *pXZ18Z* were utilized in this study. Plasmids *pXZ18* and *pXZ18Z,* and *E. coli* ML103 were obtained from Rice University, Houston, Texas. The plasmid *pXZ18* contained an acyl-ACP thioesterase (*TE*) from *Ricinus communis* (Zhang et al., 2011) and *pXZ18Z* carried *TE* with the native (3R)-hydroxyacyl-ACP dehydrase (*fadZ*). Each plasmid was transformed into *E. coli* ML103 (*fadD* mutant) (Wu et al., 2015) using a traditional heat-shock method. Recombinant *E. coli* ML103 *pXZ18* and *E. coli* ML103 *pXZ18Z* were grown in the liquid LB media (LB broth, Difco, Detroit, MI, USA) with a 100 μg/mL of ampicillin at 37 ˚C and 250 rpm.

### *Culture media and fermentation conditions*

The seed culture was prepared by inoculating a single colony from an LB plate into 5 mL of LB media containing a 100 mg/L of ampicillin, and incubated overnight at 30 ˚C and 250 rpm. Shake-flask fermentation was conducted in the liquid LB media with a supplementation of 15 g/L

glucose. Also, switchgrass hydrolysates, containing a total of 57 g/L sugars composed of 40 g/L of glucose and 17 g/L of xylose, were utilized as carbon sources for fermentation. The initial pH was adjusted to 7 using 10 N sodium hydroxide, and *TE* and *fadZ* genes were induced by adding 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) into the fermentation media. Cells were incubated at 30 ˚C and 250 rpm for 72 h.

Batch fermentation and fed-batch fermentation were carried out in a 7 L fermenter (Bioflo 110, New Brunswick Scientific Inc., Enfield, CT, USA) with a 4 L working volume. The fermentation broth was an LB media, supplemented by 15 g/L of initial glucose or switchgrass hydrolysates containing a total of 15 g/L sugars as carbon sources. Temperature was kept at 30 ˚C, agitation was 300 rpm, and pH was maintained at 7.0 by 5 N of sodium hydroxide. Air was supplied at 0.5 vvm during the fermentation.

### *Analytical methods*

Cell density was determined by measurement of dry-cell weight (DCW) and optical density. Dry-cell weight (DCW) was investigated by drying cells at 80 ˚C overnight, and optical density was estimated at 600 nm (Thermo Fisher Scientific, Lenexa, KS).

Concentrations of sugars and acetic acid were measured *via* high-performance liquid chromatography (HPLC; Shimadzu Scientific Instruments, Inc., Columbia, MD, USA), equipped with a refractive index detector (RID) and a Rezex ROA organic acid column (150 x 7.8 mm, Phenomenex Inc., Torrance, CA, USA). A mobile phase (0.005 N sulfuric acid) was pumped at a rate of 1.0 mL/min, and oven temperature was maintained at 80 ˚C.

For the free fatty acid analysis, 1 mL of fermentation media was taken into a 16 x 150 mm Kimax glass tube, and 0.5 g/L of pentadecanoic acid (C15) was added into each sample as an internal standard. A 7.5 mL of methanol was added into the each Kimax glass tube containing

sample solutions and vortexed for 1 min. A 700 μL of 10 M potassium hydroxide solution was added to the sample mixtures to hydrolyze *E. coli* cells and samples were incubated at 60 ˚C for 1.5 h. Samples were vortexed every 20 min to properly permeate and dissolve the maximum free fatty acids. After 1.5 h of incubation, 580 μL of 24 N sulfuric acid was added, and incubated at 60 ˚C for 1.5 h to methylate the free fatty acids. Samples were vortexed every 30 min for 2 min during the 1.5 h incubation. Semi-polar compounds were dissolved by adding 2 mL of water, and 2 mL of hexane was added to solubilize fatty acid methyl esters (FAME). FAME were harvested *via* centrifugation at 4000 rpm for 10 min. Concentrations of FAME were analyzed using a gas chromatograph (GC-2014, Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a flame-ionization detector (FID) and an aqueous-stable polyethylene glycol capillary column (Zebron ZB-Wax$_{plus}$ 30m x 0.25 mm x 0.25 μm, Phenomenex, Torrance, CA, USA). The oven temperature was initially set at 160 ˚C and gradually increased to 200 ˚C at a rate of 5 ˚C/min, and held for 17 min. Detector temperature was set at 250 ˚C for identifying the fatty acid composition and the FAME mixture (Supelco, 37 component FAME mix) was utilized as an external standard. Also, a mass spectrometer (GC/MS-QP 2010 SE, Shimadzu Scientific Instruments, Columbia, MD, USA) was utilized to confirm the species of fatty acids.

### *Statistical methods*

All experiments were conducted in triplicate, and the data were statistically analyzed using SAS software (SAS v9.1, SAS institute, Cary, NC, USA) by performing PROC GLM for the least-significant difference (LSD) test at a 95% confidence level ($P<0.05$).

# Results and discussion

## *Composition of switchgrass and concentrations of monomer sugars released from switchgrass*

Figure 5.2 shows the composition of switchgrass used in this study. The ground switchgrass contented 35.2 ± 1.2% (w/w) of glucan, 29.2 ± 1.2% (w/w) of xylan and 16.9 ± 0.1% (w/w) of lignin, respectively. The switchgrass was utilized as a feedstock for FFA production using recombinant strains. Total 57 g/L sugars composed of 40 g/L glucose and 17 g/L xylose were generated from switchgrass. Also, 15.6 g/L of citric acid was also identified and it was included during enzymatic hydrolysis to adjust pH (Figure 5.2). Other pretreatment-induced inhibitory compounds such as hydroxymethyl furfural, furfural, acetic acid, were not detected in the switchgrass hydrolysates, probably due to washing of the pretreated biomass with excess of water.

## *Free fatty acid production at the flask level*

Free fatty acid (FFA) production using switchgrass hydrolysates and synthetic media by two types of recombinant *E. coli* (ML103 *pXZ18* and ML103 *pXZ18Z*) was conducted at the flask level. Figure 5.3 shows FFA distributions produced from switchgrass hydrolysates and synthetic media by *E. coli* ML103 *pXZ18* and *E. coli* ML103 *pXZ18Z*. Fatty acids species produced by both recombinant *E. coli* were myristic acid (C14), palmitic acid (C16), palmitoleic acid (C16:1), and oleic acid (C18:1). Major FFAs produced by *E. coli* ML103 *pXZ18* were myristic acid and palmitoleic acid, whereas *E. coli* ML103 *pXZ18Z* produced mainly myristic acid and palmitic acid. The FFA composition observed in switchgrass hydrolysates was similar to that of synthetic medium. These results were consented with the previous studies using same recombinant *E. coli* strains by Wu and coworkers (Wu et al., 2014). Plasmid *pXZ18Z* is carrying *fabZ* overexpressing

(3R)-hydroxyacyl-ACP dehydrase, which is involved in fatty acid elongation, whereas *pXZ18Z* contains only a TE gene (Wu, Karanjikar and San, 2014). The catalytic reaction of β-hydroxyacyl-ACP dehydratase is dehydration of their substrates such as, short chain β-hydroxyacyl-ACP, long chain saturated β-hydroxyacyl-ACP and unsaturated β-hydroxyacyl-ACP. Overexpression of β-hydroxyacyl-ACP dehydratase might cause decreasing the percentage of unsaturated fatty acids (Wu, Karanjikar and San, 2014). Therefore, *E. coli* ML103 *pXZ18Z* produced lower percentage of oleic acid and palmitoleic acid compared with *E. coli* ML103 *pXZ18*. It might affect the composition of FFAs produced by both recombinant *E. coli*.

Fermentation performance of recombinant *E. coli* during FFA production has shown in Table 5.1. Totals of 2.8 g/L and 3.3 g/L of FFAs were achieved by *E. coli* ML103 *pXZ18* and *E. coli* ML103 *pXZ18Z*, respectively, using switchgrass hydrolysates. The results showed that the *E. coli* ML103 *pXZ18Z* produced 14% higher amount of FFA using switchgrass hydrolysate compared with synthetic sugars. The *E. coli* ML103 *pXZ18* also produced 8% higher amount of FFA in switchgrass hydrolysate than in synthetic sugars; however, the difference was not statistically significant at the 95% confidence level. The FFA yield per gram of sugar (Yps) showed that synthetic sugar and switchgrass hydrolysates media did not affect FFA production for both E. coli strains. Further, compared with FFAs produced from woody hydrolysates in the previous study by Wu and coworkers (2015), 80% and 35% higher level of FFAs were produced from switchgrass by *E. coli* ML103 *pXZ18* and *E. coli* ML103 *pXZ18Z*, respectively.

Both *E. coli* strains produced higher amounts of acetic acid than FFA in the switchgrass hydrolysates; a total of nearly 6 g/L of acetic acid was produced by both strains during 72 h of fermentation. Normally, microbes produce more carboxylic acid, such as acetic acid, to generate more ATP under severe stress conditions (Cray et al., 2015). This indicatedthat switchgrass

hydrolysates provided more stress conditions for recombinant *E. coli* compared with synthetic media. Since a total of 4 g/L citric acid remained at a constant level in the medium during the fermentation using switchgrass hydrolysates, it could be the major stress factor for *E. coli* strains. It is because organic acids are known as toxic compounds for microoraganisms such as *E. coli*. Weak acids accumulate with deprotonated form, and affect intracellular pH and activity of enzymes associated with metabolism or ATP production (Warnecke and Gill, 2005; Trček, Mira and Jarboe, 2015). Citric acid is a non-lipophilic acid; so, its antibacterial effect is lower than acetic acid, but it shows antibacterial effects against gram-negative bacteria such as *E. coli* (Erkmen and Bozoglu, 2016). However, further investigation is required to confirm that the citric acid in the switchgrass hydrolyzates was that main stress factor. In addition, a significantly higher DCW was produced using switchgrass hydrolysates compared with the synthetic medium. Therefore, lower Ypx and higher Yxs were obtained using switchgrass hydrolysates.

To sum up, *E. coli* strains produced different types of FFA, and *E. coli* ML103 *pXZ18Z* strain showed better fermentation performance compared with *E. coli* ML103 *pXZ18* strain. In addition, both *E. coli* strains produced FFA using switchgrass hydrolysates as efficient as synthetic sugars.

### *Overall yield of free fatty acids from switchgrass*

Overall yields of FFA production by *E. coli* ML103 *pXZ18* and *E. coli* ML103 *pXZ18Z* were evaluated to investigate the efficiency of the bioconversion process (Figure 5.4). Starting from 100 g of switchgrass, 49 g of total monomer sugars were achieved via pretreatment and the enzymatic hydrolysis process. With the conversion factor of 0.9 from glucose to glucan, and 0.88 from xylose to xylan, monomer sugar recoveries during the process were investigated (Guragain et al., 2014). Glucose and xylose recoveries from raw switchgrass were 88% and 46% of the theoretical maximum value, respectively. Lower level of xylose was recovered compared with glucose

recoveries. This might be due to hemicellulos loss during the pretreatment process. Taking the normalized conversion factors of 0.35 from glucose to FFAs and 0.29 from xylose to FFAs (Lennen and Pfleger, 2012), 55% and 72% of maximum theoretical yields were achieved from hydrolysates by *E. coli* ML103 *pXZ18* and *E. coli* ML103 *pXZ18Z*, respectively. With the combined conversion factors of glucose (or xylose) to glucan (xylan) and glucose (or xylose) to FFAs, the overall theoretical maximum yield from 1g switchgrass to FFAs was determined to be 0.23. This FFA production process from switchgrass achieved 39% and 51% of theoretical maximum yields by *E. coli* ML103 *pXZ18* and *E. coli* ML103 *pXZ18Z,* respectively.

### *Free fatty acid production using E. coli ML103 pXZ18Z at the bioreactor scale*

FFA production using *E. coli* ML103 *pXZ18Z* was carried out in the 7 L fermenter with 4 L working volume. Initially, 15 g/L glucose was added as a carbon source, and an additional 15 g/L glucose was fed into the fermenter during the fermentation. Fermentation profiles in Figure 5.5A show that FFA production by recombinant *E. coli* was the mixed growth associated product formation; product formation was observed during all the phase of cell growth. A total of 2.2 g/L of FFAs were obtained from the initial 15 g/L glucose at 36 h. A slightly lower level of FFAs was obtained compared with results of the shake flask. All glucose was consumed at 100 h and a total of 4.2 g/L of FFAs were achieved using a total of 30 g/L glucose. Changes of FFA composition during fermentation in the synthetic medium were shown in Figure 5.5C. As the results in the flask level, myristic acid and palmitic acid were major FFAs. Interestingly, the relative percentage of these major FFAs (myristic acid and palmitic acid) was continuously increased during fermentation, and their final composition was up to 90%. Whereas, concentrations of oleic and linoleic acid were constant during fermentation. Even though sugars were consumed until 100 h,

FFA production was stopped and kept at the same level after 78 h. These results indicated that sugars were utilized for purposes other than product formation after 78 h.

Table 5.2 shows that most yields (Yxs, Yps) and productivity (Qp) were higher during the first stage of fermentation. Additional glucose was fed into the fermenter at the stationary phase of the growth, and this led to a substantially lower value of Yxs in the second stage of fermentation. After more feeding of 15 g/L glucose, most fermentation parameters were decreased, except for FFA titers and Ypx. Lennen and Pfleger1 mentioned that yields and productivity are key metrics for judging FFA production to meet low price targets (Lennen and Pfleger, 2012). Based on these results, FFA production using 15 g/L glucose in the batch culture would be the appropriate fermentation condition for obtaining high productivity and yields.

Batch fermentation using switchgrass hydrolysates containing 15 g/L total sugars was conducted with the 4 L working volume at 30 ˚C and 300 rpm. After all glucose was consumed, xylose consumption was started at 12 h, and a significant amount of acetic acid was produced by recombinant *E. coli* until 16 hours of fermentation compared with synthetic media as shown in Figure 5.6A. All sugars were completely consumed, and acetate switch was observed at 22 h. Acetate switch occurred by activating AMP-forming acetyl-CoA synthetase (AMP-ACS), when the sugars in the medium were almost exhausted and cells began the transition to stationary phase; this is common phenomena in *E. coli* metabolism (Nystrom and Neidhardt, 1993). An acetate switch leads to an increase in the acetyl-CoA pool, and activates AMP-ACS, which is involved in FFA synthesis (Wolfe, 2005). Therefore, it was anticipated that the acetate switch would improve FFA production. After complete consumption of all sugars, FFA titers were slightly increased, but the effect of acetate switch was insignificant. Our results showed that 5 g/L acetic acid did not

significantly influence improvement of FFAs containing hydrocarbons. Further molecular level investigation is required to confirm the effect of acetate switch during FFA production by *E. coli*.

The change of pH during fermentation shown in Figure 5.6B reflected the acetate switch. As acetic acid production by *E. coli* rapidly increased, sodium hydroxide was pumped into the fermenter to maintain constant pH. When acetic acid consumption by *E. coli* was initiated at 20 h, pH started increasing, and nitric acid was pumped into the fermenter since 23 h.

Total 2.1 g/L FFAs was achieved using switchgrass hydrolysates containing 15 g/L total sugars in the bioreactor level. Lower level of FFAs were produced in the fermenter compared with the shake flask. This might be due to the fact that high amounts of base and acid were continuously added into the fermenter to maintain constant pH, leading to the dilution ofthe product concentrations. Even though fermentation performance at the bioreactor levels were lower than at the flask level, same value of productivity was obtained. This was due to increased rates of sugar consumption and product formation reducing fermentation periods by two-fold. Since the productivity is one of the major factors for developing FFA production at the industrial level (Lennen and Pfleger, 2012), reduction of fermentation time is a meaningful result.

The compositional changes of FFAs during fermentation using switchgrass hydrolysates are shown in Figure 5.6C. Similar to the other composition results, myristic acid and palmitic acid were major FFAs produced by *E. coli* ML103 *pXZ18Z*, showing a composition of nearly 70 to 80%. Interestingly, *E. coli* ML103 *pXZ18Z* produced higher amounts of palmitic acid than myristic acid in the switchgrass hydrolysates, whereas myristic acid were higher than palmitic acid in the synthetic medium.

# Conclusion

In this study, a bioconversion process for producing FFAs from switchgrass was developed using engineered *E. coli* strains of ML103 *pXZ18* and ML103 *pXZ18Z*. Both *E. coli* strains efficiently produced FFAs using switchgrass hydrolysates; higher concentrations of FFAs were achieved in switchgrass hydrolyzates compared with synthetic sugar media. Overall yield assessment of FFA production from raw switchgrass by *E. coli* ML103 *pXZ18Z* showed that our bioconversion process achieved 51% of maximum theoretical yield. Additionally, the processes were successfully scaled up to a laboratory fermenter level for FFA production using switchgrass hydrolysates; consequently, fermentation time was reduced by two-fold. To the best of our knowledge, this study is the first attempt of FFA production from switchgrass hydrolysates at the fermenter level. While further improvement in product titer level is essential for commercial viability, targeted FFA has potential applications as bio-lubricant and in specialty chemical industry.

Table 5-1 Fermentation performance of engineered *E. coli* during FFA production in shake flasks

| Strains | FFA (g/L) | Yxs[1] (g/g) | Yps[2] (g/g) | Ypx[3] (g/g) | Qp[4] (g/L/h) | DCW (g/L) |
|---|---|---|---|---|---|---|
| *Synthetic sugars* | | | | | | |
| *E.coli ML103 pXZ18* | $2.6 \pm 0.1^D$ | $0.11 \pm 0.0^F$ | $0.19 \pm 0.0^B$ | $1.76 \pm 0.3^A$ | $0.04 \pm 0.0^B$ | $1.5 \pm 0.3^D$ |
| *E.coli ML103 pXZ18Z* | $2.9 \pm 0.1^C$ | $0.13 \pm 0.0^{E,F}$ | $0.23 \pm 0.0^A$ | $1.79 \pm 0.4^A$ | $0.04 \pm 0.0^B$ | $1.7 \pm 0.3^D$ |
| *Switchgrass hydrolysates* | | | | | | |
| *E.coli ML103 pXZ18* | $2.8 \pm 0.1^{C,D}$ | $0.3 \pm 0.0^A$ | $0.19 \pm 0.0^B$ | $0.62 \pm 0.0^{B,C}$ | $0.04 \pm 0.0^B$ | $4.6 \pm 0.2^B$ |
| *E.coli ML103 pXZ18Z* | $3.3 \pm 0.2^B$ | $0.32 \pm 0.0^A$ | $0.24 \pm 0.0^A$ | $0.75 \pm 0.1^B$ | $0.05 \pm 0.0^B$ | $4.3 \pm 0.1^B$ |

FFA = free fatty acid, DCW = dry cell weight. The data represent average values of triplicate experiments ± sample standard deviation. Values with the same letters, in superscripts, within the same column, are not significantly different at the level of ($P<0.05$).

[1]Yxs was calculated by dividing the amount of dry cell mass by the amount of consumed glucose.
[2]Yps was calculated by dividing the amount of lipids by the amount of consumed glucose.
[3]Ypx was calculated by dividing the amount of lipids by the amount of dry cell mass.
[4]Qp was defined as the amount of lipids produced per liter per hour.

Table 5-2 Fermentation performance of *E. coli* ML103 *pXZ18Z* in the fermenter scale

| Sugars (g/L) | FFA (g/L) | Yxs[1] (g/g) | Yps[2] (g/g) | Ypx[3] (g/g) | Qp[4] (g/L/h) | DCW (g/L) |
|---|---|---|---|---|---|---|
| *Synthetic medium* | | | | | | |
| 15 | $2.2 \pm 0.1^A$ | $0.15 \pm 0.0^A$ | $0.17 \pm 0.0^A$ | $1.2 \pm 0.1^A$ | $0.07 \pm 0.0^A$ | $1.9 \pm 0.2^A$ |
| 30 | $4.2 \pm 0.2^B$ | $0.07 \pm 0.0^B$ | $0.14 \pm 0.0^B$ | $2.0 \pm 0.1^B$ | $0.04 \pm 0.0^B$ | $2.1 \pm 0.1^B$ |
| *Switchgrass hydrolysates* | | | | | | |
| 15 | $2.1 \pm 0.0^C$ | $0.38 \pm 0.0^C$ | $0.14 \pm 0.0^B$ | $0.37 \pm 0.0^C$ | $0.05 \pm 0.0^C$ | $5.7 \pm 0.2^C$ |

FFA = free fatty acid, DCW = dry cell weight

The data represent average values of triplicate experiments ± sample standard deviation. Values with the same letters, in superscripts, within the same column, are not significantly different at the level of ($P<0.05$).

[1]Yxs was calculated by dividing the amount of dry cell mass by the amount of consumed glucose.

[2]Yps was calculated by dividing the amount of lipids by the amount of consumed glucose.

[3]Ypx was calculated by dividing the amount of lipids by the amount of dry cell mass.

[4]Qp was defined as the amount of lipids produced per liter per hour.

Figure 5-1 Simplified metabolic pathway of the metabolically engineered *E. coli* strains for fatty acid synthesis using monomer sugars.  TE: gene for expressing the thioesterase derived from R. comunis; *fabZ*: gene for expressing the (3R)-hydroxymyristoryl-ACP dehydrase from *E. coli*
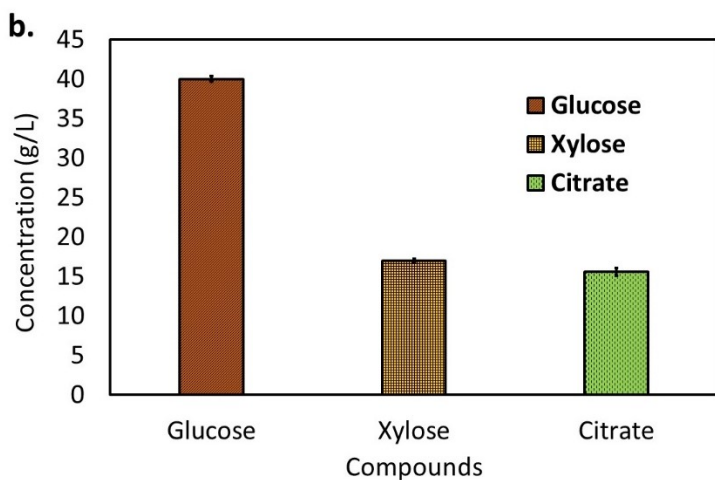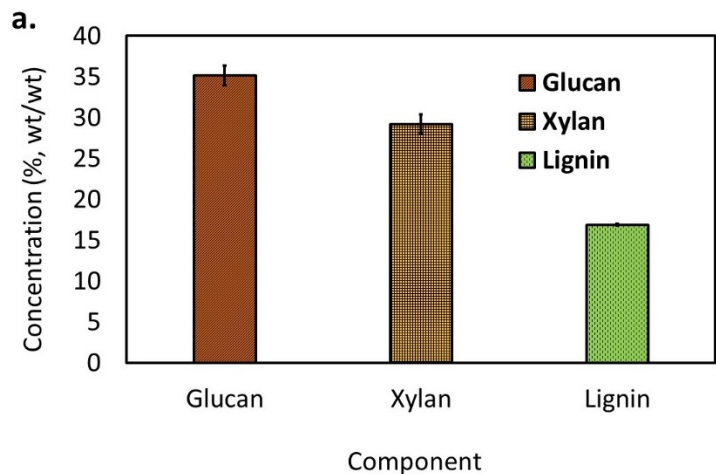
Figure 5-2 Composition of (A) switchgrass and (B) switchgrass hydrolysates. The data shows average value of triplicate experiments and error bars representing sample standard deviation.
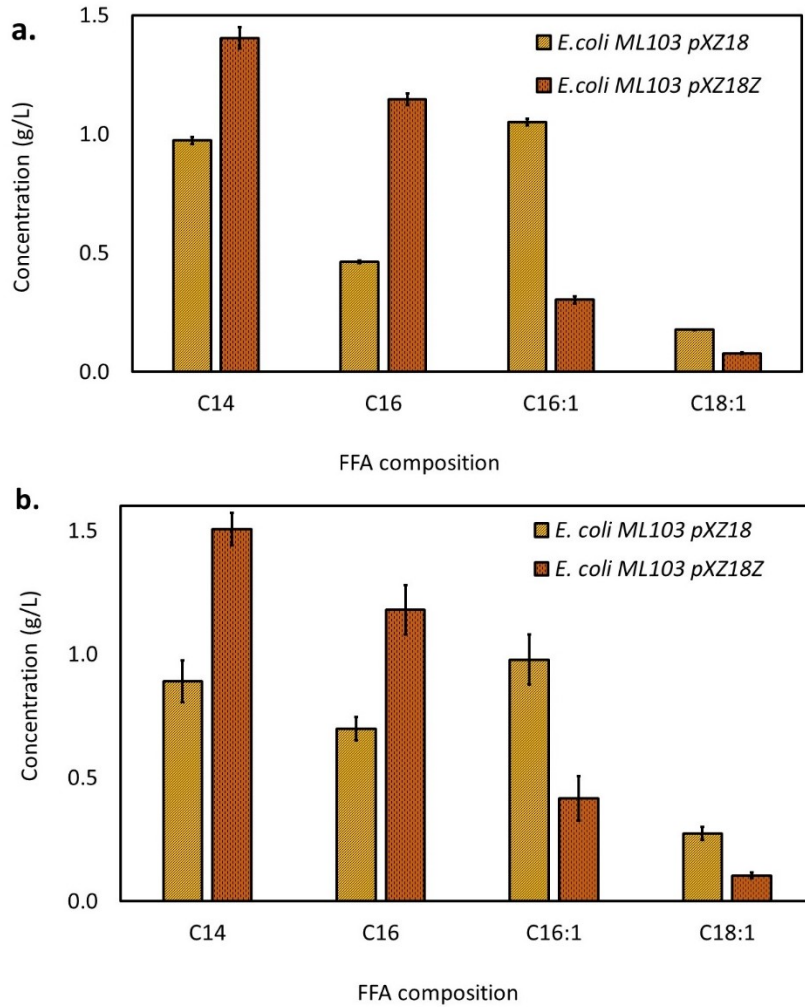
Figure 5-3 FFA production using recombinant *E. coli* (A) using synthetic sugars (B) using switchgrass hydrolysates. The data shows average value of triplicate experiments and error bars representing sample standard deviation.
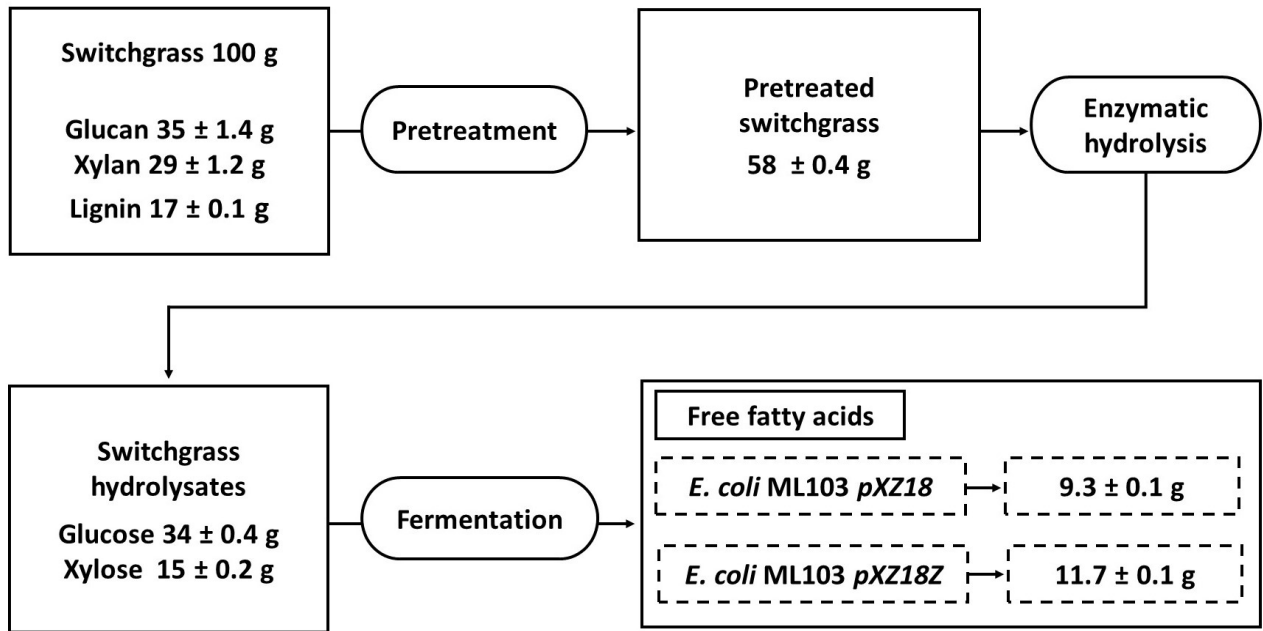
Figure 5-4 Mass balance of FFAs from switchgrass. The data shows average value of triplicate experiments and error bars representing sample standard deviation.
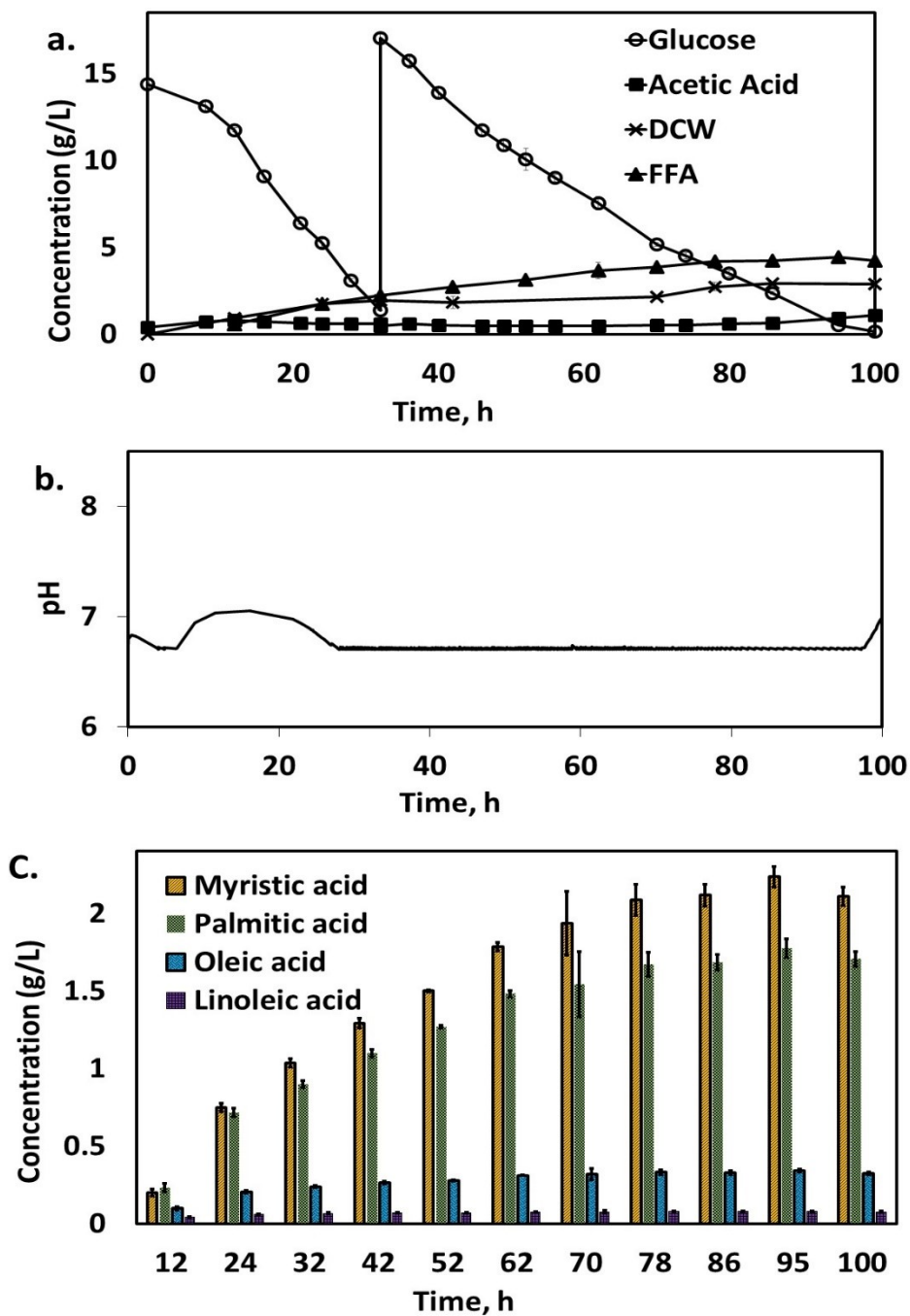
Figure 5-5 FFAs production using synthetic sugars by *E. coli* ML103 *pXZ18Z* in the fermenter scale (A) fermentation profile; (B) pH observation; (C) distribution of FFAs. The data shows average value of triplicate experiments and error bars representing sample standard deviation.
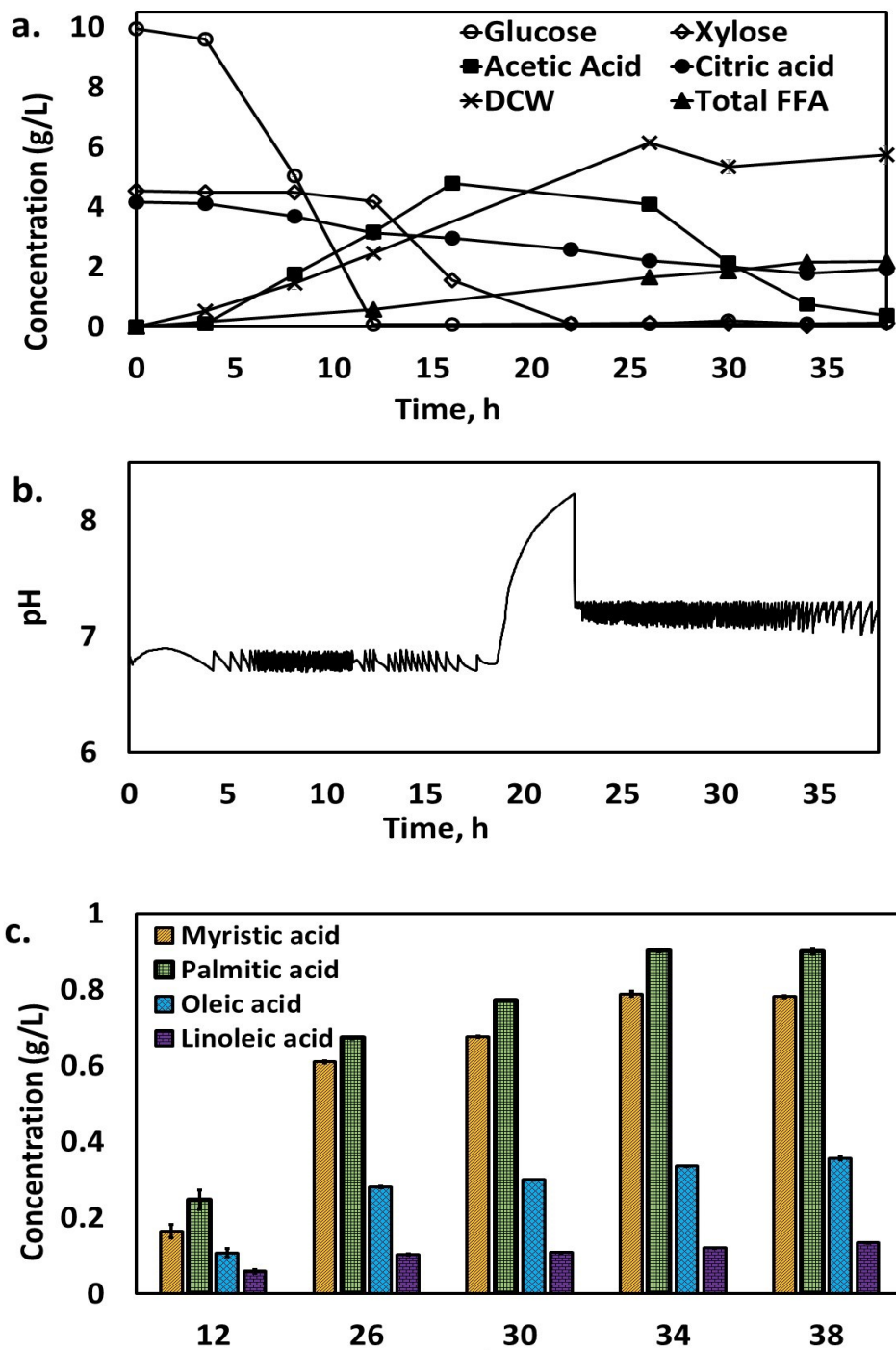
Figure 5-6 FFAs production using switchgrass hydrolysates by *E. coli* ML103 *pXZ18Z* in the fermenter scale (A) fermentation profile; (B) pH observation; (C) distribution of FFAs. The data shows average value of triplicate experiments and error bars representing sample standard deviation.

# Chapter 6 - Microbial lipid production from lignocellulosic biomass using promising oleaginous yeast systems[2]

## Abstract

Microbial lipids derived from oleaginous yeast could be a promising resource for biodiesel and other oleochemical materials. The objective of this study was to develop an efficient bioconversion process from lignocellulosic biomass to microbial lipids using three types of robust oleaginous yeast: *T. oleaginosus, L. starkeyi, and C. albidus*. Sorghum stalks and switchgrass were utilized as feedstocks for lipid production. Among oleaginous yeast strains, *T. oleaginous* showed better performance for lipid production using sorghum stalk hydrolysates. Lipid titers of 13.1 g/L were achieved by *T. oleaginosus*, using sorghum stalk hydrolysates with lipid content of 60% (wt/wt) and high lipid yield of 0.29 g/g, which was substantially higher than the value reported in literature. Assessment of overall lipid yield revealed a total of 14.3 g and 13.3 g lipids were produced by *T. oleaginosus* from 100 g of raw sorghum stalks and switchgrass, respectively. This study revealed that minimization of sugar loss during pretreatment and selection of appropriate yeast strains would be key factors to develop an efficient bioconversion process and improve the industrial feasibility in a lignocellulose-based biorefinery.

## Introduction

Microbial lipids are promising candidates for replacing traditional oil sources in the production of biodiesel, oleo-chemicals, and nutraceuticals, due to their similar chemical composition and energy value (Mazzobre et al., 2005). Research by (Suh, Lee and Chung, 1997) estimated that cost

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of microbial lipids would be $3.4/kg, excluding a feedstock price, and $5.5/kg, including glucose as a feedstock, whereas cost of vegetable oil is $1.5-3/kg less (Wu et al., 2014). A supply of low-cost carbohydrates for microbes is required for sustainable and cost-effective production of bio-based lipids.

Lignocellulosic biomass, such as agricultural residues and woody crops, is a strong alternative substrate for microbial lipid production due to low-cost investment, and high content of polysaccharides (up to 75%) and their abundancy (Singh, Gamlath and Wakeling, 2007). More than 90% of global production of plant biomass is lignocellulosic biomass, which is composed of cellulose, hemicellulose, and lignin (Breitenbach, 2002). Recalcitrant lignocellulosic biomass is converted to monomer sugars *via* pretreatment and enzymatic hydrolysis. Many pretreatment methods maximize exposure of carbohydrate polymers (cellulose and hemicellulose) with effective separation of the lignin portion, which is an interference biopolymer during bioconversion. Among many attempts, an alkaline pretreatment is known to efficiently remove lignin from plant cell wall structures (Bondeson and Oksman, 2007). Cellulose and hemicellulose are depolymerized to monosaccharides by synergetic actions of enzyme mixtures (Smith et al., 2003). The most abundant monomer sugars derived from lignocellulosic biomass are D-glucose, since cellulose represents 70% of total plant cell walls, repeating the $\beta$-(1$\rightarrow$4) glycosidic bond (Holic et al., 2012). However, species of lignocellulosic-based monomer sugars depend on biomass types.

Several challenges remain for successful bioconversion of lignocellulosic biomass to microbial lipids. A broad array of monomer sugars is generated from lignocellulosic biomass including glucose, xylose, mannose, and arabinose. Typically, the ratio of hexoses to pentoses ranges from 1.5:1 to 3:1 (Carvalho and Mitchell, 2000). However, some species of microbes only utilize limited

types of monomer sugars as carbon sources. In addition, a number of by-products, such as furans, aldehydes, and organic acids, are generated during pretreatment and enzymatic hydrolysis (Chen et al., 2014; Choi and Oh, 2012). These compounds are known to inhibit microbes' growth and product formation during fermentation. Particularly, acetic acid is inevitable compound, which are normally released from acetyl groups of hemicellulose during enzymatic hydrolysis (Karunanithy, Muthukumarappan and Gibbons, 2012). Acetic acid adversely affects the integrity of the cell membrane by accumulating in deprotonated form (Yoo et al., 2012).

Oleaginous yeast, which has an inherent ability to accumulate lipids from 20% to 70% as a percentage of cell dry weight, offers many advantages to overcome challenges associated with lignocellulose-based lipid production (Duque et al., 2014). Basidiomycetous yeast species such as *Cryptococcus albidus* and *Trichosporon oleaginosus* are known to use a variety of carbon sources, and can be grown without costly supplemental nutrients (Rosentrater and Muthukumarappan, 2006; Spiehs, Whitney and Shurson, 2002). In addition, oleaginous yeasts are tolerant to toxic compounds compared with bacteria. Previous studies reported some types of oleaginous yeast consumed weak acids, including acetic acid and formic acid (Hoover et al., 2012; Riesenberg, 1991). Utilization of diverse monomer sugars and organic acids derived from lignocellulosic biomass are directly related to product yields of lipid production. Also, their fast and higher density growth are positives associated with productivity and product titers during fermentation.

In this study, production of lignocellulose-based microbial lipids was investigated using three oleaginous yeast strains: *Trichosporon oleaginosus* ATCC20509, *Lipomyces stariey*i ATCC 56304, and *Cryptococcus albidus* ATCC10672. Sorghum stalks and switchgrass, which are typical bio-energy crops, were utilized as sugar suppliers for microbial lipid production. In addition, fermentation performance of *T. oleaginosus*, *L. stariey*i, and *C. albidus* were evaluated using

sorghum stalks and switchgrass hydrolysates. To our knowledge, *C. albidus* ATCC 10672 has not previously been evaluated for lipid production using lignocellulosic hydrolysates. Also, overall yield of microbial lipids from raw biomass was studied to evaluate the lipid production process.

## Materials and Methods

### *Lignocellulosic biomass and composition analysis*

Sorghum stalks were obtained from Texas A&M University, College Station, Texas, and ground by Mesa Associate Inc., Knoxville, Tennessee. Switchgrass was obtained from the Kansas State University agronomy farm, Manhattan, Kansas, and ground at a size of less than 1 mm, using a Tomas-Wiley laboratory mill (Model 4). Biomass composition was determined following the protocol of NREL/TP-510-42618 (Kim and Oh, 2013).

### *Pretreatment and enzymatic hydrolysis of lignocellulosic biomass*

Figure 6.1 shows a schematic diagram of the process for lignocellulosic hydrolysate preparation. The ground biomass was mixed with 1.25% (w/v) sodium hydroxide (NaOH), at the rate of 10% (w/v) solid loading, in a 500 mL flask for pretreatment. Sorghum stalks and switchgrass were pretreated at 121 °C for 30 min and 1 h, respectively. The pretreated biomass was washed with about 5 L of water until the residue of NaOH was completely removed and dried at room temperature for five days.

The pretreated biomass was slurried with 50 mM of a citrate buffer (pH 4.8), at the rate of 5% (w/v) solid loading, for the enzymatic hydrolysis. Commercial cellulolytic (Cellic C-Tec2) and hemicellulotyic (Cellic H-Tec2) enzymes, which were obtained from Novozymes Inc., Franklinton, North Carolina, were added into the pretreated biomass slurry at the rate of 5.4% and 0.6% (w/v) of biomass, respectively. Enzymatic hydrolysis was conducted in the shaking incubator at 50 °C and 140 rpm for 48 h (Innova 4300, New Brunswick Scientific, NJ). The sorghum stalks

and switchgrass hydrolysates, which is a supernatant after centrifugation (Sorvall Super T21, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 8500 rpm for 20 min, were harvested.

### *Yeast strains, medium, and culture conditions*

*Trichosporon oleaginosus* ATCC20509, *Lipomyces starkeyi* ATCC 56304, and *Cryptococcus albidus* ATCC 10672 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and cultivated in a yeast mold broth (YM broth, Difco, Detroit, MI, USA) at 25 °C and 200 rpm. All yeast cultures were preserved in YM agar plates at 4 °C, and transferred to fresh plates once a month.

### *Fermentation*

Starter cultures of all yeast strains were begun by inoculating a single colony from a YM agar plate. *T. oleaginosus, L. starkeyi, and C. albidus* were grown in a YM broth at 25 °C and 200 rpm for 12 h, and cells were transferred into a 500 mL shake flask containing 100 mL of fermentation media. Sorghum stalks and switchgrass hydrolysates, containing a total of 50 g/L sugars, were utilized as carbon sources for lipid production. Yeast extract and peptone were supplemented into the fermentation media to achieve the initial molar ratio of carbon and nitrogen sources to 76. Fermentation was carried out at 25 °C and 200 rpm for 120 h.

### *Analysis of sugar and organic acid*

Dry-cell weight (DCW) was used to determine cell concentrations. Cell pellets were washed with water two times, dried at 80 °C overnight, and measured for weight.

Sugars and organic acid concentrations were analyzed *via* a high-performance liquid chromatography (HPLC; Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) equipped with a refractive index detector (RID) and a Rezex ROA organic acid column (150 x 7.8 mm,

Phenomenex Inc., Torrance, CA, USA). Oven temperature was kept at 80 ˚C, and 0.005 N sulfuric acid was utilized as a mobile phase, with a pumping rate of 1.0 mL/min.

### *Yeast cell lysis and lipid extraction*

Yeast cells were harvested *via* centrifugation (Sorvall Super T21, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 8500 rpm for 20 min. Cells were washed two times with water and concentrated to $10^9$ cells/mL. The concentrated cells were preserved at -80 ˚C for one day prior to lipid extraction. Thawed cell pellets (0.5 mL) were transferred into a 2.5 mL polypropylene microvial, followed by adding 0.5 mL of methanol, 0.5 mL of chloroform, and 1 mL of 0.5 mm cubic zirconia beads. Bead beating was performed using a bead-beater homogenizer (Mini-Beadbeater-24, BioSpec Products, Inc., Bartlesville, OK, USA) in 45 sec intervals, with a cooling of 10 min on ice repeated six times.

Lipid extraction was conducted by following a modified Bligh and Dyer method (Uemura, 2012). The cell lysate after bead beating was transferred into a 7 mL Kimax tube, and chloroform:methanol:water were added with a ratio of 1:2:0.8, respectively. Tubes containing cell lysate mixtures were vortexed and centrifuged at 4000 rpm for 20 min. The lipid layer of the mixture was transferred into a clean tube using a Pasteur pipette, and 1 mL of chloroform was added into the mixture followed by vortexing and centrifugation. Lipid extraction was repeated three times and the combined lipid layers were filtered using PTFE filters with 0.22 µM pore size (Whatman, Fisher Scientific, Waltham, MA). The filtrates were washed two times with a 1 M potassium chloride solution, followed by drying under nitrogen gas at 40 °C until 1 ml of mixture was left in the Kimax tube. The residue was transferred into a glass vial and dried down under nitrogen gas, again to completely remove chloroform and measure the lipid weight. After determination of lipid weight, 1mL of chloroform was added into each glass vial and kept at -80

°C for further compositional analysis of the lipids. Lipid content in the yeast cells was determined by dividing weight of lipids from yeast cells by weight of concentrated cells. Cell weight was determined by measuring DCW of the concentrated cell.

## *Analysis of lipid composition*

Fatty acids in the lipids were converted to fatty acid methyl esters (FAMEs) *via* transesterification for compositional analysis. Lipid samples were transferred into a 7 mL Kimax tube with 25 nmol of internal standard (pentadecanoic acid) and the chloroform was evaporated under nitrogen gas at 40 °C. For transesterification, 1 mL of methanolic hydrochloric acid (3 M) was added into each tube and incubated at 78 °C for 30 min in the heating block. After cooling down the samples, 2 ml of water were added, followed by 1.6 mL of chloroform and 0.4 mL of hexane. The layers were then separated *via* centrigufation at 4000 rpm for 5 min. The lower layer was transferred into a clean Kimax tube and the organic phase was dried down under nitrogen gas. One hundred µL of hexane were added to solubilize FAMEs, and then transferred into a glass vial.

FAMEs were analyzed by injecting 1 µL of the sample into a gas chromatograph (GC-2014, Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a flame-ionization detector (FID) and an aqueous-stable polyethylene glycol capillary column (Zebron ZB-Wax$_{plus}$ 30 m x 0.25 mm x 0.25 µm, Phenomenex, Torrance, CA, USA). The initial oven temperature of 160 ˚C was gradually increased to 200 ˚C at a rate of 5 ˚C/min, and detector temperature was 250 ˚C. The FAME mixture (Supelco, 37 component FAME mix) was utilized as an external standard to identify fatty acid composition in the lipids.

### *Plasmid construction*

Plasmid *pRS2u2* and *pOleo-RnACLY* were obtained from the Department of Biochemistry and Biophysics, Kansas State University, Manhattan, Kansas. Plasmid *pRS2u2* was used as expression vector for oleaginous yeast with the digestion using SalI.

Target gene RnACLY derived from *Rattus norvegicus* was applified using Phusin Flash High-Fidelity PCR Master Mix (Thermo Scientific, Waltham, MA). RnACLY gene specific primers were used for this amplification (F-AGCGTCGACATGTCAGCCAAGGCAATTTC and R-TGAGTCGACTTACATGCTCATGTGTTCCG). The amplified 3.3 kb DNA fragment was digested with SalI and ligated into SalI digested *pRS2u2* harboring TEF promoter. Ligation was confirmed via colony PCR (1 cycle of pre-denaturation for 10 sec at 98 °C, 30 cycle of annealing for 5 sec at 75 °C and extension for 49 sec at 72 °C, 1 cycle of final extention for 1 min at 72 °C).

### *Statistical methods*

SAS software (SAS v9.4, SAS institute, Cary, NC, USA) was used to analyze all data by performing PROC GLM for the least-significant difference (LSD) test at a 95% confidence level ($P<0.05$).

## Results and discussion

### *Sugar recoveries from sorghum stalks and switchgrass*

The composition of ground sorghum stalks and switchgrass is shown in Figure 6.2. Sorghum stalks had a higher content of lignin (20%) compared with switchgrass (16.9%). Sorghum stalks contained three types of polysaccharides: 28.4% glucan, 19.4% xylan, and 1.7% arabinan. Switchgrass structure was 35% glucan and 29% xylan; containing a higher amount of total polysaccharides compared with sorghum stalks. Sorghum stalks and switchgrass were deconstructed using a 1.25% (w/v) sodium hydroxide solution, following the optimized conditions

in the previous study (Meesapyodsuk and Qiu, 2008). Pretreated biomass of 58.6 g and 58.4 g were obtained from sorghum stalks and switchgrass, respectively. Alkaline pretreatment was utilized to effectively eliminate lignin compounds without substaintial loss of polysaccharides (Bondeson and Oksman, 2007). After saccharification of each pretreated biomass, sugar recoveries from lignocellulosic biomass were investigated. Also, maximum theoretical yields of sugar recoveries were determined using a conversion factor of 0.9 from glucose to glucan, and 0.88 from xylose (arabinose) to xylan (arabinan) (Kim and Oh, 2013). Figure 6.3A shows sugar yields released from 100 g of each lignocellulosic biomass. Total 29.8 g of glucose, 17.8 g of xylose, and 1.7 g of arabinose were released from 100 g of raw sorghum stalks. This was 94%, 81%, and 89% of maximum theoretical yields (TY) for glucose, xylose, and arabinose, respectively. Similar amounts of fermentable sugars were achieved from sorghum stalks and switchgrass, although they had a different content of polysaccharides. Total sugar yield from 100 g of switchgrass was 34 g of glucose and 15 g of xylose. Sugar recoveries from raw switchgrass were 88% and 45% of maximal TY for glucose and xylose, respectively. Even though switchgrass content showed higher amounts of polysaccharides, lower sugar recovery was obtained due to hemicellulose loss during pretreatment. Xylose recovery was substaintially low because harsher conditions were applied for pretreatment of switchgrass compared with sorghum stalks. Sugar yield from 100 g of each pretreated biomass is shown in Figure 6.3.B. Sugar yields of glucose, xylose, and arabinose from 100 g of pretreated sorghum stalks were 51 g, 30 g, and 2.9 g, respectively. Whereas, 58 g of glucose and 26 g of xylose were released from 100 g of switchgrass. In spite of higher content of xylan in switchgrass, xylose yield from pretreated biomass was lower than from sorghum stalks. This also reflects substantial loss of hemicellulose during pretreatment. Total sugar yields from pretreated sorghum stalks and switchgrass were similar (84.1 g/100 g).

***Microbial lipid production from lignocellulosic hydrolysates***

Sorghum stalks and switchgrass hydrolysates were utilized as feedstocks for lipid production using *T. oleaginosus, L. starkeyi, and C. albidus*. Both lignocellulosic hydrolysates contained acetic acid and citric acid. Acetic acid was released from acetylated hemicellulose (Karunanithy, Muthukumarappan and Gibbons, 2012), and citric acid was included to maintain pH during enzymatic hydrolysis. Figure 6.4 shows the fermentation profile of each oleaginous yeast using sorghum stalks and switchgrass hydrolysates. Sugar consumption rate of *T. oleaginosus* was fastest, compared with the other two strains. *T. oleaginosus* consumed all sugars in sorghum stalks and switchgrass hydrolysates at 72 h. *L. starkeyi* consumed all glucose in the biomass hydrolysates at 72 h, and started using xylose. *C. albidus* slowly consumed only glucose for 120 h. Other sugar sources were not utilized; this might be due to carbon catabolite repression. Citrate utilization was only observed by *T. oleaginosus*. *T. oleaginosus* consumed a total of 6 g/L citrate in both biomass hydrolysates after all glucose was utilized at 48h. *L. starkeyi* and *C. albidus* did not use citrate as nutrients. Instead of utilization, citrate accumulation was observed during lipid production by *C. albidus*. A total of 3 g/L of citric acid was produced as a secondary metabolite. As was our expectation, all strains utilized acetic acid as nutrients. Sorghum stalks and switchgrass hydrolysates contained 0.5 g/L acetic acid and completely consumed all yeast strains.

Table 6.1 shows fermentation parameters of oleaginous yeast during lipid production. *T. oleaginosus* showed the best performance of lipid production among yeast strains. *T. oleaginosus* accumulated a total of 60% and 58% of lipids using sorghum stalk and switchgrass hydrolysates, respectively. Similar levels of DCW (about 21 g/L) were achieved from sorghum stalk and switchgrass hydrolysates. Although utilized sugar concentration was higher in the switchgrass hydrolysates, higher levels of lipid concentrations (13 g/L) were attained in the sorghum stalk

hydrolysates. Therefore, sugar utilization for product formation was more efficient in the sorghum stalk hydrolysates. A high lipid yield of 0.29 g/g was obtained by *T. oleaginosus* using sorghum stalk hydrolysates. It was a similar value to the economically feasible lipid yield suggested by (Kourist et al., 2015). *L. starkeyi* also produced higher concentrations of lipids with higher lipid content in the sorghum stalk hydrolysates. However, lower lipid yield was obtained in the sorghum stalk hydrolysates because fewer amounts of sugars were consumed in the switchgrass hydrolysates. Results of statistical analysis showed that lipid accumulation of *C. albidus* was similar to *L. starkeyi* in the sorghum stalks hydrolysates, but *C. albidus* produced the lowest concentration of lipids in both biomass hydrolysates. This was because lower amounts of DCW were obtained using both biomass hydrolysates. These results demonstrated that both lipid content and DCW were important factors to achieve high titers of lipids by oleaginous yeast, because lipids are intracellular products.

Figure 6.5 shows the composition of fatty acids produced by oleaginous yeast using sorghum stalks and switchgrass hydrolysates. Different species of fatty acids were produced by *T. oleaginosus, L. starkeyi, and C. albidus*. Major fatty acids of *T. oleaginosus* were palmitic acid (C16:0) and oleic acid (C18:1). This result was consistent with previous studies (Kim and Oh, 2013). Myristic acid (C14) was only produced by *T. oleaginosus,* but the amount was marginal. The most abundant fatty acid of *L. starkeyi* was oleic acid, accounting for more than 60%. (Jaworski and Cahoon, 2003) also reported that *L. starkeyi* contented relatively high levels of oleic acid (up to 70%), which is preferable in the oleochemical industry. Oleic acid was a major fatty acid for all yeast strains because most yeast species include a Δ9 desaturase, which incorporates a double bond at Δ9 position of stearic acid or palmitic acid (Probst et al., 2015). *T. oleaginosus* and *C. albidus* produced relatively higher levels of linoleic acid (C18:2n6) and linolenic acid

(C18:3n3) compared with *L. starkeyi*. In addition, *L. starkeyi* did not produce linolenic acid. In the fatty acid elongation cycle, oleic acid can be further desaturated to linoleic acid and linolenic acid by $\Delta^{12}$desaturase and $\omega^3$ desaturase, respectively (Probst et al., 2015). It was assumed that *L. starkeyi* does not have $\omega^3$ desaturase, and $\Delta^{12}$desaturase enzyme activity would be insubstantial. Therefore, the highest amount of oleic acid, which is a substrate of both desaturase enzymes ($\Delta^{12}$desaturase and $\omega^3$ desaturase), contented in the *L. starkeyi.* Also, it is anticipated that a desaturase enzyme produced by *T. oleaginosus* or *C. albidus,* can be utilized to develop microbial strains for polyunsaturated fatty acid production.

### *Lipid yield from lignocellulosic biomass*

Our process of lignocellulosic-based microbial lipid production was evaluated by calculating the overall yield of lipid from raw sorghum stalks and switchgrass (Figure 6.6). The highest lipid yield was achieved by *T. oleaginosus* using sorghum stalk as a feedstock. Lipid yields from sorghum stalks and switchgrass were not substantially different because similar amount of sugars (about 49 g) were recovered from 100 g of both biomasses. This result revealed that species of yeast and their fermentation performance directly affected total lipid yield from sorghum stalks and switchgrass. The highest lipid yield was achieved by *T. oleaginosus* from both lignocellulosic biomasses, since *T. oleaginosus* showed the best fermentation performance among other yeast strains during lipid production. *T. oleaginosus* produced 8% higher amount of lipids from sorghum stalks containing a 14% lower content of polysaccharides compared with switchgrass. This might be due to a substantial hemicellulose loss during pretreatment of switchgrass. It showed another key factor to attaining high lipid yields from biomass was to maximize sugar recoveries during pretreatment and enzymatic hydrolysis for hydrolysate production. Lipid yields obtained by *C. albidus* and *L. starkeyi* were not substantially different because of their similar fermentation

performance. *C. albidus* produced higher amounts of lipids using sorghum stalks, although low-lipid concentrations were obtained since higher product yield was achieved during fermentation using sorghum stalk hydrolysates. Lower amounts of lipids were obtained by *L. starkeyi* using sorghum stalks, even though higher lipid concentrations and contents were attained during fermentation of *L. starkeyi* using sorghum stalk hydrolysates. This was because lower sugar consumptions and product yields were observed in sorghum stalk hydrolysates. To sum up these results, maximization of sugar recoveries during pretreatment and enzymatic hydrolysis, and selection of proper microbial strains for lipid production, were key factors to achieve high yields of microbial lipids from lignocellulosic biomass.

### *Strategy to enhance the TAG level in oleaginous yeast systems*

Lipid titer and yields from wild strains were still not substantial for the viable production of lignocellulose-based lipids. To improve TAG levels in the oleaginous yeast cultures, a plasmid *pRS2u2-ACL* was constructed. Plasmids *pRS2u2* (Figure 6.7) and *pOleo-ACL* (Figure 6.8) were obtained from the Department of Biochemistry and Molecular Biophysics, Kansas State University, to construct *pRS2u2-ACL*. ATP citrate lyase (*ACL*) catalyzes the cleavage of citrate to yield acetyl-CoA and oxaloacetate as described in the Introduction (Figure 1.4). *ACL,* which exists as an activated form in the oleaginous microorganisms, has a responsibility to increase cytosolic Acetyl-CoA pool, which is an important substrate for carbon flow to TAG synthesis. Therefore, our hypothesis that the overexpression of *ACL* would improve TAG level in the oleaginous yeast system. Construction of *pRS2u2-ACL* was confirmed by colony PCR (Figure 6.9). First line and last line are ladder to check gene size and second line is control of *ACL* gene; PCR product from *pOleo-RnACLy* was used as control marker. Size of *ACL* gene derived from *Rattus norvegicus* is 3.3 Kb between fifth and sixth line in the ladder. Positive colonies were confirmed by colony PCR;

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therefore, it seemed that ligation of plasmid *pRS2u2-ACL* was confirmed. However, disgestion using different enzymes and sequencing will be required to check direction of *ACL* gene in the plasmid.

One of the challenges to express *ACL* genes in the oleaginous yeast system is that the transformation method was not developed for each strain. The chemical method using lithium acetate is widely used for yeast transformation. Lithium acetate is known to neutralize charges on DNA molecules and generate small holes in the plasma membrane. ssDNA was used to transfer DNA into the cells and polyethylene glycerol (PEG) helps bring the DNA into closer position with the membrane during lithium acetate transformation. Calvey and coworkers optimized the transformation protocol using lithium acetate for *L. starkeyi* (Calvey, Willis and Jeffries, 2014). Results showed that incubation time and heat shock temperature were key factors to increase transformation rates. Li and coworkers, which optimized transformation protocol for *Trichosporonoides oedocephalis* (Li et al., 2016), reported that incubation time and calcium chloride concentration were the critical factors. Another common transformation method recently used for oleaginous yeast is transfection; *Agrobacterium* mediated transformation. *Agarobacterium* harboring the plasmid are co-cultivated with oleaginous yeast on Hybond-N$^+$ membranes in the IMAS plates and transferred to yeast media supplemented with cefotzxime in order to kill the Agrabacterium and select transformants (Görner, 2016). Recent researches successfully expressed a heterologous gene in the oleaginous yeast system using transfection (Salunke et al., 2015; Görner, 2016).

## Conclusions

Microbial lipid production from sorghum stalks and switchgrass was investigated using oleaginous yeast strains. High-sugar recoveries (89% of TY) from sorghum stalks were obtained

*via* an alkaline pretreatment. *T. oleaginosus* showed the best fermentation performance using both biomass hydrolysates. Lipid titers of 13.2 g/L and lipid yield of 0.29 g/g were achieved by *T. oleaginosus* using sorghum stalk hydrolysates. Results of overall lipid yield assessment revealed a key matrix to improve industrial feasibility of lignocelluosic-based microbial lipid production is maximal recovery of fermentable sugars from raw biomass and microbial strain development to attain better fermentation performance. Also, we anticipate that *ACL* expression will improve TAG levels in the oleaginous yeast.

Table 6-1 Fermentation performance of oleaginous yeast during lipid production.

| | Lipid content[a] (%) | Lipid concentration (g/L) | Lipid yield[b] (g/g) | DCW (g/L) | Sugar consumption (g/L) |
|---|---|---|---|---|---|
| Sorghum stalk hydrolysates | | | | | |
| *T. oleaginosus* | $60 \pm 2.5^A$ | $13.1 \pm 0.7^A$ | $0.29 \pm 0.0^A$ | $21.7 \pm 0.3^A$ | $45 \pm 0.7^B$ |
| *L. starkeyi* | $44 \pm 2.0^B$ | $7.9 \pm 0.3^C$ | $0.16 \pm 0.0^C$ | $18.1 \pm 0.1^B$ | $48 \pm 0.7^A$ |
| *C. albidus* | $42 \pm 2.0^{B, C}$ | $4.6 \pm 0.2^E$ | $0.17 \pm 0.0^D$ | $11.1 \pm 0.1^D$ | $27 \pm 0.6^E$ |
| Switchgrass hydrolysates | | | | | |
| *T. oleaginosus* | $58 \pm 2.6^A$ | $12.3 \pm 0.2^B$ | $0.27 \pm 0.0^B$ | $21.1 \pm 0.6^A$ | $46 \pm 1.1^B$ |
| *L. starkeyi* | $39 \pm 0.1^C$ | $6.5 \pm 0.3^D$ | $0.17 \pm 0.0^D$ | $16.6 \pm 0.4^C$ | $38 \pm 0.9^C$ |
| *C. albidus* | $44 \pm 0.0^B$ | $4.7 \pm 0.1^E$ | $0.16 \pm 0.0^C$ | $10.7 \pm 0.3^D$ | $29 \pm 1.4^D$ |

DCW=dry-cell weight

The data represent average value of triplicate experiments ± sample standard deviation. Values with the same letters, in superscripts, within the same column, are not significantly different at the level of $P<0.05$.

[a] Lipid content was defined as weight of extractable lipid relative to weight of dry cell mass.

[b] Lipid yield was calculated by dividing amount of lipids by amount of sugar consumed.

**Ground lignocellulosic biomass**

- Sorghum stalks or Switchgrass
- Size ≤ 1 mm

**Pretreatment**

- 10% (w/v) solid loading
- 1.25% (w/v) sodium hydroxide
- 121 °C, 30 min or 1 h

**Pretreated lignocellulosic biomass**

Wash with water
Dry at room temperature

**Enzymatic hydrolysis**

- 10% (w/v) solid loading
- C-Tec2 and H-Tec2
- 50 °C, 48 h

Centrifuge at 8500 rpm for 20 min

**Lignocellulosic hydrolysates**
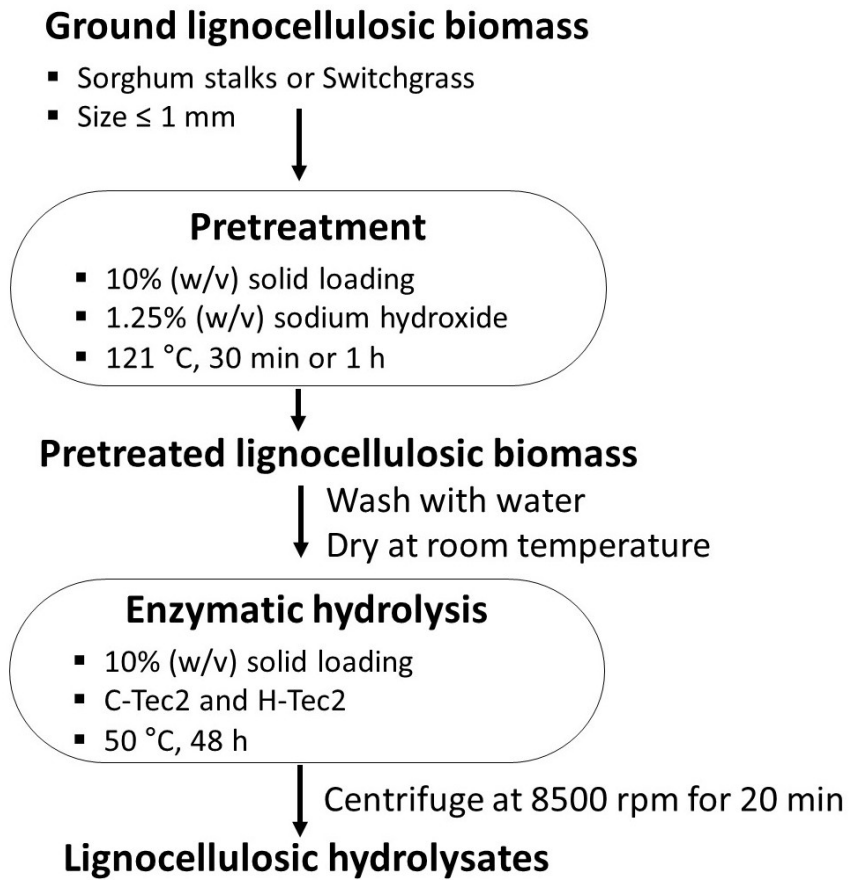
Figure 6-1 Schematic diagram of the overall process to produce fermentable sugars from lignocellulosic biomass
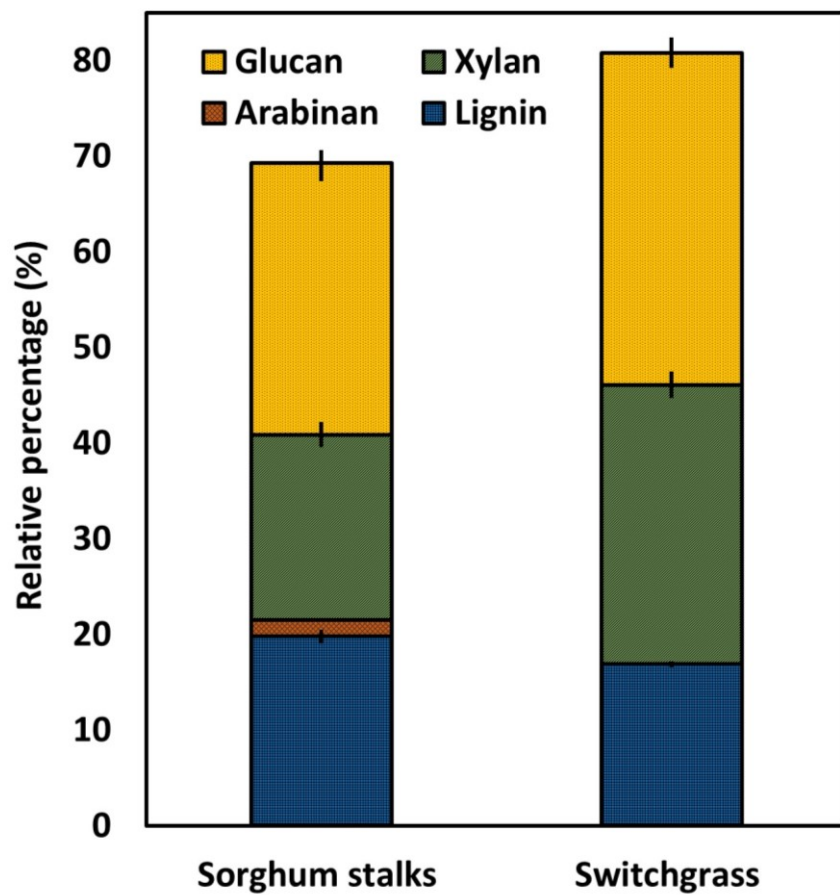
Figure 6-2 Composition of lignocellulosic biomass. The data shows average value of triplicate experiments and error bars representing sample standard deviation.
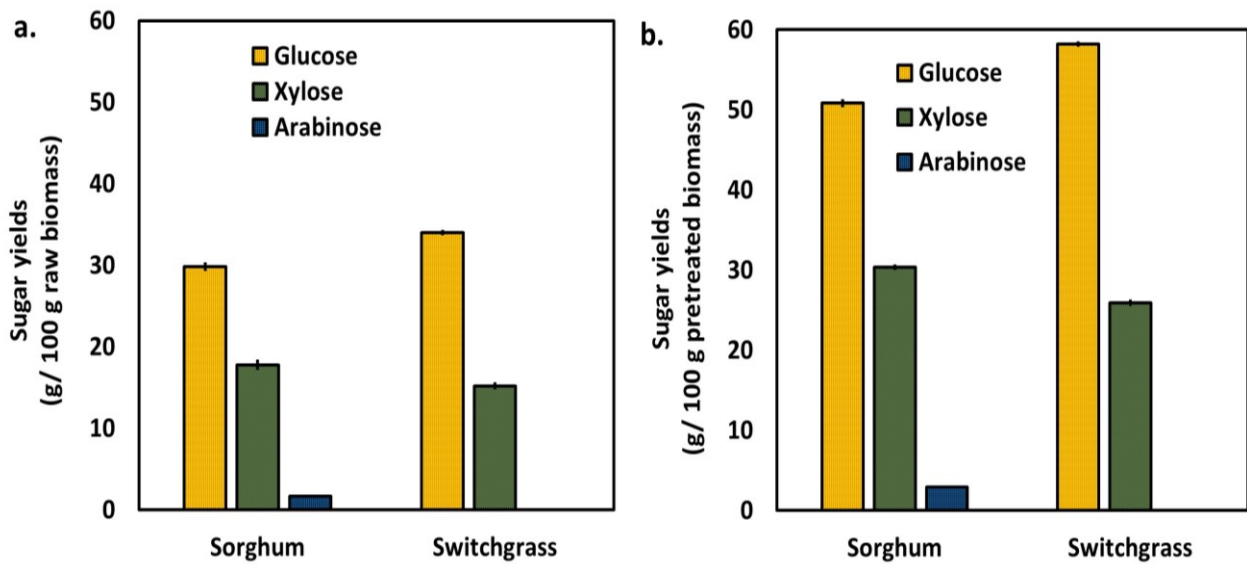
Figure 6-3 Sugar yield from (A) raw lignocellulosic biomass; (B) pretreated lignocellulosic biomass. The data shows average value of triplicate experiments and error bars representing sample standard deviation.
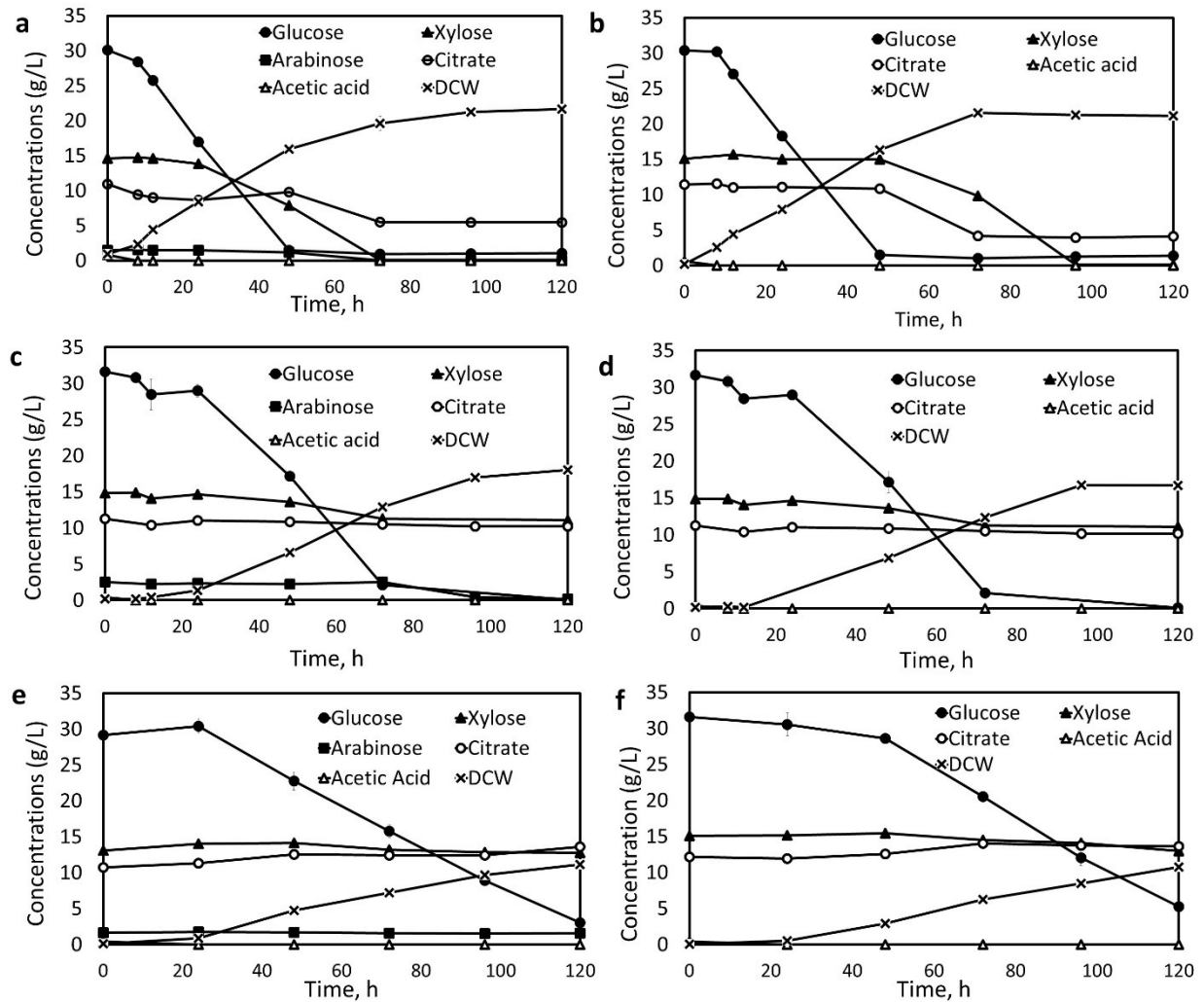
Figure 6-4 Fermentation profile during lipid production by (A) *T. oleaginosus* using sorghum stalk hydrolysates; (B) *T. oleaginosus* using switchgrass hydrolysates; (C) *L. starkeyi* using sorghum stalk hydrolysates; (D) *L. starkeyi* using switchgrass hydrolysates; (E) *C. albidus* using sorghum stalk hydrolysates; (F) *C. albidus* using switchgrass hydrolysates, The data shows average value of triplicate experiments and error bars representing sample standard deviation.
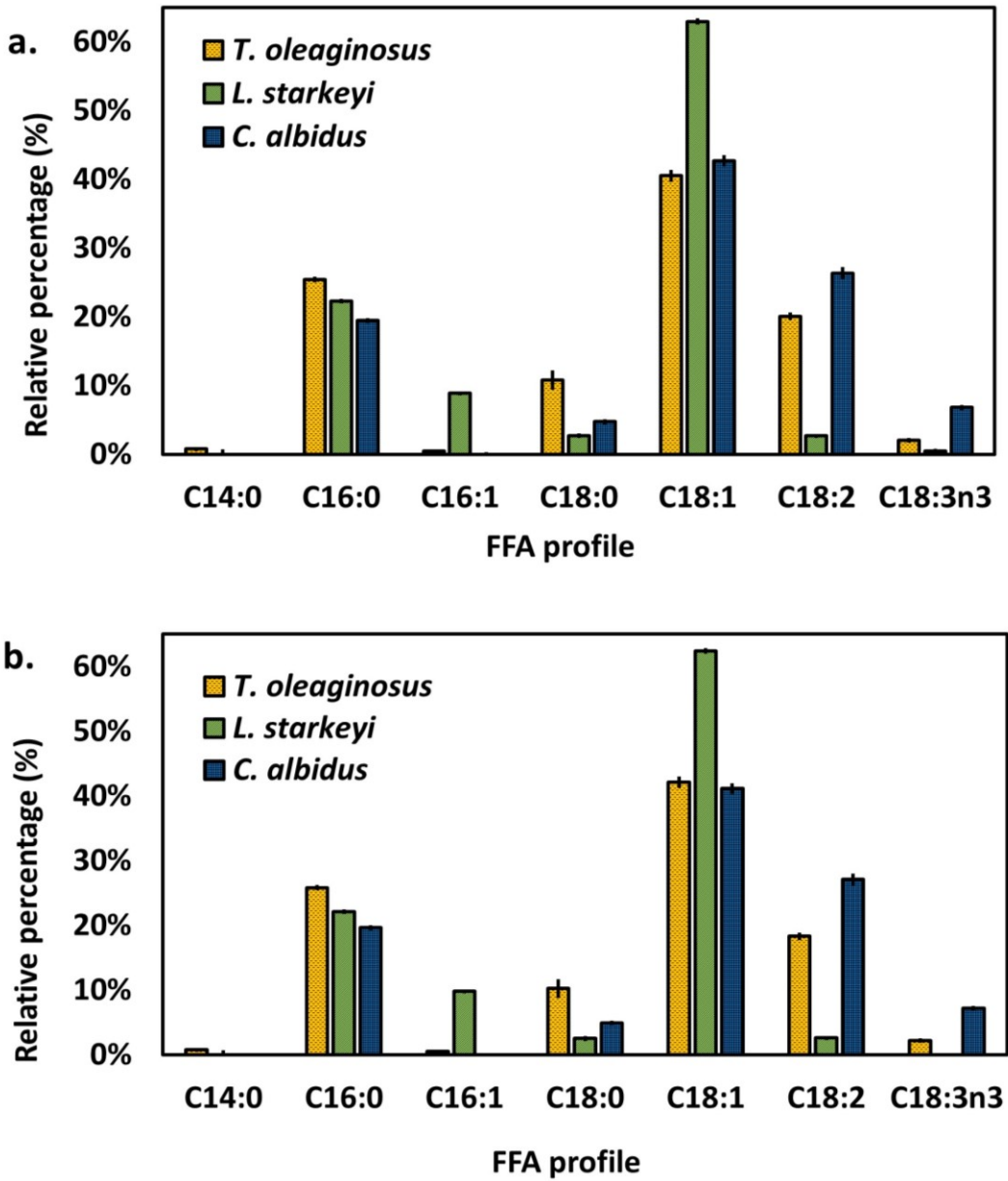
Figure 6-5 Composition of fatty acid produced from (A) sorghum stalk hydrolysates; (B) switchgrass hydrolysates. The data shows average value of triplicate experiments and error bars representing sample standard deviation.

**a.**

Sorghum stalks
100g

Pretreatment
Enzymatic hydrolysis

Sorghum stalks
hydrolysates

Total sugars : 49.3 ± 0.4 g

Fermentation

Microbial lipids

*T. oleaginosus* : 14.3 ± 0.2 g
*L. Starkey* : 7.9 ± 0.1 g
*C. Albidus* : 8.4 ± 0.2 g

**b.**

Switchgrass
100g

Pretreatment
Enzymatic hydrolysis

Switchgrass
hydrolysates

Total sugars : 49.2 ± 0.6 g

Fermentation

Microbial lipids

*T. oleaginosus* : 13.3 ± 0.0 g
*L. Starkey* : 8.4 ± 0.2 g
*C. Albidus* : 7.9 ± 0.1 g
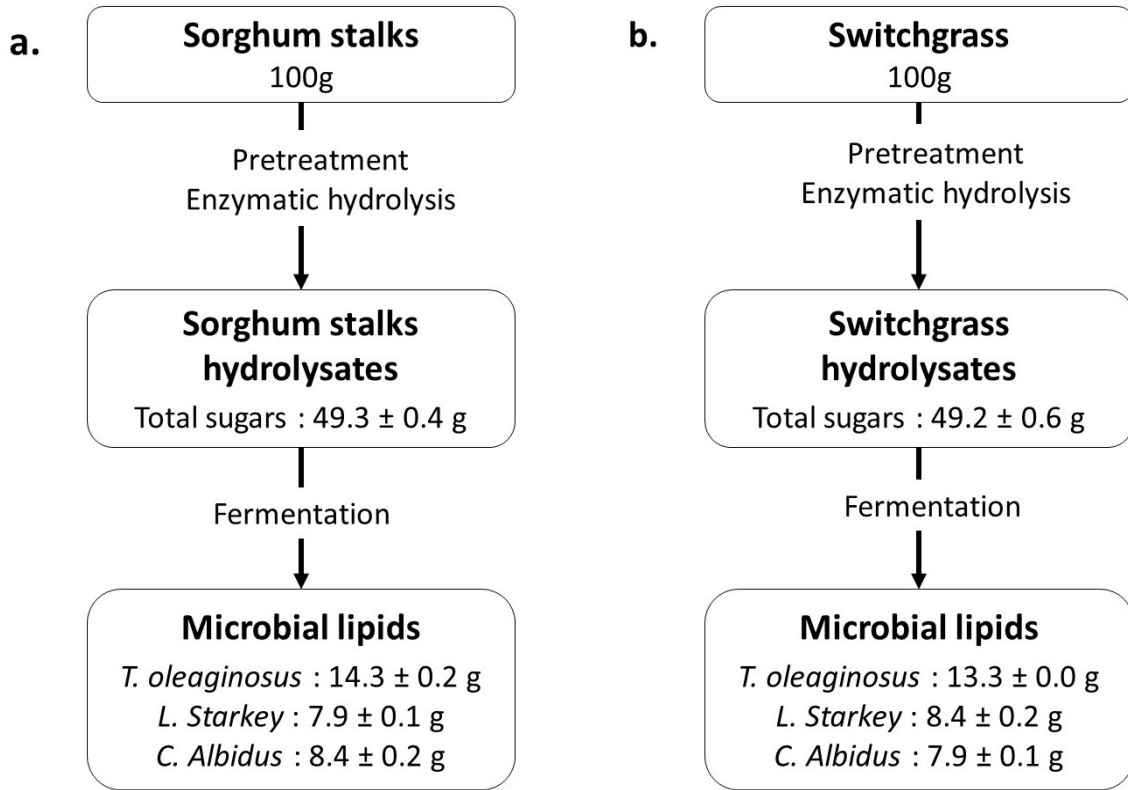
Figure 6-6 Lipid yields from (A) sorghum stalk; (B) switchgrass. The data shows average value of triplicate experiments and error bars representing sample standard deviation.
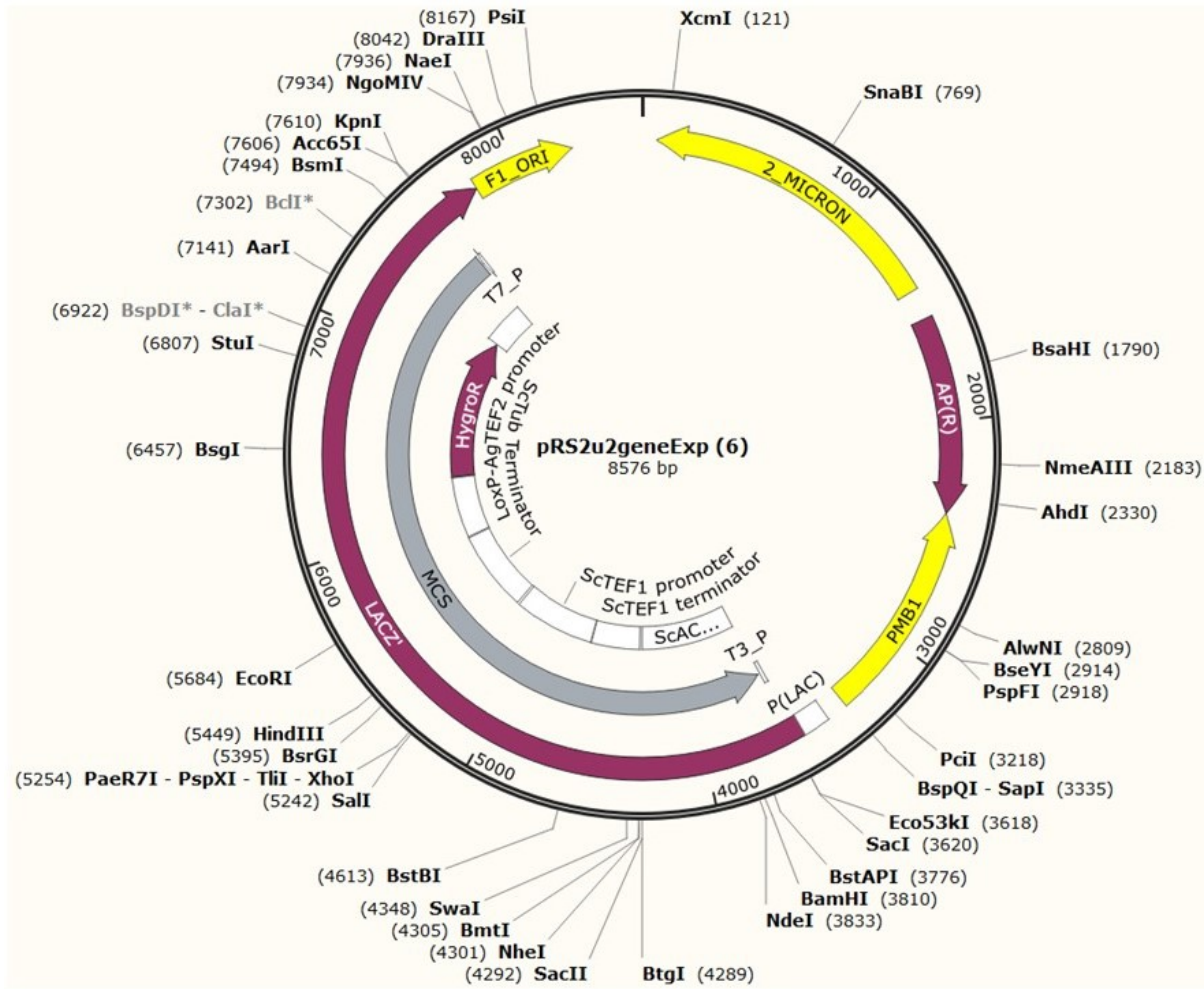
Figure 6-7 Expression vector *pRS2u2*

F1 ORI: origin of replication; HygroR: hygromycein B resistance gene; AP(R): ampicillin resistance gene; ScTEF1 promoter: TEF promoter derived from *Saccharomyces cerevisiae;* ScTEF1 terminater: TEF terminater derived from *S. cerevisiae*; MCS: multiple cloning site; SsACT: ACT promoter derived from *S. cerevisiae*; scTub terminater: Tub terminater derived from *S. cerevisiae*; LoxP-AgTEF2 promoter: TEF promoter derived from *Ashnya gossypii*
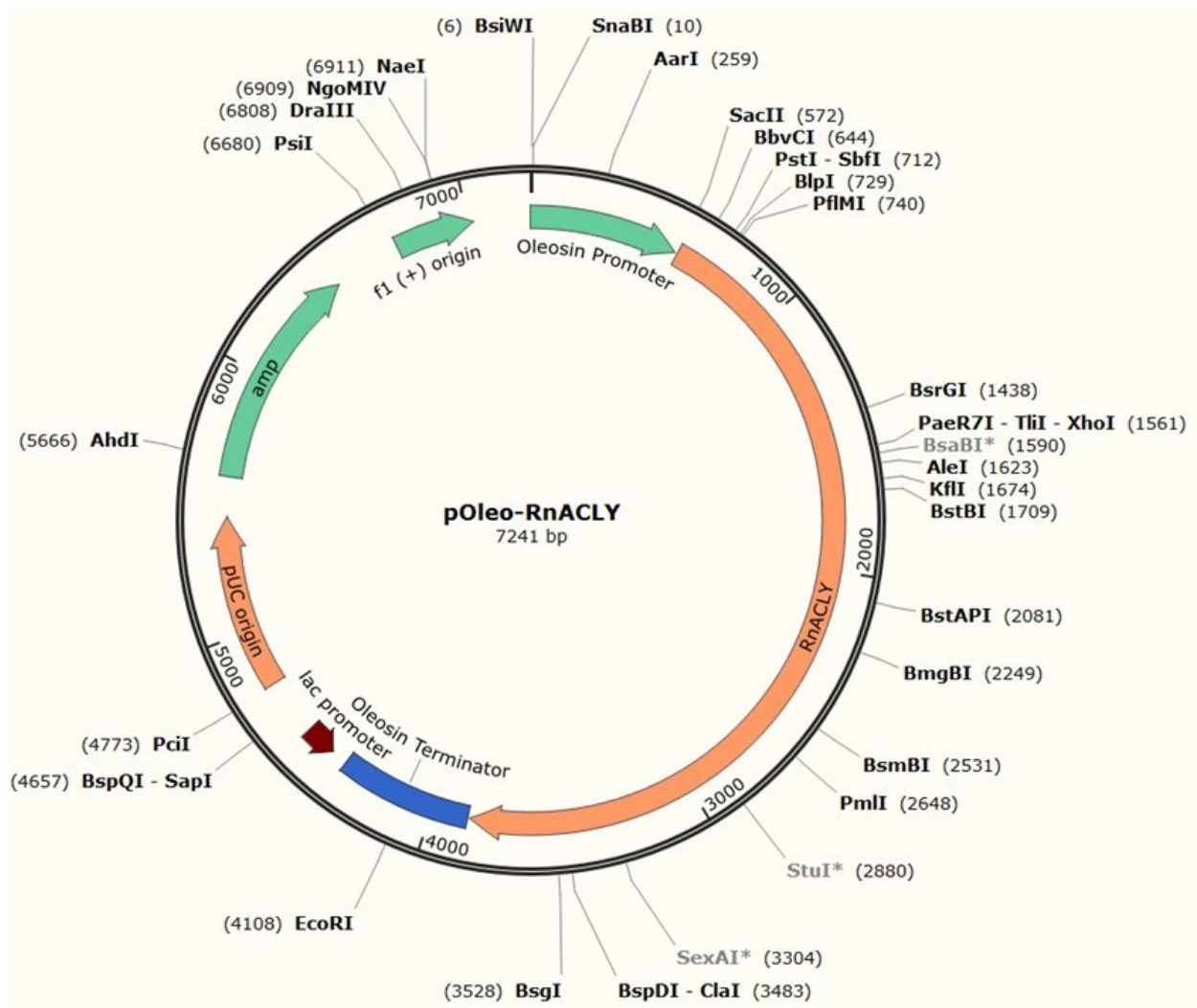
Figure 6-8 Plasmid harboring *ACL* gene *pOeo-RnACLY*

f1 (+) ORI: origin of replication; amp: ampicillin resistance gene; RnACLY: ATP-citrate lyase derived from *Rattus norvegicus*
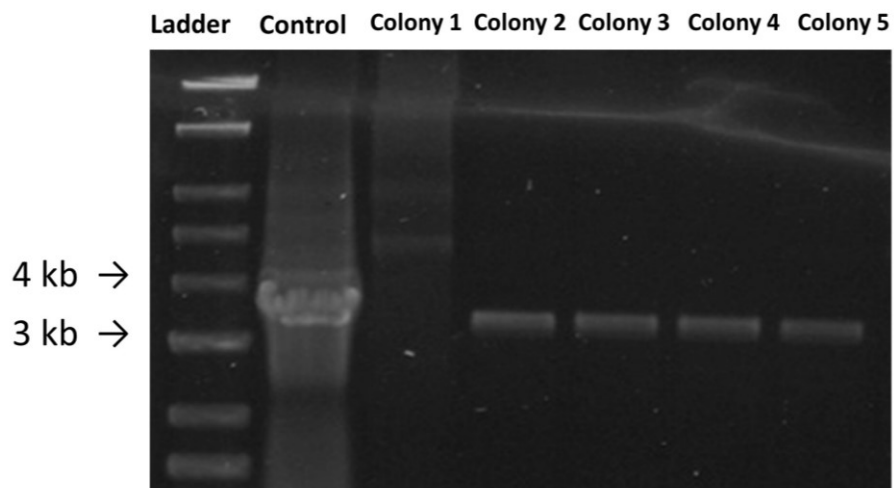
Figure 6-9 Colony PCR to confirm plasmid construction of *pRS2u2-ACL*. Control was the PCR product of plasmid *pOleo-RnACLy*. Colony 1 to 5 were randomly selected after ligation and confirmed the plasmid construction using colony PCR.

# Chapter 7 - Corn bran bioprocessing: development of an integrated process for microbial lipids production

## Abstract

Corn bran is known as a low value co-product after dry or wet milling process due to their unappealing taste and flavor, as well as its technical drawback in the food industry. However, corn bran, which is a polysaccharides-rich material, is an attractive feedstock for bio-based chemical production. In this study, the potential of corn bran as a feedstock for microbial lipid production using oleaginous yeast, *T. oleaginosus* ATCC20509, was investigated. This study found the direct effect of pretreatment conditions on the lipid accumulation of *T. oleaginosus* during fermentation using response surface methodology (RSM). In addition, overall lipid yield from raw corn bran was calculated to evaluate our corn bran bioconversion process with different pretreatment conditions. Compared with synthetic media, up to 50% higher lipid accumulations in *T. oleaginosus* was achieved using corn bran hydrolysates during the fermentation process. Among pretreatment conditions, solid loading significantly affected the fermentation process for lipid accumulation. Overall yield assessment showed that the highest sugar yields (0.53 g/g of de-starched corn bran) and lipid yields (216 mg/g of de-starched corn bran) were obtained at 5% solid loading and 1% acid loading at 30 min of pretreatment. This study demonstrated that corn bran can be a viable sugar supplier for bio-based chemical production in biorefineries. Also, the RSM model in this work can provide useful information to design the integrated bioconversion platform for lipid production using corn bran.

# Introduction

Microbial lipids are preferable to alternate plant oils in the bio-diesel and an oleo-chemical industry, due to their fatty acid composition, environmental impact, year-round production, and no requirement of broad lands (Sitepu et al., 2014; Zhou et al., 2016). Oleaginous yeast efficiently accumulates lipids of at least 20 % (w/w of dry-cell mass), mainly as a form of triacylglycerides (TAG), using a broad array of agricultural wastes (Vieira et al., 2016; Matsakas et al., 2014; Lin et al., 2013; Kerkhoven et al., 2016) that can be utilized as host strains for microbial lipid production. Economics would be the most critical factor for successful microbial lipid production in a biorefinery (Jin et al., 2015; Lennen and Pfleger, 2012). Many endeavors to improve economically feasible production of microbial lipids have been attempted such as exploration of new sugar suppliers and development of bioconversion processes to reduce costs. Previous studies have successfully produced microbial lipids using renewable biomass — such as corn stover, wheat straw, and switchgrass — as substrates (Gong et al., 2014; Slininger et al., 2016; Yu et al., 2011).

Bran, the outer layer of cereals, is too often discarded during the milling process instead of being used as a food application, due to consumers' sensory expectations and technological drawbacks in the food industry (Coda, Katina and Rizzello, 2015). Frequent corn by-products of dry milling are corn flour, corn bran, and hominy feed, and their economic disposal is the main concern of the food industry in fulfilling environmental regulations (ElMekawy et al., 2013). Corn bran is the most abundant, low-valued co-product of the industrial corn milling process in spite of high amounts of polysaccharide content with marginal amounts of lignin (Yadav et al., 2015). Corn bran is produced in yields of 60-70 g/kg, with a total production of 3 x $10^6$ dry tons per year (Rose, Inglett and Liu, 2010). Corn bran contains a large percentage of hemicellulose and has an

arabinoxylan structure consisting of a β-1,4 linked D-xylopyranosyl backbone and α-L-arabinofuranosyl residues as side units linked (1 → 2) or (1 → 3) to the main chain (Rose, Inglett and Liu, 2010; Yadav et al., 2015). Therefore, hemicellulose can be hydrolyzed into pentose (xylose and arabinose) and hexose (glucose, galactose, and mannose) (Peng et al., 2012), and is a promising substrate for microbial lipid production. Development of an effective pretreatment process is responsible for the recalcitrant of biomass structure toward hydrolysis of carbohydrate polymer (Guragain et al., 2014). Another bottleneck for integrated production of microbial lipids from renewable biomass is the generation of inhibitory compounds such as furfural and hydroxyfurfural (Keshav et al., 2016; Cavalaglio et al., 2016). Acetic acid is also an inevitable inhibitory compound during bioprocessing of hemicellulosic bio-polymers. Harsh conditions should be applied to expose the structure of lignocellulosic biomass *via* a pretreatment process. Therefore, generation of inhibitory compounds cannot be avoided in a biorefinery. To maximize utilization of corn bran hydrolysates, selection of an appropriate yeast strain, which has high tolerance to toxic compounds and enables use of diverse monomer sugars as a carbon source — including xylose and arabinose, would be critical.

*Trichosporon oleaginosus* ATCC 20509, which have been recently classified as basidomycetous, are known to accumulate up to 70% (w/w of dry mass) lipids using a variety of carbon sources such as pectin-derived sugar acids, *N*-acetylglucosamine, and whey permeate (Görner et al., 2016). Also, many studies reported that *T. oleaginosus* consume carboxylic acids, which are known as inhibitory compounds during the fermentation process. *T. oleaginosus* efficiently produced 8 g/L of lipids using acetate-based nutrients, with a yield of 0.15 g/g and productivity of 0.64 g/L/h (Gong et al., 2015). Lian and coworkers reported that acetate and

formate were good energy sources for contribution to growth and lipid production of *T. oleaginosus* (Lian et al., 2012).

This study developed the integrated process for microbial lipid production from de-starched corn bran. First, pretreatment conditions were optimized to obtain high sugar recovery and subsequent lipid yields. Fifteen runs of pretreatment conditions were applied for de-starched bran, and their sugar recoveries were investigated. Also, fifteen runs of corn bran hydrolysates were evaluated as nutrients for lipid production by *T. oleaginosus*. In addition, overall lipid yield from raw corn bran were calculated to investigate corn bran utilization as feedstocks for lipid production. Furthermore, the relationship between pretreatment conditions and lipid accumulation in *T. oleaginosus* was investigated using response surface methodology (RSM). Our study revealed that corn bran can be an alternative sugar supplier in bio-based chemical production, and optimization of pretreatment conditions would be a critical factor to improve viable production of microbial lipids.

## Materials and methods

### *Microorganisms and fermentation*

*Trichosporon oleaginosus* ATCC20509 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and grown in a yeast mold broth (YM broth, Difco, Detroit, MI, USA). Culture conditions were 25 °C at 200 rpm. Yeast cultures were preserved in a YM agar plate at 4 °C, and re-cultivated to a fresh plate once a month.

### *Preparation of de-starched corn bran*

Corn bran was obtained from LifeLine Foods, St. Joseph, Missouri, USA, and milled using a Fitz-Mill (Fitzpatrick Company, Elmhurst, IL, USA) fitted with a 2.36-mm screen. After milling, corn brans were dried at 40 °C for 72 h.

The overview of corn bran hydrolysate preparation is shown in Figure 7.1. Alpha-amylase (Liquozyme, Novozymes Inc. Franklinton, NC, USA) and glucoamylase (GC480, Genencor International Inc., Palo Alto, CA, USA) were provided from MGP Ingredients (Atchison, KS, USA) to remove starch *via* liquefaction and saccharification. Corn bran was mixed with distilled water at a rate of 15% (w/v) solid loading, and α-amylase was added with a concentration of 1 μL/g starch. Liquefaction was conducted at 80 °C for 1h. Corn bran slurry was cooled to 65 °C and saccharification was performed by adding 5 μL/g starch of glucoamylase at 65 °C for 2h. De-starched corn bran was washed with water to completely remove starch and dried at 40 °C until moisture content was below than 10 % (w/w).  Raw corn bran contented 9.6% cellulose, 25.8% hemicellulose, and 32.2% starch; de-starched corn bran was composed of 14.2% cellulose and 38% hemicellulose (Probst and Vadlani, 2015).

### *Pretreatment and enzymatic hydrolysis of de-starched corn bran*

Table 7.1 shows 15 runs of experimental conditions to optimize pretreatment for de-starched bran. Different solid loading (5, 10, 15%), acid loading (0.5, 1, 1.5%), and pretreatment times (30, 45, 60 min) were applied for each run. Corn bran was mixed with water in a 250 mL flask at a solid loading of 5, 10, or 15% (w/v). Sulfuric acid was added into each flask at a rate of 0.5, 1, or 1.5 % (w/v) and pretreated at 121 °C for 30, 45, or 60 min. After pretreatment, flasks were cooled at room temperature for 2 h and pH was neutralized to 5.0 using 10 M sodium hydroxide. Cellic C-Tec2 and Cellic H-Tec2 (Novozyme Inc, Franklinton, NC) were added into each flask at a rate of 2.7 and 0.3 % (w/w of dry biomass), respectively. Enzymatic hydrolysis was conducted at 55 °C and 140 rpm for 48 h. Corn bran hydrolysates were separated *via* centrifugation at 8500 rpm for 20 min (Sorvall Super T21, Thermo Fisher Scientific Inc., Waltham, MA, USA) and sterilized using filters with 0.22 μm pore size.

## *Fermentation conditions*

A starter culture was prepared by inoculating a single colony from a plate and growing it in a YM broth at 25 °C and 200 rpm for 12 h. Two mL of seed culture were transferred into a 250 mL flask containing 20 mL of corn bran hydrolysates. Fermentation was performed at 25 °C, 200 rpm, for 120 h. After fermentation, cells were harvested *via* centrifugation at 8500 rpm for 20 min and washed two times with $dH_2O$ to completely remove residue of nutrient media. Cells were concentrated to ~ $10^9$ cells/mL and preserved at -80 °C for 1 day prior to lipid extraction.

## *Lipid extraction and fatty acid analysis*

Concentrated cells were thawed and 0.5mL of the product was transferred into a 2.5 mL microvial, followed by an addition of 0.5 mL of chloroform, 0.5 mL of methanol, and 1 mL of 0.5 mm beads (BioSpec, Bartlesville, OK, USA). Bead beating was performed to lysate cells using a bead-beater homogenizer (Mini-Beadbeater-24, BiosSpec Products, Inc., Bartlesville, OK, USA). Six cycles of bead beating (45 sec intervals, 10 min cooling on ice) were conducted, and cell lysates were transferred into a Kimax tube. Additionally, chloroform, methanol, and water were added to make the final ratio 1:2:0.8. Cell mixtures were vortexed for 1 min and centrifuged at 4000 rpm for 15 min. The bottom layer was transferred to a clean Kimax tube and an additional 1 mL of chloroform was added to the previous cell mixtures. This procedure was repeated three times and combined all chloroform layers. One mL of potassium phosphate was added and centrifuged at 4000 rpm for 10 min. Chloroform layers were dried under nitrogen gas — 1 mL at 40 °C until the remaining portion was 1 mL. The remaining chloroform layer containing lipids was transferred into a pre-weighed microvial and completely dried at 40 °C. Lipids in the microvial were weighed to calculate final lipid weight. One mL of chloroform was added to the microvial and preserved at -80 °C for further fatty acid analysis.

Transesterification was performed to convert fatty acids in the lipids to fatty acid methyl ester. Internal standard (C15) and lipid samples were transferred into Kimax tubes, and the chloroform layer was evaporated under nitrogen gas. Transesterification was conducted by adding 1 mL of 3 M methanolic hydrochloric acid. Tubes were incubated at 78 °C for 30 min. After cooling, 2 mL of water, 1.6 mL of chloroform, and 0.4 mL of hexane were added and vortexed. The mixtures were centrifuged at 4000 rpm for 5 min to obtain separated layers. The lower layers were collected and the organic phase was evaporated, followed by an addition of 100 μL of hexane. Fatty acid composition was analyzed using a gas chromatograph (GC-2014, Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a flame ionization detector (FID) and a capillary column (Zebron ZB-Waxplus 30 m x 0.25 mm x 0.25 μm, Phenomenex, Torrance, CA, USA). Temperatures of injector and FID were set at 220 °C and 250 °C, respectively. The relative retention time of fatty acids was determined using an external standard (Supleco 37 Component FAME Mix).

### *Analysis of sugars and inhibitory compounds*

Sugar and inhibitory compounds were identified and quantified using high-performance liquid chromatography (HPLC), equipped with a Rezex ROA organic acid column (300 x 7.8 mm, Phemomenex Inc., Torrance, CA, USA) and a refractive index detector (RID). Column and RID were set at 80 °C and 40 °C, respectively. Sulfuric acid amounts of 0.005 N were utilized as the mobile phase with a flow rate of 1 mL/min.

### *Experimental design*

Response surface methodology was used to optimize pretreatment conditions (solid loading, pretreatment time and acid loading) of corn bran. The effect of pretreatment conditions on lipid content during fermentation was investigated to identify the optimum composition of corn bran hydrolysates for lipid accumulation by *T. oleaginosus*. The experimental design was generated by

the software Design Expert V. 8.0.7.1 (Stat-Ease Inc., Minneapolis, MN, USA). Box-Behnken design (Box and Behnken, 1960) was used to optimize the levels of independent input factors, which were solid loading, pretreatment time and acid loading (Table2), and lipid content (w/v) of *T. oleaginosus* was the response. A total of 13 runs comprising 3 replicates in central point was performed at the random order. Models and regression coefficients were validated with an Analysis of Variance (ANOVA), and significance for statistical results was established for $P < 0.05$.

### *Statistical methods*

Design Expert software (Version 8.0.7.1, Stat-Ease Inc., Minneapolis, MN, USA) was used to generate experimental designs for 15 runs of pretreatment conditions. A total of 15 runs was carried out in triplicate, in random order. SAS software (SAS v9.4, SAS institute, Cary, NC, USA) was used to perform PROC GLM for the least-significant-difference (LSD) test at a 95% confidence level ($P<0.05$).

## Results and discussion

### *Effect of pretreatment conditions on production of fermentable sugars and inhibitors*

Previous study regarding lipid production from bran showed that higher lipid yields were obtained from de-starched-bran hydrolysates compared with whole bran hydrolysates since de-starched bran caused production of lower amount of inhibitory compounds (Probst and Vadlani, 2015). Therefore, starch fraction was removed via liquefaction and saccharification before pretreatment process (Fiure 7.1). A total of 15 runs of pretreatment conditions were generated using Box-Behnken design to optimize the pretreatment conditions of de-starched corn bran. Pretreatment conditions with different solid loading (5, 10, 15%), acid loading (0.5, 1, 1.5 %) and pretreatment times (30, 45, 60min), were applied to disrupt the structure of de-starched corn bran

(Table 7.3). The pretreated corn bran was converted to monosaccharides *via* enzymatic hydrolysis using cellulytic and hemicellulytic enzymes, and sugar recoveries were analyzed to investigate the effect of pretreatment conditions. Table 7.4 shows sugar yields of fifteen types of corn bran hydrolysates from de-starched corn bran. The major sugar species released from de-starched corn bran was xylose. Xylose yield were normally 15 to 20% higher than glucose yields, depending on pretreatment conditions. Compositional analysis results showed 2.7% higher content of hemicellulose than cellulose (Table 7.1). Therefore, it was conceivable to achieve higher yields of xylose than glucose. The highest sugar yields of 0.53 g/g were obtained from corn bran hydrolysates applied Run 9 condition. The lowest sugar yields of corn bran hydrolysates applied Run 6 condition might have been due to pretreatment conditions of 0.5% acid loading for 45 min being insufficient to disrupt corn bran structures at 15% solid loading.

Table 7.5 shows inhibitor yields from de-starched corn bran. HMF, furfural, acetic acid, and formic acid, produced from corn bran. Acetic acid — a result from the hydrolysis of the acetyl group of hemicellulose — were the most abundant compounds (Guragain, Wang and Vadlani, 2016; Chen et al., 2012). HMF and furfural were typical by-products derived from pentose sugars under acidic conditions (Chandel, Da Silva and Singh, 2013). They can be further dehydrated to furfurals and formic acid under severe pretreatment conditions due to their unstable structure (Jönsson and Martín, 2016). HMF, furfural, and formic acid were minor compounds compared with acetic acid. However, a synergic effect of those compounds during fermentation was reported by (Mussatto and Roberto, 2003; Chandel, Da Silva and Singh, 2013). Marginal inhibitor yields were observed in the corn bran hydrolysates applied Run 6 condition, and this result also showed the pretreatment condition of Run 6 did not efficiently disturb corn bran structures; therefore, not enough sugars and minimal inhibitors were obtained. Sugar and inhibitor concentrations of corn

bran hydrolysates are shown in Figure 7.2. Although the highest sugar yields were achieved from corn bran applied Run 9 condition, total sugar concentrations were not that significant because of lower levels of solid loading (5%). When a 5% solid loading was considered during pretreatment, 23 to 27 g/L of total sugars were produced. Highest sugar concentrations (78 g/L) were obtained from corn bran applied Run 5 condition, and these were three times higher than the results of corn bran hydrolysates applied pretreatment conditions of Run 9, 10, 14, and 15. This might be due to the three times higher solid loading used in the pretreatment conditions for Run 9, 10, 14, and 15. Even if a 15% solid was utilized for Run 6, only 28 g/L of sugars were obtained. This showed that 1% acid loading with 60 min of pretreatment time was effective, but 0.5 % acid loading with 45 min of pretreatment time was not enough conditions for 15% solid loading. The correlation between pretreatment conditions and compound generation is shown in Figure 7.3. As higher sugar concentrations were achieved, concentration of inhibitory compounds also increased showing a significant relationship with solid loading. With higher solid loading, higher sugars and inhibitors were produced. There was not a significant correlation between acid loading or pretreatment duration, with the generation of sugars and inhibitors.

### *Effect of pretreatment conditions of corn bran on cell growth and lipid production by T. oleaginosus*

For integrated lipid production using corn bran hydrolysates, 10% (v/v) inoculum of *T. oleaginosus* was directly transferred into filter-sterilized corn bran hydrolysates without any additional supplement of nutrients. Fermentation was performed at 25 °C and 200 rpm for 5 days, and harvested to investigate lipid production of *T. oleaginosus* from 15 runs of corn bran hydrolysates. *T. oleaginosus* only grew in the Run 9, 14 and 16 corn bran hydrolysates which a 5% solid loading was applied during pretreatment. It might be because of that corn bran

hydrolysates applied pretreatment conditions of Run 9, 14 and 15 contained lower concentrations of inhibitors compared with other runs of hydrolysates. Higher solid loading led to higher concentrations of sugars, but also caused higher levels of inhibitory compounds. Therefore, higher levels of solid loading during pretreatment adversely affected both sugar yields and cell growth.

Fermentation performance of *T. oleaginosus* using corn bran hydrolysates was investigated and compared with lipid production using synthetic media (YM broth) (Table 7.6). *T. oleaginosus* accumulated higher levels of lipids using corn bran hydrolysates compared with synthetic media. Oleaginous yeasts are known to preferably store high level of lipids under severe stress conditions (Calvey et al., 2016). Therefore, it was assumed that corn bran hydrolysates, which provided stress conditions, induced high level of lipid accumulations in *T. oleaginosus*. Among the three runs of fermentations (Run 9, 14 and 15), the highest lipid contents and concentrations were achieved from Run 9, despite its lower levels of dry-cell weight. The lowest sugar utilization for cell growth was obtained from hydrolysates applied Run9 condition. Therefore, Ypx showed that *T. oleaginosus* most efficiently produced lipids using hydrolysates applied Run 9 condition. Compared with the previous study by Probst et al (Probst and Vadlani, 2015), which produced microbial lipids from de-starched corn bran hydrolysates using *Lipomyces starkeyi*, 1.6-fold higher lipid content and 5-fold higher Yps were achieved from hydrolysates applied Run 9 conditions, even though lower level of dry-cell mass was produced; 28% (w/w) of lipid content and 70 mg/g lipid yields were obtained from de-starched corn bran hydrolysates. It revealed that optimization of pretreatment condition and selection of robust yeast culture led to significant enhancement of lipid yields from corn bran.

Fatty acid species of lipids produced by *T. oleaginosus* are presented in Figure 7.4. Major fatty acid species of *T. oleaginosus* were palmitic acid and oleic acid, regardless of nutrient media,

which aligned with the results included in the previous studies (Thiru, Sankh and Rangaswamy, 2011; Tchakouteu et al., 2015). No significant differences of fatty acid composition were observed among all corn bran hydrolysates. Most yeast thioesterases specifically catalyze saturated fatty acid, such as palmitic acid and stearic acid, and undergo further elongation and desaturation to produce unsaturated fatty acids (Probst et al., 2015). Therefore, this may be the reason why palmitic acid and oleic acid were the major fatty acids of most oleaginous yeast strains.

### *Overall product yield of microbial lipids from corn bran*

Overall yield of lipids from raw corn brans were calculated to evaluate the optimized pretreatment and fermentation processes (Figure 7.5). After liquefaction and saccharification of raw corn bran, a total of 40.9 g of de-starched corn bran was obtained. The de-starched corn bran was utilized to produce fermentable sugar solution *via* pretreatment and enzymatic hydrolysis. Sugar and lipid yields of Run 9, 14, and 15 were calculated because *T. oleaginosus* grew only in those hydrolysates. The value of Ypx showed that sugar utilization for lipid production by *T. oleaginosus* was same in the three types of corn bran hydrolysates during fermentation. Therefore, lipid yield from corn bran (Yps) depended on sugars yield from corn bran. The highest sugar yield was achieved using pretreatment condition of Run 9 (0.53 g/g of de-starched bran), and *T. oleaginosus* accumulated the highest lipid contents (46%) and concentrations (3.8 g/L). This result showed that 5% solid loading and 1% acid loading, at 121 °C for 30 min, would be appropriate pretreatment condition of de-starched corn bran for lipid production by *T. oleaginosus*. Also, this study demonstrated that pretreatment condition is the key factor in achieving high yield of lipids.

***Response surface methodology for improving lipid content of T. oleaginosus***

This study investigated the influence of pretreatment conditions on lipid accumulation performance by *T. oleaginosus* to provide useful information towards the integrated bioconversion process. Pretreatment conditions were used as input factors and the lipid contents of *T. oleaginosus* were response variables (Table 7.2). Response surface methodology using Box-Behnken design was used to optimize the pretreatment conditions of de-starched corn bran for obtaining high accumulation of lipids by *T. oleaginosus*. Box-Behnken design allows the efficient estimation of the first- and second- order coefficients of the mathematical models (Bezerra et al., 2008). A linear regression model was developed based on the experimental results and the estimated lipid content was Eq. (1).

Equation (1): Lipid content (%) = $(7.63 - 0.47 \times A - 2.73 \times B + 0.03 \times C)^2$

(A: solid loading, B: acid loading, C: pretreatment time)

An analysis of variance indicated that the linear models for lipid content was significant as the $P$ value was lower than 0.05. The results indicated that solid loading was the highest significant factor determining lipid content whereas the effect of pretreatment time was insignificant because its $P$ value was higher than 0.05. The three-dimensional response surface plot for modeling the results was shown in Figure 7.6. Surface plot presented the effect of solid loading and acid loading on lipid accumulation of *T. oleaginosus* at the constant pretreatment time. Since pretreatment condition was not the significant factor, surface plot was not much changed at the different pretreatment time. The plots predicted that higher lipid content was achieved at the lower solid loading and lower acid loading during pretreatment. Also, the effect of solid loading was more significant rather than acid loading. The response surface model demonstrated that pretreatment conditions, specifically solid loading and acid loading, directly affected lipid accumulation by *T.*

*oleaginosus* during fermentation process. This model could be used for mathematical estimation of pretreatment conditions for lipid production.

## Conclusion

This study demonstrated that corn bran can be a valuable sugar supplier for microbial lipid production. During the fermentation process, *T. oleaginosus* accumulated higher lipid content compared with synthetic media. In addition, the pretreatment conditions were optimized to obtain high lipid accumulation during fermentation process. Higher sugar and lipid yields were obtained at lower levels of solid loading (5%). The highest sugar yield of 0.53 g/g and lipid yield of 216 mg/g were achieved from de-starched corn bran. Also, the correlation between pretreatment conditions and fermentation process was identified using RSM. The RSM model showed that solid loading and acid loading were the significant factors for lipid accumulation by *T. oleaginosus*. The optimized pretreatment condition and selection of robust yeast culture substantially enhanced the lipid production from corn bran compared with previous study. This work showed corn bran can be used as alternative feedstocks for microbial lipid production in biorefineries. It is anticipated that this study can provide invaluable information for integrated bioconversion process for lipid production using corn bran.

Table 7-1 Composition of corn bran and de-starched corn bran (Probst and Vadlani, 2015)

| Component (%, w/w) | Corn bran | De-starched corn bran |
|---|---|---|
| Cellulose | $9.6 \pm 0.2$ | 14.2 |
| Hemicellulose | $25.8 \pm 1.3$ | 38.0 |
| Starch | $32.2 \pm 3.3$ | - |
| Acid detergent lignin | $1.4 \pm 0.1$ | 2.1 |

Table 7-2 Factors and levels of variables in the central composite design

| Levels | Solid loading (%, w/v) | Acid loading (%, w/v) | Pretreatment time (min) |
|---|---|---|---|
| Lower limit | 5 | 0.5 | 30 |
| Upper limit | 15 | 1.5 | 60 |

Table 7-3 Experimental runs to optimize pretreatment conditions of de-starched corn bran

| Run | Solid loading (%, w/v) | Acid loading (%, v/w) | Pretreatment time (min) |
|---|---|---|---|
| R1 | 10 | 1 | 45 |
| R2 | 15 | 1.5 | 45 |
| R3 | 10 | 1 | 45 |
| R4 | 10 | 1.5 | 60 |
| R5 | 15 | 1 | 60 |
| R6 | 15 | 0.5 | 45 |
| R7 | 10 | 1 | 45 |
| R8 | 10 | 0.5 | 30 |
| R9 | 5 | 1 | 30 |
| R10 | 5 | 1.5 | 45 |
| R11 | 10 | 0.5 | 60 |
| R12 | 10 | 1.5 | 30 |
| R13 | 15 | 1 | 30 |
| R14 | 5 | 1 | 60 |
| R15 | 5 | 0.5 | 45 |

Table 7-4 Sugar yields from de-starched corn bran

| Experimental runs | Glucose (g/100g bran) | Xylose (g/100g bran) | Arabinose (g/100g bran) |
|---|---|---|---|
| R1 | $16.9 \pm 0.2^{C,D,E}$ | $20.2 \pm 0.5^{D,E}$ | $7.8 \pm 0.3^{D,E}$ |
| R2 | $15.9 \pm 0.2^{E}$ | $18.8 \pm 0.5^{E,F}$ | $7.8 \pm 0.3^{D,E}$ |
| R3 | $17.2 \pm 0.2^{B,C,D,E}$ | $20.7 \pm 0.4^{C,D}$ | $8.0 \pm 0.3^{D,E}$ |
| R4 | $18.2 \pm 0.1^{A,B,C}$ | $21.9 \pm 0.2^{B,C,D}$ | $9.0 \pm 0.3^{A,B,C}$ |
| R5 | $18.5 \pm 0.7^{A,B,C}$ | $22.6 \pm 0.9^{A,B}$ | $9.4 \pm 0.4^{A}$ |
| R6 | $10.0 \pm 0.1^{F}$ | $4.3 \pm 0.1^{E,FG}$ | $3.5 \pm 0.1^{F}$ |
| R7 | $17.1 \pm 0.3^{C,D,E}$ | $20.4 \pm 0.4^{C,D,E}$ | $7.9 \pm 0.2^{D,E}$ |
| R8 | $17.9 \pm 0.7^{A,B,C,D}$ | $18.2 \pm 0.8^{F}$ | $8.5 \pm 0.1^{B,C,D,E}$ |
| R9 | $19.4 \pm 0.8^{A}$ | $24.2 \pm 1.0^{A}$ | $9.4 \pm 0.5^{A}$ |
| R10 | $17.0 \pm 0.6^{C,D,E}$ | $20.2 \pm 0.7^{D,E}$ | $8.1 \pm 0.4^{C,D,E}$ |
| R11 | $17.6 \pm 1.4^{C}$ | $20.3 \pm 1.6^{D,E}$ | $8.5 \pm 0.6^{B,C,D,E}$ |
| R12 | $16.7 \pm 0.1^{C,D,E}$ | $23.1 \pm 0.3^{A,B}$ | $9.2 \pm 0.2^{A,B}$ |
| R13 | $16.6 \pm 0.1^{D,E}$ | $20.0 \pm 0.3^{D,E}$ | $8.4 \pm 0.1^{B,C,D,E}$ |
| R14 | $18.7 \pm 0.3^{A,B}$ | $22.3 \pm 0.5^{B,C}$ | $8.7 \pm 0.1^{A,B,C,D}$ |
| R15 | $17.1 \pm 0.2^{B}$ | $20.2 \pm 0.3^{D,E}$ | $7.6 \pm 0.1^{E}$ |

The data represent average values of triplicate experiments ± sample standard deviation. Values with the same letters, in superscripts, within the same column, are not significantly different at the 95% confidence level.

Table 7-5 Inhibitor yields from de-starched corn bran

| Experimental runs | HMF (g/100g bran) | Furfural (g/100g bran) | Acetic acid (g/100g bran) | Formic acid (g/100g bran) |
|---|---|---|---|---|
| R1 | $0.05 \pm 0.0^B$ | $0.22 \pm 0.0^E$ | $2.8 \pm 0.0^B$ | $0.06 \pm 0.0^{B,C,D}$ |
| R2 | $0.02 \pm 0.0^E$ | $0.29 \pm 0.0^C$ | $2.7 \pm 0.0^B$ | $0.09 \pm 0.0^{A,B}$ |
| R3 | $0.05 \pm 0.0^B$ | $0.21 \pm 0.0^E$ | $2.9 \pm 0.0^B$ | $0.06 \pm 0.0^{B,C,D}$ |
| R4 | $0.07 \pm 0.0^A$ | $0.40 \pm 0.0^A$ | $3.1 \pm 0.0^A$ | $0.10 \pm 0.0^A$ |
| R5 | $0.05 \pm 0.0^B$ | $0.29 \pm 0.0^C$ | $3.1 \pm 0.1^A$ | $0.08 \pm 0.0^{A,B,C}$ |
| R6 | $0.01 \pm 0.0^F$ | $0.02 \pm 0.0^J$ | $0.8 \pm 0.0^C$ | $0.0 \pm 0.0^F$ |
| R7 | $0.05 \pm 0.0^B$ | $0.24 \pm 0.0^D$ | $2.8 \pm 0.0^B$ | $0.06 \pm 0.0^{B,C,D}$ |
| R8 | $0.02 \pm 0.0^E$ | $0.06 \pm 0.0^I$ | $2.7 \pm 0.1^B$ | $0.0 \pm 0.0^F$ |
| R9 | $0.04 \pm 0.0^C$ | $0.14 \pm 0.0^G$ | $3.2 \pm 0.1^A$ | $0.05 \pm 0.0^{D,E}$ |
| R10 | $0.03 \pm 0.0^D$ | $0.36 \pm 0.0^B$ | $2.8 \pm 0.1^B$ | $0.08 \pm 0.0^{A,B,C}$ |
| R11 | $0.03 \pm 0.0^D$ | $0.11 \pm 0.0^{G,H}$ | $2.8 \pm 0.2^B$ | $0.04 \pm 0.0^{D,E}$ |
| R12 | $0.04 \pm 0.0^C$ | $0.18 \pm 0.0^F$ | $3.2 \pm 0.0^A$ | $0.06 \pm 0.0^{B,C,D}$ |
| R13 | $0.02 \pm 0.0^E$ | $0.12 \pm 0.0^{G,H}$ | $2.8 \pm 0.0^B$ | $0.10 \pm 0.0^A$ |
| R14 | $0.04 \pm 0.0^C$ | $0.26 \pm 0.0^D$ | $3.1 \pm 0.1^A$ | $0.07 \pm 0.0^{A,B,C}$ |
| R15 | $0.03 \pm 0.0^D$ | $0.10 \pm 0.0^H$ | $2.7 \pm 0.0^B$ | $0.0 \pm 0.0^F$ |

HMF=hydroxymethylfurfural
The data represent average values of triplicate experiments ± sample standard deviation. Values with the same letters, in superscripts, within the same column, are not significantly different at the 95% confidence level.

Table 7-6 Fermentation performance of *T. oleaginosus*

| Nutrients | Lipid content (%) | Lipid titers (g/L) | DCW (g/L) | Yps (g/g) | Yxs (g/g) | Ypx (g/g) |
|---|---|---|---|---|---|---|
| YM | $30 \pm 0^{C}$ | $2.4 \pm 0.0^{D}$ | $7.9 \pm 0.2^{C}$ | $0.1 \pm 0.0^{A}$ | $0.4 \pm 0.0^{A}$ | $0.3 \pm 0.0^{C}$ |
| Run 9 | $46 \pm 3^{A}$ | $3.8 \pm 0.2^{A}$ | $8.4 \pm 0.3^{B,C}$ | $0.1 \pm 0.0^{A}$ | $0.2 \pm 0.0^{B}$ | $0.5 \pm 0.0^{A}$ |
| Run 14 | $32 \pm 2^{C}$ | $2.9 \pm 0.2^{C}$ | $9.3 \pm 0.5^{A}$ | $0.1 \pm 0.0^{A}$ | $0.4 \pm 0.0^{A}$ | $0.3 \pm 0.0^{C}$ |
| Run 15 | $39 \pm 3^{B}$ | $3.4 \pm 0.2^{B}$ | $8.7 \pm 0.4^{B}$ | $0.1 \pm 0.0^{A}$ | $0.4 \pm 0.0^{A}$ | $0.4 \pm 0.0^{B}$ |

DCW=dry-cell weight
The data represent average values of triplicate experiments ± sample standard deviation. Values with the same letters, in superscripts, within the same column, are not significantly different at the level of ($P<0.05$).
[1]Yps was calculated by dividing the amount of lipids by the amount of consumed glucose.
[2]Yxs was calculated by dividing the amount of dry cell mass by the amount of consumed glucose.
[3]Ypx was calculated by dividing the amount of lipids by the amount of dry cell mass.

Corn Bran

**De-starch**

- Liquefaction at 80˚C for 1 h
- Saccharification at 65˚C for 2 h

Starch ←

De-starched corn bran

**Pretreatment:** 15 Runs (Table 1)

**Enzymatic hydrolysis**

- 5.4% (v/v) of C-Tec2 & 0.6% (v/v) of H-Tec2
- 55˚C for 48 h

**Centrifugation** at 8500rpm for 20min

Solids ←

Corn bran hydrolysates
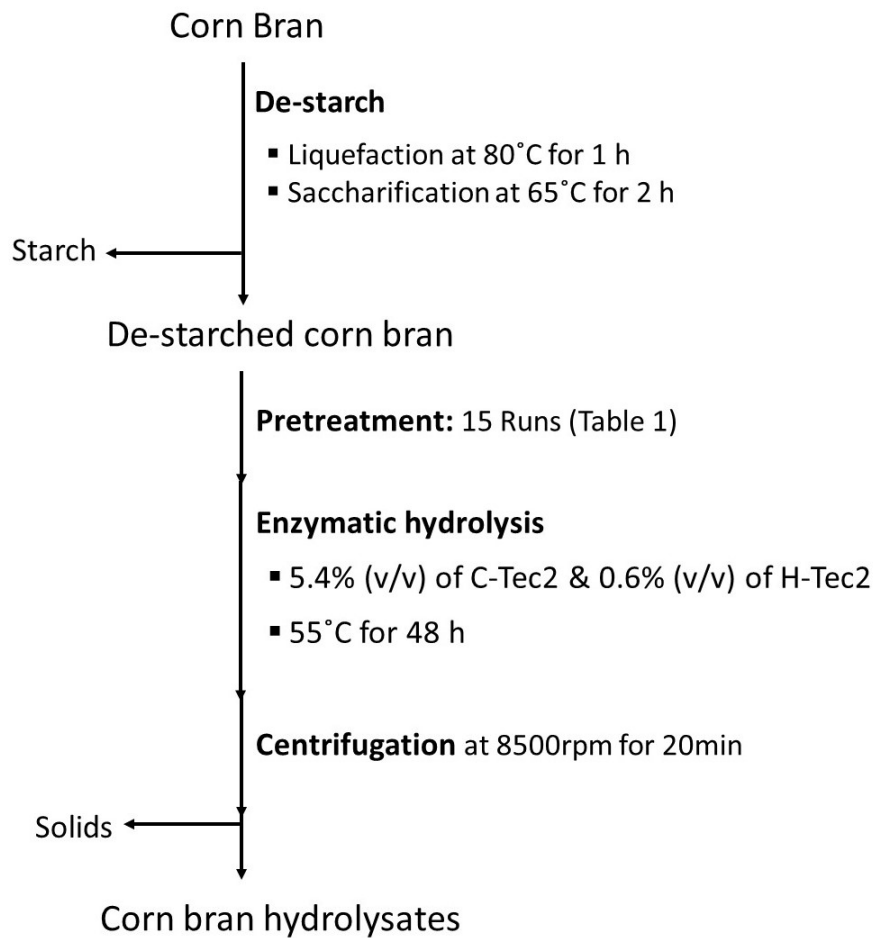
Figure 7-1 Schematic of corn bran hydrolysates production
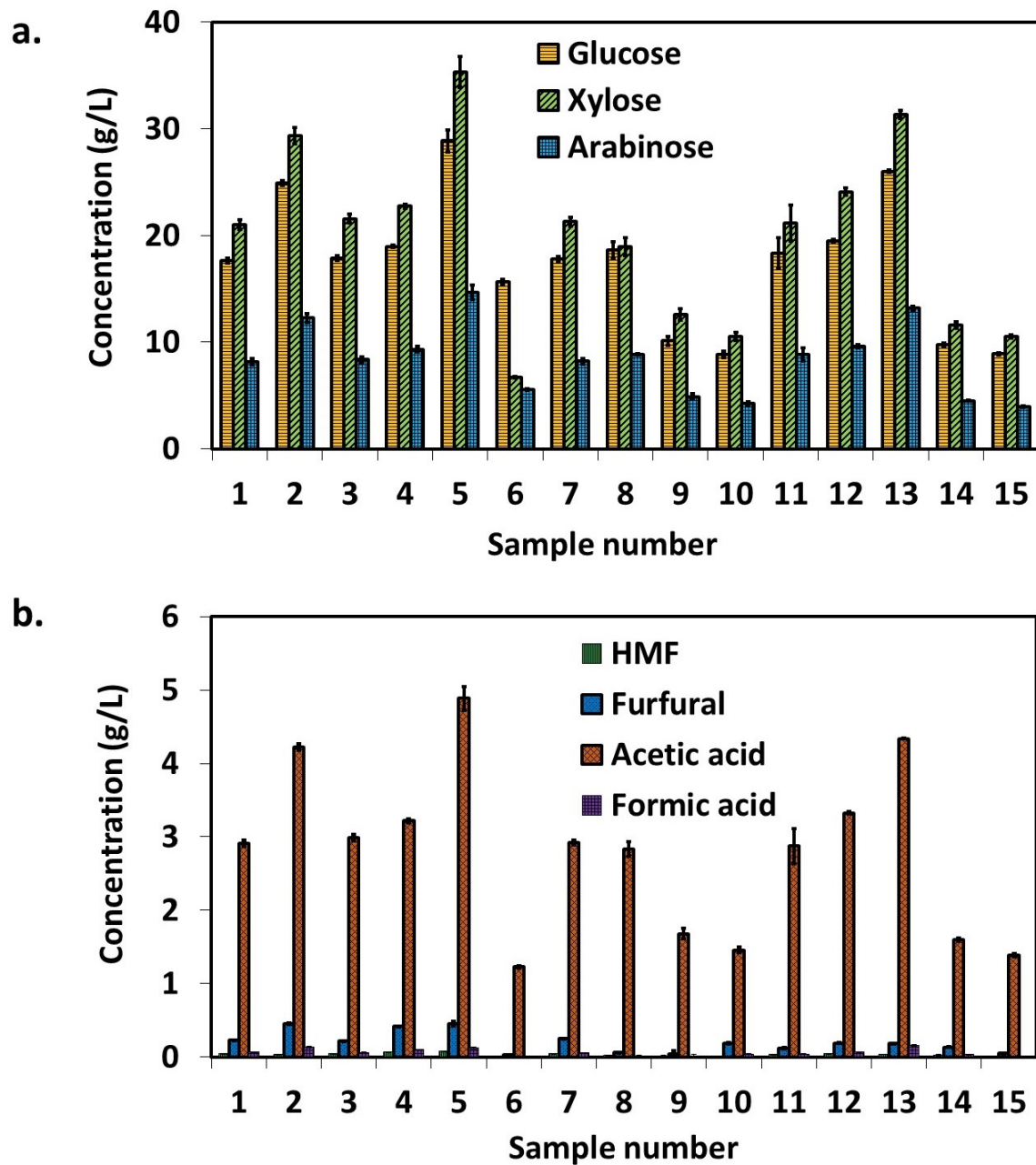
Figure 7-2 Released compounds from de-starched corn bran (A) sugar concentrations; (B) inhibitor concentrations. The data shows average value of triplicate experiments and error bars representing sample standard deviation.
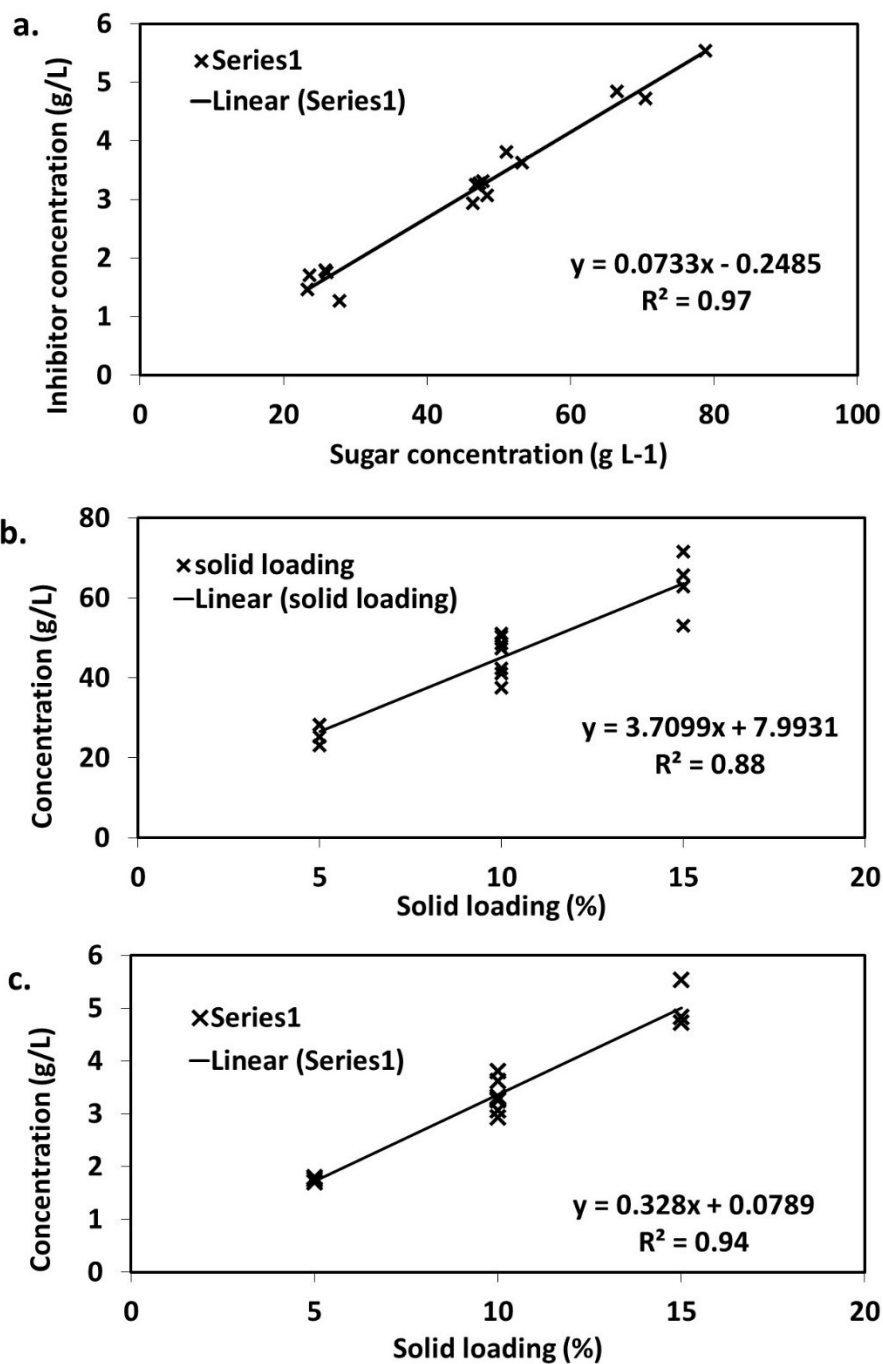
Figure 7-3 Effect of pretreatment condition on generation of sugars and inhibitors (A) correlation between sugar generation and inhibitor generation; (B) effect of solid loading on sugar generation; (C) effect of solid loading on inhibitor generation. The data shows average value of triplicate experiments and error bars representing sample standard deviation.
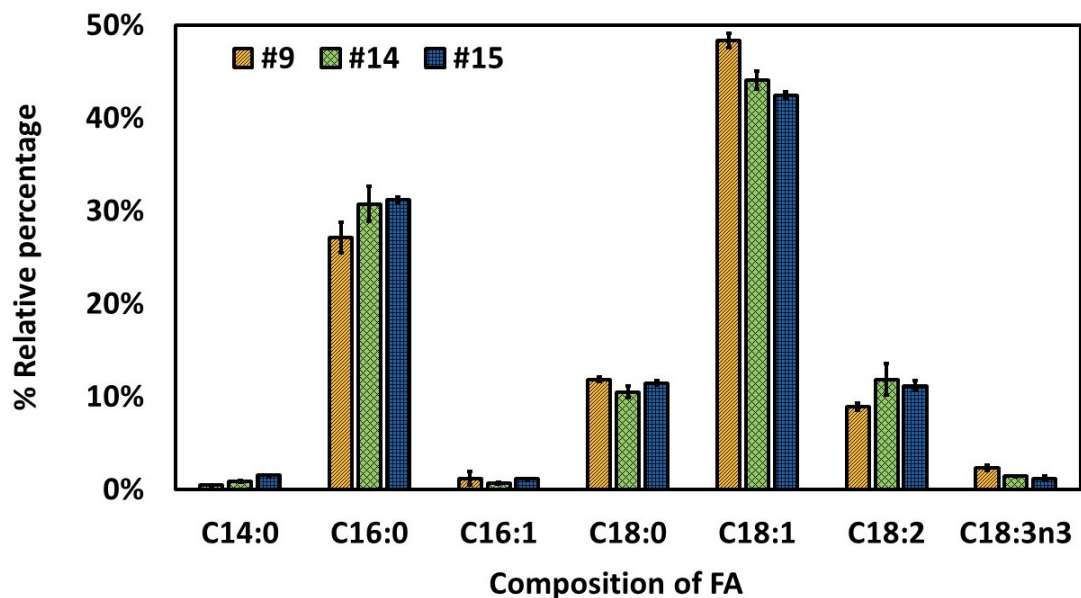
Figure 7-4 Fatty acid profile of lipid produced from corn bran hydrolysates by *T. oleaginosus*. The data shows average value of triplicate experiments and error bars representing sample standard deviation.
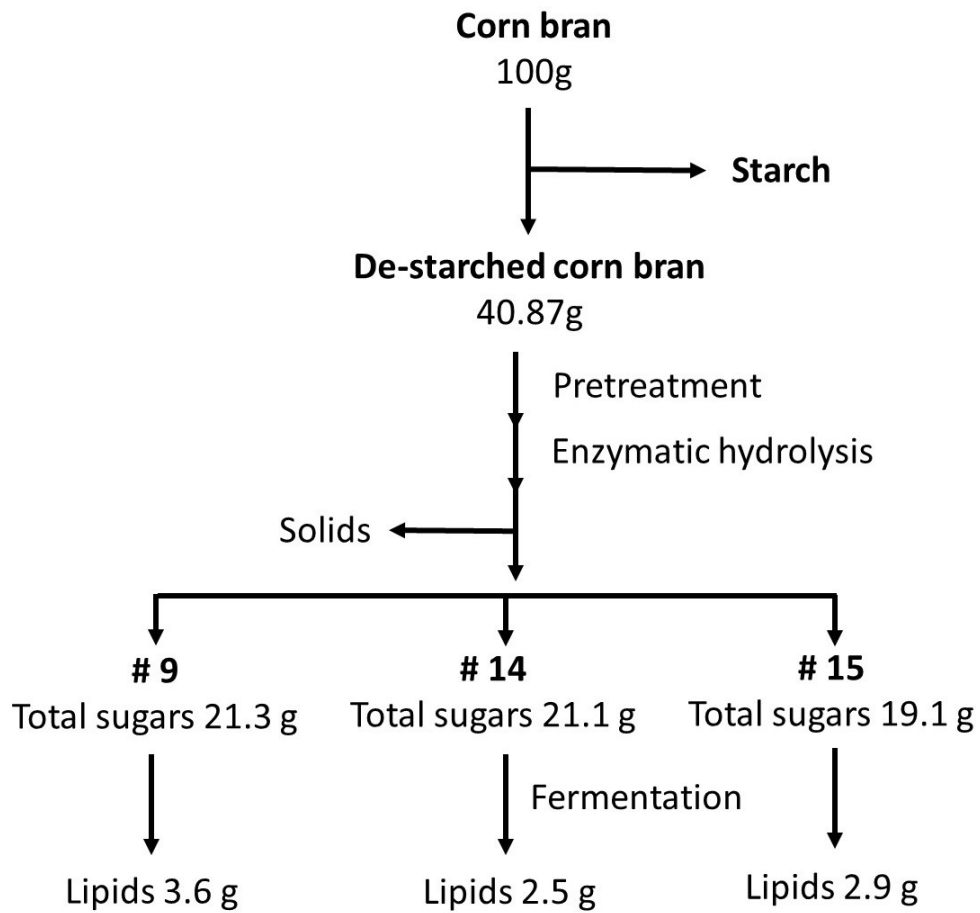
Figure 7-5 Lipid yields from raw corn bran
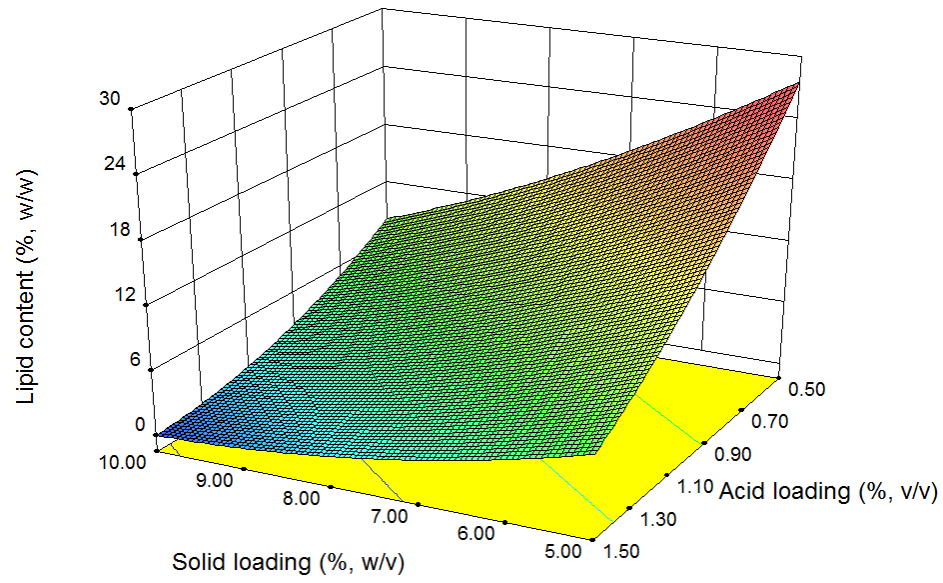
Figure 7-6 Surface plot of predicted lipid contents in *T. oleaginosus*

# Chapter 8 – Conclusions and Future Research

Microbial lipids produced by oleaginous yeast are attractive feedstocks to synthesize a variety of fatty acid-based materials in an oleochemical industry. Economics would be the critical factor for viable production of lignocellulose-based microbial lipids. The goal of this research was to produce microbial lipids from renewable biomass, as well as to evaluate the overall bioconversion process for an integrated platform of microbial lipid production. The following conclusions were drawn from this doctoral research:

1) Acid pretreatment induced the generation of xylose-rich sugar streams from lignocellulosic biomass. Activated charcoal was effective to remove hydrophobic inhibitors such as furfural and HMF from the pretreatment stream of sorghum stalk hydrolysates. Resin mixtures in a 7:3 ratio of cationic and anionic exchangers completely removed most of the inhibitory compounds from sorghum stalk hydrolysates.

2) Optimized conditions for *T. oleaginosus* fermentation provided high yield and productivity of lipid production, using lignocellulosic hydrolysates.

3) Microbial lipids were successfully produced from lignocellulosic biomass by oleaginous yeast cultures such as *T. oleaginosus, L. starkeyi, and C. albidus*. Highest lipid yield from sorghum stalks were achieved by *T. oleaginosus*.

4) Corn bran, which is a low-value by-product of the corn milling industry, was utilized as a substrate for microbial lipid production using *T. oleaginosus.* Optimization of the pretreatment condition was the key factor to achieve high yields of lipids from corn bran.

5) The bioconversion process from switchgrass to FFAs was demonstrated using engineered *E. coli* strains, and high yields of FFAs were achieved by *E. coli* ML103 *pXZ18Z*.

Based on the investigations from this doctoral research, the sustainable platform for lignocellulose-based microbial lipids was suggested (Figure 8.1). Lipid production using oleaginous yeast requires high amount of carbon sources. Therefore, efficient utilization of lignocellulosic feedstocks is essential to improve sustainability and feasibility of microbial lipid production. Chapter 3 showed that liquid fraction after pretreatment, normally discarded due to high amount of inhibitory compounds including phenolics and aliphatics, was rich in xylose solution and can be used as a carbon source for lipid production by purification with a novel pretreatment method developed in this study. Oleaginous yeast initiates lipid accumulation at the stationary phase. Therefore, the biphasic condition for growth and lipid accumulation were followed, and this was demonstrated in earlier work from our lab (Probst, 2014; Probst and Vadlani, 2017). Glucose-rich streams can be primarily utilized for yeast growth since oleaginous yeast prefer to use glucose for cell-biomass production. Purified xylose streams can be fed into the bio-reactor containing a high density of cell mass to induce more of lipid synthesis during the lipid accumulation stage.

## Future Research

Future work should be directed toward improving the sustainability of lignocellulose-based microbial lipids by increasing fermentation efficiency of oleaginous yeast, as well as integrating several processes into a one-step conversion to maximize feedstock utilization.  The following areas of research can be explored to improve sustainable production of microbial lipids:

1) Metabolic flux analysis (MFS) could be used to identify the limiting step and required cofactors for lipid synthesis in oleaginous yeast metabolism — to improve lipid production efficiency of *T. oleaginosus* during fermentation. MFS is an attractive tool to identify carbon distributions and the limiting step of product formation, by observing intermediates in the metabolic pathway. Vadlani and coworkers successfully identified carbon distribution of six *Lactobacillus* strains and this research provided useful information to reduce byproduct formation during lactic acid fermentation (Zhang et al., 2016). Limited studies have investigated metabolic flux of oleaginous yeast strains. Also, substantial variations are dependent on the oleaginous yeast strains. Therefore, MFA for a specific oleaginous yeast strain is required to identify the unique attributes in its metabolic pathway.

2) Development of a novel down-stream process method for an environmentally friendly and economical lipid extraction could be researched for further sustainable microbial lipid production. The lipid-extraction method developed by Bligh and Dyer (1959), which requires use of hazardous chemicals, is widely utilized to efficiently separate and extract lipids from oleaginous yeast cells (Wales et al., 2016). Limited lipid accessibility, blocking effects from insoluble biomass residue and formation of stable emulsion are known as main challenges for lipid extraction efficiency (Dong et al., 2016). To improve sustainability of lignocellulose-based microbial lipids, it is necessary to develop environmentally friendly extraction methods.

3) Strain development for high-valued lipid production will improve economic feasibility of microbial lipid production. To improve economic feasibility of microbial lipid production, cost reduction of feedstock development and process is essential. Another way to improve the feasibility of lignocellulose biorefinery is the development of a high value product,

which is not easy to synthesize using chemical reaction, *via* synthetic biology. The biggest advantage of using biological synthesis for chemical production is a substrate-specific and a regio-specific reaction. Enzymes in the microbial metabolism selectively catalyze substrates for the reaction and it prevent the generation of unnecessary intermediate byproducts and waste. Production of high-value fatty acids would be suitable for market demand, economics and sustainability in a biorefinery.

4) Development of gene expression system for targeted oleaginous yeast strain

Recently, lignocellulose-based lipid production using oleaginous yeast have been gaining great attention because of their robustness to toxic compounds, utilization of diverse carbon sources and a native ability to accumulate lipids. However, limited genetic information is available for most oleaginous strains except *Yarrowia lipolytica*, which is a model oleaginous species. Development of the gene expression system for oleaginous yeast strains is essential to commercialize the various industrial processes. Lately, various studies have been explored to develop expression and transformation systems for the common industrial oleaginous species; *Trichosporon oleaginosus, Lipomyces starkeyi and Rhodotorulla glutinis*. Gönors and coworkers developed expression and transformation systems for *T. oleaginosus* (Görner et al., 2016). The glyceraldehyde-3-phosphate dehydrogenase (GPD, EC1.2.1.12) and tryptophan synthase (TrpC - derived from *Aspergillus nidulans*) were chosen as the promoter and terminator, respectively, and utilized for expression system (Figure 8.2). This expression cassette, the *pRF-HU2*, was successfully expressed in the heterologous gene '*YFP*' to produce hydroxylated fatty acids in *T. oleaginosus*. Targeted heterologous or homologous genes can be expressed using the expression cassette "*PRF-HU2(GPD)-targeted gene*" in *T. oleaginosus* to manipulate the

metabolic pathway. Also, application of CRISPR/Cas9 mediated gene additing technology, which is an important new approach for generating RNA-guided nucleases with customizable specificities, for metabolic engineering purposes would allow for a substantial improvement of strain construction system (Jakočiūnas et al., 2015; Sander and Joung, 2014)
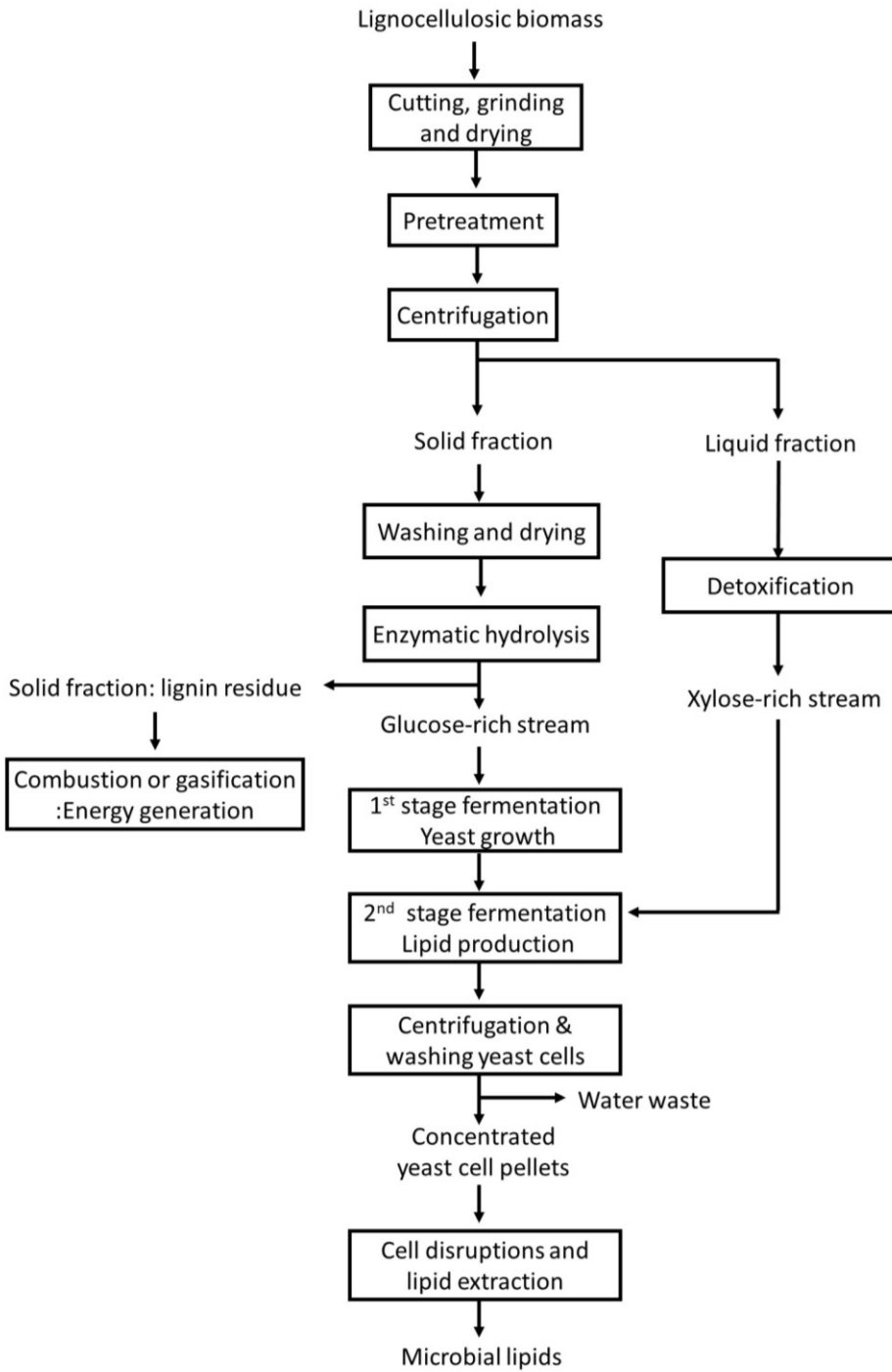
Figure 8-1. Diagram of the platform for microbial lipid production
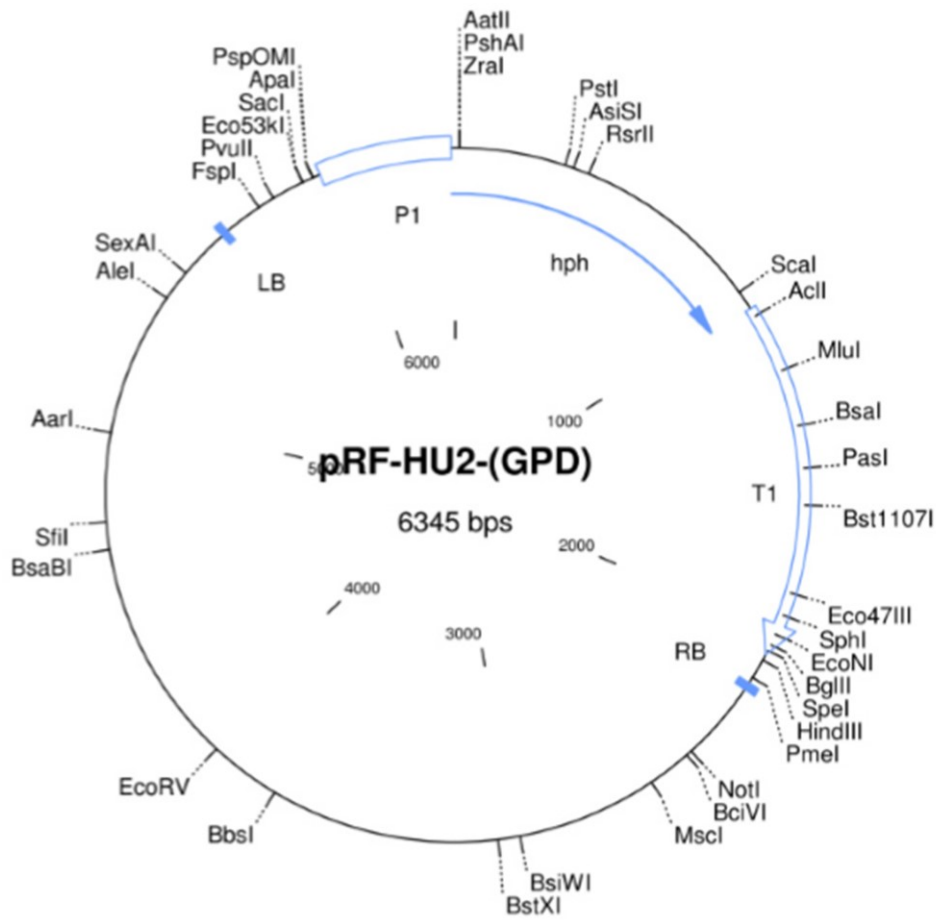
Figure 8-2 Expression vector for *T. oleaginosus*

*hph*: hygromycein B resistance gene; *P1*: GPD promoter derived from *T. oleaginosus* 390 bp; *T1*: TrypC terminator from *Aspergillus nidulans* (Görner et al., 2016)

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