

SINGLE CELL OIL PRODUCTION USING *LIPOMYCES STARKEYI*: FERMENTATION,  
LIPID ANALYSIS AND USE OF RENEWABLE HEMICELLULOSE-RICH FEEDSTOCKS

by

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Department of Grain Science and Industry  
College of Agriculture

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## Abstract

As the world population continues to grow and the uncertainty of petroleum and food availability transpires, alternative resources will be needed to meet our demands. Single cell oil (SCO) from oleaginous yeast is a renewable noncrop-based resource that can be used for the production of petroleum counterparts. Currently, commercial production is limited, mainly due to high production costs and competition from cheaper alternatives. As a result, improved fermentation techniques, utilization of low-valued feedstocks and efficient downstream processing would be highly valuable. The major objectives of this study were to: 1) optimize fermentation conditions for the development of a novel fed-batch fermentation to enhance oil production using *Lipomyces starkeyi*, 2) determine the major lipids produced by *L. starkeyi*, 3) utilize low-valued hemicellulose-rich feedstocks for oil production, and 4) demonstrate the use of 2-methyltetrahydrofuran (2-MeTHF) and cyclopentyl methyl ether (CPME) as greener solvents for oil extraction.

Under optimized fermentation conditions, the oil yield increased from 78 to 157 mg oil/g sugar when supplying xylose rather than glucose as the major carbon source. A novel repeated fed-batch fermentation supplying glucose for growth and xylose for lipid accumulation generated the highest oil yield of 171 mg oil/g sugar, oil content of 60% (dry mass basis) and oil productivity of 143 mg oil/L/hr. Oleic acid accounted for 70% of the total fatty acid profile indicating that oil from *L. starkeyi* is a naturally high source of oleic acid; an added benefit for the biofuel, cosmetic, food, and oleochemical industries. Hemicellulose-rich corn bran and wheat bran were successfully used to produce oil; oil yields of 125 and 71 mg oil/g sugar were reported for whole and de-starched bran hydrolysates, respectively. Compared to traditional methods, biphasic oil extraction systems of 2-MeTHF and CPME had an 80 and 53% extraction efficiency and 64 and 49% selectivity, respectively.

The information from this study will be useful for the development of an integrated approach to improve the viability of SCO biochemical platforms for the production of advanced biofuels and renewable chemicals.

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## List of Abbreviations

ACCase: acetyl-CoA carboxylase  
ACL: ATP citrate lyase  
AMP: adenosine monophosphate  
DAG: diacylglycerol  
DCW: dry cell weight  
DGAT: acyl-CoA:diacylglycerol acyltransferase  
DHA: docosahexanoic acid  
DHAP: dihydroxyacetone phosphate  
FA: fatty acid  
FAC: fatty acyl-CoA  
FAEE: fatty acid ethyl ester  
FAME: fatty acid methyl ester  
FFA: free fatty acid  
G3P: glycerol-3-phosphate  
GRAS: generally recognized as safe  
GSXF: glucose start:xylose feed  
IL: ionic liquid  
LB: lipid body  
PA: phosphatidic acid  
PC: phosphatidylcholine  
PDAT: phospholipid:diacylglycerol acyltransferase  
PE: phosphatidylethanolamine  
PI: phosphatidylinositol  
PS: phosphatidylserine  
PUFA: polyunsaturated fatty acid  
SCO: single cell oil  
TAG: triacylglycerol  
TCA: tricarboxylic acid  
TFA: total fatty acid

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## **Dedication**

I would like to dedicate this dissertation to my grandfather Mr. Benjamin Z. Miller who passed away in late June of 2013. A chemical engineering graduate from Purdue University who worked 40 years for DuPont and a 2<sup>nd</sup> Lieutenant in the US Marine Corps, Mr. Miller was an amazing man, one who I truly loved and admired. I will always strive to adopt the diligence and passion he had towards both work and life. His ‘work hard, play hard’ attitude is something that I have subconsciously inherited, a quality that I will cherish during my lifetime. Grandpa Miller, you are truly missed!

In Loving Memory of Benjamin Z. Miller 1930-2013

# **Chapter 1 - Oleaginous yeast: a value added platform for renewable oils**

## **Abstract**

Yeast single cell oil (SCO) is a noncrop-based, renewable oil source that can be used for the production of bio-based oleochemicals. Stand-alone production of SCO for oleochemicals currently is infeasible, and lower-cost alternatives from petroleum and crop-based resources are available. Utilizing low-valued nutrient sources, implementing cost-efficient downstream processes, and adopting biotechnological advancements such as metabolic engineering could prove valuable in making an SCO platform a reality in the emerging bio-based economy. This chapter aims to cover key biochemical pathways for storage lipid synthesis, possible pathways for SCO yield improvement, previously used bioprocessing techniques for SCO production, challenges in SCO commercialization, and advantages of adopting a renewable SCO platform.

## **Introduction**

Single cell oil (SCO) has gained attention in recent years as a biochemical conversion option for the biotechnology industry. SCO is commonly defined as neutral storage lipids accumulated by oleaginous eukaryotic microorganisms (e.g. yeast, mold, microalgae) and is often synonymous with lipid, oil, and triacylglycerol (TAG). Oleaginous prokaryotic organisms (i.e., bacteria) will not be considered in this review, but the authors acknowledge that certain bacteria, especially those belonging to the actinomycetes group, accumulate TAG in high amounts. Please refer to publications by Wältermann et al. (2007) and Alvarez and Steinbüchel (2002) for review of oleaginous prokaryotes.

Oleaginous yeasts are viable SCO producers that are able to accumulate oil up to 70% (dry mass basis) of their cellular biomass. They are unique compared with other oleaginous microorganisms in their ability to produce SCO heterotrophically from a variety of low-value inputs such as agricultural residues, food waste streams and industrial co-products (Ageitos et al., 2011). Species of oleaginous yeast include but are not limited to *Lipomyces starkeyi*, *Yarrowia lipolytica*, *Rhodoturula glutinis*, *Rhodospiridium toruloides*, *Cryptococcus curvatus* and *Trichosporon fermentans* (Ageitos et al., 2011; Beopoulos et al., 2011; Kosa & Ragauskas, 2011; Subramaniam et al., 2010; Li et al., 2008; Ratledge, 1991). The lipid profiles of yeast SCO mainly

include TAG and lesser amounts of phospholipids, sphingolipids, sterols and free fatty acids (FAs) (Subramaniam et al., 2010; Ratledge et al., 2006). SCO from oleaginous yeast may serve as a renewable source of edible oil and as an intermediate “building block” for oleochemicals such as fuels, soaps, plastics, paints, detergents, textiles, rubber, surfactants, lubricants, additives for the food and cosmetic industry and many other chemicals (Steen et al., 2010).

The production of yeast SCO for renewable oleochemicals is still in its infancy. Production of SCO has been successfully commercialized only for specialty oils containing polyunsaturated fatty acids (PUFAs) used in the food and supplement industries; these include docosahexanoic acid (DHA), arachidonic acid (ARA), and eicosapentaenoic acid (EPA) (Ratledge, 2010). SCO as a commodity-type oil has been hindered by competition from oilseed crops, which cost 10 to 20 times less (Ratledge & Cohen, 2008). Although the use of SCO as a feedstock for biodiesel has received interest in recent years, high manufacturing costs prevent the stand-alone production of biodiesel from SCO (Ratledge & Cohen, 2008). As more emphasis is placed on advancement of integrated biorefineries, the possibility of incorporating a yeast-based SCO biochemical platform for renewable fuels, chemicals, power, and products may become a reality.

In this chapter, we first highlight the biochemical pathway of storage lipid or TAG synthesis, propose biochemical engineering strategies to increase TAG yield, and evaluate fermentation processes used for SCO production. Then we expose the challenges of commercial production and the need to utilize low-valued nutrient sources and alternative technologies to lower production costs. The final portion of the review covers the demand for renewable oils and the various value-added oleochemicals that can be produced from yeast SCO. Overall, the objective of this paper is to review the use of an oleaginous yeast biochemical platform to make renewable oleochemicals.

### **Biochemistry of SCO accumulation**

The biochemistry of SCO or TAG accumulation in microorganisms has been reviewed previously (Ratledge & Wynn, 2002). In oleaginous fungi, the initiation of TAG accumulation often occurs under nitrogen-deficient conditions. Upon nitrogen limitation, carbon (i.e. sugar or glucose) is diverted from energy production via the tricarboxylic acid cycle (TCA) to TAG synthesis (Figure 1.1). The change in carbon flux is initiated upon activation of nitrogen-scavenging enzymes. One enzyme that has been identified is AMP deaminase; this enzyme

deaminates adenosine monophosphate (AMP); thus, freeing ammonia, which can be utilized by the cell as a nitrogen source when necessary. The reduced cellular AMP content results in inhibition of isocitrate dehydrogenase, the enzyme responsible for the conversion of isocitrate to oxoglutarate in the TCA cycle (Boulton & Ratledge, 1981; Botham & Ratledge, 1979). This inhibition causes isocitrate to accumulate and equilibrate back to citrate, which is transported into the cytoplasm via a citrate/malate translocase integral membrane protein. This antiport protein facilitates the passage of citrate out of the mitochondria and into the cytosol in exchange for malate (Ratledge, 2004). Oleaginous yeasts were observed to have higher activity of the citrate/malate translocase, resulting in a citrate efflux of up to eight times greater than non-oleaginous species (Evans et al., 1983). After citrate accumulates in the cytosol, ATP citrate lyase (ACL) catalyzes citrate into acetyl-CoA and oxaloacetate. ACL is an enzyme required for oleaginousity, without it, the flux of carbon to FA synthesis would be limited (Boulton & Ratledge, 1981; Botham & Ratledge, 1979). Cytosolic oxaloacetate is converted to malate and recycled back into the TCA cycle via two different routes: translocation across the mitochondrial membrane to facilitate citrate shuttling or catalysis into pyruvate, NADPH and CO<sub>2</sub> via the malic enzyme. A recent comparison of genomes from different oleaginous (*Y. lipolytica*, *Aspergillus oryzae*, *Rhizopus oryzae* and *Mucor circinelloides*) and non-oleaginous (*Saccharomyces cerevisiae*, *Candida albicans* and *Ashbya gossypii*) microorganisms has identified additional pathways for acetyl-CoA generation in the oleaginous group, such as the degradation of leucine and lysine (Vorapreeda et al., 2012).

The cytosolic acetyl-CoA produced from the indirect action of AMP deaminase can be directed to *de novo* FA synthesis. The first committed step begins with the conversion of acetyl-CoA to malonyl-CoA by the biotin-dependent acetyl-CoA carboxylase (ACCase) enzyme. After malonyl-CoA is synthesized, it binds the acyl carrier protein (ACP) of the FA synthase (FAS) complex. A repeated series of condensation, reduction and dehydration reactions adds two carbon units to a growing FA chain. Termination of FA synthesis is catalyzed by a thioesterase enzyme, which releases the FA from the ACP. NADPH, the reductant for the elongation-condensation cycle, is often provided malic enzyme activity in oleaginous yeast species. The malic enzyme forms a metabolon complex, with FAS and ACL supplying NADPH by catalysis of malate to pyruvate; however, the malic enzyme is missing from some yeast species, so it has been proposed that other NADPH generating enzymes are associated with the metabolon (Ratledge, 2004).

Fatty acyl-CoA (FAC) units often undergo elongation and desaturation. Most yeast species include a  $\Delta^9$ desaturase, which synthesizes the monounsaturated FA oleic acid (C18:1) (Tehlivets et al., 2007). A detailed description of all enzymes involved in these FA modifications is beyond the scope of this article. The reader is guided to articles by Uemura (2012), Tehlivets et al. (2007), Warude et al. (2006), and Ratledge and Wynn (2002).

After modification, FACs can be directed toward synthesis of phospholipids or TAGs; the two pathways share a number of early reactions. Glycerol-3-phosphate (G3P) serves as the glycerol backbone and is formed by glycolysis intermediate dihydroxyacetone-phosphate (DHAP) through the action of G3P-dehydrogenase. It is important to note that G3P can be converted back to DHAP or glycerol; this is dependent on growth conditions during fermentation (Beopoulos et al., 2009). Two acyltransferases, G3P acyltransferase (GPAT) and lysophosphatic acid acyltransferase (LPAT), sequentially add two FAs to G3P to form phosphatidic acid (PA). PA is dephosphorylated by phosphatidate phosphatase (PAP), producing diacylglycerol (DAG). PAP is an important regulator of *de novo* TAG synthesis; *S. cerevisiae* mutants lacking the PAP gene had irregular lipid metabolism and decreased TAG formation (Pascual & Carman, 2013). In the model yeast, *S. cerevisiae*, four different enzymes potentially catalyzed the final acylation of DAG to TAG. A type 2 diacylglycerol acyltransferase (DGAT) encoded by *DGA1* used an acyl-CoA to perform this reaction (Sorger & Daum, 2003; Oelkers et al., 2002). In the context of this article, it is interesting to note the first type 2 DGAT enzyme isolated was from the oleaginous fungus *Mortierella ramanniana* (Lardizabal et al., 2001). In addition, a phospholipid diacylglycerol acyltransferase (PDAT) encoded by *LROI* transferred the *sn*-2 acyl group from phospholipids to DAG to form TAG (Dahlqvist et al., 2000; Oelkers et al., 2000). The latter acyl-CoA-independent schemes are most often associated with exponential growth to maintain cellular membrane homeostasis, whereas the former acyl-CoA dependent pathway occurs during stationary growth (i.e. as the primary method for storage lipid accumulation). In addition to DGAT and PDAT activity, two acyl-CoA cholesterol acyltransferase (ACAT) enzymes encoded by *ARE1* and *ARE2* are also capable of synthesizing TAG from DAG, but their contribution appears minimal (Sandager et al., 2002).

Although *S. cerevisiae* is a useful genetic and biochemical model system to understand TAG biosynthesis in oleaginous yeasts and fungi, it is apparent that various species of these oil-producing microbes possess different enzymes to synthesize TAGs; for example, in addition to a

PDAT and a type 2 DGAT, *Y. lipolytica* also utilizes a type 1 DGAT for TAG biosynthesis (Beopoulos et al., 2012; Zhang et al., 2012). *R. glutinis* possesses a soluble type 3 DGAT that is presumably part of the 10S cytosolic triacylglycerol biosynthetic complex of this species (Rani et al., 2013; Gangar et al., 2001).

TAG serves as energy reserves, providing the cell usable carbon during times of nutrient deprivation; thus, mechanisms for TAG breakdown and utilization are imperative for cell function and survival. Lipid catabolism is characterized by the hydrolysis of TAG molecules into free FAs by lipases. TAG lipases are dependent on the species of yeast, but most of these enzymes are membrane-bound on lipid bodies (LBs). For example, *S. cerevisiae* contains at least four TAG lipases, ScTGL3, ScTGL4, ScTGL5 and ScAYR1, which are all associated with LBs and play a role in TAG mobilization (Ploier et al., 2013; Athenstaedt & Daum, 2005; Athenstaedt & Daum, 2003). Less is known about TAG lipases from oleaginous species, but considerable interest in lipases has been spurred by *Y. lipolytica*, which may have commercial value for the food processing, drug development, wastewater remediation, and detergent manufacturing industries (Fickers et al., 2011).

### **New technologies for understanding TAG accumulation**

In addition to studying individual metabolic steps and pathways, systems biology approaches have been applied to understanding the accumulation of TAG. Transcriptomic time course profiling was performed on the oleaginous yeast *Y. lipolytica* to understand changes in regulatory processes throughout fermentation (Morin et al., 2011). The authors confirmed previous findings about the biochemical changes that occur due to lipid accumulation by characterizing four different phases associated with SCO fermentation. These phases included a growth phase, a transition phase characterized by nucleic acid and protein repression, an early lipid accumulation phase with enhanced expression of alternative nitrogen-metabolizing enzymes most likely caused by nitrogen-scavenging mechanisms, and a late accumulation phase showing strong expression of enzymes responsible for directing carbon flux to lipid accumulation.

A study by Liu et al. (2009) performed a semi-quantitative proteome analysis of the oleaginous yeast *R. toruloides* Y4. The authors found that as fermentation entered lipid accumulation, protein and carbohydrate metabolizing enzymes were down-regulated, whereas nitrogen-scavenging and lipid-accumulation enzymes were up-regulated. Similar work was

performed on the yeast *L. starkeyi* AS 2.1560 and showed similar results indicating that enzymes involved with lipid accumulation are common across oleaginous species (Liu et al., 2011). A multi-omic study performed by the same authors on *R. toruloides* confirmed previous findings that the up-regulation of pathways associated with nitrogen-scavenging mechanisms and storage lipid synthesis occur under nitrogen-limited conditions (Zhu et al., 2012). The authors also further elucidated lipo- and proteolysis autophagy pathways, which serve as acetyl-CoA and amino acid recycling for lipid synthesis and nitrogen metabolism, respectively; these natural phenomena maintain cellular homeostasis under conditions of nutrient deprivation.

New technologies in lipidomics, a subset of metabolomics aiming to uncover the metabolic pathways associated with the complete lipid profile of a living species, could prove useful in further understanding lipid accumulation in oleaginous yeast. New technologies such as electron spray ionization tandem mass spectrometry (ESI-MS/MS) have made it possible to rapidly quantify complex lipid species from crude extracts (Welti & Wang, 2004). Some of the major areas of lipidomics have been highlighted, including determining the specific roles of lipids for cellular signaling, membrane formation, and nutrient metabolism (Gaspar et al., 2007). Researchers have used lipidomic strategies to characterize changes in the lipid profiles of various microorganisms under different growth conditions (Klose et al., 2012; Xia et al., 2011; Xia & Yuan, 2009). Little work has been done using lipidomics to understand the change in lipid profiles of oleaginous yeasts; this technology could be helpful in understanding the effects of different nutrient sources and fermentation conditions on storage lipid metabolism and accumulation.

## **Metabolic engineering strategies for increasing oil yield**

Metabolic engineering strategies that redirect the carbon flux toward SCO production could achieve yields close to the theoretical lipid coefficient of ~0.31 g of TAG per g of glucose (Ratledge & Wynn, 2002; Ratledge, 1988). Approaches include: increasing the cytosolic acetyl-CoA pool for *de novo* FA synthesis, enhancing the activity of FA synthesis (push mechanisms), up-regulating TAG formation (pull mechanisms), or inhibiting pathways competing with TAG generation.

### ***Increasing fatty acid synthesis***

Acetyl-CoA is a key intracellular metabolite that serves as the carbon supply for *de novo* FA synthesis. Increasing the “pool” of cytosolic acetyl-CoA could enhance the biosynthesis of

FAs. It should be noted that acetyl-CoA also serves as input for other metabolic pathways, including amino acid synthesis, respiration, and secondary metabolite formation; thus, increasing the “pool” of cytosolic acetyl-CoA might not increase FA production. To coerce acetyl-CoA into FA synthesis, pathways competing with acetyl-CoA could be down-regulated and/or knocked out. These pathways include: lysine residue acetylation, isoprenoid synthesis via the mevalonate pathway and the synthesis of sterols and polyketides (Hynes & Murray, 2010; Ratledge & Wynn, 2002). Little work has been done to limit the synthesis of secondary metabolites that compete for acetyl-CoA. Increasing FA and, ultimately, TAG production via secondary metabolite pathway knockout would be a novel, yet difficult endeavor due to the importance of these pathways for cell survival. However, a large amount of genomic information is available for various oleaginous microorganisms that would be highly valuable in identifying pathways competing for acetyl-CoA.

Overexpression of key enzymes such as ACCase and malic enzyme could also help “push” the flux of acetyl-CoA toward *de novo* FA synthesis. Past work by Zhang et al. (2007) showed that overexpression of malic enzyme increased lipid accumulation 2.5-fold in the oleaginous mold *Mucor circinelloides*. Researchers have overexpressed ACCase activity in various organisms and have met with limited success (Courchesne et al., 2009). The small increases in lipid production from ACCase overexpression may suggest that co-expression of other enzymes involved with the TAG synthesis pathway might be necessary. A recent study by Tai and Stephanopoulos (2013) increased the lipid content of *Y. lipolytica* from 8.8% to 41.4% (dry mass basis) by overexpressing both ACCase and DGAT together. This “push and pull” strategy is a novel method that can be used to divert carbon flux to storage lipid synthesis.

### ***Enhancing TAG accumulation***

Another approach would be to enhance the activity of TAG-forming enzymes. TAG molecules are formed by acyltransferases, which “pull” FAs from the FAS onto the glycerol-3-phosphate (G3P) backbone. G3P is synthesized from DHAP via the glycerol-3-phosphate dehydrogenase (GPD) shuttle mechanism. Previous studies have indicated that increased activity in the GPD shuttle also leads to enhanced TAG generation; for example, Beopoulos et al. (2008) deleted a gene encoding a GDH isomer in the oleaginous yeast *Y. lipolytica* to prevent flux of G3P to DHAP, which resulted in a 3-fold increase in lipid production. Manipulating the GPAT and LPAT enzymes has met with limited success. In plants, such approaches have led to increased oil



production in seeds, but similar results do not appear to have been replicated in yeast (Jain et al., 2000; Zou et al., 1999). One potential explanation is that these reactions are also involved in the synthesis of phospholipids, and their manipulation therefore has multiple effects beyond the production of storage lipids.

The final and committed step of TAG formation is catalyzed by the DGAT and PDAT enzymes via acylation at the *sn*-3 position of diacylglycerol (DAG). Because DGAT is one of the major enzymes influencing the rate of TAG formation, a considerable amount of past work has been performed attempting to enhance the activity of this enzyme and increase TAG production. One interesting study performed by Kamisaka et al. (2007) increased the lipid content in *S. cerevisiae* to 30% (dry mass basis), a lipid content comparable to that of oleaginous microorganisms. Tai and Stephanopoulos (2013) also reported that DGAT overexpression in *Y. lipolytica* increased lipid accumulation from 8.7 to 33.8% (dry mass basis).

### ***Inhibiting $\beta$ -oxidation***

Other work has focused on TAG mobilization and oxidation pathways. TAG mobilization via TAG lipases (TGLs) is the first committed step of breakdown. TGLs hydrolyze TAG to form free FAs and glycerol and are mostly restricted to lipid bodies; thus, TGL down-regulation may prevent TAG mobilization for  $\beta$ -oxidation. For example, compared with the wild type, deletion of either or both TGL3 and TGL4 in *Y. lipolytica* increased the TAG content from 20 to 30% (dry mass basis) (Dulermo et al., 2013). Similarly, Mlickova et al. (2004a) determined that five acyl-CoA oxidases (AOXs) unique only to *Y. lipolytica* influenced neutral lipid accumulation and storage. Using similar methods, the same authors deleted different AOXs (AOX1p to AOX5p) in *Y. lipolytica* and found that AOX2p regulated the size of lipid bodies and strains expressing only AOX2p, and both AOX2p and AOX4p accumulated higher amounts of storage lipids (Mlickova et al., 2004b). This result indicates that AOXs are important in the accumulation of storage lipids, and manipulating their activity may be useful in enhancing oil yield.

Other work has included multiple-gene constructs to increase storage lipid accumulation. Kamisaka et al. (2007) were able to increase the lipid content to 30% (dry mass basis) in the non-oleaginous yeast *S. cerevisiae*. The authors were able to engineer a lipid-producing strain of *S. cerevisiae* by supplementing the nutrient media with leucine, overexpressing DGAT and FA synthase, and disrupting the transcription factor *SNF2*. Beopoulos et al. (2008) demonstrated that

combined deletion of the G3P-degrading enzyme GDH and POX enzymes increased oil content in *Y. lipolytica*, although substantial formation of free FAs was observed. Efforts to develop multiple gene constructs may be effective approaches in increasing TAG synthesis and lipid accumulation.

Synthetic biology and genetic engineering will continue to be useful in developing novel bioprocesses for biofuels and bioproducts. Although research is ongoing for many new developments in SCO bioprocessing, industrial activity has been limited due to economic drawbacks. Biotechnological advancements may solve current issues and make yeast SCO a viable renewable oil source.

## **Fermentation conditions for SCO production**

### ***Stoichiometry***

SCO or neutral storage lipid accumulation is the biochemical process of storing carbon (often from sugars) as TAG. The biochemical steps in TAG synthesis, as mentioned earlier, can determine the stoichiometry of TAG accumulation. The theoretical yield of TAG synthesized from sugars is dependent on FA chain length and the degree of unsaturation. According to Ratledge (1988), the synthesis of one molecule (mol) of trioleoylglycerol ( $C_{57}H_{104}O_6$ ) requires 16 mol of glucose or xylose. To characterize the production of lipid or oil, researchers often refer to the yield as the mass of oil produced per mass of dry cell weight ( $Y_{px}$ ). Because oil is produced intracellularly, this “yield” is essentially the oil content or composition of the dry yeast cell. Although this can be used to describe a microorganism’s oil-accumulating ability, it does not reflect the chemical conversion yield derived from the stoichiometric balance described above. The yield of product per unit of substrate ( $Y_{ps}$ ), also known as the lipid coefficient, is a more suitable representation. From the stoichiometry above, the theoretical  $Y_{ps}$  of oil from glucose and xylose is 0.31 and 0.37 g/g, respectively. On a mass basis, xylose synthesizes more TAG than glucose, making it a favorable substrate for TAG accumulation.

### ***Conditions for SCO accumulation***

SCO is a secondary fermentation product (i.e., it is produced independent of growth). To induce SCO or lipid accumulation, a two-stage fermentation technique is often employed. The first stage aims to promote cellular growth and biomass formation; during the second stage, a stress-response is induced, causing metabolism to shift to storage lipid accumulation. As mentioned

earlier, the stress-response is accomplished by limiting a specific nutrient, most often nitrogen, but other minerals such as iron and zinc have been reported with similar effects (Evans & Ratledge, 1984; Yamauchi, 1983). When nitrogen becomes limiting, yeast diverts carbon to FA synthesis and ultimately to TAG accumulation.

### ***Nitrogen availability***

One of the most important factors in TAG accumulation is the carbon-to-nitrogen (C:N, mol/mol) ratio. This ratio is a critical factor in optimizing SCO production. As the ratio increases, excess carbon becomes available for SCO production. Too high of a ratio (i.e., limited nitrogen) will limit cellular growth, biomass production, and even result in significant secondary metabolite formation; thus, optimal C:N ratios depend on the production method, fermentation conditions and yeast species used (Beopoulos et al., 2009). Most researchers have reported C:N ratios that vary from 50-100, but values can be much higher (Ageitos et al., 2011; Ratledge, 2010). For example, Angerbauer et al. (2008) reported that *L. starkeyi* produced a lipid content of 68% (dry mass basis) when grown under a C:N ratio of 150 compared with 40% for a lower C:N ratio of 60. A critical concentration of nitrogen also may be important in preventing formation of undesirable metabolites such as citric acid (Beopoulos et al., 2009). Thus, controlling the C:N ratio during fermentation is essential to direct carbon flux to TAG formation.

### ***Nitrogen source***

Past researchers have found that some nitrogen sources may be preferred over others. The yeast *R. toruloides* preferred organic glutamate to inorganic ammonium ( $\text{NH}_4^+$ ), producing an oil content of 50% compared with 18% (dry mass basis), respectively (Evans & Ratledge, 1984a). The authors hypothesized that the increase in lipid accumulation was caused by the intracellular presence of  $\text{NH}_4^+$  formed from the deamination of complex nitrogen sources. Further work by Evans and Ratledge (1984b) found that intracellular pools of  $\text{NH}_4^+$  influence the onset of SCO accumulation; these pools are formed from nitrogen sources and require an additional deamination step for metabolism, such as urease for urea, and glutamate dehydrogenase for glutamate. Interestingly, cells grown using  $\text{NH}_4^+$  as the major nitrogen source did not accumulate lipids, most likely due to the low pooling of intracellular  $\text{NH}_4^+$ . A certain threshold of intracellular  $\text{NH}_4^+$  also seems to be required to initiate the regulatory mechanism(s) that trigger citrate accumulation and FA synthesis. This indicates that the enzymes involved in the initial deamination step to release

$\text{NH}_4^+$  may play a role in regulating lipid accumulation. These are interesting points to consider, because inorganic nitrogen is often the preferred source for industrial bioprocesses. Using  $\text{NH}_4^+$  as the major nitrogen source has been well documented by other researchers, so experimental determination of the preferred nitrogen source is recommended.

### ***Dissolved oxygen***

Dissolved oxygen content is critical to SCO productivity. Oxygen demand during the lipid accumulation stage has been shown to be variable, depending on the oleaginous yeast species and the culture conditions used. Past research found that oxygen demand during lipid production was lower than during the cellular growth stage (Pan & Rhee, 1986a; Pan & Rhee, 1986b; Ratledge & Hall, 1977). Bati et al. (1984) suggested oxygen to be detrimental for lipid accumulation. For *Y. lipolytica*, the authors maintained the dissolved oxygen content at zero to prevent carbon flux to citric acid and other secondary metabolites. On the contrary, some have suggested that oxygen supplementation leads to increased SCO yields (Pan et al., 1986; Choi et al., 1982). Oxygen-enriched air can be used to increase the oxygen transfer rate, which is useful in enhancing cellular growth. Pan et al. (1986) increased the cellular biomass of *R. glutinis* to 185 g/L, resulting in a lipid content of 74 g/L using oxygen-enriched air. Oxygen-enriched air also has enhanced SCO yield; Fei et al. (2011a) increased SCO from 25 to 33% (dry mass basis). Dissolved oxygen content also has been suggested to influence the degree of FA unsaturation (Brown & Rose, 1969). Davies et al. (1990) observed that under conditions of low dissolved oxygen, increased amounts of saturated FAs, mainly palmitic acid (C16:0) and stearic acid (C18:0), were observed, and further increasing the oxygen content produced greater degrees of unsaturated FAs, specifically oleic acid (C18:1) and linoleic acid (C18:2). Dissolved oxygen content seems to be a crucial factor that varies depending on yeast species, and because its exact role in SCO accumulation has yet to be determined, it provides an interesting research opportunity.

Although not be discussed extensively here, SCO accumulation is also influenced by other conditions, such as pH, temperature or micronutrients ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ,  $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ ) (Ageitos et al., 2011; Zhao et al., 2008).

### ***Fermentation strategies***

Fed-batch fermentation is a common technique in SCO production due to its ease of nutrient control during cellular growth and lipid accumulation phases (Beopoulos et al., 2011;

Zhang et al., 2011; Zhao et al., 2011; Yamauchi, 1983). A study by Zhao et al. (2011) used a repeated fed-batch method to increase lipid productivity to 0.55 g/L/hr using *R. toruloides*. The highest cellular concentrations were produced when the glucose content was maintained at 5 g/L rather than 30 g/L, resulting in higher lipid productivities. This result indicates that control over substrate loading is an important factor in fed-batch operation. A microfiltration fed-batch production scheme was used to enhance SCO production of the microorganism *R. glutinis* from 21.1 g/L to 37 g/L (Yen & Yang, 2012). A similar method was reported by Lin et al. (2011), demonstrating that a high-density two-stage fermentation system could be used to accumulate SCOs up to 1.6 g/L/hr, the highest reported productivity for yeast SCO production. First cells were grown to a high density of 104 g/L in a nutrient-dense environment, harvested and added to a 40-g/L solution of non-sterilized glucose without auxiliary nutrients. Using filtration or centrifugation to enhance cellular density prior to lipid accumulation has potential to enhance SCO productivity. Additionally, flocculation and other density gradient-induced separation techniques may be ideal, low-energy separation methods used to remove spent nutrient media.

Continuous chemostat operations also have been reported as an effective fermentation technique for SCO production (Papanikolaou & Aggelis, 2002; Alvarez et al., 1992; Brown et al., 1989; Ykema, 1988). Optimum dilution rates for maximum cell growth are often achieved slightly below the critical dilution rate (also known as washout) . Much lower dilution rates are used for SCO production that prevent excess nitrogen buildup and maximize lipid accumulation. As a rule of thumb, a dilution rate of one-third the critical dilution rate can be used in SCO production (Ratledge & Hall, 1977). A unique partial-recycle system used by Ykema (1988) resulted in a high lipid productivity of 1 g/L/hr using a dilution rate of 0.07/hr. Alvarez et al. (1992) used a dilution rate of 0.04/hr to achieve a lipid productivity of 0.24 g/L/hr with *R. glutinis*. Because SCO fermentation consists of different stages, a control mechanism would be useful in regulating the dilution rate during each stage of growth and lipid accumulation. In addition, other modifications to chemostat operation, such as using low-energy cell separation techniques to remove spent nutrient broth, could help increase cellular biomass density prior to lipid accumulation in two-stage systems.

## **Challenges for commercial production of SCO**

The stand-alone production of SCO for biodiesel and other commodities currently is not economically feasible (Ratledge, 2010). To enhance the feasibility of commercial SCO production, research and innovation needs to focus on utilizing alternative low-cost nutrient sources and assimilating new technologies to enhance productivity. Areas of improvement include developing robust strains that can produce high SCO yields from non-traditional feedstocks, enhancing SCO productivity using biotechnological and molecular techniques, tailoring FA profiles for specific end products, and developing efficient downstream processes for cell lysis and lipid extraction.

### *Nutrient sources*

One of the major hurdles preventing industrial scale cultivation of oleaginous yeast is the cost of nutrient media. Synthetic nutrients such as glucose can represent up to 60% of the total cost of fermentation (Fei et al., 2011b). Low-valued nutrient sources are often challenging to utilize because of their heterogeneous nature and the presence of inhibitory compounds. Oleaginous yeasts have been shown to be versatile biocatalysts able to utilize a variety of alternative nutrient sources to produce SCO, including but not limited to animal fat (Papanikolaou et al., 2002), corn cobs (Gao et al., 2014; Chang et al., 2013; Huang et al., 2012a), corn residue (Galafassi et al., 2012; Liu et al., 2012b; Hu et al., 2011), glycerol (Fontanille et al., 2012; Papanikolaou & Aggelis, 2002; Meesters et al., 1996; Meesters, 1996), molasses (Alvarez et al., 1992), monosodium glutamate wastewater (Liu et al., 2012a; Xue et al., 2008), olive oil wastewater (Yousuf et al., 2010), prickly-pear juice (Hassan et al., 1994), pyrolytic waste (Lian et al., 2012), rapeseed meal (Kiran et al., 2012), rice straw (Huang et al., 2009), sewage sludge (Angerbauer et al., 2008), sorghum bagasse (Liang et al., 2012a; Liang et al., 2012b), sugarcane bagasse (Huang et al., 2012b; Tsigie, 2011), volatile FAs (Fontanille et al., 2012; Fei et al., 2011a; Fei et al., 2011b), wheat straw (Yu et al., 2011) and whey permeate (Ykema, 1988; Floetenmeyer et al., 1985).

Low-value, carbohydrate-dense lignocellulosic feedstocks from plants have the potential to serve as low-cost sources of carbon for SCO production. The heterogeneous composition of cellulose, hemicellulose, and lignin in these feedstocks has limited its use in industrial bioprocesses. A pretreatment step is required to depolymerize the lignin and hydrolyze the hemicellulose and cellulose fractions into usable sugars. Feedstocks high in lignin require harsher pretreatment conditions that can produce inhibitory compounds such as furfural, 5-

hydroxymethylfurfural, vanillin, acetic acid, formic acid and levulinic acid (Chen et al., 2009). This could be a challenge for SCO production because these inhibitors can affect microbial growth and certain enzymes important in TAG synthesis, such as the malic enzyme (Huang et al., 2012c). Researchers have shown that various oleaginous yeasts are nevertheless able to accumulate high amounts of lipid from lignocellulosic hydrolysates, but productivity often suffers (Table 1.1). The highest reported SCO productivity was 0.21 g/L/hr, an order of magnitude greater than other reported values (Galafassi et al., 2012). This was accomplished by growing *R. graminis* on undetoxified corn stover hydrolysate, which is important because a neutralization step was not needed. Robust yeast strains able to handle the presence of inhibitory compounds are necessary to prevent a neutralization step; *C. curvata*, *R. toruloides* and *T. cutaneum* have shown the best resistance to inhibitory compounds (Yu et al., 2011; Chen et al., 2009; Hu et al., 2009).

Monosaccharides produced from the hydrolysis of cellulose and hemicellulose include a mixture of pentose and hexose sugars, mainly glucose and xylose. One of the major challenges in the production of cellulosic ethanol was developing yeast strains capable of utilizing xylose anaerobically (Yang, 2007; Jeffries, 2006). Because SCO production is aerobic, xylose metabolism proceeds naturally without the aid of genetic modification, so many oleaginous yeasts are capable of converting xylose to SCO (Fall et al., 1984). An early study performed by Evans and Ratledge (1983b) concluded that the yeast *C. curvata* produced the highest lipid contents when grown using xylose compared with ethanol, lactose, sucrose and glucose. Similar results were obtained for the oleaginous yeast *L. starkeyi*, which is able to utilize xylose to produce SCO (Gong et al., 2012; Zhao et al., 2008).

Other viable low-value carbohydrate sources may include co-products from the grain-processing industry. High fiber-containing streams from flour milling, such as wheat middlings or corn bran, have significant amounts of polysaccharides ( $\geq 70\%$ , dry mass basis), including starch, hemicellulose and cellulose fractions that can be selectively targeted. In addition, the low amount of lignin ( $< 5\%$ , dry mass basis) in these feedstocks may limit the need for harsh pretreatment conditions and minimize the presence of inhibitory compounds.

### ***Downstream processing***

Downstream processing is often the most costly step within the realm of bioprocess engineering. The high cost of extracting and purifying SCO has long been a major setback for

commercialization. Most SCO extraction methods have been demonstrated only at the laboratory scale, with little work done to develop standardized, industry-scale methods. The robustness of yeast cell walls further perturbs extraction, often requiring a lysis step to release internally accumulated lipids. This section provides a summary of current lysis/extraction methods, yeast cell wall structure, and alternate extraction techniques for use in SCO processing. Please refer to the following published reviews by Ratledge et al. (2010), Lesage and Bussey (2006), Garcia (1999) and Jacob (1992) for more in-depth information on cell wall assembly, lysis and downstream processing.

### ***Lysis***

Lysis is a cell conditioning method used to disrupt the tough, outer cell wall. Physical methods include high-pressure homogenization, solid shear, ultrasonics, freeze-thawing, and extrusion; chemical/biological methods include organic solvents, acid/basic hydrolysis, surfactants (detergents), enzymatic treatment, and autolysis (Table 1.2). High-yielding, low-energy lysis methods will be demanded for efficient commercial-scale extraction. One innovative idea patented by the Raytheon Company uses metal nanoparticles for lysis (Costas & Eck, 2011). The concept is similar to a gene gun: nanoparticles are propelled at high velocities under an electromagnetic field to penetrate the cell wall. The charged nanoparticles also could be easily separated and reused by applying a magnetic field.

### ***Cell wall***

The yeast cell wall is an important organelle designed to protect the cell; it is robust, able to adapt to changes in environmental conditions and is a challenge to lyse. Providing a tough yet elastic barrier, the cell wall is composed of polysaccharides and proteins, accounting for 15-25% of the cell (dry mass basis). The cell wall's toughness is attributed to highly elastic  $\beta$ -(1,3) glucan chains and rigid  $\beta$ -(1,6) glucan crosslinks (Lesage & Bussey, 2006; Jacob, 1992). Incidence of bud scar formation and cell aging can also contribute to cell wall strength (Stenson et al., 2011). Bud scars are formed at the site of yeast budding in mother cells, which are characterized by a ring of chitin. The chitin crosslinks with the highly elastic  $\beta$ -(1,3) glucan layer, resulting in a significantly stronger cell wall that is up to 10 times greater than the mannoprotein surface (Touhami, 2003). Furthermore, increased mannoprotein linking may occur during the stationary phase, further strengthening the cell wall (De Nobel et al., 1990). This mechanism of cell wall reinforcement at



later stages of growth continues to be a challenge in developing bioprocesses designed to produce intracellular metabolites, including SCO.

To our knowledge, no work has been done to understand oleaginous yeast cell wall synthesis and composition under different fermentation conditions, including storage lipid accumulation. Because SCOs are produced during the later stages of cellular growth, we suspect that cell wall strengthening may occur even under nitrogen limitation. Aguilar-Uscanga and Francois (2003) observed changes in the total cell wall mass of *S. cerevisiae* under nitrogen-limited conditions. The authors suggested that limited nitrogen may affect the incidence of proteins in the cell wall, but the ratio of  $\beta$ -glucan/mannan remained the same. Further investigation into the biochemical synthesis of cell walls in oleaginous yeast may provide relevant information for developing strategies to limit or inhibit cell wall strengthening.

### ***Extraction***

SCO extraction and purification is often characterized by multiple processing steps that utilize organic solvents to separate oil from other cellular components. As mentioned by Ratledge (2006), the cell biomass often will need to be dried prior to solvent extraction. Drying is an energy-intensive step, so alternative methods that use wet, undried biomass could prove beneficial in lowering energy costs. Methods developed by Folch et al. (1957) and Bligh and Dyer (1959) are effective liquid-liquid extraction systems originally developed for wet animal tissues that use chloroform as the nonpolar organic and methanol as the polar alcohol to separate lipids from other non-lipid-based molecules. These methods are used mainly for laboratory analysis purposes, but adopting chloroform, a toxic chemical banned in foods and cosmetics by the Food and Drug Administration, as an oil extraction solvent at the commercial scale is not feasible. Hexane is the industry-adopted solvent used for extraction of food-grade oils from oilseed crops. Although hexane is a Generally Recognized As Safe (GRAS) solvent that is inexpensive, readily available and reusable, it is derived from non-renewable petroleum and is highly flammable. Identifying alternative, renewable solvent systems capable of extracting oil from wet cellular biomass efficiently could prove valuable in commercial-scale operations. Using ethyl acetate as a greener extraction solvent along with pulsed electric field technology was a suitable extraction system for SCO from wet microalgae biomass (Zbinden et al., 2013).

Using supercritical CO<sub>2</sub> has gained recent attention as a renewable extraction technology because carbon dioxide is an inert, easily available and GRAS (Hegel et al., 2011). Hegel et al.

(2011) performed work on extracting lipids from yeast and found that supercritical CO<sub>2</sub> with a 9% w/w ethanol supplementation provided good neutral lipid separation. One tradeoff of using supercritical CO<sub>2</sub> extraction is the burden of high energy requirements, which render this method unattractive for large-scale processes. Others have found less harsh, renewable terpene-based solvents such as *d*-limonene,  $\alpha$ -piene, and *p*-cymene to be effective extraction agents for SCO (Tanzi et al., 2013; Tanzi et al., 2012).

Ionic liquids (ILs) may also serve as viable green solvents in lipid-extraction processes. ILs are non-aqueous solutions with low vapor pressures that remain liquid from 0 to 140°C (Cooney et al., 2009). Some possess both nonpolar and polar domains, making them suitable agents for selecting lipids from aqueous and other non-hydrophobic substances. A study by Young et al. (2010) used an IL-polar covalent solvent extraction method on wet biomass of the microalgae *Chlorella*. The researchers were able to use the same IL system mentioned above as a reaction medium to transesterify microalgae SCO into fatty acid methyl esters (FAMES) (Cooney et al., 2009). The use of ILs as sustainable extraction techniques holds much promise, but further work is needed to improve recovery and reuse of ILs because they are expensive compared with other solvent systems.

Overall, key considerations in adopting low-cost, sustainable solvent-based extraction technologies include utilizing renewable solvents, reducing the time and energy required, improving solvent recovery/recycling, and minimizing waste generation.

### **Single cell oil as a biochemical platform**

The Office of Energy Efficiency and Renewable Energy at the US Department of Energy created the Biomass Program to develop and produce renewable fuels, products and power to reduce dependence on foreign petroleum (DOE, 2011). In the future, integrated biorefineries, conceptual processing facilities capable of utilizing a range of feedstocks and conversion platforms to produce multiple products (fuels, chemicals, materials, and power), could offer an opportunity to contribute to petroleum independence (DOE, 2011; Sheldon, 2011; Carvalheiro et al., 2008; Kamm, 2007; Ohara, 2003). Cellulosic biomass is one potential feedstock; over 1 billion dry tons are available each year in the US, making it the only stand-alone renewable resource able to compete with petroleum (Perlack et al., 2005). Although integrated biorefineries are still in the

concept phase, the US has taken a step closer to a bio-based economy by building multiple pilot demonstrations and commercial-scale second-generation cellulosic biorefineries (DOE, 2010).

Incorporating oleaginous yeasts as a biochemical conversion option in integrated biorefineries has many advantages. Oleaginous yeast species are desirable industrial microorganisms. They are robust (handle high sugar loading, tolerate pretreatment inhibitors, are easily cultivable and are viable for multiple generations), versatile (can utilize a range of different carbon and nitrogen sources), accumulate high lipid contents (up to 70%, w/w) and can be genetically engineered (Ageitos et al., 2011). Existing infrastructure from first-generation biorefineries could be used for oleaginous yeast cultivation. The main considerations are retrofitting fermentation vessels with agitation and aeration systems. Due to shorter cultivation times compared with oilseed crops (e.g. days vs. months), SCO made for oils/products subject to market instability and price fluctuation could minimize investment risk.

### ***Renewable oleochemicals***

Fifteen to seventeen million tons of oleochemicals per year are produced worldwide, with end-use markets ranging from bulk chemicals to specialty products (Gunstone, 2001). SCO can be converted by a variety of chemical, physical and/or biochemical techniques to produce bulk commodity and high-value specialty oleochemicals. As shown in Figure 1.2, oleochemicals can be made from FAs using different processes, such as methylation, steam cracking, ozonolysis, hydroformylation, hydrogenation, epoxidation, amination and decarboxylation. Other processes include esterification, interesterification, ethoxylation, dehydrogenation, thiolation and sulfonation. SCO could be targeted to specific oleochemicals because some oleaginous yeast are able to naturally accumulate higher amounts of specific FAs: *Candida* species produce higher percentages of palmitic acid ranging up to 40% of the total FA profile, *Y. lipolytica* can produce over 50% linoleic acid and *Cryptococcus albidus* and *L. starkeyi* can produce up to 75% oleic acid (Beopoulos et al., 2011; Ratledge & Wynn, 2002).

The petroleum-based transportation fuel economy is valued at over \$350 billion each year (Frost, 2005). Yeast SCO can be hydrotreated to produce drop-in fuels such as diesel (C9-C23) and jet fuel (C8-C16) (Peralta-Yahya & Keasling, 2010). Yeast TAG can be transesterified to produce FAMES and fatty acid ethyl esters (FAEEs) that meet the quality criteria for the ASTM D6751-11b biodiesel standard (Saenge et al., 2011). The diverse spectrum of lipids produced by

microorganisms also could result in fuels with enhanced performance properties such as decreased freezing points and improved oxidative stability (Shi et al., 2011; Knothe, 2008).

Outside of fuels, the petrochemical industry generates about \$255 billion dollars annually (Frost, 2005). Although soaps and surfactants are the most commonly produced products from plant and animal-based oils and fats, recent work has focused on the production of oleochemicals capable of competing in the petrochemical market (Metzger & Bornscheuer, 2006). Monounsaturated FAs can be transformed via ozonolysis into dicarboxylic acids, which are important intermediates for polyester and polyamide synthesis (Ackman et al., 1961). Steam cracking of saturated FAs yields linear  $\omega$ -unsaturated FAs, which can be combined with alkanes to produce polyolefins (Kaminsky & Fernandez, 2008).  $\omega$ -hydroxy FAs, which are used as intermediates in the production of polyesters, are formed in a two-step hydroformylation process followed by hydrogenation (Guo et al., 2002). Produced at 1.5 million tons per annum, fatty alcohols, mainly derived from long-chain FAs, can be made into polyoxyethylene derivatives used in detergents, surfactants, cosmetics, pharmaceuticals, toiletries and as antifoaming agents (Gunstone, 2001; Pouilloux et al., 2000). Alkyl polyglycosides are useful surfactants created from the acid-catalyzed reaction of a fatty alcohol and a sugar (von Rybinski & Hill, 1998). Fatty amines can be used as flotation, anticaking and water-repellant agents as well as corrosion inhibitors and additives in lubricants and fuels (Corma et al., 2007).

In addition to chemically altering the end group of FAs, much work has been done to functionalize the double bond of mono- and polyunsaturated oils with an epoxy group. Epoxidized FAs have been used as PVC stabilizers and UV-curable coatings (Crivello & Narayan, 1992). Monounsaturated oils such as oleic acid have been epoxidized and used to create polymeric formulations such as polyesters and polymers for drug delivery vehicles (Nicolau et al., 2010; Luppi et al., 2005). Epoxidized FAs can also serve as starting materials for the synthesis of polyurethanes. Lligadas et al. (2006) polymerized epoxidized oleic acid molecules using partial reduction reactions to produce polyether polyols, the final building block for polyurethane formation. Polyunsaturated oils such as linoleic acid and  $\alpha$ -linolenic acid also can be used to synthesize polyurethanes as renewable replacements for petroleum-based polymers (Keles & Hazer, 2009). Polyols and polyurethanes also have been produced from soybean oil by a two-step hydroformylation followed by a hydrogenation process (Guo et al., 2002).

Glycerol, produced as a by-product of transesterification, can be used to create a variety of highly valued chemicals (Figure 1.3). In recent years, the worldwide increase in biodiesel production has flooded the market with a surplus of crude glycerol, a cheap, low-value by-product selling at about \$0.05 per pound (Yang et al., 2012). Opportunities exist to utilize crude glycerol to produce high-value products, including DHA, which is used in many skin-care products; polycarboxylates, which are used in household detergents; glycerol tertiary butyl ether, a replacement fuel oxygenate for the toxic methyl tertiary butyl ether; polyglycerol esters, which are used as wood treatments; acrylic acid esters, which are used as superabsorbent polymers; propylene glycol, which is used as a preservative in food/tobacco products and for the production of polyester resins; and glycerol carbonate, which can be used to produce renewable polycarbonates and polyurethanes (Pagliaro et al., 2007; Dimitratos et al., 2006; Ott et al., 2006; Cassel et al., 2001; Vieville et al., 1998; Kimura et al., 1997).

### ***Genetically modified oils***

Foreign gene cloning also presents novel opportunities for the production of unique lipids from oleaginous yeast. Introducing genes into yeast genomes to synthesize non-native FAs (C8-C12 and  $\geq$ C20) could be useful in developing new chemicals and improving the fuel properties of biodiesel and other drop-in biofuels. Genes encoding acyl-ACP thioesterase (*Ch FatB2*) from Mexican shrub *Cuphea hookeriana* were cloned into canola (*Brassica*); thus, directing FA production from native FAs (C16:0 and C18:1) to shorter, saturated chain lengths (C8:0 and C10:0) (Dehesh et al., 1996). A similar method was used to produce medium chain-length FAs (C12-14) in the green algae *Phaeodactylum tricornutum* (Radakovits et al., 2011). Various genetically modified microorganisms capable of producing customized FA profiles have been developed. DuPont de Nemours engineered a strain of *Y. lipolytica* able to accumulate TAG rich in non-native PUFAs by up-regulating PDAT and DGAT enzymes and expressing foreign desaturases and elongases (Yadav & Zhang, 2008; Jackson et al., 2007). Solazyme developed a recombinant strain of the microalgae *Chlorella protothecoides* to produce shorter chain-length, saturated FAs as feedstocks for renewable oleochemicals (Franklin et al., 2011). Ricinoleic acid, a valuable precursor to a variety of oleochemicals, was recently produced in high quantities using recombinant *Schizosaccharomyces pombe* by introducing the  $\Delta^{12}$ hydroxylase enzyme, which converts oleic acid to ricinoleic acid and is unique to the fungus *Claviceps purpurea*, into *S. pombe*,

a natural producer of oleic acid. This technique resulted in the production of ricinoleic acid, 53% of the total FA content (Holic et al., 2012). Genetically engineered strains of cyanobacteria capable of accumulating and secreting free FA have been developed (Liu et al., 2011). The researchers used a free FA uncoupling strategy by overexpressing thioesterase enzymes to reduce acyl-ACP feedback inhibition, which resulted in the secretion of free FA into the medium. FA secretion also has been demonstrated with mutated *S. cerevisiae* strains having inactivated AOX genes (Michinaka et al., 2003). Furthermore, an engineered strain of *Escherichia coli* with deleted AOX, a cloned acyl-ACP thioesterase from the oilseed plant *Cinnamomum camphorum*, and overexpression of ACCase produced medium-chain FAs (C14-C16) to a maximum concentration of 2.5 g/L in less than 30 hr. Although this titer is fairly low compared with that of oleaginous yeast, bacteria have shorter doubling times, an added advantage in commercial production strategies. FA secretion is a promising area of work because it relieves the difficulties of cell lysis, lipid extraction/purification and TAG hydrolysis.

Others have explored the direct synthesis of FAMES and FAEEs using genetic engineering (Kalscheuer et al., 2007; Kalscheuer et al., 2006). A recombinant strain of *E. coli* produced 400 mg/L of FAEE by knocking out native genes for  $\beta$ -oxidation, overexpressing native thioesterases and acyl-CoA ligases, and cloning enzymes for fatty alcohol and wax ester production (Steen et al., 2010). To take it a step further, the authors cloned genes encoding endoxylanase and xylanase, allowing hemicellulose to be utilized directly as a major carbon source. Kalscheuer et al. (2006) engineered a strain of *E. coli* to produce 1.28 g/L of FAEEs, coined “microdiesel,” when grown in the presence of glucose and oleic acid. This novel biodiesel production strategy holds promise to reduce the number of steps and costs involved with microbial biofuel production.

## Conclusions

Oleaginous yeasts are an attractive source of oil for the production of renewable oleochemicals. Commercial production has been successful only for specialty SCOs, but opportunity exists to improve commercial-scale adoption for commodity-type oils. Early research has improved understanding of the fermentation process and biochemical events that lead to SCO accumulation. The ability of oleaginous yeast to produce SCO from a wide variety of low-value feedstock sources, including pentose sugars from the hemicellulose portion of lignocellulosic biomass, will be key for commercial adoption. Along with the recent development of metabolic

engineering strategies to improve SCO yield and/or FA composition, these are key developments in commercial-scale adoption. Gaps remain, however, and further research is needed to improve the competitiveness of SCO as a commodity-type oil. Key areas of research in improving commercial adoption include developing improved understanding of the regulatory nature of TAG biosynthesis in oleaginous yeast, further improving SCO yield and productivity using fermentation and/or metabolic engineering techniques, developing pretreatment schemes that generate high-quality sugar streams for fermentation, and adopting cost-effective and sustainable downstream processes.

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**Table 1.1 Single cell oil production by oleaginous yeast from lignocellulosic biomass.**

Organism	Dry Cell Wt. (g/L)	Oil Content (% , dry mass basis)	Oil Yield (g oil/g substrate)	Oil Productivity (g/L/hr)	Substrate	Culture method	Reference
<i>C. curvatus</i>	17.2	33.5	0.20	0.040	Non-detoxified wheat straw hydrolysate	Batch-Flask	(Yu et al., 2011)
<i>C. curvatus</i>	6.0	43.3	0.19	0.022	Sorghum bagasse hydrolysate	Batch-Flask	(Liang et al., 2012b)
<i>C. curvatus</i>	15.5	64.0	0.11*	0.083	Microwave treated sorghum bagasse hydrolysate	Batch-Flask	(Liang et al., 2012a)
<i>C. curvatus</i>	6.9	32.0	0.11†	0.018	Detoxified/Neutralized pyrolytic aqueous waste	Batch-Flask	(Lian et al., 2012)
<i>Cryptococcus Sp.</i>	10.8	61.3	0.11	0.045	Glucose enriched corn cob hydrolysate	Fed-Batch-Flask	(Chang et al., 2013)
<i>L. starkeyi</i>	14.7	31.2	0.16	0.032	Non-detoxified wheat straw hydrolysate	Batch-Flask	(Yu et al., 2011)
<i>R. glutinis</i>	13.8	25.0	0.20	0.024	Non-detoxified wheat straw hydrolysate	Batch-Flask	(Yu et al., 2011)
<i>R. graminis</i>	48	34.0	0.16-0.20‡	0.21	Corn stover hydrolysate	Batch-20L	(Galafassi et al., 2012)
<i>R. toruloides</i>	9.9	24.6	0.08	0.017	Detoxified wheat straw hydrolysate	Batch-Flask	(Yu et al., 2011)
<i>T. cutaneum</i>	19.3	39.2	0.15	0.078	Corn stover hydrolysate	Batch-3L	(Hu et al., 2011)
<i>T. cutaneum</i>	38.4	32.0	0.13	0.103	Corn cob hydrolysate	Batch-3L	(Gao et al., 2014)
<i>T. dermatis</i>	24.4	40.1	0.17	0.058	Corn cob hydrolysate	Batch-Flask	(Huang et al., 2012a)

<i>T. fermentans</i>	28.6	40.1	0.10	0.06	Detoxified rice straw hydrolysate	Batch-Flask	(Huang et al., 2009)
<i>T. fermentans</i>	30.0	52.7	0.14	0.073	Sugarcane bagasse hydrolysate	Batch-Flask	(Huang et al., 2012b)
<i>Y. lipolytica</i>	7.8	4.6	0.01	<0.001	Non-detoxified wheat straw hydrolysate	Batch-Flask	(Yu et al., 2011)
<i>Y. lipolytica</i>	11.42	58.5	0.31§	0.073	Detox sugarcane bagasse hydrolysates+ peptone	Batch-Flask	(Tsigie, 2011)

\*Sorghum bagasse as the major substrate.

†Acetic acid as the major substrate.

‡Calculated using productivity of 0.21 g/L/hr, a fermentation time of 76hr, and a total sugar (glucose and xylose) consumption of range of 80-100 g/L.

¶Calculated using a total sugar concentration of 116.9 g/L (84.3 g/L xylose + 15.5 g/L glucose + 17.1 g/L arabinose).

§Calculated using a total sugar concentration of 20 g/L (13.5 g/L xylose + 3.9 g/L glucose + 2.6 g/L arabinose).

**Table 1.2 Cell wall lysis methods; adapted from Garcia (1999) and Jacob (1992).**

Method	Lysis Principle	Advantages	Disadvantages
High pressure homogenization	Liquid shear: pressurized cell suspensions are forced through a small valve and impact a wall at high velocity	Robust, industry use, continuous, handles concentrated cell solutions	Maintenance costs with valve wear/blockage, used for larger volumes (not useful for laboratory purposes)
Ball/bead mills	Solid shear: balls/beads are agitated and break apart cells by abrasion and impact	High degrees of cell wall breakage, useful for robust cell walls	Limited cell concentration range, heat generation, difficult bead recovery
Ultrasonic (sonication)	Sound waves generated by an oscillator produce cavitation forces that disrupt cell membranes	Low energy, can be used simultaneously with other methods, useful at both small and large scale	Heat generation, difficult to use on microbes with tough cell walls (fungi, microalgae), loud
Osmotic shock	Cells are placed in hypotonic solutions causing cell to expand and rupture	Works well for cells without cell walls, low energy, mild conditions	Difficult to use with microbes that have osmo-regulatory mechanisms, specific for separating periplasmic components
Freeze-thaw	Ice crystals puncture and disrupt the cell wall	Works best as a conditioning scheme before using another lysis method (e.g. bead milling)	Slow, energy intensive for large sample volumes, works well for laboratory purposes
Organic solvents	Solubilize components of the cell wall and membrane	Can be used as a preservative, often serves as the extraction medium (e.g. chloroform)	Destruction of other cellular compartments that release degradation enzymes, some are toxic
Detergents	Solubilize cell membrane lipids and proteins	Fast acting, inexpensive, works well in combination with other methods, various types for specific applications	May degrade proteins and lipids, cell wall polysaccharides are resistant
Enzymes	Hydrolyze cell wall polysaccharides (glucanases (zymolyase), chitinases)	Low energy, mild conditions, no need for specialized equipment	Expensive, specific to certain cell wall components, enzyme stability can be an issue

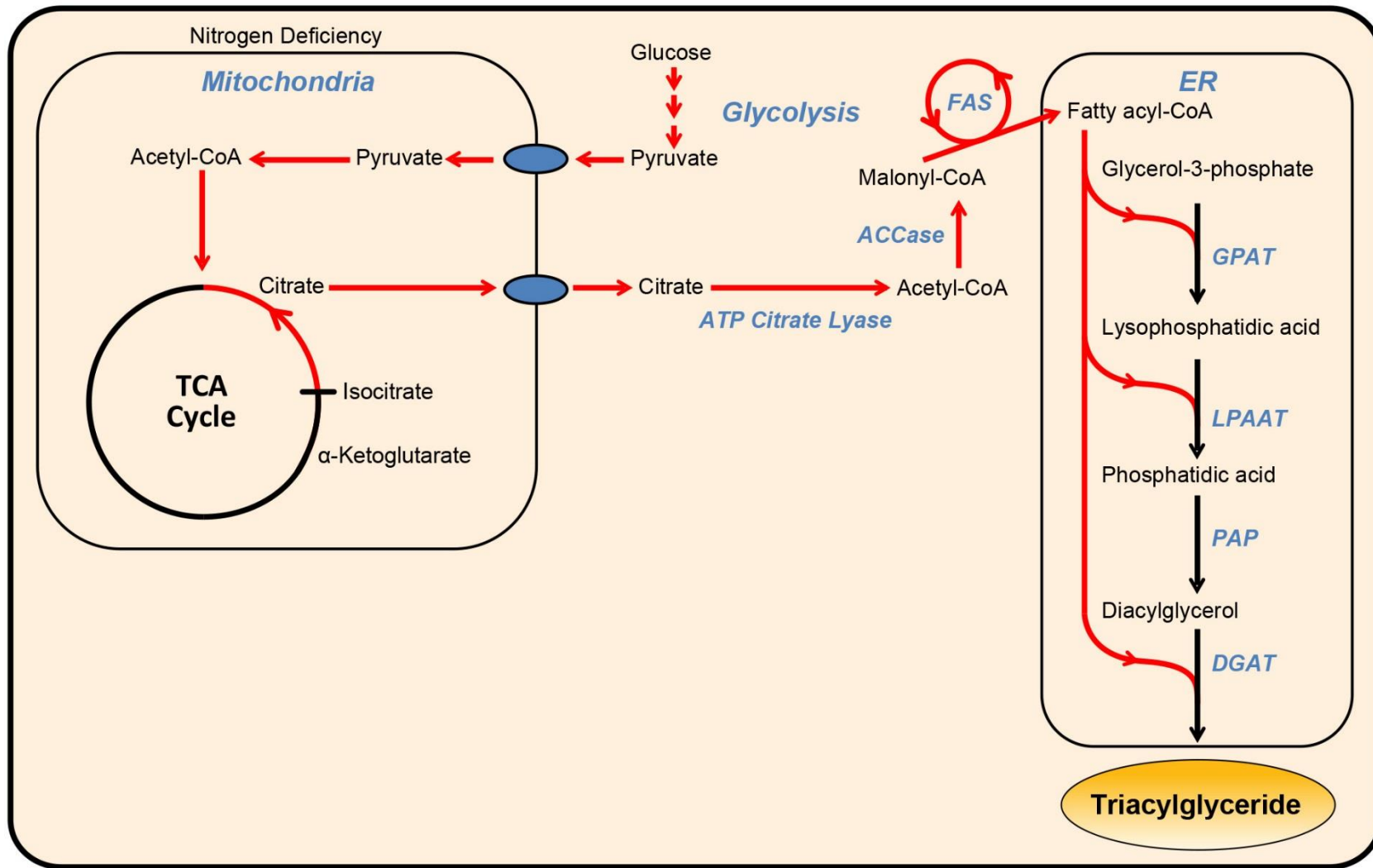


Figure 1.1 Biochemistry of triacylglyceride (TAG) accumulation in oleaginous yeast. Red arrows indicate flux of carbon from glucose to TAG; adapted from Yadav and Zhang (2008) and Picataggio et al. (2005).



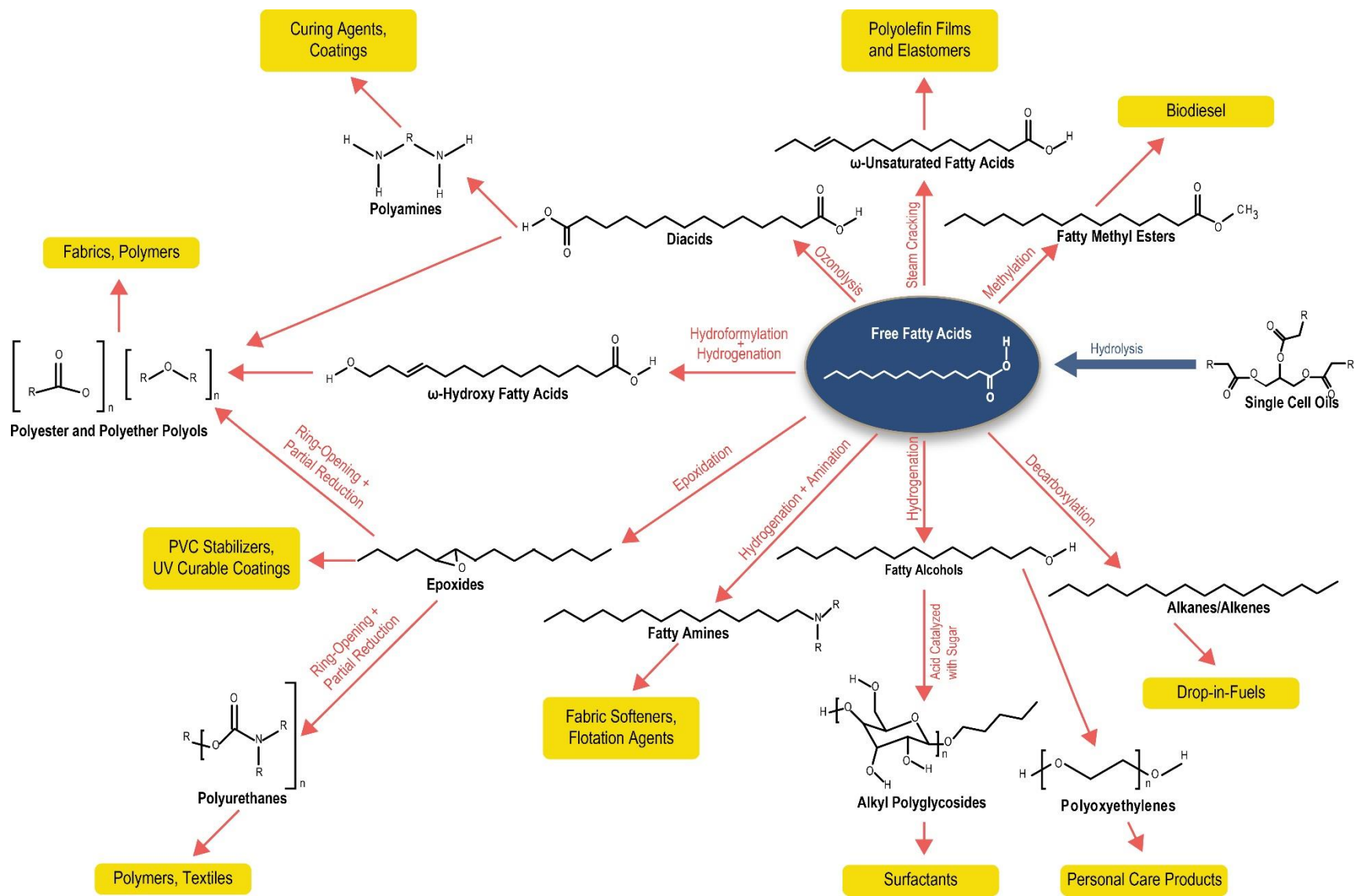
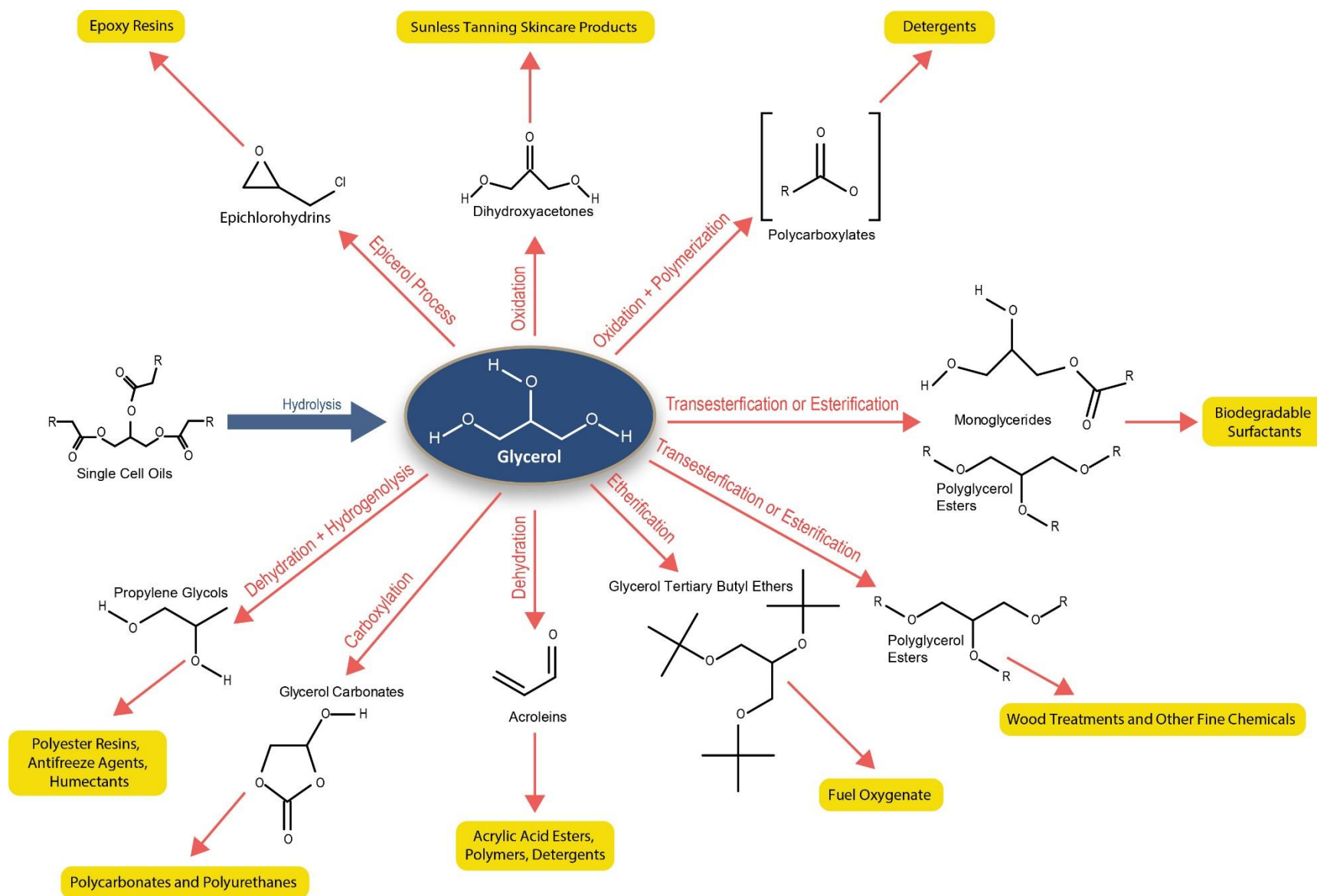


Figure 1.2 Summary of routes from fatty acids to renewable oleochemicals and their end uses.



**Figure 1.3 Summary of routes from glycerol to renewable oleochemicals and its end uses.**

## Chapter 2 - Research objectives

Oleaginous yeasts are an attractive noncrop-based renewable oil source. While commercial scale production of SCO is not widely adopted due to cost and technological limitations, an integrated approach will be useful for economically viable production platforms. Research and development that will enhance the likelihood of implementing a SCO platform include improved fermentation techniques, low-valued feedstocks utilization, sustainable and efficient oil extraction processes, and determining potential value-added uses from the oils being produced (Figure 2.1).

The major objectives of this dissertation research are:

- 1) Optimize fermentation conditions for the development of a novel fed-batch fermentation to enhance oil production using *Lipomyces starkeyi*. (Chapter 3)
- 2) Determine and quantify the major lipids produced by *L. starkeyi*. (Chapter 4)
- 3) Utilize low-valued hemicellulose-rich feedstocks from existing grain-processing industries for oil production. (Chapter 5)
- 4) Demonstrate the use of 2-methyltetrahydrofuran (2-MeTHF) and cyclopentyl methyl ether (CPME) as greener solvents for sustainable oil extraction. (Chapter 6)

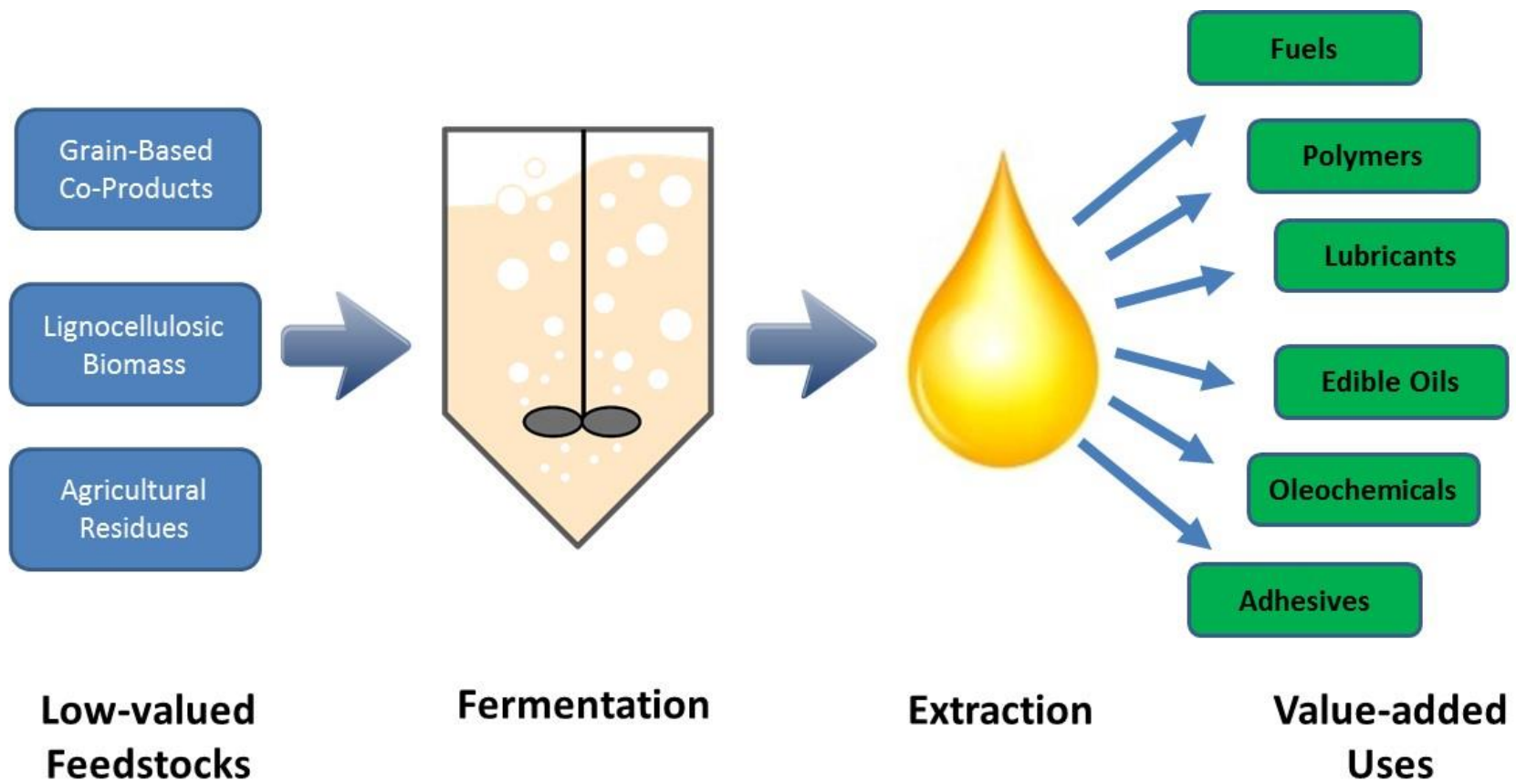


Figure 2.1 SCO biochemical platform.

## **Chapter 3 - Production of single cell oil using the xylose assimilating oleaginous yeast *Lipomyces starkeyi***

### **Abstract**

Single cell oil (SCO) from oleaginous yeast is a potential noncrop-based renewable oil source for bio-based products. *Lipomyces starkeyi* ATCC 56304 is a xylose assimilating oleaginous yeast that is able to produce SCO from low-valued substrates including lignocellulosic biomass. Shake-flask fermentations determined urea as the preferred source of inorganic nitrogen and the optimal carbon to nitrogen (C:N) ratio for both the growth and lipid accumulation stages. Fed-batch fermentation using xylose as the major carbon source generated an oil yield of 157.3 mg oil/g sugar; 2-fold greater than 78.1 mg oil/g sugar for glucose. A mixed sugar fed-batch fermentation of equal parts of glucose and xylose showed that *L. starkeyi* preferred glucose during the early growth phase. To take advantage of the preferred sugar sources for growth and lipid accumulation, a novel repeated fed-batch fermentation experiment supplied glucose for the initial growth phase and xylose for lipid accumulation phase and resulted in an oil yield of 171.3 mg oil/g sugar and oil productivity of 0.14 g/L/hr. Oleic acid accounted for 67% of the total fatty acid content indicating that SCO from *L. starkeyi* ATCC 56304 is a naturally high source of oleic acid.

### **Introduction**

In the future, renewable oil sources will be demanded due to the limited availability of petroleum reserves and an expanding oleochemical market anticipated to reach 15 million tons per year by 2018 (Transparency Market Research, 2012). Currently, crop-based plant oil (also known as vegetable oil) is the major source of renewable oil but these will be needed to feed a growing population. Single cell oil (SCO) from oleaginous yeast is a renewable, noncrop-based oil source that can be used for biofuels and oleochemicals throughout the food, pharmaceutical, cosmetics and industrial sectors.

SCO is storage lipid or oil produced from oleaginous (oil-accumulating) eukaryotic microorganisms. Oleaginous yeast are advantageous oil producers with oil content reaching 70% (dry mass basis) (Papanikolaou & Aggelis, 2011b). One particular species, *Lipomyces starkeyi* has been targeted due to its ability to produce oil from non-traditional sugar sources including xylose, a monosaccharide derived from the hemicellulose portion of plant-based materials such as

lignocellulosic biomass, agricultural residues and grain processing by-products (Huang et al., 2014; Gong et al., 2012; Liu et al., 2012; Wild et al., 2010; Yousuf et al., 2010; Angerbauer et al., 2008). Utilization of hemicellulose-derived sugars such as xylose will be a key consideration for the success of industrial SCO production.

Oleaginous yeast produce SCO during nutrient limited conditions; nitrogen is often used as the limiting nutrient. Without available nitrogen, carbon is diverted to storage lipid or triacylglycerol (TAG) accumulation, a natural mechanism for energy storage (Ratledge, 2004). To induce storage lipid accumulation, the carbon to nitrogen (C:N, mol/mol) ratio must be controlled. Too low of a ratio (excess nitrogen) will not induce SCO accumulation and too high of a ratio (i.e. limited nitrogen) will limit cellular growth, biomass production, and may even result in the formation of secondary metabolites (Beopoulos et al., 2009).

Fed-batch fermentation techniques are often used to control the C:N ratio for SCO production. This can be achieved by providing nitrogen at a lower C:N ratio during the initial stages of growth and increasing the C:N ratio by feeding additional carbon substrate into the fermenter during times of storage lipid or oil accumulation. Fed-batch fermentation has been successfully used to increase production of oil using oleaginous yeast (Galafassi et al., 2012; Fei et al., 2011; Zhang et al., 2011; Zhao et al., 2011; Wild et al., 2010; Meesters, 1996; Yamauchi, 1983).

The objectives of this study were to optimize the C:N ratio for SCO production using the oleaginous yeast *Lipomyces starkeyi* ATCC 56304; determine the fermentation performance of different carbon sources, and test a novel fed-batch fermentation scheme to improve oil yield, titer and productivity.

## **Materials and methods**

### ***Microorganism and chemicals***

Lyophilized culture of *Lipomyces starkeyi* (ATCC 56304) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), revived on yeast mold (YM) broth (Difco Laboratories, Detroit, MI, USA): 10 g/L glucose, 5 g/L peptone, 3 g/L maltose, 3 g/L yeast extract and incubated at 25°C in a temperature controlled incubator shaker (Innova 4330, New Brunswick Scientific, NJ, USA) at 200 rpm for 8 days. For long term storage, stock cultures were prepared with 50% (v/v) glycerol solution and stored at -80°C. For short term preservation,

cultures were kept on YM agar plates at 4°C; re-cultivation was performed monthly to maintain fresh culture.

All chemicals were obtained from certified suppliers and were of analytical reagent grade (Fisher Scientific, Pittsburg, PA, USA).

## ***Fermentation conditions***

### ***Inoculum preparation***

A loopful of cells from respective agar plates was inoculated into sterile YM broth (pH 5.0 adjusted with 3M hydrochloric acid). A 10% (v/v) media to flask ratio was used to ensure efficient aeration. Flasks were incubated at 25°C and 200 rpm for 72 h and sub-cultured two times for 48 h to ensure healthy growth. A 10% (v/v) inoculum was used for all fermentations. The inoculum for the fed-batch fermentations was concentrated to  $\sim 5 \times 10^8$  cells/mL by centrifuging at 5000 rpm (4070 x g) for 10 min (Sorvall Superspeed RC2-B, Thermo Fisher Scientific Inc., Waltham, MA, USA).

### ***Growth media***

Minimal media (MM) was used as the basal medium for cultivation in shake-flask and bioreactor-scale fermentations, unless mentioned otherwise, which consisted of: 0.5 g/L urea, 0.5 g/L yeast extract, 0.5 g/L  $\text{KH}_2\text{PO}_4$ , 0.5 g/L  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1 g/L  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.1 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01 g/L  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.005 g/L  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ . Nitrogen-rich (N-rich) media consisted of: 5 g/L peptone and 3 g/L yeast extract. The pH was adjusted to 5.0 using hydrochloric acid (3M). All media was sterilized at 121°C, 0.14 MPa for 15 min; sugar solutions and nitrogen containing media were sterilized separately and added aseptically once cool.

### ***Batch shake-flask fermentation***

Carbon source testing was performed using batch fermentation in 500 mL Erlenmeyer shake-flasks using MM (10%, v/v) and 50 g/L of the chosen carbon source (D-glucose, D-xylose, or L-arabinose). For nitrogen source testing, glucose was supplied as the major carbon source and the nitrogen source consisted of either ammonium sulfate (1 g/L) or urea (0.5 g/L). For the RSM optimization experiment, glucose was used as the carbon source and the amount of urea added varied depending on the C:N ratio. All shake-flask fermentations were incubated at 25°C in a temperature controlled incubator shaker at 200 rpm for 120 h unless otherwise noted.

### ***Fed-batch fermentation***

Shown in Figure 3.1, fed-batch fermentations were carried out in a 7.5 L baffled stirred-tank bioreactor with a working volume of 5 L (Bioflo 110, New Brunswick Scientific Inc., Einfield, CT, USA). Five fed-batch fermentations were performed: nitrogen rich (N-rich) and nitrogen-limited: glucose, xylose, glucose:xylose (1:1 ratio, mass basis), and glucose start:xylose feed (GSXF). The GSXF fed-batch fermentation used glucose at the start of fermentation and xylose as the feeding source. Sugar solutions (50 g/L) were sterilized separately inside the bioreactor and a concentrated solution of filter sterilized (0.22  $\mu\text{m}$ ) MM (C:N ratio of 80, mol/mol) or N-rich media (C:N ratio of 21) was pumped in aseptically after it had cooled to room temperature. The first feeding consisted of 20 g/L of sugar and 0.08 g/L of urea (C:N ratio of 100) that were sterilized separately and aseptically combined; the first feeding for N-rich fed-batch fermentation consisted of only glucose (20 g/L). Additional feedings for all fed-batch fermentations consisted of only sugar (20 g/L) unless otherwise noted. Dissolved oxygen ( $\text{dO}_2$ ) was used as an indirect indicator of sugar depletion; feedings were performed when the level of  $\text{dO}_2$  increased above 70% of air saturation. BioCommand Software (BioCommand Batch Control, Version C, New Brunswick Scientific) was used to control and monitor the fermentation conditions: agitation of 300 rpm, temperature of 26.5°C, inlet gas flow of 1 vvm. Foaming was controlled using 1% (w/v) solution of non-ionic Antifoam B emulsion (Sigma-Aldrich, St. Louis, MO). To prevent oxygen limitation during the growth stage, the  $\text{dO}_2$  content was maintained at 30% using an air/ $\text{O}_2$  two-way gas mixer. A sodium hydroxide solution (3M) was used to maintain the pH at 5.0. Sampling was performed every 6 h from 0-48 h, every 12 h from 48-72 h, and every 24 h thereafter; sampling was also performed before and after feeding.

### ***Analytical methods***

#### ***Dry cell weight and cell number determination***

Cells from a 0.5 mL aliquot of fermentation broth were harvested by centrifugation at 5000 rpm (2300 x g) for 10 min (Sorvall Super T21, Thermo Fisher Scientific Inc., Waltham, MA, USA) and washed twice with deionized water ( $\text{dH}_2\text{O}$ ). Dry cell weight (DCW) was determined gravimetrically by drying wet cells at 80°C for 12 h. Cell number was determined via cell counting of diluted cell suspensions using a hemacytometer (Hausser Scientific, Horsham, PA, USA) and a



light microscope with 20x magnification (Nikon Eclipse TS1000, Nikon Instruments Inc., Melville, NY, USA).

### ***Sugar analysis***

Fermentation broth supernatant was diluted 10x using dH<sub>2</sub>O, centrifuged at 13000 rpm (15700 x g) for 10 min to remove any remaining cell debris, and transferred to 1.5 mL HPLC vials.

Sugars were identified and quantified by a binary high-performance liquid chromatography (HPLC) system (Prominence, Shimadzu Scientific Instruments, Columbia, MD, USA). Sugars were separated using an ion exclusion column (Rezex<sup>TM</sup> RCM-monosaccharide 300 x 7.8 mm, Phenomenex, Torrance, CA, USA) and quantified with a refractive index detector (RID-10A). An isocratic mobile phase of dH<sub>2</sub>O (Direct Q, Millipore Inc., USA) was pumped a rate of 0.6 mL/min (LC-20AB). Temperature was maintained at 80°C (Prominence CTD-20A). External standards of glucose, xylose, and arabinose were prepared at 1, 2.5, 5 and 10 g/L to generate calibration curves used for quantification. HPLC data was acquired using LCsolution software (Version 1.25, Shimadzu Scientific Instruments, Columbia, MD, USA).

### ***Lysis, lipid extraction and analysis***

Yeast cells were harvested by centrifuging at 3000 rpm (2500 x g) for 15 min, washed 2x with dH<sub>2</sub>O, and concentrated to ~10<sup>9</sup> cells/mL by adding equal parts dH<sub>2</sub>O to pelleted cells. The DCW of the concentrated cell solution was determined gravimetrically and used for calculating the oil content. A 0.5 mL aliquot of concentrated cell solution was added to a 2.5 mL polypropylene screw-cap microvial with an O-ring seal. To the pelleted cells, 1 mL of 0.5 mm cubic zirconia beads, 0.5 mL of chloroform, and 0.5 mL of methanol were added. Lysis was achieved using a Beadbeater homogenizer (Mini-Beadbeater-24, BioSpec Products, Inc., Bartlesville, OK, USA) operated in 45 sec intervals with intermittent cooling on ice for 10 min for a total of six cycles.

Lipid extraction was performed using a modified method based on Bligh and Dyer (1959) with a solvent mixture of chloroform:methanol:dH<sub>2</sub>O (1:2:0.8, v/v). In summary, the cell lysate was transferred to a 7 mL glass test-tube with PTFE-lined caps. Microvials were washed 2x with chloroform and methanol to ensure all lysate was removed. The remaining chloroform, methanol, and water were added, vortexed, and centrifuged at 3000 rpm (1500 x g) for 15 min to achieve phase separation. The lower chloroform portion was removed using a Pasteur pipette and transferred to clean test tube. Extractions were repeated a total of 3x to ensure complete lipid

removal. The combined chloroform layers were filtered using a PTFE filter (0.2  $\mu\text{m}$ ) to remove any cellular debris, washed once with 1 mL of potassium chloride solution (1M), and dried down completely under nitrogen gas. Residual solvent was removed using a vacuum drier for 30 min. Total lipid weight was determined gravimetrically and used for calculating the oil content of the cells (% , dry mass basis).

Fatty acid composition was determined by a gas chromatograph (GC-2014, Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a flame ionization detector (FID) using an aqueous stable polyethylene glycol capillary column (Zebron ZB-Wax<sub>plus</sub> 30 m x 0.25 mm x 0.25  $\mu\text{m}$ , Phenomenex, Torrance, CA, USA). Fatty acids were transesterified to fatty acid methyl esters (FAMES) in methanolic HCl (3M) at 78°C for 30 min followed by addition of water and extraction with hexane. 1  $\mu\text{L}$  of sample was injected at 220°C with a split ratio of 30:1 and a column flow of 3 mL/min. The initial column temperature was set for 160°C, increased to 200°C at 5°C/min and held for 17 min. The FID detector temperature was set at 250°C. An external standard FAME mixture (Supelco® 37 component FAME mix) and a C15:0 (pentadecanoic acid) internal standard were used to ascertain the relative retention time and quantify the percentage of fatty acids, respectively (See Appendix A for the calculation). A mass spectrometer (GCMS-QP2010 SE, Shimadzu Scientific Instruments, Columbia, MD, USA) was used to confirm fatty acid species by performing a similarity search using the NIST mass spectral library. GC and GCMS data were acquired using GCsolution software (Version 2.42) and GCMSsolution software (Version 4.11, Shimadzu Scientific Instruments, Columbia, MD, USA).

### ***Elemental analysis***

Yeast cells grown in YM broth were prepared by washing with dH<sub>2</sub>O 5x to remove any remaining nutrient media and lyophilized to a powder. Elemental analysis (C, H, N, S) was determined using a combustion analyzer (PerkinElmer 2400 Series II, PerkinElmer, Waltham, MA, USA). Approximately 2 mg of sample was placed in a tared tin capsule (PerkinElmer, N2411255) and precisely weighed (PerkinElmer, AD-6 Autobalance). Oxygen was determined by difference of CHNS and ash content. Ash content was determined using a modified NREL procedure (Sluiter et al., 2008). In brief, 0.2  $\pm$  0.05 g of lyophilized yeast powder was added to a porcelain dish, dried at 105°C to account for moisture losses, heated in a muffled furnace at 575°C for 24 h and cooled in a desiccator. All analysis were done in duplicate.

### ***Experimental design and statistical analysis***

Response surface methodology was used to optimize the fermentation temperature and C:N ratio using batch shake-flask fermentation. Design Expert software (Version 8.0.7.1, Stat-Ease Inc., Minneapolis, MN, USA) was used to generate the experimental design, calculate the responses from dependent variables, perform analysis of variance (ANOVA), and generate 3D response surface plots. A rotatable  $2^2$  central composite design (CCD) with two independent variables is shown in Table 3.1. The CCD consisted of five replicates at the central point and eight axial points for a total of 13 runs with no blocking (Table 3.2). A second-order quadratic model (Equation 1) was chosen as the best fit for the experimental data which included main effects and interaction effects of each variable:

$$Y = \beta_o + \sum_{i=1}^2 \beta_i X_i + \sum_{j=i+1}^2 \beta_{ij} X_i X_j + \sum_{i=1}^2 \beta_{ii} X_i^2 \quad \text{Eq. 1}$$

where  $y$  = predicted response,  $\beta_o$  = constant coefficient,  $\beta_i$  = linear coefficient,  $\beta_{ii}$ =quadratic coefficient and  $\beta_{ij}$  = interaction coefficient.

SAS statistical software (Version 9.1, SAS Institute, Cary, NC, USA) was used to perform PROC GLM to determine means and Tukey's Honestly Significant Difference test was used for comparison between treatments ( $\alpha=0.05$ ).

## **Results and discussion**

### ***Testing of carbon and nitrogen sources***

Batch shake-flask fermentations were performed to test the effect of different nitrogen sources on biomass growth. Ammonium sulfate and urea were tested as the inorganic nitrogen sources and glucose was supplied as the major carbon source. Urea was the preferred source of inorganic nitrogen over ammonia resulting in a DCW of 15.5 g/L compared to 4.7 g/L for ammonia. The poor performance of using ammonia to produce oil using oleaginous yeast has been documented by other researchers who hypothesized that ammonia did not induce lipid accumulation due to the lack of intracellular pooling of ammonium ( $\text{NH}^+$ ) ions (Evans & Ratledge, 1984a; Evans & Ratledge, 1984c). Their findings may suggest that nitrogen sources requiring a deamination step prior to nitrogen metabolism (e.g. urea) may be better for lipid accumulation. It should be noted that other studies have successfully used ammonia as a nitrogen source (Wild et al., 2010; Zhao et al., 2008; Boulton & Ratledge, 1983; Yamauchi, 1983) . This may indicate that

the inorganic nitrogen source chosen may be species and/or strain specific and should be carefully considered.

In a separate experiment, batch flask fermentations were performed to test different carbon sources: D-glucose, D-xylose and L-arabinose. *L. starkeyi* was able to utilize both glucose and xylose; arabinose was not utilized. These results confirm the research reported in the literature that *L. starkeyi* is capable of utilizing xylose (Zhao et al., 2008; Naganuma et al., 1985). Furthermore, xylose generated an oil yield of 148.8 mg oil/g xylose, almost 3-fold greater compared to 58.0 mg oil/g glucose. This is not surprising considering xylose generates about 10% (w/w) higher theoretical yield of oil or TAG (Fakas et al., 2009; Ratledge, 1988).

### ***Elemental balance***

To further investigate the mass balance of using glucose and xylose to produce oil, stoichiometric coefficients were determined by elemental balance of carbon, nitrogen, oxygen and hydrogen. Two separate stoichiometric equations were used for each phase of fermentation: a growth phase governed by cell replication and growth, and an oil accumulation phase. The basis for the calculations included:

- Oil yield for glucose and xylose: 16 mol sugar/mol oil
- Oil MW: 865.25 g/mol (see Appendix A for calculation)
- Biomass yield for glucose and xylose: 4.13 mol biomass/mol glucose and 3.44 mol biomass/mol xylose, respectively
- Biomass MW: 23.98 g/mol (includes ash)
- No secondary metabolites are produced

The theoretical oil yields and biomass yield were obtained from Ratledge (1988) and Ykema et al. (1986). It should be noted that the theoretical oil yield is determined based on the assumption that acetyl-CoA, the carbon supply for fatty acid and ultimately TAG synthesis, is produced from glucose via glycolysis and from xylose via the phosphoketolase reaction (Papanikolaou & Aggelis, 2011a). On a molar basis, 16 mol of glucose or xylose is required to produce 1 mol of oil (triolelylglycerol).

Biomass molecular weight and formula was determined from elemental CHNS analysis on *L. starkeyi* grown under nitrogen-sufficient conditions using N-rich media. The mass balance calculated from the elemental balance is shown in Table 3.3. The most obvious difference is the

amount of oxygen required across the different sugar sources and growth phases. The calculated oxygen demand for xylose under the lipid accumulation phase was 3.4-fold less compared to glucose and to the growth phase for both sugars. This may indicate that less oxygen or air is needed during the lipid accumulation phase when using xylose as the major sugar source.

### ***Temperature and C:N ratio optimization***

Shake-flask fermentations carried out using RSM design were used to determine the optimal temperature and carbon to nitrogen (C:N) ratio for cellular growth and lipid accumulation. Using Design Expert 7.1, a fitted quadratic model was used to arrive at optimum temperature and C:N ratio. The optimized variables were found using a desirability objective function that assigns relative importance to the responses. The optimal temperature selected was 26.5°C for both DCW and oil content. The optimal C:N ratio for DCW was 80 and oil content was 100; the top three for each response are shown in Table 3.4. 3-Dimensional surface plots for DCW and oil content are shown in Figure 3.2 and Figure 3.3, respectively. Interestingly, poor growth was observed at C:N ratios lower than 60. This was unexpected because additional nitrogen is available at lower C:N ratios. For lipid accumulation, the optimal C:N ratio of 100 compares well with the values reported from literature (Ageitos et al., 2011).

### ***Fed-batch fermentations***

The optimized C:N ratio and temperature were used for performing fed-batch fermentations in a 5-Liter working volume bioreactor. Five separate fed-batch fermentations were performed using different sugar sources. Except for the N-rich fed-batch fermentation, the starting C:N ratio of the nutrient media was 80 and changed to 100 by controlling the sugar and urea content of the feed media. The N-rich fed-batch fermentation had a starting C:N ratio of 21. Performance parameters from each fermentation are shown in Table 3.5. Calculated coefficients are as follows:  $Y_{xs}$  is the cell biomass yield (g DCW/g sugar), oil content is the composition of oil within the dry cell (% , dry mass basis),  $Y_{ps}$  is the oil yield (mg oil/g sugar),  $C_p$  is the oil concentration or titer (g oil/L), and  $Q_p$  is the oil productivity (g oil/L/hr).

### ***Glucose and xylose***

The glucose fed-batch fermentation and xylose fed-batch fermentation showed similar trends; both consumed about 90 g/L of sugar within 120 h at a sugar utilization rate of about 1

g/L/h (Figure 3.4 and 3.5). As expected, oil production from xylose was substantially greater; an oil yield of 157.3 mg oil/g sugar was reached, almost 2-fold greater than 78.1 mg oil/g sugar for glucose; similar yields using glucose were reported by Wild et al. (2010). As mentioned earlier, the theoretical yield of oil from xylose is ~10% (w/w) greater than glucose but the results obtained from both shake-flask and fed-batch fermentation show that xylose is generating a yield almost 50% greater. This deviation may be due to the different biochemical pathways used for xylose and glucose metabolism. It seems that the higher conversion efficiency to oil from xylose could, in part, be due to the different pathway(s) used for xylose metabolism. As mentioned by Jeffries (2006), yeasts and other eukaryotic fungi metabolize D-xylose via xylose reductase and xylitol dehydrogenase (XR-XDH pathway) to D-xylulose and ultimately xylulose-5-phosphate. The fate of xylulose-5-phosphate is dependent upon the demand of the cell. For acetyl-CoA synthesis it enters the phosphoketolase pathway while the pentose phosphate pathway is more efficient for generating reducing power (i.e. NADPH) (Berg et al., 2002). Evans and Ratledge (1984) found that many species of yeast including *L. starkeyi* had positive phosphoketolase activity when grown in the presence of xylose. This may indicate that oleaginous yeast may use a combination of different metabolic pathways when metabolizing xylose to balance the production of acetyl-CoA and NADPH needed during fatty acid synthesis resulting in increased oil yields.

### ***Glucose:xylose (1:1)***

A glucose:xylose (Glu:Xyl, 1:1 ratio) fed-batch fermentation was performed to study the ability of *L. starkeyi* to metabolize mixed sugars. Shown in Figure 3.6, glucose was consumed readily after fermentation began while xylose experienced ~30 h lag period before being consumed. Additionally, inocula prepared using xylose required longer time to reach the required cell density compared to glucose. This may indicate that *L. starkeyi* prefers glucose to xylose during cell replication and growth. After the lag period, xylose was consumed simultaneously with glucose. Once glucose was completely utilized, a period of increased dissolved oxygen, shown by the smaller peaks in Figure 3.7, was observed prior to the complete utilization of xylose. This may be indicative of a lower oxygen demand during xylose consumption, which is in good agreement with the calculated requirement from the stoichiometric balance shown in Table 3.3. To observe the effect of doing repeated fed-batches on oil yield, the Glu:Xyl fed-batch fermentation was run for 156 h with a total of four feedings. The additional feedings were able to increase the oil concentration from 12.2 to 22.9 g/L, adjusting the oil yield from 125.5 to 170.3 mg oil/g sugar and

the lipid productivity from 0.10 to 0.14 g/L/h (Table 3.5). Providing additional substrate during the oil accumulation phase using fed-batch fermentation is an effective strategy to increase lipid yields beyond traditional batch fermentation.

### ***Glucose start:xylose feed (GSXF)***

Due to *L. starkeyi*'s preference for glucose during the growth phase and xylose for the oil accumulation phase, a fermentation scheme was performed using glucose as the starting sugar source and xylose as the feeding sugar source, also referred to as the GSXF fed-batch fermentation (Figure 3.8). After glucose was consumed (~69 h), xylose was fed into the fermenter; six feedings were performed over 255 h generating the highest oil yield of 171.3 mg oil/g sugar (Table 3.5). The additional fed-batch experiments were able to increase the oil titer 2.5-fold from 14.3 g/L at 120 h to 36.3 g/L at 255 h. Though, it should be noted, productivity did not improve. Furthermore, even after an extended fermentation time of 255 h and 210 g/L of sugar addition, oil titer never reached a plateau; it was still showing an increasing trend. Other researchers have reported this gluttonous behavior of oleaginous yeast (Fei et al., 2011; Zhao et al., 2011). One study performed by Zhao et al. (2011) showed that, even after performing a repeated fed-batch fermentation for 358 h, sugar continued to be assimilated as oil and no plateau was reached. During the final feeding (~180 h), 50 g/L of xylose was fed into the fermenter resulting in a lower utilization rate of 0.8 g/L/h compared to 1.0 g/L/h when feeding 20 g/L of xylose. The reduced utilization rate could be attributed to substrate inhibition; this has been described by Zhao et al. (2011). Controlling substrate concentration during feeding may be necessary for maintaining high conversion rates.

Comparison of each fed-batch fermentation showed that xylose had the best performance while N-rich was the poorest (Table 3.5). As mentioned previously, lower C:N ratios resulted in limited oil production; the same was observed for the N-rich fed-batch fermentation which had a starting C:N ratio of 21 (Figure 3.9). Under the lower C:N ratio, sugar utilization rates were severely impacted to 0.5 g/L/h; half of what was observed for the glucose nitrogen-limited fed-batch fermentation. It is apparent that under lipid accumulation, there is a greater demand for sugar or carbon. Prior to entering stationary phase, exponential growth proceeded for 48 h under both nitrogen-sufficient and nitrogen-limited conditions (Figure 3.4 and Figure 3.9). Due to the similar growth regime, the higher rate of sugar utilization under nitrogen limitation is most likely a result of cells accumulating additional carbon to produce storage oil. Cells under sufficient nitrogen will only accumulate the amount needed for maintenance, no additional carbon will be assimilated for

other purposes such as oil accumulation. Essentially, this suggests that cells will consume less sugar and produce less oil when nitrogen is available. This observed difference in carbon utilization reiterates the importance of maintaining the correct C:N balance during fermentation.

The use of pure xylose would ultimately be the preferred substrate for oil production, but this would not be a realistic scenario for an industrial-scale operation. Utilization of mixed sugars from low-valued feedstocks such as lignocellulosic biomass is a more likely scenario. Production of oil using a mixed sugar (Glu:Xyl) fed-batch fermentation approach resulted in a lower productivity of 0.10 g/L/h compared to 0.13 g/L/h for pure xylose. While simultaneous co-utilization of sugar sources has been reported in this study and by others; the use of xylose as the major carbon source during lipid accumulation is preferred (Gong et al., 2012; Hu et al., 2011). To achieve similar oil production from mixed sugars, a fermentation scheme capable of meeting the preferred sugar source for each stage of fermentation such as the GSXF would be preferred. From a production standpoint, separating the sugar fractions after pretreatment would be costly. Instead, novel hydrolysis strategies capable of fractionating the different components of biomass (e.g. cellulose and hemicellulose) into separate sugar streams would be preferred. To further enhance oil yield, the minimum amount of glucose needed for exponential growth could be supplied before switching over to xylose-rich streams during oil accumulation. For the GSXF, oil yields of ~200 mg oil/g xylose were achieved during the oil accumulation phase (72-255 h). This is comparable to some of the highest reported oil yields achieved for oleaginous yeast (Gong et al., 2012; Lin et al., 2011; Zhao et al., 2011).

### ***Fatty acid profiling***

As shown in Figure 3.10, the major fatty acids produced by *L. starkeyi* (ATCC 56304) included (in decreasing order) oleic acid (C18:1), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), and linoleic acid (C18:2). The fatty acid profile remained consistent across each fermentation performed. The most noticeable difference was the lower amount of oleic acid present in oil produced by the N-rich fed-batch fermentation; this indicates that oleic acid is most likely the major fatty acid accumulated as storage lipid or TAG. Compared to other oleaginous yeast, *L. starkeyi* ATCC 56304 generates a considerable amount of oleic acid, up to 67% of the total fatty acid composition (Table 3.6). This is a promising find due to the recent interest in high



oleic oils for their improved oxidative stability and cold flow properties in biodiesel and as petroleum replacements for lubricants and cosmetics (Durrett et al., 2008; Corbett, 2003).

## Conclusions

The C:N ratio is a critical factor which was first optimized for growth and oil production, and subsequently used for the fed-batch fermentation experiments. Use of fed-batch fermentation for SCO production was an effective method to increase oil yield and titer using *L. starkeyi* ATCC 56304. Glucose was a poor carbon source for oil accumulation while xylose generated a 2-fold improvement in yield and productivity at 157.3 mg oil/g xylose and 0.13 g/L/hr, respectively, when used as the major carbon source for fed-batch fermentation. A novel GSXF repeated fed-batch fermentation was able to enhance oil yield to 171.3 mg oil/g sugar, oil content to 60.1% (dry mass basis) and oil titer to 36.3 g/L using a mixture of glucose and xylose as the substrate though productivity was unchanged. Fermentation strategies such as the GSXF that take advantage of sugar utilization preference can be used to further enhance oil yields by combining the preference of glucose for cell growth with the higher oil accumulating ability of xylose. This will be an important consideration for future biorefineries since oleaginous yeast can produce oil from hemicellulose-derived sugars. The naturally high oleic acid content of the oil produced by *L. starkeyi* ATCC 56304 has potential as a value-added input for the oleochemical industry.

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**Table 3.1 Factors and input levels tested using a central composite design.**

Factor	Low actual (-1)	Mean (0)	High actual (+1)	- $\alpha$	+ $\alpha$
*Temperature (°C)	25	27.5	30	25	30
C:N Ratio	25	62.5	100	9.5	115.5

\*Temperature was not subjected to alpha level expansions due to incubator limitations.

**Table 3.2 Experimental design matrix for actual and predicted dry cell weight and oil content.**

Run	Temperature	C:N ratio	Dry cell weight (g/L)		Oil content (% , dry mass basis)	
			Actual	Predicted	Actual	Predicted
1	30.0	100.0	6.9	6.0	25.4	24.0
2	27.5	115.5	9.0	9.6	29.1	31.4
3	30.0	25.0	0.6	-0.3	6.2	7.1
4	27.5	62.5	11.4	11.5	28.9	28.4
5	30.0	62.5	4.0	5.9	20.0	20.5
6	27.5	62.5	12.5	11.5	29.5	28.4
7	25.0	62.5	10.7	10.9	22.3	24.8
8	27.5	62.5	10.9	11.5	26.4	28.4
9	27.5	9.5	0.4	1.0	6.6	5.7
10	27.5	62.5	12.1	11.5	31.6	28.4
11	25.0	25.0	4.9	4.9	10.2	10.1
12	27.5	62.5	11.4	11.5	27.3	28.4
13	25.0	100.0	10.8	10.7	31.9	29.6

**Table 3.3 Stoichiometric balance for the growth and lipid accumulation phases of fermentation.**

Growth phase	$C_xH_{2x}O_x + aO_2 + bCH_4N_2O = cCH_{1.95}N_{0.094}O_{0.53} + dCO_2 + eH_2O$					
Sugar	Oxygen, g/g (a, mol/mol)	Urea (b)	Biomass (c)	Lipid (f)	CO <sub>2</sub> (d)	H <sub>2</sub> O (e)
Glucose, x=6	0.22 (1.24)	0.065 (0.19)	0.55 (4.13)	N/A	0.51 (2.06)	0.24 (2.36)
Xylose, x=5	0.23 (1.08)	0.065 (0.19)	0.55 (3.44)	N/A	0.51 (1.75)	0.24 (2.03)
Lipid accumulation phase	$C_xH_{2x}O_x + aO_2 = fCH_{1.95}O_{0.11} + dCO_2 + eH_2O$					
Glucose, x=6	0.24 (1.31)	N/A	N/A	0.30 (0.063)	0.67 (2.72)	0.28 (2.80)
Xylose, x=5	0.068 (0.32)	N/A	N/A	0.36 (0.063)	0.51 (1.73)	0.22 (1.80)

Values in parenthesis are the molecular coefficients representative of each specific lowercase letter (a, b, c, d, e, f).

**Table 3.4 Optimized C:N Ratio for dry cell weight and oil content.**

Rank	C:N ratio	Dry cell weight (g/L)
1	80.0	12.6
2	77.5	12.7
3	86.1	12.6
Rank	C:N ratio	Oil content (% , dry mass basis)
1	100.0	32.8
2	92.5	32.7
3	93.9	32.7



**Table 3.5 Comparison of SCO fed-batch fermentation performance using different synthetic carbon sources.**

Fermentation	$Y_{xs}$ (g DCW/g sugar)	$Y_{ps}$ (mg oil/g sugar)	Oil content (% dry mass basis)	$C_p$ (oil, g/L)	$Q_p$ (oil, g/L/hr)
* <sup>A</sup> N-Rich	0.29	105.7	34.2	4.6	0.04
<sup>A</sup> Glucose	0.19	78.1	42.8	7.4	0.06
<sup>A</sup> Xylose	0.30	157.3	51.7	14.0	0.13
<sup>A</sup> Glu:Xyl (1:1, w/w)	0.26	125.5	46.4	12.2	0.10
<sup>A</sup> GluStartXylFeed	0.25	136.0	53.2	14.3	0.12
<sup>B</sup> Glu:Xyl (1:1, w/w)	0.29	170.3	58.4	22.9	0.14
<sup>C</sup> GluStartXylFeed	0.28	171.3	60.1	36.3	0.14

\*50g/L of starting glucose concentration, C:N ratio of 21, peptone used as N-source.

<sup>A</sup>120 h

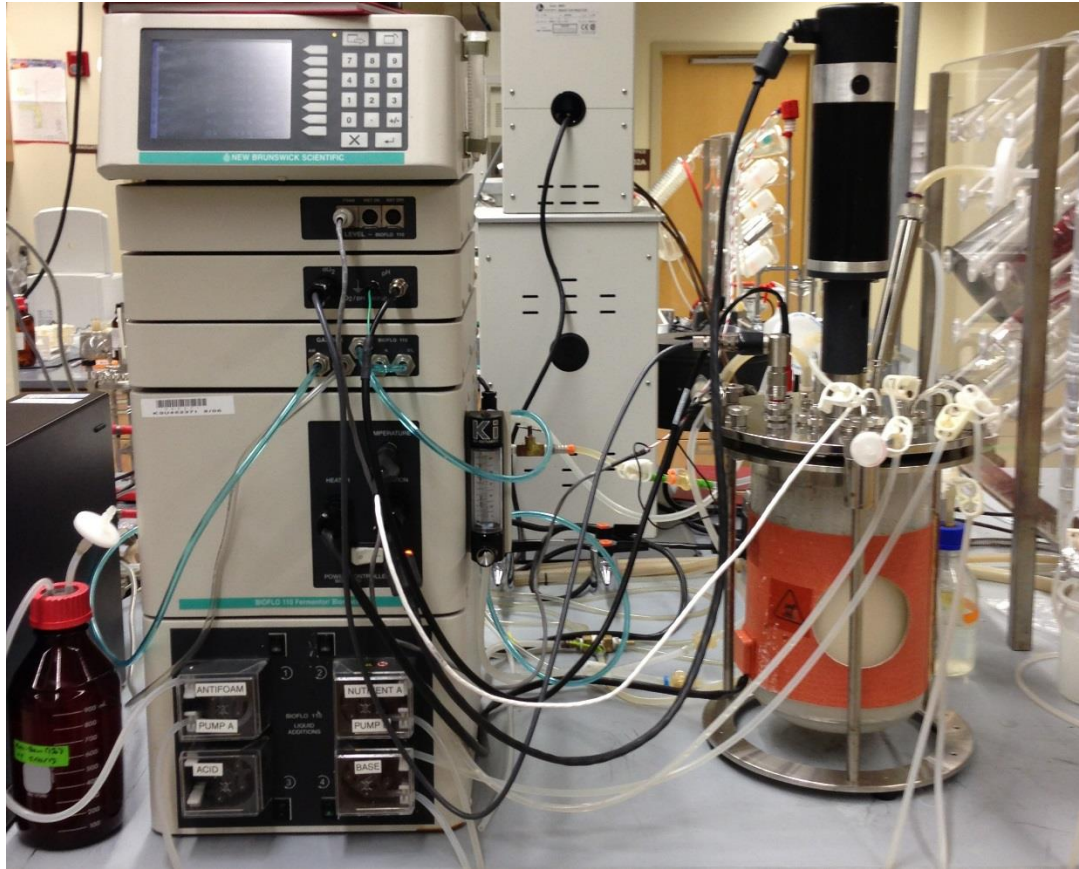
<sup>B</sup>156 h

<sup>C</sup>255 h

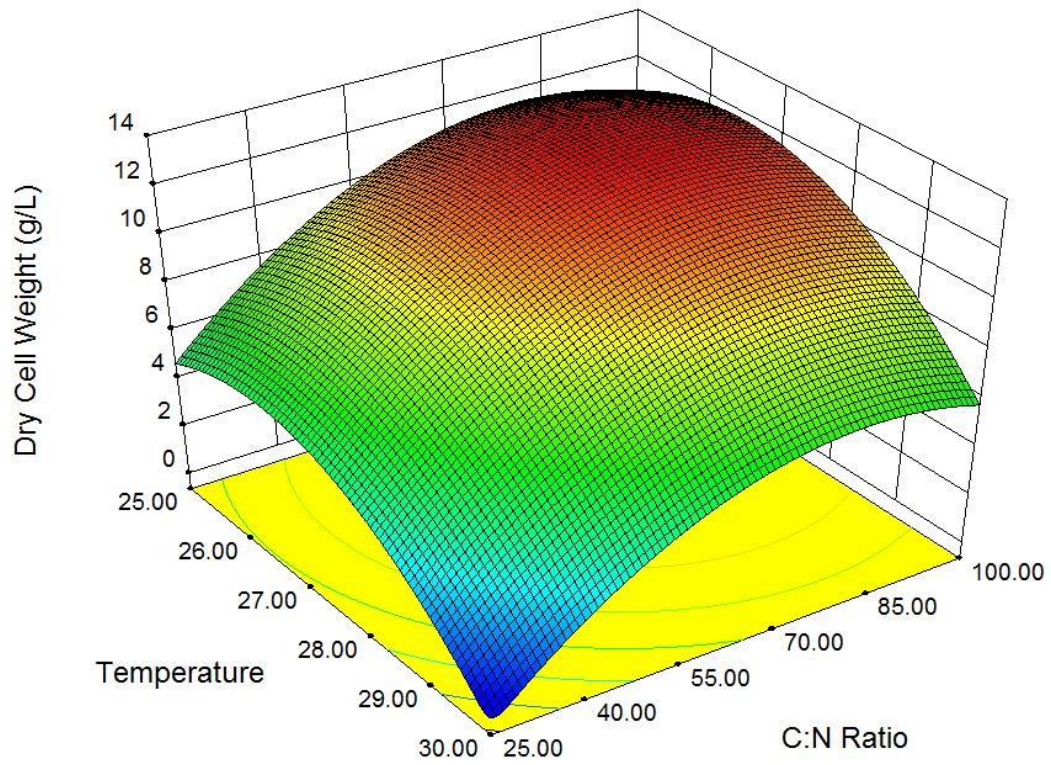
**Table 3.6 Fatty acid profiles of oleaginous yeast species.**

Yeast species	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3n3	Source
* <i>Lipomyces starkeyi</i> ATCC 56304	18.9	4.0	5.0	67.9	2.9	0.6	This study
<i>Cyptococcus curvatus</i> O3	30.1	N/A	18.5	39.3	8.3	1.2	(Zhang et al., 2011)
<i>Lipomyces starkeyi</i> AS 2.1560	37.7	3.2	4.6	51.4	1.9	N/A	(Gong et al., 2012)
<i>Rhodotorula glutinis</i> TISTR 5159	16.8	0.8	3.7	45.8	17.9	4.3	(Saenge et al., 2011)
<i>Rhodospiridium toroloides</i> Y4	29.8	1.1	5.9	53.2	5.8	0.7	(Zhao et al., 2011)
<i>Trichosporon cunatum</i> AS 2.571	27.8	0.8	20.2	48.2	3.0	N/A	(Hu et al., 2011)
<i>Yarrowia lipolytica</i> MUCL 28849	14.8	5.9	7.5	36.5	25.8	N/A	(Fontanille et al., 2012)

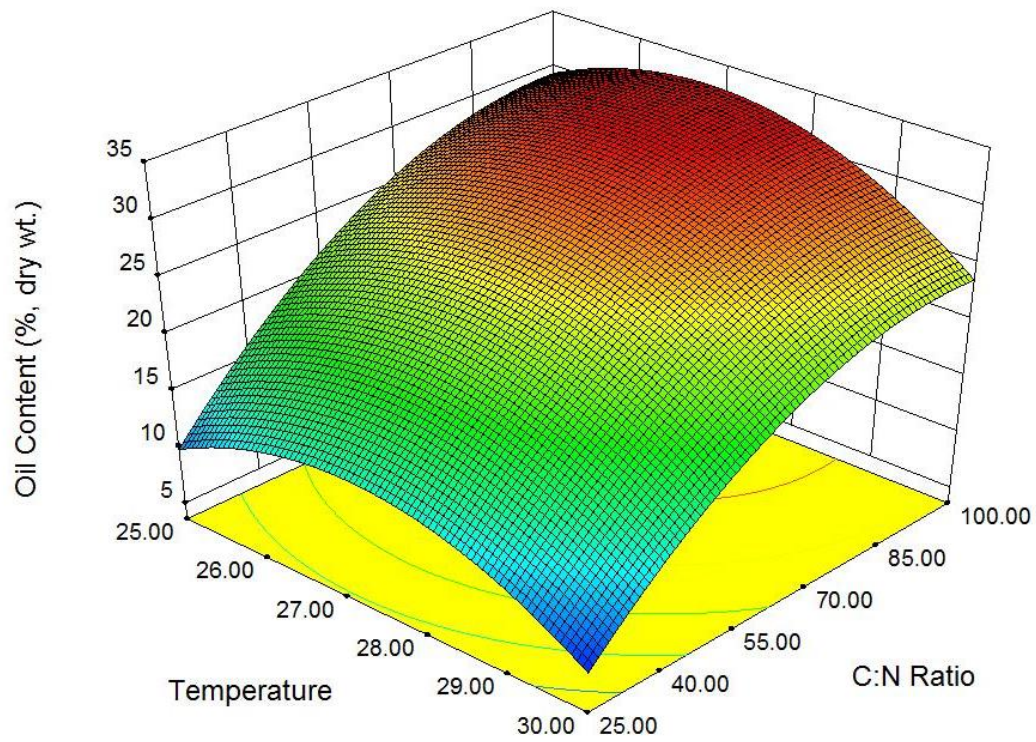
\*Fatty acid profile of total lipid extract produced from the xylose fed-batch fermentation at 120hr.



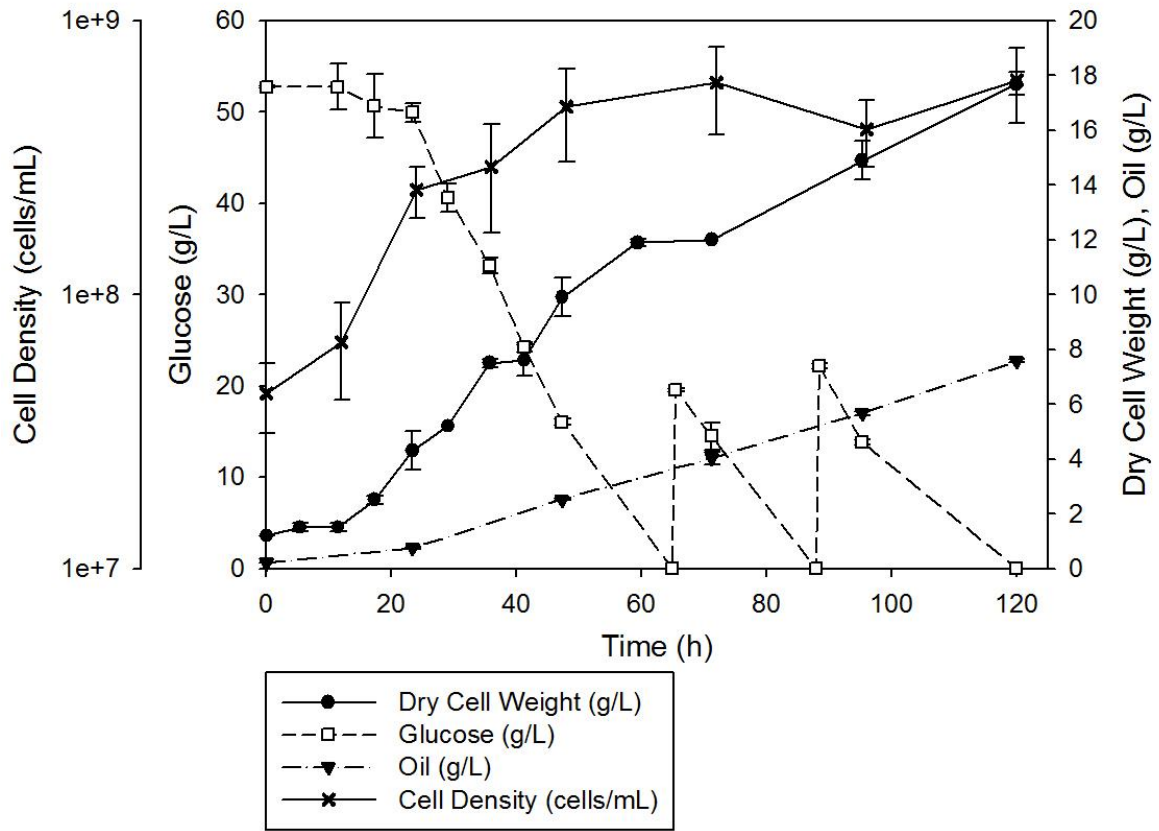
**Figure 3.1 Bench-scale bioreactor used for the fed-batch fermentation experiments.**



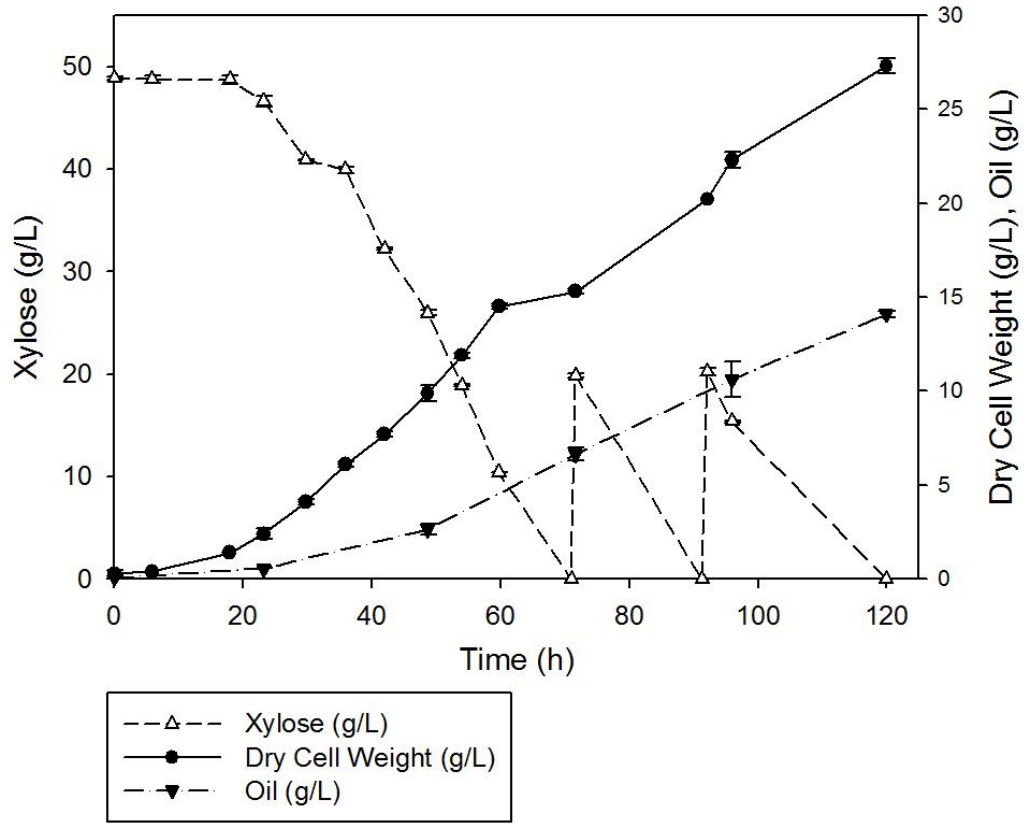
**Figure 3.2 3D surface plot of dry cell weight as a function of C:N ratio and temperature.**



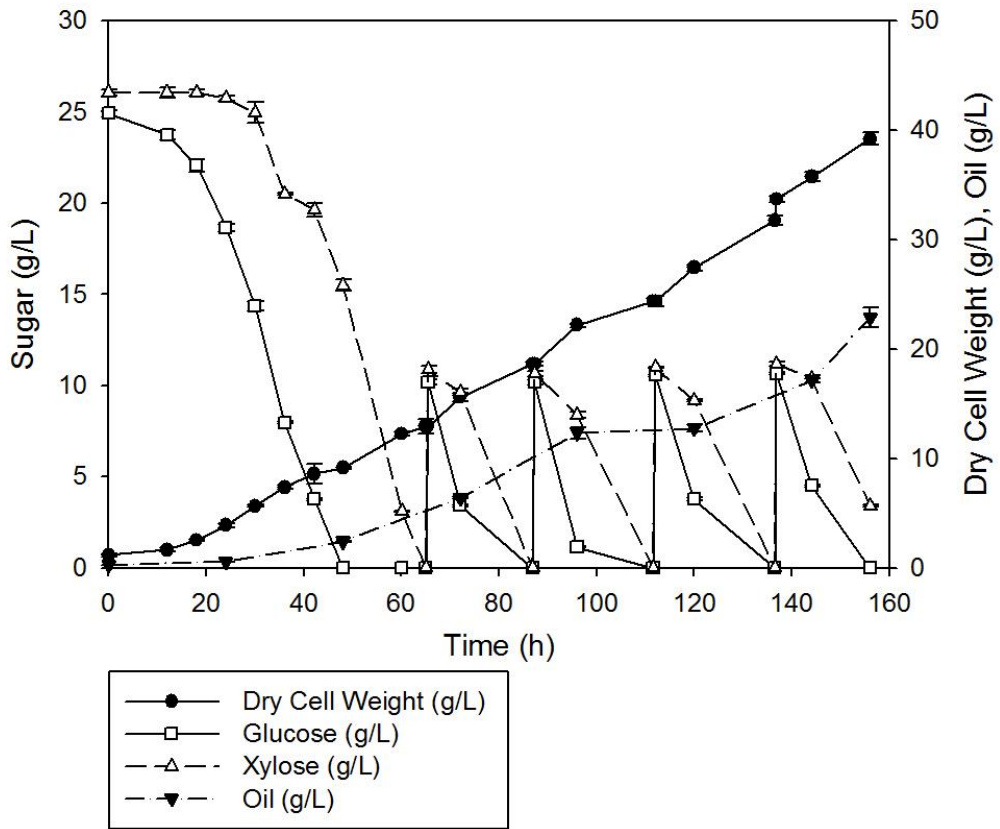
**Figure 3.3 3D surface plot of oil content as a function of C:N ratio and temperature.**



**Figure 3.4 Glucose fed-batch fermentation.**

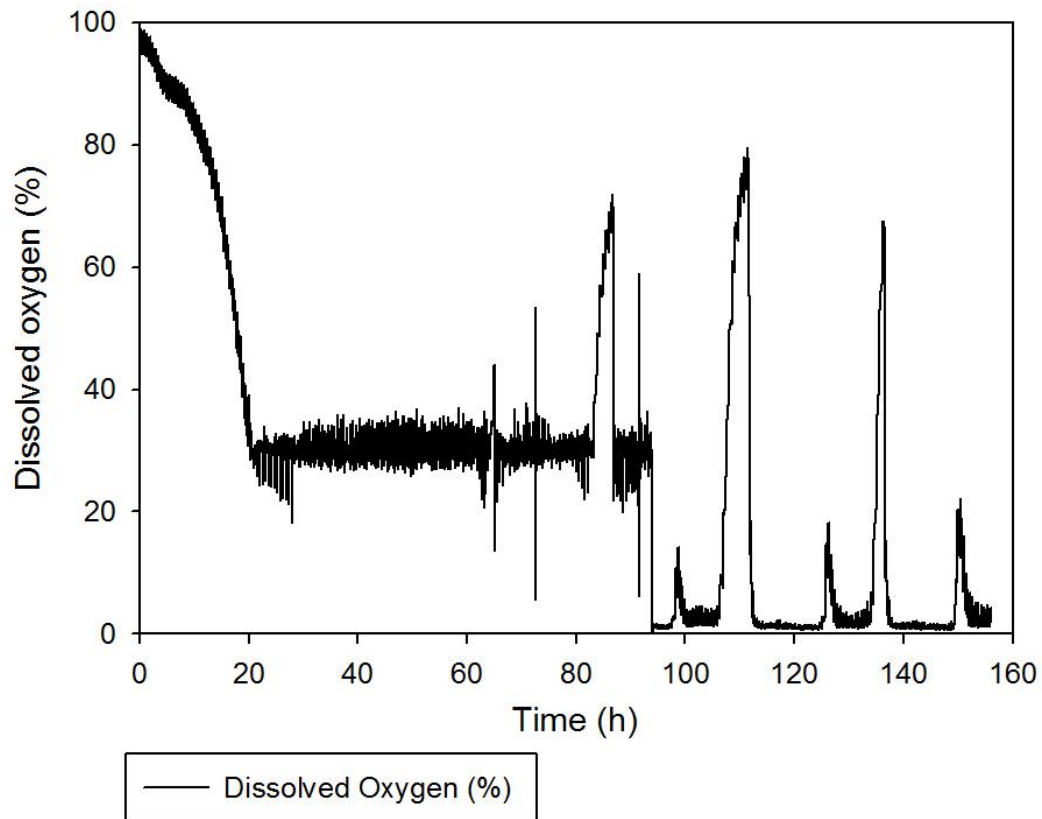


**Figure 3.5 Xylose fed-batch fermentation.**

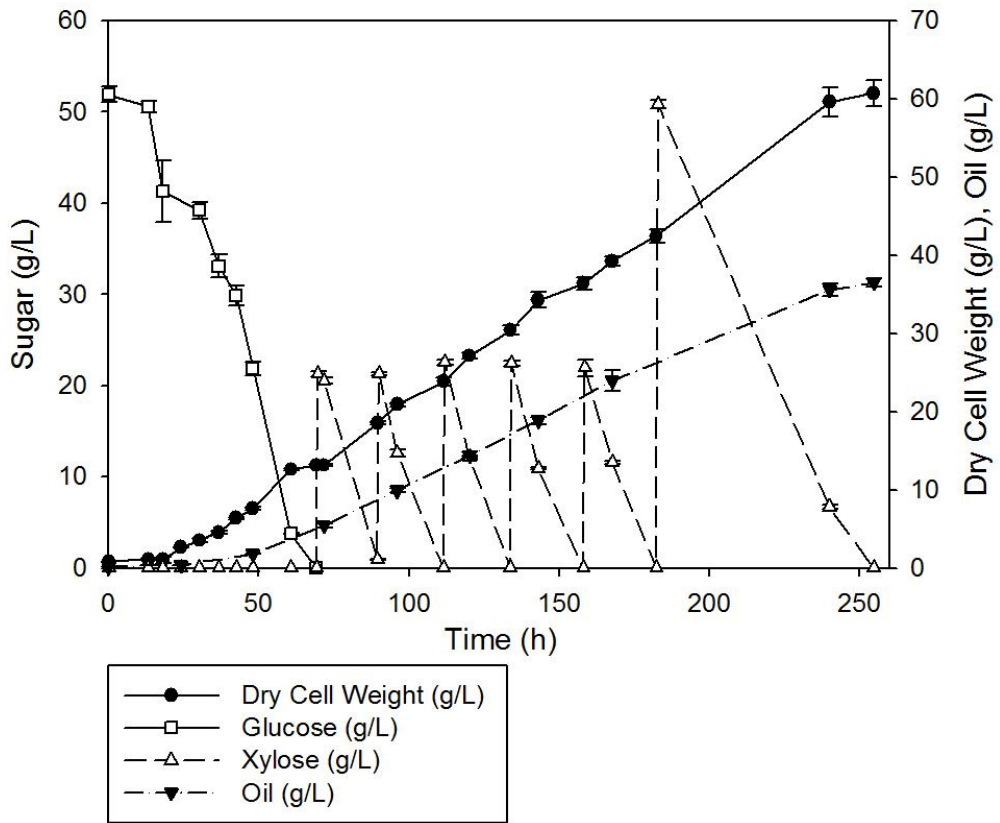


**Figure 3.6 Glucose and xylose (Glu:Xyl, 1:1 ratio) fed-batch fermentation.**

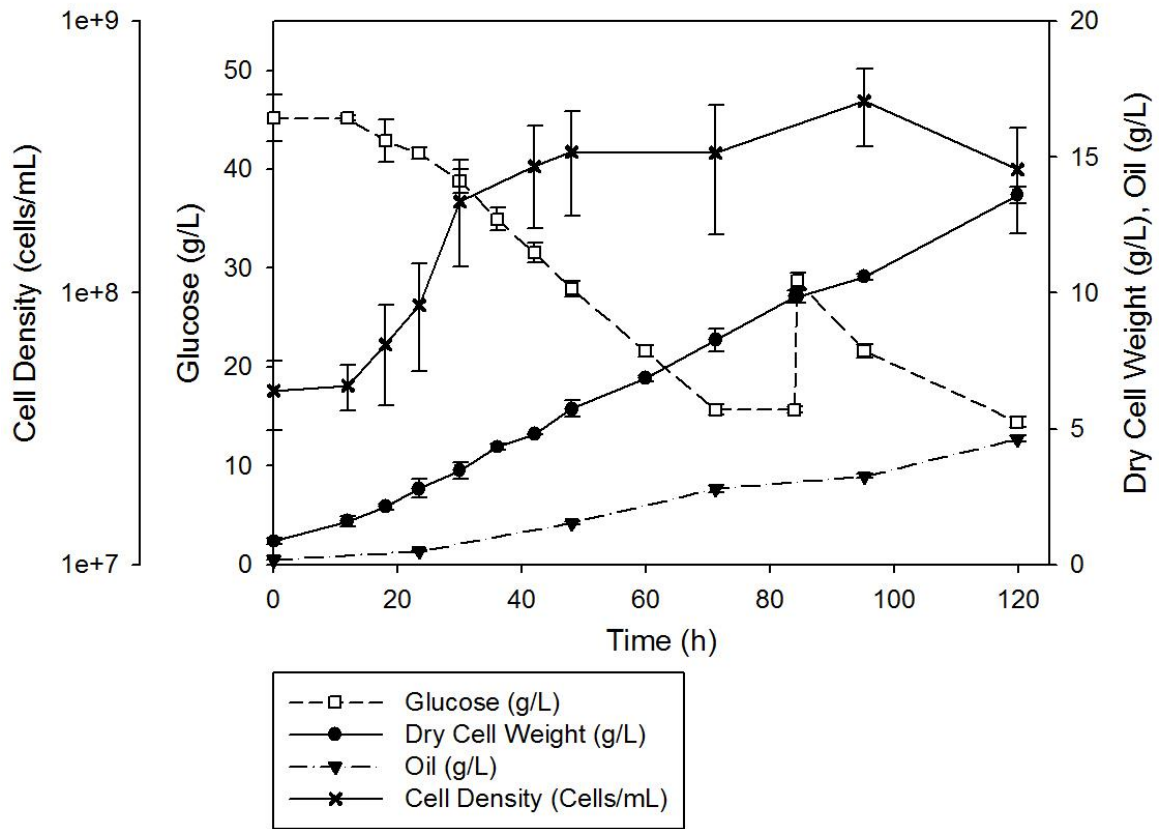




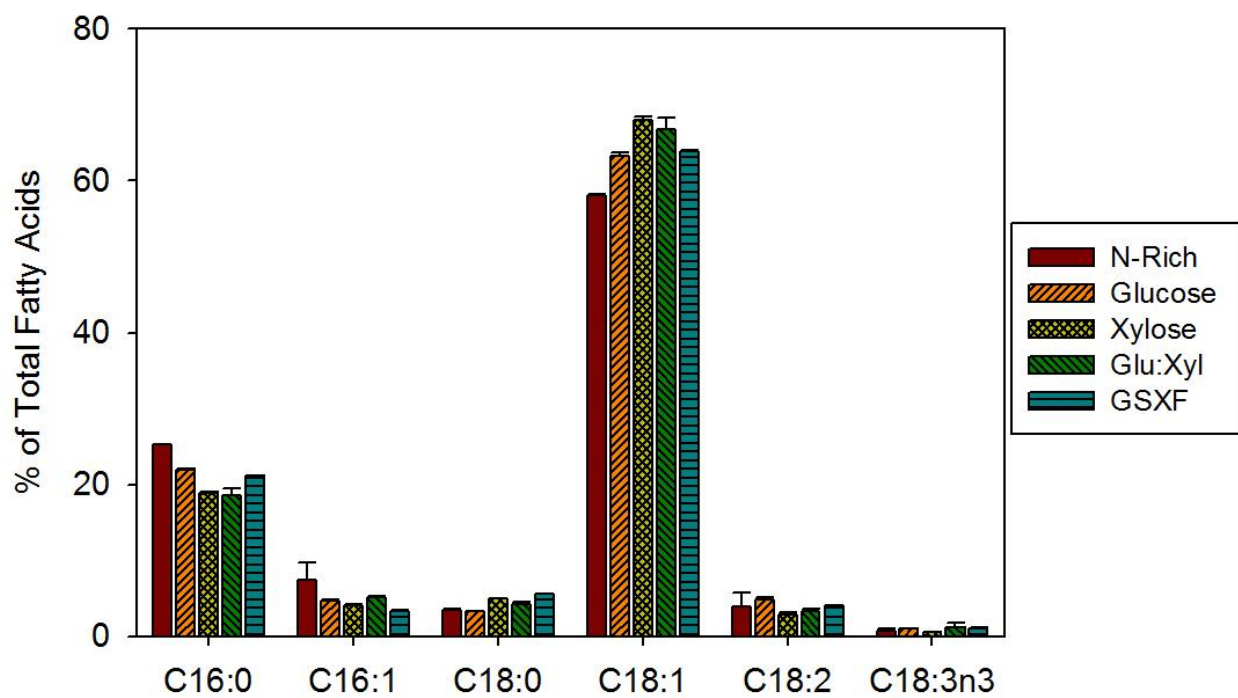
**Figure 3.7 Dissolved oxygen content for the mixed sugar (Glu:Xyl, 1:1 ratio) fed-batch fermentation. Dissolved oxygen was maintained at 30% for 96 h. Large peaks are representative of complete utilization of both glucose and xylose while the smaller peaks indicate complete glucose utilization.**



**Figure 3.8 Glucose start:xylose feed (GSXF) fed-batch fermentation.**



**Figure 3.9 Nitrogen rich (N-rich) fed-batch fermentation.**



**Figure 3.10 Comparison of fatty acid profiles across fed-batch fermentations (all compared at 120 h).**

## Chapter 4 - Lipid profiling of *Lipomyces starkeyi*

### Abstract

Lipid species were analyzed from *Lipomyces starkeyi* ATCC 56304 grown under nitrogen-limited and nitrogen-sufficient fermentation conditions. The major lipid produced was triacylglycerol (TAG), accounting for 65.8 to 84.0% of the total lipid content of the cell. Fermentations using xylose as the major carbon source during lipid accumulation generated the highest amounts of TAG, 424 mg/g DCW were achieved for the GSXF fed-batch fermentation. Under nitrogen-limited conditions, TAG accumulation became apparent from 24 to 72 h increasing almost 7-fold as cells transitioned from growth to lipid accumulation. Lipid bodies were observed during later stages of fermentation (96 h) confirming lipid accumulation. The increased presence of free fatty acids and phosphatidic acid early on (24 h) indicates that fatty acid turnover and phospholipid synthesis is prominent during growth. Palmitic acid (C16:0) and oleic acid (C18:1) were the major constituents of TAG and phospholipid species (mainly phosphatidylcholine and phosphatidylethanolamine) demonstrating the interconnected nature of these major lipid synthesis pathways.

### Introduction

*Lipomyces starkeyi* is an oleaginous ascomyceteous yeast of the class Saccharomycetes first isolated from soil (Starkey, 1946). It is a widely studied yeast due to its ability to accumulate oil or lipid (up to 60%, dry mass basis) and produce dextranases (Millson & Evans, 2007; Koenig & Day, 1988; Lodder & Kreger-van Rij, 1952). *L. starkeyi* can utilize a variety of low-valued nutrient sources including: lignocellulosic biomass, olive oil waste, sludge, and spent yeast (Yang et al., 2014; Gong et al., 2012; Yu et al., 2011; Yousuf et al., 2010; Angerbauer et al., 2008). Additionally, its genome has recently been sequenced, which can be useful for identifying and/or developing improved industrial strains (Grigoriev et al., 2012).

The biosynthesis of storage lipids in *L. starkeyi* and other oleaginous yeast occurs as a result of nutrient starvation. Nitrogen typically serves as the limiting nutrient and upon exhaustion from the growth medium carbon is diverted to triacylglycerol (TAG) accumulation. The biochemical processes involved with TAG biosynthesis in oleaginous yeast has been previously studied by Ratledge and Wynn (2002) and described elsewhere in Chapter 1. In brief, the onset of

nitrogen limitation activates adenosine monophosphate (AMP) deaminase, a nitrogen scavenging enzyme which deaminates AMP to free ammonia as utilizable nitrogen. The reduced cellular AMP content results in the build-up of citrate from isocitrate dehydrogenase inhibition. The accumulated citrate is converted to acetyl-CoA by ATP citrate lyase, an essential enzyme for TAG accumulation (Boulton & Ratledge, 1981). Acetyl-CoA carboxylase directs acetyl-CoA towards fatty acid synthesis which is catalyzed by the fatty acid synthase complex (FAS). The fatty acyl-CoA (FAC) units produced from FAS can be further elongated and/or desaturated (Tehlivets et al., 2007). After modification, FACs are transferred onto glycerol-3-phosphate through the sequential action of acyltransferase enzymes: glycerol-3-phosphate acyltransferase, lysophosphatic acid acyltransferase, phosphatidate phosphatase, and diacylglycerol acyltransferase to form TAG. Phosphatidic acid (PA), a key intermediate in TAG biosynthesis also serves as the starting point for phospholipid production; the pathway chosen is dependent on the demands of the cell (Carman & Han, 2011). TAG is stored in lipid bodies (LBs), organelles that serve as energy reserves for the cell and phospholipids are directed to membrane formation and maintenance (Sorger & Daum, 2003; Homann et al., 1987).

The aim of this study was to determine and compare the neutral and polar lipid profiles of *L. starkeyi* ATCC 56304 produced from different fed-batch fermentations.

## **Materials and methods**

### ***Microorganism and chemicals***

*Lipomyces starkeyi* (ATCC 56304) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and kept on yeast mold (YM) agar (10 g/L glucose, 5 g/L peptone, 3 g/L maltose, 3 g/L yeast extract; pH of 5.0) at 4°C. Re-cultivation was performed monthly to maintain fresh culture. All chemicals and media were obtained from certified suppliers and were of analytical reagent grade (Fisher Scientific, Pittsburg, PA, USA).

### ***Media preparation***

Fed-batch fermentation media preparation has been described elsewhere in Chapter 3. Briefly, minimal media (MM) consisted of: 50 g/L sugar (glucose or xylose) and nitrogen/trace minerals (0.5 g/L urea, 0.5 g/L yeast extract, 0.5 g/L KH<sub>2</sub>PO<sub>4</sub>, 0.5 g/L Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 0.1 g/L CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.1 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 g/L ZnSO<sub>4</sub>·7H<sub>2</sub>O, and 0.005 g/L FeSO<sub>4</sub>·7H<sub>2</sub>O. MM was

prepared to a C:N ratio of 80 by sterilizing sugar solution (50 g/L sugar) separately inside bioreactor and pumping in concentrated solution of filter sterilized (0.22  $\mu\text{m}$ ) nitrogen/trace mineral solution. The first feeding media had a C:N ratio of 100 consisted of pumping in sterilized solution of sugar (20 g/L) and urea (0.08 g/L) and subsequent feedings consisted of only sugar.

Nitrogen-rich media (N-rich) had a starting C:N ratio of 21 and composed of: 50 g/L of glucose, 5 g/L of peptone and 3 g/L of yeast extract. Pure glucose (20 g/L) was used as the feeding substrate.

### ***Fermentation conditions***

Fermentation details are described elsewhere in Chapter 3. To summarize, a 10% (v/v) inoculum grown in YM broth for 48 h at 200 rpm and 26°C was concentrated to  $\sim 5 \times 10^8$  cells/mL. Fed-batch fermentations were carried out in a 5-Liter working volume bioreactor (Bioflo 110, New Brunswick Scientific Inc., Einfield, CT, USA). Fermentation conditions were controlled: 300 rpm, 26.5°C, inlet air/O<sub>2</sub> flow of 1 vvm, dO<sub>2</sub> of 30% for the first 72 h, and a pH of 5.0.

### ***Analytical methods***

#### ***Lipid extraction and preparation***

Yeast cells were concentrated to  $\sim 5 \times 10^8$  cells/mL and lysed using a Beadbeater homogenizer (Mini-Beadbeater-24, BioSpec Products, Inc., Bartlesville, OK, USA). The cell lysate was extracted using a modified Bligh and Dyer method (Bligh & Dyer, 1959). The filtered and washed chloroform layer containing the lipids of interest was dried under nitrogen gas, vacuum dried for 30 min to remove any residual solvent and weighed; this was known as the total lipid extract. Total lipid extract was re-dissolved in 1 mL of chloroform and stored at -80°C until further analysis. A total of 5 replicates were performed.

Dry cell weight (DCW) was determined gravimetrically. Cells were harvested via centrifugation (12000 rpm for 15 min), washed 3x using dH<sub>2</sub>O and dried at 80°C for 12 h. DCW was used to calculate the total lipid content of dry cells.

Total lipid extract was fractionated into neutral and polar lipids using cyanopropyl SPE columns (Alltech Extract-Clean, Grace Discovery Sciences, Deerfield, IL, USA). Neutral lipids were eluted using a solvent mixture of hexane:diethyl ether:acetic acid (80:20:1, v/v). Polar lipids containing phospholipids were eluted using a mixture of chloroform:methanol:dH<sub>2</sub>O (4:1:0.1, v/v).

Both fractions were dried down under nitrogen gas and weighed to determine neutral and polar lipid content.

### ***Lipid analysis***

Neutral lipids were further separated on a K6 silica thin-layer chromatography (TLC) plate using a solvent mixture of hexane:diethyl ether:acetic acid (70:30:1, v/v). The plate was briefly exposed to sublimated iodine to detect lipids (Figure 4.1). Bands that co-migrated with TAG and free fatty acids (FFAs) were scraped from the plate and eluted off the silica with chloroform:methanol (99:1, v/v), dried under nitrogen gas and dissolved in chloroform. Prior to scraping, a known amount of triheptadecanoin (TAG of C17:0) was added to the TAG and FFA bands as an internal standard.

Fatty acid composition of the neutral lipid, TAG and FFA fractions were determined by gas chromatography using a polyethylene glycol capillary column (Zebron ZB-Wax<sub>plus</sub> 30m x 0.25mm x 0.25µm, Phenomenex, Torrance, CA, USA). Lipids were transesterified to fatty acid methyl esters (FAMES) and analyzed using a gas chromatograph (GC-2014, Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a flame ionization detector (FID). An external standard mixture of FAME (Supelco® 37 component FAME mix, Sigma-Aldrich, St. Louis, MO, USA) was used to determine the relative retention time for each FAME species. Quantification was performed by normalizing peak area to fatty acid carbon number and calculated using the internal standard of either C15:0 or C17:0. Verification of FAME species was performed using a mass spectrometer (GCMS-QP2010 SE, Shimadzu Scientific Instruments, Columbia, MD, USA). The same GC conditions were used as mentioned above. MS conditions were as follows: detector voltage of 0.88 kV; scan mode with a 50-500 mass/charge (m/z) range and a scan speed of 1000 scans per second.

Phospholipids were analyzed using electrospray ionization (ESI) triple quadrupole mass spectrometry (API 4000, AB SCIEX, Framingham, MA, USA). The methodology performed in this study has been previously described in Lee et al. (2011). In summary, polar lipids were identified by the polar-fragment and mass to charge (m/z) ratio of the intact ion fragment. Quantification was performed using polar lipid internal standards.



## ***Microscopy***

Microscopic analysis was performed using a laser scanning confocal microscope with a 100x oil immersion lens (ZEISS LSM 5 Pascal, Carl Zeiss AG, Oberkochen, Germany). For visualization of lipid constituents inside the cell, a lipid intercalating dye (Invitrogen Dil C18, Life Technologies Corp., Carlsbad, CA, USA) was added to a suspension of yeast cells and incubated at room temperature for 30 min.

## **Results and discussion**

### ***Neutral lipids***

The neutral lipid content of each fed-batch fermentation, which is mostly composed of storage lipid TAG with lesser amounts of diacylglycerols, monoacylglycerols, sterols and FFAs, varied from 89.6 to 96.9% (dry mass basis) of the total lipid fraction (Table 4.1). This was expected because oleaginous yeast mainly accumulate TAG as the major storage lipid. TAG accumulation was influenced by the fed-batch fermentation conditions and compared well with the fermentation performance that was previously described in Chapter 3. The xylose, Glu:Xyl and GSXF fed-batch fermentations produced 392.3, 390.2, 424.5 mg TAG/g DCW, respectively; 1.5-fold more compared to the N-rich and glucose fed-batch fermentations (263.8, 274.7 mg/g DCW, respectively).

The fatty acid profile of the neutral lipid fraction is shown in Figure 4.2. The major fatty acid species are mostly composed of saturated and monounsaturated species: oleic acid (C18:1) and palmitic acid (C16:0) accounting for roughly 65 and 20% of the total fatty acid content, respectively, with minor amounts of palmitoleic acid (C16:1) and stearic acid (C18:0). Linoleic acid (C18:2) and linolenic acid (C18:3) were present in small amounts ( $\leq 5\%$ ); this is interesting to note because polyunsaturates are not produced by the common yeast *Saccharomyces cerevisiae* (Tehlivets et al., 2007; Sandager et al., 2002). Minor differences were observed in the fatty acid profile across different nutrient conditions. The most noticeable was the N-rich fed-batch fermentation which had a higher amount of palmitic acid and palmitoleic acid, and a lower amount of oleic acid. The difference in fatty acid profile is most likely related to the poor lipid accumulation under nitrogen-sufficient conditions.

The change in neutral lipid composition was observed over the course of the GSXF fed-batch fermentation. During exponential growth (24 h) the TAG content of the cell was fairly low

at 30.9% of the total lipid content (Table 4.2). As cells enter stationary phase and lipid accumulation begins, the TAG content increased 10-fold from 45.8 mg/g DCW at 24 h to 435.6 mg/g DCW at 120 h. Coincidentally, the oleic acid content of TAG increased confirming that it is the major fatty acid accumulated as TAG (Figure 4.3). Additionally, palmitic acid and stearic acid also increased suggesting they are incorporated as TAG albeit in smaller amounts. The fatty acid profile of the TAG fractions are shown in Figure 4.4 with, again, the major constituents being oleic acid and palmitic acid. Upon lipid accumulation (72 h), the oleic acid content increased from 52.2 to 65.5% of the total fatty acid profile while palmitic acid decreased from 27.4 to 21.9%. It is apparent that oleic acid is the major fatty acid synthesized and stored as TAG during lipid accumulation while palmitic acid is present in smaller amounts as shown by Figure 4.4. Suzuki et al. (1974) found that the most abundant TAG species in *L. starkeyi* were comprised of C16:0-C18:1-C18:1 and C16:0-C16:0-C18:1; this is in good agreement with the findings presented here.

Changes in the composition and amount of FFA were also observed over the course of the GSXF fermentation (Figure 4.5). During the early stage of growth, the FFA content of the cell was 38.6 mg/g DCW and dropped to 27.0 and 13.7 mg/g DCW at 72 and 120 h, respectively (Table 4.2). It is not readily apparent why the FFA content was high during the early stages of growth. One possibility is the increased lipid turnover as a result of membrane formation in actively replicating cells (Tehlivets et al., 2007). Palmitic acid and oleic acid were the major FFA species (Figure 4.3). Palmitic acid followed the same decreasing trend as the total FFA content but, interestingly, oleic acid actually increased from 11.7 to 13.6 mg/g DCW from 24 to 72 h, respectively. This observation could likely be caused by the increased synthesis of oleic acid as a result of lipid accumulation. Furthermore, stearic acid remained relatively constant across the course of fermentation. This could also be related to the increased accumulation of oleic acid as TAG because stearic acid is a key intermediate in oleic acid synthesis.

### ***Polar lipids***

The composition of phospholipids from each fed-batch fermentation include species of: phosphatidylcholine (PC), phosphatidylethanolamine (PE) phosphatidylinositol (PI), and phosphatidylserine (PS) with minor amounts phosphatidylglycerol (PG), lysoPE, and lysoPC (Table 4.1). The polar lipid content was a minor constituent varying from 3.1 to 11.1% of the total lipid content across all fermentations. The xylose fed-batch fermentation, the only fermentation

that did not utilize glucose, was about 2- to 2.8-fold less compared to the other fermentations. One possibility for the difference is the pathway(s) used for phospholipid biosynthesis may be different for xylose and glucose under nitrogen limitation. This could be a likely hypothesis because both carbon source and nutrient availability are known to control phospholipid synthesis (Carman & Han, 2011).

In yeast, PC, PE, PI, PS, and PG mainly compose the cell and organelle membranes. The proportion of each is often dependent on the growth conditions (nutrients, growth phase, temperature, etc.) (Carman & Han, 2011). From the results in Table 4.1, it is apparent that PE was not influenced by the different nutrient conditions because its composition remained constant around 25% of the polar lipid fraction. Another interesting observation was the low PI content of the glucose fed-batch fermentation and the high content of PI in the N-rich fed-batch fermentation. One hypothesis is that PI is affected by glucose availability and nitrogen limitation. Inositol, the precursor for PI, is formed from glucose-6-phosphate (G6P), an intermediate in glucose metabolism. Under nitrogen limitation, inositol synthesis could be inhibited due to limited G6P because glucose is being directed toward TAG synthesis. This would account for the increased PI under nitrogen-sufficient conditions; more glucose would be available to form inositol.

The major fatty acids present in the phospholipids are similar to the neutral lipid fraction consisting of palmitic acid and oleic acid which include species with 34:1, 34:2 and 36:2 acyl carbons:total acyl double bonds (Figure 4.6). There was also a considerable amount of the polyunsaturated linoleic acid (C18:2) and linolenic acid (18:3) mainly present in PC and PE which included: PC 34:3, 36:3, 36:4, 36:5 and PE 36:3. This would explain the presence of polyunsaturates in the neutral lipid fraction, as mentioned earlier, since PA is responsible for lipid turnover by shuttling fatty acids across phospholipid and TAG species.

It is apparent that as fermentation proceeds and lipids are accumulated, TAG is produced at the expense of phospholipids (Table 4.2). As the TAG content increases, the content of polar lipids remains constant at approximately 30 mg/g DCW. Over the course of fermentation, the most noticeable changes in phospholipid composition were the decrease in PA from 21.2% to 3.5% and the increase in PE from 3.3 to 25.3% of the total polar lipid content. PC and PI composition remained relatively constant at about 35 and 31% of the total polar lipid content, respectively. The increased presence of PA early on during fermentation (24 h) is most likely a result of increased phospholipid synthesis for membrane formation. PA serves as the major starting point for

phospholipid synthesis forming CDP-diacylglycerol, an activated intermediate that forms PI, PG and PS; PS can be further synthesized into PE and PC (Carman & Han, 2011).

The high oleic acid content of the oil produced by *L. starkeyi* is a promising find. High oleic oil is currently sought after in a variety of markets as an input for: biodiesel with improved oxidative stability and cold flow properties, high-performance/specialty lubricants, emollients for the cosmetic industry, polymers including polyurethane, and many others (Durrett et al., 2010; Corbett, 2003)

### ***Microscopic analysis***

Microscopic imaging was performed on yeast cells sampled over the course of the glucose fed-batch fermentation. Shown in Figure 4.7, lipids and LBs were stained red using a lipid intercalating dye to aid visualization. The initial stages of the fermentation ( $\leq 48$  h) showed lipids, most likely phospholipids, dispersed evenly throughout the yeast membrane. As the cells entered lipid accumulation, LB formation became apparent and during later stages of fermentation ( $>96$  h) the LBs became larger confirming TAG accumulation and storage. From the images, it was observed that during the later stages of growth, LBs were localized along the outer perimeter of the cell due to the formation of a large void within the cell (Figure 4.7, 72 h image). We speculate that this void is a vacuole, though, this has not been confirmed. Large vacuoles are often formed as a response to undesirable, stressful conditions such as nutrient starvation (Gasch, 2003). A previous study found that vacuole formation in oleaginous yeast occurs as a result of nitrogen limitation (Gill et al., 1977). The formation of vacuoles is not desirable, as less space is available for storage lipids. Additionally, vacuole formation may be indicative of cell growth shifting towards autophagy. Recent transcriptomic and proteomic analysis of oleaginous yeast have identified up-regulated genes and proteins associated with autophagy during lipid accumulation (i.e. nitrogen limitation) (Zhu et al., 2012; Liu et al., 2009). Autophagy is also associated with lipolysis or the breakdown of storage lipid into fatty acids and volatile aromas (Singh & Cuervo, 2012; Romero-Guido et al., 2011; Wang et al., 1996). The presence of volatile aromas were observed for *L.starkeyi* during later stages of fermentation under nitrogen limitation with the detection of a pleasantly sweet odor. We hypothesize that the presence of volatile aromas may be a result of lipolysis. While lipolysis degradation may negatively affect SCO production, volatile aromas such as  $\gamma$ -decalactone are highly valuable products for the food and cosmetic industries

(Braga & Belo, 2014). Further experimentation would be needed to confirm this hypothesis and investigate the possible relationship between vacuole formation, autophagy and lipolysis. This would be useful for identifying biotechnological schemes to improve oil production and/or produce valuable aromas.

## Conclusions

The results from this study show that TAG of oleic acid (C18:1) and palmitic acid (C16:0) is the major lipid produced from *L. starkeyi* accounting for roughly 80% of the total lipid content when using xylose as the major carbon source during lipid accumulation. The increased presence of FFAs and PA during early stages of fermentation indicate that lipid turnover is prevalent as cells are rapidly replicating and growing. As fermentation proceeds and cells enter stationary phase, oleic acid synthesis and TAG formation becomes apparent indicating carbon is being directed from growth and membrane synthesis to TAG production. Overall, the high oleic acid content of the SCO produced by *L. starkeyi* is a promising asset as a value-added input for high-value renewable products and oleochemicals.

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**Table 4.1 Lipid profiles of *L. starkeyi* grown under different nutrient sources using fed-batch fermentation<sup>1</sup>.**

Lipid (mg/g DCW)	N-rich	Glucose	Glu:Xyl	Xylose	GSXF
Total Lipid Content	340.2 ± 8.6	417.5 ± 15.5	464.4 ± 26.2	514.7 ± 5.7	531.6 ± 27.5
Neutral Lipid	304.7 ± 10.8 (89.6%)	371.3 ± 10.4 (88.9%)	430.3 ± 30.9 (92.6%)	498.7 ± 6.0 (96.9%)	503.0 ± 26.5 (94.6%)
TAG <sup>2</sup>	263.8 ± 30.1 (77.5%)	274.7 ± 9.8 (65.8%)	390.2 ± 26.2 (84.0%)	392.3 ± 20.1 (76.2%)	424.5 ± 38.5 (79.9%)
Polar Lipid	35.5 ± 3.0 (10.4%)	46.2 ± 7.6 (11.1%)	34.1 ± 8.3 (7.3%)	16.0 ± 2.0 (3.1%)	28.6 ± 1.8 (5.4%)
Phosphatidylcholine (PC)	6.3 ± 0.8 (17.8%)	26.4 ± 2.3 (57.3%)	8.2 ± 3.5 (23.9%)	7.6 ± 0.6 (47.6%)	10.0 ± 6.3 (34.9%)
Phosphatidyl-ethanolamine (PE)	8.2 ± 0.5 (23.0%)	11.2 ± 6.9 (24.2%)	8.9 ± 1.8 (25.8%)	3.7 ± 0.5 (23.4%)	7.2 ± 2.8 (25.3%)
Phosphatidylinositol (PI)	20.5 ± 1.9 (57.6%)	4.9 ± 0.5 (10.7%)	15.2 ± 2.4 (44.6%)	3.2 ± 1.8 (19.9%)	8.1 ± 2.5 (28.3%)
Phosphatidic acid (PA)	0.08 ± 0.03 (0.2%)	2.6 ± 1.8 (5.6%)	0.06 ± 0.03 (0.2%)	0.2 ± 0.1 (1.4%)	1.0 ± 0.9 (3.5%)
Phosphatidylserine (PS)	0.06 ± 0.03 (0.2%)	0.8 ± 0.3 (1.7%)	1.5 ± 0.7 (4.4%)	1.2 ± 0.4 (7.3%)	1.8 ± 1.1 (6.2%)
Phosphatidylglycerol (PG)	0.2 ± 0.05 (0.6%)	0.09 ± 0.01 (0.2%)	0.2 ± 0.01 (0.6%)	0.06 ± 0.02 (0.4%)	0.1 ± 0.1 (0.5%)
LysoPE	0.2 ± 0.06 (0.5%)	0.08 ± 0.04 (0.2%)	0.1 ± 0.08 (0.4%)	0.03 ± 0.01 (0.2%)	0.3 ± 0.4 (1.2%)
LysoPC	0.05 ± 0.02 (0.2%)	0.05 ± 0.01 (0.1%)	0.07 ± 0.01 (0.2%)	0.01 ± 0.002 (0.08%)	0.06 ± 0.04 (0.2%)

N=5, values in parenthesis for neutral lipid, TAG and polar lipid represent percentage of total lipid, for PC, PE, PI, PA, PS, PG, lysoPE, and lysoPC represent percentage of polar lipid.

<sup>1</sup>All compared at 120 hr.

<sup>2</sup>TAG was calculating from the total fatty acid content of the neutral lipid fraction (Appendix B).

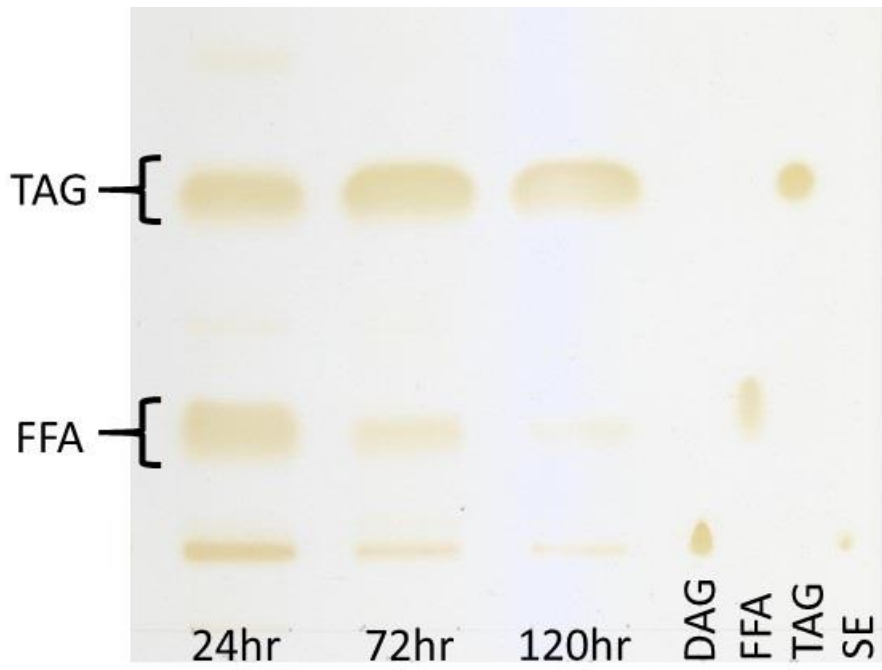


**Table 4.2 Changes in TAG, FFA and phospholipid composition over the course of the nitrogen-limited GSXF fed-batch fermentation.**

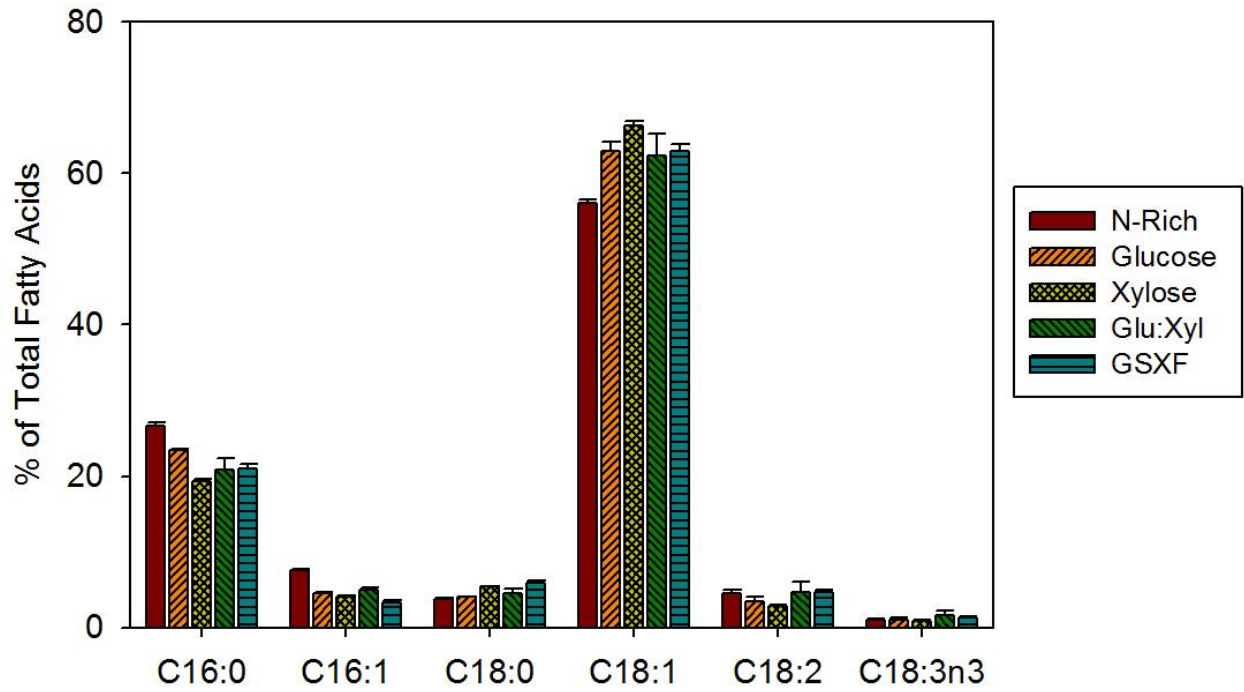
Lipid (mg/g DCW)	24 h	72 h	120 h
Total Lipid	148.4 ± 11.6	411.3 ± 39.9	531.6 ± 27.5
Neutral Lipid	127.6 ± 12.5 (86.0%)	373.2 ± 40.0 (90.7%)	503.0 ± 26.5 (94.6%)
TAG <sup>1</sup>	45.8 ± 6.5 (30.9%)	318.8 ± 18.3 (77.5%)	435.6 ± 46.4 (82.9%)
FFA <sup>1</sup>	38.6 ± 1.7 (26.0%)	27.0 ± 1.3 (6.6%)	13.7 ± 2.8 (2.6%)
Polar Lipid	20.8 ± 2.2 (14.0%)	38.1 ± 3.3 (9.2%)	28.6 ± 1.8 (5.4%)
Phosphatidylcholine (PC)	7.0 ± 1.6 (33.7%)	14.0 ± 8.1 (36.7%)	10.0 ± 6.3 (34.9%)
Phosphatidyl-ethanolamine (PE)	0.7 ± 0.1 (3.3%)	5.4 ± 2.4 (14.2%)	7.2 ± 2.8 (25.3%)
Phosphatidylinositol (PI)	7.7 ± 2.7 (37.1%)	11.1 ± 4.7 (29.3%)	8.1 ± 2.5 (28.3%)
Phosphatidic acid (PA)	4.4 ± 0.7 (21.2%)	3.1 ± 2.1 (8.3%)	1.0 ± 0.9 (3.5%)
Phosphatidylserine (PS)	0.8 ± 0.2 (3.7%)	3.9 ± 1.5 (10.2%)	1.8 ± 1.1 (6.2%)
Phosphatidylglycerol (PG)	0.2 ± 0.04 (0.8%)	0.2 ± 0.1 (0.5%)	0.1 ± 0.1 (0.5%)
LysoPE	0.03 ± 0.003 (0.1%)	0.3 ± 0.2 (0.8%)	0.3 ± 0.4 (1.2%)
LysoPC	0.01 ± 0.002 (0.1%)	0.07 ± 0.05 (0.2%)	0.06 ± 0.04 (0.2%)

<sup>1</sup>TAG and FFA were separated using TLC prior to quantification.

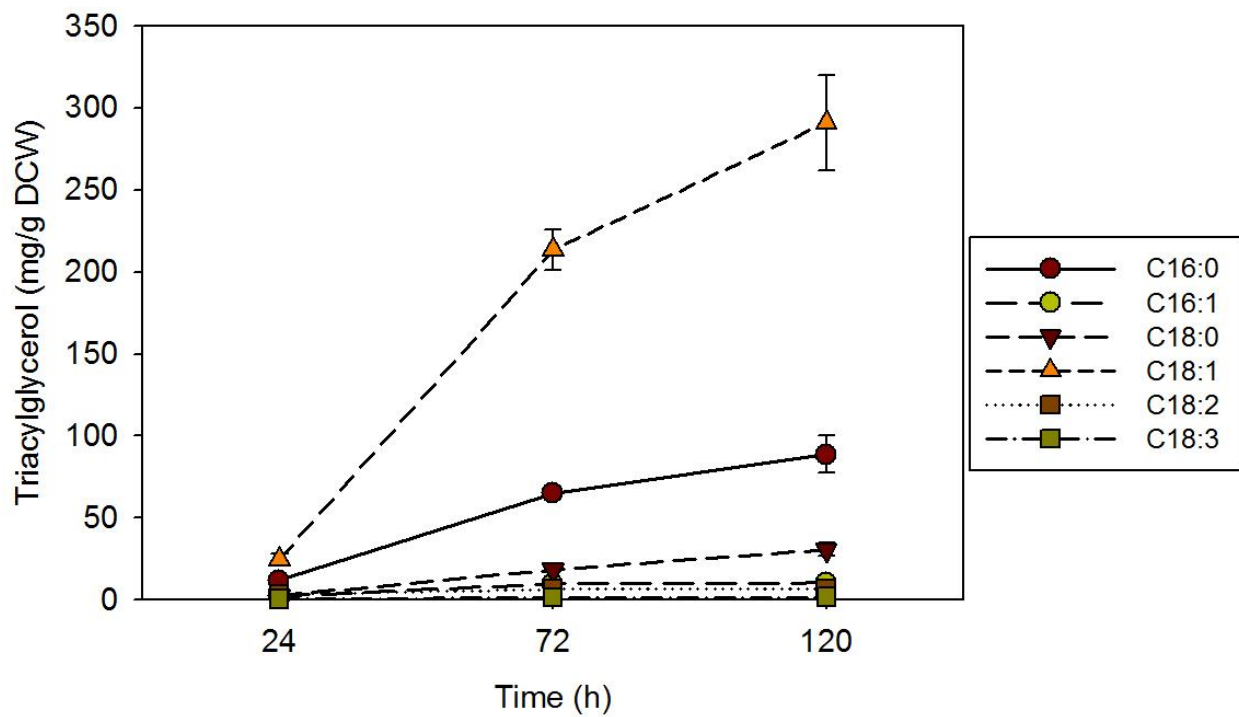
N=5 (except for TAG and FFA), values in parenthesis for neutral lipid, TAG, FFA and polar lipid represent percentage of total lipid. Values in parenthesis for PC, PE, PI, PA, PS, PG, lysoPE, and lysoPC represent percentage of polar lipid.



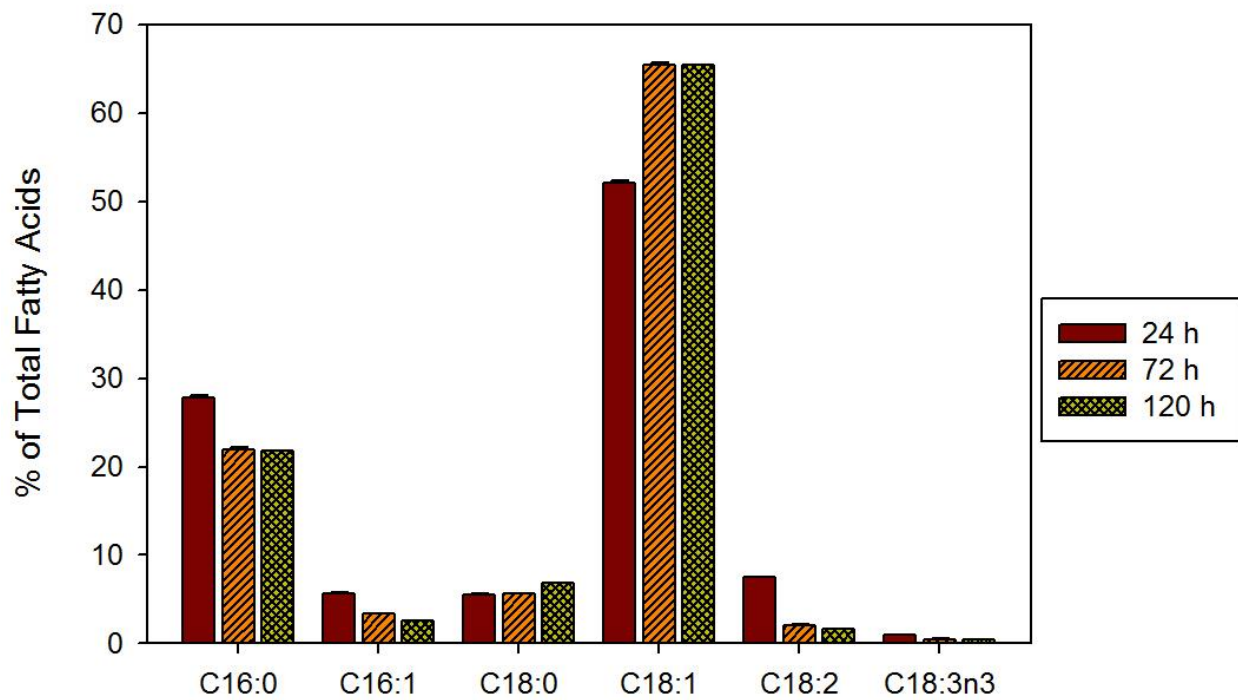
**Figure 4.1 Neutral lipid separation using thin layer chromatography (TLC).**



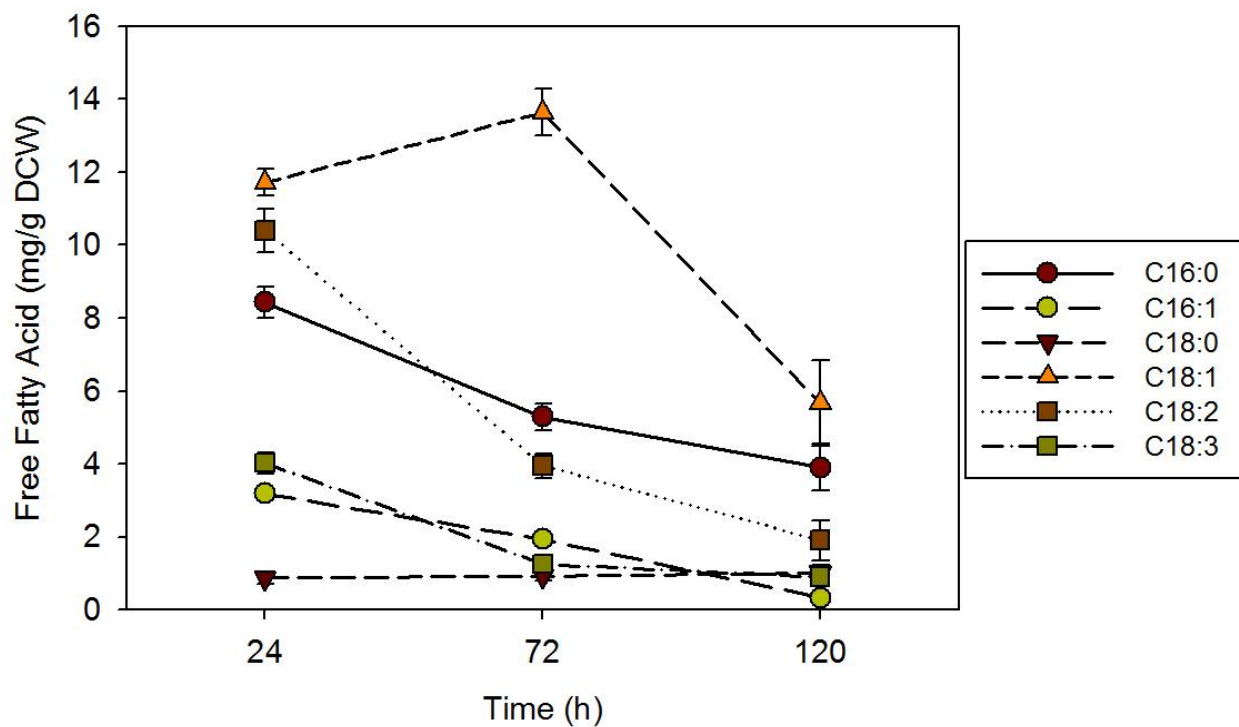
**Figure 4.2 Fatty acid profile of neutral lipid fractions from different fed-batch fermentations (compared at 120 h).**



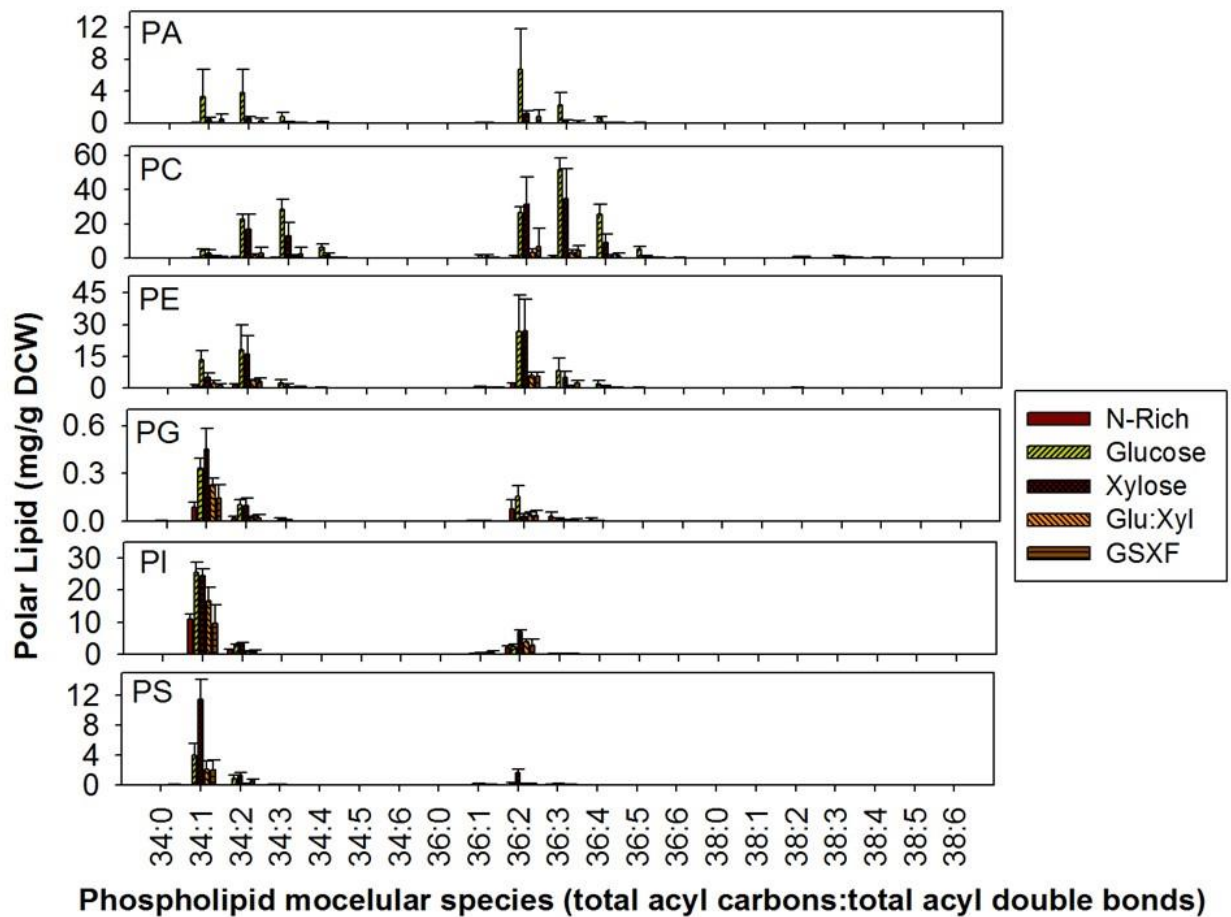
**Figure 4.3** Change in fatty acid content of triacylglycerol (TAG) over the course of the nitrogen-limited GSXF fed-batch fermentation.



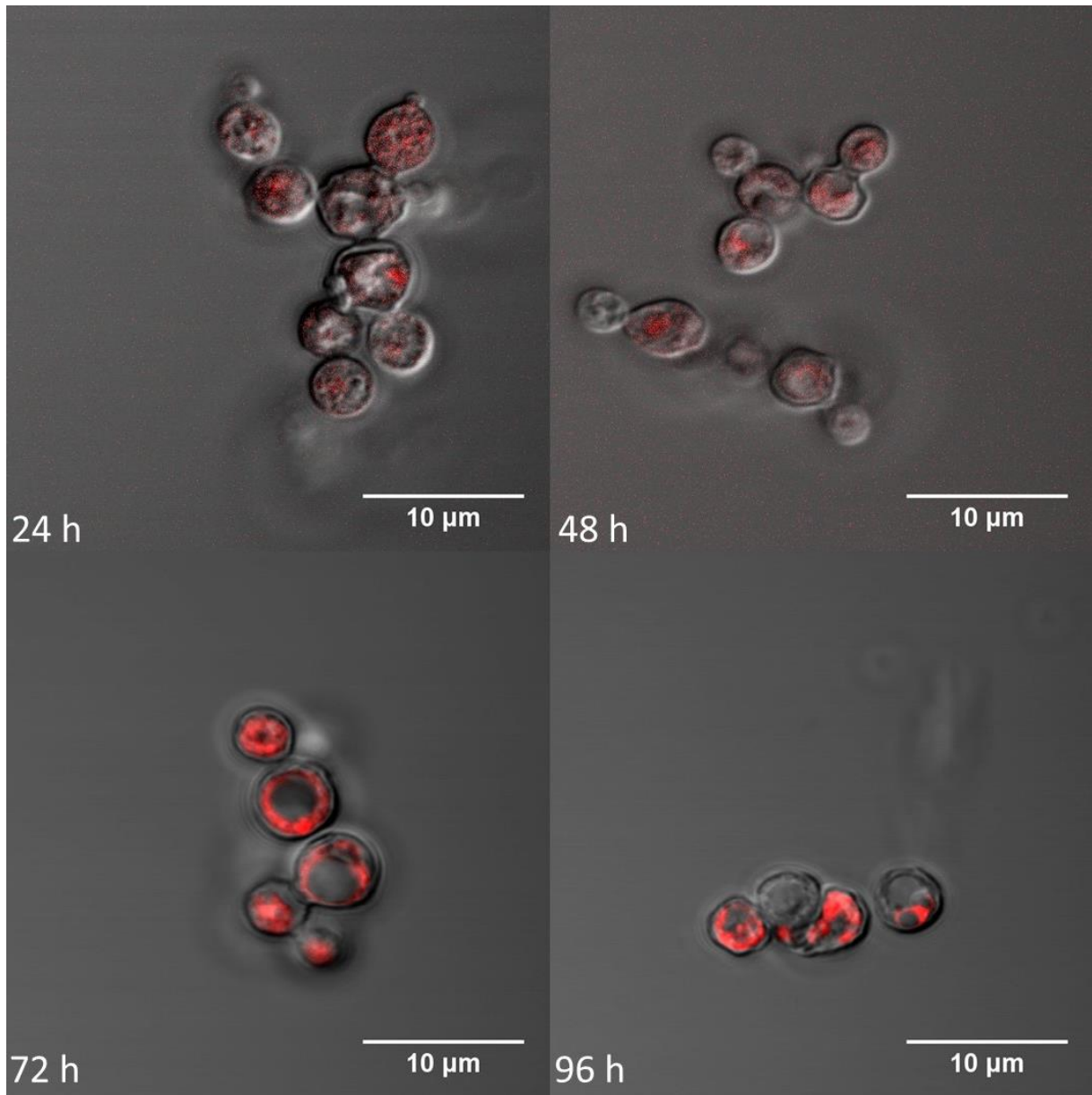
**Figure 4.4 Fatty acid profile of TAG fractions over the course of the nitrogen-limited GSXF fed-batch fermentation.**



**Figure 4.5** Change in free fatty acids (FFAs) over the course of the nitrogen-limited GSXF fed-batch fermentation.



**Figure 4.6** Phospholipid molecular species of *L.starkeyi* grown under different nutrient sources using fed-batch fermentation. PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; and PS, phosphatidylserine.



**Figure 4.7** Microscope images (100x magnification) of *L. starkeyi* showing different stages of lipid accumulation. Lipids are shown in red. Notice the appearance of lipid bodies at 72 and 96 h.



## **Chapter 5 - Single cell oil production from hemicellulose-rich milling by-products using *Lipomyces starkeyi***

### **Abstract**

Single cell oil (SCO) is a valuable noncrop-based renewable oil source. Hemicellulose derived sugars including D-xylose and L-arabinose can be utilized to produce SCO using the oleaginous yeast *Lipomyces starkeyi* ATCC 56304. Bran by-products from the corn and wheat milling industries were tested as hemicellulose-rich feedstocks for the production of SCO. Whole and de-starched corn bran and wheat bran hydrolysates were produced using dilute sulfuric acid (0, 0.5, 1.0%, v/v) pretreatment along with enzymatic hydrolysis. Acid pretreatment noticeably improved hemicellulose and cellulose hydrolysis generating, on average, a 2- to 3-fold higher sugar yield compared to hydrothermal (0% acid) pretreatment. Formation of 5-hydroxymethylfurfural (HMF), furfural and acetic acid was apparent in the acid pretreated hydrolysates, dilution was performed to lessen the inhibitory effects during fermentation. Whole bran hydrolysates outperformed the de-starched bran for SCO production. The presence of fermentable sugars and limited inhibitor formation from hydrothermal and enzyme treated hydrolysates generated the highest average oil yields of 126.7 and 124.3 mg oil/g sugar for both wheat bran and corn bran, respectively. 1.0% acid pretreatment was effective for the de-starched bran generating a hemicellulose hydrolysis efficiency of 94 and 84% for wheat bran and corn bran, respectively, resulting in the highest oil yield of 70.7 mg oil/g sugar. This study was able to demonstrate that sugars produced from the pretreatment of corn bran and wheat bran could be used as viable feedstocks for the production of SCO.

### **Introduction**

Single cell oil (SCO) from oleaginous yeast can serve as a noncrop-based renewable replacement for petroleum-based fuels, chemicals, and power. Currently, oilseed crops serve as the major renewable oil source but, as the world population increases, edible oil sources will be needed to feed the growing population. Utilization of low-valued inputs such as inedible plant-based biomass will be necessary for the commercial production of SCO. Plant-based biomass is a low-valued, readily available feedstock source available at over 1 billion dry tons per year in the US (Perlack et al., 2005). Biomass-derived sugars can be readily fermented by oleaginous yeast.

Some examples include: corn cobs (Gao et al., 2014; Chang et al., 2013; Huang et al., 2012a), corn residue (Galafassi et al., 2012; Liu et al., 2012; Hu et al., 2011), rapeseed meal (Kiran et al., 2012), rice straw (Huang et al., 2009), sorghum bagasse (Liang et al., 2012a; Liang et al., 2012b), sugarcane bagasse (Huang et al., 2012b; Tsigie, 2011), and wheat straw (Yu et al., 2011). The ability of oleaginous yeast to utilize biomass-derived sugars including D-xylose and L-arabinose is a major advantage. Efficient use of hemicellulose remains a challenge for the development of cellulosic ethanol because the ethanol-producing yeast *Saccharomyces cerevisiae* is unable to utilize pentose sugars. Since hemicellulose comprises 10-30% (dry mass basis) of plant-derived biomass; using oleaginous yeast as a value-added bioconversion option could provide additional revenue streams for future biorefineries (Saha, 2003).

Hemicellulose-rich biomass from the wheat flour and corn milling industries is readily available in the US Midwest and may serve as viable feedstock for SCO production. Wheat bran is a by-product of the flour milling industry. It represents 25-30% of total flour production with approximately  $7 \times 10^6$  dry tons produced annually in the US (Horner & Sexten, 2014; Blasi et al., 1998). In principle, flour milling is performed to separate the valuable endosperm portion from the germ and bran. The bran, covering the outside of the wheat kernel, is fractionated after the break and reduction steps using aspiration and/or sieving, often into different by-product streams. These bran-rich streams vary across milling operations but are commonly referred to as wheat mill run or wheat middlings and sold as animal feed for \$130-170 per ton (Blasi et al., 1998).

Corn bran and corn fiber are by-products of the corn milling industry produced at  $3 \times 10^6$  dry tons per year (Rose et al., 2010). Both corn bran and corn fiber have similar compositions accounting for 7-10% (dry mass basis) of the corn kernel; the major difference between each is the milling process used (Rose et al., 2010). Corn bran is produced from corn dry-milling, a by-product after the dehulling step. Corn fiber is produced from corn wet-milling, often combined with steeping solids to produce corn gluten feed. It should be noted that most corn bran/fiber is sold in the animal feed market but food-grade corn bran is becoming a prevalent dietary fiber supplement in many processed foods.

Corn bran and wheat bran constitute the outer pericarp and aleurone of the grain kernel and are composed of a significant amount of cellulose (15-20%, dry mass basis) and hemicellulose (30-35%, dry mass basis) (Saha, 2003; Saulnier & Thibault, 1999)). Hemicellulose is a complex polysaccharide that can vary widely among plant sources; bran from grains such as corn and wheat

are mostly composed of arabinoxylans and  $\beta$ -glucans (Ebringerova, 2006). Arabinoxylan is a highly water insoluble polysaccharide consisting of a xylan backbone of  $\beta$ -(1,4)-linked D-xylose units substituted with side chains of xylose, arabinose, and galactose (Ebringerova, 2006; Saha, 2003).  $\beta$ -glucans are water soluble polymers comprising of  $\beta$ -(1,4) and  $\beta$ -(1,3)-linked D-glucose units (Li et al., 2006). Comparatively, corn bran can be challenging to hydrolyze as it is nearly water insoluble while wheat bran, with a greater amount of water-soluble  $\beta$ -glucans, often has higher solubility. Regardless, each is composed of a significant amount of insoluble fiber and a pretreatment step is often needed for successful sugar hydrolysis.

Two common processes used for biomass deconstruction are acid and alkali pretreatment. Alkali pretreatment is commonly used for targeting the cellulose portion of biomass feedstocks (Rigdon et al., 2013; Guragain et al., 2013). In principle, alkali solubilizes the hemicellulose and lignin portions, exposing the cellulose which can be further hydrolyzed by cellulases (Kumar et al., 2009). Acid is an effective method for hydrolyzing the hemicellulose fraction into fermentable sugars but it can also lead to sugar degradation (Saha, 2003). To combat sugar degradation, use of dilute acid at lower temperatures combined with enzymatic hydrolysis can achieve high conversion yields. Dilute sulfuric acid has been successfully used to hydrolyze wheat and corn bran (Agger et al., 2011; Palmarola-Adrados et al., 2005; Choteborska et al., 2004; Dien et al., 1999; Saha & Bothast, 1999; Grohmann & Bothast, 1997; Osborn & Chen, 1984). The highest reported hydrolysis efficiencies with limited inhibitor formation were produced using dilute sulfuric acid (0.5-1%, v/v) at moderate temperatures (120-130°C) with enzymatic hydrolysis (Choteborska et al., 2004; Saha & Bothast, 1999).

The aim of this work is to test the use of hemicellulose-rich corn bran and wheat bran as feedstocks for the production of SCO using the oleaginous yeast *Lipomyces starkeyi* ATCC 56304. To our knowledge, no work has been done using milling by-products to produce SCO using oleaginous yeast. Previous work showed that *L. starkeyi* ATCC 56304 produced significant quantities of SCO using xylose (Chapter 3 and 4); thus, demonstrating the ability to use biomass-derived sugars as a low-cost feedstock will be important for commercialization.

## Materials and methods

### *Microorganisms, media and chemicals*

Lyophilized culture of *Lipomyces starkeyi* (ATCC 56304) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and propagated in yeast mold (YM) broth (Difco Laboratories, Detroit, MI, USA): 10 g/L glucose, 5 g/L peptone, 3 g/L maltose, 3 g/L yeast extract at 200 rpm at 25°C for 8 days. Cultures were preserved on YM agar plates at 3°C and re-cultivated monthly to maintain fresh culture. All chemicals were obtained from certified suppliers and were of analytical reagent grade (Fisher Scientific, Pittsburg, PA, USA).

### *Hemicellulose-rich bran feedstocks*

Wheat bran and corn bran (shown in Figure 5.1) were generously provided by Phibro Animal Health Corporation, Manhattan, KS, USA and by LifeLine Foods, St. Joseph, MO, USA, respectively. Each were milled (Fitz-Mill, Fitzpatrick Company, Elmhurst, IL, USA) to pass through a 2.36 mm screen, dried in a forced-air oven at 40°C for 72 h and stored at room temperature. Geometric mean diameter (ASABE S319.4) was determined by sieving through a Ro-Tap shaker (Model RX-29, W.S. Tyler Inc., Mentor, OH, USA).

For preparation of de-starched bran, commercial-grade alpha-amylase (Liquozyme®, Novozymes Inc. Franklinton, NC, USA) and glucoamylase (GC480, Genencor International Inc. Palo Alto, CA, USA) were generously provided by a dry-grind corn ethanol facility (MGP Ingredients, Atchison, KS, USA) and used for liquefaction and saccharification. Bran was slurried in deionized water (dH<sub>2</sub>O) at a 15% (dry w/v) solid loading and the pH was adjusted to 5.5 via hydrochloric acid (3M). Liquefaction was performed using alpha-amylase (1 µL/g starch) at 80°C for 1 h; saccharification was performed using glucoamylase (5 µL/g starch) at 65°C for 2 h. De-starched bran was washed with dH<sub>2</sub>O and dried at 40°C for 72 h.

### *Acid pretreatment and enzymatic hydrolysis*

Bran was slurried in dH<sub>2</sub>O with the required amount of sulfuric acid (0-1.0%, v/v) at a 15% (dry w/v) solid loading in shake-flasks. Pretreatment was performed at 121°C for 1 h. After pretreatment, the pH was neutralized and adjusted to 5.5 using sodium hydroxide (10M) prior to addition of commercial-grade cellulolytic (Cellic® CTec2, Novozymes) and hemicellulolytic (Cellic® HTec2, Novozymes) enzymes. A 3% (w/w of dry biomass) enzyme dosage was used

with a 9:1 ratio of CTec2:HTec2. Enzymatic hydrolysis was performed at 55°C at 150 rpm for 48 h. After hydrolysis, solids were removed via centrifugation at 5000 rpm (2300 x g) for 20 min (Sorvall Super T21, Thermo Fisher Scientific Inc., Waltham, MA, USA) and filter sterilized (0.22 µm) prior to being aseptically transferred to a pre-sterilized shake-flask for fermentation.

### ***Fermentation***

A 10% (v/v) inoculum, sub-cultured 2x in YM broth at 26°C and 200 rpm for 48 h was concentrated to  $\sim 5 \times 10^8$  cells/mL and used for all fermentations. Batch shake-flask fermentations were carried out in 250 mL Erlenmeyer shake-flasks using 10% (v/v) of hydrolysate to provide sufficient surface area for aeration. Flasks were incubated at 26°C and 200 rpm for 120 h unless otherwise noted.

### ***Analytical methods***

#### ***Composition analysis***

Composition analysis was conducted on corn bran and wheat bran including: proximate (moisture (AOAC Official Method 934.01), crude fat (acid hydrolysis, AOAC 954.02), crude protein (Kjedahl, AOAC 984.13 (A-D)), crude fiber (AOAC 978.10), and ash (AOAC 942.05)), acid detergent fiber (ADF, AOAC 973.18 (A-D)), neutral detergent fiber (NDF, AOAC 973.18 (A-D)), starch (AACC Approved Methods No. 76-13), acid detergent lignin (AOAC 973.18 (A-D)), and hydrolyzed sugar profile (glucose, xylose, arabinose, mannose, fucose, galactose, ribose). Representative samples were collected in triplicate and analyzed at an external laboratory (Agricultural Experimental Station Chemical Laboratories, University of Missouri, Columbia, MO, USA).

#### ***Dry cell weight determination***

Dry cell weight (DCW) was determined gravimetrically after washing cells 2x with dH<sub>2</sub>O and drying the wet cells at 80°C for 12 h.

#### ***HPLC analysis of sugars and inhibitors***

Sugars (D-glucose, D-xylose, L-arabinose, and alpha-maltose) and inhibitors (acetic acid, 5-hydroxymethylfurfural (HMF), and furfural) were identified and quantified using high performance liquid chromatography (HPLC, Prominence LC20AB, Shimadzu Scientific

Instruments, Columbia, MD, USA). Samples were diluted 10x with dH<sub>2</sub>O and filtered (0.22 μm membrane) before injection. Sugars were separated using an ion exclusion column (Rezex™ RCM-monosaccharide 300 x 7.8 mm, Phenomenex, Torrance, CA, USA) and quantified using a refractive index detector (RID) with an isocratic mobile phase of dH<sub>2</sub>O (Direct Q, Millipore Inc., USA) pumped at a rate of 0.6 mL/min at a column temperature of 80°C. Inhibitors were separated using an ion exclusion column (Rezex™ ROA-Organic Acid 150 x 7.8 mm, Phenomenex) with an isocratic mobile phase of 0.005N sulfuric acid pumped at a rate of 0.6 mL/min at a column temperature of 80°C and quantified with a photodiode array detector (PDA); acetic acid at 199 nm and HMF/furfural at 254 nm. LCsolution software (Version 1.25, Shimadzu Scientific Instruments) was used to identify and quantify each analyte from retention times and calibration curves generated from known standards of glucose, xylose, arabinose, maltose, acetic acid, HMF, and furfural.

#### ***Lipid extraction and fatty acid analysis***

The lipid extraction and fatty acid analysis procedures are described elsewhere in Chapter 3. In brief, yeast cells were concentrated to ~5x10<sup>8</sup> cells/mL and lysed using a Beadbeater homogenizer (Mini-Beadbeater-24, BioSpec Products, Inc., Bartlesville, OK, USA). The cell lysate was extracted using a modified Bligh and Dyer method (Bligh & Dyer, 1959). The filtered and washed chloroform layer containing the lipids of interest was dried under nitrogen gas, vacuum dried for 30 min to remove any residual solvent and weighed; this was known as the total lipid extract and used for calculating the oil content. The total lipid extract was re-dissolved in 1 mL of chloroform and stored at -80°C until further analysis.

Fatty acids were transesterified into fatty acid methyl esters (FAMES), separated on a capillary column (Zebron ZB-Wax<sub>plus</sub> 30m x 0.25mm x 0.25μm, Phenomenex, Torrance, CA, USA) and identified using a gas chromatograph equipped with a flame ionization detector (FID) (GC-2014, Shimadzu Scientific Instruments, Columbia, MD, USA).

#### ***Experimental design and statistical analysis***

Separate experiments were performed for whole bran and destarched bran to test the effect of bran source (corn bran and wheat bran) and sulfuric acid concentration (0, 0.5, 1.0%, v/v) on sugar hydrolysis, inhibitor generation and fermentation performance of SCO production. Design

Expert software (Design Expert 8.0.7.1, Stat-Ease Inc., Minneapolis, MN, USA) was used to generate factorial designs. Three replicates for each treatment was performed.

Statistical software SAS (SAS v9.1, SAS Institute, Cary, NC, USA) was used to perform PROC GLM to determine means and Tukey's Honestly Significant Difference test for comparison between treatments ( $\alpha=0.05$ ).

## **Results and discussion**

### ***Bran composition***

The composition of each bran feedstock is shown in Table 5.1. Both corn bran and wheat bran have three major types of polysaccharides present: starch, cellulose and hemicellulose which account for about 65% (w/w) of the bran. Corn bran had a considerable amount of crude fat (9.7%, w/w) indicating the presence of germ; oil pooling on the surface of hydrolysates was noticeable and became problematic during the filter sterilization step. Starch was evident in both feedstocks. When completely removed, the calculated hemicellulose content increased to 30.9 and 29.2% (w/w) for de-starched corn bran and wheat bran, respectively (Table 5.1). The low lignin content (<4%, w/w) of the bran sources is an added benefit preventing the need for harsh pretreatment conditions.

### ***Bran pretreatment and hydrolysis***

For a schematic overview of the pretreatment process, please refer to Figure 5.2. To summarize, both whole (scheme B) and de-starched (scheme A) wheat bran and corn bran were subjected to varying degrees of sulfuric acid pretreatment (0, 0.5, and 1.0%, v/v) followed by enzymatic hydrolysis using cellulases and hemicellulases.

Hydrothermal (0%) and acid (0.5, 1.0%) pretreatment with enzyme hydrolysis were able to hydrolyze the starch, hemicellulose, and cellulose fractions of the corn bran and wheat bran. From the results shown in Figure 5.3 and Figure 5.4, the use of acid pretreatment is critical for generating efficient sugar hydrolysis across both whole bran and de-starched bran sources.

### ***Whole bran***

Starch hydrolysis was evident in the whole bran and contributed to the presence of glucose in the hydrolysates, even under the hydrothermal pretreatment suggesting the hemicellulases

and/or cellulases have a certain degree of glucoamylase activity (Saha & Bothast, 1999). As reported by others, use of hydrothermal pretreatment and enzyme hydrolysis did not readily hydrolyze the hemicellulose portion indicated by the low xylose and arabinose yields (Figure 5.3). This is most likely due to the naturally high resistance of hemicellulose to enzymatic hydrolysis (Rose et al., 2010). Acid treatment was much more effective. 0.5% acid pretreatment significantly increased the combined xylose and arabinose content of corn bran and wheat bran from 2.6 to 14.0 g/100 g bran and 4.4 to 13.7 g/100 g bran, respectively. The residual starch was problematic generating significant amounts of HMF, an inhibitory compound formed from the degradation of glucose in the presence of heat and acid. During acid pretreatment up to 0.4 and 0.8 g/L of HMF was formed for wheat bran and corn bran, respectively (Figure 5.5).

### ***De-starched bran***

To reduce the formation of HMF and increase the release of pentose sugars from hemicellulose, the starch was removed from both corn bran and wheat bran using alpha-amylase and glucoamylase prior to pretreatment (Figure 5.2). Results from the de-starched bran sugar hydrolysis are shown in Figure 5.4 which, again, indicate the presence of acid is imperative for hemicellulose hydrolysis. As expected, increasing the acid content from 0% to 0.5% showed a greater release of combined xylose and arabinose from 2.8 to 27.6 g/100 g and 6.8 to 21.8 g/100 g for corn bran and wheat bran, respectively. The continuing trend of poor hydrolysis for corn bran using hydrothermal and enzyme hydrolysis is, again, most likely due to the resistance of corn bran to enzymatic breakdown (Agger et al., 2010; Rose et al., 2010; Saha, 2003). Corn bran arabinoxylans are highly cross-linked via di-ferulic acid bridges, which further resist attack from degrading enzymes (Agger et al., 2010). Addition of acid facilitates the breakdown of these crosslinks, exposing the xylan backbone for enzymatic attack and greater sugar hydrolysis. Starch removal substantially reduced the glucose content to roughly 3-fold compared to the whole bran hydrolysates. Furthermore, acid pretreatment also improved cellulose hydrolysis from the de-starched bran, generating 10.5 g/100 g and 16.3 g/100 g of glucose for wheat bran and corn bran, respectively.

Starch removal was able to minimize HMF formation but furfural, produced from pentose (xylose and arabinose) sugar degradation, increased substantially up to 165.3 and 219.3 mg/L for wheat bran and corn bran, respectively (Figure 5.5). Compared to the whole bran hydrolysates, the presence of acetic acid also became readily apparent in the de-starched bran hydrolysates,



increasing 2-fold from approximately 0.5 to 1.3 g/L and 1.5 to 3 g/L for wheat bran and corn bran, respectively. Acetic acid is naturally present in both wheat bran and corn bran arabinoxylans as esterified linkages to the xylan backbone (up to 3-5% in corn bran); this may account for its increased presence in the de-starched hydrolysates (Agger et al., 2010).

It should be noted that the content of arabinose in the acid treated wheat bran hydrolysates for both the whole and de-starched bran was higher than the theoretical value (Figure 5.3 and 5.4). This may be due to the different methods of analysis used for quantifying arabinose in the hydrolysates versus the composition of arabinose in the wheat bran. It may also suggest that the calculated values of arabinose in the de-starched bran may be different from the actual composition. Composition testing of the de-starched bran would be needed to confirm this observation.

### ***Fermentation performance***

Fermentation performance using corn bran and wheat bran hydrolysates varied considerably under different pretreatment conditions (Table 5.2). Fermentation proceeded unaided for hydrolysates generated from the hydrothermal pretreatment but was inhibited when using acid pretreatment for both whole bran and de-starched bran (with the exception of 0.5% acid for de-starched wheat bran). To allow the fermentation to proceed, the hydrolysates were diluted 2x with dH<sub>2</sub>O, filter sterilized and re-fermented.

*L. starkeyi* ATCC 56304 was capable of utilizing, on average, 95-98% of the total sugars (glucose, xylose, arabinose, and maltose). Maltose, a disaccharide present due to incomplete starch hydrolysis, was readily utilized by *L. starkeyi* which had not been previously recorded in our lab. Additionally, arabinose was also utilized, though some residual amounts remained that was unconsumed during fermentation. Contradictory to earlier findings, this suggests that *L. starkeyi* ATCC 56304 is capable of utilizing arabinose from bran hydrolysates. It is not readily apparent why this occurred because an earlier study found that arabinose was not readily utilized when provided as the sole carbon source. One possibility is that arabinose may only be consumed for lipid accumulation (i.e. during stationary phase) albeit at very low rates once cell growth has been established. Further experimental testing would be needed to confirm this hypothesis. The ability of *L. starkeyi* to utilize arabinose from bran hydrolysates is an exciting find indicating that a greater portion of the hemicellulose can be used for SCO production.

### ***Whole bran***

As shown in Table 5.2, whole bran hydrolysates from both corn bran and wheat bran generated higher oil contents and yields compared to de-starched bran hydrolysates. Within whole bran, hydrolysates produced from the hydrothermal pretreatment generated the highest oil yields of 126.7 and 124.3 mg oil/g sugar for corn bran and wheat bran, respectively. The hydrothermal pretreatment was able to generate high amounts of glucose (~50-60 g/L) from the highly water soluble starch portion with little to no inhibitor formation; this provided the necessary conditions for improved oil accumulation. It is apparent the oil yield was significantly affected by the use of acid pretreatment due to the presence of inhibitors. Dilution did not remove HMF and acetic acid, it only reduced the concentration; thus, growth and lipid accumulation were most likely still affected. Furthermore, dilution also limited the amount of available sugar that could be utilized for lipid accumulation, resulting in lower oil yields.

### ***De-starched bran***

The fermentation performance of de-starched bran hydrolysates was diminished compared to whole bran hydrolysates. The presence of furfural and acetic acid along with less fermentable sugar as a result of starch removal were the plausible reasons for this performance. Unlike the whole bran hydrolysates, 1.0% (v/v) acid pretreated de-starched bran hydrolysates resulted in the highest oil yield of 70.7 mg oil/g sugar for both corn bran and wheat bran. Even after dilution and the presence of furfural and acetic acid, acid pretreated bran generated the best fermentation performance. Hydrothermal pretreatment of de-starched corn bran was ineffective producing the lowest oil yield of 32.4 mg oil/g sugar while the more water soluble wheat bran had a significantly higher oil yield of 66.5 mg oil/g sugar. The use of acid pretreatment was an effective method for both bran sources leading to improved sugar hydrolysis and oil production.

### ***Fermentation comparison***

Compared to batch shake-flask fermentation of synthetic sugar sources, both the whole and de-starched bran hydrolysates, on average, generated higher oil yields compared to synthetic glucose (58 mg oil/g glucose). Oil yields produced from the whole bran hydrolysates (126.7 mg oil/g sugar) were comparable to synthetic xylose (148.8 mg oil/g xylose). These results compare well to another study using *L. starkeyi* grown on non-detoxified wheat straw hydrolysate (Yu et al., 2011). The authors were able to produce an oil yield of 160 mg oil/g sugar by using dilute

sulfuric acid pretreatment without enzyme hydrolysis. This technique generated higher proportion of xylose by minimizing the formation of glucose which improved oil yields. This further reinforces the notion that xylose utilization during lipid accumulation is key for generating high oil yields.

Overall, it is evident that fermentation performance was affected by the amount of available sugars and the presence of inhibitory compounds. The deleterious effect of inhibitory compounds including acetic acid, HMF, and furfural on oleaginous yeast growth and lipid accumulation has been well-documented by others (Huang et al., 2012; Huang et al., 2011; Chen et al., 2009; Hu et al., 2009) and confirmed by our studies. While the effect of each inhibitor may vary across oleaginous yeast species and cultivation conditions, multiple studies have reported that furfural has a higher degree of inhibition compared to HMF (Zhao et al., 2012; Huang et al., 2011; Chen et al., 2009; Hu et al., 2009). This may account for the lower oil yields obtained using de-starched hydrolysates which had a higher level of furfural compared to the whole bran hydrolysates (Figure 5.5). To minimize the effect of inhibitors, a detoxification step after pretreatment can be used, often using liming and/or activated carbon (Zhao et al., 2012; Yu et al., 2011). While detoxification can improve fermentation performance, sugar losses can occur. The dilution approach used in this experiment proved to be a useful method not requiring a detoxification step, but the addition of water reduced the sugar concentration which is not desirable. Developing hydrolysis techniques to generate high-quality sugar streams will be key for economically viable industrial fermentation platforms including SCO from yeast.

### ***Fatty acid analysis***

The major fatty acid species produced from *L. starkeyi* included palmitic acid (C16:0) and oleic acid (C18:1). The fatty acid profile remained constant for both the whole bran and de-starched bran hydrolysates and were comparable to profiles from synthetic sugar sources shown in Chapter 3 and 4 (Figure 5.7). Oleic acid accounted for about 70% of the total fatty acid profile for *L. starkeyi*. The large abundance of oleic acid may provide additional value for SCO produced from *L. starkeyi* ATCC 56304. Recently, there has been considerable interest in high oleic oils for biofuels with improved oxidative stability and cold-flow properties, and as an input for many renewable polymers, lubricants, coatings, and elastomers (Durrett et al., 2008; Corbett, 2003).

## Conclusions

This study demonstrated that corn bran and wheat bran can be utilized to produce SCO using *L. starkeyi* ATCC 56304. The highest oil yields of 126.7 and 124.3 mg oil/g sugar were produced from the whole corn bran and wheat bran hydrolysates, respectively, using hydrothermal pretreatment which was effective for hydrolyzing the starch portion and limiting inhibitor formation. Acid pretreatment was effective for the de-starched bran producing the highest oil yields of 70.7 mg oil/g sugar for both corn bran and wheat bran, but the presence of inhibitors such as acetic acid and furfural limited fermentation performance when compared to the whole bran hydrolysates. Both whole bran and de-starched bran hydrolysates outperformed synthetic glucose as a substrate for oil production and the whole bran hydrolysates were comparable to oil yields produced using synthetic xylose. To further increase oil production, adopting an appropriate fermentation scheme will be necessary. For batch fermentation, providing the ideal amount of sugar is a delicate balance; surplus sugar needs to be provided beyond what is needed for growth so it will be available for the later stages of lipid accumulation. Often fed-batch fermentation is used to improve oil yields through sugar addition. As mentioned in Chapter 3, a fed-batch technique that utilizes different sugars for the growth and lipid accumulation stages could be used for bran-based feedstocks because the starch, hemicellulose, and cellulose fractions can be selectively hydrolyzed to produce separate streams of glucose and pentose (xylose and arabinose) sugars. Additionally, the presence of HMF and furfural is undesirable; thus, development of pretreatment methods that minimize the amount of sugar degradation and/or selectively separate sugars once they are released to prevent inhibitor formation would be highly valuable for the commercialization of SCO biochemical platforms.

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**Table 5.1 Composition of whole and de-starched corn bran and wheat bran.**

Component (% , w/w)	Whole bran		De-starched bran <sup>C</sup>	
	Corn bran <sup>A</sup>	Wheat bran <sup>B</sup>	Corn bran	Wheat bran
Moisture (% , w/w)	3.7 ± 0.2	10.5 ± 0.3	-	-
Crude protein*	11.6 ± 0.2	16.5 ± 0.1	17.2	23.9
Crude fat	9.7 ± 0.1	2.3 ± 0.4	14.3	3.4
Crude fiber	10.0 ± 1.4	8.2 ± 0.6	14.7	11.9
Ash	3.4 ± 0.03	7.4 ± 0.04	5.0	10.8
Cellulose†	9.6 ± 0.2	8.0 ± 0.3	14.2	11.6
Hemicellulose <sup>§</sup>	25.8 ± 1.3	23.5 ± 1.0	38.0	34.1
Starch	32.2 ± 3.3	31.1 ± 1.0	-	-
Acid detergent lignin	1.4 ± 0.1	2.5 ± 0.1	2.1	3.6
<b>Sugars (% , w/w)‡</b>				
Glucose	34.2 ± 3.1	35.4 ± 1.2	15.8	12.8
Xylose	13.5 ± 1.1	12.6 ± 0.6	19.9	18.3
Arabinose	8.8 ± 0.7	3.4 ± 0.2	13.0	5.0
Galactose	1.8 ± 0.2	-	2.7	-

<sup>A</sup>Geometric mean diameter of 417±12µm

<sup>B</sup>Geometric mean diameter of 440±15µm

<sup>C</sup>Calculated based on complete starch removal.

\*By Kjeldahl (%N x 6.25) w/w% = grams per 100 grams of sample

†Cellulose (calculated) = acid detergent fiber (ADF) - acid detergent lignin (ADL)

<sup>§</sup>Hemicellulose (calculated) = neutral detergent fiber (NDF) - acid detergent fiber (ADF)

‡Sugars produced from acid hydrolysis.



**Table 5.2 SCO fermentation performance of whole bran and de-starched bran hydrolysates.**

<b>Whole bran</b>	Sugar utilized (g/L)†	Dry cell wt. (g/L)	Oil content (% , dry mass basis)	Y <sub>ps</sub> (mg oil/g sugar)
Wheat, 0% Acid	51.3 ± 1.6 <sup>A</sup>	17.1 ± 1.0 <sup>A</sup>	37.3 ± 1.3 <sup>A</sup>	124.3 ± 12.1 <sup>A</sup>
*Wheat, 0.5% Acid	40.5 ± 2.9 <sup>B</sup>	12.7 ± 0.9 <sup>B</sup>	31.7 ± 0.8 <sup>B</sup>	99.7 ± 2.4 <sup>BC</sup>
*Wheat, 1.0% Acid	39.4 ± 1.1 <sup>B</sup>	10.9 ± 0.3 <sup>B</sup>	31.3 ± 0.3 <sup>B</sup>	87.1 ± 0.4 <sup>CD</sup>
Corn, 0% Acid	61.7 ± 1.9 <sup>C</sup>	23.5 ± 0.1 <sup>C</sup>	33.3 ± 1.1 <sup>AB</sup>	126.7 ± 7.0 <sup>A</sup>
*Corn, 0.5% Acid	37.2 ± 4.5 <sup>B</sup>	12.3 ± 0.1 <sup>B</sup>	32.5 ± 2.8 <sup>B</sup>	108.5 ± 12.0 <sup>AB</sup>
*Corn, 1.0% Acid	47.4 ± 7.0 <sup>B</sup>	12.9 ± 2.4 <sup>B</sup>	25.4 ± 1.5 <sup>C</sup>	68.8 ± 3.4 <sup>D</sup>
<b>De-starched bran</b>				
Wheat, 0% Acid	22.4 ± 5.5 <sup>ZY</sup>	6.4 ± 0.6 <sup>Z</sup>	22.6 ± 1.3 <sup>Z</sup>	66.5 ± 8.1 <sup>Z</sup>
Wheat, 0.5% Acid	38.2 ± 12.6 <sup>YX</sup>	9.6 ± 1.2 <sup>Z</sup>	24.5 ± 6.6 <sup>Z</sup>	62.4 ± 1.3 <sup>ZY</sup>
*Wheat, 1.0% Acid	29.5 ± 2.4 <sup>ZYX</sup>	7.5 ± 0.3 <sup>ZY</sup>	27.5 ± 1.8 <sup>Z</sup>	70.7 ± 7.8 <sup>Z</sup>
Corn, 0% Acid	16.7 ± 0.15 <sup>Z</sup>	5.5 ± 0.5 <sup>Z</sup>	9.8 ± 0.6 <sup>Z</sup>	32.4 ± 4.4 <sup>Y</sup>
*Corn, 0.5% Acid	40.3 ± 6.0 <sup>X</sup>	10.1 ± 1.0 <sup>X</sup>	28.0 ± 1.0 <sup>Z</sup>	70.7 ± 6.3 <sup>Z</sup>
*Corn, 1.0% Acid	35.5 ± 0.4 <sup>YX</sup>	10.5 ± 0.6 <sup>X</sup>	22.9 ± 6.5 <sup>Y</sup>	68.1 ± 22.5 <sup>Z</sup>
<b>Synthetic sugars</b>				
Glucose, pure				58.0 ± 12.5
Xylose, pure				148.8 ± 12.9

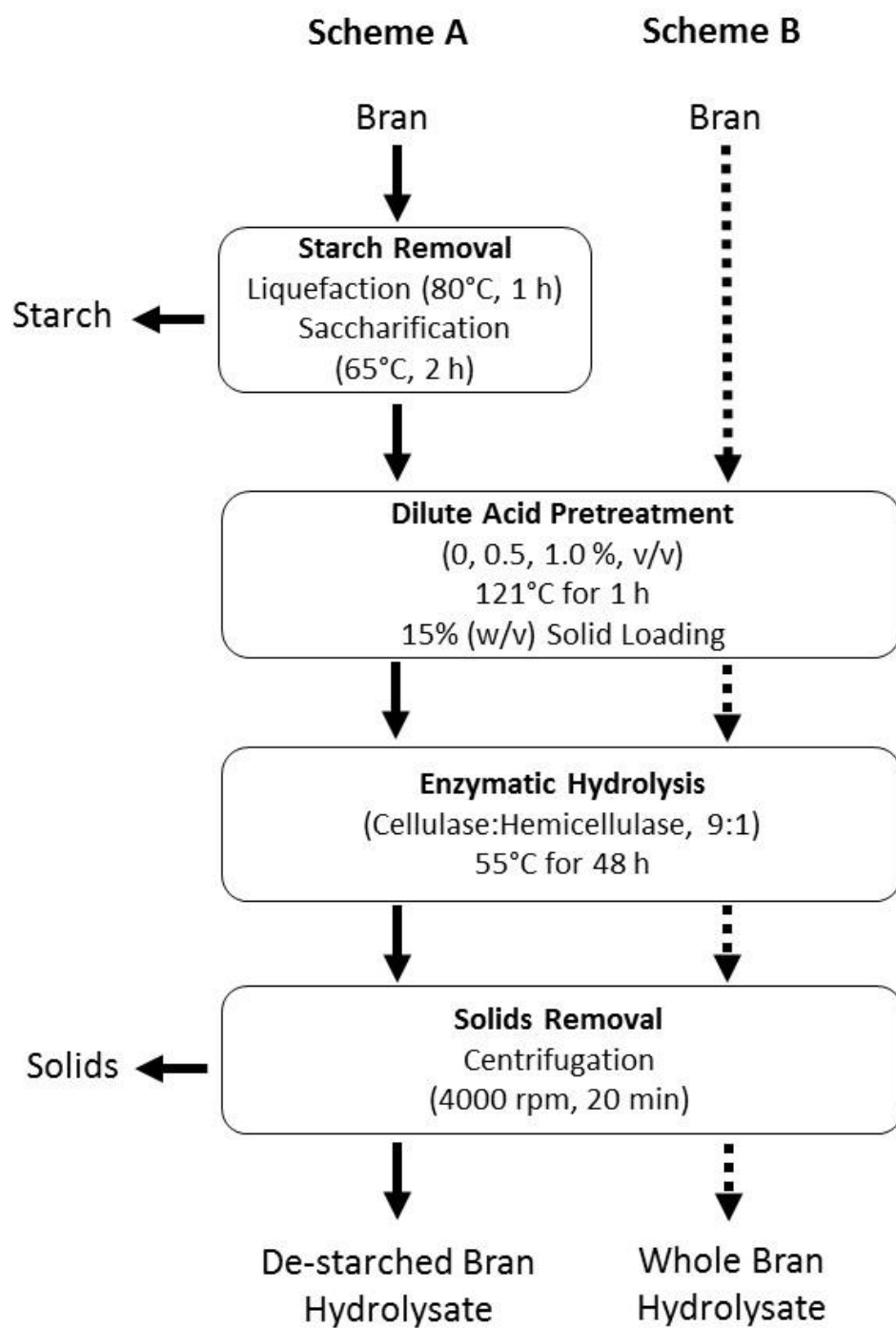
N=3, mean values with the same letters (A-D for whole bran and W-Z for de-starched bran) in the same column are not significantly different ( $P \geq 0.05$ ), values after ± are standard deviation.

\*Samples diluted 2x with dH<sub>2</sub>O before fermentation.

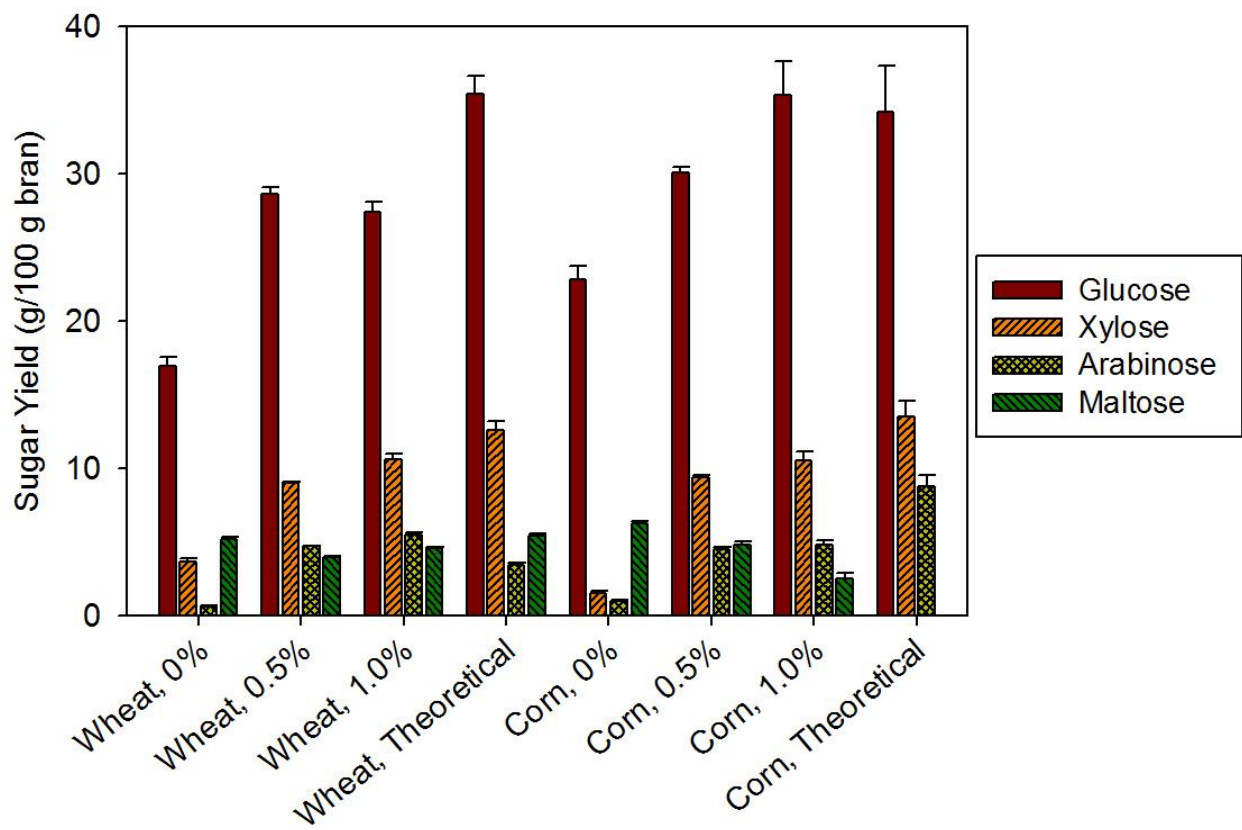
†Included glucose, xylose, maltose, and arabinose. Over 95-98% of sugars were completely utilized during fermentation across all treatments.



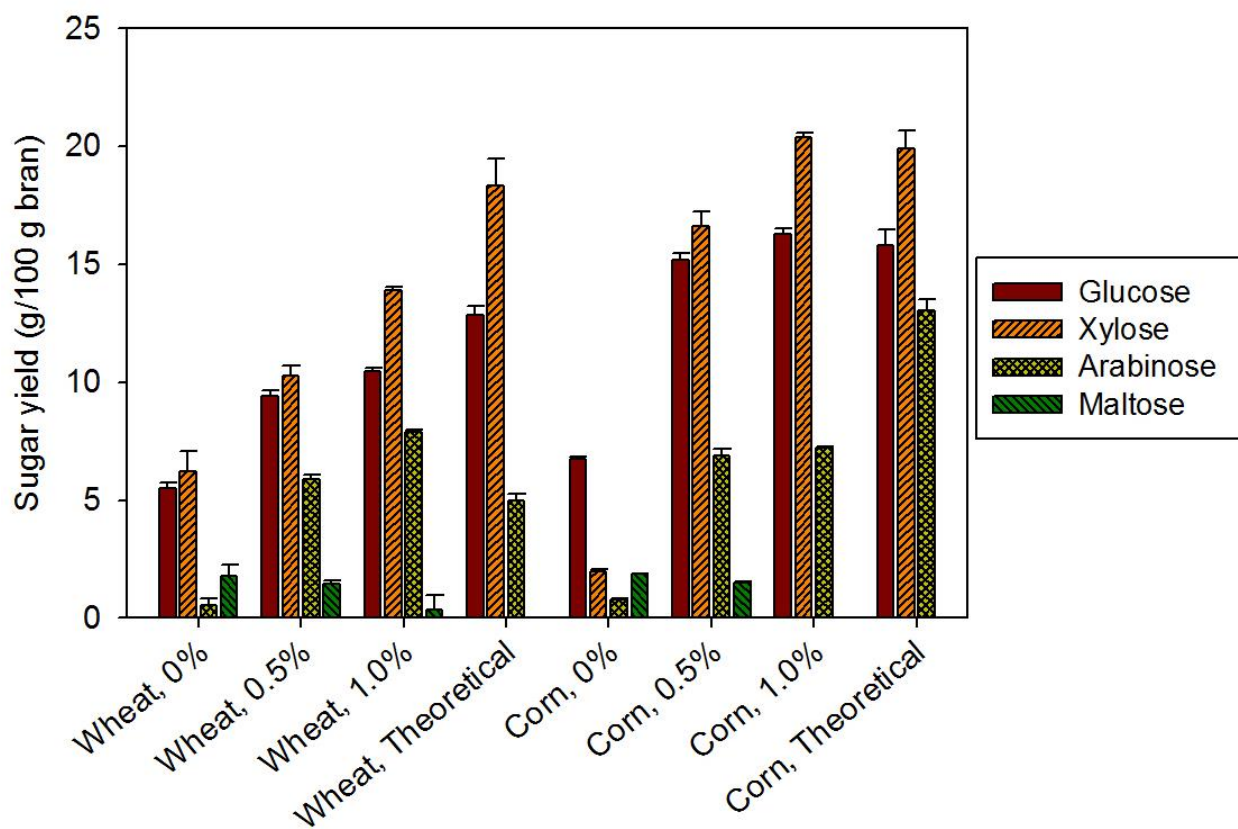
**Figure 5.1 Wheat bran (left) and corn bran (right).**



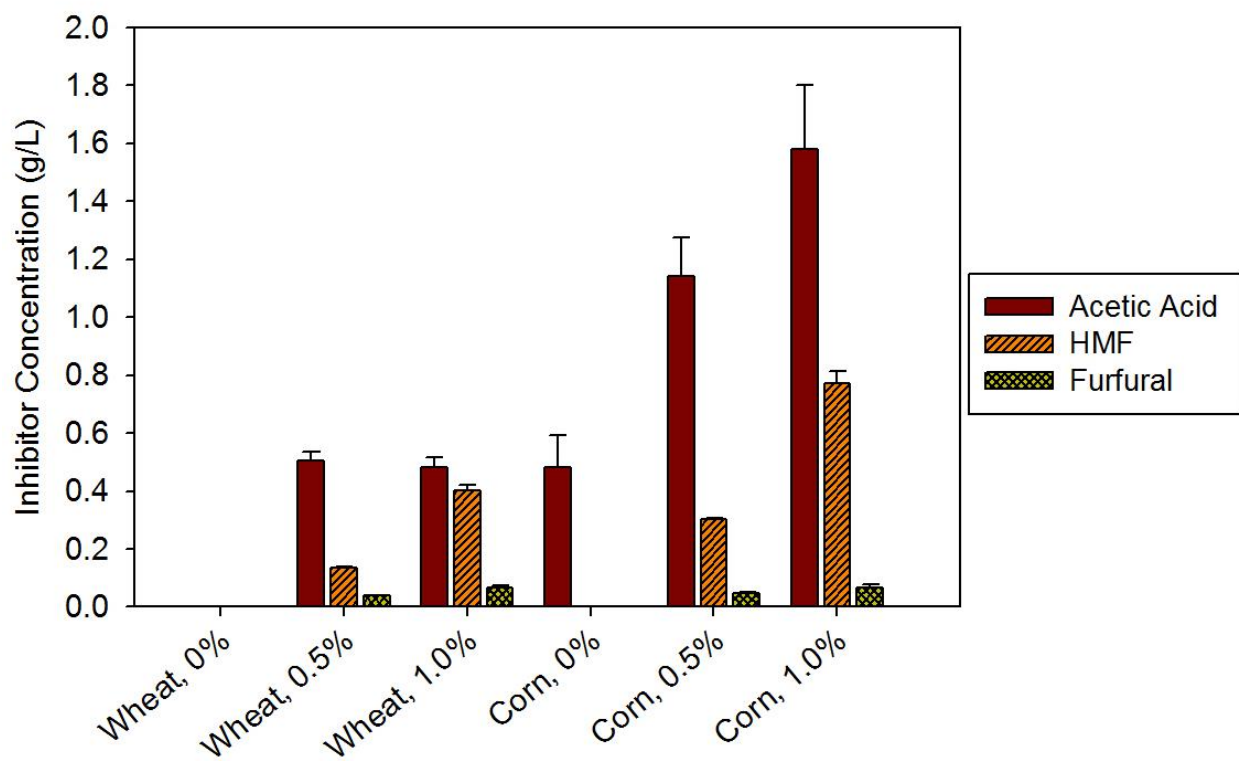
**Figure 5.2 Schematic of bran hydrolysate preparation: A) De-starched bran hydrolysate, B) Whole bran hydrolysate.**



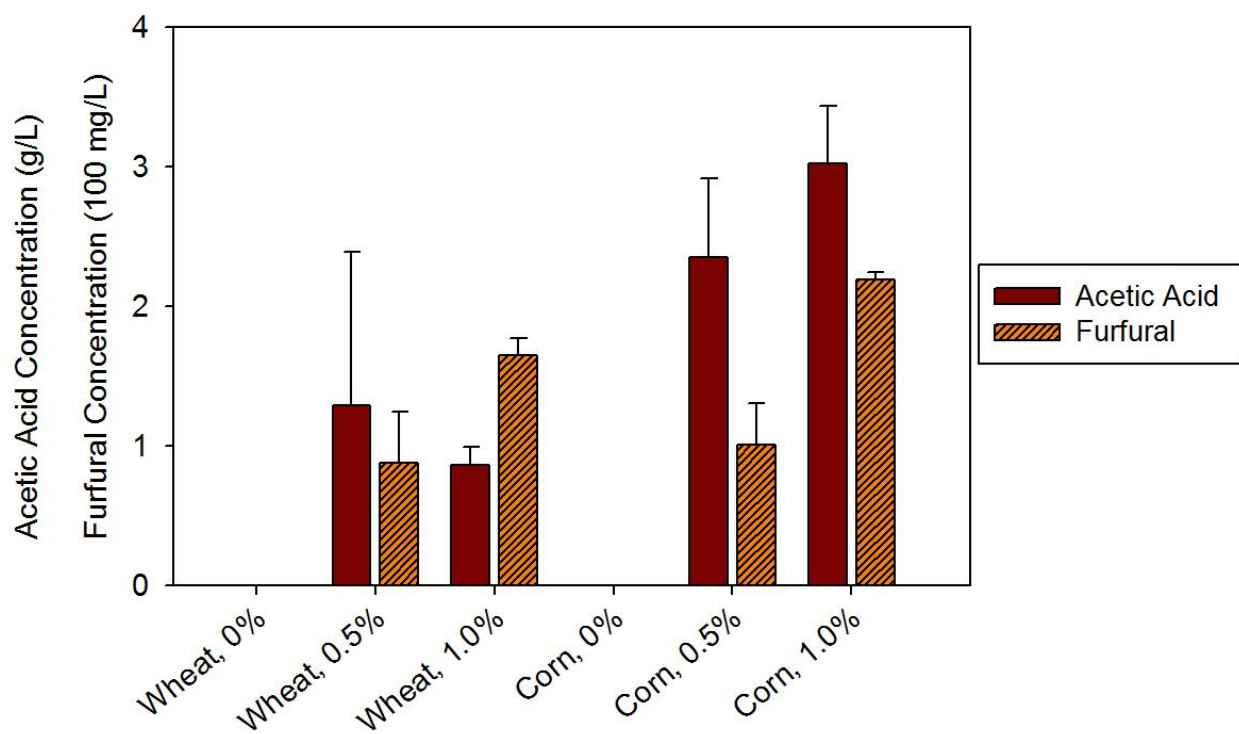
**Figure 5.3 Sugar yield of whole corn bran and wheat bran after sulfuric acid pretreatment (0, 0.5, and 1.0%, v/v) and enzymatic hydrolysis.**



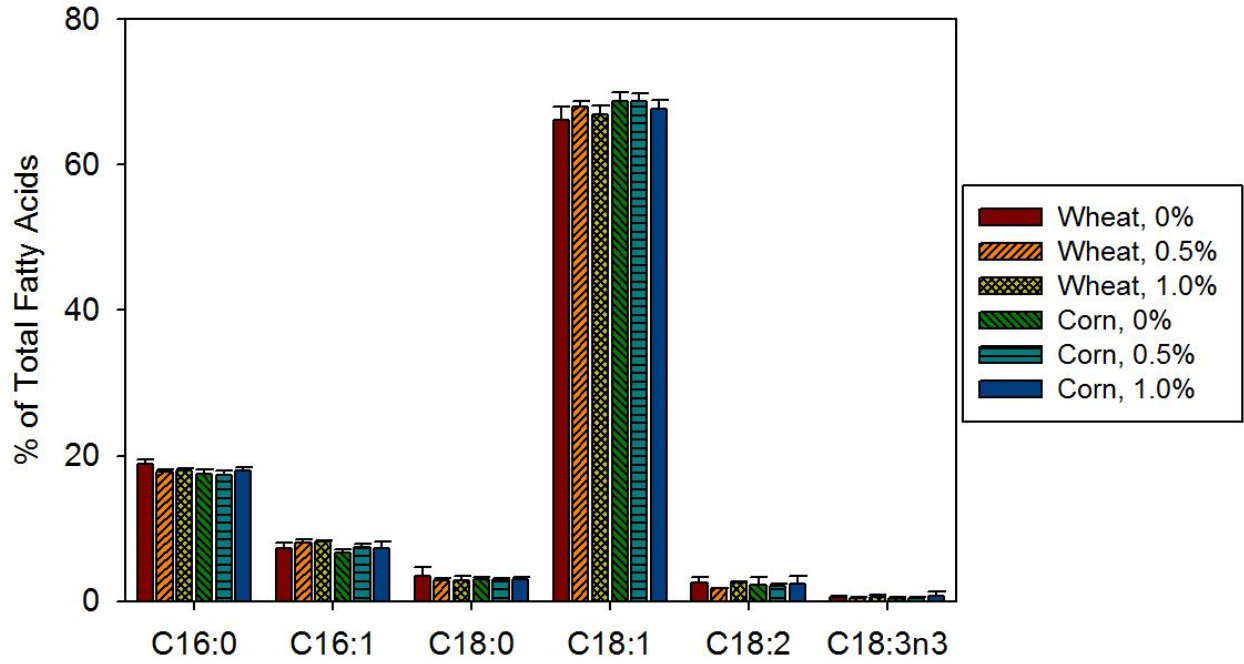
**Figure 5.4 Sugar yield of de-starched corn bran and wheat bran after sulfuric acid pretreatment (0, 0.5, and 1.0%, v/v) and enzymatic hydrolysis.**



**Figure 5.5 Inhibitor concentration of whole corn bran and wheat bran hydrolysates produced from sulfuric acid pretreatment (0, 0.5, and 1.0%, v/v).**

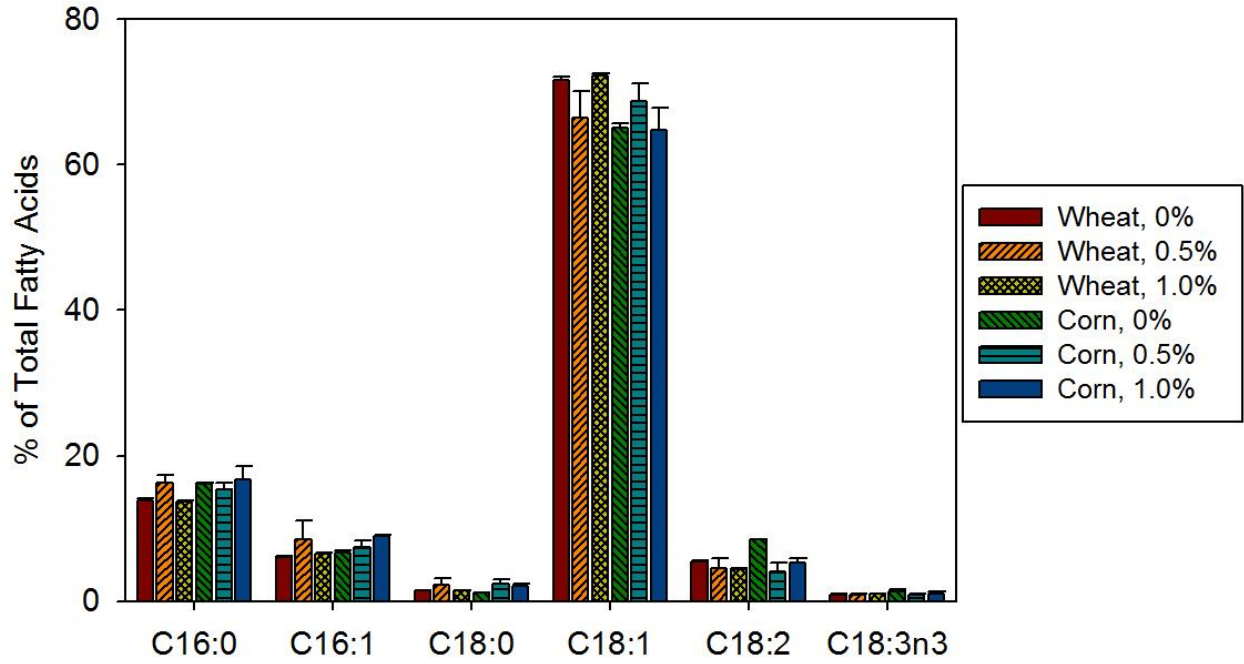


**Figure 5.6 Inhibitor concentration of de-starched corn bran and wheat bran hydrolysates produced from sulfuric acid pretreatment (0, 0.5, and 1.0%, v/v).**



**Figure 5.7 Fatty acid profile of SCO produced from whole bran hydrolysates.**





**Figure 5.8 Fatty acid profile of SCO produced from de-starched bran hydrolysates.**

## **Chapter 6 - Extraction of oil from oleaginous yeast using the green solvents 2-methyltetrahydrofuran (2-MeTHF) and cyclopentyl methyl ether (CPME)**

### **Abstract**

2-methyltetrahydrofuran (2-MeTHF) and cyclopentyl methyl ether (CPME) were evaluated as alternative solvents for extracting oil from concentrated wet cells of the oleaginous yeast *Lipomyces starkeyi* (ATCC 56304). Monophasic systems of only 2-MeTHF and CPME, and biphasic systems of CPME:water and 2-MeTHF:water (1:0.67, v/v) were tested against the laboratory adopted Bligh and Dyer method which served as the baseline and monophasic systems of hexane and chloroform. The 2-MeTHF:water biphasic system obtained an 80.0% extraction efficiency normalized to the Bligh and Dyer method, the highest out of all the solvent systems. Selectivity of 2-MeTHF and CPME were relatively low around 50-60% indicating that other constituents were being extracted from the cell lysate. In comparison, *n*-hexane, though typically not used for extracting oil from wet materials, was superior for lipid extraction as a low cost, simple monophasic system that had an extraction efficiency of 77.3% and selectivity of 80.3%. Overall, this study was able to demonstrate that 2-MeTHF and CPME could be used for oil extraction, though further testing would be needed to improve oil extraction efficiency and selectivity.

### **Introduction**

Single cell oil (SCO) has sparked recent interest as input for renewable fuels and chemicals. Oleaginous yeast are advantageous SCO producers able to accumulate oil contents up to ~70% of the dry cell mass. After fermentation, oil extraction and separation remains a major set-back for industry scale-up and production. Often the cell biomass needs to be dried prior to extraction, a step that is energy and cost intensive (Ratledge, 2010). Currently no industrial standard process exists for the extraction of oil from wet cell biomass. Criteria for selecting the proper solvent extraction method should include high selectivity and extraction efficiency for the oil from the other cellular components, non-reactivity with the oil of interest and easy separability (Halim et al., 2012). For biphasic systems, limited solubility of the extraction solvent in the 2<sup>nd</sup> phase and vice versa is important for efficient separation and minimal co-extraction of other cellular

constituents. Additional considerations that are important include adopting solvents that are low-cost, non-toxic and renewable.

The most common and effective methods for extracting oil from wet materials include those developed by Folch (1957) and Bligh and Dyer (1959) which utilize biphasic systems of the nonpolar organic solvent chloroform and the polar solvents methanol and water. While these methods are often the benchmark for laboratory practices; scalability to an industrial process would be challenging since chloroform is highly regulated due to its classification as a probable human carcinogen (EPA, 2000). *n*-Hexane is the industry standard for extracting edible oil from dry matter mainly because of its low cost, ease of recovery, and high oil solubility (Johnson & Lusas, 1983). Comparable alternatives to hexane have been investigated since it is a non-renewable, petroleum-based solvent that has been identified by the EPA as a hazardous air pollutant, and has been shown to have neurotoxic effects at high levels of exposure (ATSDR, 1999; Chang, 1987). Many attempts have demonstrated successful use of other solvent systems but none have been adopted as a replacement for hexane (Liu & Mamidipally, 2005; Wan et al., 1995; Johnson & Lusas, 1983; Rao & Arnold, 1958). To address these issues, developing greener solvent-based oil extraction systems for wet cellular biomass would help improve the efficiency and sustainability of SCO extraction.

2-methyltetrahydrofuran (2-MeTHF) and cyclopentyl methyl ether (CPME) are emerging alternative, green solvents and their adoption at the industrial level is being encouraged by the ACS Green Chemistry Pharmaceutical Roundtable (Antonucci, 2011). Green chemistry was coined to encourage the design of products and processes that minimize environmental impact (EPA, 2014). According to Capello et al. (2007), there are four major areas of focus for developing greener solvents: 1) improving environmental, health, and safety properties, 2) sourcing renewable inputs and substituting for, 3) supercritical fluids, and 4) ionic liquids. One should be aware that for a solvent to be deemed “green” it may only need to meet one of these criteria; thus, not all green solvents are created equal. Regardless, testing and demonstrating the use of alternative, greener chemicals will be important for adopting environmentally friendly chemistry practices.

2-MeTHF is a biodegradable ethereal solvent synthesized from biomass (Figure 6.1). 2-MeTHF is approved for use as an additive in P-series fuels and recently has been demonstrated in applications including biomass fractionation, protein extraction, olefin synthesis and as a reaction medium for bio-/organo-catalysis (Smolen et al., 2014; Tenne et al., 2013; Pace et al., 2012; vom

Stein et al., 2011). 2-MeTHF is well-known for its use as a cost-effective green replacement to tetrahydrofuran (THF) and is praised for having improved recyclability, lower volatility, reduced carbon emissions, and enhanced stability (Aul & Comanita, 2007). Toxicological assessment of 2-MeTHF is still pending but a recent study by Antonucci (2011) found that exposure is not associated with genotoxicity and mutagenicity. Due to the beneficial properties of 2-MeTHF, industrial applications beyond THF replacement may be possible.

CPME is another alternative ethereal solvent that has gained interest as a suitable industrial solvent. Though it is derived from petroleum, it is deemed green due to its improved characteristics compared to other ethereal solvents including: better stability to peroxide formation, low volatility, low water solubility, and acidic stability (Watanabe et al., 2007). Furthermore, according to Sakamoto (2103), CPME formation is 100% atom efficient (Figure 6.1). CPME has been used in many applications including furfural synthesis, protein purification, and radical additions (Kobayashi et al., 2013; Tenne et al., 2013; Campos Molina et al., 2012). Like 2-MeTHF, CPME exposure was not found to cause genotoxicity or mutagenicity but further assessment is needed to determine its toxicological profile (Antonucci, 2011).

The objective of this work was to test the use of 2-MeTHF and CPME as alternative, green solvents for the extraction of oil from wet cells of *Lipomyces starkeyi* ATCC 56304. Demonstrating the ability to use CPME and 2-MeTHF for oil extraction from wet cell concentrates may be useful for the future development of efficient, cost-effective, and sustainable SCO processing practices.

## **Materials and methods**

### ***Materials***

All chemicals were of reagent grade or higher, unless otherwise noted, and purchased from a certified supplier (Fisher Scientific, Pittsburg, PA, USA).

### ***Yeast preparation***

Oil-rich yeast cells (*Lipomyces starkeyi* ATCC 56304) were produced using 7.5 L baffled stirred-tank bioreactor with a working volume of 5 L (Bioflo 110, New Brunswick Scientific Inc., Einfield, CT, USA) in nitrogen-limited media with xylose as the major carbon source (50 g/L xylose, 0.5 g/L urea, 0.5 g/L yeast extract, 0.5 g/L  $\text{KH}_2\text{PO}_4$ , 0.5 g/L  $\text{Na}_2\text{HPO}_4$ , 0.1 g/L  $\text{CaCl}_2$ , 0.1

g/L MgSO<sub>4</sub>, 0.01 g/L ZnSO<sub>4</sub>, and 0.005 g/L FeSO<sub>4</sub>). Harvested yeast cells were washed 5x with deionized water (dH<sub>2</sub>O) and concentrated to ~10<sup>9</sup> cells/mL. Dry cell weight (DCW) was determined gravimetrically. Cells were harvested via centrifugation (12000 rpm for 15 min), washed 3x using dH<sub>2</sub>O and dried at 80°C for 12 h.

### ***Oil extraction***

Treatments consisted of different solvent systems: four monophasic (CPME-only (100%), 2-MeTHF-only, *n*-hexane-only, and chloroform-only), and two biphasic systems (CPME:water (1:0.67, v/v), 2-MeTHF:water (1:0.67, v/v)), and a modified Bligh and Dyer method (chloroform:methanol:dH<sub>2</sub>O (1:2:0.8, v/v)) which served as the control. The total volume of solvent used for extraction was fixed at 7.5 mL across all treatments. Using the dielectric constant of the control as a baseline for the biphasic systems of 2-MeTHF and CPME, water was added to adjust the dielectric constant to approximately 36. To calculate the ratio of water and solvent needed, the equation below was used to calculate the dielectric constant of the solvent mixture ( $\epsilon_{mix}$ ):

$$\epsilon_{mix} = \sum \epsilon_i * \phi_i + \dots \epsilon_n * \phi_n \quad \text{Eq. 1}$$

for  $n$  number of solvents,  $\epsilon_i$  is the dielectric constant for each solvent and  $\phi_i$  is the volume fraction of each solvent.

A schematic of the oil extraction process for each solvent system is shown in Figure 6.2. All extractions were performed at ambient conditions. A 0.5 mL aliquot of concentrated wet cells was added to a 2.5 mL polypropylene screw-cap microvial with an O-ring seal. To the wet, pelleted cells, 1 mL of 0.5 mm cubic zirconia beads, and 1 mL of the respective solvent system were added (e.g. for the Bligh and Dyer, 0.5 mL of chloroform and 0.5 mL of methanol); no water was added at this time. Lysis was achieved using a Beadbeater homogenizer (Mini-Beadbeater-24, BioSpec Products, Inc., Bartlesville, OK, USA) operated in 45 sec intervals with intermittent cooling on ice for 10 min for a total of six cycles. The cell lysate was transferred to a glass test-tube with PTFE-lined caps. Microvials were washed 2x with 1 mL of the respective solvent system to ensure all lysate was removed. For the biphasic systems, the remaining solvent(s) and water were added (for the Bligh and Dyer - 1.5 mL of methanol, 1 mL of chloroform and 2 mL of water; for the 2-MeTHF/CPME:water - 2.5 mL of 2-MeTHF/CPME and 3 mL of water) to achieve a total volume

of 7.5 mL. For the monophasic systems, 2.5 mL of each respective solvent was added to achieve a total volume of 4.5 mL. Solvent systems were further prepared by adding one drop of hydrochloric acid (3M) to prevent emulsion formation, vortexed and centrifuged at 3000 rpm for 15 min to achieve phase separation in the biphasic system and separate cell debris/beads in the monophasic system (Figure 6.3). A Pasteur pipette was used to remove the solvent of interest (for biphasic systems: lower chloroform layer for Bligh and Dyer; upper layer for CPME and 2-MeTHF) and transferred to clean test tube. All extractions were repeated a total of 3x to ensure complete lipid removal. The combined solvent of interest (7.5 mL) was filtered using a PTFE filter (0.2  $\mu\text{m}$ ) to remove any cellular debris, washed once with a potassium chloride solution (1M), and dried down completely under nitrogen gas. Residual solvent was removed using a vacuum drier for 30 min and weighed; this was known as the total lipid extract. The total lipid extract was re-dissolved in 1 mL of chloroform and stored at  $-80^{\circ}\text{C}$  until further analysis. Extractions were performed in triplicate.

### ***Fatty acid analysis***

The fatty acid analysis procedures are described elsewhere in Chapter 3. In brief, extracted lipids were transesterified to fatty acid methyl esters (FAMES) using methanolic hydrochloric acid (3M) at  $78^{\circ}\text{C}$  for 30 min followed by addition of water and extraction with hexane. A gas chromatograph (GC-2014, Shimadzu Scientific Instruments, Columbia, MD, USA) with a polyethylene glycol capillary column (Zebron ZB-Wax<sub>plus</sub> 30m x 0.25mm x 0.25 $\mu\text{m}$ , Phenomenex, Torrance, CA, USA) equipped with a flame ionization detector (FID) was used for separation and detection. Retention times were determined by comparison with a FAME 37 standard mixture (Supelco 37 component FAME mix, Sigma Aldrich, St. Louis, MO, USA). Internal and external standards of pentadecanoic acid (C15:0) were used to quantify total fatty acid content of the total lipid extract.

### ***Statistical analysis***

Statistical analysis was conducted using SAS (SAS v9.1, SAS Institute, Cary, NC, USA) PROC GLM to determine means and Tukey's HSD for comparison between treatments ( $\alpha = 0.05$ ).

### ***Determination of extraction efficiency and selectivity***

For comparison purposes, the total fatty acid (TFA) content of the total lipid extract was used as an indicator of oil composition since fatty acids and fatty acid containing lipid species (e.g. triacylglycerol) are the major products of interest; it is understood that the total lipid extract may comprise limited amounts of other lipid species that do not contain fatty acids. The modified Bligh and Dyer served as the basis (considered to be 100%) for calculating the normalized TFA extraction efficiency of each solvent system.

$$\begin{aligned} &TFA \text{ extraction efficiency (\%)} \\ &= \frac{\text{Total fatty acid content}}{\text{Total fatty acid content of Bligh and Dyer}} \end{aligned} \quad \text{Eq. 2}$$

TFA selectivity was used to represent the effectiveness of a solvent system to select oil from the other undesirable constituents present in the cell lysate. Essentially, this is the TFA content of the total lipid extract. Values deviating from 100% represent contamination of other undesirables and; thus, lower selectivity.

$$TFA \text{ selectivity (\%)} = \frac{\text{Total fatty acid content}}{\text{Total lipid extract}} \quad \text{Eq. 3}$$

## **Results**

The properties of 2-MeTHF and CPME are shown in Table 6.1. 2-MeTHF and CPME are hydrophobic in nature, have a similar dielectric constant compared to chloroform and are completely miscible in vegetable oil (data not shown). 2-MeTHF and CPME each have a lower density than water and form the upper phase in the biphasic system. Emulsion formation, which can occur as a result of surfactants such as phospholipids/proteins naturally present in the cell lysate, was problematic during phase separation with 2-MeTHF. Lower pH reduced surfactant interaction and broke the emulsion allowing good phase separation.

The total lipid extract and TFA extracted from wet, concentrated cells of *L. starkeyi* using each solvent system is shown in Table 6.2. The Bligh and Dyer method extracted 399.6 mg TFA/g DCW, the highest compared to the other methods. 2-MeTHF:water and hexane also extracted a considerable amount of TFA at 319.6 and 308.9 mg TFA/g DCW with extraction efficiencies of 80.0 and 77.3%, respectively. The CPME:water, CPME, and 2-MeTHF systems had the lowest extraction efficiencies of 52.6, 57.0 and 63.9%, respectively.

Furthermore, it was observed that the total lipid extract was not a good indicator of the TFA content; except for the modified Bligh and Dyer, which had a 93% TFA selectivity. Total lipid extracts from both the mono- and biphasic CPME and 2-MeTHF systems were significantly greater than others but had a lower proportion of TFA resulting in poor selectivity. In some cases the weight of the total lipid extract was 2-fold higher compared to the TFA content. Undesirable constituents other than oil may be present in the total lipid extract such as cellular components (protein, nucleic acids, and polysaccharides) and even residual solvent or water. Hexane had an 80.3% selectivity, the highest compared to the monophasic systems.

Shown in Figure 6.4, the fatty acid profile of the total lipid extract for *L. starkeyi* was similar to previous findings (Chapter 3, 4 and 5) and remained constant across all solvent systems. This suggests that the same lipid species were extracted regardless of the solvent system being used.

## Discussion

Effective oil extraction systems are characterized by the ability to extract and separate oil from other unwanted non-lipid components (Schmid & Hunter, 1971). Biphasic systems employing the use of chloroform:methanol:water, such as those developed by Folch et al. (1957) and Bligh and Dyer (1959), are some of the most effective methods to date, hence both are widely adopted as standard laboratory oil extraction methods from wet materials. The modified Bligh and Dyer method achieved the highest extraction efficiency and selectivity suggesting that this method was effective for the extraction and rectification of oil from wet cell concentrates. In comparison to the Bligh and Dyer method, the monophasic system of chloroform performed poorly without methanol and water. Addition of a polar organic solvent such as methanol increases the extraction efficiency by allowing hydrogen bonding to occur and help disrupt lipid-protein complexes (Medina et al., 1998; Schmid, 1973). This notion has been further reinforced by Halim et al. (2011) who reported that the addition of isopropanol to hexane was able to improve the total lipid extraction yield by more than 300%. The addition of water facilitates phase separation of the polar and nonpolar layers. This improves selectivity by allowing the chloroform to separate, partitioning the oil from other undesirable cell contaminants. This explains why the monophasic systems of CPME and 2-MeTHF were met with limited extraction efficiency and selectivity compared to the Bligh and Dyer method. The monophasic system of hexane had a high oil extraction efficiency



and selectivity. This is most likely due to its highly nonpolar nature and limited degree of water solubility (0.01 g water/100 g of hexane) which probably improved lipid-solvent interactions and minimized contamination of water and other undesirable components.

The biphasic systems of 2-MeTHF and CPME used water as a less toxic substitute for methanol. The amount of water used was calculated based on the dielectric constant for the modified Bligh and Dyer system. The highest performance came from the 2-MeTHF:water system which had a TFA extraction efficiency of 80.0% compared to 57.0% for CPME. While the difference between these two solvents is not readily apparent, the greater water solubility (4.4 g water/100 g of 2-MeTHF) of 2-MeTHF may have improved disruption and extraction of bound lipid complexes as a result of hydrogen bonding, similar to the role of methanol in the Bligh and Dyer method. Additionally, the limited selectivity of both the 2-MeTHF:water (64.2%) and CPME:water (48.7%) systems indicate that other undesirable non-lipid components are being extracted. It is apparent that using water as the polar phase was met with limitations. Testing of other polar phases may help improve both extraction efficiency and selectivity.

## **Conclusions**

Overall, the choice of solvent system for oil extraction is quite clear. The Bligh and Dyer system is a highly effective method for laboratory purposes while hexane is a low cost simple extraction solvent further reinforcing its adoption at an industrial level. The use of 2-MeTHF and CPME as monophasic and biphasic oil extraction systems was met with limitations; however, further studies are required to improve the extraction efficiency and selectivity. Additionally, use of ethanol as a polar organic solvent, which is less toxic than methanol, could serve as a greener substitute without the limitations of only using water. More testing is needed to validate this hypothesis.

Development of effective CPME and 2-MeTHF extraction systems could be valuable for the oleochemical industry. The beneficial properties of CPME and 2-MeTHF could enhance the processing efficiency by serving as mediums for oil storage and chemical reactions (e.g. direct transesterification). From a SCO production standpoint, efficient downstream processes would be highly valuable because oil extraction and purification remains to be one of the major costs in the production process.

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**Table 6.1 Solvent properties of CPME, 2-MeTHF, chloroform, hexane and water.**

Property	CPME <sup>1</sup>	2-MeTHF <sup>1</sup>	Chloroform <sup>2</sup>	Hexane <sup>2</sup>	Water <sup>2</sup>
Price (\$/kg) <sup>†</sup>	11	5	0.6-1.0	0.46	-
Dielectric Constant (25°C)	4.76	6.97	4.81	1.89	80.00
Molecular Weight (g/mol)	100.16	85.13	119.38	86.18	18.02
Density (g/cm <sup>3</sup> , 25°C)	0.86	0.85	1.48	0.66	1.00
Boiling Point (°C)	106	80	61	69	100
Flash Point (°C)	-1	-11	-	-22	-
Heat of Vaporization (kcal/kg)	69.2	89.1	59.3	87.6	540.0
Explosion Range (% vol)	1.1-9.9	1.5-8.9	-	1.2-7.7	-
Solubility in water (g/100 g, 20°C)	1.1	14	0.8	0.01	-
Solubility of water in solvent (g/100g, 20°C)	0.3	4.4	0.06	0.01	-
Azeotropic temperature with water (°C)	83	71	53.3	-	-
Azeotropic composition (solvent/water, %, wt)	83.7/16.3	89.4/10.6	97.0/3.0	-	-
DOT Classification	2, Flammable	2, Flammable	6.1, Poisonous	3, Flammable	Not regulated
LD50, oral (mg/kg)	1000-2000	4500	908	25000	-

<sup>1</sup>Adapted from (Watanabe et al., 2007)

<sup>2</sup>Data sourced from Lange's Handbook of Chemistry and the CRC Handbook of Chemistry and Physics.

<sup>†</sup>CPME and 2-MeTHF pricing obtained from (Zeon Corporation, 2011). Hexane and chloroform pricing obtained from (ICIS, 2006).

**Table 6.2 Oil extraction of different solvent systems.**

Solvent System	Dielectric Constant <sup>1</sup>	Total Lipid Extract (mg/g DCW)	Total Fatty Acids (mg/g DCW)	Total Fatty Acid Extraction Efficiency (%) <sup>2</sup>	Total Fatty Acid Selectivity (%) <sup>3</sup>
Bligh and Dyer†	36.1	428.1 ± 6.4 <sup>D</sup>	399.6 ± 30.0 <sup>A</sup>	100.0	93.3
2-MeTHF:Water (1:0.67)	36.1	498.2 ± 10.6 <sup>B</sup>	319.6 ± 41.9 <sup>AB</sup>	80.0	64.2
CPME:Water (1:0.67)	34.9	431.6 ± 22.7 <sup>CD</sup>	210.3 ± 22.2 <sup>B</sup>	52.6	48.7
2-MeTHF-Only	7.0	543.1 ± 24.2 <sup>A</sup>	255.4 ± 28.8 <sup>AB</sup>	63.9	47.0
CPME-Only	4.8	454.7 ± 14.2 <sup>CD</sup>	227.6 ± 9.2 <sup>B</sup>	57.0	50.1
Hexane-Only	1.9	384.5 ± 6.3 <sup>E</sup>	308.9 ± 36.9 <sup>AB</sup>	77.3	80.3
Chloroform-Only	4.8	472.0 ± 3.0 <sup>BC</sup>	301.1 ± 29.9 <sup>AB</sup>	75.4	63.8

N=3, mean values with the same letters in the same column are not significantly different ( $P \geq 0.05$ ), values after  $\pm$  are standard deviation.

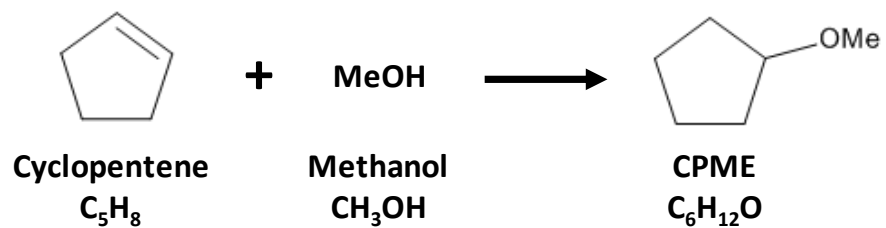
†Bligh and Dyer system consisted of chloroform:methanol:water (1:2:0.8).

<sup>1</sup>Calculated using Equation 1.

$$^2\text{Extraction efficiency} = \frac{\text{Total fatty acids}}{\text{Total fatty acids from Bligh and Dyer}}$$

$$^3\text{Total fatty acid selectivity} = \frac{\text{Total fatty acids}}{\text{Crude oil extract}}$$

## CPME



## 2-MeTHF

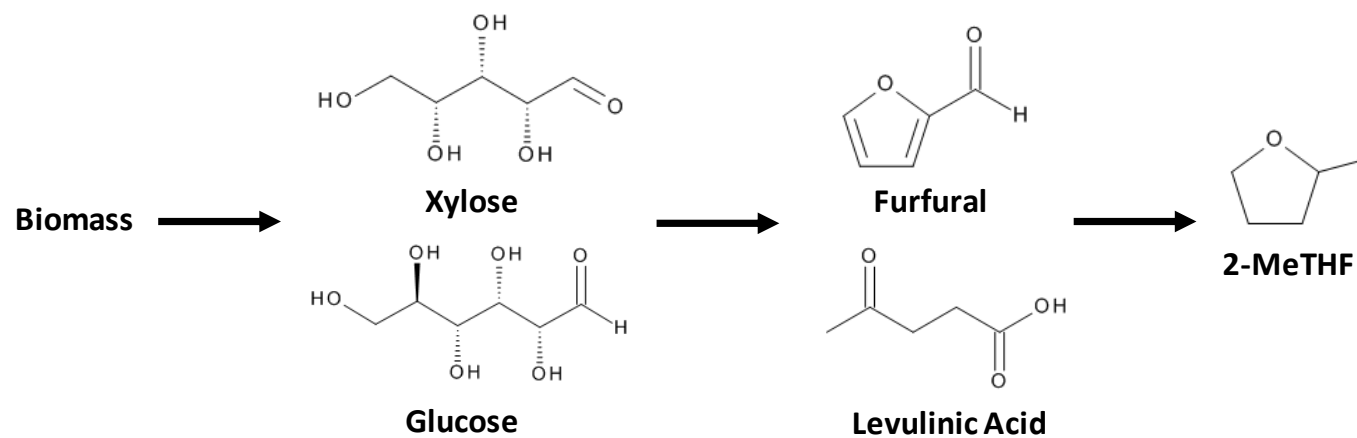


Figure 6.1 Overview of CPME and 2-MeTHF synthesis; adapted from Sakamoto (2013) and Pace (2012).

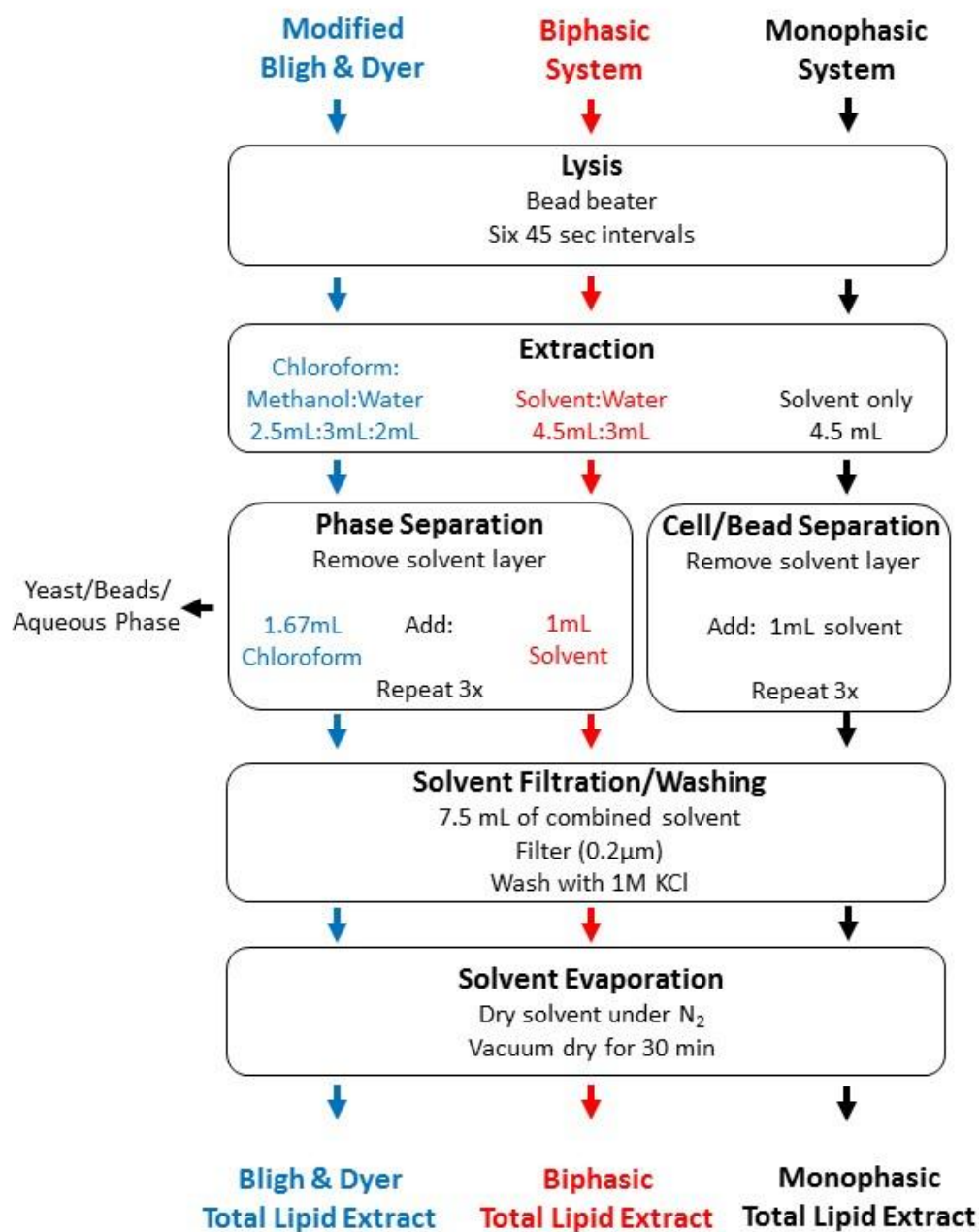
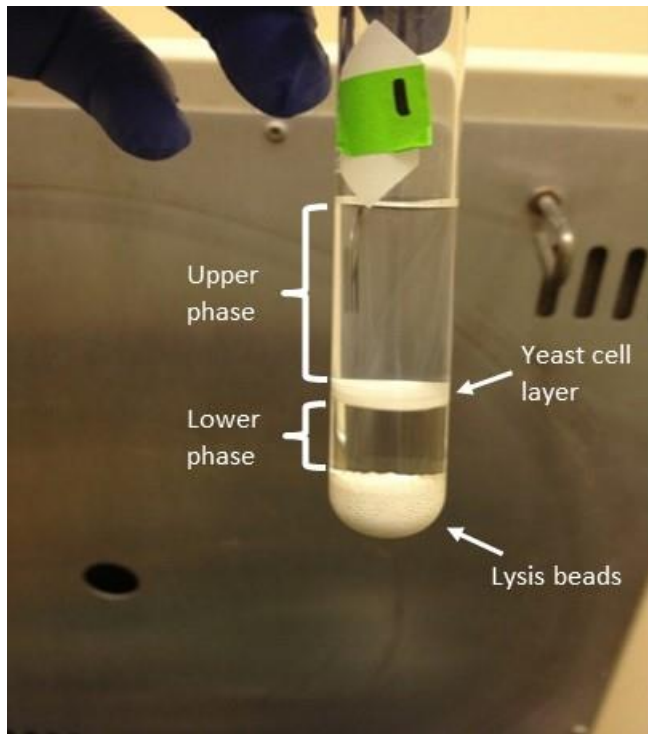
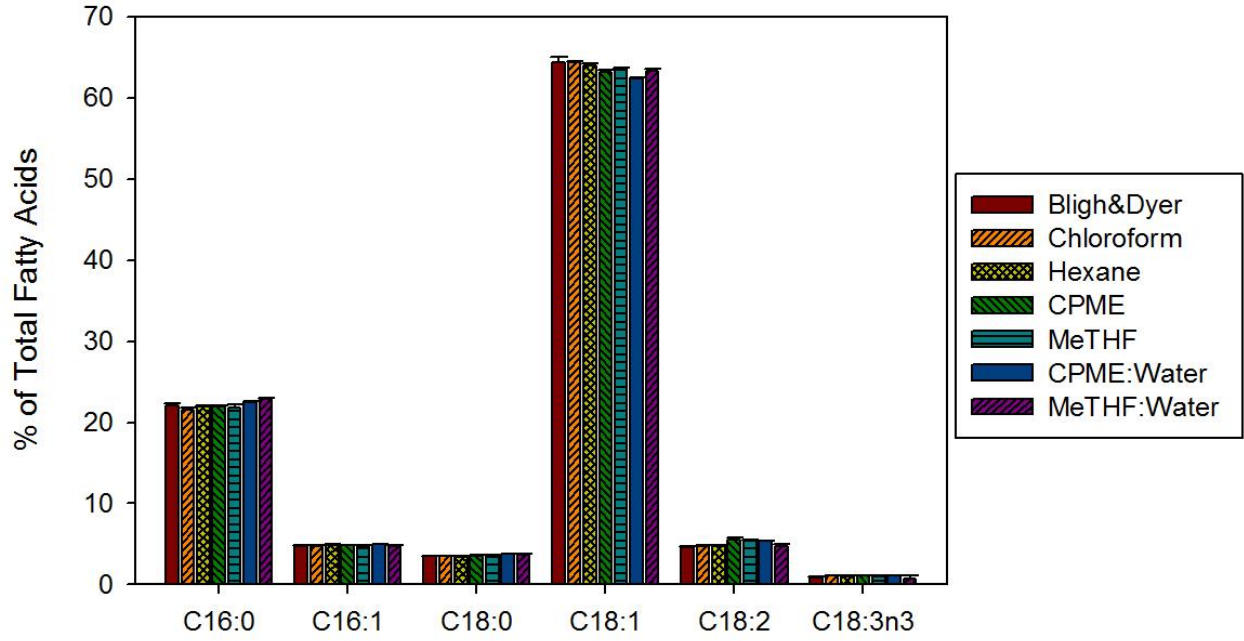


Figure 6.2 Schematic of lipid extraction for each solvent system.





**Figure 6.3 Visual representation of biphasic separation. Note volume of each phase is dependent on the solvent system used.**



**Figure 6.4 Fatty acid profile of the total lipid extract from each solvent system.**

## Chapter 7 - Conclusions and future research

Single cell oil (SCO) from oleaginous yeast is a renewable noncrop-based oil source that can be used for the production of bio-based fuels, chemicals and other petroleum counterparts. Development of an integrated approach will be necessary for economical production including: improved fermentation, utilization of low-valued feedstocks, and efficient downstream extraction and separation. The purpose of this doctoral research was to evaluate the important steps for an integrated approach for efficient SCO production from low-cost renewable resources. Key findings from our studies include:

- 1) The development of a novel fed-batch fermentation with improved utilization of glucose and xylose for SCO production;
- 2) SCO from *Lipomyces starkeyi* is naturally high in oleic acid; a key feature for improved biofuel performance and as an input for promising oleochemicals;
- 3) Hemicellulose-rich by-products from grain and bioprocessing industries such as wheat and corn milling industry can be used as low-cost feedstocks for SCO production and;
- 4) Green solvents, such as 2-methyltetrahydrofuran (2-MeTHF) and cyclopentyl methyl ether (CPME), have potential as sustainable and efficient oil extraction systems. While hexane is a cost-effective solvent for industrial extraction processes.

One of the most promising aspects of oleaginous yeast is their ability to utilize hemicellulose. As mentioned earlier, this has been a major challenge for the cellulosic ethanol industry. With the advent of biorefineries capable of generating multiple products including fuels, chemicals and power from plant-derived biomass feedstocks, oleaginous yeast could be adopted as a value-added platform for hemicellulose bioconversion (Figure 7.1).

### Future research

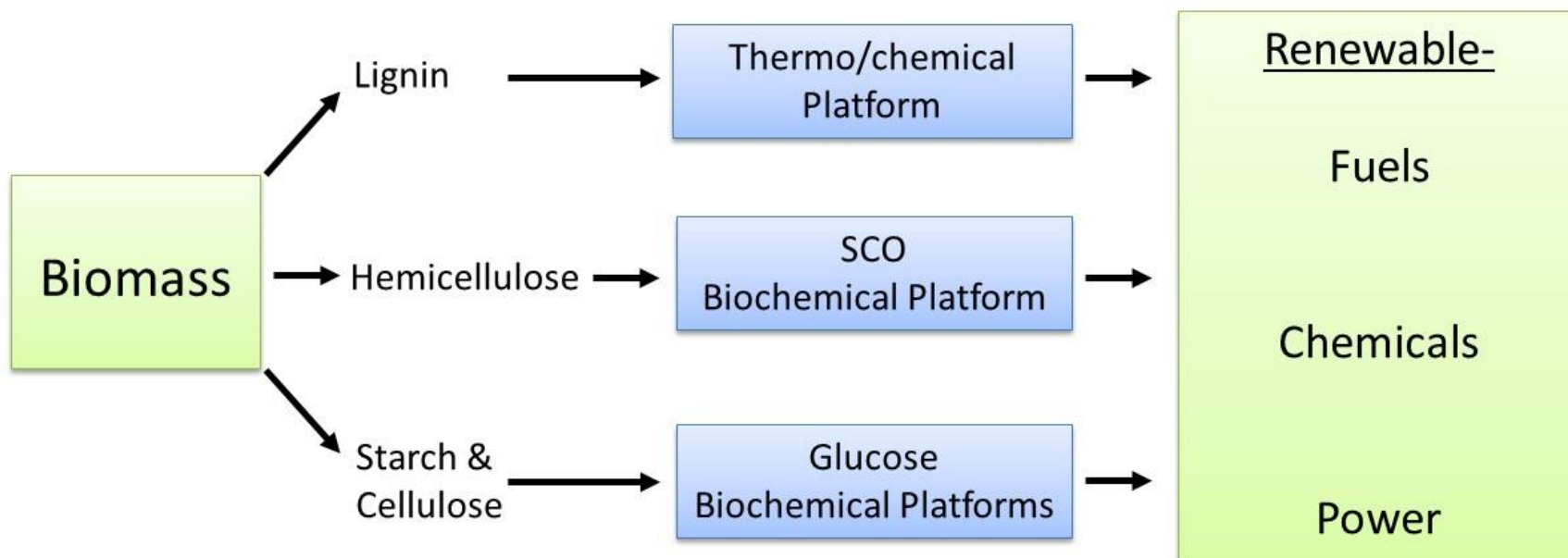
Further work is required to commercialize SCO biochemical platforms for renewable fuels, chemicals and polymer products. Currently, lower cost alternatives from petroleum and oilseed crops have limited the production of SCO as a commodity-type oil. Developing processing technologies that improve the productivity and cost competitiveness of SCO will be important for industrial scale implementation. To address this, innovative approaches will be needed across the major processing steps including effective pretreatment techniques, improved SCO fermentation

and efficient downstream processing. Additionally, performing economic and life cycle analysis would be helpful to determine the profitability and sustainability of SCO platforms. Key areas of future research include:

- 1) Fermentation systems – to improve SCO productivity by developing novel fermentation techniques to meet the different demands of growth and oil accumulation;
- 2) Metabolic engineering – to increase oil yield and titer by using biochemical pathway engineering to enhance lipid biosynthesis and tailored lipid profiles for specialty oils;
- 3) Biomass fractionation – to produce high quality sugar streams with minimal inhibitors using novel pretreatment methods;
- 4) Other promising wild-type oleaginous yeasts – to identify and characterize robust and efficient yeast species and strains for industrial-scale SCO production;
- 5) Downstream processing – to improve oil extraction and purification by adopting efficient, cost-effective and sustainable extraction systems; and
- 6) Sustainable process development – to determine the economic and environmental impact of a renewable SCO biochemical platform by performing economic and life cycle analyses.

### **Final thought**

Oleaginous yeast are remarkable microorganisms capable of producing some of the highest oil contents across the eukaryotic kingdom. Their ability to utilize a wide range of substrates including hemicellulose makes them highly valuable for the future of sustainable bioprocessing. It would be a real shame if this gluttonous fungi's ability to produce noncrop-based renewable oil was left untapped!



**Figure 7.1 SCO as a key biochemical platform for the bioconversion of biomass to renewable products.**

## Appendix A - Chapter 3 calculations

### Relative fatty acid percentage

The relative percentage of each fatty acid was determined using the amount of each fatty acid methyl ester (FAME) over the total amount of all FAME. Amount (nmol) of each FAME was determined using the proportion of peak area (integrated using GCsolution software) for each FAME to peak area for the known internal standard (C15:0):

$$\begin{aligned} & \text{Fatty acid amount (nmol)} \\ &= \frac{\text{nmol of internal standard} * \text{MW of internal standard} * \text{Area of FAME peak}}{\text{Area of internal standard peak} * \text{MW of FAME}} \end{aligned}$$

For example,

To calculate the relative percentage of C16:0, use a C16:0 (MW = 270 g/mol) peak area of 200000, a C15:0 (MW = 256 g/mol) peak area of 20000 and an internal standard amount of 25 nmol.

$$C16:0 \text{ (nmol)} = \frac{25 \text{ nmol} * 256 * 200000}{20000 * 270} = 2370.37 \text{ nmol}$$

Using the calculated C16:0 and the total amount of FAME (summation of individual FAME amounts), the relative percentage can be determined:

$$C16:0 \text{ (\%)} = \frac{C16:0 \text{ amount (nmol)}}{\sum \text{ FAME amount (nmol)}} = \frac{2370.37}{12000} = 19.75\%$$

## Elemental balance

### *Molecular weight of oil*

The molecular weight of oil (product) was calculated using the fatty acid profile of total lipid extracted from *Lipomyces starkeyi* averaged across four different fermentations (Glucose, Xylose, Glucose:Xylose (1:1), GSXF) at 120 h. The molecular formula for the average fatty acid was calculated using the spreadsheet below:

Fatty Acid	%, Total FA	Carbon	Hydrogen	Oxygen	C	H	O
C14:0	0.0523	14	28	2	0.00732	0.014646	0.001046
C16:0	20.1241	16	32	2	3.21984	6.439697	0.402481
C16:1	4.3002	16	30	2	0.688035	1.290066	0.086004
C18:0	4.4985	18	36	2	0.809721	1.619442	0.089969
C18:1	65.4610	18	34	2	11.78298	22.25674	1.30922
C18:2	3.7713	18	32	2	0.678839	1.206825	0.075427
C18:3n3	0.9648	18	30	2	0.173658	0.28943	0.019295
C20:0	0.2708	20	40	2	0.054167	0.108334	0.005417
C20:1	0.2112	20	38	2	0.042245	0.080266	0.004225
C22:0	0.3131	22	44	2	0.06888	0.137759	0.006262
				<b>Summation</b>	<b>17.5257</b>	<b>33.4432</b>	<b>1.9993</b>

Relative contribution of carbon, oxygen and hydrogen to the average molecular weight was calculated using:

$$\frac{\%, Total\ FA * atoms\ of\ C, H, or\ O}{100}$$

For example:

To determine the contribution of C18:1 to the carbon balance:

$$C18:1 = \frac{65.4610 * 18}{100} = 11.78$$

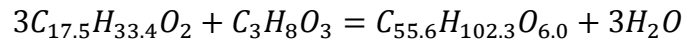
Summing each C, H, and O provides the molecular formula for the average fatty acid.

Average fatty acid = C<sub>17.5</sub>H<sub>33.4</sub>O<sub>2</sub>

$$\text{MW} = 275.74 \text{ g/mol}$$

Using the average fatty acid molecular formula, the molecular weight of oil can be calculated.

It is assumed that the oil is triacylglycerol (TAG): 3 fatty acids esterified to a glycerol backbone:



Molecular formula for oil =  $C_{55.6}H_{102.3}O_{6.0}$

Average MW of oil = 865.25 g/mol



## Appendix B - Chapter 4 calculations

### Quantification of TAG and FFA

The neutral and polar lipid fractions were assumed to be the only constituents of the total lipid content (i.e. the sum of neutral fraction and polar fraction will be 100%). The neutral fraction and polar fractions were used to calculate the amount of polar and neutral lipids for comparison purposes across fed-batch fermentations.

$$\text{Neutral Fraction (\%)} = \frac{\text{Neutral Lipid wt. (mg)}}{\text{Neutral Lipid wt. (mg)} + \text{Polar Lipid wt. (mg)}}$$

Peak area for each fatty acid was normalized based on total carbon number to account for the flame ionization detector (FID) signal increase as a function of carbon number (e.g. C15:0 would be normalized to 15 carbons, C16:0 to 16 carbons, and so on). The fatty acid content was determined using the proportion below:

$$\text{Fatty acid content (mmol)} = \frac{\text{mmol of internal std} * \frac{\text{Area of FAME peak}}{\text{FAME Carbon \#}}}{\frac{\text{Area of internal std peak}}{\text{Internal Std Carbon \#}}}$$

Fatty acid weight was determined using the fatty acid content (mmol) and the molecular weight.

$$\text{Fatty acid amount (mg)} = \text{Fatty acid amount (mmol)} * \text{MW Fatty acid} \left( \frac{\text{mg}}{\text{mmol}} \right)$$

If calculating TAG, the fatty acid content (mmol) was converted to TAG by dividing by 3 (3 mol of fatty acid per 1 mol of TAG) and using the molecular weight of the TAG molecule.

$$\text{TAG amount (mg)} = \frac{\text{Fatty acid content (mmol)}}{\frac{3 \text{ mmol Fatty acid}}{1 \text{ mmol TAG}}} * \text{MW TAG} \left( \frac{\text{mg}}{\text{mmol}} \right)$$

It should be noted that the TAG amount is only for one species of TAG (i.e. TriC16:0, TriC18:0, etc.); thus, the TAG amount was summed prior to determining the total amount of TAG. The total amount of TAG per dry cell weight was determined by correcting for any dilution factors, and using the neutral content of the total lipid fraction and the total lipid fraction of the dry cell weight.

$$\begin{aligned} \text{Total TAG} & \frac{\text{mg}}{\text{g dry cells}} \\ & = \frac{\sum \text{TAG Amount (mg)} * \text{Dilution Factor}}{\text{Neutral Lipid wt (mg)}} * \frac{\text{Neutral Lipid wt (mg)}}{\text{Total Lipid wt (mg)}} \\ & * \frac{\text{Total Lipid wt. (mg)}}{\text{Dry Cell wt. (g)}} \end{aligned}$$

For example, after analysis of a 50uL sample from 1000uL of extract containing 10mg of neutral lipid, the total amount of TAG present was 350ug. The amount of TAG in dry cell mass that contains 350mg of total lipid that is 90% neutral lipid is calculated below:

$$\begin{aligned}
 &= \frac{0.35mg \text{ TAG} * \frac{1000uL \text{ Extract}}{50 uL \text{ sample}}}{10mg \text{ Neutral Lipid wt.}} * 90\% \left( \frac{\text{Neutral Lipid mg}}{\text{Total Lipid mg}} \right) * \frac{350mg \text{ Total Lipid}}{\text{Dry cell wt. (g)}} \\
 &= 220.5 \text{ Total TAG} \left( \frac{mg}{g \text{ dry cells}} \right)
 \end{aligned}$$