Characterization and Comparison of Different Oleaginous Yeasts and Scale-Up of Single-Cell Oil Production Using *Rhodosporidium diobovatum*

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

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Abstract

Oleaginous yeasts are able to produce a high percentage of their weight as lipids, which can be used as the starting material for biodiesel production, producing a fuel with many of the same properties as petroleum-based diesel. The objective of this research was to compare three oleaginous yeast species, *Rhodosporidium babjevae*, *Rhodosporidium diobovatum*, and *Yarrowia lipolytica* to determine which species would be the best candidate for larger-scale production. Using a nitrogen-limiting medium, the three species were compared in terms of their rate of growth, highest cell densities, and lipid production capabilities, while simultaneously analyzing the composition of the lipids produced.

Following the comparison work, it was determined that *R. diobovatum* was the best candidate for scale-up. Subsequent experiments used batch cultures in bioreactors at a volume of 3.5 L, followed by a 25x fold increase to 90 L production. The results of this scale-up showed that the high levels of production and growth continued in a reactor system. As such, *R. diobovatum* could be a possible organism to use in the production of lipids from waste glycerol for biodiesel production.

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List of Abbreviations

FAME	Fatty acid methyl ester
TAG	Triacylglyceride
C16, C18	Number of carbon units in the fatty acid chain of the FAME (C16 = 16 carbon units)
C18:1, C18:2	Unsaturated FAMEs (Second number indicates the presence and number of double bonds)
NaOH	Sodium hydroxide
KOH	Potassium hydroxide
SCO	Single-cell oil
CO_2	Carbon dioxide
cdw	Cell dry weight
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
OD_{600}	Optical density measured at 600 nm
GC	Gas Chromatograph
HPLC	High-performance liquid chromatography
CE	Cetane number
IO	Iodine value

SA	Saponification value
LCS	Long-chain saturation factor
CFP	Cold filter plugging point
REG80	Biodiesel-derived waste glycerol used in this study
PBS	Phosphate buffer solution
PI	Post inoculation

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Chapter 1: Lipid Production for Biodiesel Using Single-Celled Organisms

1.1) The call for alternate fuel sources

Clean alternative fuels have been explored for many years, most commonly as a result of environmental concerns. Recently, however, fluctuating prices of crude oil caused an even greater call for renewable and predictable sources. Though the price of crude has recently dropped, due to increased worldwide production and the implementation of new technologies to extract oil from previously untapped sources (such as the Alberta tar sands or via hydraulic fracturing), this has highlighted the volatility of the oil market and the devastating effects it can have on the global economy. Many oil-producing countries are now facing deficits where they were once predicting a budget surplus. The Conference Board of Canada is now predicting a loss of $4.5 billion in 2015 from provincial royalties. Despite the current low prices, costs will eventually increase again. Biofuels could help alleviate the dependence on foreign oil sources and minimize the impact shifting global economies will have on the domestic markets.

Environmental concerns have also been a driving force behind the search for alternative energy sources, as research indicates that greenhouse gasses produced largely by the burning of fossil fuels have had a dramatic impact on global warming (Montzka et al., 2011). Higher levels of carbon dioxide in the atmosphere have resulted in some of the hottest years on record since 1880 (NASA, 2015). Even prior to the burning of the fuel, extraction of crude oil has had widespread environmental effects as well. Ranging from earthquakes as a result of hydraulic fracturing in Oklahoma (Keranen et al., 2014), to the energy intensive processes used in Alberta to extract and purify the oilsands, many of the processes used to extract the oil have widespread environmental impacts. As well, catastrophic failures of equipment used to extract or transport

oil have damaged entire ecosystems and cost billions of dollars in damages. Most notably in recent years have been the oil released into the Gulf of Mexico, causing upwards of $40 billion in damages and unimaginable environmental effects, and recent train disasters in Lac-Megantic, Quebec resulting in fires, environmental damage, and loss of life (Lin & Mendelssohn, 2012).

In short, biofuel production has increased as a result of a combination of environmental and economic concerns associated with petroleum-based fuel extraction and use.

1.2) Biofuels

In response to the environmental and economic stresses caused by petroleum fuels and their production, many governments have implemented policies dictating a minimum amount of renewable fuels to be blended into the petroleum counterparts. This reduces the overall volume of petroleum fuels used while ensuring a market for biofuel producers. In Canada, the federal government has mandates through the 'Renewable Fuels Regulations' that gasoline must contain at least 5% ethanol, and diesel must be blended with at least 2% biodiesel (NRC, 2010). However, many provinces have mandates requiring even higher biofuel blends be used. For example, Manitoba mandates that gasoline be 8.5% ethanol.

The sources of these biofuels can vary, depending on both regional and environmental conditions. Ideally, producers would want to utilize readily available materials to reduce capital costs and simplify logistics. The majority of biofuels being produced fall under the category of 'first-generation' biofuels, while current research is advancing the production of 'second-generation' biofuels. The increased demand and interest in biofuels has resulted in increased research worldwide, ranging on topics from new sources to improved production/refining.

1.2.1) First-generation biofuels

First-generation biofuels are commonly biodiesel and bioethanol produced using commodities that can also be used for food. First-generation biodiesel is most commonly derived from vegetable oils, such as canola, soy, or sunflower, and these oils are transesterified into fatty acid methyl esters (FAMEs) (Figure 1). Transesterification is a step-wise processes whereby a triacylglyceride (Samori et al.) molecule reacts with a short alcohol (usually methanol) in the presence of a catalyst (strong base – NaOH or KOH) and heat. The process is driven to completion due to the fact that the FAMEs that are produced are not miscible with glycerol, meaning the reversible reactions are highly unlikely to occur. As well, the reaction equilibrium is heavily weighted towards the production of the end-products, as according to Le Chatelier's principal when the product of one of the reactions becomes the substrate for the next step, the reaction is driven to completion (Moser, 2011). The end result of this is that for every mol of TAGs at the start of the reaction, three mols of FAMEs and one mol of glycerol are produced (Figure 1). These FAMEs can then be used in a blend with petroleum diesel without any modification of conventional diesel engines, or with some slight modification to an engine, the FAMEs can be used without any blending and cause no adverse effects on the engine.

Bioethanol, a substitute for gasoline, is primarily produced using easily fermented crops such as corn or sugarcane. However, the use of these commodities comes with higher material costs, which increase the price of the resulting bioethanol. Cellulose is being investigated as a potential feedstock for bioethanol production, given the abundance of waste streams containing cellulose. However cellulose must first be digested into shorter sugar chains in order to be fermented into alcohols. As cellulose has a strong, rigid structure, one or more enzymes, alkali treatment, or mechanical processes are required to convert it into a more easily metabolized form

(Gao et al., 2014; Rosgaard et al., 2007). Once the chains have been converted into sugars, they can be fermented into ethanol by microorganisms and subsequently used as fuel. Alternatively, the ethanol can be used as a feedstock to produce ethyl-*tert*-butyl ether, another compound that can be added to gasoline that displays certain advantages over ethanol itself (Cataluña et al., 2008). Another alcohol currently being investigated for its possibility of being blended into gasoline is butanol. As a four carbon alcohol, butanol causes less wear and tear in an engine and also has a higher energy density when compared to ethanol. Bio-butanol can be produced via anaerobic fermentation of cellulosic wastes, and it is also possible to blend butanol into gasoline at any ratio (Dürre, 2007; Gao et al., 2014).

The use of food crops to produce fuels has unintended consequences. For example, a debate surrounding the use of food crops to produce fuel in the midst of a global food crisis (food vs. fuel) has begun (Headey & Fan, 2008). There are groups that believe the highly visible allocation of food resources into fuel production reflects poorly on developed society. Additionally, using highly-desired food resources as the feedstock for biofuel production increases the price of food regardless of whether it is used for biofuel production or consumption. As such, the search for alternate processes and feedstocks has subsequently resulted in increased research into second-generation biofuels.

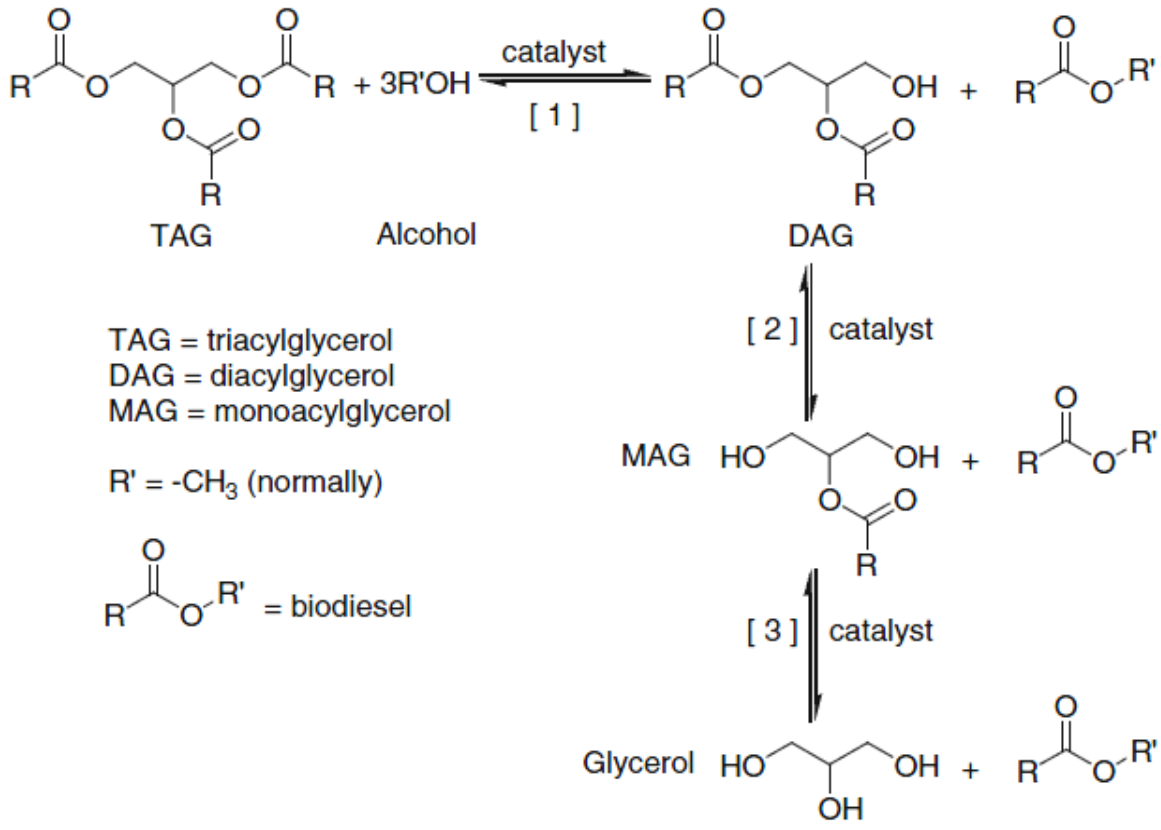

Figure 1: Schematic overview of biodiesel production from TAGs (Moser, 2011).

1.2.2) Second-generation biofuels

Second generation biofuels are fuels produced using non-conventional sources, specifically non-food sources. The use of alternate sources allows for the use of 'waste' materials as the primary feedstock for the various processes that are currently using 'food' products. Some of these sources can be the non-edible components of existing food sources, such as the cellulose in the cob of corn (Gao & Rehmann, 2014), or materials completely unrelated to food sources such as cattails (Zhang et al., 2012). This both eliminates the use of food crops for fuel production, and can lower costs involved in the fuel production, thereby allowing biofuels to be competitive with petroleum-based products.

1.3) Single celled biodiesel

In the field of biodiesel, growing interest in using single-celled organisms to produce biofuels has resulted in new technologies. A distinct advantage to using cellular organisms for biodiesel production is that very little, if any, agricultural land is used. Research on producing oils from single-celled organisms (Single-celled oils – SCO) focuses primarily on algae, bacteria, and yeast cells.

1.3.1) Second generation biodiesel from microalgae

Microalgae are being explored as an alternative, renewable source of biodiesel. Many species of algae are obligate phototrophs – requiring light to grow and conduct photosynthesis, and are able to photosynthesize at a rate comparable to most plants found on land (Pirt, 1986). This, in turn, removes CO_2 from the atmosphere and turns it into lipids which can be processed into biodiesel, making this process even more attractive in an environmental context. The

process of growing algae for biodiesel production using only light and atmospheric CO_2 is referred to as autotrophic growth. Some other algae species are able to utilize carbon sources for growth in the absence of light, called heterotrophic growth, and are grown in a conventional bioreactor. However, species of algae that can grow as obligate heterotrophs are rare, though bioprospecting has been identifying more species that are attractive for heterotrophic growth. A combination of the two growth strategies is referred to as mixotrophic growth, utilizing both a carbon source as well as photosynthesis to produce lipids. Proposals have been made to trap escaping CO_2 from flue gases in power plants and use it in mixotrophic microalgae biodiesel production, thus reducing the production of greenhouse gases while providing an inexpensive carbon source for the microalgae growth (Patil et al., 2008).

Large-scale growth of autotrophic or mixotrophic algae requires specialized reactor systems that can account for the light requirements specific to photosynthetic organisms. Two distinct systems have been developed: closed and open systems. In a closed system, where the amounts of light, CO_2, and other nutrients are all directly controlled and monitored, the production costs will be higher when compared to using an open system, though risks of contamination are lower and production is far more tightly controlled. An open system is similar to a pond or a ditch in design, and is open to the air and sunlight (which acts as the source of energy for photosynthesis). For large-scale production of lower value products, an open system is economically more favourable (Weisz, 2004). Aeration and CO_2 are obtained largely from the water-surface interface, with additional nutrients and CO_2 being added in as required. Typically, 1 kg of algae is capable of fixing 1.6-1.8 kg of CO_2 (Patil et al., 2008). However, the open design has its own noticeable drawbacks, such as lack of environmental control (being open to the elements), introduction of possible contaminants, and high energy costs required to remove the

water from the cellular biomass. In either growth strategy, achieving high cell densities is difficult due to the eventual occurrence of cell shading – cell densities become too high for light to reach some cells.

1.3.2) Second generation biodiesel from bacteria

Certain bacteria are also capable of producing large amounts of TAG molecules. Species such as *Acinetobacter calcoaceticus*, *Rhodococcus opacus*, and *Arthrobacter sp.* can produce from 20% to >40 % dry cell weight in TAG forms (Meng et al., 2009). The advantages to using bacterial species are that they have an extremely rapid doubling time and can grow to high densities, and do not require a light source. However, a problem when using bacteria for TAG production is the production of 'unusual' TAG molecules that are unusable as a fuel source. These may take the form of branched fatty acids (4,8,12-trimethyl tridecanoic acid, for example) or aromatics (phenyldecanoic acid has been detected) (Alvarez & Steinbüchel, 2002). As a result of these differences, most bacteria are used for the production of polyunsaturated fatty acids, or branched-chain fatty acids rather than TAG molecules (Patnayak & Sree, 2005).

Howard et al. worked with up-stream processing in *E. coli* for biodiesel production (Howard et al., 2013). Via genetic modification, the profile of end-products, specifically alkanes, was altered to reduce the amount of heptadecene (C17:1), which is unusable as a fuel source. In its place, alkanes and alkenes commonly found in diesel were produced. Though this is very early work, it demonstrates the viability of bacterial systems for biodiesel production, though genetic modification and other upstream processing will be required to optimize the FAME profile produced for biodiesel production.

1.4) Second generation biodiesel from yeast

To date, over 40 species of yeast have been identified as oleaginous, spanning both the ascomycete and basidiomycete genera (Sitepu et al., 2013). Oleaginous yeasts differ from non-oleaginous yeast species by storing excess carbon in the form of TAGs, while non-oleaginous species primarily store excess carbon as polysaccharides (Ageitos et al., 2011). These TAGs can then be converted to FAMEs via methylation, the same method currently used for conventional biodiesel production. Yeast do not have the light requirement as algae would, allowing them to grow in conventional bioreactors to high cell densities, and are still able to metabolize a number of carbon sources. The lipids produced are very similar to the ones found in vegetable oils, which would allow for easy utilization of the resultant biodiesel in a conventional diesel engine (Munch et al., 2015).

There are many species of oleaginous yeast that have been examined for their lipid production potential. Recently, Sitepu et al. (2013) compared sixty-nine different oleaginous yeast strains when grown on dextrose under both nutrient-rich and nitrogen-deficient conditions. It was observed that lipid production was increased under nitrogen-deficient conditions in most oleaginous species.

1.4.1) Nutrient starvation to increase lipid production

While oleaginous yeasts are naturally able to accumulate over 20% of their cell dry weight (cdw) as lipids, there are methods to cause this number to increase without using expensive and difficult genetic manipulation. The synthesis and accumulation of intracellular lipids in oleaginous yeasts generally commences once an essential nutrient is exhausted from the medium. Most commonly, nitrogen is deprived to induce lipid accumulation, though phosphorus

or sulphur limitations have also been demonstrated to induce lipid synthesis in *Rhodosporidium toruloides* (Wu et al., 2010; Wu et al., 2011). Prior to nutrient exhaustion, the lipids formed are mostly polar and are likely found in the cellular membranes (Fakas et al., 2008). Once extracellular nitrogen is exhausted from the medium, intracellular levels of AMP quickly decrease, leading to the enzyme iso-citrate dehydrogenase being inactivated. The result in non-oleaginous organisms is large amounts of citrate and iso-citrate being generated and either secreted from the cell or stored in the cytoplasm. However, oleaginous organisms have a key enzyme not found in non-oleaginous organisms, ATP-citrate lyase. This enzyme cleaves citric acid into acetyl-CoA and oxaloacetate, thereby generating the acetyl-CoA building blocks which are used to synthesize intracellular lipids.

Acetyl-CoA molecules (found in the cytosol) serve as the starting point for fatty acid synthesis. The fatty acid chain is elongated through the addition of malonyl-CoA molecules, each addition requiring the use of two NADPH molecules for energy. Once the fatty acid chains are the appropriate length, TAG synthesis follows through what is known as the Kennedy pathway (Kennedy, 1961), and has been extensively reviewed elsewhere (Beopoulos et al., 2009). Certain genes involved in the synthesis, as well as genes involved in the breakdown, of TAG molecules have been examined as possible targets for genetic manipulation to increase TAG yields in species such as *Y. lipolytica* (Tai & Stephanopoulos, 2013).

1.4.2) Low-cost carbon substrates to grow oleaginous yeast

In SCO production, as with production of many other bioproducts, the use of low-cost or waste substrates is a strategy used to reduce overall production costs. Though using pure substrates would allow for tighter control over the medium and carbon to nitrogen (or another

essential nutrient) ratio, use of pure-substrates would be cost-prohibitive. As a result, carbon sources such as cheese whey waste, olive mill waste water, and biodiesel-derived glycerol have all been explored as low-cost substrates for oleaginous yeast growth (Chatzifragkou et al., 2011; Gonçalves et al., 2009; Vamvakaki et al., 2010). As mentioned in section 1.2.1, the process of methylation of vegetable oils to produce biodiesel (in the form of FAMEs) also produces an impure form of glycerol. This waste substrate has been explored extensively as a viable carbon source for yeast growth (Chatzifragkou et al., 2011; Papanikolaou & Aggelis, 2002; Xu et al., 2012a).

One of the major complications of using waste products as substrates is the presence of impurities, which can be toxic to the cells or alter growth conditions. In the case of biodiesel-derived waste glycerol, the impurities present are mostly water, fatty acid residues, leftover base (KOH or NaOH), and various salts and trace compounds. Xu et al. (2012a) published a detailed study investigating the effects of several individual impurities present, and found that several actually improved lipid production in *R. toruloides*. For example, trace amounts of oleic acid present in the growth medium is believed to act as a surfactant during growth, allowing more efficient nutrient uptake. This results in increased biomass when grown on waste-glycerol when compared to pure glycerol.

The search for lower cost substrates and medium components is not limited to only the carbon sources. Yang et al. conducted a study demonstrating the possibility of 'recycling' yeast cell mass hydrolysates following lipid extraction, as well as the remaining growth medium, to feed further growth of the same type of cells (Yang et al., 2015). The process was repeated three times before the concentration of harmful metabolites in the growth medium became too high to

sustain growth. However, techniques such as this could provide further measures reduce the production cost of biodiesel, allowing for more competitive prices on the market.

1.4.3) Scale-up of SCO using oleaginous yeast

Another component to the cost-effective production of biodiesel using SCO is the issue of scale. As mentioned in section 1.4, cultivation of yeast species does not require one to address issues such as light requirements of the cells. However, this does not mean that scale-up of processes is a simple, linear process. More cells require more oxygen for aerobic fermentation. Therefore, providing adequate oxygen to the cells at high densities and during high lipid production is an essential requirement (Garcia-Ochoa & Gomez, 2009). Proper mixing levels that move the cells throughout the reactor while avoiding high enough shear stresses that could damage the cells must be found. Ideally, proper mixing would avoid any heterogeneities within the reactor itself, keeping the entire culture homogenous in regards to both nutrient and oxygen transfer. Sparging levels must also be high enough to provide enough oxygen, yet not generate damaging levels of pressure or excessive foaming. All of these considerations must be addressed, as well as others while moving up in scale, as the stresses of increased scale can cause stress-triggered changes in cells basic metabolism (Schmidt, 2005).

The majority of current work being done with oleaginous yeast has been at the laboratory scale, using bioreactors from 5 to 20 litres in volume (Galafassi et al., 2012; Li et al., 2007; Xu et al., 2012a). Multiple culturing conditions and strategies have been attempted with various species of yeast, with promising results. Li et al. (2007) achieved cdw of 151.5 g/L when using *R. toruloides* and a fed-batch strategy. These cells also had a lipid content of 67.5%, resulting in an overall lipid concentration of 102.3 g/L (Li et al., 2007). Larger-scale studies would be at the

pilot-scale level, and would require specialized reactor and monitoring equipment not found in many laboratory settings. Xu et al. (2012a) grew *Rd. glutinis* at a 300 L scale, using starch wastewater as the carbon substrate, with promising results. In a semi-continuous culture, the resulting biomass was 40 g/L containing 35% lipids.

However, aside from complications involving scale-up itself, another problem is the downstream processing of such large volumes of cell solution, much the same as encountered when using algae for SCO production. Concentrating and drying the biomass in order to extract and process the lipids is currently a major bottleneck in the process (Singh et al., 2011). Centrifugation at such large volumes is far too energy-intensive, which eliminates lyophilisation as a means of storing the biomass. Filtration has been considered as an option, however membrane fouling is a common occurrence at such large volumes (Christenson & Sims, 2011).

The objectives of this thesis research were to compare three oleaginous yeast species in order to identify which species would be the highest producer of lipids suitable for biodiesel production, and the subsequent scale-up of lipid production using that species to larger scales to determine if the changes in the system negatively effects produciton.

Chapter 2: Materials and Methods

This chapter contains descriptions of the materials and methods common to all experiments in subsequent chapters. Each chapter contains its own materials and methods section specific to that experiment; however common methodologies will be referred back to the appropriate section of this chapter.

2.1) Microorganisms and media

All reagents and media components were obtained from Sigma-Aldrich (Oakville, Ontario, Canada) or Thermo Fisher Scientific (Markham, Ontario, Canada), unless otherwise stated. *Rhodosporidium diobovatum* 08-225 (isolated from sea water off the coast of Florida) and *Rhodosporidium babjevae* 05-775 (isolated from drying sap on an olive tree in Winters, CA) were graciously donated by the Phaff Yeast Culture Collection, at the University of California at Davis (UCDFST). *Yarrowia lipolytica* 20460 was obtained from the American Type Culture Collection (ATCC). Cultures were maintained on agar plates containing 10 g/L yeast extract, 20 g/L peptones, 40 g/L glycerol and 10 g/L agar, grown at 30^{o}C and stored at 4^{o}C.

Some experiments were conducted using YPD medium, containing 10 g/L of yeast extract, 20 g/L peptones, and the carbon source. The other medium used was GMY medium consisting of 3 g/L yeast extract, 8 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \bullet 7H_2O$, and the carbon source, pH adjusted to 5.5 prior to sterilization as described by Amaretti et al., 2010.

2.2) Growth and lipid experimental set-up

Pre-cultures were grown from a single colony of each species for 24 hours, then used to seed growth flasks with a volume of inoculum resulting in an initial optical density of 0.02

(OD_{600}) measured using a Thermo Fisher Biomate 3 Spectrophotometer (CAT 335905) at a wavelength of 600 nm. Growth curve experiments were conducted using 100 mL of medium, with samples taken every three hours following inoculation and OD measured at 600 nm. Experiments were done in triplicate. Shake-flask experiments were conducted using 100 mL of medium in 500 mL Erlenmeyer flasks incubated at 30^{o}C and 150 RPM for the entire duration.

Experiments using biodiesel-derived waste glycerol were done using GMY medium containing 60 g/L of waste glycerol donated by the Renewable Energy Group in Danville, Illinois in GMY medium. This waste substrate, containing 78–86% glycerol, 0.3% methanol, 6–13% water, 7% ash, and 1% fatty acids, was a by-product of vegetable oil-based biodiesel production.

2.3) Lipid composition determination via Gas Chromatograph (GC)

Lipids were extracted and converted to FAMEs using a modified procedure outlined by Bligh and Dyer (Bligh & Dyer, 1959). Pyrex reaction vials containing 10 – 20 mg dried biomass, 2 mL of a 15% H_2SO_4:methanol solution, and 2 mL of a solution of 0.5 mg/mL heptadecanoic acid internal standard (Nu-Chek Prep Inc., USA) in chloroform were placed in a boiling water bath for 5 hours. Reactions were quenched by addition of 1 mL of double distilled H_2O (ddH_2O) and left overnight. A 1 mL aliquot of the FAMEs in chloroform was extracted, evaporated under nitrogen, re-suspended in 300 µL of Hexane, and analyzed using a Varian 450 GC with a DB225MS column (30 m X 0.25 mm diameter X 0.25 µm film thickness; Agilent Technologies Canada Inc.). GC analysis of the FAMES was conducted by Dennis Labossiere from the University of Manitoba Department of Human Nutritional Sciences at the University of Manitoba.

2.4) Determination of residual nitrogen and glycerol in supernatants

Supernatants were analyzed in some experiments to determine the concentrations of ammonium and glycerol remaining. Ammonium was measured using a QuikChem 8500 Flow Injection Analyzer (Lachat Instruments), and the data was processed with Omnion software (Lachat Instruments). Nitrogen depletion was confirmed using a Total Nitrogen Reagent Set (HACH product number 2714100). Glycerol was measured using a Waters HPLC, using a 300 mm x 7.8 mm HPX-87H Ion Exclusion column (Aminex) with 5 mM H_2SO_4 mobile phase, and the data was processed with Breeze 2 software (Waters).

2.5) Mathematical estimation of biodiesel properties using FAME profiles

Following GC analysis of the FAMEs, the molecular structures of the FAMEs were used to estimate several important properties of biodiesel made with oil derived from these oleaginous yeast strains. Cetane number (CE), iodine value (IO), the saponification value (SA), and the cold filter plugging point (CFP) were calculated using previously derived equations (Krisnangkura, 1986; Nascimento et al., 2013). In brief, the value of the CE was estimated using both the IO value as well as the SA. The IO and SA were estimated using equations 1 and 2, where P is the percentage of each individual FAME component by weight, M is the molecular mass of the FAME, and D is the number of double bonds present in the FAME:

$$SA = \Sigma(560 \text{ x } / M) \quad (1)$$

$$IO = \Sigma(254 \text{ x } DP) / M) \quad (2)$$

Once these two values were obtained, equation 3 was used to estimate the CE:

$$CE = 46.3 + (5458 / SA) – (0.255 \text{ x } IO) \quad (3)$$

In order to calculate the CFP, the long-chain saturation factor (LCS) is required. It is estimated using equation 4:

$$LCS = (0.1 \times C16) + (0.5 \times C18) + (1 \times C20) + (1.5 \times C22) + (2 \times C24) \quad (4)$$

Using this value, the CFP can be estimated according to equation 5:

$$CFP = (3.1417 \times LCS) - 16.477 \quad (5)$$

Chapter 3: Characterization and comparison of different oleaginous yeasts

3.1) Introduction

This section is devoted to the initial characterization of the two under-characterized *Rhodosporidium* species, and the comparison between *R. babjevae, R. diobovatum,* and *Y. lipolytica* in terms of biomass and lipid production.

3.1.1) Comparative analysis of oleaginous yeasts

Three yeast species were selected, based on the work of Sitepu et al. (2014), to be compared in terms of overall biomass and lipid production. The initial work was a carbon screening, done to determine which carbon sources each organism was capable of metabolizing. The broader the range of metabolism, the wider array of waste products can potentially be used. Following the carbon screen, the yeast were next compared by lipid and biomass production when grown on first pure glycerol, and subsequently biodiesel-derived waste glycerol (referred to hereafter as REG80). The use of REG80 in lieu of pure glycerol is intended to keep production values down as it is far less expensive. The three species selected were *Rhodosporidium babjevae*, *Rhodosporidium diobovatum,* and *Yarrowia lipolytica*. In a previous study, these three species were some of the highest producers of biomass and lipids (Sitepu et al., 2013). However, this study represents the first time an in-depth comparison of the three species in terms of their growth rate, lipid and biomass production potential on pure glycerol as well as REG80, profile of FAMEs produced, and carbon utilization efficiency was conducted. Despite using waste carbon

substrates, it is still advantageous to utilize as much of the available carbon for biomass growth and desirable end-products as possible.

Lipid production in oleaginous yeast is a secondary metabolite, meaning that it is mostly produced following the exponential phase of growth, therefore rapid growth could allow for shorter batch runs and decreased turnover time. High biomass and lipid production capabilities, combined with efficient use of the available carbon in the medium, will allow for higher yields during these runs.

3.2) Research Objectives

In the following experiments, the three yeast species were compared when grown in shake-flasks, based on the criteria of substrate utilization range, growth rate, biomass and lipid production capabilities, carbon utilization efficiency, and FAMEs produced. The objective of this comparison is to identify the top candidate for further scale-up of production.

3.3) Materials and Methods

3.3.1) Microorganisms and media

Microorganisms and media are all as described in Section 2.1.

3.3.2) Carbon Screening

Tubes containing 4 mL of water were autoclaved, followed by addition of 0.5 mL filter-sterilized 10x Difco™ Yeast Nitrogen Base (BD catalogue #239210). A 10x stock of each carbon source was prepared and filter sterilized, with 0.5 mL of a single carbon source being added to a single tube (final tube volume of 5 mL). Carbon sources examined were dextrose,

glycerol, xylose, xylan, cellulose, lactose, lactic acid, arabinose, acetate, galactose, and REG80. Non-soluble carbon sources (cellulose and xylose) were autoclaved for sterilization. Concentrations of each carbon source were adjusted to match the carbon found in 5 g/L dextrose. A negative control was created by adding an additional 0.5 mL filter sterilized water in lieu of a carbon source. Pre-cultures were grown in 250 mL baffled Erlenmeyer flasks containing 50 mL YPD media (10 g/L yeast extract, 20 g/L peptones, 10 g/L dextrose) for 24 hours before being centrifuged at 4500 rpm (4190 x g) for 5 minutes, washed with 1x phosphate buffer solution (PBS) buffer, recentrifuged, then suspended again in 1x PBS to prevent carry-over of nutrients. A 50 µL inoculum was added to each tube, incubated for 96 hours, then passaged to a tube containing the same media and incubated another 96 hours. Growth was reported positive if medium in the second tube became turbid.

3.3.3) Growth, Lipid and Biomass production experiments

Experiments were conducted as described in Chapter 2.

3.3.4) Analytical Methods

Analysis of the biomass, FAMEs, and supernatant were all as described in Chapter 2. Estimation of biodiesel properties was done as described in Chapter 2.

3.4) Results and Discussion

3.4.1) Carbon Screen

Several carbon sources were chosen in order to include common substrates used in different studies, and include a broad range of molecule size (from 2 carbon molecules to

complex polysaccharides). *Y. lipolytica* and *R. babjevae* showed the most diverse range of substrate utilization (4 of the 10), while *R. diobovatum* was able to grow on three (Table 1). Results from Sitepu et al. (2014) show that both *Rhodosporidium* species are also able to metabolize other carbon sources such as cellobiose, mannose, and galactose. Further research could uncover the ability of these organisms to utilize a variety of carbon-rich waste products as a source of nutrients for low-cost production at a larger scale in various regions.

Table 1: Ability of *R. babjevae, R. diobovatum,* and *Y. lipolytica* to metabolize and grow on various carbon sources. See text for criteria used to derermine if growth occurred (+) or did not (-).

	Glucose	**Glycerol**	**Xylose**	**Xylan**	**Cellulose**	**Lactose**	**Lactic acid**	**Arabinose**	**Acetate**	**Galactose**
R. babjevae	+	+	+	-	-	-	-	-	-	+
R. diobovatum	+	+	-	-	-	-	+	-	-	-
Y. lipolytica	+	+	-	-	-	-	+	-	+	-

3.4.2) End-point biomass and lipid production

All three yeast species were originally grown on rich YPD medium in order to determine the upper limits of biomass production for comparison with the minimal media tested. A control culture of GMY medium containing no glycerol (GMY^-) was examined to determine the effect of nutrients found in the yeast extract on culture growth and lipid production.

While the culture in YPD medium had significantly higher biomass, typically more than twice what was observed in GMY, the lipid production was reduced in all three species (Table 2). The GMY medium, while showing less than 50% biomass compared to YPD medium, had more than double the lipid production for *Y. lipolytica* and *R. babjevae*. *R. diobovatum* had the greatest difference in lipid production in GMY medium compared to YPD medium (63.66 ± 4.47 vs. 12.04 ± 0.82 % DCW, respectively). The high production of lipids in the GMY medium compared to the YPD medium indicates that GMY medium became nutrient-deficient at late stages of growth, despite carbon excess, causing cells to begin rapidly converting excess carbon into lipids. The low biomass produced when grown in GMY medium lacking any carbon source (GMY^-) indicates that the presence of the yeast extract in the medium did not provide a significant amount of carbon for growth, but was primarily a source of nitrogen and micronutrients.

Table 2. Biomass and lipid production by *R. babjevae* and *R. diobovatum* grown on various media. Results shown are the result of six independent replicates with the standard deviation shown.

	YPD medium		**GMY medium**		**GMY$^-$ medium**	
Yeast Species	**Biomass (g/L)**	**Lipid (% cdw)**	**Biomass (g/L)**	**Lipid (% cdw)**	**Biomass (g/L)**	**Lipid (% cdw)**
R. babjevae	14.42 ± 0.11	14.1± 0.79	9.39 ± 0.79	34.88 ± 3.05	0.70 ± 0.02	11.5 ± 0.04
R. diobovatum	16.45 ± 1.4	12.1 ± 0.82	10.96 ± 0.43	63.66 ± 4.47	0.90 ± 0.09	11.8 ± 0.94
Y. lipolytica	17.12 ± 0.36	10.9 ± 0.65	8.61 ± 0.66	28.81 ± 2.73	0.46 ± 0.2	9.83 ± 0.36

3.4.3) Comparison of growth curves between species on pure glycerol

The growth rates showed that both *Y. lipolytica* and *R. diobovatum* had similar growth patterns, both finishing exponential growth within 24 hours, while *R. babjevae* had a longer and more gradual exponential phase, taking up to 36 hours to reach stationary phase (Figure 2). The specific growth rates (in h^{-1}) for *R. babjevae, R. diobovatum,* and *Y. lipolytica* were 0.27, 0.51, and 0.88, respectively. Previous work has shown *R. babjevae* to have a slower growth rate than many other oleaginous yeasts (Sitepu et al., 2013). However, as TAGs are primarily produced once growth has ceased, reaching stationary phase in less time is desirable with respect to TAG production. For this reason, *R. diobovatum* and *Y. lipolytica* would be more favourable than *R. babjevae* for larger-scale production of TAGs, when considering growth rate.

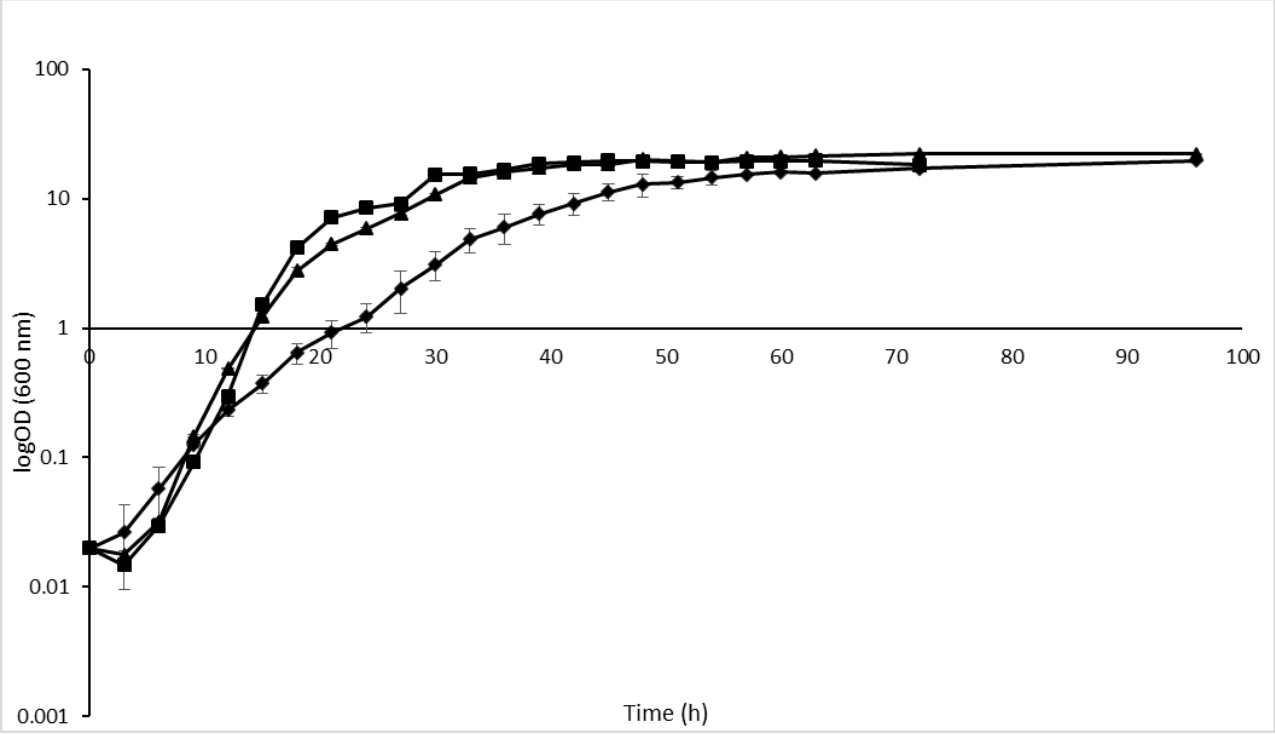

Figure 2: Growth curve analysis using OD (λ=600 nm) of *R. babjevae* (♦), *R. diobovatum* (▲), and *Y. lipolytica* (■) when cultivated on GMY media with pure glycerol as the carbon source.

3.4.4) Kinetics of biomass and lipid production

All three organisms were grown under identical conditions, allowing for a direct comparison of their growth, glycerol consumption, and lipid production in nitrogen limiting conditions. Nitrogen analysis showed the levels of free ammonia in the medium, which presumably are a result of hydrolysis of the proteins found in the yeast extract (no free ammonia was added to the medium). Total nitrogen analysis showed the medium initially contained 0.34 ± 0.04 mg/L nitrogen. In the case of *Y. lipolytica*, the lack of any detectable ammonia ions in the medium indicate the cells are most likely utilizing the ions as they are produced, or are able to use nitrogenous components in the yeast extract without releasing free ammonia. For both *Rhodosporidium* species, the depletion of the free ammonia in the medium occurred simultaneously with exhaustion of available nitrogen in the medium, thus marking an end to growth while the rate of lipid accumulation increased (occurring at 48 hours for *R. babjevae* and at 24 hours for *R. diobovatum*). In the case of *Y. lipolytica*, the loss of free ammonia did not coincide with depletion of nitrogen in the medium, confirmed using total nitrogen analysis, although nitrogen was depleted by 30 hours post inoculation (PI). Based on these results, it is clear that both *R. babjevae* and *R. diobovatum* respond to nutrient limitation in the manner typical of oleaginous yeasts, which is to cease growth and begin accumulating lipid molecules, while *Y. lipolytica* simultaneously produces biomass and accumulates lipids.

The biomass accumulation for each species followed the trends shown in the growth curves, with *R. diobovatum* and *Y. lipolytica* biomass accumulation slowing between hours 24 and 36 PI, and *R. babjevae* growing slower, eventually slowing between hours 48 and 60 (Figure 3). In all cases the exhaustion of the available nitrogen supply coincided with the decrease in biomass production rates as cell numbers remained the same and lipid accumulation increased.

Production of lipids and glycerol consumption was markedly different for all three species. While *Y. lipolytica* produced TAGs and consumed glycerol throughout growth, it was able to do so at a significantly higher rate than the *Rhodosporidium* species. All available glycerol was consumed by 72 hours PI, resulting in lipid accumulation plateauing at 29.55 ± 2.65 % cdw of 9.52 ± 0.10 g/L biomass. Following the exhaustion of the glycerol supply, biomass began to decline as cells presumably entered death phase. *R. diobovatum* utilized glycerol at a nearly steady rate for the full 120 hours, though lipid accumulation was most pronounced following 36 hours of incubation, increasing in production rate until 84 hours PI and eventually reaching 63.69 ± 1.94 % cdw of 12.14 ± 0.75 g/L biomass. *R. babjevae*¸ the highest lipid producer as previously reported by Sitepu et al. (2014) showed the lowest biomass production, though lipid production was still higher than that found in *Y. lipolytica*. The lipids produced by *R. babjevae* following 120 hours of incubation were 39.9 ± 4.3 % cdw of 8.6 ± 0.29 g/L biomass. Put in terms of mass/volume, *R. babjevae*, *R. diobovatum,* and *Y. lipolytica* produced 3.4 ± 0.29, 7.73 ± 0.75, and 2.59 ± 0.10 g/L of lipids, respectively, following 120 hours of incubation.

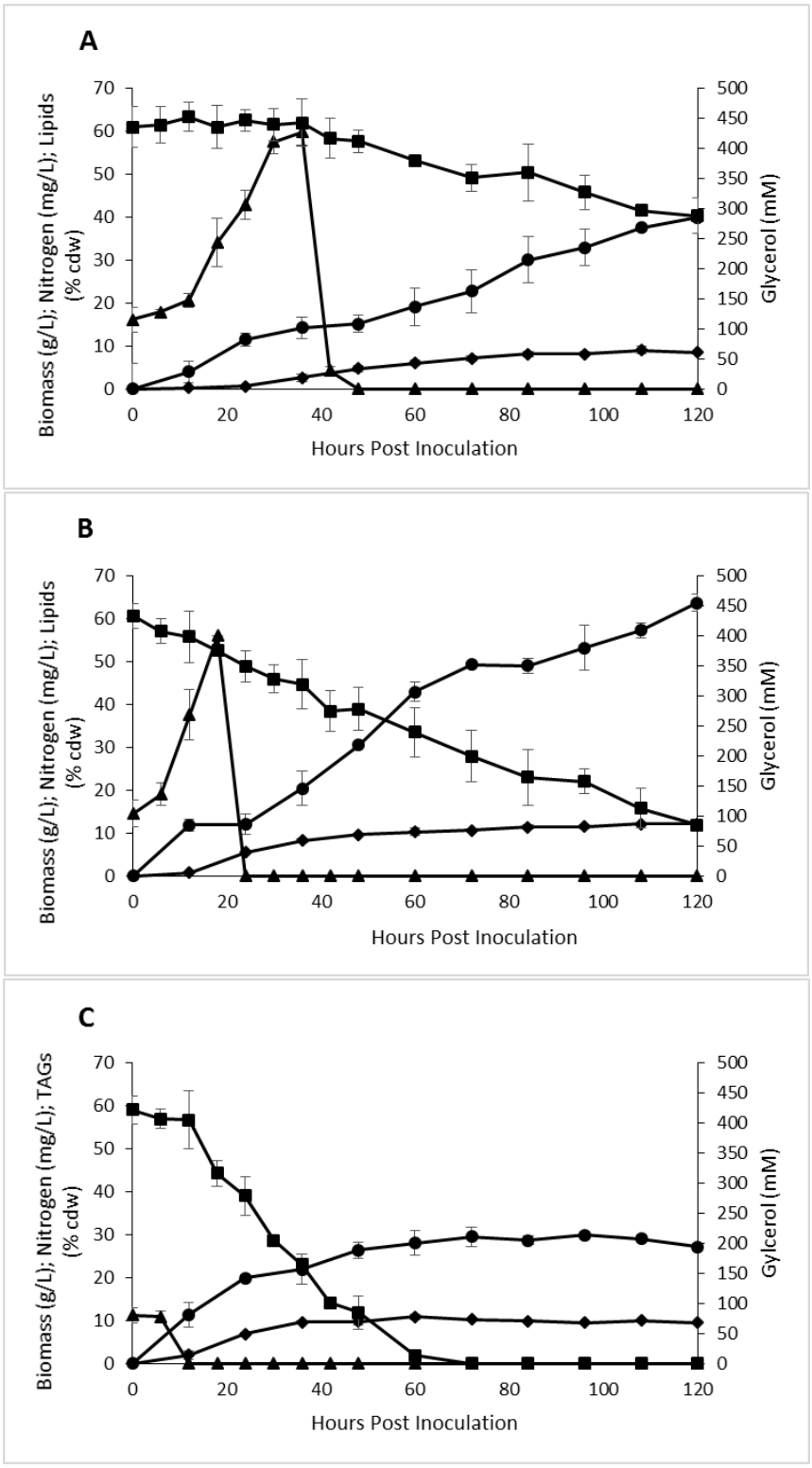

Figure 3: Comparison between *R. babjevae* (A), *R. diobovatum* (B), and *Y. lipolytica* (C) in terms of biomass (♦) and TAG (●) production while consuming pure glycerol (■) and ammonia (▲) in GMY media

3.4.5) Glycerol (Carbon) Utilization efficiency

One of the most striking differences between *Y. lipolytica* and the two *Rhodosporidium* species is that *Y. lipolytica* was able to metabolize all of the available carbon in the medium well within the 120 hours of growth. However, despite metabolizing more carbon than either *R. diobovatum* or *R. babjevae*, *Y. lipolytica* produced less biomass and TAGs per gram of glycerol consumed (Table 3). An estimate of the carbon utilization breakdown was performed, showing that 68.8% of carbon metabolized by *Y. lipolytica* was used for either respiration or excreted side-products (such as carbon dioxide and other metabolic products), as opposed to 43.9% for *R. diobovatum* and 24.6% for *R. babjevae* (Figure 4). *R. diobovatum* displays the highest conversion of carbon to neutral lipids once biomass accumulation has plateaued, using 15.2% of metabolized carbon for biomass while using 40.9% for TAG production. *R. babjevae* displayed an almost even distribution of carbon used for biomass or TAGs, using 37.6.4% carbon for biomass and 37.8.1% for neutral lipids, though this efficiency was exchanged for slow growth. The high carbon use efficiency of the *Rhodosporidium* species indicates a lack of alternate products being produced, allowing for more carbon to be put into TAG accumulation.

Comparing these three species in terms of TAG production shows that *R. diobovatum* produces a concentration of TAGs that is over twice the amount of TAGs produced by *R. babjevae* and *Y. lipolytica* (7.73 g/L vs 3.43 and 3.05 g/L, respectively). The amount of TAGs produced by *Y. lipolytica* in this study is consistent with what has been previously reported, as *Y. lipolytica* was shown to produce between 25-35% of its cdw as TAGs (Sestric et al., 2014; Sitepu et al., 2013). While *R. diobovatum* has a lower specific growth rate than *Y. lipolytica* (0.51 h^{-1} vs 0.88 h^{-1}, respectively), the combination of the higher TAG production rate with the high carbon use efficiency allowed for rapid accumulation of TAGs to concentrations much higher than seen

with *Y. lipolytica*. *R. babjevae* showed the highest efficiency for converting the carbon in the medium to both TAGs and biomass, though the low rates of specific growth and glycerol consumption would require longer incubation times to reach a high concentration of biomass and TAGs. When considering the goals of producing a high concentration of TAGs in a short amount of time, while still using the carbon source efficiently, *R. diobovatum* has the best balance of these three yeasts.

Table 3: Results of growth, glycerol consumption, biomass, and lipid production for *R. babjevae*, *R. diobovatum*, and *Y. lipolytica* when grown in GMY medium for 120 hours

Yeast	**μ (h^{-1})**	**Ψ (g/h)**	**X (g/L)**	**L (g/L)**	**$Y_{B/G}$ (g/g)**	**$Y_{L/G}$ (g/g)**	**Q_B (g/g/h)**	**Q_L (g/g/h)**
R. babjevae	0.27	0.014	5.18	3.43	0.38	0.25	0.043	0.029
R. diobovatum	0.51	0.027	6.74	7.73	0.21	0.24	0.14	0.064
Y. lipolytica	0.88	0.054	7.84	3.05	0.20	0.08	0.13	0.051

(μ=Specific growth rate; ψ=glycerol consumption rate; X=lipid free biomass produced; L=lipids produced; $Y_{B/G}$=biomass yield (gram biomass/gram glycerol consumed); $Y_{L/G}$=lipid yield (gram lipid/gram glycerol consumed); Q_B=Specific production rate of biomass; Q_L=Specific production rate of lipid)

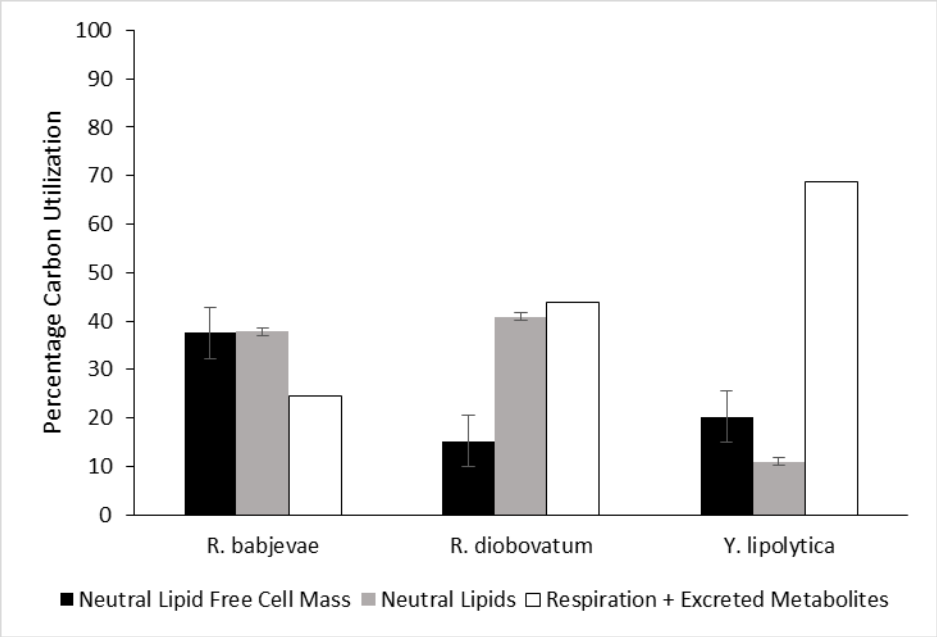

Figure 4: Comparison of the carbon usage between the three species tested following 120 hours incubation on GMY media

3.4.6) Estimation of biodiesel properties using FAME analysis

Conversion of the lipid fatty acids to FAMEs followed by GC analyses revealed that the lipids synthesized by all three yeast species were primarily C16 (palmitic), C18:1 (oleic), and C18:2 (linoleic). These are consistent with the range of lipids generally found in other oleaginous yeast species (Table 4). Major properties of any biodiesel that are directly influenced by the FAME composition are the cetane number (CE), iodine value (IO), and the cold-filter plugging point (CFP). The CE is considered the main factor of biodiesel quality – it represents the ignition quality of a diesel fuel. For most diesel engines, a value above 40 to 50 is considered acceptable (Knothe, 2008). The IO is a measure of the tendency of the biodiesel to react with oxygen, and is dependent on both the number and position of double bonds in the FAMEs. The IO is the mass of iodine that reacts with 100 grams of a substance. The CFP is used to predict the behaviour of the biodiesel at lower temperatures, where FAME molecules begin to precipitate, causing blockage in fuel lines and filters. In order to calculate the values for the CE, IO, and CFP, the saponification value (SA) and long-chain saturation factor (LCS) were also required. The SA value represents the milligrams of KOH needed to saponify 1 g of oil, while the LCS estimates the impact of each long saturated FAME on the CFP (Knothe, 2008; Ramos et al., 2009).

A mathematical estimate of the FAMEs derived from the yeast species lipids was conducted following the same methodology used for estimating algal and vegetable biodiesel, and compared with other oleaginous yeasts (Table 5). For the *Rhodosporidium* species, both were found to have good estimated CE values (57.3 for *R. babjevae* and 56.7 for *R. diobovatum*), which would place them above the accepted value for CE values. *Y. lipolytica* had the highest CE value at 58.1. However, the estimated CFP for many yeast species was higher than those found in many vegetable oils. The CFP values for *Rd. graminis* and *Y. lipolytica* were much lower than

other yeast species, especially the *Rhodosporidium*, at 0.8 and 1.1, respectively. The higher CFP values could be the result of higher levels of palmitic acid produced by the oleaginous yeast species such as the *Rhodosporidium*. However, the estimates for the CFP for both *Rhodosporidium* were lower than the values found for peanut oil (17 ^{o}C) and *R. diobovatum* was only 1.3 ^{o}C higher than palm oil, which has a CFP value of 10 ^{o}C (Ramos et al., 2009). All three species had an IO value of 78 g I_2/100g, lower than what has been found in several vegetable oils, showing that the biodiesel from SCO could not have a high tendency of oxidation, which could lead to polymerization of the fatty acids and complications within a diesel engine. Therefore, based on the fatty acid profiles recovered from the three three yeast species and the subsequent estimation of the various biodiesel properties**,** it can be assumed that biodiesel produced from any of the yeasts tested could also be used as a substitute for petroleum-based diesel fuel in a conventional diesel engine.

Table 4. Fatty acid composition (wt. %) of lipids found in various oleaginous yeasts grown on pure glycerol in shake-flasks for 120 hours.

	C14	**C16**	**C16:1**	**C18**	**C18:1**	**C18:2**	**C18:3**	**C24**	**Misc.**	**Source**
R. babjevae	2	24	1	7	38	16	5	1	6	This work
R. diobovatum	1	28	1	6	33	20	3	1	7	This work
Y. lipolytica	-	11	5	9	55	10	-	-	10	This work
	-	18	7	10	47	15	-	-	3	(Sestric et al., 2014)
R. toruloides	2	29	-	15	42	10	2	-	-	(Xu et al., 2012b)
Rd. graminus	-	20	-	7	43	20	2	-	8	(Galafassi et al., 2012)
Rd. glutinis	-	16	-	22	18	16	2	-	26	(Easterling et al., 2009)

Table 5: Estimates of biodiesel properties from various oleaginous yeasts when grown on pure glycerol. Cells were grown in shake-flasks for 120 hours.

Lipid Source	SA	IO (g I_2/100g)	CE	LCF (wt. %)	CFP (ºC)	Source
R. babjevae	191.1	78.0	57.3	7.3	6.5	This work
R. diobovatum	195.4	78.0	56.7	8.9	11.4	This work
Y. lipolytica	185.8	78.0	58.1	5.6	1.1	This work
	197.5	76.4	56.7	6.8	4.9	(Sestric et al., 2014)
R. toruloides	204.7	61.4	59.2	10.4	16.2	(Xu et al., 2012b)
Rd. graminis	186.7	80.4	57.5	5.5	0.8	(Galafassi et al., 2012)
Rd. glutinis	149.9	50.6	71.3	12.6	23.1	(Easterling et al., 2009)
Canola Oil	190.7	105.5	51.2	1.29	-12.4	(Ramos et al., 2009)
Soybean Oil	216.7	135.3	41.0	3.13	-6.6	(Ramos et al., 2009)

SA, Saponification Value; IO, Iodine Value; CE, Cetane number; LCF, Long-Chain saturation factor; CFP, Cold-filter plugging properties.

3.4.7) Growth on biodiesel-derived waste glycerol (REG80)

For the initial characterization work, pure glycerol was used as the carbon source in order to assess growth and lipid production in the *Rhodosporidium* species without possible interference of contaminants. However, using a pure form of glycerol as a feedstock for yeast biodiesel production would be cost-prohibitive for larger-scale production. As a result, the use of biodiesel-derived waste glycerol as the feedstock for oleaginous yeasts to produce lipids is under investigation (Amaretti et al., 2010; Papanikolaou & Aggelis, 2002; Papanikolaou et al., 2008; Sestric et al., 2014). In this study, no negative effects on growth were observed for either *R. diobovatum* or *R. babjevae* when metabolizing biodiesel-derived glycerol, even when using a higher concentration of waste glycerol compared to pure glycerol (60 g/L compared to 40 g/L). In fact, biomass accumulation was higher for both species (Table 6), with *R. babjevae* producing 9.9 ± 0.2 g/L of biomass and *R. diobovatum* producing 14.1 ± 0.2 g/L of biomass with waste glycerol compared to 9.39 ± 0.79 g/L for *R. babjevae* and 10.96 ± 0.43 for *R. diobovatum* on pure glycerol. In both species, the production of lipids was lower when grown on waste glycerol. *R. diobovatum* produced 50.3 ± 0.5% cdw in lipids, and *R. babjevae* produced 24.2 ± 1.1% cdw as lipids, or 7.10 and 2.40 g/L lipids, respectively. However, as a result of the increased biomass, the overall amounts of lipids produced were only slightly lower to those found when grown on pure glycerol, especially in the case of *R. diobovatum*.

The ability of *Rhodosporidium* species to produce more biomass on biodiesel-derived glycerol over pure glycerol was also observed by Xu et al. (2012) with *R. toruloides*. It was shown that certain impurities such as oleic acid present in the medium (as part of the waste glycerol used) improved the levels of biomass produced. It is believed that the oleic acid acted as a surfactant during fermentation, and subsequently aided nutritional uptake of the cells. The higher concentration of inorganic salts was

also attributed to higher biomass production. Combined, the positive effects of these impurities outweighed the negative effects of others, and resulted in increased biomass production.

Table 6: Comparison of oleaginous yeast biomass and lipid production using waste glycerol as the carbon source. Samples from this work were grown in flasks for 120 hours.

Organism	Type of Culture	Biomass (g/L)	Lipids (% cdw)	Lipids (g/L)	Reference
R. babjevae	Batch in Flask	9.9	24.1	2.4	This work[a]
R. diobovatum	Batch in Flask	14.1	50.3	7.1	This work[a]
Y. lipolytica	Batch in Flask	5.1	31.0	1.6	(Sestric et al., 2014)
Rd. graminis	Batch in Reactor	19	54	10.3	(Xu et al., 2012b)
Rd. glutinis	Batch in Flask	8.17	52.9	4.33	(Galafassi et al., 2012)
R. toruloides	Batch in Flask	19.2	47.7	9.2	(Easterling et al., 2009)

3.5) Conclusion

The three oleaginous species were compared using a number of criteria including the range of carbon sources they are able to metabolize, growth rate, the biomass and lipid production potential on both pure and biodiesel-derived waste glycerol, and the carbon utilization efficiency of each organism. All were able to metabolize a range of carbon substrates, as well as produce amounts of lipids in excess of 20% cdw. However, using all of the criteria used to compare the species, *R. diobovatum* displayed the most desirable traits for large-scale lipid production using REG80 as the sole carbon source. Further work to scale-up production was conducted using *R. diobovatum*, which will be covered in the following section.

Chapter 4: Scale-up of lipid production using *Rhodosporidium diobovatum*

4.1) Introduction

The production potential of *R. diobovatum*, having been displayed at the shake-flask level, was then explored at a larger scale. Typically, the change in scale has effects on the production and growth of single-celled organisms (Schmidt, 2005). Therefore, the same characteristics investigated at the flask level needed to be determined at a bioreactor level. This chapter details the biomass and lipid production of *R. diobovatum* when grown in two different laboratory-scale bioreactor systems.

4.2) Materials and Methods

4.2.1) Microorganism and medium

Microorganisms and media are all as described in Section 2.1.

4.2.2) Culture Conditions

Culture conditions in the reactor were selected to mimic those used in shake-flask experiments. Reactor experiments were conducted using a 7 L glass, autoclavable, stirred-tank bioreactor (Applikon Biotechnology) equipped with an on-line data acquisition and control system and a 3.5 L working volume of GMY medium, containing 60 g/L of biodiesel-derived waste glycerol as the carbon source. The reactor was equipped with three baffles and mixed using a single Rushton impeller. Sparging was through a porous gas diffuser to increase oxygen transfer. Culture pH and dissolved oxygen were monitored using a pH meter (Applikon AppliSens gel electrode) and an oxygen probe (Applikon AppliSens polarographic sterilizable). The cultivation conditions were as follows: inoculum was added to result in a starting OD of

0.02 (600nm), initial pH 5.5, temperature 30ºC, and aeration with compressed filter-sterilized air at a rate of 1 v/v/m. Dissolved oxygen was maintained above 25% by cascading impeller speed between 300 - 600 rpm. pH was monitored but not regulated. 15 mL aliquots were taken every sampling time and handled in the same manner as samples from flask experiments.

Pilot-scale growth was conducted in a refurbished 150 L, steam-sterilizable pilot reactor with a 90 L working volume and four Rushton impellers for mixing. Sparging, inoculum size, pH, and temperature conditions were identical to experiments conducted in the smaller reactor system. Impeller speed was maintained at 250 rpm. Samples were taken using a sterilized sampling port.

4.2.3) Analytical Methods

For experiments in the 7 L bioreactor, analysis of the biomass, FAMEs, and supernatant were all as described in sections 2.3 and 2.4. Estimation of biodiesel properties was done as described in section 2.5. Four separate reactor experiments were conducted, with samples being taken in duplicate to reduce the effect of sampling errors and variations.

For experiments done at the pilot-scale, samples were centrifuged for 10 minutes at 4000 rpm using a Thermo Scientific Sorvall ST 40R centrifuge, rinsed once with ddH_2O, and centrifuged again. Pellets were freeze-dried using a Labconco Freezone 4.5 and stored at room temperature until further analysis. Lipids were extracted by mixing the freeze-dried cell mass in a 60:40 hexane:isopropanol mixture overnight. Residual glycerol was determined by HPLC using an Agilent 1260 liquid chromatography system (Agilent Technologies, Inc., CA, USA) equipped with a Hi-plex H column (7.7 x 300 mm). Lipids were moved from the biomass to the solvents via passive diffusion. Cellular biomass was filtered out, solvent was allowed to

evaporate in a pre-weighed dish, and from this the lipid content of cells was determined. Lipid fractions were re-suspended in hexane to correspond to a concentration of 10 mg/L. One hundred µL of 2 M KOH:methanol was used to methylate the fatty acids. Four hundred µL of the FAMEs was mixed with 100 µL of 1.092 g/L undecanoic acid (C:11) as an internal standard. FAME samples were analyzed using a Agilent 7890A GC system.

The differences in methodology between the 7 L and 150 L systems are due to the work being done in differing laboratories. Work done in the 7 L system, as well as the shake-flask experiments, were done at the University of Manitoba. Work on the 150 L system was conducted at Western University, in the laboratory of Dr. Lars Rehmann. As such, samples were collected and analyzed according to the methodology locally used. However, a GC analysis of biomass produced at Western University was conducted at the University of Manitoba, and matched the FAME concentrations and composition obtained in London.

4.3) Results and Discussion

4.3.1) Growth and lipid production of *R. diobovatum* in a bioreactor

As mentioned, the rapid growth and robust lipid production of *R. diobovatum* caused it to be selected for larger-scale work over *R. babjevae* and *Y. lipolytica*. The reactor conditions were selected to best match those used in the shake-flask experiments in order to compare growth and lipid production at different scales. Conditions in the reactor were selected to be completely aerobic during all points of growth and lipid production. High levels of agitation (cascading between 300-600 rpm) were used to provide adequate mixing, and Antifoam 204 (Sigma-Aldrich) was added (5% anti-foam solution added at 1 mL/L of medium) to prevent excessive foaming of the medium. These experiments were carried out over a longer period (8 days) in

order to allow for complete consumption of the available glycerol in the medium. Peak biomass and lipid production was observed at 168 hrs pi, after which both the biomass and lipid levels began decreasing following exhaustion of the available glycerol in the medium.

The lipid and biomass production of *R. diobovatum* in the reactor closely followed the patterns observed in shake-flasks (Figure 5). At 168 hrs pi, biomass levels reached 13.6 ± 0.4 g/L, with 50.7 ± 2.2% cdw lipid accumulation. This corresponds to a lipid concentration of 6.9 g/L, only slightly less than the 7.1 g/L observed in shake-flask experiments (Table 6). Further optimization and improvements to the conditions within the reactor could result in greater biomass and lipid production. The low standard deviation at each data point indicate that the process is highly reproducible, and these experiments can serve as an accurate baseline on which to attempt improvements on the system.

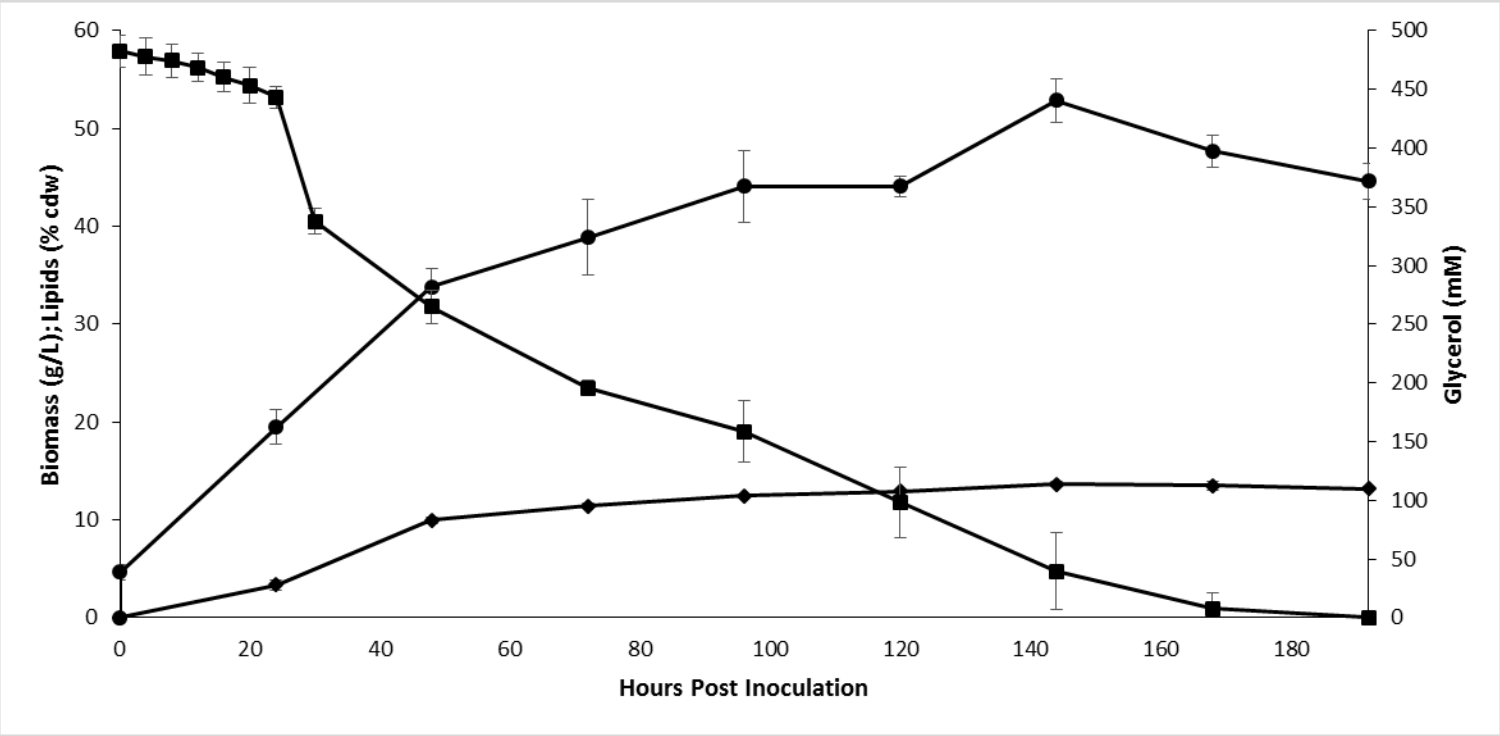

Figure 5: Biomass (♦) and lipid production (●), with glycerol (■) consumption by *R. diobovatum* grown in a 7 L bioreactor with biodiesel-derived waste glycerol as the carbon source.

4.3.2) Changes in FAME composition and resulting changes in biodiesel properties

The FAMES obtained when grown *R. diobovatum* using waste glycerol in the reactor differed from those produced when grown in shake-flasks with pure glycerol, resulting in different estimated values for the CE, IO, and CFP. The FAMEs produced contained a greater proportion of longer chain, unsaturated lipids, resulting in higher estimated CE values for the resulting biodiesel. FAMEs were comparable with those derived from the flask experiments with pure glycerol (60.6 vs. 56.7). The lower concentration of palmitic acid in the lipids derived from bioreactor grown cells also resulted in a lower estimated CFP value of 7.8 ^{o}C, a significant improvement compared to 11.4 ^{o}C from shake-flask work with pure glycerol. This change in the FAMEs and the subsequent change in the CFP can be attributed to the presence of impurities in the waste glycerol, as was seen with the increased levels of biomass produced when *R. diobovatum* was grown in shake-flasks (14.1 g/L grown with waste glycerol compared to only 10.96 g/L when grown on pure glycerol). Specific impurities such as methanol, glyceryl monooleate, and methyl oleate were found to individually reduce the proportion of palmitic acid produced by *R. toruloides*, while increasing the proportion of linoleic acid when compared to a control of pure glycerol (Xu et al. 2012). The cumulative effects of all the impurities present in the medium could have resulted in the changes observed in the FAMEs produced by *R. diobovatum.*

The composition of the FAMEs changed over time (Figure 6), which resulted in a change in the estimated biofuel properties over time as well. These different FAME compositions indicate a change in metabolism at different phases of growth. This is of interest for biodiesel production as the composition of the FAMEs harvested directly affects the qualities of the biodiesel produced, indicating that there could possibly be an ideal time to harvest biomass in

order to extract only the most desirable biodiesel. As discussed in section 3.4.6, the major properties (measurable at this point) are the cetane number and cold filter plugging point, which are calculated using other related properties. The changes in these properties when *R. diobovatum* was grown in a reactor using REG80 as the carbon source are given in Table 7. The increase in unsaturated FAME moieties (reduced amounts of C18 and C18:1, as well as greatly increased amounts of C18:2) resulted in a increased IO value, which in turn reduced the CE number as growth continued. The slightly reduced C18 also lowered the CFP. Based on FAME profile alone, the ideal time to harvest the biomass would be between hours 120 and 144 PI, as these timepoints had the highest CE and CFP. However, the higher lipid concentration at 144 hrs PI compared to 120 hrs PI suggest that this may be the optimal timepoint at which to harvest the *R. diobovatum* biomass.

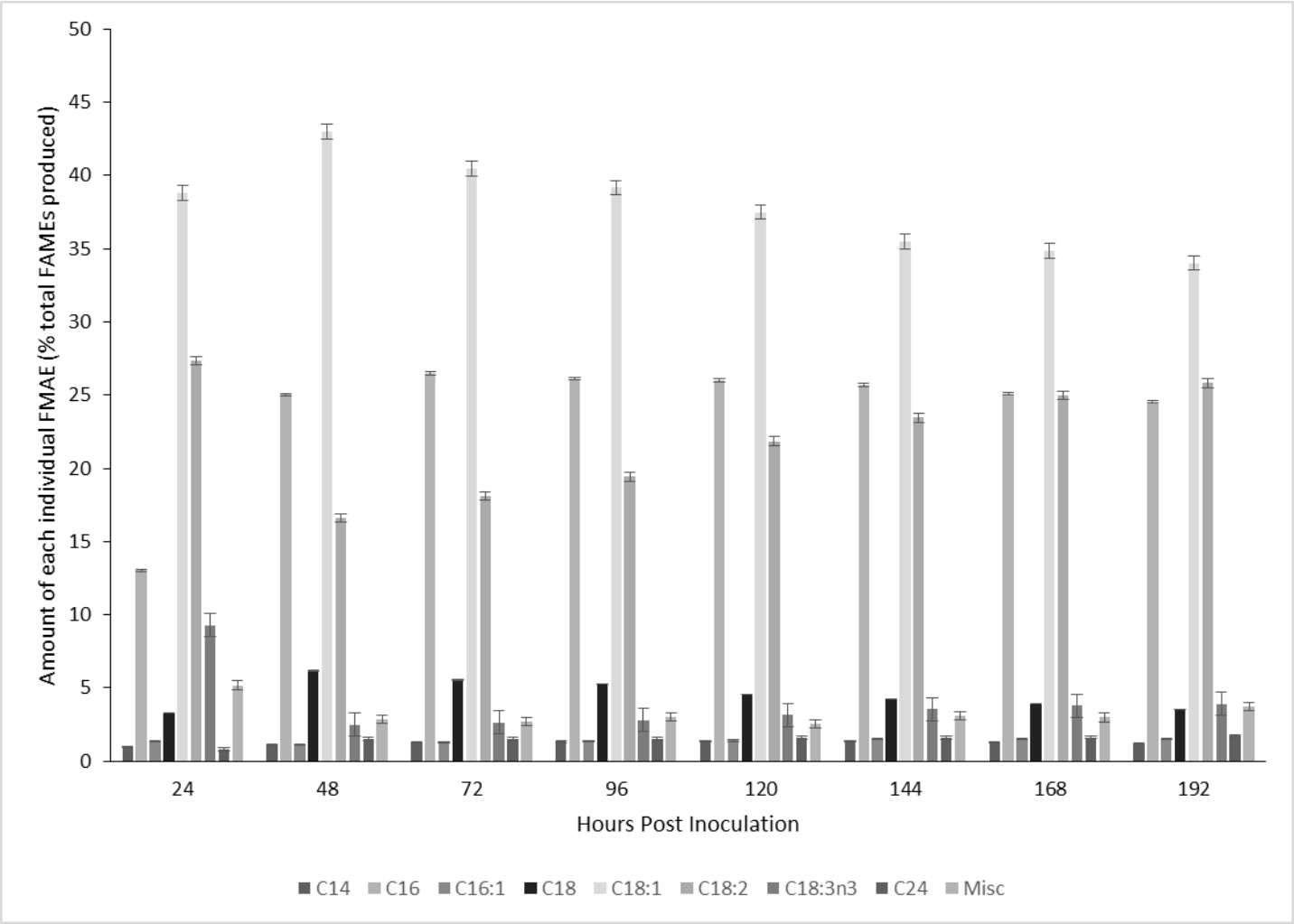

Figure 6: Changes in the FAME composition produced by *R. diobovatum* when grown in a 7 L reactor using REG80 as the sole carbon source.

Table 7: Changes in estimated biodiesel properties over time when *R. diobovatum* is grown in a 7 L bioreactor with REG80 as the carbon source. The working volume of the reactor was 3.5 L.

Time (h)	**SA**	**IO (g I_2/100g)**	**CE**	**LCF (wt. %)**	**CFP (^oC)**
24	191.47	111.18	49.78	4.53	-2.22
48	197.74	76.72	56.63	8.68	10.79
72	198.45	77.65	56.33	8.42	10
96	197.82	79.38	56.02	8.32	9.68
120	198.75	83.27	55.02	8.12	9.05
144	197.69	85.63	54.64	7.9	8.37
168	197.78	88.42	53.99	7.73	7.81
192	196.16	89.56	53.97	7.69	7.7

4.3.3) Growth of *R. diobovatum* at pilot-plant scale

The growth and lipid production characteristics of *R. diobovatum* were further tested at the pilot-scale to determine if any changes would result from the increase in scale from a 3.5 L to a 90 L working volume. Figure 7 displays the time course of the growth and lipid production experiments. At the larger scale, providing enough oxygen and adequate mixing in order to keep the medium homogenous is critical. The oxygen levels in the pilot-scale reactor did not fall below 20% saturation (or 4.9% O_2 in the medium), showing that even during periods of peak biomass and lipid productivity sparging with compressed air was sufficient to provide oxygen to the cells. Foaming was not observed, though a 5% anti-foam solution was added prior to inoculation in the concentration of 1 mL/L of medium. pH followed the same pattern as in smaller batch reactors, initially rising to 5.93 ± 0.20 before falling to 5.04 ± 0.04 by 144 hrs PI.

Biomass and lipid production over time followed a similar pattern observed in previous experiments using a 7 L bioreactor, and are shown in Figure 7. The final biomass produced reached 16.6 g/L, a 3 g/L improvement over the 7 L reactor experiments. However, this increase in biomass came at the cost of reduced lipid production, down to 37.2% cdw. Overall, the lipid production was 6.2 g/L, which is similar to those observed in 7 L reactors (6.9 g/L at the bench reactor scale). As mentioned in section 1.4.3, this drop in productivity could be a result of many issues related to scale-up, such as increased shear forces involved in mixing larger volumes or a difference in dissolved oxygen maintenance. These differences could lead to metabolic changes resulting in more biomass being produced at the expense of biomass.

The composition of the FAMEs collected over time also followed patterns previously observed at a smaller scale (Figure 8). As the fermentation continued, there was increased

amounts of unsaturated fatty acid moieties produced (particularly C18:2). C18:2 was produced as the levels of C18 decreased steadily. Of note is that the levels of C18:1 remained largely constant following 48 hours of fermentation, differing from trends at the smaller scale. Experiments with 3.5 L volume showed concentrations of this species decreased significantly over time. However, it must be noted that due to limitations of the time and materials available, any data on the amount and composition of the FAMEs are from a single experiment, and has not been reproduced. The different FAME profile resulted in different property estimates, which are summarized in Table 8. The largest difference observed was in the CFP, hypothetically a result of the higher levels of unsaturation, dropping from 14.0 ^{o}C in the 7 L reactor to 5.7 ^{o}C in the larger scale. The CE was similar between both scales, ranging between 53 and 56. The increased amount of unsaturated moieties also increased the IO value to 89 g I_2/100g, indicating that oxidative damage and polymerization of the biodiesel could be more of an issue, though these values are still lower than what has been observed in many vegetable oils.

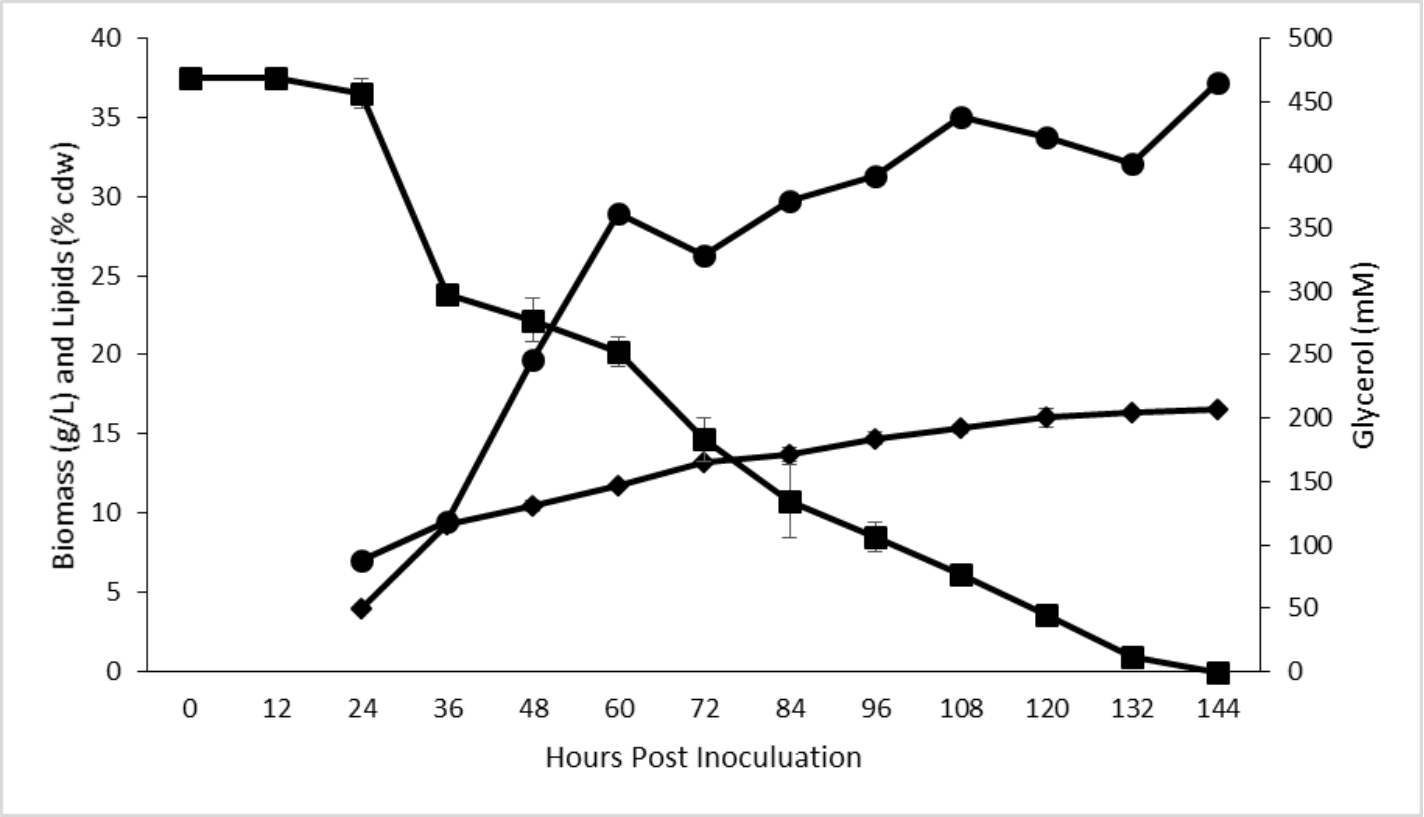

Figure 7: Biomass (♦) and lipid production (●), with glycerol (■) consumption by *R. diobovatum* grown in a 100 L bioreactor with biodiesel-derived waste glycerol as the carbon source.

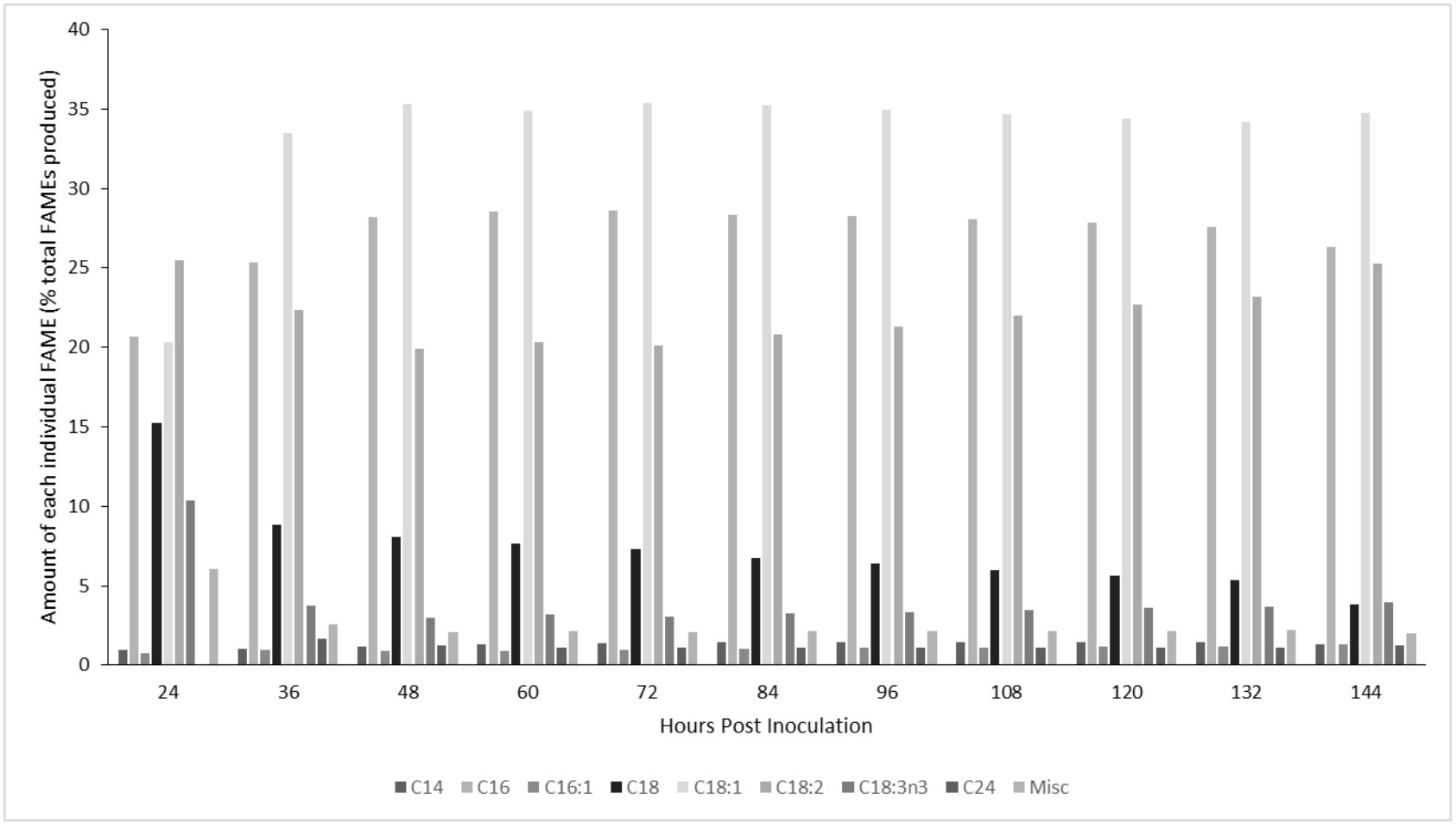

Figure 8: Changes in the FAME composition produced by *R. diobovatum* when grown in a 100 L reactor using REG80 as the sole carbon source.

Table 8: Changes in estimated biodiesel properties over time when *R. diobovatum* is grown in a 90 L bioreactor with REG80 as the sole carbon source. The working volume of the reactor was 90 L.

Time (h)	**SA**	**IO (g I_2/100g)**	**CE**	**LCF (wt. %)**	**CFP (^oC)**
24	191.3	93.7	53.7	9.7	14.0
36	198.3	81.7	55.4	10.2	15.7
48	200.1	77.0	56.3	9.3	12.9
60	200.2	77.9	56.0	8.9	11.3
72	200.2	77.6	56.1	8.7	10.9
84	200.2	79.2	55.7	8.4	9.9
96	200.2	80.2	55.5	8.2	9.3
108	200.2	81.7	55.2	8.0	8.6
120	200.2	83.1	54.9	7.8	7.9
132	200.1	84.0	54.7	7.7	7.5
144	200.1	89.1	53.5	7.1	5.7

4.4) Integration of SCO production with conventional biodiesel production

In order for biodiesel, and biofuels in general, to be competitive with their petroleum-based counterparts, they must cost the same (if not less) to produce. Current estimates show that the costs to produce a pound of biodiesel using soybean oil would be $0.41 USD ($0.90 USD/kg) (Wisner, 2015). This price only covers the costs of the production of the biodiesel itself, and does not account for the logistics of distribution. As this places the price of biodiesel generally higher than petroleum diesel, many aspects of the process could be investigated for possible cost reductions. The research detailed in this thesis addresses two costs that can be reduced directly by converting a waste product into valuable products – purchasing feedstocks and waste disposal. Essentially, the waste product becomes an auxiliary feedstock, allowing for a reduction of both areas of cost. While SCOs using biodiesel-derived waste glycerol could not be self-sufficient on its own, it could be an effective tool to reduce the costs of biodiesel production regardless of the starting feedstock used. There would be no additional logistics costs, as the entire process could be conducted within the biodiesel production facility and uses available infrastructure to produce the biodiesel and separate the fuel from the waste products. The only additional equipment required would be for the fermentations and the post-processing of the cellular biomass, all of which is commercially available and easy to obtain.

Waste glycerol is produced in a 1:1 molar ratio compared to the TAGs used as the feedstock, which corresponds to being approximately 0.476 kg of glycerol for every gallon of biodiesel produced (Yang et al., 2012). This high availability of waste glycerol has reduced the selling price to approximately $0.007/kg (Wisner, 2015), which places it far below the selling price of the biodiesel, making it a less-desirable product. Assuming that production of SCO is scaled up another 10x to a 900 L working volume, and all the conditions are kept constant, each

batch run would consume 54 kg of waste glycerol and produce 5.6 kg of biodiesel in 144 hours. Using the estimates from above, that converts $0.357 USD worth of waste glycerol into $5.04 USD worth of biodiesel, approximately a 1400% increase in value. By utilizing alternate feeding and cultivation strategies, it is a safe assumption that the yields in terms of biomass and lipid production, as well as glycerol consumption, can still be increased. A strategy such as the one used by Rywińska and Rymowicz (2010) could be adopted, whereby a portion of the cells and medium are removed and replaced with fresh medium, reaching a biomass concentration of 154 g/L using *Y. lipolytica.* At a sufficiently large scale, this process could remove the majority of waste glycerol produced and convert it back into useable lipids.

Additional areas of cost recovery can also be explored using oleaginous yeast, in particular *R. diobovatum*. Several species in the *Rhodosporidium* genus produce carotenoids as well as lipids, which are a high value product (Mata-Gómez et al., 2014). In the case of *R. diobovatum*, carotenoid production of 69 µg/g cdw was observed by Buzzini et al. (2007), with the major species being torularhodin, torulene, and γ-carotene. However, they also noted that the high biomass production of *R. diobovatum* resulted in a high volumetric concentration of carotenoids, over 1000 µg/L, indicating that carotenoids can be recovered in significant amounts from the cultures. The global market for carotenoids is currently between $1.2 and 1.4 billion due to growing interest in the anti-oxidant actions of carotenoids, which could also aid in the prevention of certain types of cancer (Mata-Gómez et al., 2014). Saenge et al. (2011) have explored the dual production of lipids and carotenoids using *Rd. glutinis*, and found that the levels of both species produced simultaneously can be high enough to warrant extractions. If further development of a downstream process for simultaneous dual extraction of both the lipids and carotenoids can be developed, then the combination of low-volume/high value and high

volume/low value products can greatly increase the economic viability of the *R. diobovatum* fermentation using waste glycerol as a feedstock.

4.5) Conclusion

The overall growth and lipid productivity of *R. diobovatum* observed at the laboratory reactor scale was very similar to the behaviour observed in flask experiments. Though the lipid productivity was slightly lower, the increased biomass produced compensated for this and the volumetric concentration of the lipids produced was only slightly lowered from flask experiments. Consumption of the waste glycerol was again at a near steady pace, with a slight increase in rate at the approximate time of nitrogen depletion. The FAMEs produced in the 3.5 L reactor were more unsaturated than those found in flasks, though this resulted in more favourable estimates for the resulting biodiesel qualities.

When the fermentation was carried out at a larger, pilot-scale (90 L volume), once again the behaviour was similar to what was observed at smaller scales, with lipid productivity decreasing and biomass production increasing. Once again, this meant that the volumetric concentration of lipids was not drastically reduced. The FAMEs produced contained more unsaturated moieties than found at smaller scales, giving estimates of a better CFP for the biodiesel while still not reducing the CE below acceptable levels. However, the point of interest is the closely mirrored behaviour of both systems despite the difference in scale. This could indicate that improvements made to the system at the smaller scale can be reasonably assumed to result in similar improvements at the larger scale. Once the system is optimized at the laboratory setting, it could be implemented with confidence at the larger scale.

The economics of using oleaginous yeast to produce SCO from biodiesel-derived waste glycerol, though estimated in very general terms, show a significant increase in the value of the products. Further optimization of the process could increase the yields and allow for production levels to match the output of waste glycerol from larger facilities, and become a profitable venture in terms of equipment and labour costs. Incorporating the extraction of alternate, high value, low-volume products (such as carotenoids) as well as the lipids could further increase the profitability of carrying out fermentations on-site at a biodiesel refinery.

Chapter 5: Concluding Remarks

This study characterizes the biomass and lipid production capabilities of several oleaginous yeasts while grown on glycerol, either pure or biodiesel-derived. Based on previous experiments, the three species were selected as it was believed they would be the highest producers from a large pool of oleaginous yeast species. The experimental design consisted of an initial screening and comparison of the three oleaginous yeast: *Rhodosporidium babjevae, Rhodosporidium diobovatum,* and *Yarrowia lipolytica.* Once the most favourable organism was determined, the process of scale-up of production was investigated.

In Chapter 3, the comparisons of the three species carbon utilization, growth, and lipid production is reported. All three species were able to utilize the various carbon sources, including pure and biodiesel-derived glycerol, and produce lipids over 20% of their cell dry weight. The three species also produced fatty acid profiles that resulted in favourable estimates for the resulting biodiesel properties. However, differences in the growth rate, carbon utilization efficiency, and especially the amounts of lipids produced resulted in *R. diobovatum* being selected as the organism for further study at larger scales.

Chapter 4 describes the scale-up efforts at a 3.5 L and 90 L working volume using *R. diobovatum*. At the 3.5 L scale, *R. diobovatum* continued to grow and produce lipids at a high rate, though the cdw of the lipids was reduced. An increase in biomass compensated for the lower lipid production, keeping the overall amounts of lipids produced similar to what was observed at the smaller scale. The fatty acids produced at a larger scale contained more unsaturated moieties, which altered the estimates of the biodiesel properties but did not do so in a way that was unfavourable. Once the behaviour of *R. diobovatum* in batch cultures in a bioreactor had been established, the process was further scaled up to 90 L. Once again, scale-up

resulted in increased amounts of biomass being produced at the expense of the amount of lipids produced per cell. The higher biomass caused the overall amounts of lipids produced to still be comparable to what was observed at the smaller scales. That the behaviour of the systems was so similar at different scales shows that it can be believed that improvements made to the system at the smaller scale will also hold true when scaled up further. A brief examination of the economics of the conversion from waste glycerol to biodiesel show the possible profitability of this process when carried out at a large enough scale.

The growth and production levels of *R. diobovatum* indicate that a system in which the waste glycerol produced during biodiesel production is metabolically converted into lipids for further biodiesel production can be developed. The costs involved could be mitigated by the lowered materials costs for production and the higher selling point of biodiesel compared to the waste glycerol.

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