Utilization of Biodiesel-Derived Crude Glycerol by Fungi for Biomass and Lipid Production

by

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ABSTRACT

UTILIZATION OF BIODIESEL-DERIVED CRUDE GLYCEROL BY FUNGI FOR BIOMASS AND LIPID PRODUCTION

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University of Guelph, 2012

Advisors: Dr. R. Nicol, Dr. W.D. Lubitz, Dr. H. Lee

Rapid expansion of the biodiesel industry has led to a surplus of crude glycerol. This thesis investigates the ability of fungi to utilize crude glycerol as an alternative to conventional carbon substrates for growth and lipid production. Screening revealed that 40 of the 61 isolates tested had increased biomass yield, compared to glucose, when crude glycerol was utilized; 29 of these isolates possessed the ability to completely metabolize 14 g·L\(^{-1}\) of glycerol after 7-14 days. The top four candidates belonged to the genera Galactomyces and Mucor. Overall, Galactomyces sp. proved to be better suited for lipid production. In addition to producing biomass with a high lipid content (up to 45 % w/w), Galactomyces sp. also exhibited high biomass yields (up to 25 g·L\(^{-1}\)). The results obtained in this study compare favourably, and in some cases exceed, other literature reported values for biomass and lipid production using glycerol.
Acknowledgements

I would first like to thank my supervisor Dr. Robert Nicol. His interest in renewable energy technologies and how it can be coupled with microbiology sparked my own interest in this field of study. Thank you for providing me with the right balance of independent learning and constant support when I needed it. I couldn’t have imagined a more down-to-earth supervisor.

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To my parents: Thank you for supporting me throughout my six year university journey. Though I know you still have no clue what I studied, your constant approval of my life’s directions made these past few years possible.
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<tr>
<td>Blast</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
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<tr>
<td>CIT</td>
<td>Citric acid</td>
</tr>
<tr>
<td>CARES</td>
<td>Centre for Agricultural Renewable Energy and Sustainability</td>
</tr>
<tr>
<td>CDW</td>
<td>Cellular dry weight</td>
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<tr>
<td>CFMM</td>
<td>Carbon free minimal medium</td>
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<tr>
<td>CG</td>
<td>Crude glycerol</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>EC</td>
<td>Electrical conductivity</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GLA</td>
<td>γ-linolenic acid</td>
</tr>
<tr>
<td>GC-FID</td>
<td>Gas chromatography with flame ionization detection</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>ICP-OES</td>
<td>Inductively coupled plasma optical emissions spectrometer</td>
</tr>
<tr>
<td>kJ</td>
<td>Kilojoule</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MEL</td>
<td>Mannosylerythritol</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
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<td>MJ</td>
<td>Megajoule</td>
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CHAPTER 1. INTRODUCTION

1.1 Glycerol Background

Glycerol (1,2,3-propanetriol) was first discovered in 1779 when it was produced from the saponification of olive oil, though it was not until the mid-1800’s that an industrial scale application, namely in the production of dynamite, was developed (Christoph et al. 2006). Glycerol is a three carbon polyol which is hygroscopic, colourless and sweet tasting in its pure form (Christoph et al. 2006). Some of the physical characteristics of this compound are listed in Table 1.

Table 1. Physical properties of pure glycerol.

<table>
<thead>
<tr>
<th>Chemical Structure</th>
<th>HO ( \begin{array}{c} \text{OH} \ \text{OH} \end{array} )</th>
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<tr>
<td>Chemical Formula</td>
<td>( \text{C}_3\text{H}_6\text{O}_3 )</td>
</tr>
<tr>
<td>Molecular Mass</td>
<td>( 92.09 \text{ g} \cdot \text{mol}^{-1} )</td>
</tr>
<tr>
<td>Density (at 20 °C)</td>
<td>( 1.261 \text{ g} \cdot \text{cm}^{-3} )</td>
</tr>
<tr>
<td>Caloric Value</td>
<td>( 18 \text{ kJ} \cdot \text{g}^{-1} )</td>
</tr>
<tr>
<td>Melting Point</td>
<td>( 18.0 \text{ °C} )</td>
</tr>
<tr>
<td>Boiling Point (at 101.9 kPa)</td>
<td>( 290.0 \text{ °C} )</td>
</tr>
<tr>
<td>Electrical Conductivity (at 20 °C)</td>
<td>( 0.1 \text{ µS} \cdot \text{cm}^{-1} )</td>
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Modified from Christoph et al. 2006

Pure glycerol now finds a range of applications primarily in the food, beverage, pharmaceutical and cosmetic industries (Christoph et al. 2006; Johnson and Taconi...
In addition to its direct use, glycerol can also serve as a platform chemical for catalytic conversions to other valuable compounds. These conversions are performed by means of oxidation reactions, reduction reactions or the reaction of glycerol with other compatible molecules and are reviewed by Johnson and Taconi (2007).

Today, the bulk of glycerol available on the market is found in one of two forms: synthetic glycerol (10 % of the market) produced by the chemical conversion of propylene, or natural glycerol (90 % of the market) which is purified from co-products generated by the oleochemical industry (Christoph et al. 2006; Patel et al. 2006). The oleochemical industry, especially biodiesel production, is on the verge of making the industry for synthetically glycerol production a thing of the past. Biodiesel is a renewable fuel which can be produced by splitting fats and oils and reacting them with a simple alcohol (Lin et al. 2011; Morrison 2000). The primary co-product of this process is a form of crude glycerol. The only US supplier of synthetic glycerol, Dow Chemical of Freeport Texas, closed its US plant in January 2006 due to the influx of this biodiesel-derived crude glycerol (Niles 2006).

1.2 Sources of Crude Glycerol

The majority of glycerol available on the market results from the purification of the crude glycerol co-product derived from processing natural lipid sources to soap, free fatty acids (FFA) and fatty acid methyl/ethyl esters (i.e. biodiesel) (Christoph et al. 2006; Johnson and Taconi 2007; Morrison 2000; Patel et al. 2006). Less industrially important natural glycerol production processes include the enzymatic degradation of lipids using
lipase, the microbial fermentation of sugars and the hydrogenation of carbohydrates (Patel et al. 2006).

Animal and plant fats are predominately made up of triglycerides (TAGs): three fatty acids bonded to a glycerol backbone (Figure 1) (Dellomonaco et al. 2010; Feofilova et al. 2010). The glycerol portion of lipids, which accounts for 8 % to 14 % of their total weight, is derived primarily by three different industrial processes: (1) high-pressure splitting, (2) saponification and (3) transesterification (Christoph et al. 2006; Werpy et al. 2004). The amount of glycerol present in the crude fractions from these different processes, along with the impurities which must be removed to obtain pure glycerol, varies widely.

1.2.1 High-pressure splitting

High-pressure splitting is a hydrolysis technique which is used to obtain FFAs from TAGs. Lipids at high temperatures (220 °C to 260 °C) and pressure (2 MPa to 6 MPa) are fed through a column with a countercurrent flow of water, releasing the FFAs from the glycerol backbone (Christoph et al. 2006; Morrison 2000; Patel et al. 2006). The FFAs are removed from the top of the column, while the phase which is retrieved from the bottom, termed sweet water, contains approximately 15 % to 20 % (w/v) glycerol, water and slight amounts of ash (<0.1 %) (Christoph et al. 2006; Morrison 2000). Subsequent purification steps help to concentrate the glycerol by evaporating the water until a solution of 70 % to 90 % (w/v) glycerol, termed hydrolysis crude, is obtained (Christoph et al. 2006; Morrison 2000).
1.2.2 Saponification

Saponification is a technique used to produce soap from feedstocks containing neutral lipids (Christoph et al. 2006). Saponification is performed by reacting lipids with an alkali solution (NaOH, alkali carbonates etc.) and salt. The lower phase which is produced, termed spent lye, is composed of glycerol (approximately 8 % to 15 % w/v), water, salt, and excess base at various concentrations (Morrison 2000). Israel et al. (2008) analyzed four spent lye samples and found they contained 6 % to 16 % glycerol, 10 % to 12 % salt, 0.3 % to 0.8 % caustic soda, 0.4 % fatty matter and 39 % to 40 % water.

1.2.3 Transesterification

The transesterification of fats and oils with alcohol (ethanol or methanol) and an alkali catalyst (NaOH or KOH) is a third source of crude glycerol (Lin et al. 2011; Morrison 2000). The reaction proceeds in a stepwise fashion in which the TAG is converted to a diglyceride, monoglyceride and finally glycerol (Naik et al. 2010). The methyl/ethyl esters which are produced can be used as a replacement for diesel fuel, reduced to fatty alcohols or marketed as food grade fatty esters (Morrison 2000; Scott et al. 2011).

Oleochemical industries, such as fish oil processing, use a transesterification reaction with ethanol to generate ethyl esters of fatty acids which can be subsequently purified into food grade omega-3 fatty acids (Scott et al. 2011). On the other hand, biodiesel is predominantly produced by the alkali-catalyzed transesterification reactions of TAG with methanol as depicted in Figure 1 (Lin et al. 2011; Shi et al. 2011).
Figure 1. Transesterification reaction for biodiesel production using plant or microbial lipids (Modified from Shi et al. 2011).

The crude glycerol which is produced, regardless if it is from biodiesel production or oil processing, typically contains more impurities than the high-pressure splitting and saponification processes due to the presence of unused reactants and soap. These impurities may be removed by acidulation to break soap and precipitate salts and evaporation to remove methanol (Morrison 2000). It is the crude glycerol obtained during the production of biodiesel which is the focus of the present work and described in further detail below.

1.3 Biodiesel Production

Rationalizing the continued search for petroleum reserves is becoming progressively difficult due to dwindling fossil fuel resources, negative environmental impacts, waste recycling challenges and increased petroleum oil prices (Ageitos et al. 2011; Subramaniam et al. 2010). Consequently, public interest in renewable fuel sources has helped increase both the popularity of and demand for biodiesel as an alternative to conventional petroleum diesel (Lin et al. 2011; Rymowicz et al. 2010).

In 2005, Canadian biodiesel production was estimated at 11 million US gallons (CRFA 2010). This value increased five-fold by 2010, when biodiesel production in
Canada had grown to over 55 million US gallons (CRFA 2010). In the US, biodiesel production is higher than in Canada. Reported US sales in 2006 were 250 million gallons, with production peaking at 691 million gallons in 2008 (NBB 2007; NBB 2010). As a result of uncertainty in the US federal government’s incentive plans (e.g. alternative fuel infrastructure tax credits), decreased sales in the US for 2009 and 2010 were reported; only 545 and 315 million gallons of biodiesel were produced (NBB 2010). Extension of the incentive plans, coupled with the Renewable Fuel Standard’s target production goal of 1 billion gallons of biodiesel in 2012, are expected to create an upward shift in annual biodiesel production in the US. As new biodiesel facilities continue to emerge, projected production rates above 10 billion US gallons a year are anticipated by 2019 (OECD-FAO 2011).

1.4 Crude Glycerol from Biodiesel Production

Projected estimates for crude glycerol from biodiesel production in the US place approximately 635 thousand tonnes of this co-product on the market between 2006 and 2015 (Nilles 2006). Globally, crude glycerol from biodiesel manufacturing has a reported annual production rate around 1.9 Mtonnes and rising (Amaral et al. 2009). An estimated annual consumption rate around 1.0 Mtonnes of glycerol is likely, based on the 3 % annual increase in global glycerol use reported in the 1980’s and 1990’s (Christoph et al. 2006). This suggests that glycerol from biodiesel production alone would provide an annual surplus of 900 000 tonnes above global market requirements (Christoph et al. 2006).
As the biodiesel industry continues to grow, the increased amount of glycerol in the market is becoming a burden to producers who now have limited options for managing this co-product (Johnson and Taconi 2007; Thompson and He 2006). While some large scale producers are able to refine this co-product for the industrial applications described in section 1.1, small scale producers are unable to justify refining costs and instead pay a fee for glycerol removal (Athalye et al. 2009; Thompson and He 2006).

1.4.1 Market Value of Glycerol

Purified glycerol has historically been valued between US $ 0.27 and $ 0.41 per kg (Johnson and Taconi 2007; Werpy et al. 2004). It has been proposed that a price reduction below US $ 0.23 per kg would be required to open up the possibility of using glycerol as a biorefinery feedstock chemical (Werpy et al. 2004). Prior to the large scale production of biodiesel, the use of glycerol for this purpose was therefore not thoroughly investigated, as the global consumption of glycerol in various markets was relatively balanced with its production thus maintaining a high value for this compound (Rywinska et al. 2010a). As biodiesel producers began establishing themselves, glycerol was still recognized as a valuable co-product which could be purified and sold to increase the profitability of the process (Yazdani and Gonzalez 2007).

Unfortunately, as biodiesel production continued to rise, glycerol prices weakened. Between 2004 and 2006 the market value of glycerol dropped ten-fold due to the increased supply from biodiesel producers and between 2005 and 2006 crude glycerol prices hovered around US $ 0.02 per kg (Nilles 2006; Yazdani and Gonzalez 2007).
2007). A more recent evaluation of 80 % purity glycerol, from September 2009 to August 2011, showed fluctuating values ranging from US $ 0.02 and $ 0.07 per kg, with current trading prices around US $ 0.04 per kg (Sims 2011).

It is predicted that a drop below $ 0.02 per kg is unlikely to occur since large markets would open up at such a low price, for example steam reforming to hydrogen or the use of glycerol in animal feed after the removal of impurities (Werpy et al. 2004). Alternatively, the crude glycerol could be burned to provide 4.3 MJ per kg of energy (Johnson and Taconi 2007). At US $ 0.02 per kg, the use of crude glycerol for this purpose would be slightly more economical than natural gas, although salt and water would first have to be removed (Johnson and Taconi 2007). Another approach to utilize the large quantities of glycerol would be the use of this co-product as a carbon substrate for microbial bioconversions to value-added compounds if industrial processes could be developed (Levinson et al. 2007).

1.4.2 Characterization of Crude Glycerol

Crude glycerol from transesterification reactions contains a variety of contaminants such as salt, soaps and unused reactants (Table 2). The primary components of this co-product include glycerol, methanol, salt, water and soap/FFAs. Literature reported values for these components (% w/w) range from 49 % to 92 % glycerol, 0.01 % to 38 % methanol, 1 % to 12 % salt, 6 % to 36 % water and 1 % to 25 % soap/FFAs (Table 2). Other reported impurities include ash, heavy metals and lignin, although these are present in much smaller amounts. Due to the common practice of using alkaline catalysts, a high pH above 8 is characteristically observed for this co-
product. Coupled with the contaminants presented in Table 2, this carbon source presents certain challenges for bioconversion processes.
Table 2. Composition (% weight) of crude glycerol from the transesterification of various lipid feedstocks.

<table>
<thead>
<tr>
<th>Lipid Feedstock</th>
<th>Glycerol</th>
<th>Na and K Salts</th>
<th>Methanol</th>
<th>Organic Impurities</th>
<th>Water</th>
<th>Soap/Free Fatty Acids</th>
<th>Ash</th>
<th>Heavy Metals &amp; Lignin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow grease</td>
<td>48.7</td>
<td>22.7</td>
<td>25.6</td>
<td>3.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liang et al. 2010</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>4-5</td>
<td>1.3</td>
<td>1.6-7.5</td>
<td>36</td>
<td></td>
<td></td>
<td></td>
<td>Saenge et al. 2011</td>
</tr>
<tr>
<td>Canola Oil, Chicken fat, Soybean Oil</td>
<td>56.5-62.4</td>
<td>12.8-28.3</td>
<td>15.3-25.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pyle et al. 2008</td>
</tr>
<tr>
<td>Vegetable Oil, Yellow grease</td>
<td>62.5-76.6</td>
<td>23.4-37.5</td>
<td>0.25-5.50</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Thompson and He 2006</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>4-5</td>
<td>3</td>
<td>0.5</td>
<td>26</td>
<td></td>
<td></td>
<td></td>
<td>Papanikolaou et al. 2008</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>4-5</td>
<td>1</td>
<td>0.5</td>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td>Papanikolaou and Aggelis 2002</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>12</td>
<td>2</td>
<td>1</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td>André et al. 2009</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>12</td>
<td>2</td>
<td>1</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td>André et al. 2010</td>
</tr>
<tr>
<td></td>
<td>Percentage of Total Fat</td>
<td>Amount (mg/dL)</td>
<td>Mean (mg/dL)</td>
<td>Standard Deviation (mg/dL)</td>
<td>Source</td>
<td></td>
<td></td>
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<td>----------------</td>
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<td>---------------------------</td>
<td>--------------------------------------------</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Rapeseed Oil</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>1</td>
<td>5</td>
<td>12.7</td>
<td>1, Kamzolova et al. 2011</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>4</td>
<td>1</td>
<td>0.5</td>
<td>13.5, Fakas et al. 2009b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>80.1</td>
<td>5.6</td>
<td>&lt;0.2</td>
<td>12.9</td>
<td>1.2, Chatzifragkou et al. 2011 (ADM Industries)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Yellow grease</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>83.4</td>
<td></td>
<td></td>
<td></td>
<td>11.6, 1.28, 2.69, Liu et al. 2011</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>85.0</td>
<td>4.5</td>
<td>1.5</td>
<td>6.0</td>
<td>3.0, Chatzifragkou et al. 2011 (Agro-Invest SA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>86</td>
<td>6.5</td>
<td>&lt;0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>92</td>
<td>2.3</td>
<td>0.01</td>
<td>0.5</td>
<td>6, Rywinska et al. 2010b</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6, Rywinska and Rymowicz 2010</td>
<td></td>
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</tr>
</tbody>
</table>
1.5 Bioconversion of Glycerol by Bacteria

The availability of cheaper carbon sources has previously limited the widespread use of glycerol for industrial microbial processes and the use of crude glycerol as the exclusive carbon source for this purpose is still in its infancy (Papanikolaou et al. 2008; Tang et al. 2009). One of the primary issues when using crude glycerol for bioconversions is acquiring microbial hosts which are able to tolerate batch-to-batch variations and the impurities which are found in this co-product (Choi et al. 2011). These inconsistencies are largely due to differences in the quality of the initial oil feedstock used and the presence of contaminants as shown in Table 2 (Thompson and He 2006).

There has been considerable interest in research on the anaerobic bioconversion of glycerol by bacteria for the production of 1,3-propanediol (PDO), an important polymer building block, as a way to utilize this abundant co-product. The anaerobic production of PDO has focused largely on species within the family of Enterobacteriaceae, with particular interest in Klebsiella and Citrobacter, and members of the genera Enterobacter, Clostridium, Lactobacillus and Bacillus (Amaral et al. 2009; Dellomonaco et al. 2010; Hao et al. 2008; Yazdani et al. 2007). Other important bioproducts which have been given considerable attention include dihydroxyacetone, butanol, propionic acid and succinic acid. The anaerobic production of these compounds by bacteria has been reviewed in a number of publications and is beyond the scope of this work (Amaral et al. 2009; Johnson and Taconi 2007; Yazdani and Gonzalez 2007).
Although promising results have been obtained with bacteria, scaling up to industrially relevant levels is complicated. Many of the bacteria under investigation are considered pathogens and require high nutrient medium, anaerobic growth conditions and tolerate only low-levels of crude glycerol (Dellomonaco et al. 2010). Furthermore, low bioproduct concentrations dissolved in the culture broth creates additional challenges for product recovery and purification.

Complex treatment procedures for crude glycerol have been proposed in an attempt to improve bioproduct yields from bacterial cultures (Asad-ur-Rehman et al. 2008). For example, in an effort to increase PDO yields from *Clostridium butyricum*, glycerol was treated with phosphoric acid, filtered to remove phosphate salts, vacuum distilled for methanol recovery and washed with n-hexanol to removal other impurities (Asad-ur-Rehman et al. 2008). While treating the crude glycerol resulted in a PDO yield comparable to the pure reagent grade glycerol control (10.7 g·L⁻¹ versus 10.6 g·L⁻¹), scaling up of this process is impractical due to the many steps required. A more practical solution is to find organisms which can tolerate the impurities found within this co-product.

When screening microbial hosts to identify candidates for the bioconversion of industrial feedstocks, a substrate-based approach rather than a product-oriented approach, has been proposed as a way to improve the success of the process (Rumbold et al. 2009). Using this type of screening procedure, microorganisms with natural abilities to utilize crude feedstocks can be identified. This in turn will help avoid the need to try and engineer microorganisms to adapt them to use these co-products. Using this approach, Rumbold et al. (2009) determined that fungi were more resilient to
inhibitors in industrial feedstocks than bacteria. Using a synthetic minimal medium, these authors added increasing concentrations of various inhibitors commonly found in industrial feedstocks, including crude glycerol, such as furfural and 5-hydroxymethyl furfural ($0.5\text{ g}\cdot\text{L}^{-1}$ to $5\text{ g}\cdot\text{L}^{-1}$), acetate ($1\text{ g}\cdot\text{L}^{-1}$ to $20\text{ g}\cdot\text{L}^{-1}$), sodium chloride ($5\text{ g}\cdot\text{L}^{-1}$ to $100\text{ g}\cdot\text{L}^{-1}$) and magnesium sulphate ($20\text{ g}\cdot\text{L}^{-1}$ to $80\text{ g}\cdot\text{L}^{-1}$). Compared to bacteria, which have been the primary focus for glycerol bioconversion to date, fungi were more effective at utilizing this carbon substrate for growth and growth inhibition due to impurities was minimal; a 50 % reduction in growth (measured as optical density or CO$_2$ production) was not observed until the highest concentrations of sodium chloride (a potential crude glycerol contaminant) was tested.

1.6 Bioconversion of Glycerol by Filamentous Fungi and Yeast

Studies of the bioconversion of glycerol by various filamentous fungi and yeast have only recently begun to appear in the literature. The United States Department of Energy (US DOE) released a list identifying the top 12 value added building block chemicals (Werpy et al. 2004). These chemicals represent interesting research opportunities owing to their market size, value and potentially diverse applications. The bioconversion of glycerol by filamentous fungi and yeast to a select number of the chemicals listed by the US DOE is possible. Crude glycerol was recently proven to be a successful carbon substrate for arabitol production by the yeast Debaryomyces hansenii (Koganti et al. 2011). Pure glycerol has also been used for the production fumaric acid by Rhizopus sp. (Moon et al. 2004) and succinic acid by a recombinant strain of Yarrowia lipolytica (Yuzbashev et al. 2010). It has also been used as a co-substrate for xylitol production by various species of Candida (Arruda and Felipe 2009; Ko et al.
2006). Other products not covered in the US DOE report, such as lipase (Mafakher et al. 2010), single cell protein (Mafakher et al. 2010), pigments (Mantzouridou et al. 2008; Martelli et al. 1992; Kusdiyantini et al. 1998), pyruvic acid (Finogenova et al. 2005), mannitol and erythritol (André et al. 2009; Chatzifrakou et al. 2011; Rywinska et al. 2010b; Rywinska et al. 2011) and acetic acid (Chatzifrakou et al. 2011) have also been identified as potential bioconversion products. Unfortunately, extensive research on the production of these compounds from crude glycerol is not yet available. Contrary to this, the production of fungal lipids, lipid derivatives and citric acid have garnered a great deal of attention in recent years. Not only has the use of pure glycerol proven to be effective for producing these substances, but crude glycerol is also being explored as a readily available carbon feedstock for the production of these compounds.

1.6.1 Single Cell Oil Production

Rising costs and limited availability of traditional lipid feedstocks for the manufacturing of biodiesel has led researchers to investigate the use of novel lipid sources (Shi et al. 2011). One alternative which is receiving considerable attention is the production of biodiesel using microbial lipids termed single cell oil (SCO). The concept for the commercial production of SCO is not a recent development and has been investigated for nearly 100 years (Ratledge and Wynn 2002). Single cell oil research has focused predominantly on the production of lipids suitable for use as biofuel feedstocks, lipids which are structurally similar to higher value oils (e.g. cocoa butter) and lipids which can be used as food supplements such as rare polyunsaturated fatty acids (PUFAs) (Beopoulos et al. 2008; Papanikolaou and Aggelis 2009; Ratledge and Wynn 2002).
Single cell oils are microbial lipids with similar compositions to oils and fats obtained from plant and animal sources (Beopoulos et al. 2009; Vicente et al. 2009). Although the fatty acids can range from lauric acid (C12:0) to docosahexaenoic acid (C22:6), many microorganisms produce lipids which contain fatty acids profiles similar to those found in plants (Papanikolaou and Aggelis 2002; Subramaniam et al. 2010). These include myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) (Fei et al. 2011; Subramaniam et al. 2010). Microorganisms can be classified as oleaginous if their cellular lipid content is in excess of 20 % of their total cellular dry weight (CDW), although some oleaginous microorganisms have been identified with the ability to produce lipids in excess of 70 % of their CDW (Meng et al. 2009; Papanikolaou and Aggelis 2009; Ratledge and Wynn 2002).

Microbial lipid production for biodiesel manufacturing has focused predominantly on microalgae and only recently has research begun to investigate other microorganisms such as fungi, yeast and bacteria (Vicente et al. 2009). Table 3 lists various microorganisms and their lipid contents. Unlike algae, heterotrophic microbes are easier to grow and the culture systems used for their growth takes advantage of existing bioreactor designs (Vicente et al. 2009).
Table 3. Oil content of various oleaginous microorganisms.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Oil Content (% CDW)</th>
<th>Microorganism</th>
<th>Oil Content (% CDW)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microalgae</strong></td>
<td></td>
<td><strong>Yeast</strong></td>
<td></td>
</tr>
<tr>
<td>Botryococcus braunii</td>
<td>25-75</td>
<td>Candida curvata</td>
<td>58</td>
</tr>
<tr>
<td>Cylindrotheca sp.</td>
<td>16-37</td>
<td>Cryptococcus albidus</td>
<td>65</td>
</tr>
<tr>
<td>Nitzschia sp.</td>
<td>45-47</td>
<td>Lipomyces starkeyi</td>
<td>64</td>
</tr>
<tr>
<td>Schizochytrium sp.</td>
<td>50-77</td>
<td>Rhodotorula glutinis</td>
<td>72</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td><strong>Fungi</strong></td>
<td></td>
</tr>
<tr>
<td>Arthobacter sp.</td>
<td>&gt;40</td>
<td>Aspergillus oryzae</td>
<td>57</td>
</tr>
<tr>
<td>Acinetobacter calcoaceticus</td>
<td>27-38</td>
<td>Mortierella isabellina</td>
<td>86</td>
</tr>
<tr>
<td>Rhodococcus opacus</td>
<td>24-25</td>
<td>Humicola lanuginose</td>
<td>75</td>
</tr>
<tr>
<td>Bacillus alcalophilus</td>
<td>18-24</td>
<td>Mortierella vinacea</td>
<td>66</td>
</tr>
</tbody>
</table>

*a CDW = Cell dry weight  Modified from Meng et al. 2009

1.6.1.1 Pathway for Microbial SCO Production

Lipid production in oleaginous microorganisms is triggered by a depletion of certain nutrients in the medium while an excess of the carbon substrate is still available (Beopoulos et al. 2009; Fakas et al, 2009; Meng et al. 2009). While the depletion of a number of different elements can result in lipid accumulation, nitrogen limitation is the most efficient at inducing lipid production (Beopoulos et al. 2009).

When all nutrients are readily available, the carbon substrate (e.g. glycerol) is taken up by the cells and distributed for the production of carbohydrates, lipids, nucleic acids and proteins (Beopoulos et al. 2009). Upon nitrogen depletion, nucleic acid and protein synthesis is reduced, inhibiting cellular division. In oleaginous microorganisms,
the carbon substrate continues to be taken up by cells and is channelled into the lipid biosynthesis pathway, increasing TAG production and its storage in lipid bodies (Ageitos et al. 2011; Beopoulos et al. 2009; Meng et al. 2009).

The pathway depicted in Figure 2 represents the route which most filamentous fungi and yeast use for glycerol assimilation and conversion to lipids. More details regarding this process are reviewed by Ratledge and Wynn (2002). Lipid accumulation is largely dependent on the adenosine monophosphate (AMP) regulated activity of the enzyme isocitrate dehydrogenase of the citric acid cycle. A cascade of events leading to citrate accumulation in the cytosol, followed by cleavage of the citrate molecule to produce oxaloacetate and acetyl-CoA, promotes fatty acid synthesis and lipid production (Ratledge and Wynn 2002). It is important to note that the pathway depicted in Figure 2 is an important metabolic process which occurs regardless of the type of carbon substrate available. A second route, where the glycerol is first oxidized to dihydroxyacetone followed by phosphorylation to dihydroxyacetone phosphate, is also possible (Wang et al. 2001).

The final TAG resulting from the sequence of events in Figure 2 are produced by means of a dehydration synthesis reaction wherein three fatty acids combine with a molecule of glycerol through ester linkages between the carboxyl groups of fatty acids and the hydroxyl groups of the glycerol (Tortora et al. 2007). The glycerol molecule in TAG is derived from an intermediate of glycolysis, dihydroxyacetone phosphate, while the fatty acids are produced by successively combining the 2-carbon units derived from acetyl-CoA molecules (Tortora et al. 2007).
Figure 2. Pathway for the production and accumulation of lipids from glycerol in oleaginous fungi. The sequence of events leading to lipid accumulation after nitrogen limiting conditions are reached is described in the text. Key enzymes involved in the process are italicized (pathway modified from Ratledge and Wynn, 2002).
1.6.1.2 Single Cell Oil Production from Glycerol

Filamentous fungi and yeast with high cellular lipid contents have been successfully cultured using crude glycerol (Table 4). For example, lipid content above 70 % (w/w) of the CDW was reported in batch cultures using the fungus *Thamnidium elegans* (Chatzifragkou et al. 2011). The yeast *Rhodotorula glutinis* has also been shown to accumulate 60.7 % (w/w) lipid using a fed-batch system (Saenge et al. 2011). Though high lipid contents have been observed in biomass, the overall amount of lipid produced per litre of medium used for most microorganisms remains relatively low, with the latter two microorganisms producing total lipids of only 11.6 g·L$^{-1}$ and 6.1 g·L$^{-1}$ from media containing 90 and 95 g·L$^{-1}$ crude glycerol respectively (Table 4). Furthermore, the overall yield of lipid produced per gram of glycerol consumed is often low in cultures. Ratledge and Wynn (2002) reported a theoretical maximum lipid yield from glycerol of 0.3 (g lipid per g glycerol consumed), however, much lower values are often observed. For example, Papanikolaou and Aggelis (2009) obtained a maximum lipid yield from glycerol of only 0.08 for *Y. lipolytica* using 50.5 g·L$^{-1}$ to 135.1 g·L$^{-1}$ crude glycerol. Fakas et al. (2009) did only slightly better using cultures of *Cunninghamamella echinulata* and *Mortierella isabellina*. Reported yields from glycerol for these isolates were 0.10 and 0.15 when crude glycerol was used.

Batch experimentations have been predominantly used to investigate lipid production from glycerol, although reports on fed-batch systems are increasing as they enable an increase in cell density and lipid content (Beopoulos et al. 2009; Subramaniam et al. 2010). This is largely due to a better control of substrate levels preventing substrate inhibition at higher glycerol concentrations (Saenge et al. 2011).
Nutrient rich batch cultures of *Cryptococcus curvatus*, for example, were negatively impacted by the impurities found in crude glycerol compared to pure glycerol (Liang et al. 2010). Growth was reduced by 2.5 times when crude glycerol was increased from 20 g·L⁻¹ to 40 g·L⁻¹ and almost no growth was observed at 60 g·L⁻¹. Switching culture systems to a fed-batch setup allowed crude glycerol grown *C. curvatus* to reach comparable cell densities to those observed in pure glycerol batch cultures. Despite the use of different culture systems, culture parameters appear to be similar. Moderate temperatures (28 °C) and a slightly acidic pH (5.0 to 6.0) are often reported (Table 4).
Table 4. Culture conditions and lipid production from the bioconversion of glycerol by filamentous fungi and yeast.

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Biomass (g·L⁻¹)</th>
<th>Lipid Content (% CDW)a</th>
<th>Lipid Concentration (g·L⁻¹)b</th>
<th>Temp (°C)</th>
<th>Time (day)</th>
<th>pH</th>
<th>Carbon Source (g·L⁻¹)c</th>
<th>Nitrogen Source (g·L⁻¹)d</th>
<th>Culture Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus niger</em> LFMB 1</td>
<td>5.4</td>
<td>57</td>
<td>3.1</td>
<td>28</td>
<td>4</td>
<td>4.8</td>
<td>CG (60)</td>
<td>(NH₄)₂SO₄ (0.5)</td>
<td>Batch</td>
<td>André et al. 2010</td>
</tr>
<tr>
<td><em>A. niger</em> NRRL 364</td>
<td>8.2</td>
<td>41</td>
<td>3.4</td>
<td>28</td>
<td>6.8</td>
<td>4.8</td>
<td>CG (60)</td>
<td>(NH₄)₂SO₄ (0.5)</td>
<td>Batch</td>
<td>André et al. 2010</td>
</tr>
<tr>
<td><em>Cryptococcus curvatus</em> ATCC 20509</td>
<td>32.9</td>
<td>52.9</td>
<td>28</td>
<td>28</td>
<td>12</td>
<td>5.5</td>
<td>CG (32)</td>
<td>YE (0.1)</td>
<td>2-stage fed Batch</td>
<td>Liang et al. 2010</td>
</tr>
<tr>
<td><em>C. curvatus</em> ATCC 20509</td>
<td>118</td>
<td>25</td>
<td>29.5</td>
<td>28</td>
<td>2.1</td>
<td>5.5</td>
<td>PG (16)</td>
<td>YE (0.1)</td>
<td>Fed-batch</td>
<td>Meesters et al. 1996</td>
</tr>
<tr>
<td><em>C. curvatus</em> ATCC 20509</td>
<td>31.2</td>
<td>44.6</td>
<td>28</td>
<td>28</td>
<td>12</td>
<td>5.5</td>
<td>CG (25.8)</td>
<td>YE (0.1)</td>
<td>1-stage fed-Batch</td>
<td>Liang et al. 2010</td>
</tr>
<tr>
<td><em>Cunninghamella echinulata</em> CCRC 31840</td>
<td>~13</td>
<td>~12</td>
<td>20</td>
<td>20</td>
<td>5</td>
<td>PG (100)</td>
<td>YE (5)</td>
<td></td>
<td>Batch</td>
<td>Chen and Chang 1996</td>
</tr>
<tr>
<td><em>C. echinulata</em> ATHUM 4411</td>
<td>5.8</td>
<td>25.6</td>
<td>2.0</td>
<td>28</td>
<td>14.2</td>
<td>6</td>
<td>CG (80)</td>
<td>(NH₄)₂SO₄ (0.5)</td>
<td>Batch</td>
<td>Fakas et al. 2009b</td>
</tr>
<tr>
<td>Species</td>
<td>Culture</td>
<td>pH</td>
<td>Dry wt</td>
<td>Temperature</td>
<td>pH</td>
<td>Sugar</td>
<td>Initial Amount</td>
<td>Type</td>
<td>Growth media</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------</td>
<td>-------</td>
<td>--------</td>
<td>-------------</td>
<td>----</td>
<td>-------</td>
<td>----------------</td>
<td>--------------</td>
<td>---------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>Mortierella isabellina ATHUM 2935</td>
<td></td>
<td>8.5</td>
<td>51.7</td>
<td>4.4</td>
<td>28</td>
<td>17.5</td>
<td>6.1 CG</td>
<td>Batch</td>
<td>(NH₄)₂SO₄ (0.5) YE (0.5)</td>
<td>Papanikolaou et al. 2008</td>
</tr>
<tr>
<td>M. isabellina ATHUM 2935</td>
<td></td>
<td>2.9</td>
<td>53.2</td>
<td>3.3</td>
<td>28</td>
<td>11</td>
<td>6 CG (80)</td>
<td>Batch</td>
<td>(NH₄)₂SO₄ (0.5) YE (0.5)</td>
<td>Fakas et al. 2009b</td>
</tr>
<tr>
<td>Mucor mucedo 1384</td>
<td></td>
<td>24.9</td>
<td>18.6</td>
<td>28</td>
<td>6.1</td>
<td>PG (30)</td>
<td>YE (5)</td>
<td>Batch</td>
<td>NH₄Cl (2.0) YE (0.5)</td>
<td>Sajbidor et al. 1988</td>
</tr>
<tr>
<td>Rhodotorula glutinis ATCC 204091</td>
<td></td>
<td>24.4</td>
<td>~37</td>
<td>35</td>
<td>2</td>
<td>6 PG (20)</td>
<td>YE (0.3)</td>
<td>Batch</td>
<td>(NH₄)₂SO₄ (1.1) NH₄Cl (2.0)</td>
<td>Easterling et al. 2009</td>
</tr>
<tr>
<td>R. glutinis TISTR 5159</td>
<td></td>
<td>10.1</td>
<td>60.7</td>
<td>6.1</td>
<td>30</td>
<td>3</td>
<td>6 CG (95)</td>
<td>Fed-batch</td>
<td>(NH₄)₂SO₄ (1.1) YE (0.5)</td>
<td>Saenge et al. 2011</td>
</tr>
<tr>
<td>Thamnidium elegans CCF 1465</td>
<td></td>
<td>16.3</td>
<td>71.1</td>
<td>11.6</td>
<td>28</td>
<td>22.9</td>
<td>5.0 CG (90)</td>
<td>Batch</td>
<td>(NH₄)₂SO₄ (0.5) YE (0.5)</td>
<td>Chatzifragkou et al. 2011</td>
</tr>
<tr>
<td>Yarrowia lipolytica ACA-DC 50109</td>
<td></td>
<td>4.57</td>
<td>20.4</td>
<td>28</td>
<td>6</td>
<td>PG (104.9)</td>
<td>YE (0.5)</td>
<td>Repeated-batch cultures</td>
<td>(NH₄)₂SO₄ (0.5) YE (0.5)</td>
<td>Makri et al. 2010</td>
</tr>
</tbody>
</table>
Table 4 continued

<table>
<thead>
<tr>
<th>Y. lipolytica</th>
<th>CDW (g/L)</th>
<th>Lipid (g/L)</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>VFA (%)</th>
<th>FFA (%)</th>
<th>Growth medium</th>
<th>Growth condition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACA-DC 50109</td>
<td>7.0-7.9</td>
<td>6-14</td>
<td>28</td>
<td>3.8-25</td>
<td>6.1</td>
<td>CG (20.5-164.0)</td>
<td>(NH₄)₂SO₄ (0.5) YE (0.5)</td>
<td>Batch</td>
<td>Papanikolaou et al. 2008</td>
</tr>
<tr>
<td>ACA-YC 5033</td>
<td>6.5</td>
<td>31</td>
<td>2.0</td>
<td>16.5</td>
<td>6</td>
<td>CG (120)</td>
<td>(NH₄)₂SO₄ (0.5) YE (0.5)</td>
<td>Batch</td>
<td>André et al. 2009</td>
</tr>
<tr>
<td>LGAM S(7)1</td>
<td>8.1</td>
<td>43</td>
<td>3.5</td>
<td>0.5-1</td>
<td>6</td>
<td>CG from fat saponification (50)</td>
<td>(NH₄)₂SO₄ (0.5) YE (0.5)</td>
<td>Continuous</td>
<td>Papanikolaou and Aggelis 2002</td>
</tr>
<tr>
<td>ACA-DC 50109</td>
<td>4.68</td>
<td>22.3</td>
<td>28</td>
<td>2.1</td>
<td>6</td>
<td>PG (27.8)</td>
<td>(NH₄)₂SO₄ (0.5) YE (0.5)</td>
<td>Repeated-batch cultures</td>
<td>Makri et al. 2010</td>
</tr>
</tbody>
</table>

*a CDW = Cell dry weight

*b Lipid concentration based on total volume of lipid extracted from the biomass produced in 1L of medium

*c CG = Crude glycerol PG = Pure glycerol

*d YE = Yeast extract
1.6.2 Lipid Derivative Production: Polyunsaturated Fatty Acids

Polyunsaturated fatty acids are becoming increasingly popular in dietary supplements and health foods which target both adult and infant health (Beopoulos et al. 2009; Ratledge and Wynn 2002; Subramaniam et al. 2010). Research on the production of eicosapentanoic acid, arachidonic acid and dihomo-γ-linolenic acid from glycerol by fungi have been reported (Athalye et al. 2009; Hou 2008). Nonetheless it is the production of γ-linolenic acid (GLA) from glycerol which is receiving the most attention (Chatzifragkou et al. 2011; Chen and Chang 1996; Fakas et al. 2009b; Papanikolaou et al. 2008; Sajbidor et al. 1988).

1.6.2.1 Polyunsaturated Fatty Acid Production from Glycerol

Successful production of GLA has been reported from crude glycerol in recent work (Table 5). For example, Chatzifragkou et al. (2011) detected GLA in the lipid fractions of six fungal species cultured using crude glycerol. The GLA content of lipids reached 1.3 % to 14.6 % (w/w) and was most abundant in the phospholipid fraction of the total extracted lipids. In their study, *Thamnidium elegans* was able to produce 371 mg·L⁻¹ at an initial crude glycerol concentration of 90 g·L⁻¹ (Chatzifragkou et al. 2011). In another study, *C. echinulata* and *M. isabellina* produced GLA at a concentration of 190 mg·L⁻¹ and 116 mg·L⁻¹, respectively, from crude glycerol (Fakas et al. 2009b). It is important to note that these values were lower than those obtained using other inexpensive carbon substrates, such as xylose, where a GLA concentration of 1119 mg·L⁻¹ was reported in cultures of *C. echinulata* (Fakas et al. 2009b). This demonstrates the need for more research to improve GLA production using glycerol.
### Table 5. y-linolenic acid (GLA) production from glycerol batch cultures by selected fungal strains.

<table>
<thead>
<tr>
<th>Fungus</th>
<th>GLA Concentration (mg·L⁻¹)ᵃ</th>
<th>GLA Content (% total lipid)</th>
<th>Temp (°C)</th>
<th>Time (day)</th>
<th>pH</th>
<th>Carbon Source (g·L⁻¹)ᵇ</th>
<th>Nitrogen Source (g·L⁻¹)ᶜ</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Thamnidium elegans</em> CCF 1465</td>
<td>371</td>
<td>28</td>
<td>28</td>
<td>22.9</td>
<td>5.0-6.0</td>
<td>CG (90)</td>
<td>(NH₄)₂SO₄ (0.5)</td>
<td>Chatzifragkou et al. 2011</td>
</tr>
<tr>
<td><em>Cunninghamella echinulata</em> ATHUM 4411</td>
<td>190</td>
<td>9.5</td>
<td>28</td>
<td>14.2</td>
<td>6</td>
<td>CG (80)</td>
<td>(NH₄)₂SO₄ (0.5)</td>
<td>Fakas et al. 2009b</td>
</tr>
<tr>
<td><em>C. echinulata</em> CCRC 31840</td>
<td>≈ 250</td>
<td>20</td>
<td>20</td>
<td>5</td>
<td></td>
<td>PG (100)</td>
<td>YE (5)</td>
<td>Chen and Chang 1996</td>
</tr>
<tr>
<td><em>Mortierella isabellina</em> ATHUM 2935</td>
<td>116</td>
<td>9.7</td>
<td>28</td>
<td>11</td>
<td>6</td>
<td>CG (80)</td>
<td>(NH₄)₂SO₄ (0.5)</td>
<td>Fakas et al. 2009b</td>
</tr>
<tr>
<td><em>M. isabellina</em> ATHUM 2935</td>
<td>120</td>
<td>8.3</td>
<td>28</td>
<td>2</td>
<td>6.1</td>
<td>CG (100.5)</td>
<td>(NH₄)₂SO₄ (0.5)</td>
<td>Papanikolaou et al. 2008</td>
</tr>
<tr>
<td><em>Mucor mucedo</em> 1384</td>
<td>14.3</td>
<td>28</td>
<td>7</td>
<td>6.1</td>
<td></td>
<td>PG (30)</td>
<td>YE (5)</td>
<td>Sajbidor et al. 1988</td>
</tr>
</tbody>
</table>

ᵃ GLA concentration based on total volume of GLA extracted from the biomass produced in 1L of medium  
ᵇ CG= Crude glycerol  PG=Pure glycerol  
ᶜ YE = Yeast extract
1.6.3 Lipid Derivative Production: Biosurfactants

Surfactants represent a group of amphiphilic, petroleum-based products produced by means of chemical reactions (Van Bogaert et al. 2007). Surfactants function by accumulating along the interface of hydrophobic and hydrophilic solutions which reduces the surface tension and increases the solubility of immiscible fluids (Amaral et al. 2010). Due to their ability to act as solubilizers, emulsifiers and dispersants, a number of applications exist for their use in a range of industries including textile, cosmetic, food, pharmaceutical and detergent manufacturing (Van Bogaert et al. 2007).

Structurally comparable compounds, termed biosurfactants, can be produced by microorganisms. These compounds may be attached to the cell or produced extracellularly from both hydrophobic substrates and carbohydrates (Amaral et al. 2010). Bacterial species have dominated research as producers of biosurfactants, however, their pathogenic nature has limited their large scale use for this purpose (Amaral et al. 2010).

Increasingly, fungal and yeast species are being identified with the ability to produce glycolipids, an important group of biosurfactants. These species include members from Candida, Pseudozyma, Yarrowia and Ustilago genera (Amaral et al. 2010; Liu et al. 2011). Glycolipids are an important group of biosurfactants and include compounds such as rhamnolipids, sophorolipids, cellobioselipids, trehaloselipids and mannosyl erythritol lipids (Develter and Lauryssen, 2010). They are predominantly made up of a carbohydrate bound to one or more lipophilic moiety (Liu et al. 2011). Recently, interest in the production and use biosurfactants has increased due to their low toxicity,
biodegradability and density of potential applications (Ageitos et al. 2011; Ashby and Solaiman 2010; Liu et al. 2011).

1.6.3.1 Production of Biosurfactants from Glycerol

*Candida bombicola* ATCC 22214 has been identified with the ability to produce relatively large quantities of sophorolipids from glycerol (Ashby et al. 2005). Interestingly, crude glycerol proved to be a better substrate for sophorolipid production, resulting in 43 g·L⁻¹ sophorolipids, while pure glycerol cultures resulted in only 9 g·L⁻¹ of sophorolipid production. It was suggested that the presence of hexane soluble compounds within the crude glycerol resulted in the higher production of sophorolipids (Ashby et al. 2005). Indeed, sophorolipid production in pure glycerol increased to 43 g·L⁻¹ when medium was supplemented with 12.5 g·L⁻¹ biodiesel.

The production of other biosurfactants from glycerol has also been reported. Due to difficulties in separating biosurfactant mannosylerythritol lipids (MEL) from certain substrates used in their production (e.g. soybean oil), the use of glycerol has been proposed as an alternative which could simplify downstream processing (Morita et al. 2007). Morita et al. (2007) assessed the ability of ten strains of *Pseudozyma* to produce biosurfactants from pure glycerol. *Pseudozyma antarctica* JCM 10317 was identified with the ability to produce 16.3 g·L⁻¹ of MEL when 100 g·L⁻¹ pure glycerol and 2 % (w/v) mannose were both added to the culture intermittently.

Crude glycerol has also proven to be effective for MEL production. Liu et al. (2011), for example, were successfully able to produce MEL and ustilagic acid biosurfactants using 50 g·L⁻¹ crude glycerol obtained from the transesterification of waste cooking oil.
Growth of the fungus *Ustilago maydis* in batch cultures was significantly better in crude glycerol than pure glycerol, and total glycolipid yield was comparable to what was obtained in glucose cultures. After optimal culture parameters were established (pH 4, 50 g·L⁻¹ crude glycerol, 30 ºC, addition of trace elements) a total glycolipid concentration of 32.1 g·L⁻¹ was attained after 8 days (Liu et al. 2011).

**1.6.4 Organic Acid Production from Glycerol**

Organic acid production from glycerol is also showing promise as a way to utilize crude glycerol. Organic acids represent important building-block chemicals which can be produced from a variety of carbon substrates using the natural metabolic activities of microorganisms (Amaral et al. 2009; Sauer et al. 2008). As previously stated, the production of organic acids (e.g. fumaric acid, itaconic acid, succinic acid) from renewable resources has been identified as an important area of research by the US DOE (Werpy et al. 2004), though limited research on the production of organic acids from glycerol exist. Low amounts of fumaric acid have been produced by *Rhizopus* sp. using a combination medium containing glycerol and rice bran (Moon et al. 2004), while succinic acid production from a recombinant *Y. lipolytica* strain has also been reported (Yuzbashev et al. 2010). Contrary to the limited number of reports for the aforementioned organic acids, citric acid production using glycerol is beginning to emerge as a potential fungal bioproduct which can be produced using this substrate.

**1.6.4.1 Citric Acid Production**

Citric acid (CIT) represents a valuable industrially produced organic acid with applications in chemical, pharmaceutical, beverage and food industries (Imandi et al.
Annual production of CIT surpasses 800,000 tonnes globally and is increasing at a rate of 5 % per year (Amaral et al. 2009; Finogenova et al. 2005).

Historically, CIT was purified from citrus fruits, however 90 % of modern CIT production is now performed by means of batch fermentation, as this is the most economical production system available (Sauer et al. 2008; Soccol et al. 2006). A number of microorganisms, including filamentous fungi, yeast and bacteria have all been used as CIT producers, though it is the submerged fermentation of various carbohydrates by Aspergillus niger which is now the principal production system (Soccol et al. 2006).

The rising demand for CIT is fuelling a search for alternative substrates for its production. Increasingly more agricultural and industrial waste products have been investigated for this purpose including fruit pomace, brewery wastes and citrus and kiwi fruit peelings (Rywinska and Rymowicz 2010; Rymowicz et al. 2010; Soccol et al. 2006). Though glycerol has proven to be suitable for biomass accumulation in A. niger cultures, this increasingly available carbon substrate does not promote good CIT production by this fungus (Xu et al. 1989).

1.6.4.2 Pathway for Citric Acid Production

Citric acid is an important intermediate of the tricarboxylic acid pathway and is essential for cellular functioning (Kamzolova et al. 2011). Production of CIT by yeast and filamentous fungi follows the same pathway that oleaginous microorganisms use for lipid production, up to the point where citrate is expelled from the mitochondria into the
cytosol (Figure 2). Rather than being cleaved by acetyl-CoA lyase to produce oxaloacetate and acetyl-CoA for lipid production, non-oleaginous microorganisms simply release the excess CIT into the surrounding medium (Papanikolaou and Aggelis 2009; Ratledge and Wynn 2002).

1.6.4.3 Citric Acid Production from Glycerol

Research on a suitable replacement for A. niger has predominantly focused on yeast strains due to their resistance to high substrate concentrations, good conversion rates and tolerance to impurities permitting the use of low quality substrates (Kanzolova et al. 2011). While many species of yeast have biotechnological applications, high yield organic acid production using glycerol has primarily been documented in strains of Yarrowia lipolytica (Amaral et al. 2009). Research conducted by Kamzolova et al. (2011) highlights this finding. Fifty-nine natural and seven mutant yeast strains belonging to the genera Debaryomyces, Candida, Pichia, Saccharomyces, Yarrowia and Torulopsis were investigated by these authors for acid production on solid medium with glycerol as the carbon substrate (Kamzolova et al. 2011). Yeast belonging to the genera Yarrowia were almost the exclusive producers of organic acids, with the exception of a single Candida strain, C. paludigena VKM Y-2443, which exhibited only weak production. Similarly, Chatzifragkou et al. (2011) also screened a number of microorganisms, including both natural strains of yeast and filamentous fungi, for their ability to utilize glycerol. Among the nine yeast and six filamentous fungi tested only one, Yarrowia lipolytica LFMP 19, produced extracellular organic acids.
When Levinson et al. (2007) tested 27 strains of *Y. lipolytica* for their ability to produce CIT from 40 g·L\(^{-1}\) pure glycerol, all strains produced this organic acid at concentrations ranging from 1.4 g·L\(^{-1}\) to 21.6 g·L\(^{-1}\). These concentrations correspond to yields between 0.035 and 0.54 (g CIT per g glycerol consumed) and are some of the highest yields reported to date.

The production of this organic acid from glycerol has proven to be comparable to that of glucose, with some strains showing preference for glycerol over glucose (Papanikolaou and Aggelis 2009; Papanikolaou et al. 2002; Rywinska et al. 2010b). Papanikolaou et al. (2002) cultured *Y. lipolytica* LGAM S(7)1 in medium containing various mixtures of crude glycerol and glucose. When both glucose and glycerol were simultaneously supplied to this yeast, glycerol was preferentially taken up by the cells. Results from Rywinska et al. (2010b) are also in agreement with these findings. The yeast *Y. lipolytica* A-101 first utilized glycerol and subsequently consumed glucose after its depletion in culture broths.

Three distinct phases of growth are often observed during the metabolism of glycerol by *Y. lipolytica*, especially in repeated-batch cultures (Makri et al. 2010). Initial growth phase (0 h to 24 h) is characterized by an increase in biomass production through the conversion of glycerol to cellular material. Once the nitrogen source is depleted the second lipogenic phase (30 h to 52 h) begins. Citric acid production remains low during this phase but cellular lipids accumulate. Finally, in the third phase of growth, the CIT production phase (55 h to 130 h), stored lipids are degraded for cell maintenance while glycerol is taken up by cells to produce CIT. Due to lipid turnover during the CIT production phase, simultaneous production of these two bioproducts is difficult to
achieve. Many researchers report CIT production without noteworthy lipid accumulation (Chatzifragkou et al. 2011; Papanikolaou and Aggelis 2002; Papanikolaou et al. 2002) although André et al. (2009) were able to simultaneously produce CIT (50.1 g·L⁻¹) and lipids (30 % w/w and 2.0 g·L⁻¹) from Y. lipolytica using liquid submerged batch cultures supplemented with 120 g·L⁻¹ glycerol and both ammonium sulfate and yeast extract at 0.5 g·L⁻¹ as nitrogen sources.

As Table 6 shows, natural strains of Y. lipolytica cultured using crude glycerol from biodiesel manufacturing typically achieve similar yields (g CIT per g glycerol consumed) as those obtained from pure glycerol. Yields from crude glycerol ranged from 0.42 to 0.56 while those from pure glycerol ranged from 0.44 to 0.55. Similarly, the concentration of CIT in culture broths is comparable. Maximum values of 77.4 g·L⁻¹ and 66.5 g·L⁻¹ have been reported in the literature for crude and pure glycerol from cultures of natural strains (Table 6). These values are similar to the concentrations described for A. niger (see Soccol et al. 2006 for a more extensive review).

Similar to the fungal production of lipids, CIT production from glycerol by Y. lipolytica has been investigated in batch, fed-bath and repeated-batch culture systems (Rywinska et al. 2011). Although Rymowicz et al. (2010) suggested that using a repeated-batch mode or cell recycling could minimize expenses associated with sterilization and inoculum preparation, batch cultures are typically utilized with moderate temperatures (28 °C) and a slightly acidic pH (5.0 to 6.0) (Table 6). Few reports exist on continuous cultures as they often require high dilution rates to obtain good CIT production. This results in lower final CIT concentrations in the medium which hinders purification steps (Rywinska et al. 2010a; Rywinska et al. 2011).
Table 6. Culture conditions and citric acid production from the bioconversion of glycerol by *Yarrowia lipolytica* strains.

<table>
<thead>
<tr>
<th><em>Yarrowia lipolytica</em> strain</th>
<th>Biomass (g·L(^{-1}))</th>
<th>Citric Acid (g·L(^{-1}))</th>
<th>Citric Acid Yield(^a)</th>
<th>Temp (°C)</th>
<th>Time (day)</th>
<th>pH</th>
<th>Carbon Source (g·L(^{-1}))(^b)</th>
<th>Nitrogen Source (g·L(^{-1}))(^c)</th>
<th>Culture Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRRL YB-423</td>
<td>21.6</td>
<td>0.54</td>
<td>28</td>
<td>10</td>
<td>6.0</td>
<td>PG (40)</td>
<td>YE (0.25)</td>
<td>Batch</td>
<td>Levinson et al. 2007</td>
<td></td>
</tr>
<tr>
<td>NRRL YB-423</td>
<td>21.8</td>
<td>0.55</td>
<td>28</td>
<td>11</td>
<td>6.0</td>
<td>PG (40)</td>
<td>YE (0.1)</td>
<td>Batch</td>
<td>Levinson et al. 2007</td>
<td></td>
</tr>
<tr>
<td>1.22</td>
<td>24.0</td>
<td>54.0</td>
<td>30</td>
<td>7</td>
<td>3.0</td>
<td>CG (300)</td>
<td>NH(_4)Cl (1.0)</td>
<td>Fed-batch</td>
<td>Rymowicz et al. 2009</td>
<td></td>
</tr>
<tr>
<td>ACA-YC 5033</td>
<td>6.5</td>
<td>50.1</td>
<td>28</td>
<td>15.6</td>
<td>4.5-6.0</td>
<td>CG (120)</td>
<td>(NH(_4))(_2)SO(_4) (0.5)</td>
<td>Batch</td>
<td>André et al. 2009</td>
<td></td>
</tr>
<tr>
<td>LGAM S(7)1</td>
<td>5.8-6.8</td>
<td>33.6-35.1</td>
<td>28</td>
<td>9.8</td>
<td>5.0-6.0</td>
<td>CG (80-120)</td>
<td>(NH(_4))(_2)SO(_4) (0.5)</td>
<td>Batch</td>
<td>Papanikolaou et al. 2002</td>
<td></td>
</tr>
<tr>
<td>LGAM S(7)1</td>
<td>6-7.5</td>
<td>11-30</td>
<td>28</td>
<td></td>
<td></td>
<td>CG (45-120)</td>
<td>(NH(_4))(_2)SO(_4) (0.5)</td>
<td>Batch</td>
<td>Papanikolaou and Aggeli, 2002</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Yarrowia lipolytica biomass yield.
### Table 6 continued

<table>
<thead>
<tr>
<th>Strain</th>
<th>pH</th>
<th>OD</th>
<th>Temp</th>
<th>Time</th>
<th>Co-substrate</th>
<th>Co-micronutrient</th>
<th>Growth Phase</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACA-DC 50109</td>
<td>7.4</td>
<td>62.5</td>
<td>0.56</td>
<td>28</td>
<td>25</td>
<td>5.0-6.2</td>
<td>CG (164)</td>
<td>(NH₄)₂SO₄ (0.5)</td>
</tr>
<tr>
<td>ACA-DC 50109</td>
<td>5.9</td>
<td>33.6</td>
<td>0.52</td>
<td>28</td>
<td>10</td>
<td>6</td>
<td>PG (105)</td>
<td>(NH₄)₂SO₄ (0.5)</td>
</tr>
<tr>
<td>A-101</td>
<td>19.9</td>
<td>66.8</td>
<td>0.43</td>
<td>30</td>
<td>3.5</td>
<td>5.5</td>
<td>CG (150)</td>
<td>NH₄Cl (3.0)</td>
</tr>
<tr>
<td>A-101</td>
<td>17.1</td>
<td>66.5</td>
<td>0.44</td>
<td>30</td>
<td>4.3</td>
<td>5.5</td>
<td>PG (150)</td>
<td>NH₄Cl (3.0)</td>
</tr>
<tr>
<td>A-101</td>
<td>82.2</td>
<td>0.52</td>
<td>30</td>
<td>~4</td>
<td>5.5</td>
<td>1:1</td>
<td>CG:Glucose (150 each)</td>
<td>NH₄Cl (3.0)</td>
</tr>
<tr>
<td>A-101</td>
<td>19.0</td>
<td>22.0</td>
<td>30</td>
<td>7</td>
<td>3.0</td>
<td>3.0</td>
<td>CG (300)</td>
<td>NH₄Cl (1.0)</td>
</tr>
<tr>
<td>NCIM 3589</td>
<td>77.4</td>
<td>30</td>
<td>3</td>
<td></td>
<td>CG (54.4)</td>
<td>YE (0.3)</td>
<td>Batch</td>
<td>Imandi et al. 2007</td>
</tr>
<tr>
<td>NCIM 3589</td>
<td>13.41</td>
<td>30</td>
<td>3</td>
<td></td>
<td>CG (38.8)</td>
<td>(NH₄)₂SO₄ (0.2)</td>
<td>Batch</td>
<td>Kumari et al. 2008</td>
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Table 6 continued

<table>
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<tr>
<th>LFMB 19</th>
<th>5.9</th>
<th>9.4</th>
<th>0.11</th>
<th>28</th>
<th>12.9</th>
<th>6.0</th>
<th>CG (90)</th>
<th>(NH₄)₂SO₄ (0.5)</th>
<th>YE (0.5)</th>
<th>Batch</th>
<th>Chatzifragkou et al. 2011</th>
</tr>
</thead>
</table>

*a g citric acid per g glycerol consumed  
*b CG = Crude glycerol  PG = Pure glycerol  
*c YE = Yeast extract
Regardless if the search for a new bioproduct includes research on lipid production or organic acid production, many authors continue to emphasize the need for an inexpensive carbon feedstock if a viable industrial process is to be developed (Amaral et al. 2009; Sauer et al. 2007). With the large quantities of inexpensive glycerol currently available on the market, this biodiesel production co-product may provide a good feedstock option to develop an industrial process and is the focus of the research described in this thesis.
1.7 Research Objectives

The economics of the biodiesel production could be improved if new value-added compounds could be produced from the glycerol arising from the transesterification reaction, resulting in new markets for crude glycerol sales (Fakas et al. 2009). Finding uses for crude glycerol will assist not only in increasing the profitability of the process, but also reduce expenses associated with the disposal of this co-product (Rywinska et al. 2010).

The research presented in this study aimed to broaden crude glycerol bioconversion options by screening environmental isolates of filamentous fungi and yeast for those that:

- Utilize crude glycerol from biodiesel manufacturing as a carbon substrate for biomass production
- Tolerate the impurities found in this co-product
- Produce new and useful bioproducts as the result of glycerol metabolism
- Grow aerobically using minimal medium
- Are non-pathogenic

It has been stated that the discovery and development of new technologies which utilize the large quantities of crude glycerol is an area of research in need of immediate attention (Saenge et al. 2011). It is the goal of the research presented here to address this issue.
CHAPTER 2. MATERIALS AND METHODS

2.1 Crude Glycerol Collection and Characterization

2.1.1 Crude Glycerol Source and General Composition

Crude glycerol used in this study was kindly provided by the Centre for Agricultural Renewable Energy and Sustainability (CARES) biodiesel research facility at the University of Guelph, Ridgetown Campus (Ridgetown, ON). The crude glycerol was generated as the primary co-product from the alkali-catalyzed transesterification reaction of waste vegetable oil with methanol. Due to the high free fatty acid content of the feedstock, a sulphuric acid-catalyzed esterification reaction was performed prior to the transesterification step (Lamers 2010). This was completed as a preventative measure to reduce soap and emulsion production. A flow diagram depicting biodiesel production at the CARES facility is shown in Figure 3. Crude glycerol is retrieved from the process after the transesterification step, prior to washing the final biodiesel product. The amber-coloured crude glycerol which was obtained (approximately 20 L) was stored in a sealed plastic container at 20 °C to 25 °C for the duration of the trials described in this work. Prior to removing a sample, the container was vigorously shaken by hand to ensure an even distribution of its contents. All trials were conducted using the same batch of crude glycerol.
Figure 3. Process flow diagram for the production of biodiesel at the Centre for Agricultural Renewable Energy and Sustainability.

The composition of the crude glycerol was established by determining the concentration of glycerol, methanol and FFAs present in the sample. Quantitative
analysis of crude glycerol to determine the true glycerol content was conducted using the BioVision Free Glycerol Assay Kit (BioVision Inc., Mountain View, CA) according to the manufacturer’s directions. This enzyme system works by enzymatically oxidizing the glycerol to a product which interacts with the glycerol probe provided in the kit. The resulting product of this reaction shows an optimum absorbance at 570 nm and is directly proportional to the amount of glycerol present in the sample. The concentration of residual methanol in the crude glycerol was determined by Maxxam Analytics (Mississauga, ON) via gas-chromatography with flame ionization detection (GC-FID).

To estimate the FFA concentration, equal volumes (5 mL) of crude glycerol and distilled water were combined with 0.1 mL of 10 M HCl in a 15 mL centrifuge tube. When acidified, soaps which are present in the crude glycerol degrade, releasing their FFAs which can be isolated for analysis (Pyle et al. 2008). Once acid adjusted, the contents of the tube were mixed for 30 seconds using a fixed speed vortex mixer (Fisher Scientific) and centrifuged for 10 minutes at 90 x g. A clear separation of the lower aqueous phase from the upper, dark brown, fatty acids phase resulted, the latter of which was removed and weighed to determine the concentration of FFA as a percent of the total weight.

2.1.2 Elemental and Physical Analysis

Further characterization of crude glycerol was accomplished by sending a sample to the University of Guelph, Laboratory Services, Soil and Nutrient Laboratory (Guelph, ON). Nitrogen was determined as a percentage of wet weight using total Kjeldahl nitrogen based on Thomas et al. (1967) and macronutrients (Ca, K, Mg, Na, P,
S) were analyzed using an inductively coupled plasma optical emissions spectrometer (ICP-OES). Electrical conductivity (EC) was also assessed to determine sample salinity using an EC meter and the “Standard Methods for the Examination of Water and Wastewater” as described by Greenberg et al. (1992).

In addition to nutrient analysis, the pH of the crude glycerol co-product was measured by taking the average of the pH reported by Laboratory Services (pH 9.1) and a measurement recorded in the Ridgetown campus lab (pH 9.7). Measurements were made using the same crude glycerol sample. The pH of crude glycerol was determined in lab using a HANNA Instruments HI 211 pH/ORP meter (Hanna Instruments, Laval, QC) calibrated with buffers 7.01 and 10.01. The pH probe (model HI 1131) was directly submerged into 20 mL of crude glycerol and allowed to equilibrate until a constant reading was obtained (approximately five minutes).

2.2 Isolation of Environmental Microorganisms

A collection of 33 environmental fungal isolates, available from a previous collection (Lamers 2010) and stored at 4 °C on potato dextrose agar (PDA), were available for experimentation (Appendix A). New fungi were purified from environmental water samples collected across southern Ontario (see Appendix A). An additional three fungal isolates were obtained from biodiesel waste-stream samples and two fungal isolates were purified as contaminants from algal cultures growing in biodiesel wash water at the Ridgetown campus lab.

Environmental water samples were collected between the months of May and August, 2009. Fungal isolates were purified from these samples as outlined in the flow
diagram presented in Figure 4. Pure cultures were stored as stock plates at 4 °C on PDA. Prior to beginning a trial, a fungal plug (28 mm diameter) was removed from stock plates and incubated on fresh medium at 20 °C to 25 °C. Long term storage of microorganisms was done at -80 °C by freezing a fungal plug removed from the edge of actively growing mycelium with 10 % (v/v) reagent grade glycerol in distilled water. All isolates were frozen in duplicate and tested for viability after a minimum of one week of storage.
Collect environmental samples in clean 50 ml tubes

Prepare $10^0$ - $10^{-3}$ dilutions using distilled water

Spread plate 500 µl of each dilution on PDA

Incubate plates at 20 °C to 25 °C

Subculture and purify individual colonies

Store samples at 4 °C if immediate plating is not possible

If overgrown, prepare fresh $10^{-4}$ to $10^{-5}$ dilutions and replate

Store pure cultures on PDA at 4 °C or in 10 % (v/v) glycerol at -80 °C

**Figure 4.** Procedure for the isolation of fungi from environmental and biodiesel waste samples.
2.3 Screening Microbial Cultures for Growth in Crude Glycerol

2.3.1 Microorganisms and Media

Sixty-one fungal isolates were used in preliminary trials to identify isolates with the ability to utilize crude glycerol as the primary carbon substrate for growth. Two types of medium were utilized during the study. Yeast extract medium (YE) was used for initial screening trials and was prepared by combining 10 g·L\(^{-1}\) yeast extract (Fisher Scientific) with distilled water, as described by Athalye et al. (2009). Carbon free minimal medium (CFMM) was also utilized. This chemically defined synthetic medium, contained (in g·L\(^{-1}\)): Na\(_2\)HPO\(_4\) 2.2; KH\(_2\)PO\(_4\) 0.3; NH\(_4\)NO\(_3\) 3.0; MgSO\(_4\)·7H\(_2\)O 0.2; FeSO\(_4\)·7H\(_2\)O 0.01; CaCl\(_2\)·2H\(_2\)O 0.01 (Habe et al. 2004).

Depending on the trial, media were supplemented with carbon substrates (glucose or crude glycerol) between 50 g·L\(^{-1}\) and 100 g·L\(^{-1}\). Due to the presence of additional contaminants in the crude glycerol, the true glycerol content which represents 28 \% (w/v) in treatment cultures was 14 g·L\(^{-1}\) and 28 g·L\(^{-1}\). Reagent grade glucose was used in control cultures to test the effectiveness of crude glycerol as a carbon and nutritional source for fungal growth. Media were autoclaved at 121 °C for 30 minutes. A serial dilution (10\(^{-1}\) to 10\(^{-3}\)) was prepared of autoclaved medium and plated out on PDA. This test ensured that any contaminating microorganisms which might be present in the crude glycerol or glucose were sterilized. After cooling, pH adjustments were made to the medium using a 10 M solution of HCl or KOH. All pH measurements were performed using the HANNA Instruments HI 211 pH/ORP meter calibrated with buffers with pH 7.01 and 10.01.
2.3.2 Culture Conditions

Screening trials investigating the ability isolates to utilize crude glycerol for growth were performed as liquid batch cultures in 50 mL Erlenmeyer flasks with a working volume of 20 mL. Trials were completed at least twice, with the exception of the initial screen of the 61 isolates. Prior to beginning a trial, fungi were pre-cultured at 20 °C to 25 °C on fresh PDA plates. Inoculations were performed using a fungal plug (5.8 mm diameter) aseptically removed from the actively growing outer edge of the mycelium. Incubations were carried out for seven to 15 days at 20 °C to 25 °C with orbital shaking at 150 rpm. Initial screening of the culture collection (61 isolates) was performed as single flask trials (n=1). All other trials were conducted with three replicate flasks per treatment and this datum was used to calculate means and standard errors.

2.3.3 Analytical Methods

Fungal growth yield, which was based on biomass yield (g biomass L⁻¹ culture broth), was assessed by harvesting cells at the end of the cultivation period. Dry weights were obtained by filtering culture broths through pre-weighed Fisherbrand P8 filter paper (Fisher Scientific) or 0.45 µl membrane filters (Magna nylon filters or Millipore nitrocellulose filters). When membrane filters were used, the Millipore Sterifil aseptic system (Millipore, Billerica MA) connected to a DistiVac Plus vacuum (Brinkmann Instruments Inc., Westbury NY) was employed to increase the speed of the filtering process. Harvested biomass was dried at 65 °C to 85 °C in an Isotemp oven (Model 630F, Fisher Scientific) until a constant weight was attained. Final biomass
yields were corrected for the effect of uninoculated medium on weight and relative
growth yield (RG) was calculated as [1]

\[
RG = \left( \frac{T-C}{C} \right) \times 100 \quad [1]
\]

where \( T \) represents the crude glycerol treatment weight and \( C \) represents the glucose
control weight of dried fungal biomass.

Carbon substrates and metabolites were detected in culture broths by thin layer
chromatography (TLC) using a protocol adapted from Bansal et al. (2008). Culture
broths were either analyzed directly or by combining 200 µl of broth with 800 µl of
acetone prior to analysis. Samples were spotted onto silica-coated aluminum plates (AL
SIL G/UV, Whatman Ltd, England) and chromatographic separations were performed
using water saturated 1-butanol as the solvent. After thoroughly air-drying at 20 °C to 25
°C, compounds were detected by briefly dipping plates into a solution of 0.5 % (w/v)
KMnO₄ in 1 M NaOH. Glycerol and glucose appeared as yellow spots on a pink
background. Retention factor (\( R_f \)) values were calculated from the centre of spots as [2].

\[
R_f = \frac{Distance \ traveled \ by \ compound}{Distance \ traveled \ by \ solvent \ front} \quad [2]
\]

Because the colors on the plate rapidly fade, a digital image was recorded within the
first ten minutes.
2.4 Characterization of Fungal Isolates

2.4.1 DNA Extraction

DNA isolation and polymerase chain reaction (PCR) amplification of target DNA was performed with the help of Sukhdeep Sidhu from Dr. Hung Lee’s laboratory at the University of Guelph (Guelph, ON) to determine the identity of leading candidates identified in section 2.3. Fungal cultures of F24, BF1, V19 and V22 were prepared in 20 mL of potato dextrose broth (PDB) inoculated with a loop of fungal culture scraped from PDA plates. Cultures were incubated overnight at 28 °C with orbital shaking at 200 rpm.

Genomic DNA isolations were performed using a QIAGEN DNeasy extraction kit (Mississauga, Canada). Two millilitres of each culture was centrifuged for 10 minutes at 6000 x g to pellet cells. After removal of the supernatant, cells were lysed by suspending the pellets in 0.2 mL of lysis buffer (1M sorbitol, 0.1M EDTA, pH 7.4) along with 50 units of lyticase per 10^7 cells (Sidhu 2011). Tubes were incubated for 30 minutes at 30 °C in a water bath. Once cell lysis was performed, all subsequent steps for DNA extraction were performed according to the DNeasy kit manufacturer’s directions.

2.4.2 PCR Amplification of the 18S rRNA gene

Polymerase chain reaction amplification of the 18S ribosomal RNA gene (18S rRNA) was carried out on extracted genomic DNA using a Mastercycle® Gradient thermal cycler (Eppendorf Canada, Mississauga, ON). A PCR master mix (Fermentas Canada Inc., Burlington, ON) was combined with 200 nM of each primer and approximately 50 ng of template DNA for each isolate (Sidhu 2011). Positive and negative control reactions were prepared using DNA from Candida shehatae (positive
control) or water (negative control) as the reaction template. Forward 18sF2 (GCCATGCATGTCTAAGTATAA) and reverse 18sR4 (CCTCGTTAAGGGATTTAAATTGT) primers were selected from Muir et al. (2009). The reaction included one cycle at 94 °C for three minutes, 35 cycles at 94 °C for 45 seconds, 58 °C for 45 seconds and 72 °C for 45 seconds and finally an extension cycle at 72 °C for five minutes (Sidhu 2011).

Analysis of the PCR products was performed by running 50 µl of each reaction on a 2 % (w/v) agarose gel for 30 minutes at 85 Volts (Sidhu 2011). DNA bands were visualized under UV light after staining with ethidium bromide. The size of the amplified fragments was determined using a molecular ladder run alongside the PCR reactions.

2.4.3 DNA Sequencing

The PCR products obtained from each isolate were sent for sequencing by the University of Guelph, Laboratory Services (Guelph, ON). Sequencing reactions were performed using 3 ng to 20 ng of the template DNA and the ABI Prism® BigDye® Terminator Cycle Sequencing Ready Reaction kit v3.1, following the manufacturer's directions (Applied Biosystems, Foster City, CA). Prior to sequencing analysis, dye terminators were removed from the reaction using Sephadex G-50 (Sigma, Oakville, ON) in Multiscreen-HV plates (Millipore, Mississauga, ON). Chromatograms produced from the clean products were finally analyzed using the ABIPrism® DNA Sequencing Analysis Software Version 3.7 (Applied Biosystems). Both forward and reverse sequences were obtained for each isolate.
The identity of each fungal isolate was determined by conducting a sequence homology search of the nucleotide database on the National Center for Biotechnology Information website (NCBI, 2011). This was performed using the Basic Local Alignment Search Tool (BLAST) available online. The identity of each fungus was concluded based on the highest homology of each sequence to known sequences within the NCBI database.

**2.5 Process Development for Biomass Production**

Culture optimization trials were performed in CFMM using the top performing fungal isolates (F24, BF1, V19 and V22) selected from the initial screen of the culture collection. These were selected based on biomass yield, time required for complete glycerol metabolism and their ability to produce new bioproducts. Culture conditions and analytical methods were the same as described in section 2.3. For solid media growth trials, CFMM was prepared and 15 g·L⁻¹ of agar was added prior to autoclaving. In addition to glucose, reagent grade glycerol was also used as a control to assess the effectiveness of crude glycerol as a carbon substrate for fungal growth. Trials were conducted using three replicate flasks per treatment (crude glycerol or pure glycerol) and this datum was used to calculate means and standard errors.

**2.5.1 Effect of Initial Carbon Substrate, pH and Methanol on Growth Yield**

The optimal glycerol concentration for fungal growth yield was determined from initial carbon substrate concentrations of 25 g·L⁻¹ to 400 g·L⁻¹ and pH 8.5. Due to the presence of other contaminants, crude glycerol culture broths contained less carbon than controls. The true glycerol content for treatments cultures was between 7 g·L⁻¹ and
112 g·L\(^{-1}\). Both solid medium (three week trials) and liquid cultures (one week trials) were used. Using a crude glycerol concentration of 100 g·L\(^{-1}\) (glycerol content 28 g·L\(^{-1}\), C:N 41), the optimal culture pH for growth was subsequently determined in liquid cultures. This was performed by testing isolates biomass yield after seven days of incubation at pH values of 5, 6, 7, 8 and 9.

In order to determine the tolerance of microorganisms used in this study to methanol, fungi were grown for one week in medium containing zero to 116.6 g·L\(^{-1}\) of added methanol. This corresponded to volumetric concentrations between zero and 12 % (v/v). Medium was supplemented 100 g·L\(^{-1}\) crude glycerol (glycerol content 28 g·L\(^{-1}\); treatment) or 100 g·L\(^{-1}\) glycerol (control) and pH adjusted to 8.0. Due to the presence of residual methanol in the crude glycerol, the actual methanol content was higher in treatment cultures by 23.9 g·L\(^{-1}\). Culture conditions and analytical methods were the same as described in section 2.3.

### 2.6 Bioconversion of Crude Glycerol to Bioproducts

#### 2.6.1 Large Scale Growth of Isolates

In an effort to determine the identity of the new fungal bioproducts detected by TLC, 400 mL batch cultures (n=1) were prepared for isolates which were identified as promising candidates for crude glycerol bioconversions. Isolates F24, BF1, V19 and V22 were grown in CFMM supplemented with 100 g·L\(^{-1}\) of crude glycerol (glycerol content 28 g·L\(^{-1}\); treatment) or 100 g·L\(^{-1}\) glucose (control). No pH adjustments were performed for crude glycerol containing medium, resulting in a pH of 8.9. Control flasks were therefore adjusted with 10 M KOH after autoclaving to 8.9.
Cell suspensions for each isolate were prepared by washing the surface of six day old actively growing cultures on PDA (fungi covered approximately ¾ of the plate’s surface) with 10 mL sterile distilled water, similar to the protocol described by Athalye et al. (2009). The surface of each plate was gently scratched with a sterile needle to increase the concentration of the cell suspension and 4 mL (1 % v/v inoculum) was used to inoculate the CFMM. A second BF1 culture was also prepared by inoculating 400 mL of CFMM with a BF1 fungal “ring.” This was prepared by removing the center from a six day old BF1 culture using the top edge of a flamed 125 mL Erlenmeyer flask to produce a ring of fungal mycelium. This ring was cut into five rough segments which were used as inoculum. All incubations were carried out at 20 °C to 25 °C with orbital shaking at 100 rpm. Once a week, for a duration of six weeks, a 200 µl sample of culture broth was removed from each flask for TLC analysis as described in section 2.3.3.

2.6.2 Bioproduct Extraction and Column Purification

Cultures described above were incubated until crude glycerol was no longer detected on TLC plates (six weeks). Half of the culture broth (200 mL, including fungal biomass) was decanted from each culture flask into a separatory funnel and extracted three times with 150 mL of water saturated 1-butanol. Extractions were performed by adding the water saturated 1-butanol solvent to the culture broth and gently rocking the funnel for 30 seconds to mix. Phase separation between the water saturated 1-butanol and culture broths occurred in less than five minutes for the first solvent extraction. During the second and third extractions, phase separation proceeded more slowly and funnels were allowed to sit at least 30 minutes to obtain two distinct layers. After phase
separation, the upper butanol layer was removed using a pipette and the process was repeated for a total of three extractions. The butanol extracts were pooled together and concentrated using a Heidolph Collegiate Laboratora 4000 rotary evaporator (Heidolph Instruments, Germany). Evaporation occurred under vacuum at 90 °C and 90 rpm until solvent was no longer condensing on the cooling coil. The resulting extracts were stored at 4 °C.

Two mL of each concentrated sample was purified by column chromatography in an attempt to separate the bioproduct detected by TLC from other compounds. Guidelines for preparing and running columns were established using information from Williams and Hill (2009) and Murphy (2010). Chromatography columns were prepared using a 30 cm glass column (KIMAX, USA) with an internal diameter of 2.5 cm. A short length of plastic tubing was fitted to the bottom of the column to control solvent flow by means of a hemostat.

Columns were prepared and run following the directions in Figure 5. As a result of the dark colour of the fungal extract, it was possible to visually track the progression of the latter down the silica (Figure 6). When the coloured front reached the bottom of the column, 5 mL aliquots were collected in labelled test tubes. Thin layer chromatography, as described in 2.3.3, was used to ensure that the desired bioproduct spot was still present after the extraction and column purification. Aliquots which showed the presence of the bioproduct of interest were pooled together and the solvent removed by rotary evaporation as previously described.
Plug the base of column with cotton and add sand to a depth of 1 cm

Gently add 10 mL of n-hexane

Prepare a slurry by combining 20 g of Whatman Purasil 230-400 mesh silica powder with 30 mL of n-hexane

Gently add the silica slurry in 5 mL volumes to the column. Tap the side to remove air bubbles

Once silica is packed, top the column off with sand to a depth of 1 cm to protect the silica

Prepare the column for samples by rinsing with 2 volumes of the mobile phase (water saturated 1-butanol)

Load 2 mL of extracted bioproduct, open the hemostat and allow the extract to run down the column

Collect 5 mL aliquots in labelled test tubes and analyze for bioproducts by TLC

Do not allow the mobile phase to drop below the top layer of sand. Top up column with more mobile phase as needed

**Figure 5.** Procedure for the purification of fungal bioproducts by column chromatography.
Figure 6. Column chromatography of extracted fungal bioproducts. The components in 2 mL of each fungal extract were separated using a silica column eluted with water saturated 1-butanol as the mobile phase. The black arrow indicates the progression of the fungal extract through the column.

2.6.3 Qualitative Analysis of Bioproducts

Initially, the identification of bioproducts was conducted by running the bioproducts alongside standards on TLC plates as described in 2.3.3. Standards included a variety of sugars, organic acids and specialty compounds such as 1,3-propanediol, at a concentration of 10 mg·mL⁻¹ in water or ethanol. BF1, which produced a non-polar compound, was also compared with the FFAs separated from crude glycerol in 2.1.1 by directly spotting the FFA onto the TLC plate. This was performed to
ensure that the spot being detected on TLC plates was not simply due to soap splitting in the crude glycerol.

Further qualitative analysis was conducted by the University of Guelph, Laboratory Services (Guelph, ON) using the gas chromatography–mass spectrometry (GC-MS) protocol described by Kim et al. (2010). Bioproducts obtained for isolates BF1, V19 and V22 were diluted in 80 % (v/v) methanol to give a bioproduct concentration between 10 mg·mL\(^{-1}\) and 100 mg·mL\(^{-1}\) of methanol prior to analysis.

2.6.4 Quantitative Analysis of Citric Acid Production

Qualitative analysis of culture broths by GC-MS analysis revealed the presence of CIT. The ability of isolates V22 and BF1 to produce extracellular CIT was therefore assessed quantitatively. Batch cultures (n=3) of these fungi were prepared by inoculating 400 mL of YE medium. Treatment cultures contained 50 g·L\(^{-1}\) crude glycerol (glycerol content 14 g·L\(^{-1}\); C:N ratio 217) while control cultures contained 50 g·L\(^{-1}\) glucose. Furthermore, control cultures were pH adjusted with 10 M KOH to match the pH of crude glycerol containing medium (pH 8.3). Flasks were inoculated with 10 % (v/v) of a liquid inoculum produced by adding 40 mL of 4 to 5 day old liquid cultures growing in PDB to 360 mL of YE medium. Cultures were incubated for seven days at 20 °C to 25 °C with orbital shaking at 150 rpm. In addition to the above experiment, culture broths for isolates V22 and F24 from culture optimization trials described in section 2.5.1 were also analyzed for this metabolite.

Quantitative analysis of extracellular CIT production was conducted using Megazyme Citric Acid enzyme assay kit (Megazyme International Ireland Ltd., Wicklow,
Ireland). This enzyme kit function by first producing oxaloacetate and acetate from CIT by the activity of the enzyme citrate lyase. When NADH is present, the subsequent conversion of oxaloacetate to L-malate by the enzyme L-malate dehydrogenase occurs. The consumption of NADH in this reaction is measured at 340 nm and is proportional to amount of CIT initially present in the sample. Analyses were directly performed on culture broths after biomass removal by filtration using Whatman P8 coarse filter paper or centrifugation at 1160 x g for 10 minutes to pellet cells.

### 2.7 Bioconversion of Crude Glycerol to Single Cell Oil

#### 2.7.1 Culture Conditions

In addition to investigating CIT production, the ability of isolates V22 and BF1 to produce SCO was assessed. Lipid extractions were performed on V22 and BF1 cultures after five, seven or 28 days of incubation. Batch cultures of these fungi were prepared in 1 litre flasks by inoculating 400 mL of YE medium supplemented with 50 g·L⁻¹ crude glycerol (glycerol content 14 g·L⁻¹) giving a C:N ratio of 217. Glucose or pure glycerol (50 g·L⁻¹) was used as a control to test the effectiveness of crude glycerol as a carbon substrate for lipid production. Controls were pH adjusted with 10 M KOH to match the pH of crude glycerol containing medium (pH 8.3). Flasks were inoculated with 6 to 8 fungal plugs removed from actively growing cultures on PDA for the 28 day trial. All other trials were conducted using medium inoculated with 10 % (v/v) of a liquid inoculum. This was achieved by adding 40 mL of 4 to 5 day old liquid cultures growing in PDB to 360 mL of YE medium. Cultures for all of the described trials were incubated
at 20 °C to 25 °C with orbital shaking at 150 rpm. Trials were conducted in triplicate and this data was used to calculate means and standard errors.

2.7.2 Lipid Extractions

After the incubation period, biomass was harvested from cultures by centrifuging broths at 1160 x g for 10 minutes to pellet cells. Pellets were dried to a constant weight at 60 °C to 80 °C and lipids were extracted from biomass, after grinding to a fine powder in a food processor, using chloroform:methanol (2:1 v/v) and the protocol described by Zhu et al. (2009). Residual glycerol was detected in culture broths after the incubation period using the TLC technique described in section 2.1.1.

2.8 Analysis of Data

In addition to calculating means and standard errors, more in depth data analysis was conducted using the statistical analysis software SYSTAT® version 13. Significant differences between calculated means for crude glycerol versus glucose or pure glycerol controls were determined using a Student’s t-test. When samples belonged to an asymmetric distribution or possessed unequal variances they were analyzed using a Mann-Whitney u-test.
CHAPTER 3. RESULTS

3.1 Crude Glycerol Characterization

3.1.1 General Composition of Crude Glycerol

Enzymatic analysis of the crude glycerol revealed that glycerol represented only one of three major components within this co-product. Glycerol was detected at a concentration of 280 g·L\(^{-1}\) (28 % w/v) (Table 7). Free fatty acids made up the largest portion and accounted for 297 g·L\(^{-1}\) (30 % w/v), while methanol was slightly lower at 260 g·L\(^{-1}\) (26 % w/v) of the total crude glycerol sample analyzed (Table 7). When autoclaved, an 8 % (w/v) reduction in the methanol content of the crude glycerol was observed (i.e. the methanol content dropped from 26 % to 18 % w/v). Compared to other components in Table 1, FFA and methanol represented the most abundant contaminants in the analyzed sample.

3.1.2 Elemental and Physical Analysis

Further analyses were completed in order to determine if additional nutrients or inhibitors of fungal growth yield were present in the crude glycerol. Nitrogen was detected at a very low concentration, with total nitrogen being present at a concentration of 190 µg·g\(^{-1}\) (Table 7). The two most abundant elements in the crude glycerol sample included potassium at 28,000 µg·g\(^{-1}\) and sulphur at 1,400 µg·g\(^{-1}\) (Table 7). In addition to these elements, in order of decreasing concentrations, sodium, phosphorus, magnesium and calcium were also detected by ICP-OES analysis (Table 7).

The pH obtained for the crude glycerol was 9.4 and the salinity of the crude glycerol, measured as EC, was moderate at 7.73 mS·cm\(^{-1}\) (Table 7).
Table 7. Compositional analysis of crude glycerol obtained from the Centre for Agricultural Renewable Energy and Sustainability biodiesel research facility.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>4.3 µg·g⁻¹</td>
</tr>
<tr>
<td>Magnesium</td>
<td>1.3 µg·g⁻¹</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>7.8 µg·g⁻¹</td>
</tr>
<tr>
<td>Potassium</td>
<td>28,000 µg·g⁻¹</td>
</tr>
<tr>
<td>Sodium</td>
<td>230 µg·g⁻¹</td>
</tr>
<tr>
<td>Sulfur</td>
<td>1,400 µg·g⁻¹</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>190 µg·g⁻¹</td>
</tr>
<tr>
<td>Electrical Conductivity</td>
<td>7.73 mS·cm⁻¹</td>
</tr>
<tr>
<td>Glycerol</td>
<td>280 g·L⁻¹</td>
</tr>
<tr>
<td>Methanol</td>
<td>260g·L⁻¹</td>
</tr>
<tr>
<td>Free Fatty Acids</td>
<td>297g·L⁻¹</td>
</tr>
<tr>
<td>pH</td>
<td>9.4</td>
</tr>
</tbody>
</table>

The crude glycerol appeared to be stable over the timeframe of the trials described in this work. Visually, no physical changes were observed in the crude glycerol and the spot detected by TLC appeared to be consistent from the beginning to the end of trials described over the two year period.
3.2 Isolation of Environmental Microorganisms

Twenty-eight new fungal isolates were successfully purified from environmental water and biodiesel waste stream samples (appendix A). Combined with the isolates already available, 61 fungi were available for trials. This collection encompassed a variety of morphologies, with the majority of isolates showing hyphal growth, although the collection also included yeast isolates (Figure 7). A list of microbial isolates, including the location from which they were obtained, can be found in Appendix A.

Figure 7. Morphological variations of fungi isolated from environmental and biodiesel waste samples. Cultures were grown on potato dextrose agar for seven days at 20 °C to 25 °C. From left to right and starting at the top row isolates include: F29, S125, F13, F21, F16, F3, S115 and F18.
3.3 Screening Microbial Cultures for Growth in Crude Glycerol

3.3.1 Initial Screening of Fungal Isolates in Undefined Medium

The fungal collection, encompassing 61 fungal isolates purified from environmental samples, biodiesel waste streams and algal culture contaminants were screened for their ability to grow in crude glycerol. Trials were performed over a seven to 15 day period in the undefined YE medium and growth yield was assessed as the total yield of dry biomass.

Screening of the culture collection revealed that growth yield of the environmental isolates covered a large range, with biomass yields varying from as much as 34.3 g·L⁻¹ (F10) to as little as 2.3 g·L⁻¹ (V24) in crude glycerol containing medium (Figure 8). Interestingly, 40 of the 61 isolates produced higher biomass yields when grown in the treatment medium containing 50 g·L⁻¹ crude glycerol (glycerol content 14 g·L⁻¹) compared to their corresponding glucose controls (Figure 8 and Table 8). Based on their relative growth yield compared to glucose controls (see equation [2] in section 2.3.3), these 40 isolates could be divided into three categories:

1. Isolates showing a biomass yield increase, compared to controls, between 5 % and 50 % (18 isolates)
2. Isolates showing a biomass yield increase, compared to controls, between 51 % and 100 % (10 isolates)
3. Isolates showing a biomass yield increase, compared to controls, above 100 % (12 isolates).
**Figure 8.** Effect of carbon substrate on dry biomass yield of environmental fungal isolates. Batch cultures (n=1) were performed in yeast extract medium supplemented with 50 g·L⁻¹ of crude glycerol (glycerol content 14 g·L⁻¹) or 50 g·L⁻¹ glucose (control) and pH adjusted to 6.5. Biomass yields were determined after 14 days of incubation by filtering biomass through 0.45 µm membrane filters and drying to a constant weight.
Table 8. Growth yield of fungal isolates in crude glycerol relative to glucose control.

Fungi were grown in yeast extract medium (n=1) using 50 g·L⁻¹ of crude glycerol (glycerol content 14 g·L⁻¹) or glucose (control).

<table>
<thead>
<tr>
<th>Fungal Isolate</th>
<th>Complete Glycerol Metabolism</th>
<th>Growth Yield Relative to Control (%)</th>
<th>Fungal Isolate</th>
<th>Complete Glycerol Metabolism</th>
<th>Growth Yield Relative to Control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X4</td>
<td>N</td>
<td>269.7</td>
<td>F8</td>
<td>Y</td>
<td>32.1</td>
</tr>
<tr>
<td>F7</td>
<td>Y</td>
<td>225.5</td>
<td>F16</td>
<td>N</td>
<td>26.5</td>
</tr>
<tr>
<td>F26</td>
<td>N</td>
<td>206.6</td>
<td>S125</td>
<td>N</td>
<td>22.3</td>
</tr>
<tr>
<td>F12</td>
<td>N</td>
<td>171.5</td>
<td>S36</td>
<td>Y</td>
<td>20.2</td>
</tr>
<tr>
<td>F23</td>
<td>Y</td>
<td>168.5</td>
<td>F13</td>
<td>N</td>
<td>16.3</td>
</tr>
<tr>
<td>T2R15a</td>
<td>N</td>
<td>143.7</td>
<td>S101</td>
<td>Y</td>
<td>12.1</td>
</tr>
<tr>
<td>S141</td>
<td>Y</td>
<td>136.0</td>
<td>S83</td>
<td>Y</td>
<td>7.7</td>
</tr>
<tr>
<td>EB Pp</td>
<td>Y</td>
<td>131.0</td>
<td>F35</td>
<td>Y</td>
<td>5.9</td>
</tr>
<tr>
<td>BF2</td>
<td>Y</td>
<td>117.9</td>
<td>T1 Can</td>
<td>N</td>
<td>5.9</td>
</tr>
<tr>
<td>F9</td>
<td>N</td>
<td>115.3</td>
<td>F36</td>
<td>Y</td>
<td>-1.4</td>
</tr>
<tr>
<td>F6</td>
<td>Y</td>
<td>114.9</td>
<td>X29</td>
<td>N</td>
<td>-1.9</td>
</tr>
<tr>
<td>F32</td>
<td>N</td>
<td>100.8</td>
<td>EB Pk</td>
<td>Y</td>
<td>-3.4</td>
</tr>
<tr>
<td>F29</td>
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<td>99.0</td>
<td>F15</td>
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<td>-18.4</td>
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<tr>
<td>BF1</td>
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<td>90.8</td>
<td>S98</td>
<td>N</td>
<td>-19.5</td>
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<tr>
<td>F10</td>
<td>N</td>
<td>89.3</td>
<td>F1</td>
<td>Y</td>
<td>-19.6</td>
</tr>
<tr>
<td>F14</td>
<td>Y</td>
<td>89.0</td>
<td>F18</td>
<td>N</td>
<td>-24.2</td>
</tr>
<tr>
<td>ConF</td>
<td>Y</td>
<td>87.1</td>
<td>F17</td>
<td>Y</td>
<td>-25.1</td>
</tr>
<tr>
<td>S108</td>
<td>N</td>
<td>83.7</td>
<td>S121</td>
<td>Y</td>
<td>-25.8</td>
</tr>
<tr>
<td>F19</td>
<td>N</td>
<td>83.7</td>
<td>T7R15a</td>
<td>N</td>
<td>-38.6</td>
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<tr>
<td>S1</td>
<td>Y</td>
<td>77.8</td>
<td>F4</td>
<td>N</td>
<td>-39.0</td>
</tr>
<tr>
<td>V19</td>
<td>Y</td>
<td>57.1</td>
<td>X2</td>
<td>N</td>
<td>-45.0</td>
</tr>
<tr>
<td>F11</td>
<td>N</td>
<td>51.5</td>
<td>X3</td>
<td>N</td>
<td>-48.8</td>
</tr>
<tr>
<td>V22</td>
<td>Y</td>
<td>47.3</td>
<td>F3</td>
<td>N</td>
<td>-54.4</td>
</tr>
<tr>
<td>S93</td>
<td>N</td>
<td>44.7</td>
<td>S70</td>
<td>N</td>
<td>-59.0</td>
</tr>
<tr>
<td>S64</td>
<td>Y</td>
<td>43.5</td>
<td>F21</td>
<td>N</td>
<td>-68.0</td>
</tr>
<tr>
<td>S9</td>
<td>Y</td>
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<td>X14</td>
<td>N</td>
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</tr>
<tr>
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<td>N</td>
<td>-72.3</td>
</tr>
<tr>
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<td>N</td>
<td>38.2</td>
<td>S75</td>
<td>N</td>
<td>-77.3</td>
</tr>
<tr>
<td>F27</td>
<td>Y</td>
<td>36.8</td>
<td>S122</td>
<td>N</td>
<td>-86.6</td>
</tr>
<tr>
<td>S124</td>
<td>Y</td>
<td>32.8</td>
<td>V24</td>
<td>N</td>
<td>-92.7</td>
</tr>
<tr>
<td>S115</td>
<td>Y</td>
<td>32.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Glycerol metabolism was based on TLC analysis. Y = Isolate showed complete metabolism of crude glycerol; N = Isolate did not completely metabolise the crude glycerol.

b Growth yield relative to control was calculated as 100*(T-C)/C, where T = treatment weight and C = control weight. Negative values indicate a biomass yield which was less than the control.
Analysis of culture broths by TLC revealed that glucose and glycerol appeared on plates as bright yellow spots with retention factors of 0.45 and 0.28 respectively (data not shown). Figure 9 shows a typical chromatography plate from this initial screen. For example, after two weeks incubation, it is easily observed that the glycerol is no longer detected in ConF and S115 cultures.

Thin layer chromatography screening identified 29 fungal isolates with the capability to completely consume the crude glycerol over the course of the incubation period (Table 8). Complete metabolism of the 50 g·L⁻¹ crude glycerol (glycerol content 14 g·L⁻¹) was detected after seven days (17 isolates) or 14 days (12 isolates) of incubation (Table 9).

Improved growth yield relative to control could not be used as an indicator for an isolate’s ability to completely metabolize crude glycerol. Isolate X4, for example, grew much better in the treatment culture (biomass yield was 23.8 g·L⁻¹) than its glucose control (6.4 g·L⁻¹) however, complete metabolism of crude glycerol was not achieved over the duration of the incubation period (Table 8). Alternatively, although isolate S121 showed reduced growth yield in the treatment culture (16.7 g·L⁻¹) compared to its control (22.5 g·L⁻¹), glycerol was not detected in its culture broth (Table 8 and 9). In order to maintain a shorter incubation time, the subset of 17 isolates which completely utilized glycerol after one week was selected for further analysis.
Figure 9. Example of thin layer chromatography screening of microbial cultures for crude glycerol metabolism. Batch cultures (n=1) were prepared in yeast extract medium supplemented with 50 g·L⁻¹ of crude glycerol (glycerol content 14 g·L⁻¹) or 50 g·L⁻¹ glucose (control) and pH adjusted to 6.5. Residual glycerol in culture broths was detected after seven days by thin layer chromatography alongside pure glycerol (P), crude glycerol (C) and uninoculated medium (M) standards. Separations were performed using water saturated 1-butanol as the solvent and 0.5 % (w/v) KMnO₄ in 1 M NaOH as the indicator. Biodiesel appears as a spot just below the solvent front.
Table 9. List of fungi able to metabolize crude glycerol completely after seven or 14 days of incubation. Cultures (n=1) were grown in yeast extract medium supplemented with 50 g·L⁻¹ crude glycerol (glycerol content 14 g·L⁻¹), pH 6.5.

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>7 days</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>BF1</td>
<td></td>
<td>F8</td>
</tr>
<tr>
<td>F14</td>
<td></td>
<td>F6</td>
</tr>
<tr>
<td>S1</td>
<td></td>
<td>ConF</td>
</tr>
<tr>
<td>F24</td>
<td></td>
<td>S101</td>
</tr>
<tr>
<td>V19</td>
<td></td>
<td>S64</td>
</tr>
<tr>
<td>S115</td>
<td></td>
<td>S9</td>
</tr>
<tr>
<td>V22</td>
<td></td>
<td>S36</td>
</tr>
<tr>
<td>BF2</td>
<td></td>
<td>F23</td>
</tr>
<tr>
<td>F7</td>
<td></td>
<td>F35</td>
</tr>
<tr>
<td>EB Pp</td>
<td></td>
<td>F1</td>
</tr>
<tr>
<td>S141</td>
<td></td>
<td>F27</td>
</tr>
<tr>
<td>F17</td>
<td></td>
<td>S124</td>
</tr>
<tr>
<td>F15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EB Pk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S121</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S83</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.3.2 Growth Yield Comparison of Isolates in Undefined and Defined Minimal Medium

The 17 glycerol metabolizing isolates identified in the initial screening trial (Table 9, left column) were further assessed for their ability to utilize crude glycerol as a carbon substrate for growth at an increased concentration of 100 g·L⁻¹ (glycerol content 28 g·L⁻¹) and pH 8.5. Using these culture conditions, both the YE and CFMM were effective
media for fungal growth using crude glycerol, although high biomass yield in one type of medium did not guarantee a good yield in the other (Figure 10). Biomass yield from isolate F7, for example, was 37.0 g·L$^{-1}$ in YE but only 10.3 g·L$^{-1}$ in CFMM treatment cultures.

Fourteen of the 17 isolates cultured in YE medium had higher biomass yields when crude glycerol was used as the carbon substrate rather than glucose (Figure 10a). Overall, isolate BF2 achieved the highest biomass yield among the crude glycerol treatment cultures (38.6 g·L$^{-1}$) while S121 grew best among the controls (34.5 g·L$^{-1}$). Contrary to this, isolate S115 showed the lowest biomass yield among the glycerol treatment cultures (1.5 g·L$^{-1}$) while F7 had the lowest biomass among the controls (4.5 g·L$^{-1}$).

Similar to the results obtained in YE medium, when growth yield was compared among isolates cultured in CFMM, 13 of the 17 isolates achieved higher biomass yields when cultured in the presence of crude glycerol (Figure 10b). Overall isolate EB Pp achieved the highest biomass yield in the glycerol treatment cultures (32.0 g·L$^{-1}$) while F14 grew best among the controls (36.3 g·L$^{-1}$). The lowest biomass yield in CFMM was recorded for isolate S121 among the glycerol treatment cultures (3.5 g·L$^{-1}$) and EB Pk for the controls (2.6 g·L$^{-1}$). With the exception of isolates F14, S83 and S121, CFMM was not an effective growth medium when glucose was used as the carbon substrate.
**Figure 10.** Effect of medium type and carbon substrate on biomass yield by glycerol metabolizing fungal isolates. Batch cultures (n=1) were performed in yeast extract medium (a) or carbon free minimal medium (b) supplemented with 100 g·L\(^{-1}\) of crude glycerol (glycerol content 28 g·L\(^{-1}\)) or 100 g·L\(^{-1}\) glucose (control) and pH adjusted to 8.5. Biomass yields were determined after complete metabolism of glycerol was observed (sampling conducted every two days), or at the end of the 15 day incubation period. Isolates marked by an * completely metabolized the glycerol.
Thin layer chromatography analysis of the culture broths was first conducted after seven days of incubation, followed by sampling every two days, for a total incubation period of 15 days. Unlike the initial screen of fungal isolates at 50 g·L\(^{-1}\) presented in Table 9, not all of the fungi listed in Figure 10 completely metabolize crude glycerol after the incubation period. At 100 g·L\(^{-1}\) crude glycerol (glycerol content 28 g·L\(^{-1}\)), only six of the 17 isolates tested completely metabolized the crude glycerol. These isolates are marked with an asterisk in Figure 10.

Overall, an increased number of fungal isolates were able to utilize the crude glycerol when grown in YE compared to CFMM. The incubation time required to achieve complete metabolism of glycerol, along with the medium in which isolates were cultured and their growth yield relative to controls are shown in Table 10. All six isolates which completely metabolized the glycerol grew better in the treatment cultures compared to their corresponding controls (Table 10). After seven days of growth, YE grown isolates V19 and V22 had completely utilized the available glycerol in the medium, followed by isolate F24 in both YE and CFMM after 11 days of incubation. Fungal isolates F7, F36 and F15 in YE and V22 in CFMM were also identified with the ability to completely metabolize the crude glycerol by day 13.
**Table 10.** Growth yield and incubation time required for complete glycerol metabolism by a subset of fungi under different culture conditions. Fungi were grown in yeast extract medium (YE) or carbon free minimal medium (CFMM) using 100 g·L\(^{-1}\) of crude glycerol (glycerol content 28 g·L\(^{-1}\)) or 100 g·L\(^{-1}\) glucose (control) and pH 8.5 (n=1).

<table>
<thead>
<tr>
<th>Fungal Isolate</th>
<th>Medium</th>
<th>Days</th>
<th>Growth Yield Relative to Control (%)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V19</td>
<td>YE</td>
<td>7</td>
<td>135.9</td>
</tr>
<tr>
<td>V22</td>
<td>YE</td>
<td>7</td>
<td>87.5</td>
</tr>
<tr>
<td>F24</td>
<td>YE</td>
<td>11</td>
<td>95.5</td>
</tr>
<tr>
<td>F24</td>
<td>CFMM</td>
<td>11</td>
<td>228.9</td>
</tr>
<tr>
<td>F7</td>
<td>YE</td>
<td>13</td>
<td>723.6</td>
</tr>
<tr>
<td>F36</td>
<td>YE</td>
<td>13</td>
<td>425.9</td>
</tr>
<tr>
<td>F15</td>
<td>YE</td>
<td>13</td>
<td>19.1</td>
</tr>
<tr>
<td>V22</td>
<td>CFMM</td>
<td>13</td>
<td>396.8</td>
</tr>
</tbody>
</table>

\(^a\) Growth yield relative to control was calculated as 100*(T-C)/C, where T= treatment weight and C= control weight.

Glycerol metabolism in cultures of F24, BF1, V19 and V22 coincided with the production of new bioproducts which could be detected by TLC. These products were identified based on their ability to produce a detectable spot on TLC plates with different \(R_f\) values than glycerol or biodiesel. Retention factor values for each of the detected bioproducts, along with the \(R_f\) of glycerol, are shown in Table 11. Isolate BF1 produced a bioproduct with the largest \(R_f\) value (0.65). This bioproduct traveled close to the solvent front, suggesting the production of a non-polar compound by this fungus. Isolates F24 and V22 each produced a bioproduct spot with an \(R_f\) equal to 0.25, while V19 produced two new bioproduct spots with \(R_f\) values of 0.25 and 0.19. These
bioproducts all traveled below the spot representing glycerol ($R_f$ 0.45) suggesting the production of more polar compounds.

**Table 11.** Retention factor values for glycerol and detected bioproduct spots identified by thin layer chromatography for fungal isolates V19, V22, F24 and BF1. Samples were developed using water saturated 1-butanol and developed in a solution of 0.5 % (w/v) KMnO$_4$ in 1 M NaOH.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bioproduct Retention Factor ($R_f$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>0.45</td>
</tr>
<tr>
<td>Biodiesel</td>
<td>0.68</td>
</tr>
<tr>
<td>V19 Culture Broth</td>
<td>0.25 0.19</td>
</tr>
<tr>
<td>V22 Culture Broth</td>
<td>0.25</td>
</tr>
<tr>
<td>F24 Culture Broth</td>
<td>0.25</td>
</tr>
<tr>
<td>BF1 Culture Broth</td>
<td>0.65</td>
</tr>
</tbody>
</table>

Fungal isolates F24, BF1, V19 and V22 were selected for more in depth analysis based on three different criteria:

1. Their ability to metabolize the crude glycerol, without any pre-treatment, more rapidly than other isolates in the culture collection (F24, V19 and V22; Table 4).
2. The disappearance of the glycerol spot on TLC plates corresponds with the production of new bioproduct spots (Table 5).

3. Biomass yield in CFMM was comparable to growth yield in YE medium when crude glycerol was used as the carbon substrate (F24, BF1 and V22).

These four candidates were isolated from deciduous forests (V19 and V22), biodiesel wash water (F24) and CARES crude glycerol sludge (BF1).

3.3.3 Analysis of Leading Candidates

Growth yield of isolates F24, BF1, V19 and V22 in both YE and CFMM was compared in triplicate after 14 days of incubation (Figure 11). Once again, when cultured using crude glycerol as the primary carbon substrate, the final biomass yield obtained in treatment cultures was higher for all isolates tested with the exception of BF1 in CFMM (Figure 11b). Overall, isolates F24 and V22 achieved the highest biomass yields in treatment cultures regardless of medium type used. When grown in YE medium, the biomass yields for these isolates were 31.3 g·L\(^{-1}\) ± 3.3 g·L\(^{-1}\) and 25.4 g·L\(^{-1}\) ± 3.4 g·L\(^{-1}\) respectively, while biomass yields for CFMM grown treatment cultures for these two fungi were 23.2 g·L\(^{-1}\) ± 1.1 g·L\(^{-1}\) and 24.1 g·L\(^{-1}\) ± 1.0 g·L\(^{-1}\) (Figure 11). Generally, BF1 had the lowest overall biomass yield, accumulating only 9.2 g·L\(^{-1}\) ± 0.6 g·L\(^{-1}\) and 8.9 g·L\(^{-1}\) ± 0.8 g·L\(^{-1}\) of biomass dry weight, respectively, in YE and CFMM supplemented with crude glycerol. Isolate V19, which showed moderate growth yield in treatment cultures, accumulated 20.1 g·L\(^{-1}\) ± 0.4 g·L\(^{-1}\) and 12.2 g·L\(^{-1}\) ± 0.3 g·L\(^{-1}\) biomass dry weight in YE and CFMM, respectively.
Figure 11. Comparison of biomass yield from isolates F24, BF1, V19 and V22 after 14 days growth in either yeast extract medium (a) or carbon free minimal medium (b).

Media were supplemented with 100 g·L\(^{-1}\) crude glycerol (glycerol content 28 g·L\(^{-1}\)) or 100 g·L\(^{-1}\) glucose (control) as the primary carbon substrates at an initial pH of 8.5. Error bars represent the standard error of the mean (n=3).

Although a moderate reduction in growth yield was observed when YE was replaced with the synthetic CFMM, results from Figure 11 suggest that the synthetic minimal medium is still effective for biomass production. In addition, bioproducts could still be detected by TLC when cultures of these fungi were grown in this synthetic...
medium. The CFMM was therefore used in the majority of subsequent trials, with the exception of lipid trials, aimed at investigating bioproduct production by these isolates.

3.4 Characterization of Fungal Isolates

3.4.2 PCR Amplification of the 18S rRNA gene

Environmental fungal isolates F24, BF1, V19 and V22 were identified in this work as leading candidates for the bioconversion of crude glycerol. In order to determine the identity of these fungi, genomic DNA extractions and molecular characterization of their 18S rRNA gene was performed. Analysis of PCR products by gel electrophoresis revealed a single DNA fragment approximately 490 base pairs in length which could be amplified for each isolate. These fragments were similar in size to the band present in the Candida shehatae positive control. No band was observed for the negative control which contained water in place of a DNA template.

3.4.3 DNA Sequencing

The forward sequences for 18S rRNA genes, obtained with the help of the University of Guelph, Laboratory Services (Guelph, ON), were used for homology searches. Sequencing analysis was performed for each 18S rRNA fragment using the Blastn nucleotide search on the NCBI website. Interestingly, the fragments analyzed from three of the four isolates (F24, V19 and V22) were identified as having high homology (98% nucleotide similarity or better) to 18S rRNA genes from strains of Galactomyces geotrichum (Table 12).
Morphologically, isolates V19, V22 and F24 show similar creamy-white, yeast-like growth patterns on PDA (Figure 12). After six days of growth on this medium, the colony diameter of each fungus was nearly identical, varying from 60.1 to 60.8 mm. Nonetheless, slight morphological differences could be observed between isolates. Isolate V22, for example, produced concentric growth rings shortly after inoculation and isolate F24 demonstrated some lighter radial lines extending through the colony (Figure 12). Due to the fact that isolates V19 and V22 were isolated from the same location and their 18S rRNA gene sequence was identical, V19 was subsequently removed from the list of candidates for further trials, with the exception of CIT trials, to reduce the number of isolates being used.

Unlike the Galactomyces species identified in this study, isolate BF1 showed a much different morphology. This fungus displayed thick, grey, mycelial growth on PDA medium and was able to cover the plate’s entire surface (85 mm diameter) within 4 to 6 days of incubation (Figure 12). Blast search analysis of the PCR amplified partial 18S rRNA gene sequence for this isolate revealed 99 % homology to an 18S rRNA gene sequence from a strain Mucor circinelloides f. circinelloides (Table 12).
Table 12. Blastn sequence analysis of 18S rRNA from fungal isolates F24, BF1, V19 and V22.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Closest match in NCBI database a</th>
<th>GenBank Accession No.</th>
<th>Percent Sequence Similarity b</th>
</tr>
</thead>
<tbody>
<tr>
<td>F24</td>
<td><em>Galactomyces geotrichum</em></td>
<td>GQ458018.1</td>
<td>100</td>
</tr>
<tr>
<td>BF1</td>
<td><em>Mucor circinelloides f. circinelloides</em></td>
<td>HM641689.1</td>
<td>99</td>
</tr>
<tr>
<td>V19</td>
<td><em>Galactomyces geotrichum</em></td>
<td>GQ458033.1</td>
<td>98</td>
</tr>
<tr>
<td>V22</td>
<td><em>Galactomyces geotrichum</em></td>
<td>GQ458033.1</td>
<td>98</td>
</tr>
</tbody>
</table>

a Closest match was determined using the Blastn search on the National Center for Biotechnology Information nucleotide database (NCBI, USA).

b Percent sequence similarity was based on sequence alignment of the 18S rRNA from isolates F24, BF1, V19 and V22 with known sequences from the NCBI nucleotide database.
Figure 12. Colony morphology of *Galactomyces* (F24, V19 and V22) and *Mucor* (BF1) isolates. Cultures were grown on potato dextrose agar for 3 days (BF1) or 6 days (F24, V19 and V22) at 20 °C to 25 °C.
3.5 Process Development for Biomass Production

3.5.1 Effect of Initial Carbon Substrate Concentration on Growth Yield

Initially, the influence of carbon substrate on growth yield was assessed for isolates BF1, F24 and V22. These trials were performed using solid CFMM with crude glycerol (treatment) or glucose (control) at concentrations ranging from 100 g·L⁻¹ to 400 g·L⁻¹ (glycerol content from 28 g·L⁻¹ to 112 g·L⁻¹ in treatment cultures). A plateau in the surface area value was observed when the mycelia reached the outer edge of the plate.

Above 100 g·L⁻¹ of crude glycerol (glycerol content from 28 g·L⁻¹), the growth of all three isolates was negatively impacted, and no growth was observed in treatment cultures above 200 g·L⁻¹ of this carbon substrate (Figure 13, top panels). Control cultures, on the other hand, were more tolerant to higher carbon substrate (glucose) concentrations. Although growth of isolates was delayed at 200 g·L⁻¹, by day 21 the final colony surface area recorded were similar (F24 and BF1), or slightly better (V22), than what was recorded at 100 g·L⁻¹ glucose (Figure 13, bottom panels). With the exception of BF1 control cultures, growth was reduced above 200 g·L⁻¹ for the isolates tested in the glucose control medium.
Figure 13. Time course of fungal growth on solid medium in response to increasing concentrations of carbon substrate. Fungal plugs for isolates BF1 (a,b), F24 (c,d), and V22 (e,f) were placed onto solid carbon free minimal medium supplemented with 100 g·L⁻¹, 200 g·L⁻¹, 300 g·L⁻¹ or 400 g·L⁻¹ of crude glycerol (glycerol content of 28 g·L⁻¹, 56 g·L⁻¹, 84 g·L⁻¹ and 112 g·L⁻¹; top panels) or glucose (bottom panels). Growth was assessed daily by recording the colony diameter using digital callipers and calculating the surface area. Error bars represent the standard error of the mean (n=3).
Figure 13 continued: Isolate F24
Figure 13 continued: Isolate V22

![Graph](image-url)

**Graph Legend:**
- 100 g L⁻¹
- 200 g L⁻¹
- 300 g L⁻¹
- 400 g L⁻¹

**Axes:**
- Vertical: V22 Surface Area (mm²)
- Horizontal: Incubation time (days)
Based on the concentrations tested, the upper crude glycerol range for growth was determined to be 100 g·L⁻¹ (glycerol content 28 g·L⁻¹) as shown in in Figure 13. Liquid trials using CFMM were performed in order to determine the lower crude glycerol range and reagent grade glycerol was also used in place of glucose as the control. After seven days of growth, higher biomass yields were obtained in treatment cultures containing an initial crude glycerol concentration up to 100 g·L⁻¹ (glycerol content 28 g·L⁻¹) for isolate V22 and up at 200 g·L⁻¹ (glycerol content 56 g·L⁻¹) for isolate F24 (Figure 14). Isolate BF1 had higher biomass yield in treatment cultures at all concentrations tested.

When fungi were cultured using pure glycerol (control) each isolate showed optimal growth yield at a different concentration (Figure 14). Isolate V22 achieved the highest biomass yield (18.0 g·L⁻¹ ± 2.6 g·L⁻¹) at an initial glycerol concentration of 100 g·L⁻¹, whereas 50 g·L⁻¹ proved to be best for isolate F24 (11.7 g·L⁻¹ ± 0.8 g·L⁻¹) and 150 g·L⁻¹ for isolate BF1 (29.7 g·L⁻¹ ± 1.0 g·L⁻¹). When cultured using crude glycerol (treatment), both V22 and F24 achieved their highest biomass yields (25.6 g·L⁻¹ ± 0.4 and 29.9 g·L⁻¹ ± 2.6 g·L⁻¹) at an initial glycerol concentration of 100 g·L⁻¹ (glycerol content 28 g·L⁻¹) while BF1 grew best at 150 g·L⁻¹ (glycerol content 42 g·L⁻¹) and produced 30.8 g·L⁻¹ ± 0.6 g·L⁻¹ biomass yield (Figure 14). Unlike V22 and F24, BF1 appeared to grow at a broader range of glycerol concentrations. These results support the observations made using solid medium in section 3.5.1 where the best growth yields of fungal isolates occurred around 100 g·L⁻¹ of crude glycerol (Figure 13). This corresponds to a glycerol content of 28 g·L⁻¹ and a C:N ratio of 41.
**Figure 14.** Effect of initial glycerol concentration on biomass yield of fungal isolates V22, F24 and BF1 after seven days of growth. Batch cultures (n=3) were performed in carbon free minimal medium (pH 8.5) supplemented with 25 g·L⁻¹, 50 g·L⁻¹, 100 g·L⁻¹, 150 g·L⁻¹ or 200 g·L⁻¹ of crude glycerol (glycerol content of 7 g·L⁻¹, 14 g·L⁻¹, 28 g·L⁻¹, 42 g·L⁻¹ or 56 g·L⁻¹ respectively) or pure glycerol (control). Error bars represent the standard error of the mean (n=3).
3.5.2 Effect of Initial pH on Growth Yield

In addition to analyzing the effect of initial glycerol concentration on biomass yield, the effect of initial culture pH on growth yield was assessed. After one week of incubation in liquid CFMM, V22 and F24 showed optimal biomass yields at pH 8 in both treatment and control cultures (Figure 15). When grown at this pH, isolates V22 and F24 achieved biomass yields of 10.9 g·L⁻¹ ± 0.6 g·L⁻¹ and 12.5 g·L⁻¹ ± 1.1 g·L⁻¹ in pure glycerol cultures and 20.1 g·L⁻¹ ± 1.9 g·L⁻¹ and 27.7 g·L⁻¹ ± 3.2 g·L⁻¹ in crude glycerol cultures (Figure 15). Above or below this pH, growth yield of isolates drops off in treatment cultures. Similar results were observed when BF1 was cultured at pH 8.5 and 6.5 in YE medium supplemented with 50 g·L⁻¹ crude glycerol. At pH 8.5, BF1 attained a final biomass yield of 10.5 g·L⁻¹ ± 0.8 g·L⁻¹ while at 6.5 only 6.6 g·L⁻¹ ± 0.5 g·L⁻¹ was achieved (data not shown).

After seven days of incubation, the final pH of V22 and F24 cultures from Figure 15 were measured to determine the influence of fungi on this parameter. The pH of both V22 and F24 dramatically dropped in glycerol control cultures with an initial pH of 8 or less (Figure 16). The final pH of culture broths for these two isolates ranged from pH 2.3 to 2.6 in medium with an initial pH between 5 and 8. At pH 9, higher final pH values were recorded for these isolates (Figure 16).

Similar to the glycerol control cultures, a decrease in the final pH of treatment cultures was also observed. With the exception of F24 grown at pH 9, the final pH of treatment cultures were higher than those recorded for control cultures. Analysis of the
culture broth from treatment cultures showed final pH values between 2.7 and 7.3 for isolate V22 and from 2.5 to 7.3 for isolate F24 (Figure 16).

Figure 15. Effect of initial culture pH on biomass yield of fungal isolates V22 and F24 after seven days of growth. Batch cultures (n=3) were performed in carbon free minimal medium supplemented with 100 g·L⁻¹ of crude glycerol (glycerol content 28 g·L⁻¹) or 100 g·L⁻¹ pure glycerol (control) and pH adjusted to 5, 6, 7, 8 or 9. Error bars represent the standard error of the mean (n=3).
Figure 16. Effect of initial medium pH on final culture pH of isolates V22 and F24. Batch cultures were performed in carbon free minimal medium supplemented with 100 g·L\(^{-1}\) of crude glycerol (glycerol content 28 g·L\(^{-1}\)) or 100 g·L\(^{-1}\) pure glycerol (control) and the initial pH adjusted to 5, 6, 7, 8 or 9. Final pH measurements were recorded after seven days of growth. Error bars represent the standard error of the mean (n=3).

3.5.3 Effects of Methanol on Growth Yield

Unlike isolate BF1, above 100 g·L\(^{-1}\) of crude glycerol, the biomass yields of isolates V22 and F24 abruptly decreased (Figure 14), possibly due to methanol inhibition. The crude glycerol obtained for this study contains residual methanol from the transesterification reaction (Table 7) which may negatively impact fungal growth. Using the optimized culture conditions determined in Figure 14 and 15 (100 g·L\(^{-1}\) crude glycerol and pH 8.0) the tolerance of V22 and F24 to methanol was assessed by adding
methanol to cultures broths after medium was autoclaved to prevent evaporation. Residual methanol already present in the crude glycerol was not taken into account when adding methanol after autoclaving.

When pure glycerol was used as a carbon substrate, the addition of methanol above 9.7 g·L\(^{-1}\) and 4.9 g·L\(^{-1}\) negatively impacted the biomass yield of isolates V22 and F24, respectively. This corresponds to a volumetric methanol concentration of 1 % and 0.5 % (v/v). These methanol concentrations are less than what would be present in autoclaved medium prepared using 100 g·L\(^{-1}\) crude glycerol. At this concentration, 23.9 g·L\(^{-1}\) methanol would be expected. Compared to the methanol control (zero methanol added), biomass yield in cultures with 19.4 g·L\(^{-1}\) methanol dropped by 56.7 % and 49.5 % (w/v) for isolate V22 and F24. Interestingly, when crude glycerol was used in treatment cultures the impact of adding methanol was minimal. Compared to the zero methanol control cultures, where no additional methanol was provided, biomass yield in cultures with 19.4 g·L\(^{-1}\) added methanol dropped by only 2.5 % (w/v) for V22 and increased by 14.8 % (w/v) for F24 (data not shown). Taking into account the methanol already present in the crude glycerol, these microorganisms could tolerate up to 43.3 g·L\(^{-1}\) of this alcohol. In order to determine the upper limit of methanol tolerance, the concentration at which these fungi were cultured was tested at 0 g·L\(^{-1}\), 19.4 g·L\(^{-1}\), 38.9 g·L\(^{-1}\) and 116.6 g·L\(^{-1}\) of added methanol which corresponds to a volumetric methanol concentration of 0 %, 2 %, 4 % and 12 % (v/v).

Similar to the results recorded in the initial trial, growth yield of V22 and F24 in pure glycerol control cultures was negatively impacted at an added methanol concentration of 19.4 g·L\(^{-1}\) or greater (Figure 17). Compared to control cultures with no
added methanol, biomass yield in medium with 19.4 g·L\(^{-1}\) methanol dropped from 14.1 g·L\(^{-1}\) ± 0.2 g·L\(^{-1}\) to 6.1 g·L\(^{-1}\) ± 0.4 g·L\(^{-1}\) for V22 and from 9.7 g·L\(^{-1}\) ± 0.5 g·L\(^{-1}\) to 4.9 g·L\(^{-1}\) ± 0.5 g·L\(^{-1}\) for F24 (Figure 17). Treatment cultures which used crude glycerol were much more tolerant to the addition of this alcohol, showing good growth yield at 19.4 g·L\(^{-1}\). Compared to the zero added methanol treatment cultures, a slight yield drop was observed from 20.3 g·L\(^{-1}\) ± 1.0 g·L\(^{-1}\) to 19.8 g·L\(^{-1}\) ± 0.7 g·L\(^{-1}\) for V22 while an increase from 22.3 g·L\(^{-1}\) ± 1.1 g·L\(^{-1}\) to 25.6 g·L\(^{-1}\) ± 0.9 g·L\(^{-1}\) for F24 was recorded (Figure 17). Nonetheless, tolerance of these isolates to methanol did not exceed 19.4 g·L\(^{-1}\) of added methanol and growth was almost completely inhibited at a methanol concentration of 38.8 g·L\(^{-1}\) or greater. As previously observed, when the residual methanol in the crude glycerol containing culture broth is taken into account, V22 and F24 tolerate a total methanol concentration of 43.3 g·L\(^{-1}\).
Figure 17. Influence of methanol concentration on biomass yield of fungal isolates V22 and F24. Batch cultures (n=3) were performed in carbon free minimal medium supplemented with 100 g·L⁻¹ of crude glycerol (glycerol content 28 g·L⁻¹) or 100 g·L⁻¹ pure glycerol (control) and pH adjusted to 8.0. Methanol was added to culture broth after the medium was autoclaved at 0 g·L⁻¹, 19.4 g·L⁻¹, 38.9 g·L⁻¹ or 116.6 g·L⁻¹ (corresponds to a volumetric concentration of 0 %, 2 %, 4 % or 12 % v/v). Error bars represent the standard errors of the mean (n=3).

3.6 Bioconversion of Crude Glycerol to Bioproducts

Thin layer chromatographic analysis of culture broths taken from F24, BF1, V19 and V22 cultures demonstrated the ability of these isolates to metabolize the crude glycerol in media and produce bioproducts with new Rᵢ values (Table 11). Both
qualitative and quantitative approaches were attempted to determine the identity and quantity of bioproducts produced by these fungi.

### 3.6.1 Large Scale Growth of Isolates

Scaling up from 20 mL cultures to 400 mL culture for bioproduct extraction trials resulted in a much longer incubation period. Complete metabolism of glycerol within these larger cultures was not observed until after six weeks of incubation. Fungal cultures of isolates F24, V19 and V22 which were inoculated with a cell suspension, and BF1 inoculated with a fungal "ring", had completely consumed the glycerol found in their culture broths at this time. Analysis of medium at the end of the incubation period for these large scale trials showed that isolates V19, V22 and BF1 all produced a new bioproduct from glycerol. Unlike previous small scale trials, F24 did not appear to have produced a bioproduct based on TLC results and its broth was consequently not extracted.

### 3.6.2 Bioproduct Extraction and Column Purification

Rotary evaporation of solvent extracts from large scale cultures resulted in extracts which were a thick, dark amber-coloured syrup. The amount obtained from each culture varied, with the final extract volume obtained for each isolate after solvent removal being 2 mL, 8 mL and 6 mL for isolates BF1, V19 and V22, respectively (Table 13).

After aliquots of column purified bioproducts were pooled together and concentrated, the final dry weight obtained for each bioproduct was 602.7 mg for BF1 and 55.5 mg for V22. Isolate V19 appeared to produce two bioproducts which could be
separated by column chromatography and these were designated as V19i and V19ii. The final weight obtained for these bioproducts were 29.8 mg for V19i and 19.1 mg for V19ii (Table 13).

**Table 13.** Solvent extraction and column purification of bioproducts from BF1, V19 and V22 culture broths. Bioproducts were extracted from 200 mL of culture broth after six weeks growth in carbon free minimal medium supplemented with 100 g·L⁻¹ crude glycerol (n=1). Extracts were purified by column chromatography and dried by rotary evaporation.

<table>
<thead>
<tr>
<th>Fungal Isolate</th>
<th>Volume of Butanol Extracted Bioproduct</th>
<th>Weight of Column Purified Bioproduct</th>
</tr>
</thead>
<tbody>
<tr>
<td>BF1</td>
<td>2 mL</td>
<td>602.7 mg</td>
</tr>
<tr>
<td>V19</td>
<td>8 mL</td>
<td>29.8 and 19.1 mg</td>
</tr>
<tr>
<td>V22</td>
<td>6 mL</td>
<td>55.5 mg</td>
</tr>
</tbody>
</table>

Thin layer chromatography of the column purified bioproducts is shown in Figure 18. Non-polar compounds were the first to elute, followed by compounds of increasing polarity. The two bioproducts produced by isolate V19 were detected in aliquots 23 to 34 (V19i) and the second in 35 to 43 (V19ii), although some carry-over of the first bioproduct appeared to have occurred into aliquots containing the second (Figure 18, lanes 2 and 3). Isolate V22’s bioproduct was identified in aliquots 15 to 30. Although all of the biodiesel appeared to have been removed, when aliquots were concentrated
together a slight amount of biodiesel could still be detected in the sample (Figure 18, lane 4). The bioproduct of interest for isolates BF1 was identified in aliquots 2 to 14. Due to the nonpolar nature of this bioproduct, the latter could not be separated from the biodiesel present in the extract using this technique (Figure 18, lane 5).

**Figure 18.** Detection of column purified bioproducts from the metabolism of crude glycerol by fungal isolates V22, V19 and BF1. Culture broths (n=1) were extracted with water saturated 1-butanol and concentrated. Two milliliters of each concentrated sample were separately purified by column chromatography using silica as the stationary phase and water saturated 1-butanol as the mobile phase. Bioproducts were detected by thin layer chromatography alongside glycerol (G) using water saturated 1-butanol as the solvent system and 0.5 % (w/v) KMnO₄ in 1 M NaOH as the indicator. Biodiesel appears as a spot just below the solvent front.
3.6.3 Qualitative Analysis of Bioproducts

Bioproducts shown in Table 13 were run alongside known standards using the glycerol TLC protocol previously described in this work. None of the unknown bioproducts had \( R_f \) values which corresponded to those of the tested standards (data not shown). Compared to the FFA standard, the BF1 bioproduct ran differently on the TLC plates, suggesting a different polar bioproduct was being produced (Figure 19). The BF1 bioproduct, which had an \( R_f \) of 0.61 ran lower on the plate than both the biodiesel spot (\( R_f \) 0.80) and the FFA spot (\( R_f \) 0.73).

Figure 19. Thin layer chromatography analysis of the non-polar BF1 bioproduct and free fatty acids found in crude glycerol. Crude glycerol (G), the column purified BF1 bioproduct (BF1) and free fatty acids (FFA) were separated on silica plates using water saturated 1-butanol as the solvent and 0.5 % KMnO\(_4\) in 1 M NaOH as the indicator. Biodiesel appears as a spot just below the solvent front and the BF1 bioproduct is indicated by arrow 1 and FFAs by arrow 2.
Although TLC plates only showed one or two spots in the purified bioproduct samples, the more sensitive GC-MS analysis conducted by Laboratory Services (Guelph, ON) revealed a number of compounds in the extracted samples (Table 1). Very different metabolites were produced by each of the isolates tested, although V19i and V19ii did have identical profiles. The presence of organic acids, such as CIT, in the analyzed fungal bioproduct samples was suspected prior to receiving the analysis results due to reduction in pH of the medium during growth as previously reported (Figure 1). The ability of isolates to produce CIT and lipid was further investigated using isolates V22, F24 and BF1.

**Table 14.** Qualitative analysis of fungal metabolites produced from the bioconversion of 100 g·L⁻¹ crude glycerol. Bioproducts were detected by GC-MS analysis by the University of Guelph Laboratory Services.

<table>
<thead>
<tr>
<th><strong>Fungal Isolate</strong></th>
<th><strong>Fungal Metabolite</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>V19 i &amp; ii</td>
<td>Glycerol</td>
</tr>
<tr>
<td></td>
<td>Arabinofuranose</td>
</tr>
<tr>
<td></td>
<td>Citric Acid</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td></td>
<td>Linoleic Acid</td>
</tr>
<tr>
<td></td>
<td>Stearic Acid</td>
</tr>
<tr>
<td>V22</td>
<td>Phosphoric Acid</td>
</tr>
<tr>
<td></td>
<td>Citric Acid</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td></td>
<td>Glucopyranose</td>
</tr>
</tbody>
</table>
3.6.4 Quantitative Analysis of Citric Acid Production

Quantitative enzymatic analysis of CIT production was performed on the culture broths of isolates V22 and BF1. After seven days of incubation, only very low concentrations of this organic acid was detected in cultures broths, regardless of carbon substrate utilized. Isolate V22 produced 0.08 g·L⁻¹ ± 0.01 g·L⁻¹ and 0.14 g·L⁻¹ ± 0.01 g·L⁻¹ of CIT in medium containing crude glycerol and glucose, respectively. Likewise, BF1 produced 0.08 g·L⁻¹ ± 0.00 g·L⁻¹ and 0.11 g·L⁻¹ ± 0.00 g·L⁻¹ of CIT.

Similar results were obtained when culture broths from the optimization trials for isolates V22 and F24, described in section 3.5, were analyzed. A maximum CIT concentration (0.23 g·L⁻¹) was reported for isolate F24 cultured for seven days using 100 g·L⁻¹ crude glycerol (glycerol content 28 g·L⁻¹, C:N 41) and pH adjusted to 6.0. Due to the very low concentrations detected, the bioconversion of crude glycerol to this bioproduct was not further pursued.

3.7 Bioconversion of Crude Glycerol to Single Cell Oil

Along with CIT production, SCO production by fungal isolates was also investigated. As was shown in Figure 15, isolate BF1 produced a non-polar product when cultured in the presence of crude glycerol. The production of non-polar lipids was therefore assessed for this isolate, along with isolate V22.

3.7.1 Bioconversion of Crude Glycerol to Single Cell Oil after 28 Days

Complete metabolism of crude glycerol, as detected by TLC, was determined to occur after 28 days of incubation for both fungi and it was at this time that lipid contents
were first analyzed. When crude glycerol was used as the carbon substrate, the lipid content in both isolates was much higher than the glucose control (Figure 20). Biomass from isolate BF1 crude glycerol treatment cultures had an average lipid content of 12.0 % ± 0.5 % (w/w) while biomass from glucose control cultures contained only 2.0 % ± 0.6 % (w/w) lipids. Isolate V22 proved to be a slightly more effective lipid producing microorganism, producing biomass with a lipid content of 22.5 % ± 0.9 % which was 1.9 times higher than isolate BF1 in treatment cultures. Average lipid content from V22 control cultures was much lower, having only 4.7 % ± 0.3 % (w/w) lipid content (Figure 20).
Figure 20. Effect of carbon substrate on lipid content in BF1 and V22 fungal biomass. Batch cultures were grown for 28 days in yeast extract medium supplemented with 50 g·L⁻¹ crude glycerol (glycerol content 14 g·L⁻¹) or 50 g·L⁻¹ glucose (control). Lipid was extracted from 1 gram of dried biomass using chloroform:methanol (2:1). Error bars represent the standard errors of the mean (n=3).

3.7.2 Bioconversion of Crude Glycerol to Single Cell Oil after Seven Days

The influence of reducing the incubation time from 28 days to seven on the ability of these fungi to produce lipids was analyzed next. Similar to the 28 day trials, after one week of incubation, both isolates V22 and BF1 cultured in the presence of crude glycerol accumulated significantly higher cellular lipid contents compared to their corresponding glucose controls (Figure 21). Overall, higher lipid contents were also
observed in seven day old biomass compared to 28 day old biomass for both isolates tested. Lipid analysis revealed that isolate V22 produced biomass with an average lipid content of 31.7 % ± 1.1 % (w/w) in treatment cultures as opposed to 4.7 % ± 0.4 % (w/w) in the control (Figure 21). Isolate BF1 produced biomass with a slightly higher lipid content of 36.6 % ± 1.3 % (w/w) in treatment cultures and 2.5 % ± 1.1 % (w/w) in controls (Figure 21). These values represent an increase in the lipid content of treatment cultures which was seven and 15 times higher than the lipid content of biomass harvested from control cultures for isolates V22 and BF1, respectively.
**Figure 21.** Effect of carbon substrate on lipid content in BF1 and V22 fungal biomass after seven days. Cultures were grown in yeast extract medium supplemented with 50 g·L⁻¹ crude glycerol (glycerol content 14 g·L⁻¹) or 50 g·L⁻¹ glucose (control). Lipid was extracted using chloroform:methanol (2:1). Error bars represent the standard error of the mean (n=3). Values marked with * are significantly different from control as determined by a Student’s t-test.

While reducing the incubation time from 28 days to one week increased lipid content, it dramatically reduced the biomass yield of isolate BF1 in treatment cultures. As Figure 18 shows, biomass yield for isolate BF1 dropped from 20.5 g·L⁻¹ ± 0.7 g·L⁻¹ to 0.44 g·L⁻¹ ± 0.0 g·L⁻¹ (Figure 22). As opposed to this, isolate V22 exhibited only a slight
reduction in the yield of biomass from 11.3 g·L⁻¹ ± 0.2 g·L⁻¹ to 10.2 g·L⁻¹ ± 0.0 g·L⁻¹ (Figure 22).

**Figure 22.** Effect of incubation time on biomass yield for isolates BF1 and V22.

Cultures were grown for 7 or 28 days in yeast extract medium supplemented with 50 g·L⁻¹ crude glycerol (glycerol content 14 g·L⁻¹). Error bars represent the standard error of the mean (n=3).

Although the overall cellular lipid content (as percent dry weight) produced by isolate BF1 was higher than that observed for V22 (Figure 21), the former produced 23 times more biomass from crude glycerol after one week of incubation (Figure 22). This
resulted in a higher overall lipid yield as grams of lipid per litre of medium used; lipid yield in treatment cultures after seven days was 0.2 g·L$^{-1}$ for BF1 and 3.2 g·L$^{-1}$ for V22. In order to maintain short incubation times, further analyses were conducted using only isolate V22.

### 3.7.3 Bioconversion of Crude Glycerol to Single Cell Oil after Five Days

When liquid inoculum was prepared, it was observed that an increase in biomass yield (as g·L$^{-1}$) for isolate V22 occurred up to day 4 to 5 of incubation, after which point stationary phase was reached (data not shown). For this reason, the ability of isolate V22 to produce lipid after only five days of incubation was therefore assessed.

Reducing the incubation period to five days was beneficial for isolate V22 in terms of cellular lipid content. A significant increase the lipid content of crude glycerol treatment cultures compared to both glucose and pure glycerol controls was observed. Treatment cultures produced biomass with average lipid contents between 43.7 % ± 1.2 and 45.4 % ± 1.3 % (w/w) (Figure 23a & b). This represents a 1.4 times increase compared to the lipid content of biomass obtained after seven days (Figure 17). Changing the carbon source from glucose (Figure 21a) to pure glycerol (Figure 23b) also resulted in an increase in the cellular lipid content from 4.3 % ± 0.3 % to 26.9 % ± 1.6 % (w/w) respectively, however, the use of crude glycerol still proved to be most beneficial for lipid production.
Figure 23. Effect of carbon substrate on lipid content in V22 biomass. Cultures were grown for five days in yeast extract medium supplemented with 50 g·L\(^{-1}\) crude glycerol (glycerol content 14 g·L\(^{-1}\)), 50 g·L\(^{-1}\) glucose (control; 19a) or 50 g·L\(^{-1}\) pure glycerol (control; 19b). Lipid was extracted using chloroform:methanol (2:1). Error bars represent the standard error of the mean (n=3). Values marked with * are significantly different from control as determined by a Student’s t-test.

Overall the biomass yield for V22 treatment cultures exhibited only a slight decrease from the 10.2 g·L\(^{-1}\) ± 0.0 g·L\(^{-1}\) reported after seven days (Figure 22) to 9.6 g·L\(^{-1}\) ± 0.2 g·L\(^{-1}\) and 9.8 g·L\(^{-1}\) ± 0.1 g·L\(^{-1}\) after five days of incubation. Although the biomass yield was slightly reduced, the increased cellular lipid content produced after five days gave overall higher lipid yields; between 4.3 and 4.4 grams of lipid per litre of medium used was recorded. Overall reducing the incubation period resulted in higher lipid yields per litre of medium throughout the lipid trials. After 28, seven and five days of
incubation, recorded lipid yields for isolate V22 grown in the presence of crude glycerol were 2.5 g·L⁻¹, 3.2 g·L⁻¹ and finally 4.4 g·L⁻¹.

In addition to lipid production, glycerol consumption was monitored by enzymatic analysis over the course of the trial. At the end of the five day incubation period, residual glycerol was still detected in the medium of crude glycerol treatment cultures. As Figure 24 shows, a gradual daily decrease in the glycerol concentration of the medium was observed, however, at the end of the incubation period 8.7 g·L⁻¹ ± 0.2 g·L⁻¹ of glycerol remained unconsumed. Day zero data was not available, however, medium prepared using 50 g·L⁻¹ of crude glycerol should contain approximately 14 g·L⁻¹ glycerol. This indicates that 62.3 % (w/v) of the glycerol added to the medium remained at the end of the trial.
Figure 24. Time course of glycerol consumption by isolate V22. Cultures were grown for five days in yeast extract medium supplemented with 50 g·L\(^{-1}\) crude glycerol giving medium with an initial glycerol content of 14 g·L\(^{-1}\). Residual glycerol was determined by enzymatic analysis using the BioVision Free Glycerol Assay Kit (BioVision Inc., Mountain View, CA). Error bars represent the standard error of the mean (n=3).
CHAPTER 4. DISCUSSION

4.1 Crude Glycerol Characterization

The effectiveness of using crude glycerol from biodiesel production as an inexpensive carbon substrate for fungal growth and bioproduct production was assessed in the present study.

Though the use of inexpensive carbon feedstocks generated as co-products from various industries may reduce culturing costs, they are often contaminated with a variety of other compounds. The multiple purification steps which may be required to remove these impurities can negatively impact the original cost savings associated with their use (Sauer et al. 2008). This emphasizes the importance of understanding the composition of a feedstock to determine if it can feasibly be used in microbial bioconversions.

4.1.1 General Composition of Crude Glycerol

The crude glycerol used in the present investigation was analyzed to determine both its general composition (glycerol, FFAs and methanol) and to detect the presence of available nutrients and inhibitors of fungal growth. No pre-treatment, including autoclaving, was performed prior to the characterization of this co-product.

The composition of crude glycerol obtained from biodiesel processing varies widely as was observed in the literature reported samples (Table 2). The variations from one sample to the next are the result of production practices, the use of various lipid feedstocks, and completeness of the reaction (Thompson and He 2006; Pyle et al.
The two main impurities which are commonly detected in crude glycerol from biodiesel production are methanol and soap (Athalye et al. 2009). Common elements which are also detected include calcium, potassium, phosphorus, magnesium, sulfur and sodium (Athalye et al. 2009). Characterization of the crude glycerol from the CARES facility (Table 7) revealed that this co-product was primarily made up of glycerol (28 % w/v), FFAs (30 % w/v) and methanol (26 % w/v).

Overall the glycerol content was low compared to other reported values, where 49 % to 92 % (w/v) glycerol has previously been reported (Table 2). The low glycerol content suggests that an incomplete conversion of the lipid feedstock to biodiesel occurred. This is possible as the crude glycerol used in this study was obtained shortly after the CARES plant was commissioned, when the processing protocol for the facility was still under development.

Pyle et al. (2008) analyzed the composition of crude glycerol obtained from the transesterification of three different lipid feedstocks: soybean oil, chicken fat:soybean oil (50:50) and canola oil. The concentration of methanol (13 % to 28 %) and soap (15 % to 25 %) varied from one batch to the next but differed only slightly from the results obtained in the work presented here. The high FFAs concentration observed in the crude glycerol from the CARES facility is indicative of high soap content, another consequence of process development occurring at the time when the crude glycerol was obtained. When present at high concentration both methanol and FFAs have been known to act as inhibitors of microbial growth (Beopoulos et al. 2008; Liu et al. 2011). Microorganism need to be identified which can tolerate the impurities in crude glycerol from biodiesel production. This will allow the direct use of crude glycerol for microbial
processes without the need for purification to remove various contaminants such as FFAs.

### 4.1.2 Elemental and Physical Analysis

A range of various elements and nitrogen were also present in the CARES facility crude glycerol, in addition to methanol and FFAs (Table 7). As expected, the concentration of potassium (28,000 µg·g⁻¹) was high compared to other elements analyzed. This is likely due to the presence of residual KOH catalyst and is comparable to what has been previously observed (27,300 µg·g⁻¹ and 31,250 µg·g⁻¹) when crude glycerol was produced using a KOH catalyst (Pyle et al. 2008). Glycerol produced from a NaOH catalyzed reactions has been shown to contain very low concentrations of potassium and high sodium levels (Pyle et al. 2008; Thompson and He 2006). The work presented by Thompson and He (2006) supports the conclusion that residual catalyst remains in the glycerol. The high residual catalyst concentration is also responsible for the alkaline pH of this co-product (Table 7).

The concentrations of other elements reported in Table 7 differ compared to other studies. For example, the concentrations of calcium and phosphorus (4.3 µg·g⁻¹ and 7.8 µg·g⁻¹) were lower than those reported by Thompson and He (2006) from seven analyzed crude glycerol samples. However, the sulfur concentration (1,400 µg·g⁻¹) was much higher than the maximum concentration (129 µg·g⁻¹) reported by these same authors. This may be the result of the sulfuric acid catalyzed esterification reaction which was performed at the CARES facility as a pre-treatment prior to the transesterification reaction. As with the soaps present in the crude glycerol co-product,
residual sulfur can potentially be harmful for microbial growth. It has been reported, for example, that a concentration of elemental sulfur in the range of 1 µg·mL⁻¹ to 12 µg·mL⁻¹ can negatively impact the growth of various fungal pathogens (Cooper and Williams 2004). The medium used in this study would likely have around 140 µg·mL⁻¹ of sulphur when prepared using 100 g·L⁻¹ crude glycerol which could negatively impact microbial growth.

High salt content (e.g. above 0.8 mS·cm⁻¹), as determined using EC, may also inhibit growth. Medium prepared at a 100 g·L⁻¹ concentration using the CARES crude glycerol would be expected to have an EC value of 0.77 mS·cm⁻¹. This is a similar conductivity to domestic tap water which typically ranges from 0.5 mS·cm⁻¹ to 0.8 mS·cm⁻¹ (Hanna 2011) and likely would not cause any issues for microbial growth.

4.2 Isolation of Environmental Microorganisms

It has been reported that various fungi and yeast appear to possess increased tolerance to industrial feedstocks such as crude glycerol, along with the impurities which they contain, compared to bacterial isolates (Rumbold et al. 2009). Both filamentous fungi and yeast were selected for the glycerol bioconversion trials detailed in this thesis based upon these findings. New fungi were purified from environmental water samples collected across southern Ontario and from glycerol-containing waste samples taken from biodiesel facilities in an effort to increase the number of isolates available in the lab collection. Some of the morphologies of these environmental isolates are shown in Figure 7 and Figure 12. It was hypothesized that wastes from a biodiesel facility could potentially provide an excellent resource for the isolation of new microorganisms which
can utilize the crude glycerol as a result of their pre-existing exposure to this carbon substrate.

4.3 Screening Microbial Cultures for Growth in Crude Glycerol

Research into the bioconversion of crude glycerol to date has focused on fungi belonging to a limited number of genera (Section 1.6; Tables 4 to 6). Identifying new fungi with the inherent ability to utilize the crude glycerol co-product was one of the goals set forth by this study. Using newly isolated fungal isolates, along with those already available in lab (Appendix A) the present investigation aimed to identify of isolates which could use crude glycerol as a primary substrate for growth.

Although the need for new fungal species for the bioconversion of crude glycerol is evident, certain limitations exist when taking a discovery based research approach. Due to the unknown identity of the isolates used, conditions which favour growth are unknown and need to be established. Nutrient rich YE medium was therefore used in the initial screening trials. The growth yield of isolates in the initial screen was also compared to glucose as this carbon substrate has been frequently used in the literature and therefore provides a good reference for comparison (Papanikolaou et al. 2002).

4.3.1 Initial Screening of Fungal Isolates in Undefined Medium

As described in Table 7, three primary impurities of the crude glycerol used in the present investigation include methanol, FFAs, potassium and sulfur. In order to remain a low cost alternative to conventional carbon substrates, and to improve the economics of the process, it has been proposed that crude glycerol should not require any purification steps prior to its use (Mu et al. 2006). The crude glycerol was not purified
when used in this study, except for autoclaving. Initial screening of environmental fungal isolates was conducted using 50 g·L$^{-1}$ of crude glycerol (glycerol content 14 g·L$^{-1}$). This preliminary screen revealed that crude glycerol was an effective substrate for fungal growth; 65.6 % of tested isolates had increased biomass yields compared to their control (Figure 8). In addition to good growth yield, isolates which fully metabolized the glycerol present in the medium were targeted. A thin layer chromatography (TLC) protocol was employed to select for candidates that possessed the ability to utilize the crude glycerol most rapidly and seventeen of the fungi in the tested culture collection showed promise for this purpose (Table 9).

4.3.2 Growth Yield Comparison of Isolates in Undefined and Defined Minimal Medium

In addition to a low tolerance to the impurities found in crude glycerol, the need for complex, and expensive, nutrient media is another problem which limits the large scale application of microbial bioconversions of crude glycerol (Dellomonaco et al. 2010). One advantage of using fungi is their ability to grow in minimal medium without a large number of inputs (e.g. vitamins) thereby decreasing medium costs (Dellomonaco et al. 2010; Rumbold et al. 2009). The use of synthetic minimal medium can greatly reduce costs if an industrial scale application were to be developed. Although based on pricing from a scientific supply company, Table 15 implies a 37 % reduction in medium cost can be obtained by substituting YE medium with CFMM.
### Table 15. Cost comparison of yeast extract medium and carbon free minimal medium.

<table>
<thead>
<tr>
<th>Medium Components</th>
<th>Cost per g&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cost for 1 L medium</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Yeast Extract Medium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>$0.09</td>
<td>$0.90</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>$0.90</strong></td>
</tr>
<tr>
<td><strong>Carbon Free Minimal Medium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;HPO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>$0.04</td>
<td>$0.09</td>
</tr>
<tr>
<td>KH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>$0.36</td>
<td>$0.11</td>
</tr>
<tr>
<td>NH&lt;sub&gt;4&lt;/sub&gt;NO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>$0.12</td>
<td>$0.36</td>
</tr>
<tr>
<td>MgSO&lt;sub&gt;4&lt;/sub&gt;·7H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>$0.04</td>
<td>$0.01</td>
</tr>
<tr>
<td>FeSO&lt;sub&gt;4&lt;/sub&gt;·7H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>$0.05</td>
<td>$0.00</td>
</tr>
<tr>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt;·2H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>$0.04</td>
<td>$0.00</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>$0.57</strong></td>
</tr>
</tbody>
</table>

<sup>a</sup> Cost based on Fisher Scientific and Sigma Aldrich retail/institutional low volume 2011 pricing

Following the initial screen, the 17 isolates identified as crude glycerol metabolizing isolates (Table 9) were further assessed for their ability to utilize crude glycerol as a carbon substrate for growth at an increased concentration of 100 g·L<sup>-1</sup> (glycerol content 28 g·L<sup>-1</sup>). Fungal cultures were simultaneously cultured using the synthetic and defined CFMM in order to determine if the undefined YE medium could be replaced with a synthetic minimal medium. Furthermore, the initial culture pH was maintained at 8.5 rather than 6.5, as this is the pH of unadjusted medium prepared using the crude glycerol biodiesel co-product.
The majority of fungal isolates grew better than glucose controls when crude glycerol was used as the carbon substrate for growth regardless of medium type tested (Figure 10). Isolates V22 and F24 were identified as the only two isolates with the ability to completely metabolize the crude glycerol in both types of medium (Table 10). In addition to their ability to metabolize the crude glycerol, the metabolism of this carbon substrate coincided with the production of new bioproducts which could be detected by TLC (Table 11). This ability was also seen in isolates V19 and BF1 where new bioproducts were also detected by TLC (Table 11).

Up to this point in the experimental process, only single flask cultures were used and triplicate trials were now conducted in order to better assess the ability of the fungi to utilize crude glycerol for growth and bioproduct production. Batch culture of isolates F24, BF1, V19 and V22 emphasized again that a high yield of biomass could be effectively produced when crude glycerol was used (Figure 11). While biomass production in YE medium was slightly higher, CFMM still proved to be an effective culture medium for these isolates when crude glycerol was used as a carbon substrate.

As described throughout this thesis, the presence of impurities in the crude glycerol results in a reduced glycerol content when it is used to prepare treatment culture medium. Medium prepared using 100 g·L⁻¹ of this co-product would be expected to contain an actual glycerol concentration of 28 g·L⁻¹. Previously published studies using similar culture conditions and glycerol concentrations provided much lower biomass yields than what was observed in the present work, especially for V22 and F24 which produced 24.1 ± 0.9 g·L⁻¹ and 23.2 ± 1.1 g·L⁻¹ biomass yield in CFMM from crude glycerol (Figure 11). When natural strains of *Yarrowia lipolytica* were cultured in shake
flasks at a glycerol concentration of 30 g·L⁻¹, using crude glycerol, dry biomass yields ranged from 4.2 g·L⁻¹ to 8.2 g·L⁻¹ after incubation periods up to one week in duration (André et al. 2009). In their recent work, Liu et al. (2011) compared growth yield of the fungus *Ustilago maydis* in medium containing crude glycerol between 10 g·L⁻¹ and 180 g·L⁻¹. After five days of incubation, the fungus was unable to attain a biomass yield above 8 g·L⁻¹. Similar results were obtained when the use of pure glycerol was assessed as opposed to crude glycerol. Six day old batch cultures of *Mortierella alpina* and *M. zychea*, using pure glycerol concentrations between 2.5 g·L⁻¹ and 150 g·L⁻¹, resulted in cell dry weights between 1.5 g·L⁻¹ and 7.2 g·L⁻¹ (Hou 2008). Compared to fungal growth yield in these previously reported studies, the isolates identified in the present work show promise as high biomass producers.

### 4.4 Characterization of Fungal Isolates

Molecular characterization through gene sequencing is an effective way to determine the identity of unknown microorganisms purified from environmental samples. Knowing the fungal species used in a study is important if industrial scale applications are to be developed to ensure that the species of interest are non-pathogenic or to determine if genetic tools are available.

Molecular analysis of the four leading candidates revealed that three (V19, V22 and F24) all belonged to the genus *Galactomyces*, while isolate BF1 was a species of *Mucor* (Table 12; Figure 12). Although the bioconversion of crude glycerol has been reported several times for *Mucorales* (Chatzifragkou et al. 2011; Fakas et al. 2009b; Sajbidor et al. 1988) only one report mentions the use of *Galactomyces* for this purpose.
(Koganti et al. 2011). Since V22 and V19 were isolated from the same deciduous forest location, and molecular characterization showed that their 18S rRNA were identical (Table 12), only V22 was selected for further experimentation due to its slightly better biomass yields.

4.5 Process Development for Biomass Production

In order to use microorganisms to their full potential when using industrial feedstocks for bioconversion processes, optimization of the culture conditions in which they are grown is an important part of process development. Saenge et al. (2011) demonstrated that statistical modelling effectively determined optimal culture conditions for the yeast *Rhodotorula glutinis*. Values which were obtained for biomass, lipid content, lipid yield and carotenoid production could be improved by 3.3, 3.8, 12.4 and 2.1 times, respectively when optimized and non-optimized cultures were compared. In addition to optimizing culture parameters, identifying isolates which show tolerance to known inhibitors when industrial feedstocks are used can benefit processes in which the feedstock quality is variable. For this reason, one week batch trials were conducted to investigate culture optimization for biomass production, along with the tolerance of selected isolates to methanol. Pure glycerol was also used in place of glucose in the control cultures to determine if the improved biomass yield in preliminary tests was due to a preference of these microorganisms for glycerol as a carbon substrate.

4.5.1 Effect of Initial Carbon Substrate Concentration on Growth Yield

Determining the optimum carbon substrate concentration for fungal growth is important and may vary from one microorganism to the next. Results obtained from
these trials are shown in Figures 13 and 14. An initial concentration of 100 g·L⁻¹ crude glycerol (glycerol content 28 g·L⁻¹) proved to be best when culturing isolates V22 and F24 in, while BF1 showed a slightly higher optimum at 150 g·L⁻¹ (glycerol content 42 g·L⁻¹). Just as previously observed with glucose, treatment cultures grown in crude glycerol typically resulted in higher biomass yields compared to their corresponding pure glycerol control. The only exception was for V22 cultures grown in pure glycerol at a concentration above 100 g·L⁻¹ (Figure 14).

Other studies have described species of fungi which exhibit improved growth yield using crude glycerol as a carbon substrate. For example, when biomass yield was compared between cultures of *Yarrowia lipolytica* strains grown in glucose, pure glycerol and crude glycerol, higher yields of biomass were consistently obtained in cultures which utilized crude glycerol as their carbon substrate (Rywinska et al. 2010b). Maximal biomass yields obtained for these three carbon substrates were 21.6 g·L⁻¹, 18.3 g·L⁻¹ and 24.8 g·L⁻¹ for glucose, pure glycerol and crude glycerol, respectively. In other similar studies, the fungus *Ustilago maydis* had significantly improved biomass production in crude glycerol (approximately 14 g·L⁻¹) compared to glucose (8 g·L⁻¹) after 12 days of incubation while the fungus *Trichoderma reesie* was able to grow in crude glycerol even though no growth was observed when pure glycerol was tested (Liu et al. 2011; Rumbold et al. 2009).

It has been suggested that additional nutrients which are easily available in the crude glycerol co-product may contribute to improved biomass yield. Table 7 shows how the crude glycerol from the present study does in fact contain various elements and a slight amount of nitrogen (190 μg·g⁻¹) which may be utilized by the fungi. (Celik et al.
The ability of fungi to use nutrients present in crude glycerol is further supported by the observation that the yeast *Rhodotorula glutinis* was able to produce 3.06 g·L\(^{-1}\) biomass in water and crude glycerol with no further nutrient supplementation (Saenge et al. 2011).

It has been speculated that using equivalent amounts of pure glycerol to crude glycerol may result in osmotic stress for the cultures grown in pure glycerol, resulting in lower biomass. This occurs since the overall glycerol content would be higher in the pure glycerol medium than what is present in the crude glycerol co-product due to impurities (Ashby et al. 2005). Alternatively, in carbon-limited conditions, cellular metabolism directs carbon substrates into the production of biomass, while carbon-excess and limited nitrogen directs the production of lipids (André et al. 2010).

**4.5.2 Effect of Initial pH on Growth Yield**

Culturing microorganisms at their optimal pH is another important step in microbial bioconversion development as pH directly affects biomass and bioproduct production capabilities of most microorganisms (Saenge et al. 2011). The presence of residual KOH catalyst from the transesterification reaction gives crude glycerol an alkaline pH. When media was prepared using this co-product, the final pH of the culture broth was therefore quite high, characteristically between 8 and 8.5. Although some fungi exhibit a broad pH tolerance for growth, many fungi prefer medium which is slightly acidic (Moore-Landecker 1990).

Fungal culture screening in this study sought to identify those which could be successfully grown at a pH above 8. This screening approach appears to have been
successful since the three leading candidates from this study, namely V22, F24 and BF1, all grew best when the initial pH of the medium was between 8.0 and 8.5 (Figure 15). These isolates therefore represent good candidates for crude glycerol bioconversions as they should not require pH adjustments to be made when preparing medium with glycerol from alkali-catalyzed transesterification reactions.

The influence of culture pH on growth varies from one microorganism to the next and can even be variable within species and strains from the same genus. Growth yield and arachidonic acid production by *Mortierella alpina* from glycerol, for example, was not significantly affected by a change in culture pH ranging from 5.0 to 7.5, however in this same study the growth of *M. zychae* was influenced by changing this parameter (Hou 2008).

### 4.5.3 Effects of Methanol on Growth Yield

Residual methanol from the transesterification process is another reactant which can be found in the crude glycerol co-product (Table 7). The transesterification reaction resulting in biodiesel production is a reversible process, therefore an excess of methanol is used to drive the reaction to completion. This results in the presence of methanol in the crude glycerol (Lin et al. 2011). The actual concentration of methanol varies widely between batches and is also influenced by whether a methanol recovery system employed at the production facility. Analysis of crude glycerol produced by the transesterification of seven different oil feedstocks, Thompson and He (2006) obtained glycerol with a methanol concentration ranging from 23.4 % to 37.5 % (w/w), while the methanol content in glycerol from the study by Pyle et al. (2008) ranged from 12.8 % to
28.3 % (w/w). Characterization of various glycerol samples from a number of literature sources demonstrate methanol concentrations ranging from less than 0.01 % up to 37.5 % (w/w) in crude glycerol (Table 2). The crude glycerol in the present investigation contained 260 g·L⁻¹, or approximately 23.6 % (w/w), methanol. This relatively high concentration is likely due to the absence of a methanol recovery system at the CARES facility.

When medium was prepared at 100 g·L⁻¹ crude glycerol, the methanol concentration prior to autoclaving was 26.0 g·L⁻¹. While some authors have found that autoclaving media is sufficient to remove the methanol (Athalye et al. 2009; Liu et al. 2011) others have shown its presence after sterilization. In the study performed by Liang et al. (2010), the methanol concentration in medium containing crude glycerol was only reduced from 252.9 g·L⁻¹ to 111.3 g·L⁻¹ after autoclaving. Analysis of autoclaved crude glycerol samples in the present study revealed an 8 % (w/v) decrease in the methanol content. Assuming this 8 % drop, the resulting medium would have approximately 23.9 g·L⁻¹ of methanol present. Fungal isolates which exhibit some tolerance to this alcohol would therefore be beneficial since autoclaving this co-product does not completely remove this alcohol.

It was hypothesized that the abrupt drop in growth yield for isolates V22 and F24 at crude glycerol concentrations above 100 g·L⁻¹ was possibly due to methanol inhibition (Figure 14). For this reason, these two isolates were tested for their ability to tolerate the addition of methanol to culture broths.
Because the majority of biodiesel producers do methanol recovery, in the initial screen medium was supplemented with methanol up to 19.4 g·L\(^{-1}\) (which corresponds to a volumetric concentration of 2 % (v/v)). It has been suggested that a concentration of this alcohol approaching even 15 g·L\(^{-1}\), or 1.5 % (v/v), in crude glycerol is unlikely to occur in most biodiesel facilities, therefore identifying fungi with the ability to tolerate 2 % (v/v) should enable the use of crude glycerol from variable sources, even those with higher methanol contents (Ashby and Solaiman 2010). Residual methanol present in the crude glycerol was not accounted for when adding methanol, therefore treatment cultures actually had higher methanol contents than control cultures. While V22 and F24 control cultures grown using pure glycerol were negatively impacted by this addition, treatment cultures grown in medium supplemented with crude glycerol demonstrated little impact to the addition of methanol up to 19.4 g·L\(^{-1}\) (data not shown). These results demonstrate that crude glycerol represents a better carbon substrate for fungal growth in minimal medium as it improves the fungal isolates' ability to tolerate the residual methanol which may be present, though the mechanisms which allow this remain unknown.

In an effort to determine the upper limit of methanol tolerance, isolates V22 and F24 were grown in medium supplemented with methanol concentrations up to 116.6 g·L\(^{-1}\), excluding residual methanol in the crude glycerol. This corresponds to a volumetric concentration of 12 % (v/v). Crude glycerol treatment cultures could grow at an added methanol concentration of 19.4 g·L\(^{-1}\), however growth yield was adversely affected when methanol was added at concentration of 38.9 g·L\(^{-1}\) and no growth was observed at 116.6 g·L\(^{-1}\) (Figure 17). The results obtained in this study reflect those
found in the work performed by Liu et al. (2011). When grown in 0 g·L⁻¹, 19.4 g·L⁻¹, 48.6 g·L⁻¹ and 97.2 g·L⁻¹ methanol, the fungus *Ustilago maydis* showed reduced growth in cultures supplemented with both crude and pure glycerol at an added methanol concentration of 19.4 g·L⁻¹, with complete inhibition of growth being observed at a methanol concentration of 48.6 g·L⁻¹ or higher (Liu et al. 2011).

Taking into account residual methanol present after autoclaving, it can be said that the upper limit of tolerance for V22 and F24 is approximately 43.3 g·L⁻¹. The inhibition of growth at high crude glycerol concentrations (Figure 14) could therefore be due to the presence of methanol. Medium prepared using 150 g·L⁻¹ crude glycerol (the upper limit for crude glycerol concentrations for V22 and F24) would contain 35.9 g·L⁻¹ methanol after autoclaving. This is nearing to the upper limit of methanol tolerance determined from Figure 17. Knowing the tolerance level of microorganisms to this impurity will help when developing bioconversion processes, especially if crude glycerol will be obtained from facilities which do not do methanol recovery.

**4.6 Bioconversion of Crude Glycerol to Bioproducts**

Previous trials identified three fungal isolates (one filamentous fungi and two yeast) possess the ability to utilize crude glycerol as a carbon substrate for growth. These isolates show good biomass production compared to other species reported in the literature, they exhibit tolerance to growth inhibitors and the metabolism of glycerol coincides with the production of a new bioproducts (Table 11). For a bioconversion project to truly be successful it is important to identify the products which are being
produced, the quantity and concentration at which they are made and whether or not they provide a benefit to industry.

3.6.1 Qualitative and Quantitative Analysis of Citric Acid

The small culture volumes initially used did not provide a large enough volume to enable solvent extractions and identification. For this reason, larger volume cultures were prepared using 400 mL of medium in 1 L flasks to enable both qualitative and quantitative analyses to be conducted. Scaling up to the larger culture volume dramatically increased incubation time and complete metabolism of glycerol was not observed until 28 days after inoculation took place. Furthermore, despite the fact that F24 previously showed a bioproduct based on TLC analysis (Table 1), no bioproduct was detected in the large scale culture and qualitative analysis could not be conducted for this isolate.

After solvent extracting and column purifying the bioproducts detected in the culture broths (Table 13, Figure 18), samples were sent away to the University of Guelph, Lab Services (Guelph, ON) for qualitative GC-MS analysis. Although only one primary bioproduct spot could be detected by TLC in the samples (Figure 18) a variety of compounds were detected (Table 14). Most notable was the detection of CIT in the Galactomyces culture broths.

During growth yield optimization trials, a drop in the final pH of medium was recorded (as low as 2.5; Figure 16) for the isolates under investigation after one week of incubation. When high levels of CIT accumulate within cells, CIT is released into the surrounding medium (Papanikolaou et al. 2001). The production and release of organic
acids into the culture broth is known to cause a reduction in medium pH (André et al. 2009). The discovery that CIT could be detected in V19 and V22 bioproduct fractions (Table 14) lead research to focus on this particular bioproduct.

Species of *Yarrowia* have been extensively studied as CIT producers from glycerol (Table 6). Wild type strains of *Yarrowia lipolytica* have been shown to produce up to 77 g·L⁻¹ of CIT when crude glycerol is used as the primary carbon substrate (Imandi et al. 2007). Genetically modified isolates, such as acetate-negative mutants of *Y. lipolytica* have been shown to produce even higher levels of CIT. Concentrations of CIT in excess of 150 g·L⁻¹ have been reported from both crude and pure glycerol (Rymowicz et al. 2009; Rywinska and Rymowicz, 2010; Rywinska et al. 2010a; Rywinska et al. 2011). *Galactomyces* and *Yarrowia* belong to the same family (Dipodascaceae), therefore it was hypothesized that if any of the tested isolates could produce CIT, it would likely be a *Galactomyces* sp.

Citric acid could be detected in the culture broths from all isolates tested. Unfortunately, the concentration of CIT present was extremely low. None of the isolates were able to produce CIT at a level above 0.23 g·L⁻¹, even when culture broths from the optimization trials were analyzed.

Levinson et al. (2007) compared 27 strains of *Yarrowia lipolytica* along with five strains from three other *Yarrowia* species to determine if the CIT producing capabilities extended to other relatives. As with our study, these yeast were obtained from a variety of sources and geographic locations including food, food production facilities, insects and environmental and clinical sites. The production of CIT varied and covered a wide
range of concentrations between 1.4 g·L\(^{-1}\) and 21.6 g·L\(^{-1}\) when 40 g·L\(^{-1}\) pure glycerol was used as the carbon substrate. Interestingly, only the strains of *Yarrowia lipolytica* produced CIT, with all other species lacking the ability to produce this organic acid altogether (Levinson et al., 2007).

One of the challenges with CIT fermentations is determining the most effective substrate C:N ratio to use for organic acid product. Levinson et al. (2007), for example, tested the effect of various substrate C:N ratios on their *Y. lipolytica* cultures. The authors found that increasing the C:N ratio from 172 to 343 improved not only the production rate of CIT, but also the overall yield (Levinson et al. 2007). These ratios are much higher than that tested using the optimized CFMM which had a C:N ratio of 41 and gave only low levels of CIT. Nonetheless, using YE medium supplemented with 50 g·L\(^{-1}\) of crude glycerol, and which had a much higher C:N ratio of 217, did not improve the CIT production capabilities of these isolates. Taken together with the findings from the Levinson et al. (2007), it is unlikely that optimizing the culture broth for the C:N ratio will have much effect on CIT production by these isolates.

**4.7 Bioconversion of Crude Glycerol to Single Cell Oil**

Single cell oils produced by microorganisms could potentially displace some plant and animal-based oils as a feedstock for biodiesel production (André et al. 2009; Meng et al. 2009; Vicente et al. 2009). Results from published work suggest that high lipid contents typically occur only with low biomass production, which is not favourable for large scale applications (Ageitos et al. 2011). If SCO were to replace plant and animal-based oils, high biomass production and high lipid yields must be achieved.
simultaneously (Ageitos et al. 2011). Small scale trials described in the present work have shown that high biomass yields (g biomass dry weight per g culture broth) are possible with the isolates under investigation (e.g. Figure 11). In addition to this, analysis of the BF1 bioproduct spot (Table 11; Figure 19) suggests the production of a non-polar compound.

As with species of *Mucor*, the *Yarrowia* relative of *Galactomyces* has been identified with the ability to produce SCO from glycerol (Table 4). Isolate V22 was consequently also assessed for SCO production. The use of yeast, such as isolate V22, for SCO production also offers a number of advantages compared to filamentous fungi as they are unicellular, show rapid growth rates and rapidly accumulate in lipid bodies (Saenge et al. 2011).

Various lipid extraction protocols exist for harvesting SCO from cell cultures. Effective lipid extractions were often achieved through the use of solvent systems comprising both polar and non-polar solvents. This enables membrane bound lipids to be removed via the polar solvent while also extracting the nonpolar lipids fractions from cells (Somashekar et al. 2001). Vicente et al. (2009) tested three different solvent systems, chloroform:methanol, chloroform:methanol:water and n-hexane for extracting lipids from *Mucor* biomass. Yield comparisons showed that the chloroform:methanol extraction was most effective, resulting in the highest amount of lipids being extracted with minimal loss. Work conducted by Somashekar et al. (2001) was also in agreement with these finding, therefore this solvent system was used in the trials described here.
As with the production of organic acids, lipid production is often associated with higher C:N ratios. Typically SCO production requires C:N ratio above 50 (Kosa and Ragauskas 2011). Since the optimized medium (CFMM 100 g·L⁻¹, pH 8) has a C:N of only 41, YE medium supplement with 50 g·L⁻¹ crude glycerol was used in lipid trials to provide isolates with a medium having a C:N ratio of 217 as previously discussed.

4.7.1 Bioconversion of Crude Glycerol to Single Cell Oil after 28 Days

Microorganisms can be classified as oleaginous if their cellular lipid content is in excess of 20 % (w/w) of their total CDW (Meng et al. 2009; Papanikolaou and Aggelis 2009; Ratledge and Wynn 2002). As Figure 20 shows, when grown using crude glycerol as the carbon substrate, isolate V22 produces biomass with a lipid content above the 20 % (w/w) cut-off value (22.5 % ± 0.9 % w/w) used to classify oleaginous microorganisms. As opposed to this, isolate BF1 produced biomass with a lipid content below this value (12.0 % ± 0.5 % w/w).

Molecular characterization of isolate BF1 (Table 12) identified this fungus as a member from *Mucor* genus. The production of lipids using species of *Mucor* and various carbon substrates is known. Roux et al. (1994), for example, compared the lipid production capabilities of 28 different *Mucor* strains (representing 22 species) after five days using 50 g·L⁻¹ glucose as the carbon substrate. Lipid content of biomass varied from 0.5 % to 47.7 % (w/w) in this study, with the average being 13.3 %. Interestingly, this is close to the value obtained for the 28 day old BF1 cultured using crude glycerol (12.0 % w/w, Figure 20), but is much higher than the 2.0 % (w/w) lipid content recorded for control cultures which used an identical concentration of glucose as the published
study (Figure 16). In another study investigating lipid production by *Mucor mucedo* using 30 g·L⁻¹ pure glycerol, a slightly higher lipid content of 18.6 % (w/w) was reported (Sajbidor et al. 1988). Lipid production using *Galactomyces* strains has yet to be published in the literature, therefore comparing the lipid production capabilities of isolate V22 to other species being studied is not possible.

According to weekly TLC analysis (data not shown), glycerol was detected in media until 28 days of incubation. Long incubation times are not atypical for SCO production because nitrogen limitation must first occur to induce lipid production. Additional nitrogen present in the crude glycerol was not expected to increase the incubation time to reach the limiting concentration required for lipid production. As reported in Table 7, the crude glycerol from the CARES facility has a very low total nitrogen content (190 µg·g⁻¹). The length of time required to achieve complete glycerol metabolism (28 days) and lipid production by isolates BF1 and V22 is in agreement with the timeline reported by other authors investigating lipid production from this carbon substrate. As Table 4 demonstrates, batch cultures supplemented with crude glycerol ranging from four to 25 days of incubation have been reported for lipid production using filamentous fungi and yeast, with the average being around 15 days. Fakas et al. (2009b), for example, reported that 14 and 11 days of incubation were needed to achieve 25.6 % and 53.2 % (w/w) lipid contents in *Cunninghamella echinulata* and *Mortierella isabellina* cultured using crude glycerol and 1 g·L⁻¹ total nitrogen.

Due to the fact that lipid production was not monitored throughout the incubation period, it is possible that as the carbon substrate began to deplete in the medium and some re-consumption of cellular reserve lipids occurred. This would reduce the final
lipid content measured in the biomass. The influence of time on lipid content in cells is evident in the work by Chatzifragkou et al. (2011). In their trials, the lipid content of *Pichia membranifaciens* dropped from 15 % (w/w) at the start of the trial to 1.4 % after 5.8 days of incubation when biomass reached its highest value, suggesting a re-consumption of lipids for cellular growth. The re-consumption of cellular lipids has also been demonstrated using fatty acid profile studies. For example, André et al. (2010) demonstrated using cultures of *Aspergillus niger* that C18:0 (stearic) and Δ9C18:1 (oleic) acids decreased over the incubation period while Δ9,12C18:2 (linoleic) acid increased in crude glycerol cultures. This suggests that when microorganisms consume cellular lipids, saturated and mono-unsaturated fatty acids were preferred.

In order to investigate the possibility that biomass with higher lipid contents can be achieved, two new lipid trials were conducted where biomass was harvested after seven or five days of incubation to determine the effect of reducing the incubation period while maintaining all other culture parameters consistent to the 28 day trial.

### 4.7.2 Bioconversion of Crude Glycerol to Single Cell Oil after Seven Days

Reducing the incubation period from 28 days to one week resulted in an overall increase in the lipid content of dried biomass for both BF1 and V22 treatment cultures (Figure 21). Similar to the 28 day trial, significantly higher lipid contents were observed in the crude glycerol treatment cultures compared to their corresponding controls. Considering the results from this trial, both BF1 and V22 can be classified as oleaginous microorganisms. These two isolates had average lipid contents of 36.6 % ± 1.3 % and 31.7 % ± 1.1% (w/w) respectively. These values compare favourably to the average
lipid content of 37.8% produced by the species listed in Table 4 when both crude glycerol and pure glycerol cultures are considered.

A major disadvantage of the reduced incubation time is that BF1 biomass is dramatically decreased from 20.5 g·L⁻¹ ± 0.7 g·L⁻¹ (after 28 days) to 0.44 g·L⁻¹ ± 0.0 g·L⁻¹ in crude glycerol cultures (Figure 22). Results from published work suggest that high lipid contents typically occur with low biomass production rates as was observed in the present work (Ageitos et al., 2011). Even with the higher lipid content in the biomass, the overall low biomass yield does not make BF1 a practical isolate to utilize unless long incubations times were employed. Unlike isolate BF1, isolate V22 biomass yield was only slightly reduced in treatment cultures after one week. As Figure 22 shows, biomass yield decreased from 11.3 g·L⁻¹ ± 0.2 g·L⁻¹ after 28 days to 10.2 g·L⁻¹ ± 0.0 g·L⁻¹ after seven days. The slight increase in biomass obtained after 28 days from this isolate does not justify the longer incubation period. A seven day turnaround time for this culture process could be performed four separate times over a 28 day timeframe, consequently amounting to a total combined biomass yield 40.8 g·L⁻¹. Further experimentation was therefore conducted on isolate V22 as it was selected as a better candidate for the bioconversion of crude glycerol to SCO.

4.7.3 Bioconversion of Crude Glycerol to Single Cell Oil after Five Days

Liquid inoculum preparation using YE medium for isolate V22 demonstrated that biomass for this yeast increases until day four to five, at which point stationary phase begins. The results from initial lipid trials discussed in section 4.7.1 and 4.7.2 supports these findings. Very little change in growth yield was observed for this yeast when
biomass from seven day old cultures was compared to that of 28 day old cultures (Figure 22). For this reason, lipid production by isolate V22 was assessed after five days, the time required for cells to reach stationary phase. Due to the increased lipid content and biomass in crude glycerol, pure glycerol was used in addition to glucose as a control carbon substrate to determine if isolate V22 simply favours glycerol over glucose as a carbon substrate for SCO production.

Cellular lipid contents after five days were higher than those obtained after seven or 28 days (Figure 23). The highest lipid content obtained was 45.4 % ± 1.3 % (w/w) from crude glycerol grown cultures. As previously observed, glucose did not produce biomass with a high lipid content (4.3 % ± 0.3 % w/w), however, exchanging glucose for pure glycerol did improve the ability of this yeast to produce lipids (Figure 23). Pure glycerol control cultures attained an average lipid content of 26.9 % ± 1.6 % (w/w) (Figure 23b). Biomass from crude glycerol cultures was slightly lower than what was observed after seven days. Biomass yield decreased from 10.2 g·L⁻¹ ± 0.0 g·L⁻¹ to between 9.6 g·L⁻¹ ± 0.2 g·L⁻¹ and 9.8 g·L⁻¹ ± 0.1 g·L⁻¹.

Scaling up the culture volume from 20 mL to 400 mL gave overall lower biomass yields. Nonetheless, similar research has shown comparable biomass and lipid values for other fungi and yeast to what was observed in the 400 mL cultures described in this work. Batch cultures of the fungus *Aspergillus niger* produced 8.2 g·L⁻¹ of biomass with a lipid content of 41 % (w/w) after 6.8 days of incubation using biodiesel derived crude glycerol (André et al. 2010). Similarly, Chatzifragkou et al. (2011) demonstrated that *Thamnidium elegans* could produce 7.0 g·L⁻¹ biomass with a lipid content of 40.1 % (w/w).
Analysis of the glycerol consumption over the course of the five day trial (Figure 24) revealed that residual glycerol (62.3 %) remained in the medium. Unfortunately, no data point was available for day zero. Assuming an initial glycerol content of 14 g·L⁻¹ at the start of the trials, the decrease in glycerol content to just above 10 g·L⁻¹ after 24 hours is slightly concerning and could benefit from additional investigation. Regardless, it is still clear that the glycerol is being consumed by the cells. Other studies have shown residual glycerol in culture broths after growth cessation. In the study performed by Fakas et al. (2009) the concentration of crude glycerol remaining in cultures after 12.5 days of incubation was above 40 g·L⁻¹ when strains of Mucorales were grown at an initial glycerol concentration of 80 g·L⁻¹. Papanikolaou et al. (2008) also reported glycerol remaining in culture broths even after 25 days of incubation in their study. They suggest that a microorganism's tendency not to consume all of the available carbon substrate is strain-dependent and can be influenced by a variety of parameters aside from incubation time (Papanikolaou et al. 2008).

4.8 Significance of Research

The successful development of large scale bioconversion processes using microorganisms is not only influenced by yield and productivity of the process, but also by the substrate costs and downstream purification steps required (Sauer et al. 2008). Biomass harvesting and lipid extraction could potentially offer a simple alternative to more complex purification processes for bioproducts which are dissolved in the medium such as CIT.
The work presented in this thesis provides new insight in the area of SCO production using *Galactomyces* sp., a newly identified oleaginous yeast. In addition to providing a new market for crude glycerol, the use of SCO as a second generation feedstock for biodiesel production also addresses the need for a more sustainable lipid supply for biofuel production.

The CARES biodiesel research facility at the University of Guelph, Ridgetown Campus, is working on improving the overall economic and environmental sustainability of the biodiesel production process. One aspect of this research is the discovery of new uses for underutilized co-products such as crude glycerol. Research such as the work presented here helps to address this need and broadens the field of glycerol bioconversion for future process development.
CHAPTER 5. CONCLUSIONS

5.1 Research Conclusions

The influx of large quantities of crude glycerol into the market has created a relatively inexpensive supply of carbon substrate for microbial applications (Amaral et al., 2009). Integrating processes for the conversion of crude glycerol by microorganisms with existing biodiesel manufacturing facilities is an important direction on which research initiatives should focus due to the large quantities of glycerol which are already available (Xu et al., 1989). In order to achieve a productive process, the cost of production and recovery of final products both have to be economically favourable. Harvesting biomass and extracting lipids is an example of a simple process which could potentially be implemented into a biorefinery model. The trials outlined in this thesis provide evidence of an environmental isolate belonging to the Galactomyces genus which shows promise for this purpose.

At the onset of this research, many objectives were put in place to address the need for new glycerol bioconversion options. It was initially proposed that this could be achieved by screening environmental fungal isolates to identify those which could:

- Utilize crude glycerol from biodiesel manufacturing as a carbon substrate for biomass production
- Tolerate the impurities found in the crude glycerol co-product
- Produce new and useful bioproducts as the result of glycerol metabolism
- Grow aerobically using minimal medium
- Are non-pathogenic
The results obtained from this research have helped to realize these objectives and have provided a platform for future research.

In the present study, biodiesel derived crude glycerol was effectively utilized as the primary carbon substrate for biomass production using filamentous fungi and yeast. Preliminary screening trials identified new fungal isolates to broaden glycerol bioconversion options and take advantage of this inexpensive carbon substrate. A fungal isolate showing yeast-like growth (V22) and belonging to the genus *Galactomyces* has been identified as a promising candidate for the bioconversion of crude glycerol. This isolate possessed the natural ability to:

- Utilize crude glycerol up to 28 g·L\(^{-1}\) at a pH which is realistic for media prepared using this alkaline co-product for lipid production
- Produce high biomass yields (up to 25 g·L\(^{-1}\)) using minimal medium in an aerobic environment
- Tolerate the methanol impurity (up to 43 g·L\(^{-1}\)) at a concentration above what would be expected in this co-product (15 g·L\(^{-1}\) or less)
- Produce biomass with a high lipid content in a timeframe (five days) similar or better than what has been reported for other fungi and yeast

Taking into account the report by Koganti et al. (2011) this thesis represents only the second report identifying *Galactomyces* sp. as a candidate for the bioconversion of glycerol and the first report identifying this fungus as a lipid producer from crude glycerol.
5.2 Recommendations

Until recently, only a few reports have taken into account filamentous fungi and yeast for the bioconversion of glycerol. Although increased studies are now being published, an effective solution to manage the excess crude glycerol from biodiesel manufacturing still does not yet exist. Given the knowledge obtained from the present work, a few prospective trials to further this field of research are possible:

- Studies using a more regulated fed-batch setup should be performed to determine if growth yield and lipid production can be improved by regulating growth inhibition due to high carbon substrate concentrations and the presence of impurities.
- More in depth analysis on the influence of crude glycerol C:N ratios, the addition of trace elements and the effect of using alternative nitrogen sources on lipid production needs to be addressed.
- Low pH was shown to inhibit growth of Galactomyces in this study. The effect of regulating this parameter to maintain an optimum pH for lipid production should be investigated.
- Given the knowledge that Galactomyces belongs to the same family (Dipodascaceae) as another genus of lipid producing microorganisms (Yarrowia), prospective studies should include an investigation of other genera within this family determine if the lipid producing capabilities extend to other members.
- Investigation of biomass uses after lipid extraction should be pursued. For example, waste water remediation, protein for animal feed, pigment sources, etc. (Fei et al. 2011; Ratledge and Cohen 2008).
CHAPTER 6. REFERENCES


single cell oil production by *Cunninghamella echinulata* and *Mortierella isabellina*. Biomass Bioeng. **33**: 573-580.


Morita, T., Konishi, M., Fukuoka, T., Imura, T., Kitamoto, D. 2007. Microbial conversion of glycerol into glycolipid biosurfactants, mannosylerythritol lipids, by a


Available online at http://www.oecd.org/document/9/0,3746,en_36774715_36775671_45438665_1_1_1_1,00.html. (accessed online September, 2011).


(accessed online July, 2011).


Sidhu, Sukhdeep. Personal Communications, March 2011 through September 2011. Dr. Hung Lee Research Laboratory, University of Guelph, Guelph, ON.


### CHAPTER 7. APPENDIX

#### 7.1 Appendix A

**Table 16.** List of fungal isolates assessed in glycerol bioconversion studies.

<table>
<thead>
<tr>
<th>Fungal ID</th>
<th>Sample Origina</th>
<th>Fungal ID</th>
<th>Sample Origin</th>
</tr>
</thead>
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<tr>
<td>X2</td>
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<td>EB Pp</td>
<td>Nicol- Lab stock</td>
</tr>
<tr>
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<td>Nicol- Lab stock</td>
<td>EB Pk</td>
<td>Nicol- Lab stock</td>
</tr>
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</tr>
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<td>F3</td>
<td>Northside Pond, Tilbury</td>
</tr>
<tr>
<td>X29</td>
<td>Nicol- Lab stock</td>
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</tr>
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<td>F21</td>
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<td>S124</td>
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<td>Algal flask contamination</td>
</tr>
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<td>Nicol- Lab stock</td>
<td>BF 2</td>
<td>Biodiesel glycerol sludge (CARES)</td>
</tr>
</tbody>
</table>

*a Isolates from the Nicol lab stock were previously isolated from soil samples taken from a deciduous forest in London Ontario, or from a long term ecological research site in Michigan.