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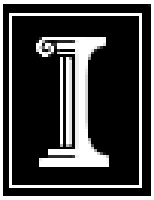

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List of Abbreviations

ASTM	American Society for Testing and Materials
ATCC	American Type Culture Collection
DI	Deionized
FAEE	Fatty Acid Ethyl Ester
FAME	Fatty Acid Methyl Ester
FFA	Free Fatty Acid
ISU	Illinois State University
MSTFA	N-methyl-N-trimethylsilytrifluoracetamide
RPM	Rotations Per Minute
WVO	Waste Vegetable Oil

Abstract

This research examined the use of whole-cell biocatalysts to produce biodiesel at room temperature (25^{o}F). *Rhizopus oryzae* (ATCC 10260) was used to catalyze the conversion of virgin and waste oils (triglycerides) into biodiesel fuel (fatty acid methyl ester, FAME) in the presence of 15% water. Results indicate that the whole-cell biocatalyst can produce about a 90% yield of FAME from virgin oil, and nearly complete conversion of the remaining oil into free fatty acid (FFA), using a 96-hour reaction at room temperature (25^{o}F). In a 72-hour reaction, FAME yields were about 75% for virgin oil, 80% for waste vegetable oil, and 55% for brown (trap) grease.

Increasing reaction temperature to 35^{o}C dramatically *reduced* FAME yield. Separating the hydrolysis and methanolysis reactions did not increase yields, nor did the substitution of ethanol for methanol in the reaction. However, there is some evidence that ethanol may either esterify FFA more quickly than methanol, or result in a more stable ester.

Whole-cell biocatalysts may be an effective way to transesterify waste oils or greases that are high in FFAs and difficult to dewater. Brown (trap) grease and similar degraded or complex greases are good candidates for further whole-cell biocatalyst research. Additional research is also needed on reactor design and type of micro-organism that will increase yield and decrease reaction time. Culturing the whole-cell biocatalyst on industrial or residential waste should be explored as a means to reduce costs.

Executive Summary

This research examined the use of whole-cell biocatalysts derived from *Rhizopus oryzae* (ATCC 10260) to catalyze the conversion of virgin oils and brown and yellow grease (triglycerides) into biodiesel fuel (fatty acid methyl ester, FAME). The objectives of the research were to answer the following questions:

1. How well can a whole-cell biocatalyst transesterify triglycerides, including high-FFA (free fatty acid) waste greases, if grown and reacted at room temperature, without immobilization?
2. How much can the yield of transesterification be improved through alteration of:
   - Reaction temperature
   - Additional alcohol and reaction time
   - Additional transesterification reaction
3. How much can the yield of transesterification be improved through separation of the hydrolysis and methanolysis reactions?
4. What effect does the use of ethanol, in place of methanol, have on the yield of the reaction?

The whole-cell biocatalysts were prepared by cultivating *Rhizopus oryzae* at room temperature (25^{o}C) without immobilization and then air drying for a minimum of 48 hours. These catalysts were then used in a 72-hr, room temperature, transesterification reaction that contained 15% water (as phosphate buffer). Methanol was added in three equal doses at 0 hr, 24hr and 48 hr, for a total dose equal to the stoichiometric ratio (3:1 methanol/triglyceride molar ratio). FAME yields were about 75% for virgin oil, 80% for used fryer oil, and 55% for brown (trap) grease.

To improve the yield of the 72-hour transesterification reaction, several alternatives were tested. Increasing reaction temperature to 35^{o}C dramatically *reduced* FAME yield. An initial 72-hour transesterification reaction was also followed by either 1) an additional dose of methanol and 24 hours of reaction time or 2) a second 72-hour transesterification reaction. Both of these treatments increased FAME yield to close to 90% with nearly complete conversion of the remaining oil into FFA.

Separating the hydrolysis and methanolysis reactions did not increase yields, nor did the substitution of ethanol for methanol in the reaction. However, there is some evidence that ethanol may either esterify FFA more quickly than methanol, or result in a more stable ester.

Overall, this work demonstrated that a fungal biocatalyst, grown and reacted at room temperature, can convert almost 100% of combined glycerides into biodiesel, FFA and glycerine. This suggests several possible implications:

1. Whole-cell biocatalysts may be an effective way to transesterify waste oils or greases that are high in FFAs and difficult to dewater. Brown (trap) grease and similar

degraded or complex greases are good candidates for further whole-cell biocatalyst research.

2. Three factors have the potential to significantly decrease reaction time and increase yield for whole-cell biocatalytic production of biodiesel: a) reactor design; b) type of micro-organism; and c) conditions of cultivation/immobilization of the micro-organism.
3. The ability to grow the whole-cell biocatalyst on inexpensive media – potentially industrial or residential waste – could dramatically lower the cost of biocatalyst production. However, it is unclear at full scale how the costs would compare to alternative methods of processing high-FFA oils.

I. Introduction and Background

I.1. Limitations of Current Biodiesel Production Methods

Current technology for biodiesel production, using an alkali catalyst, has several important limitations. First, it cannot be used for feedstocks with free fatty acid (FFA) content greater than a few percent (Canakci and Van Gerpen, 2001). Second, feedstocks must be free of water. These two limitations mean that some waste oils cannot be processed without pretreatment to remove FFAs and water. The use of acid catalysts has shown promise as an approach for esterifying FFAs prior to reaction with base catalyst, but still requires feedstock free of water (WMRC, 2006). Both alkali and acid catalytic methods require the use of excess methanol, which must be recovered; produce salts that must be removed from the product; and generate glycerin as a low-grade by-product (Van Gerpen, 2004).

I.2. Lipase as a Catalyst

Biological enzymes, particularly lipases, can be used in place of alkali or acid as a catalyst. Biodiesel production using such enzymes would offer several potential advantages (Haas et al., 2002):

- It requires little or no heating.
- The presence of FFAs in the feedstock increases yield, without soap production, creating opportunities for using a variety of lower-quality, lower-cost feedstocks.
- It works even in the presence of water.
- It requires less alcohol and produces no salts.

A number of lipases have been explored as catalysts in biodiesel production. In the transesterification of rapeseed oil with 2-ethyl-1-hexanol, 97% conversion of esters was obtained using *Candida rugosa* lipase powder (Linko et al., 1998). De et al. (1999) investigated the conversion of fatty alcohol esters using immobilized *Mucor miehei* lipase (Lipozyme IM-20) with yields in the range of 86.8-99.2%. Taiwanese researchers achieved a yield of 92% using *Rhizomucor miehei* lipase (Shieh et al., 2003). Researchers in China, testing a variety of lipases, achieved yields of up to 94% (Deng et al., 2003; Du et al., 2004). Research in Italy tested several lipases and found that one produced from the bacterium *Pseudomonas cepacia* produced 100% yield in six hours (Salis et al., 2005). Similar work was performed in the United States (Nourreddini et al., 2005).

The U.S. Department of Agriculture has investigated a wide variety of lipases and found many to be very effective catalysts (Nelson et al., 1996; Abigor et al., 2000; Hsu et al., 2001; Haas et al., 2002). In almost all cases, reactions occurred at temperatures under 100^{o}F (38^{o}C).

Unfortunately, lipase and other enzymes tend to be high in cost due to purification procedures, making the process uneconomical (Van Gerpen et al., 2004; Nourreddini et al., 2005). To extend the life of the enzyme, thus reducing its cost, a number of researchers have “immobilized” the

lipase in or on a physical structure to stabilize the enzyme and allow its reuse. Research suggests that the most effective immobilization technique is the use of a silica gel, greatly extending lipase life without losing yield (Hsu et al., 2002). However, construction of the gel/lipase structure can be time consuming and expensive (Fukuda et al., 2001). In addition, even with the ability to reuse the enzymes for several reaction cycles, the cost continues to be high.

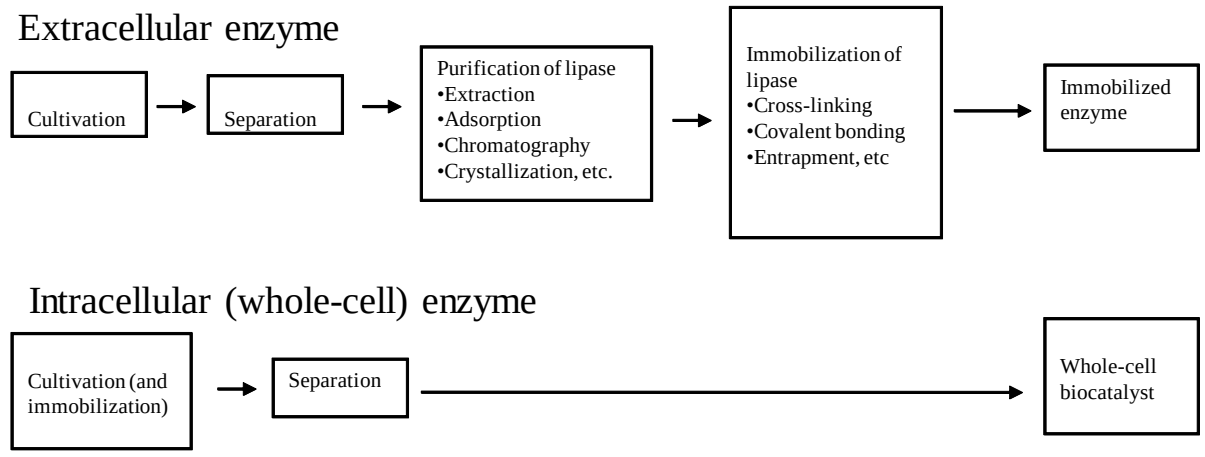
I.3. Whole-cell Lipase

An alternative to purified lipase enzyme is to use the organisms that produce the enzyme. In essence, intracellular enzymes expressed on the cell wall or membrane are used as "whole-cell" biocatalysts instead of extracellular enzymes that require extraction and purification from the culture medium. Figure 1 illustrates the differences in using extracellular and intracellular lipases. Considerably fewer steps are required to produce whole-cell lipase. Immobilization can be accomplished as part of the culturing process (Oda et al., 2005). If organisms that produce lipases can be readily and cheaply cultured in large quantities, use of such whole-cell biocatalysts offers the promise of inexpensive biocatalysts that can transesterify high-FFA wastes, such as yellow and brown grease.

Whole-cell enzymes have been used to catalyze a variety of reactions (Fukuda et al., 1996; Yamaji and Fukuda, 1997; Chen and Wang et al., 1997; Liu et al., 1998b, 1999, 2000; Kondo et al., 1999; Yamaji et al., 2000). However, only four research groups have published on the whole-cell production of fatty acid esters (Ban et al., 2001, 2002; Matsumoto et al., 2001; Oda et al., 2005; Shiraga et al., 2005). Chen and Wang (1997) used a whole-cell biocatalyst to produce a wax ester, but their use of long-chain alcohols limits the applicability of their work for biodiesel production. All four groups focused on the lipase produced by the filamentous fungi *Rhizopus oryzae.* Ban et al. (2001, 2002) and Oda et al. (2005) used the fungi directly, while Matsumoto et al. (2001) and Shiraga et al. (2005) genetically engineered the yeast *Saccharomyces cerevisiae* to produce the lipase enzyme from *Rhizopus oryzae.*

In the laboratory, whole-cell biocatalysts are frequently immobilized in porous biomass support particles (BSPs), a technique developed by Atkinson et al. (1979). This allows the biocatalysts to be reused in subsequent reactions. This was the approach used by Ban et al. (2001, 2002) and Oda et al. (2005). However, both Matsumoto et al. (2001) and Shiraga et al. (2005) added the biocatalyst directly to the reaction without immobilization. While this approach means the biocatalyst cannot be reused, it eliminates the time and expense of immobilization.

Figure 1. Comparison of lipase production processes using extracellular (commercial) and intracellular (whole-cell) lipases (modified from Fukuda et al., 2001).



Evidence suggests that some of the lipase is bound to the membrane on the inside of the cell wall. Hama et al. (2006) used western blot analysis to show that *Rhizopus oryzae* cells produce two types of lipases. One was bound to the cell wall and the other bound to the cell membrane. Because of this, cells are often treated to increase permeability prior to use. Ban et al. (2001) pretreated cells with a number of organic solvents. Matsumoto et al. (2001) and Shiraga et al. (2005) used freeze-drying to increase permeability. However, Ban et al. (2002) and Oda et al. (2005) found that simple air drying for 24-48 hours was adequate.

Ban et al. (2001) found that immobilized *Rhizopus oryzae* cells could produce close to a 90% biodiesel yield even in the presence of 15% water. Ban et al. (2002) pretreated the immobilized *Rhizopus oryzae* cells with glutaraldehyde and found that lipase activity showed no significant decrease during six batch cycles, with the biodiesel yield of 70-83% in each cycle. Oda et al. (2005) achieved a methyl ester yield of greater than 80% and showed that immobilized *Rhizopus oryzae* cells produced in a 20-liter air-lift bioreactor had a higher durability in the biodiesel reaction compared to cells obtained from shake-flask cultivation.

Because fungi have the potential to be inexpensively grown, these results suggest potential for the use of such whole-cell biocatalysts in biodiesel production from high-FFA waste greases without the need to dewater. In all of the above studies, organisms were grown and reacted at elevated temperatures – generally 30-50^{o}C. One way to potentially reduce costs would be to grow and react the organisms at room temperatures, provided yields can be maintained or increased.

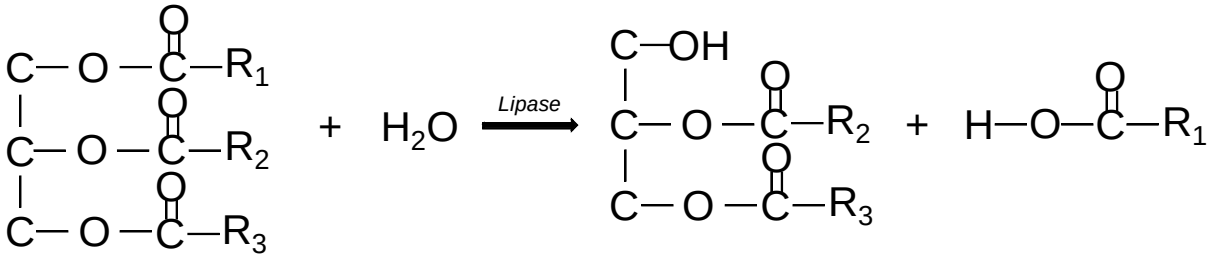
I.4. Separate Hydrolysis and Methanolysis Reactions

It has been observed that in enzymatic conversion of triglyceride into fatty acid ester, two types of reactions appear to occur (Tan et al., 2006). The first is removal of a fatty acid chain from the triglyceride, producing diglyceride and free fatty acid (see Figure 2). This type of reaction is generally referred to as hydrolysis. It proceeds with subsequent removal of a fatty acid chain from the diglyceride, and further removal of the final fatty acid chain from the monoglyceride to produce glycerin. A second type of reaction is generally referred to as methanolysis (alcoholysis) or esterification. In this reaction, the methyl group from the methanol is added to free fatty acid to produce an ester and water (see Figure 2).

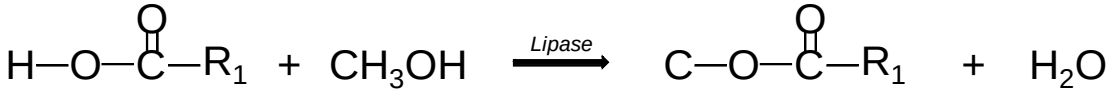
One advantage of performing both of these reactions simultaneously in a single reaction vessel is that methanolysis removes one reaction product of the first reaction (free fatty acid), stimulating continued hydrolysis. However, there are two potential disadvantages. The first is that water, needed for hydrolysis, is a product of methanolysis, inhibiting further methanolysis (Fukuda et al., 2001). Second, methanol, needed for methanolysis, is known to inhibit lipase activity, limiting hydrolysis (Hsu et al., 2002). This suggests that yields might be improved by conducting hydrolysis and methanolysis separately. Hydrolysis could be performed first in the absence of methanol. After removing excess water, methanolysis could be conducted. This could be followed by further hydrolysis and so on, in an iterative process.

Figure 2. Hydrolysis and methanolysis reactions.

Hydrolysis



Methanolysis



I.5. Ethanol Instead of Methanol

Some studies have observed better yield of transesterification or esterification using ethanol as compared to methanol (Mittelbach, 1990; Nelson et al., 1996; Abigor et al., 2000; Hsu et al., 2001). The reason for a better yield may be that lipases are more tolerant of ethanol than methanol (Fukuda et al., 2001), or that lipases act on long-chain fatty alcohols better than on short-chain ones (Shimada et al., 1997; Shimada et al., 1998).

I.6. Project Objectives

The primary objectives for this project were to answer the following research questions:

1. How well can a whole-cell biocatalyst transesterify triglycerides, including high-FFA waste greases, if grown and reacted at room temperature, without immobilization?
2. How much can the yield of transesterification be improved through alteration of:
   - Reaction temperature
   - Additional alcohol and reaction time
   - Additional transesterification reaction
3. How much can the yield of transesterification be improved through separation of the hydrolysis and methanolysis reactions?
4. What effect does the use of ethanol, in place of methanol, have on the yield of the reaction?

II. Materials and Methods

II.1. Preparation of Whole-Cell Biocatalysts and Oils

II.1.1. Whole-cell Biocatalysts

Based on previous studies using whole-cell biocatalysts, three filamentous fungal strains were used in this study: *Rhizopus oryzae* (ATCC 10260), *Rhizopus oryzae* (ATCC 96382) and *Rhizopus oryzae* (ATCC 34612). *Rhizopus oryzae* (IFO 4697, Institute for Fermentation, Osaka, Japan) has been previously used as a whole-cell biocatalyst for biodiesel production with 80 – 90% yield (Ban et al., 2001, 2002; Oda et al., 2005). Since we were not able to get this strain from Japan and it was not clear if any American Type Culture Collection (ATCC) strain corresponds to this particular one, it was decided to use the ATCC *Rhizopus oryzae* strains (10260, 96382, 34612) which have documented lipase activity (Nakashima et al., 1990; Salleh, 1993). Because these fungi produce high lipase activities and are significantly easier to harvest than bacteria, no bacterium was examined in this study.

Difco™ potato dextrose agar, Difco™ potato dextrose broth, and basal medium were used for fungi propagation. The basal medium contained, in 1L of deionized (DI) water: 70 g polypepton; 1.0 g $NaNO_3$; 1.0 g KH_2PO_4; 0.5 g $MgSO_4 \cdot 7H_2O$; and 30 g olive oil (Ban et al., 2001, 2002). Polypepton and other ingredients were purchased from Fisher Scientific.

Following the methods of Ban et al. (2001, 2002), fungi cultivation was carried out using basal medium inoculated by transferring spores from a fresh potato dextrose agar plate. However, this resulted in limited cell growth in our study. We then developed a two-step process of pre-incubation and incubation that showed 3-4 times more cell growth as compared to single-step cultivation. For pre-incubation, Erlenmeyer flasks (125 ml) containing 50 ml of the potato dextrose broth were inoculated by aseptically transferring spores from a fresh potato dextrose agar plate and kept at room temperature (25^{o}C) for 24-72 hours on a reciprocal shaker at 250 RPM. For the incubation step, the filamentous fungi were aseptically transferred from these flasks to other Erlenmeyer flasks (125 ml) containing 50 ml of the basil medium and incubated at room temperature on a reciprocal shaker at 150-350 rpm for another 24-72 hours. Effects of culturing conditions on lipase activity and fungi morphology are presented in Appendix I and II.

The filamentous fungi were separated from the broth by filtering through a strainer. After washing with tap water for 1 minute (Ban et al., 2002), they were air-dried at room temperature for at least 96 hours. Oven drying at 103^{o}C indicated less than 0.3% water remained following the above treatment.

While cultivation on an immobilization structure has been found to extend the life of whole-cell enzymes, we followed the method of Matsumoto et al. (2001) and Shiraga et al. (2005) by adding the organism directly to the reaction without immobilization. This increased the amount of fungi needed for the research, but avoided the cost of materials and equipment for the production of biomass support particles.

Based on its lipase activity performance as well as its superior transesterification yield, ATCC 10260 was selected as the fungi to be used for all subsequent reactions (see Appendix III and IV for details).

II.1.2. Oils

Three types of oil were used in this study: canola oil purchased from local grocery store (Wal-Mart), waste vegetable oil (0.6 - 3.7% FFA and water content of about 1%), and brown (trap) grease. The latter two were both from Illinois State University (ISU) campus dining services. The campus dining services uses canola oil for cooking. No pretreatment was applied to any of the oils. However, to liquefy the brown grease, it was mixed with a 1:1 ratio of methyl ester (produced from soybean oil) prior to being used in a reaction. Discussion of how this dilution was taken into account when evaluating results is presented in Appendix V.

II.2. Reactions

II.2.1. Standard 72-Hour Transesterification Reaction

Transesterification of canola oil was carried out at room temperature (25^{o}C) in a 25 ml screw-cap bottle using a magnetic stir bar for 72 hours. The reaction mixture contained: 12 ml/10.98 g of canola oil, 1.65 ml of 0.1M phosphate buffer (pH 6.8) solution (i.e., water content of 15 wt% of oil), 1.389 ml of methanol, and 0.5 g dry weight of whole-cell biocatalyst. A dose of 0.463 ml of methanol was added stepwise to the reaction mixtures three times: at 0 hr, 24 hr and 48 hr. Total methanol resulted in a 3:1 stoichiometric ratio with triglyceride (no excess methanol), in contrast to the 6:1 ratio typically used in biodiesel production. During the reaction, 0.5 ml of reaction mixture was taken for FFA analysis at approximately 8hr, 24 hr, 32 hr, 48 hr, 56 hr and 72 hr.

The above reaction conditions were adopted from Ban et al. (2002) with a few modifications as summarized in Table 1.

Table 1. Comparison of transesterification reaction conditions by Ban et al. (2002) and by authors (current study).

Conditions		**Ban et al. (2002)**	**Authors (current study)**
Oil type		Soybean	Canola
Whole-cell Biocatalyst	Immo-bilizations	Immobilized using Biomass Support Particle	Non-immobilized
Mixing		Reciprocal shaker (150 RPM)	Magnetic stir bar and plate
Temperature (^{o}C)		35	25

Two exceptions to the above reaction were used to check the appropriateness of (1) stepwise addition of methanol and (2) the presence of water. To check the appropriateness of stepwise addition of methanol, a modified procedure was used in which 1.389 ml methanol was added only once at 0 hr instead of at 0, 24, and 48 hr. To check the effect of the presence of water, reactions were run using no added buffer.

In addition, we tested the effect of using various amounts of whole-cell biocatalysts on transesterification yield. These results are presented in Appendix VI.

Duplicate reactors were used for each experimental condition. At the end of the reaction, products were centrifuged at 3000 RPM for 45 minutes. The oil layer was analyzed by acid titration and gas chromatography as discussed below. A screening test for bound glycerin (the "methanol test") was also conducted. However, due to the limitations of the methanol test (see Appendix VII), results are not presented.

II.2.2. Extension/Alteration of the Standard 72-hr Transesterification Reaction

In an attempt to drive the transesterification reaction closer to completion, we tested the following three approaches: (1) one additional dose of methanol (0.463 ml) was added to the reactor at 72 hours and the reaction continued for another 24 hours for a total of 96 hours; (2) a second 72-hr transesterification reaction was conducted using the oil-phase reaction products from an initial 72-hr transesterification reaction; and (3) reaction temperature was increased to 35^{o}C. These experiments were carried out using virgin canola oil.

II.2.3. Separate Hydrolysis and Methanolysis Reactions

The hydrolysis reaction was conducted by reacting 0.5 g of fungi, 12 ml of oil, and 15% water for 24 hours. The methanolysis reaction was conducted on the resulting centrifuged oil layer by adding 0.5 g of fungi and 0.463 ml of methanol (one-third of the stoichiometric amount) and mixing for 48 hours.

The use of separate hydrolysis and methanolysis reactions was explored in two ways:

1. Three iterations of hydrolysis/methanolysis reactions, described in the paragraph above.
2. A single set of hydrolysis/methanolysis reactions as a follow-on to the standard 72-hour transesterification reaction. This second approach had three variations:
    a. The standard hydrolysis/methanolysis reaction.
    b. A three-angstrom molecular sieve (purchased from Sigma/Aldrich) was added to the methanolysis reaction to remove water. The amount of molecular sieve added was based on the theoretical production of water assuming all residual FFA would be converted to FAME.
    c. Methanolysis was conducted using a higher dose of methanol and fungus (2 times the stoichiometric ratio of methanol and five times the normal amount of fungus - added in order to compensate for possible enzyme deactivation caused by methanol).

II.2.4. Ethanol Instead of Methanol

The substitution of ethanol for methanol was examined in two ways. First, ethanol was used instead of methanol in the standard 72-hr transesterification and followed by one hydrolysis and one ethanolysis reaction. In the 72-hr transesterification reaction, ethanol was added in three doses at the usual times: 0hr, 24hr and 48hr. However, the amount added each time was 50% greater (so 1 1/2 times the stoichiometric amount total) than with methanol in order to increase yield, given evidence that ethanol is less likely to inhibit lipase than methanol.

In the second approach, ethanol was used following a standard 72-hr transesterification with methanol and one hydrolysis reaction. In this ethanolysis reaction, ethanol was added at the 50% excess dose every 24 hrs for a total of 96 hrs.

II.3. Analyses

II.3.1. Lipase Hydrolysis Activity

Lipase hydrolysis activity of the whole-cell biocatalysts was determined by how efficiently they catalyzed hydrolysis of triglycerides. We defined one unit of hydrolysis activity as the release of 1 μmol of fatty acid per hour per mg of whole-cell biocatalyst. The hydrolysis reaction was carried out by stirring 30 ml of DI water with 6 ml of canola oil and 0.5 g dry weight of whole-cell biocatalysts under room temperature for 1 hour. Centrifugation at 3000 rpm for 45 minutes was used to separate oil from water and whole-cell biocatalysts. FFA content in the oil phase was determined by titration using phenolphthalein indicator (see FFA titration section). This is a modified form of the procedure described in Fukumoto et al. (1964).

II.3.2. Methanol Test: A Screening Test for Combined Glycerin

Fatty acid methyl ester (FAME) is relatively soluble in methanol, while di- and triglycerides are not. Thus, it has been recommended by some “home brewers” of biodiesel that this test can be used to evaluate the completeness of the transesterification reaction. Given its speed and low cost, it is an attractive option for screening reaction results.

In our approach, 1 ml of oil phase reactant was mixed with 9 ml methanol in a 15 ml graduated (up to 0.1 ml resolution) centrifuge tube. Phase separation was easily read and could be used as an estimation of percent triglyceride remaining. A more detailed description of this method and its limitations, especially related to high FFA levels, is presented in Appendix VII. Given its limitations, only gas chromatography results are presented for combined glycerin in this report.

II.3.3. FFA Titration

FFA in samples was quantified by titrating with 0.025M NaOH. Isopropanol in the amount of 9-10 ml was used to dissolve 0.5 -1.0 ml of sample and phenolphthalein was used as an indicator (Abigor et al., 2000). The difference between test results obtained under the same operating conditions on an identical sample was less than 0.075 ml titrant (i.e., approximately

0.061 mass% differences in FFA), so a single titration was considered adequate. Since buffer was added to the transesterification reaction mixture, it was important to check whether the buffer interferes with FFA determination by titration. Results presented in Appendix VIII indicted that the buffer in the reaction mixture does not interfere with titration.

II.3.4. Gas chromatography for quantification of FAME, FAEE, glycerides, and glycerol

Mono-, di- and triglycerides, as well as FAME, FAEE (fatty acid ethyl ester) and glycerol, were quantified by gas chromatography (GC) following ASTM-6584 protocol. Approximately 100-mg samples were mixed with 100 ul N-methyl-N-trimethylsilyltrifluoracetamide (MSTFA) and two internal standards. The mixture was then placed at room temperature for 30 min. The trimethylsilyl derivatives were then diluted in 8 ml n-heptane and analyzed by using high temperature GC. A Hewlett-Packard Model 6890 GC equipped with a HT-5 column (12 m x 0.32 mm, I.D. x 0.1 μm df, Supelco, Cat. No. 25002) was used along with a HT-5 precolumn (6 m x 0.53 mm, I.D. x 0.1 μm df, Supelco, Cat. No. 25004) to facilitate cool-on-column injection. FID detector temperature was 380^{o}C. The carrier gas was helium at a flow rate of 3 mL/min measured at 50^{o}C. The column conditions were initial temperature 50^{o}C (hold 1 min); 15^{o}C/min to 180^{o}C (rate 1); 7^{o}C/min to 230^{o}C (rate 2); 30^{o}C/min to 380^{o}C (hold 10 min).

The two internal standards used were 1,2,4-Butanetriol - (internal Standard 1 for quantification of glycerol) and 1,2,3-Tridecanolylglycerol (tricaprin) – (internal standard 2 for quantification of mono, di- , triglycerides FAEE and FAME).

Mono-, di-, and triglycerides were determined by calibrating against monoolein, diolein, and triolein standards, respectively. Commercial B100 (ASTM-certified B100, produced at Incobrasa Industries, Ltd., Gilman, Illinois (www.incobrasa.com)) was used to generate a calibration curve for FAME quantification. Ethyl oleate was used to generate a calibrate curve for quantification of fatty acid ethyl ester (FAEE). R^2 values for all calibration curves exceeded 0.99. Standards and reagents for GC analysis were purchased from Supelco/Sigma-Aldrich.

Since the difference between successive test results obtained by the same operator with the same apparatus under constant operating conditions on identical test materials was less than 0.05 - 0.10 mass% for glycerol, mono-, di-, and triglycerides, a single injection was considered adequate.

III. Results and Discussions

III.1. Transesterification at Room Temperature

III.1.1. Confirming Methanol Dosing Strategy and Water Content

According to Ban et al. (2001, 2002) and Matsumoto et al. (2001), transesterification with the highest yield was carried out with stepwise additions of methanol in the presence of 15% of water. However, many other researchers that use lipases directly in transesterification or esterification use no water at all and have indicated that higher water activity will inhibit the reaction since water is a product (Du et al., 2004; Soumanou and Bornscheuer, 2003; Hsu et al., 2002; Haas et al., 2002; Abigor et al., 2000; Nelson et al., 1996).

We evaluated the effect of water content (0% vs. 15% as buffer) and methanol addition (single dose vs. three doses) in the standard 72-hr transesterification reaction. Table 2 summarizes the results.

Results suggest that a single methanol dose or a lack of water (as buffer) produced a very poor yield. The combination of stepwise addition of methanol and 15% buffer content was superior. Therefore, our transesterification reactions were carried out with stepwise methanol addition and 15% buffer content.

III.1.2. Effect of Oil Type

In addition to virgin canola oil, we tested waste vegetable oils (WVOs) with two levels of FFA. WVO 1 had 0.6% FFA and a relatively clear appearance; WVO 2 had 3.7% FFA and a cloudy appearance. WVO 2 contained some hydrogenated oil and solidified at room temperature. In addition, we tested brown (trap) grease with an FFA content of about 80%. Results of transesterification of the waste vegetable oil and trap grease in comparison with canola oil are

Table 2. Effect of water (buffer) content and methanol addition in 72-hr transesterification of canola oil.

Reaction Conditions[1]		**Reaction Products**	
Methanol addition	**Buffer (% of oil)**[2]	**FAME (mass%)**	**FFA (mass%)**
Stepwise	0.0	0.0	5.2
Stepwise	15.0	57.9	8.1
Once	0.0	0.0	5.2
Once	15.0	1.1	13.9

[1] *Rhizopus oryzae* (ATCC 10260) was used; other conditions are specified in Materials and Methods; duplicate reactors were not used.

[2] % is defined as g/100 ml of sample.

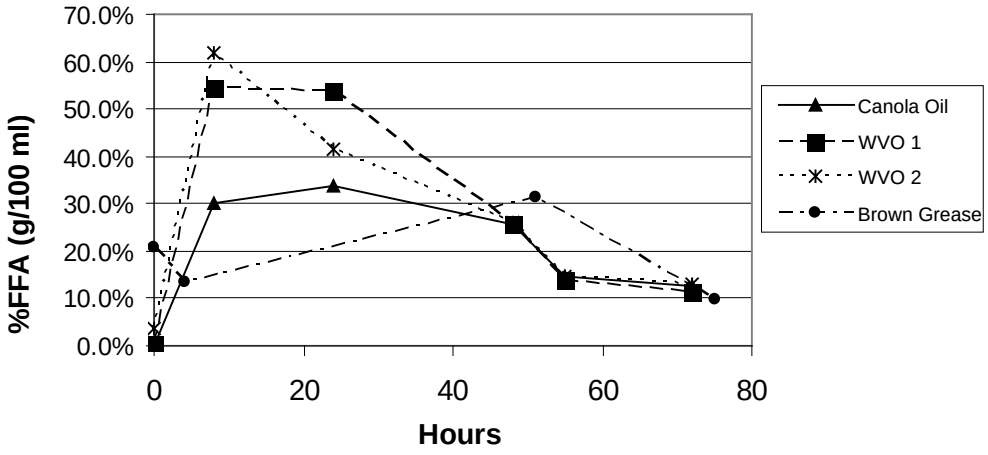
summarized in Table 3. The results indicate that about a 75% FAME conversion for virgin canola oil, about an 80% conversion for each WVO, and about a 55% conversion for brown grease.

FFA content was also monitored over time during the reaction. Results are presented in Figure 3. The pattern of FFA results over time suggests that transesterification may involve two separate reactions – hydrolysis, followed by methanolysis (esterification). In addition, it appears that initial hydrolysis of WVOs may proceed more rapidly than hydrolysis of virgin oil. This may be because a fatty acid chain is more easily removed from di- and monoglyceride than from triglyceride (Darnoko and Cheryan, 2000). Since waste vegetable oils have a higher content of di- and monoglycerides, hydrolysis of these molecules may proceed more rapidly. The brown grease used in the reaction tracked in Figure 3 had an FFA content of approximately 50%. The initial FFA test of approximately 20% is due to the dilution of the brown grease with methyl ester and buffer.

Table 3. Effect of oil type on 72-hour transesterification reaction.

	Reaction Products (mass %) (values in parentheses are results of duplicate reactions)				
Oil Type	**FAME**	**FFA**	**Mono-glycerides**	**Di-glycerides**	**Tri-glycerides**
Canola	73.9 (77.4, 70.4)	16.0 (13.1, 18.9)	0.9 (1.0, 0.8)	4.3 (5.0, 3.6)	4.9 (3.5, 6.3)
WVO 1 (0.7% FFA)	80.9 (81.1,80.7)	12.3 (12.2, 12.4)	1.8 (1.7, 1.8)	3.6 (3.5, 3.6)	1.6 (1.6, 1.5)
WVO 2 (3.7% FFA)	78.7 (77.3, 80.0)	16.1 (17.9, 14.3)	1.3 (1.2, 1.4)	2.6 (2.3, 2.8)	1.5 (1.4, 1.5)
Brown Grease (80% FFA)	57.2 (56.9, 57.4)	42.1 (41.8, 42.4)	0.3 (0.2, 0.3)	0.4 (0.4, 0.3)	0.5 (0.5, 0.5)

Figure 3. FFA content during 72-hr transesterification reaction by types of oil.



III.2. Increasing Transesterification Yield

Results from the extension or alteration of the standard 72-hr transesterification reaction are presented in Table 4, along with the standard 72-hr transesterification reaction results for canola oil from Table 3. Virgin canola oil was used in all of the reactions listed in Table 4.

Following a standard 72-hr transesterification reaction, an extra dose of methanol or a second 72-hr reaction resulted in FAME yield approaching 90%. Elevated temperature, however, resulted

Table 4. Effect of extension or alteration of 72-hour transesterification reaction.

Reaction Conditions[a, b]	**Reaction Products (mass %)** (values in parentheses are results of duplicate reactions)				
	FAME	**FFA**	**Mono-glycerides**	**Di-glycerides**	**Tri-glycerides**
Standard 72-hr (Table 3)	73.9 (77.4, 70.4)	16.0 (13.1, 18.9)	0.9 (1.0, 0.8)	4.3 (5.0, 3.6)	4.9 (3.5, 6.3)
Additional methanol dose (following standard 72-hr)	89.7 (90.1, 89.3)	7.1 (6.5, 7.7)	0.6 (0.7, 0.5)	1.5 (1.6, 1.4)	1.1 (1.1, 1.1)
Second 72-hr reaction (following standard 72-hr)	87.8 (86.5, 89.0)	9.8 (11.8, 7.7)	0.4 (0.4, 0.3)	1.2 (0.6, 1.7)	1.0 (0.7, 1.3)
Standard 72-hr conducted at 35°C	3.5 (3.8, 3.2)	12.4 (9.7, 15.1)	1.0 (1.0, 0.9)	9.5 (10.7, 8.2)	73.7 (74.8, 72.6)

[a] Unless otherwise specified, reaction temperature is 25°C.
[b] Canola oil is used for all reaction conditions in Table 4.

in dramatically lower yield. Most studies on enzymatic transesterification or esterification using pure lipases (powder or immobilized form) observed optimum yields at elevated temperatures in the range of 37°C to 45°C (Nelson et al., 1996; Hsu et al., 2001; He et al., 2002; Hass et al., 2002; Radzi et al., 2005). One possible explanation for our finding is the different temperatures at which the fungi were cultivated. Previous research used lipase from organisms grown at an elevated temperature, and thus may have stimulated production of enzymes that are stable at elevated temperatures. Our fungus was cultivated at room temperature and may have produced a lipase that is not stable at an elevated temperature.

III.3. Separating Hydrolysis and Methanolysis Reactions

During the 72-hr transesterification reactions, we observed an FFA profile that generally demonstrated a rapid increase early in the reaction, and then a slow decrease as the reaction progressed (Figure 3). This pattern is consistent with the theory that transesterification occurs in two reactions. In the first, hydrolysis, lipase facilitates the reaction of oil and water to remove a fatty acid chain, producing FFA. In the second, methanolysis, lipase facilitates the reaction of FFA and methanol to produce FAME. The pattern of FFA illustrated in Figure 3 suggests that hydrolysis proceeds more rapidly than methanolysis.

As discussed previously, there is reason to believe that yields could be improved by separating these two reactions. This was explored in two ways: (1) by conducting a set of three hydrolysis/methanolysis iterations over a period of 216 hours; and (2) by following the standard 72-hr transesterification with hydrolysis/methanolysis.

III.3.1. Three iterations of hydrolysis/methanolysis

GC results indicated that by the third iteration almost all of the combined glycerides had been converted to FAME or FFA. FFA content during each iteration is presented in Appendix IX. Biodiesel yield is presented in Table 5. FAME yield was not significantly better than the 72-hr transesterification, which is shorter duration and more convenient. However, the iterative approach successfully converted about 99% of triglycerides into FAME and FFA.

III.3.2. One hydrolysis/methanolysis following a standard 72hr transesterification

Results of a standard 72-hr transesterification reaction followed by one 24-hr hydrolysis and one 48-hr methanolysis indicated that this approach produces about an 83% yield, and about a 97% conversion of triglycerides into FAME and FFAs (Table 6). An unanticipated result occurred

Table 5. Biodiesel yield of three iterations of hydrolysis and methanolysis.

	Reaction Products (mass %) (values in parentheses are results of duplicate reactions)				
Reaction Conditions	**FAME**	**FFA**	**Mono-glycerides**	**Di-glycerides**	**Tri-glycerides**
3 Iterations of hydrolysis and methanolysis	80.7 (79.5, 81.9)	18.4 (20.1, 16.7)	0.2 (0.1, 0.3)	0.4 (0.2, 0.6)	0.4 (0.2, 0.5)

during the hydrolysis. At the end of the 72-hr transesterification, the products were approximately 70% FAME, 10% glycerides and 20% FFA. During hydrolysis, more residual glycerides were converted into FFA, but some FAME was apparently also converted back to FFA (note decrease of glycerides from 10% to 6%, decrease of FAME from 73% to 59% and increase of FFA from 16.5% to 34.4%). The last methanolysis then converted most of the FFA into FAME (note decrease of FFA from 34% to 14% and increase of FAME from 59% to 83%).

The effects of adding a molecular sieve are presented in Table 7. A molecular sieve has been commonly used to improve the yield of esterification/transesterification reactions since it is effective in adsorbing water and, therefore, reduces the inhibitive effect of water on esterification (Duan et al., 2006; Unal, 1998; Yahya et al., 1998; Liu et al., 1998a; Chen and Wang, 1997). However, we found no significant improvement in FAME yield by using the molecular sieve. A higher dose of methanol and fungus also did not improve the yield as shown in Table 7.

III.4. Ethanol Instead of Methanol

Some studies have observed better yield of transesterification and alcoholysis when ethanol was used as the alcohol as compared to methanol (Abigor et al., 2000; Hsu et al., 2001). The reason

Table 6. Biodiesel yield of 72-hr transesterification followed by one hydrolysis and one methanolysis.

	Reaction Products (mass %) (values in parentheses are results of duplicate reactions)				
Reaction Conditions*	**FAME**	**FFA**	**Mono-glycerides**	**Di-glycerides**	**Tri-glycerides**
72-hr transesterification	73.1 (72.2, 74.0)	16.5 (17.4, 15.6)	1.4 (1.3, 1.4)	5.0 (4.8, 5.1)	4.2 (4.4, 4.0)
72-hr transesterification + 1 hydrolysis	59.3 (59.3, 59.3)	34.4 (34.5, 34.2)	0.6 (0.6, 0.6)	2.6 (2.6, 2.5)	3.2 (3.2, 3.2)
72-hr transesterification + 1 hydrolysis and 1 methanolysis	83.0 (83.2, 82.7)	14.5 (14.3, 14.6)	0.2 (0.2, 0.2)	1.5 (1.4, 1.5)	1.0 (0.9, 1.0)

*Canola oil is used for reaction conditions in Table 6.

Table 7. Effect of molecular sieve, extra dose of methanol/fungus on methanolysis reaction.

	Reaction Products (mass %) (values in parentheses are results of duplicate reactions)				
Reaction Conditions	**FAME**	**FFA**	**Mono-glycerides**	**Di-glycerides**	**Tri-glycerides**
Baseline condition	83.0 (83.2, 82.7)	14.5 (14.3, 14.6)	0.2 (0.2, 0.2)	1.5 (1.4, 1.5)	1.0 (0.9, 1.0)
Molecular sieve	83.8 (84.7, 82.8)	14.4 (13.3, 15.5)	0.5 (0.5, 0.4)	0.9 (0.9, 0.9)	0.5 (0.6, 0.4)
Extra methanol/fungus	76 (75.6, 76.4)	18.9 (18.9, 18.8)	1.2 (1.3, 1.1)	2.4 (2.6, 2.2)	1.6 (1.7, 1.4)

for a better yield may be that lipases are more tolerant of ethanol than methanol (Fukuda et al., 2001), or that lipases act more effectively on longer-chain fatty alcohols (Shimada et al., 1997, 1998). Other potential advantages of using ethanol instead of methanol are that ethanol is less hazardous than methanol and that ethanol can be produced from renewable resources. Though ethanol is more expensive, recent technological innovations and increasing production scale are bringing ethanol production costs down.

We explored the impact of using ethanol in the 72-hr transesterification followed by one hydrolysis and one ethanolysis reaction. Results are summarized in Table 8. Compared to methanol (Table 6), ethanol produced a somewhat lower ester yield at the end of the 72-hr reaction, yet a slightly higher yield after ethanolysis. Ethanol also tended to leave a higher combined glycerin residual. Interestingly, there was an increase in ethyl ester (FAEE) during hydrolysis, whereas there was a decrease in methyl ester (FAME) when methanol was used. This pattern of results suggests an interesting possibility: that, as compared to methanol, ethanol shifts the FAA/ester equilibrium toward ester. This could either be due to faster conversion of FFA into ester, or the production of an ester that is better able to resist hydrolysis. However, it is also possible that the increased dose of ethanol, along with the greater ethanol residual in the hydrolysis reaction, accounts for the observed results. Further research will be needed to explore this and confirm the actual cause.

Ethanol was also used in a final 96-hr ethanolysis reaction on the product of a standard 72-hr transesterification (with methanol) and subsequent hydrolysis reaction. As indicated in Table 9, this produced an almost 90% FAME/FAEE yield, with 99% of triglyceride converted to either FAME/FAEE or FFA. Very little combined glycerin remained.

Table 8. Results of 72-hr transesterification with ethanol followed by one hydrolysis and one ethanolysis.

	Reaction Products (mass %) (values in parentheses are results of duplicate reactions)				
Reaction Conditions*	**FAEE**	**FFA**	**Mono-glycerides**	**Di-glycerides**	**Tri-glycerides**
72-hr transesterification w/ ethanol[a]	66.1 (65.5, 66.7)	13.5 (14.6, 12.4)	10.2 (10.2, 10.1)	7.6 (7.9, 7.3)	2.7 (2.8, 2.5)
72-hr transesterification + 1 hydrolysis[b]	71.7 (69.8, 72.6)	17.9 (18.6, 17.1)	2.9 (3.1, 2.6)	6.1 (6.4, 5.8)	2.2 (2.4, 2.0)
72-hr transesterification + 1 hydrolysis and 1 ethanolysis[c]	85.2 (84.4, 86.0)	7.9 (8.5, 7.3)	4.9 (4.8, 4.9)	1.8 (1.9, 1.6)	0.4 (0.4, 0.3)

*Canola oil is used for all reaction conditions in Table 8.

Table 9. Results of ethanolysis following transesterification and hydrolysis.

	Reaction Products (mass %) (values in parentheses are results of duplicate reactions)				
Reaction Conditions	**FAME/FAEE**	**FFA**	**Mono-glycerides**	**Di-glycerides**	**Tri-glycerides**
72-hr transesterification w/ MeOH+ 1 hydrolysis + 1 ethanolysis	89.5 (89.4, 89.5)	9.5 (9.4, 9.5)	0.3 (0.3, 0.3)	0.5 (0.4, 0.5)	0.3 (0.4, 0.2)

IV. Conclusions

Whole-cell biocatalysts, grown and reacted at room temperature and without immobilization, can achieve biodiesel yields of about 90%, and can convert almost 100% of triglycerides into either biodiesel or FFA. However, this required a reaction time of at least 96 hours.

Conclusions with respect to the specific objectives of this project are:

1. Whole-cell biocatalyst derived from *Rhizopus oryzae* (ATCC10260) was able to produce biodiesel with a yield of about 75% for virgin oil, 80% for waste vegetable oil, and 55% for brown (trap) grease with a 72-hr transesterification reaction using methanol in a solvent-free, water-containing system at room temperature.
2. An additional dose of methanol along with another 24 hours of reaction time at room temperature increased the yield for virgin canola oil to about 90%. A second 72-hr transesterification reaction did not perform better than this. Elevating the reaction temperature to 35^{o}C significantly diminished the yield.
3. The separation of hydrolysis and methanolysis reactions did not improve yield. Also, the use of a molecular sieve to reduce water concentration during methanolysis did not improve the yield. Similarly, increasing the dose of biocatalyst and methanol during methanolysis did not improve the yield.
4. The use of ethanol instead of methanol did not improve the yield. However, results suggest that ethanol may produce a more stable ester in the presence of the *Rhyzopus oryzae* lipase.

Overall, this work demonstrated that a whole-cell fungal biocatalyst, grown and reacted at room temperature, can convert almost 100% of combined glycerides into biodiesel and FFA. This suggests several possible implications:

1. Whole-cell biocatalysts may be an effective way to transesterify waste oils or greases that are high in FFA and difficult to dewater. Brown (trap) grease and similar degraded or complex greases are good candidates for further whole-cell biocatalyst research.
2. Three factors have the potential to significantly decrease reaction time and increase yield for whole-cell biocatalytic production of biodiesel: a) reactor design; b) type of micro-organism; and c) conditions of cultivation/immobilization of the micro-organism.
3. The ability to grow the whole-cell biocatalyst on inexpensive media – potentially industrial or residential waste – could dramatically lower the cost of biocatalyst production.
   (Note: An economic analysis for this process was not conducted. Optimum reactor design, growth media, and types of organisms have not been established and these factors strongly influence the economics of the process.)

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Appendix I

Lipase Activity of the Whole-Cell Biocatalysts by Culturing Conditions

Lipase activity of *Rhizopus oryzae* (ATCC 10260) was first examined under various culture conditions including number of pre-incubation hours, number of incubation hours, and shaker rpm. Results are summarized in Table I-1.

As indicated in Table I-1, pre-incubation time of 72 hours, incubation time of 72 hours, and shaker RPM at 250 for incubation appears to provide highest lipase activity. As a result, these conditions were utilized for growing cultures used in subsequent reactions.

Table I-1. Lipase Activity of *Rhizopus oryzae* (ATCC 10260)[1].

Culturing Conditions			**Lipase Hydrolysis Activity (μmole FFA released/mg cell)**
Pre-incubation (hrs)	**Incubation (hrs)**	**Shaker RPM for incubation**	
24	24	250	3.60
24	48	250	4.08
24	72	250	5.76
48	48	250	5.10
48	72	250	6.48
48	96	250	5.34
72	48	250	10.80
72	72	250	14.82
72	96	250	7.62
72	72	150	5.64
72	96	150	5.52
72	72	350[2]	4.50

[1] Pellet-like growth was observed in all samples.
[2] Significantly lower cell mass yield was observed.

Appendix II

Fungi Morphology

We observed three types of fungi growth morphology in our culturing conditions: suspended growth, pellet growth and cotton-like growth. Both pellet growth and cotton-like growth provided similar lipase activity per unit dried cell mass (approximately 5 – 15 μmole FFA released/mg cell). Pellet growth and cotton growth were tested in standard 72-hr esterification reactions with approximately 70-80% yield and were the primary whole-cell biocatalysts used in this study.

Suspended growth provided the greatest challenge in terms of separating cell mass from culture broth. The broth together with fungi cells was tested in the 72-hr esterification reaction and the yield was very poor.

Fungi morphology is a function of the size and maturity of the inoculum, agitation, medium composition, temperature and pH, etc. (Zhou et al., 2000; Znidarsic and Pavko, 2001; Wang et al., 2005). Although a number of factors play a role, it appears from our observations that size and maturity of the inoculum are the most critical. However, due to the filamentous nature of the fungi used, variation in the amount of inoculum and maturity is common and therefore result in morphology differences within the same batch.

Unless otherwise specifically stated, whole-cell biocatalysts used in our studies were derived from pellet growth or cotton-like growth.

Appendix III

Lipase Activity of the Whole-Cell Biocatalysts by Types of Fungi

In addition to *Rhizopus oryzae* (ATCC 10260), *Rhizopus oryzae* (ATCC 96382) and *Rhizopus oryzae* (ATCC 34612) were considered for this research. All three were examined for lipase activity. Results are presented in Table III-1.

As indicated in Table III-1, the other two fungi species did not appear to have superior lipase activity when compared to *Rhizopus oryzae* (ATCC 10260), taking into account both incubation times.

Table III-1. Lipase Activity of *Rhizopus oryzae* (ATCC 96382) and *Rhizopus oryzae* (ATCC 34612) as compared to *Rhizopus oryzae* (ATCC 10260).

Species/Culturing Conditions			**Lipase Hydrolysis Activity (μmole FFA released/mg cell)**
Species	**Pre-incubation (hrs)**	**Incubation (hrs)**	
Rhizopus oryzae (ATCC 10260)	48	48	5.10
	48	72	6.48
Rhizopus oryzae (ATCC 34612)	48	48	3.54
	48	72	7.98
Rhizopus oryzae (ATCC 96382)	72	72	3.42

Appendix IV

Effect of Types of Fungi and Lipase Activity on Transesterification Yield

The effect of different types of fungi in catalyzing transesterification was examined and results are summarized in Table IV-1. As indicated in the table, the other two fungi species did not appear to have superior transesterification activity when compared to *Rhizopus oryzae* (ATCC 10260), especially for FAME reaction product. Therefore, *Rhizopus oryzae* (ATCC 10260) was used for our studies, unless otherwise specified.

We also examined whether and how lipase activity of different batches of *Rhizopus oryzae* (ATCC 10260) affected the 72-hr transesterification reaction. Results are presented in Table IV-2 and Figure IV-1.

Lipase activity of the fungi did not appear to be associated with transesterification conversion. This could be due to the fact that lipase activity is defined and measured by hydrolysis reactions as opposed to transesterification reactions. Another explanation could be that once lipase activity exceeds a certain level, greater lipase activity does not improve transesterification. The level at which we dosed our reaction may have exceeded this level. This is consistent with the observation that much lower cell mass resulted in similar transesterification conversions (discussed later in Appendix VI).

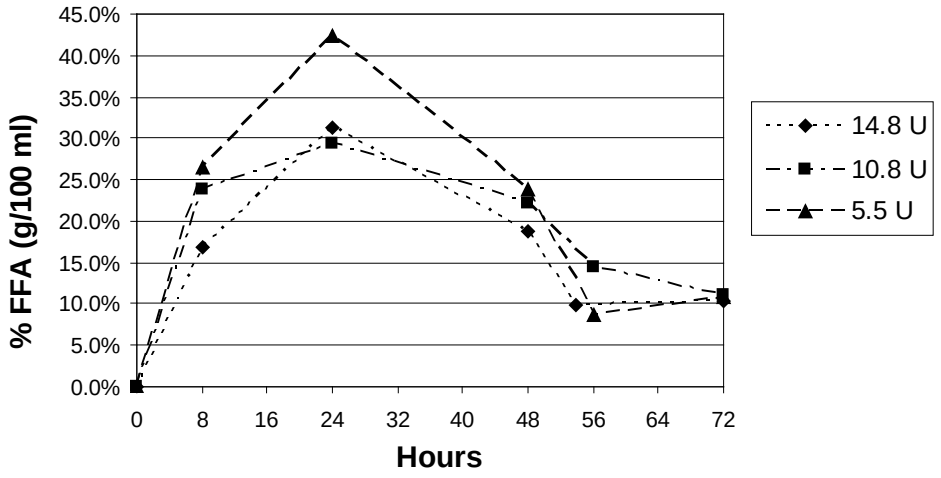
Table IV-1. Effects of types of fungi in 72-hr transesterification of canola oil.

Reaction Conditions		**Reaction Products** (values in parentheses are results of duplicate reactions)	
Types of Fungi	**Lipase Hydrolysis Activity (μmole FFA released/mg cell)**	**FAME (mass %)**	**FFA (mass %)**
Rhizopus oryzae (ATCC 10260)	5.68	75.7 (72.4, 79.0)	15.6 (14.1, 17.1)
Rhizopus oryzae (ATCC 34612)	7.98	55.0 (51.2, 58.8)	28.4 (26.7, 30.1)
Rhizopus oryzae (ATCC 96382)	3.42	52.4 (54.7, 50.1)	16.1 (17.1, 15.1)

Table IV-2. Effect of lipase activity in 72-hr transesterification of canola oil (using three different batches of ATCC 10260).

Batches of ATCC (10260)	**Lipase Activity (μmole FFA released/mg cell)**	**Results** (values in parentheses are results of duplicate reactions)	
		FAME (mass %)	**FFA (mass %)**
1	14.8	66.6 (68.5, 64.7)	12.5 (12.1, 12.8)
2	10.8	68.3 (70.6, 66.0)	11.0 (11.4, 10.5)
3	5.7	75.7 (72.4, 79.0)	15.6 (14.1, 17.1)

Figure IV-1. FFA content during 72-hr transesterification by lipase activity of fungi. (1 U = 1umole FFA released /mg cell).

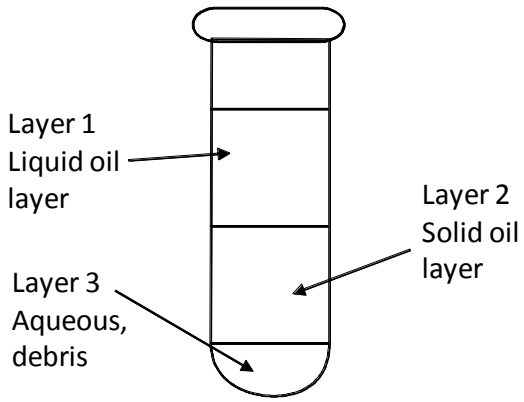


Appendix V

Estimating Biodiesel Yield from Brown Grease

1. Reaction Condition

2. Twenty-four g of brown grease (including debris) were mixed with 20 ml soy biodiesel (B100) as feedstock for transesterification reaction at room temperature (25^{o}C). After the reaction was complete, the reaction mixture was centrifuged and separated into three layers. Layer 1 and Layer 2 are oil phases containing methlesters (from B100 and converted from brown grease) and FFA (from brown grease). Layer 1 is clear liquid and Layer 2 is solid at room temperature. Layer 3 is an aqueous phase that includes buffer solution, fungus and debris from the brown grease. While it was difficult to weigh each layer accurately, we estimated that the oil layers each weighed approximately 16 g. We ran duplicate reactors, but for simplicity we used one reactor's data to show the procedures we used to estimate yield.



2. Estimation of weight of grease available for biodiesel conversion in the reaction

- We measured the weight of empty centrifuge tube (Wt_a); it was 13.55 g.
- We measured the weight of centrifuge tube with only Layer 3 (Wt_b); it was 23.22 g.
- Weight of buffer solution and fungus added is known; they were 3.4 g and 1.0 g, respectively.
- Weight of debris = Wt_b - Wt_a -Wt_{buffer} - Wt_{fungus} = 23.22-13.55-3.4-1.0 = 5.27 g.
- Weight of brown grease added = 24 g - Wt_{debris} = 24-5.27 = 18.73 g.
- Brown grease contains 20.2% water (based on GC analysis), so actual grease available for conversion is 18.73 g x 80.2% = 15 g.

3. Weight of soy biodiesel added in the reaction

We measured the weight of 20 ml of soy biodiesel and it turned out to be 17 g.

4. GC Analysis Results

We used GC to analyze the composition (mass%) of Layer 1 and Layer 2. Results are presented in Table V-1.

Table V-1. Composition (mass%) of Layer I and Layer II.

	FAME	FFA	Mono-glycerides	Di-glycerides	Tri-glycerides
Layer 1	82.4	17.4	0.05	0.03	0.04
Layer 2	77.2	21.8	0.2	0.4	0.5

5. Estimation of brown grease to biodiesel yield and unreacted FFA in brown grease

a. Assuming X mass% of brown grease is converted to biodiesel, then

FAME from Brown grease + FAME from B100 = FAME in Layer 1 + FAME in Layer 2

15 g x X% + 17 g = 16 g x 77.2% + 16 g x 82.4% ; X = 56.9%

b. Assuming Y mass% of unreacted FFA in brown grease, and since soy biodiesel contains no FFA, then

Unreacted FFA from Brown grease = FFA in Layer 1 + FFA in Layer 2

15 g x Y% = 16 g x 21.8% +16 g x 17.4%; Y = 41.8%

Appendix VI

Effect of Cell Mass on Transesterification Yield

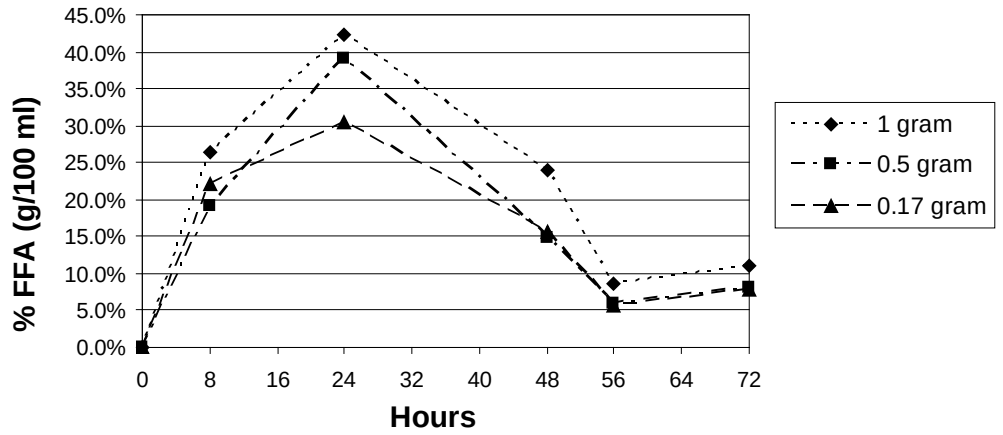
To explore the effect of different amounts of whole-cell biocatalyst on transesterification yield, we used 1.0 g, 0.5 g and 0.17 g of biocatalysts in 72-hr transesterifications using canola oil. Results are presented in Table VI-1 and Figure VI-1.

As shown in Table VI-1 and Figure VI-1, cell mass of 0.5 g provided comparable yield to cell mass of 1.0 g, particularly for the FAME reaction. Therefore, we used 0.5 g cell mass in the rest of the studies unless otherwise specified.

Table VI-1. Effect of cell mass in 72-hr transesterification of canola oil.

Reaction Conditions		**Reaction Products** (values in parentheses are results of duplicate reactions)	
Cell Mass (gram)	**Lipase Activity (μmole FFA released/mg cell)**	**FAME (mass %)**	**FFA (mass%)**
1.0	5.5	77.3 (78.8, 75.8)	13.3 (12.9, 13.6)
0.5	5.5	79.3	9.0
0.17	5.5	72.9	9.3

Figure VI-1. FFA content during 72-hr transesterification by cell mass.



Appendix VII

Methanol Test - Screening Test for Combined Glycerin

The methanol test is commonly used in “home brew” biodiesel production to test for residual combined glycerin – an indicator of the completeness of reaction. One ml of oil phase reactant is mixed with 9 ml of methanol in a 15 ml graduated (up to 0.1 ml resolution) centrifuge tube. Phase separation can be easily read and can be used as an estimation of the percent of triglycerides remaining. This test is based on the recognition that triglyceride and diglyceride are not soluble in methanol, while FAME and monoglyceride are soluble (Zhou et al., 2006). Reportedly, diglyceride will dissolve in warm methanol, while triglyceride will not (Morrison and Smith, 1964).

We tested this approach using virgin canola oil and commercial B100 and confirmed that it was a good quantitative screening tool. Canola oil solubility was not significantly increased when the methanol was heated. Thus, the volume of the oil layer in the methanol test was taken as an approximation of the tri- and diglyceride remaining in the reaction products. The extent to which the volume decreased with heating was taken as an indication of the amount of diglyceride present. Since the difference between test results obtained under the same operating conditions on an identical sample was less than 0.03 ml (i.e., as accurate as the method allows), a single test was considered adequate.

However, we have recently found that the methanol test is confounded by the presence of high FFA. While we do not yet understand the mechanism, high FFA levels tend to make tri- and diglyceride more soluble in methanol, producing a false negative test result. Therefore, this method will underestimate tri- and diglyceride presence unless only small amounts of FFA are present.

Appendix VIII

Impact of Buffer Solution on FFA Titration

To test whether 0.1 M phosphate buffer solution used in the reaction mixture interferes with FFA determination by titration method, we designed the following experiment:

1. Mix 16 ml of canola oil containing 47.6% FFA with 15 % of DI water in reactor A and continue to mix on the stir plate for 24 hours.
2. Mix 16 ml of canola oil containing 47.6% FFA with 15% of 0.1 M phosphate buffer in reactor B and continue to mix on the stir plate for 24 hours.
3. Take 0.5 ml sample from each reactor and compare FFA by titration.
4. Centrifuge the reaction mixture from each reactor and compare FFA by titration.

Results are summarized in the Table VIII-1. Based on the data, we concluded that there was minimal interference from the buffer solution on FFA determination by titration.

Table VIII-1. Impact of Buffer Solution on FFA Determination by Titration.

Type of solution	**FFA content (%) prior to centrifugation**	**FFA content (%) after centrifugation**
DI water	54.8	67.0
Buffer	54.0	67.3

Appendix IX

Hydrolysis and Methanolysis

In this appendix, we present the following information:

1. FFA content during initial hydrolysis and methanolysis (Figure IX-1 and Figure IX-2, respectively). This is intended to show that: (1) hydrolysis for 24 hours has reached equilibrium and (2) methanolysis for 48 hours has reached equilibrium.
2. FFA content after each iterative hydrolysis/methanolysis reaction (Table IX-1).
3. Change of oil volume during iterative hydrolysis/methanolysis reactions (Table IX-2).

1. FFA content during initial hydrolysis and methanolysis

Figure IX-1. FFA contents during initial hydrolysis.

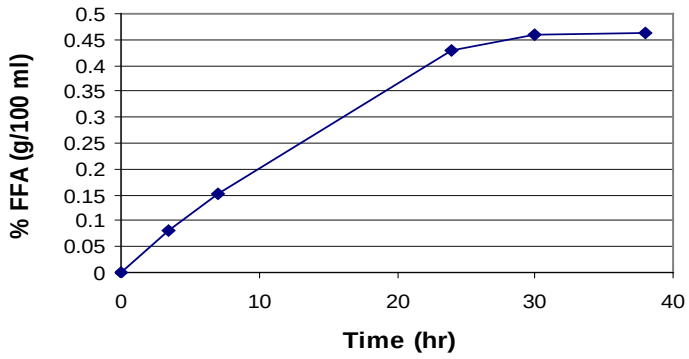
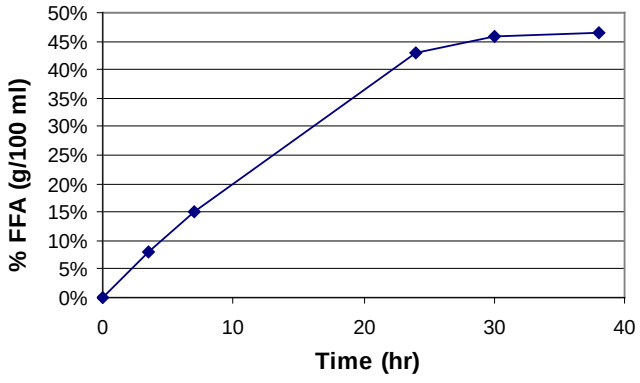


Figure IX-2. FFA contents during initial methanolysis.



2. FFA content after each iterative hydrolysis/methanolysis reaction (virgin oil)

Table IX – 1. FFA content at end of each iterative hydrolysis/methanolysis reaction.

	1st Hydrol.	1st Methanol.	2nd Hydrol.	2nd Methanol.	3rd Hydrol.	3rd Methanol.
% FFA content (g/100 ml)	46% (45%, 48%)	12% (12%, 11%)	35% (32%, 38%)	17% (18%, 16%)	30% (27%, 32%)	16% (14%, 18%)

Note: values in parentheses are results of duplicate reactions

3. Change of oil volume during iterative hydrolysis/methanolysis reactions

It should be noted that the following conditions were applied to data presented in the table below:

a. Separations of oil layer between reactions were accomplished by centrifugation at 3000 rpm for 45 minutes.
b. Due to the emphasis on quality of reaction mixture as opposed to quantity, about 1-2 ml of oil was not collected at the oil/water interface after each centrifugation (1 ml was assumed in the table below).
c. 4 x 0.5 ml samples were collected for analysis during the 1st hydrolysis.
d. 5 x 0.5 ml samples were collected for analysis during the 1st methanolysis.
e. 2 x 0.5 ml samples were collected for analysis during the 2nd hydrolysis.
f. 3 x 0.5 ml samples were collected for analysis during the 2nd methanolysis.
g. 2 x 0.5 ml samples were collected for analysis during the 3rd hydrolysis.
h. 2 x 0.5 ml samples were collected for analysis during the 3rd methanolysis.

Table IX – 2. Oil volumes at the end of each iterative hydrolysis/methanolysis reaction.

	1st Hydrol.	1st Methanol.	2nd Hydrol.	2nd Methanol.	3rd Hydrol.	3rd Methanol.
Starting Volume (ml)	30 (30, 30)	22 (22, 22)	17.5 (17.5, 17.5)	13.6 (13.5, 13.6)	9.6 (10.2, 9.0)	7.4 (7.6, 7.1)
Volume sampled or left (ml)	~3	~3.5	~1	~2.5	~1	~2
Ending Volume (ml)	22 (22, 22)	17.5 (17.5, 17.5)	13.6 (13.5, 13.6)	9.6 (10.2, 9.0)	7.4 (7.6, 7.1)	5.1 (5.3, 4.9)
Volume not accounted for (ml)	**~5**	**~1**	**~3**	**~1.5**	**~1.2**	**~.3**