

Adding value to renewable biodiesel production from the oleaginous yeast *Lipomyces starkeyi*

by
Jack R. Brosy

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Honors Baccalaureate of Science in Biochemistry and Biophysics (Honors Scholar)
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Abstract approved:

Alan T. Bakalinsky

Global warming and on-going depletion of fossil fuels have stimulated research to develop renewable sources of energy. Current renewable energy use comprises only 10% of the energy consumed in the US and is focused mostly on generating electricity. The majority of the energy consumed by the US transportation sector is in the form of petroleum-based fuel. One potential renewable source is biodiesel, produced mainly from plant oil and animal fat. Production of biodiesel from plant oil competes with land use for food and feed crops. One alternative source of biodiesel that does not compete for agricultural land directly is oleaginous yeast, defined as yeast capable of producing >20% lipid as dry weight. This study tested a simplified approach for culturing the oleaginous yeast *Lipomyces starkeyi* 78-23 for lipid production, and tested a by-product of the lipid extraction process for value as a substitute for commercial yeast extract. The lipid yield and composition were found to be comparable between cells grown on glucose or xylose. The aqueous phase recovered from the lipid extraction was tested as an alternative to commercial yeast extract in media used to culture *S. cerevisiae* S288c and *L. starkeyi* 78-23. On a weight basis, the extract was found to have about 4 times more nutritional value than commercial yeast extract for *L. starkeyi* and 3 time more nutritional value for *S. cerevisiae*.

Key words: oleaginous yeast, *L. starkeyi* 78-23, renewable energy, biodiesel, yeast extract

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Honors Baccalaureate of Science in Biochemistry and Biophysics and Honors Baccalaureate of Science in Psychology project of Jack R. Brosy presented on May 29th, 2015.

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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Introduction

Global warming and diminishing oil supplies caused by the consumption of fossil fuels and subsequent release of greenhouse gases are motivating efforts to develop alternative renewable sources of energy (Schubert, 2006). Renewable energy comes from a source that is either replenished or not depleted over a time scale comparable to its usage. In spite of efforts to develop renewable energy, usable sources constitute only ~10% of the total energy used in the United States. Furthermore, while many sources of renewable energy such as solar, wind, hydro-, and geothermal energy can readily generate electricity, the transportation sector requires renewable liquid fuels. One source of renewable petroleum is biodiesel, currently produced mainly from vegetable oil and animal fats, but also potentially available from microbial lipids (Li et al., 2008; Meng et al., 2009).

According to the Intergovernmental Panel on Climate Change (IPCC) Fifth Assessment Report, greenhouse gases from anthropogenic sources are currently being emitted at the highest rate in human history, mostly caused by the use of fossil fuels (IPCC, 2013). These emissions have a clear and direct influence on human and natural systems globally; concentrations and emissions of atmospheric carbon dioxide, methane, and nitrous oxide between 2000 and 2010 have been the highest in recorded history and a majority of the emissions have been driven by economic and population growth. Nearly 78% of total CO₂ emissions can be attributed to the consumption of fossil fuels between 1970 and 2010. These greenhouse gas emissions have also caused a warming of both the atmospheric and oceanic environments causing drastic melting of snow and ice in the Greenland and Antarctic ice sheets which have raised sea levels and altered global ecosystems. Warming of the arctic permafrost has also created a feedback loop

because of the characteristically high methane content within the ice. The methane is presumably of microbial origin and is stored as a hydrate trapped in clathrates, a structure of water molecules surrounding the methane molecules. Methane is 29 times more potent than carbon dioxide as a greenhouse gas which triggers more melting of the permafrost and more release of methane (Zimov et al., 2006).

In 2005, U.S. Congress enacted the Energy Policy Act which mandated that 7.5 billion gallons of renewable fuel were to be blended into gasoline by 2012. The Renewable Fuel Standard (RFS) program was created to accomplish this goal. In 2007, the RFS was expanded by the Energy Independence and Security Act. Renewable fuel blends were to include diesel in addition to gasoline, the target volume of renewable fuel was increased to 9 billion gallons by 2008 and to 36 billion gallons by 2022, and a standard was set that mandated that the renewable fuel that was replacing petroleum needed to have lower greenhouse gas emissions than petroleum (EPA, 2005; EISA, 2007). These two acts lay the groundwork for the federal requirement to develop renewable fuel to supplement and eventually replace petroleum-based fuels.

In the United States, 82% of the energy consumed is derived from non-renewable fossil fuels (petroleum, natural gas, and coal), 8% is from nuclear electric power, and 10% is renewable energy (Figure 1). Biomass energy, a renewable fuel derived from organic matter, comprises half of the renewable energy. This can be separated into first- and second-generation biomass. First-generation biomass is unprocessed organic material that is primarily burned for cooking and heating. Second-generation biomass is processed into biofuels such as ethanol and biodiesel (Nigam and Singh, 2011).

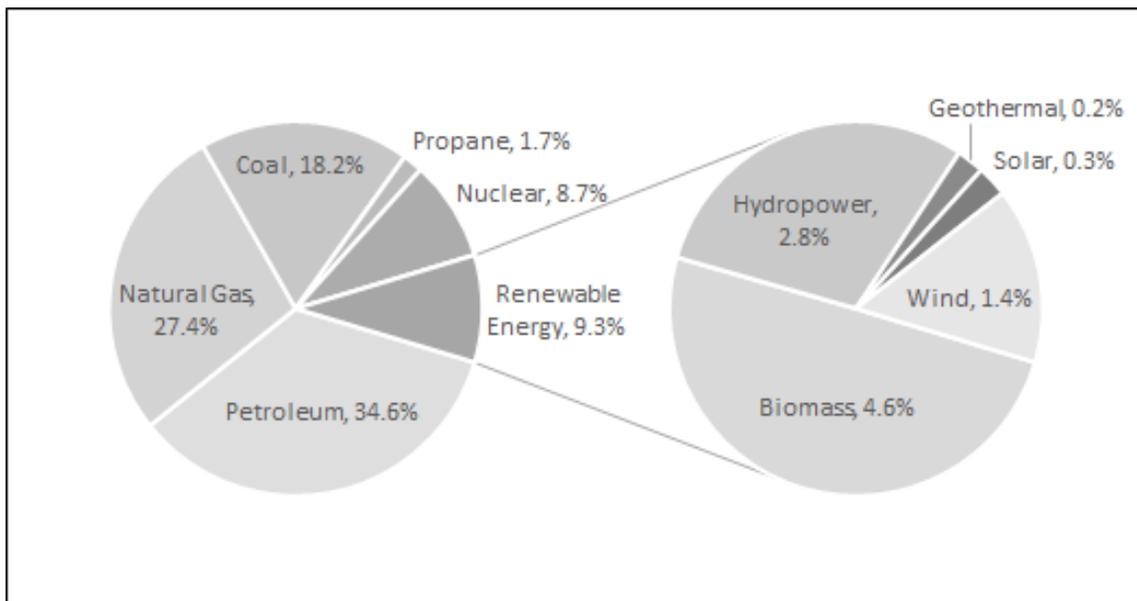


Figure 1. U.S. Energy Consumption by Source (Adapted from U.S. EIA, Annual Energy Review 2011).

The transportation sector uses 28% of the total energy consumed in the U.S., 95% of which is petroleum-based (U.S. Energy Information Administration, 2011; Yan et al. 2009). Figure 2 shows total U.S. energy consumption by sector. The transportation sector uses a disproportionate amount of petroleum-based fuels relative to other sectors and requires a renewable energy source that can replace petroleum. Biodiesel, which has the potential to replace petroleum-based fuels for the transportation sector, is a renewable fuel made by the transesterification of oils and fats (Nigam and Singh, 2011). However, since most biodiesel is made from plant oils, there is direct competition for land needed to grow food and feed crops which inflates the costs of producing biodiesel and places competition on land-use (Gallagher, 2008).

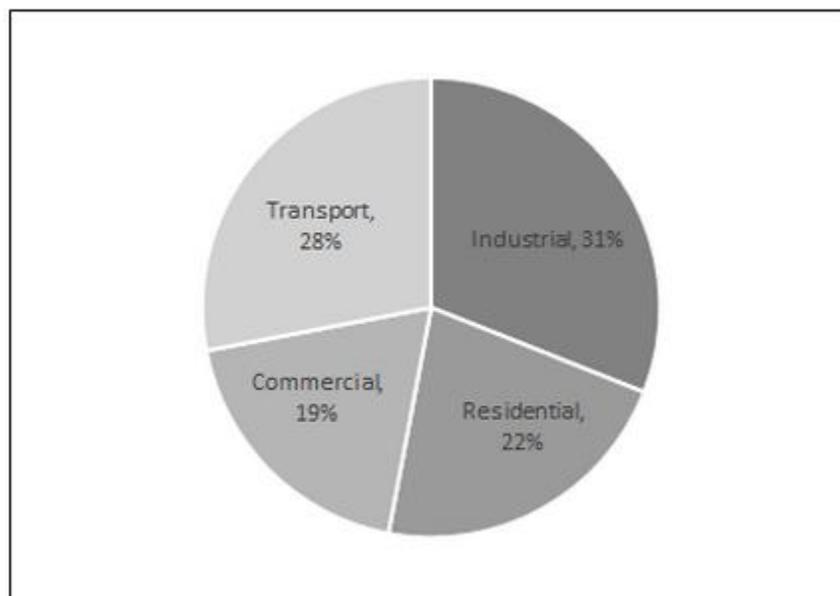


Figure 2. U.S. Energy Consumption by Sector (Adapted from U.S. EIA, Annual Energy Review 2011). Top sources of energy include petroleum, natural gas and biomass.

This competition with agriculture has led to a search for an alternative source of biodiesel that does not compete for agricultural lands. One potential source of biodiesel is oleaginous yeast, defined as a yeast able to produce >20% of its dry weight as lipid (Ratledge and Wynn, 2002; Pan et al., 2009). Oleaginous yeasts have garnered interest for use in producing biodiesel because yeast lipids are similar to plant oils and the cultivation methods are relatively simple and amenable to scale-up compared to the cultivation of oil crops (Ageitos et al. 2011; Papanikolaou and Aggelis, 2011).

However, producing biodiesel is still more expensive than conventional diesel made from fossil fuels and thus, research has been focused on developing methods to produce lipid from cheap feedstocks such as lignocellulosic biomass (Chen et al., 2009; Yu et al., 2011).

Lignocellulose is the main component of plant cell walls and has three main components: lignin,

cellulose, and hemicellulose (Rubin, 2008). Cellulose consists of linear chains of glucose and is crystalline in structure (Saha, 2004). In contrast, hemicellulose is a polymer containing both pentoses and hexoses (Saha, 2004). Lignin is a polymer that holds together cellulose and hemicellulose by a three-dimensional cross-linking of phenylpropanoid polymers (Rubin, 2008). In order to make cellulose less refractory to enzymatic degradation, the lignocellulose needs to be pretreated, commonly by steam explosion, high-pressure liquid ammonia, lime, and/or dilute sulphuric acid treatments (Saha, 2004). Pentose and hexose monomers recovered following subsequent enzymatic hydrolysis can then be used as a feedstock to produce cellulosic ethanol or biodiesel via fermentation.

Lignocellulosic hydrolysates are an inexpensive substrate for the production of biodiesel. Zhao et al. (2008) optimized a medium for lipid accumulation by *Lipomyces starkeyi* and found that the yeast grown on the optimized medium produced greater than 60% lipid per dry weight biomass. An important condition for lipid accumulation in oleaginous yeasts is a medium with a high carbon-to-nitrogen ratio (Angerbauer et al., 2008; Holdsworth and Ratledge, 2008). Lin et al. (2011) took advantage of this property and optimized lipid production in *L. starkeyi* by separating the cell growth phase of the fermentation from the lipid accumulation phase. Cells were first grown on YEPD (yeast extract, peptone and dextrose) medium for 36 hours and were then transferred to a glucose solution for 36 hours which resulted in a yield of 65% lipid on a dry weight basis.

The focus of this thesis was to extend experiments described by Lin et al. (2011) to simplify the process of obtaining lipid from the oleaginous yeast *L. starkeyi*. Specifically, we compared xylose to glucose as a carbon source for growing a different strain of *L. starkeyi* to

produce lipid, and tested the aqueous phase of the lipid extract as a substitute for commercial yeast extract. Lipid yield and composition from cultures of *L. starkeyi* grown on glucose or xylose were found to be comparable. When used as a substitute for commercial yeast extract in YEPD medium, the recovered aqueous material from the lipid extraction was found to have 3.8-fold greater nutritional value for *L. starkeyi* 78-23 and 3.2-fold greater nutritional value for *S. cerevisiae* S288c.

Materials and Methods

Strains and Media

Lipomyces starkeyi 78-23 (Phaff Yeast Culture Collection, UC Davis) and *Saccharomyces cerevisiae* S288c (ATCC 204508 from Manassas, VA) were used throughout this study. Strains were stored at -70°C in YEPD liquid media supplemented with 20% glycerol. Strains were grown on YEPD plates or in liquid culture. The YEPD medium contained (per liter) glucose (20g), peptone (20g), and yeast extract (10g). The YEPX medium contained (per liter) xylose (30g), peptone (20g), and yeast extract (10g). The PD medium contained (per liter) glucose (20g) and peptone (20g). All media were adjusted to pH 6 using HCl or NaOH and sterilized by autoclaving at 121°C for 15 minutes.

Culture Conditions

Lipid production was separated into two stages: a growth phase and a lipid accumulation phase. The growth phase involved growing *L. starkeyi* in a nutrient-rich broth, followed by a lipid accumulation phase during which harvested cells were suspended in a sugar solution to stimulate production of lipid. The method was adapted from Lin et al. (2011).

Briefly, cultures were started from 1mL seed cultures of YEPD and YEPX inoculated from an isolated colony on a YEPD plate. Seed cultures were incubated for 24 hours on a rotary shaker (200rpm) at room temperature and then were added to 100mL of YEPD and YPX, respectively, in 1000mL Erlenmeyer flasks capped with aluminum foil. Cultures were incubated for 48 hours on a rotary shaker (200rpm) at room temperature after which cells were harvested by centrifugation (10 minutes at 850x g). Cells were washed once and resuspended in a final

volume of 800mL of YEPD or YEPX. These cultures were incubated in 2800mL Fernbach flasks for 3 days on a rotary shaker (200rpm) at room temperature and capped with aluminum foil. The foil was widened around the neck of these flasks and secured with tape to preserve sterility while increasing aeration. Cells were harvested by centrifugation for 20 minutes at 850x g. Cells were washed once with sterile dH₂O, transferred to 800mL solutions containing 4% glucose or 4% xylose, respectively, in 2800mL Fernbach flasks, and incubated in the sugar solutions for 3 days at room temperature at 200rpm on a rotary shaker. Cultures were transferred to pre-weighed 50mL Falcon tubes in 50mL fractions and centrifuged for 10 minutes at 850x g. The supernatant was discarded and the wet cell pellet weight was determined gravimetrically. Cell pellets were freeze-dried for 2 days, weighed, and stored at room temperature in a desiccator filled with CaSO₄.

Lipid Extraction

The modified Folch's method for lipid extraction was used which combines a 2:1 chloroform:methanol solvent extraction with glass bead-beating followed by a final addition of 0.9% NaCl to facilitate phase separation (Sitepu et al., 2012). Solvent addition was done in two steps such that the bead-beating was done in the presence of just one quarter of the total amount of solvent used. Post-beating, the remaining three quarters of solvent were added together with 0.2 volumes of 0.9% NaCl (relative to the total volume of solvent).

Briefly, nine aliquots of the freeze-dried cell pellets from the single 800mL yeast cultures grown on glucose and xylose were added to pre-weighed glass vials. Aliquots weighed between 80-100mg. The volume of solvent to be added to each aliquot was then calculated at a rate of

20mL per gram of dried cells. Acid-washed 0.45mm glass beads (0.2 volumes relative to the total volume of solvent) were added to each aliquot followed by a quarter of the solvent volume. After addition of the first quarter of the chloroform:methanol solvent, aliquots were vortexed for a total of 10 minutes in 30 second increments followed by 30 seconds increments on ice. After vortexing, the remaining three quarters of the solvent and 0.2 volumes of 0.9% NaCl (relative to the total volume of solvent) were added to each aliquot then briefly vortexed. Aliquots were centrifuged for 5 minutes to separate the phases. The lower chloroform phase was transferred to another pre-weighed glass vial and placed under an N₂ stream to evaporate the chloroform. Lipid mass and yield were determined gravimetrically.

Gas Chromatographic Analysis

Gas chromatographic analysis of the lipid extracted from the freeze-dried yeast cells allows for a quantitative analysis of the fatty acid composition of the triacylglycerols. Three 100mg aliquots of lipids obtained from the single 800mL glucose-grown and 800mL xylose-grown *L. starkeyi* 78-23 cultures were analyzed. Prior to analysis, the lipid samples were washed with 0.9% NaCl to remove phospholipids and dried under N₂ gas. The lipids were subjected to transesterification to make fatty acid methyl esters (FAMES) (using methanolic sulfuric acid) which were separated by GC, and identified by mass spectrometry, essentially as described (Schlechtriem et al., 2008). Three GC runs were performed per sample for a total of 9 analyses. The following reference standards were included in the analyses (12.5% each of C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, and C20:0 methyl esters, Nu-chek prep #GLC-65).

Recovery and Analysis of the Aqueous Layer of the Lipid Extract

Typically, the aqueous fraction of the lipid extraction is discarded. This aqueous soluble material may contain nutrients of a composition similar to commercial yeast extract and therefore may be suitable as a replacement for commercial yeast extract in media used to grow yeast such as YEPD. This possibility was tested directly. The aqueous phase was recovered from the lipid extraction, dried under nitrogen gas, dissolved in a known amount of distilled water, sterile-filtered through a 0.45 μM membrane, and stored at 4°C. The potency of this aqueous extract was tested by comparing it to commercial yeast extract in a growth assay. The procedure resembled a bioassay where *S. cerevisiae* S288c or *L. starkeyi* 78-23 was grown in YEPD initially, washed and resuspended in distilled water, and was then grown in PD medium (lacking yeast extract) to starve cells of yeast extract-specific components. The starved cells were then used to inoculate PD containing commercial yeast extract at a range of concentrations to determine the relationship between added commercial yeast extract and cell yield, measured as A_{600} values. Once a linear relationship was determined by plotting A_{600} values vs added yeast extract, the experimental aqueous extract was tested at concentrations that spanned the linear range.

The following experiment was conducted to determine whether the aqueous extract derived from the lipid extraction of *L. starkeyi* could serve as a substitute for commercial yeast extract. The experimental yeast extracts derived from the aqueous phases from lipid extractions of four aliquots of dried cells obtained from a single 800mL YEPD culture, were pooled, dried, weighed, and used as a substitute for commercial yeast extract in the formulation of PD +

experimental aqueous extract. This experimental "YEPD" was then tested as a growth medium for both *S. cerevisiae* S288c and *L. starkeyi* 78-23. Initially, a standard curve was generated that related growth (A_{600}) of *S. cerevisiae* S288c and *L. starkeyi* 78-23 to the amount of commercial yeast extract in the growth medium as described above. Cells were then grown on PD + two different concentrations of added aqueous extract in parallel to determine the growth yield. Growth yields were quantified in terms of yields observed as a function of commercial yeast extract.

Briefly, four aliquots of freeze-dried cell pellet from the single 800mL glucose-grown *L. starkeyi* 78-23 cultures were extracted by the modified Folch's method. Aliquots weighed ~400mg each for a total weight of 1634mg. The aqueous phases were pooled into a single container, freeze-dried, and weighed. The dried aqueous extract weighed 129mg (7.9% of the total dry weight). The extract was dissolved in 4mL of dH₂O, sterile filtered, and stored at 4°C.

Cells of either *S. cerevisiae* S288c or *L. starkeyi* 78-23 were then grown for 24 hours in 1mL of standard YEPD at room temperature at 200rpm, harvested, washed twice, and added to 2mL of PD (2% peptone, 2% glucose) medium that was inoculated at 4×10^5 cells/mL and incubated for 16 hours to starve cells of yeast extract-specific components. The starved cells were then used to inoculate 1mL samples of PD medium supplemented with known amounts of commercial yeast extract to prepare a standard curve or with known amounts of the experimental aqueous extract to compare to the standard curve. An inoculum of 2×10^5 cells/mL was used because this corresponded to an A_{600} of 0.0. Cultures grown to generate the standard curves were done in triplicate (*S. cerevisiae* S288c) or duplicate (*L. starkeyi* 78-23). Tests of the experimental aqueous extracts were performed in duplicate for both *S. cerevisiae* S288c and *L. starkeyi* 78-23.

Cultures for generating the standard curves and for determining the nutritional value of the aqueous extracts were incubated for 24 (*S. cerevisiae* S288c) or 48 hours (*L. starkeyi* 78-23). Following incubation, cell density was measured (A_{600}) and diluted as necessary such that readings ranged between 0.1 and 0.3. Values based on diluted samples were corrected by multiplying by the dilution factor. A_{600} values were then plotted as a function of added commercial yeast extract. The nutritional value of the experimental aqueous extracts was determined by converting observed growth of *S. cerevisiae* S288c or *L. starkeyi* 78-23 (as A_{600} values) to a corresponding amount of commercial yeast extract based on the standard curve. The potency of the experimental aqueous extract was defined in terms of "commercial yeast extract equivalents."

Results and Discussion

Preliminary Experiments

The purpose of the preliminary experiments was to choose 1) an appropriate growth medium, 2) an appropriate growth vessel, and 3) a lipid extraction method.

Growth Medium

Preliminary experiments were conducted with *L. starkeyi* 78-23 to test growth in various media used in previous studies (data not shown). Several formulations of media were tested including various forms of nitrogen (NH₄Cl and yeast extract), iron supplements (FeSO₄ and FeCl₃), and a supplement-rich medium, "Medium A": (per liter) glucose (30g), yeast extract (1.5g), NH₄Cl (0.5g), KH₂PO₄ (7.0g), Na₂HPO₄•12H₂O (5.0g), MgSO₄•7H₂O (1.5g), FeCl₃•6H₂O (0.08g), ZnSO₄•7H₂O (0.01g), CaCl₂•2H₂O (0.1g), MnSO₄•5H₂O (0.1mg), CuSO₄•5H₂O (0.1mg), Co(NO₃)₂•6H₂O (0.1mg) (Zhao et al., 2008; Kimura et al., 2004). *L. starkeyi* 78-23 grown in a medium containing yeast extract as a nitrogen source produced 2-fold more cells (measured by hemocytometer) than a medium with ammonium chloride. Negligible differences were observed in cell yield when *L. starkeyi* 78-23 was grown in media supplemented with iron. While cells yields were about 2.5-higher in "Medium A" than in YEPD, preparation of Medium A was laborious, and hence, YEPD was chosen as growth medium.

Growth Vessel

Lipid production by oleaginous yeasts is an oxygen-dependent process. Increasing the amount of agitation of the cultures increases overall aeration and therefore lipid yield. A 24-well microtiter shaker was tested as a potential vessel because agitation rates could be increased to a maximum

of 2000rpm, providing a simple way to compare multiple culture conditions in parallel at the microscale (~3mL max/well). However, we found that agitation rates were limited to 500rpm and volumes to 2mL because faster agitation or larger volumes would cause splashing of medium out of wells. The 24-well microtiter plate was tested at an agitation rate of 500rpm using volumes of 1 and 2mL. A significant amount of condensate was found to accumulate on the lid over the time-scale of cell growth. Over a 48-hour period, up to 15% of the medium in a single well was found to evaporate. Furthermore, this evaporation was not constant across each wells. More evaporation occurred from wells at the periphery of the plate (10-15%) than in the center (2-5%). Because the evaporation confounded assessment of cell yields, the microtiter plate was not chosen as a growth vessel for subsequent experiments. Instead, we used glass test tubes for experiments with volumes up to 2mL and Erlenmeyer flasks for larger volumes.

Extraction Method

An enzymatic extraction process was tested for the extraction of lipid and aqueous soluble materials. We used zymolyase, an enzyme that digests the yeast cell wall to facilitate release of lipids without use of solvents. Cells were incubated with zymolyase at 45°C for 30 minutes. Quantification of lipid yield was unsuccessful because lipids could not be separated from the mixture following the digestion. Microscopic observation of cells after lysis revealed large aggregates of cells that interfered with subsequent processing. In an effort to disaggregate the clumps, 0.5% SDS was added during enzymatic lysis in potassium phosphate buffer. This approach was not successful. The cell aggregates persisted with possible formation of potassium dodecyl sulfate that interfered with spectrophotometric measurement of cell density, the indicator

of cell lysis. Thus, the modified Folch's method of lipid extraction was used instead of enzymatic extraction.

Lipid Yield and Composition

L. starkeyi 78-23 was grown in YEPD and YEPX as described in Materials and Methods. Cells were harvested, freeze-dried, and extracted by a modified Folch's method. Nine aliquots of the freeze-dried cells pellets from single 800mL glucose- and xylose-based cultures were used to quantify lipid yields (Table 1). Lipid from the glucose-grown culture reached 37% of the dry mass while lipid from the xylose-grown culture reached 35% of the dry mass.

Table 1. Lipid yield from *L. starkeyi* 78-23 grown on YEPD or YEPX.

Carbon Source	Lipid Yield per Biomass	Standard Deviation	Percent Standard Deviation	Lipid Yield per Sugar (g/g)	Energy Yield (J lipid/J sugar)
Glucose	37.4%	2.09	5.6%	0.04	0.09
Xylose	35.3%	1.64	4.6%	0.04	0.16

Nine aliquots (80-100mg) of dried cells harvested from a single 800mL culture of *L. starkeyi* 78-23 grown in either YEPD or YEPX were analyzed for lipid composition. Lipid yield is grams of lipid per grams of sugar. Energy yield is joules of lipid per joules of sugar.

The fatty acid composition of the lipids was determined by GC-MS of the methyl esters. Table 1 compares data from the present study with previous studies. Three aliquots from each culture were extracted for lipids, converted to fatty acid methyl esters, and analyzed by gas chromatography in three runs per aliquot. Figure 3 compares the fatty acid compositions this study and the following table (Table 2.) includes the data collected from the GC analysis and comparisons of previous research conducted on lipid collected from *L. starkeyi*.

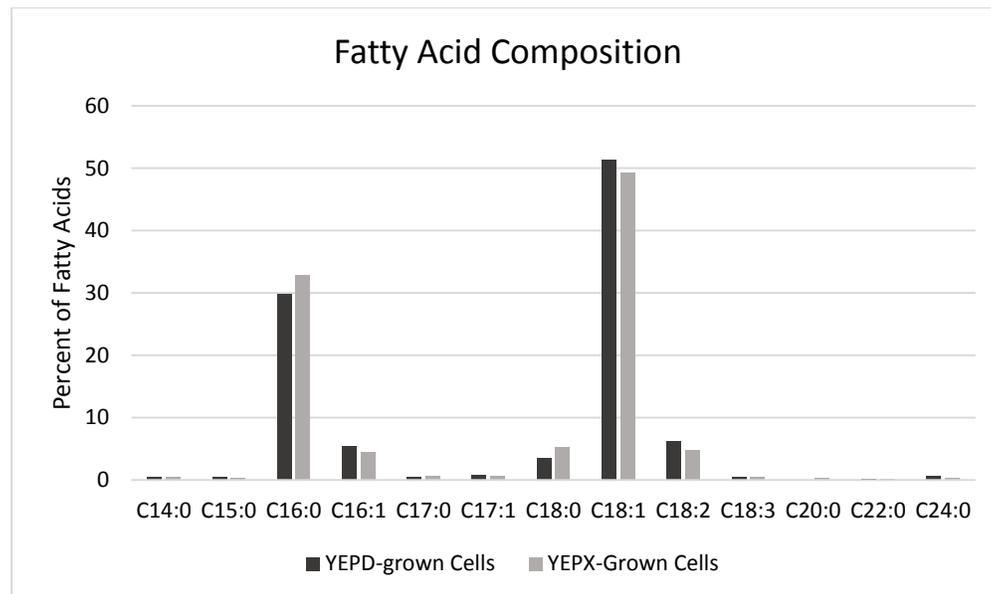


Figure 3. Fatty acid composition of YEPD- and YEPX-grown cells (glucose and xylose, respectively) from the 800mL cultures.

Table 2. Fatty acid composition of triacylglycerols from *L. starkeyi* samples.

C14:0	C15:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	C18:2	C18:3	C20:0	C22:0	C24:0	Ref.
0.51	0.39	29.88	5.42	0.39	0.87	3.47	51.27	6.25	0.51	0.15	0.19	0.68	YEPD-grown; this study
0.45	0.26	32.94	4.48	0.54	0.68	5.28	49.36	4.78	0.39	0.28	0.21	0.36	YEPX-grown; this study
0.5	-	36.5	3.6	-	-	5.4	52.8	1.2	-	-	-	-	[1]
0.5	-	36.6	4.3	-	-	6.2	48.9	1.1	-	-	-	-	[2]
-	-	35.30	-	-	-	-	49.06	4.28	0.39	-	-	-	[3a]
-	-	37.27	-	-	-	-	48.18	2.71	0.26	-	-	-	[3b]
-	-	39.0	3.0	-	-	3.0	55.0	-	-	-	-	-	[4]
Trace	-	33.9	5.8	-	-	5.1	50.6	3.4	0.2	-	-	-	[5]

YEPD- and YEPX-grown cells (glucose and xylose, respectively) from the 800mL cultures. [1] Lin et al. (2011); *L. starkeyi* AS 2.1560, [2] Zhao et al. (2008); *L. starkeyi* AS 2.1560, [3a] Sha (2013), *L. starkeyi* CBS 1807, [3b] Sha (2013), (xylose-grown); *L. starkeyi* CBS 1807, [4] Wild et al. (2010); *L. starkeyi* NRRL Y-11557 [5] Viljoen et al. (1986); *L. starkeyi* CBS 2516. All cultures from the cited works were glucose-grown unless specified otherwise.

The most abundant acyl groups found in *L. starkeyi* 78-23 were oleic acid (C18:1) and palmitic acid (C16:0) which accounted for about 50% and 30-35%, respectively, of the fatty acids in both the present study, and in other *L. starkeyi* strains in previous experiments. Palmitoleic (C16:1), stearic (C18:0), and linoleic acid (C18:2) each accounted for about 5% of the total acyl groups found in both glucose- and xylose-grown *L. starkeyi* 78-23, similar to what has been observed in other studies, with the exception of C18:2, which was somewhat higher in the present work. All other acyl groups were found to be minor components (<1%).

The differences in lipid content between studies may be attributed in part to use of different *L. starkeyi* strains and in part to different growth conditions. *L. starkeyi* 78-23 was used in the present study; Sha (2013) used *L. starkeyi* CBS 1807; Wild et al. (2010) used *L. starkeyi* NRRL Y-11557; and Zhao et al. (2008) and Lin et al. (2011) used *L. starkeyi* AS 2.1560. The strain AS 2.1560 was used in two studies where the lipid yield was >60%.

All experiments excluding ours and those of Lin et al. (2011) were single batch experiments (cell growth and lipid accumulation were not separated into two phases). The experiment conducted by Zhao et al. (2008) was performed to optimize medium composition for lipid production. Their final medium had the following composition: (per liter) glucose (48.9g), xylose (24.4 g), yeast extract (7.9 g), FeSO₄ (4.0 mg), KH₂PO₄ (7.0 g), Na₂HPO₄ (2.0 g), MgSO₄•7H₂O (1.0 g), and NH₄Cl (0.5 g).

Yeast grown on this optimized medium reached a lipid content of 61.5%. The media used by Sha (2013) was similar to that used by Zhao et al. (2011), but contained more CaCl₂, citric acid, ZnSO₄, and MnSO₄. The experiment from Wild et al. (2010) was unique in that lipid accumulation was compared following growth on medium containing glucose or potato starch as

carbon sources. Both the glucose- and potato starch-containing media incorporated several supplements similar to those used in the experiments described by Sha (2013) and Zhao et al. (2008). However, lipid content in these latter two media only reached only 40%. Lin et al. (2011) conducted a two-stage fermentation (cell growth phase separated temporally from lipid accumulation phase) similar to that used in the present study where cells were grown on YEPD to allow cells to grow, and then were subsequently incubated in a 4% glucose solution to allow lipid accumulation. Cells reached a lipid content of 65.2%.

In the present, lipid content and composition was found to be about the same when *L. starkeyi* 78-23 was grown on media containing either glucose or xylose similar to what was reported by Sha (2013). To our knowledge, the present study is the first in which lipid yields and composition were compared in a two-stage fermentation that used both glucose and xylose as carbon sources. Lin et al. (2011) previously demonstrated that a different strain of *L. starkeyi* could produce about 65% lipid (compared to 35% in the present study) in YEPD-grown cells that were subsequently incubated in a glucose solution (nitrogen-free).

Nutritional value of aqueous extract obtained from the lipid extraction

To test cell growth as a function of added commercial yeast extract, cells were initially starved of yeast extract in PD medium. Determining the appropriate length of the starvation period was accomplished by growing *L. starkeyi* 78-23 and *S. cerevisiae* S288c in YEPD for 24 hours. Cells were then harvested and resuspended in PD medium (lacking yeast extract) and incubated between 6 and 24 hours in order to starve them of yeast extract-specific components. The starved

cells were then used to inoculate PD medium with known amounts of added commercial yeast extract.

Cells that were incubated in PD medium between 6 and 14 hours exhibited no differences in cell yield as a function of added yeast extract. That is, all cultures reached a titer of 2×10^8 cells/mL as though that had not been depleted of all yeast extract-specific components. Cells that were incubated in PD medium between 18 and 24 hours failed to grow when transferred to PD medium with known amounts of added yeast extract. One interpretation is that prolonged starvation for some yeast extract-specific component(s) was lethal. Cells starved for 16 hours prior to transfer to PD medium containing known additions of yeast extract were found to grow in proportion to the addition. Therefore, a 16-hour starvation period was chosen.

The aqueous phases from four aliquots of the lipid extraction of the freeze-dried cell pellet of the single 800mL glucose-grown *L. starkeyi* 78-23 culture were recovered, pooled, dried, and redissolved in 4mL of dH₂O. A total of 129mg of aqueous soluble material was recovered from 1634mg of freeze-dried cell pellet. This aqueous extract was used to determine if the material could serve as a replacement for commercial yeast extract in YEPD formulations.

Standard curves were generated that related cell growth to known amounts of added commercial yeast extract. Three replicates were performed for *S. cerevisiae* S288c and two for *L. starkeyi* 78-23. In parallel, cells were grown in duplicate on PD medium with known amounts of added experimental aqueous extract from *L. starkeyi*. Figure 4 shows a representative standard curve for *L. starkeyi* 78-23. The mean slope for the standard curve based on *L. starkeyi* 78-23 was 6.41 A₆₀₀/% [w/v] yeast extract. The mean slope for the standard curve based on *S. cerevisiae* S288c was 6.59 A₆₀₀/% (w/v) yeast extract. R² values were greater than 0.98 for both

yeasts. The cell density of cultures to which no commercial yeast extract had been added were subtracted from all data points before standard curves were generated. This value was about 0.1 for *L. starkeyi* 78-23 and 0.08 for *S. cerevisiae* S288c. Inclusion of data points between 0.25 and 1% added yeast extract reflected a linear correlation with A_{600} values. The "zero yeast extract" data point (0,0) did not fit this linear relationship and was not included in the calculation of the slope.

The experimental aqueous extract was tested at at two concentrations, 0.16 and 0.32%, in duplicate, for both *L. starkeyi* 78-23 and *S. cerevisiae* S288c. The cell density (A_{600}) of these cultures were compared to the slopes from the cultures grown with known additions of commercial yeast extract to calculate the potency of the experimental aqueous extracts. Table 3 shows the slopes of the standard curves and the mean potency of the experimental aqueous extracts. For *L. starkeyi* 78-23, the mean potency of the experimental aqueous extract was found to be 3.8-fold greater than commercial yeast extract. For *S. cerevisiae* S288c, the mean potency of the experimental aqueous extract was 3.2-fold greater than commercial yeast extract. This means that the aqueous extract recovered from *L. starkeyi* was a better supplement than yeast extract for YEPD formulation.

Table 3. Slopes of the standard curves from cultures grown on commercial yeast extract.

Experiment	Strain	Slope ($A_{600}/\%$ [w/v] yeast extract)	R ² Value	Mean Potency of Aqueous Extract ¹
1	<i>S. cerevisiae</i> S288c	7.48	0.983	2.84
2	<i>S. cerevisiae</i> S288c	5.98	0.993	3.40
3	<i>S. cerevisiae</i> S288c	6.32	0.987	3.32
4	<i>L. starkeyi</i> 78-23	6.95	0.991	3.43
5	<i>L. starkeyi</i> 78-23	5.87	0.988	4.23

¹The potency of the aqueous extract is the ratio of the measured cell density of the experimental cultures to the expected cell density from the slope (standard curve). The mean potency is based on the average of the growth response to the 0.16 and 0.32% additions of the experimental aqueous extract.

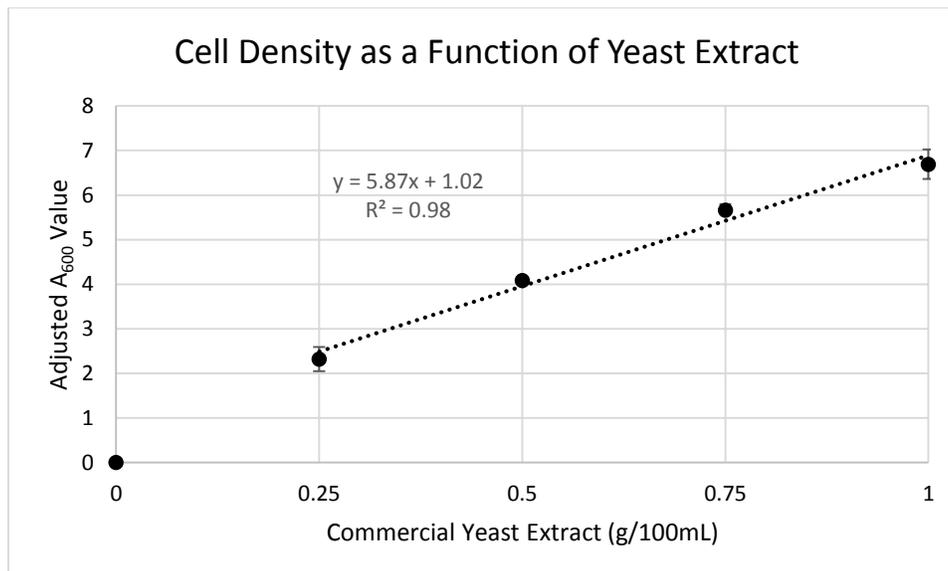


Figure 4. Representative standard curve from growth of *L. starkeyi* 78-23 on commercial yeast extract. All data points were adjusted by the dilution factor and by subtracting the A₆₀₀ value of the 0% yeast extract addition, 0.11.

Conclusion

The purpose of these experiments was to test methods to increase the value of using *L. starkeyi* as a source of biodiesel. In the first experiment, we expanded on the work of Lin et al. (2011) and compared the growth and lipid accumulation of *L. starkeyi* on media containing glucose or xylose. We temporally separated the cultures into a growth phase and a lipid accumulation phase. Following lipid extraction, lipid yields were quantified and acyl group composition were analyzed by gas chromatography-mass spectrometry. Lipid yields of cultures grown on glucose were comparable to cultures grown on xylose (37 and 35%, respectively). Lipid composition was also comparable between the two cultures with oleic and palmitic acids being the predominant acyl groups. This lipid composition was consistent with previous reports. However, strain differences may account for differences in lipid yield. For example, in previous work with *L. starkeyi* AS 2.1560, lipid yield was reported to be over 60% by dry weight (Lin et al. 2011; Zhao et al. 2008).

In our second experiment, we recovered the aqueous phase from the modified Folch's method for lipid extraction of *L. starkeyi*. This aqueous phase was dried, weighed, redissolved in dH₂O, and used as an alternative to yeast extract in formulations of YEPD. When cells were grown on PD medium containing the aqueous extract, *L. starkeyi* 78-23 grew 3.8 times more than on PD containing an equivalent amount of commercial yeast extract. When *S. cerevisiae* S288c was grown on PD containing the aqueous extract, it grew 3.2 times more than cells grown on PD containing an equivalent amount of commercial yeast extract. To our knowledge, this represents a previously unexplored means of increasing the potential value of using *L. starkeyi* as a source of lipid for production of biodiesel.

Further experiments that could build on our work could test the potency of the experimental aqueous extract over multiple cultures and extractions. Furthermore, because we did not test the lipid produced from cells grown on the recovered aqueous extract from *L. starkeyi*, it would be important to confirm high yields and acceptable acyl group composition.

With respect to the temporal separation of the yeast growth phase from the lipid accumulation phase, it will be important to replace laboratory media with realistic lignocellulosic hydrolysates as feedstock and to confirm that the same lipid accumulation occurs. While the feedstock will contain glucose and xylose, we anticipate that the potentially inhibitory byproducts produced during pretreatment of lignocellulose will be a barrier to yeast growth for biodiesel production.

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