Nitrogen limitation, oxygen limitation, and lipid accumulation in

Lipomyces starkeyi

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Abstract

Lipid production by oleaginous yeasts is optimal at high carbon-to-nitrogen ratios. In the current study, nitrogen and carbon consumption by *Lipomyces starkeyi* were directly measured in defined minimal media with nitrogen content and agitation rates as variables. Shake flask cultures with an initial C:N ratio of 72:1 cultivated at 200 rpm resulted in a lipid output of 10 g/L, content of 55 %, yield of 0.170 g/g, and productivity of 0.06 g/L/h. All of these values decreased by $\approx$ 50-60% when the agitation rate was raised to 300 rpm or when the C:N ratio was lowered to 24:1, demonstrating the importance of these parameters. Under all conditions, *L. starkeyi* cultures tolerated acidified media (pH $\approx$ 2.6) without difficulty, and produced considerable amounts of alcohols; including ethanol, mannitol, arabitol, and 2,3-butanediol. *L. starkeyi* also produced lipids from a corn stover hydrolysate, showing its potential to produce biofuels from renewable agricultural feedstocks.

Keywords

Oleaginous yeast; aeration; C:N ratio; polyols; hydrolysate

1. Introduction

Replacing non-renewable fossil fuels with environmentally sustainable biofuels remains a major challenge for our civilization. Oleaginous yeasts, defined by their ability to accumulate from 20 to 70% of their total dry biomass as lipids, are being investigated for their potential to produce a variety of valuable chemicals (Sitepu et al., 2014b). Metabolic engineering and bioprocess optimization efforts may lead to economically competitive manufacturing of biodiesel fuel using these organisms. *Lipomyces starkeyi* is a particularly well suited host given its impressive native abilities, including the capability to utilize a wide variety of carbon sources and to grow in media without vitamin supplementation (Sitepu et al., 2014a). *L. starkeyi* is capable of simultaneously utilizing mixed sugars, including glucose and xylose (Anschau et al., 2014), glucose and mannose (Yang et al., 2014), and cellobiose and xylose (Gong et al., 2012). This species is also known for its considerable ability to degrade extracellular polysaccharides by secreting glycosidases (Kang et al., 2004; Angerbauer et al., 2008; Wild et al., 2010), a trait that could be useful for generating valuable oils from low-cost food waste substrates (Ratledge and Cohen, 2008). Finally, *L. starkeyi* has a sequenced genome (Grigoriev et al., 2011) and a recently developed transformation system (Calvey et al., 2014), providing tools for genetic manipulation which are lacking in most oleaginous yeast species.

The future of biofuels depends upon developing strains that are capable of growth on affordable and environmentally sustainable feedstocks. Lignocellulosic hydrolysates

primarily contain glucose and xylose, so microbial conversion of pentose sugars is essential (Jin et al., 2015). Previous studies have demonstrated that *L. starkeyi* can produce lipids from hydrolysates derived from spent yeast cells (Yang et al., 2014), sewage sludge (Angerbauer et al., 2008), wheat straw (Yu et al., 2011), sugarcane bagasse (Anschau et al., 2014), corncobs (Huang et al., 2014), and corn stover (Sitepu et al., 2014c). Lipid accumulation proceeded despite the presence of moderate concentrations of inhibitors released during the hydrolysis of hemicellulosic biomass; including acetic acid up to 4 g/L (Yu et al., 2011), hydroxymethylfurfural up to 2 g/L (Sitepu et al., 2014a), and up to 0.5 g/L furfural (Chen et al., 2009). The ability of *L. starkeyi* to produce lipids despite the presence of these inhibitors fulfills an essential prerequisite for “second generation” biofuel production.

Oleaginous yeasts are known to accumulate lipids when grown in a nitrogen-deficient or other nutrient-limited medium (Ratledge and Wynn, 2002). Nitrogen limitation can be induced by growing cultures in media with a high molar ratio of carbon to nitrogen; typical C:N ratios reported in the literature are in the range of 50:1 to 150:1 (Subramaniam et al., 2010). In experiments that investigate multiple C:N ratios, lipid accumulation commonly increases as the C:N ratio increases (Angerbauer et al., 2008; Wild et al., 2010). However, nitrogen concentrations are rarely measured directly in these studies. Here, several *L. starkeyi* batch shake flasks with defined minimal media were studied, in which the nitrogen content was altered while keeping all other variables constant. Additionally, a persulfate digestion method for measuring media nitrogen levels was employed, to directly investigate the relationship between nitrogen limitation and lipid production over time.

Lipid accumulation is the dominant metabolic activity of oleaginous yeasts during nitrogen limitation (Ratledge and Wynn, 2002). However, few studies have examined the influence of aeration on lipid productivity in these species (Sitepu et al., 2014b). Further, the effects of oxygen limitation appear to differ widely among lipogenic yeasts. For example, in *Apiotrichum curvatum* (*Cryptococcus curvatus*) grown on casein whey, lipid yields decreased with lower oxygenation rates (Davies et al., 1990). In one *L. starkeyi* study, dissolved oxygen concentrations had no significant effect on lipid yields (Naganuma et al., 1985). Conversely, in *Yarrowia lipolytica* cultivated with stearin, lipid accumulation occurred at low oxygen saturation, while cell mass was produced predominantly in highly aerated cultures (Papanikolaou et al., 2002). High dissolved oxygen concentrations also consistently reduced lipid accumulation in *Rhodotorula glutinis* (Yen and Zhang, 2011). Compared to cells grown in airlift bioreactors, which typically exhibit low aeration rates, *R. glutinis* cells cultivated in highly aerobic agitated fermenters had a faster growth rate and higher cell mass, yet a nearly 50% reduction in average lipid content (Yen and Liu, 2014). In the following studies, evidence indicates that oxygen limitation plays an important role in promoting lipid accumulation in *L. starkeyi*.

2. Materials and Methods

2.1 Strains and media

Lipomyces starkeyi NRRL Y-11557 (ATCC 58680, CBS 1807) was used throughout these experiments. Unless otherwise noted, reagents were purchased from Sigma-Aldrich (St. Louis,

MO). *L. starkeyi* cultures were maintained on YPD plates (m/v: 1% yeast extract, 2% peptone, 2% glucose, 2% agar) with subculturing once per month.

All starter cultures were grown in 50 mL of defined minimal media (DMM) with 20 g/L of glucose, as described by (Long et al., 2012). Lipid accumulation experiments were carried out in modified DMM, containing 60 g/L of glucose and nitrogen provided as a mixture of urea and ammonium chloride with a 1:1 ratio of atomic nitrogen (Section 3.4). Carbon-to-nitrogen (C:N) ratios were varied as indicated: media with an initial C:N ratio of 24:1 contained 1.07 g/L of urea and 1.91 g/L of NH₄Cl, corresponding to a total atomic nitrogen content of 1.0 g/L. Media with an initial C:N ratio of 48:1 contained 0.54 g/L of urea and 0.96 g/L of NH₄Cl, corresponding to a total atomic nitrogen content of 0.5 g/L. Media with an initial C:N ratio of 72:1 contained 0.36 g/L of urea and 0.64 g/L of NH₄Cl, corresponding to a total atomic nitrogen content of 0.33 g/L. All media were sterilized by passage through a 0.2 µm filter.

Corn stover hydrolysate treated using the ammonia fiber expansion (AFEXTM) process was obtained from Michigan State University (Balan et al., 2009). The AFEX-treated corn stover hydrolysate (referred to herein as “AFEX”) contained approximately 49 g/L glucose and 20.5 g/L xylose. Nitrogen content of the hydrolysate was not determined, but the C:N ratio was estimated to be approximately 20:1 based on the composition of synthetic AFEX as described by (Sitepu et al., 2014c).

2.2 Culturing conditions

All shake flask experiments were conducted in 500 mL Erlenmeyer flasks containing 200 mL of media, incubated at 30 °C and shaken at 200 revolutions per minute (rpm), unless

otherwise noted. For experiments in defined media, starter cultures were grown in DMM for 48 to 72h, and cells were inoculated at 1 to 2% by volume in order to yield initial OD₆₀₀ readings targeted at 0.75. AFEX flasks were also inoculated with DMM cultures, to a starting OD₆₀₀ of 5. No increase in OD₆₀₀ was observed after 4 days, and the flasks were reinoculated with a second starter culture to a higher density (OD₆₀₀ of 8) and a final inoculation volume of $\approx$ 13% (v/v).

2.3 Analytical methods

For all experiments, analytical methods described below were performed once per day. Cell concentration was determined with an Agilent 8453 spectrophotometer (Santa Clara, CA) by taking optical density readings at 600 nm (OD₆₀₀). Dry cell weight (DCW) was determined by filtering 5 to 7 mL of culture through a pre-weighed PALL Corporation 0.2 μ m pore size polyethersulfone membrane (Port Washington, NY), drying the cells by microwaving at low power for 10 to 15 minutes, and weighing. All sugar (glucose, xylose) and alcohol (ethanol, mannitol, arabitol, 2,3-butanediol) concentrations were determined by high performance liquid chromatography (HPLC) as described by (Su et al., 2014). Media pH was measured using a Fisher Scientific Accumet AB15 Basic meter (Hampton, NH).

To determine lipid content, 2 mL of cells were washed, centrifuged, and frozen at -20°C prior to extraction. Thawed cell pellets were resuspended in 2 mL of water, and lipids were liberated by addition of 200 μ L of concentrated HCl, with heating at 95 °C for 1 h. Lipids were collected using a Bligh & Dyer type extraction with 7.5 mL of 2:1 (v:v) methanol:chloroform and three extractions in 2 mL of chloroform (Bligh and Dyer, 1959). Pooled chloroform layers were evaporated to dryness in a 40 °C heating block under a constant stream of N₂ prior to

127 transesterification. To convert lipids to fatty acid methyl esters (FAMES), 1.0 mL of anhydrous
128 HCl in methanol was added to the dried extract, and the mixture was heated at 50 °C overnight.
129 Then, 1.0 mL n-Hexane and 10 mL of 50 mg/mL NaHCO₃ were added to the samples, vortexed
130 thoroughly, and centrifuged to separate the layers. The hexane layer was collected and the
131 extracted FAMES were analyzed immediately using a LECO Corp. Inc., (Saint Joseph, MI)
132 Pegasus4DGCxGC TOFMS with an Agilent Technologies (Santa Clara, CA) 7890A gas
133 chromatograph and aStabilwax-DA 30m x .25 mm I.D., x 0.25µm primary column. Helium was
134 used as the carrier gas with a flow rate of 1.0 mL/min. Inlet temperature was 225 °C. The
135 primary GC oven temperature program began at 100 °C held for 1.0 minute, increased at 10 °C
136 per minute up to 250 °C, and was held there for 5.0 min. The TOF acquisition rate was 10/sec,
137 and spectra were recorded from 10-500 atomic mass units.

138 For nitrogen analysis, 1 mL of culture was centrifuged and the supernatant was frozen at
139 -20 °C until ready for sampling. Nitrogen content was assayed via a persulfate-mediated
140 digestion method using the Hach Company (Loveland, CO) Low Range Total Nitrogen Reagent
141 Set (0.5 - 25.0 mg/L N; catalog #2672245) and protocol, with the following modifications. The
142 media supernatant was diluted as necessary in deionized water, typically 1:30 to 1:50 fold, to
143 lower the initial total atomic nitrogen concentration to below 25 mg/L. The persulfate digestion
144 step was conducted in boiling water for 30 min, and then allowed to equilibrate to room
145 temperature for 20 min. The reaction was followed by measuring the formation of a yellow
146 complex with an absorbance peak at 420nm. After the final 5 min incubation, OD₄₂₀ samples
147 were taken every 30 seconds up to 8 min and averaged. A calibration curve was developed to
148 enable conversion of OD₄₂₀ readings into mg/L atomic nitrogen.

3. Results and Discussion

3.1 C:N Ratio and Nitrogen Limitation

To investigate the relationship between nitrogen limitation and lipid accumulation in *L. starkeyi*, cultures were grown at 200 rpm in defined minimal media containing 60 g/L glucose and initial C:N ratios of 24:1, 48:1, or 72:1. A persulfate digestion protocol was used to obtain a more quantitative analysis of nitrogen limitation, and to directly correlate nitrogen concentration with changes in the metabolic characteristics and physiology of a culture.

Figure 1 displays the consumption of nitrogen and glucose, along with production of cell mass and lipids, under each condition. Flasks with a C:N ratio of 24:1 began with 1000 mg/L of atomic nitrogen, and about half of the original nitrogen still remained in the media after all of the glucose had been consumed (Fig. 1B). The C:N ratio dropped over time, as glucose was consumed faster than the nitrogen source, and the final C:N ratio fell to under 10:1 (Fig. 1E). Cells cultivated under this condition did not experience nitrogen limitation, and the final lipid content of these cells was less than 20% (Table 1). Flasks that began with 500 mg/L nitrogen and a moderate initial C:N ratio of 48:1 had insignificant lipid accumulation over the first four days, despite a C:N ratio which increased to >60:1 and nitrogen concentrations that dropped below about 200 mg/L (Fig. 1B, 1D, 1E). A period of rapid lipogenesis started after 96 hours, and continued even as the C:N ratio began to drop (Fig. 1D, 1E). This suggests that the nitrogen limitation response did not occur immediately in these cells; rather, a persistent metabolic shift towards lipid accumulation was triggered only after nitrogen availability fell below a certain critical point. Flasks growing in medium with a initial C:N ratio of 72:1 and a total atomic

nitrogen concentration of 333 mg/L likely experienced nitrogen limiting conditions soon after inoculation. The C:N ratio increased over time, as the nitrogen was consumed much faster than carbon, and the culture eventually reached a C:N ratio > 100:1 (Fig. 1E). Thus, a sustained high rate of lipid production was observed throughout the entire course of this experiment.

Nitrogen limitation blocks cell division and initiates lipid accumulation in oleaginous yeasts (Ageitos et al., 2011). This process involves enzymes including AMP deaminase and isocitrate dehydrogenase, which are uniquely sensitive to nitrogen limitation in oleaginous yeasts (Ratledge, 2004). When nitrogen is limited, flux through the citric acid cycle is reduced, and acetyl-CoA is instead funneled toward lipid accumulation. As expected, lipid contents (%), lipid yields (g/g), and specific lipid productivities (g/L/h) all progressively increased as the C:N ratio increased (Table 1). Conversely, higher nitrogen content enabled sustained cell reproduction; leading to higher ODs (data not shown) and DCWs (Fig. 1C). Cells grown in media with a 72:1 initial C:N ratio reached maximal levels of about 10 g/L lipids, 55% lipid content, 0.17 g/g lipid yield, and 0.06 g/L/h productivity, each of which was 2-3x higher than the respective values obtained from cells grown with a 24:1 ratio (Table 1).

These results illustrate that consideration of only the initial C:N ratio is not always sufficient. For cultures beginning with a 48:1 ratio, the presence of excess nitrogen resulted in a relatively low level of lipid production. Thus, increasing the C:N ratio by adding additional carbon sources may not be an effective strategy. Cells only enter into a lipid accumulation phase once the concentration of available nitrogen is sufficiently low. Therefore, high lipid yields are often best achieved in two-stage fermentations, in which cell division and lipid

accumulation modes are spatially and/or temporally separated, and lipids are produced in second stage media containing little to no nitrogen (Lin et al., 2011).

3.2 Aeration and Oxygen Limitation

In a preliminary experiment, *L. starkeyi* was cultivated in fully aerated 2-L bioreactors with DMM containing 80 g/L of glucose and a C:N ratio of 80:1. Despite the nitrogen-limited conditions, this culture accumulated only about 5 g/L of lipids with a 25% lipid yield (data not shown). Oxygen availability, monitored by a dissolved oxygen probe, was maintained at 100% saturation by vigorous aeration (750 rpm agitation, 0.25 vvm air flow). Microscopic examinations showed an abundance of abnormally diminutive cells, suggestive of rapid cell division. Similarly, *L. starkeyi* cultivated in another highly aerobic bioreactor (1000 rpm, 1 vvm) achieved only 23.7% lipid content in media with a C:N ratio of 61.2:1, while higher lipid yields were obtained in batch shake flasks with identical media agitated at 140 rpm (Wild et al., 2010). Several fed-batch *L. starkeyi* bioreactors (400 rpm, 1 vvm) failed to exceed 30% lipid content, despite containing media with a C:N ratio of 800:1 (Anschau et al., 2014). The highly oxygenated conditions in these bioreactors may have contributed to the low yields, but further testing of the influence of aeration on lipid accumulation in *L. starkeyi* was required.

Figure 2 presents the results obtained when *L. starkeyi* was cultured in DMM shake flasks with an initial C:N ratio of 72:1 and agitation rates controlled at 150, 200, or 300 rpm. The rate of glucose consumption increased progressively as agitation increased (Fig. 2A, Table 1). Similarly, increasing agitation rates corresponded with more rapid cell production, resulting in higher final dry cell weights and DCW productivities (Fig. 2C, Table 1). However, these results

confirmed that high aeration rates lead to reduced lipid production. At 300 rpm, about 5.4 g/L of lipids were accumulated, at 28% by weight, a 0.09 g/g yield, and 0.035 g/L/h productivity (Table 1). Lipid content, yield, and productivity at 300 rpm were all approximately 50% lower than the respective values achieved at 200 rpm (Fig. 2D, Table 1). These results are consistent with those obtained from *R. glutinis*, where growth rates and biomass production improved as the aeration rate progressively increased, while the values for all lipid accumulation metrics were reduced by higher aeration rates (Yen and Zhang, 2011; Yen and Liu, 2014).

The results at 150 rpm reveal the tradeoffs between cell division and lipid accumulation caused by oxygen limitation. The decreased agitation rates slowed cell reproduction, causing the 150 rpm flasks to lag behind in glucose consumption and DCW production (Fig. 2A and 2C). The reduced number of cells hampered the total amount of lipid that could be produced in the time allotted. However, the specific lipid productivity and cellular lipid contents were almost identical between cultures grown at 150 and 200 rpm (Fig. 2D, Table 1). Given an additional 24 h of incubation time, flasks grown at 150 rpm eventually reached lipid contents comparable to flasks grown at 200 rpm (data not shown).

Additionally, an important association between agitation rates and the accumulation of polyols was discovered (Section 3.3). All of the accumulated ethanol, mannitol, arabitol, and 2,3-butanediol were summed together at each timepoint to yield an assessment of the total alcohol content (Fig. 2B). At higher agitation rates, a reduction in the accumulation of alcohols was observed. Under all conditions tested, concentration of alcohols increased over time, and catabolism of accumulated alcohols only began after the exhaustion of glucose. Due to their

faster glucose utilization, higher agitation rates were also associated with a more complete assimilation of previously produced alcohols. Flasks incubated at 300 rpm consumed nearly all of the alcohols present in the media, while flasks growth at 150 rpm were unable to metabolize any alcohols in the time allotted (Fig. 2B). Given additional incubation time, the 150 and 200 rpm flasks were able to consume alcohols and increase their lipid content (Section 3.3). These results are consistent with those of (Su et al., 2014), who found that higher oxygen transfer rates were associated with faster growth rates and lower ethanol production in two pentose fermenting yeast species. However, in that study, no significant correlation was observed between aeration and accumulation of other polyols.

Interestingly, a recent study on a second-stage *L. starkeyi* culture accumulating lipids in a stirred-tank bioreactor found that the dissolved oxygen concentration quickly dropped to zero and remained undetectable throughout the experiment (Lin et al., 2011). Yet, the lipid contents, yields, and productivities achieved (67.9 g/L, 64.9%, and up to 2.0 g/L/h, respectively) are among the highest ever reported for this species. Lin et al. hypothesized that higher productivities could be achieved with increased aeration, but it is likely that the oxygen limited conditions contributed to the excellent productivity in the first place.

There appears to be a synergistic effect between oxygen limitation and nitrogen limitation for inducing lipid accumulation in certain oleaginous yeast species. In highly aerobic conditions, glucose is preferentially used to generate ATP via oxidative phosphorylation. However, when oxygen becomes limited, it can no longer be used as a terminal electron acceptor, so less ATP is generated and excess NADH is produced from glycolysis. The cofactor

imbalance can be resolved by recycling NADH back to NAD⁺ via alternative reduction reactions, such as those leading towards the accumulation of reduced alcohols (Section 3.3). Meanwhile, even though reduced ATP availability lowers the growth rate, carbon can instead be directed toward lipid accumulation. This is possible because, compared to the ATP requirements of DNA replication and protein synthesis during cell growth, lipid accumulation requires relatively little ATP. Thus, oxygen limitation reduces the growth rate, leads to incomplete glucose oxidation, and may enhance lipid production.

3.3 Polyols

Pentose fermenting yeasts have been reported to accumulate a variety of polyols, including xylitol, ribitol, arabitol, glycerol, acetoin, and 2,3-butanediol (Su et al., 2014). Among the oleaginous yeasts, only *Y. lipolytica* is known to be a significant producer of polyols (Workman et al., 2013). Here, for the first time, *L. starkeyi* is shown to produce various alcohols; including ethanol, mannitol, arabitol, and 2,3-butanediol. Trace amounts of acetoin (<0.2 g/L) were also detected in all flasks (data not shown).

Alcohol production was observed under all conditions tested. Data from three representative experiments are shown in Figure 3. Accumulation of arabitol began immediately, while mannitol and ethanol were detectable after 24 h, and 2,3-butanediol after 72 h. Concentrations of each alcohol increased progressively, reaching maximum concentrations near 2.5 g/L for mannitol and ethanol (Fig. 3A, 3B), while arabitol and 2,3-butanediol peak concentrations reached about 1 g/L (Fig. 3C, 3D). After the exhaustion of glucose, some alcohols were consumed by cells and used for the production of more lipids or

cell mass. Catabolism of alcohols followed a similar pattern in all conditions tested. Ethanol was the first to be consumed, followed by mannitol. Arabitol and 2,3-butanediol were not catabolized until nearly all the ethanol present was consumed (Fig. 3).

While alcohols were produced under all conditions tested, there was a correlation between oxygen limitation and polyol production (Section 3.2). The maximum concentrations of mannitol, arabitol, 2,3-butanediol, and ethanol at 300 rpm were all about 30-40% lower than their respective levels at 200 rpm (Fig. 3). Higher aeration rates also led to more rapid catabolism of all alcohols after glucose exhaustion, resulting in even lower final concentrations of polyols. Flasks with low agitation rates were unable to re-utilize accumulated polyols in the time allotted. However, all polyols were eventually consumed, after the course of several additional days (data not shown). Low agitation rates create fermentative conditions, which leads to higher concentrations of all alcohols. As noted in *Y. lipolytica* by Workman et al., oxygen limiting conditions create cofactor imbalances, particularly an excess of NADH, which are restored by the reduction of sugars into polyols or by the reduction of pyruvate into ethanol or butanediol.

Typically, oleaginous yeast cultures are monitored only for glucose concentration, which can be easily be measured by a variety of methods. Lipid production continues up to the point of glucose exhaustion, upon which the experiment is terminated. However, under certain nitrogen limiting conditions, total alcohol concentration can reach nearly 7 g/L at the point of glucose depletion (Fig. 2B). These byproducts represent a significant carbon sink, which will adversely impact lipid yields, unless they are consumed. For example, in flasks incubated at 200

rpm with a 72:1 C:N ratio, lipid content increased by about 1 g/L or 3.5% in samples taken between 168 and 192 h (Fig. 2D). In this same period, only 2.6 g/L of glucose remained to be consumed, indicating a conversion which exceeds the maximum theoretical yield of lipids from glucose. This can be explained because about 1.8 g/L of ethanol, along with trace amounts of arabitol and mannitol, were also consumed during this same period (Fig. 3). This effect was also confirmed in flasks incubated at 150 rpm (data not shown). However, it should be noted that flasks agitated at 300 rpm did not show a similar increase in lipid content despite consuming over 3 g/L of alcohols during this period (Fig. 2B, 2D), providing further evidence that highly aerated conditions do not support lipid accumulation. Thus, these results indicate that extending the fermentation by an additional 24 h after glucose exhaustion can potentially improve lipid yields by 5-10%, under appropriate conditions. These results are consistent with the observations of (Huang et al., 2014), who noted that lipid accumulation in *L. starkeyi* continued 24-48 h after the exhaustion of glucose and xylose, although the significance of this result was not discussed.

3.4 pH

The optimal initial pH for lipid accumulation in *L. starkeyi* depends upon the carbon and nitrogen sources present in the media (Angerbauer et al., 2008). In preliminary experiments, it was observed that high concentrations of urea led to alkalization of the media within a few days, which retarded cell growth and led to premature death (data not shown). Urea may be ineffective as a sole nitrogen source because the *L. starkeyi* urease enzyme hydrolyzes urea into ammonia at a rate faster than cells can consume, leading to buildup of this base. Thus, *L.*

starkeyi does not well tolerate pH values above 6.5, as has been reported previously (Naganuma et al., 1985; Angerbauer et al., 2008). However, mixing ammonium chloride with urea at a 1:1 atomic nitrogen ratio counteracted alkalization of the media, so this ratio was utilized in subsequent experiments. In this study, each of the shake flasks containing DMM began at an initial unadjusted pH near 5.0. In all cases, a rapid drop in the media pH to about 3.0 over the course of the first 24 h was observed (Fig. 1F). Beyond the first timepoint, pH continued to decrease slowly to a final value near 2.6. While the effect oleaginous yeasts have on culture pH varies and is widely dependent on strain and on the media used (Sitepu et al., 2014c), culture acidification in nitrogen limited media without pH control has been previously observed in *L. starkeyi* (Starkey, 1946; Wild et al., 2010).

The pH of oleaginous yeast cultures is an important consideration, as lipid production is significantly reduced at low pH values in some species (Ageitos et al., 2011). These results indicate that *L. starkeyi* is able to thrive and accumulate lipids under very acidic conditions (Fig. 1F). This may be beneficial in consideration of biodiesel economics, as pH control adds to the production cost of any fermentation process (Sitepu et al., 2014b). Furthermore, acidic pH can help to suppress the growth of bacterial contaminants, which is a major concern in industrial microbiology (Ratledge and Cohen, 2008).

3.5 Lipid Properties

A summary of the fatty acid profiles obtained in these experiments is shown in Table 2. On average, the profile of total accumulated lipids consisted of approximately 36% palmitic

acid (C16:0), 3% palmitoleic acid (C16:1), 14% steric acid (C18:0), 43% oleic acid (C18:1), and 2% linoleic acid (C18:2). These results are similar to lipid profiles obtained by others for this species(Anschau et al., 2014;Yang et al., 2014; Gong et al., 2012;Lin et al., 2011; Wild et al., 2010), although slight differences can be explained by variability between *L. starkeyi* strains and culturing conditions(Sitepu et al., 2014b).For example, the oxygen limited conditions in low aeration flasks led to a slight increase in the degree of saturation of fatty acids. This is due to the oxygen requirements of fatty acid desaturases, and is consistent with previous observations in *C. curvatus*(Davies et al., 1990). The lipid profile of *L. starkeyi* is remarkably similar to that of palm oil, one of the most common vegetable oil feedstocks used for creating biodiesel (Table 2). This suggests that a biodiesel produced from lipids extracted from this species would have desirable fuel properties and would be compatible with existing diesel engine technology. This is not the case in most oleaginous fungi and algae, which have elevated levels of polyunsaturated fatty acids that are undesirable in biodiesel fuels(Meng et al., 2009; Ratledge, 2004).

L. starkeyi cells were examined by light and fluorescent microscopy to investigate the morphological changes which occur during lipid accumulation. In nitrogen limited minimal media, cells were spherical, very large($\approx 10\ \mu\text{m}$ in diameter),and contained a single lipid body which encompassed nearly the entire volume of the cell. Upon staining with Nile Red, these globular organelles fluoresced brilliantly, confirming the presence of lipids. These results are in agreement with the first recorded observations of this species (Starkey, 1946).

3.6 AFEX

Previous studies have investigated the growth of *Lipomyces* and other oleaginous yeasts on various agricultural hydrolysates. In one comparative study of dozens of oleaginous yeasts, *L. starkeyi* accumulated more lipids than any other species when cultivated on a synthetic hydrolysate similar to AFEXTM-treated corn stover (Sitepu et al., 2014c). Therefore, growth of *L. starkeyi* on authentic corn stover hydrolysate produced by the AFEX procedure was further examined.

This hydrolysate initially contained about 48 g/L of glucose and 20 g/L of xylose, which were consumed over the course of 8 days. An initial lag period was observed, as noted by (Huang et al., 2014), likely due to the inhibitors present in hydrolysates. Acetic acid was consumed during the lag period, and cell growth and sugar utilization lagged until the acetic acid was consumed. Similarly, (Sitepu et al., 2014c) observed that a variety of oleaginous yeast species grown on AFEX hydrolysates all consumed acetic acid within the first 48 h after inoculation. Most xylose was consumed only after the exhaustion of glucose, consistent with the typical glucose repression response (Gong et al., 2012; Zhao et al., 2008). However, some simultaneous utilization did occur (Fig. 4). Simultaneous utilization of glucose and xylose by *L. starkeyi* in hydrolysates was also observed by (Huang et al., 2014) and (Sitepu et al., 2014c), similarly in conditions where glucose concentrations were limited. About 9 g/L of lipids were produced in this study, accounting for a lipid content of 38%, a yield of .14 g/g sugar, and a productivity rate of .05 g/L/h (Table 1).

Based on these findings, *L. starkeyi* has a promising innate ability to convert minimally processed agricultural hydrolysates into a palm oil substitute. Lipid production from existing

agricultural waste residue, like corn stover, has the potential to generate renewable biodiesel fuel without commitment of any additional arable land or competition with the food supply (Subramaniam et al., 2010).

4. Conclusions

Although the wide taxonomic diversity of oleaginous yeasts prohibits generalizing results from any single species, this investigation revealed several parameters which are often overlooked. First, direct measurements of nitrogen concentrations can help reveal changes in the C:N ratio during cultivation, and determine the conditions in which lipid accumulation is initiated. Second, aeration levels should be optimized for every oleaginous species, as lipid production significantly decreases at high agitation rates in *L. starkeyi*. Third, accumulation of alcohol byproducts can detract from lipid yields. *L. starkeyi* produces large amounts of polyols under lipogenic conditions, which are re-consumed after the exhaustion of glucose.

Figures and Tables

Fig. 1. Effect of initial C:N ratio on growth and lipid production. *L. starkeyi* cultures were grown in minimal media at 200 rpm with an initial C:N ratio of 24:1, 48:1, or 72:1. Media glucose (A), nitrogen (B), biomass (C), intracellular lipid (D), C:N ratio (E), and pH levels (F) are shown; data points represent the average of duplicate flasks, errors bars denote the range of values. Final timepoint omitted from Figure 1E, due to glucose exhaustion.

Fig. 2. Effect of agitation rate on growth and production of secondary metabolites. *L. starkeyi* cultures were grown in minimal media with a 72:1 initial C:N ratio, with agitation at 150, 200, or 300 rpm. Consumption of glucose (A), production of alcohols (B) and biomass (C), and cellular lipid content (D) are shown; data points represent the average of duplicate flasks, errors bars denote the range of values.

Fig. 3. Effect of agitation rate on alcohol production and consumption. *L. starkeyi* cultures were grown with variable C:N ratios and agitation rates, as indicated. Accumulation and re-assimilation of alcohols including mannitol (A), ethanol (B), 2,3-butanediol (C), and arabitol (D) are shown; data points represent the average of duplicate flasks, errors bars denote the range of values.

Fig. 4. Lipid production from AFEX-treated corn stover hydrolysate. *L. starkeyi* cultures were grown at 200 rpm in hydrolysate with an estimated C:N ratio of $\approx 20:1$. Consumption of glucose and xylose, and production of biomass and lipids were monitored; data points represent the average of triplicate flasks, errors bars denote the standard deviation.

Table 1:

Lipid production capabilities of *L. starkeyi* at variable C:N ratios and agitation rates.

Agitation Rate (rpm)	Initial C:N Ratio	Culture Time (h)	Total Sugars (g/L)	DCW Produced (g/L)	Lipids Produced (g/L)	Lipid Content (%)	Lipid Yield (g/g Sugar)	Lipid Productivity (g/L/h) ^a	DCW Productivity (g/L/h) ^a	Sugar Consumption (g/L/h) ^a	Specific Lipid Productivity g/(g DCW h ⁻¹) ^a
200	24:1	160	59.99	20.64	3.87	18.74	0.064	0.026	0.139	0.414	0.001
200	48:1	169	57.59	18.37	5.51	30.00	0.096	0.036	0.114	0.374	0.002
200	72:1	192	58.90	18.28	10.03	54.85	0.170	0.060	0.112	0.376	0.004
150	72:1	196	60.33	17.56	7.84	44.63	0.130	0.044	0.086	0.316	0.004
300	72:1	192	58.90	19.08	5.42	28.43	0.092	0.035	0.125	0.392	0.002
200	≈ 20:1 ^b	193	69.32	24.63	9.38	38.07	0.135	0.049	0.128	0.359	0.002

^a Productivities calculated using measurements taken between 24-168 h.^b C:N ratio for AFEX hydrolysate is estimated. All other conditions utilize defined minimal medium with glucose. (See Methods)

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Table 2:

Comparison of fatty acid profiles of *L. starkeyi* at variable C:N ratios and agitation rates.

Agitation Rate (rpm)	Initial C:N Ratio	Fatty Acid Content (%)										
		Lauric (12:0)	Myristic (14:0)	Palmitic (16:0)	Palmitoleic (16:1)	Steric (18:0)	Oleic (18:1)	Linoleic (18:2)	Linolenic (18:3)	Arachidic (20:0)	Behenic(22:0)	Nervonic(24:1)
200	24:1	0.0	0.4	37.5	3.1	12.2	44.6	1.5	0.1	0.1	0.0	0.5
200	48:1	0.2	0.9	30.3	3.1	15.8	46.3	1.6	0.0	0.4	0.2	1.2
200	72:1	0.0	0.5	36.9	2.4	15.1	42.9	1.2	0.0	0.2	0.3	0.5
150	72:1	0.0	3.4	36.5	5.5	16.0	36.4	0.9	0.0	0.7	0.6	0.0
300	72:1	0.0	0.9	37.8	3.2	13.0	41.8	2.5	0.1	0.1	0.0	0.5
200	≈ 20:1 ^a	0.0	0.9	35.2	3.0	10.3	46.5	2.7	0.0	0.8	0.6	0.0
Average		0.0	1.2	35.7	3.4	13.7	43.1	1.7	0.0	0.4	0.3	0.5
Palm Oil^b		0.1	0.7	36.7	0.1	6.6	46.1	8.6	0.3	0.4	0.1	0.0

^aC:N ratio for AFEX hydrolysate is estimated. All other conditions utilize defined minimal medium with glucose. (See Methods)^b Palm Oil fatty acid profile as reported by (Ramos et al., 2009).

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References

- Ageitos, J.M., Vallejo, J.A., Veiga-Crespo, P., and Villa, T.G. (2011). Oily yeasts as oleaginous cell factories. *Appl. Microbiol. Biotechnol.* **90**, 1219–1227.
- Angerbauer, C., Siebenhofer, M., Mittelbach, M., and Guebitz, G.M. (2008). Conversion of sewage sludge into lipids by *Lipomyces starkeyi* for biodiesel production. *Bioresour. Technol.* **99**, 3051–3056.
- Anschau, A., Xavier, M.C.A., Hernalsteens, S., and Franco, T.T. (2014). Effect of feeding strategies on lipid production by *Lipomyces starkeyi*. *Bioresour. Technol.* **157**, 214–222.
- Balan, V., Bals, B., Chundawat, S.P.S., Marshall, D., and Dale, B.E. (2009). Lignocellulosic Biomass Pretreatment Using AFEX. In *Biofuels*, J.R. Mielenz, and J.M. Walker, eds. (Humana Press), pp. 61–77.
- Bligh, E.G., and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917.
- Calvey, C.H., Willis, L.B., and Jeffries, T.W. (2014). An optimized transformation protocol for *Lipomyces starkeyi*. *Curr. Genet.* **60**, 223–230.
- Chen, X., Li, Z., Zhang, X., Hu, F., Ryu, D.D.Y., and Bao, J. (2009). Screening of oleaginous yeast strains tolerant to lignocellulose degradation compounds. *Appl. Biochem. Biotechnol.* **159**, 591–604.
- Davies, R.J., Holdsworth, J.E., and Reader, S.L. (1990). The effect of low oxygen uptake rate on the fatty acid profile of the oleaginous yeast *Apiotrichum curvatum*. *Appl. Microbiol. Biotechnol.* **33**, 569–573.
- Gong, Z., Wang, Q., Shen, H., Hu, C., Jin, G., and Zhao, Z.K. (2012). Co-fermentation of cellobiose and xylose by *Lipomyces starkeyi* for lipid production. *Bioresour. Technol.* **117**, 20–24.
- Grigoriev, I.V., Nordberg, H., Shabalov, I., Aerts, A., Cantor, M., Goodstein, D., Kuo, A., Minovitsky, S., Nikitin, R., Ohm, R.A., et al. (2011). The Genome Portal of the Department of Energy Joint Genome Institute. *Nucleic Acids Res.* **40**, D26–D32.

- 489 11. Huang, C., Chen, X.-F., Yang, X.-Y., Xiong, L., Lin, X.-Q., Yang, J., Wang, B., and Chen, X.-D. (2014).
490 Bioconversion of Corn cob Acid Hydrolysate into Microbial Oil by the Oleaginous Yeast *Lipomyces*
491 *starkeyi*. Appl. Biochem. Biotechnol. 172, 2197–2204.
- 492 12. Jin, M., Slininger, P.J., Dien, B.S., Waghmode, S., Moser, B.R., Orjuela, A., Sousal, L. da C., and Balan,
493 V. (2015). Microbial lipid-based lignocellulosic biorefinery: feasibility and challenges. Trends
494 Biotechnol. 33, 43–54.
- 495 13. Kang, H.K., Lee, J.H., Kim, D., Day, D.F., Robyt, J.F., Park, K.-H., and Moon, T.-W. (2004). Cloning and
496 expression of *Lipomyces starkeyi* α -amylase in *Escherichia coli* and determination of some of its
497 properties. FEMS Microbiol. Lett. 233, 53–64.
- 498 14. Lin, J., Shen, H., Tan, H., Zhao, X., Wu, S., Hu, C., and Zhao, Z.K. (2011). Lipid production by *Lipomyces*
499 *starkeyi* cells in glucose solution without auxiliary nutrients. J. Biotechnol. 152, 184–188.
- 500 15. Long, T.M., Su, Y.-K., Headman, J., Higbee, A., Willis, L.B., and Jeffries, T.W. (2012). Cofermentation
501 of Glucose, Xylose, and Cellobiose by the Beetle-Associated Yeast *Spathaspora passalidarum*. Appl.
502 Environ. Microbiol. 78, 5492–5500.
- 503 16. Meng, X., Yang, J., Xu, X., Zhang, L., Nie, Q., and Xian, M. (2009). Biodiesel production from
504 oleaginous microorganisms. Renew. Energy 34, 1–5.
- 505 17. Naganuma, T., Uzuka, Y., and Tanaka, K. (1985). Physiological factors affecting total cell number and
506 lipid content of the yeast, *Lipomyces starkeyi*. J. Gen. Appl. Microbiol. 31, 29–37.
- 507 18. Papanikolaou, S., Chevalot, I., Komaitis, M., Marc, I., and Aggelis, G. (2002). Single cell oil production
508 by *Yarrowia lipolytica* growing on an industrial derivative of animal fat in batch cultures. Appl.
509 Microbiol. Biotechnol. 58, 308–312.
- 510 19. Ramos, M.J., Fernández, C.M., Casas, A., Rodríguez, L., and Pérez, A. (2009). Influence of fatty acid
511 composition of raw materials on biodiesel properties. Bioresour. Technol. 100, 261–268.
- 512 20. Ratledge, C. (2004). Fatty acid biosynthesis in microorganisms being used for Single Cell Oil
513 production. Biochimie 86, 807–815.
- 514 21. Ratledge, C., and Cohen, Z. (2008). Microbial and algal oils: Do they have a future for biodiesel or as
515 commodity oils? Lipid Technol. 20, 155–160.
- 516 22. Ratledge, C., and Wynn, J. (2002). The biochemistry and molecular biology of lipid accumulation in
517 oleaginous microorganisms. In Advances in Applied Microbiology, (Elsevier), pp. 1–44.
- 518 23. Sitepu, I., Selby, T., Lin, T., Zhu, S., and Boundy-Mills, K. (2014a). Carbon source utilization and
519 inhibitor tolerance of 45 oleaginous yeast species. J. Ind. Microbiol. Biotechnol. 41, 1061–1070.
- 520 24. Sitepu, I.R., Garay, L.A., Sestric, R., Levin, D., Block, D.E., German, J.B., and Boundy-Mills, K.L.
521 (2014b). Oleaginous yeasts for biodiesel: Current and future trends in biology and production.
522 Biotechnol. Adv. 32, 1336–1360.

523 25. Sitepu, I.R., Jin, M., Fernandez, J.E., Sousa, L. da C., Balan, V., and Boundy-Mills, K.L. (2014c).
524 Identification of oleaginous yeast strains able to accumulate high intracellular lipids when cultivated
525 in alkaline pretreated corn stover. *Appl. Microbiol. Biotechnol.* **98**, 7645–7657.

526 26. Starkey, R.L. (1946). Lipid Production by a Soil Yeast. *J. Bacteriol.* **51**, 33–50.

527 27. Su, Y.-K., Willis, L.B., and Jeffries, T.W. (2014). Effects of aeration on growth, ethanol and polyol
528 accumulation by *Spathaspora passalidarum* NRRL Y-27907 and *Scheffersomyces stipitis* NRRL Y-
529 7124. *Biotechnol. Bioeng.* **112**, 457–469.

530 28. Subramaniam, R., Dufreche, S., Zappi, M., and Bajpai, R. (2010). Microbial lipids from renewable
531 resources: production and characterization. *J. Ind. Microbiol. Biotechnol.* **37**, 1271–1287.

532 29. Wild, R., Patil, S., Popovi, M., Zappi, M., Dufreche, S., and Bajpai, R. (2010). Lipids from *Lipomyces*
533 *starkeyi*. *Food Technol. Biotechnol.* **48**, 329–335.

534 30. Workman, M., Holt, P., and Thykaer, J. (2013). Comparing cellular performance of *Yarrowia lipolytica*
535 during growth on glucose and glycerol in submerged cultivations. *AMB Express* **3**, 58.

536 31. Yang, X., Jin, G., Gong, Z., Shen, H., Song, Y., Bai, F., and Zhao, Z.K. (2014). Simultaneous utilization of
537 glucose and mannose from spent yeast cell mass for lipid production by *Lipomyces starkeyi*.
538 *Bioresour. Technol.* **158**, 383–387.

539 32. Yen, H.-W., and Liu, Y.X. (2014). Application of airlift bioreactor for the cultivation of aerobic
540 oleaginous yeast *Rhodotorula glutinis* with different aeration rates. *J. Biosci. Bioeng.* **118**, 195–198.

541 33. Yen, H.-W., and Zhang, Z. (2011). Effects of dissolved oxygen level on cell growth and total lipid
542 accumulation in the cultivation of *Rhodotorula glutinis*. *J. Biosci. Bioeng.* **112**, 71–74.

543 34. Yu, X., Zheng, Y., Dorgan, K.M., and Chen, S. (2011). Oil production by oleaginous yeasts using the
544 hydrolysate from pretreatment of wheat straw with dilute sulfuric acid. *Bioresour. Technol.* **102**,
545 6134–6140.

546 35. Zhao, X., Kong, X., Hua, Y., Feng, B., and Zhao, Z. (Kent) (2008). Medium optimization for lipid
547 production through co-fermentation of glucose and xylose by the oleaginous yeast *Lipomyces*
548 *starkeyi*. *Eur. J. Lipid Sci. Technol.* **110**, 405–412.

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