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*Research article*

# Identification of superior lipid producing Lipomyces and Myxozyma yeasts

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**Abstract:** Oleaginous yeasts are of interest for production of single cell oils from sugars. Eighteen members of the *Lipomyces* and *Myxozyma* clade were screened for lipid production when cultured on 10%w/v glucose. The highest ranking yeasts included *L. tetrasporus* (21 g/L), *L. spencer-martinsiae* (19.6 g/L), and *L. lipofer* (16.7 g/L). By contrast, *Rhodosporidium toruloides*, which was included as a positive control, produced 16.7 g/L. The *L. tetrasporus* and *L. lipofer* were further characterized for growth and lipid production on sugars present in biomass hydrolysates. These included L-arabinose, xylose, and an equal glucose and xylose mixture. *L. tetrasporus* had lipid titers of 16.3–20.8 g/L and *L. lipofer* 12.5–17.0 g/L. When both strains were grown on an equal mixture of glucose and xylose, xylose was consumed immediately following glucose. Lipid contents for the yeasts consisted primarily of C18:1 and C16:0, which makes them a promising source of lipids for fuel applications.

**Keywords:** oleaginous yeasts; single cell oils; xylose; lipid

## 1. Introduction

It is historically known that some yeasts become highly enriched for lipids when grown in

media with excess sugar and a deficiency of essential macro element(s), typically nitrogen [1]. Yeasts that are able to accumulate at least 20% of their biomass as lipids are termed oleaginous yeasts [2]. These lipids consist of triglycerides (TAGs) and are observed as prominent granule(s) occurring within the yeast cell when viewed microscopically. These TAGs act as a carbon and energy reserve that is advantageous for yeasts living in niches that oscillate between feast and famine nutrient regimes [1]. The yeast extracted TAGS are similar in composition to vegetable oils and are of interest as use in sustainable production of fuel, food ingredients, and industrial chemical and polymer applications [3,4].

The oleaginous phenotype appears to be uncommon as it is observed in approximately 70 of the known 1500 yeast species [1]. Production of lipids for storage involves a biochemical cascade mechanism that has been elucidated for a few representative strains [5,6]. The trait appears to be randomly distributed across multiple clades within the Ascomycota and Basidiomycota phyla [1]. Several exceptional lipid producers occur within the *Lipomyces* clade; as might be expected from the genus name. Reported results for *Lipomyces starkeyi* strains, for example, are among the highest ever reported (41.8 g/L–67.9 g/L) when cultivated in 2-stage or fed batch cultures [7-9]. Despite these exceptional titers, *Lipomyces* yeasts have not been systemically screened for lipid production. There is merit in having results in which the yeasts have been challenged with a uniform culturing method because differences in the C:N ratio, nitrogen source, and aeration are known to influence lipid production [4]. A uniform set of data allows for trends to be uncovered as to how lipid production varies across and within species of this notable clade. An important question that we seek to address here is if a yeast's evolutionary relationship within the clade predicts its relative ability to accumulate lipids.

We also have another purpose in choosing the *Lipomyces* clade to screen. We are seeking to identify yeasts suitable for producing single cell oil (SCO) from lignocellulosic biomass. Using fibrous biomass as a feedstock is advantageous because it is sustainable, abundant, and has a low green house footprint compared to glucose originating from row crops [10,11]. It would also afford the economic opportunity to utilize agricultural waste (e.g. corn stover) and production of perennial biomass crops grown on marginal farmland, which is unsuitable for growing food and feed crops [12]. We think that *Lipomyces* yeasts are promising candidates for this purpose because they have a broad sugar range and herbaceous lignocelluloses contains a mixture of sugars including glucose, xylose, and L-arabinose [13]. Many yeasts do not or are limited in their ability to grow and produce lipids on L-arabinose and xylose, including *Yarrowia lipolyitca* [14]. This is notable because *Y. lipolyitca* Po1f has been genetically engineered to produce lipids on glucose to high titers (e.g., 40 g/L) [15] and health promoting polyunsaturated lipids [16]. Furthermore, we have observed in a more limited screening of *Lipomyces* on authentic biomass hydrolyzates that many were robust for growth and lipid production [17].

Measurement of lipid production by yeasts is problematic in part because they are stored within the yeast cells. Here we chose to use the sulfo-phospho-vanillin (SPV) [18-21] assay because it is sensitive (10 μg lipids) [18], convenient, and only requires access to a standard laboratory spectrophotometer. Other methods for measuring lipids include gravimetric, Nile red or BODIPY fluorescent staining [22-24], time-domain NMR [25], thin layer chromatography, and gas chromatography of FAMEs with flame ionization or mass spectrometry detection [26]. These alternatives are either tedious, require too much sample for small flask cultures, or require specialized instrumentation. The SPV assay was originally developed for human serum samples but

has been applied to bacterial [18], algal [21], and even yeast samples [20]. However, the single yeast study only included *R. glutinis* [20]. Herein the SPV assay is validated on multiple yeast species and tested for interference from non-lipid components.

In the current study, 18 members of the *Lipomyces*/*Myxozyma* clade were screened for lipid production on glucose medium with a high C:N ratio. In addition, four unrelated but commonly reported oleaginous yeast species (*Cryptococcus aerius, Torulaspora delbrueckii, Rhodosporidium toruloides,* and *Y. lipolytica*) are included for comparison. Finally, two of the exceptional yeasts from this screen are further characterized for growth on L-arabinose, glucose, xylose, and an equal mixture of the later two.

## 2. Methods

### *2.1. Source and maintenance of cultures*

All yeast cultures were generously supplied by the ARS culture collection (Peoria, IL) and strains are identified by accession number. Yeast cultures were routinely stored in glycerol solution (20% v/v) at −80 ℃.

### *2.2. Media formulations*

Yeasts were grown on Lipid Production Medium (LPM, 3 g/L peptone and 8 g/L yeast extract) and supplemented (unless stated otherwise) with 100 g/L glucose. Yeast inocula were grown on YP2D (20 g/L peptone, 10 g/L yeast extract, 20 g/L glucose) supplemented with 15 g/L Difco agar for solid cultures.

### *2.3. Phosphoric acid–Vanillin lipid assay*

Lipids were measured using the phosphoric vanillin lipid (PVL) assay modified from Izard and Limberger (2003). The yeast suspension (50 µl) was mixed with 1 mL of 18 M sulfuric acid in a threaded 13 × 100 mm Pyrex™ test tube (Corning # 9826-13) and heated at 100 ℃ for 10 min in a dry heating bath. The solution was cooled for 5 min in an ambient water bath. Next, 2.5 mL of the vanillin-phosphoric acid was added and reacted for 15 min at 37 ℃. The test tube was cooled for 10 min in water bath at ambient temperature. The absorbance of each reaction was read at 530 nm against a reference sample prepared with 50 µl water. It is believed that sulfuric acid reacts with the unsaturated lipid to form a carbonium ion that reacts with the activated carbonyl group of the phospho-vanillin to produce a colored complex that has a maximum absorption at 525 nm [19].

Absorbance measurements were converted to lipid concentration using a calibration curve prepared using refined corn oil. Corn oil (100 mg) was dissolved in 2:1 chloroform:methanol (20 mL) and the stock solution was loaded into the assay mixture at 50–250 µg. A standard curve was run with each set. The vanillin-phosphoric acid solution was prepared that day by dissolving 0.12 g vanillin in 20 mL $dH_2O$, and adjusting the volume to 100 mL with 85% o-phosphoric acid.

*2.4. Flask cultivation*

Lipid production flask cultures were routinely cultivated in 250 mL baffled Erlenmeyer flasks capped with breathable silicone sponge closures (Bellco, Vineland, NJ) and filled with 50 mL of LPM supplemented with 100 g/L sugar. Cultures were inoculated to 0.1 $OD_{600}$ (optical density at 600 nm). Flasks were incubated at 25 ℃ and mixed at 200 rpm in a refrigerated shaker (Innova 4230, New Brunswick, NJ). Precultures were prepared by transferring a single colony grown on solid YP2D to a 250 mL Erlenmeyer flask filled with 50 mL of YP2D and incubating (200 rpm, 25 ℃) for 48 h.

Lipid production cultures were sampled every 1–2 days by withdrawing 1 mL of broth to a microfuge tube. Samples were clarified by centrifuging (10 min, 16,000x g). The liquid was transferred to a HPLC target vial with the appropriate dilution. The pellet was washed with 1 mL of $dH_2O$ and re-suspended to 1 mL volume and stored at −20 ℃ to await lipid analysis.

*2.5. Micro growth cultures*

Growth curves were conducted using the BioLector® bench top micro bioreactor (m2p-labs, Aachen, Germany) equipped with 48 well FlowerPlate® plates. The BioLector is designed for enhanced aeration and equipped with automated backscatter measurement of cell biomass. The settings used were: 0.80 mL LPM cultures, 25 ℃, 85–90% humidity, 1500 rpm mixing, and backscatter (620 nm) was measured at gains of 1, 10, and 20 with each well sampled every 30 min. Well cultures were inoculated to a beginning $OD_{600}$ of 0.1. The seed culture was prepared as described above for the flask cultures.

*2.6. Bioreactor runs*

Larger volumes of yeast to produce extracted lipids were grown in computer controlled 2.5 L working volume Biostat B bioreactors (Sartorius AG, Goettingen, Germany). The medium used was 1.5 L of LPM supplemented with 100 g/L glucose and the culture was inoculated with 50 mL of a flask culture. Similar culture conditions were used for all yeasts: pH 6 (controlled with 4 N NaOH), 25 ℃, 800 rpm mixing, and 1.0 L/min aeration. Sugar consumption was monitored and following its exhaustion, the cell metabolism was halted by heating at 55 ℃ for 30 min. Cells were harvested by centrifugation 16,800 x g, 10 min; Eppendorf 5418 centrifuge, Hamburg, Germany ), freeze dried (freeze dry system, Labconco Corporation, Kansas City, MO), and stored at −20 ℃. Yeast inocula were grown in a foam capped 250 mL Erlenmeyer baffled flasks filled with 50 mL of YP2D with mixing at 200 rpm at 25 ℃ in a refrigerated shaker (Innova 4230, New Brunswick) for 3 days.

*2.7. Extraction of lipids*

Freeze dried cells (10 g) were ruptured by ball milling for 1 min (settings: 30 cycles per sec, 25 mL 316 stainless steel bottles filled with 20 mm balls, Retsch Mill model MM301). Ground cells were resuspended in 80 mL of $dH_2O$. Lipid extraction solvent (200 mL methanol and 100 mL chloroform) was added and the mixture was agitated in an ultrasonic bath for 30 min (Branson ultrasound bath model 2510). The suspension was then mixed at 4 ℃ for 10 min using a magnetic

stirrer and 100 mL of chloroform and 100 mL of $dH_2O$ was added. The mixture was centrifuged for 15 min at 3000 rpm and 20 ℃ (Sorvall floor centrifuge RC5C Plus, rotor SLA 1500, 867 x g) to separate the mixture into two layers. The lower layer (e.g. chloroform) was recovered using a glass pipette and transferred to a separatory funnel, which was allowed to settle overnight. The lower layer was removed to a pre-weighed beaker. The solvent was evaporated at room temperature and the oil weighed to determine the yield.

### *2.8. Analytical methods*

Fatty acid composition was determined using extracted lipids. Derivatization to FAMEs (via methanolic KOH) was performed as described previously [1] and analyzed using a PerkinElmer (Waltham, MA) Clarus 580 GC equipped with an FID and an HP88 capillary column (30 m × 0.25 mm i.d., 0.20 μm film thickness). Carrier gas was $H_2$ with a flow rate of 15.0 mL/min. The temperature program was: hold at 100 ℃ for 5 min, ramp from 100 ℃ to 220 ℃ at 10 ℃/min and hold at 220 ℃ for 15 min. Injection volume was 1.0 μl with a split ratio of 10.0:1. The concentration of sample in hexane was approximately 20 mg/mL. The injector and detector temperatures were 240 ℃ and 280 ℃, respectively. FAME peaks were identified by comparison to reference standards (>99%, Nu-Chek Prep, Inc., Elysian, MN).

Sugar concentrations were measured in culture broths using a SpectraSYSTEM™ liquid chromatography system (Thermo Electron Corporation, Waltham, MA equipped with an automatic sampler, column heater, isocratic pump, refractive index detector, and computer based integrator running Chromquest ver. 2.5 (Thermo Electron Corporation). Samples (20 μl) were injected onto a sugar analysis column (Aminex HPX-87H Column, 300 × 7.8 mm, Bio Rad Laboratories, Inc., Hercules, CA) and eluted with 5 mM sulfuric acid at 0.6 mL/min and 65 ℃ as previously described.

## 3. Results and Discussion

### *3.1. Lipid quantification assay*

Our first aim was to validate a simple and sensitive assay for measurement of yeast lipids. Quantification of the SPV assay was first tested by extracting lipids from *L tetrasporus* Y-11562 and *L. lipofer* Y-11555. The responses of the assay were correlated to the amounts of added lipid (Figure 1A). Refined corn oil was used an external control because it has a similar fatty acid profile. The assay was quantitative for determining lipids for both yeasts ($R > 0.99$) and furthermore the absolute responses virtually overlapped with the refined corn oil.

Next the assay was adapted for use with intact cells. Six yeast strains were cultured and harvested following lipid production. The yeasts were washed and the amount of lipid measured at 5 concentrations. Assay linearity was tested for each of the strains by plotting amounts of measured lipids versus amounts of added biomass (example plot: Figure 1B). Over the entire set, the correlations between measured lipid contents and added yeast cells were all 1.00 and the response factor varied from 0.85–1.00 (Table 1).

The SPV assay had been earlier evaluated for responses from non-lipid substrates, including DNA, protein, minerals, and glycerol [18]. We broaden this list to include non-lipid compounds associated with yeast cultures (Table 2). Pentose showed no interference and hexose a small amount

(4.5–4.6%) on a weight basis. Next the nitrogen sources (ammonium sulfate, peptone, and yeast extract) were evaluated and each showed less than 1% interference with the assay. The overall medium had a background of 5.9%. This amount of interference should be easily eliminated provided the yeasts are washed prior to the assay. Finally, yeast extract can be used to model low lipid containing yeast biomass and this as well had a minor response compared to lipids (0.9%). Therefore the lipid assay appears robust to various yeasts and provided the yeast cells are washed interference should not be a concern.

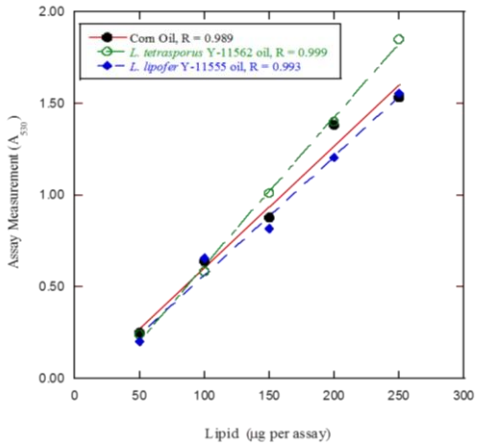


**Figure 1A. Validation of phosphoric vanillin lipid (PVL) assay for measurement of yeast lipids.** Measurements for the PVL assay (absorbance 500 nm) were linear with the amounts of extract lipid added to the assay for both yeasts and (control) corn oil.

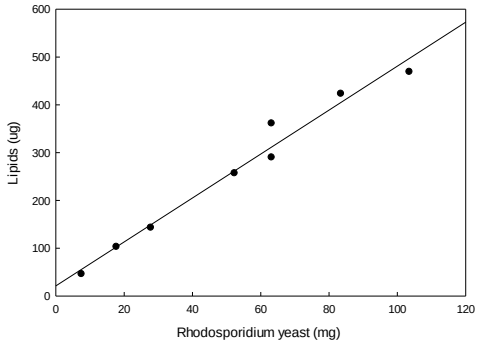


**Figure 1B. Validation of phosphoric vanillin lipid (PVL) assay for measurement of yeast lipids.** Assay results were also correlated to lipid contents when whole yeast cells were added to the assay at varying amounts (dry weight basis); also see Table 1. Corn oil was used as an external standard for conversion of absorbance units to µg of lipids.

**Table 1. Response of phosphoric vanillin assay for six lipid producing yeasts.**

| Yeast | Strain | Slope | Correlation |
|---|---|---|---|
| *Yarrowia lipolytica* | YB437 | 0.93 | 1.00 |
| *Cryptococcus aerius* | Y1399 | 0.99 | 1.00 |
| *Lipomyces lipofer* | Y11555 | 0.85 | 1.00 |
| *Rhodosporium toruloides* | Y1091 | 1.00 | 1.00 |
| *Lipomyces tetrasporus* | Y11562 | 0.99 | 1.00 |
| *Lipomyces tetrasporus* | Y27496 | 1.00 | 1.00 |

**Table 2. Testing various media ingredients for interference with lipid assay.**

| Compound | A530[1] | % Interference[2] |
|---|---|---|
| *Sugar* | | |
| L-Arabinose | 0 | 0.0% |
| Galactose | 0.0673 | 4.5% |
| Glucose | 0.0683 | 4.6% |
| Xylose | 0 | 0.0% |
| *Nitrogen* | | |
| Ammonium sulfate | 0 | 0.0% |
| Peptone | 0.0047 | 0.3% |
| Yeast extract | 0.013 | 0.9% |
| *Other* | | |
| Medium + Glucose | 0.089 | 5.9% |
| Corn oil | 1.499 | 100.0% |

Notes: (1) uniform 200 μg samples were used;

(2) % interference = A530 of compound/A530 of oil × 100.

### *3.2. Screening lipid producing yeasts*

*Lipomyces* yeasts, as the genus name implies, are oleaginous but have yet to be systemically screened for lipid production. Eighteen lipid producing yeasts were chosen from the *Lipomyces*/*Myxozyma* clade (Figure 2) for screening based upon their availability from the ARS culture collection and ability to use L-arabinose and xylose. In addition, four other lipid producing yeast strains (*C. aerius, T. delbrueckii*, *R. toruloides,* and *Y. lipolytica*), which have been widely reported for their exceptional lipid production, were included for comparison. The yeasts were cultured with glucose (100 g/L) in nitrogen limited growth medium and monitored for cell biomass and lipid contents (Table 3). Maximum lipid titers were 1.4–21.0 g/L. *Y. lipolytica* had the highest rate of glucose consumption and produced 10.8 g/L lipids. Most of the other yeasts took 6-7 days to consume the glucose. The top ranked lipid producing yeast strains were *L. tetrasporus* Y-11562, *L. spencer-martinsiae* Y-7042 (formally *L. koneonenkoae*), *L. lipofer* Y-11555, and *R. toruloides* Y-1091. These yeasts were also the most enriched for lipid contents with 68.6% g/g biomass, 62.0% g/g, 66.7% g/g, and 53.9% g/g, respectively. Assuming that the maximum yield of lipids from glucose is 0.33 g/g [27], the highest conversion efficiency was observed for *L. tetrasporus* Y-11562 (63.6% of theoretical). Lipid content (% g/g) was correlated with maximum lipid titer (g/L,

$R^2$ = 0.87), but not total cell biomass (g/L; $R^2$ = 0.33). These trends are expected as total cellular biomass would be determined in part by the nitrogen content of the medium.

The most noticeable trend from this study is that *Myxozyma* strains were weaker lipid producers than the major *Lipomyces* grouping (*L. lipofer* to *L. tetrasporus*) (Figure 2; Table 3). The average lipid contents for the *Lipomyces* and *Myxozyma* groupings were 42.4 ± 19.7 % w/w (*n* = 11) and 15.9 ± 8.4 % w/w (*n* = 7), respectively. Also, 5 of the *Myxozyma* and only 2 of the *Lipomyces* yeasts did not meet the oleaginous criterion (e.g. > 20% lipid content).

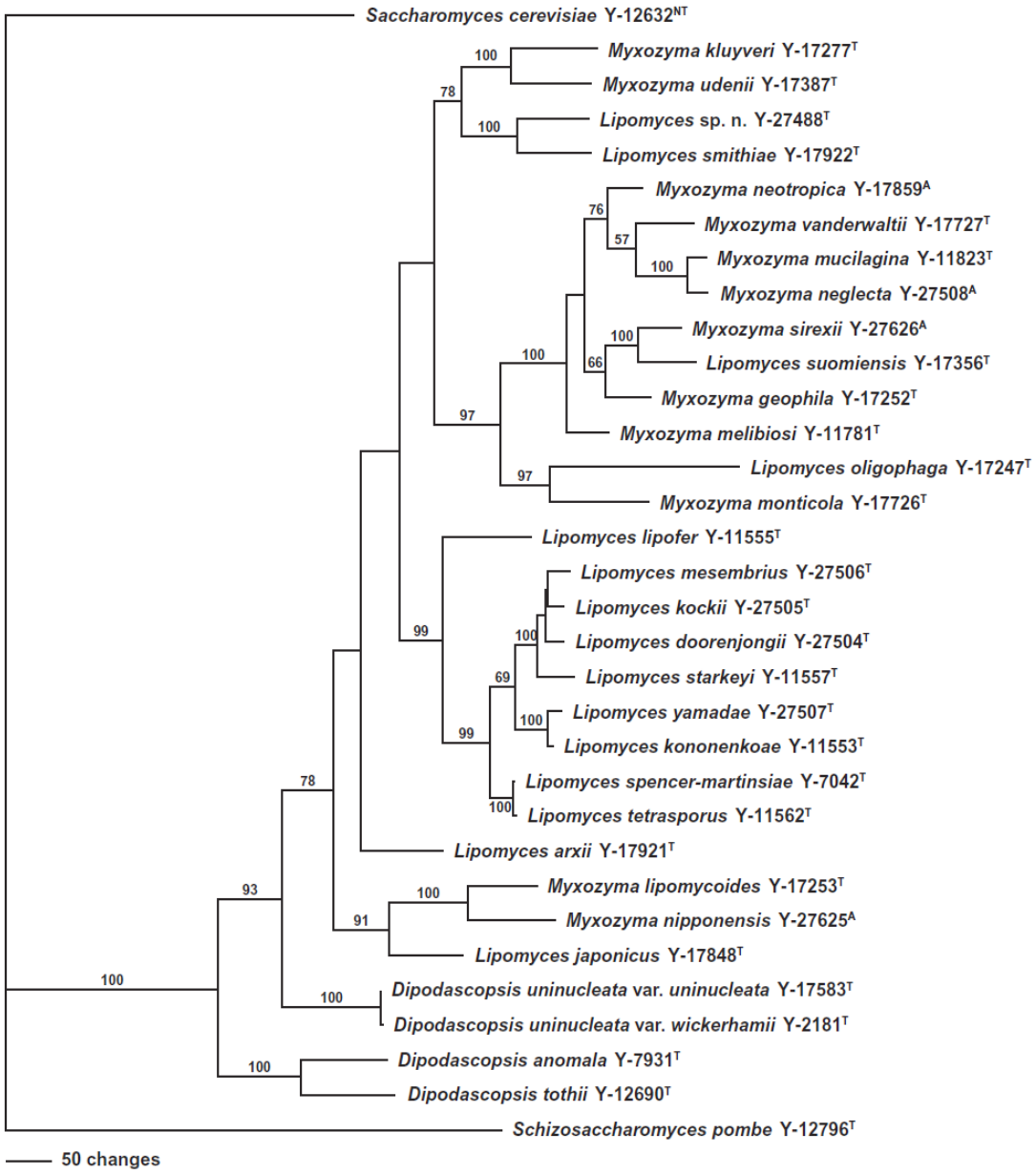


**Figure 2. Phylogeny chart for *Lipomyces*/*Myxozyma* clade. Adapted from [14].**

**Table 3. Comparison of lipid production for *Lipomyces* and *Myxozyma* clade.**

| Species | Strain | Lipids (g/L) | % Lipids (% wt/wt) | Time (days) | Cell Biomass (g/L) | Efficiency[a] (% maximum) |
|---|---|---|---|---|---|---|
| *Cryptococcus aerius* | Y-1399 | 9.1 ± 1.2 | 43.3 ± 5.0 | 6 | 20.9 ± 0.5 | 27.6 |
| *Lipomyces arxii* | Y-17921 | 1.4 ± 0.1 | 8.0 ± 1.3 | 6 | 17.7 ± 2.6 | 4.2 |
| *Lipomyces doorenjongii* | Y-27504 | 10.7 ± 0.61 | 39.6 ± 0.8 | 7 | 26.9 ± 0.86 | 32.4 |
| *Lipomyces oligophaga* | Y-17247 | 7.65 ± 0.8 | 31.4 ± 2.4 | 8 | 24.4 ± 0.3 | 23.2 |
| *Lipomyces spencer-martinsiae* | Y-7042 | 19.6 ± 1.3 | 62.0 ± 7.0 | 5 | 31.7 ± 0.7 | 59.4 |
| *Lipomyces kononenkoae* | Y-11553 | 2.9 ± 0.3 | 16.6 ± 1.9 | 11 | 14.8 ± 0.5 | 8.8 |
| *Lipomyces lipofer* | Y-11555 | 16.7 ± 1.4 | 66.7 ± 2.8 | 6 | 25.0 ± 1.0 | 50.6 |
| *Lipomyces starkeyi* | Y-11557 | 3.0 ± 1.3 | 12.3 ± 4.9 | 4 | 24.7 ± 0.4 | 9.1 |
| *Lipomyces starkeyi* | Y-27495 | 15.4 ± 1.2 | 51.2 ± 1.2 | 7 | 30.7 ± 3.0 | 46.7 |
| *Lipomyces starkeyi* | Y-27494 | 15.0 ± 0.1 | 49.0 ± 0.5 | 6 | 29.6 ± 6.2 | 45.5 |
| *Lipomyces starkeyi* | Y-27493 | 11.5 ± 1.0 | 36.3 ± 2.1 | 6 | 43.9 ± 4.2 | 34.8 |
| *Lipomyces tetrasporus* | Y-11562 | 23.6 ± 0.2 | 68.6 ± 3.5 | 6 | 30.6 ± 1.8 | 63.6 |
| *Lipomyces tetrasporus* | Y-27496 | 12.5 ± 0.5 | 44.1 ± 0.1 | 6 | 28.3 ± 0.7 | 37.9 |
| *Lipomyces tetrasporus* | Y-27497 | 2.7 ± 0.2 | 20.4 ± 2.3 | 6 | 13.1 ± 1.5 | |
| *Myxozyma geophila* | Y-17252 | 2.3 ± 0.2 | 11.4 | 7 | 20.0 ± 1.1 | 7.0 |
| *Myxozyma lipomycoides* | Y-17253 | 3.9 ± 0.7 | 19.5 ± 0.0 | 7 | 20.1 ± 3.4 | 11.8 |
| *Myxozyma mucilagina* | Y-11823 | 1.9 ± 0.1 | 9.3 ± 0.4 | 7 | 20.3 ± 0.05 | 5.8 |
| *Myxozyma udenii* | Y-17387 | 4.2 ± 0.51 | 20.6 ± 1/8 | 6 | 20.8 ± 0.5 | 12.7 |
| *Myxozyma vanderwaltii* | Y-17727 | 1.9 ± 0.10 | 11.1 ± 0.0 | 5 | 17.3 ± 0.2 | 5.8 |
| *Rhodosporidium toruloides* | Y-1091 | 16.7 ± 1.3 | 53.9 ± 2.8 | 11 | 31.0 ± 0.7 | 50.6 |
| *Torulaspora delbrueckii* | Y-63011 | 5.5 ± 0.4 | 39.6 ± 6.4 | 3 | 13.9 ± 3.2 | 16.7 |
| *Trigonopsis varaibilis* | Y-1579 | 7.9 | 28.9 | 7 | 27.5 | 23.9 |
| *Yarrowia lipolytica* | YB-437 | 10.8 ± 0.0 | 39.3 ± 0.83 | 4 | 27.4 ± 0.58 | 32.7 |

[a] efficiency refers to the % of the maximum metabolic yield for lipids realized, which is 0.33 g lipids/g glucose.

**Table 4. Literature-cited results for lipid production by various members of the *Lipomyces* clade in simple batch cultures.**

| Yeast | Strain ID | Carbon Source | Lipid Content (% g/g) | Lipid Conc. (g/L) | Lipid Yield (g/g) | Reference |
|---|---|---|---|---|---|---|
| *Lipomyces anomalus* | CBS 6740 | glucose | 7.5 | na | na | [34] |
| *Lipomyces doorenjongii* | CBS 7542 | glucose | 72.3 | 6.11 | na | [46] |
| *Lipomyces japonicus* | CBS 7319 | glucose | 9.0 | na | na | [34] |
| *Lipomyces kockii* | CBS 7729 | glucose | 78.0 | 16.0 | 0.16 | [46] |
| *Lipomyces kockii* | CBS 7731 | glucose | 50.0 | 13.2 | 0.13 | [46] |
| *Lipomyces kononenkoae* | CBS 2514 (NRRL Y-11553) | glucose | 18.0 | na | na | [34] |
| *Lipomyces lipofer* | IBPhM y-281 | ethanol | 37.1 | na | 0.11 | [29] |
| *Lipomyces lipofer* | IBPhM y-693 | ethanol | 51.5 | na | 0.18 | [29] |
| *Lipomyces lipofer* | CBS 944 (NRRL Y-11555) | glucose | 40.0 | na | na | [35] |
| *Lipomyces lipofer* | CBS 944 | glucose | 27.5 | na | na | [34] |
| *Lipomyces lipofer* | NRRL Y-11555 | glucose | 36.0 | 0.48 | na | [44] |
| *Lipomyces mesembrius* | CBS 7737 | glucose | 44.2 | 3.07 | 0.03 | [46] |
| *Lipomyces mesembrius* | CBS 7661 | glucose | 9.6 | 0.79 | 0.10 | [46] |
| *Lipomyces mesembrius* | CBS 7600 | glucose | 11.0 | 0.97 | 0.11 | [46] |
| *Lipomyces mesembrius* | CBS 7601 | glucose | 12.9 | 1.26 | 0.13 | [46] |
| *Lipomyces starkeyi* | CBS 1807 | glucose | 27.0 | na | na | [34] |
| *Lipomyces starkeyi* | IBPhM y-282 | ethanol | 43.1 | na | 0.07 | [29] |
| *Lipomyces starkeyi* | IBPhM y-694 | ethanol | 46.6 | na | 0.15 | [29] |
| *Lipomyces starkeyi* | DSM 70295 | glucose | 56 | 7.5 | na | [47] |
| *Lipomyces starkeyi* | DSM 70296 | glycerol | 35.9 | 12.3 | na | [48] |

| Yeast | Strain ID | Carbon Source | Lipid Content (% g/g) | Lipid Conc. (g/L) | Lipid Yield (g/g) | Reference |
|---|---|---|---|---|---|---|
| *Lipomyces starkeyi* | DSM 70296 | glycerol | 12.7–17.4 | 1.6–5.1 | na | [49] |
| *Lipomyces starkeyi* | AS 2. 1560 | glucose | 13.3 | 8.3 | na | [8] |
| *Lipomyces starkeyi* | AS 2. 1560 | glucose | 53.3 | 12.6 | 0.18 | [50] |
| *Lipomyces starkeyi* | UCDFST 78-23 | glucose | 25.3 | 0.51 | na | [44] |
| *Lipomyces tetatrasporus* | CBS 1808 | glucose | 7.0 | na | na | [34] |
| *Lipomyces tetrasporous* | IBPhM y-695 (NRRL Y-11562) | ethanol | 66.5 | na | 0.26 | [29] |
| *Myxozyma cf. melibiosi* | UCDFST 76-318.3 | glucose | 9.4 | 0.25 | na | [44] |
| *Myxozyma melibiosis* | NRRL Y-11781 | glucose | 19.5 | 0.39 | na | [44] |
| *Myxozyma mucilagina* | UCDFST 76-214.2 | glucose | 11.1 | 0.22 | na | [44] |

Within species, there was no observed trend. While *L. lipofer* was the third highest ranking yeast by lipid content, it was also the most distantly related to the two top ranked yeasts (*L. tetrasporus* and *L. spencer-martinsiae*) [14]. Furthermore, a wide range of lipid values was observed when multiple isolates were screened in this study (Table 3). Therefore, species identification is not predictive of an isolate's ability to produce lipids. Still it is of interest that the two top ranked *Lipomyces* strains (*L. tetrasporus* and *L. spencer-martinsiae*) are closest neighbors (Figure 2). Results from prior studies reporting on *Lipomyces* and *Myxozyma* yeasts are summarized in Table 4. In agreement with the trends observed in the present study, yeasts that ranked highest for lipid production are scattered among the clade members and yeast falling with the Myxozyma group were poor lipid producers.

Lipid titers reported here for glucose flask culture are among the highest reported for flask cultures of Lipomyces (Table 4) or other yeast [28]. The top ranking 5 strains produced 15.0–23.6 g/L (Table 3), while prior reported results were 12.4–16.0 g/L. Lipid yields were also very high for the top 5 yeasts: 0.150–0.236 g/g. The highest previously reported for flask cultures were 0.16 and 0.18 g/g for growth on glucose. However, comparison of various studies needs to be treated with caution because media, including glucose concentration, varied widely. Furthermore, as noted in the introduction, lipid titers were much higher for fed- or 2 stage- batch cultures (41.8 g/L–67.9 g/L) [7-9] than observed for single-stage flask cultures.

Our top lipid producer, *L. tetrasporus* has also been previously identified as a high lipid producing strain. *L. tetrasporus* was originally discovered in a screen of lipid producing yeasts grown on ethanol that was collected from Russian soil [29], where this yeast was observed to accumulate lipid to a respectful 66.5% g/g content. This result is somewhat tempered by the consideration that the maximum lipid yield on ethanol (0.33–0.35 g/g; [27,30]) is considerably higher than on glucose (0.54 g/g; [30]). *L. spencer-martinsiae* has attracted interest for its ability to degrade native starch granules and was explored as a possible candidate for production of single cell protein, presumably in nitrogen rich medium [31-33]. We were unable to find previous reference to its favorable potential for lipid production. We were also unable to find previous papers reporting on lipid producing cultures of *L. starkeyi* strains Y27495 and Y27494. These strains are worthy of further investigation because *L. starkeyi* DSM70296 and AS2. 1560, neither of which was screened here, are among the top yeast lipid producers described in the literature (Table 4). This was not the case for *L. lipofer*, which has been previously explored for lipid production [34,35]. *L. kockii* is the only *Lipomyces* yeast species not included in this study that has been previously reported to have a high lipid titer (Table 4). In summary, of the top 5 ranked lipid producing yeast strains only two had been previously characterized for lipid production in flask cultures.

### *3.3. Lipid production on sugar mixtures*

Two of the top producing *Lipomyces* yeast strains *(L. lipofer Y-11555* and *L. tetrasporus* Y-11562 ) were selected for further characterization. The major sugars associated with herbaceous biomass include L-arabinose, glucose, and xylose. Specific growth rates were measured for yeasts cultured in each of these sugars (Table 5) using a specialized micro-titer culture system equipped with an optical sensor used for monitoring cell growth (Figure 3). The mini-culture system allows for high aeration rates and continuous measurement of cell density; cell density is measured using backscatter, which remains linear at high biomass concentrations [36]. Both yeasts strains had similar

specific growth rates on glucose (0.087 and 0.091 $h^{-1}$). It is notable that both strains grew nearly as well on xylose as they did on glucose (0.083 and 0.075 $h^{-1}$). Growth rates on L-arabinose were slowest for both yeasts. This might be expected because the pathway for L-arabinose metabolism involves an added set of redox coupled reactions compared to that of xylose [37].

Lipid production cultures are usually characterized by separate growth and lipid phases. The yeasts initially grow exponentially and produce yeast biomass. Once the cells exhaust an essential macro-nutrient, typically nitrogen, excess carbon is funneled into producing triglycerides, which are stored as intracellular globule(s). Therefore, both yeasts were cultured in flask cultures with sampling for biomass ($OD_{600}$), lipid production, and sugar consumption.

**Table 5. Summary of lipid production results for *L. tetrasporus* and *L. lipofer*.**

| Sugar | Growth Rate[1] ($h^{-1}$) | Lipids (g/L) | Biomass (g/L) | Culture Time (days) | Lipids Cell Content (%wt/wt) | Efficiency (% of max) |
|---|---|---|---|---|---|---|
| *L. lipofer* Y-11555 | | | | | | |
| L-Arabinose | 0.047 | 13.48 ±1.44 | 23.11 ±0.72 | 12–14 | 56.9 ±0.0 | 40.5 ±0.0 |
| Glu+Xyl | na | 16.29 ±0.31 | 26.05 ±1.34 | 7 | 62.2 ±0.0 | 48.9 ±0.0 |
| Glucose | 0.091 | 15.91 ±1.35 | 24.57 ±1.64 | 6.2 | 61.6 ±0.0 | 47.8 ±0.0 |
| Xylose | 0.075 | 16.99 ±0.99 | 27.55 ±0.27 | 7 | 54.8 ±0.0 | 51.0 ±0.0 |
| *L. tetrasporus* Y-11562 | | | | | | |
| L-Arabinose | 0.050 | 16.33 ±1.38 | 28.66 ±0.62 | 8 | 56.9 ±3.6 | 49.0 ±4.2 |
| Glu+Xyl | na | 20.75 ±1.10 | 33.37 ±0.37 | 5 | 62.2 ±4.0 | 62.3 ±3.3 |
| Glucose | 0.087 | 19.62 ±1.12 | 31.87 ±1.64 | 4–5 | 61.6 ±0.4 | 58.9 ±3.4 |
| Xylose | 0.083 | 18.01 ±1.39 | 32.88 ±0.45 | 5 | 54.8 ±5.0 | 54.1 ±4.2 |

[1]Initial growth rate based upon mean of three micro-cultures.

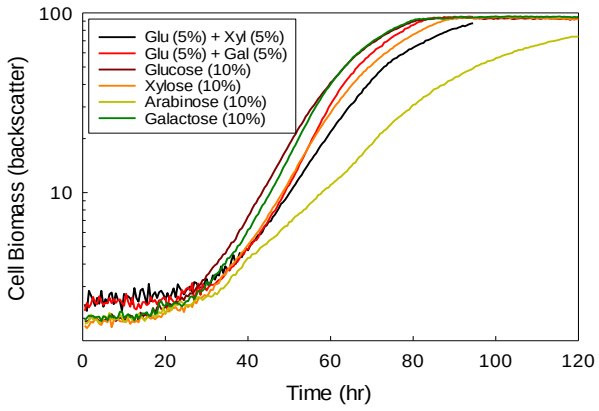


**Figure 3. Growth curves for L. tetrasporus Y-11557 grown on individual and binary sugar mixtures.**

*L. lipofer* Y-11555 was cultured on L-arabinose, glucose, and xylose (Figure 4A). Lipid production on 100 g/L of glucose or xylose was completed in 6 days. In both cases, growth preceded lipid production. The arabinose culture took twice as long to finish. Lipid production was lowest for

L-arabinose (13.48 g/L) and similar for the other sugars (15.91–16.99 g/L) (Table 5). Cell biomass followed a similar pattern. When *L. lipofer* Y-11555 was cultured on a mixture of glucose and xylose, glucose was exhausted prior to xylose consumption, indicating glucose repression of xylose transport and/or metabolism (Figure 4B). Additionally, the duration and final lipid titer was similar to those recorded for the cultures containing either glucose or xylose (Table 5).

*L. tetrasporus* Y-11562 was also cultured on L-arabinose, glucose, and xylose (Figure 5A). Glucose and xylose cultures were completed within 5 days and L-arabinose within 12-14 days. Lipid production was the lowest for L-arabinose (16.33 g/L) and similar for the other sugars (18.01–20.8 g/L). *L. tetrasporus* Y-11562 was also evaluated for lipid production on a binary mixture of glucose and xylose. There is no lag phase between glucose and xylose consumption and it appears that the two sugars might be co-consumed (Figure 5B), albeit xylose was consumed slower than glucose. Though this aspect requires further characterization, co-utilization of glucose and xylose is a rare trait for yeast because of catabolite repression [38]. An exception is the oleaginous yeast *Trichosporon cutaneum*, which has been observed to co-consume glucose and xylose [39]. *T. cutaneum* had a higher lipid yield on glucose than xylose [39] in contrast to *L. tetrasporus*, which was observed to have similar lipid yields for growth on either sugar (Table 5).

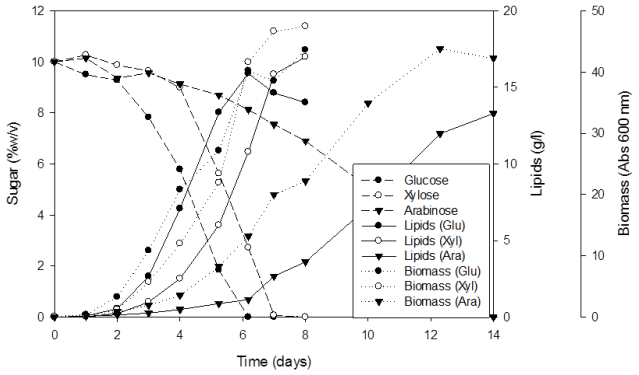


**Figure 4A. Growth and lipid production profiles for *L. lipofer* cultured on individual sugars.**

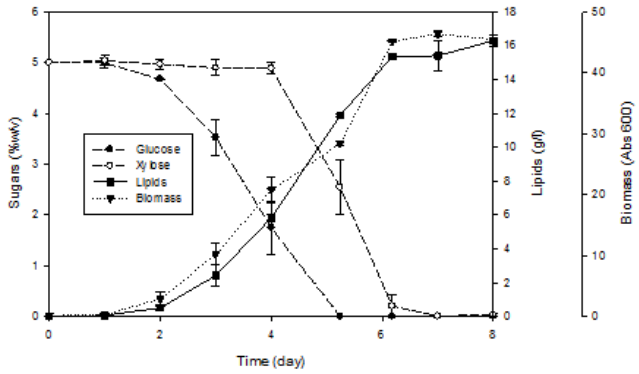


**Figure 4B. Growth and lipid production profiles for *L. lipofer* cultured on an equal mixture of glucose and xylose sugars.**

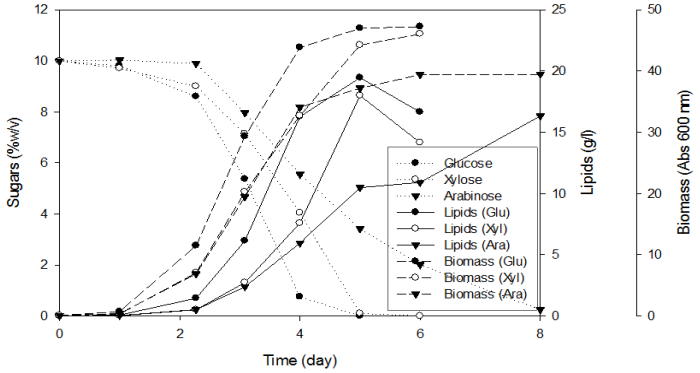


**Figure 5A. Growth and lipid production profiles for *L. tetrasporus* cultured on individual sugars.**

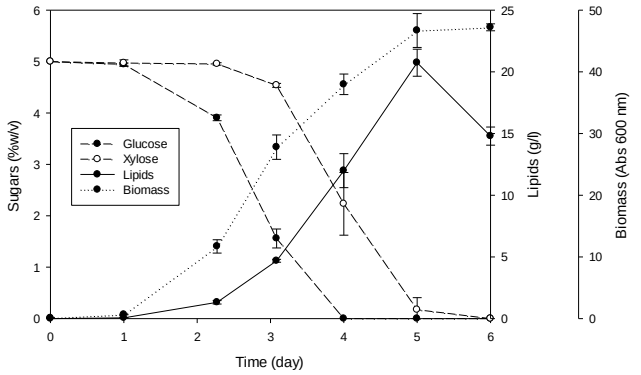


**Figure 5B. Growth and lipid production profiles for *L. tetrasporus* cultured on and an equal mixture of glucose and xylose. Each curve is the average of duplicate runs.**

*L. tetrasporus* Y-11562 consistently outperformed *L. lipofer Y-11555* as measured by lipid production yield and rate (Table 5). Lipid yields were 47.0% and 56.8% of maximum for *L. lipofer* Y-11555 and L. *tetrasporus* Y-11562, respectively. In addition to producing more lipids, *L. tetrasporus* Y-11562 also produced lipids faster. Most *L. tetrasporus* Y-11562 cultures were completed in 5 days versus 6–7 days for *L. lipofer Y-11555*. The difference was particularly pronounced for L-arabinose consumption (8 vs. 12–14 days for *L. tetrasporus* Y-11562 vs *L. lipofer* Y-11555). No significant metabolic intermediates (e.g., arabitol and xylitol) were detected in the culture for growth on either pentose, perhaps because they were grown in highly aerobic cultures.

Most oleaginous yeasts are able to metabolize xylose and L-arabinose. In a screen of 45 type strains, only 5 failed to grow to a high cell titer on xylose and 14 on L-arabinose [40]. As noted earlier, *Y. lipolytica* was one of those that was not observed to grow, though other isolates can consume xylose, albeit weakly [17]. However, this survey of 45 strains did not measure lipid production. Of those evaluated in the past for lipid production using xylose and L-arabinose media [28], only *L. starkeyi* AS 2. 1390 produced comparable lipid titers (14 and 20.9 g/L, respectively). *R. toruloides* As 2. 1389 produced only 7.2 and 4.8 g/L from xylose and L-arabinose, respectively. However, that is not to suppose that the top ranking yeasts identified herein are not

exceptional. These yeasts (*L. lipofer*, *L. tetrasporus*, and *L. spencer-martinsiae*) have been successfully evaluated on hydrolysates prepared from corn stover, Douglas firs, and switchgrass for lipid production [17,41].

**Table 5. Summary of lipid production results for *L. tetrasporus* and *L. lipofer*.**

| Sugar | Growth Rate[1] ($h^{-1}$) | Lipids (g/L) | Biomass (g/L) | Culture Time (days) | Lipids Cell Content (%wt/wt) | Efficiency (% of max) |
|---|---|---|---|---|---|---|
| *L. lipofer* Y-11555 | | | | | | |
| L-Arabinose | 0.047 | 13.48 ± 1.44 | 23.11 ± 0.72 | 12–14 | 56.9 ± 0.0 | 40.5 ± 0.0 |
| Glu+Xyl | na | 16.29 ± 0.31 | 26.05 ± 1.34 | 7 | 62.2 ± 0.0 | 48.9 ± 0.0 |
| Glucose | 0.091 | 15.91 ± 1.35 | 24.57 ± 1.64 | 6.2 | 61.6 ± 0.0 | 47.8 ± 0.0 |
| Xylose | 0.075 | 16.99 ± 0.99 | 27.55 ± 0.27 | 7 | 54.8 ± 0.0 | 51.0 ± 0.0 |
| *L. tetrasporus* Y-11562 | | | | | | |
| L-Arabinose | 0.050 | 16.33 ± 1.38 | 28.66 ± 0.62 | 8 | 56.9 ± 3.6 | 49.0 ± 4.2 |
| Glu+Xyl | na | 20.75 ± 1.10 | 33.37 ± 0.37 | 5 | 62.2 ± 4.0 | 62.3 ± 3.3 |
| Glucose | 0.087 | 19.62 ± 1.12 | 31.87 ± 1.64 | 4–5 | 61.6 ± 0.4 | 58.9 ± 3.4 |
| Xylose | 0.083 | 18.01 ± 1.39 | 32.88 ± 0.45 | 5 | 54.8 ± 5.0 | 54.1 ± 4.2 |

[1] Initial growth rate based upon mean of three micro-cultures.

### *3.4. Fatty acid compositions*

The fatty acid compositions were measured for *L. lipofer*, *L. tetrasporous*, and (for comparison) *R. tortuloides* (Table 6). Their fatty acid profiles were similar and consisted largely of oleic (C18:1 9c) and palmitic (C16:0) acids followed by minor amounts of linoleic (C18:2), palmitoleic (C16:1 9c), and stearic (C18:0) acids. These accounted for 97.9–99.1% of the lipids measured for the yeasts. Lipid yields from oleaginous yeasts are typically enriched for oleic, palmitic, stearic, and linoleic acid as observed here [6,42]. The fatty acid profile for *L. tetrasporus* was previously reported in order of abundance to be: oleic (42.5%), palmitic (31%,), steric (14.5%), and linoleic (5.5%) [43]. While both *L. tetrasporus* strains were observed to be also enriched for oleic and plamitic acids, strain Y11562 was observed to be unusually high in oleic acid (74.3%, Table 6). The lower temperature used here (25 ℃ vs. 30 ℃) would favor unsaturated fatty acids [34]. Results for *L. lipofer* were similar to those observed earlier for this species grown in three different media [44]. The high oleic content observed here, and common for yeasts, is considered favorable for biodiesel applications [45]. Furthermore, based upon a statistical model, it has been concluded that yeast oils would be appropriate for use as biodiesel at a similar blending ratio as biodiesel originating from vegetable oil [42]. The lipids would also be of interest for other applications including as a feedstock for jet fuel [4].

**Table 6. Fatty acid composition for selected yeasts.**

| Species | *L. tetrasporous* | | *L. lipofer* | *R. tortuloides* |
|---|---|---|---|---|
| Isolate: | Y11562 | Y27496 | Y11555 | Y1091 |
| Fatty Acid | | % lipid composition | | |
| C14:0 | | 0.1 | | 0.8 |
| C15:0 | | 0.1 | | |
| C16:0 | 16.6 | 32.8 | 25.5 | 27.3 |
| C16:1 9c | 2.3 | 3.4 | 4.6 | 0.4 |
| C17:0 | 0.4 | 0.1 | | |
| C17:1 9c | | 0.2 | | |
| C18:0 | 3.2 | 4.4 | 1.1 | 1.3 |
| C18:1 9c | 74.3 | 55.7 | 63.3 | 59 |
| C18:2 | 2 | 2 | 4.6 | 9.9 |
| C19:1 9c | | 0.2 | | |
| C20:0 | 0.5 | 0.4 | 0.7 | |
| C20:1 | | | | |
| C20:4 | 0.2 | 0.2 | 0.1 | 0.3 |
| C22:0 | | | | |
| C24:0 | 0.4 | 0.4 | 0.1 | 0.7 |
| C24:1 | | | | |
| UNK | 0.1 | | | 0.3 |
| Total | 100 | 100 | 100 | 100 |

## 4. Conclusion

The oleaginous phenotype was conserved among *Lipomyces* but not *Myxozyma* yeasts. However, the extent of lipid production appeared niche dependent as it varied among isolates belonging to the same species. Three yeasts from this clade were found to be of particular interest because of their unusually high lipid production on glucose in comparison to other oleaginous yeasts reported for their exceptional lipid production. Furthermore, *L. lipofer* and *L. tetrasporus* were further characterized for growth on lignocellulosic biomass associated sugars and found to grow and produce lipids equally well on glucose, xylose, or an equal mixture of the two.

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## Conflict of interest

All authors declare no conflicts of interest in this paper.

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