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Journal

Journal of Industrial Microbiology and Biotechnology, 50(1)

Authors

Lopes, Daiane

Dien, Bruce

Hector, Ronald

et al.

Publication Date

2023-02-17

DOI

10.1093/jimb/kuad040

Peer reviewed

Journal of Industrial Microbiology and Biotechnology, 2023, 50, kuad040
https://doi.org/10.1093/jimb/kuad040
Advance access publication date: 21 November 2023
Bioenergy, Biofuels and Biochemicals – Original Paper

Determining mating type and ploidy in *Rhodotorula toruloides* and its effect on growth on sugars from lignocellulosic biomass

Daiane Dias Lopes [1,2], Bruce S. Dien [1,2], Ronald E. Hector[1], Vijay Singh[2,3], Stephanie R. Thompson[1], Patricia J. Slininger[1], Kyria Boundy-Mills[4], Sujit S. Jagtap[2,5], Christopher V. Rao[2,5]

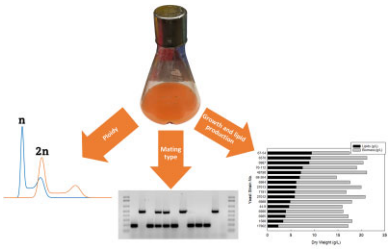
[1]USDA, Agricultural Research Service, National Center for Agricultural Utilization Research, (Bioenergy Research), 1815 N University, Peoria, IL 61604, USA
[2]DOE Center for Advanced Bioenergy and Bioproducts Innovation (CABBI), University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA
[3]Department of Agricultural and Biological Engineering, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA
[4]Phaff Yeast Culture Collection, Food Science and Technology, University of California Davis, One Shields Ave, Davis, CA 95616, USA
[5]Department of Chemical and Biomolecular Engineering, University of Illinois at Urbana-Champaign, 600 S. Mathews Ave., Urbana, IL 61801, USA
Correspondence should be addressed to: Ronald E. Hector at Ronald.Hector@usda.gov; Bruce S. Dien at Bruce.Dien@usda.gov

Abstract: *Rhodotorula toruloides* is being developed for the use in industrial biotechnology processes because of its favorable physiology. This includes its ability to produce and store large amounts of lipids in the form of intracellular lipid bodies. Nineteen strains were characterized for mating type, ploidy, robustness for growth, and accumulation of lipids on inhibitory switchgrass hydrolysate (SGH). Mating type was determined using a novel polymerase chain reaction (PCR)-based assay, which was validated using the classical microscopic test. Three of the strains were heterozygous for mating type (A1/A2). Ploidy analysis revealed a complex pattern. Two strains were triploid, eight haploid, and eight either diploid or aneuploid. Two of the A1/A2 strains were compared to their parents for growth on 75%v/v concentrated SGH. The A1/A2 strains were much more robust than the parental strains, which either did not grow or had extended lag times. The entire set was evaluated in 60%v/v SGH batch cultures for growth kinetics and biomass and lipid production. Lipid titers were 2.33–9.40 g/L with a median of 6.12 g/L, excluding the two strains that did not grow. Lipid yields were 0.032–0.131 (g/g) and lipid contents were 13.5–53.7% (g/g). Four strains had significantly higher lipid yields and contents. One of these strains, which had among the highest lipid yield in this study (0.131 ± 0.007 g/g), has not been previously described in the literature.

Summary: The yeast *Rhodotorula toruloides* was used to produce oil using sugars extracted from a bioenergy grass.

Keywords: oleaginous yeast, PCR assay, single cell oil, lignocellulose, biofuels

Graphical abstract



Rhodotorula toruloides yeast were characterized for ploidy, mating type, and growth and lipid production on switchgrass hydrolysate.

Introduction

Rhodotorula toruloides is a red colored strictly aerobic basidiomycetous yeast that is generally self-sterile (heterothallic), which has been isolated from terrestrial plants, soils, and marine environments (Kurtzman et al., 2011). It is of growing interest to the biotechnology industry because the yeast is oleaginous, produces carotenoids (e.g., source of red color), and is a source of specialized enzymes used by the pharma and chemical industries (Jin et al., 2015; Park et al., 2018). It is the second most cited oleaginous yeast species (Abeln and Chuck, 2021). Select isolates of *R. toruloides* are also among the best yielding lipid producers described in the literature (Li et al., 2007; Fei et al., 2016; Slininger et al., 2019), second to *Lipomyces starkeyi* (Abeln & Chuck, 2021). Though challenging to work with genetically, continued advances in developing molecular tools have enhanced its popularity (Yu & Shi, 2023). *Rhodotorula toruloides* is also widely used by researchers developing processes

Received: September 22, 2023. **Accepted:** November 13, 2023.

to microbially convert lignocellulosic-based sugars into biofuels and chemicals. These researchers prize its favorable physiology for industrial bioprocesses (Huang et al., 2022). Desirable traits include minimal nutrient requirements, broad metabolism in terms of both carbon and nitrogen compounds, hardiness, and ability to achieve high cell densities (Zhao et al., 2022). Specifically, the yeast is able to utilize hexose and pentose sugars and acetate (Kurtzman et al., 2011). It even metabolizes lignin related aromatics that are released by many pretreatment processes (Yaguchi et al., 2020; Osorio-Gonalez et al., 2022).

Hydrolysates prepared from lignocellulose are chemically complex, which reflects both the chemical nature of plant cell walls and the multi-staged processing steps used to release structural carbohydrates as monomeric sugars. These chemicals include furans generated from the dehydration of hexoses and pentoses, organic acids, especially acetate, and aromatics released from the partial solubilization of lignin (Chen et al., 2006; Du et al., 2010). All are inhibitory to the growth of yeast (Klinke et al., 2004; Hu et al., 2009). An important area of continuing research is identifying and/or engineering yeast strains that are robust for growth and product yield on unrefined hydrolysate sugars (e.g., Mertens et al., 2018; Quarterman et al., 2018). Adaptative laboratory evolution has also been applied to improve yeast hardiness (Slininger et al., 2015; Liu et al., 2021). Tolerance to inhibitors varies dramatically between yeast species and across isolates within a species (Sitepu et al., 2014). Our laboratory has developed a model medium prepared using dilute-acid pretreated switchgrass to compare inhibitor tolerance and have applied it to *Lipomyces* and *Yarrowia* species with success (Slininger et al., 2016; Quarterman et al., 2017). This study extends the survey to *R. toruloides*. Besides adding knowledge on the growth hardiness of *R. toruloides*, this work allows for comparisons among three clades of leading oleaginous yeast.

We also seek to clarify how the ploidy of a strain affects its ability to grow and produce lipids. While *R. toruloides* are most commonly found with a single mating type, it does possess a self-sporulating diploid cycle (Abe & Sasakuma, 1986). In fact, the Type-strain for *R. toruloides* is diploid and quite stable (personal observation). There is ample evidence in case of Ascomycete yeast species that polyploids have improved hardiness to environmental stresses compared to their haploids. Brazil, the second largest fuel ethanol producer in the world, runs continuous fermentations with yeast recycle. The commercial yeast strains employed are heterothallic diploid strains with the exception of a homothallic diploid and a polyploid strain (Della-Bianca et al., 2013). A screen of 160 *Saccharomyces* yeast strains isolated from breweries, distilleries, and rugged environments, yielded three with exceptional tolerance to inhibitors present in corn stover hydrolysates and all were diploids (Mertens et al., 2018; personal communication). There is also direct evidence that diploids can be more rugged than haploid strains for growth in hydrolysate. For example, (Lopes et al., 2017) compared the growth of isogenic haploid and diploid *Saccharomyces* strains with commercial genetic backgrounds for growth in hydrolysate cultures. The isogenic diploid strain had shorter lag time than the relevant haploid strain. In another study, *Saccharomyces* yeast were evolved over the course of 100 generations in cultures with added ethanol (3–9%) or 1 M KCl. The diploids had faster growth rates and eventually grew to dominate the populations (Harari et al., 2018). Interestingly, diploid yeast cells arose through both whole-genome duplication and mating type switching, even though the beginning strains were heterothallic (e.g., self-sterile). Much less is known about how ploidy effects culture growth in non-*Saccharomyces* yeast. Both *Scheffersomyces* (*Pichia*) *stipitis* and *Scheffersomyces* (*Candida*) *shehatae* grown on woody hydrolysate with cell recycle were observed to at least double their ploidy (Talbot & Wayman, 1989). Also, for *Yarrowia lipolytica* (Li & Alper, 2020), it was observed that mating a haploid strain engineered for xylose metabolism with its wild-type parental yeast strain resulted in a diploid strain that grew faster and had greater sugar consumption compared to the engineered haploid strain.

Herein, we seek to provide the needed tools and preliminary results to determine if ploidy is an important consideration for *R. toruloides*. In this study, a set of nineteen *R. toruloides* isolates, gleaned from public collections, is characterized for mating type and ploidy. To facilitate mating type determination for this dimorphic yeast, a polymerase chain reaction (PCR)-based assay was developed based upon the presence of mating pheromone receptors *STE3.A1* and *STE3.A2* in mating type A1 and A2 cells, respectively. The molecular assay was verified, where possible, based on the classical microscopic mating assay using testing strains with known mating types. Ploidy analysis was based on single-cell analysis of cells stained using the deoxyribonucleic acid (DNA) propidium iodide (PI) fluorescent stain. Two A1/A2 strains and their parents were screened for growth in concentrated biomass sugars prepared from dilute-acid pretreated switchgrass. Finally, all nineteen strains were compared for growth and lipid production in less concentrated hydrolysate cultures, which provides further information on the influence of genetic background on lipid production from unrefined hydrolysate sugars.

Materials and Methods

Chemicals and Media Ingredients

All chemicals were of analytical grade and unless mentioned otherwise were purchased from (Thermo-Fisher Scientific, Waltham, MA, USA). Media ingredients were purchases from Sigma (St. Louis, MO, USA), except Difco Vitamin Assay Casamino Acids (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Carbohydrase enzyme preparations were gifted from Novozymes (Bagsvaerd, Denmark).

Yeast Strains

All the *R. toruloides* strains used in this study are available from public yeast collections (Table 1). Yeast strains were stored as glycerol stocks (25%v/v) at −80°C. Strains were revived on solid Yeast Extract–Peptone–Dextrose (YPD) medium (per L: 10 g yeast extract, 20 g peptone, 20 g dextrose, and 20 g Difco agar) by incubating at 28°C until individual colonies formed before being stored at 4°C. Yeast strains were replated every two weeks or less.

Yeast Mating Type Assays

Mating type was determined by PCR amplification of mating type specific pheromone receptors *STE3.A1* and *STE3.A2* from genomic DNA. Genomic DNA was isolated essentially as described previously (Looke et al., 2011) with incubation at 70°C for 30 min prior to ethanol precipitation. Precipitated genomic DNA was resuspended in 100 µL of 10 mM Tris pH 8.0 buffer and pelleted again to remove cell debris. 2 µL of genomic DNA was used as template for PCR using Phusion Hot Start polymerase (NEB, Ipswich, MA, USA). Primers to amplify *R. toruloides* A1 and A2 specific pheromone receptors (*STE3.A1*, GenBank accession # JN246662 and *STE3.A2*, GenBank accession # JN246595) were designed using Primer-BLAST (Ye et al., 2012) to limit non-specific products as well as identify primers that hybridize to all genomes in the Refseq

Table 1. Mating types and ploidies

Yeast #	Mating types(s)[1]	Visual confirmation	G1 DNA peak (RFU)[2]	G2 DNA peak (RFU)	Ploidy *n*	Source[3]	Other names
NRRL Y-6984	A1	+	25 156	48 364	2.0	ARS	NRRL 1091
NRRL Y-6985	A2	+	12 279	23 038	1.0	ARS	NRRL 1588
NRRL Y-6986	A1	+	12 986	26 529	1.0	ARS	NRRL 6672
NRRL Y-6987	A1/A2	na	36 388	72 138	2.9	ARS	NBRC 8766, NRRL 1091 x NRRL 1588
NRRL Y-6988	A1/A2	na	25 116	47 263	2.0	ARS	NBRC 8767; NRRL 6985 x NRRL 6986
NRRL Y-1091	A1	+	25 120	48 229	2.0	ARS	CBS14; NBRC 0559
NRRL Y-1588	A2	+	15 034	30 163	1.2	ARS	CBS 349; NBRC 0880
NRRL Y-7191	A1	+	25 746	51 529	2.1	ARS	ML-142
NRRL Y-27012	A1	+	14 092	26 753	1.1	ARS	
NRRL Y-27013	A1	+	12 822	24 746	1.0	ARS	
NRRL Y-48190	A2	+	13 680	25 957	1.0	ARS	YS 69
NRRL Y-17902[4]	-	-	11847	22 737	0.9	ARS	CBS 6681
CBS 6570	A1	+	13 781	25 826	1.0	CBS	
UCDFST 44–7	A2	+	27 166	50 804	2.3	Phaff	QM J604
UCDFST 44–9	A2	+	29 698	55 368	2.4	Phaff	QM 1718
UCDFST 67–54	A1	+	37 617	74 228	3.2	Phaff	
UCDFST 68–264	A1	+	30 002	57 982	2.5	Phaff	CBS 315
UCDFST 68–269	A1	+	32 341	63 490	2.7	Phaff	CBS 2370
UCDFST 70–112	A1/A2	na	28 817	53 930	2.3	Phaff	

[1]Mating type detected by multiplex PCR assay performed using genomic DNA.
[2]Relative fluorescent units based on PI DNA staining.
[3]Collections: Agricultural Research Service (ARS) Culture Collection (NRRL strains, National Center for Agricultural Utilization Research, Peoria, IL); Westerdijk Institute (formerly called Centraalbureau voor Schimmelculture-CBS, Utrecht, The Netherlands); Phaff Yeast Culture Collection (UCDFST, University of California, Davis, CA); U.S. Army Quartermaster Culture Collection (QM); and Institute for Fermentation Osaka (IFO, collection transferred to the NBRC, the National Institute of Technology and Evaluation Biological Resource Center, Tokyo, Japan).
[4]Identified as a *R. mucilaginosa* (see text).

representative genomes collection for *R. toruloides* (taxid:5286). MAT A1 specific primers, Rt-STE3.A1 (forward) and Rt-STE3.A1 (reverse), amplify a 187 bp fragment from *STE3.A1*. MAT A2 specific primers, Rt-STE3.A2 (forward) and Rt-STE3.A2 (reverse), amplify at 503 bp product from *STE3.A2*. Primers for MAT A1 and A2 were tested as individual primer pairs for each mating type and in a multiplex PCR reaction using both primer pairs. The multiplex PCR amplified products from both mating types with no additional amplification products observed. Primer sequences are as follows:

Rt-STE3.A1 (forward, 5′-GCCTAGGTCTTCCCCTGTTG-3′),
Rt-STE3.A1 (reverse, 5′- GACAAGCAAAGCTTACCGGC-3′),
Rt-STE3.A2 (forward, 5′- GTGTGGGTCGTGATAGCGAA-3′),
and Rt-STE3.A2 (reverse, 5′- CCAACCGCAAAAATGGGAGG-3′).

Yeast mating types were confirmed visually. A colony of the yeast strain to be tested was separately mixed with A1 (NRRL Y-6984) and A2 (NRRL Y-6985) yeast cells on a YPD plate using sterile toothpicks in triplicate. The plate was incubated at 28°C checked daily for the formation of hyphae with clamp connections by microscopic examination, which in the case of a positive outcome takes 2–3 days (Banno, 1967).

Rhodotorula Species Identification

Rhodotorula species designations were determined by sequence analysis of the internal transcribed spacer (ITS) region PCR-amplified using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). PCR amplification was performed using Phusion Hot Start Flex (NEB) with the following thermocycler conditions: 30 s at 98°C, 30 cycles of [10 s at 98°C, 20 s at 61°C, and 15 s at 72°C], followed by a final extension of 5 min. at 72°C. Genomic DNA used as template for the PCR reactions was isolated as described above. The amplified PCR products were purified using a QIAquick PCR Purification column (Qiagen, Germantown, MD, USA) and sequenced with primer ITS1 and ITS4 using the BigDye Terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA, USA). DNA sequences were then used to perform individual nucleotide–nucleotide searches as described in (Romanelli et al., 2014). *Rhodotorula* species identifications were made based on maximum identities ≥98% and query coverages ≥90%.

Preparation of Switchgrass Hydrolysate

The pelletized switchgrass used in this study (Dien et al., 2018) and its dilute-acid pretreatment and enzymatic hydrolysis (Quarterman et al., 2017) have been previously described in the cited articles. The pelletized switchgrass contained [g/kg dry pellets]: 338 glucans, 221 xylan, 36 acetate, and 204 lignin (Dien et. al., 2018). The remaining components include aqueous extractables and ash. Briefly, the switchgrass pellets were ground in a knife mill to pass through a 4 mm screen, mixed with 0.936%v/v H_2SO_4 and 37.5 mg/L Pluronic F-68 at 20%w/w solids. The slurry was heated to 160°C at 2.6°C/min, held at temperature for 15 min, and water cooled, all while mixing at 50 rpm. The pretreated slurry was neutralized by adding $Ca(OH)_2$ followed by sodium citrate buffer (50 mM) to a pH of 4.5 and digested for 72 h at 50°C using 2.7 mL Ctec3 cellulase (Novozymes, NA) and 0.5 mL Htec2 hemicellulase (Novozymes, NA).

Hydrolysate Lipid Production Cultures

Yeast hydrolysate cultures were grown using the Biolector culturing system in 48 micro-well FlowerPlates (Beckman Coulter GambH, Germany). All cultures were incubated at 28°C. A 16 mL snap cap polystyrene sterile culture tube containing 2 mL of YPD was inoculated from a culture plate using a sterile loop. The preseed culture was grown while shaking at 250 rpm for 18 h. One-half mL was transferred to a 50 mL baffled Erlenmeyer flask containing 10 mL of 50%v/v hydrolysate supplemented with 5.6% v/v casamino acids nutrients (per L: 18.1 g Difco Vitamin Assay

Casamino Acids, 23.1 g $(NH_4)_2SO_4$,180 mg DL-Tryptophan, and 720 mg L-cysteine) and adjusted to pH 6.0 with NaOH. The seed culture was grown for 48 h and shaken at 250 rpm. The culture was harvested, centrifuged, and the cell pellet resuspended in phosphate buffered saline to an OD_{600} of 50. The concentrated yeast stock was used to inoculate each well on the culture plate to an OD_{600} of 1.0. The culture well contained 800 µL of either 60%, 75%, or 90%v/v concentrated hydrolysate supplemented with 6.7%, 8.3 %v/v, 10.0%v/v casamino acids nutrient, respectively, to a uniform C: N of approximately 60. The plate was mixed at 800 rpm for approximately 96 h and yeast biomass (e.g., backscatter) was measured every 30 min.

The final micro-culture was transferred to a microcentrifuge tube and centrifuged for 10 min at 15 000 x g. The supernatant was used for high-performance liquid chromatography (HPLC) analysis to determine residual sugars. The cell pellet was washed twice and resuspended to a volume of 1 mL with dH_2O. An aliquot (800 µL) of the cell suspension was transferred to a tared FastPrep tube (MP Biomedicals, Solon, OH, USA), stored at −80°C, and used for determination of dry weight and fatty acid composition. The reminder was stored at −20°C and used for determination of total lipid titer.

Analytical Methods

Yeast ploidy: yeast strains were cultured overnight on YPD and transferred with 10%v/v inoculum to fresh YPD (at 28°C and 250 rpm) and grown for an additional 6 h. One mL of yeast cells was harvested, pelleted, and fixed with chilled 70% v/v ethanol and stored at −20°C. Fixed cells were stained and analyzed using flow cytometry according to (Todd et al., 2018) with slight modifications. Cells were washed with phosphate buffer (PB, 100 mM sodium phosphate, pH 7.2), and incubated for 10 min in a detergent solution (per 100 mL: 0.5 g Triton x-100, 1.75 g NaCl, 2.5 mL 4 N HCl) on ice to denature chromatin (Dien et al., 1994) followed by a PB wash. The cells were treated with RNAse A (20 µL, 500 U/mL, Cat #2286, Ambion) at 37°C for 1 h. Cells ($\approx 1 \times 10^7$) were resuspended in 150 µL of a PI solution for 40 min at 37°C (PI cell cycle kit, Product #: CSK0112, Nexcelom, Lawrence, MA, USA), and stored overnight at 4°C. Cells were resuspended into PB containing 25 µg/mL PI for flow cytometry. The flow cytometer (Bio Rad S3e Cell Sorter, Hercules, CA, USA) was equipped with a 488 nm laser and a 615/25 filter and set to run 500 cells/min. All samples were collected at the same electronic gains (forward scatter = 348 and PI = 736) and G1 and G2 DNA peak values were estimated by fitting the DNA histograms using FCS Express software (De Novo Software, Pasadena, CA, USA) after gating out yeast doublets based upon DNA fluorescence area versus peak values. Ploidy was determined by normalizing either G1 or G2 DNA contents based on the DNA contents of haploid (Y-6986) and diploid (Y-6988) yeast. Autofluorescence was determined to be minimal by running unstained fixed yeast cells. The same stained yeast sample was run as the first and last runs and the G1 fluorescent peaks were within 0.1% of each other. An isogenic series of *S. cerevisiae* yeast ranging in ploidy form 1n to 4n were stained in parallel and used to confirm that fluorescence increased linearly with genomic content (Supplementary Fig. S1). Cell sizes were measured from growth cultures without fixing using a Cellometer X2 (Nexcelom Bioscience, Lawrence, MA, USA) according to the protocol supplied by the manufacturer.

Yeast dry weights and fatty acid composition: washed cells were dried for 48 h in a freeze dryer (Labconco, cat. no. 7 960 046, Kansas City, MO, USA) in a tared FastPrep tube and re-weighed to determine cell dry weights. Lipids were extracted, converted to fatty acid methanol esters, and analyzed for fatty acids using a gas chromatography equipped with a flame ionization detector as previously described (Quarterman et al., 2017).

Lipid titers: lipid concentrations were measured using the sulfo-phospho-vanillin colorimetric assay as previously described (Dien, Slininger et al., 2016; Slininger et al., 2019). Briefly, an aliquot of washed yeast cells was digested in a concentrated H_2SO_4 solution at 100°C, cooled, and the lipids reacted with a vanillin-phosphoric acid solution at 37°C. The absorbance was measured at 530 nm and adjusted for background absorbance using a similarly prepared water blank. Readings were converted to lipids using a calibration curve prepared with corn oil constructed daily.

Sugars and furans: sugars and furans were measured using a Thermo HPLC system equipped with a refractive index (RI) and ultra-violet detectors. Samples (20 µL) were injected onto a Bio-Rad HPX-87H column heated to 65°C and eluted with 0.6 ml/min of 5 mM sulfuric acid. Sugars and organic acids were determined from the RI signals and furans from their absorbance at 277 nm using external standards.

Statistical Methods

All growth and lipid production hydrolysate cultures results reported are based on triplicate experiments. Analysis of variance (ANOVA) and multiple comparisons using the Turkey's range test were performed using Design Expert version 13 (State-Ease, Inc. Minneapolis, MN, USA) and results are reported in the text.

Results

Mating and Ploidy Results

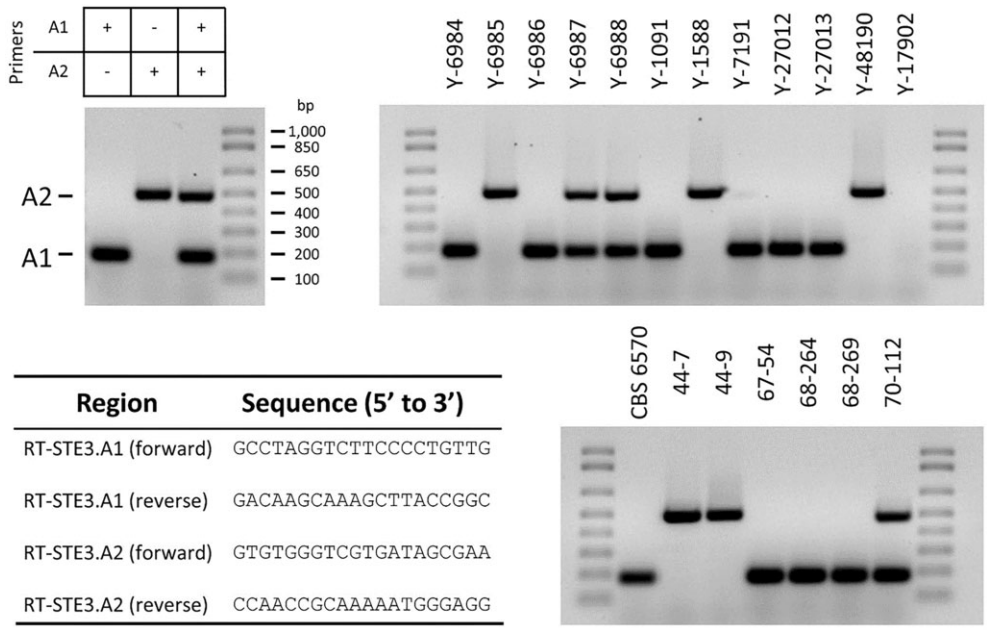
The PCR assay successfully identified A1 and A2 mating types and could be run in multiplex (Fig. 1). There were no detectable background amplification products because the primers have been designed to minimize off-target amplification products in the *R. toruloides* genome. For strain NRRL Y-17902, PCR amplification of *STE3.A1* and *STE3.A2* gene fragments failed, and the strain did not mate with either A1 or A2 tester strains. Since we were unable to confirm the presence of pheromone receptors for this strain, we next sequenced the ITS region and performed BLAST analysis to determine if strain NRRL Y-17902 was misclassified as *R. toruloides*. Sequence comparison of the amplified ITS region revealed that strain NRRL Y-17902 was *R. mucilaginosa*.

Nine of the yeast strains were identified as A1, five as A2, and three as A1/A2 (Table 1). Mating types were verified microscopically using A1 and A2 tester strains by watching for induced formation of hyphae in the presence of the opposite mating type.

The ploidies of the strains were measured by using flow cytometry in conjunction with a standard cell cycle model (Table 1). Eight strains were haploid (0.9–1.0 n), 9 strains were either diploid or aneuploidy (2.0–2.7n), and two were triploids (3.1 and 3.2 n). The exponential growing cultures were also measured for cell diameter using a Cellometer. The log mean weighted diameters were estimated and as expected, the polyploid yeast cells were larger than the haploids. The mean diameters were 5.51 ± 0.50 µm for the haploids, 6.74 ± 0.0.63 µm for the diploids/aneuploids (2.0–2.7 n), and 7.05 ± 0.31 µm for triploids (data not shown).

Growth and Lipid Production on Switchgrass Hydrolysate

Pelletized switchgrass was pretreated at 20%w/w solids with dilute sulfuric acid, neutralized, and digested with commercial cellulases and hemicellulases. The neutral sugar concentration was



Region	Sequence (5' to 3')
RT-STE3.A1 (forward)	GCCTAGGTCTTCCCCTGTTG
RT-STE3.A1 (reverse)	GACAAGCAAAGCTTACCGGC
RT-STE3.A2 (forward)	GTGTGGGTCGTGATAGCGAA
RT-STE3.A2 (reverse)	CCAACCGCAAAAATGGGAGG

Fig. 1. Mating-type electrophoretic profile in 1 % agarose gel: amplification products around 187 bp for MAT-A1 and 503 bp for MAT-A2; 1 Kb Plus DNA Ladder (Invitrogen). Freshly grown colonies provided the DNA template as described in the methods section. Multiplex reactions were performed in duplicate using the Phusion® Hot Start Flex 2X Master Mix kit (NEB). The PCR assay failed to determine mating type for NRRL Y-17902 (see text).

Table 2. Chemical composition of undiluted switchgrass hydrolysate

	Concentrations [g/L]			
Chemical	**Batch #1**	**Batch #2**	**Batch #3**	**Average**
Glucose (g/L)	68.7	68.2	68.6	68.5 ± 0.3
Xylose (g/L)	50.0	45.8	48.7	48.2 ± 2.2
Arabinose (g/L)	4.8	5.6	2.9	4.4 ± 1.4
Neutral sugars (g/L)[1]	123.5	119.6	120.2	121.1 ± 3.8
Acetate (g/L)	6.3	6.9	5.6	6.3 ± 0.7
HMF (g/L)	0.32	0.32	0.43	0.36 ± 0.07
Furfural (g/L)	1.5	1.8	2.4	1.9 ± 0.5

[1]Sum of glucose, xylose, and arabinose.

120 g/L (Table 2). The biological inhibitors measured here included acetate, furfural, and 5-hydroxymethylfurfural (HMF). Results are listed for the three separate batches of hydrolysate used in this study. The sum of the neutral sugars only varied by 3.11% across the three batches. Concentrations of HMF and furfural varied by small amounts 0.07 and 0.5 g/L, respectively. The yeast strains were unable to grow on undiluted hydrolysate fortified with nutrients and, therefore, the hydrolysate was diluted to 50–75%v/v. Strain NRRL Y-6987 was run with each yeast culture plate and used as an external control.

The NRRL yeast strains were challenged for growth on 75 and 90% v/v concentrated hydrolysate. The seed cultures were grown on 50%v/v concentrated switchgrass hydrolysate (SGH) to acclimate the cells to hydrolysate. Acclimation had been observed to be beneficial for production of lipids from hydrolysate in a prior study (Dien, Zhu et al., 2016). Seed cultures for strains Y-1588 and Y-6985 did not grow. None of the other strains were able to grow in 90%v/v cultures. All of the rest of the yeast strains except Y-6986 and Y-48190 grew on 75%v/v hydrolysate sugars (Supplementary Fig. S2). Of particular interest is comparing A1/A2 strains Y-6988 (Y-6985 x Y-6986) and Y-6987 (Y-1091 x Y-1588) to their parents (Fig. 2). For the parental strains that grew, their lag times were twice that of the progeny (Fig. 2 and Supplementary Fig. S2). Extended lag phases are commonly observed when microbes are grown on unrefined hydrolysate sugars. More broadly, the haploids took on average 9.3 h longer than the polyploids to begin growing (41.9 ± 14.6 h vs. 51.2 ± 6.5 h). Final lipid titers ranged from 0.626 to 8.23 g/L and were significantly different across the set of yeast ($p < 0.001$). The highest producing yeast were the polyploid yeasts Y-6984, Y-6987, Y-1091, and haploid Y-27013.

This experiment was repeated with a new batch of hydrolysate using a limited set of NRRL strains: Y-1091, Y- 6986, Y-6987, Y-17902, and Y-27013. The yeast strains were challenged with 60%, 75%, and 90% v/v concentrated hydrolysate. None grew on 90%v/v hydrolysate, in agreement with the previous result. However, for the 75%v/v hydrolysate cultures, only the triploid strain (Y-6987) grew (data not shown). In contrast, all but Y-1091 grew in the 60%v/v hydrolysate cultures. Likewise, the non-NRRL strains were challenged for growth on 75%v/v concentrated SGH and NRRL Y-6987 was included as a control. Once again, only NRRL Y-6987 grew (data not shown). The failure of the other strains to grow on 75%v/v hydrolysate sugars is likely because the batches varied slightly in inhibitory potency. It can be observed that the potent inhibitor furfural varied from 1.5 to 2.4 g/L across hydrolysates (Table 2). However, hydrolysate contains numerous organic inhibitors and, therefore, it is not possible to pinpoint the precise source of inhibition. The high sensitivity to small changes in inhibitors is not surprising, given the very long lag phases observed for the original 75%v/v cultures.

The rest of yeast strains were subsequently characterized for yeast growth and lipid production on 60%v/v concentrated hydrolysate (Supplementary Fig. S3A and B). Lag phases varied significantly among the yeast cultures ($p < 0.001$). Three yeast strains (UCDFST 44–7, UCDFST 68–269, and NRRL Y-1091) out of the 17 tested were unable to grow. Five other yeast strains (Table 3) had

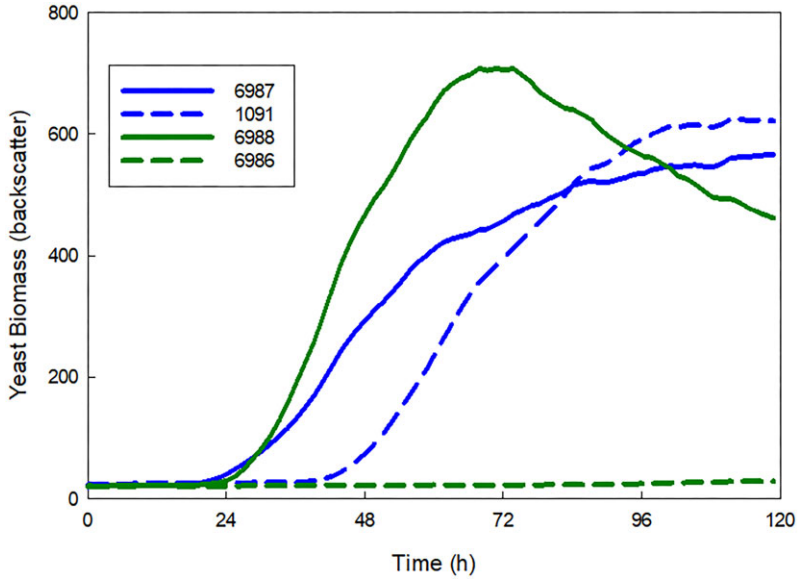


Fig. 2. Comparisons of conjugated yeast NRRL Y-6988 and Y-6987 and their parents on 75%v/v concentrated SGH.

Table 3. Growth kinetics for hydrolysate cultures

Yeast strain	Hydrolysate Batch	Growth rate[2] h^{-1}	Lag phase[3] h
NRRL Y-1588	2	0.063 ± 0.002	32.5 ± 1.5
NRRL Y-17902	2	0.153 ± 0.020	31.5 ± 0.9
NRRL Y-27012	2	0.165 ± 0.014	18.1 ± 0.5
NRRL Y-27013	2	0.148 ± 0.011	44.5 ± 1.5
NRRL Y-48190	2	0.161 ± 0.005	17.7 ± 0.3
NRRL Y-6984	2	0.185 ± 0.004	11.7 ± 0.1
NRRL Y-6985	2	0.113 ± 0.006	18.3 ± 0.4
NRRL Y-6986	2	0.120 ± 0.011	62.9 ± 3.3
NRRL Y-6987	2	0.105 ± 0.009	20.3 ± 0.6
NRRL Y-6987	3	0.111 ± 0.012	20.3 ± 0.4
NRRL Y-6988	2	0.136 ± 0.016	15.3 ± 0.8
NRRL Y-7191	2	0.143 ± 0.009	16.3 ± 0.1
CBS 6570	3	0.157 ± 0.012	48.4 ± 1.7
UCDFST 44–9	3	0.164 ± 0.012	38.3 ± 1.0
UCDFST 67–54	3	0.109 ± 0.008	23.4 ± 0.6
UCDFST 68–264	3	0.089 ± 0.007	58.0 ± 1.6
UCDFST 70–112	3	0.152 ± 0.011	28.3 ± 0.4

[1]UCDFST strains 44–7 and 68–264 and NRRL Y-1091 did not grow on 60%v/v hydrolysate.
[2]Linear correlations for ln(yeast biomass) vs. time were >0.98
[3]Time to achieve first doubling.

lag times in excess of 38 h. When yeast cells are introduced to hydrolysate, a lag time is often observed during which the furans are reduced to less toxic alcohols (Liu et al., 2004). Herein, yeast cultures grown on hydrolysate batch 3 were tested for the presence of HMF and furfural after 96 h. The furans had disappeared from all except for UCDFST 44–7 and UCDFST 68–264, which were largely unchanged (data not shown). Among the remaining eleven yeast strains, the median lag time was 18.4 h and the shortest lag times were observed for NRRL Y-6984 and diploid NRRL Y-6988 (11.7 and 15.3 h; $p < 0.05$). The triploid yeast (NRRL Y-6987) had a lag time of 20.4 h.

The median growth rate was 0.146 h^{-1} and the difference between the fastest and slowest growing strains was 0.122 h^{-1} (Table 3). Strain NRRL Y-6984 grew the fastest and NRRL Y-1588 and UCDFST 68–264 grew the slowest ($p = 0.016$). Neither of the slowest growing strains had particularly long lag phases. In general, growth rate and lag phase were uncorrelated ($r^2 = 0.08$).

The NRRL and other yeast strains were received at two different times and, therefore, evaluated using different batches of hydrolysate. Both batches were prepared under identical conditions and were similar in composition (Table 2). Strain NRRL Y-6987 was included as a control for all experiments and its lag phases ($p = 1.00$) and growth rates ($p = 0.567$) were the same when grown on both hydrolysates. However, there was a significant difference for NRRL Y-6987 grown on either hydrolysate for lipid titer ($p = 0.0433$), and dry weight ($p < 0.0001$), but not lipid content ($p = 0.236$). It is possible that lipid titers and dry weights were more sensitive to differences between the two batches because they relied on a single time point as opposed to backscatter, which was measured throughout the experiments. Whatever the cause, statistical analysis was performed for each set of hydrolysate cultures separately.

The final lipid titers ranged widely from 2.33 to 9.40 g/L and the median was 6.12 g/L (Fig. 3; Table 4). As discussed, the experiments using NRRL and UCDFST/CBS strains were each analyzed for statistical differences separately. Statistical differences ($p < 0.0001$) were present in both sets of experiments for lipid concentration, cell biomass, lipid yield, and cellular lipid content. The best lipid producing NRRL strains were Y-6987 and Y-48190 ($p < 0.05$). For the CBS and UCDFST strains, the best lipid producers were UCDFST 67–54 and CBS 6570 as well as the external control strain NRRL Y-6987 ($p < 0.05$). Lipid content (%w/w of dry yeast biomass) is the next most critical parameter because it helps to determine the ease of oil recovery. Lipid contents ranged from 13.5% to 53.7%w/w and the median was 33.7% (Table 4). The NRRL yeast strains most enriched for lipids were Y-6987, Y-7191, Y-6984, and Y-48190 ($p < 0.05$). The results for the CBS and UCDFST experiment were more interesting. Strain UCDFST 67–54 had the maximum lipid content (53.7%w/w, $p < 0.05$). However, strains UCDFST 68–264, CBS 6570, and NRRL Y-6987 all had respectable lipids contents that exceeded 43%w/w. Strains UCDFST 68–264 and NRRL Y-7191 were of lesser interest because both had lower lipid production and, therefore, were more enriched for lipids simply because they produced less non-lipid biomass. In contrast, strains

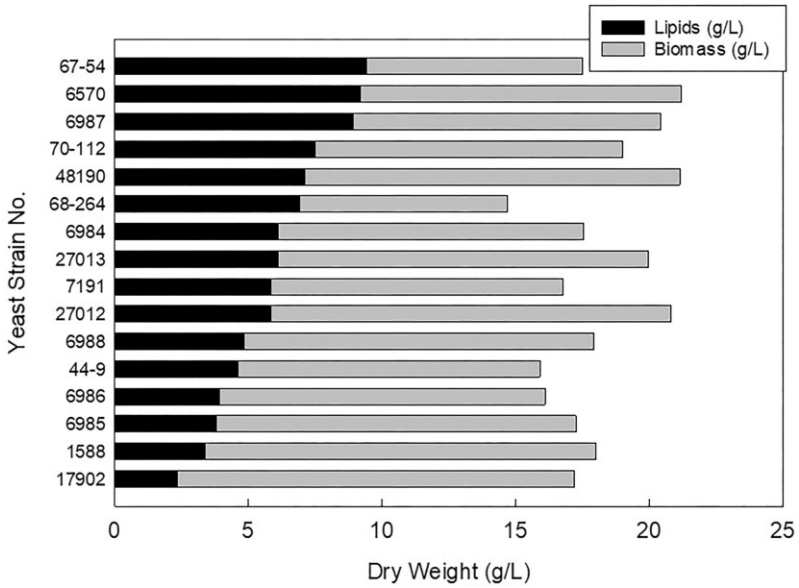


Fig. 3. Comparison of final total lipid titers for yeast grown on 60%v/v SGH.

Table 4. Yeast biomass and lipid production results from hydrolysate cultures at 96 h[1]

Yeast strain	Hydrolysate batch	Lipid titer g/L	Residual xylose[2] g/L	Yeast biomass g/L	Lipids %w/w	Lipid yield g/g[3]
NRRL Y-1588	2	3.36 ± 0.87	11.1 ± 0.1	14.98 ± 2.06	22.4 ± 2.0	0.047 ± 0.012
NRRL Y-17902	2	2.33 ± 0.36	n.d.	17.18 ± 0.22	13.5 ± 2.1	0.032 ± 0.005
NRRL Y-27012	2	5.81 ± 0.61	n.d.	19.54 ± 0.78	29.8 ± 4.9	0.081 ± 0.006
NRRL Y-27013	2	6.12 ± 0.20	n.d.	19.96 ± 0.72	30.7 ± 1.8	0.085 ± 0.003
NRRL Y-48190	2	7.11 ± 0.18	n.d.	21.14 ± 1.15	33.7 ± 1.5	0.099 ± 0.002
NRRL Y-6984	2	6.12 ± 0.34	n.d.	17.54 ± 0.2	34.9 ± 2.1	0.085 ± 0.005
NRRL Y-6985	2	3.81 ± 0.06	n.d.	17.25 ± 0.59	22.1 ± 1.0	0.053 ± 0.001
NRRL Y-6986	2	3.92 ± 0.14	n.d.	16.10 ± 1.44	24.4 ± 1.6	0.054 ± 0.002
NRRL Y-6987	2	7.60 ± 1.30	n.d.	19.30 ± 0.60	39.0 ± 6.3	0.103 ± 0.018
NRRL Y-6987	3	9.16 ± 0.03	n.d.	21.2 ± 0.2	43.3 ± 3.2	0.127 ± 0.009
NRRL Y-6988	2	4.85 ± 0.53	n.d.	17.92 ± 0.76	27.1 ± 3.7	0.067 ± 0.007
NRRL Y-7191	2	5.82 ± 0.61	n.d.	16.78 ± 1.15	34.9 ± 5.6	0.081 ± 0.008
CBS 6570	3	9.19 ± 0.03	1.5 ± 0.1	21.2 ± 0.5	43.4 ± 2.9	0.128 ± 0.009
UCDFST 44–9	3	4.65 ± 0.02	5.5 ± 0.1	16.0 ± 0.1	29.1 ± 2.3	0.065 ± 0.005
UCDFST 67–54	3	9.40 ± 0.02	n.d.	17.5 ± 0.2	53.7 ± 2.2	0.131 ± 0.007
UCDFST 68–264	3	6.93 ± 0.01	12.6 ± 0.3	14.8 ± 0.7	47.0 ± 1.1	0.096 ± 0.003
UCDFST 70–112	3	7.55 ± 0.02	n.d.	19.1 ± 0.3	39.6 ± 1.8	0.105 ± 0.005

[1]Strains, UCDFST 44–7, UCDFST 68–264, and NRRL Y-1091 did not grow on 60%v/v hydrolysate.
[2]Strains that grew exhausted glucose.
[3]Grams of lipid produced per gram of starting neutral sugars (glucose, xylose, and arabinose).

NRRL Y-6987, NRRL Y-48190, UCDFST 67–54, and CBS 6570 are of primary interest because they ranked high for both lipid production and content.

The yeast strains were also analyzed for fatty acid contents of the lipids. Fatty acid composition is important for determining market value and suitability for use in biodiesel. The fatty acids for the yeast were enriched for (in order of abundance): C18:1, C16:0, C18:2, and C18:0 (Fig. 4). Strains NRRL Y-17902 and NRRL Y-1588 showed the most variation from the group. Strain NRRL Y-17902 was enriched for C18:1 and strain NRRL Y-1588 was enriched for C18:0. The yeast lipids are expected to be suitable for manufacturing biodiesel or for use in the general oil market because they are similar in composition to canola oil and low in content of polyunsaturated fatty acids.

Discussion

Mating Type and Ploidy

Rhodotorula toruloides was an early model for understanding the life cycle of Basidiomycetous yeast (Banno, 1967). Its lifecycle was solved by studying the progeny of mating yeast with a combination of inheritable markers, microscopic examination, and single-cell nuclear content measurements (Abe & Sasakuma, 1986). While the life cycle is quite complex, it can be simplified for our purposes. There are three types of vegetive budding yeast: haploids, single mating type aneuploids (e.g., A1 or A2), or diploids. Diploids are self-sporulating and can form unnucleated hyphae, which give rise to blastospores and teliospores. Three strains were determined to be A1/A2 conjugates (Table 1) and the remainder single mating type strains.

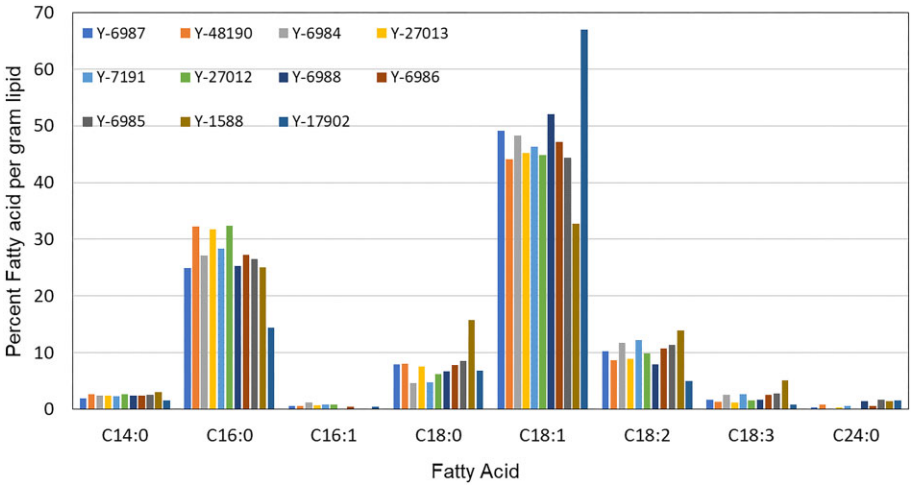


Fig. 4. Fatty acid profiles for yeast grown on 60%v/v SGH.

The molecular mating type assay developed here is an important improvement over the classical microscopic method. The most obvious advantage is that the molecular method readily distinguishes between A1, A2 and A1/A2 types. In contrast, the microscopic method test relies on a negative response to A1 and A2 tester strains or evidence of sporulation. The assay can also be used to conveniently track the stability of A1/A2 strains.

An auxiliary benefit is that the assay is specific for *R. toruloides* and highly related species. In our case, NRRL Y-17902 was shown to be misidentified as a *R. toruloides*. Molecular testing revealed that it is instead *R. mucilaginosa*. NRRL Y-17902 (ATCC 26194 = CBS 6681 = CCRC 20327 = CCY 20–2-16 = NCYC 819 = VTT C-82132) was received by the ARS culture collection from American Type Culture Collection (ATCC). The original strain was deposited at the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands) by M. Hofer, who used it for a series of sophisticated metabolic studies, albeit originally under the name *R. gracilis* (Hauer & Hofer, 1978; Woost & Griffin, 1984; Janda et al., 1993). While not a *R. toruloides*, growth and lipid results have been retained because the strain had not been previously characterized for these properties.

The yeast strains were also characterized for ploidy by measuring single-cell DNA content using the fluorescent DNA stain PI and a flow cytometer (Table 1). NRRL strains Y-6988 and Y-6987 are identified as conjugates (Table 1). Strain NRRL Y-6988 is a diploid and was created using two haploid strains (NRRL Y-6985 x Y-6986). Strain NRRL Y-6987 is triploid and was created by crossing the haploid NRRL Y-1588 to the diploid NRRL Y-1091. Identifying NRRL Y-6987 as triploid is of interest because it is the Type strain for *R. toruloides* and has been extensively reported on for production of lipids (discussed below). Strain UCDFST 70–112 was the only other A1/A2 type yeast. This is notable because the other two mated strains described in this study were both constructed in a laboratory. For the set of haploids, A1 and A2 mating types had equivalent DNA contents, which agrees with an earlier report (Suh et al., 1993). Finally, seven of the single-mating type strains contained greater than 1n DNA contents. This result is not wholly unexpected because *R. toruloides* is heterothallic and has been observed to form aneuploid cells during sporulation at a high frequency (Abe & Sasakuma, 1986). Furthermore, aneuploidy and polyploidy have been observed in *Saccharomyces* populations (Gilchrist & Stelkens, 2019) where it is hypothesized to serve as a route for rapid adaptive evolution (Gerstein & Berman, 2015; Harari et al., 2018) and to have played an important role in domestication of *Saccharomyces* (Gorter de Vries et al., 2017). Therefore, the existence of polyploid yeast in nature may play a role in the evolution of *R. toruloides*.

Polyploid versus Haploid Fitness for Growth Under Environmental Stress

As discussed in the introduction, there is both indirect and direct evidence from *Saccharomyces* that diploids are selected for when growing populations are subjected to stress. Within this context, the growth properties of A1/A2 strains (NRRL Y-6987 and Y-6988) were compared to that of their parents (NRRL strains Y-1091, Y-1588, Y-6985, and Y-6986) on 75%v/v concentrated SGH. Only the triploid (NRRL Y-6987) and diploids (NRRL Y-6988 and Y-1091) grew under these challenging conditions (Fig. 2). More generally among the NRRL yeast, the polyploid yeast averaged a 22% shorter lag time than those haploids that grew (Supplementary Fig. S2).

When the hydrolysate concentration was lowered to 60%v/v, NRRL Y-6988 and NRRL Y-6987 still had shorter lag phases and produced more lipids than its parents (Tables 3 and 4). Zhan and collaborators (Zhang et al., 2022) compared an A1/A2 *R. toruloides* strain to one of its haploid progenies for growth rate and lipid production. They also found that the A1/A2 strain (Y4) culture out produced the haploid culture; 12.0 g/L versus 6.9 g/L, respectively. However, in this study the mean lag times and final lipid titers across all haploids and polyploids were not significantly different: lag times were 34.6 ± 17.9 h versus 25.8 ± 14.4 h and final lipid titers were 6.88 ± 1.70 g/L and 5.21 ± 2.27, respectively. It is also notable that NRRL Y-1091 failed to grow in a 50%v/v SGH seed culture, belying the complexity of growth on SGH.

In conclusion, the two conjugated yeast strains were more robust for growth on concentrated hydrolysate than their parents. It is tempting to relate their superior performance to physical factors. The polyploid yeast were larger than the haploids and, therefore, have a reduced ratio of surface area to volume. It is commonly supposed that this will benefit haploids when nutrients are scarce and polyploids in the presence of toxins (Gerstein & Berman, 2015). However, this hypothesis was rejected from a study of a large and genetically diverse set of haploid and auto-diploid *Saccharomyces* strains exposed to various nutrient

limitations and toxins (Zorgo et al., 2013). While ploidy did have an effect across most of the treatments, the relative fitness of diploids versus haploids was unpredictable. Therefore, it is unknown if the superiority of the A1/A2 observed here is a general rule or arose from hybrid vigor. However, the lack of significant differences between the polyploid and haploid strains on 60%v/v SGH supports the later explanation or at the least signifies the importance of genetic factors. Still, the superiority of NRRL Y-6987 and NRRL Y-6988 compared to their parents recommends developing fusion protocols as a strategy for strain improvements.

Survey of Lipid Production Cultures on 60%v/v Concentrated SGH

Sixteen of the 19 *R. toruloides* yeast strains challenged on 60%v/v SGH were able to grow, which testifies to the robustness of this species in hydrolysate cultures. Switchgrass hydrolysate (60%v/v) on averaged contained 11.8 mM furfural and 2.8 mM HMF. This agrees with a study that characterized tolerance of *R. toruloides* Y4 (an adapted version of NRRL Y-6987) for growth in glucose media containing varying concentrations of furfural, HMF, acetic acid, and various lignin-based aromatics (Hu et al., 2009). Similar to this study, the yeast grew well in the presence of 10.2 mM furfural and 15.0 mM HMF. However, a more recent study using NRRL Y-1091 observed that this strain was able to withstand only up to 4 mM of HMF and 8 mM of furfural (Liu et al., 2021). Our yeast might have been more robust because the yeast were pre-adapted by growing the seed culture on 50%v/v SGH.

While the *R. toruloides* strains were generally robust, lipid yields varied widely within the species. We have observed similar behavior for *Lipomyces* (Slininger et al., 2016) and *Yarrowia* (Quarterman et al., 2017) and, therefore, it appears that genetic background should be a strong consideration when selecting a strain. Our laboratory has surveyed the *Yarrowia* and *Lipomyces* clades on the same SGH. *Yarrowia lipolytica* was generally a weaker lipid producer with final titers of 0.1–5.1 g/L (Quarterman et al, 2017). However, a member of the related species *Candida phangngensis* PT1-17 produced 9.8 g/L, which is comparable to the best yield observed here, albeit this yield was much higher than that observed for the other *Yarrowia* yeast strains. Another survey of primarily *Lipomyces* yeast (Slininger et al., 2016), challenged a set of 32 strains on a less challenging hydrolysate prepared with ammonia and based upon these results selected a *Lipomyces tetrasporus* and a *Lipomyces konoenkoe* yeast strains for evaluation on 75%v/v and 100%v/v SGH. The maximum lipid titer for each was 11.7 g/L and 10.8 g/L. Therefore, while both strains demonstrated exceptional tolerance to inhibitors, the end lipid titers in simple batch cultures were only slightly higher than those observed here for the best performing *R. toruloides* strain. Notably, these two strains and *R. toruloides* NRRL Y-1091 were evaluated in a two-stage culture and lipid titers were amplified to 26.2–29.0 g/L, which suggests that the yields reported here can be further improved using a more elaborate culture scheme. Other studies using *R. toruloides* to produce lipids from hydrolysates (in batch cultures) yielded 2.4–11.7 g/L of lipids starting with a wide variety of lignocellulosic substrates (for a review: Zhao et al., 2022). Therefore, in general the results reported here are comparable to those found in the literature for single batch cultures.

The best performing strains in terms of lipid titer and content on SGH were NRRL Y-6987, NRRL Y-48190, UCDFST 67–54, and CBS 6570. Strains NRRL Y-6987 (DSZM 4444, CBS 6016, IFO 8766, NBRC 8766, NRRL Y-6987, and CGMCC 2.1389) and its related domesticated strain Y4 (Yong-Hong et al., 2006; Zhao et al., 2010) are the most well studied *R. toruloides* yeast strains for lipid production. Most notably, strain Y4 was grown in a fed-batch culture on refined glucose and achieved 71.6 g/L of lipids and 106.5 g/L total cell mass in 5.6 days (Li et al., 2007). Strain Y-6987 was also among the best performing strain for a comparison of five NRRL *R. toruloides* strains (Y-1091, Y-6984, Y-6987, Y-27012, and Y-27013) for growth and lipid production on 75% v/v concentrated bioenergy sorghum hydrolysate (Cheng et al., 2021). Strain NRRL Y-1091 did not grow on the 75%v/v hydrolysate and the others produced 5–8 g/L of lipids. NRRL strains Y-6987 and Y-27013 were subsequently chosen for a two-stage culture. The lipid titers were increased to 19.1 and 17.8 g/L for NRRL Y-6987 and NRRL Y-27013, respectively. The highest lipid titers for strain NRRL Y-6987 were achieved with fed-batch cultures feeding either corn stover hydrolysate (100 g/L glucose and 10 g/l xylose) (Fei et al., 2016) or wheat-based glucose waste (94 g/L glucose) (Tsakona et al., 2016). The final lipid titers were an impressive 32 and 38 g/L with lipid contents around 60% w/w. Both groups of researchers credit the very high lipid titers to maintaining the glucose concentration at 12–17 g/L. Another determining factor was the choice of concentrated glucose syrups free of lignocellulosic inhibitors. Finally, strain CBS 6570 is notable because it was engineered for hyper-lipid production (Zhang et al., 2016). Even though only tested in fed-batch glucose cultures, the optimized engineered strains produced an impressive 89.4 g/L of lipids compared to 18.0 g/L for the control strain. Given the successes using NRRL Y-6987 and CBS 6570, it would be of interest to explore the potential of UCDFST 67–54 because it has not been previously reported on.

Fatty Acids Profiles in Hydrolysate Cultures

The properties of the yeast oils and their suitability for use in the biodiesel, food, and chemical markets is determined by their fatty acid profile. The TAGS here were enriched for the following fatty acids (type, median value): oleic acid (C18:1, 45.6%), palmitic acid (C16:0, 27.2%), linoleic acid (C18:2, 10.4%), and stearic acid (C18:0, 7.9%). The shortest and longest fatty acids measured in amounts greater than 1% were C14:0 and C24:0. These results are similar to a prior screen of 13 *R. toruloides* yeast strains from the CBS collection, which observed, from approximately high to low, C18:1, C18:2, C16:0, and C18:0 (Perrier et al., 1996). Broadly, these fatty acid profiles are similar to those reported for other well-known oleaginous yeast species, which consist primarily of C18:1 followed by C16:0, C18:0, and C18:2 (Ratledge, 2004; Poontawee et al., 2023). In yeast, palmitoyl-CoA and sterolyl-CoA are formed by type I fatty acid synthase complex, where they are exported to the endoplasmic reticulum to form TAGs. Oleic, linoleic, and others are formed in the endoplasmic reticulum by desaturases and (two-carbon) elongases (Ratledge, 2004; Adrio, 2017). The ratios of fatty acids synthesized is impacted by media and culture conditions, especially growth temperature (Sitepu et al., 2013; Wu et al., 2023).

Recently, Zhang and colleagues (Zhang et al., 2022) compared the fatty acid profile for a haploid (NP11) versus it parental diploid strain (CGMCC 2.1389) and found that there were no significant differences between them and that C18:1, C16:0, and C18:0 accounted for over 90% of the fatty acids present. Likewise, fatty acids for the diploids and haploids described here were similar.

Based on the fatty acid profile, TAGS from *R. toruloides* should be a good substitute for vegetable oils because the later are likewise enriched for C16:0, C18:0, C18:1, and C18:2 fatty acids (Sajjadi et al., 2016). The TAGS should also be suitable for use in producing biodiesel because a review of the composition of biodiesel from 28 sources of edible oils suggested they had a similar general

composition to the TAGs observed here: C18:1 > C18:2 > C16:0 > stearic acid C18:0 (ibid). More specifically, TAGS from *R. toruloides* are perhaps most similar to that of canola and rapeseed oils which share a high C18:2 content.

All the yeast strains included in this study reside in public collections. We would be remiss in not crediting the decades of dedication by numerous yeast researchers who collected strains and deposited them in formal repositories to be preserved for unknown future uses. For example, strain UCDFST 70–112 was isolated in 1970 from a bee by a student taking a yeast ecology course taught by Herman Phaff at the University of California Davis. UCDFST strains 44–7 and 44–9 were isolated in 1944 from decaying tents in New Guinea and sent to Phaff for identification, as part of a U.S. Army study of microbes that produced cellulases that caused deterioration of military materials in tropical climates. Hundreds of microbes from that study were preserved in the Quartermaster Culture Collection (QM) (Reese et al., 1950). That same project generated the *Trichoderma reesei* strain that is well-known for cellulase discoveries and numerous industrial applications. Strain UCDFST 67–54, which exhibited particularly strong lipid yield and appealing lipid composition in this study, was acquired by Herman Phaff in 1967 from Isao Banno, who named the species *R. toruloides* in that year, because it was used in his early studies of *Rhodotorula* sexuality (Banno, 1963, 1967). This study is a testament to the importance of public culture collections for the advancement of industrial microbiology as it progresses beyond the use of model yeast species.

Summary

A new molecular assay has been developed to determine mating type in *R. toruloides* and validated on a set of 19 strains. The assay also uncovered that NRRL Y-17902 was misidentified and belongs to *R. mucilaginosa*. Three of the strains were A1/A2. The set had a complex pattern of ploidies that included two triploids, eight haploids, and eight diploids or aneuploids. This set was further characterized for lipid production on SGH. Among the best four ranked strains, one has not been previously described and should be of interest for future studies. Finally, two of the A1/A2 yeast were compared to their parents in SGH challenge cultures and found to be more robust toward inhibitors. This result suggests that yeast fusion might be an effective strategy for improving future performance of *R. toruloides*.

Acknowledgments

The authors acknowledge the excellent technical help provided by Ms. Katie Card for help obtaining the PCR results, Ms. Kristina Glenzinski for operating the flow cytometer and advice on the nuclear DNA staining protocol, and Dr. Irnayuli Sitepu for molecular typing the Phaff yeast strains. The yeast strains used in this study were provided by the ARS culture collection (Peoria, IL, USA) and Phaff Yeast Culture Collection (University of California, Davis, CA, USA) and biomass hydrolysis enzymes by Novozymes North America.

Supplementary Material

Supplementary material is available online at *JIMB* (*www.academic.oup.com/jimb*).

Conflict of Interest

The authors declare no conflict of interest.

Funding

Authors are thankful to the DOE Center for Advanced Bioenergy and Bioproducts Innovation (U.S. Department of Energy, United States, Office of Science, Office of Biological and Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the U.S. Department of Energy. Environmental Research under Award Number DE-SC0018420) for funding this research work.

R. Hector, B. Dien, and P. Slininger received financial support from the U.S. Department of Agriculture, Agricultural Research Service, United States (CRIS Numbers 5010-41000-189 or 190-00D). Mention of trade names or commercial products in this article is solely for the purpose of providing scientific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

References

Abe, K. & Sasakuma, T. (1986). Identification of a diploid self-sporulating cycle in the basidiomycetous yeast *rhodosporidium toruloides*. *Journal of General Microbiology*, 132, 1459–1465. ≤Go to ISI≥://WOS:A1986C822200004.

Abeln, F. & Chuck, C. J. (2021). The history, state of the art and future prospects for oleaginous yeast research. *Microbial Cell Factories*, 20(1), 1–31. https://doi.org/10.1186/s12934-021-01712-1

Adrio, J. L. (2017). Oleaginous yeasts: promising platforms for the production of oleochemicals and biofuels. *Biotechnology and Bioengineering*, 114(9), 1915–1920. https://doi.org/10.1002/bit.26337

Banno, I. (1963). Preliminary report on cell conjugation and mycelial stage in *rhodotorula* yeasts. *Journal of General and Applied Microbiology*, 9(2), 249–251. https://doi.org/10.2323/jgam.9.249

Banno, I. (1967). Studies on sexuality of *Rhodotorula*. *Journal of General and Applied Microbiology*, 13(2), 167–196. https://doi.org/10.2323/jgam.13.167

Chen, S. F., Mowery, R. A., Castleberry, V. A., van Walsum, G. P., & Chambliss, C. K. (2006). High-performance liquid chromatography method for simultaneous determination of aliphatic acid, aromatic acid and neutral degradation products in biomass pretreatment hydrolysates. *Journal of Chromatography A*, 1104(1–2), 54–61. https://doi.org/10.1016/j.chroma.2005.11.136

Cheng, M. H., Dien, B. S., Jin, Y. S., Thompson, S., Shin, J., Slininger, P. J. W., Qureshi, N., & Singh, V. (2021). Conversion of high-solids hydrothermally pretreated bioenergy sorghum to lipids and ethanol using yeast cultures. *ACS Sustainable Chemistry & Engineering*, 9(25), 8515–8525. https://doi.org/10.1021/acssuschemeng.1c01629

Della-Bianca, B. E., Basso, T. O., Stambuk, B. U., Basso, L. C., & Gombert, A. K. (2013). What do we know about the yeast strains from the Brazilian fuel ethanol industry? *Applied Microbiology and Biotechnology*, 97(3), 979–991. https://doi.org/10.1007/s00253-012-4631-x

Dien, B. S., Mitchell, R. B., Bowman, M. J., Jin, V. L., Quarterman, J., Schmer, M. R., Singh, V., & Slininger, P. J. (2018). Bioconversion of pelletized big bluestem, switchgrass, and low-diversity grass mixtures into sugars and bioethanol. *Frontiers in Energy Research*, 6, 1–15.. https://doi.org/10.3389/fenrg.2018.00129

Dien, B. S., Peterson, M. S., & Srienc, F. (1994). Cell-cycle analysis of *Saccharomyces cerevisiae*. In *Methods in Cell Biology*. (Vol. 42), (pp. 457–475). Elsevier.

Dien, B. S., Slininger, P. J., Kurtzman, C. P., Moser, B. R., & O'Bryan, P. J. (2016). Identification of superior lipid producing lipomyces and myxozyma yeasts. *AIMS Environmental Science*, 3(1), 1–20.

Dien, B. S., Zhu, J., Slininger, P. J., Kurtzman, C. P., Moser, B. R., O'Bryan, P. J., Gleisner, R., & Cotta, M. A. (2016). Conversion of SPORL pretreated Douglas fir forest residues into microbial lipids with oleaginous yeasts. *RSC Advances*, 6(25), 20695–20705. https://doi.org/10.1039/C5RA24430G

Du, B. W., Sharma, L. N., Becker, C., Chen, S. F., Mowery, R. A., van Walsum, G. P., & Chambliss, C. K. (2010). Effect of varying feedstock-pretreatment chemistry combinations on the formation and accumulation of potentially inhibitory degradation products in biomass hydrolysates. *Biotechnology and Bioengineering*, 107(3), 430–440. https://doi.org/10.1002/bit.22829

Fei, Q., O'Brien, M., Nelson, R., Chen, X. W., Lowell, A., & Dowe, N. (2016). Enhanced lipid production by *Rhodosporidium toruloides* using different fed-batch feeding strategies with lignocellulosic hydrolysate as the sole carbon source. *Biotechnology for Biofuels*, 9(1), 430–440. https://doi.org/10.1186/s13068-016-0542-x

Gerstein, A. C. & Berman, J. (2015). Shift and adapt: the costs and benefits of karyotype variations. *Current Opinion in Microbiology*, 26, 130–136. https://doi.org/10.1016/j.mib.2015.06.010

Gilchrist, C. & Stelkens, R. (2019). Aneuploidy in yeast: segregation error or adaptation mechanism? *Yeast*, 36(9), 525–539. https://doi.org/10.1002/yea.3427

Gorter de Vries, A. R., Pronk, J. T., & Daran, J.-M. G. (2017). Industrial relevance of chromosomal copy number variation in *Saccharomyces* yeasts. *Applied and Environmental Microbiology*, 83(11), e03206–03216.

Harari, Y., Ram, Y., Rappoport, N., Hadany, L., & Kupiec, M. (2018). Spontaneous changes in ploidy are common in yeast. *Current Biology*, 28(6), 825–835.e4. https://doi.org/10.1016/j.cub.2018.01.062

Hauer, R. & Hofer, M. (1978). Evidence for interactions between energy-dependent transport of sugars and membrane-potential in yeast *Rhodotorula gacilis* (*Rhodosporidium toruloides*). *Journal of Membrane Biology*, 43(4), 335–349. https://doi.org/10.1007/Bf01871695

Hu, C. M., Zhao, X., Zhao, J., Wu, S. G., & Zhao, Z. B. K. (2009). Effects of biomass hydrolysis by-products on oleaginous yeast *Rhodosporidium toruloides*. *Bioresource Technology*, 100(20), 4843–4847. https://doi.org/10.1016/j.biortech.2009.04.041

Huang, Q. T., Kamal, R., Shen, H. W., Lu, H. B., Song, J. Y., Chu, Y. D., Xue, C., & Zhao, Z. K. (2022). Pilot-scale conversion of corn stover into lipids by the red yeast *Rhodosporidium toruloides*. *Journal of Environmental Chemical Engineering*, 10(6), 108858. https://doi.org/10.1016/j.jece.2022.108858

Janda, S., Sigler, K., & Hofer, M. (1993). Endogenous respiration reflects the energy load imposed by transport of nonmetabolizable substrates and by induced Denovo protein-synthesis in *Rhodotorula-glutinis*. *Archives of Microbiology*, 159(6), 541–544. https://doi.org/Doi 10.1007/Bf00249033

Jin, M. J., Slininger, P. J., Dien, B. S., Waghmode, S., Moser, B. R., Orjuela, A., Sousal, L. D., & Balan, V. (2015). Microbial lipid-based lignocellulosic biorefinery: Feasibility and challenges. *Trends in Biotechnology*, 33(1), 43–54. https://doi.org/10.1016/j.tibtech.2014.11.005

Klinke, H. B., Thomsen, A. B., & Ahring, B. K. (2004). Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. *Applied Microbiology and Biotechnology*, 66(1), 10–26. https://doi.org/10.1007/s00253-004-1642-2

Kurtzman, C., Fell, J. W., & Boekhout, T. (2011). *The Yeasts: a Taxonomic Study*. Elsevier.

Li, H. B. & Alper, H. S. (2020). Producing biochemicals in *Yarrowia lipolytica* from Xylose through a strain mating approach. *Biotechnology Journal*, 15(2), e1900304. https://doi.org/10.1002/biot.201900304

Li, Y. H., Zhao, Z. B., & Bai, F. W. (2007). High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture. *Enzyme and Microbial Technology*, 41(3), 312–317. https://doi.org/10.1016/j.enzmictec.2007.02.008

Liu, Z. J., Radi, M., Mohamed, E. T. T., Feist, A. M., Dragone, G., & Mussatto, S. I. (2021). Adaptive laboratory evolution of *Rhodosporidium toruloides* to inhibitors derived from lignocellulosic biomass and genetic variations behind evolution. *Bioresource Technology*, 333, 125171. https://doi.org/10.1016/j.biortech.2021.125171

Liu, Z. L., Slininger, P. J., Dien, B. S., Berhow, M. A., Kurtzman, C. P., & Gorsich, S. W. (2004). Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethlfuran. *Journal of Industrial Microbiology & Biotechnology*, 31(8), 345–352. https://doi.org/10.1007/s10295-004-0148-3

Looke, M., Kristjuhan, K., & Kristjuhan, A. (2011). Extraction of genomic DNA from yeasts for PCR-based applications. *Biotechniques*, 50(5), 325–328. https://doi.org/10.2144/000113672

Lopes, D. D., Rosa, C. A., Hector, R. E., Dien, B. S., Mertens, J. A., & Ayub, M. A. Z. (2017). Influence of genetic background of engineered xylose-fermenting industrial *Saccharomyces cerevisiae* strains for ethanol production from lignocellulosic hydrolysates. *Journal of Industrial Microbiology & Biotechnology*, 44(11), 1575–1588. https://doi.org/10.1007/s10295-017-1979-z

Mertens, J. A., Kelly, A., & Hector, R. E. (2018). Screening for inhibitor tolerant *Saccharomyces cerevisiae* strains from diverse environments for use as platform strains for production of fuels and chemicals from biomass. *Bioresource Technology Reports*, 3, 154–161. https://doi.org/10.1016/j.biteb.2018.07.006

Osorio-Gonalez, C. S., Saini, R., Hegde, K., Lefebvre, A., Avalos-Ramirez, A., & Brar, S. K. (2022). Inhibitor degradation by *Rhodosporidium toruloides* NRRL 1588 using undetoxified wood hydrolysate as a culture media. *Biomass & Bioenergy*, 160, 106419. https://doi.org/10.1016/j.biombioe.2022.106419

Park, Y.-K., Nicaud, J.-M., & Ledesma-Amaro, R. (2018). The engineering potential of *Rhodosporidium toruloides* as a workhorse for biotechnological applications. *Trends in Biotechnology*, 36(3), 304–317. https://doi.org/10.1016/j.tibtech.2017.10.013

Perrier, V., Dubreucq, E., & Galzy, P. (1996). Fatty acid and carotenoid composition of Rhodotorula strains. *Archives of microbiology*, 164, 173–179. http://doi.org/10.1007/BF02529968

Poontawee, R., Lorliam, W., Polburee, P., & Limtong, S. (2023). Oleaginous yeasts: Biodiversity and cultivation. *Fungal Biology Reviews*, 44, 100295. https://doi.org/10.1016/j.fbr.2022.11.003

Quarterman, J., Slininger, P. J., Kurtzman, C. P., Thompson, S. R., & Dien, B. S. (2017). A survey of yeast from the *Yarrowia* clade for lipid production in dilute acid pretreated lignocellulosic biomass hydrolysate. *Applied Microbiology and Biotechnology*, 101(8), 3319–3334. https://doi.org/10.1007/s00253-016-8062-y

Quarterman, J. C., Slininger, P. J., Hector, R. E., & Dien, B. S. (2018). Engineering *Candida phangngensis*-an oleaginous yeast from the Yarrowia clade-for enhanced detoxification of lignocellulose-derived inhibitors and lipid overproduction. *Fems Yeast Research*, 18(8), 1–13. https://doi.org/10.1093/femsyr/foy102

Ratledge, C. (2004). Fatty acid biosynthesis in microorganisms being used for single cell oil production. *Biochimie*, 86(11), 807–815. https://doi.org/10.1016/j.biochi.2004.09.017

Reese, E. T., Siu, R. G. H., & Levinson, H. S. (1950). The biological degradation of soluble cellulose derivatives and its relationship to the

mechanism of cellulose hydrolysis. *Journal of Bacteriology*, 59(4), 485–497. https://doi.org/10.1128/Jb.59.4.485-497.1950

Romanelli, A. M., Fu, J., Herrera, M. L., & Wickes, B. L. (2014). A universal DNA extraction and PCR amplification method for fungal rDNA sequence-based identification. *Mycoses*, 57(10), 612–622. https://doi.org/10.1111/myc.12208

Sajjadi, B., Raman, A. A. A., & Arandiyan, H. (2016). A comprehensive review on properties of edible and non-edible vegetable oil-based biodiesel: Composition, specifications and prediction models. *Renewable & Sustainable Energy Reviews*, 63, 62–92. https://doi.org/10.1016/j.rser.2016.05.035

Sitepu, I., Selby, T., Lin, T., Zhu, S., & Boundy-Mills, K. (2014). Carbon source utilization and inhibitor tolerance of 45 oleaginous yeast species. *Journal of Industrial Microbiology & Biotechnology*, 41(7), 1061–1070. https://doi.org/10.1007/s10295-014-1447-y

Sitepu, I. R., Sestric, R., Ignatia, L., Levin, D., German, J. B., Gillies, L. A., Almada, L. A. G., & Boundy-Mills, K. L. (2013). Manipulation of culture conditions alters lipid content and fatty acid profiles of a wide variety of known and new oleaginous yeast species. *Bioresource Technology*, 144, 360–369. https://doi.org/10.1016/j.biortech.2013.06.047

Slininger, P. J., Dien, B. S., Kurtzman, C. P., Moser, B. R., Bakota, E. L., Thompson, S. R., O'Bryan, P. J., Cotta, M. A., Balan, V., Jin, M., Sousa, L. D., & Dale, B. E. (2016). Comparative lipid production by oleaginous yeasts in hydrolyzates of lignocellulosic biomass and process strategy for high titers. *Biotechnology and Bioengineering*, 113(8), 1676–1690. https://doi.org/10.1002/bit.25928

Slininger, P. J., Dien, B. S., Quarterman, J. C., Thompson, S. R., & Kurtzman, C. P. (2019). Screening for oily yeasts able to convert hydrolysates from biomass to biofuels while maintaining industrial process relevance. *Methods in Molecular Biology*, 1995, 249–283. https://doi.org/10.1007/978-1-4939-9484-7_16

Slininger, P. J., Shea-Andersh, M. A., Thompson, S. R., Dien, B. S., Kurtzman, C. P., Balan, V., Sousa, L. D., Uppugundla, N., Dale, B. E., & Cotta, M. A. (2015). Evolved strains of *Scheffersomyces stipitis* achieving high ethanol productivity on acid- and base-pretreated biomass hydrolyzate at high solids loading. *Biotechnology for Biofuels*, 8(1), 60. https://doi.org/10.1186/s13068-015-0239-6

Suh, S.-O., Kuroiwa, T., & Sugiyama, J. (1993). Quantitative differences of nuclear DNA contents and their taxonomic implications in Leucosporidium scottii, Rhodosporidium toruloides, and related basidiomycetous yeasts. *The Journal of General and Applied Microbiology*, 39(3), 295–302. https://doi.org/10.2323/jgam.39.295

Talbot, N. J. & Wayman, M. (1989). Increase in ploidy in yeasts as a response to stressing Media. *Applied Microbiology and Biotechnology*, 32(2), 167–169. https://doi.org/10.1007/Bf00165882

Todd, R. T., Braverman, A. L., & Selmecki, A. (2018). Flow cytometry analysis of fungal ploidy. *Current Protocols in Microbiology*, 50(1), e58. https://doi.org/10.1002/cpmc.58

Tsakona, S., Skiadaresis, A. G., Kopsahelis, N., Chatzifragkou, A., Papanikolaou, S., Kookos, I. K., & Koutinas, A. A. (2016). Valorisation of side streams from wheat milling and confectionery industries for consolidated production and extraction of microbial lipids. *Food Chemistry*, 198, 85–92. https://doi.org/10.1016/j.foodchem.2015.11.031

Woost, P. G. & Griffin, C. C. (1984). Transport and metabolism of 2-deoxy-D-glucose by *Rhodotorula-glutinis*. *Biochimica Et Biophysica Acta*, 803(4), 284–289. https://doi.org/10.1016/0167-4889(84)90119-8

Wu, C. C., Honda, K., & Kazuhito, F. (2023). Current advances in alteration of fatty acid profile in *Rhodotorula toruloides*: A mini-review. *World Journal of Microbiology & Biotechnology*, 39(9), 234. https://doi.org/10.1007/s11274-023-03595-3

Yaguchi, A., Franaszek, N., O'Neill, K., Lee, S. H., Sitepu, I., Boundy-Mills, K., & Blenner, M. (2020). Identification of oleaginous yeasts that metabolize aromatic compounds. *Journal of Industrial Microbiology & Biotechnology*, 47(9–10), 801–813. https://doi.org/10.1007/s10295-020-02269-5

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics [Electronic Resource]*, 13(1), 134. https://doi.org/10.1186/1471-2105-13-134

Yong-Hong, L., Bo, L., Zong-Bao, Z., & Feng-Wu, B. (2006). Optimization of culture conditions for lipid production by *Rhodosporidium toruloides*. *Chinese Journal of Biotechnology*, 22(4), 650–656. https://doi.org/10.1016/S1872-2075(06)60050-2

Yu, Y. & Shi, S. B. (2023). Development and perspective of *Rhodotorula toruloides* as an efficient cell factory. *Journal of Agricultural and Food Chemistry*, 71(4), 1802–1819, https://doi.org/10.1021/acs.jafc.2c07361

Zhang, S., Ito, M., Skerker, J. M., Arkin, A. P., & Rao, C. V. (2016). Metabolic engineering of the oleaginous yeast Rhodosporidium toruloides IFO0880 for lipid overproduction during high-density fermentation. *Applied Microbiology and Biotechnology*, 100, 9393–9405. http://doi.org/10.1007/s00253-016-7815-y

Zhang, Y., Kamal, R., Li, Q., Yu, X., Wang, Q., & Zhao, Z. K. (2022). Comparative fatty acid compositional profiles of *Rhodotorula toruloides* haploid and diploid strains under various storage conditions. *Fermentation-Basel*, 8(9), 467. https://doi.org/10.3390/fermentation8090467

Zhao, X., Wu, S. G., Hu, C. M., Wang, Q., Hua, Y. Y., & Zhao, Z. B. K. (2010). Lipid production from Jerusalem artichoke by *Rhodosporidium toruloides* Y4. *Journal of Industrial Microbiology & Biotechnology*, 37(6), 581–585. https://doi.org/10.1007/s10295-010-0704-y

Zhao, Y., Song, B. C., Li, J., & Zhang, J. F. (2022). *Rhodotorula toruloides*: An ideal microbial cell factory to produce oleochemicals, carotenoids, and other products. *World Journal of Microbiology & Biotechnology*, 38(1), 13. https://doi.org/10.1007/s11274-021-03201-4

Zorgo, E., Chwialkowska, K., Gjuvsland, A. B., Garre, E., Sunnerhagen, P., Liti, G., Blomberg, A., Omholt, S. W., & Warringer, J. (2013). Ancient evolutionary trade-offs between yeast ploidy states. *Plos Genetics*, 9(3), e1003388. https://doi.org/10.1371/journal.pgen.1003388

Received: September 22, 2023. **Accepted:** November 13, 2023