

A survey of yeast from the *Yarrowia* clade for lipid production in dilute acid pretreated lignocellulosic biomass hydrolysate

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Received: 22 September 2016 / Revised: 30 November 2016 / Accepted: 8 December 2016 / Published online: 23 December 2016
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Abstract *Yarrowia lipolytica* is an oleaginous yeast species that has attracted attention as a model organism for synthesis of single cell oil. Among over 50 isolates of *Y. lipolytica* identified, only a few of the strains have been studied extensively. Furthermore, 12 other yeast species were recently assigned to the *Yarrowia* clade, and most are not well characterized in terms of cell growth and lipid accumulation, especially in industrially relevant conditions. In the present study, we investigated biomass and lipid production by 57 yeast isolates, representing all 13 species in the *Yarrowia* clade, on a non-detoxified dilute acid-pretreated switchgrass hydrolysate under highly aerobic conditions. The objective was to compare yeast physiology during growth in an abundant, low-cost biomass feedstock and to expand diversity of genetically tractable, oleaginous yeasts available for lipid research. Screening of 45 *Y. lipolytica* isolates demonstrated considerable variation within the species in terms of lipid accumulation (min = 0.1 g/L; max = 5.1 g/L; mean = 2.3 g/L); three strains (NRRL YB-420, YB-419, and YB-392) were especially promising for cellulosic biomass conversion with average improvements of 43, 57, and 64%, respectively, in final lipid titer as compared to control strain W29. Subsequently, evaluation of strains from 13 distinct species in the *Yarrowia* clade identified

Candida phangngensis PT1-17 as the top lipid producer with a maximum titer of 9.8 g/L lipid, which was over twofold higher than the second-best species in the clade (*Candida hollandica* NRRL Y-48254). A small set of the most promising strains from the screenings was further characterized to evaluate inhibitor tolerance, lipid production kinetics, and fatty acid distribution. We expect that the results of this study will pave the way for new biotechnological applications involving previously overlooked and under-characterized strains within the *Yarrowia* clade.

Keywords Oleaginous yeast · Single cell oil · *Yarrowia lipolytica* · Lignocellulosic hydrolysate · Lipid

Introduction

For almost a century, there has been commercial interest in harvesting yeast lipids as an alternative source of oil especially for food and feed applications (Lindner 1922). More recently, concerns about petroleum prices and greenhouse gas emissions have extended microbial lipid research toward sustainable production of fatty acid derived fuels and chemicals. Triacylglycerols (TAGs), which make up the majority of storage lipids in yeast, can be readily transesterified to biodiesel (i.e., fatty acid methyl esters) or hydrolyzed to release free fatty acids (FFA) for chemical transformation (Jin et al. 2015). Though similar in composition to plant oils, yeast lipids have several important advantages including a shorter process cycle, relative ease of scale-up, and potential for year-round production independent from season or climate (Beopoulos et al. 2011). The identification of suitable host strains and species for optimal lipid production from low-cost feedstocks is a critical aspect of industrial process development and has been the focus of several extensive yeast

Electronic supplementary material The online version of this article (doi:10.1007/s00253-016-8062-y) contains supplementary material, which is available to authorized users.

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screening experiments (Pan et al. 2009; Kitcha and Cheirsilp 2011; Sitepu et al. 2012, 2013; Slininger et al. 2016).

Among over 1600 identified yeast species, less than 5% are known to be oleaginous, i.e., accumulate lipids at greater than 20% of dry weight (Sitepu et al. 2013). The best lipid producers among the known oleaginous yeasts come from several genera including *Cryptococcus*, *Lipomyces*, *Rhodotorula*, *Rhodospiridium*, and *Trichosporon*; these have been shown to accumulate lipids at levels exceeding 60–70% of biomass under conditions of nutrient limitation (Ratledge and Wynn 2002). *Yarrowia lipolytica*, the most intensively studied and well characterized of all the oleaginous yeasts, can generate lipids up to approximately 40% of cell mass in similar nutrient-limited culture conditions (Beopoulos et al. 2009). Although mediocre in terms of lipogenesis, *Y. lipolytica* has emerged as a model lipid-producing microorganism with notable advantages over the aforementioned yeasts for industrial applications. This ascomycete, which attracted commercial attention in the 1970s for its ability to synthesize organic acids (Nicaud 2012), has become an established microorganism in the food industry with generally recognized as safe (GRAS) status for citric acid production and a recent assessment assuring safety of the yeast biomass for human consumption (Groenewald et al. 2014). *Y. lipolytica*'s ability to assimilate a wide variety of hydrophobic substrates makes feasible the bioconversion of agro-industrial waste residues, such as glycerol or animal fat, to higher value lipids and other useful products (Papanikolaou et al. 2002a, b; Fickers et al. 2005). Furthermore, the availability of an annotated genome sequence (Dujon et al. 2004), efficient transformation methods (Chen et al. 1997), and genetic tools, such as specialized expression vectors and selectable markers (Barth and Gaillardin 1996; Thevenieau et al. 2009), has facilitated advanced genetic engineering techniques. Thus, *Y. lipolytica* is often preferred as a host organism in metabolic engineering studies for overproduction of single-cell oil (Tai and Stephanopoulos 2013; Blazeck et al. 2014; Qiao et al. 2015) and synthesis of other oleochemicals (Ledesma-Amaro and Nicaud 2016).

Despite numerous advantages of *Y. lipolytica* for biotechnology, native strains have demonstrated little or no capacity for assimilation of pentoses, such as xylose or arabinose (Kurtzman 2011). These five-carbon sugars constitute a significant portion of polysaccharides in lignocellulosic biomass, an inexpensive and abundant feedstock that does not compete directly with food commodities. Furthermore, the yeast has been reported to have a fairly low tolerance to furfural (Sitepu et al. 2014), a degradation product from xylose and common fermentation inhibitor generated during biomass pretreatment. The inherent challenges to mixed sugar utilization and inhibitor tolerance have limited the number of research studies evaluating performance of the model oleaginous yeast in lignocellulosic hydrolysates. To our knowledge, only five studies have reported lipid accumulation by wild-type *Y. lipolytica* strains

during growth in various hydrolysates—made from wheat straw (Yu et al. 2011), sugarcane bagasse (Tsigie et al. 2011), rice bran (Tsigie et al. 2012), corn stover (Slininger et al. 2016), or woody biomass (Dien et al. 2016b). However, substantial variations in hydrolysate preparation and culture conditions make any comparison of data among the studies difficult or impossible. Also, the previous work has included only a limited number of *Y. lipolytica* strains (six wild-type strains and one auxotrophic mutant) among the scores of isolates (>50) available in culture collections around the world. The screening study by Slininger et al. (2016) did include five strains of *Y. lipolytica* producing between 2.5 and 5.8 g/L lipids in ammonia fiber expansion (AFEX)-pretreated corn stover hydrolysate. Still, little or no phenotypic data are available for the majority of isolates to describe growth, lipid production, sugar utilization, or inhibitor tolerance. Prior research on *Saccharomyces cerevisiae* suggests that yeast strains can vary widely in their tolerance to common inhibitors in cellulosic hydrolysates (Nilsson et al. 2005; Almeida et al. 2007).

Y. lipolytica was the only identified species in its genus for several decades (Nicaud 2012). In 2005, Kurtzman first defined a potential *Yarrowia* clade and proposed two species from genus *Candida* as new members based on phylogenetic analysis of 26S rDNA sequences (Kurtzman 2005). Over the past 11 years, the total number of member species in the *Yarrowia* clade has grown to 13 (Fig. 1) with the new strains isolated from various environments including meat or dairy products, sea water, and insects (Knutsen et al. 2007; Limtong et al. 2008; Chang et al. 2013; Groenewald and Smith 2013; Nagy et al. 2013, 2014). Non-fermentative metabolism and limited growth on non-glucose sugars are traits common to the clade. However, no phenotypic data are available for the *Yarrowia* clade, with the exception of a few *Y. lipolytica* isolates, relating to tolerance of common fermentation inhibitors (i.e., furfural, hydroxymethylfurfural (HMF), and acetic acid) or potential for lipid accumulation in lignocellulosic biomass hydrolysates.

In the present study, we investigated growth and lipid production by 57 yeast isolates, representing 13 species in the *Yarrowia* clade, in an enzyme saccharified dilute acid-pretreated switchgrass hydrolysate (SGH). The objective was to identify and characterize oleaginous strains in the clade that are most suitable for accumulating lipids using abundant, low-cost biomass feedstocks. “Liberty” switchgrass, the first high-yielding perennial adapted to the US Great Plains and Midwest region (Vogel et al. 2014), was chosen as the feedstock to represent a prototypical lignocellulosic biomass energy crop. The switchgrass was pretreated in dilute sulfuric acid, which is a lower cost option as compared to other alternatives; this method has long been considered to be at the forefront of biomass pretreatment technologies (Slininger et al. 2016). A suitable C/N ratio for the hydrolysate was identified by surveying growth and lipid production by a representative *Y. lipolytica* strain in synthetic media across a wide range of

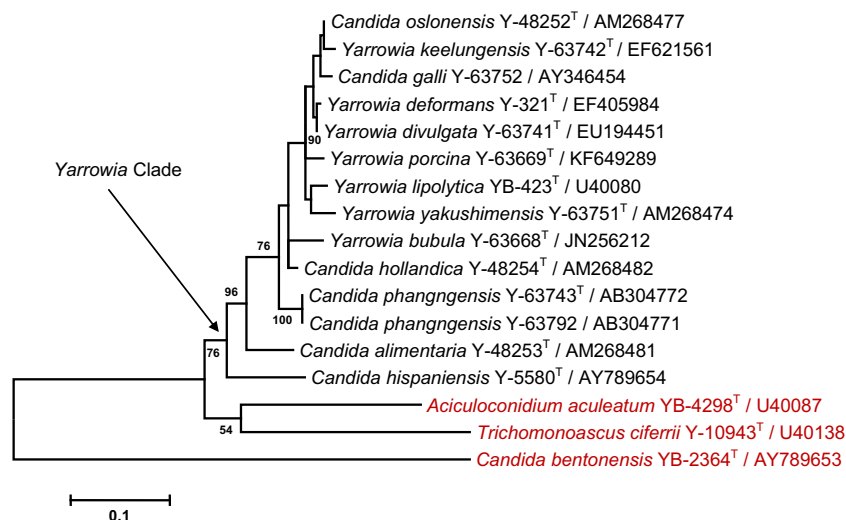


Fig. 1 Phylogenetic relationships among species of the *Yarrowia* clade (black) and reference species (red) determined from maximum likelihood analysis of D1/D2 LSU rRNA gene sequences using MEGA 5.2 (Tamura et al. 2011). Bootstrap values >50% are given at nodes and based on 1000 replicates. Strain numbers designated with *superscript T* are type strains

possible C/N values (data not shown). The initial screenings—comparing 45 *Y. lipolytica* isolates and type strains from all other species in the *Yarrowia* clade—were conducted in SGH under aerobic conditions with *Y. lipolytica* W29 (= CBS 7504) included as the control strain. The W29 strain, which is quite prevalent in the literature, was originally isolated from wastewater in Paris, France (Barth and Gaillardin 1996), and several of its auxotrophic derivatives (e.g., Po1f, Po1g) have been widely used for genetic manipulation of the species (Madzak et al. 2000; Liu and Alper 2014). The results of the initial screening provide meaningful physiological data for a large set of phylogenetically related yeasts and demonstrate surprising phenotypic diversity across the clade (and within the *Y. lipolytica* species) in terms of lipid titer, cell mass, sugar utilization, and sugar alcohol production. Subsequently, the most promising strains for lipid accumulation were further characterized to evaluate other relevant traits—tolerance to fermentation inhibitors, kinetic data for growth and lipid production, and distribution of fatty acids. We expect that the results of this study will open the way for new biotechnological applications involving previously under-characterized strains in the *Yarrowia* clade.

Materials and methods

Preparation of SGH

Switchgrass hydrolysates were prepared from pelleted Liberty switchgrass (Vogel et al. 2014) that was passed through a 4-mm knife mill. The SGH preparation at 20% w/w solids was based on a protocol described previously (Slininger et al.

of the species. Species presently placed in *Candida* need to be transferred to *Yarrowia* to conform to the International Code of Nomenclature for algae, fungi, and plants (Melbourne Code) (McNeill et al. 2012) (color figure online)

2016) with some modifications as noted below. Dilute acid pretreatment was conducted by loading 20 g dry weight of biomass into a stainless steel vessel with 80 mL of 0.936% v/v H₂SO₄ solution and 0.3 g Pluronic F-68. The vessels were mounted in a Mathis Labomat IR Dyer Oven and rotated at 50 rpm during heating to 160 °C at 2.6 °C/min, a 15-min hold at 160 °C, and cooling to 40 °C at 6.0 °C/min; the direction of rotation was reversed every minute. Subsequently, the pretreated product was neutralized by adding 3.6 mL of 30% Ca(OH)₂ solution and 4.5 mL of 1 M citric acid buffer (pH = 4.5) to each reactor vessel and mixing for 30 min in the Labomat. For enzymatic saccharification, hydrolysates were transferred to 250-mL pyrex bottles with addition of 2.7 mL Ctec2 and 0.5 mL HTec2 (Novozymes, Bagsvaerd, Denmark) to provide cellulases and hemicellulases. Capped bottles were incubated at 50 °C and 175 rpm for approximately 72 h. The resulting product was adjusted to pH 6 with 10 M NaOH and then filter sterilized through a 0.2- μ m Nalgene filter unit. An enzyme-based assay kit (Megazyme International, Wicklow, Ireland) was used to measure available nitrogen in the form of primary amino nitrogen (PAN), ammonia, and urea. For all experiments, the SGH was adjusted to C/N = 65 by supplementing with 2.31 g/L (NH₄)₂SO₄ (Fisher Scientific, Waltham, MA, USA) and a vitamin-free casamino acid mix containing 1.81 g/L Difco Vitamin Assay Casamino Acids (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), 0.018 g/L L-tryptophan (Sigma, St. Louis, MO, USA), and 0.072 g/L L-cysteine (Sigma, St. Louis, MO, USA). Amended SGH was diluted with sterile water as specified for each experiment. Table 1 describes the concentration of sugars, inhibitors, and nitrogen compounds in a representative batch of SGH before and after nitrogen amendment.

Table 1 Composition of enzyme saccharified dilute acid pretreated switchgrass hydrolysate (SGH) with or without nitrogen amendments

	Component	Units	SGH (Unamended)	SGH (N-amended)	60% SGH (N-amended)
Sugars	Glucose	g/L	64.2 ± 0.1	61.0	36.4
	Xylose	g/L	50.1 ± 0.0	47.6	28.3
	Arabinose	g/L	5.4 ± 0.1	5.1	3.0
Inhibitors	Acetic acid	g/L	5.4 ± 0.1	5.1	2.9
	Furfural	mM	15.9 ± 0.1	15.1	8.8
	HMF	mM	4.4 ± 0.0	4.2	2.5
Nitrogen	PAN	mg/L	160.6 ± 3.2	225.3	135.2
	Ammonia	mg/L	120.9 ± 1.8	710.3	426.2
	Urea	mg/L	12.7 ± 0.0	12.1	7.3
	C/N ratio	–	209.9	65.0	65.0

HMF 5-hydroxymethylfurfural, PAN primary amino nitrogen

Yeast strains and culture conditions

Y. lipolytica strain W29 (= CBS 7504) was obtained from the CBS-KNAW Culture Collection in Utrecht, the Netherlands and used as the control in this study. Forty-four other *Y. lipolytica* isolates, including the type strain NRRL YB-423, were selected from the ARS (NRRL) Culture Collection in Peoria, IL, USA. The strain accession numbers for all 45 *Y. lipolytica* strains evaluated in this study are included in Table 2. In addition, the type strains of 12 other species in the *Yarrowia* clade were obtained from the CBS and ARS Culture Collections for comparison with the model oleaginous species *Y. lipolytica*. The strain number, abbreviated name, and source habitat of all isolates used in the *Yarrowia* clade screening and subsequent experiments are described in Table 3. A second isolate of *Candida phangngensis* (strain TM2-16) was generously provided to us by Dr. Savitree Limtong (Kasetsart University, Bangkok, Thailand) and deposited in the ARS Culture Collection (NRRL Y-63792). All strains used in this study are wild-type, prototrophic yeast strains.

After being received from the culture collections, all strains were maintained in 20% glycerol at –80 °C. Yeast strains were revived from cryo-preserved stocks by streaking onto yeast extract-peptone-dextrose (YPD) agar plates, incubating for 48–72 h, and refrigerating for short-term use within 1 week. All *Yarrowia* strains were cultured at 28 °C on either solid or liquid media. For the rich media preculture, a single colony was inoculated into 10 mL liquid YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) in a 50-mL baffled flask and incubated at 250 rpm for approximately 24 h. A 5% (v/v) inoculum of this preculture was transferred into SGH (C/N = 65; pH = 6) diluted to 50% (v/v) strength with sterile water and then 0.5 mL per well was dispensed into deep, 96-well plates (Applikon Biotechnology, Delft, the Netherlands) with at least one empty row between strains. Deep well plates were fitted with standard aeration covers and incubated at

400 rpm for 48 h. Next, hydrolysate precultures were harvested and washed with sterile water before inoculating cells to OD_{620 nm} ≈ 1 in fresh SGH (C/N = 65; pH = 6) diluted to 60, 75, or 90% (v/v) strength as specified for the experiment. Test cultures were dispensed into deep well plates (0.5 mL per well) and incubated as described above for the SGH precultures (400 rpm). For each time point evaluated, yeast samples were harvested in duplicate by pipetting the entire content of two separate wells and analyzing for dry weight, lipids, and HPLC. For the screening of 45 *Y. lipolytica* strains, there were two minor modifications to the above procedure in order to accommodate a large number of cultures. First, the rich media preculture was conducted by inoculating a single colony into 5 mL YPD and dispensing 0.5 mL per well into deep well plates, which were incubated for ~24 h at 400 rpm. Second, test cultures were inoculated by a 3.2% (v/v) transfer from the hydrolysate preculture into fresh 60% strength SGH (C/N = 65; pH = 6) in order to achieve initial OD_{620 nm} ≈ 1 for the best-growing strain. Test cultures were grown in deep well plates as described previously.

Time-course profiles of select strains were obtained at larger scale in duplicate flask cultures. For this purpose, precultures were grown up from a single colony in 10 mL YPD in a 50-mL baffled flask as before. After 24 h, a 5% (v/v) inoculum was transferred into 10 mL of 50% strength SGH (C/N = 65; pH = 6) in 50-mL baffled flasks and incubated for 24 h at 250 rpm. Hydrolysate precultures were harvested and washed with sterile water. Then, washed cells were inoculated to OD_{620 nm} ≈ 1 in 25 mL of fresh 50% strength SGH (C/N = 65; pH = 6) in 125-mL baffled flasks. Flask cultures were incubated with 250 rpm shaking and periodically sampled in duplicate for dry weight, lipids, and HPLC.

Lipid quantification

Lipids were quantified using a sulfo-phospho-vanillin (SPV) colorimetric assay modified from Izard and Limberger (2003)

Table 2 Screening results for 45 *Yarrowia lipolytica* strains grown in SGH

Strain	Dry cell weight (g/L)	Lipid (g/L)	Lipid content ^a (%)	Lipid yield ^b (g/g)	Consumed xylose (g/L)	Sugar alcohol ^c (g/L)
W29	15.0	3.1 ± 0.1	20.8 ± 0.5	0.089	19.7	7.0 (93)
YB-271	13.6	2.2 ± 0.1	16.1 ± 0.7	0.062	25.0	17.7 (97)
YB-272	3.3	0.1 ± 0.0	2.1 ± 0.5	0.015	0	0
YB-275	7.7	0.7 ± 0.0	8.8 ± 0.6	0.032	1.5	0.2 (100)
YB-276	15.1	2.6 ± 0.1	17.3 ± 0.8	0.074	7.0	0.6 (100)
YB-279	10.1	0.4 ± 0.2	4.0 ± 2.0	0.013	0	0
YB-281	13.2	1.1 ± 0.3	8.2 ± 2.0	0.032	20.1	10.0 (55)
YB-387	12.1	2.1 ± 0.0	17.3 ± 0.2	0.060	22.6	14.4 (95)
YB-392	15.8	4.5 ± 0.3	28.2 ± 1.8	0.127	6.1 ± 0.4	2.0 ± 0.2 (33)
YB-419	17.2	4.9 ± 0.0	28.5 ± 0.0	0.139	17.1 ± 0.2	7.1 ± 0.3 (100)
YB-420	13.4	5.1 ± 0.2	37.9 ± 1.3	0.152	4.0 ± 0.0	0
YB-421	9.8	1.5 ± 0.1	15.2 ± 0.9	0.042	17.6	10.4 (95)
YB-422	11.6	0.7 ± 0.2	6.2 ± 1.6	0.021	8.4	2.1 (91)
YB-423	13.7	2.4 ± 0.0	17.9 ± 0.0	0.072	5.6	4.0 (65)
YB-437	15.3	2.5 ± 0.4	16.2 ± 2.7	0.070	19.8	10.3 (90)
YB-523	11.1	0.2 ± 0.0	1.5 ± 0.2	0.005	0	0
YB-566	12.1	2.2 ± 0.1	18.4 ± 1.0	0.063	25.7	20.7 (60)
YB-567	12.2	2.3 ± 0.2	18.6 ± 1.8	0.065	25.7	19.9 (59)
YB-618	17.7	2.4 ± 0.7	13.4 ± 4.0	0.067	12.3	1.6 (65)
YB-3381	16.3	3.4 ± 0.7	20.7 ± 4.2	0.096	2.9	1.2 (0)
Y-1094	14.6	1.3 ± 0.0	9.2 ± 0.1	0.038	19.2	5.5 (93)
Y-1095	14.9	2.1 ± 0.0	14.3 ± 0.2	0.061	14.7	5.7 (100)
Y-5383	10.0	0.6 ± 0.0	5.9 ± 0.1	0.030	0	0
Y-5384	18.3	3.8 ± 0.2	20.9 ± 0.9	0.109	14.1	3.2 (61)
Y-5385	15.9	2.0 ± 0.1	12.8 ± 0.7	0.060	1.3	0.4 (0)
Y-5386	18.2	4.1 ± 0.3	22.6 ± 1.6	0.117	12.3	4.1 (42)
Y-7207	18.1	4.0 ± 0.0	21.8 ± 0.0	0.112	10.6	2.8 (9)
Y-7208	16.8	2.4 ± 0.0	14.0 ± 0.1	0.067	8.4	1.9 (31)
Y-7212	18.1	3.6 ± 0.1	20.1 ± 0.3	0.103	9.7	2.6 (9)
Y-7318	6.8	0.3 ± 0.1	3.9 ± 1.1	0.030	0	0
Y-7484	16.8	2.6 ± 0.4	15.4 ± 2.2	0.074	10.4	2.1 (36)
Y-7751	15.8	2.0 ± 0.5	12.8 ± 3.1	0.057	9.2	3.9 (19)
Y-7752	16.3	1.9 ± 0.5	11.6 ± 3.0	0.054	9.7	3.6 (23)
Y-7753	17.4	3.2 ± 0.1	18.6 ± 0.7	0.096	9.7	2.1 (36)
Y-7754	18.6	3.8 ± 0.2	20.5 ± 1.1	0.108	10.1	2.5 (34)
Y-7755	14.2	2.3 ± 0.2	16.4 ± 1.7	0.066	14.3	3.6 (59)
Y-7756	15.1	3.1 ± 0.1	20.6 ± 0.9	0.104	1.3 ± 0.1	0
Y-7757	15.1	3.3 ± 0.4	22.2 ± 2.7	0.111	2.4 ± 0.2	0
Y-7817	14.6	2.0 ± 0.2	13.6 ± 1.1	0.056	13.9 ± 0.2	4.0 (77)
Y-9635	9.1	1.3 ± 0.2	14.5 ± 1.8	0.049	7.3 ± 0.1	0.1 (0)
Y-11853	11.1	1.1 ± 0.1	9.7 ± 1.3	0.055	0	0
Y-17536	17.3	2.9 ± 0.5	16.9 ± 2.6	0.083	9.3 ± 0.2	1.0 (52)
Y-63529	10.4	1.3 ± 0.5	12.1 ± 5.1	0.036	2.5 ± 0.9	2.4 (96)
Y-63530	12.4	1.3 ± 0.2	10.6 ± 1.8	0.039	14.8 ± 0.3	8.5 (98)
Y-63532	13.8	2.2 ± 0.2	15.6 ± 1.2	0.061	8.5 ± 0.1	5.7 (51)

Cells were harvested in duplicate at 72 h during growth on 60% (v/v) strength N-amended SGH

^a Lipid content is calculated as a percent of dry weight

^b Lipid yield is calculated per gram of glucose consumed

^c Sugar alcohol is the sum of xylitol and arabitol produced. The number in parentheses indicates percent of total sugar alcohol that is xylitol

and previously described for tracking intracellular lipid accumulation by oleaginous yeast (Wang et al. 2009; Dien et al. 2016a, b; Slininger et al. 2016). Briefly, washed cell pellets (20 µL) were mixed with 0.4 mL concentrated sulfuric acid in glass test tubes and heated for 10 min at 100 °C. The reaction was cooled for 10 min in an ambient water bath. Next, 1 mL of a vanillin-phosphoric acid solution was added to each tube and the reaction was allowed to proceed for 15 min at 37 °C. After cooling for 10 min, the absorbance of each

reaction was measured at 530 nm against a reference sample prepared with 20 µL water. For creating a calibration curve, three lipid standards were prepared on the same day by dissolving corn oil in 2:1 (v/v) chloroform/methanol and duplicate samples of each standard (20 µL each) were included in the assay. The vanillin-phosphoric acid solution was prepared fresh for each assay by dissolving 0.12 g vanillin in 20 mL distilled water and 80 mL of 85% o-phosphoric acid. The assay was validated previously for various oleaginous yeast

Table 3 Description of species and strains used in *Yarrowia* clade screening and subsequent experiments

Species	Strain ^a	Abbreviation	Source habitat
<i>Yarrowia lipolytica</i>	CBS 7504 = W29	YL29	Wastewater, France
<i>Y. lipolytica</i>	YB-392	YL392	Gluten settler, IL, USA
<i>Y. lipolytica</i>	YB-419	YL419	Milled corn, IL, USA
<i>Y. lipolytica</i>	YB-420	YL420	Milled corn, IL, USA
<i>Y. lipolytica</i>	YB-567	YL567	Gluten settling water, IL, USA
<i>Candida alimentaria</i>	Y-48253 ^T	CA	Cured ham, Norway
<i>Candida galli</i>	Y-63752	CG	Chicken liver, GA, USA
<i>Candida hispaniensis</i>	Y-5580 ^T	CHi	Larva of beetle, Spain
<i>Candida hollandica</i>	Y-48254 ^T	CHo	Back of cow, Netherlands
<i>Candida oslonensis</i>	Y-48252 ^T	CO	Kiwi yogurt, Norway
<i>Candida phangngensis</i>	Y-63743 ^T = PT1-17	CP	Seawater, Thailand
<i>C. phangngensis</i>	Y-63792 = TM2-16	CP2	Seawater, Thailand
<i>Yarrowia bubula</i>	Y-63668 ^T	YB	Beef, Hungary
<i>Yarrowia divulgata</i>	Y-63741 ^T	YDi	Bacon, Denmark
<i>Yarrowia deformans</i>	Y-321 ^T	YDe	Fingernail, Austria
<i>Yarrowia keelungensis</i>	Y-63742 ^T	YK	Seawater, Taiwan
<i>Yarrowia porcina</i>	Y-63669 ^T	YP	Minced pork, Hungary
<i>Yarrowia yakushimensis</i>	Y-63751 ^T	YY	Termite gut, Japan

^a Strain numbers designated with superscript T are type strains of the species

lipids, and common media ingredients were found to provide minimal interference (Dien et al. 2016a). The SPV assay shows a highly linear colorimetric response across a wide range of oil concentrations (Supplementary Fig. S1).

Lipid extraction and fatty acid profiling

Lipids were extracted from yeast by following a procedure based on that described previously by Schneiter and Daum (2006). Briefly, washed cells from 5 mL yeast cultures were lyophilized over two nights in a freeze dryer (Labconco, cat. no. 7960046, Kansas City, MO, USA). Dry cells were lysed in a FastPrep-24 homogenizer (M.P. Biomedicals, Santa Ana, CA, USA) by agitation for 1 min with 0.5 mm zirconia/silica beads. Then, 1 mL of extraction solvent (2:1 v/v, chloroform/methanol) was added to each tube followed by agitation for 1 h in a multi-reaction vortexer (Heidolph, Schwabach, Germany). After centrifugation, 0.6 mL of the extract was transferred to 0.2 mL of 0.3% NaCl for washing to remove water-soluble contaminants. The organic layer was transferred to a separate vial, and the solvent was evaporated overnight at ambient temperature. Transesterification of lipid extracts was performed by adding 2 mL of hexane and 0.2 mL of 2 M KOH in methanol to each sample and mixing for 2 min. For analysis of fatty acid composition, the resulting FAME samples were injected into a GC-FID (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent HP-88 capillary column (30 m × 0.25 mm). The following settings were used for the GC analysis: injection

volume = 2 μL; inlet = splitless mode; detector temp = 280 °C; H₂ flow = 35 mL/min; air flow = 400 mL/min; and oven temp = 135 °C for 5 min, increased at 2 °C/min to 210 °C, increased at 50 °C/min to 220 °C, and held 5 min at 220 °C. To identify peaks, a commercial reference standard containing 31 saturated and unsaturated fatty acid methyl esters (Nu-Chek Prep, Inc., cat. no. GLC-411, Elysian, MN, USA) was also analyzed by GC as above. Total fatty acid content of each sample was based on the sum of all individual FAME peak areas identified in the chromatogram.

Biomass determination

Dry weight was measured from 0.5 mL yeast samples that were washed and resuspended in distilled water. Washed cells were transferred into a preweighed aluminum pan with an equal volume of distilled water for rinsing. Samples were dried in an oven at 80 °C for 24 h before determining gross weight on an analytical balance. Optical density (OD) was measured by absorbance at 620 nm in 1 cm path length cuvettes with an Evolution 60 spectrophotometer. Samples were diluted to obtain absorbance values in the linear range for the instrument.

HPLC analysis

The concentration of extracellular sugars, acetic acid, and aldehyde inhibitors (HMF and furfural) was determined by a Waters high-performance liquid chromatography system

equipped with an Aminex HPX-87H column (Biorad Laboratories, Inc., Hercules, CA, USA). Column temperature was kept constant at 60 °C, and the elution was performed with 15 mM HNO₃ at a flow rate of 0.6 mL/min. Sugars and acetic acid were detected with a refractive index detector (RID), and inhibitors were detected with an ultraviolet absorbance detector at 245 nm. Sugar alcohols, including xylitol and arabitol, were analyzed by a SpectraSystem liquid chromatography system (Thermo Electron Corporation, Waltham, MA, USA) equipped with an Aminex HPX-87P column (Biorad Laboratories, Inc., Hercules, CA, USA) and RID. Column temperature was maintained at 80 °C, and elution was performed with water at 0.6 mL/min.

Results

Screening of 45 *Y. lipolytica* strains

The initial screen included *Y. lipolytica* strains only. All 45 isolates available from the ARS and CBS culture collections were grown in 60% (v/v) strength SGH (Table 1) using deep, 96-well plates for a comparative evaluation of strains within the species. After 72 h, yeast samples were harvested in duplicate and analyzed for growth, lipid accumulation, sugar utilization, and metabolite production (i.e., xylitol and arabitol). Table 2 summarizes the key parameters for all strains evaluated. The reference strain W29 (= CBS 7504) accumulated 3.1 g/L lipid and 15.0 g/L cell mass for a total lipid content of 20.8% dry weight (DW). Among the remaining isolates, 20 out of 44 produced more biomass than the control (W29) and the highest dry weight of 18.6 g/L was observed for NRRL Y-7754. The poorest growing strain (NRRL YB-272) generated 3.3 g/L cell mass and consumed only 12.7% of the initial glucose (35.2 g/L) during the course of the experiment. Also, NRRL YB-272 was the lowest lipid producer with 0.1 g/L total lipids and a lipid content of 2.1% DW. Although 25% of the isolates evaluated (11 out of 44) demonstrated a higher lipid titer than the control, only eight of the 11 (NRRL YB-392, YB-419, YB-420, Y-5384, Y-5386, Y-7207, Y-7212, and Y-7754) were statistically significant improvements ($P < 0.05$). Among these, the best three strains (NRRL YB-420, YB-419, YB-392) produced 40–65% more lipids than the reference W29. NRRL YB-420 was the top lipid producer among all strains in terms of both titer (5.1 g/L) and cell content (37.9% DW), although its cell mass (13.4 g/L) was below average for the species. In addition, NRRL YB-419 and YB-392 were promising strains for lipid accumulation with final lipid output of 4.9 and 4.5 g/L, respectively, and lipid content of 28.5 and 28.2% DW, respectively. Overall, 12 of the 45 strains tested generated intracellular lipids at greater than 20% of dry cell weight, which is the threshold to be classified as oleaginous (Ratledge 1991).

In terms of sugar utilization, most strains (28 of 45) consumed all glucose in the hydrolysate (35.2 g/L) within 72 h and only nine isolates consumed less than 90% of the initial glucose during this time. As expected, xylose uptake was inhibited in the presence of glucose due to carbon catabolite repression (Gancedo 1998). However, the 28 strains that did exhaust glucose in the hydrolysate showed a surprisingly large variability in xylose consumption, ranging from 2.5 to 25.7 g/L (median = 12.3 g/L). This result not only could be due to a variation in xylose transport or aldose reductase activity within the species but also may be explained by other factors such as glucose consumption rate or inhibitor tolerance. YB-566 and YB-567 were the best xylose-utilizing isolates of *Y. lipolytica* even though their cell growth and lipid production were below average for the species. For these two strains, almost 75% of consumed pentose was reduced to xylitol and arabitol, and they secreted sugar alcohols at the highest overall titers (20.7 and 19.9 g/L, respectively). The best three lipid producers (YB-420, YB-419, and YB-392) showed surprising variation in terms of xylose consumption (4.0, 17.1, and 6.1 g/L, respectively) and xylitol production (0, 7.1, and 0.7 g/L, respectively) although they were isolated from similar habitats. For comparison, the reference W29 consumed 19.7 g/L xylose and generated 6.6 g/L xylitol during the course of the experiment. Although we originally hypothesized that the xylose assimilation pathway and fatty acid synthesis pathway may be competing for the co-factor NADPH in oleaginous yeast, there was no significant correlation (negative or positive) between xylose consumption and lipid production within the set of 45 isolates evaluated ($R^2 = 0.04$). However, there was a positive correlation between xylose and arabinose consumption ($R^2 = 0.70$), which is likely due to overlap in activity toward the two pentoses by some sugar transporters and aldo-keto reductases (Ellis 2002; Träff et al. 2002; Sanchez et al. 2010).

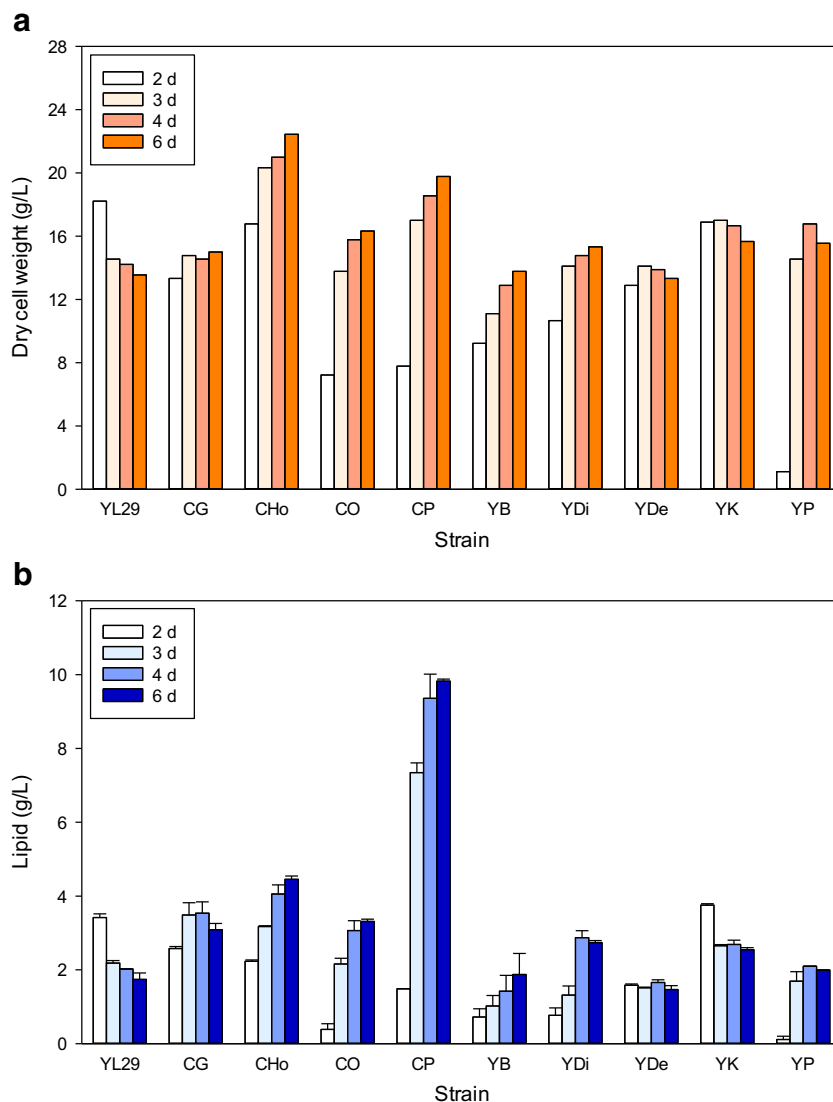
Comparative evaluation of 13 species in the *Yarrowia* clade

To broaden the search, screening was extended to the entire *Yarrowia* clade. The reference strain *Y. lipolytica* W29 and type strains from 12 other species in the clade (Fig. 1) were subsequently evaluated for growth and lipid production in cellulosic hydrolysate. Table 3 lists the abbreviation and source habitat for all strains included in the clade screening. Although *Candida alimentaria* (CA) was viable on YPD plates and normal-sized colonies appeared within 3 days, there was no growth in liquid YPD flask cultures at 28 °C within 1 week. A previous study reported that this strain showed optimum growth in YPD at 21 °C, but cell growth was inhibited at 25 °C; nevertheless, all other *Yarrowia* strains evaluated, representing eight different species, grew normally at 28 °C (Michely et al. 2013). Thus, CA was the first to be

eliminated in our clade screening due to its temperature sensitivity at ≥ 25 °C, which is unfavorable for commercial scale-up. The remaining strains were all viable in seed cultures of 50% (v/v) strength SGH, and washed cells were subsequently transferred to fresh 60% (v/v) strength SGH test cultures for periodic monitoring in aerobic conditions. Duplicate wells were harvested at 2, 3, 4, and 6 days, and samples were analyzed for lipids, dry cell weight, and sugars. Two strains (*CHi* and *YY*) were unable to tolerate the 60% (v/v) SGH test culture and remained at low cell mass (<1.7 and <0.5 g/L, respectively) with little or no sugar utilization even up to 6 days. We suspect that poor inhibitor tolerance may have been a factor limiting cell growth in the hydrolysate by these two strains. For the remaining ten species, Fig. 2 summarizes the results for growth and lipid accumulation at the four sample times. The control *YL29* reached a maximum dry weight of 18.2 g/L at 2 days with lipid production at 3.4 g/L and 18.8% DW. Between 2 and 3 days of incubation, cell mass dropped

significantly to 14.6 g/L with one third of the weight loss due to lipid catabolism; thereafter, biomass remained fairly steady for the duration of the experiment. Only two of nine *Yarrowia* species reached a higher cell mass than the reference strain—*Candida hollandica* (*CHo*) and *C. phangngensis* (*CP*) achieved a maximum of 22.4 and 19.8 g/L biomass, respectively, at 6 days. *CP* was by far the top lipid producer in the clade with lipid titer (and cell growth) surpassing the control strain between days 2 and 3 and then steadily increasing to a maximum of 9.8 g/L (49.7% DW) at day 6. *Yarrowia keelungensis* (*YK*) and *CHo* also showed a significant improvement in maximum lipid accumulation as compared to the reference strain ($P < 0.05$) with maxima occurring at day 2 and day 6, respectively. Similar to the control, *YK* grew up quickly to 16.9 g/L dry weight and accumulated 3.8 g/L lipids within 2 days, but 30% of total lipid was consumed between days 2 and 3. *CHo* was the second-best lipid producing strain in the clade under these conditions, but the maximum titer

Fig. 2 Comparative evaluation of species in the *Yarrowia* clade on SGH. Cells were cultured in 60% (v/v) strength N-amended SGH in deep, 96-well plates and sampled at four time points for analysis of **a** dry weight and **b** lipid accumulation. Error bars indicate standard deviation from the mean of duplicate samples. Strains are represented by an abbreviation of their species name, as indicated in Table 3. Three *Yarrowia* strains were unable to grow in this condition and are not included in the figure (*CA*, *CHi*, and *YY*)



(4.5 g/L) was still less than half that of the *CP* strain. Overall, 4 out of 13 species that we tested in this experiment achieved a maximum lipid content of greater than 20% of dry weight (*CG*, *CO*, *CP*, and *YK*); the oleaginicities of these strains has not been demonstrated previously on non-hydrophobic substrates.

Two strains—*YL29* and *YK*—had the fastest glucose consumption rate in the clade, and both were able to consume all glucose in the hydrolysate within 2 days. After depletion of glucose, these two yeasts consumed 24.0 and 18.0 g/L xylose, respectively, at the 3 day sample time—tops for the clade. The best lipid-producing strain (*CP*) showed a longer lag time (≈ 40 h) after inoculation to SGH test cultures and consumed only 0.6 g/L xylose after 3 days; xylose consumption steadily increased to 9.6 g/L after 6 days. All ten *Yarrowia* species that grew in SGH test cultures showed some capacity for transporting xylose into the cell in the absence of glucose, although to varying degrees. However, cell growth generally slowed or plateaued after glucose depletion, and we found little or no evidence for assimilation of xylose to produce biomass. Supplementary Fig. S2 describes sugar utilization and xylitol production over time for all strains evaluated. After uptake, intracellular xylose was reduced to xylitol and then primarily secreted by the cells rather than being further metabolized to xylulose-5-phosphate for entry into the pentose phosphate pathway. Among the set of 13 *Yarrowia* strains evaluated, *YK* produced the most xylitol with a titer of 12.9 g/L at day 3 and yield of 0.72 g/g of xylose consumed. At the same sample time, the control *YL29* produced 10.6 g/L xylitol at a yield of 0.44 g/g of xylose consumed. Table 4 summarizes the results for lipid and sugar alcohol production by all strains in the clade.

Physiological characterization of select strains

After screening a total of 57 *Yarrowia* isolates, we wanted to further characterize the most promising yeasts for current and future applications involving bioconversion of lignocellulosic hydrolysates. Seven strains were selected based on the screening results, including five *Y. lipolytica* isolates (*YL29*, *YL392*, *YL419*, *YL420*, and *YL567*) and two other species from the clade (*CHo* and *CP*). First, we evaluated capacity for growth and lipid accumulation at various strengths of hydrolysate in order to test for tolerance to common fermentation inhibitors in SGH (i.e., furfural, HMF, and acetic acid). Yeasts were cultured in either 60, 75, or 90% (*v/v*) strength SGH in deep 96-well plates, and cells were harvested at 3 days for analysis (Fig. 3). All seven strains grew to >10 g/L cell mass in the 60% SGH, and six of the seven were also able to propagate in the higher strength 75% SGH. Unfortunately, the best lipid producer from the screening experiments, *CP*, was the only isolate unable to grow in more concentrated hydrolysate; thus, the type strain of *C. phangngensis* appears to have heightened

sensitivity to fermentation inhibitors as compared to other yeasts in the clade. Both *YL419* and *YL420* accumulated 6.7 g/L lipid in the 75% SGH—the highest lipid titer observed for *Y. lipolytica* in any condition used in this study—while cell density (22.6 and 17.3 g/L, respectively) and xylitol production (12.1 and 0 g/L, respectively) were quite different between the two isolates. In comparison, control *YL29* grew to 18.6 g/L biomass with 4.1 g/L lipid in the same condition. In the 90% SGH, the control strain did not grow and only three of the seven strains were able to achieve cell mass >5 g/L (*YL392*, *YL420*, and *YL567*). Among these three, *YL392* reached the highest cell density (17.3 g/L) and lipid output (4.1 g/L) but both parameters were lower than those for the same strain grown in 75% SGH (19.6 g/L DW and 6.2 g/L lipid). In general, the jump from 60% SGH to 75% SGH was beneficial for boosting lipid titers and yields by *Y. lipolytica* strains, while the subsequent increase to 90% strength was detrimental to productivity—most likely due to increased toxicity from inhibitors. The only exception to this trend was *YL567*, which grew to similar cell density and lipid content regardless of the hydrolysate concentration. The best pentose-reducing strain from the *Y. lipolytica* screening, *YL567*, generated its highest sugar alcohol titer of 29.2 g/L in the 75% SGH, and the titer dropped slightly to 25.7 g/L in the 90% SGH.

To validate microtiter plate results at a larger scale, we cultured select *Yarrowia* strains using 50% (*v/v*) strength SGH in highly aerated baffled shake flasks. Duplicate flasks were sampled every 12–16 h throughout the exponential growth phase in order to obtain reliable kinetic data for cell growth and lipid production. A second isolate of *C. phangngensis* called TM2-16—abbreviated *CP2* in this study—was included at this stage to compare its lipogenesis with the type strain of the species (*CP*). Figure 4 and Supplementary Fig. S3 show time-course profiles for the seven strains included in the flask experiments. Lipid and dry weight numbers were noticeably lower in flask cultures as compared to those in deep well plates likely due to decreased oxygen transfer rate at 250 rpm (versus 400 rpm) and lower total sugars in 50% *v/v* SGH (versus 60% strength). Still, general trends were very similar among strains at the two different scales. In flasks, *CP* attained a maximum lipid titer of 6.1 g/L in stationary phase (40.6% DW), which was greater than twofold higher than the control strain at its peak (2.8 g/L). *CP2* accumulated lipid to a similar level (5.9 g/L) as the type strain, but lag phase appeared to be approximately 8–12 h shorter for this isolate. Both strains grew to similar cell mass (14.8–15.0 g/L) and consumed xylose slowly after glucose depletion. As compared to the two *C. phangngensis* isolates, the five *Y. lipolytica* strains had significantly shorter lag time—likely due to greater tolerance to inhibitors (Fig. 3)—and all showed dry weights in the range of 2.4–5.3 g/L within 13 h. Control *YL29* reached a maximum glucose consumption

Table 4 Comparative evaluation of 13 species in the *Yarrowia* clade grown in SGH

Strain ^a	Dry cell weight (g/L)	Lipid (g/L)	Lipid content ^b (%)	Lipid yield ^c (g/g)	Consumed xylose (g/L)	Sugar alcohol ^d (g/L)
YL29	14.6	2.2 ± 0.1	15.0 ± 0.5	0.060	24.0	11.4 (93)
CA ^e	–	–	–	–	–	–
CG	14.8	3.5 ± 0.3	23.6 ± 2.3	0.097	4.8	3.1 (53)
Chi ^e	1.7	0.2 ± 0.0	11.6 ± 0.7	~0	0	0
CHo	20.3	3.2 ± 0.0	15.6 ± 0.1	0.088	2.3	0.7 (0)
CO	13.8	2.2 ± 0.2	15.7 ± 1.1	0.068	0	0
CP	17.0	7.3 ± 0.3	43.2 ± 1.6	0.205	0.6	0.2 (44)
YB	11.1	1.0 ± 0.3	9.2 ± 2.6	0.035	0	0
YDi	14.1	1.3 ± 0.2	9.3 ± 1.7	0.038	2.6	1.3 (0)
YDe	14.1	1.5 ± 0.0	10.7 ± 0.1	0.042	12.3	4.6 (84)
YK	17.0	2.7 ± 0.0	15.6 ± 0.2	0.074	18.0	13.0 (99)
YP	14.6	1.7 ± 0.3	11.6 ± 1.8	0.079	0	0
YY ^e	<0.5	0	0	0	0	0

Cells were harvested in duplicate at 71 h during growth in 60% (v/v) strength N-amended SGH

^a Strains are represented by an abbreviation of their species name, as indicated in Table 3

^b Lipid content is calculated as a percent of dry weight

^c Lipid yield is calculated per gram of glucose consumed

^d Sugar alcohol is the sum of xylitol and arabitol produced. The number in parentheses indicates percent of total sugar alcohol that is xylitol

^e CA did not grow in YPD at 28 °C and thus was eliminated from the clade screening. Chi and YY grew in 50% (v/v) strength SGH precultures but showed little or no growth in 60% (v/v) SGH test cultures

rate of 1.26 g/L/h in exponential phase and quickly grew to 13.3 g/L cell mass at 28 h. However, lipids began to be consumed gradually after this sample time and dry weight had dropped to 10.1 g/L at 96 h. YL419 was the only strain to accumulate greater than 3 g/L lipid within 28 h, and thus, this isolate had the highest lipid productivity in the set (0.12 g/L/h). For YL420, cell growth and glucose consumption rate were lower than other *Y. lipolytica* strains, but steady lipid accumulation until 96 h eventually led to the maximum lipid titer (4.7 g/L) and cell content (32.1% DW) for the species. The best pentose-reducing strain YL567 was able to consume all glucose and xylose within 72 h and secrete up to 14.7 g/L total sugar alcohols, but maximum lipid titer was still low at 2.1 g/L. Table S1 shows xylitol and arabitol production by all seven strains in the flask cultures at maximum sugar alcohol titer.

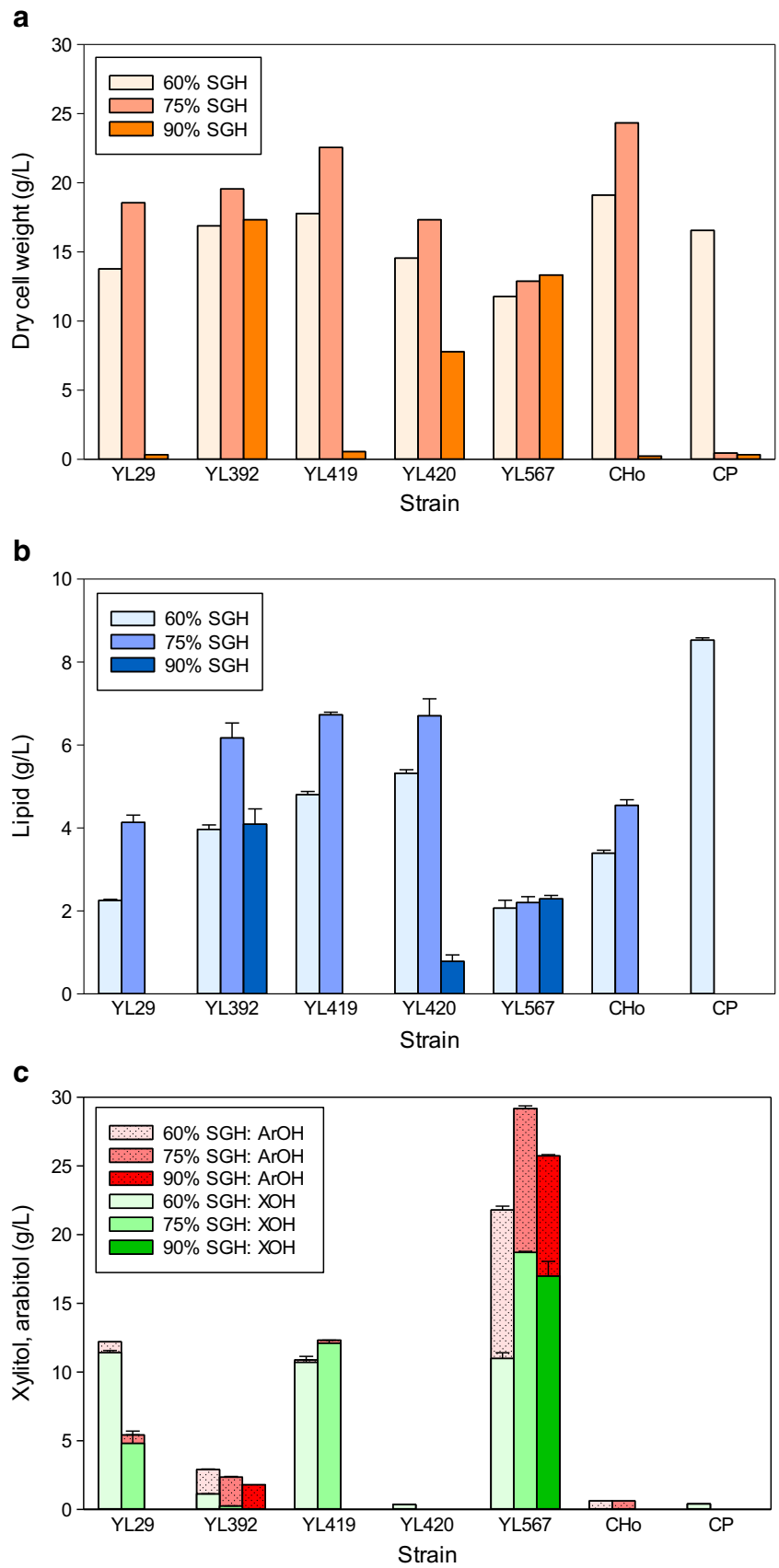
Fatty acid composition was determined by GC analysis of transesterified lipid extracts from select strains grown in flask cultures of SGH. Greater than 90% of the total fatty acids were in the form of palmitic acid, palmitoleic acid, stearic acid, oleic acid, and linoleic acid (Fig. 5). Oleic acid was the major component in all lipid extracts constituting 45–57% of total fatty acids. Lipids high in oleic acid content have been recommended for producing biodiesel with desirable fuel properties due to the optimal chain length and degree of unsaturation (Knothe 2005). There were a few notable differences between the fatty acid profiles of the five *Y. lipolytica* strains evaluated as compared to the two *C. phangngensis* strains. Mainly,

palmitic acid (C16:0) and palmitoleic acid (C16:1) contents were up to twofold higher in the *C. phangngensis* strains, while oleic acid (C18:1) content was lower by about 5–10% of total fatty acids. Thus, *C. phangngensis* generated a significantly higher proportion of 16-carbon fatty acids as compared to *Y. lipolytica* (35 and 18% of total fatty acids, respectively). Composition of fatty acids was very similar for cultures grown in either deep 96-well plates (Supplementary Fig. S4) or baffled shake flasks (Fig. 5).

Discussion

Phylogenetic relationships have been used to select relevant strains for evaluation based on the phenotype of a reference strain or species; this strategy has guided screening of yeasts for various applications (Libkind et al. 2005; Kurtzman et al. 2010; Dien et al. 2016a). In the case of the *Yarrowia* clade, there exist some added benefits to discovery of superior strains with similar phylogeny to *Y. lipolytica*—most notably a link to

Fig. 3 Test of strength tolerance for select strains in the *Yarrowia* clade on SGH. Cells were cultured in either 60, 75, or 90% (v/v) strength N-amended SGH in deep, 96-well plates and sampled for analysis of **a** dry weight, **b** lipid accumulation, and **c** sugar alcohol production. Error bars indicate standard deviation from the mean of duplicate samples. Strains are represented by an abbreviation of their species name and culture collection ID, as indicated in Table 3



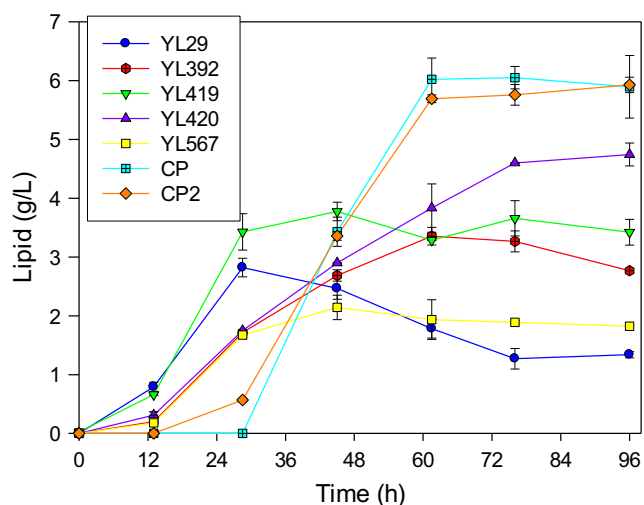


Fig. 4 Time course profiles of select strains in the *Yarrowia* clade on SGH. Yeast strains were cultured in 50% (v/v) strength N-amended SGH in baffled shake flasks and sampled periodically for analysis of lipid accumulation. Error bars indicate standard deviation from the mean of duplicate samples. Strains are represented by an abbreviation of their species name and culture collection ID, as indicated in Table 3

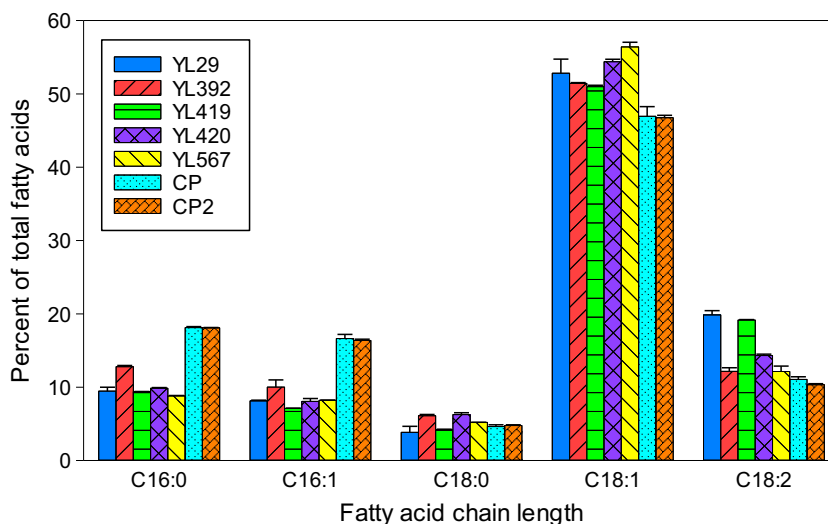
safe use, prior commercial-scale applications, and genetic tractability. Thus, several studies have focused on comparative evaluation of multiple species within the *Yarrowia* clade. For example, Michely et al. (2013) published an interesting study comparing growth parameters and lipid content by nine *Yarrowia* species in defined media containing either oleic acid or glucose. For all strains, maximum lipid content ranged from 30 to 67% DW on oleic acid but only 4–7% DW on glucose (Michely et al. 2013). Another recent study evaluated 12 species of the *Yarrowia* clade for sugar alcohol production in a minimal media with glucose, fructose, or glycerol. It was reported that *Candida oslonensis* was the best producer of erythritol and mannitol with titers up to 69.8 g/L total polyols (Rakicka et al. 2016). The

forementioned work has shown substantial phenotypic diversity within the growing *Yarrowia* clade and demonstrates unexpected utility by a few previously uncharacterized strains.

Lignocellulosic biomass, which includes agricultural waste, wood and pulp waste, and dedicated energy crops, can yield a sustainable, low carbon footprint harvest of over 1 billion t per year in the USA (Perlack et al. 2011). To date, there has been no thorough screening of the *Yarrowia* clade for growth and production of lipids using hydrolysates of lignocellulosic biomass. Thus, we have evaluated a total of 57 yeast isolates, including all that were publicly available from the ARS and CBS culture collections, in a non-detoxified dilute acid pretreated switchgrass hydrolysate. Those strains identified as exceptional were further characterized in shake flasks by generating time profiles. The study directly addresses the range of lipogenesis within the *Yarrowia* clade, the ability to consume pentoses and the sugars' metabolic fates, and the range of robustness to inhibitors.

In the initial screen, 45 *Y. lipolytica* isolates were evaluated using the same hydrolysate and culture conditions, and we were able to rank the strains in terms of lipid titer and biomass yield. Lipid accumulation varied widely from 0.1 to 5.1 g/L and lipid content ranged from 2.1 to 37.9% DW (Table 2). NRRL YB-420 emerged from the screen as the top lipid-producing *Y. lipolytica* isolate in terms of both titer and cell content. However, shake flask studies determined that the second-best from the screening (NRRL YB-419) had faster growth rate and higher lipid production rate than YB-420 through the early stationary phase (Fig. 4). NRRL YB-420 and YB-419 both reached a species high of 6.7 g/L lipid after 3 days in 75% (v/v) SGH, and they grew to cell mass of 17.3 and 22.6 g/L, respectively (Fig. 3a, b). For comparison, other reports have highlighted lipid accumulation by a few of the available *Y. lipolytica* isolates during growth in cellulosic hydrolysates. For example, *Y. lipolytica* W29 grew to 7.8 g/L cell

Fig. 5 Fatty acid profile of lipid extracts from select strains after 4-day culture in baffled shake flasks with 50% (v/v) N-amended SGH. Fatty acids are designated as follows: C16:0, palmitic acid; C16:1, palmitoleic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid. Error bars represent standard deviation of biological duplicates. Strains are represented by an abbreviation of their species name and culture collection ID, as indicated in Table 3



mass in flask cultures of wheat straw hydrolysate (initial pH 5.5) supplemented with 1.5 g/L yeast extract; however, lipid titer was low at only 0.4 g/L (Yu et al. 2011). Lipid production was improved to 6.7 g/L (58.5% DW) by culturing auxotrophic strain *Y. lipolytica* Po1g (derived from W29) in a sugarcane bagasse hydrolysate (initial pH 6.5) amended with 5 g/L each of yeast extract and peptone (Tsigie et al. 2011). It should be noted that initial furfural concentration was much lower in the hydrolysate of sugarcane bagasse (0.9 mM) as compared to that of the wheat straw prepared by Yu et al. (4.5 mM). More recently, an extensive screening of oleaginous yeasts—including five strains of *Y. lipolytica* from the ARS culture collection—was conducted in AFEX-pretreated corn stover hydrolysate (initial pH 6) with the only nitrogen amendment coming from ammonia added in pretreatment (Slininger et al. 2016). In this condition, all five *Y. lipolytica* strains grew to high optical density and accumulated 2.5–5.8 g/L lipid. NRRL YB-392 and YB-437 tied for the top spot within the species in terms of maximum lipid titer (5.8 g/L) although the former showed higher lipid productivity (0.096 g/L/h). The enhanced lipogenesis of these two strains in the corn stover hydrolysate screening as compared to our SGH cultures can be attributed to lower initial furfural concentration (0.1 versus 8.8 mM) associated with AFEX pretreatment and higher total sugars without need for diluting inhibitors. *Y. lipolytica* W29 and NRRL YB-420, among other strains, were not included in the oleaginous yeast evaluation by Slininger et al. (2016).

Besides *Y. lipolytica*, oleaginicities has not been previously demonstrated for other species in the *Yarrowia* clade by de novo synthesis from non-hydrophobic substrates. Here, we were able to obtain higher lipid content from mixed sugars in hydrolysate than what was observed in a previous *Yarrowia* screening in glucose minimal media (Michely et al. 2013) by using highly aerobic conditions and tuning initial C/N ratio. In this way, 10 out of 13 species were able to proliferate in hydrolysate test cultures and maximum lipid content for these strains ranged from 9.1 to 50.4% of dry weight. Furthermore, we identified four species that were able to accumulate lipids at $\geq 20\%$ of dry weight: *Candida galli* CBS 9723 (24.3% DW), *C. oslonensis* Y-48252 (20.3% DW), *C. phangngensis* PT1-17 (50.4% DW), and *Y. keelungensis* CBS 11062 (22.2% DW). Since metabolic sensitivity to inhibitors was likely a limiting factor to growth and lipid production, other *Yarrowia* species may also prove to be oleaginous in a less inhibitory hydrolysate or synthetic media with similar aeration. *C. phangngensis* was the top lipid-producing species in the clade with lipid titers up to 9.8 g/L in the deep well plate screening (Fig. 2b). In flask cultures, both isolates of *C. phangngensis* (PT1-17 and TM2-16) had similar time profiles and maximum lipid titers were over twofold higher than that for *Y. lipolytica* W29 (Fig. 4). A comprehensive genomic and transcriptomic

analysis of both *C. phangngensis* strains is suggested as an avenue of future research to explain the enhanced capacity for lipogenesis in hydrolysate as compared to other strains in the clade.

Y. lipolytica cannot grow on xylose as a sole carbon source (Kurtzman 2011; Sitepu et al. 2014), and thus, a pentose assimilation pathway was long thought to be missing or incomplete. However, several recent studies have reported of xylose consumption after glucose depletion in hydrolysates containing a mixture of cellulosic sugars (Tsigie et al. 2011; Slininger et al. 2016). To rectify the apparent discrepancy, two independent research groups have employed bioinformatics tools to examine xylose metabolism in *Y. lipolytica* at the molecular level. Their results demonstrate the presence of a complete and functional xylose pathway in this yeast, but the enzymes are poorly expressed and the pathway is limited by the xylitol dehydrogenase step (Rodriguez et al. 2016; Ryu et al. 2016). Based on these findings, we can attribute two general observations in our screening of *Yarrowia* isolates—little or no growth during the pentose consumption phase and accumulation of sugar alcohols—to weak, unbalanced expression of xylose metabolic genes and a bottleneck at the XDH step. Most strains did produce some sugar alcohols, but xylitol titer and yield varied significantly within the clade and even among *Y. lipolytica* isolates (Tables 2 and 4). This observation may be related to variable aldose reductase activity and could be better understood by future work involving enzymatic activity assays and transcriptional evaluation. *Y. keelungensis* CBS 11062 was the top xylitol producer in the clade with titers reaching 19.0 g/L after 4 days in the deep well plate screening at a yield of ~ 0.75 g/g of xylose consumed (Supplementary Fig. S2c). Moreover, this strain was also above average in terms of maximum lipid content (22.2% DW) and lipid yield (0.10 g/g of glucose consumed). With some strain engineering and/or process optimization, xylitol could be a promising co-product to offset the costs of biodiesel production from lignocellulosic biomass. Xylitol has a substantial market in the food industry as a sugar substitute since it is low in calories and helps to prevent dental caries (Mäkinen 1992). Also, xylitol is a versatile building block for chemical conversion to xylaric acid, glycols, and other value-added chemicals (Werpy et al. 2004). Current demand for xylitol exceeds 125,000 t per year and is expected to increase steadily in the coming years (de Albuquerque et al. 2014).

Lignocellulosic biomass is recalcitrant and requires extensive pretreatment before enzymatic hydrolysis to release sugars. In the process of pretreatment, various compounds are generated that have been shown to inhibit yeast growth, such as organic acids, phenolics, and furans (Jönsson and Martín 2016). Identification of robust yeast strains that resist common fermentation inhibitors is a longstanding barrier to microbial transformation of crude biomass-derived sugars. In this work, we intended to fill in gaps in the literature regarding

the robustness of *Yarrowia* strains to the complex mixture of inhibitors found in a real biomass hydrolysate. In the initial screening, all 45 *Y. lipolytica* isolates and type strains from 9 of 12 other species in the clade were able to propagate in 60% (v/v) SGH, which contained 8.8 mM furfural, 2.5 mM HMF, and 2.9 g/L acetic acid (Table 1). Subsequently, we observed that six of seven *Yarrowia* strains grew to high cell density in 75% (v/v) SGH cultures, and *Y. lipolytica* NRRL YB-392 was the most tolerant strain among the set with final cell mass of 17.3 g/L in 90% (v/v) SGH (Fig. 3a). The results are noteworthy since the 75 and 90% (v/v) SGH contained furfural at 11.0 and 13.2 mM, respectively, and no previous work has demonstrated growth of *Y. lipolytica* in a hydrolysate with furfural above 4.5 mM. Furfural is known to be the major inhibitor interfering with cell growth of many oleaginous yeasts (Yu et al. 2011), and thus, we paid particular attention to its concentration as an indicator of toxicity. Even in a minimal media, *Y. lipolytica* UCDFST 51-30 (not included in our study) was unable to grow with addition of only 5.2 mM furfural, but high optical density was achieved in the same media with 15.9 mM HMF (Sitepu et al. 2014). In our SGH microplate experiments, we used more highly aerobic conditions (400 rpm, square wells) than previous studies, and the results may suggest that increasing oxygen transfer to the cultures can enhance inhibitor tolerance. Higher oxygen supply has been shown to improve cell viability and tolerance of *S. cerevisiae* (Ryu et al. 1984). Also, *C. phangngensis* strains PT1-17 and TM2-16, the best lipid producers in the clade (Figs. 2b and 4), appear to have heightened sensitivity to fermentation inhibitors as compared to other *Yarrowia* strains. Strain PT1-17 was unable to grow in 75% (v/v) SGH (Fig. 3a), and both isolates of *C. phangngensis* showed significantly longer lag time in flask cultures of 50% (v/v) SGH than *Y. lipolytica* strains (Fig. 4; Supplementary Fig. S3). Still, the native capacity for conversion of cellulosic sugars to lipid makes this species interesting for future study.

In summary, we have identified and described previously under-characterized *Yarrowia* strains that outperformed reference *Y. lipolytica* W29 in actual hydrolysate. Three *Y. lipolytica* strains (YB-392, YB-419, and YB-420) and *C. phangngensis* (PT1-17 and TM2-16) were found to be advantageous for lipid production from cellulosic sugars. Fatty acid profiles of select strains were comparable to those of rapeseed (canola) oil or other oleaginous yeast lipids; thus, these yeasts are predicted to be well suited for biodiesel production. Three other *Y. lipolytica* strains (YB-271, YB-566, YB-567) and *Y. keelungensis* CBS 11062 may be promising hosts for conversion of pentoses to xylitol and/or arabinol, which are commodity chemicals and potential co-products. Future work will focus on understanding the genetic and regulatory factors underlying

these phenotypes and transferring the genetic system developed for the reference strain to newly identified strains in this study.

Acknowledgements We would like to acknowledge Novozymes for the kind donation of enzymes and the ARS Culture Collection and Mr. James Swezey for providing many of the yeast strains used in this study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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