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Carbon source utilization and inhibitor tolerance of 45 oleaginous yeast species

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Abstract Conversion of lignocellulosic hydrolysates to lipids using oleaginous (high lipid) yeasts requires alignment of the hydrolysate composition with the characteristics of the yeast strain, including ability to utilize certain nutrients, ability to grow independently of costly nutrients such as vitamins, and ability to tolerate inhibitors. Some combination of these characteristics may be present in wild strains. In this study, 48 oleaginous yeast strains belonging to 45 species were tested for ability to utilize carbon sources associated with lignocellulosic hydrolysates, tolerate inhibitors, and grow in medium without supplemented vitamins. Some well-studied oleaginous yeast species, as well as some that have not been frequently utilized in research or industrial production, emerged as promising candidates for industrial use due to ability to utilize many carbon sources, including *Cryptococcus aureus, Cryptococcus laurentii, Hannaella* aff. *zeae*, *Tremella encephala,* and *Trichosporon coremiiforme.* Other species excelled in inhibitor tolerance, including *Candida* aff. *tropicalis, Cyberlindnera jadinii, Metschnikowia pulcherrima*, *Schwanniomyces occidentalis* and *Wickerhamomyces ciferrii*. No yeast tested could utilize all carbon sources and tolerate all inhibitors tested. These results indicate that yeast strains should be selected based on characteristics compatible with the composition of the targeted hydrolysate. Other factors to consider include the production of valuable co-products such as carotenoids, availability of genetic tools, biosafety level, and flocculation of the yeast strain. The data generated in this study will aid in aligning yeasts with compatible hydrolysates for conversion of carbohydrates to lipids to be used for biofuels and other oleochemicals.

Keywords Oleaginous yeast · Biodiesel · Lignocellulosic hydrolysate · Inhibitor tolerance · Triacylglycerols · TAG · Screening · Carbohydrate utilization

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Introduction

Oleaginous yeasts are a renewable, sustainable means to produce biodegradable oil for oleochemicals including fuels, chemicals and nutritional oils. Dozens of high-oil yeast species have been described that can accumulate between 20 and 70 % intracellular oil in the form of lipid bodies, containing primarily triacylglycerols (TAGs). These oils are similar in composition to those of plant oils currently used for human consumption and for biodiesel [1–4]. While the most commonly studied yeasts include *Yarrowia lipolytica, Rhodotorula glutinis, Lipomyces starkeyi, Cryptococcus curvatus,* and *Rhodosporidium toruloides,* there are dozens of other yeast species that accumulate oil when grown on glucose [4–8]. Some of these less frequently studied species may have superior properties for specific applications such as conversion of a particular feedstock.

Some oleaginous yeast species accumulate oil when grown on hydrolysates of lignocellulosic materials such as wood or grass energy crops, forestry waste, agricultural residues, food processing waste, and municipal solid waste [9–16]. Some of the less frequently studied oleaginous yeast species could have useful properties for research and

industrial applications such as utilization of a broader spectrum of carbohydrates, or tolerance of inhibitors associated with specific types of lignocellulosic hydrolysates. Because oleaginous yeasts are found in many taxonomic clades, they may utilize multiple routes of inhibitor tolerance.

Recent studies of the inhibitor tolerance and sugar utilization by oleaginous yeast species have utilized a small number of yeast strains and species [17]. Expansion of these studies to a broader panel of oleaginous yeast species would therefore be useful for development of more robust industrial yeast strains able to utilize a broader range of nutrients, and tolerate higher levels of inhibitors.

The purpose of this study was to compare industrially relevant characteristics of a wide panel of oleaginous yeasts including utilization of various carbon sources, and tolerance of predominant inhibitors commonly found in lignocellulosic hydrolysates. Furfural and 5-(hydroxymethyl) furfural (HMF) are formed from degradation of pentoses and hexoses, and acetic acid are formed from breakdown of hemicellulose. Carbon sources were selected that are known constituents of lignocellulosic hydrolysates [18–20] or are present in waste streams or byproducts. In addition, ability to grow in the absence of supplemented vitamins was tested, as this property could reduce production costs.

Materials and methods

Yeast strains

The 48 yeast strains used in this study were obtained from the Phaff Yeast Culture Collection, University of California Davis (http://phaffcollection.ucdavis.edu), and were selected based on known oil accumulating potential, as indicated in Table 1. Strains were revived from cryopreserved stocks stored in 20 % glycerol at −80 °C by streaking onto PD agar (potato dextrose, Difco™, Sparks, MD, USA), and were incubated at 23 °C. Inoculum was prepared by suspending one loop full (2–4 μL) of ≤7 day-old colony into 5 mL sterile ultrapure water and vortexing briefly.

Reagents

Glucose (ACS, cat# D16-3), ammonium chloride (ACS, cat# A661-500), and magnesium sulfate heptahydrate (BioReagents, cat# BP213-1) were from Fisher Scientific (Fair Lawn, NJ, USA). BBL yeast extract (cat# 211929) and DIFCO yeast nitrogen base (cat# 239210) were from Becton, Dickinson and Company (Sparks, MD, USA). Potassium phosphate monobasic (purity >99.0 %, cat# RC-084) was from G-Biosciences (St. Louis, MO, USA). Sodium phosphate dibasic (ACS, cat# LC24774-1) was from LabChem Inc (Pittsburgh, PA, USA). Furfural (minimum 98 % purity, cat# F0073) was from TCI America (Portland, OR, USA). 5-Hydroxymethyl furfural (HMF) (98 % purity, cat# A12475) was from Alfa Aesar (Lancashire LA3 2XY, UK). Sucrose (ACS, cat#SX1075-3) was from EM Science (Gibbstown, NJ, USA). Vitamin free yeast base (cat#M208-500G) was from HiMedia Labs (India).

Test media

Assimilation of carbon sources was tested using yeast nitrogen base as described [21]. Media consisted of 0.67 % yeast nitrogen base (YNB) and 0.5 % of one carbon source: glucose as positive control, xylose, L-arabinose, D-arabinose, cellobiose, mannose, galactose, rhamnose, or galacturonic acid. A concentration of 0.4 % was used for glycerol. In addition, blank YNB medium without carbon source was used as a negative control. Media were sterile filtered with BP Millipore Express (0.22 μm), and 5 mL aliquots were dispensed into sterile capped 16 × 160 mm culture tubes.

Ability to grow in the absence of supplemented vitamins was tested using Vitamin Free Media as recommended by the manufacturer. This medium contains glucose as the sole carbon source. Medium was sterile filtered, and 5 mL aliquots were dispensed into sterile capped culture tubes.

Medium A [22] consists of (% w/v): 3 % glucose, 0.01 % $CaCl_2{\cdot}H_2O$, 0.05 % NH_4C1, 0.15 % yeast extract (Difco Labs, Detroit, MI, USA), 0.7 % KH_2PO_4, 0.25 % $Na_2HPO_4{\cdot}2H_20$, 0.15 % $MgSO_4{\cdot}7H_2O$, 0.008 % $FeC1_3{\cdot}6H_2O$, 0.001 % $ZnSO_4{\cdot}7H_2O$, 0.000007 % $MnSO_4{\cdot}H2O$, 0.00001 % $CuSO_4{\cdot}5H_2O$, and 0.0000063 % $Co(NO_3)_2$.

Tolerance of three commonly encountered inhibitors was tested. Inhibitors were suspended in Medium A with 3 % glucose [23]. The three concentrations of 5-hydroxymethyl furfural (HMF) were 0.5, 1.0, and 2.0 g/L. The two concentrations of furfural were 0.5 and 1.0 g/L. The initial pH of these media was adjusted to 5.5 with 6 N HCl. The concentration of acetic acid was 2.5 g/L, and the initial pH was 3.5. Media were sterile filtered, and 5 mL aliquots were dispensed into sterile culture tubes.

Growth observation

Twenty microliters of the cell suspension from each strain were used to inoculate each medium. All cultures were incubated in a roller drum at room temperature (23 °C). Relative growth was visually scored at 3, 7, 10 and 14 days after inoculation, as illustrated in Fig. 1 [21].

For each vitamin-free media tube, a 20 μL aliquot was removed from the tube that showed positive growth after 5 days growth, and inoculated into a fresh tube of sterile

Table 1 Yeast strains and origin of isolations

Species name	Phaff collection ID	Other collection ID	Strain, lipid content and reference for this species	Habitat and geographic source
Candida aff. *tropicalis*	UCDFST 10-1087	PL3F2	(na): 23 % [38]	Leaf litter, Protected Forest Papalia, South Konawe, Sulawesi, Indonesia
Candida diddensiae	UCDFST 10-168	CBS 2214; ATCC 15541	Strain 2: 37 % [39]	Shrimp, Aransas Bay, TX, USA
Cryptococcus aerius	UCDFST 73-135		IBPhM y-229: 63.3 % [40]	Mushroom top with *Drosophila* eggs and larvae, Near Orleans, CA, USA
Cryptococcus aff. *taibaiensis*	UCDFST 73-750		UCDFST 73-750: 37.4 % [4]	Isolated from Freycin leaf axil, Lanai, HI, USA
Cryptococcus albidus	UCDFST 63-203		UCDFST 63-203: 34.0 % [4]	Fales hot spring effluent, Mono Lake, CA, USA
Cryptococcus cf. *aureus*	UCDFST 81-663.4		(na): 21.1 % [41]	*Opuntia lindheimeri,* Galveston Beach, TX, USA
Cryptococcus curvatus	UCDFST 76-559		Strain D: 57 % [42]	Mushrooms, AZ, USA
Cryptococcus humicola	UCDFST 10-1004	P016AD1; FORDACC 579	UCDFST 10-1004: 35.5 % [4]	Fungal fruiting body, Protected Forest Papalia, South Konawe, Sulawesi, Indonesia
Cryptococcus humicola	UCDFST 12-717	PL1F4; FORDACC 1608	UCDFST 10-1004: 35.5 % [4]	Leaf litter, Protected Forest Papalia, South Konawe, Sulawesi, Indonesia
Cryptococcus laurentii	UCDFST 12-803	PLE1112RB; FORDACC 4429	UCDFST 68-684.1: 31.3 % [4]	*Solanum torvum* leaf surface, Protected Forest Papalia, South Konawe, Sulawesi, Indonesia
Cryptococcus oeirensis	UCDFST 05-864		UCDFST 05-864: 25.8 % [4]	Olive fly, Davis, CA, USA
Cryptococcus ramirezgomezianus	UCDFST 54-11.224		UCDFST 54-11.224: 40.0 % [4]	Fruiting body of *Pleurotus* fungus, Aspen Valley, Yosemite, CA, USA
Cryptococcus terreus	UCDFST 61-443		UCDFST 61-443: 51.7 % [4]	Soil, CA, USA
Cryptococcus victoriae	UCDFST 10-939	T2002RA; FORDACC 768	UCDFST 10-939: 22.1 % [36]	Lucanid beetle larva gut, Protected Forest Papalia, South Konawe, Sulawesi, Indonesia
Cryptococcus wieringae	UCDFST 05-544		UCDFST 05-544: 52.6 % [4]	*Prunus cerasus* nectar, Wolfskill Experimental Orchard, Dixon, CA, USA
Cyberlindnera saturnus	UCDFST 68-1113		CBS 5761: 25 % [43]	Soil near Portage glacier, Kenai Peninsula, AK, USA
Cyberlindnera jadinii T	UCDFST 76-80	CBS 1600; NRRL Y-1542	NRRL Y-1289: 22 % [44]	Pus of a human abscess
Geotrichum fermentans	UCDFST 89-29	CBS 409.34; ATCC 28578; CBS 5057	IBPhM y-481: 19.5 % [40]	Wood, Sweden
Hannaella aff. *zeae*	UCDFST 92-112		UCDFST 92-112: 25.6 % [4]	Rotten bamboo shoot of *Phyllostachys pubescens,* Hseto Park, Taiwan
Kodamaea ohmeri	UCDFST 54-7	CBS 1038; NRRL Y-2080	BYA-523: 53.3 % [45]	Isolated from sambal ulek (Indonesian fermented chili peppers), Indonesia
Kurtzmaniella cleridarum T	UCDFST 76-729.2		UCDFST 76-729.2: 33.3 % [4]	Mushrooms, Patrick Point State Park, Trinidad, CA, USA
Leucosporidiella creatinivora	UCDFST 62-1032		UCDFST 62-1032: 48.6 % [4]	Exudate of alder tree, Marin County, CA, USA
Lipomyces lipofer T	UCDFST 78-19	CBS 944; NRRL Y-11555	UCDFST 78-19: 51.3 % [4]	Garden soil, The Netherlands
Lipomyces starkeyi T	UCDFST 78-23		UCDFST 78-23; 40.00 % [4]	*Opuntia stricti* cactus, Australia
Lipomyces tetrasporus T	UCDFST 78-28	ATCC 32372; CBS 5910; NRRL Y-11562	IBPhM y-695: 66.5 % [40]	Soil, Russia

Table 1 continued

Species name	Phaff collection ID	Other collection ID	Strain, lipid content and reference for this species	Habitat and geographic source
Metschnikowia gruessii	UCDFST 11-1106		(na): 34 % [46]	Pacifica raspberries, Davis, CA, USA
Metschnikowia gruessii	UCDFST 11-1130		(na): 34 % [46]	Pacifica raspberries infested with *Drosophila suzukii*, CA, USA
Metschnikowia pulcherrima	UCDFST 11-1039		J2-4: 30 % [47]	Rainier cherries infested with *Drosophila suzukii,* Corvallis, OR, USA
Myxozyma melibiosi T	UCDFST 52-87	ATCC 24226; CBS 2102; NRRL Y-11781	UCDFST 52-87: 23.4 % [4]	Isolated from bark beetle *Dendroctonus monticolae* in *Pinus ponderosa*, Lassen County, CA, USA
Pseudozyma aphidis	UCDFST 11-1358	M076LWD; FORDACC 1264	Candida sp. 107, NCYC 911: 44 % [48]	*Rubus moluccanus* leaf surface, Mekongga mountain range, North Kolaka, Sulawesi, Indonesia
Rhodosporidium paludigenum	UCDFST 09-163	CBS 3044	39.7 % [4]	Leaf of *Desmodium repens*, The Netherlands
Rhodosporidium babjevae	UCDFST 04-877		UCDFST 04-877: 46.9 [4]	Olive fly, Davis, CA, USA
Rhodosporidium babjevae	UCDFST 05-775		UCDFST 05-775: 65.3 % [4]	Drying sap scraped off olive tree *Olea europaea*, Wolfskill Experimental Orchard, Dixon, CA, USA
Rhodosporidium cf. *fluviale*	UCDFST 81-485.4		UCDFST 81-485.4: 15.0 % [4]	*Opuntia ficus-indica*, Tucson, AZ, USA
Rhodosporidium diobovatum	UCDFST 04-830		UCDFST 04-830: 40.9 % [36]	Olive fly, Wolfskill Experimental Orchard, Dixon, CA, USA
Rhodosporidium toruloides	UCDFST 68-264	CBS 315	UCDFST 68-264: 45.5 % [36]	Isolated from the air in Tokyo, Japan
Rhodotorula glutinis T	UCDFST 50-309	ATCC 2527; CBS 20	UCDFST 50-309: 19.3 % [4]	Isolated from the atmosphere by Saito
Rhodotorula graminis	UCDFST 04-862		UCDFST 04-862: 39.9 % [36]	Olive fly, Davis, CA, USA
Rhodotorula minuta	UCDFST 78-281		H3-2: 24.6 % [47]	*Opuntia stricta*, Yarrawonga, NSW, Australia
Rhodotorula mucilaginosa	UCDFST 40-129		UCDFST 40-129: 32.7 % [4]	Isolated from soil in CA, USA (1942)
Schwanniomyces occidentalis var. *occidentalis* T	UCDFST 73-1	ATCC 2322; CBS 819; NRRL Y-10	(na): 23 % [49]	Soil
Tremella encephala	UCDFST 68-887.2		UCDFST 68-887.2: 41.7 % [4]	Bark of *Salix* sp., Prince George, BC, Canada
Trichosporon coremiiforme	UCDFST 88-108.4		CH005: 37.8 % [10]	*Cephalocereus royenii* rot, Island of St. Martin, Caribbean
Trichosporon dermatis	UCDFST 63-110		ATCC 20506: 60.8 % [50]	Mono Lake water, high salinity, CA, USA
Trichosporon guehoae	UCDFST 60-59		UCDFST 60-59: 37.5 % [4]	Slime flux of a chestnut tree, Winschoten, The Netherlands
Trigonopsis variabilis T	UCDFST 75-19	ATCC 10679; CBS 1040; NRRL Y-1579	CBS 1040: 43.7 % [51]	Beer, Germany
Wickerhamomyces ciferrii	UCDFST 04-836		NRRL Y-1031: 22 % [52]	Olive fly, Wolfskill Experimental Orchard, Dixon, CA, USA
Yarrowia lipolytica	UCDFST 51-30	CBS 1073; IFO 1746	NRRL Y-1094: 54.8 % [53]	Isolated from olives, Italy

T type strain

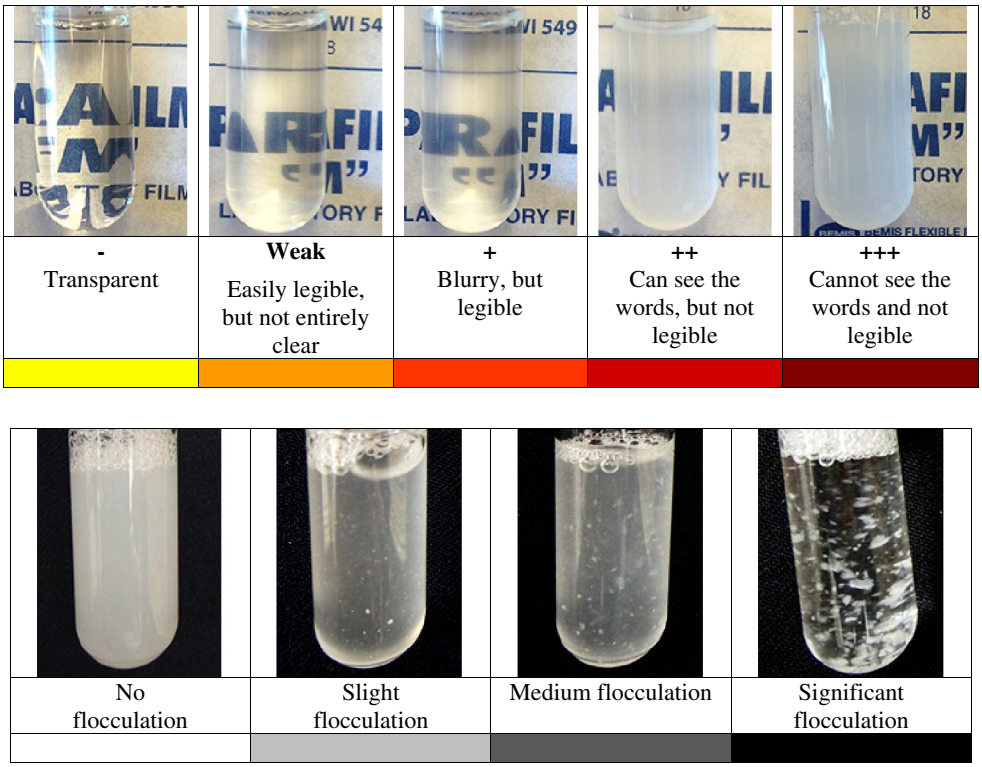

Fig. 1 Key for growth and flocculation as depicted in Fig. 2. Yeast cultures were grown in yeast nitrogen base plus glucose

vitamin-free media. The second inoculated tube was used to validate growth.

Results

A broad range of oleaginous yeast species, including both ascomycetes and basidiomycetes, were tested for growth on various carbon sources, tolerance of inhibitors, and growth without supplemented vitamins. Results of growth studies are depicted in Fig. 2. Sugars and inhibitors were selected that are present in various lignocellulosic hydrolysates [20], waste streams, or byproducts. Because the concentrations of inhibitors in hydrolysates depend on the feedstock and on the pretreatment and hydrolysis conditions, multiple inhibitor concentrations were used to correspond with common ranges of concentrations in hydrolysates of various types [20]. For example, the concentration of furfural in hydrolysates ranges from undetectable to 2880 mg/L, most commonly around 400–800 mg/L [20]. Concentrations of 800 mg/L or higher inhibit *S. cerevisiae* growth [20]. A few of the yeasts used in this study could grow in 500 mg/L furfural; a small number could grow in 1000 mg/L.

All yeasts grew in 0.5 % glucose, the positive control. None of the yeasts grew in media YNB without a supplemented carbon source, as expected for the negative control (data not shown).

Most of the strains tested were able to grow on D-xylose. Notable exceptions include the model organism *Yarrowia lipolytica,* which is known to grow on very few carbon sources [24]. About half of the strains tested were able to grow in galacturonic acid, a product of the breakdown of polygalacturonate (pectin). Some yeasts were observed to have delayed growth in the presence of certain substrates. If utilization of these substrates is needed for a given hydrolysate, pre-conditioning of the seed culture on these substrates may result in more prompt growth.

Tolerance of growth inhibitors varied widely among the yeasts tested. Five strains, *Wickerhamomyces ciferrii* UCDFST 04-836, *Candida* aff. *tropicalis* UCDFST 10-1087, *Metschnikowia* cf. *pulcherrima* UCDFST 11-1039, *Schwanniomyces occidentalis* UCDFST 73-1, and *Cyberlindnera jadinii* UCDFST 76-80, could grow well in the presence of each of the three inhibitors at the highest tested concentration. All yeast strains tested grew well in 0.5 g/L HMF. Four and ten strains were unable to grow in 1.0 and 2.0 g/L HMF, respectively. About half the strains tested grew well in 0.5 g/L furfural, and only seven strains could grow well in 1.0 g/L furfural. Only twelve strains could grow in 2.5 g/L acetic acid; most of these are ascomycetes. Because the pH of this test medium was lower

Species	UCDFST Strain ID#	Flocculation	Carbon Sources											Inhibitors					
			Glucose	D-Xylose	L-Arabinose	D-Arabinose	Cellobiose	Mannose	Galactose	Rhamnose	Sucrose	Galacturonic Acid	Glycerol	Vitamin Free Media	Furfural (0.5g/L)	Furfural (1 g/L)	Acetic Acid (2.5 g/L)	HMF (0.5g/L)	HMF (1g/L)	HMF (2g/L)
Candida aff. *tropicalis*	10-1087																			
Candida diddensiae	10-168																			
Cryptococcus aerius	73-135		D						D		D			D					D	D
Cryptococcus aff. *taibaiensis*	73-750		D			D					D								D	
Cryptococcus albidus	63-203		D								D		D							
Cryptococcus cf. *aureus*	81-663.4					D							D	D						D
Cryptococcus curvatus	76-559												D	D						D
Cryptococcus humicola	10-1004														D					
Cryptococcus humicola	12-717												D		D					
Cryptococcus laurentii	12-803														D					D
Cryptococcus oeirensis	05-864					D													D	
Cryptococcus ramirezgomezianus	54-11.224												D		D					D
Cryptococcus terreus	61-443					D			D		D									
Cryptococcus victoriae	10-939								D	D					D					
Cryptococcus wieringae	05-544																			
Cyberlindnera jadinii	76-80		D				D	D			D		D		D	D	D		D	D
Cyberlindnera saturnus	68-1113														D					
Geotrichum fermentans	89-29				D		D								D					
Hannaella aff. *zeae*	92-112							D		D			D		D				D	D
Kodamaea ohmeri	54-7					D									D					
Kurtzmaniella cleridarum	76-729.2									D			D							D
Leucosporidiella creatinivora	62-1032						D		D				D		D					D
Lipomyces lipofer	78-19		D								D				D					D
Lipomyces starkeyi	78-23				D	D	D			D	D		D					D	D	D
Lipomyces tetrasporus	78-28			D	D		D		D		D		D	D					D	D
Metschnikowia cf. *gruessii*	11-1106																			D
Metschnikowia cf. *gruessii*	11-1130																			D
Metschnikowia cf. *pulcherrima*	11-1039																			
Myxozyma melibiosi	52-87					D									D				D	D
Pseudozyma aphidis	11-1358					D	D			D			D						D	D
Rhodosporidium paludigenum	09-163								D	D			D							D
Rhodosporidium babjevae	04-877										D								D	
Rhodosporidium babjevae	05-775																		D	D
Rhodosporidium diobovatum	04-830													D						
Rhodosporidium fluviale	81-485.4				D	D			D		D			D						D
Rhodosporidium toruloides	68-264				D	D					D		D							
Rhodotorula glutinis	50-309						D				D		D	D						
Rhodotorula graminis	04-862			D			D			D					D					D
Rhodotorula minuta	78-281																		D	
Rhodotorula mucilaginosa	40-129					D			D											D
Schwanniomyces occidentalis	73-1																			
Tremella encephala	68-887.2														D				D	D
Trichosporon coremiiforme	88-108.4													D	D					
Trichosporon dermatis	63-110												D	D	D					
Trichosporon guehoae	60-59														D					D
Trigonopsis variabilis	75-19																			
Wickerhamomyces ciferrii	04-836						D													
Yarrowia lipolytica	51-30																			

◀**Fig. 2** Assimilation of carbon sources, tolerance of inhibitors, and growth without supplemented vitamins by various oleaginous yeast species. Relative turbidity at the end of 10 days growth as described under "growth observation" is diagrammed as shown in Fig. 1. *D* delayed growth, reaching maximum turbidity after 7 days

than the others, it is not clear whether growth was repressed by the acetic acid or simply the lower pH, though the fact that many of these yeasts were originally isolated on acidified medium of pH 3.8 suggests the former. Determination of the nature and concentrations of inhibitors in the hydrolysate, and the inhibitor tolerance of candidate oleaginous yeasts, are therefore critical steps in the process of selecting appropriate oleaginous yeast strains for cultivation on a specific hydrolysate.

Many yeast strains exhibited delayed growth under higher concentrations of HMF, with most of the strains having delayed growth in the 2.0 g/L concentration. Many of the strains that grew in 0.5 g/L furfural also showed delayed growth.

Ability of a yeast species to grow without supplemented vitamins is relatively rare, with 20 % or fewer of known yeasts possessing this property [25]. It is clade specific, being more common among basidiomycetous species than ascomycetous species. In our evaluation, twenty-one strains tested grew well on medium lacking vitamins after the second trial, though some of these had delayed growth. These strains may be suitable for cultivation in hydrolysates that lack vitamins.

Dewatering, or separation of yeast cells from spent media, is required for current oil extraction strategies. Ability of yeasts to form cohesive multicellular clusters may aid in this process. Strain *Trichosporon coremiiforme* UCDFST 88-108.4 was the only one that showed significant flocculation, with clear separation of media and yeast cells, as shown in Fig. 2. Seven yeast strains were observed to have slight flocculation.

Discussion

A broad range of properties contribute to the robustness of an industrial microorganism: prompt and high yield production of a desired product, ease of genetic manipulation, ability to grow quickly, to utilize available nutrients, to grow independently of costly nutrients such as vitamins, and to tolerate stresses such as pH, osmolarity, temperature and inhibitors. Some combination of these characteristics may already be present in wild precursor strains. In the current era of powerful DNA sequencing and genetic manipulation tools, study and use of microbial species beyond the small set of commonly used laboratory or model strains is becoming more feasible. Industrial microbiologists are now tapping culture collections and nature to select parent strains that already have a number of promising characteristics [26–28]. These yeasts can either be developed as industrial organisms, or their properties can be engineered into model or industrial organisms such as *Saccharomyces cerevisiae* or *Escherichia coli* [3].

In this study, several industrially relevant characteristics were profiled in a large number of oleaginous yeast strains belonging to a broad taxonomic range: 48 strains belonging to a 45 species. The authors are not aware of a comparable published study. There have been reports of the effects of selected inhibitors on growth and lipid production by selected yeast species, such as the effect of several inhibitors on *Rhodosporidium toruloides* [29], aldehydes and organic acids on *Trichosporon fermentans* [30, 31] several inhibitors on *Cryptococcus curvatus* [32], and screening of the effect of several inhibitors on growth and lipid production by five oleaginous yeast species: *R. glutinis, T. cutaneum, R. rubra, R. toruloides,* and *L. starkeyi* [17]. These studies demonstrated that in addition to inhibiting growth, presence of inhibitors correlated with decreased cellular lipid content. This effect varied with different inhibitors: furfural often decreases both growth and lipid accumulation more severely than HMF. Options for mitigating these effects include use of hydrolysates with decreased inhibitor concentrations, such as AFEX™—pretreated corn stover [33], detoxification of hydrolysates [34], or selection of yeasts that are naturally resistant to these inhibitors. This study presents support for those wishing to pursue the third option.

Inhibitor concentrations were selected for this study that represent ranges often found in hydrolysates. Concentrations of inhibitors vary widely with the nature of the feedstock, and the pretreatment process used. For example, acetic acid concentrations range from 200 to 7800 g/L in various feedstocks, with concentrations in corn stover hydrolysate ranging from 2300 to 7800 g/L. Reported concentrations of furfural range from 0.2 to 2800 mg/L in hydrolysates of various feedstocks, and 510–710 mg/L in corn stover [20]. Furfural concentrations are generally higher than HMF in a given hydrolysate, especially in dilute-acid and hydrothermal pretreated hydrolysates: HMF ranges from 0 to 3400 mg/L in various hydrolysates, with corn stover hydrolysates containing 100–560 mg/L. Our data indicate that oleaginous yeasts tend to be more sensitive to furfural than to HMF at the same concentration: most of the tested yeasts could grow in 2 g/L HMF, but few could grow in 0.5 g/L furfural. This is consistent with observations in *T. fermentans* [30]: furfural had much more detrimental effects on biomass than HMF, and also suppressed intracellular lipid accumulation more severely. Some strains tested appear more tolerant of HMF and furfural than *Saccharomyces cerevisiae*, which shows growth

inhibition at a concentration of 1 g/L of either of these compounds [35]. Side-by-side testing is needed to confirm these results. Delayed growth in HMF by many yeast strains suggests that preconditioning in the presence of this inhibitor before inoculation in the test media may improve early growth performance.

Data presented in this study will aid in selection of yeasts naturally resistant to the types and concentrations of inhibitors present in a target hydrolysate. Further validation of the effects of relevant inhibitors on biomass and lipid production by a promising yeast candidate will assist in process development. One model to follow is the work performed by Huang et al. [30, 31], in which they demonstrated the effects of many aldehydes and organic acid inhibitors on biomass production and lipid content by *T. fermentans* CICC 1368. They observed inhibitory concentrations of 2.1 mM for furfural, and 15.1 mM for HMF.

It must be pointed out that additional strains of the tested species may behave differently, as there are often considerable strain-to-strain differences within a species. For example, we recently demonstrated that four strains of *Rhodosporidium diobovatum* had lipid content ranging from 20 to 40 % (w/w) when grown under identical conditions [36]. This suggests that examination of additional strains of selected species may uncover strains that more closely fit desired profiles. The Phaff Yeast Culture Collection is an ideal resource for this type of survey, as it contains over 7,000 strains belonging to over 800 different species, with up to 500 independently isolated, wild-type strains per species [26, 27].

It is important to screen multiple strains of a species to select one with the desired properties, particularly those that are described as "variable" in taxonomic literature, as this means some strains have this property and some do not. For example, the two tested strains of the species *Metschnikowia gruessii* performed differently on vitamin-free medium: strain UCDFST 11-1106 was not able to grow, but strain UCDFST 11-1130 was. Growth without vitamins by this species is summarized as "Variable" in the primary taxonomic treatise [25]. The strain of *Lipomyces lipofer* that was tested in this study did not grow on D-xylose as a sole carbon source, but some other strains of this species are able to grow [37].

Several strains tested were able to grow on all or most of the carbon sources tested. These include *Cryptococcus humicola, C. laurentii, C. ramirezgomezianus, C. terreus, C.* cf. *aureus, Trichosporon coremiiforme, Tremella encephala,* and *Hannaella* aff. *zeae.* However, the inhibitor tolerance of these species is not promising: few of these were able to grow in furfural or acetic acid. Identification of yeasts able to use the specific carbon sources present in a given hydrolysate may be more achievable than identification of one strain able to utilize all potential carbon sources. Furthermore, the proportion of different sugars varies depending on the lignocellulosic feedstock and pretreatment and hydrolysis procedures. Glucose is released by hydrolysis of cellulose, and is the predominant sugar in hydrolysates. Several pentoses are also abundant in hydrolysates. Xylose is second in concentration to glucose in most hydrolysates, produced by hydrolysis of hemicellulose, and thus ability to utilize xylose is a high priority. Although two isomers of arabinose were tested in this study, the L-isomer is found in hemicellulose. L-Arabinose is less abundant than xylose, and almost all yeasts that can utilize xylose can also utilize L-arabinose [25] because the metabolic pathways converge. Ability to utilize cellobiose would be useful during simultaneous saccharification and fermentation of plant biomass, because less beta-glucosidase would be required for enzymatic hydrolysis.

Additional carbon sources used in this study are related to other potential feedstocks. Glycerol is a byproduct of biodiesel production, and is available in crude form in bulk quantities. Sucrose is present in molasses, a byproduct of sugar refining. It is also produced in large volumes for ethanol production in Brazil. Galacturonic acid is a product of degradation of pectin. High-pectin food processing wastes include sugar beet pulp, tomato peels and citrus rinds. Data generated in this study may aid in development of processes to convert these waste streams to value-added products.

It must be emphasized that this study simply evaluated growth on the selected carbon sources. Further analysis is needed to determine the conversion efficiency of these carbon sources to yeast cell biomass and lipids, and the composition of the lipids produced.

Tests for ability to grow without supplemented vitamins produced encouraging results: roughly half of strains tested could grow well in vitamin-free medium. Use of these strains could reduce or eliminate the need to supplement hydrolysates with expensive nutrients. Alternatively, spent yeast biomass after oil extraction could be used to provide vitamins.

Additional factors not tested in this study also influence the choice of yeast strain. For example, despite the fact that *Yarrowia lipolytica* utilizes relatively few carbon sources and has low tolerance of furfural, it is very genetically tractable, and has a long history of safe and productive use in industrial applications. Some oleaginous yeasts, including some used in this study, belong to clades known to include human pathogens, such as *Trichosporon coremiiforme* and *Candida tropicalis,* and should be tested for safety before industrial deployment. Furthermore, many of the yeasts tested in this study are pink due to production of carotenoid pigments. These may be co-extracted with TAGs, necessitating an additional separation step, but providing valuable co-products. Carotenoids may stabilize TAGs due to their

antioxidant activity. Some strains used in this study exhibit mild to significant flocculation. This may facilitate cell harvesting, but could complicate mixing and aeration.

A large number of factors contribute to the selection of yeasts suitable for a specific industrial application. Fortunately, multiple yeast culture collections around the world carry large numbers of species, and large numbers of strains within these species, that are available for screening [26, 27].

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