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BIOENERGY/BIOFUELS/BIOCHEMICALS - ORIGINAL PAPER

Identification of oleaginous yeasts that metabolize aromatic compounds

Allison Yaguchi[1] · Nicole Franaszek[1] · Kaelyn O'Neill[1] · Stephen Lee[1] · Irnayuli Sitepu[2] · Kyria Boundy-Mills[2] · Mark Blenner[1]

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Abstract

The valorization of lignin is critical for the economic viability of the bioeconomy. Microbial metabolism is advantageous for handling the myriad of aromatic compounds resulting from lignin chemical or enzymatic depolymerization. Coupling aromatic metabolism to fatty acid biosynthesis makes possible the production of biofuels, oleochemicals, and other fine/bulk chemicals derived from lignin. Our previous work identified *Cutaneotrichosporon oleaginosus* as a yeast that could accumulate nearly 70% of its dry cell weight as lipids using aromatics as a sole carbon source. Expanding on this, other oleaginous yeast species were investigated for the metabolism of lignin-relevant monoaromatics. Thirty-six oleaginous yeast species from the Phaff yeast collection were screened for growth on several aromatic compounds representing S-, G-, and H- type lignin. The analysis reported in this study suggests that aromatic metabolism is largely segregated to the *Cutaenotrichosporon*, *Trichosporon*, and *Rhodotorula* clades. Each species tested within each clade has different properties with respect to the aromatics metabolized and the concentrations of aromatics tolerated. The combined analysis suggests that *Cutaneotrichosporon* yeast are the best suited to broad spectrum aromatic metabolism and support its development as a model system for aromatic metabolism in yeast.

Keyword Lignin · Oleaginous yeast · Aromatic · *Cutaneotrichosporon* · *Rhodotorula*

Introduction

The biomass economy has largely focused on the sugars derived from the cellulose and hemicellulose fractions of lignocellulose; however, recent studies by the US Department of Energy show that valorization of lignin is necessary to make biofuels economical [1–3]. A billion-ton biomass economy would produce approximately 225 million tons of lignin by-product [3, 4]. Proposed uses for this lignin are largely low-value, including polyurethane products [5], and phenolic adhesives [6], or are niche markets requiring significant lignin purification, such as carbon fibers [7–9]. Several barriers prevent the valorization of lignin, including its heterogenous composition and the resulting heterogeneity of its depolymerized components [10–13]. Another use for this lignin is to use it as a feedstock for bioproduction of fuels and other chemicals. While many catalytic processes require fairly pure feedstocks, microbial systems have sufficient funneling pathways to metabolize a wide variety of feedstocks [14–16]. Therefore, lignin depolymerization followed by the microbial valorization of the small oligomers and monomers is increasingly studied [17–20]. The majority of microbial systems studied for their aromatic metabolism are from a limited number of genera, including the *Pseudomonas*, *Rhodococcus*, and *Sphingobium*, as most bacteria are not capable of aromatic metabolism [16]. Synthetic biology and metabolic engineering tools have been successfully applied to improve the metabolism of aromatics as well as the production of compounds such as muconic acid, vanillin, and polyhydroxyalkanoates [21–24].

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✉ Mark Blenner
blenner@clemson.edu

[1] Department of Chemical and Biomolecular Engineering, Clemson University, 206 S. Palmetto Blvd, Clemson, SC 29634, USA

[2] Phaff Yeast Culture Collection, Food Science and Technology, University of California Davis, One Shields Ave, Davis, CA 95616, USA

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Yeasts are far less studied for aromatic metabolism; however, they offer many advantages over bacteria, including resistance to phage infection and tolerance to extreme pH, high osmolarity, and solvents. Even more compelling is the eukaryotic cell physiology that enables the production and quality control of fungal enzymes for the modification and breakdown of polymeric lignin [25–27]. Approximately 100 of the over 1600 known yeast are considered oleaginous due to their accumulation of greater than 20% of their biomass as neutral lipids, primarily triacylglycerides (TAGs) [28, 29]. These yeast have been increasingly studied for the sustainable production of biofuels and oleochemicals [30, 31]. Since the products of aromatic metabolism via the ortho-cleavage pathway (acetyl-CoA and pyruvate) are the precursors for fatty acid biosynthesis, identification of oleaginous yeasts able to metabolize aromatic compounds could increase product yields. Unfortunately, like most microbes, metabolism of aromatic compounds is an uncommon phenotype exhibited by yeasts.

Previous studies in bioremediation identified *Trichosporon cutaneum* (now *Cutaneotrichosporon cutaneum*) as an aromatic metabolizer [32–38], but the cells were not shown to retain oleaginous behavior when grown on aromatics. We previously reported *Cutaneotrichosporon oleaginosus*, a closely related yeast, grew on phenol, 4-hydroxybenzoic acid (pHBA) and resorcinol as sole carbon sources. This yeast was shown to accumulate almost 70% of dry weight as lipids using resorcinol as the sole carbon source [39]. Another recent study of the oleaginous red yeast, *Rhodosporidium toruloides* (now *Rhodotorula toruloides*) identified metabolism of *p*-coumaric acid, ferulic acid, vanillic acid, and pHBA as sole carbon sources, but did not describe resulting lipid accumulation. The same study also demonstrated *Cutaneotrichosporon guehoae*'s ability to metabolize pHBA as the sole carbon source [40]. Other reports of aromatic metabolism in yeast come from *Candida* sp. [41–43] and some species of heterobasidiomycetous yeasts, particularly those in the order Sporidiobolales [44].

To gain a wider appreciation of the prevalence of aromatic metabolism amongst oleaginous yeast, thirty-six oleaginous yeast were screened from the Phaff Yeast Culture Collection. This yeast were screened for growth on aromatics as sole carbon sources, including phenol, resorcinol, *p*-coumarate, pHBA, syringate, and ferulate. Most of the yeast that were able to utilize several aromatic compounds were from genera *Cutaneotrichosporon* and *Trichosporon* (class Tremellomycetes, order Trichosporonales) and genera *Rhodotorula* and *Rhodosporidiobolus* (class Microbotryomycetes, order Sporidiobolales). Overall, metabolism of S- and G-type lignin was rare and phenol toxicity limited its consumption. A deeper analysis revealed that *C. oleaginosus* accumulated approximately 40–50% of its dry weight as lipids using several aromatic compounds as sole carbon sources. These data support the use of yeasts in the genus *Cutaneotrichosporon*, *Cutaneotrichosporon oleaginosus* in particular, for aromatic conversion to oleochemicals.

Materials and methods

Cell culture

Thirty-six oleaginous ascomycete and basidiomycete yeasts were obtained from the Phaff Yeast Culture Collection (Table 1) at the University of California Davis (https://phaffcollection.ucdavis.edu). These strains were selected based on results of the previous analysis of lipids accumulated under various conditions, including assimilation of several carbon compounds, though not aromatics [28, 45, 46]. All yeasts were pre-cultured overnight in 2 mL YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose). Cells were washed three times with YSC buffered with 50 mM phosphate buffer, pH 6.5, containing no carbon source. Cell washing entailed centrifuging cells at 1100×*g* for 4 min at 4 °C, decanting supernatant, and resuspending in 50 mM phosphate buffered YSC (pH 6.5) media. YSC was made according to manufacturer formulation (6.7 g/L DB Difco™ Yeast Nitrogen Base (YNB) without Amino Acids, 0.69 g/L CSM-Leu (Sunrise Scientific cat. #1005–010), 100 mg/L leucine) and then buffered with 0.5 M potassium phosphate buffer (pH = 6.5) to a final concentration of 50 mM. Carbon sources included glucose, phenol, resorcinol, *p*-coumaric acid, *p*-hydroxybenzoic acid (pHBA), ferulic acid, and syringic acid. The pH of all media was adjusted to 6.5 using potassium hydroxide. Wells of a 48-well plate (Nunclon® cat. # 150687) were filled with 250 µL of media and inoculated to a starting OD of 0.3. Plates were sealed with Breathe-Easy breathable film (Diveresified Biotech cat. # BEM-1). Plates were incubated in a VWR/Shel Lab 1565 T General Purpose Incubator on a VWR Micro Plate Shaker 120 V at 28 °C for 72 h. Absorbance at 600 nm was measured every 6 h using a Biotek® Epoch™ 2 microplate reader.

Selected strains were chosen for further analysis in flasks. As before, cells were pre-cultured overnight in 2 mL YPD. Cells were washed as before with YSC buffered with 50 mM phosphate buffer, pH 6.5, containing no carbon source. Washed cells were used to inoculate 15 mL of low nitrogen media (50 mM phosphate buffer, 1.7 g/L YNB without Amino Acids or Ammonium Sulfate, 0.69 g/L CSM-Leu, 100 mg/L leucine) to an initial OD of 0.3 in 50 mL baffled flasks. Ammonium sulfate was supplemented to achieve a C:N molar ratio of 200 across all carbon sources and carbon concentrations. Flasks were shaken at 250 rpm and 28 °C for 72 h and monitored every 6 h by measuring OD600 using a cuvette on a Thermo Scientific Nanodrop™ 2000.

Table 1 Yeast strains used in this study. Yeasts used in this study were obtained from the Phaff Yeast Culture Collection, Food Science and Technology, University of California Davis (UCDFST, https://phaffcollection.ucdavis.edu)

UCDFST strain ID	ID in other collections	Species	Geographic origin	Source material	Genbank accession (Genome)	Assembly level	GenBank accession (26S)
Phylum: Ascomycota							
UCDFST 48–23.8^T	ATCC 15541, CBS 2214, NRRL Y-2000	*Candida diddensiae*	Gulf of Mexico, Texas	Surface of shrimp	–	–	MN920654
UCDFST 74–62	CBS 1517, NRRL Y-1082, DBVPG 7306	*Cyberlindnera jadinii*. Syn: *Candida utilis*	Unknown	Unknown	GCA_001245095.1	Scaffold	MN920655
UCDFST 68–1113	NRRL Y-17396	*Cyberlindnera suaveolens*	Kenai Peninsula, Alaska, USA	Soil near Portage glacier	GCA_003709245.2	Scaffold	MH595044
UCDFST 78-19^T	CBS 944, NRRL Y-11555	*Lipomyces lipofer*. Syn: *Waltomyces lipofer*	The Netherlands	Garden soil	GCA_003705915.1	Scaffold	MN920656
UCDFST 78–23	NRRL Y-11557	*Lipomyces starkeyi*	N.S.W., Australia	*Opuntia stricta* cactus rot	GCA_001661325.1	Scaffold	MN920653
UCDFST 78–28	ATCC 32372, CBS 5910, NRRL Y-11562	*Lipomyces tetrasporus*	Russia	Soil	–	–	MN920657
UCDFST 11–1039	–	*Metschnikowia chrysoperlae*	Corvallis, Oregon, USA	Rainier cherries infested with *Drosophila suzukii*	–	–	KY037807
UCDFST 72–48	ATCC 22590, CBS 6246, NRRL Y-7535	*Schwanniomyces vanrijiae* var. *yarrowii*. Syn: *Debaromyces vanrijiae* var. *yarrowii*	Madrid, Spain	Excrements of insects on *Paulownia imperialis*	–	–	MN920658
UCDFST 73-1^T	ATCC 2322, CBS 819, NRRL Y-10	*Schwanniomyces occidentalis* var. *occidentalis*	Unknown	Soil	–	–	KY037810
UCDFST 10-162^T	ATCC 22214, CBS 6009, NRRL Y-17069	*Starmerella bombicola*. Syn: *Candida bombicola*	Canada	Honey of a bumblebee	GCA_004124885.1	Contig	KU609484
UCDFST 04–836	–	*Wickerhamomyces ciferrii*. Syn: *Pichia ciferrii*	Wolfskill Experimental Orchard, Dixon, California, USA	Male olive fly	GCA_000313485.1	Contig	MH130228
UCDFST 51–30	CBS 2073, IFO 1746, NRRL Y-224	*Yarrowia lipolytica*	Italy	Olives	GCA_000002525.1	Chromosome	KY037831
Phylum: Basidiomycota							
UCDFST 60–59	–	*Cutaneotrichosporon guehoae*	Winschoten, Netherlands	Slime flux of a chestnut tree	GCA_001600415.1	Scaffold	KY037828
–	ATCC 20509	*Cutaneotrichosporon oleaginosus*	Iowa, USA	Dairy farm drain	GCA_001027345.1	Scaffold	HM802135.1
UCDFST 68–280	ATCC 10658, CBS 319, NRRL Y-1589	*Cystobasidium minutum*	Saito, Japan	Air	–	–	KU609482

Table 1 (continued)

UCDFST strain ID	ID in other collections	Species	Geographic origin	Source material	Genbank accession (Genome)	Assembly level	GenBank accession (26S)
UCDFST 06–227	CBS 140, ATCC 24171, NRRL Y-2537	*Filobasidium magnum.* Syn: *Cryptococcus magnus*	Davis, California, USA	*Drosophila melanogaster*	–	–	MN920662
UCDFST 68–887.2	–	*Naematelia encephala.* Syn: *Tremella encephala*	Prince George, British Columbia, Canada	Bark of *Salix* sp.	GCA_002105065.1	Contig	KY037826
UCDFST 63–203	–	*Naganishia albida.* Syn: *Cryptococcus albidus*	Mono Lake, California, USA	Hot spring effluent	GCA_001599735.1	Scaffold	KY037812
UCDFST 68–934.2	–	*Occultifur externus*	Vancouver, Canada	Exudate from *Acer* sp.	–	–	KU609481
UCDFST 10–219	NRRL Y-50378	*Papiliotrema flavescens.* Syn: *Cryptococcus flavescens*	Davis, California, USA	*Anisomorpha buprestoides*—whole gut	GCA_000442785.1	Scaffold	MN920652
UCDFST 67–20	ATCC 18809, CBS 4101, NRRL Y-12675	*Rhodotorula bogoriensis.* Syn: *Pseudohyphozyma bogoriensis*	Indonesia	Leaf surface of *Randia malleifera*	–	–	KU609480
UCDFST 69–61	ATCC 22078, CBS 6031, NRRL Y-17376	*Rhodotorula araucariae*	Chile	Rotting *Araucaria araucana* tree	–	–	KU609441
UCDFST 68–274	CBS 4406, IFO-0415	*Rhodotorula dairenensis*	Saito, Japan	Air	–	–	MN920659
UCDFST 08-225^{T}	CBS 6085	*Rhodotorula diobovata*	Southeastern Barrier Reef, Florida, USA	Sea water	–	–	KU609432
UCDFST 05–503	–	*Rhodotorula graminis*	Davis, California, USA	Female olive fly	GCA_001329695.1	Scaffold	KU609446
UCDFST 68–329	CBS 482, ATCC 24216, NRRL Y-6683	*Rhodotorula mucilaginosa*	France	Smut-infected leaves	GCA_002806785.1	Scaffold	KU609449
UCDFST 67–52	CBS 349, NRRL Y-1588, IFO 0880	*Rhodotorula toruloides.* Syn: *Rhodosporidium toruloides*	Koshikawa Bota, Japan	Soil by K. Okunuki	GCA_000222205.2	Scaffold	KU609440
UCDFST 06–583	–	*Rhodosporidiobolus* aff. *lusitaniae*	Davis, California, USA	*Bactrocera olea* (olive fruit fly)	–	–	KU609443
UCDFST 09–1303	–	*Rhodosporidiobolus odoratus.* Syn: *Sporobolomyces odoratus*	Davis, California, USA	Sunflower tree leaf	–	–	KU609473
UCDFST 67–68	CBS 5000	*Rhodosporidiobolus ruineniae.* Syn*: Sporidiobolus ruineniae*	Bogor, Indonesia	Unknown	–	–	MN920660
UCDFST 61–443	–	*Solicoccozyma phenolicus.* Syn: *Cryptococcus phenolicus*	California, USA	Soil	GCA_001600015.1	Scaffold	KY037819
UCDFST 05–893	–	*Sporidiobolus metaroseus*	Ventura, California, USA	Olive fruit fly-infested olive	–	–	KU609534

Table 1 (continued)

UCDFST strain ID	ID in other collections	Species	Geographic origin	Source material	Genbank accession (Genome)	Assembly level	GenBank accession (26S)
UCDFST 68–346	CBS 2637	*Sporobolomyces carnicolor*	Delft, The Netherlands	Air	–	–	KU609456
UCDFST 67–65	–	*Sporobolomyces johnsonii*. Syn: *Sporidiobolus johnsonii*	Unknown	Tropical foliage	–	–	KU609451
UCDFST 88–108.4	–	*Trichosporon coremiiforme*	Island of St. Maarten	*Cephalocereus royenii* cactus rot	GCA_001752605.1	Scaffold	MN920661
UCDFST 76–729.2	–	*Vanrija musci*. Syn: *Cryptococcus musci*	Patrick Point State Park, Trinidad, California, USA	Mushrooms	–	–	KY037828

The GenBank accession numbers for draft genomes and sequence identifiers are listed

CBS Westerdijk Institute, Utrecht, Netherlands, *NRRL* USDA-ARS, Peoria, IL, USA, *ATCC* American Type Culture Collection, Manassas, VA, USA

[T]Indicated type strain of the species

–: Indicates unavailable

Dry cell weight and lipid analysis

Cells were harvested for dry cell weight by washing 10 mL of cell culture with 20 mL of Milli-Q water three times and drying overnight at 40 °C under vacuum in aluminum pans. Dry cell weights were measured using an analytical balance. To identify and quantify lipids in cell biomass, extracted cellular lipids were transesterified to FAMEs as described previously [47] with minor modifications. Briefly, 1 mL of cell culture was harvested and centrifuged at 13,000 rpm for 3 min at 25 °C. Glyceryl triheptadecanoate was added as internal standard (100 µL of a 2 mg/mL stock in methanol) to the cell pellet before extraction. Lipids were transesterified to FAMEs with 500 µL of 0.5 N sodium methoxide followed by 30 min of vortexing at 2000 rpm. The solution was neutralized with 40 µL sulfuric acid. FAMEs were extracted by adding 850 µL hexane followed by 20 min of vortexing at 2000 rpm. The mixture was centrifuged for 1 min at 8000 rpm, and 800 µL of the organic layer was collected for GC-FID analysis using an Agilent 7890B system.

Substrate utilization

Aromatic substrate utilization was studied as previously reported [39]. Briefly, a Waters 600E high-performance liquid chromatography (HPLC) system was used in conjunction with a BioRad Fast Acid Analysis HPLC column and Waters 996 PDA detector. The running buffer was 10% v/v acetonitrile and 0.01 N H_2SO_4 in a 1:1 mixture, run at a flow rate 0.6 mL/min and at 65 °C. Wavelengths for various substrates were as follows: phenol at 270 nm, *p*-coumarate at 325 nm, resorcinol at 274 nm, and pHBA at 254 nm. Concentrations were calculated from standard curves created for each carbon source in the appropriate medium.

Phylogenetic tree

The partial large ribosomal subunit (26S) sequence of *C. oleaginosus* was accessed from GenBank. The remaining sequences were generated using Phaff collection yeast strains. GenBank accession numbers for all species are listed in Fig. 1 and Table 1. Phylogenetic distances of the strains used in this study were based on a parsimony analysis on the 26S ribosomal rRNA sequences using an online server called Phylogeny.fr [48]. Briefly, alignment was performed by MUSCLE, curation with Gblocks, and the phylogenetic map was constructed using PhyML. All settings were left as default and Shimodaira-Hasegawa (SH)-like approximate likelihood ratio tests provided statistical tests for branch support. The output Newick file was input to an online map

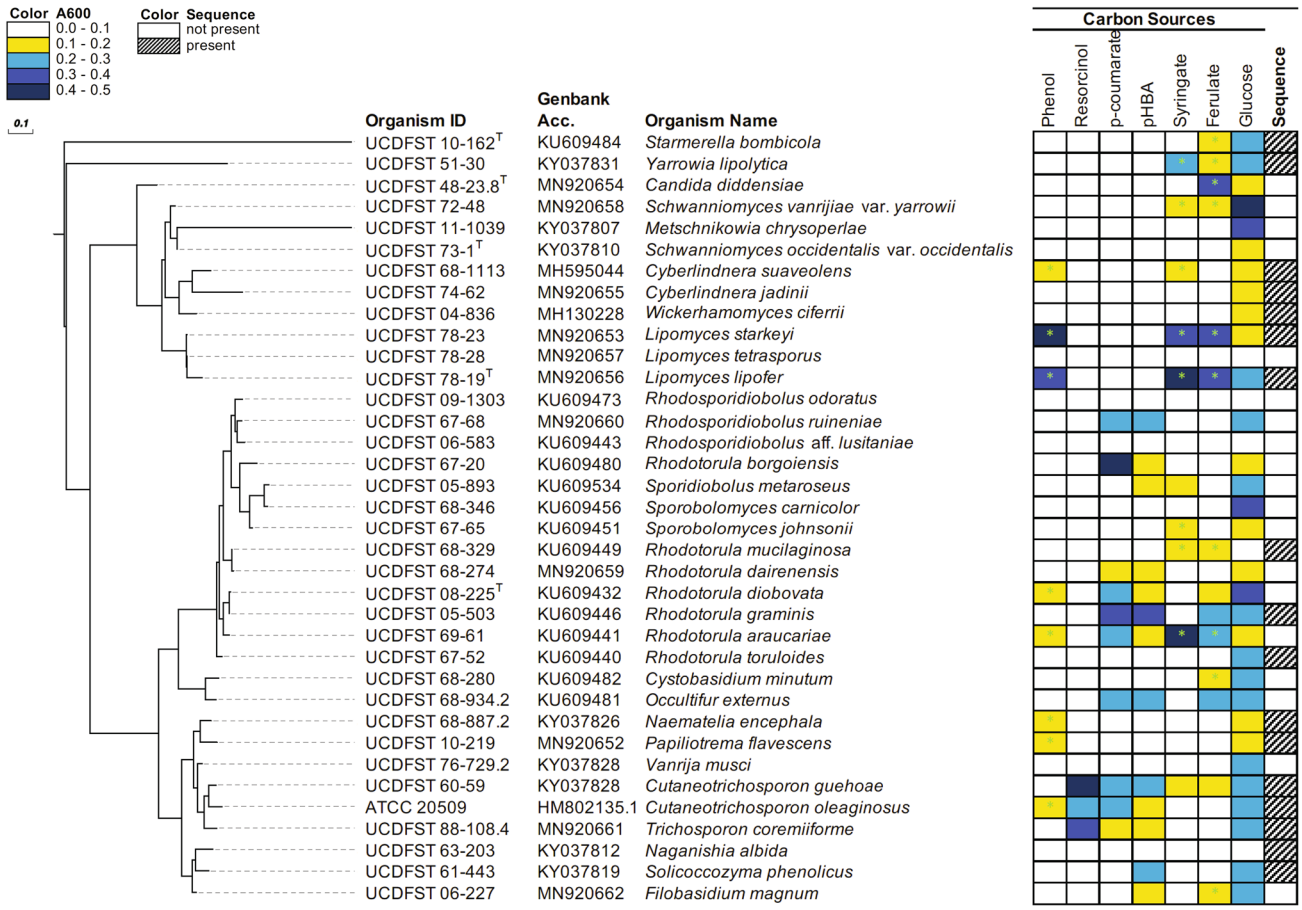

Fig. 1 Phylogenetic analysis of the 36 yeast strains used in this study with their ability to grow in 2 g/L aromatic compound, indicated by column name, as the sole carbon source. Boxes containing an asterisk (*) represents a carbon concentration of 1 g/L. Cells are ranked by bins of 0.1 OD with white indicating the lowest ODs and the darkest blue indicating the highest ODs. Data were collected from biological triplicates. Branch length corresponds to distance between species. The last column represents availability (patterned box) or absence (open white box) of a draft genome in GenBank

viewer called EvolView v2 [49]. Bootstrap scores and branch lengths were not shown for visibility.

Results

Aromatic screen to identify aromatic metabolizing yeast

Figure 1 summarizes the data of all thirty-six yeasts grown with phenol, resorcinol, *p*-coumarate, pHBA, syringate, ferulate, and glucose as sole carbon sources. The first seven columns of the figure categorize maximum A600 reached from cells grown with each carbon source while the far right column marks presence (patterend black boxes) or absence (open white boxes) of a draft genome on GenBank. Accession numbers of draft genomes and the 26S sequences used to generate the phylogenetic tree, as well as other organism information, are listed in Table 1. Boxes with an asterick indicate maximum A600 values achieved by cells grown with 1 g/L carbon source rather than 2 g/L.

Of the yeasts studied, 58% of the ascomycete yeasts were able to grow on aromatic carbon sources and 79% of the basidiomycete yeasts were able to grow. Importantly, none of the ascomycete yeasts were able to grow in concentrations greater than 1 g/L. Metabolism of H-lignin compounds was exclusively segregated to basidiomycetes. Ten yeast species grew in media containing 2 g/L *p*-coumaric acid. Most of the yeasts belonged to genera *Rhodotorula*, *Rhodosporidiobolus, Trichosporon*, or *Cutaneotrichosporon*. The only yeast not in these genera was *Occultifur externus*. Thirteen yeasts grew in 2 g/L pHBA. Eight of these species were in genera *Rhodotorula*, *Rhodosporidiobolous*, *Trichosporon*, and *Cutaneotrichosporon*. The four not in these were *Filobasidium magnum*, *Occultifur externus*, *Solicoccozyma phenolicus*, and *Sporidiobolus metaroseus*, the latter of which is classified in order Sporidiobolales [50].

None of the yeast grew in media containing 2 g/L phenol, which is known to be toxic to most microbes; however, during a subsequent experiment, eight yeast grew in media containing 1 g/L phenol—*Cutaneotrichosporon oleaginosus*, *Cyberlindnera suaveolens*, *Lipomyces lipofer*, *Lipomyces starkeyi*, *Naematelia encephala*, *Papiliotrema flavescens*, *Rhodotorula araucariae*, and *Rhodotorula diobovata*. Interestingly, results show phenol metabolism is more divergently found across ascomycetes and basidiomycetes than *p*-coumarate and pHBA. Metabolism of 2 g/L of the aromatic diol resorcinol, on the other hand, was very narrowly found in two closely related genera, *Trichosporon* and *Cutaneotrichosporon*.

Only two yeasts, *Cutaneotrichosporon guehoae* and *Sporidiobolus metaroseus*, were able to grow on 2 g/L syringate. When the syringate concentration was dropped to 1 g/L, eight more yeasts were able to grow, including *Yarrowia lipolytica*, *Schwanniomyces vanrijiae* var. *yarrowii*, *Cyberlindnera suaveolens*, *Lipomyces starkeyi*, *Lipomyces lipofer*, *Sporobolomyces johnsonii*, *Rhodotorula mucilaginosa*, and *Rhodotorula araucariae*.

Only four yeasts, *Rhodotorula diobovata*, *Rhodotorula graminis*, *Occultifur externus*, and *Cutaneotrichosporon guehoae* were able to grow in media containing 2 g/L ferulate. Ten more yeasts were able to grow when ferulate concentration was lowered to 1 g/L, though many did not grow to an absorbance higher than 0.1. These yeast are *Starmarella bombicola*, *Yarrowia lipolytica*, *Candida diddensiae*, *Schwanniomyces vanrijiae* var. *yarrowii*, *Lipomyces starkeyi*, *Lipomyces lipofer*, *Rhodotorula mucilaginosa*, *Rhodotorula araucariae*, *Cystobasidium minutum*, and *Filobasidium magnum*. Unlike H- lignin monomer, the metabolizers of G- and S- lignin monomers were split roughly evenly between ascomycetes and basidiomycetes, but growth on compounds at 2 g/L was exclusive to basidiomycetes.

Collectively, aromatic metabolism is enriched among oleaginous basidiomycetes compared to oleaginous ascomycetes, even when the concentration of substrates was lowered to 1 g/L. Many of the *Rhodotorula*, *Trichosporon*, and *Cutaneotrichosporon* species were able to grow in at least half of the aromatic compounds, even when substrate concentrations of 2 g/L were used. A single species with promising characteristics was selected to connect aromatic catabolism to an oleaginous phenotype. The availability of draft genomes of all the listed *Trichosporon* and *Cutaneotrichosporon* species was an advantage over the *Rhodotorula* species. Furthermore, these results indicated that *Cutaneotrichosporon* could grow faster in several substrates compared to *Rhodotorula* (data not shown). To consider which yeast would be best to investigate further, we considered the genetic tools available for both yeasts. The genetic transformation system for *C. guehoae* seems limited to spheroplast fusion, whereas *C. oleaginosus* has *Agrobacterium*-mediated transformation and electroporation developed. All three methods result in random genomic integration. Auxotrophic strains and heterologous drug resistance genes exist for both species, but neither have stable plasmid expression [39, 51–55]. However, heterologous expression of several proteins for enhanced TAG production and novel lipid synthesis was performed in *C. oleaginosus*, demonstrating practical use of a more advanced genetic toolkit [52, 53]. As such, we continued with *C. oleaginosus* for the remainder of the experiments in this study.

Cutaneotrichosporon oleaginosus remains oleaginous when grown on several aromatics

While all previous growth assays were conducted in media containing up to 2 g/L of substrate, higher concentrations were tested to determine the maximum concentration cells could tolerate before growth was inhibited (Fig. 2). Cells were grown in 48-well plates over 48 h in phosphate-buffered YSC media. Cells were able to grow with no detriment in 4 g/L of both pHBA and *p*-coumarate. Higher concentrations of pHBA and *p*-coumarate could not be tested due to solubility limitations. Cells were able to grow in up to 3.5 g/L resorcinol; however, the cells showed an increased lag phase and decreased growth rate. Therefore, in shake flask experiments, 4 g/L pHBA, 4 g/L *p*-coumarate, 3 g/L resorcinol, and 1 g/L phenol were used in a phosphate-buffered YSC media, pH 6.5. To induce lipogenesis, a carbon to nitrogen (C:N) molar ratio of 200 was used for all the substrates. All aromatic compounds were fully metabolized by 72 h, as determined by HPLC–UV-vis (Fig. 3a). All media types and carbon concentrations reached the same

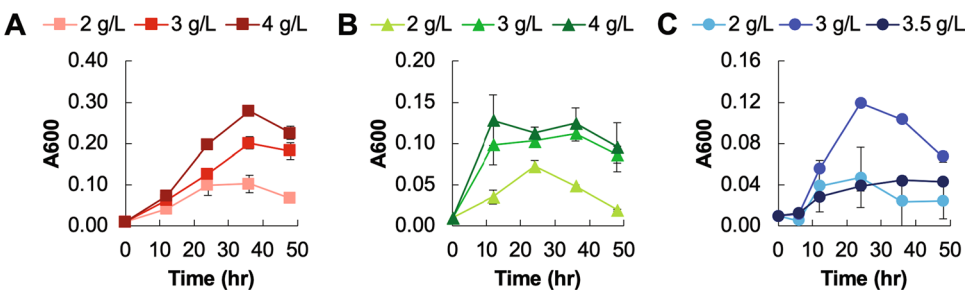

Fig. 2 Growth charts of *C. oleaginosus* grown with increasing concentrations of **a** pHBA (red squares), **b** *p*-coumarate (green triangles), and **c** resorcinol (blue circles) over 48 h. The data are the mean and error bars are the standard deviation of biological triplicates. Lines serve as visual aid only (colour figure online)

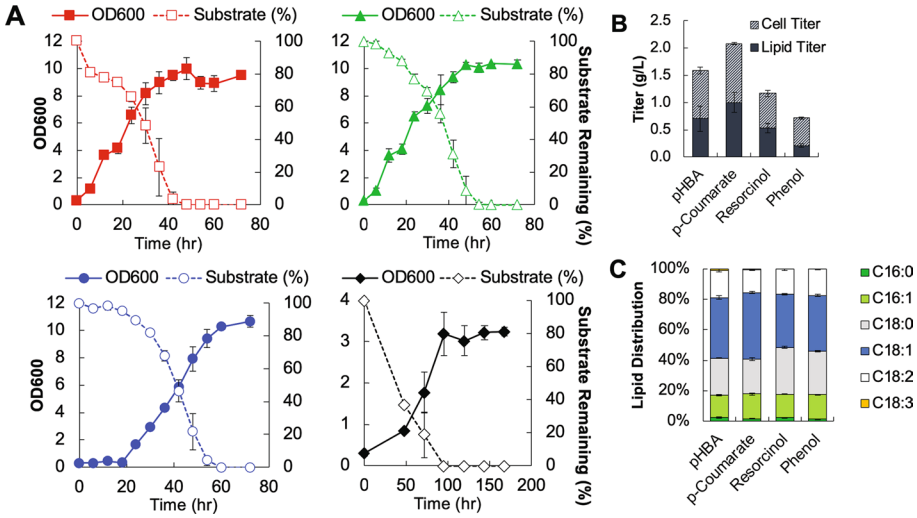

Fig. 3 Flask growth of *C. oleaginosus* cells grown in low nitrogen media (C:N = 200) over 72 h. **a** OD600 (left axis, solid markers, solid lines) and substrate utilization (right axis, open markers, dashed line) for pHBA (red squares), *p*-coumarate (green triangles), resorcinol (blue circles), and phenol (black diamonds, **b** biomass (dashed fill) and lipid titer (solid fill), **c** lipid profile distribution. The data are the mean and error bars are the standard deviation of biological triplicates. Lines serve as visual aid only (colour figure online)

Table 2 Biomass titer, lipid titer, and lipid accumulation of *C. oleaginosus* cells grown in flask studies

Substrate	Biomass titer (g/L)	Lipid titer (g/L)	Lipid accumulation (%)
pHBA	1.58 ± 0.06	0.70 ± 0.23	44.9 ± 16.2
p-Coumarate	2.08 ± 0.02	1.00 ± 0.18	48.2 ± 9.0
Resorcinol	1.17 ± 0.06	0.53 ± 0.09	45.6 ± 8.9
Phenol	0.72 ± 0.02	0.21 ± 0.03	29.2 ± 5.4

Errors are reported as the standard deviation of biological triplicates

final OD600 of about 10, except for cells grown with phenol, which reached OD600 just over 3.0. The highest biomass resulted from cells grown with 4 g/L *p*-coumarate, followed by cells grown with 4 g/L pHBA, 3 g/L resorcinol, and 1 g/L phenol, in that order (Fig. 3b; Table 2). Surprisingly, growth on *p*-coumarate resulted in 0.5 g/L more cells than cells grown on pHBA. Despite differences in final biomass titer achieved, all cells grown with each aromatic substrate remained highly oleaginous at nearly 50% lipid accumulation on a dry biomass basis, except for phenol-grown cells (Table 2). Cells grown on phenol reached 29.2% lipid on a biomass basis, still retaining oleaginous behavior.

The majority of the fatty acid was oleic acid for cells grown on each substrate. Both pHBA and *p*-coumaric acid resulted in a linoleic component of approximately 1% of the total fatty acid profile, but cells grown on resorcinol and phenol accumulated oleic acid rather than linoleic acid (Fig. 3c; Table 3).

Discussion

Overall, a higher percentage of basidiomycete yeasts than ascomycetes are able to grow in lignin-derived monoaromatic compounds as sole carbon sources. This is in agreeance with Sampaio [56], who screened 227 yeast strains isolated from terrestrial and aquatic habitats on guaiacol, protocatechuic acid, vanillic acid, ferulic acid, and veratric acid as sole carbon sources. Not all of the yeasts screened were oleaginous yeasts, as they were in the present study; however, a number of them fell similar clades examined in this study, such as *Candida*, *Cryptococcus*, *Rhodosporidium*, *Rhodotorula*, and *Trichosporon* species. Mills et al. screened 68 yeasts from six genera isolated from sewage against 15 hydroxy derivatives of phenol and benzoic acid as sole carbon sources at 1 g/L and concluded no obvious relationship between utilization of the compounds and phylogentic classificiation [57]. However, only two ascomycete yeast species were tested, one of which was not oleaginous, and the rest were basidiomycetes of the *Candida*, *Cryptococcus*,

Table 3 Lipid profile of *C. oleaginosus* cells grown in flask studies

	% Total fatty acids					
Substrate	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
pHBA	2.3 ± 0.5	14.7 ± 0.3	24.4 ± 0.2	39.7 ± 1.2	17.9 ± 1.0	1.1 ± 0.1
p-Coumarate	1.6 ± 0.2	16.3 ± 0.7	22.9 ± 1.1	43.7 ± 0.7	14.9 ± 0.3	0.8 ± 0.0
Resorcinol	2.3 ± 0.1	15.4 ± 0.1	30.8 ± 0.7	34.9 ± 0.4	16.6 ± 1.0	0.0 ± 0.0
Phenol	1.4 ± 0.1	16.1 ± 0.2	28.5 ± 0.3	36.7 ± 0.5	17.5 ± 0.8	0.0 ± 0.0

Errors are reported as the standard deviation of biological triplicates

Rhodotorula, and *Trichosporon* genera. We notice that the choice of carbon source varies the prevalanece of ascomycete versus basidiomycete yeasts among successful growers, in agreeance with Sampaio's conclusions.

Of the yeasts capable of aromatic catabolism in this study, some had previously been reported by others to be capable of aromatic catabolism. A study of *Occultifur externus* sp. nov. reports carbon assimilation of a number of aromatic compounds, including 3-hydroxybenzic acid, pHBA, vanillyl alcohol, vanillic acid, ferulic acid, protocatechuate, *p*-coumaric acid, and caffeic acid, though a much lower concentrations of 0.1% (w/v) was used to avoid toxicity [58]. *Lipomyces starkeyi* ATCC 56304 has been shown to remove 62% of the phenolic content of diluted palm oil mill effluent, but cells only accumulated 21.3% lipid content [59]. In another study of olive oil mill effluent, *L. starkeyi* reduced phenolic content by approximately 50% and accumulated just under 30% of its biomass as lipid [60]. In both studies, the oil mill wastes also included sugars, so lipid content cannot be attributed to purely phenolic catabolism, nor do these works necessarily support catabolism of phenolics into central metabolites. While also not sole carbon sources, *L. starkeyi* NBRC10381 cells were shown to uptake vanillin and syringaldehyde present at concentrations of approximately 0.1 mM when mixed in a synthetic lignocellulosic hydrolysate blend. Because the synthetic hydrolysate had multiple carbon sources in it, lipid accumulation cannot be attributed to just the phenolics, but cells were highly oleaginous at 68% despite the presence of inhibitors [61].

An extensive study by Sànchez i Nogué et al. screened 31 oleaginous yeasts for their ability to grow on corn stover hydrolysate and determined *C. oleaginosus* ATCC 20509, *R. toruloides* DSM-4444, and *C. guehoae* UCDFST 60–59 were top performers. These three yeasts were screened for their growth utilizing 20 mM ferulic acid, vanillic acid, *p*-coumaric acid, and pHBA as sole carbon sources in YNB. *Cutaneotrichosporon oleaginosus* and *C. guehoae* were able to metabolize pHBA fully but did not grow when cultured with the other compounds. *Rhodotorula toruloides* was able to fully metabolize all four compounds [40]. These results agree with Yaegashi et al. who analyzed *R. toruloides* strain IFO 0880, the same strain used in the present study [62]. Differences between the studies may be attributed to media variations, as a potassium phosphate-buffered synthetic complete media was used in the present study, Sanchez i Nogue et al. used a potassium hydrogen phthalate buffered YNB media, and Yaegashi et al. used an unbuffered synthetic complete media. It is acknowledged that media choice may impact these results. YSC was chosen in this study due to its defined formulation and general use across a variety of yeast species. The ionization of the acidic substrates (syringate, ferulate, *p*-coumarate, and pHBA) motivated the use of a buffer. However, it is possible that some yeasts will grow better in media specifically formulated for its nutritional requirements. Due to the number of yeasts in the present study, the initial down-selection was intentionally based on a single media formulation. The results conclusively show which yeast appear to grow on the various aromatic substrates as a sole carbon source, but does not rule out the possibility that others could grow on aromatics if provided the right nutrients.

Some species formerly classified in the genus *Trichosporon* have been reclassified as *Cutaneotrichosporon* [50]. In this discussion, they will be referred to as *Cutaneotrichosporon*. *Cutaneotrichosporon cutaneum* ATCC 20271 is a closely related yeast to *C. oleaginosus* that is well described for tolerance and metabolism of several lignocellulosic inhibitors, including phenolics. *Cutaneotrichosporon cutaneum* R57 was able to assimilate hydroxyquinone, resorcinol, phenol, 2,6-dinitrophenol, and *p*-cresol at 0.2 g/L and 3-nitrophenol and 3-chlorophenol at 0.1 g/L as sole carbon sources. Lipid titers were not provided, but degradation kinetics were described [63]. A strain isolated from a soil suspension, collected from olive tree fields irrigated by olive mill wastewater for three successive years, was able to grow in media containing phenol as the single carbon source up to 2 g/L, though lipid profiles are also not reported [64]. Phenolic aldehydes are particularly toxic as compared to the alcohol versions, but *C. cutaneum* suffered negligible growth defects when grown with 2.5 g/L syringaldehyde as the sole carbon source. 4-hydroxybenzaldehyde at 1.5 g/L resulted in significant growth defects and vanillin only partially inhibited cell growth at 2.0 g/L [65]. The lipid titers and profiles were again not reported. A separate study grew cells in batch cultures with the same three compounds and found only 4-hydroxybenzaldehyde resulted in oleaginous behavior even when concentrations reached 2 g/L. A 120 h fed-batch fermentation with 4-hydroxybenzaldehyde resulted in 39% accumulation of biomass as lipids [66]. Lipid profiles were not provided. Mills et al. studied 68 different yeast species and six of the ten *C. cutaneum* species grew on the widest variety of substrates supplied as sole carbon sources at 1 g/L, including phenol, catechol, hydroquinone, hydroxyquinol, phloroglucinol, benzoic acid, salicylic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, protocatechuic acid, gentisic acid, 2,4-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, and 3,5-dihydroxybenzoic acid [57]. *Cutaneotrichosporon guehoae* CBS 8521 type strain assimilates phenol, hydroquinone, resorcinol, phloroglucinol, *m*-cresol, 4-methylcatechol, salicylate, 2,3-hydroxybenzoate, 4-hydroxyacetophenone, 3-hydroxycinnamate, and 4-hydroxycinnamate [67]. Lipids titers or accumulation are not reported. *Cutaneotrichosporon oleaginosus* ATCC 20509 was previously reported as being able to metabolize phenol, pHBA, and resorcinol as sole carbon sources. A similar fed-batch study was performed, in which cells were

grown with resorcinol over 156 h and accumulated 69.5% of yeast cell biomass as lipids. Fifty percent of the accumulated lipids were C18:1 [39]. In the present study, *C. oleaginosus* demonstrated oleaginous behavior with every aromatic compound under batch culturing conditions, although cells cultivated in phenol accumulated less intracellular lipid than in resorcinol, pHBA, and *p*-coumarate. A previous name for *C. oleaginosus* is *Cryptococcus curvatus*. *Cryptococcus psychrotolerans* IITRFD, now called *Vishniacozyma psychrotolerans*, was found to metabolize a range of aromatic hydrocarbons, such as phenol, anthracene, naphthalene, and pyrene, although it was in media containing 4% glucose. Because the latter three compounds are petroleum-based aromatic hydrocarbons, we will not discuss them and instead highlight their reports of phenol metabolism, since the authors demonstrate clear utilization of the phenol. Over the course of 144 h, *C. psychrotolerans* was capable of accumulating 46.54% lipids per dry cell weight for a final titer of 6.4 g/L and productivity of 0.0444 g/L/h. Lipid accumulation, titer, and productivities are higher than that of the cells grown with glucose-only containing media. Lipid profiles show a near-even split across C16:0, C18:1, and C18:2 fatty acids, a similar profile to the glucose-only control [68].

Metabolism of lignocellulosic hydrolysates and tolerance and/or assimilation of lignin phenolic monomers has been studied in the *Rhodotorula* genera; this discussion focuses on assimilation rather than tolerance. *Rhodotorula* sp. ZM1, isolated from an acid mine drainage, was found to metabolize phenol in acidic conditions. While a lag phase was present, ZM1 cells could metabolize up to 1.3 g/L of phenol as the sole carbon source by 120 h. Su et al. describe predicted ortho-cleavage pathways based on gene predictions performed on their whole-genome sequencing and functional annotation. While many *Rhodotorula* strains are oleaginous, Su et al. did not examine lipid production [69]. Jarboui et al. found that a *R. mucilaginosa* strain isolated from olive mill wastewater was shown to grow in and fully metabolize protocatechuic acid, *p*-coumaric acid, and vanillic acid as sole carbon sources at 1 g/L, though lipid titers were not reported. It was noted this strain behaved differently from other reported *R. mucilaginosa* strains, which did not metabolize aromatics [70]. In another study, a different *R. mucilaginosa* strain, isolated from top soil, metabolized *p*-coumaric acid and ferulic acid present in a base-catalyzed depolymerized (BCD) lignin liquor at approximately 7 g/L and 0.5 g/L, respectively. Lipid titers were not reported [18]. Rodriguez et al. studied a *R. mucilaginosa* J31 and *R. toruloides* IFO0880 strains ability to metabolize pHBA, protocatechuic acid, vanillic acid, ferulic acid, *p*-coumaric acid, and benzoic acid as single carbon sources at a concentration of 1 g/L. The authors also show both cell lines completely metabolize protocatechuic acid and pHBA present in hydrolysates derived from two engineered lines of *Arabidopsis thaliana*. Generally, *R. mucilaginosa* grew to higher biomass than *R. toruloides*, except when grown with ferulic acid. No growth was seen for vanillin or 4-hydroxybenzaldehyde as sole carbon sources for either species [71]. Lipid titers were not reported, as the strains were engineered to produce bisaboline, similarly to Yaegashi et al. [62]. In the present study, *R. mucilaginosa* grew poorly in syringate and ferulate when the concentration was less than 1 g/L. This emphasizes a recurring concept: that different strains of the same species often have very different phenotypes such as metabolic capabilities.

Toxicity mechanisms are difficult to probe, but some studies observed an increase of unsaturated bonds in fatty acids in response to aromatic compounds. An increase of C18:1 was observed when *C. fermentans* CICC 1368 was grown with increasing concentrations of vanillyl alcohol in the presence of a glucose/xylose blend, though the C18:1 increase was not as dramatic with hydroquinone or catechol [72]. *Rhodotorula kratochvilovae* (formerly classified as *Rhodosporidium kratochvilovae*) HIMPA1 exhibited an increase of monounsaturated fatty acids from 51.87 to 62.98% with increasing phenol concentrations, presumably due to the conversion of C18:0–C18:1 by a Δ-9 desaturase [73]. The same increase of C18:1 content of cells grown in resorcinol, pHBA, and phenol relative to cells grown in glucose was observed in *C. oleaginosus* cells [39]. Since *C. oleaginosus* accumulates high intracellular lipids when cultivated in phenolic conditions, and the lipids produced have a significant amount of unsaturated C18:1 fatty acids, this yeast may be particularly suited to overcome aromatic toxicity.

Conclusions

Significant effort has been made to engineer model organisms, such as *Saccharomyces cerevisiae* and *Escherichia coli*, for aromatic conversion to industrially relevant products [21–24, 74, 75]. Engineering new substrate metabolism is exceedingly difficult since this complex phenotype is directly tied to central metabolism. An increasingly popular approach is to identify microbes that inherently exhibit more of the desired properties and develop the tools to engineer them, as described in several recent reviews and opinions [76–78]. There is significant variation in metabolic capabilities between species, and among independently isolated strains of the same species; however, building biodiversity collections has become complicated due to habitat loss and the Nagoya Protocol [79, 80]. Therefore, it is important to preserve microorganisms in professionally managed culture collections such as the Phaff Yeast Culture Collection, to ensure that these microorganisms continue to be available for future studies [81, 82].

Although yeast genomes are being sequenced at a rapid pace, functional annotation is lagging. Genomes of bacteria, such as *Rhodococcus opacus* PD630 and *Pseudomonas putida*, have been more thoroughly annotated, but the low identity to eukaryotic genes yields few successful BLAST homology results. Therefore, functional data are required to even begin to explore aromatic metabolism in yeast. This study, which identified promising aromatic metabolizing yeasts, will aid in identifying the functional genes responsible for aromatic metabolism in yeast. The major findings of this study include identification of over a dozen potential aromatic metabolizing oleaginous yeast species and a better understanding of the phylogenetic distribution of aromatic metabolism pathway branches.

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