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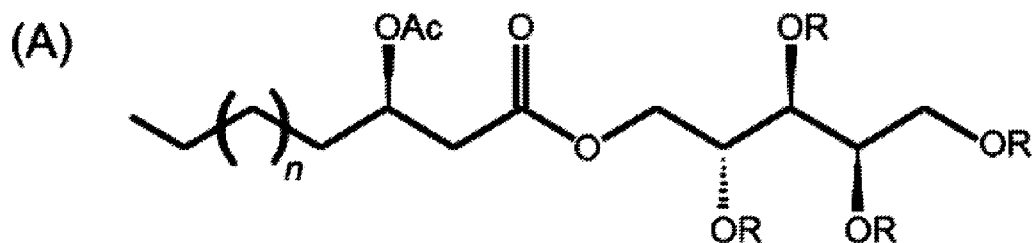


Fig. 13A

(57) Abstract: Provided are methods and yeast cultures for producing polyol lipids and polyol lipid compositions.

METHODS OF PRODUCING POLYOL LIPIDS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Appl. No. 62/325,859, filed on April 21, 2016, which is hereby incorporated
5 herein by reference in its entirety for all purposes.

BACKGROUND

[0002] Surfactants, or surface active agents, are amphiphilic organic molecules used as detergents, emulsifiers, wetting agents, dispersants, foaming agents, antifoam agents, and humectants. The annual global production of surfactants was 13 million metric tons in 2008
10 [Ashby (2013) J Surfactants Deterg 16:683-691]. Between 40 and 65% of surfactants are derived from petroleum [Deleu (2004) C R Chim 7:641-646; Rust (2008) Surfactants: a market opportunity study update. OmniTech International Ltd, Midland, MI]. The remaining feedstocks are primarily plant oils such as palm or coconut oil. Due to environmental and food security concerns, renewable and more environmentally friendly
15 alternatives are being sought. Naturally produced microbial biosurfactants are appealing alternatives because they are produced renewably and sustainably [Makkar (2011) AMB express 1:1-19], have low ecotoxicity [Renkin (2003) Riv Ital Sostanze Gr 80:249-252], are highly biodegradable [Mulligan (2005) Environ Pollut 133:183-198; Renkin, *supra*], and are active under a broad range of conditions [Khan (2014) Biosurfactants: Production and
20 Utilization—Processes, Technologies, and Economics 159:269; Marchant (2012) Trends Biotechnol 30:558-565]. Microbial biosurfactants display a unique combination of market attractiveness and high demand growth, and market prices range \$10–\$30 per kg, depending on the type, the market and the application [Microbial biosurfactants market: Global industry analysis, size, share, growth, trends and forecast 2014 - 2020. Transparency Market
25 Research, City, pp 1-74]. Microbial biosurfactants are used in numerous applications including industrial and household cleaning products, cosmetics, lubricants, adhesives, agro- chemicals, mining, petroleum extraction and cleanup, and in pulp and paper industries.

[0003] Yeast glycolipids (GL, see Fig. 1) are a group of microbial biosurfactants
30 comprising sophorolipids (SL), mannosylerythritol lipid (MEL), cellobiose lipids (CL) and polyol lipids (PL) [Marchant, *supra*]. PL comprise two subgroups: (1) liamocins (LM),

which consist of a single partially acetylated polyol head group with three or four 3,5-dihydroxydecanoic tails polyesterified through the 5-hydroxy group and are currently the best understood (Fig. 1e) [Price (2016) J Antibiot DOI 10.1038/ja.2016.92; Price (2013) Carbohydr Res 370:24-32], and (2) polyol esters of fatty acids (PEFA) produced by yeasts taxonomically close to the *Rhodotorula glutinis/graminis* clade [Cajka (2016) J Nat Prod 79:2580-2589; Tulloch (1964) Can J Chem 42:830-835]. PEFA are amphiphilic molecules composed of an acetylated (R)-3-hydroxy fatty acid esterified through the carboxyl end to a 5 or 6 carbon polyol, typically D-mannitol or D-arabitol, with varying degrees of acetylations (Fig. 1d) [Cajka, *supra*]. They differ from LM in that the $\Delta 5$ carbon is not hydroxylated and thus, only have a single acetylated (R)-3-hydroxylated acyl chain of varying numbers of carbons, usually C16:0 and C18:0. LM polyol head group is also non-acetylated, while PEFA's polyol head group can present different degrees of acetylation. Commercial production of SL, MEL and CL requires provision of both a hydrophobic carbon source such as vegetable oil, and a hydrophilic carbon source such as glucose. In contrast, PL could be synthesized in commercially relevant amounts without a hydrophobic carbon source. A few ascomycete and basidiomycete yeast species have been reported to produce PL (see Table 1). Interestingly, all the basidiomycetes reported to produce PEFA are taxonomically close to the *Rhodotorula glutinis/graminis* clade [Kurtzman (2011) The Yeasts: A Taxonomic Study. Elsevier, Oxford]. *Rhodotorula babjevae* UCDFST 04-877 secreted five times more PEFA than its phylogenetic neighbors [Cajka, *supra*], raising the question on whether this feature could be present in related species as well.

[0004] Recommended strategies for improving biosurfactant production include identifying new hyper-producing microorganisms, genetically modifying production strains, optimizing media and growth conditions, using wastes or other low-cost raw materials, improving downstream processing, and capturing multiple co-products [Dhanarajan (2014) Biosurfactants: Production and Utilization—Processes, Technologies, and Economics 159:153]. Addressing the first strategy to improve SL production, Kurtzman [Kurtzman (2010) FEMS Microbiol Lett 311:140-146] analyzed 26 strains from the USDA-ARS Culture Collection (<http://nrrl.ncaur.usda.gov>) belonging to 18 yeast species within the *Starmerella* clade. This clade contains *Starmerella* species, several related *Candida* species, and numerous species that currently lack valid species names. They confirmed that clade member *Candida apicola* produces SL, which had been previously known [Gorin (1961) Can J Chem 39:846-855; Stodola (1967) Bacteriol Rev 31:194; Tulloch (1968) Can

J Chem 46:345-348]. They found three additional SL-secreting yeast species within the Starmerella clade [Kurtzman (2012) Intl J Syst Evol Microbiol 62:2307-2311; Kurtzman (2010) FEMS Microbiol Lett 311:140-146]. Targeting this taxonomic clade was therefore a successful strategy. It is important to point out that SL production was strain-specific. For
5 example, of two strains of *Candida apicola* that were analyzed, one produced very high levels of SL, and one produced no detectable SL.

[0005] The main disadvantages of the previous art were that no detailed chemical structures of PEFA from any basidiomycetous yeast was previously described, and that the yields within the basidiomycetous yeast were low, and not suitable for commercialization.
10 In all cases, yeast cultures were grown in media where no iron was supplemented, and the level of aeration was not as high as in cultures and methods described herein.

TABLE 1
Yeast Species Previously Reported To Synthesize And Secrete Polyol Lipids: Polyol Esters of Fatty Acids (PEFA) or Liamocins

Species	Strain	Publ. Year	Environmental source of the strain	Reference
Basidiomycota (PEFA)				
<i>Rhodosporiobolus azoricus</i> (previously identified as <i>Rhodotorula glutinis</i>)	CBS 4648	1964	Leaf surface of Cacao plants, Ghana	Tulloch, et al., <i>Can J Chem</i> (1964) 42:830–835
<i>Rhodotorula glutinis</i>	16A8	1964	Flowers, Canada	
<i>Rhodotorula toruloides</i> (previously identified as <i>Rhodotorula glutinis</i>)	IP-30 = ATCC 204091	1994	Hydrocarbon contaminated soil, India	Johnson, et al., <i>Biotechnol Lett</i> (1992) 14:487–490
<i>Rhodotorula graminis</i>	6CB	1961	Flowers, Canada	Tulloch, et al., <i>Can J Chem</i> (1964) 42:830–835
<i>Rhodotorula babjevae</i> (syn. <i>Rhodosporiidium babjevae</i>)	UCDFST 04-877	2016	Olive fly, California, USA	Cajka, <i>J Nat Prod</i> (2016) 79:2580–2589
Ascomycota (Liamocins)				
<i>Aureobasidium</i> sp.	A-2	1994	Natural sources	Kurosawa, et al., <i>Biosci Biotechnol Biochem</i> (1994) 58:2057–2060
<i>Aureobasidium</i> sp.	A-21 M	1994	Natural sources	
<i>Aureobasidium pullulans</i> (syn. <i>Pullularia pullulans</i>)	272	1964	Phyllosphere of tropical foliage, Java	Ruinen, et al., <i>Antonie Van Leeuwenhoek</i> (1964) 30:377–384
<i>Aureobasidium pullulans</i>	CU 39 = NRRL 58551	2009, 2011	<i>Mimusops elengi</i> L. (Spanish cherry), Thailand	Manitchotpsit, et al., <i>Mycol Res</i> (2009) 113:1107–1120; Manitchotpsit, et al., (2011) <i>Biotechnol Lett</i> 33:1151–1157
<i>Aureobasidium pullulans</i>	CU 43 = NRRL 50380	2011, 2013	Leaf of <i>Cassia fistula</i> L. (golden shower tree), Udonthani, Thailand	Manitchotpsit, et al., (2011) <i>Biotechnol Lett</i> 33:1151–1157; Price, et al., <i>Carbohydr Res</i> 370:24–32

CBS: Centraalbureau voor Schimmelcultures, The Netherlands; ATCC: American Type Culture Collection, VA, USA; NRRL: USDA-ARS Culture Collection, IL, USA

SUMMARY

[0006] In one aspect, a yeast culture is provided. In varying embodiments, the yeast culture comprises a population of basidiomycetous yeast cells, one or more hydrophilic (*e.g.*, non-hydrophobic) carbon sources, and/or at least about 1 g/L polyol lipid (*e.g.*, polyol ester of fatty acid (PEFA)), *e.g.*, at least about 2 g/L, 3 g/L, 4 g/L, 5 g/L, 6 g/L, 7 g/L, 8 g/L, 9 g/L, 10 g/L, 11 g/L, 12 g/L, 13 g/L, 14 g/L, 15 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L, 40 g/L, 45 g/L, 50 g/L, 100 g/L, 150 g/L, 200 g/L, 250 g/L, 300 g/L, 350 g/L, 400 g/L polyol lipid, *e.g.*, up to about 450 g/L of polyol lipid. In varying embodiments, the culture does not comprise one or more hydrophobic carbon sources. In varying embodiments, the culture does not comprise one or more hydrophobic carbon sources selected from the group consisting of oils, alkanes, fatty acids, fatty esters and mixtures thereof. In varying embodiments, the yeast culture comprises a population of basidiomycetous yeast cells, one or more hydrophilic (*e.g.*, non-hydrophobic) carbon sources, one or more hydrophobic carbon sources, and at least about 1 g/L polyol lipid (*e.g.*, polyol ester of fatty acid (PEFA)), *e.g.*, at least about 2 g/L, 3 g/L, 4 g/L, 5 g/L, 6 g/L, 7 g/L, 8 g/L, 9 g/L, 10 g/L, 11 g/L, 12 g/L, 13 g/L, 14 g/L, 15 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L, 40 g/L, 45 g/L, 50 g/L, 100 g/L, 150 g/L, 200 g/L, 250 g/L, 300 g/L, 350 g/L, 400 g/L polyol lipid, *e.g.*, up to about 450 g/L of polyol lipid. In varying embodiments, total polyol lipids are as measured after 1, 2, 3, 4, 5, 6, 7 or 8 days growth. In varying embodiments, the culture is a liquid culture. In varying embodiments, the volume of the culture is at least about 0.5L, 1L, 2L, 3L, 4L, 5L, 10L, 25L, 50L, 75L, 100L, 250L, 500L, 1000L, or more. In varying embodiments, the one or more hydrophilic carbon sources comprise non-hydrophobic or hydrophilic waste stream and/or hydrolysates. In varying embodiments, the culture comprises less than about 2% (w/v) nitrogen, *e.g.*, in the range of about 0.005% (w/v) to about 2% (w/v) nitrogen. In varying embodiments, the nitrogen source is selected from the group consisting of ammonia, ammonium salt, nitrate, nitrite, nucleotides, nucleosides, proteins, peptides, amino acids, urea and its derivatives, and mixtures thereof. In varying embodiments, the culture comprises about 0.05% (w/v) ammonium chloride. In varying embodiments, the yeast culture comprises an iron salt, *e.g.*, an iron salt selected from the group consisting of iron chloride (II) & (III), iron sulfate (II) & (III), iron nitrate (II) & (III), iron phosphate (II) & (III), fumarate, iron edetate, iron citrate, iron malate, iron oxalate, iron tartrate, iron succinate, iron acetate, in their anhydrous or their hydrated states, and organometallic complexes thereof. In some embodiments, the yeast culture comprises the iron salt in a

concentration in the range of about 0.001 mg/L to about 10 g/L, *e.g.*, from about 1 mg/L to about 0.5 g/L, *e.g.*, from about 0.01 g/L to about 0.5 g/L. In varying embodiments, the culture comprises one or more hydrophilic carbon sources at a concentration in the range of about 0.2% (w/v) to about 70% (w/v), *e.g.*, in the range of about 0.2% (w/v) to about 10%, 20%, 30%, 40%, 50% or 60% (w/v). In varying embodiments, the yeast culture is supplemented with extra hydrophilic carbon source (*e.g.*, a mono- or di- saccharide, *e.g.*, glucose and/or sucrose) to a concentration of at least about 50 g/L, *e.g.*, at least about 75 g/L, 100 g/L, 125 g/L, 150 g/L, 175 g/L, 200 g/L, 225 g/L, 250 g/L, 275 g/L or 300 g/L. In varying embodiments, the culture comprises a carbon to nitrogen ratio of about 5:1 to about 400:1, *e.g.*, from about 10:1 to about 200:1, from about 20:1 to about 100:1, *e.g.*, from about 25:1 to about 75:1, *e.g.*, from about 30:1 to about 70:1, *e.g.*, from about 40:1 to about 60:1, *e.g.*, from about 30:1 to about 40:1, *e.g.*, using a nitrogen source that can be consumed or utilized by the yeast cells. In some embodiments, the culture comprises dissolved oxygen at a concentration of at least about 10%, *e.g.*, at least about 15%, 20%, 25%, 30%, 35%, 40%, 45%, and up to 60%, 70%, 80%, 90%, 100% dissolved oxygen, *e.g.*, from about 15% to about 80%, *e.g.*, from about 20% to about 60% dissolved oxygen. In varying embodiments, the hydrophilic carbon source is selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, sugar alcohols, polyols, polyol lipids (including polyol esters of fatty acid (PEFAs)), organic acids, esters, aldehydes, ketones, alcohols, waste streams, plant materials, lignocellulosic hydrolysates, industrial co-products and mixtures thereof. In varying embodiments, the hydrophilic carbon source is a monosaccharide, an oligosaccharide or a polysaccharide and comprises one or more sugar moieties selected from the group consisting of glucose, sucrose, xylose, galactose, rhamnose, arabinose, mannose, cellobiose, galacturonic acid, lactose, sophorose, glycerol, and mixtures thereof. In varying embodiments, the population of basidiomycetous yeast cells comprises cells in the taxonomic class Microbotryomycetes. In varying embodiments, the population of basidiomycetous yeast cells comprises cells in the taxonomic order Sporidiobolales. In varying embodiments, the population of basidiomycetous yeast cells is from genera classified within the taxonomic order Sporidiobolales selected from the group consisting of *Rhodosporidiobolus*, *Rhodotorula*, and *Sporobolomyces*. In varying embodiments, the population of basidiomycetous yeast cells comprises *Rhodotorula* cells. In varying embodiments, the *Rhodotorula* cells are *Rhodotorula babjevae* (*syn. Rhodosporidium babjevae*) cells. In varying embodiments, the population of basidiomycetous yeast cells comprises one or more species selected from the group

consisting of *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*), *Rhodotorula diobovata* (syn. *Rhodosporidium diobovatum*), *Rhodotorula kratochvilovae* (syn. *Rhodosporidium kratochvilovae*), *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*), *Rhodotorula* aff. *paludigena* (syn. *Rhodosporidium* aff. *paludigenum*), and
 5 *Rhodotorula dairenensis*. In varying embodiments, the population of basidiomycetous yeast cells comprises one or more strains selected from the group consisting of:

- a) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain NRRL Y-67018 (also deposited as UCDFST 04-877),
- b) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain NRRL Y-
 10 67017 (also deposited as UCDFST 05-775),
- c) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 68-916.1,
- d) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 67-458,
- e) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 05-736,
- 15 f) *Rhodotorula diobovata* (syn. *Rhodosporidium diobovatum*) strain UCDFST 04-830,
- g) *Rhodotorula diobovata* (syn. *Rhodosporidium diobovatum*) strain NRRL Y-67015 (also deposited as UCDFST 08-225),
- h) *Rhodotorula kratochvilovae* (syn. *Rhodosporidium kratochvilovae*) strain
 20 NRRL Y-67016 (also deposited as UCDFST 05-632),
- i) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain NRRL Y-67012 (also deposited as UCDFST 09-163),
- j) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain UCDFST 81-492,
- 25 k) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain UCDFST 82-646.2,
- l) *Rhodotorula* aff. *paludigena* (syn. *Rhodosporidium* aff. *paludigenum*) strain NRRL Y-67009 (also deposited as UCDFST 81-84), and

m) *Rhodotorula dairenensis* strain NRRL Y-67011 (also deposited as UCDFST
 30 68-257). In varying embodiments, the population of basidiomycetous yeast cells comprises one or more strains selected from the group consisting of: a) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 68-916.1, b) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 67-458, c) *Rhodotorula babjevae* (syn.

Rhodospiridium babjevae) strain UCDFST 05-736, d) *Rhodotorula diobovata* (syn. *Rhodospiridium diobovatum*) strain UCDFST 04-830, e) *Rhodotorula paludigena* (syn. *Rhodospiridium paludigenum*) strain UCDFST 81-492, and f) *Rhodotorula paludigena* (syn. *Rhodospiridium paludigenum*) strain UCDFST 82-646.2. In varying embodiments, the cells do not comprise *Pseudohyphozyma bogoriensis* (syn. *Rhodotorula bogoriensis*). In varying embodiments, the polyol lipids comprise polyol esters of fatty acids (PEFAs). In varying embodiments, the polyol lipid comprises an (*R*)-3-hydroxy fatty acid moiety comprising from 10 to 24 carbon atoms in length, e.g., from 14 to 24 carbon atoms in length. In varying embodiments, the polyol lipid comprises a sugar alcohol moiety esterified to the carboxyl end of the (*R*)-3-hydroxy fatty acyl moiety. In varying embodiments, the (*R*)-3-hydroxy fatty acyl moiety can be acetylated. In varying embodiments, the polyol lipid sugar alcohol moiety comprises non-esterified hydroxy groups that can be acetylated. In varying embodiments, the polyol lipid sugar alcohol moiety comprises a backbone selected from D-mannitol and D-arabitol. In varying embodiments, the polyol lipid sugar alcohol moiety does not comprise xylitol.

[0007] In varying embodiments of the yeast culture, the polyol lipid comprises one or more polyol esters of fatty acids (PEFAs) listed in Table 4.

[0008] In varying embodiments, the yeast culture comprises a polyol lipid (e.g. PEFA) profile comprising:

i) at least about 20%, e.g., at least about 25%, 30%, 35%, 40%, up to about 45% of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂);

ii) at least about 5%, e.g., at least about 6%, 7%, 8%, 9%, 10%, 15%, 20%, up to about 25%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂); and/or

iii) at least about 5%, e.g., at least about 6%, 7%, 8%, 9%, 10%, 15%, up to about 20% of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₄H₅₈O₁₃).

[0009] In varying embodiments, the yeast culture comprises a polyol lipid (e.g. PEFA) comprising at least about 25%, e.g., at least about 30%, 35%, 40%, 45%, up to about 50% of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂).

[0010] In varying embodiments, the yeast culture comprises a polyol lipid (e.g. PEFA) comprising:

i) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, up to about 18%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol

5 comprising 3 acetylations (Molecular Formula: C₃₂H₅₆O₁₂);

ii) at least about 1%, *e.g.*, at least about 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, up to about 20%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₂H₅₄O₁₃);

10 iii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, 20%, up to about 25%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₄H₅₈O₁₃);

iv) at least about 15%, *e.g.*, at least about 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, up to about 50% of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂); and/or

15 v) at least about 10%, *e.g.*, at least about 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 25%, 30%, up to about 35% of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂).

[0011] In varying embodiments, the yeast culture further comprises a polyol lipid (e.g. PEFA) comprising:

20 vi) at least about 2%, *e.g.*, at least about 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₄H₅₆O₁₄); and/or

25 vii) at least about 1%, *e.g.*, at least about 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol (Molecular Formula: C₃₆H₆₀O₁₄).

[0012] In varying embodiments, the yeast culture comprises a polyol lipid (e.g. PEFA) comprising:

30 i) at least about 1%, *e.g.*, at least about 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₄H₅₈O₁₃);

ii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, up to about 45%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₄H₅₆O₁₄);

iii) at least about 10%, *e.g.*, at least about 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, up to about 25%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₆H₆₀O₁₄);

iv) at least about 15%, *e.g.*, at least about 20%, 25%, 30%, 35%, 40%, up to about 45%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂); and/or

v) at least about 15%, *e.g.*, at least about 20%, 25%, 30%, 35%, 40%, up to about 45% of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂).

[0013] In varying embodiments, the yeast culture comprises a polyol lipid (*e.g.* PEFA) comprising:

i) at least about 10%, *e.g.*, at least about 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, up to about 25%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₄H₅₈O₁₃);

ii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, up to about 30%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to

D-mannitol comprising 5 acetylations (Molecular Formula: C₃₄H₅₆O₁₄);

iii) at least about 10%, *e.g.*, at least about 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, up to about 25%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₆H₆₀O₁₄);

iv) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂);

v) at least about 15%, *e.g.*, at least about 20%, 25%, 30%, 35%, 40%, up to about 45%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂); and/or

vi) at least about 0.5%, *e.g.*, at least about 1%, 2%, 3%, 4%, 5% up to about 6% of an acetylated C14:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₂₉H₄₈O₁₂).

[0014] In varying embodiments, the yeast culture comprises a polyol lipid (e.g. PEFA) comprising:

i) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising
5 4 acetylations (Molecular Formula: C₃₄H₅₈O₁₃);

ii) at least about 10%, *e.g.*, at least about 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, up to about 25%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₄H₅₆O₁₄);

10 iii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₆H₆₀O₁₄);

iv) at least about 15%, *e.g.*, at least about 20%, 25%, 30%, 35%, 40%, up to about 45%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂);

15 v) at least about 35%, *e.g.*, at least about 36%, 37%, 38%, 39%, 40%, up to about 45% of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂); and/or

20 vi) at least about 0.5%, *e.g.*, at least about 1%, 2%, 3%, 4% up to about 5%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 3 acetylations (Molecular Formula: C₃₀H₅₂O₁₂).

[0015] In varying embodiments, the yeast culture comprises a polyol lipid (e.g. PEFA) comprising:

i) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, up to about 20%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol
25 comprising 4 acetylations (Molecular Formula: C₃₂H₅₄O₁₃);

ii) at least about 45%, *e.g.*, at least about 46%, 47%, 48%, 49%, up to about 50% of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂); and/or

30 iii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, up to about 12% of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂).

[0016] In varying embodiments, the one or more hydrophobic carbon sources are selected from the group consisting of oils, alkanes, fatty acids, fatty esters and mixtures

thereof. In varying embodiments, the one or more polyol lipids are present in the medium in a form that can be harvested without solvent extraction.

[0017] Further provided is a bioreactor comprising a yeast culture, as described above and herein.

5 [0018] In a further aspect, provided are methods of producing one or more polyol lipids. In some embodiments, the methods comprise culturing a population of basidiomycetous yeast cells in a yeast cell culture comprising one or more hydrophilic (*e.g.*, non-hydrophobic) carbon sources, whereby the basidiomycetous yeast cells produce one or more polyol esters of fatty acids (PEFAs). In varying embodiments, the culture does not
10 comprise one or more hydrophobic carbon sources. In some embodiments, the methods comprise culturing a population of basidiomycetous yeast cells in a yeast cell culture comprising one or more hydrophilic (*e.g.*, non-hydrophobic) carbon sources and one or more hydrophobic carbon sources, whereby the basidiomycetous yeast cells produce one or more polyol lipids (*e.g.*, polyol esters of fatty acids (PEFAs)). In varying embodiments,
15 additional hydrophilic carbon source is added, *e.g.*, during any stage of growth including exponential growth, and after the population of basidiomycetous yeast cells reaches stationary phase. In varying embodiments, at least about 1 g/L polyol lipid (*e.g.*, polyol esters of fatty acids (PEFAs)), *e.g.*, at least about 2 g/L, 3 g/L, 4 g/L, 5 g/L, 6 g/L, 7 g/L, 8 g/L, 9 g/L, 10 g/L, 11 g/L, 12 g/L, 13 g/L, 14 g/L, 15 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L, 40 g/L, 45 g/L, 50 g/L, 100 g/L, 150 g/L, 200 g/L, 250 g/L, 300 g/L, 350 g/L, 400 g/L polyol
20 lipid, *e.g.*, up to about 450 g/L of polyol lipid, is produced. In some embodiments, a polyol lipid yield in the range of about 0.5 g/L to about 30 g/L can be obtained from yeast cultures comprising in the range of about 10 g/L to about 250 g/L glucose. In some embodiments, a polyol lipid yield of at least about 14 g/L, *e.g.*, from about 14 g/L to about 17 g/L, can be
25 obtained from yeast cultures comprising about 50 g/L glucose. In varying embodiments, total polyol lipids are as measured after 1, 2, 3, 4, 5, 6, 7 or 8 days growth. In varying embodiments, the volume of the culture is at least about 0.5L, 1L, 2L, 3L, 4L, 5L, 10L, 25L, 50L, 75L, 100L, 250L, 500L, 1000L, or more. In varying embodiments, the culture comprises less than about 2% (w/v) nitrogen, *e.g.*, in the range of about 0.005% (w/v) to
30 about 2% (w/v) nitrogen. In varying embodiments, the nitrogen source is selected from the group consisting of ammonia, ammonium salt, nitrate, nitrite, nucleotides, nucleosides, proteins, peptides, amino acids, urea and its derivatives, and mixtures thereof. In varying embodiments, the culture comprises about 0.05% (w/v) ammonium chloride. In varying

embodiments, the yeast culture comprises an iron salt, *e.g.*, an iron salt selected from the group consisting of iron chloride (II) & (III), iron sulfate (II) & (III), iron nitrate (II) & (III), iron phosphate (II) & (III), fumarate, iron edetate, iron citrate, iron malate, iron oxalate, iron tartrate, iron succinate, iron acetate, in their anhydrous or their hydrated states, and

5 organometallic complexes thereof. In some embodiments, the yeast culture comprises the iron salt in a concentration in the range of about 0.001 mg/L to about 10 g/L, *e.g.*, from about 1 mg/L to about 0.5 g/L, *e.g.*, from about 0.01 g/L to about 0.5 g/L. In varying embodiments, the culture comprises one or more hydrophilic carbon sources at a concentration in the range of about 0.2% (w/v) to about 70% (w/v), *e.g.*, in the range of

10 about 0.2% (w/v) to about 10%, 20%, 30%, 40%, 50% or 60% (w/v). In varying embodiments, the culture comprises a carbon to nitrogen ratio of about 5:1 to about 400:1, *e.g.*, from about 10:1 to about 200:1, from about 20:1 to about 100:1, *e.g.*, from about 25:1 to about 75:1, *e.g.*, from about 30:1 to about 70:1, *e.g.*, from about 40:1 to about 60:1, *e.g.*, from about 30:1 to about 40:1, *e.g.*, using a nitrogen source that can be consumed or utilized

15 by the yeast cells. In some embodiments, the yeast culture is maintained at a temperature in the range of between about 24°C to about 27°C. In varying embodiments, the hydrophilic carbon source is selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, sugar alcohols, polyols, polyol lipids (*e.g.*, polyol esters of fatty acids (PEFAs)), organic acids, esters, aldehydes, ketones, alcohols, waste streams, plant

20 materials, lignocellulosic hydrolysates, industrial co-products and mixtures thereof. In varying embodiments, the hydrophilic carbon source is a monosaccharide, an oligosaccharide or a polysaccharide and comprises one or more sugar moieties selected from the group consisting of glucose, sucrose, xylose, galactose, rhamnose, arabinose, mannose, cellobiose, galacturonic acid, lactose, sophorose, glycerol, and mixtures thereof.

25 In varying embodiments, the population of basidiomycetous yeast cells comprises cells in the taxonomic class Microbotryomycetes. In varying embodiments, the population of basidiomycetous yeast cells comprises cells in the taxonomic order Sporidiobolales. In varying embodiments, the population of basidiomycetous yeast cells is from genera classified within the taxonomic order Sporidiobolales selected from the group consisting of

30 *Rhodosporidiobolus*, *Rhodotorula*, and *Sporobolomyces*. In varying embodiments, the population of basidiomycetous yeast cells comprises *Rhodotorula* cells. In varying embodiments, the *Rhodotorula* cells are *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) cells. In varying embodiments, the population of basidiomycetous yeast cells comprises one or more species selected from the group consisting of *Rhodotorula babjevae*

(syn. *Rhodosporidium babjevae*), *Rhodotorula diobovata* (syn. *Rhodosporidium diobovatum*), *Rhodotorula kratochvilovae* (syn. *Rhodosporidium kratochvilovae*), *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*), *Rhodotorula* aff. *paludigena* (syn. *Rhodosporidium* aff. *paludigenum*), and *Rhodotorula dairenensis*. In varying
 5 embodiments, the population of basidiomycetous yeast cells comprises one or more strains selected from the group consisting of:

a) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain NRRL Y-67018 (also deposited as UCDFST 04-877),

b) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain NRRL Y-67017 (also deposited as UCDFST 05-775),
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c) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 68-916.1,

d) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 67-458,

e) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 05-736,

f) *Rhodotorula diobovata* (syn. *Rhodosporidium diobovatum*) strain UCDFST
 15 04-830,

g) *Rhodotorula diobovata* (syn. *Rhodosporidium diobovatum*) strain NRRL Y-67015 (also deposited as UCDFST 08-225),

h) *Rhodotorula kratochvilovae* (syn. *Rhodosporidium kratochvilovae*) strain NRRL Y-67016 (also deposited as UCDFST 05-632),

i) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain NRRL Y-67012 (also deposited as UCDFST 09-163),
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j) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain UCDFST 81-492,

k) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain
 25 UCDFST 82-646.2,

l) *Rhodotorula* aff. *paludigena* (syn. *Rhodosporidium* aff. *paludigenum*) strain NRRL Y-67009 (also deposited as UCDFST 81-84), and

m) *Rhodotorula dairenensis* strain NRRL Y-67011 (also deposited as UCDFST 68-257). In varying embodiments, the population of basidiomycetous yeast cells comprises

30 one or more strains selected from the group consisting of: a) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 68-916.1, b) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 67-458, c) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 05-736, d) *Rhodotorula diobovata* (syn. *Rhodosporidium diobovatum*) strain UCDFST 04-830, e) *Rhodotorula paludigena* (syn.

Rhodospiridium paludigenum) strain UCDFST 81-492, and f) *Rhodotorula paludigena* (syn. *Rhodospiridium paludigenum*) strain UCDFST 82-646.2. In varying embodiments, the cells do not comprise *Pseudohyphozyma bogoriensis* (syn. *Rhodotorula bogoriensis*). In varying embodiments, the polyol lipid comprises one or more polyol esters of fatty acids (PEFAs). In varying embodiments, the polyol lipid comprises a 3-hydroxy fatty acid comprising from 10 to 24 carbon atoms in length, e.g., from 14 to 24 carbon atoms in length. In varying embodiments, the polyol lipid comprises a sugar alcohol moiety esterified to the carboxyl end of the 3-hydroxy fatty acyl moiety. In varying embodiments, the 3 hydroxyl fatty acyl moiety can be acetylated. In varying embodiments, the polyol lipid sugar alcohol moiety comprises non-esterified hydroxy groups that can be acetylated. In varying embodiments, the polyol lipid sugar alcohol moiety comprises a backbone selected from D-mannitol and D-arabitol. In varying embodiments, the polyol lipid sugar alcohol moiety does not comprise xylitol. In varying embodiments of the yeast culture, the polyol lipid comprises one or more polyol esters of fatty acids (PEFAs) listed in Table 4.

[0019] In varying embodiments, the methods produce a yeast culture comprising a polyol lipid (e.g. PEFA) comprising:

i) at least about 20%, e.g., at least about 25%, 30%, 35%, 40%, up to about 45% of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂);

ii) at least about 5%, e.g., at least about 6%, 7%, 8%, 9%, 10%, 15%, 20%, up to about 25%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂); and/or

iii) at least about 5%, e.g., at least about 6%, 7%, 8%, 9%, 10%, 15%, up to about 20% of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₄H₅₈O₁₃).

[0020] In varying embodiments, the methods produce a yeast culture comprising a polyol lipid (e.g. PEFA) comprising at least about 25%, e.g., at least about 30%, 35%, 40%, 45%, up to about 50% of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂).

[0021] In varying embodiments, the methods produce a yeast culture comprising a polyol lipid (e.g. PEFA) comprising:

i) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, up to about 18%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 3 acetylations (Molecular Formula: C₃₂H₅₆O₁₂);

5 ii) at least about 1%, *e.g.*, at least about 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, up to about 20%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₂H₅₄O₁₃);

iii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, 20%, up to about 25%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₄H₅₈O₁₃);

10 iv) at least about 15%, *e.g.*, at least about 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, up to about 50% of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂); and/or

v) at least about 10%, *e.g.*, at least about 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 25%, 30%, up to about 35% of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂).

[0022] In varying embodiments, the methods produce a yeast culture comprising a polyol lipid (*e.g.* PEFA) comprising:

vi) at least about 2%, *e.g.*, at least about 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₄H₅₆O₁₄); and/or

vii) at least about 1%, *e.g.*, at least about 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol (Molecular Formula: C₃₆H₆₀O₁₄).

25 **[0023]** In varying embodiments, the methods produce a yeast culture comprising a polyol lipid (*e.g.* PEFA) comprising:

i) at least about 1%, *e.g.*, at least about 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₄H₅₈O₁₃);

30 ii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, up to about 45%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₄H₅₆O₁₄);

iii) at least about 10%, *e.g.*, at least about 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, up to about 25%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₆H₆₀O₁₄);

iv) at least about 15%, *e.g.*, at least about 20%, 25%, 30%, 35%, 40%, up to about 45%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂); and/or

v) at least about 15%, *e.g.*, at least about 20%, 25%, 30%, 35%, 40%, up to about 45% of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂).

10 **[0024]** In varying embodiments, the methods produce a yeast culture comprising a polyol lipid (*e.g.* PEFA) comprising:

i) at least about 10%, *e.g.*, at least about 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, up to about 25%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₄H₅₈O₁₃);

15 ii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, up to about 30%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₄H₅₆O₁₄);

iii) at least about 10%, *e.g.*, at least about 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, up to about 25%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₆H₆₀O₁₄);

20 iv) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂);

v) at least about 15%, *e.g.*, at least about 20%, 25%, 30%, 35%, 40%, up to about 45%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂); and/or

25 vi) at least about 0.5%, *e.g.*, at least about 1%, 2%, 3%, 4%, 5% up to about 6% of an acetylated C14:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₂₉H₄₈O₁₂).

30 **[0025]** In varying embodiments, the methods produce a yeast culture comprising a polyol lipid (*e.g.* PEFA) comprising:

i) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₄H₅₈O₁₃);

ii) at least about 10%, *e.g.*, at least about 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, up to about 25%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₄H₅₆O₁₄);

iii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₆H₆₀O₁₄);

iv) at least about 15%, *e.g.*, at least about 20%, 25%, 30%, 35%, 40%, up to about 45%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂);

v) at least about 35%, *e.g.*, at least about 36%, 37%, 38%, 39%, 40%, up to about 45% of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂); and/or

vi) at least about 0.5%, *e.g.*, at least about 1%, 2%, 3%, 4% up to about 5%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 3 acetylations (Molecular Formula: C₃₀H₅₂O₁₂).

[0026] In varying embodiments, the methods produce a yeast culture comprising a polyol lipid (*e.g.* PEFA) comprising:

i) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, up to about 20%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₂H₅₄O₁₃);

ii) at least about 45%, *e.g.*, at least about 46%, 47%, 48%, 49%, up to about 50% of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂); and/or

iii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, up to about 12% of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂).

[0027] In varying embodiments of the methods of producing, the one or more hydrophobic carbon sources are selected from the group consisting of oils, alkanes, fatty acids, fatty esters and mixtures thereof. In varying embodiments, the method does not comprise actively pH-adjusting the culture. In some embodiments, the culture volume to

container volume ratio is 1:3 or greater, *e.g.*, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10 or greater. In some embodiments, the container is a baffled flask, *e.g.*, covered in such a manner to allow air/oxygen exchange, *e.g.*, with a foam stopper. In some embodiments, the culture comprises dissolved oxygen at a concentration of at least about 10%, *e.g.*, at least about 15%, 20%, 25%, 30%, 35%, 40%, 45%, and up to 60%, 70%, 80%, 90%, 100% dissolved oxygen, *e.g.*, from about 15% to about 80%, *e.g.*, from about 20% to about 60% dissolved oxygen. In varying embodiments, the methods further comprise purifying and/or isolating the polyol lipid. In varying embodiments, the polyol lipid does not need to be purified from a hydrophobic carbon source. In varying embodiments, the polyol lipid can be purified and/or isolated without cell lysis, *e.g.*, directly from the media. In some embodiments, the yeast cells secrete the one or more polyol lipids into the medium in a form that can be harvested without solvent extraction.

[0028] In a related aspect, provided are polyol lipid compositions produced according to the methods described above and herein. In another aspect, provided are compositions comprising one or more polyol esters of fatty acids (PEFAs) listed in Table 4. In varying embodiments, the polyol lipid compositions are selected from the group consisting of a cleanser, a detergent, a surfactant (*e.g.*, for recovery of oil), a wetting agent, an antifoam agent, an emulsifier, an emollient, a dispersant (*e.g.*, for cleanup of oil including spilled petroleum), a humectant, an antibacterial agent, an antiviral agent, an antifungal agent, a spermicide, an insecticide, a lubricant, an adhesive, a crystal modifier, an instantizer, a viscosity modifier, a mixing/blending aid, a release agent, a cream, a foam, a mousse, a lotion, a balm, and an ointment. In varying embodiments, polyol lipid composition is free of any hydrophobic carbon source. In varying embodiments, the method is performed as a batch, fed batch or continuous-feed process.

[0029] In varying embodiments, the composition comprises a polyol lipid (*e.g.* PEFA) comprising:

i) at least about 20%, *e.g.*, at least about 25%, 30%, 35%, 40%, up to about 45% of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂);

ii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, 20%, up to about 25%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂); and/or

iii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, up to about 20% of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₄H₅₈O₁₃).

[0030] In varying embodiments, the composition comprises a polyol lipid (*e.g.*

5 PEFA) comprising at least about 25%, *e.g.*, at least about 30%, 35%, 40%, 45%, up to about 50% of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂).

[0031] In varying embodiments, the composition comprises a polyol lipid (*e.g.* PEFA) comprising:

10 i) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, up to about 18%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 3 acetylations (Molecular Formula: C₃₂H₅₆O₁₂);

ii) at least about 1%, *e.g.*, at least about 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, up to about 20%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to
15 D-mannitol comprising 4 acetylations (Molecular Formula: C₃₂H₅₄O₁₃);

iii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, 20%, up to about 25%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₄H₅₈O₁₃);

iv) at least about 15%, *e.g.*, at least about 16%, 17%, 18%, 19%, 20%, 25%,
20 30%, 35%, 40%, 45%, up to about 50% of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂); and/or

v) at least about 10%, *e.g.*, at least about 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 25%, 30%, up to about 35% of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula:
25 C₃₃H₅₆O₁₂).

[0032] In varying embodiments, the compositions further comprise a polyol lipid (*e.g.* PEFA) comprising:

vi) at least about 2%, *e.g.*, at least about 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to
30 D-mannitol comprising 5 acetylations (Molecular Formula: C₃₄H₅₆O₁₄); and/or

vii) at least about 1%, *e.g.*, at least about 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol (Molecular Formula: C₃₆H₆₀O₁₄).

[0033] In varying embodiments, the composition comprises a polyol lipid (*e.g.* PEFA) comprising:

i) at least about 1%, *e.g.*, at least about 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₄H₅₈O₁₃);

ii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, up to about 45%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₄H₅₆O₁₄);

iii) at least about 10%, *e.g.*, at least about 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, up to about 25%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₆H₆₀O₁₄);

iv) at least about 15%, *e.g.*, at least about 20%, 25%, 30%, 35%, 40%, up to about 45%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂); and/or

v) at least about 15%, *e.g.*, at least about 20%, 25%, 30%, 35%, 40%, up to about 45% of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂).

[0034] In varying embodiments, the composition comprises a polyol lipid (*e.g.* PEFA) comprising:

i) at least about 10%, *e.g.*, at least about 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, up to about 25%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₄H₅₈O₁₃);

ii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, up to about 30%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₄H₅₆O₁₄);

iii) at least about 10%, *e.g.*, at least about 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, up to about 25%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₆H₆₀O₁₄);

iv) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂);

v) at least about 15%, *e.g.*, at least about 20%, 25%, 30%, 35%, 40%, up to about 45%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂); and/or

vi) at least about 0.5%, *e.g.*, at least about 1%, 2%, 3%, 4%, 5% up to about 6% of an acetylated C14:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₂₉H₄₈O₁₂).

10 **[0035]** In varying embodiments, the composition comprises a polyol lipid (*e.g.* PEFA) comprising:

i) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₄H₅₈O₁₃);

15 ii) at least about 10%, *e.g.*, at least about 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, up to about 25%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₄H₅₆O₁₄);

iii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₆H₆₀O₁₄);

iv) at least about 15%, *e.g.*, at least about 20%, 25%, 30%, 35%, 40%, up to about 45%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂);

v) at least about 35%, *e.g.*, at least about 36%, 37%, 38%, 39%, 40%, up to about 45% of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂); and/or

vi) at least about 0.5%, *e.g.*, at least about 1%, 2%, 3%, 4% up to about 5%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 3 acetylations (Molecular Formula: C₃₀H₅₂O₁₂).

30 **[0036]** In varying embodiments, the composition comprises a polyol lipid (*e.g.* PEFA) comprising:

i) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, up to about 20%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₂H₅₄O₁₃);

ii) at least about 45%, *e.g.*, at least about 46%, 47%, 48%, 49%, up to about 50% of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂); and/or

iii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, up to about 12% of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂).

10 [0037] In a further aspect, provided is a yeast cell of a yeast strain selected from the group consisting of:

a) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain NRRL Y-67018 (also deposited as UCDFST 04-877),

b) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain NRRL Y-67017 (also deposited as UCDFST 05-775),

c) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 68-916.1,

d) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 67-458,

e) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 05-736,

20 f) *Rhodotorula diobovata* (syn. *Rhodosporidium diobovatum*) strain UCDFST 04-830,

g) *Rhodotorula diobovata* (syn. *Rhodosporidium diobovatum*) strain NRRL Y-67015 (also deposited as UCDFST 08-225),

h) *Rhodotorula kratochvilovae* (syn. *Rhodosporidium kratochvilovae*) strain NRRL Y-67016 (also deposited as UCDFST 05-632),

i) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain NRRL Y-67012 (also deposited as UCDFST 09-163),

j) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain UCDFST 81-492,

30 k) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain UCDFST 82-646.2,

l) *Rhodotorula aff. paludigena* (syn. *Rhodosporidium aff. paludigenum*) strain NRRL Y-67009 (also deposited as UCDFST 81-84), and

m) *Rhodotorula dairenensis* strain NRRL Y-67011 (also deposited as UCDFST 68-257). In another aspect, provided is a yeast cell of a yeast strain selected from the group consisting of: a) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 68-916.1; b) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 67-458; c) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 05-736; d) *Rhodotorula diobovata* (syn. *Rhodosporidium diobovatum*) strain UCDFST 04-830; e) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain UCDFST 81-492; and f) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain UCDFST 82-646.2.

- 10 [0038] Further provided is a population of yeast cells comprising one or more yeast strains as described above and herein. Further provided is a yeast culture comprising one or more yeast strains as described above and herein.

DEFINITIONS

- [0039] The term “population of yeast cells” refers to two or more yeast cells.
- 15 [0040] The term “hydrophobic carbon source” refers to an organic compound that is insoluble in water or has a solubility in water of less than 1 g/L.
- [0041] The term “hydrophilic carbon source” refers to an organic compound that is soluble in water at concentrations greater than 1 g/L.

BRIEF DESCRIPTION OF THE DRAWINGS

- 20 [0042] Figures 1A-E depicts structure of the five known yeast secreted GL biosurfactants: (A) sophorolipids, (B) cellobiose lipids, (C) mannosylerythritol lipids, (D) polyol esters of fatty acids and (E) liamocins.
- [0043] Figures 2A-C illustrate (A) Overlay of RPLC-ESI(+)-MS extracted ion chromatograms of extracellular lipids isolated from *Rhodotorula paludigena* UCDFST 81-492. For masses and peak annotations see Table 4; remaining unannotated lipids in this figure were either not detected or at low signal intensity. (B) MS/MS spectrum acquired at a collision energy of 20 eV and predicted fragment structures corresponding to (C) acetylated 3-hydroxypalmitic acid condensed with completely acetylated D-arabitol (C₃₁H₅₂O₁₂; peak 12 in Fig. 2A).
- 25
- 30 [0044] Figure 3 illustrates unsupervised hierarchical clustering and heat map of yeast strains and PEFA species. The row z-score represents the deviation from the mean by

standard deviation unit; red color indicates high percentage of PEFA; blue color indicates low percentage of PEFA. Yeast strain ID numbers are from the Phaff Yeast Culture Collection, University of California, Davis (color figure available online at <https://link.springer.com/article/10.1007%2Fs10295-017-1919-y>).

5 **[0045]** Figure 4 illustrates General workflow of PEFA analysis. (FA, fatty acid; FAME, fatty acid methyl ester; GC-EIMS, gas chromatography–electron ionization mass spectrometry; HILIC-ESI(–)MS, hydrophilic interaction chromatography–negative electrospray ionization mass spectrometry; HMDB, Human Metabolome Database; LLE, liquid–liquid extraction; MTPA, (R)-(–)- α -methoxy- α -trifluoromethylphenylacetyl
10 derivatives; RPLC-ESI(+)MS/MS, reversed-phase liquid chromatography–positive electrospray ionization–tandem mass spectrometry; TFA, trifluoroacetyl derivatives; TMS, trimethylsilyl derivatives).

15 **[0046]** Figure 5 illustrates an overlay of RPLC-ESI(+)MS extracted ion chromatograms of extracellular lipids isolated from *Rhodotorula babjevae* UCDFST 04-877. For masses and peak annotations see Table 5.

[0047] Figures 6A-B illustrates a separation of PEFA extracts after acidic hydrolysis using HILIC-ESI(–)MS with extracted ion chromatograms of (A) 3-hydroxy fatty acids and (B) C5- and C6-sugar alcohols. Both classes of compounds were detected as deprotonated molecules $[M - H]^-$.

20 **[0048]** Figures 7A-D illustrate a GC-MS chromatogram of 3-hydroxy fatty acids detected in PEFA extracts after acidic hydrolysis. (A) Derivatized 3-hydroxy fatty acids detected as methyl esters (m/z 103). (B) EI spectrum of methyl palmitate with highlighted specific fragment m/z 103. (C) Derivatized 3-hydroxy fatty acids detected as 3-trimethylsiloxy methyl esters (m/z 175). (D) 3-[(Trimethylsiloxy)]methyl palmitate with
25 highlighted specific fragment m/z 175.

[0049] Figure 8A-D illustrate GC-MS chromatograms (m/z 217) of polyols detected in PEFA extracts after acidic hydrolysis and a series of standards: (A) xylitol, arabitol (mixture of D-/L- forms), ribitol standards; (B) detected arabitol in PEFA hydrolysate; (C) mannitol (mixture of D-/L- forms), dulcitol, glucitol standards; (D) detected mannitol in
30 PEFA hydrolysate.

[0050] Figures 9A-B illustrate enantioselective analysis of polyols using GC-EIMS. Extracted ion chromatograms (m/z 69) of (A) a standard mixture of D-/L-arabitol and D-/L-

mannitol and (B) detected D-arabitol and D-mannitol in hydrolyzed PEFA. Compounds were detected as TFA derivatives.

[0051] Figures 10A-C illustrate enantioselective analysis of 3-hydroxy fatty acids using GC-EIMS. Extracted ion chromatograms (m/z 189) of (A) 3-hydroxy C14:0, (B) 3-hydroxy C16:0, and (C) 3-hydroxy C18:0. Compounds were detected as MTPA-O-fatty acid methyl esters.

[0052] Figures 11A-B illustrate predicted fragment structures of polyol esters of fatty acids based on MS/MS spectra acquired in ESI(+): (A) acetylated 3-hydroxypalmitic acid condensed with completely acetylated arabitol (C₃₁H₅₂O₁₂; peak 5 in Figure 5); (B) acetylated 3-hydroxystearic acid condensed with completely acetylated arabitol (C₃₃H₅₆O₁₂; peak 10 in Figure 5).

[0053] Figure 12A-B illustrates predicted fragment structures of polyol esters of fatty acids based on MS/MS spectra acquired in ESI(+): (A) acetylated 3-hydroxypalmitic acid condensed with partially acetylated mannitol (C₃₂H₅₄O₁₃; peak 4 in Figure 5); (B) acetylated 3-hydroxystearic acid condensed with partially acetylated mannitol (C₃₄H₅₈O₁₃; peak 9 in Figure 5).

[0054] Figures 13A-B illustrate a general structure of polyol esters of fatty acids with (A) arabitol and (B) mannitol as a hydrophilic moiety secreted by *Rhodotorula babjevae* UCDFST 04-877. The value for n can vary from 8 to 12. R can be either hydrogen –H or an acetyl group –COCH₃.

[0055] Figures 14A-I illustrate the data obtained during Runs 1-9 of benchtop bioreactor cultivation of yeast strains *Rhodotorula babjevae* UCDFST 04-877, *Rhodotorula* aff. *paludigena* UCDFST 81-84, using either glucose or sucrose as main carbon sources, dosed in different amounts and fashions. The end point results and sugar dosing are given in Table 5.

[0056] Figure 15 illustrates a microscopic visualization using Brightfield mode and fluorescence mode at 100 X magnification of the water in oil emulsion formed when 1 mL of octane is mixed with 1mL of water and 5 mg of the mixture of PEFA produced by strain *Rhodotorula* aff. *paludigena* UCDFST 81-84, and mixed for 2 minutes using S8220 Deluxe mixer (EVANSTON, Illinois, 60201) in the mode of “FAST” at room temperature.

DETAILED DESCRIPTION

1. Introduction

[0057] Methods, yeast cultures and polyol lipid compositions are described herein, based in part on the discovery of yeast species that convert hydrophilic carbon sources (*e.g.*,
5 simple sugars) into lipids, and then secrete them into the medium in a form that can be harvested without solvent extraction—a breakthrough that addresses obstacles of harvesting and extraction.

[0058] The methods, yeast cultures and polyol lipid compositions described herein enable development of yeast-based processes to convert lignocellulosic hydrolysates into a
10 suite of products, including easily harvested lipids, *e.g.*, for use in the sustainable production of biodiesel and other oleochemicals.

[0059] The methods, yeast cultures and polyol lipid compositions described herein overcome the disadvantages of the prior art in that polyol lipids are produced by these yeasts in commercially relevant quantities without provision of hydrophobic substrate or
15 substrates. The methods, yeast cultures and polyol lipid compositions described herein entail the use of yeasts in phylum Basidiomycota, class Microbotryomycetes, order Sporidiobolales, genera *Rhodotorula*, *Rhodospiridiobolus* and *Sporobolomyces* for conversion of glucose or other carbohydrates to polyol lipids Polyol lipids are produced by these yeasts in commercially relevant quantities without provision of hydrophobic substrate
20 or substrates.

[0060] Yeasts have been used as model organisms for decades. The first eukaryotic genome to be sequenced was that of the yeast *Saccharomyces cerevisiae* [26], in part due to its long history of use as a model organism for genetics research, and for food, beverage and biofuel production. *Starmerella bombicola* is a model yeast for conversion of carbohydrate
25 plus fatty acids or vegetable oil to secreted glycolipids (*e.g.*, sophorolipids) [18, 27, 28].

[0061] We identified basidiomycetous yeast strains that are able to secrete polyol lipids, particularly, polyol esters of fatty acids (PEFAs). In addition to producing between 0.7 and 12 g/L intracellular lipids, primarily triacylglycerols (TAGs), these yeasts are able to secrete up to an estimated 25 - 27 g/L crude polyol lipids.

30 [0062] Basidiomycetous yeasts in the order Sporidiobolales are able to directly convert less expensive simple sugars into significant quantities of secreted polyol lipids (*e.g.*, polyol esters of fatty acids (PEFAs)), opening up opportunities to use these polyol

lipids for a broader range of consumer and industrial surfactant applications. The present yeast cultures, methods and polyol lipid compositions facilitate the development of an economically favorable industrial process for biosurfactant production.

[0063] The advantages of these basidiomycetous yeasts for production of polyol lipids include without limitation:

• Tulloch, *et al*, partially determined glycolipid structures produced in low yields (between 1 – 2 g /L) by basidiomycetous species. *See* Tulloch, *et al*, 1964, *supra*. Tulloch 1964 determined the components of the secreted glycolipids: the polyols arabitol and mannitol plus trace amounts of xylitol, hydroxy fatty acid and acetate. But Tulloch 1964 did not determine how these pieces were assembled or the mixture of PEFA molecules, as provided herein. Moreover, the basidiomycetous yeast and yeast cultures described herein produce and secrete significant quantities of polyol esters of fatty acids, as much as to 25 – 27 g/L, or more, when fed only hydrophilic carbon sources (*e.g.*, simple sugars such as glucose, xylose or sucrose).

• The polyol lipids are secreted, so cell harvesting and lysis are not necessary.

• The polyol lipids are heavier than water and yeast cells, so inexpensive gravimetric techniques can be used to harvest them.

• The oils are extracellular and mostly insoluble in water, so organic solvent extraction is not required. This greatly reduces both facility construction and processing costs, decreases environmental impact and increases operational safety.

• Because the cells producing the polyol esters of fatty acids need not be harvested or lysed, a continuous or fed-batch culture system further reduces costs.

2. Methods of Producing Polyol Lipids

[0064] Generally, the methods entail culturing a population of basidiomycetous yeast cells in a yeast culture containing relatively high concentrations of a hydrophilic (*e.g.*, non-hydrophobic) carbon source and low concentrations of nitrogen, and with or without the presence of a hydrophobic carbon source.

a. Yeast Cultures

[0065] Provided are yeast cultures for producing one or more polyol lipids (*e.g.*, polyol esters of fatty acids (PEFAs)) according to the methods described herein. In varying embodiments, the yeast culture comprises a population of basidiomycetous yeast cells, a hydrophilic (*e.g.*, non-hydrophobic) carbon source, and at least about 1 g/L total polyol lipid, *e.g.*, at least about 2 g/L, 3 g/L, 4 g/L, 5 g/L, 6 g/L, 7 g/L, 8 g/L, 9 g/L, 10 g/L, 11 g/L, 12 g/L, 13 g/L, 14 g/L, 15 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L, 40 g/L, 45 g/L, 50 g/L, 100 g/L, 150 g/L, 200 g/L, 250 g/L, 300 g/L, 350 g/L, 400 g/L total polyol lipid, *e.g.*, up to about 450 g/L of total polyol lipid. The one or more polyol lipids are extracellularly secreted by the yeast cells into the media and can be harvested without cell lysis and extracted without use of organic solvent.

[0066] In varying embodiments, the culture does not comprise one or more hydrophobic carbon sources. In varying embodiments, the culture comprises one or more hydrophobic carbon sources. In varying embodiments, the one or more hydrophobic carbon sources are selected from the group consisting of oils, hydrocarbons, unsaturated hydrocarbons, fatty acids, fatty esters including glycerides and mixtures thereof, alcohols, diols, sterols, waste streams, hydrolysates, and mixtures thereof. In varying embodiments, the one or more hydrophobic carbon sources include an oil, *e.g.*, a mineral oil, animal oil, a plant oil, a microbial oil (*e.g.*, an algal oil, a yeast oil, a bacterial oil), a fish oil, a vegetable oil or a nutseed oil, *e.g.*, canola oil, rapeseed oil, olive oil, almond oil, walnut oil, peanut oil, coconut oil, or others, or mixtures thereof. In varying embodiments, the one or more hydrophobic carbon sources comprise at least 8 carbon atoms, *e.g.*, an alkane, fatty acid, fatty ester, alcohol, diol comprising 8 or more carbon atoms, *e.g.*, from 8 to 24 carbon atoms. In varying embodiments, the one or more hydrophobic carbon sources are selected from a primary or secondary alcohol or diol having from about 4 to about 24 carbon atoms, *e.g.*, having 8 or more carbon atoms, *e.g.*, from 8 to 24 carbon atoms or from 8 to 14 carbon atoms. In varying embodiments, the one or more hydrophobic carbon sources are selected from an aliphatic linear or branched hydrocarbon, which may contain one or more substituents selected from the group consisting of -OR, -COOH, and an ester with a carbon chain length of about 4 to about 24 carbon atoms, *e.g.*, having 8 or more carbon atoms, *e.g.*, from 8 to 24 carbon atoms or from 8 to 14 carbon atoms.

[0067] In varying embodiments, the culture comprises less than about 2% (w/v) nitrogen, *e.g.*, in the range of about 0.005% (w/v) to about 2% (w/v) nitrogen. In varying

embodiments, the nitrogen source is selected from the group consisting of ammonia, ammonium salt, nitrate, nitrite, nucleotides, nucleosides, proteins, peptides, amino acids, urea and its derivatives, and mixtures thereof. In varying embodiments, the culture comprises about 0.05% (w/v) ammonium chloride.

- 5 **[0068]** In varying embodiments, the culture comprises one or more hydrophilic carbon sources at a concentration of at least about 0.2% (w/v), *e.g.*, in the range of about 0.2% (w/v) to about 70% (w/v), *e.g.*, in the range of about 0.2% (w/v) to about 10%, 20%, 30%, 40%, 50% or 60% (w/v). In varying embodiments, the culture comprises a carbon to nitrogen ratio of about 5:1 to about 400:1, *e.g.*, from about 10:1 to about 200:1, from about 10 20:1 to about 100:1, *e.g.*, from about 25:1 to about 75:1, *e.g.*, from about 30:1 to about 70:1, *e.g.*, from about 40:1 to about 60:1, *e.g.*, from about 30:1 to about 40:1, *e.g.*, using a nitrogen source that can be consumed or utilized by the yeast cells.

- [0069]** In varying embodiments, the hydrophilic carbon source is selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, sugar alcohols, polyols, polyol lipids (*e.g.*, polyol esters of fatty acids (PEFAs)), organic acids, esters, 15 aldehydes, ketones, alcohols, waste streams, plant materials, lignocellulosic hydrolysates, industrial co-products and mixtures thereof. In varying embodiments, the hydrophilic carbon source is a monosaccharide, a disaccharide, an oligosaccharide or a polysaccharide and comprises one or more sugars selected from the group consisting of glucose, sucrose, 20 xylose, galactose, rhamnose, arabinose, mannose, cellobiose, galacturonic acid, lactose, sophorose, glycerol, and mixtures thereof. In varying embodiments, the amount of air is increased by culturing the yeast in containers designed to create turbulent flow (*e.g.* baffled flasks). In varying embodiments, a device capable of simultaneously allowing gas exchange with the outer environment (*e.g.* a foam stopper in case of using Erlenmeyer 25 baffled flasks) and maintaining the culture sterile is used.

- [0070]** In varying embodiments, the population of basidiomycetous yeast cells in the yeast culture comprises basidiomycetous yeast cells within the taxonomic class Microbotryomycetes. In varying embodiments, the population of basidiomycetous yeast cells comprises cells within the taxonomic order Sporidiobolales. In varying embodiments, 30 the basidiomycetous yeast cells are within the *Rhodotorula glutinis* clade. In varying embodiments, the population of basidiomycetous yeast cells is from genera classified within the taxonomic order Sporidiobolales selected from the group consisting of *Rhodosporidium sp.*, *Sporidiobolus*, *Rhodotorula*, and *Sporobolomyces*. In varying embodiments, the

population of basidiomycetous yeast cells comprises *Rhodospiridium* cells. In varying embodiments, the *Rhodospiridium* cells are *Rhodotorula babjevae* (syn. *Rhodospiridium babjevae*) cells. In varying embodiments, the population of basidiomycetous yeast cells comprises one or more species selected from the group consisting of *Rhodotorula babjevae* (syn. *Rhodospiridium babjevae*), *Rhodotorula diobovata* (syn. *Rhodospiridium diobovatum*), *Rhodotorula kratochvilovae* (syn. *Rhodospiridium kratochvilovae*), *Rhodotorula paludigena* (syn. *Rhodospiridium paludigenum*), *Rhodospiridium* aff. *paludigenum*, and *Rhodotorula dairenensis*. In varying embodiments, the cells do not comprise *Pseudohyphozyma bogoriensis* (syn. *Rhodotorula bogoriensis*), a species in class Microbotryomycetes but not in order Sporidiobolales [29].

[0071] In varying embodiments, the yeast culture comprises hydrophilic carbon source (e.g., a mono- or di- saccharide, e.g., glucose and/or sucrose) (30 g/L), yeast extract (1.5 g/L), ammonium chloride (0.5 g/L), potassium phosphate monobasic (7.0 g/L), sodium phosphate dibasic (5.0 g/L), magnesium sulfate hexahydrate (1.5 g/L) and micronutrient solution comprised of various salts (10 mL/L). In varying embodiments, the yeast culture comprises an iron salt. Illustrative iron salts that find use include without limitation iron chloride (II) & (III), iron sulfate (II) & (III), iron nitrate (II) & (III), iron phosphate (II) & (III), fumarate, iron edetate, iron citrate, iron malate, iron oxalate, iron tartrate, iron succinate, iron acetate, in their anhydrous or their hydrated states, and organometallic complexes thereof. In varying embodiments, the culture comprises an iron salt in a concentration in the range of about 0.001 mg/L to about 10 g/L, e.g., from about 1 mg/L to about 0.5 g/L, e.g., from about 0.01 g/L to about 0.5 g/L. In varying embodiments, the yeast culture is supplemented with extra hydrophilic carbon source (e.g., a mono- or di- saccharide, e.g., glucose and/or sucrose) to a concentration of at least about 50 g/L, 75 g/L, 100 g/L, 125 g/L, 150 g/L, 175 g/L, 200 g/L, 225 g/L, 250 g/L, 275 g/L or 300 g/L.

b. Methods of Producing Polyol Lipids

[0072] Provided are methods of producing one or more polyol lipids (e.g., polyol esters of fatty acids (PEFAs)), comprising culturing a population of basidiomycetous yeast cells in a yeast cell culture comprising a hydrophilic carbon source, wherein the culture does not comprise one or more hydrophobic carbon sources, whereby the basidiomycetous yeast cells produce and secrete one or more polyol lipids. As discussed above, the yeast cells are grown in a yeast cell culture media having a high carbon to nitrogen (C/N) ratio. In varying embodiments, the hydrophilic carbon source (e.g., a mono- or di- saccharide, e.g., glucose

and/or sucrose) is added after the population of basidiomycetous yeast cells reaches stationary phase. Embodiments of the yeast culture are as described above. When culturing yeast cells in the herein described yeast cultures and according to the methods described herein, at least about at least about 1 g/L polyol lipid, *e.g.*, at least about 2 g/L, 3 g/L, 4 g/L, 5 g/L, 6 g/L, 7 g/L, 8 g/L, 9 g/L, 10 g/L, 11 g/L, 12 g/L, 13 g/L, 14 g/L, 15 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L, 40 g/L, 45 g/L, 50 g/L, 100 g/L, 150 g/L, 200 g/L, 250 g/L, 300 g/L, 350 g/L, 400 g/L polyol lipid, *e.g.*, up to about 450 g/L of polyol lipid, are secreted into the yeast culture medium.

[0073] The population of basidiomycetous yeast cells is cultured in the yeast cell culture medium under conditions sufficient for yeast cell growth and polyol lipid production and secretion. The embodiments of illustrative yeast cultures are as described above and herein. For example, in certain embodiments, the yeast cells are cultured at a temperature of about 24°C with continuous agitation (*e.g.*, in shake flasks, *e.g.*, baffled shake flasks, *e.g.*, at about 200 rpm) for time period sufficient for yeast cell growth and polyol lipid production and secretion, *e.g.*, for about 6 hours to about 168 hours post inoculation with yeast cells, *e.g.*, about 6, 12, 24, 48, 72, 96, 120, 144, 168 hours post inoculation with yeast cells. The yeast cells are further provided with a sufficient amount of a hydrophilic carbon source, *e.g.*, as described above. The yeast cells are supplied with sufficient levels of oxygen to metabolize the hydrophilic carbon source into a polyol lipid containing a hydroxylated fatty acid as part of its structure, *e.g.*, in baffled flasks stoppered with porous stoppers or membranes designed to allow passage of gas without compromising the sterility of the system, or in bioreactors capable of controlling the amount of oxygen (*e.g.* in the form of air or mixtures of air and oxygen) through regulation of gas supply and/or agitation. In varying embodiments, the yeast cells are grown in a buffered media that requires no pH adjustment. For example, in certain embodiments the yeast cells are cultured at pH values of about 6.5. In varying embodiments, total polyol lipids are measured and determined after 1, 2, 3, 4, 5, 6, 7 or 8 days growth.

[0074] During and after growth in shake flasks, a hydrophobic liquid settles to the bottom of the growth flask or bioreactor. In varying embodiments, the one or more polyol lipids secreted by the yeast cells has density in the range of about 1.00 g/mL to about 1.10 g/mL, *e.g.*, from about 1.08 g/mL to about 1.09 g/mL. The secreted extracellular polyol lipids have significantly lower processing costs because the costs of lysing of cells and separation of oil from cell debris can be eliminated or greatly reduced.

[0075] In varying embodiments, the yeast populations are cultured under conditions that promote aeration of the cultures and oxygenation of the cells. Such strategies can include culturing lower culture volumes in larger flasks, *e.g.*, employing a culture volume to container volume ratio of 1:3 or greater, *e.g.*, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10 or greater, 5 employing baffled flasks; covered the culture flask in such a manner to allow air/oxygen exchange, *e.g.*, with a foam stopper; and/or exposing the yeast cultures to a super-oxygenated atmosphere. In some embodiments, the yeast culture comprises dissolved oxygen at a concentration of a least about 10%, *e.g.*, at least about 15%, 20%, 25%, 30%, 35%, 40%, 45%, and up to 60%, 70%, 80%, 90%, 100% dissolved oxygen, *e.g.*, from about 10 15% to about 80%, *e.g.*, from about 20% to about 60% dissolved oxygen. When cultured in a bioreactor, the yeast culture can fill as much as 90% of the container volume, and the oxygen can be bubbled into the culture.

[0076] In varying embodiments, the methods further comprise the step of isolating and/or purifying the one or more polyol lipids from the yeast culture. Because the polyol 15 lipids are extracellularly secreted from the yeast cells, and the yeast cell culture does not contain a hydrophobic carbon source, the one or more polyol lipids can be isolated and/or purified from the yeast cell culture without cell lysis or extraction requiring an organic solvent. The secreted polyol lipid compounds are denser than water and can be recovered inexpensively using separation methods based on density, including without limitation 20 centrifugation, continuous decanting, or passive settling. The extracellular polyol lipids can be washed from the rest of the cells and culture components by subsequent washing steps using water, yielding a product containing moisture to up to 8%. The moisture content does not affect the extracellular polyol lipid performance or its functionality. In varying embodiments, the moistened extracellular polyol lipid can be further dried using known 25 techniques, *e.g.*, tumble drying, plate drying, flash drying, freeze drying and other known techniques. In varying embodiments, both wet and dry extracellular lipids can be further fractionated to obtain pure molecular species by thin layer chromatography, HPLC, preparative chromatography, and other known techniques. Accordingly, in varying embodiments, the basidiomycetous yeast cells can be cultured under batch, fed-batch or 30 continuous feed processing conditions. In varying embodiments, using batch, fed-batch or continuous feed cultivation conditions in which the culture is repeatedly or continuously fed allowing the cells to continue producing product, up to several hundred grams of substrate *e.g.*, 100 g, 150 g, 200 g, 250 g, 300 g, 350 g, 400 g, 450 g or 500 g) per liter can be

obtained. In varying embodiments, polyol lipid yields of about 0.5 g/L to about 30 g/L can be obtained from yeast cultures comprising in the range of about 10 g/L to about 250 g/L glucose. In varying embodiments, polyol lipid yields of about 14 g/L to about 17 g/L can be obtained from yeast cultures comprising about 50 g/L glucose.

- 5 [0077] Embodiments of the one or more polyol lipids and polyol lipid composition profiles produced according to the methods are as described below.

3. Polyol Lipid and Polyol Lipid Profiles Produced According to the Methods

- [0078] Polyol lipid biosurfactants include polyol esters of fatty acids (PEFAs) and liamocins. In varying embodiments, the one or more polyol lipids in the yeast cultures,
10 produced according to the methods, and/or in the polyol lipid compositions isolated and/or purified from the yeast cultures comprise one or more polyol esters of fatty acids (PEFAs). In varying embodiments, the one or more polyol lipids are free of any hydrophobic carbon source.

- [0079] Polyol esters of fatty acids are amphiphilic molecules comprising a sugar
15 alcohol, *e.g.*, a D-mannitol and/or a D-arabitol, esterified to the carboxyl end of a 3-hydroxy fatty acyl moiety, which may or may not be acetylated. The non-esterified hydroxy groups of the sugar alcohol may or may not be acetylated as well (*See*, Figure 1). In varying embodiments, the one or more polyol lipids produced are a mixture of similar compounds containing (*R*)-3-hydroxy fatty acyl moieties with varying chain lengths, in the range of
20 about 10 to 24 carbons, preferably in the range between 12 to 20 carbons. In varying embodiments, the (*R*)-3-hydroxy fatty acyl moieties can present varying degrees of unsaturation in the range of about 0 to 6, *e.g.*, in the range between 2 to 5. In varying embodiments, the non-esterified hydroxy groups of the sugar alcohol can be esterified to acetyl groups. In varying embodiments the sugar alcohol can be fully acetylated. In varying
25 embodiments, the sugar alcohol can be non-acetylated. In varying embodiments, acetylations can range between these two states.

- [0080] In varying embodiments, the one or more polyol lipids in the yeast cultures, produced according to the methods, and/or in the polyol lipid compositions isolated and/or purified from the yeast cultures comprise one or more polyol esters of fatty acid (PEFAs)
30 listed in Table 4, below. In varying embodiments, the polyol lipid sugar alcohol moiety comprises a 5 carbon polyol, which can be D-arabitol or L-arabitol. In varying embodiments, the polyol lipid sugar alcohol moiety comprises a 6 carbon polyol, which can

be D-mannitol or L-mannitol. In varying embodiments, the one or more polyol lipids comprise a fatty acid comprising from 10 to 24 carbon atoms in length. In some embodiments, the polyol lipid sugar alcohol moiety is attached or bound carboxyl end of the fatty acid. In some embodiments, the fatty acid is hydroxylated at position Δ -3 from the carboxyl end. In some embodiments, the hydroxyl group in position Δ -3 may or may not be acetylated.

[0081] In varying embodiments, the one or more polyol lipids in the yeast cultures, produced according to the methods, and/or in the polyol lipid compositions isolated and/or purified from the yeast cultures comprise a polyol lipid (*e.g.*, PEFA) profile comprising:

10 i) at least about 20%, *e.g.*, at least about 25%, 30%, 35%, 40%, up to about 45% of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂);

15 ii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, 20%, up to about 25%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂); and/or

iii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, up to about 20% of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₄H₅₈O₁₃).

[0082] In varying embodiments, the one or more polyol lipids in the yeast cultures, produced according to the methods, and/or in the polyol lipid compositions isolated and/or purified from the yeast cultures comprise a polyol lipid (*e.g.*, PEFA) profile comprising at least about 25%, *e.g.*, at least about 30%, 35%, 40%, 45%, up to about 50% of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂).

25 **[0083]** In varying embodiments, the one or more polyol lipids in the yeast cultures, produced according to the methods, and/or in the polyol lipid compositions isolated and/or purified from the yeast cultures comprise a polyol lipid (*e.g.*, PEFA) profile comprising:

30 i) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, up to about 18%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 3 acetylations (Molecular Formula: C₃₂H₅₆O₁₂);

ii) at least about 1%, *e.g.*, at least about 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, up to about 20%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₂H₅₄O₁₃);

iii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, 20%,
 5 up to about 25%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₄H₅₈O₁₃);

iv) at least about 15%, *e.g.*, at least about 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, up to about 50% of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂); and/or

10 v) at least about 10%, *e.g.*, at least about 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 25%, 30%, up to about 35% of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂).

[0084] In varying embodiments, the one or more polyol lipids in the yeast cultures,
 15 produced according to the methods, and/or in the polyol lipid compositions isolated and/or purified from the yeast cultures comprise a polyol lipid (*e.g.* PEFA) profile comprising:

vi) at least about 2%, *e.g.*, at least about 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₄H₅₆O₁₄); and/or

20 vii) at least about 1%, *e.g.*, at least about 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol (Molecular Formula: C₃₆H₆₀O₁₄).

[0085] In varying embodiments, the one or more polyol lipids in the yeast cultures,
 25 produced according to the methods, and/or in the polyol lipid compositions isolated and/or purified from the yeast cultures comprise a polyol lipid (*e.g.* PEFA) profile comprising:

i) at least about 1%, *e.g.*, at least about 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₄H₅₈O₁₃);

30 ii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, up to about 45%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₄H₅₆O₁₄);

iii) at least about 10%, *e.g.*, at least about 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, up to about 25%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₆H₆₀O₁₄);

iv) at least about 15%, *e.g.*, at least about 20%, 25%, 30%, 35%, 40%, up to about 45%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂); and/or

v) at least about 15%, *e.g.*, at least about 20%, 25%, 30%, 35%, 40%, up to about 45% of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂).

10 **[0086]** In varying embodiments, the one or more polyol lipids in the yeast cultures, produced according to the methods, and/or in the polyol lipid compositions isolated and/or purified from the yeast cultures comprise a polyol lipid (*e.g.* PEFA) profile comprising:

i) at least about 10%, *e.g.*, at least about 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, up to about 25%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₄H₅₈O₁₃);

ii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, up to about 30%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₄H₅₆O₁₄);

iii) at least about 10%, *e.g.*, at least about 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, up to about 25%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₆H₆₀O₁₄);

iv) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂);

v) at least about 15%, *e.g.*, at least about 20%, 25%, 30%, 35%, 40%, up to about 45%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂); and/or

vi) at least about 0.5%, *e.g.*, at least about 1%, 2%, 3%, 4%, 5% up to about 6% of an acetylated C14:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₂₉H₄₈O₁₂).

[0087] In varying embodiments, the one or more polyol lipids in the yeast cultures, produced according to the methods, and/or in the polyol lipid compositions isolated and/or purified from the yeast cultures comprise a polyol lipid (*e.g.* PEFA) profile comprising:

i) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₄H₅₈O₁₃);

5 ii) at least about 10%, *e.g.*, at least about 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, up to about 25%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₄H₅₆O₁₄);

iii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, up to about 12%, of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 5 acetylations (Molecular Formula: C₃₆H₆₀O₁₄);

10 iv) at least about 15%, *e.g.*, at least about 20%, 25%, 30%, 35%, 40%, up to about 45%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂);

v) at least about 35%, *e.g.*, at least about 36%, 37%, 38%, 39%, 40%, up to about 45% of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol
15 comprising 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂); and/or

vi) at least about 0.5%, *e.g.*, at least about 1%, 2%, 3%, 4% up to about 5%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 3 acetylations (Molecular Formula: C₃₀H₅₂O₁₂).

[0088] In varying embodiments, the one or more polyol lipids in the yeast cultures,
20 produced according to the methods, and/or in the polyol lipid compositions isolated and/or purified from the yeast cultures comprise a polyol lipid (*e.g.* PEFA) profile comprising:

i) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, 15%, up to about 20%, of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-mannitol comprising 4 acetylations (Molecular Formula: C₃₂H₅₄O₁₃);

25 ii) at least about 45%, *e.g.*, at least about 46%, 47%, 48%, 49%, up to about 50% of an acetylated C16:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising 4 acetylations (Molecular Formula: C₃₁H₅₂O₁₂); and/or

iii) at least about 5%, *e.g.*, at least about 6%, 7%, 8%, 9%, 10%, up to about 12% of an acetylated C18:0 (*R*)-3-hydroxy fatty acid esterified to D-arabitol comprising
30 4 acetylations (Molecular Formula: C₃₃H₅₆O₁₂).

[0089] In varying embodiments, the one or more polyol esters of fatty acid (PEFAs) are selected from those listed in Table 4. In varying embodiments, the population of yeast

cells in the yeast cell culture produces one or more polyol lipids having a polyol lipid (e.g. PEFA) profile according to the polyol lipid (e.g. PEFA) profiles provided in Tables 4A-C.

4. Compositions Comprising Polyol Lipids

[0090] Further provided are compositions comprising the one or more polyol lipids and/or having a polyol lipid (e.g. PEFA) profile as described above and herein. Surfactants (both petroleum and bio-based) are used in a broad variety of agricultural, nutritional, cosmetic, veterinary, therapeutic, and industrial applications due to their many activities. Activities include cleansers, detergents, wetting agents, antifoam agents, emulsifiers, dispersants (e.g., for cleanup of oil including spilled petroleum), and humectants. They are used in household and industrial cleansers and detergents, textiles, agrochemicals, photo chemicals, petroleum extraction, construction materials, adhesives, lubricants, mining, and the pulp and paper industry. Surfactants used in household and laundry detergents end up in the environment. The poor performance of petroleum-based surfactants with regard to sustainability, bio-accumulation, eco-toxicity and/or biodegradability is pushing development of bio-based replacements, or biosurfactants, which can be produced microbially from renewable feedstocks, have lower toxicity, and are biodegradable.

[0091] Major biosurfactants on the market include surfactin and emulsin, and a variety of glycolipids which are the major class of biosurfactants. Polyol lipids, including polyol ester of fatty acids (PEFAs, have potential to be renewable, low-toxicity, biodegradable alternatives to petroleum-based surfactants. Rhamnolipids are produced by a pathogenic bacterium, *Pseudomonas aeruginosa*. Sophorolipids, produced by non-pathogenic yeasts, are currently on the market as detergents, emulsifiers, wetting agents, dispersants and other activities. To our knowledge, at the time of filing of this application, polyol lipids are not commercially available.

[0092] Similar to sophorolipids and acetylated lactone forms of sophorolipids, the present polyol lipid compositions can find use as effective additives in shampoos, body washes, detergents, and cosmetic products. Certain polyol lipids may have anti-bacterial activity. The acidic forms can be effective ingredients in skin treatments and as moisturizing agents. Due to skin-friendly properties, the present polyol lipids can be used in cosmetics and pharmaceuticals. Glycolipid esters have proven to be excellent moisturizers for cosmetic uses (See, e.g., U.S. Patent No. 4,297,340). Additional uses include spermicides and virucides (Shah, *et al.*, *Antimicrob Agents Chemother.* (2005) 49(10):4093-100), septic shock antagonists, anticancer agents and protein

inducers/repressors in microbial systems (*See, e.g.*, Intl. Publ. No. WO 2007/073371 A1). Derivatives and modified polyol esters of fatty acids further have been shown to have antifungal activity (*See, e.g.*, Intl. Publication No. WO2011/127101 and U.S. Patent Publ. No. 2012/022241).

- 5 **[0093]** Accordingly, further provided are polyol lipid compositions, *e.g.*, comprising one or more of the polyol lipids described above and herein, and produced by the methods described herein. Also contemplated are derivatives of the polyol lipids described above and herein, and produced by the methods described herein, for example, derivatives of either the sugar alcohol or lipid moiety, including hydroxylated fatty acids, D-mannitol,
- 10 D-arabitol and their derivatives, etc. In varying embodiments, the polyol lipid composition is one or more of a cleanser, a detergent, a wetting agent, an antifoam agent, an emulsifier, a surfactant, an emollient, a dispersant, a humectant, an anti-bacterial agent, an anti-viral agent, an anti-fungal agent, a spermicide an insecticide, a lubricant, an adhesive, a crystal modifier, an instantizer, a viscosity modifier, a mixing/blending aid, and/or a release agent.
- 15 **[0094]** The polyol lipid compositions find use in numerous applications, including without limitation, household and industrial cleansers and detergents, textiles, agrochemicals such as control of fungal and insect pests, food processing such as cleaning agents for fresh and frozen fruits, vegetables, meats and processed foods, food processing such as rheologic modifiers in food applications including doughs, pastas and emulsions
- 20 such as mayonnaise, dressings, and syrups, food processing such as antiadherents, photo chemicals, petroleum extraction such as release agents for fracking, construction materials such as lubricants and demolding agents for brick, ceramic, cement and concrete, mining such as adjuvants in the coal industry, pulp and paper industry, cosmetics such as creams, foams, mousses, balms, ointments , personal care formulations such as shampoos, body
- 25 washes, conditioners, soaps, creams, skin treatments, and moisturizing agents, therapeutics such as ointments and creams, spermicides, anti-viral and anti-cancer agents, leather auxiliary agents, fuel oil emulsification for improved atomization, yielding a more complete combustion, bioremediation of contaminated soils, groundwater and surface water, dust suppression in mines and quarries, release agents for asphalt truck beds, facilitates castable
- 30 aqueous emulsions for the manufacture of explosives, ingredient in metal working fluids, and oleochemicals such as biodiesel, platform chemicals. Additional uses of the polyol lipid compositions produced by the present methods are described, *e.g.*, in U.S. Patent

Publication Nos. 2012/0022241 and 2013/0072414, hereby incorporated herein by reference for all purposes.

EXAMPLES

[0095] The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1

Discovery Of Synthesis And Secretion Of Polyol Esters Of Fatty Acids By Four Basidiomycetous Yeast Species In The Order Sporidiobolales

[0096] This Example is published as Garay, *et al.*, J Ind Microbiol Biotechnol. (2017) Mar 13 [PMID: 28289902], which is hereby incorporated herein by reference in its entirety for all purposes.

MATERIALS AND METHODS

[0097] *Chemicals and yeast strains.* All chemicals were of analytical grade, except glucose (catalog number S25295B, Fisher Science Education PA, USA) which was technical grade.

[0098] Sixty-five yeast strains listed in Table 2. They were revived from cryopreserved stocks in the Phaff Yeast Culture Collection, University of California Davis (<http://phaff-collection.ucdavis.edu>) by streaking onto potato dextrose agar and incubating at room temperature up to 7 days. It must be noted that the species names of many yeast strains used in this study, and recent publications [11, 37–41] have been updated due to recent taxonomic revisions [53]. The strain ID number is unchanged. For example, yeast strain UCDFST 04-877 was formerly called *Rhodospiridium babjevae* [11, 37–41], and is now called *Rhodotorula babjevae*. The former species name is listed as a synonym in Table 2.

TABLE 2
Description of the yeast strains used for the present study.

Strain species and Phaff collection ID	Synonym ^a	ID number in other collections	Source habitat	Genbank Accession numbers
<i>Cystobasidium minutum</i> UCDFST 68-280	<i>Rhodotorula minuta</i>	CBS 319, ATCC 10658, NRRL Y-1589	Air, Tokyo, Japan	KU609482, KU609540
<i>Hamamotaea singularis</i> UCDFST 60-79	<i>Sporobolomyces singularis</i>	CBS 5109	<i>Tsuga heterophylla</i> (western hemlock), Siletz river, OR, USA	KU609483
<i>Occultifur externus</i> UCDFST 68-934.2			Exudate of <i>Acer</i> sp., BC, Canada	KU609481, KU609539
<i>Rhodospiridiobolus</i> aff. <i>lusitaniae</i> UCDFST 06-583	<i>Rhodotorula</i> aff. <i>lusitaniae</i>		Olive fly, Davis, CA, USA	KU609443, KU609501
<i>Rhodospiridiobolus colostri</i> UCDFST 05-609	<i>Rhodotorula colostri</i>		Olive <i>Olea europaea</i> flower, Winters, CA, USA	KU609442, KU609500
<i>Rhodospiridiobolus</i> cf. <i>fluvialis</i> UCDFST 81-485.4	<i>Rhodospiridium</i> cf. <i>fluviale</i>		<i>Opuntia ficus-indica</i> , Tucson, AZ, USA	KU609434, KU609492
<i>Rhodospiridiobolus odoratus</i> UCDFST 04-870	<i>Sporobolomyces odoratus</i>		Olive fly, Davis, CA, USA	KU609474, KU609532
<i>Rhodospiridiobolus odoratus</i> UCDFST 09-1303	<i>Sporobolomyces odoratus</i>		Sunflower leaf, Davis, CA, USA	KU609473, KU609531
<i>Rhodospiridiobolus poonsookiae</i> UCDFST 10-441	<i>Sporobolomyces poonsookiae</i>	FORDACC 1642	Leaf litter, Sulawesi, Indonesia	KU609475, KU609533
<i>Rhodospiridiobolus ruineniae</i> UCDFST 10-1058	<i>Sporidiobolus ruineniae</i>	FORDACC 552	Leaf litter, Sulawesi, Indonesia	KU609462, KU609520
<i>Rhodospiridiobolus ruineniae</i> UCDFST 10-1109	<i>Sporidiobolus ruineniae</i>	FORDACC 540, InaCC 0476	Leaf surface, Sulawesi, Indonesia	KU609463, KU609521

TABLE 2
Description of the yeast strains used for the present study.

Strain species and Phaff collection ID	Synonym ^a	ID number in other collections	Source habitat	Genbank Accession numbers
<i>Rhodosporiobolus ruineniae</i> UCDFST 12-776	<i>Sporidiobolus ruineniae</i>	FORDACC 1885	Soil, Sulawesi, Indonesia	KU609460, KU609518
<i>Rhodosporiobolus ruineniae</i> UCDFST 67-67 ⁽¹⁾	<i>Sporidiobolus ruineniae</i>		Leaves of <i>Malpighia coccigera</i> . Bogor, Indonesia	KU609461, KU609519
<i>Rhodosporiobolus ruineniae</i> UCDFST 78-84	<i>Sporidiobolus ruineniae</i>		Plant material, Panama	KX771208 KX771223
<i>Rhodotorula araucariae</i> UCDFST 69-61 ⁽¹⁾		CBS 6031, ATCC 22078, NRRL Y-17376	<i>Araucaria araucana</i> tree in Chile	KU609441, KU609499
<i>Rhodotorula babjevae</i> UCDFST 04-830	<i>Rhodosporidium babjevae</i>		Male olive fly, Winters, CA, USA	KX771211, KX771226
<i>Rhodotorula babjevae</i> UCDFST 04-877	<i>Rhodosporidium babjevae</i>		Olive fly, Davis, CA, USA	KU609429, KU609487
<i>Rhodotorula babjevae</i> UCDFST 05-613	<i>Rhodosporidium babjevae</i>		Olive fly, Davis, CA, USA	KX771204, KX771219
<i>Rhodotorula babjevae</i> UCDFST 05-736	<i>Rhodosporidium babjevae</i>		Female olive fly, Davis, CA, USA	KX771209, KX771224
<i>Rhodotorula babjevae</i> UCDFST 05-775	<i>Rhodosporidium babjevae</i>		Dry sap of olive tree, Winters, CA, USA	KU609430, KU609488
<i>Rhodotorula babjevae</i> UCDFST 06-542	<i>Rhodosporidium babjevae</i>		Olive-fly infested olive, Winters, CA, USA	KX771212, KX771227
<i>Rhodotorula babjevae</i> UCDFST 67-102	<i>Rhodosporidium babjevae</i>		Sea water, Point Reyes, CA, USA	KX771203, KX771218

TABLE 2
Description of the yeast strains used for the present study.

Strain species and Phaff collection ID	Synonym ^a	ID number in other collections	Source habitat	Genbank Accession numbers
<i>Rhodotorula babjevae</i> UCDFST 67-436	<i>Rhodosporidium babjevae</i>		Exudate of <i>Pterocarya rhoifolia</i> , Kaida, Kiso, Japan	KX771205, KX771220
<i>Rhodotorula babjevae</i> UCDFST 67-458	<i>Rhodosporidium babjevae</i>		Exudate of <i>Betula ermani</i> , Mt. Fuji, Japan	KU609433, KU609491
<i>Rhodotorula babjevae</i> UCDFST 67-478	<i>Rhodosporidium babjevae</i>		Exudate of <i>Prunus sargentii</i> , Jatani, Yamagata, Japan	KX771210, KX771225
<i>Rhodotorula babjevae</i> UCDFST 67-506	<i>Rhodosporidium babjevae</i>		Exudate of <i>Ulmus davidiana</i> var. <i>japonica</i> . Hibara (Fukushima), Japan	KX771206, KX771221
<i>Rhodotorula babjevae</i> UCDFST 68-916.1	<i>Rhodosporidium babjevae</i>		Insect frass in <i>Alnus</i> sp. (alder) tree, BC, Canada	KU609431, KU609489
<i>Rhodotorula dairenensis</i> UCDFST 68-257		CBS 347, NRRL Y-1596	Air, Tokyo, Japan	KU609444, KU609502
<i>Rhodotorula dairenensis</i> UCDFST 68-275 ⁽¹⁾		CBS 2826, ATCC 32768	Pasture grass, New Zealand	KU609448, KU609506
<i>Rhodotorula diobovata</i> UCDFST 08-225	<i>Rhodosporidium diobovatum</i>	CBS 6085	Sea water, FL, USA.	KU609432, KU609490
<i>Rhodotorula glutinis</i> UCDFST 50-309 ⁽¹⁾		CBS 20, ATCC 2527	Atmosphere, Japan	KX771201, KX771216
<i>Rhodotorula graminis</i> UCDFST 05-503			Olive fly, Davis, CA, USA	KU609446, KU609504
<i>Rhodotorula kratochvilovae</i> UCDFST 05-632	<i>Rhodosporidium kratochvilovae</i>		<i>Chrysoperla careae</i> (green lacewing), Winters, CA, USA	KU609435, KU609493

TABLE 2
Description of the yeast strains used for the present study.

Strain species and Phaff collection ID	Synonym ^a	ID number in other collections	Source habitat	Genbank Accession numbers
<i>Rhodotorula mucilaginosa</i> UCDFST 05-218			<i>Drosophila melanogaster</i> fly in apple. Davis, CA, USA.	KX771215, KX771230
<i>Rhodotorula mucilaginosa</i> UCDFST 10-221			<i>Anisomorpha buprestoides</i> (2-stripe stick insect), Davis, CA, USA	KX771214, KX771229
<i>Rhodotorula mucilaginosa</i> UCDFST 13-478			Forest ecosystem: Rawa Aopa, Sulawesi, Indonesia	KU609450, KU609508
<i>Rhodotorula mucilaginosa</i> UCDFST 67-64			Unknown.	KX771213, KX771228
<i>Rhodotorula mucilaginosa</i> UCDFST 68-329 ⁽¹⁾		CBS 482, ATCC 24216, NRRL Y-6683	Smut-infected leaves, France	KU609449, KU609507
<i>Rhodotorula mucilaginosa</i> UCDFST 68-854.2		CBS 6462	Exudate of <i>Populus tremuloides</i> . BC, Canada	KX771207, KX771222
<i>Rhodotorula mucilaginosa</i> UCDFST 78-54			Lake water, Wisconsin, USA	KU609445, KU609503
<i>Rhodotorula</i> aff. <i>paludigena</i> UCDFST 81-84	<i>Rhodosporidium</i> aff. <i>Paludigenum</i>		<i>Opuntia</i> sp., Bahamas	KU609438, KU609496
<i>Rhodotorula paludigena</i> UCDFST 09-163	<i>Rhodosporidium paludigenum</i>	CBS 3044	Leaf of <i>Desmodium repens</i> , Netherlands	KU609436, KU609494
<i>Rhodotorula paludigena</i> UCDFST 81-492	<i>Rhodosporidium paludigenum</i>		<i>Opuntia ficus-indica</i> cactus; AZ, USA	KX771202, KX771217
<i>Rhodotorula paludigena</i> UCDFST 82-646.2	<i>Rhodosporidium paludigenum</i>		<i>Melocactus intortus</i> , Prickly Pear Island, British Virgin Islands	KU609437, KU609495

TABLE 2
Description of the yeast strains used for the present study.

Strain species and Phaff collection ID	Synonym ^a	ID number in other collections	Source habitat	Genbank Accession numbers
<i>Rhodotorula sphaerocarpa</i> UCDFST 68-43	<i>Rhodospiridium sphaerocarpum</i>		Auxotrophic mutant of strain UCDFST 48-23	KU609439, KU609497
<i>Rhodotorula toruloides</i> UCDFST 67-52 (NT)	<i>Rhodospiridium toruloides</i>	CBS 349, NRRL Y-1588	Soil, Tokyo, Japan	KU609440, KU609498
<i>Sporidiobolus paraseus</i> UCDFST 09-1305			Sunflower leaf surface, Davis, CA, USA	KU609457, KU609515
<i>Sporidiobolus paraseus</i> UCDFST 44-17			Hemp rope, Panama	KU609454, KU609512
<i>Sporidiobolus paraseus</i> UCDFST 67-552			Exudate of Monarch birch <i>Betula maximowicziana</i> , Tomakomai, Japan	KU609453, KU609511
<i>Sporobolomyces bannaensis</i> UCDFST 10-421		FORDACC 1727, InaCC 0478	Leaf surface, Sulawesi, Indonesia	KU609458, KU609516
<i>Sporobolomyces bannaensis</i> UCDFST 10-451		FORDACC 4481, InaCC 0521	Leaf surface, Sulawesi, Indonesia	KU609459, KU609517
<i>Sporobolomyces bannaensis</i> UCDFST 10-453		FORDACC 1700, InaCC 0523	Leaf surface, Sulawesi, Indonesia	KU609468, KU609526
<i>Sporobolomyces bannaensis</i> UCDFST 11-470		FORDACC 937, InaCC 0521	Leaf surface, Sulawesi, Indonesia	KU609469, KU609527
<i>Sporobolomyces aff. beijingsensis</i> UCDFST 11-1143			Cherry, Corvallis, OR, USA	KU609477, KU609535
<i>Sporobolomyces carnicolor</i> UCDFST 03-573			Rubiaceae fruit, Nicaragua	KU609471, KU609529

TABLE 2
Description of the yeast strains used for the present study.

Strain species and Phaff collection ID	Synonym ^a	ID number in other collections	Source habitat	Genbank Accession numbers
<i>Sporobolomyces carnicolor</i> UCDFST 68-346		CBS 2637	Air, Delft, the Netherlands	KU609456, KU609514
<i>Sporobolomyces carnicolor</i> UCDFST 68-348		CBS 4215	Unknown, Nagano, Japan	KU609472, KU609530
<i>Sporobolomyces johnsonii</i> UCDFST 67-65	<i>Sporidiobolus johnsonii</i>		Tropical foliage, Netherlands	KU609451, KU609509
<i>Sporobolomyces johnsonii</i> UCDFST 68-29	<i>Sporidiobolus johnsonii</i>	CBS 5470, ATCC 10765, NRRL Y-17303	Infected Raspberry plant <i>Rubus idaeus</i> , WA, USA	KU609452, KU609510
<i>Sporobolomyces metaroseus</i> UCDFST 05-893			Olive fly-infested olive, Ventura, CA, USA	KU609476, KU609534
<i>Sporobolomyces salmonicolor</i> UCDFST 10-159	<i>Sporidiobolus salmonicolor</i>		Soil in the permafrost area, Siberia,	KU609447, KU609505
<i>Sporobolomyces salmonicolor</i> UCDFST 44-3	<i>Sporidiobolus salmonicolor</i>		Deteriorated quartermaster items, FL, USA	KU609465, KU609523
<i>Sporobolomyces salmonicolor</i> UCDFST 67-1029	<i>Sporidiobolus salmonicolor</i>		Elm exudate in soil, Davis, CA, USA	KU609464, KU609522
<i>Sporobolomyces salmonicolor</i> UCDFST 72-182	<i>Sporidiobolus salmonicolor</i>		Tree, Kyoto, Japan	KU609467, KU609525
<i>Trigonosporomyces aff. hylophilus</i> UCDFST 79-1005	<i>Rhodotorula aff. hylophilus</i>		Substrate unknown, Davis, CA	KU609478, KU609536

UCDFST: Phaff Yeast Culture Collection, University of California Davis. FORDACC: Ministry of Forestry Culture Collection, Bogor, Indonesia. InaCC: Indonesian National Culture Collection, Cibinong, Indonesia. CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. NRRL: USDA-ARS Culture Collection, Peoria, IL, USA. ATCC: American Type Culture Collection, Maryland, USA. (T): Type strain. (NT) neotype strain. See, [53] for information on recent taxonomic changes.

[0099] *Molecular identification.* Strain identities were confirmed using ITS and partial 26S ribosomal sequencing by colony PCR using primers ITS1, ITS4 [54], NL1 and NL4 as previously described [12, 14]. Strains *Rhodotorula babjevae* UCDFST 67-478, UCDFST 06-542, UCDFST 04-830, UCDFST 67-102, *Rhodotorula mucilaginosa* UCDFST 10-221, UCDFST 67-64, and UCDFST 05-218 did not amplify with the cell lysis method previously described. Therefore, DNA was extracted and purified using the DNA Wizard Kit® (Cat. No. Promega, NY, USA) from 5 mL liquid PDB cultures inoculated with a loopful of fresh cells (≈ 0.02 g) streaked from a potato dextrose agar and incubated in 16 cm $\times$ 150 mm disposable borosilicate glass culture tubes (cat. number 14-961-31 Fisher Scientific, CA, USA) for 20 h. DNA extracts were successfully amplified and the corresponding PCR products were sequenced with the primers described above at The College of Biological Sciences UCDNA Sequencing Facility (University of California, Davis, CA, USA). The rest of the amplified sequences from the strains not requiring DNA extraction with the kit were sequenced with the primers described above at Beckman Coulter Genomics (Danver, MA, USA). In both cases, single pass Sanger sequencing techniques [16] were used, and identification was performed with NCBI-BLAST [2] as previously described [12, 14].

[0100] *Yeast growth conditions.* A loopful of fresh cells (≈ 0.2 g) from a freshly grown plate was suspended in 5.0 mL of sterile deionized water. To prepare a seed culture, 500 μ L of this inoculum were inoculated into 50 mL bioreaction tubes (part number 229475, Celltreat Scientific Products, Shirley, MA, USA) containing 9.5 mL of Medium A, a medium with high C:N ratio (68:1) known to induce lipid accumulation in oleaginous yeasts [11, 45]. Modified Medium A contains 50 g L⁻¹ glucose, 0.1 g L⁻¹ calcium chloride, 0.5 g L⁻¹ ammonium chloride, 1.5 g L⁻¹ yeast extract, 7.0 g L⁻¹ KH₂PO₄, 5 g L⁻¹ Na₂HPO₄·2H₂O, 1.5 g L⁻¹ MgSO₄·7H₂O, 0.08 g L⁻¹ FeCl₃·6H₂O, 10.0 mg L⁻¹ Zn SO₄·7H₂O, 0.1 mg L⁻¹ MnSO₄·H₂O, 0.1 mg L⁻¹ CuSO₄, and 0.1 mg L⁻¹ Co(NO₃)₂. The cultures were then grown for 24 h at 24 °C and 200 rpm in a rotary shaker incubator (Series 25, New Brunswick Scientific Co., Edison, NJ, USA). Five mL of this seed culture were then inoculated into 500 mL baffled Erlenmeyer flasks containing 95 mL Medium A containing 50 g L⁻¹ glucose, and stoppered with foam stoppers (Cat.# L800-D, Identiplugs®, Jaece Industrie, Inc., NY, USA). The cultures were incubated for 7 days at 24 °C and 200 rpm.

[0101] Cell pellets were harvested by centrifugation at 3220×g for 10 min and washed with sterile deionized water two times by centrifugation. The cell pellets were stored over- night at −80 °C and freeze-dried at −46 °C, 0.133 mbar (Freez-one® 4.5 L Freeze Dry System Model 7750020, Labconco®, Kansas City, MO, USA). The dry cells
5 were weighed to estimate dry cell weight (DCW) in terms of grams of recovered dry cells per liter of culture. PEFA were recovered by one of the following methods:

[0102] When PEFA droplets were observed in the culture, 10 mL of culture were each transferred to three tared 15 mL conical tubes and centrifuged at 3220×g for 10 min at room temperature. PEFA that sedimented to the bottom of the bottles were aspirated using
10 9 inch Pasteur pipettes and transferred to tared 15 mL conical tubes. Because some PEFA mix with cells during pipetting, deionized sterile water was added to the mixture, which was then vortexed and centrifuged to further separate the PEFA layer from the cells. The cells were carefully resuspended in water and pipetted out from the PEFA. The PEFA were freeze dried overnight to remove moisture and weighed to estimate grams of recovered
15 PEFA per liter culture.

[0103] If PEFA droplets were not observed in the culture, then 10 mL of whole culture were extracted twice with 40 mL of ethyl acetate in duplicate and the solvent was removed overnight using a vacuum speed concentrator (miVac Duo®, Genevac Inc., Stone Ridge, NY, USA) programmed for non-freezing solvents (e.g. ethyl acetate) at minimum
20 working pressure (≤ 6 mm Hg) and 25 °C. The dry weight of the PEFA residue was recorded.

[0104] *Product characterization.* PEFA products were analyzed as previously described [7]. Native PEFA molecules were analyzed using reversed-phase liquid chromatography-electrospray ionization in positive mode with a quadrupole/time-of-flight mass spectrometer (RPLC-ESI(+)-QTOFMS) in MS and MS/MS modes. Aliquots
25 containing 10 $\mu\text{g mL}^{-1}$ of crude PEFA in methanol were run through a system consisting of an Agilent 1290 Infinity LC system (Agilent Technologies) with a pump (G4220A), a col- umn oven (G1316C), an autosampler (G4226A), and an Agilent 6550 iFunnel QTOFMS. Diluted samples were separated on an Acquity UPLC CSH C18 column ($100 \times$
30 2.1 mm; 1.7 μm) coupled to an Acquity UPLC CSH C18 VanGuard pre-column (5×2.1 mm; 1.7 μm) (Waters). The column was maintained at 65 °C at a flow-rate of 0.6 mL min^{-1} . The mobile phases consisted of (A) acetonitrile/water (60:40, v/v) with ammonium formate (10 mM) and formic acid (0.1%) and (B) isopropanol/acetonitrile (90:10, v/v) with

ammonium formate (10 mM) and formic acid (0.1%). The separation was conducted under the following gradient: 0 min 15% (B); 0–2 min 30% (B); 2–2.5 min 48% (B); 2.5–11 min 82% (B); 11–11.5 min 99% (B); 11.5–12 min 99% (B); 12–12.1 min 15% (B), 12.1–15 min 15% (B). A sample volume of 1 μL was used for the injection. Sample temperature was maintained at 4 °C. The QTOF instrument was operated in ESI(+) with the following parameters: MS1 mass range: m/z 50–1700; MS/MS mass range: m/z 50–1700; collision energy: 20 eV; capillary voltage: 3 kV; nozzle voltage: 1 kV; gas temperature: 200 °C; drying gas (nitrogen): 14 L min^{-1} ; nebulizer gas (nitrogen): 35 psi; sheath gas temperature: 350 °C; sheath gas flow (nitrogen): 11 L min^{-1} ; acquisition rate MS1: 10 spectra s^{-1} ; acquisition rate MS/MS: 13 spectra s^{-1} ; total cycle time: 0.508 s; number of precursor ions per cycle: 4; mass range for selection of precursor ions: m/z 500–1200. The instrument was tuned using an Agilent tune mix (mass resolving power $\sim 20,000$ FWHM). A reference solution (m/z 121.0509, m/z 922.0098) was used to correct small mass drifts during the acquisition. Mass Hunter Qualitative (B.05.00) and Quantitative (B.05.01) Analysis (Agilent) software programs were used for the data processing. For the identification of the structural fragments and structural assembly the software Mass Frontier 7.0 (Thermo Scientific, Palo Alto, CA, USA) was used.

RESULTS

[0105] *Screening of yeasts for PEFA synthesis and secretion.* Sixty-five yeast strains were used in this study, including PEFA-secreting basidiomycetes *Rhodotorula babjevae* UCDFST 04-877 [7]. Table 3 lists the 19 strains belonging to 6 species that produced more than 1 g L^{-1} net PEFA out of the 65 candidate yeasts. The LC–MS data revealed that overlap of triacylglycerols occurred in both strains whose PEFA was separated by physical sedimentation and water washing as well as those extracted with ethyl acetate. Therefore, a “purity percentage” is also included in Table 3, to indicate what percent of the crude extract is PEFA. Some strains carried over a conspicuously large amount of triacylglycerols, and little to no PEFA. Examples include 15 strains from the species *R. ruineniae*, *R. sphaerocarpa*, *R. aff. lusitaniae*, *S. salmonicolor*, *R. dairenensis*, *R. glutinis*, *S. pararoseus*, *S. johnsonii*, *C. minutum*, and *O. externus* which displayed purity coefficients of less than 80%, ranging from less than 1–71.6%.

TABLE 3
Yeast strains that secreted $\geq 1 \text{ g L}^{-1}$ net PEFA

Strain species and Phaff collection ID	ID number in other collections	Source habitat	Dry Cell weight (g L^{-1})	Crude extract containing PEFA (g L^{-1})	Estimated PEFA purity (%)	Net PEFA produced (g L^{-1})
<i>R. babjevae</i> UCDFST 04-830		Male olive fly, Winters, CA, USA	13	8.5	99.9	8.5
<i>R. babjevae</i> UCDFST 04-877		Olive fly, Davis, CA, USA	14.9	8.7	99.1	8.6
<i>R. babjevae</i> UCDFST 05-613		Olive tree, Davis, CA, USA	13.6	4.3	99.8	4.3
<i>R. babjevae</i> UCDFST 05-736		Female olive fly, Davis, CA, USA	12.5	5.5	99.9	5.5
<i>R. babjevae</i> UCDFST 05-775		Dry sap of olive tree, Winters, CA, USA	14.1	6.5	98.4	6.4
<i>R. babjevae</i> UCDFST 06-542		Olive-fly infested olive, Winters, CA, USA	13	5.1	99.9	5.1
<i>R. babjevae</i> UCDFST 67-102		Sea water, Point Reyes, CA, USA	11.4	5.8	99.9	5.8
<i>R. babjevae</i> UCDFST 67-436		Exudate of <i>Pterocarya rhoifolia</i> , Kaida, Kiso, Japan	13.3	4	99.8	4
<i>R. babjevae</i> UCDFST 67-458		Exudate of <i>Betula ermani</i> , Mt. Fuji, Japan	13.4	2.7	92.5	2.5
<i>R. babjevae</i> UCDFST 67-478		Exudate of <i>Prunus sargentii</i> , Jatani, Yamagata, Japan	13.5	4.6	99.8	4.6
<i>R. babjevae</i> UCDFST 67-506		Exudate of <i>Ulmus davidiana</i> var. <i>japonica</i> . Hibara (Fukushima), Japan	12.8	5.1	99.8	5.1

TABLE 3
Yeast strains that secreted $\geq 1 \text{ g L}^{-1}$ net PEFA

Strain species and Phaff collection ID	ID number in other collections	Source habitat	Dry Cell weight (g L^{-1})	Crude extract containing PEFA (g L^{-1})	Estimated PEFA purity (%)	Net PEFA produced (g L^{-1})
<i>R. babjevae</i> UCDFST 68-916.1		Insect frass in <i>Alnus</i> sp. (alder) tree, BC, Canada	13.9	1.1	97.2	1.1
<i>R. diobovata</i> UCDFST 08-225	CBS 6085	Sea water, FL, USA.	9.6	2.1	96.9	2
<i>R. graminis</i> UCDFST 05-503		Olive fly, Davis, CA, USA	13.9	3.8	99.6	3.8
<i>R. kratochvilovae</i> UCDFST 05-632		<i>Chrysoperla careae</i> (green lacewing), Winters, CA, USA	15.1	3.1	98.4	3.1
<i>R. aff. paludigena</i> UCDFST 81-84		<i>Opuntia</i> sp., Bahamas	11.9	12.7	97.4	12.4
<i>R. paludigena</i> UCDFST 09-163	CBS 3044	Leaf of <i>Desmodium repens</i> , Netherlands	13.7	2.2	96.8	2.1
<i>R. paludigena</i> UCDFST 81-492		<i>Opuntia ficus-indica</i> cactus; AZ, USA	11.1	11.8	99.8	11.7
<i>R. paludigena</i> UCDFST 82-646.2		<i>Melocactus intortus</i> , Prickly Pear Island, British Virgin Islands	14.9	3.1	98.5	3.1

The yeast strains were sourced from the Phaff Yeast Culture Collection, University of California Davis. Abbreviation “cf.” (Latin “confer”): the species is quite similar to the indicated known species. Abbreviation “aff.” (Latin “affinis”): the species is significantly different from the indicated known species, and is probably a novel species based on ribosomal sequence analysis. Strains identified as “T” are the type strain of the species, “NT” is the neotype strain. The first two digits of the ID number are the year the strain was deposited in the Phaff collection. New PEFA secreters are in bold. N/A: Not applicable.

[0106] The PEFA were detected visually when the PEFA phase separated from the culture with simple gravity separation such as centrifugation, or when extracted with ethyl acetate. The PEFA that settled in the bottom of the tubes had a density higher than water, approximately 1.08 g/L. A similar behavior was observed previously for LM [24] and SL [52]. Fifteen strains produced at least 3 g/L net PEFA. *Rhodotorula aff. paludigena* UCDFST 81-84 produced 12.4 g/L PEFA and *Rhodotorula paludigena* UCDFST 81-492 produced 11.7 g/L when grown in 50 g/L glucose, ranking as the top two PEFA producers identified in the present study. All nineteen PEFA producing strains identified in the present study except *Rhodotorula kratochvilovae* UCDFST 05-632, and *Rhodotorula diobovata* UCDFST 08-225, produced at least 1 g/L net PEFA that appeared as visible droplets in the culture. For the latter two strains, PEFA was successfully extracted with ethyl acetate.

[0107] *Identification of PEFA products.* Each PEFA-secreting yeast produced a mixture of nine to thirteen structurally similar PEFA, differing in the type of sugar alcohol attached (either D-mannitol or D-arabitol), the degree of acetylation in the sugar alcohol, and the chain length of the 3-hydroxy fatty acid. The hydroxy group on position 3 of the fatty acid is the only one present throughout the fatty acid chain and is acetylated in all PEFA congeners. A total of 19 different PEFA molecular congeners with different molecular weights and retention times were detected (Table 4). An example of a chromatogram showing the PEFA profile for *R. paludigena* UCDFST 81-492 and the MS/MS spectra of PEFA 12 (acetylated C16:0 3-hydroxy fatty acid esterified to D-arabitol with 4 acetylations) with fragment annotations are provided in (Fig. 2a, b). PEFA were detected and PEFA profiles were determined for 20 strains that produced less than 1 g L⁻¹ PEFA, with varying purity percentages. The PEFA profiles of the yeast species fall into 3 clusters of PEFA species (Fig. 3). Details of relative abundance of each PEFA species for the 39 strains that secreted detectable amounts are presented in Table 5.

TABLE 4

Chemical formula, molecular weight, retention time (tR), description, and systematic name of the 19 PEFA detected in this study

PEFA no.	Chemical formula	m/z [M + NH ₄] ⁺	tR (min)	Description
PEFA 1	C ₂₇ H ₄₆ O ₁₁	564.3384	2.05	Acetylated C12:0 3-hydroxy fatty acid esterified to D-arabitol with 3 acetylations
PEFA 2	C ₃₀ H ₅₀ O ₁₃	636.3590	2.10	Acetylated C14:0 3-hydroxy fatty acid esterified to D-mannitol with 4 acetylations
PEFA 3	C ₂₈ H ₅₀ O ₁₁	580.3697	2.29	Acetylated C16:0 3-hydroxy fatty acid esterified to D-mannitol with 2 acetylations
PEFA 4	C ₂₇ H ₄₈ O ₁₀	550.3591	2.45	Acetylated C16:0 3-hydroxy fatty acid esterified to D-arabitol with 2 acetylations
PEFA 5	C ₂₉ H ₄₈ O ₁₂	606.3490	2.49	Acetylated C14:0 3-hydroxy fatty acid esterified to D-arabitol with 4 acetylations
PEFA 6	C ₃₂ H ₅₂ O ₁₄	678.3695	2.52	Acetylated C14:0 3-hydroxy fatty acid esterified to D-mannitol with 5 acetylations
PEFA 7	C ₃₀ H ₅₂ O ₁₂	622.3803	2.61	Acetylated C16:0 3-hydroxy fatty acid esterified to D-mannitol with 3 acetylations
PEFA 8	C ₂₉ H ₅₀ O ₁₁	592.3697	2.86	Acetylated C16:0 3-hydroxy fatty acid esterified to D-arabitol with 3 acetylations
PEFA 9	C ₃₂ H ₅₄ O ₁₃	664.3903	2.87	Acetylated C16:0 3-hydroxy fatty acid esterified to D-mannitol with 4 acetylations
PEFA 10	C ₃₀ H ₅₄ O ₁₁	608.4004	2.99	Acetylated C18:0 3-hydroxy fatty acid esterified to D-mannitol with 2 acetylations
PEFA 11	C ₃₄ H ₅₆ O ₁₄	706.4008	3.10	Acetylated C16:0 3-hydroxy fatty acid esterified to D-mannitol with 5 acetylations
PEFA 12	C ₃₁ H ₅₂ O ₁₂	634.3803	3.11	Acetylated C16:0 3-hydroxy fatty acid esterified to D-arabitol with 4 acetylations
PEFA 13	C ₃₂ H ₅₆ O ₁₂	650.4110	3.12	Acetylated C18:0 3-hydroxy fatty acid esterified to D-mannitol with 3 acetylations
PEFA 14	C ₃₄ H ₅₈ O ₁₃	692.4216	3.31	Acetylated C18:0 3-hydroxy fatty acid esterified to D-mannitol with 4 acetylations
PEFA 15	C ₃₁ H ₅₄ O ₁₁	620.4010	3.29	Acetylated C18:0 3-hydroxy fatty acid esterified to D-arabitol with 3 acetylations
PEFA 16	C ₃₆ H ₆₀ O ₁₄	734.4321	3.49	Acetylated C18:0 3-hydroxy fatty acid esterified to D-mannitol with 5 acetylations
PEFA 17	C ₃₃ H ₅₆ O ₁₂	662.4116	3.47	Acetylated C18:0 3-hydroxy fatty acid esterified to D-arabitol with 4 acetylations
PEFA 18	C ₃₄ H ₆₀ O ₁₂	678.4423	3.52	Acetylated C20:0 3-hydroxy fatty acid esterified to D-mannitol with 3 acetylations
PEFA 19	C ₃₆ H ₆₂ O ₁₃	720.4529	3.69	Acetylated C20:0 3-hydroxy fatty acid esterified to D-mannitol with 4 acetylations

PEFA were detected as [M + NH₄]⁺ and [M + Na]⁺ adducts using RPLC-ESI(+)-QTOFMS

Table 5

All polyol esters of fatty acids (PEFA) detected, as a percent of total PEFA produced by those yeast strains whose PEFA crude extracts gave signals above the detection level.

Strain species and Phaff collection ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Other s (N/I)
<i>Cystobasidium minutum</i> UCDFST 68-280	0.4	0.1	0.1	0.2	1.1	0.1	0.4	27.7	3.4	0.6	0.6	28.0	1.6	3.7	13.0	0.5	6.2	4.0	1.6	4.7
<i>Occultifur externus</i> UCDFST 68-934.2	0.4	0.3	0.3	0.6	1.1	0.2	0.5	35.6	15.2	0.5	0.8	19.2	1.3	6.3	8.7	0.6	3.3	0.2	0.5	3.3
<i>Rhodospordiobolus aff. lusitaniae</i> UCDFST 06-583	0.2	0.4	0.6	0.8	0.8	0.4	2.6	7.3	7.8	0.9	4.0	25.3	5.4	11.3	7.0	2.1	11.9	1.3	0.7	6.0
<i>Rhodospordiobolus odoratus</i> UCDFST 09-1303	0.6	0.7	1.3	1.5	1.6	0.0	3.6	7.2	6.7	1.0	3.3	17.2	5.8	10.2	6.6	2.2	6.4	2.3	1.9	13.8
<i>Rhodospordiobolus ruineniae</i> UCDFST 10-1058	0.3	0.4	0.8	1.2	0.4	0.2	4.3	8.0	9.2	1.4	2.5	13.4	10.1	18.6	8.2	2.6	13.0	0.8	0.4	2.4
<i>Rhodospordiobolus ruineniae</i> UCDFST 12-776	1.2	0.9	1.0	1.1	1.0	0.7	1.5	4.3	6.5	0.9	3.0	16.7	3.5	13.3	10.3	1.7	12.5	1.9	1.2	10.4
<i>Rhodospordiobolus ruineniae</i> UCDFST 67-67 ⁽¹⁾	0.1	0.1	0.2	0.6	0.6	0.2	1.4	7.6	3.2	0.5	4.3	29.2	5.7	5.6	15.0	1.9	21.0	0.3	0.2	1.5
<i>Rhodospordiobolus ruineniae</i> UCDFST 78-84	1.0	0.5	1.8	1.4	2.0	0.4	0.7	19.9	12.4	0.7	1.8	14.2	2.9	8.0	9.2	1.0	5.4	1.2	1.0	8.2

Table 5

All polyol esters of fatty acids (PEFA) detected, as a percent of total PEFA produced by those yeast strains whose PEFA crude extracts gave signals above the detection level.

Strain species and Phaff collection ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Other s (N/I)
<i>Rhodotorula</i> <i>babjevae</i> UCDFST 04-830	0.2	0.1	0.3	1.5	1.2	0.2	12.4	13.6	10.0	0.2	2.9	18.8	6.9	9.3	8.2	0.9	12.9	0.0	0.0	0.0
<i>Rhodotorula</i> <i>babjevae</i> UCDFST 04-877	0.1	0.3	0.2	0.4	1.1	0.2	1.6	4.2	12.8	0.2	3.9	31.4	3.5	12.2	5.4	2.1	19.9	0.1	0.3	0.1
<i>Rhodotorula</i> <i>babjevae</i> UCDFST 05-613	0.0	0.3	0.0	0.1	1.0	0.1	3.4	5.5	17.7	0.1	3.1	26.9	4.3	12.7	5.6	1.4	17.4	0.0	0.1	0.0
<i>Rhodotorula</i> <i>babjevae</i> UCDFST 05-736	0.05	0.46	0.05	0.23	1.18	0.16	3.38	8.39	21.71	0.07	3.07	22.11	2.99	15.31	5.78	1.19	13.60	0.01	0.02	0.02
<i>Rhodotorula</i> <i>babjevae</i> UCDFST 05-775	0.2	0.4	0.8	1.2	1.7	0.2	4.2	8.1	12.0	0.8	3.7	22.2	6.6	13.3	7.9	1.9	14.1	0.1	0.2	0.0
<i>Rhodotorula</i> <i>babjevae</i> UCDFST 06-542	0.1	0.2	0.3	0.4	0.9	0.2	10.2	7.6	16.2	0.3	3.1	19.0	8.6	13.7	6.2	1.1	11.6	0.0	0.0	0.0
<i>Rhodotorula</i> <i>babjevae</i> UCDFST 67-102	0.1	0.1	0.1	0.2	0.8	0.4	11.5	6.6	10.7	0.1	5.6	20.6	9.6	11.7	5.6	2.0	13.9	0.0	0.1	0.0
<i>Rhodotorula</i> <i>babjevae</i> UCDFST 67-436	0.0	0.2	0.0	0.0	0.7	0.3	6.5	3.4	16.0	0.1	5.7	26.2	6.1	12.7	4.4	2.0	15.3	0.0	0.1	0.0

Table 5

All polyol esters of fatty acids (PEFA) detected, as a percent of total PEFA produced by those yeast strains whose PEFA crude extracts gave signals above the detection level.

Strain species and Phaff collection ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Other s (N/I)
<i>Rhodotorula</i>																				
<i>babjevae</i>	0.0	0.2	0.0	0.0	0.8	0.3	2.7	3.4	19.2	0.1	7.3	22.2	4.7	17.5	4.6	2.6	13.8	0.0	0.4	0.0
UCDFST 67-458																				
<i>Rhodotorula</i>																				
<i>babjevae</i>	0.1	0.1	0.2	0.8	1.1	0.2	11.7	11.3	7.0	0.2	3.4	24.5	9.8	5.7	8.5	1.1	14.1	0.0	0.0	0.0
UCDFST 67-478																				
<i>Rhodotorula</i>																				
<i>babjevae</i>	0.1	0.1	0.4	1.5	0.6	0.1	10.2	13.0	9.8	0.3	2.1	20.3	8.0	11.0	9.0	0.8	12.5	0.0	0.0	0.0
UCDFST 67-506																				
<i>Rhodotorula</i>																				
<i>babjevae</i>	0.1	0.0	2.4	1.3	0.1	0.2	3.3	7.2	1.9	2.3	11.2	7.9	16.9	5.3	13.2	7.2	18.7	0.3	0.1	0.2
UCDFST 68-916.1																				
<i>Rhodotorula</i>																				
<i>dairiensis</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2	2.9	0.1	12.4	23.1	0.4	6.1	1.7	9.1	43.8	0.0	0.0	0.1
UCDFST 68-257																				
<i>Rhodotorula</i>																				
<i>diobovata</i>	0.0	0.0	0.0	0.0	0.0	0.2	0.1	0.4	0.8	0.1	30.7	7.8	1.2	16.9	0.9	22.3	18.4	0.0	0.1	0.0
UCDFST 08-225																				
<i>Rhodotorula glutinis</i>	0.0	0.2	0.2	0.1	0.7	0.7	9.3	4.3	15.3	0.2	10.5	15.2	9.4	14.0	4.2	4.5	10.5	0.0	0.0	0.0
UCDFST 05-503																				
<i>Rhodotorula glutinis</i>	0.0	0.1	0.1	0.1	0.2	0.5	0.7	1.1	4.9	0.2	36.1	11.8	3.6	13.2	2.9	15.4	8.2	0.1	0.4	0.2
UCDFST 50-309 ⁽¹⁷⁾																				

Table 5

All polyol esters of fatty acids (PEFA) detected, as a percent of total PEFA produced by those yeast strains whose PEFA crude extracts gave signals above the detection level.

Strain species and Phaff collection ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Other s (N/I)
<i>Rhodotorula</i>																				
<i>daiirensis</i> UCDFST 68-275 ⁽¹⁾	0.5	1.1	0.9	1.4	0.6	0.5	3.4	3.3	10.2	0.9	3.4	14.5	5.9	14.3	6.0	2.1	8.4	2.6	1.9	10.1
<i>Rhodotorula</i> <i>kratochvilovae</i> UCDFST 05-632	0.1	0.0	0.0	0.5	5.6	3.5	0.8	10.1	8.1	0.2	11.5	11.0	2.4	12.4	10.1	9.9	13.1	0.2	0.4	0.0
<i>Rhodotorula</i> <i>mucilaginoso</i> UCDFST 05-218	1.5	1.1	1.0	2.0	3.1	0.8	1.5	13.3	3.2	1.1	1.9	35.8	1.1	1.4	4.7	0.9	7.9	1.3	0.9	8.1
<i>Rhodotorula</i> <i>mucilaginoso</i> UCDFST 10-221	0.7	0.2	0.7	1.8	2.7	0.6	1.2	16.9	5.8	0.3	2.3	32.5	2.4	3.8	8.0	1.3	8.2	0.7	0.3	5.9
<i>Rhodotorula</i> <i>mucilaginoso</i> UCDFST 67-64	1.1	0.7	1.1	1.1	1.3	0.7	1.0	18.4	9.6	1.0	2.2	15.9	2.4	5.5	9.7	1.4	7.0	0.4	1.1	11.0
<i>Rhodotorula</i> <i>mucilaginoso</i> UCDFST 68-854.2	0.8	0.6	1.1	0.9	1.2	0.3	1.5	29.5	23.1	0.5	1.4	16.0	2.1	4.3	7.3	0.9	3.9	0.2	0.2	2.1
<i>Rhodotorula</i> aff. <i>paludigena</i> UCDFST 81-84	1.9	0.0	0.2	1.0	6.0	0.0	0.5	33.3	0.6	0.1	0.0	34.4	0.2	0.1	15.0	0.0	5.8	0.0	0.0	0.0
<i>Rhodotorula</i> <i>paludigena</i> UCDFST 09-163	0.1	0.4	0.1	0.3	1.8	0.1	2.1	6.2	16.4	0.2	1.5	47.7	2.4	6.9	5.7	0.2	7.4	0.0	0.1	0.1

Table 5
All polyol esters of fatty acids (PEFA) detected, as a percent of total PEFA produced by those yeast strains whose PEFA crude extracts gave signals above the detection level.

Strain species and Phaff collection ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Other s (N/I)
<i>Rhodotorula</i>																				
<i>paludigena</i> UCDFST 81-492	2.7	1.3	1.7	2.9	3.5	0.1	5.6	34.5	16.4	0.3	0.2	18.0	1.1	1.8	5.0	0.0	0.9	0.0	0.0	0.2
<i>Rhodotorula paludigena</i> UCDFST 82-646.2	0.3	0.2	0.3	1.2	2.2	0.0	2.6	24.6	15.4	0.5	0.3	22.2	2.1	5.2	15.9	0.0	6.3	0.0	0.1	0.0
<i>Rhodotorula sphaerocarpa</i> UCDFST 68-43	0.6	1.2	1.0	1.1	1.2	1.1	2.0	7.6	8.0	0.8	2.6	18.1	3.8	11.2	7.4	2.1	10.3	1.2	1.6	10.8
<i>Sporidiobolus pararoseus</i> UCDFST 67-552	0.1	0.2	0.2	0.2	0.5	0.1	0.3	5.7	25.8	1.9	1.4	22.9	3.1	18.6	5.0	0.9	10.3	0.4	0.5	1.5
<i>Sporobolomyces carnicolor</i> UCDFST 68-346	0.4	0.3	1.8	1.3	1.1	0.3	0.6	38.8	5.3	0.5	1.5	15.9	3.0	6.5	11.6	0.7	4.8	0.5	0.4	2.8
<i>Sporobolomyces johnsonii</i> UCDFST 67-65	0.7	0.5	0.8	0.7	1.2	0.5	0.4	27.3	8.1	1.7	0.7	20.9	1.7	4.8	9.5	0.6	5.0	0.3	0.6	8.2
<i>Sporobolomyces salmonicolor</i> UCDFST 10-159	0.2	0.5	0.5	0.7	0.9	0.3	3.1	5.3	11.1	0.3	2.5	24.3	4.9	14.0	9.1	1.2	11.0	1.0	0.3	6.0

PEFA 1, PEFA 2, etc: PEFA species listed in Table 4. N/I: not identified. Each value is the percent of total PEFA for that strain. “aff.”

(affinis) means a new species most closely related to the named species.

[0108] The most abundant and prevalent PEFA species among all the yeast PEFA profiles is PEFA 12 (C₃₁H₅₂O₁₂), present in at least 5% relative abundance across all strains that secreted trace or higher amounts of PEFA, and up to 12% relative abundance in 34 out of the 39 strains. Four more PEFA, namely PEFA 8 (C₂₉H₅₀O₁₁), PEFA 9 (C₃₂H₅₄O₁₃), PEFA 14 (C₃₄H₅₈O₁₃), and PEFA 17 (C₃₃H₅₆O₁₂) follow PEFA 12 in terms of abundance and prevalence. They are all present in at least 5% relative abundance in 30 strains and 12% relative abundance in 14 strains. PEFA 8, 12 and 17 contain D-arabitol in the structure, and PEFA 9 and 14 have D-mannitol. PEFA 12 and 17 are fully acetylated, while PEFA 8, 9 and 14 have one available hydroxy group. These characteristics are consistent with more hydrophobic physical properties.

[0109] The 19 PEFA identified in this study can be grouped into three categories in terms of their free hydroxy groups: (a) a more polar group with at least two free hydroxy groups. Six molecules belong to this group: PEFA 3 (C₂₈H₅₀O₁₁), PEFA 4 (C₂₇H₄₈O₁₀), PEFA 7 (C₃₀H₅₂O₁₂), PEFA 10 (C₃₀H₅₄O₁₁), PEFA 13 (C₃₂H₅₆O₁₂) and PEFA 18 (C₃₄H₆₀O₁₂). The strain producing the most abundant polar PEFA was *Rhodotorula babjevae* UCDFST 68-916.1, with 26.6% relative abundance; (b) a less polar group with only one free hydroxy group. Seven out of the 19 molecules are grouped here, namely PEFA 1 (C₂₇H₄₆O₁₁), PEFA 2 (C₃₀H₅₀O₁₃), PEFA 8 (C₂₉H₅₀O₁₁), PEFA 9 (C₃₂H₅₄O₁₃), PEFA 14 (C₃₄H₅₈O₁₃), PEFA 15 (C₃₁H₅₄O₁₁) and PEFA 19 (C₃₆H₆₂O₁₃). The strain producing the most abundant fraction of these molecules was *Occultifur externus* UCDFST 68-934.2, with 66.9% relative abundance; (c) a non-polar group with all the hydroxy groups being acetylated. Six molecules classify in this group: PEFA 5 (C₂₉H₄₈O₁₂), PEFA 6 (C₃₂H₅₂O₁₄), PEFA 11 (C₃₄H₅₆O₁₄), PEFA 12 (C₃₁H₅₂O₁₂), PEFA 16 (C₃₆H₆₀O₁₄) and PEFA 17 (C₃₃H₅₆O₁₂). The strain displaying the highest abundance is *Rhodotorula dairenensis* UCDFST 68-257 with 88.4% relative abundance. Seven out of the 19 molecules contain D-arabitol, while the rest contain D-mannitol. No other sugar alcohols were detected. Fatty acid chain length ranged from 12 to 20 carbons, in even number increments. The most abundant chain length identified was hexadecanoate (C₁₆:0), and the second most abundant was octadecanoate (C₁₈:0). The highest abundance of medium chain length (C₁₂:0 and C₁₄:0) molecules was seen in *Rhodotorula kratochvilovae* UCDFST 05-632, while the highest abundance of C₂₀:0 was observed for *Cystobasidium minutum* UCDFST 68-280. In average, seven molecular species accounted for 78.5% of PEFA abundance across the whole panel, namely PEFA 12 (C₂₉H₅₀O₁₁), PEFA 8 (C₃₂H₅₄O₁₃), PEFA 17

(C₃₄H₅₆O₁₄), PEFA 9 (C₃₁H₅₂O₁₂), PEFA 14 (C₃₄H₅₈O₁₃), PEFA 15 (C₃₁H₅₄O₁₁) and PEFA 11 (C₃₃H₅₆O₁₂) with decreasing relative abundance percentages: 21.4, 12.6, 11.5, 10.5, 9.7, 7.7 and 5.1%, respectively. No unsaturated fatty acyl chains were present in the 19 PEFA identified. This is a striking difference from the intracellular oils reported for these strains, where the bulk of the acyl chains in the intracellular triacylglycerols are unsaturated [11, 41], in some cases with up to 3 double bonds in the acyl chains. The nineteen structures are described in Table 4.

[0110] Unsupervised hierarchical clustering and heat map of strains and PEFA species revealed three types of patterns (see Fig. 3). The first denotes an association between *Rhodotorula paludigena*/*Rhodotorula aff. paludigena* species and a PEFA profile where PEFA 8 is highly predominant, followed by PEFA 12. These PEFA contain D-arabitol. The second pattern involves *Rhodotorula babjevae* strains and a PEFA profile dominated by PEFA 12, which is fully acetylated and contains D-arabitol as well. The third pattern includes *Rhodotorula kratochvilovae* and *Rhodotorula diobovata*, the other two newly identified secreting yeasts, and a high percentage of PEFA 11, 16 and 17. The first two PEFA contain D-mannitol. There are two exceptions to these tendencies, namely *R. babjevae* UCDFST 68-916.1 and *R. paludigena* UCDFST 09-163, which display different patterns compared to the other strains from the same species. These results show that PEFA secretion is primarily species specific, but both qualitative and quantitative strain differences exist as well. The applications for the different PEFA compositions may differ: PEFA from *R. babjevae* should be more hydrophobic than those coming from *R. paludigena* due to fewer free hydroxy groups. More research is needed to identify optimal applications for each PEFA profile.

DISCUSSION

[0111] Before this study, only seven species of yeasts were known to naturally synthesize and secrete PL (see Table 1). This study increased the number of known species by adding species *Rhodotorula diobovata*, *Rhodotorula kratochvilovae*, *Rhodotorula paludigena*, *Rhodotorula aff. paludigena*. The taxonomic term “aff.” or *affinis* means it is a new species; the closest valid species is *R. paludigena*. The UCDFST (Phaff Yeast Culture Collection) strain used in this study is the only known representative of this novel species. These yeast species belong to a taxonomic clade that was recently revised [53], which resulted in genus name changes, as described above.

[0112] These four yeasts produce at least 1 g L⁻¹ with PEFA purities above 80%. Additional yeasts producing trace amounts of PEFA are also reported. The two highest PEFA producers in the present study, namely *R. aff. paludigena* UCDFST 81-84 and *R. paludigena* UCDFST 81-492, achieved glucose conversions of 0.25 and 0.23 g of PEFA per gram of glucose respectively. Cajka et al. obtained values equivalent to a glucose conversion of 0.086 g of PEFA per gram of glucose from *R. babjevae* UCDFST 04-877 and Tulloch et al. in the 1960s documented values equivalent to a glucose conversion of 0.05 g of PEFA per gram of glucose. from *R. azoricum* (previously called *R. glutinis* or *R. graminis*) CBS 4648 [7, 46]. The *R. paludigena* and *R. aff. paludigena* strains thus converted 2.9 times and 2.7 times more glucose into PEFA compared to Cajka et al. and 5 times and 4.6 times more compared to Tulloch et al. [7, 46] All these new PEFA-secreting yeasts present significant opportunity to expand our knowledge of synthesis and secretion of structurally complex products, which may be beneficial for development of tools for synthesis and secretion of a broad variety of oleochemicals beyond PEFA and other GL.

[0113] The function of PEFA as well as other yeast GL in natural ecosystems has been the subject of speculation. For example, the GL may aid in modifying leaf surface permeability, possibly facilitating uptake of hydrophobic carbon sources such as the long chain waxes present in the cuticle on the surface of leaves [6, 35]. Other theories include secretion of yeast GL as an external form of carbon storage [51]. Others have suggested the use of GL as anti-microbial and anti-fungal compounds [36, 42]. Indeed, LM were shown to display selective antibacterial activity against six *Streptococcus* species [4], even with different polyol head groups [28]. Many of the PEFA-secreting yeasts identified in this study were isolated from plant surfaces and insects, consistent with the first theory. PEFA production is more apparent in the *Rhodotorula glutinis/graminis* clade, comprising *R. glutinis*, *R. graminis*, *R. babjevae* and *R. diobovata* species, as well as in the *R. paludigena* clade, which appear in the top branches the phylogenetic tree (See, Figure 4 of Garay, *et al.*, J Ind Microbiol Biotechnol. (2017) Mar 13 [PMID: 28289902]). PEFA production was negligible or undetected in other phylogenetic branches, suggesting an evolutionary pattern. Furthermore, the highest three producers (*R. aff. paludigena* UCDFST 81-84, *R. paludigena* UCDFST 81-492 and *R. babjevae* UCDFST 04-877) in the present work were originally isolated from plant surfaces (Table 3). Six more PEFA producing strains (*R. paludigena* UCDFST 09-163, *R. babjevae* UCDFST 67-458, *R. graminis* 05-503, *R. paludigena* 82-646.2, *R. babjevae* UCDFST 68-916.1, *R. graminis* UCDFST 05-613, see

Table 3) originated from plant surfaces/plant surface interactions as well. All these strains agglomerate in the *R. glutinis/graminis/babjevae* and the *R. paludigena* clades, which, as mentioned before, are located in the top branches of the phylogenetic tree depicted in Fig. 4 of Garay, *et al.*, J Ind Microbiol Biotechnol. (2017) Mar 13 [PMID: 28289902]. Only two
5 PEFA secreters identified in this study had source habitats unrelated to plant surfaces/plant surface interactions.

[0114] The production of PEFA by these yeasts differs significantly from that of previously known GL secreting yeasts such as SL-secreting *Starmerella bombicola* [51, 52] in that significant levels of PEFA are produced when cultivated on glucose as the carbon
10 source, without a hydrophobic co-substrate. Similar results were reported previously for LM, where yields of up to 6.0 g L⁻¹ were obtained from 50 g L⁻¹ sucrose by *Aureobasidium pullulans* strain CU 39 (NRRL 58551) [24], and up to 35 g L⁻¹ from 120 g L⁻¹ glucose by *Aureobasidium* sp. strain A-21M (although the latter value came from collecting 30 mL cultures to add up a total volume of about 1 L) [18]. The consequences of this property are
15 quite significant for commercial production of PEFA for two reasons. First, the cost of glucose and other carbohydrates is significantly lower per kilogram than fatty acids and other hydrophobic co-substrates. Second, because residual lipids do not mingle with the GL product, purifying the product would involve simpler, less costly downstream processing. Commercial production of SL requires an ethyl acetate extraction to separate the SL and
20 other hydrophobic materials from the spent media, then a hexane extraction to remove residual input vegetable oils or fatty acids from SL [3, 51, 52]. Elimination of one or both of these steps would reduce both capital expenses for constructing facilities appropriate to handle flammable solvents, and operating expenses. Phase separation of PEFA produced by the basidiomycete yeasts described in the study suggests that a production technology with
25 fewer or no solvent extraction steps is feasible.

[0115] These newly identified PEFA-secreting yeast species all are classified in phylum Basidiomycota. Basidiomycetes are significantly different from ascomycetes morphologically, genetically, and physiologically. The phylum names derive from differences in spore formation. Basidiomycetes have much higher GC content, often over
30 60%, which can lead to difficulties with PCR and DNA sequencing. Basidiomycetes have a very different cell wall structure [33], which affects methods used to harvest intracellular products as well as genetic transformation techniques. As a result, fewer genome sequences and genetic tools are available for basidiomycetous yeasts [26]. However, some genomes

have been sequenced, and genetic tools are being developed for some species with biotechnology value such as oleaginous yeast *Rhodotorula toruloides* [55], which, like the PEFA-secreting yeasts reported here, is in the taxonomic order Sporidiobolales.

[0116] Basidiomycetes do have some advantages. In general, basidiomycetes, including those in the order Sporidiobolales, are able to assimilate a broader range of carbon sources, particularly pentoses that are utilized by few of the GL-secreting ascomycete species [37]. Some basidiomycetous yeasts can also grow without supplemented vitamins, reducing culture medium costs.

[0117] The basidiomycetous yeasts identified in this study simultaneously synthesize high levels of intracellular triacylglycerols, accumulating 40–65% oil by dry weight [11, 41], indicating efficient citrate flux from mitochondria to generate a rich pool of acetyl CoA, that will maintain the cytosolic fatty acid synthase actively producing fatty acids. This might explain why the yeast uses fatty acids as building blocks for PEFA synthesis [10]. In addition there are three main questions that remain unsolved and required further work towards elucidating the biosynthetic pathway of PEFA. First, further research is needed to understand how the hydroxylation on position $\Delta 3$ of the fatty acyl moiety occurs. In other yeast GL systems, like SL production by species *Starmerella bombicola*, hydroxylation on the ω or $\omega-1$ position occurs through a cytochrome P-450 enzyme that presumably is bound to the outer leaflet of the endoplasmic reticulum [48, 49]. Second, there might be a reductive step in the biosynthetic pathway to convert glucose to D-mannitol. Stodola et al. reported the existence of an NADP dependent mannitol dehydrogenase displaying high activity in cell free extracts from extracellular PEFA producing strains of *Rhodotorula glutinis* [44]. More research is needed to identify the particular location of the enzyme, and how glucose is reduced to mannitol as part of the biosynthetic pathway [44]. In the case of LM, it was shown that *Aureobasidium pullulans* strain NRRL 50380 was able to directly incorporate (D-arabitol, D-xylitol, D-ribitol and L-threitol) into the LM structures, whereas dosing of sugars like sucrose, D-fructose, D-mannose and D-arabinose resulted in LM having mannitol head groups [28]. Therefore *A. pullulans* is able to convert the sugars to mannitol, and incorporate certain polyols into the LM backbone. It is not yet known whether the basidiomycetous yeasts involved in the present study are capable of such behavior, though structural similarities between LM and PEFA suggest this may be possible. Finally, the genomes of those yeasts secreting either SL, MEL or CL have been sequenced, and genes responsible for synthesis of GL have been

identified [31], as has the transporter responsible for secretion in the case of SL [43]. The SL transporter protein has been described as an ABC transmembrane transporter [50]. Further analysis is required to determine whether these newly identified PEFA-secreting yeasts possess similar synthesis and/or secretion gene clusters.

5 **[0118]** This study validates the strategy of screening taxonomic relatives to identify additional yeast species with targeted properties. For example, Kurtzman measured SL production by 26 strains belonging to 18 yeast species in the taxonomic clade that includes *Starmerella bombicola* [21]. SL production was not detected in most strains tested. Because *Candida* is a polyphyletic genus, with hundreds of species spread across numerous
10 taxonomic clades within Ascomycota, a strong knowledge of yeast taxonomy enabled selection of appropriate species within the targeted clade. Access to a large and diverse culture collection (the USDA-ARS Culture Collection, <http://nrrl.naur.usda.gov/cgi-bin/usda/>) also enabled that study.

15 **[0119]** There were many parallels in the current study. A large and diverse culture collection was tapped to acquire a diversity of native yeast strains. Roughly half of the known yeast species in the targeted clade within the order Sporidiobolales [19] were available from the Phaff Yeast Culture Collection (<http://phaffcollection.udcavis.edu>). Taxonomic expertise enabled selection of appropriate yeast species for this study, as the genus *Rhodotorula* is polyphyletic. Novel species currently lacking valid species names
20 were discovered to produce PEFA in the current study. To indicate new species, the taxonomic term “affinis” (abbreviated “aff.”) is placed between the genus and the species names of the closest valid species. A novel species found to synthesize and secrete PEFA in this study is *Rhodotorula aff. paludigena*. The Phaff collection strain is the only known strain of this species. Further work is needed to fully describe these species and assign a
25 valid species name.

30 **[0120]** Variation of properties among strains of a given yeast species is often observed, such as enzymatic activity [12], inhibitor tolerance [40] and lipid content [11, 41]. When Kurtzman measured SL production by two strains of *Candida apicola*, one produced the highest levels seen in their study, and the other did not produce detectable levels. Similarly, we found different PEFA secretion levels when comparing multiple strains of *Rhodotorula babjevae*. Strain UCDFST 04-877 secreted 8.7 g L⁻¹, strain UCDFST 05-775 secreted 6.3 g L⁻¹ and strain UCDFST 68-916.1 secreted 1.1 g L⁻¹ crude PEFA extract, all with purity above 97% suggesting different phenotypes within the same species.

[0121] The first two digits of the Phaff collection ID number indicate the year the strain was deposited in the collection. Yeasts listed in Table 3 were acquired over the last five decades. These observations dramatically demonstrate the importance of preserving a broad variety of microbes in professionally managed collections to enable future discoveries [5], of clearly designating the yeast collection and strain ID number in publications, and of utilizing the same strain in subsequent studies to ensure consistent results.

[0122] Discovery of these PEFA-secreting yeasts may aid in improving production of renewable, sustainable, environmentally friendly surfactants for use in household and industrial cleaning products, as well as many other applications. Further studies including optimization of production, technoeconomic analysis and examination of surfactant activities will help determine whether these yeasts have the potential to alter global surfactant production and use.

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Example 2

Multiplatform Mass Spectrometry-Based Approach Identifies Extracellular Glycolipids of the Yeast *Rhodotorula babjevae* UCDFST 04-877

[0123] This example is published as Cajka, *et al.*, *J Nat Prod.* (2016) Oct 28;
79(10):2580-2589, hereby incorporated herein by reference in its entirety for all purposes.

20

[0124] In this example, a multiplatform mass spectrometry-based approach was
used for elucidating extracellular lipids with biosurfactant properties produced by the
oleaginous yeast *Rhodotorula babjevae* UCDFST 04-877. This strain secreted 8.6 ± 0.1 g/L
extracellular lipids when grown in a benchtop bioreactor fed with 100 g/L glucose in
medium without addition of hydrophobic substrate, such as oleic acid. Untargeted reversed-
25 phase liquid chromatography–quadrupole/time-of-flight mass spectrometry (QTOFMS)
detected native glycolipid molecules with masses of 574–716 Da. After hydrolysis into the
fatty acid and sugar components and hydrophilic interaction chromatography–QTOFMS
analysis, the extracellular lipids were found to consist of hydroxy fatty acids and sugar
alcohols. Derivatization and chiral separation gas chromatography–mass spectrometry (GC-
30 MS) identified these components as D-arabitol, D-mannitol, (R)-3-hydroxymyristate, (R)-3-
hydroxypalmitate, and (R)-3-hydroxystearate. In order to assemble these substructures back
into intact glycolipids that were detected in the initial screen, potential structures were in-

silico acetylated to match the observed molar masses and subsequently characterized by matching predicted and observed MS/MS fragmentation using the Mass Frontier software program. Eleven species of acetylated sugar alcohol esters of hydroxy fatty acids were characterized for this yeast strain.

5 MATERIALS AND METHODS

[0125] *General Experimental Procedures.* LC-MS-grade solvents, mobile-phase modifiers, derivatives, and other reagents were obtained from Fisher Scientific, Hampton, NH, USA (water, acetonitrile, methanol), and Sigma-Aldrich/Fluka, St. Louis, MO, USA (2-propanol, hexane, dichloromethane, methyl tert-butyl ether, formic acid, ammonium formate, N-methyl-N-(trimethylsilyl)trifluoroacetamide, methoxyamine hydrochloride, trifluoroacetic anhydride, (R)-(-)- α -methoxy- α -(trifluoromethyl)phenylacetyl chloride, K₂CO₃, pyridine, hydrochloric acid, sodium hydroxide). Standards of ribitol, xylitol, D-(+)-arabitol, L-(-)-arabitol, dulcitol, glucitol, D-mannitol, D-(+)-glucose, and 17-L-[(2'-O- β -D-glucopyranosyl- β -D-glucopyranosyl)oxy]-cis-9-octadecenoic acid 1',4''-lactone 6',6''-diacetate were from Sigma-Aldrich, while 3-hydroxytetradecanoic acid (racemate), 3-hydroxypalmitic acid (racemate), and 3-hydroxystearic acid (racemate) were purchased from Matreya LLC, State College, PA, USA. (S)-3-Hydroxymyristic acid and (R)-3-hydroxymyristic acid were from Santa Cruz Biotechnology (Dallas, TX, USA). L-Mannitol was from Carbosynth (Compton, UK).

[0126] *Extracellular Lipids.* The oleaginous yeast *Rhodotorula babjevae* UCDFST 04-877 was obtained from the Phaff Yeast Culture Collection (UCDFST), University of California Davis,(32, 33) and its identity was confirmed by ITS and 26S ribosomal sequencing as described previously.(18) The GenBank accession numbers are KR149271 and KU609429, respectively.(18) The yeast was revived from cryopreserved stocks and plated on potato dextrose agar plates for up to 7 days at 26 °C. A colony was suspended in 5 mL of deionized sterile water, and 500 μ L of the suspension was used to inoculate 9.5 mL of medium A(34) (with 50 g/L glucose) in 50 mL bioreaction tubes (Celltreat Scientific Products, Shirley, MA, USA) and incubated at 26 °C (room temperature) for 24 h. Samples of 5 mL of the culture were used to inoculate 95 mL of fresh medium A (with 50 g/L glucose) in 500 mL baffled flasks and incubated at 26 °C for 24 h. The contents of two flasks (corresponding to 200 mL of culture) were used to inoculate a 7 L bioreactor (Bioflo 3000, New Brunswick, Edison, NJ, USA) containing a working volume of 4 L of fresh medium A (with 100 g/L glucose) and incubated for 7 days at 26 °C with no pH adjustment.

Constant dissolved oxygen of 40% was maintained throughout the fermentation. A time series sampling of 10 mL aliquots was extracted twice with 40 mL of ethyl acetate and dried overnight using a modular concentrator (Mi Vac Duo, Gardiner, NY, USA) set at room temperature and minimum working pressure (oscillating between 4–6 mbar) to recover 8.6 ± 0.1 g/L PEFA from the culture at harvest. Around 50 mg of the crude extract was resuspended in 1 mL of ethyl acetate and stored at -80°C for the chemical structure characterization.

[0127] *RPLC-MS Analysis of Native Glycolipids.* (1) Sample preparation: A 10 $\mu\text{g/mL}$ solution was created via a two-step dilution, by first diluting 2 mg of the sample to 2 mL with methanol to an initial concentration of 1 mg/mL and vortexing for 10 s. In a second step, 10 μL of this solution was taken to a final concentration of 10 $\mu\text{g/mL}$ by adding 990 μL of methanol. (2) RPLC-MS analysis: The system consisted of an Agilent 1290 Infinity LC system (Agilent Technologies) with a pump (G4220A), a column oven (G1316C), an autosampler (G4226A), and an Agilent 6550 iFunnel QTOFMS. Diluted samples were separated on an Acquity UPLC CSH C18 column (100×2.1 mm; $1.7 \mu\text{m}$) coupled to an Acquity UPLC CSH C18 VanGuard precolumn (5×2.1 mm; $1.7 \mu\text{m}$) (Waters). The column was maintained at 65°C at a flow rate of 0.6 mL/min. The mobile phases consisted of (A) acetonitrile–water (60:40, v/v) with ammonium formate (10 mM) and formic acid (0.1%) and (B) 2-propanol–acetonitrile (90:10, v/v) with ammonium formate (10 mM) and formic acid (0.1%). The separation was conducted under the following gradient: 0 min 15% B; 0–2 min 30% B; 2–2.5 min 48% B; 2.5–11 min 82% B; 11–11.5 min 99% B; 11.5–12 min 99% B; 12–12.1 min 15% B; 12.1–15 min 15% B. A sample volume of 1 μL was used for the injection. Sample temperature was maintained at 4°C . The QTOFMS instrument was operated in electrospray ionization (ESI) in positive mode with the following parameters: MS1 mass range, m/z 50–1700; MS/MS mass range, m/z 50–1700; collision energy, 20 eV; capillary voltage, +3 kV; nozzle voltage, +1 kV; gas temperature, 200°C ; drying gas (nitrogen), 14 L/min; nebulizer gas (nitrogen), 35 psi; sheath gas temperature, 350°C ; sheath gas flow (nitrogen), 11 L/min; acquisition rate MS1, 10 spectra/s; acquisition rate MS/MS, 13 spectra/s; total cycle time, 0.508 s; number of precursor ions per cycle, 4; mass range for selection of precursor ions, m/z 500–1200. The instrument was tuned using an Agilent tune mix (mass resolving power $\sim 20\,000$ fwhm). A reference solution (m/z 121.0509, m/z 922.0098) was used to correct small mass drifts

during the acquisition. For the data processing, MassHunter Qualitative B.05.00 and Quantitative B.05.01 Analysis (Agilent) software programs were used.

[0128] *Untargeted Analysis of Hydrolyzed Samples Using HILIC-MS.* (1) Sample preparation: Samples (1 mg) were hydrolyzed in a 1.5 mL Eppendorf tube with (i) 0.3 mL of 1 M HCl in methanol at 55 °C for 2 h and (ii) 0.3 mL of 1 M NaOH in methanol at 55 °C for 2 h. After cooling to room temperature, both extracts were neutralized to pH 7. An aliquot of 10 µL was added to 1990 µL of an acetonitrile–water (80:20, v/v) mixture. After brief vortexing and centrifugation (13 400 rcf for 2 min), a 100 µL aliquot was transferred to a glass vial and submitted to HILIC-MS analysis. The reagent blanks were run in the same manner. (2) HILIC-MS analysis: The system consisted of an Agilent 1290 Infinity LC system (Agilent Technologies) with a pump (G4220A), a column oven (G1316C), an autosampler (G4226A), and an Agilent 6550 QTOFMS. Extracts were separated on an Acquity UPLC BEH Amide column (150 × 2.1 mm; 1.7 µm) coupled to an Acquity UPLC BEH Amide VanGuard precolumn (5 × 2.1 mm; 1.7 µm) (Waters). The column was maintained at 45 °C at a flow rate of 0.4 mL/min. The mobile phases consisted of (A) water with ammonium formate (10 mM) and formic acid (0.125%) and (B) 95:5 (v/v) acetonitrile–water with ammonium formate (10 mM) and formic acid (0.125%). The separation was conducted under the following gradient: 0 min 100% B; 0–2 min 100% B; 2–7.7 min 70% B; 7.7–9.5 min 40% B; 9.5–10.25 min 30% B; 10.25–12.75 min 100% B; 12.75–17.75 min 100% B. A sample volume of 1 µL was used for the injection. Sample temperature was maintained at 4 °C. The QTOFMS instrument was operated in electrospray ionization in negative mode with the following parameters: mass range, m/z 50–1700; capillary voltage, –3 kV; nozzle voltage, –1 kV; gas temperature, 200 °C; drying gas (nitrogen), 14 L/min; nebulizer gas (nitrogen), 35 psi; sheath gas temperature, 350 °C; sheath gas flow (nitrogen), 11 L/min; acquisition rate, 2 spectra/s. For the data processing, the MassHunter Qualitative B.05.00 (Agilent) software program was used.

[0129] *GC-MS Analysis of Free Fatty Acids.* (1) Sample preparation: Samples were prepared similarly to those in “Untargeted Analysis of Hydrolyzed Samples Using HILIC-MS”. After cooling to room temperature, 800 µL of hexane was added to each tube. The tube was vortexed for 10 s followed by centrifugation at 13 400 rcf for 2 min. For direct analysis of fatty acid methyl esters, 10 µL of the extracts was further diluted with hexane (90 µL) and injected. For analysis of 3-trimethylsiloxy methyl esters, 10 µL of the hexane extracts was evaporated and 100 µL of MSTFA was added, followed by shaking at 37 °C

for 30 min. The contents were transferred to a glass vial and submitted to GC-MS analysis. The reagent blanks were run in the same manner. (2) GC-MS analysis: The system consisted of an Agilent GC-MS system (Agilent Technologies) with a 7683 Series autosampler, a split/splitless injector, a 6890N GC system, and a quadrupole mass spectrometer 5975C. Injection parameters were as follows: injection volume, 0.2 μ L; injector temperature, 225 $^{\circ}$ C; helium carrier gas flow, 1 mL/min for 28 min, 4 mL/min² to 2 mL/min (hold for 8.5 min); splitless period, 0.5 min. For GC separation, a 30 m $\times$ 0.25 mm, 0.25 μ m DB-225ms (Agilent) capillary column was used with an oven temperature program: 60 $^{\circ}$ C (0.5 min), 15 $^{\circ}$ C/min to 135 $^{\circ}$ C (hold 5 min), 15 $^{\circ}$ C/min to 235 $^{\circ}$ C (hold 10 min). MS detection parameters were as follows: acquisition rate, 3.2 scans/s; mass range, m/z 50–500; MS ion source temperature, 230 $^{\circ}$ C; MS quadrupole temperature, 150 $^{\circ}$ C; electron multiplier voltage, 2060 V. For the data processing, the MSD ChemStation E.02.00 (Agilent) software program was used. The NIST 14 spectral library (NIST, Gaithersburg, MD, USA) was used for identification of fatty acids.

[0130] *GC-MS Analysis of Polyol Composition.* (1) Sample preparation: Samples were prepared similarly to those in “Untargeted Analysis of Hydrolyzed Samples Using HILIC-MS”. An aliquot of 10 μ L of neutralized extracts was submitted to a two-step derivatization. First, 10 μ L of 40 mg/mL methoxyamine hydrochloride in pyridine was added to dry extracts followed by shaking at 30 $^{\circ}$ C for 90 min. Second, 90 μ L of MSTFA was added to the extracts followed by shaking at 37 $^{\circ}$ C for 30 min. The contents were diluted 100-fold with MSTFA, transferred to a glass vial, and submitted to gas chromatography–mass spectrometry (GC-MS) analysis. The reagent blanks were run in the same manner. (2) GC-MS analysis: The system consisted of an MPS2 automatic liner exchange system (Gerstel, Mülheim an der Ruhr, Germany), an Agilent 7890A GC system, and a time-of-flight Pegasus III mass spectrometer (Leco, St. Joseph, MI, USA). Injection parameters were as follows: injection volume, 0.5 μ L; injector temperature, 50 $^{\circ}$ C ramped to 250 $^{\circ}$ C; helium carrier gas flow, 1 mL/min; splitless period, 25 s. For GC separation a 30 m $\times$ 0.25 mm, 0.25 μ m Rtx5Sil MS (Restek, Bellefonte, PA, USA) capillary column including an additional 10 m integrated guard column (Restek) was used with an oven temperature program: 50 $^{\circ}$ C (1 min), 20 $^{\circ}$ C/min to 330 $^{\circ}$ C (5 min). MS detection parameters were as follows: acquisition rate, 17 spectra/s; mass range, m/z 85–500; MS ion source temperature, 250 $^{\circ}$ C; transfer line temperature, 280 $^{\circ}$ C; MCP detector voltage, 1600 V. For the data processing, ChromaTOF 4.50 (Leco) software was used.

[0131] *GC-MS Enantioselective Analysis of 3-Hydroxy Fatty Acids.* (1) Sample preparation: Samples were prepared similarly to those in “Untargeted Analysis of Hydrolyzed Samples Using HILIC-MS”. After cooling to room temperature, 800 μL of hexane was added to each tube. The tube was vortexed for 10 s followed by centrifugation at 13 400 rcf for 2 min. An aliquot of 20 μL was evaporated and submitted to derivatization. Pyridine (100 μL) and (R)-(-)-MTPA-Cl (10 μL) were added to dry extracts. The mixture was allowed to react at room temperature for 2 h. Then, water (700 μL) and solid K_2CO_3 (one spatula tip) were added, and the mixture was vortexed for 20 s followed by addition of MTBE (700 μL). The tube was vortexed for 20 s followed by centrifugation at 13 400 rcf for 2 min. An aliquot of 100 μL of the organic phase with methylated hydroxy fatty acids as their MTPA derivatives was evaporated to dryness. The residues were dissolved in 200 μL of MTBE and subjected to GC analysis. The reagent blanks were run in the same manner. Stock solutions of (R)-3-hydroxymyristic acid, (S)-3-hydroxymyristic acid, (R,S)-3-hydroxytetradecanoic acid (racemate), (R,S)-3-hydroxyhexadecanoic acid (racemate), and (R,S)-3-hydroxyoctadecanoic acid (racemate) were prepared in methanol (1 mg/mL). An aliquot of 10 μL was treated in the same way as yeast glycolipid samples. (2) GC-MS analysis: The system consisted of an Agilent GC-MS system (Agilent Technologies), a 7693 Series autosampler, a split/splitless injector, a 7890A GC system, and a quadrupole mass spectrometer 5977A. Injection parameters were as follows: injection volume, 1 μL ; injector temperature, 250 $^{\circ}\text{C}$; helium carrier gas flow, 1 mL/min; splitless period, 1.5 min. For GC separation a 30 m $\times$ 0.25 mm, 0.25 μm SLB-5ms (Supelco) capillary column was used with an oven temperature program: 60 $^{\circ}\text{C}$ (1.5 min), 40 $^{\circ}\text{C}/\text{min}$ to 180 $^{\circ}\text{C}$ (hold 2 min), 2 $^{\circ}\text{C}/\text{min}$ to 220 $^{\circ}\text{C}$ (hold 35 min), 10 $^{\circ}\text{C}/\text{min}$ to 250 $^{\circ}\text{C}$ (hold 15 min), 20 $^{\circ}\text{C}/\text{min}$ to 300 $^{\circ}\text{C}$ (hold 10 min). MS detection parameters were as follows: acquisition rate, 2.3 scans/s; mass range, m/z 50–600; MS ion source temperature, 230 $^{\circ}\text{C}$; MS quadrupole temperature, 150 $^{\circ}\text{C}$; electron multiplier voltage, 1700 V. For the data processing, the MSD ChemStation E.02.00 (Agilent) software program was used.

[0132] *GC-MS Enantioselective Analysis of Polyols.* (1) Sample preparation: Samples were prepared similarly to those in “Untargeted Analysis of Hydrolyzed Samples Using HILIC-MS”. An aliquot of 20 μL of neutralized extracts was dried and submitted to derivatization. Dichloromethane (100 μL) and trifluoroacetic anhydride (100 μL) were added, the glass tubes were closed, and the samples were heated at 50 $^{\circ}\text{C}$ for 30 min. The reagents were evaporated to dryness, and the samples were dissolved in 200 μL of

dichloromethane and submitted to GC-MS analysis. The reagent blanks were run in the same manner. Stock solutions of D- and L-arabitol and D- and L-mannitol were prepared in water (1 mg/mL). An aliquot of 10 μ L was evaporated and submitted to derivatization. (2) GC-MS analysis: The system consisted of an Agilent GC-MS system (Agilent Technologies), a 7683 Series autosampler, a split/splitless injector, a 6890N GC system, and a quadrupole mass spectrometer 5975C. Injection parameters were as follows: injection volume, 0.2 μ L; injector temperature, 200 $^{\circ}$ C; helium carrier gas flow, 1 mL/min; splitless period, 0.5 min. For GC separation a 25 m $\times$ 0.25 mm, 0.25 μ m Chirasil-Dex CB (Agilent) capillary column was used with an oven temperature program: 50 $^{\circ}$ C (0.5 min), 15 $^{\circ}$ C/min to 90 $^{\circ}$ C (hold 10 min), 1 $^{\circ}$ C/min to 100 $^{\circ}$ C, 20 $^{\circ}$ C/min to 200 $^{\circ}$ C (hold 10 min). MS detection parameters were as follows: acquisition rate, 1.26 scans/s; mass range, m/z 50–650; MS ion source temperature, 230 $^{\circ}$ C; MS quadrupole temperature, 150 $^{\circ}$ C; electron multiplier voltage, 2060 V. For the data processing, the MSD ChemStation E.02.00 (Agilent) software program was used.

[0133] *Structure Elucidation of Polyol Esters of Fatty Acids*. Mass Frontier v. 7.0 (Thermo Scientific, Palo Alto, CA, USA) was used for structure elucidation. MS/MS spectra were submitted in MSP format (text files containing spectra in the NIST MS Search format) and structures in MOL format prepared in ACD/ChemSketch software (Advanced Chemistry Development, Toronto, Ontario, Canada).

RESULTS AND DISCUSSION

[0134] *R. babjevae* UCDFST 04-877 was isolated from a female olive fly trapped in an olive tree at UC Davis Campus, California, USA, in 2004. This yeast is of commercial interest since it has been described to produce high amounts of intracellular lipid mainly in the form of triacylglycerols(18) as well as being able to grow in carbon sources different from glucose (e.g., glycerol) and the presence of inhibitors.(20) *R. babjevae* is a close relative of *R. glutinis* and *R. graminis*, which were identified in the 1960s to produce a heavy oil.(9) Given the analytic capabilities of the time, researchers were able to identify only broadly the constituents of the oil, but failed to provide a detailed profile of the molecules and the exact structures as the present study is providing. These glycolipids are observed as droplets that sink when grown in a nutrient-limited media under aeration conditions like the one used in the present study.

[0135] A multiplatform mass spectrometry-based approach was used for identification and characterization of extracellular lipids produced by yeast *R. babjevae*

UCDFST 04-877. Figure 4 summarizes the workflow integrating the analysis of native molecules and the detailed characterization of hydrolyzed glycolipid precursors with the interpretation of mass spectra by in-silico software for structure elucidation. As discussed in subsequent sections, combining information obtained from both liquid chromatography–mass spectrometry (LC-MS) and gas chromatography–mass spectrometry (GC-MS) was essential for this task.

[0136] Structural Elucidation of Glycolipids by Chemical Analysis. The isolated extracts of glycolipids were separated using reversed-phase liquid chromatography (RPLC) on a C18 column followed by mass spectrometric (MS) detection in positive electrospray ionization (ESI) mode. Several features in the mass range of m/z 592–734 (as ammonium adducts) were found in the chromatogram (Figure 5). For some of them, calculated elemental compositions matched those of published sophorolipid species.(21) However, follow-up analysis of a commercially available standard of sophorolipids containing dominantly 17-L-[(2'-O- β -D-glucopyranosyl- β -D-glucopyranosyl)oxy]-cis-9-octadecenoic acid 1',4"-lactone 6',6"-diacetate (>70% purity) showed that this glycolipid detected as $[M + NH_4]^+$ and $[M + Na]^+$ molecular species had a retention time of 1.48 min, while the unknown glycolipid with the same elemental composition ($C_{34}H_{56}O_{14}$) and detected as the same molecular species eluted later at 3.16 min. Additional MS/MS analysis confirmed also differences in MS/MS spectra of these compounds. Because most of the elemental compositions obtained by the analysis of these native glycolipids did not provide satisfactory results in databases such as PubChem (22), SciFinder (23), or ChemSpider,(24) further experiments were conducted in order to determine the structure of these extracellular lipids.

[0137] Typical glycolipids consist of both lipophilic (e.g., fatty acid) and hydrophilic (e.g., sugar) constituents (25). Individual substructural components of glycolipids must be characterized by hydrolysis, under either acidic, alkaline, or enzymatic conditions (26). Hydrolysis using HCl and NaOH was used, both in methanol. The hydrolysis of sophorolipids consisting mainly of 17-L-[(2'-O- β -D-glucopyranosyl- β -D-glucopyranosyl)oxy]-cis-9-octadecenoic acid 1',4"-lactone 6',6"-diacetate was used as a positive control, which correctly provides 17-hydroxyoctadecenoic acid and glucose after hydrolysis treatment. This standard was used for quality control to ensure that the hydrolysis and derivatization reactions were working properly.

[0138] The hydrolysis was conducted at 55 °C for 2 h using (i) 1 M HCl in methanol and (ii) 1 M NaOH in methanol. The hydrolysates were neutralized for initial untargeted screening. A small volume of the extract was further diluted with acetonitrile–water (80:20, v/v) and separated using hydrophilic interaction chromatography (HILIC). Accurate mass detection in negative ESI was used because it permits detection of both fatty acids and sugars, typical constituents of glycolipids, including the determination of sum formulas, which is very hard to do in GC-MS. Using this method, both free fatty acids and polar constituents of these glycolipids were annotated (Figure 6). Querying the Metlin (27) and HMDB (28) databases for the detected accurate masses, the fatty acid constituents were annotated as saturated hydroxy fatty acids with elemental compositions of $C_{14}H_{28}O_3$, $C_{16}H_{32}O_3$, and $C_{18}H_{36}O_3$. The polar constituents were found to be $C_5H_{12}O_5$ and $C_6H_{14}O_6$, indicating sugar alcohols (polyols), rather than the hexoses ($C_6H_{12}O_6$) observed in the sphorolipid control.

[0139] The position of the hydroxy group in the hydroxy fatty acids could not be identified by LC-MS/MS because current databases, including Metlin, lack MS/MS spectra for these compounds. In addition, the fragment ions of fatty acids are in general less abundant compared to precursor ions, making the identification even more difficult. Similarly, common sugar alcohols provide almost identical MS/MS spectra in LC-MS analyses. When retention time was used for reliable identification of different sugar alcohol standards under HILIC conditions, most sugar alcohols did not separate chromatographically under the conditions used in the present study. Gas chromatography–mass spectrometry (GC-MS) was therefore used, permitting improved peak capacity and availability of more comprehensive electron ionization (EI) libraries (e.g., NIST 14) for compounds such as fatty acids.

[0140] The hydrolyzed extracts were submitted to liquid–liquid extraction using hexane in order to isolate hydroxy fatty acid methyl esters. Because the hydrolysis was conducted in methanol solution, it was possible to inject the hexane extracts directly and detect the hydroxy fatty acids as methyl esters. However, for confirmation purposes, follow-up derivatization using N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was conducted for derivatization of the free hydroxy group of particular hydroxy fatty acids. Separation of both types of derivatized extracts was conducted on a GC column with a DB-225ms polar stationary phase. The lipids were unambiguously assigned as hydroxy fatty acids with the hydroxy group in the β -position (C3), interpreting the characteristic

fragments m/z 103 and m/z 175 for 3-hydroxy fatty acids detected as methyl esters or 3-trimethylsiloxy methyl esters, respectively (Figure 7). These assignments were confirmed by analyzing authentic standards of 3-hydroxypalmitic and 3-hydroxystearic acids with matching electron ionization spectra and retention times

5 **[0141]** For analysis of polar constituents, the reaction mixture was neutralized, followed by evaporation of small aliquots, conducting a two-step derivatization (methoximation and silylation) and separation of derivatives on a GC column with a nonpolar stationary phase (Rtx-5Sil MS). A series of authentic standards of sugar alcohols (C5-polyols: xylitol, D-/L-arabitol, ribitol; C6-polyols: D-/L-mannitol, dulcitol, glucitol)
10 were derivatized and analyzed in the same way. Although only silylation is needed for derivatization of sugar alcohols, methoximation was also used because the hydrolysates might contain less volatile matrix components containing a carbonyl group. Indeed, methoximation was needed for derivatization of glucose during analysis of a sophorolipid standard (quality control). Matching retention time and mass spectra confirmed that the
15 glycolipids contain arabitol and mannitol in their structures (Figure 8).

[0142] Finally, an enantioselective analysis was performed using GC-MS in order to determine the D- or L-configuration of arabitol and mannitol and the R- or S-configuration of 3-hydroxy fatty acids. For enantioselective analysis of polyols, the chiral GC Chirasil-Dex CB phase was used, which permitted excellent separation of both D-/L-arabitol and D-
20 /L-mannitol as trifluoroacetyl (TFA) derivatives. Analysis of the extracellular lipid hydrolysates confirmed that polyols are D-arabitol and D-mannitol (Figure 9).

[0143] Under these conditions, the R- or S-configuration of 3-hydroxy fatty acids did not separate, mainly because they eluted at the upper temperature limit of the GC column (200 °C), where the effect of the chiral phase for separation is negligible. In order to
25 verify the stereoconfiguration, 3-hydroxy fatty acids were converted into methyl esters followed by derivatization of the free 3-hydroxy group using (R)-(-)- α -methoxy- α -trifluoromethylphenylacetyl chloride [(R)-(-)-MTPA-Cl, Mosher's reagent]. Corresponding MTPA-O-fatty acid methyl ester diastereomers (Mosher's esters) were then separated on a nonpolar GC column.(29) As Figure 10 shows, extracellular lipid hydrolysates contained all
30 detectable 3-hydroxy fatty acids in the R-configuration. When running R- and S-standards of 3-hydroxy C14:0, the S-diastereomer eluted earlier compared to its R-counterpart, as reported earlier (29, 30).

[0144] *Structural Elucidation of Glycolipids by in-Silico Assembly*. Similar components of extracellular glycolipids were reported for *R. glutinis* more than 50 years ago,(9) albeit without proving absolute configurations and without detailing the masses of the intact lipids. As stated before, the masses of the intact glycolipids were observed in the
5 initial untargeted RPLC-QTOFMS screen in a mass range of m/z 592–734 (as ammonium adducts, Table 6). Condensing 3-hydroxystearate (or -palmitate) to mannitol or arabitol resulted in masses ranging between 406 and 464 Da. The difference from the observed masses indicated that these glycolipids were acetylated by 4–6 acetyl groups. In all cases, the hydroxy groups of the fatty acids were acetylated, as evidenced by treating 3-acetoxy
10 fatty acid polyol esters with alkaline sodium methoxide, which led to the formation of α,β -unsaturated palmitic and stearic esters via β -elimination of acetic acid.

TABLE 6
Identified Extracellular Lipids (Polyol Esters of Fatty Acids) Secreted by *Rhodotorula babjevae* UCDFST 04-877

peak number ^a	<i>t_R</i> (min)	<i>m/z</i> [M+NH ₄] ⁺	elemental composition	acetylated (<i>R</i>)-3-hydroxy fatty acid	D-polyol	degree of acetylation of polyol
1	2.43	606.349	C29H48O12	C14:0	arabitol	4
2	2.48	622.3803	C30H52O12	C16:0	mannitol	3
3	2.8	592.3697	C29H50O11	C16:0	arabitol	3
4	2.87	664.3908	C32H54O13	C16:0	mannitol	4
5	3.07	634.3803	C31H52O12	C16:0	arabitol	4
6	3.1	706.4014	C34H56O14	C16:0	mannitol	5
7	3.12	650.4116	C32H56O12	C18:0	mannitol	3
8	3.27	620.401	C31H54O11	C18:0	arabitol	3
9	3.29	692.4221	C34H58O13	C18:0	mannitol	4
10	3.44	662.4116	C33H56O12	C18:0	arabitol	4
11	3.46	734.4327	C36H60O14	C18:0	mannitol	5

[0145] In order to confirm the complete, intact structures of the observed glycolipids, Mass Frontier software was used to evaluate and interpret mass spectral data based on acquired MS/MS spectra and proposed structures.(31) Structures were first submitted consisting of acetylated (R)-3-hydroxypalmitic acid and acetylated (R)-3-hydroxypalmitic stearic acid condensed to completely acetylated D-arabitol (Table 6 and Figure 5; peaks 5 and 10). Predicted fragment structures matched accurate masses (<5 ppm) of fragments. This was further confirmed by comparing fragment structures differing by 28 Da (corresponding to C₂H₄ increase in the fatty acyl chain), containing fatty acid moieties such as m/z 497 (peak 5 in Figure 5) vs m/z 525 (peak 10 in Figure 5) or m/z 237 (peak 5, Figure 5) vs m/z 265 (peak 10, Figure 5), and fragments originating from acetylated D-arabitol, which were identical for both glycolipids (e.g., m/z 141, m/z 201, and m/z 303); see Figure 11.

[0146] Less abundant glycolipids (Table 6 and Figure 5; peaks 4 and 9) consisted of acetylated (R)-3-hydroxypalmitic acid or acetylated (R)-3-hydroxystearic acid, each condensed to partially acetylated D-mannitol with one nonacetylated hydroxy group in the sugar alcohol. All possible structures containing one free hydroxy group were probed; however, MS/MS prediction results using Mass Frontier were ambiguous with respect to the exact location of the nonacetylated hydroxy group. In fact, the observed peaks 4 and 9 in Figure 5 might be a mixture of isomers. Fragment structures were observed differing in a 28 Da fatty acid moiety (e.g., m/z 237 vs m/z 265; m/z 467 vs m/z 495; m/z 527 vs m/z 555; m/z 569 vs m/z 597) and fragments originating from partially acetylated mannitol, which were identical for both glycolipids (e.g., m/z 153, m/z 231, m/z 273, m/z 333) (Figure 12).

[0147] Combining all information collected, the full structures (3-hydroxy fatty acid, polyol, and degree of acetylation) of all detected glycolipids were completed (Figure 13 and Table 6). Polyol lipids containing 3-hydroxypalmitic acid were the most abundant, followed by (R)-3-hydroxystearic acid and (R)-3-hydroxymyristic acid (detectable as trace amount, see peak 1 in Figure 5). D-Arabitol was the dominating polyol unit in these extracellular lipids (Table 6 and Figure 5).

SUMMARY AND CONCLUSIONS

[0148] This study demonstrated the combined use of several chromatography–mass spectrometry-based methods that together enabled the complete identification and characterization of 11 extracellular lipids produced by the yeast *R. babjevae* UCDFST 04-877, including absolute configurations. Use of LC–accurate mass fragmentation analysis

was indispensable to obtain knowledge of the intact glycolipids, including final validation of complete structures by software-guided interpretation of the MS/MS spectra. However, LC-MS analysis alone was incapable of providing sufficient information for full elucidation, either with or without hydrolysis of the glycolipids into substructure components. GC-MS with various derivatization methods showed superior ability to distinguish sugar alcohol isomers, readily identified the hydroxy fatty acids, and proved to be required for defining absolute configuration analysis. This workflow shows the importance of combining different mass spectrometry-based methods together with structure elucidation using in-silico fragmentation software programs in order to determine structures of intact glycolipids.

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Example 3

Scaling Up Into 7L (4L Culture Volume) Fed Batch Fermentors

[0149] Yeast strains *Rhodospiridium aff. paludigenum* (UCDFST 81-84) and *Rhodospiridium babjevae* (UCDFST 04-877) were cultured in 4L culture volume in batch fermentors. A total of 11 runs were done. The plots for each run are depicted in Figure 14, and values at the time of harvest for cell dry weight, crude extracellular lipid and total microbial conversion are summarized in Table 7.

[0150] For all 11 runs, a seed culture was prepared by inoculating two 500 mL baffled flasks containing 95 mL of Medium A (50 g/L of either glucose or sucrose, depending on the run) with 5 mL cell suspension prepared as described in Example 1, and incubating it at room temperature for 24 hours at 200 RPM. The seed culture was transferred to a 7 L bioreactor containing 4 liters of Medium A with either glucose or sucrose with varying sugar concentrations depending on the run (see Table 7). Some of the runs had additional glucose dosings at 48 hours. Cultures were incubated for 7 days at 27°C, 0.5 VVM (air) and agitation looped to the dissolved oxygen (DO) sensor to maintain a minimum of 40% DO during the entire fermentation. The starting pH of the media was

6.5 in all cases, and no acid or base were added during the course of the fermentation, because it was observed that adjusting the pH resulted in decreased PEFA production (results not shown). The pH values decreased during the course of the fermentation to final values of 6.3, 6.2 and 6.2. Temperature was kept constant at 27°C. No antifoam was added during the course of fermentation, because when PEFA production began, the initial foam present on the surface of the cultures decreased until it disappeared completely.

[0151] Recovery of cells and secreted PEFA in bioreactors: Fifty mL samples were collected to monitor cell growth, glucose consumption and PEFA production. Samples were shaken thoroughly and divided into two sets of 10 mL aliquots. The first set was extracted twice with 40 mL of fresh ethyl acetate in duplicate, the solvent was removed using a vacuum speed concentrator (miVac Duo®, Genevac Inc., Stone Ridge, NY, USA), and the dry weight of the PEFA residue was recorded. The second set of 10 mL cultures were centrifuged in duplicate as described above and washed with DSW twice, and lyophilized as described above to obtain the dry weight. CMP was then determined by subtracting the PEFA output to the CMP values. Glucose concentration was determined via hydrophilic interaction chromatography–mass spectrometry (HILIC–MS). A volume of 10 µL of sample (sugar solution) was mixed with 990 µL acetonitrile/water (80:20, v/v) mixture containing *d*₇-glucose (Sigma–Aldrich, St. Louis, MO) at a concentration of 250 µg/mL. After brief vortexing and centrifugation (12,000 rpm for 2 min) 100 µL aliquot was transferred to a glass vial and submitted to HILIC–MS analysis. Quantification was conducted analyzing glucose and sucrose (Sigma–Aldrich) solutions (1–1500 µg/mL) in acetonitrile/water (80:20, v/v) mixture containing *d*₇-glucose (internal standard) at a concentration of 250 µg/mL. The system consisted of an Agilent 1290 Infinity LC system (Agilent Technologies, Santa Clara, CA) with a pump (G4220A), a column oven (G1316C), an autosampler (G4226A), and an Agilent 6550 QTOFMS. Diluted media samples were separated on an Acquity UPLC BEH Amide column (150 × 2.1 mm; 1.7 µm) coupled to an Acquity UPLC BEH Amide VanGuard pre-column (5 × 2.1 mm; 1.7 µm) (Waters). The column was maintained at 45°C at a flow-rate of 0.4 mL/min. The mobile phases consisted of (A) water with ammonium formate (10 mM) and formic acid (0.125%) and (B) 95:5 (v/v) acetonitrile/water with ammonium formate (10 mM) and formic acid (0.125%). The separation was conducted under the following gradient: 0 min 100% (B); 0–2 min 100% (B); 2–7.7 min 70% (B); 7.7–9.5 min 40% (B); 9.5–10.25 min 30% (B); 10.25–12.75 min 100% (B); 12.75–17.75 min 100% (B). A sample volume of 3 µL was used for the injection.

Sample temperature was maintained at 4°C. The QTOF instrument was operated in electrospray ionization in negative mode with following parameters: mass range: m/z 50–1700; capillary voltage: –3 kV; nozzle voltage: –1 kV; gas temperature, 200°C; drying gas (nitrogen), 14 L/min; nebulizer gas (nitrogen), 35 psi; sheath gas temperature, 350°C; 5 sheath gas flow (nitrogen), 11 L/min; acquisition rate: 2 spectra/s. For the data processing MassHunter Qualitative (B.05.00) and Quantitative (B.05.01) Analysis (Agilent) software programs were used.

[0152] Samples from run 6 and 7 were used to determine the physicochemical and surfactant properties of the PEFA mixtures, as described in Example 3.

TABLE 7

Run number	Yeast Strain	UCDFST Strain ID	Type of carbon source used	Amount and Feeding Strategy	Dry Cell weight (g/L)	Crude extracellular lipid (g/L)	Total Microbial Conversion (g/L)
1	<i>R. babjevae</i>	04-877	Glucose	50 g/L initial	16.59	3.98	20.6 (41.1% conversion)
2	<i>R. aff. paludigemum</i>	81-84	Glucose	50 g/L initial	14.39	7.69	22.1 (44.16% conversion)
3	<i>R. aff. paludigemum</i>	81-84	Glucose	100 g/L initial 50 g/L at 48 hours	22.71	15.22	37.9 (25.28% conversion)
4	<i>R. babjevae</i>	04-877	Glucose	100 g/L initial, 50 g/L at 48 hours	32.77	7.32	40.1 (26.73% conversion)
5	<i>R. babjevae</i>	04-877	Glucose	100 g/L initial	29.22	8.55	37.8 (37.77% conversion)
6	<i>R. aff. paludigemum</i>	81-84	Glucose	100 g/L initial	21.87	14.48	36.4 (36.35% conversion)
7	<i>R. aff. paludigemum</i>	81-84	Sucrose	50 g/L initial	16.66	9.53	26.2 (52.38% conversion)
8	<i>R. aff. paludigemum</i>	81-84	Sucrose	100 g/L initial	28.84	19.44	48.3 (48.28% conversion)
9	<i>R. aff. paludigemum</i>	81-84	Sucrose	150 g/L initial, 50 g/L at 48 hours,	23.24	21.48	44.7 (22.36% conversion)
10	<i>R. paludigena</i>	81-492	Glucose	100 g/L initial	18.6	17.5	36.1 (36.1% conversion)
11	<i>R. aff. paludigena</i>	81-84	Glycerol	100 g/L initial	20.3	18.7	39.0 (39.0% conversion)

[0153] The results are consistent with the conclusion that increasing amounts of product with increasing amount of carbon source. However, the efficiency with increasing amount of carbon source tends to drop in this particular condition. Adjusting the glucose concentration and feed rate, and aeration, can increase yields even more.

5

Example 4

Identifying the Physicochemical and Surfactant Properties of the PEFA Mixtures

[0154] The solubility, surface tension, surfactant particle size, emulsification index and size distribution under the microscope were analyzed for the crude PEFA mixtures obtained in runs 5 and 6. Sophorolipid (Sigma–Aldrich, catalog number 53735, 1',4"-Sophorolactone 6',6"-diacetate), one of the most well understood biosurfactants from yeast, was used as control.

[0155] **Solubility:** The water solubility of the PEFA was investigated by visual and turbidimetric detection (Transilluminator, FBTIV-816, Fisher Scientific). A blender (Ultra Turrax T25, IKA Labortechnik) was used to make a stock mixture of sophorolipids and water in a concentration of 1000 mg/L. The blending time was 1 minute and the blend mode was set to 11000 / min. The mixtures of run 5 and run 6 were soluble to up to 10 mg/L, which was below the solubility of the control.

[0156] **Surface Tension:** Surface tensions were measured, using Wilhelmy plate method (Digital Tensiometer K10ST, KRÜSS) for different concentrations of PEFA in aqueous solution. The stabilization time was from 30 minutes in higher concentration to as long as 2 hours in lower concentration (below 2.5 mg/L). The final data was recorded when the measured surface tension became stable. The results are summarized in Table 8.

TABLE 8					
Surfactants	Minimum surface tension (mN/m)	Critical Micelle Concentration (mg/L)	Slope of linear part for surface tension line (mN/m)	Surface excess concentration (mol/m ²)	Average area occupied by each molecule (Å ² /molecule)
<i>R. babjevae</i> UCDFST 04-877	30.4	1.5	−11.14	4.5×10^{-6}	35.7
<i>R. aff. paludigenum</i> UCDFST 81-84	33.4	1.3	−31.94	1.29×10^{-5}	12.5
Sophorolipid	36.8	15.2	−11.14	2.93×10^{-6}	54.7

[0157] From a practical standpoint, a smaller dose of surfactant for both the mixture produced by strain *R. babjevae* UCDFST 04-877 and *R. aff. paludigenum* UCDFST 81-84 was required to achieve the desired surfactant activity when compared to the control.

[0158] **Surfactant Particle Size Analysis:** Zetasizer (Malvern Instruments Inc. Westborough, MA) was used to measure the surfactant particle size in water by dynamic light scattering. The measurement angle was 90°. The temperature was set to be 25°C. The refractive index was set to be 1.52. The results showed that both biosurfactants were able to create smaller particles than the control, as shown in Table 9. Smaller averages of diameters relate to a higher interfacial surface, which is a desired trait in surfactants.

TABLE 9	
Surfactants	Z-Average of diameter (nm)
<i>R. babjevae</i> UCDFST 04-877	298.2
<i>R. aff. paludigenum</i> UCDFST 81-84	491.9
Commercial sophorolipid	749.2

[0159] **Size distribution under the microscope:** Firstly, Nile Red was added in octane in the concentration of 0.05 mg/mL (0.005 wt%). The actual amount of dissolved Nile Red is much smaller than the added amount. Methylene Blue was added into the water in the concentration of 0.004 mg/mL (0.0004 wt%). Then 1 ml of dyed water and 1 ml of dyed octane were mixed in a 2-ml centrifuge tube. Then the tube is vortexed for 2 minutes using S8220 Deluxe mixer (EVANSTON, Illinois, 60201) in the mode of “FAST”. Optical microscope (Carl Zeiss AG) was used for observation. The emulsions were observed immediately after they are prepared (within 30 minutes, see Figure 15).

[0160] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

CLAIMS

What is claimed is:

1. A yeast culture comprising: a population of basidiomycetous yeast cells, one or more hydrophilic carbon sources, and/or at least about 1 g/L polyol lipid.

5 2. The yeast culture of claim 1, wherein the culture does not comprise one or more hydrophobic carbon sources.

3. The yeast culture of any one of claims 1 to 2, wherein the culture does not comprise one or more hydrophobic carbon sources selected from the group consisting of oils, hydrocarbons, unsaturated hydrocarbons, fatty acids, fatty esters
10 including glycerides and mixtures thereof, alcohols, diols, sterols, waste streams, lignocellulosic hydrolysates, industrial co-products and mixtures thereof.

4. A yeast culture comprising: a population of basidiomycetous yeast cells, one or more hydrophilic carbon sources, one or more hydrophobic carbon sources and at least about 1 g/L polyol lipid.

15 5. The yeast culture of any one of claims 1 to 4, wherein the culture is a liquid culture.

6. The yeast culture of any one of claims 1 to 5, wherein the culture comprises less than about 2% (w/v) nitrogen.

7. The yeast culture of any one of claims 1 to 6, wherein the culture
20 comprises nitrogen in the range of about 0.005% (w/v) to about 2% (w/v).

8. The yeast culture of any one of claims 1 to 7, wherein the nitrogen source is selected from the group consisting of ammonia, ammonium salt, nitrate, nitrite, nucleotides, nucleosides, proteins, peptides, amino acids, urea and its derivatives, and mixtures thereof.

25 9. The yeast culture of any one of claims 1 to 8, wherein the culture comprises about 0.05% (w/v) ammonium chloride.

10. The yeast culture of any one of claims 1 to 9, wherein the culture comprises an iron salt.

11. The yeast culture of claim 10, wherein the iron salt is selected from the group consisting of iron chloride (II) & (III), iron sulfate (II) & (III), iron nitrate (II) & (III), iron phosphate (II) & (III), fumarate, iron edetate, iron citrate, iron malate, iron oxalate, iron tartrate, iron succinate, iron acetate, in their anhydrous or their hydrated states, and organometallic complexes thereof.

12. The yeast culture of any one of claims 10 to 11, wherein the culture comprises the iron salt in a concentration in the range of about 0.001 mg/L to about 10 g/L.

13. The yeast culture of any one of claims 1 to 12, wherein the culture comprises one or more hydrophilic carbon sources at a concentration in the range of about 0.2% (w/v) to about 70% (w/v).

14. The yeast culture of any one of claims 1 to 13, wherein the culture comprises a carbon to nitrogen ratio of about 5:1 to about 400:1.

15. The yeast culture of claim 14, wherein the culture comprises a carbon to nitrogen ratio of about 30:1 to about 70:1.

16. The yeast culture of any one of claims 1 to 15, wherein the hydrophilic carbon source is selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, sugar alcohols, polyols, organic acids, esters, aldehydes, ketones, alcohols, waste streams, plant materials, lignocellulosic hydrolysates, industrial co-products and mixtures thereof.

17. The yeast culture of any one of claims 1 to 16, wherein the hydrophilic carbon source is a monosaccharide, an oligosaccharide or a polysaccharide and comprises one or more sugar moieties selected from the group consisting of glucose, sucrose, xylose, galactose, rhamnose, arabinose, mannose, cellobiose, galacturonic acid, lactose, sophorose, glycerol, and mixtures thereof.

18. The yeast culture of any one of claims 1 to 17, wherein the population of basidiomycetous yeast cells comprises cells within the taxonomic class Microbotryomycetes.

19. The yeast culture of any one of claims 1 to 18, wherein the population of basidiomycetous yeast cells comprises cells within the taxonomic order Sporidiobolales.

20. The yeast culture of any one of claims 1 to 19, wherein the population of basidiomycetous yeast cells is from genera classified within the taxonomic order Sporidiobolales selected from the group consisting of *Rhodosporidiobolus*, *Rhodotorula*, and *Sporobolomyces*.

21. The yeast culture of any one of claims 1 to 20, wherein the population of basidiomycetous yeast cells comprises *Rhodotorula* cells.

22. The yeast culture of claim 21, wherein the *Rhodotorula* cells are *Rhodotorula babjevae* cells.

23. The yeast culture of any one of claims 1 to 20, wherein the population of basidiomycetous yeast cells comprises one or more species selected from the group consisting of *Rhodotorula babjevae*, *Rhodotorula diobovata*, *Rhodotorula kratochvilovae*, *Rhodotorula paludigena*, *Rhodotorula* aff. *paludigena*, and *Rhodotorula dairenensis*.

24. The yeast culture of any one of claims 1 to 20, wherein the population of basidiomycetous yeast cells comprises one or more strains selected from the group consisting of:

- a) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain NRRL Y-67018 (also deposited as UCDFST 04-877),
- b) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain NRRL Y-67017 (also deposited as UCDFST 05-775),
- c) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 68-916.1,
- d) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 67-458,
- e) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 05-736,
- f) *Rhodotorula diobovata* (syn. *Rhodosporidium diobovatum*) strain UCDFST 04-830,
- g) *Rhodotorula diobovata* (syn. *Rhodosporidium diobovatum*) strain NRRL Y-67015 (also deposited as UCDFST 08-225),

h) *Rhodotorula kratochvilovae* (syn. *Rhodosporidium kratochvilovae*) strain NRRL Y-67016 (also deposited as UCDFST 05-632),

i) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain NRRL Y-67012 (also deposited as UCDFST 09-163),

5 j) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain UCDFST 81-492,

k) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain UCDFST 82-646.2,

10 l) *Rhodotorula* aff. *paludigena* (syn. *Rhodosporidium* aff. *paludigenum* strain NRRL Y-67009 (also deposited as UCDFST 81-84), and

m) *Rhodotorula dairenensis* strain NRRL Y-67011 (also deposited as UCDFST 68-257).

25. The yeast culture of any one of claims 1 to 20, wherein the population of basidiomycetous yeast cells comprises one or more strains selected from the group consisting of:

a) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 68-916.1,

b) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 67-458,

c) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 05-736,

20 d) *Rhodotorula diobovata* (syn. *Rhodosporidium diobovatum*) strain UCDFST 04-830,

e) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain UCDFST 81-492, and

f) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain UCDFST 82-646.2.

25 26. The yeast culture of any one of claims 1 to 25, wherein the polyol lipid comprises a 3-hydroxy fatty acid moiety comprising from 10 to 24 carbon atoms in length.

27. The yeast culture of claim 26, wherein the polyol lipid comprises a sugar alcohol moiety esterified to the carboxyl end of the 3-hydroxy fatty acyl moiety.

30 28. The yeast culture of any one of claims 26 to 27, wherein the 3-hydroxy fatty acyl moiety can be acetylated.

29. The yeast culture of any one of claims 1 to 28, wherein the polyol lipid sugar alcohol moiety comprises non-esterified hydroxy groups that can be acetylated.

30. The yeast culture of any one of claims 1 to 29, wherein the polyol lipid sugar alcohol moiety comprises a backbone selected from mannitol and arabitol.

5 31. The yeast culture of any one of claims 1 to 30, wherein the polyol lipid sugar alcohol moiety does not comprise xylitol.

32. The yeast culture of any one of claims 1 to 31, wherein the polyol lipid comprises one or more polyol esters of fatty acid (PEFAs).

10 33. The yeast culture of any one of claims 1 to 32, wherein the polyol lipid comprises one or more polyol esters of fatty acid (PEFAs) listed in Table 3.

34. The yeast culture of any one of claims 1 to 33, wherein the culture comprises a polyol lipid profile comprising:

i) at least about 20% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$);

15 ii) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{33}H_{56}O_{12}$); and/or

iii) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 4 acetylations (Molecular Formula: $C_{34}H_{58}O_{13}$).

20 35. The yeast culture of any one of claims 1 to 33, wherein the culture comprises a polyol lipid profile comprising at least about 25% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$).

36. The yeast culture of any one of claims 1 to 33, wherein the culture comprises a polyol lipid profile comprising:

25 i) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 3 acetylations (Molecular Formula: $C_{32}H_{56}O_{12}$);

ii) at least about 1% of an acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 4 acetylations (Molecular Formula: $C_{32}H_{54}O_{13}$);

30 iii) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 4 acetylations (Molecular Formula: $C_{34}H_{58}O_{13}$);

iv) at least about 15% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$); and/or

v) at least about 10% of an acetylated C18:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{33}H_{56}O_{12}$).

5 37. The yeast culture of claim 36, further comprising:

vi) at least about 2% of an acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{34}H_{56}O_{14}$); and/or

vii) at least about 1% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol (Molecular Formula: $C_{36}H_{60}O_{14}$).

10 38. The yeast culture of any one of claims 1 to 33, wherein the culture comprises a polyol lipid profile comprising:

i) at least about 1% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 4 acetylations (Molecular Formula: $C_{34}H_{58}O_{13}$);

15 ii) at least about 5% of an acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{34}H_{56}O_{14}$);

iii) at least about 10% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{36}H_{60}O_{14}$);

iv) at least about 15% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$); and/or

20 v) at least about 15% of an acetylated C18:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{33}H_{56}O_{12}$).

39. The yeast culture of any one of claims 1 to 33, wherein the culture comprises a polyol lipid profile comprising:

25 i) at least about 10% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 4 acetylations (Molecular Formula: $C_{34}H_{58}O_{13}$);

ii) at least about 5% of an acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{34}H_{56}O_{14}$);

iii) at least about 10% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{36}H_{60}O_{14}$);

30 iv) at least about 5% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$);

v) at least about 15% of an acetylated C18:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{33}H_{56}O_{12}$); and/or

vi) at least about 0.5% of an acetylated C14:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{29}H_{48}O_{12}$).

5 40. The yeast culture of any one of claims 1 to 33, wherein the culture comprises a polyol lipid profile comprising:

i) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 4 acetylations (Molecular Formula: $C_{34}H_{58}O_{13}$);

10 ii) at least about 10% of an acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{34}H_{56}O_{14}$);

iii) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{36}H_{60}O_{14}$);

iv) at least about 15% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$);

15 v) at least about 35% of an acetylated C18:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{33}H_{56}O_{12}$); and/or

vi) at least about 0.5% of an Acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 3 acetylations (Molecular Formula: $C_{30}H_{52}O_{12}$).

20 41. The yeast culture of any one of claims 1 to 33, wherein the culture comprises a polyol lipid profile comprising:

i) at least about 5% of an acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 4 acetylations (Molecular Formula: $C_{32}H_{54}O_{13}$);

ii) at least about 45% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$); and/or

25 iii) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{33}H_{56}O_{12}$).

42. The yeast culture of any one of claims 1 to 41, wherein the one or more polyol lipids are present in the medium in a form that can be harvested without solvent extraction.

30 43. A bioreactor comprising a yeast culture of any one of claims 1 to 42.

44. A method of producing one or more polyol lipids, comprising culturing a population of basidiomycetous yeast cells in a yeast cell culture comprising one or more hydrophilic carbon sources, wherein the culture does not comprise one or more hydrophobic carbon sources, whereby the basidiomycetous yeast cells produce one or more polyol lipids.

45. The method of claim 44, wherein the culture does not comprise one or more hydrophobic carbon sources selected from the group consisting of oils, alkanes, fatty acids, fatty esters and mixtures thereof.

46. A method of producing one or more polyol lipids, comprising culturing a population of basidiomycetous yeast cells in a yeast cell culture comprising one or more hydrophilic carbon sources and one or more hydrophobic carbon sources, whereby the basidiomycetous yeast cells produce one or more polyol lipids.

47. The method of any one of claims 44 to 46, wherein additional hydrophilic carbon source is added.

48. The method of any one of claims 44 to 47, wherein at least about 1 g/L polyol lipid is produced.

49. The method of any one of claims 44 to 48, wherein a polyol lipid yield in the range of about 0.5 g/L to about 30 g/L can be obtained from yeast cultures comprising in the range of about 10 g/L to about 250 g/L glucose.

50. The method of any one of claims 44 to 49, wherein a polyol lipid yield of at least about 14 g/L can be obtained from yeast cultures comprising about 50 g/L glucose.

51. The method of any one of claims 44 to 50, wherein the culture comprises less than about 2% (w/v) nitrogen.

52. The method of any one of claims 44 to 51, wherein the culture comprises nitrogen in the range of about 0.005% (w/v) to about 2% (w/v).

53. The method of any one of claims 44 to 52, wherein the nitrogen source is selected from the group consisting of ammonia, ammonium salt, nitrate, nitrite,

nucleotides, nucleosides, proteins, peptides, amino acids, urea and its derivatives, and mixtures thereof.

54. The method of any one of claims 44 to 53, wherein the culture comprises about 0.05% (w/v) ammonium chloride.

5 55. The method of any one of claims 44 to 54, wherein the culture comprises an iron salt.

56. The method of claim 55, wherein the iron salt is selected from the group consisting of iron chloride (II) & (III), iron sulfate (II) & (III), iron nitrate (II) & (III), iron phosphate (II) & (III), fumarate, iron edetate, iron citrate, iron malate, iron oxalate, iron
10 tartrate, iron succinate, iron acetate, in their anhydrous or their hydrated states, and organometallic complexes thereof.

57. The method of any one of claims 55 to 56, wherein the culture comprises the iron salt in a concentration in the range of about 0.001 mg/L to about 10 g/L.

58. The method of any one of claims 44 to 57, wherein the culture
15 comprises one or more hydrophilic carbon sources at a concentration in the range of about 0.2% (w/v) to about 50% (w/v).

59. The method of any one of claims 44 to 58, wherein the culture comprises a carbon to nitrogen ratio of about 5:1 to about 400:1.

60. The method of claim 59, wherein the culture comprises a carbon to
20 nitrogen ratio of about 30:1 to about 70:1.

61. The method of any one of claims 44 to 60, wherein the yeast culture is maintained at a temperature in the range of between about 24°C to about 27°C.

62. The method of any one of claims 44 to 61, wherein the hydrophilic carbon source is selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, sugar alcohols, polyols, organic acids, esters, aldehydes, ketones, alcohols,
25 waste streams, plant materials, lignocellulosic hydrolysates, industrial co-products and mixtures thereof.

63. The method of any one of claims 44 to 62, wherein the hydrophilic carbon source is a monosaccharide, an oligosaccharide or a polysaccharide and comprises one or more sugar moieties selected from the group consisting of glucose, sucrose, xylose, galactose, rhamnose, arabinose, mannose, cellobiose, galacturonic acid, lactose, sophorose, glycerol, and mixtures thereof.

64. The method of any one of claims 44 to 63, wherein the population of basidiomycetous yeast cells comprises cells within the taxonomic class Microbotryomycetes cells.

65. The method of any one of claims 44 to 64, wherein the population of basidiomycetous yeast cells comprises cells within the taxonomic order Sporidiobolales cells.

66. The method of any one of claims 44 to 65, wherein the population of basidiomycetous yeast cells is from genera classified within the taxonomic order Sporidiobolales selected from the group consisting of *Rhodosporiobolus*, *Rhodotorula*, and *Sporobolomyces*.

67. The method of any one of claims 44 to 66, wherein the population of basidiomycetous yeast cells comprises *Rhodotorula* cells.

68. The method of claim 67, wherein the *Rhodotorula* cells are *Rhodotorula babjevae* cells.

69. The method of any one of claims 44 to 66, wherein the population of basidiomycetous yeast cells comprises one or more species selected from the group consisting of *Rhodotorula babjevae*, *Rhodotorula diobovata*, *Rhodotorula kratochvilovae*, *Rhodotorula paludigena*, *Rhodotorula* aff. *paludigena*, and *Rhodotorula dairenensis*.

70. The method of any one of claims 44 to 66, wherein the population of basidiomycetous yeast cells comprises one or more strains selected from the group consisting of:

a) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain NRRL Y-67018 (also deposited as UCDFST 04-877),

b) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain NRRL Y-67017 (also deposited as UCDFST 05-775),

- c) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 68-916.1,
- d) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 67-458,
- e) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 05-736,
- 5 f) *Rhodotorula diobovata* (syn. *Rhodosporidium diobovatum*) strain UCDFST 04-830,
- g) *Rhodotorula diobovata* (syn. *Rhodosporidium diobovatum*) strain NRRL Y-67015 (also deposited as UCDFST 08-225),
- h) *Rhodotorula kratochvilovae* (syn. *Rhodosporidium kratochvilovae*) strain
- 10 NRRL Y-67016 (also deposited as UCDFST 05-632),
- i) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain NRRL Y-67012 (also deposited as UCDFST 09-163),
- j) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain UCDFST 81-492,
- 15 k) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain UCDFST 82-646.2,
- l) *Rhodotorula* aff. *paludigena* (syn. *Rhodosporidium* aff. *paludigenum*) strain NRRL Y-67009 (also deposited as UCDFST 81-84), and
- m) *Rhodotorula dairenensis* strain NRRL Y-67011 (also deposited as UCDFST
- 20 68-257).

71. The method of any one of claims 44 to 66, wherein the population of basidiomycetous yeast cells comprises one or more strains selected from the group consisting of:

- a) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 68-916.1,
- 25 b) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 67-458,
- c) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 05-736,
- d) *Rhodotorula diobovata* (syn. *Rhodosporidium diobovatum*) strain UCDFST 04-830,
- e) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain UCDFST 81-
- 30 492, and
- f) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain UCDFST 82-646.2.

72. The method of any one of claims 44 to 71, wherein the polyol lipid comprises a 3-hydroxy fatty acid moiety comprising from 10 to 24 carbon atoms in length.

73. The method of claim 72, wherein the polyol lipid comprises a sugar alcohol moiety esterified to the carboxyl end of the 3-hydroxy fatty acyl moiety.

5 74. The method of any one of claims 72 to 73, wherein the 3-hydroxy fatty acyl moiety can be acetylated.

75. The method of any one of claims 44 to 74, wherein the polyol lipid sugar alcohol moiety comprises non-esterified hydroxy groups that can be acetylated.

10 76. The method of any one of claims 44 to 75, wherein the polyol lipid sugar alcohol moiety comprises a backbone selected from mannitol and arabitol.

77. The method of any one of claims 44 to 76, wherein the polyol lipid sugar alcohol moiety does not comprise xylitol.

78. The method of any one of claims 44 to 77, wherein the polyol lipid comprises one or more polyol esters of fatty acid (PEFAs).

15 79. The method of any one of claims 44 to 78, wherein the polyol lipid comprises one or more polyol esters of fatty acid (PEFAs) listed in Table 4.

80. The method of any one of claims 44 to 79, wherein the culture comprises a polyol lipid profile comprising:

20 i) at least about 20% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$);

ii) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{33}H_{56}O_{12}$); and/or

iii) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 4 acetylations (Molecular Formula: $C_{34}H_{58}O_{13}$).

25 81. The method of any one of claims 44 to 79, wherein the culture comprises a polyol lipid profile comprising at least about 25% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$).

82. The method of any one of claims 44 to 79, wherein the culture comprises a polyol lipid profile comprising:

i) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 3 acetylations (Molecular Formula: $C_{32}H_{56}O_{12}$);

5 ii) at least about 1% of an acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 4 acetylations (Molecular Formula: $C_{32}H_{54}O_{13}$);

iii) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 4 acetylations (Molecular Formula: $C_{34}H_{58}O_{13}$);

10 iv) at least about 15% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$); and/or

v) at least about 10% of an acetylated C18:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{33}H_{56}O_{12}$).

83. The method of claim 82, further comprising:

15 vi) at least about 2% of an acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{34}H_{56}O_{14}$); and/or

vii) at least about 1% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol (Molecular Formula: $C_{36}H_{60}O_{14}$).

84. The method of any one of claims 44 to 79, wherein the culture comprises a polyol lipid profile comprising:

20 i) at least about 1% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 4 acetylations (Molecular Formula: $C_{34}H_{58}O_{13}$);

ii) at least about 5% of an acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{34}H_{56}O_{14}$);

25 iii) at least about 10% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{36}H_{60}O_{14}$);

iv) at least about 15% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$); and/or

v) at least about 15% of an acetylated C18:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{33}H_{56}O_{12}$).

30 85. The method of any one of claims 44 to 79, wherein the culture comprises a polyol lipid profile comprising:

i) at least about 10% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 4 acetylations (Molecular Formula: $C_{34}H_{58}O_{13}$);

ii) at least about 5% of an acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{34}H_{56}O_{14}$);

5 iii) at least about 10% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{36}H_{60}O_{14}$);

iv) at least about 5% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$);

10 v) at least about 15% of an acetylated C18:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{33}H_{56}O_{12}$); and/or

vi) at least about 0.5% of an acetylated C14:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{29}H_{48}O_{12}$).

86. The method of any one of claims 44 to 79, wherein the culture comprises a polyol lipid profile comprising:

15 i) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 4 acetylations (Molecular Formula: $C_{34}H_{58}O_{13}$);

ii) at least about 10% of an acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{34}H_{56}O_{14}$);

20 iii) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{36}H_{60}O_{14}$);

iv) at least about 15% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$);

v) at least about 35% of an acetylated C18:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{33}H_{56}O_{12}$); and/or

25 vi) at least about 0.5% of an Acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 3 acetylations (Molecular Formula: $C_{30}H_{52}O_{12}$).

87. The method of any one of claims 44 to 79, wherein the culture comprises a polyol lipid profile comprising:

30 i) at least about 5% of an acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 4 acetylations (Molecular Formula: $C_{32}H_{54}O_{13}$);

ii) at least about 45% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$); and/or

iii) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to

arabitol comprising 4 acetylations (Molecular Formula: $C_{33}H_{56}O_{12}$).

88. The method of any one of claims 44 to 87, wherein the method does not comprise actively pH-adjusting the culture.

89. The method of any one of claims 44 to 88, wherein the culture
5 volume to container volume ratio is 1:3 or greater.

90. The method of claim 93, wherein the container is a baffled flask.

91. The method of any one of claims 44 to 90, wherein the culture comprises dissolved oxygen at a concentration of a least 10% and up to 100%.

92. The method of any one of claims 44 to 91, further comprising
10 purifying and/or isolating the polyol lipid.

93. The method of claim 92, wherein the polyol lipid does not need to be purified from a hydrophobic carbon source.

94. The method of any one of claims 92 to 93, wherein the polyol lipid can be purified and/or isolated without cell lysis.

95. The method of any one of claims 44 to 94, wherein the yeast cells
15 secrete the one or more polyol lipids into the medium in a form that can be harvested without solvent extraction.

96. The method of any one of claims 44 to 95, wherein the method is performed as a batch, fed batch or continuous-feed process.

97. A polyol lipid composition produced according to the method of any
20 one of claims 44 to 96.

98. The polyol lipid composition of claim 97, wherein the composition is selected from the group consisting of a cleanser, a detergent, a surfactant, a wetting agent, an antifoam agent, an emulsifier, an emollient, a dispersant, a humectant, an antibacterial
25 agent, an antiviral agent, an antifungal agent, a spermicide, an insecticide, a lubricant, an adhesive, a crystal modifier, an instantizer, a viscosity modifier, a mixing/blending aid, a

release agent, a cream, a foam, a mousse, a lotion, a balm, an ointment, and an oleochemical composition.

99. The polyol lipid composition of any one of claims 97 to 98, wherein the polyol lipid composition is free of any hydrophobic carbon source.

5 100. A composition comprising one or more polyol esters of fatty acid (PEFAs) listed in Table 4.

101. The composition of claim 100, comprising a PEFA profile comprising:

- 10 i) at least about 20% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$);
- ii) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{33}H_{56}O_{12}$); and/or
- iii) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 4 acetylations (Molecular Formula: $C_{34}H_{58}O_{13}$).

15 102. The composition of any one of claims 100 to 101, wherein the culture comprises a PEFA profile comprising at least about 25% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$).

103. The composition of claim 100, comprising a PEFA profile comprising:

- 20 i) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 3 acetylations (Molecular Formula: $C_{32}H_{56}O_{12}$);
- ii) at least about 1% of an acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 4 acetylations (Molecular Formula: $C_{32}H_{54}O_{13}$);
- iii) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to 25 mannitol comprising 4 acetylations (Molecular Formula: $C_{34}H_{58}O_{13}$);
- iv) at least about 15% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$); and/or
- v) at least about 10% of an acetylated C18:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{33}H_{56}O_{12}$).

104. The composition of claim 103, further comprising:

vi) at least about 2% of an acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{34}H_{56}O_{14}$); and/or

vii) at least about 1% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol (Molecular Formula: $C_{36}H_{60}O_{14}$).

105. The composition of claim 100, comprising a PEFA profile comprising:

i) at least about 1% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 4 acetylations (Molecular Formula: $C_{34}H_{58}O_{13}$);

ii) at least about 5% of an acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{34}H_{56}O_{14}$);

iii) at least about 10% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{36}H_{60}O_{14}$);

iv) at least about 15% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$); and/or

v) at least about 15% of an acetylated C18:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{33}H_{56}O_{12}$).

106. The composition of claim 100, comprising a PEFA profile comprising:

i) at least about 10% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 4 acetylations (Molecular Formula: $C_{34}H_{58}O_{13}$);

ii) at least about 5% of an acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{34}H_{56}O_{14}$);

iii) at least about 10% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{36}H_{60}O_{14}$);

iv) at least about 5% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$);

v) at least about 15% of an acetylated C18:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{33}H_{56}O_{12}$); and/or

vi) at least about 0.5% of an acetylated C14:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{29}H_{48}O_{12}$).

107. The composition of claim 100, comprising a PEFA profile

comprising:

i) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 4 acetylations (Molecular Formula: $C_{34}H_{58}O_{13}$);

5 ii) at least about 10% of an acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{34}H_{56}O_{14}$);

iii) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to mannitol comprising 5 acetylations (Molecular Formula: $C_{36}H_{60}O_{14}$);

iv) at least about 15% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$);

10 v) at least about 35% of an acetylated C18:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{33}H_{56}O_{12}$); and/or

vi) at least about 0.5% of an Acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 3 acetylations (Molecular Formula: $C_{30}H_{52}O_{12}$).

15 108. The composition of claim 100, comprising a PEFA profile comprising:

i) at least about 5% of an acetylated C16:0 3-hydroxy fatty acid esterified to mannitol comprising 4 acetylations (Molecular Formula: $C_{32}H_{54}O_{13}$);

ii) at least about 45% of an acetylated C16:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{31}H_{52}O_{12}$); and/or

20 iii) at least about 5% of an acetylated C18:0 3-hydroxy fatty acid esterified to arabitol comprising 4 acetylations (Molecular Formula: $C_{33}H_{56}O_{12}$).

109. The composition of any one of claims 100 to 108, wherein the composition is selected from the group consisting of a cleanser, a detergent, a surfactant, a wetting agent, an antifoam agent, an emulsifier, an emollient, a dispersant, a humectant, an antibacterial agent, an antiviral agent, an antifungal agent, a spermicide, an insecticide, a lubricant, an adhesive, a crystal modifier, an instantizer, a viscosity modifier, a mixing/blending aid, a release agent, a cream, a foam, a mousse, a lotion, a balm, an ointment, and an oleochemical composition.

30 110. The composition of any one of claims 100 to 109, wherein the PEFA composition is free of any hydrophobic carbon source.

111. A yeast cell of a yeast strain selected from the group consisting of:

- a) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain NRRL Y-67018 (also deposited as UCDFST 04-877),
- b) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain NRRL Y-67017 (also deposited as UCDFST 05-775),
- 5 c) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 68-916.1,
- d) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 67-458,
- e) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 05-736,
- f) *Rhodotorula diobovata* (syn. *Rhodosporidium diobovatum*) strain UCDFST
- 10 04-830,
- g) *Rhodotorula diobovata* (syn. *Rhodosporidium diobovatum*) strain NRRL Y-67015 (also deposited as UCDFST 08-225),
- h) *Rhodotorula kratochvilovae* (syn. *Rhodosporidium kratochvilovae*) strain NRRL Y-67016 (also deposited as UCDFST 05-632),
- 15 i) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain NRRL Y-67012 (also deposited as UCDFST 09-163),
- j) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain UCDFST 81-492,
- k) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain
- 20 UCDFST 82-646.2,
- l) *Rhodotorula* aff. *paludigena* (syn. *Rhodosporidium* aff. *paludigenum*) strain NRRL Y-67009 (also deposited as UCDFST 81-84), and
- m) *Rhodotorula dairenensis* strain NRRL Y-67011 (also deposited as UCDFST 68-257).

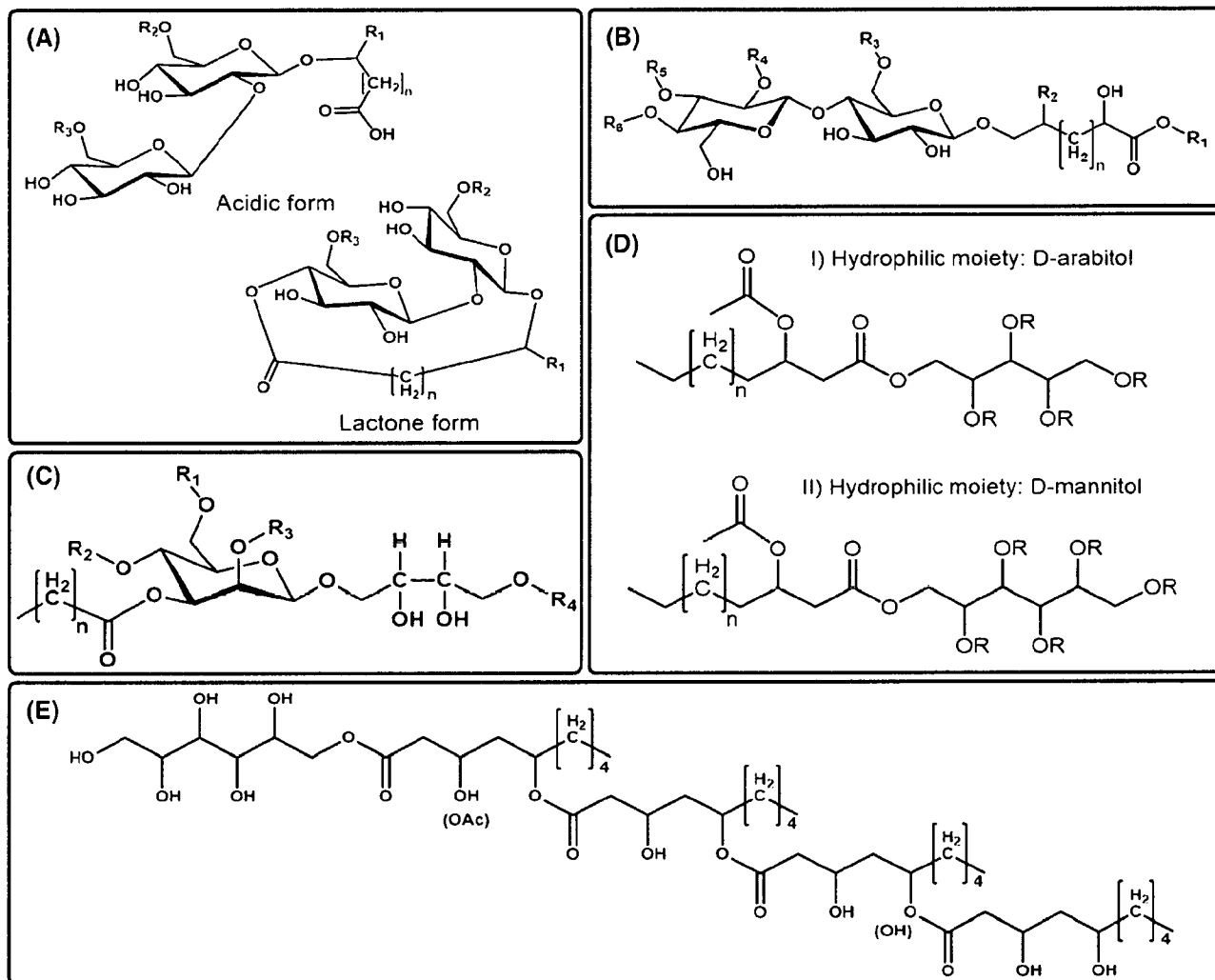
- 25 112. A yeast cell of a yeast strain selected from the group consisting of:
- a) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 68-916.1,
 - b) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 67-458,
 - c) *Rhodotorula babjevae* (syn. *Rhodosporidium babjevae*) strain UCDFST 05-736,
 - d) *Rhodotorula diobovata* (syn. *Rhodosporidium diobovatum*) strain UCDFST 04-
 - 30 830,
 - e) *Rhodotorula paludigena* (syn. *Rhodosporidium paludigenum*) strain UCDFST 81-492, and

f) *Rhodotorula paludigena* (syn. *Rhodospiridium paludigenum*) strain UCDFST 82-646.2.

113. A population of yeast cells comprising one or more yeast strains of any one of claims 111 to 112.

5 114. A yeast culture comprising one or more yeast strains of any one of claims 111 to 112 or a population of yeast cells of claim 113.

1/17

**Fig. 1A-E**

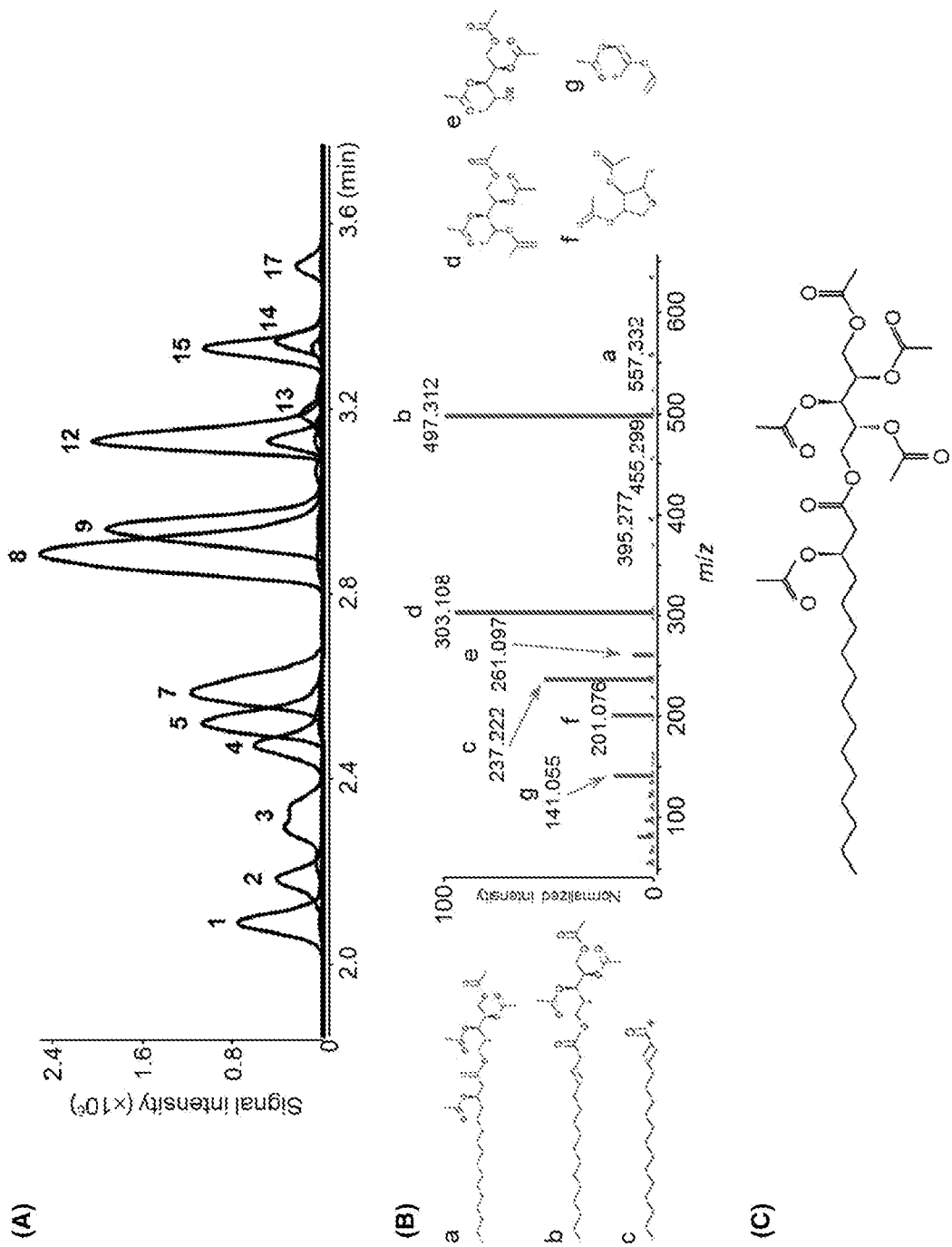


Fig. 2A-C

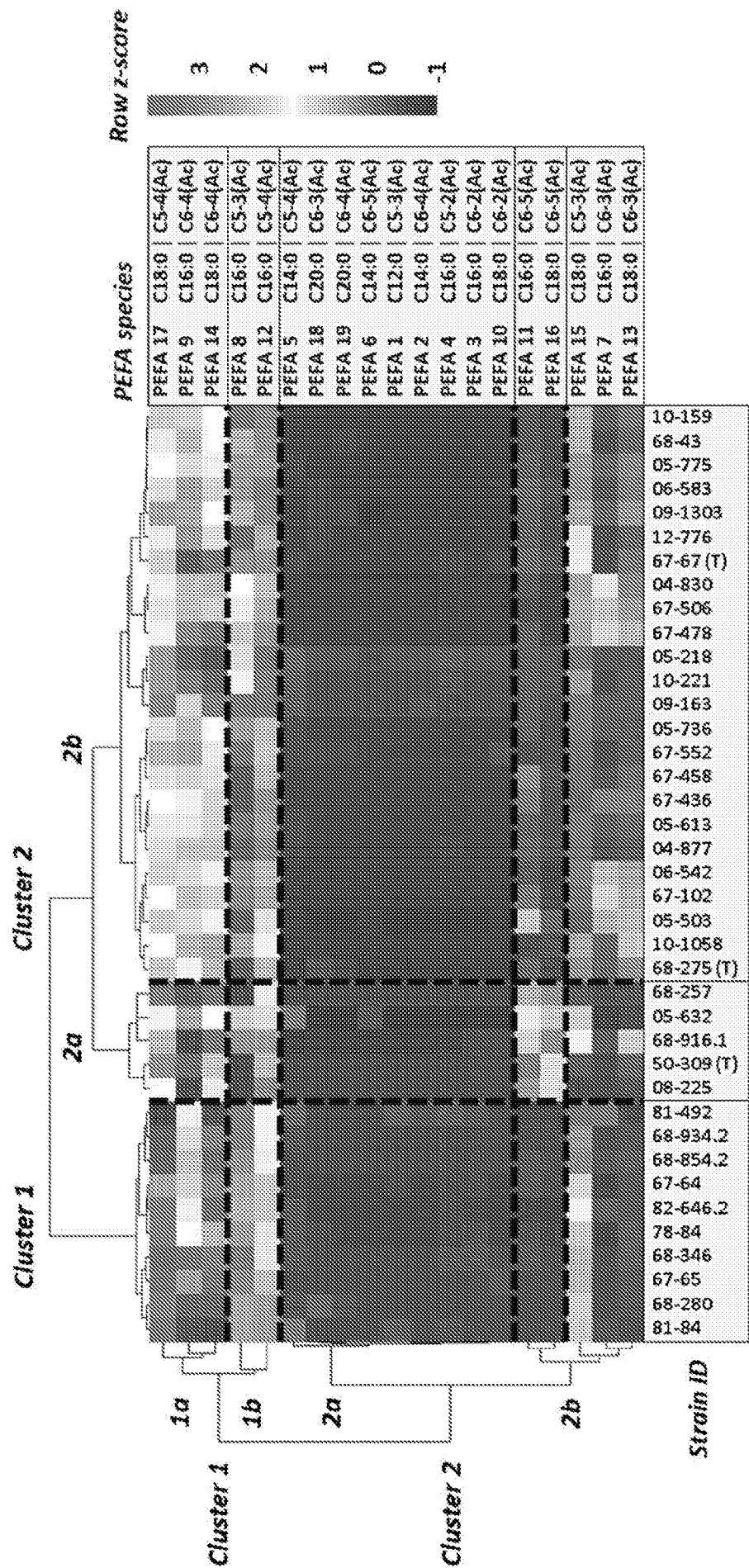


Fig. 3

4/17

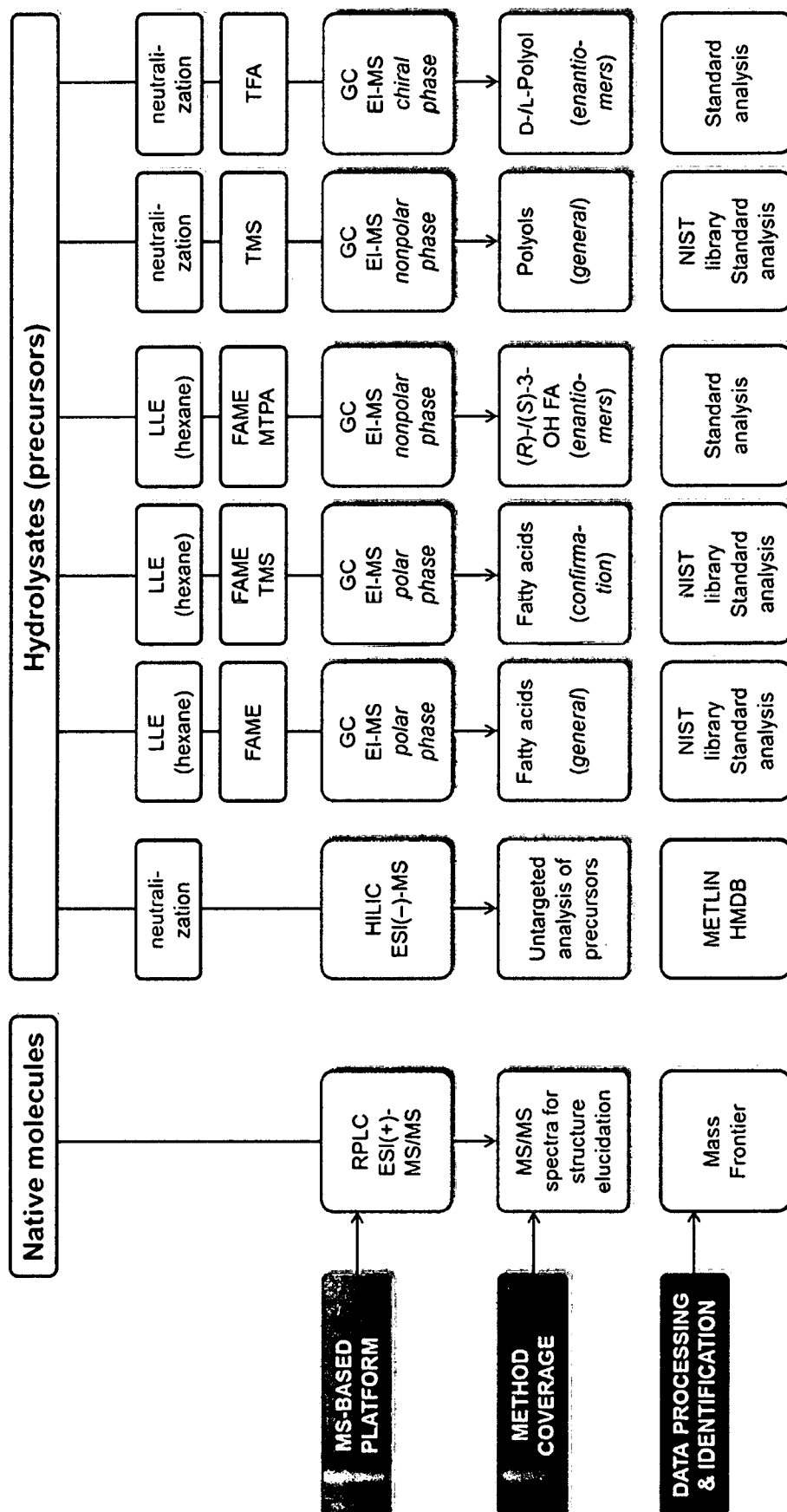
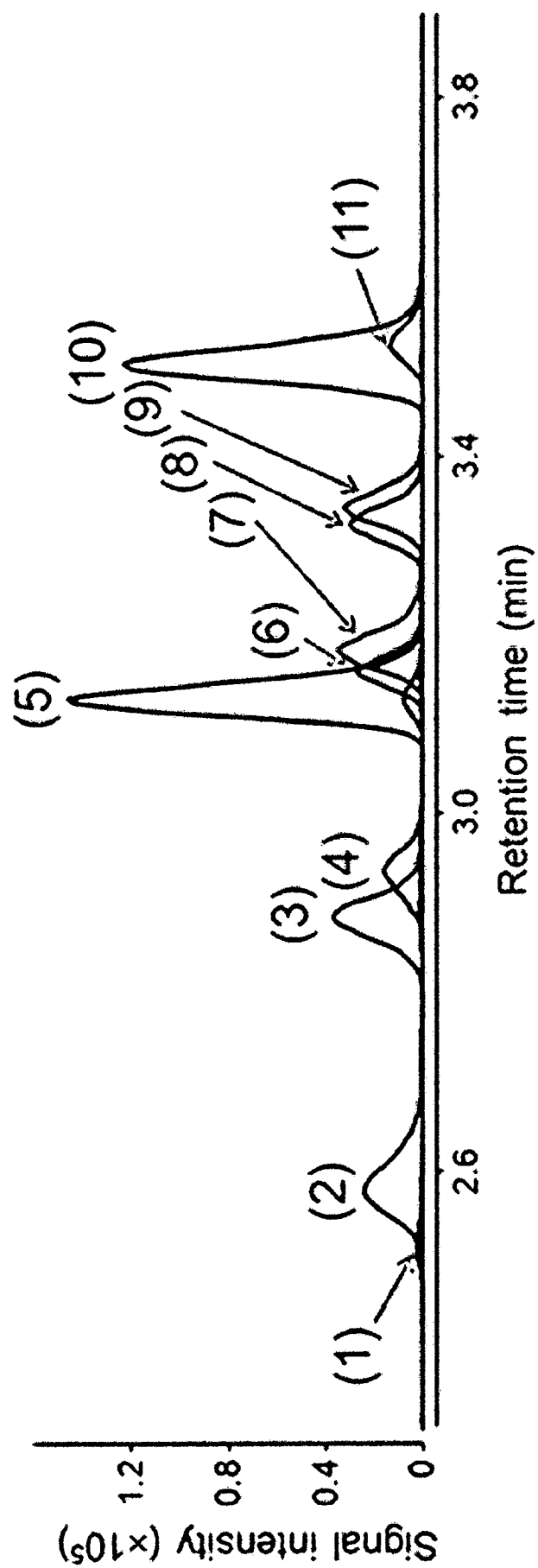
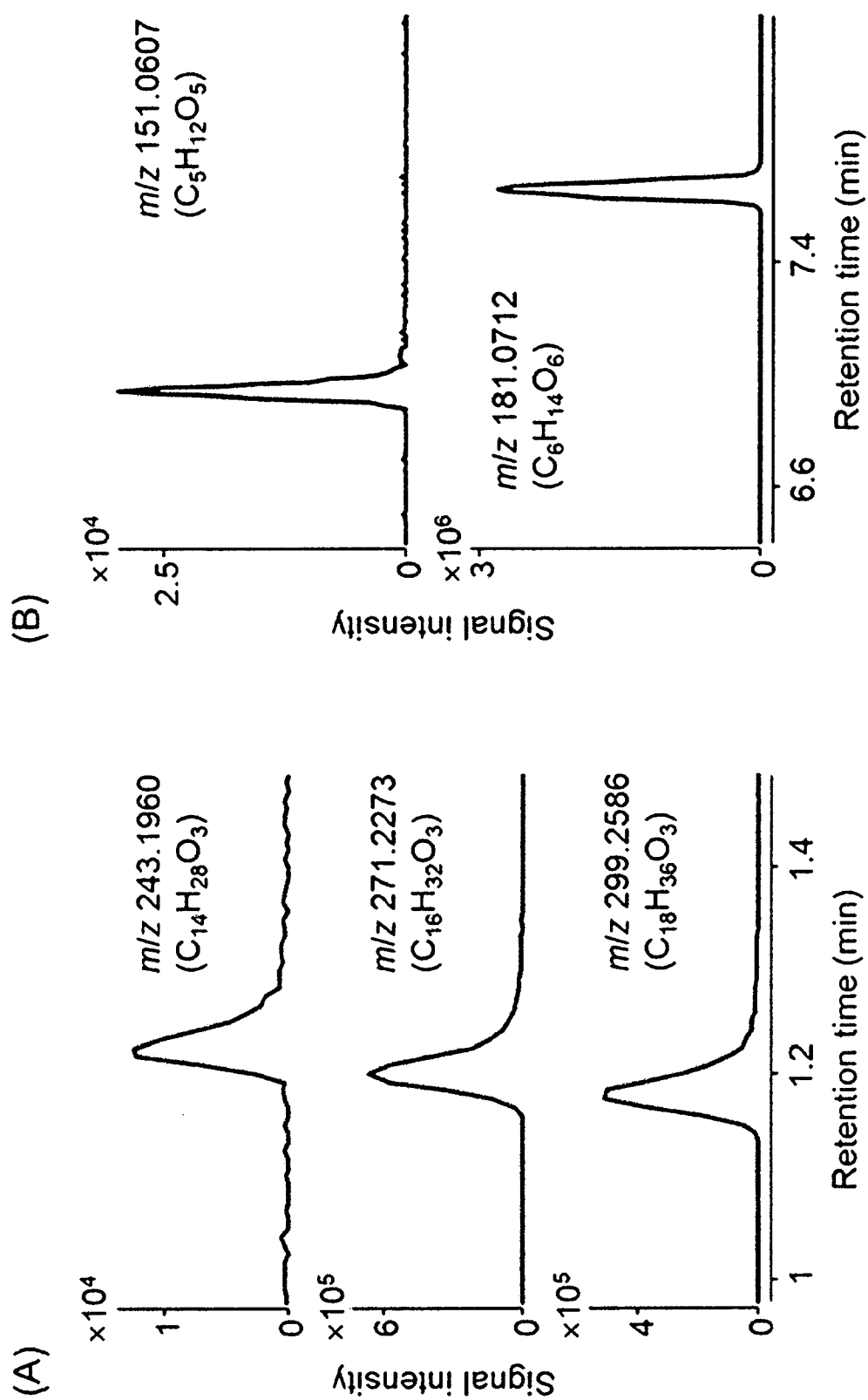


Fig. 4

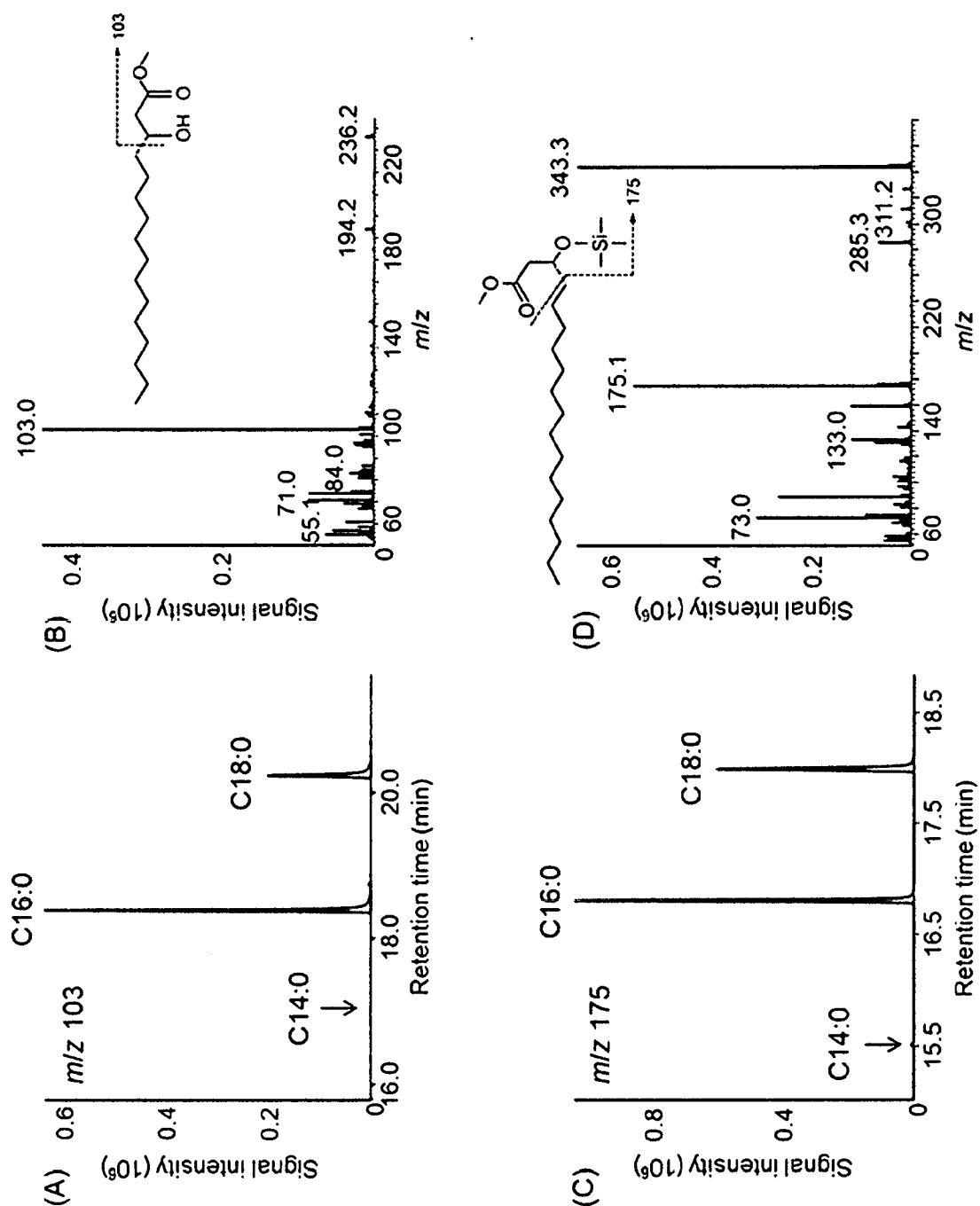
5/17

**Fig. 5**

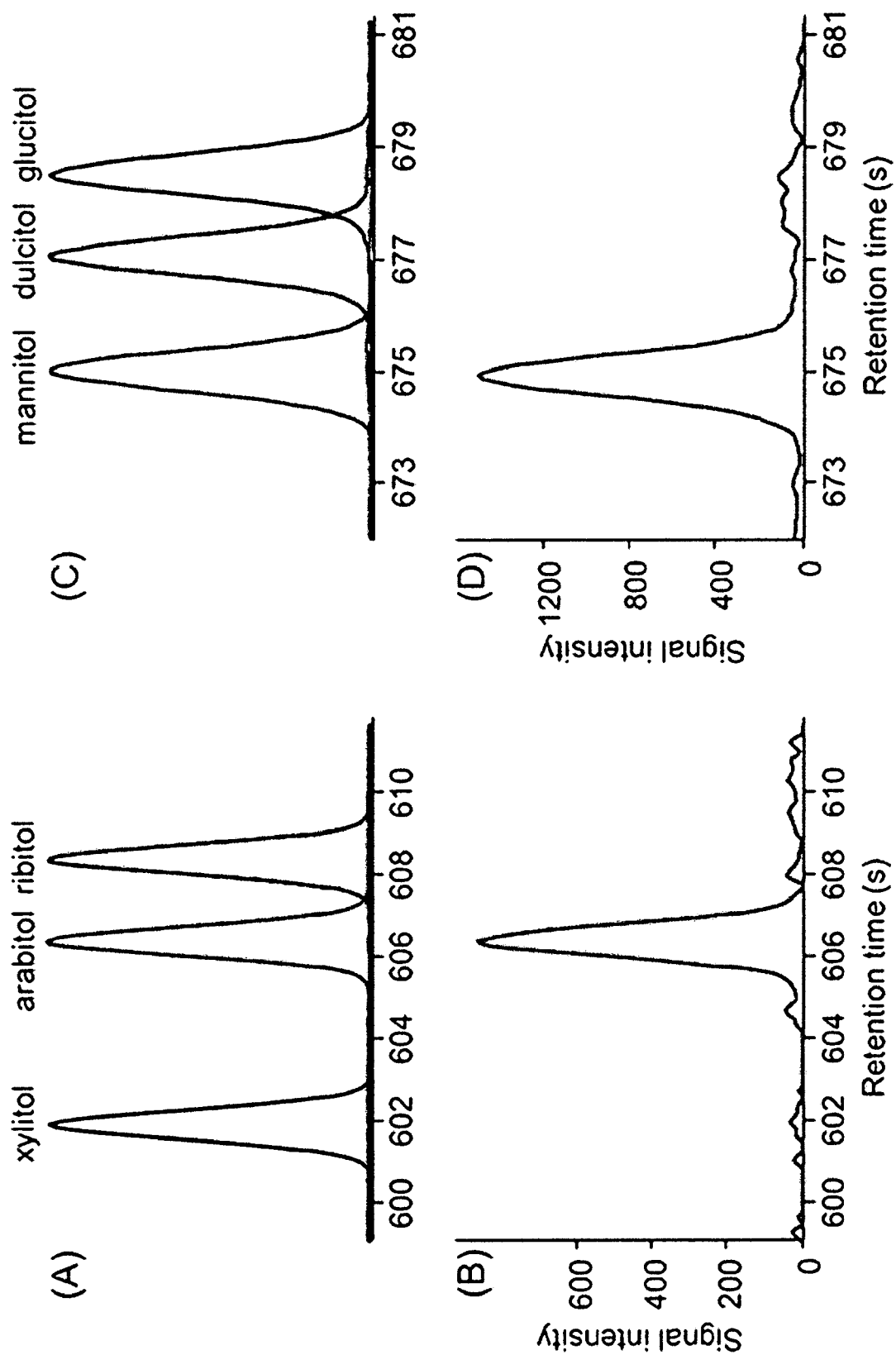
6/17

**Fig. 6A-B**

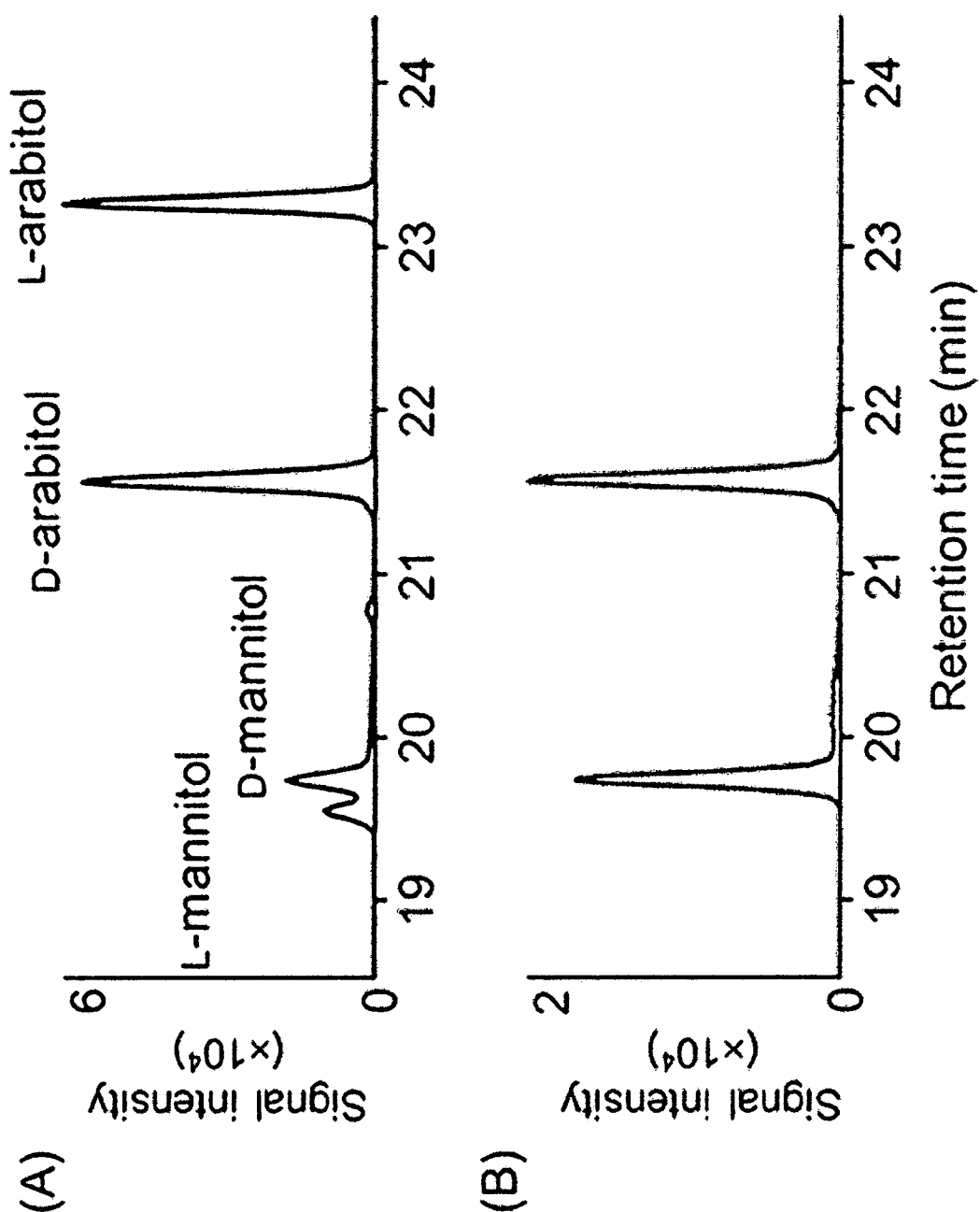
7/17

**Fig. 7A-D**

8/17

**Fig. 8A-D**

9/17

**Fig. 9A-B**

10/17

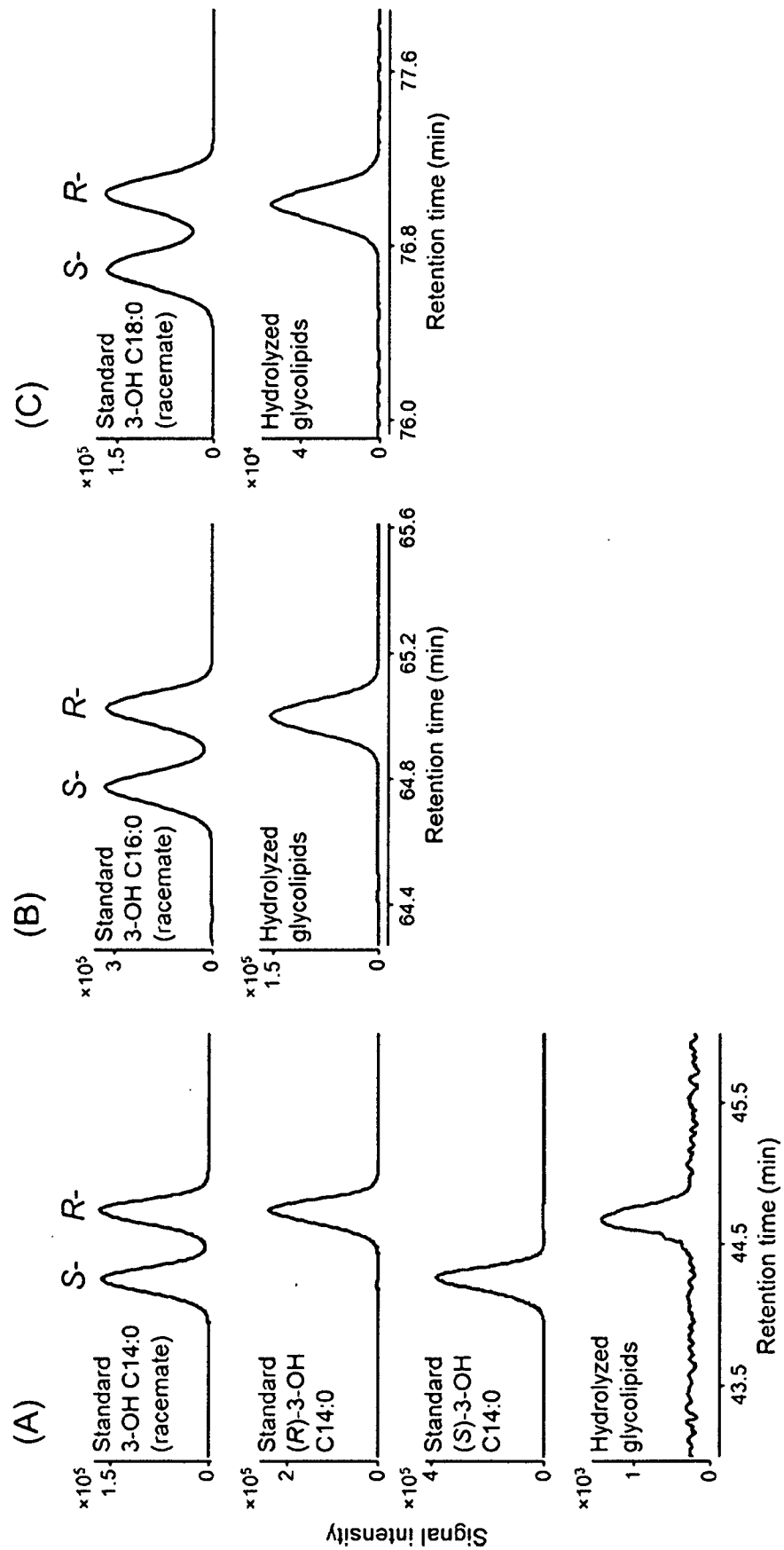
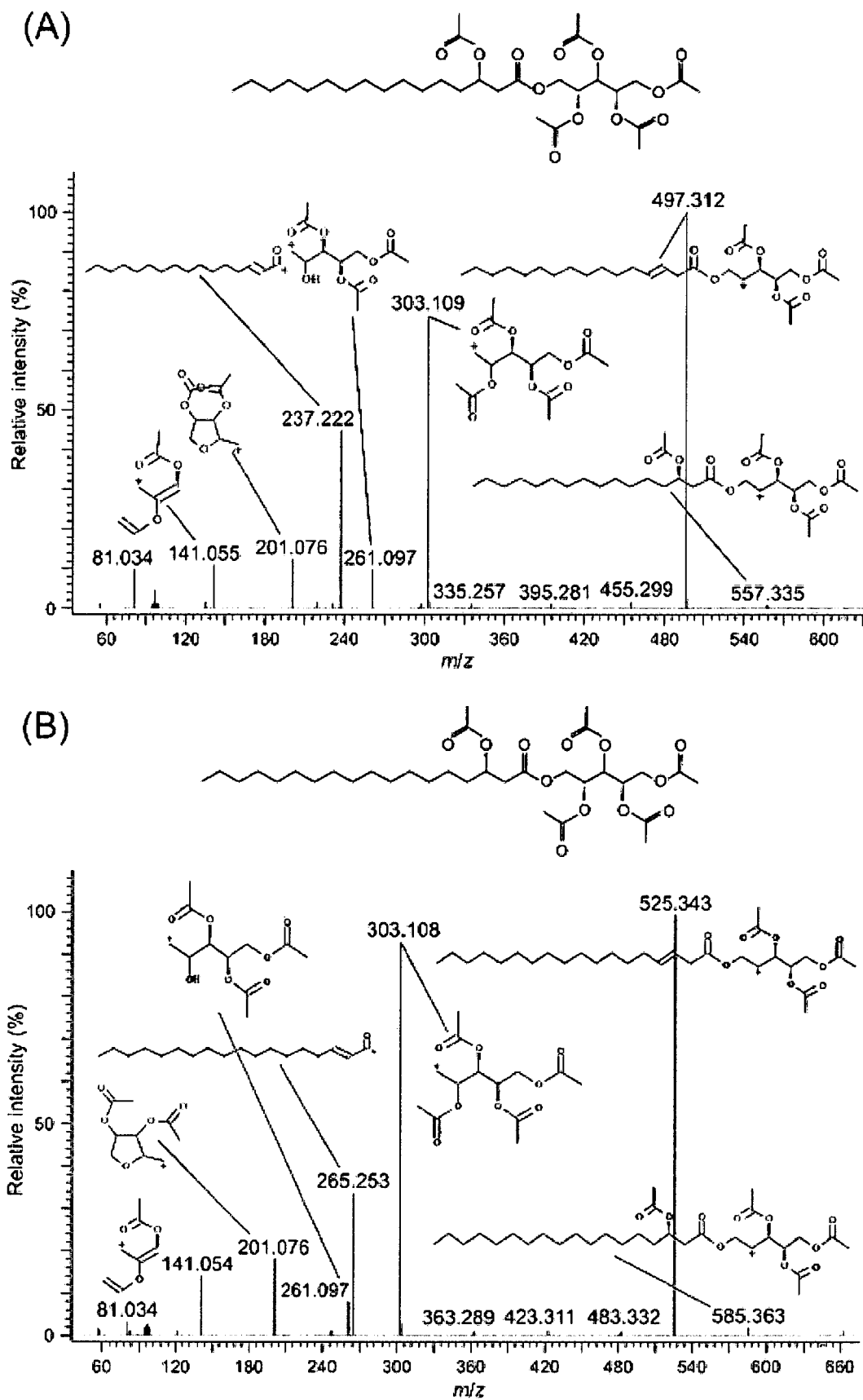
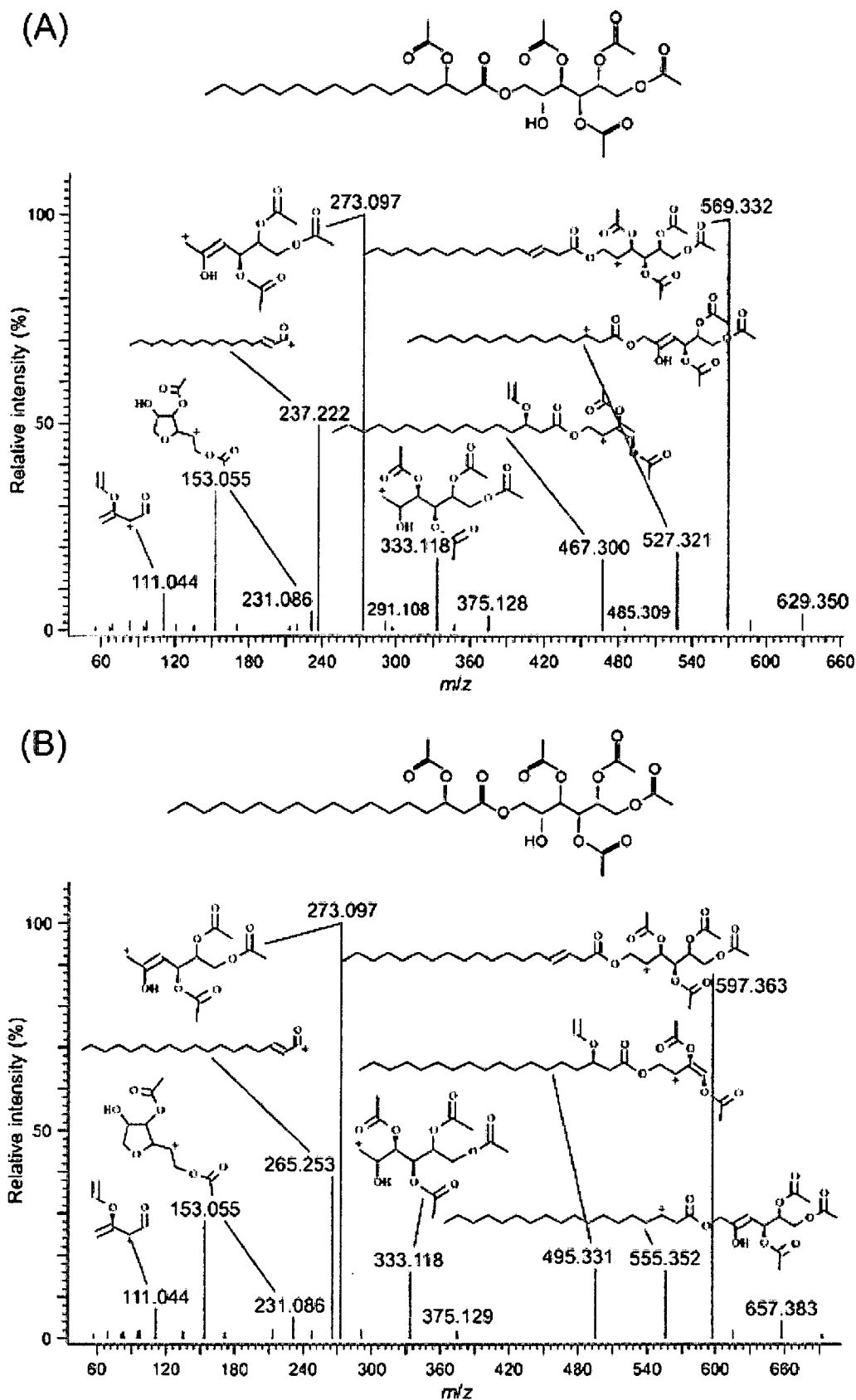
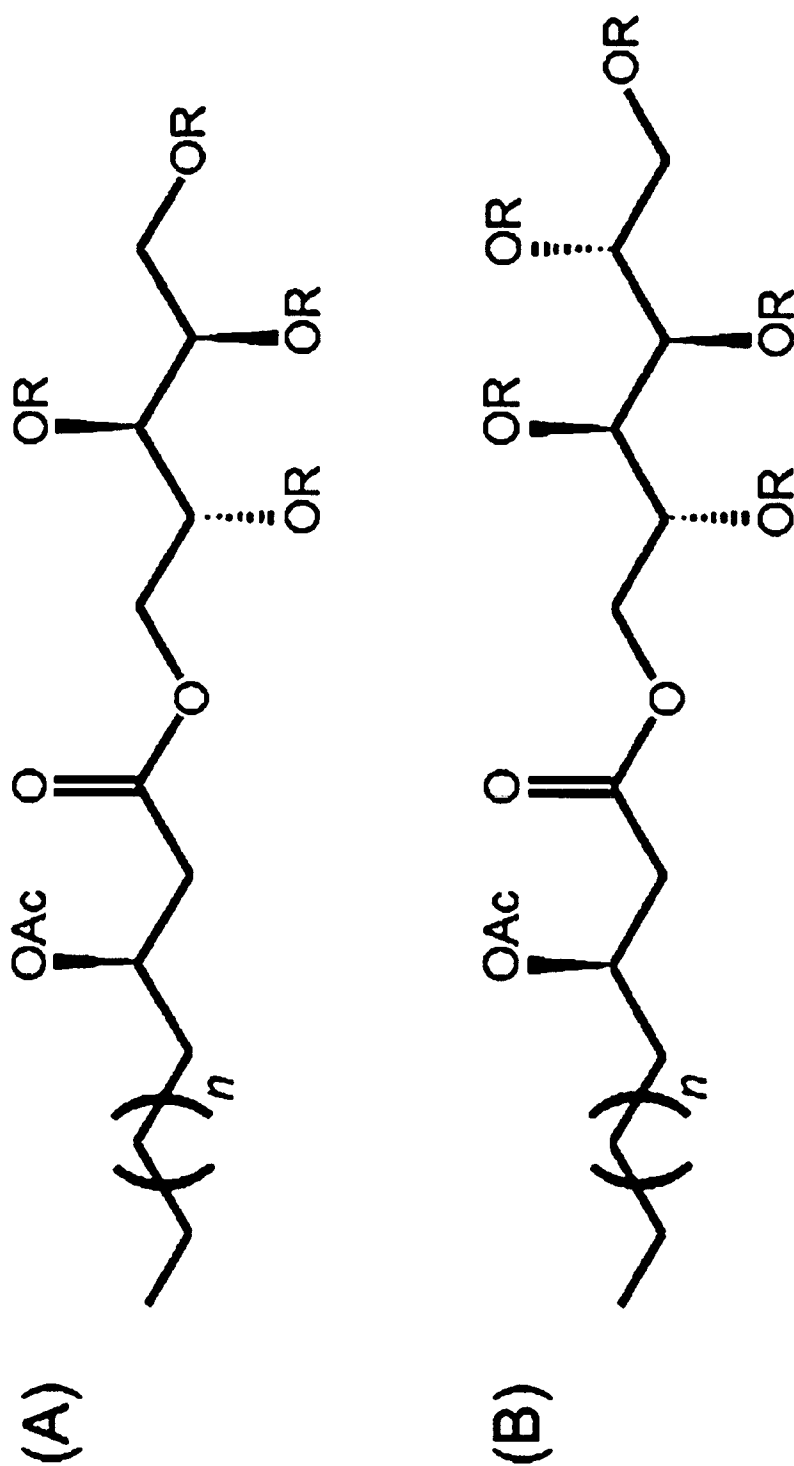


Fig. 10A-C

**Fig. 11A-B**

12/17**Fig. 12A-B**

**Fig. 13A-B**

14/17

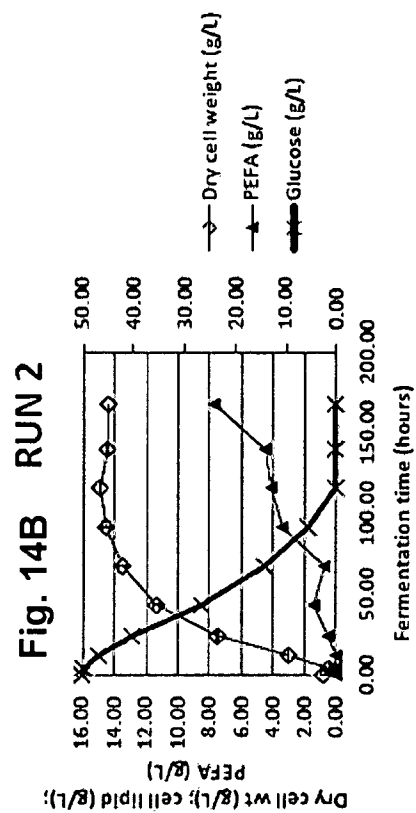
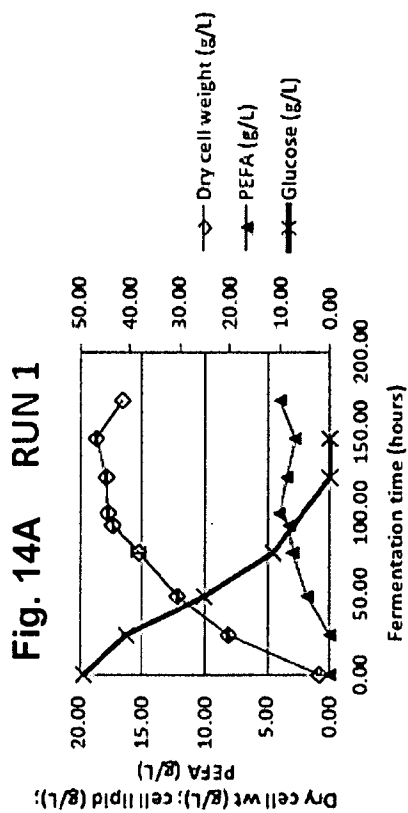
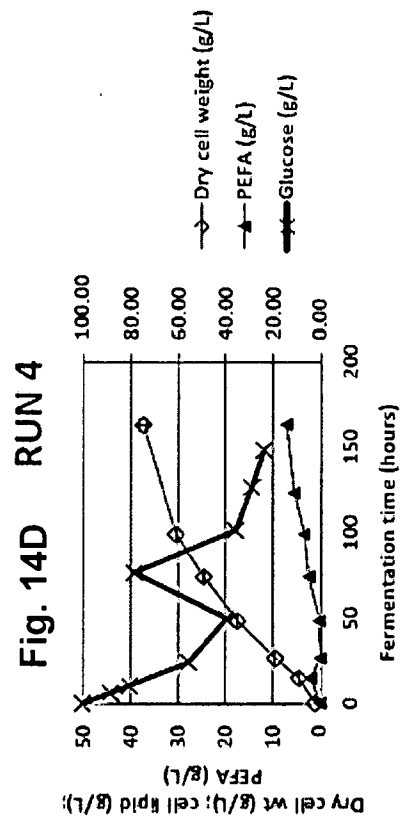
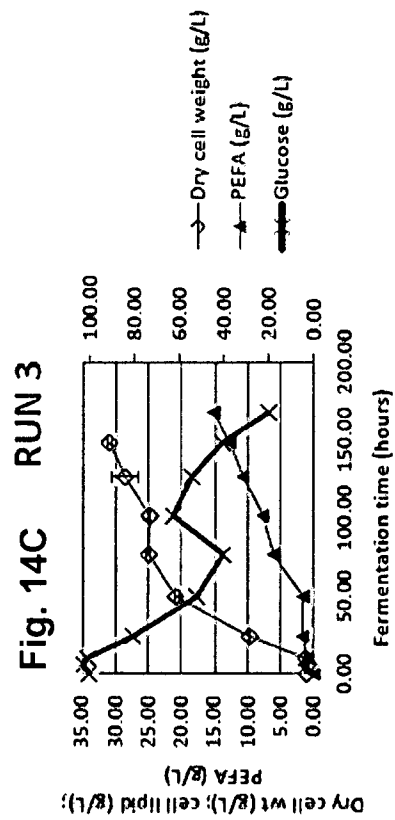
**Fig. 14A-D**

Fig. 14E RUN 5

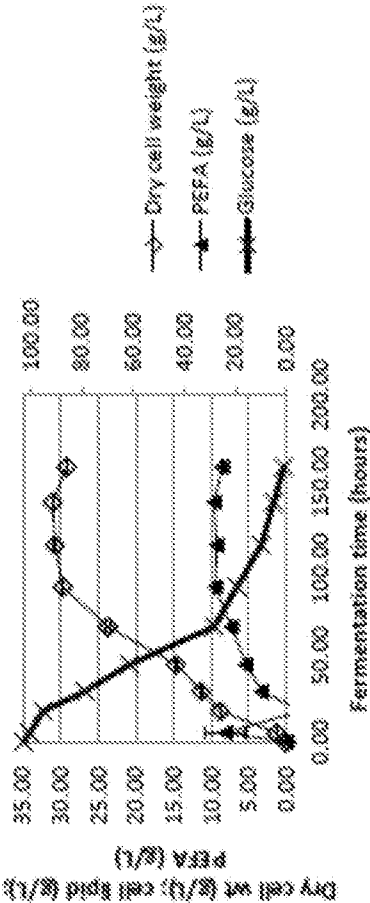


Fig. 14F RUN 6

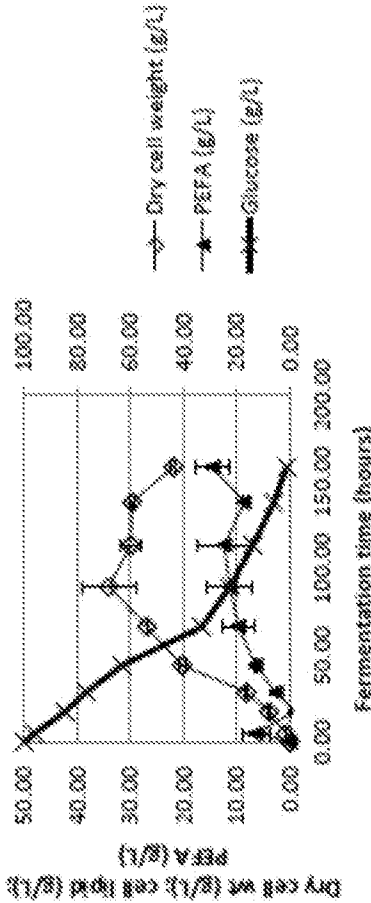


Fig. 14G RUN 7

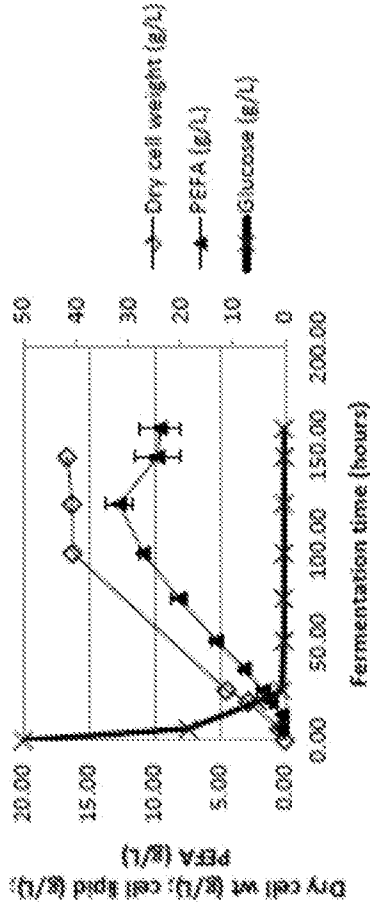


Fig. 14H RUN 8

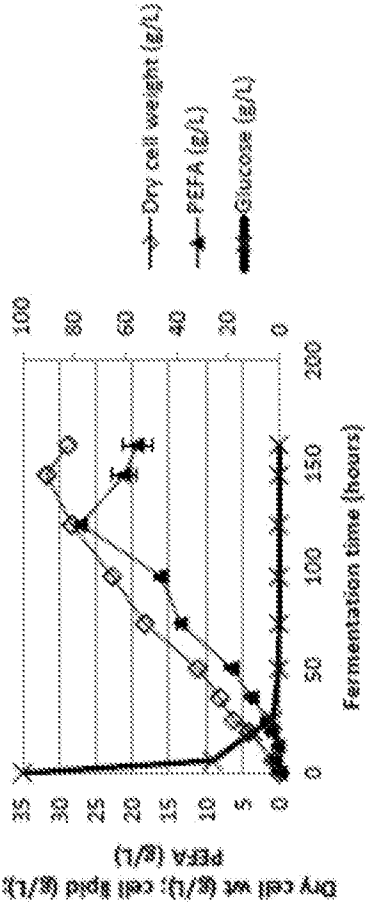


Fig. 14E-H

16/17

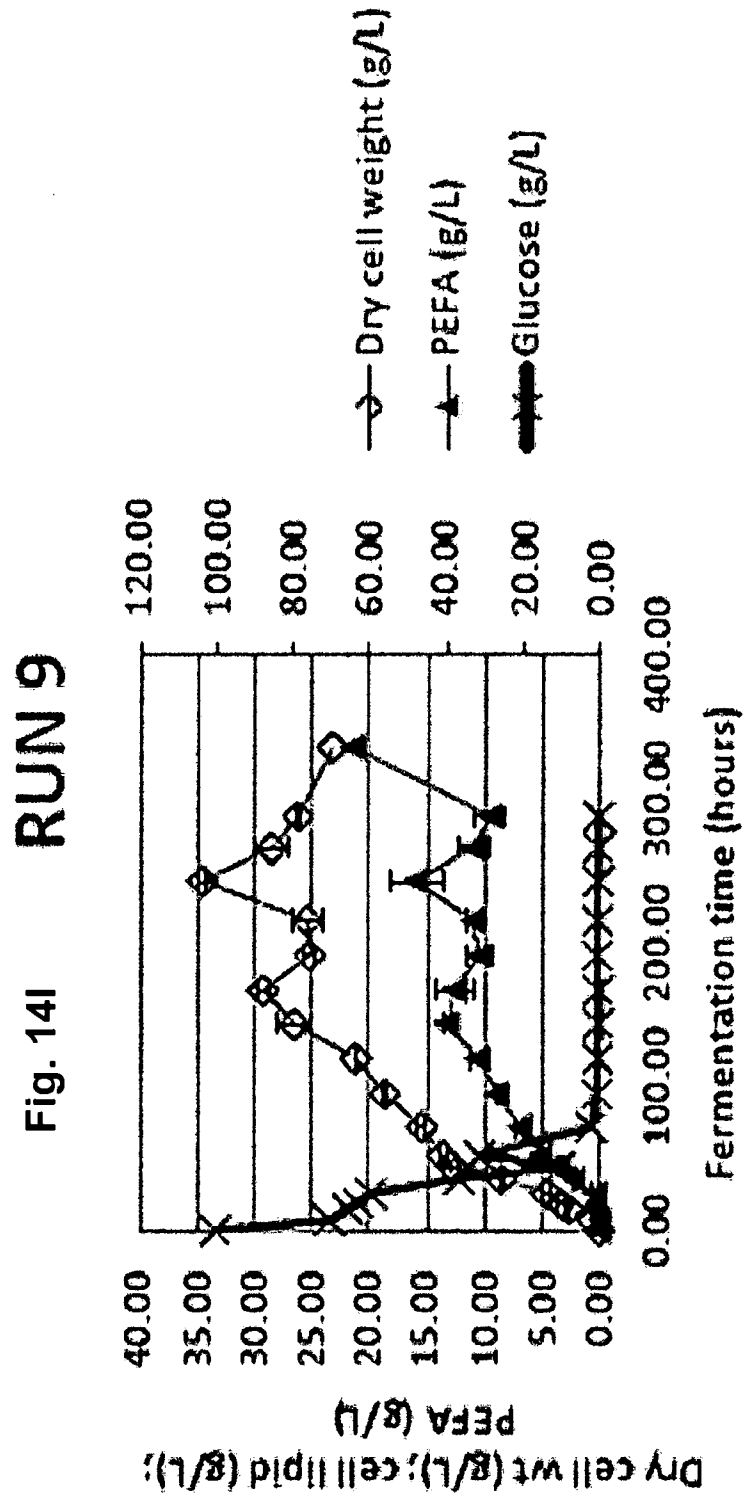


Fig. 14I

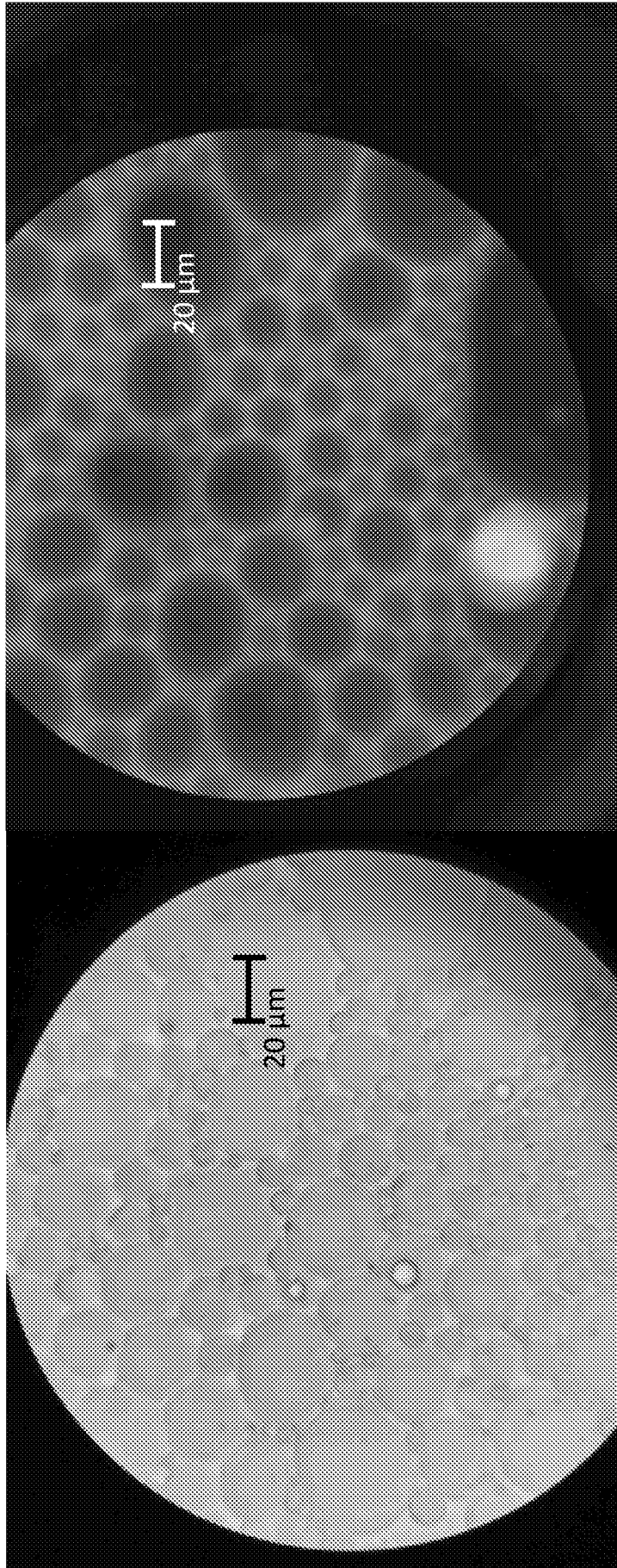


Fig. 15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/028670

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/70; C07H 15/06; C12P 7/64; C12R 1/645 (2017.01)

CPC - A61K 31/7032; C07K 14/375; C07K 14/39; C12P 7/6409; C12P 7/6436; C12P 7/6463; C12R 1/645 (2017.02)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 435/72; 435/134; 435/254.1; 435/255.1 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015/153476 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 08 October 2015 (08.10.2015) entire document	1-4, 44-47, 100-108, 111, 113
X	SITEPU et al. "Manipulation of culture conditions alters lipid content and fatty acid profiles of a wide variety of known and new oleaginous yeasts species," Bioresource Technology, 28 June 2013 (28.06.2013), Vol. 144, Pgs. 360-369. entire document	112
A	WO 2015/075443 A1 (CRODA INTERNATIONAL PLC) 28 May 2015 (28.05.2015) entire document	1-4, 44-47, 100-108, 111-113
A	WO 2015/142736 A1 (THE UNITED STATES OF AMERICA, AS REPRESENTED BY THE SECRETARY OF AGRICULTURE) 24 September 2015 (24.09.2015) entire document	1-4, 44-47, 100-108, 111-113
A	US 2011/0136110 A1 (VAN BOGAERT et al) 09 June 2011 (09.06.2011) entire document	1-4, 44-47, 100-108, 111-113

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

14 June 2017

Date of mailing of the international search report

14 JUL 2017

Name and mailing address of the ISA/US

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Authorized officer

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/028670

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 5-43, 48-99, 109, 110, 114
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.