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Short Communication

Lipid production on free fatty acids by oleaginous yeasts under non-growth conditions

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HIGHLIGHTS

- Microbial lipids production on free fatty acids under non-growth conditions.
- Production of lipids of exceptionally high fatty acid relative contents.
- Oleaginous yeasts use a broad range of long-chain free fatty acids effectively.

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ABSTRACT

Microbial lipids produced by oleaginous yeasts serve as promising alternatives to traditional oils and fats for the production of biodiesel and oleochemicals. To improve its techno-economics, it is pivotal to use wastes and produce high quality lipids of special fatty acid composition. In the present study, four oleaginous yeasts were tested to use free fatty acids for lipid production under non-growth conditions. Microbial lipids of exceptionally high fatty acid relative contents, e.g. those contained over 70% myristic acid or 80% oleic acid, were produced that may be otherwise inaccessible by growing cells on various carbon sources. It was found that *Cryptococcus curvatus* is a robust strain that can efficiently use oleic acid as well as even-numbered saturated fatty acids with carbon atoms ranging from 10 to 20. Our results provided new opportunity for the production of functional lipids and for the exploitation of organic wastes rich in free fatty acids.

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1. Introduction

Lipids including vegetable oils and animal fats serve as renewable alternatives to reduce dependence on petroleum feedstock for a sustainable production of biodiesel and other oleochemicals including surfactants, lubricants (Biermann et al., 2011) and even pharmaceuticals (Villamor et al., 2007). The ever growing demand and limited supply of lipids have resulted in intensive competition with food, controversial arable land use and environmental concerns. To find different lipids resources, efforts have been paid to use microorganisms for the production of lipids. In particular, lipids production by oleaginous yeasts is of great interest in terms of titre, production rate and yield. When sugar and related substrates are used as carbon sources, oleaginous yeasts produce lipids via *de novo* biosynthesis with compositional profiles resembling those of vegetable oils and animal fats which contain mainly long chain fatty acids having 16 and 18 carbon atoms (Papanikolaou and Aggelis, 2011; Li et al., 2011; Sitepu et al., 2013).

However, microbial lipids suffer from low economic acceptability, to prompt which tremendous efforts have been focused on exploration of low-priced feedstock such as lignocellulosic biomass and industrial wastes (Huang et al., 2013). Besides the sugar-based resources, free fatty acids (FFA) containing derivatives from oleochemical industry and slaughter houses serve as another promising feedstock for microbial lipid manufacturing (Papanikolaou et al., 2002). It is also of great environmental and economic benefits to explore these fatty acid containing wastes. More significantly, it is appealing to produce high-valued microbial lipids contained special fatty acid compositional profiles, such as with rare fatty acid species or dedicated composition (Beopoulos et al., 2014; Xue et al., 2013). To this end, it is imperative to investigate the capacity of oleaginous yeasts to accumulate lipids of special compositional profiles.

Oleaginous yeasts tend to initiate *ex novo* lipogenesis instead of *de novo* lipid biosynthesis when feed with hydrophobic substrates such as fats, alkanes and fatty acids, as carbon source for cell

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growth and lipid production (Papanikolaou and Aggelis, 2011). In particular, *Yarrowia lipolytica* can use alkanes and FFA as sole carbon sources for the production of lipids as reviewed elsewhere (Papanikolaou and Aggelis, 2010) and organic acids (Kamzolova et al., 2011). However, there was little information available to use FFA by cells under non-growth conditions, where cells may behave differently in terms of lipid metabolism and accumulate lipids with special compositional profiles. In the present study, cells of 4 representative oleaginous yeasts were fed with FFA as feedstock for *ex novo* lipid production in buffered media in the absence of nitrogen sources. It was found that those oleaginous yeasts were capable of accumulating neutral lipids to high lipid contents and incorporating a number of saturated long chain fatty acids as well as the unsaturated one oleic acid into neutral lipids to high fatty acid contents under non-growth conditions. Our results indicated that *Cryptococcus curvatus* is the most robust specie that can effectively use oleic acid as well as all even-numbered saturated fatty acids with carbon atoms ranging from 10 to 20. More interestingly, this study provided a new strategy to produce designed microbial lipids with exceptionally high contents of individual fatty acid that may be otherwise inaccessible by growing oleaginous species on various carbon sources (Sitepu et al., 2013).

2. Methods

2.1. Strains, reagents and media

Yeast strains *Rhodosporidium toruloides* AS 2.1389, *Lipomyces starkeyi* AS 2.1560 and *Trichosporon cutaneum* AS 2.571 were from the China General Microbiological Culture Collection Center. *C. curvatus* ATCC 20509 was from the American Type Culture Collection. All the strains were maintained at 4 °C on YPD agar slant containing (g/L) glucose 20; yeast extract 10; peptone 10 and agar powder 15, pH 6.0.

Standards for decanoic acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and arachidic acid (C20:0) were products of Sigma. Tween 80 and 3-(N-morpholino)propanesulfonic acid (MOPS) were purchased from Gen-View (Galveston, USA). All other reagents mentioned in this study were obtained from local supplier.

YEPD media contained (g/L): glucose 20; yeast extract 10 and peptone 10, pH 6.0. The media were sterilized by autoclaving at 121 °C for 18 min.

Unless otherwise specified, lipid production media contained (g/L): fatty acid 20; Tween 80, 20, MOPS 10.5, pH 6.0.

2.2. Lipid production procedure

A loop of yeast cells was cultivated in YEPD media at 30 °C, 200 rpm until the OD_{600} reached 10–12. The resulted cultures were re-inoculated in YEPD media with a ratio of 10% (v/v), and cultivated under the same conditions for 24 h. Cells were collected by centrifugation at 6000*g* for 3 min, washed twice with distilled water before used for lipid production experiments.

Cells were transferred under ambient environment into 50 mL of non-sterile lipid production media in 250-mL Erlenmeyer flasks with an initial optical density at 600 nm (OD_{600}) of 16–18, that was roughly 5.4–6.0 g/L dry cell weight (DCW). Cultures were held under non-sterile conditions at 30 °C, 200 rpm for 72 h.

All culture experiments were performed in triplicate, and the results were presented as the mean ± standard derivation.

2.3. Analytical methods

Cell mass was expressed as DCW. Cells from 30 mL of the culture media were harvested by centrifugation at 8000*g* for 5 min, washed with ethanol (95%) and hexanes to remove extracellular fatty acids (Papanikolaou et al., 2002) and dried at 105 °C for 24 h to a constant weight. Lipid content was expressed as g lipid per g DCW.

Total cellular lipid was extracted by a mixture of chloroform and methanol according to the reported procedure (Li et al., 2007). Lipid content was expressed as g lipid per g DCW multiplied by 100%. All values were the average of 3 independent experiments.

Fatty acid compositional profiles of microbial lipid samples were analyzed upon transesterification by using the gas chromatography (GC) method (Li et al., 2007). Briefly, 70 mg lipids were stirred with 0.5 mL of 5% KOH methanol solution at 65 °C for 50 min, followed by the addition of 0.2 ml of BF_3 diethyl etherate and 0.5 ml of methanol, refluxed for 10 min, cooled, diluted with distilled water and extracted with hexanes. The organic layer was washed twice with distilled water and subjected to GC analysis. Fatty acids were profiled with a 7890F GC instrument (Techcomp Scientific Instrument Co. Ltd., Shanghai, China) equipped with a cross-linked capillary FFAP column (30 m × 0.32 mm × 0.4 mm) and flame ionization detector. Operating conditions: N_2 carrier as 40 ml/min, injection port temperature 250 °C, oven temperature 190 °C and the detector temperature was 280 °C. Fatty acids were identified by comparing their retention times with those of standards (Sigma, USA) and quantified based on their respective peak areas and normalized.

Thin layer chromatography (TLC) analysis was performed on $HSGF_{254}$ silica gel plates by using hexane–diethyl ether–acetic acid (80:20:1, v/v/v) as the developing system.

3. Results and discussion

3.1. Lipid production from common saturated long-chain fatty acids

Myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0) are common saturated fatty acids found in vegetable oils and microbial lipids. To test whether oleaginous yeasts can assimilate FFA for lipid production under non-growth conditions, cells were resuspended in MOPS buffer supplemented with 20 g/L FFA in the absence of nitrogen and phosphorus sources. Upon being held at 30 °C, 200 rpm for 72 h, cells were recovered, fully washed with ethanol and hexanes. Total cellular lipids were extracted and the majority of the lipid samples were neutral lipids based on thin layer chromatography analysis (Papanikolaou et al., 2002). It was found that when C14:0 was used, cells of *C. curvatus*, *L. starkeyi*, *R. toruloides* and *T. cutaneum* accumulated lipids to final contents of 76.3%, 64.3%, 37.9% and 43.4%, respectively (Table 1), and that C14:0 relative contents of these lipids were 75.3%, 65.3%, 27.0% and 77.8%, respectively (Fig. 1A). The C14:0 relative contents were at least 30-, 130-, 15- and 100-fold higher than those samples obtained by cultivation of the corresponding yeasts using glucose as the carbon source (Table 1) and 2- to 6-fold higher than those of palm kernel oil (Cardoso Bejan et al., 2014). When C16:0 was fed, the C16:0 relative contents of the total lipids were 65.1%, 55.3%, 51.9% and 80.4% (Fig. 1B), at least 2.0-, 1.5-, 1.4- and 1.7-fold those of the literature data (Table 1) and 1.3- to 2.0-fold those of palm oil (Cardoso Bejan et al., 2014). Interestingly, however, when C18:0 was employed, these strains accumulated C18:0 to no more than 12% in lipid fractions (Fig. 1C). Instead, cells of *C. curvatus*, *L. starkeyi* and *R. toruloides* converted C18:0 mainly into C16:0 and C18:1. For *T. cutaneum*, there was little cell mass

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