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High-efficiency extracellular release of free fatty acids from *Aspergillus oryzae* using non-ionic surfactants

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ABSTRACT

Free fatty acids (FFAs) are useful for generating biofuel compounds and functional lipids. Microbes are increasingly exploited to produce FFAs *via* metabolic engineering. However, in many microorganisms, FFAs accumulate in the cytosol, and disrupting cells to extract them is energy intensive. Thus, a simple cost-effective extraction technique must be developed to remove this drawback. We found that FFAs were released from cells of the filamentous fungus *Aspergillus oryzae* with high efficiency when they were cultured or incubated with non-ionic surfactants such as Triton X-100. The surfactants did not reduce hyphal growth, even at 5% (w/v). When the *faaA* disruptant was cultured with 1% Triton X-100, more than 80% of the FFAs synthesized *de novo* were released. When the disruptant cells grown without surfactants were incubated for 1 h in 1% Triton X-100 solution, more than 50% of the FFAs synthesized *de novo* were also released. Other non-ionic surfactants in the same ether series, such as Brij 58, IGEPAL CA-630, and Tergitol NP-40, elicited a similar FFA release. The dry cell weight of total hyphae decreased when grown with 1% Triton X-100. The decrement was 4.9-fold greater than the weight of the released FFAs, implying release of other intracellular compounds. Analysis of the culture supernatant showed that intracellular lactate dehydrogenase was also released, suggesting that FFAs are not released by a specific transporter. Therefore, ether-type non-ionic surfactants probably cause non-specific release of FFAs and other intracellular compounds by increasing cell membrane permeability.

1. Introduction

With the increasing worldwide demand of fossil fuels and the consequent progress of global warming, biofuels derived from plant biomass have been focused as a promising substitute due to less effect on global environment by the carbon neutral characteristics (Lennen and Pfleger, 2013; Liang and Jiang, 2013). Additionally, because some pharmaceuticals and dietary supplements produced from rare natural lipid resources are expensive, their heterologous production in the engineered microorganisms has been focused for lowering the prices (Delarue and Guriec, 2014; Tamano, 2014).

Microbial lipid production has been researched in bacteria [*Escherichia coli* (Steen et al., 2010; Zheng et al., 2012), *Corynebacterium glutamicum* (Plassmeier et al., 2016)], yeast [*Saccharomyces cerevisiae* (Chen et al., 2014; Leber et al., 2015; Shi et al., 2016), *Yarrowia lipolytica* (Pomraning et al., 2015; Wasylenko et al., 2015)], filamentous fungi [*Aspergillus oryzae* (Tamano and Miura, 2016; Tamano et al., 2015, 2013), *Mortierella alpina* (Sakuradani et al., 2013), *Mucor circinelloides* (Rodríguez-Frómeta et al., 2013; Zhao et al., 2016) *etc.*], and photosynthetic microorganisms such as cyanobacteria and microalgae (Liu et al., 2011; Mallick et al., 2016; Ruffing, 2013). Free fatty acid (FFA), acylglycerol, fatty acid methyl ester (FAME), fatty acid ethyl ester (FAEE), and alkane have been major targets of the microbial production. Among them, FFA can be used as a feedstock for production of biofuels (*e.g.*, FAME, FAEE, and alkane) and functional lipids (*e.g.*, eicosapentaenoic acid, and

Abbreviations: FFA, free fatty acid; FAME, fatty acid methyl ester; FAEE, fatty acid ethyl ester; CTAB, cetyltrimethylammonium bromide; SDS, sodium dodecyl sulfate; CD, Czapek–Dox; HPLC, high performance liquid chromatography; RT, room temperature; LDH, lactate dehydrogenase; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; DCW, dry cell weight; cmc, critical micelle concentration.

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docosahexaenoic acid) (Lennen and Pfleger, 2013; Zhou et al., 2016).

The *A. oryzae* is a filamentous fungus that is used in the production of fermented foods [*e.g.*, Japanese alcohol (*sake*), soy paste, and soy sauce]. Because the fungus is known not only for its efficient degradation of macromolecules such as amylose and polypeptide but also for its efficient generation of metabolites such as malic acid and kojic acid, we have been investigating methods to increase the production of FFAs (Tamano and Miura, 2016; Tamano et al., 2015, 2013). We previously attained a 9.2-fold increase in FFA productivity by disruption of the acyl-CoA synthetase gene, *faaA* (Tamano et al., 2015). However, FFAs still accumulate inside the cells, and for industrial use, they must then be extracted by mechanical disruption or lysis of the cells using organic solvents. However, these extraction processes are costly and energy-intensive. Therefore, a simpler method for recovering FFAs from *A. oryzae* cells is required.

Surfactants solubilize compounds that are otherwise insoluble in aqueous solvents. They also permeabilize cell membranes, resulting in increased presentation of microbial metabolites and enzymes outside cells when they are added to microbial culture media (Miozzari et al., 1978). For example, when Triton X-100 was added to the culture media of *Y. lipolytica*, secretory production of citric acid increased by 1.8-fold (Mirbagheri et al., 2011). Also, various surfactants are reported to have similar activities of increasing secretory production in various microorganisms such as lipase in *Aspergillus niger* (Mahadik et al., 2004), pigments in *Monascus* sp. (Hu et al., 2012), and phytase in *Pichia anomala* (Kaur and Satyanarayana, 2010).

Surfactants are classified as non-ionic and ionic surfactants. Non-ionic surfactants are further classified into ether and ester types, while ionic surfactants are further classified into cationic, anionic, and zwitterionic types. Triton X-100, Brij 58, IGEPAL CA-630, and Tergitol NP-40 are commonly used ether-type non-ionic surfactants. Tween 20, 40, and 80 are well-known ester-type non-ionic surfactants. Cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulfate (SDS) are cationic and anionic surfactants, respectively, while CHAPS is a zwitterionic surfactant.

In this study, we identified that application of ether-type non-ionic surfactants to *A. oryzae* is effective in high-efficiency extracellular release of FFAs. We also performed a detailed analysis of the composition of released lipids including FFAs.

2. Materials and methods

2.1. Fungal strains and culture

We used an *A. oryzae* wild-type RIB40 strain distributed by the National Research Institute of Brewing (NRIB, Hiroshima, Japan) and the derivative *ΔfaaA* strain (RIB40 *ΔligD::ptrA ΔniaD::niaD of A. nidulans ΔpyrG::sC* of *A. nidulans ΔfaaA::pyrG* of *A. nidulans*) constructed previously (Tamano et al., 2015). These strains were maintained on Czapek–Dox (CD) minimal agar medium (Tamano et al., 2007). The CD agar medium contained 2% glucose, while the glucose concentration was increased to 10% for the CD liquid medium. Cultures were prepared by inoculating 2.5×10^7 spores of each *A. oryzae* strain to 50 ml of CD liquid medium in a 250-ml flask, followed by incubation at 30 °C, with shaking at 200 rpm for 120 h. An additional incubation of hyphae was done by standing or shaking (at 200 rpm) in 20 ml of surfactant solution at room temperature (RT, 24 °C) or 4 °C for 60 min in a 250-ml flask after the hyphae of 5-d culture without surfactants were collected and washed by 100 ml of Milli-Q water twice. Triton X-100 (Code No. 648466, Merck Millipore, Billerica, MA, USA), Brij 58 (Code No. P5884, Sigma, St. Louis, MO, USA), IGEPAL CA-630 (Code No. I8896, Sigma), Tergitol NP-40 (Code No. NP40S, Sigma), Tween 20 (Code No. 166-21213, Wako, Osaka, Japan), CHAPS (Code No. 347-04723, Wako), and sodium cholate (Code No. 196-13722, Wako) were used as the surfactants.

2.2. Quantification of intracellular and extracellular FFAs

Sample preparation of intracellular FFAs was described in our previous report (Tamano et al., 2015). For FFA quantification, five microliters of each intracellular and extracellular (culture supernatant itself) FFA sample was applied to the FFA enzyme assay kit, Free Fatty Acids Half-micro Test Kit (Roche Applied Science, Mannheim, Germany).

2.3. Composition analysis of intracellular and extracellular FFAs

Sample preparation and subsequent composition analysis of intracellular FFAs using high performance liquid chromatography (HPLC) were described in our previous report (Tamano et al., 2015). Extracellular FFA samples were prepared as follows. The *A. oryzae* culture supernatant (5 ml) was transferred to a 10-ml screw-cap glass centrifuge tube, followed by addition of 5 ml of chloroform. After vigorous shaking, the sample solution was centrifuged at $5000 \times g$ at RT for 2 min. The upper aqueous layer was removed by pipette, and 4.5 ml of the lower chloroform layer was transferred to a new 10-ml screw-cap glass centrifuge tube. The transferred chloroform fraction was evaporated by incubating at 50 °C overnight on a heating block in a fume hood. The resultant precipitate containing extracellular FFAs was applied to the FFA composition analysis similarly to the intracellular FFA samples.

2.4. Lactate dehydrogenase (LDH) assay

The LDH quantity in the culture supernatant of *A. oryzae* was determined using a cytotoxicity assay kit (Roche Applied Science). A 50-μl aliquot of culture supernatant was mixed with 100 μl of the reaction solution included in the kit and incubated at RT for 15 min. During incubation, lactate molecules in the reaction solution are converted to pyruvate molecules, generating $NADH^+/H^+$ molecules from NAD^+ molecules. The generated $NADH^+/H^+$ molecule transfers two hydrogens to the tetrazolium salt (which is yellow) contained in the reaction solution, producing a formazan salt (which is red). After addition of 50 μl of the stop solution provided in the kit, absorbance at 490 nm derived from the formazan salt was measured in order to determine the level of LDH activity.

2.5. Lipid analysis by thin-layer chromatography (TLC)

The *A. oryzae* liquid culture was separated to culture supernatant and hyphae by Miracloth (Merck Millipore). Culture supernatant was mixed with 100 ml of Milli-Q water and 150 ml of chloroform:methanol (2:1). The mixture was placed in a separatory funnel and shaken vigorously. After standing for 30 min, the lower chloroform fraction was transferred to a new flask. Then, 50 ml of chloroform:methanol (2:1) was added to the upper aqueous layer, and the chloroform fraction was transferred to the initial chloroform fraction after vigorous shaking and separation. Twenty grams of anhydrous magnesium sulfate were added to the chloroform fraction for dehydration, followed by incubation for 30 min at RT and filtration using filter paper. The filtrated chloroform fraction was evaporated using a rotary evaporator. The resultant extract was dissolved in 1.6 ml of chloroform and stored at −30 °C as an extracellular lipid sample.

Hyphae were washed twice with 100 ml of Milli-Q water, frozen in liquid nitrogen, and lyophilized. Lyophilized hyphae were disrupted at 4200 rpm for 1 min by a micro homogenizing system (MicroSmash MS-100, TOMY, Tokyo, Japan) using three zirconia