Korean J. Chem. Eng., 36(6), 903-908 (2019)
DOI: 10.1007/s11814-018-0229-7

pISSN: 0256-1115
eISSN: 1975-7220

Engineering *Trichosporon oleaginosus* for enhanced production of lipid from volatile fatty acids as carbon source

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(*Received 21 September 2018 • accepted 17 January 2019*)

Abstract−*Trichosporon oleaginosus* is one of the most promising hosts for microbial lipid production owing to its high-productivity. In an effort to develop an economical production process, we engineered *T. oleaginosus* towards high-lipid production from volatile fatty acids (VFA) derived from anaerobic fermentation of food waste. First, we established a method for labeling intracellular lipid with lipophilic BODIPY fluorescent dye. Next, a random library was constructed by treatment with a chemical mutagen, and high-lipid producers were screened using fluorescence-activated cell sorting. Subsequently, one clone, N14, was successfully isolated, which exhibited 3-fold higher lipid production (19.4%) in VFA (6 g/L) media than the wild-type strain, and also showed increased lipid production in higher concentrations of VFA (18 or 24 g/L). Based on fatty acid methyl ester (FAME) analysis, N14 contained higher stearic acid (C18:0) and oleic acid (C18:1) content compared with those of the wild-type strain.

Keywords: *Trichosporon oleaginosus*, Volatile Fatty Acids, Fluorescence-activated Cell Sorting, Microbial Lipid, BODIPY

INTRODUCTION

Trichosporon oleaginosus, previously termed *Cryptococcus curvatus*, is an oil-producing microorganism classified under the phylum Basidiomycota [1]. It is especially known for its ability to accumulate high levels of triglycerides by utilizing a broad range of carbon sources [2]. To achieve increased lipid productivity by *T. oleaginosus*, various approaches have been used, which include attempts to minimize biolipid-production costs by utilizing cheap carbon sources, or by optimizing culture conditions at different production scales [3,4]. A dry cell mass of more than 70% lipid has been achieved in a number of studies [5-7]. Because of these positive results, *T. oleaginosus* is regarded as a promising host for the sustainable production of biodiesel and other valuable chemicals.

Lowering the production costs remains as an important challenge in the research area of microbial lipid production. As a breakthrough, volatile fatty acid (VFA)-based biofuel production using microorganisms has become one of the most promising next-generation biofuel technologies. It is well known that VFA, generally including acetic acid, propionic acid and butyric acid, can be produced efficiently from the anaerobic digestion of organic wastes such as food wastes [8-11]. Thus, applying VFA as low-cost base material is expected to dramatically reduce overall production costs. In recent years, there have been extensive studies describing the feasibility of using VFA as feedstock in microbial lipid production [12,13]. The economic analysis of the VFA model suggests that biodiesel production from VFA can compete with the current dominant sources, such as palm oil, soy bean oil, microalgae oil, and others [12].

With the whole-genome sequence of *T. oleaginosus* being reported [14], there have been major attempts to understand and manipulate this fungus and its metabolic pathways related to lipid synthesis by means of genomic and transcriptomic analyses [15]. However, to date, there has been only one report describing genetic engineering of *T. oleaginosus*, where Gorner et al. [16] successfully produced high-value non-natural fatty acids by the heterologous expression of enzymes [16]. As an alternative strategy for strain engineering, a fluorescence-activated cell sorting (FACS)-based screening strategy may be considered. Because FACS technology allows for high-throughput screening, randomly generated libraries on a large scale can be easily analyzed according to individual cell-fluorescence phenotypes, which can be generated through a genetic circuit designed for various target biomolecules [17,18]. To visualize the intracellular lipids and to apply a FACS-based strategy for screening, staining methods using lipophilic dyes such as Nile red and BODIPY have been described for microalgal species [19]. By applying this strategy for the adaptive evolution of *Chlamydomonas* sp., which lacks genetic tools for engineering, high-lipid producers were successfully isolated [20].

In this work, in order to develop a more efficient platform for microbial lipid production, we sought to engineer *T. oleaginosus* toward enhanced lipid-production from low-cost carbon sources (VFA). We established a method of lipid staining using lipophilic

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BODIPY, which was then used to screen a random library that was constructed using random mutagenesis with the chemical mutagen ethylmethane sulfonate (EMS). Using FACS-based high-throughput screening, a mutant was successfully isolated that was able to accumulate higher levels of intracellular lipids from VFA as a sole carbon source, compared to levels accumulated by the parental wild-type strain.

MATERIALS AND METHODS

1. Strain and Media

Wild-type *T. oleaginosus* (ATCC 20509) was purchased from the Korean Collection for Type Cultures (KCTC). All strains used in the study were maintained at 4 °C on YM (yeast extract, 3 g/L; malt extract, 3 g/L; peptone, 5 g/L; dextrose, 10 g/L) agar plates, and were subcultured every 15 days. For cell culture, cells were first cultivated in YPD liquid media (yeast extract, 10 g/L; peptone, 10 g/L; glucose, 20 g/L), and then transferred into minimal medium (KH_2PO_4, 2.7 g/L; Na_2HPO_4, 0.95 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; citric acid·H_2O, 0.52 g/L; yeast extract, 0.1 g/L; $CaCl_2 \cdot 6H_2O$, 40 mg/L; $FeSO_4 \cdot 7H_2O$, 5.5 mg/L; $ZnSO_4 \cdot 7H_2O$, 4 mg/L; $MnSO_4 \cdot H_2O$, 3 mg/L; 18 M H_2SO_4 (aq), 1 μL/L, pH 6.0) supplemented with glucose or VFA (acetic acid : propionic acid : butyric acid at volume ratios of 5 : 1 : 1, respectively) as carbon sources. Ammonium chloride (NH_4Cl) was supplied as a nitrogen source for molar C/N ratio of 20. The pH was adjusted to 6.0 with 10 M NaOH, and the temperature was maintained at 25 °C. Cells were incubated in a rotary shaker at 200 rpm and were cultured in 50 mL of minimal medium in 250 mL flasks. A final concentration of 50 μg/mL of streptomycin was added as the sole antibiotic. Cell growth was determined by measuring the optical density (OD) at 600 nm, using a spectrophotometer (Optizen POP; Mecasys, Daejeon, Republic of Korea).

2. Random Mutagenesis

EMS and its inactivating reagent sodium thiosulfate were purchased from Sigma Aldrich (St. Louis, MO, USA). The mutagenesis involved modifying an online method from the Hahn laboratory (http://research.fhcrc.org/hahn/en/methods.html). Wild-type cells were cultured in 30 mL of YPD broth in 250 mL flasks, and after culturing for 24 h, 5 mL of the culture broth was centrifuged at 8,000 rpm for 2 min. The harvested cells were washed with 100 mM sodium phosphate buffer (pH 7.0) and were resuspended in 1.7 mL of the same buffer. The cells were transferred to 16×125 mm glass tubes, and 50 μL of EMS was added. After incubation at 30 °C in a rotary shaker at 200 rpm for 1 h, 8 mL of 5% sodium thiosulfate was added to stop the mutagenesis. The cells were washed with 100 mM sodium phosphate buffer (pH 7.0) and then transferred into minimal medium (50 mL) containing 6 g/L of the mixture of VFA.

3. Intracellular Lipid Staining

For the FACS analysis of intracellular lipid, cells were stained with fluorescent BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene). The staining was performed by modifying a previous protocol used for staining microalgae [19]. A 5 mM stock solution was prepared by dissolving BODIPY in dimethyl sulfoxide (DMSO; Sigma Aldrich). After culturing, cells were harvested by centrifugation (8,000 rpm, 2 min, 25 °C), and the cell pellets were resuspended in 1 mL of 1× phosphate-buffered saline (PBS; NaCl, 8 g/L; KCl, 0.2 g/L; Na_2HPO_4, 1.42 g/L; KH_2PO_4, 0.24 g/L). The cells were mixed with a final concentration of 5 μM BODIPY and incubated at 25 °C in a rotary shaker at 200 rpm for 5 min. The cells were washed twice using 1 mL of 1× PBS each time. Finally, the cells were resuspended in 1 mL of 1× PBS and directly used for FACS analysis and library screening.

4. Library Screening

FACS-based library screening was performed using a MoFlo™ XDP cell sorter (Beckman Coulter, Inc., Miami, FL, USA) equipped with a 488 nm laser and a 530/40 nm band pass filter. The top 1% of the cells with high-fluorescence signals were sorted using the purify mode. The sorted cells were collected directly into liquid minimal medium, and the collected cells were cultured for the subsequent round of screening.

5. Fatty Acid Methyl Ester (FAME) Analysis

After culturing, cells were harvested by centrifugation (8,000 rpm, 5 min, 4 °C). Cell pellets were freeze-dried at −50 °C for four days. For FAME analysis, intracellular lipids were extracted from 10 mg of dried cells using 2 mL of a chloroform-methanol mixture (2 : 1, v/v) followed by 5 min of vigorous agitation. For the transesterification of extracted fatty acids, each sample was treated with 1 mL of methanol and 300 μL of sulfuric acid, and heated at 115 °C for 20 min. After cooling to 25 °C, 1 mL of distilled water was added and mixed. Following centrifugation (4,000 rpm, 5 min, 25 °C), the organic phase was collected and filtered using a 0.20 mm RC-membrane syringe filter (Sartorius Stedim Biotech, Germany). FAME content was analyzed using gas chromatography (HP6890, Agilent Technology Inc., Santa Clara, CA, USA) coupled with a flame-ionized detector and an INNOWAX capillary column (30 m × 0.32 mm × 0.5 μm; Agilent). FAMEs were identified and quantified by comparing the retention times and peak areas against mixtures of FAME standards.

6. Nutrient Analysis

The concentrations of acetic acid, propionic acid and butyric acid in the culture broth were determined using high-performance liquid chromatography (HPLC) with an LC-20AD pump, CTO-20A column oven, and SPD-20A UV-Vis detector (Shimadzu, Kyoto, Japan) equipped with a Zorbax Eclipse AAA column (4.6×150 mm, 3.5-micron; Agilent) [21]. During the culturing, supernatant samples were prepared by centrifugation (13,000 rpm, 1 min, 4 °C), and filtered using 0.22 μm PVDF syringe filters (Futecs Co., Ltd., Daejeon, Republic of Korea). Chromatography was performed at 50 °C with a 0.45 $mL min^{-1}$ flow rate using 5 mM H_2SO_4 as a single mobile phase. VFA concentrations were detected at 210 nm. Glucose concentration was determined using a glucose analyzer (YSI 2700 SELECT™ Biochemistry Analyzer; YSI Life Sciences, Yellow Springs, OH, USA).

RESULTS AND DISCUSSION

1. Evaluation of the VFA Media

Although the VFA composition from anaerobic fermentation of food waste can vary because of various factors, including temperature and pH, in most cases acetic acid remains the major component [22]. Also, it is generally known that microorganisms, including

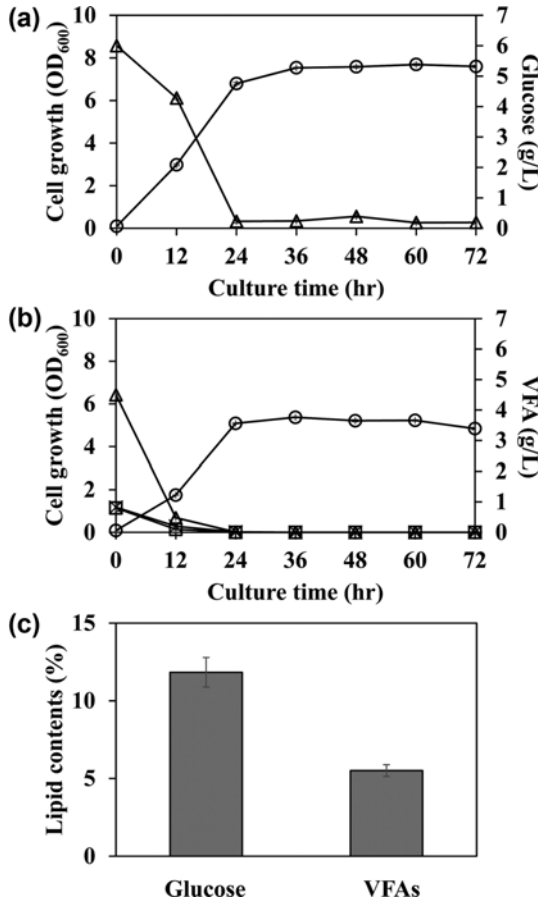

Fig. 1. Time profiles of cell density (OD_{600}) and nutrient concentrations during cultivation of wild type *T. oleaginosus* in 6 g/L glucose (a) and VFAs (b). Symbols: open circle (○), cell density (OD_{600}); open triangle (△), glucose (a) or acetic acid (b); open square (□), propionic acid; cross (×), butyric acid. (c) Content of lipid accumulated in cells during cultivation in each carbon source (glucose or VFAs).

T. oleaginosus, prefer acetic acid over propionic and butyric acid for both lipid accumulation and cell growth [23,24]. Based on previous report [21], each VFA (acetic acid, propionic acid, and butyric acid) was mixed at a volume ratio of 5 : 1 : 1, respectively, and the VFA mixture was used as a sole carbon source for the cultivation of the *T. oleaginosus*. To verify the feasibility of using the VFA-based media, the growth and lipid production of the wild-type strain were examined. When 6 g/L of total VFA or glucose was supplemented as the sole carbon source, the VFA media demonstrated no benefit in terms of cell growth (Fig. 1(a) and 1(b)). In the media containing glucose (6 g/L), the wild-type *T. oleaginosus* consumed the glucose within 24 h and grew to OD_{600}=7.7 (Fig. 1(a)). However, in the media containing VFA, cell density reached only OD_{600}= 5.3, even though all the VFA were consumed within 24 h (Fig. 1(b)). From the analysis of total lipid production, we also found that wild-type *T. oleaginosus* cells synthesized more lipid in glucose media than in VFA media. In VFA media, the lipid content was 5.5% (w/w), but culturing in the equivalent concentration of glucose as the carbon source, the lipid content was as high as 12% (w/w) (Fig. 1(c)). These results supported the argument that it is necessary to develop a high-lipid producer, capable of a more efficient and economic production of biodiesel precursors using VFAs as carbon sources.

2. Staining Intracellular Lipids Using BODIPY Fluorescent Dye

To isolate high-lipid producers from the random library, we designed a FACS-based high-throughput screening method that could isolate high-lipid producing cells based on fluorescence intensity. It is known that lipids synthesized in microbial cells can be efficiently stained with lipophilic fluorescent dyes such as Nile red and BODIPY, and that the resulting fluorescence intensity is highly correlated to the intracellular lipid content [19,20,25]. Using flow cytometry analysis, the fluorescence intensity of individual cells can be analyzed at high speeds (>10^5 cells/sec), and the cells demonstrating a higher level of fluorescence which represent high-lipid production, can be isolated from large populations with high accuracy. Using a lipophilic BODIPY dye, we evaluated the fluorescent staining of lipid synthesized in *T. oleaginosus*. First, to find the optimal concentration of BODIPY for lipid staining, six different concentrations of BODIPY (0.25, 1, 2.5, 5, 10, and 50 μM final concentrations) were examined by staining cells with the same lipid content, and the fluorescence signals were compared using flow cytometry analysis. As shown in Fig. S1, higher fluorescence signals were generated as the BODIPY concentrations increased. Quantitatively, the fluorescence signals were not linearly correlated with BODIPY concentrations, rather the signals seemed to become saturated as the concentrations were increased. We believe that excess amounts of BODIPY caused non-specific accumulation, while too small of amounts of the dye may lead to a reduction of the minimal range of detection. Based on these findings, 5 μM was determined to be the optimal concentration of BODIPY for staining intracellular lipid in *T. oleaginosus*. In addition, at this concentration, no significant effects on cell viability from the BODIPY staining were observed (Table S1), which means the labeling with BODIPY at 5 μM is allowed for cell labeling without cell damage.

Next, the fluorescence signals from cells containing different lipid levels were compared. Therefore, cells were cultivated in minimal media with different initial concentrations of glucose (5, 10, 15, and 30 g/L) in which cells could synthesize different amounts of lipid. The actual content of the intracellular lipid from each culturing condition was also analyzed by gas chromatography (GC), and it was clearly confirmed that the lipid content also gradually increased, in concordance with the increases of the initial glucose concentrations. The lipid content was 8.53, 19.09, 30.07, and 39.4% for cells cultured with glucose concentrations of 5, 10, 15, and 30 g/L, respectively. From the same cultivations, the fluorescence intensities of cells after BODIPY (5 μM) staining were analyzed by flow cytometry. As a result, higher fluorescence signals were detected as the initial glucose concentrations were increased (Fig. 2(a)). The plot of mean fluorescence intensities (MFI) relative to lipid content demonstrated a linear correlation for the lipid range under 40% (R^2=0.9891) (Fig. 2(b)). This result indicated that the FACS-based high-throughput screening method could be used for the isolation of high-lipid producing cells from a random library.

3. Isolation of a High-lipid Producing Mutant from a Random Library

For the screening of high-lipid producer, a random library was

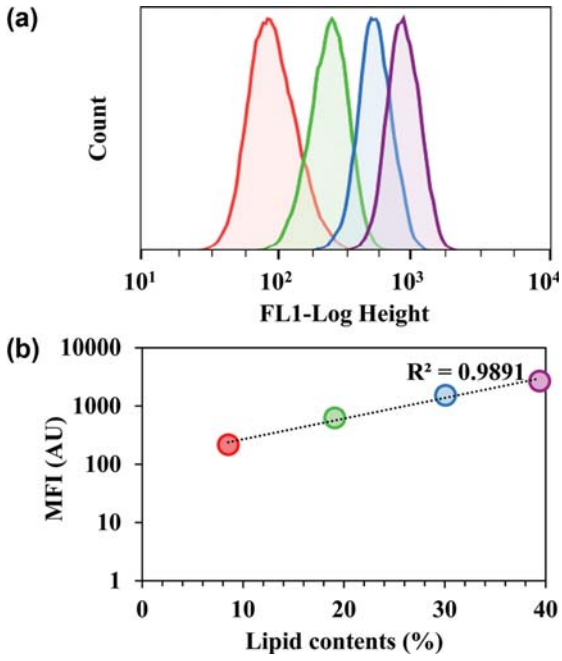

Fig. 2. (a) FACS analysis of intracellular lipids synthesized in 5, 10, 15 and 30 g/L glucose media, which is represented in red, green, blue and purple colors, respectively. (b) The mean fluorescence intensity (MFI) values were plotted against the same cells' actual lipid contents. Each color refers to the peaks with the same color in (a). The dashed line represents the linear trend.

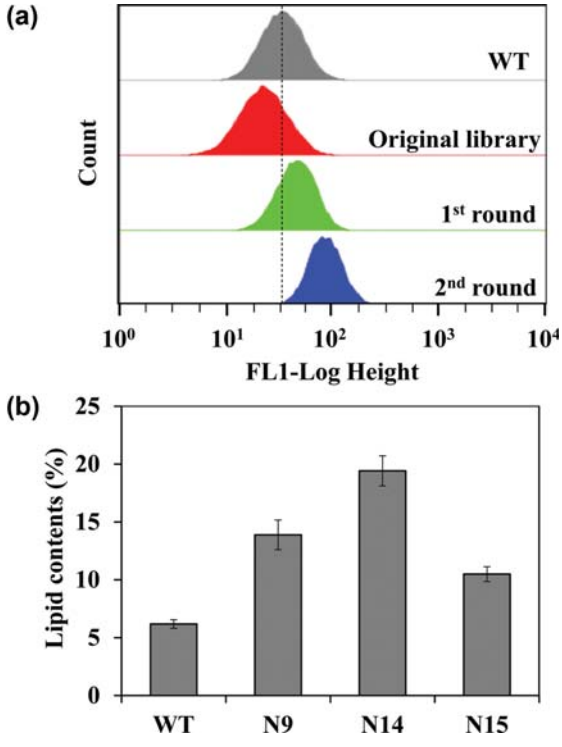

Fig. 3. FACS-based high throughput screening of library (a) the fluorescence signals of wild-type, original mutant library, 1st-sorted population, and 2nd-sorted population. (b) Lipid contents (%) in the isolated mutants and wild type strain (WT).

constructed by mutagenesis of wild-type *T. oleaginosus* using EMS treatment as described in materials and methods section. After culturing, cells from the random library were stained with BODIPY (5 μM), and the high-fluorescence cells were selectively sorted using a flow cytometer. In each round, the population with high fluorescence (top 1% of the total cells) was selectively sorted and recovered. The original library exhibited a slightly lower mean intensity than that of the wild-type cells; however, the mean fluorescence of the cells gradually, but noticeably, increased throughout the two consecutive rounds of sorting (Fig. 3(a)). After the second round of sorting, the sorted cells were spread on YM agar plates and incubated. Fifteen clones were randomly picked for analysis of lipid production in minimal media containing 6 g/L of VFA. After culturing and staining with BODIPY, the fluorescence signal of each clone was analyzed by flow cytometry. Among the 15 clones, three candidates (clones N9, N14, and N15) demonstrated considerably higher peaks of fluorescence intensity compared to that of wild-type cells (Fig. S3). The actual lipid content in those three candidates was determined and all showed higher lipid content than that of

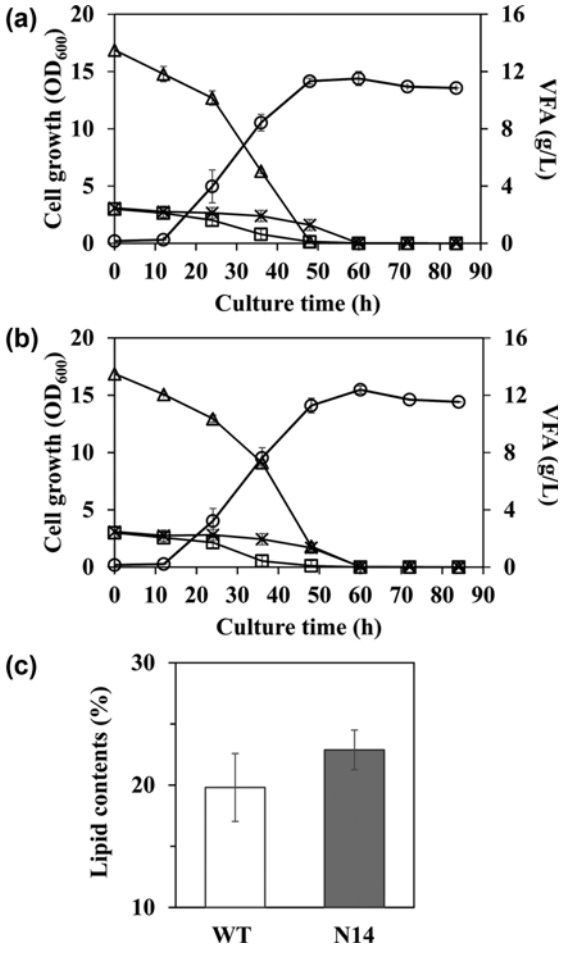

Fig. 4. Time profiles of cell density (OD_{600}) and nutrient concentrations of wild-type (a) and N14 (b) during culturing in 18 g/L VFA. Symbols: open circle (○), cell density (OD_{600}); open triangle (△), acetic acid; open square (□), propionic acid; cross (×), butyric acid. (c) Content of lipid accumulated in cells during each cultivation.

wild type (Fig. 3(b)). Among them, clone N14 exhibited the highest lipid content (19.4%), which was approximately 3-fold higher than that of wild-type cells (6.2%), while showing similar growth and VFA utilization profiles (Fig. S4). Based on these results, N14 was selected as a final candidate for further characterization.

4. Lipid Production in Higher Concentrations of VFA

To verify the potential usefulness of the N14 strain, cell growth and lipid production were examined in higher concentrations of VFA (18 g/L and 24 g/L). When cells were cultivated in the media containing 18 g/L of VFA, the growth characteristics of N14 strain were similar to those of the wild-type strain (Fig. 4(a) and 4(b)). However, N14 demonstrated a 17% higher lipid accumulation (22.9±1.6%) compared to that of the wild-type strain (19.8±2.8%) (Fig. 4(c)). In the media containing 24 g/L of VFA, N14 exhibited a relatively longer lag phase and a lower final cell density compared with the wild-type strain (Figs. 5(a) and 5(b)), while the mean lipid level in the N14 strain was 37.4±1.6% of the total cellular content, which was 18% higher than that in the wild-type strain (31.7±3.5%), as shown in Fig. 5(c). When we compared our results of isolated N14 strain. From this result, lipid content (wt%), productivity (g/L/h) and yield (g/g) were compared with previous literatures where mixed volatile fatty acids were used (Table S2). Comparison results show that it is possible to achieve higher lipid production without any genetic modification. It is plausible that the VFA composition and cultivation conditions (media component, temperature, etc.) can greatly affect the growth and lipid production ability. Thus, further characterization and optimization is needed for N14.

5. Analysis of Lipid Synthesized in the N14 Strain

For further characterization of lipid synthesized in the N14 strain, cells were cultured in media containing two different carbon sources (glucose and VFA) at a concentration of 24 g/L, and the major FAME composition of the lipids synthesized in the N14 strain were compared with those in the wild-type strain. With both carbon sources, the N14 strain showed higher lipid content compared to that of the wild-type strain (Fig. 6(a)). Particularly, the N14 strain developed greater levels of lipid production during culturing in VFA medium compared to culturing in glucose medium. In contrast, the wild-type strain exhibited lower levels of lipid production in VFA compared to culturing in glucose. Additionally, the fatty acid profiles of the lipid were analyzed. When glucose was provided as the sole carbon source, both wild type and N14 strains showed similar profiles; C18 fatty acids comprised more than 80% of the five fatty acids, with oleic acid (C18:1) being the major component (Fig. 6(b)). In contrast, when VFA were provided as the sole carbon source, different fatty acid profiles were observed between the wild-type and N14 strains. Specifically, the N14 strain exhibited similar profiles as those when cultured in glucose medium. In

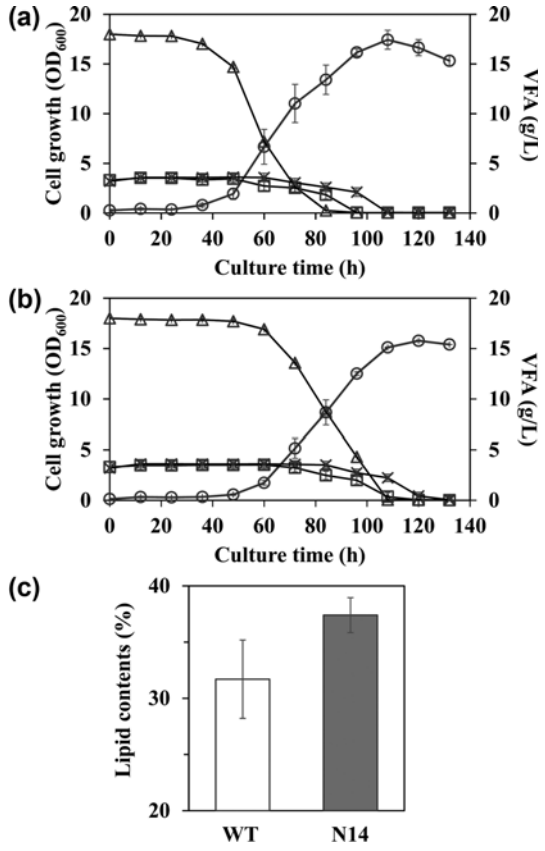

Fig. 5. Time profiles of cell density (OD_{600}) and nutrient concentrations of wild-type (a) and N14 (b) during culturing in 24 g/L VFA. Symbols: open circle (○), cell density (OD_{600}); open triangle (△), acetic acid; open square (□), propionic acid; cross (×), butyric acid. (c) Content of lipid accumulated in cells during each cultivation.

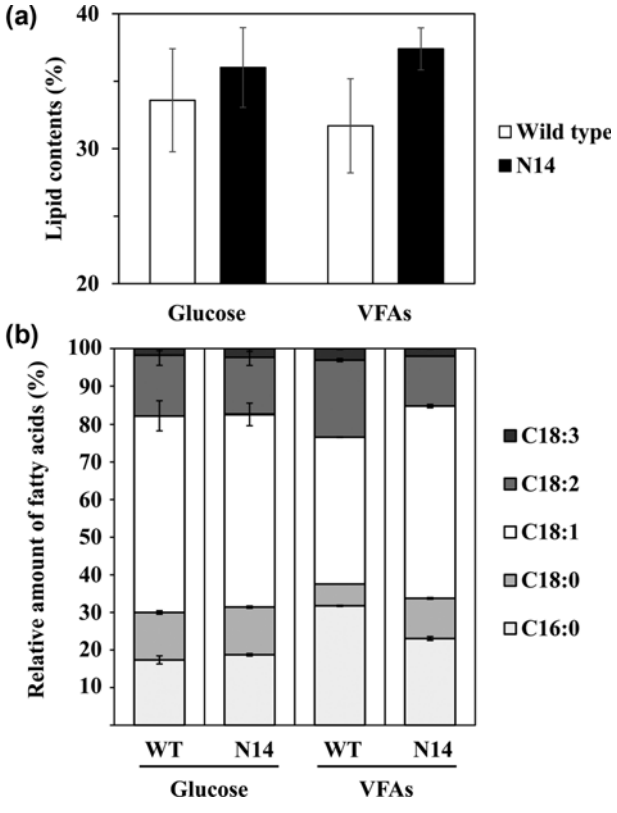

Fig. 6. (a) The lipid contents in wild-type (white bar) and N14 (black bar) in 24 g/L of glucose and VFA. (b) The relative amounts (w/w) of fatty acids.

contrast, the wild-type strain demonstrated differences in FAME composition; stearic acid (C18:0) and oleic acid (C18:1) levels were decreased, while palmitic acid (C16:0) and linoleic acid (C18:2) levels were increased. Because lipid composition directly affects biodiesel quality, including physical and chemical properties [26], various efforts have been made to modify lipid composition of microorganisms by genetically engineering the producer strains [27,28]. These efforts have primarily focused on increasing the proportion of oleic acid and decreasing the levels of linoleic acid [28] because oleic acid is favorable in terms of fuel ignition properties (Cetane number), and because the amount of mono-unsaturated fatty acids (MUFAs) highly correlates to the cold-flow properties of a biodiesel [26]. In this perspective, we propose that the N14 strain of *T. oleaginosus* has advantages in both lipid titer and quality, as they relate to use as a biofuel.

CONCLUSIONS

We successfully isolated high lipid-accumulating *T. oleaginosus* mutant (N14) by the FACS-based screening of a random mutant library. The isolated N14 produced higher levels of lipids by utilizing VFA as sole carbon sources. We also demonstrated that BODIPY could be applied as a reliable indicator of the amount of intracellular lipids. To the best of our knowledge, this is the first report on the successful application of FACS-based screening to *T. oleaginosus*. In combination with the FACS-based strategy, the continuing efforts in genetic engineering will ultimately lead to a sustainable biodiesel produced from low cost carbon sources.

ACKNOWLEDGEMENTS

This work was supported by the Advanced Biomass R&D Center (ABC) of Global Frontier Project funded by the Ministry of Science and ICT (Grant No. 2017018586) and Commercializations Promotion Agency for R&D Outcomes (Grant No. 2016K000192).

SUPPORTING INFORMATION

Additional information as noted in the text. This information is available via the Internet at http://www.springer.com/chemistry/journal/11814.

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