



Highly selective isolation and characterization of *Lipomyces starkeyi* mutants with increased production of triacylglycerol

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Abstract

The oleaginous yeast *Lipomyces starkeyi* is an attractive organism for the industrial production of lipids; however, the amount of lipid produced by wild-type *L. starkeyi* is insufficient. The study aims to obtain *L. starkeyi* mutants that rapidly accumulate large amounts of triacylglycerol (TAG). Mutagenized yeast cells at the early stages of cultivation were subjected to Percoll density gradient centrifugation; cells with increased production of TAG were expected to be enriched in the resultant upper fraction because of their lower density. Among 120 candidates from the upper fractions, five mutants were isolated that accumulated higher amounts of TAG. Moreover, when omitting cells with mucoid colony morphology, 11 objective mutants from 11 candidates from the upper fraction were effectively (100%) isolated. Of total 16 mutants obtained, detailed characterization of five mutants was performed to reveal that five mutants achieved about 1.5–2.0 times TAG concentration (4.7–6.0 g/L) as compared with the wild-type strain (3.6 g/L) at day 5. Among these five mutants, strain E15 was the best for industrial use because only strain E15 showed significantly higher TAG concentration as well as significantly higher degree of lipid to glucose and biomass to glucose yields than the wild-type strain. Thus, Percoll density gradient centrifugation is an effective method to isolate mutant cells that rapidly accumulate large amounts of TAG. It is expected that by repeating this procedure as part of a yeast-breeding program, *L. starkeyi* mutants suitable for industrial lipid production can be easily and effectively obtained.

Keywords Oleaginous yeast · *Lipomyces starkeyi* · Triacylglycerol · Percoll · Lipid droplet

Introduction

Global energy demand is constantly increasing, and excessive use of fossil fuels has caused increases in greenhouse gas

emissions. As such, there is an urgent need to reduce emissions of greenhouse gases that cause global warming. Future energy demands compel us to explore the production of biofuels, i.e., clean fuels produced through the harnessing of biological processes, as a suitable alternative source of energy. For example, biodiesel is already an alternative fuel to petroleum diesel used widely in Europe. However, since biofuels are currently made primarily from edible oils, the production of biofuels is competitive to feed and food production, leading to a global food supply imbalance, and requires large areas of land for production, which may cause deforestation and destruction of the ecosystem (Gui et al. 2008).

To overcome this problem, oils produced by microorganisms are becoming an attractive alternative to petroleum because their production and use do not affect food supply or require a large amount of space. It has been reported that many microorganisms can accumulate large amounts of lipid per cell (Ratledge 2004; Liang and Jiang 2013). Among these microorganisms, oleaginous yeasts, including *Yarrowia*

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lipolytica, *Cryptococcus albidus*, *Rhodospodium toruloides*, *Rhodotorula glutinis*, and *Lipomyces starkeyi*, are the most promising candidates for industrial lipid production because they can accumulate large amounts (greater than 20% of their dry weight) of lipids in the form of triacylglycerol (TAG) per cell, and have ability to use a wide variety of carbon sources, such as hexose and pentose, derived from wood, grass, energy crops, and even sewage sludge (Ageitos et al. 2011; Sitepu et al. 2014; Mcneil and Stuart 2018; Sutanto et al. 2018). Among these oleaginous yeasts, *L. starkeyi* has great industrial potential, because the amount of TAG it accumulates accounts for up to 70% of its dry weight. Recently, Juanssilfero et al. (2018) demonstrated that wild-type *L. starkeyi* NBRC10381 cells cultured under nitrogen-limited conditions accumulated a lipid content constituting 85% of their dry weight.

In some oleaginous yeasts, mutants with large amount of TAG have already been obtained by random mutagenesis and pre-selection with cerulenin, an inhibitor of fatty acid synthase (Wang et al. 2009; Tapia et al. 2012; Yamada et al. 2017). Wang et al. (2009) irradiated *Rhodotorula glutinis* cells with carbon ions and subsequently obtained mutants with high lipid content by pre-selection with cerulenin. Yamada et al. (2017) improved the cerulenin screening in which they mutagenized *Rhodospodium toruloides* cells by ultraviolet irradiation and subsequently selected ethanol- and H₂O₂- or cerulenin-tolerant mutants. In *L. starkeyi*, Tapia et al. (2012) reported that they mutagenized cells with ultraviolet irradiation and subsequently obtained a mutant that accumulated more lipid than the wild-type control strain through cerulenin screening. However, these methods are still time- and/or cost-consuming because some mutagenized cells after pre-selection did not show significant increase in TAG content. Moreover, in the cerulenin selection, mutant cells were not screened in terms of shortening cultivation time to accumulate higher amount of TAG as compared to a wild-type strain. Saving time for accumulating TAG is thought to be the need for industrial use.

In the present study, wild-type *L. starkeyi* CBS1807 cells were mutagenized using ethyl methanesulfonate, and then Percoll density gradient centrifugation was used to enrich the low-density cells at the early stages of cultivation, which were expected to rapidly accumulate large amounts of TAG. After the isolation of cells from the low-density fractions, 11 colonies were obtained that did not show a mucoid colony morphology, and it was confirmed that all 11 isolated mutants accumulated larger amounts of TAG per cell than the wild-type control cells, indicating that the combination of low-density cell enrichment via Percoll density gradient centrifugation and omitting colonies having mucoid morphology is a very effective strategy for isolating mutant cells that produce large amounts of TAG.

Materials and methods

Strain and media

Lipomyces starkeyi strain CBS1807 (Centraalbureau voor Schimmelcultures) was used as a wild-type strain. Cells were cultured in YPD medium (1% yeast extract (Kyokutou, Tokyo, Japan), 2% HIPOLYPEPTON (Nihon Pharmaceutical, Tokyo, Japan), and 2% glucose), semi-defined medium (S medium) (0.5% (NH₄)₂SO₄, 0.1% KH₂PO₄, 0.01% NaCl, 0.1% yeast extract (Kyokutou, Tokyo, Japan), 0.05% MgSO₄·7H₂O, 0.01% CaCl₂·2H₂O, and 5% glucose), and semi-defined glycerol medium (SG medium) (0.5% (NH₄)₂SO₄, 0.1% KH₂PO₄, 0.01% NaCl, 0.1% yeast extract (Kyokutou, Tokyo, Japan), 0.05% MgSO₄·7H₂O, 0.01% CaCl₂·2H₂O, and 2% glycerol). The pH of S and SG mediums were adjusted to 5.5 with 1 N NaOH before adding 5% MgSO₄·7H₂O and 1% CaCl₂·2H₂O and autoclaving. Reagents were obtained from Wako (now renamed FUJIFILM Wako Pure Chemical) (Osaka, Japan) unless otherwise mentioned. Solid medium was made by adding 2% agarose to liquid medium. Cells were cultured at 30 °C.

Determination of cell concentration

Cell cultures were adequately diluted with Cell Pack (Sysmex, Kobe, Japan), sonicated with a Vibra Cell sonicator (Sonics & Materials, Danbury, CT, USA) for 20 s (cycles of 5 s “on,” 5 s “off”) and then subjected to counting in a CDA-1000 particle counter (Sysmex, Kobe, Japan).

Ethyl methanesulfonate treatment

Wild-type *L. starkeyi* cells were cultured to the logarithmic phase (1.0×10^7 cells/mL) in YPD medium and were collected via centrifugation at 2270×g for 5 min at room temperature. After washing twice with 10 mL of 0.2 M sodium phosphate buffer (pH 7.0), 2.0×10^8 cells were suspended in 9660 μL of 0.2 M sodium phosphate buffer (pH 7.0) in a 1.5-mL tube, and then 340 μL of EMS solution (Sigma-Aldrich, St Louis, MO, USA) for EMS-treated cells or 340 μL of 0.2 M sodium phosphate buffer (pH 7.0) for EMS-untreated cells was added to the tube. The tube was then rotated at 30 °C for 30 min for the first and the second trials or 20 min for the third one. Cells were then collected via centrifugation at 5000×g for 4 min at room temperature, washed twice with 1 mL of 5% sodium thiosulfate solution to inactivate the EMS, washed once with 1 mL of YPD medium, and then suspended in 1 mL of YPD medium. A total of 100 μL of the resulting cell suspension was used for a cell viability count, and the remainder of the cell suspension (900 μL) was subjected low-density cell enrichment using Percoll density gradient centrifugation.

Estimation of cell viability after EMS treatment

Cells from the EMS-treated and the untreated cell suspensions (100 μL) were diluted 10, 100, and 1000 times with YPD medium, and 100 μL of each diluted cell suspension was inoculated on solid YPD medium. After 4 days of incubation, the number of colonies was counted, and the estimated cell viability (CV) of the EMS-treated cells was calculated using the following formula: $\text{CV} = (\text{the number of colonies of the EMS-treated cells}) / (\text{the number of colonies of the untreated cells}) \times 100$.

Enrichment of low-density cells using Percoll density gradient centrifugation

Cells from the EMS-treated and the untreated cell suspensions (900 μL) produced in the previous section were washed once with SG medium and then incubated in 7 mL of SG medium in L-shaped test tubes at 30 °C for 3 days. Part of the culture described above was added to 75 mL of S medium in a 200-mL baffled flask to yield a total cell count of 1.25×10^6 cells/mL. After a 24-h incubation at 30 °C with rotation at 160 rpm, part of the resultant culture was again added to 75 mL of S medium in a 200-mL baffled flask giving a total cell count of 1.25×10^6 cells/mL. After an incubation of 2–3 days at 30 °C, 1.0×10^9 cells were collected via centrifugation at $3000 \times g$ for 5 min at room temperature, and then suspended in 1 mL of PBS (0.8% NaCl, 0.29% $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.02% KCl, and 0.02% KH_2PO_4). This cell suspension was mixed with 8 mL of Percoll (GE Healthcare). The mixture was centrifuged in a 70.1 Ti rotor (Beckman Coulter) at 25 °C for 20 min at 12,000 rpm for the first and second trials or at 22,000 rpm for the third one. After Percoll density gradient centrifugation, the resultant upper fractions including the low-density cells were collected and cultivated on SG medium of the next cultivation. This enrichment of low-density cells was repeated four times in the first trial or three times in the second and third one; the uppermost fractions isolated, which were thought to include many low-density cells, were streaked on solid medium.

Small-scale cultivation conditions

The resulting colonies from the previous section were picked and re-cultured in test tubes containing 2 mL of SG medium at 30 °C for 2 days. Part of the culture described above was added to 5 mL of S medium in a 50-mL tube in which the optical density at 660 nm of the final culture was 0.2. After a 24-h incubation at 30 °C with rotation at 160 rpm, part of the resultant culture was again added to 5 mL of S medium in a 50-mL tube in which the optical density at 660 nm of the final culture was 0.2. After a 48-h incubation at 30 °C with rotation

at 160 rpm, cultures were collected for fluorescence-activated cell sorting (FACS) analysis.

Fluorescence-activated cell sorting analysis

Cells incubated for 2 days in S medium as described above were collected via centrifugation for 1 min at $5000 \times g$ at room temperature. The cells were then suspended in 1 mL of PBS to yield a total cell count of 5.0×10^6 cells/mL, and 50 μL of 0.1 mg/mL Nile Red (Wako, Osaka, Japan) was added to the cell suspension. After 3 min, the cell suspension was analyzed using a FACS Calibur Flow cytometer (Becton Dickinson, San Jose, CA, USA) with an FL2 channel detector and CellQuest Software (Becton Dickinson).

Large-scale cultivation conditions

Among the mutants isolated during the trials, the colonies designated A42, E15, E47, K13, and K14 were selected and characterized using large-scale cultivation. The resulting colonies were picked and re-cultured in 7 mL of SG medium in an L-shaped test tube at 30 °C for 3 days. Part of the culture described above was added to 75 mL of S medium in a 200-mL baffled flask to yield a total cell count of 1.25×10^6 cells/mL. After a 24-h incubation at 30 °C with rotation at 160 rpm, part of the resultant culture was again added to 75 mL of S medium in a 200-mL baffled flask to yield a total cell count of 1.25×10^6 cells/mL. After a 24-h incubation at 30 °C with rotation at 160 rpm, cultures were collected (day 1) and subsequently sampled every 24 h.

Extraction and quantification of TAG

Extraction and quantification of the amount of intracellular lipid (mainly triglycerides) were essentially described (Naganuma et al. 1982; Oguri et al. 2012; Oguro et al. 2017). Cells were harvested from 1 mL of liquid culture via centrifugation at $5000 \times g$ for 1 min at room temperature, washed once with 1 mL of PBS, and re-suspended in 1 mL of PBS in a 2-mL tube. The optical density of the cell suspension at 660 nm was adjusted to < 10 . After centrifugation at $5000 \times g$ for 1 min at room temperature, the resultant cell pellet was incubated at 70 °C for 5 min, and then stored at -30 °C. Subsequently, 500 μL of PBS and 1 g of glass beads (0.5 mm in diameter, AS ONE) were added to the 2-mL tube, and the cells were homogenized at room temperature using a bead beater instrument (Multi-Beads Shocker, Yasui Kikai, Osaka, Japan) for 30 cycles (30 s of agitation and 30 s of rest) at 2500 rpm. After 500 μL of PBS was added to the 2-mL tube, the tube was incubated at room temperature with rotation at a maximum speed using Micro Mixer (TAITEC, Saitama, Japan). The total amount of TAG and glycerol in the homogenized cell suspension was enzymatically analyzed using a TG E-test (Wako,

Osaka, Japan) according to the manufacturer's instructions. Briefly, TAG in a sample was treated with lipoprotein lipase and the released glycerol concentration was determined using the absolute calibration method. Assuming that all TAGs are triolein, TAG concentration was calculated from glycerol concentration. The amount of glycerol in the homogenized cell suspension was enzymatically analyzed using an F-kit for glycerol (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. For the third trial, the amount of TAG was determined as the value obtained using the TG E-test. On the other hand, for the characterization of the obtained mutants, A42, E15, E47, K13, and K14, the amount of TAG was determined as the difference in the values obtained using the TG E-test and the F-kit for glycerol.

Measurement of dry cell weight

Cells were harvested from 1 mL of liquid culture via centrifugation at $5000\times g$ for 1 min at room temperature in a 1.5-mL tube and re-suspended in 1 mL of PBS. The cell suspension was centrifuged for 1 min at $5000\times g$ at room temperature and the resultant cell pellet was stored at $-30\text{ }^{\circ}\text{C}$. Thereafter, the cell pellet was freeze-dried using a Freeze Dryer (FD-1000, Eyela, Tokyo, Japan) to measure the weight.

Determination of glucose concentration of culture supernatant

The culture obtained after large-scale cultivation was centrifuged at $5000\times g$ for 1 min at room temperature. The D-glucose concentration of the obtained culture supernatant was measured using the Wako Glucose C-II Test (Wako, Osaka, Japan) according to the manufacturer's instructions.

Lipid extraction for comparison of fatty acid composition of TAG

The extraction of total lipids was performed by modifying Bligh and Dyer's method (Bligh and Dyer 1959). Overall, 6.0×10^9 cells were harvested from the liquid culture at day 5 via centrifugation at $6000\times g$ for 5 min at room temperature in a 2-mL test tube, washed once with 1 mL of 0.75% saline solution, and freeze-dried using a Freeze Dryer (FD-1000, Eyela, Tokyo, Japan). The freeze-dried cells were homogenized with 475 μL of chloroform:methanol:distilled water (1:2:0.8 (v/v/v)) solution using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) with 600 mg of glass beads (0.5 mm in diameter, AS ONE, Osaka, Japan) at 2500 rpm for 15 min at room temperature, and subsequently, 125 μL of chloroform and 125 μL of distilled water were added to the solution. The solution was mixed using the Multi-Beads Shocker at 2500 rpm for 5 min at room temperature. After centrifugation at $18,000\times g$ for 5 min, the upper aqueous layer was removed,

and the bottom layer was transferred to a 1.5-mL test tube and evaporated by impinging with nitrogen at room temperature. The extracted samples were treated with a fatty acid methylation kit for Glyceride (Nacalai Tesque, Kyoto, Japan) to convert fatty acids of TAG to fatty acid methyl esters (FAMES) according to the manufacturer's instruction.

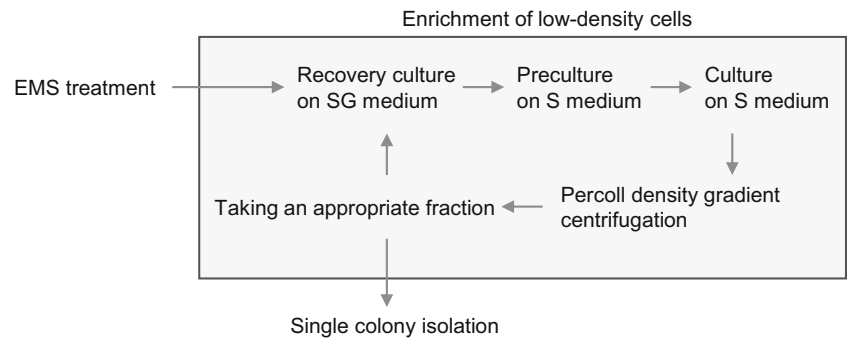
GC-FID analysis

The composition analysis of FAMES using gas chromatography with a flame ionization detector (GC-FID) was performed by modifying David et al.'s (2005) method using a Shimadzu GC-2014 (Shimadzu, Kyoto, Japan) instrument equipped with FID and a capillary column DB-23 (60 m \times 0.25 mm, 0.25- μm film thickness, J and W Scientific, Folsom, CA, USA). The injector port temperatures were maintained at $250\text{ }^{\circ}\text{C}$. Helium was used as the carrier gas at a flow rate of 1.11 ml/min. Split ratio of 10:1 was used, and 1 μL of sample was injected in GC-FID for the analysis. The oven temperature cycle was set as follows: $50\text{ }^{\circ}\text{C}$ (1 min), $25\text{ }^{\circ}\text{C}/\text{min}$ to $175\text{ }^{\circ}\text{C}$, and $4\text{ }^{\circ}\text{C}/\text{min}$ to $230\text{ }^{\circ}\text{C}$ (10 min). The detector gas was hydrogen and air, and the detector temperature was set at $280\text{ }^{\circ}\text{C}$. The individual FAMES were identified by their retention times in comparison with commercial FAME standards (Food industry FAME mix, Restek, Bellefonte, PA, USA). Peaks not identified above were confirmed by gas chromatography–mass spectrometry (GC-MS). Each carbon atom of the aliphatic compounds capable of hydrogenation yields the same signal and the overall FID response to each substance is proportional to the sum of these hydrogenable effective carbon atoms (Poole 2015), thereby allowing a direct comparison of the peak areas to determine the sample composition. The percentages of quantity of individual FAMES were calculated using the following formula: (Percentage of individual FAME) = (Area of the individual FAME)/(Sum areas under all peaks of all individual FAME) \times 100.

GC-MS analysis

The identification of unidentified FAMES on GC-FID analysis by GC-MS was performed by modifying David et al.'s (2003) method using a Shimadzu GCMS-QP2010 SE apparatus (Shimadzu, Kyoto, Japan) with a DB-23 column. The injection port, ion source, and interface temperatures were $250\text{ }^{\circ}\text{C}$, $200\text{ }^{\circ}\text{C}$, and $280\text{ }^{\circ}\text{C}$, respectively. Helium gas was used as the carrier gas at a constant flow rate of 1.32 mL/min. The oven temperature, split ratio, and injection volume were according to the GC-FID condition. The MS was operated in electron impact ionization mode (EI, 70 eV), using the scan mode. All mass spectra were acquired over the m/z range of 45–500. The identification of compounds was performed using a NIST08 Mass Spectral Library (National Institute of Standards and Technology, MD, USA) and the Shimadzu GC/MS Metabolite Database ver. 2 (Shimadzu, Kyoto, Japan).

Fig. 1 Schematic diagrams illustrating the work flow for isolation of low-density cells that accumulate larger amounts of TAG. EMS-treated cells were subjected to a low-density cell enrichment procedure three or four times per trial, and then isolated on solid media



Statistical analysis

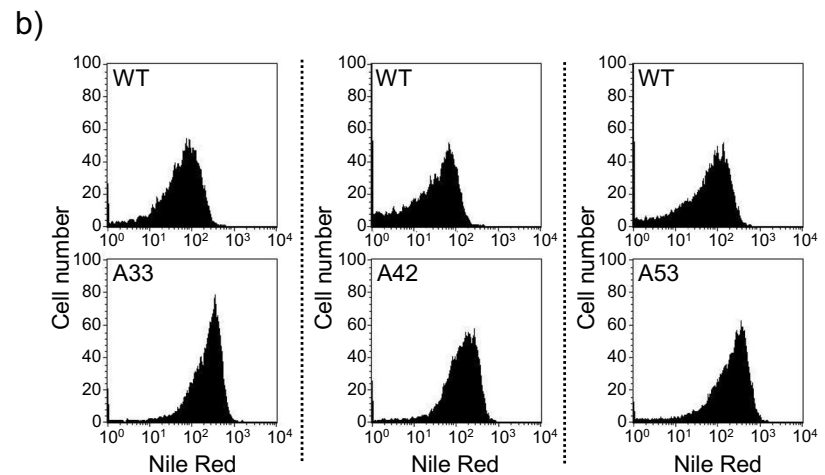
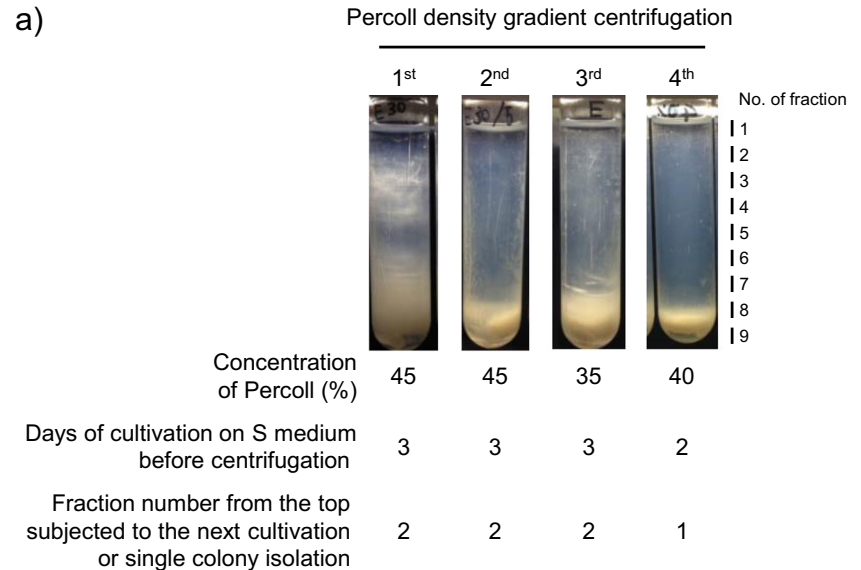
Statistical analysis was performed using Dunnett’s test and Tukey’s test with GraphPad Prism software, version 8 (GraphPad Software Inc., San Diego, CA, USA). Differences were considered to be significant when the *P* value was less than 0.05.

Results

For the first trial to obtain mutants with increased TAG production per cell, wild-type *L. starkeyi* cells were mutagenized using EMS. CV tests performed after mutagenesis showed 6%. The low-density cells were enriched using Percoll density gradient centrifugation four times (for details, refer to the

Fig. 2 Isolation of strains A33, A42, and A53 in the first trial. **a** Enrichment of low-density cells by performing Percoll density gradient centrifugation four times after EMS treatments.

Concentration (%) of Percoll and days of cultivation on S medium before Percoll density gradient centrifugation is shown. The centrifuged samples were divided into nine fractions and the fractions from the top subjected to the next cultivation or single colony isolation are also shown. **b** FACS analysis of the wild-type (WT), A33, A42, and A53 cells stained with Nile Red after small-scale cultivation for 2 days. Histograms show relative fluorescence of Nile Red (*x* axis) and cell number (*y* axis). Each histogram represents 10,000 cells. The representative histograms of duplicate experiments are shown



“Materials and methods” section and Fig. 1). After the fourth centrifugation, nine fractions were collected and the cells in the uppermost fractions were isolated (Fig. 2a). After small-scale cultivation of 50 isolated strains for 48 h, the amount of TAG produced by each colony was analyzed by staining the cells with Nile Red and conducting FACS analysis. Among these, three mutants, designated A33, A42, and A53, showed stronger fluorescence than the wild-type strain (Fig. 2b), suggesting that these mutants accumulated more TAG than the wild-type strain.

For the second trial, another batch of mutagenized *L. starkeyi* cells was used. CV tests conducted after mutagenesis showed 44%. The low-density cells were enriched using Percoll density gradient centrifugation three times. After the third centrifugation, cells in the 700- μ L uppermost fraction were subjected to single colony isolation (Fig. 3a). After the small-scale cultivation of 70 isolated strains for 48 h, the

amount of TAG produced by each colony was analyzed by staining the cells with Nile Red and conducting FACS analysis. Among these, three mutants, designated E15, E16, and E47, showed stronger fluorescence than the wild-type strain (Fig. 3b), suggesting that these mutants accumulated more TAG than the wild-type strain. Of the 120 isolated strains during the first and second trials, six strains were obtained that accumulated larger amounts of TAG than the wild-type strain.

For the third trial, the other batch of mutagenized *L. starkeyi* cells was used. CV tests conducted after mutagenesis showed 44%. The low-density cells were enriched using Percoll density gradient centrifugation three times. After the third centrifugation, cells in the 3.0-mL uppermost fraction were subjected to single colony isolation (Fig. 4a). To more efficiently obtain the mutants that accumulated large amounts of TAG, 11 colonies were picked that did not show mucoid colony morphology, as these cells may produce less

Fig. 3 Isolation of strains E15, E16, and E47 in the second trial. **a** Enrichment of low-density cells by performing Percoll density gradient centrifugation three times after EMS treatments. Concentration (%) of Percoll and days of cultivation on S medium before Percoll density gradient centrifugation is shown. The centrifuged samples were divided into nine fractions and the top fraction (mL) was subjected to the next cultivation or single colony isolation, as shown. **b** FACS analysis of the wild-type (WT), E15, E16, and E47 cells stained with Nile Red after small-scale cultivation for 2 days. Histograms show relative fluorescence of Nile Red (x axis) and cell number (y axis). Each histogram represents 10,000 cells. The representative histograms of triplicate experiments are shown

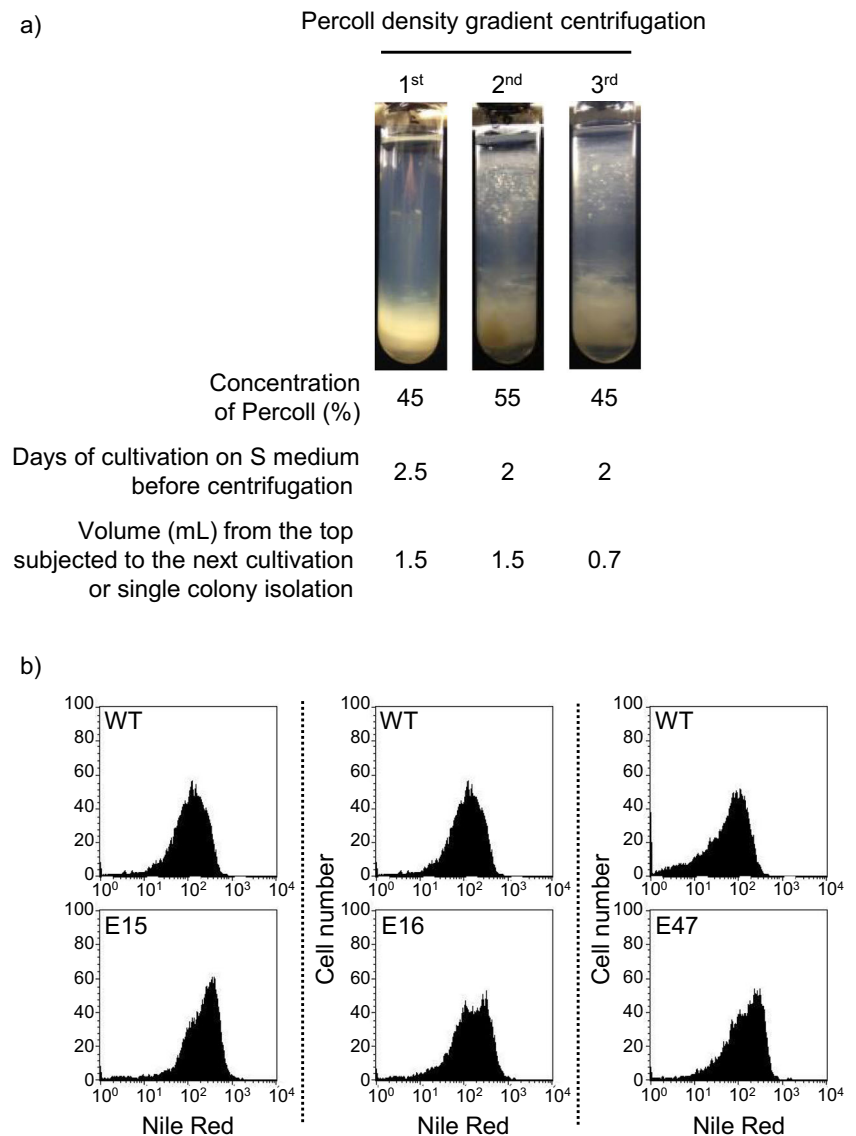
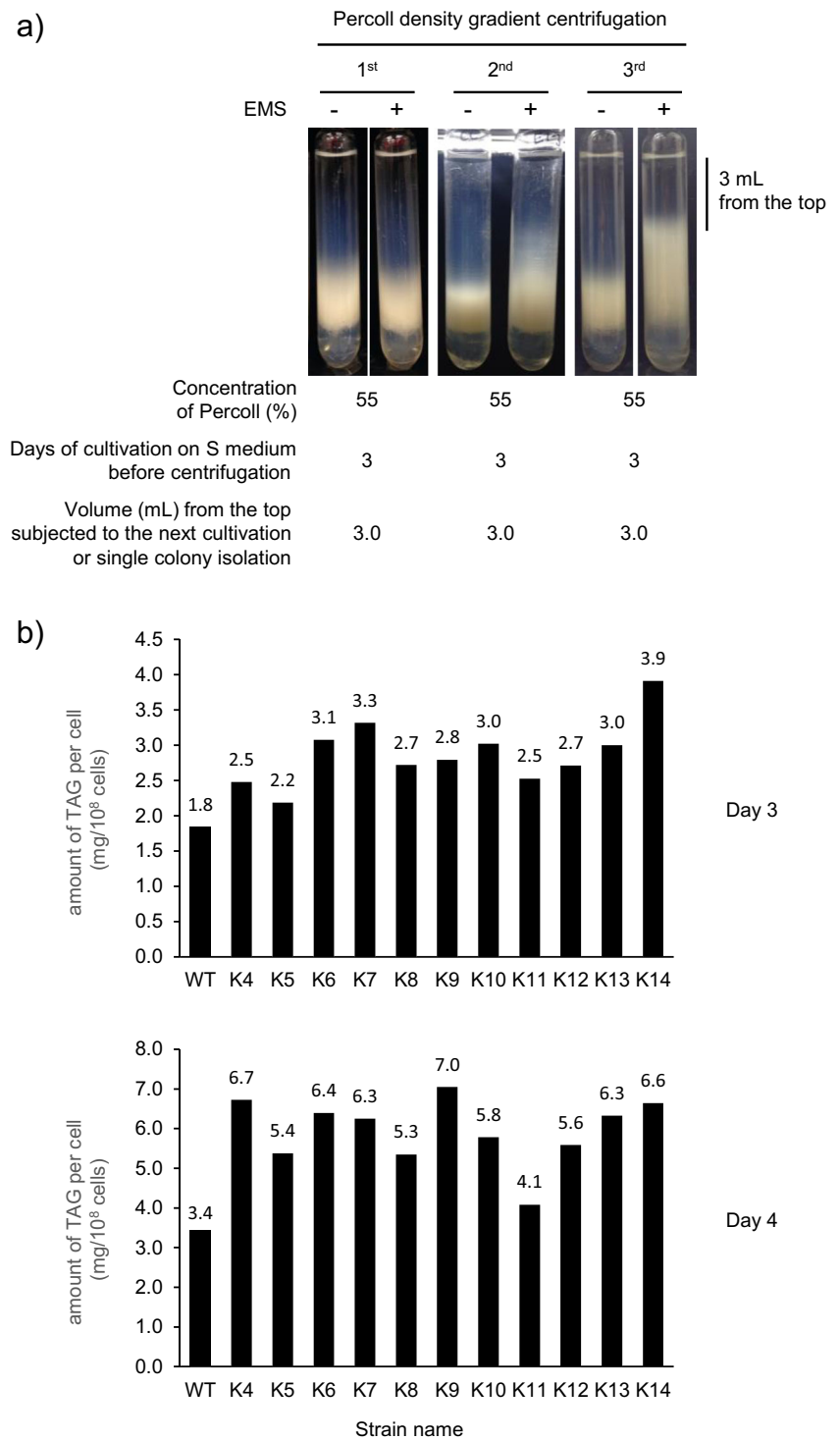


Fig. 4 Isolation of strains K4–K14 in the third trial. **a** Enrichment of low-density cells by performing Percoll density gradient centrifugation three times after treatments with (+) or without (-) EMS. Concentration (%) of Percoll and days of cultivation on S medium before Percoll density gradient centrifugation is shown. The centrifuged samples were divided into nine fractions and the top fraction (mL) subjected to the next cultivation or single colony isolation, as shown. **b** Amount of TAG per cell (mg/10⁸ cells) in the wild-type (WT), and K4–K14 cells after large-scale cultivation for 3 (upper panel) or 4 (lower panel) days. The amount of TAG was determined as the value obtained using the TG E-test



extracellular polysaccharide and more TAG. These mutants were designated K4–K14 and characterized using small-scale cultivation. All of the mutant cells accumulated larger amounts of TAG than the wild-type cells (Fig. 4b). These results indicated that the combination of the low-density cell enrichment procedure and the omitting of colonies with a mucoid morphology efficiently isolate mutant cells with higher amount of TAG.

Among the mutants isolated during the three trials, A42, E15, E47, K13, and K14 were picked and characterized using large-scale cultivation. The cell concentration of these mutants was not much different from that of the wild-type strain on day 1, but was slightly lower than that of the wild-type strain after day 2 when cells were thought to be in a stationary phase, except strain A42 (Fig. 5a). In particular, the cell concentration of strain E15 was about 80% of that of the wild-type strain

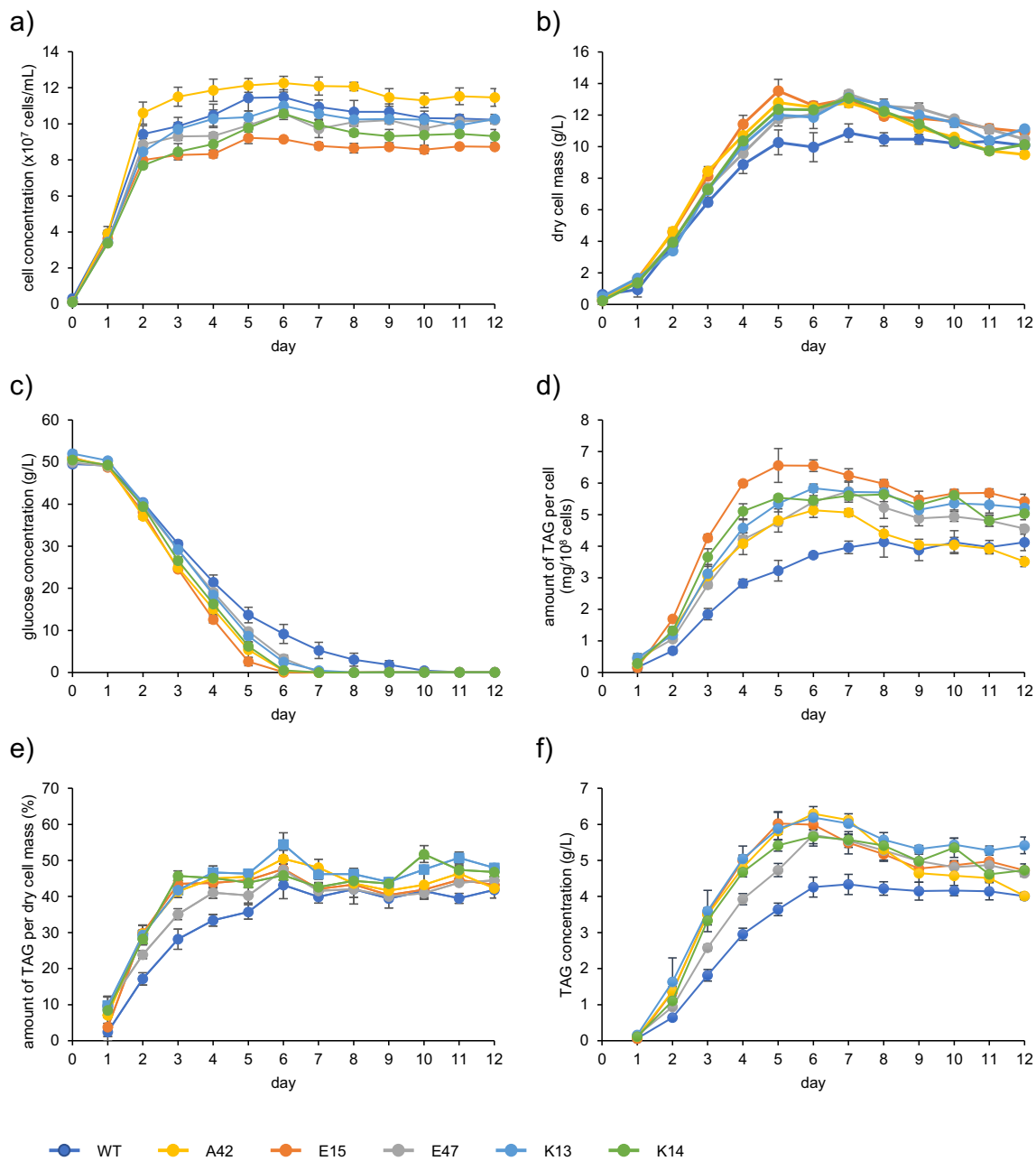


Fig. 5 Characterization of strains A42, E15, E47, K13, and K14. The wild-type (WT), A42, E15, E47, K13, and K14 cells were cultured according to large-scale cultivation condition. **a** Cell concentration (cells/mL), **b** dry cell mass (g/L), **c** glucose concentration (g/L) of culture supernatant, **d** amount of TAG per cell (mg/10⁸ cells), **e** TAG amount per dry cell mass (%), and **f** TAG concentration (g/L) are shown. The

amount of TAG was determined as the difference in the values obtained using the TG E-test and the F-kit for glycerol. Data are presented as the means \pm S.E.M. of three independent experiments. Statistical analysis was performed using Dunnett's test. The results of the statistical test are described in the text

at days 4 and 5 (Dunnett's test, $P < 0.05$) and that of strain K14 was about 85% of that of the wild-type strain at day 5 (Dunnett's test, $P < 0.05$). The cell concentration of strain A42 was slightly higher than that of the wild-type strain. However, the dry cell weights of all five mutants, especially strains A42 and E15 (Dunnett's test, $P < 0.05$), were heavier than those of the wild-type strain at day 5 when glucose still existed in all the media of the wild-type and mutant strains

(Fig. 5b). Moreover, all five mutants consumed the glucose in the medium faster than the wild-type strain (Fig. 5c). The amount of TAG per cell in all five mutants was significantly higher than that of the wild-type strain at days 2 to 5 (Dunnett's test, $P < 0.05$; Fig. 5d), supporting the notion that the mutant cells were isolated from the upper fraction in Percoll density gradient centrifugation during the trials at days 2 or 3. The amounts of TAG per dry cell mass of these

mutants, especially strains A42, E15, K13, and K14 (Dunnett's test, $P < 0.05$), were also higher than those of the wild-type strain at day 5 (Fig. 5e). The TAG concentrations of all five mutants, which were calculated by multiplying the cell concentration by the amount of TAG per cell, were also much higher than those of the wild-type strain at day 5 (Dunnett's test, $P < 0.05$; Fig. 5f).

The highest amounts of TAG per cell, the highest TAG content per dry cell mass, and the highest TAG concentrations are listed in Table 1. Regarding the highest TAG content per cell, all mutants tended to accumulate more TAG per cell than the wild-type strain, although only the differences between the wild-type and E15 or K13 strains were significant (Tukey's test, $P < 0.05$). The degree of the highest TAG content per cell was not significant among these mutants (Tukey's test, $P > 0.05$). Regarding the highest TAG content per dry cell mass, all mutants tended to accumulate more TAG per dry cell mass than the wild-type strain; however, the difference was not significant (Tukey's test, $P > 0.05$). Moreover, the degree of the highest TAG content per dry cell mass was not significant among these mutants (Tukey's test, $P > 0.05$). The degree of the highest TAG concentration in all mutants significantly increased when compared with that of the wild-type strain (Tukey's test, $P < 0.05$); however, the degree of the highest TAG content per cell was not significant among these mutants (Tukey's test, $P > 0.05$). The material balances $Y_{L/S}$ (lipid to substrate yield) and $Y_{X/S}$ (biomass to substrate yield) at the day when the highest TAG concentrations of the wild-type strain and all mutants were achieved are listed in Table 2. Strain E15 showed significantly higher $Y_{X/S}$ than the wild-type strain (Tukey's test, $P < 0.05$), but the other mutants did not show the significance (Tukey's test, $P > 0.05$). $Y_{X/S}$ was not significant among these mutants (Tukey's test, $P > 0.05$). In addition, strain E15 showed significantly higher $Y_{X/S}$ than all the other mutants (Tukey's test, $P < 0.05$). Moreover, all mutants showed significantly higher $Y_{L/S}$ than the wild-type strain (Tukey's test, $P < 0.05$). $Y_{L/S}$ was not significant among these mutants (Tukey's test, $P > 0.05$).

The fatty acid profiles of the TAGs accumulated by the wild-type and mutant strains are listed in Table 3. The fatty

acid profiles of TAG in *L. starkeyi* resembled those of palm oil, in which palmitic and oleic acids are the major components (Deffense 1985). The degree of palmitic acid in strains A42, E15, and K14 and oleic acid in strain A42 was significantly higher and lower, respectively, than that of the wild-type strain (Dunnett's test, $P < 0.05$), although the major fatty acids were still palmitic acid and oleic acid in these mutants.

Discussion

In the present study, an efficient method to obtain *L. starkeyi* mutants accumulating large amounts of TAG was established. These mutants were isolated using Percoll density gradient centrifugation and the enriched upper fraction containing high lipid cells were collected. In the first and second trials, total 120 candidates were obtained from the resultant upper fractions, which were subsequently stained with Nile Red, and subjected to FACS analysis to measure the amount of TAG per cell. From the 120 candidates, six mutants were considered to accumulate more TAG than the wild-type strain (Figs. 2 and 3). In the third trial, after single colony isolation of cells from the upper fraction, colonies that showed mucoid colony morphology were omitted because mucoid colony formation is thought to be frequently associated with the production of extracellular polysaccharides (Kurtzman et al. 2011), which could compete with TAG synthesis in terms of carbon source utilization. Presently, it is unknown why the cells with mucoid morphology on the agar plate floated to the upper fractions in the Percoll density gradient centrifugation. All cells without mucoid colony morphology are not always expected to accumulate higher amount of TAG than that of the wild-type strain because the cell density is affected by many factors, including cell cycle phases (Allen et al. 2006; Bryan et al. 2010). Nevertheless, 11 *L. starkeyi* mutants that accumulated higher amount of TAG than the wild-type strain were efficiently (100%) isolated (Fig. 4).

Some mutant strains that accumulated large amount of TAG have already been reported in some oleaginous yeasts (Wang et al. 2009; Tapia et al. 2012; Yamada et al. 2017);

Table 1 Highest amount of TAG per cell and TAG concentration of the wild-type and mutant strains

Strain	Highest amount of TAG per cell (mg/10 ⁸ cells)	Highest TAG content per dry cell mass (% (w/w))	Highest TAG concentration (g/L)
WT	4.1 ± 0.5 (day 8)	42.0 ± 4.1 (day 8)	4.3 ± 0.3 (day 7)
A42	5.1 ± 0.2 (day 6)	50.4 ± 2.4 (day 6)	6.3 ± 0.2 (day 6)*
E15	6.6 ± 0.2 (day 5)*	47.6 ± 1.5 (day 6)	6.0 ± 0.3 (day 5)*
E47	5.7 ± 0.3 (day 7)	47.3 ± 1.6 (day 6)	5.7 ± 0.3 (day 6)*
K13	5.8 ± 0.1 (day 6)*	54.4 ± 3.2 (day 6)	6.2 ± 0.1 (day 6)*
K14	5.7 ± 0.2 (day 8)	51.8 ± 2.4 (day 10)	5.7 ± 0.2 (day 6)*

Data are presented as the means ± S.E.M. of three independent experiments. Statistical analysis is performed using Tukey's test. *Significant difference ($P < 0.05$) to WT

Table 2 Material balance at the day when the highest TAG concentration was achieved

Strain (day)	$Y_{L/S}$ (TAG (g)/glucose (g))	$Y_{X/S}$ (dry cell mass (g)/glucose (g))
WT (day 7)	0.11 ± 0.01 (4.3/44.3)	0.24 ± 0.01 (10.9/44.3)
A42 (day 6)	0.12 ± 0.00 (6.3/50.5)*	0.25 ± 0.01 (12.5/50.5)
E15 (day 5)	0.12 ± 0.00 (6.0/48.6)*	0.28 ± 0.01 (13.5/48.6)*†
E47 (day 6)	0.12 ± 0.01 (5.7/46.6)*	0.26 ± 0.00 (12.0/46.6)
K13 (day 6)	0.13 ± 0.01 (6.2/49.5)*	0.24 ± 0.00 (11.9/49.5)
K14 (day 6)	0.11 ± 0.00 (5.7/50.0)*	0.25 ± 0.00 (12.3/50.0)

Data are presented as the means ± S.E.M. of three independent experiments. Statistical analysis is performed using Tukey's test. *Significant difference ($P < 0.05$) to WT. † Significant difference ($P < 0.05$) to A42, K13, and K14. $Y_{L/S}$ lipid to substrate yield, $Y_{X/S}$ biomass to substrate yield

Wang et al. (2009) obtained high lipid-producing mutants of an oleaginous yeast, *Rhodotorula glutinis*, using cerulenin for the pre-selection of cells after mutagenesis using ion irradiation. Cerulenin is a potent inhibitor of fatty acid biosynthesis; thus, the fatty acid biosynthesis enzymes of cerulenin-tolerant mutants are expected to have higher activities, resulting in higher lipid content per cell. Among the 33 mutants that produced larger colonies in cerulenin-supplemented media, 22 mutants showed increased lipid concentrations. Tapia et al. (2012) also isolated a higher lipid-producing *L. starkeyi* mutant using cerulenin for the pre-selection after UV irradiation. They obtained a total of 90 cerulenin-tolerant mutants, and among them, they picked six cerulenin-tolerant mutants that produced the largest colonies. Among these six mutants, one mutant showed a significant increase in both biomass and lipid productivity. Yamada et al. (2017) improved the cerulenin screening in which they selected ethanol- and H₂O₂- or cerulenin-tolerant mutants of *R. toruloides*. They reported that approximately 75% of ethanol- and H₂O₂-

tolerant and 42% of cerulenin-tolerant mutants produced larger amounts of lipid. Among these, they selected ethanol-, H₂O₂-, and cerulenin-tolerant mutants exhibiting the highest lipid production. Altogether cerulenin screening is also a feasible strategy to obtain mutants showing high lipid production. Compared with these strategies, the third trial was efficient (100%) in obtaining mutants with large amount of TAG (Fig. 4). Moreover, by using this method, a variety of mutants are expected to be efficiently obtained in the future. When mutants that accumulate TAG more rapidly are needed, as in the present study, the cultivation time before Percoll density gradient centrifugation is adjusted to be short, and when mutants are expected to improve the maximum amount of TAG in the late stage of culture, before Percoll density gradient centrifugation mutagenized cells are needed to grow until the day just before glucose is exhausted. These mutants are expected to have several mutations in their genome; thus, the mechanism of accumulation and degradation of TAG in *L. starkeyi* can be uncovered by analyzing the responsible genes in the mutants.

All five mutants significantly accumulated higher amount of TAG more rapidly than the wild-type strain (Fig. 5d, f), supporting the notion that the mutant cells were isolated from the low-density fractions in the early stages of the cultivations. Considering the industrial use of these mutants, strain E15 is thought to be the best mutant because (1) only strain E15 showed significantly higher TAG concentration in the medium as well as significantly higher degree of $Y_{L/S}$ and $Y_{X/S}$ than the wild-type strain, when each strain achieved the highest TAG concentration (Tables 1 and 2) and (2) the cultivation time to achieve the highest TAG concentration in strain E15 was the shortest among the mutants. Regarding the fatty acid profile of TAG, strains A42 and E15 showed higher degree of palmitic acid and strain A42 showed lower degree of oleic acid than the wild-type strain; however, the major fatty acids

Table 3 Fatty acid profile (%) of TAG accumulated in the wild-type and mutant strains

Strain	Palmitic acid (C16:0)	Palmitoleic acid (C16:1)	Stearic acid (C18:0)	Oleic acid (C18:1)	Linoleic acid (C18:2)	α-Linolenic acid (C18:3)	Other fatty acids
WT	35.74 ± 0.23	3.14 ± 0.13	5.50 ± 0.14	46.51 ± 0.46	3.70 ± 0.17	0.07 ± 0.01	5.33 ± 0.28
A42	39.32 ± 0.44*	3.25 ± 0.04	4.68 ± 0.06	42.73 ± 1.14*	5.44 ± 0.50	0.09 ± 0.00	4.49 ± 0.23
E15	40.07 ± 0.27*	3.65 ± 0.05	3.68 ± 0.08	44.65 ± 0.42	4.42 ± 0.10	0.06 ± 0.01	3.47 ± 0.04
E47	36.21 ± 0.49	2.90 ± 0.03	5.85 ± 0.06	47.14 ± 0.56	3.37 ± 0.08	0.06 ± 0.01	4.47 ± 0.04
K13	37.46 ± 0.22	3.97 ± 0.05	3.23 ± 0.09	48.83 ± 0.45	3.21 ± 0.16	0.04 ± 0.01	3.25 ± 0.10
K14	38.21 ± 0.68*	3.90 ± 0.20	3.02 ± 0.21	48.64 ± 1.05	3.19 ± 0.16	0.04 ± 0.00	2.98 ± 0.19

FAME analysis with GC-FID was identified by comparison with commercial FAME standards. The peaks of methyl esters of C14:0, C15:0, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1n9, C18:2n6, C18:3n6, C18:3n3, C20:0, C20:1, C22:0, C23:0, C24:0, and C24:1n9 were identified by their retention times in comparison with commercial FAME standards. Peaks not confirmed above were identified by GC-MS as methyl esters of polyunsaturated fatty acids (approximately C18–C20) and C25:0, and the summation of the percentage of these peaks are indicated in others. Data are presented as the means ± S.E.M. of three independent experiments. Statistical analysis is performed using Tukey's test in palmitic and oleic acids. *Significant difference ($P < 0.05$) to WT

were still palmitic acid and oleic acid in these mutants (Table 3), suggesting that TAG accumulated in these mutants could be an alternative to vegetable oils.

A system has been recently developed to integrate multiple copies of heterologous genes in *L. starkeyi* (Oguro et al. 2014). In addition, a DNA homologous recombination system with high efficiency has been developed by creating a $\Delta lig4$ mutant (Oguro et al. 2017). The future aim is to produce *L. starkeyi* mutants suitable for industrial use by breeding the obtained mutants using the system reported herein and genetically improving them using the aforementioned gene recombination techniques.

An efficient method is established to isolate *L. starkeyi* mutants accumulating large amount of TAG by enriching low-density mutagenized cells with Percoll density gradient centrifugation, subsequently omitting cells with mucoid colony morphology. Five mutants achieved about 1.5–2.0 times TAG concentration (g/L) as compared with the wild-type strain. One of the mutants, E15, showed significant increase in TAG concentration in the medium as well as the degree of lipid to glucose yield ($Y_{L/S}$) and biomass to glucose yield ($Y_{X/S}$). The cultivation time to achieve the highest TAG concentration in strain E15 was the shortest among the mutants. The major fatty acids of TAG accumulated in these mutants are palmitoleic acid and oleic acid, suggesting that TAG accumulated in these mutants is used as a substitute for vegetable oils. Some *L. starkeyi* mutants that are suitable for industrial use could be created by this method in the near future.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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