

Development of a Screening Method for Isolation of Microalgae Strains That Accumulate Lipids Under Nitrate-replete Conditions

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Research

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Abstract

Background: Microalgae biofuels have attracted global attention as an alternative to fossil fuels as an energy resource. Microalgae generally accumulate lipids under nitrogen-depleted conditions, but cell growth is depressed under these conditions which causes decrease in lipid productivity. To realize one-step cultivation for biofuel production, microalgae that highly accumulate lipids even under nitrogen-replete conditions are needed. This study aimed to develop a screening method for microalgae mutants with high lipid content even in the presence of a nitrogen source.

Results: Mutant cells were generated by irradiating the oleaginous green microalga *Chlamydomonas* sp. KOR1 with carbon ion beams, cultured under nitrate-replete conditions, and then subjected to FACS-based screening for lipid-rich cells. By repeatedly performing the sequential procedures of cultivation and selection, strains KAC1710 and KAC1801, which highly accumulate lipids under nitrate-replete conditions, were successfully obtained. These mutants formed significant lipid droplets in the cells even in the presence of abundant nitrate and achieved 1.5- and 2.1-fold greater lipid content compared to KOR1, respectively.

Conclusion: This study developed a novel nitrogen-conditioned screening method for microalgae mutants that accumulate lipids in the presence of a nitrogen source. This method should contribute to microalgae biofuel production via one-step cultivation under nitrogen-replete conditions.

Background

Microalgae can photosynthetically produce biofuel feedstocks such as triacylglycerol (TAG) directly from atmospheric carbon dioxide. Thus, microalgae biofuels that have a low environmental burden have attracted global attention as a sustainable energy resource [1, 2]. Some terrestrial plants, such as corn, soybeans, and rapeseed, can also produce biofuel feedstocks photosynthetically. However, biomass yields per unit area are much higher with microalgae than terrestrial plants, and furthermore, microalgal biofuel production does not compete with food production [3, 4]. Some oleaginous microalgal strains in the genera *Chlorella*, *Nannochloropsis*, *Scenedesmus*, and *Chlamydomonas* have been reported as promising biofuel producers because they demonstrated lipid accumulation of >50% per dry cell weight (DCW) [5-11].

Lipid accumulation in microalgae is regulated by environmental factors such as light [12, 13], salinity [14, 15], and nutrient availability [16, 17]. Among these factors, nitrogen depletion is a common and strong trigger for boosting lipid accumulation in cells. The cell composition of microalgae changes depending on environmental nitrogen conditions. These organisms generally accumulate protein under nitrogen-replete conditions [14, 18], whereas lipids are accumulated under nitrogen-depleted conditions [14, 18]. As lipid accumulation generally occurs under nitrogen-depleted conditions, cultivation of microalgae for lipid production must be divided into two steps: a nitrogen-replete step for cell growth and a nitrogen-depleted step for lipid accumulation, which is unsuitable for cell growth [19].

Previous studies have identified target genes for metabolic engineering aimed at improving lipid accumulation in the presence of nutrients. In *Nannochloropsis gaditana*, a transcription factor downregulated under nitrogen deficiency, Zn (II)₂Cys₆, was identified, and down-regulation of this transcription factor improved lipid production under growth condition [20]. In *Cyanidioschyzon merolae*, overexpression of glycerol-3-phosphate acyltransferase (GPAT) induced a 56.1-fold increase in TAG productivity in the growth phase [21, 22]. However, the regulatory mechanism of lipid accumulation under nitrogen-replete conditions remains poorly understood, even though it is important for identifying target genes for metabolic engineering. Information regarding this regulatory mechanism could also be obtained from comparative analyses of lipid-rich microalgae mutants.

In previous studies, microalgae lipid accumulation was improved by random mutagenesis [23-27]. Irradiation with heavy-ion beams functions as a useful mutagen that induces drastic changes in the genome, and this approach has been applied to microalgae such as *Nannochloropsis* [28], *Euglena* [29], and *Chlamydomonas* [30]. Fluorescence-activated cell sorting (FACS) is a powerful tool for screening lipid-rich cells from a large number of random mutants. Boron-dipyrromethene (BODIPY) and Nile red, fluorescent dyes that stain lipid droplets in living cells, are commonly used in combination with FACS [29, 31-32]. Although high lipid-producing strains have been obtained using these tools, the screenings were only conducted under conditions suitable for lipid production, for example, nitrogen-deficient conditions. Thus, a screening method for microalgae that accumulate lipids under nitrogen-replete conditions has not been established.

This study aimed to develop a screening method to obtain microalgae mutants that accumulate lipids under nitrogen-replete conditions. As the parent for mutational breeding, the oleaginous microalga *Chlamydomonas* sp. KOR1 was exposed to carbon ion beam for random mutagenesis, and the resultant mutant cells were cultured in the presence of abundant nitrate until just before FACS-based screening for lipid-rich cells. This sequential screening approach led to the isolation of strain KAC1710, which highly accumulated lipids under nitrate-replete conditions. By repeating the nitrogen-conditioned screening procedure using KAC1710 as the parent, KAC1801, in which lipid accumulation under the nitrate-replete conditions was further improved compared to KAC1710, was obtained. In the presence of nitrate, the lipid productivity of KAC1710 and KAC1801 was 1.3- and 1.8-fold higher than that of KOR1, respectively. Thus, this study developed a novel nitrogen-conditioned screening method that is useful for obtaining lipid-accumulating mutants under nitrogen-replete conditions.

Results

First-round breeding using *Chlamydomonas* sp. KOR1 as the parent

For one-step lipid production, microalgae that can accumulate lipids under nitrogen-replete conditions are needed. It was assumed that screening of lipid-rich cells from randomly mutated cells cultured in the presence of an abundant source of nitrogen would be useful to obtain such mutants. To examine this hypothesis, mutational breeding was performed using *Chlamydomonas* sp. KOR1 as the parent. KOR1

was irradiated with carbon ion ($^{12}\text{C}^{5+}$) beams to induce random mutations. The mutant cells were cultured under the nitrate-replete condition in MB 12N medium containing 2% (w/v) sea salt for 3 days. MB12N medium contains abundant NaNO_3 (1588.4 mg/L) than previously used medium [11]. Nitrate concentration in the medium after 3 days cultivation was approximately 800 mg/L which shows nitrate in the medium was replete. Then, mutants were subjected to FACS-based screening of lipid-rich cells highly stained by BODIPY 505/515.

In the first sorting experiment, the chlorophyll fluorescence and BODIPY fluorescence intensity of most cells were $1.0 \times 10^5 \sim 1.0 \times 10^6$ (a.u.) and $1.0 \times 10^4 \sim 2.0 \times 10^5$ (a.u.), respectively (Fig. 1a). From these, 1,000 cells exhibiting high BODIPY fluorescence of $1.0 \times 10^5 \sim 1.0 \times 10^6$ (a.u.) were sorted (Additional file1: Fig. S1 and Table S1). The sorted cells were subjected to repeated cultivation under nitrate-replete conditions and FACS-based sorting, for a total of five times. In the fifth sorting experiment, a cell population exhibiting higher BODIPY fluorescence than the original cells was observed (Fig. 1b), suggesting that mutant cells that accumulate lipids in the presence of nitrate were successfully screened. Then, the mutant colonies were isolated by inoculating the cells obtained in the fifth-sorting on Tris-acetate-phosphate (TAP) agar plates. For the secondary screening, 18 mutant strains were randomly selected (designated as KAC17s), cultured under nitrate-replete conditions for 3 days, at which time the lipid content was measured using gas chromatography-mass spectrometry (GC-MS). Among these mutants, KAC1710 exhibited the highest lipid content (18.3%), compared with lipid content of only 7.2% for KOR1 (Fig. 1c).

Second-round breeding using *Chlamydomonas* sp. KAC1710 as the parent

To further increase the lipid content under nitrate-replete conditions, second-round breeding was conducted using KAC1710 as the parent. Similar to the first-round breeding, KAC1710 was irradiated with a heavy-ion beam, and the nitrogen-conditioned screening of lipid-accumulating cells was performed using FACS. In the first sorting experiment, chlorophyll fluorescence and BODIPY fluorescence of most cells were $1.0 \times 10^5 \sim 10^6$ (a.u.) and $1.0 \times 10^5 \sim 1.0 \times 10^6$ (a.u.), respectively (Fig. 2a). From these, 1,000 cells exhibiting higher BODIPY fluorescence of approximately 3.0×10^6 (a.u.) were sorted (Additional file1: Fig. S2 and Table S2). The sorted cells were repeatedly subjected to cultivation under nitrate-replete conditions and sorting of high BODIPY fluorescence cells, for a total of four times. In the fourth sorting experiment, most cells exhibited BODIPY fluorescence higher than 1.0×10^6 (a.u.) (Fig. 2b), suggesting that further improvement in lipid accumulation under nitrate-replete conditions was achieved. The cells obtained in the fourth sorting were then spread on TAP agar plates to isolate mutant strains. For the secondary screening, 15 mutant strains were randomly selected (designated as KAC18s), cultured under nitrate-replete conditions, and then their lipid content was analyzed by GC-MS. Among these mutants, KAC1801 exhibited the highest lipid content (23.1%), compared to 15.2% for the parental strain KAC1710 (Fig. 2c).

Increased formation of lipid droplets in KAC1710 and KAC1801

Transmission electron microscopy (TEM) analysis revealed that KAC1710 and KAC1801 cells accumulated lipid droplets even in the presence of nitrate, and KAC1801 accumulated more lipid droplets than KAC1710 (Fig. 3). In addition, KAC1710 and KAC1801 cells were larger in size than KOR1 cells, which might have been caused by the increased lipid droplet formation in these mutants. These results suggested that formation of lipid droplets under nitrate-replete conditions was increased by the two random mutagenesis and nitrate-conditioned screening steps.

Evaluation of lipid productivity of KAC1710 and KAC1801

To evaluate lipid productivity under nitrate-replete conditions, KOR1, KAC1710, and KAC1801 were cultured in MB 12N medium containing 2% (w/v) sea salt, and cell density, biomass, nitrate concentration in the medium, and lipid content were measured. Maximum cell density of KOR1, KAC1710, and KAC1801 during 6 days of cultivation was 8.0×10^6 cells/mL, 5.7×10^6 cells/mL, and 5.3×10^6 cells/mL, respectively (Fig. 4a). Maximum biomass of KOR1, KAC1710, and KAC1801 was 4.1 g-DCW/L, 3.6 g-DCW/L, and 3.3 g-DCW/L, respectively (Fig. 4b). These results suggest that delayed cell division in KAC1710 and KAC1801 led to the lower biomass production. In addition, nitrate consumption was decreased in KAC1710 and KAC1801 (Fig. 4c). KAC1710 and KAC1801 consumed 79.8% and 62.9% of the nitrate in the MB 12N medium, respectively, whereas KOR1 completely consumed the nitrate in the medium over 6 days. The lipid content of KAC1710 and KAC1801 at day 5 was approximately 19.1% and 26.6%, respectively, which was 1.5- and 2.1-fold higher than that of KOR1 (12.5%) (Fig. 4d). Although biomass production was reduced, lipid production and productivity of KAC1710 and KAC1801 were improved based on the increase in lipid content. Lipid production of KOR1, KAC1710, and KAC1801 at day 5 was 457.8 mg/L, 605.4 mg/L, and 810.4 mg/L, respectively (Fig. 4e). Lipid productivity of KAC1710 and KAC1801 at day 5 was 1.3-fold (121.1 mg/L/day) and 1.8-fold (162.1 mg/L/day) higher, respectively, compared to KOR1 (91.6 mg/L/day) (Fig. 4f). Thus, by increasing lipid accumulation, lipid productivity under nitrate-replete conditions was improved.

Lipid productivity of KAC1710 and KAC1801 under nitrate-deficient conditions was also investigated using MB 6N medium containing 2% (w/v) sea salt, which contains half the amount of nitrate compared to MB 12N medium (Additional file1: Fig. S3). Biomass production of KAC1710 and KAC1801 after 10 days of cultivation was 5.1 g-DCW/L and 4.4 g-DCW/L, respectively, whereas that of KOR1 was 5.7 g-DCW/L (Additional file1: Fig. S3a). Nitrate in the medium was completely consumed in 3 days by KOR1, whereas 4 days were required by KAC1710 and KAC1801 (Additional file1: Fig. S3b). The lipid content of KOR1, KAC1710, and KAC1801 at day 10 was 43.5%, 50.7%, and 37.1%, respectively (Additional file1: Fig. S3c); thus, under nitrate-depleted conditions, improvement was observed only in KAC1710. Also, lipid production of KOR1 and KAC1710 was 249.7 mg/L/day and 259.8 mg/L/day, respectively, whereas in KAC1801, it decreased to 163.5 mg/L/day (Additional file1: Fig. S3d).

Discussion

Microalgae such as *Chlorella*, *Nannochloropsis*, *Scenedesmus*, and *Chlamydomonas* are promising biofuel producers, but lipids are generally accumulated under adverse environmental conditions such as nitrogen deficiency [19]. To examine lipid production under nitrogen-replete conditions, this study developed a novel nitrogen-conditioned screening method to obtain lipid-accumulating mutants. Previous research also succeeded in screening for lipid-rich mutant microalgae [28, 29, 31, 32]; however, those screenings were performed only under conditions suitable for lipid accumulation. This study employed nitrogen-replete conditions in which microalgae generally do not accumulate lipids and conducted nitrogen-conditioned screening in reference to previous studies involving random mutagenesis and FACS-based screening. Strains KAC1710 and KAC1801 obtained in the present screening formed many lipid droplets in the presence of nitrate (Fig. 3) and accumulated 1.5- and 2.1-fold more lipids than KOR1, respectively (Fig. 4d). This is the first study that successfully screened lipid-accumulating strains under nitrogen-replete conditions from randomly mutated microalgae.

The lipid productivity of KAC1801 under nitrate-replete conditions was 162 mg/L/day (Fig. 4f and Table 1), higher than that of *Nannochloropsis* sp. (55 mg/L/day) and *Scenedesmus obliquus* (78 mg/L/day) [33, 34] and comparable to that of *Isochrysis zhangjiangensis* (141 mg/L/day) and *Nannochloropsis oculata* (142 mg/L/day) under nitrogen-depleted conditions [35, 36]. Although lipid productivity was still lower than that of *Dunaliella salina* (240 mg/L/day) and *Chlamydomonas* sp. (306 mg/L/day) cultured in the absence of a nitrogen source [37, 11], these results suggest that KAC1801 is a promising strain that can produce a comparable amount of lipids to previously reported microalgae under nitrate-replete conditions.

Under nitrate-depleted conditions, on the other hand, the lipid productivity of KAC1710 and KAC1801 did not change or even decreased compared to that of KOR1 (Additional file1: Fig. S3). Thus, it was suggested that the response to nitrate was modified in these mutants, whereas the lipid synthesis pathway itself was not generally improved. Strains KAC1710 and KAC1801 exhibited decreased nitrate consumption compared to KOR1 (Fig. 4c). This suggested that nitrate assimilation was reduced in these mutants. Target of rapamycin (TOR) signaling reportedly plays an important role in nutrient sensing in *Chlamydomonas reinhardtii*. Previous research reported that TOR inactivation mimics nitrogen-deficient conditions and induces lipid accumulation despite the presence of a nitrogen source in the medium. It was also reported that TOR-inactivation condition increases the expression of lipid synthesis related genes such as diacylglycerol acyltransferase [21]. It is hypothesized that partial dysfunction of TOR in KAC1710 and KAC1801 enabled the cells to accumulate lipid droplets under nitrate-replete conditions. In *Nannochloropsis gaditana*, the transcriptional factor Zn (II)₂Cys₆ was identified as a negative regulator of lipid accumulation. Zn (II)₂Cys₆ down-regulated mutant showed biosynthesis switching from proteins to lipids. In Zn (II)₂Cys₆ down-regulated mutant confirmed to decrease the expression levels of nitrogen assimilation genes such as nitrate transporter (NRT2), nitrate reductase (Nir), glutamine synthetases (GS1 and GS2), and ammonium transporter (AMT1) than wild-type strain [20]. KAC strains also decreased nitrogen assimilation and increased lipid accumulation (Fig. 4 c-d). This result also suggested that suppression of nitrogen assimilation may restrict carbon flux to protein, resulting in allocating carbon to

lipids. Further studies are required to elucidate the lipid accumulation mechanism in KAC1710 and KAC1801, however.

Conclusions

This study developed a screening method to identify valuable microalgae mutants that can accumulate lipids under nitrate-replete conditions. The mutants KAC1710 and KAC1801 were obtained by nitrogen-conditioned screening with FACS. Under nitrate-replete conditions, KAC1710 and KAC1801 formed significant lipid droplets in the cells and accumulated 1.5- and 2.1-fold more lipid than the parent strain, respectively. For microalgae biofuel production, the results of this study should contribute to the establishment of a method for one-step cultivation under nutrient-replete conditions.

Methods

Strains

Chlamydomonas sp. KOR1, a lipid-rich mutant derived from *Chlamydomonas* sp. JSC4 [38], and its mutants were used in this study. Microalgae were maintained on BG-11 plates containing 1.5% agar under continuous illumination at $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and 25°C .

Mutagenesis

Microalgae were cultured for 2 days in TAP medium [39] using double-deck photobioreactors constructed using two flasks [40]; the upper stage contained 70 mL of cell culture, and the lower stage contained $\text{K}_2\text{CO}_3/\text{KHCO}_3$ solution adjusted to supply 2% CO_2 gas to the upper stage under continuous illumination at $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 30°C . The optical density at 750 nm (OD_{750}) was measured using a UV mini-1240 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). The cell culture was diluted with TAP medium to make cell suspension of $\text{OD}_{750} = 0.5$, and 100 μL of the diluent was seeded onto TAP agar plates. The plates were covered with polyimide film Kapton 30EN (Du Pont-Toray CO. Ltd., Aichi, Japan) and irradiated with 50 Gy of heavy-ion beams ($^{12}\text{C}^{5+}$, accelerated energy; 220 MeV, surface LET; 107 keV/ μm) accelerated by an azimuthal varying field cyclotron at Takasaki Ion Accelerators for Advanced Research Application of the National Institutes for Quantum and Radiological Science and Technology [30].

Nitrogen-conditioned screening

For primary screening, mutant cells prepared above were cultured for 3 days under continuous illumination at $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 30°C using Modified Bold (MB) 12N medium (18.6 mM NaNO_3 , 0.22 mM K_2HPO_4 , 0.3 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.17 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.43 mM KH_2PO_4 , 0.43 mM NaCl , and metals described in a previous report [41]) containing 2% (w/v) sea salt (Sigma-Aldrich Co., St. Louis, MO, USA) and 2% CO_2 . A total of 5.0×10^6 cells were collected by centrifugation at $8,000 \times g$ for 1 min at

25°C and resuspended in 1 mL of PBS. Then, 50 µM BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene, Thermo Fisher Scientific, MA, USA) was added to the cell suspension. After incubation for 5 min at room temperature in the dark, cells with the highest BODIPY fluorescence were sorted using a fluorescence-activated cell sorter SH-800 (SONY, Tokyo, Japan). To perform the screening procedures repeatedly, sorted cells were subjected to the next cultivation and FACS-based sorting described above. After the final sorting, sorted cells were seeded on TAP agar plates and cultured for 1 week until colony formation.

For secondary screening, cells from isolated colonies were cultured for 3 days under nitrate-replete conditions using 12-well plates under continuous illumination of 100 µmol photons m⁻² s⁻¹ supplying 2% CO₂ at 30°C with rotary shaking at 100 rpm. Lipid content of the cells was measured as described below.

Measurement of nitrate concentration

Nitrate concentration was measured as previously reported [14, 42]. The culture broth was centrifuged at 8,000 × *g* for 1 min. The supernatant was diluted with distilled water, and the optical density at 220 nm was measured using a UV mini-1240 UV-VIS spectrophotometer (Shimadzu). Nitrate concentration was calculated using a calibration curve.

Lipid analysis

Lipid content was measured as described in a previous study [11]. Cells in culture broth were collected by centrifugation at 8,000 × *g* for 1 min at 25°C and washed once with distilled water. Cell pellets were stored at -30°C until subjected to freeze-drying. A total of 2-3 mg of dried cells was fractured with 300 µL of 0.5-mm glass beads YGB05 using a multi-beads shocker MB1001C (S) (Yasui Kikai, Osaka, Japan) at 2,700 rpm for 1 min: On, 1 min; Off × 30 cycles, 4°C. Released lipids were esterified using a fatty acid methylation kit (Nacalai Tesque, Kyoto, Japan) and analyzed on a GCMS-QP2010 plus (Shimadzu) instrument equipped with a DB-23 capillary column (60 m, 0.25 mm internal diameter, 0.15 µm film thickness; Agilent Technologies, CA, USA) for identifying and quantifying fatty acids. Heptadecanoic acid (Sigma-Aldrich Co.) was used as an internal standard. The intracellular lipid content was shown by calculating lipid weight per dry cell weight.

TEM analysis

Cells were fixed overnight with 2% paraformaldehyde, 2% glutaraldehyde, and 50 mM cacodylic acid. After dehydration in graded ethanol solutions (50-100%), samples were infiltrated with propylene oxide and transferred to 100% resin and polymerized at 60°C for 2 days. The specimens were sectioned and stained with 2% uranyl acetate at room temperature for 15 min. Observation was performed using a JEM-1400 plus electron microscope with a CCD camera EM-14830RUBY2 (JEOL Ltd., Tokyo, Japan).

Evaluation of the lipid productivity of KOR1 and its mutants

Batch culture for evaluating lipid productivity was performed using the KOR1, KAC1710, and KAC1801 strains. Pre-culture and primary culture were performed using MB 12N medium containing 2% (w/v) sea salt for 3 days and 6 days under continuous illumination at $250 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ at 30°C. During the primary culture period, the cell density, biomass density, nitrogen source concentration in the medium, and intracellular lipid content were analyzed every day. The cell density was analyzed using a TC20™ Automated Cell counter (Bio-Rad, USA), and the other parameters were analyzed by the above method.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data set obtained in this study can be confirmed from the main article and supplementary information.

Competing interest

The authors declare no competing interests.

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Author contributions

T.O. designed the research, conducted the experiments, and drafted the manuscript. Y.K. designed the research and revised the manuscript. K.S. and Y.O. performed mutagenesis and revised the manuscript. T.H. designed the research, revised the manuscript, and supervised the study. A.K. commented on the research and assisted with the laboratory management. All authors read and approved the final version of the manuscript.

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Tables

Table 1. Lipid productivity in previous reports and this study.

Strain	Nitrogen source	Lipid content (%)	Lipid productivity (mg/L/day)	Reference
<i>Chlamydomonas</i> sp. JSC4	Deficient	57	306	[11]
<i>Dunaliella salina</i>	Deficient	44	240	[37]
<i>Nannochloropsis oculata</i> NCTU-3	Deficient	30	142	[36]
<i>Isochrysis zhangjiangensis</i>	Deficient	41	141	[35]
<i>Scenedesmus obliquus</i>	Deficient	39	78	[34]
<i>Nannochloropsis</i> sp. F&M-M24	Deficient	31	55	[33]
<i>Chlamydomonas</i> sp. KOR1	Replete	12	92	This study
<i>Chlamydomonas</i> sp. KAC1710	Replete	19	121	This study
<i>Chlamydomonas</i> sp. KAC1801	Replete	26	162	This study

Figures

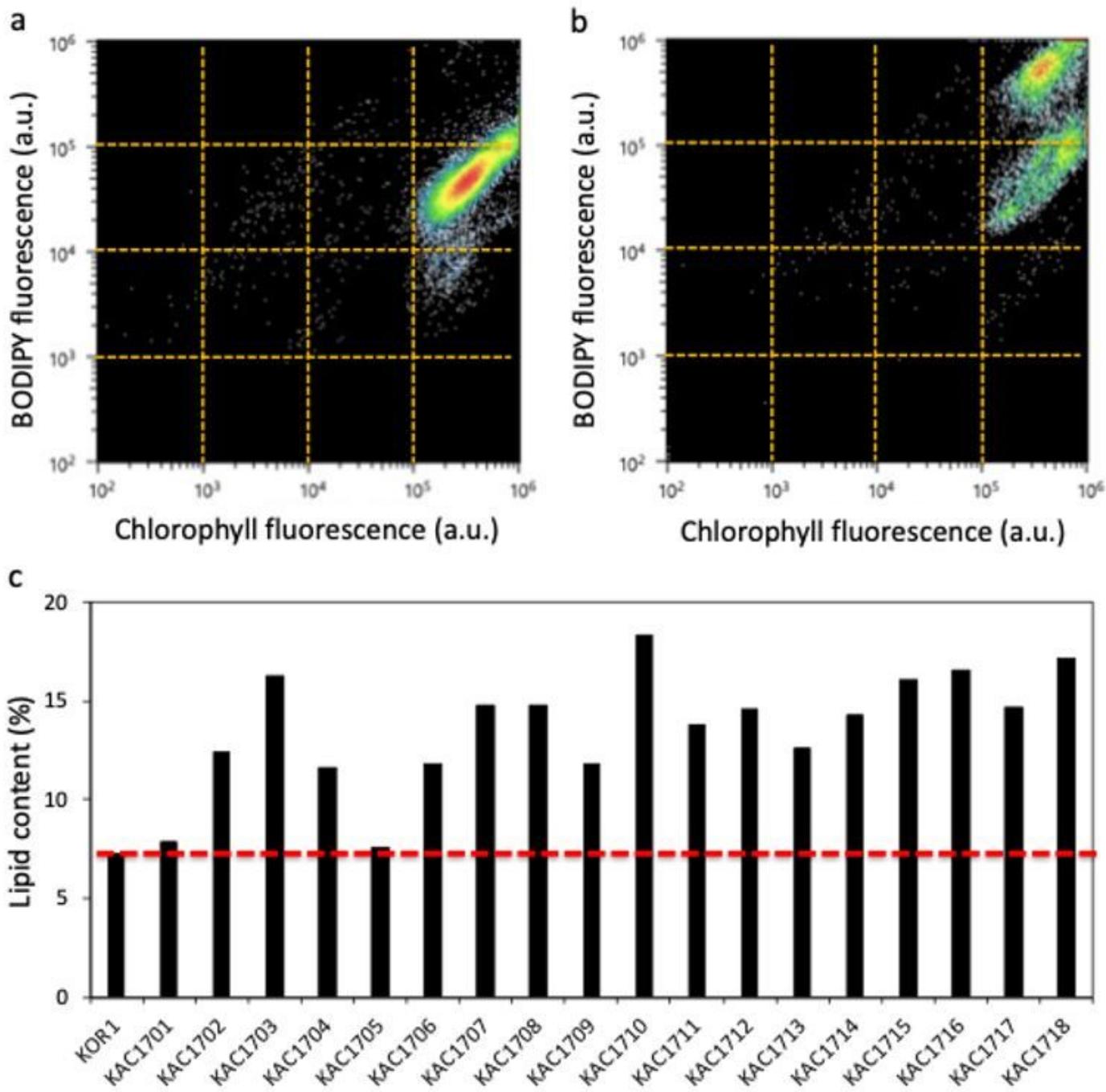


Figure 1

First-round screening using *Chlamydomonas* sp. KOR1 as the parent strain. a Fluorescence plot of the first sorting experiment. b Fluorescence plot of the fifth sorting experiment. Red, yellow, green, blue, and white spots indicate increasing number of events. c Lipid content of KOR1 and KAC17 mutants cultured under nitrate-replete conditions. Red-dotted line indicates lipid content of KOR1 (7.2%).

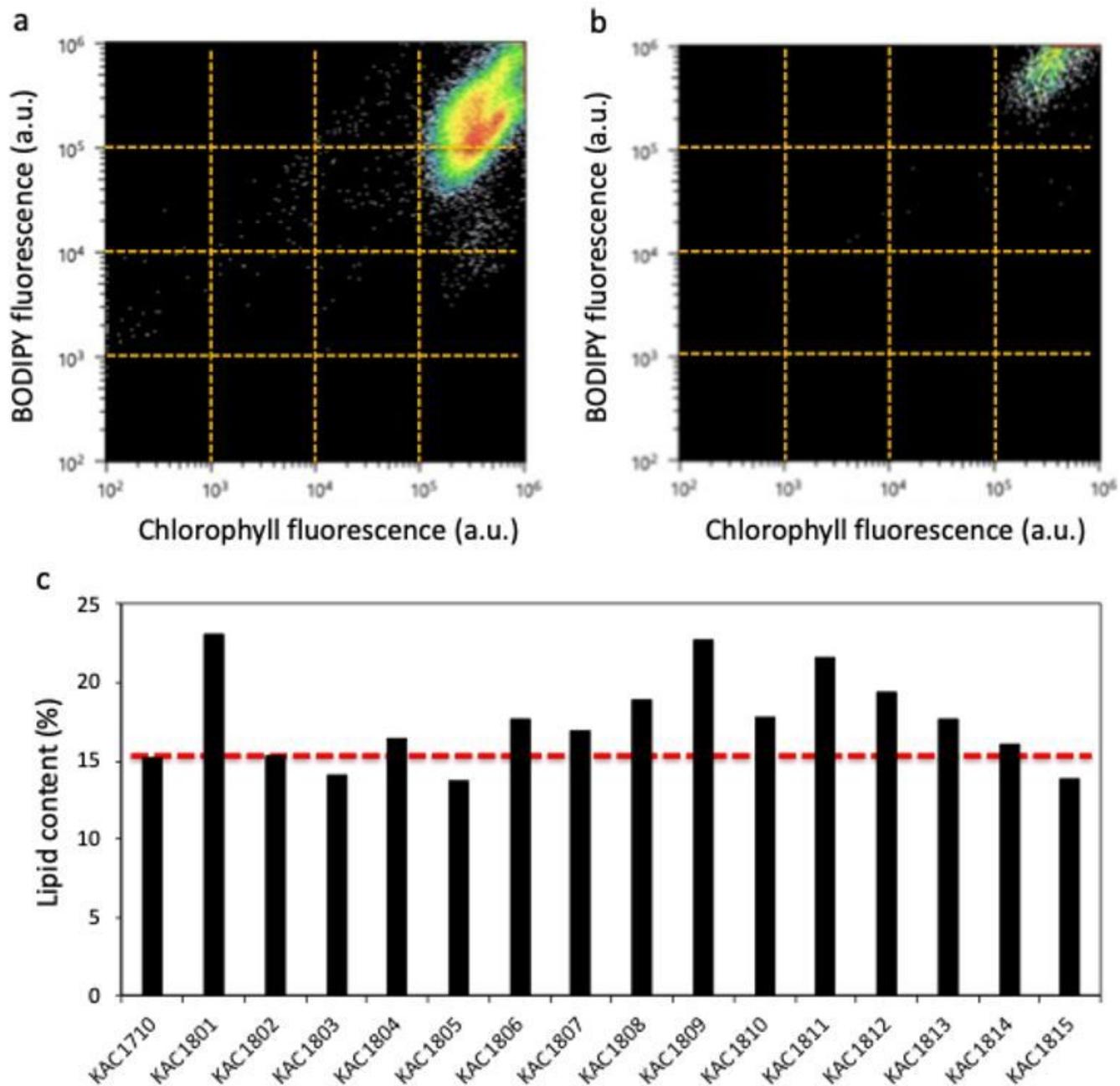


Figure 2

Second-round screening using *Chlamydomonas* sp. KAC1710 as the parent strain. a Fluorescence plot of the first sorting experiment. b Fluorescence plot of the fourth sorting experiment. Red, yellow, green, blue, and white spots indicate increasing number of events. c Lipid content of the KAC1710 and KAC18 mutants cultured under nitrate-replete conditions. Red-dotted line indicates the lipid content of KAC1710 (15.2%).

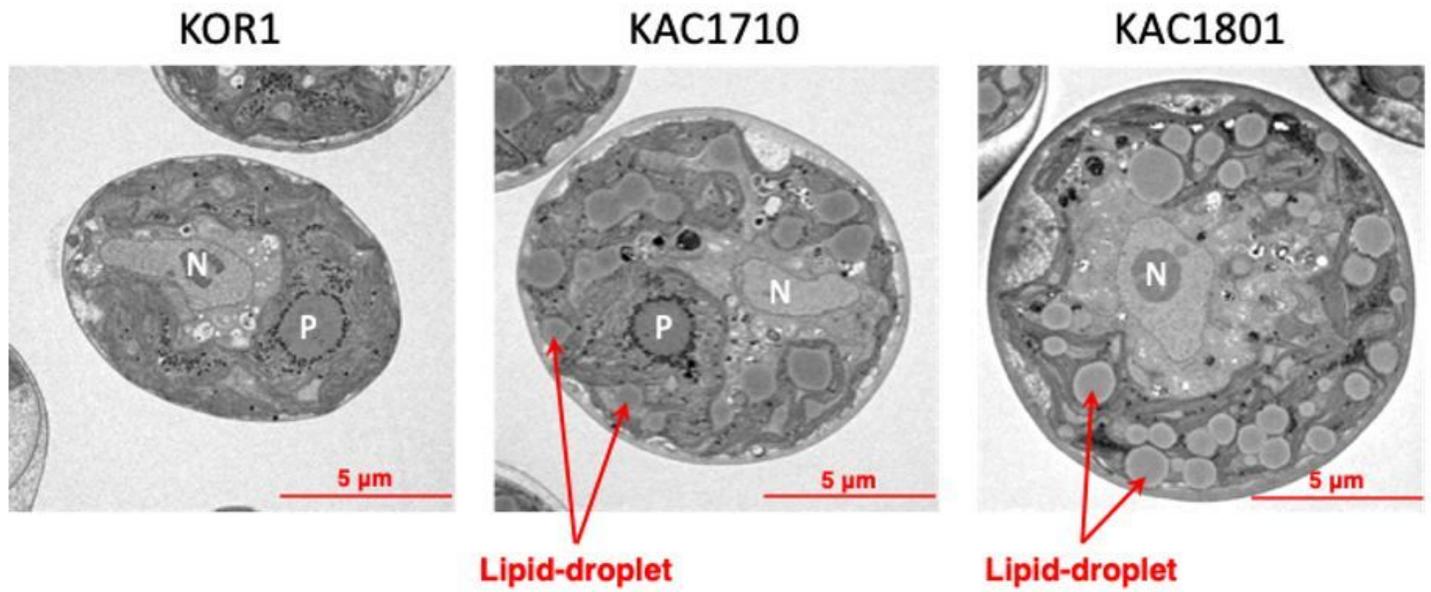


Figure 3

TEM images of KOR1, KAC1710, and KAC1801 cells. Before TEM analysis, microalgal cells were cultured for 4 days in the presence of abundant nitrate using MB 12N medium containing 2% (w/v) sea salt. N: Nucleus, P: Pyrenoid

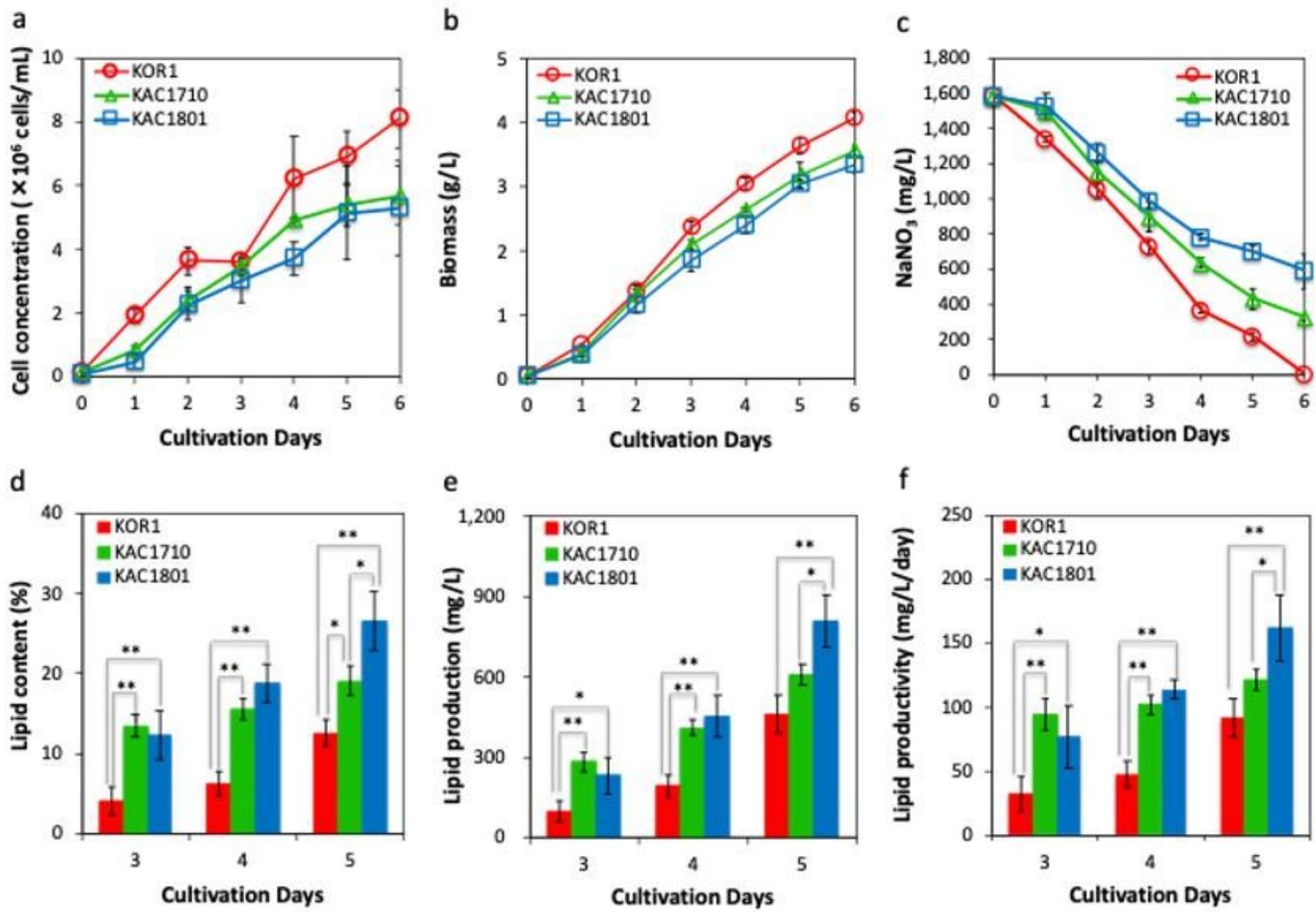


Figure 4

Evaluation of lipid productivity in KAC1710 and KAC1801 under the nitrate-replete conditions a Cells density. b Biomass. c Nitrate concentration. d Lipid content. e Lipid production. f Lipid productivity. Error bars indicate the standard deviation of three replicate experiments (*: $p < 0.05$, **: $p < 0.01$ by Tukey test).

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