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Transgressive, reiterative selection by continuous buoyant density gradient centrifugation of *Dunaliella salina* results in enhanced lipid and starch content

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ABSTRACT

Microalgae can serve as useful feedstocks for biofuel production as they can be grown with fresh, brackish, or salt water and their lipid and starch contents can be manipulated to create customized feedstocks for different classes of biofuels. Continuous buoyant density gradient centrifugation (CBDGC) was used to perform reiterative, transgressive selection to isolate wildtype and ethyl methanesulfonate-mutagenized *Dunaliella salina* cells with enhanced lipid and starch production. Sixty rounds of transgressive selection resulted in the isolation of cell populations with significantly lower or higher buoyant densities. Lipid content in the low-density populations was enhanced by 1.2- to 2.9-fold in wildtype cells and 1.3- to 2.3-fold in mutagenized cells as measured by Nile Red dye staining, but the lipid content differences were not significant when quantified by liquid chromatography–tandem mass spectroscopy possibly due to the composition of the lipid pools measured by these contrasting techniques. In contrast, starch content in the high-density populations was increased by 2-fold in wild type cells and 1.4- to 1.6-fold in mutagenized cells, respectively. The observed alterations in lipid and starch contents appeared to be stable after more than 70 weeks (392 cell generations). CBDGC-based selection provides a useful and accessible technological alternative to genetic engineering approaches for the customization of microbial biofuel feedstocks.

1. Introduction

Microalgae are promising renewable bioenergy feedstocks due to their high photosynthetic efficiency, their high annualized biomass production capacity compared with most terrestrial crops, and their ability to accumulate large amounts of lipid and starch [1,2]. Although strategies to increase microalgae productivity by improving their solar energy capture or conversion efficiency have been developed [3], novel strategies to enhance lipid or starch production are critical to improving the economic viability of a microalgae-based biofuel industry [4,5]. Manipulation of cultivation conditions, including temperature, light, salinity, pH, and heavy metal concentrations can alter the lipid or starch biosynthesis and composition of microalgae [6]. However, nutrient limitation (i.e., nitrogen) is the most common strategy reported to increase lipid or starch accumulation [6,7]. Genetic mutagenesis of populations of microalgae cells followed by high-throughput screening of individual cells or clonal populations for altered lipid content has also been performed [8–14]. Spectrophotometer or a flow cytometer screening assays in combination with lipid-specific dyes or starch-specific stains have been employed [9,13–17]. For example, *Chlamydomonas reinhardtii* mutants with insertional disruptions in starch biosynthesis have been shown to preferentially accumulate lipids under nitrogen deprivation [18–21].

Buoyant density gradient centrifugation (BDGC) exploits the differences in the relative density of biological constituents within a gradient medium to separate cells, sub-cellular organelles, and macromolecular complexes on the basis of buoyant force. Continuous or discontinuous (step) high (bottom)-to-low (top) concentration density gradients are typically made using cesium chloride (CsCl), sucrose, or Percoll® media within analytical or preparative scale centrifuge tubes. Following application of the samples to the top of the tube, the centrifugation process allows each component of the sample to occupy a position in the

Abbreviations: CBDGC, continuous buoyant density gradient centrifugation; TAG, triacylglycerol; FFA, free fatty acid; ASW, artificial sea water; EMS, ethyl methanesulfonate; NR, Nile Red; FCM, flow cytometer; ASE, accelerated solvent extraction; WT, wild type; MT, mutant; DW, dry weight.

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gradient that is equivalent to their buoyant density. Thus, constituents that sediment near the bottom of the gradient will have a higher density, whereas those near the top of the gradient will have a lower density [22]. Discontinuous or continuous BDGC has been used to quantify the *in vivo* buoyant densities of microbial cells with altered lipid, hydrocarbon, or biopolymer contents under different environmental stress or culture conditions [23,24].

The unicellular green alga from the genus *Dunaliella* have been proposed as a feedstock for the generation of biofuels due to its remarkable ability to grow in a wide range of extreme environments. *Dunaliella* is one of the most halotolerant eukaryotic organisms known and is able to withstand a range of salinities from 0.05 to 5.5 M NaCl. Furthermore, this alga is able to tolerate high irradiance levels (1500 μE m^{-2} s^{-1}), low temperatures (4 °C), and also low nutrient concentrations [25]. Additionally, the relatively high growth rate (doubling time of 0.4–1.25 days for *Dunaliella salina*) and its ability to produce lipids and starch under nutrient deprivation make this alga a potential feedstock for biofuel production [26–29].

In this study, reiterative and transgressive selection of wildtype (WT) and ethyl methanesulfonate (EMS)-mutagenized (MT) *D. salina* cell populations using CBDGC resulted in the isolation of cells with lower and higher buoyant densities resulting in either enhanced lipid or starch accumulation, respectively. Following such selection, the cell populations appeared to stably express these selected compositional changes for at least 70 weeks (392 cell generations). This innovative approach provides a useful and easily accessible technological alternative to genetic engineering strategies for the selection and isolation of algae cells with altered feedstock composition suitable for renewable biofuel production schemes.

2. Materials and methods

2.1. Growth conditions

D. salina (CCAP 19/18) cells obtained from the Culture Collection of Algae and Protozoa (CCAP) (Argyll, Scotland) were grown in 1.0 M NaCl artificial seawater (ASW) medium under a photon flux density of 150 μmol m^{-2} s^{-1} (16 h light (26 °C)/8 h dark (20 °C) cycle under aeration with sterile air humidified by bubbling the air through a sterile-water-filled trap.

2.2. Chemical mutagenesis

To determine the optimal ethyl methanesulfonate (EMS) concentration for mutagenesis studies, cells were treated with 0, 50, 100, 150, 200, or 250 mM EMS as described [30]. After treatment with EMS, cell density was monitored for 10 days to determine the degree to which cells were killed by the treatment. Then, the cells were re-cultured and their growth was examined for 31 days to determine the effect of EMS on cell survival. Cell growth was examined by absorbance at OD_{680}.

2.3. Reiterative, transgressive selection of WT and MT populations by CBDGC

Wildtype (WT) and EMS mutant (MT) *D. salina* cells were grown to early-exponential phase (1 week) reaching a cell density of ~0.1 (OD_{680}). Cells were harvested by centrifugation at 2000 ×*g* for 6 min and placed atop a continuous Percoll® gradient. These continuous 10% (top) to 70% (bottom) Percoll® gradients were prepared using 5 M NaCl artificial seawater (ASW) diluted to a final concentration of 1 M NaCl resulting in osmotically equilibrated media. All gradient materials and tubes were sterilized prior to use in order to maintain axenic *D. salina* cultures. After the 24th round of selection, the Percoll® gradient concentration was adjusted to 0–90% to allow further rounds of selection to be performed, as the 10–70% gradient range no longer permitted the separation of the least and most dense cells, respectively. The gradient mixture was then centrifuged at 7000 ×*g* for 30 min at room temperature. In each experiment, identical Percoll® gradients were loaded with colored density marker beads (GE Healthcare, Pittsburgh, PA, USA) to determine the sample's buoyant density. Additionally, mock controls in which cells were applied to and collected from the Percoll® gradients, but never selected based on density, were included. After centrifugation, the distances of the cell populations and the density marker beads were measured to calibrate each band to its approximate buoyant density. Assuming a linear distribution, the geometric center of the population density distribution was used to indicate the average density of the culture. For selection, 10% of the cells of higher or lower buoyant densities were carefully removed with serological pipettes and traces of Percoll® were removed by washing the cells with 1 M NaCl ASW media. Mock controls and selected-cell populations were re-cultured and collected reiteratively for a period of 60 weeks. See Fig. 1A for an overview of this method. After 60 weeks, selected and unselected WT and MT populations were maintained under identical culture conditions and were sub-cultured every month over a period of 70 weeks (~392 cell generations). After 70 weeks, samples from the 60th round of selection and from the unselected WT and MT populations were used for transmission electron and confocal microscopy studies. Additionally, samples from the 20th, 40th, 60th rounds of selection, and from the unselected WT and MT populations were grown to stationary phase (3 weeks, ~0.35 OD_{680}) in triplicate and analyzed for their lipid, FFA, TAG, and starch contents (Fig. 1B).

2.4. Transmission electron and confocal microscopy

Samples from the 60th round of selection and unselected WT and MT populations were used to determine changes in morphology produced by the CBDGC selection process. For the transmission electron microscopy (TEM) evaluation, cells were fixed with Tousimis® Fixative (1.5% v/v glutaraldehyde/1% v/v formaldehyde in 0.12 M Sorensen's buffer at pH 7.35, isotonic) (Tousimis Inc., Rockville, MD). Samples were prepared and processed at the Electron Microscopy Lab, Department of Medical Pathology, University of California, Davis (Davis, CA). Briefly, samples were placed in Karnovsky's fixative, fixed in osmium tetroxide, and embedded in an epoxy resin using a hybrid microwave-bench method [31,32].

To collect fluorescent images, aliquots containing 10^5–10^6 cell/ml were stained with 5 μg/ml Nile Red (NR) (9-diethylamino-5H-benzo[α]phenoxazine-5-one) (in acetone) for 10 min at 37 °C. The stained cells were observed using a laser-scanning confocal microscope (Olympus FluoView 1000, Olympus Inc., Center Valley, PA). To visualize the neutral lipids, the cells were excited using a 488 nm laser and emission was measured at 585/42 nm. To determine the localization of the lipids and the specificity of NR for lipid droplets, chlorophyll fluorescence was viewed using a 633 nm laser and emission was measured at 660/20 nm. To determine the differences in lipid bodies and starch granules between the selected and unselected populations, five different EM and confocal images were used, and one representative image was selected for qualitative imagery.

2.5. Lipid analysis by flow cytometry (FCM)

The lipid content of microalgae was assessed using Nile Red (NR) and flow cytometry (FCM). WT and MT cells were grown to early-exponential phase (one week) to a cell density of ~0.1 (OD_{680}). To determine the dye concentration that resulted in uniform fluorescence intensities for *D. salina*, an aliquot of WT and MT non-selected cells containing 10^5–10^6 cell/ml were stained for 10 min at 37 °C with different final concentrations of NR (0.25, 0.5, 2.5, 5, and 10 μg/ml) in acetone. The optimal concentration was found to be 5 μg/ml, which was used for further analysis. Cells were stained and analyzed with an LSR II Flow Cytometer (BD Biosciences, San Jose, CA) configured as depicted in Supplemental Table B.1 (Appendix B). The NR signal for the neutral