Abstract

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Chlamydomonas reinhardtii is the model organism for the production of high-value bioenergy feedstocks including starch, lipids, chlorophyll, carotenoids, and proteins. Synthesis of these bio-products escalates under abiotic stress conditions that are deleterious for cell survival. To address this problem, we evaluated the beneficial effects of the innovative cultivation strategies, i.e., two-stage and gradient application of salt stress and hetero/mixotrophy to *C. reinhardtii*. The study included 20 different possible cultivation methods, where gradient mixotrophy containing 5 g/L sodium acetate is found to yield the highest biomass content of 9.82 ± 0.06 million cells/mL (3.6-fold higher than autotrophic control). It also resulted in the maximum production of chlorophyll, and carotenoid, i.e., 36.17 ± 1.74 mg/mL, and 8.85 ± 0.52 mg/mL, respectively. Additionally, single-stage mixotrophy with equivalent carbon concentration yields the highest triacylglycerol (TAG) content, 3-fold higher than autotrophy, while gradient heterotrophy with 5 g/L acetate serves as the most favorable condition producing 81-fold increased starch content. Temporal-based production of starch and TAG was observed and found to be regulated by carbon partitioning and starch-to-lipid switching. Gene expression analysis showed that autophagy, starch degradation activity, and *de novo* lipid synthesis together cause enhanced TAG production under gradient salt stress and single-stage mixotrophy conditions. Furthermore, 2D-epifluorescence microscopy revealed that the lipid droplet (LD) size and the number of droplets in a cell get altered under salt stress. Single-stage salt stress showed a proportionate increase in the number of small-sized LDs while the gradient cultivation caused large-sized LDs to increase. On the other hand, single-stage mixotrophy exhibited an increase in the large-sized LD. The formation and growth of LDs were found to be governed by liquid-liquid phase separation (LLPS). The increased phase separation in LD formation resulted in two-phase LDs in the single-stage mode of cultivation under both salt stress and mixotrophy. Altogether, LD biogenesis is influenced by LLPS and further regulated by the cell's proteome and thermodynamic factors. The image analysis further revealed the thick palmelloid formation and highly-ordered 8-celled arrangement in single-stage salt stress. Gradient cultivation under salt stress resulted in delayed/absent palmelloid formation and also caused no significant change in the cell size and shape. Understanding the molecular details of lipid droplet formation would, thus, play a crucial role in refining biofuel

quality in large-scale production. Overall, gradient cultivation proved to be a favorable growth condition for bioenergy feedstock production.

This study also displays the importance of an appropriate cultivation strategy for the optimum production of desirable bioenergy feedstock. This would require screening of multiple variations in cultural conditions and environmental factors. This led us to optimize the development of micro-Raman spectroscopy for multivariate, rapid, and non-invasive screening of cultivation methods. Here, a discrete wavelet transform (DWT) algorithm was used to resolve the Raman spectral peaks which showed high heterogeneous distribution in lipid and carotenoid composition in salt stress-exposed single *Chlamydomonas* cells. The increased lutein/β-carotene ratio under two-stage salt stress, higher unsaturation content in the fatty acids at the stationary growth, and imbalances in cellular homeostasis under stress formed the key interpretations of micro-RS data. These results show that micro-RS serves as a state-of-the-art tool for future screening of multiple cultivation strategies. Overall, this study highlights that with the correct modifications in the cultivation methods and the use of proper tools and targets, microalgal research can be revolutionized for the optimum production of bioenergy feedstocks.