Synopsis of the thesis on

Energy feedstock production in *Chlamydomonas reinhardtii* as a function of abiotic stress

Submitted to

The Maharaja Sayajirao University of Baroda

For the degree of

Doctor of philosophy in Microbiology

By

Shubhangi Pandey

Under the guidance   and co-guidance of

Prof. G. Archana   **Dr. Debjani Bagchi**

PROFESSOR and HEAD   ASSISTANT PROFESSOR
DEPARTMENT OF MICROBIOLOGY   DEPARTMENT OF PHYSICS
AND BIOTECHNOLOGY CENTRE

Introduction

Microalgae and biofuel

Microalgae, especially green algae, are found mostly in freshwater or marine water and generally grow autotrophically. Microalgae offer many advantages over traditional oil crops for bioenergy feedstock production including high-value products like omega-3 fatty acids, vaccines, bioplastics, pharmaceuticals and cosmetics. They serve as the third-generation source for the production of biofuels. They offer robust environmental adaptability, no competition with food or arable land, rapid fixation of environmental carbon, cultivation on wastewater and year-round cultivation (Khan et al. 2009; Kumar, Sharma, and Dwivedi 2013). Microalgal biomass is known to have a high caloric value, low viscosity and low density (Yu et al. 2013).

Precursors of bioenergy feedstock: Lipids, starch and photosynthetic pigments

Triacylglycerides (TAG) are the precursor for biodiesel. These belong to the class of neutral lipids and are the intermediary product of lipid metabolism, de novo synthesized in the chloroplast or endoplasmic reticulum from acyl Co-A. These are present in the form of lipid droplets (LDs) mostly accumulated inside the chloroplast, although they might localize in the cytoplasm as well. These LDs also consist of certain proteins called MLDPs (major lipid droplet proteins) (Li-Beisson, Beisson, and Riekhof 2015). Other than the anabolic enzymatic de-novo lipid synthesis (Lv et al. 2013), catabolic breakdown of lipids or starch also result in accumulation of TAG. Carbon partitioning, starch-to-lipid switching, increasing carbon/nitrogen ratio are the mechanisms which favor lipid accumulation (Jia et al. 2015; Johnson and Alric 2013). Microalgae synthesize triacylglycerol (TAG) more when they are exposed to abiotic stress such as limitation of nutrients including phosphorus and nitrogen, supra-optimal conditions of temperature, light, carbon dioxide and salinity (Cheng and He 2014).

Fermentation of starch leads to production of bioethanol or biogas. It is observed that during starvation, the microalgal cell division is arrested and macromolecular compounds are recycled or sent to the plastid for accumulation of starch in the form of granules (Ball et al. 1990). Starch is over-produced when microalgae are grown with additional source of carbon or when exposed to nutrient limitation or hyper-saline condition (Gardner et al. 2013; Nagarajan et al. 2017).

Photosynthetic pigments, chlorophyll and carotenoids, are good source of natural food coloring agents and food additives. Carotenoids form a rich source of nutraceuticals because of their antioxidant properties. As a result, carotenoid production is accelerated in presence of environmental stress in order to neutralize reactive oxygen species (Campenni' et al. 2013).

Role of autophagy in production of bioenergy feedstock precursors

Autophagy, a cellular defense mechanism, is known to catabolically synthesize starch and lipids under stress (Couso et al. 2017). Stress induces ROS production in cells, which trigger autophagy for combating unfavorable growth conditions. It involves autophagosome formation which then fuses with the lysosome where cellular contents are degraded and the generated monomers are recycled for starch and tri-acyl glycerol production (Couso et al. 2017; Glick, Barth, and Macleod 2010). This autophagic response is controlled by photosynthetic pigments like carotenoids and a fall in the level of carotenoids due to improper biosynthesis can further trigger autophagic response (Pérez-Pérez, Couso, and Crespo 2012). Thus, carotenoids can be a good indicator of the stress levels in the cells, which in turn can be a good indicator of lipid accumulation.

Biofuel production under salt stress

Salt stress induces an increase in reactive oxygen species (ROS) and cause oxidative damage to membrane lipids resulting the disruption of the membrane integrity and cellular homeostasis. As a result, there is an increased production of carotenoids when microalgae are subjected to hyper-saline conditions. The photosynthetic activity is negatively affected (Shetty, Gitau, and Maróti 2019). Salt stress effectively increases the starch and lipid production in *Chlamydomonas reinhardtii* when grown autotrophically as well as heterotrophically (Fan and Zheng 2017). Cultivation of *Scenedesmus* sp. in both single-stage and two-stage, results in ROS mediated increased biofuel production under salt stress (Pancha et al. 2015). An innovative salinity gradient strategy is known to deliver improved lipid accumulation in salt-tolerant *Chlamydomonas* sp. JSC4 along with good biomass yield (Ho et al. 2014).

Heterotrophic and mixotrophic cultivation for biofuel production

Carbon availability is a key factor in the control of carbon partitioning between starch and TAG (Fan et al. 2012). The highest biofuel potential was observed in *C. reinhardtii* when 5% CO_2 was used as the carbon substrate (Gardner et al. 2013). This study also points out the phenomenon of carbon reallocation from starch to TAG based carbon storage. A two-stage

heterotrophic cultivation has proved to be better for higher biomass and biofuel production from *Chlorella* sp. (Zheng et al. 2012). Two-stage mixotrophy with glucose has shown to increase the biomass, lipids and carotenoids in *Chlorella* sp. (Chen et al. 2021; Yen and Chang 2013). Mixotrophic cultivation of *C. reinhardtii* too results in higher biomass and FAME yield (Moon et al. 2013). A study performed with *Chlorella* sp. shows that among different types of carbon source used in mixotrophy, xylose is the least preferable one for growth and sucrose yields maximum lipid content (Lin and Wu 2015).

High-resolution techniques to study biofuel production

The most commonly used technique to study lipid production is Gas chromatography coupled with mass spectrometer, which allows detailed analysis of lipid composition along with the amount produced. Not only such techniques are laborious, they demand high amount of starting culture and can become non-economical at many a times. Raman spectroscopy is non-invasive and rapid, and minimum supply of culture and high-resolution topography allows single-cell analysis too. Raman spectroscopy of lipids details out the lipid composition, including lipid unsaturation degree and chain length (Czamara et al. 2015; Graham et al. 2018), important aspects of biodiesel quality. Single-cell Raman spectroscopy has also been applied to microalgae in order to study starch, lipids, photosynthetic pigments and proteins under different growth conditions (Chiu et al. 2017; He et al. 2017), but despite an industrial importance, very less focus is laid on characterizing the heterogeneity in the population, which can serve as one of the bottlenecks in industrial productivity.

Another powerful and economical technique used to carry out single-cell studies is microscopy. Microscopical observation of cells stained with a fluorescent dye, Nile red is widely used to observe lipid droplet distribution in a microalgal cell. Nile red staining is a well-optimized method which can also be used to quantify the cellular lipids using fluorescence spectroscopy (Chen et al. 2009; Kou et al. 2013). High-resolution single-cell analysis has recently gained a lot of importance. It is been used to study lipid density, lipid amount, chlorophyll distribution, starch granules accumulation, or even cell morphological changes subjected to the stressful environment (Lee et al. 2013; Sandmann et al. 2018; Trampe et al. 2011).

Microscopy allow observation of cells at a much more intricate level. A majority of biological studies employ population averaged assays which can only depict the dominant biological behavior. However, cell-to-cell differences always occur in a population and

phenotypic heterogeneity can occur even in an isogenic population, like those in cell growth, stress resistance and metabolite production (Wang et al. 2014). Such variations in phenotypes are crucial for cells to adapt to stress conditions (Lidstrom and Konopka 2010; Martins and Locke 2015). The averaged phenotypes in different populations could turn out be identical, however, their distribution patterns at a single-cell level can be vastly varying, and this can have an impact on how the population reacts to sudden stress and also its stability (Altschuler and Wu 2010). Thus, such analysis can be a key to cellular heterogeneity, which can also prove to be essential in optimizing the biofuel feedstock at an industrial scale.

Chlamydomonas reinhardtii: a model organism to study biofuel production

Chlamydomonas reinhardtii is a single-celled, motile, freshwater, green microalgae. It has a simple life cycle and has a completely sequenced genome. It grows at ambient temperature of 25 °C thus avoiding majority of bacterial and fungal contamination, making the laboratory based controlled studies easier to perform. Apart from being a photosynthetic organism, it can grow mixotrophically as well as heterotrophically. The process of lipid production, starch synthesis and pigment formation under abiotic stress is well-studied in *C. reinhardtii* (Harris 2001). These features altogether make this organism an ideal candidate as the model system to understand the complexity of biofuel production and to explore the effect of genetic modifications or changes in the usual cultivation methods (Scranton et al. 2017).

Challenges in the algal biofuel industry

Owing to the fast-depleting current energy reserves and exponentially increasing demand of alternative energy source, third generation microalgal source offers economical and environment-friendly solution. However, there are several challenges faced by the current research scenario in algal biofuel production. Making algal growth and harvesting more efficient and crop protection from abiotic and biotic stress factors forms the first and top-priority challenge. Scaling-up from bench to pond cultivation to increase the production, oil extraction and downstream processing are some other important challenges faced at the large-scale production level. With a proper understanding of the population-scale heterogeneity existing in a single culture, unanimous production of good quality biofuel can be obtained. Avoiding bacterial and fungal contamination at large-scale production is another factor important at large-scale production. The population heterogeneities would also help to understand the biological process of adaptation to stress eventually leading to evolution of

new resistant species. Thus, an optimization of these challenges would directly affect the biofuel productivity at the industrial scale increasing the cost-effectiveness of the process.

Rationale

Considering the main challenges put to algal biofuel industries, the aim of this study is to optimize the cultivation of microalgae under stress in order to increase the biomass as well as biofuel yield. *Chlamydomonas reinhardtii* CC-125 is used as a model to study the effect of two different abiotic stress conditions, salt stress and hetero- and mixo-trophic modes of cultivation. By employing single-stage, two-stage and gradient strategies of cultivation, the impact of stress on production of starch, lipids, chlorophyll and carotenoids is studied to explore the favorable condition for an efficient biomass and biofuel production. This study further targets to understand the prevailing population-scale heterogeneity in a single culture using high-resolution micro-Raman spectroscopy. With the aim of cutting down the tedious and cumbersome procedures involved in biochemical analytical methods, micro-RS is applied to characterize lipids and pigments produced as a function of stress. On the scale of single-cell studies, it would also be interesting to study the phenomena of lipid droplet distribution and further characterize the lipid droplets using fluorescence microscopy, as a function of mode of cultivation.

Objectives

1. Effect of salt stress and varying cultivation strategies on bioenergy feedstock production in *Chlamydomonas reinhardtii*

2. Role of carbon concentration and mode of cultivation on growth and accumulation of lipid, starch and photosynthetic pigments in *Chlamydomonas reinhardtii* under heterotrophy and mixotrophy

3. Application of micro-Raman spectroscopy for non-invasive determination of biomolecular composition and cellular heterogeneity in *Chlamydomonas reinhardtii*

Results

Objective-1 Effect of salt stress and varying cultivation strategies on bioenergy feedstock production in _Chlamydomonas reinhardtii_

Chlamydomonas reinhardtii CC-125 was procured from Chlamydomonas Resource Centre, Minnesota. Cells were cultivated in Tris-acetate-phosphate (TAP) medium, at neutral pH 7.0 at a constant temperature of 25 °C, shaking at 200 rpm, with a 12h: 12h light: dark cycle using white light of ~ 50 μmol photons $m^{-2} s^{-1}$ intensity in the presence of 40 μg/ml ampicillin to avoid any bacterial contamination. _C. reinhardtii_ CC-125 was exposed to salt stress, with three different sodium chloride concentrations, 100 mM, 150 mM and 200 mM applied in single-stage (total amount of salt added in the beginning of the culture), two-stage (salt is added either during early log-phase or mid-log phase of the culture) and gradient-stage (salt is added in varying step sizes to achieve final concentration).

Firstly, the effect of 100 mM NaCl on growth of CC-125 was checked. Single-stage (SS 100), two-stage (D2 100, salt added on 2nd day of growth and D4 100, salt added on 4th day of growth) were the test cultures, along with an autotrophic control. Cell density was found to remain unaffected by changing salt stress stages. Total chlorophyll and carotenoids per 10^6 cells were found to be significantly higher in SS 100 and D2 100 by stationary phase. Highest carotenoid and chlorophyll yield is obtained in SS_100, 6.3 ± 0.64 mg/ml and 30.45 ± 3.6 mg/ml respectively. Starch, after staining with Lugol's iodine, was found to be significantly higher in single-stage at stationary phase, to be 0.46 ± 0.1 units/ml, compared to 0.0045 ± 0.0001 units/ml in Control. Thus, 100 mM NaCl imposes no significant change on growth of microalgae. Single-stage induces highest production of carotenoids, chlorophyll and starch followed by two-stage, D2_100.

Since, 100 mM was shown to have no impact on growth and single-stage cultivation resulted in significantly high photosynthetic pigments, higher salt concentrations were studied. Single-stage SS_150 and SS_200, two-stage D4_150 and D4_200, and two different gradient-strategies were used, i.e., (i) g15_10_150 and g20_10_200 where 15 mM and 20 mM of salt is added daily for 10 consecutive days, respectively and (ii) g30_10_150 and g40_10_200 where 30 mM and 40 mM of salt is added on alternate days, respectively. In case of 150 mM, g30_10_150 was found to accumulate maximum biomass. SS 200 was shown to be lethal to cell growth, D4_200 showed improved cell density and both the gradient cultivations proved to be even better at biomass accumulation, although lesser than the control. Highest carotenoid and chlorophyll content are observed in g30_10_150, 6.02 ± 1.1 mg/ml and 28.9 ±

2.8 mg/ml respectively. There is a significant drop in the carotenoid and chlorophyll formation under 200 mM salt, especially in D4_200. SS_150 shows maximum starch production. D4_150 also show a significant increase in starch, followed by gradient cultivation, compared to control. Highest starch yield of 0.3 ± 0.04 units/ml is obtained in SS_150. On the contrary, g15_10_150 show an increased lipid production with maximum yield of 36.18 ± 4.6 units/ml at day 6. Under 200 mM, maximum starch yield is obtained at the log-phase, 0.2 ± 0.04 units/ml in g20_10_200, which is not significantly different than Control. There is no significant difference in the yields of lipid content either. Thus, gradient cultivation improved biomass accumulation under both 150 mM and 200 mM NaCl. Under 150 mM while single-stage results in increased starch content, gradient diverts the pathway towards lipid accumulation. Hence, changing mode of cultivation plays an important role in differentiating the cellular pathways and diverting the carbon pool between starch and lipids. This knowledge becomes more crucial for large-scale industrial production of preferential biofuel.

Secondly, lipid droplets containing neutral lipids were stained with Nile red and ~100 individual cells were observed with an epifluorescence microscope. Intensity and size of the cell and lipid droplets individually was assessed using Fiji software. Lipid droplets were found to be larger in size in D2_100 throughout the growth cycle. Presence of more evenly distributed droplets in SS_100 defines the liquid-liquid phase co-existence of lipid molecules in single-stage, while two-stage observes the presence of phase separation. The involvement of liquid-liquid phase separation (LLPS) in lipid droplet formation in Chlamydomonas is a novel finding of this study. In addition, reduced cell size and cells enclosed in a palmelloid was another distinct feature of SS 100, which lacks in two-stage. This aligns with a previous study which reports the palmelloid formation under hypersaline condition (Khona et al. 2016; Shetty et al. 2019). This single-cell approach yields information on the lipid droplet distribution and the corresponding effect on cell morphology under salt stress. Analysis on lipid droplet distribution and characterization under 150 mM are ongoing.

Objective-2 Role of carbon concentration and mode of cultivation on growth and accumulation of lipid, starch and photosynthetic pigments in *Chlamydomonas reinhardtii* under heterotrophy and mixotrophy

To study heterotrophy, the current study deploys the use of sodium acetate (NaAc) as the external carbon source in three concentrations, 1 g/L, 5 g/L and 10 g/L added in single-stage *viz.* SS_H 1 gL^{-1}, SS_H 5 gL^{-1} and SS_H 10 gL^{-1} respectively. Sodium acetate has been shown to yield maximum biomass and lipids in *C. reinhardtii* when grown in single-stage (Moon et al. 2013), making it the preferable choice of carbon source to study gradient cultivation. Gradient-strategy of carbon source introduction was applied with steps of 0.5 g/L added daily, 1 g/L added alternately and 1 g/L NaAc added consecutively in G_H 0.5_10, G_H 1_10 and G_H 1_5, respectively. A prolonged lag phase in heterotrophy was observed, which increases with the carbon concentration, making SS_H 10 gL^{-1} as the most affected condition attaining stationary phase at 21 days *versus* 6 days in autotrophy. Gradient cultivation was observed to promote the biomass yield irrespective of the step size. Hence, changing the cultivation condition under heterotrophy alters the growth cycle of CC-125. Since, cultures were grown in dark, total chlorophyll content significantly dropped with respect to the autotrophic culture. Carotenoid content was also found to be adversely affected in all the conditions. Acetate concentration and age of the culture both were found to directly regulate the starch concentration. Maximum yield of starch is obtained from gradient mode of cultivation, i.e., G_H 1_10 by 15th day of growth, at 6.2 ± 1.2 units/ml. SS_H 5 gL^{-1} yields maximum lipid content, 249 ± 35 units/ml. Therefore, while gradient strategy of cultivation can efficiently be used to generate good yields of bio-feed and bioethanol, single-stage is a better option to harvest lipids and hence biodiesel.

To check the effect of light along with the above concentrations of sodium acetate, mixotrophic cultivation of CC-125 was also performed; the test conditions were- SS_M 1 gL^{-1}, SS_M 5 gL^{-1} and SS_M 10 gL^{-1} and G_M 1_10. Mixotrophy was also found to result in an increased lag phase and hence increased sustainability of the culture with increasing acetate concentration. Also, the amount of biomass was found to be highest in G_M 1_10. Significantly higher yields of both chlorophyll and carotenoids, i.e., 36.2 ± 1.7 mg/ml and 8.84 ± 0.5 mg/ml respectively, can be obtained from G_M 1_10 as compared to 21.13 ± 2.14 mg/ml and 6.5 ± 0.46 mg/ml in SS_M 5 gL^{-1}, respectively. SS_M 10 gL^{-1} yields highest amount of starch by the end of its stationary phase at day 21. Lipids are observed to accumulate most in SS_M 5 gL^{-1} yielding 359.6 ± 45.6 units/ml. Since, there was a maximum yield of biomass and lipids in mixotrophy, expression of genes responsible for autophagy and lipid production were studied. Significantly high upregulation of acetyl-coA carboxylase (ACCase), di-acyl glycerol acetyl transferase (DGAT) and starch phosphorylase (SPase) were observed in SS_M

5 gL^{-1} at stationary phase, denoting the role of de-novo lipid synthesis and starch-to-lipid switching in excessive lipid production. There was an increase in gene expression of ATG4 and ATG8 in SS$_M$ 5 gL^{-1} at stationary phase, but the fold change was significantly low. It was further intriguing to study the lipid droplet distribution and their characteristics in single cells. Single-cell epifluorescence microscopy was performed and we found an interesting phenomenon of liquid-liquid phase co-existence in case of stationary phase culture while phase separation was observed at log phase. The co-existence was largely pronounced in single-stage cultivation. Thus, lipid concentration, age of the culture and growth phase of the carbon addition altogether defines the phase boundary in lipid droplet formation in CC-125 under mixotrophy. Hence, while SS$_M$ 5 gL^{-1} showed increased lipid concentration, SS$_M$ 10 gL^{-1} showed enhanced starch content and G$_M$ 1_10 is favorable for biomass and pigment accumulation. Like salt stress and heterotrophy, mixotrophy as well offers scope for manipulation of cultivation strategy to direct the production of desired bioenergy feedstock.

Objective-3 Application of micro-Raman spectroscopy for non-invasive determination of biomolecular composition and cellular heterogeneity in *Chlamydomonas reinhardtii*

In order to study cell-to-cell heterogeneity in biomolecular composition of *C. reinhardtii* under stress, micro-Raman spectroscopy was used. Salt stress at 100 mM (single-stage and two-stage) was selected as the condition of preference, since this condition leaves no significant impact on the growth of Chlamydomonas cells and liberates equally good amount of starch, lipids as well as the photosynthetic pigments. Micro-RS with the incident beam of 50 mW power attenuated from a 532 nm diode pumped solid state laser (Laser Quantum) was passed through a 50X objective lens (N.A. 0.6), and the back-scattered Raman signal was collected from the focal spot of about 500 nm for the acquisition time of 2 s. Discrete wavelet transform (DWT) algorithm was applied to denoise the Raman spectra acquired for ~ 30 single cells for each condition. Reproducible Raman peaks were obtained at ~864 cm^{-1} for starch, 1350 cm^{-1} for proteins, ~1288 and ~1186 cm^{-1} for chlorophylls, ~1006, ~1156 and ~1523 cm^{-1} for carotenoids and ~1064, ~1086, ~1440, ~1656 and ~1750 cm^{-1} for lipids. The heterogeneity index was calculated for carotenoids, chlorophylls, lipid chain length and lipid unsaturation content, and distribution plots were drawn. Heterogeneity in the Raman peak center corresponding to three carotenoid peaks (~1006, ~1156 and ~1523 cm^{-1}) was found to increase at stationary phase. Deconvolution of Raman peak at ~1523 cm^{-1} shows the presence

of two close-by Raman peaks, centered at ~1520 and ~1530 cm^{-1} and coinciding with *β*-carotene and lutein, respectively (Osterrothová et al. 2019). Interestingly, we observed an increase in the wavenumber of later peak at the stationary phase and in D2 100. This study highlights the effect of mode of salt stress application and age of the culture on the type of carotenoid produced. The mean of Raman intensity corresponding to carotenoid peak was also observed to be greatly affected by the age of the culture in two-stage salt stress. On further analyzing the ratiometric intensity distribution of *β*-carotene normalized by the peak intensity at 1156 cm^{-1}, a significant increase in the distribution mean was observed in two-stage, both D2 100 and D4 100. Hence, two-stage salt stress is the most affected condition facing the change in carotenoid composition.

Single-cell heterogeneities in peak center defining fatty acid chain length (1086 cm^{-1} to 1094 cm^{-1}) increased under salt stress. Chain shortening was observed at stationary phase in SS 100, while D4 100 shows to produce short chain FA (12-13C) at both end-log and stationary phase. This could be due to the activation of β-oxidation pathway under stress. Salt stress was found to increase the heterogeneity in the unsaturation content too, with maximum HI observed in two-stage, D4 100. This heterogeneity is also enhanced as the culture ages. On further analyzing peaks at ~1440 and ~1656 cm^{-1}, an increase in bi-tri unsaturation content in stationary phase was observed, prominent in D4 100 at both growth phase. This response of the Chlamydomonas population to salt stress remained undetected in average measurements, as only mono-unsaturation content was obtained similar to that of the standard Triolein. Also, in principal component analysis for lipid unsaturation, D4 100 was seen to form a separate cluster. Thus, D4 100 was observed to have highest heterogeneity in its population which can be attributed to the population-based adaptation strategy to the salt stress, which lacks otherwise in the single-stage. This study thus throws light on the cultivation-governed adaptation of population towards environmental stress which in long run can result in evolution.

Lastly, the impact of salt stress on cellular homeostasis was also studied. Since, the Raman intensities obtained under the given experimental conditions could not be directly correlated to the actual cellular concentration of the biomolecules, correlations among starch, lipids and photosynthetic pigments were determined on a relative scale. Single-stage salt stress was shown to exhibit more negative correlations in comparison to two-stage cultures. Deteriorating correlation values on day 10 further suggests poor growth towards the stationary phase. Hence, Raman intensity correlations are found sensitive to the age and

mode of salt stress applied to the culture and can serve as a rapid assay to determine the physiological state of cells under stress.

Conclusion

The present study employs changes in cultivation strategy under stress, single-cell analysis of lipids and pigments by micro-Raman spectroscopy and lipid droplet characterization by epi-fluorescence microscopy. The first approach shows that the gradient application of carbon and salt increases the biomass accumulation. G_M 1_10, gradient application of sodium acetate with 1 g/L step size in mixotrophy, generates maximum biomass, chlorophyll and carotenoids, while under heterotrophy, G_H 1_10 yields maximum starch. Single-stage mixotrophic growth in 5 g/L sodium acetate, SS_M 5 gL^{-1}, results in the highest lipid accumulation. This single-stage cultivation observes a joint participation of autophagy, de-novo lipid synthesis and starch degradation involved in escalating the lipid production. Micro-Raman signals are shown to be sensitive to changes in cultivation modes under stress and can detect the changes in cellular composition at single-cell level. By elaborating on the cell-to-cell heterogeneity observed in pigment formation, lipid chain length and unsaturation content, it helps to understand the strategy of microalgae towards stress adaptation. Two-stage, D4_100 salt stress condition, yields maximum heterogeneity in lipid composition. The current work further highlights the phenomenon of liquid-liquid phase separation involved in microalgal lipid droplet formation, which in turn is shown to depend on the severity of stress, age of culture and lipid concentration.

Thus, better understanding of microalgal response to changing cultivation strategies can help the industries to modulate their bioreactors based on the requirement of preferrable bioenergy feedstock. This study, hence, brings out the wholesome use of microalgae in biofuel production without any requirement of genetic modification, which makes it an economical bio-factory.

References

Altschuler, Steven J., and Lani F. Wu. 2010. "Cellular Heterogeneity: Do Differences Make a Difference?" *Cell* 141(4):559–63. doi: 10.1016/j.cell.2010.04.033.

Ball, Steven G., Léon Dirick, André Decq, Jean Claude Martiat, and RenéF F. Matagne. 1990.

"Physiology of Starch Storage in the Monocellular Alga Chlamydomonas Reinhardtii." *Plant Science* 66(1):1–9. doi: 10.1016/0168-9452(90)90162-H.

Campenni', L., B. P. Nobre, C. A. Santos, A. C. Oliveira, M. R. Aires-Barros, A. M. F. Palavra, and L. Gouveia. 2013. "Carotenoid and Lipid Production by the Autotrophic Microalga Chlorella Protothecoides under Nutritional, Salinity, and Luminosity Stress Conditions." *Applied Microbiology and Biotechnology* 97(3):1383–93. doi: 10.1007/s00253-012-4570-6.

Chen, Jih Heng, Yuichi Kato, Mami Matsuda, Chun Yen Chen, Dillirani Nagarajan, Tomohisa Hasunuma, Akihiko Kondo, and Jo Shu Chang. 2021. "Lutein Production with Chlorella Sorokiniana MB-1-M12 Using Novel Two-Stage Cultivation Strategies – Metabolic Analysis and Process Improvement." *Bioresource Technology* 334(April):125200. doi: 10.1016/j.biortech.2021.125200.

Chen, Wei, Chengwu Zhang, Lirong Song, Milton Sommerfeld, and Qiang Hu. 2009. "A High Throughput Nile Red Method for Quantitative Measurement of Neutral Lipids in Microalgae." *Journal of Microbiological Methods* 77(1):41–47. doi: 10.1016/j.mimet.2009.01.001.

Cheng, Dan, and Qingfang He. 2014. "Assessment of Environmental Stresses for Enhanced Microalgal Biofuel Production – an Overview." 2(July):1–8. doi: 10.3389/fenrg.2014.00026.

Chiu, Liang Da, Shih Hsin Ho, Rintaro Shimada, Nan Qi Ren, and Takeaki Ozawa. 2017. "Rapid in Vivo Lipid/Carbohydrate Quantification of Single Microalgal Cell by Raman Spectral Imaging to Reveal Salinity-Induced Starch-to-Lipid Shift." *Biotechnology for Biofuels* 10(1):1–9. doi: 10.1186/s13068-016-0691-y.

Couso, Inmaculada, María Esther Pérez-Pérez, Enrique Martínez-Force, Hee-Sik Kim, Yonghua He, James G. Umen, José L. Crespo, and Peter Bozhkov. 2017. "Autophagic Flux Is Required for the Synthesis of Triacylglycerols and Ribosomal Protein Turnover in Chlamydomonas." *Journal of Experimental Botany* (January). doi: 10.1093/jxb/erx372.

Czamara, K., K. Majzner, M. Z. Pacia, K. Kochan, Agnieszka Kaczor, and M. Baranska. 2015. "Raman Spectroscopy of Lipids: A Review." *Journal of Raman Spectroscopy* 46(1):4–20. doi: 10.1002/jrs.4607.

Fan, Jianhua, and lvhong Zheng. 2017. "Acclimation to NaCl and Light Stress of Heterotrophic Chlamydomonas Reinhardtii for Lipid Accumulation." *Journal of Bioscience and Bioengineering* 124(3):302–8. doi: 10.1016/j.jbiosc.2017.04.009.

Fan, Jilian, Chengshi Yan, Carl Andre, John Shanklin, Jörg Schwender, and Changcheng Xu. 2012. "Oil Accumulation Is Controlled by Carbon Precursor Supply for Fatty Acid Synthesis in Chlamydomonas Reinhardtii." *Plant and Cell Physiology* 53(8):1380–90. doi: 10.1093/pcp/pcs082.

Gardner, Robert D., Egan Lohman, Robin Gerlach, Keith E. Cooksey, and Brent M. Peyton. 2013. "Comparison of CO2and Bicarbonate as Inorganic Carbon Sources for Triacylglycerol and Starch Accumulation in Chlamydomonas Reinhardtii." *Biotechnology and Bioengineering* 110(1):87–96. doi: 10.1002/bit.24592.

Glick, Danielle, Sandra Barth, and Kay F. Macleod. 2010. "Autophagy: Cellular and Molecular Mechanisms." *Journal of Pathology* 221(1):3–12. doi: 10.1002/path.2697.

Graham, Duncan, Lauren E. Jamieson, Angela Li, Karen Faulds, and Duncan Graham. 2018. "Ratiometric Analysis Using Raman Spectroscopy as a Powerful Predictor of Structural Properties of Fatty Acids."

Harris, Elizabeth H. 2001. "Chlamydomonas As a Model Organism." *Annu. Rev. Plant Physiol. Plant Mol. Biol* 52:363–406. doi: 10.1146/annurev.arplant.52.1.363.

He, Yuehui, Peng Zhang, Shi Huang, Tingting Wang, Yuetong Ji, and Jian Xu. 2017. "Label-Free,

Simultaneous Quantification of Starch, Protein and Triacylglycerol in Single Microalgal Cells." *Biotechnol Biofuels* 10:1–18. doi: 10.1186/s13068-017-0967-x.

Ho, Shih Hsin, Akihito Nakanishi, Xiaoting Ye, Jo Shu Chang, Kiyotaka Hara, Tomohisa Hasunuma, and Akihiko Kondo. 2014. "Optimizing Biodiesel Production in Marine Chlamydomonas Sp. JSC4 through Metabolic Profiling and an Innovative Salinity-Gradient Strategy." *Biotechnology for Biofuels* 7(1):1–16. doi: 10.1186/1754-6834-7-97.

Jia, Jing, Danxiang Han, Henri G. Gerken, Yantao Li, Milton Sommerfeld, Qiang Hu, and Jian Xu. 2015. "Molecular Mechanisms for Photosynthetic Carbon Partitioning into Storage Neutral Lipids in Nannochloropsis Oceanica under Nitrogen-Depletion Conditions." *Algal Research* 7:66–77. doi: 10.1016/j.algal.2014.11.005.

Johnson, Xenie, and Jean Alric. 2013. "Central Carbon Metabolism and Electron Transport in Chlamydomonas Reinhardtii: Metabolic Constraints for Carbon Partitioning between Oil and Starch." *Eukaryotic Cell* 12(6):776–93. doi: 10.1128/EC.00318-12.

Khan, Shakeel A., Rashmi, Mir Z. Hussain, S. Prasad, and U. C. Banerjee. 2009. "Prospects of Biodiesel Production from Microalgae in India." *Renewable and Sustainable Energy Reviews* 13(9):2361–72. doi: 10.1016/j.rser.2009.04.005.

Khona, Dolly K., Seema M. Shirolikar, Kanak K. Gawde, Erik Hom, Manjushree A. Deodhar, and Jacinta S. D'Souza. 2016. "Characterization of Salt Stress-Induced Palmelloids in the Green Alga, Chlamydomonas Reinhardtii." *Algal Research* 16:434–48. doi: 10.1016/j.algal.2016.03.035.

Kou, Zheng, Shuqing Bei, Juan Sun, and Junmin Pan. 2013. "Fluorescent Measurement of Lipid Content in the Model Organism Chlamydomonas Reinhardtii." *Journal of Applied Phycology* 25(6):1633–41. doi: 10.1007/s10811-013-0011-x.

Kumar, Mukesh, M. P. Sharma, and Gaurav Dwivedi. 2013. "Algae Oil as Future Energy Source in Indian Perspective." *INTERNATIONAL JOURNAL of RENEWABLE ENERGY RESEARCH* 3(4):913–21.

Lee, Do Hyun, Chae Yun Bae, Jong In Han, and Je Kyun Park. 2013. "In Situ Analysis of Heterogeneity in the Lipid Content of Single Green Microalgae in Alginate Hydrogel Microcapsules." *Analytical Chemistry* 85(18):8749–56. doi: 10.1021/ac401836j.

Li-Beisson, Yonghua, Fred Beisson, and Wayne Riekhof. 2015. "Metabolism of Acyl-Lipids in Chlamydomonas Reinhardtii." *Plant Journal* 82(3):504–22. doi: 10.1111/tpj.12787.

Lidstrom, Mary E., and Michael C. Konopka. 2010. "The Role of Physiological Heterogeneity in Microbial Population Behavior." *Nature Chemical Biology* 6(10):705–12. doi: 10.1038/nchembio.436.

Lin, Tse Shih, and Jane Yii Wu. 2015. "Effect of Carbon Sources on Growth and Lipid Accumulation of Newly Isolated Microalgae Cultured under Mixotrophic Condition." *Bioresource Technology* 184:100–107. doi: 10.1016/j.biortech.2014.11.005.

Lv, Hexin, Ge Qu, Xizhen Qi, Lina Lu, Chaoguang Tian, and Yanhe Ma. 2013. "Transcriptome Analysis of Chlamydomonas Reinhardtii during the Process of Lipid Accumulation." *Genomics* 101(4):229–37. doi: 10.1016/j.ygeno.2013.01.004.

Martins, Bruno M. C., and James C. W. Locke. 2015. "Microbial Individuality: How Single-Cell Heterogeneity Enables Population Level Strategies." *Current Opinion in Microbiology* 24:104–12. doi: 10.1016/j.mib.2015.01.003.

Moon, Myounghoon, Chul Woong Kim, Won Kun Park, Gursong Yoo, Yoon E. Choi, and Ji Won Yang. 2013. "Mixotrophic Growth with Acetate or Volatile Fatty Acids Maximizes Growth and Lipid Production in Chlamydomonas Reinhardtii." *Algal Research* 2(4):352–57. doi:

10.1016/j.algal.2013.09.003.

Nagarajan, Dillirani, Duu Jong Lee, Akihiko Kondo, and Jo Shu Chang. 2017. "Recent Insights into Biohydrogen Production by Microalgae – From Biophotolysis to Dark Fermentation." *Bioresource Technology* 227(1):373–87. doi: 10.1016/j.biortech.2016.12.104.

Osterrothová, Kateřina, Adam Culka, Kateřina Němečková, David Kaftan, Linda Nedbalová, Lenka Procházková, and Jan Jehlička. 2019. "Analyzing Carotenoids of Snow Algae by Raman Microspectroscopy and High-Performance Liquid Chromatography." *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy* 212:262–71. doi: 10.1016/j.saa.2019.01.013.

Pancha, Imran, Kaumeel Chokshi, Rahulkumar Maurya, Khanjan Trivedi, Shailesh Kumar Patidar, Arup Ghosh, and Sandhya Mishra. 2015. "Salinity Induced Oxidative Stress Enhanced Biofuel Production Potential of Microalgae Scenedesmus Sp. CCNM 1077." *Bioresource Technology* 189:341–48. doi: 10.1016/j.biortech.2015.04.017.

Pérez-Pérez, María Esther, Inmaculada Couso, and José L. Crespo. 2012. "Carotenoid Deficiency Triggers Autophagy in the Model Green Alga Chlamydomonas Reinhardtii." *Autophagy* 8(3):376–88. doi: 10.4161/auto.8.3.18864.

Sandmann, Michael, Michaela Sch, Martin Lippold, and Sascha Rohn. 2018. "Analysis of Population Structures of the Microalga Acutodesmus Obliquus during Lipid Production Using Multi-Dimensional Single-Cell Analysis." (April):1–9. doi: 10.1038/s41598-018-24638-y.

Scranton, Melissa A., Joseph T. Ostrand, Francis J. Fields, and Stephen P. Mayfield. 2017. "Production." 82(3):523–31. doi: 10.1111/tpj.12780.Chlamydomonas.

Shetty, Prateek, Margaret Mukami Gitau, and Gergely Maróti. 2019. "Salinity Stress Responses and Adaptation Mechanisms in Eukaryotic Green Microalgae." *Cells* 8(12):1–16. doi: 10.3390/cells8121657.

Trampe, Erik, Jörg Kolbowski, Ulrich Schreiber, and Michael Kühl. 2011. "Rapid Assessment of Different Oxygenic Phototrophs and Single-Cell Photosynthesis with Multicolour Variable Chlorophyll Fluorescence Imaging." *Marine Biology* 158(7):1667–75. doi: 10.1007/s00227-011-1663-1.

Wang, Tingting, Yuetong Ji, Yun Wang, Jing Jia, Jing Li, Shi Huang, Danxiang Han, Qiang Hu, Wei E. Huang, and Jian Xu. 2014. "Quantitative Dynamics of Triacylglycerol Accumulation in Microalgae Populations at Single-Cell Resolution Revealed by Raman Microspectroscopy." *Biotechnology for Biofuels* 7(1):58. doi: 10.1186/1754-6834-7-58.

Yen, Hong Wei, and Jung Tzu Chang. 2013. "A Two-Stage Cultivation Process for the Growth Enhancement of Chlorella Vulgaris." *Bioprocess and Biosystems Engineering* 36(11):1797–1801. doi: 10.1007/s00449-013-0922-6.

Yu, Shuiyan, Quanyu Zhao, Xiaoling Miao, and Jiping Shi. 2013. "Enhancement of Lipid Production in Low-Starch Mutants Chlamydomonas Reinhardtii by Adaptive Laboratory Evolution." *Bioresource Technology* 147:499–507. doi: 10.1016/j.biortech.2013.08.069.

Zheng, Yubin, Zhanyou Chi, Ben Lucker, and Shulin Chen. 2012. "Two-Stage Heterotrophic and Phototrophic Culture Strategy for Algal Biomass and Lipid Production." *Bioresource Technology* 103(1):484–88. doi: 10.1016/j.biortech.2011.09.122.