Chapter 5

Summary and Future Directions

5 Summary and Future Directions

5.1 Summary

The potential of microalgae as a resource for a sustainable and cost-effective alternative for biofuel production is faced with certain challenges, the most important being the compromised growth rates under conditions that favor high lipid content. The present study was focused on the outcome of microalgal growth and associated apportioning of carbon into different metabolites in presence of abiotic stress conditions with special attention on cultivation strategies that could result in good cell count as well as lipid content. Salt stress and carbon availability under hetero/mixotrophy have been addressed in this study as the inducers of biofuel production in *Chlamydomonas reinhardtii* CC-125, a green freshwater microalga. The present study has broadly focused on three main aspects of bioenergy feedstock production, i.e., (1) changing the cultivation strategy to increase the net productivity and yield of the feedstock (cell density, pigments, starch, and lipids), (2) analyzing lipid droplet formation in microalgae with respect to gene expression and phase separation kinetics, and (3) optimizing micro-Raman spectroscopy for high-resolution and high-throughput characterization of feedstock production and fast, non-invasive metabolite profiling.

Apart from the usual single-stage cultivation, two alternative cultivation modes were assessed in the first aspect of this study, i.e., two-stage and gradient strategy, to induce adaptivity in cells to stress factors. A total of 20 different growth strategies were employed, 10 of salt stress, and 10 of different trophic conditions with changing carbon concentrations (**Table 5.1**). The yields of bioenergy feedstocks under all the conditions tried in this study are represented in **Figure 5.1**. Mixotrophic gradient, G_M 5 gL^{-1} yields maximum cell density with 9.82 ± 0.06 million cells/mL which is 3.6-fold higher than the autotrophic control. It also generates the highest chlorophyll and carotenoid content of 36.17 ± 1.74 mg/mL and 8.85 ± 0.52 mg/mL, respectively. The mixotrophic single-stage, SS_M 5 gL^{-1}, on the other hand, produces the highest lipid content, 3-fold higher than the autotrophic control. Heterotrophic gradient cultivation resulted in maximum starch production and G_H 1_10 (the heterotrophic analog of G_M 5 gL^{-1}) showed an 81-fold increase compared to the control. Overall, mixotrophy serves as the most favorable growth condition to generate bio-feed, nutraceuticals, pharmaceuticals, and biodiesel, while heterotrophic cultivation boosts the production of bioethanol and bio-hydrogen. The escalated lipid production in SS_M 5 gL^{-1} is observed due to the increased carbon

pool collectively contributed by de-novo lipid synthesis, starch degradation, and autophagic activities. Thus, there is a differential response of *Chlamydomonas* cells towards changing modes of cultivation and a better understanding can help the industries in modulating their bioreactors based on the requirement of preferable bioenergy feedstock. This study is relevant to the use of native microalgal strains without requisite genetic modification.

In the second aspect of the thesis, the contribution of liquid-liquid phase separation in lipid droplet formation in the model microalga, *Chlamydomonas reinhardtii* CC-125 was addressed. Using 2D epifluorescence microscopy, size, distribution, and the number of lipid droplets (LD) formed under salt stress and mixotrophy were determined. Under single-stage salt stress, the proportion of small LDs increased, while in gradient salt stress, major proportion of large-sized LDs was found. Single-stage mixotrophy, on the other hand, produced large-sized LDs. The oxidative state of the cell, lipid concentration, lipid breakdown and turnover, droplet fusion, the protein composition of the droplets, and the cellular location of initiation of droplet formation are the major governing factors of droplet size and the phase separation phenomena. The stressful growth conditions imposed on the cells also modulate the thermodynamic factors contributing to the energy barriers involved in droplet growth and dynamics. The high salt concentration of 150 mM applied in the single-stage caused cell size reduction due to osmotic shock to the cells. The single-stage cells also produced a thick palmelloid layer enclosing about 8 cells. Gradient cultivation caused no significant alterations in cell morphology and also resulted in delayed palmelloid development. This study showed how the cultivation strategy alters the cell morphology and lipid droplet growth dynamics, the details of which are necessary for efficient large-scale cultivation of microalgae. To the best of our knowledge, this is the first report of lipid droplet morphology assessment in microalgae.

In the third Chapter, micro-Raman spectroscopy was applied for the rapid detection and characterization of bioenergy feedstock in microalgae. Salt stress with 100 mM NaCl served as the best growth condition for the optimization since they caused an insignificant effect on the growth. Micro-Raman spectroscopy was particularly used to detect the biomolecular profile of microalgae at the single-cell level, which revealed cell-to-cell heterogeneities. The two-stage salt stress culture was found to exhibit maximum heterogeneity in the lipid and carotenoid composition. The increase in the lutein content in two-stage salt stress and the presence of good biodiesel quality 18:0 fatty acids in the microalgal lipids are the main highlights of this study. Overall, this part of the study observes the presence of adaptive mechanisms in microalgal

cells, which differ among the cultivation methods and are often overlooked by bulk measurements.

Table 5.1. Nomenclature and description of cultivation conditions to culture *C. reinhardtii* under salt stress and hetero/mixotrophy

Cultivation nomenclature	Cultivation environment	Concentration of the abiotic element	Cultivation strategy	Time of addition of abiotic element
SS_100	Salt stress	100 mM NaCl	Single-stage	0^{th} day
D2_100			Two-stage	2^{nd} day
D4_100			Two-stage	4^{th} day
SS_150		150 mM NaCl	Single-stage	0^{th} day
D4_150			Two-stage	4^{th} day
g15_10_150			Gradient	15 mM NaCl added daily to achieve 150 mM
g30_10_150			Gradient	30 mM NaCl added on alternate days to achieve 150 mM
D4_200		200 mM NaCl	Two-stage	4^{th} day
g20_10_200			Gradient	20 mM NaCl added daily to achieve 200 mM
g40_10_200			Gradient	40 mM NaCl added on alternate days to achieve 200 mM
SS_M 1 gL^{-1}	Mixotrophy	1 g/L NaAc	Single-stage	0^{th} day
SS_M 5 gL^{-1}		5 g/L NaAc	Single-stage	0^{th} day
SS_M 10 gL^{-1}		10 g/L NaAc	Single-stage	0^{th} day
G_M 5 gL^{-1}		5 g/L NaAc	Gradient	1 g/L acetate added on alternate days to achieve 5 g/L
SS_H 1 gL^{-1}	Heterotrophy	1 g/L NaAc	Single-stage	0^{th} day
SS_H 5 gL^{-1}		5 g/L NaAc	Single-stage	0^{th} day
SS_H 10 gL^{-1}		10 g/L NaAc	Single-stage	0^{th} day
G_H 0.5_10		5 g/L NaAc	Gradient	0.5 g/L acetate added daily to achieve the final concentration of 5 g/L
G_H 1_10			Gradient	1 g/L acetate added on alternate days to achieve the final concentration of 5 g/L
G_H 1_5			Gradient	1 g/L acetate added daily to achieve the final concentration of 5 g/L

NaCl- Sodium chloride, NaAc- Sodium acetate

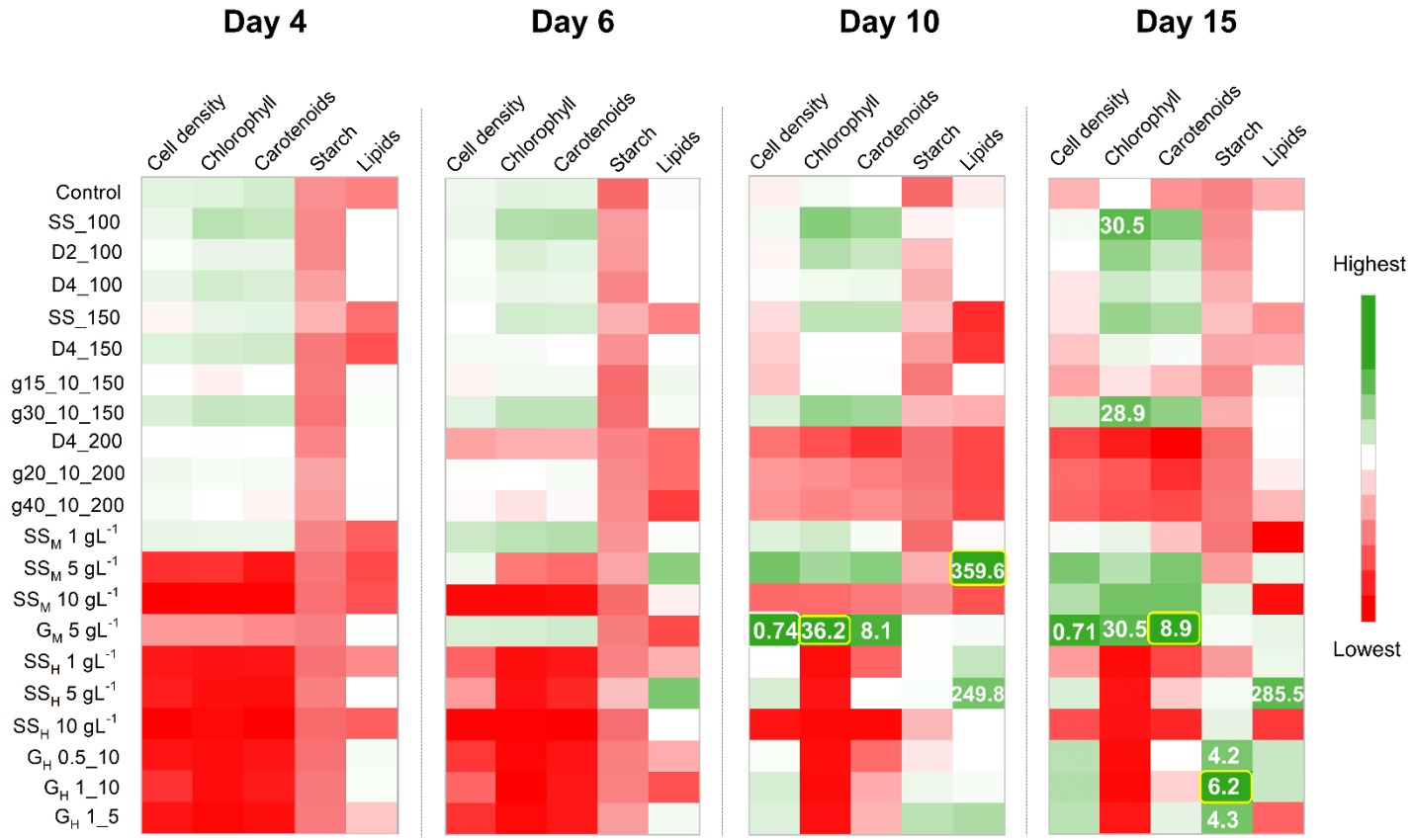

Figure 5.1. Heat map representing the total yield of bioenergy feedstock obtained from *C. reinhardtii* grown under salt stress and hetero/mixotrophic cultivations.

The feedstocks under consideration are cell density, chlorophyll, carotenoid, starch, and lipid production. The values mentioned in the green boxes have units, *viz.* million cells/mL for cell density, mg/mL for chlorophyll and carotenoids, a.u. /mL for starch, and f.u. /mL for lipids. The green boxes highlighted in yellow represents the highest value for that particular feedstock. The nomenclature for the culture conditions is explained in the **Table 5.1**.

5.2 Future Directions

A thorough understanding of how the manipulation of stress and mixotrophy in cultivation strategies for microalgae can result in varying distribution of carbon resources into energy feedstock is valuable for improving yields at the industrial level. It would also be interesting to explore more variations in the step size during gradient cultivation. A detailed analysis of the metabolic pathways involved in lipid and starch production can reveal more insights into the differential behavior of microalgae as a function of changing cultivation strategy. Micro-Raman spectroscopy can be used as the state-of-the-art tool for multivariate analysis of starch and protein also, apart from pigments and lipids that are being studied here. The technique and the analysis offer great prospects in the future for optimization of various other stress conditions such as high light intensity, light/dark cycle, nutrient concentration, and temperature, with single stage or gradient mode changes. An important question which remains to be analyzed is the mechanism of the adaptive processes which come into play under gradient mode of stress administration. Are there any inherent plasticity introduced in the cells or would the microalgae recover and show similarities with the Control culture if the stress conditions are removed and normal conditions are restored? This study brings to the forefront the possible involvement of ROS in regulating the phase separation in lipid droplet assembly and growth. What is the kinetics governing the phase separation? What are the roles of the cellular-scale heterogeneities in governing the quality of industrial scale biodiesel production? What are the key regulatory proteins involved in the liquid-liquid phase separation kinetics, droplet assembly and coexistence with the cytosol as a two-phase state? These are some unanswered questions in the field of microalgal research that demand more attention in the future.