Appendix

Appendix 1

Composition of Tris-Acetate Phosphate (TAP) medium

A1.1. Composition of Tris-Acetate Phosphate (TAP) medium

Tris-Acetate Phosphate medium is a synthetically composed medium containing many trace elements with a pH adjusted to 7.0. The source for TAP medium composition is Chlamydomonas Resource Centre (https://www.chlamycollection.org). The stock solution composition is given in **Table A1.1**. The composition of each component of stock solution is given in **Tables A1.2-A1.4**.

Table A1.1. Composition of stock solutions for 1 liter of TAP medium

Content	Volume (for 1 Liter)
1 M Tris base	20 mL
Phosphate Buffer II	1 mL
Solution A	10 mL
Hutner's Trace Elements	1 mL
Glacial acetic acid	1 mL

Table A1.2. Composition of 100 mL Phosphate Buffer II

Content	Volume (for 100 mL)
K_2HPO_4	10.8 g
KH_2PO_4	5.6 g

Table A1.3. Composition of 500 mL Solution A

Content	Volume (for 500 mL)
NH_4Cl	20 g
$MgSO_4.7H_2O$	5 g
$CaCl_2.2H_2O$	2.5 g

Table A1.4. Composition of 1 Liter Hutner's Trace Elements (HTE) solution

Salt	Amount	Volume of water to dissolve
EDTA disodium salt	50 g	250 mL
$ZnSO_4.7H_2O$	22 g	100 mL
H_3BO_3	11.4 g	200 mL
$MnCl_2.4H_2O$	5.06 g	50 mL
$CoCl_2.6H_2O$	1.61 g	50 mL
$CuSO_4.5H_2O$	1.57 g	50 mL
$(NH_4)_6Mo_7O_{24}.\ 4H_2O$	1.10 g	50 mL
$FeSO_4.7H_2O$	4.99 g	50 mL

To prepare 1 liter of HTE solution, first dissolve every component in the given volume of water. EDTA should be dissolved in boiling water with constant stirring. Prepare $FeSO_4.7H_2O$ at the end to avoid any oxidation. Mix all the components, except EDTA. Bring it to a boil and cool down the solution to 70 ℃. At this point, add 85 mL of hot 20 % KOH solution. Cool down the solution and make up the volume to 1 liter with water. Initially, the solution is green in color, which turns to dark red, and then finally turns to deep purple in color in a few days. Keeping the solution on constant stirring fastens the process. At the end of the process, the solution leaves rust-brown colored precipitates which should be filtered out before use. The final solution must be stored in aliquots in a refrigerator for longer use.

Appendix 2

qRT-PCR: Primer specificity, amplification, and melting plots

A2.1. Primer specificity

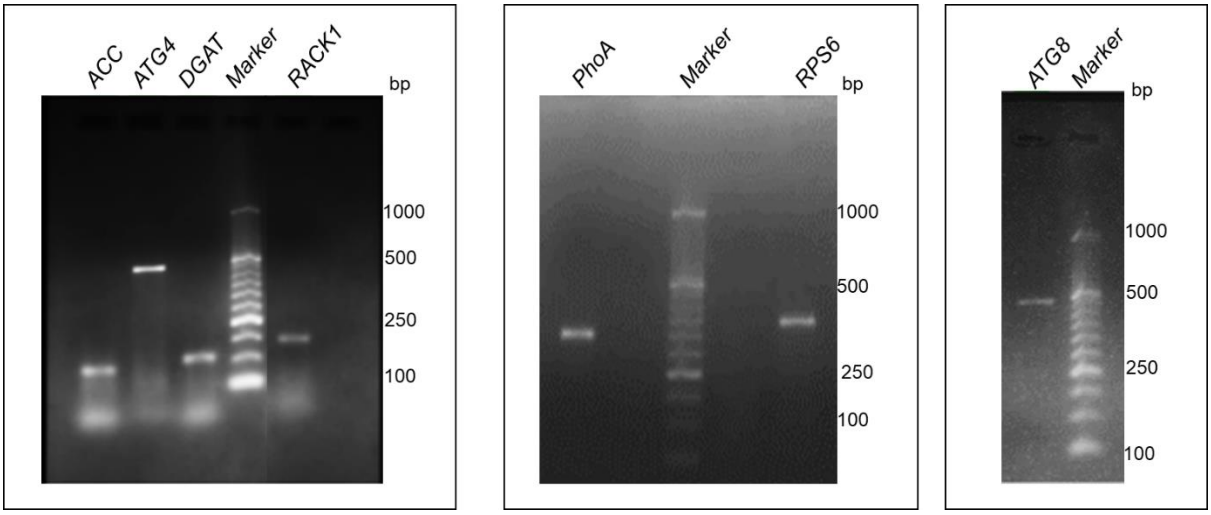

Figure A2.1. Gene amplification using mRNA-specific primers on *C. reinhardtii* genome

Gene specific primers were designed as mentioned in the **Table 2.1** and their specificity was checked by amplifying genes from whole genome of *C. reinhardtii* CC-125. The PCR product was seperated on 2.5% agarose gel and the product size was checked for specific amplification.

A2.2. Amplification plots obtained in qRT-PCR

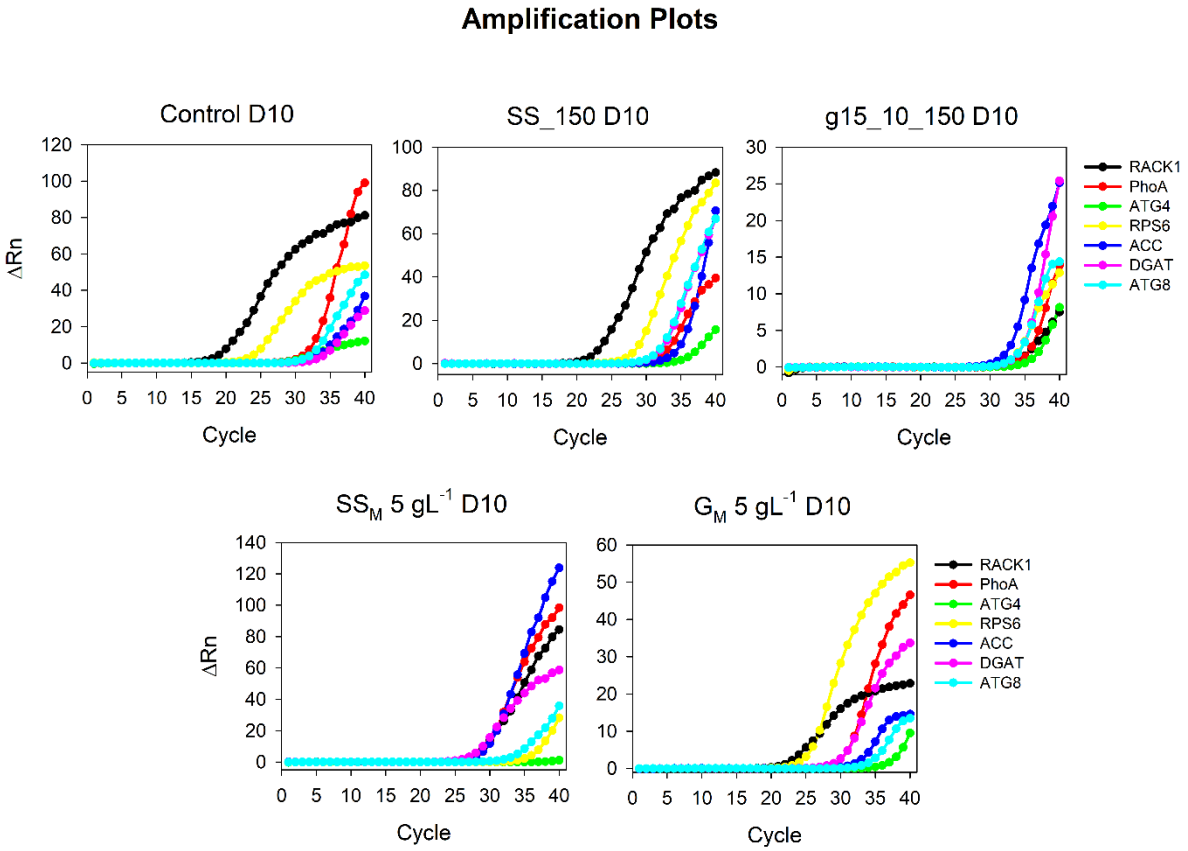

Figure A2.2. Representative amplification plots of qRT-PCR.

Amplification plots for Control, salt stress (SS_150 and g15_10_150), and mixotrophy (SS_M 5 gL^{-1} and G_M 5 gL^{-1}) on 10th day of cultivation are shown with primers for *RACK1*, *ACC*, *DGAT*, *ATG4*, *ATG8*, *PhoA*, and *RPS6*. Here, ΔRn is the Rn value of the experimental signal minus the Rn value of the baseline signal generated by the instrument.

A2.3 Melting plots obtained in qRT-PCR

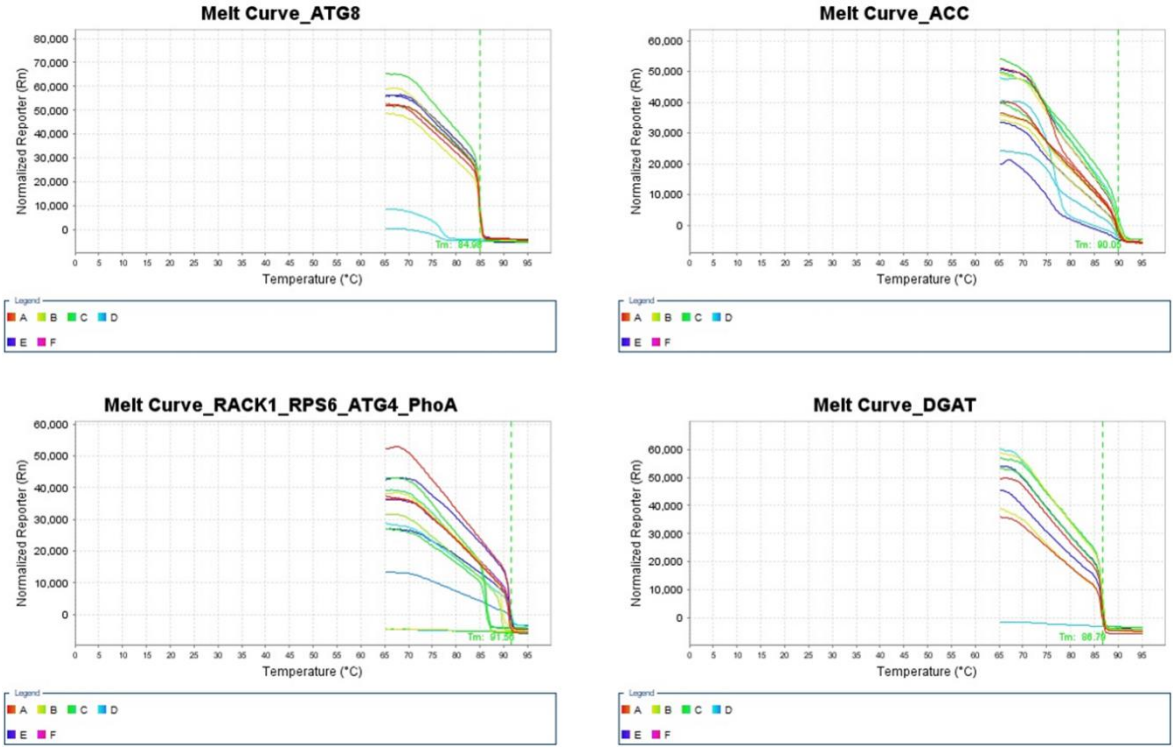

Figure A2.3. Representative melting plots of qRT-PCR.

Melt curves for 6 RT-PCR genes, i.e., *RACK1*, *ACC*, *DGAT*, *ATG4*, *ATG8*, *PhoA*, and *RPS6* are shown for selective growth conditions, including Control, salt stress (SS_150 and g15_10_150), and mixotrophy (SS_M 5 gL^{-1} and G_M 5 gL^{-1}).

Appendix *3*

Heterogeneities in the ν(C-C) Raman mode under salt stress

A3.1. Heterogeneities in the ν(C-C) Raman mode in *C. reinhardtii* cultivated under salt stress

Contour plots illustrating variations in the wave number of peak maxima for lipid C–C stretch for different growth conditions and days of harvesting are given in **Figure A3.1.** Two peaks, 1063 and 1086 cm^{-1} are displayed and the most probable peak centers are marked with a magenta line. Hardly any variation is seen in the 1063 cm^{-1} peak, but the 1086 cm^{-1} peak shows large variations with sub-populations having different peak centers. These variations become more pronounced in the case of stress conditions D2 100 and D4 100, especially on the 10^{th} day.

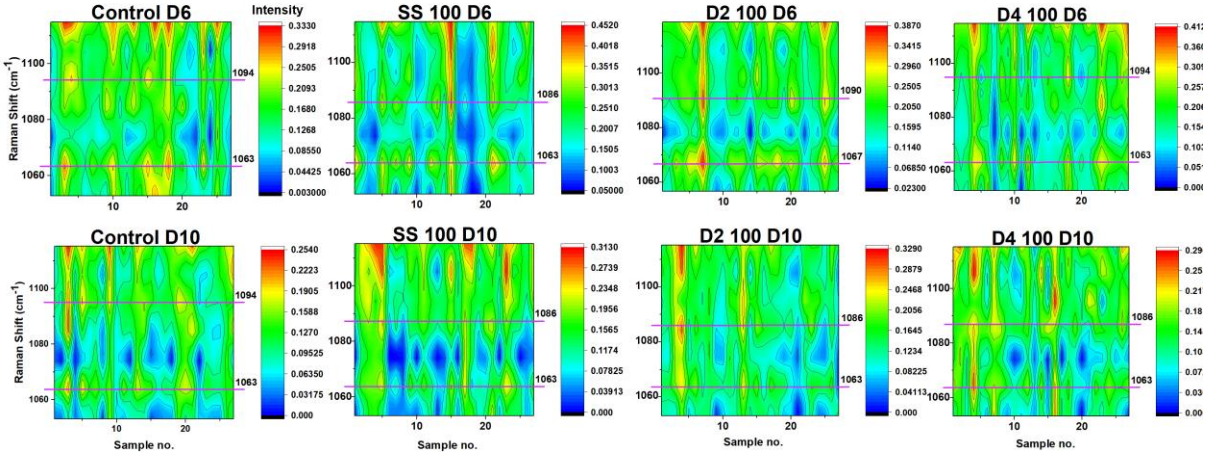

Figure A3.1. Heterogeneities in the ν(C-C) Raman mode in a population of 100 cells of *C. reinhardtii* cultivated under salt stress.

Appendix 4

Pearson's correlation analysis for cellular components under salt stress

A4.1. Pearson's correlation coefficients for cellular components in *C. reinhardtii* under salt stress obtained from Raman spectroscopy

Pearson's correlation coefficients were calculated between any given two cellular components among starch, protein, carotenoids, chlorophyll, saturated lipid, and unsaturated lipid using custom-made commands in MATLAB. The values are shown in **Figure A4.1.,** for all the cultures, Control (no NaCl), SS 100 (100 mM NaCl added on Day 0), D2 100 (100 mM NaCl added on Day 2), and D4 100 (100 mM NaCl added on Day 4 of culture) when sampled on **A**. Day 6 and **B.** Day 10 of their growth. These coefficient values demonstrate the relationship between any two components, it can be either a positive correlation or a negative one. A positive correlation means that the concentration of both components would either increase or decrease. A negative correlation means that the concentration of either one of the two components would increase, while the other would decrease.

A

Day 6

					Control	
Starch	1					
Protein	0.5	1				
Caro	0	0.17	1			
Chloro	0.5	0.68	0.09	1		
Sat. lipid	0.78	0.38	-0.06	0.32	1	
Unsat. lipid	0.79	0.34	-0.31	0.45	0.72	1
	Starch	Protein	Caro	Chloro	Sat. lipid	Unsat. lipid

					SS 100	
Starch	1					
Protein	0.67	1				
Caro	0.07	0.27	1			
Chloro	0.67	0.73	0.27	1		
Sat. lipid	0.76	0.71	0.09	0.65	1	
Unsat. lipid	0.65	0.44	-0.1	0.49	0.67	1
	Starch	Protein	Caro	Chloro	Sat. lipid	Unsat. lipid

					D2 100	
Starch	1					
Protein	0.77	1				
Caro	-0.09	0.22	1			
Chloro	0.76	0.78	-0.03	1		
Sat. lipid	0.83	0.61	-0.25	0.66	1	
Unsat. lipid	0.84	0.63	-0.13	0.78	0.66	1
	Starch	Protein	Caro	Chloro	Sat. lipid	Unsat. lipid

					D4 100	
Starch	1					
Protein	0.57	1				
Caro	0.02	0.44	1			
Chloro	0.64	0.63	0.16	1		
Sat. lipid	0.82	0.45	-0.05	0.51	1	
Unsat. lipid	0.38	0.14	-0.21	0.29	0.44	1
	Starch	Protein	Caro	Chloro	Sat. lipid	Unsat. lipid

B

Day 10

					Control	
Starch	1					
Protein	0.58	1				
Caro	-0.03	-0.14	1			
Chloro	0.61	0.65	0.02	1		
Sat. lipid	0.66	0.46	0.17	0.46	1	
Unsat. lipid	0.59	0.39	-0.12	0.45	0.48	1
	Starch	Protein	Caro	Chloro	Sat. lipid	Unsat. lipid

					SS 100	
Starch	1					
Protein	0.03	1				
Caro	-0.39	0.16	1			
Chloro	-0.12	0.52	0.1	1		
Sat. lipid	0.64	0.18	-0.43	0.05	1	
Unsat. lipid	0.54	-0.04	-0.42	-0.23	0.39	1
	Starch	Protein	Caro	Chloro	Sat. lipid	Unsat. lipid

					D2 100	
Starch	1					
Protein	0.17	1				
Caro	0.32	-0.1	1			
Chloro	0.15	0.4	-0.04	1		
Sat. lipid	0.37	0.38	0.13	-0.06	1	
Unsat. lipid	0.29	0	-0.22	-0.01	0.18	1
	Starch	Protein	Caro	Chloro	Sat. lipid	Unsat. lipid

					D4 100	
Starch	1					
Protein	0.41	1				
Caro	0.03	0.15	1			
Chloro	0.22	0.38	0.1	1		
Sat. lipid	0.37	0.17	0.07	-0.06	1	
Unsat. lipid	0.36	0.11	-0.20	0.26	-0.32	1
	Starch	Protein	Caro	Chloro	Sat. lipid	Unsat. lipid

Figure A4.1. Relationship among the cellular components as a function of culture age and salt stress in *C. reinhardtii*.

The Raman wavenumbers used for the analysis are 864 cm^{-1} for starch, 1349 cm^{-1} for proteins, 1524 cm^{-1} for carotenoids, 1280 cm^{-1} for chlorophylls, 1440 cm^{-1} for saturated lipids, and 1656 cm^{-1} for unsaturated lipids.

Appendix 5

Publications and Presentations

A5.1. List of publications

1. Pandey, S., Kumar, P., Dasgupta, S., Gattupalli, A., and Bagchi, D. Gradient Strategy for Mixotrophic Cultivation of *Chlamydomonas reinhardtii*: Small Steps, a Large Impact on Biofuel Potential and Lipid Droplet Morphology. *Bioenerg. Res.* **16**, 163–176 (2023). https://doi.org/10.1007/s12155-022-10454-w

2. Pandey, S., Gattupalli, A., and Bagchi, D. Micro-Raman spectroscopy of the light-harvesting pigments in *Chlamydomonas reinhardtii* under salinity stress. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* **281**, 12613 (2022). https://doi.org/10.1016/j.saa.2022.121613

BioEnergy Research
https://doi.org/10.1007/s12155-022-10454-w

Gradient Strategy for Mixotrophic Cultivation of *Chlamydomonas reinhardtii*: Small Steps, a Large Impact on Biofuel Potential and Lipid Droplet Morphology

Shubhangi Pandey[1] · Palak Kumar[1] · Swarnali Dasgupta[1] · Gattupalli Archana[1] · Debjani Bagchi[2]

Received: 31 January 2022 / Accepted: 10 April 2022

Abstract

Mixotrophic cultivation of microalgae is an economical and environment-friendly approach to benefit biofuel production by increasing biomass. In this study, a novel strategy of gradient addition of carbon source is used in *Chlamydomonas reinhardtii* to obtain better biomass yields. Gradient strategy leads to low autophagy levels in microalgae, yielding the highest biomass of 9.42 ± 0.21 million cells/mL after 15 days of cultivation. This approach produces highest chlorophyll (36.17 ± 1.74 mg/mL) and carotenoids (8.85 ± 0.52 mg/mL). At 5 g/L sodium acetate, gradient mode results in increased starch accumulation at the stationary phase, while the single-stage produces the highest triacylglycerol (TAG) content at the log phase. TAG production is mediated by the combined action of high autophagy, de novo lipid synthesis, and starch degradation process. Increased autophagy indicates high oxidative stress in single-stage which results in liquid–liquid phase separation (LLPS) of TAG from the cytosol, forming lipid droplets (LDs) for cellular redox maintenance. The LD-cytosol phase coexistence boundary for single-stage reveals complete LD demixing from cytosol above a saturated volume fraction (φ_{sat}) due to LD growth. In the gradient mode, LDs are small and dispersed in the cytosol. These differences in LD size and density are attributed to the cell's proteome and thermodynamic factors. For the first time, LLPS is observed to influence LD biogenesis in *Chlamydomonas reinhardtii*. Thus, this study unravels the metabolic regulation of mixotrophic biofuel production in *Chlamydomonas*, demonstrating gradient strategy as a promising approach for improving yields of various bioenergy products.

Keywords *Chlamydomonas reinhardtii* · Mixotrophy · Gradient strategy of cultivation · Biofuel potential · Autophagy · Liquid–liquid phase separation

Abbreviations

NaAc	Sodium acetate
SS	Single-stage
Chl	Chlorophyll
TAG	Triacylglycerol
LD	Lipid droplet
LLPS	Liquid–liquid phase separation

Introduction

Microalgae are the third-generation sources of biomass, biodiesel, bioethanol, biogas, and high-value products like nutraceuticals [1]. The biodiesel generated from microalgae is an environment-friendly alternative source of energy capable of meeting the growing demands of the world population [2]. Microalgae produce enhanced amounts of bioenergy products like carotenoids, starch, hydrogen, or lipids under abiotic stress conditions like nitrogen or sulfur deprivation, elevated temperature, changing light intensity, and light/dark cycle or high salinity [3]–[6]. However, microalgal growth gets severely compromised under stress, resulting in poor biomass production and low yields of biofuel, a potential bottleneck for industries. In comparison, employing the mixotrophic growth of microalgae has proved to be a more economically viable alternative because of the improved productivity of biomass, lipids, and carotenoids [7]. In mixotrophy, proper selection of the carbon source is

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Micro-Raman spectroscopy of the light-harvesting pigments in *Chlamydomonas reinhardtii* under salinity stress

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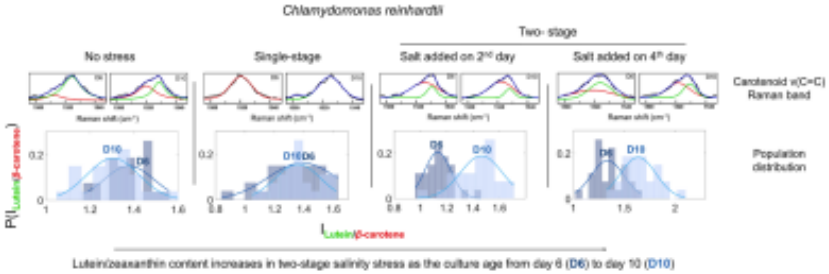
HIGHLIGHTS

- Micro-Raman spectroscopy captures varying compositional heterogeneity in carotenoids produced in *C. reinhardtii* as a function of culture age and salinity stress.
- Cellular variability in pigments, as probed by random sampling of a subpopulation in a culture, gets enhanced with salinity stress and age of culture.
- A decrease in relative Raman intensity of chlorophylls with respect to carotenoids correlates with a concomitant decrease in chlorophyll content in the cells observed with bright-field microscopy.
- Well-maintained chlorophyll-carotenoid cooperativity is observed from the correlations of Raman intensity of these pigments in presence of 100 mM NaCl. This correlation coefficient can be a good marker for detecting the maximum salt concentration allowing stable photosynthesis and hence good microalgal growth.

GRAPHICAL ABSTRACT



ARTICLE INFO

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Carotenoids
Chlorophyll

ABSTRACT

Microalgae are a rich source of carotenoids with enhanced yields during biotic or abiotic stresses, which often impose survival challenges on the cells. Using a non-invasive pigment profiling approach with micro-Raman spectroscopy, we have analyzed the effect of salinity stress on carotenoids in autotrophic *Chlamydomonas reinhardtii*. Raman spectral analysis of ν(C = C) mode indicates an increase in the carotenoids with lower conjugation length (lutein and zeaxanthin) compared to β-carotene, as the function of culture age and salinity stress, but especially when salinity stress was imposed in two-stage mode (stress imposed on 2nd day, D2_100, and 4th day, D4_100, during exponential phase). Population-scale heterogeneities in carotenoid Raman mode peak center,

Abbreviations: **RS**, Raman spectroscopy; **TAP**, Tris-acetate phosphate; **Car**, Carotenoids; **Chl**, Chlorophyll; **LHC**, Light-harvesting complex; **PS**, Photosystem; **ROS**, Reactive oxygen species; **HI**, Heterogeneity index.

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A5.2. List of presentations

1. Pandey, S., Kumar, P., Dasgupta, S., Gattupalli, A., and Bagchi, D. Gradient application of sodium acetate decides the bioenergy feedstock production in mixotrophically growing *Chlamydomonas reinhardtii*. Poster presented at International Conference on Biotechnology for Resource Efficiency, Energy, Environment, Chemicals and Health (BRE3CH), CSIR- Indian Institute of Petroleum, India (2021).
2. Pandey, S., Gattupalli, A., and Bagchi, D. Properties of photosynthetic pigments and lipids probed at single-cell level in *Chlamydomonas reinhardtii* exposed to saline stress using micro-Raman spectroscopy and fluorescence microscopy. Poster presented at Algal Biomass, Biofuels, and Bioproducts in collaboration with "Algal Research", Hawaii, USA (2021).
3. Pandey, S., Kumar, P., Patil, V., Mishra S., Gattupalli, A., and Bagchi, D. Inter-relationships among the cellular components of *Chlamydomonas reinhardtii* under salt stress. Presented at Conclave 2020, MSU Baroda, India (2020).
4. Pandey, S., Kumar, P., Patil, V., Mishra S., Gattupalli, A., and Bagchi, D. The relationship of triacylglycerol production with phenotypic characteristics in *Chlamydomonas reinhardtii* under salt stress. Poster presented at Prospects of microalgae: past, present and future, CSIR- CSMCRI, Bhavnagar, India (2019).

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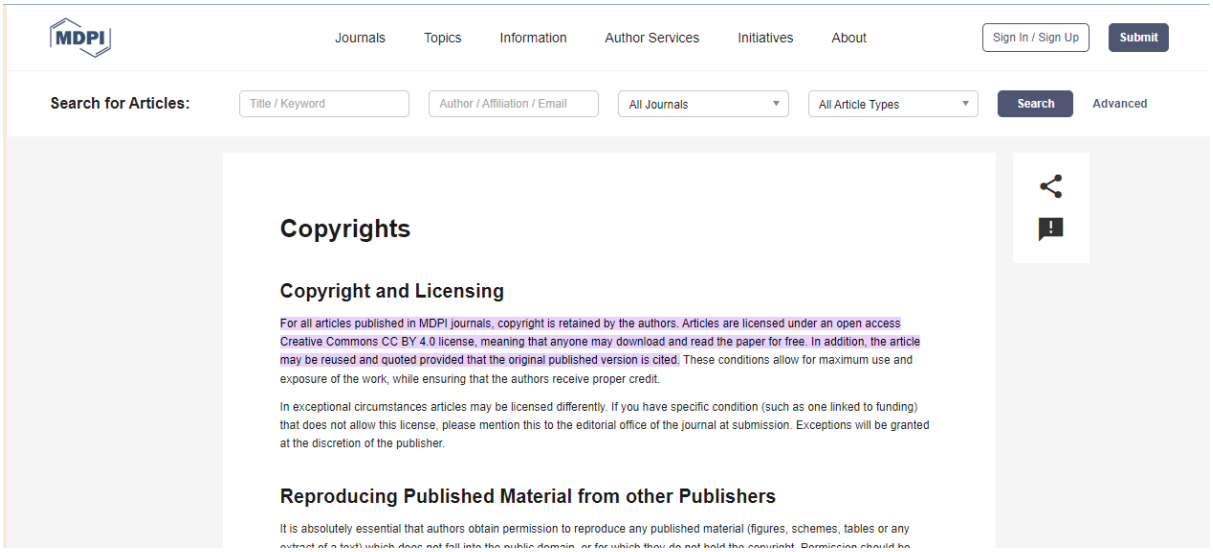

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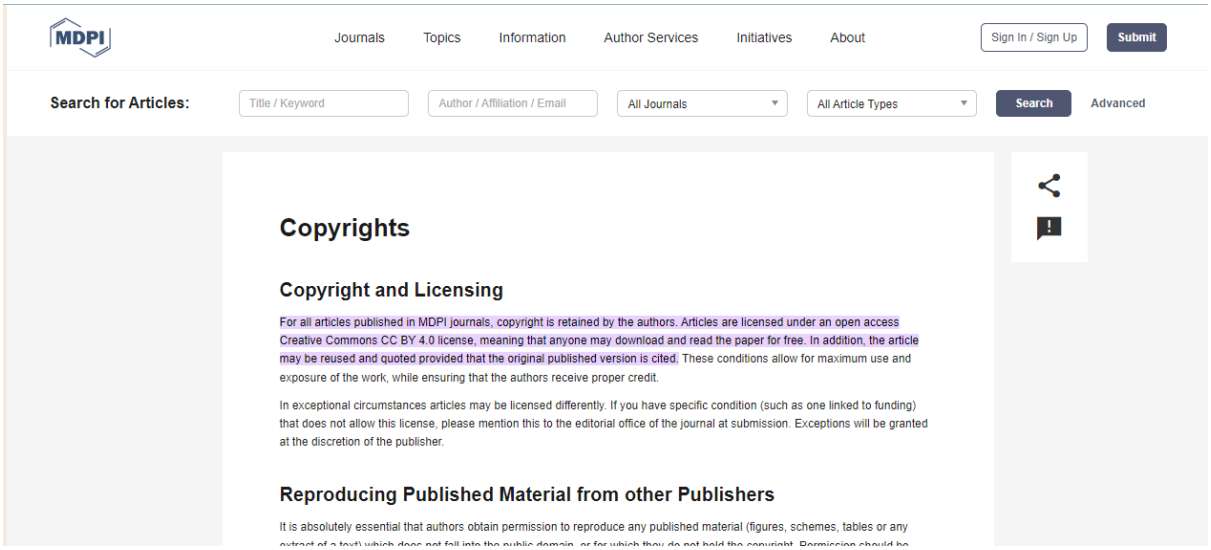

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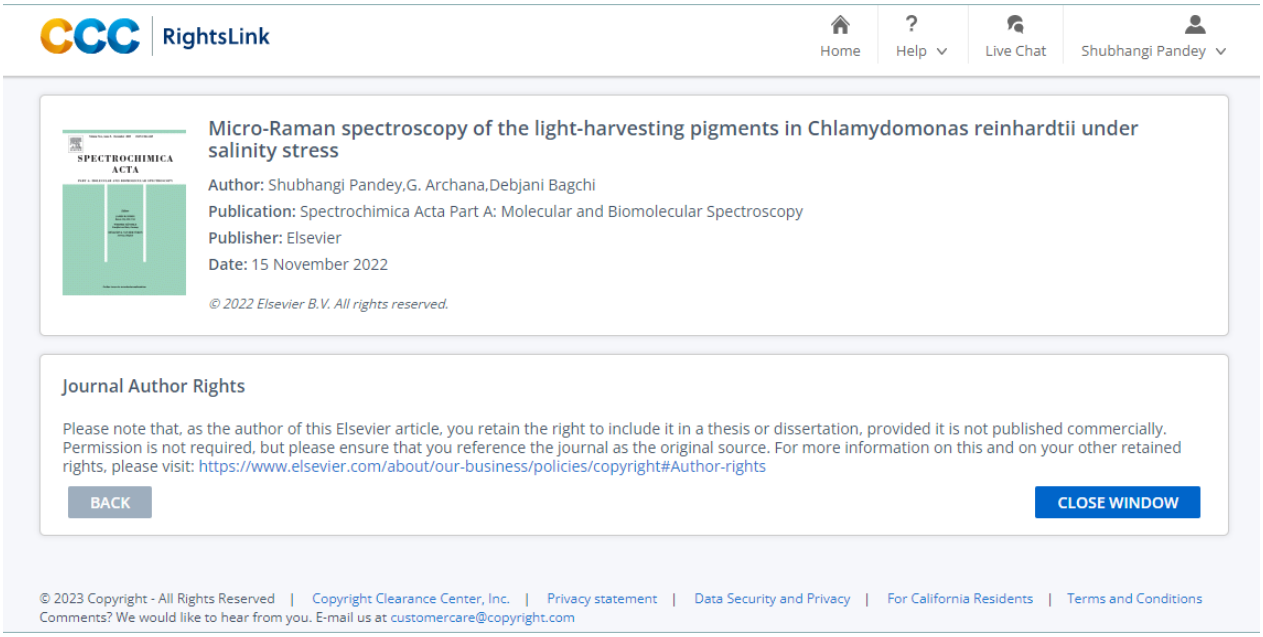

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Micro-Raman spectroscopy of the light-harvesting pigments in Chlamydomonas reinhardtii under salinity stress

Author: Shubhangi Pandey,G. Archana,Debjani Bagchi

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