



Original Research Paper

Systematic metabolome profiling and multi-omics analysis of the nitrogen-limited non-model oleaginous algae for biorefining

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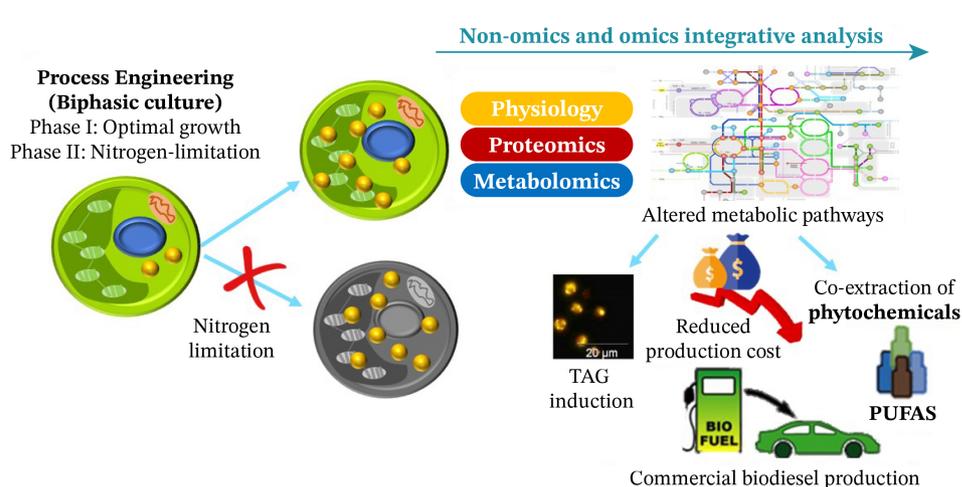
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HIGHLIGHTS

- 'Algomics' of non-model nitrogen-limited microalgae to realize metabolic tug-of-war for biomass and TAG assimilation.
- MS/MS annotation reveals accumulation of phytochemicals: TAGs, PUFAs, phytosterols, and terpenoids.
- 4× and 6× increase of two different PUFAs: 9(S)HpOTRE and dihomogamma-linolenic acid, respectively, during nitrogen-limitation.
- Severity modulation of nitrogen-limitation results in varied TAG species.
- Biorefining TAG and PUFA for economic feasibility in large-scale biodiesel production.

GRAPHICAL ABSTRACT



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ABSTRACT

Oleaginous microalga *Chlorella* is a promising microbial cell factory for producing lipids, transesterifiable to biodiesel, and phytochemical, high-value molecules (HVMs). To better understand the stress-induced oleaginous mechanism of *Chlorella* sp. FC2 IITG that closely matches with *Chlorella sorokiniana* based on 18s rRNA gene sequence, we performed 'Algomics' by systematically integrating metabolomics and proteomics data in response to time-resolved nitrogen-limitation: 40 h (mild), 88 h (moderate), and 120 h (severe). Ten HVMs belonging to four biological classes: triacylglycerol (TAG), polyunsaturated fatty acids (PUFA), phytosterols, and terpenoids were annotated using untargeted metabolomics and MS/MS fragmentation pattern match to the spectral library. In particular, the study evidenced 4× and 6× increased accumulation of two different PUFA: 9(S)HpOTrE and dihomo-gamma-linolenic acid, respectively, in nitrogen-limitation conditions. Co-extraction of TAG and PUFA could lower the biodiesel production cost for feasible commercialization. The investigation found a maximum accumulation of TAG 59:10 at 40 h, while that for TAG 54:4 was recorded at 88 h, which suggests different TAG species could be induced by regulating nitrogen-limitation severity. Elevated β-oxidation, glycolysis, and tricarboxylic acid (TCA) cycle identified in proteomics analysis could provide the substrates, phosphoenolpyruvate, pyruvate, and acetyl CoA, for different phytochemical accumulation in response to nitrogen limitation. The multiomics data unraveled a metabolic tug-of-war ongoing between biomass and storage lipid (TAG) accumulation during nitrogen-limitation, which involved multiple processes including hindered CO₂ fixation, the supply of energy, reductants, and carbon reallocation from proteins and membrane lipids. These findings provide distinct oleaginous mechanisms in non-model microalgae, *Chlorella* sp. FC2 IITG, and engineerable targets for microalgal trait improvements.

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Abbreviations

ACCase	Acetyl-CoA carboxylase	LC-MS	Liquid chromatography-mass spectrometry
AGPase	ADP-gluco-pyro-phosphorylase	PEP	Phosphoenolpyruvate
ALDO	Fructose-1, 6-bisphosphate aldolase	PGK	Phosphoglycerate kinase
CV	Coefficient of variance	PK	Pyruvate kinase
CID	Collision-induced dissociation	PLS-DA	Partial least squares-discriminant analysis
DCW	Dry cell weight	PRK	Phosphoribulokinase
DGLA	Dihomo-gamma-linolenic acid	PUFA	Polyunsaturated fatty acid
ENL	Enolase	Q-TOF	Quadrupole time-of-flight
ENR	Enoyl-ACP reductase	RPI	Ribose-5-phosphate isomerase
ESI	Electrospray ionization	rPPP	Reductive pentose phosphate pathway
FAS	Fatty acid synthase	RT	Retention time
FC2	<i>Chlorella</i> sp. FC2 IITG	SBP	Sedoheptulose-1,7-bisphosphatase
HAD	Hydroxy acyl-ACP dehydrogenase	TAG	Triacylglycerol
HPLC	Liquid performance liquid chromatography	TPI	Triosephosphate isomerase
HVMs	High-value molecules	TCA	Tricarboxylic acid

1. Introduction

The global energy demand increased by 2.3% in 2018 (highest since 2010) and is projected to grow by ~50% between 2018 and 2050 (US Energy Information Administration, 2018). Sustained fossil fuel combustion emits greenhouse gases that contribute significantly to global warming and climate-changes. The global temperature has already risen by 1 °C, and the global temperature is likely to break the 1.5 °C threshold as early as 2030 if greenhouse gas emissions continue at the current rate. To keep the rising temperatures in check, we need to shift the trajectory of greenhouse gas emissions by 2050 and switch to greener fuels like microalgae-based third-generation biofuels. Microalgae can be used to produce a variety of biofuels, including bioethanol and biodiesel (Colin et al., 2011). However, bioethanol is low in energy density and incompatible with the existing fuel infrastructure, hence encouraging a shift in research focus from algae-based bioethanol to biodiesel production. Biodiesel is an ideal candidate for various reasons: 20% biodiesel (B20) or lower is suited for current diesel engine models with reliability and durability, superior lubrication properties, non-toxic, biodegradable, and produces lower levels of most air pollutants than petroleum-based diesel fuel. Biodiesel consumption/demand increased exponentially (200x) from about 10 million gallons in 2001 to about 2 billion gallons in 2016 (US Energy Information Administration, 2019). The feedstocks currently used for biodiesel production are plant-based, mostly vegetable oils from soybean, corn, sunflower, palm, and rapeseed (US Energy Information Administration, 2018). However, these could jeopardize food security by triggering competition over water, land, and fertilizer consumption.

Microalgae photosynthesize sunlight and carbon dioxide to energy-rich biomolecules, triacylglycerol (TAG), transesterifiable into biodiesel. In addition to biodiesel production, microalgae sequester greenhouse gases (Ono and Cuello, 2007; Packer, 2009), pivotal in circumventing climate change, are capable of bioremediating wastewaters (Markou and Georgakakis, 2011; Rawat et al., 2011), and synthesizing high-value pharmaceuticals and nutraceuticals as co-products (Raja et al., 2008; Shah et al., 2020). Several microalgae genera such as *Phaeodactylum* (Molina Grima et al., 1999), *Nannochloropsis* (Sukenik, 1999), *Porphyridium* (Su et al., 2016), *Schizochytrium* (Morita et al., 2006), and *Chlorella* (Levasseur et al., 2020), are cultivated heterotrophically for the production of high-value products. Microalgae offer significant advantages over yeast and bacteria, as they are photosynthetic and do not require any addition of organic sources of carbon, which is an essential criterion for establishing long-term sustainability for any industrial processes (Kazamia and Smith, 2014).

High production cost is the major caveat in the commercialization of microalgae-based biodiesel technology. Strain selection and improvement, lipid extraction to its fullest (Halim et al., 2012), identification and co-extraction of valuable co-products (Song et al., 2015; Lin and Eudes, 2020), and synchronizing biomass and lipid accumulation (Rai et al., 2016) are the active areas of research. Biomass and lipid induction are inversely related and depend extensively on the growth conditions: optimal conditions support biomass while stress induces lipid accumulation. Stress conditions including light intensity (Mahong et al., 2012), temperature (Valledor et al., 2013; Abdelaziz et al., 2014), high CO₂ (Lv et al., 2010), salinity (Mastrobuoni et al., 2012), oxidative stress (Wang et al., 2004), UV-irradiation (Wijffels and Barbosa, 2010), and nutrient limitation especially nitrogen (Longworth et al., 2012; Guarnieri et al., 2013), are associated with this phenotype. Prior knowledge and understanding of algal stress physiology, particularly carbon allocation between different biosynthetic pathways and regulatory mechanisms controlling the distribution of desired metabolites, is imperative to cut down the biodiesel production cost.

Availability of whole-genome sequences of several algae and associated technological advancements in analytical sciences and data analysis has facilitated multidimensional 'Algomics' analysis to pursue the microalgal system biology. Numerous transcriptomics and proteomics studies have been performed to investigate the stress-associated lipid accumulation in *Chlamydomonas reinhardtii* (Longworth et al., 2012; Blaby et al., 2013), *Tisochrysis lutea* (Garnier et al., 2014), *Ostreococcus tauri* (Martin et al., 2012), *Haematococcus pluviialis* (Recht et al., 2012), *Nannochloropsis* sp. (Dong et al., 2013; Karthikaichamy et al., 2017), *Phaeodactylum tricornutum* (Valenzuela et al., 2012; Yang et al., 2014), and *Chlorella* sp. (Cao et al., 2014; Rai et al., 2016 and 2017). However, limited metabolomic investigations are

performed to understand the underlying molecular mechanisms involved in oil production from microalgae (Hirth et al., 2017; Shaikh et al., 2019).

Chlorella is one of the most widely studied microalgae and an industrial strain for biodiesel development (Ma et al., 2018). *Chlorella* is fast-growing, accumulates 50-70% lipids (TAG)/g dry cell weight (DCW), could be a source of functional foods, nutraceuticals, food supplements, and is genetically manipulatable. Thus, *chlorella* biomass could be co-extracted for TAG and high-value pharmaceuticals and nutraceuticals to lower the biodiesel production cost. Here we report on a comprehensive metabolomics analysis of the *Chlorella* sp. FC2 IITG to get a holistic overview of the biochemical process involved in nitrogen-limitation-induced TAG and phytochemical accumulation. Data from the present study highlights the crosstalk between metabolic changes and stress physiology. High-value nutraceutical by-products are identified that could support the cut-down of biodiesel prices.

2. Methods

2.1. Study design

The objective of this work is to define the metabolomic analysis of the microalgae, *Chlorella* sp. FC2 IITG (here onwards referred to as FC2) due to nitrogen-limitation. Unbiased metabolomics analysis was performed for the FC2 during three nitrogen-limitation timepoints (40, 88, and 120 h), representing different nitrogen-limitation severity (mild, moderate, and severe, respectively), based on the varied neutral lipid accumulation. Features are annotated by exact mass and MS/MS spectra corresponding to the spectral libraries publicly available, MoNA, METLIN, and GNPS. The data was also integrated with the proteomics findings reported previously by our group (Fig. 1). Microalgal culture conditions and physiological analysis are described previously (Rai et al., 2017).

2.2. Strain selection, stress-physiology, and proteomics analysis

The indigenous oleaginous green microalga FC2 was isolated by our research group from the North-Eastern part of India (Muthuraj et al., 2014). FC2 was cultured under a photoautotrophic condition in a 5.0 L automated bioreactor (Biostat B plus, Sartorius, Gettingen, Germany) containing 4.0 L of slightly modified BG11 medium following the previously described growth conditions (Rai et al., 2017). To classify the microalgae strain, 18S rRNA gene sequencing was performed. For optimal growth, we maintained nitrogen levels of ~1.6 g/L, whereas nitrogen was removed entirely from media for nitrogen-limitation conditions. Synchronization of the yield and TAG accumulation in nitrogen-limited non-model FC2 was achieved by process engineering the FC2 cultivation through growing it in biphasic photoautotrophic conditions (first phase: optimal growth and second phase: nitrogen-limitation induced lipid accumulation). Neutral lipid accumulation was confirmed by Nile red staining following the previously described protocol (Rai et al., 2017). Furthermore, we performed unbiased global quantitative protein profiling to unravel the underlying post-transcriptional mechanisms associated with the phenotype (Rai et al., 2017).

2.3. Metabolite extraction

Metabolites were extracted from four biological replicates of FC2 cells from each group: control (0 h), different nitrogen-limitation conditions: mild (40 h), moderate (88 h), and severe (120 h) (Fig. 1). Four hundred µL of chilled methanol: chloroform: water (4:3:1, Optima LC-MS grade; Fisher Scientific) with 2 µM caffeine (Analytical Standard, Sigma-Aldrich) was added to 100 mg ground FC2 cells, vortexed for 30 s, and incubated at -80°C for 8 h. The samples were centrifuged at 14,000 × g for 15 min at 4°C to pellet down the precipitated proteins. Polar metabolites were separated in the methanol and water phase, while non-polar metabolites were extracted in chloroform, non-polar solvent (Bligh and Dyer, 1959). Both polar and non-polar fractions were pooled together and evaporated to dryness in a vacuum concentrator. The dried extracts were then reconstituted in 50% acetonitrile and 0.1% formic acid (100 µL), filtered through a 0.2-micron syringe filter, and stored at -80°C before liquid chromatography-mass spectrometry (LC-MS) analysis.

2.4. Metabolomics analysis and profiling

A high-resolution LC-MS based comprehensive untargeted metabolomics profiling of FC2 extracts for four biological replicates from each group: control (0 h), different nitrogen-limitation conditions: mild (40 h), moderate (88 h), and severe (120 h) was performed (Fig. 1).

and mobile phase B is 0.1% formic acid in 90% acetonitrile (LC-MS grade solvents, Fisher Scientific). Initial conditions were restored in 2 min ahead of 5 min of column re-equilibration. The HPLC system was coupled with an Agilent 6550 ifunnel Q-TOF LC-MS/MS system controlled by Agilent MassHunter™ software packages and equipped with an ESI source. MS1 spectra were acquired in positive electrospray ionization (+ESI) mode with

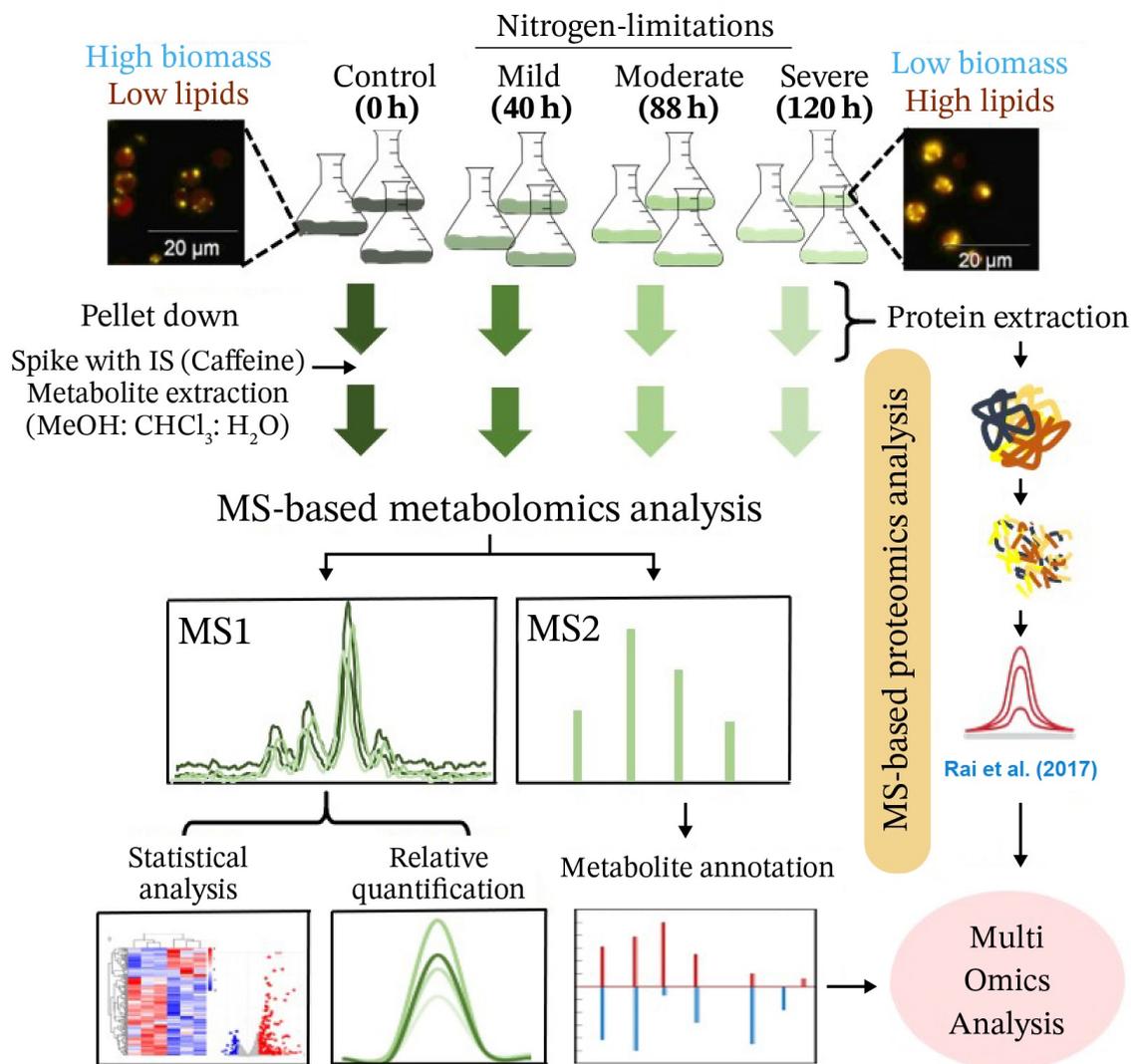


Fig. 1. Multi-omics workflow for metabolomics analysis and proteomics data integration of microalgae *Chlorella* sp. FC2 IITG. FC2 was grown biphasically (phase I: optimal condition, phase II: nitrogen-limited), and three different nitrogen-limitation severity conditions: mild (40 h), moderate (88 h), and severe (120 h) was selected for metabolomics analysis. Features were prioritized based on univariate and multivariate analysis and annotated using MS/MS fragmentation patterns. Proteomics data from the same samples published previously (Rai et al., 2017) was integrated for multi-omics analysis to better understand metabolic tug-of-war between biomass and TAG assimilation during nitrogen-limitation.

2.4.1. Instrumentation and data acquisitions

MS and MS/MS analysis of the metabolites were performed as per our laboratory protocol (Patel et al., 2019). FC2 metabolite extracts (6 μL) were chromatographically separated with an Agilent 1260 Infinity HPLC system with a Zorbax SB-AQ C18 column (100x2.1 mm) packed with 1.8 μm particles (Agilent Technologies, Santa Clara, CA) using a gradient of 5% mobile phase B increasing linearly to 80% B in 25 min at a flow rate of 0.3 mL/min, and temperature at 40°C. The mobile phase A comprises 0.1% formic acid in water,

a mass range of 50-1000 m/z. The instrument was externally calibrated, and mass spectra were recorded at 20K resolution with ESI-L Low Concentration Tuning Mix (Agilent Technologies) daily. MS/MS analyses were conducted using nitrogen as collision gas and collision-induced dissociations (CID). Ten most abundant ions per MS1 scan were fragmented for data-dependent acquisition, and the spectra were collected. The samples were randomized to eliminate any biases, and a pooled quality control sample (0, 40, 88, and 120 h) was run after every four samples to determine run-to-run variation.

2.4.2. Quality assessment of the data collection

Agilent raw data files were converted to “.mzXML” files using MSconvert software (Holman et al., 2014). Raw data files were manually inspected in Agilent MassHunter™ software. The coefficient of variance (CV) for resuspension standard caffeine ($[M+H]^+$, m/z 195.0882) was determined.

2.4.3. Feature finding

MS feature detection was performed using MZmine 2.53 in batch mode following the reported method (Gauglitz et al., 2020). The mzXML files were imported and cropped based on retention time (0–23 min). The mass detection noise level for MS was set at 4.0E3 and 5.0E2 for MS/MS. Chromatograms were built with ADAP chromatogram builder, a min-height of 3.0E3, a min gr size in # of scans of 3, a gr intensity threshold of 9.0E3, an m/z tolerance of 20.0 ppm. Chromatograms were deconvoluted using minimum local search (chromatographic limit: 60%; search minimum in RT range (min): 0.05; minimum relative height: 15%; min absolute height: 3.0E3, min ratio of peak top/edge: 1.5 and peak duration range (min) of 0.00 - 2.00). Isotopic peaks were grouped with an m/z tolerance of 20.0 ppm, a retention time tolerance of 0.1, and a maximum charge of 3. Features were aligned with m/z tolerance of 20.0 ppm, weight or m/z 75%, and weight for RT 25%, with a retention time tolerance of 0.1 min. Gap filling was done with an intensity tolerance of 10.0% and a retention time tolerance of 0.15 min. Feature tables were filtered to include only features that contain a minimum of 8 peaks in a row. Duplicate peaks were filtered, and the feature table was exported.

2.5. Data filtering, analysis, and visualization

The data filtering was performed using MetaboAnalyst v4.0 (<https://www.metaboanalyst.ca/>). The data were filtered to create feature lists based on the number of entities detected in at least two conditions for the control (0 h) and nitrogen-limitation conditions (40 h, 88 h, and 120 h). For a given condition of nitrogen-limitation, filtered entity lists were created based on the number of entities that passed 80% sample replicate thresholds. Four biological replicates were analyzed as one group after the ion intensity for each molecular ion was averaged across the replicates. Partial least squares-discriminant (PLS-DA) analysis and volcano plots were generated to visually demonstrate the variance of the metabolic phenotypes within the group on the sample files based on the variable: nitrogen-limitation. One-way ANOVA ($p < 0.05$) analysis resulted in lists of matrix table showing the comparison results for each pair of conditions as a p-value. Fold change ≥ 1.5 , and p-value ≤ 0.05 was the criterion to select differentially expressed metabolites. Missing values were excluded from the calculations, and the results were compared. Features were annotated based on MS/MS spectra. All annotations are level 2 or 3, according to the 2007 metabolomics standards initiative (Sumner et al., 2007). A heat map (K-mean clustering) was generated for the metabolites.

2.6. Statistical analyses

One-way ANOVA was used to determine the statistical significance of the results. p-value ≤ 0.05 was considered statistically significant.

2.7. Data and software availability

LC-MS/MS data from this paper have been deposited to massIVE with the dataset identifier MSV000085689 (<ftp://massive.ucsd.edu/MSV000085689/>).

3. Results and Discussion

3.1 Nitrogen-limitation induces TAG accumulation in FC2 cells

Unicellular microalgae are at the forefront of research efforts to develop technologies and model systems for renewable biodiesel production. Abiotic stresses strongly induce lipid accumulation in the form of TAG, convertible to biodiesel. A comprehensive understanding of the molecular mechanisms underlying the stress physiology to achieve the high yield and simultaneous accumulation of phytochemicals, including TAGs and polyunsaturated fatty acids (PUFA) is inevitable to strengthen the scope for microalgal biodiesel commercialization. Our group previously isolated the indigenous oleaginous

green microalga FC2 from the North-Eastern part of India (Muthuraj et al., 2014). To classify the microalgae, 18S rRNA gene of FC2 (Accession no.: JX154075.1) was sequenced, and as per the latest update in the 18S rRNA gene database, the strain could potentially be *Chlorella sorokiniana*. A phylogenetic tree was constructed with closely related species (*C. vulgaris*, *C. pyrenoidosa*, *C. sorokiniana*, *C. singularis*, etc.) and found that the strain FC2 falls close to *C. sorokiniana* as depicted in the phylogenetic tree (Fig. S1). The novel microalgal strain "*Chlorella* sp. FC2 IITG" has been deposited to the National Collection of Industrial Microorganisms (NCIM) culture collection, National Chemical Laboratories (NCL), Pune, India, with a safe deposit number NCIM-5666.

Process engineering strategies employed in our previous study resulted in a maximum biomass titer of 17.7 g/L and total lipid accumulation of 313 mg/L.d, the highest reported to date among *Chlorella* sp. (Muthuraj et al., 2015). The lipid accumulation is higher than that reported for *C. vulgaris* grown in continuous nitrogen-limitation (Liu et al., 2016). Further, a 50.34% (w/w, DCW) higher neutral lipid storage in severe nitrogen-limited FC2 (120 h), as compared to 1% in optimal growth conditions, was reported (Fig. S2) (Rai et al., 2017). Also, nitrogen-limitation leads to time-dependent down-regulation of protein, carbohydrate, and chlorophyll contents in FC2 (Fig. S2). The time points, i.e., three different nitrogen-limitation severity conditions: 40 h (mild), 88 h (moderate), and 120 h (severe), which represent the critical stages for neutral lipid accumulation, were selected for downstream 'Algomics' analysis. The major findings from the proteomics analysis are published previously (Rai et al., 2017). The present study highlights the metabolomics analysis and multi-omics data integration.

3.2. Metabolome profile of nitrogen-limited FC2 cells

Microalgal FC2 extracts were analyzed by a high-resolution Q-TOF mass spectrometer. The base peak MS chromatogram of all the conditions, control (0 h), and nitrogen-limitations (mild, moderate, and severe) were highly reproducible (Fig. 2a). A similar trend was noted with merely 14.7% coefficient of variance of caffeine (m/z 194.08), spiked as internal standard, for all biological replicates (n=4) and experimental FC2 samples (control and nitrogen-limited; depicted in Figure 2a), which further confirms the reproducibility in sample processing and the LC-MS runs. PLS-DA clustered all the treatments and control conditions into four groups (Fig. 2b), based on significantly altered features (fold changes ≥ 1.5 , and p-value ≤ 0.05). Nitrogen-limitation induced modulations of 430 significant features, of which 84 showed overlap in mild, moderate, and severe conditions. In contrast, 5, 52, and 113 features are expressed exclusively in mild, moderate, and severe nitrogen-limitation, respectively. Still, other features assist in the algal adaptation to more stringent nitrogen-limitation conditions (Fig. 2c). Similar patterns were found in the proteomics and metabolomics analysis, where the maximum number of proteins (57) (Rai et al., 2017) and features (132) were regulated during the transition from moderate to severe nitrogen-limitation (Fig. 2c), suggesting drastic metabolic re-organization to combat extreme nitrogen-limitation stress and enhance the chance of survival. Volcano plots showing p-values vs. log₂ fold change of 40/0, 88/0, and 120/0 h are depicted in Figure 3a (list of significantly altered features are shown in Table S1). The data also shows a similar trend, i.e., a higher number of features are dysregulated in severe (339) nitrogen-limitations (Fig. 3a iii) followed by moderate (302) (Fig. 3a ii) and mild (133) (Fig. 3a i) stress conditions (Table S1).

3.3. High-value molecules amass in nitrogen-limited FC2 cells

Microalgae are promising third-generation biofuel candidates and future bio-based food/feedstocks with a unique lipid composition, high protein content, and a source of unlimited bioactive phytochemicals, referred to as "High-value molecules (HVMs)". Although produced in fewer amounts, these products have much higher economic value than whole, dried microalgal biomass. HVMs range from PUFA, pigments, proteins to carbohydrates, with diverse applications in food, feed, chemical, cosmetic, energy, and health industries (Eppink et al., 2019; Bhowmick et al., 2020). The content and compositions of HVMs are rooted in their genetic, biochemical, and physiological diversity, which provides

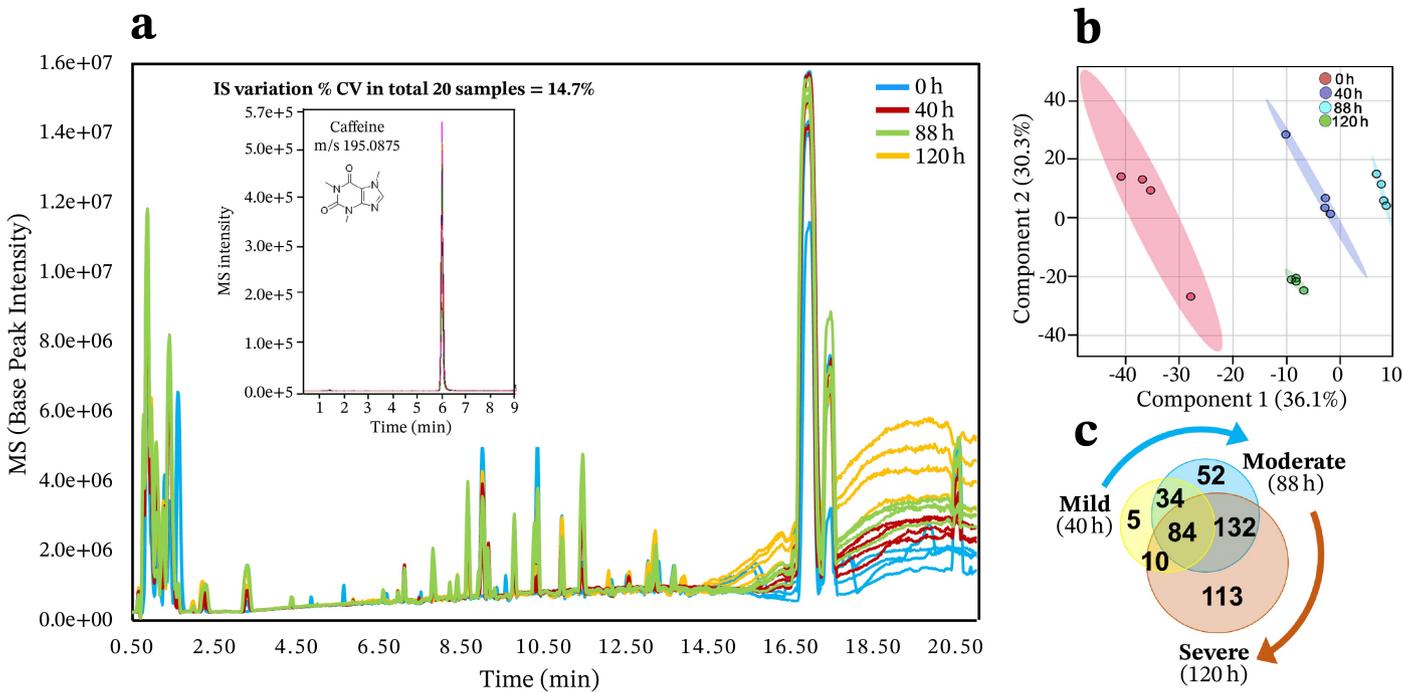


Fig. 2. Metabolomics analysis of microalga *Chlorella* sp. FC2 IITG, exposed to increasing nitrogen-limitation severity (mild, moderate, and severe). **(a)** Positive mode full scan base peak MS chromatogram of the biphasic metabolite extract (chloroform, methanol, and water) of the FC2. The data was acquired from Q-TOF LC-MS/MS in +ESI mode. The subset shows the caffeine (m/z 194.0803) spiked as internal standard, the co-efficient of variance for the 16 samples was 14.7%. **(b)** 2D partial least square-discriminant analysis (PLS-DA) differentiates the metabolomes of nitrogen replete and deplete FC2 into four groups. Component 1 (31.6%) and Component 2 (30.3%) are plotted. MS1 features are TIC normalized per sample, and the PSLDA analysis was performed using MetaboAnalyst. **(c)** Venn diagram showing the unique and overlapping differentially abundant features (one-way ANOVA, FDR ≤ 0.05) in different nitrogen limitation severity. Consistently, more mass features are significantly different due to nitrogen limitation severity. Number of mass features that differ in mild (yellow), moderate (blue), or severe (orange) nitrogen limitation is shown in the colored circles, with overlaps.

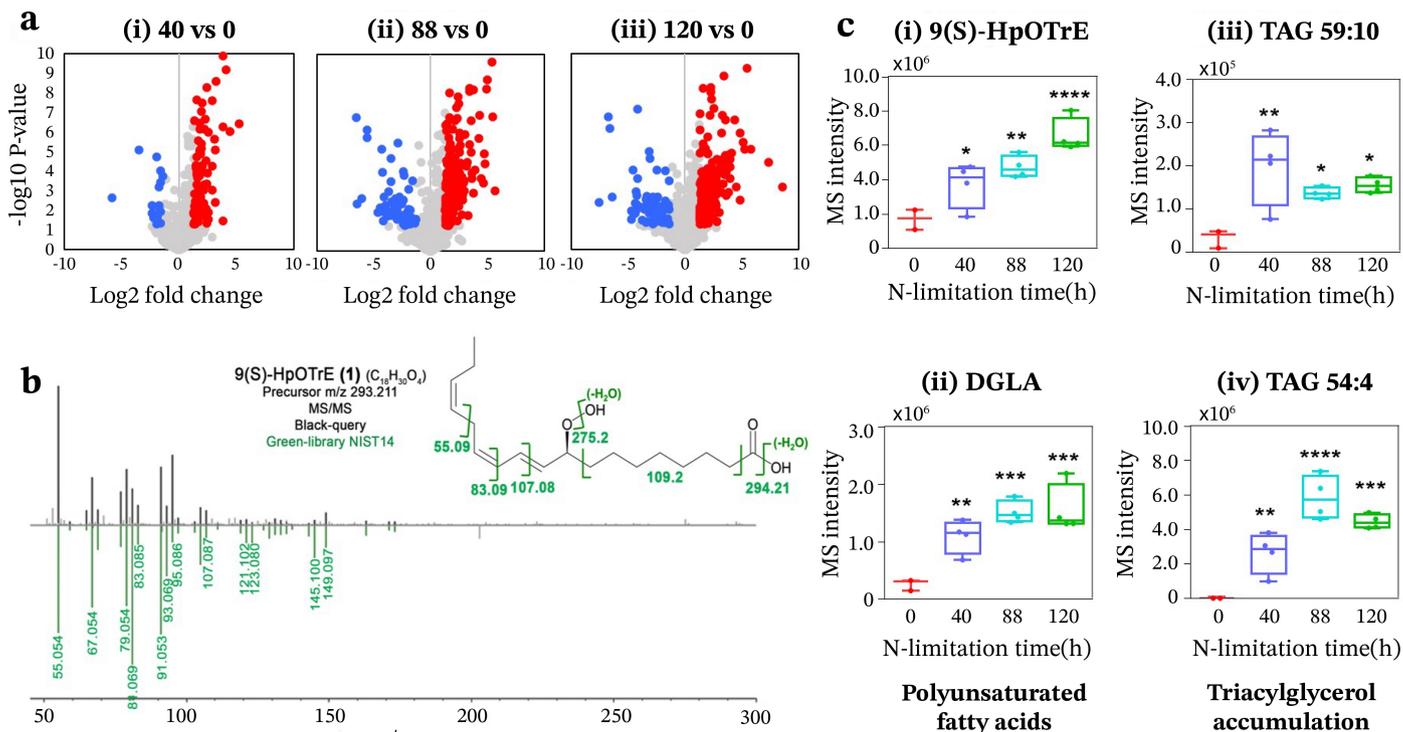


Fig. 3. Dysregulation of features/metabolites in nitrogen limited FC2. **(a)** Volcano plots showing P values ($-\log_{10}$) vs. feature ratio of (\log_2) (i) 20 vs. 0, (ii) 88 vs. 0, and (iii) 120 vs. 0. Blue, down-regulated; red, up-regulated; and grey, no significant change ($p\text{-value} > 0.05$). **(b)** Mirror plot showing MS/MS spectra match, subset shows chemical structure, and structural fragments of 9(S)-HpOTrE (m/z 293.211). Black, identified in our dataset; green, NIST14 library match. **(c)** Box plot showing the individual differential expression of (i) 9(S)HPOT, (ii) DGLA, (iii) TAG 59:10, and (iv) TAG 54:4. Results are shown as biological replicates and mean \pm SD. One-way ANOVA showed significant differences between groups. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

opportunities to discover novel small molecules for industrial and health applications (Kim and Chojnacka, 2015). More than 4000 studies on microalgal phytochemicals were published between 1926 and 2016 (Michalak et al., 2017). Co-extraction of HVMs and TAG has immense potential to lower the microalgal biodiesel production costs, a major snag in the commercialization of this biofuel (Khoo et al., 2019). The first step towards isolating these bioactive molecules in a cost- and energy-efficient manner is their identification and quantitation. In the present study, we annotated ten significantly altered metabolites in the nitrogen-limited FC2 cells (detailed in Table 1) by mining online available MS/MS spectral libraries. The MS/MS spectral match of one such metabolite is shown in Figure 3b, and the subset shows the corresponding structural fragments. These ten metabolites are grouped into four biological classes: PUFAs, TAG, phytosterols, and terpenoids. We annotated two TAG species, namely, TAG 59:10 and TAG 54:4 (Table 1). Interestingly, a 200-fold higher storage of TAG 54:4 was noted (Fig. 3c iv) in moderate nitrogen-limitations (88 h) compared to the optimal growth. Simultaneously, severe conditions led to the reduction in TAG 54:4, suggesting, scope to optimize nitrogen-limitation duration to sync FC2 yield and TAG induction. Also, we found a substantial accumulation of Dihydrotestosterone in moderate (88 h) and severe (120 h) nitrogen-limited FC2 cells (Table 1 and Fig. 4). Salinity stress-induced accumulation of phytosterols, ergosterol, and 7-dehydroperiferasterol, was reported in two different species of *Dunaliella* (Francavilla et al., 2010). Further, we found that nitrogen-limitation led to the accumulation of several diterpenoid and diterpene glycosides metabolites (listed in Table 1 and Fig. 4). However, terpenoids' extraction is technically challenging, with low or moderate yields (Moser and Pichler, 2019).

3.4. Biorefining of TAGs and PUFAs could lower biodiesel production cost

Microalgae are recognized as a rich source of PUFAs. Several PUFAs have penetrated the markets, while other classes of molecules, including polyphenols, phytosterols, terpenoids, and hormones, are still under active research. Fatty acids from microalgae have higher commercial importance than fish oils due to lower contaminations of methyl mercury, dioxins, and polychlorinated biphenyls (Ryckebosch et al., 2014). PUFAs from microalgae have an estimated market value of 140 USD/kg (Borowitzka, 2013).

We found 4x and 6x higher accumulation of two different PUFA: 9(S)HpOTrE (Fig. 3c i) and dihomo-gamma-linolenic acid (Fig. 3c ii), respectively, in severe nitrogen-limitation conditions (120 h). 9(S)HpOTrE is a downstream product of alpha-linolenic acid (ALA), omega-3 fatty acid. The global omega-3 market size was valued at USD 2.49 billion in 2019 and is expected to expand at a compound annual growth rate of 7.7% over the forecast period (Grand View Research, 2020). Likewise, dihomo-gamma-linolenic acid (DGLA) is a precursor for several physiologically essential acids: arachidonic (ARA; C20:4n-6), eicosapentaenoic acid (C20:5n-3), and docosahexaenoic (C22:6n-3). The fact that FC2 accumulates PUFAs during nitrogen-limitation could be exploited to lower the biodiesel production cost by co-extracting with TAG. Industrially-compatible method for co-extracting phytochemicals was attempted (Delbrut et al., 2018; Santoro et al., 2019). Still, extensive research is needed to optimize the PUFA accumulation in stress-condition and extraction of phytochemicals to its fullest.

Table 1.

List of differentially abundant metabolites identified in nitrogen-limited *Chlorella* sp. FC2 IITG.

No.	Biological class	Compounds	Monoisotopic mass	m/z	Adduct	RT (min)	Nitrogen-limitation timepoints			Chemical formula
							40 h	88 h	120 h	
1	Polyunsaturated fatty acids (PUFA)	9(S)HpOTrE	310.2144	293.2091	M+H-H ₂ O	12.15	2.2	2.8	3.9	C18H30O4
2		Dihomo-gamma-linolenic acid (DGLA)	306.2559	307.1493	M+H	9.96	4.2	5.8	6.0	C20H34O2
3	Triacylglycerides	TAG 59:10	940.6581	963.4636	M+Na	21.14	6.05	4.20	4.8	C63H88O6
4		TAG 54:4	882.7676	905.4246	M+Na	15.61	100.9	224.3	170.7	C57H102O6
5	Steroids	Dihydrotestosterone	290.2246	291.1934	M+H	11.63	2.8	4.3	4.6	C19H30O2
6	Diterpenoid	14-Deoxyandrographolide	334.2144	335.2159	M+H	13.03	3.65	4.64	6.5	C20H30O4
7		13-Labdene-2,8,15-triol	324.2664	347.1791	M+Na	11.54	4.2	6.3	9.2	C20H36O3
8		Annosquamosin C	292.2402	293.2417	M+H	19.43	7.5	2.8	7.9	C19H32O2
9	Diterpene glycosides	(2R,3R,4S,5R,6R)-2-[[7-[(2R,3R,4R,5S)-3,4-Dihydroxy-5-(hydroxymethyl)oxolan-2-yl]oxy-2-ethenyl-2,4b,8,8-tetramethyl-4,4a,5,6,7,8a,9,10-octahydro-3H-phenanthren-3-yl]oxy]-6-methyloxane-3,4,5-triol	582.3404	605.3279	M+Na	11.45	5.84	9.51	10.91	C31H50O10
10		c1-15(9-11-38-23(34)13-22(32)33)6-7-17-16(2)18(12-20-28(3,4)21(31)8-10-29(17,20)5)39-27-26(37)25(36)24(35)19(14-30)40-27/h9,17-21,24-27,30-31,35-37H,2,6-8,10-14H2,1,3-5H3,(H,32,33)/b15-9-	570.3040	571.3110	M+H	10.15	2.76	3.82	7.26	C29H46O11

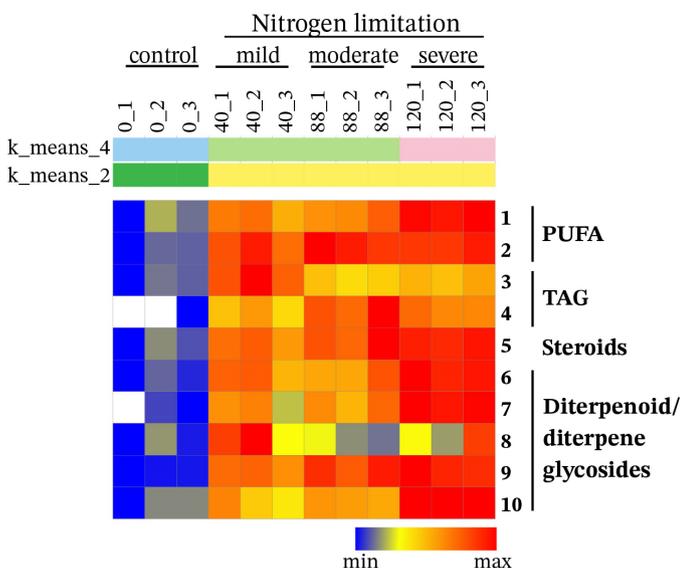


Fig. 4. Heat map illustrating the post-transcriptional dynamics of individual metabolites in response to nitrogen limitation (see details in Table 1). Blue, down-regulated; and red, up-regulated.

3.5. Nitrogen-limitation induces TAG synthesis in non-model microalgae FC2

TAG is storage neutral lipids that have essential roles in energy homeostasis and carbon storage. TAG is considered a viable, sustainable source of biodiesel with commercial applications. Various abiotic stresses, including nitrogen-limitation, lead to TAG biosynthesis, which is a complex process. Systems biology studies revealed the readjustment of metabolism with a shift of carbon flux to TAG.

3.5.1. Impaired carbon dioxide fixation

Our physiological and proteomic analysis indicates that nitrogen-limitation reduces photosynthesis in FC2 cells. Considerable decline in the chlorophyll content and chlorophyll biosynthetic protein abundance, including porphobilinogen deaminase, was noted (Rai et al., 2017). Also, we found reduced regeneration of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and significant downregulation of two of the rate-limiting enzymes phosphoribulokinase (PRK) and sedoheptulose-1,7-bisphosphatase (SBP), suggesting that carbon dioxide fixation is severely impaired in nitrogen-limited FC2 (Rai et al., 2017), which correlates well with the reduced overall yield. The nitrogen-limitation-induced decline in chlorophyll content is linked to rapid cessation in its synthesis and dilution by cellular growth rather than its degradation, as reported in *C. reinhardtii* (Juergens et al., 2015). We found upregulation in several enzymes, including peptidase, proteasomes, and ubiquitins, supporting the net degradation of protein and, therefore, the considerably decreased protein level (Rai et al., 2017). This would imply that the enormous carbon demand for TAG synthesis in nitrogen-limited FC2 cells is met by catabolizing and reallocating carbon of proteins and membrane lipids. We also found overexpression of several of β -oxidation pathways enzymes that promote membrane lipids breakdown to provide both carbon and energy (Rai et al., 2017). The distribution of macromolecules such as carbohydrates, proteins, chlorophyll, and neutral lipids or TAG under different nitrogen-limitation ages (0, 40, 88, and 120 h) is depicted in Figure S2. This shows a drastic increase in the neutral lipid content with a concomitant reduction in the protein, carbohydrate, and chlorophyll levels within the biomass, thus explaining the rearrangement of macromolecular compositions to form TAG. Besides, activity screening of enzymes such as ADP-glucopyrophosphorylase (AGPase), acetyl-CoA carboxylase (ACCase), glycerol-3-phosphate dehydrogenase, and diacyl-glycerol transferase showed varied activity under nutrient-limitation and replete conditions in FC2 (Muthuraj et al., 2019). For instance, the activity of AGPase was reduced with a concomitant increase in

ACCase, suggesting the reduction of fluxes towards carbohydrate biosynthesis and associated redirection of carbon towards TAG biosynthesis under nitrogen limitation conditions (Muthuraj et al., 2019). Apart from their energy production role, these proteins, amino acids, and membrane lipids play an essential role as intermediates in replenishing the tricarboxylic acid (TCA) cycle for carbon recycling, and energy and reductant generation to meet the enhanced synthesis of fatty acids/TAG. Fatty acid biosynthesis is an expensive process; for instance, the biosynthesis of one unit of C16:0 requires eight molecules of NADPH and seven ATP molecules. Thus, nitrogen-limited FC2 must have robust biosynthesis pathways to fulfill the energy and reductant demand for oleagenicity. Simultaneously, the salvaged nitrogen is likely to be repartitioned to those most needed functions for maintaining cell viability against nitrogen-limitation and producing storage lipids (Park et al., 2015; Chen et al., 2017; Rai et al., 2017).

3.5.2. Activation of glycolysis, TCA cycle, and reductive pentose phosphate pathway (rPPP)

All organisms are capable of synthesizing lipids, but only the oleaginous species accumulate significant quantities (>30% DCW), primarily due to the production of high levels of acetyl-CoA, the basic building block of fatty acids (Ratledge, 1994). Acetyl CoA is produced by oxidative decarboxylation of pyruvate, the end-product of glycolysis. We hypothesize that the pyruvate production is favored in nitrogen-limited FC2 by the significant up-regulation of several glycolysis pathway enzymes, namely, phosphoglycerate kinase (PGK), pyruvate kinase (PK), enolase (ENL), and triosephosphate isomerase (TPI) (Rai et al., 2017). Acetyl CoA also connects glycolysis and the citric acid cycle to yield citrate. It is possible that citrate is accumulated into the cytoplasm but could not be identified in our metabolomics study as we used a reverse-phase column for analysis. While the upregulation of isocitrate lyase in our proteomics study suggests the conversion of citrate to acetyl-CoA, thus providing substrates for *de novo* lipid biosynthesis (Fakas et al., 2009). Dynamic metabolic flux balance analysis conducted during the growth of the FC2 strain under both replete and nutrient limitation conditions also depicted an increase in the fluxes towards acetyl-CoA biosynthesis, which are further utilized by the organism for neutral lipid biosynthesis (Muthuraj et al., 2013). This redirection of fluxes to acetyl-CoA formation is characterized by increased fluxes in the glycolytic pathway during nutrient-limited photoautotrophic growth. The study showed a 57% increase in the flux towards acetyl-CoA biosynthesis from pyruvate and 63% reduction in flux towards citrate formation from acetyl-CoA under nutrient limitation conditions as compared to replete conditions (Muthuraj et al., 2013). Further enhanced phosphoenolpyruvate (PEP), pyruvate, and acetyl CoA led to the production of HVM (Fig. 5). Overall, nitrogen-limited FC2 cells maintained a flux to Pyruvate \rightarrow Acetyl CoA \rightarrow fatty acids *via* enhancing glycolysis, TCA cycle, and reductive pentose phosphate pathway (rPPP).

3.5.3. Fatty acid synthesis and TAG assembly

We found that nitrogen-limitation enhances the expressions of several enzymes of fatty acid synthase (FAS) like acyl carrier proteins, hydroxy acyl-ACP dehydrogenase (HAD), and enoyl-ACP reductase (ENR) in FC2 (Rai et al., 2017). The up-regulation of these enzymes in a harmonized manner strongly stimulates the integration of acetyl-CoA into *de novo* synthesized fatty acids. Over 100 genes are identified to be involved in lipid metabolism in microalgae *C. reinhardtii* (Li-Beisson et al., 2015) and diatom *P. tricornutum* (Muhlroth et al., 2013) based on sequence analysis. Several genes involved in lipid metabolism are engineered to improve lipid accumulation in algae and higher plants; however, limited success is achieved to increase TAG accumulation (Iskandarov et al., 2016; Ajjawi et al., 2017), possibly due to the multifunctional roles of several of the enzymes. Surprisingly, overexpression of acetyl-CoA carboxylase (ACCase) in rapeseed has no significant change in seed lipid content, but a 5-fold higher lipid content in potatoes was reported (Klaus et al., 2004). Also, overexpression of 3-ketoacyl-acyl-carrier protein synthase III (KASIII) reduces the rate of lipid synthesis in Arabidopsis and rapeseed (Dehesh et al., 2001), which is consistent with our study, as no significant changes in KAS were seen in nitrogen-limited FC2. Also, using FAME

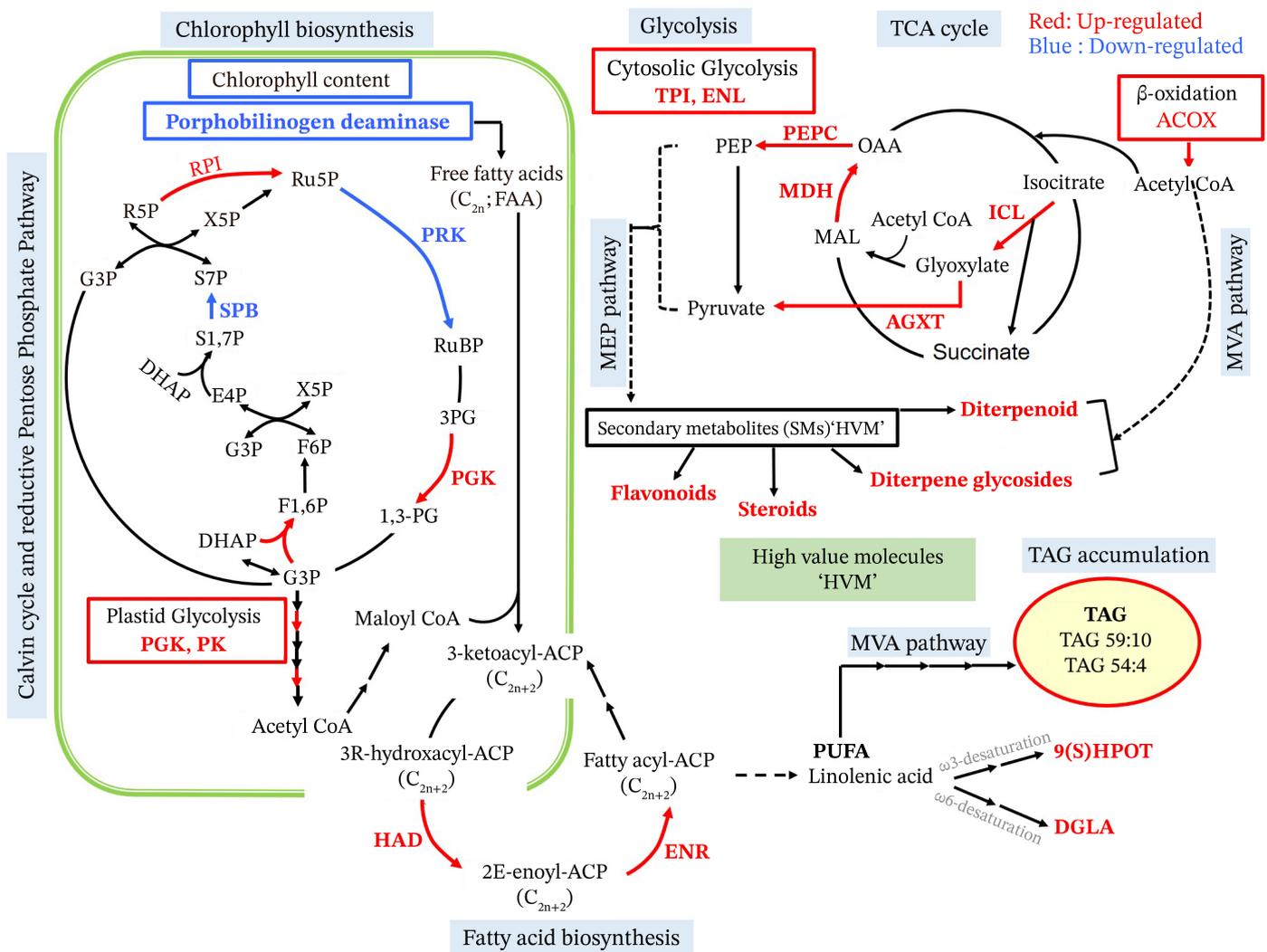


Fig. 5. Multi-omics analysis reveals the regulation of central carbon and nitrogen metabolic pathways related to TAG biosynthesis in nitrogen-limited FC2. The regulatory proteins are indicated by blue (down-regulated) and red (up-regulated) arrows, respectively. The metabolites accumulated during nitrogen limitation is shown in red.

profiling, we found a higher accumulation of C16:0 (palmitic acid), C18:1 (oleic acid), and C18:2 (linoleic acid) in nitrogen-limited FC2 (Muthuraj et al., 2014). C18:3 (LA) was ~1–7% weight fraction of total fatty acids, which is less than the permissible limit of 12 mol% as per European standards (Chisti, 2007). The *de novo* fatty acids are further desaturated to different levels by a series of desaturases, including omega-6 fatty acid desaturase, and omega-3 fatty acid desaturase. The ratio of unsaturated (monounsaturated plus polyunsaturated) to saturated fatty acid fractions in FC2 cells was ~1.2 (Muthuraj et al., 2014). We identified a significant up-regulation in two PUFAs, namely 9(S)HpOTrE and DGLA in nitrogen-limited FC2 cells. Synergistic actions of several of the enzyme combinations, namely, GPDH, PGK, ENL, FBP, ALDO, TPI, and PK belonging to the glycolytic pathway, and RPI and RPE from rPPP provide cells with the flexibility to modulate energy levels, reducing power or a combination of these functions (Cantor and Sabatini, 2012). A similar pathway organization is reported in *Scenedesmus dimorphus*, *Scenedesmus quadricauda* (Sharma and Chauhan, 2016), and *Mucor circinelloides* (Tang et al., 2016), suggesting their strong role in redirecting the carbon-flux to TAG synthesis in nitrogen-limitations in oleaginous microalgae.

4. Conclusions and future prospects

Despite the extensive research on lipid production in microalgae, it is still challenging to pin-point precise molecular mechanism(s) driving nitrogen-limitation-induced TAG accumulation. This study focussed on the integrated analysis of non-omics (physiology) and omics (proteome and metabolome) of the non-model microalgae *Chlorella* sp. FC2 IITG metabolism (Fig. 5), cultivated in highly controlled parameter photobioreactors, under nitrogen-limitation conditions in a time-dependent manner. Metabolically, there is a tug-of-war ongoing between biomass and TAG assimilation during nitrogen-limitation. The reduced carbon flux limits TAG biosynthesis through central metabolism. Hence, overexpression of two of the rate-limiting enzymes PRK and SBP, under the control of the nitrogen-limitation induced promoter, could enhance CO₂ fixation and enable better biomass and TAG assimilation.

From a commercialization point of view, more investigations are required to elucidate the target pathways, enzymes, and isoform(s) for increasing TAG productivity. In this regard, a more in-depth study of linolenic acid metabolic pathway enzymes, activity, and selectivity may

help to unveil TAG formation and accumulation. Further, employing metabolomics, we annotated ten high-value molecules in the nitrogen-limited FC2, which could be co-extracted with TAG to lower the biodiesel production cost. In particular, nitrogen-limited FC2 cells accumulated 4-6x PUFAs. Our data suggest that FC2 is a promising microalgae strain for producing TAG and PUFAs simultaneously under moderate to severe nitrogen-limitation and could be an industrial strain of choice. Still, extensive research is needed to optimize the PUFA accumulation in stress-condition and extraction of phytochemicals to its fullest. A yet another attractive alternative from an environmental and technological point of view is to convert waste materials into fuels. The microalgae-based conversion of agro-industrial and municipal wastes into storage lipids could further lower biodiesel production costs and challenges for upcoming years.

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Supplementary data

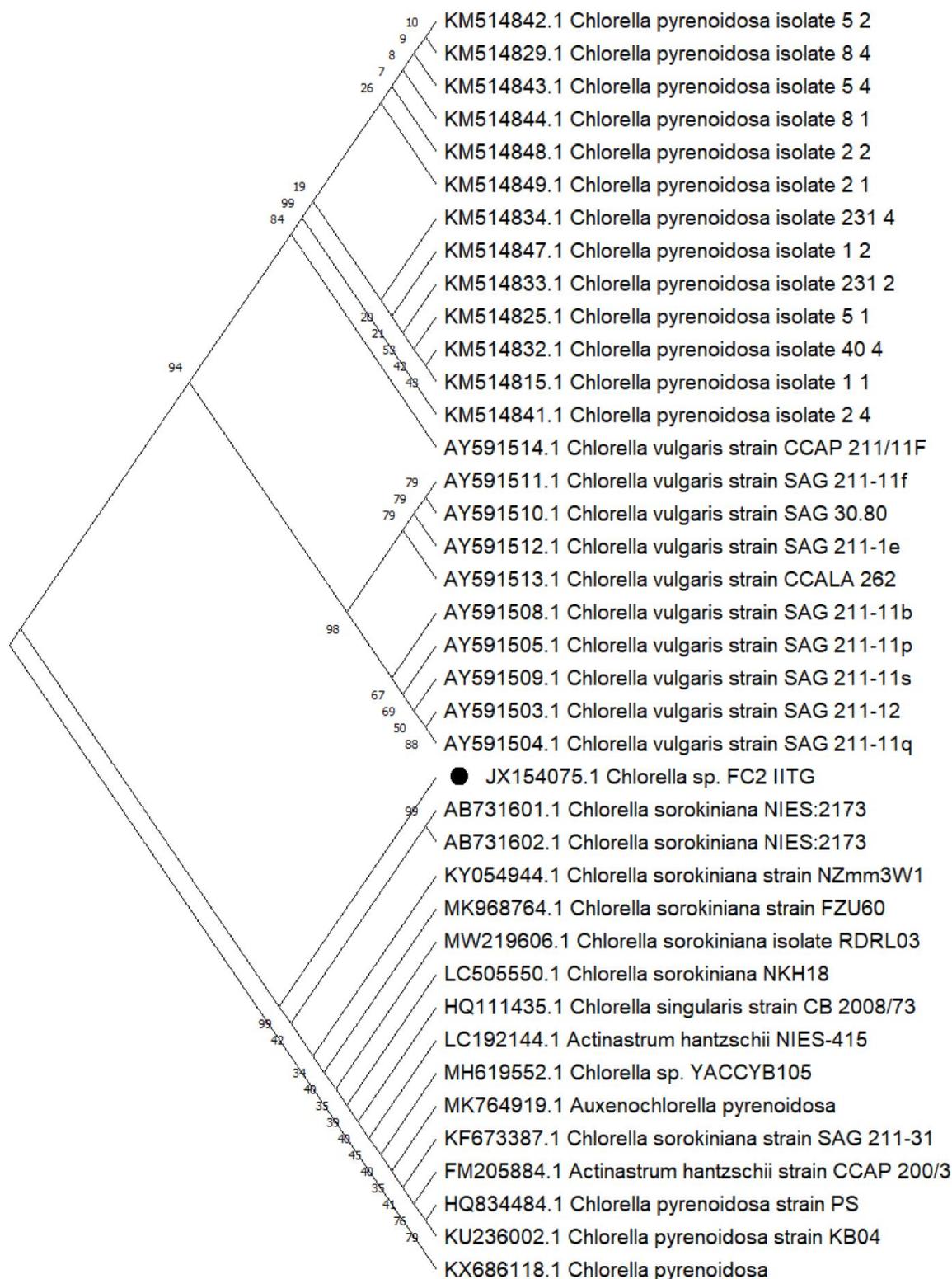


Fig. 1S. Molecular identification of *Chlorella* sp. FC2 IITG as *Chlorella sorokiniana* based on phylogenetic analysis of 18S rRNA gene sequence. The phylogenetic tree constructed based on the neighbour joining method with bootstrap analysis (1,000 replicates in %) is shown. The strain used in the present study is marked with a filled circle.

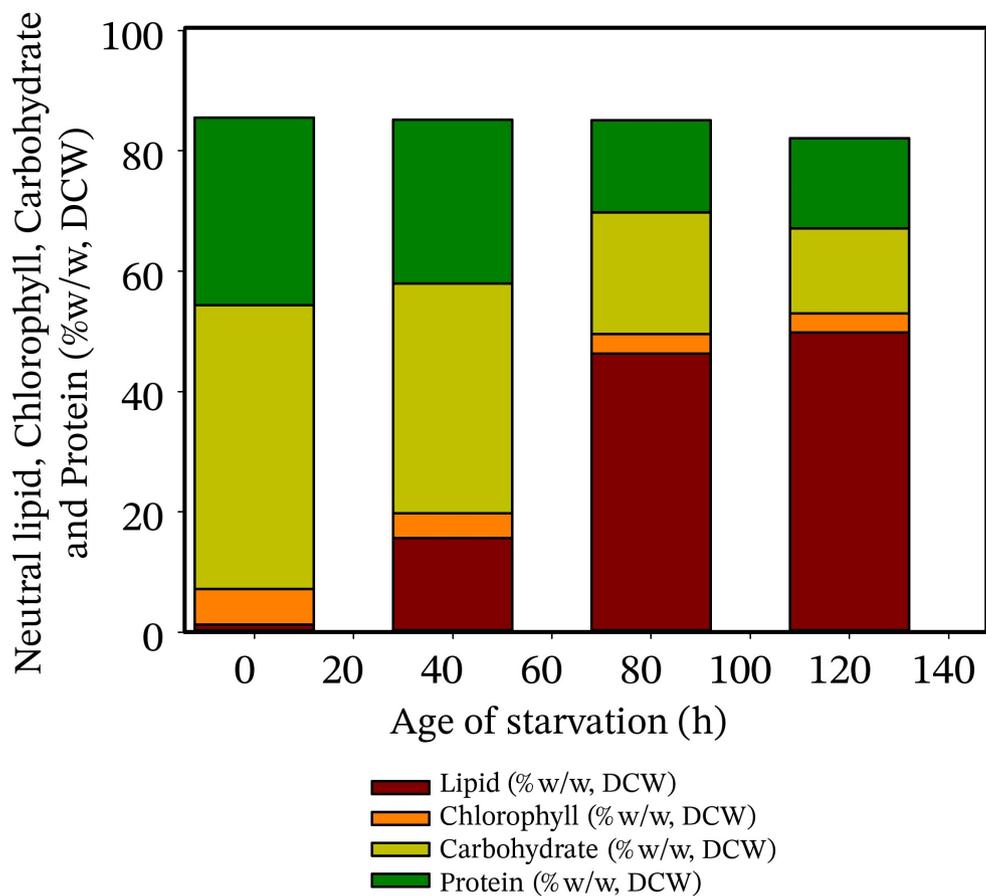


Fig. 2S. Adjustments in macromolecular compositions of microalgal biomass grown under optimal condition (0 h) and nitrogen-limitation conditions (44, 80, and 120 h) in %w/w, DCW.

Table S1.

Complete list of significantly altered features in mild (40 h), moderate (88 h), and severe (120 h) nitrogen-limitation as compared to optimal growth (0 h) in *Chlorella* sp. FC2 IITG.
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