Stress Biology in Microalgae Depicts Molecular Insights for Simultaneous Production of Lipids and High Value Precursors

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Abstract

Microalgae are the promising resource of renewable energy to replace decreasing oil reserves as a source of lipids and high value precursors for biofuels. These algal oils are rich in the triacylglycerols (TAGs) that serve as material for conversion to biofuels. Studies on the biosynthetic pathways and rate limiting steps of triacylglycerols formation in microalgae are limited. Hence, microalgae have been considered as potential feedstock to produce higher biomass and lipid content that is more suitable for biofuel production than traditional oleaginous crop plants, thus seems to be a niche for accumulating energy reserves to produce next generation renewable such as biofuels and high-value chemicals, an essential alternative for diminishing fossil fuels. Evaluation of growth and lipid profiles of oleaginous freshwater microalgae under nutrient deprivation will be the method to identify best industrial strain for production of biofuel and high value precursors at the commercial level. In the present study, we have evaluated two freshwater microalgae strains i.e. *Chlorella* sp. and *Tetraselmis* sp. to find out their metabolic responses on growth and lipid profiles under different nutrient-limited (nitrogen, phosphorous and/or sulfur) conditions. Our results demonstrate that all these strains showed severe growth hampering by stress phenomenon under nutrient deprivation, with few exceptions. Henceforth, depicting molecular insights triggered by nutrient stress biology in these microalgae demonstrated primarily increased TAG content (~up to 17-18 mg L⁻¹ D⁻¹) in both freshwater strains. In conclusion, the simultaneous production of lipids and high-value precursors among these oleaginous strains will be further characterized, which may be a critical step towards making algae-derived biofuels economically competitive for industrial production.

Keywords: Biomass; Lipid; Microalgae; Oleaginous; Triacylglycerols

Abbreviations: TAGs: Triacylglycerols; FAMES: Fatty Acid Methyl Esters; GC-MS: Gas Chromatography-Mass Spectrometry; SPV: Sulfo Phospho Vanillin

Introduction

Nearly 40-50% of total global photosynthesis occurring on earth is primarily due to the presence of these algal populations [1]. Microalgae have been identified as a viable feedstock for biofuels due to their efficient capabilities to convert sunlight and CO₂ to biomass (which is of great importance for the feed, fuel and chemical industries), can thrive in fresh to saline waters, and the ability to grow on non-arable lands that has led their path towards the replacement of fossil fuels with sustainable renewable biofuels pertaining to ever increasing energy demands [2].

Several oleaginous strains of microalgae have been identified which are capable to produce naturally high levels of lipids (e.g. 20-75% dry mass) in the form of triacylglycerols (TAGs), which can be converted for use as biodiesel via transesterification process. Many research findings demonstrated that stress responses in these microalgae could induce the formation of neutral lipids (triacylglycerols-TAGs, the precursors for biofuel production) under various stress conditions like starvation, temperature changes, salt concentration but the major hurdle being hindered cell growth [3,4]. Microalgae are considered as key players in producing oils (biofuel precursors) compared to other terrestrial crops (e.g. palm, rapeseed, and soybean) and thus have gained importance as economical feedstock with certain challenges [5]. Microalgae biofuels are considered as one of the viable alternative sources of energy as they are renewable, sustainable and environmental-friendly and reconstruction of these metabolic pathways for increasing oil content is foreseen as a niche in algal biology [6].
Although microalgae biofuels hold great promises, still considerable challenges exist for their commercialization. Generally, the lipid composition among microalgae varies between 20-75% (dw) depending on the species as well as the environmental conditions [7-10]. Recently, various biochemical strategies have been employed to improve lipid accumulation and biomass production [11,12]. Nutrient availability has a significant impact on growth of microalgae as well as broad effects on their lipid and fatty acid composition [3,13-15]. Henceforth, nutrient deprivation is one of the most widely used and applied lipid induction techniques followed in enhancing microalgal lipid and high value precursor production where they tend to change their metabolic profiles and biochemical composition. Producing these value-added biorenewable in addition to biofuels, fatty acid methyl esters (FAMEs), and lipids has the potential to improve microalgal-based biorefineries by employing either the autotrophic or the heterotrophic mode, which could be an offshoot of biotechnology [16].

In the present study, we focussed on screening and characterization of two different freshwater oleaginous microalgal strains depending on their growth (biomass) and lipid profiles under different nutrient limitations, viz. nitrogen, phosphorous and sulphur for biofuel production and the overview of this study is to assess the effect of nutrient deprivation i.e., stress biology for simultaneous production of microalgal lipids and high value precursors.

Materials and Methods

Strains and culture conditions

Two freshwater microalgal strains were used in this study namely Chlorella sp and Tetraselmis sp procured from Institute of bioresources and Sustainable Development (IBSD), Imphal, Manipur, India. Cultures were incubated under continuous illumination (~120 µmol m⁻² s⁻¹ photo synthetically active radiation [PAR]) on an orbital shaker (200 rpm) at 25 °C and ambient levels of CO₂ Cells were initially grown photoautotrophically to the middle of the logarithmic phase in concentration of nitrate (NO₃⁻), phosphorus (PO₄³⁻), and sulfur (SO₄²⁻) in growth media. Cultures were incubated under illumination (~120 µmol m⁻² s⁻¹) at continuous illumination (~120 µmol m⁻² s⁻¹) on an orbital shaker (200 rpm) at 25 °C and ambient levels of CO₂. The effect of nutrient deprivation on the growth of freshwater microalgae Chlorella sp and Tetraselmis sp subjected to nutrient replete (control) and nutrient deprivation i.e., stress biology for simultaneous production of microalgal lipids and high value precursors.

Quantification of total lipids by sulfo-phospho-vanillin (SPV) assay

The standard lipid stock (s) were prepared using commercial canola oil (2 mg mL⁻¹) in chloroform, which was subsequently stored at -20 °C before use. Different concentrations of lipid in microfibers of standard oil solution were added in the empty tube. The tubes were incubated at 60 °C for 10 min to evaporate the solvent and 100µl of water was added to the lipid standard. Further sample was prepared by following SPV reaction methods. Teflon-covered glass vials were used throughout all experiments. Phospho-vanillin reagent was prepared by initially dissolving 0.6 g vanillin in 10ml absolute ethanol; 90ml deionised water and stirred continuously. Subsequently 400ml of concentrated phosphoric acid was added to the mixture and the resulting reagent was stored in the dark coloured bottle until use. To ensure high activity, fresh phospho-vanillin reagent was prepared shortly before every experiment run. For SPV reaction of the algal culture for lipid quantification, a known amount of biomass in 100 µl of water, which are either suspended in a known volume of liquid culture or harvested via centrifugation at 4,000 RPM for 5 min. 2 ml of concentrated (98%) sulphuric acid was added to the sample and heated for 10 min at 100 °C and cooled for 5 min in ice bath. 5 ml of freshly prepared phospho-vanillin reagent was added and the sample was incubated for 15 min at 37 °C; incubator shaker at 200 rpm. The absorbance reading at 530 nm was taken in order to quantify the lipid within the sample [18,19]. For blank, culture extracts replaced with water (100 µl), sulphuric acid (2 ml) and 5 ml of SPV reagent was used.

FAME analysis by gas chromatography-mass spectrometry

For fatty acid methyl esters (FAME) analysis, lipids in the microalgal cells (approximately ~1 x 10⁸ cells) were hydrolysed and methylsterified in 300 µl of a 2% H₂SO₄ solution and methanol solution for 2 h at 80 °C. Prior to the reaction, 50 µg of heptadecanoic acid (Alfa Aesar, USA) was added as internal standard. After esterification step, 300 µl of 0.9% (w/v) NaCl solution and 300 µl of hexane was added and mixed thoroughly for 20s. To separate the phase, samples were centrifuged at 3,000 x g for 3 min. A total of 1µl of hexane layer was injected into an Agilent 6890 gas chromatograph (GC) coupled to mass spectrometer (MS) [3,20]. The running conditions for GC-MS were described by Agilent’s RTL DBWax method [21].

Result and Discussion

The effect of nutrient deprivation on the growth of Chlorella sp and Tetraselmis sp

The growth of freshwater microalgae Chlorella sp. and Tetraselmis sp. subjected to nutrient replete (control) and...
medium deprived of different nutrients (N, P, and S) in minimal BG-11 media under continuous illumination of light was shown in Figures 1A & 1B. Both species of microalgae had initial lag phase and exponential growth phase followed by the late log phase at day 10 in all medium, although some of them were not very obvious. Previous studies on the total nutrient deprivation resulted in the largest reduction of algal growth, followed by deprivation of nitrogen, phosphate and sulfur from the medium [22]. The influence of nutrient deprivation on the microalgal growth seemed to be species-specific or have different performance in different culture conditions [23]. Deprivation of phosphorus from the medium reduced the growth rate of both Chlorella sp and Tetraselmis sp than control (Figure 1A & 1B). However, the phosphorus deprivation significantly reduced the optical densities of Chlorella sp. and Tetraselmis sp. from day 6 to day 10 compared to the BG-11 medium (Figures 1A & 1B).

Nitrogen limitation of Chlorella sp and Tetraselmis sp had drastic effect on the growth with the complete nutrition deprivation. sulfur deprivation had different effect on the growth i.e. in Chlorella sp shown drastic reduction in growth whereas Tetraselmis sp significantly reduced the optical densities which varied from day 6 to day 10 compared to control. These results suggested that different microalgae respond differently to the different nutrient deprivation. Although nutrient deprivation can increase the lipid content of microalgae, it reduced the growth and biomass of the microalgae more or less, which is not desirable in the lipid production.

Table 1: *Average of three biological replicates ± standard deviations.

| Species    | Biomass Production (g L\(^{-1}\)) | Specific Growth Rate µ (day\(^{-1}\)) | Doubling Time (h) |
|------------|---------------------------------|-------------------------------------|-------------------|
| Chlorella sp | 0.98                            | 0.23                                | 69.6              |
| Tetraselmis sp | 0.76                           | 0.25                                | 64.3              |

Figure 1A: Growth profiles of freshwater microalgae Chlorella sp. subjected to continuous illumination of light under nutrient replete and deplete [-N; -P; -S] conditions in minimal (BG-11) media.

Figure 1B: Growth profiles of freshwater microalgae Tetraselmis sp. subjected to continuous illumination of light under nutrient replete and deplete [-N; -P; -S] conditions in minimal (BG-11) media.

Nutrient availability is critical for cell division and intracellular metabolite cycling and once nutrients such as N, P, and S become depleted or limited in the medium, invariably, a steady decline in reproduction rate ensues. Once this occurs, the activated metabolic pathways responsible for biomass production are down-regulated and cells instead divert and deposit much of the available C into lipid [24,25]. In the present study, two freshwater microalgal strains were subjected to nutrient (N, P, and S) deprivation to understand their effect on the growth and simultaneous production of lipids and high value precursors, to determine potential candidate for biofuel production. Table 1 shows the growth profiles of two freshwater microalgae Chlorella sp. and Tetraselmis sp. in controlled growth conditions. The biomass production of Chlorella sp. and Tetraselmis sp. were 0.98 and 0.76 g L\(^{-1}\) respectively and exhibited similar specific growth rates of 0.23 and 0.25 day\(^{-1}\). Studies demonstrate that under optimal growth conditions (i.e. adequate supply of nutrients including C, N, P, S and sunlight), biomass productivity can exceed 30g dry weight per square meter per day with very low (<5%) w/w lipid content and is species dependent [26]. In principle, biomass production and lipid biosynthesis compete for photosynthetic assimilation of inorganic carbon, and a metabolic shift is required to switch from biomass synthesis to energy storage metabolism [3,25]. Thus, lipids are typically believed as storage reserves within the cell that enables the organism to survive adverse environmental and/or nutrient-limited conditions [27]. To analyze the growth profile changes under different nutrient deprivations such as nitrogen (-N), phosphorus (-P), and sulfur (-S), these strains were grown in strict photoautotrophic conditions in minimal BG-11 media following sampling at time intervals of 24h. To determine the biochemical/metabolic changes under similar conditions in these microalgae, the samplings for other experiments were collected at time intervals of 0, 48, 96 and 144 h. Our results demonstrate that Chlorella sp. had severe effect on growth in nitrogen (-N) and sulfur (-S) depletion after 3rd day whereas
under phosphorus (-P) depletion decline in growth was seen after 6th day (Figure 1A). In *Tetraselmis* sp. under sulfur (-S) and phosphorous (-P) depletion, the growth is stunted after 7th day while under nitrogen (-N) depletion growth is severely inhibited by 3rd day onwards as shown in Figure 1B. The effect of N, P, and S deprivation on growth of freshwater microalgae *Chlorella* sp. and *Tetraselmis* sp. subjected to 10 days of nutrient deprivation with continuous illumination of light in minimal BG-11 media triggered a wide range of different unique responses in these microalgae.

**Lipid analysis and profiling in two freshwater microalgae *Chlorella* sp. and *Tetraselmis* sp. subjected to nutrient deprivation**

Stress-induced strategies for enhancing lipid accumulation, such as nutrient depletion is the most prevalent method employed in microalgae [23,28-30]. This is primarily due to two factors:

1. Lack of requisite nutrients such as N, P, and S limits the cellular division forcing the organism to take advantage of alternative pathways for inorganic carbon fixation, where de novo fatty acids (in the form of TAGs) are synthesized and stored [29]

2. Photosynthesis and the electron transport chain produce ATP and NADPH as energy storage and electron carrier metabolites, respectively in eukaryotic microalgae which is consumed during biomass production [31].

Under normal growth conditions, the balanced ratio of reduced and oxidized metabolites is maintained; however under nutrient starvation, due to lack of requisite nutrients, the pool of NADP+ and ADP can become depleted [27]. Fatty acid synthesis consumes NADPH and ATP, therefore increased fatty acid synthesis replenishes the pool of required electron acceptors in the form of NADP+ and de novo fatty acids are stored as lipids [21].

Total lipids were extracted from both the microalgal strains and analysed by phosphovanillin assay and GC-MS as described in materials and methods, to understand the formation of TAGs under different nutrient starvation conditions. The total lipid was estimated using phosphovanillin assay (Figures 2A & 2B) and fatty acid methyl esters (FAMEs) also known to be neutral lipid were quantitatively determined by GC-MS (Figures 3A & 3B). Also our data has shown relation between these methods that can be reproducible i.e. GC-MS derived quantitative values will be ~3 times more than that of phosphovanillin assay (Figures 2A & 2B). In *Chlorella* sp. the neutral lipid content seems to be drastically enhancing under nutrient deprivation although FAME productivity was higher in nitrogen (-N) and sulfur (-S) starvation than phosphorus (-P) (Figure 3A). In *Tetraselmis* sp. nutrient limitation has profound effect on accumulation of lipids in all stress conditions (Figure 3B). However, the FAME productivity was higher under nitrogen (-N) starvation in *Chlorella* sp. (~17.4mg L⁻¹ D⁻¹) whereas *Tetraselmis* sp. showed highest FAME productivity in phosphorus starvation (~16 mg L⁻¹ D⁻¹) at the end of day 6. The highest productivity under phosphorus (-P) deprivation in case of *Tetraselmis* sp. can be attributed to its increased growth as well as lipid accumulation with compared to *Chlorella* sp. which did not accumulate much lipid under phosphorus (-P) deprivation. Thus, this phenomenon can be an alternative approach to enhance lipid content among specific microalgal strains without compromising growth. In both these strains, nitrogen starvation had a severe effect on growth but also lead to increase in neutral lipid production by onset of nutrient depletion (2nd day) (Figures 3A & 3B). This demonstrates that most of the metabolic responses and biochemical changes initiates immediately upon nutrient deprivation.
Nitrogen is critical for protein synthesis, but under limiting conditions most of the carbon fixed in photosynthesis is used for producing lipid or carbohydrates, instead of proteins [23,29,32-35]. Thus, nitrogen is considered most important nutrient affecting lipid metabolism in microalgae. sulfur is one of the significant nutrients that affect the biohydrogen production in microalgae [6,33]. Our studies have also shown that sulfur deprivation also led to increased total lipid content in the Chlorella sp. and Tetraselmis sp. Phosphorous is involved in many cellular metabolic processes and results in accumulation of lipids. In Scenedesmus sp LX1, lipid accumulates up to 53% under phosphorus-limited conditions [36,37]. Consequently, thus desired biofuels precursors can be achieved by manipulating nutrient conditions for enhanced production of sustainable renewables.

**Conclusion**

In the present study, two oleaginous microalgae Chlorella sp. and Tetraselmis sp. were assessed for understanding the stress biology that can simultaneously produce lipids and other high value precursors useful for different applications in the areas of bioenergy including others such as pharmaceutical and nutraceutical industries. Nutrient limitation in both these strains led to a severe growth arrest, with few exceptions under phosphorous-limiting. Nitrogen (-N), phosphorus (-P), and sulfur (-S) starvation increased the lipid content in both freshwater strains. Lipid profiling shows a recycling of lipids and change in the saturation and instauration level of fatty acids under different nutrient-limiting conditions. Nitrogen limitation increased the lipid content to higher levels and the saturation and instauration level were found to be favourable for biofuel precursors. In conclusion, the biomass and lipid productivity among these freshwater strains seems to be competitive, and further characterization may be a critical step towards making these algae-derived biofuels economically competitive for industrial production. With these desirable properties, Chlorella sp. and Tetraselmis sp. would be highly suitable candidates for simultaneous production of lipids and biorenewables (B~3~ Biomass, Biofuels and Biorenewables) without compromising growth. Understanding the entire system through integrated omics research will lead to identify relevant enzyme-encoding genes, and reconstruct the metabolic pathways involved in the biosynthesis and degradation of precursor molecules that may have potential for biofuel production, aiming towards the vision of tomorrow’s bioenergy needs.

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