



RESEARCH ARTICLE

Evaluation of growth pattern and biochemical components of *Chlamydomonas reinhardtii* Dangeard

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Abstract

Chlamydomonas reinhardtii Dangeard is the simplest motile, unicellular fresh water alga of class Chlorophyceae. It also functions as the most efficient model system for converting solar energy to chemical energy in the form of various metabolites. The objective of the present work is to deal with the growth conditions/growth kinetics of *Chlamydomonas reinhardtii* Dangeard, required for optimal biomass production. The parameter used to study growth of algae was, photosynthetic measurement, biomass, proteins and lipid measurement, which vary with the change in the cultural conditions. Present investigations reveal that change in protein content is positively correlated with the increase in biomass, revealing that the algae can grow rapidly in laboratory/cultural conditions. Lipid content shows a negative correlation with proteins and biomass. Lipids are known to have a role as structural components, in hydration and also in signaling events. Lipids, mainly the triacyl glycerides (TAGs) act as storage compounds enabling the microalgae to survive in adverse environmental conditions. Lipidic content increases in *Chlamydomonas reinhardtii* increases with optimal light and nutrient system. The increase is in the form of triacyl glycerides which serve as precursors for the production of biodiesel and bioethanol. Conclusion-Further research is required to investigate the interactions of biomolecules and growth of algae.

Keywords

Algae, *Chlamydomonas reinhardtii*, Lipids, Pigment system, Proteins

Introduction

Algae are one of the oldest forms of plants present on earth. They have been mentioned in ancient Roman, Greek and Chinese literature. Algae can occupy diverse habitats and are present in almost all places of the planet. Algae can be microscopic or can be large macroscopic like the seaweeds. Algae play an important role in ecosystem providing food for fisheries, playing an important role in the food chain and also by providing oxygen to various living forms. Among all the member of algae, green algae, Chlorophytes are the most diverse form of algae, with nearly 7000 taxa (1, 2). It is a well-known fact, that fossil fuels are limited and continuous use of fossil fuel causes the production of carbon dioxide and greenhouse gases. An accumulation of greenhouse gases has caused environmental related issues, which have to be solved through advances in science and technology. This includes, the formation of new cleaner type of energy resources. Microalgae are fast growing photosynthetically efficient and can be used as plant re-

source for generation of cleaner alternative fuel, biodiesel. Biodiesel is environmental friendly, renewable and biodegradable fuel originating from unprocessed/ recycled vegetable oil chemically. Microalgae like *Chlamydomonas reinhardtii* Dangeard (3) convert carbon dioxide into carbohydrates and lipids. They cause carbon dioxide sequestration and reduce air pollutants. Due to the simple life cycle which can be easily manipulated, *C. reinhardtii* has been used as a model to study photosynthesis, cell division, flagella synthesis, mitochondrial activity and more recently its use as a biofuel (4). In order, to understand the use of *C. reinhardtii* as a biofuel it is important to know the biochemical components of the algae and the growth conditions associated with it. Growth of *C. reinhardtii* can also be assessed by different processes/biochemicals associated with it, including chlorophyll content.

Proteins are important biomolecules and have a role in cell differentiation (5), cell signalling pathways (6) and cellular adhesion (7). Many glycoproteins play a structural, defensive, signaling, adhesive, nutritive and guidance function (8, 9). Biochemical analysis of proteins will display the different proteins required for locomotion, differentiation, adhesion and signalling pathways. Lipids are also biochemical components having an important function in algae. *Chlamydomonas* spp. is efficiently able to convert water, carbon dioxide and light into products suitable for renewable energy including hydrogen, carbohydrates and lipids (10). An accumulation of lipids also helps the algae to overcome different environmental stress, mainly nitrogen deficiency stress (11). Lipids are known to have a role as structural components, in hydration and also signalling events. An understanding of the growth parameters of *C. reinhardtii* and the nutrient conditions that allow the growth of algae are required before the algae can be explored for biomass/lipid production. Present studies deal with the growth kinetics of *C. reinhardtii* culture, through spectrophotometric studies and its biochemical analysis.

Materials and Methods

Culture of microalgae

Cultures of *C. reinhardtii* were obtained from Visva-Bharti culture collection of Algae, Shantiniketan and were cultured in laboratory conditions. The cultures were in inactive form and were subcultured to form actively dividing cells. Algal cultures were cultured in N 11 medium (12). The composition of the media included micronutrients, KNO₃ (1 g l⁻¹), Na₂HPO₄·H₂O (0.083 g l⁻¹), KH₂PO₄ (0.052 g l⁻¹), MgSO₄·7H₂O (0.05 g l⁻¹), CaCl₂·H₂O (0.01 g l⁻¹). Trace metal mix included MnCl₂·4H₂O (0.099 g l⁻¹), NiSO₄·6H₂O (0.0236 g l⁻¹), ZnSO₄·7H₂O (0.063 g l⁻¹), CuSO₄·5H₂O (0.005 g l⁻¹), NH₄NO₃ (0.0029 g l⁻¹), (NH₄)₆Mo₇O₂₄·4H₂O (0.0018 g l⁻¹), CoSO₄·7H₂O (0.0028 g l⁻¹). Fe-EDTA stock- 10 g chelate per liter, autoclaved separately and added (1 ml) to the cool sterilized medium. Temperature was kept constant at 25±2 °C, with a photoperiod of 14:10 hr., light intensity 70 μm and photon m⁻²sec⁻¹ and pH of 6.8. Batch/closed culture were maintained and light microscopy studies were carried out after

every five days.

Determination of growth kinetic parameter

The Growth kinetic of the *C. reinhardtii* culture was determined by calculating the optical density at 683nm, of the culture every day. The specific growth rate was calculated at the exponential growth rate:

$$\text{Specific growth rate } (\mu) = \frac{\ln(n_2 - n_1)}{t_2 - t_1}$$

where n₁-absorbance of culture suspension at the beginning (t₁) n₂- absorbance of culture suspension at the end (t₂) of selected time.

Experiments were designed to have representative of each phase of growth of the algae. Therefore, different physiological parameters were studied after every five days of culture growth.

Determination of microalgae cell concentration

Cell concentration was determined by measuring the optical density at wavelength (685 nm) using UV/VIS spectrophotometer. The concentration was also determined using a haemocytometer and counting the number of cell at 50X.

Calculation of the dry cell weight/biomass

The dry cell weight of the microalgae was measured by conventional oven drying method (13). Briefly, one hundred millilitres aliquots of culture was passed through a cellulose acetate membrane filter (0.45 μm pore size, 47 mm in diameter). Each loaded filter was dried at 105 °C until the weight was invariant. Dry weight of the blank filter was subtracted from the loaded filter to obtain the microalgae dry cell weight.

Estimation of total protein content

Total protein content was estimated (14). 500 μl of algae was taken and was digested in 500 μl of 1N NaOH for 10 min in boiling water bath. After cooling 2.5 ml of reagent B (prepared by adding 48 ml of 5% Na₂CO₃ and 2 ml of 0.5% CuSO₄·5H₂O in 1% Sodium potassium tartarate) and kept undisturbed for 10 min. After 10 min 500 μl of folin reagent (prepared by diluting phenol folin with water in 1:2 ratio) was added. Absorbance was read at 700 nm after 30 min incubation in dark. A standard curve was generated using stock of Bovine Serum Albumin (BSA; 1 mg ml⁻¹) for serial dilutions ranging from 10-100 μg of BSA.

Estimation of total lipid content

Lipids were extracted according to standard procedure (15). A mixture of 4 ml methanol and 2 ml of chloroform were added to glass vials containing a known amount of dry biomass. After 24 hr the mixture was agitated in a vortex for 2 min and two millilitres of chloroform was added. The mixture was again shaken vigorously for 1min and then 3.6 ml of millipore water was added. The mixture was again vortexed and centrifuged at 2000 rpm for 10 min. The supernatant was filtered through Whatman No 1 filter paper into a previously weighed clean vial and evaporated on a water bath. The residue was further dried in an oven at 104 °C. The residue left represents total lipids and its contents

were determined gravimetrically. Extracted lipids were dissolved in equal volume of chloroform and boarded on silica plates for ascending chromatography. Neutral lipids were separated using hexane: diethyl ether: acetic acid (80:20:1).

Estimation of total chlorophyll content

Total chlorophyll content was determined according to standard procedure (16). Two ml. of the culture suspension was centrifuged at 10000 rpm for 5 min. The pellet was dissolved in two ml of 90% acetone and incubated overnight at 4 °C. The mixture was again centrifuge at 10000 rpm. for 5 min and the supernatant was used for pigment estimation. The chlorophyll content was estimated according to the following equation:

All experiments were done in triplicates with at least three technical replicates in each experiment undertaken. Statistical analysis, wherever required, was undertaken by calculating standard deviations and errors along the mean data from replicates. .

$$\text{Chl a} = \frac{(14.21 \times \text{OD}_{663} - 3.01 \times \text{OD}_{645}) \times V}{W}$$

$$\text{Chl b} = \frac{(25.23 \times \text{OD}_{645} - 5.16 \times \text{OD}_{663}) \times V}{W}$$

$$\text{Chl (a+b)} = \frac{(9.05 \times \text{OD}_{663} + 22.2 \times \text{OD}_{645}) \times V}{W}$$

Results

Chlamydomonas reinhardtii shows a simple life cycle

Batch cultures of *C. reinhardtii* were prepared from the algal collection center and were studied for different physiological parameter after an interval of 5 days. The growth kinetics of the algal culture was also investigated separately. Batch cultures/closed cultures of *C. reinhardtii* were prepared with the N-11 nutrient media. It was observed that in the given nutrient, light and temperatures conditions, *Chlamydomonas* reproduced at a fast rate (Fig. 1E). Studies carried out by light microscopy revealed the presence of spherical cells of *C. reinhardtii* with two anteriorly placed flagella and a thick cell wall. The cell wall encloses a plasma membrane, nucleus, cytoplasm, contractile vacuoles, cup shaped chloroplast, pyrenoid, eyespot. In the center a nucleus was clearly visible along with the cup shaped chloroplast and pyrenoids (Fig. 1A). As the number of *C. reinhardtii* culture days increase, clumping of the algae was evident. This was mainly due to the presence of mucilage which allowed the cells to stick to one another (Fig. 1B, C). Fresh medium was not provided during incubation, which led to a decline in nutrient concentrations and an increase in concentration of waste. This stress led to the various changes in *C. reinhardtii* which was observed in the later stages of the cultural studies. In the given conditions, *C. reinhardtii* reproduced at a fast rate as could be visualized in 5 day and 15 day culture. The specific growth rate at the exponential phase was 1.18 (Fig. 1E). After 15 days of culture there was a slight decrease the density. The cell density is principally

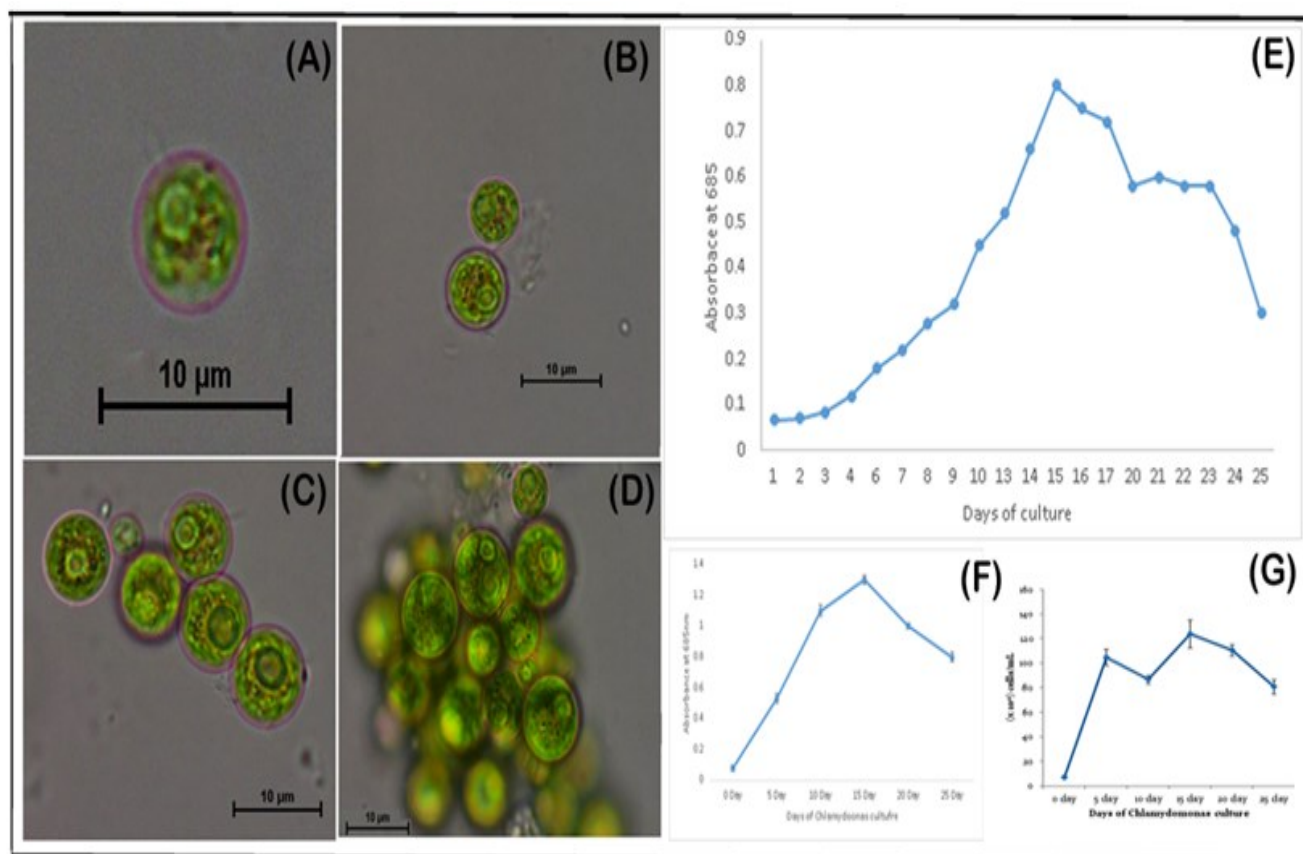


Fig. 1. Light microscopy of *Chlamydomonas reinhardtii* (A-F). (A): *C. reinhardtii* showing cup shaped chloroplast and nucleus (Magnification: 630×). (B); *C. reinhardtii*, morphology at 5 day culture (Magnification: 400×). (C): *C. reinhardtii*, showing clustering at 10 day culture (Magnification: 400×) (D): *C. reinhardtii* showing clustering and clumping (Magnification: 400×). (E): Growth curve of *C. reinhardtii* in cultural conditions. (F): Growth measure of *C. reinhardtii* as measured by spectrophotometer. (G); Growth/cell density as measured by haemocytometer.

guided by light penetration, corresponding to the light penetration (OD of the cultures).

Microalgae cell concentration

The microalgae concentration was determined by two methods, by absorbance at 685 nm and by haemocytometer (Fig. 1F). As can be observed, the cells enter the log phase after 5 day of culture. The cell count also increases. Studies by haemocytometer, however, revealed that the number of cells decline after 5 day to 10 day and then further decreased (Fig. 1G). This is observed due to the formation of clumps/colonies. *C. reinhardtii* is a fresh water motile algae, however since it has been cultured (closed) in flasks, *C. reinhardtii* may form clumps or colonies (Fig. 1D). It is also known that when water is limiting, cells which may be present as palmelloid stage, which is group of non flagellate cells grouped in a common hydroxyproline and sugar rich mucilage. When palmelloid aggregates are exposed to water, they transform to flagellate forms.

Pigments system

Pigments refer to the substances that help in the absorption of a particular wavelength of visible light. The pigment extracted in *C. reinhardtii*, correspond to maximum absorption at 683 nm (corresponding to the chlorophylls) and 433 nm (corresponding to the carotenoids; (Fig. 2A). Further investigations revealed that chlorophyll a is the major form of chlorophyll present in *C. reinhardtii* (Fig. 2B). Chlorophyll b is lesser in as compared to chlorophyll a. As the days of algal culture increase, the chlorophyll content also increases, and thereafter remains stable.

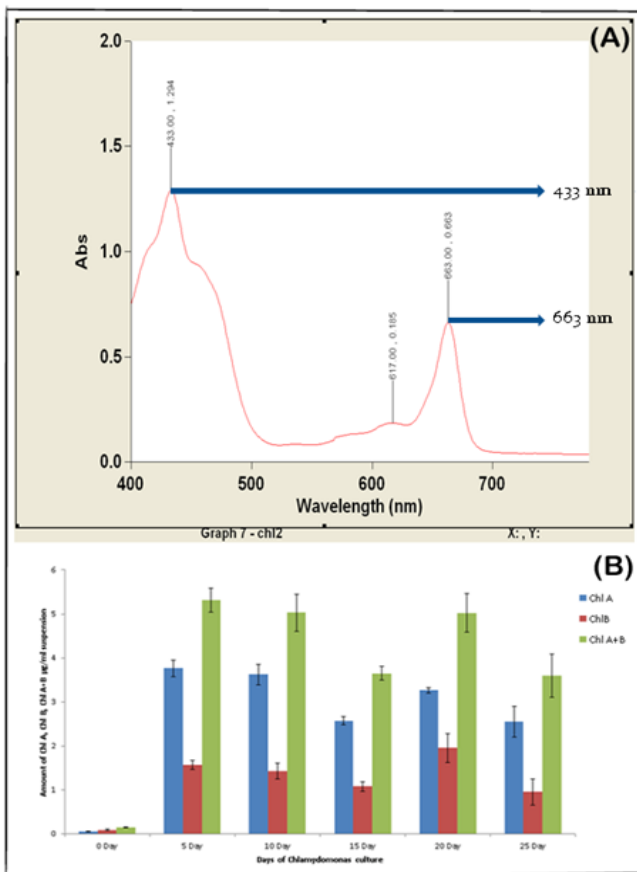


Fig. 2. Estimation of chlorophyll content. (A): Absorption spectra of chlorophyll in *C. reinhardtii*. (B): Total chlorophyll content (a and b) in *C. reinhardtii*.

Protein content increases in cultural conditions

Proteins are required for growth and reproduction. As can be correlated with growth kinetics, proteins show a sharp increase from 0 day to 5 day of algal culture (Fig 3A). The increase further continues until 15 days of culture and then a decline in the protein content is observed in the 20 day of culture. It can be concluded that proteins required for growth and reproduction are active till the 15th day and there are some physiological changes occurring in the 20 day leading to a decrease in the protein content.

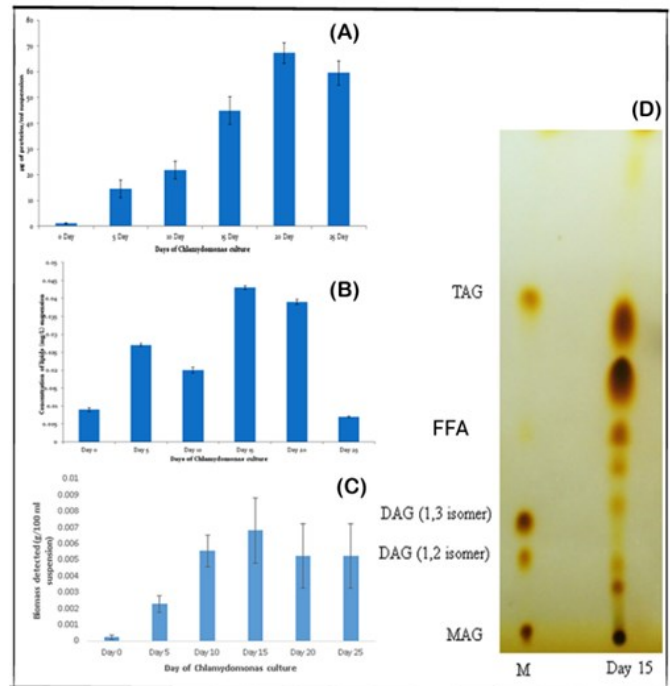


Fig. 3. Biochemical detection in *C. reinhardtii*. (A): Total protein content in *C. reinhardtii* in cultural conditions. (B): Gravimetric estimation of total lipid content in cultural condition of *C. reinhardtii*. (C): Total biomass of *C. reinhardtii* in cultural condition (D): TLC analysis of neutral lipids in *C. reinhardtii* after 15 days of culture.

Dry Cell Weight

The dry cell weight or the biomass of *C. reinhardtii* increased as the days of algal culture increase (Fig. 3C). The dry weight or biomass is mainly the proteins, cell wall carbohydrates and membrane lipids. Present investigations reveal that the biomass increases even though the growth declines. This can be attributed to the fact that algal culture has started facing stress after 15 days of culture and has led to increase palmelloid stage of *C. reinhardtii*. Biochemical pathway may be directed towards the formation of cell wall carbohydrates, mucilage and other products, leading to slight increase in the biomass.

Lipid content increases even after a decline in the protein content

The lipid content of *C. reinhardtii* reveals an increased level, as the days of culture increase. Present investigations reveal that the lipid content initially increase, however, shows a sharp decline after 20 days of culture (Fig. 3B). It may be concluded that the algae the lipids may be initially used as food storage. Later on, however, the algal culture faces some stress after 20 days thereby altering its biochemical pathways. After 20 days, the lipids may be catabo-

lized for the formation of mucilaginous sugars, leading to high occurrence of palmelloid stage in algal cultures. Quantitative studies through thin layer chromatography of *Chlamydomonas* culture in 15 days reveal the presence of monoacylglycerides, diacylglycerides and free fatty acids (Fig. 3D).

Discussion

Chlamydomonas reinhardtii grows exponentially in N-11 media

Chlamydomonas reinhardtii Dangeard is a fresh water microalgae and shows a simple life cycle. Structurally the algae is pear shaped/spherical with two anterior flagella, a thick cell wall, plasma membrane, nucleus, cytoplasm, two contractile vacuoles, enlarged cup shaped chloroplast, pyrenoid, eyespot and other cellular organelles like mitochondria, golgi bodies, endoplasmic reticulum, ribosomes, vesicles and dense granules called volutin granules (17). The flagella originate from blepharoplast, and show 9+2 arrangement. Flagella are connected to nucleus by neuromotor apparatus. At the base of flagella, contractile vacuoles are present which function as osmoregulators and regulate the water content in *C. reinhardtii*. The cell wall of *C. reinhardtii* is complex composed of seven layers of hydroxyprolien rich glycoproteins (18). A single cup shaped chloroplast in which contain the chlorophyll and an eyespot or stigma is embedded and is involved in the light dependent movement of *C. reinhardtii*. The lower part of the chloroplast is occupied by pyrenoids which aids in the carbon accumulating mechanism increasing the efficiency of enzyme RuBisCo (Ribulose 1, 5-bisphosphate carboxylase oxygenase) (18). Growth curve when plotted against absorbance of 685 nm revealed the presence of lag phase, exponential (log) phase, stationary phase and declining phase. Various cultural conditions, mainly light intensity and nutrient availability have an effect on growth rate and cell density. Excessive solar radiation cause photoinhibition, while low light due to cultural density may lead to reduced photosynthetic activity and growth rates (19). The initial phase was the lag phase, where cell division was present, however, there was no net increase in the mass. The cells possibly synthesize new components, mainly ATP, essential cofactor, ribosome etc. for cell division. After the lag phase the cells enter the log phase, where the growth rate is constant, and cells divide at regular intervals. In the log phase there is an increase in protein and DNA synthesis resulting in an increased rate of reproduction, thereby increased biomass. The cultures in log or exponential growth respond to precise, coordinated and quick changes to the environment. As the nutrient conditions deplete in the cultures, the cells enter the stationary phase, where the growth rate decreases. Earlier studies have indicated that nitrogen starvation triggers signalling pathway to sexual cycle (20). The zygote formed undergoes a resting period, hence a decrease in the growth of the culture. As the cell density increase, the OD increases reaching to its peak and then slowly lowers down due to cell conglomeration of dead cells, nutrient depletion and bacterial infection. Soon after the stationary phase, the

cells enter the decline stage, which is mainly due to starving nutrients. The cell growth declines and the cells undergo programmed cell death. Present investigations reveal that in *C. reinhardtii* specific growth rate in the culture was 1.18.

Palmelloid stage of Chlamydomonas is formed in cultural condition

Structural studies have revealed that *C. reinhardtii* possesses a large chloroplast enabling it effectively trap solar radiation and also makes it a highly photosynthetically efficient model. Chlorophyll causes light absorption at wavelength of 645 and 663. Absorption of chlorophyll is also a measure of the health of culture, as damaged cell decolorize (21). Absorption at 443 corresponds to the absorption by carotenoids, which are involved in absorption of light, reduction in the production of radical oxygen species (ROS) and energy dissipation (22). As per the increase in biomass, an increase in chlorophyll content was also expected. A zig zag pattern of chlorophyll content, is observed after 5th day of culture. It can be postulated that it is due to the formation of palmelloid stage observed after 5 days of culture which does not allow the total extraction of the chlorophyll pigment. A decline in the latter period (15 days of culture) shows a deteriorating cultural conditions of the microalgae.

Protein content tends to increase in cultural conditions

Among the different biomolecules, proteins are indicators of growth in *C. reinhardtii*. Proteins are required for cell division, protein maturation and other processes till the culture reaches the stationary phase. There is also a correlation of an increase of the proteins along with the biomass of the algae. It can be postulated that decline of nitrogen in the culture may cause stress and initiate biochemical pathways, giving rise to toxic substances, resulting in decline of the algal culture. Various enzymes play a regulatory role in physiological processes of *Chlamydomonas* spp. Enzyme phosphoenol pyruvate carboxylase (PEPC) regulates photosynthesis and photorespiration. The protein has a negative role in lipid production and blocking the enzymes results in increased accumulation of lipids (23). Unique novel protein including hydrogenase is also present in *Chlamydomonas* spp. which allows the production of hydrogen instead of oxygen (24). The enzyme hydrogenase has been used for biofuel purposes and further profiling of proteins will be helpful to indicate the activity of this enzyme in relation to the days of *C. reinhardtii* culture.

Stress in the algal cultures, has led to an increase in accumulation of lipids

An increase in lipids is correlated with a decrease in biomass. Initially there is an increase in the lipids as correlating with the proteins and biomass. In the latter stages, however, after 20 days of culture, there is a decrease in the protein and biomass. Earlier studies have indicated unfavorable conditions cause accumulation of lipids. Lipids accumulate in the chloroplast and inside the lipid bodies. A breakdown of protein tends to release lipids in culture (25). Lipids in the form of fatty acids are synthesized in the plastids, and are then transported for further modification and assembly. It has been established that nitrogen deprivation

induces accumulation of TAGs and starch. TAGs, act as a storage of carbon rich compounds, a resource for future recovery (26). Concurrent to earlier research it has been observed that *Chlamydomonas* spp. form TAGs when it is nutrient deprived (27) or salt stress, as has been observed in 15-20 days of culture. Present results indicate an increase in TAGs, in 15 days of culture. Lipids from algae are rich in saturated and unsaturated fatty acids mainly oleic acid, palmitic, stearic and linoleic acid. TAGs can be used in the production of biodiesel (28) and bioethanol (29). The composition of lipids as revealed in present study show that *C. reinhardtii* can be used for the production of biodiesel.

Conclusion

Present studies undertaken in algal culture indicate that the nutritive media N-11 is favorable for the growth of *C. reinhardtii*. This can be observed by the increased rate of growth in 5 days (initial stages of the culture growth). As *C. reinhardtii* days of culture increase the amount of biomass, chlorophyll, proteins and lipid also increase. After 10 day, however, palmelloid stage in *C. reinhardtii*, is observed, which hinders in the total chlorophyll extraction. The total protein content increases and shows a decline only after the 15th day of culture revealing certain physiological changes in the culture of *C. reinhardtii*. The lipid content initially increases and later shows a decreasing trend after the 15-20 days revealing, stress and changes in biochemical pathway in the algal culture. A detailed characterization of protein and lipid will further aid in understanding the exact change that has occurred in the cultural situation of *C. reinhardtii*.

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Compliance with ethical standards

Conflict of interest: The author declares that there are no conflicts of interest.

Ethical issues: None.

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