

Effects of Culture Conditions on Growth and Biochemical Profile of *Chlorella Vulgaris*

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Abstract

The effects of culture conditions at different temperature and light regimes on growth and the contents of chlorophyll-a, chlorophyll-b, total carotenoids, total protein and total free amino acids of *Chlorella vulgaris* were determined. The growth of *C. vulgaris* in terms of optical density (0.42 at 670 nm), cell count (440 x 10⁴ cells/ml) and dry weight (30.2 mg/50 ml), and the amount of chlorophyll- a (2.16%), chlorophyll- b (0.59%) and total protein, was found higher at the temperature 25-30°C and natural day light receiving through the north facing window of the growth room. Although, the amount of total carotenoids (0.440%) and free amino acids (834 µg/gm fresh weight) were found maximum in continuous light at 30-35°C, not much differences in the amount of carotenoids (0.385%) and free amino acids (822 µg/gm fresh weight) were found at 25-30°C and natural day light. The natural day light at 25-30°C was also proved proficient, as distinct banding pattern with unique polypeptides such as 15KDa, 47KDa and 50KDa, on the other hand, 23KDa, 26KDa and 36KDa appeared in all samples, these bands were not affected by light and temperature. Our results indicate that among all five culture conditions tested, the cultures kept at north facing window receiving natural day light at temperature 25-30°C, show best growth and higher contents of biochemicals that will be beneficial to use *Chlorella* for high nutritive purpose.

Keywords: *Chlorella*; Culture condition; Growth; Chlorophylls; Carotenoids; Proteins; Amino acids

Abbreviations: CC: Cell Count; Chl-a: Chlorophyll-a; Chl-b: Chlorophyll-b; DW: Dry Weight; FW: Fresh Weight; OD: Optical Density

Introduction

Chlorella is the most cultivated eukaryotic green micro alga, as it is widely used as a health food and feed supplement, as well as in the pharmaceutical and cosmetics industry. It contains proteins, carotenoids, lipids, immunostimulator compounds, polysaccharides, vitamins, antioxidants and minerals. The growth of algae is a function of many factors, including nutrients, pH, salinity, temperature and light (duration and intensity). Among these factors, the light that directly influences photosynthetic mechanism is an important factor in defining optimal conditions for the culture [1]. In the presence of non-limiting nutrients, the efficiency of microalgal culture remains controlled, mainly by light intensity and temperature. Photosynthesis of phytoplankton is influenced by natural factors, such as temperature and irradiance [2]. These factors influence the nutritional value of phytoplankton such as protein, carbohydrate, lipid, amino acid and pigments composition [3]. These specific chemicals attributes will not follow similar trends with changing temperature and light conditions [4]. The effects of irradiance and photoperiod on the biomass and fatty acid composition of *Chlorella vulgaris*, were also examined [5]. It has been long known that direct sunshine is harmful to algal cultures. Under natural conditions, receiving direct rays of the sun rarely fall on an alga, and a few centimeters of interposed water are sufficient to reduce the harmful effects. In the natural habitats, algae grow predominantly in diminished light; hence cultures should be placed in the window, where direct sun light could be avoided.

Refer to the previous work in *Prochlorococcus sp.*, the effect of illumination on cell growth cycle were examined [6], and in *Chlamydomonas geileri* the growth to be dependent on a wide range of temperature [7]. The growth of *Chlamydomonas ulvaensis* and

its polysaccharide contents, to be dependent on light intensity, and temperature range [8] and the effects of various lights on the growth of *Pithophora kewensis*, *Cladophora flexuosa*, *Chaetomorpha melagonium* and *Rhizocloium riparium*, were also observed [9]. The temperature dependent sensitivity of growth and photosynthesis of *Scenedesmus obliquus* and *Navicula pelliculosa* [10], and the effect of varied light intensities and temperature on desmids, have been reported [11,12]. These workers have observed that temperature as well as illumination conditions, play an important role in defining varied morphological factors.

The aim of the present study is to evaluate the influence of different culture conditions of temperature and illumination on the growth and biochemical profile of *C. vulgaris*, and to optimize the best culture condition.

Materials and Methods

Test organism and culture conditions

The experimental organism *Chlorella vulgaris* was isolated from Mawtha, a fresh water pond, pH 7.3, near Amber Fort in Jaipur, Rajasthan (India), cultured on Modified Chu-10 medium and maintained on the same medium by regular subculturing in every two weeks, as previously described [13]. Experiments to evaluate the effect

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of different culture conditions on *C. vulgaris*, were carried out in the departmental laboratory.

In order to find out the optimum culture condition, the cultures were subjected to five different conditions of temperature and light regimes. In the growth room, light was provided by fluorescent tube lights (40 W) having 2500 Lux intensity, and were fixed at a distance of 64 cm from the cultures in constant and alternate light conditions. One set of five different culture conditions were placed in the laboratory in window, which was facing north, for providing natural day and dark periods. The intensity of natural day light was as an average 2700 Lux. The following culture conditions set in the growth room were used in the present study:

1. Constant light at temperature 25-30°C (Set I)
2. Alternate light and dark period (12:12 hr), at 25-30°C (Set II)
3. Constant light at 30-35°C (Set III)
4. Alternate light and dark period (12:12 hr), at 30-35°C (Set IV)
5. Natural day and dark period, at north facing window 25-30°C (Set V)

Growth measurement

Three test tube sets for each culture condition, containing 10 ml of the culture medium and 2 ml of freshly growing cultures were subjected to different culture conditions, and their growth were followed through optical density (OD), cell count (CC) and dry weight (DW). Optical density was recorded by using colorimeter at 670 nm, and cell count examination was performed using haemocytometer (Neubauer improved). Dry weight was determined using 50 ml algal sample of culture which was filtered on a Whatman GF/C Filter, rinsed with distilled water, and weighed after drying at 60°C for overnight. Simultaneously, five conical flasks containing 250 ml of each medium and 50 ml *C. vulgaris* were subjected for estimation of pigments, total protein and total free amino acids. All medium in the flask and test tubes were sterilized in an autoclave at 121°C for 20 min., before inoculation. Cultures were shaken gently, thrice a day to avoid clumping and accelerate the growth process. Experiment for each medium was carried out in triplicates. Observations were carried out over a period of five weeks, after initial readings.

Estimation of pigments

Chlorophyll content of the samples were extracted in 90% (v/v) acetone, and chlorophyll-a, b estimated by Parson and Strickland method [14]. Carotenoid content of the samples were extracted in 80% (v/v) acetone, and estimated by Jensen method [15].

Estimation of proteins

Protein content of the samples was estimated quantitatively by Lowry [16] by dry biomass, using bovine serum albumin (BSA) as standard. Qualitatively, the proteins were estimated by the SDS-PAGE analysis, which was carried out according to Laemmli [17]. For extraction of proteins, the samples were homogenized with lysis buffer containing 0.5M Tris-HCl, 8M Urea, 5% (w/v) SDS, 20% (v/v) Glycerol and 10% (v/v) β -Mercaptoethanol; final pH 6.8 and centrifuged at 4°C for 20 min at 10,000 rpm. Protein extract was used for SDS-PAGE analysis, using a 12% polyacrylamide gel containing 0.1% SDS and the buffer system of Laemmli. Gels were run at 20°C at a constant current of 15 mA, for approximately 4 h. Gels were stained with 1% Coomassie brilliant blue, for protein visualization.

Estimation of amino acids

Quantitative estimation of free amino acid was carried out, by method of Lee and Takahashi [18]. One milliliter aliquot of the alcoholic extract was mixed with 5 ml of ninhydrin reagent and boiled in a boiling water bath for 20-25 minutes, and after cool down, absorbance was recorded at 570 nm with spectrophotometer. Glycine was used as a standard.

Results

Biomass production

Estimation of growth through OD, CC and DW of *Chlorella vulgaris* in different culture conditions shows different growth pattern, in spite of all culture conditions started with similar initial inoculums (Figures 1-3). Among all five culture conditions, north facing window receiving natural day light at temperature 25-30°C shows best growth for nutritive purpose of *Chlorella*, and followed by alternate light and dark period at 25-30°C, continuous light at 25-30°C and poor growth was observed in continuous light at 30-35°C.

In alternate light and dark (12:12 hr) in both the sets of temperature, i.e. 25-30°C and 30-35°C, growth was higher at 25-30°C, where OD was increased 3 times the initial record and in 30-35°C, OD was only 2.8 times (Figure 1). The CC and DW also support our results (Figure 2 and 3). Higher number of cells and dry weight were observed at 25-30°C, which showed an increase of about 2.9 and 3.2 times, respectively, from the initial cultures. On the other hand, at 30-35°C the number of cells and dry weight increased only 2.7 times.

The two sets of temperature i.e. 25-30°C and 30-35°C under

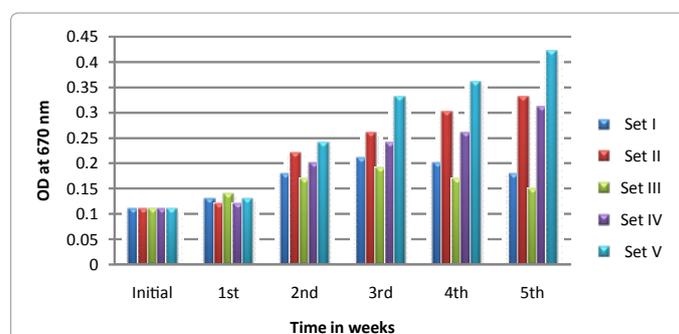


Figure 1: Effects of different culture conditions on growth (OD at 670 nm) of *Chlorella vulgaris*.

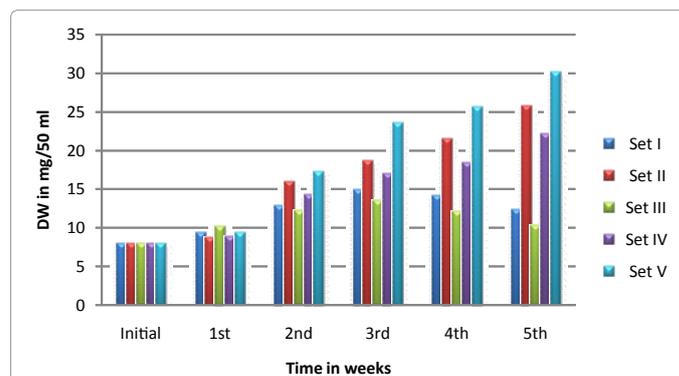
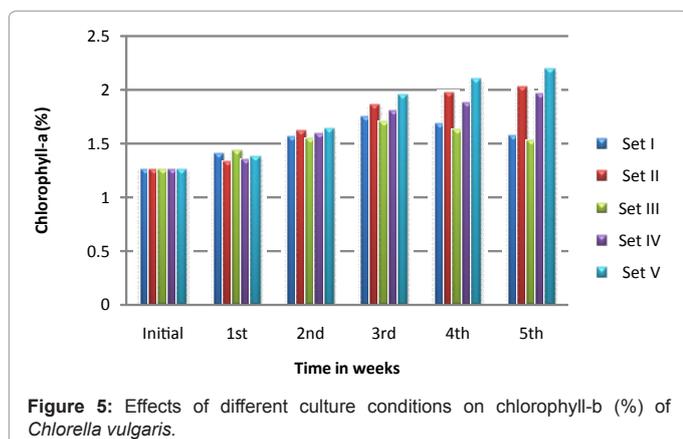
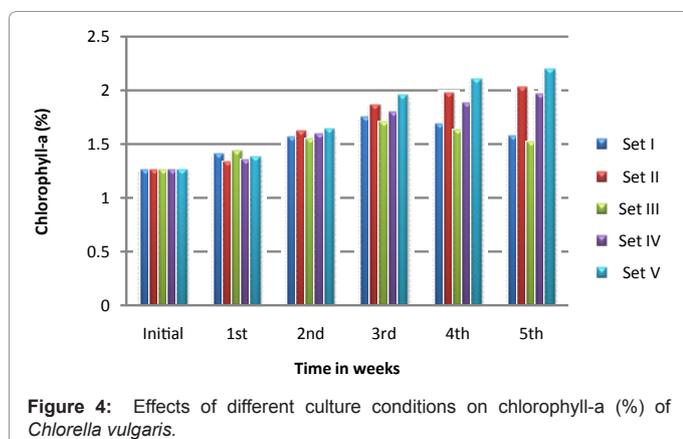
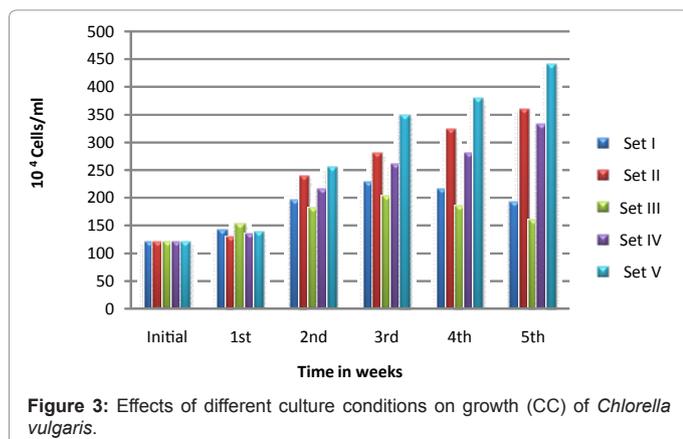


Figure 2: Effects of different culture conditions on growth (DW, in mg/50ml) of *Chlorella vulgaris*.



continuous illumination, the higher growth rate showed in 25-30°C, where OD and DW observations indicated an increase of 1.6 and 1.5 times, respectively, from the initial record at the end of 5th week. The CC also supports these data. The number of cells increased at 25-30°C showed about 1.6 times from the initial count, whereas at 30-35°C the OD and DW raised 1.3 and 1.2 times correspondingly, and the CC increased 1.3 times from the initial record.

The maximum biomass concentration as OD, DW and CC were observed under natural day light condition in north facing window at 25-30°C, where OD increased 3.8 times the initial record and DW showed an increase of about 3.7 times from the initial observation. Cell

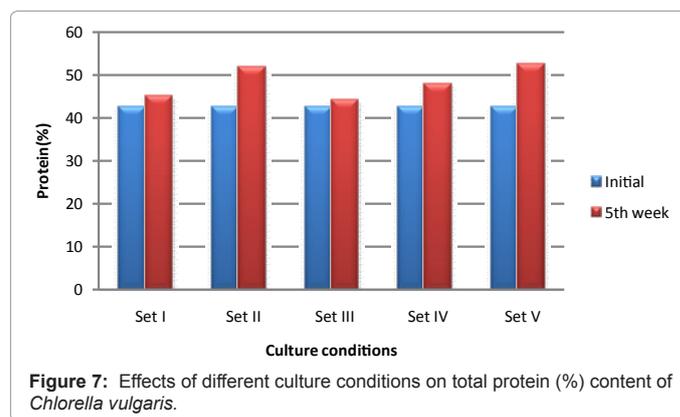
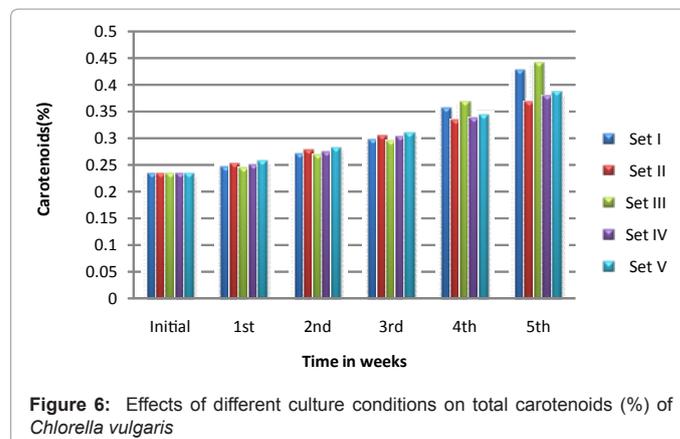
count also correlates the above result and increased about 3.6 times the initial number (Figure 3).

Biochemical production

The pigment content of the algae also correlates with the growth of *Chlorella vulgaris* in normal culture condition. The higher amount of Chl-a and Chl-b were found in cultures receiving natural day light in north facing window at 25-30°C i.e. 2.16% and 0.59% correspondingly, after a period of five weeks, followed by 2.03% and 0.52% in alternate light and dark period at 25-30°C, 1.96% and 0.50% in alternate light and dark period at 30-35°C, 1.57% and 0.42% in continuous light at 25-30°C and least amount of Chl-a and Chl-b content were observed in continuous light at 30-35°C i.e. 1.52% and 0.39%, respectively (Figures 4 and 5).

All the cultures receiving different culture conditions show dissimilar change in total carotenoids accumulation. In all culture conditions, carotenoid content enhanced up to the 5th week, but in continuous light with both temperature sets, carotenoid content significantly increased after 3rd week onwards, therefore, higher amount of carotenoid content was shown in continuous light at 30-35°C i.e. 0.440%, followed by in continuous light at 25-30°C, natural day light at 25-30°C, alternate light and dark period at 30-35°C and alternate light and dark period at 25-30°C i.e. 0.427%, 0.385%, 0.378 % and 0.367%, respectively (Figure 6).

The total protein contents were directly proportionate to the growth and chlorophyll contents. Continuous light and higher temperature negatively affected protein concentration. The highest amount of protein was found in natural day light at 25-30°C i.e. 52.6% followed by



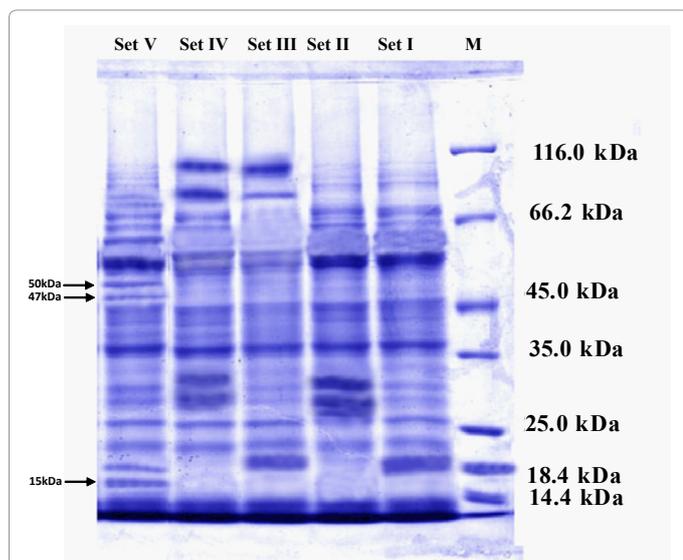


Figure 8: SDS-PAGE of *Chlorella vulgaris*, where M is a marker.

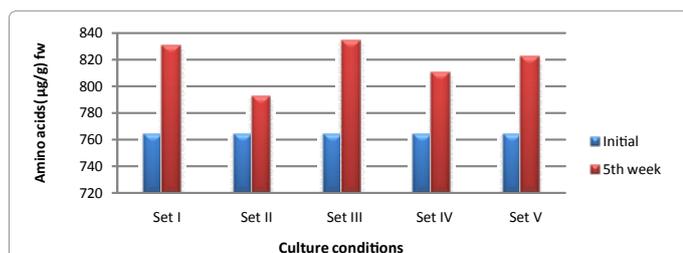


Figure 9: Effects of different culture conditions on total free amino acids ($\mu\text{g/g fw}$) of *Chlorella vulgaris*.

51.9% in alternate light and dark period at 25-30°C, 47.9% in alternate light and dark period at 30-35°C, 45.2% in continuous light at 25-30°C, and lowest protein content was observed in continuous light at 30-35°C i.e. 44.2% (Figure 7).

Qualitative estimation of protein by SDS PAGE showed different polypeptide profile, at different culture conditions. Some bands were common in all culture conditions, like 23 KDa, 26 KDa and 36 KDa. Furthermore, some were specific for light condition and temperature conditions. 28 KDa to 32 KDa band with higher density were found common in both sets of alternate light, and with modest lesser density found in natural day light condition. 19 KDa to 21 KDa band with elevated concentration were found common in both sets of continuous light, and 19 KDa was also present in natural day light condition. In both 25-30°C temperature sets, 55 KDa, 75 KDa, 76 KDa band were present, and approximate 75 KDa and 83 KDa feadible bands were also present in natural day light condition. 83 KDa, 99 KDa to 101 KDa band were present with higher density, in both sets of 30-35°C temperature condition. Some unique bands like 15 KDa, 47 KDa and 50 KDa were present in natural day light at 25-30°C (Figure 8).

The total free amino acids showed inverse trend from proteins, higher amount of free amino acids were found in continuous light at 30-35°C i.e. 834 $\mu\text{g/g fw}$, followed by 830 $\mu\text{g/g fw}$ in continuous light at 25-30°C, 822 $\mu\text{g/g fw}$ in natural day light at 25-30°C, 810 $\mu\text{g/g fw}$ in alternate light and dark period at 30-35°C and 792 $\mu\text{g/g fw}$ in alternate light and dark period at 25-30°C (Figure 9) (Table 1).

Discussion

Highest biomass concentration (OD, CC and DW) were observed at natural day light and 25-30°C temperature. These observations were also supported by higher contents of Chl-a, Chl-b and total proteins, however, total carotenoids and free amino acids showed different trend. Changes in irradiances and photoperiods, the growth of *Chlorella vulgaris* respond differently [19]. Natural day light and 25-30°C temperature were favorable for overall growth of *C. vulgaris*. Maximum specific growth rate of *C. pyrenoidosa*, increased uniformly with enhanced temperature, in the range 22°C to 30°C [20], and more increase of temperature resulted in a drop of specific growth rate and cells are unable to grow at temperature above 33°C [21], so the optimum temperature for *C. vulgaris* is 25-30°C.

It was observed in our study that in the 1st week of experiment, the growth of *Chlorella* under continuous illumination, was greater than the alternate light and natural day light. This is for the reason that the incidence of adequate light energy under continuous light in the 1st week of cultivation, during cell metabolism process, therefore, *C. vulgaris* is able to grow speedily. Similar observations were reported by scientists [22].

After 3rd week onwards, in both sets of continuous light, the growth and chlorophyll content were reduced and carotenoid content was increased rapidly. This is because of photooxidation reaction in the cells, owed to excess light that cannot be absorbed by the photosynthetic apparatus. The changes in pigments are related to an adaptation mechanism, chlorophyll was reduced due to photooxidation, and carotenoids were increased to protect photooxidative damage of the cell [19], in addition to that, in higher light the algae synthesized smaller photosynthetic units, most probably to prevent photo damage, however, in low light larger photosynthetic units are found probably to aid light harvesting [22].

In general, rapidly growing microalgal cells were exhibited by a higher protein and low carbohydrate content [4], so the rapidly growing cells of natural day light at 25-30°C showed higher amount of protein. Similar observations found in *Ankistrodesmus fusiformis* [23]. Higher temperature showed decrease in protein content and a concurrent accumulation of carbohydrates in *Spirulina sp* [24], and lower degree of illumination favors higher protein in *Chlorella* [25]. In light-dark cycle, light favors the accumulation of carbohydrate and in the absence of light, cells obtain their energy by metabolizing carbohydrate, and this energy is used to synthesis protein.

Not too much variation found in the total free amino acid concentration at different culture conditions, though, amino acids showed inverse trend from the protein. Similar observations were noted that suppressed protein biosynthesis encouraged free amino acid accumulation in *C. vulgaris* [26], due to degradation of protein

Culture Conditions	Chlorophyll-a (%)	Chlorophyll -b (%)	Carotenoids (%)	Protein (%)	Amino Acids ($\mu\text{g/g fw}$)
Set I	1.57 \pm 0.025 ^a	0.42 \pm 0.025 ^a	0.427 \pm 0.004 ^a	45.2 \pm 1.84 ^a	830 \pm 2.5 ^a
Set II	2.03 \pm 0.035 ^b	0.52 \pm 0.045 ^b	0.367 \pm 0.004 ^b	51.9 \pm 1.40 ^b	792 \pm 5.68 ^b
Set III	1.52 \pm 0.030 ^c	0.39 \pm 0.045 ^a	0.440 \pm 0.002 ^c	44.2 \pm 1.27 ^a	834 \pm 3.60 ^a
Set IV	1.96 \pm 0.025 ^d	0.50 \pm 0.040 ^b	0.378 \pm 0.004 ^d	47.9 \pm 1.62 ^c	810 \pm 2.51 ^c
Set V	2.16 \pm 0.025 ^e	0.59 \pm 0.045 ^c	0.385 \pm 0.002 ^e	52.6 \pm 1.10 ^b	822 \pm 3.00 ^d

Table1: Effect of different culture conditions on biochemical contents of *Chlorella vulgaris* in 5th week. Values are means \pm Standard deviation (n = 3). For each individual experiment, variable means with the same letter are not significantly different (p < 0.05).

molecules [27] or different environmental stresses [28].

Enhancement of protein expression occurred in a large number of protein species, under stress conditions. 28 KDa to 32 KDa band were found similar to *Dunaliella salina* and these bands were highly reduced in both sets of continuous light. It may be related to the proteins of the LHC-II (Light harvesting complex of photosystem II), and in high irradiance stress is the down-sizing of the chl-a and chl-b LHCII antenna by unknown mechanism [29]. This is one adaptive mechanism of green algae to stress. Protein bands of 19 KDa to 21 KDa were similar to ELIPs (Early light inducible proteins) of land plants, and *Dunaliella Cbr* (Carotenoid binding protein), which serves to protect the photosystem against too much radiation and possibly anti-photooxidative role of this protein [30]. The 55 KDa band may be related to RuBP carboxylase, which was also found in *C. protothecoides* [31] and 75 KDa, 76 KDa bands may be related to normal optimum temperature (25°C-30°C). In higher temperature i.e. 30-35°C, 83 KDa, and 99 KDa to 101 KDa bands were seen, which may be related to heat shock proteins (HSPs). HSPs prevent the cell from thermo-damage and help to survive in higher temperature. In our experiment, the natural day light at 25-30°C temperature was proved proficient, as distinct banding pattern with unique polypeptides such as 15 KDa, 47 KDa and 50 KDa. On the other hand, 23 KDa, 26 KDa and 36 KDa appeared in all samples, these bands were not affected by light and temperature.

Light/dark cycle was more supportive for growth than other regimes, because cell number is sustained longer in exponential phase longer [19], and photoperiodicity also save the consumption of light energy and increase light energy efficiency [22].

Conclusion

Due to high nutritional value, natural pigments and anti-oxidant activity, *Chlorella vulgaris* is used in food and pharmaceuticals industries. Therefore, objective of this research was to optimize the best culture condition for its high biomass, chlorophyll contents, total protein, total carotenoids and total free amino acids. It has been mentioned above that natural day light at 25-30°C showed highest concentration of biomass, chlorophyll and protein contents, but carotenoids and amino acids were found little lower from the maximum in natural day light at 25-30°C. A slight stress condition is developed in natural day light due to sun light intensity or photoperiod, which favors the accumulation of carotenoids and free amino acids, without affecting the concentration of biomass, chlorophyll and protein contents. Therefore, we recommend culturing *Chlorella* for its high nutritive purpose at natural day light at 25-30°C.

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