



## Proximate Composition, Chlorophyll *a*, and Carotenoid Content in *Dunaliella salina* (Dunal) Teod (Chlorophyceae: Dunaliellaceae) Cultured with Cost-Effective Seaweed Liquid Fertilizer Medium

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**Abstract:** Growth, proximate composition, chlorophyll *a*, and carotenoid content were estimated in static cultures of micro alga *Dunaliella salina* (Dunal) Teod (Chlorophyceae: Dunaliellaceae) to compare the quality and quantity of biomass produced with seaweed liquid fertilizer (SLF) against Walne's medium. Culture of micro algae was performed with controlled temperature of  $27 \pm 1$  °C, salinity of 33, and an irradiance of  $120 \pm 3 \mu \text{mol m}^{-2} \text{s}^{-1}$ , and daily samples were taken to estimate the above-mentioned parameters. Mean cellular density from samples of both medium were similar and growth rates were 0.44 and 0.42 doubling per day, respectively. Maximum values of protein content 5.2 and 5.5 pg per cell were estimated on day 5 in Walne's medium and SLF, respectively. Carbohydrate and lipid content decreased during the first 4 days corresponding to the culture exponential growth. Higher carbohydrate content was found in both media during the first 2 days and thereafter reduced partially compared to their concentrations. Generally lipid contents in cultures with SLF were significantly higher ( $P \leq 0.05$ ) compared to Walne's medium. Both pigments also increased exponentially and their concentration was same in both experiments. In conclusion, all the parameters tested were similar when using either media, and hence SLF can be used as an alternate media for micro algal culture.

**Key Words:** Seaweed liquid fertilizer, *Dunaliella salina*, Algal culture

### Introduction

The marine unicellular green micro alga *Dunaliella salina* (Dunal) Teod (Chlorophyceae: Dunaliellaceae) has been used as non-conventional live feed for clam *Villorita cyprinoides* (Gireesh et al., 2001) as well as a potential source of  $\beta$ -carotene (Ben-Amotz, 1999; Garcia-Gonzalez et al., 2000). This species also gives indispensable energy and whole nutrients for the growth and development of larvae and juveniles of invertebrates. Hence, micro algal culture is an important activity that influences the nutritional value of aquatic herbivores (Whyte et al., 1989), as well as the economic aspects of their culture (Coutteau & Sorgeloos, 1992). The use of nonconventional media based on organic matter is an alternative to reduce production costs in the hatchery system, which is cheaper than the analytical grade ones and usually used in the culture of micro algae.

Various organic nonconventional media uses for the culture of micro algae include agricultural fertilizers and soil extracts (Herrero et al., 1991; Lopez-Ruiz et al., 1995; Nieves et al., 1996). Others have investigated the biochemical composition of the micro algae as a response to the medium (Wikfors, 1986; Brown, 1991; Herrero et al., 1991; Lopez-Elias & Voltolina, 1993; Kaladharan et al., 1999). Valenzuela-Espinoza et al. (1999) prepared a low-cost alternative to the f/2 medium that may be useful for cheaper production of micro algae. However, the biochemical compositions, which are important for animal nutrition, have only been analyzed at a specific time of the growth phase. The present study aims at a comparison between 2 culture media to evaluate daily changes in proximate composition (protein, carbohydrate, and lipid), chlorophyll *a*, and carotenoid pigment of *Dunaliella salina*

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cultured with seaweed liquid fertilizer medium and Conway or Walne's media, based on the proposition that the nutrient source can change the biochemical content of marine micro algae.

## Materials and Methods

### Algal Culture

The marine micro algae *Dunaliella salina* was obtained from the Algology laboratory of CMFRI, Kochi and culture maintained at the temperature of  $27 \pm 1$  °C in seaweed liquid fertilizer (SLF) medium and with Walne's or Conway medium (Walne, 1974) as control in 3 different levels of culture experiments. A procedure for seaweed extract preparation was described by Kaladharan et al. (2002) and its composition is presented in Table 1. The first culture level was sterilized in an Erlenmeyer flask with 150 ml of media with triplicate for each treatment. Cultures began with an algal inoculum of  $5.7 \times 10^6$  cells ml<sup>-1</sup>. The culture period was 7 days with continuous illumination at the irradiance of  $120 \mu\text{mol m}^{-2} \text{s}^{-1}$ , provided by fluorescent lamps. The second experiment conducted in 3 l flasks with 1.5 l media in triplicate for each treatment. Each flask was inoculated with 150 ml of micro algae from the previous level to provide an initial concentration of  $5.5 \times 10^5$  cell ml<sup>-1</sup>. Micro algal culture was maintained for 7 days under continuous light with an irradiance of  $122 \mu\text{mol m}^{-2} \text{s}^{-1}$ .

The third culture experiment was conducted in a 15 l glass tank with 10 l medium in triplicate. Cultures were prepared with natural filtered seawater and chlorinated and neutralized with Sodium thiosulfate prior to the

addition of both medium and inoculum. The micro algae in 1.5 l culture were inoculated to each experimental unit of 10 l of media under controlled conditions.

The culture was carried out at a constant temperature ( $27 \pm 1$  °C), under stable light with 4 fluorescent lamps of about  $110 \pm 3 \mu\text{mol m}^{-2} \text{s}^{-1}$ , air flow of  $4.5 \text{ l min}^{-1}$  through dry air compressor, and salinity of 33‰. The pH was measured every day with a pH meter (Altex). Culture was quantified for daily cellular density with a Neubauer counting chamber of 0.1 mm in depth in duplicate.

### Analytical Methods

Samples of 250 ml from each of the experimental conditions in 10 l of culture were harvested at the initiation of the culture and after 24 h period daily. Thereafter, only 150 ml were filtered for the 2<sup>nd</sup> day, 100 ml on the 3<sup>rd</sup> and 4<sup>th</sup> days, and 50 ml on the 5<sup>th</sup>, 6<sup>th</sup>, and 7<sup>th</sup> days. The change in the filtered volume was due to changes in the cellular concentrations. Filtering was made with Whatman GF/C filters of 1.2  $\mu\text{m}$  and 47 mm in diameter, preheated at 450 °C for 6 h and rinsed with ammonium formate (2%). Daily samples of the media were taken throughout the experiment period to determine protein, carbohydrate, lipid (fresh weight), chlorophyll *a*, and carotenoid content. Samples were immediately stored at -60 °C for later analysis. Proteins were estimated following the procedure of Lowery et al. (1969). Carbohydrates were quantified as glucose by phenol-sulfuric acid method (Kochert, 1978). Lipids were extracted following the method of Bligh and Dyer (1959), and quantified by the method of Pande et al. (1963).

For analysis of chlorophyll *a* and total carotenoid, daily samples of 10 ml were filtered in triplicate through filter paper (GF/C) of 0.45  $\mu\text{m}$  and 25 mm in diameter. The filters were placed in McCartney tube with plastic caps and covered with aluminium foil to prevent light penetration and frozen at -20 °C for later analysis. Chlorophyll *a* and carotenoid pigment concentration was determined by the method described by Parson et al. (1985).

Cell density and biochemical data were compared statistically by one-way analysis of variance (ANOVA) ( $P \leq 0.05$ ).

## Results

### Algal Growth

The temperature in 10 l of culture in both treatments varied between 27 and 28 °C, with a mean value of  $27 \pm$

Table 1. Physico-chemical properties of seaweed liquid fertilizer.

Parameters	Seaweed extract
Physical parameters Colour	Yellow
pH	7.3
Chemical parameters (mg/l)	
Sodium	185
Potassium	113
Magnesium	108.3
Calcium	195.26
Phosphorous	51.35
Iron	0.37
Chloride	415.55
Sulphate	16.84
Silica	38.12
Copper	0.38
Zinc	1.01
Nitrate	19.05

0.72 °C. Initial pH in the control and fertilizer media was 7.6 and 7.7, respectively. The final pH values were 8.9 in the Walne's medium (control), while in the SLF it was 8.5. Salinity remained at 33‰ throughout the experiment in both media.

Growth of *D. salina* under the above descriptions is shown in Figure 1. The average initial cellular density in the Walne's control medium was  $3.6 \times 10^5$  and  $3.7 \times 10^5$  cells  $\text{ml}^{-1}$  for the medium with fertilizers. From day 1, cell density increased significantly, as the growth rate increased from 0.2 to 0.6 per day for both treatments. In the same way exponential growth was observed during the first 5 days in the culture. Average final densities were  $6.8 \times 10^5$  –  $6.6 \times 10^5$  cell  $\text{ml}^{-1}$  for cells cultured in seaweed liquid fertilizer or control media, respectively. The analysis of variance (ANOVA) showed no significant difference ( $P \leq 0.05$ ) between the cellular density during the 7 days of culture, and the average division rate was 0.44 and 0.42 doubling per day. After the 5th day, the growth rate remained constant with 0.1 division per day for the control and 0.2 division per day for the seaweed fertilizer. Doubling time was inversely proportional to the parameters described for both treatments. Results indicate that the exponential growth phase for both treatments occur between days 1 and 5.

#### Proximate Chemical Composition and Chlorophyll *a*

The results presented in Figure 2 showed that it was not possible to detect cellular protein content at the beginning of the culture with the methodology used. While the initial fresh weight average value of 13.9 pg per cell of carbohydrates was quantified from the organic cell content in control medium against 10.5 pg per cell in medium with

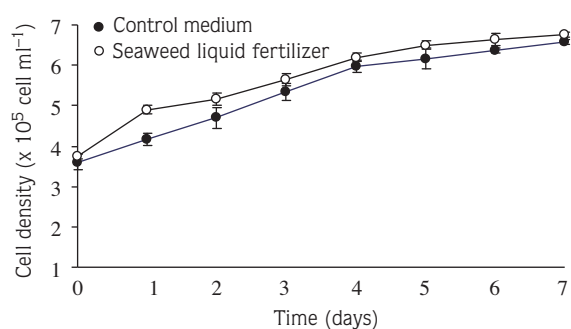


Figure 1. Average growth of *Dunaliella salina* in 10 l of culture with control medium (Walne's media) and seaweed liquid fertilizer. The vertical bars indicate standard error. (n = 3).

SLF. Lipid content showed average values of 9.5 pg per cell and 10.4 pg per cell for control and seaweed liquid fertilizer, respectively. After 24 h of culture, a progressive increase of proteins, until the 5th day, was found (Figure 2). Significant differences ( $P \leq 0.05$ ) were found between the control and experimental culture for the 2nd day. Unlike carbohydrate, lipid content decreased during the same period of time indicated above (Figures 3 and 4). The increase of protein content corresponds with the increase of cellular density observed during the exponential phase, which had the duration of 5 days in both treatments. Maximum protein content of 5.2 pg per cell and 5.5 pg per cell were found on the 5<sup>th</sup> day of culture in control and SLF; however, carbohydrate and lipids were 5.0 pg per cell, 6.3 pg per cell for Walne's medium and 5.0 pg per cell, 6.9 pg per cell for SLF, respectively. After that time, protein content decreased whereas carbohydrate and lipid showed a slight increase during the stationary growth. Both carbohydrates and lipids were higher for seaweed liquid fertilizer compared to the control medium ( $P \leq 0.05$ ) (Figure 2).

During the period of culture, concentration of chlorophyll *a* per cell varied from  $0.19 \times 10^{-1}$  to  $4.2 \times 10^{-1}$  pg per cell in the Walne's medium (control), while in the cultures with seaweed fertilizers, the range was between  $0.67 \times 10^{-1}$  and  $4.1 \times 10^{-1}$  pg per cell (Figure 5). The production of chlorophyll *a* per cell increased growth rate during 5 days. In the same way the maximum chlorophyll *a* content per cell was  $4.2 \times 10^{-1}$  pg per cell on the 6<sup>th</sup> day for the culture with seaweed fertilizer, and after then started to decrease. However, in control medium, this value remained constant until the 7<sup>th</sup> day ( $4.1 \times 10^{-1}$  pg per cell) of culture. The result showed increase in

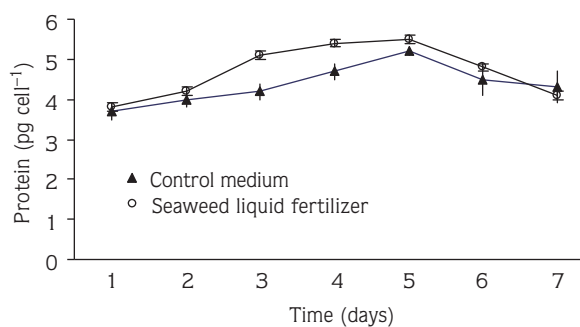


Figure 2. Average content on proteins per cell in 10 l culture of *Dunaliella salina* with control (Walne's media) and seaweed liquid fertilizer. The vertical bars indicate standard error. (n = 3).

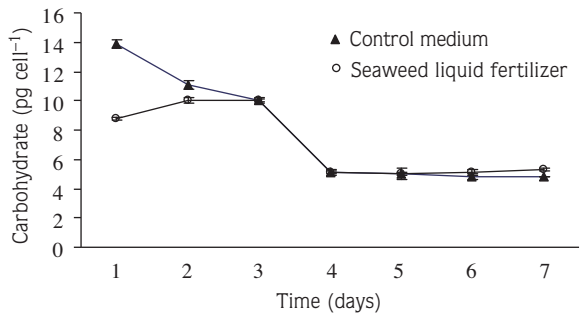


Figure 3. Average content on carbohydrates per cell in 10 l culture of *Dunaliella salina* with control (Walne's media) and seaweed liquid fertilizer. The vertical bars indicate standard error. (n = 3).

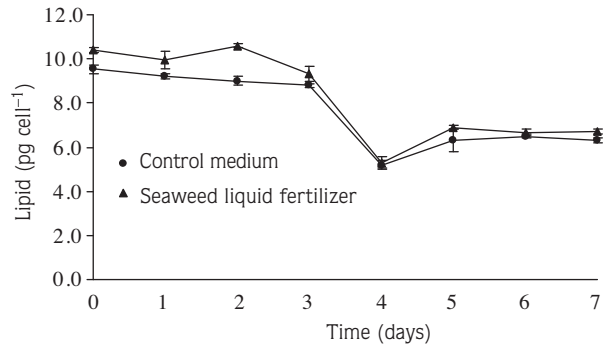


Figure 4. Average content on lipids per cell in 10 l culture of *Dunaliella salina* with control (Walne's media) and seaweed liquid fertilizer. The vertical bars indicate standard error. (n = 3).

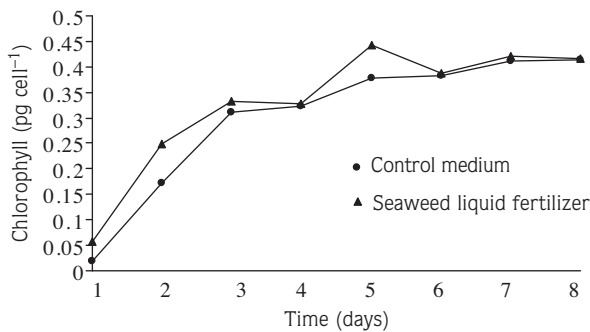


Figure 5. Average content of chlorophyll *a* on *Dunaliella salina* in culture of 10 l with control (Walne's media) and seaweed liquid fertilizer.

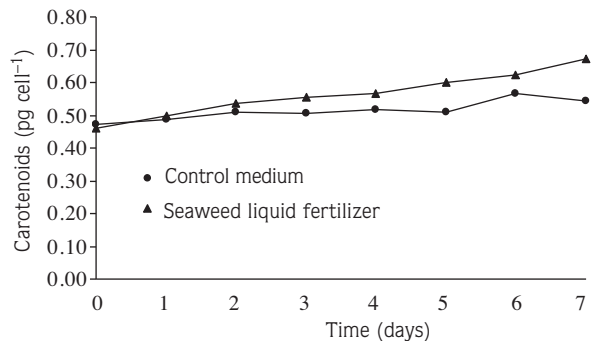


Figure 6. Average content of carotenoid on *Dunaliella salina* in culture of 10 l with control (Walne's media) and Seaweed liquid fertilizer.

carotenoid content as the growth progressed in both medium (Figure 6). However, high carotenoid content is observed in SLF medium.

## Discussion

### Algal Growth

The results show that there was no significant difference between the growths of *Dunaliella salina* cultured with both media (Figure 1). This indicates that changes in the nutrient source did not affect the growth rate of *Dunaliella salina* during 7 days of culture. This result agrees with earlier works reporting that the biomass production can be affected by the concentration of nutrients but not by the compound utilized (Fabregas et al., 1985; Herrero et al., 1991). Jin et al. (2003) has

reported mutant green alga, *Dunaliella salina*, accumulates zeaxanthin under all growth conditions while Stella and Ami (1986) reported cadmium uptake during high NaCl concentration in cultures. However, all these experiments concentrated on physiological or biochemical aspects in culture medium based on inorganic chemicals. The author suggests that seaweed liquid fertilizer is an excellent substitute that can be used for the mass culture of micro algae, due to the fact that there is no significant difference between biochemical compositions and cell concentrations after the 5<sup>th</sup> day of culture when using both media.

### Proximate Cellular Composition, Chlorophyll *a* and Carotenoid

With respect to the biochemical composition of *Dunaliella salina*, carbohydrates and lipids were high at the

beginning of culture period in both treatments. However, no protein content was detected (Figure 2). The high proportion of storage product (carbohydrate and lipid) (Figure 2) was due to the algal population used as inoculum being limited by nitrogen and phosphorus, although the cells were metabolically active in the culture. Brown et al. (1993) found in stationary-phase cultures of *Isochrysis* sp. significantly more carbohydrates and lipids than protein. The similar pattern was also reported for carbohydrates in diatom cultures (Myklestad, 1974). Protein content, after the nutrients were added, showed an increase as the culture was growing, with maximum values of 5.2 pg per cell and 5.5 pg per cell on the 5<sup>th</sup> day for the control medium and the SLF, respectively. Protein content with Walne medium was higher than the seaweed liquid fertilizer culture on the 2<sup>nd</sup> day and reverse was the case on the 6<sup>th</sup> day. A similar trend was reported by Whyte (1987), with 33.44 dry weight% for protein in the exponential phase and 42.31 dry weight% for the stationary phase. Brown (1991) found a protein content of 6.8 pg per cell at the end of the log phase in *Isochrysis* sp. The same was reported by Herrero et al. (1991) for protein values of 9.57 pg per cell, for *I. galbana* in culture with a commercial medium and 8.3 pg per cell with f/2 medium. The results obtained in the present study confirm the hypothesis that culture of *Dunaliella salina* with seaweed liquid fertilizer produces the same biomass and protein content as with Walne or Conway medium.

Carbohydrates were higher in the control during the 1<sup>st</sup> day of culture, and lipids were higher in the seaweed liquid fertilizers, but both were lower from the 5<sup>th</sup> to 7<sup>th</sup> day of culture. Both constituents decreased during the exponential growth phase until the 5th day. A small increase in carbohydrate content and a higher increase in lipids were detected during the final part of the exponential phase. These results suggest that changes in the cellular biochemical composition were influenced by the nutrient concentration in the medium. Carbohydrates have been found as intermediary reserves in some algae, due to the fact that they are required when the nitrogen becomes limited in the lipid synthesis. In the present study, when protein content in *Dunaliella salina* decreases, lipid increases and carbohydrate remains constant (Figure 2). These changes in the constituents agreed with earlier reports on protein content, which was always higher in the exponential phase (Renaud et al., 1999). Conversely,

carbohydrate and lipid tend to accumulate in the stationary phase (Brown et al., 1993; Zhu et al., 1997). However, Fernandez-Reiriz et al. (1989), in *Isochrysis galbana* (T-Iso), found 1.8 and 5.9 pg per cell of carbohydrates and lipids, respectively, representing 12.9% and 23% dry weight. These results are consistent with this study and show a clear relation between nutrient concentration and the biochemical composition as well as physiological state of the cells in culture.

Pigments are one of the cellular compounds, especially chlorophyll, that is used for estimating biomass of micro algae in culture and can be used to measure growth as well as productivity. The results from this study indicate that cell yield increased proportionally to the growth rate of *Dunaliella salina* (Figures 1 and 3). An exponential increase in the cellular chlorophyll *a* and total carotenoid content were observed till the second day. This confirms that there is a good relationship between growth rate and efficient photosynthetic apparatus during the culture. However, after the 2<sup>nd</sup> day there was no significant difference in the pigment content per cell in either treatment ( $P \leq 0.05$ ). On the other hand, chlorophyll content does not depend only on the cellular density, but also on the irradiance.

## Conclusions

It is concluded that there is no significant difference between the proximate composition obtained with seaweed liquid fertilizers and Walne's medium as both media produced similar amounts of protein, carbohydrate, lipid, and pigment content during the culture period. The use of seaweed liquid fertilizer constitutes a possible substitute to reduce manufacture costs, equal to 8 times lower than Walne's or Conway medium. Seaweed fertilizers can alternate the conventional media (Walne, 1974) used commonly for culture in commercial hatcheries.

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