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Optimization of media and culture conditions and changes in the levels of major biochemical constituents during growth stages of *Nannochloropsis oculata* in the laboratory

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Original Article

Abstract

Comparison of media and a few culture conditions for growing *Nannochloropsis oculata*, in laboratory is the major objective of this communication. Axenic stock culture was inoculated to different culture media and were evaluated for biomass production and pigment content. Of the three media studied, the f/2 medium was found to produce maximum cell density. Culture conditions like pH, temperature and salinity, optimized in f/2 medium revealed that pH 9, temperature 20°C and salinity 25 ppt were found to yield optimum output. Estimation of biochemical constituents showed more lipid content in the stationary phase, indicating the right harvesting time and this live feed organisms can also be considered for biofuel in the future.

Keywords: *Live feed organism, log phase, chlorophyll a, PUFA, biofuel*

Introduction

Microalgae, the live feeds in aquaculture are photosynthetic organisms living in aquatic environments. They are cultivated and used as food in some Asian countries but are commercially utilized worldwide in the production of cosmetics (Mourelle *et al.*, 2017), pharmaceuticals (Barbosa *et al.*, 2014) besides as feed in aquaculture, for biofuel production and as biofilters to remove nutrients and other pollutants as well as contaminants from waste water (Sirakov and Velichkova, 2014). *Nannochloropsis oculata* is a unicellular marine microalga, having small (2-3μ), spherical to slightly ovoid shaped cells which are non-motile. The genus *Nannochloropsis* only contains chlorophyll *a* and completely lacks chlorophyll *b* and *c.* In addition, they have higher concentrations of commercially important pigments such as β-carotene, zeaxanthin and other xanthophylls such as astaxanthin, violaxanthin, vaucheraxanthin (Lubian *et al.*, 2000), antheraxanthin and canthaxanthin.

Nannochloropsis has an important role to play in industrial applications, as it is a rich source of poly unsaturated fatty acids. It accumulates significant amounts of eicosapentaenoic acid (Boussiba *et al.*, 1987). This paved the way for growing interest in the studies of biofuel production using photosynthetic organisms. The sequencing of this algal genome had helped to develop nuclear transformation tools needed for genetic engineering (Kilian *et al.*, 2011). Currently, it is commonly used as live feed for rotifers and larvae of crustaceans, molluscs and finfishes. High reproduction was reported for rotifers which feeds on *Nannochloropsis* sp. than other microalgae (Prema *et al.,* 2006). Studies by Gopakumar (2004) also proved that rotifers

fed with *Nannochloropsis* have higher growth rate and lipid content. The dried biomass is also used as a supplement for the production of pellet feed for adult fishes. A study conducted by Tamaru *et al.* (1993) concluded that the requirements of fatty acids for mullets be satisfied with the rotifers fed by a combination of yeast and *N. oculata*. The present study was aimed to find out the most suitable media for culturing *N. oculata,* the optimum culture conditions such as temperature, salinity and pH and to assess the suitable harvesting time by estimating the basic biochemical constituents of the microalga during different growth stages.

Material and methods

Pure and axenic stock culture of the microalga *Nannochloropsis oculata* (Hibberd, 1981) maintained at the Marine Botany Laboratory of Department of Marine Biology, Microbiology & Biochemistry, Cochin University of Science and Technology, at its log phase was used for the present study. Cultures of *N. oculata* were raised in 1000ml Erlenmeyer flasks containing 500ml of Guillard's f/2 medium, with 10% inoculum at a salinity of 28ppt. Illumination was provided by cold white fluorescent light for 12:12 L/D photoperiod with an irradiance of 2000 lux. Cultures were maintained at the room temperature. Three common algal culture media, along with its dilutions, were used for the present study such as Chu#10 medium (Chu, 1942), Conway Walne's medium (Walne, 1970) and f/2 medium (Guillard and Ryther, 1962). Different dilutions of f/2 medium such as its full strength, half (f/4) and one fourth the concentration (f/8) of f/2 were used. Similarly full strength and half the concentration of Walne's medium (W/2) were also used for the study.

For pigment estimation, 10ml algal culture was filtered under vacuum through 0.45μ glass filter paper (Whatman) and washed with double distilled water to remove salts. The filter paper containing pigments were soaked in 10 ml 90% acetone (v/v) in separate vials with screw cap and is refrigerated overnight in dark. Later it was centrifuged and the supernatant was used for the quantification (Kumar and Saramma, 2013; Strickland and Parsons, 1972).

The optimization studies were done for temperature, pH and salinity using f/2 medium and volume was reduced to 150ml (triplicates). For temperature, the algal culture flasks were incubated at varying temperatures like 20°C (algal culture room), 25°C and 30°C (both kept in different incubators) and 35°C (temperature-controlled shaker). The pH of medium was adjusted using 1N NaOH and 1N HCl for obtaining required levels ranging from 5 to 9. The salinity tolerance of *N. oculata* was studied by growing the cultures in different salinities such as 20, 25, 30, 35 and 40 ppt. Cell counting was done using a haemocytometer to calculate growth rate.

The biochemical constituents such as carbohydrate, protein and lipid content of the livefeed alga during different growth stages (log, stationary and decline phase) were determined using standard methods. 1.0 ml of the algal cultures was filtered through GF/C filter paper for the estimation of carbohydrates and proteins. Whereas, 5mL culture was filtered for lipid estimation. The estimation of protein involved an extraction in Trichloroacetic acid (TCA) and NaOH allowing the chemical lysis of the algal sample followed by the addition of Folin-Ciocalteau reagent and measuring the optical density at 540nm after 45 minutes (Lowry *et al.*, 1951). For carbohydrate, Phenol-Sulphuric acid method was adopted by measuring the absorbance at 490nm (Dubois *et al.,* 1956). For lipid estimation, Chloroform-Methanol solvents were used with Phosphovanillin reagent and the colour developed after 30 minutes was read at 520nm (Barnes and Blackstock, 1973).

Results and discussion

The microalga *N. oculata* proliferated in the usual characteristic pattern consisting of lag, log, stationary and declining phases. The cell count was taken for 18 days, by the time the algae were found to have entered the declining phase in all the media studied (Fig.1). The growth was observed increasing from 6th to 12th day in all media and obtained a maximum cell count on the 12th day and among those media, f/2 showed the maximum value (Fig. 2). Higher growth rate was also obtained in f/2 medium (0.17/day) followed by Walne's (0.16/day). Hence f/2 medium can be considered as the most suitable for *N.oculata* culture under

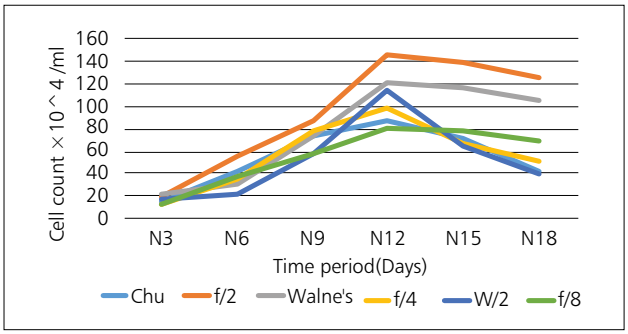

Fig. 1. Growth curve of *N. oculata* in different media

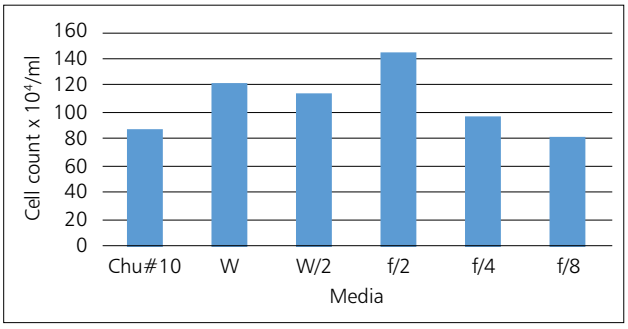

Fig. 2. Cell count of *N. oculata* in different media on 12th day (log phase)

laboratory conditions than f/4 and f/8. In a study conducted by Spolaore *et al.* (2006) the optimum growth conditions for *N. oculata* were estimated as 21°C, 52 μmol photons/m^2/S and pH 8.4 and a maximum growth rate (0.0359/h) was achieved. According to Stottrup and McEvoy (2003), most microalgal species used in aquaculture shows a satisfactory growth in f/2 and Walne's medium. In the present study, after f/2 medium, comparatively higher rate of growth and cell count was obtained for Walne's medium, than the dilutions of f/2 medium (f/4 and f/8 media), which shows least adaptation of *N. oculata* to oligotrophic conditions. Contradicting this, higher growth rates of *N. salina* were obtained from f/4 followed by f/8 medium (Kumar and Saramma, 2018) indicating its adaptation to oligotrophic conditions.

Fig. 3 shows the effect of the media used for *N.oculata* culture on Chl *a* production. The pigment production was high in f/2 media (2.539mg/m^3) when compared to its dilutions, Walne's and Chu#10 media. The Chl *a* concentration obtained from Chu#10 media was the lowest (1.707mg/m^3). A similar trend was observed in carotenoid concentration also (Fig.4), which showed a maximum value of 0.905mg/m^3 in f/2 media followed by 0.853mg/m^3 in Walne's. The results obtained from the present study are well agreeing with earlier studies on *N.oculata* (Gitelson *et al.*, 2000; Simionato *et al.*, 2011).The maximum Chlorophyll *a* production in f/2 medium may be attributed to higher growth rate of the alga in the same medium. The synthesis of carotenoids is modulated by varying culture conditions such as lowering light intensity or low penetration of light due to high cell density can increase carotenoid production (Rebolloso-Fuentes *et al.,* 2001). Another study conducted by Fae'Neto *et al.* (2018) also obtained

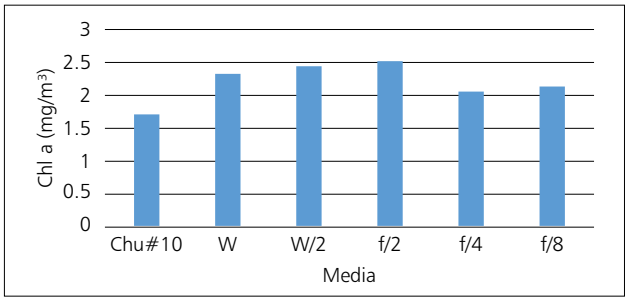

Fig. 3.Chl a concentration of *N. oculata* in different media

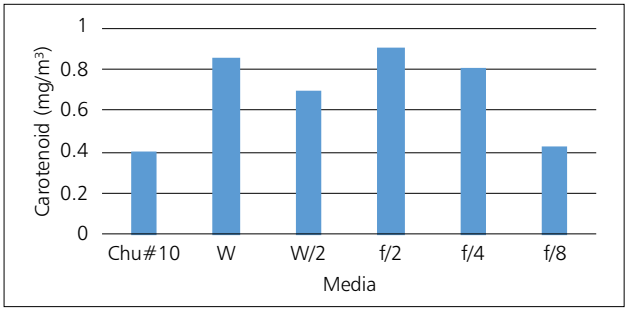

Fig. 4.Carotenoid concentration of *N. oculata* in different media

N. oculata biomass with carotenoids ranging from 0.4 to 2.9mg/g DW during 17 days of culture in f/2 medium.

According to Likun *et al.* (2014) optimization of the culture temperature is very important for the improvement of biodiesel of microalgae. In the present study *N. oculata* showed temperature tolerance from 15-35°C. Of these, 20°C was found to be the optimal temperature for the algal culture, where the cell count attained the maximum (Fig. 5). A similar finding can be traced from Spolaore *et al.* (2006), that under 21°C, the microalga showed the maximum growth rate. Converti *et al.* (2009) reported that the variation in temperature greatly affects the lipid content of the microalgae studied (*N. oculata and Chlorella vulgaris*) and according to them temperature ranging from 20–25°C could increase the lipid content considerably from 7.90 to 14.92% of the total dry biomass. At the same time, growth rate was more than halved for temperature below the threshold (Converti *et al.,* 2009). Sukenik *et al.* (1989) revealed that a temperature above 32°C was detrimental for *N. oculata* with cell proliferation being unable to keep with the rate of death of cells. In the present study also, there was no appreciable growth beyond 35°C, as the stock culture was maintained or acclimatized at 20°C. The bio flocculation efficiency was also found maximum upto 30°C and started decreasing afterwards (Surendhiran and Vijay, 2014).

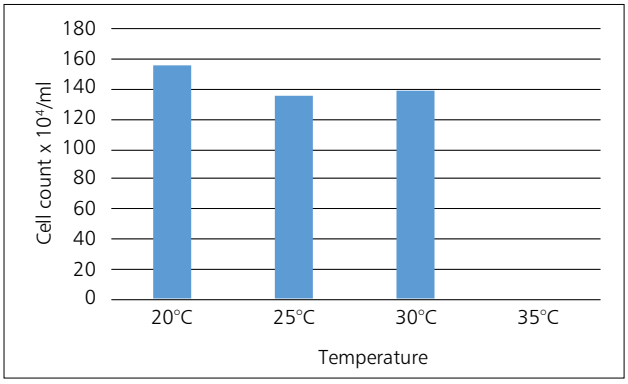

Fig. 5. Effect of temperature on growth of *N.oculata*

When *N. oculata* was cultured at different salinities, maintaining all other conditions controlled, the microalgae showed tolerance to a range of salinities between 20 and 40 ppt. The results demonstrated that the cell count of *N. oculata* was the highest at a salinity of 25 ppt (Fig. 6). Lin *et al.* (2012) found that the dry biomass of *N.oculata* obtained at a salinity of 25ppt was the highest. Similarly, the chlorophyll *a* and lipid contents were also found to be the highest in this salinity (Sen *et al.,* 2005) and also the alga had the highest content of chlorophyll and biomass at a salinity of 25ppt. Cho *et al.* (2007) studied the influence of temperature and salinity on growth of green algae *Chlorella ellipsoidea* and *N. oculata.* They found that maximum density of *N. oculata* was significantly affected by temperature and not salinity. The specific growth rate of this microalga was

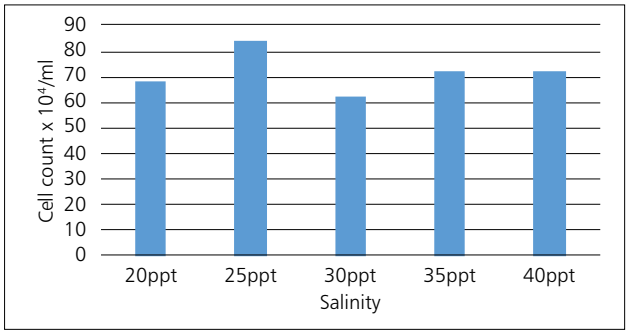

Fig. 6 Effect of salinity on growth of *N. oculata*

maximum at 25°C and 10ppt but the maximum density was obtained at 25°C and a salinity of 30 ppt. Studies by Abu-Rezq *et al.* (1999) also defined optimum culture conditions ranges between 20 to 40 ppt and 19-21°C.

The results represented in Fig. 7 shows that the cell count was high at pH 8 and 9. From this observations, it is clear that *N. oculata* prefers a slightly alkaline medium for maximum growth. Spolaore *et al.* (2006) optimized the growth conditions of *N.oculata* using response surface method and found that the optimum pH for this microalga is 8.4. Bartley *et al.* (2013) also made similar observations that in addition to the maximum cell count, growth rate and biofuel production (lipid content) will be maximum at a pH between 8 and 9. In general, pH values of 8 to 9 might be the most suitable for maximum algal growth and minimal contamination. Georgianna and Mayfield (2012) identified that *N. oculata* can tolerate fluctuations in alkaline pH. The experimental variations in the study conducted by Tamburic *et al.* (2014) were also within the above range of pH tolerance, which indicate that variations in the pH did not limit the growth of *N. oculata*. Maximum growth rate of *N. gaditana* were also observed at 25°C, pH 8 and at 25ppt (Shyni Markose *et al.*, 2020).

The biochemical estimation in the present study deals with the analysis of protein, carbohydrates and lipid concentration (Fig. 8).The biochemical composition of an alga depends on the availability of nutrients and the age of culture (Harrison *et al.*, 1997). In log phase, till 11th day, carbohydrate content was very less (22μg/mg), when compared to proteins and lipids (45 and 62 μg/mg respectively). According to Becker (1994), carbohydrate or lipid may increase in its content as a reserve for carbon, depending on the algal species cultured. The carbohydrate production in 10 species of Chlorophyceae and Prasinophyceae during stationary phase was found to be increased by 5.9- 16.7% in a study conducted by Brown and Jeffrey (1992). Since proteins are the building blocks of an organism, its content in the microalgae is very much important as they are used as aquaculture feeds. Higher concentration of protein is obtained from microalgal cultures during log phase, as the proliferation of cells is maximum. As the nutrient depletes, the concentration of protein is also decreased (Ogbonna and Tanake, 1996; Lourenco *et al.,*1997). The protein concentration was high in the log phase (85 μg/mg). As it enters stationary phase, that is on 12th day, it starts decreasing (65 μg/mg). The lipid content was maximum during the late stationary phase (102 μg/mg) and was only slightly decreased in the declining phase. *Nannochloropsis* cultures accumulate energy reserve molecules in stationary and decline phases as the cell proliferation become slow (Rebelloso-Fuentes *et al.,* 2001). Similar observations were made by Olofsson *et al.* (2014), that by lowering the concentrations of macronutrients such as phosphorus and nitrogen, biomass lipid content can be increased. Another study by Dunstan *et al.* (1993) revealed that as there is intense cell proliferation during the log phase of culture, the fatty acid concentration was also high. Microalgal diets including *Chlorella, Isochrysis* and *Nannochloropsis* proved that there is an increase in neutral lipids and phospholipids (Loveson *et al.*, 2020)

The f/2 medium was found to be the most suitable among the media studied, as it provides maximum growth and pigment concentrations. The culture conditions studied, showed that a temperature of 20-21°C, pH of 8-9 and salinity of 25ppt was optimum for this microalga. Since there is an appreciable amount of lipids among the biochemical constiuents was observed, *N.oculata* during stationary phase can be harvested for biofuel production.

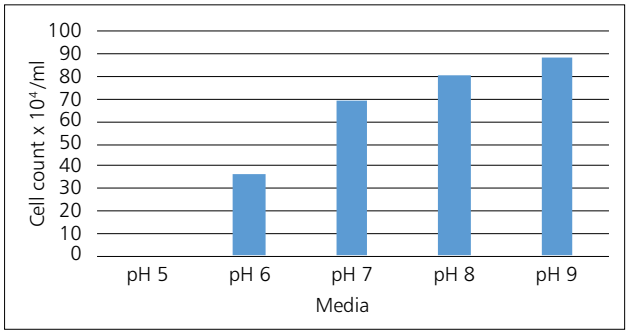

Fig. 7. Effect of pH on growth of *N. oculata*

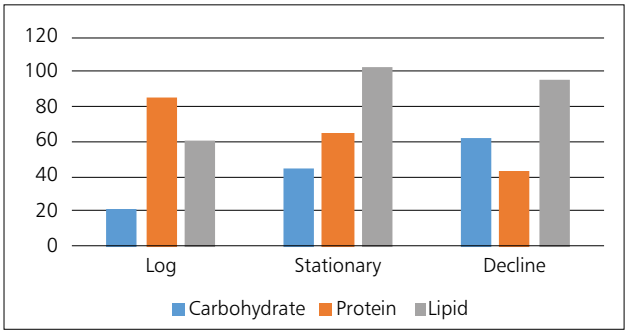

Fig. 8. Biochemical composition of *N. oculata* during different phases of growth

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