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RESEARCH ARTICLE

OPTIMIZATION AND EFFECT OF CULTURE MEDIUM AND CONCENTRATION ON THE GROWTH AND BIOCHEMICAL COMPOSITION OF MARINE MICROALGA- *NANNOCHLOROPSIS SALINA*

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ABSTRACT

Nannochloropsis salina is a marine free living nanoplanktonic alga with biotechnological potential. Among the three Common algal culture media studied, Guillard's f/2 medium produced better biomass, growth rate and production of Chl *a* when compared to Walne's and Chu#10 media. There was not much difference among the three media when it came to the amount of carotenoids produced. Protein production was more in Walne's medium during the log phase whereas, high production of carbohydrate and lipid were seen during the decline phase in f/2 medium. Growth rate analysis of *N. salina* in different dilutions of Guillard's F medium; f/2, f/4 and f/8, revealed a comparatively high growth rate and a shorter lag phase in f/4 medium.

Key words: Nannochloropsis salina, Microalgae, Culture medium, Medium concentration.

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INTRODUCTION

The criterion of the usefulness of a medium has been its final yield of algal material (Fogg, 1966). Media for microalgae cultures consist of seawater base and analytical grade nutrients (Bold and Wynne, 1985). A culture medium should be economical, produce high growth rates, satisfy the needs of microalgae and easy to prepare (Fabregas *et al*., 1985). A change in nutrient availability influences the nutritional composition of microalgae (del Pilar and Voltolina, 1996). In microalgal cultures, chemically complete media such as f/2 and Walne's are used and these media provide satisfactory growth for most of the species of microalgae used as aquaculture feed (Tzovenis *et al*., 1997; Støttrup and McEvoy, 2008). Rafiqul *et al*. (2005) studied the growth and biochemical profile of *Spirulina fusiformis* in three different culture media. Zarouk medium was proven to be better when compared to NPK medium. Lopez-Elias *et al*. (2008) reported that f/2 medium of Guillard is a common medium used in hatcheries, but it is not verified if the quantities of nutrients are adequate for algal growth. They found that f/2 medium was more significant in terms of *Chaetoceros muelleri* cultures. The organic content was slightly superior in cultures with f/2 medium.

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Studies of El Nabris (2012) on development of cheap and simple culture medium for the microalga *Nannochloropsis* sp. based on agricultural grade fertilizers available in the market of Gaza strip (Palestine) revealed that the *Nannochloropsis* growth was limited when NPK fertilizer was used. Their results were also similar to the findings of Kanlis *et al.* (2004). Studies with *N. gaditana* revealed that the nitrogen in the form of nitrate and phosphorus in the form of KH_2PO_4 was best for algal growth (Sanchez-Silva *et al*., 2013). The physiology of microalgae is affected by the nutrient concentration of the culture medium (Maloney, 1966). Fogg (1966) observed greater log phase when algal cells were sub-cultured into a medium containing high concentration of a particular substance. Concentration of the medium is an important factor which influences the growth of microalgae because of differences in nutritional requirement. Berges *et al*. (2001) found that low nutrient concentration supports growth of oceanic species. Lee (2002) observed best growth of *Isochrysis galbana* at 50% of f/2 medium followed by 25% and 100%. In variable concentrations of the same medium, growth rate was different. The maximum biomass was also observed in 50% dilution of f/2 medium than that of 100%. Lopez-Elias *et al*. (2011) observed higher cellular concentrations of microalgae cultured in f/2 medium with high inoculum density. However, this effect was not observed in microalgae cultured in 2-F medium.

MATERIALS AND METHODS

Nannochloropsis salina culture maintained in the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Kerala was used for the study. *N. salina* cultures were raised in 1000 mL Erlenmeyer flasks containing 600 mL f/2-Si medium (Guillard, 1975) with a salinity of 30 ppt. Illumination was provided by cold white fluorescent light of 2000 lux for a light/dark period of 12:12 hours. Cultures were maintained at room temperature (30± 2ºC). Three common algal culture media were used in the present study, f/2 medium, Conway Walne's medium and Chu#10 medium. Different dilutions of f/2 medium viz., full strength, half, and one fourth the concentrations of f/2 were also used in the present study.

Measurement of growth rate
Cell counting was done by using a Haemocytometer.
Growth rate was calculated using Guillard's equation (Guillard, 1973).

$$r = \frac{\ln N_t - \ln N_0}{\Delta t}$$

Where

r = exponential growth rate
$\ln N_t$ = population size at the end of the time interval
$\ln N_0$ = population size at the beginning of the time interval
Δt = length of the time interval.

Growth rate was expressed as growth/day. Biomass was determined routinely by measuring the absorbance of the samples at 690 nm (OD 690) (Hu *et al*., 2015) against culture medium as reference in a spectrophotometer (Hitachi U-2001). OD 690 values were multiplied with a predetermined conversion factor converting it in to dry weight (DW). The conversion factor was established by plotting OD 690 values versus dry weight (determined gravimetrically) of different biomass concentrations. For the gravimetric determination of the dry weight standards, cultures of different biomass concentrations were filtered using GF/C filter and washed with isotonic solution of ammonium bicarbonate (0.5 M). The filter paper was dried overnight at 50°C in an oven (Illman *et al*., 2000).

Estimation of pigments

1 mL culture samples were withdrawn and filtered through 13 mm Whatman GF/C filter paper (gentle vacuum filtration). The filter papers with algal cells were introduced into a screw capped test tube containing 5 mL of 90% (v/v) acetone. The test tube was covered with aluminium foil to prevent the entry of light. Samples were ground in a glass homogenizer and refrigerated for 1 hour. The samples were then centrifuged at 3000 rpm for 5 minutes. The clear supernatant was taken, made up to 5 mL with 90% acetone and used for pigment quantification. The experiments were performed at dark in triplicate (Strickland and Parsons, 1972; Kumar and Saramma, 2013). Absorbance of the sample was noted using 90% acetone as blank, at 750, 665, 645, 630 and 480 nm in a spectrophotometer (Hitachi U-2001). The extinction at 750nm was subtracted from the extinctions at 665, 645, 630 and 480 nm (Jeffrey and Humphrey, 1975). The amount of Chl *a* in the sample was estimated using the equation:

C_a = 11.85 (OD 665) - 1.54(OD 645) - 0.08 (OD 630) (Jeffrey and Humphrey, 1975)
Chlorophyll *a* µg/L = C_a × Extract volume (mL)/Volume of sample (L) × Path length of the cuvette (cm)
C_{Car} = 4 (OD 480) (Strickland and Parsons, 1972).
Total carotenoids µg/L = C_{Car} × extract volume (mL)/ Volume of sample (L) × Path length of the cuvette (cm)

Biochemical analysis

In order to determine the biochemical composition of the alga, cultures in log, stationary and decline phases were used. 1 mL cultures were filtered through GF/C filter paper for the estimation of carbohydrate and protein whereas; 5 mL cultures were filtered for estimation of lipid. Total carbohydrates were estimated using phenol sulphuric acid method (Dubois *et al*., 1956). 1 mL of 1 N NaOH was added to the test-tubes containing algal samples. The tubes were heated in a boiling water bath for 10 min. The samples were cooled and the supernatant was taken for analysis. Total protein was analysed using Lowry's method (Lowry *et al*., 1951). 5 mL of organic solvent (chloroform : methanol, 2:1) was added to the filtered sample and centrifuged at 2500 rpm for 5 min. Supernatant was taken and made up to 5 mL using chloroform : methanol 2:1 (Folch *et al*., 1957). 1 mL of the sample was taken into a clean test tube and allowed to dry overnight in a desiccator. After the evaporation of all the traces of solvent, 1 mL sulphuric acid was added and incubated in a water bath at 60°C for 10 minutes. Total lipid was estimated by phosphovanillin method (Barnes and Blackstock, 1973). One way analysis of variance (ANOVA) was done with SPSS version 17. The values were compared using Tukeys Test ($p < 0.05$).

RESULTS

In the present study, three common algal culture media were used, f/2, Walne's and Chu#10. Fig. 1reveals that until the 12th day there was only a gradual increase in cell count in all these media. After 12 days, the mean cell count in the f/2 medium increased when compared to Walne's and Chu#10 media. On the 15th day after inoculation the cell count in f/2 medium was higher when compared to Walne's medium and Chu#10 medium. Thus f/2 medium was found to be the optimum medium for biomass production.

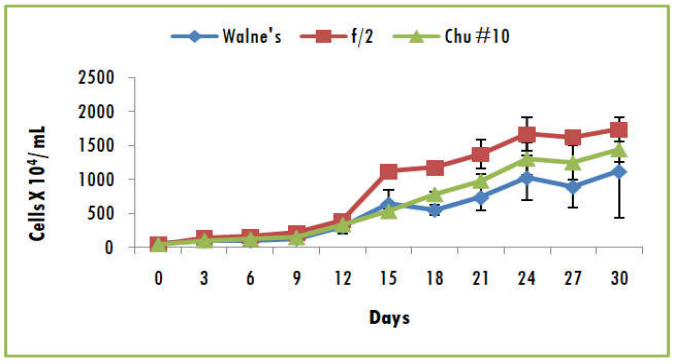

Fig. 1 Effect of culture media on cell number of *N. salina*

Higher growth rate was obtained in f/2 medium (0.17/day). In Walne's medium growth rate was 0.16/day and in Chu#10 medium it was 0.134/day. Statistical analysis revealed that growth rate varied significantly in cultures with different media ($p = 0.031$, $p<0.05$) (Fig. 2).

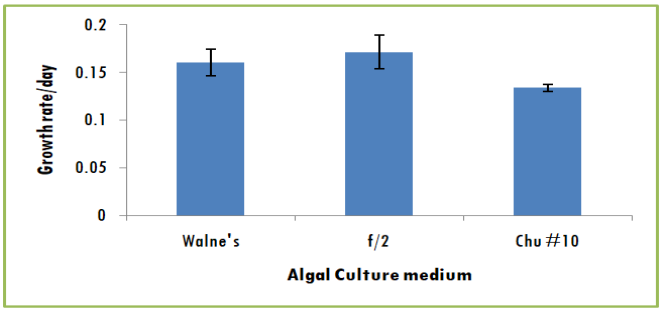

Fig. 2 Effect of algal culture media on growth rate in *N. salina*

Cell count was maximum in f/4 medium followed by f/8 and f/2. After 12 days a decrease in cell count was noted both in f/4 and f/8 medium, which also had a considerably lesser lag phase. A gradual increase in growth was noted in f/2 medium (Fig. 3).

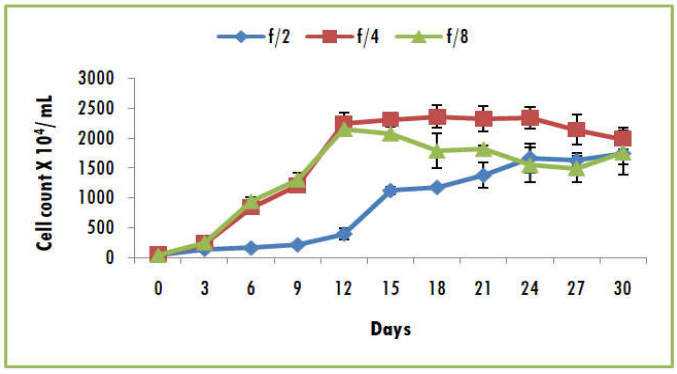

Fig. 3 Effect of strength of algal culture media on cell number of *N. salina*

Growth rate was maximum in f/4 medium (0.189/day) and in f/2 medium the growth rate was 0.17/day. There was no significant difference in growth rate in these three different media concentrations ($p = 0.068$, $p>0.05$) (Fig. 4).

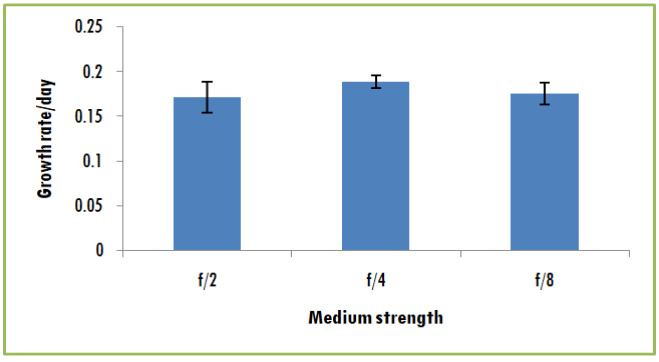

Fig. 4 Effect of strength of culture media on growth rate in *N. salina*

Fig. 5 shows the effect of 3 major algal culture media on production of Chl *a* by *N. salina*. Chl *a* production was high in f/2 medium in comparison to Walne's and Chu#10 media. Lowest chlorophyll production was noted in Chu#10 medium. Effect of different algal culture media on total carotenoid production was also studied (Fig. 6). In Walne's medium maximum carotenoid production was noticed on the 30th day (3042 µg/L) where as in f/2 medium it was on the 27th day (2919 µg/L), and in Chu#10 on 24th day (1757 µg/L).

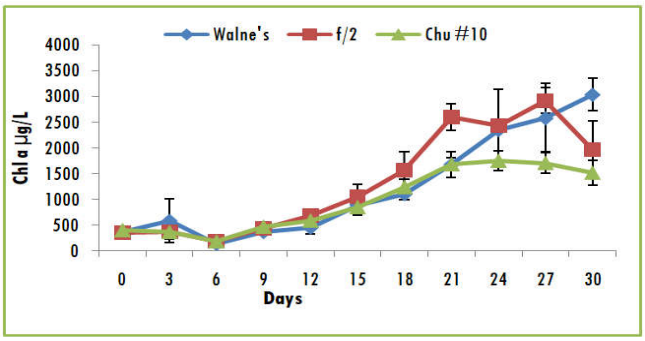

Fig. 5 Effect of culture media on production of Chl *a* in *N. salina*

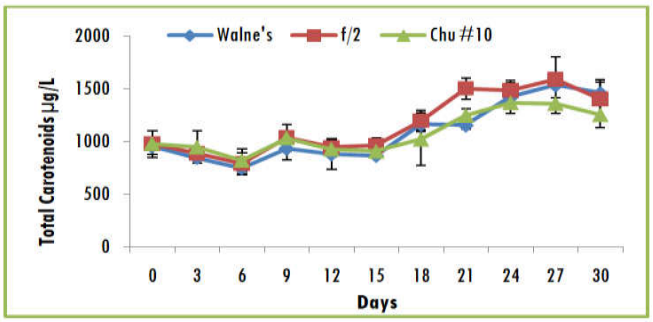

Fig. 6 Effect of culture media on total carotenoids production in *N. salina*

Effect of medium strength was studied using different dilutions of f/2 medium (f/2, f/4 and f/8). In f/4 medium, Chl *a* production was highest on 15th day of incubation (2086 µg/L). In f/2 medium, on the 15th day the Chl *a* content was less (1050 µg/L), however, there was a gradual increase thereafter and the maximum yield was observed on the 27th day (2919 µg/L) (Fig. 7).

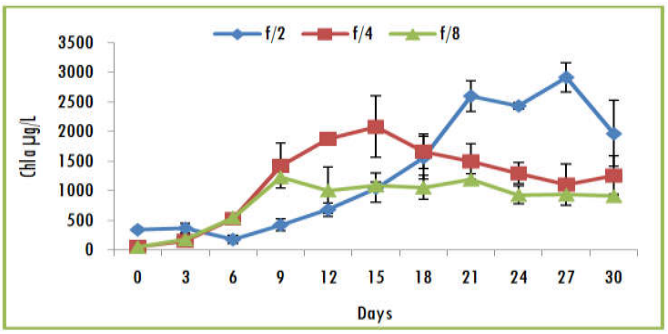

Fig. 7 Effect of medium strength on Chl *a production* in *N. salina*

On the 15th day, carotenoid production was maximum in the f/4 medium but it later decreased (Fig. 8). In f/2 and f/8 media, maximum production of carotenoid was noticed on 27th day.

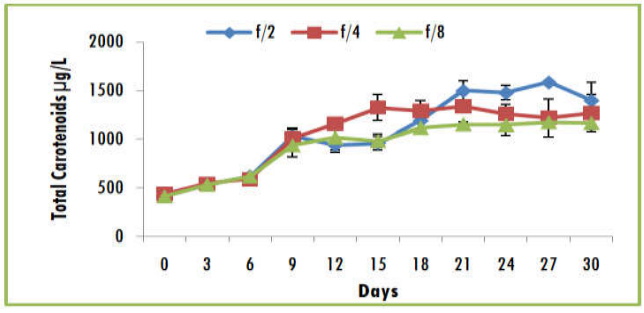

Fig. 8 Effect of medium strength on carotenoid production in *N. salina*

Protein content was higher in the log phase in all the three media, among which Walne's medium showed the maximum (Fig. 9). There was no significant difference in protein production in these three different media ($p = 0.167$, $p>0.05$).

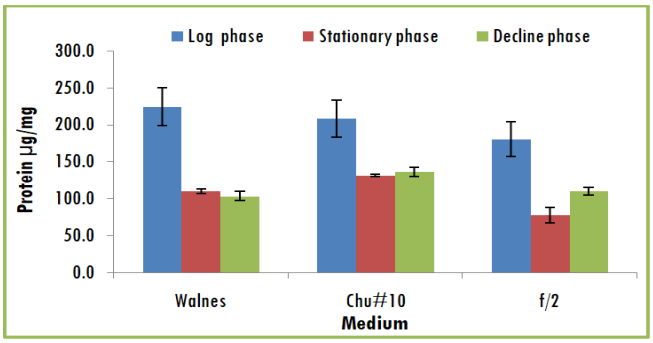

Fig. 9 Production of protein by *N. salina* in different algal culture media

In all the medium concentrations, log phase cultures produced more protein compared to other phases (Fig. 10). f/8 medium produced more protein in log phase (215 µg/mg) compared to f/2 and f/4. There was no significant difference in production of protein in these three different media concentrations ($p = 0.174$, $p>0.05$).

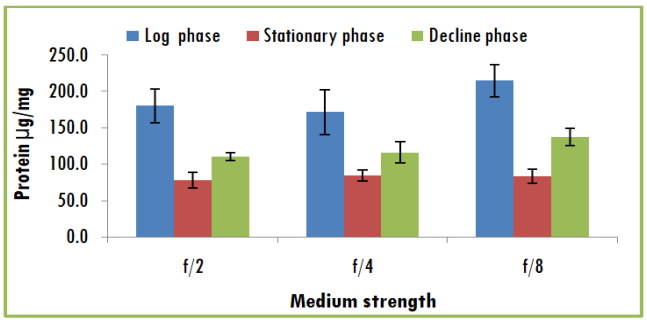

Fig. 10 Production of protein by *N. salina* in different strengths of f/2 medium

Carbohydrate production was found to be highest in f/2 medium in all the 3 phases of growth when compared with other two media (Fig. 11).

In the log phase, in all the three media, carbohydrate content was very less but it increased in the stationary and decline phases. There is significant difference in carbohydrate production by *N. salina* in the three different media ($p = 0.007$, $p<0.05$).

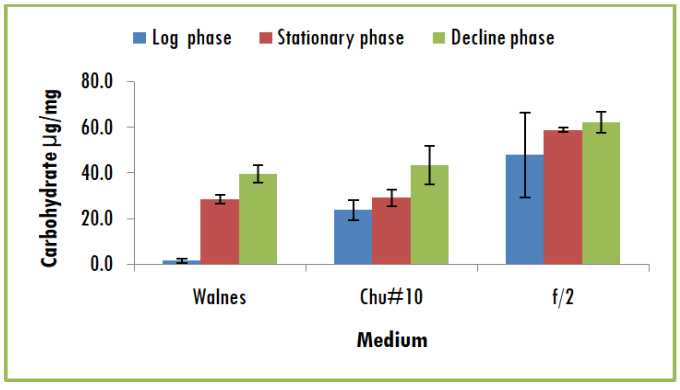

Fig. 11 Production of carbohydrate by *N. salina* in different culture media

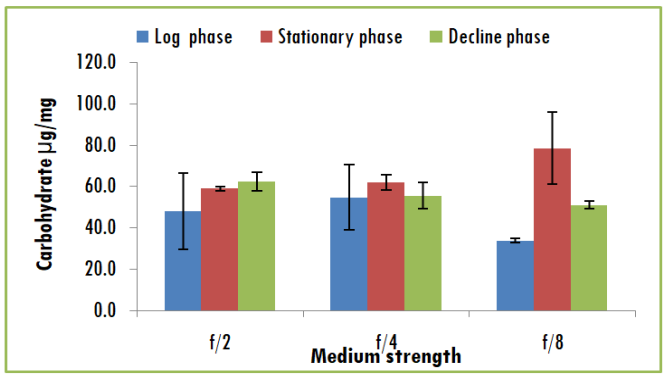

Fig. 12 Production of carbohydrate by *N. salina* in different medium strength

Carbohydrate content was comparatively higher at stationary phase in all the media concentrations. Maximum production of carbohydrate was noted at stationary phase in f/8 medium (Fig. 12). Results of ANOVA showed that there is no significant difference in production of carbohydrate in these three various media concentrations ($p = 0.174$, $p>0.05$).

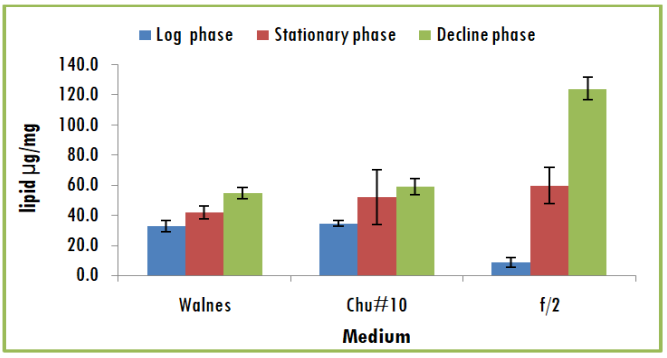

Fig. 13 Production of lipid by *N. salina* in different algal culture media

Lipid content was highest in f/2 medium in the decline phase of growth (Fig. 13). In f/2 medium the culture produced only 8.6 µg/mg in log phase but it increased up to 59.7 µg/mg in stationary phase and 124.2 µg/mg in decline phase. Statistical analysis showed that there is significant difference in lipid production between f/2 medium and the other two media ($p = 0.000$, $p<0.05$).

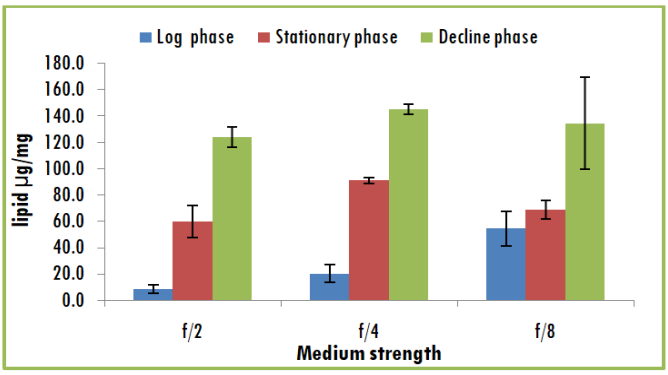

Fig. 14. Production of lipid by *N. salina* in different concentration of f/2 media

Fig. 14 shows the effect of medium concentration on lipid production. Decline phase cultures produced more lipid than cultures in other phases. Maximum lipid production was in f/4 medium (145.4 µg/mg) during the decline phase. Statistical

analysis revealed that there is no significant difference in production of lipid in these three different media concentrations ($p = 0.499$, $p>0.05$).

DISCUSSION

In the present study, three common algal culture media were used. Walne's Medium (Walne, 1970), f/2 medium (Guillard and Ryther, 1962; Guillard, 1975) and Chu#10 medium (Chu, 1942). In all the three media, a lag phase of 12 days was noted for *N. salina*. The acclimation period of microalgae is dependent on the physiological condition of the cells, physical factors and the chemical composition of the medium. In enriched media, nutrient supply imposes a level of stress on cellular physiology of the organism (Parkhill *et al*., 2001). In the present study, f/2 medium was observed to induce high cell biomass in comparison with Walne's and Chu#10 medium. f/2 medium is a commercial medium most frequently used for microalgal cultures (Johansen *et al*., 1990). Hibbered (1981) cultured *N. gaditana* in f/2 medium for better biomass. The unavailability of chelater and vitamins in the Chu#10 medium might be the reason for lower growth in the medium.

The present results are in agreement with Støttrup and McEvoy (2008) who revealed that f/2 and Walne's media provide satisfactory growth for most of the species of microalgae used in aquaculture. Tzovenis *et al*. (1997) also found that F, f/2 and Walne's medium are the chemically complete media which are commonly used to culture microalgae (Lopez-Elias *et al*., 2008) but for the large scale culture of microalgae f/2 medium is inadequate. This is because the preparation of the growth medium represents a large share of the running cost of aquaculture farms (Zhang *et al*., 2001). In the present study, three concentrations of f/2 medium were used for culturing *Nannochloropsis salina*; f/2, f/4 and f/8. Usually, commercial laboratories use f/2 medium, which is an enriched medium for microalgal culture (Guillard and Ryther, 1962; Tzovenis *et al*., 1997). Media may be evaluated on the basis of growth rate of each algal species in order to improve the microalgal biomass production and to decrease the cost (Lopez-Elias *et al*., 2008). Lopez-Elias *et al*. (2011) observed high growth rate (1.03± 0.03/day) in the case of *Tetraselmis chuii* in f/2 medium when compared with 2F medium. This indicated that a stress may be induced by the highly enriched medium in algal culture.

The nutrient requirement of each species of algae changes (Richmond, 2004). In the present study, a comparatively high growth rate was observed in f/4 medium, followed by f/8 medium. The higher growth rate in f/4 medium may be due to the possible adaptation of marine microalgae to oligotrophic condition. The best growth of *Isochrysis galbana* was obtained at 50% of f/2 medium and followed by 25% and 100% respectively (Lee *et al*., 2002). Since *N. salina* was found to grow and produce biomass maximally in f/4 medium, the cost of biomass production can be reduced considerably. Chl *a* production in f/2 medium was higher compared to both Walne's and Chu#10 media. This may be due to the high growth rate of *N. salina* in f/2 medium. Carotenoid production was not much different in the different media used. After 21 days of culture an increase of carotenoid concentration was noticed in all the three media. Fogg (1966) postulated that an increase in carotenoid concentration in algal cultures may be due to the deficiency in nitrate and phosphate in the medium. Similar observations were made by Dipak and Lele (2005) and Solovchenko *et al*. (2008).

Medium strength influenced pigment production in *N. salina*. In f/2 medium a prolonged 'lag' phase was noticed and maximum chl *a* production was on the 27th day, whereas in f/4 medium maximum production was on the 15th day. Chlorophyll production was comparatively very less in f/8 medium and the maximum production was on the 9th day. This may be due to nutrient depletion in the medium. Thus, a long lag phase with long steady growth is obtained in the f/2 medium which helps to maintain the cultures in laboratory condition for preservation but not for mass culturing (Tzovenis *et al*., 1997). In all the culture states, f/8 medium produced comparatively less carotenoids. This may be due to the low cell biomass and nutrient depletion. In f/2 medium the undepleted nitrate and phosphate may be the reason for low carotenoid production up to 27th day and the depletion of the nutrients may be the reason for the rate of increase of carotenoid production in f/2 medium after 27th day. In the present study, in all the three culture media Walne's, f/2 and Chu#10, protein production was high in log phase compared to the other two growth phases.

It is found that in microalgal cultures, protein will be high in log phase and it declines when the cells enter into stationary and decline phases where nutrients are depleted (Ogbonna and Tanake, 1996; Lourenco *et al*., 1997; Zhu *et al*., 1997). The superiority of Walne's medium in protein production may be due to high amount of nutrients in the medium compared to that of f/2 medium. Proteins are the building blocks for tissue biosynthesis in animals. So protein content of microalgae is important when microalgae are used as an aquaculture feed (Wikfors *et al*., 1992; Kreeger, 1993). A protein concentration range of 19-70% of cellular dry weight depending on growth stage was observed in microalgae (Gatenby *et al*., 2003). Factoring this, Radhakrishnan *et al*. (2009) cultured *N. salina* in Walne's medium to apply it as an aquaculture feed. The strength of the culture medium is an important parameter to be considered while developing a suitable medium for algal growth. In f/8 medium the protein production was very high in log phase compared to other concentrations of F medium. Similar quantity of protein was noted in f/2 and f/4 medium. An increase in protein production in log phase of nutrient deficient cultures may be due to the increased production of proteinaceous substance like chelater (Andersen, 2005).

Carbohydrate production was high in f/2 medium in all the stages of algal growth. Biochemical composition of an algal cell depends on the culture age and nutrient availability (Morris *et al*., 1983; Harrison *et al*., 1997). As the cultures enter the stationary phase, the cultures produce more carbohydrate than protein (Zhu *et al*., 1997). 5.9-16.7% increase in carbohydrate production was detected in 10 species of Chlorophyceae and Prasinophyceae in stationary phase (Brown and Jeffrey, 1992). In f/2 medium, carbohydrate concentration was high in the decline phase. But, maximum carbohydrate was produced in f/8 medium in the stationary phase. It is generally accepted that depending on the algal species in stationary and decline phase, carbohydrate or lipid may increase as a reserve for carbon (Becker, 1994). A high growth rate with nutrient depletion may activate cells in stationary phase to store more carbohydrate than protein as shown by Tomaselli *et al*. (1987).The production of lipid was also influenced by the culture medium and the culture phases. Microalgal lipid is important for gametogensis, gonad maturation and embryo development in aquatic animals (Pollero, 1983). In the present study, lipid production rate was

very low in log phase cultures but a gradual increase in lipid production was noticed in stationary phase and it attained maximum in decline phase. In microalgae, total lipid increases in decline phase (Lourenco *et al*., 1997). A high rate of microalgal growth in f/2 medium produces nitrogen depletion in the medium. This may be the reason for high lipid production in decline phase in f/2 medium. Zhu *et al*. (1997a) postulated that nutrient limiting conditions in decline phase will trigger the carbon incorporation into algal cells as carbohydrates and lipids. Yeesang and Cheirslip (2011) also found that nitrogen deficiency in the medium may activate algal cells to accumulate carbon metabolites as lipid. Lipid production was high in the decline phase, irrespective of the medium strength. In log phase, lipid production was very low. A considerable increase in lipid production in the log phase was seen in f/8 medium. This may be due to the low nutrient content in f/8 medium. Decline phase culture in f/4 medium produced the maximum amount of lipid compared to other media.

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