

Effect of Salinity and pH Ranges on the Growth and Biochemical Composition of Marine Microalga- *Nannochloropsis salina*

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ABSTRACT

Nannochloropsis salina is a free living unicellular marine microalga. The present study analyse the influence of salinity and pH on the growth and biochemical composition of this microalga. The alga could tolerate a wide range of salinities. Maximum growth rate and production of chlorophyll, carotenoids and protein was noted in cultures with 40ppt salinity. Cultures with a salinity of 50ppt recorded maximum carbohydrate and lipid production. However, the organism could not tolerate low salinity levels as no growth was observed at zero and 10ppt salinity. The optimum pH required for the maximum production of biochemicals varied considerably. While maximum growth rate and Chl *a* production were noted at pH 9, carotenoid production was more in cultures with pH 6. Production of protein and carbohydrate was high at pH 10, and lipid at pH 7.

Highlights

- Effect of salinity and pH on growth and biochemical composition of microalgae.

Keywords: *Nannochloropsis salina*, Microalgae, salinity, pH

Microalgae are the key primary producers on earth and form the base of aquatic food chain. They have been the subject of biotechnological research since early 1950's. Microalgae are targeted as a source of health foods, aquaculture feed, biofuels and bioactive substances (Burlew 1953; Spolaore *et al.* 2006). These tiny plants have high biomass production capacity, faster growth rate and high CO₂ fixation rates compared with higher plants. Microalgae are a source of high-value products such as fatty acids, natural colourants, therapeutics and biopolymers. With the development of aquaculture and other branches of biotechnology, including biofuel production, the necessity of algal biomass production gained much interest (Wahidin *et al.* 2013). Ecophysiological studies are helpful in determining environmental

conditions which are optimal, favourable or merely tolerable for the growth of algal species.

Salinity is an important environmental factor influencing microalgal growth in marine algal cultures. In nature, salinity influences the distribution and abundance of phytoplankton species in the aquatic ecosystem. With variation in salinity, changes in cellular volume and osmotic adjustments were noticed in microalgae (Lee 2008). Algae differ in their adaptability to salinity, and based on their tolerance levels they are grouped as halophilic (salt requiring for optimum growth) and halotolerant (having response mechanism that permits their existence in the saline medium). In both these conditions, microalgae produce some metabolites to protect



themselves from salt injury and as a balance to the surrounding osmotic pressure (Chen and Jiang 2009). The growth rate and metabolic activity in most marine microalgae is checked below salinity of 20ppt (Andersen 2005). Optimum salinity of microalgae is species dependent. The optimum growth and tolerance to variations on salinity depend mainly on the species and its natural habitats. Hence, documentation of optimum salinity and salinity range of each species is significant. pH also influences the growth rate of microalgae. The optimum pH of a majority of microalgae ranges from pH 7-9. There are not many reports available on the optimum pH and salinity for the growth of *N. salina* (Bartley *et al.* 2014). Optimization of these parameters is essential for the cultivation of this economically important group of alga. Variation in salinity causes cellular biochemical changes (Kirst 1989).

pH is also one of the most influential factors in algal culture. Microalgal metabolism depends on pH of the culture medium. Microalgal cell membrane is completely permeable to H⁺ and OH⁻ ions. Extreme concentrations of H⁺ and OH⁻ ions (low pH and very high pH) can affect algal growth and development. The optimum pH of most of the microalgae is between 7 and 8 (Andersen, 2005). Abu-Rezq *et al.* (1999) found that carbon dioxide incorporation in *Nannochloropsis* sp. culture medium induced a decrease in pH which adversely affected the growth of *Nannochloropsis*. Most of the species used in aquaculture require a pH between 6 and 9 with an optimum close to neutrality (Støttrup and McEvoy 2008). A change in the extracellular pH will affect not only the growth rate but also the biochemical composition of the cells, including extracellular production (Kirkvold, 1994; Mykkestad and Swift, 1998). Rocha *et al.* (2003) found that an important factor affecting the growth of *Nannochloropsis* is pH of the medium. Lopez-Elias *et al.* (2008) observed that pH tends to increase depending on the age of *Chaetoceros muelleri* cultures.

Moisander *et al.* (2002) found that changes in salinity of water affects the growth and biochemical composition of estuarine planktonic algae. Pigments are the most obvious characteristics of microalgae. Each phylum has its own particular combination of pigments. Chlorophyll is the primary photosynthetic pigment in microalgae. Chl *a* is reported from all microalgae whereas, chl *b* is reported from

Chlorophyceae and Euglenophyceae. Chl *c*, *d* and *e* are found in several marine algae. Microalgae are also rich in carotenoids. The average concentration of carotenoids in algae is about 0.1-0.2% of the dry weight. In *Dunaliella* up to 14% per dry weight β-carotene was detected under appropriate culture conditions (Becker 2004). Microalgal carotenes are used as food additives and possess high antioxidant potential.

Protein is the major organic component of microalgae (15-25% of dry weight) followed by lipid (5-20%) and carbohydrates (5-12%) (Brown *et al.* 1997). The food demand in the coming decades may not be covered by conventional agriculture. Therefore efforts must be taken to find alternate unconventional protein source (Becker and Venkataraman 1984). Due to high growth rate and rich protein content microalgae are superior to agriculture crops for protein production. Tropical countries like India are well suited for algal cultivation. In microalgae, carbohydrate can be found in the form of sugars, starch, cellulose and other polysaccharides. Among carbohydrates, glucose is the predominant sugar in all the species of microalgae. In Chlorophyceae, the cell wall is made of cellulose, a polymer of glucose.

Lipids and fatty acids function as membrane components, as a source of energy, as metabolites and storage products. In microalgae, the average lipid content varies between 1 and 40% and under some unfavourable conditions it may be as high as 85% of the dry weight. Algal lipids are composed of fatty acids having carbon number C12-C22. They may be either saturated or unsaturated. The value of microalgae as a food source depends on characteristics such as cell size and biochemical composition. The biochemical composition of algae varies with species, light intensity, duration of light, temperature, culture medium, pH, salinity and growth stage. Variation in biochemical profile due to growth stage is frequently related to culture age and nutrient depletion, in batch culture (Morris *et al.*, 1983). It is well known that microalgae usually respond with physiological and biochemical alterations to the culture conditions where they grow (Scragg *et al.* 2002). Therefore, it is important to study the influence of environmental and nutritional factors on the biochemical composition of microalgae. However, information related to the influence of environmental characters on algal



chemical composition is still scarce (Garcia *et al.* 2012).

Nannochloropsis is popular as a source of high value pigments, proteins, carbohydrates and lipids including PUFA. Studies on the biochemical profile of *N. salina* in different culture conditions would be useful in microalgal biotechnology, aquaculture and biofuel industry. The present study analyses the influence of salinity and pH on the growth and biochemical profile *N. salina*.

MATERIALS AND METHODS

Nannochloropsis salina culture maintained at 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 30ppt. 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± 2°C).

Salinity tolerance of *N. salina* was studied by growing the cultures in *f/2* medium having different salinity. The salinity range of 0-50ppt was used in the present study.

Effect of pH was studied in *f/2* medium with pH 6, 7, 8, 9 and 10. pH of the medium was adjusted using 1 N NaOH and 1 N HCl.

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:

$$Ca = 11.85 (\text{OD } 665) - 1.54(\text{OD } 645) - 0.08 (\text{OD } 630) \text{ (Jeffrey and Humphrey, 1975)}$$

Chlorophyll *a* $\mu\text{g/L} = Ca \times \text{Extract volume (mL) / Volume of sample (L)} \times \text{Path length of the cuvette (cm)}$

CCar = 4 (OD 480) (Strickland and Parsons, 1972).

Total carotenoids $\mu\text{g/L} = \text{CCar} \times \text{extract volume (mL) / Volume of sample (L)} \times \text{Path length of the cuvette (cm)}$



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 AND DISCUSSION

Effect of salinity on cell density was studied by growing the cultures in media having salinity that ranged from 0 to 50ppt (Fig. 1). *N. salina* could not grow in media with salinity 0 and 10ppt. However, good growth was observed in the salinity range 30 to 50ppt.

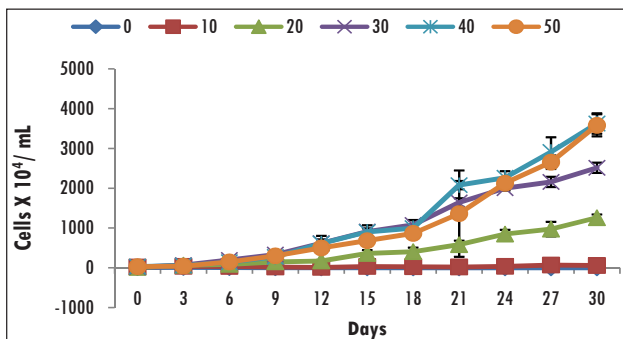


Fig. 1: Effect of salinity on cell number of *N. salina*

The mean growth rate of *N. salina* at salinity 0 and 10ppt was noted as zero as there was no growth in both the cultures (Fig. 2). At salinity 20ppt the growth rate was 0.144/day. Maximum mean growth rate was obtained at salinity 40ppt. Statistical analysis showed that there is significant difference in growth rate in different salinity ($p = 0.000, p < 0.05$).

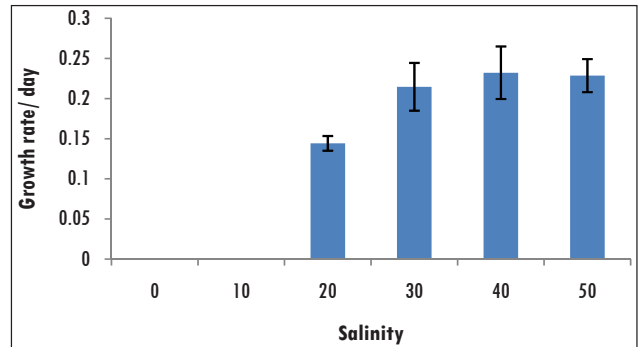


Fig. 2: Effect of salinity on growth rate in *N. salina*

Effect of pH on the cell density was studied by adjusting the initial pH of the media over a range of 6 to 10. *N. salina* showed a wide tolerance to pH ranging from 6-9. However, cell number was very low at pH 10. Maximum cell number was observed at pH 8. Results are presented in Fig. 3.

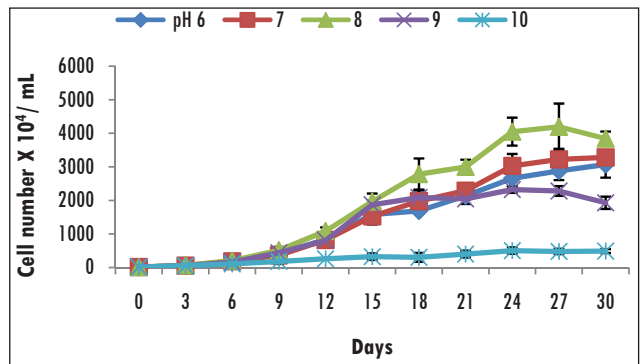


Fig. 3 Effect of pH on cell number of *N. salina*

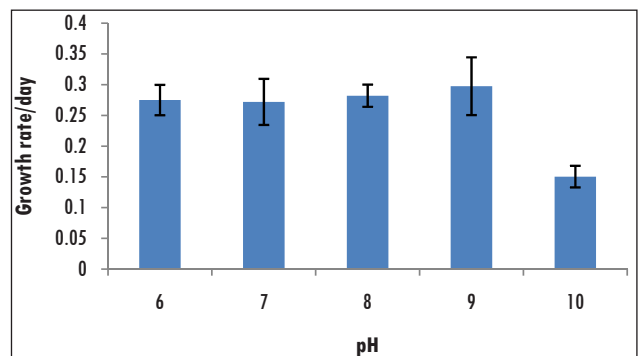


Fig. 4: Effect of pH on growth rate in *N. salina*

Fig. 4 shows the growth rate of *N. salina* in different pH. Maximum growth rate was obtained at pH 9 (0.297/day), and the lowest at pH 10 (0.15/day). ANOVA results showed that there is significant difference between growth rates of *N. salina* at different pH levels ($p = 0.001$, $p < 0.05$).

Salinity also influenced the production of Chl *a* in *N. salina* (Fig. 5). Maximum production was at 40ppt (2308 $\mu\text{g/L}$ on 30th day). At 50ppt, there was considerable reduction in Chl *a* production after 18th day of incubation.

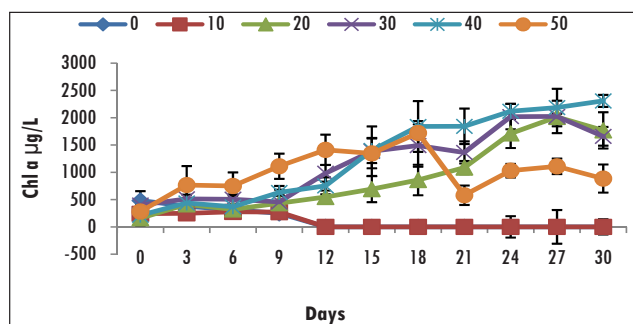


Fig. 5: Effect of salinity on Chl *a* in *N. salina*

There was not much difference in Chl *a* production in cultures grown in the pH range from 6 to 9 (Fig. 6). However, at pH 10 the chlorophyll production was less.

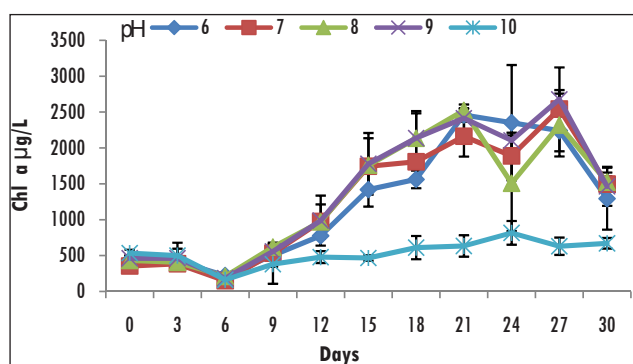


Fig. 6: Effect of pH on Chl *a* production in *N. salina*

Maximum carotenoid production was noticed at 40ppt on the 27th day (1766.7 $\mu\text{g/L}$) followed by 30 ppt on the 27th day (1547 $\mu\text{g/L}$) and at 20ppt on the 30th day (1333 $\mu\text{g/L}$). Production of carotenoid decreased considerably after the 18th day at 50ppt (Fig. 7).

There was not much variation in carotenoid production under the different pH tested (6-10) (Fig. 8). However, carotenoid production was comparatively lesser at pH 10.

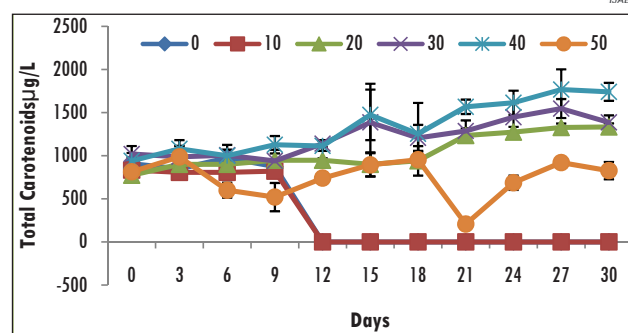


Fig. 7: Effect of salinity on total carotenoid production in *N. salina*

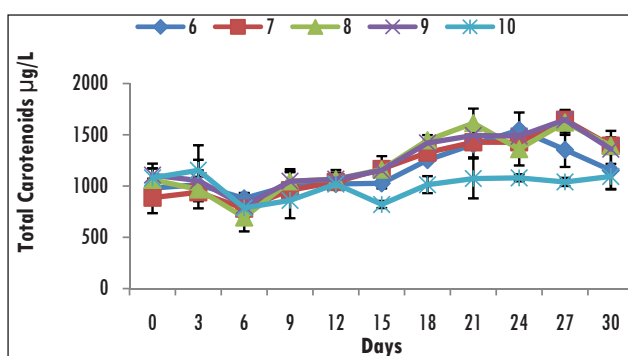


Fig. 8: Effect of pH on carotenoid production in *N. salina*

Biochemical composition was determined at the log, stationary and decline phases of growth. Change in salinity of the medium influenced the production of protein in *N. salina* (Fig. 9). Maximum protein production was observed in the log phase culture at 40ppt, followed by 30ppt and 50ppt. Statistical analysis showed significant difference in protein production in different salinities ($p = 0.000$, $p < 0.05$).

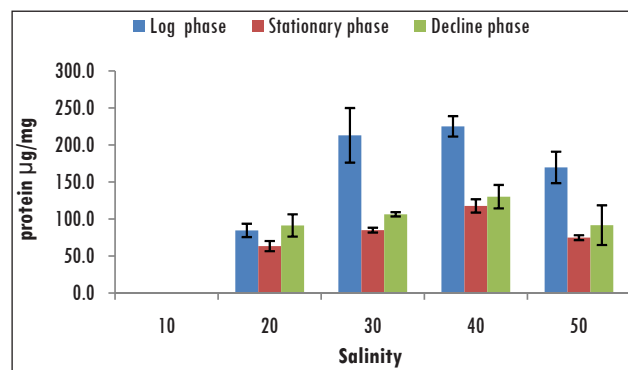


Fig. 9: Production of protein by *N. salina* under different salinity

pH influenced the production of proteins by *N. salina* (Fig. 10). At all the pH levels the production of protein was highest in log phase. Maximum protein



content was observed in the log phase culture at pH 10. Statistical analysis showed significant difference in protein production at different pH levels ($p = 0.000, p < 0.05$).

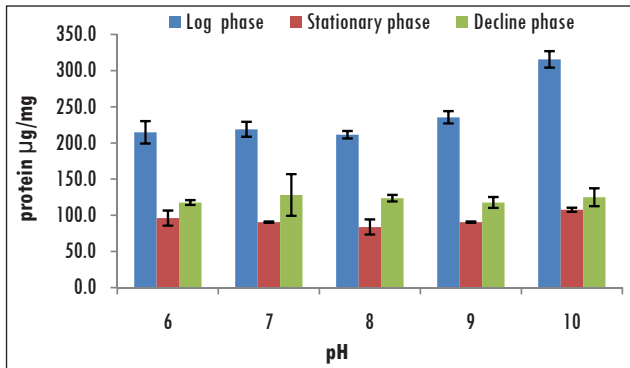


Fig. 10: Production of protein by *N. salina* in different pH

Carbohydrate production was highest at 50ppt in the log phase (Fig. 11). At 50ppt, the log phase culture produced 134.8 µg/mg carbohydrate but it decreased to 86.1 µg/mg in stationary phase. Statistical analysis revealed that there is difference in carbohydrate production in various salinity ($p = 0.000, p < 0.05$).

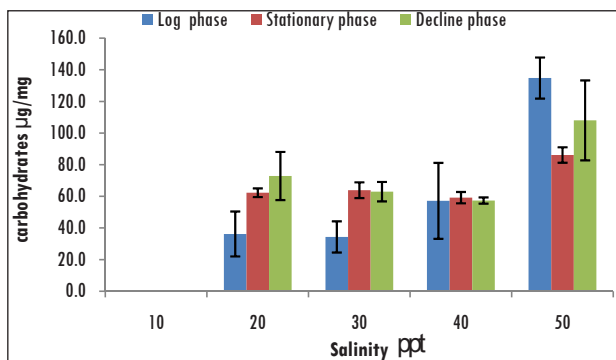


Fig. 11: Production of carbohydrate by *N. salina* in different levels of salinity

Carbohydrate production under different pH levels was studied (pH 6-10) (Fig. 12). At pH 6, 8 and 10 the stationary phase cultures produced more carbohydrate, but at pH 7 and 9 the cultures produced more carbohydrate in decline phase. ANOVA results showed that there is no significant difference in carbohydrate production in various pH ($p = 0.064, p > 0.05$).

The highest concentration of lipid was noted in cultures of salinity 50ppt during the decline phase (Fig. 13). During log and stationary phases, the lipid content was low compared to decline phase at all

salinity levels. Statistical analysis showed that there is no significant difference in lipid production in different salinities ($p = 0.228, p > 0.05$).

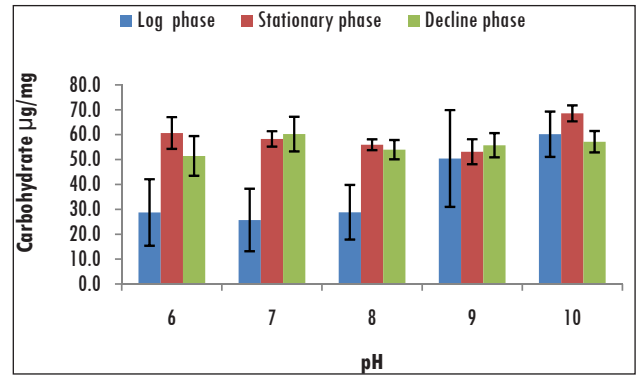


Fig. 12: Production of carbohydrate by *N. salina* under different pH

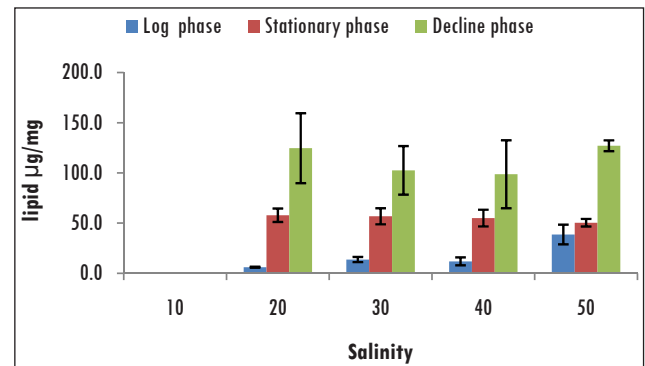


Fig. 13: Production of lipid by *N. salina* in different salinity

Fig. 14 presents the effect of pH on lipid production in different culture phases. In decline phase all the cultures produced more amount of lipid compared to stationary and log phase. In decline phase the maximum lipid production was noted at pH 7 (124.8 µg/mg). ANOVA results showed that there is no significant difference in lipid production in different pH ($p = 0.032, p > 0.05$).

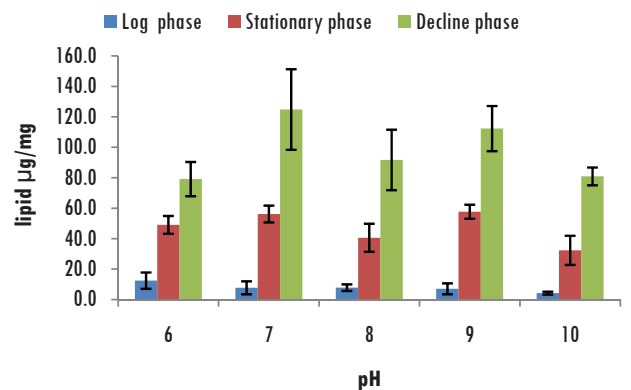


Fig. 14: Production of lipid by *N. salina* in different pH



Salinity is one of the most important environmental factors that influence the growth, biochemical composition and distribution of marine microalgae. Additionally, it has an influence on the density, viscosity and solubility of diverse gases in the water column (Johansen *et al.* 1990). Most of the microalgae have high tolerance to salinity change and can grow well in a wide range of salinity (Brown *et al.* 1996). In the present study *N. salina* was cultured in media having a salinity range of 0ppt to 50ppt. There was no growth at 0 and 10ppt. At 20ppt, growth was very slow and at 30, 40 and 50ppt, the alga showed better growth. Maximum growth was noted at 40 ppt. Salinity tolerance capacity, and optimum salinity of microalgae are species specific (Thessen *et al.* 2005). Cho *et al.* (2007) observed maximum growth of *N. oculata* at 30 ppt salinity. The present results agree with Abu-Rezq *et al.* (1999) who found that growth rate of *N. salina* will change with change in salinity and *N. salina* will grow better between 20 and 40ppt salinity.

Salinity is a factor which controls the productivity in natural aquatic habitats (Booth and Beardall 1991). In the present study, production of chlorophyll was maximum at 30 and 40ppt salinity. A decrease in photosynthetic rate was noted in microalgae in high salinities, which might be the reason for decrease in the growth rate in higher salinities (Hart *et al.* 1991). High salinity in the medium will negatively influence the production of chlorophyll content in algae (Rai *et al.* 2015). Changes in salinity also affect the photosynthesis rate of phytoplankton (Moisander *et al.* 2002; Lartigue *et al.* 2003).

Maximum production of carotenoids was noted at 40ppt salinity. Dipak and Lele (2005) found that with increase in salinity chlorophyll amount decreases and carotenoid content in the cell increases. This increase in carotenoid with increase in salinity may be a protective measure of the cell against increased salinity stress (Sunilkumar and Dharmaraj 2003).

Salinity showed considerable influence on protein production. Changes in salinity affect the biochemical composition of microalgae (Moisander *et al.* 2002a). Protein production was more at 30 and 40ppt salinity, compared to other salinity ranges. Maximum production of protein was noted at 40ppt salinity. Irrespective of salinity, protein concentration was high in log phase and was low in stationary phase. In the present study protein

production at 50ppt salinity was low compared to 30 and 40ppt. Richmond (1986) and Rai *et al.* (2015) also reported that protein synthesis in most microalgae cells are affected by high salinities. Vonshak *et al.* (1996) reported that the algae cultivated with high salt concentrations had low chlorophyll and protein content.

Salinity influenced carbohydrate production considerably. Maximum carbohydrate production was observed in the log phase cultures at 50ppt salinity. Carbohydrate content was low in actively growing cultures at 30 and 40 ppt salinity. Warr *et al.* (1985) and Tomaselli *et al.* (1987) have reported that carbohydrate synthesis was stimulated by stress conditions. According to Gill *et al.* (2002) soluble sugars in cells play an osmotic regulatory function in high salinity ranges, which could be the reason for high carbohydrate production at 50ppt salinity in the present study. It may be an adaptive measure of algal cells to survive under high saline conditions. Similar to the present study, an increase in carbohydrate content was observed in *Scenedesmus quadricauda* when the algae are exposed to high salinity (Kirroliia *et al.* 2011).

Production of lipid was influenced by salinity. Maximum lipid production was noted at 50ppt salinity in the decline phase. In all salinity ranges, lipid production was low in log phase and increased gradually and maximum was observed in the decline phase. Similar to the present study, a considerable increase in lipid production was noted at high salinities in some species of microalgae (Kirst, 1989). When *N. oculata* was cultured with increasing salinities, highest percentage of lipid was found at increased salinities (30-35ppt) (Renaud and Parry, 1994). Abu-Rezq *et al.* (1999) and Guschina and Harwood (2006) also have shown that lipid content of microalgae changes with change in salinity. In the present study, algal lipid production was high at low salinity also (20ppt). Present results of high lipid in low salinity were supported by findings of Griffiths and Harrison (2009) where they found high lipid production in *T. weissflogii* at a low salinity range. High lipid content at high and low salinity may be due to adaptation under stress conditions (Takagi *et al.* 2006).

pH is another important factors to consider for microalgae culture. Algal cell membrane is completely permeable to H⁺ and OH⁻ ions, and



the concentration of such ions may affect cellular functions, causing death at extreme concentrations of H⁺ (very low pH). High levels of OH⁻ (high pH) can affect microalgal growth, but it is not lethal as a low pH condition (Johansen *et al.* 1990). The optimum pH for most of the microalgae ranges from 7 to 8 (Abalde *et al.* 1995). An important variable that affected *Nannochloropsis* growth was pH value (Rocha *et al.* 2003). Most species of microalgae used in aquaculture industry require a pH between 6 and 9, with an optimum close to pH 7 (Støttrup and McEvoy 2008). However, studies of Abu-Rezq *et al.* (1999) with *Tetraselmis* showed that pH is not significantly affecting growth rate. Fabregas *et al.* (1985) and Montaini *et al.* (1995) also found that pH of the medium was not significantly affecting the growth of *Tetraselmis*. In the present study, the maximum growth rate of *N. salina* was noted at pH 9 followed by pH 8. Lowest growth rate was noted at pH 10. It is in agreement with the observations of Spolaore *et al.* (2006a) who obtained a good growth for *Nannochloropsis* sp. at a pH of 8.4; similar results were also obtained by Rocha *et al.* (2003). The results also agree with Perez (1994) who found that pH significantly affected the growth of *Isochrysis galbana*.

Chl *a* production was also influenced by pH. Maximum production was noted at pH 9. Chl *a* production was very low at pH 10. An increase in Chl *a* production at pH 9 may be due to the high growth rate of *N. salina* at pH 9. The actively dividing cells in good physiological condition produce maximum amount of chlorophyll (Balode *et al.* 1998). Maximum carotenoid production was noted at pH 6. Except at pH 10, carotenoid production was almost similar at different pH. Stress condition in the culture increases the production of carotenoids (Borowitzka *et al.* 1984). In the present study the production of chlorophyll was very high at pH 9, whereas carotenoid production was higher at low pH (pH 6 and 7).

When the effect of pH was studied (pH 6-10), protein production was found to be more in the log phase irrespective of the pH of the media. Maximum protein production was shown by the log phase culture at pH 10. The organism might be producing more protein to overcome the stress caused by high pH.

At pH 6, 8 and 10 the cultures produced more

carbohydrate in the stationary phase whereas at pH 7 and 9 the carbohydrate production was more in the decline phase. pH 6 and 10 produced maximum amount of carbohydrates. Except at higher pH the log phase cultures produced less amount of carbohydrate. Studies by Warr *et al.* (1985) and Tomaselli *et al.* (1987) have shown that carbohydrate synthesis is stimulated by stress conditions.

Lipid production was also influenced by the culture pH. At all the pH conditions lipid production was maximum in decline phase and was very low in log phase. Maximum lipid production was noted at pH 7 in decline phase. But at pH 6 and pH 10 the lipid production was very low compared with other pH ranges. Usually an increased lipid production was usually seen in stressed condition however, Dayananda *et al.* (2007) found that hydrocarbon content of *Botryococcus braunii* was not affected by the culture pH.

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