CHAPTER - 5

DISCUSSION
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The bright and beautiful orange colour of *Glaucocystis nostochinearum* Itzigsohn and *Oocystaenium elegans* Gonzalves et Mehra in their natural habitat drew the attention to collect it from their natural habitat and carried out preliminary investigations for their coloured pigments. As the preliminary investigations were very encouraging in terms of carotenoid content, field observations were made at different locations of Assam to ascertain the availability of both the algae. The algae were found to grow in the ponds and 'beels' throughout Assam.

The alga *Glaucocystis nostochinearum* Itzigsohn (Geitler, 1923) is classified under division-Chlorophyta, class-Chlorophyceae, order-Chlorococcales (Phillipose, 1967), family-Oocystaceae (Bohlin, 1901); sub family-Oocystoideae. The other species, *Oocystaenium elegans* Gonzalves et Mehra, (Gonzalves and Mehra, 1959)) is also classified under the division-Chlorophyta, class-Chlorophyceae, order-Chlorococcales (Phillipose, 1967), family-Oosystaceae (Bohlin, 1901), sub-family-Eremosphaeroideae. Both these algae are fresh water aquatic forms and grow in the North Eastern regions of India.

The nutritional requirements of algae are essentially the same as those of the higher plants. For algal culture, some well-defined nutrient media have been suggested by different workers for different groups of algae (Chu, 1942; Fogg, 1965; Bold, 1942; Rao et al., 1981). Factors like pH of the media, intensities of light, different temperatures as well as different growth hormones play an important role in
artificial algal cultures. Since pH has regulatory effects on various cellular enzymes (Dwivedi et al., 1992), pH of the medium is essential for maintaining the active metabolism of cells in culture. An alga growing in natural habitat is expected to grow in culture medium having the same pH value as that of the natural habitat (Pringsheim, 1972). Thus the selection of most suitable pH range can ensure maximum yield of algae in artificial conditions. Light intensities play a significant role in pigmentation as well as in the growth of algae (Takatori and Imahori, 1971; Lipps, 1973). Significant change in pigmentation was observed in algae when cultured under continuous illumination for prolonged periods (Srivastava and Nizam, 1986). Accumulation of carotenoids in algae could be enhanced by exposing the algae to Ultraviolet-A (320-400 nm) radiation with 0.5 to 0.8M salinity (Jahnke, 1999). The environmental conditions like changes in temperature have drastic effects on growth and development as well as on yield of pigments in plants (Reddy et al., 1995). A light and dark cycle of 14 and 10 hours and 25° ± 1°C temperatures are the most favourable conditions for algal growth (Jose, 1978). When organisms like algae are collected from their natural habitat and grown in artificial media they are adversely affected due to deficiencies in nutritional ingredients (Chu, 1942; Rao et al., 1981). It is possible to provide required environmental conditions during artificial cultures through appropriate levels of different mineral nutrients. In a similar study nine inorganic media were employed to determine the optimal growth of two species of Scenedesmus algae and Juller's and Bold's media were reported to
be most suitable for the maximum growth of both the species (Bajaj and Srivastava, 1985).

In the present investigation artificial cultures of *Glaucocystis nostochinearum* Itzigsohn and *Oocystaenium elegans* Gonzalves et Mehra were initiated in Bold Basal Medium (BBM), Modified Chu-10 Medium and Bristol Medium. Growth in terms of increase in the total chlorophyll content was studied after 160 days of inoculation of the algal cells in the media. It was noteworthy to observe that *Glaucocystis nostochinearum* Itzigsohn and *Oocystaenium elegans* Gonzalves et Mehra responded differentially to the growth media. It is evident from the experiments on growth that *Glaucocystis nostochinearum* Itzigsohn can grow well in Bold Basal Medium (BBM) while *Oocystaenium elegans* Gonzalves et Mehra grow well in Modified Chu-10 Medium. Suitable growth of the experimental algae in these media was because there was a perfect balance of all the minerals in the media and laboratory conditions. Hence BBM was selected for growing and maintaining *Glaucocystis nostochinearum* Itzigsohn and Modified Chu-10 Medium was selected for growing and maintaining *Oocystaenium elegans* Gonzalves et Mehra. Since Bristol Medium did not help in initiating algal growth of any kind, the medium was not used during the investigations.

*Glaucocystis nostochinearum* Itzigsohn was grown in Bold Basal Medium and was maintained in batch cultures under controlled illumination with 1200 llux light intensity from cool-white fluorescent bulbs on a 14:10 hours light and dark cycle at 30 ±1°C. The pH of the growth medium was maintained at 6.0 and
the cultures were shaken twice a day with the help of a vortexer. Larger volumes of culture medium under similar conditions were also inoculated with cells and maintained as stocks for subsequent experiments.

*Oocystaenium elegans* Gonzalves et Mehra was cultured in Modified Chui-10 medium with same culture conditions as used for *Glaucocystis nostochinearum* Itzigsohn.

Following the culture conditions as mentioned above both the algae were produced at large scale (mass culture) for obtaining sufficient experimental material to undertake the biochemical analysis.

Growth measurements in artificial cultures of *G. nostochinearum* and *O. elegans* were carried out considering the parameters like total chlorophyll content, fresh weight, cell dry weight and packed cell volume (PCV) of the algae etc.

The total chlorophyll content was measured as an index of growth. For total chlorophyll content biomass of *G. nostochinearum* was harvested at 10 days of growth interval for 90 days and total chlorophyll content was estimated spectrophotometrically. In the experiment it was found that the total chlorophyll content of the algae exhibits an increasing trend up to 30 days of growth and reached an optimal level at 40 days of growth beyond which it had shown a decreasing trend up to 90 days in Bold Basal Medium (BBM). In *O. elegans* it was found that the total chlorophyll content of the algae increased up to 30 days of growth and reached an optimal level at 40 days of growth in Modified Chu-10 Medium beyond which the amount did not increase.
In the present investigation chlorophyll estimation was taken as a measure of growth, as chlorophyll is directly responsible for harvesting light used for generation of assimilatory power essential for CO₂ fixation (Raoof and Kaushik, 2002). The photosynthetic activity in the course of the life cycle has been found to vary with the developmental status of cell (Tamiya et al., 1953; Nihei et al., 1954; Lorenzen, 1959). The observed changes in photosynthetic range may greatly depend on environmental conditions, both those existing during the experiments and those of periods prior to actual measurement of photosynthesis (Sargent, 1940; Sorokin, 1958).

Fresh weight of the algae was considered as an index of algal growth. The fresh weight of *G. nostochinearum* cultured in Bold Basal Medium (BBM) increased up to 80 days of culture and gradually decreased to a steady state. Since BBM provided all the necessary requirements for favourable growth of the alga, it was possible to harvest an appreciable amount of fresh biomass of *G. nostochinearum* with a short span of time. In case of *O. elegans*, gain in fresh weight was up to 50 days of growth and the growth was gradually slowed down and finally attained a steady state. During the investigation it was observed that the growth rate of *O. elegans* was not like that of *G. nostochinearum* and therefore further study on the culture conditions may help in enhancing biomass production in *O. elegans*.

Measurement of growth in the experimental algae was also taken by considering dry weight of cells as a parameter. The cell dry weight of *G. nostochinearum* cultured in Bold Basal Medium (BBM) increased up to 80 days of
culture and gradually decreased to a steady state. In *O. elegans* gain in cell dry weight was observed up to 50 days of growth and it gradually decreased and finally attained a steady state after 90 days of culture.

The growth rate of the algae was also measured in terms of packed cell volume (PCV). The packed cell volume (PCV) of *G. nostochinearum* cultured in Bold Basal Medium (BBM) increased rapidly up to 60 days of culture and gradually decreased and reached steady state after 80 days. In *O. elegans* the packed cell volume (PCV) of the algae exhibited an increasing trend up to 60 days of growth in Modified Chu-10 Medium and thereafter the packed cell volume (PCV) became steady up to 90 days.

Sorokin (1960) computed the photosynthetic activity on the developmental stage of the cells on the basis of packed cell volume and cell dry weight of *Chlorella pyrenoidosa* against light intensity. Bongers (1958) compared the photosynthetic rates computed on the basis of packed cell volume and cell dry weight and concluded that the amplitude of fluctuation in the course of cell development is much smaller for rates calculated on the dry weight basis. The ratio between and packed cell volume of cells does not remain constant in cell development (Sorokin, 1957; Bongers, 1958). It changes in favour of dry weight.

In general both *G. nostochinearum* and *O. elegans* in artificial culture conditions exhibited growth up to 60 days beyond which the growth remained static as well as decline. This may be attributed to the availability of the nutrients in the media which gradually depleted due to utilization by the organisms. This clearly
suggests that in artificial cultures of these algae, the nutrient media should be changed after 60 days of inoculation.

The microalgae are important source of carotenoids. Many of them have already been investigated for carotenoid production. For example *Dunalie\textit{lla salima}* is the most suitable organism for the mass production of $\alpha$-carotene and $\beta$-carotene (Chidambara Murthy \textit{et al.}, 2005) and *Haematococcus pluvialis* is a rich source of astaxanthin (Margalith, 1999; Cysewski and Todd Lorenz, 2004). In the present investigation an attempt has been made to isolate and characterize the carotenoids in *G. nostochinearum* and *O. elegans*. The algae collected from natural habitat and as well as from artificial cultures were dried under shade and powdered. Extraction of carotenoids was carried out under yellow light in the laboratory. The algal powder was homogenized and sonicated to rupture the thick cell wall so that organic solvents can penetrate inside the cell. The carotenoid pigments along with chlorophyll were extracted by dichloromethane ($\text{CH}_2\text{Cl}_2$). The estimation was carried outt spectrophotometrically by following the procedure of Britton (1985) with the help of a Shimadzu spectrophotometer.

Chromatographic methods were used to separate individual carotenoids from the mixtures. The modern method of thin layer chromatography (TILC) has been found wide application in the field of carotenoid estimation. The popularity of this method is due to its speed and efficiency in separation. High performance liquid chromatography (HPLC) is also widely used as an analytical tool in the studies of algal pigments (Jeffrey \textit{et al.}, 1997). The advantage of HPLC over
other conventional methods of analysis such as column chromatography and TLC techniques include shorter analysis time, greater resolution, ease of quantitation and lower limit of detection.

The analysis of carotenoids in the experimental algae namely *G. nostochinearum* and *O. elegans* collected from both natural habitat and culture conditions was highly interesting. The amounts of total carotenoids as well as number of individual carotenoids were found to be varied according to the place of origin i.e. natural habitat and culture conditions.

The total carotenoid estimated in *G. nostochinearum* collected from natural habitat was found to be $3.0 \pm 0.09\text{mg}/100\text{gm}$ of dry algae. The major carotenoids in this alga were lutein- $0.638\pm0.06\text{mg}$, echinenone-$0.196\pm0.03\text{mg}$, $\alpha$-carotene-$0.070\pm0.01\text{mg}$ and $\beta$-carotene-$0.281\pm0.04\text{mg}$ per 100 gm of dry algae. Contrarily the total carotenoid of the same alga grown in artificial cultures was found to be $131.1 \pm 0.07\text{mg}/100\text{gm}$ of dry algae. Under such conditions the major carotenoids were lutein- $33.315\pm0.09\text{mg}$, astaxanthin- $40.826\pm0.1\text{mg}$, echinenone-$2.898\pm0.07\text{mg}$, $\alpha$-carotene-$1.679\pm0.06\text{mg}$ and $\beta$-carotene-$1.661\pm0.06\text{mg}$ per 100 gm of dry algae.

Comparative analysis showed that *G. nostochinearum* collected from natural habitat synthesizes about $3 \pm 0.09\text{mg}$ of carotenoid per 100 gm of dry algae. The same algae cultured in Bold Basal Medium could accumulate about $131.1 \pm 0.07\text{mg}$ of carotenoid per 100 gm of dry algae, which was almost 43 times more carotenoid than that of the algae collected from the natural habitat.
From the investigations it was observed that the algae synthesize lutein, echinenone, α-carotene and β-carotene in natural habitat as well as in artificial culture conditions but amounts of carotenoid in artificial culture conditions increased by many folds. The algae synthesize astaxanthin in artificial culture conditions, which was not found in the algae collected from natural habitat.

The total carotenoid in *O. elegans* collected from natural habitat was found to be 1.535 ±0.4 mg per gm of dry algae. The major carotenoids found in this alga was fucoxanthin- 0.233 ±0.04 mg, zeaxanthin- 0.64 ±0.3 mg, lutein- 0.228 ±0.09 mg, astaxanthin- 0.0026 ±0.0003 mg, echinenone-0.005 ±0.0001 mg and β-carotene- 0.02 ±0.001 mg per gm of dry algae. The total carotenoid in *O. elegans* grown in artificial cultures was found to be 4.546 ±0.5 mg per gm of dry algae. In artificial culture conditions, the alga synthesized fucoxanthin- 0.236 ±0.05 mg, zeaxanthin- 1.663 ±0.09 mg, lutein- 0.355 ±0.04 mg, astaxanthin- 0.0026 ±0.0001 mg, echinenone- 0.848 ± 0.01 mg and β-carotene- 0.016 ±0.001 mg per gm of dry algae.

Comparative analysis showed that *O. elegans* collected from natural habitat synthesized 1.535 ±0.4 mg of carotenoid per gm of dry algae. The same alga when cultured in Modified Chu-10 Medium produced 4.546 ±0.5 mg of carotenoid per gm of dry algae, which was almost three times more than that of the algae collected from the natural habitat. From the investigations it was observed that the algae could synthesize fucoxanthin, zeaxanthin, astaxanthin, lutein, echinenone and
β-carotene in natural habitats as well as in artificial culture conditions but amounts of carotenoid in artificial culture conditions increased by many folds.

Maximum carotenoid accumulation depends upon the important factors, which include high light intensity (Massyak and Radchenko, 1970; Semenko and Abdullars, 1980), extreme temperature (Henley et al., 2002), high salinity (Mortain-Bertmd et al., 1994; Henley et al., 2002) and deprivation of minerals nutrient including nitrate, sulphate and possibly phosphate (Ben-Amotz, 1987; Vorsa et al., 1994; Shelly et al., 2002). These factors can be provided in artificial culture conditions for maximum carotenoid accumulation.

The pigment, which provide the natural colour to the algal thallus constitute one of the most effective criteria in algal taxonomy. Each algal class or division has its own particular combination of pigment (Sharma, 1992). As more and more algae are examined by modern tools, an increasing number of exceptions to the general pattern are being uncovered. Dunaliella salina has been reported to have higher amount of β-carotene under conditions of high light intensity and high salt concentration (Chidambara Murthy et al., 2005). In some stress conditions, for example nitrogen deficiency, certain algae accumulate large amount of β-carotene in the oily droplets or in the cell wall (Goodwin, 1992). Thus it has been observed that high light intensity and limited growth rate accumulate high amount of carotenoids. Therefore, further works on culture conditions as well as analysis of carotenoids would be needed to get further enhanced amount of carotenoids in G. nostochinearum and O. elegans.
Identification of lipids requires the determination of their fatty acid components. Photosynthetic eukaryotic organism possesses poly unsaturated fatty acids (PUFA's) as acyl lipids concentrated in chloroplast (Fatma and Sultan, 1999). Extraction and estimation of lipids were carried out in *G. nostochinearum* and *O. elegans* harvested from both natural habitat and artificial cultures.

The amount of lipids of *G. nostochinearum* collected from natural habitat was found to be 2.61 ±0.044gm/100gm of algae. The same algae in culture conditions produced 11.4 ±0.276gm/100gm of lipids, which is four times more than that produced by the alga in natural habitat. The only fatty acid of *G. nostochinearum* in natural habitat was myristic acid while under artificial culture conditions the same alga synthesizes palmitic acid as the only fatty acid. It was interesting to note the absence of Poly Unsaturated Fatty Acids (PUFAs) in *G. nostochinearum* either in natural habitat or in artificial culture conditions.

Extraction and estimation of lipids was also carried out in *O. elegans* harvested from natural habitat as well as from artificial cultures. The amount of lipids in *O. elegans* collected from natural habitats was found to be 15.616 ±0.072 gm/100gm of algae. The same alga under artificial culture conditions produced more lipids (19.8 ±0.202 gm/100gm of dry algae). The only fatty acid of *O. elegans* in natural habitat was myristic acid while under artificial culture conditions the major fatty acids were found to be myristic acid, palmitic acid and linolenic acid.

*O. elegans* collected from natural habitat was deficient of poly unsaturated fatty acids (PUFAs). Interestingly when the same alga grown in
Modified Chu-10 Medium synthesized linolenic acid as the only poly unsaturated fatty acids (PUFAs). This clearly indicates the necessity of further investigations on the factors responsible for synthesizing poly unsaturated fatty acids in *O. elegans* under artificial conditions.

Biosynthesis of lipids increases under unfavourable conditions retarding growth of culture (Solovchenko et al., 2008). Unfavourable conditions and stresses, e.g., deficiency of mineral (nitrogen) with excessive intensive illumination, in some unicellular algae promotes biosynthesis of lipids. Lipids are assumed to be the sink for the excessive photoassimilates, which can not be utilized in the absence of nitrogen for synthesis of protein and other compounds (Thompson, 1996)

In recent years, fatty acids (FAs) in marine algae have aroused considerable interest among researchers. This is because marine plants can produce C18 and C20 poly unsaturated fatty acids (PUFAs) (Kayama et al., 1989). The ability of cell to accumulate PUFAs is intrinsically limited in most algae, since these fatty acids are generally components of membranal lipids whose content is strictly regulated. According to the results, both the microalgae investigated in this study were not similar in fatty acid profile. The fatty acids content of microalgae differed according to taxonomic group and the growth conditions (Brown, 2002). Composition of lipid in phytoplankton have a major effect on the health and reproductive success of many marine organisms, specifically zooplankton (Gioedkoop et al., 1998; Suzuki et al., 2002), mussel (Freites et al., 2002) and crab (Miezianie et al., 2002). Environmental conditions, such as phosphorous levels, light
availability and temperature can affect lipid composition in microalgae (Chelf, 1990). However, due to complex ecosystem dynamics, it is difficult to determine the degree to which specific abiotic factors influence changes in lipids in aquatic organisms (Wainman and Smith, 1997). Few studies have systematically examined these factors using controlled conditions (Al-Hasan et al., 1990; Dybdahl, 1995). Johansen et al., (1990) observed considerable variability in type and amount of fatty acids among the strains they investigated. These results indicate that observed differences in fatty acid profiles among studies might be attributable to differences in individual algal strains within a species. These fatty acids are essential for nutrition of many animals, including humans (Uki et al., 1986), and are of interest in biotechnology, in food chain studies and in cosmetics (Servl et al., 1994). Fatty acids of marine macrophytes have also attracted the attention of chemotaxonomists. Fatty acids compositions of red, brown and green macrophytic algae, belonging to different taxonomic classes, orders or families and genera, have distinguishing features of taxonomic value (Miralles et al., 1990; Aknin et al., 1992; Khvatimchenko, 1993, 1995). Two marine green algae, Codium dwarkense and Coelium taylorii, were analyzed for fatty acids by GC-MS using serially coupled capillary columns with different stationary phase polarities. This method showed the presence of more than 40 volatiles, including low molecular and dioic compounds (Dembitsky et al., 2003).

The present study established that Glaucocystis nostochinearum Itziigsohn and Oocystaenium elegans Gonzalves et Mehra may be considered as a
cheaper source of carotenoids and if any protocol permits their mass artificial culture, these two algae may be exploited for commercial production of natural carotenoids and fatty acids.