CHAPTER IV
CULTURE OF MICROALGAE

Microalgae have been advanced as the solution to many problems and as the potential source of many products. However, the cost associated with their culture and harvesting where ever necessary extracting and purifying the product of interest, preclude many of these suggestions at the outset. Estimates of the cost of producing micro-algae range from $(US) 0.5 to $(US) 1.5 kg^{-1} (Richmond and Priess, 1980). There has been very little applied research carried out with the blue green algae, despite their potential significance to biotechnology. Most of the applied research that has been done is an attempt to explain the dominance of Blue green algae in the surface waters of eutrophic lakes.

Three Blue green algae that have been studied in continuous cultures are Oscillatoria (Zevenboom and Mur, 1981), Anacystis (Frischknecht and Schneider, 1979) and Gomphosphaeria (Wilmot and Martin, 1977). Light of low intensity favours the growth of many blue green algae. Oscillatoria for example is able to survive at a lower light intensity than green algae such as Scenedesmus (Mur and Beijdorff, 1978). Ranges of temperature suitable for the growth of blue green algae are much wider than those for other algae and in general the optimum temperature for blue green algae is about 35°C. Thermophilic strains of Synechococcus grow at 73°C (Fogg et al., 1973).

High salinity is not generally conducive to the growth of blue green algae and of those species that do tolerate high
salinities, none is able to fix nitrogen. It seems that nitrogen fixation, as a process, is more sensitive than photosynthesis to high salinity (Tel-Or, 1980). One of the most interesting characteristics of many blue green algae is their ability to grow heterotrophically in the dark or in the light, though the phenomenon has not yet been systematically studied. Nostoc and Tolypothrix have been grown heterotrophically in the dark. Tolypothrix tenuis grow in the dark on glucose, fructose and sucrose in the presence of ammonium salts or amino acids, but not in the presence of nitrate (Fogg et al., 1973).

Important determinants of the economics of algal culture are the growth rate and/or final biomass yield, the selective advantage or disadvantage of the strain used, and the case of harvesting the biomass. A corollary of faster growth has improved competitive advantage over contaminating algae. A further advantage could be gained by genetically modifying the starting strain so it could grow well in conditions under which the original organism grows poorly or not at all. However the manipulated environmental parameter would have to be cheap and simple to apply to large ponds.

Examining the engineering design of algal cultivation systems of a size sufficient to produce tonnes of algae or algal products daily involves consideration of not only the application desired but also a host of factors, many of which are uncontrollable or not as controllable in the natural environment as in laboratory cultures and some of which are probably not yet known.
Some known factors requiring consideration in design are:

1) The specific application

2) a) Media requirements of the species of algae to be grown to produce a desired biomass, product, products or process.
   
b) Various media inputs as a function of quality and availability for economical production.

3) Local climatological conditions, including geographical and seasonal variations in illumination, temperature, precipitation, evaporation, relative humidity and other factors.

4) Physical properties of the design area including soil type, slopes, drainage, water quantity and quality.

5) Specific physical requirements for cultures: mixing, depth, residence time and power inputs to attain needed productivity of algae or products under a given set of conditions.

6) Attainable efficiencies and productivities

7) Harvesting, processing and their costs.

Many of the above items are more or less interrelated and few can be dealt with independently. Nevertheless, in the following we shall deal first with existing and proposed applications of algae and then with each factor in the order mentioned, pointing out relevant interactions and problems as and when a case arises.
There are many existing and potential applications of large-scale micro-algal cultivation systems and the most often suggested is the production of quality protein for use as human food in developing countries (Chastel and Clement, 1975). Next is the cultivation of algae for organic residues as a source for fish production (Edminster, 1947). The most important one is the nitrogen fixation. The nitrogen fixing *Anabaena* spp. that grow symbiotically with *Azolla* in rice paddies can be grown as intensive culture near rice paddies are used locally to provide nitrogen (Venkataraman and Becker, 1986).

Only in arsenic laboratory cultures is one able to distinguish subtle species differences in organic or mineral requirements, nevertheless, specific environments do support diverse species of algae. Decisive factors appear to be the total dissolved salts, Na:Ca ratios (Provasoli *et al.*, 1954), the presence or absence of fixed nitrogen (Stewart, 1968) and the presence or absence of organic substances (Provasoli, 1963). Media replete with all life essentials except fixed nitrogen will encourage growth of nitrogen fixing blue green algae such as *Anabaena* (Fogg, 1971). Nichols (1973) has summarized the range of freshwater media for algal growth.

An artificially compounded media, of the strength recommended, would be prohibitively expensive unless the product produced is very high in value. Attempts to use water enriched with lower cost agricultural fertilizers for mass algal cultivation is a risky alternative since these may contain copper or other toxic substances seriously sufficient enough to impair
algal growth as well as defile the product (Anon, 1984). One is left with the conclusion that, for economical mass production of microalgal and algal products or processes, media costs are high on the list of dominant factors in determining their economic feasibility.

Next is the wide range of tolerance for media composition by micro-algae to pesticide, herbicide, insecticide, commercial fertilizers, fungicide, to different salinity ranges and under natural and artificial light conditions. In addition to tolerance the algal production technology involves three steps ie. cultivation, harvesting and processing. No last word has yet been said on the most ideal conditions to produce maximal yield which depend on the location of algal plants, temperature, light, agitation used, supply of nutrients, pH etc. The basic requisite for successful algal cultivation is the construction of a suitable pond which should be efficient, easy to operate, durable and cheap (Richmond and Priess, 1980).

The success in commercial production of algae depends on the method of harvesting. Several options are available now, centrifugation sedimentation, filtration, screening, strouning and flocculation. The processing can be done by the method of Drum drying, Spray drying, Sun drying. Comprehensive literature is available on the above conditions (Venkataraman, 1983; Richmond and Becker, 1986; Venkataraman and Becker, 1986; Kaplan et al., 1986; Venkataraman and Fatima, 1990; Bongale and Singh, 1990 and Pushparaj et al., 1997).
Several works have been reported on the tolerance aspect of the blue green algae (Stulp and Stam, 1984; Reed and Stewart, 1985; Gabbay and Tel-Or, 1985; Reddy et al., 1990; Anand et al., 1990a; Pabbi and Vaishya, 1990; Dikshit and Tiwari, 1990; Parameshwaran, 1995; Caiola et al., 1996; Ibelings, 1996; Tomaselli et al., 1997; Kebede, 1997; Vargas et al., 1998; Francisco et al., 1999; Rai and Tiwari, 1999; Gayathri, 2001; Hobson, 2001 and Qiu et al., 2003).

In the present study the effect of commercial fertilizers, biofertilizer, pesticide, fungicide, insecticide and the response of salinity tolerance of the isolated alga \textit{(Chroococcus tenax)} and its growth performance under \textit{BG11} medium in natural and artificial light has been analysed.

4.2. Materials and Methods

4.2.1. Culture

During the study Cyanobacteria were the dominant forms. Among them 4 genera were recorded with high frequency percentage. One among the four was \textit{Chroococcus tenax} which was isolated for culture studies (Plate 2). This strain was made arsenic and the systematic position is as follows.

\begin{center}
\begin{tabular}{ll}
Class & Cyanophyta \\
Order & Chroococcales \\
Family & Chroococcaceae \\
Genus & \textit{Chroococcus} \\
Species & \textit{tenax} (Kirchn.) Hieron.
\end{tabular}
\end{center}
The experiment (IV. Fig.1) was done with the alga by the following sequences:

i) Isolation of the alga

ii) Preparation of stock culture for the selected alga

iii) Growth pattern of the alga in a pesticide, insecticide, fungicide, biofertilizer (Azospirillum), nitrogenous fertilizer, non-nitrogenous fertilizer, under different salinities(%) and in BG11 medium (under natural and artificial light).

iv) Study the growth pattern and cell density (IGR and cell doublings) in the various media mentioned.

v) Fixing the optimum nutrient concentrations in the tested media.

vi) Analysis of biochemical constituents of the algae at the stationary phase of the best grown medium.

vii) Statistical analysis (S.D and Student ‘t’ test) of the results obtained to find out the best medium and the suitable concentration or ppm for the growth of the blue green algae.

4.2.2. Culture conditions necessary for growth:

Microalgae require light, optimum temperature (27°C, pH 7.1) and dissolved nutrients for their successful growth (Laing,
ALGA CULTURE

Chroococcus tenax

MEDIA FORMULATIONS

MF₁, MF₂, MF₃, MF₄, MF₅,
MF₆, MF₇, MF₈, MF₉

BG₁₁ under natural light (0.1 to 1%)
BG₁₁ artificial light (0.1 to 1%)
Azospirillum (0.1 to 1%)
Nitrogenous fertilizer - Urea (0.1 to 1%)
Non-nitrogenous fertilizer-Potash (0.1 to 1%)
Bavistin (0.1 to 1%)
Phosphomidon (0.1 to 1%)
Carbaryl (0.1 to 1%)
Salinity (1 to 7%)

PARAMETERS STUDIED

IGR
Cell doubling
Chlorophyll ‘a’
Carotenoid
Phycocyanin
Phycoerythrin
Allophycocyanin

Protein
Lipid
Carbohydrate

Fig. 1: Experimental design for the culture of alga during the study
1991). In addition, microalgal growth can be influenced by external environmental condition and the method of culture employed. Growth is inhibited when the culture lack sufficient nutrients, optimum temperature and pH (Liao et al., 1991).

The isolated alga was grown in BG$_{11}$ medium (Rippka et al., 1979) without any nitrogen source at 27 ± 1°C under the fluorescent illumination of 30 to 40 mE m$^{-2}$ s$^{-1}$. Alternate light and dark phases were provided. The cultures were grown in 500ml conical flasks and was used throughout the study as stock, which was checked regularly for the bacterial contamination.

Throughout the study the experiments were carried out in test tubes with the liquid cultures and always with triplicates. Usually test tubes were shaken once in two days inorder to avoid the clumping of cells.

4.2.3. Various medias of algal culture:

In the present study the growth characteristics of the selected alga was assessed by culturing it in different nutrient sources such as Urea (0.1 - 0.9%), Potash (0.1 - 0.9%), Bavistin (0.1 - 0.9%), Phosphomidon (0.1 - 0.9%), Carbaryl (0.1 - 0.9%), Azospirillum (0.1 - 0.9%), Salinity (1 to 7%), in BG$_{11}$ medium under natural and artificial light (2000 lux - under laboratory) condition. The pH of the media was adjusted to 7.1. In all the experiments 50ml basic media (BG$_{11}$ medium) was amended with the other nutrient sources. Cultures grown in BG$_{11}$ media alone
was used as control and the inoculum was from the exponential phase only. After inoculation the following growth characters were measured.

4.2.4. Determination of growth:

1) Cell density (James and Al-Khars, 1990)

Cell counts of the alga cultured in various experimental media were monitored by using a "Neubauer" improved "Haemocytometer" at 11.00 hrs in the morning on alternate days. The specific growth rates of dividing cells in the photostage of the reaction chamber is quantified by respective formulae.

i) The Instantaneous growth rate (K)

\[ K = \ln N_t - \ln N_0 / t \]

Where

- \( N_t \) is the final cell count
- \( N_0 \) is the initial cell count
- \( t \) is the number of days.

Instantaneous growth rate (K) is a derived specific growth rate constant factor.

2) Growth velocity (doublings) per day

\[ X = \ln N_n - \ln N_i / \ln 2 (t_n - t_i) \]

Where

- \( N_n \) is the final cell count
- \( N_i \) is the initial cell count
- \( t_n \) is the final time in days
- \( t_i \) is the initial time in days.

It is defined as the unit cell doubling rate factor per day.
4.2.5 Chlorophyll 'a' and Carotenoid

From the experimental cultures known quantity of the samples were taken during the stationary phase and centrifuged at 5000 rpm for 10 minutes. The pellets were added with few ml of 100% acetone and left in the dark for 24 hours for complete extraction of pigment at 4°C in a refrigerator. Optical density was measured at 664 nm, 630 nm and 480 nm in a Bauch and Lomb spectrophotometer separately for the cultures kept in triplicates following the method given by Strickland and Parsons (1972) and Jeffrey and Humphrey (1975).

\[
\text{Chlorophyll 'a' } = 11.47 \times 10^6 E_{664} - 0.40 \times E_{630} \\
\text{Carotenoid } = E_{480} \times 100
\]

('E' denotes extinction at respective wave length)

Determination of phycobilin pigments:

Phycobilin pigment components viz., Phycocyanin (PC), Phycoerythin (PE) and Allophycocyanin (APC) were analysed using the method of Bennett and Bogoard (1973).

Algal pellet after extract in 80% (for the study of chlorophyll 'a') was washed and centrifuged twice with distilled water and was resuspended in 50 ml phosphate buffer (pH 6.7). After repeated freezing and thawing in phosphate buffer cells were disrupted for 5 minutes using disintegrator. The homogenised suspension was centrifuged at 5000 rpm for 5 minutes in a centrifuge. Optical density was measured at 562 nm, 615 nm and 652 nm in Bedsmann DU20 spectrophotometer.
$$PC = \frac{Abs_{615} \cdot 0.474 (Abs_{652})}{5.34}$$

$$APC = \frac{Abs_{652} \cdot 0.208 (Abs_{615})}{5.09}$$

$$PE = \frac{Abs_{562} \cdot 2.41 (PC) \cdot 0.849 (APC)}{9.62}$$

4.2.6. Estimation of protein: (Lowry et. al., 1951)

Known quantity of the cultures from the various media were harvested at stationary phase and centrifuged at 5000 rpm for 10 minutes. From the supernatent collected total amount of protein was estimated after hydrolysis in 0.5N sodium hydroxide for 24 hrs at 30°C.

4.2.7. Estimation of carbohydrate: (Kochert, 1978)

Total carbohydrate content of the selected alga cultured in various media from the stationary phase was estimated by phenol sulphuric acid method.

4.2.8. Estimation of Lipid

For the quantitative estimation of lipid, the harvested algal cultures were (from stationary phase) dried at 60°C separately and made to fine powder. Lipids were first extracted with Chloroform · Methanol (1:2) mixture (Bligh and Dyer, 1959). The sediment was again extracted with chloroform and methanol mixture. The supernatant was transferred into a previously weighed Petridish and the solvents were allowed to evaporate at 80°C (Rouser et al., 1967). The percentage of lipid was then calculated.
4.2.9. Energy pond concept

*Chroococcus tenax* when grown in pits of 18cm depth resulted in a good growth of the cells. From the pit on the 20\textsuperscript{th} day by filtering 40 gm / F.w. of *Chroococcus tenax* was harvested (Plate 2).

The isolated alga was cultured in energy ponds of 18cm depth and 40cm length and width ratio. The ponds were created in green house (If they are taken in agriculture fields they can be covered by small mesh). The pit was lined by polyethylene sheet. The method adopted for pond cultivation is based on Venkataraman and Mahadevaswamy (1992). The pond was provided with well water and farm yard manure (Gupta and Changwal, 1992). The alga *Chroococcus tenax* grown in BG\textsubscript{11} medium was introduced and the pH of the medium was maintained to 7.8 - 8.1. The temperature of the pond was 25 - 37°C. The light intensity is 1000 m EM\textsuperscript{2} S\textsuperscript{-1}. Regular agitation was given for the even dispersal to keep the cells in active growth phase and to remove oxygen produced during photosynthesis.

During growth, periodical monitoring of microbial contamination is very important in maintaining quality and safety of algal biomass. Contamination is due to other green algae, diatoms, bacteria, fungi and insects. In conditions of high level of contamination it is better to flocculate the contaminants before recycling the effluent both for energy pond concept and mass cultivation of the algae. To avoid contamination the pH was adjusted to alkaline range (7.8 - 8.1). In the 20\textsuperscript{th} day the alga was harvested by filtering.
4.2.10. Mass culture of *Chroococcus tenax*:

After the analysis of growth in various media, the alga was mass cultured by "Pit method" (Bongale and Singh 1986). Pits of 1m x 90 cm size with a depth of 15 cm were covered with polythene sheets and were filled with 20 litres of well water. Treatment combination of soil (150 mg/l), superphosphate (50mg / l) and farmyard manure (50mg / l) were applied in the pit.

*Chroococcus tenax* (Kirchn.) Hieron. strains isolated from the ponds cultured in stock was inoculated (20 ml) in to the pit (Plate 2). Periodical monitoring of contamination was done for other algae, fungi and bacteria. Algal cells after 20 days were manually harvested by filtration and growth was measured in terms of fresh weight of the algal bio-mass.

4.3. Results

4.3.1. Growth and biochemical characters of *Chroococcus tenax* in BG 11 medium

A normal sigmoid growth curve (IV. Fig. 2) was obtained. The maximum growth rate was recorded on the 12th day. *Chroococcus* cell growth performance behavior expressed as IGR and cell velocity doublings depicted a high IGR of 0.192 at 0.6% of BG 11 and high cell density of 0.45 at 0.6% of BG 11 under natural day light conditions (IV. Table 1).

Under artificial light levels cell growth rate monitored as IGR and cell doublings evidenced beneficial levels (0.14 and 0.23, respectively) at 3% (IV. Fig. 3).
Similarly under natural light the alga showed a chlorophyll ‘a’ content of 7.86 µg chl ‘a’ ml⁻¹/d.w., carotenoid 4.73 µg ml⁻¹/d.w., phycocyanin 6.99 µg ml⁻¹/d.w., phycoerythrin 4.1 µg ml⁻¹/d.w., allophycocyanin 5.3 µg ml⁻¹/d.w., protein 75 µg ml⁻¹/d.w., carbohydrate 30 µg ml⁻¹/d.w. and lipid content 15%.

The biochemical analysis of *Chroococcus tenax* grown under artificial light (IV. Table 2) showed chlorophyll ‘a’ content of 7.4 µg chl ‘a’ ml⁻¹/d.w., carotenoid 4.92 µg ml⁻¹/d.w., phycocyanin of 6.34 µg ml⁻¹/d.w., phycoerythrin 3.9 µg ml⁻¹/d.w., allophycocyanin 5.1 µg ml⁻¹/d.w., protein content of 68.6 µg ml⁻¹/d.w., carbohydrate of 27.4 µg ml⁻¹/d.w. and lipid content of 14.3%.

### 4.3.2. Growth in commercial fertilizers

The isolated *Chroococcus tenax* was grown in commercial fertilizers such as urea and potash. Addition of urea to the medium was found to affect the growth of alga in all the concentrations (IV. Fig. 4). In higher concentrations the alga failed to grow (<0.5%). There was considerable cell growth enhancement at 0.1% doses (IGR of 0.172) and cell doubling vigour was observed to be high (0.2%) at 0.1% (IV. Table 1).

Biochemical determination of the isolate, grown in the commercial fertilizer urea in the stationary phase resulted in chlorophyll ‘a’ content of 6.86 µg chl ‘a’ ml⁻¹/d.w., carotenoid 3.1 µg ml⁻¹/d.w., phycocyanin of 6.54 µg ml⁻¹/d.w., phycoerythrin of 5.79 µg ml⁻¹/d.w., allophycocyanin of
2.86 µg ml⁻¹ / d.w., protein content of 48 µg ml⁻¹ / d.w., carbohydrate of 24 µg ml⁻¹ / d.w., and a lipid content of 9.8% (IV. Table 3).

Likewise the biological growth activity of *Chroococcus tenax* with non nitrogenous fertilizer (potash) showed considerable cell doublings (0.357) at 0.4% dose and promoting a better IGR of 0.11 at 0.4% (IV. Table 1).

Potash amendment was a good supplement for the growth of the isolate. The isolate grew well in all the concentration of potash, but growth was slightly affected in higher concentration (IV. Fig. 5).

The biochemical analysis of *Chroococcus tenax* grown in potash showed chlorophyll ‘a’ content of 6.94 µg chl ‘a’ ml⁻¹, carotenoid 3.86 µg ml⁻¹ / d.w., phycocyanin 6.73 µg ml⁻¹ / d.w., phycoerythrin 5.96 µg ml⁻¹ / d.w., 2.94 µg ml⁻¹ / d.w., allophycocyanin, 52 µg ml⁻¹ / d.w. of protein, lipid content of 11% and carbohydrate content of 24.8 µg ml⁻¹ / d.w. (IV. Table 3).

### 4.3.3. Growth Response of *Chroococcus tenax* in biofertilizer (Azospirillum)

Appreciable cell photosynthetic activity of *Chroococcus tenax* with varying biological concentrations (IV. Fig. 6) of Azospirillum was noticed. The amount of chlorophyll ‘a’ and cell density (0.19) recorded was lower than that of control. However the growth rate was good (mean IGR of 0.09) at lower concentration (0.1 to 0.5) than that of control and commercial fertilizer (IV. Table 1 and 3).
Biochemical responses of *Chroococcus tenax* with the augmenting agent Azospirillum has the chlorophyll 'a' content of 7.74 μg chl 'a' ml⁻¹ / d.w., carotenoid 3.91 μg ml⁻¹ / d.w., phycocyanin of 7.51 μg ml⁻¹ / d.w., phycoerythrin of 6.91 μg ml⁻¹ / d.w., 3.12 μg ml⁻¹ / d.w. of allophycocyanin, 61 μg ml⁻¹ / d.w. of protein, 28.6 μg ml⁻¹ / d.w. of carbohydrate and 11.3% of lipid content (IV. Table 3).

4.3.4. Growth activity of *Chroococcus tenax* to fungicide, pesticide and insecticide

Growth responses of *Chroococcus tenax* was uniformly good (IV. Fig. 7). The Intensive growth rate recorded was 0.07 and cell doubling as 0.15 above 0.3% subjection exposure (IV. Table 1). Addition of fungicide Bavistin did not bring considerable variation in photosynthesis or pigments. Biochemical determination for *Chroococcus tenax* with Bavistin proved measurable reduction in the pigments, protein and carbohydrate (IV. Table 4). Protein content of 58 μg ml⁻¹ / d.w. and carbohydrate of 30 μg ml⁻¹ / d.w. was recorded which was more, than that obtained from cells grown without any amendment.

Growth and cell tolerance of *Chroococcus* sps. to Phosphomidon, recorded better tolerance at 0.1% exposure levels. However at high concentration the growth (IV. Fig. 8) and cell doubling were drastically affected (0.11) at 0.7%. But IGR was satisfactory (0.08) until 0.2%. The biochemical analysis also revealed a low protein (50 μg ml⁻¹ / d.w.), carbohydrate (25 μg ml⁻¹ / d.w.) and lipid (10.1%) contents. The pigment contents recorded were also low (IV. Table 4).
*Chroococcus* cell growth performance cultured with the media containing the insecticide (carbaryl) resulted a low IGR of 0.07 and cell doubling 0.32 at 2%. The protein and carbohydrate contents observed was also very low (IV. Fig. 9).

### 4.3.5. Salinity Tolerance of *Chroococcus tenax*

Stress tolerance of *Chroococcus tenax* to salinity flux was quantified in terms of IGR and cell doublings (IV. Fig. 10). The tolerance was found to be very poor at 5% indicating a poor IGR and cell doubling of 0.04 and 0.06, respectively. Best performance level was attained at 3% (IGR of 0.08 and cell doubling of 0.155) (IV. Table 1).

Biochemical analysis on the stationary phase also revealed, better pigments, protein, lipid and carbohydrate contents at 3% (IV. Table 5).
Fig. 2: Growth pattern of *Chroococcus tenax* in BG$_{11}$ medium (natural light)

Fig. 3: Growth pattern of *Chroococcus tenax* in various concentration of the BG$_{11}$ (under artificial light) medium
Fig. 4: Growth pattern of *Chroococcus tenax* in various concentration of the nitrogenous fertilizer (Urea)

Fig. 5: Growth pattern of *Chroococcus tenax* in various concentration of the non-nitrogenous fertilizer (Potash)
Fig. 6: Growth pattern of *Chroococcus tenax* in various concentration of the biofertilizer (Azospirillum)

Fig. 7: Growth pattern of *Chroococcus tenax* in various concentration of the fungicide (Bavistin)
Fig. 8: Growth pattern of *Chroococcus tenax* in various concentration of the pesticide (Phosphomidon)

Fig. 9: Growth pattern of *Chroococcus tenax* in various concentration of the insecticide (Carbaryl)
Fig. 10: Growth pattern of *Chroococcus tenax* in various salinity ranges
Table 1: Instantaneous growth rate and cell doubling of *Chroococcus tenax* in various culture medias (Mean ± SD)

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Various Medias in which the alga was cultured</th>
<th>Concentration at which best growth occurred</th>
<th>Mean IGR µd⁻¹</th>
<th>Mean doublings d⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BG₁₁ medium (Natural light)</td>
<td>0.6%</td>
<td>0.19±0.03</td>
<td>0.45±0.40</td>
</tr>
<tr>
<td>2</td>
<td>BG₁₁ medium (Lab conditions)</td>
<td>0.3%</td>
<td>0.14±0.01</td>
<td>0.23±0.03</td>
</tr>
<tr>
<td>3</td>
<td>Biofertilizer (Azospirillum)</td>
<td>0.2%</td>
<td>0.18±0.04</td>
<td>0.35±0.20</td>
</tr>
<tr>
<td>4</td>
<td>Commercial fertilizer (Urea)</td>
<td>0.1%</td>
<td>0.17±0.02</td>
<td>0.22±0.03</td>
</tr>
<tr>
<td>5</td>
<td>Non nitrogenous fertilizer (Potash)</td>
<td>0.4%</td>
<td>0.11±0.01</td>
<td>0.36±0.04</td>
</tr>
<tr>
<td>6</td>
<td>Pesticide (Bavistin)</td>
<td>0.3%</td>
<td>0.07±0.01</td>
<td>0.15±0.03</td>
</tr>
<tr>
<td>7</td>
<td>Fungicide (Phosphomidon)</td>
<td>0.1%</td>
<td>0.08±0.03</td>
<td>0.11±0.02</td>
</tr>
<tr>
<td>8</td>
<td>Insecticide (Carbaryl)</td>
<td>0.3%</td>
<td>0.07±0.04</td>
<td>0.32±0.01</td>
</tr>
<tr>
<td>9</td>
<td>Salinity (0 to 8%)</td>
<td>3%</td>
<td>0.08±0.002</td>
<td>0.17±0.04</td>
</tr>
</tbody>
</table>
Table 2: Biochemical analysis of *Chroococcus tenax* grown under BG11 medium

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Natural light (µg ml⁻¹) / dry weight</th>
<th>Artificial light</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll 'a' (µg chl 'a' ml⁻¹)</td>
<td>7.86</td>
<td>7.4</td>
</tr>
<tr>
<td>Carotenoid (µg ml⁻¹)</td>
<td>4.73</td>
<td>4.92</td>
</tr>
<tr>
<td>Phycocyanin (µg ml⁻¹)</td>
<td>6.9</td>
<td>6.34</td>
</tr>
<tr>
<td>Phycoerythrin (µg ml⁻¹)</td>
<td>4.1</td>
<td>3.9</td>
</tr>
<tr>
<td>Allophycocyanin (µg ml⁻¹)</td>
<td>5.3</td>
<td>3.9</td>
</tr>
<tr>
<td>Protein (µg ml⁻¹)</td>
<td>75</td>
<td>68.6</td>
</tr>
<tr>
<td>Carbohydrate (µg ml⁻¹)</td>
<td>30</td>
<td>27.4</td>
</tr>
<tr>
<td>Lipid (%)</td>
<td>15</td>
<td>14.3</td>
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</tbody>
</table>
Table 3: Determination of Biochemical parameters of *Chroococcus tenax* grown in BG_{11} medium amended with, Urea, Potash and Azospirillum

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (BG_{11} medium)</th>
<th>BG_{11} + Urea</th>
<th>BG_{11} + Potash</th>
<th>BG_{11} + Azospirillum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll 'a' (µg chl 'a' ml^{-1})</td>
<td>7.4</td>
<td>6.86</td>
<td>6.94</td>
<td>7.74</td>
</tr>
<tr>
<td>Carotenoid (µg ml^{-1})</td>
<td>4.92</td>
<td>3.1</td>
<td>3.86</td>
<td>3.91</td>
</tr>
<tr>
<td>Phycocyanin (µg ml^{-1})</td>
<td>6.34</td>
<td>6.54</td>
<td>6.73</td>
<td>7.51</td>
</tr>
<tr>
<td>Phycoerythrin (µg ml^{-1})</td>
<td>3.9</td>
<td>5.79</td>
<td>5.96</td>
<td>6.91</td>
</tr>
<tr>
<td>Allophycocyanin (µg ml^{-1})</td>
<td>5.1</td>
<td>2.86</td>
<td>2.94</td>
<td>3.12</td>
</tr>
<tr>
<td>Protein (µg ml^{-1})</td>
<td>68.6</td>
<td>48</td>
<td>52</td>
<td>61</td>
</tr>
<tr>
<td>Carbohydrate (µg ml^{-1})</td>
<td>27.4</td>
<td>24</td>
<td>24.8</td>
<td>28.6</td>
</tr>
<tr>
<td>Lipid (%)</td>
<td>14.3</td>
<td>9.8</td>
<td>11</td>
<td>11.3</td>
</tr>
</tbody>
</table>
Table 4: Biochemical parameters of *Chroococcus tenax* grown in BG11 medium amended with an insecticide, fungicide and pesticide.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pesticide, Insecticide and Fungicide Applied</th>
<th>Control (BG11 medium)</th>
<th>BG11 + Carbaryl</th>
<th>BG11 + Bavistin</th>
<th>BG11 + Phosphomidon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll 'a' (µg chl ‘a’ ml⁻¹)</td>
<td></td>
<td>7.4</td>
<td>5.86</td>
<td>6.99</td>
<td>6.97</td>
</tr>
<tr>
<td>Carotenoid (µg ml⁻¹)</td>
<td></td>
<td>4.92</td>
<td>3.7</td>
<td>5.3</td>
<td>3.6</td>
</tr>
<tr>
<td>Phycocyanin (µg ml⁻¹)</td>
<td></td>
<td>6.34</td>
<td>6.31</td>
<td>6.86</td>
<td>6.06</td>
</tr>
<tr>
<td>Phycoerythrin (µg ml⁻¹)</td>
<td></td>
<td>3.9</td>
<td>5.87</td>
<td>6.07</td>
<td>5.96</td>
</tr>
<tr>
<td>Allophycocyanin (µg ml⁻¹)</td>
<td></td>
<td>5.1</td>
<td>2.84</td>
<td>3.73</td>
<td>2.02</td>
</tr>
<tr>
<td>Protein (µg ml⁻¹)</td>
<td></td>
<td>68.6</td>
<td>57</td>
<td>58</td>
<td>50</td>
</tr>
<tr>
<td>Carbohydrate (µg ml⁻¹)</td>
<td></td>
<td>27.4</td>
<td>28.4</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>Lipid (%)</td>
<td></td>
<td>14.3</td>
<td>10.2</td>
<td>11.3</td>
<td>10.1</td>
</tr>
</tbody>
</table>
Table 5: Biochemical analysis of *Chroococcus tenax* grown under Salinity

<table>
<thead>
<tr>
<th>Parameters</th>
<th>(µg ml⁻¹) / dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll ‘a’</td>
<td>7.53</td>
</tr>
<tr>
<td>Carotenoid</td>
<td>5.1</td>
</tr>
<tr>
<td>Phycocyanin</td>
<td>6.54</td>
</tr>
<tr>
<td>Phycoerythrin</td>
<td>4.2</td>
</tr>
<tr>
<td>Allophycocyanin</td>
<td>5.6</td>
</tr>
<tr>
<td>Protein</td>
<td>71</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>27</td>
</tr>
<tr>
<td>Lipid (%)</td>
<td>13</td>
</tr>
</tbody>
</table>
Table 6: Chlorophyll ‘a’ content estimated as a function of growth for *Chroococcus tenax*

<table>
<thead>
<tr>
<th>Chlorophyll ‘a’ content (Mean)</th>
<th>Media</th>
<th>Df</th>
<th>'t' value</th>
<th>Level and Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.86 μg chl ‘a’ ml⁻¹</td>
<td>BG₁₁ (NL)</td>
<td>2</td>
<td>6.74*</td>
<td>S</td>
</tr>
<tr>
<td>7.4 μg chl ‘a’ ml⁻¹</td>
<td>BG₁₁ (AL)</td>
<td>2</td>
<td>5.26*</td>
<td>S</td>
</tr>
<tr>
<td>6.73 μg chl ‘a’ ml⁻¹</td>
<td>Salinity</td>
<td>4</td>
<td>20.91*</td>
<td>S</td>
</tr>
<tr>
<td>6.86 μg chl ‘a’ ml⁻¹</td>
<td>Urea</td>
<td>3</td>
<td>25.67*</td>
<td>S</td>
</tr>
<tr>
<td>6.94 μg chl ‘a’ ml⁻¹</td>
<td>Potash</td>
<td>3</td>
<td>21.72*</td>
<td>S</td>
</tr>
<tr>
<td>7.74 μg chl ‘a’ ml⁻¹</td>
<td>Azospirillum</td>
<td>4</td>
<td>1.99</td>
<td>NS</td>
</tr>
<tr>
<td>5.86 μg chl ‘a’ ml⁻¹</td>
<td>Carbaryl</td>
<td>3</td>
<td>43.43*</td>
<td>S</td>
</tr>
<tr>
<td>6.99 μg chl ‘a’ ml⁻¹</td>
<td>Bavistin</td>
<td>3</td>
<td>14.35*</td>
<td>S</td>
</tr>
<tr>
<td>6.97 μg chl ‘a’ ml⁻¹</td>
<td>Phosphomidon</td>
<td>4</td>
<td>28.74</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Significant at P>0.05 level.

NL - Natural Light  AL - Artificial Light
Energy pond

Stock culture of *Chroococcus tenax*

Mass culture of *Chroococcus tenax* by Pit method
4.4. Discussion

The Cyanobacteria are the group of microorganisms that are mostly connected to rice cultivation (Venkataraman, 1975, 1979; Roger and Kulasooriya, 1980). Many factors like light, temperature, pathogens, antagonism, pH, nutritional status of soil, water and toxic substances affect the growth of Cyanobacteria. The use of pesticides against the target systems may also affect the growth of Cyanobacteria (Pabbi and Vaishya, 1990; Dikshit and Tiwari, 1990). Venkataraman and Rajalakshmi (1972) reported that coloran, dalapon and dithane inhibited the growth of certain species of Anabaena, Nostoc, Aulosira and Tolypothrix. At lower concentrations stimulatory effect was observed on Tolypothrix tenuis and Calothrix brevissima (Ibrahim, 1972; Gangawane, 1980; Anand and Veerappan, 1980; Bharati and Angadi, 1981; Kaushik and Venkataraman, 1983 and Tarar and Shewale, 1984).

Blue green algae are oxygen evolving photosynthetic prokaryotic organisms many of which are capable of nitrogen fixation (Stewart, 1980). Besides being able to reduce the elemental nitrogen blue green algae utilize various type of nitrogen sources (both organic and inorganic) efficiently for their growth (Kratz and Myers, 1955; Horne and Fogg, 1970; Rhee and Timoth, 1983; Elder and Parker, 1984 and Rawson, 1985). Singh (1975) studied the effect of fertilizers on 12 nitrogen fixing blue greens and reported high concentrations of urea, affected the growth. Lower concentrations of urea and potash promoted the growth of nitrogen fixing strains and non nitrogen fixing strains (Anand and Karuppasamy, 1987).
In the present study the response of *Chroococcus tenax* grown in BG$_{11}$ medium was noticed under natural and artificial light conditions. Under natural light the growth was faster (IV. Fig. 2 and IV. Table 2) and the amount of pigments, protein, lipid and carbohydrate content was also high when compared with the cells grown in artificial light. Light is an important factor which affect the population of Cyanobacteria in an ecosystem (Pabbi and Vaishya, 1990). *Nostoc calcicola* preferred a lower light regime and it grows well in 1200 lux units of light intensity (Anand *et al.*, 1990a). Likewise the isolate requires a high light intensity for its growth.

Physiological response of the alga (*Chroococcus tenax*) to amendment of urea and potash indicated that at lower concentrations there was good growth (IV. Fig. 4 and 5). At higher concentration the growth rate and biochemical components were also, less (IV. Table 3). Preference of blue green algae to a lower concentration of fertilizers in the milieu for good growth was found in two of the strains of *Nostoc musicola* and *Anabaena torulosa* (Anand and Karuppasamy, 1987). Singh (1975) showed that higher concentration of urea and potash reduced the growth of various blue green algae. External source of nitrogen has been good for algal growth, (Fogg *et al.*, 1973) as in the case of *Anabaena torulosa* which grew better in nitrogen fixing condition.

Another important point is the capacity of certain blue green algal strains to carry on with nitrogen fixation unabated even in the presence of a nitrogen source. Though in the presence of external source of nitrogen, the process of nitrogen fixation
may be redundant, these strains exhibit a unique behavior. The result obtained in the present study is similar to the earlier report of Anand and Karuppusamy (1987) on the work based on the physiological response of nitrogen fixing blue green algae to commercial fertilizers.

*Chroococcus tenax* when grown in the biofertilizer Azospirillum showed a very good growth, when compared with other fertilizers (IV. Fig. 6). However the growth rate and cell density was very low. But at lower concentrations of 0.1% to 0.5% the growth rate was good. This is due to the presence of nitrogen source. Turpin (1991) have reported that supplementation of nitrogenous source in the culture medium enhanced the microalgal growth.

When grown in different concentration of fungicide, pesticide and insecticide it was noticed that at low levels (IV. Fig. 7, 8 and 9) they enhanced the metabolic activities and also add on to the suitability of the isolate to become a prospective biofertilizer strain. The concentrations at which the alga grew in the pesticide did not affect the morphology (IV. Table 4) and was found to be more resistant to the pesticide. Very low concentration of these may be effectively used to prevent the destruction by insects. Similar type of result has been observed by Singh (1973); Anand and Veerappan (1980); Dikshit and Tiwari (1990). Pabbi and Vaishya (1990) stated that at lower concentration of the insecticide, the growth was very less and delayed, resulting in less protein and chlorophyll content. Similar to carbaryl, growth and chlorophyll inhibition
has been reported in several Cyanobacterial species due to the presence of 2, 4, 5T, MCPA, Propazine, etc (Roychoudhry and Kaushik, 1986). Depending on the concentration and nature of the pesticide, workers have reported a stimulatory, neutral or inhibitory effect (Holst et al., 1982; Kaushik and Venkataraman, 1983 and Straton, 1984). Biochemical determination of *Chroococcus tenax* with Bavistin, Phosmomidon and Carbaryl contents also revealed a measurable reduction in the pigments, protein, carbohydrate and lipid contents (IV. Table 4).

Characteristics of *Chroococcus tenax* when grown in varying concentrations of sodium chloride under laboratory conditions gave ample evidence of its halotolerant nature, surviving salt concentration at 4% (IV. Fig. 10). Salt not only sustained but enhanced its growth after the initial adaptation period. Several investigators have recently shown positive growth responses of blue green to external concentrations of sodium chloride (Apte and Thomas, 1974; Kessler, 1976 and Talpasayi and Rao, 1979). The fact that no significant morphological changes were observed under a relatively strong stress in agreement with the observations of Stam and Holleman (1975).

Chlorophyll 'a' content is often considered as an index of growth; although this pigment was measured only during the peak growth of the alga, it can be presumed that the decreasing tendency in its content with increasing salinity will be observed at every stage of growth. Apparently therefore, the corresponding faster growth rates of the cells photosynthetic processes were not disturbed despite a loss in chlorophyll content. A possible
explanation lies in the fact that phycocyanin showed a sharp increase with an increase in salinity, the ratio of phycocyanin to chlorophyll was of a high order in cells grown in saline media when compared to those grown in control (IV. Table 6). It has been shown by Haxo and Blinks (1950) that phycocyanin is a principal photosynthetic pigment in these alga and it is well established that biliproteins like phycocyanin transfer absorbed light energy to chlorophyll with high efficiency. Similar result has been noted on the other biochemical aspects also (protein, lipid and carbohydrate). This is in accordance with the earlier report of Anantani and Vaidya (1983) on the growth characteristics in a halotolerant Cyanophyte.

Basically, the algal production technology is comparable to agriculture and involves three well defined steps i.e. cultivation, harvesting and processing. Upto this day, the last word has not been said on the most ideal conditions required to produce algae and this depends on various factors such as the species being cultivated, location of the algal plant, climatic conditions, mode of agitation, supply of nutrients etc. The basic requisite for successful algal cultivation is the construction of a suitable pond which should be efficient, easy to operate, durable and cheap (Venkataraman, 1983).

*Chroococcus tenax* was produced in outdoor ponds which necessitates effective pond management to obtain reproducible yields, quality and cost benefit economics. To overcome the hazardous effect of water logging and salinity as growth of crop plants, scientists in various laboratories are engaged to raise salt
tolerant blue greens or Cyanobacteria. Hence the native autochthonous Cyanobacteria was isolated and cultured on open ponds (Energy pond concept). In order to increase the yield of algal powder, the medium was fertilized with the organic manure (farm yard manure 5%) which is cheap and easily available to the rural people i.e., to develop a low cost rural biotechnology and to obtain abundant biomass. However the contamination due to other groups (bacteria, fungi, algae) exists in the culture. But it was minimal due to alkaline pH (Venkataraman, 1981; Venkataraman and Becker, 1986). The medium was used in unsterilized deep well water. The earthen trenches of (18cm depth, 40cm length and width ratio) resulted in a 15% increase in total biomass (Plate 2). The mass production of *Chroococcus tenax* in salt free media during present investigation is quite significant for use as bio-fertilizer. A lot of publications are present regarding the open pond culture of microalgae (Gupta and Bajaj, 1983; Benemann, 1985; Richmond, 1988; Gupta and Changwal, 1992 and Venkataraman and Mahadevaswamy, 1992). Thus cultivation of isolate appears to be rather easy in outdoor open conditions.

Majority of methods proposed for mass cultivation of algae involved number of chemicals. But in the present study for mass cultivation only the organic manure, agriculture field soil and superphosphate are added (Plate 2). Algal colonies covered the surface entirely by 20 days. The yield was 90 g/l fresh weight of algal material. This results revealed that well water with organic manure (cow dung), agriculture field soil and superphosphate supported good growth of the alga. This work is concordant with
the earlier report of Bongale and Singh (1986, 1990) on the mass cultivation of *Nostoc piscinale* supplemented with paddy straw ash and superphosphate. It is concluded that organic manure (cow dung), superphosphate and agriculture field soil in deep well water serves as a suitable medium for field multiplication of algae. Formulation of such simple media by utilizing chemicals commonly known and easily available, will help in popularising algal biofertilizer at farmer’s level more efficiently.