CHAPTER II

CULTURING OF ALGAE FOR THE PHYSIOLOGICAL INVESTIGATION

A BRIEF REVIEW OF WORKS ON CULTURES OF ALGAE

Living organisms depend upon lot of factors during their life cycle. Main factor for surviving in the nature is nutrition, for which specific raw materials are required. In nature microbes and other photosynthetic organisms are found in ponds, puddles or as small patches on wet soil. In these habitats different communities of algae are thriving in association. In laboratory cultures, sometimes they all need special ingredients for their individual growth. Culture studies enable one to establish the absolute requirements of their nutrition for the growth. Laboratory cultures also serve as tools for taxonomical, physiological, biochemical and genetical studies. Hence the 'unialgal' and 'pure' cultures of certain species were grown for the investigation.

Beijerinck (1890) applied the term 'pure culture' for that in which there was the presence of only one species in a culture. Urskov (1922) defined 'pure culture' as one which consists of individuals, those all descended only from a single cell. Smith (1914) used the term 'unialgal'
culture for the first time. The 'unialgal' culture is that in which only one species of algae is present and the presence of other organisms such as bacteria and fungi are not taken into consideration. 'Pure culture' indicates the presence of only one species and it is completely free from bacteria and other organisms. The term 'axenic culture' is used for that culture in which the organism is grown along with its very natural associates. There are volumes of literature for getting pure cultures of various algal species. These methods are reviewed by Venkatraman (1969).

De (1939) and Singh (1942) employed silica gel plating; because bacteria can be removed by using inorganic media. Laloraya and Mitra (1964) also used this technique. Algal material was treated by chlorine water, by Fogg (1942). Allison et. al. (1930 and 1937); Ferloff et. al. (1950); Watanbe (1959); Venkatraman and Saxena (1963) used the technique of ultraviolet irradiation for getting pure culture. Van Baalen (1968) studied the effect of UV radiation on coccoid-blue green algae to study the survival, photosynthesis and photoreactivation. Kumar (1963) studied the UV resistant strain of Anacystis nidulans. Asato and Folsome (1969); Winkin (1969); Srivastava and Kumar (1969, 1971); Kumar (1970) studied the mutagenic effect of UV radiation on blue-green algae.
The time of irradiation required for making the algae free from bacteria varies with the organisms. Antibiotics were widely used for preparing 'pure-culture'. Watanabe and Kiyohara (1963) used various concentration of antibiotics in algal cultures. Gupta et. al. (1956) used mercuric chloride 1:10,000 diluted solution for getting 'pure-culture'. Aleshina (1961) tried to obtain bacteriologically pure cultures of flagellated-halophilic green algae. Coler Robert et. al. (1969) outlined the technique for the isolation of ciliated protozoa, blue-green algae and green algae from bacteria, by using the electrophoretic mobility of ciliated protozoa and ciliated forms of algae. They have shown apparently greater vulnerability of bacteria over protozoa and algae to lethal effects of electric filed.

For the preparation of media, many bacteriological methods were adopted for the separation of particular alga from its associates. Solid media are preferable. In early days gelatine was used as a solidifying agent, but now agar-agar is widely used for the cultivation of algae. Main difficulty to use agar is that the other organisms like bacteria and fungi grow along with alga, because agar itself is an organic substance, which provides nutrition to other organisms to grow. Silica gel is also used to isolate algal species from its associates. Venkatraman (1969)
suggested a modified method for preparing silicagel plates and making them sterile. Sodium silicate is also used sometimes, for isolating algal species from bacteria like organisms. Venkatraman (1969) had reviewed several methods used for this purpose. Algae grow better in nature. But in laboratory, algae should be provided the almost natural condition, and hence the special ingredients of inorganic salts, organic substances, vitamins, other chemicals, and air are supplied for their growth. Culture medium for algae can be prepared by adding major ionic compositions. Many workers have made some natural water like media. Chu (1942); Scott (1945); Myers and Clark (1944); Witsch (1946); Arnon et. al. (1955); Watanbe (1960); Spektorov et. al. (1963); Gorham et. al. (1964) etc.

Carbon, sulfur, phosphorus, nitrogen, potassium, magnesium and other elements are essential for the nutrition of algae. Trace elements or micronutrients are supplemented in the main media (Arnon, 1938). Some algae require vitamins and other growth promoting substances in very little amount.

Algae are able to synthesize their own food material through photosynthesis; because they are autotrophic in nature. Light is one of the important factor for the growth of autotrophes (algae). Tamiya et. al. (1953)
studied the effects of light intensities on the growth rate of alga *Chlorella ellipsoidea*. Jorgensen (1960) also observed that the strong light has variable effects on cells which were previously grown in low light intensity. Jaroszynska (1971) studied growth of *Chlorella vulgaris* and *Chlorella pyrenoidosa* in permanent and periodic illuminated cultures. Sirnoval and Kandler (1958) showed that very intense light is able to bleach the cells. Hattori and Fujita (1959) studied the effects of various wavelengths of light on the pigments.

Kiyohara et. al. (1960) demonstrated that *Toxopthrix tenusis* grows heterotrophically when exogenous energy source like glucose and casaminoacids were supplied in dark. Lord and Merrett (1971) showed that glycollate stimulates the growth of *Chlorella pyrenoidosa*. Lewin and Guillard (1963) studied the interrelations between temperature and light.

Temperature is also a valuable factor for the algal growth. Mesophilic algae are not able to survive at the temperature higher than 40°C, but some strains of *Chlorella* isolated by Sorokin and Myers (1953) can survive in higher temperature.

The hydrogen ion concentration of the medium is also responsible for the growth; because it has direct effect on the protoplasm of the cell.
For the physiological studies of algae, carbon dioxide concentration in the cultural atmosphere is an important factor. Generally 5% CO₂ mixed air is aerated to the cultures, as suggested by Warburg (Myers, 1960).

Carbon may be provided to most algae, in the form of glucose, acetate, and glycollate. The simple method of producing Chlorella on large scale, is to use the large cotton stoppered flasks, cultures are provided with glucose, illuminated and mechanically shaken or aerated (Myers, 1960; Matsuka, Mistsuo and Hiji Hase, 1970, 1971) studied several aspects of metabolism of glucose and acetate in Chlorella protothecoides. Piskunkova et. al. (1971) studied acetate and pyruvate requirement of Scenedesmus quadricauda. Kyc (1970) studied the sodium-humate and increase cells in Scenedesmus quadricauda.

Algae can grow in presence of continuous light as well as in altering periods of light and dark (12 hr each) Sueoka (1960), Sager and Granick (1953). To provide sufficient light and aeration, shaking of the culture media is required; for continuous shaking, shakers are in wide use in laboratories.

When people are diverting their attention towards using algae as food not only in underdeveloped countries but also in affluent space crafts the culturing of algae
in laboratory and also on large scale is a problem of research of the present day and it will always going to remain a green subject of investigation.

MATERIALS AND METHODS

The cultivation of algae in our laboratory has been undertaken with the aim to study:

i. physiological relationship between two groups, namely blue-green, the prokaryotic algae and green, the eukaryotic algae,

ii. to evaluate their role in crop fields,

iii. sea-coast of this part of the country viz. Okha, Dwarka and Veraval is endowed by the natural wealth. Colourful flora of the marine algae, may be of economic importance.

Following algae were cultured for the experimentation in the present investigation:

<table>
<thead>
<tr>
<th>Blue-green algae</th>
<th>Thallus structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Chroococcus minutus, Nag.</td>
<td>Unicellular</td>
</tr>
<tr>
<td>3. Nostoc (Strain-222 IARI)</td>
<td>Filamentous with heterocyst.</td>
</tr>
</tbody>
</table>
Green algae

5. *Chlorella pyrenoidosa*, Chick Unicellular
6. *Scenedesmus* sp. Coenobium
7. *Pithophora* sp. Filamentous branched
8. *Ulva fasciata*, Delile Marine, paranchymatous thalloid
10. *Chara corallina*, Klein ex Willdenow Filamentous but highly evolved
11. *Chara globularis*, Thurlier Filamentous but highly evolved

Thus these organisms cover: primitive and advanced; unicellular and multicellular; fresh water and marine; nitrogen fixing and non-nitrogen fixing forms.

a. Cleaning of glass-ware:

Glass wares were washed with tap water and soaked in cleaning solution of chromic acid for overnight. Next day they were cleansed first by tap water and then by 'Tipol' detergent solution. After this glass wares were washed with tap water and distilled water simultaneously and then they were dried in oven.
b. Preparation of Media:

For the preparation of media, double distilled glass-water was used. In most of the cases, analar chemicals were used. Weighing of ingredients were made on Mettler micro balance.

It is by several trials of various prescriptions for the media by various authors, we could select some of them and applied for the preparation of culture media of algae. Media prepared for the cultivation of algae are as follows:

1. Oscillatoria jasovensis, Vouk.

Kiyohara et. al. (1960) prepared the medium for growing Toxopothrix tenuis, heterotrophically. The same medium was slightly modified by eliminating casaminocids, glucose and adding potassium nitrate. Its composition is as follows:

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>gm/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>1.0 - 3.5</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.25</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>0.5</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>0.02</td>
</tr>
<tr>
<td>FeSO₄.7H₂O</td>
<td>0.02</td>
</tr>
<tr>
<td>Arnon's A₅</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

pH of the medium was adjusted to 7.5
Watanabe (1960)

Ingredients gm/l
KNO₃ 3.0
Na₂HPO₄·12H₂O 0.5
MgSO₄·7H₂O 0.5
CaCl₂·2H₂O 0.02
FeSO₄·7H₂O 0.02
A₅ (micronutrient) 1.0 ml

Another medium was also practiced, and that is formulated by Watanabe (1960) (Venkatraman, 1969) and the "Cyanophycean medium" of Thomas and David (1969). Composition of ingredients are as follows:

Ingredients gm/l
KNO₃ 1.00
K₂HPO₄ 1.0
MgSO₄·7H₂O 0.25
Ca(NO₃)₂·4H₂O 0.025
Na-Citrate 0.165
FeSO₄·7H₂O 0.02
A₅ (micronutrient) 1.0 ml
pH of the medium was adjusted to 8.0
Ingredients  
\[ \begin{align*} 
\text{KNO}_3 & \quad 1.25 \\
\text{Na}_2\text{HPO}_4 & \quad 1.00 \\
\text{Na-EDTA} & \quad 0.50 \\
\text{Citric acid} & \quad 0.006 \\
\text{CaCl}_2 \cdot 2\text{H}_2\text{O} & \quad 0.084 \\
\text{H}_3\text{BO}_3 & \quad 0.114 \\
\text{FeSO}_4 \cdot 7\text{H}_2\text{O} & \quad 0.05 \\
\text{ZnSO}_4 \cdot 7\text{H}_2\text{O} & \quad 0.088 \\
\text{MnCl}_2 \cdot \text{H}_2\text{O} & \quad 0.014 \\
\text{MoO}_3 & \quad 0.007 \\
\text{CuSO}_4 \cdot 5\text{H}_2\text{O} & \quad 0.016 \\
\text{Co(NO}_3\text{)}_2 \cdot 6\text{H}_2\text{O} & \quad 0.005 \\
\end{align*} \]

pH of the medium was adjusted to 8.0

3. Medium used for *Nostoc* (strain-222- IARI). Venkatraman suggested nitrogen free medium for this *Nostoc*-222 and hence Nitrate free Fogg's medium was used.

Fogg *(1949)*

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>gm/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td></td>
</tr>
<tr>
<td>[ \text{KH}_2\text{PO}_4 ]</td>
<td>0.2</td>
</tr>
<tr>
<td>[ \text{MgSO}_4 \cdot 7\text{H}_2\text{O} ]</td>
<td>0.2</td>
</tr>
<tr>
<td>[ \text{CaCl}_2 \cdot 2\text{H}_2\text{O} ]</td>
<td>0.1</td>
</tr>
<tr>
<td>[ \text{Fe-EDTA} ]</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

To the above medium 1 ml/l was added, of the following medium:
Ingredients S.g/1.

- $\text{Na}_2\text{MoO}_4$ 0.1
- MnCl$_2$ 0.1
- $\text{H}_3\text{BO}_3$ 0.1
- CuSO$_4$ 0.1
- ZnSO$_4$ 0.1

pH of the medium was adjusted to 7.6.

B. Composition of A5 micronutrient solution:

- a. $\text{H}_3\text{BO}_3$ 2.860
- b. MnCl$_2$ 1.810
- c. ZnSO$_4 \cdot 7\text{H}_2\text{O}$ 0.222
- d. $\text{Na}_2\text{MoO}_4$ 0.0177
- e. CuSO$_4 \cdot 5\text{H}_2\text{O}$ 0.079

C. Preparation of Fe-EDTA stock solution:

Dissolved 26.1 g of EDTA (ethylene diamine tetraacetic acid) in 268.0 ml of 1N KOH solution, and added 2.9 g. of ferrous sulfate and made up the volume to one litre. The solution was aerated overnight to produce a stable complex, marked by a change in colour to dark brown. Made up the volume to one litre. 1 ml of this solution in
1 litre gave 5 ppm iron concentration.

4. Medium used for *Chlorella pyrenoidosa*, Chick. were those of Spektorov et. al. (1963), Venkatraman (1969) and Watanbe (1960).

a. **Prings-82 medium (Spektorov et. al., 1963):**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>gm/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>5.0</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>2.5</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.25</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>0.003</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.037</td>
</tr>
</tbody>
</table>

**Composition of Arnon's A₄ micronutrient solution:**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>gm/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄VO₃</td>
<td>0.02296</td>
</tr>
<tr>
<td>NH₄MoO₄</td>
<td>0.0229</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>2.86</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>1.81</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>0.222</td>
</tr>
</tbody>
</table>

Only 1.0 ml of A₄ solution was added to 1 litre of main cultural solution. NH₄VO₃ was not available to us. So it was omitted from the composition of the medium. But due
to this the algae did not show the distinct effect on the growth to visible extent.

b. **Myers' medium for Chlorella (Myers, 1947):**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>gm/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>1.21</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>2.46</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.23</td>
</tr>
<tr>
<td>Fe₂(SO₄)₃</td>
<td>0.052</td>
</tr>
<tr>
<td>Na-citrate</td>
<td>0.195</td>
</tr>
</tbody>
</table>

In all above mentioned media for *Chlorella pyrenoidosa*, Myers' medium was preferable for its optimum growth.
4. Medium used for Scenedesmus sp. Composed by Arnon et al. (1955):

Ingredients

- **KNO\textsubscript{3}** 0.02 M
- **MgSO\textsubscript{4}.7H\textsubscript{2}O** 0.001 M
- **CaCl\textsubscript{2}** 0.005 M
- Phosphate 0.002 M

The phosphate was supplied as a mixture of KH\textsubscript{2}PO\textsubscript{4} and K\textsubscript{2}HPO\textsubscript{4}. This medium was supplemented by A\textsubscript{5} micromnutrient solution 1.0 ml.

pH was adjusted to 6.7

5. *Pithophora* was grown in soil extract culture.

The garden-pond soil on which *Pithophora* was growing naturally was used for the purpose. *Pithophora* sp. grew in long filamentous form. Our efforts to grow *Pithophora* on inorganic synthetic media are not successful so far.

6. Cultivation of marine forms *Ulva fasciata*, *Delise* and *Enteromorpha flexuosa* (Wulff) J.Ag.

Algae were cultured in natural sea water for the experiments as well as in artificial sea water medium. Ahluwalia (1967). Composition of artificial sea water is as follows:
i. **Ingredients**  

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>gm/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>27 - 28</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>0.3 - 0.5</td>
</tr>
<tr>
<td>CaSO₄</td>
<td>1.0 - 1.5</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>1.5 - 2.0</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.2 - 0.25</td>
</tr>
<tr>
<td>KCl</td>
<td>0.3 - 0.5</td>
</tr>
</tbody>
</table>

pH was adjusted to 7 - 7.3 and sp. gravity was about 1.025 - 1.028.

ii. 30.33 gms of raw salt was dissolved in one litre of distilled water and was filtered. This could also serve as a good culture medium for marine algae.

7. **Chara**: Species of charophytes were grown in glass aquarium, which was filled with sterile sand and garden soil. The algae were planted in the aquarium soil-substratum.

C. **Collection of algae**:

*Oscillatoria jasorvensis, Chlorella pyrenoidosa* were collected from the paddy fields and ponds in University Campus area. *Pithophora* was also collected from Botanical garden of University School of Sciences, Ahmedabad.

Algal material was washed in the glass jars with running tap water and then algal mass was collected in buchner funnel, and allowed to stand on the surface of the
filter paper. After the identification, all the materials were purified by series of reculturing, in respective media.

*Nostoc* (Strain-222, IARI) and *Anabaena variabilis* were kindly supplied by Venkatraman-IARI, Delhi and Thomas from Trombay respectively.

d. Preparation of Solid medium

2 gm of agar was added in the above mentioned media and agar solution (Melted) was poured in the flasks. These flasks were plugged with non-absorbant cotton and were capped by paper. These flasks were then autoclaved for 20 minutes at 15 lbs/sq.inch pressure. These flasks were sterilized and kept oblique overnight so the slant was prepared for the plating or inoculation.

The media which were not solidified with agar-agar were sterilized by autoclaving at 15 lbs/sq.inch pressure for 20 minutes. The flasks were then put in running water-tray for cooling of medium. By cooling, the precipitates could be dissolved. In some media having high amount of phosphate, the phosphates were added separately after cooling of medium and under aseptic condition.

f. Bacteria free cultures

In spite of aseptic conditions and autoclaving, cultures of blue-green algae were some times not free from bacteria. Additional measures were taken to remove bacteria
from the culture. The inoculum was exposed to germicidal Philips Ultraviolet tube 2537 Å for varying time of 5 to 10 minutes. It was constantly shaken by magnetic stirrer to achieve thorough exposure of all algal suspension. This removed bacteria from culture of algae. This much exposure was not found harmful to algae but killed bacteria if any.

*Oscillatoria jasorvensis* has been found resistant to antibiotic treatment and hence removal of bacteria is easier in this case. The inoculum was kept in the streptomycin-sulphate solution (200 mg/l) for eight hours under constant shaking.

g. Inoculum

Algae were inoculated in culture medium under aseptic conditions by platinum wire loop between two burners. It was then inoculated in the culture medium.

First inoculation was done by surface plating method (Streaking). After growth of 4-5 days the isolated unialgal mass was inoculated to liquid medium.

h. Bacteriological test for inoculum

The purity of an inoculum was tested by following bacteriological medium:
Ingredients

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>gm/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0</td>
</tr>
<tr>
<td>Meat extract or Beef extract</td>
<td>3.0</td>
</tr>
</tbody>
</table>

pH of the medium was adjusted to 7.6.

Autoclaved culture tubes were inoculated by algal inocula, and the tubes were kept at 37°C in incubator for 72 hrs. The absence of turbidity indicated that there was no bacterial contamination in the algal culture. The algal samples were also examined microscopically and stained with Gram's iodine method for checking the presence of bacteria.

1. Temperature

All cultures were maintained at 25°C. However, cultures of *Anabaena variabilis; Oscillatoria jasorevensis*, *Ulva fasciata* and *Enteromorpha flexuosa* grew better at room temperature (30°C). *Nostoc - 222* grew better at 25°C in temperature controlled room.

1. Light

Philips fluorescent tube light 40W were used for illumination. The light falling on culture flasks was about 70 foot candles.
EXPERIMENTAL FINDINGS

It was aimed to make intensive study of growth behaviour of unicellular blue-green and green alga in different cultural conditions. Which will be discussed in the next chapter. Algae other than unicellular forms were not studied for the growth as such. Some observations are reported here:

1. Oscillatoria jasovensis, Vouk.
   i. Medium suggested by Kiyohara et. al. (1960) in which alga did show the poor growth whereas in medium composed by Watanbe (1960) alga showed better growth.
   ii. Room temperature 30±2°C is very effective for the better growth of blue green algae.
   iii. After 10-15 days algal mass become spongy balls like structures.
   iv. After 10-15 days of growth algal medium turns yellow-brown in colour. This may be due to some exudate or decomposition of old algal filaments.

   1. This alga grew better in Myers 'G' medium for blue-green algae.
ii. Algae showed better growth in the month of June-July during the year because of favourable room temperature.

iii. Production of heterocyst and spores are also more common at optimum temperature.

iv. On agar-solid media after inoculation slant became bluish. This bluish colour due to reaction between ammonium molybdate and some reducing substance, will be discussed elsewhere.

3. *Chlorella pyrenoidosa*, *Chick* and *Scenedesmus* sp.
   i. In control experiment algae found resistant to 200 mg/l streptomycin.
   
   ii. Continuous shaking culture flasks give better growth.

4. *Pithophora* sp.
   
   i. Alga grew better in submerged pot surface.
   
   ii. Flask cultures did not show active growth for more than 3-days.

5. *Ulva fasciata*, and *Enteromorpha flexuosa*.

   These marine algae grew better in sea water than in artificial culture medium.


