II. MATERIALS AND METHODS

MATERIALS AND METHODS

The materials for the present investigation include three freshwater microalgae *Chlorococcum humicola*, (Naeg.) Rabenhorst, *Chlorella ellipsoidea*, Gerneck and *Scenedesmus bijuga*, (Turp.) Lagerheim - all belonging to the class Chlorophyceae.

2.1 Isolation of Test Organisms

The test organisms were isolated from water samples collected from different freshwater ponds in and around Ernakulam district. Dilution method was adopted for isolation. For this, small quantities of water samples were kept undisturbed in sterilised petridishes after adding a pinch of KNO_3 as basic nutrient. These were exposed to suitable conditions of light and temperature. After two weeks, the discrete algal colonies

developed in the petridishes were examined under light microscope and identified with the help of the descriptions given by Fritsch (1935) and Prescott (1982).

Different colonies were subjected to serial dilution independently. By successive serial dilution two unicellular green algae *viz. Chlorococcum humicola,* (Naeg.) Rabenhorst and *Chlorella ellipsoidea,* Gerneck and one colonial green alga *Scenedesmus bijuga,* (Turp.) Lagerheim were obtained in pure form and were selected as the study materials.

Systematic position of the isolated algae as proposed by Fritsch (1935) is as follows:

Chlorococcum humicola, (Naeg.) Rabenhorst

Division	-	Chlorophyta
Class	-	Chlorophyceae
Order	-	Chlorococcales
Family	-	Chlorococcaceae
Genus	-	*Chlorococcum,* Fries
Species	-	*humicola,* (Naeg.) Rabenhorst

Chlorella ellipsoidea, Gerneck

Division	-	Chlorophyta
Class	-	Chlorophyceae
Order	-	Chlorococcales

Family	-	Chlorellaceae
Genus	-	*Chlorella*, Beyerinck
Species	-	*ellipsoidea*, Gerneck

Scenedesmus bijuga, (Turp.) Lagerheim

Division	-	Chlorophyta
Class	-	Chlorophyceae
Order	-	Chlorococcales
Family	-	Coelastraceae
Genus	-	*Scenedesmus*, Meyen
Species	-	*bijuga*, (Turp.) Lagerheim

2.2 Culture Conditions

Various nutrient solutions like Miquel's medium (Miquel, 1890), Walne's medium (Walne, 1974) and Ward and Parish medium (Ward and Parish, 1982) were tried for culturing the isolated algae. Maximum growth of test organisms was observed in Ward and Parish medium. Hence the stock cultures and the experimental cultures were raised in this medium.

The normal constituents of Ward and Parish medium are:

Macronutrients

$NaNO_3$	-	25.5 g/l
$MgCl_2.6H_2O$	-	12.2 g/l
$MgSO_4.7H_2O$	-	14.7 g/l
$CaCl_2.2H_2O$	-	4.41 g/l
$NaHCO_3$	-	15.0 g/l
K_2HPO_4	-	1.044 g/l

Micronutrients

1. $CoCl_2$ - 0.78 g/l
2. $CuCl_2$ - 0.9 g/100ml

1ml of each of this solution was diluted to 1 litre for making stock solution.

3. a) H_3BO_3 - 0.1855 g
   b) $MnCl_2$ - 0.2643 g
   c) $ZnCl_2$ - 0.327 g
   d) $Na_2MoO_4.H_2O$ - 0.0073 g
   e) $FeCl_3$ - 0.0960 g
   f) Na_3EDTA - 0.300 g
   g) Micronutrient solutions 1 and 2 - 1 ml each
   h) Distilled water - 1 litre

The maintenance medium was prepared by adding 1 ml of each macronutrient solution and 1 ml of micronutrient stock solution number (3) to 1 litre double distilled deionised water.

The stock cultures were maintained in sterilised "Borosil" conical flasks plugged with non-absorbent cotton (Plate 2.1a). Reculturing was done after every 14 days to keep the cells in the exponential phase. The mother cultures and the experimental cultures were exposed to a photoperiod of 10 hrs light and 14 hrs darkness. Illumination was provided

by day light fluorescent tubes with the intensity of 2000 lux. The ambient temperature ranged from 27^0C to 32^0C.

Aeration was not provided to the cultures. Instead, they were hand shaken manually to give three or four rotations regularly to avoid the settling of the cells. Cultures were not bacteria free.

2.3 Test Procedure

The experimental investigations conducted in the laboratory conditions were concerned with the role of macronutrients phosphorus, magnesium and calcium and micronutrients iron, manganese and zinc on the growth of the test organisms.

Test cultures were kept in sterilised "Borosil" conical flasks of one litre capacity plugged with non-absorbent cotton. 500 ml of the culture medium was taken in each conical flask. All sample containers and glasswares were washed with detergent, rinsed with tap water, distilled water and soaked in nitric acid overnight. They were washed again in double distilled water to reduce the degree of contamination.

In order to evaluate the requirements of each selected macronutrient, the experiments were carried out using Ward and Parish medium containing various concentrations of the concerned element. The medium deficient of the test element was kept as control. Double distilled deionised water was used for the preparation of the medium.

The modified medium of Ward and Parish (Asma, 1993) which contains lesser number of micronutrients was used for the assessment of trace elements. The composition of the modified medium is as follows:

Macronutrients

$NaNO_3$	-	6.37 g
$MgCl_2 \cdot 6H_2O$	-	3.05 g
$MgSO_4 \cdot 7H_2O$	-	3.675 g
$CaCl_2 \cdot 7H_2O$	-	1.1 g
K_2HPO_4	-	0.261 g
Distilled water	-	1 litre

Micronutrients

1. a) $CoCl_2$ - 0.078 g/100ml

   b) $CuCl_2$ - 0.9 g/100ml

   Dilute 1 ml of each to 1 litre for working stock solution.

2. a) H_3BO_3 - 0.185 g

   b) $MnCl_2$ - 0.264 g

   c) $FeCl_3$ - 0.096 g

   d) Na_3EDTA - 0.3 g

   e) Distilled water - 1 litre

   To this add 1 ml of each micronutrient stock solution

3. Na_2SiO_3 - 1.2g

   Distilled water - 100 ml

The maintenance and test medium were prepared by adding 4 ml of macronutrient solution and 1 ml each of micronutrient stock solutions (2) and (3) to 1 litre sterilised double distilled deionised water.

The role of manganese (Mn) and iron (Fe) was assessed using the modified medium by adopting the same method as used for the study of macronutrients. The influence of zinc (Zn) was studied by exposing the test organisms to different concentrations of $ZnCl_2$. The dilution for each concentration was done with the medium.

The inoculum for all the experiments was taken from the mother cultures during the exponential phase. The cells to be inoculated were starved in the medium devoid of the particular element for a week, prior to the inoculation. The initial concentration of test organisms in all the experiments was 10 x 10^4 cells/ml.

Each experiment was conducted in triplicate for a period of 21 days. The test cultures were periodically hand shaken to keep the cells in suspension. The experimental set up was as shown in plate 2.1b.

2.4 Growth Measurements

2.4.1 Measurement of cell concentration

About 1 ml of culture from each conical flask was fixed in Lugol's iodine in every alternate days and counted with a calibrated haemocytometer and represented as cells x 10^4/ml.

2.4.2 Quantitative estimation of algal pigments by spectrophotometry

The concentrations of chlorophylls and carotenoids were determined by spectrophotometric analysis of pigment extract (Parsons *et al.*, 1984).

10 ml of the culture from each experimental flask was filtered through Millipore HA filters of pore size 0.45μ. One to two drops of $MgCO_3$ were added to the samples while filtering to prevent acidification. The pigments were extracted by adding 10 ml of 90% acetone. The extraction was carried out at low temperature in a refrigerator for 24 hrs. After the extraction was completed, samples taken out of the refrigerator were allowed to warm to room temperature. The extracts along with the blank were centrifuged after making the final volume to 10 ml. The absorbance of pigment extract was measured against the blank at different wavelengths as 480, 510, 630, 647, 664 and 750 nm. The concentration of various pigments was calculated using the equations given by Parsons *et al.* (1984).

(C*a*) Chlorophyll *a* $= 11.85\ E_{664} - 1.54\ E_{647} - 0.08\ E_{630}$

(C*b*) Chlorophyll *b* $= 21.03\ E_{647} - 5.43\ E_{664} - 2.66\ E_{630}$

(Cc) Chlorophyll *c* $= 24.52\ E_{630} - 1.67\ E_{664} - 7.60\ E_{647}$

(Cp) Plant carotenoids $= 7.6\ (E_{480} - 1.49\ E_{510})$

Where E stands for the absorbance at different wavelengths corrected by the 750 nm reading.

2.4.3 Productivity measurement

Productivity measurements were made using light and dark bottle method (Gaarder and Gran, 1927). From each experimental flask 3 samples - initial, light and dark - filled in 60 ml BOD bottles were analysed for dissolved oxygen content. BOD bottles were filled with algal cultures without introducing any air bubble and stoppered quickly to avoid contact with the air. The dissolved oxygen content of the initial bottles were determined by Winkler's method. The light and dark bottles were incubated in light for 5 hours and the oxygen content in both the sets of bottles were determined by the same procedure.

From the differences in the oxygen content of the initial, light and dark bottles GPP and NPP were calculated (Strickland and Parsons, 1972).

The oxygen values were then converted into their carbon equivalents using the formula,

$$\text{mg C / l / hr} = \frac{O_2 \text{ ml} \times 0.536}{PQ \times T}$$

where,

PQ (photosynthetic quotient) - 1.25, T- time

2.4.4 Estimation of biochemical compounds

1. Carbohydrate (Kochert, 1978a)

Carbohydrate measurements were made using phenol-sulphuric acid method as described by Kochert (1978a) based on the procedures developed by Dubois *et al.* (1956).

Algal pellet collected after centrifugation was homogenised in 1 ml 80% H_2SO_4 as proposed by Myklestad and Haug (1972). After adding the required volumes of 5% phenol and con. H_2SO_4, the intensity of the colour developed was read against the blank at 485 nm in a spectrophotometer. The carbohydrate content of the experimental sample was determined from the standard graph prepared with different concentrations of glucose.

2. Protein (Dorsey *et al.*, 1978)

10 ml of the culture from each conical flask was centrifuged to obtain the algal pellet. After washing the pellet with an isotonic solution of ammonium molybdate, the extraction of protein was carried out at 100^0C for 100 minutes using appropriate reagents. The concentration and final volumes of the reagents were as suggested by Winkfors *et al.* (1984). After the development of biuret-folin colour on heating, the absorbance was measured against the blank at 660 nm in a spectrophotometer. The unknown concentration of the protein was calculated from the standard graph obtained with prepared solutions of Bovine Serum Albumin.

2.5 Photomicrography

Photomicrographs of three test organisms were taken in order to expose the morphological changes that had been occurred in control cells when compared to those supplied with optimum amount of concerned element. The algal cells were fixed in 50% glycerine and their photomicrographs were taken using an inverted microscope (Leica, Germany). Kodak (200) colour films were used for taking photographs.

2.6 Statistical analysis

In order to evaluate the effect of various concentrations of different nutrients on three microalgae, two way analysis of variance (ANOVA) technique was employed (Snedecor and Cochran, 1967). The F value was taken at 5% and 1% significant level. In Anova Tables, different concentrations of nutrient were considered as treatments and between days as replicates.

Based on the findings of the present study, a modified Ward and Parish medium for each alga was prepared and the same is proposed for the better culturing of the studied species. The constituents of the medium for each species is given in the following pages.

Modified Ward and Parish Medium for *Chlorococcum humicola,* (Naeg.) Rabenhorst

Macronutrients

1.	$NaNO_3$	-	25.5 g in 1 litre Dist. H_2O
2.	$MgCl_2.6H_2O$	-	12.2 g in 1 litre Dist. H_2O
3.	$MgSO_4.7H_2O$	-	14.7 g in 1 litre Dist. H_2O
4.	$CaCl_2.2H_2O$	-	4.41 g in 1 litre Dist. H_2O
5.	$NaHCO_3$	-	15.0 g in 1 litre Dist. H_2O
6.	KH_2PO_4	-	1.044 g in 1 litre Dist. H_2O

Micronutrients

1.	$CoCl_2$	-	0.78 g in 1 litre Dist. H_2O
2.	$CuCl_2$	-	0.9 g in 100 ml Dist. H_2O

Dilute 1ml of both (1) and (2) to 1 litre for working stock solution.

3.	H_3BO_3	-	0.1855 g
	$MnCl_2$	-	0.2643 g
	$ZnCl_2$	-	0.1308 g
	$Na_2MoO_4 . H_2O$	-	0.0073 g
	$FeCl_3$	-	0.024 g
	Na_3EDTA	-	0.300 g
	Dist. H_2O	-	1 litre

Micronutrient stock solutions (1) and (2) - 1 ml each

To prepare maintenance and test medium, add 1 ml of each macronutrient solution and 1 ml of micronutrient solution number (3) to 1 litre Dist. H_2O.

Modified Ward and Parish Medium for *Chlorella ellipsoidea*, Gerneck

Macronutrients

1. $NaNO_3$ - 25.5 g in 1 litre Dist. H_2O
2. $MgCl_2.6H_2O$ - 6.1 g in 1 litre Dist. H_2O
3. $MgSO_4.7H_2O$ - 7.35 g in 1 litre Dist. H_2O
4. $CaCl_2.2H_2O$ - 2.205 g in 1 litre Dist. H_2O
5. $NaHCO_3$ - 15.0 g in 1 litre Dist. H_2O
6. KH_2PO_4 - 1.044 g in 1 litre Dist. H_2O

Micronutrients

1. $CoCl_2$ - 0.78 g in 1 litre Dist. H_2O
2. $CuCl_2$ - 0.9 g in 100 ml Dist. H_2O

Dilute 1ml of both (1) and (2) to 1 litre for working stock solution.

3. H_3BO_3 - 0.1855 g

   $MnCl_2$ - 0.132 g

   $ZnCl_2$ - 0.1743 g

   $Na_2MoO_4{\cdot}H_2O$ - 0.0073 g

   $FeCl_3$ - 0.048 g

   Na_3EDTA - 0.300 g

   Dist. water - 1 litre

   Micronutrient stock solutions (1) and (2) - 1 ml each

To prepare maintenance and test medium, add 1 ml of each macronutrient solution and 1 ml of micronutrient solution number (3) to 1 litre Dist.H_2O.

Modified Ward and Parish Medium for *Scenedesmus bijuga,* (Turp.) Lagerheim

Macronutrients

1. $NaNO_3$ - 25.5 g in 1 litre Dist. H_2O
2. $MgCl_2.6H_2O$ - 12.2 g in 1 litre Dist.H_2O
3. $MgSO_4.7H_2O$ - 14.7 g in 1 litre Dist. H_2O
4. $CaCl_2.2H_2O$ - 2.205 g in 1 litre Dist. H_2O
5. $NaHCO_3$ - 15.0 g in 1 litre Dist. H_2O
6. KH_2PO_4 - 1.044 g in 1 litre Dist. H_2O

Micronutrients

1. $CoCl_2$ - 0.78 g in 1 litre Dist. H_2O
2. $CuCl_2$ - 0.9 g in 100 ml Dist. H_2O

Dilute 1 ml each of (1) and (2) to 1 litre for working stock solution.

3. H_3BO_3 - 0.1855 g
   $MnCl_2$ - 0.132 g
   $ZnCl_2$ - 0.1308 g
   $Na_2MoO_4.H_2O$ - 0.0073 g
   $FeCl_3$ - 0.048 g
   Na_3EDTA - 0.300 g
   Dist.H_2O - 1 litre

   Micronutrient stock solutions (1) and (2) - 1 ml each

Add 1 ml of each macronutrient solution and 1 ml of micronutrient solution number (3) to prepare maintenance and test medium.

PLATE -2.1

a. Stock cultures of test organisms

A. *Chlorococcum humicola,* (Naeg.) Rabenhorst

B. *Chlorella ellipsoidea*, Gerneck

C. *Scenedesmus bijuga*, (Turp.) Lagerheim

b. Experimental set up.

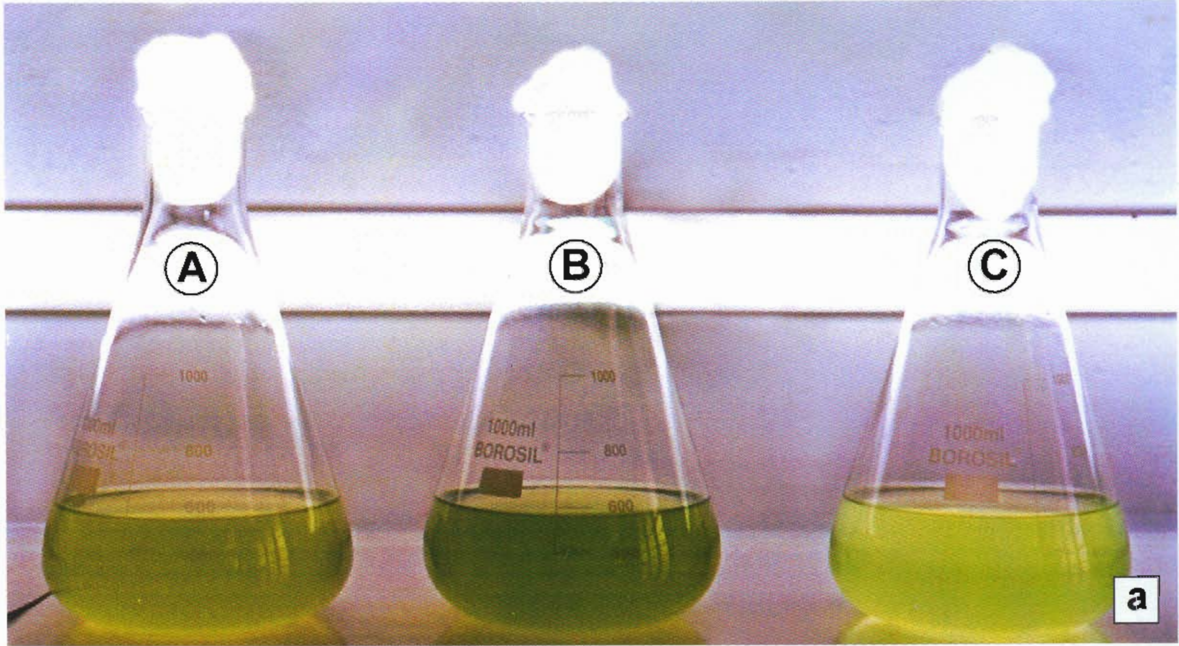

PLATE - 2.1