CHAPTER - III

MATERIALS AND METHODS

A. Test conditions:

The algal culture room:

It is a small room measuring 8' x 6' x 8' (high) located at the entrance to the existing cold storage room which is being maintained at about 5°C. A glass table on wheels measuring 3' x 2' x 3' (high) is placed at the opposite corner of the entrance. The table has two transparent glass sheets of 0.5 cm thick, each measuring 3' x 2' and are placed one below the other. One of them is placed at the top and the other is at a distance of 1' - 5" below the top glass sheet. The lower sheet of the glass is at a height of 1' - 5" above the ground floor.

Two pairs of cool white transperent day-light tube-lights each of 4 ft. in length, 40 Watts and 2 ft. apart were placed just below the top glass sheet and another similar pair just below the second glass sheet for illuminating the ground floor which was covered with white sheets of paper for reflecting the light into the culture medium. The intensity of illumination measured outside

the flask at the liquid level on the ground floor was 375 lux as measured by a representative of Philips Company. Illumination was continuous day and night. The temperature of the room on the ground floor ranged between 28.4 to 29.9°C.

Isolation, acclimatization and maintenance of stock algal cultures for the laboratory scale experiments:

The pure algal specimens studied were obtained from the Algal Division of the Department of Microbiology, Indian Agricultural Research Institute, New Delhi. All of them were maintained as pure cultures on Fogg's autotrophic agar slants and liquid medium on the middle glass sheet in the culture room for use in the experiments to follow:

Composition of Fogg's algal nutrient medium:-

Fogg's medium (with N):		A_5 micronutrient solution:	
KH_2PO_4	= 0.2 g/l	H_3BO_3	= 0.86 g/l
$MgSO_4$	= 0.2 g/l	$MnCl_2$	= 1.81 g/l
$CaCl_2$	= 0.1 mg/l	$ZnSO_4.7H_2O$	= 0.222 g/l
KNO_3	= 0.25 mg/l	$NaMoO_3$(85%)	= 0.0177 g/l
A_5 solution	= 1.0 ml/l	$CuSO_4.5H_2O$	= 0.079 g/l
Fe EDTA	= 1.0 ml/l		
Agar	= 1.5% for solid medium		

Fe. EDTA was prepared as follows:

26.1 gm. of EDTA was dissolved in 268 ml of 0.1N KOH, 24.9 gm of Fe $SO_4.7H_2O$ was added to it and the volume was made up to one litre. The solution was aerated overnight to produce the stable complex (the colour changed to dark brown).

From agar slants each alga was transferred aseptically to three flasks containing sterilised Fogg's liquid medium and incubated in the algal culture room for 7 - 10 days when good algal growths were obtained. Then to each of three flasks containing Fogg's liquid medium with 10% of raw, settled and strained (through cotton) sewage collected from Wadi Sewage Disposal Works, Baroda, 25% of algal culture grown already in Fogg's liquid medium were added. These flasks were kept on the ground floor in the algal culture room. In about a week there was good growth of algae. From these flasks, algal cultures (25%) were again transferred to another set of three flasks each containing 10% of raw, settled and strained sewage in Fogg's liquid culture medium. There was again good growth in about a week's time on incubation in the algal culture room. A third time the same process was repeated when the alga was found to grow in Fogg's liquid medium containing 10% raw sewage. The same process

was adopted making use of 25%, 50% and 75% sewage in Fogg's liquid media and finally 100% sewage.

When the alga was found to grow in 100% fresh raw strained sewage, 5%, 10%, 15% and 25% of the algal culture were added to each of 1500 ml of fresh raw, settled and strained sewage in growth culture flasks of three litre capacity; and they were kept for incubation on the ground in the lighted room. Twice a day the flasks were vigorously shaken for five minutes in order to keep the algal cells suspended in the liquid medium. The algae developed nicely in all the culture flasks in about a week's time. It was found that the flasks to which 10% of the algal culture was added gave optimum results as was indicated by turbidity measurements and cell counts (The algal cells were counted by means of a haemocytometer and the turbidity was measured in a Klett-summerson Colorimeter at 660 m/u). So, a 10%, week old culture was always used in subsequent growth culture experiments. In this way the algae to be used in the following experiments were acclimatised to the light and temperature conditions in the laboratory and thus also maintained.

Ten lots of 10% of the algal culture containing the alga to be investigated in a good physiological condition

proportionate to the quantity of raw sewage used, were centrifuged for 20 minutes at 2000 rpm at room temperature. The supernatant was thrown out, the algal pellets at the bottom were mixed in sterile distilled water and were thoroughly agitated to break up the pellets for resuspending the algae and centrifuged again for 20 minutes at 2000 rpm at room temperature. The supernatant was again thrown out. The operation was repeated thrice in order to reduce the nutrient carry over. After washing, the algal pellets were transferred to a sterilised standard measuring flask of 500 ml capacity. After sufficient agitation the volume was made up to 500 ml with sterile distilled water. 50 ml aliquots were used for each of the experimental culture flasks, which were placed in the lighted room. The algal dry weights were also estimated in 50 ml of aliquots which were deducted from the corresponding total estimated algal biomass for each alga on each detention period.

Laboratory set up of the apparatus for experiments on algal-bacterial symbiosis:

Adamse (1968) has shown a distinct similarity in the bacteriological composition of the activated sludge formed in a newly established oxidation ditch fed with dairy waste water and that developed in the laboratory apparatus

consisting of a series of culture flasks, which resembled our own.

So the laboratory apparatus for multiple batch tests consisted of a series of three litre flat-bottomed culture flasks of wide form, pyrex brand glass, compressed Erlenmeyer type, with a high ratio of surface area to volume. The average diameter at the bottom of each flask was 24.5 cm, thickness 0.25 cm, surface area of the shallow portion at the bottom when filled everytime with 1.5 litre of sewage was 452.5 sq. cm. and the depth with 1500 ml liquid was 3.5 cm. The empty flasks were first sterilised with non-absorbent cotton.

The growth culture units consisting of twelve culture flasks were used for each experiment. Four of them were used for control and the remaining eight for algal treatment. Therefore, for each detention period there were three culture flasks one serving the purpose of control and the other two, for algal treatment for duplicate tests. Each of the control flasks contained 1.5 litres of fresh, raw settled and strained sewage, which were collected from Wadi Sewage Disposal Works, Baroda, while each of the algal flasks contained 1450 ml of fresh raw, settled and strained sewage and 50 ml of the algal aliquots as mentioned previously.

Chemicals and glasswares:

Chemicals used were mostly of A.R. quality. Glasswares used were of Pyrex or Corning quality.

B. Methods:

Three sets of samples were drawn from the middle of the liquid portion in the culture flasks on zero, second, fourth and sixth day for each experiment in the following order: first for biological examination then for physico-chemical tests and lastly for biochemical tests.

Biological examination:

The aim of the biological examination was to obtain an idea of the degree of purification by knowing the type of rotifers and protozoans present.

Microscopic examination:- Samples of sediments were examined under high and low magnifications for the presence of protozoans, filamentous bacteria, organic debris etc.

Dry weight of algal biomass:- This method was done according to Algal Assay Procedure Bottle Test (1971, p. 51). 500 ml. of algal suspension was centrifuged, the

sedimented cells were carefully washed thrice in distilled water containing 15 mg of $NaHCO_3$/litre without loss of cells, transferred to tarred crucibles, dried overnight in a hot air oven at 105°C and weighed.

Elementary analysis for nitrogen and phosphorus in algal biomass:-

This was done according to AOAC (1945). The algal biomass is not pure but is mixed with a negligible amount of organic matter.

Physico-chemical examination:

The aim of the physico-chemical examination was to obtain an idea of the degree of purification from the reduction in chemical and biological oxygen demand and the nutrients removal.

Physical conditions:

Colour was recorded as it appeared to the naked eye. pH was measured with a Beckman pH meter and temperature with a thermometer 0°C to 50°C calibrated to 0.2°C.

Chemical conditions:

American standard methods - 10th and 13th Editions - were generally used for estimating the chemical conditions.

Phenolphthalein alkalinity:- It was estimated using 0.02 N H_2SO_4 with phenolphthalein as an indicator according to Standard Methods, 13th Ed. (p.52).

BOD_5 at 20°C:- It was estimated by Winkler's Azide modification (13th Ed. p. 489).

COD :- It was estimated by dichromate reflux method according to 13th Ed. (p. 495).

Phosphate:- It was estimated by colorimetric stannous chloride method. (13th Ed. p. 530).

Ammonia nitrogen:- It was estimated by direct Nesslerisation method (13th Ed. p. 240).

Nitrite nitrogen:- It was estimated by α-naphthylamine hydrochloride method. (13th Ed. p.240).

Nitrate nitrogen :- It was estimated by reduction method using sodium hydroxide and aluminium foil and direct nesslerisation (10th Ed. p. 151).

Biochemical tests:

Biochemical tests were performed to find out how the metabolic changes affected by bacteria help in purification of sewage. The following tests were performed:

Carbohydrate:- Total sugar and free sugar were estimated by Hane's method. (Hawk, 13th Ed.).

Protein and amino nitrogen:- 200 ml of sewage samples were concentrated on a water bath at 60°C, to 25ml and from the supernatant protein was estimated by the method of Lowry Rosenbrough. (Lowry et al. 1951, p. 265) and amino nitrogen was estimated by Russel's Colorimetric method (Russel, 1944, p. 147).

Volatile acids (lower organic fatty acids):- They were estimated by the tentative methods. (Standard methods, 13th Ed. p. 577).