CHAPTER - III

MATERIALS AND METHODS

A. Test conditions:

i) 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 about 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 transparent 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 (PAAP, 1969).

The temperature of the room on the ground floor ranged between 27.5 to 28.6°C.

ii) 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 culture 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	= 0.25mg/l	$NaMoO_3$(85%)	= 0.0177 g/l
A_5 Soln.	= 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 $FeSO_4.7H_2O$ was added 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 sterilized 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, 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 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 mμ). 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 re-suspending the algae and centrifuged again for 20 minutes at 2000 rpm. 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 weight was also estimated in 50 ml of aliquots which was deducted from the total estimated algal biomass for each alga on each detention period.

iii) 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 diary 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 3 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 sterilized with non-absorbent cotton.

The growth culture units consisted 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, strained and homogeneous sewage, while each of the algal flasks contained 1450 ml of fresh, raw settled, [redacted] strained and homogeneous sewage and 50 ml of the algal aliquots as mentioned previously.

Three algal specimens used in the experiments were thus acclimatized and seeded in the culture flasks so

that there was a vigorous growth in the culture flasks in 6 days. The algae used in the experiments were (a) Scenedesmus obliqus; (b) Microcystis aeruginosa and (c) Mixed algae. [redacted]

Chemicals and Glasswares:

Chemicals used were mostly of A.R. quality; and for bacteriological examination, bacteriological grade chemicals were used. Glasswares used were of Pyrex or Corning quality.

B. Methods:

Sample collection for Analysis:

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

Sterility of the Laboratory equipment and media:

Sterilized media were tested by incubation at 37°C for 48 hours followed by storage at room temperature for

48 hours prior to use every time. Glasswares like pipettes, petridishes, test tubes, sample bottles, culture flasks etc., were sterilized in a hot air oven and later tested for sterility according to the directions of Harrigan and Mc Cance (1966, p. 74).

i) Bacteriological examination:

The aim of the bacteriological examination was to obtain (a) an idea of the degree of purification attained from the sanitary aspect; (b) types of bacteria in the effluents and (c) the morphological and physiological traits of the dominant heterotrophic bacteria developing on different detention periods. Three types of tests were done:

1. Coliform group:

Coliform group tested by multiple tube fermentation technique using MacConkey's neutral red-lactose-broth (5 tubes of 5.0 ml broth for each dilution) according to the British Bacteriological Examination of Water Supplies (1957).

2. Total colonies count at 37°C temperature after 24 hours:

Sewage samples were serially diluted in sterile distilled water and plated on nutrient agar medium

contained in sterile glass petridishes. The actual procedure consisted of spreading 1.0 ml of the diluted sample on the surface of the agar with bent glass rods, and incubated at 37°C. The colonies were counted in a colony counter. (Prakasam, 1966).

3. Isolation of heterotrophic non-photosynthetic bacteria from sewage agar :

All the samples taken from algae-treated flasks only were serially diluted (ten-fold) in the sterile distilled water and dispersed by shaking vigorously in test tubes after 0, 2, 4 and 6 days. Volumes of 1.0 ml from the last two dilutions were measured into each of the two sterile petridishes. For culturing the dominant bacteria a non-selective medium as suggested by Dias and Bhat (1964) was used. Sewage agar (Sewage solidified with 2% agar and neutralised, after autoclaving with dilute phosphoric acid) was employed. The plates inoculated with diluted samples were incubated for 7 - 10 days at room temperature (25 - 27°C). All colonies in a sector of a plate having a total of 50 - 60 colonies were selected (in preference to selecting a few dissimilar colonies), and cultures were made in PPYE

present on the isolation plates. A sector of a plate containing about 50 colonies was chosen in each case for the purpose of this investigation.

a) Typing and characterization of the 200 bacterial isolates:

The tests were done generally according to the Manual of Microbiological Methods (1957).

1) Morphological traits for colour, shape, diameter margin and surface were identified according to Harry Seeley and Vandemark (1970);

2) Staining reactions and reserve food materials: Gram, acid fast, spores and flagella were done according to the Manual of Microbiological Methods (1957). Cell wall, capsule and reserve food materials were done according to Harry Seeley and Vandemark (1970).

3) Biochemical tests such as carbohydrate fermentation test, citrate utilization, nitrate reduction, ammonia oxidation, H_2S production, MR and VP test, starch hydrolysis, geletin hydrolysis, fat hydrolysis and catalase activity, were done according to the Manual of Microbiological Methods (1957).

b) Classification and Identify of the genera:

The main objects of the bacteriological examination were four-fold: (1) to get an idea of the reductions in coliforms on each of the detention periods for the sanitary quality of the treated samples; (2) to obtain an approximate estimate of the total number of bacterial flora; (3) to determine the morphological and chief bio-chemical characteristics of the dominant types for classification and identity of the genera, and (4) to find out if the generic types of bacteria differ on different days of the detention period indicating assimilatory or endogenous flora.

Identifying all the bacteria present was an impossible task and the number was limited to ... individual strains isolated from the 8th, 9th or 10th dilution plate which showed independent colonies. As in many cases the boundaries of bacteria are ill-defined and so the characteristics of the bacterial isolates are stressed rather than the names of well defined species.

Our preliminary examination of the hundreds of bacterial isolates showed that the bacterial flora of the high-rate aerobic oxidation pond consisted mainly

of aerobic Gram-negative, non-spore forming rods; and Ingram and Shewan (1960) have proposed various schemes for their classification. In Bergey's Manual (1957) an important distinction is made between polar flagellates and peritrichous flagellates. The former are placed in the order Pseudomonadales and the later under Eubacteriales. Further biochemical characteristics are used primarily for differentiation of generic types. The polarly flagellated genera Pseudomonas and Xanthomonas are defined as attacking carbohydrates oxidatively with the formation of acid; Aeromonas and and Zymomonas, also polar flagellated, attack sugars fermentatively with the formation of acid and gas.

The peritrichously flagellated Achromomonaceae with the genera Achromobacter, Flavobacterium, and Alcaligens are characterised by attacking carbohydrates (if at all) oxidatively (with or without the production of acid) which distinguishes them from the fermentative Enterobacteriaceae.

Bacteria which do not utilize glucose are often classified as Alcaligens, thereby overlooking the fact that these organisms should have peritrichous flagella.

The polar-flagellated gram-negative bacteria which did not produce acidity in Hugh and Leifson's medium are

classified in Bergey's Manual (1957) in the genus Zoogloea, which resembles in most respects Comamonas (Davis and Park, 1962). Dias and Bhat (1964, p. 415) have shown the important characteristics of Comamonas on which we have based our generic classification of Comamonas.

A number of non-motile rods with marked diversity of form were also found and they are placed under Corynbacteriaceae or Coryneforms.

ii. Biological examinations:

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.

Three types of examinations are possible:

a) Microscopic :

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

b) Algal biomass:

Dry weight:- This method is particularly useful for assessing the growth of filamentous and mixed algal

cultures according to directions given in Appendix (Algal Assay Procedure Bottle Test, 1971 p. 51). A suitable portion of algal suspension about 500 ml in replicate is centrifuged for 20 minutes, the sedimented cells carefully washed thrice in distilled water containing 15 mg $NaHCO_3$/l without loss of cells, transferred to tarred crucibles, dried overnight in hot air oven at 105°C and weighed. The algal biomass is not pure but may be mixed with a very negligible amount of organic matter. From the results, the original weight of algae added on zero day has been deducted so that the actual increased dry weight in biomass are shwon.

c) Elementary analysis for N and P in algal-biomass:

Elementary analysis for nitrogen (N) and phosphorus (P) in algal biomass was done according to AOAC (1945).

iii) Physico-chemical examinations:

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.

a) 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° to 50°C calibrated to 0.2°C.

b) Chemical conditions:

American Standard Methods - 13th Edition was generally used for estimating the chemical conditions.

1. Ammonia nitrogen : It was estimated by direct Nesslerization method (13th Ed. p. 226).

2. Phosphate: By Colorimetric stannous chloride method (13th ed. p. 530).

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

4. Chemical oxygen demand (COD): By dichromate reflux method according to Standard Methods (13th ed. p. 495).

5. Biological oxygen demand (BOD_5 at 20°C): By Winkler's Azide modification (13th ed. p.489).

iv) Biochemical tests:

To find out how the metabolic changes were effected by bacteria in the purification of sewage, the following tests were performed:

a) Carbohydrate : Total sugar and free sugar were estimated by Hane's method (Hawk, 13th ed. p. 923).

b) Protein and amino nitrogen: In order to separate the particulate matter from the samples, the procedure of successive sedimentation, centrifugation was followed. 200 ml of sewage sample was sedimented and freed from suspended matter by centrifuging at 5000 x g. for 20 minutes. The supernatant thus obtained was concentrated upto 10.0 ml on steam water bath at nearly 60°C. The final volume was made up to 25.0 ml and then again centrifuged. The supernatant was used for the estimation of protein and amino nitrogen.

Protein was estimated by the method of Lowry and Rosebrough (Lowry et al. 1951,p. 265) and amino nitrogen was estimated by Russel's Colorimetric method (Russel, 1944, p. 147).

c) Volatile acids(Lower fatty acids): Volatile acids were estimated by the tentative method (Standard Methods, 13th ed. p. 577).