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M A T E R I A L S _ A N D _ M E T H O D S

C O N T E N T S

Contents (contd.) Page

Contents (contd.) Page

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MATERIALS AND METHODS

Test Conditions:

(a) 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 and each measuring 3' x 2', and placed one below the other. One of them is placed at the top and the other at a distance of 1'-6" below the top glass sheet. The lower sheet of glass is at a height of 1'-6" above the ground. The ground floor is covered with a white sheet of paper for reflecting the light into the bottom portion of culture flasks.

(b) Lighting Arrangements in Algal Culture Room:

Two fluorescent day-light tube lights each of 4 ft. in length, 40 watts and 2' apart are hanging at a height of 9" above the top glass sheet. Another similar pair of tube lights is placed just below the top glass

sheet and third pair just below the second glass sheet for illuminating the ground floor. All three pairs of tube light can be operated individually.

(c) Temperature of the Algal Culture Room:

The temperature at different heights on the glass table i.e. top glass platform — 29.8 - 30.5°C, middle glass platform - 28 - 29°C and the ground floor was 27- 29°C.

(d) The Intensity of Illumination:

The intensities of illumination measured outside the flask at the liquid level at

the ground floor = 375 lux. Illumination was continuous and did not simulate the diurnal pattern existing in nature (PAAP 1969).

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

More than a dozen pure algal specimens of green and blue-green varieties growing on Fogg's autotrophic agar slants were obtained from the Algal Division of the Department of Microbiology, Indian Agricultural

Research Institute, New Delhi; a few were isolated from the local tanks and ponds when they were having algal blooms; and Chlorella vulgaris was isolated from Baroda sewage during the course of our previous studies. All of them are being maintained as pure cultures in Fogg's slants and liquid medium in the laboratory for use in the experiments to follow:

Fogg's medium (with N):-	A_5 Micro nutrient solution:-
KH_2PO_4 = 0.2 g/l	H_3BO_3 = 0.86 g/l
$MgSO_4,7H_2O$ = 0.2 g/l	$MnCl_2$ = 1.81 g/l
$CaCl_2$ = 0.1 g/l	$ZnSO_4,7H_2O$ = 0.222 g/l.
KNO_3 = 0.25 g/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 = 1.5% for Solid medium.

Preparation of Fe EDTA:

Dissolve 26.1 g of EDTA in 268 ml of 1.0 N KOH. Add, 24.9 g. of $FeSO_47H_2O$ and dilute to one litre.

Acclimatization procedure :

From agar slants the algae were transferred asceptically to several flasks containing sterilized Fogg's liquid medium and incubated in the algal culture room for 7 - 10 days (or more in some cases) when good algal growths were obtained.

Then, to each of three flasks containing the algal culture in Fogg's liquid medium, 10% of settled strained (through cotton) sewage were added; and the sewage-added flasks were kept on the ground floor in the algal culture room. In about a week there was a good growth of the 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 10% raw sewage. The process was repeated making use of 25%, 50% 75% Sewage in Fogg's liquid media and finally in 100% sewage.

When the alga was found to grow in 100% fresh raw strained sewage, 5%, 10% 15% and 25% of the algal culture corresponding to the quantity of sewage used were added to each of 1500 ml of raw, strained sewage in growth culture flasks of 3 litre capacity; and they were kept for incubation on the ground and in the lighted room. Twice a day the flasks were vigourously shaken for about 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. The algal cells in each of these concentrations on the sixth day were counted by means of a haemocytometer and at the same time the turbidity measurements of the algal growths of each concentration were also made in a Klett Summerson Colorimeter at 660 m u and it was found that the 10% culture growth gave optimum results consistently. So, 10% of the culture was used in our experiments and the dry algal biomass in 10% culture amounted to about 35 to 60 mg per litre and the corresponding quantity of algal biomass deducted from the total estimated algal biomass for each alga on each detention period. 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 acclimatized to the light and temperature conditions in the laboratory and thus also maintained Ten lots of 10% of the algal cells in good physiological condition proportionate to the quantity of sewage used, were centrifuged for 20 minutes at 2000 rpm; the supernatant thrown out; the algal pallets at the bottom were mixed in sterile distilled water, thoroughly agitated to break up the pallets for resuspending the algae, and centrifuged again for 20 minutes at 2000 rpm and the supernatant again thrown out. The operation was repeated thrice to reduce (a) the nutrient-carry over and (b) the bacterial load from the stock culture to the batch growth culture experimental flasks. The pallets were transferred to a sterilized standard measuring flask of 500 ml and were resuspended after sufficient agitation in sterile distilled water.

Aliquots of 50ml each were used for each of the eight experimental algal culture flask, which were placed in the constant temperature lighted room and from the remaining lots of the 50 ml each of the algal dry weight was estimated.

(f) Laboratory set up of 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 resemble our own.

So, the laboratory apparatus for multiple batch tests consisted of a series of culture flasks each of which contained 1.5 litre of fresh raw settled sewage strained through sterilized cotton(for removal of suspended matter) and operated on a fill-and-draw basis. Four of them were serving as control, containing only raw sewage and the remaining eight culture flasks were treated with the tested algal culture in duplicate. Therefore for each detention period there were three culture flasks one serving the purpose of control and the other two for duplicate tests. The results expressed for the algal culture represent the average of duplicate test. The growth culture units consisted of twelve culture flasks were tested at a time in the experiment. The culture flasks were of wide form, Pyrex-brand glass, compressed Erlenmeyer type with the high ratio of surface area to volume and each of three litre capacity. They were sterilized and pluged with

sterilized non-absorbant cotton.

Details of the culture flasks used:

Average diameter at the bottom of the culture flask = 24.5 cm.

Average thickness of the glass culture flask = 0.25 cm.

Average surface area of the shallow portion at bottom with 1500 ml = 452 sq.cm.

Average depth of the culture fluid with 1500 ml liquid = 3.5 cm.

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:

3.2 METHODS:

A. Sample collection for analysis:

Four sets of samples were drawn from the middle of the liquid portion in the flask in the following order. First for bacteriological examination, then for

biological examination, for physico-chemical tests and lastly for biochemical tests.

i) Sterility test of the laboratory equipment and media:

Sterilized media were tested by incubation at 37°C 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 given on page 74 of Harrigan and McCance (1966 p.74).

B. Bacteriological examination:

(a) Object of the examination:

The aim of the bacteriological examination was to obtain a) an idea of the degree of purification attained from the sanitary aspects; b) morphological and physiological traits of the dominant heterotrophic bacteria developing on different detention periods.

(b) Three types of Tests:

(i) Coliform group:

Coliform group was 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 (1956).

(ii) Total Colonies count at 37°C Temperature after 24 hours:

Samples of sewage were serially diluted in sterile distilled water and plated on nutrient agar [redacted] contained in sterile glass petri-dishes. The actual procedure consisted of spreading 1 ml of the diluted sample on the surface of the agar with sterilized bent glass rods, and incubated at 37°C. The colonies were counted in a colony counter and estimated according to the dilution and the number of colonies recorded.

(iii) Isolation of heterotrophic non-photosynthetic bacteria from sewage agar:

All the samples taken from algae treated flasks were serially diluted tenfold in sterile distilled water contained in test tubes and dispersed by shaking vigourously after 0, 2, 4 and 6 days. Volumes of 1.0 ml from the last two dilutions were inoculated into each of two sterile Petri-dishes. 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. 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 broth (Protease peptone 0.5%, Yeast extract (Difco) 0.1%). The survival of isolates in PPYE broth was nearly cent percent. The purification of the isolate was made by the usual single colony technique on PPYE agar (PPYE broth + agar).

The main objects of the bacteriological examination were two fold: (a) to determine the morphological and chief biochemical characteristics of the predominant bacteria for classification and identity of the genera so that the characteristics of the bacterial isolates were stressed rather than the names of well defined species and (b) to find out if the generic types of bacteria differed on different days of the four detention periods (0, 2, 4 and 6 days) indicating assimilatory and endogenous flora.

(iv) Classification and identity of the genera:

In the Bergey's Manual (1957) an important distinction is made between polar and petrichous flagellates. The former are placed in the order Pseudomonadales and the latter under Eubacteriales. Further bacterial 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 Zymomonas also polarly flagellats attack sugars fermentatively with the formation of acid and gas.

The petrichously flagellated Achromobacteriaceae with the genera Achromobacter, Flavobacterium and Alcaligenes are characterised as 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 Alcaligenes, thereby overlooking the fact that these organisms should have petrichous flagella (Van Gils 1964).

The polar-flagellated Gram-negative bacteria which do not produce acidity in Hugh and Leifson's medium are classified in Bergey's Manual under the genus Zoogloea which resembles in most respects Comamonas (Davis and Park, 1962). Dias and Bhatt (1964) have shown the important attributes of Comamonas on which we have based our generic classification of Comamonas.

(v) Typing or characterization of the heterotrophic non-photosynthetic bacterial isolates :

Typing and characterization of bacterial isolates were generally made according to the Manual of Microbiological Methods (1957): and they are briefly stated below:

Tests	Methods
(a) Staining reactions	
1. Gram	Burk and Kopeloff-Beerman Modification, page 16
2. Acid fast	Ziehl-Neelsen page 18
3. Spore	Synder's Modification of Dornor's page 21
4. Flagella	Bailey's method as modified by Conn page 27
5. Fat-droplets	Burden's (1946) technique page 30
6. Capsule	Mckinney's (1962) method

Tests		Methods
7.	The cell-wall	Seeley and Vandemark (1970)
(b)	Reserve Food materials:	
8.	Lipid inclusions	by Burden's stain
9.	Glycogen	by staining with Lugol's iodine Seeley and Vandemark (1970)
10.	Volutin	by staining with a drop of acidified aqueous methylene blue solution. Seeley and Vandemark (1970).
(c)	Enzyme activity:	
11.	Catalase activity	According to American Manual of Microbiology, (1957).
(d)	Physiological:	
12.	Nitrate reduction	Protease peptone yeast extract broth containing 0.1% KNO_3 was used. Nitrate reduction was tested using sulfanilic acid and -Napthylamine and sodium acetate reagents.
13.	Indole production	Protease peptone broth containing 0.1% tryptophan was used. Indole produced was detected by Kovac's reagent.
14.	H_2S production	Protease peptone yeast broth with lead acetate paper was used.
15.	Gelatin hydrolysis	Nutrient agar containing 0.2% gelatin was used. The Zone around the inoculum was detected by 15% $HgCl_2$ in Con. HCl.

Tests		Methods
16.	Starch hydrolysis.	Nutrient agar containing 0.2% starch was used. Hydrolysis was detected, by Lugol's iodine Soln.
17.	Fermentation tests.	Protease peptone yeast extract broth containing 0.004% bromo-cresol purple was used. Sugars, e.g. glucose, sucrose, lactose, mannitol were added at 1% level. Gas production was noted in inverted Durham's tubes.
18.	Methyl red and Voges Proskaur Test.	Glucose-phosphate medium was used. Acetylmethyl-carbinol, produced was detected by O'Mears tests as modified by Levine, Epstein and Vaughn.
*19.	Citrate Utilization	Koser's Citrate medium No. 100 of Fred & Waksman (1928) was used.
*20.	Nitrogen fixation	Nitrogen free Manitol medium No. 77 of Fred & Waksman (1928) was used. The result of the test was judged by visual turbidity.
*21.	Ammonia oxidation	Medium 52 of Fred and Waksman (1928) was used. Sulfanilic acid and α-napthylamine and Na-acetate reagents were added followed by zinc powder addition to detect nitrate formation.

Tests	Methods
20. Tributyrin hydrolysis	Nutrient agar containing 0.1% tributyrin was used. Hydrolysis was detected by appearance of clear zone around the growing colony.

* Citrate utilization and nitrogen fixation tests were judged by visual turbidity. To obviate any response due to carry over of nutrient with the medium, the tubes were serially transferred twice before considering the tests as positive. PPYE broth bacteria are inoculated 3% H_2O_2 added and Vapour.

21. Hugh and Leifson's technique :-

The isolated bacteria were classified according to their attack of carbohydrates. Breakdown of sugars formed the basis of classification. Hugh and Leifson's technique was employed. Hugh and Leifson's medium is a tryptone agar medium with 1% sugar. The test was performed aerobically as well as anaerobically by covering the agar with a layer of sterile paraffin. Production of acid is indicated by a change in the colour of bromothymol blue indicator.

C. Physical Examination:

Colour was recorded as it appears to the naked eye. Turbidity was measured at 660 mμ using distilled water as blank in a Klett-Summerson colorimeter, Hydrogen ion concentration expressed in terms of pH, was measured by means of a Systronic pH meter. Temperature was measured with a thermometer (0-110°C) calibrated to a tenth of a degree.

D. Chemical Examination:

Standard methods (1971) were generally used for the tests mentioned below:

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

COD : by Dichromate Reflux Method according to (13th Ed. p. 495)

Phosphate : by Colorimetric Stannous Chloride Method (13th Ed. p. 240)

Nitrite nitrogen: by -naphthylamine hydrochloride Method (13th Ed. p. 240).

Nitrate nitrogen: by reduction method using Sodium hydroxide and aluminium foil and direct necslerization. (10th Ed. p. 151)

Ammonia nitrogen: Direct Necslerization method (13th Ed. p. 226)

ALKALINITY: (Page No. 371-376)

(a) Phenolphthelein alkalinity:

Phenolphthelein and total alkalinity were determined by titration with a standard solution of a strong mineral acid (H_2SO_4) to the successive bicarbonate and carbonic acid equivalance points by means of colour. Phenolphthelein indicator enable the measurement of that and half of the carbonate.

E. Biochemical Examination:

For the estimation of soluble free sugar, total sugar, amino acid nitrogen and protein in the samples of sewage drawn from high rate oxidation ponds on different days the following procedures were adopted. In order to separate the particulate matter from the samples, the procedure of successive sedimentation, centrifugation was followed, page 489 (1971).

i) 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 sample was sedimented and freed from suspended matter by centrifuguing at 500 x g for 20 minutes or by

filtration through whatman No. 40 filter paper. The supernatant thus obtained was concentrated upto 10.0 ml on steam water bath at 60°C. The final volume is made upto 25.0 ml and then centrifuged or filtered. The supernatant was used for the estimation of protein and amino nitrogen.

ii) Protein:

This was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951, p. 265), Bovine albumin was used as a standard in the range of 10 - 100 mg.

iii) Amino-acid nitrogen:

Using the above samples, this was estimated according to Russel (1944, p.147). Mixed glycine-glutamic acid standard was used in the range of 0 to 30 ug.

iv) Carbohydrates:

a.1) Total Sugar
a.2) Free sugar

} By Hane's Method in Hawk (Philiph et al.) 1954.

The method is based on the reduction of alkaline potassium ferricyanide to ferrocynide by reducing

sugar. The ferrocynide formed is precipitated as a double salt of K and Sn by treating with Hanes's, B.

The residual ferricyanide is treated with KI and iodine liberated is treated with KI and the iodine liberated titrated against Standard Sodium thiosulfate.

v) Volatile acids (Standard methods 13th Ed. p. 577-580).

Total volatile acids were estimated in samples acidified to pH 1 to 1.2 kept at 60°C for 30 minutes. After digestion, the sample was distilled in 4 aliquotes of 50 ml each, and titrated against 0.1N NaOH with phenolphthalein as an indicator.

F. Biological Examination:

(i) Microscopic:

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

(ii) Algal biomass:

(a) Dry weight:-This method has been suggested in (Provincial Algal Assay Procedure Bottle test, 1971, p.51) for the dry weight of algal biomass estimation.

A suitable portion of algal suspension was centrifuged in duplicates, the sedimental cells carefully washed thrice in distilled water containing 15 mg $NaHCO_3$/l without loss of cells, transferred to tarred crucibles, dried overnight in a hot air oven at 105°C and weighed. The dry weight thus obtained represent the true dry weight of the algal biomass although it may contain also a microscopic amount of undeterminable percentage of organic matter. From these dry weights which represent the weight of algae on particular detention period, the original weight of algae added on zero day has been substrated so that the actual weight shown in the tables represent the actual increase in dry algal bio-mass. These results represent the mean of duplicate test.

(b) Elementary analysis for N and P.

in algal biomass:- This was done according to AOAC (Ed. 1945) Page 27 for N and Page 127 for P.