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I. Test Conditions:

(a) The algal culture room:

It is a small room measuring 8'x6'x8' (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'x2'x3' (high) is placed at the opposite corner of the entrance. The table had was two white transparent glass sheets of 0.5 cm thick and each measuring 3'x2', 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 floor, which is covered with big white sheets of paper for reflecting light into the bottom of the culture flasks.

(b) Lighting arrangements in the algal culture room:

~~Two fluorescent day-light tube lights each of 4' in length, 40 watts and 2' apart are hanging at a height of 9" above the top glass sheet. Another similar~~ A pair of tube lights is placed just below the top glass sheet and a third pair just below the second glass sheet for illuminating the ground floor. All the ~~three~~ two pairs of tube lights 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 =29.0-30.5°C, middle glass plate each =29-28°C and the ground floor=24-25°C,~~ was 18-19°C.

(d) The intensity of illumination:

The intensities of illumination on the ground floor is 395 lux. Illumination was continuous and did not simulate the dilunal 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 experiments. All of them were maintained as pure-cultures in Fogg's agar slants and in liquid medium in the laboratory for use in the experiments as follow:

Composition of the Fogg's Algal nutrient medium:
Fogg's medium (with Nitrogen)

		A_5 Micro nutrient solution
KH_2PO_4	- 0.2g/1	
$MgSO_4$	- 0.2g/1	H_3BO_3 -0.86g/1
$CaCl_2$	- 0.1g/1	$MnCl_2$ -1.81 g/1
KNO_3	- 0.25g/1	$ZnSO_4.7H_2O$ -0.222g/1
A_5 solution	- 1.0ml/1	$NaMoO_3$(85%) =0.0117 g/1
FeEDTA	- 1.0m1/1	$CuSO_4.5H_2O$-0.079 g/1
Agar	- 1.5% for solid medium.	

Preparation of Fe-EDTA:

Dissove 26.1 g of EDTA in 268 ml. of 1.0N KOH Add 24.9 g of $FeSO_4$, $7H_2O$ and dilute to one liter. Aerate the solution overnight to produce the stable complex (change of colour to dark brown). Make up the volume to one liter.

Acclimatization procedure:

From agar slants the algae were transferred aseptically to several flasks containing sterilized Fogg's liquid medium and incuba-ted 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 bottom in the algal culture room. In about a week there was 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 same process was adopted 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 this algal culture corresponding to the quantity of sewage used were added to each of 1500 ml. fresh raw, strained sewage in growth culture flasks of 3 litres capacity, and they were kept for incubation on the ground and in the lighted room. Twice a day the flasks were vigorously shaken for ten 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. A 10% week old culture was considefed sufficient and was always used in subsequent growth culture experiments. In this way, the algae to be used in the following experiments were acclimati-zed to the light, temperature and Baroda settled sewage conditions

in the laboratory and thus also were maintained. 10% of the algal cells in good physiological condition proportionate to the quantity of raw sewage used, were centrifuged for 20 minutes at 2000 rpm, the supernatant was thrown out, the algal pellets at the bottom were mixed in sterile distilled water, thoroughly agitated to breakup the pellets for resuspending the algae, and centrifuged again for 20 minutes at 2000 rpm, and the supernatant was again thrown out. The operation was repeated thrice in order to reduce (a) the nutrient carry-over and (b) the bacterial load from the stock culture to the batch growth culture experimental flasks. 10 such pellets were transferred to a sterilized standard measuring flask of 1500 ml capa--city and were resuspended after sufficient agitation in sterile distilled water and then made up to 500 ml. Aliquots of 50 ml. each with special broken tipped pipettes were used for the experimental culture flasks, which were placed in the constant temperature lighted room.

(f) Laboratory set up of the apparatus for experiments on algal bacterial symbiosis:

Admse (1968) has shown a distinct similarity in the bacterio--logical composition of the activated sludge formed in a newly establi--shed oxidation ditch fed with dairy wastewater 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 culture tests consisted of a series of twelve culture flasks each of which containing 1.5 liters of fresh raw settled sewage strained through sterilized cotton (for removal of suspended matter). The culture four flasks were used for control and other 8 flasks for algal treatment for duplicate tests and for algal-biomass determination.

flasks were of wide form, pyrex brand glass, compressed Erlenmeyer type with a high ratio of surface area to volume and each of three liters capacity. They were sterilized and plugged with sterilized non-absorbent cotton. As already stated the culture flasks were shaken twice a day for about 10 minutes. And they were loosely plugged.

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 the bottom with 1500 ml.=452.5 sq.cm.

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

Chemicals and glass wares:

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.

2. (a) Sample Collection for analysis:

Four sets of samples were drawn from the middle of the liquid portion in the flasks in the following order. First, for bacteriological examination, then for biological examination, for physico-chemical tests and lastly for bio-chemical tests.

(b) (i) Sterility of tests 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. Glasswares like pipettes, Petri dishes, test tubes, sample bottles, culture flasks etc. were sterilized in a hot air oven and later tested for sterility according to direction given on page 74 of Harrigan and McCance (1966)

3. <u>Bacteriological examination:</u>

(i) <u>Object of the examination:</u>

The aim of the bacteriological examination was to obtain

(a) an idea of the degree of purification attained from the sanitary aspect; and

(b) the morphological and physiological traits of the dominant hetero trophic bacteria developing on sewage agar plates at different detention periods.

(ii) <u>Three types of tests:</u>

(ii-1) <u>Coliform group:</u>

Coliform group is tested by multiple tube fermentation technique using Mc-Conkey'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-2) <u>Total Colonies count at 37°C after 24 hours:</u>

Sewage samples, were serially diluted in sterile distilled water and platted 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.

(ii-3) Isolation of heterotrophic non-photosynthetic bacteria/on <u>sewage agar at room temperature:</u>

~~Two methods are~~ The method is available for characterizing the heterotrophic bacterial flora: ~~(a)~~ to examine every individual organism on an agar plate which involves the process of isolation, purification and testing of the isolates. ~~The other is the replica plating technique of Lederberg and Lederberg (1952) and Lochhead's nutritional method. Lochhead (1956) has discussed the validation and limitation of the two techniques and the laborious task involved in both. The former method has been followed through out in this investigation.~~ Dias and Bhat (1964) concluded that sewage agar was a non specific medium

useful for the cultivation of aerobic heterotrophs. So, sewage agar medium for the isolation and study of the predominent functional bacteria was used in all our experiments.

To obtain an estimate of the relative numbers of the different kinds of microorganisms which develop in sewage agar plates and to calculate the percentage distribution of the various organisms present in a sample several statistical methods (Harrigan and McCance 1966) for the selection are described. One of the methods recommended is the selection of every colony occuring in either a single sector or in opposite sectors of a plate depending upon the number of colonies required for examination and on the numbers of colonies 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.

(ii-4) Typing and characterization of heterotrophic non-photosynthetic bacterial isolates:

The methods given in the Manual of Microbiological Methods (Society of American Bacteriologists 1957) were followed in the tests carried out for the identification of bacteria unless otherwise sta-ted, the various tests and the methods used are described in a tabular form below:

(ii-5.1) Morphological traits:

Tests	Method
(a) Color	(Seeley and Vandemark 1970)
(b) Shape	"
(c) Diameter	"
(d) Margin	"
(e) Surface	"

(ii-5.2) Staining reactions:

(a) Gram - Hucker modification:
This staining was done by preparing the smear on the slide fixing and staining

with crystal violet, Iodine and finally with saffranin solution (Manual of Micro--biological methods 1957).

(b) Acid fast .. (Ziehl - Neelson Method)
This staining was done by staining the smear first by Ziehl's carbol fuchsin and finally by methylene blue solution (American Manual of Microbiological Methods 1957 p.18)

(c) Spore .. (Dorner's method -syndron's modification)
This was done by covering a smear with a small piece by blotting paper and stain--ing with freshly prepared Ziehl's carbol fuchsin and finally a saturated aeueous nigrosin solution.

(d) The cell-wall .. According to (Seeley and Vandemark 1970 p.48)
This was done by applying cetyl pyridinium chloride on the smear and staining the smear by a saturated aeueous congored and finally by methylene blue solution.

(e) The capsule .. According to Seely and Vandemark 1970 p.49)
This was done by staining the dried smear with crystal violet and washing it with 20% aeueous copper sulfate solution.

(f) Flagella .. This was done by staining the smear of fresh culture with solution A containing tannic acid and ferric chloride and solution B containing solution A, basic fuchsin, con.HCL and formaldehyde and finally with Ziehl's carbol fuchsin.

Tests for reserve materials or storage products:

(a) Glycogen - According to Seeley and Vandemark 1970-p.51) This was done by staining the smear with Lugol's iodine solution. Glycogen stained reddish brown with iodine.

(b) Volutin The dried and fixed smear was stained with one drop of acidified aeueous methylene blue solution (Seeley and Vandemark 1970.p.51).

(c) Fat (Burdon's method)
The smear was stained by Sudan-Black B solution prepared in 70% ethylalcohol. The slide was comple--tely dried by blotting paper and washed with xylene and finally stained by 0.5% aqueous saffranin solution. (Seeley and vandemark 1970).

(ii-5.4) Biochemical Tests: (Manual of Microbiological methods 1957)

(a) Fermentation test in Hugh and Leifson's carbohydrate medium using glucose.

(b) Carbohydrate fermentation test using the same sugar as mentioned above.

(c) Citrate utilization

(d) Action on nitrates

(e) Ammonia oxidation

(f) H_2S production

(g) MR and VP test

(h) Starch hydrolysis

(i) Gelatin hydrolysis

(j) Fat hydrolysis

(k) Catalase activity

A loop of the fresh, 18 hrs. old culture of the organism prepared in nutrient broth was inoculated into the above mentioned media and incubated them according to the methods given in American Manual of Microbiology (1957).

(ii-5.5) Classification and identity of the Genera of the bacteria;

The main objects of the bacteriological examination were four fold: (a) To get an idea of the reductions in coliforms on each of the detention period for the sanitary quality of the treated sam--ples; (b) To obtain an approximate estimate of the total numbers of bacterial flora; (c) To determine the morphological and chief biochemical characteristics of the dominant types for classification and identify of the genera, and (d) To find out if the generic types of bacteria differ on different days of the detention period indica--ting 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 10th dilution plates showing 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 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 latter 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 Zymomonas, also polar flagellated, attack sugars fermen--tatively with the formation of acid and gas.

The peritrichously flagellated Achromobacteriaceae with the genera Achromobacter. Flavobacterium and Alcaligenes 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 utilise glucose are often classified as Alcaligenes, there by overlooking the fact that these organisms should have peritrichous flagella (Van Gils 1964).

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 genus Zooglea, which resembles in most respects Comamonas (Davis and Park 1962). Dias and Bhat (1964 p 415) have ref 192 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 Corynebacteriaceae or "Corynforms."

4. Biological Examination:

(i) Microscopic: Samples of the sediments were examined with high and low magnifications for the presence of protozoans, algae, filamentous bacteria, organic debri etc.

(ii) Algal biomass:

(a) Dry weight: This method has been followed according to directions given in Appendix (Algal assay procedure

Bottle test 1971-p-51.

A suitable portion of algal suspension, is centrifuged, the sedimented cells carefully washed thrice in distilled water containing 15 mg. $NaHCO_3$ per liter without loss of cells, transferred to crucibles, dried over night in a hot air oven at 105°C and weighed. The dry weight thus obtained does not represent the true dry weight of the algal bio-mass as it contains also a micro amount of undeterminable percentage of organic matter. The dry weight of alga added on 0 day is deducted from these weights so that the results recorded for these weights represents the average ~~algal weight~~ of duplicate of the increased algal weight.

(b) N and P content:

Elementary analysis for N and P in algal bio-mass was done according to AOAC (1945). The determined values are not from pure algal samples but from slightly impure samples of algae containing ~~of~~ negligible amount of organic matter (vide microscopic examination).

5. Physico chemical conditions:

(a) Physical conditions:

i. Color: as it appeared to the naked eye.

ii. Temperature: by a thermometer 50 x ½°C

iii. PH: By PH meter.

(b) Chemical conditions American standard Methods 1971 10th and 13th edition:

(b1) Ammonia nitrogen -(Direct Nesslerization method page 226).

The sample was treated with Zinc-sulfate and alkali to pre-cipitate calcium, iron, magnesium and sulfide and then treated with Nessler reagent which formed a yellow color and the intensity of the color was measured at 420 mu in a Klett-Summerson colorimeter.

(b2) Nitrite-nitrogen (Naphthylamine-hydrochloride method.)
page 241.

The nitrite concentration was determined through the formation of a reddish purple azo-dye produced at pH 2.0 to 2.5 by the coupling of diazotised sulfanilic acid with naphthylamine hydrochloride, The brown color was measured at 540 mu in a Klett- Summerson colorimeter.

(b_3) Nitrate Nitrogen:-(Reduction method, American Standard-Methods, 10th Ed)

100 ml. of sewage sample was treated with 2 ml. of nitrate free hydroxide solution and concentrated by boiling in a china dish to about 20 ml. to remove free ammonia. The nitrate-nitrogen which is converted into ammonia nitrogen is determined by Nesslerization method.

(b4) Phosphate (PO_4) (By stannous chloride method p.530)

The formation of molybdophosphoric acid is reduced to the intensely coloured complex, molybdenum blue, by stannous chloride. the colour intensity was measured at 540 mu.

(b5) Alkalinity

(b51) Phenolphthalein alkalinity (p.375)

(b52) Total alkalinity (p.374)

Alkalinity is determined by titration with a standard soluti--on of a strong mineral acid (H_2SO_4) to the successive bicarbonate and carbonic acid equivalence points by means of colour. Phenolph--thalein indicator enables the measurement of that alkalinity fracti--on contributed by the hydroxide and half of the carbonate.

(b6) BOD5 at 20°C (Azide modification p.489)

BOD is the relative oxygen requirements of waste waters, effluents, and polluted waters. For this, the sample is added into BOD bottles containing aerated water. The difference between the immediate BOD and after incubation after 5 days at 20°C is BOD5.

(b7) COD (p.495)

A known volume of sample is refluxed with known amounts of potassium dichromate and sulfuric acid, and the excess dichromate is titrated with ferrous ammonium sulfate. The amount of oxidi zable organic matter, measured as oxygen equivalent is proportional to the potassium dichromate consumed.

6. Biochemical Tests for Microcystis aeruginosa:

1. Carbohydrates:

Total sugar and reducing sugar (Hane's method)

(Practical Physiological chemistry 13th ed.)

The method is based on the reduction of alkaline potassium ferri-cynide to ferrocynide formed which is precipitated as a double salt of potassium and zinc by treating with Hane's B. The residual ferricynide is then treated with potassium iodide and the iodine liberated is titrated against standard thiosulfate.

ii. Protein: (By folin ciocalteo's method)

This was estimated by the method of Lawry Rosebrough. Bovin albumin was used as a standard in the range of 10-100 ug.

iii. Amino-nitrogen (Russel, 1944)

Amino groups combine with B-naphthoquinone sulfonate in alkaline solution to form highly coloured compounds. Upon addition of acid, an orange red colour is produced which is determined colourimetrically. Mixed glycine, glutamic acid standard was used in the range of 0.30 ug.

For the estimation of protein and amino-nitrogen in sewage sample the following procedure was adopted.

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 rpm for 20 minutes or by filtration through whatman No.40 filter paper. The supernatant thus obtained was concentrated upto 10 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 aminoacid nitrogen.

(iv) 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 degestion, then distilled in 4 aliquts of 50 ml. each and titrated against 0.1N NaOH, with phenolphthalein as indicator.