C
H
A
P
T
E
R

III

MATERIALS AND METHODS

MATERIALS

Literature review of laboratory models

In their laboratory studies on "algal-bacterial symbiosis " in oxidation ponds, Oswald, Gotaas, Ludwig and Lynch (1953 a; 1953 b) had used four types of culture apparatus. In one case they used "57-mm pyrex tubes having a liquid volume of 1200 ml, equipped with an internal cooling tube and peripheral 30-W daylight fluorescent lights. Appurtenances permit feeding and withdrawal of liquid on a continuous basis and provide for bubbling air through the culture to maintain homegeneity. The assembly is designed to facilitate aseptic techniques in handling of the liquids."

"During the series of tests herein reported the following factors were maintained at the following constant values: light 1200 fc; temperature 25°C; air bubbling rate 500 ml per minute; BOD of influent (5 day, 25°C, Warburg, 110 p.p.m.). The single independent variable was the rate of application of sewage and equivalent withdrawal of the algal suspension. Since the total culture volume was held constant, the period that sewage was retained in the system

was determined by the daily volume of sewage applied. This period has been designated as the retention period R, defined as R = V/v in which V is the culture volume in ml, v is the volume of feed or withdrawal in ml per day and R is expressed in days. "

" In performing any single test the retention period was held constant over the entire testing period, usually from 20 to 30 days. At the start of the test, the 1200 ml tube was filled with sewage that had been inoculated with a pure culture of C. pyrenoidosa previously developed in sterile sewage in a separate culture flask. The content of the tube was then inoculated with 10 ml suspension containing approximately equal amounts of inoculum of 22 strains of bacteria. In the growth units at the start Chlorella populations were about 0.1×10^6 cells per ml and the bacterial populations about 1×10^6 colonies per ml."

In another case they carried out 20-day laboratory tests in a specially designed culture tube called "symbiocon" which is a closed continuous system simulating a "balanced aquarium" for bacteria and algae in which the atmosphere is eliminated as a factor in gas exchange."

In the third case, they carried out laboratory experiments in "open continuous systems" termed "growth

units" which are large culture tubes having facilities for light, temperature, feed rate, and sterility control (Ludwig, Oswald, Gotaas and Lynch, 1951).

In the fourth case Oswald, Golueke, and Gee (1961) used a culture unit consisting of an oval shaped polythylene bath, approximately 14 cm in depth, 38 cm wide and 64 cm long and having a capacity of 16 litres at a depth of 12.7 cm. The surface area of the culture at this level was 1450 sq.cm.

These "growth units" differ from symbiocons in that instead of being sealed from the atmosphere, bubbling air is utilized to maintain mixing. Open units such as these are more simply operated than symbiocons. They are more versatile than symbiocons for such physical variables as light, temperature and retention periods."

Temperature in all of the series ranged between 24° and 26°C and individual series varied ± 0.5°C from the mean. Light intensities were estimated on the basis of the number of new 30-watt day light fluorescent lights used.

"A definite volume of culture is removed each day, depending upon the retention period studied and is replaced by an equal volume of settled sewage. The portions of settled sewage employed each day were all derived from a single 100-gallon sample, which had been previously

settled, pasturised, bottled in half-gallon lots, sterilized, stored at low temperature and reseeded with sewage bacteria just prior to use." Evaporation losses were made up with sterile distilled water before withdrawal (Ludwig, Oswald, Gottas and Lynch, 1951).

Keshavan, Behn and Ames (1964) in their study of the kinetics of aerobic removal of organic wastes by the activated sludge process stored the remainder of the sludge in Winchester-quarts bottles of 2.5 litres capacity each in order to lower the metabolic activity of the microorganisms. This sludge was used periodically to replenish the liquid in the reservoir. The temperature of the cold sludge was always brought to the working level by squirting warm water over the container.

The laboratory experiments carried out by Oswald and his associates on "algal-bacterial symbiosis" differed fundamentally in several respects from the operation of an oxidation pond under field conditions and resembled more of an activated sludge process as will be evident from their obtaining "maximum bacterial activity corresponding to maximum uptake of oxygen at R = 0.25 day, which checked closely with the detention time of 4 to 6 hours in the operation of activated sludge plants." (Oswald, Gotaas, Ludwig

and Lynch, 1953b). In a conventional oxidation pond, there is no bubbling of air (excepting in the recent so-called aerated lagoons), no addition of individual uni-algal cultures such as Euglena or Chlorella and the bacterial population structure is not well defined in quality of quantity to begin with and the eco-system is not also enclosed as in "symbiocon" so that the atmosphere is not "eliminated as a factor in gas exchange."

So, our laboratory experiments were designed in such a way as to follow the conditions existing in natural conventional oxidation ponds as far as possible. There are about 25 oxidation ponds working in series treating about 30 mgd of sewage in the Pirana and Vasana Sewage Farms on the banks of the river Sabarmati at Ahmedabad. Ganapati, Prasada Rao, Godbole, Kothandaraman and Koshy (1965) and Jayangoudar (1967) have described the working of conventional oxidation ponds in Ahmedabad. Their method of operation was adopted in our laboratory experiments. When an oxidation pond is started in the Sewage Farm, the aerobic pond is first filled as rapidly as possible to a depth of about three feet and allowed to rest undisturbed for a period of 10 to 20 days until the pond turns greenish or bluish-green naturally. This period represents two phases: the bacterial phase I followed by the algal phase II after 7 to 10 days.

Then the inlets and outlets are opened and it represents the second stage when the actual operation of oxidation ponds at desired rates of flow or retention periods is started. Chapter 4 of this thesis describes the conditions of existence in the first stage of operation representing the bacterial phase I and the algal phase II of an aerobic oxidation pond under laboratory conditions.

Laboratory scale oxidation pond

A laboratory model was constructed with a glass aquarium, a reservoir of polyethylene carboy of eleven gallons capacity, a constant head inflow arrangement, effluent collection system, new 30 watt daylight fluorescent lights, and auxiliary control pipes. The design data are given below :

The aquarium measures 24" long, 12" broad and 12" deep. Sewage was filled upto a depth of 9.5" leaving a free-board of 2.5". Surface area was 2 sq.ft. and the volume at 9.5" depth was about 10 gallons.

The constant factors were : (a) Intensity of light maintained at 1200 fc throughout; (b) the laboratory temperature 28°C - 30°C ; (c) 5 day BOD at 20° C of the original sewage about 300 ppm; (d) the aquarium filled at the beginning with 10 gallons of raw settled sewage free

from suspended matter, and this volume kept constant throughout the experimental period.

A definite volume of 3.5 litres was removed from near the surface area by siphoning through a sterilized rubber tubing on 4th, 7th, 14th, 21st and 28th day for physico-chemical, biochemical, bacteriological and biological tests hereafter described in detail; and was replaced by an equal volume of the same sewage which had been preserved in about a dozen clean glass bottles each of about 3.0 litres capacity in the cold room. (0-5°C). Each time two bottles were removed from the cold room a few hours before the withdrawal of the liquid, tested for temperature and if necessary adjusted to the temperature of the sewage in the aquarium by pouring warm water over the bottles; so that the made up sewage was of the same temperature as that in the aquarium. Also, evaporation losses were made up with sterile distilled water everytime before withdrawal.

Raw sewage used

Flow, strength and constituents of raw sewage in a sewage disposal works vary from hour to hour, day to day and season to season. So, attention was paid to these considerations when the sampling procedure was decided so

that a true representative sewage of almost the same quality was drawn.

Raw sewage from the Baroda Sewage Disposal Works was always obtained during dry weather flow between 10 and 11 a.m. when there was a peak flow. Samples were collected in clean Winchester-quarts bottles. The settleable solids were removed as per directions given in the "Standard Methods" A.P.H.A. (1955) which allow fresh domestic sewage to stand under quiescent conditions for one hour. At the end of the settling period, the supernatant was siphoned off, filtered through a layer of sterilised cotton wool and used in all the experiments described in this thesis.

Chemicals and Glasswares

Chemicals used were mostly of analytical grade and/or laboratory grade for the chemical analysis and for bacteriological studies, bacteriological grade chemicals of Difco, U.S.A. were used.

Glasswares used were generally of Pyrex Brand or its equivalent.

METHODS

Sample Collection

Four sets of samples were drawn from near the sufface area of the stored sewage in the aquarium in the following order: For bacteriological examination, for

biological examination, for physico-chemical analysis and lastly for biochemical tests under aseptic conditions.

Bacteriological examination

Multiple tube dilution technique. Tests were done for coliforms at 37°C, E.coli Type I at 44°C, Faecal streptococci at 45°C, and citrate utilizers at 37°C according to the British Technique (vide Report - Bacteriological Examination of Water Supplies,1956). Citrate utilizers were estimated after 48 hours (Keller 1960). This medium is specific for identification of the members of the coliform group, which are non-faecal or usually non-faecal in origin and which are capable of utilizing an ammonia salt as the sole source of nitrogen and sodium citrate as the sole source of carbon.

All the results presented in tables usually refer to the Most Probable Number (MPN) of orgahisms per 100 ml of sample.

Total colonies count and isolation of individual strains. We find selective media being used by a few workers in their attempt to evaluate the nature and quality of microorganisms in their studies of different systems of sewage treatment. Allen (1944) tried various media and recommended nutrient agar as being the best for isolation of bacteria from activated sludge. Jasewicz and Porges(1956)

used nutrient agar with skim milk for the same purpose; Van Gils (1964) used tryptone-glucose agar. Dias and Bhat (1964) stated sewage agar as being the most satisfactory non-selective medium for their studies on several types of activated sludges. So, in this work sewage agar was used as a non-selective medium.

Sewage agar was prepared as follows : Fresh settled sewage after separation from suspended matter by filtering through a thin layer of cotton was solidified with 2% agar and autoclaved for 30 minutes at 15 psi., neutralised if necessary with sterile dilute phosphoric acid under asceptic conditions to pH 7.0 - 7.2. The solidified sewage agar in sterile petri dishes were inoculated with 0.1 ml samples serially diluted sewage and spread on to the surface of agar with sterile bent glass rod. All the plates were incubated at room temperature which varied between 28° to 30°C for 10 to 15 days. All colonies were first counted. After taking the total count, the plates with a total of 100 to 200 colonies were selected and from a sector of a plate having about 40 to 50 individual colonies, the individual colonies were transferred to peptone-yeast extract agar slants. The purification of the individual strain was made by the usual single colony isolation on the same peptone-yeast agar, on which they were maintained. It is assumed that the organisms thus isolated reflect the dominant bacterial composition of

the sewage in the laboratory model oxidation pond.

Typing and characterization of bacterial isolates. The methods given in Manual of Microbiological Methods, Society of America Bacteriologists (1957), were followed in the tests carried out for the identification of bacteria, unless otherwise stated. The various tests and the methods used are described in a tabular form below :

Tests	Method
A. Staining	
1. Gram	Burk and Kopeloff - Beerman's modification
2. Acid fast	Ziehl - Neelsen
3. Spore	Synder's modification of Dornor's
4. Flagella	Bailey's method as modified by Fisher and Conn
5. Fat-droplets	Burdeon's (1946) technique
6. Capsule	McKinney's (1962) method
B. Physiological	
7. Nitrate Reduction	Nutrient broth containing 0.1% KNO_3 was used. Nitrate reduction was tested using sulfanilic acid and α - napthyl-amine reagents.
8. Indole Production	Nutrient broth containing 0.1% tryptophan was used. Indole produced was detected by Kovac's reagent.

Tests	Method
9. H_2S Production	Nutrient broth with lead acetate paper was used.
10. Gelatin liquefaction	Nutrient agar containing 0.4% gelatin was used. The liquefaction was detected with Smith's reagent.
11. Starch hydrolysis	Nutrient agar containing 0.2% starch was used. Hydrolysis was detected using Lugol's iodine solution.
12. Fermentation tests	Nutrient broth containing 0.004% bromo-cresol purple was used. Sugars were added at 1% level. Gas production was noted in inverted Durham's tubes.
13. Litmus milk	Medium No.90 of Fred & Waksman (1928) was used. Change in the colour of litmus was noted to detect acid (red) or alkali (blue) production.
14. Methyl-red and voges-Proskaur tests	Glucose-phosphate medium was used. Acetyl-methyl-carbinol produced was detected by O'Mear's test as modified by Levine, Epstein, and Vaughn.
*15. Citrate utilization	Koser's citrate medium No.100 of Fred and Waksman (1928) was used.
*16. Nitrogen fixation	Nitrogen-free mannitol medium No.77 of Fred and Waksman (1928) was used.

* 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.

Tests	Method
17. Ammonia oxidation	Medium 52 of Fred and Waksman (1928) was used. Sulfanilic acid and α- napthylamine reagents were added followed by zinc powder addition tod detect nitrate formation.
18.Tributyrin hydrolysis	Nutrient-agar containing 0.1% tributyrin was used. Hydrolysis was detected by appearance of clear zone around the growing colony.

Biological Examination

Microscopic examination of the surface scum and layer was done as follows : A loopful (0.5 cm. diam.) of the viscous scum or surface layer and a drop of the liquid were examined first qualitatively for identification of the organisms; and then their counts made. To maintain uniformity loopful of the scum was spread over an area of 25 sq.mm. using diluted glycerine water. Five such loops or one drop of the liquid were spread carefully over the area marked on a clean glass slide and examined microscopically using a magnification of 100. This was repeated five times, the samples being taken from different places on the surface of the aquarium. Frequencies of the organisms present are expressed in symbols of Fritsch and Rich(1913). The

values of the various symbols are from counts of individuals found in 25 fields so that rrr = 1 ; rr = 1-50 ; r = 50-100; C = 100-200; CC = 200 - 500 and CCC = over 500.

Physico-chemical examination

Physical. Colour was recorded as it appeared 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 Beckman pH meter ; and temperature with a thermometer 0-50°C calibrated to a fifth of a degree.

Chemical. Alkalinity was determined by titration with a standard acid (0.02N H_2SO_4) using phenolphthalein and methyl orange as indicators; dissolved oxygen and 5-day B.O.D. at 20°C by the Pomeroy-Kirochman-Alsterberg modification of the original Winkler's method; inorganic phosphates using aminonapthol sulfonic acid reagent; ammoniacal nitrogen by direct Nesslerization method; nitrogen nitrite by diazotization method; and nitrogen nitrate by reduction and direct Nesslerization. Standard Methods A.P.H.A. (1955) were generally followed for the above tests excepting for the acid permanganate values which were done according to methods of chemical analysis as applied to sewage and sewage effluents (Ministry of Housing and Local Government, 1956)

Biochemical examination

Estimation of soluble organic constituents. For the estimation of soluble free sugar, total sugar, amino acids and protein in the samples of sewage drawn from the aquarium on different days, the following procedures were adopted. In order to separate the particulate matter from the samples, the procedure of successive sedimentation, centrifugation and lyophilization (freeze-drying) was followed. According to Hunter and Heukelekian (1965) freeze-drying is least likely to affect adversely the soluble organic matter constituents.

One litre of the sample was sedimented, and freed from suspended matter by centrifuging at 5000 x g for twenty minutes. The supernatant was collected separately. The residue was thrice washed with glass distilled water and the washings were carried over to the supernatant. The final liquid thus obtained was then concentrated to dryness by freeze-drying in a lyophilizer. The dried mass was resuspended in 20 to 30 ml of glass distilled water and homogenized in a Potter-Elvehjem homogenizer at 4000 rpm for two to three minutes, and the volume made up to 50 ml; and again centrifuged. The final supernatant was used for estimation of free sugar, total sugar, amino acid nitrogen and protein.

Free sugar. Free sugar was estimated according to the method of Marais, Dewit and Quicke (1966). Different aliquots of the concentrated samples were taken and made up to 1.0 ml with glass distilled water. 1.0 ml of alkaline copper sulphate reagent was added to each, and then the tubes were kept in boiling water for 20 minutes. The tubes were then cooled immediately in running tap water; and 2.0 ml of arseno-molybdate reagent was added and the contents mixed thoroughly. The final volume was made upto 10.0 ml and the colour developed was read at 750 mμ on Beckman Spectrophotometer model DU after 1.5 hours and before two hours. Standard curve was drawn by using glucose in the range of 0 to 30 μg. The results are expressed in terms of glucose.

Total sugar. 1.0 ml of the concentrated sample was mixed with 1.0 ml of 1.0 N HCI and hydrolysed by keeping the tubes in boiling water for one hour. To minimise loss due to evaporation the tubes were loosely covered with marbles. Then they were cooled and the contents neutralised by adding anhydrous sodium carbonate. The free sugar liberated was estimated as detailed above. The results are expressed in terms of glucose.

Amino acid nitrogen. Using the concentrated samples, this was estimated according to Russel (1944). Mixed glycine-

glutamic acid standard was used in the range of 0 to 30 µg.

Protein. This was estimated by the method of Lowry, Rosebrough, Farr and Rendall (1951). Bovine albumin was used as a standard in the range of 10-100 µg.

Total fat

One litre of each of the samples drawn on the days mentioned was used for this purpose. The particulate matter in the sample was removed by sedimentation and centrifuging at 5000 x g for 20 minutes. The supernatant was collected, acidified to pH 2.5 to 3.0 using bromo-phenol-blue as indicator. Then the fatty matter was extracted using 50 ml solvent ether. The extraction was made in three lots. The ether fractions were pooled together and concentrated to 5 ml over a water bath at 40°C. The final concentrate was passed through anhydrous sodium sulphate so that all traces of moisture were removed. The ether extract was collected in a previously ether washed, weighed, clean beaker of 50 ml capacity, and evaporated to dryness over a water bath at 40°C. The last traces of ether were removed under vaccum. Then the beaker with the final residue was weighed. The difference in weight of the beaker alone and beaker plus fat gave the total weight of fat in one litre of the original sample.

Estimation of chlorophyll-a,

Chlorophyll-a was measured according to the original

method of Richards with Thompson (1952). Depending upon the amount of algae present in the sample, volumes varying from 10 to 1000 ml were centrifuged as described in presence of 0.1 gm. of magnesium carbonate per litre of the sample. The supernatant was discarded; and the residue was dried in the dark under vaccum. The dried residue was then extracted in 90% acetone by keeping overnight at room temperature in the dark in glass stoppered test tubes. The tubes were again shaken thoroughly, centrifuged and the clear coloured supernatant was used for measuring absorptions in a Beckman Spectrophotometer model DU. Readings were taken at 665 mμ, 645 mμ and 630 mμ, from which absolute amounts of chlorophyll-a, can be calculated.

VISCOUS SCUM

Isolation

About 50 litres of cotton-filtered, sedimented raw sewage were stored in each of four glass aquaria measuring 24" x 12" x 12" which were loosely covered and left undisturbed for four days during which a viscous scum was formed on the surface of each aquarium.

A small portion of the scum was used for microscopic examination and for isolation of the pure strains. Several bits of the viscous scum were removed from one of the aquariam by means of sterile long needles and transferred in series through sterile distilled water containing

sterile glass beads to wash them free from extraneous bacteria and foreign matter through 10 to 12 changes depending upon the sizes of the bits. Finally the clumps were transferred to sterile distilled water flasks in which they were effectively dispersed by means of sterile glass beads added to the dilution blanks and were shaken vigorously on rotary shaker for 15 minutes in order to disintegrate the bits so as to form a homogeneous suspension. Then it was serially diluted; and 0.1 ml of each dilution was plated on sewage agar; and the plates were incubated at the room temperature for 10-15 days. Individual colonies were transferred to nutrient agar for maintenance as described earlier. The bacteria were identified to the genus level as already described.

To identify the zoogloeal organisms, the isolated strains were grown in autoclaved sewage (Butterfield 1935), in Unz and Dondero's medium (1964), in Cabtree's medium* (personal communication); and in nutrient broth. Butterfield (1935) has stated that "the peculiar branched tree-like form of zoogloea growth and the peculiarly shaped colonial forms in liquid media - this one characteristic is almost sufficient for identification as Zoogloea ramigera." Crabtree and McCoy (1967) have also

*Crabtree's Medium: Casein Hydrolysate 1.0g, yeast extract 0.5g, K_2HPO_4 1.0g, KH_2PO_4 = 0.5g, glucose 0.5g, distilled water 1 litre, pH = 6.8 - 7.1.

confirmed it. So, those which showed the formation of finger - like projections were classified as zoogloeas and used for further studies.

Biochemical examination of the scum

The scum was analysed for free sugar, total sugar, protein, amino-nitrogen and total fat. The scum was removed and dried in a freeze-dryer. A known amount of the dried scum powder was extracted with solvent ether in a Soxhlet apparatus for 10-16 hours. The difference in the weight of the scum before and after the fat extraction gave the weight of fat. The ether containing fat was also evaporated and the residue weighed for verification of the quantity of fat. The fat content is expressed in percent dry weight of the scum.

The powdery scum after fat extraction was homogenised in distilled water as described earlier and free and total sugar, amino acid nitrogen and protein were estimated by the method described earlier.

Preparation of acetone powder from the viscous scum for enzyme studies

The surface scum formed in another glass aquarium was carefully collected. The wet weight of the scum was noted. The entire scum was transferred to a waring blender and double the volume of pre-chilled (- 20^{o}C) acetone

was added; and the blender immediately started and kept at the maximum speed for 30 seconds at 4°C. The suspension was then filtered through cheese cloth, and the residue was collected and the process repeated. The powder was collected and spread on a filter paper and dried at room temperature till it was free from acetone. Weight of the dry acetone powder was noted and stored at 4°C until further use.

Enzyme preparation. 20 mg. of acetone powder were taken for each ml of glass distilled water and homogenized in a Potter-Elvehjem homogeniser for 30 seconds at 4000 rpm. at 0°C. The homogenate was centrifuged in a Sorvall refrigerated centrifuge at 5000 x g for 20 minutes at 0°C and the supernatant collected was used as the enzyme source.

Amylase activity was estimated by the method of Benfield (1955); Protease according to Bergmeyer (1963) and Lipase according to McDonald and Lefave (1962).

Isolation of poly-β-Hydroxy-Butyric acid (PHB) from the viscous scum. The method of isolation was based on that of Dias and Bhat (1964).

PURE CULTURE STUDIES WITH BACTERIA

On page4-26 Table 4-7 of Chapter 4, the types of bacteria present on different days are shown. From a study of the table, it is possible to state which groups of bacteria were dominant on different days of isolation and they are

given below :

Days on which isolated from the laboratory model oxidation pond	Names of the Genus	Percentage of the total isolates on each day
0	Micro-coccus	40
	Sarcina	30
4	Brevibacterium	38
	Micrococcus	20
7	Corynebacterium	35
	Flavobacterium	21
14	Corynebacterium	36
	Brevibacterium	24
21	Flavebacterium	28
	Micro-coccus	28
	Brevibacterium	21
28	Micro-coccus	42
	Brevibacterium	32

All the groups of bacteria comprising 296 strains have been studied for their physiological characteristics. From these studies it was possible to find out which of the bacterial strains belonging to each genus gave positive reactions for most of the tests, and which gave for the least number of tests. One strain from each of these groups for each genus was selected and screened for pure culture studies as detailed below.

Screening of the bacteria for pure culture studies

The 26 strains selected in this way were grown on sewage agar slants for 48 hours at room temperature (28 - 30°c). The bacterial growth was suspended in sterile distilled water and the turbidity for each strain was adjusted to 50 klett units so that approximately a uniform number of organisms may be inoculated in each case. 5 ml. of the individual suspensions were inoculated into 100 ml of sterilised sewage taken in 250 ml. capacity conical flasks. All the inoculated and a few un-inoculated flasks were incubated at room temperature.

The flasks inoculated with the bacterial strains isolated on 0, and 4th days were removed after 4 days along with one un-inoculated flask. The contents of the flasks were thoroughly shaken and filtered through a layer of cotton wool. The filtrates were analysed for turbidity, pH, alkalinity, acid- $KMnO_4$ value, inorganic phosphate, ammonia-nitrogen and relative stability.

Similarly, flasks inoculated with the strains isolated on 7, 14, 21 and 28 days were removed after their respective periods of storage along with one control flask on each day. The filtrates were examined for the different physico-chemical characteristics as described above.

The above experiment was repeated thrice with the 26 strains. From the results thus obtained, 13 strains were

selected which gave positive proofs of purification. The selected strains are listed below and they were used for further detailed studies.

Names of the bacterial strains selected

Serial No.	Days on which Isolated	Strain No.	Generic name
1	0	R - 66	Micro-coccus sp.
2	4	0 - 96	Brevibacterium sp.
3	7	0 - 137	Corynebacterium sp.
4	7	0 - 140	Flavobacterium sp.
5	14	0 - 143	Brevibacterium sp.
6	14	0 - 149	Corynebacterium sp.
7	21	0 - 156	Micro-coccus sp.
8	21	0 - 166	Brevibacterium sp.
9	28	0 - 195	Micro-coccus sp.
10	28	0 - 201	Brevibacterium sp.

Actual pure culture studies with the ten selected strains of bacteria

5.0 ml suspensions of 48-hour old cultures of each of the above strains were inoculated into a set of 6 conical flasks, each containing 100 ml sterile sewage. Also, one more set of flasks was inoculated with a mixture of all the strains. All the inoculated flasks along with a set of un-inoculated flasks were incubated at room temperature.

One flask from each set was removed on 0, 4, 7, 14, 21 and 28 days. The contents of the flasks were analysed for the various physico-chemical tests already mentioned. The 0 day flasks were examined immediately after inoculation.

The above experiments were repeated thrice, and the results are given as averages of the three experiments in tabular statements, each table representing the results given by the ten bacterial strains and their mixture for each test.

Pure culture studies with algae and bacteria

Isolation and maintenance of pure cultures of algae. During the course of our experiments, it was possible to isolate four algal specimens which were found to thrive well in sewage. They were : (i) Chlorella vulgaris; (ii) Scenedesmus quadricauda ; (iii) Oscillatoria obscura and (iv) Oscillatoria chalybea.

There were two stages in the isolation of pure bacteria-free cultures of the above algae. (i) free from other algae; and (ii) free from bacteria.

Free from other algae

When a particular algae was found dominant, 10 ml of the algal suspension were taken and centifuged at 3000 g for 10 minutes. Later, the supernatant was discarded; and the sediment which was suspended in distilled water, was inoculated

into flasks containing sterile sewage. The inoculated flasks were exposed continuously to fluorescent light. When the liquid turned greenish or bluish-green a loopful was examined microscopically for the purity of the alga. Chlorella vulgaris and Scenedesmus quadricauda were found to grow as pure cultures free from other algae. So, they were maintained in sterile sewage by subculturing once in a week.

As for the other two algae: O.Obscura and O.chalybea, they were found to be associated with Chlorella; and they were purified by methods based on their growth characteristics.

The two species of Oscillatoria being filamentous are found to grow on the surface, while Chlorella easily settled down at the bottom. Secondly, Chlorella grows faster than Oscillatoria. So, when a mixture of the two algae is allowed to grow in sterile sewage without disturbance, Chlorella grows first and settles down while Oscillatoria starts growing slowly after about 7 days and attains maximum growth in 14 days. During this period most of the Chlorella settles down leaving a few entrapped in the filaments of Oscillatoria. So, after about 14 days, a loopful of the Oscillatoria from the top surface of the culture flasks, was removed without disturbance and was suspended in a drop of distilled water placed on a microscopic slide. The suspension was examined microscopically and with the help of two fine pointed needles a bit of the alga was broken into smaller threads and the

threads were thoroughly agitated so that the globular Chlorella cells which were attached to the threads of Oscillatoria became free and were easily drained off. This operation was repeated several times till on microscopic examination, the Oscillatoria filaments were found free from Chlorella cells. Then the threads were inoculated into 10 ml. of sterile sewage taken in 25 ml conical flasks and were exposed to light. When the growth was at its maximum, a loopful was again examined microscopically for the presence of Chlorella cells. It was found necessary to repeat the above procedure several times before Oscillatoria was completely freed from Chlorella. Then they were maintained by sub-culturing once in a week in sterile sewage.

Bacteria-free algal cultures. The four types of algae mentioned, though found to be free from other algae, were not free from bacteria. So, bacteria-free cultures of the above 4 species of algae were prepared as detailed below.

Week old cultures of alga was taken in sterile test tube and centrifuged at 3000 g for 10 minutes. The supernatant was discarded and repeating the same procedure a fairly good amount of each algal specimen was collected.

The algal sediments were washed thrice with 10 ml. portions of sterile distilled water and finally suspended in 1.0 ml of sterile distilled water. Five drops of chlorine water were added to each and the tubes were set aside for 5 minutes

for the chlorine to act on the remaining bacteria. The volume was again made up to 10 ml, with sterile distilled water and the algae were centrifuged, and re-suspended in 1.0 ml, of sterile distilled water. The process of chlorination was repeated thrice and a loop of the suspension was transferred to nutrient agar slants which were incubated in the dark for 48 hours at 37°C. The remaining suspension was inoculated into sterile sewage and the flasks were exposed to light continuously. When the flasks became greenish or bluish-green, a loopful was again inoculated into nutrient agar slant and incubated in the dark for 48 hours at 37°C. If there were no bacterial growth on the agar slants, then the algal culture was considered to be free from bacteria. The bacteria-free algal cultures, thus obtained, were maintained by sub-culturing once a week. Occasionally, the cultures were tested for their freedom from bacteria.

In addition to the above 4 species of algae which were actually isolated from Baroda raw sewage, two other pure nitrogen fixing species were also studied. They were obtained from the Indian Agricultural Research Institute, New Delhi. They are : Aulosira fertilisima and Nostoc pyriforms. As the two species had to be acclimatised to sewage the following procedure was adopted.

The two algae were maintained in the laboratory on the two synthetic media described below :

Medium for Aulosira

K_2HPO_4 = 0.2 g

$CaCl_2$ = 0.1 g

$MgSO_4.7H_2O$ = 0.2 g

KNO_3 = 0.2 g

1% $FeCl_3$ Soln = 1.0 ml

A_5 Soln = 1.0 ml

Distilled water = 1000 ml

Composition of A_5 solution

H_3BO_4 = 2.90 g

$MnCl_2.H_2O$ = 1.81 g

$ZnCl_2$ = 0.11 g

$CuSO_4.5H_2O$ = 0.08 g

Amm.molybdate = 0.018 g

Dist. Water = 1000 ml

pH was adjusted to 7.5 and the medium was distributed in 100 ml. quantities in 250 ml. conical flasks and autoclaved at 10 psi. for 10 minutes. This alga was sub-cultured once a fortnight.

Medium for Nostoc

The alga was maintained in 4 strength Chou's No.10 modified medium. (Gerloff, Fitzgerald and Skoog 1950).

This alga was sub-cultured in the above medium once in a fortnight.

Acclimatisation. Solutions containing 10%, 25%, 50%, 75% and 100% concentrations of sewage were prepared in tap water, and sterilized at 15 psi. for 30 minutes.

To begin with, both the algae were inoculated into 10% sewage, and incubated in presence of fluroscent light. They took nearly a month to grow. Next, two more sub-cultures in 10% sewage were done. From the third sub-culture, the algae were inoculated into 25% sewage. Two more transfers were done in 25% sewage as stated above. Thus for each concentration of sewage, three transfers were made and finally they were cultured in 100% sterile sewage. Algae acclimatised to sewage were maintained by sub-culturing once in 20 days in sterile sewage.

Pure culture studies with algae and bacteria

The objects of the following experiments were two fold: (a) to find out how much purification of sewage each of the 6 species of algae alone was able to bring out in the absence of bacteria; and (b) to find out how much purification of sewage each of the six species of algae brought about in presence of the mixture of 10 bacteria used already in pure culture studies. For this purpose, the six species of algae were divided into two groups: (I) fast growing algae- Chlorella, and the two species of Oscillatoria; (II) the slow growing algae: Scenedesmus, Aulosira and Nostoc.

Experiments with Group I algae : (Chlorella and 2 species of Oscillatoria)

Four day old cultures of each of the three species of algae were taken and centrifuged at 3000 g for 10 minutes

in sterile test tubes. The supernatant was discarded and the sedimented algae were washed thrice with sterile distilled water, re-suspended in water and the turbidity was adjusted to 50 Klett units.

Forty-eight hour old cultures of the 10 bacterial strains already used for pure culture studies were also taken and the growth of each culture was suspended in sterile distilled water and the suspensions of all the ten cultures were mixed together. The turbidity of the mixture was adjusted to 50 Klett units.

Next 5 ml of each individual algal suspensions and mixed bacterial suspensions were inoculated in different sets of 250 ml capacity flasks each containing 100 ml of sterilized sewage.

Observations expected in each set are briefed below :

Sr.No.	Inoculated with	Observations expected to show
I	Chlorella vulgaris	Changes brought about by C.vulgaris only.
II	C.vulgaris along with mixture of bacteria	Changes brought about by C.vulgaris in presence of bacteria.
III	Oscillatoria chalybea	Changes brought about by O.chalybea alone.
IV	O.chalybea with mixture of bacteria	Changes brought about by O.chalybea in presence of bacteria
V	Oscillatoria obscura	Changes brought about by O.obscura only.
VI	O.obscura with mixture of bacteria	Changes brought about by O.obscura in presence of bacteria
VII	Mixture of all the three algae.	Changes brought about by mixed algae alone.
VIII	Mixture of all the three algae with mixture of bacteria	Changes brought about by mixed algae in presence of bacteria
IX	Uninoculated flasks	Control.

On 0, 4, 7, 14, 21 and 28th day one flask from each of the nine sets of flasks was removed shaken thoroughly and filtered through a layer of cotton. The filtrates were analysed for the several physico-chemical characteristics. The 0 day flasks were examined immediately. The experiment was repeated thrice and the results are furnished as averages of the three experiments in tabular statements, each table representing the changes for a single test.

Similar experiments were done in the case of the three slow growing algae viz. : Scenedesmus, Aulosira, and Nostoc.