CHAPTER VI

Identification of microbes and study of their efficacy:

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Total treatment of tannery effluent is generally divided into three categories viz. primary, secondary and tertiary. The secondary treatment is normally by bacterial action. It is therefore felt necessary to study bacterial action on tannery effluent. In general a large number of bacteria are present in effluent. They all have different rate of growth and different capabilities of BOD reduction. In the present work an attempt has been made to isolate bacterial strains and to study their growth rate and BOD reduction capabilities. For this purpose a few points on the effluent flow line identified as explained in the following few lines.

VI.1.1 Selections of spots for study of effluent quality :

The primary treated effluent was the first sample taken for the characterization of the influent to secondary treatment plant. The next sample is taken from the secondary treatment unit to determine the change, if any, during the biological treatment of the influent. The next sampling spot is from the discharge point of the ETP to determine the change in the treated effluent after the secondary treatment. Final sampling point is after the clarification of the secondary treated wastewater. These sampling points give a comparative indication of the tannery effluent quality at different phases of treatment as shown in figure V.12.

VI.1.2 Spot Testing of effluent sample at selected points :

Various parameters viz. DO, NO_3^-, PO_4^{3-}, pH and temperature of the treated effluent indicative of the effluent quality at four selected spots as mentioned under section VI.1.1 are determined on spot. Temperature was measured with the help of a thermometer, while NO_3^-, PO_4^{3-} were measured colorimetrically by Aquamerck field testing kits (E.Merck, Germany) and DO was measured by both DO meter and titrimetrically by Aquamerck field testing kits (E.Merck, Germany). Four

samples drawn from each spot for testing above parameters and the mean values of the parameters were noted in table VI.1

The effluent samples from each spot separately collected in sterile container and brought to the laboratory in ice packed insulated boxes

Table VI.1: Parameters at Activated sludge plant (ASP) of Mokamehghat ETP:

Sl No	Spot	Temp	pH	DO (mg/l)	NO_3^- (mg/l)	PO_4^{3-} (mg/l)
1	Inlet of ASP	30°C	6.5	1.1	10	NF
2	ASP	32°C	6.5-7	2.9-4.4	25	0.5
3	Outlet of ASP	32°C	6.8-7	4.8	25-50	0.25
4	Outlet of 1st clarifier	30°C	5.5-6	5.7	25-50	Below 0.25
5	Outlet of 2nd clarifier	30°C	5.5-6	5.7	25-50	Below 0.25

VI.1.3 Procedure for determination of Bacterial count:

Culture media was prepared using the guidelines from the 'Standard Methods' of American Public Health Association (APHA). In order to arrive at a suitable dilution ratio, seven sets of experiments were conducted on the sample from Activated sludge plant, which contains maximum bacterial population.

The microbial load of the samples collected from secondary treatment plant at Mokamehghat Activated sludge plant was determined and shown in Table VI.2.

Table VI.2: Microbial count at Activated sludge plant (ASP) of Mokamehghat ETP :

Sl No	Bacterial Count (ml^{-1})	Bacterial Mean Count (ml^{-1})
1	11.7×10^6	
2	6.5×10^6	
3	10.2×10^6	9.7×10^6
4	8.4×10^6	
5	12.0×10^6	

The mean count result as shown above signifies that, there is a huge load of micro organisms in the activated sludge treatment system which needs to be isolated in order to assess their relative efficiency in decomposing the organic matters into simpler form and thereby reducing the pollution load which can act as an index for the BOD removal of the tannery effluent. For measurement of BOD, COD standard methods were followed and given in detail in Appendix-I including materials and machines/apparatus.

VI.2. Method of isolating pure culture

For isolation of pure culture the first step is to prepare the media and then cultivation of bacteria and isolation as explained in the following paragraph:

Preparation of media :

For the cultivation of heterotrophs certain complex raw materials such as peptones, meat extract and yeast extract were used and the resulting media support the growth of a wide variety of heterotrophic bacteria. Agar is included as a non-

nutritive solidifying agent when a solid medium is required. Nutrient broth and Nutrient agar are relatively simple liquid and solid media that support the growth of many common heterotrophs. The addition of yeast extract to each of these formulas improves the nutrient quality, since yeast extract contains several of the B vitamins and other growth-promoting substances. 3 gms Beef extract, 5 gms Peptone, 15 gms Agar, 3 gms Yeast extract are dissolved in 1000 ml distilled water (except Agar) and pH was adjusted to 7. Then agar was added and the medium was boiled to dissolve the agar. The medium was dispensed into tubes or flasks and sterilized by autoclaving at 15 lb pressure for 15-20 minutes. After sterilization the hot medium was poured in petridishes and allowed to cool which will provide solid media.

Cultivation of bacteria :

The sample containing mixed culture was first diluted in sterile distilled water to contain only a few cells per milliliter before being used to inoculate the vergin sterile agar media prepared in the laboratory. Since the number of bacteria in the specimen is not known, a series of dilutions were made so that at least one of the dilutions will contain a suitably sparse concentration of cells from which cell count can be obtained easily. Effluent from activated sludge treatment process containing bacteria was diluted to 10,100,1000,10000 times in sterile distilled water. From each dilution 0.1 ml was spread on the surfaces of separate cooled agar plates and incubated at 37°C for 24 hours to get distinct colonies.

Isolation :

After cultivation of bacteria, by means of a transfer loop, a portion of the distinct colony was placed on the surface of a sterile agar medium and streaked across the surface. This manipulation thins out the bacteria on the agar surface so

that some individual bacteria were separated from each other. When streaking was properly performed, the bacterial cells will be sufficiently far apart in some areas of the plate to ensure that the colony developing from one cell will not merge with that growing from another. Pure cultures have been isolated by several times streaking.

By following the same process 65 strains were isolated in pure form from the sample of Activated sludge plant.

VI.3. Determination of efficiency of 65 isolated pure strain:

The Primary treated tannery effluent samples were collected separately in sterilized bottle and brought back to the laboratory in an ice packed container for determination of relative efficiency of each 65 strains so isolated and purified.The collected samples were distributed in BOD bottle and inoculated with isolated and purified strains individually. The initial dissolved oxygen content was measured at 4 hrs interval for 1½ days and 8 hrs interval for another day. In the same pulse simultaneously NO_3^- and PO_4^{3-} concentration were determined. The results are shown in table VI.3 in Appendix-II. BOD values are presented graphically in figures B1-B65 in Appendix-III.

The efficiency of each strain of bacteria was evaluated with reference to the following parameters - BOD, NO_3^- and PO_4^{3-}. BOD efficiency of each bacteria was measured as described in Appendix-I. NO_3^- and PO_4^{3-} measurements were carried out according to following processes.

VI.3.1 Procedure for nitrate (NO_3^-) determination:

Nitrate determination was carried out by semi-quantitative method by reducing to nitrite reacting with sulfanilic acid in an acidic medium to form diazoniam salt.

This salt was then coupled with benzoic acid to produce orange-yellow colour, which was compared with precalibrated colour card. For this purpose nitrate field testing kit (Aquamerck) was used for nitrate estimation of the samples.

Procedure :

The test bottle was rinsed with the sample tannery waste water and filled to the 5-ml mark. Then 1 level microspoon of reagent was added to the test vessel. The test vessel was closed with the lid and shaken well for 1 minute. After 5 minutes the test vessel was placed on the precalibrated colour card and matched with a colour comparison value to find the quantity of nitrate.

VI.3.2 Procedure for phosphate (PO_4^{3-}) determination :

It was determined by the isopoly acids which occur in acidic solution after reacting with ammonium heptamolybdate. These isopoly acids are then converted to corresponding heteropoly acids with phosphate. This yellow coloured solution containing phosphomolibdic acids were subsequently converted by reduction into blue coloured solutions and determined colorimetrically. Phosphate field testing kit (Aquamerck) was used to determine the phosphate content of tannery waste water.

Procedure :

The test vessel was rinsed with the sample waste water and filled to the 5-ml mark. 5 drops of reagent No.1 was added and swirled. Then 1 level of microspoon of reagent No. 2 was added and dissolved by swirling. After 2 minutes the test vessel was placed on the colour card and matched with a colour comparison value which gives the indication of phosphate content in the effluent.

VI.4. Identification of species name of 4 efficient bacteria :

From the above mentioned studies it has been found that out of the 65 species isolated four were found the very efficient in BOD reduction. For identification of four pure bacterial strains, morphological tests, physiological tests, biochemical tests etc. were done. These tests were carried out following the standard procedures of General Characterization by Smibert and Krieg (1981). The results of the different tests carried out are shown in table VI-5. The colony picture of four pure bacterial strains are given in Appendix IV. The species name and generation time of four pure bacterial strains were identified and are as given in the following table VI-4

Table VI-4. Species name of bacteria:

Bacterial sample no.	Bi-nomial name of bacteria	Generation time of bacteria at 35^{0} C temperature.
1	*Enterobacter aerogens*	30 minute
2	*Pseudomonas aeruginosa*	36 minute
3	*Bacillus megaterium*	35 minute
4	*Bacillus subtillis*	30 minute

Table-VI.5. Characteristics features of isolated four pure bacterial strains:

Table –VI.5.1. Morphological Tests:

Tests	Results			
	Enterobacter aerogens	*Pseudomonas aeruginosa*	*Bacillus megaterium*	*Bacillus subtillis*
Colony Morphology: Configuration	Round	Round	Round	Round
Margin	Entire	Entire	wavy	wavy
Elevations	Convex	Convex	Convex	Convex
Surface	Smooth	Smooth	Rough	Rough
Density	Trans lucent	Trans lucent	Trans lucent	Trans lucent
Pigments	-	Greenish	-	-
Gram's Reaction:	-	-	+	+
Shape	Rods	Rods	Rods	Rods
Size	Short	Moderate	Long	Long
Arrangement	Single	Single	Single	Single
Spore: Endospore	-	-	+	+
Position			Central	Central
Shape			Oval	Oval
Motility:	+	+	+	+
Fluorescence(UV) :	-	+	-	-

Table VI.5.2. Physiological Tests:

Growth at temperatures:	Results			
	Enterobacter aerogens	*Pseudomonas aeruginosa*	*Bacillus megaterium*	*Bacillus subtillis*
4^0C	-	-	-	-
10^0C	-	-	-	-
15^0C	-	-	-	-
22^0C	+	+	+	+
26^0C	+	+	+	+
30^0C	+	+	+	+
37^0C	+	+	+	+
42^0C	+	+	+	+
55^0C	-	-	-	-
65^0C	-	-	-	-
Growth at pH:				
pH 5.0	+ -	+ -	+ -	+
pH 5.7	+	+	+	+
pH 6.8	+	+	+	+
pH 8.0	+	+	+	+
pH 9.0	+ -	+ -	+ -	+ -
pH 11.0	-	-	-	-
Growth on NaCl(%) :				
2.5	+	+	+	+
5.0	+	+	+	+
7.0	+	-	+	+
8.5	-	-	+	+
9.0	-	-	+	+
10.0	-	-	+	+
Growth under Anaerobic condition:	+	-	-	-

Table VI.5.3. Biochemical Tests:

Tests	Results			
	Enterobacter aerogens	*Pseudomonas aeruginosa*	*Bacillus megaterium*	*Bacillus subtillis*
Growth on MacConkey agar: non-Lacfermenter	+	+	-	-
Indole Test	-	-	-	-
Methyl Red Test	-	-	-	-
Voges Proskauer test	+	-	-	-
Citrate Utilization	+	+	+	+
Casein hydrolysis	+	+	+	+
Starch hydrolysis	-	-	+	-
Urea hydrolysis	-	-	-	-
Nitrate Reduction	+	+	-	-
Nitrite Reduction	+	-	-	-
H_2S Production	-	+ -	-	-
Cytochrome Oxidase test	-	+	-	+
Catalase test	+	+	+	+
Oxidation/Fermentation(O/F)	F	O	F	F
Gelatin liquifaction	-	+	+	+
Arginine dihydrolase	-	+	-	-
Lysine decarboxylase	+ -	-	-	-
Ornithine decarboxylase	+ -	-	-	-

Acid production from carbohydrates	**Results**			
	Enterobacter aerogens	*Pseudomonas aeruginosa*	*Bacillus megaterium*	*Bacillus subtillis*
Adonitol	+	-	-	-
Arabinose	+	-	-	-
Cellobiose	+	-	+	+
Dextrose	+	+	+	+
Fructose	+	+	+	+ -
Galactose	+ -	-	+	+ -
Inositol	+	-	+ -	+
Inulin	+	-	+	+
Lactose	+	-	-	-
Maltose	+	-	+	+
Mannitol	+	+	+	+
Melibiose	+ -	-	-	-
Raffinose	+	-	+ -	-
Salicin	+	-	-	-
Sorbitol	+	-	+	+ -
Sucrose	+	-	+	+
Trehalose	+	+	+	+
Xylose	+	+	+	-

VI.5. Efficiency determination of 4 isolated strains singly and in combination under atmospheric condition with respect to BOD reduction:

Effluent was disinfected with UV light in laminar flow bench as shown in Appendix-IV. Effluent was taken in sterile BOD bottle. A small amount of a pure culture was added to the effluent. Then aeration was done with the help of aerators (porous materials which provide very small bubbles of air to the effluent). Sample BODs were measured following standard method at certain time interval in the usual way. Ultimate oxygen contents of the effluent after aeration were measured and maximum %BOD reduction were calculated and this observation are presented graphically as shown in figure B-66 in Appendix-III. The above results for each bacterial strain singly and in combination are shown in table VI.-6 in Appendix-II and presented graphically in figures B 67 - B 78 in Appendix-III. From the above observation ultimate DO content of treated effluent is obtained and the maximum BOD reduction is determined. From the table it is evident that increased number of bacterial strains will increase BOD reduction and also the DO content of the sample.