Characterization and Identification of Microflora in Activated Sludge Process.

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Abstract: The study was carried out for identifying microorganisms present in the extended aeration activated sludge process at Common Effluent Treatment Plant (CETP), Thane. The stabilization of biological foams results from production of extra cellular materials like lipids, peptides, proteins and carbohydrates which have the properties of surface active agents and bulking phenomenon has been recognized for a long time and is known to be caused by at least 14 types of filamentous microorganisms.

The microbial population from treated sludge was isolated on nutrient medium, MacConkey's agar and Sabauroud's agar using streak plate method. The isolated colonies were then Gram stained and isolates were used further for biochemical tests to confirm the organism upto probable genus level.

Diverse microflora was observed in the water sample collected from aeration tank at CETP. The microscopic examination of water samples showed the presence of *Diatoms* (Bacillariophycophyta), *Paramoecium species, Vorticella species, Nematodes, Trypanosomes,* and motile filamentous algae. The activated sludge at CETP showed the presence of various types of bacterial and fungal species *viz., Pseudomonas, Klebsiella, Alcaligenes, Sphaerotilus natans, Beggiatoa species, Escherichia coli, Staphylococcus aureus* and *Aspergillus*.

***Keywords**:* CETP, Activated sludge, Streak plate method, Biochemical tests.

Introduction:

Wastewater is defined as the water carrying wastes collected from residences, institutions and various industries. Wastewater released in the natural water bodies without any treatment, leads to cause diseases. The wastewater is treated by primary, secondary and tertiary treatments which include physical, chemical and biological processes. In primary treatment, the solids from wastewater are removed by sedimentation and filtration as physical process. Secondary treatment involves the use of trickling filters, activated sludge, lagoons and anaerobic digesters to remove the organic decomposable matter. Activated sludge or extended aeration treatment involves a continuous system where aerobic microorganisms are mixed with waste water and then separated in a gravity clarifier. Therefore, waste treatment system such as the activated sludge system depends on the activities of communities of living organism (Sharifi *et al*., 2001). In this process, the sewage or industrial waste water is aerated in an aeration tank in which microbial floc is suspended. The bacterial flora grows and remains suspended in the form of floc, which is called as activated sludge.

In the aeration tank, there is a chain of microorganisms which actually degrade the organic matter. These microorganisms are highly active in degradation of xenobiotics which are otherwise non biodegradable thus reducing the chemical oxygen demand (COD) value of water. It also helps to reduce the biological oxygen demand (BOD) value. Microbial population developing in the form of biofilm is closely linked with each other with respect to their metabolism. Generally these are decaying, facultative anaerobic and aerobic organisms.

The microbial floc formed in this process is zoogleal mass of living organisms embedded with their food and slime material which act as centers for biological oxidation. Hence, it is called as activated sludge. The microorganisms should be provided with essential nutrients such as Nitrogen, Phosphorus which are supplied in the form of urea and mono-or di- ammonium hydrogen phosphate. Other nutrients such as Potassium, Magnesium, and Calcium are generally present in the waste. Other important factors which determine the efficiency of activated sludge are pH, temperature and oxidation reduction potential. The optimum pH range for process is 6.5 to 9.0. The extended activation process can be assessed with the help of microbial indicators as observed by regular microscopic examination of activated sludge. A good activated sludge contains a relatively high number of free swimming and stalked ciliates apart from rotifers. The presence of such diverse microflora in activated sludge makes its use inevitable in the wastewater treatment to increase the efficiency of treatment plant.

Therefore, use of this activated sludge is more common in treatment of effluent generated in large scale or small scale industries. As per guideline given by Maharashtra Pollution Control Board (MPCB) and subsequent reference of Memorandum of article and Tripartite agreement, all medium and large scale units have to give full-fledged treatment involving primary, secondary and detoxification treatment to the effluent and small scale units are supposed to give primary and detoxification treatment, before it is discharged to sewer system leading to Common Effluent Treatment Plant (CETP). CETP unit collectively treats the wastewater receiving from various industries. Such effluent

consists of varied organic and inorganic components which in turn regulate the development of zoogleal film.

In the present study, attempts have been made to study the microbial diversity present in activated sludge used at CETP, Thane, Maharashtra, which treats the wastewater from varied industries *viz.,* chemical, pharmaceuticals, dyes, pigment manufacturing, petrochemical, electronics, textile processing, engineering etc.

Materials and Methods: The effluent generated from different zones of industrial area is being collected in to three different collection pumps provided at Rabale, Pawane and Sanpada. The effluent collected at Rabale and Sanpada is pumped to the Pawane (KoparKhairane) pump. The ultimate equalisation of entire waste water takes place at Pawane and equalized wastewater is then further treated by adopting extended aeration activated sludge process. The effluent is disposed to Vashi creek and solid waste generated is sent to Trans Thane Creek for secured land filling. The existing treatment facility having capacity of 12 Million Liters per Day (MLD) and 15 MLD is divided in two plots namely Plot P-18 and P-60. They are about 750 meter away from each other. In CETP, the effluent is treated for 36hours. The aeration tank is divided in 3 bays at 12 MLD plant where each bay consists of 5 aerators. Whereas aeration tanks in 15 MLD are divided in 4 bays where each bay consisting of 4 aerators.

The sample for analysis was collected from outlet of 12 MLD aeration tanks from all the three bays. Also, the splashing liquid from each aerator as well as sludge sample from side walls of each aerator of each bay was collected for microbiological analysis. The sludge was analyzed by wet mount method to determine the presence of microflora. Collected water sample from aeration tank was centrifuged at 3000 revolution per minutes for 15 minutes. The supernatant was discarded and the precipitate was resuspended in sterile distilled water. This sample was then examined microscopically under 40X magnification.

After the wet mount analysis the sludge samples were used to culture the microflora present in it. Each sludge sample was diluted 1 : 10 with sterile distilled water, and loopful suspension of each was streaked on sterile plates of Nutrient agar and MacConkey's agar using T-streak or side streak technique and the plates were incubated at room temperature for 24hours. Colony characteristics were recorded for each isolate and it was characterized by various biochemical tests *viz.,* Indol, Methyl red, Voges-Proskauer, Triple Sugar Iron butt (TSI), Citrate utilization, production of certain enzymes like catalase, urease and oxidase. These tests were performed to identify the organism upto genus.

The fungal strains were cultivated on sterile Sabaurouds agar plates. The plates were incubated at room temperature for about 3 days. The tentative genus identification was carried out by lactophenol blue wet mount method. The fungal species were identified based on the specific types of spores they formed.

Results and Discussion: The wet mount of all the different sludge samples revealed the presence of different microorganism likes protozoans, rotifers, bacteria, algal filaments and fungal mycelia. Different types of protozoa and rotifers observed were *Vorticella, Neviculata, Trypanosoma* and *Paramoecium*.

The agar plates after incubation of 24 hours at room temperature showed number of well isolated colonies. Table 1 gives the comparative colony characteristics of certain isolates on Nutrient agar plates and table 2 represents the colony characteristics of certain isolates on MacConkeys agar plates which are species representative.

The fungal strains were grown on sabaurouds agar plate after 3 days of incubation. The plates were continued to incubate for spores to form. Depending on the mycelial and spore characteristics it was confirmed that the growing fungi was of *Aspergillus species* and *Geotrichum species*.

Table.1: Comparative colony characteristics of isolates on Nutrient agar plate

Colony characteristics	BAY 1			BAY 2		BAY 3	
	colony 1	colony 2	colony 3	colony 1	Colony2	colony 1	colony 2
Size	1 mm	>1 mm	1 mm	1 mm	1 mm	1 mm	>1 mm
Shape	Circular	Pin Point	Circular	Circular	Circular	Circular	Pin Point
Colour	Bluish Green Pigment	Colourless	White	Bluish Green	Golden Yellow	Bluish Green	White
Elevation	Concave	Concave	Concave	Flat	Flat	Flat	Flat
Consistency	Smooth	Smooth	Mucoid	Smooth	Smooth	Smooth	Smooth
Opacity	Opaque	Opaque	Translucent	Transparent	Opaque	Transparent	Transparent
Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire
Gram Nature	Gram Negative Bacilli	Gram Positive Cocci In Cluster	Gram Negative Bacilli	Gram Negative Bacilli	Gram Positive Cocci In Cluster	Gram Negative Rods	Gram Negative Bacilli

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Table.2: Comparative colony characteristics of isolates on MacConkey's agar plate

Colony characteristics	Bay 1		Bay 2		Bay 3	
	Colony 1	**Colony 2**	**Colony 1**	**Colony 2**	**Colony 1**	**Colony 2**
Size	1mm	1mm	1 mm	1 mm	1 mm	1 mm
Shape	Circular	Circular	Circular	Circular	Circular	Circular
Colour	Pink (Mucoid)	Pink (Dry)	Pink (Dry)	Pink (Mucoid)	Pink	Coloueless
Elevation	Entire	Entire	Concave	Concave	Concave	Flat
Consistency	Smooth	Rough	Smooth	Smooth	Smooth	Smooth
Opacity	Translucent	Translucent	Opaque	Opaque	Opaque	Transparent
Margin	Entire	Entire	Entire	Entire	Entire	Entire
Gram Nature	Gram Negative Bacilli	Gram Negative Bacilli	Gram Negative Bacilli	Gram Negative Bacilli	Gram Negative Rods	Gram Negative Rods

The isolated colonies were further analysed for various biochemical tests to determine the possible genus of the isolate. Table 3 represents the comparative analysis of different biochemical tests performed to characterize the isolates from Nutrient agar and MacConkeys agar plates. Table 4 gives the presence of various microorganisms in activated sludge and they were identified on the basis of colony characteristics, Gram nature and biochemical tests.

Table. 3 : Biochemical tests for detection of microorganisms.

Biochemical test	1st Colony	2nd Colony	3rd Colony	4th Colony	5th Colony
Indole	+	-	-	-	-
Methyl Red	+	-	-	-	-
Voges-Proskauer	-	-	+	+	-
Citrate	-	-	+	+	-
Urease	-	-	+	+	-
TSI : BUTT **: SLANT** **: GAS** **: H_2S**	A A + -	- - - -	A A + -	ALK ALK - -	N ALK - -
Oxidase	-	-	+	+	+
Catalase	+	+	+	+	+
Possible Organism	***E. coli***	***S. aureus***	***Klebsiella***	***Pseudomonas***	***Alcaligenes***

Key: +: Positive test, -: Negative test, A: Acid production, A & G: Acid and Gas production.

Table.4 Different types of microorganisms present in activated sludge.

Protozoa and Rotifers	Bacteria	Fungi
Diatoms (Bacillariophycophyta)	*Pseudomonas spp*	*Aspergillus spp*
Paramecium spp	*Escherichia coli*	*Geotrichum spp*
Vorticella spp	*Staphylococcus aureus*	
Nematodes	*Klebsiella spp*	
Trypanosomes	*Alcaligenes spp*	
Motile algal filaments	*Sphaerotilus natans*	
	Beggiatoa spp	

Protozoa are important micro-organisms taking part to the ecosystem balance in wastewater treatment plants. A procedure for their semi-automated identification and counting based on image analysis is proposed. The main difficulty is the segmentation of the protozoa as most of them are in contact with the sludge. The protozoa are characterized by the size of their silhouette (area and length) and three shape factors (elongation, circularity and eccentricity). Table 5 represents the relationship between commonly occurring protozoans with the efficacy of treatment plant.

Table.5: Some relations between protozoa and plant efficiency

Predominant group Efficiency	Possible cause
Small flagellates very low	Bad oxygenation of the sludge, too high loading, presence of fermenting substances
Large swimming ciliates (> 50 µm) low	Too high loading
Crawling ciliates	Good condition
Crawling + attached ciliates	Good condition
Amoebae with shell	good Low loading, diluted mixed liquor, good nitrification

It was observed that if the oxygen supply in relation to the demand becomes inadequate, sphaerotilus and other filamentous organisms attain the ascendency and the sludge becomes bulking. Biochemical activities of these organisms would bring about the purification in a way similar to the desirable sludge organisms except with lower and more efficient oxygen utilization at lower tensions. In other words when the sludge is diffuse and filamentous, it exposes more surface which might enable the sludge to obtain the limited amount of oxygen present in the medium immediately surrounding it. This type of sludge however, usually produces a sparkling effluent.

Two major types of bacteria were observed during the 24 hours of aeration. They were dispersed, short, thick, round ended rods; approx size 2-2.5 microns x 1 micron. Some of these organisms appeared as finger like capsules. As the aeration period progressed there was an apparent increase in the no. of slime- enmeshed bacteria. *Sphaerotilus* like organisms often as unsheathed forms; these were more abundant after 6 hours of aeration and reached an apparent maximum after 24 hours.

Alcaligenes species which has heterotrophic nitrification and aerobic denitrification abilities was used to treat actual wastewater containing high-strength ammonium under aerobic conditions.

Aerobic bacteria live in colonial structures called floc and are kept in suspension by the mechanical action used to introduce oxygen into the wastewater. This mechanical action exposes the floc to the organic material while treatment takes place. Following digestion, a gravity clarifier separates and settles out the floc.

The total aerobic bacterial counts in standard activated sludge are in the order of 10^8 colony forming units/ mg of sludge. When culture based techniques are used, it was found that the major genera in the flocs are *Zooglea, Pseudomonas, Alcaligenes, Enterobacter, Bacillus species* etc. These microorganisms decompose the organic matter as a part of their metabolism. Biological degradation of organic wastes generates the simple sugars, inorganic salts like nitrates, phosphates, sulphates and carbon dioxide which is used by the growing organisms to enrich the sludge. Decomposed organic matter can then be used as biofertilizer.

Majority of filamentous organisms are bacteria, although some of them are classified as algae, fungi or other life forms. There are a number of types of filamentous bacteria which proliferate in the activated sludge process. Filamentous organisms perform several different roles in the process, some of which are beneficial and some of which are detrimental. When filamentous organisms are in low concentrations in the process, they serve to strengthen the floc particles. This effect reduces the amount of shearing in the mechanical action of the aeration tank and allows the floc particles to increase in size. Larger floc particles are more readily settled in a clarifier. Larger floc particles settling in the clarifier also tend to accumulate smaller particulates (surface adsorption) as they settle, producing an even higher quality effluent.

e.g. *Sphaerotilus* (sheathed bacteria), *Beggiatoa* (gliding bacteria) Moderately polluted water may carry cells or spores of three types; however it has fewer true aquatic fungi and aquatic hyphomycetes, soil fungi are more numerous. Heavily polluted water has large no. of soil fungi. The group - designated as soil fungi includes yeast like fungi, many species of which have been isolated from polluted waters.

The association between fungal densities and organic loading suggests that fungi may be useful indicators of pollution. Because fungi possess broad enzymatic capabilities, they can degrade actively most complex natural substances and certain synthetic compounds, including some pesticides.

Conclusion:

The waste water collected in CETP is heavily polluted as it contains effluents of many industries. The treatment of such water is a tedious job. The Activated sludge process or the Extended Aeration system employed in CETP gives reduction in COD. This is the result of presence of wide variety of microorganisms in activated sludge. Thus, after identifying different microorganisms such as bacteria, protozoa and rotifers, fungi and studying the role played by each microorganism in degradation of organic matter, it can be concluded that the treatment unit is working efficiently.

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