Investigation of Sludge Characteristics and Bacterial Diversity of Membrane Bioreactor Systems

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2010-NUST-MSPhD-EnvS-08

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By

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I dedicate this thesis to the best parents in the world

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List of Abbreviations

A/O-MBR	Oxic/anoxic MBR
AOB	Ammonium oxidizing bacteria
API	Analytical profile Index
CASP	Conventional activated sludge treatment plant
CFV	Cross flow volume
C-MBR	Conventional MBR
COD	Chemical oxygen demand
CT	Capillary tube
DNA	Deoxyribonucleic acid
DO	dissolved oxygen
EMB	Eosin methylene blue
EPS	Extracellular polymeric substances
FC	Pleated filter cartridge (FC)
FI	Filamentous index
FS	Flat sheet

HF	Hollow fiber
HRT	Hydraulic retention time
MB-MBR	Moving bed MBR
MBR	Membrane bioreactor
MF	Microfiltration
MLSS	Mixed liquor suspended solids
MT	(Multi) tubular
NOB	Nitrite oxidizing bacteria
PAO	Polyphosphate accumulating organisms
PCR	Polymerase chain reaction
RH	Relative hydrophobicity
SMBR	Submerged membrane bioreactor
SND	Simultaneous nitrification and denitrification
SRB	Sulfate reducing bacteria
SRT	Sludge retention time
SW	Spiral Wound
TN	Total Nitro

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Abstract

Bench-scale conventional membrane bioreactor (C-MBR), moving bed membrane bioreactor (MB-MBR) and anoxic/oxic membrane bioreactor (A/O-MBR) being operated under similar feed, environmental and operating conditions were evaluated for their bacterial diversity. Serial dilutions were performed followed by plating and colony count. The maximum number of colonies was found in A/O-MBR (8.3×10^8) followed by MB-MBR (1.5×10^8) and C-MBR (0.5×10^8) respectively. Streak plate technique was used to isolate phynotypically different bacteria and their morphologies were observed. Eleven bacteria were isolated from C-MBR and A/O-MBR while ten isolates were obtained from MB-MBR. Eight isolates were obtained from cake layer and eight isolates from the media in A/O-MBR and MB-MBR while four isolates were obtained from effluents of all three reactors leading to a total of 52 isolates. Reactors were majorly dominated by Gram negative bacteria (≥90%) therefore API 20E kit was used for identification of the bacteria. Activated sludge in all three reactors was dominated by *Enterobacteriaceae*, followed by *Pseudomonadaceae* family. *Psuedomonas aeruginosa* selected as representative of denitrifying bacteria was isolated from A/O-MBR only using citrimide followed by amplification on PCR. DNA of the sludge was isolated using DNA fast spin kit for detection of *N. europea* and *N. winogradskyi*. Through PCR detection of nitrifying bacteria *Nitrosomonas europaea* was done from MB-MBR only and *N. winograskyi* was detected in all three reactors.

Chapter 1

Introduction

1 Background

Wastewater contains nutrients, a number of pathogenic bacteria that dwell in human intestinal tract and other toxic compounds that pose various health and environmental impacts (Metcalf and Eddy 2003). Nitrogen containing wastewater has adverse impact on the water bodies and contribute to dissolved oxygen (DO) depletion, toxicity, eutrophication and methemo-globinemia (Lim et al., 2008). It is important to treat wastewater before it finds its way to fresh water bodies. The need for cleaner water is increasing day by day therefore the effluent limits go stringent and increase the need for an advance wastewater treatment system (Liang et al., 2010).

1.1 Conventional Wastewater Treatment

Activated sludge treatment is most widely applied for wastewater treatment. In activated sludge process soluble and insoluble organic contents are removed from wastewater by conversion into a flocculent microbial suspension that settles under gravity (Ramothokang, 2003). Three major steps involved in conventional activated sludge treatment are primary, secondary and tertiary treatment. In primary treatment the sewage is simply retained in a basin that results in settling of heavy solids leaving only light solids and oil to come on surface. The settled material is left behind in the basin while rest of the wastewater moves for secondary clarification. Dissolved and suspended biological matter is removed in secondary treatment and is sent for tertiary

treatment. In tertiary treatment sewage is further treated by physical or chemical disinfection. The water can then be discharged into the stream, used for ground water recharge or irrigation (Bhatti et al., 2009).

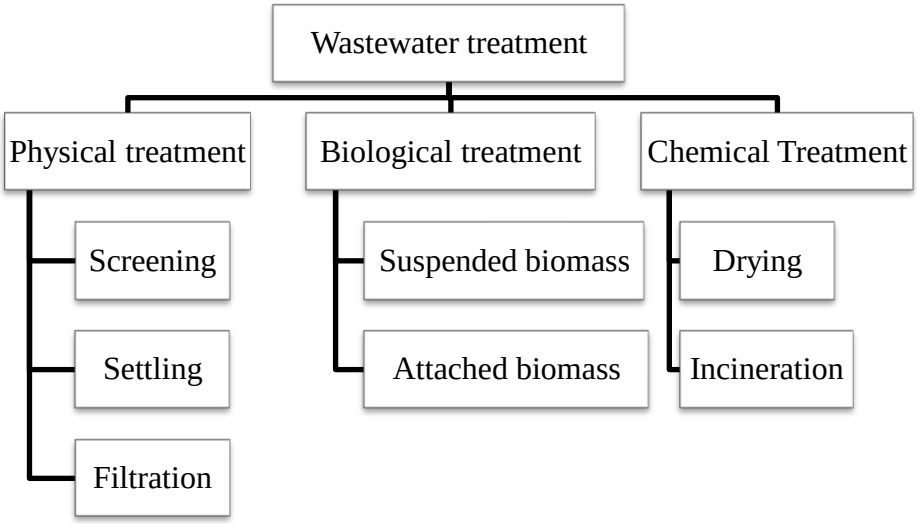


Figure 1.1 Wastewater treatment (adapted from Henze et al., 2008)

1.2 Biological Wastewater Treatment

Treatment system in which natural role of bacteria is utilized for bioconversion; the biological flocs and biofilms are used for degrading or adsorbing dissolved colloidal, settleable and particulate matter (Henze et al., 2008). Biological treatment processes include both aerobic and anaerobic systems. Aerobic biological wastewater treatment systems make use of mixed microbial consortia to transform organic and inorganic pollutants to harmless byproducts that can be released easily into the environment (Dias et al., 2003). Aerobic technologies are mostly implied for treatment of municipal and industrial wastewaters. But use of anaerobic systems has now increased because of its low construction, operation and maintenance cost.

However the biomass production is low and the effluent requires post treatment because of high COD along with nutrients and pathogens (Gašpariková et al., 2005).

1.3 Membrane Bioreactors

Modification of aerobic suspended growth system by addition of membrane module is known as a membrane bioreactor (MBR) (Hasar, 2009). Membrane bioreactor is becoming widely applicable for biological wastewater treatment (Duan et al., 2009) because of various advantages that include

- It can be operated to ensure simultaneous nitrification and denitrification and phosphorus removal by precipitation (Melin et al., 2006).
- Use of membrane eliminates need for secondary and tertiary treatment (Bhatti et al, 2009) resulting in smaller footprint.
- Operational conditions are more controlled as an independent sludge retention time (SRT) and hydraulic retention time (HRT) can be maintained. High sludge concentration allows better treatment of wastewater.
- Effluent is of high quality.

While the disadvantages include

- MBR is expensive to install and operate.
- Frequent monitoring and maintenance of membrane is required.
- Certain limitations are caused by temperature, pressure and pH to meet membrane tolerances and the sensitivity of membranes to some chemicals.

- Oxygen transfer may be less efficient because of high MLSS concentration and also if there is surplus sludge its treatability is doubtful (Melin et al., 2006).
- Membrane fouling reduces membrane filtration capacity by reducing filtration flux (Dias et al., 2003). Microbes responsible for treatment of wastewater are also responsible for biofouling of the membrane (Wagner and Loy, 2002).

The advancement in molecular techniques has enabled detection and reliable quantification of bacteria in wastewater (Silyn-Roberts and Lewis, 2001). Methods such as PCR-DGGE have been used for evaluation of bacterial diversity in MBRs but the knowledge of structure and diversity of bacterial community and its understanding in MBRs treating municipal waste water is not well understood (Duan et al., 2009).

1.4 Objectives

The aims of the study were to compare three membrane bioreactor setups for the microbial consortium present in the reactor and to relate it with the performance of the reactor so the following objectives were established:

- Isolation and characterization of microbes from three MBRs installed at Wastewater Laboratory IESE-SCEE (NUST).
- Isolation of *Pseudomonas aeruginosa* as representative of denitrifying microorganisms from the MBRs and detection of nitrifiers (*Nitrosomonas* and *Nitrobacter*) through PCR as representative of ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) respectively.
- Establish a relation between microbial communities and treatment performance of MBRs

Chapter 2

Literature Review

2.1 Membrane Bioreactor

Membrane bioreactor technology combines the activated sludge treatment with conventional activated sludge treatment where removal is achieved by filtration rather than gravity. MBRs are considered to be modification of conventional activated sludge process. MBRs now have various configurations and designs to ensure maximum filtration. It can filter the water by removing suspended matter, dissolved matter and all pathogens as shown in Figure 2.1

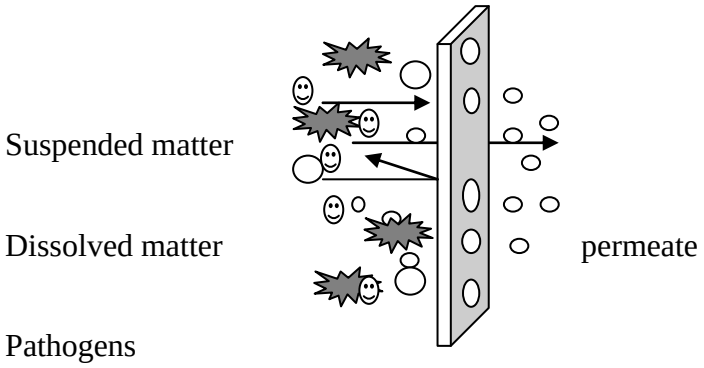


Figure 2.1 Membrane filtration

The first generation of MBR had cross flow operated membranes placed outside the treating tank (Fig 2.2b). Large amount of energy was required for high cross flow velocity maintenance to avoid cake layer formation. Submerged membranes (Fig 2.2a) were an important development of MBR that significantly reduced energy consumption as the pressure applied for permeate extraction was decreased. In submerged membrane bioreactor (SMBR) low pressure air diffusion is

used for both cleaning of membrane and supplying of air for biomass (Van der Roest et al., 2002). Therefore SMBRs are applied more for municipal wastewater treatment (Melin et al., 2006).

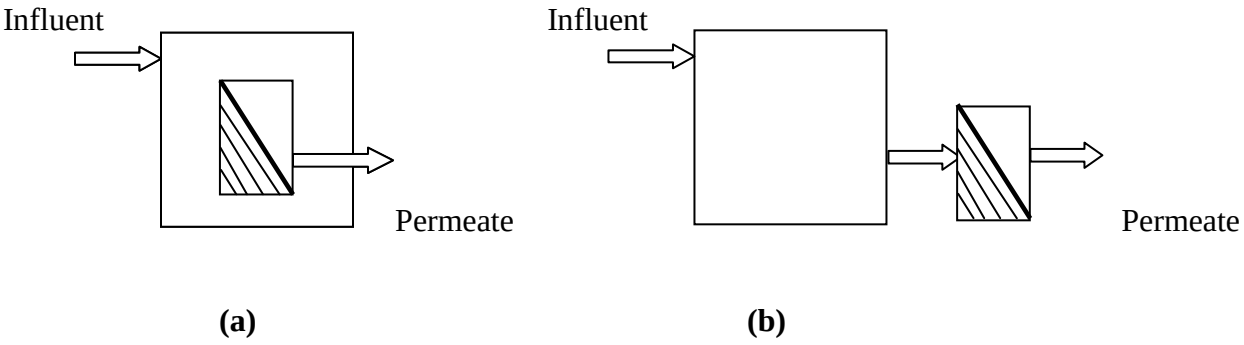


Figure 2.2 Schematic diagram for (a) Submerged membrane bioreactor (b) external membrane bioreactor

2.1.1 Membrane Filtration

MBRs depend on the ability of a membrane unit to pass all the flow coming to membrane so their configuration is very important. Six configurations currently employed for MBR are

- Plate and frame/ flat sheet (FS)
- Hollow fiber (HF)
- (Multi) tubular (MT)
- Capillary tube (CT)
- Pleated filter cartridge (FC)
- Spiral wound (SW)

FS, HF and MT are the most suited to MBR because they permit turbulence and regular cleaning (Judd, 2011). For better understanding their images are provided in Fig 2.3

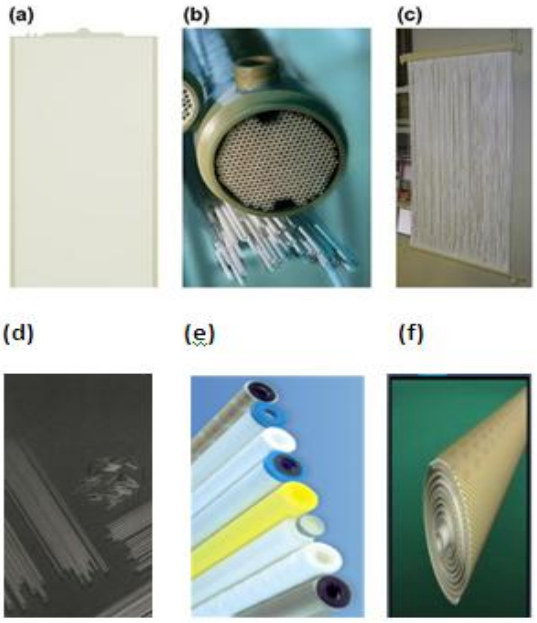


Figure 2.3 Membrane configurations a) FS b) MT c)HF d) CT e) FC f) SW

(Judd et al.,2011)

Filtration can be done at various levels in MBR and those include micro filtration, ultra filtration, nano filtration and reverse osmosis. The pore size for micro filtration ranges from 0.1 to 1 micron and it can achieve greater than 90% reduction in turbidity. Ultrafiltration has a reduced pore size of 0.001 to 0.1 micron. Turbidity can be sharply reduced to greater than 99%. Nano filtration separates salts and sugars from water and is thus used for water softening. Reverse osmosis can remove almost

everything from water lending in ultrapure for potable usage. It is however important to know that because of the smaller pore size, the operating pressure increases for microfiltration and utrafiltration membranes and the operational cost increases (Naveed et al., 2006). Usually the low pressure micro or ultra filtration is used with membrane being immersed in the aeration tank (Bhatti et al., 2009). Details of membrane filtration, their sizes and pathogen removal are given in Table 2.1

Table 2.1 Membrane filtration and pathogen removal

Membrane Filtration	**Size (µm)**	**Removal**
Micro filtration (MF)	0.1	Removes suspended or colloidal particles and can retain Bacteria
Ultra filtration (UF)	0.01	Removes organic macro molecules and has the ability to remove viruses
Nano Filtration (NF)	0.001	Can remove dissolved contaminants and renders water soft
Reverse Osmosis (RO)	0.0001	Designed to remove dissolved contaminants and remove almost everything from water

Adapted from (EPA, 2003)

2.1.2 Optimization of MBR Performance

MBR performance is studied in terms of nutrient removal in various MBR setups for example a moving bed membrane bioreactor (MB-MBR) and a conventional membrane bioreactor were evaluated for simultaneous carbon and nitrogen removal and compared for their performance. Operating at an SRT of 60

days, HRT of 12 hours and COD/TN ratio of 8.9–22.1, showed that membrane fouling behavior was more alarming in MBMBR and TN removal was better in MBMBR (65.3%) (Zhang et al., 2009).

Requirement of oxygen for the activity of AOB and NOB increases the energy required for wastewater treatment, therefore if nitrite is accumulated instead of nitrate the energy cost required for aeration of wastewater can be reduced. Nitrite accumulation increases the availability of carbon for denitrification (You and Chen, 2008). Various MBR designs are tried for treatment of wastewater and proper nitrification and denitrification. However researchers have brought in shortcut biological nitrogen removal (SBNR) concept where instead of converting nitrite to nitrate and then back to nitrite for denitrification the nitrite formed by AOB is subjected to denitrification with a challenge of ensuring nitrite accumulation (Zhang et al., 2009).

A number of processes have now been developed to improve wastewater treatment. Wastewaters having lesser C/N ratio and greater ammonium are now treated in two steps with the first being partial nitrification followed by anaerobic ammonium oxidation or annamox. Moles of oxygen required for partial nitrification is 0.75 moles as compared to 2 moles required for complete nitrification. Equation for partial nitrification (50%) is given here

$$NH_4^+ + 0.75O_2 \rightarrow 0.5NH_4^+ + 0.5NO_2^- + 0.5H_2O + H^+$$

Annamox converts nitrite to nitrogen gas using ammonium as electro donor. Alongwith nitrogen nitrate is also produced in this step. Annamox can be summarized using the following equation.

$$NH_4^+ + 1.32NO_2^- + 0.066HCO3^- + 0.13H^+ \rightarrow 1.02N2 + 0.26NO3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O$$

However pH, DO, substrate concentration and HRT have to be maintained to control production of nitrate (Feng et al., 2007).

2.2 Microorganisms in Wastewater Treatment

Wastewater is mostly discharged into streams without treatment thinking that the self-purification ability would take care of it. But most of the receiving bodies are already overcharged. Therefore effluent treatment must be done before discharge, so that the physico-chemical parameters of receiving water body are not harmed.

Major microorganisms present in wastewater are bacteria, protozoa, metazoa, algae and fungi but bacteria makeup (95%) most of all the wastewater microorganisms in activated sludge and have important role in wastewater treatment (Gerardi, 2006). Nutrient removal is done through two major processes

- Fixed film processes
- Suspended growth processes

The fixed films processes are based on ability of microorganisms to grow on surfaces because of availability of food, protection from high velocity currents and other

environmental conditions. Physical forces such as adhesion and adsorption etc might also be responsible for attached growth.

As the adsorbed microorganisms grow and reproduce, EPS is produced, a gel matrix layer is formed on the surface and this film is known as biofilm. Removal of wastewater nutrients in fixed film processes is only attained when the wastewater is brought in contact with biofilm. Simultaneous nitrification and denitrification (SND) occurs within flocs or inner zones of biofilm that allows heterotrophic denitrifiers to produce nitrogen gas (Yang et al., 2009).

In suspended growth the bacterial flocs are in continuous contact with wastewater. Bacteria, protozoa and metazoan dominate suspended growth processes (Curtis, 2003). Most of the bacteria are Gram negative hetrotrophic rod shaped in aerobic conditions including *Pseudomonas*, *Chromobacter*, *Achromobacter*, *Alcaligenes* and *Flavobacterium*. *Coliforms* are said to enter wastewater from influent and are not considered indigenous. Nitrifying bacteria as well as filamentous bacteria (*Beggiatoa, Thiothrix and Sphaerotilus*) are also present in wastewater and form biofilms.

Various kinds of bacteria play their role in treating wastewater and the important of them are filamentous bacteria, methanogenic bacteria, poly phosphate accumulating bacteria, sulfate reducing bacteria, nitrifying bacteria, denitrifying bacteria. A brief review of these microbes is as under

2.2.1 Nitrification

Ammonoum oxidising bacteria (AOB) and nitrite oxidizing bacteria (NOB) are obligatory chemolithoautotrophs and are responsible for causing nitrification in wastewater (Bothe et al., 2000). In the first step ammonia is oxidized to nitrite by ammonia oxidizing bacteria (AOB) while in the next step nitrite is oxidized to nitrate by nitrite oxidizing bacteria (NOB). Denitirification is the last step that involves conversion of nitrate to nitrous oxide or nitrogen gas (Geets et al., 2007). The process of nitrification is explained by reaction 2.1 that describes the reaction of AOB such as *Nitrosomonas* while reaction 2.2 describes role NOB such as *Nitrobacter* in nitrite oxidation (Andriany, 2007).

$$NH_4^+ + 1.5O \rightarrow NO^-_2 + 2H^+ + H_2O + \text{energy} \qquad (2.1)$$

$$NO^-_2 + 0.5O \rightarrow NO^-_3 + \text{energy} \qquad (2.2)$$

The whole process of nitrification can be summarized as

$$NH_4^+ + 2O_2 \rightarrow NO^-_3 + 2H^+ + H_2O + \text{energy}$$

In environments where the growth of nitrifying bacteria is hindered because of unfavorable environment, heterotrophic bacteria and fungi may also cause nitrification. This type of nitrification can take place when reactor is in its exponential phase (Lin et al., 2007).

Nitrifying bacteria are very slow growing and belong to autotrophic group of bacteria (Wagner and Loy, 2002). Traditional classification system defined AOB in five genera *Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosovibrio* and *Nitrosolobus* but advanced molecular techniques helped merge *Nitrosospira,*

Nitrosovibrio and *Nitrosolobus* in one common genus the *Nitrosospira.* All these microorganisms are categorized into two subclasses of *Proteobacteria* while *Nitrosococcus* species are categorized into either beta or gamma subclass of *Proteobacteria*.

AOB are very sensitive to various environmental factors and operational parameters such as pH, temperature, retention times and toxic chemicals etc therefore the oxidation of ammonia is the rate limiting factor for biological nitrification process (Bae et al., 2011). The genes contained in AOB responsible for oxidation of ammonia are ammonia monooxygenase (AMO) (Geets et al., 2007). Detailed description of enzyme involved, reactions and responsible gene is given in Table 2.2

Table 2.2 Enzymes of nitrification

Enzyme	**Reaction**	**Gene**	**Gene size (bp)**
Ammonia Monooxygenase	$NH_3+2[H]+O_2 \rightarrow NH_2OH+H_2O$	amoA	744-825
		amoB	1250-1262
		amoC	813-880
Hydroxylamine Oxidoreductase	$NH_2OH+H_2O \rightarrow HNO_2+4[H]$	Hao	1713

Source: (Bothe et al., 2000)

None of the bacteria discovered so far have the capacity to convert ammonia directly to nitrate. Some heterotrophic bacteria and fungi can also oxidise ammonia or

reduce nitrogen from organic compounds to hydroxyleamine, nitrite and nitrate (Bothe et al., 2000).

2.2.2 Denitrification

Denitrifying bacteria cause removal of nitrogen compounds from wastewater. Nitrate is first converted to nitrite by nitrate reductase while in the second step of denitrification, nitrite is reduced by nitrite reductase (Nir). Nitrite reductase is the key enzyme of the denitrification process. Nitrate and nitrite are converted via nitric oxide to nitrous oxide or dinitrogen. The reduction of nitrate to nitric oxide (NO) is catalyzed by copper nitrite reductase (Nirk) or cytochrome cd1 nitrite reductase (NirS) with nirS gene being more common than nirK (Harbi et al., 2010). Nitrate reductases, nitrite reductases, nitric oxide reductases and nitrous oxide reductase are the four reactions by which nitrate is reduced to dinitrogen. Two types of nitrite reductase are cupper nitrite reductase encoded by nirK gene and cytochrome cd1 nitrite reductase encoded by nirS gene (Henry et al., 2004).

Denitrifying bacteria are very diverse with most belonging to different subclasses of *Proteobacteria*, Gram positive bacteria and even *Archea* (Geets et al., 2007). Most denitrifying bacteria can cause complete denitrification. A number of microorganisms can cause denitrification in various environments and *Pseudomonas* is reported to dominate such kind of microorganisms. *Achromobacter, Agrobacterium, Alcaligenes, Bacillus, Chromobacterium, Flavobacterium* and *Hyphomicrobium* are also reported to cause denitrification (Lim et al., 2008). Some bacteria can denitrify aerobically as well (Hallin and Lindgren, 1999).

Hetrotrophic denitrifiers have been reported for their ability to simultaneously nitrify and denitrify. Autotrophic microorganisms are also said to have caused SND if grown under oxygen limitation (Bothe et al., 2000). Details of the enzymes involved and the reactions of denitrification are given in Table 2.3

Table 2.3 Enzymes of denitrification

Enzymes	**Reaction**	**Gene**	**Gene size (bp)**
NO_3^- reductase	$NO3^- + 2e^- + 2H^+ \rightarrow NO^-_2 + H_2O$	narG	3588-3788
		narH	1464-1677
		narJ	516-741
		narI	672-741
Heme NO^-_2 reductase	$NO^-_2 + 2e^- + 2H^+ \rightarrow NO + H_2O$	nirS	1665-1791
Cu NO^-_2 reductase	$NO^-_2 + 2e^- + 2H^+ \rightarrow NO + H_2O$	nirK	1092-1140
NO reductase	$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$	norB	1341-1542
		norC	441-453
N_2O reductase	$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O$	nosZ	1905-1959

Source: Bothe et al., 2000

2.2.3 Polyphosphate Bacteria

Polyphosphate or phosphorus accumulating bacteria (PAO) such as

Acinobacter, Aerobacter, Beggiatoa, Klebsiella and many others remove orthrophosphates from wastewater greater than the cellular needs (Gerardi, 2006). Enhanced biological phosphorus removal (EBPR) is achieved by enriching PAOs in a wastewater treatment system. Its application in wastewater treatment is important because it is economical and environment friendly (Gunther et al., 2009).

Higher biomass yield and relatively shorter retention time is required for phosphorus removal and biological P removal requires different operation conditions than C and N removal. MBR also facilitates phosphorus removal from wastewater. The increased phorsoprus removal in MBR is related to growth of PAOs in MBR. As PAOs can survive better in starvation condition and compete non P-accumulating microorganisms (Monclús et al., 2010).

2.2.4 Filamentous Bacteria

Filamentous bacteria have long been known for their ability to cause bulking and foaming. Growth of floc forming bacteria is important in CASP because they settle waste water organic under the force of gravity and thickened sludge return in activated sludge treatment system. However sludge bulking is detrimental to wastewater treatment.

Ramothokang et al. (2003) reported that various filamentous microorganisms are isolated as an indicator for

- Lower DO
- Low F/M ratio

- Presence of septic waste
- Nutrient deficiency and
- Low pH

Filamentous bulking can influence operation in MBR and should be minimized for optimal performance. Increase in filamentous index (FI) increase growth of ciliates→ vorticella → rotifiers → Epistylis → arcella and EPS and relative hdrophobicity (RH) increases. It was also observed that at nitrogen deficiency, COD removal also decreased and filamentous index increased (Zhang and Cao, 2010).

2.2.5 Methanogenic Bacteria

Methanogenic bacteria are strict anaerobes and chemolithotrophic autotrophs in nature. These bacteria utilize acetate, hydrogen and carbon dioxide to form methane. Breakdown of organic matter and accumulation of end products of acid producing bacteria and utilization of organic matter would not be possible without methanogens. These bacteria best operate at a pH 6.5-8 and are sensitive to acidic environment. Methanogens are called actoclastic methanogens and hydrogen utilizing methanogens based upon the food they uptake.

Methanosaeta and *Methanosarcina* are acetate utilizing bacteria while *Methanosarcina* can also utilize methanol, methyl amine and sometimes H and CO_2. Hydrogen utilizing bacteria also contribute in methane production up to as much as 30%. The hydrogen produced by hydrolytic bacteria and acid forming bacteria is utilized by methanogens to reduce carbon dioxide, formate, methanol and methylamines (Curtis, 2003).

Anaerobic membrane bioreactors were evaluated for their methanogenic activities. Membrane bioreactor operating at a lower temperature (15˚C) had decreased methanogenic activity as compared to the one operating at 25˚C. The sludge attached on membrane had reduced methanogenic and overall removal capability (HO and Sung, 2010).

2.2.6 Sulfate Reducing Bacteria

Sulfate is a component of urine and is found in municipal wastewater and may also be produced by oxidation of sulfide and mixed liquor aeration. Sulfate reducing bacteria (SRB) such as *Desulfovibrio* are anaerobic in nature and remain inactive or die in the presence of free molecular oxygen. These bacteria utilize sulfate to degrade a small amount of substrate. Utilization of sulfate also termed as sulfate reduction results is production of hydrogen sulfide or sulfide (Gerardi, 2010). However H_2S production by sulfate reduction is a major drawback of the process (Ben-Dov et al., 2007).

In a study intermittent aeration was used to indentify SRB species by PCR method. The membrane bioreactor had two compartments. Compartment one was anaerobic while the second compartment with MF membrane was maintained in aerobic conditions. Longest periods of non aeration modes in the second compartment showed highest level of biological sulfate removal. Physical filtration by membrane module and biological treatment leads to better sulfate removal or active SRB (Diwiranti-Hadiwinoto et al., 2004).

2.3 Observation of Bacterial Communities

A number studies exist for observation and monitoring of bacterial communities responsible for wastewater treatment in activated sludge and a few about membrane bioreactors, some of them are reported here.

In order to check the colonies related to fouling, slices were taken from different locations from the fouled membranes of a membrane bioreactor. DNA was extracted from the cake sample and PCR amplification was done. The gel pattern was analyzed. Specific nitrification rate (SNR) and specific oxygen uptake rate (SOUR) was recorded to evaluate bacterial activity (Huang et al., 2008).

Subramanian et al. (2010) isolated the mucoidal colonies from municipal wastewater sludge due to their significant role in sludge flocculation. Isolated strains and the overall consortium were grown in mineral medium in orbital shaker until the broth became viscous and were studied further by various staining techniques and flocculation. Likewise Jihong and Zhi-sheng (2007) isolated phenotypically different colonies from membrane bioreactor. Synthetic medium was inoculated with the isolated single and mixed strains. The COD decrease was plotted against time to check efficiency of COD degrading bacteria.

Activated sludge sample from food, paper, livestock, textile and sewage wastewater was analyzed. Genomic DNA extraction was done using ‘Fast DNA Spin Kit’. Universal primer set 9F and 1392R were used for nested PCR. Amplification was done using these set of primers. Fragments were re-amplified using 341F-GC and

518R set of primers. For AOB the PCR product of first step was used as template and amplified using CTO189f and CTU654R. To generate DGGE product a third amplification was performed using DGGE primers 341F-GC and 518R were used for PCR amplicon of AOB. AOB's abundance was found using Real time PCR. Results showed that the total bacterial 16S rRNA gene copy of both samples was not much different and similarly AOB population was almost similar in all samples. However number of amoA gene was greater in tannery, livestock and sewage wastewater as compared to food and paper (Kyunghwa et al., 2010).

In another study two MBR configurations i.e. external and immersed were characterized by different operating conditions and their effect was observed on microbial activity and sludge characteristics. COD was observed and biological activity was evaluated using respirometry test. And the results depicted that SMP release was greater in external MBR and so was heterotrophic bacterial development while immersed MBR allowed better autotrophic bacterial development. COD removal was also greater for immersed MBRs (Clouzot et al., 2011).

2.4 Membrane Fouling

Membrane fouling is the phenomenon in which the membrane pores are clogged. It can either be reversible or irreversible. Physical cleaning is required for reversible fouling and it can be eliminated by backwashing or simple surface cleaning. However chemical cleaning is required for irreversible fouling (Miyoshi, et al., 2009). In a submerged MBR, fouling control can also be approached by aeration (Andriany, 2007). The nature and extent of membrane fouling in MBR are strongly influenced by

operating conditions, and biomass characteristics. The operating conditions include organic loading rate (OLR), Hydraulic retention time (HRT), sludge retention time(SRT), cross flow volume(CFV) and aeration while biomass characteristics are explained using EPS, MLSS, floc size. Fouling is caused by many factors such as sludge particle deposition, adhesion of macromolecules to the membrane surface and pore clogging by small molecules, among which the cake layer formation by sludge particle deposition is the most common reason for the flux decline. Three mechanisms of cake formation include

- Polarization,
- Pore plugging and
- Pore narrowing

In polarization due to size exclusion a lot of matter accumulates on the membrane surface. Organic macromolecules i.e. EPS, some ions and small bacteria enters into the pores and causes fouling while small bacteria and soluble EPS enters the pore and builds up on the wall causing narrowing and therefore membrane fouling (Sombatsompop, 2007).

2.4.1 Control membrane fouling

In order to reduce membrane fouling regular cleaning of membrane is required. Membrane cleaning strategies vary with the composition of wastewater of a reactor.

The two main cleaning methods include:

- Mechanical Cleaning
- Chemical Cleaning

Mechanical cleaning involves physical removal of particles deposited on the membrane material while chemical cleaning involves the following methods

- Chemically enhanced back-flush (in-situ)
- Intensive cleaning outside MBR (ex-situ) (Li et al., 2008)

Isolation of autotrophic microorganisms is very time consuming (Hiorns et al., 1995). Traditional techniques to check microbial communities include light microscope observation and cultivation techniques (Wagner & Loy, 2002). Several methods are now available to check the abundance of nitrifying communities that include denaturing gradient gel electrophoresis (DGGE), fluorescence in situ hybridization (FISH) and immunofluorescence probing. While because of the taxonomic diversity of denitirfying bacteria instead of 16S rRNA based techniques the specified genes are targeted such as amoA, nirS and nirK etc (Geets et al., 2007).

Chapter 3

Methodology

3.1 Experimental Setup

Three lab scale MBRs 1) Conventional MBR (C-MBR), 2) Moving bed MBR (MB-MBR) and 3) Oxic/Anoxic MBR (A/O-MBR) having submerged hollow fiber microfiltration membrane module (Mitsubishi Rayon, Japan), 0.1µm pore size and 0.2 m^2 surface area were studied for their bacterial diversity. The acrylic reactors were divided into three compartments for C-MBR and MB-MBR with the membrane module submerged in middle compartment while A/O-MBR was divided into two compartments with mechanical mixer (Cole-Parmer, USA) placed in one compartment and membrane module in the other. Reactors were aerated using diffusers. MB-MBR and A/O-MBR unlike C-MBR had plastic (Kaldness) media in the reactor and C-MBR served as a control reactor to this study. Wastewater was fed into the reactor using gravity. Peristaltic pump(Master Flex, Cole-Parmer, USA) with two minutes filtration and two minutes relaxation time was used to draw the permeate. Synthetic wastewater was used as an influent for all reactors its composition is given in Table 3.1. The operating condition were kept similar for all three reactors and are listed in Table 3.2

Table 3.1 Composition of synthetic wastewater

Ingredients	**Formula**	**Quantity (g/l)**
Hydrated Glucose	$C_6H_{12}O_6.H_2O$	216.5625
Ammonium Chloride	NH_4Cl	80.25
Potassium Di-Hydrogen Phosphate	KH_2PO_4	23.03
Trace elements		
Calcium chloride	$CaCl_2$	2.1
Magnesium Sulphate	$MgSO_4.7H_2O$	2.1
Ferric Chloride	$FeCl_3$	0.63
Manganese chloride	$MnCl_2.4H_2O$	0.42
pH buffer	$NaHCO_3$	168

Table 3.2 Operating conditions of all MBRs

Parameters	**Condition**
Sludge retention time (SRT)	30 days
Hydraulic retention time (HRT)	8 hours
Organic loading rate (OLR)	1.5 $Kg/m^3/d$
Nitrogen loading rate (NLR)	0.15 $Kg/m^3/d$
Food to microorganism ratio (F/M)	0.2 ± 0.03
Power of hydrogen (pH)	6-7
Mixed liquor suspended solids (MLSS)	6-8 g/L

Schematic diagram for the reactor is given in Fig 3.1 and three reactors are shown separately in Fig 3.2

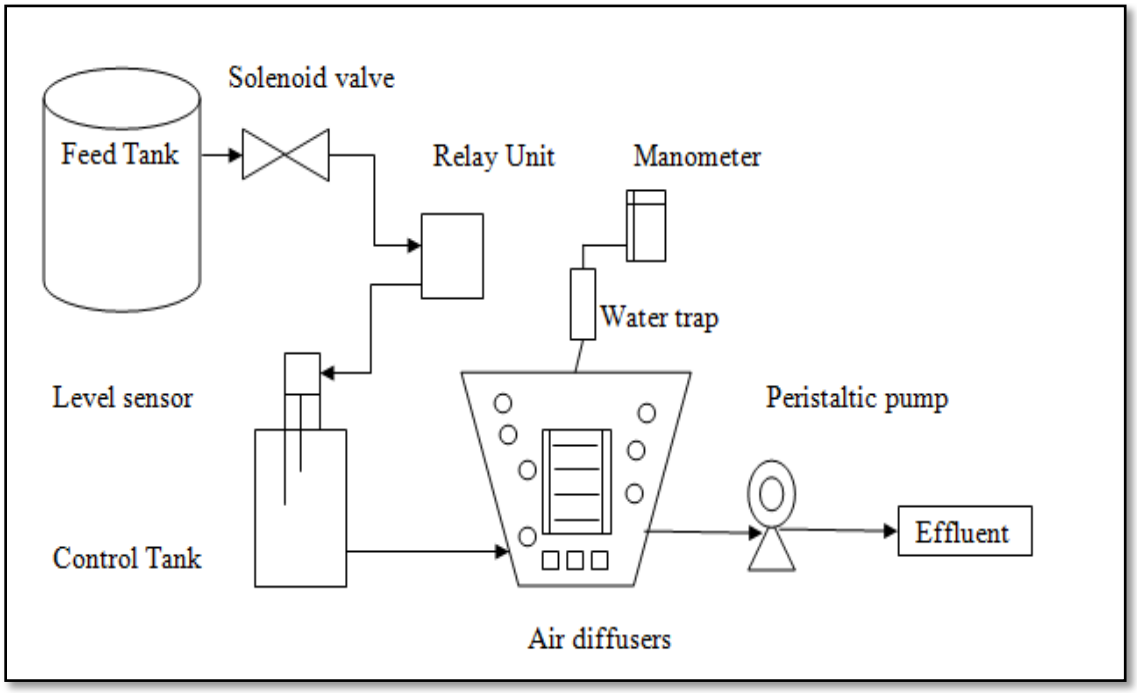
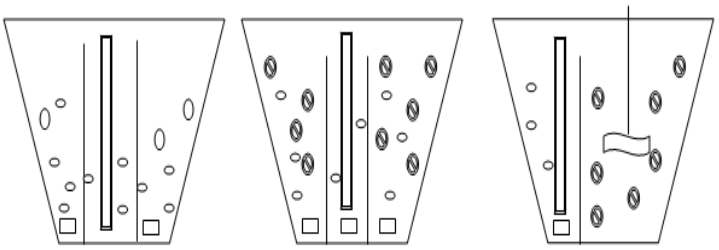


Figure 3.1 Schematic diagram of MBR setup



(a) (b) (c)

Figure 3.2 Three reactors a) C-MBR b) MB-MBR c) A/O-MBR

In the Microbiology teaching laboratory IESE, SCEE-NUST all glass, plastic ware and the media were autoclaved at 121° C and 15 psi for 15 minutes and oven dried before use.

3.2 Sample Selection

Sludge samples were collected from three membrane bioreactors installed at Wastewater Lab IESE, SCEE NUST. In order to have a true representation of overall consortium present in MBRs various sampling points were selected.

- Samples were collected from within the bioreactors.
- Samples were also collected from the effluents of the reactors to observe any bacteria escaping membranes.
- Samples were collected from the cake formed on membranes to observe microbes responsible for membrane fouling, lastly
- The slime layer formed on media of A/O-MBR and MB-MBR was also observed.

3.3 Sample Collection

Samples were collected from reactor and the effluents thrice in the beginning and three times during steady state condition and towards the end. Samples were collected from membrane when trans membrane pressure went beyond 50 Kpa. Cake sample was taken on 1st November and 22nd December from all three membranes of the reactors. While towards the end of our study a thin layer formation was observed on media so it was sonicated three times in autoclaved distilled and used for isolation.

3.4 Isolation of Bacterial Strains

Samples of reactors and cake layer were taken in sterilized flasks. Serial dilution technique was performed as per standard procedure. After preparation of dilution and mixing of the test tubes 0.1 ml of the sample was taken and plated onto nutrient agar and tryptone soy agar plates. Spread plate technique was performed to plate the sample and allowed to grow in the incubator for 24-48 hours at 37°C. Colony counting was done after 24 hours of incubation. The effluents were however plated on both nutrient agar and Eosin methylene blue agar. Media with biofilm formation was sonicated and the sample was plated on nutrient agar.

3.5 Purification of Bacteria

Maximum possible bacteria were marked on the basis of their morphological characteristics such as shape, size and color and isolated on fresh agar plates. Selected colonies were single colony streaked. Streak plate technique was performed to isolate the colonies. Plates were incubated for 24-48 hours at 37°C. Colonies were streaked for 3-5 rounds or more till assured of having obtained a pure colony. Each pure colony was stored in the refrigerator for further use.

3.6 Isolation of *Pseudomonas aeruginosa*

Pseudomonas Citrimide agar (PCA) was used for the isolation of desired microorganism from all reactors. PCA plates were plated with reactor samples upto 10^{-3} dilution. The process was repeated ten during the study.

3.7 Morphological Characterization

3.7.1 Colony Morphology

Single colonies were studied for their color, shape, size, margin, elevation, texture etc to observe the characteristics of the isolated strains (Annexure A)

3.7.2 Cell Morphology

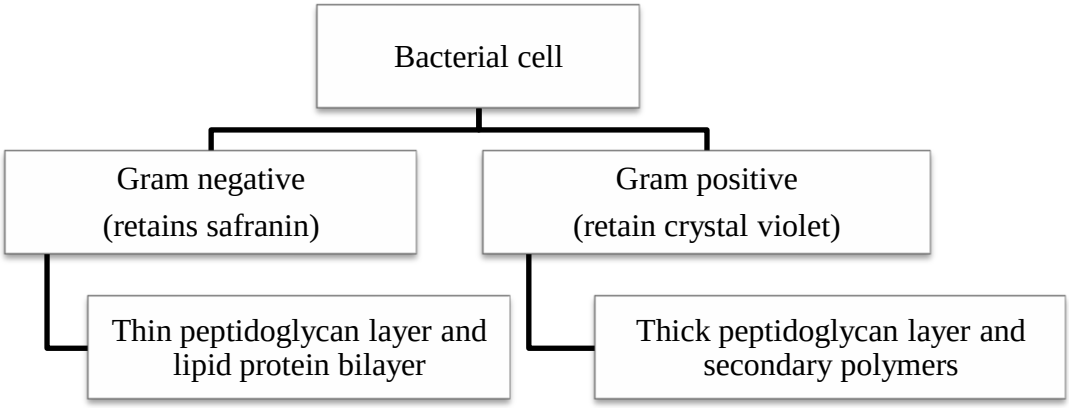


Figure 3.3 Bacterial cell staining

Gram staining was performed as per standard method for all the isolates of the wastewater sample.

3.8 Analytical Profile Index (API)

API 20E (Biomeurix, Canada) is a test kit used for the identification of enteric and other non-fastidious bacteria. It comprises of a plastic strip that has 20 mini cupules in it. Each cupule contains a specific medium for biochemical characterization. For performing the test a saline suspension (0.85% NaCl) was prepared and autoclaved. Saline suspensions were formed for fresh colonies; the suspensions were added in the cupules of API strips till the end except for citrate

utilization (CIT), voges–proskauer (VP) and gelatin liquefaction (GEL), where the cupule was filled completely. A drop of mineral oil was added in the cupules filled to neck to avoid drying out. The strip was covered with the lid provided and placed in incubator overnight. Color changes were noted and results was recorded (table 3.3). In the entire carbohydrates test, fermentation is shown by acid production and is indicated by yellow color. Few cupules have to be provided with reagents, supplied by the manufacturer. TDA reagent is added into TDA cupule. James/Kovacs reagent was added to IND while VP1 and VP2 were added to VP. The test was allowed to develop for a few minutes and results were recorded.

An additional oxidase test was performed to develop seven digit code required for API web software. In order to perform this test tryptic soy agar plates were prepared. Colonies were grown and 1% *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride was added. A color change to purple was noted as positive while no color change as negative result

Table 3.3 API result indicator color

Cupule Medium	Results	
	Positive	**Negative**
O-Nitrophenyle-B-D-galactoside (ONPG)	Light yellow to yellow	Colorless
Arginine Dehydrolase (ADH)	Light to dark red	Yellow
Lysine Decarboxylase (LDC)	Light to dark red	Yellow
OnthinineDecarbolyxase (ODC)	Light to dark red	Yellow
Citrate Utilization (CIT)	Blue green to Blue	Pale green to yellow
HydregenSulfide (H_2S)	Black	Grey to colorless
Tryptophan deaminase (TDA)	Deep red	Brown
Indole (IND)	Pink	Colorless/pale green/yellow
Voges–Proskauer (VP)	Red/Pink	Colorless/ slight pink
Gelatin liquefaction (GEL)	Goes Black (digested)	No change
Glucose (GLU)	Yellow	Yellow green, green, blue
Mannitol	Yellow	Yellow green, green, blue
Inositol	Yellow	Yellow green, green, blue
Sorbitol	Yellow	Yellow green, green, blue
Rhamnos	Yellow	Yellow green, green, blue
Sucrose	Yellow	Yellow green, green, blue
Melibiose	Yellow	Yellow green, green, blue
Amygdaline	Yellow	Yellow green, green, blue
Arabinose	Yellow	Yellow green, green, blue

3.9 DNA extraction from *Pseudomonas aeruginosa*

Both manual and kit methods were used and their description is as under

3.9.1 Phenol chloroform method

A simple method that does not require usage of any expensive enzyme for cell lysis was used. Piotr Chomczynski and Nicoletta Sacchi devised this method in 1987. For DNA extraction 10-12 agar slants were prepared and streaked with *Pseudomonas* specie isolated from A/O-MBR and allowed to grow in incubator at 37°C for 24 hours. The slants were then washed with 0.85% NaCl for preparation of suspension for DNA extraction. The following steps were followed.

1. Bacterial culture was centrifuged for 3 minute at 12,000 rpm.
2. Saturated culture was harvested and resuspended in 200 µl of lysis buffer.
3. 66 µl of 5M NaCl solution was added to remove protein and cell debris. It was centrifuged at 12,000 rpm for 10 min at 4°C, the tubes was inverted several times.
4. Supernatant was transferred to a new vial and equal amount of chloroform was added followed by mixing.
5. Centrifugation at 12,000 rpm for 3 min was done and supernatant was saved in a new vial.
6. DNA was precipitated with 100% ethanol, washed twice with 70% ethanol, dried and redissolved in 50µl 1xTE buffer

Gel documentation (Mupid one Advance and Cleaver scientific limited microdoc with UV trans-illuminator) and nano drop (Eppendorf biophotometer plus) results were used to check DNA extraction. The loading dye and sample was taken in a ratio of 1:5 and was inserted into the gel vials for gel documentation and ladder was also added followed by recording the observations in pictorial format.

3.9.2 Extraction using Kit

PrepEase kit (Affymetrix, Canada) and the provided protocol was used for followed for DNA extraction. Steps of DNA extraction are as under

1. Addition of 0.24 ml of homogenization buffer after preparation of bacterial suspension followed by vortex mixing.
2. 0.2 ml of chloroform/isoamyl alcohol and 0.8 ml of protein precipitation buffer was added.
3. Centrifugation was done at 13,000 rpm for 4 min and 0.88 ml of supernatant was transferred to new vial with 0.62 ml of isopropanol.
4. It was mixed followed by centrifugation at 13,000 rpm for 4 min. DNA precipitated out.
5. The pellet was washed with 70% ethanol followed by centrifugation at 12000 rpm for 2 min.
6. Supernatant was aspirated and DNA pellet was dried followed by addition of 50-300 µl of DNA resuspension buffer.
7. The vial was vortex mixed and stored at -4°C.

Results were noted using nano drop and gel documentation.

3.9.3 DNA Extraction for Detection of Nitrifying Bacteria

Soil DNA Isolation Kit (Norgen, Canada) was used for nitrifying bacteria. This kit is provided with the ability to remove all traces of humic acid content such as manure and is therefore best suited for isolation of DNA from soil and activated sludge samples.

Four major steps of DNA extraction are

1. Lysate preparation
2. Binding to column
3. Column wash
4. DNA elution

The details of the DNA extraction steps are discussed hereunder

Lysate Preparation

1. Wastewater Sample was transferred to eppendorf tube and centrifuged at 14000 rpm. Pellet was resuspeded in lysis buffer and added to bead tube.
2. Lysis additive was added and sample was centrifuged after vortexing briefly for a minute at 14000 rpm.
3. Binding solution was added to supernatant, mixed well and incubated for 5 minute.

4. It was then centrifuged at 14000 rpm and supernatant was transferred to new vials. 70% ethanol was added in equal volume and vortexed briefly.

Binding Column

1. 600µl of clear lysate was put into spin column combined with collection tube followed by centrifugation and the follow through of the collection tube was discarded.
2. The process was repeated depending upon lysate volume.

Column Wash

1. 500µl of wash solution-I was added in column and centrifuged and wash solution II was added.
2. It was centrifuged again followed by spinning to dry the resin.

DNA Elution

1. The spin column was placed in fresh eppendorf tube and 50µl elution buffer was added to it.
2. It was centrifuged at 2000 rpm for 2 minutes followed by 1 min centrifugation at 14000 rpm.
3. The eluted volume was stored at -20°C for further use.

3.10 Selection of Primer

Primers (Affymetrix, Canada) used for detection of selected species are listed in Table 3.4. PCR amplification was carried out in 25µl reaction mixture containing

1.25 unit of taq polymerase (BioBasic, Canada) with manufacturers reaction buffer and 25 Mm $MgSO_4^-$, 10µM of each primer (Table 3.8) and 2.5mM of dNTPs. The PCR mixture was placed in PCR (9600 TE Thermocycler, Taiwan) for amplification. It was run at an initial denaturation of 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 1 minute, annealing, elongation at 72°C and a final extension of 10 minutes at 72°C.

Primers selected for amplification of bacteria important in wastewater treatment were as under

Table 3.4 Primers selected for this study

Primer	**Sequence (5′-3′)**	**Target**	**Annealing temp (°C)**	**Product size (bp)**	**Reference**
PA-GS-F	GACGGGTGAGTAATGCCTA	*Pseudomonas* species	54	618	Spilker et al., 2004
PA-GS-R	CACTGGTGTTCCTTCCTATA				
Nwi70F	GGCGTAGCAATACGTCAG	*Nitrobacter winogradskyi*	55	59	Ludwig et al., 2004
Nwi165R	ATCCCGTATTAGCCCAAG				
amoA-1F	GGGGTTTCTACTGGTGGT	*Nitrosomonas europaea*,	61	481	Mintie et al., 2003
amoA-1FamoA-2R	CCCCTCTGCAAAGCCTTCTTC				

3.11 PCR Amplification

For amplification of DNA template a complete mixture was formulated that comprises of the following ingredients, purpose of each ingredient is presented in Table 3.5

Table 3.5 PCR ingredients and their purpose

Ingredients	Purpose
Taq DNA polymerase	Produces an enzyme DNA polymerase that amplified the DNA from primers by polymerase chain reaction
Mg ions (Magnesium Sulphate/chloride)	Serve as cofactor
Buffer	Provides optimal pH and salt conditions
dNTP	Follow standard base pairing rule
DNA template	Provides the DNA to be amplified
Primers	For each target sequence at the end of the DNA, both strands are copied simultaneously in both directions.

Steps vary for different microorganisms and enzymes of bacteria however a general range of a PCR cycle has been provided in table 3.6

Table 3.6 steps of a PCR reaction

Step	Temperature	Time (minutes)
Denaturation	92-95ºC	2:00-5:00
		00:30-1:00
Annealing	Varies	1:00-3:00
Elongation	72ºC	1:00-3:00
Final Extension	72ºC	7:00-10:00

After the reaction was completed gels were prepared for the amplicons and results were recorded in the form of a picture.

Chapter 4

Results and Discussion

4.1 Floc Characteristics

Activated sludge flocs are a mixture of living and dead bacterial cells and may include filamentous bacteria, precipitated salts and trapped organic fibers and inorganic particles. They are held together by polymeric compounds surrounding the cells and may be aided by chemical bonding force. Floc formation is also important because they can survive in nutrient poor environment and also protect themselves against protozoans (Eikleboom, 2000).

Flocs of C-MBR were round, compact and firm while for MB-MBR and A/O-MBR the flocs were irregular, open, and weak probably due to addition of media. The floc size was small for all three reactors as shown in table 4.1. This smaller size may be related to aeration in the reactors. Smaller floc size in a reactor may result in a higher mass transfer for C and N and result in better effluent quality (Abdessemed et al., 2009).

Table 4.1 Floc morphology of C-MBR, MB-MBR and A/O-MBR

Characteristics	**C-MBR**	**MB-MBR**	**A/O-MBR**
Shape	Round	Irregular	Irregular
Structure	Compact	Open	Open
Strength	Firm	Weak	Weak
Size	Small	Small	Small

Diffused aeration and relatively higher sludge loading results in irregular shaped flocs in a reactor and have reduced settling velocity (Eikleboom, 2000). In our study presence of media in A/O-MBR and MB-MBR along with aeration and high sludge loading rate may have added in formation of irregular flocs in the two reactors. Although it is important to control filamentous bacteria in MBR, if the flocs do not have significant filamentous bacteria they may lead so severe fouling as presence of filamentous bacteria leads to formation of a porous cake layer over the membrane that does not cause pore blocking (Meng et al., 2005) thus floc formation cannot always be taken in negative terms.

4.2 Colony Count and Cell Characteristics

The extent and nature of activity in a bioreactor is found out by taking into the account the total number of bacteria and types of bacteria present in it. The CFU/ml, found out by spread plate technique indicates that the bacterial activity was greatest in A/O-MBR followed by MB-MBR and then C-MBR. Thus it can be anticipated that

the performance of A/O-MBR would be greatest and least for C-MBR as bacteria utilize the wastewater nutrients as their food. The colony count for all reactors is given in Table 4.2

Table 4.2 CFU/ml count of the reactors

Reactor	**Number of bacteria**
C-MBR	5.1×10^7
MB-MBR	1.5×10^8
A/O-MBR	8.3×10^8

Eleven bacteria were isolated from C-MBR and A/O-MBR while ten isolates were obtained from MB-MBR. Each isolate was given a code according to the reactor like C-1, MB-1, A/O-1 and so on. Total eight isolates were obtained from cake layer and eight isolates from the media in A/O-MBR and MB-MBR while four isolates were obtained from effluent of all three reactors leading to a total 52 isolates. The results for cell morphology, colony morphology for all isolates and API of the Gram negative non filamentous isolates are given in appendix and discussed here.

The Gram staining result for all three reactors (Figure 4.1) showed that the Gram negative cocci were dominating all three reactors and rod shaped bacteria were very less in percentage. Addition of media has increased the percentage of Gram negative bacilli in MB-MBR and A/O MBR.

Researches show that activated sludge is dominated by Gram negative bacteria. Even if a homogenized sample is examined microscopically after Gram staining, it reveals major flora to be Gram negative (Eikleboom, 2000). Gram negative bacteria are phylogentically more diverse than Gram positive bacteria and this can be used as an explanation of their dominance in biological wastewater treatment in other environments (Seviour et al., 2000).

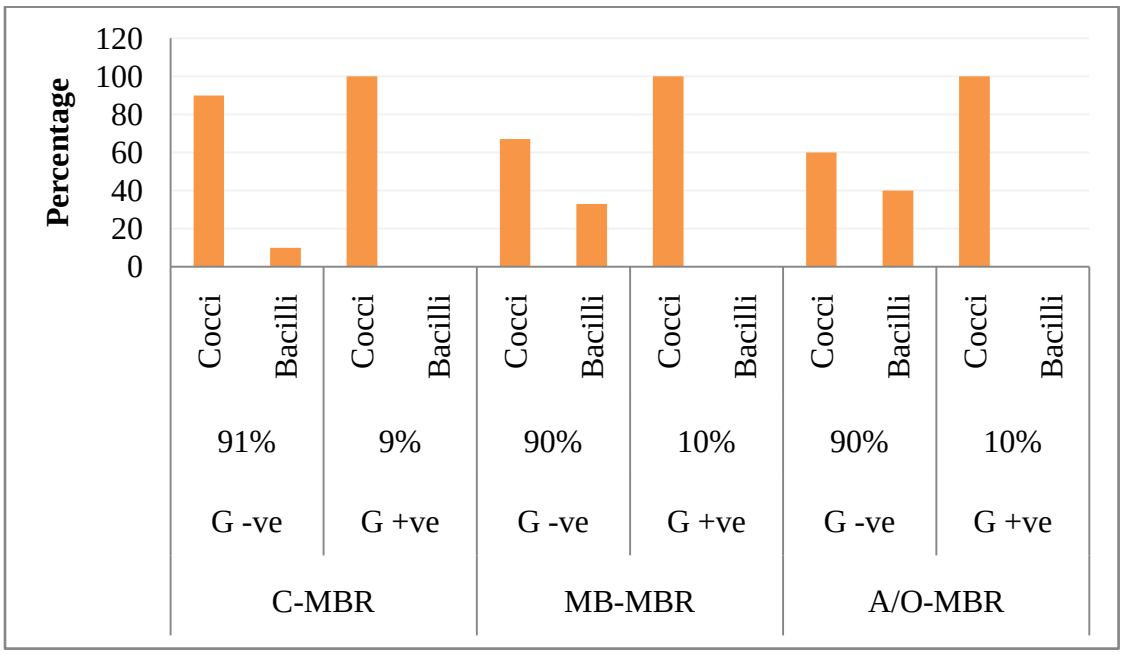


Figure 4.1 Gram staining for all the MBRs

Similar to the reactor isolates the isolates obtained from media present in all three reactors was dominated by Gram negative cocci with the percentages being 75% for A/O-MBR and 100% for MB-MBR as shown in Figure 4.2

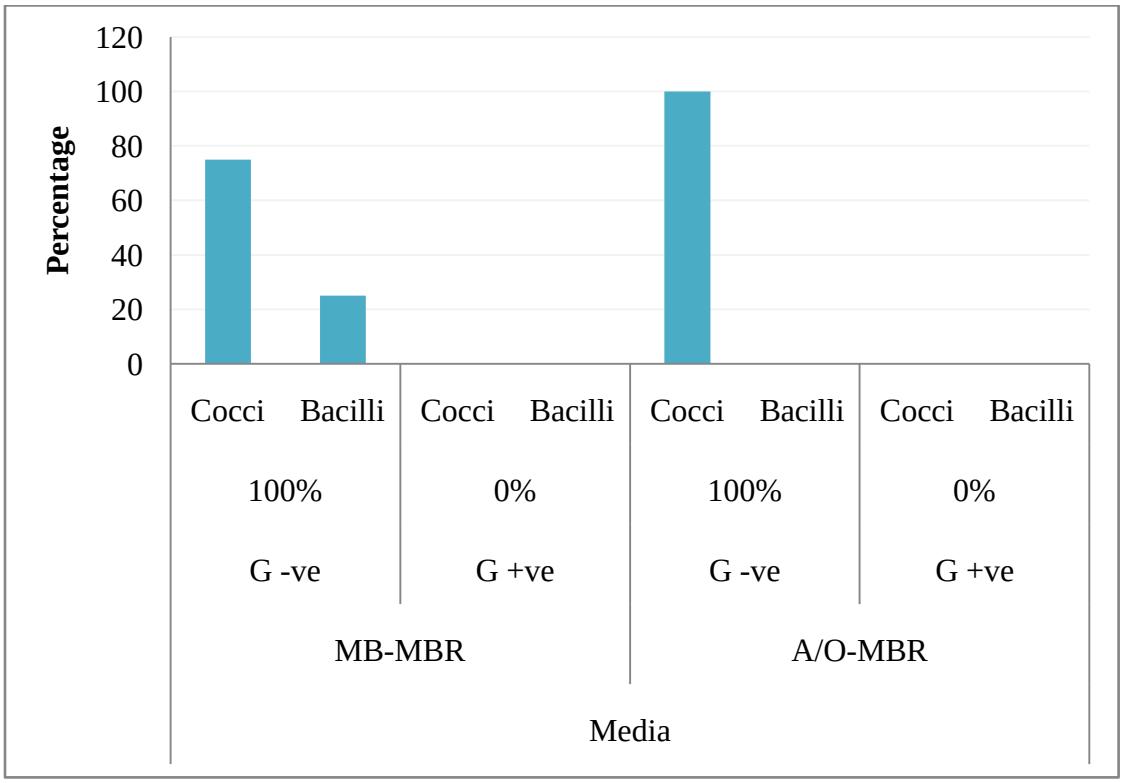


Figure 4.2 Gram staining for isolates obtained from kaldness media

Compared to Figure 4.1 and 4.2, the results for isolates of cake layer for all three reactors showed a different result. Not only did the number of Gram positive increase but rod shaped bacteria were also greater in number as shown in Figure 4.3

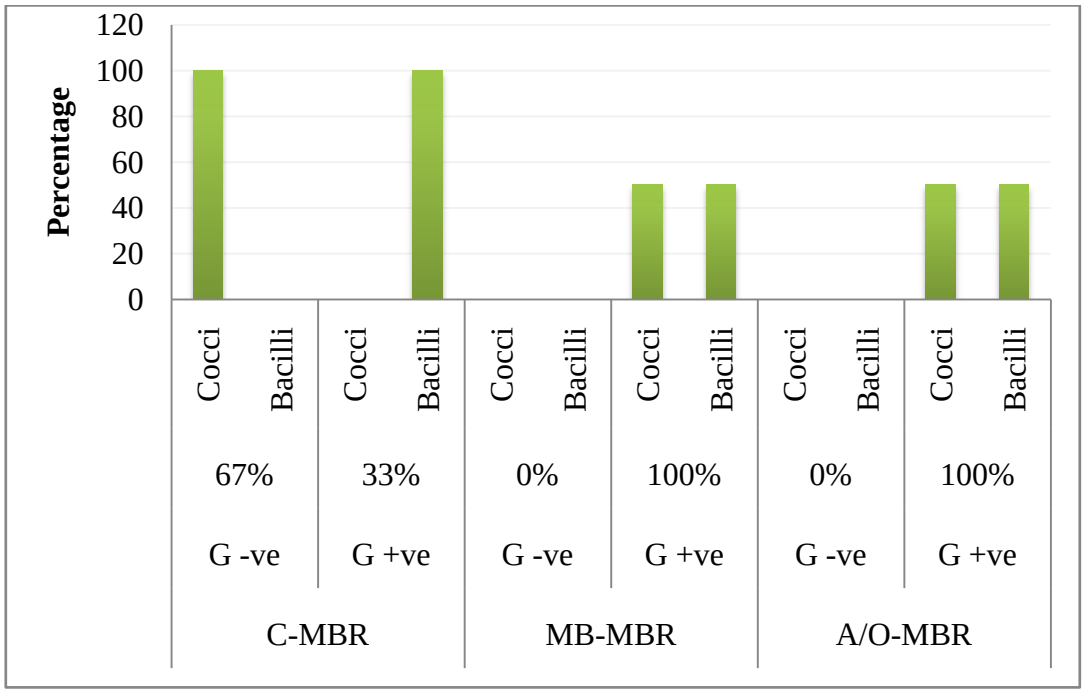


Figure 4.3 Gram staining for isolates of cake layer

The colony morphology was observed for isolates from the reactor, fouled membrane cake layer of all three reactors and the media of MB and A/O-MBR. All three reactors was majorly dominated by off white colonies as C-MBR had 63.6% off white colonies, MB-MBR had 80% off white colonies while A/O-MBR had 73% off white colonies. Circular shape and smooth colonies dominated all reactors. As far as texture is concerned the colonies that were creamy in nature and form a thread when they are picked with a loop may contribute to EPS formation (Sabramanian et al., 2008). The percentage of creamy colonies were found to be highest in MB-MBR (40%), followed by C-MBR (27.3%) and was lastly for A/O-MBR (18.1%) as shown in Figure 4.4. A comparison for the creamy colonies in all reactors, their cake layers and media is given in Figure 4.5

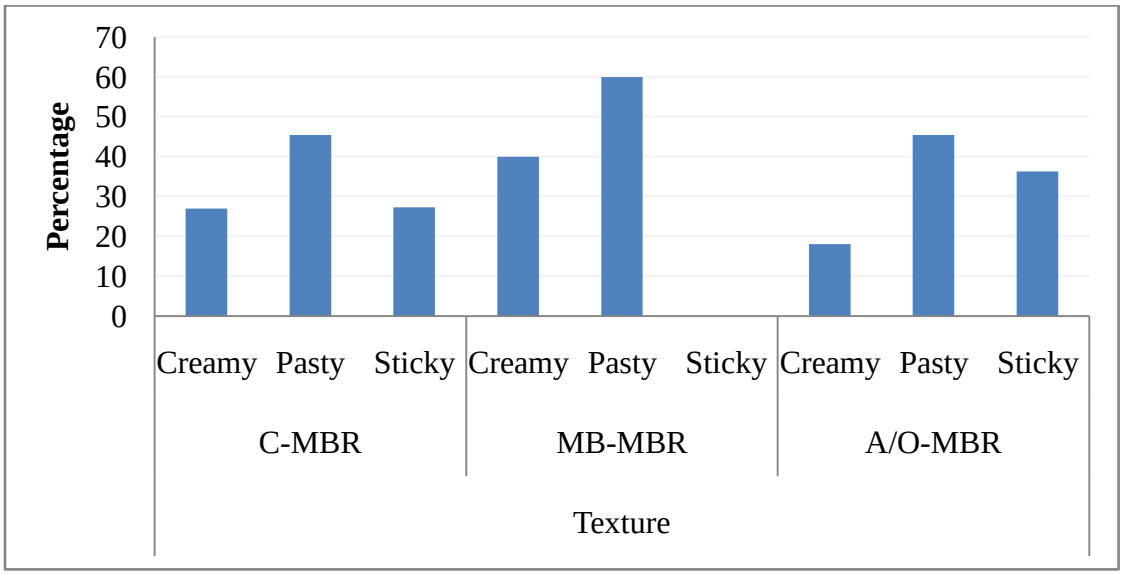


Figure 4.4 Texture of isolated colonies in all three reactors

More creamy colonies were found in the cake layer of all reactors than the reactor itself. Similarly the percentage of creamy colonies is also greater in media isolates, which infers that creamy colonies may also contribute biofilm formation.

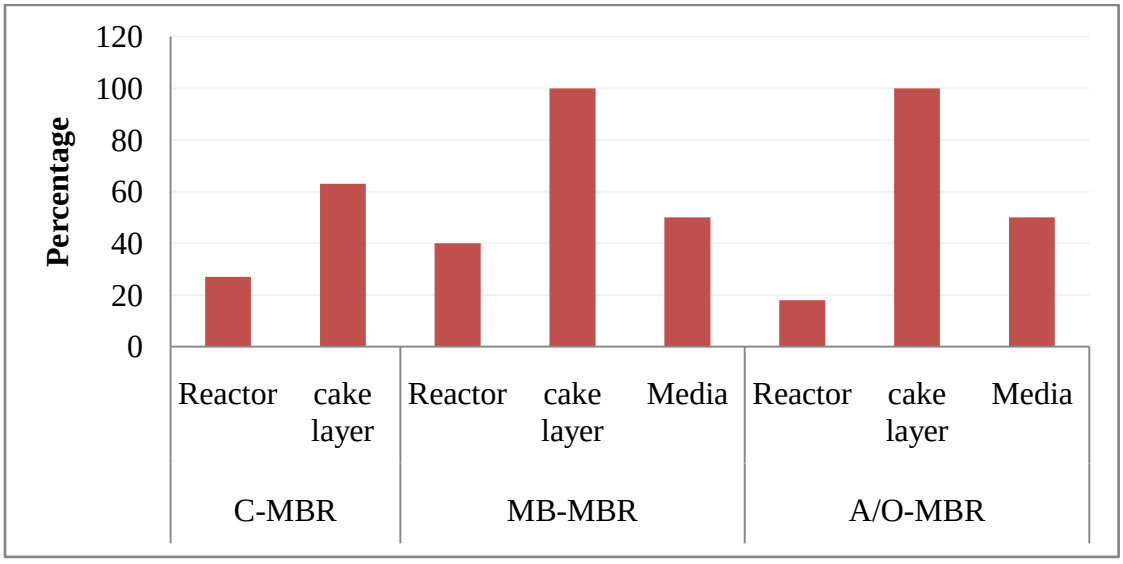


Figure 4.5 Creamy colonies in reactor, cake layer and media

4.2.1 Isolation of *Pesudomonas aeruginosa*

Pseudomonas aeruginosa was isolated from A/O-MBR only that gave yellow green colonies on Citrimide agar. The isolated colony was further identified by API 20E kit and the seven digit code generated also confirmed the isolate to be *Pseudomonas aeruginosa*.

4.3 Analytical Profile Index (API) 20E Identification

Normally Bergey's manual is used for identification of activated sludge however various commercial products are also being applied for identification. Juang and Morgan (2001) reported that API 20E system can be applied for identification of dominating microorganisms i.e. Gram negative bacteria. API web software used confirmed many isolates. The isolates of the effluent dominated by Gram negative were identified as *Enterobacteriaceae*. *Enterobacteriaceae* includes various pathogenic bacteria such as *E. coli, Salmonella, Shigella, Yersina* along with many harmless symbiotic bacteria (Williams et al., 2010). The isolates of the effluent may have entered the MBR effluent after it passes through the membrane and were not pathogenic in nature.

API identification in C-MBR, MB-MBR and A/O-MBR is given in Table 4.3

Table 4.3 Isolates identified by API from all three reactors

C-MBR	**MB-MBR**	**A/O-MBR**
Pseudomonas fluorescens/putida	*Klebsiella pneumonia ssp pneumoniae*	*Burkholderia cepacia*
Pseudomonas oryzihabitans	*Grimontia hollisae*	*Pseudomonas aeruginosa*
Klebsiella oxytoca	*Pseudomonas oryzihabitans*	*Pseudomonas fluorescens/putida*
Pantoea spp	*Pasteurella pneumotropica*	*Yersinia ruckeri*(possibility)
Klebsiella Pneumonia ssp ozaenae	*Klebsiella oxytoca*	*Raoultella terrigena*
Erwinia spp (possibility)	*Vibrio fluvialis*(possibility)	*Klebsiella oxytoca*
		Bordetella/Alcaligenes/Moraxella spp
Bordetella/Alcaligenes/Moraxella spp	*Mannheimia haemolytica*	*Myroidesspp/Chryseobacterium indologenes*

Pseudomonas, a major denitrifying bacteria, are known for their diversity and their growth in all kinds of environment (Peix et al., 2009). From the membrane bioreactors almost all kinds of *Pseudomonas* species were isolated that include

Pseudomonas fluorescens/putida, Pseudomonas oryzihabitans and *Pseudomonas aeruginosa* with the first two isolated from all reactors while last from A/O-MBR only. This also implies that *Pseudomonas fluorescens/putida* and *Pseudomonas oryzihabitans* can grow at higher DO as compared to *Pseudomonas aeruginosa*.

Biosorption of heavy metals by microorganisms is considered as a positive option for recovery of heavy metals from wastewater (Hussein et al., 2004). *Pseudomonas fluorescens* is one of the soil dwelling microorganisms. Presence of *Pseudomonas fluorescens* in soil helps soil remediation by adsorbing toxic metals and other xenobiotics (Wasi et al., 2010). *Pseudomonas putida* too is known as a plant growth promoting bacteria and dwells in rhizosphere of the plant. Its role in degrading benzene, toluene, ethylbenzene, and xylenes was evaluated and better contribution was observed in terms of plant growth and bioremediation (Lee et al., 2011).

Another bacterium that was isolated from three MBRs was *Klebsiella oxytoca.* It is a nitrifying bacterium and has been studied for its ability to oxidize nitrite (Abd-al-haleem et al., 2007). *Pantoea* spp has been isolated as EPS producing bacteria and can therefore contribute to fouling in C-MBR **(**Subramanian et al., 2010**).** *Klebsiella pneumonia ssp ozaenae* and *pneumonia* have been isolated from paper and pulp wastewater and are studied for their ability to fix nitrogen for wastewater treatment and are found in aerated stabilized basins of paper and pulp wastewater (Bowers et al., 2008). *Klebsiella pneumonia* is the clinically most important specie of genus *klebsiella* (Postgate, 1998). It has been found out that *Klebsiella pneumonia* is capable of bioflocculant production that results in formation of flocs. Bioflocculation is important

in terms of solid liquid separation in wastewater (Nie et al., 2011). Pentachlorophenol (PCP) is recalcitrant organic compound that usually pass untreated from municipal wastewater treatment plant. *Bordetella/Alcaligenes/Moraxella* spp have been studied for their ability to treat PCP and are found to dominate in their treatment (Khong et al., 2004).A study confirmed that *Pasteurella* along with many *Psuedomonas* species are responsible for denitrification (Drysdale et al., 2001). *Raoultella terrigena* biodegrades lipid and has been studied for its better efficiency (Raud et al., 2010)**.** *Bukholderia cepacia* has been studied for its ability to degrade 6-chlorovanillin (Yeber et al., 2000).

Many other bacteria such as *G.hollisae*. *P.oryzihabitans*, *M.haemolytica* etc have been reported for their pathogenic nature only. However the isolates of the effluent were not identified as pathogens. It is because the membrane pore size of 0.1µm enables an MBR to retain almost all kinds of bacteria and render the water harmless for non potable purpose.

4.4 DNA Extraction

For genomic DNA extraction of *Pseudomonas* both kit and manual method were used. Results were recorded as pictures using UV illuminator and as ng/µl readings from nanodrop. It was observed that although lesser DNA concentration is achieved from Kit extraction but it was free from all debris. Because of lesser debris, DNA did not degrade early and amplification was achieved easily. The gel picture saved for manual DNA extraction is given in Figure 4.6

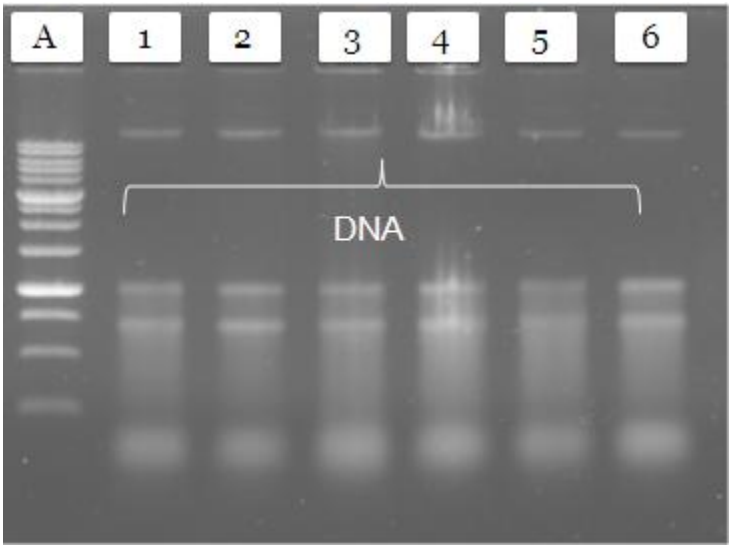


Figure 4.6 Gel picture for DNA extraction by phenol chloroform method a)1kb ladder followed by DNA extraction samples

The pictures for kit extraction showed a very light line in the section where genomic DNA rests (around 10,000 bp). This was furthur confirmed by nano drop readgins where the DNA concentration reached to a maximum of 79 ng/µl as compared to 113 ng/µl achieved by manual method. This is because nanodrop counts any DNA present in sample. Similarly the DNA extraction followed for DNA fast spin kit was evaluated

4.5 PCR Amplification

Various PCR mixtures combination and concentration as well as temperature variations were used for amplifying nitrifying bacteria. The final concentration and quantity that proved to be helpful is given in Table 4.4

Table 4.4 PCR mixture composition used for amplification

Reagent	Final Concentration	Quantity (µl)
10x Taq reaction buffer	1x	2.5
Magnesium Sulphate	2.5mM	2.5
dNTP	200µM	2
Primer, Forward	2µM	1
Primer, Reverse	2µM	1
Taq DNA polymerase	1U	0.2
Template DNA	> 10ng/µl	3
PCR Water		12.8
Total Volume		25

The PCR mixture is subject to PCR thermocycler conditions and amplification can only be achieved at a specific condition. The amplification conditions for all three bacteria studied is discussed hereafter.

4.6 *Nitrosomonas europaea*

The DNA spin kit extraction of all three reactor samples taken at the same time were subjected to PCR. *Nitrosomonas* was detected from MB-MBR only that has

more aeration and media to support biofilm formation. The thermocylcer PCR condition for *Nitrosomonas* and *Nitrobacter* is given in Table 4.5

Table 4.5 PCR thermocycler condition for *Nitrosomonas* and *Nitrobacter*

Steps	**Temperature**	**Time (minutes)**
Denaturation	95ºC	5:00
		1:00
Annealing	59.5ºC (*Nitrosomonas)* 57ºC *(Nitrobacter)*	1:00
Elongation	72ºC	1:00
Final Extension	72ºC	10:00

N=35

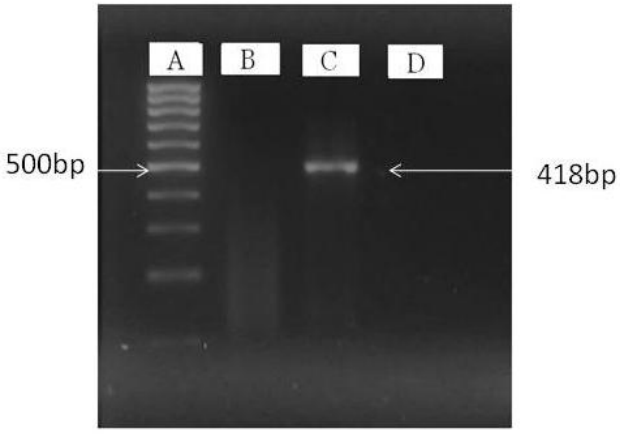


Figure 4.7 Agarose gel picture of *N.europaea*, detection from three MBRs a) lane 1 shows 100bp ladder followed by PCR amplicon of three reactors b) C-MBR, c) MB-MBR, d) A/O- MBR

4.7 *Nitrobacter winogradskyi*

The agarose gel picture of *Nitrobacter winogradskyi* is shown in fig 4.8

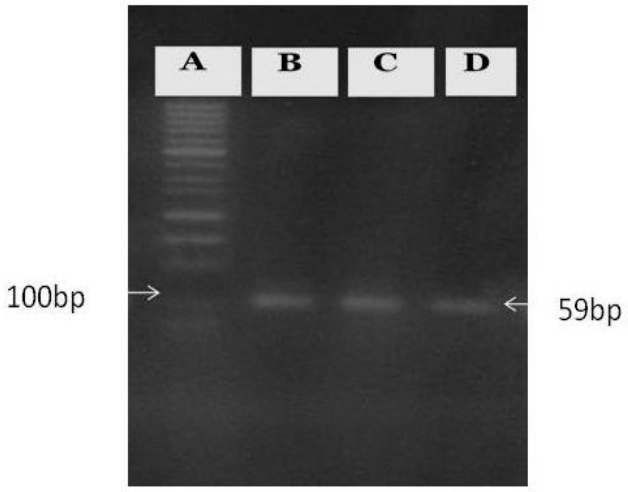


Figure 4.8 Agarose gel picture for detection of *N. winogradskyi* from three MBRs a) lane 1 shows 50 bp ladder, followed by PCR amplicon of three reactors b) C-MBR, c) MB-MBR, d) A/O- MBR.

4.8 *Pseudomonas aeruginosa*

Table 4.6 Thermocycler PCR conditions for Pseudomonas aeruginosa

Step	**Temperature**	**Time (minutes)**
Denaturation	95ºC	10:00
		00:30
Annealing	51-59 ºC	00:30
Elongation	72ºC	1:00
Final Extension		10:00

n=40

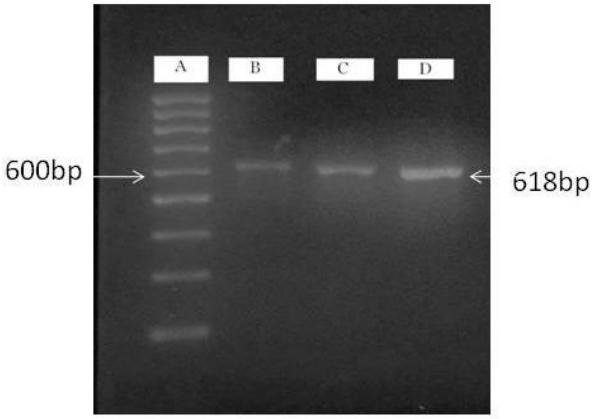


Figure 4.9 Agarose gel picture of *P.aeruginosa* amplification a) lane 1 represents100bp ladder followed by its amplification at three temperatures b) 51ºC, c) 57ºC, d) 59ºC

The API kit could only be used for identification of bacteria that are not fastidious. However bacteria such as *N.europaea* and *N.winogradskyi* are difficult to isolate. Through PCR detection of *Nitrosomonas* from MB-MBR only suggested that provision of media along with aeration helps simultaneous nitrification and denitrification. While *Nitrobacter* was detected in all three reactors which means that nitrite oxidizing bacteria are relatively easy to grow as compared to *Nitrosomonas* in MBR. The isolation of *P.aeruginosa* from A/O-MBR only is indication of the fact that lesser DO is required for better nitrification in MBRs. The API kit also identified many *Psuedomonas* species from all three reactors which means that intermittent aeration does allow growth of species that cause denitrification even if the overall DO is relatively higher. The general consortium in all three reactors was relatively similar because the food was similar. It is only because of addition of media in MB-MBR that better nitrification had been achieved and because of mechanical mixing in one compartment that better denitrification was achieved in A/O-MBR and their performances were improved as compared to C-MBR.

Chapter 5

Conclusions and Recommendations

This study investigated bacterial isolates obtained from three reactors (C-MBR, MB-MBR and A/O-MBR), their effluents, the cake layer formed during membrane fouling and the thin layers formed on media. Most of the isolates obtained from streak plate technique had similar characteristics however treatment performance varied for reactors because of their configuration that allowed growth of bacteria important for wastewater treatment. An additional advanced PCR study was conducted to verify their uniformity in bacterial consortium. *N. europaea* was detected from MB-MBR only. *N. winograskyi* was found in all three reactors. *P.aeruginosa* was isolated from A/O-MBR only. The bacterial activity dominated in A/O-MBR where the colony count always remained greatest.

5.1 Conclusions

Following conclusions were drawn from this study:

1. Wastewater treatment microorganisms majorly comprise of Gram negative bacteria. The API results showed that activated sludge in all three reactors was dominated by *Enterobacteriaceae*, followed by *Pseudomonadaceae* family.
2. Creamy colonies, an indicator to EPS formation were dominated in MB-MBR and may result in frequent membrane fouling.
3. The general consortium identified by API did not show a great variation however when individual isolate were studied through PCR it was revealed

that change in setup of an MBR can support a complete different group of microorganisms. More aeration and media supports nitrification while lesser DO in A/O-MBR supported denitrification.

4. *N. europaea* was detected in MB-MBR only as it had both enough aeration and media for simultaneous nitrification and denitrification (SND) reaction.
5. Better denitrification was observed in A/O-MBR and *Psuedomonas aeruginosa* was isolated from A/O-MBR only.
6. *N. winograskyi* was detected in all three reactors showing that *Nitrosomonas* is more fastidious than *Nitrobacter*.
7. Greater colony count and denitrification proved A/O-MBR to be better option as compared to other two reactors.

5.2 Recommendations

Following recommendations are noteworthy for further study

1. Isolation of *Nitrosomonas* and *Nitrobacter* using enrichment culture technique
2. Bacterial amplification and then identification through sequencing particularly for filamentous microorganisms
3. Phase contrast microscopy for identification of filamentous microorganisms directly from sludge.

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Annexure A

Colony morphology and solution preparation

Colony Morphology

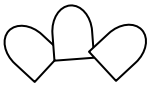
a. Shape

Circular 

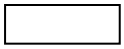
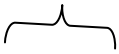
Irregular 

b. Margin

Smooth  , Dentate  , Rhizoidal  , Undulate 

Lobate 

c. Elevation

Flat , Raised  , Umborate  , Convex 

Concave  , Doom 

d. Texture

Creamy: That forms a thread with loop

Sticky: pellicle forming

Pasty: non thread forming

Solutions used for DNA extraction in phenol chloroform method

1) **1x TBE**

    1) 10.8g of Tris Base
    2) 5.5g of Boric acid
    3) 0.93g of EDTA

    It was dissolved in 1L distilled water.

2) **Lysis Buffer**

    1) 1% SDS
    2) 1mM EDTA, 40mM Tris Acetate (1.19g/100ml)
    3) 20mM sodium acetate

Mixed all three solutions and maintained a pH 7-8.

Calculations:

Tris

40mM = 11.9g/l

160mM = (11.9*160)/40

= 47.6 g/l

For 100 ml = 47.6/10 = 4.76/100ml

$M_1V_1 = M_2V_2$

V_1 = (40*30)/160 = 7.5ml

5% SDS

5% x V_1 = 10*30 = 6ml

For 40 mM V_1 = (20*30)/40 = 15ml

Sodium Acetate

1M = 136.08g

1mM = 136.08 *40* 10^{-3}

= 5.4432g

= 0.54432g/100ml

3) TE Buffer

1) Tris base 121g/L
2) 0.25M EDTA 93.05g/L

Dissolved these solutions in 990ml distilled water

4) 5M NaCl

14.61g dissolved into 50ml water

5) 0.85% NaCl

Annexure B

Results

Table 1B Bacterial Isolates obtained from C-MBR

Bacterial Isolates
C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9,C-10, C-11

Table 2B Bacterial Isolates obtained from MB-MBR

Bacterial Isolates
MB-1. MB-2, MB-3, MB-4, MB-5, MB-6 , MB-7, MB-8, MB-9, MB-10, MB-11

Table 3B Bacterial isolates obtained from A/O-MBR

Bacterial Isolates
A/O-1, A/O-2, A/O-3, A/O-4, A/O-5, A/O-6, A/O-7, A/O-8, A/O-9, A/O-10, A/O-11,A/O-12

Table 4B Bacterial isolates obtained from cake layer

S.no	Bacterial Isolate
1	MF-C-1, MF-C-2, MF-C-3
2	MF-MB-1, MF-MB-2
3	MF-A/O-1, MF-A/O-2, MF-A/O-3

Table 5B Bacterial isolates obtained from membrane sludge

S.no	Bacterial Isolate
1	EC-1
2	EMB-1
3	EA/O-1, EA/O-2

Table 6B Isolates obtained from media

S.no	Bacterial Isolate
1	MB-a. MB-b. MB-c, MB-d
2	A/O-a. A/O-b, A/O-c, A/O-d

Table 7B Colony morphology of bacterial isolates obtained from C-MBR

Isolate	Shape	Size (cm)	Margin	Elevation	Texture	Color	
						Naked eye	Microscope
C-1	Irregular	0.2	Undulate	Umborate	Pasty	Luminescent yellow	Dark golden brown
C-2	Irregular	0.1-0.3	Lobate	Concave	Sticky	Dirty off-white	Dark golden brown
C-3	Circular	0.2-0.4	Smooth	Raised	Pasty	Off-white	Soil brown
C-4	Irregular	0.1-0.2	Lobate	Concave	Sticky	Dirty off-white	Dark golden brown
C-5	Irregular	0.1-0.2	Undulate	Umborate	Sticky	Yellowish off-white	Dark olive green
C-6	Circular	0.1	Smooth	Raised	Creamy	Off-white	Dark grey
C-7	Circular	0.2	Smooth	Flat	Creamy	Off-white	Dark grey
C-8	Circular	0.1	Smooth	Dome	Pasty	Lemon yellow	Golden brown
C-9	Circular	0.1	Smooth	Flat	Pasty	Off-white	Soil brown
C-10	Circular	0.1	Smooth	Flat	Pasty	Dark yellow	Soil brown
C-11	Circular	0.1-0.2	Smooth	Convex	Creamy	Yellow	Soil brown

Table 8B Colony morphology of bacterial isolates obtained from MB-MBR

Isolate	Shape	Size (cm)	Margin	Elevation	Texture	Color	
						Naked eye	Microscope
MB-1	Circular	0.2-0.3	Smooth	Convex	Creamy	Off-white	Golden brown
MB-2	Circular	<0.1	Smooth	Flat	Creamy	Lemon yellow	Dark golden brown
MB-3	Circular	0.1	Smooth	Flat	Creamy	Dirty offwhite	Golden brown
MB-4	Circular	≤0.1	Smooth	Flat	Pasty	Off-white	Soil brown
MB-5	Circular	≤0.1	Smooth	Flat	Pasty	Off-white	Soil brown
MB-6	Circular	0.1	Smooth	Raised	Pasty	Milky offwhite	Soil brown
MB-7	Circular	0.3-0.4	Smooth	Convex	Pasty	Off-white	Dark brown
MB-8	Circular	<0.1	Smooth	Convex	Pasty	Milky offwhite	Dark grey
MB-9	Circular	0.1-0.2	Smooth/ Undulate	Umborate	Creamy	Luminescent Off-white	
MB-10	Circular	0.1-0.3	Smooth	Raised	Pasty	Off-white	Golden brown

Table 9B Colony morphology of bacterial isolates obtained from A/O-MBR

Isolate	Shape	Size (cm)	Margin	Elevation	Texture	Color	
						Naked eye	Microscope
A/O-1	Irregular	0.1-0.2	Undulate	Umborate	Sticky	Yellowish Off-white	Soil brown
A/O-2	Circular	<0.1	Smooth	Convex	Sticky	Dirty off-white	Dark soil brown
A/O-3	Irregular	0.2-0.4	Undulate	Concave	Sticky	Dirty Yellow	Light golden brown
A/O-4	Irregular	0.1-0.2	Undulate	Raised	Sticky	Yellowish Off-white	Dark golden brown
A/O-5	Circular	0.1	Smooth	Convex	Pasty	Light Off-white	Golden brown
A/O-6	Irregular	0.2-0.4	Undulate	Raised	Pasty	Off-white	Dark brown
A/O-7	Circular	0.1-0.2	Smooth	Flat	Creamy	Light dirty Off-white	Light golden brown
A/O-8	Circular	0.2-0.4	Smooth	Convex	Pasty	Off-white	Dark grey
A/O-9	Circular	<0.1	Smooth	Flat	Pasty	Milky offwhite	Soil brown
A/O-10	Circular	<0.1	Smooth	doom	Pasty	dirty Off-white	Light brown
A/O-11	Circular	0.2	Smooth	Flat	Creamy	Milky off-white	Dark grey

Table 10B Colony morphology of isolates obtained from effluent and cake layer

Isolate	Shape	Size (cm)	Margin	Elevation	Texture	Color	
						Naked eye	Microscope
MF-C-1	Circular	< 1	rhizoidal	Flat	Pasty	Offwhite	soil brown
MF-C-2	circular	≤ 1	smooth	Flat	Creamy	Translucent off-white	Light golden brown
MF-C-3	irregular	2-3	Smooth	Flat	Pasty	Off-white	Dark grey brown
MF-MB-1	circular	1-2	Dentate	Flat	Creamy	Luminescent yellow	Light golden brown
MF-MB-2	Irregular	2	rhizoidal	Flat	Creamy	off-white	Dark grey brown
MF-A/O-1	irregular	3-4	rhizoidal	Flat	Creamy	Dirty Off-white	Golden brown
MF-A/O-2	irregular	3-4	Smooth	Flat	Creamy	Dirty Off-white	Dark grey
MF-A/O-3	irregular	2	rhizoidal	Flat	Pasty	Off white	Soil brown
EC-1	Circular	1-2	Smooth	Raised	creamy	Translucent yellow	Very light Soil brown
EMB-1	Circular	< 1	Smooth	Raised	creamy	Translucent yellow	Colorless
EA/O-1	Circular	< 0.1	Smooth	Raised	pasty	Yellow	Soil brown
EA/O-2	Circular	< 0.1	smooth	Concave	pasty	Yellow	Golden brown

Table 11B Colony morphology for isolates of media from MB-MBR and A/O-MBR

Isolate	Shape	Size (cm)	Margin	Elevation	Texture	Color	
						Naked eye	Microscope
MB-a	Irregular	2-3	Dentate	Flat	Pasty	Offwhite	Dark grey
MB-b	Irregular	2-3	Dentate	Flat	Creamy	Offwhite	Dark golden brown
MB-c	Circular	<0.5	Smooth	Convex	Pasty	Colorless	Dark golden brown
MB-d	Circular	<0.5	Smooth	Convex	Creamy	Dirty off white	Dark brown
A/O-a	Irregular	1-2	Rhizoidal	Flat	Creamy	Offwhite	Dark grey
A/O-b	Irregular	2-3	Dentate	Raised	Pasty	Offwhite	Dark brown
A/O-c	Circular	1-1.5	Smooth	Flat	Creamy	Offwhite	Golden brown
A/O-d	Circular	<1	Smooth	Flat	Pasty	Colorless	Golden brown

Table 12B Gram staining of bacterial isolates obtained from C-MBR

Bacterial Isolates	**Gram Staining**	**Shape**	**Arrangement**
C-1	Gram negative	Bacilli	Diplo &strepto-bacilli
C-2	Gram negative	Cocci	Single &strepto-cocci
C-3	Gram negative	Bacilli	Diplo &strepto-bacilli
C-4	Gram negative	Cocci	Strepto-cocci
C-5	Gram negative	Cocci	Strepto-cocci
C-6	Gram negative	Cocci	Strepto-cocci
C-7	Gram negative	Cocci	Single &strepto-cocci
C-8	Gram negative	Cocci	Staphylococci
C-9	Gram negative	Cocci	Tetrad &staphylococci
C-10	Gram negative	Cocci	Staphylococci
C-11	Gram positive	Cocci	Diplo &strepto-cocci

Table 13B Gram staining of bacterial isolates obtained from MB-MBR

Bacterial Isolates	**Gram Staining**	**Shape**	**Arrangement**
MB-1	Gram negative	Cocci	Diplo &strepto-cocci
MB-2	Gram negative	Cocci	Strepto-cocci
MB-3	Gram negative	Bacilli	Strepto- bacilli
MB-4	Gram negative	Bacilli	Strepto- bacilli
MB-5	Gram negative	Cocci	Diplo &strepto-cocci
MB-6	Gram negative	Cocci	Diplo &strepto-cocci
MB-7	Gram negative	Cocci	Staphylococci
MB-8			
MB-9	Gram positive	Cocci	Diplo, tetrad &staphylococci
MB-10	Gram negative	Bacilli	Strepto- bacilli

Table 14B Bacterial isolates obtained from A/O-MBR

Bacterial Isolate	Gram Staining	Shape	Arrangement
A/O-1	Gram negative	Bacilli	Strepto- bacilli
A/O-2	Gram negative	Bacilli	Diplo &strepto-bacilli
A/O-3	Gram negative	Bacilli	Strepto- bacilli
A/O-4	Gram negative	Cocci	Strepto-cocci
A/O-5	Gram negative	Cocci	Diplo &strepto-cocci
A/O-6	Gram negative	Cocci	Strepto-cocci
A/O-7	Gram negative	Bacilli	Diplo-bacilli
A/O-8	Gram negative	Cocci	Diplo &strepto-cocci
A/O-9	Gram negative	Cocci	Tetrad &staphylococci
A/O-10	Gram negative	Cocci	Single
A/O-11	Gram positive	Cocci	Tetrad &staphylococci

Table 15B Gram staining of fouling and effluent strains

Bacterial Isolate	Gram Staining	Shape	Arrangement
MF-C-1	Gram positive	Cocci	Streptococci
MF-C-2	Gram negative	Cocci	Streptococci
MF-C-3	Gram positive	Cocci	Streptococci
MF-MB-1	Gram negative	Cocci	Single
MF-MB-2	Gram positive	Cocci	Streptococci
MF-A/O-1	Gram negative	Cocci	Streptococci
MF-A/O-2	Gram positive	Cocci	Streptococci
MF-A/O-3	Gram negative	Cocci	Single
EC-1	Gram negative	Cocci	Diplo and streptococci
EMB-1	Gram negative	Cocci	Single
EA/O-1	Gram negative	Bacilli	Diplobacilli
EA/O-2	Gram negative	Cocci	Single

Table 16B Gram staining of medium isolates

Bacterial Isolate	Gram Staining	Shape	Arrangement
MB-a	Gram negative	Cocci	Sreptococci
MB-b	Gram negative	Cocci	Streptococci
MB-c	Gram negative	Cocci	Staphylococci
MB-d	Gram negative	Cocci	Staphylococci
A/O-a	Gram negative	Cocci	Streptococci
A/O-b	Gram negative	Cocci	Streptococci
A/O-c	Gram negative	Bacilli	Diplobacilli
A/O-d	Gram negative	Cocci	Staphylococci

Table 17B API Strip Results for all isolates

MB1	000404
MB2	121577
MB4	004100
MB5	000502
MB6	000502
MB7	731112
MB8	704577
MB9	524577

C1	000004
C2	020000
C3	020004
C4	725577
C5	524577
C6	000404
C7	000100
C8	100416
C9	101317
C10	301417

A/O1	000104
A/O2	000200
A/O3	000004
A/O4	731300
A/O5	500777
A/O6	001200
A/O7	525577
A/O8	000202

EC1	621107
MFA2	611245
EA/O2	000577
EA/O1	000073
MFC2	725577
MFC1	725577
MFA1	733657
MFC3	621300
EMB1	221100

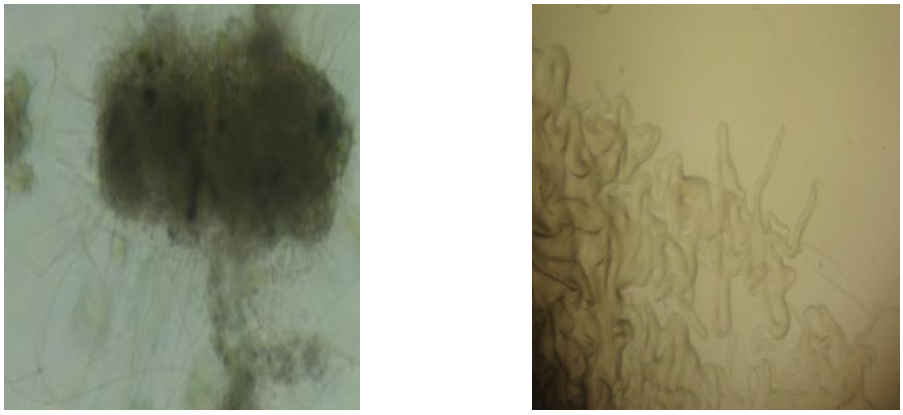
Table 18B DNA Extraction using NanoDrop

Extraction	**Amount (ng/µl)**
P1	56
P2	30
P3	81
P4	100
P5	67
P6	56
P7	58
P8	95
P9	+++
P10	23
P11	107
P12	47
P13	97
P14	94
P15	54
P16	29
P17	32
P18	36
P19	113
P20	+++
P21	+++
P22	80
P23	104
P24	82
P25	58

Annexure C

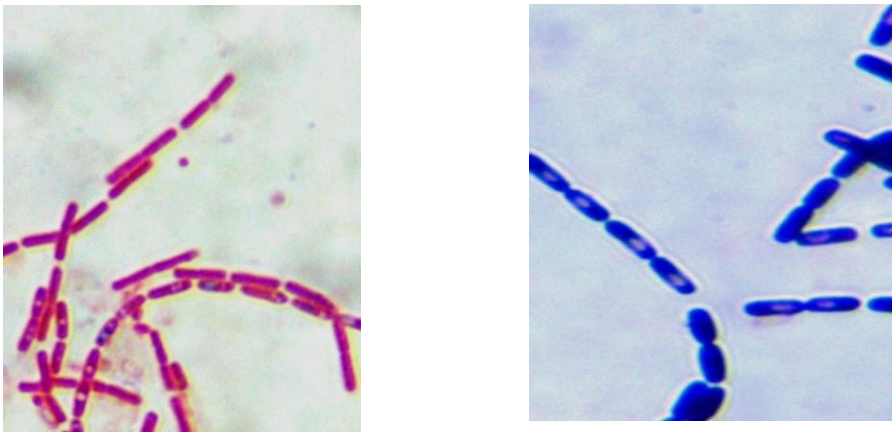
Figures

Morphology



(a) (b)

Figure 1C Morphology a) floc morphology b) colony morphology (margins)

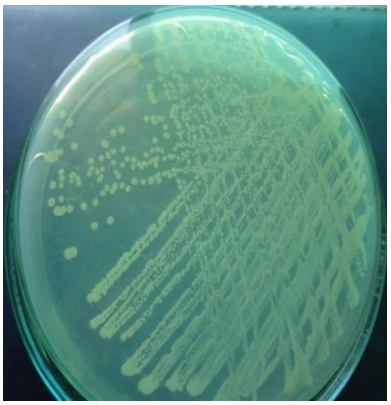


(a) (b)

Figure 2C Gram negative reaction a) Gram negative cell b) Gram positive cell

Plating

(a)



(b)

Figure 3C a) Standard plate count b) Streak plate

API

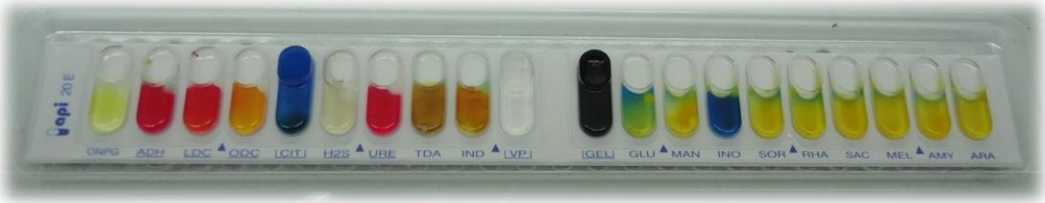


Figure 4C API 20E strip after use

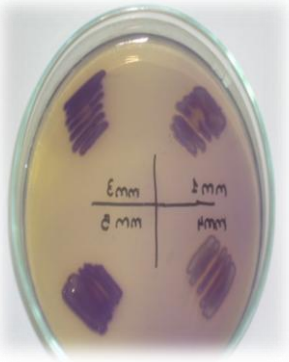


Figure 5C Oxidase test