MICROBIAL CHARACTERIZATION OF SEWAGE TREATMENT PLANT, I-9 ISLAMABAD

BY

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2010-NUST-MS PhD-Env S-19

Institute of Environmental Science and Engineering (IESE)
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A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science in Environmental Science

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"In the Name of Allah, the most Beneficent, the most Merciful"

Dedication

Dedicated to the unfathomable
depths of love
and
ever strengthening prayers of

My Grand Parents

who have brought my hopes
to attain the destination…..

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All acclamation and appreciation for **ALMIGHTY ALLAH** the Benevolent and Clement, who directed my thoughts and emboldened my ambitious in the form of this humble write up.

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HIRA WAHEED

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List of Abbreviations

AOB	Ammonia Oxidizing Bacteria
API	Analytical Profile Index
ASP	Activated Sludge Process
BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
DO	Dissolved Oxygen
DNA	Deoxyribo Nucleic Acid
DGGE	Denaturing Gradient Gel Electrophoresis
FISH	Fluorescence In Situ Hybridization
GHG	Green House Gas
HRT	Hydraulic Retention Time
NOB	Nitrite Oxidizing Bacteria
PCR	Polymerase Chain Reaction
PRS	Profile Recognition System
TSS	Total Suspended Solids
TN	Total Nitrogen
TOC	Total Organic Carbon
TSS	Total Suspended Solids
rRNA	Ribosomal Nucleic Acid
STP	Sewage Treatment Plant
Sp.	Specie
US EPA	United States Environment Protection Agency
WWTP	Wastewater Treatment Plant

ABSTRACT

Presence of different forms of ammonia in the wastewater treatment plants (WWTP) are toxic to aquatic life and promote eutrophication in receiving water bodies. It becomes imperative to lower the ammonia concentration in the effluents for which characterization of microbial diversity of the WWTP is foremost objective. The study was planned to identify the diversity of nitrifiers (ammonia oxidizing and nitrite oxidizing bacteria) and denitrifers using PCR technique. Activated sludge samples of complex mixture of microorganisms were collected from sewage treatment plant treating domestic wastewater. High bacterial diversity was found in the aeration tank where 7, 35 and 57 % isolates belongs to class α, β and γ proteobacteria respectively. On the basis of gram reaction, only 17 % of isolated species were found to be gram positive bacteria. Total nitrifiers and denitrifiers bacterial numbers in the mixed liquor were in the range of 2.2 to 2.5 x 10^{14} and 5.06 to 10.1 x 10^{13} copies/L respectively. To confirm the specifity of the amoA and 16S rRNA primers, the PCR product obtained from MLSS was sequenced. *Nitrosomonas europaea* was found to be the most important ammonia oxidizing baceria using 16S rRNA and amoA primers.The prominent members of the nitrite oxiding bacteria in activated sludge were most related to *Nitrosospira* sp. and *Nitrobacter winogradskyi* using 16S rRNA and NxrB primers respectively. *Pseudomonas* genera were isolated as a part of the denitrifying microbial flora. The use of cultural techniques in combination with PCR analysis with 16S rRNA and nirS approach revealed *Pseudomonas aeruginosa* as important denitrifier. Average effluent concentraion of NH_4^+, NO_2^-, NO_3^- and TN was found to be 1.2, 0.4, 11.3 and 19 mg/L. The study indicated that molecular technique including PCR using bacterial

16S rRNA and NxrB gene is useful for the comparison of the population abundance in wastewater samples, offering high analytical sensitivity and precision.

Chapter 1

INTRODUCTION

The biological wastewater treatment, which uses activated sludge for the transformation of organic and inorganic pollutants, has contributed greatly to the improvement of the aquatic environment worldwide, and is still the very commonly employed process for the treatment of municipal wastewater because of its low operation cost and high performance. For more than 100 years endless efforts have been made by numerous researchers to improve this process, leading to the creation of many types of activated sludge technologies with more stable performance and new functions such as biological removal of phosphorus and nitrogen, In spite of these developments, however, many municipal treatment plants are still facing some troubles like sludge bulking, foaming, failures in nitrification or phosphate removal, etc. (Seviour et al.,2000). The reasons for these troubles might be very different, varying from improper design, to improper operation, exceptional influents, or some load shocks, etc., but almost all of the troubles in function or performance are related to the changes of microbial community structures.

Activated sludge process generally contains numerous microbial species i.e. viruses, protozoa, metazoan, bacteria, fungi, and algae, while bacteria comprise approximately 95% of the total microbial population (Jenkins et al., 1993) and play a key role in the purification of water quality. Besides, importance of activated sludge, knowledge of microbial ecology in wastewater treatment systems has been fairly inadequate.

Activated sludge process can achieve nitrogen and phosphorus removal in addition to the removal of organic carbon constituents. Nitrogen in wastewater exists in four types; i) organic nitrogen (Org-N), ii) ammonia nitrogen (NH_4-N), iii) nitrite nitrogen (NO_2-N) and vi) nitrate nitrogen (NO_3-N). Different forms constitute the total nitrogen content. Although nitrogen is an essential nutrient for biological growth and is one of the main constituents in all living organism, an excessive presence of it in the effluent wastewater should be avoided for several reasons. In these various forms, nitrogen can have several adverse effects on the aquatic environment: (i) nitrogen in the reduced forms will exert oxygen demand in the receiving water; (ii) ammonia and nitrite nitrogen are toxic to fish (iii) ammonia nitrogen requires large amounts of chlorine for disinfection during treatment of water to potable standards; (iv) nitrite and nitrate nitrogen can cause eutrophication lakes and landlocked water bodies resulting in the uncontrolled growth of algae and other aquatic plants; (v) nitrate nitrogen in excess of 10 mg/L is unsuitable for consumption by babies because of the risk of methaemoglobinaemia.

By nitrification and denitrification, nitrogenous products could be removed from wastewater biologically. When untreated wastewater arrives to the treatment plant, most nitrogen is present in the ammonia form, which can be removed through the process called nitrification. Nitrification is oxidation of ammonia to nitrite by ammonia oxidizing bacteria (AOB) and then nitrite is subsequently oxidized to nitrate by nitrite-oxidizing bacteria (Siripong and Rittmann, 2007).

Ammonia oxidizing bacteria and nitrite oxidizing bacteria are described as nitrifying bacteria or nitrifiers. *Nitrobacter* and *Nitrosomonas* have found

important bacteria for nitrite and ammonium oxidation, respectively. However, number of studies indicates the presence of *Nitrosospira* (Coskuner and Curtis, 2002) alongside with *Nitrosomonas*, and *Nitrospira* are the dominant nitrite oxidizing bacteria in ASP (Dionisi et al., 2002; Harms et al., 2003). Concurrence of various nitrifiers undertaking the similar functions indicates functional termination, which permit microbial communities to keep physiological capabilities when conditions are changed. Thus, an elevated level of nitrifier diversity is presumed to confer performance constancy (Kowalchuk et al., 2000). Consequently, physiological activity as well as abundance of nitrifying bacteria are believed to be the rate limiting parameters for the nitrogen conversion in wastewater (Wagner and Loy, 2002), as well as in drinking water treatment plants.

Activated sludge systems and biofilm on granular activated carbon are capable of nitrification in wastewater treatment plant (WWTP) and drinking water treatment plant respectively. Because of the slow growth rates and poor yields of the organisms involved, nitrification is generally regarded as the rate limiting step of ammonium nitrogen removal efficiencies in the nitrogen removal process (Coelho et al., 2000).

Denitrification is generally considered as a special case of dissimilation in which gaseous NO, N_2O and N_2 are the end products. On a biochemical level, the denitrification process is rather complex in which various reaction intermediates are involved. More often, the process is considered as comprising two simplified steps. Initially, nitrate is converted to nitrite and then in the second step, nitrite is further reduced to N_2. Most denitrifiers are facultative heterotrophs which can utilize energy for their growth from organic compounds using nitrate as the

hydrogen acceptor. *Pseudomonas* is considered to be one of the most important bacteria that cause denitrification. Therefore, it is imperative to examine the relationship among functional stability and microbial community dynamics at large scale in the wastewater treatment systems (Wang et al., 2011).

Molecular techniques are getting more frequent to detect a specific group of microbes without their cultivation. DNA probes in complementary to 16S rRNA are increasingly employed to study the microbial population of a complex systems (Kelly et al., 2005).

1.1 Sewage Treatment Plant, I-9 Islamabad

Recognizing the potential of wastewater for reuse, Pakistan has taken initiative to treat and reuse the wastewater. Government of Pakistan has established a centralized sewage wastewater treatment plant capable of treating 17 million gallons per day (MGD) using standard activated sludge process. This treatment plant is located at sector I-9 Islamabad. It has 4 phases out of which Phase 1 and 2 were constructed in 1964 and 1966 respectively with a treatment capacity of 4 MGD. However with the increasing population of the city it was decided in 1986 to increase the plant capacity by installing Phase 3 with treatment capacity of 3 MGD. The phase 4 was built in 2007 with an average daily flow of 10 MGD. The catchment area of phase 4 is sector 10 and 11 of D, E, F, G and H.

The plant consists of following phases:

- Pretreatment including fine bar screening and grease grit removal
- Primary sedimentation tank
- Aeration tank
- Secondary sedimentation tank

- Sludge treatment process

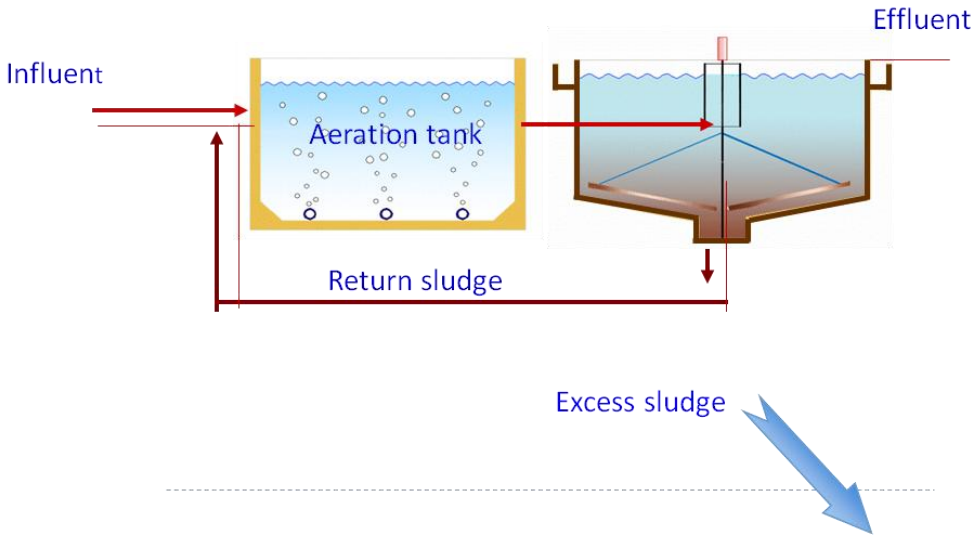

Figure 1.1: Activated sludge process

1.2 Objectives

The main objective of this work was to investigate the nitrifying and denitrifying bacterial composition in ASP by using culture dependent as well as culture independent methods. Whereas functional genes of nitrification and denitrification were targeted to examine the abundance and diversity of nitrifying and denitrifying bacteria using polymerase chain reaction (PCR).

The specific objectives were:

- To examine the diversity of microbial community in sewage treatment plant to assess the dominant indigenous microbes.
- To identify and characterize nitrifying and denitrifying bacterial communities in order to identify the treatment potential of the plant.

Chapter 2

LITERATURE REVIEW

2.1. Water and Wastewater

Water is the most important and fundamental ingredient of the life on earth. However, increasing population growth coupled and unsustainable water practices in the field of agriculture and industry stressed the water availability all over the world. The unhealthy relationship of industrial activity, urbanization, economic growth, unsustainable development, climate change and environmental pollution has increased the fear of water scarcity, which is serious and matter of great fear in recent times.

Estimates exposed the fact that Pakistan had declined from 5000 m^3 per capita of fresh to less than 1,000 m^3 per capita to be declared as water stressed country (PSCEA, 2006). The water table is dropping at the rate of 3 meters per year in the Kirthar. Groundwater table is also dropping all over the country. Furthermore, around 200 MG domestic WW is being cleared to surface waters daily. While collection level is no more than 50 % nationally (<20 % in rural), however, only 10 % of collected WW is effectively being treated (World Bank, 2006). Importantly, absence of regular monitoring programme and NEQS are increasing complexity of the problem.

2.2. Wastewater Treatment Options

Industrial, agricultural and domestic wastewater treatment has an important role in solving the global problem of water pollution. Wastewater treatment plants uses physical and biochemical procedures to minimize the organic matter,

eliminate pathogenic organisms and enhance water quality, so that water can be reused or discharged into the environment with nominal concerns.

Various wastewaters treatment technologies are being applied such as coagulation, flocculation, membrane separation, ultrafiltration, reverse osmosis etc. Use of activated carbon produces a secondary pollutant, whereas biological treatment is not a complete solution to the problem due to biological resistance of some dyes (Arslan et al., 2000). However, biological processes can remove above 90% of the biological oxygen demand (BOD) in wastewater (Sheng et al., 2008).

Aerated lagoons are among widely used wastewater treatment processes because of their low capital and maintenance costs, less sludge production and integration in the environment. The process works on the decay and consumption of organic material by microbes under aerobic conditions. The key players in the transformation of complex organic compounds are prokaryotes in WWTP. The studies on the microbial performance concluded that changes in the diversity of communities can compromise the whole process (Forster et al., 2003).

2.3. Activated Sludge Process (ASP)

ASP is amongst biological treatment systems, which is employed in sewage wastewater treatment and/or as a secondary treatment option in the industrial wastewater treatment. Aerobic digestion is utilized in various processes ranging from conventional to auto thermal (Riley and Forster, 2002). Most of the biological wastewater plants work on the principle of activated sludge process which converts organic substances into microbial biomass (Ritchelita et al., 1998). Suspended bacteria produce effluent within legal standards by oxidising the carbonaceous and nitrogen compounds ensuring minimal environmental impacts.

Activated sludge process is consistent due to various reasons; i) presence of bacteriophage can lead to rapid and large change in dominant bacterial species. Several bacterial species always exist, if one is destroyed by a phage, another can substitute it quickly so that major disorder in treatment efficiency is not noticed. This sets the main basis of activated sludge to works reliably. ii) Severance in microbial ecosystems and the major fight for energy resources, minor variations in the treatment process can lead to major changes in the microbial population composition and the floc physical characteristics. Thus, redundancy allows the microbial strains most adept of remaining to dominate rapidly as conditions change. iii) the growth and maintenance of a large, diverse, and active population of bacteria over time developed firm and dense mature floc particles which ensure suitable oxidation of carbonaceous BOD and nitrogenaceous BOD (Tizghadam et al., 2007).

The main cost associated with the ASP is the electrical energy required to operate the aerating instruments to maintain necessary aeration and mixing (Balku, 2007). The real oxygen demand for proper aerobic digestion is supposed to be higher because it depends on different factors including pH, temperature, inclusion of primary sludge and retention time (Metcalf and Eddy, 2003).

Activated sludge system is capable of achieving a substantial reduction of COD and TSS effluent quality with residual values of only 35 and 14 mg/L respectively when operated at hydraulic retention time (HRT) of 2 h (Tawfik et al. 2007). Aerobic digestion can be exploited at temperature going from room to thermophilic i.e. 50-60 ^{o}C (Messenger et al., 1990). Zupancic and Ros (2007) stated that sludge treated with O_2 was degraded between 32 ^{o}C and 65 ^{o}C. The best

temperature for aerobic nitrification is 30-35 ^{o}C (Willers et al., 2002). Best efficiency of soluble COD removal was reported at 30 ^{o}C (62 %), however COD removal rate declined as temperature rises above 60^{o}C (38 %) (Lapara et al., 2001).

Kornaros and Lyberatos (2006) studied the wastewater treatment in activated sludge process; the microbes produced in the trickling filter were capable to remove COD level up to 36000 mg/L, at different pH ranging from 5.5 to 8.0. The COD removal efficiency was 65 percent at pH value of 5.5. The removal efficiency was 74 percent, when the pH was increased up to 7.5 (by adding buffer solution). In aerobic process, the optimum range of pH lie between 6.5 to 7.5, as most of the bacterial communities cannot survive pH levels above 9.5 or below 4.0 (Tabrizi and Mehrvar, 2004).

The degree of organic removal for the activated sludge process is considerably high (Puyol et al., 2011). COD and colour removal efficiencies were observed in between 97 and 91 % and between 84 and 91 % at HRT of 19.17 hrs and 1.22 days in combined anaerobic and aerobic systems (Isik and Sponza, 2007).

2.3.1. Microbial Diversity of ASP

Prokaryotic microorganisms are dominant in all plant types and found responsible for the conversions. Certain microorganisms, however, create frequently encountered problems of sludge bulking and foaming. Subsequently, the efficiency of the WWTP mostly depends on the composition and functioning of microbial community. Although biological wastewater treatment is historic, however, research on the microbiology of this process suffered from severe methodological limitations (Wagner and Loy, 2002). After the introduction of molecular techniques in wastewater microbiology (Purkhold et al., 2000), it

became likely to determine the composition and dynamics of microbial communities and to identify the potential microbes for different process types.

Rani et al. (2008) discovered the dominant cultured bacteria revealing microbial diversity in functional effluent treatment plant of Delhi, India were *Alcaligenes* ,*Bacillus* and *Pseudomonas*. *Brevundimonas*, *Citrobacter*, *Pandoraea* and *Stenotrophomonas* were specific to pesticide effluent treatment plant and *Agrobacterium*, *Brevibacterium*, *Micrococcus*, *Microbacterium*, *Paracoccus* and *Rhodococcus* were specific to pharmaceutical treatment plant. They suggested that microbes can be kept and exploited for efficient working and maintenance of treatment plants.

Environmental factors affect the degree of AOB diversity in wastewater treatment systems. Selection for either predominance of a single AOB population or several different AOB populations can occur together. Recently, level of AOB diversity found in a reactor relates to the stability of the reactor. Hence engineering a system with a better diversity may increase performance and stability. However, it is imperative to proliferate the diversity of the best suitable AOB.

2.3.2. GHG Emissions

Wastewater treatment plants are considered as potential sources of the GHG emissions. Among many, nitrous oxide (N_2O) is very significant greenhouse gas which is 300 times more effective than carbon dioxide (CO_2). Nitrous oxide is a main sink for stratospheric ozone (Ravishankara et al., 2009; IPCC, 2007). Two third of nitrous oxide is released by microbial processes occurring mainly in agriculture as well as in biological wastewater treatment (US EPA, 2009). Nitrous oxide emissions dominate the total GHG emissions from biological wastewater

treatment (Wunderlin et al., 2010). It is very urgent to limit the anthropogenic N_2O emissions. In addition to N_2O, nitric oxide (NO) could be present along with the nitrous oxide, which is toxic for microbes (Zumft, 1993) and plays a significant role in the destruction of the ozone layer present in the stratosphere (Crutzen, 1979).

Release of nitrous oxide in biological wastewater treatment is linked with autotrophic nitrification and heterotrophic denitrification. Denitrification is the reduction of nitrate (NO_3) to atmospheric nitrogen (N_2). The process is carried out by heterotrophic denitrifiers (HET) with obligatory intermediates like nitrite (NO_2), nitric oxide (NO) and nitrous oxide (N_2O). There are three major pathways for N_2O production (Hallin et al., 2005; Kampschreur et al., 2009).

- Hydroxylamine oxidation
- Nitrifier denitrification
- Heterotrophic denitrification

The major processes in biological wastewater treatment are autotrophic nitrification and heterotrophic denitrification however, the fundamental routes remain uncertain. Nitrite dependent N_2O production is resulted when nitrite is added under aerobic conditions which is mainly by nitrifier denitrification of ammonia oxidizing bacteria (AOB). Furthermore, addition of hydroxylamine resulted in N_2O production via hydroxylamine oxidation

Sümer et al. (1995) estimated the N_2O emission using direct gas collection method and concluded as 4.5×10^3 kg N_2O nitrogen was released annually from waste-water treatment plants in Germany. Similarly, N_2O emissions from a Belgium wastewater treatment plant have also been determined by incubating

samples of raw and settled sewage in stationary sealed flasks (Derbuyn et al., 1994).

2.3.3. Nitrification

Nitrification is carried out in two steps; it is the oxidative process in which ammonia (NH_4^+) is transformed to nitrite (NO_2^-) and then subsequently oxidized to nitrate (NO_3^-). Oxygen is added to the ions by the nitrifying bacteria during the oxidation of ammonium ions and nitrite ions. This process is crucial part of global nitrogen cycle and also a critical step in many WWTPs (Kelly et al., 2005). The ammonia and the nitrite oxidizing bacteria convert ammonia to nitrite and nitrite to nitrate, respectively. Since most of the nitrogen in the wastewater is present either in form of urea or ammonium/ammonia. It is crucial to minimize the ammonia concentrations in the effluent of WWTPs since this is toxic to aquatic life and supports eutrophication in the receiving waters (Gerardi, 2002).

In aeration tank of ASP, the amounts of ammonium ions and ammonia depends upon pH and temperature of the activated sludge. Approximately 95 % of the reduced form of nitrogen is present as ammonium ions at the temperature range of 10 to 20^oC and at pH from 7 to 8.5. High temperatures can inhibit ammonia oxidation. Oxygen solubility decreases as temperature increases, and although nitrification can occur at dissolved oxygen (DO) level as low as 0.05 mg/L (Abeliovich, 1994). However, low oxygen concentrations are not favorable. High temperatures also increase heterotrophic respiration requirements, further reducing oxygen concentrations.

Although ASPs are employed for nitrification, but these processes are not perfect for nitrification. As the population size of nitrifying bacteria gets diluted,

makes difficult to obtain and maintain required nitrification. Activated sludge includes almost 90 to 97 % bacteria as organotrophs, while the remaining 3 to 10 % of the bacteria are nitrifiers (Gerardi, 2002).

2.3.3.1. Nitrifying Bacteria

As described by Gerardi (2002), there are numerous organisms that are adept of oxidizing ammonium and nitrite ions including actinomycetes (*Mycobacterium*, *Nocardia*, *Streptomyces*), Algae (*Chlorella*), Bacteria (*Arthobacter*, *Bacillus*, *Nitrosomonas*, *Nitrobacter*, *Proteus*, *Vibrio*), Fungi (*Aspergillus*) and Protozoa (*Epistylis*, *Vorticella*). But the principal organisms responsible for most of the nitrification belong to two genera: nitrosofying bacteria and nitrifying bacteria (Schmidt, 1982). Contributing microbial genera found in natural ecosystems and engineered treatment systems include *Nitrosomonas, Nitrosospira* and *Nitrosococcus*. Common genera responsible for nitrification in natural and constructed systems comprise *Nitrobacter* and *Nitrospira* (Kelly et al., 2005).

Recent studies show that *Nitrospira* were the dominant NOB under the favorable growth conditions in WRPs (Burrell et al., 1999). *Nitrospira* have high affinity towards oxygen (Schramm et al., 1999) and they can maintain their ribosome content for a long period of inactivity (Morgenroth et al., 2000). However, Juretschko et al. (1998) studied industrial WRP samples with high ammonia concentration. Further, Burrell et al. (1999) used a reactor spike with nitrite, and Schramm et al. (1999) studied NOB in biofilms.

Nitrifying bacteria are very slow growing microbes even the fastest growing *Nitrosomonas europaea* has a specific growth rate of about 2 d (Prosser, 1989) and are recalcitrant to cultivation attempts. Nitrifying bacteria are very

sensitive to the disturbances in several environmental and engineering factors such as temperature shift, extreme pH, low DO, and a wide variety of chemical inhibitors (Rittmann and Mccarty, 2001).

Table 2.1: Oxidation reaction for nitrifying bacteria

Oxidation reactions	Responsible Genus
$NH^+ + 1.5O_2 \longrightarrow NO_2^- + H_2O + 2H^+$	*Nitrosomonas*
$NO_2^- + 0.5O_2 \longrightarrow NO_3^-$	*Nitrobacter*

Ammonia monooxygenase subunit A (amoA) gene and 16S rRNA genes are broadly used to investigate the variety and abundance of AOB in WWTPs (Otawa et al., 2006). 16S rRNA gene is more suited than amoA gene and suggested similarity thresholds of 16S rRNA and amoA genes to define different AOBs are ninety seven and eighty percent respectively. The comparative studies of phylogeny depicted that phylogenetic trees produced based on AOB amoA and 16S rRNA genes were consistent (Dionisi et al., 2002). The growth studies show that *Nitrsomonas* oxidize 30 pounds of ammonium ions for the production of one pound of dry cells, while *Nitrobacter* oxidize 100 pounds of nitrite ions. In contrast, organotrophic bacterium *E. coli* oxidize 2 pounds of glucose to achieve the same growth of one pound of dry cells.

Regardless of the significance of ammonia oxidizers in wastewater treatment, their slow growth and heterogeneous nature of activated sludge have made it difficult to investigate the AOB in wastewater treatment systems using traditional culturing methods.

2.3.4. Denitrification

Denitrification process utilized in wastewater treatment systems has specific objective to convert the products of nitrification into gaseous nitrogen compounds (NO_2), ultimately to remove them from the sewage. Although denitrification often combines with the ditrification to eliminate different types of nitrogenous compounds from wastewater that arises whenever an anoxic conditions exists (Gerardi, 2002). Mostly identification of denitrifiers in activated sludge is based on cultivation dependent methods.

Actually, nitrification process does not eliminate nitrogen from wastewater it just transforms ammonium ions to nitrate ions. However, denitrification is the process which removes nitrogen from wastewater by converting it to insoluble gases that is then released to the atmosphere. Besides N_2, N_2O gas is also produced during the denitrification process from nitrate ion and nitrite ions. It then escapes to the atmosphere because of being insoluble in wastewater.

Basically there are four fundaments steps of denitrification by which nitrate is reduced into N_2 by the help of different enzymes that includes metaloenzymes nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase (Nos) (Philippot, 2002).

Denitrification performs an important function in waste treatment by removing excess oxygen in local environment and by degrading organic pollutants anaerobically (Hallin et al., 2005).

2.3.4.1. Denitrifying Bacteria

Denitifying bacteria has rich taxonomic diversity and mostly belong to subclasses of the proteobacteria while others belong to gram positive bacteria and

also Archaea (Philippott, 2005). In many researches members of the genera *Pseudomonas*, *Bacillus*, *Alcaligenes*, *Methylobacterium*, *Hyphomicrobium* and *Paracoccus* were detected having share in the denitrifying microbial flora from WWTPs (Vedenina and Govorukhina 1988).

Gerardi (2002) found *Pseudomonas*, *alcaligenes* and *Bacillus* to be the largest denitrifying bacteria. He also reported that *Pseudomonas* do not accomplish denitrification completely and believed to produce N_2O instead of N_2 as their gaseous end products.

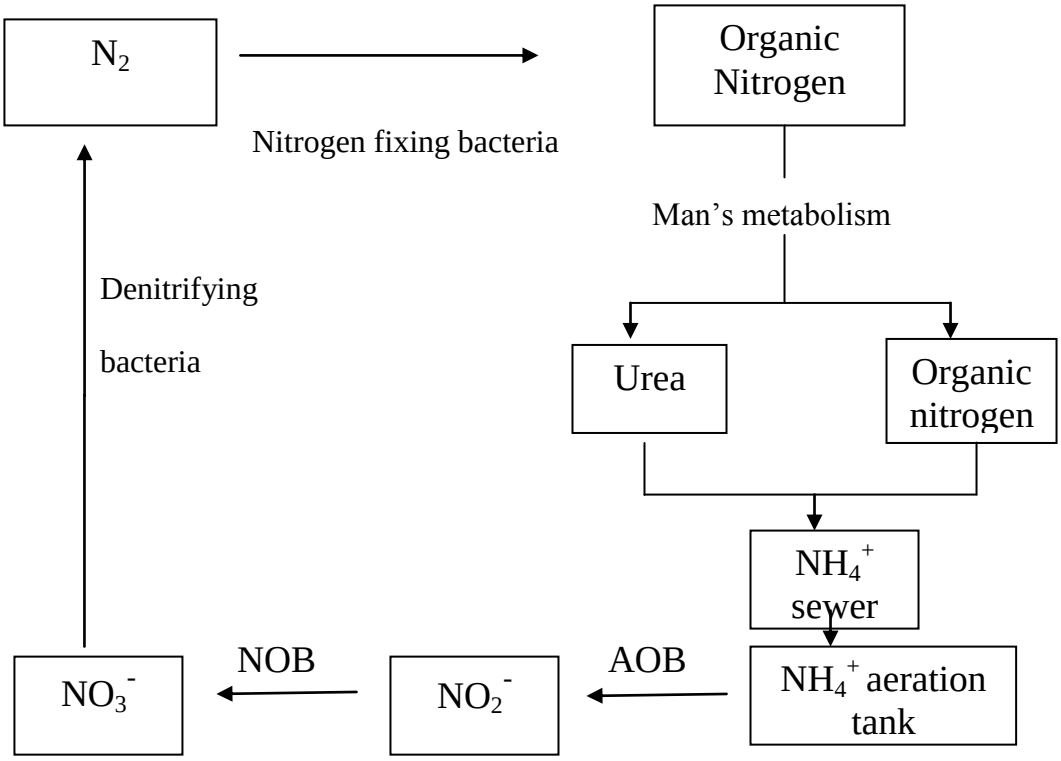

Figure 2.1: Wastewater nitrogen cycle

Zumft (1993) found almost 130 denitrifying bacteria within more than 50 genera, and the denitrification pathway give them a competitive advantage in low-oxygen environment. Wang et al (2011) detected *Thauera sp.* as major denitrifying bacteria in the WWTP of Beijing. They found it at a high percentage of 27.18 % in the clone library and showed a significant role in the removal of NO_3-N.

Neef et al (1996) used a specific FISH probes and succeeded to detected significant numbers of *Hyphomicrobium spp.* and *Paracoccus spp.* in a denitrifying sand filter which was augmented with methanol for the purpose to reduced carbon compound for nitrate reduction. Both of the detected genera were present less than 0.1% of the total cell counts in a non denitrifying sand filter which were run in parallel without addition of methanol. The findings indirectly suggests an active participation of both genera in the denitrification process.

Molecular studies for the composition of denitrifying community are difficult to execute because the denitrifying phenotype cannot be concluded from the phylogeny of a microorganism. However, the combination of FISH and micro auto radiography makes possible to identify of denitrifiers in situ by performing two different types of experiments in parallel (Lee et al., 2006).

Bacterial species which use substrate under anoxic environments entirely in the presence of nitrate or nitrite are most likely denitrifiers. The use of FISH in combination with the full cycler RNA method exposed that novel, still uncultured Azoarcus related bacteria are important denitrifiers in an industrial nitrifying/ denitrifying systems (Juretschko et al., 1998).

Denitrifying ability is pervasive among bacteria because of lateral gene transfer in contrast to the nitrifying. Denitrifying bacteria are very diverse

facultative anaerobes which possess the ability to switch from O_2 to NO_2 as terminal electron acceptors under the conditions where oxygen is limited or absent (Tamegai et al., 2002).

2.4. Polymerase Chain Reaction (PCR)

Over the last couple of decades molecular detection techniques have revealed a massive reservoir of unexplored microbes. This enormous genetic diversity could be used as a resource for the development of novel biotransformations, bioremediation processes and bioenergy generation (Lee et al., 2006). For better utization of these diverse genetic potentials there is a need to culture these unexplored microbes in controlled laboratory environment which further requires the knowledge of their nutrient and growth conditions (Kalia et al., 2003).

PCR has been successfully used in different fields of microbial studies to investigate the diversity of microbial communities and their response to deviations in the environmental conditions. Studies regarding bacterial diversity in wastewaters has also been carried out using a DGGE based approach for reactors systems and ASP (Gilbride et al., 2006), discovering the presence of highly complex bacterial communities.

Analytical techniques which targets 16S rDNA or functional genes were commonly utilized for microbial quantification. These may include hybridization based techniques like membrane hybridization and fluorescence in situ hybridization (FISH) (Okabe et al., 1999) as well as polymerase chain reaction (PCR) based techniques. Hybridization methods are generally less sensitive these may have detection limits in the order of 105 DNA/RNA copies or greater. These can only be used for the investigation of environmental samples of relatively high

microbial concentrations. On the other hand PCR based methods are proficient of detecting DNA/RNA at very low concentrations. However, the precision of PCR methods could be compromised due to a number of factors. These factors may include primarily the operating conditions such as reagent depletion, competition of amplicons with primers, and the loss of polymerase activity (Schneegurt and Kulpa, 1998).

In the PCR process the target DNA sequence is intensified over a number of denaturation annealing extension cycles. Conventional PCR can only monitor the final concentration of the amplicon using a DNA binding fluorescent dye. The qRT PCR is capable of monitoring the concentration of the amplicon throughout the amplification cycles using a group of new fluorescent reagents. These reagents have the special ability to bind with the amplicon without causing damage at the end of each cycle ensuring that amplification may continue to proceed. The fluorescence intensity produced in the PCR process has corresponding concentration of the amplicon in real time. More recently, real-time PCR was investigated for enumerating nitrifying bacteria in wastewater sludge and soil. Real time PCR permits reliable and continuous observation of wide range of samples during PCR used along with hybridization probe sets (Geets et al., 2007).

Chapter 3

MATERIALS AND METHODS

3.1. Sample Collection

i. Composite wastewater samples were collected from three locations i.e. inlet, aeration tank and outlet of sewage treatment plant, I-9 Islamabad from September 2011 to March 2012. In order to have a true representation of overall consortium present in activated sludge process, sampling was done twice a month during 10:00 am to 02:00 pm.

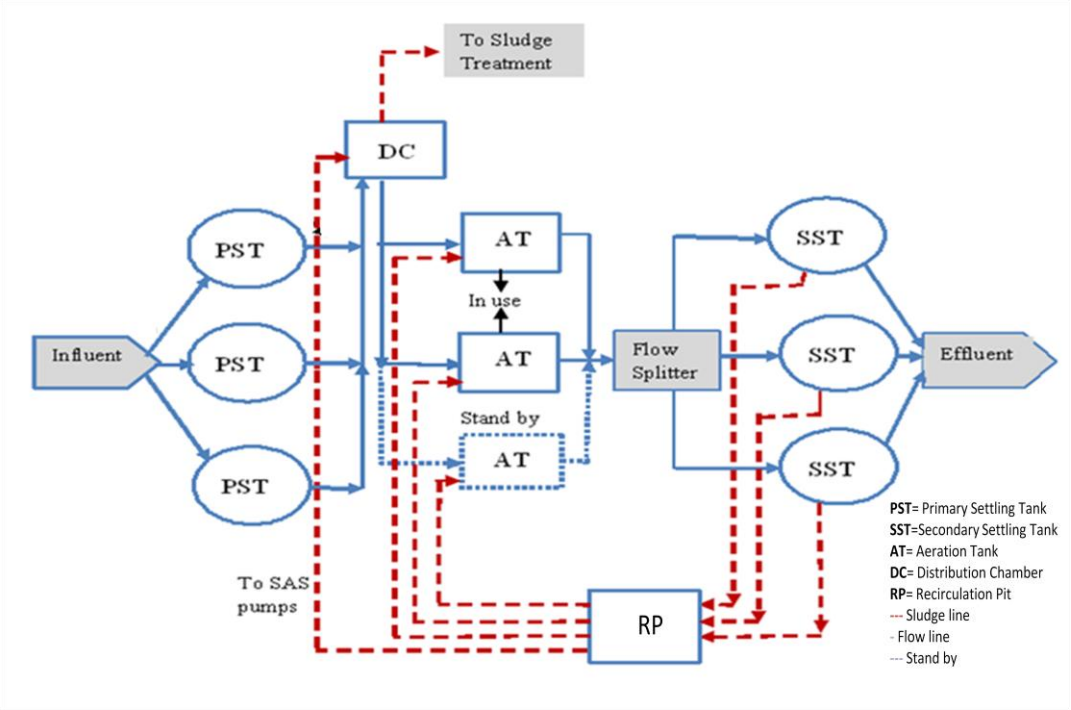

Figure 3.1: Detailed schematic of sewage treatment plant, Islamabad (Fatima, 2011)

ii. Activated sludge was sampled from the outlet of secondary sedimentation tank. Temperatures of the wastewater and sludge samples were measured on site and stored at 4 and -20 ^{o}C respectively, in the laboratory for the analysis of

microbial and physico-chemical parameters.

3.2. Isolation of General Microbial Species

Serial dilutions of each sample were prepared and 1 ml from each of the dilution was used in spread plate technique using nutrient agar, tryptone soy agar, simmon citrate agar and EMB agar for isolation of microbial colonies. Distinct colonies were selected from each of the spread plate by visual observation and each was subjected repeatedly (4 to 5 cycles) to streak plate technique to get different colonies separated.

3.2.1. Total Bacterial Counts

Total bacterial counts for each sample were determined. Nine test tubes contained 9 ml saline blanks were taken. Wastewater sample was vortexed to ensure even dispersal of cells.

3.2.2. Serial Dilution Technique

Dilutions of each sample upto 9 were made as follows:

1. With a sterile pipette, 1 ml of sample was transferred aseptically into water blank tube number 1. The sample has been diluted 10 times to 10^{-1}.
2. 1 ml of 10^{-1} dilution was mixed in 9 ml of autoclaved distilled water to from 10^{-2} dilution.
3. 1 ml of 10^{-2} dilution was mixed in 9 ml of autoclaved distilled water to get 10^{-3} dilution.
4. Similarly, 10^{-4},10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} dilutions were made for each of the three wastewater samples.
5. 0.1 ml from each dilution was spread on different nutrient agar plates with the help of sterilized glass spreader.

6. The Nutrient agar plates were incubated at 37^{o}C for 24 hours.
7. The plates showing countable colonies (30-300) were taken and colony were counted with the help of colony counter.
8. Total bacterial counts/ 0.1 ml were calculated by the following method:

Calculations

Colonies per plate = N

Volume of dilution added to plate = 0.1 ml

Number of colonies / 0.1 ml = number of colonies x dilution factor

3.2.3. Bacterial Identification

Thirty five distinct colonies were selected using spread plate technique by visual observation using colony counter. A small amount of bacterial growth was picked and single colony streaking was done. Same procedure was followed to pick all the selected strains. Plates were incubated for twenty 24-48 hours at 37°C. Each colony was subjected repeatedly (4 to 5 cycles) to streak plate technique to get different colonies separated. Each pure colony was stored in the refrigerator for further use.

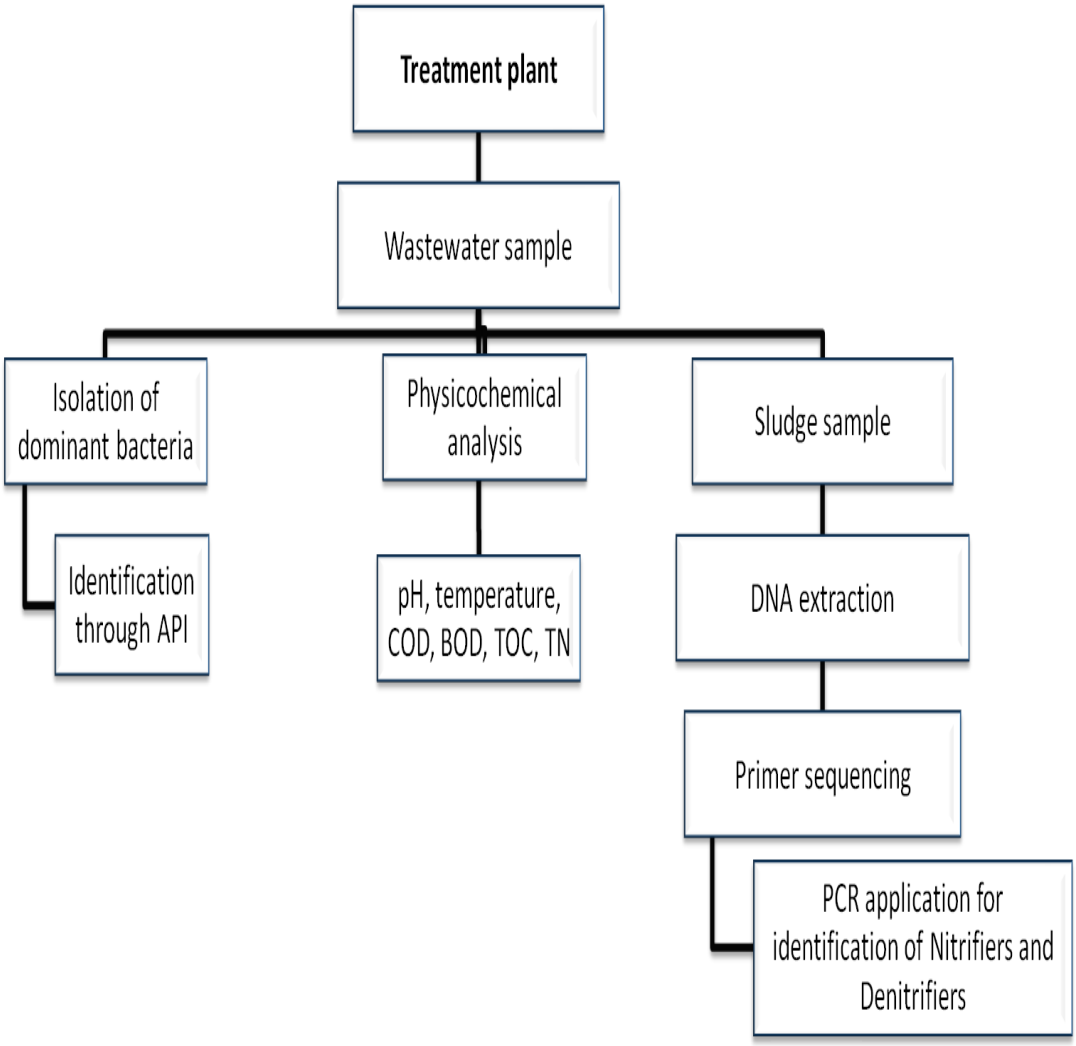

Figure 3.2: Research methodology

3.2.3.1. Study of Morphological Characteristics

Morphology of separated colony was studied by noting their form, elevation, size, margin, surface, odor, color, pigmentation, opacity and Gram reaction using standard techniques (Appendix A). Gram staining was performed as per methods described in standard methods (APHA, 2005).

Table 3.1: Attributes to study colony morphology

S. No	Morphological Attributes	Measuring Techniques
1.	Form	Circular, irregular, rhizoid
2.	Elevation	Flat, raised, convex, umbonate
3.	Size	Pinpoint, small, moderate or large
4.	Margin	Entire, lobate, undulate, filamentous
5.	Surface	Dull, shiny
6.	Pigmentation	Intracellular, extracelluar, non chromogenic
7.	Opacity	Opaque, translucent, transparent
8.	Gram reaction	Gram negative or positive

3.2.3.2. Analytical Profile Index

Identified microbes were confirmed using biochemical confirmation test and were counter checked by API kits. The API 20 NE employs plastic strip composed of 20 individual microtubes, each containing a dehydrated medium in the bottom and an upper cupule. The media become hydrated during suspension inoculation of the test organisms, and the strip was then incubated in a plastic covered tray to prevent evaporation. In this manner, 20 biochemical tests were performed. Following incubation, identification of the organisms was made by means of computer assisted

system called PRS (profile recognition system). PRS includes an API coder, profile register and selector (Appendix B).

Figure 3.3: API 20 NE strip

3.3. Primers Sequence

The PCR conditions and primers used in this experiment are listed in Table 3.2. The sequences were compared with the GeneBank database using BLASTN function (http://www.ncbi.nlm.nih.gov/).

3.4. Isolation and Identification of Nitrifying Bacteria

3.4.1. DNA Extraction from Sludge

Genomic DNA was isolated from activated sludge using a Norgen's Soil DNA Isolation Kit (Cat no. 26500, Canada) following the manufacturer's instructions.

i. Lysate preparation

250 mg of sludge sample was added to a provided bead tube and lysis solution of 750 µl was added into it. Vortexed briefly to mix sludge with lysis solution. 100 µl of lysis additive was added and mixed well. Tube was centrifuged for 1 minute at 14000 rpm. Upto 450 µl of supernatant was transferred to a

DNAase free microcentrifuge tube. 100 µl of binding solution was added to the tube and homogenized the tube by inverting it few times and incubated for 5 minutes on ice. The lysate was spinned for 1 minute at 14000 rpm to pellet any protein and sludge particles. Using a pipette, 450 µl of supernatant was transferred into a DNAase-free microcentrifuge tube. An equal volume of a 70 % ethanol was added to the lysate collected above and vortexed to mix.

Table 3.2: Targeted genes and their primer sequence details

Primers	**Sequence (5` ⟶ 3`)**	**Target genes**	**Anneal-ing Temp. (^{o}C)**	**References**
Pseudomonas aeruginosa				
nosZ-F	CGT TGT TCA TCG ACA GCC AG	N_2 O reductase (nosZ)	54	Throback et al., 2004
nosZ 1622R	CGC ACC TTC TTG CCC TTG C	N_2 O reductase (nosZ)	55	
nirScd3aF	GTC AAC GTC AAG GAA ACC GG	Nitrite reductase	54	Srinandan et al., 2011
nirS R3 cd	GAC TTC GGA GTC TTG AC	Nitrite reductase	54	
PA-SS-F	GGG GGA TCT TCG GAC CTC A	16S rRNA	58.7	Figuerola and Erijman, 2010
PA-SS-R	TCC TTA GAG TGC CCA CCC G	16S rRNA	59.2	
Nitrosomonas europaea				
Neu1265F	GCCAATCTCAGAAAGCAC	16S rRNA	48	Montras et al., 2008
Neu1422R	TCTGGTGAAAACCACTCC	16S rRNA	48	
Nitrobacter winogradskyi				
NxrB 1F	ACGTGGAGACCAAGCCGGG	norB	57.6	Montras et al., 2008
NxrB 1R	CCGTGCTGTTGATCTCGTTGATCT	norB	57.4	
Nitrospira sp.				
NSR 1113F	CCTGCTTTCAGTTGCTACCG	16S rRNA	53.8	Figuerola, and Erijman, 2010
NSR 1264R	GTTTGCAGCGCTTTGTACCG	16S rRNA	53.8	

ii. Binding to a column

A spin column was assembled with one of the provide collection tubes. Up to 600 µl of the clarified lysate with ethanol was applied onto the column and centrifuged for 1 minute at 14000 rpm. The flow through was discarded and reassembled the spin column with the collection tubes.

iii. Column wash

500 µl of wash solution I was applied to the column and centrifuged for 1 minute. Discarded the flowthrough and reassembled the spin column with its collection tube. 500 µl of wash solution II was applied to the column and centrifuged for 1 minute. The flowthrough was discarded and reassembled the spin column with its collection tube. Column was spined for 2 minutes in order to thoroughly dried the resin. The collection tube was discarded.

iv. DNA elution

The column was placed into a fresh 1.7 ml elution tube provided with the kit. 50 µl of elution buffer was added to the column and centrifuged for 2 minutes at 2000 rpm followed by a 1 minute spin at 14000 rpm.

v. Storage of DNA

The purified genomic DNA was stored at -20^{o}C. The quantity and purity of DNA obtained from the sludge were determined using Nanodrop (Eppendorf biophotometer Plus).

3.4.2. Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out to analyse the amplified DNA sample. 1 % (w/v) agarose gel was prepared by melting 0.6 g of agarose in 60 ml of 1x TBE buffer (10.8 g of Tris base, 5.5 g of boric acid and 0.93 g of EDTA

dissolved in 1 L of distilled water) in microwave oven for 2 minutes. Ethidium bromide solution (0.5 µg/ml final concentration) was added to stain DNA.

Amplified DNA sample were mixed with loading dye and loaded into the wells. Electrophoresis was performed at 100 volts for half an hour in 1x TBE running buffer. After electrophoresis, amplified products were visualized by placing the gel on UV transilluminator.

3.4.3. *Nitrosomonas europaea*

PCR was performed to amplify DNA. The reaction mixture (10 µl) for PCR was prepared having composition given in Table 3.3.

The reaction mixture was processed through thermocycling condition consisting of 95^{o}C for template denaturation followed by 40 cycles of amplification for each consisting of three steps: 60 sec at 95^{o}C for DNA denaturation into single strand, 1 minute at 52.2^{o}C for primer to hybridize or anneal to their complementary sequences on either side of the target sequence and 1 minute at 72^{o}C for extension of complementary DNA strand from each primer followed by 10 minutes at 72^{o}C for Taq DNA polymerase to synthesize any unextended strand left.

Figure 3.4: PCR (Company: Extra Gene 9600)

PCR was performed using thermo cycler (Extra Gene 9600).Finally, PCR products were subjected to agarose gel (1%) electrophoresis against 100bp DNA marker, stained with loading dye and visualized using UV transluminator.

Table 3.3: PCR mixture for *Nitrosomonas europaea*

Reagents	Concentration	Volume (µl)
$MgSO_4$	20 mM	0.5
dNTPs	200 µM	0.7
Taq buffer	10 X	1
Forward primer	1 µM	2
Reverse primer	1µM	2
DNA template	1-100 ng/µl	1
Taq polymerase	500 U/ µl	0.3
PCR water	-------	2.5

3.4.4. *Nitrobacter winogradskyi*

PCR amplification of nitrite oxidoreductase β subunit (norB) of *Nitrobacter winogradskyi* was performed in a total volume of 25 µl with composition mentioned in Table 3.4. The PCR thermocycling steps for nxrB primer sets were as follows: 95^{o}C for 5 min, and 40 cycles at 95^{o}C for 1 min, 57.4^{o}C for 1 min, 72^{o}C for 1 min and final elongation at 72^{o}C for 10 min. Negative controls and a blank (no DNA added) were included in all of the PCR amplification experiments. PCR was performed using thermo cycler (Extra Gene 9600).

3.4.5. *Nitrospira sp.*

Detection of 16S rRNA of *Nitrospira sp.* was performed in a total volume of 10 µl having recipe mentioned in Table 3.5. For the 16SrRNA gene detection of *Nitrospira sp.*, the PCR program includes 5 min at 95°C, and 40 cycles at 95°C for 1 min, 61°C for 1 min, 72°C for 1 min and final elongation at 72°C for 10 min.

Table 3.4: PCR mixture for *Nitrobacter winogradsky*i

Reagents	Concentration	Volume (µl)
$MgSO_4$	20 mM	2.5
dNTPs	2.5 mM	2
Taq buffer	10 X	2.5
Forward primer	2 µM	1
Reverse primer	2 µM	1
DNA template	1-100 ng/µl	4
Taq polymerase	500 U/ µl	0.3
PCR water		11.7

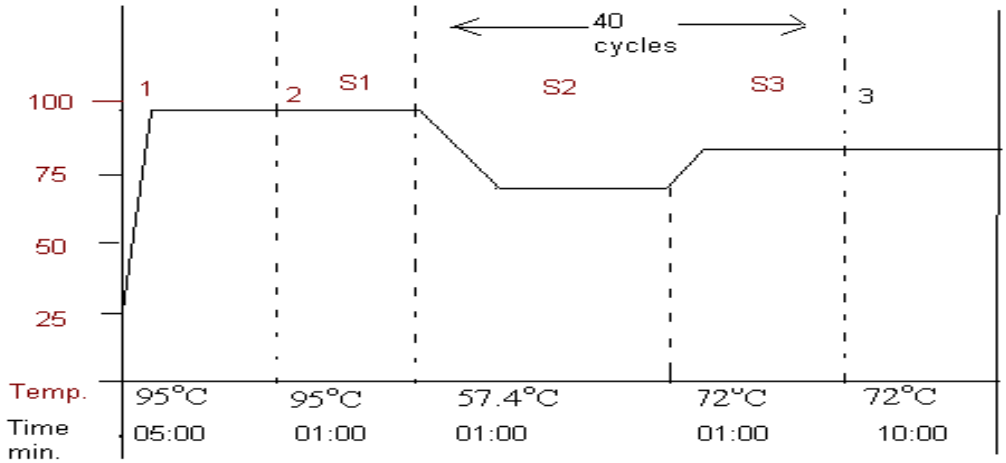

Figure 3.5: PCR programme for *Nitrobacter winogradskyi*

Table 3.5: PCR mixture for *Nitrospira sp.*

Reagents	Concentration	Volume (µl)
$MgSO_4$	20 mM	0.5
dNTPs	2.5 mM	0.7
Taq Buffer	10 X	1
Forward primer	1 µM	1
Riverse primer	1 µM	1
DNA template	1-100 ng/µl	1
Taq	500 U/ µl	0.3
PCR water		4.5

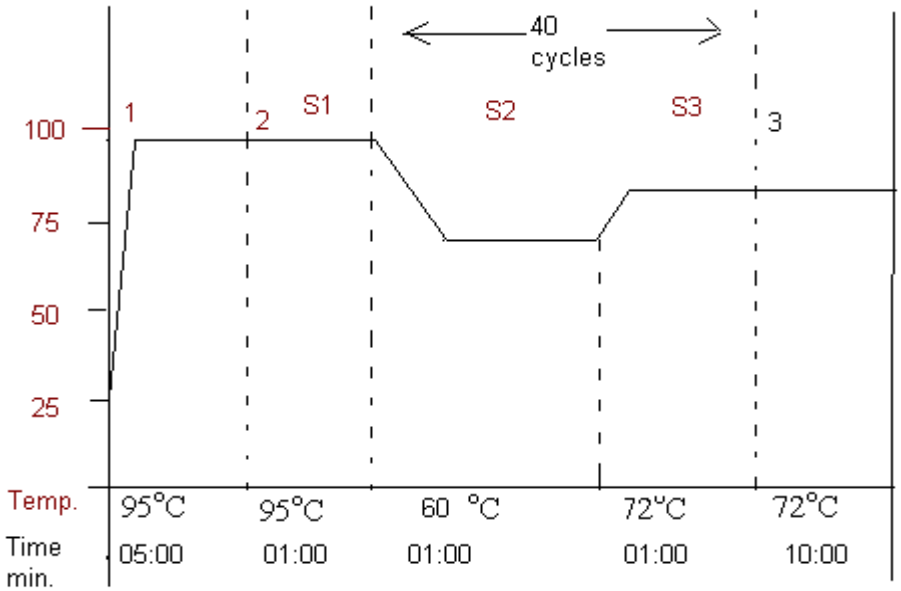

Figure 3.6: PCR programme for *Nitrospira sp.*

3.5. Isolation and Identification of Denitrifying Bacteria

3.5.1. Isolation of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa was isolated from aeration tank using Cetrimideagrar which is selective agar for said specie. Pure culture of *Pseudomonas aeruginosa* (ATCC 27853) was also used as a reference in the study.

3.5.2. DNA Extraction from Wastewater

Genomic DNA was extracted from pure cultures using the following technique (Phenol chloroform method).

A simple and rapid method was used for extraction of bacterial genomic DNA. The bacterial culture after making suspension with 0.85 % NaCl, was centrifuged at 12, 000 rpm for 3 minutes. 1.5 ml of a saturated culture was harvested after centrifugation. The cell pellet was resuspended and lysed in 200 µl of lysis buffer (40 mM Tris-acetate pH 7.8, 20 mM sodium-acetate, 1 mM EDTA, 1% SDS) by vigorous pipetting. To remove most proteins and cell debris, 66 µl of 5M NaCl solution was added and mixed well, The viscous mixture was centrifuged at 12,000 rpm for 10 minutes at 4°C. The clear supernatant was transferred into a new vial and equal volume of chloroform was added.

The tube was gently inverted at least 50 times when milky solution was completely found. Following centrifugation at 12,000 rpm for 3 min, the extracted supernatant was transferred to another vial. DNA was precipitated with 100 % ethyl alcohol and left for an hour and then centrifuged for five minutes at 12000 rpm. DNA pellet was washed twice with 70 % ethyl alcohol and centrifuged for 2 minutes at 12000 rpm. It was dried in speed-vac, and finally the pellet was re-suspended in 50 µl 1 x TE buffer. The size distribution of genomic DNA was

determined by electrophoresis on a 1% agarose gel. Ethidium bromide was used as a dyeing agent.

3.5.3. *Pseudomonas aeruginosa*

Detection of 16S rRNA of *Pseudomonas aeruginosa* was performed in a total volume of 25 µl having recipe mentioned in Table 3.7.

Table 3.6: Mixture for 16SrRNA gene detection in *Pseudomonas aeruginosa*

Reagents	Concentration	Volume (µl)
$MgSO_4$	20 mM	2.5
dNTPs	2.5 mM	2
Taq buffer	10 X	2.5
Forward primer	2 µM	1
Riverse primer	2 µM	1
DNA template	1-100 ng/µl	3
Taq polymerase	500 U/ µl	0.3
PCR water		12.7

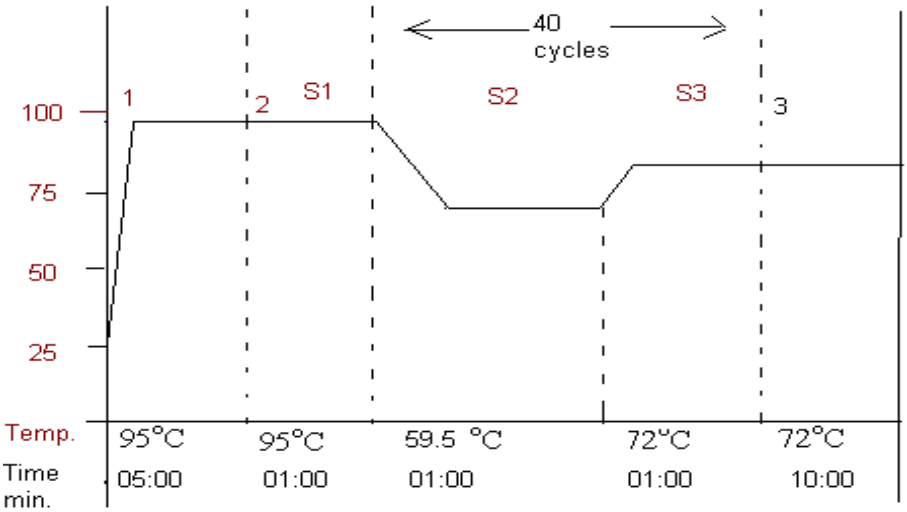

Figure 3.7: PCR programme for *Pseudomonas aeruginosa*

Chapter 4

RESULTS AND DISCUSSION

4.1. Microbial Identification through API

Despite there are other microorganisms than bacteria in activated sludge process, a review of bacteria was of special interest of this study. The bacterial population is proven to be diverse in the activated sludge process. The extent and nature of activity 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 the presence of high bacterial diversity in aeration tank.

Table 4.1: Isolation of predominant colonies

Serial No.	Sample	No of isolates	No. of bacteria (CFU/ml)
1.	Inlet	07	51.0×10^5
2.	Aeration tank	14	780.0×10^5
3.	Outlet	07	0.00033×10^5

Molecular technique has shown that the dominant organisms were those of the beta class of the Proteobacteria, whereas the culture methods had shown that the gamma sub-class of proteobacteria was dominant in the activated sludge process (Figure 4.2). Generally, this group of bacteria may be involved in carbon oxidizers, nitrogen oxidizers, floc-formers, non floc-formers, predators, nuisance organisms, aerobes and facultative anaerobes. Table 4.2, 4.3 and 4.4 shows a summary of some principal genera of bacteria found in activated sludge process. Some of these genera were also being reported by Rittman and Mc Carty (2001) and Gerardi (2002).

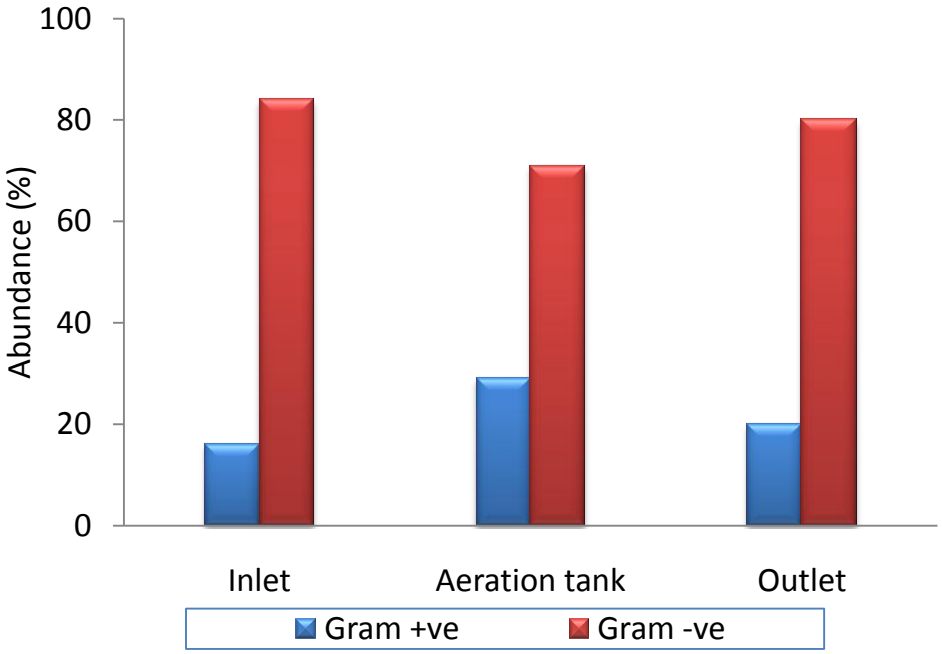

Figure 4.1: Gram staining for isolates of sewage treatment plant

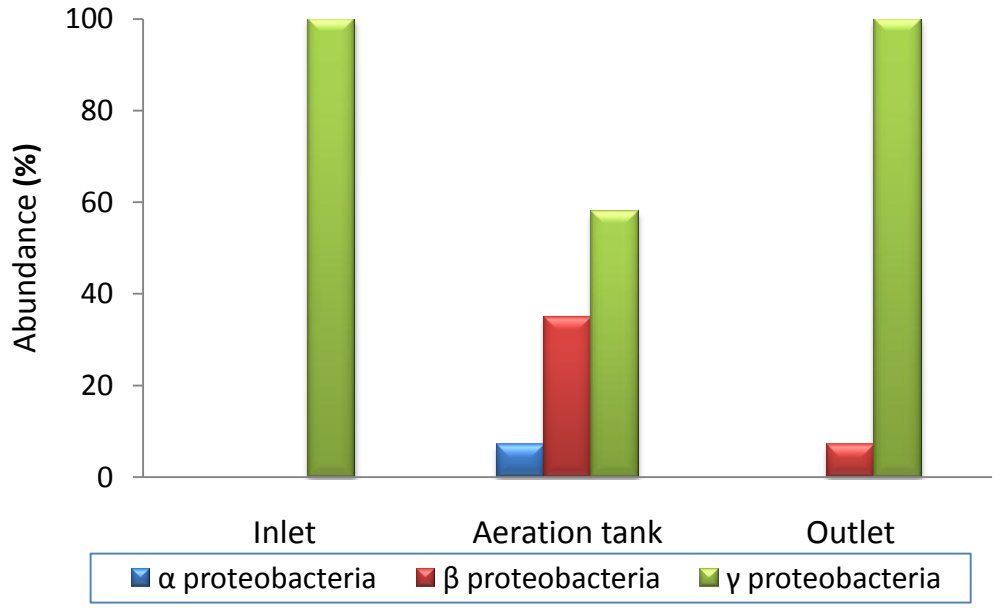

Figure 4.2: Classification of isolates extracted from sewage treatment plant

Twenty eight distinct colonies were selected from the spread plate and streak plate technique by visual observation and their colony morphology was studied (Appendix A). On the basis of gram reaction, only 17 % of colonies were found to be gram positive bacteria (Figure 4.1). All gram negative bacteria were subjected for API analysis. Almost all isolates from inlet (Table 4.2) belonged to γ proteobacteria (family: *Enterobacteriaceae, Pseudomonadaceae*). While high bacterial diversity was found to be in aeration tank where 7, 35 and 57 % isolates belongs to class α, β and γ proteobacteria respectively. So, γ proteobacteria generally found to dominate the sludge habitats, suggesting the adaptability of this group (Srinandan et al., 2011).

Table 4.2: Isolated species from influent of STP identified through API

Isolates	**API code**	**Species**	**Family (Class)**
1.	2002002	*Pseudomonas aeruginosa*	Pseudomonadaceae (γ)
2.	0004052	*Shigella sp.*	Enterobacteriaceae (γ)
3.	2002110	*Aeromonas salmonicida sp. salmonicida*	Aeromonadeceae (γ)
4.	2002113	*Pantoea sp.*	Enterobacteriaceae (γ)
5.	6206253	*Serratia odorifera*	Enterobacteriaceae (γ)
6.	5215773	*Klebsiella pneumonia sp. ozaenae*	Enterobacteriaceae (γ)
7.	5144552	*Escherichia coli*	Enterobacteriaceae (γ)

Klebsiella pneumonia is the clinically most important specie of genus *klebsiella.* 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). *Psuedomonas* species are responsible for denitrification while *Raoultella terrigena* biodegrades lipid and has been studied for its better efficiency.

Table 4.3: Species isolated from aeration tank of STP identified through API

Isolates	**API code**	**Species**	**Family (Class)**
1.	0005042	*Acinetobacter baumannii*	Moraxellaceae(γ)
2.	2006004	*Chromobacterium violaceum*	Neisseriaceae (β)
3.	1067167	*Aeromonas hydrophila*	Aeromonadeceae (γ)
4.	6303002	*Burkholderia cepacia*	Burkholderiaceae (β)
5.	0003004	*Bordetella parapertussis*	Alcaligenaceae(β)
6.	2002002	*Pseudomonas aeruginosa*	Pseudomonadaceae (γ)
7.	6202000	*Stenotrophomonas maltophilia*	Xanthomonadaceae (γ)
8.	7316573	*Serratia liquefaciens*	Pseudomonadaceae (γ)
9.	3002024	*Vibrio fluvialis*	Vibrionaceae (γ)
10.	0002020	*Pasteurella pneumotropica*	Pasteurellaceae (γ)
11.	1203004	*Brucella sp.*	Brucellaceae (α)
12.	1002004	*Chryseobacterium meningosepticum*	Flavobacteriaceae (β)
13.	1006767	*Serratia plymuthica*	Enterobacteriaceae (γ)
14.	1202004	*Burkholderia cepacia*	Burkholderiaceae (β)

Fourteen different gram negative strains were isolated from aeration tank and identified. Their details along with family information given in Table 4.3. belongs to family *Xanthomonadaceae, Enterobacteriaceae, Pseudomonadaceae,*

Neisseriaceae, Moraxellaceae, Burkholderiaceae, Brucellaceae, Vibrionaceae and Pasteurellaceae. The culturable approach revealed *Pseudomonas spp*. to be the main group at all sources. Several studies have reported the species of this genus as being the most commonly isolated in treatment plants. Their ability to grow supported by a wide range of organic substrates without any specific nutritional requirements, explain their ubiquity and the high number of strains isolated. Presence of some betaproteobacteria in aeration tank may help in degradation of different hydrocarbons and the respiration of nitrogenous oxide in anoxic conditions may enhanced their capability of thriving in activated sludge environments (Parales, 2010).

Table 4.4: Isolated species from Outlet of STP identified through API

Isolates	**API code**	**Species**	**Family (Class)**
1.	1046042	*Pseudomonas luteola*	Pseudomonadaceae (γ)
2.	5215773	*Klebsiella pneumoniae sp. ozaenae*	Enterobacteriaceae (γ)
3.	4004672	*Raoultella terrigena*	Enterobacteriaceae (γ)
4.	1002004	*Chryseobacterium meningosepticum*	Flavobacteriaceae (β)
5.	2002002	*Pseudomonas aeruginosa*	Pseudomonadaceae (γ)
6.	7002000	*Stenotrophomonas maltophilia*	Enterobacteriaceae (γ)
7.	5144552	*Escherichia coli*	Enterobacteriaceae (γ)

All dominant species of effluent were from family *Xanthomonadaceae, Enterobacteriaceae, Pseudomonadaceae* (Table 4.4).

4.2. 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 4.3.

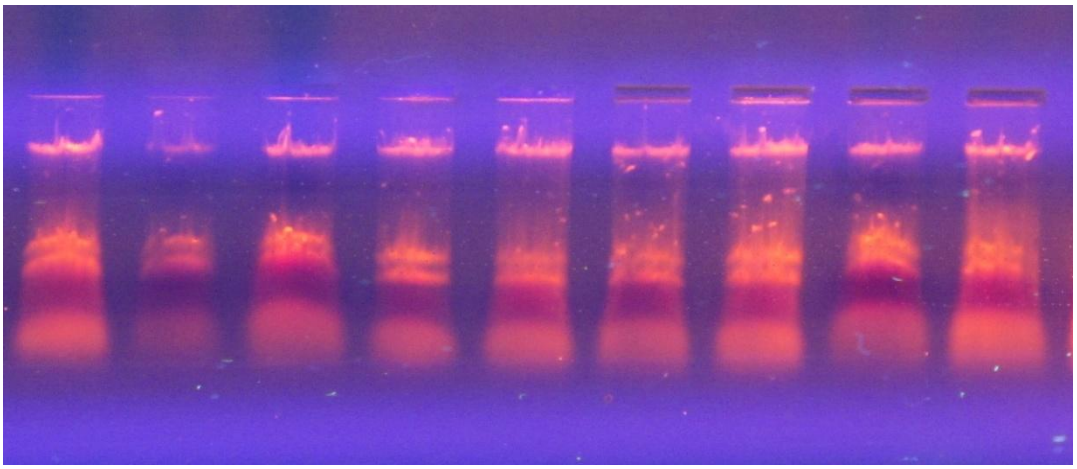

Figure 4.3: Gel picture for DNA extraction by phenol chloroform method

4.3. Detection of Nitrifying Bacteria through PCR

All the primers used in this study were rigorously tested for specificity by their designers. These were tested with the objective that would these primer sets maintain their specificity in case the annealing temperature was changed. To increase the amplification efficiency, the primer concentration in the reaction mixture targeting 16S rRNA gene of *Nitrosomonas europaea* and norB gene of *Nitrobacter winogradskyi* was raised from 1 µM to 2 µM respectively. Application of the selected primers to PCR using DNA of targeted organisms caused single band of the theoretically expected size. Figures 4.4-4.7 shows the results on agarose gels.

The primers Neu 1265F and Neu1422R (Montras et al., 2008) were used to amplify a 400 base pairs (bp) fragments of 16SrRNA gene of ammonia oxidizing bacteria (AOB). The PCR products were examined on 2 % (w/v) ethidium bromide-stained agarose gel (Figure 4.4).

The suitability of molecular biological techniques to detect major nitrifiers and denitrifiers was assessed. *Nitrosomoans europeae* was found to be the major ammonia oxidizing bacteria that causes nitrification. The activated sludge plant was successfully performing nitrification and AOB are removing larger part of the ammonia by nitrification. Validity of this assumption is confirmed by the abundance of AOB (Typically 2.2×10^{14} to 2.5×10^{14}) and the absence of NH_4^+-N. The increased nitrate concentration is another justification of the assumption. Relying on the physic-chemical data (Table 4.5), the AOB and NOB are confirmed as responsible for ammonia removal efficiency, mostly by coupled process of nitrification and denitrification.

Nitrospira sp. were also detected as the dominant NOB in most of the sludge samples. A combination of longer SRT and low temperature, winter operational conditions, may favor *Nitrospira sp*. to enhance the activity and efficiency. The *Nitrospira sp*. population was observed less significant than *Nitrobater sp*. Their presence may be an element for maintaining good nitrification, specifically, when the conditions are not suited for nitrifier growth in the winter (Siripong and Rittmann, 2007).

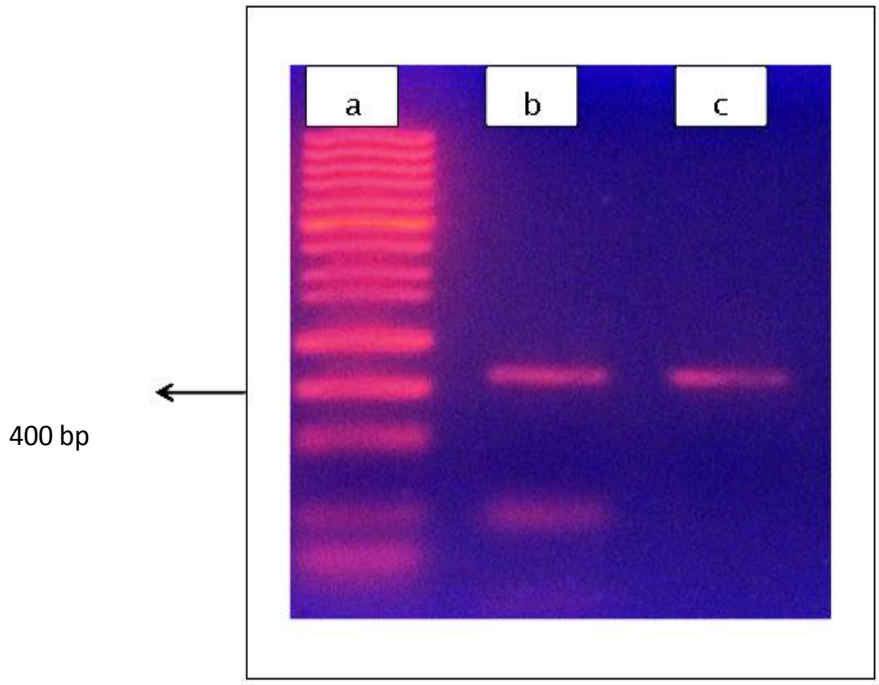

Fig. 4.4: (a) 100bp ladder (b) & (c) amplified AOB 16SrRNA gene in activated sludge samples

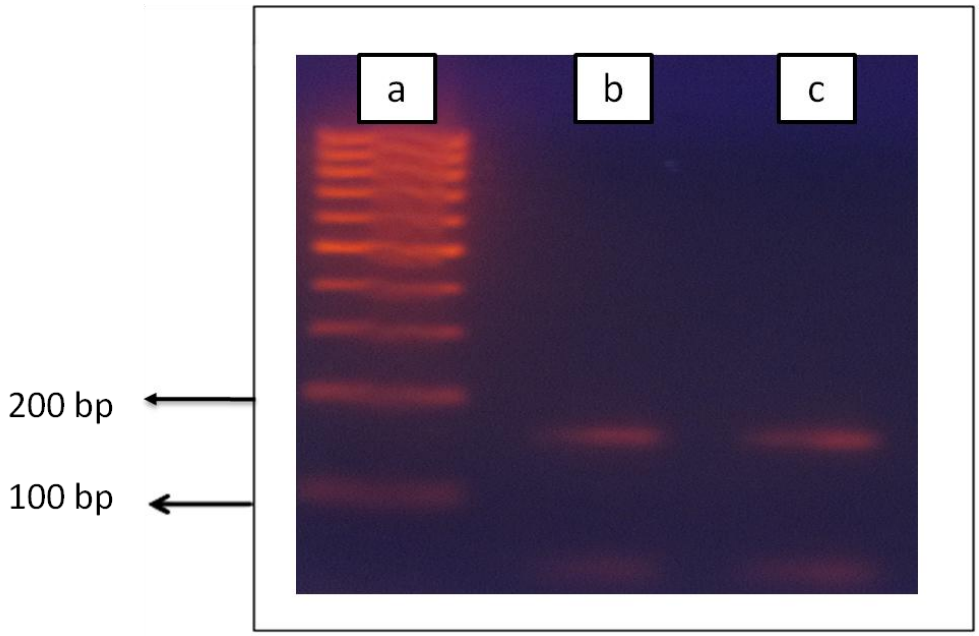

Figure 4.5: (a) 100 bp ladder (b) & (c) amplified 16SrRNA gene in activated sludge samples

The primers NSR 1113F and NSR 1264R (Figuerola and Erijman, 2010) were used to amplify a 158 base pairs (bp) fragments of 16S rRNA gene of *Nitrospira sp.* (NOB). PCR product was examined by electrophoresis on 1.5 % agarose gel (Figure 4.5).

Results also demonstrated the presence of *Nitrobacter winogradskyi* in the treatment plant as nitrite oxidizing bacteria (NOB) as Nxr B gene has been detected by PCR (Figure 4.6). The result was in accordance with the results of activated sludge from other researchers (Montras et al., 2008; Layton et al., *2005).* The primers NxrB 1F and NxrB1R (Montras et al., 2008) were used to amplify 98 base pairs (bp) fragments of norB gene of nitrite oxidizing bacteria (NOB). Finally, PCR products were subjected to agarose gel (2%) electrophoresis against 50bp DNA marker, stained with loading dye and visualized using UV transluminator.

PCR may give relatively rapid results, into the bacterial activity importantly with biological gradient. Because of the abundance of nitrifers and denitrifiers, it seems that they play crucial role in the high removal of NH_3 and NH_4^+-N, NO_3^-, NO_2^- form the effluents (Milner et al., 2008). Recent studies also confirmed that AOB and NOB have a complex metabolism that includes both aerobic and anaerobic ammonia oxidation and denitrification (Schmidt et al., 2003).

In the activated sludge process, nitrifying bacteria are able to increase in number only if their reproductive rate is greater than their removal rate through sludge wasting and discharge in the final effluent. Therefore a high MCRT is required to increase the number of nitrifying bacteria in the ASP. The nitrifier found to be strict aerobes, so nitrification occurs only under aerobic conditions at dissolved oxygen levels of 1.0 mg/L or more. Nitrification requires a long retention

time, a low food to microorganism ratio (F:M), a high mean cell residence time, and adequate buffering (alkalinity).

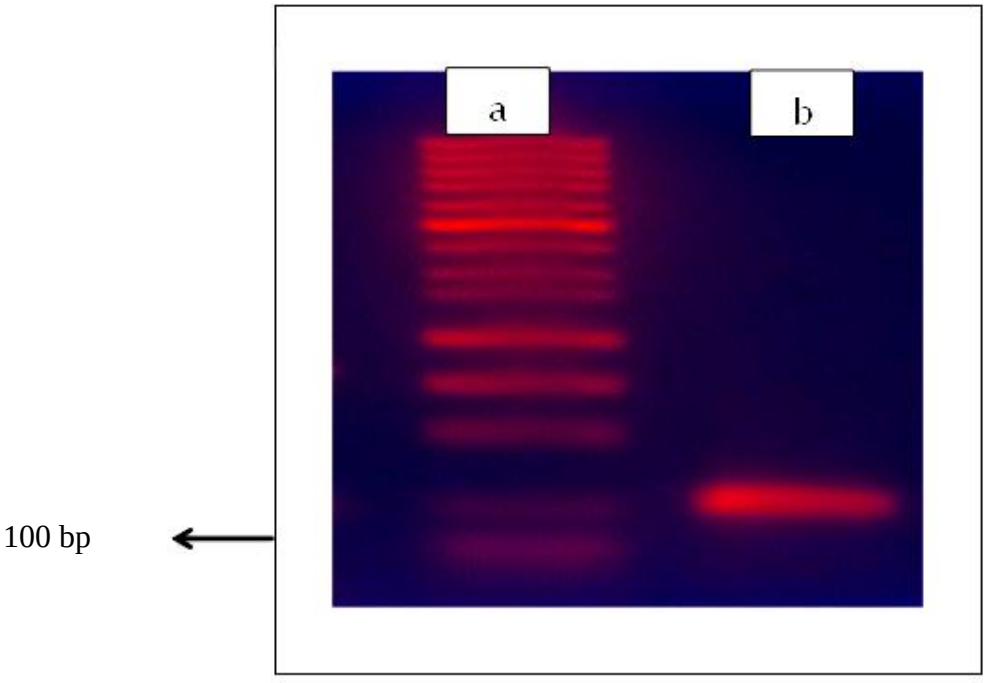

Figure 4.6: (a) 50 bp ladder (b) amplified Nxr B gene in activated sludge samples

The nitrification process produces acid. This acid formation may lowers the pH of the biological population in the aeration tank and can cause a reduction of the growth rate of nitrifying bacteria. The optimum pH for *Nitrosomonas* and *Nitrobacter* was found to be between 7.5 and 8.5.

4.4. Detection of Denitrifying Bacteria through PCR

The process may occur under anoxic condition specially when oxygen levels are depleted and nitrate becomes the primary oxygen source for microorganisms. Conditions that affect the efficiency of denitrification may include nitrate concentration, anoxic conditions, presence of organic matter, pH, temperature, alkalinity and the effects of trace metals. Denitrifying organisms

found to be generally less sensitive to toxic chemicals than nitrifiers, and recover from toxic shock loads quicker than nitrifiers.

Denitrifying bacteria like Pseudomonas sp. was at a high percentage and played a significant role in removal of NO_3 and other organic compounds. *Pseudomonas aeruginosa* was always detected in all activated sludge samples. The three sets of primers including nosZ, nirS and PA-SS were used to amplify N_2 O reductase, Nitrite reductase and 16S rRNA gene of *Pseudomonas aeruginosa* (denitrifier).

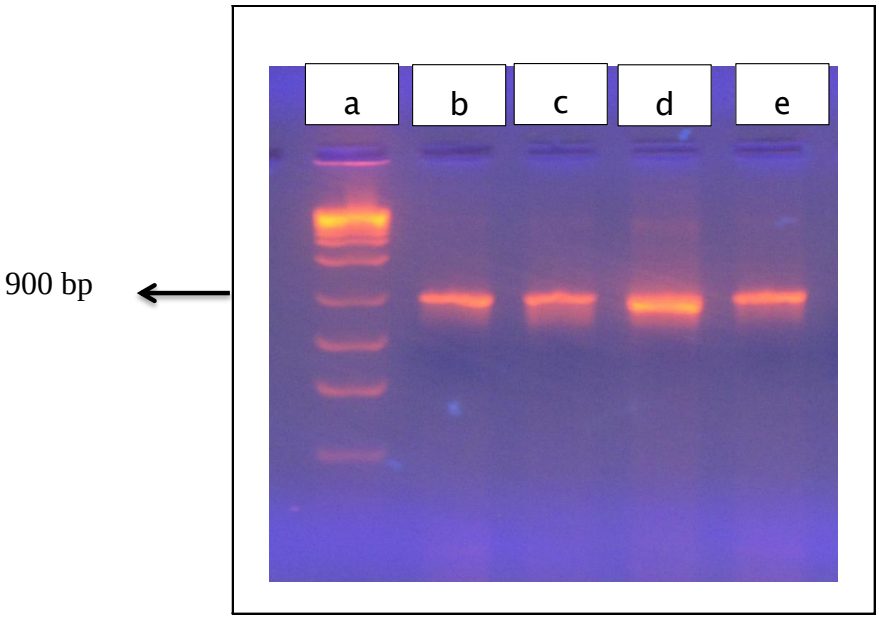

Figure 4.7: (a) 1 kb ladder (b) amplified 16SrRNA gene of *Pseudomonas aeruginosa* (ATCC 27853) (c, d & e) samples from STP

The three sets of primers including nosZ, nirS and PA-SS were used to amplify N_2 O reductase, Nitrite reductase and 16S rRNA gene of *Pseudomonas aeruginosa* (denitrifier). PCR amplification products of 910 bp were obtained (Figure 4.7.) for all the isolates and the reference organism (ATCC 27853). Only

with the nirS and nosZ primer pair, no amplification was detected in the sludge samples. Therefore, these primers were not used for further analyses. Denitrifying bacteria like *Pseudomonas sp*. was at a high percentage and played a significant role in removal of NO_3^- and other organic compounds. Among all *Pseudomonas sp*., *Pseudomonas aeruginosa* was always detected in all activated sludge samples.

Efficiencies were observed highest at SRT of 7 days as shown in table 4.5, whereas data on SRT of 5 and 6 days were taken from the recent study conducted by Shehzad, (2012). It was taken for the purpose of comparison. Efficiency is well depicted by, the COD and BOD removal i.e. 77 and 81 percent respectively, 52 percent removal of total nitrogen shows the dominance of the nitrifiers and denitrifiers in the system. As nitrifying bacteria are slow growing so Longer SRT is required with the fact that crucial nitrifying bacteria requires extended growth period that should be more than 5 days.

Table 4.5: Physico-chemical analysis of STP I-9, Islamabad

Parameters	**Removal efficiency (%)**		
	5d	**6d**	**7d**
TSS (mg/L)	93	90	91
COD (mg/L)	78	80	77
BOD (mg/L)	83	86	81
TN (mg/L)	46	48	52
TOC (mg/L)	72	78	67

Chapter 5

Conclusions and Recommendations

The proper knowledge of the composition and dynamics of microbes from aerated compounds of ASP is crucial to assure the proper working of wastewater treatment plants. PCR based approach used in this study has demonstrated to be effective in getting new information concerning the growth and dynamics of these communities with accuracy and rapidity. Study discovered dominant microbial community making ground for enhancing efficiency of treatment plant resulting in low input cost. Through the application of molecular techniques, in particular, PCR, the culture techniques commonly used are not capable of providing full details of the bacterial population in activated sludge process. The bacterial population proven to be diverse in the activated sludge process. PCR results has shown that the dominant organisms were those of the beta class of the Proteobacteria, whereas the culture methods had shown that the gamma sub-class of proteobacteria was dominant in the activated sludge process.

5.1. Conclusions

Following conclusions were drawn from this study:

- Dominance of the γ-proteobacteria was observed as 7, 35 and 57 % isolates belonged to class α, β and γ proteobacteria respectively in aeration tank.
- *Nitrosomoans europaea* was detected as nitrifier which causes ammonia oxidation and helps in conversion of ammonium ion into nitrite ion.
- *Nitrobacter winogradskyi* and *Nitrospira sp.* were detected as a part of nitrite oxidizing bacteria.
- *Pseudomonas aeruginosa* was detected as denitrifier.

- 52 % removal of total nitrogen shows the dominance of the nitrifiers and denitrifiers in the system. As higher SRT is necessary to set up a population of nitrifying microorganisms that are capable to perform effective nitrification.
- PCR technique using 16S rRNA approach found to be rapid and useful for the comparison of the population abundance in wastewater samples.

5.2. Recommendations

Following recommendations are important for further study:

- Real time PCR along with DGGE is required to study quantitative analysis of nitrifying and denitrifying activated sludge system.
- Phylogenetic analysis of all detected genes should be constructed for a deeper understanding of the complex variables and communities involved in the N-cycling of sewage treatment plant.
- Seasonal variations of bacterial community should be observed to understand better community composition of activated sludge.

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Appendix A

Appendix A

Table A1 : Colony morphology of species isolated from Inlet

Isolates	Shape	Elevation	Margin	Color	Pigmentation	Odor	Surface	Gram reaction	Structure	Opacity	Shine
1.	Circular	Flat	Lobute	Creamy	Yes	Smelly	Rough	Pink	Chained rods	Opaque	No
2.	Circular	Flat	Entire	Yellowish creamy	No	Smelly	Smooth	Pink	Individual coccoi	Opaque	Yes
3.	Circular	Slightly raised	Entire	Yellow	No	Smelly	Smooth	Pink	Individual coccoi	Opaque	Yes
4.	Circular	Flat	Entire	Creamy	No	Smelly	Rough	Pink	Individual + chained coccoi	Translucent	No
5.	Circular	Flat	Entire	Creamy	No	Smelly	Smooth	Pink	Cocco bacillus	Translucent	No
6.	Circular	Raised	Entire	Yellow	Yes	Smelly	Smooth	Pink	Individual coccoi	Translucent	Yes

Table A2: Colony morphology of species isolated from effluent

Isolates	Shape	Elevation	Margin	Color	Pigmentation	Odor	Surface	Gram reaction	Opacity	Shine
1.	Irregular	Flat	Undulate	Creamy	Yes	Smelly	Rough	Pink	Transparent	No
2.	Circular	Raised	Lobate	Off white	Yes	Smelly	Smooth	Pink	Translucent	No
3.	Circular	Convex	Lobate	Yellow	No	Smelly	Wrinkled	Pink	Opaque	No
4.	Irregular	Convex	Entire	Creamy	No	Smelly	Smooth	Pink	Opaque	No
5.	Circular	Flat	Entire	Creamy	No	Smelly	Smooth	Pink	Translucent	No
6.	Circular	Raised	Entire	Yellow	Yes	Smelly	Smooth	Pink	Translucent	Yes

Table A3: Colony morphology of species isolated from Aeration Tank

Sample no.	Shape	Elevation	Margin	Color	Pigmentation	Odor	Surface	Gram reaction	Opacity	Shine
1.	Circular	Flat	Lobate	Off white	No	Smelly	Smooth	Pink	Translucent	Yes
2.	Circular	Raised	Entire	Yellowish creamy	No	Odorless	Smooth	Pink	Translucent	Yes
3.	Circular	Flat	Undulate	Creamy	No	Odorless	Rough	Pink	Opaque	No
4.	Circular	Raised	Entire	Yellow	No	Smelly	Smooth	Pink	Opaque	No
5.	Circular	Flat	Loabte	Off white	No	Smelly	Wrinkled	Pink	Translucent	No
6.	Circular	Convex	Entire	Creamy	Yes	Smelly	Dull	Pink	Translucent	Yes
7.	Irregular	Flat	Entire	Creamy	No	Odorless	Smooth	Pink	Opaque	Yes
8.	Circular	Raised	Entire	Pale white	No	Bit smelly	Rough	Pink	Opaque	No
9.	Irregular	Raised	Entire	Off white	No	Smelly	Smooth	Pink	Opaque	No
10.	Irregular	Convex	Lobate	Pale yellow	Yes	Odorless	Smooth	Pink	Translucent	No
11.	Circular	Flat	Lobate	Yellow	No	Smelly	Rough	Pink	Transparent	No
12.	Circular	Raised	Lobate	Off white	No	Smelly	Rough	Pink	Translucent	No
13.	Circular	Flat	Lobate	Creamy	No	Smelly	Wrinkled	Pink	Opaque	No
14.	Circular	Raised	Entire	Creamy	Yes	Smelly	Smooth	Pink	Translucent	Yes
15.	Irregular	Raised	Lobate	Creamy	No	Smelly	Smooth	Pink	Opaque	No
16.	Circular	Flat	Undulate	Yellow	Yes	Smelly	Smooth	Pink	Opaque	Yes
17.	Circular	Flat	Entire	Off white	No	Bad smell	Rough	Pink	Opaque	No

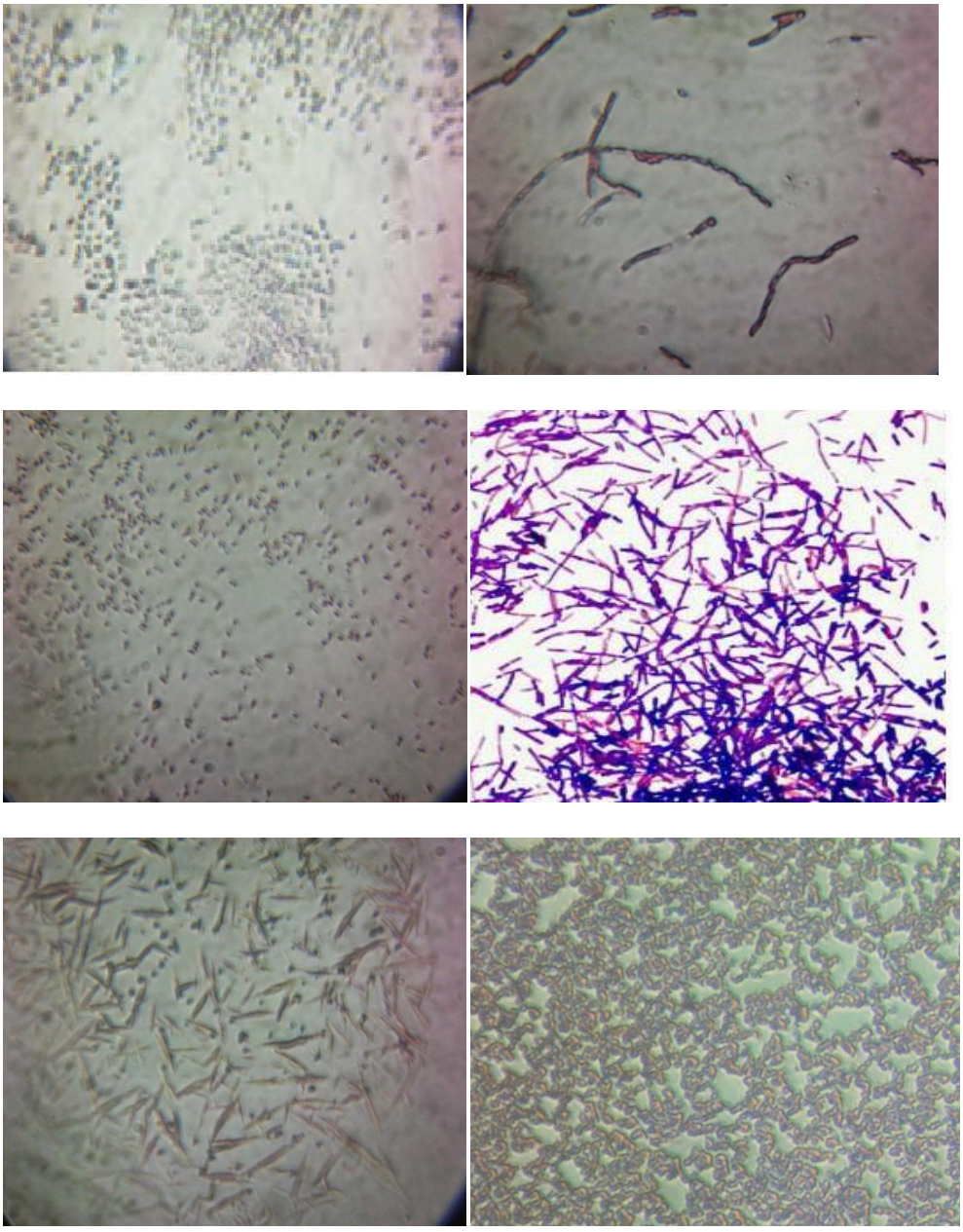

Figure A1: Results of gram reaction

Appendix B

Appendix B

Protocol for Analytical Profile Index 20NE

1. The elongated flap on the incubation tray was labelled with the number of unknown culture.
2. With a pipette, approximately 5 ml of distilled water was added to the incubation tray.
3. Using a sterilized loop, an isolated colony on a streak plate culture was touched and transferred the inoculum to a 5 ml tube of sterile saline, and mixed well to get a uniform suspension.
4. Using a Pasteur pipette containing the bacterial saline suspension, the tube section of each compartment was filled by placing the tip of the pipette against the side of the cupule.
5. The cupules in the CIT, VP and GEL microtubes was completely filled with saline. While the remaining microtubes was filled using sterile mineral oil to provide an anaerobic environment. The inoculated strip was covered with the tray lid and incubated for 24 hrs at 37^{o}C.
6. The test reagants were added in the following order: kovac's reagent to IND, VP reagent 1 and 2 into VP, ferric chloride reagent to TDA.
7. After 15 minutes, color changes were observed and interpreted observation by determining the seven digit profile number. Unknown organisms were identified by referring to the profile recognition system.
8. 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% reagent was added. A color change to purple was noted as positive while no color change as negative result.
9.

Table B1: 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)	Black (digested)	No change
Glucose (GLU)	Yellow	Yellow green to blue
Mannitol	Yellow	Yellow green to blue
Inositol	Yellow	Yellow green to blue
Sorbitol	Yellow	Yellow green to blue
Rhamnos	Yellow	Yellow green to blue
Sucrose	Yellow	Yellow green to blue
Melibiose	Yellow	Yellow green to blue
Amygdaline	Yellow	Yellow green to blue
Arabinose	Yellow	Yellow green to blue

Appendix C

Appendix C

Table C1: API profile for species isolated from inlet

Biochemical tests	**Inlet**					
	1	**2**	**3**	**4**	**5**	**6**
ONPG	–	–	–	–	–	–
ADH	+	–	+	+	+	+
LDC	–	–	–	–	+	+
ODC	–	–	–	–	–	–
CIT	–	–	–	–	+	+
H_2S	–	–	–	–	–	–
URE	–	–	–	–	–	–
TDA	–	–	–	–	–	–
IND	–	–	–	–	–	–
VP	–	–	–	–	–	–
GEL	+	–	+	+	+	+
GLU	–	+	–	–	+	+
MAN	–	–	+	+	–	–
INO	–	–	–	–	+	+
SOR	–	–	–	–	–	–
RHA	–	+	+	+	+	+
SAC	–	–	–	–	–	–
MEL	–	+	–	–	+	+
AMY	+	–	–	+	+	+
ARA	+	+	–	+	+	+
OX	+	–	–	–	–	+
API Code	2002002	0004052	2002110	2002113	6206253	6206257

Table C2: API profile for species isolated from Aeration tank

Biochemical tests	Aeration pond							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
ONPG	–	–	+	–	+	–	–	+
ADH	–	+	–	+	+	+	+	+
LDC	–	–	–	+	+	+	+	+
ODC	–	–	–	+	–	–	–	+
CIT	–	–	–	+	–	+	+	+
H_2S	–	–	–	–	–	–	–	–
URE	–	–	–	–	–	–	–	–
TDA	–	–	+	–	–	–	–	+
IND	–	–	+	–	+	–	–	+
VP	+	–	+	+	–	–	–	–
GEL	–	+	+	+	+	+	+	–
GLU	+	+	+	–	+	–	–	–
MAN	–	–	+	–	–	–	–	+
INO	–	–	–	–	+	–	–	+
SOR	–	–	–	–		–	–	+
RHA	–	–	–	–	–	–	–	+
SAC	–	–	–	–		–	–	+
MEL	+	–	–	–	+	–	–	+
AMY	–	–	–	–	–	–	–	+
ARA	+	–	+	+	+	–	–	+
OX	–		+	–	–	+	–	–
API Code	0005042	2006004	1067106	6303002	7046662	6202004	6202000	7360773

Table C3: API profile for species isolated from Aeration tank

Biochemical tests	Aeration pond								
	9	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
ONPG	+	+	–	–	+	+	+	–	+
ADH	+	+	–	–	–	–	–	–	–
LDC	+	–	–	–	–	–	–	–	–
ODC	+	–	–	–	–	–	–	–	–
CIT	+	–	–	–	–	–	+	–	–
H_2S	–	–	–	–	–	–	–	–	–
URE	+	–	–	–	–	–	–	–	–
TDA	–	–	–	–	–	–	–	–	–
IND	–	–	–	–	–	–	–	–	–
VP	–	–	–	+	–	–	–	–	–
GEL	+	+	+	+	+	+	+	+	+
GLU	+	–	–	–	–	+	–	–	–
MAN	+	–	–	–	–	+	–	–	–
INO	–	–	–	–	–	+	–	–	–
SOR	+	–	–	–	–	+	–	–	–
RHA	+	–	–	–	–	–	–	–	–
SAC	+	+	+	–	–	+	–	–	–
MEL	+	–	–	–	–	+	–	–	–
AMY	+	–	–	–	+	+	–	–	–
ARA	+	–	–	–	–	+	–	–	–
OX	–	+	+	+	+	+	+	+	–
API Code	7316573	3002024	0002020	0003004	1002004	1006767	1202004	0002004	1002000

Table C4: API profile for species isolated from effluent

Biochemical tests	**Effluent**					
	1	**2**	**3**	**4**	**5**	**6**
ONPG	+	–	–	–	+	+
ADH	–	–	–	+	+	+
LDC	–	+	+	+	+	+
ODC	–	–	–	–	–	-
CIT	–	–	–	–	–	-
H_2S	–	–	–	–	–	-
URE	–	–	–	–	–	-
TDA	–	–	–	–	–	-
IND	+	–	–	–	–	-
VP	–	–	+	–	–	-
GEL	+	–	+	+	+	+
GLU	+	+	+	+	+	-
MAN	–	–	–	–	–	-
INO	–	+	+	+	+	-
SOR	–	+	+	+	+	-
RHA	–	+	+	+	+	-
SAC	–	+	+	+	+	-
MEL	+	+	+	+	+	-
AMY	–	–	–	–	–	-
ARA	+	+	+	+	+	-
OX	–	–	–	+	–	-
API Code	1046042	4004672	4007672	6006676	7006672	7002000