Chapter – 2

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Introduction
2.1 INTRODUCTION

One of the key issues in the water-resource management is the (indirect) re-use of waste water for drinking water supply or for industrial or agriculture purposes because of the scarcity of pristine waters. In this context, the fate and effects of organic contaminants in wastewater entering the environment has gained more attention. For more than one hundred years wastewater treatment has become one of the world largest technologies for environmental protection (Fuhs and Chen, 1975, Nielsen et al., 2010).

The role of microorganisms is elaborated further here because they are also important in the treatment of wastewater. What is waste for humans and higher vertebrates becomes a useful food substrate for the microorganisms. In both natural and engineered treatment systems microorganisms such as bacteria, fungi, protozoa, and crustaceans play an essential role in the conversion of organic waste to more stable less polluting substances. They form what is termed a food chain (Crocetti et al., 2002, Thomas et al., 2003, Oehmen et al., 2005; Burow et al., 2007 and Nielsen et al., 2010).

Microorganisms play a major role in decomposing waste organic matter, removing carbonaceous BOD, coagulating nonsetttable colloidal solids, and stabilizing organic matter. These microorganisms convert colloidal and dissolved carbonaceous organic matter into various gases and cell tissue. The cells having a specific gravity greater than water, can then be removed from treated water through gravity settling (Elkelboom and Geurkink, 2002; Kragelund et al., 2006; Kragelund et al., 2007).

In the treatment of wastewater three types of overall processes are distinguished to represent the conversion of organic wastes by microorganisms. The classification is based on whether the environment where the process takes place is aerobic, anaerobic or photosynthetic. Under aerobic conditions, micro-organisms utilize oxygen to oxidize organic substances to obtain energy for maintenance and the synthesis of cellular material (Kong et al., 2001). Under anaerobic conditions, the microorganisms utilize nitrates, sulphates and other hydrogen acceptors to obtain energy for the synthesis of cellular material from organic substances.

Photosynthetic organisms use carbon dioxide as a carbon source, inorganic nutrients as sources of phosphate and nitrogen and utilize light energy to drive the conversion process. Microorganisms also produce waste products, some of which are desirable and some undesirable.
Gases such as CO$_2$ and N$_2$ are desirable, since they can be easily separated and do not produce pollution. Gases such as H$_2$S, although easily separated require treatment for odor (Mobarry et al., 1996; Juretschko et al., 2002).

Microorganisms are significant in water and wastewater because of their roles in disease transmission and in biological treatment processes. Water, wastewater, and other water practitioners must have considerable knowledge of the microbiological characteristics of water and wastewater (Gieseke et al., 2003).

Several studies have been carried out to investigate the occurrence of organic contaminants in wastewater. Wastewater treatment (domestic or sewage water) is the process of removing the contaminants from wastewater. Industrial sources of wastewater often require specialized treatment processes. It includes physical, chemical and biological processes. Its objective is to produce a treated effluent and a solid waste or sludge suitable for discharge. This sludge may also be reused (Wagner et al., 1996). The sludge is often inadvertently contaminated with toxic organic and inorganic compounds.

Primary treatment is intended to reduce oils, grease, fats, sand, grit, and settle-able solids. This step is done entirely mechanically by means of filtration and sedimentation.

The secondary treatment is designed to substantially degrade the organic content of the sewage. In this secondary or advanced treatment step, very often microorganisms are used in the purification step. This biological treatment is an efficient method for the removal and reduction of both organic contaminants as well as for the reduction of the nutrient load. In this purification step, dissolved organic matter is progressively converted into a solid mass by using indigenous, waterborne bacteria (Kragelund et al., 2007). Several methods are being used in modern Wastewater Treatment plants (WWTP’s), but the most economical and simple method is Rotating Biological Contactor (RBC). RBCs use a variety of mechanisms and processes to use dissolved oxygen to promote the growth of a biological floc that substantially removes organic material.

2.1.1 Role of Microorganisms in Wastewater Treatment

Microorganisms require certain nutrients for growth. The basic nutrients of abundance in normal wastewater are carbon (C), nitrogen (N), phosphorus (P), with the ratio of C: N: P approximately equal to 100:10:1. In addition to C, N and P, trace amounts of sodium (Na),
Potassium (K), magnesium (Mg), iron (Fe), and many others are required. The stabilization of organic matter is accomplished biologically using a variety of microorganisms.

Figure 2.1.1 Carbon – Building block for Cell Synthesis

The relationship between the source of the carbon and the source of the energy for micro-organism is important. Carbon is the basic building block for cell synthesis. Energy must be obtained from outside the cell to enable synthesis to proceed. Different organisms have different ways to obtain carbon and energy (Figure 2.1.1).

<table>
<thead>
<tr>
<th>Carbon requirement</th>
<th>Energy source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autotrophs - use CO₂</td>
<td>Phototrophs - use light</td>
</tr>
<tr>
<td>Heterotrophs - use organic carbon</td>
<td>Chemotrophs - use Glucose, inorganics &amp; Sulphur</td>
</tr>
</tbody>
</table>

2.1.2 Relationship to Oxygen

Different organisms have different requirement in O₂ for metabolic reactions. Those that use O₂ as electron acceptor in energy producing pathways are aerobes. Organisms that don’t use O₂ are anaerobes. Facultative anaerobes can adapt to anaerobic conditions.

2.1.3 Relationship to Temperature

Each species of bacteria reproduces best within a limited range of temperatures.

<table>
<thead>
<tr>
<th>Temperature range of bacteria</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 200°C</td>
<td>Psychrophiles</td>
</tr>
<tr>
<td>25- 400°C</td>
<td>Mesophiles</td>
</tr>
<tr>
<td>45- 600°C</td>
<td>Thermophiles</td>
</tr>
</tbody>
</table>
2.1.4 The Role of Enzymes

Enzymes are compounds that are made by living organisms. Their purpose is to help biochemical reactions to occur. Almost all biochemical reactions require the presence of enzymes to cause the reaction to occur. Enzymes help bacteria in the process of breaking down nutrients, and in rebuilding broken down nutrients into the new compounds that they require for growth and reproduction. Enzymes only do what they are supposed to when environmental conditions are right. If the conditions are not right the enzymes will not function properly, thus, the bacteria will not function properly, and they will not survive. If conditions are right the bacteria will live and prosper.

2.1.5 Impact of 16S rDNA Gene Sequence Analysis for Identification of Bacteria

The traditional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on genotypic methods. Comparison of the bacterial 16S rDNA gene sequence has emerged as a preferred genetic technique. 16S rDNA gene sequence analysis can better identify poorly described, rarely isolated, or phenotypically aberrant strains, can be routinely used for identification of mycobacteria, and can lead to the recognition of novel pathogens and noncultured bacteria.

The 16S rDNA gene sequencing technique has several advantages over phenotypic and biochemical identification. Nevertheless, none of these approaches is 100% accurate for the bacterial identification. One of the advantages of using the 16S rDNA gene analysis is that this gene is present in all bacteria.

Hence in this chapter the work was done to identify and isolate the indigenous organism responsible for this efficient treatment of washwater with satisfactory water qualities of the effluent coming out from the outlet of RBC.
Review of Literature
2.2 REVIEW OF LITERATURE

The discovery of microorganisms in 1684, is usually ascribed to Antony van Leeuwenhoek, who was the first person to publish microscopic observations of bacteria. Although the most common mode of growth for microorganisms on earth is in surface associated communities, the first reported findings of microorganisms “attached in layers” were not made until the 1940s.

2.2.1 Role of microorganisms in wastewater treatment:

Biological wastewater treatment is mainly carried out by prokaryotes, even if fungi, protozoa, algae and rotifers may also be represented (Alaerts et al., 1996). The microorganisms remove carbon and other nutrients from sewage by employing various metabolic and respiratory processes. The most frequently found prokaryotes in biological wastewater treatment systems belong to the classes Alpha, Beta and Gamma proteobacteria, Bacteroidetes and Actinobacteria (Ahn et al., 2001).

Generally wastewater is composed of organic material, i.e. proteins, carbohydrates, fats and oils; nutrients, mainly nitrogen and phosphorus; as well as trace amounts of recalcitrant organic compounds and metals. Biodegradable organic material is biochemically oxidized by heterotrophic bacteria under aerobic conditions resulting in production of carbon dioxide, water, ammonia and new biomass. Under anaerobic conditions methanogenic archaea, partially oxidizes organic material to yield carbon dioxide, methane and new biomass (Neytzell-De Wildes, 1991).

2.2.2 Biofilm:

During the 1960s and 70s the research on “microbial slimes” accelerated but the term “biofilm” was not unanimous formulated until 1984. Various definitions of the term biofilm have been proposed over the years. According to the omniscient encyclopedia, Wikipedia a biofilm is “a structured community of microorganisms encapsulated within a self-developed polymeric matrix and adherent to a living or inert surface”. Dental plaque, surfaces of slippery stones and pebble in a stream, slimy coatings in showers or on boat hulls, gunge on infected wounds or the mass clogging water distribution pipes are examples of biofilms that may be encountered in one's everyday life.

Biofilm reactor configurations applied in wastewater treatment include trickling filters, high rate plastic media filters, rotating biological contactors, fluidized bed biofilm reactors, airlift
reactors, granular filters and membrane immobilized cell reactors. A general division between fixed and moving bed processes based on the state of the support material is usually done. Fixed bed systems include all systems where the biofilm is formed on static media such as rocks, plastic profiles, sponges, granular carriers or membranes (Hall-Stoodle et al., 2004). The liquid flow through the static media supplies the microorganisms with nutrients and oxygen. Moving bed systems comprise all biofilm processes with continuously moving media maintained by high air or water velocity or mechanical stirring. Biofilm carrier material (media) is selected based on size, porosity, density and resistance to erosion (Lear and Lewis, 2012).

2.2.3 Microorganisms in biofilms:

Microorganisms in biofilms formed over a substrate like Rotating Biological Contactors (RCBs) produce extracellular polymeric substances (EPS) that hold the cell aggregates together and form the structural biofilm matrix scaffold. The fact that EPS is produced even under growth-limiting conditions, despite the high energy consumption it requires, emphasizes the advantages for bacterial cells to be in biofilm. The biofilm matrix shelters the bacterial cells from antimicrobial agents and environmental stress by acting as a physical barrier (Juretschko et al., 2002).

Other ecological advantages of the biofilm lifestyle are metabolic cooperation, presence of microniches and facilitated gene transfer. Efficient metabolic cooperation or mutual dependence (syntrophism) frequently evolves within biofilms due to interspecies substrate exchange facilitated by the spatial proximity of the cells. Development of microniches with diverse oxygen and nutrient concentrations within biofilms creates favorable conditions for a great variety of species. Enhanced gene transfer rates, often detected in biofilm communities, guarantees a progressive evolution and genetic diversity increasing the competitiveness of the bacterial cells.

Biofilms were initially thought of as homogenous systems of cells entrapped in slime but recent research findings point in the opposite direction. Nowadays, the perception of physiologic and genetic heterogeneity in biofilms is generally accepted in the research community (Momba et al., 2000; Donlan et al., 1994). Natural biofilms usually harbour a multitude of microbial species forming complex differentiated populations capable of developing highly convoluted structures, often separated by a network of water channels.

Bacterial cells adapted to a surface-associated lifestyle express phenotypic traits distinct
from those expressed during planktonic growth. For example, increased tolerance to antimicrobial agents, altered metabolic or biochemical reaction rates, enhanced degradation ability of toxic chemicals and changed synthesis of biomolecules have been observed (Dawes, 1985).

Wastewater treatment with biofilm systems has several advantages compared to suspended growth systems. Operational flexibility, low space requirements, reduced hydraulic retention time, resilience to changes in the environment, increased biomass residence time, high active biomass concentration, enhanced ability to degrade recalcitrant compounds as well as a slower microbial growth rate resulting in lower sludge production are some of the benefits with biofilm treatment processes (Rex and Michael Stenstrom, 1981). Biofilm systems also permit enhanced control of reaction rates and population dynamics.

2.2.4 Biofilm characterization:

Besides primary, secondary and tertiary wastewater treatments, biofilm systems have also been successfully used to treat industrial wastewaters. Biofilms used in wastewater treatment take advantage of a number of removal mechanisms such as biological degradation, biosorption, bioaccumulation and biomineralisation.

![Image of biofilm characterization](image)

**Figure 2.2.2 Biofilm characterization**

Nutrients in wastewater are abundant in the bulk water; hence limitation mostly occurs in biofilms. Different ratios of nutrients available determine the type of biofilm that develops (Loosdrecht et al., 2000). Higher concentration of biodegradable organic substances usually favors the growth of heterotrophs. Microorganisms consume nutrients in their vicinity creating a
nutrient gradient. The gradient causes nutrient replenishment, which is advantageous to the fast growers normally found on biofilm surfaces. The slow growers are usually relegated to the base (Loosdrecht et al., 2000) and due to mass transfer limitations; they may enter endogenous respiratory state and be sloughed (Lewandoski and Beyenal, 2003). Increase in nutrients results in increased growth (Cowan et al., 1991) as long as other factors do not become limiting.

2.2.5 Biofilm development:

Cations are a vital component in biofilm development. They increase bacterial attachment to surfaces by either physiology-dependant mechanisms or by reducing negative repulsive forces between bacteria and surfaces. Electrolytes such as calcium and magnesium are important cellular cations and cofactors for enzymatic reactions. These play a role of enhancing attachment indirectly. Alternatively, cations improve bacterial attachment by reducing repulsive forces between negatively charged bacterial cells and glass surfaces through neutralizing the charges. Fletcher, (1988) was able to demonstrate this phenomenon in experiments on the effects of Sodium, Calcium, Lanthanum and Iron (III) on attachment of *Pseudomonas fluorescens* to glass. It was found that the cations reduced repulsive forces between any two groups of adhesive polymers found in slimes.

One of the most important factor for attached growth is the surface characteristic. Rough and porous surfaces have been found to be suitable for microbial growth. These provide increased surface area for attachment and protection against hydraulic shear forces (Oliveira et al., 2003). A diversity of microorganisms will colonize rough and porous surfaces more rapidly due to the variety of microenvironments created (Characklis et al., 1990 a & b).

Substrata with higher hydrophobicity or wettability are known to favor microbial attachment. Hydrophilic substances attract water, apparently bringing the cells closer to the substratum. Hydrophilic interactions can also prevail between the cells and the substratum thereby reducing repulsive forces (Oliveira et al., 2003). Non-polar substances like Teflon and plastics attach microorganisms more rapidly than glass and metals.

2.2.6 Extracellular polymeric substance (EPS):

Attachment cannot be possible without a matrix upon which cells are deposited. This is provided by a substance known as ‘extracellular polymeric substances’ (EPS) made by the bacteria. EPS are composed of nucleic acids, proteins and other organic matter (85-90%). Their development depends on the nutritional status of the surrounding media. EPS are highly hydrated
substances due to the hydrogen bond formation with water. This property enables them to prevent cells from desiccation. EPS are also known to protect microorganisms against toxic substances and anti-biotics (Bishop et al., 2003).

EPS have unique properties of possessing negatively charged groups on their surface. This permits binding to cations such as calcium and magnesium, which are known to form crosslinks with polymer strands providing greater binding force in biofilms (Fletcher, 1988; Bishop et al., 2003). EPS are essential in wastewater treatment, variations in its biological, chemical and physical properties make treatment technologies like activated sludge, trickling filters, rotating biological contactors, fluidized or submerged fixed-bed reactors depend on them (Bryers and Characklis 1990; Metcalf and Eddy, 2003).

For organisms to attach, the rate of attachment should be greater than the washout rate. These two processes are greatly influenced by velocity. The zone adjacent to the substratum-liquid interface is termed as the hydrodynamic boundary layer. Its thickness depends on linear velocity; the higher the velocity, the thinner it becomes. Increasing flow velocity exerts mechanical stress on the biofilm thus wearing it out (Esterl et al., 2003). Although beyond a certain threshold it may erode and abraise the biofilm (Morgesin and Schinner, 2001), some degree of velocity may have a positive effect. Rijnaarts et al., (1993) and Donlan et al., (1994) have shown that fluid movement aids transportation of cells to the substratum for deposition.

2.2.7 Micro – organisms present in natural habitat like water:

*Enterobacter* species are found in the natural environment in habitats such as water, sewage, vegetables, and soil. Before the widespread use of antibiotics, *Enterobacter* species were rarely found as pathogens, but these organisms are now increasingly encountered, causing nosocomial infections such as urinary tract infections and bacteremia (Eichoff et al., 1966). In addition, they occasionally cause community-acquired infections. In 1975 in the United States, *Enterobacter* species accounted for 4.6% of all pathogens causing infections and accounted for 5.7% of all cases of primary bacteremia (Center for Disease Control, 1977). In 1984, *Enterobacter* species accounted for 5.9% of all nosocomial infections in U.S. hospitals and 6.3% of all nosocomial bacteremia (Centers for Disease Control, 1984).

The genus *Enterobacter* was first proposed by Hormaeche and Edwards (1960). However, the history of some species now placed in the genus *Enterobacter* can be traced, albeit with some confusion, to the end of the 19th century. “*Bacillus lactis aerogenes*” was isolated by
Escherich in 1885 from milk and renamed “Bacillus aerogenes” by Kruse in 1896 and “Aerobacter aerogenes” by Beijerinck in 1900. Until 1955, differentiation of this organism from Friedlander’s bacillus (now called Klebsiella pneumoniae) was not clear, and most authors considered “B. lactis aerogenes” or “Aerobacter aerogenes” to be either nonmotile or to contain both motile and nonmotile strains (Edwards and Mary, 1955). This led them to state that “A. aerogenes” strains were in fact Klebsiella strains.

“Bacterium cloacae” was described by Jordan, (1890) and transferred to a new genus “Cloaca” as “Cloaca cloacae” by Castellani and Chalmers, (1920). In the first edition of Bergey’s Manual (Bergey et al., 1923), this species was transferred to the genus “Aerobacter” as “A. cloacae.” Since “Aerobacter aerogenes” was at that time indistinguishable from Klebsiella pneumoniae, Edwards and Mary, (1955) proposed that the species “A. aerogenes” not be used, although disappearance of the type species (“A. aerogenes”) implied disappearance of the genus (“Aerobacter”). Because of this, two solutions were suggested for “A. cloacae”: 1) the reintroduction of the genus “Cloaca” with the species “Cloaca cloacae” (reference is not an exact match Kauffman, 1954); and 2) the redefinition of the genus “Aerobacter” with “A. cloacae” as type species (Edwards and Mary, 1955). However, this latter proposal did not conform to the rules of nomenclature.

A significant step forward occurred when Moller, (1955) devised some simple methods for testing amino acid decarboxylases. Since the “Cloaca” group was arginine-positive it could now easily be distinguished from the Klebsiella group, which was arginine-negative. This led to the finding of motile strains of the “Cloaca” group, which were arginine-negative and produced gas from inositol and glycerol (Hormaeche and Munilla, 1957). These strains were called “Cloaca B” (arginine-positive strains forming the “Cloaca A group”). Then, after reexamination of many cultures using the decarboxylase test, Hormaeche and Edwards, (1958) redefined the genus “Aerobacter” to include two species, “A. aerogenes” (“Cloaca B”) and “A. cloacae” (“Cloaca A”), with the type species reaffirmed as “A. aerogenes.”

One of the species now classified in the genus Enterobacter which have been described after delineation by DNA-DNA hybridization is Enterobacter aerogenes. Hormaeche and Edwards 1960a; “Bacillus aerogenes”; “Aerobacter aerogenes”. Hormaeche and Edwards 1958; Enterobacter aerogenes (Hormaeche and Edwards, 1960); Klebsiella mobilis, Bascomb et al.,1971. The type strain is strain ATCC 13048 (= CDC 819–56, NCTC 10006, CIP 60.86).
2.2.8 Use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria

In the last decade, as a result of the widespread use of PCR and DNA sequencing, 16S rDNA sequencing has played a pivotal role in the accurate identification of bacterial isolates and the discovery of novel bacteria in clinical microbiology laboratories. For bacterial identification, 16S rDNA sequencing is particularly important in the case of bacteria with unusual phenotypic profiles, rare bacteria, slow growing bacteria, uncultivable bacteria and culture-negative infections.

2.2.9 Identification of rare bacteria and bacteria with unusual phenotypic profiles.

16S rDNA sequencing is particularly useful in identifying unusual bacteria that are difficult to identify by conventional methods, providing genus identification in >90% of cases, and identification of 65–83% of these at the species level (Drancourt et al., 2000 and Mignard and Flandrois, 2006). The MicroSeq 500 16S rDNA-based identification system was also able to identify 81% of clinically significant bacterial isolates with ambiguous biochemical profiles and 89.2% of unusual aerobic Gram-negative bacilli to the species level (Woo et al., 2003 and Tang et al., 1998).

Unlike phenotypic identification, which can be affected by the presence or absence of nonhousekeeping genes or by variability in expression of characters, 16S rDNA sequencing provides accurate identification of isolates with a typical phenotypic characteristics.
Materials and Methods
2.3 MATERIALS AND METHODS

2.3.1 Sample collection

A small amount of biofilm was scrapped from the Rotating biological contactor (RBC) treating washwater of Trichy distillery Unit in a sterile glass containers, tapered tightly, processed for biochemical parameters and stored at −20°C for further analyses.

2.3.2 Bacterial analysis of biofilm sample from Rotating Biological Contactor (RBC)

Isolation of bacteria

Approximately one gram of scrapped biofilm sample from the Rotating Biological Contactor (RBC) was taken in 9 ml of sterile distilled water and mixed to a homogenous solution. Different dilutions of the sample were prepared as 10^-3, 10^-5 and 10^-6 and were applied onto agar plates. Isolation of bacteria was performed by dilution plate technique (Waksman and Fred, 1992) and direct plate technique, all samples were properly diluted and spread on nutrient agar medium (Appendix I).

The agar plates were prepared by addition of approx. 20 ml molten medium. After gently rotating, the plates were incubated at 28°C for 24 to 48 h. After incubation selected colonies of bacteria were transferred from mother culture plates onto respective agar plates, incubated at 37°C for 24-48h. Pure cultures were stored at 4°C until further examination.

Bacterial colonies were characterized morphologically, biochemically and physiologically following the directions given by the Bergey’s Manual of Systematic Bacteriology (Schleifer, 1989 and Aneja, 2005). Cultural characteristics of pure isolates in Nutrient agar media were recorded after incubation for 24-48h at 37°C. The patterns of growth to be considered were evaluated in the following manner: size, pigmentation, configuration, margin and elevation.

2.3.3 Nutrient agar cultivation

Nutrient agar is a solid medium that contains nutrient for cultivation of bacteria and fungi (Madigan and Martinko, 2005). Less than 1% of all existing bacteria can be successfully cultivated, and nutrient agar can grow most of these microbes (Madigan and Martinko, 2005). Nutrient agar has been used for enumeration of total microorganisms in water, beverages and biological products (Madigan and Martinko, 2005).
The culture was inoculated by an aseptic transfer of 0.1 ml sample aliquot into the medium. After spreading, the medium was allowed to dry and then incubated at 37°C for 24 hours. Grown cells were enumerated to obtain heterotrophic bacteria counts.

2.3.4 Gram Staining

The Gram stain is most consistent when done on young cultures of bacteria (less than 24 hours old). When bacteria die, their cell walls degrade and may not retain the primary stain, giving inaccurate results. Because Gram staining is usually the first step in identifying bacteria, the procedure should be memorized.

2.3.5 Biochemical Characterization

Enzymatic activities are widely used to differentiate bacteria. Even closely related bacteria can usually be separated into distinct species by subjecting them to biochemical test, such as one to determine their ability to ferment an assortment of selected carbohydrates. For one example of the use of biochemical tests is to identify bacteria. Moreover, biochemical tests can provide insight into a species niche in the ecosystem. For example, a bacterium that can fix nitrogen gas or oxidize elemental sulfur will provide important nutrients for plants and animals (Kundu and Gaur, 1894).

2.3.6 Enumeration

The enumeration of microbial populations was accomplished using Total Viable Count (TVC) method. TVC was performed on nutrient agar media by means of serial dilution agar plating method (Williams, 1989). Dilutions $10^{-6}$, $10^{-7}$ and $10^{-8}$ of water samples were used for agar plating. One milliliter of appropriate diluted suspension was transferred in petriplates containing molten agar medium (45°C). The experimental petriplates were reared in replicates for each sample. The plates were incubated at 37°C for 24 hrs (Aneja, 2004) to obtain viable colony.

2.3.7 Isolation

The isolation was performed following serial dilution, spreading and pour-plate methods (Aneja 2004). The viable bacterial colonies were then transferred into broth for further cultivations. The cultivation was performed with appropriate incubation, temperature and time required for growth. Isolation of pure cultures was completed by the streak plate method on various agar media, i.e. NA (nutrient agar, pH = 7.0), MCA (MacConkeys’ agar) and EMB (eosin–methylene blue agar). Gram staining was used on each step after the transfer
of a single colony to check the purity of the culture. The bacterial isolates were subcultured on agar slants of their respective media at regular intervals to maintain viability and metabolic activities. Agar slants were stored at a temperature of 4°C, which shows growth, protects from damage due to evaporation of medium and preserves the cultures. The isolates were maintained in replicates; one as the working culture to be used as a source for identification tests and other as stock culture from which new working cultures were prepared whenever required.

Colonies were transferred to Petri dishes containing selective culture media like, EMB agar, Simmons Citrate agar, Cetrimide agar, Pseudomonas agar, Mac Conkey agar, SS agar, Blood agar, KF agar and other culture media like Wilson and Blair media, TCBS agar for identification purpose. Then every colony was transferred to nutrient agar for preservation of stock cultures. For provisional identification of bacteria Gram staining, Endospore staining, Capsule staining, Motility test were done. In order to study the morphology of bacteria, cells were heat killed and fixed on the slide. The fixed bacteria were stained and studied for size, shape, arrangement, spore formation and capsulation etc. Hanging drop method was performed to study motility of bacteria.

2.3.8 Biochemical analyses

Biochemical analyses were done by performing the catalase test, oxidase test, triple sugar iron reactions, citrate utilization test, urease test, indole test, motility test, Voges-Proskaur test, methyl red test and sugars fermentation test (Bergey et al., 1923).

2.3.9 Antibiotics resistance

Plate dilution technique was used for determining the minimal inhibitory concentration (MIC) to the selected antibiotics, Amoxicillin (Amx), Tetracycline (Tet) and Ciprofloxacin (Cip), on nutrient agar plates (Sambrook et al., 2001) by incubating at 37°C for 24 h.

2.3.10 Bacterial identification

The primary identification of the isolates was carried out on the basis of their cultural characteristics on agar plates and microscopic observations. The secondary identification of the isolates was carried out on the basis of their biochemical characteristics, the detection of which aid in the identification and classification of bacteria those were found morphologically identical (Greenberg et al., 1985).
Five isolates were taken from the highest dilution plates and continually streaked on agar to obtain pure colonies. *Enterobacter aerogenes, E. cloace* and *Escherichia coli* were selected as test organisms due to their well flourished growth. Other isolates showed a very poor growth and were contaminated, which made it very difficult to identify. Each bacterial species was grown on 50 ml nutrient broth cultures in Erlenmeyer flasks and incubated for 1 day at 30°C. A dilution series of each culture was prepared aseptically to study the growth (density) of each bacterium. Dilution $10^{-3}$ (which contained about $2 \times 10^3$ cells/ml) was selected as the most suitable dilution. A set of 15 nutrient agar plates were inoculated with 0.5 ml of selected dilution of each bacterium and the inoculum was spread evenly with the aid of a sterile glass rod. Plates were incubated for 30 min.

### 2.3.11 Sulphide, indole and motility tests

The sulphide, indole and motility (SIM) tests is a three in one method using a single medium to detect microorganisms’ motility and their ability to break down specific compounds from the medium to produce sulphide and indole. The principle of the sulphide test is to detect the ability of the microorganisms to produce sulphide from sulphate (Perry *et al.*, 2002).

The medium in the test tubes was autoclaved at 121°C for 15 minutes. Aseptic 1.0 ml sample aliquot inoculation was performed, followed by incubation at 35°C for 18-24 hours. Motility was indicated by turbidity of the culture medium. Hydrogen sulphide formation was indicated by the production of black precipitates. The indole test was performed by covering the medium with a layer of Kovac’s indole reagent, resulting in production of a purple colour, indicating indole production.

### 2.3.12 Spore staining

Spore production is an important characteristic of some bacteria, allowing them to resist adverse environmental conditions such as desiccation, chemical exposure and extreme heat (Dragon and Rennie, 1995).

The sample for analysis was smeared onto a microscope slide and fixed through drying over a Bunsen burner flame. The slide was placed on a 1000 ml glass beaker and flooded with malachite solution and boiled on a hotplate for 20 seconds. A 30 seconds reaction time was allowed. The slide was rinsed with tap water and then re-stained by flooding it with eosin solution for one minute and then Safranine solution for 30 seconds. The
slide was rinsed with tap water and softly dried with a paper towel. Positive spore identification was observed as an emerald green colour under an Olympus BX51 microscope.

2.3.13 Lactose Utilisation

The ability of bacteria to utilize lactose as a source of energy and carbon can be tested by the ability of the bacteria to grow on MacConkey agar with salt and crystal violet. Lactose medium selects a wide range of total coliform microbes (APHA, 1998).

About 0.1 ml subsample of the culture from the sample was aseptically inoculated and spread over the medium with a hockey stick spreader. Inoculated medium was allowed to dry, and then incubated at 37°C for 48 hours. Microbial cells grown were enumerated.

2.3.14 Catalase test

Catalase is an enzyme that splits hydrogen peroxide into water and oxygen. The principle of this test is to detect the presence of catalase in the microorganisms.

A culture growing on a Nutrient agar plate was tested for catalase activity by adding 0.5 ml of 3% hydrogen peroxide. Positive results were observed through bubbles forming in response to microbial activity.

2.3.15 Oxidase test

The oxidase test differentiates between the families of Pseudomonadaceae (oxidase positive) and Enterobacteriaceae (oxidase negative). The reagent’s active agent is tetramethyl-p-phenylenediamine, which is utilised by the enzyme cytochrome oxidase, acting as an electron donor during the electron transport chain in the microorganism (Steel, 1962; Health Protection Agency, 2008).

A colony from a Nutrient agar plate was picked onto filter paper. A drop of the oxidase reagent was added, and the reaction was observed within 20 seconds.

2.3.16 Methyl red and Voges-Proskauer tests

These tests detect the ability of the microorganisms to ferment glucose. For the methyl-red test, glucose is fermented to produce acid. For the Voges-Proskauer test, glucose is fermented to acetoin, and this test enables differentiation of *Bacillus* species from enterics. These tests have been used to detect characteristics of microorganisms such as *Klebsiella* spp., *Pseudomonas* spp. and *Enterobacter* spp.

Two test tubes containing MR-VP medium were each inoculated with 1.0 ml of the culture from the same sample and incubated at 35°C for 4 days. After incubation, the methyl-
red test was conducted by adding about five drops of the methyl-red indicator solution to the first tube. A positive result was indicated by the medium changing colour to red.

2.3.17 Starch hydrolysis

Some microorganisms contain amylase, an enzyme that can hydrolyse starch into glucose. Amylase is excreted into the media and initiates starch breakdown. The starch hydrolysis test is used to identify the reactions correlated with growth on a starch agar plate and this reaction has been recorded in aquatic microbiology to indicate characteristics of genera such as *Acetobacter* and *Acinetobacter* (Garrity *et al.*, 1984; 2005).

Samples being analysed were streaked onto starch medium and incubated upside down at 37°C for 24 hours. Iodine solution was flooded over microbial colonies after the incubation period. In the presence of the enzyme amylase and subsequent starch hydrolysis a yellow/gold zone around the growth was observed and its absence indicated negative results.

2.3.18 Citrate test

The citrate test identifies the use of citrate as a sole carbon source in the absence of other nutrients in this test medium. The end products cause the bromo-thymol blue indicator in the medium to turn from forest green to royal blue. This reaction has been used in testing for the characteresitics of genera such as *Nitrobacter* spp., *Rhizobium* spp. and *Klebsiella* spp. (Garrity *et al.*, 2005).

The citrate medium was dispensed into 9 cm sterile Petri dishes and allowed to solidify. An aliquot of 0.1 ml of the culture from the sample was aseptically transferred onto the plate and aerobically incubated for 24 - 48 hours at 35°C. Cell growth was enumerated.

2.3.19 Nitrate reduction test

The principle of this method is to determine the ability of a microorganism to reduce nitrate to nitrite or free nitrogen gas. This denitrification process can be undertaken by bacteria that use nitrate as the final electron acceptor in anaerobic respiration. Groups that have been recorded to facilitate this reaction include *Nitrobacter* spp., *Klebsiella* spp. and *Pseudomonas* spp. (Garrity *et al.*, 1984; 2005).

Cooled nitrate broth medium was aseptically inoculated with a 1.0ml culture from the sample and incubated for 5 days at 37°C. After incubation, nitrite production was determined using standard method 354.1 (APHA, 1998). The presence of nitrite was indicated by the colour change to red after approximately 15 minutes, demonstrating that nitrate was reduced.
to nitrite. When there was no colour change, few particles of a zinc metal powder were added. A positive colour change after zinc addition indicated that nitrate was present in the sample and had not been reduced. No colour change after addition of zinc meant that nitrite was produced and may have transformed to nitrogen gas, which was not measured.

2.3.20 DNA isolation of Enterobacter

Microbial cells were harvested by centrifugation, and cell pellets were suspended in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) containing 2 mg of lysozyme per ml in a total volume of 5 ml. Samples were incubated at 30°C for 30 min, which was followed by addition of 1.2 ml of 0.5 M EDTA and protease (final concentration, 0.2 mg/ml) and an additional 30 min of incubation. After addition Cell Lysis buffer of 0.7 ml of 10% (w/v) sodium dodecyl sulfate (SDS), 1.8 ml of 5 M NaCl, and 1.5 ml of 10% CTAB-NaCl solution (10% [w/v] cetyl trimethyl ammonium bromide in 0.7 M NaCl), the mixtures were incubated at 65°C for 20 min. Samples were extracted with chloroform-isoamyl alcohol (24:1, by volume), and the supernatants were transferred to clean tubes. DNA was precipitated by addition of 0.6 volume of isopropanol and pelleted by centrifugation (48,000 x g, 15 min). DNA pellets were rinsed with 70% (w/v) ethanol, dried, dissolved in TE buffer, and quantified by absorption spectrophotometry at 260 nm.

2.3.21 Primer details and PCR conditions.

Each PCR mix contained ~250 ng DNA, 100 ng each forward and reverse primer, 200 mM dNTPs (Invitrogen), 25 mM MgCl₂ and 2.5 U Platinum Taq DNA polymerase (Invitrogen) in the manufacturer’s buffer. All reactions were done with a 3 min 95 °C hot start followed by 35 cycles of 95 °C for 1 min, the primer-specific annealing temperature for 45 min at 59 °C, and 72 °C for 2 min, followed by a 30 min 72°C extension. Primers designed to anneal to conserved regions in cyanobacteria were used to amplify bacterial methyl transferase gene and it can amplify 0.9 to 1.0 kb regions in extracted DNA.

2.3.22 Phylogenic tree construction

Sequences were aligned using Clustal X (Thompson et al., 1997). To compare with other Enterobacter sp. sequences were retrieved from Genbank. All sequence alignments were pruned to 566 bp. No insertions or deletions were detected. Finally Neighbour-joining (Saitou and Nei, 1987) tree was constructed with MEGA 6.0 (Tamura et al., 2007).
Results and Discussions
2.4 RESULTS AND DISCUSSIONS

The rotating biological contactor is a fixed (attached) biomass system comprising rotating discs (Plate 2.4.1). Biofilm gradually forms on the disc surface. The constant rotation of the disc causes mixing of the liquid. Also, the rotating disc surface alternately comes into contact between air and wastewater and thus acts as an aeration device for wastewater treatment. However, the practical use of rotating biological contactor (RBC) was introduced, on the basis that the dissolved oxygen (DO) in the reactor did not have significance on treatment efficiency because adequate amount of oxygen could be supplied during the air exposure cycle. Thereafter most of the mathematical models have been developed considering the biological step to be the rate limiting step (Kornegay and Andrews, 1968; Clark et al., 1978).

Plate 2.4.1 Development of thick biofilm on the surface of discs.

In the current study the fast growing microorganisms enhanced the treatment and reduced the hydraulic retention time. The RBC with the biofilm was quickly stabilized and
then used for wastewater treatment. Thus the RBC may act as a reservoir of bacterial communities capable of degrading organic waste present in wash water (Banerjee, 1998). The washwater samples taken from the inlet and outlet of the RBC used for treating the washwater in the distillery plant were characterized. An average COD concentration of 1300mg/l, BOD concentration of 360mg/l, total solids (TS) of 65000, total suspended solids (TSS) of 15000, and DO of 5.1 mg/l were obtained based on experimental data resulting from the analysis.

Attached growth of microbial film was developed on the surface of discs for an incubation period of 24 hours. Mounted disc shafts with a sufficient load of biomass were rotated for 13 and 11 rpm, respectively. Since the process is aerobic, the DO concentration was detected in the entire treatment process. The constant DO level, which was in a reasonable range, represents the system being sufficiently aerated and the growth of the biofilm on the discs proved that the attached growth occurred while the biofilm was exposed to the air.

Wastewater has a wide range of pH variations making it suitable for diverse growth of microorganisms. Nitrifiers and denitrifiers have been reported to grow well in pH ranges from 7.2 to 9.0 and 7.0 to 8.0 respectively (Metcalf and Eddy, 2003).

The DO showed a constant increment ranging from 5 to 7mg/l. On the other hand, other parameters like BOD, COD Total Solids etc showed reductions ranging from 50 to 65% by 24 hours.

A properly designed RBC is very reliable based on the amount of biomass attached to the discs. The biomass permits the RBC to more effectively withstand hydraulic and organic surges. The hydraulic characteristic of a bench scale model is determined by liquid flow rate, the rotational speed of the disc and the percentage of submerged surface (Banerjee, 1998).

The bulk of the microbial film on the RBC discs consists of bacteria, protozoa, metazoa, etc. (Nahid et al., 2001). The key factor in the RBC’s performance is to maintain biofilm stability. It is therefore important to know the physical properties, composition and activity of the biofilm.

Hence in order to identify and isolate the indigenous organism responsible for this efficient treatment of washwater with satisfactory water qualities of the effluent coming out from the outlet of RBC, some biofilm was scrapped from the RBC in a sterile condition. From the scrapped biofilm five different microbial colonies were isolated (Plate 2.4.2 and Plate
2.4.3) and were subjected to microscopic examination and various biochemical tests. The result clearly indicated that among the five different organisms the gram negative rods *Escherichia coli*, *Enterobacter aerogenes* and *E. cloacae* were found to be dominant (Plate 2.4.4 and plate 2.4.5) and plays a major role in the reduction of organic load in the washwater released from the various units of distillery. The other colonies were contaminated and were difficult to identify due to very poor growth.

![Plate 2.4.2 Petri dish showing bacterial isolates from RBC](image1)

![Plate 2.4.3 Petri dish showing purified culture bacteria from RBC](image2)
Plate 2.4.4 Colonies of *Enterobacter aerogenes* and *Enterobacter cloacae*

Plate 2.4.5 Colonies of *E.coli*
2.4.1 Morphological, Physiological and Biochemical identification of the isolate:

*Enterobacter aerogenes* and *E. cloacae* isolated from the sample are examples of non-fecal coliform and can be found in vegetation and soil which serve as source by which the pathogen enters the water. *E coli* is a well established index of fecal contamination (WHO, 1982).

Plate 2.4.6 Gram–negative Microscopic view (1000x)

Plate 2.4.7 *Enterobacter aerogenes* negative staining
Plate 2.4.8 *E. aerogenes* gram staining

Plate 2.4.9 *E. coli* grams staining

*E. aerogenes*, *E. cloacae* and *E. coli* are gram negative pink slender rods (Plate 2.4.6, 2.4.7, 2.4.8 and 2.4.9) They have a two-layered cell wall, composed of lipids and carbohydrates. The gram stain turned the bacteria pink, indicating that the crystal purple couldn't penetrate the second outer layer, while the Safranin could. On nutrient agar, *E. aerogenes* as well as *E. cloacae* form colonies that are round, 2–3 mm in diameter, and slightly iridescent or flat with irregular edges. *E. coli* showed smooth, regular and white
coloured colonies and measures around 1 – 3 x 0.4 - 0.7µm in size. Based on biochemical tests, *E. aerogenes* and *E. cloacae* showed positive reaction to Voges Proskauer test, whereas Citrate utilization test, Glucose fermentation, Lactose fermentation, Fructose fermentation and Catalase test, whereas showed negative reaction to Indole test and Methyl red test. *E. coli* showed positive response to Indole test, Methyl red test and the entire Carbohydrate test except, Voges Proskauer test, Citrate utilization and sucrose test. The biochemical test which differentiates *E.aerogenes* and *E.cloacae* from *E.coli* is growing the bacterial colonies in EMB agar. *Enterobacter aerogenes* and *E.cloacae* showed pink coloured colonies without sheen while *E.coli* colonies showed good growth of dark blue-black colonies with metallic green sheen indicating vigorous fermentation of lactose and acid production which precipitates the green metallic pigment (Table 2.4.1, 2.4.2 & 2.4.3 & Plates 2.4.10 to 2.4.18).

Table 2.4.1 Biochemical Tests - *Enterobacter aerogenes*

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Biochemical Tests</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Simple staining</td>
<td>Rod shaped</td>
</tr>
<tr>
<td>2</td>
<td>Gram’s staining</td>
<td>Gram negative pink slender rods</td>
</tr>
<tr>
<td>3</td>
<td>Indole</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>Methyl red</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>Voges proskauer</td>
<td>Positive</td>
</tr>
<tr>
<td>6</td>
<td>Citrate Utilization</td>
<td>Positive</td>
</tr>
<tr>
<td>7</td>
<td>Sugar Fermentation Glucose</td>
<td>Positive</td>
</tr>
<tr>
<td>8</td>
<td>Lactose</td>
<td>Positive</td>
</tr>
<tr>
<td>9</td>
<td>Fructose</td>
<td>Positive</td>
</tr>
<tr>
<td>10</td>
<td>Sucrose</td>
<td>Negative</td>
</tr>
<tr>
<td>11</td>
<td>Catalase</td>
<td>Positive</td>
</tr>
<tr>
<td>12</td>
<td>MacConkey</td>
<td>Pink coloured colonies</td>
</tr>
<tr>
<td>13</td>
<td>EMB</td>
<td>Pink coloured without sheen</td>
</tr>
<tr>
<td>14</td>
<td>Pseudomonas agar</td>
<td>No fluorescence</td>
</tr>
</tbody>
</table>
Table 2.4.2 Biochemical Tests - *Enterobacter cloacae*

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Biochemical Tests</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>Methyl red</td>
<td>Negative</td>
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<tr>
<td>5</td>
<td>Voges proskauer</td>
<td>Positive</td>
</tr>
<tr>
<td>6</td>
<td>Citrate Utilization</td>
<td>Positive</td>
</tr>
<tr>
<td>7</td>
<td>Sugar Fermentation Glucose</td>
<td>Positive</td>
</tr>
<tr>
<td>8</td>
<td>Lactose</td>
<td>Positive</td>
</tr>
<tr>
<td>9</td>
<td>Fructose</td>
<td>Positive</td>
</tr>
<tr>
<td>10</td>
<td>Sucrose</td>
<td>Negative</td>
</tr>
<tr>
<td>11</td>
<td>Catalase</td>
<td>Positive</td>
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<tr>
<td>12</td>
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</tr>
<tr>
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<td>EMB</td>
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</tr>
<tr>
<td>14</td>
<td>Pseudomonas agar</td>
<td>No fluorescence</td>
</tr>
</tbody>
</table>

Plate 2.4.10  A – *E. aerogenes*;  B - *E. cloacae*  C – *E. coli*
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Biochemical Tests</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Simple staining</td>
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<tr>
<td>3</td>
<td>Indole</td>
<td>Positive</td>
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<tr>
<td>4</td>
<td>Methyl red</td>
<td>Positive</td>
</tr>
<tr>
<td>5</td>
<td>Voges proskauer</td>
<td>Negative</td>
</tr>
<tr>
<td>6</td>
<td>Citrate Utilization</td>
<td>Negative</td>
</tr>
<tr>
<td>7</td>
<td>Sugar Fermentation Glucose</td>
<td>Positive</td>
</tr>
<tr>
<td>8</td>
<td>Lactose</td>
<td>Positive</td>
</tr>
<tr>
<td>9</td>
<td>Fructose</td>
<td>Positive</td>
</tr>
<tr>
<td>10</td>
<td>Sucrose</td>
<td>Negative</td>
</tr>
<tr>
<td>11</td>
<td>Catalase</td>
<td>Positive</td>
</tr>
<tr>
<td>12</td>
<td>MacConkey</td>
<td>Pink coloured colonies</td>
</tr>
<tr>
<td>13</td>
<td>EMB</td>
<td>Metallic green sheen</td>
</tr>
<tr>
<td>14</td>
<td>Pseudomonas agar</td>
<td>No fluorescence</td>
</tr>
</tbody>
</table>
Plate 2.4.11  A – *E. aerogenes*;  B - *E. cloacae*  C – *E. coli*

Plate 2.4.12  A – *E. coli*  B – *E. aerogenes*;  C - *E. cloacae*
Plate 2.4.13  A – *E. aerogenes*  B - *E. cloacae*  C – *E. coli*

Plate 2.4.14  A - *E. aerogenes*; B - *E. cloacae*  C – Control
Plate 2.4.15  
A – *E. coli*  
B – Control

Plate 2.4.16  
Fermentation of glucose - *Enterobacter cloacae*
Plate 2.4.17  Fermented of sugars - Enterobacter aerogenes

Plate 2.4.18  Fermentation of Sugars - Escherichia coli
### Table 2.4.4 Fermentation Test of *E. coli*

<table>
<thead>
<tr>
<th>Fermentation Type</th>
<th>Position</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose fermentation</td>
<td>left</td>
<td>gas+</td>
</tr>
<tr>
<td>Glucose fermentation</td>
<td>middle</td>
<td>gas+</td>
</tr>
<tr>
<td>Sucrose fermentation</td>
<td>right</td>
<td>no gas-</td>
</tr>
</tbody>
</table>

**Plate 2.4.19** *Enterobacter aerogenes* and *Escherichia coli* in EMB agar

**Plate 2.4.20** *Enterobacter aerogenes* and *E. cloacae* both form pink colonies on EMB
Plate 2.4.21 *Enterobacter aerogenes* and *Escherichia coli* in Macconkey agar

Plate 2.4.22 *Escherichia coli* on MacConkey agar
Thus *Enterobacter aerogenes* and *E. cloacae* are Gram negative rod-shaped bacteria in the same family as *Escherichia coli*. They can grow on many of the same selective media as *Escherichia coli*, including: MacConkey Agar, EMB agar and Lauryl-Tryptose broth. *E. aerogenes* and *E. cloacae* ferments lactose, producing acid and gas like *Escherichia coli* and is classified as an example of coliform bacteria. There are several significant differences between *E. aerogenes*, *E. cloacae* and *Escherichia coli*: 1) Most strains of *E. coli* are able to grow and produce acid from lactose in a medium such as lauryl tryptose broth or brilliant green bile broth at 44.5 degrees C. while most strains of *E. aerogenes* and *E. cloacae* do not grow well at that temperature, *E. aerogenes* and *E. cloacae* grows better at temperatures between 34 - 40 degrees C. 2) *E. aerogenes* and *E. cloacae* carries out 2,3-butanediol fermentation and thus give a positive test in the Voges-Proskauer test while *E. coli* is negative. 3) *E. coli* is positive in the methyl red test while *E. aerogenes* and *E. cloacae* are usually, but not always, negative (this is not the best test to rely on). 4) *E. coli* is positive for the indole test while *E. aerogenes* and *E. cloacae* are negative, this is a very reliable test.

Of these three species, *E. aerogenes* and *E. cloacae* have their special property of synthesizing polyhydroxyalkanoates (PHA) under nutrient limiting condition and in presence of
excess of carbon sources (Reema et al., 2013). Hence in order to study this interesting character of *Enterobacter aerogenes* and *E. cloacae*, they have been subjected to molecular identification as described below.

**2.4.2 Antibiotic susceptibility testing**

Antibiotic susceptibility testing will be done using Kirby Bauer disc diffusion method (Bauer et al., 1966) as described below. The antibiotics used were given in Table 2.4.5.

<table>
<thead>
<tr>
<th></th>
<th>Ampicillin (10µg)</th>
<th>Tetracycline (30µg)</th>
<th>Ciprofloxacin (5µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gentamicin</strong></td>
<td>(10µg)</td>
<td>Cefotaxime (30µg)</td>
<td></td>
</tr>
</tbody>
</table>

A single colony of the test strain was transferred into 3ml of sterile physiological saline. Turbidity of the cell suspension was adjusted to 0.5 Mc Farland’s standard, either by adding new colonies or adding more sterile saline. With the help of a sterile cotton swab, a uniform bacterial smear was made on to Mueller-Hinton agar (HiMedia, India) plate (Appendix I). Antibiotic discs were placed on the plate, each plate holding not more than five discs. The discs were spaced to provide room for the development of the zone of inhibition. The plates were incubated at 37°C for 24 hours before examination. The result was interpreted as resistant, intermediate or sensitive based on the size of the inhibition zones around each disc as provided by the manufacturer (HiMedia, India).
Escherichia coli isolates exhibited resistance to ampicillin. They also showed resistance to Gentamicin, Ciprofloxacin, and Cefotaxime. Tetracycline is most effective in killing Enterobacter aerogenes and E. cloacae. The Tetracycline had the largest zone of inhibition (ZOI), 15 mm in diameter, while Streptomycin came in second, with a 10mm ZOI in diameter. The Penicillin has 0mm ZOI. Penicillin was not very effective in killing the bacteria, because it typically works best on Gram positive bacteria, Enterobacter aerogenes and E. cloacae are Gram negative (Plate 2.4.19).

2.4.3 16SrDNA based identification of the isolate

The identification of bacteria is fundamental to understanding the biodiversity in an ecosystem as well as the ecological processes. Bacteria are very important to the environment because they interact with life on Earth by their metabolic activities.

It is possible to extract DNA from microorganisms and identify them using the Genebank database in ncbi.nlm.gov. (Dahllof et al., 2000). There are more than 15 000 16S rDNA sequences that can be used to identify microorganisms by using phylum and even subclass specific probes. The 16S and 23S rDNA genes code for the rRNA molecules required for protein
synthesis. They are approximately 1500 to 3000 base pairs long and contain both highly variable and conserved regions (Wilderer et al., 2002). By using specific primers, it is possible to amplify specific DNA regions using the polymerase chain reaction (PCR).

It is possible to extract DNA from environmental samples without the need to culture microorganisms. PCR is highly sensitive and can detect as few as ten copies of the DNA in a complex mixture if the DNA is ‘clean’. Based on the 16S rDNA sequences, phylogenetic trees are compiled and used to classify bacteria using their 16S rDNA. Similarly, 18S rRNA sequences can be used for the identification of yeast and fungi using the methods mentioned above (Cappa and Cocconcelli, 2001).

The plate was send to the Scientific Synergy Laboratory, Chennai for sequencing. The sequences were received and the reverse sequences were turned around with [http://bioinformatics.org/sms/rev_comp.html](http://bioinformatics.org/sms/rev_comp.html). The forward and reverse compliment sequences were compared by aligning them with [http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html](http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html). Any mistakes were repaired and the samples were blast against a GenBank and identified using [http://isolate.fusariumdb.org/index.php](http://isolate.fusariumdb.org/index.php) and [http://blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)

### 2.4.4 Bacterial isolation and identification

Sequencing confirmed the identification of *Enterobacter cloacae* in the biofilm. The 16S RNA sequence was submitted in genbank and the GenBank accession number is “KP276148”.

The generated sequences were compared to sequences in the National Centre Biotechnology Information database using BLAST search option to determine the closest known related species (Altschul et al., 1997).
2.4.5 Phylogenic tree construction

Figure 2.4.25 Phylogenetic Tree Construction - Neighbour-joining analysis of a 566 bp fragment of the 16S gene using K2P parameter. Numbers at nodes indicated bootstrap values from 1000 replicates. Numbers before each species meant the bootstrap values among same species. Values below 50 were not showed. Enterobacter sp. BAB-3361 (KF984470.1); Enterobacter cloacae strain BR3 (KJ522786.1); Enterobacter cloacae strain AZ-3 (KJ675625.1); Enterobacter mori strain R18-2 (NR_116430.1); Enterobacter oryzendophyticus strain REICA_082 (JF795011.1); Enterobacter oryzendophyticus strain REICA_032 (JF795010.1); Enterobacter kobei (AB616140.1); Enterobacter soli strain LF7 (GU814270.1).

Clustering of Enterobacter cloacae (this study) with other Enterobacter cloacae and not with any other Enterobacter sp. indicates that this organism is Enterobacter cloacae. The 16S RNA sequence is submitted to the GenBank under the accession number “KP276148”.

Buranasilp and Charoenpanich, (2011) in their work in biodegradation of acrylamide identified the presence of Enterobacter aerogenes from domestic wastewater in Chonburi, Thailand.
Nur Ceyhan and Guven Ozdemir, (2011) also reported originally newly isolated strain of *Enterobacter aerogenes* from cooling towers of a petrochemical industry plant, in Turkey in their study.

Osode and Augustina Nwabuje, (2007) isolated 7 species (viz.), *Aeromonas hydrophila, Enterobacter aerogenes, Klebsiella pneumoniae, Klebsiella ozonae, Proteus mirabilis, Providencia rettgeri* and *E. coli* from the Dimbaza wastewater treatment plant.

Olinda and Naresh Magan, (2002 & 2003) in their work to examine the potential of using e-nose technology to differentiate water samples on the basis of qualitative volatile production pattern due to microbial or metal interaction isolated different microbial species like *Enterobacter aerogenes, Escherichia coli* and *Pseudomonas aeruginosa* in the presence of low concentrations of different heavy metals (As, Cd, Pb and Zn) in bottled, reverse osmosis (RO) and tap water.

Osode and Augustina Nwabuje, (2007) reported the presence of *Escherichia coli* and *Enterobacter aerogenes* during their work to test the numbers of fecal coliform bacteria in Lake Michigan, the site being located on South Shore Water Treatment Facility.

Ilona et al., (2009) in their work confirmed the presence of *Pseudomonas aeruginosa, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Enterobacter aerogenes, Staphylococcus aureus, Bacillus cereus* and *Citrobacter freundii* in the brewery effluent entering in most Nigerian rivers cause environmental pollution.

Saleem et al., (2011) inveterate that highly polluted surface waters are abundant in various types of bacteria among which, *E. coli* (15.77%) followed by *Enterobacter aerogenes* (12.19%) as well as *K. pneumoniae, C. freundii, Vibrio cholerae, Salmonella* spp and *P. aeruginosa* were predominant.

Zaika, Kot and Kharina, (2011) in their study of phages that inhabit water treatment systems described that *E. coli* is not the only microorganism that inhabits wastewater. *Enterobacter aerogenes*, a close relative of *E. coli* are also found in bulk water purification systems. As *E. coli*, this bacterium is conditionally pathogenic to humans and does not cause disease in healthy people. But unlike *Escherichia coli, Enterobacter aerogenes* and *E. cloacae* were studied very poorly.
Tasnim Farasat et al., (2012) in their work identified the presence of \textit{E.coli} in industrial wastewater effluents collected from different sources in Lahore.

In Jae Park et al., (2003) in their work in Characterization of the proteolytic activity of bacteria Isolated from a Rotating Biological Contactor reported four proteolytic bacteria isolated and identified from a rotating biological contactor.

Singh and Mittal, (2012) in their work reported the presence of \textit{Paracoccus pantotrophus}, nitrifiers and other heterotrophs in the biofilm of Rotating Biological Contactor treating synthetic wastewater.

Alicia van der Merwe, (2008) in his work isolated three different \textit{Pseudomonas} sps., like \textit{P.aeruginosa}, \textit{P.putida} and \textit{P.fluorescens} from biofilm treating wastewater.

Ebtesam Bestawy et al., (2005) isolated eight bacterial species from vegetable oil and grease-contaminated industrial wastewater of which only four namely, \textit{Pseudomonas} sp., \textit{P. diminuta}, \textit{P. pseudoalcaligenes} and \textit{Escherichia} sp. were found to have the ability to degrade oil and grease in the contaminated wastewater.

Sofia Andersson, (2009) in their research performed at the Division of Environmental Microbiology over the last years resulted in the isolation of possible bacterial key-organisms like \textit{Comamonas denitrificans}, \textit{Brachymonas denitrificans}, \textit{Aeromonas hydrophila} with efficient nutrient removal properties.

Kris Pynaert et al., (2003) in their study on lab-scale rotating biological contactor (RBC) treating a synthetic NH4\(^+\) wastewater devoid of organic carbon and showing high N losses characterized the biofilm where \textit{Nitrosomonas}-like species and \textit{Kuenenia stuttgartiensis} dominated.
Conclusion
2.5 CONCLUSION

The goal of this study was to isolate the microorganisms responsible for the degradation of the organic matter and the stabilization of organic wastes present in washwater released from Trichy Distilleries and chemicals ltd., Sangalliyandapuram, Trichy, Tamilnadu.

In our study five different colonies of microorganisms were isolated of which *Enterobacter aerogenes*, *E. cloacae* and *Escherichia coli* flourished well in the biofilm scrapped from RBC. Biochemical tests were done to identify the strains. 16S rDNA based identification of the isolate was done with the help of Scientific Synergy Laboratory, Chennai. The generated sequences were compared with sequences in the National Centre Biotechnology Information database using BLAST search option to determine the closest known related species and submitted to GenBank.