SCREENING, ISOLATION AND IDENTIFICATION OF MICROORGANISMS FROM POST-TREATED SEWAGE WATER

Thesis submitted in partial fulfillment of the requirements for the degree of

Masters of Philosophy (M. Phil)

IN

MICROBIOLOGY

By

MEERA ONGMU BHUTIA

Roll No: 12PDMB03

Registration No: 12SU6397

Department of Microbiology, School of Life Sciences

Sikkim University

Tadong, Gangtok, Sikkim 737102

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सिक्किम विश्वविद्यालय

(भारतीय संसद के अधिनियमद्वारा स्थापित केन्द्रीय विश्वविद्यालय)

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I declare that the thesis entitled **"Screening, Isolation and Identification of Microorganisms from Post-Treated Sewage Water"** submitted by me for the award of **Master of Philosophy (M.Phil) Degree in Microbiology** of Sikkim University is my original work. The content of this thesis is based on the experiments which I have performed myself. This thesis has not been submitted for any other degree to any other University.

Date: 22nd July 2014

Meera Ongmu Bhutia

Roll No: 12PDMB03

Regn.No: 12SU6397

We recommend that the thesis be placed before the Examiners for evaluation.

Signature of Head of the Department

Dr H K Tiwari

Signature of the Supervisor

Dr Bimala Singh

6 माईल, सामदुर, तादोंग, गगंटोक - 737102 सिक्किम, भारत

दूरभाष : 00-91-3592 - 251067, 251403, फैक्स :- 251067/251757

6th Mile, Samdur, PO Tadong 737102, Gangtok, Sikkim, India

Phones : 00-91-3592-251067, 251403, Fax - 251067/251757

website : www.sikkimuniversity.in/www.sikkimuniversity.ac.in

Email : sikkimuniversity@gmail.com

सिक्किम विश्वविद्यालय
(भारतीय संसद के अधिनियमद्वारा स्थापित केन्द्रीय विश्वविद्यालय)
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CERTIFICATE

This is to certify that the thesis entitled **"Screening, Isolation and Identification of Microorganisms from Post-Treated Sewage Water"** submitted to the **Sikkim University** for the award of **Master of Philosophy (M.Phil) Degree in Microbiology,** embodies the results of *bona fide* research work carried out by Ms. Meera Ongmu Bhutia under my guidance and supervision. No part of the thesis has been submitted for any other degree, diploma, associate-ship and fellowship.

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Bimala Singh

Place: Gangtok, Sikkim

Date: 22nd July 2014

Dr. Bimala Singh

(Supervisor)

Department of Microbiology

6 माईल, सामदुर, तादोंग, गगंटोक - 737102 सिक्किम, भारत
दूराभाष : 00-91-3592 - 251067, 251403, फैक्स :- 251067/251757

6th Mile, Samdur, PO Tadong 737102, Gangtok, Sikkim, India
Phones : 00-91-3592-251067, 251403, Fax - 251067/251757
website : www.sikkimuniversity.in/www.sikkimuniversity.ac.in
Email : sikkimuniversity@gmail.com

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Dedication

Dedicated to the sweet memories of my loving Father........

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List of Abbreviations Used

IND	International Nomenclature of Diseases
UN	United Nations
WHO	World Health Organisation
WWTP	Waste water treatment plant
PHED	Public Health and Engineering department
IL&FS	Infrastructure Leasing & Financial Services Limited
SDR	Sikkim Development Report
ADB	Asian Development Bank (ADB)
CPCB	Central Pollution Control Board
STP	Sewage treatment plant
WII	Winrock International India
ICDDR	International Centre for Diarrhoeal Disease Research
EPA	Environmental Protection Agency
NICD	National Institute of Communicable diesease
MLD	Million litres per day
UNESCO	United Nations Educational, Scientific and Cultural Organization
UNICEF	United Nations International Children's Emergency Fund
µS/cm	Micro Siemens/centimeter
mg/L	Milligram/litre
CFU/mL	Colony forming unit per millilitre

Chapter 1

Introduction

1. INTRODUCTION

Due to rapid population growth and increasing industrialization, the demands of natural resources of earth mainly water is extremely increasing (UNESCO, 2009). To ensure adequate public health, the quality and quantity of these resources are important (Gupta et al., 2004). The main global cause of mortality due to scarcity of safe drinking water and poor sanitation is estimated to be approximately 4,000 per day (Bartram et al., 2005). Despite meeting the Millennium Development Goals regarding access to potable water and proper sanitation, there will still be 547 million people without a safe drinking water supply and 2.4 billion people without access to an improved sanitation facility in 2015 (UN, 2012; UNICEF, 2014). Waterborne infections occur more in developing nations than in developed nations due to lower levels of sanitation, less public health awareness and low socioeconomic conditions (Toze, 1997; Elimelech, 2006).

Riverine system is the fundamental resource of water in many parts of the world (Thorvat et al., 2011). At the source of a river, the water is relatively pure, but when it flows towards downstream, many pollutants like human sewage, animal waste, industrial effluents and urban runoff enter river water, thus polluting it (Khan et al., 2009; Ahmed et al., 2005).

Domestic wastewater is a combination of human and animal excreta consisting mainly of proteins, carbohydrates, fats and oils, urea derived from urine and gray water resulting from washing, bathing, and cooking (Bitton, 2005; Naidoo et al., 2014).
It also consists of heavy metals, nonmetals, chlorinated compounds and benzene compounds (Metcalf and Eddy, 2004). Today acute pollution prevails in Indian rivers like Ganga, Yamuna, Bramhaputra, Krishna, Hoogly etc. which are getting contaminated day by day (Thorvat et al., 2011). Deteriorating water quality poses a great risk to the health of downstream communities who use river water for various purposes like drinking, washing clothes, and bathing with no prior treatment (Raschid et al., 2005).

Wastewater treatment plants is being recognized as the key sources of microbial pollution of water, atmospheric air and soil which pose a greater risk to the environment

and humans (Douwes et al., 2001; Fracchia et al., 2006; Grisoli et al., 2009; Reinthaler et al., 2010; Korzeniewska, 2010). Hospital and municipal wastewaters may contain pathogenic viruses, multidrug-resistant bacteria, yeasts, protozoa, and parasite eggs (Lin et al, 2004; Yong et al, 2009). Therefore, pollutants in waste water need to be brought down to permissible limits for safe disposal in order to reduce the environmental and health hazards (Boots et al., 1978; Manju et al., 1998).

Current knowledge of the need for sanitation and treatment of polluted water began when there was an outbreak of cholera in London in the year 1885 and John Snow proved that a cholera outbreak was due to sewage contaminated river water (Toze et al., 1997). Hence, Biological wastewater treatment is very important for its reuse in different activities (Chen, 1997; Raj et al., 1997). A heterotrophic bacterium oxidizes the organic material biochemically under aerobic condition to yield carbon dioxide, ammonia, water and new biomass. Methanogenic archaea, under anaerobic condition, partly oxidizes organic material to produce methane, carbon dioxide etc (Madigan et al., 2003)

The most frequently found bacterial community in sewage belongs to *Enterobacteriaceae* family as these are natural microbiota of human gastrointestinal tract, (Wery et al., 2008; Korzeniewska et al., 2009). The degree of efficiency of wastewater treatment plant (WWTP) is indicated by the number of *Enterobacteriaceae* bacteria present in effluents (Filipkowska, 2003; Espigares et al., 2006). Hence, coliforms represent important indicator organisms for evaluating bacteriological safety of drinking water, recreational area, and food. (Nwachukwu et al., 2006).

In Sikkim, Gangtok is the only town to have a piped sewer system and sewage treatment plant which was established in 1980 and is maintained by the Public Health Engineering Department (PHED) (IL&FS, 2003). The main trunk line of 300mm to 750mm diameter which has been laid along NH31 A from the center of town to treatment plant site at Adampool covers a distance of almost 12 km. The present treatment capacity of the plant at Adampool is 10 million litres per day (MLD) only although the liquid waste generation is to tune of 20 MLD (Annual Report of Water Security and Public Health Engineering Department, 2010-2011). The treated sewage effluent is being discharged into the river (Rongni Chu). However, the treatment plant is poorly

maintained and the leakage of pipe joints often occurs especially during the monsoons resulting in contamination of ground water and soil. Hence, this pose a severe threat on public health affecting 60 percent of household with 140,000 cases of gastrointestinal disease reported annually (SDR, 2008).

`Therefore the present study of the dissertation emphasizes on the microbial analysis of Post Treated Sewage effluent, Adampool as it is of greater importance to estimate the hazard due to fecal pollution containing pathogenic organisms.

1.1. Rationale and scope of study

In India almost 70% of the water has become polluted due to the discharge of domestic sewage and industrial effluents into natural water source, such as rivers, streams as well as lakes (Sangu, 1987). The improper management of water systems may cause serious problems in terms of availability and quality of water (Subbarao et al., 1995). Since water quality and human health are closely related, analysis of wastewater effluent is of prime importance before it is disposed off into river water. Certain physical, chemical and microbiological standards are designed to ensure the efficiency of wastewater treatment (Tebutt, 1983; Mara, 2004).

In Sikkim, Gangtok is the only town to have a full-fledged sewerage system and it caters to approx 25,000 people. Sewage is treated at Adampool Treatment Plant, Gangtok (East Sikkim) and finally disposes off into the river body (Rongni chu) (SDR, 2008). The river water where the treated sewage effluent is being disposed off, may be the sourceof irrigation, drinking and recreation purposes for downstream communities. Hence, it is of great importance to analyse the quality of wastewater before it is being disposed off as it may pose a health risk to humans as well as environment. With this intent, the present study was an attempt to evaluate the general bacteriological and physico-chemical parameters of wastewater from Adampool sewage treatment plant. The presumptive identification of these isolated bacterial population were done based on morphological and biochemical characterization.

1.2. AIM AND OBJECTIVES OF THE STUDY

- To study some physico-chemical parameters of the post-treated sewage water.
- To isolate and characterize some bacterial population present in post treated sewage.
- To identify the isolated microorganisms based on morphological and biochemical characteristics (Presumptive identification).

Chapter 2

Review of Literature

2 REVIEW OF LITERATURE

Both in the developed and developing world, water-borne diseases continue to pose a major threat to human health (Ford, 1999). Despite widespread employment of sewage treatment facilities and water treatment practice design to ensure safe water supplies, outbreak of diarrhoeal diseases still occur in developed world (Colwell, 1996). About 1.7 million people worldwide die from infectious diarrhoeal disease every year and the scenario is more severe in developing nations with high mortality rate among children due to lack of sanitation, hygiene and unsafe water (WHO, 2002).

Water pollution and freshwater depletion are currently viewed as the top environmental problem in Asian region (ADB, 1997). The availability of fresh water is less than 3% of the world's total water supply and due to this veracity water that was earlier used for drinking, cleaning and bathing will ultimately end up back in a river or lake for it to be consumed once again (Ellis, 2004).

Wastewater can be divided into two categories - industrial wastewater and municipal (domestic) wastewater. Generally, the industrial wastewater undergoes in-plant pretreatment and is reused before being discharged into a public water bodies. However, the domestic wastewater consisting of human waste and grey water from house activities are usually connected to a sewerage pipeline and finally is disposed off into the surface water after being treated at the sewage treatment plant (Patankar, 2001).

In the developing world, the main source for irrigation, recreational, drinking, and other domestic purposes are surface water (Ashbolt et al., 2004; Begum et al., 2005; Obi et al., 2004). In India, there are around 234 sewage treatment plants (STPs). Most of these were developed under various river action plans (from 1978-79 onwards) and are located in cities along the banks of major rivers (CPCB, 2005). This clearly signifies that the main source of water pollution is municipal waste (Patankar, 2001).

2.1 Waste Water Treatment

There are three major steps of waste water treatment, namely, primary secondary and tertiary treatment. Primary treatment separates large solids from waste stream (Maier

et al., 2009). Secondary treatment is one of the key components of a wastewater treatment plants as it reduces the biochemical oxygen demand (BOD), suspended solids (SS) and toxicity of industrial wastewaters and the production environmental friendly outgoing effluent (Sahlstrom et al., 2004). Tertiary treatment involves coagulation, disinfection, reverse osmosis which further reduces turbidity, nitrogen, phosphorus, metals etc. (Maier et al., 2009). Processing of the effluent also reduces the number of pathogenic organisms that may be present (Betancourt et al., 2004). However, most treatment plants are designed to eliminate organic matter only which clearly indicates the prevalence of pathogens in waste water effluents as the degree of pathogenic bacteria in wastewater totally depends on the duration of the process and the treatment principles applied (aerobic/anaerobic) (Curtis, 2003).

2.2 Waste Water Reuse

Recycled wastewater have been used in variety of applications which includes agricultural irrigation, recreational, industrial process etc (Patankar, 2001). Agriculture irrigation is the biggest use of recycled wastewater with an estimated 80% of wastewater being used for irrigation by India and China (Winrock International India, 2007). In areas like Vadodara and Gujarat, the sale of waste water is common due to lack of alternative sources of water (Bhamoriya, 2004). It has been reported that irrigation with waste water results in higher crop productivity, over the normal waters (Van der Hoek et al., 2002; Cornish et al., 2001; Ensink et al., 2002; Danso et al., 2002; Buechler et al., 2003) The farmers of Andhra Pradesh uses urban and industrial untreated wastewater diluted with fresh river water for irrigation chiefly during the monsoon season (Buechler et al., 2003). In Karnataka, open wastewater drains flowing out of cities are the source of water for agriculture (Raschid et al., 2004). Reuse of waste water without treatment can lead to significantly surplus disease as well as economic losses (Shuval et al., 1986; Swerdlow et al., 1992).

2.3 Health and Environmental concerns in Wastewater Management

In India, due to lack of infrastructure and resources, more than 80% (out of 16,662.5 MLD of wastewater generated, only 4037.2 MLD) of wastewater generated is discharged into natural water bodies without any treatment (CPCB, 2000; WII, 2007). Twenty-seven cities have only primary treatment facilities and only forty-nine have primary and secondary treatment facilities.

The river Ganga and its tributaries meet 40% of the water requirement for drinking and irrigation in India (CPCB, 2002). Inefficient treatment processes leads to release of microorganisms into the aquatic environment and become a major source of faecal contamination (George et al, 2002; Hendricks, et al, 2012). Practices of holy dip and crematory processes along the banks also have serious impacts on physicochemical and microbiological water quality of the river (Sikander, 1986). High density of coliform bacteria has been reported in the river Ganga throughout its course from Himalaya to Bay of Bengal (CPCB, 2005). Faecal contaminants pose a health risk to humans and animals upon exposure to contaminated water (George et al., 2002).

Waterborne pathogens have a devastating effect on public health, especially in the developing countries of sub-Saharan Africa and Southeast Asia (Pitman, 2002). Waterborne infectious agents responsible for these diseases include a variety of helminthes, protozoa, fungi, bacteria, rickettsiae, viruses and prions (WHO, 2003).

The infectious diseases which can be transmitted from water are summarized in Table 1.

Table 2.1: Prevalence of common water-related diseases

Disease	Cause and route of transmission	Geographic Extent	Number of Cases	Deaths per year
Major water-borne diseases				
Diarrheal disease (including amoebic and bacillary dysentery)	Various bacteria, viruses and protozoa travel the fecal-oral route via contaminated water, food and person to person contact	Worldwide	4 billion currently	3-4 million
Cholera	Bacteria travel the fecal-oral route via contaminated water, food, person to person contact	South America, Africa, Asia	384000 per year	20000
Hepatitis A	Virus travels the fecal-oral route via contaminated water, food, person to person contact	Worldwide	600000 to 3 million	2400-12000
Typhoid and Paratyphoid	Bacteria travel fecal-oral route via contaminated water, food, person to person contact	20% Latin America, Africa 80% in Asia	16 million currently	600000
Polio	Virus travels fecal-oral route via contaminated water, food, person to person contact	66% in India, 34% in near East Asia ,Africa	82000 currently	9000

Source: WHO 1996, WHO 1998

Nematode infections, particularly from the roundworm *Ascaris lumbricoides,* can be spread by effluent reuse practices (WHO, 1989). Giardiasis is the most commonly reported intestinal protozoan parasite infection worldwide and prevalence surveys of infection among children range from 1 to 68% (Chen et a., 2007). The analysis of waste water and water for the presence of pathogens such as *Salmonella species, Shigella species and Vibrio cholera* is of particular concern because they have been associated with gastrointestinal infections, therefore, is not in agreement with EPA water standard for recreational use (EPA, 2003). Other Pathogenes including Enteropathogenic strains of *Eschericha coli*, *Pasteurella*, *Campylobacter, Brucella*, *Clostridium,* and many others can be isolated from sewage (Sobsey, 1989; Reasoner, 1982; Hill et al., 1996).

Many are enteric, however *Legionella, Mycobacterium species* and *Leptospira* have also been isolated from wastewater which are non-enteric illness causing bacteria (Wilson et al., 1995; Fliermans, 1996; Neuman et al., 1997). Every decade or so, due to globalization, changing population demographics, travel and the application of new detection technologies a "new pathogens" have been identified which are called as 'Emerging Pathogens' (Nwachcuku et al., 2004). Emerging waterborne pathogens are those micro-organisms that have become suddenly or gradually more important as a cause of waterborne disease, particularly those which contaminate surface waters via waste water effluents (Hoogenboezem, 2007). One such example is *Helicobacterium pylori,* a major cause of peptic ulcer which are tolerant to high pH (Bergey et al., 1994). Others include highly infectious *Escherichia coli* O157:H7, a virulent strain of *Escherichia coli* is a causative agent of Haemorrhagic colitis and Haemolytic uremic syndrome (Collier et al., 1998; Muniesa et al., 2000). One of important feature of *Escherichia coli* O157 is that it can survive for very long time in water (Geldrich et al., 1998). The strain *Escherichia coli* O157:H7 has been isolated from both untreated and treated water sources from North and South America (Martins et al., 1992; Tsai et al., 1993; Grant et al., 1996).

Vibrio cholera which is the main cause of cholera are more resistant to disinfection and may be present in non-endemic carriers that may contribute to contamination of waste water without medical treatment (Lechevallier et al., 1999;

Curtis, 1996). *Vibrio cholera* O139, a toxigenic strain of *Vibrio cholera*, also known as "Bengal strain" was reported to have been emerged from India in October 1992, causing a new pandemic to Asia (ICDDR, 1993).

Water washed infection occur due to poor domestic and personal hygiene and the disease is not due to presence of infectious agent in water but rather due to lack of readily accessible water. *Shigella species* is such agent which causes such disease by faeces contaminated hands and utensils (Moe, 2007). Typhoid fever is caused *by Salmonella enterica serovar typhi* was once the most common form of waterborne disease in developed countries, occurring far more commonly than cholera (Chin, 2000). Globally, it is estimated that every year 600 000 deaths occurs due to typhoid fever and over 16 million cases of illness are reported (Wain et al., 2003).

Sewage often contains heavy metals which have serious health hazard on in the long term exposure that can lead to cancer and kidney disease (Shuval et al., 1986; Nriagu, 1990; Ghafoor et al., 1995; Blumenthal et al., 2000). Sewage discharges drastically alters the ecology of environment by lowering dissolved oxygen conditions and nutrient loading of the water bodies such as nitrogen and phosphorous resulting in toxic algal blooms, a condition called Eutrophication (Morrison et al., 2001, Jeffrey, 2014). One of the important aspects of current environmental research in water is the emergence of contaminants from sewage such as endocrine disrupting chemicals (EDCs), pharmaceuticals and personal care products (PPCPs) which interfere with the endocrine system of wildlife and human (Sorensen et al., 1998; Sumpter et al., 2005).

2.4 Indicator of Pathogens

To monitor bacterial pathogen removal in waste water treatment, faecal coliforms (*Escherichia coli*) are typically employed (Curtis, 2003), which is universally accepted for monitoring and assessing the micro-biological safety of water supplies (Dissanayake et al., 2004). In 2004, *Enterococcus* species took the place of fecal coliform as the new USA Federal standard for water quality at public salt water beaches and *Escherichia coli* at fresh water beaches (EPA, 2004). It is believed to provide a higher correlation than

faecal coliform with many of the human pathogens often found in city sewage (Jin G et al., 2004).

The removal of *Vibrio cholera* O1 from the treatment plant is still not clear because this non pathogenic *Vibrio cholera* have been found to grow in waste stabilization ponds. Recently, it has been discovered that the toxin gene that distinguish the pathogenic organisms from non- pathogenic form is encoded by a bacteriophage which is induced by sunlight. This study indicates that this phage may constitute part of a natural mechanism for the origination of new toxigenic strains of *V. cholera* (Faruque et al., 2000).

2.5 Outbreak of waterborne infection

Widespread outbreak of enteric bacterial disease would not be possible if there would have been an improved waste water treatment plant (Cabe, 1970). The first investigator who showed a distinct relation between faecal contamination of drinking water and the occurrence of Cholera was Dr. John Snow in Victorian London (Richardson et al., 1936). The work of Snow is considered as the first epidemiological study. This study further clearly indicates the relation between feacal contaminations as a transmission route for illness. In the year 1990 an epidemic of cholera caused widespread suffering and deaths in South America (Daniel et al., 2005) and during rainy seasons the disease spread even further. Recently, epidemics of cholera have been reported from different parts of India, Nigeria and Zimbabwe. The outbreak was caused by *Vibrio cholera* O1 isolated from municipal taps and wells (Sur et al., 2006). In England, every year over 50 cases of cholera is being reported to public health authority (CDSE, 2005).

Since time immemorial cholera has been endemic in the Ganges delta. There were annual epidemics in West Bengal and Bangladesh (NICD, 2008). The burden of cholera in India is miscalculated due to lack of surveillance and proper laboratory support (Deen et al., 2008). The occurrence of cholera is far more than the annual number which is reported by Government of India to the World Health Organisation (WHO). This findings clearly indicates that cholera is an under-recognized problem in India (Sarkar et al., 2012). Payment et al, in the year 2000, in their study found that both in drinking water

sources and sewage from Canada have been shown to contain microorganisms such as Total coliforms, faecal coliforms, *Giardia lamblia, Cryptosporidium,* human enteric viruses, and *Clostridium perfringens*.

Several outbreaks of *Escherichia coli* O157:H7 associated with drinking water have been reported in U.S with first recognize outbreak in Missouri community in1986 with 243 cases (Herwaldt et al., 1991). It has also been associated with recreational water exposure (CDC, 1993; CDC, 1996) which involve lake water and treated swimming pool (CDC, 2002).Reported recreational water outbreaks have involved *Giardia, Cryptosporidium, Norovirus, Shigella sonnei* etc. enter gastrointestinal tract by ingestion (CDC, 1993; CDC, 2002; CDC, 2004). Other recreational waterborne outbreaks have involved ingestion, inhalation or contact of organisms like *Pseudomonas, Legionella* (CDC, 1993), *Staphylococcus aureus* (Charoencaet al., 1993), *Vibrio* species and Mycobacterium species (Dufour, 1986). Number of waterborne disease outbreak associated with recreational water has been increasing with highest being reported in 2000 and 2001 since 1978 (CDC, 2004).

Few cases of Typhoid have been reported from the United States and most of the cases are imported from endemic areas (Chin, 2000). Typhoid fever is endemic in most parts of Central America (Olarte and Galindo, 1973), Southeast Asia (Mirza et al., 1996; Ling et al., 2000) and the Indian subcontinent (Shanahan et al., 1998; Rahman et al., 2002) and recently increasing numbers of cases have been reported in Africa (Kariuki et al., 2000, Mills-Robertson et al., 2002). Outbreaks of typhoid fevers and dysentery were linked to unsanitary mixing of some water supplies and sewage. It has been reported that 80% of sicknesses and deaths among children in the world are caused by unsafe drinking water (WHO, 2003).

Recently *Helicobacterium pylori* have been detected in USA drinking water from private wells. A significant relation between people using drinking water containing *H. pylori* and the number of ulcers has been observed (Baker, 1999).

2.6 Antibiotic resistance, the need for global solutions

.The emergence of multi-drug resistance in bacterial human pathogens is one of the most serious challenges for health care globally (Rizzo, 2013). Pathogens that earlier were sensitive to antibiotics are becoming resistant by acquiring resistant genes or by mutation in DNA which is preexisted (Deplano et al., 1997). One of the main sources of antibiotics release into the environment is wastewater treatment plants (WTPs) and the treatment facilities can be a hotspot for horizontal resistance gene transfer between the bacteria (Bouki et al., 2013).

The major worldwide public health issues which needs serious action is antibiotic resistance Enterobactericeae (Levy, 2002, Wright, 2007). Antibiotic resistance genes (ARGs) have been found in Enterobactericeae isolated from sewage treatment plants (Auerbach et al., 2007), groundwater (Sandford et al., 2001), river (Thompson et al., 2007) and sediments (Pei et al., 2006). It has been demonstrated that hospital wastewater contains a higher number of resistant bacteria (Schwartz et al., 2003; Stieber et al., 2004).

Extended spectrum β- lactamase (ESBL) and New Delhi metallo β- lactamase (NDM-1) enzymes produced by Enterobactericeae and *Klebsiella pneumonia* carbapenemase (KPC) are spreading worldwide (Rolain et al., 2010; Schlesinger et al., 2011). In the year 2011, Walsh et al findings in New Delhi showed NDM-1-producing bacteria (including *Vibrio cholera* and *Shigella boydii)* in two (4%) of 50 drinking water samples and fifty one (30%) of 171 seepage samples that clearly suggests the possibility of acquiring resistance outside health-care facilities. The human activities on the banks of rivers, ponds, and lakes also contribute to contamination of surface waters by multiple drug resistant *E. coli* harboring virulence genes (Lin et al., 2004; Ram et al., 2004; Edge et al., 2005).

An epidemic of chloramphenicol resistant *Salmonella typhi* emerged in 1972 and another epidemic with concurrent resistance for chloramphenicol, co-trimoxazole and ampicillin in 1992 (Saha et al., 1992). *Enterococci* is one of the leading nosocomial pathogens that have been related to human diseases (Murray et al, 1990; Devriese et al., 1994; Jett et al., 1994; Flahaud et al., 1997). Vancomycin-resistant *Enterococci* (VRE) is an emerging international threat to public health (Donald et al., 1997). Prevalence of

vancomycin resistant *Enterococcus* is high from sewage (Iversen et al., 2002, Talebi et al., 2008) The chief cause for the emerging multi drug resistance (MDR) in sewage is heavy use of antibiotics and indiscriminate release of hospital wastewater into public sewage (Chitnis, 2002, Ekhaise et al., 2008).

2.7 Research on different aspects of wastewater

2.7.1 Waste water treatment using microalgae:

Microalgae play a major role in aerobic treatment of waste in the secondary treatment process, such as for the removal of nitrogen and phosphorus, coliform bacteria, heavy metals and reduction of biochemical oxygen demand (Raouf et al., 2012). It can also generate biomass that can be a tremendous source of 'organic' fertilizers (Kaur et al., 2012) as well as biofuel production (Greenwell et al., 2010).

Currently major interest is developed in some advanced world nations such as USA, Australia, Thailand, Taiwan and Mexico (Borowitzka, 1988; Moreno et al., 1990, Wong et al., 1990 and Renaud et al., 1994). A system such as the Advanced Integrated Wastewater Pond Systems (AIWPS) technology for algae based wastewater treatment was commercialized by Oswald (1991) and Green, (1996) in the United States.

2.7.2 Waste water treatment using membrane bioreactor:

Membrane bioreactors technology is implemented with immersed microfiltration or ultra filtration membranes that replace gravity sedimentation and clarify the wastewater effluent (Judd, 2006). Membrane bioreactors (MBRs) can produce high-quality effluent that is appropriate for unlimited industrial and irrigation applications especially prospective for use in developing countries where there is a need for improved sanitation (Daiger et al., 2005). One of the growing applications of MBRs is as pretreatment for reverse osmosis, which, then followed by ultra violet radiation that can produce water for direct or indirect potable use (Mark et al., 2008).

2.7.3 Wastewater treatment using constructed wetlands:

Constructed wetlands are the recently proven efficient technologies for wastewater treatment. Compared to conventional treatment systems, constructed wetlands are low cost, are easily operated and maintained, and have a strong potential for application in developing countries (Amelia, 2001). There have been a several reports of faecal coliform and pathogen removal in wetlands in different formats e.g. reed beds (Coombes et al., 1995; Cooper et al., 1996; Green et al., 1997). However, removal of Vibrio cholera was as slow as removal of the indicators (Stott et al., 1996)

Indian experience with constructed wetland systems is still on an experimental scale, treating different kinds of wastewater (Juwarkar et al., 1995; Billore et al., 1999, 2001, 2002; Jayakumar et al., 2002). One of the major restraints of wetland systems is the requirement of a large land area that is not readily available in developing countries like India (Kaur et al., 2012).

Chapter 3

Materials & Methods

3. MATERIALS AND METHODS

3.1 Materials

All the chemicals used in this study were obtained from HiMedia and Merck, Mumbai and Stanbio, Kolkata.

3.1.1 Instruments used

Instrument	Code
Autoclave	Instrumentation India .
Hot air oven	Nainstrument naha0/031/09
Laminar air flow	Remi/rofv170
Incubator	Remi/csi-24bl
Micro pipette	Pipetman
Weighing machine	Pl-202-s/03
Micro oven	Samsung rtz4/2009
Refrigerator	Samsung
pH meter	8102nuwp(Thermoscientific)

3.1.2 Culture media and media components

Thiosulphate-citrate-bile salts-sucrose agar (TCBS)	M189, Hi-Media
MacConkey Sorbitol Agar	M298, Hi-Media
Salmonella-Shigella Agar (SS)	M1032, Hi-Media

Preparation of the following media were done by referring Cappuccino and Sherman (Microbiology Laboratory Manual) 2009.

Nutrient agar medium	Composition
Beef extract	3g
Peptone	5g
Sodium chloride	5g
Agar	15g

Distilled water	1000 mL
pH	7.4± 0.2 at 25°C

Nutrient Broth	**Composition**
Peptone	5g
Sodium chloride	3g
Beef extract	3g
Distilled water	1000mL
pH	7.4± 0.2 at 25°C

MacConkey Agar	**Composition**
Peptone	20 g
Trehalose	10 g
Bile salts	1.5 g
Sodium chloride	5 g
Neutral red	0.05 g
Crystal violet	0.001 g
Agar	15 g
Distilled water	1000 mL
pH	7.4

Eosin Methylene Blue Agar	**Composition**
Peptone	10g
Lactose	5g
Dipottasium phosphate	2g
Agar	13.5g
Eosin Y	0.4g
Methylene blue	0.065g
pH	7.2

3.1.3 Media for the biochemical tests

Media	**Code**
Sim Medium	M181 Hi-Media
MR-VP Medium	M070, Hi-Media

Simmons Citrate Agar	**Composition**
Ammonium dihydrogen phosphate	1g
Dipottasium phosphate	1g
Sodium chloride	5g
Sodium citrate	2g
Magnesium sulphate	0.2g
Bromothymol blue	0.08g
Agar	15g
Distilled water	1000 mL
pH	7

Triple Sugar Iron Agar	**Composition**
Beef extract	3g
Yeast extract	3g
Peptone	15g
Proteose peptone	5g
Lactose	10g
Saccharose	10g
Dextrose	1g
Ferrous sulphate	0.2g
Sodium chloride	5g
Sodium thiosulphate	0.3g
Phenol red	0.024g
Agar	12g
pH	7.4

Arabinose Fermentation Broth	**Composition**
Tripticase	10g
Arabinose	5g
Sodium chloride	5g
Phenol red	0.018g
Distilled water	1000 mL

Rhamnose Fermentation Broth	**Composition**
Tripticase	10g
Rhamnose	5g
Sodium chloride	5g
Phenol red	0.018g
Distilled water	1000 mL

Raffinose Fermentation broth	**Composition**
Tripticase	10g
Raffinose	5g
Sodium chloride	5g
Phenol red	0.018g
Distilled water	1000 mL

Arabinose Fermentation Broth	**Composition**
Tripticase	10g
Arabinose	5g
Sodium chloride	5g
Phenol red	0.018g
Distilled water	1000 mL

Xylose Fermentation Broth	**Composition**
Tripticase	10g
Xylose	5g
Sodium chloride	5g
Phenol red	0.018g
Distilled water	1000 mL

Inositol Fermentation Broth	**Composition**
Tripticase	10g
Inositol	5g
Sodium chloride	5g
Phenol red	0.018g
Distilled water	1000 mL

Dulcitol Fermentation Broth	Composition
Tripticase	10g
Dulcitol	5g
Sodium chloride	5g
Phenol red	0.018g
Distilled water	1000 mL

3.1.4 REAGENTS

Gram's Crystal Violet	S012, (Hi Media)
Safranin	S027 (Hi Media)
P-Amino-N,N dimethylanilineine Oxalate	RM641 (HiMedia)
Phenol red	MH5M552152 (Merck)
Neutral red	1008 (Hi Media)
Safranine	10225 (Stanbio)
Grams iodine	RM1315 (Hi Media)

Gram's iodine

Iodine	1g
Potassium iodide	2g
Distilled water	300 mL

Kovac's reagent

p-Dimethylaminobenzaldehyde	5g
Amyl alcohol	75 mL
Hydrochloric acid	25 mL

Barrit's reagent

Solution A

α- Napthanol	5g
Ethanol	95 mL

Solution B

Potassium hydroxide	40 g
Creatinine	0.3 g
Distilled water	1000 mL

Methyl Red Solution

Methyl red	0.1g
Ethyl alcohol	300 mL
Distilled water	200 mL
p- aminodimethylalanine oxalate	0.5g
Distilled water	50 mL

5 % Aqueous Malachite Green

Malachite green	100g
Distilled water	2 L

0.25 % Methylene blue

Methylene blue	0.25 g
Glacial acetic acid	1 mL
Distilled water	99 Ml

Carbol fuschin

Basic fuchsin	1g
Phenol crystal	5g
Absolute ethanol	10 mL
Distilled water	100 mL

3.1.5 Chemicals

Concentrated Hydrochloric acid	HG8H580592 (MERCK)
Absolute ethyl alcohol	MB106 (HI MEDIA)
Hydrogen peroxide	HI6H560662 (MERCK)
Urea Broth Base	M111, HiMedia
Urea GR	MJ7M572681,Merck

3.2. METHODOLOGY

3.2.1 Sample location site

Samples were collected from sewage treatment plant (STP) Adampool, located 10 kms away from Gangtok, East Sikkim, 27.3065°N, 88.5828°E.

3.2.2 Collection of samples

Post treated sewage sample were collected from Adampool Sewage Treatment Plant, Gangtok, East Sikkim. The samples were collected in sterile 200 mL plastic bottles and immediately transported to the laboratory for analysis and stored at a 4-8°C until microbial analysis (Atieno et al., 2013). All samples were analyzed within 2 hours of collection and were collected in duplicate during each sampling. A total of four sets of sample were collected during the month of November (2013), February (2014), May (2014) and June (2014). The samples collection was done in the morning time from 9 am-12 am.

3.2.3 Period of study

The samples were studied between the months of November 2013 to June 2014.

3.2.4 Physico-chemical analysis of sample

The value of temperature, total dissolved solids and conductivity of post treated sewage sample were measured by using electronic portable Pocke Tester (La Motte Tracer) (code: 1766). The pH of effluent was measured using automatic pH meter (Orion Z Star Thermoscientific).

3.2.5 Isolation of microorganisms

1 mL of the post treated sewage sample collected in duplicate (sample A and sample B) was transferred in tube containing 9 mL sterile distilled water. Serial dilutions (10^{-1} – 10^{9}) were prepared for both samples taking the initial dilution as 10^{-1}. From the dilution range of 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}, 0.1 mL of the sample was inoculated in nutrient

agar (NA), Eosin Methylene Blue agar (EMB), MacConkey agar, Salmonella-Shigella agar, Tri-citrate bile sugar media and Sorbitol MacConkey agar using spread plate technique (Kumar et al., 2012; Eze, 2009; Sherman et al., 2009). The inoculated plates were incubated at 37^0 C for 24 hrs (Eze, 2009). After incubation individual colonies were isolated in pure culture using streak plate technique (Eze, 2009; Sherman et al., 2009).

3.2.6 Preservation of pure culture

Pure cultures isolated by streak plating technique from nutrient agar and various selective media were preserved in nutrient broth using 15% (v/v) glycerol at -80^0 C. Slants of pure isolates were prepared in nutrient agar and preserved at 4^0 C for working culture.

3.2.7 Determination of microbial load

a. Plate count method

R2A agar was used for the determination of microbial load in terms of CFU/mL (Reasoner, 1985). The post treated sewage sample was serially diluted up to a dilution of 10^{-9}. Dilutions of 10^{-3}, 10^{-4}, 10^{-5} and10^{-6} were selected and 0.1 mL of the above dilution was plated in R2A agar in triplicates using spread plate technique. The plates were incubated at 37°C for 24 hours (Reasoner, 1985). After 24 hours the plates were observed for growth and the number of bacteria was counted in terms of CFU/mL for each dilution and average number of bacteria in the sample was calculated.

b. Most probable number (MPN)

According to the standards of Environmental Protection Agency (EPA, 2003), the MPN method was used to estimate the concentration of viable microorganisms in the sewage sample. In this method five tubes of each of the three dilutions 10 mL, 1 mL and 0.1 mL of post treated sewage sample was taken and inoculated in lactose broth with Durham's tube to check for lactose fermentation and gas production. Only the presumptive test was performed. Growth of the bacteria was observed as turbidity in lactose broth and gas production as formation of gas bubble inside the inverted Durham's tube. Tubes with bacterial growth (turbid) and gas production were taken as positive tube

and the results were tabulated. The MPN index and 95 % confidence limit per 100 mL is calculated by referring the results with the standard chart of MPN index (EPA, 2003; American Public Health Association, 1916).

3.3 Physiological characteristics of bacterial isolates

a. Production of the enzyme catalase

The production of enzyme catalase by the isolates was detected as gas bubbles on addition of 3% hydrogen peroxide solution to the cultures (Taylor et al., 1972). A smear of each isolate was prepared on glass slides on top of which a drop of 3% hydrogen peroxide solution was added (Taylor et al., 1972). Detection of gas bubble was interpreted as a positive reaction indicating that the isolate produces the enzyme catalase.

b. Production of enzyme oxidase

The oxidase test is used to analyze the ability of bacteria to produce the enzyme cytochrome oxidase. This enzyme catalyses the transfer of electrons from cytrochrome c to molecular oxygen and is usually found in bacteria which uses oxygen as the terminal electron acceptor during respiration (Brown, 2005). The oxidase test is most useful in characterization of gram negative bacteria (Blackman et al., 1978). In this test artificial electron acceptor *p*- aminodimethylalanine oxalate is used which turns purple in color from yellow when it is reduced by transfer of electrons from cytochrome c. Production of oxidase enzyme in bacterial isolates was detected by production of purple color on exposure to the chemical *p*- aminodimethylalanine oxalate. The isolated colonies were picked on a sterile cotton swabs and the inoculums were allowed to dry. The cotton swabs were then tamped lightly for 10 times on the filter paper wet with *p*-aminodimethylalanine oxalate solution (Tarrand et al., 1982). Detection of purple colour on the tip of cotton swab was interpreted as a positive reaction (Brown, 2005).

c. Production of enzyme urease

This test is used for the identification of several genera and species of Enterobactericeae including the genera *Proteus*, *Providencia*, *Klebsiella* and *Morganella*. Urease broth was used for the test, which contains the substrate urea. The urease enzyme

breaks down urea releasing ammonia which converts the pH of the medium alkaline. Increase in pH causes the phenol red indicator present in the media to turn from yellow to bright pink indicating a positive reaction (Brown, 2005).

3.4 Phenotypic Characterization of bacterial isolates

a. Gram staining

A smear of 24 hr old bacterial culture of all isolates was prepared on a grease free glass slide. The smear was first air dried and then heat fixed (Bartholomew, 1962). The heat fixed smear was then flooded with crystal violet stain for 1 minute and then washed with distilled water for 5 seconds. The washed slides were then flooded with Gram's iodine solution for 1 minute and then again washed for 5 seconds with distilled water. The slides were then washed with 95% ethanol in drop wise manner. After washing with distilled water again the smear was flooded with safranin for 1 minute (Bartholomew, 1962). The stained slides were finally washed with distilled water, air dried and observed under microscope. All the observation was done under oil immersion objective.

b. Endospore staining

A loop full culture of the isolates was taken on a grease free slide and a smear was prepared. The smear was air dried and heat fixed. The heat fixed slides were then placed over a steaming water bath with a piece of paper towel on the slides to prevent the stain from drying. The slides were then flooded with 5% aqueous malachite green (Mormak et al., 1985). Malachite green was added periodically to prevent the drying of the stain. The slides were then heated till the dye started to steam. The slides were then washed with water and counterstained with crystal violet for two minutes (Mormak et al., 1985). Subsequently the slides were washed with water, dried and observed under microscope at 100 x magnification.

c. Acid fast staining

Smear of the isolates were made in a grease free glass slides. The slides were air dried and then heat fixed. The smear was then flooded with carbol fuchsin and then

steamed on a water bath for 5 minutes (Brown, 2005). The stain was added to the slides at regular intervals to avoid the stain from drying. After cooling the slides were then decolorized with acid alcohol (3% hydrochloric acid in 95% ethyl alcohol) (Ellis et al., 1993) until no more color ran off from the slide. The slides were then washed with water and counterstained with methylene blue (0.25% methylene blue in 1% acetic acid) for 30 seconds (Ellis et al., 1993). Subsequently the slides were washed with water, dried and observed under oil immersion (Brown, 2005).

d. Motility test

The motility tests of the isolates were done by two methods namely stab inoculation in SIM agar and by Hanging Drop Technique. In SIM agar the motility of the bacteria can be determined by observing the spreading growth of organism from the line of inoculation. If the organism is motile it swims away from the line of inoculation into the uninoculated surrounding medium (Brown, 2005). In Hanging Drop Technique, a drop of culture was placed on a cover slip which was then placed over a special glass slide that has a concave depression at the center. The glass slide was held in place by applying petroleum jelly on the ends of cover slip. The swimming motility of the organism was then observed under phase contrast microscope (Brown, 2005).

3.4.1. Biochemical tests

1. IMViC TEST

a. Indole production test

SIM agar medium was used to evaluate the production of indole by the isolates. SIM agar contains the amino acid tryptophan which is converted to indole by the enzyme tryptophanase. The indole is detected by the addition of Kovac's reagent which produces cherry red colour if indole is present. The appearance of cherry red color gives a positive reaction (Sherman and Cappuccino, 2009). Tubes containing SIM agar was inoculated with the isolates using stab inoculation. The inoculated tubes were then incubated for 24

hours at 37^0 C. After 24 hours the tubes were checked for indole production using Kovac's reagent.

b. Methyl red test

This test is used to differentiate a number of enteric bacteria. Methyl red voges proskauer (MRVP) broth was used which contains glucose. This test evaluates the organism's ability to ferment glucose. On fermentation of glucose various acidic end products are produced such as acetic acid, succinic acid etc which lowers the pH of the medium. The methyl red indicator added to the broth turns red as the pH of the medium lowers, indicating a positive reaction. The broth however turns yellow on addition of methyl red if glucose fermentation has not taken place (Sherman and Cappuccino, 2009; Brown, 2005). A loop full of culture was inoculated in tubes containing MRVP broth for all the isolates and incubated at 37^0 C for 24 hours. After the incubation, the glucose fermentation was checked by addition of methyl red indicator in all the tubes.

c. Voges proskauer test

Some of the organisms produce non acidic products such as 2, 3-butanediol ethanol and acetoin on fermentation of glucose, which gives a negative test for methyl red. Voges Proskauer test evaluates the organism's ability to produce these non acidic products on fermentation of glucose. Barrit's reagent A and B is used in this test which gives a Red coloration when these non acidic products are oxidized to diacetyl compounds, indicating a positive reaction (Sherman and Cappuccino, 2009; Brown, 2005). Loop full of culture was inoculated in MRVP broth and incubated for 24 hours at 37 ^{0}C for all the isolates. After the incubation 15 drops of Barrit's reagent A was added followed by the addition of 15 drops of Barrit's reagent B. The tubes were shaken after every few minutes and results were observed after 15 minutes.

d. Citrate utilization test

This test is used to differentiate some enteric bacteria such as *Enterobacter aerogenes* and *Salmonella typhimurium,* based on their ability to use citrate as a sole carbon source (Brown, 2005). Simmon's citrate agar was used in this test which contains

sodium citrate as a sole carbon source and bromothymol blue as a pH indicator which turns green to blue at an alkaline pH. A loop full of culture was taken and streaked on the surface of citrate agar slants (Sherman and Cappuccino, 2009). The inoculated tubes were then incubated for 24 hours at 37^0 C. The slants were observed for colour change after 24 hours (Brown, 2005).

2. Triple Sugar Iron Agar Test

TSI agar is a modified agar media resulting from the combination of Russel's double sugar agar (Russell's, 1911) with the Kliger's Iron agar (Kliger, 1917). It comprises of three main sugars, glucose (0.1%) sucrose (1%) and lactose (1%). Besides carbohydrate it contains beef extract, yeast extract, and peptone which are the source of nitrogen, vitamins and minerals. Phenol red is the pH indicator. During preparation the tubes containing the molten agar are angled. The slant is aerobic, while the butt is anaerobic. The tubes were inoculated by first stabbing at the butt, which was followed by streaking at the slant and were incubated at 37^0 C for 24 hours. If any of the carbohydrate is fermented the drop in pH will cause the medium to change from reddish orange to yellow. A deep red colour indicates alkalization of the peptones. Sodium thiosulphate in the medium is reduced by some bacteria to hydrogen sulphide, a colorless gas. The hydrogen sulphide thus upon reaction with ferric ions in the medium, produce iron sulphide, a black insoluble precipitate. The bacteria are said to be glucose fermenter if the reaction tube produce an alkaline slant (red) and an acid butt (yellow), signifying that only glucose is metabolized. Since, this substrate is present in minimal concentration; the small amount of acid produced on the slant surface is oxidized rapidly, whereas in the butt, acid reaction is maintained because of reduced oxygen tension and slower growth of the organism. When the reaction tube is overlaid with acid, over acid in both the slant (yellow) and the butt (yellow) then the bacteria are said to be glucose, lactose and / or sucrose fermenters. When there is no acid produced in both the slant and the tube the bacteria are said to be glucose, lactose and sucrose non fermenter.

3. Production of Hydrogen Sulphide

The production of hydrogen sulphide by the isolates was observed by making a stab inoculation of the isolates in tubes containing SIM agar. SIM agar contains iron salts that react with hydrogen sulphide to form dark precipitates of iron sulphide. The inoculated tubes were incubated for 24 hrs at 37^0 C. Appearance of black colour in SIM agar stabs indicated a positive reaction (Brown, 2005)

4. Sugar Fermentation Test

The isolates were screened for their ability to ferment 8 different carbohydrates. Tubes containing 5 mL carbohydrate broth (Tripticase 10g/l, 5g/L of different carbohydrate, Sodium chloride 5g/L, and Phenol red 0.018g/L) were inoculated with the isolates and incubated at 37^0 C for 24 hours. For the observation of gas production Durham's tubes were used. Color change from red to yellow indicates that that the carbohydrate has been utilized along with the production of acidic products which causes the lowering of the pH. This causes the phenol red indicator to turn yellow in color. The appearance of yellow color indicates a positive reaction (Sherman and Cappuccino, 2009). The gas production is also observed as a gas bubble or a void inside the inverted Durham's tube (Brown, 2005).

Chapter 4

Results

4. RESULTS

4.1 Physico-chemical analysis of post-treated sewage water:

The value of pH, conductivity, total dissolved solids and temperature were analysed. A total of four sets of sample were collected in different months. The results are tabulated and shown in Table 4.1. The results of physico-chemical parameter of post treated sewage were compared with the general standard discharge of effluents, shown in Table 4.2 (CPCB, 1998). The graphical representation of analysis of variation in physico-chemical parameters are shown in figure such as Figure 4.1.1: Analysis of variation in temperature and pH of post treated sewage water in different months; Figure 4.1.2: Analysis of variation in total dissolved solids (TDS) of post treated sewage water in different months; Figure 4.1.3: Analysis of variation in Conductivity of post treated sewage water in different months. A linear regression of conductivity on total dissolved solids is also plotted in Figure 4.1.4.

Figure 4.1: ***Physico-chemical analysis of post treated sewage water by electronic portable Pocke Tester***

Table: 4.1: Results based on the Physico-chemical parameters of post treated sewage water

SAMPLES	PARAMETERS					
	Colour	Odour	pH	Temperature (^0C)	TDS (mg/L)	Conductivity (µS/cm)
Sample I	Light brown	Musty smell	6.25	18.4	210	420
Sample II	Faint yellow	Pungent smell	6.73	16.5	220	450
Sample III	Gray	Pungent smell	7.02	21.6	250	525
Sample IV	Light yellow	Musty smell	7.01	28.5.	230	430

TDS: Total dissolved solids

Sample I: collected during the month of November 2013

Sample II: collected during the month of February 2014

Sample III: collected during the month of May 2014

Sample IV: collected during the month of June 2014

Table 4.2: General standards for discharge of effluents (CPCB, 1998)

Parameters	Into inland surface waters Indian Standards: 2490 (1974)	On land for irrigation Indian Standards: 3307 (1974)
Colour	N2	N2
Odour	N2	N2
pH	5.5-9	5.5-9
Temperature (^0C)	40	-
TDS (mg/L)	2100	2100
Conductivity (µs/cm)	1000	2250

N2: All efforts should be made to remove colour and unpleasant odour as far as practicable

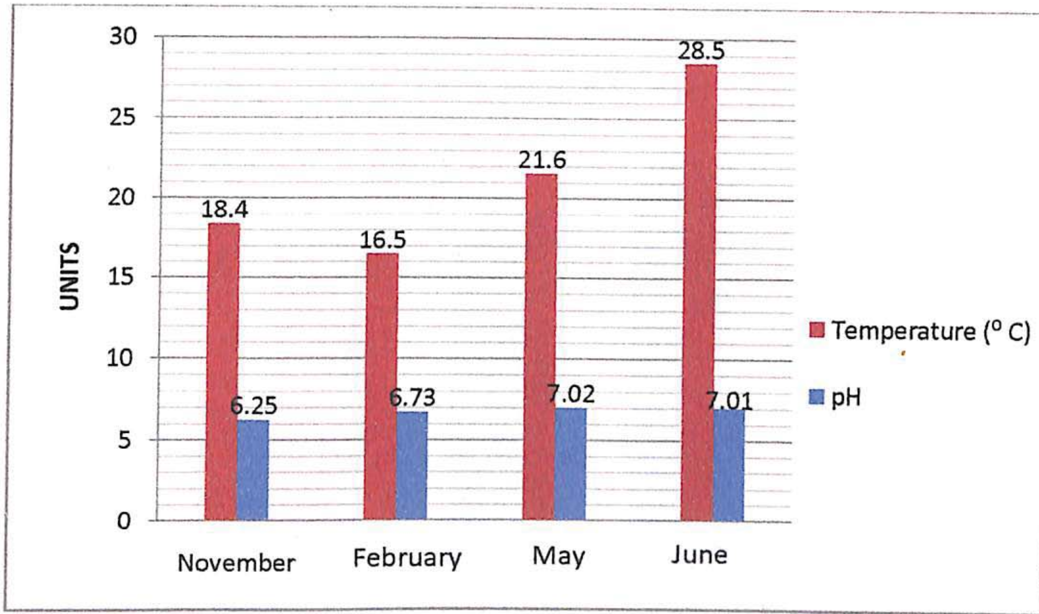


Figure 4.2: Analysis of variation in temperature and pH of post treated sewage water in different months

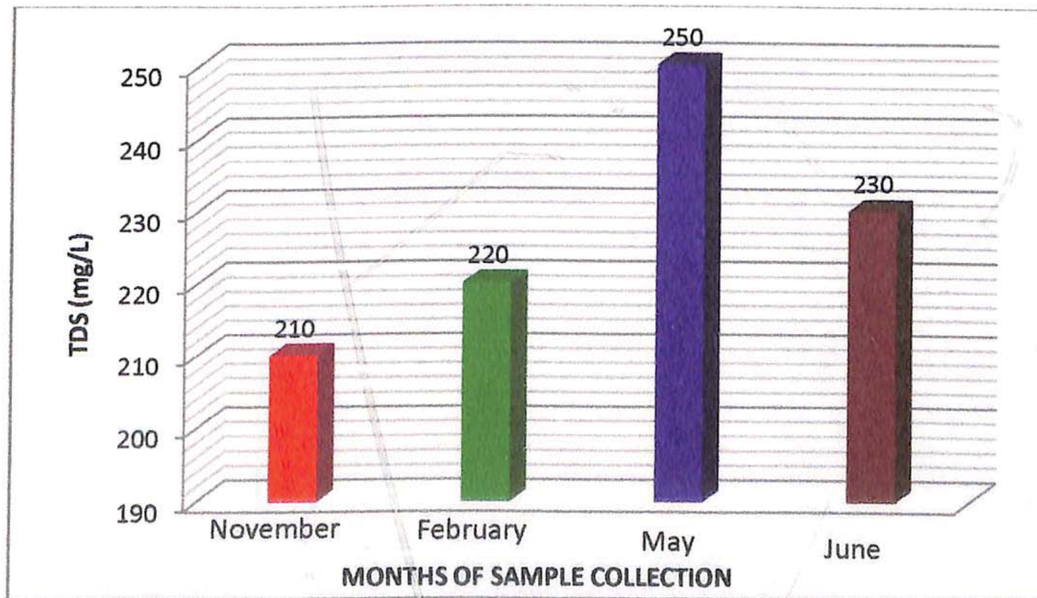


Figure 4.3: Analysis of variation in total dissolved solids (TDS) of post treated sewage water in different months

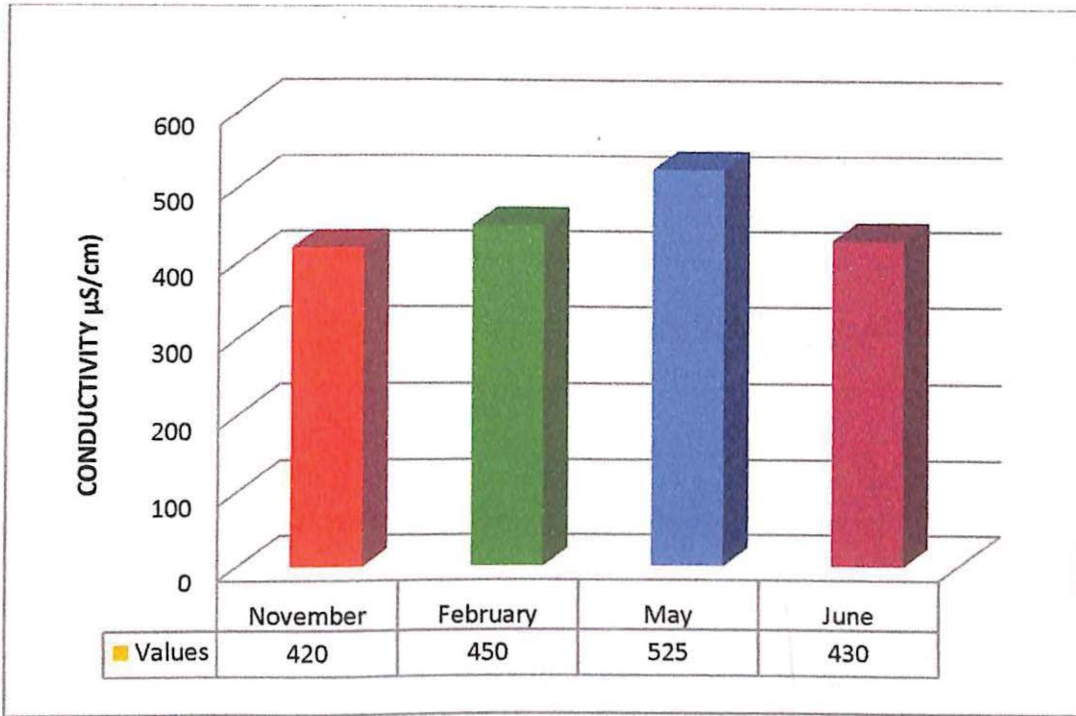


Figure 4.4: Analysis of variation in Conductivity of post treated sewage water in different months

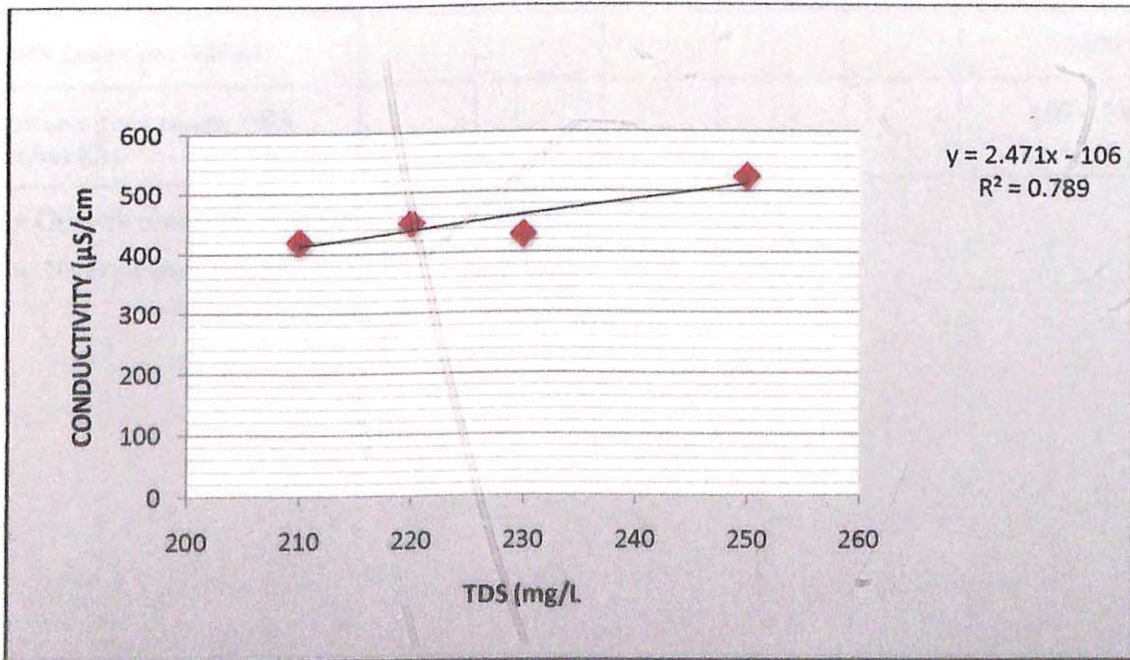


Figure 4.5: Linear regression of conductivity on TDS

4.2. Analysis of microbial load of post treated sewage sample

The microbial load of post treated sewage sample were analysed by using Multiple Tube Most Probable Number Method (MPN) and Agar Plate count method.

4.2.1. Analysis of microbial load using Multiple Tube Most Probable Number Method (MPN)

The microbial load of the post treated sewage sample was evaluated using the Multiple Tube Most Probable Number Test (MPN) technique. The MPN index per 100 ml was found to be 1600 as compared with the standard chart given in Table 4.4. The results of MPN are tabulated in Table 4.3.

Table 4.3: Analysis of microbial load by Most Probable Number (MPN) Method

Sample dilutions	Tubes					Tubes with Positive results
	TUBE 1	TUBE 2	TUBE 3	TUBE 4	TUBE 5	
PT2X-10 ml	+	+	+	+	+	5
PT1X-1ml	+	+	+	+	+	5
PT1X-0.1ml	+	+	+	-	+	4
MPN Index per 100ml						1600
Lower-upper range 95% probability						600 – 5300

+ = Growth observed with gas

- = No growth observed

Table 4.4. Standard chart for the MPN index and 95 % confidence limits for various combinations of positive results when five tubes are used per dilution (10mL, 1mL and 0.1 mL portions of sample).

COMBINATION OF POSITIVES	MPN INDEX PER 100 mL	95 % CONFIDENCE LIMITS		COMBINATION OF POSITIVES	MPN INDEX PER 100 mL	95 % CONFIDENCE LIMITS	
		UPPER	LOWER			UPPER	LOWER
0-0-0	<2	-	-	4-2-1	26	12	65
0-0-1	2	1.0	10	4-3-0	27	12	67
0-1-0	2	1.0	10	4-3-1	33	15	77
0-2-0	4	1.0	13	4-4-0	34	16	80
1-0-0	2	1.0	11	5-0-0	23	9	86
1-0-1	4	1.0	15	5-0-1	30	10	110
1-1-0	4	1.0	15	5-0-2	40	20	140
1-1-1	6	2.0	18	5-1-0	30	10	120
1-2-0	6	2.0	18	5-1-1	50	20	150
2-0-0	4	1.0	17	5-1-2	60	30	180
2-0-1	7	2.0	20	5-2-0	50	20	170
2-1-0	7	2.0	21	5-2-1	70	30	210
2-1-1	9	3.0	24	5-2-2	90	40	250
2-2-2	9	3.0	25	5-3-0	80	30	250
2-3-0	12	5.0	29	5-3-1	110	40	300
3-0-0	8	3.0	24	5-3-2	140	60	360
3-0-1	11	4.0	29	5-3-3	170	80	410
3-1-0	11	4.0	29	5-4-0	130	50	390
3-1-1	14	6.0	35	5-4-1	170	70	480
3-2-0	14	6.0	35	5-4-2	220	100	580
3-2-1	17	7.0	40	5-4-3	280	120	690
4-0-0	13	5.0	38	5-4-4	350	160	820
4-0-1	17	7.0	45	5-5-0	240	100	940
4-1-0	17	7.0	46	5-5-1	300	100	1300
4-1-1	21	9.0	55	5-5-2	500	200	2000
4-1-2	26	12.0	63	5-5-3	900	300	2900
4-2-0	22	9.0	56	5-5-4	1600	600	5300
				5-5-5	>1600	-----	-------

Source: APHA 1992

4.2.2 Analysis of microbial load by agar plate count method (CFU/mL)

The bacterial count for post-treated sewage sample were done using Reasoner's 2A agar. A dilution of 10^{-4}, 10^{-5} and 10^{-6} were taken for counting. All the experiments were performed in triplicates. The number of colonies was counted using the colony counter pen (Cole Parmer). The bacterial load of the samples collected during the four months for each dilution is shown in Table 4.5. The highest bacterial count was observed in the month of May with $11.5X10^6$ CFU/mL and the lowest count with $5.3X\ 10^6$ CFU/mL in the month of November.

Table 4.5: Analysis of microbial load by agar plate count method

MONTH OF SAMPLE COLLECTION	MICROBIAL LOAD (CFU/m/L)		
	DILUTION 10^{-4}	DILUTION 10^{-5}	DILUTION 10^{-6}
November	$1.5x10^7$	$4.3x10^7$	$1x10^7$
February	$0.8x10^7$	$3.5x10^7$	$1.5x10^7$
May	$2.5x10^7$	$7x10^7$	$2x10^7$
June	$2.2x10^7$	$6x10^7$	$2x10^7$

4.3. Results based on phenotypic characterization of bacteria

4.3.1 Morphological and cultural characteristics of bacterial isolates of post treated sewage water:

The cultural characteristics of all the isolates were analyzed in Nutrient Agar and MacConkey Agar media. The Gram staining was done to observe the morphological characteristics of the isolates shown in.The results are tabulated in Table 4.6. Out of 75 bacteria isolated from the post treated sewage effluents, the percentage of Gram negative bacilli, Gram positive cocci and Gram positive bacilli were 81%, 16% and 2.60% respectively, shown in Figure 4.13.

Various selective media were used to isolate enteric bacteria and some gastrointestinal pathogens. This includes Eosin methylene blue agar (EMB), Salmonella Shigella agar (SS), Thiosulphate-citrate-bile salts-sucrose agar (TCBS) agar and MacConkey Sorbitol agar. The various cultural characteristics shown by Gram negative bacteria for their identification in these selective media are as follows:

a) EMB agar: This media is mainly used for the identification of *Escherichia coli* which is characterized by the colonies showing green metallic sheen. *Klebsiella* species, on the other hand, shows purple colonies with dark centre in this media.

b) SS agar: This media is used for the identification of *Salmonella* which shows black colouration of the colonies and *Shigella* which gives colourless colonies in this media.

c) TCBS: This selective media is used for the identification of various species of *Vibrio*. The identification of *Vibrio cholera* is done by producing yellow colouration of the colonies.

d) MacConkey Sorbitol agar: This media is selective for identification of enteropathogenic *Escherichia coli* O157:H7, which produces colourless colonies. Colony morphology of isolates using different culture media are shown in Figure 4.7 to 4.12.

Table 4.6: Morphological and cultural characteristics of the isolates

ISOLATE	GROWTH CHARACTERISTICS IN NUTRIENT AGAR	GROWTH CHARACTERISITCS IN MACCONKEY AGAR	GRAM STAINING
SI:EF:03	Circular, moist, smooth with entire margin and non mucoid.	Pink coloured colonies.	GNB
SI:EF:04	Circular, smooth, convex, golden yellow 2-3mm in diameter.	Small pink coloured colonies	GPC
SI:EF:05	Circular, smooth, bright red colonies.	Colourless colonies .	GNB
SI:EF:06	Round, cream, mucoid colonies with 3-4mm in diameter.	Large, mucoid, pink colour	GNB
SI:EF:07	Round, cream, mucoid colonies, 3-4mm in diameter,	Large, mucoid, pink colour	GNB
SI:EF:08	Circular, smooth, opaque, and golden yellow pigment, 2-3mm in diameter	Very Small pink coloured colonies	GPC
SI:EF:09	Circular, smooth, opaque, and golden yellow pigment, 2-3mm in diameter	Very Small pink coloured colonies	GPC
SI:EF:10	Circular,moist,smooth with entire margin and non mucoid	Pink coloured colonies	GNB
SI:EF:11	2-4mm in diameter, circular, smooth, convex, opaque, easily emulsifiable, and golden yellow pigment	Small pink coloured colonies	GPC
SI:EF:12	Circular, moist, smooth with entire margin and non mucoid	Pink coloured colonies	GNB
SI:EF:13	Circular, moist, smooth with entire margin and non mucoid	Pink coloured colonies	GNB
SI:EF:14	Smooth convex colonies, 2-4mm in diameter	Pale colonies	GNB
SI:EF:15	Large, cream, convex smooth, mucoid colonies	Pink coloured colonies	GNB
SI:EF:16	3-4mm in diameter, round, cream, mucoid colonies	Large, mucoid, pink to red in colour	GNB
SI:EF:17	3-4mm in diameter, round, cream, mucoid colonies	Large, mucoid, pink to red in colour	GNB
SI:EF:18	Circular,moist,smooth with entire margin and non mucoid	Pink coloured colonies	GNB
SI:EF:19	Large, cream, convex smooth, mucoid colonies	Pink colonies	GNB

SI:EF:20	Circular, entire, low convex, smooth and translusent	Tiny deep pink coloured colonies	GPC
SI:EF:21	Circular, moist, smooth with entire margin and non mucoid	Pink coloured colonies	GNB
SI:EF:22	2-4mm in diameter, smooth, large, translucent, low convex shaped, greenish blue pigment	Pale or colourless colonies	GNB
SI:EF:24	3-4mm in diameter, round, cream, mucoid colonies	Large, mucoid, pink to red in colour	GNB
SI:EF:25	Large, cream, convex smooth, mucoid colonies	Pink coloured colonies	GNB
SI:EF:26	Circular, moist, smooth with entire margin and non mucoid	Pink coloured colonies	GNB
SI:EF:27	Circular, smooth, bright red colonies.	Pale colonies	GNB
SII:EF:28	Round, raised, opaque, and greyish white coloured, 2-3mm in diameter	No growth	GPB
SII:EF:29	Round, raised, opaque, and greyish white coloured, 2-3mm in diameter	No growth	GPB
SII:EF:31	2mm in diameter, circular, convex, smooth, and translucent	Colourless colonies	GNB
SII:EF:32	Circular, moist,smooth with entire margin and non mucoid	Pink coloured colonies	GNB
SII:EF:33	Round, cream, mucoid colonies, 3-4mm in diameter	Large, mucoid, pink to red in colour	GNB
SII:EF:34	Circular, moist, smooth with entire margin and non mucoid	Pink coloured colonies	GNB
SII:EF:35	Circular, moist, smooth with entire margin and non mucoid	Pink coloured colonies	GNB
SII:EF:38	2mm in diameter, circular, convex, smooth, and translucent	Colourless colonies	GNB
SIII:EF:40	Circular, moist, smooth with entire margin and non mucoid	Pink coloured colonies	GNB
SIII:EF:41	Circular, entire, low convex, smooth and translusent	Tiny deep pink coloured colonies	GPC
SIII:EF:42	Circular,moist,smooth with entire margin and non mucoid	Pink coloured colonies	GNB
SIII:EF:43	2-3mm in diameter, circular, transculent, low convex and smooth	Colourless colonies	GNB
SIII:EF:44	2mm in diameter, circular, convex, smooth, and translucent	Colourless colonies	GNB

SIII:EF:45	Large, cream, convex smooth, mucoid colonies	Pink coloured colonies	GNB
SIII:EF:46	3-4mm in diameter, round, cream, mucoid colonies	Large, mucoid, pink to red in colour	GNB
SIII:EF:47	Circular, entire, low convex, smooth and translusent	Tiny deep pink coloured colonies	GPC
SIII:EF:48	2mm in diameter, circular, convex, smooth, and translucent in appearance	Colourless colonies	GNB
SIII:EF:49	2mm in diameter, circular, convex, smooth, and translucent in appearance	Colourless colonies	GNB
SIII:EF:50	Produced a swarming colonies	Colourless colonies	GNB
SIII:EF:51	Produced a swarming colonies	Colourless colonies	GNB
SIII:EF:52	Produced a swarming colonies	Colourless colonies	GNB
SIII:EF:53	1-2mm in diameter, moist, translucent, round disks colonies in appearance, bluish tinge in transmitted light	Colourless or pale colonies	GNB
SIII:EF:54	2-3mm in diameter, circular, translucent, low convex and smooth in appearance	Colourless colonies	GNB
SIII:EF:55	Circular,moist, smooth with entire margin and non mucoid	Pink coloured colonies	GNB
SIII:EF:57	1-2mm in diameter, moist, translucent, round disks colonies in appearance, bluish tinge in transmitted light	Colourless or pale colonies	GNB
SIII:EF:58	Produced a swarming colonies	Colourless colonies	GNB
SIII:EF:59	2mm in diameter, circular, convex, smooth, and translucent in appearance	Colourless colonies	GNB
SIII:EF:60	2-4mm in diameter, circular, smooth, convex, opaque, easily emulsifiable, and golden yellow pigment	Small pink coloured colonies	GPC
SIII:EF:61	1-2mm in diameter, moist, translucent, round disks colonies in appearance, bluish tinge in transmitted light	Colourless or pale colonies	GNB
SIII:EF:62	1-2mm in diameter, moist, translucent, round disks colonies	Colourless or pale colonies	GNB

	in appearance, bluish tinge in transmitted light		
SIII:EF:63	Circular, translucent, cream colonies	Colourless colonies	GNB
SIII:EF:64	Large, cream, convex smooth, mucoid colonies	Pink coloured colonies	GNB
SIII:EF:65	Circular ,moist, smooth with entire margin and non mucoid	Pink coloured colonies	GNB
SIII:EF:66	Large, cream, convex smooth, mucoid colonies	Pink coloured colonies	GNB
SIII:EF:67	Large, cream, convex smooth, mucoid colonies	Pink coloured colonies	GNB
SIV:EF:68	Circular, convex, cream colonies, 2-3mm in diameter	Pale colonies	GNB
SIV:EF:69	Circular, moist, smooth with entire margin and non mucoid	Pink coloured colonies	GNB
SIV:EF:70	Large, cream, convex smooth, mucoid colonies	Pink coloured colonies	GNB
SIV:EF:72	Circular, smooth, convex, opaque, easily emulsifiable, and golden yellow pigment	Small pink coloured colonies	GPC
SIV:EF:73	2-4mm in diameter, smooth, large, translucent, low convex shaped, greenish blue pigment	Colourless colonies	GNB
SIV:EF:74	Produced a swarming colonies	Colourless colonies	GNB
SIV:EF:75	Large, cream, convex smooth, mucoid colonies	Pink coloured colonies	GNB
SIV:EF:76	Smooth, round, convex, bright red colonies,	Pale colonies	GNB
SIV:EF:77	Circular, entire, low convex, smooth and translusent	Tiny deep pink coloured colonies	GPC
SIV:EF:78	Smooth, round, convex, bright red colonies,	Pale colonies	GNB
SIV:EF:79	Circular, entire, low convex, smooth and translusent	Tiny deep pink coloured colonies	GPC
SIV:EF:80	Circular, moist, smooth with entire margin and non mucoid	Pink coloured colonies	GNB
SIV:EF:81	Circular, entire, low convex, smooth and translusent	Tiny deep pink coloured colonies	GPC
SIV:EF:82	1-2mm in diameter, moist, translucent, round disks colonies in appearance, bluish tinge in transmitted light	Colourless or pale colonies	GNB

GPC: Gram positive cocci, GPB: Gram positive bacilli, GNB: Gram negative bacill

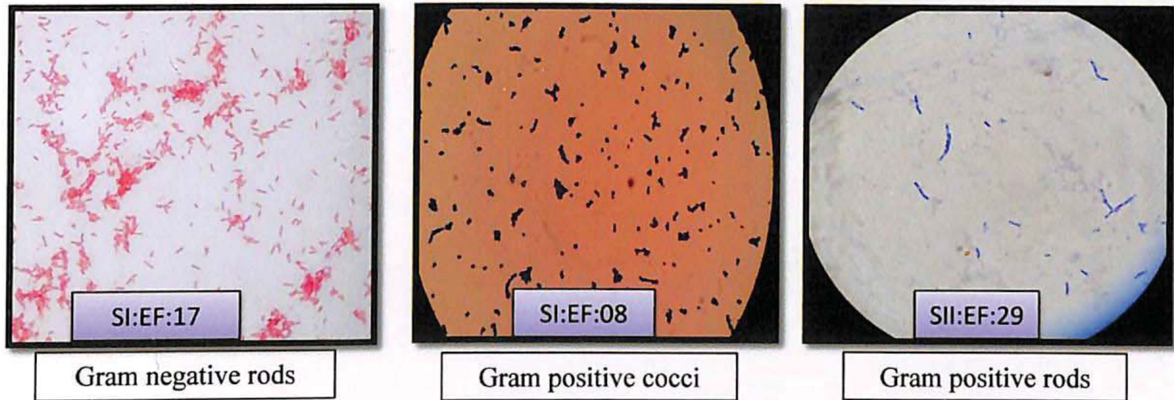


Figure 4.6: Morphology of Gram's stained bacteria under compound microscope (100x)

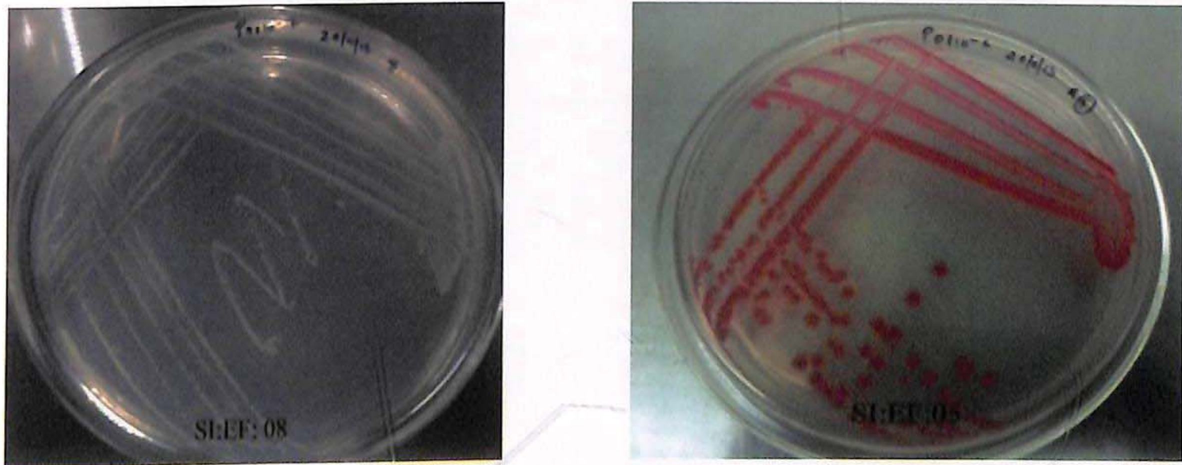


Figure 4.7: Colony morphology of some bacterial isolates on Nutrient agar

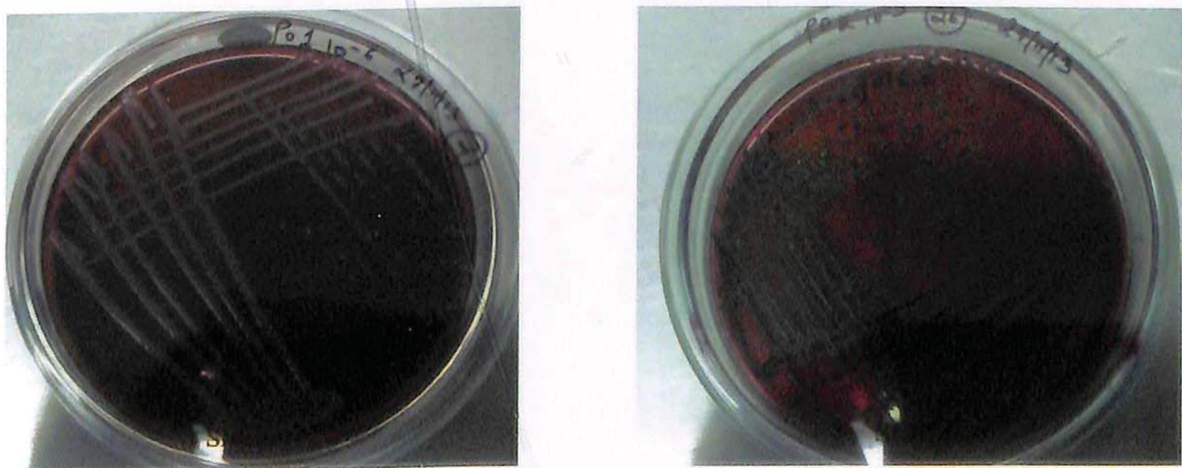


Figure 4.8: Colony morphology of some bacterial isolates on EMB agar

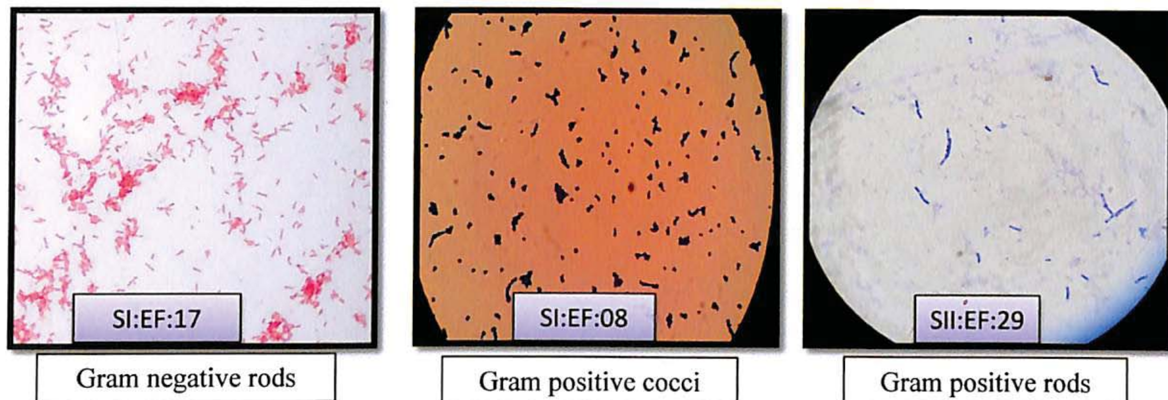


Figure 4.6: Morphology of Gram's stained bacteria under compound microscope (100x)

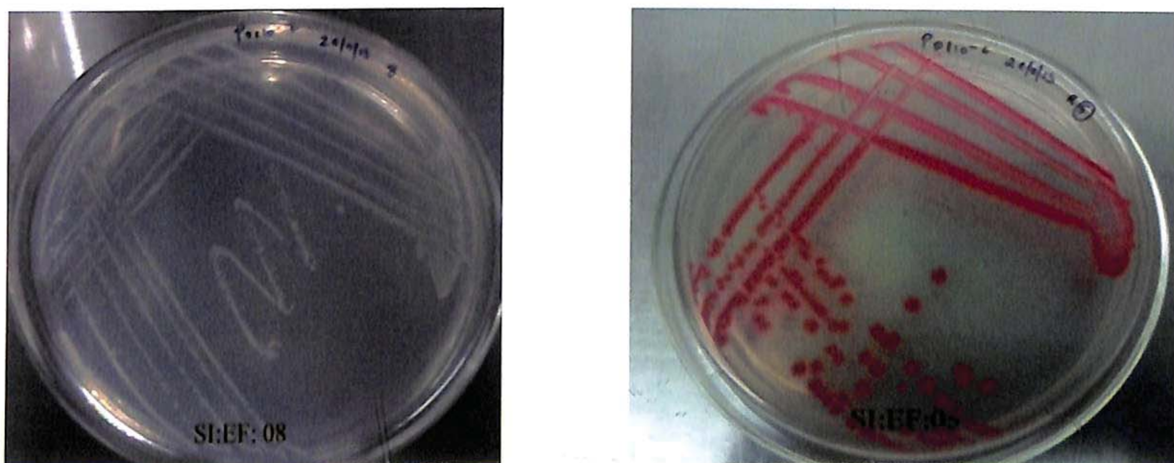


Figure 4.7: Colony morphology of some bacterial isolates on Nutrient agar

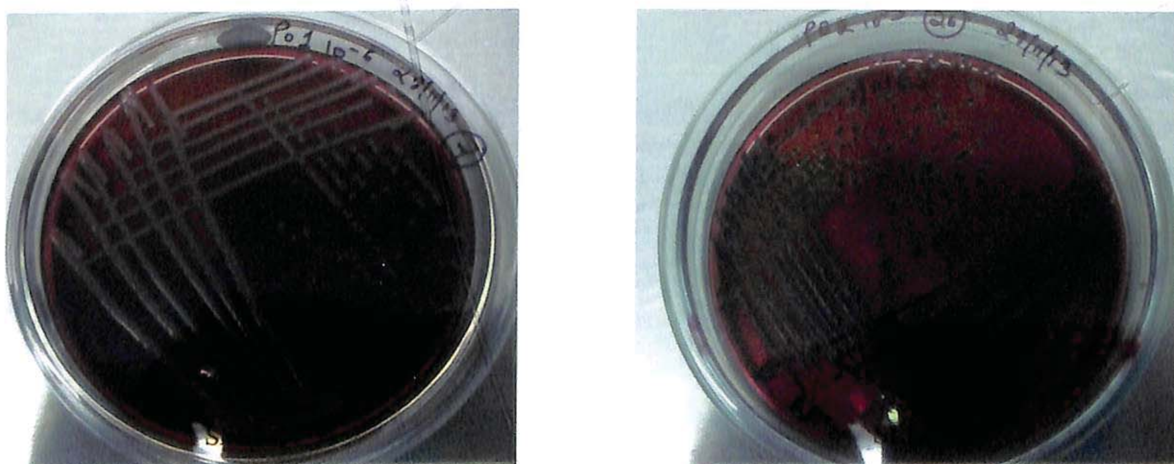


Figure 4.8: Colony morphology of some bacterial isolates on EMB agar

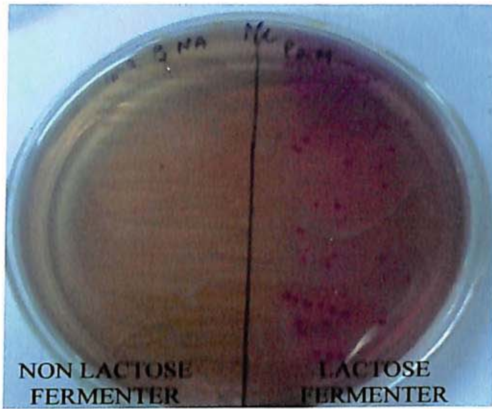


Figure 4.9: Isolate on MacConkey agar

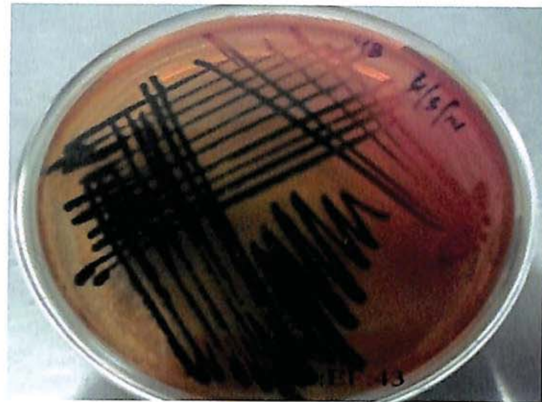


Figure 4.10: Isolate on SS agar

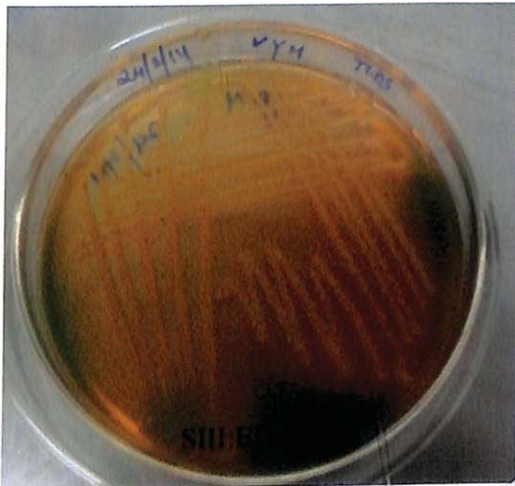


Figure 4.11: Isolate on TCBS agar

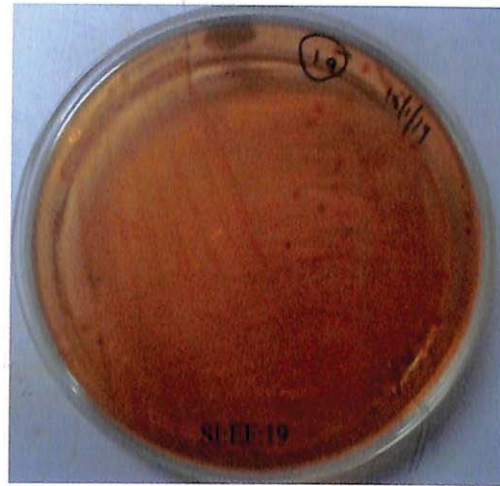


Figure 4.12: Isolate on Sorbitol MC agar

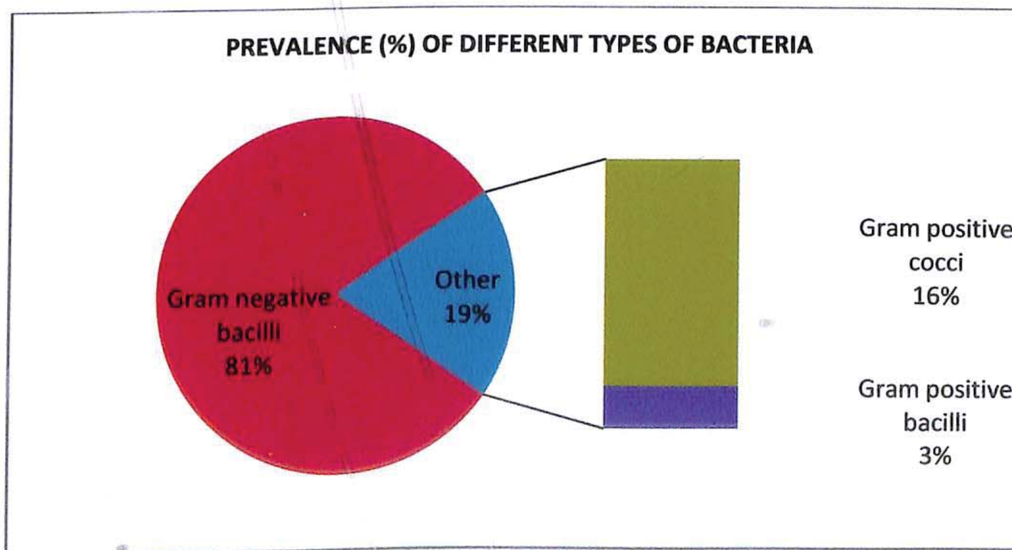


Figure 4.13: Prevalence of different types of bacteria based on Gram staining in post treated sewage water

4.3.2 Grouping of gram positive isolates based on various staining properties and catalase test

On the basis of various staining properties and catalase test, Gram positive isolates were grouped into three groups using Bergey's Manual. Endospore producing Gram positive rods were grouped under Group I which consist of *Bacillus, Clostridium and Sporolactobacillus.* All the non spore forming, catalase positive, irregular and tetrad arrangement Gram positive cocci were grouped under Group VI which consist of *Micrococcus, Planococcus* and *Staplylococcus.* The non spore forming, catalase negative, pairs and chain arrangement Gram positive cocci were grouped under Group VII consisting of *Streptococcus* and *Enterococcus.* The results are tabulated in Table 4.7.

Table 4.7: Grouping of gram positive isolates based on various staining properties and catalase test

ISOLATES	MOTILITY	GRAM STAIN	ACIDFAST STAIN	CATALASE	ENDOSPORE STAIN	GROUP
SI:EF:04	-	GPC	-	+	-	Group VI
SI:EF:08	-	GPC	-	+	-	Group VI
SI:EF:09	-	GPC	-	+	-	Group VI
SI:EF:11	-	GPC	-	+	-	Group VI
SI:EF:20	-	GPC	-	-	-	Group VII
SII:EF:28	-	GPB	-	+	+	Group I
SII:EF:29	-	GPB	-	+	+	Group I
SIII:EF:41	-	GPC	-	-	-	Group VII
SIII:EF:47	-	GPC	-	-	-	Group VII
SIII:EF:60	-	GPC	-	+	-	Group VI
SIV:EF:72	-	GPC	-	+	-	Group VI
SIV:EF:77	-	GPC	-	-	-	Group VII
SIV:EF:79	-	GPC	-	-	-	Group VII
SIV:EF:81	-	GPC	-	-	-	Group VII

GPC: Gram positive cocci
Group I: *Bacillus, Clostridium, Sporolactobacillus*
Group VI: *Micrococcus, Planococcus, Staphylococcus*
Group VII: *Streptococcus, Enterococcus*

4.3.3 Biochemical tests for the presumptive identification of Gram negative bacteria

Various biochemical tests were done for the identification of Gram negative bacteria. The test included IMViC, Triple sugar iron (TSI), urease, catalase, motility, oxidase, and various carbohydrate fermentation tests. Eight sugars were used for the evaluation of the ability of isolates to ferment various carbohydrates. The results for presumptive identification of the Gram negative bacterial isolates based on these biochemical tests are represented in the respective tables as Biochemical test for presumptive identification of *Escherichia coli* (Table 4.8), *Enterobacter* species (Table 4.9), *Klebsiella* species (Table 5.0), *Shigella* species (Table 5.1), *Proteus* species (Table 5.2), *Serratia* species (Table 5.3), *Vibrio* species (Table 5.4), *Pseudomonas* species (Table 5.5), *Salmonella* species (Table 5.6) and *Citrobacter* species (Table 5.7).

Figure 4.3.4 shows various biochemical tests done for the identification of Gram negative isolates.

Table 4.8: Biochemical test for presumptive identification of *Escherichia coli*

ISOLATES	IMVIC TEST				Triple Sugar Iron Agar Test	Catalase Test	CARBOHYDRATE FERMENTATION TEST								PRESUMPTIVE IDENTIFICATION
	Indole	Methyl red	Voges proskauer	Citrate			Sucrose	Mannose	d-Mannitol	Dulcitol	Arabinose	Inositol	d-Raffinose	Xylose	
SI:EF:03	+	+	-	-	A/A/G	+	-	+	+	-	+	-	-	+	*Escherichia coli*
SI:EF:10	+	+	-	-	A/A/G	+	-	+	+	-	+	-	+	+	*Escherichia coli*
SI:EF:12	+	+	-	-	A/A/G	+	-	+	+	-	+	-	+	+	*Escherichia coli*
SI:EF:13	+	+	-	-	A/A	+	-	+	+	-	+	-	-	+	*Escherichia coli*
SI:EF:18	+	+	-	-	A/A	+	+	+	+	-	+	-	-	-	*Escherichia coli*
SI:EF:21	+	+	-	-	A/A	+	+	+	+	-	+	-	-	+	*Escherichia coli*
SI:EF:26	+	+	-	-	A/A	+	-	+	+	-	+	-	-	+	*Escherichia coli*
SII:EF:32	+	+	-	-	A/A/G	+	-	+	+	+	+	-	-	+	*Escherichia coli*
SII:EF:34	+	+	-	-	A/A	+	-	+	+	+	+	-	-	+	*Escherichia coli*

SII:EF:35	+	-	-	-	A/A/G	+	+	+	+	+	+	-	+	+	*Escherichia coli*
SIII:EF:40	+	+	-	-	A/A/G	+	+	+	+	+	+	-	+	+	*Escherichia coli*
SIII:EF:42	+	+	-	-	A/A	+	-	+	+	-	-	-	-	+	*Escherichia coli*
SIII:EF:55	+	+	-	-	A/A/G	+	-	+	+	+	+	-	+	+	*Escherichia coli*
SIV:EF:69	+	+	-	-	A/A	+	+	+	+	+	+	-	+	+	*Escherichia coli*
SIII:EF:65	+	+	-	-	A/A	+	-	+	+	+	+	-	+	+	*Escherichia coli*
SIV:EF:80	+	+	-	-	A/A/G	+	-	+	+	-	+	-	-	+	*Escherichia coli*

A: Acid production indicated by yellow colouration of the media.

G: Gas production.

A/A: Acidic slant/Acidic butt= glucose, lactose and/or sucrose fermenter

A/A/G: Acidic slant/acidic butt with gas production

Table 4.9: Biochemical tests for presumptive identification of *Enterobacter* species

ISOLATES	IMVIC TEST				Triple Sugar Iron AgarTest	Catalase Test	CARBOHYDRATE FERMENTATION TEST								PRESUMPTIVE IDENTIFICATION
	Indole	Methyl red	Vogess proskauer	Citrate			Sucrose	Mannose	d-Mannitol	Dulcitol	Arabinose	Inositol	d-Raffinose	Xylose	
SI:EF:15	-	-	+	+	A/A	+	+	+	+	+	+	+	+	+	*Enterobacter* species
SI:EF:19	+	+	+	+	A/A	+	+	+	+	+	-	-	+	-	*Enterobacter* species
SI:EF:25	-	-	+	+	A/A/G	+	+	+	+	-	+	+	+	+	*Enterobacter* species
SIII:EF:45	-	-	+	+	A/A	+	+	+	+	-	-	-	+	+	*Enterobacter* species
SIII:EF:64	+	+	+	+	A/A/G	+	+	+	+	-	-	-	+	+	*Enterobacter* species
SIII:EF:66	-	-	+	+	A/A	+	+	+	+	-	-	-	-	+	*Enterobacter* species
SIII:EF:67	+	+	+	+	A/A	+	+	+	+	+	-	+	+	+	*Enterobacter* species
SIV:EF:70	-	+	-	+	A/A/G	+	+	+	+	+	+	-	-	+	*Enterobacter* species
SIV:EF:75	-	+	+	+	A/A	+	+	+	+	-	+	+	+	+	*Enterobacter* species

A/A/G: acidic slant/Acidic butt/Gas production

Table 5.0: Biochemical test for presumptive identification of *Klebsiella species*

ISOLATES	IMVIC TEST				Triple Sugar Iron Agar Test	Catalase Test	Urease Test	CARBOHYDRATE FERMENTATION TEST								PRESUMPTIVE IDENTIFICATION
	Indole	Methyl red	Voges proskauer	Citrate				Sucrose	Mannose	d-Mannitol	Dulcitol	Arabinose	Inositol	d-Raffinose	Xylose	
SI:EF:06	+	+	+	+	A/A/G	+	-	+	+	+	+	+	+	+	+	*Klebsiella* species
SI:EF:07	+	+	-	+	A/A/G	-	-	+	+	+	-	-	+	+	+	*Klebsiella* species
SI:EF:16	+	+	+	+	A/A	+	-	+	+	+	+	-	+	+	+	*Klebsiella* species
SI:EF:17	-	-	+	+	A/A	-	-	+	+	+	+	-	-	+	+	*Klebsiella* species
SI:EF:24	+	+	+	+	A/A	+	-	+	+	+	+	+	+	+	+	*Klebsiella* species
SII:EF:33	+	-	+	+	A/A	+	+	+	+	+	+	+	-	+	+	*Klebsiella* species
SIII:EF:46	-	+	+	-	A/A	+	-	+	+	+	-	-	+	+	+	*Klebsiella* species

A/A/G: acidic slant/Acidic butt/Gas production

Table 5.1: Biochemical tests for presumptive identification of *Shigella species*

ISOLATES	IMVIC TEST				Triple Sugar Iron Agar Test	Catalase Test	Urease Test	CARBOHYDRATE FERMENTATION TEST								PRESUMPTIVE IDENTIFICATION
	Indole	Methyl red	Voges proskauer	Citrate				Sucrose	Mannose	d-Mannitol	Dulcitol	Arabinose	Inositol	d-Raffinose	Xylose	
SII:EF:31	-	-	-	+	K/A	-	-	-	+	+	-	-	+	+	+	*Shigella* species
SII:EF:38	+	+	+	-	K/A	-	-	+	+	+	-	+	-	-	-	*Shigella* species
SIII:EF:48	+	+	-	-	K/A	+	-	-	+	+	+	-	-	-	+	*Shigella* species
SIII:EF:49	+	+	-	-	K/A	+	-	+	+	-	-	-	-	-	+	*Shigella* species
SIII:EF:59	+	-	-	-	K/A	+	+	+	+	-	-	-	-	+	+	*Shigella* species
SIII:EF:44	+	+	-	-	K/A	-	+	+	+		-	-	-	-	-	*Shigella* species

A= Acid production indicated by yellow colour

K= No acid production indicated by red colour.

K/A= Alkaline slant/ acidic butt= glucose fermentation only

Table 5.2: Biochemical tests for presumptive identification of *Proteus species*

ISOLATES	IMVIC TEST				Triple Sugar Iron Agar Test	Catalase Test	Urease Test	CARBOHYDRATE FERMENTATION TEST								PRESUMPTIVE IDENTIFICATION
	Indole	Methyl red	Voges proskauer	Citrate				Sucrose	Mannose	d-Mannitol	Dulcitol	Arabinose	Inositol	d-Raffinose	Xylose	
SIII:EF:50	-	+	-	-	K/AB	+	+	-	-	-	-	+	-	-	+	*Proteus* species
SIII:EF:51	-	+	-	-	K/A/B	-	+	-	-	-	-	+	-	-	+	*Proteus* species
SIII:EF:52	+	+	-	-	K/A/B	+	+	-	+	-	-	+	-	+	+	*Proteus* species
SIII:EF:58	+	+	-	-	K/A	+	+	+	+	-	-	+	-	-	-	*Proteus* species
SIV:EF:74	-	+	-	+	A/A/B	+	+	-	-	-	-	+	-	-	+	*Proteus* species

A= Acid production indicated by yellow colour

K= No Acid production indicated by red colour.

B= production of hydrogen sulphide indicated by black colour

K/A/B= Alkaline slant/ acidic butt/ hydrogen sulphide production

A/A/B= Acidic slant/acidic butt/ hydrogen sulphide production

Table 5.3: Biochemical tests for presumptive identification of *Serratia species*

ISOLATES	IMVIC TEST				Triple Sugar Iron Agar Test	Catalase Test	Urease Test	CARBOHYDRATE FERMENTATION TEST								PRESUMPTIVE IDENTIFICATION
	Indole	Methyl red	Voges proskauer	Citrate				Sucrose	Mannose	d-Mannitol	Dulcitol	Arabinose	Inositol	d-Raffinose	Xylose	
SI:EF:05	-	+	-	-	A/A	+	-	+	+	+	-	-	-	-	-	*Serratia* species
SI:EF:27	-	+	+	+	A/A/G	+	-	+	+	+	-	-	+	+	+	*Serratia* species
SIII:EF:63	-	+	+	+	A/A	+	-	+	+	+	-	-	-	+	+	*Serratia* species
SIV:EF:68	-	+	+	+	A/A	+	-	+	+	+	-	-	-	-	-	*Serratia* species
SIV:EF:76	-	+	+	+	A/A	+	-	+	+	+	-	-	+	+	+	*Serratia* species
SIV:EF:78	-	+	+	+	A/A	+	-	+	+	+	-	-	-	+	+	*Serratia* species

A/A= Acidic slant/ acidic butt

A/A/G= Acidic slant/acidic butt/gas production

Table5.4: Biochemical tests for presumptive identification of *Vibrio species*

ISOLATES	IMVIC TEST				Triple Sugar Iron Agar Test	Catalase Test	Oxidase Test	CARBOHYDRATE FERMENTATION TEST								PRESUMPTIVE IDENTIFICATION
	Indole	Methyl red	Voges proskauer	Citrate				Sucrose	Mannose	d-Mannitol	Dulcitol	Arabinose	Inositol	d-Raffinose	Xylose	
SIII:EF:53	-	+	+	-	A/A	-	+	+	+	+	-	-	-	-	+	*Vibrio* species
SIII:EF:61	+	+	+	-	A/A/G	-	+	+	+	+	-	-	-	-	-	*Vibrio* species
SIII:EF:57	+	-	+	+	A/A	-	+	+	+	+	-	-	-	+	+	*Vibrio* species
SIII:EF:62	+	+	-	-	A/A	-	+	+	+	+	-	-	+	-	+	*Vibrio* species
SIV:EF:82	-	+	-	+	A/A	-	-	+	+	+	-	-	-	+	+	*Vibrio* species

A/A= Acidic slant/acidic butt

A/A/G= Acidic slant/ acidic butt/gas production

Table 5.5: Biochemical tests for presumptive identification of *Pseudomonas species*

ISOLATES	IMVIC TEST				Triple Sugar IronAgar Test	Catalase Test	Oxidase Test	CARBOHYDRATE FERMENTATION TEST								PRESUMPTIVE IDENTIFICATION
	Indole	Methyl red	Voges proskauer	Citrate				Sucrose	Mannose	d-Mannitol	Dulcitol	Arabinose	Inositol	d-Raffinose	Xylose	
SI:EF:02	-	-	-	+	K/K	+	+	-	+	-	+	+	+	+	+	*Pseudomonas* species
SI:EF:22	-	-	-	+	K/K	+	+	-	+	-	+	+	+	+	+	*Pseudomonas* species
SIV:EF:73	-	-	-	+	K/K	+	+	-	+	-	+	+	+	+	+	*Pseudomonas* species

K= no acidic production indicated by red colouration of media

K/K= Alkaline slant/Alkaline butt= glucose, lactose and sucrose nonfermenter.

Table 5.6: Biochemical tests for presumptive identification of *Salmonella species*

ISOLATES	IMVIC TEST				Triple Sugar Iron Agar Test	Catalase Test	Oxidase Test	CARBOHYDRATE FERMENTATION TEST								PRESUMPTIVE IDENTIFICATION
	Indole	Methyl red	Voges proskauer	Citrate				Sucrose	Mannose	d-Mannitol	Dulcitol	Arabinose	Inositol	d-Raffinose	Xylose	
SII:EF:36	-	+	-	+	A/B	-	+	-	-	-	-	-	+	+	+	*Salmonella* species
SIII:EF:43	-	+	-	-	A/B	-	+	-	-	-	-	-	+	+	-	*Salmonella* species
SIII:EF:54	-	+	-	-	K/A	-	+	-	-	-	-	-	-	+	-	*Salmonella* species

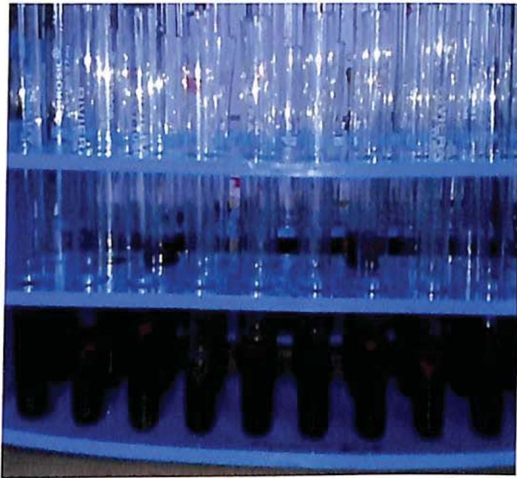
A/B=Acidic slant/Black butt (H_2S production)

K/A= Alkaline slant/ Acid butt

Table 5.7: Biochemical tests for presumptive identification of *Citrobacter species*

ISOLATES	IMVIC TEST				Triple Sugar Iron Agar Test	Catalase Test	Oxidase Test	CARBOHYDRATE FERMENTATION TEST								PRESUMPTIVE IDENTIFICATION
	Indole	Methyl red	Voges proskauer	Citrate				Sucrose	Mannose	d-Mannitol	Dulcitol	Arabinose	Inositol	d-Raffinose	Xylose	
SI:EF:14	+	+	-	+	A/A	+	-	-	+	+	-	+	-	-	+	*Citrobacter* species

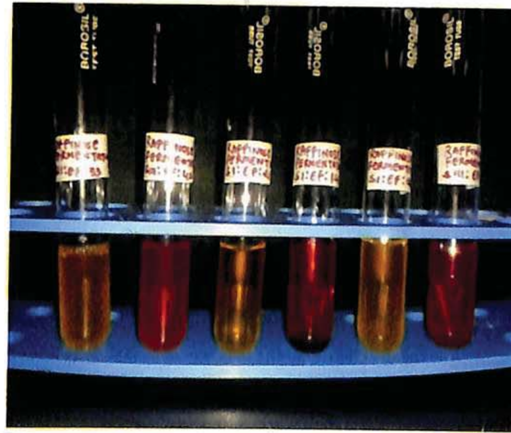
A/A= Acidic slant/Acidic butt



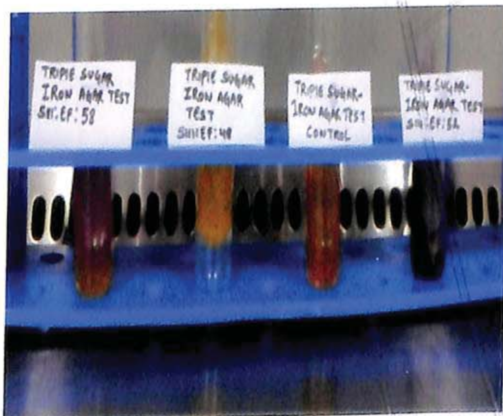
INDOLE

METHYL RED

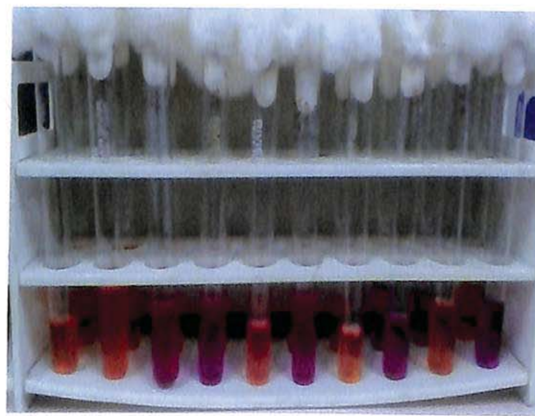
VOGES PROSKAUER



SUGAR FERMENTATION



TRIPLE SUGAR IRON



UREASE TEST

Figure 4.14: Results of Bochemical Tests

4.3.4 Prevalence of various Gram negative bacteria present in the post treated sewage water

A total of 61 isolates were found to be Gram negative bacilli. *E. coli* was most prevalent among these isolates followed by *Enterobacter* species, *Klebsiella* species, *Shigella* species, *Serratia* species, *Proteus* species, *Pseudomonas* species, *Salmonella* species and *Citrobacter species* in decreasing order. The number and percentage of various gram negative bacilli are shown in Table 5.8. Graphical representation is also shown in Figure: 4.15.

Table 5.8: Prevalence of Gram negative bacilli isolated from post treated sewage

Escherichia coli	***Enterobacter* species**	***Klebsiella* species**	***Shigella* species**	***Serratia* species**
16 (26.2%)	9 (14.7%)	7 (11.4%)	6 (9.8%)	6 (9.8%)

***Proteus* species**	***Vibrio* species**	***Pseudomonas* species**	***Salmonella* species**	***Citrobacter* species**
5 (8.1%)	5 (8.1%)	3 (4.9%)	3 (4.9%)	1(1.7%)

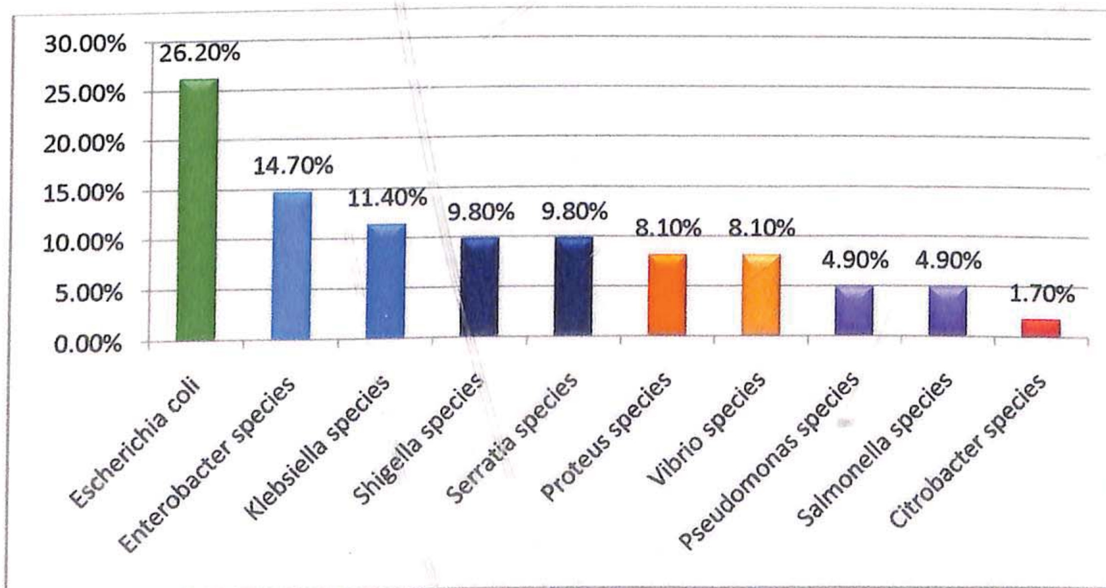


Figure 4.15: Percent prevalence of various gram negative bacilli isolated from post treated sewage water

Chapter 5

Discussion

5. DISCUSSION

Poor sanitation and unhygienic water results in the loss of 0.4 million lives annually in India (WHO, 2007). With nearly 900 million people affected by diarrhoea each year and an equal number suffering from disease caused by various microorganism, unclean water ranks at top of the world's population problem (Sharma 2001). Traditionally, the waste water has been extensively studied with regard to physical and chemical characteristics only. However, lately microbiological quality of the river has come under greater focus owing to deleterious effects of pollution on human health, especially in the context of Designated Best Use (DBU) of the river waters.

The present study was carried out to isolate and identify the bacterial load related to human health from post treated sewage water of Adampool sewage treatment plant at Adampool, East Sikkim.as well as to analyse some physico-chemical parameters that are present in it, A total of four sets of post treated sewage water samples were collected during the months of November 2013, February 2014, May 2014 and June 2014. Analysis of physico-chemical parameters included temperature, pH, total dissolved solids (TDS) and conductivity. Temperature is the most important factor which influences the biochemical and biological characteristics of the aquatic system. The present investigation revealed that the temperature varies from a minimum of 16.5°C in January to a maximum of 28.5°C in June (Table 4.1). The hydrogen-ion concentration is an important quality parameter of wastewater. The pH value of the collected sample varies from 6.25 to 7.02 in post treated sewage water. The minimum value was observed in the month of November while the maximum value was observed in the month of May (Table 4.2). In a similar study carried out by Tripathi et al, (1991) on the physico-chemical parameters of city sewage of Varanasi discharged into Ganga river the pH value was found to be within the range of 7.3-7.7 and temperature varies from 22.2 to 32.8. There are neither major industries nor mining activities in the area that could cause extreme changes in the pH of Adampool sewage effluents. Thus, the results obtained for pH measurements in the effluent were as expected and were under the permissible limits as per standard disposal of wastewater effluent (CPCB, 2008).

The value of total dissolved solids (TDS) ranges from 210-250 mg/L, the maximum value being obtained in the month of May 2014. The electrical conductivities of the water samples generally varied significantly and ranged from 420µS/cm to 525µS/cm throughout the period of study. Higher conductivity was observed in the month of May 2014. Conductivity in water is directly proportional to the total dissolved solids as well as temperature: the warmer the water, the higher the conductivity (EPA, 2012). Hence electrical conductivity is a useful indicator of mineralization and salinity in sewage. The linear regression of conductivity and TDS being represented in figure 4.1.4 indicated that there was a positive and proportionate increase in both the parameters. The conductivity and TDS gave a correlation coefficient of R^2=0.789 for the four months. Values of correlation coefficient can vary between +1 and -1 and a perfect correlation has a coefficient of +1. The regression analysis carried out to correlate the two parameters with each other revealed that the conductivity and TDS gave a coefficient of 0.789 which lies between +1 and -1. This shows that a positive correlation exist between the two parameters of post treated sewage sample. The results showed that the conductivity of the sewage water depends on the ionic solutes present in it (Adams, 1990).

Multiple fermentation tube technique was performed for the detection of total coliform from the sewage effluent. This technique is based on the most probable number of bacteria present in a sample that produces gas in a number of fermentation tubes with various volumes of diluted samples In the presumptive test experiment, the number of total coliform was found to be 1600 MPN/100 ml with reference to standard charts based on statistical studies of known concentration of bacteria (EPA, 2003; American Public Health Association, 1916). Total coliform were found maximum in domestic wastewater which is due to discharge of excreta from human beings. Therefore a potential health risk exists due to presence of microbial pathogens in water. In another study by Rathore et al (2014), the value of total coliform was found to be 240 – 918 MPN/100 ml from sewage water at Udaipur, Rajasthan. Thus, the value obtained in present study is not consistent with the previous finding by Rathore et al (2007).

Enumeration of bacteria by plate count technique for the post treated sewage sample was also carried out. Determination of microbial load of sewage effluent is an

important parameter to study the percentage reduction of microorganisms by the treatment plant and thus to evaluate its working efficiency. In the present study, the microbial load was calculated for all the samples collected during the four months. Values obtained are tabulated in Table 4.5. The highest bacterial load was observed in the month of May with $7x10^7$ CFU/mL and the lowest count with $3.5x\ 10^7$ CFU/mL in the month of February. In the present study, it was found that the value of TDS, PH and Conductivity were highest in the month of May which directly correlates with the microbial load as this was also found to be highest in the same month.

Bacterial analysis of the present study have shown that the maximum population of bacteria belongs to gram negative rods followed by gram positive cocci and then by gram positive bacilli in decreasing order of their percentage occurrence in post treated sewage sample. Out of the total isolates 81% were found to be gram negative rods , 16 % belonged to gram positive cocci and 2.60%,were gram positive rods. The isolates belonging to gram positive cocci and gram positive rods were categorized into three groups based on endospore staining, acid fast staining, and catalase test in accordance to Bergey's manual (Brown, 2005). All the non spore forming, catalase positive, gram positive cocci were grouped under Group VI which consists of *Micrococcus, Planococcus* and *Staphylococcus.* Endospore producing Gram positive rods were grouped under Group I which consist of *Bacillus, Clostridium and Sporolactobacillus.* The non spore forming, catalase negative Gram positive cocci were gouped under Group VII consisting of *Streptococcus and Enterococcus.*

The present study shows that the maximum number of organisms found in post treated sewage samples were gram negative bacilli and among them the prevalence of bacteria belonging to members of *Enterobactericeae* family was highest as these are the common microflora of human guts. The final identification of the isolates was done based on the morphological characteristics and biochemical properties in accordance to the Bergey's Manual. Out of the total gram negative rods, the prevalence of *Escherichia coli* was found to be 26.2% followed by *Enterobacter species* (14.7%), *Klebsiella* species (11.4%), *Shigella* species and *Serratia* species (9.8%), *Proteus* and *Vibrio* species each (8.1%), *Pseudomonas* and *Salmonella* species each (4.9%) and *Citrobacter* species (1.7%).

Focus were given more on Enterobactericeae members as degree of efficiency of wastewater treatment plant (WWTP) is indicated by the number of Enterobacteriaceae present in effluents (Filipkowska 2003; Espigares et al., 2006). In the present study the prevalence of *E. coli* was highest among the Enterobactericeae. *E. coli* being a common human gut microflora, the identification of this genus upto the strain level is very important as enterohemorrhagic *E. coli* O157:H7 is a pathogenic strain and has the highest mortality rate of all waterborne disease (Moe, 2007). *Salmonella*, *Shigella* and *Vibrio*, which are considered as pathogens and major cause of gastrointestinal infections were isolated from the sewage effluent. Apart from domestic wastewater, Adampool sewage treatment plant also receives hospital waste. Hence the prevalence of pathogenic bacteria may be high. There are reports that the hospital treating cholera patients may have more toxigenic *Vibrio* than *E. coli* (Curtis, 2003).However in the present study occurrence of *E. coli* was high compared to *Vibrio*.

There are reports regarding the prevalence of multi-drug resistant bacteria among *Enterobactericeae* family which has been has been demonstrated by Betina, (1994). Even more dangerous has been the emergence of another New Delhi metallo-beta-lactamase, or NDM-1 reported in isolate of *K. pneumoniae* from a patient who had been hospitalized in New Delhi in 2008, NDM-1 was soon found throughout India, in Pakistan, and in the United Kingdom (Yong et al., 2009). Enterobacteriaceae spread easily between humans by hand carriage and water and have a tendency to acquire genetic material through horizontal gene transfer, mediated mostly by transposons and plasmids (Partridge, 2011; Stokes et al., 2011; Toleman et al., 2011). The regular management of waste water treatment plant is important. Due to improper design, poor maintenance, frequent electricity break downs and lack of technical man power, the facilities constructed to treat wastewater do not function properly and remain closed most of the time (CPCB, 2007).

Chapter 6

Summary

6. Summary

The present study is based on the determination of some physico-chemical parameters and microbiological analysis of post treated sewage effluent which was collected from Adampool sewage treatment plant, Sikkim. The study was done for four months November, February, May and June. The sample was collected in a sterile collection container and was processed in laboratory within two hours of collection. The physico-chemical analysis included colour, odour, pH, TDS, conductivity and temperature.The values obtained ranges from 16.5° C-28.5° C for temperature, 6.25 to 7.02 for pH, 210-250 mg/L for TDS and 420-525 μS/cm for conductivity. Linear correlation of conductivity over TDS showed a positive correlation between the two parameters with correlation coefficient of $R^2 = 0.789$ for the four months. The total coliform load was measured using Multiple Tube Fermentation (MPN) technique and bacterial counting was done using plating technique on R2A media. Maximum bacterial load were observed in the month of May and minimum for the month of November.

For the bacteriological analysis of post treated sewage water, serial dilutions of sample were made and isolation of bacteria was done using the spread plate technique on nutrient agar (NA), EMB agar, MacConkeys agar, Salmonella-Shigella agar, Thiosulphate-citrate-bile salts sucrose media (TCBS) and Sorbitol MacConkeys agar.

Phenotypic characterization of these isolates were done based on Gram staining, endospore staining, Acid fast staining, different biochemical tests and carbohydrate tests. Various biochemical test carried out were IMViC, urease test, catalase test, oxidase test, and triple sugar iron agar test. Eight carbohydrates (Inositol, Dulcitol, Sucrose, Raffinose, Mannose D-Mannitol, L-Arabinose and D-Xylose) were used to test the ability of the isolates to ferment them. Growth characteristics of the isolates on various specific media were also checked to aid the identification of these isolates. Gram negative bacteria was found to be dominant in the sewage sample and the percentage occurrence of various members were found to be 26.2% for *E. coli,* 14.7% for *Enterobacter* species, 11.4% *Klebsiella* species, 9.8% each for *Shigella* and *Serratia*, 8.1% each for *Proteus* and *Vibrio*, 4.9% each for *Salmonella* and *Pseudomonas* and 1.7% *Citrobacter* species.

Chapter 7

Conclusion

7. Conclusion

Microbial analysis of post treated sewage water is extremely important as it reflects the degree of efficiency of wastewater treatment plant. The treated effluent of Adampool sewage treatment plant which is being discharged into the river (Rongni Chu) may be the source of drinking, bathing, or irrigation for the people living in downstream localities. Poorly treated wastewater may be hazardous to humans as well as to the environment. From this study on the physico-chemical parameters and bacteriological analysis of post treated sewage water the following conclusions were made:

- The value of pH, temperature, TDS and conductivity were under the permissible limits as per CPCB guidelines.
- Microbial analysis revealed the prevalence of various types of bacteria. Among them the prevalence of bacteria belonging to Enterobactericeae family was more.
- Presence of pathogenic bacteria such as *Salmonella, Shigella* and *Vibrio* even after treatment of sewage was also detected.

The present study involves only a preliminary analysis to check the microbial diversity of sewage effluent. Further analysis has to be undertaken for bacterial identification upto the species level as well as strain identification and also examine the antibiotic susceptibility test with respect to different antibiotics. A field survey needs to be done in and around the treatment plant to analyse the various diseases suffered by the local people which may be directly or indirectly linked to exposure of wastewater.

Future prospect also includes research for the microbial analysis of treated sewage sludge as it is being used by farmers for crop irrigation. The sewage sludge may consist of wide variety of disease causing bacteria and protozoan that may pose a health risk to the farmers and local populace. It is well said that prevention is better than cure. The challenge thus is to find low-cost technology, user friendly methods, which on one hand must avoid threatening our substantial wastewater dependent livelihoods and on the other hand protect degradation of our valuable natural resources.

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