Physiochemical and Microbial Analysis of Soil and Water of Dhobiajharan Village (Proposed Coal Mine Site - Tubed)

A Project Thesis Submitted in Partial Fulfillment of The

Requirements

For The Degree in

Bachelor of Technology in Biotechnology

Submitted by:

SIBARAM BEHERA

(109BT0020)

Under Guidance of

Prof. Mukesh Kumar Gupta

Department of Biotechnology and Medical Engineering

National Institute of Technology

Rourkela-769008

2012-2013

CERTIFICATE

This is to certify that, the thesis entitled "Physiochemical and Microbial analysis of Soil and Water of Dhobiajharan Village (Proposed Coal Mine Site - Tubed)" submitted by Sibaram Behera, in partial fulfillment of the requirement for the award of Bachelor of Technology degree in Biotechnology at National Institute of Technology Rourkela, is an authentic work carried out by him under my supervision. To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University/Institute for award of any Degree/Diploma.

Date:

Prof. /Dr. Mukesh Kumar Gupta

Place: Rourkela

Dept. of Biotechnology & Medical Engineering

NIT Rourkela

Contents

List of Figures

List of Tables

Abbreviations

EIA: Environmental Impact Assessment

IAIA: International Association for Impact Assessment

EC: Electrical Conductivity

TDS: Total Dissolved Solids

DNA: Deoxyribonucleic acid

rRNA: Ribosomal ribonucleic acid

HCl: Hydrochloric acid

CFU: Colony Forming Unit

DMAB: p-dimethylaminobenzaldehyde

MR-VP: Methyl Red -Voges-Proskauer

ABSTRACT

Baseline data collection is an important component of Environmental impact analysis which is a decision making step for a proposed project. The objective of this study was to collect baseline data of Dhobiajharan village which comes under a proposed coalmine site. In this study we have isolated and analyzed the environmental status of Dhobiajharan. Microbial analysis focusing on bacteria was done quantitatively. Identification of isolated bacteria was done by performing several Biochemical tests. Some physiochemical parameters were also analyzed. CFU values show that it comes in the normal range. Analyzing biochemical test results of two isolated bacteria strain (D_2 and D_1), it was predicted that these two strains might belongs to Enterobacter *spp.* and *Streptococcus spp.* respectively.

Key Words: Environment, EIA, CFU, Microbial analysis

Chapter-1

INTRODUCTION

Environment and humans are are highly dependent on each other. The term natural environment, commonly referred as 'environment', is used to denote everything that surrounds us. It therefore, includes the living things like all types of animals (including humans) and plants, as well as the non-living things (i.e. everything other than the living organisms) including the three basic elements of nature, i.e. land, water and air. The living world is usually called the 'biotic world'; while the non-living things are referred as the 'abiotic world'. As a matter of fact, every entity in an environment interacts with its environment, causing its influence on the environment; accordingly gets influenced by its environment. Humans being a part of the biotic component of the environment eventually get affected by their natural environment and accordingly cause an impact on the environment [1].

In the natural environment in the biosphere, there exists a perfect balance or equilibrium between the various organisms and this is known as the ecological balance. In this equilibrium state, the relative numbers of different organisms in a particular environment remain constant. However, this ecological balance may get disturbed when the changes take place in the natural environment. When the balance get disturbed, all the components of environment get affected by this. The disturbance in the balance may be due to various reasons (e.g. species variation, population variation, natural hazards or man-made causes etc.). Now-a-days Man made cause is prominent over the other cause for the disturbance in ecological balance disturbance and biodiversity loss.

Earth's most unique feature is the existence of life, and the most extraordinary feature is diversity in life. Approximately 9 million types of animals, plants, protists and fungi inhabit the Earth. At the first Earth Summit, it was declared by majority of the world's nations that human actions were dismantling the Earth's ecosystems, eliminating species, genes and biological traits at an alarming rate [2]. The impact of human activity on environment is also known as anthropogenic impact on environment. The human activities such as industrialization, urbanization, mining activity etc. affect the environment most. For example the ecological effects of roads which is reviewed by Seiler Andreas [3]. The number of reservoir construction is increasing as the demand of water rising worldwide. Constructions of large freshwater reservoirs are associated with serious impacts upon natural environment. Morley Neil J [4] reviewed the anthropogenic effects of a reservoir construction particularly focusing on parasite fauna of aquatic wildlife.

An environmental impact assessment (EIA) is an approach of assessment of the possible positive or negative impact that a proposed project may have on the environment, together considering the environmental, economic and social aspects. The purpose of the assessment is to ensure that decision makers consider the ensuing environmental impacts when deciding whether to proceed with a project. In simple word this is a decision making step. The International Association for Impact Assessment (IAIA) defines an environmental impact assessment as "*the process of identifying, predicting, evaluating and mitigating the biophysical, social, and other relevant effects of development proposals prior to major decisions being taken and commitments made"* [5]. This decision making process was introduced with the purpose of identifying or evaluating the potential beneficial and adverse impacts of the proposed projects on the environment, taking into account environmental, socioeconomic, cultural and aesthetic considerations. These aspects are critical for determination of the viability of a project and to decide if a project should be granted environmental clearance.

An EIA concentrate on conflicts natural resource constraints and problems which might affect the future of a project. It also involves the prediction of how the project could harm to people, their livelihoods, and the other nearby developmental activities. After potential impact prediction, the EIA identifies mitigation approaches to minimize the impacts and to improve the project viability. An EIA aims to identify the impacts at an early stage of the project planning and design. So that the project can be modified accordingly in order to reduce the adverse effects of the project.

Baseline data collection is an important segment of environmental impact assessment (EIA).It describes the existing environmental status of the identified study area. There are various areas (e.g.-water, air, land, noise, biological etc.) which are primarily concerned for EIA.

The population and kinds of microorganisms present in soil depend on many environmental factors; nutrients availability, available moisture, degree of aeration, pH, temperature etc. Soil bacteria and fungi play pivotal roles in various biochemical cycles and are responsible for the recycling of organic compounds [6]. Therefore result obtained from microbial analysis give information about soil health. Bacteria make up the most abundant group of microorganisms in the soil ($3.0 \times 10^6 – 5.0 \times 10^8$) per gram of soil, followed by the actinomycetes ($1.0 \times 10^6 – 2.0 \times 10^7$), fungi ($5.0 \times 10^3 – 9.0 \times 10^6$), yeast ($1.0 \times 10^3 – 1.0 \times 10^6$), algae and protozoa ($1.0 \times 10^3$-

5.0 x 10^5) and nematodes (50 – 200) counts per gram of soil [6]. Therefore microbial analysis of soil is essential to know about soil health.

Physicochemical analysis of soil and water provides information about the current environmental status, for example pH of water.

1.1 Site Location

The village (Dhobiajharan) comes under the proposed coalmine site-tubed, Jharkhand. The tubed coal block is located at Latehar district of the state of Jharkhand.The full village comes in the proposed coal mine area. A map showing Dhobiajharan is presented below.

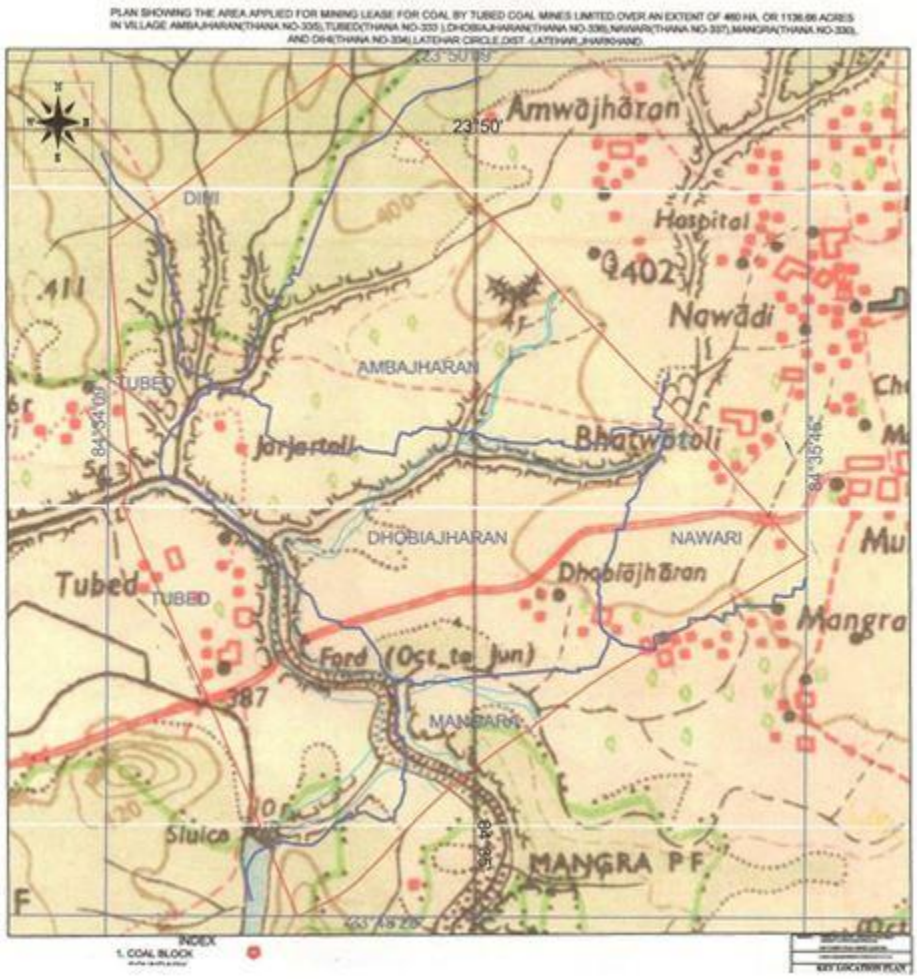


Figure-1: Map showing site location

1.2 Objectives

The present work focused on baseline data collection for EIA of Dhobiajharan village. The detailed objectives were:

- Quantitative analysis of soil living Bacteria.
- Identification of isolated bacteria.
- Physiochemical analysis of soil
- Physiochemical analysis of water

Chapter-2

LITERATURE REVIEW

2.1 Soil

Soils are particulate materials of the outer crust of the earth surface formed from the continuous weathering of the underlying parental rocks. Therefore, the type of soil is a function of the nature of the underlying rocks. Soil formation has been reported to be combination of various interrelated factors of parental materials, climate, organisms, topography and time [7]. Soil is important to everyone directly or indirectly.

Soil is a complex ecosystem where living organisms play a key role in the maintenance of its properties. Soil is a highly complex medium influence by environmental and physicochemical parameters, creating a varied habitat for a diverse range of soil microorganisms. Soil quality can be assessed by analyzing different physiochemical parameters with the analysis of microbial diversity .These are the various indicators which provide the actual condition, nature and quality of the soil.

2.2 Soil Color

Soil color does not affect the behavior and use of soil; however it can indicate the composition of the soil and give clues to the conditions that the soil is subjected to. Soil can exhibit a wide range of color; gray, black, white, reds, browns, yellows and under the right conditions green [8].Color and distribution pattern of soil results from both chemical and biological processes, especially redox reactions. As the soil contains various minerals, organic compounds so the combination lead into new and colorful compounds. Reducing environment produce disrupted color pattern but aerobic environment result in uniform pattern of color change. Yellow or red soil indicates the presence of iron oxides. Dark brown or black color in soil indicates that the soil has high organic matter content. Due to presence of water wet soil appears darker than dry soil. Soil color is get affected by oxidation rate which is dependent upon water content. High water content means less air in the soil, specifically less oxygen. In well drained (oxygen rich soils) red and brown colors caused by oxidation are more common, as opposed to in wet (low oxygen) soils where the soil usually appears grey. The presence of specific minerals can also affect soil color. Manganese oxide causes a black color, glauconite makes the soil green and calcite can make soil appearance white [8].

2.3 Soil types

Soil is basically of 5 types [9], these are:-

- Sandy Soil:

  This is light and dry in nature. Approximately no moisture content and absorb heat quickly. This is good for the production of early crops. It is fit for cultivation any time of the year but it need to be watered frequently.

- Clay Soil:

  Clay soil is also called ‘late’ soil. The soil serves as an excellent retort for the dry season, as it has a high water retention quality. For improving texture, it is necessary to drain clay soil frequently. The soil becomes unmanageable during rainy season, as it becomes ‘sticky’. On the other hand, during draught, it becomes ‘rock solid’.

- Loam Soil:

  Loamy soil is a combination of all the three - sandy soil, clay soil and silt soil, in the ratio of 40:40:20. It is suitable for any and every kind of crops. loam soil has best of the characteristic of all. It has high nutrients content, warms up quickly in summers and rarely dries out in the dry weather. It has become the ideal soil for cultivation.

- Peaty Soil:

  Peaty soils are acidic in content, which makes them sour. This is the most exceptional feature of Peaty soils. Usually found in low-lying areas, these soils require proper drainage, as the place is accustomed to a lot of water clogging. Though peaty soils have less nutrient content, they warm up quickly in the spring, making them excellent if right amount of fertilizers are added.

- Chalky Soil:

  Chalky soil is alkaline in nature and usually poor in nutrients. It requires nourishment, in the form of additional nutrients and soil improvers, for better quality. The soil becomes dry in summers, making it very hard, and would require too much of watering for the plants to grow. The only advantage which such a soil has is its lime content. When deep-rooted, Chalky soil becomes excellent for plant growth and favors good growing conditions as well.

2.4 Physicochemical parameters of Soil

These parameters provide the physical and chemical status of the soil. Rabah et *al*. (2010) assessed different physicochemical parameters of the soil contaminated with abattoir effluents. Zaiad Galal M (2010) analyzed different physicochemical parameters Al-Khums city, Libya. The parameters which were studied by different groups are summarized and represented in a table. (Table-1)

Table 1. Different physiochemical parameters of soil

Sl.No	Physicochemical parameters	Reference
1	pH, Temperature, Nitrogen, Magnesium, Phosphorus, Potassium, Calcium, Sulphide, Organic matter, Cation exchange capacity	Rabah et al. (2010) [10]
2	moisture contents, conductivity, TDS, pH, and chloride contents	Zaiad Galal M (2010) [11]
3	pH, Conductivity, Total alkalinity, Total chloride, Sulphate, Bulk density, Moisture content, Organic matter, Na, K	Narkhede *et al.*(2011) [12]
4	pH, Bulk density, Specific gravity, N,P,K, Carbon Nitrogen ratio	Pal and Lalwani (2011) [13]
5	pH, EC ,N, P, K, Cu, Fe, Mn ,Zn Ni, Cd	Pujar *et al.*(2012) [14]

2.5 Soil Bacteria

Different reports show the presence of various kinds of bacteria playing various roles (e.g. Decomposers, Nitrogen fixers etc.) in soil ecological system. Diverse kinds of bacteria belonging to different genus and species are found in soil commonly. Summarizing findings of different studies a list of genus of common soil living bacteria was prepared which is presented below in tabular format.

Table 2. List of common soil bacteria

Sl No.	Genus	Reference
1	*Arthobacter spp.*	15
2	*Streptomyces spp.*	15
3	*Pseudomonas spp.*	6,13,15
4	*Bacillus spp.*	6,13,15
5	*Corynebacterium spp.*	6
6	*Flavobacterium spp.*	6
7	*Aeromonas spp.*	6
8	*Staphylococcus spp.*	6
9	*Nocardia spp.*	6
10	*Streptococcus spp.*	6,17
11	*Lactobacillus spp.*	6
12	*Enterobacter spp.*	13,16

2.6 Identification of Bacteria

There are two approaches which are widely used for the identification of unknown bacteria. One approach is the biochemical approach which involves different tests which provide information about biochemical characteristics of bacteria. Bergey's manual described these tests in systematic manner.

The other approach is the molecular approach in which bacteria identification was done according to the information of 16SrRNA sequence. Delmont *et al.* (2011) [18], Rachid *et al.* (2012) [19] and Maciel *et al*. (2009) [20] used this approach for the identification of bacteria. In this approach DNA from the soil sample is extracted by direct or indirect methods. Then 16SrRNA segment of isolated DNA is amplified and sequenced. Fatima et al. (2011) [21] reported a comparative study of different DNA extraction methods from soil.

2.7 Bergey's Manual

In 1923, David Bergey, professor of bacteriology at the University of Pennysylvania, and four colleagues published a classification of bacteria that could be used for identification of bacterial species, the Bergey's Manual of Determinative Bacteriology. This manual is now in its ninth edition [22]. In 1984, the first edition of Bergey's Manual of Systematic Bacteriology was published. It contained descriptions of all prokaryotic species then identified. There has been enormous progress in prokaryotic taxonomy since the first volume was published. In particular, the sequencing of rRNA, DNA and proteins has made phylogenetic analysis of prokaryotes feasible.

2.8 Water

Water is a chemical compound having two hydrogen and one oxygen atom linked by covalent bond. Its molecular formula is H_2O.Water is a liquid at standard ambient temperature and pressure. It is also found in solid (ice) and gaseous (vapour) state. 71% of the total Earth surface is covered by water. On Earth water is found as ocean, river, lake, groundwater and glacier etc. Water is a vital element of all the biological systems. Water is needed for day to day activity. For various production processes at industries water is also essential. Water plays a pivot role in the field of agriculture. Water is also a huge ecological system which houses various kinds of organisms. Safe drinking water is essential to humans and other life forms even though it provides no calories or organic nutrients. Water is highly essential for day to day activities of human. The physical and chemical parameters of water (e.g. pH, Dissolved Oxygen, TDS, alkalinity etc.) provide the quality of water and for which purpose it can be used.

Chapter- 3

MATERIALS AND METHODS

3.1 Sample Collection

Samples were obtained from random plots at the depth of 15cm in sterilized zip lock as described by Arotupin et.al (2008) [7]. A soil auger was used to obtain volume samples with a minimum of 0.5 kg of soil per sampling area. GPS coordinates of the sampling plots were noted. Soil samples were collected in tightly sealed plastic bags and kept at 4°C to keep them field moist and to preserve biological properties. Water sample was collected from the tube well and pond/open well for ground water and surface water respectively.

GPS coordinates of soil sample collection: 84^0 35' 12" E and 23^0 49' 56" N

3.2 Physiochemical analysis

Physiochemical parameters of soil and water are analyzed by following the methods described in the respective Indian Standards. The soil specimen obtained from field was prepared in accordance with IS: 2720 (part 1) – 1983 before performing test for physiochemical parameters.

3.2.1 pH

The pH value which is a measure of the hydrogen or hydroxyl ion activity of the soil water system indicates whether the soil is acidic, neutral or alkaline in reaction. Crop growth suffers much both under very low as well as high pH. The instrument for pH measurement commonly used is a digital pH meters have single electrode assembly. The instrument being a potentiometer, the pH scale has to be calibrated before use with buffer solutions of known pH values. 30g of soil is taken in a 100ml beaker to which 75 ml of distilled water is added .The suspension is stirred at regular intervals for 30 minutes and the pH is recorded. The suspension is stirred well just before the electrode are immersed and readings are taken.

pH of water sample was taken by using pH meter. Three readings were observed and then mean of it was calculated.

3.2.2 Organic matter

The soil is grounded and completely passed through 0.2 mm sieve (80mesh) and 1gm is placed at the bottom of a dry 500 ml conical flask. Add 10 ml of potassium dichromate (1N) in the 500 ml conical flask, swirled and conical flask gently to disperse the soil in the dichromate solution. Then 20 ml of sulphuric acid is run in run in and swirled again two or three times. The flask is allowed to stand for 30 minutes and there after 200 ml of distilled water along with 10 ml of

ortho-phosphoric acid is added and 1ml of diphenylamine indicator. The whole contents are titrated with ferrous ammonium sulphate solution till the color flashes from blue – violet to green. For a final calculation, a blank is run without soil.

3.2.3 Calcium Carbonate

Five gram of soil was weighed accurately and transferred into a 150 ml beaker.100 ml of HCl was added to it. It was kept at Room Temperature for 1 hour with vigorous stirring. After settling, 20 ml of supernatant liquid was taken and 6 to 8 drops of bromothymol blue indicator was added to it. Titration was performed with sodium hydroxide solution.

3.2.4 Water content

The container with lid was weighed for this study petridishes were used. Required quantity of soil specimen was taken and the weight was noted down. The specimen is dried in oven at 110^0C for 24 hours without the lid of petridish. Every time the container is taken out for weighing .The lid of petridish was replaced and the final constant reading was obtained.

The percent of water content (w) was calculated using the following formula,

$$w= (w2-w3)/ (w3-w1)$$

Where w = water content percent

$w_{1=}$ mass of container with lid with wet soil

w_2= mass of container with lid with dry soil

w_3 = mass of container with lid

3.2.5 Dissolved Oxygen

This was performed by Wrinkler method.2 ml of manganous sulfate solution and 2ml of alkaline potassium iodide was added to the water. The solutions were mixed thoroughly.2 ml of conc. Sulfuric acid was added to it. From the above solution 200 ml was transferred into a conical flask. Few drops of starch indicator were added. Then it was titrated against sodium thiosulfate till blue color turns violet. The amount of titrants used gives DO value. Three readings were taken and mean of it was calculated.

3.3 Microbial analysis

These are the tests or steps which were followed for analysis and identification of bacteria.

3.3.1 Isolation and Total Bacterial Count

This was achieved by serial dilution or log dilution method. Five test tubes containing 9ml of sterile distilled water were taken. One test tube containing 10 ml of sterile distilled water was taken.1 g of soil was added to the test tube with 10 ml of sterile distilled water. Mixing was done properly. Then 1 ml of microbial suspension was added to another test tube containing 9 ml of sterile distilled water. Again mixing was performed properly.1ml of microbial suspension was added to another test tube containing 9ml sterile distilled water. This step was repeated serially for other test tubes. In this way the microbial suspension get 10 fold serially diluted.100 µl of diluted suspension was poured into the surface of Nutrient agar plate .and spread by 'L' shaped spreader. The bacteria can thus be isolated and counted by C.F.U i.e. Colony Forming Unit.

C.F.U=No. of colonies/inoculum size (g) X Dilution Factor

Pure culture was isolated from this plate by streak plate method using inoculation loop.

3.3.2 Gram Staining

The Gram stain is a differential stain that allows classifying bacteria. Gram +ve as well as Gram –ve cells take up the deep violet color of the primary stain crystal violet. When treated with a mordant (Iodine) a crystal violet-Iodine (CVI) complex is formed resulting deep purple color in all cells. When alcohol is applied, it quickly removes the crystal violet-Iodine complex of Gram –ve cells with ease. This is attributed to the dissolving of excess lipids present in the outer membrane of Gram –ve bacteria. The complex takes a longer time to be removed in Gram +ve cells due to thick and impermeable peptidoglycan layer in their walls. Thus controlled treatment of alcohol only decolorizes the entire Gram –ve cells while the Gram positive retains purple color. A counter stain such as safranine is then used to stain the colorless Gram negative cells.

Table 3. Steps of Gram Staining

S. No	Particulars	Holding Time	Treatment	Gram +ve	Gram - ve
1	Flood the smear with primary stain crystal violet	1min	Wash gently with distilled water	Deep Violet	Deep Violet
2	Flood the smear with mordant Gram's Iodine	1 min	Wash gently with distilled water	Deep Purple	Deep Purple
3	Flood the smear with decolorizer alcohol	10-15 sec	Wash gently with distilled water	Deep Purple	Colorless
4	Flood the smear with counter stain Safranine	1 min	Wash gently with distilled water	Deep Purple	Red

3.3.3 Catalase test

The catalase test determines the presence of the enzyme catalase in bacteria. It is essential for differentiating Gram + ve coccus bacteria (e.g. Staphylococcus and Streptococcus).The catalase enzyme serves to neutralize the bactericidal effects of Hydrogen Peroxide .Catalase expedites the breakdown of H_2O_2 into water and oxygen.

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \uparrow$$

To test the catalase activity few drops of 3% H_2O_2 was applied over the bacterial colony of 18-24 hrs. Bubble formation indicates Catalase positive.

3.3.4 Oxidase Test

The oxidase test is a biochemical reaction that assays for the presence of enzyme cytochrome oxidase**.** A small piece of filter paper was soaked in 1% Kovács oxidase reagent and dried**.** The

composition of Kovács oxidase reagent is 1% tetra-methyl-p-phenylenediamine dihydrochloride in distilled water. A well isolated colony was picked from a fresh bacterial plate (18 to 24 hours) and rubbed into the filter paper soaked with Kovács oxidase reagent. Color change indicates the result of this test. Microorganisms are oxidase positive when the color changes to dark purple within 5 to 10 seconds. Microorganisms are delayed oxidase positive when the color changes to purple within 60 to 90 seconds. Microorganisms are oxidase negative if the color does not change or it takes longer than 2 minutes.

3.3.5 Indole Test

The indole test screens for the ability of an organism to degrade the amino acid tryptophan and produce indole by the activity of tryptophanase enzyme.

Tryptophan + water = indole + pyruvic acid + ammonia

Indole test was performed as described by Abdulkadir and Waliyu (2012) [16]. One percent tryptophan broth was taken in a test tube and inoculated with bacteria colony. After 48 hours of incubation period at 37°C, one militre (1ml) of chloroform was added to the broth. The test tube was shaken gently. 5 drops of Kovács reagent was added directly to the tube. This was also shaken gently and allowed to stand twenty (20) minutes. The formation of red coloration at the top layer indicated positive and yellow coloration indicates negative. Composition of Kovacs reagent:

Table 4. Composition of Kovacs reagent

Ingredient	Amount
Amyl or isoamyl alcohol	150.0 ml
p-dimethylaminobenzaldehyde (DMAB)	10.0 g
HCl (concentrated)	50 ml

3.3.6 Citrate Test

The citrate test screens a bacterial isolate for the ability of utilization citrate as its carbon and energy source. Citrate utilization test was performed as described by Abdulkadir and Waliyu

(2012) [16].This test was performed by inoculating the bacteria into Simmon's citrate medium. The composition of Simmon's citrate medium is given in the table. The inoculated medium was incubated for 48 to 72 hours .The color of the medium indicates the result. If the color of media changes from green to blue then the bacteria is citrate positive. If the media retain the green color after incubation period then the bacteria is citrate negative.

Table 5. Simon's Citrate medium composition

Ingredients	Amount
Magnesium sulfate (heptahydrate)	0.2 g
Ammonium dihydrogen phosphate	1.0 g
Dipotassium phosphate	1.0 g
Sodium citrate (dehydrate)	2.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Bromothymol blue	0.08 g
Deionized water	1000ml

3.3.7 Methyl Red test

Methyl red test was performed as described by Abdulkadir and Waliyu (2012) [16].A tube of MR-VP broth was inoculated by a fresh pure culture (18-25 hours). The composition of MR-VP broth is given in Table 6. The inoculated MR-VP broth was incubated for 48-72hours at 37°c after which, one milliliter (1ml) of the broth was transferred into a small tube. Small quantities (2-3 drops) of methyl red were added to it. Color change of the medium provides the result. If the color is red then the bacteria is positive otherwise if color is yellow, the bacteria is negative.

Table 6. Composition of MR-VP broth

Ingredients	Amount
Buffered peptone	7.0 g
Dipotassium phosphate	5.0 g
Dextrose	5.0 g
Deionized water	1000ml

3.3.8 Urease test

Presence of enzyme urease which splits urea into ammonia CO_2 was detected by inoculating bacterial cultures into tubes containing urease broth Incubated at 30 $\pm$ 0.1^0C for 72 hours. Purplish pink coloration of the medium indicated positive reaction. Composition of the urease broth medium:

Table 7. Composition of urease broth medium

Ingredients	Grams/Litre
Peptone	1
NaCl	5
K_2HPO_4	2
Glucose(Sterilized Separately)	1
Urea(Filter sterile)	20
pH	6.8
Phenol red(Filter Sterilized)	6 ml

Chapter-4

RESULTS AND DISCUSSION

4.1 Soil Characteristics

The color of the soil collected from Dhobiajharan is yellowish. The obtained values of different physiochemical parameters of soil are represented in tabular format which is given below.

Table 8. Physicochemical parameters of Soil

S.No	Parameter Name	Obtained Value
1	pH	6.51
2	Organic Matter	1.17%
3	$CaCO_3$ Content	16.1%
4	Moisture Content	8.68%

4.2 Total Bacterial Count

The isolated bacteria was quantified by calculating Colony Forming Unit (C.F.U) i.e. Colony Forming Unit. The obtained C.F.U values are represented in the following table.

Table 9. CFU values

Dilution	No.of Colonies	Dilution factor	CFU(per g)
10^{-4}	67	10^4	$67x10^4$
10^{-5}	32	10^5	$32x10^5$
10^{-6}	26	10^6	$26x10^6$

Two types of bacterial colonies were found in the plates. By streak plate method pure bacteria culture is isolated. The two types of bacteria were named as D1 and D2, Which will be used for the identification tests.

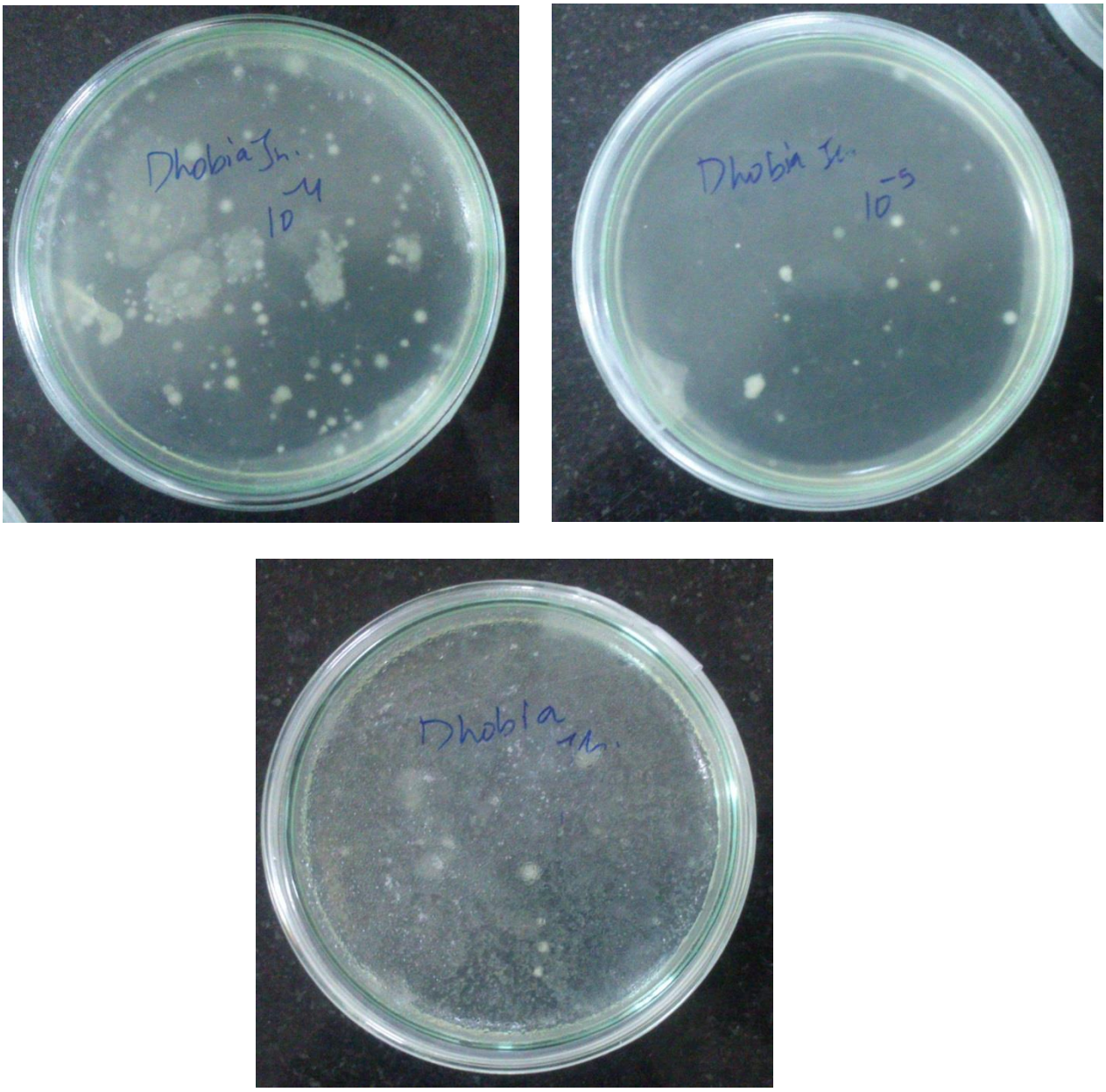


Figure 2. Isolated bacteria colonies

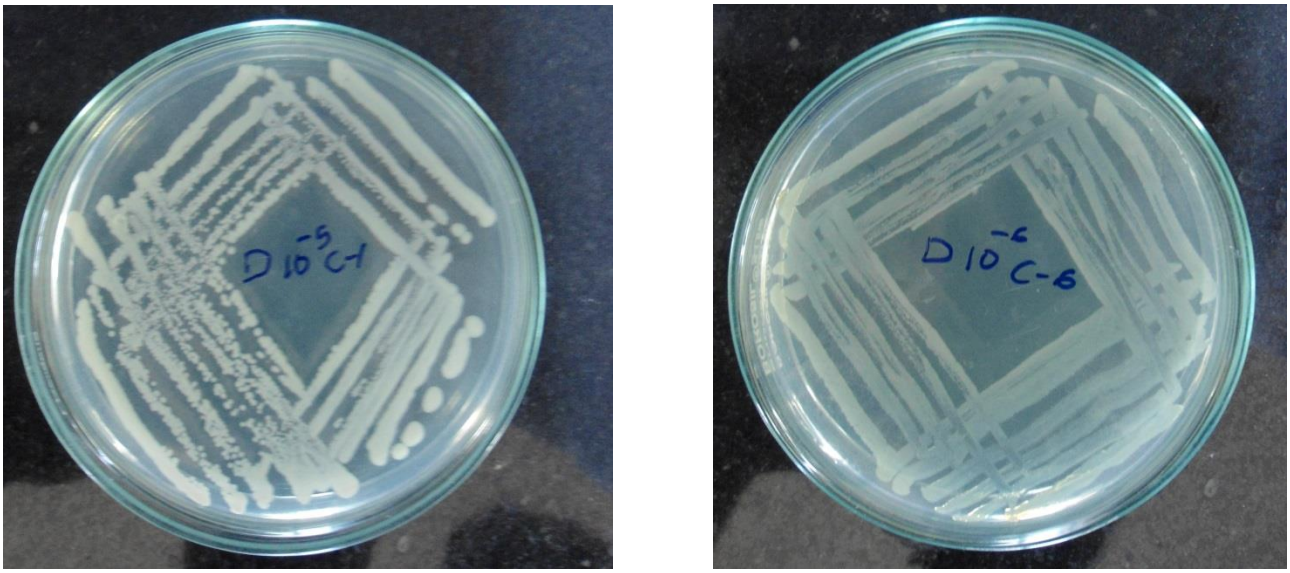


Figure 3. Pure culture isolation by streak plate method

4.3 Biochemical Test result

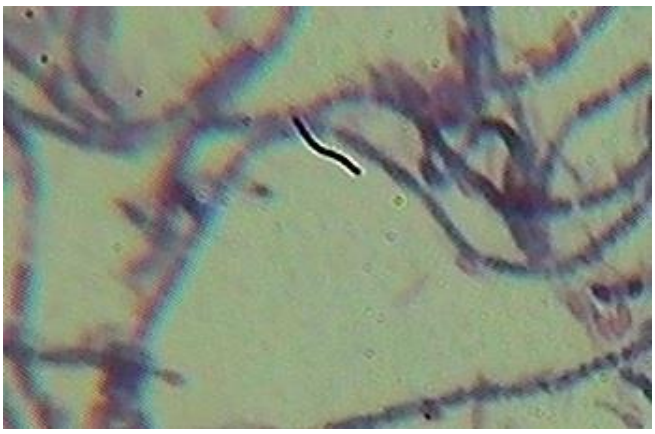
The obtained results of biochemical tests are given below. These are required for identification of bacteria genus.

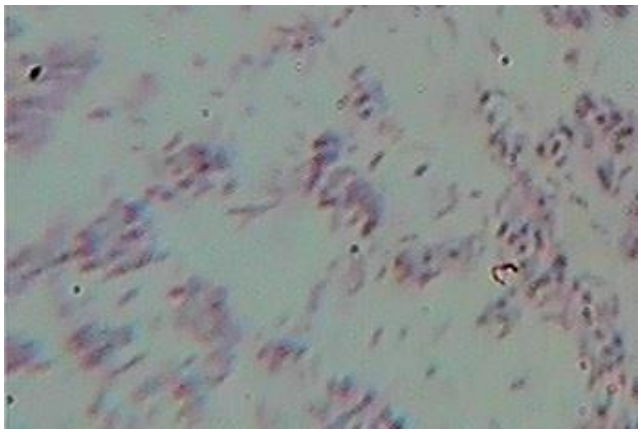
4.3.1 Gram Staining

D_1 gives purple color and D_2 gives Red color when observed under microscope. Microscopic observation of D1 shows that shape is coccus and found in chains. The shape of D_2 is bacilli in nature.

Table 10.Gram stain results

D_1	Gram +ve	Coccus
D_2	Gram -ve	Bacilli





D_1 *D_2*

Figure-4: Gram staining result

4.3.2 Catalase Test

D_1 does not form bubbles when H_2O_2 is applied.D2 forms bubble when H_2O_2 is applied.

Table 11.Catalase test results

D1	No bubble	Catalase - ve
D2	bubbles	Catalase +ve

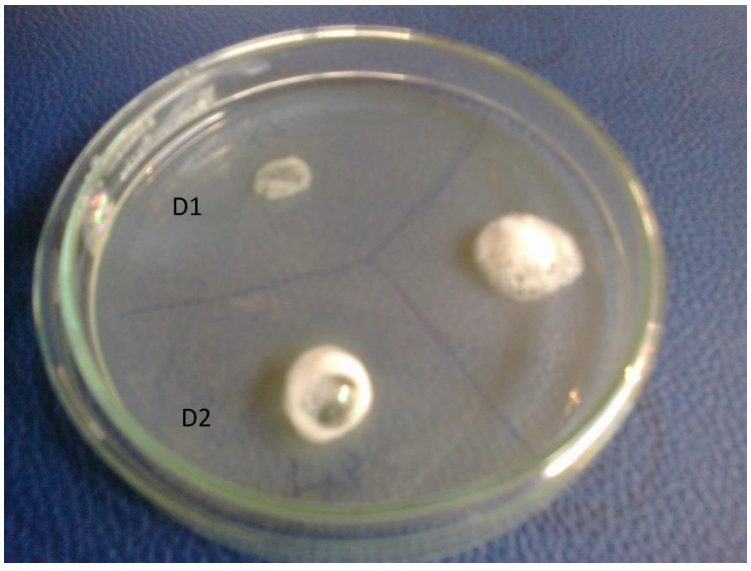


Figure-5.Catalase test result

4.3.3 Oxidase Test

Both D_1 and D_2 do not change color when rubbed into kovacs oxidase reagent treated filter paper. Therefore both D_1 and D_2 are oxidase negative**.**

Table 12.Oxidase test results

D1	No color change	Oxidase -ve
D2	No color change	Oxidase -ve

4.3.4 Indole Test

Both D1 and D2 give yellow color when Kovacs reagent is applied. Therefore both D_1 and D_2 are Indole negative.

Table 13.Indole test results

D1	Yellow color	Indole -Ve
D2	Yellow color	Indole -Ve

Figure-6. Indole test result

4.3.5 Citrate utilization test

Both for D_1 and D_2 the media color changes from green to blue. Therefore both D1 and D2 are citrate positive.

Table 14.Citrate test results

D1	Blue color media	Citrate +ve
D2	Blue color media	Citrate +ve

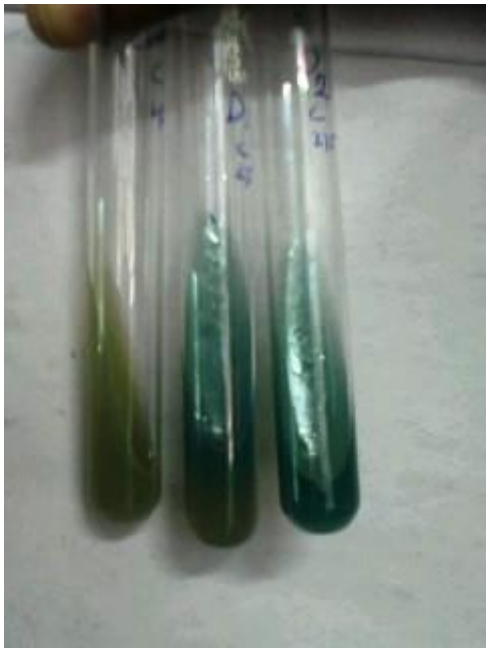


Figure-7. Citrate utilization test result

4.3.6 Methyl Red test

Both for D_1 and D_2 the color is yellow. Therefore D_1 and D_2 are methyl red negative.

Table 15. MR test results

D1	Yellow color	MR –ve
D2	Yellow color	MR -ve

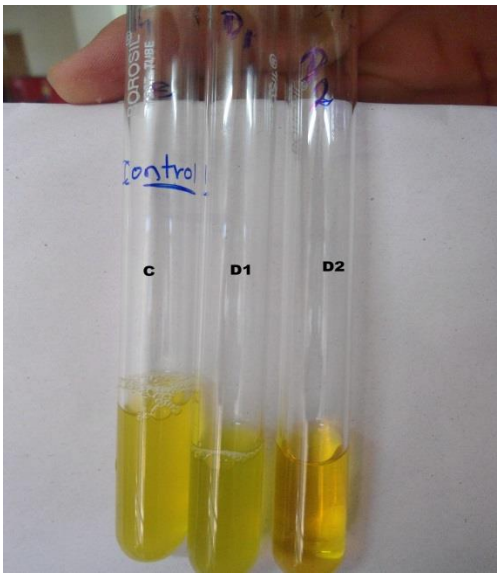


Figure-8. Methyl Red test result

4.3.7 Urease Test

The color of D1 changed therefore it is D_1 is urease positive while the color of D_2 does not change so Urease negative**.**

Table 16.Urease test results

D_1	Urease + ve
D_2	Urease - ve

Figure-9: Urease test result

4.4 Water parameter

Dissolved oxygen and pH of water was calculated and represented in tabular format.

Table-17.water parameter result

S. No.	Parameter	Obtained value
1	pH	6.4
2	Dissolved Oxygen(DO)	7.45 mg/ml

4.4 Discussion

The soil color of Dhobiajharan was yellowish. According to Brady and Weil (2006) [8], yellow color of a soil indicates the iron oxide content may be high. The soil is clayey in nature so the water content is high. From the analysis of moisture content it was obtained that moisture content of the soil is around 8.7% which is quite high. Water affects soil formation, structure, stability and erosion but is of primary concern with respect to plant growth. Water is essential to plants for four reasons:

- It constitutes 85%-95% of the plant's protoplasm.
- It is essential for photosynthesis.
- It is the solvent in which nutrients are carried to, into and throughout the plant.

- It provides the turgidity by which the plant keeps itself in proper position.

The pH of the soil of Dhobiajharan was found to be 6.51.According to the United States Department of Agriculture [23] soil pH range classification is represented in tabular format. From the table, it was obtained that the nature of the soil is slightly acidic. Organic matter content of the soil was found to be 1.17%.Calciumcarbonate content of the soil was found to be above 16% which is quite high.

By analyzing the CFUs it was observed that the no. of bacteria comes in the normal range. Ogunmwony et.al. (2008) reported that Bacteria are most abundant group of soil. One gram of normal soil contains approximately 3.0×10^6-5.0×10^8 of bacteria. Bacteria play various roles in soil for examples it act as decomposers, Nitrogen fixers etc. Therefore Bacteria no. shows the status of soil health. From this study it was observed that soil of Dhobiajaharan is healthy and supports the growth of bacteria.

Comparing the results of the biochemical test results with the Bergey's manual it is predicted that the D1 strain may belongs to Streptococcus genus and D2 strain may belongs to *Enterobacter* genus. Biochemical test profile of *Enterobacter* which was reported by Pal and Lalwani et al. (2011) and Abdulkadir and Waliyu (2012) was compared with the obtained result of D2.From the comparison it was observed that there are high similarity between D2 strain and *Enterobacter*. Therefore D2 might be a species of genus *Enterobacter*.

Table -18. Soil classification according to pH

Denomination	pH range
Ultra acid	< 3.5
Extreme acid	3.5–4.4
Very strong acid	4.5–5.0
Strong acid	5.1–5.5
Moderate acid	5.6–6.0
Slight acid	6.1–6.5
Neutral	6.6–7.3
Slightly alkaline	7.4–7.8
Moderately alkaline	7.9–8.4
Strongly alkaline	8.5–9.0
Very strongly alkaline	> 9.0

Table 19: Comparison of Enterobacter and unknown D2

Biochemical Test	***Enterobacter***	**D_2**
Gram staining	Negative	Negative
Shape	Bacilli	Bacilli
Catalase	+	+
Oxidase	-	-
Indole	-	-
Citrate	+	+
Methyl Red	+/-	-
Urease	-	-

Similarly By analyzing the result for D_1 it was found that the shape, Gram staining result, Catalase and oxidase test result it was predicted that the D_1 strain might be a species of the genus *Streptococcus.*

Table-20: Comparison of Streptococcus and unknown D1

Biochemical Test	***Streptococcus***	**D_1**
Gram Stain	+	+
Shape	coccus	coccus
Catalase	-	-
Oxidase	-	-

Chapter 5

CONCLUSION AND FUTURE WORK

By this study microbial analysis focusing on bacteria of the soil collected from Dhobiajharan was performed. Analyzing various biochemical test results the genus of isolated strains were predicted as *Enterobacter* and *Streptococus* corresponding to D2 and D1.Quantitavie analysis of bacteria show the soil health status. Physiochemical parameters also provide the soil health status. This study is mainly aimed on the baseline data collection aspect of Environmental Impact Assessment (EIA).By this studies some aspects of baseline data collection for the EIA of proposed coal mine site was obtained.

In order to get information about environmental status, more number of physicochemical parameter needs to be studied. Microbial analysis of water need to be performed to study the kinds of microbes grow.

REFERENCES

1. Garg et al., Environmental Science and Ecological Studies, New Delhi: Khanna Publishers, 2007
2. Cardinale et al., Biodiversity loss and its impact on humanity, Nature, Vol 486, (2012), pg-59.
3. Seiler Andreas, Ecological effects of roads a review, Introductory Research Essay No 9,(2001), Department of Conservation Biology, SLU, Uppsala
4. Morley Neil J., Anthropogenic Effects of Reservoir Construction on the Parasite Fauna of Aquatic Wildlife, Eco Health,4,(2007), 374-383
5. "Principle of Environmental Impact Assessment Best Practice." International Association for Impact Assessment. 1999.
6. Ogunmwony et.al., Microbial analysis of different top soil samples of selected site in Obafemi Awolowo University,Nigeria,Scientific Research and Essay.vol3(3),(2008),pp.120-124
7. Arotupin and Akinyosoye, Micobiological and physicochemical charecterstics of Cassava cultivated soils, Research Journal of Microbiology,3(1),(2008);pp-41-46
8. Brady Nyle C. & Weil Ray R., Elements of the Nature and Properties of Soils, New Jersey : Prentice Hall (2006), page 95.
9. Burh Priya Monalisha, Microbial Analysis of Soil and Water Samples from Koel River in Rourkela, Odisha (2011)
10. Rabah et al., microbiological and physico-chemicalassessment of soil contaminated with abattoir effluents in sokoto metropolis, Nigeria., Science World Journal, Vol 5 (No 3) ,(2010)
11. Zaiad Galal M, Physico-Chemical Analysis of Soils in Al-Khums city, Libya, Journal of Applied Sciences Research, Vol 6(8),(2010),Pg:1040-1044
12. Narkhede et al. , Physico-Chemical Analysis Of Soil Collected From Chorwad, Tehsil – Bhusawal, Dist. Jalgaon (M.S.), Int. J. Chem. Sci.: 9(4), (2011),Pg:1973-1978

13. Pal and Lalwani, A brief comparative study of bacterial population of different soils of Bhopal, Bioscience Biotechnology Research Communication, Vo l 4,No 2,(2011) ,Pg:,211-215

14. Pujar et al., Analysis Of Physico-Chemical And Heavy Metal Concentration In Soil Of Bijapur Taluka, Karnataka, Science. Revs. Chem. Commun.: 2(1), (2012),Pg: 76-79

15. Mohapatra Pradipta K,Textbook of Environmental Microbiology, New Delhi: I K International Publishing House,2008

16. Abdulkadir and Waliyu, Screening and Isolation of the Soil Bacteria for Ability to Produce Antibiotics, European Journal of Applied Sciences 4 (5),(2012),Pg.: 211-215

17. Gledhill and Casida, Predominant Catalase-negative Soil Bacteria, Applied Microbiology, Vol. 17, No.2,(1969), p. 208-213

18. Delmont et al., Accessing the soil metagenome for studies of Microbial diversity, Applied and Environmental Microbiology, Vol 77,No-4,(2011),p.1315-1324

19. Rachid et al. ,Physical-chemical and microbial changes in Cerrado soil under differing sugarcane harvest management system, BMC Microbiology,12,(2012),2012

20. Maciel et al., Simple DNA extraction protocol for a 16SrDNA study of bacterial diversity in tropical landform soil used for bioremediation of oil waste ,Genetics and Molecular research8(1),(2009),Pg:375-388

21. Fatima et al., Microbial DNA extraction from soil by different methods and its PCR amplification, Biochem. cell arch., Vol 11,No 1(2011)

22. Willey et al. , Prescott, Harley and Klein's Microbiology, New York: The McGraw-Hill publications,2008

23. Soil Conservation Service, U.S. Department of Agriculture Handbook 18