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ISOLATION AND IDENTIFICTION OF MICROORGANISMS FROM DIFFERENT SOIL SAMPLES OF BILASPUR(C.G).

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ABSTRACT

Present investigation is the isolation and identification of microorganisms' i.e bacteria and fungi from different soil samples (soil samples taken from Ashoknagar, near Maharana Pratap Chowk, Rajkishor Nagar, near C.M.D Chowk.) of Bilaspur in Chhattisgarh. The bacteria identified from soil samples are *E.coli, Enterobacter aerogenes, Klebsiella, Proteus vulgaris, Bacillus cereus, Bacillus subtilis, Clostridium, Staphylococcus aureus, Pseudomonas aeruginosa, Shigella,* and *Salmonella* and fungi are *Aspergillus, Rhizopus, Mucor, Candida,* Penicillium, *fusarium* etc.Out of them, some bacteria were commonly found in each sample such as *E. coli, Staphylococcus aureus, Clostridium, some fungi were commonly found in all samples such as Aspergillus, Rhizopus, Mucor. Pseudomonas aeruginosa* was isolated from garden soil as well as *Enterobacter aerogenes, Klebsiella, Proteus vulgaris,* were found in garden soil. *Staphylococcus aureus, Pseudomonas aeruginosa* were found in road side (traffic area) and *Bacillus subtilis, Salmonella, Shigella, Staphylococcus aureus were found in industrial area. Fusarium* were found in garden soil. Candida was found in road side soil sample. Aspergillus, *Penicillium* were observed in industrial area.

KEYWORDS: Culture media, Gram stain, soil samples, lab, microscope, biochemical test.

INTRODUCTION

soil is the outer region of earth-crust consisting of loose material formed by weathering of rock, and gives to plant both mechanical and nutritional support. Soil can be defined as the space time continuum forming the upper part of the earth crust. Thus, soil is a complex product of parental material, or geology, topology, climate, time and biological activity on

anthopogenic activity (Griffin 1972).Microbial life has been present at least 3,500 million years, and the earth itself was only formed 4,600 million year ago. "For much of its history'' earth was a planet of microbes.

Bacteria are the smallest unicellular prokaryotes (0.5-1×1.0-2.0µm), the most abundant group and usually more numerous than others, the number of which varies between 10^8 and 10^{10} cell per gram soil. However, in an agriculture field heir number goes o about 3×10^9/g soil which accounts for about 3 tones weight per acre. Based on regular presence, bacteria are divided in two groups: (a) Soil indigenous (i.e true resident) or auochthonous, and (b) soil invader or allochthonous.

Moreover, the number and types of bacteria are influenced by soil types and their microenvironment, organic matter, cultivation practices, etc. They are found in high number in cultivation than virgin land, maximum in rhizosphere soil possibly due to aeration and nutrient availability (Rovira,1965; Alexander,1977).

MATERIALS AND METHODS

A) Soil sample: Soils were collected from different area of Bilaspur at the depth of 6-10 cm with polythene bag by sterile method.

B) Laboratory media:- Dehydrated chemically defined medium was used and prepared as per manufacture instrumentation.

Nutrient Agar medium, Potato Dextrose Agar, Mueller Hinton Agar , Mac Conkey Agar, Sabouraud Dextrose Agar, Manitol Salt Agar, Glycerol Yeast Agar, Litmus Milk Broth, Phenol Red Lactose Broth, SIM Agar, Simon Citrate Agar, MR-VP Broth, Starch Agar, Nutrient Gelatin, Urea Broth.



Fungus on sabouraud Bacteria on nutrient agar Mueller Hinton agar. Catalase test Nutrient Agar.

Dextrose agar medium.

C) Other Requirements:- Petri plate, test tube, test tube strand, sprit lamp, inoculation loop, spreader, conical flask, culture tube, thread, distil water, cover slip, cotton, digital balance, marker pen, slide, alcohol.

D) Instrumentation:- Autoclave, Hot Air Oven, Laminar Air Flow, Digital Balance, Microscope, Incubator, pH Metter.

METHODS:- To identify the unknown bacteria and fungi from mixed culture by morphological and biochemical methods. It is a systematic and careful process by which we could be identifies bacteria and fungi.

a) Soil Sample Preparation:-Collected some soil samples from our study area and samples were placed in inoculating chamber for air dried. Then the samples were crashed within polythene bag to make fine powder. Weighed 1 gm from each sample. Dissolved in a test tube containing 10 ml of normal saline water. The solution is called stock solution and used to serial dilution.

b) Serial dilution technique

Principle:-The quality of microbes depends on soil nature. The method is based upon the principle that the material containing microorganisms are cultured. Each viable microorganism will developed into a colony. Hence, the microorganisms from soil sample by serial dilution is made easy

Procedure:-Prepared 10 dilution series from 10^{-1} to 10^{-10} and 1ml of stock solution transfer to first tube containing 10ml normal saline water labeled as 10^{-1} and then 1ml of solution transfer 10^{-1} to 10^{-2} and up to last tube as the same process and from these selected 10^{-5}, 10^{-6}, 10^{-7}, and10^{-8} and then 0.5ml of solution taken from selected tube and placed on nutrient media.

c) Identification of microorganisms

Gram Staining

Principle of Gram Staining:-Gram stain divides bacterial cells into two major groups, gram-positive and gram-negative, which makes it an essential tool for classification and differentiation of microorganisms.

REQUIREMENTS

Primary stain: Crystal Violet (Hucker's). **b)** Gram's Iodine**. c) Decolorizing Agent:** Ethyl Alcohol 95% **d) Counter sain:** Safranin.

Procedure:-Using sterile technique, prepare a smear on the clean slide. Transferring organisms to the slide. Mix and spread both organisms. Allow smears to air-dry and then heat. Gently flood smears with crystal violet and let stand 1minute. Gently wash with tap water. Gently flood smears with the Gram's iodine mordant and let stand 1minute. Gently wash with tap water. Decolorize with 95% ethyl alcohol. Gently wash with tap water. Counterstain with safranin for 45 seconds. Gently wash with tap water. Blot dry with bibulous paper and examine under oil immersion.

d) BIOCHEMICAL TEST

1) IMViC test.

Principle: IMViC is an acronym that stands for four different tests such as Indole test, Methyl red test, Voges-Proskauer test, Citrate utilization test. IMViC tests are employed in the identification of members of family enterobacteriaceae. Cultures of any members of Enterobacteriace have to grow for 24 to 48 hours at 37°C and the respective tests can be performed.

Indole test: Indole test is performed on sulfide-indole-motility (SIM) medium or in Tryptophan broth. Result is read after adding Kovac's reagent.

Methyl Red (MR)Test:-Positive methyl red test are indicated by the development of red color after the addition of methyl red reagent. A negative methyl red test is indicated by no color change.

Voges-Proskauer (VP) test:-Negative test is indicated by lack of color change after the addition of Barritt's A and Barritt's B reagents. A positive Voges-Proskauer test is indicated by the development of red-brown color after the addition of Barritt's A and Barritt's B reagents.

Citrate utilization test is performed on Simmons citrate agar:

Negative citrate utilization test is indicated by the lack of growth and color change in the tube. A positive citrate result as indicated by growth and a blue color change.

2) Carbohydrate fermentation test

Principle:-Some organisms are capable of fermenting sugars such as glucose anaerobically, while other use the aerobic pathway.

Procedure:-Aseptically inoculate each labeled carbohydrate broth with bacterial culture. (keep uninoculated tubes as control tubes). Incubate the tubes at 18-24 hours at 37^{o}C. Observe the reaction.

3) H_2S production test

Principle:-The SIM medium contains peptone and sodium thiosulfate as the sulfur substrates; ferrous sulfate (F_eSO_4), which behaves as the H_2S indicator, and sufficient agar to make the medium semisolid and thus enhance anaerobic respiration. Regardless of which pathway is used, the hydrogen sulfide gas is colorless and therefore not visible. Ferrous ammonium sulfate in the medium serves as an indicator by combining with the gas, forming an insoluble black ferrous sulfide precipitate that is seen along the line of the stab inoculation and is indicative of H_2S production. Absences of the precipitate are evidence of negative reaction.

Procedure:-Take two sterile SIM agar tubes, one named Test and the other Control. Remove the cap of the SIM agar tube named 'Test' and flame the neck of the tube. Inoculate the SIM agar with the inoculation loop containing the inoculums from the culture plate. Again flame the neck of the SIM agar tube and place it in the test tube rack. Inoculate only the broth in the tube named 'Test' using aseptic technique. Leave the broth in the tube named 'Control' uninoculated. Incubate both the tubes (Test and Control) for 24 to 48 hours at 37°C. Remove the SIM agar tubes from the incubator and observe.

4) Litmus milk test

Principle:-The major milk substrates capable of transformation are the milk sugar lactose and the milk proteins casein, lacto-albumin, and lacto-globulin. To distinguish among the metabolic changes produced in milk, a P^H indicator, the oxidation-reduction indicator litmus, is incorporated into the medium.

Procedure:- Using sterile technique, inoculate experimental organisms into its appropriately labeled medium by means of loop inoculation. Incubate all the tubes (Test and Control) for 24 to 48 hours at 37°C.

5) Urease test

Principle:-Urea is a nitrogen containing compound that is produced during decarboxylation of the amino acid arginine in the urea cycle. Some bacteria have the ability to produce an enzyme urease as part of its metabolism to break down urea to ammonia and carbon dioxide.

Procedure:-Take two sterile tube containing urea broths (UB), one named Test and the other Control. Remove the cap of the tube named 'Test' and flame the neck of the tube. Inoculate the tube (UB) with the inoculation loop containing the inoculums from the culture plate. Again flame the neck of the tube (UB) and place it in the test tube rack. Inoculate only the broth in the tube named 'Test' using aseptic technique. Leave the citrate agar in the tube named 'Control' uninoculated. Incubate both the tubes (Test and Control) for 24 to 48 hours at 37°C.

6) Starch hydrolysis test

Principle:-Starch agar is used to demonstrate the hydrolytic activities of these exoenzymes. The medium is composed of nutrient agar supplemented with starch, which serves as the polysaccharide substrate. If the starch has been hydrolyzed, a clear zone of hydrolysis will surround the growth of the bacteria. This is a positive result.

Procedure:-Take two sterile starch agar plates, one named Test and the other Control. Slightly open upper part of starch agar plate named 'Test' and flame the neck of the plate. Inoculate the starch agar late with the inoculation loop containing the inoculums from the bacterial culture plate. Incubate for 48 hours. Flood both plates with iodine. Blue color indicates no hydrolysis, while a clear zone indicates hydrolysis.

7) Catalase test

Principle:-Catalase production can be determined by adding the substrate H_2O_2 to an appropriately incubated nutrient agar slant culture. If catalase is present, the chemical reaction mentioned is indicated by bubbles of free oxygen gas. This is a positive catalase test; the absence of bubble formation is a negative catalase.

Procedure:-Dip a capillary tube into 3% H_2O_2. Touch a colony. Observe the tube for bubble indicating a positive reaction.

8) Gelatin hydrolysis test

Principle:-Gelatin is a protein produced by hydrolysis of collagen. Liquefaction is accomplished by some microorganisms capable of producing a gelatinase.

Procedure:-Using sterile technique, inoculate experimental organisms into its appropriately labeled medium by means of loop inoculation. Incubate all the tubes (Test and Control) for 24 to 48 hours at 37°C.

RESULT AND DISCUSSION

RESULT

Isolation and identification of bacteria and fungi from different soil samples of Bilaspur (C.G). The present investigation was identified on the basis of their morphological (with the help of Gram staining) and biochemical characteristics'

ISOLATION OF SOME BACTERIA AND FUNGI FROM SOIL SAMPLES

After the four month study from January to May, eleven strains of bacteria and six stains of fungi were observed and isolated from different soil samples of Bilaspur (C.G). The isolated bacteria are *E.coli, Enterobacter aerogenes, Klebsiella, Proteus vulgaris, Bacillus cereus, Bacillus subtilis, Clostridium, Staphylococcus aureus, Pseudomonas aeruginosa, Shigella,* and *Salmonella* and fungi are *Aspergillus, Rhizopus, Mucor, Candida,* Penicillium, *fusarium.*

IDENTIFICATION OF BACTERIA AND FUNGAL STAIN FROM SOIL SAMPLES

The bacteria are isolated on the basis of cultural, morphological, gram staining and biochemical characteristics and fungi are isolated on the basis of cultural, morphological characteristics (Stain and compound microscope).

Table no. 1. Bacteria and fungi were isolated from varies sites of Bilaspur (C.G).

Sampling around in each month	Sampling site
January(1,2)	Ashoknagar (garden soil)
February (1,2)	Maharana Pratap Chowk (road side soil)
March(1,2)	Rajkishor Nagar (agricultural area's soil)
April(1,2)	Near C.M.D Chowk (industrial soil)

Table no. 2. Cultural characteristics of isolated bacteria from soil samples

S.No	Form	Color	Margin	Elevation	Odor
B1	Circular	White	Entire	Raised	Faecal
B2	Circular	White	Entire	Convex	N.S.O
B3	Irregular	White,	Entire	Raised	N.S.O

		somewhat translucent			
B4	Circular	Grayish	Entire	Convex	N.S.O
B5	Circular	Blue-gray	Entire	Convex	N.S.O
B6	Circular	White	Entire	Convex	Fruity
B7	Circular	Golden	Entire	Convex	N.S.O
B8	Irregular	White waxy	undulate	Flat	N.S.O
B9	Irregular	White	Undulate	Flat	N.S.O
B10	Irregular	Pink	Entire	Umbonate	N.S.O
B11	Circular	Grayish	Entire	Raised	N.S.O

Note: N.S.O= No special odor.

Table no. 3. Morphological characteristics of isolated bacteria.

S.No	Size(µm)	Gram Staining	Shape	Arrangement
B1	2	-	Rod	Large clump
B2	1-2	-	Rod	Single
B3	1-2	-	Rod	Single
B4	1-3	-	Rod	Single
B5	1-5	-	Rod	Single
B6	2-8	-	Rod	Single
B7	1-5	+	Cocci	Clump & few Single
B8	3-10	+	Rod	Single
B9	3-10	+	Rod	Single
B10	3-10	+	Rod/spindle	Single
B11	2-8	-	Rod	

Note: + indicate gram positive cell & - indicate gram negative cell.

Table no. 4. Biochemical characteristics of isolated bacteria.

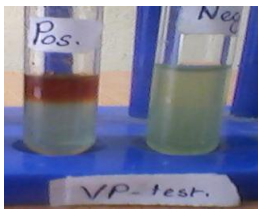
S.No	Litmus Milk Reaction	Lactose fermentation	Indole production	MR Reaction	VP Reaction	Citrate Use	Urease Activity	Catalase Activity	Gelatin Liquefaction	Starch Hydrolysis	H_2S production
B1	A	AG	+	+	-	-	-	+	-	-	-
B2	A	AG	-	-	+	+	-	+	-	-	-
B3	AG	AG	-	-	-	+	+	+	-	-	-
B4	Alkine	-	+	+	-	-	-	+	-	-	-
B5	Alkine	-	+	+	-	-	+	+	+	-	+
B6	Rapid peptonization	-	-	-	-	+	-	+	+ Rapid	-	-
B7	A	A	-	+	+	-	-	+	+	-	-
B8	Peptonization	-	-	-	+	-	-	+	+ Rapid	+	-
B9	Peptonization	-	-	-	+	-	+	+	+ Rapid	+	+

B10	Alkine	-		+	-	-	-	-	-	-	-
B11	Alkine	-	-	+	-	+	-	+	-	-	-

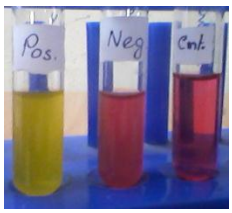
Note: + indicate positive test & - indicate negative test, A indicate acid production & G indicate gas production.

Indole test.

MR-test



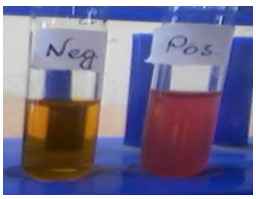
P-test.



Lactose fermentation.

H_2S test.

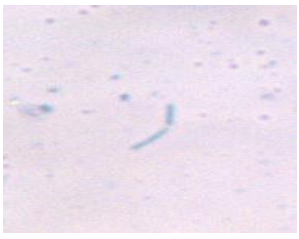
Catalase test



Urease test

Table no. 5. On the basis of all above characteristics, isolated bacteria were identified as shown in the table.

S.No	Identified bacterium
B1	*Escherichia coli*
B2	*Enterobacter aerogenes*
B3	*Klebsiella sp*
B4	*Shigella sp*
B5	*Proteus valgaris*
B6	*Pseudomonas aeruginosa*
B7	*Staphylococcus aureus*
B8	*Bacillus cereus*
B9	*Bacillus subtilis*
B10	*Clostridium sp*
B11	*Salmonella sp*



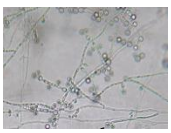
Diplobacillus

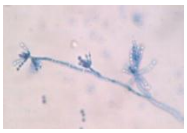
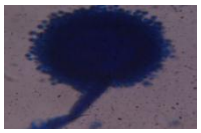
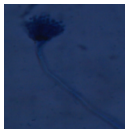
Table no. 6. Cultural and microscopic characteristics of isolated fungi from soil samples

S.No	Colonial Morphology	Microscopic Appearance
F1	Rapidly growing white colored fungus swarms over entire plate; aerial mycelium cottony.	Spores are oval, nonsepate mycelium gives rise to single sporangiophores with globular sporangium containing a columella; there are no rhizoids.
F2	Rapidly growing white colored fungus swarms over entire plate; aerial mycelium cottony.	Spores are oval, colorless, or brown; nonsepate mycelium gives rise to straight sporaniophores that terminate with black sporangium containing a columella; rootlike hyphae (rhizoids) penetrate the medium.
F3	White colonies become greenish-blue, black, or brown as mature cultures.	Single-celled spores (conidia) in chains developing at the end of the sterigma arising from the terminal bulb of the conidiophores arise from a septate mycelium.
F4	Woolly, white, fuzzy colonies changing color to pink, purple or yellow.	Multicelled spores (conidia) are oval or crescent-shaped and attached to conidiophores arising from a septate mycelium.
F5	Mature cultures usually greenish or blue-green.	Single-celled spores (conidia) in chains develop at the end of the sterigma arising from the medula of the conidiophore; branching conidiophore arise from a septate mycelium.
F6	Colonies are small, round, moist, and colorless, with unbroken, even edges.	Yeastlike fungus produces pseudomycelium.

Table no. 7. On the basis of above characteristics, isolated fungi were identified as shown in the table.

F1	*Mucor*
F2	*Rhizopus*
F3	*Aspergillus*
F4	*Fusarium*
F5	*Penicillium*
F6	*Candia*


Fusarium. *Candida* *Mucor.* *Penicillium.* *Aspergillus.* *Aspergillus* *Rhizopus*

Table:8. Monthly average temperature of Bilaspur (C.G).- January13 °C, February15°C, March 20.5, April-27°C.

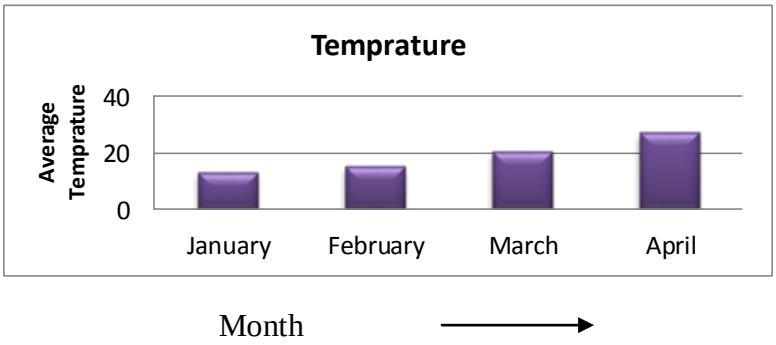


Fig.1. Monthly average temperature of Bilaspur (C.G).

Number of isolated bacteria and fungi from soil samples.- Bacteria- 11, Fungi-6 (fig 2)

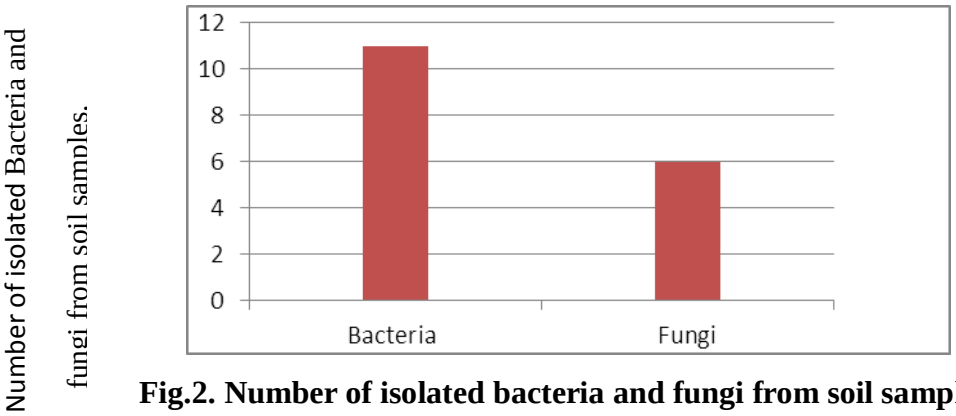


Fig.2. Number of isolated bacteria and fungi from soil samples.

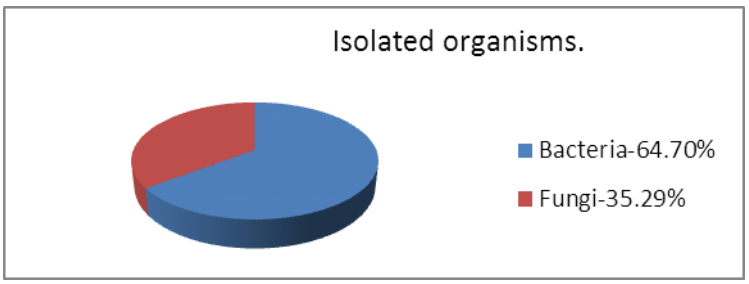


Fig.3. Isolated Bacteria, Fungi.

Table:-9. Average Bacterial colonies on Nutrient Agar Medium.

Dilution factor	10^{-5}	10^{-6}	10^{-7}	10^{-8}
No. of Bacterial colony	23	7	1	0

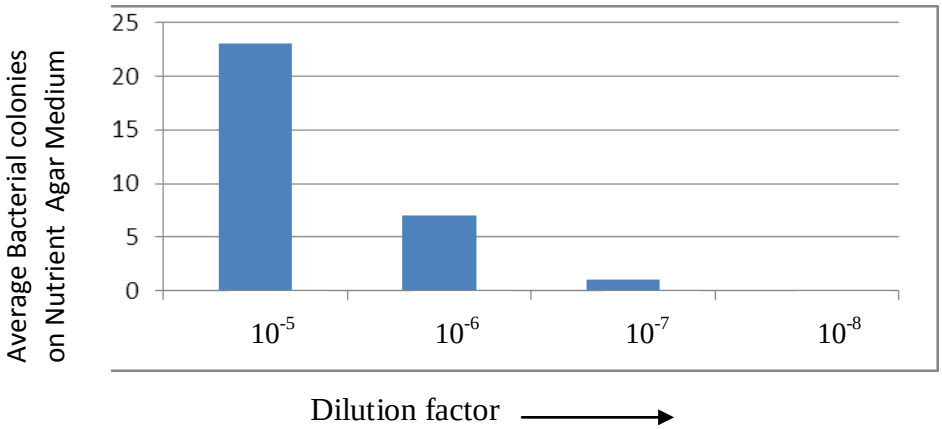


Fig.4. Average Bacterial colonies on Nutrient Agar Medium.

Table.10. Average Fungal colonies on Sabouraud Dexrose Agar Medium.

10^{-5}	10^{-6}	10^{-7}	10^{-8}
19	4	1	0

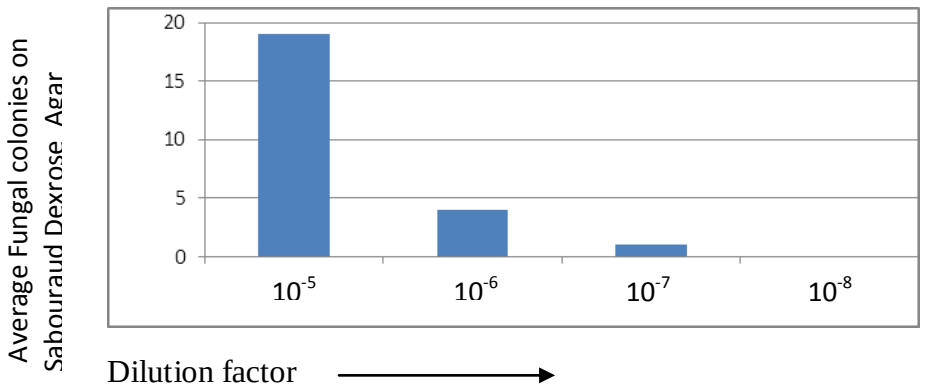


Fig.5. Average Fungal colonies on Sabouraud Dexrose Agar Medium.

Isolated Gram +ve - 4 and Gram-ve – 7 bacteria.(Fig.6)

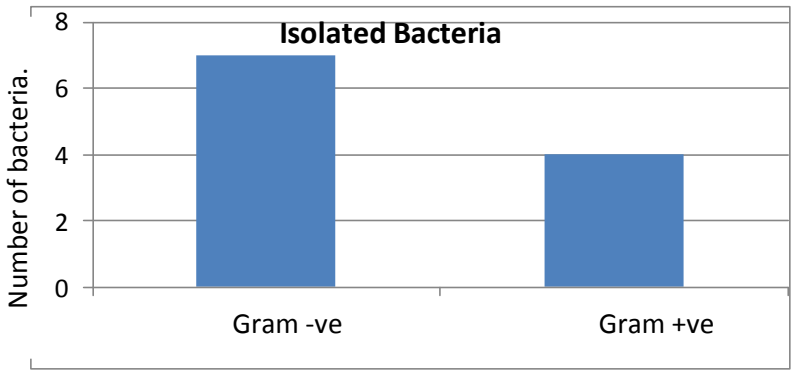


Fig.6. Total gram +ve and gram –ve Bacteria isolated from soil samples

DISCUTION

The isolated bacteria are *E.coli, Enterobacter aerogenes, Klebsiella, Proteus vulgaris, Bacillus cereus, Bacillus subtilis, Clostridium, Staphylococcus aureus, Pseudomonas aeruginosa, Shigella,* and *Salmonella* and fungi are *Aspergillus, Rhizopus, Mucor, Candida, fusarium,* Penicillium.

Eleven bacteria and six fungi were found from four selected area of Bilaspur(C.G). Out of them, some bacteria were commonly found in each sample such as *E. coli, Staphylococcus aureus, Clostridium,* some fungi were commonly found in all samples such as *Aspergillus, Rhizopus, Mucor.*

Pseudomonas aeruginosa was isolated from garden soil as well as *Enterobacter aerogenes, Klebsiella, Proteus vulgaris,* were found in garden soil. *Staphylococcus aureus, Pseudomonas aeruginosa* were found in road side (traffic area) and *Bacillus subtilis, Salmonella, Shigella, Staphylococcus aureus* were found in industrial area.

Fusarium were found in garden soil. Candida was found in road side soil sample. *Aspergillus, Penicillium* were observed in industrial area (sugar industry) because of easily and abundantly available carbon source by product of sugar industries (Ali-2004). Bacterial isolates recovered from all fourth side showed slightly difference in MICs for Cu, Cd, Pb, Zn, Hg, Ni. Similar observation was reported by the two group of researcher in different time. (Kunito *et al.*-1986; Choudhary and kumar-1996).

In industrial area, road side soil (traffic area) soil samples, the minimum required nutritional condition are not observed due to heavy metal pollution, such as lead, copper, nickel, nilufer, cevic, ayten, kareca. Effect of cadmium, zinc, copper, and fluoranthene on soil bacteria (Turkey). Even through oxigen is not prerequisite for the growth of *Pseudomonas aeruginosa,* at least NO_3 must be available as respiratory electron acceptor which is minimum in those soil samples.

Some microbial strains posses' genetic determinants that confer the resistance. In bacteria, those determinants are often found on plasmid, which have facilitated their study at the molecular level (Cervanter et al-1094).

Monitoring of antibiotic resistant bacteria in soil sample can be used as an indicator of industrial and urban pollution. Several studies have found that metals influence

microorganisms by harmfully affecting their growth morphology and biochemical activities resulting in decreased biomass diversity (Baath- 1998; Robert-1992; Ahmed-2002).

CONCLUSION

Different type of soil collected from the different area of Bilaspur in C.G and isolated different types of microorganisms (Bacteria and Fungi) such as the bacteria are *E.coli, Enterobacter aerogenes, Klebsiella, Proteus vulgaris, Bacillus cereus, Bacillus subtilis, Clostridium, Staphylococcus aureus, Pseudomonas aeruginosa, Shigella,* and *Salmonella* and fungi are *Aspergillus, Rhizopus, Mucor, Candida, fusarium,* Penicillium.

Some microorganisms such as *Actinomycetes* and economically important fungi to demonstrate the potential of these organisms for bio-control of pathogenic bacteria and other pathogenic organisms which may be helpful in medical microbiology and agricultural field in future.

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