IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN: 2278-3008.Volume 5, Issue 6 (Mar. – Apr. 2013), PP 80-89
www.iosrjournals.org

"Isolation and Biochemical Characterization of Antibiotic Producing Microorganism from Waste Soil Samples of Certain Industrial areas of India"

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Abstract: *The search for new antibiotics continues in a rather overlooked hunting ground. In the course of screening for new antibiotic-producing microorganisms, isolates showing antimicrobial activity were isolated from waste soil samples from various habitats in the Industrial Areas in Dheradun, Uttarakhand, India. Existing methods of screening for antibiotic producers together with some novel procedures were reviewed. Both modified agar-streak and agar-plug methods were used in the primary screens. The use of selective isolation media, with or without antibiotic incorporation and/or heat pretreatment, enhanced the development of certain actinomycete colonies on the isolation plates. Antibiotics have long been considered the "magic bullet" that would end infectious disease. Although they have improved the health of countless numbers of humans and animals, many antibiotics have also been losing their effectiveness since the beginning of the antibiotic era. Bacteria have adapted defenses against these antibiotics and continue to develop new resistances, even as we develop new antibiotics. In recent years, much attention has been given to the increase in antibiotic resistance. As more microbial species and strains become resistant, many diseases have become difficult to treat, a phenomenon frequently ascribed to both indiscriminate and inappropriate use of antibiotics in human medicine. However, the use of antibiotics and antimicrobials in raising food animals has also contributed significantly to the pool of antibiotic resistant organisms globally and antibiotic resistant bacteria are now found in large numbers in virtually every ecosystem on earth. Dual culture bioassays were used to screen seven selected Bacillus isolates for activity against four plant pathogenic fungi in vitro. All isolates were able to inhibit the pathogens to varying degrees. Two isolates, R29 and B81, were selected for further testing and characterization. Further bioassays were performed on five complex nutrient media which were adjusted to pH S.S and 7, and both incubated at 2SoC and 30°C" respectively. It was found that pH and media composition showed significant influences on the antifungal activities of the isolates tested, but that a SoC temperature difference in incubation temperature did not. Tryptone soy agar was found to give rise to the largest inhibition zones. Both isolates were tentatively identified using standard biochemical and morphological tests. Based on its phenotypic characteristics, R29 was identified as a strain of B. subtilis. B81 proved to be more difficult to assign to a specific group or species of Bacillus, though B. subtilis and B. licheniformis were considered to be the nearest candidates. Genomic DNA was extracted from both isolates and a portion of each of their 16s rDNA genes were amplified and sequenced for homology testing against the GeneBank database. Homology testing confirmed that both isolates were members of the genus Bacillus and most probably strains of B. subtilis. The DNA fragment used for sequencing proved to be too small to give conclusive identification of the isolates.*

Key Words: *Collection of soil samples, Isolation techniques for bacterial and fungal spp by different microbiological methods, Dilutions, Biochemical characterization.*

I. Introduction

In the beginning of 20th century, the idea of growth inhibition of one microorganism present in the vicinity of other one came into existence. Later, it was demonstrated that growth inhibition of the former micro-organism was mediated by secretion of toxic metabolites by the later. This toxic metabolite was termed as 'antibiotic' and the phenomenon of act of growth inhibition by antibiotics as 'antibiosis. The antibiotics are defined as "the complex chemical substances, the secondary metabolites which are produced by microorganisms and act against other microorganisms". In nature, there is universal distribution of antibiosis among the microorganisms owing to which they are involved in antagonism. Those microorganisms which have capacity to produce more antibiotics can survive for longer time than the others producing antibiotics in less amount. However, antibiotics produced by microorganisms have been very useful for the cure of certain human diseases caused by bacteria, fungi and protozoa. Due to continuous endeavor made in this field, the antibiotics discovered at present are about 5,500. Total world production of antibiotics is more than one million tons per annum. This success has been possible only due to continuous researches made during the last 4 decades.

The diversity of soil microorganisms were of great significance as a factor promoting the early discovery of antibiotics [1]. Many types of microorganisms such as moulds, bacteria, protozoa and algae, all competing for limited nutrients in the soil, have to devise strategies to survive. Among these microbes are autotrophs, free living nitrogen fixers, thermopiles, acidophiles, pathogens and saprophytes [1]. Natural product antimicrobials have been used for centuries by native peoples all around the world, but it wasn't until the late 1800s that people began to search for single compounds that could be used to kill disease causing bacteria. Soil and plants had been used to prevent wounds from becoming infected or to treat rashes as far back as the Roman and Byzantine empires. All cultural groups have medicinal folklore that has some level of effectiveness for any number of ailments. Many modern common therapeutic agents have their origins in natural sources, such as: Paclitaxel, also known as Taxol, which was derived from the yew tree Morphine, is derived from the opium poppy; Atropine comes from the belladonna leaf; and Aspirin is from white willow bark [1].

The actinomycetes yielded about 70 % of these, and the remaining 30 % are products of filamentous fungi and non-actinomycete bacteria [2-5]. Most of the bioactive compounds from actinomycete sort into several major structural classes such as amino glycosides (e.g., streptomycin and kanamycin), ansamycins (e.g., rifampin), anthracyclines (e.g., doxorubicin), b-lactam (cephalosporins), macrolides (e.g., erythromycin), and tetracycline. The objectives of this experiment were to survey for antibiotic-producing bacteria found in soil and isolate and characterize antibiotics produced by soil-borne bacteria. Putative antibiotic-producing bacteria were isolated from soil and antibiotic production was confirmed. It was hypothesized that antibiotic-producing bacteria would be of the genera *Bacillus* or *Streptomyces* and that the antibiotics would be peptides that inhibited the growth of Gram-positive bacteria [6-9]. The first truly effective class of antimicrobial drugs was the sulfonamides, discovered by Gerhard Domagk. In 1932, two scientists at the Bayer Company, Mietzsch and Klarer, synthesized Prontosil red, a red dye bound to a sulfonamide group. Domagk showed, in 1935, that infections in mice caused by hemolytic streptococci were cured by Prontosil red [10, 11]. Unfortunately for Bayer, Prontosil red was shown to have no antibacterial activity *in vitro*. This lack of activity was explained by several researchers [12-18] showing that Prontosil red is split *in vivo* into its component dye and sulfanilamide, the active antibacterial agent and a previously described molecule that was already in the public domain. From that point, sulfanilamide was manufactured by a number of companies and work was begun to modify the molecule to enhance performance, leading to decreased side effects and a broader spectrum of action.

II. Materials And Methods

2.1: Collections of soil samples from different places:

The soil samples were collected from in an around Dheradun, Industrial Areas Uttarakhand, India. The soil samples were dried separately at 37^{o}C for 1 hour in hot air oven. Then they were cooled at room temperature. 1 gm of each soil sample was added to a conical flask containing 100 ml of sterile water and few drops of Tween-80 solution. All flasks were shaken for 30 minutes in orbital shaker incubator at 27^{0}C. These flasks were considered as stock cultures.

2.2: Isolation of Microbes:

The sample is mixed with 1L pure water to make solution sample. This solution sample is diluted up to 10-5 serial dilution. Serial dilution is a process of diluting a sample several times. The sterile Petri dish is label. Than 90ml of phosphate buffer is transferred into each of 5 tubes using sterile pipettes (10ml) with aseptic technique. By using sterile pipette (1ml), 1ml of water sample is transferred into 10-1 tube. Mix the test tube properly. Continue dilution with aseptic technique for test 10-2 until 10-5. After that each of serial dilution is transferred into nutrient agar plate by using spread plate method. 0.1ml of an appropriately diluted culture is spread over the surface of agar using sterile glass spreader. The plate is then incubated until the colonies appear. It is important that the surface of the plate be fairly dry so that the spread liquid soaks in.

2.3: Preliminary screening of crude antibiotic produced

Agar Streak Method: The microbial sensitivity of the soil isolates were analyzed by Agar streak method'. Each of the isolate was streaked as a straight line on SBCD medium and incubated at 27^{0}C for about 6 days After 6 days, different strains of microorganisms were streaked at right angle, but not touching to the streak and incubated at 37^{0}Cfor 24 hours in case of bacteria and 27^{0}Cfor 48 hours in case of fungi. If, the organism is sensitive against the antibiotic produced by actinomycetes, then it will not grow near the actinomyces [Table 1]..

Test of Microorganisms: The test bacteria that were used in this study include references, environmental as well as clinical isolates. The typical reference strains were as follows:

Gram Positive Bacteria: *Staphylococcus aureus* , *Streptococcus faecalis, Streptococcus pyogens, Bacillus cereus, Acinetobacter calcaoceuticus.*

Gram Negative Bacteria: *Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Klebsiella pneumonia, Bacillus subtilis.*

Table 1:
Morphological and Cultural Characterization:

Results (slant/butt)	Symbol	Interpretation
Red/yellow	Alkaline reaction/ Acid production	Glucose fermentation only; Peptone catabolized
Yellow/yellow	Acid production / Acid production	Glucose and lactose and/or sucrose fermentation
Red/red	Alkaline reaction / Alkaline reaction	No fermentation; Peptone catabolized
Red/no color change	Alkaline reaction / No Change	No fermentation; Peptone used aerobically
Yellow/yellow with bubbles	Acid production / Acid production, Gas production	Glucose and lactose and/or sucrose fermentation; Gas produced
Red/yellow with bubbles	Alkaline reaction / Acid production, Gas production	Glucose fermentation only; Gas produced
Red/yellow with bubbles and black precipitate	Alkaline reaction / Acid production, Gas production, H2S	Glucose fermentation only; Gas produced; H2S produced
Red/yellow with black precipitate	Alkaline reaction / Alkaline reaction, H2S	Glucose fermentation only; H2S produced
Yellow/yellow with black precipitate	Acid production / Acid production, H2S	Glucose and lactose and/or sucrose fermentation; H2S produced
No change/no change	No Change	No fermentation

Morphological Characterization:

Cultural Characterization: Morphological and cultural characters of the selected actinomycetes strains were studied by inoculating the selected strain into sterile International Streptomycetes Project (ISP) media like the media were sterilized and poured into sterile petridishes. After solidification of the media, culture of the selected strain was streaked on the media surface by simple method aseptically and incubated at 27^0C for 7 days. Morphological characters such as colony characteristics, type of aerial hyphae, growth of vegetative hyphae, fragmentation pattern, spore formation and nature of fermentation were observed [Table 1].

***** Tryptone – Yeast extract broth
***** Oatmeal agar
***** Inorganic salts – Starch Agar
***** Glycerol – Aspargine Agar
***** Peptone – Yeast extract agar
***** Tyrosine Agar
***** Carbon utilization agar

The media were sterilized and poured into sterile petridishes. After solidification of the media, culture of the selected strain was streaked on the media surface by simple method aseptically and incubated at 27^0C for 7 days. Morphological characters such as colony characteristics, type of aerial hyphae, growth of vegetative hyphae, fragmentation pattern and spore formation were observed

III. Results And Discussion:

Isolation Results: The isolation process is a procedure of isolation the mixture of colonies to a single colony. This process was done by using streaking method to obtain pure cultures. The soil's sample was added with 1 Liter pure water to obtain solution sample before transferred into nutrient agar plate. It is important that the numbers of colonies developing on the plates are not being too large. On crowded plates some cells may not form colonies, and some colonies may fuse, leading to erroneous measurements. So, to obtain the appropriate colony number, the samples need to be diluted. This solution samples were diluted up to 10-5. By using spread plate method, the diluted samples were transferred into nutrient agar plate and the bacteria were grown on it. From the observation, these samples take about three until four days to growth on the plate. Table 2 (A & B) and Figure 1 show the growth of the bacteria on plate after 5 days.

Collection of soil samples and number of microbial strains & Actinomycetes in isolation plates: The projection of aerial mass is well explained in Table 2 (A & B) and illustrated in Figure 1.

Table 2: (A): Number of Microbial Strains

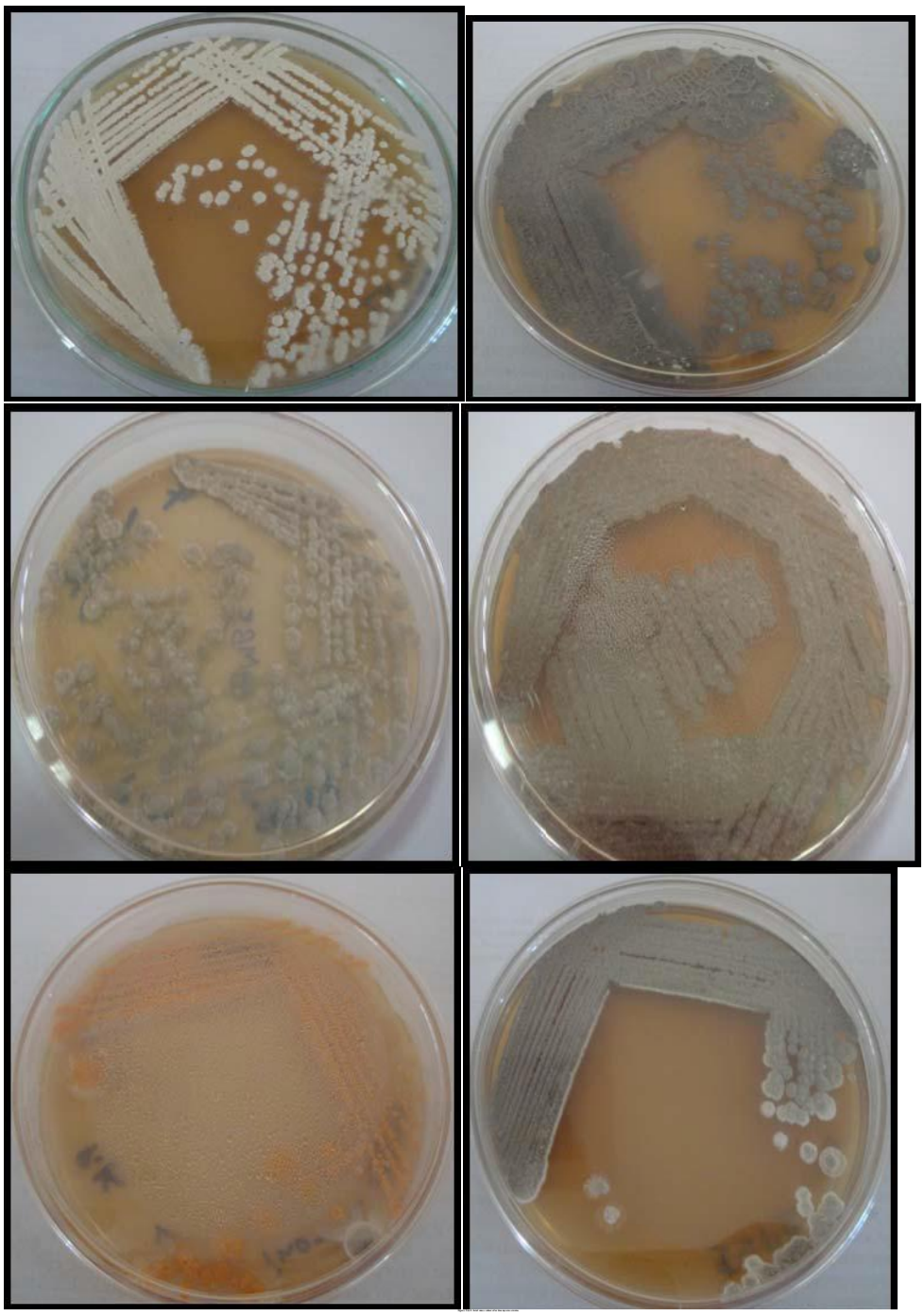
Sr. No.	Dilution of soil samples (10^{-6})	Heat treatment (45^0C for 1 hour)	No. of Actinomycetes and bacterial strains (colonies) on isolation media				Nature of soil sample (Surface & sub surface)	pH of soil
			SBCD	AIA	SCA	TSA		
1	10^{-5}	NA	--	--	--	--	Dry soil sample	7.4
2	10^{-5}	NA	--	--	--	--	Water logged	7.5
3	10^{-4}	AP	4	5	--	10	mud	7.5
4	10^{-4}	AP	3	6	--	14	Water logged	6.5
5	10^{-3}	AP	--	5	--	16	mud	7.0
6	10^{-4}	AP	--	--	--	20	Loam	6.8
7	10^{-5}	AP	--	13	15	25	Sandy	7.8
8	10^{-6}	AP	15	48	36	25	Mud	5.6
9	10^{-6}	AP	35	50	32	30	Black dry soil sample	5.7
							Black wet soil sample	
							Red dry soil sample	

NA: Not applied, AP: Applied SBCD – Soyabean casein digest medium, TSA: Tryiptase soya agar, AIA – Actinomycetes isolation agar, SCA – Starch casein agar.

Table 2: (B): Aerial Mass Distinction of Actinomyces

Sl.no	Strains	Aerial mass of colour
1	B2	W (White colour)
2	BS	Gy (Gray colour)
3	CO	Gy
4	CS	Gy
5	C1	WGy (White gray colour)
6	C 8	Gy
7	C9	(W) Gy
8	C11	W
9	C12	GyW
10	D1	Y
11	MB1	W
12	MB2	Gy
13	MB3	WGy
14	MB5	W(Gy)
15	MB9	W

Figure 1: Systematic Picture of Growth of Bacteria on Agar Plate with Specified Culture Time of 5 Days.



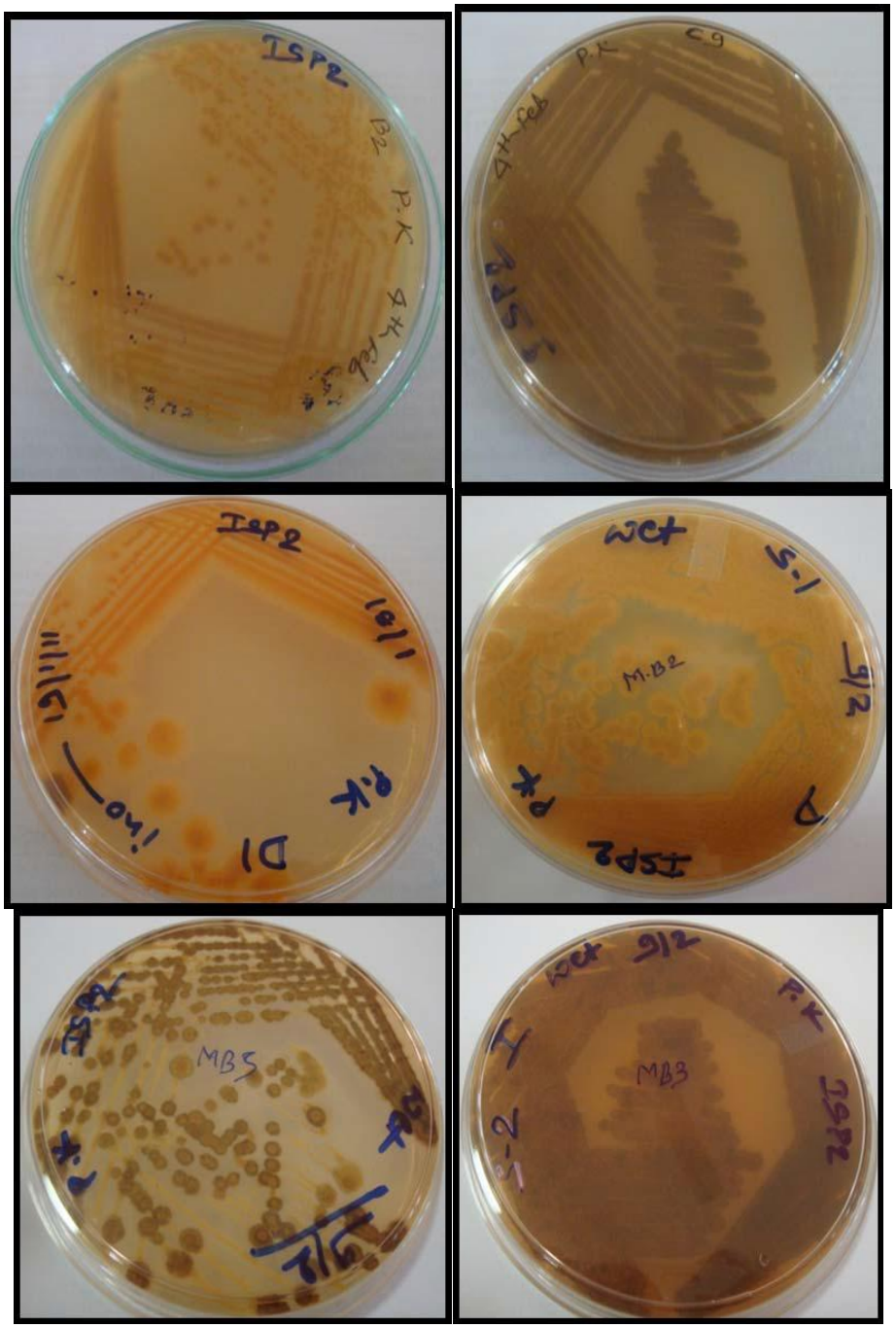


Figure 2: Sensitivity of Different Microorganisms towards the Soil Isolates by Agar Streak Method.

Table 3: (A): Growth Inhibition Status Various Microorganisms by Soil Isolates

Soil isolates	*B.subtilis*	*S.aureus*	*E.coli*	K.*pneumoniae*	*P.aeruginosa*	*A.niger*	*A.terreus*	*C.albicans*
A1	++	+	+	_	++	_	_	+
A2	+	++	+	_	++	_	_	+
A3	_	_	++	_	++	_	+	++
A4	++	++	+++	++	+	+	_	++
A5	+	_	+	+	+	_	_	_
A6	+	_	+	_	_	_	_	_
A7	++	_	+	+	+	+	_	+
A8	_	+	+	+	_	+	+	_
A9	_	+	_	+	_	_	_	_

+++ = Better inhibition, ++ = Good inhibition, + = Moderate inhibition, - = No inhibition

Table 3 (B): : Taxonomical Characterization of Soil Isolates

Soil isolates	Melanoid formation	Nitrate reduction	Proteolytic activity	Gelatin liquefication	Starch hydrolysis	Carbon assimilation	Acid production	H_2S production
A-1	Light brown pigmentation	+	Clear with acidic reaction	+	+	Glucose	+	_
A-2	Only growth	+	Clear with acidic reaction	+	+	Glucose	+	_
A-3	Only growth	+	Not clear with slightly alkaline reaction	+	+	Fructose	+	+
A-4	Light brown pigmentation	+	Not clear with acidic reaction	+	+	Lactose	+	+
A-5	Only growth	+	_	+	+	Lactose	+	_
A-6	Light brown pigmentation	+	_	+	+	Maltose	+	_

'+' Positive reaction, '-' Negative reaction

Table 4: Morphological and Cultural Characterization of the Microbial Strain.

Serial No.	Medium used for the growth of microorganisms	A-4 Microbial strains
1	Tryptone-yeast extract broth (TSP-1)	Growth occurs by the pellicle formation.
2	ISP-2	Creamish white colored colonies with clear zone around it were observed.
3	(Oatmeal agar) ISP-3	Slight black – creamish color thick colonies; no aerial mycelium formation was observed.

4	Inorganic salt-starch agar (ISP-4)	Blackish-brown colored thick colonies with waxy margin and convex surface was observed.
5	Glycerol asparagines agar base (ISP-5)	Whitish colored thin colonies striated surface; with less aerial mycelium and filamentous growth was observed.
6	Peptone yeast extract iron agar (ISP-6)	Thin transparent colonies with black colored soluble pigments were seen. No filamentous growth was seen.
7	Tyrosine agar base (ISP-7)	Cream colored, lobe shape, convex surface, little mycelium growth was observed.
8	Carbon utilization agar (ISP-9)	Thin yellowish golden colored colonies with little mycelium growth were observed.

Screening for antimicrobial activity:

This study was done by the Cross streak method against five pathogenic strains of bacteria namely *Pseudomonas*, *E.coli*, *Klebsiella*, *Bacillus and Proteus* results shown in Table (4 & 5) and Figure 3.

Table 5: Screening for Antimicrobial Activity

Sl.no.	Strain	***Pseudomonas***	***E.coli***	***Klebsiella***	***Bacillus***	***Proteus***
1.	B2	-	-	+		+
2.	BS	-	-	-	+	-
3.	CO	-	+	-	+	-
4.	CS	±	-	-	+	+
5.	C1	±	-	-	±	+
6.	C8	-	-	+	+	+
7.	C9	-	+	+	±	+
8.	C11	±	+	+	+	+
9.	C12	-	-	+	+	-
10.	D1	±	-	++	-	++
11.	MB1	-	-	-	-	+
12.	MB2	-	-	+	-	+
13.	MB3	-	-	+	-	+
14.	MB5	-	+	-	-	-
15.	MB9	-	+	+	+	+
++: Good ,		+: Positive	-: Negative		**±: Moderate**	

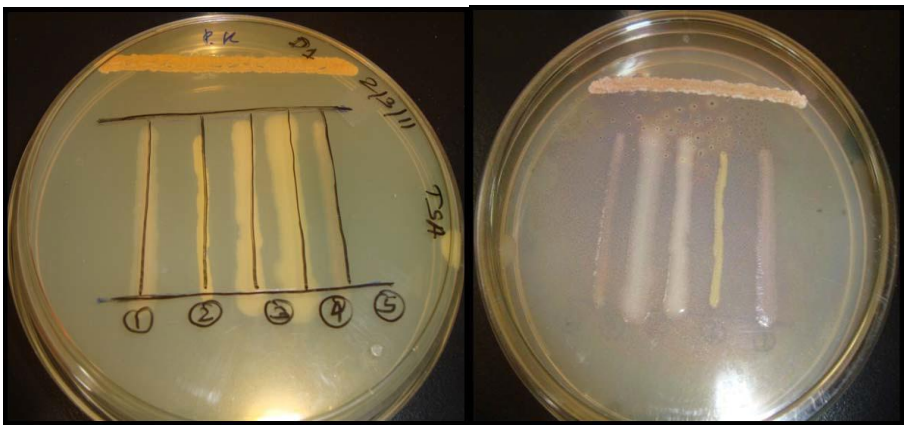


Figure 3: Screening of Antimicrobial Activity by Cross Streak Method Against Five Bacterial Pathogenic Strains.

Among all the strains only D1 has shown good antimicrobial activity against *Klebsiella* and *Proteus.* Growth of *Proteus* was inhibited by most of the strains. MB1 has shown activity against Proteus only. *Pseudomonas was* the least inhibited pathogenic strain CS, C11, C1 and D1 has shown moderate inhibition against it. E.coli has also shown very least resistance against any strains (Figure 4, A-I)..

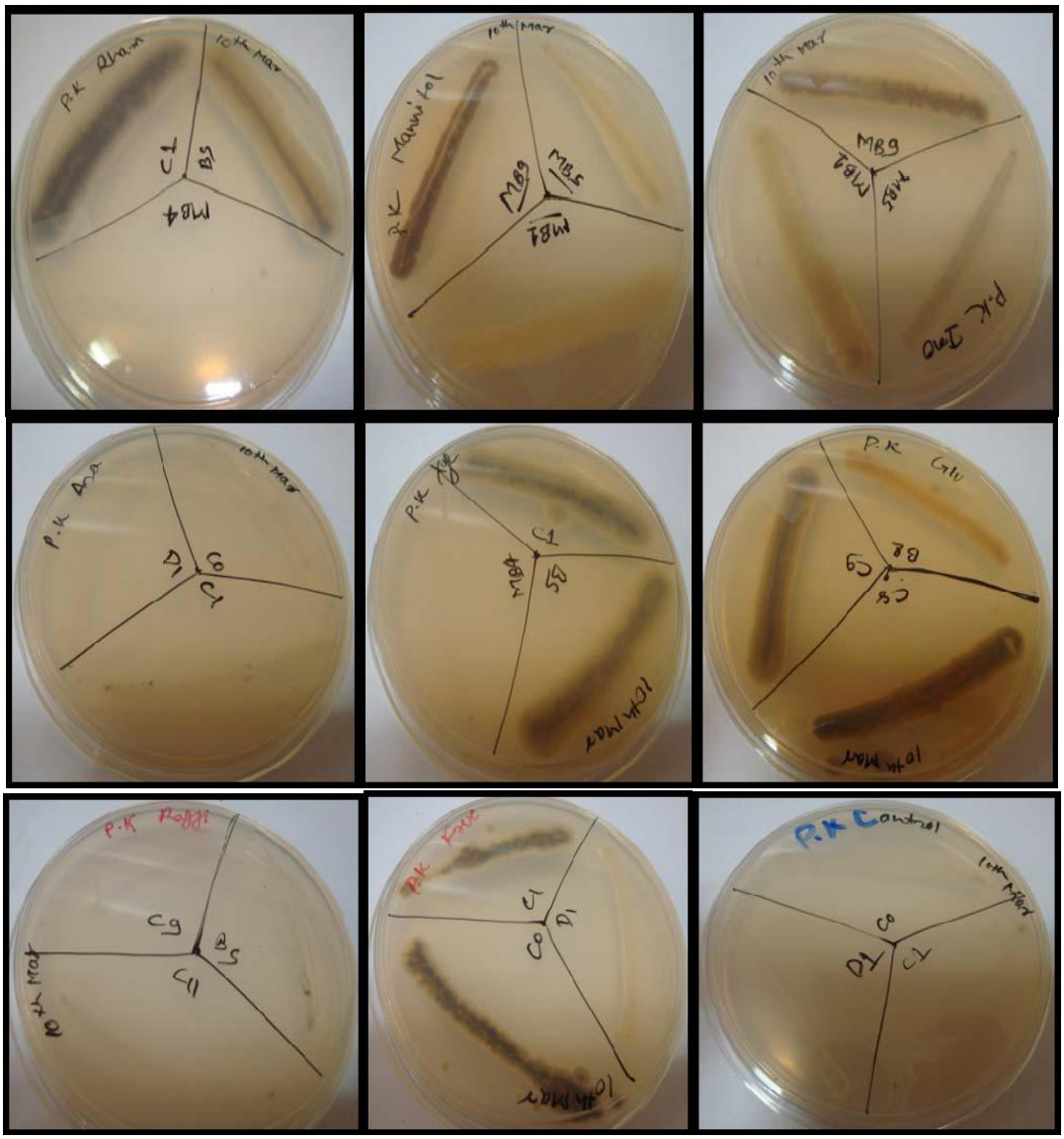


Figure 4 (A to I): Assimilation of Different Carbon Sources

IV. Concluding Remarks

Soil sediment samples collected from the Dehradun pharmaceutical companies were divided in to two parts as wet sample and dry sample. Serial dilution of wet and dry was done the no of isolates from the dry sample was more in number. Marine sediment samples are good for the isolation of actinomycetes; Goodfellow and Hynes [12] reviewed the literature on isolation of actinomycetes from waste soil sediments and suggested that the waste soil sediment may be valuable for the isolation of novel actinomycetes. 15 isolates of actinomycetes was isolated from the samples. The characterization was done by the phenotypic characterization and species affiliation by physiological and biochemical characteristics described by Das *et al.* [9].

The aerial mass colour of almost all strains were Whitish grey and only one strain D1 has shown yellow colour. Recent reports [17, 18] further entail that white colour series of actinomycetes they were the dominant forms. Colour series were also recorded in soil, morphological observation of colonial characteristics such as amount and colour of vegetative growth, and the presence and colour of aerial mycelium and spores, and again the presence of diffusible pigments are recorded for each strain studied colonial growth on agar plate [19-25]. Concluding substantial activities are concerning with cellulose, caseinase, amylase, lipase and gelitinase with certain specific variations.

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