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A COMPARATIVE STUDY OF ANTIBACTERIAL COMPOUNDS FROM SOIL BACTERIA

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ABSTRACT

All isolations were made from soils from three different locations i.e, cattle shed, euthrophic lake and garbage waste (non-biodegradable).Mother culture was prepared for all 3 by Spread Plate Method, after 1:10 Serial Dilution upto 10^{-5} dilutions .This was followed by quadrant streaking and Grams' staining. Nutrient broths were, inoculated with different colonies and checked for antibiotic activities against *Staphylococcus aureus, Pseudomonas aeruginosa* and *E.coli* .Further growth kinetics studies were done for them by taking their Optical Density at 600 nm at regular intervals (app 24 hrs) .Antibiotic production medias were prepared and inoculated with different samples and incubated for 96-120 hrs on shaker at room temperature. This media was centrifuged and the supernatant and pellet were tested for antibiotic activity. This was followed by test for MIC to get least concentration and Thin layer Chromatography separation of Protein sample.

Key words: Non-Biodegradable, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*.

INTRODUCTION

The term "antibiotic" was coined by Selman Waksman in 1942 .Broadly defined, antibiotics include a chemically heterogeneous group of small organic molecules of microbial origin that, at low concentrations, are deleterious to the growth or metabolic activities of other microorganisms **[1]**. Antibiotics are among the most frequently prescribed medications in modern medicine. Antibiotics cure disease by killing or injuring bacteria. The first antibiotic was penicillin, discovered accidentally from a mold culture. Today, over 100 different antibiotics are available to doctors to cure minor discomforts as well as life-threatening infections. Although antibiotics are useful in a wide variety of infections, it is important to realize that antibiotics only treat bacterial infections **[2]**.

Soil is rich in microorganisms capable of antibiotic synthesis is well accepted, but the frequency with which synthesis occurs at ecologically significant levels in nature has been much less clear **[3]**. Over the past decade, however, genetic and molecular techniques, coupled with sensitive and bioanalytical assays and equipment, have been applied to demonstrate conclusively that microorganisms synthesize a variety of antibiotics, even under field conditions **[4,5]**.

Antibiotics are mainly classified in two types on the basis of mode of action

A. Broad Spectrum Antibiotics

A chemical substance produced by a microorganism, which has the capacity to inhibit the growth of or to kill other microorganisms is called as an antibiotic. An antibiotic that is effective against a wide range of infectious microorganisms which includes both gram positive and gram negative bacteria is called as a Broad spectrum antibiotic **[6]**.

Broad-spectrum antibiotics are properly used in the following medical situations:

- Empirically prior to identifying the causative bacteria when there is a wide differential and potentially serious illness would result in delay of treatment. This occurs, for example, in meningitis, where the patient can become so ill that he/she could die within hours if broad-spectrum antibiotics are not initiated **[7]**.

- For drug resistant bacteria that do not respond to other, more narrow spectrum antibiotics.
- In super-infections where there are multiple types of bacteria causing illness, thus warranting either a broad-spectrum antibiotic or combination antibiotic therapy **[8]**.

B. Narrow Spectrum Antibiotics

Antibiotics may be defined as the sub-group of anti-infective that are derived from bacterial sources and are used to treat bacterial infections. An antibiotic may be classified basically as "narrow-spectrum" or "broad-spectrum" depending on the range of bacterial types that it affects **[9,10]**. Narrow-spectrum antibiotics are active against a selected group of bacterial types. Broad-spectrum antibiotics are active against a wider number of bacterial types and, thus, may be used to treat a variety of infectious diseases **[11,12,13]**.

Narrow spectrum antibiotics are used for the specific infection when the causative organism is known **[14]**.

Keeping in mind the importance of Bacterial species as a source of antimicrobial producers and the previous research work being carried out by various researchers, some of them being **[15,16,17].**

MATERIALS AND METHODOLOGY

Collection of soil sample:

Soil sample was collected in a pre-sterilized Petri plate and sterile spatula from 3 locations i.e, cattle shed, eutrophic lake and garbage waste (non-biodegradable) **[18]**.

Isolation of bacteria from soil samples:

The bacteria were isolated from soil sample by serial dilution in 0.85% NaCl solutions and spreading on sterilized nutrient agar media. Then the pure culture plates were prepared by selecting the cultures on the basis of their morphological characteristics **[19]**.

Screening of cultures for their antimicrobial activity:

The pure culture broths were prepared and then the antibacterial activity was analysed against gram positive and gram negative strains. *Escheriachia coli, Pseudomonas aeruginosa, Staphylococcus aureus.* The tests were carried out by using agar well diffusion method **[20]**.

Strain identification of isolates:

For identifying the strain numbers of biochemical tests were carried out by using Bergy's manual **[21]**.

Study of growth parameters of isolates:

The growth kinetic of the culture was performed after inoculating it in sterilized broth medium and the absorbance were taken at 620 nm in spectrophotometer at constant time intervals **[19]**.

Production and purification of antimicrobial component:

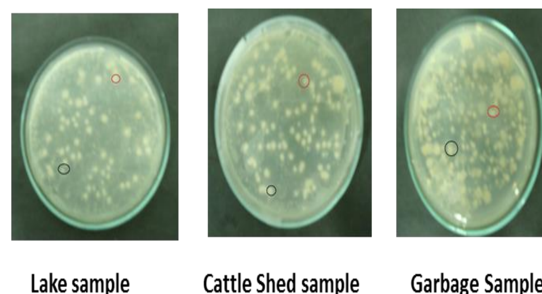
The productions of antibiotic were carried out by using shake flask fermentation method and then the purification was performed by using solvent extraction method. Further the antimicrobial component were analyzed for their antimicrobial activity by using agar well diffusion assay **[20]**.

RESULTS

Isolation of bacteria by serial dilution method:

Microbes were isolated from 3 different soil samples by Serial Dilution method using 0.8% NaCl as control for 10^{-1} to 10^{-6} dilutions.

Further , 3 Nutrient Agar (NA) plates were prepared corresponding to 10^{-1} ,10^{-3} and 10^{-5} dilutions which resulted in a continuous film of bacterial growth as shown below in figure 1.



Lake sample Cattle Shed sample Garbage Sample

Figure1- Mixed colonies in spread plate after serial dilution

As the plates of 10^{-5} dilution showed single isolated colonies so 2 colonies were chosen per soil sample (namely S1, S2, L3, L4, W5, W6) for further study .First of all the colony morphology was noted for all the cultures isolated.

Table 1: Colony morphology of cultures from S1 to W6

	Size (Mm)	Shape	Colour	Margin	Opacity
S 1	1	Circular	Off-White	Entire	Translucent
S 2	2	Circular	White	Entire	Opaque
L 3#	1.5	Irregular	Off-White	Lobed	Translucent
L 4	1.5	Irregular	Off-White	Lobed	Translucent
W 5	2.2	Circular	Off-White	Entire	Shiny
W6	2	Circular	Yellow	Entire	Translucent

Quadrant Streaking:

Bacterial isolates located from primary screening were sub-cultured using Quadrant Streaking .

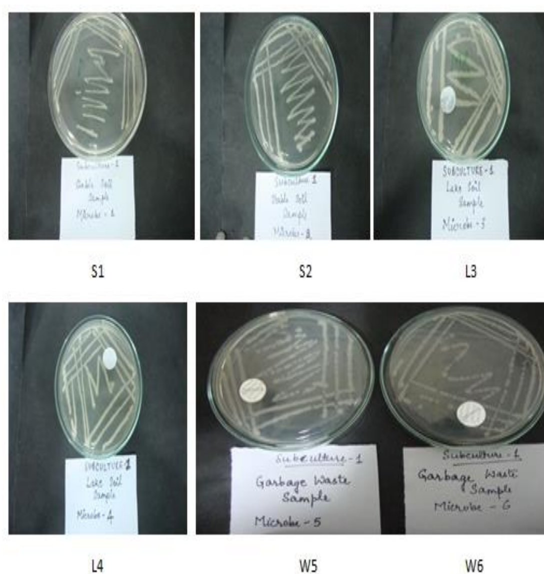


Figure 2- Pure colonies obtained through Quadrant Streaking

Grams' Staining:

It was done to identify the Gram positive or Gram negative mature of the bacterial isolates and to identify the shape of the bacterial cells in the particular isolate. Following results were observed on visualising under compound microscope.

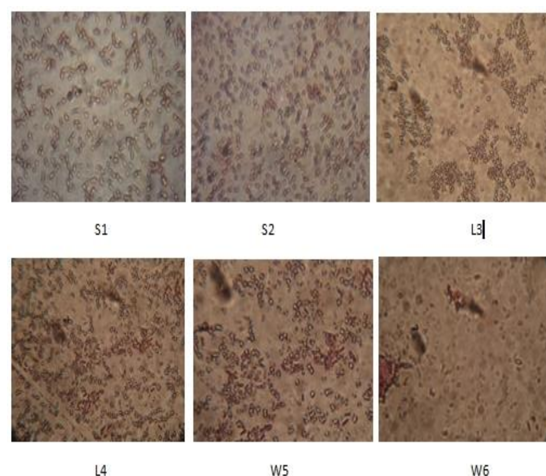


FIGURE 3 – Result of Grams' staining observed under compound microscope

Table 2: Results of Grams' staining

Sample	Gram +/-	Shape of cells observed
S1	-	Chained colonies of curved cells
S2	-	*Streptococcus* and single cocci
L3	-	Single cocci *
L4	-	Single cocci
W5	+	Single curved ,single rods and single cocci
W6	-	Chained rods ,*Streptococcus*

* # As the colony morphology and Grams' staining results of L3 and L4 were completely identical so further steps were carried out only with L4.

Antibiotic Sensitivity Test /Multiple Drug Resistance Test (MDR):

MDR Test of purified cultures S1, S2, L4, W5, W6 was performed against ***Pseudomonas aeruginosa , Staphylococcus aureus and E.coli*** . The detailed results of the same can be seen in tables 3-7 and figures 4-8.

Table 3- Antibiogram of Tetracycline, distilled water and S1 culture against various pathogens

PATHOGENS	TETRACYCLINE Diameter (mm)	DISTILLED WATER	SAMPLE Diameter (mm)
Pseudomonas aeuruginosa	26	-	0.0
Staphylococcus aureus	26	-	13
E.coli	26	-	0.0

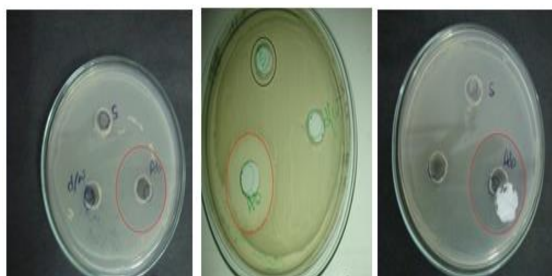


Figure 4- Antibiogram analysis of culture S1 against various pathogens

Table 4- Antibiogram of Tetracycline, distilled water and S2 culture against various pathogens

PATHOGEN	TETRACYCLINE Diameter (mm)	DISTILLED WATER	SAMPLE Diameter (mm)
Pseudomonas aeuruginosa	24	-	15
Staphylococcus aureus	24	-	12
E.coli	24	-	0.0

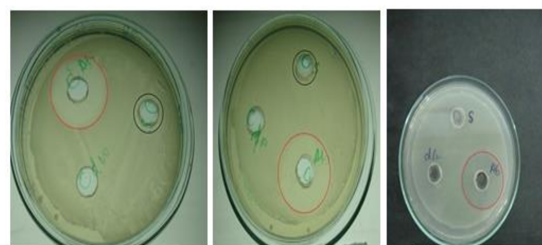


Figure 5- Antibiogram analysis of culture S2 against various pathogens

Table 5- Antibiogram of Tetracycline, distilled water and L4 culture against various pathogens

PATHOGEN	TETRACYCLINE Diameter (mm)	DISTILLED WATER	SAMPLE Diameter (mm)
Pseudomonas aeuruginosa	22	-	0.0
Staphylococcus aureus	28	-	20
E.coli	22	-	0.0

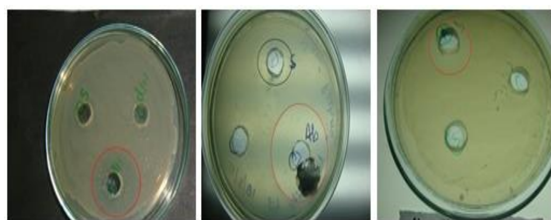


Figure 6- Antibiogram analysis of culture L4 against various pathogens

Table 6- Antibiogram of Tetracycline, distilled water and W5 culture against various pathogens

PATHOGEN	TETRACYCLINE Diameter (mm)	DISTILLED WATER	SAMPLE Diameter (mm)
Pseudomonas aeuruginosa	22	-	14
Staphylococcus aureus	24	-	14
E.coli	26	-	16

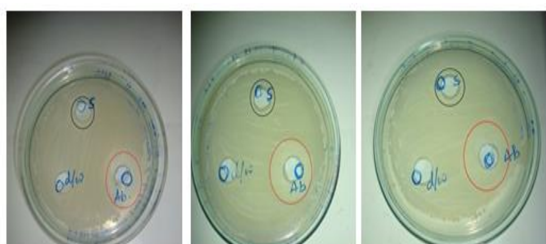


Figure 7- Antibiogram analysis of culture W5 against various pathogens

Table 7- Antibiogram of Tetracycline, distilled water and W6 culture against various pathogens

PATHOGEN	TETRACYCLINE Diameter (mm)	DISTILLED WATER	SAMPLE Diameter (mm)
Pseudomonas aeuruginosa	24	-	14
Staphylococcus aureus	24	-	14
E.coli	26	-	0.0

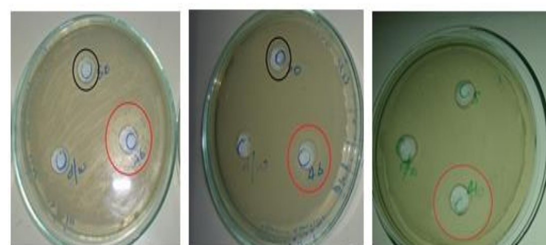


Figure 8- Antibiogram analysis of culture W6 against various pathogens

All the isolates showed positive results against atleast 1 out of the 3 test pathogens so all the 5 cultures were taken for further studies.

(NOTE: Well diameter is 8 cm)

Growth kinetics studies of the cultures s1, s2, l4, w5 and w6:

Growth kinetics of cultures was performed as shown below in Tables 8-12 and Figures 9-13 respectively

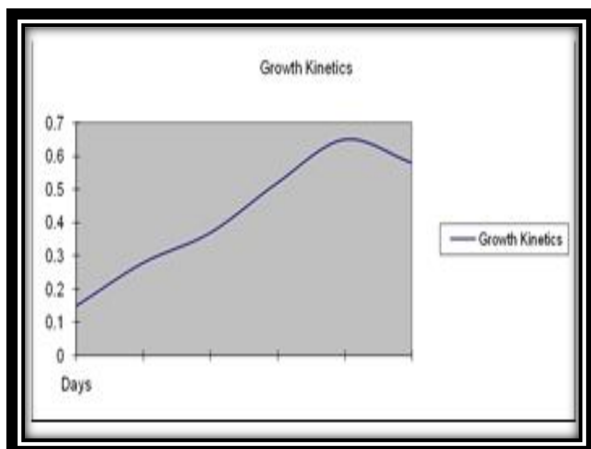


Figure 9: Growth Kinetics graph for S1

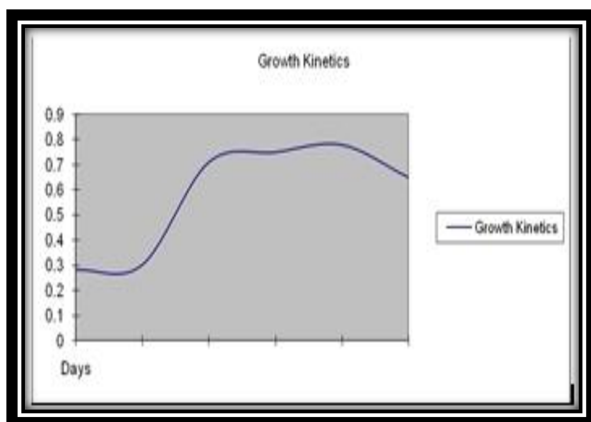


Figure 10: Growth Kinetics graph for S2

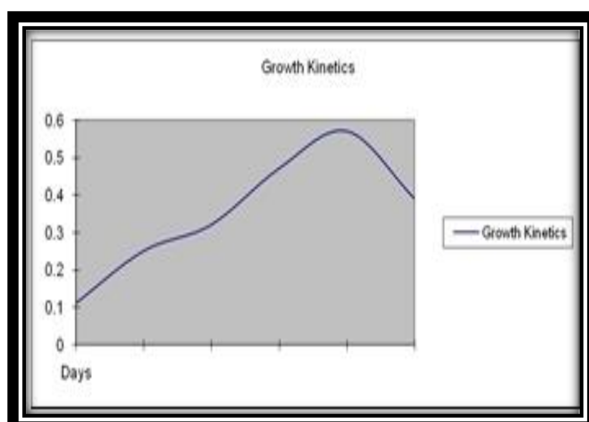


Figure 11: Growth Kinetics graph for L4

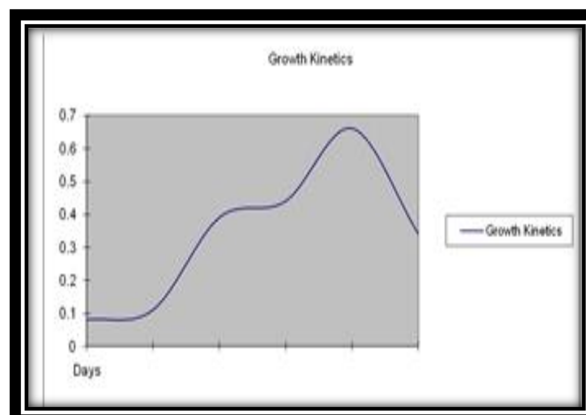


Figure 12: Growth Kinetics graph for W5

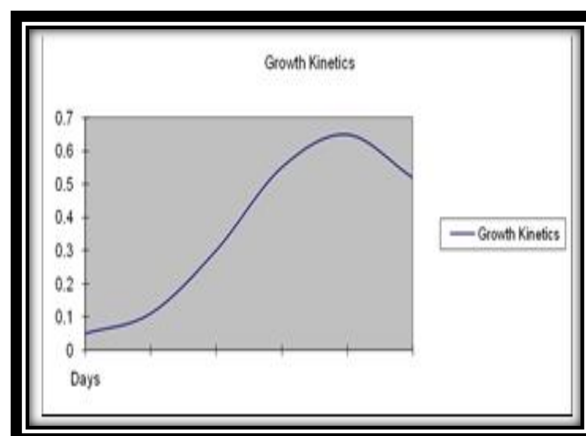


Figure 13: Growth Kinetics graph for W6

Production of crude antibiotic on lab scale by shake flask method:

The 5 flasks of production media inoculated with the 5 cultures ,resulted in growth of colonies on a large scale after shake flask fermentation. This was done to obtain maximum production of antibiotic substances by the bacterial cultures.

Partial Purification:

Intracellular extract of S1 in NYD Production medium:

The amount of antimicrobial component left in the eppendorf after air drying was measured as **0.233** gm as shown in the table below

Table 9- Intracellular antibiotic component of S1

S .NO	Wt. On day zero (gm)	Wt. After drying(gm)	Difference (gm)
1.	0.907	0.929	0.022
2.	0.913	0.914	0.001
3.	0.882	0.910	0.028
4.	0.913	0.915	0.002
5.	0.918	0.919	0.001
6.	0.921	0.926	0.005
7.	0.903	0.905	0.002
8.	0.903	0.919	0.016
9.	0.946	0.952	0.006
10.	0.913	0.914	0.001
11.	0.918	0.949	0.031
12.	0.950	0.952	0.002
13.	0.904	0.938	0.034
14.	0.923	0.926	0.003
15.	0.921	0.926	0.005
16.	0.909	0.927	0.018
17.	0.916	0.919	0.003
18.	0.893	0.929	0.036
19.	0.910	0.927	0.017
Total wt.			**0.233**

Hence , initial concentration = **245.26 mg/ml**

Intracellular extract of S2 in NYD Production medium:

The amount of antimicrobial component left in the eppendorf after air drying was measured as **0.349** gm as shown in the table below

Table 10- Intracellular antibiotic component of S2

S .NO	Wt. On day zero (gm)	Wt. After drying(gm)	Difference (gm)
1.	0.924	0.939	0.015
2.	0.914	0.926	0.012
3.	0.919	0.935	0.016
4.	0.919	0.934	0.015
5.	0.909	0.920	0.011
6.	0.914	0.926	0.012
7.	0.918	0.935	0.017
8.	0.913	0.931	0.018
9.	0.910	0.932	0.022
10.	0.922	0.937	0.015

S .NO	Wt. On day zero (gm)	Wt. After drying(gm)	Difference (gm)
11	0.917	0.933	0.016
12	0.919	0.939	0.020
13	0.933	0.949	0.016
14	0.940	0.951	0.011
15	0.909	0.918	0.009
16	0.899	0.919	0.020
17	0.910	0.921	0.011
18	0.940	0.957	0.17
19	0.919	0.934	0.015
20	0.913	0.927	0.014
21	0.907	0.922	0.15
22	0.907	0.924	0.017
Total wt.			**0.349**

Hence ,initial concentration = **317.27 mg/ml**

Intracellular extract of L4 in NYD Production medium:

The amount of antimicrobial component left in the eppendorf after air drying was measured as **0.216** gm as shown in the table below

Table11- Intracellular antibiotic component of L4

S .NO	Wt. On day zero (gm)	Wt. After drying(gm)	Difference (gm)
1.	0.924	0.927	0.003
2.	0.926	0.937	0.011
3.	0.926	0.928	0.002
4.	0.912	0.913	0.001
5.	0.915	0.932	0.017
6.	0.912	0.919	0.007
7.	0.924	0.927	0.003
8.	0.917	0.931	0.014
9.	0.906	0.912	0.006
10.	0.924	0.925	0.001
11.	0.912	0.919	0.007
12.	0.901	0.918	0.017
13.	0.922	0.923	0.001
14.	0.913	0.918	0.005
15.	0.929	0.937	0.008
16.	0.915	0.932	0.017
17.	0.927	0.930	0.003
18.	0.930	0.937	0.007
19.	0.923	0.933	0.010
20.	0.925	0.936	0.011
21.	0.913	0.937	0.024
22.	0.916	0.932	0.016
23.	0.888	0.896	0.008
24.	0.915	0.932	0.017
Total wt.			**0.216**

Hence , initial concentration = **180.00 mg/ml**

Intracellular extract of W5 in NYD Production medium:

The amount of antimicrobial component left in the eppendorf after air drying was measured as 0.547 gm as shown in the table below

Table 12- Intracellular antibiotic component of W5

S .NO	Wt. On day zero (gm)	Wt. After drying(gm)	Difference (gm)
1.	0.907	0.929	0.022
2.	0.882	0.910	0.028
3.	0.019	0.915	0.002
4.	0.921	0.926	0.005
5.	0.903	0.919	0.016
6.	0.946	0.952	0.006
7.	0.918	0.949	0.031
8.	0.904	0.938	0.034
9.	0.921	0.926	0.005
10.	0.916	0.919	0.003
11.	0.882	0.910	0.028
12.	0.924	0.927	0.003
13.	0.926	0.928	0.002
14.	0.915	0.932	0.017
15.	0.924	0.927	0.003
16.	0.906	0.912	0.006
17.	0.912	0.919	0.007
18.	0.922	0.923	0.001
19.	0.929	0.937	0.008
20.	0.927	0.930	0.003
21.	0.923	0.933	0.010
22.	0.916	0.932	0.016
23.	0.917	0.932	0.017
24.	0.929	0.937	0.008
Total wt.			**0.547**

Hence ,initial concentration = **455.83 mg/ml**

Intracellular extract of W6 in NYD Production medium:

The amount of antimicrobial component left in the eppendorf after air drying was measured as **0.298** gm as shown in the table below

Table 13- Intracellular antibiotic component of W6

S .NO	Wt. On day zero (gm)	Wt. After drying(gm)	Difference (gm)
1.	0.909	0.920	0.011
2.	0.918	0.935	0.017
3.	0.910	0.932	0.022
4.	0..917	0.933	0.016
5.	0.933	0.949	0.016
6.	0.940	0.951	0.011
7.	0.899	0.919	0.020
8.	0.940	0.957	0.017

S .NO	Wt. On day zero (gm)	Wt. After drying(gm)	Difference (gm)
9.	0.913	0.927	0.014
10.	0.907	0.927	0.017
11.	0.913	0.931	0.018
12.	0.912	0.919	0.007
13.	0.926	0.937	0.011
14.	0.901	0.918	0.017
15.	0.929	0.937	0.008
16.	0.930	0.937	0.007
17.	0.915	0.932	0.017
18.	0.888	0.896	0.008
19.	0.924	0.927	0.003
20.	0.922	0.923	0.001
21.	0.930	0.937	0.007
22.	0.924	0.925	0.001
23.	0.915	0.932	0.017
24.	0.913	0.927	0.014
Total wt.			**0.298**

Hence ,initial concentration = **248.33 mg/ml**

DISCUSSION

Bacteria were isolated & purified from the soil sample collected from cattle shed, euthrophic lake and garbage waste(non-biodegradable) on nutrient agar media .

Primary screening of the obtained bacteria for antibiotic production was performed by incubating the mixed culture plates for several days & observing for the zone of inhibition on the plates.

Secondary screening of the culture found to be positive in primary screening was done by **agar** well diffusion method as done earlier by **Awais *et al* (2007).**

Production of antimicrobials from the culture found to be positive in secondary screening was carried out in NYD production medium by flasks level fermentation, similar media and fermentation technique has been used by **Jing *et al* (2009).**

The intracellular and extracellular components of the fermentation mixture were separated by centrifugation and use of methanol and chloroform.

Antibiogram analysis of the purified antimicrobial extract performed by agar well diffusion method zone of inhibition were measured.MIC was determined for the samples showing antibiotic properties and TLC was finally conducted to check the presence of amino acids in the antibiotics substances produced.

The comparative study of antibiotic property of different soil samples collected revealed that soil is a rich source of antimicrobial compounds of different types.

CONCLUSION

Finally it can be concluded that Bacteria is a good source of Antibiotic and very useful for industrial antibiotic production.

The isolated bacteria can easily grow onto NYD Production media containing Beef-extract, Yeast extract, Glucose at 37°C appropriate temperature for better growth of bacteria. The pH 7.5 was appropriate bacterial growth and Antibiotic production.

Further prospective of work includes purification of Antibiotic in order to gain higher specific activity of Antibiotic by the help of Affinity chromatography, HPLC.

Antimicrobials can be used in addition with some cations in order to enhance the zone of inhibition.

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