"Isolation and Characterisation of Antibiotic producing Microbes present in Rhizospheric soil"

Surabhi Arora[1],Dipika Nandi[2],Namrata Prasad[3], ,Saurabh Rawat[4] ,Amit Pandey[a]
Graphic Era university,Dehradun[1,2,3,4] and MRD Life Sciences[a]

ABSTRACT

Different Soil samples of *Calotropis procera* and *Catharanthus roseus* were subjected for antibacterial activity of microbes, extraction of secondary metabolites i.e both intracellular and extracellular, and characterisation. Three type of colony were found i.e white, off-white and yellow. Further using Bergers' manual *Macrococcusluteus ,N.sicca* were obtained.
Secondary metabolites i.e intacellular and extracellular were extracted using solvents chloroform and methanol. Antibiotic sensitivity test was performed against pathogen(*E.coli,P.aeroginosa and S.aureus*) and the best Zone of inhibition was of culture of *Catharanthus roseus* i.e 28.5 mm. Different test were performed to characterise the microbes. This was performed in order to isolate microbes that produce antibiotic and then further be used for medicinal purpose.

Keywords:-Zone of Inhibition, Antibiotic Sensitivity Test, Bergey's Manual.

1. INTRODUCTION

An antibiotics (against life) is a compound or substance that kills or slows down the growth of bacteria. 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.

1.1Mode of action of antibiotics

1.1.1 Cell Wall Synthesis Inhibitors
1.1.2 Interfering with Protein Synthesis
1.1.3 Cell Membrane Inhibitors
1.1.4 Effect on Nucleic Acids
1.1.5 Competitive Inhibitors

1.2The main classes of antibiotics

1.2.1 Beta-Lactams
- **a**.Penicillin
- **b**.Cephalosporins

1.2.2 Macrolides
1.2.3 Tetracyclines
1.2.4 Aminoglycosides

2. METHODOLOGY

2.1 Collection of soil sample

2.1.1 Serial dilution: Serial dilution method is perform to get reduce number of bacterial colonies to pure colonies. It is the volume of the sample to the total volume of the sample and dilutants. For this 30ml of 0.85% NaCl was prepared and 5ml NaCl was poured in the first test tube and 4.5 ml in rest test tubes and autoclaved. 0.5grm of soil was added in 1st test tube and serial dilution method was performed (10^{-1} to 10^{-5}).

2.2 Primary screening of bacterial species for antibacterial compound production

2.2.1 Sub culturing:- was done in NA media by spreading 50 µl of culture on NA plates with spreader and incubating the plates at 37ºC for overnight.

2.2.2 Pure culturing: Derived from a mixed culture (containing many species) by transferring a small sample into new sterile growth medium in such a manner as to disperse the individual cells across the

medium. Pure culture contain a single species of organism.

A pinch of culture on a inoculation loop was taken from the spreaded plates (10^{-1}, 10^{-3} and 10^{-5}),and streaked over NA plates and incubated at 37ºC for overnight.

2.3 Secondary Screening of Bacterial species for antibacterial compound production

2.3.1 Antibiotic Sensitivity Test

With constant exposure to different environmental conditions, microbes develop self resistant and develop drug resistant against the compound. If antibiotic will be effective then they will show zone of inhibition against the pathogen and if culture will be resistive then culture will show full growth in the presence of antibody.

NB broth was prepared, inoculated and incubated at 37ºC in shaker for overnight.

NA plates were prepared and 50µl of *E.coli, P.aeruginosa, S.aureus* each culture was spreaded on different NA plates. 3 wells were prepared on each plates and 50µl of sample was pipetted in ech well and plates were incubated.

2.3.2 Growth kinetics:

It is applied to determine the time period at which the culture show its optimum activity(stationary phase). Growth of any microbe occurs in different stages: Lag phase, Log phase, Stationary phase, Decline phase. In 50 ml NB 50µl of bacterial culture was inoculated and incubated at 37°C and O.D was taken at 620 nm day by day until stationary phase is obtained.

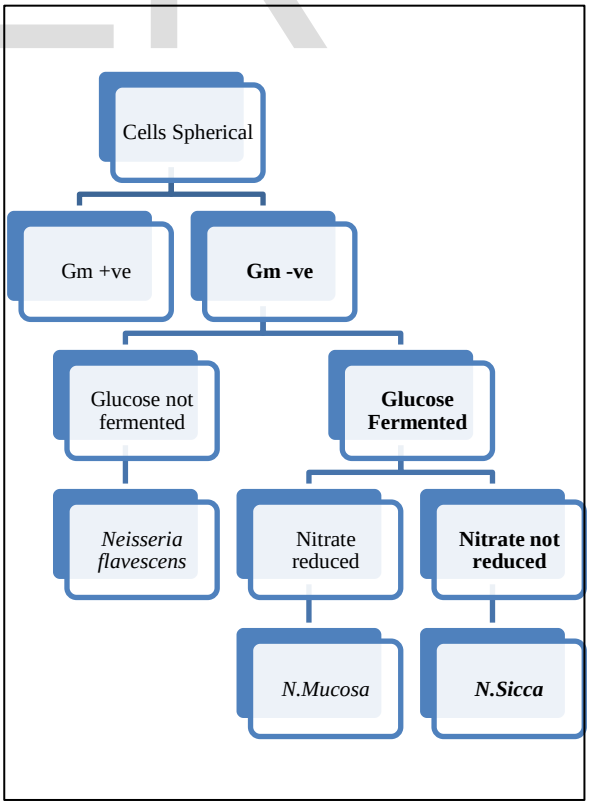
2.3.3 Extraction of secondary metabolites from isolated culture: solvents chloroform and methnol were used. Metabolites extracted due to difference in phase i.e extracellular were dissolve in chloroform and these metabolites comes in bottom layer where as the intracellular were dissolve in methnol and these metabolites were observed in top layer. Mixing of metabolites depend on the polarity of solvent.

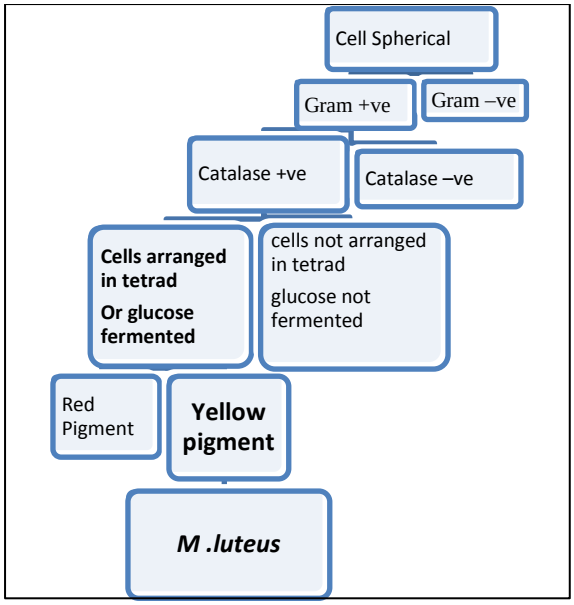
NB was prepared and inoculated. It was then incubated in shaker incubator at 37ºC for days in order to obtain stationary phase. After incubation 5 ml was transfered in falcon tube and centrifuged at 5000rpm for 5 min.

a) For Intracellular (pellet) - The pellet was dissolved in 2.5 ml of methanol and mixed properly for 30 minutes. Centrifugation was done at 10000 rpm for 10 min. Top layer was collected in a new tube and air dried, it was then dissolve in 100nM Tris HCl pH8 and again Antibiotic Sensitivity test was performed.

b) For Extracellular (supernatant) - To the supernatant equal volume of chloroform was added and mixed properly for 30 minute and centrifuged at 10000 rpm for 10 min and bottom layer was transferred to new tube and air dried. It was then dissolved in 100nM Tris Hcl pH 8, again AST was performed.

2.4 Characterization of microbes





2.4.1 Gram's staining

Gram's staining is a process to distinguish between gram +ve and gram –ve bacteria. Gram –positive cells have thick peptidoglycan cell wall that is able to retain the crystal violet-iodine complex that occurs during staining, while gram-negative cells have only thin layer of peptidoglycan. Thus Gram- positive cells do not decolorize with ethanol, and gram-negative cells do decolorize. This allows the gram-negative cells to accept the counter stain safranin. Gram-positive cells will appear purple, while gram-negative cells will appear pink.

A thin smear of culture was prepared on sterile glass slide and heat fix it. A drop of crystal violet(primary stain) was added over the smear for 60 sec and washed with distil water. Similarly, a drop of gram's iodine (Mordant-to fix), 95% alcohol (dehydrating agent), and safranin (counter stain) was added on the smear one by one, by washing with distil water in between, for 60 sec, 20-30sec and 90 sec respectively. It was then air dried and observed under microscope.

2.4.2 Catalase Test:- It is a process of characterization of bacteria through which they are classified as aerobic and anaerobic. Catalase is an enzyme which cleaves H_2O_2 and O_2. Hence on addition of H_2O_2 on bacterial culture, if there is production of O_2 during test than bacteria is aerobic otherwise the bacteria is anaerobic.

A drop of H_2O_2 was put over the smear of the sample. A pinch of culture was taken and put on it, mixed well, observed for evaluation of bubbles (catalase -positive) and if bubbles are not coming it show catalase-negative.

2.4.3 Mannitol Test:- is useful for the selective isolation of pathogenic staphylococci, since most other bacteria inhibited by the high salt concentration. Colonies of pathogenic staphylococci are surrounded by yellow halo, indicating mannitol fermentation.

Mannitol broth was prepared transferred to autoclaved test tubes, inoculated with culture and incubated at 37ºC for overnight.

2.4.4 Glucose Fermentation Test: Glucose after the cell can be catabolise either aerobically(bio-oxidations in which molecular oxygen can be serve as the final electron acceptor) or aneorobically (bio-oxidation in which inorganic ions other than oxygen can be serve as final electron acceptor. Growth of the microorganism in this medium is either by utilizing the tryptone which results in alkaline reaction(dark blue) or by utilizing glucose which results in the production of acid(turning bromo blue to yellow).

OF glucose broth medium and glucose were prepared and inoculated and kept at 35°C for 24-48 hours.

2.4.5 Nitrate Reduction Test : Bacterial species may be differentiated on the basis of their ability to reduce nitrate to nitrite or nitrogenous gases. The reduction of nitrate may be coupled to anaerobic respiration in some species.

NB media was prepared, autoclaved, cooled, inoculated with culture and kept it for overnight. α-Napthol(4-5) drops and conc. H_2SO_4(4-5) drops were added. Red

and pink colour indicate positive nitrate reduction and vice-versa.

2.4.6 Methyl Red and Voges-Proskauer test: are used to differentiate two major types of facultatively anerobic enteric bacteria that produce large amounts of acid and those that produce the neutral products acetoin as end product. If the organism produces large amount of organic acids end product from glucose the medium will remain red (positive test) after the addition of methyl red a pH indicator. If methyl red turn yellow a negative test.

MRVP broth(pH 6.9) was prepared and autoclaved. It was innoculatedand incubated at 35°C for 48 hours. 5 drops of methyl red indicator was added and observed for colour change. 12 drops of V-P reagent and 2-3 drops of V-P reagent II was added. It is then shaken gently for 30 sec. The reaction was left to complete for 15-30 min to observe color change.

2.4.7 Endospore test: An aqueous primary stain (malachite green) is applied and steamed to enhance penetration of the impermeable spore coats. Once stained the endospores do not readily decolorize and appear green within red cells.

A thin smear of culture was made and Malachite green was added in the pressure of water vapours for 5 min. After washing Safranin was added to it for 40 sec further it was again washed with distilled water, air dried and observed under the microscope.

3.RESULTS

In this paper, a total of 7 cultures of bacteria were isolated from the collected sample of soil from lakashmanpuri, Gomti Nagar, Lucknow,India. 3 were obtained from the *Caranthus roseus* and 4 were of *Calathropis procera*.

3.1 Serial Dilution :- Through serial dilution, microbes were isolates from soil sample of *Calathrophis procera* and *Catharanthus roseus*. Mixed culture were obtained by spreading different colonies like white, off-white and yellow.

Spreading results:

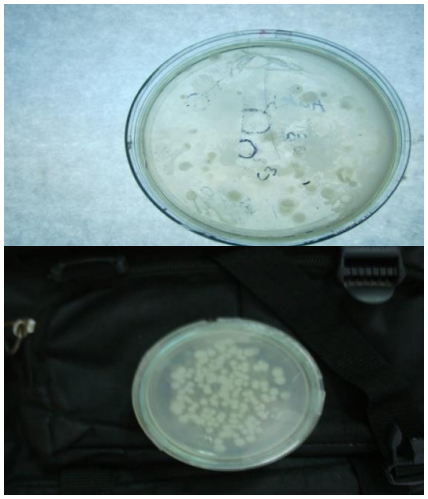


Fig 1

Fig 2

Fig1:Colonies of *Calatropis procera*
Fig 2:Colonies of *Catharanthus roseus*

Table 1:Morphology of *Calotropis procera*

Colony Morphology	C1	C2	C3
Shape	Regular	Irregular	Regular
Colour	Yellow	White	White
Texture	Smooth	Smooth	Smooth
Elevation	Flat	Flat	Flat
Margin	Entire	Lobate	Entire
Opacity	Transparent	Transparent	Transparent

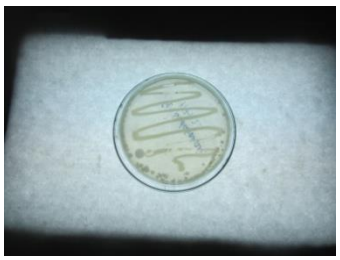
Table 2:Morphology of *Catharanthus roseus*

Colony Morphology	C1	C2	C3
Shape	Regular	Regular	Fusi form
Colour	Yellow	White	Off White
Texture	Smooth	Smooth	Gummy
Elevation	elevated	Flat	Flat
Margin	Entire	Entire	Undulant
Opacity	Opaque	Opaque	Opaque

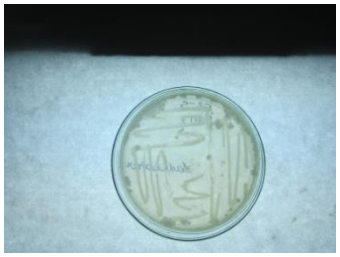
3.2 Pure culturing

With the help of streaking pure colonies were obtained through streaking from mixed colonies.

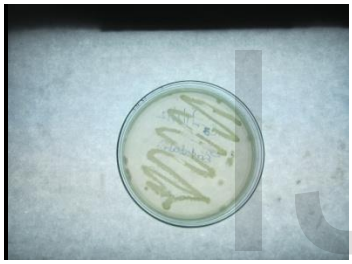
Fig 3: Streaking result of *Catharanthus roseus*



C 1



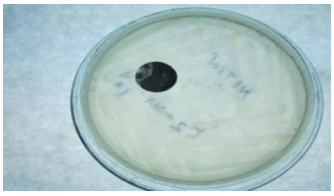
C 2



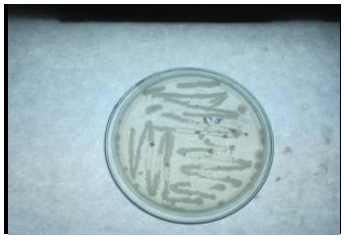
C 3

Fig 4 : Streaking result of *Calotropis procera*

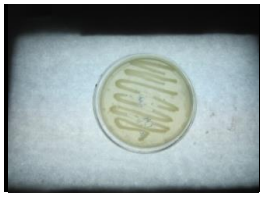
C 1



C 2



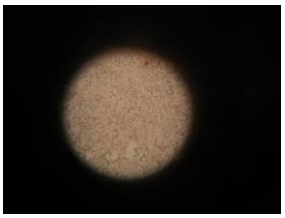
C 3



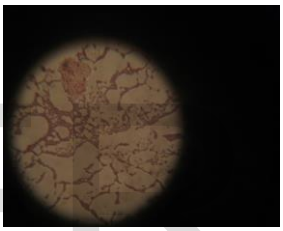
C 4

3.3 Staining results: Gram positive bacteria retain violet colour while gram negative retain pink colour. Both negative and positive bacteria were obtained.

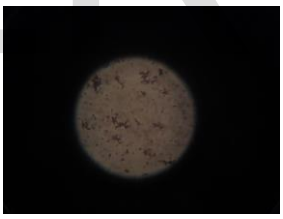
Fig 5:Microscopic view of *Calotropis procera*



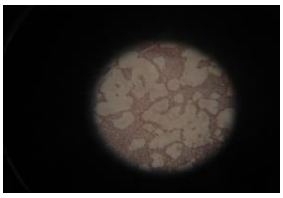
a)



b)



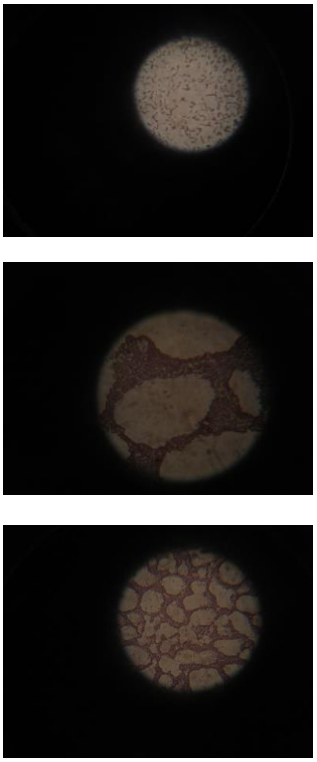
c)



d)

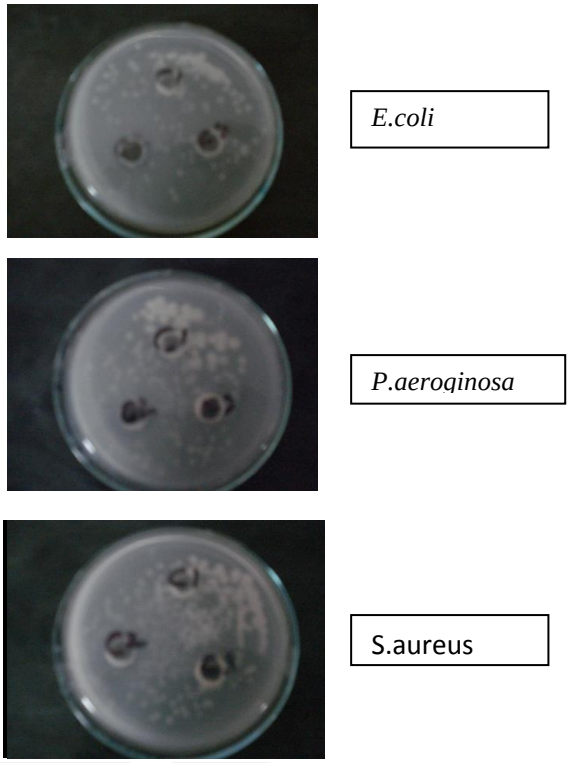
a) C1 A,Gram Negative cocci(pink colour)
b) C2 A, Gram Negative cocci(pink colour)
c) C3 A,Gram Positive rods(purple colour)
d) C4 A, Gram Negative cocci(pink colour)

Fig6: **Microscopic view of *Catharanthus roseus***



a) C1 S,Gram Positive Cocci (purple colour)
b) C2 S,Gram Positive Cocci (purple colour)
c) C1 S,Gram Positive Cocci (purple colour)

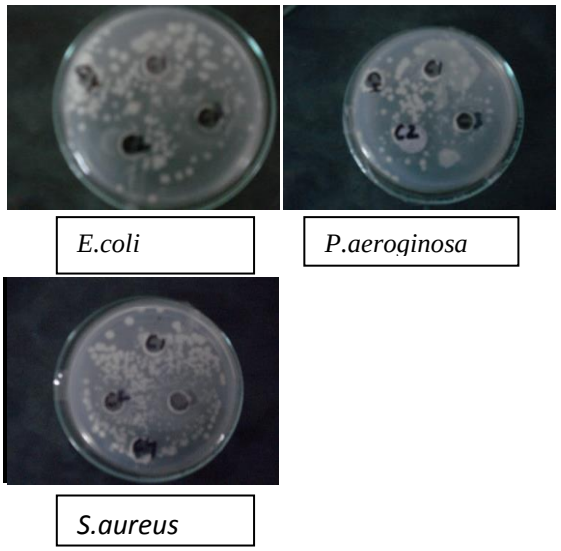
Fig 8:Results of AST→*Cathranthus roseus*



3.4 Primary Screening:

3.4.1Antibiotic sensitivity test result:-

Fig 7 :Results of AST→*Calotropis procera*



3.4.1 a) Secondary Screening:

Extraction of Secondary Metabolites→

Table 3 : Zone of Inhibition obtained during AST

Culture	Pathogen	Intracellular	Extracellular	Intra+Extra
C2-S	*Pseudomonas*	6.5mm	--	14mm
C2-S	*S.aureus*	22.5mm	11mm	11mm
C2-S	*E.coli*	15.5mm	10.5	19mm
C3-S	*Pseudomonas*	28.5mm	29mm	27.5mm
C3-S	*S.aureus*	28.5mm	11.5mm	25mm
C3-S	*E.coli*	28.0mm	29.0mm	27.0mm
C1-A	*Pseudomonas*	--	--	--
C1-A	*S.aureus*	--	--	--
C1-A	*E.coli*	--	--	--
C2-A	*Pseudomonas*	11.5mm	13.0mm	12.0mm
C2-A	*S.aureus*	15.0mm	--	13.0mm
C2-A	*E.coli*	14.5mm	13mm	14.5mm
C3-A	*Pseudomonas*	--	--	--
C3-A	*S.aureus*	--	--	--
C3-A	*E.coli*	--	--	--
C4-A	*Pseudomonas*	--	--	--
C4-A	*S.aureus*	--	--	--
C4-A	*E.coli*	--	--	--

Images of AST

Fig9.AST→C2-S

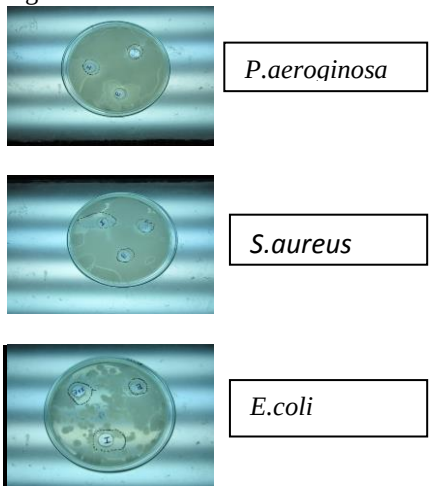


Fig10.AST→C3-S

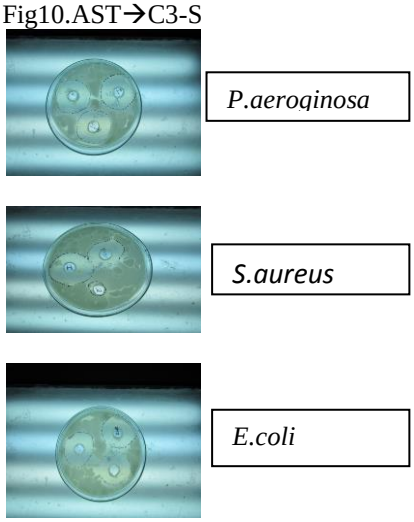


Fig11.AST→C1-A

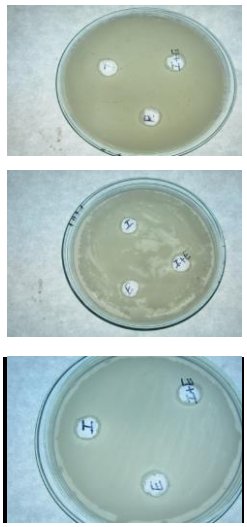


Fig12.AST→C2-A

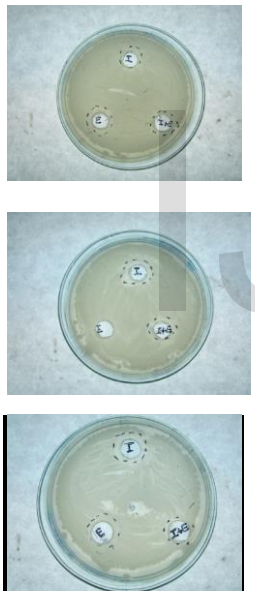
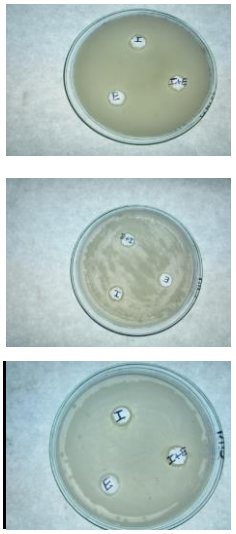


Fig13.AST→C4-A



Pseudomonas *E.coli*
S.aureus

3.5.Identification & charachterization through Bergey's manual

Table 4: Illustrates biochemical analysis of isolated Cultures

Biochemical Tests	C1-A	C2-A	C3-A	C4-A	C1-S	C2-S	C3-S
Gram Staining	-ve Cocci	-ve Cocci	+ve rods	-ve Cocci	+ve cocci	+ve Cocci	+ve Cocci
Catalase Test	--	--	+ve	--	+ve	+ve	+ve
Endospore Test	--	--	+ve	--	-ve	+ve	-ve
Glucose Fermentation	+ve	+ve	--	+ve			
MRVP test	--	--		--	--	--	--
Nitrate Test	-ve	-ve	--	-ve	-	--	--

By the help of Bergey's Manual following was observed:

Table 5: Illustrates the microbes identified through various biochemical tests.

Culture	Microbe identified:
C1-A	*N.Sicca*
C2-A	*N.Sicca*
C3-A	*B. sutilis*
C4-A	*N.sicca*
C1-S	*M. lutes*
C2-S	*M. lutes*
C2-S	*M. lutes*

4. CONCLUSION

This paper is carried out to study isolation and characterization of antibiobiotic producing microbes present in soilTotal 7 cultures were isolated through serial dilutionmethod and they were characterised using Bergey's manual and the identified cultures were *N.Sicca,M.lutes* and *B.subtilis* these cultures were subjected to antibiotic sensitivity test against bacterial pathogens(*E.coli,P.aeruginosa and S.aureus*) and a little Zone of inhibition was observed. Further Intracellular and extracellular metabolites were extracted and then they were subjected to Antibiotic Sensitivity test, the Zone of Inhibition was observed with the best
Result in intracellular metabolites of *M.letus* about 28.5mm.
These can be used to prepare medicine for human being. Earlier there was not much antibiotics for various diseases but now due to technology antibiotics can be easily produced.

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