ISOLATION, CHARACTERIZATION & PLASMID PROFILING OF MDR *BACILLUS* SPECIES USING AGRICULTURAL SOIL SAMPLES OF THREE DISCRETE ZONE, NASHIK DISTRICT, MAHARASHTRA

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ABSTRACT:

Antibiotic Resistant *Bacillus* species were isolated from the 3 different agricultural field areas i.e. field soil near industrial area (Musal gaon, MIDC, sinnar), Field soil near Construction area (Lokhande mala, nashik road, nashik), Field soil near hospital area (Sambhaji nagar, sinnar). The microscopically, biochemical test suggested it to be *Bacillus* species. Our major aim was to check the effect of isolated *Bacillus* species on the human by the hemolytic test, as how pathogenic bacteria enters into the human food chain especially *Bacillus* by the crops eaten by human from agriculture fields. The early discovery and close monitoring of MDR, XDR, or indeed PDR bacterial strains must be started by all clinical microbiology laboratories.

KEYWORDS:

Bacillus, Antibiotic Drug Resistant, MDR, Hemolytic

INTRODUCTION

Antibiotic use has been beneficial &, when prescribed & taken correctly, their value in patient care is enormous. Antibiotics are medicines that kill bacteria or slow the growth of bacteria. They are used to cure diseases. However, these drugs have been used so widely & for so long that the infectious organisms the antibiotics are designed to kill have adapted to them, making the drugs less effective. Many fungi, viruses, & parasites have done the same. Antibiotic is very different from chemotherapeutic drugs, antibiotics are natural drugs that is produced by several

fungi or bacteria but chemotherapeutics drugs are manmade substances. History of antibiotics began in 1932, (Nussbaum., et al, 2006) when the first drug sulfonamide was prepared. Sulfonamides are effective drugs. Sulfonamides have shown tremendous positive results on urinary tract infections, shigellosis & Pneumococcal pneumonia. The problems of infectious diseases suddenly increased when certain became antibiotic resistant. Certain microorganisms have the ability to become resistant to one specific antimicrobial agent (or a similar type of agent), while others have the capability to become resistant to multiple antimicrobial agents or classes. These organisms are commonly known as strains that are resistant to multiple drugs, or MDR strains. In certain situations, the microorganisms have developed such strong resistance that none of the antibiotics currently accessible can have any effect on them. Extensive use of antibiotics in human treatment led to the emergence of disease-causing bacteria that are resistant to multiple medications. Bacteria can develop multidrug resistance through one of two mechanisms. Initially, these bacteria can gather numerous genes in one cell, where each gene is responsible for conferring resistance to a specific drug. Resistance plasmids are known to commonly accumulate this collection. Additionally, the resistance to multiple drugs can also arise from an up regulation of genes responsible for producing efflux pumps that expel various types of drugs. Due to the increase of resistance to antibiotics, there is a pressing need to develop new & innovative antimicrobial agents. Plants have been extensively studied as a possible source of new substances. Due to their abundance of bioactive compounds, they possess potential therapeutic benefits. Because of their low toxicity, there is a long tradition of using dietary plants in the treatment of infectious disease & to combat the MDRs successfully.

MATERIALS & METHODS

1. SOIL SAMPLE COLLECTION

i. The agriculture field soil sample was collected from 3 different locations from different field areas.

ii. The first site corresponds to Field soil near industrial area (Musal gaon, MIDC, sinnar), Field soil near Construction area (Lokhande mala, nashik road, nashik), Field soil near farming area (Sambhaji nagar, sinnar) in Maharashtra, India.

iii. From each site approx. 10 gram of soil sample were collected and placed in sterile plastic bags, then taken to the laboratory.

(as in figure no. 1)

Field soil near Construction area (Lokhande mala, nashik road, nashik) SOIL SAMPLE 1

Field soil near hospital area (Sambhaji nagar, sinnar) SOIL SAMPLE 2

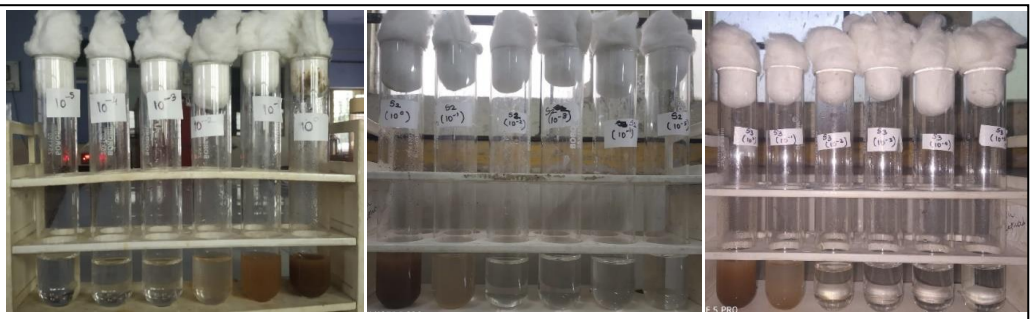
Field soil near industrial area (Musal gaon, MIDC, sinnar) SOIL SAMPLE 3

Figure no. 1 (Soil Sample Collection)

2. ISOLATION OF BACTERIA FROM SOIL SAMPLES

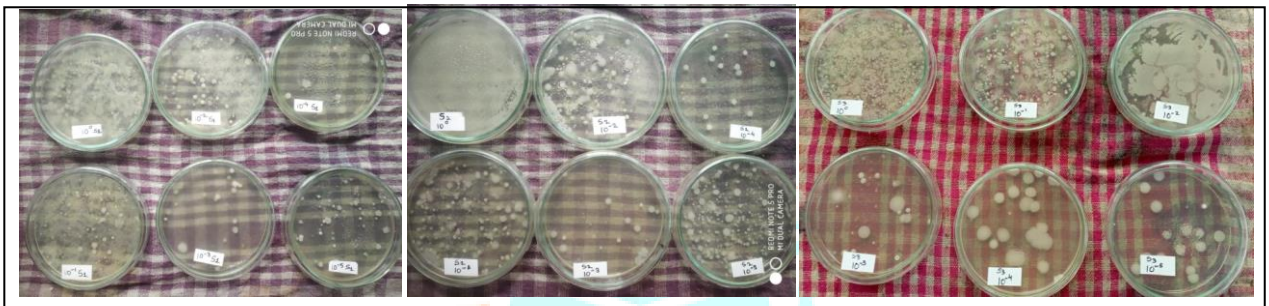
- Soil bacteria were isolated by the Standard Serial Dilution and Spread Plate method.
  - **Requirement:-**
    a. Soil sample
    b. Saline solution
    c. Sterile test-tubes
    d. Sterile Nutrient Agar plates
    e. Micropipette
    f. L-shape glass rod
    g. Ethanol

- **Protocol:-**
  i. In which 1gm of each soil sample was weighed and mixed in 10 ml of Sterile Saline.
  ii. Then the 1ml from first tube is added to the next tube containing 9 ml Sterile Saline, in this way samples were then serially diluted 5 times. (as in figure no.2)
  iii. Out of the 6 dilutions, 100µl from the each dilution (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) of each sample were spread on sterilized Nutrient Agar Plates by sterile spreader under aseptic conditions.
  iv. This plates were incubated in incubator at 37^0C for 24 hrs. for bacterial colony. (as in figure no. 3)



SOIL SAMPLE 1 | SOIL SAMPLE 2 | SOIL SAMPLE 3

Figure No. 2 (Standard Serial Dilution)



SOIL SAMPLE 1 | SOIL SAMPLE 2 | SOIL SAMPLE 3

Figure No. 3 (Spread Plate Method)

3. PURE CULTURE MAINTAINANCE BY STREAK PLATE METHOD

- **Requirement:-**
  a. Inoculating loop (wire loop)
  b. Sterile nutrient agar plates / slants
  c. Plates of serial dilutions
- **Protocol:-**
  i. The inoculating loop was sterilized by putting it in flame till red hot.
  ii. After cooling it down, the well isolated colonies were picked from the spread plates and were streaked on the Nutrient Agar plates.
  iii. Plated were incubated for 24hrs at 37^0C. Then stored at 4^0C for subsequent studies. (as in figure no.4)

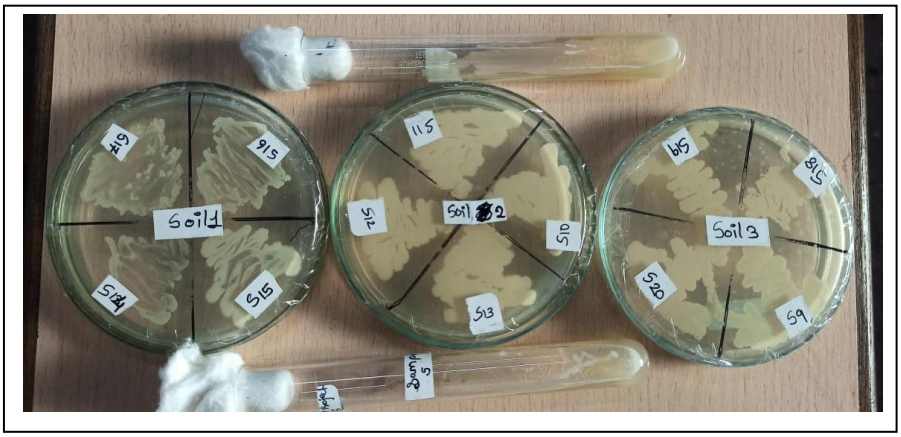


Figure No. 4 (Pure Culture)

4. MORPHOLOGICAL CHARACTERIZATION OF BACTERIA

➢ Morphological characterization of bacteria was performed by Gram's Staining.

- **Requirements:-**
  a. Bacterial suspension
  b. Wire-loop
  c. Slides
  d. Crystal violet
  e. Decolorizer (ethanol)
  f. Gram's iodine
  g. Distilled water
  h. Tap water
  i. Burners
- **Protocol:-**
  i. Bacterial suspension were made in sterile saline and were used.
  ii. Under aseptic conditions, loop full suspension was picked and smear was made on a clean grease free slide. Then they were air-dried and heat-fixed.
  iii. Stain the smear with Crystal Violet Stain for 1 minute & wash the slide in gentle and indirect stream of tap water for 2 sec.
  iv. Flood the smear with Gram's Iodine and wait for 1 minute & wash the slide in gentle and indirect stream of tap water for 2 sec.
  v. Add drop by drop Gram's Decolorizing agent to smear till slide runs clear.
  vi. Flood slide with counterstain, Safranin and wait for 30 seconds & Wash the slide in gentle and indirect stream of tap water until no color appears and then blot dry with absorbent paper.
  vii. Smear was covered with Oil Immersion and observed under microscope. (As in figure no. 5)

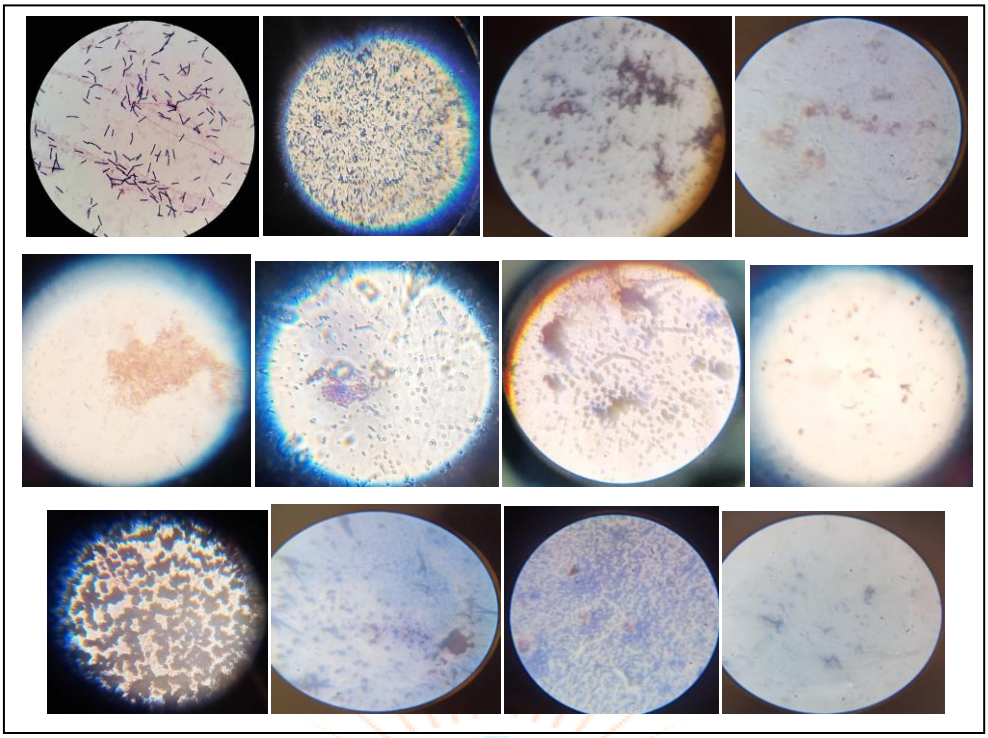


Figure No. 5 (Gram's staining)

5. BIOCHEMICAL CHARACTERIZATION OF BACTEIA

5.1. CATALASE TEST

- **Requirement:-**
  a. Wire loop
  b. Ethanol
  c. Slides
  d. 3% H_2O_2 (Hydrogen Peroxide)
  e. Pure bacterial suspension
- **Protocol:-**
  i. Taken a loop full of bacterial suspension and make smear on an ethanol cleaned grease free slide.
  ii. Then add 1 drop of 3% H_2O_2 (Hydrogen Peroxide) (do not mix or cover the slide with cover slip) and place slide in Petri plate and observe instantly. (results in figure no. 6)

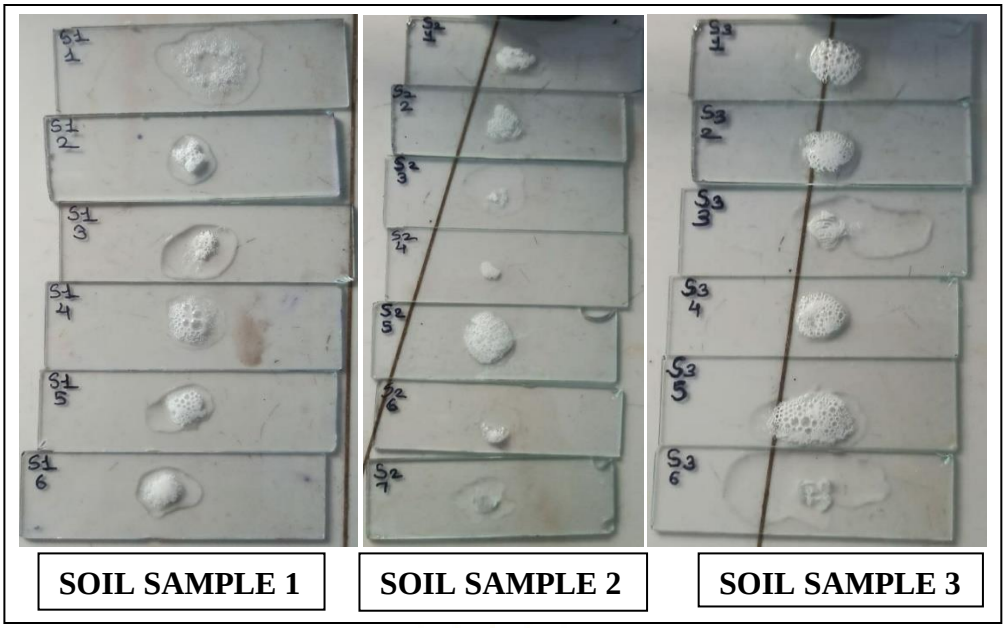


Figure No. 6 (Catalase Test)

5.2. GELATIN HYDROLYSIS TEST

- **Requirement:-**
  a. Gelatin Hydrolysis media

     Gelatin……..120 g/L

     Peptone…….5 g/L

     Beef extract……3 g/L

     pH…….6.8

  b. Sterile test-tubes
  c. Wire-loop
  d. Pure bacterial suspension
- **Protocol:-**

  i. Pick-up the colony with wire-loop from the pure culture and inoculate it by stabbing 4-5 times, half inch into Gelatin Hydrolysis Media.

  ii. Incubate tubes in incubator for 48 hrs. at 37^0C.

  iii. Remove tube from incubator and place in ice bath or refrigerator for 30 minutes or until control tube solidify.

  (Results in figure no. 7)

Figure No. 7 (Gelatin Hydrolysis Test)

5.3. INDOLE TEST

- **Requirements:-**
  a. Tryptone Broth

     Tryptone……10 g/L

     $NaCl_2$……..5 g/L

  b. Kovac's Reagent

     Amyl Alcohol ……..…….150 ml

     DMAB (p-dimethylaminobenzyldehyde).….10g

     HCl……...50 ml

  c. Sterile test-tubes
  d. Dropper
  e. Wire loop
  f. Pure Bacterial culture
- **Protocol:-**
  i. Take 2ml Tryptone Broth and inoculate it with pure bacterial culture.
  ii. Incubate tubes in incubator at 35^0C for 24-48 hrs.
  iii. Add 5 drops of Kovac's Reagent to tubes.

(Results in figure no. 8)

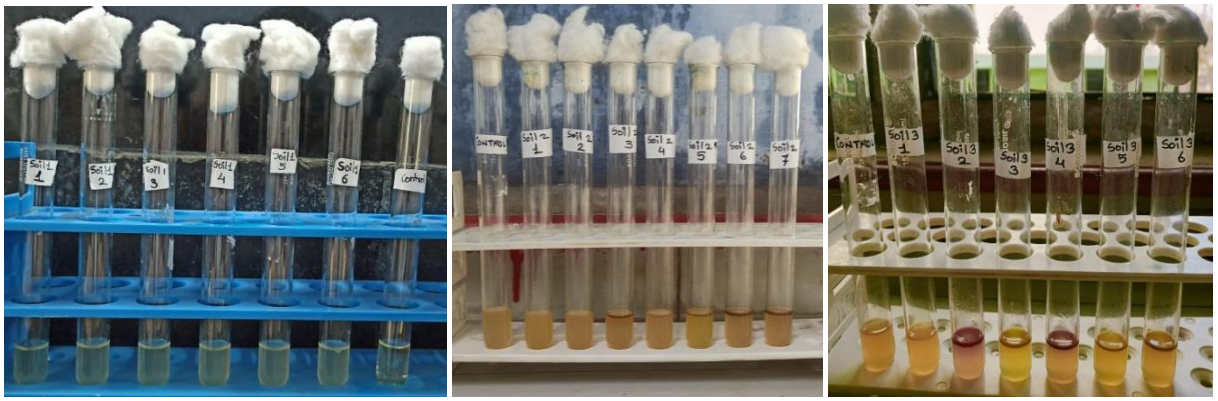
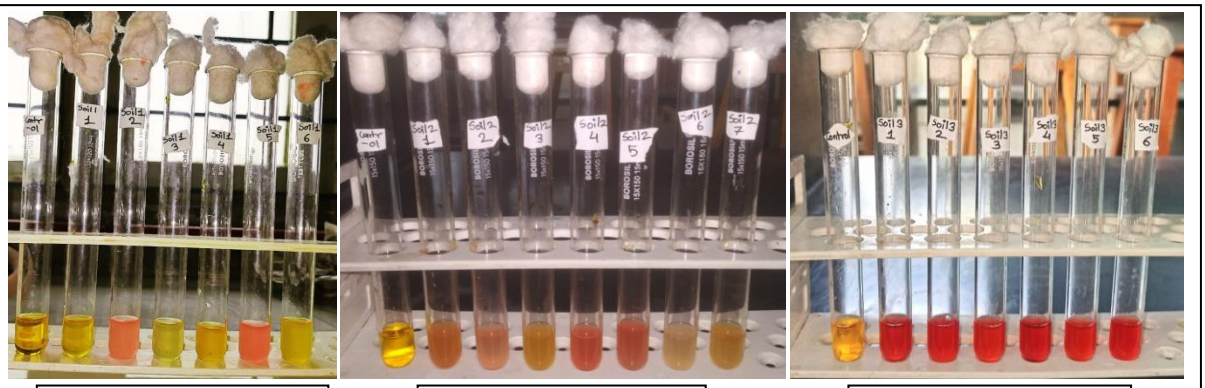


Figure No. 8 (Indole Test)

5.4. METHYL RED TEST

- **Requirement:-**
  a. MRVP Broth
     Buffered peptone……7 g/L
     Glucose……5 g/L
     Dipotassium phosphate……5 g/L
     pH……6.9
  b. Methyl Red Solution (0.02%)
     Methyl red……0.1 g
     Ethyl Alcohol……300 ml
     Distilled Water……..makeup volume to 500 ml
  c. Wire loop
  d. Pure bacterial culture
  e. Sterile test-tubes
  f. dropper
- **Protocol:-**
  i. Take 2 ml MRVP Broth in tubes and inoculate it with pure bacterial culture.
  ii. Incubate the tubes in incubator at 37^0C for 24 hrs.
  iii. Add 2-3 drops of Methyl red Indicator to tubes and observe it immediately.

(Results in figure no. 9)



SOIL SAMPLE 1 | SOIL SAMPLE 2 | SOIL SAMPLE 3

Figure No. 9 (Methyl Red Test)

5.5. MOTILITY TEST

- **Requirement:-**
  - **a.** SIM (Sulphur, Indole, Motility) Media

    Pancreatic Digest of Casein……20 g/L

    Peptic Digest of Animal Tissue…..6.1 g/L

    Agar……3.5 g/L

    $Fe(NH_4)_2(SO_4)_2.6H_2O$ …… …0.2 g/L

    $Na_2S_2O_3.H_2O$…… … …..0.2 g/L

    Ph……7.3
  - b. Wire-loop
  - c. Pure bacterial culture
  - d. Sterile test-tubes
- **Protocol:-**
  - i. Take pure bacterial culture with wire loop and stab the colony in SIM Media to 1/3 inch in middle of tube and remove.
  - ii. Incubate the tubes in incubator for 7 days at 35-37^{0}C.

(results in figure no. 10)

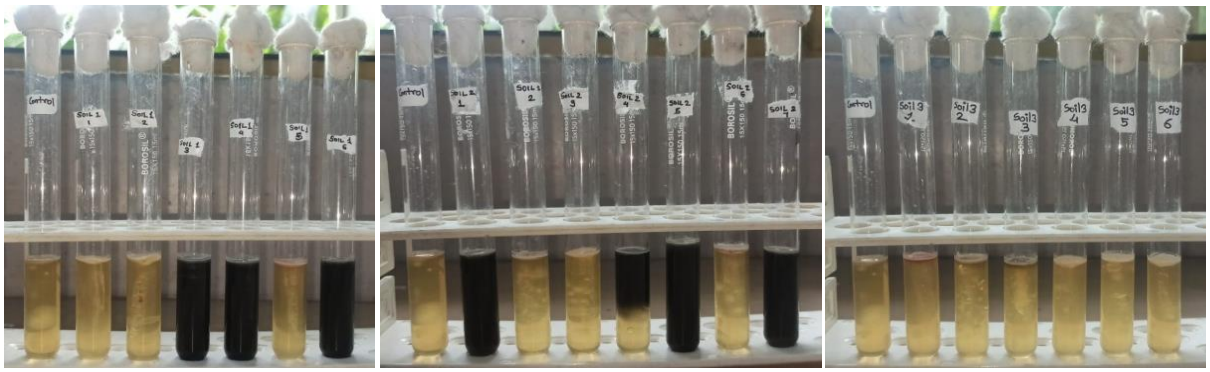


Figure No. 10 (Motility Test)

5.6. CITRATE UTILIZATION TEST

- **Requirement:-**
  a. Simmons's Citrate Agar

  $NaCl_2$.....5 g

  Sodium Citrate (dehydrate)…….2 g

  Ammonium dihydrogen phosphate…….1 g

  Dipotasium phosphate……..1 g

  Magnesium Sulphate (heptahydrate)…0.2 g

  Bromothymol Blue……...…..0.08 g

  Agar……..……15 g

  Deionized water…….....1000 ml

  Ph……6.9

  b. Pure bacterial culture

  c. Sterile test-tubes

  d. Wire-loop

- **Protocol:-**

  i. Make the Simmons's Citrate Agar slants.

  ii. Pickup pure bacterial culture with wire loop and streak from center.

  iii. Incubate the tubes in incubator for 4-7 days at 35-37^{0}C.

  (Results in figure no. 11)

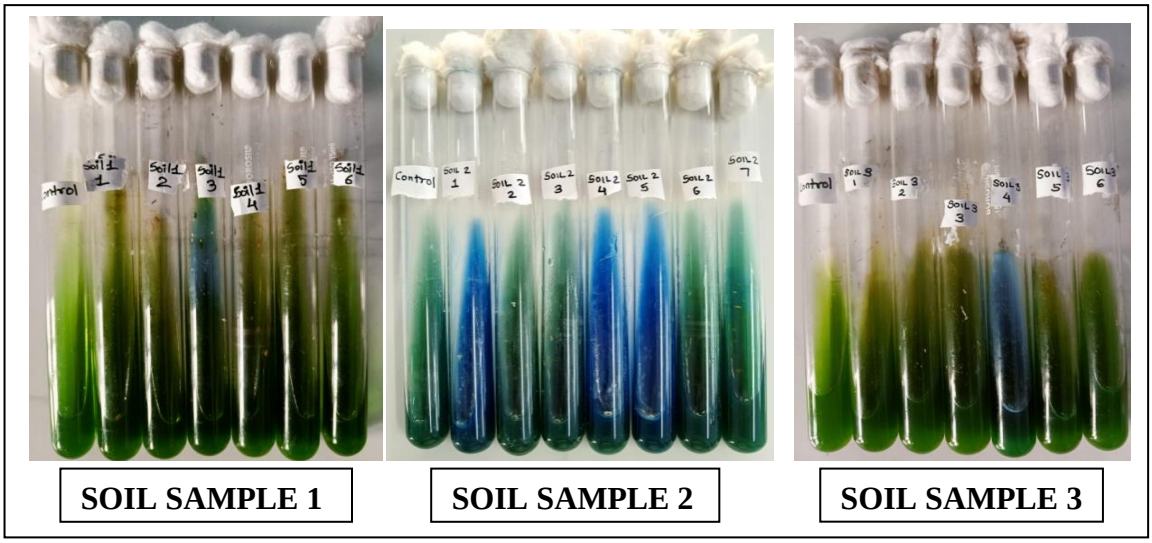


Figure No. 11 (Citrate Utilization Test)

5.7. VOGES-PROSKAUER TEST

- **Requirement:-**
  a. MRVP Broth
     Buffered peptone……7 g/L
     Glucose……5 g/L
     Dipotassium phosphate……5 g/L
     pH……6.9
  b. Voges-Proskauer Reagent A
     α-napthol 5%.....50 g
     Absolute Ethanol……..1000 ml
  c. Voges-Proskauer Reagent B
     Potassium hydroxide……400 g
     Deionized Water……1000 ml
  d. Pure bacterial culture
  e. Sterile test-tubes
  f. Wire-loop
- **Protocol:-**
  i. Take 2 ml of MRVP Broth in test-tubes and inoculate tubes with pure bacterial culture. Incubate tubes in incubator at 37^0C for 24 hrs.
  ii. Add 6 drops of Voges-Proskauer Reagent A in test-tubes and mix well to aerate & Add 2 drops of Voges-Proskauer Reagent B in test-tubes and mix well to aerate.
  iii. Observe for pink-red color at surface within 30 minutes.
  iv. Shake the tubes vigorously during 30 min period.

(Results in figure no. 12)

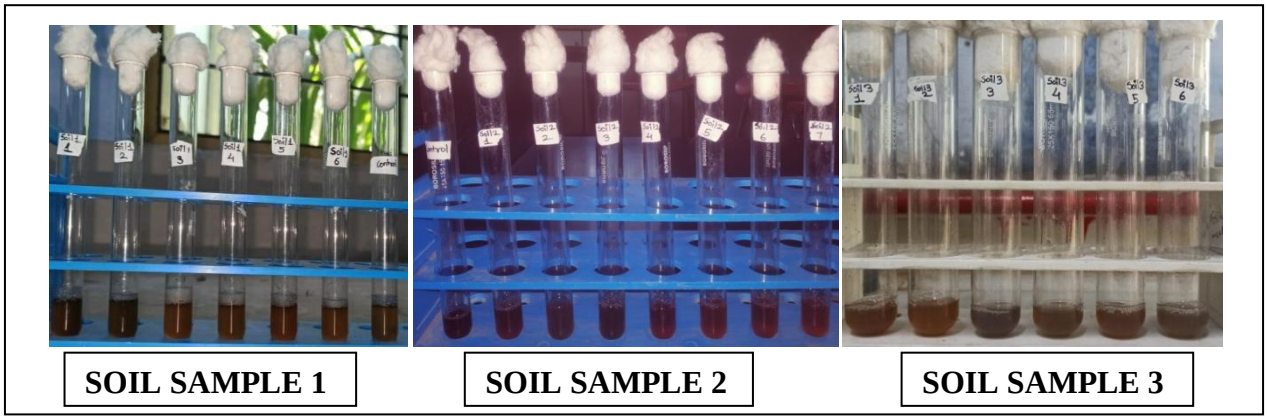


Figure No. 12 (Voges-Proskauer Test)

- **All biochemical results in table no. 1, table no. 2 & table no. 3**

Sr. no.	Gram's staining	Catalase Test	Gelatin Hydrolysis Test	Indole Test	Methyl Red Test	Motility Test	Citrate Utilization Test	Voges-Proskauer Test
1	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve
2	+ve	+ve	+ve	-ve	+ve	-ve	-ve	-ve
3	-ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve
4	-ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve
5	+ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve
6	-ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve
Control	------	------	-ve	-ve	-ve	-ve	-ve	-ve

Table No. 1 (Soil Sample 1)

Sr. no.	Gram's staining	Catalase Test	Gelatin Hydrolysis Test	Indole Test	Methyl Red Test	Motility Test	Citrate Utilization Test	Voges-Proskauer Test
1	-ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve
2	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve
3	-ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve
4	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve
5	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
6	+ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve
7	+ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve
Control	------	------	-ve	-ve	-ve	-ve	-ve	-ve

Table No. (Soil Sample 2)

Sr. no.	Gram's staining	Catalase Test	Gelatin Hydrolysis Test	Indole Test	Methyl Red Test	Motility Test	Citrate Utilization Test	Voges-Proskauer Test
1	+ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve
2	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve
3	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve
4	+ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve
5	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve
6	+ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve
Control	------	------	-ve	-ve	-ve	-ve	-ve	-ve

Table No. 3 (Soil Sample 3)

6. ANTIMICROBIAL RESISTANCE, SUSCEPTIBILITY PROFILING OF SELECTED *Bacillus* SPECIES USING ANTIBIOTICS DISCS

- **Concentrations Of Antibiotic Discs**
  a. Ampicillin (10µg)
  b. Penicillin (10µg)
  c. Chloramphenicol (30µg)
  d. Levofloxacin (5µg)
  e. Streptomycin (10µg)

f. Amoxicillin (30µg)
g. Doxycycline (30µg)
h. Ciprofloxacin (15µg)
i. Clindamycin (2µg)

➢ **Preparation Of Antibiotic Discs**

- **Requirement:-**
  a. Whatman paper no. 1
  b. Punching machine
  c. Antibiotics solutions
  d. Forceps
  e. Petri plate
  f. Burners
  g. Sterile dropper
  h. Hot plate
- **Protocol:-**
  i. Make discs of Whatman filter paper number 1 with the punching machine and autoclave it.
  ii. Make the solution of antibiotics as per the concentration, which are purchased from medicals.
  iii. With the help of sterile forceps place a single discs on a Petri plate.
  iv. Add the antibiotic solutions dropwise on the discs.
  v. Keep the petri-plate containing disc on hotplate.
  vi. After drying, the discs are ready for further use.

  (Results in figure no. 13)

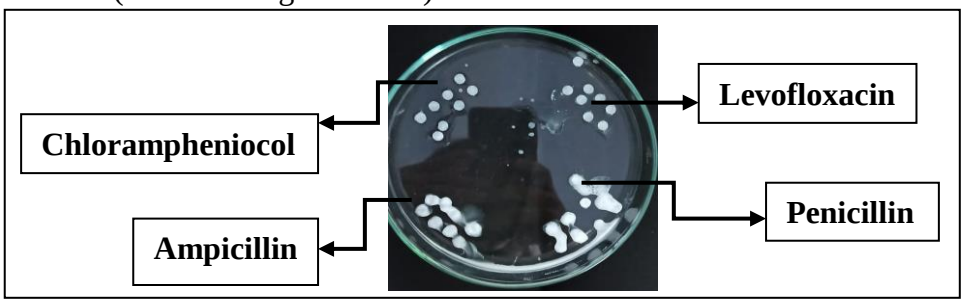


Figure No. 13 (Antibiotics Discs Preparation)

- **Kirby-Bauer Disc Diffusion Method**
  - **Requirement:-**
    a. Sterile Muller Hinton Agar plates
    b. Pure bacterial culture suspensions of selected *Bacillus* species
    c. L-shape glass rod
    d. Antibiotic discs
    e. Forceps
    f. Scale
    g. HiMedia resistance chart
    h. Sterile nutrient agar plates
    i. Wire loop
    j. Ethanol
  - **Protocol:-**
    i. Pure bacterial culture suspensions were made and spreaded on the MHA Plates.
    ii. Antibiotics discs of the above mentioned concentrations were placed carefully on spreaded bacterial culture MHA plates and left for diffusion for some time.
    iii. Then plates were incubated at 37^0C for 24 hrs. in incubator.
    iv. Later the diameter of zone of inhibition was measured using ruler and noted & compared with the resistance chart of HiMedia.
    v. The colonies which resist to more than 2 antibiotics are collected and stated as Multi-Drug resistance.
    vi. Selected colonies suspension were spreaded on the nutrient agar plates and incubated at 37^0C for 24 hrs. in incubator, for the pure culture and further studies.

    (Results in figure no. 14 & table no.4)

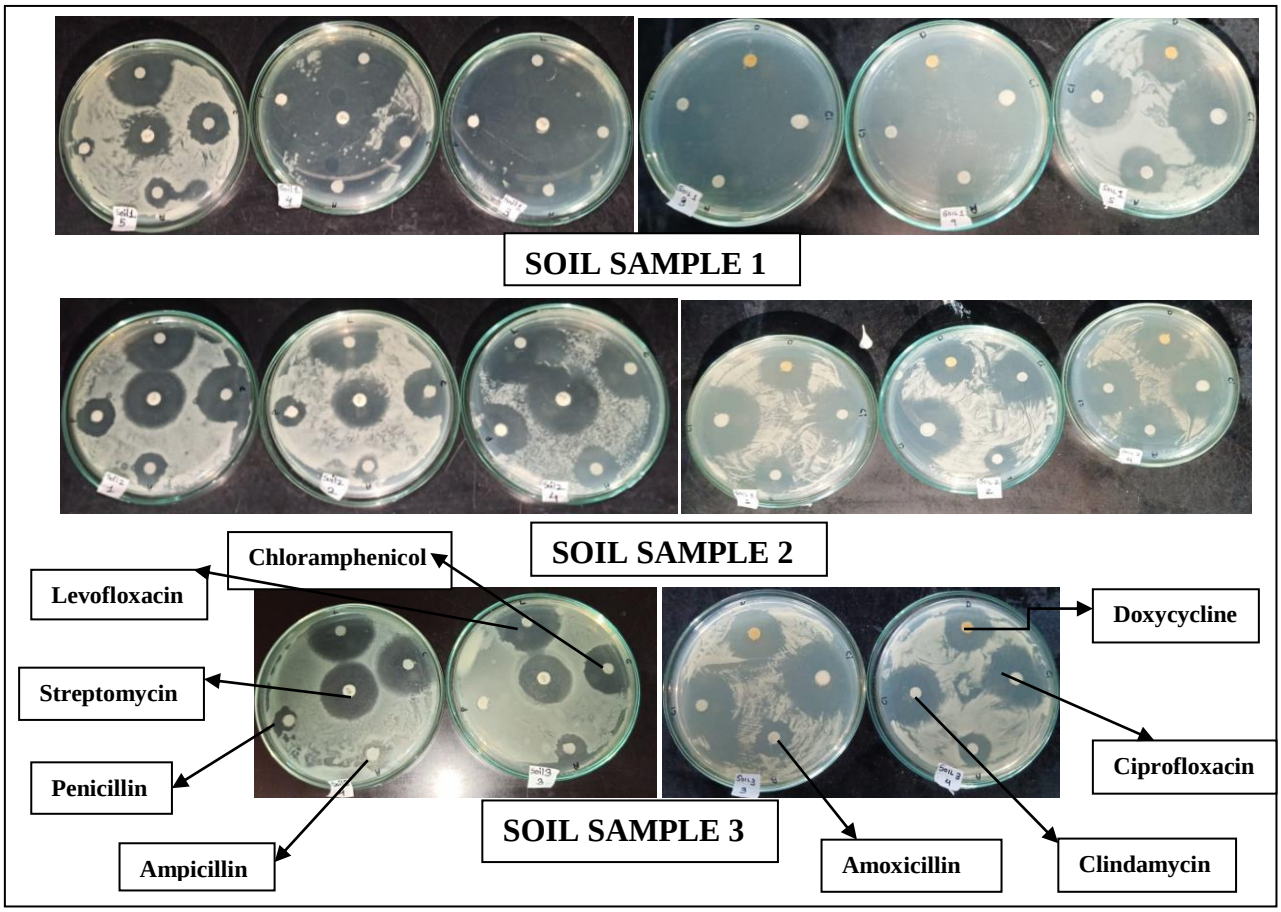


Figure No. 14 (Disc Diffusion Method)

Sr. no.	Antibiotics	Resistance diameter	S 1(3) mm	S 1(4) Mm	S 1(5) Mm	S 2(1) mm	S 2(2) mm	S2(4) Mm	S 3(3) mm	S 3(4) mm
1	Ampicillin (10μg)	≤13	-	30	12-R	14-R	8-R	22	16	10-R
2	Penicillin (10μg)	≤19	-	34	10-R	16	12-R	26	0-R	6-R
3	Chloramphenicol (30μg)	≤12	-	24	20	28	26	28	24	26
4	Levofloxacin (5μg)	≤15	-	44	34	36	32	40	32	32
5	Amoxicillin (30μg)	≤30	-	24	13-R	13-R	12-R	24	10-R	12-R
6	Ciprofloxacin (15μg)	≤45	-	42	42	28-R	24-R	36-R	36-R	28-R
7	Doxycycline (30μg)	≤12	-	24	26	26	22	24	24	22
8	Clindamycin (2μg)	≤14	-	34	24	36	32	30	32	26
9	Streptomycin (10μg)	≤11	-	30	20	32	26	32	26	30
MDR OR NOT			No	No	Yes	No	Yes	No	No	Yes

Table No. 4 (Multi-Drug Resistance)

7. CONFIRMATIONAL TEST OF *Bacillus* SPECIES BY GROWTH ON SOYABEAN CASEIN DIGEST AGAR OR TRYPTIC SOY AGAR

- **The Soybean Casein Digest Agar also known as Tryptic Soy Agar is a selective media for the *Bacillus* species.**

- **Requirement:-**
  a. Sterile Soybean Casein Digest Agar plates
  b. L-shape glass rod
  c. Ethanol
  d. Pure bacterial suspension of selected MDR species
- **Protocol:-**
  i. Soybean Casein Digest Agar plates were prepared.
  ii. 100µl of selected MDR Pure bacterial culture suspension were spreaded on plates with L-shape glass rod.
  iii. Plates are incubated in incubator at 37^0C for 24 hrs.
  iv. If growth of bacteria is seen, then *Bacillus* species are confirmed. (Results in figure no. 15)

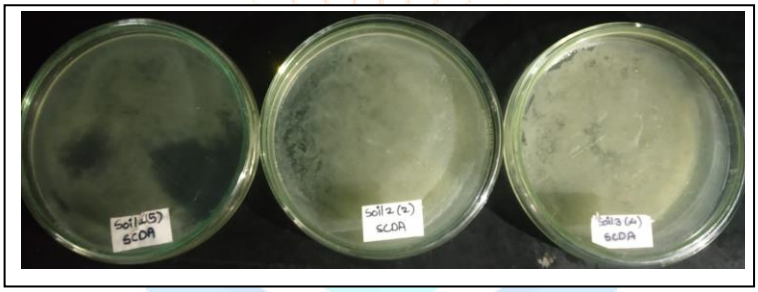


Figure No. 15 (Confirmation of *Bacillus* species by growth on soyabean casein digest agar)

8. PLASMID ISOLATION AND TO CONFIRMED PLASMID OF MDR *Bacillus* SPECIES BY USING AGAROSE GEL ELECTROPHORESIS

➢ Plasmid Isolation Of MDR *Bacillus* SPECIES

- **Requirements:-**
  a. Selected MDR *Bacillus* species suspension
  b. LB (Luria- Bertani) Broth
  c. TAE Buffer
  d. Micro-centrifuge tubes
  e. 100% Ethanol
  f. 70% Ethanol
  g. 1X TE (TRIS , EDTA) (ph-2.8)
  h. RNase
  i. Alkaline lysis solution 1
     50mM Glucose……100µl
     25mM Tris-Cl (ph-8)……...50µl
     10mM EDTA (ph-8)……20µl

Sterile double distilled water……..final volume to 2ml

j. Alkaline lysis solution 2

0.2N NaOH……40µl

1% SDS……..200µl

Sterile double distilled water……..final volume to 2ml

k. Alkaline lysis solution 3

5M Potassium acetate……..1.2ml

Glacial acetic acid……230µl

Sterile double distilled water……..final volume to 2ml

(Results in figure no. 16)

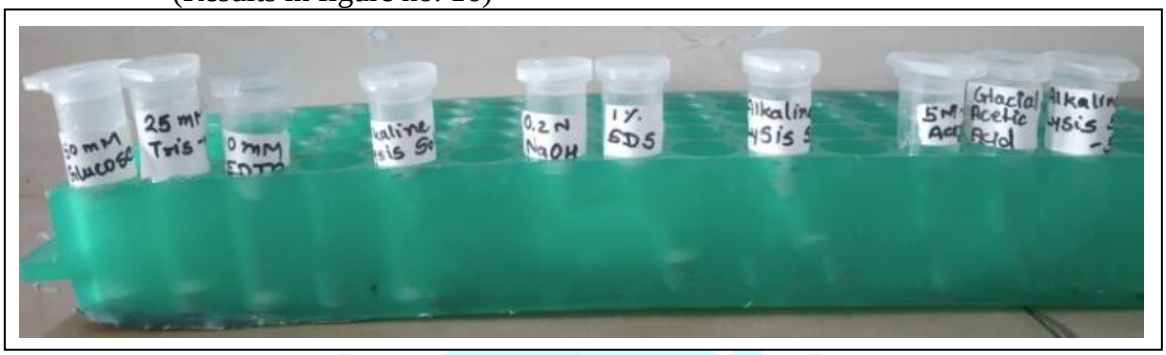


Figure No. 16 (Alkaline Lysis Solutions)

- **Protocol:-**

i. Inoculate single bacterial colony of all selected MDR species in 5ml LB Broth & Incubate the culture overnight at 37^0C by vigorous shaking.

ii. Pipette 1.5ml of each culture into micro-centrifuge tubes & centrifuge at 8000rpm at 4^0C for 10 mins.

iii. Discard supernatant and invert the tubes on blotting paper to drain out leftover supernatant. Place on ice.

iv. Resuspend the pellet in 100µl of ice cold alkaline lysis solution 1 by vigorous vertexing. Place on ice for 5 mins and shift to room temperature.

v. Add 200µl of freshly prepared alkaline lysis solution 2 to each bacterial suspension at room temperature. Close the tubes and mix content by inverting the tubes rapidly by 5 times. Do not vortex. Store tubes on ice for 5-10 mins.

vi. Add 150µl of ice cold alkaline lysis solution 3. Close the tubes and mix gently by inverting the tube several times. Store tubes on ice for 3-5 mins.

vii. Centrifuge tubes at 8000rpm at 4^0C for 10 mins. & Transfer supernatant immediately to another fresh micro-centrifuge tubes.

viii. Add 450µl of 100% ethanol to precipitate the DNA. Mix by inverting the tubes. Incubate at room temperature for 10-15 mins.

ix. Centrifuge at 6000rpm for 30 mins at 4^0C & Discard supernatant and invert the tubes on blotting paper to drain out leftover supernatant.

x. Add 1ml of 70% ethanol to pellet. Close the tubes and mix the tubes by inverting several times.

xi. Recover the DNA by centrifugation at maximum speed for 2min at 4^0C & remove supernatant by aspiration.

xii. Dry the sample by storing open tubes at 37^0C for 10-15 min till there is no traces of ethanol or until ethanol has evaporated.

xiii. Resuspend the pellet (DNA) in 50µl of TE + 20µg/ml DNase free RNase & mix it by tapping the tubes so that DNA goes into the solution.

xiv. Store DNA solution at -20^0C.

(Results in figure no. 17)

Figure No. 17 (Plasmid Isolation)

- **Preparation of 1% agarose gel**
  - **Requirement:-**
    a. Agarose
    b. Ethidium Bromide
    c. 1X TAE Buffer
    d. Gel box
    e. Well comb
    f. Beaker
    g. Pipette
  - **Protocol:-**

i. Take 100 ml of 1X TAE Buffer in beaker add 1gm agarose powder.

ii. Heat for 1-3 min until the agarose is completely dissolved (do not over boil the solution).

iii. Let agarose solution cool down to about 150^0C (about when you can comfortably keep your hand on beaker) about 5 min.

iv. Add Ethidium bromide (EtBr) to solution about 2-3µl.

v. Pour the agarose into a gel tray with the well comb in place.

vi. Let sit the gel at room temperature for 20-30min, until I has completely solidified.

vii. Remove the combs. Gel is ready to check purity of genomic DNA.

(Results in figure no. 18)

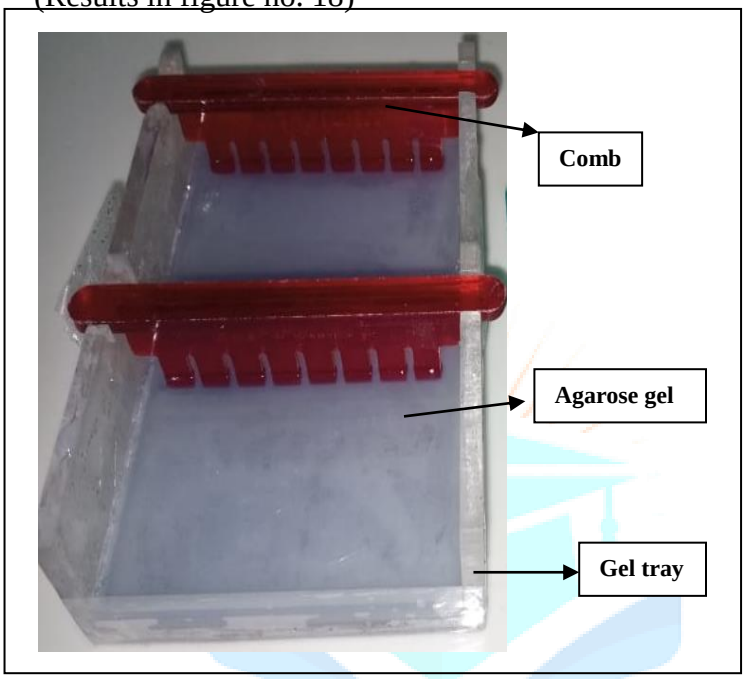


Figure no. 18 (Gel preparation)

➢ **To check the purity of genomic DNA**

- **Requirement:-**
  a. Agarose gel with wells
  b. DNA solution isolated from MDR *Bacillus* species
  c. Loading dye
  d. Micropipette
  e. 1X TAE Buffer
  f. Casting tray
  g. Voltage source
  h. Ice pack
  i. UV Trasilluminator

- **Protocol:-**
  i. Fill the casting tray with 1X TAE buffer.
  ii. Place the gel in such a position that it is near to negative electrode.
  iii. Load the well with 6µl loading dye and 12µl isolated DNA sample.
  (As in figure no. 19)
  iv. Cover the electrophoresis chamber with ice pads.

v. Run the electrophoresis at 100V for 1-2 hrs. , till it runs 3/4th.

vi. Remove the gel and observe the bands under the UV light.

(As in figure no. 20)

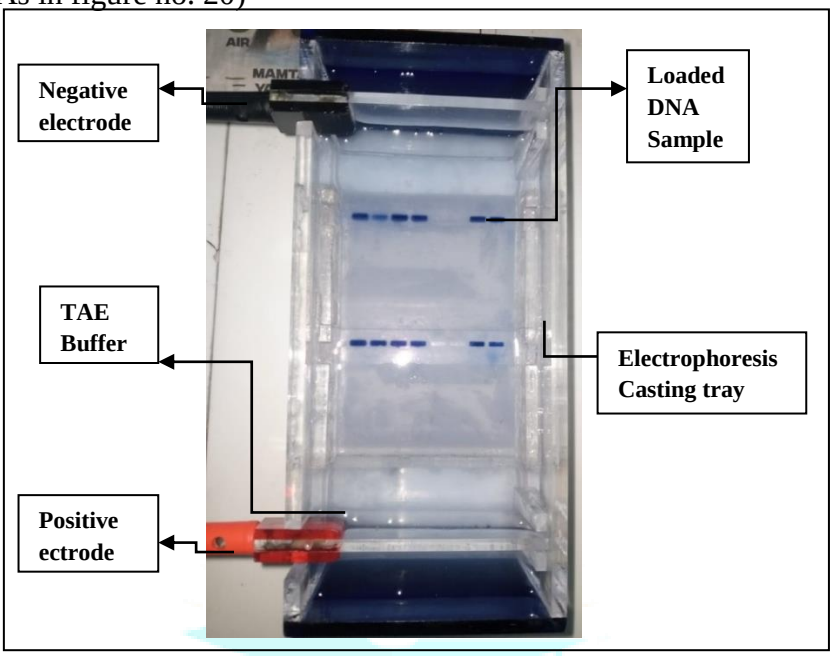


Figure no. 19(Purity Checkup of Genomic DNA)

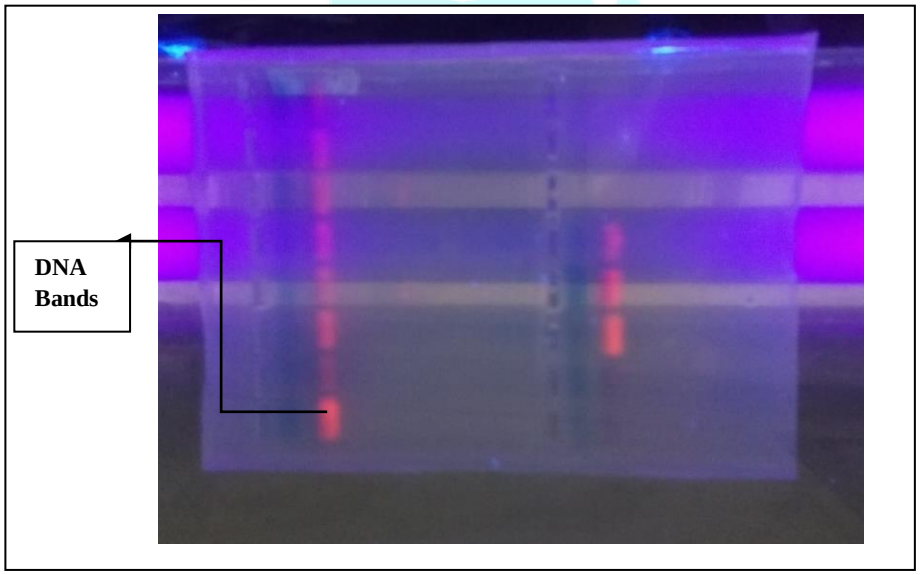


Figure No. 20 (Purity of Plasmid DNA Under UV Light)

9. HEMOLYTIC ANALYSIS

- **Hemolysis test to detect that , does the MDR *Bacillus* species rupture the RBC's & cause harm to humam**
  - **Requirement:-**
    a. Human blood sample
    b. Sterile saline solution
    c. Selected MDR *Bacillus* species culture

d. Syringe
e. Sterile test-tubes
f. Sterile pipettes

- **Protocol:-**

i. Take 3ml sterile saline in sterile test-tubes.
ii. Inoculate it with bacterial culture.
iii. Take one tube as control, in which no bacterial culture is present.
iv. Add 0.5ml blood sample to each tube, leaving control tube.
v. Incubate the tubes at 35^0C for 24 hrs. (As in figure no. 21)

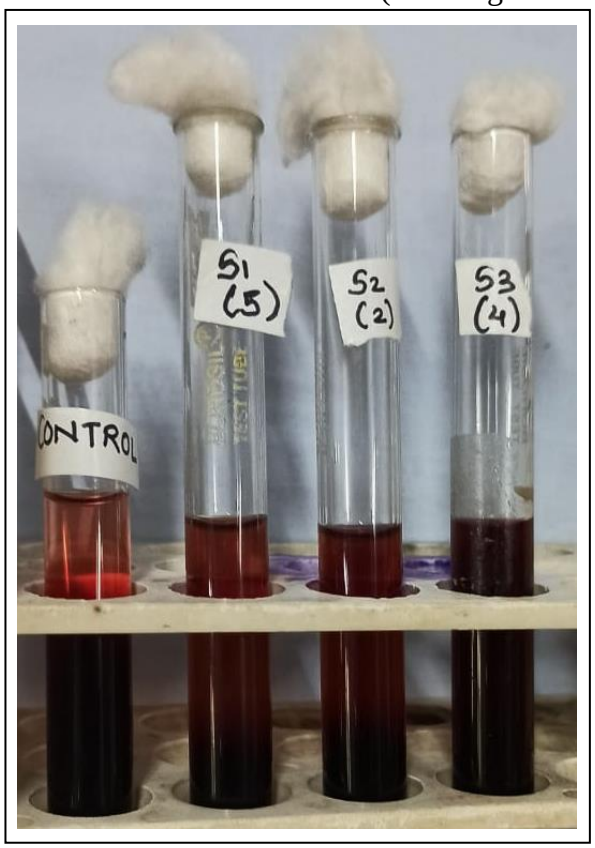


Figure No. 21 (Hemolysis Test)

RESULT & DISCUSSION :

- **Environmental and Soil Characterization of the Study Sites**

Three soil samples from various sites were collected such as, near hospital area, near industrial area and near construction area were selected for this study: To characterize the antibiogram and Hemolytic properties of these 3 various soil samples. We performed Multi-Drug Resistance test and Hemolytic test, to check whether the MDR *Bacillus* species and can become a infectious and harmful for the human life,

- **Gram Staining and Biochemical Characterization of Isolates**

  Nutrient agar were used to isolate strains from the soil samples. (For details, see Materials and Methods). Biochemical characterization were performed to select the *Bacillus* Species. To taxonomically identify selected MDR *Bacillus* species isolated, their gene was amplified and sequenced. Similar studies were observed in various research paper to collect the MDR data of bacteria. Regarding genera isolated, *Bacillus* was the most represented genus in our study, with 6 isolates from site near construction, 6 isolates from site near hospital area and 6 isolates from site near industrial area.

- **Multi-Drug Resistance Profiling**

  In the present studies, the susceptibilities of comprising *Bacillus* species against antibiotics were determined. Species associated differences in the resistance of the strains to concentrations of Ampicillin, Penicillin, Chloramphenicol, Levofloxacin, streptomycin, amoxicillin, Doxycycline, ciprofloxacin and clindamycin were observed among 3 *Bacillus* spp. The *Bacillus* spp. were predominantly resistant to Ampicillin, Penicillin, Amoxicillin, and Ciprofloxacin were observed. The high resistance trait may be attributed to an intrinsic characteristic of this spp. Due to the uniform distribution of the MIC values. As these spp. are resistant to more than two antibiotics they were considered as Multi-Drug Resistance. We perform hemolysis to check that does these spp. are harmful to human health.

- **Hemolytic property**

  Hemolysis tube method was followed to confirm species pathogenecity to human health, by performing hemolysis using human blood sample it got confirmed the multi-drug resistance species can be harmful to human life. As this species enters into the food chain the can be harmful the humans or them who eat the crops or vegetables or fruit grown in that soil. By this study it can be predicted about degree of pathogenecity of these bacterial species.

CONCLUSION :

The data derived from this study indicate that the soil sample may serve as multitudinous source of *Bacillus* species. These bacteria are considered as one of the most contaminants and infectious for human health. We hereby conclude that soil sample sever as source of *Bacillus* species resistant to multi-antibiotics. The antibiotic strains were identified as Ampicillin-resistant strains; Penicillin-resistant strains; ciprofloxacin-resistant strains and Amoxicillin-resistant strains. The result of this study highlighted the need to study antibiotic resistant data, as it is limited. All clinical microbiology laboratories should initiate the early identification and regular tracking of MDR, XDR, or even PDR bacterial strains in order to decrease the widespread issue

of antimicrobial resistance, which has become a global problem. Hence, a surveillance study is mandatory. As per the current and previous study antibiotic-resistance is prevalent among the most common Gram-negative *Bacillus* species.

REFERENCE :

1) Antibacterial activity of soil bacteria isolated from Kochi, India and their molecular identification. Davis Gislin, Dorairaj Sudarsanam, Gnanaprakasam Antony Raj, Kathirvelu Baskar, Department of Advanced Zoology & Biotechnology, Loyola College, Chennai 600 034, Tamil Nadu, India, Journal of Genetic Engineering and Biotechnology, Volume 16, Issue 2, February 2018.
2) Saha, Aindrila & Das, Rashmi & Dasgupta, Moumita & Dutta, Subhadeep & Haque, Ghazala & Mitra, Arup. (2014). Isolation and Characterization of Multi Drug Resistant Pathogen from Soil Samples Collected From Hospital Disposal Site. IOSR Journal of Environmental Science, Toxicology and Food Technology. 8. 74-80. 10.9790/2402-08547480.
3) Gaete, Alexis & Mandakovic, Dinka & González, Mauricio. (2020). Isolation and Identification of Soil Bacteria from Extreme Environments of Chile and Their Plant Beneficial Characteristics. Microorganisms. 8. 1213. 10.3390/microorganisms8081213.
4) Thapa, A., Budhathoki, A., Sapkota, A., Sainju, M., Shrestha, P., & Prasad Pant, S. (2021). Isolation, Identification and Screening of *Bacillus* species with Antimicrobial Activity from Different Soil Samples of Kathmandu Valley. Nepal Journal of Biotechnology, 9(2), 1–6.
5) Chalasani Ajay G., Dhanarajan Gunaseelan, Nema Sushma, Sen Ramkrishna, Roy Utpal. An Antimicrobial Metabolite from *Bacillus* sp.: Significant Activity Against Pathogenic Bacteria Including Multidrug-Resistant Clinical Strains. Frontiers in Microbiology, volume 6 (2015)
6) Finegold SM. Anaerobic Gram-Negative Bacilli. In: Baron S, editor. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston; 1996. Chapter 20. Available from: https://www.ncbi.nlm.nih.gov/books/NBK8438/
7) Antibiotics targeting Gram-negative bacteria. Radha Rangarajan, Rasika Venkataraman, in Drug Discovery Targeting Drug-Resistant Bacteria, 2020.
8) Biodegradative activities of some gram- negative bacilli isolated from kerosene treated soil grown with cowpea (Vigna unguiculata). D.O. Adetitun, O.D. Awoyemi, O.O. Adebisi, O.M. Kolawole, A.B. Olayemi. Vol. 16 No. 1, (2016) African Journals Online.

9) Ridgway JP, Peterson LR, Thomson RB Jr, Miller BA, Wright MO, Schora DM, Robicsek A. Sensitivity of surveillance testing for multidrug-resistant Gram-negative bacteria in the intensive care unit. J Clin Microbiol. 2014 Nov;52(11):4047-8. doi: 10.1128/JCM.02369-14. Epub 2014 Aug 20. PMID: 25143577; PMCID: PMC4313249.Introduction to Gram-Negative Bacilli. By Larry M. Bush , MD, FACP, Charles E. Schmidt College of Medicine, Florida Atlantic University; Maria T. Vazquez-Pertejo , MD, FACP, Wellington Regional Medical Center. Last review/revision Apr 2022 | Modified Sep 2022

10) Muntean D, Horhat FG, Bădițoiu L, Dumitrașcu V, Bagiu IC, Horhat DI, Coșniță DA, Krasta A, Dugăeșescu D, Licker M. Multidrug-Resistant Gram-Negative Bacilli: A Retrospective Study of Trends in a Tertiary Healthcare Unit. Medicina (Kaunas). 2018 Nov 26;54(6):92. doi: 10.3390/medicina54060092. PMID: 30486311; PMCID: PMC6307078.

11) Beyene D, Bitew A, Fantew S, Mihret A, Evans M (2019) Multidrug-resistant profile and prevalence of extended spectrum β-lactamase and carbapenemase production in fermentative Gram-negative bacilli recovered from patients and specimens referred to National Reference Laboratory, Addis Ababa, Ethiopia. PLoS ONE 14(9): e0222911. https://doi.org/10.1371/journal.pone.0222911

12) Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 1966 Apr;45(4):493-6. PMID: 5325707.

13) Mohamed N. Alanber, Naiyf S. Alharbi, Jamal M. Khaled, Evaluation of multidrug-resistant *Bacillus* strains causing public health risks in powdered infant milk formulas, Journal of Infection and Public Health, Volume 13, Issue 10, 2020.

14) Ridgway JP, Peterson LR, Thomson RB Jr, Miller BA, Wright MO, Schora DM, Robicsek A. Sensitivity of surveillance testing for multidrug-resistant Gram-negative bacteria in the intensive care unit. J Clin Microbiol. 2014 Nov; 52 (11):4047-8. doi: 10.1128/JCM.02369-14. Epub 2014 Aug 20. PMID: 25143577; PMCID: PMC4313249.

15) Muntean D, Horhat FG, Bădițoiu L, Dumitrașcu V, Bagiu IC, Horhat DI, Coșniță DA, Krasta A, Dugăeșescu D, Licker M. Multidrug-Resistant Gram-Negative Bacilli: A Retrospective Study of Trends in a Tertiary Healthcare Unit. Medicina (Kaunas). 2018 Nov 26;54 (6):92. doi: 10.3390/medicina54060092. PMID: 30486311; PMCID: PMC6307078.

16) Tropea A. Microbial Contamination and Public Health: An Overview. Int J Environ Res Public Health. 2022 Jun 17;19(12):7441. doi: 10.3390/ijerph19127441. PMID: 35742689; PMCID: PMC9224327.

17) Cross-Domain and Viral Interactions in the Microbiome. Rowan-Nash Aislinn D., Korry Benjamin J., Mylonakis Eleftherios, Belenky Peter. Volume 83(May 2023) (January 2019), American Society for Microbiology, https://doi.org/10.1128/MMBR.00044-18

18) Domenico P, Marx JL, Schoch PE, Cunha BA. Rapid plasmid DNA isolation from mucoid gram-negative bacteria. J Clin Microbiol. 1992 Nov; 30(11):2859-63. doi: 10.1128/jcm.30.11.2859-2863.1992. PMID: 1333482; PMCID: PMC270542.

Tran C, Cock IE, Chen X, Feng Y. Antimicrobial *Bacillus*: Metabolites and Their Mode of Action. Antibiotics (Basel). 2022 Jan 12;11(1):88. doi: 10.3390/antibiotics11010088. PMID: 35052965; PMCID: PMC8772736.

19) Errington J, Aart LTV. Microbe Profile: *Bacillus subtilis*: model organism for cellular development, and industrial workhorse. Microbiology (Reading). 2020 May;166(5):425-427. doi: 10.1099/mic.0.000922. Epub 2020 May 4. PMID: 32391747; PMCID: PMC7376258.

20) Fahim NAE, Ismail GAE, Abdelaleem MB, Elanany M, Saber SM, El-Ashry MAE. Identification, characterization and antibiotic susceptibility testing for *Bacillus* species. Iran J Microbiol. 2022 Aug;14(4):484-494. doi: 10.18502/ijm.v14i4.10234. PMID: 36721507; PMCID: PMC9867631.

21) Khan ZA, Siddiqui MF, Park S. Current and Emerging Methods of Antibiotic Susceptibility Testing. Diagnostics (Basel). 2019 May 3;9(2):49. doi: 10.3390/diagnostics9020049. PMID: 31058811; PMCID: PMC6627445.

22) Bayot ML, Bragg BN. Antimicrobial Susceptibility Testing. 2022 Oct 10. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan–. PMID: 30969536.

23) Alhumaid, S., Al Mutair, A., Al Alawi, Z. *et al.* Antimicrobial susceptibility of gram-positive and gram-negative bacteria: a 5-year retrospective analysis at a multi-hospital healthcare system in Saudi Arabia. *Ann Clin Microbiol Antimicrob* **20**, 43 (2021). https://doi.org/10.1186/s12941-021-00450-x

24) Clinical laboratory standards institute (CLSI). Performance standards for antimicrobial susceptibility testing; twenty fifth Informational Supplement, 2015. Available at http://clsi.org/blog/2015/01/08/clsi- publishes-new-antimicrobial-susceptibility-testing-standards/.