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ISOLATION OF *BACILLUS* SPP. FROM SOIL FOR ANTIMICROBIAL PRODUCTION AND ANTIBIOTIC RESISTANCE

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ABSTRACT:

Members of the *Bacillus* genus are generally found in soil and most of these bacteria have the ability to disintegrate proteins, namely proteolytic activity. Protease enzymes not only have important industrial uses, but also the proteases of these microorganisms play an important role in the nitrogen cycle, which contributes to the fertility of the soil. *Bacillus spp*. show higher level of resistance in supernatant and methanol. Ethyl acetate showed more effective against *E. coli* MTCC-1687.The result of this study showed that antimicrobial agent resistance was present in almost all isolates of *Bacillus spp.* isolated from different sample of soil. Isolates show higher level of resistance or reduced susceptibility to, Ampicillin and Ceftriaxone as there was less effective zone of inhibition observed. This study showed the distribution of antimicrobial agent resistance in *Bacillus spp.* isolates from a variety of sample and analysis of such pattern of resistance may prove to be useful beyond simple description.

KEY WORD: *Isolation of bacillus, Antibiotic Resistance, Antimicrobial Production, Ampicillin, Ceftriaxone.*

INTRODUCTION:

The bacteria are the most abundant group usually more numerous than the four combined. Soil bacteria can be rod, (bacilli) cocci (spherical) spirilla (spirals),

of these, *Bacillus* are more numerous than the others. They are one of the major groups of soil bacteria population and are very widely distributed (Bhagabati et al. 2004). The number and type of bacteria present in a particular soil would be greatly influenced by geographical location such as soil temperature, soil type, soil pH, organic matters contents, cultivation, aeration and moisture content (Davies and Williams, 1999). Members of the *Bacillus* genus are generally found in soil and most of these bacteria have the ability to disintegrate proteins, namely proteolytic activity. Protease enzymes not only have important industrial uses, but also the proteases of these microorganisms play an important role in the nitrogen cycle, which contributes to the fertility of the soil.In paddy field soil, most of the nitrogen source is stored as biomass protein and decomposes slowly to low molecular weight amino acids by the activity of soil protease. Soil protease is thought to be mainly supplied by soil microorganisms. There are numbers of bacteria having potential to produce antibiotic example of which is *Bacillus* species which produce antibiotic like bacitracin, pumulin and gramicidin which are active against Gram positive bacteria such as *Staphylococci, Streptococci, Corynebacter, Streptomyces* species which produce antibiotic like tetracycline, chloramphenicol, vancomycin, gentamycin which are active against Gram negative bacteria. Antibiotic resistance is a specific type of drug resistance virtually evolves via natural selection acting upon random mutation, but it can also be engineered by applying an evolutionary stress on a population. The use of antibiotics along with immunization and sterile techniques in hospitals has significantly decreased the number of lethal bacterial infections. However, antibiotics once seen as miracle drugs are now becoming useless in treating various bacterial diseases. The rapid emergence of resistance to antibiotics amongst pathogen generates visions of the "potential post antibiotics era threating present and future medical advances" (Wise 2008) the emergence of bacteria resistant to antibiotics is common in areas where antibiotics are used, but antibiotic resistant bacteria are also increasingly occurs in aquatic environment. The widespread use of antibiotics in medicine and in intensive animal husbandry is indicative of selection pressure exerted on bacteria. Intensive animal husbandry causes resistant bacteria to enter the environment directly from liquid manure and muck. There are several reports have also documented the presence, for example, of vancomycin resistant Enterococci (VRE) in the stools of asymptomatic individuals who have neither recently also been found in sewage, from stool of healthy farm animals and animal products, but also in surface water as well as soil.

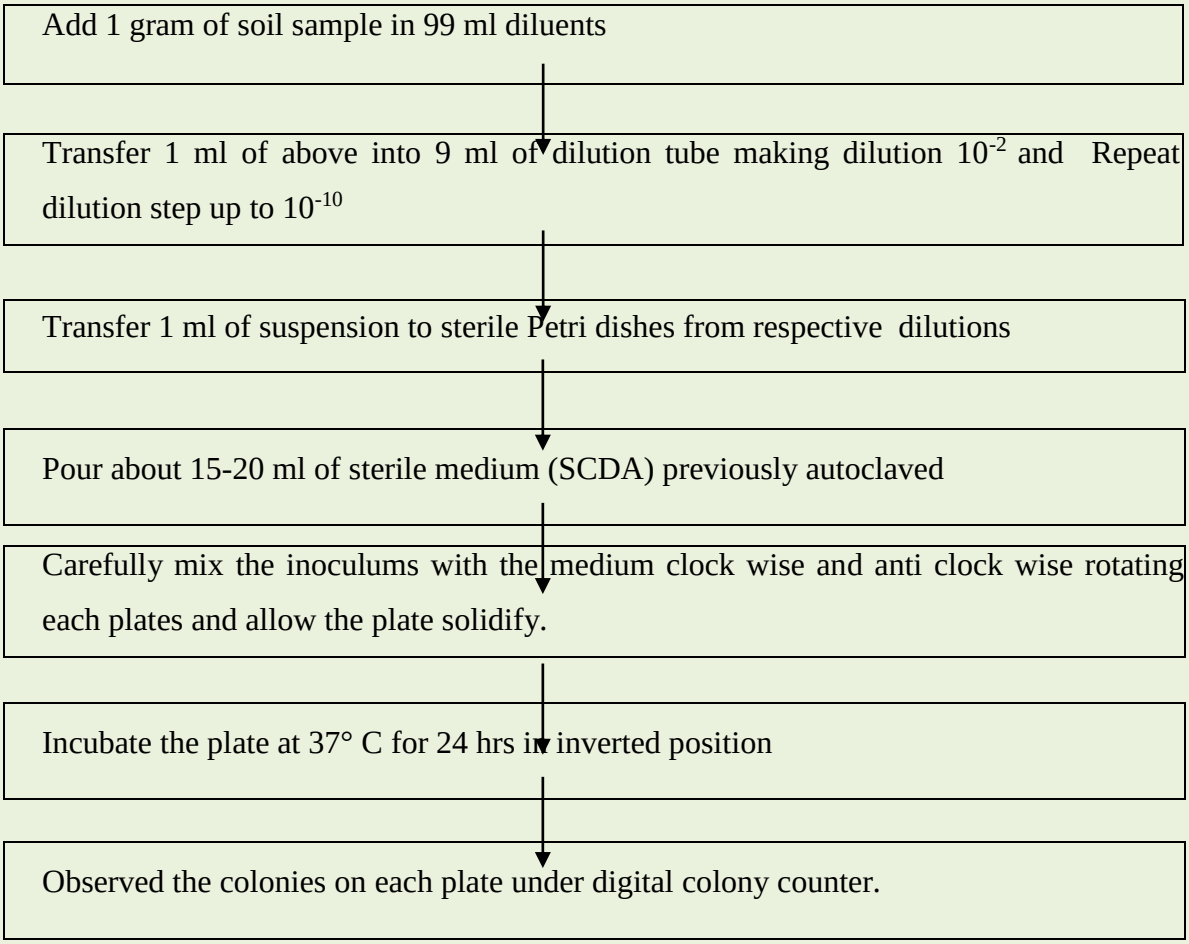
MATERIALS AND METHODS:

Collection and Preparation of Soil Sample: In systematic screening program for isolation of bacteria 20 soil samples were collected at different suburb of Delhi NCR were collected from upper layer where most of the microbial activity take place and thus where most of the bacteria population is concentrated. Soil samples (approximately 10g) were collected using clean dry and sterile container along with sterile spatula.

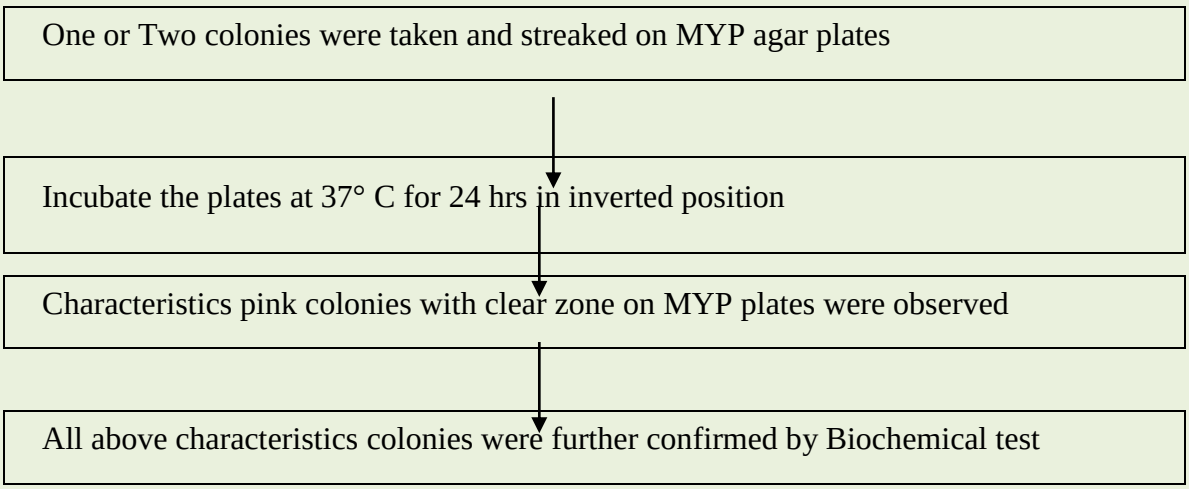
The isolation and identification of *Bacillus spp.* was done by the technique as per as per IS 5887 (Pt-6), 1999, Reaff: 2005 in test samples. Out of 20 soil samples 18 *Bacillus spp.* were isolated. further confirmation by biochemical test.

Detection and Isolation of *Bacillus* from soil sample:-

Step1: - Total Bacterial Count from soil sample



Step2: - Isolation and Identification of *bacillus spp*. By MYP Agar Selective media



After biochemical confirmation by standard biochemical tests, confirmed *Bacillus* spp. isolates were streaked on NA slants and tested for antibiotic producing against *E.coli* MTCC 739, *E.coli* MTCC 1687 and *S.aureus* MTCC 96 and antibiotic sensitivity test against four different antibiotics (Ampicillin, Meropenem, Erythromycin stearate and Ceftriaxone) by agar well diffusion assay method.

Extraction for antimicrobial compound from isolated *bacillus* strains: 24 hrs old cultures were harvested by sterile 0.85% normal saline solution and inoculate 2 ml culture in 100 ml sterile nutrient broth media flask. Incubate and shake NB flask orbital incubator shaker 37°C and 160 rpm for five days. After 5 days cultures were taken in sterile centrifuge tube from NB flask for centrifugation at 5000 rpm for 15 minutes. After centrifugation supernatant were collected in flasks. For solvent extraction 20 ml ethyl acetate and 20 ml methanol were added into the remaining biomass in centrifuge tube respectively. After centrifugation at 5000 rpm for 15 minutes ethyl acetate and methanol were collected in test tubes and solvent dried by nitrogen gas.

Inoculum Preparation: *E.coli* MTCC 739, *E.coli* MTCC 1687 and *S.aureus* MTCC 96 were sub cultured on non selective nutrient agar slants. The bacterial cultures were incubated overnight at 37°C. 0.5 McFarland density of bacterial isolates was adjusted in normal saline (0.85% NaCl) using densitometer to get bacterial population of 1.0 x 10^8 cfu /ml.

Agar Well Diffusion Assay (Zone of Inhibition Evaluation): Antimicrobial activity of isolated *Bacillus* strains was evaluated by agar well diffusion assay. 100µl of each of the adjusted cultures were mixed into separate 100 ml of sterile, molten, cool MHA, mixed well and poured into sterile petri plates. These were allowed to solidify and then individual plates were marked for each individual culture. Each plate was punched to make wells of 6 mm diameter with the help of sterile cork borer at different sites of the plates. 100 µl of supernatant and ethyl acetate, methanol solutions were pipette into the well in assay plates. Plates were incubated overnight at 37°C. Following incubation, petri-plates were observed for the inhibition zones, diameters of which were measured by using Vernier Calipers.

METHOD FOR ANTIBIOTIC SENSITIVITY TEST:

Inoculum Preparation: All 18 isolated *Bacillus cereus* culture were sub cultured on non selective nutrient agar slants. The bacterial cultures were incubated overnight at 37°C. 0.5 McFarland density of bacterial isolates was adjusted using normal saline (0.85% NaCl) using densitometer to get bacterial population of 1.0 x 10^8 cfu /ml.

Agar Well Diffusion Assay (Zone of Inhibition Evaluation): Antibiotic susceptibility and resistance were evaluated by agar well diffusion assay. 100µl of each of the adjusted cultures were mixed into separate 100 ml of sterile, molten, cool MHA, mixed well and poured into sterile petri plates. These were allowed to solidify and then individual plates were marked for each individual *Bacillus* isolates. Each plate was punched to make wells of 6 mm diameter with the help of sterile cork borer at different sites of the plates. 100 µl of respective antibiotic solutions were pipette into the well in assay plates. Plates were

incubated overnight at 37°C. Following incubation, petri-plates were observed for the inhibition zones, diameters of which were measured by using Vernier Calipers.

RESULTS AND DISCUSSION:

Antibiotic resistance and susceptibility patterns of Isolated *Bacillus spp.* were checked against four different commonly prescribed antibiotics namely Ampicillin, Erythromycin, Meropenem, and Ceftriaxone by agar well diffusion assay method. Third part was to check antimicrobial activity of isolated strains against *E.coli* MTCC 739, *E.coli* MTCC 1687 and *S.aureus* MTCC 96.

Table 1 represented the zone of inhibition in millimeter (mm) of isolated *Bacillus* strain against test four different antibiotics. Results of antimicrobial activity against *E.coli* MTCC 739, *E.coli* MTCC 1687 and *S.aureus* MTCC 96 were shown in Table 2.

CONCLUSION:

Antimicrobial activity and antibiotic resistance pattern of *Bacillus spp.* isolated from different sample of soil.The result of this study shows that many strains of the collection of *Bacillus* isolated from soil have strong antimicrobial activity against positive culture. *Bacillus spp*. show higher level of resistance in supernatant and methanol. Ethyl acetate showed more effective against *E. coli* MTCC-1687.The result of this study showed that antimicrobial agent resistance was present in almost all isolates of *Bacillus spp.* isolated from different sample of soil. Isolates show higher level of resistance or reduced susceptibility to, Ampicillin and Ceftriaxone as there was less effective zone of inhibition observed. The *Bacillus spp.* isolates showed maximum susceptibility to Meropenem as the observed zone of inhibition has more effective diameter of 30 mm. Isolates showed enter effective susceptibility to Erythromycin. The zone of inhibition measured is Erythromycin 24mm.

The microbial world has demonstrated remarkably resilience and emergence of resistance is practically invariable upon the introduction of an antibiotic into the environment. The relentless build-up of resistance may make the valuable antibiotic assets useless and a post antibiotic scenario may emerge. There are enough resources atleast in the developed world to treat their infections but the developing world is facing access issues and contributing to the resistance pool. The problem is therefore is a global and requires to be tackled on that scale.

This study showed the distribution of antimicrobial agent resistance in *Bacillus spp.* isolates from a variety of sample and analysis of such pattern of resistance may prove to be useful beyond simple description. As concern about water quality and environmental contamination by human and agricultural waster have increased it has become increasingly important to develop low cost screening tools that can be used to identify the most probable contamination. The distinct pattern of antimicrobial agent resistance may prove to be a valuable tool for the development of multivariate statistical techniques for bacterial sources identification.

Based upon these studies, it was found that most of the isolates in the present study show multiple tolerances to antibiotics. In soil there are some substances that have the potential to select for antibiotic resistances even though they are not antibiotics themselves.

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Table 1: Antibiotic resistance patterns of *Bacillus* isolates

Bacillus Isolates	Zone of inhibition*(in mm) against Antibiotics			
	Ampicillin	Erythromycin Stearate	Ceftriaxone	Meropenem
B1	18	23	15	27
B2	0	0	0	26
B3	0	11	0	25
B4	0	23	0	30
B5	21	24	18	29
B6	0	0	0	24
B7	0	0	0	0
B8	19	24	18	30
B9	20	23	17	30
B10	0	23	0	23
B11	0	0	0	23
B12	17	24	16	28
B13	0	0	0	27
B14	0	0	0	21
B15	0	20	0	22
B16	0	19	0	23
B17	0	22	17	27
B18	0	20	0	30

*Zone of inhibition in mm. Diameter including well diameter of 6.0 mm

Table 2: Antimicrobial activity patterns of *Bacillus* isolates

Bacillus Isolates	Against positive culture	Zone of inhibition*(in mm) against *E.coli* MTCC 739, *E.coli* MTCC 1687 and *S.aureus* MTCC 96		
		Supernatant	Ethyl acetate	Methanol
S-6	***E.coli* MTCC 739**	NZI	NZI	NZI
	***E.coli* MTCC 1687**	NZI	16	NZI
	***S.aureus* MTCC 96**	NZI	NZI	NZI
S7	***E.coli* MTCC 739**	NZI	NZI	NZI
	***E.coli* MTCC 1687**	NZI	11	NZI
	***S.aureus* MTCC 96**	NZI	NZI	NZI
S8	***E.coli* MTCC 739**	NZI	NZI	NZI
	***E.coli* MTCC 1687**	NZI	12	NZI
	***S.aureus* MTCC 96**	NZI	NZI	NZI
S12	***E.coli* MTCC 739**	NZI	NZI	NZI
	***E.coli* MTCC 1687**	NZI	13	NZI
	***S.aureus* MTCC 96**	NZI	NZI	NZI

*Zone of inhibition in mm. Diameter including well diameter of 6.0 mm

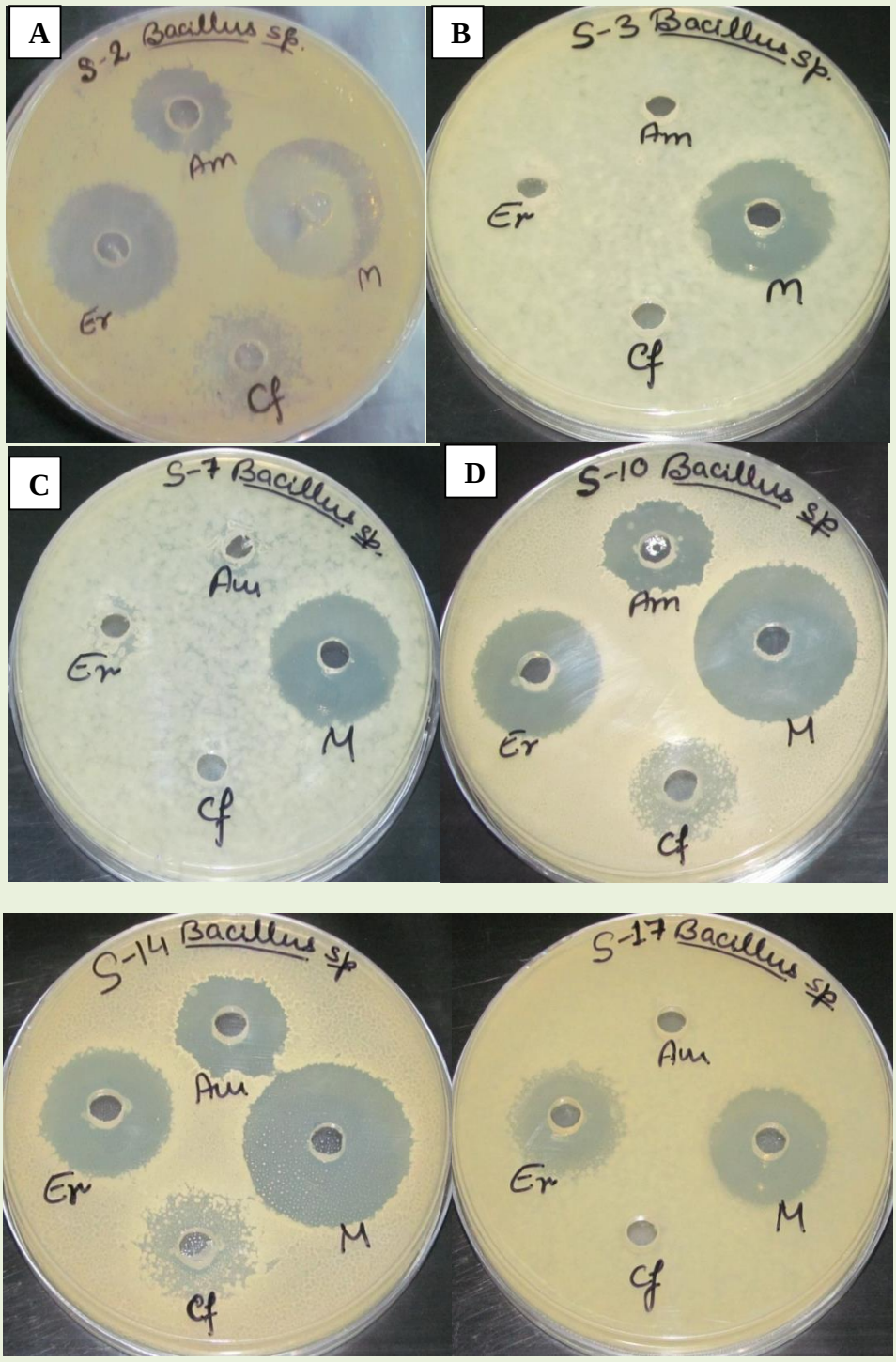

Fig. 1- Zone of inhibition against *Bacillus* isolates; Am- Ampicillin, M- Meropenem, Cf- Cefitraxone and Er. – Erythromycin

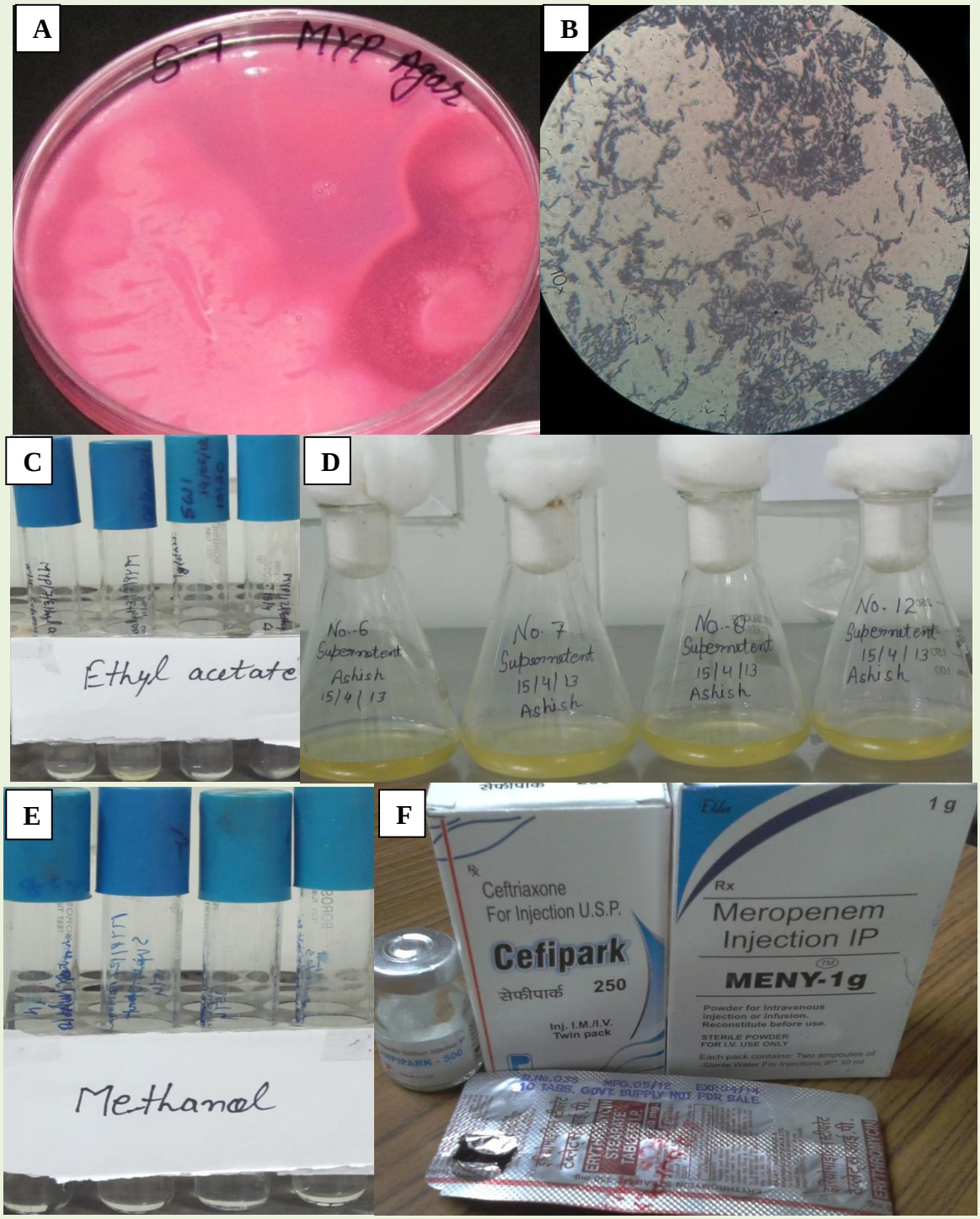

Fig 2:- Various stage of experiment; A - Characteristics colony on MYP agar plate; B - Gram staining of isolated *Bacillus spp.* C- Ethyl acetate extract; D- Supernatant of centrifugation; E - Methanol extract; F - Antibiotic used in the experiment

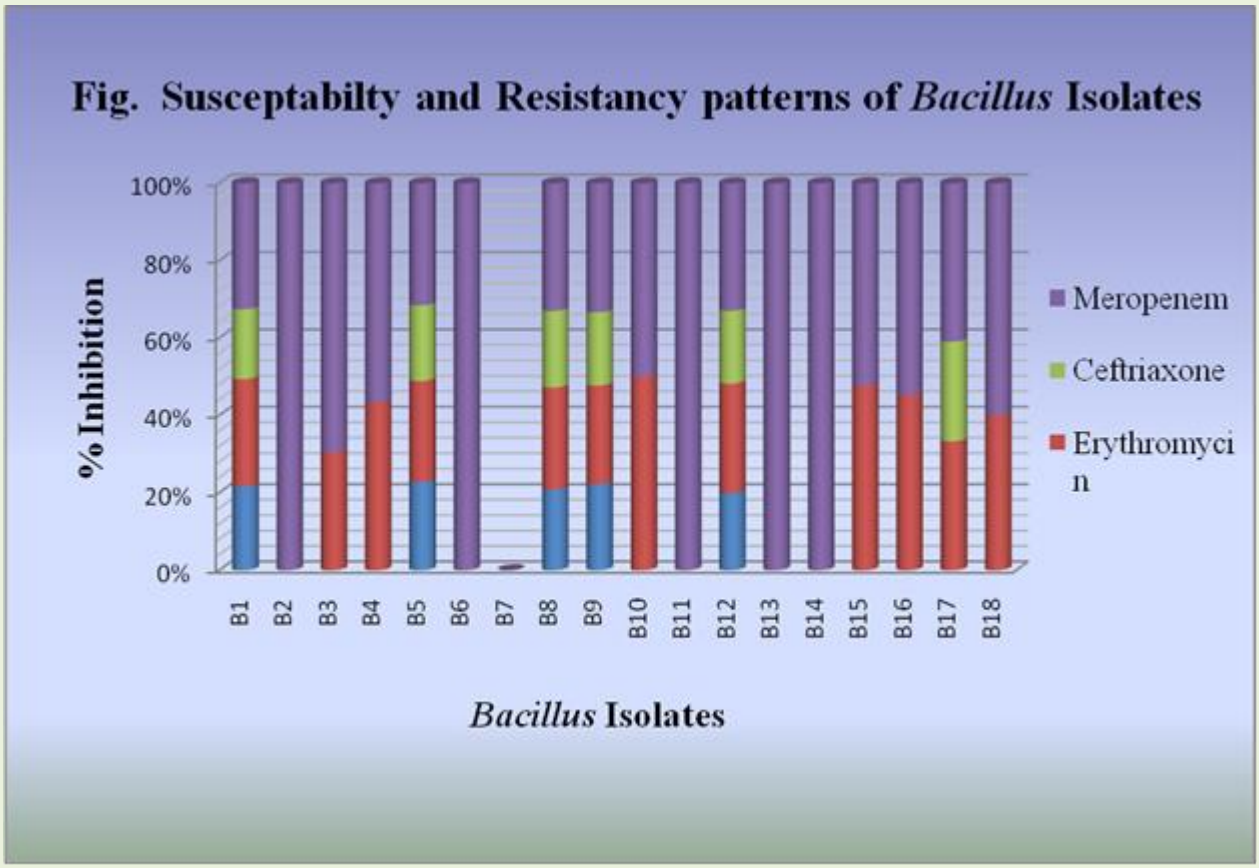
Fig. Susceptabilty and Resistancy patterns of Bacillus Isolates
% Inhibition
100%
80%
60%
40%
20%
0%
B1
B2
B3
B4
B5
B6
B7
B8
B9
B10
B11
B12
B13
B14
B15
B16
B17
B18
Bacillus Isolates
Meropenem
Ceftriaxone
Erythromycin