Isolation, Biochemical Characterization and Antibiotic Potency of Bacteria isolated from Soil Sample

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Abstract

Antibiotics are one of the most exploited secondary metabolites produced by bacteria that are employed in a range of applications. Bacteria have evolved multiple mechanisms against these antibiotics to survive. First, these bacteria may carry many genes coding for resistance to a single drug and secondly, higher expression of genes that encode for multidrug resistance. Antibiotic resistance is now a major challenge that the world is currently facing. Most of the antibiotics used today are produced by soil microorganisms. The present study aimed to isolate, biochemically characterize and assess the antibiotic potency of bacteria obtained from a soil sample. This effort screened and examined soil bacteria with antibiotic activity for morphological features that can provide valuable information about the strain. Soil, being a rich reservoir of microbial diversity, provides a valuable source for the discovery of novel microorganisms with potential applications in various fields.

In this study, microscopical, biochemical and broth microdilution (MIC) methods were carried out for the identification and antimicrobial activity of the isolates obtained from soil samples. The minimum inhibitory concentration (MIC) of antibiotics that inhibits the visible growth of the bacterium was used to determine the susceptibilities of bacteria to drugs such as antibiotics. The results highlight the diversity of bacteria in the soil and their potential contributions to the field of antibiotic discovery. The findings of this study will not only provide the evidence of prevalence of antibiotic-resistant bacteria in soil samples but will also assist in designing the appropriate dose and treatment schedule against infections.

Keywords: Antimicrobial resistance, multidrug resistance, ESKAPE pathogens, minimal inhibitory concentration.

Introduction

Bacteria are ubiquitous, single-celled microscopic living organisms. They also inhabit surfaces and substances like soil, water and food which make them key players in the ecosystems. Compared to other species, the cell structure is simpler and they usually lack membrane-bound structures and the other organelles. In contrast, bacteria contain small extra-chromosomal molecules of DNA known as plasmids that may contain genes for various functions such as antibiotic resistance, or various virulence factors[14]. Based on shapes, bacteria are classified into five groups either comma-shaped, called *vibrio*, rod-shaped, called *bacilli*, spherical, called *cocci*, spiral-shaped, called *spirilla* and coiled, called *spirochaetes*. In soil, there are 50% beneficial bacteria and 25% harmful bacteria. Conventional microbiological methods like the Gram-stain reaction, morphology and metabolic reactions have been used to identify the majority of the currently recognized bacterium species[7].

Antibiotic resistance is a persistent issue that has historically increased. Although the use of antibiotics has been beneficial, these drugs have been used so widely and for so long that the infectious organisms like bacteria, fungi, viruses and parasites are designed to kill by antibiotics. Some organisms may develop resistance to a single antimicrobial agent, while others, often referred to as multidrug-resistant or MDR strains develop resistance to several antimicrobial agents. The selection of pathogenic bacteria resistant to various medications resulted from the extensive use of antibiotics in human therapy[13]. Bacterial antimicrobial resistance (AMR) has become a global concern for human, animal and environmental health. This is due to the rise, spread and persistence of MDR organisms[24]. The necessity to develop new and novel antimicrobial agents has arisen as a result of the rise in antibiotic resistance[29].

Historical Background: Unicellular microbes, the first life forms on earth 4 billion years ago, are the ancestors of bacteria. Although bacterial fossils such as stromatolites exist, their lack of distinguishing morphology makes them ineffective for studying bacterial evolution or dating the emergence of a specific bacterial species[12]. On the other hand, gene sequences may be used to rebuild the phylogeny of bacteria and these studies show that bacteria split first from the archaeal/eukaryotic branch. It was highly likely that the most recent common ancestor of bacteria and archaea, a hyper thermophile, lived between 2.5 and 3.2 billion years ago, indicating that the bacteria may have been the first life on earth 3.22 billion years ago[2].

Multi-Drug Resistant (MDR): Bacteria that are resistant to three or more classes of antimicrobial drugs are often referred to as multidrug-resistant bacteria (MDR bacteria). Infections caused by MDR bacteria are one of the greatest challenges and pose a serious problem for public health[33]. They can be classified into 3 main categories: Gram-positive, Gram-negative and others (acid-stain). One of the primary causes for the rise in growth in MDR selection is the

overuse or improper use of antibiotics in healthcare, livestock and agricultural sectors, which resulted in the emergence of the ESKAPE bacteria[18].

The six nosocomial pathogens *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter* spp. are together referred to as "ESKAPE" due to their increasing virulence and multidrug resistance. ESKAPE pathogens are capable of escaping the biocidal action of antimicrobial agents[26]. ESKAPE is a group of both Gram-positive and Gram-negative bacteria that are highly virulent and are resistant to commonly used antibiotics. As a result, they are the leading cause of life-threatening nosocomial or hospital-acquired infections among immunocompromised and severely ill patients who are most vulnerable[26]. Two of the most prevalent ubiquitous pathogens found in healthcare are *P. aeruginosa* and *S. aureus*. Gut flora, soil, water, contamination of adjacent surfaces, equipment and hands are all common reasons and have the potential to propagate the infection.

As a result of both natural and artificial selective pressures and causes, antibiotic resistance in bacteria typically originates through genetic mutation or acquires antibiotic-resistant genes (ARGs) through horizontal gene transfer, a genetic exchange process by which antibiotic resistance can spread[16,32]. The increased resistance to routinely used medicines such as penicillin, vancomycin, carbapenems and others distinguishes ESKAPE bacteria from other pathogens[30].

Enterococcus faecium: Enterococci are Gram-positive facultative anaerobic spherically-shaped (coccus) bacteria that most usually cause healthcare-related infections (HAIs) in immunocompromised people in pairs or chains[6]. It is frequently acquired resistance to β-lactam antibiotics such as penicillin and some other antibiotics. It is the first of the ESKAPE organisms mentioned by the WHO and threatening to public health[34]. Along with an increase in *E. faecium* resistance to vancomycin antibiotics, particularly vancomycin-A, there has also been an increase in vancomycin-resistant enterococci (VRE) strains[25]. This virulent property of bacteria is also due to thick biofilm formation, which operates as a "mechanical and biochemical shield" that shields bacteria from antibiotics and is bacteria's most effective defense mechanism against therapy[30].

Staphylococcus aureus: *S. aureus* is a Gram-positive round-shaped bacterium that is widely present in the human skin microbiota and poses little threat to humans who have a healthy immune system. On the other hand, *S. aureus* can cause infections when it gets into areas of the body where it is not often prevalent such as wounds. Similar to *E. faecium*, it can cause infections on implanted medical equipment and develop biofilms that complicate antibiotic therapy. Methicillin-resistant (MRSA) is a kind of *S. aureus* that has developed resistance to certain antibiotics such as methicillin, dicloxacillin, oxacillin, cloxacillin, nafcillin and cephalosporins, making it different from another *S. aureus* strains[22].

Klebsiella pneumoniae: *K. pneumoniae* belongs to the Enterobacteriaceae family and is a Gram-negative rod-shaped (bacillus) bacterium that excels at acquiring resistance genes via horizontal gene transfer. It shows frequent resistance to phagocyte therapy because of its dense biofilm and strong adherence to surrounding cells. *K. pneumoniae* is steadily resistant to antibiotics such as penicillin and ampicillin because of its β-lactamases and belongs to the extended-spectrum β-lactamase or ESBL strains including multidrug-resistant to a wide spectrum of antibiotics such as cephalosporin, ceftazidime, or carbapenems, resulting in the emergence of carbapenem-resistant *K. pneumoniae* (CRKP), for which only a few medications are currently being developed to treat infection[20,23].

Acinetobacter baumannii: *A. baumannii* is most commonly found in hospitals where it has developed resistance to all known antibiotics. Due to its endurance for a variety of pHs, temperatures, nutritional levels and arid habitats, the Gram-negative, short-rod-shaped *A. baumannii* survives in a variety of unfavorable situations. The Gram-negative characteristics of *A. baumannii's* membrane surface, particularly the efflux pump and outer membrane, allow it to resist a larger spectrum of antibiotics[22]. MDR *A. baumannii* is resistant to several classes of antimicrobial agents including fluoroquinolones, broad-spectrum β-lactams, aminoglycosides, carbapenems and cephalosporin[1,10].

Pseudomonas aeruginosa: *Pseudomonas aeruginosa*, a Gram-negative rod-shaped (bacillus) bacteria, is a common hydrocarbon degrader that lives in the soil as well as it can survive in harsh environmental conditions. Due to its adaptability, it thrives well in the lungs of patients with late-stage cystic fibrosis (CF)[8]. Wide range of antimicrobial resistances in MDR strains of *P. aeruginosa* to antibiotics such as carbapenems, penicillins, fluoroquinolones cephalosporins, aminoglycosides, β-lactams and increased multidrug efflux pumps, makes therapy more difficult[11,28].

***Enterobacter* spp.:** *Enterobacter* refers to a group of Gram-negative, rod-shaped (bacillus) bacteria. The human population is in critical need of novel and efficient antibiotic therapies since some strains cause blood infections and urinary tract infections (UTIs) and are resistant to several antibiotics[27]. Currently, colistin and tigecycline are the only antibiotics utilized for therapy[22].

When *Enterobacter* spp. were treated with numerous steadily rising doses of benzalkonium chloride (BAC), a 5-300-fold rise in MIC was reported. Other Gram-negative bacteria of the ESKAPE group show a similar ability to adapt to the disinfectant BAC[15].

Numerous other antibiotics were discovered and developed after the discovery of penicillin in 1928. We currently assume that antibiotic therapy can cure any infectious disease. Around 100,000 tonnes of antibiotics are manufactured annually on a global scale and their use has had a substantial impact on bacterial life on earth. Pathogen strains have become more resistant to antibiotics and some have developed resistance to multiple antibiotics and chemotherapeutic drugs, known as multidrug resistance (MDR)[19]. The soil is a complex ecosystem harboring a vast diversity of microorganisms including bacteria with unique biochemical traits and potential antibiotic-production capabilities.

The present study aimed to explore soil bacteria with antibacterial activity against human pathogenic bacteria. Hence this study was undertaken to isolate, characterize, and detect the prevalence of antimicrobial resistance/MDR bacterial isolates in soil samples. Studying these microorganisms is crucial for understanding their roles in ecological processes and exploring their applications in various industries, particularly in medicine. The present study aimed to explore soil bacteria with antibacterial activity against human pathogenic bacteria.

Material and Methods

Materials: All reagents and chemicals used in this study were of high analytical grade. Other equipment used are incubator shaker, colony counter, horizontal laminar air flow and laboratory incubator.

Chemicals: 0.85% sodium saline, 70% and 95% alcohol, crystal violet (0.1g), gram's iodine (0.18 g), saffranine (0.2 g), 3% KOH, hydrogen peroxide H_2O_2 (3%), ampicillin (1mg/ml), ethanol (100%), resazurin solution (5 mg/ml) were used.

Microbial strains: For a comparison study with bacterial isolates from soil samples, the five clinical bacterial MDR strains (one Gram's positive viz. *Staphylococcus aureus* and four Gram's negative viz. *Eschericia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were used.

Sample collection, isolation and characterization of bacteria: The soil sample was collected from the grounds of Chandigarh University. The soil sample was collected by using clean, dry and sterile polybags along with a sterile spatula. Samples were stored in the polybags and transported to the laboratory, where they were stored. After that, the serial dilution of the soil sample was prepared as described previously[3]. Briefly, the sample suspension was made by adding 1g of soil sample into the stock test tube with 10 ml of sterile water under sterile conditions, vigorously shaking for at least 1 minute and then allowed to stand for a few minutes. 9 ml of the normal saline prepared was distributed into 8 test tubes for 10^{-1} through 10^{-8} sterile dilution blanks from the stock. Using a sterile pipette, 1 ml of sample from stock was transferred into a 10^{-1} dilution blank and shaken properly. For each subsequent step, 1 ml from the 10^{-1} dilution was transferred to the 10^{-2} tube, then from the 10^{-2} to the 10^{-3} and so on until 10^{-8}. 0.1ml of the sample was transferred from a 10^{-4} to 10^{-7} dilution tube into sterilized nutrient agar culture media by a sterile spreader under aseptic conditions. The plates were then incubated in an incubator at 37°C for 24 hours.

Morphology: The colony morphology was viewed with the aid of a colony counter. Morphological characteristics such as colony, edges, surface and elevation were observed.

Purification of bacteria: A number of bacterial colonies were obtained on a nutrient agar plate; the streak plate technique was used to isolate organisms from a mixed population into a pure culture on an agar plate. The inoculating loop was sterilized by putting it in the flame until it was red hot and then allowed to cool it down. Distinct isolated colonies from the spread agar plate culture were further streaked over the nutrient agar plate. The plates were then incubated for 24 hours at 37ºC.

Identification of bacteria

a) Gram staining method: The procedure is based on the reaction between peptidoglycan polymer in bacteria's cell walls. The steps involved include staining the bacteria, using a mordant to fix the color, decolorizing the cells and then applying a counterstain. A smear on a clean glass slide was made by adding a drop of water and a loop of the bacterial isolate in an aseptic condition which was then allowed to air dry and then fixed thermally. The smear was gently flooded with crystal violet and left for 1 min. before washing it with tap or distilled water. Iodine was added and allowed to stand for another 1 minute and washed away with tap water. Smear was decolorized with 95% ethanol (alcohol) or acetone until the alcohol ran almost clear and washed with tap water after 5-10 sec. The smear was counter-stained with safranin and allowed to stand for 45 sec. Gently rinse with tap water, air dry the glass slide and observe under the microscope.

b) Catalase Test: This is a test to ascertain the ability to produce catalase by bacterial isolates purified on an agar plate from a soil sample. Catalase is an enzyme that breaks down hydrogen peroxide (H_2O_2) into oxygen and water. The rapid formation of oxygen bubbles in the presence of H_2O_2 after the addition of an inoculum proves the existence of the enzyme in the bacterial isolate. The catalase test was performed by placing a drop of hydrogen peroxide on a microscope slide which contains a smear of the organism made by placing a loopful of the colony of pure culture by a sterilized wire loop.

c) KOH Solubility Test: The potassium hydroxide test (KOH test) is used to identify Gram-negative microbes. On a microscopic slide, add one drop of 3% KOH and transfer a loopful of isolated bacteria to the drop of KOH using a cooled sterile loop. Mix bacteria into the KOH solution until

an even suspension is obtained. Lift the loop and if a mucoid string can be lifted with the loop from the slide, it is a Gram-negative bacterium and if a watery suspension is produced, it is a Gram-positive bacterium.

d) Determination of Minimal Inhibitory Concentration (MIC) by broth microdilution method: The minimum inhibitory concentrations (MICs) of antibiotics against isolates and known MDR bacteria were determined by standard broth dilution assay described under CLSI guidelines using resazurin indicator solution[4]. The overnight bacterial suspensions were adjusted to achieve turbidity equivalent to a 0.5 McFarland turbidity standard with sterile nutrient broth. In a 96-well microtiter plate, for each bacteria culture, the assay consists of one vertical row of broth sterility control (only nutrient broth [NB]) and 1 vertical row of growth control (NB inoculated with bacterial culture). Plates were prepared under aseptic conditions. A 100 ul nutrient broth was poured into each of the ten wells in a vertical row. Each vertical row's first well contained 100 ul of 1 mg/ml ampicillin respectively.

The mixture was mixed thoroughly. Then, 100 µl of the mixture from the first well was transferred into the second well (2^{-2}) using a sterile pipette and thoroughly mixed. Again, 100 µl of the mixture was once more transferred from the second well into the third well (2^{-3}) and mixed properly. This serial dilution was continued to the tenth well (2^{-10}). Once all dilutions were done, 100 µl was removed from the tenth well and discarded. The final concentration of antibiotics in each well was now reduced to half of their original concentration. After that, 10 µl of diluted bacterial suspension (apart from the broth sterility well) was added into all wells and thoroughly mixed. For each bacterial species, plates were prepared in triplicate and incubated in an incubator at 37°C for 18-24 h.

After overnight incubation, 10 ul resazurin (5 mg/ml) was added to all wells and incubated at 37°C for an additional 1-2 h. The color change could be seen clearly and visually. The growth of bacterial strains was indicated by changes in color from purple to pink or colorless. The MIC value was determined as the lowest concentration before the color change. The average of three data was used to determine the MIC for both the test material and the bacterial strains.

Results

Isolation, screening and morphological characteristics of bacteria: Antibiotic resistant bacteria were isolated from the soil sample by serial dilution and streak plate methods. The growth was seen in the plates of dilution 10^{-4} to 10^{-7}. Many well-characterized colonies were obtained which were considered for further analysis. Plates having growth were sub-cultured by streak on nutrient agar media and pure colony was seen and preserved. Though it is difficult to differentiate the species based on colony morphology, the colonies of some isolates were small and large, had smooth, rough, mucoid texture, creamy white, white, pink in color, irregular, circular shape, filamentous margin and flat, raised, convex elevation (Figure 1A and 1B). The total bacteria count for the soil samples was obtained by calculating the colony forming unit per milliliter (CFU/ml) and as a result, 3×10^{10} CFU/ml was obtained.

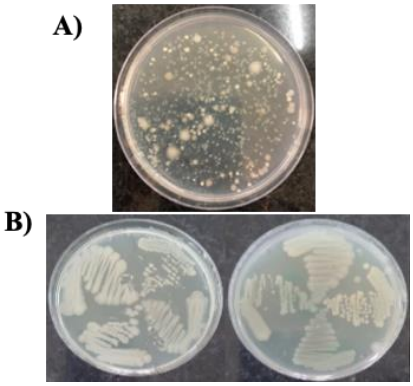

Figure 1: (A) Pictures demonstrating the colony growth of a mixture of bacterial strains after spreading in the plate. (B) Bacterial pure colonies were streaked on agar plates for further purification. The Screening was done for the isolated colonies on nutrient agar media.

Gram Staining: As a result of purification, we have obtained a pure colony from the mixture of colonies. Gram stain determination showed that the majority of the isolates had Gram-positive bacteria (1, 3 and 4) while isolates 2 and 5 were Gram-negative. The contents of isolates were found to be rods, cylindrical rods and cocci shaped based on morphological appearance as observed under the microscope. These were observed under 100x magnification in which isolates 1 (rod shape), 3 (rod shape) and 4 (cocci shape) were Gram-positive, while isolates 2 (rod shape) and 5 (cylindrical rod shape) were Gram-negative.

Gram staining of five cultures of known MDR strains viz. *S. aureus*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *A. baumannii* was also performed (Figure 2A and 2B). Among all of the bacterial strains, only *S. aureus* was Gram-positive and the rest were Gram-negative. The isolates that we collected showed morphological similarities to those of known MDR strains.

Biochemical characterization of isolated bacterial colonies from soil vis-a-vis known MDR strains

a) Catalase test: Morphology in itself was not adequate to differentiate many bacterial isolates, so it had to be complemented with some conventional microbiological biochemical characteristics. Catalase is an enzyme that converts hydrogen peroxide to water and oxygen gas. It is used to distinguish between those bacteria that produce a catalase enzyme, from non-catalase producing bacteria. The presence of the enzyme in a bacterial isolate and known MDR strains were evident when a small inoculum was added to H_2O_2 and the rapid elaboration of oxygen bubbles occured. If oxygen bubbles are generated, it is a confirmatory positive test for catalase production and if

bubbles are not generated, it is a negative sign for catalase production. The culture should not be more than 24 hrs old. Catalase test in comparison with known MDR strains, on isolates 1, 2, 3, 4 and 5 by using H_2O_2 showed that isolates 2 and 5 were positive (bubble formation) for catalase test and isolates 1, 3 and 4 were negative (no bubble formation) for catalase test (Figure 3A). The known cultures of *S. aureus*, *E. coli*, *K. pneumoniae, P. aeruginosa* and *A. baumannii* showed positive results for catalase activity (Figure 3B).

KOH Solubility Test: The Gram-negative bacteria were identified using the potassium hydroxide test (KOH). In the presence of KOH, the thin layer of peptidoglycan of the cell walls of Gram-negative bacteria disintegrates, but does not affect Gram-positive bacteria's cell walls. Disintegration of the cell walls of gram-negative bacteria results in the lysis of the cell and the release of its contents including the DNA. The DNA will make the solution very viscous and when touched, the solution will stick to the loop. Due to the presence of a thicker peptidoglycan layer in the cell wall of Gram-positive bacteria, it remains unaffected by KOH.

Thus, the cells of gram-positive bacteria will not be lysed, the DNA will not be released and no viscosity will be observed. In this study, isolates 2 and 5 showed positive KOH results as they formed the mucoid thread in the KOH solubility test indicating Gram-negative bacteria whereas isolates 1, 3 and 4 showed a negative KOH result, as no thread was formed when the loop was lifted which indicates these were Gram-positive bacteria (Figure 4A). In the case of known MDR strains, the KOH test was performed only with *S. aureus* and *E. coli* as controls (Figure 4B). In our known bacterial cultures, most of the bacteria were Gram-negative and give the KOH positive test as the thread was formed, except *S. aureus,* which is a Gram-positive bacteria.

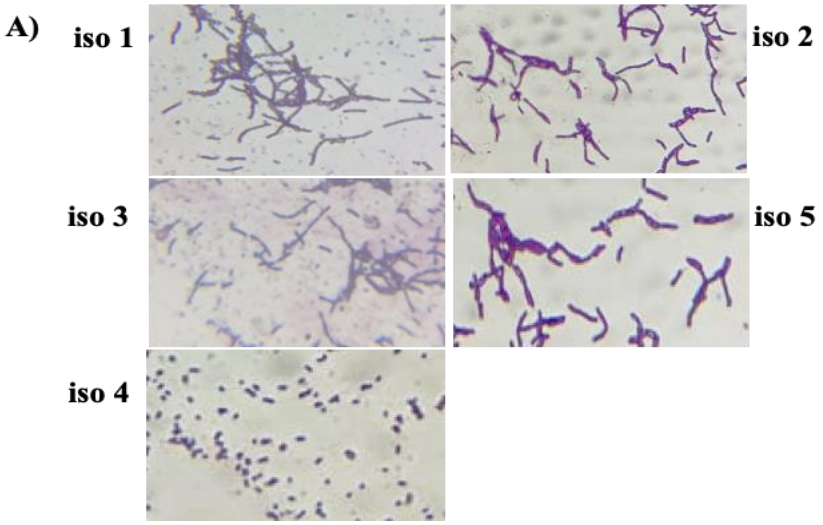

Figure 2(A): Morphological characters and gram stain for the bacterial strains isolated from soil, Chandigarh University. Bacteria cells of isolates as seen under the microscope after gram staining (Mg x 100). Isolates 1 (iso 1), 3 (iso 3) and 4 (iso 4) are gram-positive whereas isolates 2 (iso 2) and 5 (iso 5) are Gram-negative.

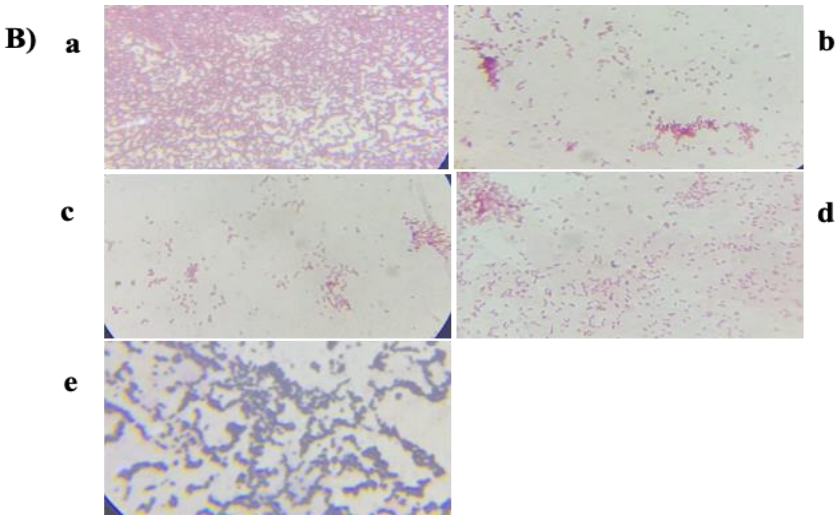

Figure 2(B): Morphological characters and gram stain for the known MDR bacterial strains. Bacteria cells as seen under the microscope after gram staining (Mg x 100). a) *E. coli,* b) *Klebsiella pneumoniae,* c) *Acinetobacter baumannii,* d) *Pseudomonas aeruginosa* and e) *Staphylococcus aureus*.

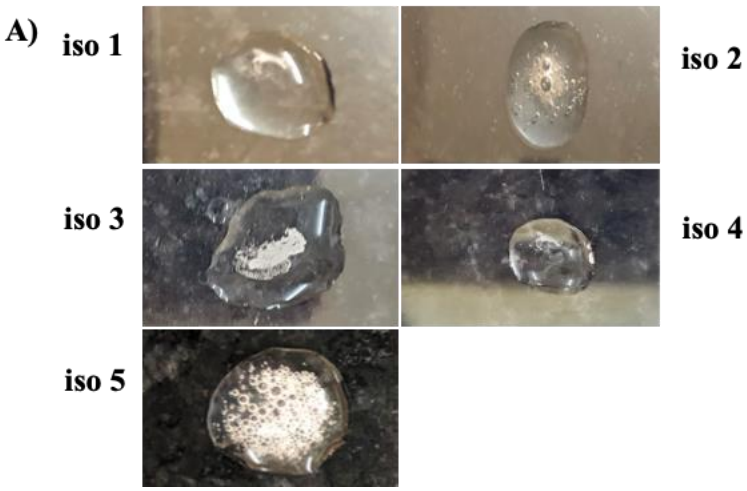

Figure 3(A): Catalase test results in bacterial isolates from a soil sample. Isolates 2 (iso 2) and 5 (iso 5) showed catalase positive whereas isolates 1 (iso 1), 3 (iso 3) and 4 (iso 4) showed catalase negative test.

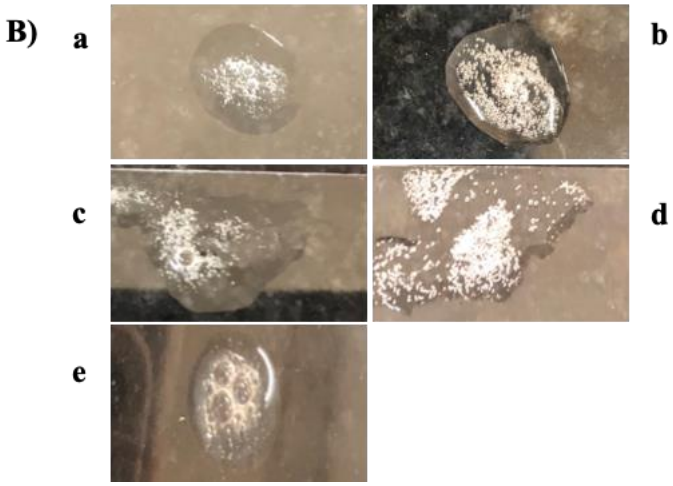

Figure 3(B): Catalase test results in known MDR strains show catalase positive test. a) *E. coli*, b) *K. pneumoniae*, c) *A. baumannii*, d) *P. aeruginosa* and e) *S. aureus*.

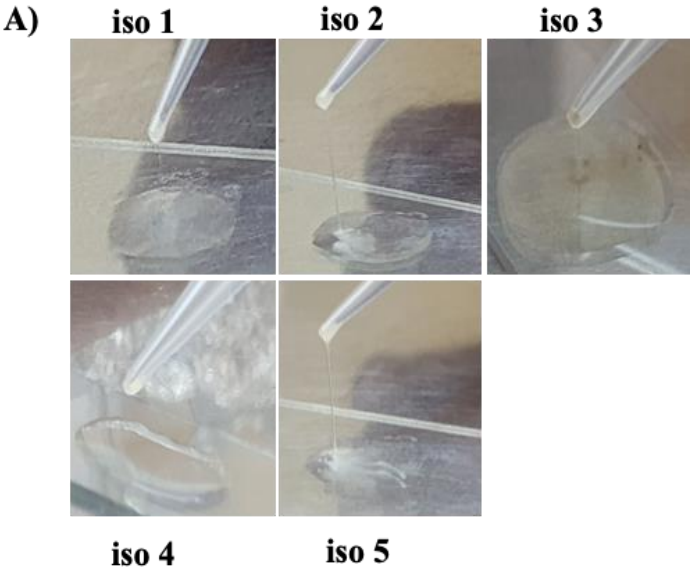

Figure 4(A): KOH solubility test of bacterial isolates from soil. Isolates 2 (iso 2) and 5 (iso 5) showed a positive test which indicates they are Gram-negative bacteria whereas isolates 1 (iso 1), 3 (iso 3) and 4 (iso 4) showed a negative test.

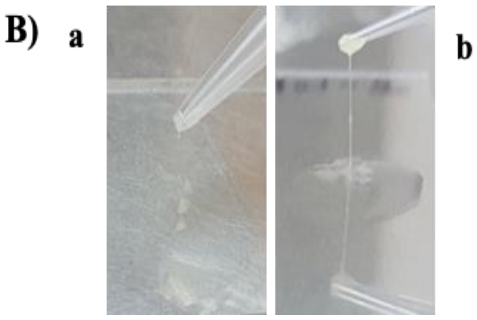

Figure 4(B): KOH solubility test of *S. aureus* and *E. coli.* a) *S. aureus* shows a negative test; b) *E. coli* shows a positive test.

Table 1
MIC (µg/ml) determination using the resazurin assay. The antimicrobial activity of ampicillin was evaluated against gram stain bacterial isolates from soil and Gram-positive (*S. aureus*) and Gram-negative (*E. coli, K. pneumoniae, A. baumannii, P. aeruginosa*) MDR bacterial strains.

Bacterial strains	Ampicillin [MIC (µg/ml)]
iso 1	12.5 ± 0.80
iso 2	25 ± 1.0
iso 3	6.25 ± 0.52
iso 4	25 ± 0.50
iso 5	25 ± 0.57
E. coli (ATCC, MIC positive control)	25 ± 0.0
P. aeruginosa (ATCC, MIC negative control)	6.25 ± 0.5
E. coli	25 ± 0.7
K. pneumoniae	25 ± 1.0
A. baumannii	12.5 ± 0.57
P. aeruginosa	6.25 ± 0.57
S. aureus	25 ± 0.0

*iso 1, Isolate 1; iso 2, Isolate 2; iso 3, Isolate 3; iso 4, Isolate 4; iso 5, Isolate 5 bacteria from soil sample. Experiments were performed individually in triplicate and the results are expressed as the mean value ± standard deviation.

Antimicrobial activity of bacterial isolates from soil and known MDR strains by MIC: An oxidation-reduction indicator known as resazurin was used for the evaluation of cell growth[17]. A direct comparison of the antibiotic's MIC as determined by the resazurin methods (Table 1) using the antibiotic ampicillin against different bacterial isolates and known MDR strains was performed. After being incubated overnight, followed by a 2 hour incubation with resazurin, all broth sterility control wells (only nutrient broth and resazurin dye, no bacteria) for all of the tested bacteria showed a blue color. In contrast, all of the wells in the growth control column (containing nutrient broth and bacteria + resazurin dye, no antibiotic) had turned to pink color or pale pink from blue.

Table 1 demonstrated that the MIC values of ampicillin against bacterial isolates observed at the wells prior to the occurrence of a color change from purple to pink were 12.5 ± 0.80 µg/ml, 25 ± 1.0 µg/ml, 6.25 ± 0.52 µg/ml, 25 ± 0.50 µg/ml, 25 ± 0.57 µg/ml against isolates 1, 2, 3, 4 and 5 respectively. On the other hand, the MICs of ampicillin against known MDR strains for the Gram-negative bacteria, *E. coli*, *K. pneumoniae, A. baumannii* and *P. aeruginosa* were 25 ± 0.7 µg/ml, 25 ± 1.0 µg/ml, 12.5 ± 0.57 µg/ml, 6.25 ± 0.57 µg/ml respectively and for the Gram-positive bacteria, *S. aureus* was 25 ± 0.0 µg/ml. The MIC values of ampicillin against ATCC control bacterial stains used for MIC, *E. coli* (ATCC, positive control) and *P. aeruginosa* (ATCC, negative control) were 25 ± 0.0 µg/ml and 6.25 ± 0.5 µg/ml respectively. The MIC of the antibiotic against all tested bacteria is summarized in table 1.

Discussion

Soil harbors diverse microorganisms playing important geoactive roles that make microbiology as one of the most significant phenomena in the biosphere requiring an interdisciplinary approach to define environmental exploitation. In this study, bacterial strains were isolated from soil samples and were screened for antibiotic resistance or susceptibility and were compared with the known MDR bacterial strains. As evidenced by changes in shape, size, color, margin and elevation, a total of 300 bacterial colonies showed diverse morphological characteristics. Based on their biochemical characterization and Gram reaction, some of the isolates belong to the Gram-negative and some to the Gram-positive species. Out of 5 isolates, 3 were Gram-positive bacteria and 2 were Gram-negative bacteria,

positive for catalase and KOH solubility tests. The findings of this study underscore the importance of soil as a reservoir of diverse bacteria with unique biochemical characteristics and antibiotic production capabilities.

The lowest concentration of the antibiotic ampicillin that resulted in color change or resazurin reduction is defined as the MIC in the present study. Accordingly, the MICs determined by the resazurin-based assay were relatively higher as compared to those via conventional assay[31], allowing results to be comparable for different bacterial isolates and the known MDR bacterial strains. Only live cells were capable of reducing resazurin. In the study by Golebiowski et al[9], the MICs of active compounds were defined as the lowest concentration of active compounds at which growth inhibition was clearly evident (a pellet at the bottom of the well and the absence of turbidity). However, this could lead to overestimating the inhibitory effects of the active compounds, as the presence of a pellet at the bottom of the well and the absence of visual turbidity may not guarantee bacterial inhibition as the growth of cells can still be observed.

In contrast, the reduction of resazurin to resorufin by live cells that results in a visible change in color from purple to pink, can be detected in as few as 80 cells[21] and hence can reduce the risk of overestimating the inhibitory action of antibiotics. MIC results from the present study show that *E. coli* and *S. aureus* were more resistant to ampicillin (Table 1). *P. aeruginosa* was more susceptible to tested antibiotic compared to *K. pneumoniae* and *A. baumannii*. On the other hand, 3 bacterial isolates (iso 2, iso 4 and iso 5) from the soil showed resistance to ampicillin, a similar effect as compared to *E. coli* and *S. aureus* whereas isolate 1 (iso 1) was moderately resistant to antibiotic[5].

This study provides evidence of the prevalence of antibiotic-resistant bacteria in soil samples as well as known MDR bacterial strains. The existence of high drug resistance to antibiotics is a result of patients' negligence, misuse and overuse of antibiotics, self-prescription, inadequate understanding and knowledge about MDR isolates and antimicrobial resistance among clinicians. As a result, MDR strain detection, prevention of transmission, updated knowledge of antimicrobial susceptibility profiles followed by infection control measures and antimicrobial surveillance, are needs of the present scenario and demands of the hour. This will not only help in the alarmingly rising menace of multiple antibiotic resistance but will also assist in designing the appropriate dose and treatment schedule against infections.

Conclusion

Globally, the effects of excessive use of drugs can cause serious damage to human and animal health as well as the environment. The present study focused on the isolation, screening and biochemically characterization of antibiotic-resistant bacterial strains inhabiting soil that harbors a large population of diverse microorganisms as well as comparison with the known MDR bacteria. As a result of the isolation of bacteria, a mixture of colonies was observed on NA plates. Then we calculated the colony-forming unit per milliliter and as a result, we obtained $3x10^{10}$ CFU/ml. The bacterial isolates purified from soil samples showed resistance against commonly used broad-spectrum antibiotic drugs.

Based upon the colony characteristics on NA, microscopical studies and biochemical tests, isolates showed traits such as Gram staining, catalase and KOH-positive tests and were classified as Gram-negative and Gram-positive bacteria. In addition, the resazurin-based assay is a simple, reliable and feasible screening assay for assessing the antibacterial activity of antibiotic ampicillin against MDR strains (*E. coli*, *P. aeruginosa*, *K. pneumoniae*, *A. baumannii* and *S. aureus*) and both Gram-positive (iso1, iso 3 and iso 4) and Gram-negative bacteria (iso 2 and iso 5). Bacterial isolates exhibited high to moderate levels of resistance against the β-lactam classes of antibiotics such as ampicillin. The MIC values of antibiotic on the experimental MDR organism and bacterial isolates from soil lie between 6.25-25µg/ml.

Therefore, in order to reduce the threat of antimicrobial resistance which is a major global issue and concern, early detection and close monitoring of antibiotic-resistant bacteria, or MDR bacterial strains, must be initiated by all clinical microbiology laboratories. Strict health policies, rules and regulations should also be implemented to restrict the use of unsupervised antibiotics as well as to ensure ongoing monitoring and reporting of antibiotic resistance. This will help in combating the increasing threat posed by the evolution of bacterial strains that are multidrug resistant. Our findings provide encouraging insights into the potential of these soil bacteria as sources of novel antibiotics. Further research could explore their applications in combating antibiotic-resistant pathogens.

Acknowledgement

The authors are thankful to Department of Biotechnology, Chandigarh University for providing the infrastructural support for this study.

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(Received 18th February 2024, revised 10th February 2025, accepted 25th February 2025)