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Segregation, Biochemical characterization, and molecular identification of soil bacteria exhibiting antibacterial activity

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ABSTRACT

This study focused on screening soil bacteria that possess antibacterial properties, sourced from various locations in Hisar, Haryana, India. To address this, the researchers employed standard techniques to isolate and characterize soil bacteria exhibiting antibacterial activity. The bacteria were tested against harmful human pathogens such as Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa by using primary and secondary screening methods. Isolates SB1, SB2, and SB3 demonstrated antibacterial properties against all three pathogens in both screening methods. The effects of filter sterilization, autoclaving, and proteinase K treatment were studied on the culture filtrates. 16S rRNA sequencing identified the isolates SB1, SB2, and SB3 as *Neomicrococcus lactis strain PR-F11, Nocardia Alba strain YIM 30243* and *Bacillus cereus NC7401*, respectively. Out of 208 soil samples, only 3 bacterial isolates displayed antibacterial activity against human pathogens S. aureus, E. coli, and P. aeruginosa. The isolates, *Neomicrococcus lactis strain PR-F11, Nocardia Alba strain YIM 30243* and *Bacillus cereus NC7401*, were identified as Gram-positive by 16S rRNA studies. Culture filtrates of these isolates were tested for efficacy A comparison of the antibacterial activity of culture filtrates and antibiotic streptomycin showed that culture filtrates produced an inhibition zone of 19.5 mm. These 3 bacterial strains, showed antibacterial activity against all the mentioned human pathogens. The antibiotic factor was found to be proteinaceous, as proteinase K significantly reduced the antibacterial activity of the culture filtrates. Based on these significant results, these 3 bacteria are promising candidates for the isolation of new antibacterial agents.

Keywords: *Antagonistic activity,* B*acterial isolates, biochemical testing 16S rRNA sequencing, Soil samples*

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INTRODUCTION

The soil has a vast microbial community as it contains a lot of organic matter (Gao et al., 2022; Andersen et al., 2013). These microorganisms are bioactive and can be mostly found in the top few inches of agricultural soil (Siciliano et al., 2014). They can exist in different habitats, including extreme conditions like inside oceanic crust rocks, cold temperatures, hot springs, and deep ocean floors (Kumar et al., 2022). The activity and diversity of soil microorganisms

are influenced by abiotic and biotic factors such as vegetation type, soil texture and chemical nature, nutrient availability, pH, moisture content, climate, and temperature (Glass et al., 2022). The presence of microbes in the soil also affects soil physiology, which varies across seasons in the same location (Mason et al., 2022). Agricultural fields' organic waste has a high nutrient content, supporting the growth of microorganisms. Specific bacterial communities, like Thiobacillus, Rhizobium, Nitrosomonas, Clostridium, Nitrobacter, Caulobacter, Pseudomonas, and Frankia, play a crucial role in nutrient cycling (Gupta et al., 2022).

Over the past few decades, numerous research groups have identified and isolated a vast array of bacteria that produce primary and secondary metabolites, enzymes, antibiotics, and other novel compounds. Microorganisms are the primary source of secondary metabolites due to their unique structure and ability to act as antimicrobial agents against pathogenic bacteria (Gupta et al., 2022) . Antibiotics are produced as a self-defense mechanism by bacteria to help them survive but are not necessary for their growth. Soil bacteria are the primary source of antibiotics, and around 500 new antibiotics are discovered each year. These antibiotics are low-molecular-mass (< 1500 kDa) products of secondary metabolism, typically produced during the late growth phase of a relatively small group of microorganisms (Vora et al., 2022). They protect bacteria from other competitive microorganisms. Currently, a small group of microorganisms belonging to the genera Penicillium, Streptomyces, Cephalosporium, Micomonospora, and Bacillus are responsible for producing most of the antibiotics in use today. Bacillus species generally produce polypeptide-type bacteriocins that are effective against several Gram-positive bacteria.

According to available data from 2010, India had the highest antibiotic usage, with 12.9 × 109 units (10.7 units per person) (Koya,2022). This is due to the increasing demand for bacterial antibiotics globally, as resistant pathogenic bacteria make existing antibiotics less

effective. Pharmaceutical and healthcare researchers are under pressure to find alternative antibacterial substances for use in clinics, food preservation, and dairy products due to this trend (Sagar et al., 2023). Improper disposal and indiscriminate use of antibiotics have led to drug resistance in pathogenic bacteria (de Souza et al., 2023). Antibiotic resistance is a significant challenge for modern medicine to combat. Antibiotics aim to inhibit the growth of or kill microbes that cause infectious diseases, and drug resistance is considered a threat to health security. Bacteria evolve mechanisms to modify or acquire new genes naturally, which makes drugs less effective. Incomplete doses of prescribed antibiotics also make it easier for pathogenic bacteria to develop resistance, which necessitates the need for new antibiotics (Correia et al., 2023). This study aimed to isolate and characterize antibiotic-producing bacteria from soil in seven different sites, including the garden, playground, canteen, and sewage sump covered with vegetation. Some soil bacteria displayed antibacterial activity and were characterized and identified using molecular methods. The current research shows encouraging results that can be utilized further. After analyzing 208 soil samples, only three bacterial isolates revealed antibacterial activity against S. aureus, E. coli, and P. aeruginosa which were identified as *Neomicrococcus lactis strain PR-F11, Nocardia Alba strain YIM 30243* and *Bacillus cereus NC7401*through 16S rRNA studies.

METHODOLOGY

Sample collection

To collect soil samples, we went to an area with abundant native vegetation at an altitude of 215 meters and a latitude and longitude of 29.47° N, 75.72° E. The soil in this location is alluvial fine loamy to clayey. Before collecting the samples, we cleared any debris from the soil surface and dug down 5-10 centimeters. We collected approximately 20 grams of soil from each site and stored them in an icebox for transport to the laboratory.

Pathogenic bacteria and culture conditions

To isolate bacteria from soil samples collected from various sites (3 sites) such as the garden, sewage sump, botany department, the standard serial dilution technique was employed. A gram of soil sample was mixed with 10 ml of sterile water and serially diluted from 10^{-1} to 10^{-4}. 100 µl of the serially diluted soil sample was then blended with warm nutrient agar medium and poured into Petri plates. To prevent fungal growth, Natamycin (Sigma-Aldrich, USA) at 20 µg/ml was added to the molten nutrient agar medium at 48°C. After 48 hours, the plates exhibited a lawn of mixed bacterial colonies, and sterile toothpicks were used to pick

individual colonies that were streaked onto fresh nutrient agar plates to obtain pure cultures. The pure culture was preserved and used to test antibacterial activity against human pathogenic bacteria such as Staphylococcus aureus (MTCC 96), Escherichia coli (MTCC 739) and Pseudomonas aeruginosa (MTCC 741). The concentration of all pathogenic bacteria was calibrated to obtain the OD = 0.75 using a UV/Vis spectrophotometer at 600 nm. (Prashanthi et al., 2021)

Primary screening

Four different sites provided soil samples that yielded a total of 208 bacterial colonies. These bacteria were screened for their ability to fight pathogenic bacteria such as Staphylococcus aureus (MTCC 96), Escherichia coli (MTCC 739), and Pseudomonas aeruginosa (MTCC 741) in vitro. Two methods were used: perpendicular streaking and seed overlay. The soil bacteria were streaked in a straight line through the center of the nutrient agar plates, while the pathogenic bacteria were streaked perpendicularly to the soil bacteria. After 24 hours of incubation, any inhibition zone between the soil and pathogenic bacteria was noted. Bacterial strains that showed an inhibition zone against the test pathogens were selected for secondary screening. (Khan, 2022)

Seed overlay method

To cultivate the isolated soil bacteria, a sterile toothpick was used to inoculate a nutrient agar plate, which was then incubated for 48 hours. To halt the growth of the bacteria, 2 ml of chloroform was added and the plate was incubated for an additional hour. This allowed only the secondary metabolites from the original bacteria to remain active on the plate. After 20 minutes of open exposure to allow for chloroform evaporation, 100 µl of each pathogenic bacterium with an OD of 0.75 was mixed with 2 ml of nutrient broth and thoroughly combined. The resulting mixture was then transferred to the agar plate and incubated for 24 hours. The effectiveness of the secondary metabolites against the pathogenic bacteria was determined by measuring the diameter of the inhibition zone. The bacteria that produced an inhibition zone were selected for secondary screening. (Gislin et al., 2018)

Secondary screening to confirm antibacterial activities

We grew the active bacteria chosen from the primary screening method individually in Nutrient broth at 30 °C under shaking conditions. After 24 hours, we adjusted the nutrient broth with cells to an OD of 0.75 at 600 nm using a UV/Visible spectrophotometer. We then

centrifuged it at 5000 × g for 10 minutes and collected the cell-free culture filtrate which we stored at 4 °C. To determine the minimum inhibitory concentrations (MICs) of the culture filtrate, we used the Agar Well Diffusion method. We inoculated Nutrient agar plates with 100 µl of the pathogenic organism using the spread plate method. We then made two wells in the agar plates at equal distances using a 6-mm-diameter cork borer. We filled the wells separately with 50 µl and 100 µl of cell-free culture filtrates of SB1, SB2, and SB3. After that, we placed a filter paper disc about 6 mm in diameter impregnated with streptomycin (20 µl) on the agar surface as a positive control. We incubated the agar plates for 2 days at 28 °C for bacterial growth. We determined the antibacterial activity of the culture filtrate by measuring the zone of inhibition (Kirby-Bauer Test) around the well. (Khan, 2022)

Effect of filter sterilization, autoclaving, and proteinase K treatment on culture filtrate

In order to determine if the culture filtrates of SB1, SB2, and SB3 can be used as a sustainable antibacterial agent, they underwent three different treatments. Firstly, they were filtered through a 0.45-µm Millipore filter. Secondly, they were autoclaved at 121°C for 20 minutes, and finally, they were treated with proteinase K at 0.02 mg/ml and heated to 50°C for 1 hour. The antibacterial activity of the resulting treatments was then tested using four different methods: (1) crude cell-free culture filtrate, (2) filter-sterilized culture filtrate, (3) filter-sterilized and heat-sterilized culture filtrate, and (4) filter-sterilized and proteinase K treated culture filtrate. The inhibition zone for each treatment was measured and recorded. (Prashanthi et al., 2021)

Morphological and biochemical analysis of bacteria

Bacteria that were cultured for 24 hours in nutrient broth were utilized for Gram staining and biochemical characterization. (Prashanthi et al., 2021)

Molecular identification of bacteria

To isolate DNA from overnight-grown cultures of SB1, SB2, and SB3, 2.0 ml of bacterial suspension was transferred to a microcentrifuge tube and centrifuged at 10,000 × g for 2 minutes to collect the pellet. This process was repeated twice to obtain enough cells. The cells were then washed with 0.9% saline and treated with 0.2 ml of protease to remove the protein and cellular materials and release the genomic DNA. After centrifuging the tubes and inverting them 5-6 times, they were boiled in a water bath for 1 hour at 55 °C. Next, 0.1 ml of DNA Salt solution was added and centrifuged at 5,000 × g for 5 minutes. Finally, 0.8 ml of

the precipitated solution was added slowly and mixed by inverting the centrifuge tube several times. The cells were washed with 70% ethanol, and the extracted DNA was dried, suspended in TE buffer, and stored at 4 °C. The purity of the isolated genomic DNA was assessed by quantifying its quality and integrity using a spectrophotometer at a wavelength of 260 and 280 nm, and by checking its purity on an 08% agarose gel.

To amplify the 16S rRNA gene fragments, universal primers (forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse primer 5′-GGTTACCTTGTTACGACT-3′) were used with the following PCR parameters: an initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 54 °C for 2 minutes, extension at 72 °C for 2 minutes, and final extension at 72 °C for 5 minutes. The amplified PCR products were then electrophoresed on an agarose gel. The amplified 16S rRNA gene fragments were purified and sequenced using a DNA sequencing service. The obtained 16S rRNA gene sequences were uploaded to the Basic Local Alignment Search Tool (BLAST) to identify nucleotide sequence matching with the reference sequences. (Sambrook JF, Russell et al., 2001)

RESULTS

Soil sample

Samples of soil were taken from three locations, then diluted and spread on Nutrient agar plates. These plates were then incubated at 30 °C for 3 days. From all three locations, a total of 208 bacterial colonies were isolated. Each individual colony was selected and introduced to Petri plates that contained nutrient agar medium. These plates were also incubated at 30 °C. A greater number of colonies were found in the garden and near the sewage sumps compared to those in the botany department samples (Table 1).

Table 1: Bacteria from soil samples

Sampling location	Dilution	CFU/ml	No. of colonies	Antibacterial activity
Garden	10^{-3}	9.8×10^{6}	50	SB1
Near Sewage sump	10^{-3}	9.9×10^{6}	54	SB2, SB3
Near botany Department	10^{-3}	4.5×10^{6}	38	Nil

Primary screening for antibacterial activity

We conducted a primary screening on 208 bacterial isolates to determine their antibacterial activity against pathogenic bacteria such as S. aureus, E. coli, and P. aeruginosa. Out of all the isolates, only one (SB1) taken from garden soil, and two (SB2 and SB3) from soil near the sewage sump were found to have antibacterial activity against all the tested bacteria, as shown in Table 2. As a result, we selected these three isolates for secondary screening.

Table 2: Antibacterial activity against pathogenic bacteria by primary screening

Isolates	*S. aureus*	*E. coli*	*P. aeruginosa*
Garden (SB1)	+	+	+
Near Sewage sump (SB2)	+	+	+
Sewage sump (SB3)	+	+	+

SB1: *Neomicrococcus lactis strain PR-F11* **SB2—** *Nocardia Alba strain YIM 30243***, SB3—** *Bacillus cereus NC7401*

Secondary screening for antibacterial activity

The 3 isolates SB1, SB2 & SB3 which were confirmed to have antibacterial activity through primary screening were subjected to secondary screening for further confirmation of their activity against human pathogens *S. aureus*, *E. coli*, and *P. aeruginosa*. All culture filtrates at 100 µl showed a maximum inhibition zone except *E. coli* indicating that Gram (+) bacteria are susceptible to antibiotics than Gram (−) bacteria. The filter-sterilized culture filtrate of SB3 showed a maximum zone of inhibition with 16.5 mm, 11.0 mm, and 15.0 mm compared to SB1 and SB2 against the tested pathogens (Table3). In antibiotic disc method also, SB3 showed a maximum zone of inhibition with 19.5 mm, 19.0 mm, and 19.0 mm compared to SB1 and SB2.

Table 3: Antibacterial activity of culture filtrate of SB1, SB2, and SB3 against pathogenic bacteria

Bacterial Isolates	Pathogenic bacteria (O.D= 7.5)	Culture filtrate 100µl	Filter Sterilized 100µl	Filtered Sterilized +Autoclaved Culture filtrate 100µl	Filter Sterilized + Proteinase K Culture filtrate 100µl	Antibiotic disc Streptomycin 20µl
SB1	*S. aureus*	11.5 ± 0.1	13.0± 0.1	8.0± 0.1	9.0± 0.2	18.5± 0.1
	E. coli	7.0± 0.2	8.0± 0.1	5.5± 0.1	6.5± 0.2	18.0± 0.2
	P. aeruginosa	10.0± 0.3	11.0± 0.1	8.5± 0.1	9.9± 0.1	18.5± 0.2
SB2	*S. aureus*	12.5± 0.1	14± 0.3	9.0± 0.2	10.0± 0.1	17.5± 0.2
	E. coli	8.5± 0.1	10.0± 0.2	6.34± 0.2	7.0± 0.2	18.0± 0.2
	P. aeruginosa	10.0± 0.2	12.0± 0.1	9.0± 0.1	10.0± 0.1	18.0± 0.1
SB3	*S. aureus*	13± 0.2	16.5± 0.2	9.5± 0.1	10.5± 0.1	19.5± 0.1
	E. coli	10.0± 0.2	11.0± 0.2	6.5± 0.2	7.5± 0.1	19.0± 0.2
	P. aeruginosa	13.5± 0.1	15.0± 0.1	10± 0.1	9.5± 0.1	19.0± 0.1

Results are expressed as antagonistic activity (mm) of the isolate bacteria against pathogenic compared to control (mean ± SD, $n = 3$). Values significantly different from control if $*\rho < 0.05$ as analyzed by Student's *t*-test. Control value for all the pathogenic bacteria 6 mm. All the isolates demonstrated antibacterial properties against pathogenic bacteria. The inhibition zone for the standard antibiotic streptomycin ranged from 17.5 to 18.5 mm, depending on the specific pathogen.

Morphological characterization of antagonistic bacteria

The morphological characterization of bacterial isolates SB1, SB2, and SB3 which showed antibacterial activity were done, and results are tabulated (Table 4)

Bacterial Isolates	Colony Characteristics	Microscopic Characteristics	Biochemical Characterization		Bacterial Species
SB1	Light yellow colonies No Spore Formation	Gram Positive Motility Negative Cocci	**Positive** Catalase Nitrate reduction Casein Hydrolysis	**Negative** Oxidase Voges-Proskauer Indole production	*Neomicrococcus lactis*
SB2	Waxy and shiny Non-motile aerial filaments	Gram Positive pleomorphic ally ranging from a bacillary to a coccoid	Catalase Nitrate reduction Casein hydrolysis	Urease Voges-Proskauer	*Nocardia alba*
SB3	Spore formation	Gram-positive	Catalase Nitrate reduction Casein hydrolysis (variable) Voges-Proskauer Salicin Fructose Sucrose	Mannitol negative Dulcitol negative	*Bacillus cereus*

Molecular identification of bacteria

The isolation of genomic DNA was carried out to obtain pure DNA from the isolates PR1, PR2, and PR3. The 16sRNA was amplified from the isolated DNA sample using PCR. 1.2% of agarose gel was used to verify the amplified products which showed a fragment of 1.5 kb. The amplified 16s r RNA of SB1, SB2, and SB3 were subjected to purification and sequencing.

The sequence of Neomicrococcus lactis strain PR-F11

GAGAATTCCACGTTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGG
AGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCG
GCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGAC
ATGGGCCGGATCGCCGCAGAAATGCGGTTTCCCTTCGGGGCCGGTTCACAGGTG
GTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA

GCGCAACCCTCGTTCTATGTTGCCAGCGGTTCGGCCGGGGACTCATAGGAGACTG
CCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTAT
GTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGTTGCGATACTGTGA
GGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGAGGTCTGCAACTCG
ACCTCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTTGGTAACACCC
GAAGCCGGTGGCCTAACCCTTTTGGGAGGGAGCCGTCGAAGGTGGGACCGGCGA
TTGGGACTAAGTCGTAACAAGGTAACCGATAAGG

The sequence of Nocardia Alba strain YIM 30243

CGGCGTGCTTAACACATGCAAGTCGAGCGGTAAGGCCCTTCGGGGTACAC
GAGCGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGTACTTCGGG
ATAAGCCTGGGAAACTGGGTCTAATACCGGATATGACCAGGAATGCATG
TTCTGTGGTGGAAAGATTTATCGGTACAGGATGGGCCCGCGGCCTATCAG
CTTGTTGGTGGGTAACGGCCTACCAAGGCGACGACGGGTAGCCGACCTG
AGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGA
CGCCGCGTGGGGGATGACGGCCTTCGGGTTGTAAACCCCTTTCGACAGGG
ACGAAGCGCAAGTGACGGTACCTGTAGAAGAAGCACCGGCCAACTACGTG
CCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTACTGG
GCGTAAAGAGCTTGTAGGCGGTCTGTCGCGTCTTCTGTGAAAACTTGGGG
CTCAACCTTAAGCTTGCAGGGGATACGGGCAGACTAGAGTACTTCAGGGGAGAC
TGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGG
CGAAGGCGGGTCTCTGGGAAGTAACTGACGCTGAGAAGCGAAAGCATGGGTAGC
AAACAGGATTAGATACCCTGGTAGTCCA TGCCGTAAACGGTGGGTACT

The sequence of *Bacillus cereus NC7401*

AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATG
CAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGA
CGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGG
GAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATT
GAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCT
AGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAG
AGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACCCTACGGGA

GGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACG
CCGCGTGAGTGATGAAGG

Discussion

Soil is a rich source of various types of bacterial communities that are highly adapted to constantly changing soil environments (Bissett et al., 2013). Due to competition for survival, soil bacteria produce antibacterial compounds to eliminate their competitors (Kennedy and Papendick, 1995). This makes soil bacteria a preferred choice for screening antibacterial activity. Many scientists have isolated countless antibiotic-producing bacteria from soil (Fierer et al., 2007). For example, Arifuzzaman et al. 2010 found that 20 soil bacterial strains were active against pathogenic microbes. Falkinham et al.2009 reported that soil bacteria are the basis for producing nearly 500 antibiotics per year. The demand for bacterial antibiotics is increasing globally because pathogenic bacteria are becoming resistant to many antibiotics and some Staphylococcus spp., Streptococcus spp., pseudomonads, and Enterobacteria, which cause several human health issues, have developed resistance to many antibiotics (Prashanthi and GK., 2021).

The purpose of this study was to find soil bacteria that have the ability to fight against harmful bacteria. After analyzing 208 bacterial colonies from soil samples, only three were found to have antibacterial properties. These three active bacteria - *Neomicrococcus lactis, Nocardia Alba* and *Bacillus cereus* - were identified through molecular methods. In our research, we utilized primary and secondary screening methods to isolate and screen bacterial isolates, which was also done by (Khan,2021). The agar well diffusion method, which involves using cell-free culture filtrates, has been commonly adopted by many scientists for secondary screening of bacteria (Prashanthi and GK., 2021). Similar studies on various bacteria were conducted by Rafiq et al., 2019 who suggested that most species belonging to the genus Bacillus have potential for producing antibiotics. Our study also identified *Bacillus cereus* as one of the active bacteria. The results of this study indicate that SB1, SB2, and SB3 have broad-spectrum activity. All culture filtrates at 100 µl inhibited the growth of bacterial pathogens, with a larger inhibition zone observed for S. aureus, and P. aeruginosa, compared to E. coli. Salim et al., 2017 had reported of antibiotic producing actinomycetes from soil samples particularly belonging to *Nocardia* genus. Furthermore, Ding and co-workers have also isolated rare secondary metabolites from *Nocardia sp.* that act as antibiotics. Similar

findings were reported by Li et al., 2015 for *Nocardia sp.* Antimicrobial activity of *Neomicrococcus lactis* was reported byPrashanthi et al., 2021.

This suggests that Gram-positive bacteria may be more susceptible to antibiotics due to differences in their membrane constituents. Specifically, the peptidoglycan layer of Gram-positive bacteria is not as effective of a barrier as the outer polysaccharide membrane possessed by Gram-negative bacteria (Holst et al., 2010). In our research, we found that the filter-sterilized culture filtrate of SB3 had the highest antibacterial activity against all the tested pathogens. This is consistent with the findings of Vaca et al., 2023, who studied bacteriocin like substances comparison produced by different species of Bacillus related to B. cereus group with specific antibacterial activity against foodborne pathogens. Bizani et al., 2005 had also reported antibacterial activity of cerein 8A, a bacteriocin-like peptide produced by Bacillus cereus.

After subjecting the culture filtrate to both autoclaving and proteinase K treatment, it was discovered that the antibacterial activity against all tested pathogens had reduced. This reduction in activity was attributed to the proteinaceous nature of the antibacterial principle, as demonstrated by the effect of proteinase K treatment. Members of the genus Bacillus have been found to produce various types of peptides that exhibit a broad spectrum of antibacterial activity against pathogenic bacteria (Lee and Kim, 2011). Proteins, whether simple or complex groups of polypeptides, create pathways with multiple enzymatic steps involving polyketide synthases and peptide synthetases to produce antibiotics. Previous research has highlighted that Bacillus species are a reliable source of antibiotics against a range of pathogens. (Stein, 2005).

In order to identify bacteria, laboratories around the world have traditionally used a method known as morphological and biochemical characterization. Microscopic identification and biochemical testing have been the standard practice for several decades. In our studies, the Gram staining results indicated that the SB1, SB2, and SB3 isolates are Gram-positive. The results of our biochemical tests were consistent with these isolates.

Conclusion

We have made progress in isolating soil bacteria with antibacterial properties. Our efforts have yielded promising results, with the identification of three bacterial isolates. These isolates are. *Neomicrococcus lactis, Nocardia Alba* and *Bacillus cereus.* We tested these isolates against a range of human pathogens, including S. aureus, E. coli, and P. aeruginosa.

While E. coli showed some resistance, the Gram-positive pathogens were more susceptible to the antibacterial effects of the isolates. Furthermore, we found that filter-sterilized culture filtrates were more effective. *Bacillus cereus* showed the strongest antibacterial activity among the three isolates. Overall, all three isolates demonstrated broad-spectrum antibiotic activity, and further research is required to compare their efficacy with available antibiotics. We believe that these isolates hold potential for further exploration of their antibacterial properties.

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