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Research Article

Isolation Of Mesophilic Bacteria And Study On Potential Properties Of Bacterial Supernatant

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ABSTRACT

Two mesophilic bacteria, Shouchella clausii and Shouchella rizhosphaerae, were isolated from a pre-treated soil sample using selective isolation techniques. Gram's staining was used to differentiate and identify the bacteria, which appeared as purple rod-shaped entities. DNA isolation, PCR amplification, and sequencing were performed to further understand their genetic makeup and identity. A comprehensive phylogenetic tree was constructed using advanced MEGA 11 software, revealing a genetic similarity of 39% and 94% between Shouchella rizhosphaerae and Shouchella clausii, respectively. The aqueous component of Shouchella clausii showed potent antibacterial activity against S. aureus, a Gram-positive strain. The antimicrobial compounds in Bacillus clausii's cell-free supernatant exhibited a narrow spectrum of action, with heightened inhibition observed only against Gram-positive species. The study successfully isolated and identified these mesophilic bacteria from pre-treated soil samples, with chymotrypsin showing promising potential as a viral protein inhibitor.

INTRODUCTION

Soil microbiology is crucial for plant growth and essential compound production. Soil bacteria, like Shouchella clausii, are known for their role in soil microbiology and their potential to produce growth regulators and antibiotics. Shouchella clausii is a rod-shaped, mesophilic gram-positive bacterium found in various environments, contributing to nutrient cycling and bioremediation. It is classified as a probiotic microbe due to its symbiotic relationship with host organisms. Antibiotics, which inhibit pathogens, can be produced from various microorganisms, with 50%-60% isolated from Shouchella clausii. They produce antibiotic compounds effective against gram-positive bacteria like Clostridium difficile, Enterococcus faecium, and Staphylococcus aureus. Their alkaliphilic nature

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has shown value in preventing and treating gastrointestinal illnesses. Shouchella clausii has been used as an antibiotic to combat pathogenic bacteria and viruses causing upper respiratory illnesses like tonsillitis and pharyngitis. It is also helpful in treating digestive system diseases like diarrhea, constipation, stomach ulcers, Crohn's disease, colitis, and mal-absorption syndrome. The current study aimed to examine the antiviral activity of Shouchella clausii and bacterial supernatant against Herpes Simplex Virus (HSV).

OBJECTIVES:

- To isolate the mesophilic bacteria from the soil sample.
- To characterize the isolated bacteria through Gram's staining and 16S rRNA sequencing.
- To interpret the results of sequencing and to construct the phylogenetic tree.
- To determine the antibacterial activity of isolated bacteria's supernatant.
- To check the antiviral activity of potential drug produced by mesophilic bacteria against herpes simplex virus.

METHODOLOGY:

Collection & pre-treatment of soil sample:

Soil samples were collected from agricultural fields, particularly vegetative fields like gardening soil, at depths of 10-20cm. The samples were stored in sterile polythene bags and shade dried to prevent moisture. The samples were homogenized to dry out and remove large components like twigs and stones. The samples were chemically treated with calcium carbonate for acidity neutralization. A sample of 5g was taken, and large debris was removed using a mesh. The sample was pre-treated with 3g of calcium carbonate in 30ml of distilled water, mixed with the soil, and incubated at room temperature for at least 4 days.

Isolation of mesophilic bacteria:

The mesophilic bacteria were isolated using serial dilution and spread plate technique. The media was prepared using specialized media, such as starch-caesin agar, which was diluted with distilled water. The media was then autoclaved at 1210C for 20 minutes. The media was then placed in a dust-free room and the spread plate technique was used to ensure even distribution and growth of the microorganisms. The prepared media was then poured into petriplates and allowed to solidify. The serially diluted sample was spread over the plates using an L-rod and rotator. The plates were then incubated at an appropriate temperature for 7 days. The colony selection or pure culture was achieved by observing the colonies growth and selecting the individual colony for sub-culturing into fresh media. Gram staining was performed, and molecular techniques were used to identify the isolated bacteria.

Characterization of isolated bacteria:

Gram staining was used to identify isolated bacteria from pure culture plates, revealing their morphological characteristics. The bacteria were then sequenced using the 16S rRNA gene, which is highly conserved in all bacteria due to its essential function in ribosome assembly. The 16S rRNA gene contains variable regions that may serve as fingerprints for particular species. The fresh culture was preserved in phosphate buffer solution and sent to the Eurofins lab in Bangalore. DNA extraction and PCR amplification with universal primers were performed. Data analysis generated a large dataset of short DNA sequences representing the variable regions of the 16S rRNA gene. The raw sequence data was trimmed using BioEdit tool, and two FASTA sequences were interpreted from the data primer 1 and primer 2. Phylogenetic analysis was performed using the NCBI Blastn program, which identified nucleotide sequences by copying and pasting sequences on the NCBI Blastn. The BLAST program was used to create a phylogenetic tree, showing the evolutionary history of biological sequences like DNA, RNA, or proteins. MEGA 11 is the most recent version of the utility, optimized for 64-bit

computer platforms. To create a phylogenetic tree, take the nucleotide sequence "copy" and open the BLAST tool on Google. Paste the sequence in the search box in FASTA format, select the species, paste the sequence in Word or Notepad, and save the sequences. Open MEGA 11 software, click on "create new alignment", insert sequences, select align, and click compute. The sequences are then saved in MEGA format, and a phylogenetic tree is constructed.

Antibacterial activity:

Antibacterial agents are chemicals that kill or inhibit the growth of bacteria locally, either bacteriostatic or bactericidal. They are crucial in fighting infectious diseases. In the well diffusion method, sterile agar plates were prepared and solidified, and bacterial cultures are inoculated. Extracts or compounds were added to the wells, and the plates were incubated at the appropriate temperature. The zone of inhibition around the wells indicated the bacterial inhibition. This method helps in understanding the effectiveness of antibacterial agents in combating infections.

Molecular docking:

Molecular docking is a computational tool used in drug design and virtual screening studies to predict experimental binding modes and affinities of small molecules within specific receptor targets. It uses energy scoring and a search algorithm to create and assess ligand postures. The antiviral property of a drug was checked using molecular docking. The process involves identifying a protein or target, downloading it in pdb format, and identifying a ligand, such as Chymotrypsin, potentially produced by Shouchella clausii. The docking was performed using Autodock Vina, and the conformations of ligands are analyzed. Structure visualization is then done using PyMOL.

RESULTS AND DISCUSSION:

Collection and pre-treatment of the soil sample:

The soil sample was collected from the field and pre-treated with calcium carbonate to neutralize and remove debris. According to Korn-Wendisch and Kutzner (1992), this sample was suitable for selectively isolating mesophilic bacteria like Shouchella clausii and Shouchella rizhosphaerae.

Isolation and screening of mesophilic bacteria: The soil sample was serially diluted and then spread plate technique was used to isolate and screen mesophilic bacteria, supplemented with SCA media, resulting in colonies of small, circular, or irregular shapes.

Figure 1: Isolation & Screening of mesophilic bacteria

Characterization of isolated bacteria:

The isolated bacteria were characterized using Gram's staining, a technique used to differentiate between Gram positive and Gram negative cell wall constituents. The mesophilic bacteria were observed as purple and concluded to be Gram positive bacteria with a rod-like structure.

16S rRNA sequencing:

In this study, the isolated bacteria were characterized to identify their species. By performing DNA isolation, PCR amplification and sequencing, they were analysed with two primers (primer 1 & primer 2). The sequence which was obtained for the isolated bacteria was observed in FASTA format.

>0224-303___Sample_With_PBS_Primer1_G12.ab1

TTTTAACTTGCGGCACTACGGGGCATCGA
AACCCCTAACACCTAGCACTCATCGTTTA
CGGCGTGGACTACCAGGGTATCTAATCCT
GTTTGCTCCCCACGCTTTCGCGCCTCAGCG
TCAGTTACAGACCAGAGAGTCGCCTTCGC
CACTGGTGTTCCTCCACATCTCTACGCATT
TCACCGCTACACGTGGAATTCCACTCTCC
TCTTCTGCACTCAAGCTCCCCAGTTTCCAA
TGGCCGCTCGGGGTTGAGCCCCGAGATTT
CACATCAGACTTAAGAAGCCGCCTGCGCG
CGCTTTACGCCCAATAATTCCGGACAACG
CTTGCCACCTACGTATTACCGCGGCTGCT
GGCACGTAGTTAGCCGTGGCTTTCTGGTG
AGGTACCGTCAAGGTACCGCCCTATTCGA
ACGGTACCGCTTCTTCCCTCACAACAGAG
CTTTACGACCCGAAGGCCTTCCTCACTCA
CGCGGCGTTGCTCCGTCAGACTTTCGTCC
ATTGCGGAAGATTCCCTACTGCTGCCTCC
CGTAGGAGTCTGGGCCGTGTCTCAGTCCC
AGTGTGGCCGATCACCCTCTCAGGTCGGC
TACGCATCGTTGCCTTGGTAAGCCGTTAC
CTTACCAACTAGCTAATGCGCCGCGGGCC
CATCCCTTAGTGATAGCCGAAGCCATCTT
TCACCCTCTCTCCAGGTGGAGAAAGGGAT
TATCCGGTATTAGCTCCGGTTTCCCGGAG
TTATCCCAGTCTAAGGGGCAGGTTGCCCA
CGTGTTACTCACCCGTCCGCCGCTAACGT
CCAAAGAAGCAAGCTCCTTATGTGTCCGC
TCGACTTGCATGTATTAGGCGCGCCGCCC
GCGTTCGTCCTAGACCCAGGATCAAATCT
ATACGGGCCCGT

>0224-303___Sample_With_PBS_Primer2_G12.ab1

CATGCCCGTAGTGCCGAAAGTTAACACAT
TAAAGCACTCCGCCTGGGGAGTACGGCCG
CAAGGCTGAAACTCAAAGGAATTGACGG
GGACCCGCACAAGCAGTGGAGCATGTGG
TTTAATTCGAAGCAACGCGAAGAACCTTA
CCAGGTCTTGACATCCTTTGACCACCCAA
GAGATTGGGCTTCCCCTTCGGGGGCAAAG
TGACAGGTGGTGCATGGTTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCG
CAACGAGCGCAACCCTTGATCTTAGTTGC
CAGCATTGAGTTGGGCACTCTAAGGTGAC
TGCCGGTGACAAACCGGAGGAAGGTGGG
GATGACGTCAAATCATCATGCCCCTTATG
ACCTGGGCTACACACGTGCTACAATGGAT
GGTACAAAGGGCAGCGAAACCGCGAGGT
GAAGCCAATCCCATAAAGCCATTCTCAGT
TCGGATTGCAGGCTGCAACTCGCCTGCAT
GAAGCCGGAATTGCTAGTAATCGCGGATC
AGCATGCCGCGGTGAATACGTTCCCGGGT
CTTGTACACACCGCCCGTCACACCACGAG
AGTTTGTAACACCCGAAGTCGGTGAGGCA
ACCTTTTGGAGCCAGCCGCCTAAGGTGGG
ACAAATGATTGGGGTGAAGTCGTAACAA
GGGTAACCGTAAGATTGTGACCCGGGACG

These two FASTA sequences were submitted to the NCBI, enabling BLAST to determine the species' similarities. Shouchella clausii was identified by the FASTA sequence of primer 1 (Nielsen et al., 1995), whereas Shouchella rizhosphaerae was identified by the same sequence for primer 2.

Phylogenetic analysis:

The phylogenetic tree was constructed using MEGA 11 software and percentage of similarities was identified as per their tree construction.

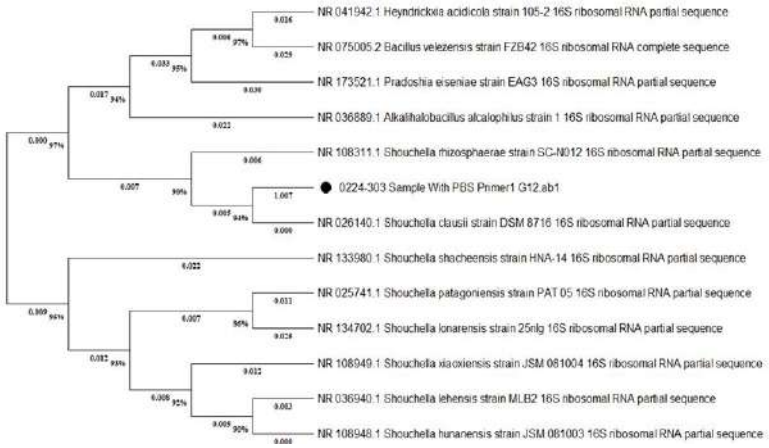

Figure 2: Phylogenetic tree of Shouchella clausii

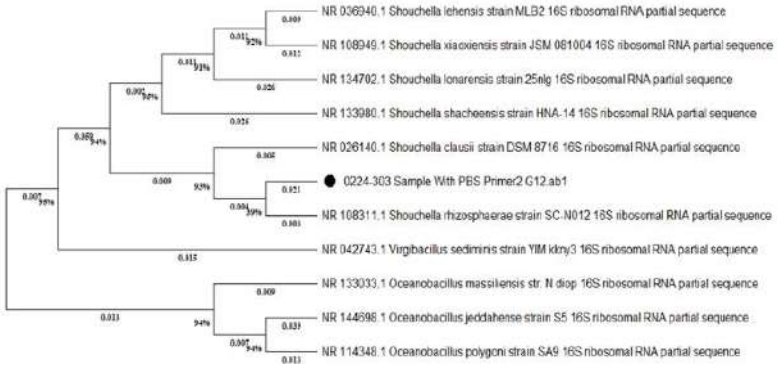

Figure 3: Phylogenetic tree of Shouchella rizhosphaerae

The sequencing data from Figures 2 and 3 were analyzed, revealing that two distinct species of mesophilic bacteria were generated from the rizhospheric soil. These species were 39% similar to Shouchella rizhosphaerae and 94% similar to Shouchella clausii. Shouchella clausii was identified as the mesophilic bacterium that was isolated and grown based on phylogenetic analysis.

Antibacterial activity:

The antibacterial efficacy of the soil sample's Shouchella clausii bacterial supernatant was evaluated against one Gram (+) and one Gram (-) bacterium. Microorganisms such as S. aureus and E. coli were collected. Following the incubation time, S. aureus exhibited the highest inhibitory activity for 24 hours at 37 °C, with a zone of inhibition of 20 mm at 50 µL and 27 mm at 100 µL concentrations.

Table 1: Antibacterial activity-Zone of inhibition

Bacterial supernatant concentration	Zone of inhibition for E.coli	Zone of inhibition for S. aureus
50 µg/mL	16mm	20mm
100µg/mL	18mm	27mm

The study by Maria & et al. (2004) found that the antimicrobial compounds in the cell-free supernatant of Bacillus clausii, also known as Shouchella clausii, displayed a narrow activity. Here, E.coli showed the highest zone of inhibition at concentrations of 50µL and 18mm at 100µL, while S. aureus showed the maximum zone of inhibition at concentrations of 20mm and 27mm at 100µL. To study about the antiviral effect of Bacterial supernatant against HSV, the docking studies has been conducted as it was a cost effective and quick method. And the HSV was also a contagious one, so that the study moved over the docking studies.

Molecular docking:

Shouchella clausii produces serine protease inhibitors, and the binding effectiveness of these inhibitors was analyzed using molecular docking (Gabrielle et al., 2016). One effective serine protease inhibitor that can break down viral proteins is chymotrypsin. Table 2 & 3 provided specifics on the antiviral activity of chymotrypsin against the herpes simplex virus, including conformational information.

Table 2: Conformational info of molecular docking

Conformation	Binding Energy	Ligand Efficiency	Inhibitory Constant uM	Intermol Energy	Vdwhb Desolvation energy	Electro Static Energy	Total internal	Torsional Energy	Unbound Energy
1	-10.96	-0.3	9.19	-14.25	-14.25	0.0	35.43	3.28	35.43
2	-8.63	-0.24	475.59	-11.91	-11.91	0.0	35.19	3.28	35.19
3	-8.04	-0.22	1.28	-11.32	-11.32	0.0	34.45	3.28	34.28
4	-7.83	-o.22	1.82	-11.11	-11.11	0.0	33.86	3.28	33.86
5	-7.15	-0.2	5.76	-10.43	-10.43	0.0	34.94	3.28	34.94
6	-7.05	-0.2	6.8	-10.33	-10.33	0.0	34.35	3.28	34.35
7	-7.04	-0.2	6.94	-10.32	-10.32	0.0	34.01	3.28	34.01

Table 3: Hydrogen bonds, targets and ligands

Conformation	No. of hydrogen bonds	Ligand-2	Target
1	1	4051:O3	B:VAL73:HN
2	0	-	-
3	1	4051:O5	B:ASP31:HN
4	1	4051:O1	B:ARG78:HH
5	2	4051:O8, O5 & 4051:O2	B:LEU111:HN2 B:GLN246:HE
6	1	4051:N1	B:TYR22:HH
7	0	-	-

The conformational information for molecular docking was obtained from tables 2 and 3, which showed maximum binding energy at -10.96 kcal/mol, intermolecular interaction capacity, and ligand efficiency at -0.3. Conformation 5 had the most hydrogen bonds, ligands, and targets. Mohammad Shayestehpour et al., 2022, found that the combination of selenium and Shouchella clausii supernatant reduced HSV-1 replication, suggesting that bacterial supernatant may be effective in docking HSV. PyMOL was used to analyze the docking structure of ligand binding to a protein, allowing for inspection of ligand poses, interactions between ligands and protein residues, and optimization of ligand binding affinity and the ligand chain.

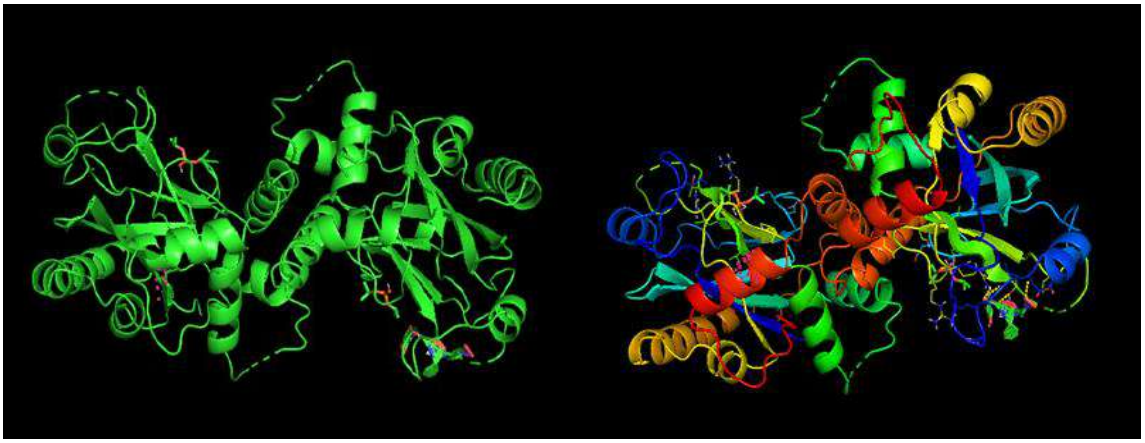

Figure 4: Protein-Ligand interaction

Therefore, the chymotrypsin a serine protease inhibitors from Shouchella clausii should be acted as inhibitory compound against Herpes simplex virus.

CONCLUSION:

In conclusion, the study successfully isolated and identified two mesophilic bacteria, Shouchella clausii and Shouchella rizhosphaerae, from pre-treated soil samples through the characterizations. The antibacterial activity of Shouchella clausii supernatant against S aureus was particularly highly potent and molecular docking showed promising potential for chymotrypsin as a viral protein inhibitor.

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