Isolation, characterization and identification of bacterial strain showing antimicrobial activity

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ABSTRACT

Natural sources such as plants, microorganisms and animals provide a range of natural medicinal mixtures for the treatment of various infectious diseases. There is increasing need to search new antibacterial agents due to the rising incidences of bacterial infections and their resistance towards antibiotics. The antimicrobial production is a common phenomenon for most bacteria. Due to this reason the aim of the present study was focused on the isolation of microbial strains showing antimicrobial activity from the soil collected near the roots of Calotropis species growing at UIET campus of M D University Rohtak. Microorganisms were isolated by serial dilution, plating method and grown on nutrient agar medium. All isolated strains were tested for antimicrobial activity using agar well diffusion method. Out of 8 isolates 7 strains showed antimicrobial activity. The characterization of isolates was carried out by morphological method, biochemical method and molecular characterization techniques. The isolate having highest antimicrobial activity was identified as Bacillus cereus by 16S rRNA sequence analysis.

Keywords: Bacillus cereus, Calotropis spp., 16s RNA sequencing.

INTRODUCTION

Antimicrobials are the natural substances that kill or inhibit the growth of microorganisms and cause little or no damage to the host. In existing situation of pharmaceutical development microbes evolve changes in their metabolism and attain genetic structure which is unaffected against the drugs used in the treatment of common infectious disease[1, 2]. These drug resistant microbes are more pathogenic with high humanity rate and become a challenge in the pharmaceutical and healthcare industry. Present solution involves the development of a new balanced approach to antibiotic use and ascertain of new antimicrobials, but the problem of antibiotic resistance is growing globally and may reduce the current antimicrobial agents insufficient to control some bacterial infections. To overcome this problem, scientists are looking for the development of substitute and different medications.

There are so many sources which show antimicrobial activity such as plant extracts, microorganisms, essential oils etc. but microorganisms are most commonly used source because it causes no or lesser side effect. The improvement of bacterial super resistant strains is ensuing in presently used antibiotic agents which are failing to end many bacterial infections. Due to this reason the research is ongoing for new antimicrobials, either by the design and synthesis of new compounds or through the search of natural products which are antimicrobial agents and not discovered yet [3].

Medicinal plants are the heritage of global importance which plays a dynamic role in world health care system of developing countries. Calotropis spp. is wild plant known for its pharmacological importance from centuries. The complete plant is investigated for various activities such as anti-inflammatory, anti-implantation, anthelmintic, wound healing[4]. Almost every part of this plant is used in various forms in different parts of the world. The root of Calotropis spp. has been found to produce various phytochemicals such as flavonoids, tannins, alkaloids, phenols, steroids, glycosides, saponins which were well studied for antimicrobial activity [5].
The current study is focused on isolation of antimicrobial activity showing microorganisms, their screening, characterization and identification by morphological, biochemical and molecular techniques.

**MATERIALS AND METHODS**

**Sample collection**
Soil sample was collected near the root of *Calotropis spp.* growing in the U.I.E.T campus, M.D University, Rohtak, Haryana. The samples were transferred in sterile zip lock plastic bag and maintained under aseptic conditions in laboratory.

**Microorganisms**
Bacterial strains of *Escherichia coli* (MTCC 118), *Staphylococcus aureus* (MTCC 7405), *Pseudomonas aeruginosa* (MTCC 2582), and *Bacillus megaterium* (MTCC 428) were procured from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh (India). The bacterial strains were maintained on Nutrient agar media and used as test strains for antimicrobial activity assay of isolates.

**METHOD**

**Isolation of bacterial strains**
Serial dilution technique was used for the isolation of microbes [6]. 1.0 gm of soil sample was dissolved in 10 ml autoclaved saline. Then it was serially diluted (10⁻¹ to 10⁻⁶) and diluted samples were evenly spread on to sterilized Nutrient Agar (NA) plates. The inoculated plates were incubated at 37 °C for 24 to 48 hours for development of colonies. The pure isolates were obtained by repeated sub culturing by streaking on NA plates. All the pure isolates were maintained on NA slants at 4° C.

**Screening of Isolates for Antimicrobial Activity**

**Sample Preparation of Bacterial Isolates**
The samples were prepared from cultures grown by aseptically transferring a loop full of cells from all the isolates maintained on agar slants in 50 ml sterilized nutrient broth. The cultures were incubated for 24hrs at 37 °C on an orbital shaker at 150 rpm. 5 ml sample was taken from each culture after the interval of every 2hrs during incubation. Then each sample was centrifuged at 10,000 rpm for 15 minutes. Cell pellet was discarded and supernatant was used for screening for antimicrobial activity.

**Antimicrobial Activity Assay**
Agar well diffusion method [10] was used to assay antimicrobial activity of pure isolates. The Petriplates containing the sterilized agar media were inoculated with test microbial strains by the cotton swab spreading method and wells of 5mm diameter were punched on inoculated agar plates using the sterile gel puncher. 30µl of samples prepared from isolate, amoxicillin (2mg/ml) and sterile distilled water were poured in the wells. The plates were incubated at 37 °C for 24 hours. The zone of inhibition as an indicator of the antimicrobial activity, formed around each sample well was measured.

**Characterization of bacteria**

**Morphological characterization**
Morphological characteristics of isolates were studied by Culture characterization and microscopic observation.

The isolated bacterial strains were observed under microscope to study the morphology i.e. color, shape, size, nature of colony and pigmentation. The isolates were stained by gram staining and observed under light microscope [11].

**Biochemical characterization**
The biochemical characterization of bacterial isolates was done by performing different tests such as starch hydrolysis, nitrate reduction, oxidase test and IMVIC tests[11].

**Molecular characterization**
The molecular characterization of bacterial isolate was done by DNA isolation followed by PCR amplification and sequencing of 16s ribosomal RNA [6-9].

**Identification of bacteria by sequencing of the16S rRNA gene**

**DNA extraction and PCR Amplification**
For isolation of bacterial genomic DNA, 50 ml LB broth was inoculated with a single bacterial colony and grown to an absorbance of 0.5–1.0 at 600 nm. Cells were collected by centrifugation at 5000 rpm, at 4°C, for 10 min. The
bacterial genomic DNA was isolated. Amplification of the 16s rRNA gene was performed using the universal primers.

**Forward primer:** 5’- AGAGTTTTGATCMTGGCTCAG-3’  
**Reverse Primer:** 5’-TACGGYTACCTTGTTACGACTT-3’  
PCR was performed as follows in a total volume of 50 µl in a 0.2 ml thin walled PCR tube.

**Sequencing**
Both strands of the rDNA region amplified by PCR were sequenced by automated DNA sequencer -3037xl DNA analyzer from Applied Biosystems using BigDye® Terminator v3.1 cycle sequencing Kit (Applied Biosystems). Sequence data were aligned and dendrograms were generated using sequence analysis software MEGA version 5.2 from applied biosystems.

**Bioinformatics analysis**
Sequences were compared to the non-redundant NCBI database by using BLASTn, with the default settings used to find the most similar sequence and were sorted by the E score. A representative sequence of 10 most similar neighbours was aligned using CLUSTAL W2 for multiple alignments with the default settings [6-9]. The multiple alignment file was then used to create phylogram using MEGA5 software.

**RESULTS AND DISCUSSION**

**Isolation, Screening and Characterization of bacterial isolates**
Total 8 different bacterial isolates were obtained and single colonies developed during sub culturing. All the pure isolates were maintained on NA plates as shown in Figure 1 and stored at 4°C till further use. Seven isolates showed antimicrobial activity against the test strains.

The isolates were characterized by morphological and biochemical testing. The isolate showing maximum antimicrobial activity was identified as *Bacillus Species* and results are presented in Table 1 and Table 2.

**Antimicrobial activity assay**
All isolates were screened for antimicrobial activity. The samples prepared from pure isolates growing at 2, 4 and 6 hours were tested for antibacterial activity. A significant decrease in antibacterial activity was noticed as growth of isolate progressed from 4 hrs to 6 hrs as presented in Fig. 2 (a) and Fig.2 (b).

The A04 isolate showed maximum zone of inhibition against test strain *B. megaterium*. No zone of inhibition was observed against *P. aeruginosa* by isolates. The strain showing maximum zone of inhibition was selected and further characterized by molecular characterization technique.

**Molecular characterization**
**Identification of bacterial isolate by 16s rRNA sequencing**
The DNA sample was run on an agarose gel. Single band was visualized when observed under the Gel doc, which confirmed the purity of sample, as the bands of DNA were single, distinct and no traces of contaminants were observed. Then, sequencing of the 16s rRNA gene of bacterium was done and the same was amplified by Taq DNA polymerase along with the DNA marker (Fig.3). This was then subjected to agarose gel electrophoresis. The sequence obtained was then blasted in NCBI database. Based on the 16s rRNA sequences, the above bacterium was confirmed as *Bacillus cereus*. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the distance method and are in the units of the number of base differences per site. The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1355 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. (Fig. 4)

**Phylogenetic Analysis**
NR 11399 *Bacillus pseudomycoids*  
NR 074540 *Bacillus cereus*  
NR 041248 *Bacillus anthracis*  
AOA *Bacillus cereus*  
NR 024697 *Bacillus weihenstephanensis*  
NR 113996 *Bacillus weihenstephanensis*  
NR 121761 *Bacillus toyonensis*  
NR 114581 *Bacillus thuringiensis*
NR 116644 *Bacillus gaemokensis*
NR 074914 *Bacillus cytotoxicus*
NR 125530 *Bacillus manliponensis*

![Phylogenetic tree](image1)

**Fig. 4** Phylogenetic tree

![Pure culture of isolates streaked on Nutrient Agar plates](image2)

**Fig. 1** Pure culture of isolates streaked on Nutrient Agar plates

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Form</td>
<td>Irregular</td>
</tr>
<tr>
<td>Color</td>
<td>Creamy</td>
</tr>
<tr>
<td>Gram staining</td>
<td>Positive</td>
</tr>
<tr>
<td>Shape</td>
<td>Rod</td>
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</tbody>
</table>

**Table 1** Morphological characterization of isolate.

<table>
<thead>
<tr>
<th>Biochemical Test</th>
<th>Results</th>
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<tbody>
<tr>
<td>Starch hydrolysis</td>
<td>Positive</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>Positive</td>
</tr>
<tr>
<td>Catalase</td>
<td>Negative</td>
</tr>
<tr>
<td>Oxidase</td>
<td>Negative</td>
</tr>
<tr>
<td>Mannitol</td>
<td>Negative</td>
</tr>
<tr>
<td>Iodole</td>
<td>Negative</td>
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**Table 2** Biochemical characterization of bacterial isolate.
CONCLUSION

Now-a-days, bacterial resistance is a major problem throughout the world. So it is necessary to search microorganism producing new, biologically safe and eco-friendly antibacterial agents. In present investigation bacterial isolate showing highest antimicrobial activity is identified as Bacillus cereus. This isolate can be further exploited for production of antimicrobials.

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REFERENCES


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