ABSTRACT

Objective: To investigate the micro-flora of the mesophilic soil of Rajasthan for isolation of novel compounds having antibacterial potentials.

Methods: In present experiments, bacterial colonies were isolated from four different regions of Jaipur, Rajasthan and screened for antimicrobial efficacy against five selected pathogens Pseudomonas aeruginosa MTCC 7093, Staphylococcus aureus MTCC 7443, Escherichia coli MTCC 40, Klebsiella pneumoniae MTCC 530, and Bacillus subtilis MTCC 121. Antimicrobial efficacy against the selected strains was performed. The potential efficacy of the extract was also screened for gas chromatography–mass spectroscopy (GC-MS) analysis for novel metabolites screening. Further, potent bacterial strains were identified at the molecular level by 16S ribosomal deoxyribonucleic acid (DNA) sequencing method.

Results: After the primary screening, 29 microbial isolates were selected for the screening of bioactivity. Results displayed zones of inhibition ranging from 5 mm till maximum 13 mm. Soil testing indicated survival conditions for microbes isolated, and biochemical tests supported the identification of screened isolates. The potentially isolated strains S-III C, S-III D and S-IV D were identified at the molecular level using 16S ribosomal DNA sequencing as Bacillus shakeletonii, Bacillus thuringiensis and Bacillus subtilis subsp. inaquosorum, respectively.

Conclusion: Extraction of active metabolites from soil microbiota, against five pathogenic bacteria, is far better, safe and economical method. This study will help in exploring new compounds against increasing number of resistant pathogenic strains with an aim to reduce demand of medicinal antibiotics, such as vancomycin, which are usually given for treating infections. However, there have been cases of vancomycin-resistant enterococci and vancomycin-resistant Staphylococcus aureus being reported. This leads to use of alternate antibiotics and prevents the use of vancomycin as the primary drug for the treatment of diarrhoea caused by Clostridium difficile [1].

INTRODUCTION

In India, the most common pathogens behind nosocomial infections are the gram-positive bacteria, with Staphylococcus aureus being the principal one. There has been a raise in the rate of antibiotic resistance in bacteria related to nosocomial infections, most importantly in intensive care units. These are nowadays the prominent sites of infections. There are many broad-spectrum antibiotics, such as vancomycin, which are usually given for treating such infections. However, there have been cases of vancomycin-resistant enterococci and vancomycin-resistant Staphylococcus aureus being reported. This leads to use of alternate antibiotics and prevents the use of vancomycin as the primary drug for the treatment of diarrhoea caused by Clostridium difficile [1].

There are different categories of microorganisms causing nosocomial infections, and the pathogen vary according to the group of people being infected, kinds of health care settings, medical facilities, and also the countries (developed and developing). Commonly reported gram positive microbe includes Staphylococcus aureus, Staphylococci coagulase negative, and Enterococci. Gram-negative ones include Acinobacter baumanii, Klebsiella pneumoniae, Escherichia coli, and Pseudomonas aeruginosa [2-4]. Fungi are also recognized as a cause of nosocomial infections, mainly in blood streams, apart from bacterial infections in India [5].

Natural products are described in simple terms as those chemical matter isolated from different kinds of living beings. These could be classified on a broad basis as primary or secondary. It has been the most suspicious field of microbiology to conclude a precise definition, describe exact functions and existence of secondary metabolites (SMs) in nature [6]. SMs are low molecular weight compounds (usually less that 3000) with diverse and complicated chemical configurations, incomprehensible functions, produced by strains of distinct microbial species, and by some plants. Although antibiotics are the best known bioactive metabolites, there are many more with significant activities of medical, nutritional and industrial importance [7]. They seem to be completely unnecessary for the microbial producers, having no any noticeable function in their life cycle, but they help in the survival of the microbial population in adverse conditions [6]. The function of SMs is opposite to that of primary metabolites, like nucleic acids, proteins, fats and carbohydrates, which are essential for microbial growth. Though the process of secondary metabolite production is based on genes, but these are highly influenced by changes in the environment of the microorganism. Apart from nutrient deficiency, other factors could be declining growth rate and the presence of inducer [8]. These factors tend to generate signals that cause a cascade of events leading to morphological and chemical changes in the microbial strains. It is considered that the cell investment in secondary metabolite production is almost the confirmation of a function that should give the organisms certain advantage against other members of the community. These are produced by the organisms to inhibit other organism’s competing for a same ecological niche. Also, they are produced after active growth of the organism and are structurally diversified. The distribution of SMs is also unique, and some metabolites are found in a range of related microorganisms, while others are only found in one or a few species. Parallel to the screening for new antibiotics, efforts have been focused in finding low molecular weight SMs with different biological activities [9-12]. The SMs isolated from microbes exhibits either antimicrobial; which includes antibacterial, antifungal, anti-protozoan action; antitumor or antiviral activities, earlier known as antibiotics. With the aid of recent knowledge, the term “antibiotic” is more or less an obsolete conception, whenever there is discussion regarding the bioactive SMs. The practical importance of antibiotics and other SMs is incredible. They have wide applications in the human therapy and veterinary therapy, agriculture, scientific research and in numerous other areas.
Antibacterial substances produced by Bacillus species to be found on the second place after laboratory due to variety in the antimicrobial peptide (AMP) due to different chemical structures [13]. The Bacillus subgroup has been reported to produce a huge number of amplifiers, and this can be a good source to explore the novel antibacterial substances [14, 15]. Compounds extracted have been shown to be active against a broad range of gram-positive bacteria [16]. An amount of reported bacteriocins is very less as in comparison to the reported microbial species, so necessitating a need to explore the properties and therapeutic applications of microorganisms such as Bacillus. The aim of the current research was going to target the bacteria producing anti-bacterial substance from the mesophilic soil. The soil is a rich source of microorganisms, as it offers an optimum environment for the growth of microorganisms, which contributes towards tremendous diversity of bacteria. In the current study, we have isolated different microorganisms which are able to produce antimicrobial substances from the soil of the arid and semi-arid region, sterile techniques and required plating methods. The significant microbes were further determined by molecular characterization.

**MATERIALS AND METHODS**

**Chemicals used**

The chemicals and media utilized in the current study were bought from Hi-Media, CDH, SRL and Sigma-Aldrich.

**Methods**

**Test pathogens and growth conditions**

Pure cultures of five test pathogens were obtained from Department of Microbiology, JECRC University, Jaipur and SMS hospital. These included Pseudomonas aeruginosa MTCC 7093, Staphylococcus aureus MTCC 7443, Escherichia coli MTCC 40, Klebsiella pneumoniae MTCC 530, and Bacillus subtilis MTCC 121. These test pathogens were cultured in nutrient broth at 36 °C for 16-18 h. Microbial isolates were tested against these five bacteria using disc diffusion, well diffusion and perpendicularly cross streak methods [17, 18].

**Isolation of antibacterial compounds producing bacteria from mesophilic soil**

Bacterial strains were isolated from soil collected from four locations of Jaipur, Rajasthan i.e. slum area of Jawahar circle, SMS hospital, Durgapura and Mansarovar, and listed in table 1. Selection of site was also based on various factors, like the temperature of the particular area, population load around the area, the presence of any organic or inorganic additive/contaminant to the soil of the particular region, ease of repetition of sample collection, and ease of accessibility of the area. Soil was collected from 0.04 m deep from the top soil layer, at each of these locations. It was then brought to the laboratories and stored at 4 °C till further processing.

Suspension of all the four soil samples was prepared using physiological methods and followed by serial dilution method. Spread plate technique on nutrient agar plates was used for the isolation of microorganisms. Serial tenfold dilution of mesophilic soil samples were spread on sterile nutrient agar plates and incubated at 36 °C for 24 h. Total 29 isolates were obtained out of which only six were morphologically dominant which were selected for further study. Nutrient agar (5 g/l peptone; 3 g/l beef extract; 5 g/l NaCl; 15 g/l agar) was prepared and autoclaved for plating the samples using warcup method and direct soil sprinkle method. After 24-36 h of incubation at 35 °C and 40 °C plates were observed for screening the required colonies [19].

**Upscaling of culture**

The colonies screened from the dilutions of soil samples were inoculated in Luria broth media. Shaker treatment for 2 h daily at 700 rpm was provided for 30 d at different temperature ranges. Regular testing of metabolites (primary/secondary) was done after 7 d, 15 d, 21 d, and 27 d [20].

Liquid-liquid extraction of bioactive compounds

After 27 d of incubation, each culture was centrifuged at 8000 rpm for 10 min and the supernatant was collected separately. Metabolites were extracted using three solvents i.e. benzene, ethyl acetate and chloroform. The supernatant was mixed in 2:1 ratio with each of the three solvents, shaken and allowed to mix properly. The mixture was left undisturbed to allow the separation of the solvent is having the dissolved metabolites from the culture. The solvent was then decanted from the culture and allowed to vaporize at 40-50 °C in the oven. The method used for separating bioactive compounds from extracellular SMs was liquid-liquid extraction. Further, these extracts were named as LLE-I, LLE-II and LLE-III for benzene, ethyl acetate and chloroform respectively [21].

**Analysis of biological activity**

The dried form of compounds collected was again mixed in 2-3 ml of respective solvents. Sterilized circular discs were cut and soaked in solvents containing bioactive compounds. Lawn of five common nosocomial infection causing pathogens i.e. Staphylococcus aureus, Klebsiella pneumonia, Bacillus subtilis, Pseudomonas aeruginosa and Escherichia coli was prepared on nutrient agar, and all are nomenclature in table 2. The antimicrobial activity was then tested against these pathogens using disc diffusion method [22]. Plates were incubated at 38°C for 48 h.

**Segmentation and categorization of biologically active compounds**

Centrifugation

Centrifugation of culture broth was performed at 8000 rpm for 10 min. Supernatants of S-III C, S-III D and S-IV C were collected after liquid-liquid extraction with benzene, ethyl acetate and chloroform as LLE I, LLE II and LLE III, respectively, and processed for antimicrobial screening. Three bio-actively rich fractions were collected and stored at 4 °C for further studies.

**Thin layer chromatography (TLC)**

Glass plates of 18 cm ×18 cm were used to perform TLC, so that approximately 4 samples could be run together. The slurry was made with silica gel and water. Mixing and shaking of silica gel in water should be proper for the homogenous and adhesive mixture. Thin layer on glass plates was formed and kept for 3-4 h on the plain surface for drying and later in the oven at 70 °C. The temperature of the oven was raised to 110 °C for 1 h for activation of the plates. The plates were taken out and allowed to cool. Mobile phase (solvent) was prepared and poured in TLC glass chamber in which plates spotted with extract were placed. The plates were placed in the chamber till it run or develop up to ¾ of the TLC plate and then again kept in oven for drying. Plates were sprayed with specific reagents and spots were observed. The developed plate was baked at 110 °C for 30 min and observed under UV light chamber and the displacement of development (mobile phase) and extract were measured and recorded [23].

**GC-MS analysis**

Shimadzu model QP-2010 plus, column-Rtx-Ms, 30 meter × 0.25 mm i.d. × 0.25 um film thickness was used for detection. Samples were prepared accordingly for analysis. Extracts were collected in 100 ml beaker and mixed with methanol. The mixture was filtered properly to remove any crystal particle. The homogenous solution was collected in ependrof after testing it with microinjection. Samples were loaded in the injector and processed. Chromatograms with compounds detected in solvent were recorded and compared compound library [24].

**Microbial identification**

The bacterial samples (S-III C, S-III D and S-IV C) were sent for identification based on 16SrRNA analysis and the recorded in table 8-10. The identification report was generated using EZ-Taxon Database and the confidence in identification is limited by both the availability and the extent of homology shown by the ~1200 bp sequence of the sample with its closest neighbor in the database. Details and sequence fasta are shown in fig. 3 [25].
Bacterial genomic DNA was isolated using the Insta Gene TM Matrix Genomic DNA isolation kit as per the kit instruction procedures. An isolated bacterial colony was picked and suspend in 1 ml sterile water in a microfuge tube, centrifuged for 1 minute at 10,000–12,000 rpm to remove the supernatant. Around 200 μl of Insta Gene matrix was added to the pellet and incubated at 56 °C for 15 min, rotated at high speed for 10 seconds, and placed in a 100 °C heat blocker boiling water bath for 8 min. Finally, the content was rotated at high speed for 10 seconds and spanned at 10,000–12,000 rpm for 2 min. In result, 20μl of the supernatant was used per 50 μl PCR reaction.

RESULTS AND DISCUSSION

Bacterial strains isolation

Four locations were selected from the vicinity of Jaipur slum area of Jawahar circle, SMS hospital, Durgapura and Mansaro var, identity number given to the particular location is S-I, S-II, S-III and S-IV respectively. The locations were selected on the basis of the quality of the soil. These areas are rich in contamination as hospitals are the prime locations and sites for the various infections and slum areas are the power plants for the same. Further, the locations are of the arid region. Samples were collected in sterile bags and 29 colonies were isolated from all the four areas were shown in table 1. These colonies were subcategorized into A, B, C, and D etc for systemic nomenclature. These colonies were collected by various streaking methods viz. warcup method and direct soil sprinkle method onto nutrient agar media.

These 29 colonies were further sub-cultured to isolate as pure colonies and used for future testing. To start with the target was to discover the organisms having a resistive action against a few pathogens and also for nosocomial infection-causing pathogens. 29 isolates were processed through various test techniques against selected 5 test pathogens i.e. E. coli, Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella pneumonia and Staphylococcus aureus. The test pathogens which were used are listed with MTCC code in table 2.

Table 1: Screened microbial isolates

| Soil sampling location | Soil sample I.D. | Total no. of bacterial isolates | Isolate I.D. |
|------------------------|------------------|--------------------------------|--------------|
| JC slum area           | S-I              | 7                              | A,B,C,D,E,F,G |
| SMS hospital           | S-II             | 8                              | A,B,C,D,E,F,G,H |
| Durgapura              | S-III            | 7                              | A,B,C,D,E,F,G |
| Mansaro var            | S-IV             | 7                              | A,B,C,D,E,F,G |

Table 2: List of test pathogens

| Soil sampling location                  | Soil sample I.D. | Total no. of bacterial isolates |
|----------------------------------------|------------------|--------------------------------|
| Pseudomonas aeruginosa                  | MTCC 7093        | P1                             |
| Staphylococcus aureus                   | MTCC 7443        | P2                             |
| Escherichia coli                        | MTCC 40          | P3                             |
| Klebsiella pneumonia                    | MTCC530          | P4                             |
| Bacillus subtilis                       | MTCC 121         | P5                             |

This limited down the search for extraction of a compound of fundamental significance. Out of 29 isolates, 3 indicated biological action against previously mentioned 5 test pathogens were listed in table 2 and shown in fig. 1. Whereas table 3 clearly shows the size zone of inhibition in millimeter and conclusive data was interpreted by mean deviation.

Fig. 1: Antimicrobial screening plates showing positive results, A=S-III C against Pseudomonas aeruginosa, B=S-III D against, Staphylococcus aureus, C=S-IV C against Escherichia coli

Table 3: Antimicrobial analysis of strains (S-IIIC, S-IIID and S-IV C) against five test pathogens i.e. P1, P2, P3, P4 and P5

| Identification of isolates shown antibiotic activity | Zone of inhibition (mm) |
|-----------------------------------------------------|-------------------------|
|                                                      | Test pathogens          |
|                                                      | P1          | P2           | P3            | P4           | P5            |
| Soil samples                                         |             |              |               |               |               |
| S-IIIC                                               | 13±0.20     | 0            | 0             | 0             | 0             |
| S-IIID                                               | 0           | 0            | 9±0.20        | 0             | 0             |
| S-IV C                                               | 0           | 10±0.3       | 0             | 0             | 0             |

Values are mean Inhibition zone (mm)±SD of quadruplicate, P1—Pseudomonas aeruginosa; P2—Staphylococcus aureus; P3—E. coli; P4—Klebsilla pneumonia and P5—Bacillus subtilis

For further screening, these 3 isolates were prepared through maturation handle with standard shaker treatment for a particular span and diverse incubation period and temperature. Centrifugation took after by extraction with solvents LLE-I, LLE-II and LLE-III
separately. Activity test is necessary for affirming which fraction is really producing SMs or compounds and under what conditions it is in charge of the movement against the test pathogens are tabulated in table 4.

| Isolates | Rf Value (Benzene) | E. A. | Chloroform | Color of spot | Benzene | E. A. | Chloroform |
|----------|--------------------|------|------------|--------------|---------|------|------------|
| S-III C  | -                  | 0.3  | -          | -            | Dark brown | -    | -          |
| S-III D  | 0.5                | 0.15 | -          | N. D.        | Dark orange | -    | -          |
| S-IV C   | -                  | -    | 0.11       | -            | Grayish   | -    | -          |

Tests and above-mentioned experiments confirmed that the isolates S-III C, S-III D and S-IV C are producing the compounds which need to be targeted. For detailed information about the compounds, they were processed through TLC with Rf value mentioned in table 5.

| Sample location | Catalase test | Of basal test | SIM test | Mac conkey test | Starch hydrolysis | Motility | Mannitol test | Urease test | Tentative microbe |
|-----------------|---------------|---------------|----------|-----------------|-------------------|----------|---------------|-------------|------------------|
| S-III C         | C             | +             | Red      | -               | +                 | +        | -             | -           | Enterobacter      |
| S-III D         | D             | +             | -        | +               | -                 | -        | +             | -           | Enterobacter      |
| S-IV C          | C             | +             | Red      | +               | -                 | -        | +             | -           | Enterobacter      |

Disengagement, determination and biochemical testing was accomplished for the essential screening of microorganisms and enlisted in table 6 and 7. Principle target was to detach a microorganism which produces a compound which can be valuable in assembling drug against nosocomial disease bringing on pathogens.

| Sample location | Shape | Color | Opacity | Elevation | Surface | Texture | Gram+ve/-ve |
|-----------------|-------|-------|---------|-----------|---------|---------|-------------|
| S-III C         | C     | Rods  | Buff    | Opaque    | Curve   | Smooth  | Butyrous negative |
| S-III D         | D     | Rods  | yellow  | Opaque    | Negligible | Smooth  | Butyrous negative |
| S-IV C          | C     | Rods  | Buff    | Translucent | Negligible | Smooth  | Viscid negative |

Applications and medical significance of every compound were analyzed. Isolates which showed biological activities against nosocomial infection causing pathogens were tested for biochemical as well as their morphological features. After the primary and secondary screening, these isolates were analyzed for 16s RNA sequences for the comprehensive identification and genome sequencing was reported in table 8-10. The results proved that the identified nucleotide sequences were having great similarities to the Bacillus species, which confirms the production of antibacterial compounds by culturing and processing confirmed microorganism.

| S. No. | Name of strain            | Code    | Accession no. | Pairwise similarity % | Reference |
|--------|---------------------------|---------|---------------|-----------------------|-----------|
| 1      | Bacillus sonorensis       | ATCC 14579(T) | ABD16077       | 99.21               | [26]      |
| 2      | Bacillus aureus           | ATCC 14578(T) | ADB190217      | 99.11               | [27]      |
| 3      | Bacillus tequilensis      | -       | AEB16079       | 99.12               | [27]      |
| 4      | Bacillus thuringiensis    | ATCC 10792(T) | ACNF01000156   | 98.84               | [28]      |
| 5      | Bacillus subtilis sub sp. | BCT-7112(T) | CP006863       | 98.54               | [29]      |

| S. No. | Name of strain            | Code    | Accession no. | Pairwise similarity % | Reference |
|--------|---------------------------|---------|---------------|-----------------------|-----------|
| 1      | Bacillus shackletonii     | ATCC 14580(T) | ABD17333      | 99.68               | [30]      |
| 2      | Bacillus sonorensis       | NBRC 10123(T) | AYTN010000016  | 99.61               | [31]      |
| 3      | Bacillus aureus           | 24KT(T) | AY831843      | 99.36               | [32]      |
| 4      | Bacillus toyonensis       | GSS04(T) | KJ018278      | 99.01               | [33]      |
| 5      | Bacillus shackletonii     | LMG 19435(T) | A250318       | 98.87               | [30]      |
GC–MS study elaborated every bit of information about the compounds extracted from isolates, like molecular weight, RT time, boiling point, structural formula, linear formula, and IUPAC names were mentioned in fig. 2. Applications and medical significance of every compound were analyzed with chemical library [37]. GC–MS analysis of S-III C, S-III D and S-IV C active rich fractions possessing four, three and four antibacterial compounds respectively. The RT of the Peaks reveals the presence of different compounds. The area covered by the peaks is directly proportional to the amount of compound present in the solvent. The peaks were selected and compared with a standard which automatically generated the list of compounds were enlisted in fig. 2.

**PCR protocol**

Using below 16S rRNA Universal primers (table 11 and 12) gene fragment was amplified using MJ Research Peltier Thermal Cycler.

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### Table 10: List of the closely related strains for sample S-IV C

| S. No. | Name of strain | Code | Accession no. | Pairwise similarity % | Reference |
|--------|----------------|------|---------------|------------------------|-----------|
| 1      | Bacillus subtilis sub sp. inaquosorum | KCTC13429(T) | AMXN01000021 | 99.72 | [34] |
| 2      | Bacillus aureus | KCTC13622(T) | AYO01000043 | 99.64 | [35] |
| 3      | Bacillus anthracis subsp. subtilis | NCIB3610(T) | ABQ01000001 | 99.51 | [34] |
| 4      | Bacillus subtilis ub sp. spizizenii | NCIB3610(T) | CP002905 | 99.34 | [36] |
| 5      | Bacillus toyonensis | DSM8802(T) | AM747812 | 99.32 | [33] |

### Table 11: Primer details

| Primer name | Sequence details | Number of base |
|-------------|-----------------|----------------|
| 27F         | AGATTTTATCCMTGGCTCAG | 20             |
| 1492R       | TGGGTACCTGTTAGACTT  | 22             |

### Table 12: Sequencing primer details

| Primer name | Sequence details | Number of base |
|-------------|-----------------|----------------|
| 785F        | GGATTTAGATCCCTTGTA | 18             |
| 907R        | CGTCAATCTTTTTRAGTTT | 20             |
The 16s rRNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of our sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences [38]. The resulting aligned sequences were used using the program G blocks 0.91b. Primer 785F and 907R was used for sequence details and the number of the nucleotide sequence are shown in fig. 3 A, B and C, the number of base are 18 and 20 as shown in table 13. This G blocks eliminates poorly aligned positions and divergent regions [39]. Finally, the program PhyML 3.0aLRT was used for phylogeny analysis and HKY85 as Substitution model. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data while being one order of magnitude faster. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering [40].

Fig. 3: 16s rRNA sequencing of isolates, A=S-III C, B=S-III D, C=S-IV C.
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