Antimicrobial and enzymatic activity of actinomycetes isolated from soils of coastal islands

Abstract

Microbiological investigation of unexplored ecosystems is crucial for discovering of antibiotic producing actinomycetes. The present study was conducted to determine antimicrobial activity and identify the most active strains. Actinomycetes were isolated using the spread plate technique following by serial dilution of samples on starch casein agar. The screening method consists of primary and secondary testing. The most active isolates were identified based on molecular and cultural methods. 42 out of 66 isolates displayed antimicrobial potential. 63% exhibited antibacterial activity, 16% antifungal activity, and 16% displayed both activities. Identified isolates, *Streptomyces scabrisporus*, *Streptomyces sparsogenes*, *Streptomyces misakiensis*, *Streptomyces cirratus*, *Streptomyces lincolnensis*, *Streptomyces endophyticus*, *Streptomyces chartreusis*, and *Streptomyces alboniger* showed a broad spectrum of enzymatic activities. The results indicated that these isolates may serve as antibiotic and enzyme-producing microbes.

Key words: Actinomycetes, human pathogens, primary and secondary screening, unexplored soils

INTRODUCTION

Although the soil is a natural reservoir of actinomycetes which produce antibiotics for pharmaceutical industry,[1] unexplored habitats and niches have attracted considerable attention in recent years.[2] Actinomycetes are Gram-positive, saprophytic filamentous bacteria[3] that are responsible for the production of over 20,000 natural products extensively used in pharmaceutical and agrochemical industry.[4] The need of new antimicrobial agents is greater than ever due to the emergence of multidrug resistance in common pathogens, and the rapid emergence of new infections.[5] Keeping these points in view, the present study was undertaken to isolate and characterize antimicrobial actinomycetes from soil samples collected in coastal areas. In accordance with the previous reports, we initiated this research program, because to obtain antimicrobials from soils, scientific are trying to investigate unexplored habitats for interesting streptomycetes as the possible candidates for the discovery of antimicrobial compounds.

MATERIALS AND METHODS

Collection of samples

The soil samples were collected from coastal islands, Mauritius, archipelago Mascarenes (20°18′32.45″S, 57°22′10.52″E), Bahamas – Nassau (25°02′12.92″N, 77°22′27.63″W), Canary Islands–Gran Canaria (28°09′37.62″N

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15°25'12.50W), and from Democratic Socialist Republic of Sri Lanka – Colombo (6°55'03.40"N 79°51'11.60E). The soil samples were air-dried for 2 days, crushed, sieved and dried for 45 min at 60°C to eliminate the fungal growth. The pH of soils was determined in water solution.

Isolation of actinomycetes
Soil samples were plated following the dilution plating method on starch casein agar[8] supplemented with cycloheximide (1 mg/mL). The plates were incubated at 30°C for 7 days. The identification of actinomycetes was done based on morphology of spore chains and presence of aerial and substrate mycelium. Isolates were purified on ISP2 medium[7] and identical colonies were scored out.

Screening of antimicrobial activity
Primary screening was determined by agar plug method.[9] The ethyl acetate extracts of active strains were subjected to secondary screening using the broth microdilution method.[9] The ethyl acetate was added to the cultures in the ratio of 1:1 (v/v), centrifuged, and evaporated in a rotary evaporator (Stuart, UK) at 40°C. Final extracts were dissolved in 1 ml of ethyl acetate:acetone:methanol solution in ratio 1:1:1 (v/v). Dilution stages of raw extracts were observed by inhibited wells (A-H).

The test microorganisms included Bacillus subtilis (B.s) (DSM 10), Micrococcus luteus (M.l) (DSM1790), Staphylococcus aureus (S.a) (Newman), Mycobacterium smegmatis (M.s) (ATCC 700084), Escherichia coli (E.c1) (DSM 1116), E. coli (E.c2) (TolC), Pseudomonas aeruginosa (Ps.a) (PA14), Chromobacterium violaceum (Ch.v) (DSM 30191), Candida albicans (C.a) (DSM 1665), Pichia anomala (P.a) (DSM 6766), and Mucor hiemalis (M.h) (DSM 2656).

Characterization of the most antagonistic isolates
Molecular taxonomy and phylogenetic analysis
The isolation of genomic DNA was done by the method described by[16] and amplified by PCR using primers according to.[11] The PCR reaction ran in thermocycler Biometra T Personal (Germany). Reaction mixture was prepared in total volume of 50 μL (5 μL of 10 × DreamTaq Green PCR buffer, 5 μL of 2 mmol.dm–3dNTP, 2 μL of each 10 μmol.dm–3 primer, 0.3 μL Taq DNA polymerase, and 0.5 μL of template DNA—approximately 20 ng). The PCR reaction ran under the following conditions: 95°C for 3 min, 40 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 90 s, and a final extension at 72°C for 10 min. PCR products were purified with exonuclease I and thermosensitive alkaline phosphatase. The sequencing was carried out in MacroGen, South Korea. The similarity of the 16S rRNA partial gene sequences was analyzed with the similar existing sequences available in the data bank of NCBI using BLAST search.

Morphological, physiological, and biochemical characterization
Morphological characteristics of the strains were determined using the methods given by the International Streptomyces Project (ISP) on ISP2-ISP7 media.[17] Physiological characteristics included the growth at different temperatures range from 4°C to 42°C, pH range from 4.0 to 10.0, sodium chloride tolerance test (2.5%, 5%, 7.5%, and 10%), and utilization of the different carbon sources (glucose, arabinose, inositol, cellulose, mannose, fructose, galactose, rhamnose, sucrose, xylose) on ISP9 medium. Biochemical characteristic was done using ApiZym® kit (bioMérieux, France). Api stripes were inoculated and evaluated according to manufacturer's manual.

RESULTS AND DISCUSSION
In the present study, actinomycetes were isolated from soil samples collected from different coastal areas of the world with a view to isolate cultures with antimicrobial activities. A total of 264 actinomycetes were isolated from 4 soil samples at mesophilic temperature. Maximum number of colonies was obtained from samples no. 1 and 2. When considering the pH of the samples, the highest viable colonies were recovered from neutral to alkaline (pH 6.7–7.5) soil while the lowest from acidic soil [Table 1].

The actinomycetes grew for 1 week thus releasing of their secondary metabolites was enabled. Based on colony morphology, 66 suspected actinomycete cultures were tested in primary screening. The formation of inhibition zones as a result of the production of secondary metabolites were exhibited at 64% of all studied actinomycetes. Tested strains showed high activity against Gram-positive bacteria, moderate activity against Gram-negative bacteria C. violaceum and E. coli (TolC), yeasts and fungus and low activity against Gram-negative bacteria, P. aeruginosa and E. coli (DSM 1116) [Figure 1a]. The results from the present study are in agreement with many studies.[12,13] They reported that actinomycetes usually show good activity against Gram-positive bacteria but lacking activity against Gram-negative bacteria. The reason for different sensitivity could be ascribed to the morphological differences of

### Table 1: Distribution of actinomycetes

| Sample | Geographical location  | pH | NI (CFU/g) | NMDC | Labeling of studied isolates |
|--------|------------------------|----|-----------|------|------------------------------|
| 1      | Mauritius              | 7.2| 86×10¹     | 20   | MR1-MR20                    |
| 2      | Bahamas-Nassau         | 7.5| 104×10¹    | 29   | BNA1-BNA25                  |
| 3      | Canary Islands-Gran Canaria | 6.7| 26×10¹    | 5    | CG1-CG5                    |
| 4      | Sri Lanka-Colombo      | 6.2| 48×10¹     | 12   | SL1-SL12                    |

NI: Number of isolates, NMDC: Number of morphologically different colonies
their outer membrane. There is also the possibility that Gram-negative bacteria might have acquired the resistant genes from the neighboring resistant bacterial cells in the previous environment.

All active strains in primary screening were subjected to secondary screening [Figure 1b]. Some of the isolates did not show any activity. This may be associated with disintegration of the ethyl acetate extracts during the extraction process, while some showed low or improved activity. During this screening, actinomycete isolates show antibiotic activity mostly on agar plates in comparison with liquid extracts. There are two explanations for these results; first, the cultivation on solid and liquid media may lead to the production of different active antibiotics. Second, some compounds may be lost during the organic solvent extraction, because the active components may become inactivated during the extraction step. Eight isolates [Table 2] showed high antimicrobial activity against both bacterial and fungal test organisms and therefore were selected for the next characterization studies.

Noticeable development was recorded when isolate MR3 inhibited almost all the bacterial as well as fungal test organisms to a greater extent. Maximum activity was recorded against B. subtilis, S. aureus, M. luteus, and M. smegmatis (32–42 mm) with inhibited wells until H.

On the other hand, isolate CGI2 proved to be more active against Gram-negative organisms, especially against C. violaceum and E. coli DSM1665. The fact that selectively active actinomycete isolates exhibited broad spectrum of antibacterial and antifungal activities signify the possible production of several antimicrobial secondary metabolites and secretion of compounds with multiple microbial targets.

**Characterization of the most potent isolates**

To clarify which species were responsible for the antimicrobial effect and understand their phylogenetic relationships, those actinomycetes were identified based on 16S rRNA gene sequences and cultural taxonomy procedure. The dendrogram of the eight isolates indicating species relatedness. The dendrogram differentiated the strains into two broad groups. Strains CGI4 and CGI5 formed the first group while CGI2, CGI3, SL11, BNA21, MR3, and BNA14 constituted the second group. Phylogenetic comparison of sequences with the databases of valid species using NCBI server is shown in Figure 2.

It could be stated that the isolates could be identified as Streptomyces scabrisporus (CGI4), Streptomyces sparsogenes (CGI5), Streptomyces misakiensis (CGI2), Streptomyces cirratus (CGI3), Streptomyces lincolnensis (SL11), Streptomyces endophyticus (BNA21), Streptomyces chartreusis (MR3), and Streptomyces alboniger (BNA14) with 99% similarity.

Figure 1: Antimicrobial activity of tested actinomycetes in (a) primary screening, (b) secondary screening

Table 2: Antimicrobial activity of the most active strains in primary and secondary screening

| Strain | B. subtilis | C. violaceum | E. coli 1 | E. coli 2 | M. luteus | P. aeruginosa | M. smegmatis | S. aureus | M. hiemalis | P. anomala | C. albicans |
|--------|-------------|--------------|-----------|-----------|-----------|---------------|-------------|-----------|------------|------------|-------------|
| MR3    | 42/H        | 16/C         | 14/B      | 18/C      | 31/H      | 14/B          | 29/C        | 35/H      | 14/B       | 16/B       | 14/A        |
| BNA14  | 22/G        | 13/A         | 11/A      | 16/C      | 22/G      | 10/A          | 24/D        | 36/H      | 12/A       | 14/A       | 14/A        |
| BNA21  | 34/H        | 18/C         | 0/-       | 18/D      | 25/G      | 14/B          | 28/G        | 32/H      | 14/B       | 18/C       | 12/D        |
| CGI2   | 16/C        | 22/D         | 16/B      | 20/D      | 18/C      | 17/C          | 16/B        | 15/D      | 0/-        | 11/A       | 11/A        |
| CGI3   | 28/G        | 12/A         | 11/A      | 14/B      | 31/H      | 10/-          | 16/B        | 32/H      | 0/-        | 0/-        | 0/-         |
| CGI4   | 18/C        | 15/C         | 14/B      | 14/B      | 32/H      | 0/-           | 14/B        | 31/H      | 18/C       | 12/A       | 10/A        |
| CGI5   | 34/H        | 12/A         | 0/-       | 16/C      | 34/H      | 0/-           | 29/H        | 28/G      | 12/A       | 13/A       | 13/A        |
| SL11   | 21/C        | 19/C         | 13/B      | 15/C      | 21/D      | 10/A          | 20/C        | 18/C      | 0/-        | 11/A       | 13/A        |

B. subtilis: Bacillus subtilis, C. violaceum: Chromobacterium violaceum, E. coli: Escherichia coli, M. luteus: Micrococcus luteus, S. aureus: Staphylococcus aureus, M. smegmatis: Mycobacterium smegmatis, P. aeruginosa: Pseudomonas aeruginosa, M. hiemalis: Mucor hiemalis, P. anomala: Pichia anomala, C. albicans: Candida albicans, P. aeruginosa: Pseudomonas aeruginosa
Direct PCR amplification of 16S rRNA sequences from the most active isolates demonstrated excellent congruence with morphological and physiological characteristics. Morphology and physiology of tested strains were compared with the most similar strain given by 16S rRNA results. Six selective media were used to observe the colony morphology of the isolates. The colors of the aerial and substrate mycelium, aerial hyphae arrangements, and spore chain ornamentation indicating variation among the tested isolates [Tables 3 and 4].

Enzymatic characteristics and quantification of studied enzymes were analyzed with ApiZym® stripes [Table 5]. We found out that all isolates exhibited high (>40 nmol) alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and glucosidase activity. According to many previous reports, mentioned activities are common among streptomycete isolates.[17,18] The use of ApiZym® stripes is providing the advantage of easy and fast determination between two isolates showing significant appearance by means of the differences in their enzyme profiles.
Table 3: Morphological features of the most active strains

| Isolates | Color of aerial mycelium | Color of substrate mycelium | Soluble pigments | Shape of sporophores |
|----------|--------------------------|-----------------------------|-----------------|---------------------|
| CGI4     | ISP2-ISP7 none           | ISP2-ISP7 ivory             | None            | Spiral              |
| CGI5     | ISP2-5 grey, ISP6-7 yellow | ISP2-7 yellow               | None            | Spiral              |
| CGI2     | ISP2-7 grey              | ISP2,3,7 red, ISP4-6 yellow | Red             | Rectus flexibilis   |
| CGI3     | ISP2-4 cream, ISP5-7 none | ISP2-7 beige               | None            | Rectus flexibilis   |
| SL11     | ISP2-7 white             | ISP2, 5-7 brown, ISP3-beige | Brown           | Rectus flexibilis   |
| BNA21    | ISP2,4,5 white, ISP3,6,7 yellow | ISP2-5 orange, ISP6-7 brown          | None            | Rectus flexibilis   |
| MR3      | ISP2-5 blue, ISP6, 7 none | ISP2-5 ivory, ISP6,7 brown | Brown           | Spiral              |
| BNA14    | ISP2-4 grey, ISP3, 5-7 sparse | ISP2-4 grey, ISP5-7 beige | Grey            | Rectus flexibilis   |

ISP: International Streptomycete Project

Table 4: Physiological features of the most active strains

| Isolate | NaCl tolerance | C-utilization | Optimal pH | Optimal temperature (°C) |
|---------|----------------|---------------|------------|--------------------------|
| CGI4    | Up to 5%       | Glucose, arabinose, inositol, sucrose | 7           | 28                       |
| CGI5    | Up to 2.5%     | Glucose, sucrose, mannitol, raffinose | 7           | 28                       |
| CGI2    | Up to 2.5%     | Glucose, arabinose, fructose           | 7-8         | 30                       |
| CGI3    | Up to 5%       | Glucose, arabinose, fructose           | 7-8         | 28                       |
| SL11    | Up to 2.5%     | Glucose, arabinose, xylose, inositol, mannose, fructose, raffinose | 7           | 28                       |
| BNA21   | Up to 2.5%     | Glucose, arabinose, mannose            | 7           | 28                       |
| MR3     | Up to 2.5%     | Glucose, arabinose, xylose, inositol   | 7           | 30                       |
| BNA14   | Up to 2.5%     | Glucose, arabinose, inositol, mannose, cellulose | 7           | 28                       |

Table 5: Enzymatic potential of selected streptomycete strains using API® ZYM stripes

| Enzymes                      | CGI4 | CGI5 | CHI2 | CHI3 | SL11 | BNA32 | MR3 | BNA14 |
|------------------------------|------|------|------|------|------|-------|------|-------|
| Alkaline phosphatase         | 5    | 5    | 5    | 5    | 5    | 5     | 5    | 5     |
| Esterase                     | 3    | 4    | 3    | 4    | 2    | 3     | 4    | 4     |
| Esterase lipase              | 4    | 5    | 4    | 3    | 3    | 2     | 3    | 3     |
| Lipase                       | 4    | 1    | 4    | 0    | 0    | 0     | 2    | 0     |
| Leucine arylamidase          | 5    | 5    | 5    | 5    | 5    | 5     | 5    | 5     |
| Valine arylamidase           | 3    | 1    | 3    | 1    | 5    | 4     | 5    | 5     |
| Cystine arylamidase          | 2    | 0    | 1    | 1    | 4    | 3     | 4    | 1     |
| Trypsin                      | 2    | 0    | 0    | 0    | 2    | 1     | 2    | 2     |
| Chymotrypsin                 | 5    | 0    | 0    | 0    | 5    | 0     | 0    | 5     |
| Phosphatase acid             | 5    | 5    | 4    | 5    | 5    | 5     | 5    | 5     |
| Naphthol-AS-BI-phosphohydrolase | 5    | 5    | 3    | 5    | 5    | 5     | 5    | 3     |
| Galactosidase                | 0    | 0    | 0    | 0    | 3    | 3     | 1    | 0     |
| Galactosidase                | 1    | 4    | 0    | 4    | 3    | 3     | 5    | 1     |
| Glucuronidase                | 0    | 0    | 0    | 0    | 0    | 0     | 0    | 0     |
| Glucosidase                  | 3    | 5    | 3    | 5    | 5    | 4     | 5    | 5     |
| Glucosidase                  | 2    | 1    | 4    | 0    | 4    | 4     | 3    | 4     |
| N-acetyl-glucose-amidase     | 1    | 3    | 5    | 5    | 1    | 5     | 5    | 0     |
| Mannosidase                  | 2    | 0    | 0    | 4    | 2    | 5     | 1    | 0     |
| Fucosidase                   | 0    | 0    | 0    | 0    | 0    | 1     | 0    | 0     |

5: >40 nmol, 4: 20 nmol, 3: 10 nmol, 2: 5 nmol, 1: 2.5 nmol, 0: No activity

For the proper identification of genera and species of streptomycetes, morphological, physiological and biochemical properties together with 16S rRNA sequencing were used to make fast and reliable identification of antimicrobial active strains, which can provide an interesting source of secondary metabolites, especially antibiotics.

CONCLUSIONS

Extensive screening of the isolates for their antibacterial and antifungal activity revealed that eight of all tested strains have strong antibiotic producing potential. Therefore, these isolates prove to be promising strains which can be further studied for its applications in the production...
of important pharmaceutical compounds. Search for the important metabolites, especially from streptomyces, requires screening from a large number of isolates to discover biological compounds and this study implies that streptomyces from coastal soils are a potential source for the development of interesting antimicrobial agents.

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Conflicts of interest
There are no conflicts of interest.

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