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Bioprospecting of actinobacteria from Yelagiri hills with special reference to antibacterial activity

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ABSTRACT

Actinobacteria are the group of ecologically and industrially important prokaryotes but their distribution and utilization from hills/forest ecosystems especially are very less interms of bioactive molecules. With this view an attempt was made for the exploitation of actinobacteria from Yelagiri Hills with special reference to antibacterial activity. Actinobacteria from Yelagiri hill soil was isolated using starch casein agar and the counts were ranged between 1.5X10^4 and 6X10^{4}cfu/gm of soil. Based on the cultural morphology about 40 actinobacterial isolates were selected for further investigations. In antibacterial screening against Staphylococcus aureus, Streptococcus species, Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa by agar plug method, about 50% of the actinobacterial isolates was inhibited atleast any one of the six bacterial pathogens tested. In particular, strain number YA3 (7-17mm zone of inhibition) and YF2 (9-16mm zone of inhibition) showed broad spectrum activity. Bioactive metabolites from YA3 and YF2 were produced by adopting submerged fermentation using ISP2 broth. In agar well diffusion method culture filtrate from strain YA3 showed promising activity when compared to YF2. Further crude extract from the YA3 culture filtrate was extracted using ethyl acetate and n-hexane and tested for antibacterial activity by disc diffusion method. Only the ethyl acetate extract showed good activity against all the test bacterial pathogens. In thin layer chromatographic separation, the crude ethyl acetate extract of strain YA3 produced two spots with Rf value of 0.76 and 0.83. In bioassay guided fractionation, both the spots showed antibacterial activity. Based on the studied phenotypic characteristics strain YA3 was suspected as Streptomyces species. Findings of the present study conclude that Yelagiri hill is the potential ecosystem for antagonistic actinobacteria which deserves for bioprospecting. The antagonistic actinobacterium YA3 will be a good candidate for the isolation of antibiotic molecule since it showed good activity in the preliminary and secondary screening experiments and it also from an underrepresented ecosystem.

Key Words: bioprospecting, actinobacteria, antibacterial activity, hill ecosystem, Streptomyces.

INTRODUCTION

Microorganism being the pioneer colonizer of this earth planet, as come to stay has cosmopolitan conglomerates of highly compatible organisms. Microorganisms with its 3.8 billion year biosynthetic experience remain nature's best chemists and treasure house for a variety of novel biologically active metabolites. Microorganisms act as mini-biological factories to produce

various high value metabolites. Since civilization, the use of microorganisms to produce natural products and processes that benefit and improve our socio-economic lifestyles had been a part of our human history. They are the easiest manipulated sources of value added products like drugs, therapeutic proteins, vaccines, diagnostics and others. The term microbial bioprospecting refers to the search of microorganism for biological products or the utilization of microbial cell as a whole for human benefit and environment applications. In general, the microbial bioprospecting starts from the collection of environmental samples to the identification and application of specific bioproducts. After the discovery of penicillin from *Penicillium notatum*, hundreds of thousands of microbial cultures was screened for their ability to produce antibiotics the first era of large scale bioprospecting had begun [1]. Among the various industrially important microorganisms, actinobacteria are of prime importance and are primarily recognizes as organisms of academic curiosity and also as potential antibiotic producers. Actinobacteria are the common inhabitants of soil with an unprecedented ability to produce numerous high value metabolites including the antibiotics of clinical importance [2]. Though more number of microbial antibiotics discovered, more than 50% of that are produced by the members of the group actinobacteria especially *Streptomyces* and *Micromonospora*. At present, the rate of novel antibiotic discovery from actinobacteria is decreasing due to the searching of routine ecosystems. This leads to the discovery of known actinobacteria which will produce known secondary metabolites. This problem can be overcome by exploitation of less explored ecosystems which can pave the way for discovery of new antibiotics. In India there are very few reports on actinobacterial study from hills and forest ecosystems [3, 4]. With this view, the present study was initiated for bioprospecting of actinobacteria from Yelagiri hill soil - an unexplored source - for actinobacteria with special reference to antibacterial activity.

EXPERIMENTAL SECTION

Soil sample collection & pretreatment

In total, soil samples were collected from four different places in Yelagiri Hills (Long. 78.6ºE, Lat. 12.6ºN) Vellore district, Tamil Nadu, India, using sterile polythene bags. All the soil samples were air-dried under room temperature for three days immediately after collection. All the dried samples were treated with dry heat at 55ºC for 10 minutes [5].

Isolation of actinobacteria

One gram (1gm) of soil sample was transferred into 9 ml of sterile distilled water (10^1) and serially diluted up to 10^5 dilutions using each 9 ml of sterile distilled water blanks. Hundred microlitre of diluted soil sample from 10^3, 10^4 and 10^5 dilutions was spreaded on starch casein agar (SCA) plates supplemented with nalidixic acid (20µg/ml) and nystatin (100µg/ml /ml). Plating was done in triplicate and all the plates were incubated at 28ºC for 1 month. The individual actinobacterial colonies were selected and inoculated on ISP2 (International Streptomyces Project) agar plates and incubated for 7 days at 28^0C. Morphologically distinct colonies were selected and subcultured on ISP2 agar slants and preserved at 4^0C until further studies [5].

Characterization of actinobacteria

Cultural characteristics of selected actinobacterial isolates were studied by inoculating them in to ISP2 agar medium. After 7 days of incubation, cultural characteristics such as growth rate, consistency, aerial mass color, reverse side pigment, soluble pigment were recorded [6].

Screening for antagonistic activity:
Antagonistic activity of actinobacterial isolates were tested by adopting agar plug method. Test bacterial pathogens used in this study include *S. aureus, Streptococcus sp., B. subtilis, E. coli, K. pneumoniae* and *P. aeruginosa.* All the bacterial isolates were obtained from the Department of Microbiology, Sri Sankara Arts & Science College, Kanchipuram. Agar plug were removed with a 5 mm diameter core from 10 days grown cultures of the actinobacteria from ISP2 agar medium. The surface growth on agar was removed with sterile knife to obtain only the diffused microbial metabolites in the agar plugs.

The agar plugs were placed onto the nutrient agar plate which was previously swabbed with the test bacterial pathogens. All the plates were then incubated at 37ºC for 24 hours. Following incubation, antimicrobial activity was indicated by the formation of an inhibition zone surrounding the agar plug which may provided an indication of diffused antimicrobial metabolites produced by the growing actinobacterial culture. The absence of an inhibition zone indicated a negative result for the production of diffusible metabolites in to the solid growth medium [7].

Production of bioactive substances from selected actinobacteria
Bioactive substance from selected actinobacterial isolates (YA3 and YF2) were produced through submerged fermentation by adopting shake flask method. About 10% of actinobacterial inoculum was transferred into each 100 ml of ISP2 broth and incubated in rotary shaker with 95 rpm at 28^0C for 7 days. After incubation the cell free supernatant was separated by centrifugation at 10000 rpm for 10 minutes [5].

The 18 hours old broth test cultures were inoculated into freshly prepared nutrient agar plates by using sterile cotton swab. Then 5mm well was made on the nutrient agar plates using well cutter and loaded with 100 µl of cell free culture supernatant and incubated at 37ºC for 24 hours. Zone of inhibition was measured after incubation and expressed as millimetre in diameter.

Extraction of bioactive substance
The cell free supernatant which showed maximum zone of inhibition in well diffusion assay was extracted using equal volume of solvents such as ethyl acetate and n-hexane for overnight. Then the solvent portion was collected and concentrated by evaporation. Antibacterial activity of crude extracts was tested by disc diffusion method at 100µg/disc concentration [4].

Partial purification of crude extract
The crude extract which showed antibacterial activity was purified using silica gel coated Thin Layer Chromatography (TLC) plates. The crude extract was dissolved in 200µl of methanol. With the help of capillary tube, the sample was spotted at the bottom of silica gel coated plate and placed in the developing chamber. The chromatogram was run using different organic solvents with varying proportions. The separated compounds were observed as spots using iodine chamber and the Rf value also calculated using standard formula [8]. Further the analytical TLC was also run to get high amount of separated compounds. The separated compounds were tested for antibacterial activity by adopting disc diffusion method as described earlier.

Characterization and identification of potential actinobacteria:
The potential actinobacteria strains were characterized by those methods described by Shirling and Gottileb [6]. The following characters were studied for the identification of actinobacteria:

cultural morphology, microscopic appearance, utilization of carbon source, physiological and biochemical characteristics [9].

RESULTS AND DISCUSSION

Isolation and characterization of actinobacteria

Colonies with actinobacterial morphology was observed on starch casein agar medium (SCA) inoculated with Yelagiri hill soil. Actinobacterial population was ranged between 1.5 X 10^4 and 6 X 10^4 CFU/ gm of soil. Selective isolation of target organisms requires certain pretreatment of sample as well as the culture medium. Pretreatment of soil by heat and the addition of antibiotic/ other inhibitory substances may favour the growth of target actinobacteria by inhibiting the growth of unwanted microorganisms [10]. The available literature revealed that the humic acid vitamin agar and soil extract were mainly used for the isolation of soil actinobacteria. There are certain reports stated that the heat treatment of soil sample at 55^0C for 10 minutes and the addition of nalidixic acid (20µg/ml) and nystatin (100µg/ml) facilitates the growth of selective actinobacterial genera especially *Streptomyces* [11] . In addition, Mayuran *et al*., [12], and Radhakrishnan *et al*., [3,5] has recommended that the starch casein agar medium was used for the isolation and enumeration of actinobacteria from unusual ecosystems like desert and forest, respectively. In the present study also the collected samples were pretreated by heat at 55^0C for 10 minutes and the starch casein agar medium was supplemented with nalidixic acid (20µg/ml) and nystatin (100µg/ml). These two pretreatment approaches facilitated the isolation of actinobacteria from Yelagiri hill soil.

Characterization of actinobacteria

About 40 morphologically different actinobacterial colonies were selected from SCA plates inoculated with four Yelagiri soil samples. Growth pattern of all the isolates were given in table 1.

Table 1: Growth pattern of actinobacteria isolated from Yelagiri Hill soil on ISP2 agar medium

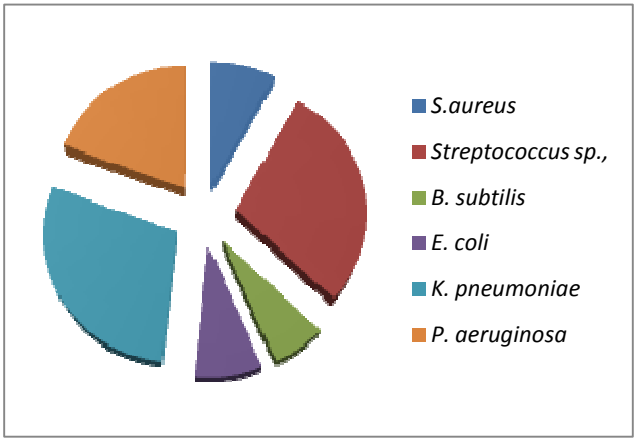
Characteristics	Growth pattern	No. of Isolates (%)
Growth	Good	12 (37%)
	Moderate	20 (62%)
Consistency	Powdery	9 (28%)
	Leathery	15 (46%)
	Mucoid	8 (25%)
Aerial Mass color	White	8 (25%)
	Gray	18 (56%)
	Yellow	5 (15%)
	Orange	1 (3%)
Reverse side Pigment	Brown	12 (37%)
	Yellow	4 (12%)
Soluble pigment	Brown	4 (12%)

Preliminary screening of actinobacteria for antibacterial activity

Antimicrobial activity of actinobacteria was preliminarily screened by agar plug method against bacterial pathogen. In total about 5 actinobacterial strains inhibited *S. aureus*, 18 strains inhibited *Streptococcus sp*, 4 strains inhibited *B. subtilis*, 5 strains inhibited *E. coli*, 18 strains inhibited *K. pneumoniae*, and 12 strains inhibited *P. aeruginosa* (Figure 1). Screening of microbial strains

for antagonistic activity is prerequisite for any natural product drug discovery programme. In general the preliminary screening method used for the detection of antagonistic activity is should be simple, less laborious and user friendly. Some common methods which are in practice from ancient times for primary screening for the detection of antagonistic activity includes crowded plate method, agar overlay method, cross streak method and agar plug method [12, 5, 7]. But each and every method has its own merits and demerits. In the present study agar plug method was used for the detection of antagonistic activity. This method allowed utilizing very small amount of medium for the culturing and production of bioactive compounds and also for the detection of antimicrobial activity of more number of actinobacterial isolates against wide range of pathogens with less investment costs. Thus the result showed that there is a hope for bioprospecting of antagonistic actinobacteria from Yelagiri hill ecosystem.

FIGURE 1: Total number of antagonistic isolates



Production of bioactive compounds from selected actinobacteria strains

Based on the antagonistic activity of actinobacteria in preliminary screening, two actinobacterial strains such as YA3 and YF2, which inhibited all the tested pathogens, was selected as for production of bioactive substances. During fermentation, both the actinobacterial strains were showed good growth on ISP2 broth. As mentioned earlier, the production of bioactive compounds from actinobacteria isolated from unique environment is less. Hence in order to initiate such a study antibiotic production from selected actinobacterial strains were carried out using ISP2 broth and the activity of culture filtrate was evaluated by agar well diffusion method.

Testing antibacterial activity of actinobacterial culture filtrate

Antibacterial activity of actinobacteria culture filtrate was given in table 2. Culture filtrate from strain YA3 showed activity against all the five test pathogens. Culture filtrate of YF2 strain showed activity against *K. pneumoniae, P. aeruginosa* and *B. subtilis* but not *against E. coli, S. aureus,* and *Streptococcus sp.* Hence the actinobacterial strain YA3 was selected as potential strain for further studies. In most of the studies, the antimicrobial activity of crude compound was tested only after extraction from culture broth using solvents like ethyl acetate [13]. Without knowing whether the antagonistic activity is mediated by intracellular or extracellular product, the extraction of bioactive compound from fermentation broth using different solvents is a resource wasting process. In the present study, before extraction, the cell free supernatant was tested for antimicrobial activity by agar well diffusion method in which the culture filtrate showed above 10 mm inhibition against the test bacterial pathogens (Table 2). This result clearly indicated that the antimicrobial activity of potential strain is due to the production of extracellular bioactive compounds. The published literature stated that most of the antibiotics

from actinomycetes are extracellular in nature [14]. Further the bioactive compound extracted from the culture broth also showed good antimicrobial activity.

Table 2: Antibacterial activity of actinobacterial cultural filtrate

Test organisms	Zone of inhibition in mm	
	YA3	YF2
S. aureus	11	-
Streptococcus sp	9	-
B. subtilis	11	7
E. coli	6	-
K. pneumoniae	12	11
P. aeruginosa	10	10

Antibacterial activity of crude extracts

Of the two different solvents used for extracellular compound extraction considerable quantity of extract was obtained both in ethyl acetate and n-hexane. In antibacterial activity testing, of the two extracts tested, only the ethyl extract of cell free supernatant was showed good activity against all the tested pathogens (Table 3).

Table 3: Antibacterial activity of actinobacteria extract

Test organism	Zone of inhibition in mm
	YA3
S. aureus	10
Streptococcus sp	8
B. subtilis	7
E. coli	11
K. pneumoniae	10
P. aeruginosa	9

Antibacterial activity of purified compounds

Totally two spots were observed in TLC and the Rf value of the first and second spot was calculated as 0.76 and 0.83, respectively. The antibacterial activity testing both the spots showed antibacterial activity (Table 4) and it indicated the presence of atleast two different bioactive compounds in the crude extract. Purification of natural product from the crude extract is the prerequisite for its characterization and structure determination. Thin layer chromatography is the very old but simple technique for the separation of bioactive compounds [8, 4]. Further some advanced purification methods are needed for large scale purification, characterization and subsequent evaluation.

Table 4: Antibacterial activity of purified compounds obtained from TLC

Test organisms	Zone of inhibition in mm	
	Spot 1	Spot 2
S. aureus	13	15
Streptococcus sp	11	11
B. subtilis	15	16
E. coli	16	12
K. pneumoniae	12	14
P. aeruginosa	14	13

Characterization of potential actinobacterial strains YA3

Under microscopic observation, strain YA3 showed the presence of substrate and aerial mycelium with rectus flexible (RF) arrangement of spore chains. Cultural characteristics of strain YA3 was given in table 6. Based on the studied phenotypic characteristics strain YA3 was suspected as member of the genera *Streptomyces*. Further chemotaxonomic and molecular characterization is needed to confirm its taxonomic position.

Table 5: Characteristics of potential actinobacteria YA3

Characters	YA3
Micromorphology	
Aerial mycelium	+
Substrate mycelium	+
Spore chain morphology	Rectus Flexible [RF]
Cultural characteristics	
Colony consistency	Powdery
Aerial mass color	Pink
Reverse side pigment	Brown
Soluble pigment	Brown
Growth on different ISP medium	
ISP1	Good
ISP2	Good
ISP3	Good
ISP4	Good
ISP5	Good
ISP6	Moderate
ISP7	-
Carbon compounds	
Glucose	+
Sucrose	-
Xylose	+
Inositol	+
Mannitol	+
Fructose	-
Rhamnose	+
Raffinose	-
Arabinose	-
Cellulose	-
Enzymatic activities	
Amylase	+
Lipase	-
Protease	+
Temperature tolerance (ºC)	
20	Moderate
30	Good
40	Good
45	Moderate
pH tolerance	
5	-
7	Good
9	Good
11	-
Anaerobic condition	Moderate

Findings of the present study conclude that Yelagiri hill is the potential ecosystem for antagonistic actinomycetes which deserves for bioprospecting. Actinobacterial strain YA3 will be a good candidate for the isolation of antibiotic molecule since it showed good activity in the preliminary and secondary screening experiments and it also from an unexplored ecosystem.

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