Research Article

Antibacterial Potentials of Actinomycetes Isolated from Gujarat

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ABSTRACT

Screening and isolation of promising strains of novel actinomycetes with potential antibiotic is still a thrust area of research because in recent time, many pathogenic bacteria are resistant to currently available antibiotics. Ninety five actinomycetes from twenty four different soil and marine samples have been isolated using pretreatment and specific media. Thirty one isolates were selected by primary screening using cross streak method and further six potent actinomycetes were screened out by secondary screening on the basis of the production of antibacterial spectrum against ten bacterial test cultures. Antibacterial substances were extracted from fermentation broth by the process of solvent extraction and separated by TLC. Solvent system has been established for extraction and separation of antibacterial substance. Antibacterial activities of the extracts were performed using Kirby-Bauer disc diffusion method. The current investigation reveals that soil and marine actinomycetes isolates can acts as potent source for novel antibacterial compounds against pathogenic bacteria.

Keywords: Actinomycetes, Screening, Fermentation, Solvent extraction, TLC

INTRODUCTION

Actinomycetes are high GC containing gram-positive, aerobic, suprageneric subgroup of bacteria well known for antibiotic production.[1] They are famous for the production of secondary metabolites and have the capacity to synthesize wide range of biologically active compound such as antibiotic, antitumor agents, immunosuppressive agents and these metabolites also possess antimicrobial, antimalarial and anti-inflammatory activities.[2,3]

Actinomycetes hold a predominant position due to their diversity and had proven their ability to provide novel substances. Most of them are free living saprophytes, widely distributed in soil and marine sediments; show marked chemical and morphological diversity and form a distinct evolutionary line of organisms.[4]

Recently, attention has been paid on screening of Actinomycetes from soil and other diverse environments such as marine sediments and water for their ability to produce novel antibiotics.

Antibiotics are generally isolated from microbes and exhibits antimicrobial, antitumor or antiviral activities to kill or inhibit the growth of other pathogenic bacteria, fungi, viruses and parasites.[3] About 80% of the naturally occurring antibiotics are produced by actinomycetes and among them two most prolific producers are *Streptomyces* and *Micromonospora.*[5]

Antibiotics which are produced by actinomycetes are of different classes including aminoglycosides, anthracyclins, glycopeptides, β-lactums, macrolides, nucleosides, peptides, polyenes, polyethers, terpenes and tetracycline's, which possess wide range of biological activities.[6-9]

According to the World Health Organization, antibiotic resistance cases against many bacterial pathogens have been increased due to over-prescription and the improper use of antibiotics.[10] Nowadays multi drug resistant strains of pathogens are emerging more quickly than the rate of discovery of antibiotics. Recently, methicillin and vancomycin resistant strain of *Staphylococcus aureus*[11] and penicillin resistant *Streptococcus pneumoniae* has been reported.[12,13] To overcome this problem, scientists and many pharmaceutical industries trying to perform new screening programmes for the discovery of novel actinomycetes from untouched environment like marine water.[14] Novel actinomycetes must be better option for the finding of unknown bioactive substances against resistant pathogens. In Gujarat, no significant studies have been reported so far to isolate and evaluate actinomycetes from different soil and marine habitats that could produce useful antibiotics. Therefore, the present work was undertaken to isolate the potent actinomycetes from diverse sources of soil, marine sediments and marine water to elucidate their antibacterial activity against various pathogenic bacteria.

MATERIALS AND METHODS

Study Area and Period

The study area was located at terrestrial and coastal regions of Gujarat, India. Soil and marine sample were collected from Agricultural soil, Industrial soil, Marine sediments & Marine water. It was collected from 11 district of Gujarat in November 2012.

Sampling and Isolation of Actinomycetes

Soil samples were collected at different depth of 5-15 cm using standard methods. The collected samples were transferred to research laboratory of Department of Biotechnology in Virani campus where the entire research work was carried out. The soil samples were sieved through 250 µm pore size sieve and were air-dried for one week at 37 °C temperature. Isolation and enumeration of actinomycetes were performed by serial dilution and spread plate technique on selective isolation media such as starch casein agar, actinomycetes isolation agar, tryptophan soya agar, humic acid vitamin agar and oat meal agar plates aseptically.[15] Nalidixic acid (50µg/ml) and cyclohexamide (50µg/ml) were added in both media to inhibit gram negative bacterial and fungal contamination.[16] The plates were incubated aerobically at 28 °C up to 7 day and observed intermittently during incubation. After incubation, actinomycetes on the plates were identified on the basis of morphological characterization. The identified colonies were purified by repeated streak plate method, maintained in glycerol stock and stored at -20 °C.

Primary Screening

Primary screening of actinomycetes isolates were performed by cross streak method on Muller Hinton agar plate to evaluate their antibacterial spectrum. The test bacteria used for primary screening were *Staphylococcus aureus* (MTCC737), *Bacillus megatarium* (MTCC3353), *Bacillus subtilis* (MTCC441), *Escherichia coli* (MTCC443), *Pseudomonas aeruginosa* (MTCC3541), *Salmonella typhi* (MTCC9289), *Proteus vulgaris* (MTCC426), *Serratia marcescens* (MTCC97) and *Enterobacter gergoviae* (MTCC621). Actinomycetes isolates were inoculated in straight line on MHA plate and incubated at 28 °C for 7 day. The test organisms were streaked perpendicular to the actinomycetes isolates on the same plate. The plates were incubated at 37 °C for 24 hours and plates were examined by determining the diameter of the inhibition zone.[17]

Fermentation Process

Submerge fermentation were performed with promising cultures of actinomycetes using Soybean casein digest broth. Flasks were lodged on the shaker incubator at the speed of 140 rpm at 28 °C for 7-11 days. After fermentation, the media was harvested, centrifuged to remove cell debris and supernatant was transferred aseptically into screw capped bottles at 4 °C for kinetic studies and further assay.[18]

Secondary Screening

Secondary antimicrobial screening of actinomycetes was detected by agar well diffusion method on Mueller Hinton agar.[19] Test cultures were incubated in nutrient broth and inoculated for 24 hours at 37 °C. The turbidity of the broth was adjusted at 0.5 optical density using spectrophotometer. Twenty hour old grown test organism were mixed with MHA media and poured over petri dishes. The wells (5 mm diameter) were made using sterile cork borer, 20 µl crude extract were added and incubated at 37 °C for 24 hour. Streptomycin (20µg/ml) was used as positive control. After incubation the zone of inhibition were measured and recorded.[20]

Extraction of Antibacterial Compounds

The antibacterial compounds were recovered from the harvested medium by solvent extraction method. The filtrate was mixed with equal volume of ethyl acetate and shaken vigorously for 1 hour in a Separatory funnel.

The solvent phase that contains antibiotic was separated from the aqueous phase after stabilization of mixture. Extracts were concentrated by evaporation of ethyl acetate using rotary evaporator.[21]

The crude extract obtained from each isolates were dissolved in methanol and used as stock concentration for determination of antimicrobial activity against test pathogens using methanol as a negative control.

Antibacterial Activity

The antimicrobial activities of those extracts were tested against three gram positive and seven different gram negative test organisms. The procedure applied in doing the sensitivity tests was in accordance with Kirby-Bauer agar disc diffusion method.[22]

Thin Layer Chromatography

Antibacterial substance was separated by thin layer chromatography (Merck Silica Gel 60 F254) on silica gel sheet using chloroform: methanol (24:1) as a solvent system. Chromatograms were observed under UV light (254 nm and 366 nm).[23]

All spots of TLC were scraped off carefully from the plate, dissolved in methanol and centrifuged at 10000 rpm for 5 min in order to remove silica. The supernatant was collected, filtered from 0.22 µm filter and used for antibacterial bioassay.

Data Analysis

All tests were conducted in triplicate. Data are reported as means ± standard deviation (SD). Results were analyzed statically by using Microsoft Excel 2007 (Roselle, IL, USA).

RESULTS AND DISCUSSION

Isolation of actinomycetes

Actinomycetes have been proven as a potential source of bioactive compounds and richest source of secondary metabolites.[24] 85 different actinomycetes have been isolated from soil samples from 11 districts (Table 1) and 10 actinomycetes were obtained from marine samples of the Gujarat (Table–2).

From these 85 soils sample isolates; 54 actinomycetes isolated from agricultural soil and 31 actinomycetes were

isolated from industrial area. Actinomycetes of agricultural area shows higher production of antibiotics among all the isolates of soil sample reveals that fertile soil possess huge number of organisms which leads to competition and increases the possibility of antibiotic production. Present study indicated that, 26 (30.58%) isolates were found to be potentially efficient, showing antimicrobial activity against gram positive and gram negative bacteria. This result (30.58%) is higher than of previous studies of Abebe Bizuye [25] but lesser than 59.09%.[26] Among marine samples, out of 10 halophilic actinomycetes, 5 (50%) were capable of biosynthesizing antimicrobial metabolites, shows great potency of halophilic isolates over soil actinomycetes.

Table 1: Description of soil samples collected from different sites of Gujarat and isolated actinomycetes

Sample sites	Districts	Specific Soil Sample area	Soil Depth (cm)	No. of isolates and their code
1	Junagadh	Agricultural university	6	S2, S10
2	Kutch	J.P. Cement	8	S1, S5, S8, S9, S15, S18
3	Kutch	Bhuj garden	8	S43, S45, S46,S47
4	Surat	Industrial area	6	S24, S25, S26, S60, S128, S131, S132, S136, S137, S138, S145, S147, S149, S150, S151
5	Surat	Agricultural area	7	S16, S75, S76, S77, S165, S170, S174, S175
6	Ankleshwar	Agricultural area	8	S21, S27, S28, S29, S70, S72, S73, S74, S113, S114, S116, S117, S118, S120, S121, S122, S123, S124, S125, S126, S127
7	Ankleshwar	Industrial area – 1	6	S30, S107, S109, S153, S154, S155, S162, S178
8	Ankleshwar	Industrial area – 2	7	S68
9	Morbi	Industrial area	9	S80
10	Rajkot	Agriculture area	6	S53, S64, S94, S95, S96, S97, S98, S101, S102, S105, S106, S176, S119
11	Amreli	Agriculture area	8	S3, S13, S81, S82, S89, S142

Table 2: Description of water samples collected from different area of Gujarat and isolated actinomycetes

Sampling sites	Districts	Specific water sample	No. of isolates and their code
1	Kutch	Khambhat	MA1
2	Kutch	Mundra	MD1, MD2
3	Veraval	Somnath	MF1, MF2, MF3
4	Porbandar	Dwarka	MG1
5	Kutch	Mandavi	MI1, MI2, MI3

Table 3: Antibacterial activity of ethyl acetate extracts of potential actinomycetes against test organism

Test Organisms	Zone of inhibition (mm)						
	S8	S16	S114	S142	MD2	MA1	Streptomycin
EC	6.50 ± 0.4	7.33 ± 0.4	7.33 ± 0.4	7.17 ± 0.2	9.83 ± 0.2	9.17 ± 0.2	16 ± 0.7
PV	8.00 ± 0.5	14.00 ± 0.4	11.00 ± 0.5	9.17 ± 0.2	7.50 ± 0.4	6.83 ± 0.2	16 ± 0.2
SA	6.17 ± 0.2	9.83 ± 0.5	8.67 ± 0.4	0.00	17.33 ± 0.2	7.33 ± 0.2	17 ± 0.1
BM	6.33 ± 0.5	6.67 ± 0.4	14.50 ± 0.4	0.00	15.83 ± 0.5	0.00	16 ± 0.8
SS	7.00 ± 0.4	12.50 ± 0.4	12.50 ± 0.4	6.17 ± 0.2	13.00 ± 0.7	8.17 ± 0.2	15 ± 0.9
KP	7.00 ± 0.6	10.17 ± 0.2	6.50 ± 0.4	7.50 ± 0.4	7.00 ± 0.4	7.50 ± 0.4	15 ± 0.4
XC	8.50 ± 0.4	10.33 ± 0.2	9.17 ± 0.5	6.50 ± 0.4	7.17 ± 0.2	6.17 ± 0.2	15 ± 0.7
BC	6.17 ± 0.2	0.00	0.00	7.33 ± 0.2	6.17 ± 0.2	9.00 ± 0.4	14 ± 0.8
ST	7.00 ± 0.4	8.33 ± 0.2	7.17 ± 0.2	9.33 ± 0.2	8.83 ± 0.5	8.17 ± 0.2	14 ± 0.5
EG	0.00	9.50 ± 0.4	6.83 ± 0.4	0.00	6.67 ± 0.2	0.00	13 ± 0.8

EC: *E. coli*, PV: *P. vulgaris*, SA: *S. aureus*, BM: *B. megaterium*, SS: *S. sonnei*, KP: *K. pneumonia*, XC: *X. campestris*, BC: *B. cereus*, ST: *S. typhi*, EG: *E. Gergoviae*.
Values are mean ± SD of three replications.

Primary Screening

Intense screening of actinobacteria especially rare actinomycetes is taking place all over the world.[27] Independent observation for zone of inhibition around well or disc on the incubated plate are indicated as antibacterial activity of secondary metabolites extracted from actinomycetes against test bacteria. Among the 95 isolates, 31 isolates showed antibacterial activity against at least one of the ten test organisms. Out of selected 31 isolates, 26 soil isolates are S1, S8, S15, S16, S25, S28, S43, S70, S75, S77, S80, S81, S113, S114, S117, S118, S119, S120, S136, S138, S142, S145, S154, S165, S170, S175 and 5 marine isolates are M, MA1, MD1, MD2, MF1. Out of 26 soils sample isolate, 34.62% isolated from industrial area of Gujarat and 65.38% isolated from agriculture area of Gujarat. Among these 26 soil isolates, 26.92% isolates have been shown antibacterial activity against gram negative test bacteria while rest 73.08% have been shown antibacterial activity against both gram negative and gram positive test bacteria. All the marine isolates showed antibacterial activity against both gram positive and gram negative test culture. There was a high significant difference among antagonistic activity of isolates against test cultures. The previous study indicated that, the inhibition zone of crude extracts from isolates against MRSAs ranged from 0-15 mm.[28] In this study, considering 20 µl of crude extract used for antimicrobial assay, inhibition zone of ethyl acetate extracts from six isolates against pathogenic microbes ranged from 0-17 mm shows good result, when compared to Yucel and Yemac's results[28]. Streptomycin antibiotic was considered as standard shows 16±2 mm against most of test organism.

Antibacterial Activity of Crude Extracts

The crude extract of submerge fermentation were subjected to secondary screening. Standardization of solvent extraction was performed on the basis of different polarities of organic solvents for the extraction of antibacterial compounds from actinomycetes.[29] The extracts from ethyl acetate showed maximum antibacterial activity against test organism. Previous studies also show that ethyl acetate shows better antimicrobial activity, other solvents extracts showed moderate activity or no activity against test organism.[30]

Six potent actinomycetes have been selected for further studies on the basis of significant antibacterial activity against ten test organisms.

Among these 6 isolates, one (S8) has been isolated from industrial area, three (S16, S114, S142) has been isolated from agricultural area and two (MD2, MA1) were halophilic actinomycetes.

These six isolates were identified and confirmed by microscopic and macroscopic examination. All are found to be gram positive, spore containing and filamentous bacteria. The macroscopic appearance of five isolate showed leathery, white powdery colonies in soybean casein digest agar where as MA1 showed yellow colony.

Antibacterial assay were performed by Kirby-Bauer techniques using disc diffusion method (Table 3). Crude extracts from selected isolates shown high antibacterial activity against *S. aureus*. MD2 shows highest activity against *S. aureus* (17.33±0.2) mm which was better than standard antibiotic streptomycin (17 ± 0.1) mm. MD2 also shows effective zone of inhibition against *B. megaterium* (15.83 ± 0.5) mm and *S. Sonnei* (13.00 ± 0.7) mm which was close to streptomycin. Crude extract from soil isolate S114 also shown highest antibacterial activity against *B. megaterium* (14.50 ± 0.4) mm, which was closely comparable to streptomycin, followed by *S. sonnei* (12.50 ± 0.4) mm and *P. vulgaris* (11.00 ± 0.5) mm. S114 and S16 extracts have shown antibacterial activity against both gram positive and gram negative but it was not shown activity against *B. cereus.* S16 shows higher antibacterial activity against *P. vulgaris* (14.00 ± 0.4) mm and *S. sonnei* (12.50 ± 0.4) mm. Crude extract from soil isolate S142, S8 and marine actinomycetes MA1 also showed effective zone of inhibition against *P. Vulgaris* (9.17 ± 0.2) mm, *X. Campestris* (8.50 ± 0.4) mm and *E. Coli* (9.17 ± 0.2) mm respectively.

Thin Layer Chromatography

Thin-layer chromatography using various solvent has been performed using various solvent systems and chromatographic profile was visualized.[31] Several mobile phases were investigated for separation of antibacterial metabolites. Solvent system chloroform: methanol (24:1) was found to be comparatively better for separation of compounds. Antibacterial bioassay of all scraped spots revealed that spot 1 and 4 having R_f value 0.12 and 0.37 possess antibacterial compound in S16. Spot 4 of S114 having R_f value 0.22, spot 5 of S8 having R_f value 0.32, spot 4 of S142 having R_f value 0.26, spot 6 of MA1 having R_f value 0.31 and spot 5 of MD2 with R_f value 0.24 possess antibacterial compounds.

CONCLUSION

Soil isolate S114 and marine MD2 shows excellent potency of antibacterial activity against most of the pathogenic test organism. Morphological observations and cell wall analysis of actinomycetes reveals that five isolate belong to the genus *Streptomyces* and one possibly belongs to *Nocardia*. Holt identified the isolated actinomycetes based on the colony morphology and gram staining.[32] Actinomycetes producing *Streptomyces* which are isolated from marine environment are identified with the help of Nonomura key (1974) and Bergey's Manual of Determinative Bacteriology.[33] The identification and production of novel antibacterial molecules from marine actinomycetes are necessary to counteract antibiotic resistance in microbial population. The results of the present study were interesting and encouraging because the crude extracts from the isolates may have promising antibiotics for treatment of antibiotic resistant bacteria.

Therefore, further purification process is significant to get pure antibacterial substance for the application of treatment of different pathogenic microorganisms.

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