Original Article

ISSN Print 2231 – 3648
Online 2231 – 3656

ISOLATION AND CHARACTERIZATION OF ACTINOBACTERIA FROM FOREST SOILS OF MAHABUBNAGAR DISTRICT OF ANDHRA PRADESH, INDIA

*Pavan Kumar Pindi, Balakrishna G, Shiva Shanker A
Dept. of Microbiology, Palamuru University,
Mahabubnagar, A.P., India - 509001.

Abstract

The aim of the present study was to isolate and identify the potential actinomycetes for their antimicrobial activity. The soil samples were collected from undisturbed soils of Nallamala Forest, Andhra Pradesh. Seven distinct morphotypes were isolated on Starch casein agar medium by using the standard serial dilution method. These isolates were screened for their antimicrobial activity against thirteen pathogenic microorganisms. All the seven isolates were unable to inhibit the growth of bacterial pathogens and where as three isolates were possessed anti fungal activity. Morphologically and biochemically distinct strain PUSt-5 selected for polyphasic identification. Further, the 16S rRNA gene sequence was carried out for the strain PUSt-5 and BLAST analysis has shown 98% similarity with streptomyces sp. The result of present investigation revealed that, soil actinomycetes from Nallamala forest of Andhra Pradesh, INDIA are potential source for antimicrobial components.

Keywords: Actinomycetes, Nallamala Forest, Antimicrobial activity, Starch Casein Agar medium.

Introduction

Actinomycetes are gram-positive filamentous bacteria, free living, saprophytic bacteria widely distributed in soil, water and colonizing plants[1]. Most of the actinomycetes are believed to be terrestrial, however some strains also distributed in marine environments. Among the microorganisms actinomycetes are the best known for their ability to produce antibiotics. Apart from the antibiotics they also produce other bioactive secondary metabolites[2-5] such as enzymes, immunomodulators, anti helmenthic, anti cancer (mytomycin, daunomycin) and immuno suppressive agents(rapamycin, FK 506). Among the actinomycetes streptomyces are the major producers of antibiotics. About 80% of the commercially and medically useful antibiotics derived from streptomyces species[6].

Author for Correspondence:
Pavan Kumar Pindi,
Dept. of Microbiology, Palamuru University,
Mahabubnagar, A.P., India - 509001.
Email: pavankumarpindi@gmail.com

The resistance of the pathogenic microorganisms to the anti microbial agents is a global issue[7]. Hence to fight with the drug resistance pathogens, there is an urgent need for newer safer and less expensive drugs from various natural resources especially from microbial origins. In this view, the present study was designed to isolate and identify potential actinomycetes for their anti microbial activity from ever green reserve forest soils of Mahabubnagar district of Andhra Pradesh.

Materials and Methods

Soil Sample Collection

Soil samples (500 g) were collected from rhizosphere region of plants in Nallamala forest of Mahabubnagar district of Andhra Pradesh, (Lat 16^0 22^1 35^{11} N, 78^0 45^1 21^{11}E). The samples were taken up to a depth of 15 cm, after removing approximately 3 cm of the soil surfaces. The soil samples were placed in a sterile poly ethylene bags, closed tightly to avoid external contamination and transported to the laboratory and maintained at 4^0C until further use.

Isolation of Actinomycetes

Starch casein agar (SCA) medium[8] (g/l: starch 10; casein 0.3; KNO_3 2; NaCl 2; K_2HPO_4 2; $MgSO_4$ 7 H_2O 0.05; $CaCO_3$ 0.02; $FeSO_4$ 7H_2O 0.01 Agar 18; pH 7.2) employed for the isolation of actinobacteria. Culture medium was prepared and sterilized at 121^0 C in 15lbs pressure for 15 min and supplemented with tetracycline (100µg/ml) to prevent bacterial growth[9, 10]. The collected soil samples (1gr) were serially diluted in distilled water up to 10^{-6} dilution, and 0.1ml of the diluted samples were spread over the starch casein agar plates. The plates were incubated at 30^0 C for 7-10 days. After incubation the actinomycetes colonies were observed. Pure cultures were obtained from selected colonies by repeated sub culturing on starch casein agar slants.

Test Microorganism

Staphylococcus aureus, Protes vulgaris, Klebsiella pneumoniae, Escherichia coli, Salmonella typhi, Salmonella paratyphi sp, Pseudomonas aureginosa, Shigella sp, Candida albicans, Aspergillus niger, Penicillium sp, Aspergillus flavus, Aspergillus fumigatus.

Screening of Actinomycetes for Antimicrobial Compounds

The screening method consists of two steps, Primary screening and secondary screening.

In the primary screening the antimicrobial activity of pure isolates were determined by cross streak method. Secondary screening was done through the agar well diffusion method[11]. Primary screening of actinomycetes were done by using starch casein agar plates and inoculated with isolates by a single streak of inoculum in the center of Petri dish and incubated at 28^0 C. After observing a good ribbon like growth of the actinomycetes on the plates the test organism were streaked at right angles to the original streak of actinomycetes. The plates were incubated at 37^{0}C for bacteria and 28^{0}C for fungus. After incubation period positive isolates were selected based on the inhibition zone against the test organism on agar plate.[12] In the secondary screening the selected isolates were inoculated into SC broth. Seven- day-old culture grown on SCA media was used to inoculate the flasks. The flasks were incubated in a rotary flask shaker at 28^0 C for seven days at 120 rpm rotation. The resulted culture broth was centrifuged at 10,000 rpm for 10 min. The supernatant obtained was used for extra cellular anti microbial activity by agar well diffusion method[13]. A lawn culture of target organism was made on the surface of specific agar medium. Nutrient agar was used for bacteria and Sabaroud's dextrose agar (SDA) was used for fungi. Wells were made by using a sterile well borer of 7 mm width, 200 micro lit of supernatant of each isolates was loaded in each well. The plates were incubated for 24 h at 37^{0}C for bacteria and 48 h at 28^{0}C for fungi. After the incubation the plates were examined for the zone of inhibition around the well.

Characterization of Actinomycetes

The selected actinomycetes were characterized by morphological and biochemical tests. Morphological tests consist of macroscopic and microscopic methods. The colony color, mycelium structure, color arrangement of spores on the mycelium and colors of colonies were observed and compared with Bergey's manual of determinative bacteriology. Moreover several biochemical tests[14] such as production of H_2S, catalase, oxidase, urease, lipase, protease, starch hydrolysis and gelatin hydrolysis were determined. Antibiotic sensitivity test against different antibiotics was performed using starch casein agar medium by paper disc method.

Molecular Assays

In the present study, white, ash, yellow, brown colored 7 distinct colonies were isolated and morphologically and biochemically characterized. Among 7 isolates PUSt-5 was shown unique morphological and biochemical features. So PUSt-5 isolate selected for sequencing and for further studies.

Molecular Taxonomy

Genomic DNA isolation

Genomic DNA isolation was conducted according to the protocol described by Corbin method with some modifications. Briefly, a single colony was cultured in 50 ml liquid ISP2 medium for 24 h in shaker incubator at 26° C. Then the culture was centrifuged for 3 min at 5000 rpm and supernatant was discarded. The bacterial cells were pulverized in liquid nitrogen, suspended in a solution-I, containing 10 mM Tris (pH:

7.4), 1 mM EDTA, 0.5% SDS and 0.1 mg/ml of proteinase K, and lysed by incubation at 37° C for 1h, then the solution -II containing 0.8 M NaCl and 1% CTAB was added to the lysates, and incubated at 65° C for 20min and extracted with equal volume of chloroform isoamylalchohol (24:1). Nucleic acid was precipitated from the aqueous phase with 0.6 volume of isopropanol and finally purified using ethanol 70%[11].

Amplification of 16 S rRNA gene

To amplify the fragment of 16S rRNA, the isolated genomic DNA was amplified by using actinospecific forward and reverse primer (St F 5' AAGCCCTGGAAA CGGGGT 3' and St R' 5' CGTGTGCAGCCCAAGACA 3'). The PCR reaction mixture (50 µl) contained 50 pmol each of forward and reverse primers, 4 dNTPs at 0.2 mM each, 2.0mM $MgCl_2$, and 0.5ng/µl bacterial genomic DNA as the template DNA and 1.5U Taq DNA polymerase. The PCR amplification was achieved with 94°C for 5 min as primary denaturing temperature, then 94°C for 1min as denaturing temperature, 54°C for 60 sec as annealing temperature72°C for 105 sec as extension time, in 35 cycles, and 72°C for 10 min as final extension time. The PCR product was used for sequencing. The 16S rRNA partial gene sequence was subjected to BLAST search in the NCBI database. The DNA sequences were aligned and phylogenetictree was constructed by neighbor joining method using Clustal W[15]. The BLAST search analysis has shown the 98% similarity with Streptomyces sp.

Table01: Biochemical Characteristics of Streptomyces Isolates

Characteristics	PUSt-1	PUSt-2	PUSt-3	PUSt-4	PUSt-5	PUSt-6	PUSt-7
Amylase	+	+	+	+	+	+	+
Protease	-	+	+	-	-	-	-
Lipase	-	-	+	-	-	+	-
Urease	-	+	+	-	-	+	-
H_2S production	-	-	-	-	-	-	-
Gelatin	+	-	+	+	+	-	-
Oxidase	+	+	-	-	+	+	-
Catalase	+	+	+	+	+	+	+

Table 02: Antibiotic Sensitivity of Streptomyces Isolates

Antibiotic disc	PUSt-1	PUSt-2	PUSt-3	PUSt-4	PUSt-5	PUSt-6	PUSt-7
Amoxicillin	R	R	R	R	R	R	S
Chloramphenical	S	S	S	R	R	R	R
Gentamycin	S	S	S	S	S	S	S
Streptomycin	S	S	S	S	S	S	S
Tetracycline	S	R	R	S	R	S	S

Table 03: Anti microbial activity of Streptomyces Isolates (in mm)

Test organisms	PUSt-1	PUSt-2	PUSt-3	PUSt-4	PUSt-5	PUSt-6	PUSt-7
Staphylococcus aureus	0	0	0	0	0	0	0
Protes vulgaris	0	0	0	0	0	0	0
Klebsiella pneumoniae	0	0	0	0	0	0	0
Escherichia coli	0	0	0	0	0	0	0
Salmonella typhi	0	0	0	0	0	0	0
Salmonella parathyphi spp	0	0	0	0	0	0	0
Pseudomonas aureginosa	0	0	0	0	0	0	0
Shigella sp	0	0	0	0	0	0	0
Candida albicans	*5*	3	0	0	0	0	0
Aspergillus niger	0	0	0	0	0	0	0
penicillium sp	0	*7*	4	0	0	0	0
Aspergillus flavus	3	*5*	0	0	0	0	0
Aspergillus fumigatus	0	0	0	0	0	0	0

Results and Discussion

The Gram positive, filamentous streptomycetes having high (G+C) (>55%) content in their DNA, are the most studied and well known group of actinomycetes. The actinomycetes groups have a great ability to produce most important secondary metabolites such as antibiotics, anti tumors, anti helmenthic and immunosuppressive agents etc. Hence the present study focused on the isolation and identification of actinomycetes with potential antimicrobial activity, from forest soils of Mahabubnagar district, Andhrapradesh. About 7 isolates were obtained from the systematic serial dilution and plating of the collected soil sample. Among 7 isolates, only 3 isolates showed antimicrobial activity. These 3 isolates showed only antifungal activity but not antibacterial activity. The strain PUSt-2 showed mild to moderate activity against Aspergellus flavus, Candida albicans, Penicillium sp. The isolate PUSt-1 showed activity against Aspergellus flavus and Candida albicans. Another isolate PUSt-3 showed moderate anti fungal activity against Penicillium sp. Results of antibiotic sensitivity test were presented in table-2. All isolates showed sensitivity to Gentamycin and Streptomycin. Most of the isolates showed resistance to Amoxicillin. The isolates PUSt-1and PUSt-7 showed resistance to only Amoxicillin and Chloramphenical respectively. The results of biochemical tests were presented in table-1. All isolates were positive for amylase production and catalase production and negative for H_2S production.

The identification of isolate was carried out by using molecular studies like PCR amplification, 16S rRNA gene Sequencing, BLAST search in NCBI data bank and construction of phylogenetic tree. This method is one of the strongest and efficient one for identification of particular organism. The BLAST search analysis has shown 98% similarity with Streptomyces sp. Out of seven three isolates i.e., PUSt-1, PUSt-2 and PUSt-3 isolates were shown moderate anti fungal activity.

Conclusion

The search for novel antibiotics especially from soil actinobacteria needed a huge population of isolates in order to discover an actionomycetes with novel compound of pharmaceutical interest. Because of this, the research will be more promising if diverse and more actinomycetes are isolated and screened. In this context, the present study as an attempt to identify and pick-out versatile strains of streptomycetes from the forest regions of Mahabubnagar district that display antimicrobial activity against a variety of microbial pathogens intrinsically. However further studies are needed with respect to the structural characterization and the biological activity of the secondary metabolite. The present investigation reveals that Nallamala forest of Andhra Pradesh is one of the potential source for antimicrobial components of actinomycetes.

Acknowledgment

Authors are thankful to Vice-Chancellor
Prof. K. Narasimha Reddy and Registrar
Prof. K. Venkata Chalam, Palamuru University,
Mahabubnagar for their encouragement and facilities.

References

1. Goodfellow M, O, Donnell AG. Search and discovery of industrially-significant actinomycetes. Proceeding of the 44th Symposium on Society for General Microbiology, (SCGM 89), Cambridge University Press , Cambridge ; 1989 . p. 384-83.
2. Vining LC. Secondary metabolism, inventive evolution and biochemical diversity – a review .Gene 1992; 115:135-40.
3. Edwards C. Isolation, properties and potential applications of thermophilic actinomycetes.Applied Biochem Biotechnology 1993; 42:161- 79.
4. Demain A. Why do microorganisms produce antimicrobials? Proceeding of the Symposium on Society of General Microbiology, 1995, Cambridge University Press, Cambridge, 1995. p: 205 -28.
5. Xu LH, Jiang Y, Li WJ, Wen ML, Li MG, Jiang CL, *Streptomyces roseoalbus sp*. Nov., an actinomycete isolated from soil in Yunnan, China. Antoine Van Leeuwenhoek 2005; 87: 189-94.
6. Miyadoh S, Research on antibiotic screening in Japan over the last decade: a producing microorganisms approach. Actinomycetologica 1993: 9:100-06.
7. Singer RS, Finch R, Wegener HC, Bywater R, Walters J, Lipsitch M, Antibiotic resistance-the interplay between antibiotic use in animals and human beings. Lancet Infect Dis 2003; 3:47-51.

8. Kuster E, Williams ST, production of hydrogen sulphide by *Streptomyces* and methods for its detection. App. Microbiol 1964; 12:46-52.
9. Williams, S.T. and Davies, F.L. (1965) Use of antibiotics for selective isolation and enumeration of Actinomycetes in soil. Journal of General Microbiology 38:251-261.
10. Porter, J.N., Tresner, H.D.(1960). A method for the preferential isolation of actinomycetes from soil. Journal of Applied Microbiology 8:174-178.
11. Adinarayana Gorajana, Ellaiah Poluri and Axel Zeeck (2010) Cytotoxic compounds from marine actinomycete, African journal of Biotechnology Vol. 9(42), pp. 7197-7202.
12. Madigan, M.T., Martinko, J.M. and Parker, J. (1997) Antibiotics: isolation and characterization. In: Brock Biology of Microorganisms, 8th (eds), Prentice-Hall International Inc. *New Jersey,* pp: 440-442.
13. Grammer, A. (1976) Antibiotic sensitivity and assay test. In: Collins, C.H. and P.N. Lyne, editors. Microbiological Methods. *London: Butterworths* 235.
14. Shirling EB, Gottlieb D, Methods for characterization of *streptomyces* species. Int J Syst Bacteriol 1996; 16:312-40.
15. Saitou N, Nei M, The neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol Biol Evol, 24, 1987, 189-204.