Int J Pharm Bio Sci 2015 Jan; 6(1): (B) 750 - 758

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975- 6299**

ISOLATION AND CHARACTERIZATION OF ANTIFUNGAL ACTINOMYCETE FROM THIRUPORUR FORESTS

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ABSTRACT

Nearly sixty isolates of bacteria, fungi and actinomycetes were selected from ten soil samples. The soil samples were collected from the Thiruporur forest area. Among the sixty isolates, we selected twenty isolates of actinomycetes. These actinomycetes were isolated from soil samples by the serial dilution method. They were subjected to primary screening. After the primary screening three actinomycetes named A3, A7 and A20 were selected on the basis of zones of inhibition produced around them. This was followed by secondary screening against *Aspergillus fumigatus* (streak culture) which was the target pathogen. The isolate A7 showed prominent antifungal activity after the secondary screening, hence it was selected for further study. It was subjected to morphological and molecular methods to reveal its identity.

KEYWORDS: **Terrestrial, *Streptomyces,* Aerial mycelia, Melanin, Antifungal activity.**

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INTRODUCTION

Actinobacteria the so called actinomycetes are mostly found in terrestrial, especially soil, and marine ecosystems (McCarthy and Williams, 1992;[1] Stach and Bull, 2005).[2] They play an important role in soil formation by gradually destroying and reducing complex compounds found in various polymers of fungal materials, dead animal and plant using extracellular enzymes (McCarthy and Williams, 1992).[3] The physiological and metabolic properties, leads to production of various volatile substances (Gust *et al*., 2003)[4]and a large number of secondary metabolites. Many of these metabolites are important and are used as, antifungal, antitumor, antiviral immunosuppressant, antibiotic or anti parasitic agents. Many species of actinomycetes, particularly those belonging to the genus Streptomyces,are well known as antifungal bio control agents (Xiao *et al*.,2002,Joo.2005,Errakhi *et al*.,2007,Li *et al*., 2010).[5] Lebrihi A *et al*.,(2007).[6] The predominance of fungal infections has increased enormously over the past few decades in immunosuppressive and immunocompromised patients. It is a major concern for the present day clinicians. Although few antifungal drugs are available in the market their efficacy to treat the invasive fungal infections is a compromise. Thus more focused efforts in antifungal drug discovery are needed to develop a more successful and effective antifungal agent in the clinical arena. The present research paper reports the identification and characterization of the antifungal actinomycete isolated from soil.

MATERIALS AND METHODS

ISOLATION FROM SOIL

The soil samples were collected from the forests of Thiruporur area, Chennai, Tamilnadu. Ten different soil samples were collected from different places at a depth of 3-10 cm. The collected soil samples were kept in sterilized zip locked polythene covers and tagged.

ISOLATION OF ACTINOMYCETES PRETREATMENTS FOR ACTINOMYCETES ISOLATION

Pretreatment was performed by incubating the soil samples in 0.1% $CaCO_3$ at room temperature for seven days (Takizawa *et al*., 1993).[7] This was followed by primary screening using serial dilution method. From the required dilution, 0.1 mL suspension was drawn and plated over the surface of SCA medium. All the plates were incubated at 28 ± 2 °C for three weeks. The spread plated Petri plates were incubated at room temperature (28 °C ± 2 °C) and monitored periodically over 5 weeks for development of actinomycetes growth. The actinomycetes colonies were recognized by powdery, thick and leathery features; initially, white colonies that adhered strongly to the agar surface (Shirling and Gottlieb, 1966)[8] were selected and given a unique alpha numeric accession number. Single-digit numerical characters prefixed with the letter “A” and the isolates were named as A1, A2,…A20 which denotes “Actinomycets1,2…to 20”.

TEST ORGANISM

The test organism *Aspergillus fumigatus* was isolated from the soil. It was identified, sequenced (18S ribosomal RNA gene, partial sequence, ITS1, 5.8S ribosomal RNA gene, and ITS2, complete sequence; and 28S ribosomal RNA gene, partial sequence) and submitted in NCBI with accession number KC119199.

SCREENING OF ANTIFUNGAL ACTINOMYCETES

The 20 actinomycetes isolates A1, A2…A20 were cultured in sabouraud dextrose agar medium and were incubated at 37 °C for about seven days. All these isolated cultures were purified by streak plate technique and identified by their morphology and screened for their antifungal property. The classical method described in the identification by (Nonomura, 1974)[9] and Bergey’s Manual of Determinative Bacteriology Buchnan and Gibbons,1974)[10] was very much useful in the identification. The three strains named A3, A7, A20 which showed encouraging results were

selected after the primary screening. These shortlisted isolates were then subjected to secondary screening with the test organism *Aspergillus fumigatus*.

MORPHOLOGICAL CHARACTERIZATION
CULTURAL CHARACTERIZATION

Morphological and cultural characters were studied by inoculating the strain A7 in various growth media like Yeast malt extract agar (ISP2), Oat meal agar (ISP3), In- organic salts starch agar (ISP4), Glycerol asparagine agar (ISP5), Peptone yeast extract agar (ISP6), Tyrosine agar (ISP7) and Glucose asparagine agar (ISP9). All these media were sterilized and poured into sterile petri dishes for solidification. After solidification of the media, the selected strain A7 was streaked and incubated at room temperature for seven days.

MICROSCOPICAL OBSERVATIONS
GRAM STAINING METHOD

The simple gram staining technique was carried out to identify the actinomycete, and with the help of light microscope it was confirmed as gram positive bacteria.

DETERMINATION OF CELL WALL AMINO ACID

The extraction of cell wall amino acid from whole-organism hydrolyzate of selected actinomycete A7 as well as the reference strain *Streptomyces avermitilis* ATCC 31271 were carried out in TLC. From the position of the spot it was observed and inferred that the isolate A7 belonged to the genus *Streptomyces.*

TAXONOMICAL CHARACTERIZATION
PRODUCTION OF MELANIN

Production of melanin pigment is one of the significant properties of actinomycetes. To study the production of melanin pigments, the isolate A7 was streaked on agar plates and supplemented with ISP–7. It was incubated at room temperature (28 °C ± 2 °C) for seven days. After the seventh day, when observed the petri plates were filled with a black pigment. This showed that the isolate A7 is a pigment producing one.

SUBSTRATE DEGRADATION
CASEIN HYDROLYSIS

Casein is a major protein found in milk. It is composed of amino acids linked together by peptide bonds. Casein hydrolysis was done by supplementing Bennett agar medium with skimmed milk. The casein in the skimmed milk interacted with the medium to produce a colloidal suspension, which made the medium opaque. The strain A7 was streaked on the agar surface of the petri plate and kept at room temperature for about 7 days. The isolate produced a clear zone adjacent to its growth. This showed that the isolate was able to hydrolyze casein.

UTILIZATION OF UREA

Urease test was performed by growing the test organism on agar medium containing the pH indicator phenol red (pH6.8).The test organism A7 was streaked on to the surface of the medium, and any evidence of urease production of the organism was detected by the change of colour from red to deep pink. No colour change was observed which indicated urea was not utilized by the isolate A7.

HYDROLYSIS OF STARCH (AMYLASE PRODUCTION TEST)

The ability to degrade starch was used as a criterion for the determination of amylase production. It was tested by performing the starch test to determine the presence or absence of starch in minimal medium by using iodine solution as indicator. The presence of starch was indicated by the formation of dark blue colour of the medium and a yellow zone around the colony. The selected isolate gave positive results for starch hydrolysis.

UTILIZATION OF GELATIN

The utilization of gelatin was carried out in modified Bennett's agar. Gelatin is a protein that is digested by an enzyme called gelatinase in actinomycetes. The inoculated plate was examined to see whether it produced any clearing, around the line of growth.

HYDROLYSIS OF AESCULIN

The hydrolysis of aesculin were carried out in Bennett's agar. The plates were inoculated with A7 and incubated at room temperature for about seven days. After incubation the plates

were examined for the presence or absence of fluorescence and colour change.

UTILIZATION OF CARBON

Carbon utilization by the actinomycete was determined after growing on carbon utilization medium (ISP–9) (Pridham and Gottlieb, 1948).[11] Different sterilized carbon sources were added to the sterile basal mineral salts agar (60 °C) to get a final concentration of 1% mixed gently and poured 20 mL of medium in each Petri plate. The carbon sources and controls were as follows: no carbon source (negative control); D-Glucose (positive control); L-Arabinose; Sucrose; D-Fructose; D-Xylose; D- Galactose; Starch; D-Mannitol and Sorbitol. A loopful of inoculum was streaked across the plate, and incubated at 30°C for seven days. The growth of actinomycete on a given carbon source was compared with both positive and negative controls.

EFFECT ON TEMPERATURE

The effect of temperature on the growth of actinomycete was determined using ISP–4 medium in a temperature gradient incubator. A loopful of the selected actinomycete A7 was streaked separately on the medium and incubated at different temperature viz., 20 °C, 30 °C, 40 °C and 55 °C. After 7 days of inoculation, the plates were observed for the growth of actinomycetes.

EFFECT OF PH ON GROWTH

The effect of pH on the growth of actinomycetes was determined using ISP–4. Acidic range (pH 3–5) was prepared with acetate buffer, neutral range (pH 6–7) was prepared with phosphate buffer and alkali range (pH 8–11) was prepared with Tris-buffer. A loopful of selected actinomycete isolate A7 was streaked separately on the above medium with different pH and incubated at room temperature (28 °C ± 2 °C). After the 7th day, the plates were observed for the growth of actinomycete.

RESULTS

SCREENING AND ISOLATION

Screening of the soil allows to discard many of the valueless microbes, and helps with the isolation of the organism of interest from a large microbial population. Many industrial screening programs for the isolation of novel antibiotic forming actinomycetes involve initial testing of any surface colonies Bushnell, (1941)[12] , Pickup *et al.,* (1990)[13] suggested that agar cultures were suitable for examining large numbers of isolates initially. It was done by inoculating the soil samples with calcium carbonate as this is the most efficient technique for the isolation of actinomycetes Alferova *et al*.,(2003).[14] The isolates of actinomycetes with raised powdery colonies and a zone of inhibition around them by crowded plate technique were shortlisted. They were also subjected to the dual streak culture method on starch casein agar media to get pure cultures. About 20 strains were selected and they were preserved in actinomycetes agar slants and also in glycerol for further study.Of the 20 strains of actinomycetes isolated and screened against the fungal pathogen *Aspergillus fumigatus* the strains A3, A7 and A20 showed, some promising activity after primary screening were selected. It was again further subjected to secondary screening to get a promising isolate. When they were subjected to secondary screening the strain A7 gave excellent activity against the pathogen *Aspergillus fumigatus* by producing a clear zone around it. Thus the primary and secondary screening revealed that the strain A7 as the, the most promising one and it was selected for further study.

MORPHOLOGIC, MICROSCOPIC AND TAXONOMIC STUDIES

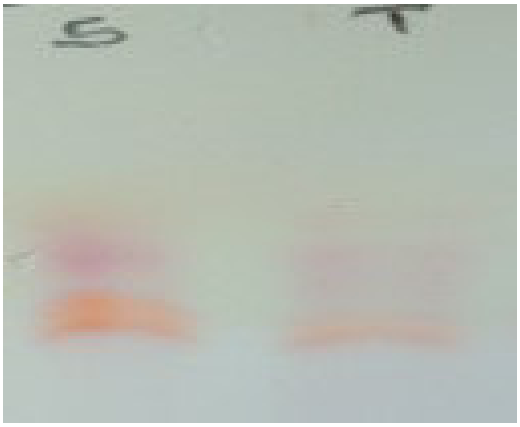
The cultural characteristics were studied in different ISP (International Streptomyces Project) media and found that ISP2 and ISP4 supported the growth of the selected isolate A7. The selected actinomycete isolate was subjected to microscopical examination. It gave violet colour by retaining the crystal violet. This showed that the isolate A7 as gram positive. During the cell wall amino acid analysis, the presence of L-Diaminopimelic acid (DAP) in its cell wall was found out, when it was compared with the standard strain *Streptomyces avermitilis*. The presence of DAP showed that the isolate belonged to Streptomyces. The isolate produced yellow coloured pigments when it was grown in ISP2 medium this showed that the isolate was a

pigment producing one. Gelatin was degraded by the isolate by producing a clear area around it when grown in minimal medium. Casein and starch were hydrolyzed by the isolate when it was grown in Benett' s agar. The isolate A7 produced a dark black coloured pigment when grown in ISP 7 this showed that the isolate was able to produce melanin pigments. The isolate when subjected to urea utilization with phenol red as indicator failed to produce pink colour this proved that the isolate was not able to utilize urea. Further when the isolate was grown in minimal media supplemented with aesculin it gave negative results which was indicated by absence of colour change in the medium.It was observed that the isolate A7 needed an optimum temperature for its growth (i.e.) between 30±3 °C with a pH range of 8-11. It gave excellent growth results when it was grown in potato dextrose broth. The antifungal activity of the strain A7 against the pathogenic fungi was good when it was grown on potato dextrose agar indicating that potato dextrose agar medium was good for inducing antifungal activity in the strain A7.The following table summarizes the results of morphologic, microscopical and taxonomic properties, described above.

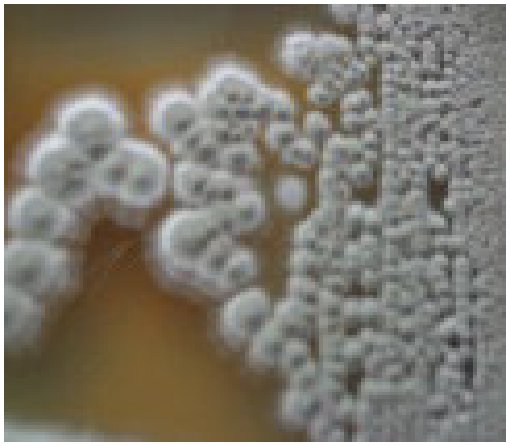
Morphological and physiological characteristics of A7 isolate

Characteristics	Result
Gram stain	+
DAP	+
Spore mass	Grey
Diffusion pigment	+
Gelatin degradation	+
Casein hydrolysis	+
Urea utilization	-
Starch hydrolysis	+
Aesculin	-
Melanin production	+
Growth in ISP medium	
ISP 2	+
ISP 3	-
ISP 4	+
ISP 5	-
Effect on carbon source	
Galactose	+
Arabinose	+
Xylose	-
Glucose	+
Mannitol	+
Sorbitol	+
Effect on temperature	
20 °C	-
30 °C	+
40 °C	±
55 °C	-
Effect on pH	
5	-
6	±
7	±
8	+
9	+
10	+
11	+
(+) Positive (–) Negative(±)Moderate	

***Cell wall amino acid*- Picture1**
S-Standard, (Streptomyces avermitilis)
T –Test, A7

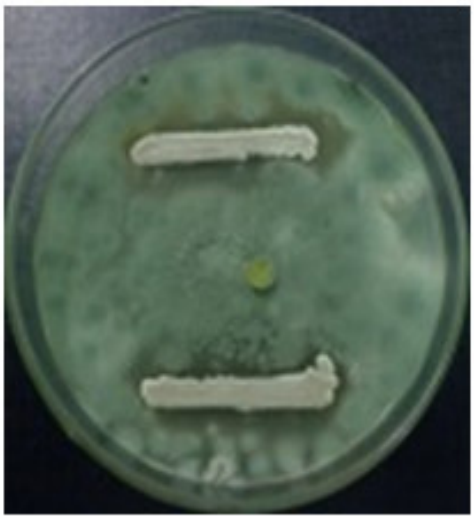


***Colonies of strain A7*- Picture 2**

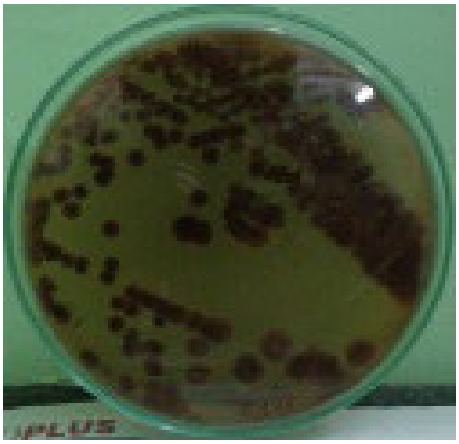


***Primary screening* –Picture 3**

Secondary screening **–Picture 4**



Melanin production **–Picture 5**



CONCLUSION

Actinomycetes have provided many important bioactive substances (Tamura *et al*., 1993).[15] These microorganisms stand out as a unique group of prokaryotic organisms in two respects, the diversity of their morphology and metabolic products (Ensign, 1978).[16]Approximately two thirds of the naturally occurring antibiotics have been isolated from actinomycetes (Okami and Hotta, 1988).[17] The need for a new, safe and more effective antifungal is a big challenge with the increase in immune compromised resistant pathogens.[18]At present most of the microbes has developed resistance to the existing antibiotics and so it provoked the necessity of a constant research for the new antibiotics in order to overcome the resistant pathogens.[19] Actinomycetes were known for their ability to produce antibiotics, and there is an urgent need for a new safe and a more effective antifungal antibiotic. It will be a challenge for the research community as well as the pharmaceutical industry.[20] Therefore ,there is a huge potential in actinomycetes for the discovery of noval bioactive natural products.[21] Initially 20 actinomycetes were selected based on the zone of clearance they produced around their colonies. Out of the 20 strains only 3 were selected based on their antifungal activity. This was followed by the taxonomical and biochemical tests to prove that their behaviors resembled actinomycetes. Further, these tests helped to choose the fermentation medium as well as for the production of antibiotic. The strain A7 was selected based on its significant antifungal

activity against the targeted ubiquitous pathogen *Aspergillus fumigatus*. From the morphological and taxonomical studies, it was observed and concluded that the strain A7 to belong to the genus Streptomyces. Further studies on metabolite extraction and purification are currently in progress.

ACKNOWLEDGEMENT

I express my sincere thanks to Dr .M. Krishna raj for providing his lab to carry out this work.

CONFLICT OF INTEREST

Declared none.

REFERENCES

1. McCarthy A.J., and Williams S.T., Actinomycetes as agents of Bio degradation in the Environment-a review, GeneVol. (115):189–192, (1992).
2. Stach J.E., Bull A.T., Antonie Van Leeuwenhoek., Estimating and Comparing the Diversity of Marine Actinobacteria.Vol. (87) :3–9, (2005).
3. McCarthy A.J., Williams S.T. ,Actinomycetes as Agents of Biodegradation in the Environment-a review.GeneVol.(115):189–192, (1992).
4. Gust B., Challis G.L., Fowler K., Kieser T., and Chater K.F., PCR- targeted *Streptomyces* gene replacement Identifies a Protein domain needed for Biosynthesis of the sesquiterpene soil odour geosmin, Proc Natl Acad Sci USA,Vol. (100):1541–1546, (2003).
5. Xiao k., kinkell L., Samac D A., Errakhi R., Bouteau., Biological control of *phytophthora* root rots on alfalfa and soyabean with *Streptomyces;* Biol Control,Vol. (23); 285-295, (2002).
6. Lebrihi A., Barakate M., Evidences of Biological Control Capacities of *sterptomyces* spp. Against *Sclerotium rolfsii* Responsible for Damping off disease in sugar beet (*Beta vulgaris* L.) World J. Microbiol Biotechnol , Vol.(23);1503-1509,(2007).
7. Takizawa S., Kojima S., Tamura S., Fujinaga S., Benno Y., Naas T., *Lactobacillus kefir* granum sp. and *Lactobacillus parakefir* sp. two New species from kefir grains. Int. J. Syst. Bacteriol ,Vol.(44):435-439, (1993).
8. Shirling E.B., Gottlieb D., Cooperative description of type strains of *Streptomyces* II. Species description from first study. Int. Syst. Bacteriol,Vol. (18):69-189, (1966).
9. Nonomura H., Key for Classification and Identification of 458 species of the Streptomycetes included in ISP. J. Ferment. Technol,Vol. (52): 78-92, (1974).
10. Buchanan R.E.,and Gibbons N.E.,Ed. Bergey's Manual of Determinative Bacteriogy. 8 th Edn, The Williams and Wilkins Co publishers; Baltimore, U.S.A,(1974).
11. Pridham T., Gottileb D., The Utilization of Carbon Compounds by Some Actinomycetes as an Aid for Species Determination Bacteriol. Vol. (56), 107-114, (1948).
12. Bushnell D L., Haas H F.,The Utilization of Certain Hydrocarbons by Microorganisms. Kansas Agricultural Experiment Station,(199):653–673, (1941).
13. Pickup R W.*et al* .,Detection of Genetically Engineered traits Among Bacteria in the Environment;Trends in Biotechnol,Vol.(8);329-333,(1990).
14. Alferova *et al* .,The Application of Succession Analysis in Combination with EHF Irradiation to the Sggelective Isolation of Actinomycetes from Soil. Microbiol. Vol. 1 (72).No.1.pp:114-117, (2003).
15. Tamura *et al*., Takizawa S., Kojima S., Fujinaga S., Benno., Nakase Y., Int. J. syt. Bacteriol.Vol. (44):435-439, (1993).
16. Ensign J C., Formation Properties and Germination of Actinomycete

Spores.Annu Rev Microbiol,(32);185-219,(1978).

17. Okami Y., Hotta K., Goodfellow M.,Williams S T., Mordarski M., Search and Discovery of new Antibiotics,Ed Actinomycetes in Biotechnol.Publisher Academic Press Inc; SanDiego, pp. 3367 (1988).
18. Sweetline C., Usha R., Palaniswamy M.,Studies on Anticandidal activity of the Actinomycetes Isolated from Coimbatore region of Tamilnadu. Accesed from Int. J. of Pharma and Biosciences. July,Vol. 5(3) B: 46-53,(2014).
19. Vengadeshprabu K., Sundaramoorthi C., Guptasaurabh., Karthick K., Tamilselvi N., Production and Characterization of Antibiotics from soil isolated actinomycetes. Accessed from Int. Research J of Pharmacy, Vol.2(4): 114-118,(2011).
20. Augustine S.K., Bhavsar S P., Baserisalehi M., Kapadnis B P., Isolation Characterization and optimization of Antifungal activity of an Actinomycete of soil origin. Accessed from Int. J. of Exp. Bio.September, Vol. (42), pp. 928-932,(2004).
21. Jose A.Salas., *et al*., Activation and Identification of five clusters for Secondary Metabolites in *Streptomyces albus* J1074 Accessed from Microbial Biotechnol. Vol.7(3): 242-256, (2014).