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Isolation and Identification of Soil derived Actinomycetes *Nocardiopsis alba.*

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ABSTRACT

The Actinomycetes were isolated from the K.S.Rangasamy College of Technology, Biotechnology garden at a depth of 15- 20 cm. It was air dried for 4 days and the impurities were removed after it. It was then subjected to serial dilution and found 10^{-4} dilution was optimum for isolation of Actinomycetes. Later based on the colony morphology a single colony was chosen and it was subjected to simple streak and then quadrant streaking to isolate pure colonies. The isolate was subjected to various biochemical tests and amylase producing capacity. 16Sr RNA sequencing was been carried out to identify out the isolate and it was found as *Nocardiopsis alba* and it was deposited in NCBI and the accession ID is KF957834.

Keywords: Actinoycetes, Biochemical tests, 16Sr RNA sequencing, *Nocardiopsis alba*, NCBI

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INTRODUCTION

Actinomycetes, a member of a heterogeneous group of gram-positive, generally anaerobic bacteria noted for a filamentous and branching growth pattern that results, in most forms, in an extensive colony, or mycelium. The mycelium in some species may break apart to form rod- or coccid-shaped forms. Many genera also form spores; the sporangia, or spore cases, may be found on aerial hyphae, on the colony surface, or freely within the environment. Actinomycetes are one among the major source for the production of Antibiotics and enzymes (Ravikumar *et al.,* 2012). Majority of this antibiotics substances are good antibacterial agent for treating against bacteria in plants and animals including human beings (Raayadapadar *et al.,* 2001). Many species of Actinomycetes occur in soil and are harmless to animals and higher plants, while some are important pathogens, and many others are beneficial sources of antibiotics. Several species of *Streptomycetes* cause the disease actinomycosis in humans and cattle. Many of the Actinomycetes are sources of antibiotics such as streptomycin (Muralisastry *et al.,* 2013)

Actinomycetes are a specific group as bacteria though morphologically they resemble fungi because of their elongated cells that branch into filaments or hyphae. During the process of composting mainly thermophilic (adapted to high temperatures) and thermo. In the initial phase of composting the intensive increase of microbial activity leads to a self-heating of the organic material. High temperatures in composting helps to kill viruses, pathogenic bacteria, e.g. coli forms, and weed seeds present. Actinomycetes live predominantly aerobically, i.e. they need oxygen for their metabolism. The compost material, should therefore be well aerated (Deepa *et al.,* 2013)

Generally, Actinomycetes grow on fresh substrates more slowly than other bacteria and fungi. During the composting process the Actinomycetes degrade natural substances such as chitin or cellulose. Actinomycetes are a non-taxonomic term for a group of common soil microorganisms sometimes called thread or ray bacteria. They are known for decomposing more resistant organic materials such as chitin, a complex sugar found in the outer skeleton of insects and elsewhere. Nucleic materials (DNA) consist of a helical structure with a sugar backbone attached through the bases adenine–thymine (A+T) and guanine–cytosine (G+C). Compared with the DNA of other organisms, Actinomycetes have a high percentage of guanine–cytosine bases. In growth habit, many Actinomycetes resemble fungi but are smaller, and terms common to both are used to describe morphological features More than one-half of the antibiotics used in human medicine, including aureomycin, chloromycetin, kanamycin, neomycin, streptomycin, and terramycin, come from soil Actinomycetes. The smell of freshly turned soil is due to metabolic end products called geosmins that are produced by these organisms and move through soil as unseen volatiles. Actinomycetes can be said as most economical and biotechnologically valuable prokaryote (Rajeshmuthu *et al.,* 2013)

The 16S rRNA gene is the most conserved DNA in all cells. Portions of the rDNA sequence from distantly related organisms are remarkably similar. Genes that encode the rRNA have been used extensively to determine taxonomy, phylogeny (evolutionary relationships), and to estimate rates of species divergence among bacteria. Thus the comparison of 16SrRNA sequence can show evolutionary relatedness among microorganisms.

Thus we can refer as “rRNA gene” or “rDNA” to designate the DNA in the genome that produces the ribosomal RNA. Most prokaryotes have three rRNA, called the 5S, 16S and 23S rRNA. Bacterial 16S, 23S, and 5S rRNA genes are typically organized as a co-transcribed operon. Ribosomal RNAs in Prokaryotes

The 16SrRNA sequence has hyper variable regions, where sequences have diverged over evolutionary time. Primers are designed to bind to conserved regions and amplify variable Regions. The DNA sequence of the 16SrRNA gene has been determined for an extremely large number of species.

MATERIALS AND METHODS

Isolation of Actinomycetes

The Soil sample was collected from the K.S.Rangasamy College of Technology Biotechnology garden at a depth of 15- 20 cm. The collected soil sample was found to be red in color with huge moisture. The moisture

content was removed by drying the sample in room temperature for 3-4 days. The collected sample was subjected to serial dilution. The serial dilution is a technique in microbiology by which the microbe's population is reduced. The dried 1 gram of soil sample was collected and it was diluted till 10^{-4} dilution (Selvakumar*et al*., 2012) because the growth of the Actinomycetes was found to be abundant in this dilution. The media used for isolating these Actinomycetes was Starch Casein agar media (SCA media) these media was found to be selective media for the growth of the Actinomycetes.

This sterilized media was supplemented with the Cyclohexamide and Nallidixic acid which inhibits the growth of the fungus and also Gram negative bacteria as these Actinomycetes were found to be Gram positive in nature. The 10^{-4} dilutions was pour plated on the media SCA media. It was then incubated for 4 days at 30ºC in incubator. The growth of the organism where observed after 4 days, lot of colonies were observed with various morphologies.

The colony which appears to be white powdery and oval shaped was alone chosen for the sub culturing process. Because the probability of Actinomycetes where more in this colony than comparing with the other colonies. This selected colony was subjected to the sub culturing, ordinary streaking procedures were carried out and it was incubated for 4 days at 30ºC in SCA media. After 4 days of incubation, the quadrant streaking was carried out in order to obtain the pure culture with SCA media.

Biochemical tests

Starch hydrolysis test

Amylase enzymes were produced by the Actinomycetes. Hence to determine whether the isolated organism is Actinomycetes this test was being performed.

Gram Straining

A loop full of culture with the help of sterilised inoculation loop in a clean sterilised glass slide. Heat fixed the sample by placing the slide on heat Bunsen flame. Flood the slide with the crystal violet solution for one minute and then flood with the tap water not more than five seconds. Flood the slide with the Grams iodine solution for one minute and then flood again with the tap water for five to ten seconds. Then 95% of alcohol solution was added so that the decolourization process will occur. This process should not take more than ten seconds because it may damage the cellular components. Flood the slide with the help of the safranin solution not more than thirty seconds. Then it should be drained completely, observation should be made under a microscope.

Casein hydrolysis test

Inorder to test the production of protease enzyme casein test was been carried out. Casein agar was prepared and used as a selective media. The isolated organism was been taken in loop full and cultured in casein agar and incubated for 4 days at 30°C. (Selvakumar *et al.,* 2012)

Urease hydrolysis test

Inorder to test whether the isolated organism degrades Urease, Urease hydrolysis test has been carried out. Christensen's Urea Agar was the selective media used here. (Selvakumar *et al.,* 2010. The media is poured on a petri dish and a loop full of culture is to be inoculated in this media and kept for incubation for 4 days at 30° C.

Lipase test

This lipase test was used to test whether the organism has the capacity to produce the lipase enzyme. This lipase enzyme degrades the fat molecules. Here the Tributryin oil serves as a fat molecule. Tributryin agar was used as a differential media. The media was sterilized and poured in petridish. The culture was inoculated in them and incubated for 4 days at 30° C.

Gelatin hydrolysis test

To test whether the isolated organism can produce gelatinase enyme, gelatin hydrolysis test was been performed. Gelatin media serves as the differential media here (Selvakumar*et al.,* 2010). The culture was incubated at 30° C for 1 week.

MacConkey agar Test

MacConkey agar was found to be the selective media to identify whether the organism is gram positive or gram negative. The selected culture was inoculated in the media and kept for incubation for 4 days at 30° C. (Selvakumar*et al., 2010)*

Salt Tolerance Test

An increased concentration of the NaCl content in the media is used to determine whether the organism survives in that condition. If the organism survives the high amount of salt and grows after the 4 days of incubation those organism was known to be salt tolerable. The media used here is Starch Casein agar media with increased amount of salt. The NaCl was increased 6.5 times of its original requirement.

16SrRNA sequencing

DNA isolation

The procedure has been carried out as suggested by Vijay kumar *et al.,* 2010. The isolated pure culture of Actinomycetes was inoculated in the sterilized Starch casein broth and kept for incubation for 4 days at 30ºC in rotary shaker at 180 rpm. After 4 days of incubation the culture was centrifuged at 10,000 rpm for 10 minutes in order to separate the mycelium. 0.1 g of mycelium was taken and placed in a sterilized dish. which was crushed with the help of liquid nitrogen and supplemented with 50 µl TE buffer and lysozyme 20mg/ml. It was incubated in water bath for 30 minutes at 37º C. Once the incubation period is over 20 µl of 10% SDS and 20 µl of proteinase K was added and incubated at 55º C for 30 minutes.

With equal volume of phenol and chloroform solution in 1:1 ratio at 10,000 rpm for 5 mins the lysate was been extracted. The aqueous phase containing nucleic acid was transferred to a fresh tube. Precipitation with ethanol was carried out in order to separate the DNA. 70% ethanol is the precipitating agent which is kept undisturbed for 30 minutes at -20º C. Centrifugation at 10,000 rpm for 10 minutes was carried out to separate the pellet. The pellet was washed thrice with 90 % ethanol and suspended in TE buffer. The 20 µl RNase solution was added and incubated at 37º C for 1 hour. It was once again extracted with phenol chloroform at 1:1 ratio to obtain the pure product.

PCR amplification and sequencing

PCR amplification of the 16SrDNA of the isolated Actinomycetes and automated sequencing was performed using primers

27F-5' AGAGTTGATCMTGGCTCAG 3'
1492R-5' TACGGYTACCTTGTTAGCGACTT 3'

Final volume of the reaction mixture is set to 25 µl containing 1X PCR buffer, 1.5 mM of $MgCl_2$, 200 µM of each dNTP, 20 pico-moles of each primer, 2.5U of Taq DNA polymerase and 100 ng of template DNA. The amplification is carried out in an Eppendorf Thermo-cycler 96. With the profile such as initial denaturation at 94º C for 2 minutes, then 30 amplification cycles of 94º C for 1 minute, 55º C for 1 minute and 72º C for 2 minutes.

The PCR product was detected by agarose gel electrophoresis and visualized by ultraviolet florescence on straining with ethidium bromide. The products were purified using HiPurA™ PCR product purification spin kit. Sequencing reactions were performed using the ABI PRISM® BigDye® Terminator version cycle 3.1 Cycle sequencing Kit.

Basic Local Alignment Search Tool (BLAST)

From the 16SrRNA studies, sequence of nucleotide was obtained. From the obtained sequence the corresponding organism was to be known, hence the BLAST tool was being used. The obtained sequence was compared with the database of NCBI so that we can able to know the exact organism. In the NCBI website nucleotide BLAST has to be selected where they obtained nucleotide sequence has to be tered in the FAST format and then the database has to be selected to 16S ribosomal RNA sequence. Based upon the maximum score among the other organism the organism was identified.

RESULTS AND DISCUSSION

ISOLATION OF ACTINOMYCETES

After the incubation period of spread plate, a single colony is chosen based upon its morphology. This identified colony was sub cultured using the simple streaking method. In order to obtain the pure culture, quadrant streaking was performed. Those colonies from the quadrant streaking were sub cultured and preserved or the future use.

BIOCHEMICAL TESTS

Gram straining

The isolated organism was confirmed to be gram positive. The presence of mycelium and spores confirms us that they are Actinomycetes.

Starch hydrolysis test.

Iodine solution was been poured, a clear zone around the organism is seen. The amylase enzyme produced by the Actinomycetes made it so. This enzyme repels the iodine solution.

Casein hydrolysis test

Protease degrades the casein present in the media. Presence of clear zone shows the isolated organism has the ability to produce protease.

Lipase Test

After the incubation period Lipase activity was examined, no change was seen around the inoculated organism. In order to obtain a positive result clear zone should be observed around the grown organism. Which clearly shows that the organism have no ability to produce the enzyme lipase.

Urease Test

The organism has not used the urea present in the media as a substrate to produce the urease enzyme because a red color zone was not seen around the organism.

Salt Tolerance Test

The ability of the organism to tolerate the maximum amount of salt was tested using the salt tolerance test. The organism has survived in high amount of salt content, hence the organism was found to tolerate the high amount of salt.

Gelatinase Test

The isolated organism has been tested for the production of gelatinase enzyme. It was kept in ice for half an hour and it is observed that the media still remains un-solidified. Hence gelatinase is produced by the organism which degrades the gelatin present in the media.

MacConkey agar test

The growth of the organism was not seen in the media which shows that the organism isolated is a Gram Positive bacterium.

16SrRNA SEQUENCING

An average amount of DNA was extracted and it was to be amplified. The extracted genomic DNA and the PCR product with the help of 1kb DNA ladder was allowed to run in 1% Agarose gel and it is found that they are in not in degraded condition. The PCR amplification of the product yielded the molecular size of DNA 1.5 kb length. Using the automated sequence the sequence was retrieved with the help of suitable primers. The obtained sequence was entered in the form of FASTA format and the corresponding organism was identified using the Basic Local Alignment Search Tool (BLAST) a similarity searching tool. Based upon the maximum identity the organism was identified as *Nocardiopsis alba.* Then those obtained sequence was submitted in NCBI and obtained the accession id as **KF957834.**

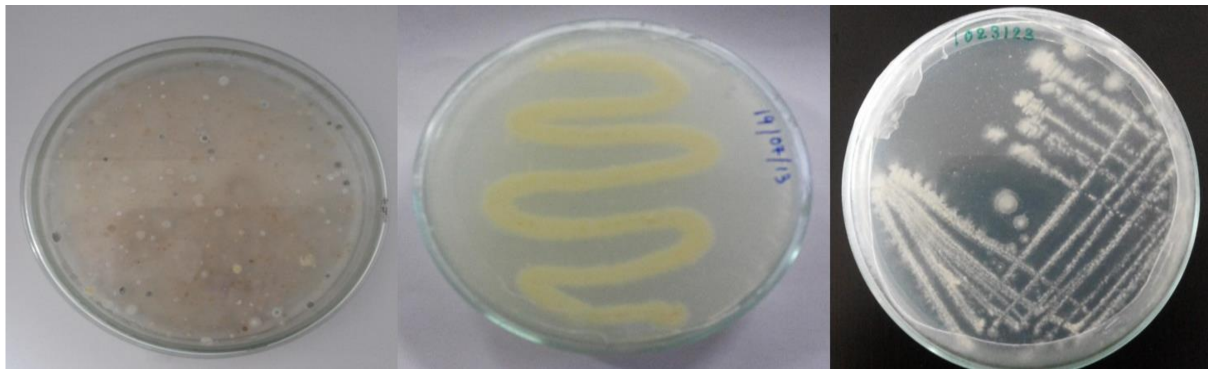

Fig 1. Spread plate **Fig 2. Streak Plate** **Fig 3. Quadrant Plate**

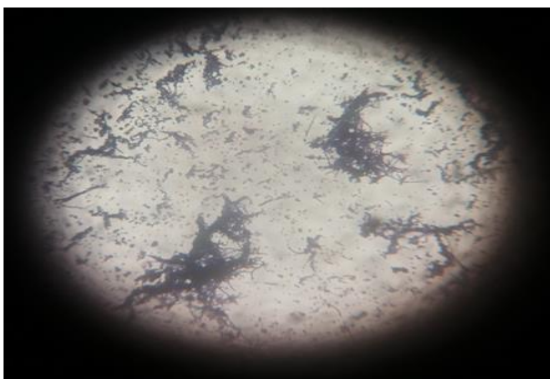

Fig 4. Gram staining

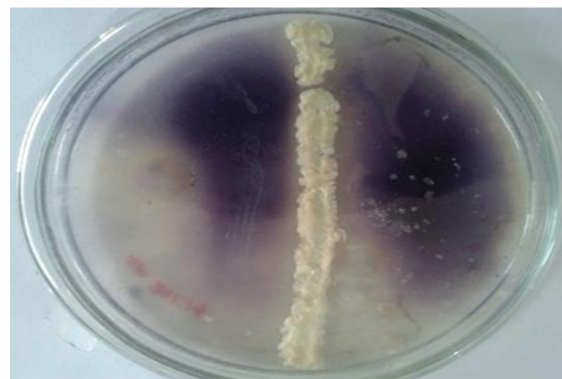

Fig 5. Starch Hydrolysis test

Fig 6. Casein hydrolysis

Fig 7. Lipase test

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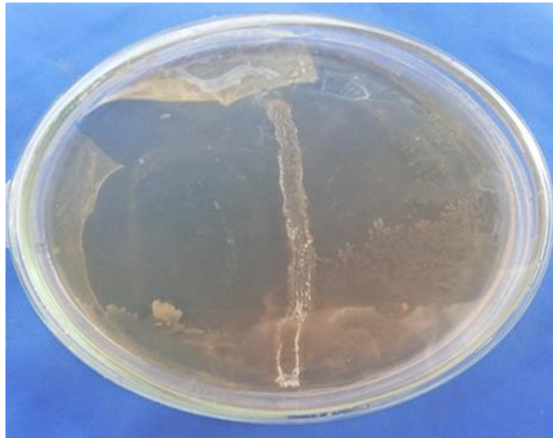

Fig 8. Urease test

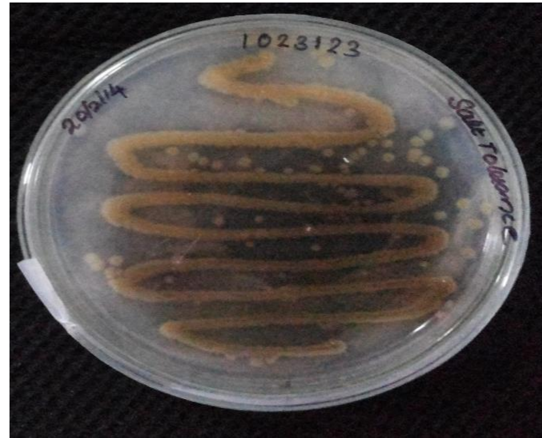

Fig 9. Salt Tolerance

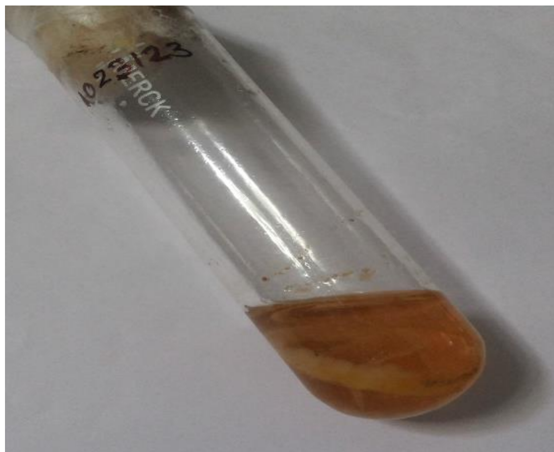

Fig 10. Gelatinase test

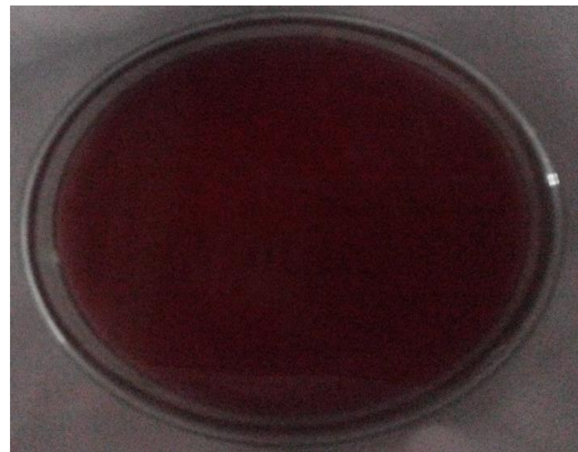

Fig 11. Maccankey Agar test

Table 1 : Biochemical Tests

	Result	Conclusion
Gram Straining	++	Gram positive bacteria with mycelium and spores
Casein hydrolysis test	++	Protease is produced to degrade the casein
Urease test	--	Urease enzyme was not produced
Lipase test	--	Lipase enzyme was not produced
Starch hydrolysis test	++	Amylase enzyme was produced
Salt tolerance test	++	Organisms were found to have salt tolerance capacity
Gelatinase Test	++	The gelatin was degraded hence the media remains unsolidified even after the incubation in ice.
MacConkey agar Test	--	The growth of the organism was not seen.

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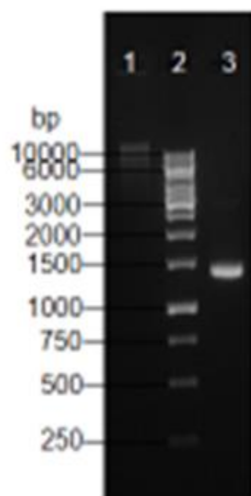

Agarose Gel (1%) showing Genomic DNA, 1Kb DNA ladder & PCR Product of **1023123**
Lane 1 – Genomic DNA
Lane 2 – 1Kb DNA Ladder
Lane 3 – PCR product

Fig 12. PCR product

DISCUSSIONS

The isolated organism was found to be *Nocardiopsis alba* using the 16SrRNA sequencing and BLAST. The obtained sequence was deposited in the NCBI and obtained the accession id as KF957834. The growth of Actinomycetes was observed in 10^{-4} dilutions as seen by Selvakumar*et al.* (2012). In the biochemical characterization such as MacConkey agar test growth of the actinomycetes was not observed, the same result was obtained for Selvakumar*et al.* (2012). In Urease test a negative result was obtained whereas for Selvakumar*et al.* (2012) he has obtained positive result which shows that the *Nocardiopsis alba* does not produces the ureases. The gelatin test yields a positive result as Selvakumar*et al.* (2012) obtains. Casein test shows positive results whereas for Selvakumar*et al.* (2012) he obtains negative result. It shows that *Nocardiopsis alba*has the capacity to produce protease enzyme.

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REFERENCES

[1] Deepa, S,Kanimozhi and Panneerselvam A (2013) “Antimicrobial activity of extracellular synthesized silver nanoparticles from marine derived actinomycetes”, International Journal of Current Microbiology and Applied science, Volume 2 Issue 9, pp. 223-230.

[2] MuraliSastry, Absar Ahmad, Islam Khan and Rajiv Kumar (2013) “Biosynthesis of metal nanoparticles using fungi and actinomycetes”, Current science, volume 85 issue 2, pp, 162-170.

[3] Rajeshmuthu.M, Subbaiya.R, Balasubramanian. M, Ponmurugan .P, Masilamani Selvam. 2013. Isolation and Identification of Actinomycetes *Isoptericola variabilis* From Cauvery River Soil Sample. International journal of current microbiology and Applied Sciences. Vol.2 pp, 236-245

[4] Ravikumar. S, Fredimoses. M, Gnanadesigan. M. 2012. Anticancer property of sediment Actinomycetes against mcf 7 and MDA MB 231 cell lines. Asian Pacific journal of Tropical biomedicine, pp. 92-96.
[5] Rayadapadar. S, Paul, A.K. 2001. Production of an antifungal antibiotics by *Streptomyces s*pecies aburaviences IDA 28. Journals of microbial research, pp, 315 323.
[6] River Soil Sample. International journal of current microbiology and Applied Sciences. Vol.2 pp, 236 245.
[7] Selvakumar P, S.Viveka, S.Prakash, S.Jasminebeaula and R.Uloganathan (2012)"Antimicrobial activity of extracellular synthesized silver nanoparticles from marine derived *Streptomyces rochei*" International journal of biological science, pp, 188-197
[8] Vijay Kumar, Alpana Bharati, Omprakash Gusain and Gajraj Singh Bisht, 2010. An "Improved method for Isolation of Genomic DNA from filamentious Actinomycetes", Journal of Sceince Engineering and Technical management, Volume 2 (2), pp 10-13