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Isolation and characterization of *Actinomycetes* from soil samples and screening their antibacterial activity.

Isolation and characterization of Actinomycetes from soil samples and screening their antibacterial activity.

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ABSTRACT

The main objective of the present study was isolation, purification and characterization of *Actinomycetes* from different soil samples, bioactive compounds produced from *Actinomycetes spp*, having antimicrobial activity against 6 selected pathogenic strains and degradation of biodegradable plastics. *Actinomycetes spp.* are the most widely distributed group of microorganisms in nature which is primarily in the soil. Soil samples were taken from different habitats in Chennai. Potential isolates of *Actinomycetes* colonies were screened and purified. Isolates of *Actinomycetes* colonies were characterized by morphological, physiological and biochemical tests. These colony isolates were subjected to extraction of bioactive compounds. Antibacterial activity of the purified extract of isolates were evaluated. The colony isolates were tested for antimicrobial activity against 6 selected pathogenic strains. Solvent Phase crude extract and Intermediate Phase crude extract showed strong inhibition of growth of bacterial organisms in *Bacillus subtilis, Escherichia coli, Klebsiella pneumonia, Streptococcus spp,* except *Staphylococcus aureus* and *Pseudomonas spp.* Degradation of Biodegradable Plastics have been evaluated against *Actinomycetes spp.* These colony isolates have antibacterial activity and can be used in the development of new antibiotics for pharmaceutical compounds and also as a biocontrol agent against pathogenic fungi.

Keywords: *Actinomycetes sp.,* Starch Casein medium and Biodegradable plastics.

RESUMEN

El objetivo principal del presente estudio fue el aislamiento, purificación y caracterización de Actinomycetes de diferentes muestras de suelo, compuestos bioactivos producidos a partir de Actinomycetes spp, con actividad antimicrobiana contra 6 cepas patógenas seleccionadas y degradación de plásticos biodegradables. Actinomicetos spp. Son el grupo de microorganismos más ampliamente distribuido en la naturaleza,

principalmente en el suelo. Se tomaron muestras de suelo de diferentes hábitats en Chennai. Se examinaron y purificaron posibles aislados de colonias de Actinomycetes. Los aislados de colonias de Actinomycetes se caracterizaron mediante pruebas morfológicas, fisiológicas y bioquímicas. Estos aislados de colonias se sometieron a extracción de compuestos bioactivos. Se evaluó la actividad antibacteriana del extracto purificado de los aislados. Se analizó la actividad antimicrobiana de los aislados de colonias contra 6 cepas patógenas seleccionadas. El extracto crudo en fase solvente y el extracto crudo en fase intermedia mostraron una fuerte inhibición del crecimiento de organismos bacterianos en *Bacillus subtilis, Escherichia coli, Klebsiella pneumonia, Streptococcus* spp, excepto *Staphylococcus aureus* y *Pseudomonas* spp. Se ha evaluado la degradación de Plásticos Biodegradables frente a Actinomycetes spp. Estos aislados de colonias tienen actividad antibacteriana y pueden usarse en el desarrollo de nuevos antibióticos para compuestos farmacéuticos y también como agente de biocontrol contra hongos patógenos.

Palabras clave: *Actinomycetes* sp., medio almidón caseína y Plásticos biodegradables.

INTRODUCTION

Soil microorganisms provide an excellent resource for the isolation and identification of therapeutically important products. Among them, *Actinomycetes* are an important group. Actinomycetales is composed of 80 genera, is nearly from the terrestrial soils, where they live primarily as saprophytes, colonizing plants and water and showing morphological and chemical diversity with a distinct evolutionary line. *Actinomycetes* perform significant biogeochemical roles in soils and are highly valued for their unparalleled ability to produce biologically active metabolites.

Actinomycetes is a non-taxonomic term for a group of common soil microorganisms, sometimes also called as “a thread or ray bacteria”. They are known for decomposing more resistant organic matter such as chitin, a complex sugar found in the outer skeleton of insects. It belongs to a group of simple prokaryotic organisms which are having a filamentous structure which resembles the fungal mycelium, which also consist of highly dense filamentous network. The internal structure of cell wall of the *Actinomycetes* is similar to a group of bacteria. Thus, *Actinomycetes* are also referred to as filamentous Actinobacteria and act as a connecting link between fungi and bacteria. *Actinomycetes* are “true bacteria”, and not fungus and thus are placed in the kingdom of “Bacteria” and a class “Actinobacteria”. They are ubiquitous and commonly found in soil and thus are soil microorganisms. *Actinomycetes* also act as “Decomposers” and carry out the decomposition of organic compounds like Complex sugar, Chitin, Hemicellulose etc. In addition to soil, these are also very common in marine habitat and considered as a treasure house of secondary metabolites. Its filamentous forms are predominantly aerobic, and few of them are anaerobic. The morphology of *Actinomycetes* is similar to fungi as it produces a dense, branched and filamentous and raised colony over the substrate but most of its features are common to bacteria than of fungi and thus are placed in the group of bacteria which also includes members like Corynebacterium, Mycobacterium,

Actinomyces and Streptomyces etc. The diameter of *Actinomycetes* is much smaller (1-2µm) than the branches of fungi which ranges from 5µm-10µm. The filamentous form of A*ctinomycetes* is aerobic and may also produce spore singly or in the form of chains. The colony appears as a powdery mass and pigmented by the formation of aerial spores. Morphology of *Actinomycetes* is considered to be related to both bacteria and fungi. It is a gram-positive anaerobic bacteria, which are now recognized as prokaryotic organisms close to the bacteria and also more susceptible to antibiotics and have cell walls that contain muramic acid. They may be defined as gram-positive bacteria that form branching hyphae. It also contains high guanine and cytosine content of 55% in their DNA which is recognized as antibiotics, secondary metabolites and bioactive compounds that affect microbial growth.

MATERIALS AND METHOD

Collection of soil samples: the soil samples were collected in sterile polythene bags from Mandaveli and Ayanavaram in Chennai. Each soil samples collection was made from 6-12 inches depth of the surface of the ground using a sterile cover. The samples were placed in sterile polythene bags sealed tightly and immediately brought to the Microbiology Laboratory. The soil sample was directly transferred into polyethylene bags to minimize moisture losses during transportation. The soil samples were air dried for 2 - 3 hrs at 37℃ crushed and sieved for isolation purposes. The remaining soil samples were labelled and stored for future analysis in the laboratory.

Isolation of pure culture of Actinomycetes: for the isolation of *Actinomycetes*, the sample was air dried for 2 – 3 hrs at 37℃ in room temperature. Isolation and enumeration were done by serial dilution, spread plate and streak plate techniques. The collected sample was subjected to pretreatment by heating in oven at 100℃ for 30 minutes. One gram of soil sample was weighed and the sample was spread on the Starch Casein Agar or Starch Nitrate Agar Medium and the plates were incubated for 6-7 days at 30℃. Another method was carried out using serial dilution. One gram of soil sample was diluted in 10ml of distilled water and further serially diluted up to 10^{-6} dilution, 0.1ml of each dilution were spread on the Starch Casein Agar Medium or Starch Nitrate Agar Medium uniformly and incubated for 6-7 days at 30℃. After 7 days incubation, whitish pin-point colonies were observed. Streptomycin 40µl/ml, Chloramphenicol and Griseofulvin 50µl/ml (Himedia) was spread to prevent bacterial and fungal contamination respectively. The emerging colonies of pigmented and non-pigmented actinomycetes were subcultured and maintained for future use.

Media composition - Starch casein agar: starch Casein Agar (SCA) is used for the detection of Saccharolytic soil and marine bacteria mostly *Actinomycetes*. This medium has Starch as the complex carbohydrate source and Casein as nitrogen source. The soil samples provide complex ionic source that makes the medium suitable for microbial flora and also buffers the medium.

Characterization of *Actinomycetes* morphology characterization: the *Actinomycetes* were characterized morphologically in ISP 1 media and incubated for 6 days at 30℃. The colonies were observed under a high-power

magnifying lens and colony morphology was noted with respect to aerial, color, branching, substrate mycelium and nature of the colony.

Colony morphology or physiological characterization: Colony morphology includes color of aerial mycelium and size of the colony. Colony morphology of the *Actinomycetes* was studied under a high power magnifying lens by observing the color of the colony and nature of spores surface. Gram Staining was also performed.

Biochemical characterization: Various biochemical tests were performed to identify the *Actinomycetes spp*. The Biochemical tests include Indole test, Methyl Red test, Vogus- Proskauer test, Citrate Utilization test, Triple sugar iron test, Catalase test, Oxidase test, Gelatin hydrolysis, Urease test, Lactose, Maltose and Sucrose Utilization tests (acid production from different sugars).

Demonstration of carbohydrate fermentation test: the Carbohydrate Fermentation Broth (glucose, lactose, sucrose) was prepared and added to three different conical flasks each containing one sugar. Durham tubes were added to each test tube in an inverted position. The Durham's tube was also fully filled with broth. The tubes were labeled and sterilized in an Autoclave for 20 minutes at 121℃. Sterilized carbohydrate broth tubes were aseptically inoculated with pure culture. The tubes were incubated for 24 – 48 hours at 37℃. After 48 hours of incubation, the tubes were observed for results.

Screening of *Actinomycetes:* The screening method of isolates consists of two steps: Primary screening and Secondary screening. In Primary screening, the antibacterial activity of pure isolates was determined by cross-streak method on Muller Hinton Agar (MHA). Secondary screening of isolates was done by agar well diffusion method with crude extract of chloroform after secondary metabolite extraction.

Extraction of bioactive compounds: Production of bioactive compound was done by submerged fermentation. *Actinomycetes* isolates were taken in 150ml of ISP1 broth or Starch Casein broth or Starch Nitrate broth in a 250ml conical flask under sterile conditions and incubated at 30℃ for 7 days at 150 rpm rotation. After fermentation, the medium was filtered and was centrifuged at 10,000 rpm for 10 minutes to remove cells and debris and was harvested for fermented broth. Resultant fermented broths were added to equal volume of Chloroform solvent. Then the samples were shaken vigorously and the solvent- extraction method was processed. The solvent phase and middle phase (pellet) was collected and evaporated in a desiccator. The completely dried residues were re-dissolved in dimethyl sulfoxide (DMSO) and lyophilized for futureuse.

Determination of actibacterial activity: Agar well diffusion method was used to determine the antimicrobial activity by using crude extracts. The crude extracts containing compounds of organisms was tested for their antibacterial activity. The test organisms were inoculated in test tubes containing nutrient broths and were labeled separately. They were incubated at 37℃ overnight obtaining broth cultures. The test organisms used in antimicrobial activity were: *Escherichia coli, Bacillus subtilis, Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas spp.* and *Streptococcus spp.* The fungal organism was *Candida spp.* After overnight incubation, bacterial cultures and fungal culture were swabbed in solidified Miller Hinton Agar Plates. The Potato Dextrose Agar medium was used for Antifungal activity. The zone of inhibition around the wells was measured and recorded.

Degradation of biodegradable plastics: The ISP1 broth, Starch Casein broth and Starch Nitrate broth were prepared and the *Actinomycetes* were inoculated in the broth. One gram of biodegradable plastics were weighed and added into the broth. This broth was incubated for 2 months in the rotatory for 37°C. After 2 months of incubation, the broth was filtered and the biodegradable plastics was washed with water with no impurities and the biodegradable plastics were spread in petriplates and was air dried in room temperature for two days. The consistency, nature and weight of the biodegradable plastics were recorded.

Weight of Biodegradable Plastics = Dry weight of the plastics - Wet weight of the plastics

RESULTS AND DISCUSSIONS

Samples collections: soil samples were collected from different places. The soil samples were collected from 6-8 inches deep from the surface of the ground and it is stored in sterile plastic bags (Figures 1 and 2).

Isolation of pure culture: the isolated organism when grown on the Starch Casein Agar medium and Starch Nitrate Agar medium showed whitish pin-point colonies with powdery form media which is specific for *Actinomycetes spp.* (Figures 3 and 4).

Staining techniques: the Simple and Gram's staining of the organism showed branched and large filamentous like hyphae and thus the microorganism was confirmed as Gram positive (Figures 5 and 6).

Biochemical tests: further, Biochemical test was done and the organism was confirmed to be an *Actinomycetes spp.*(Table 1)

Extraction of bioactive compounds: extraction of bioactive compound was carried out by Submerged Fermentation method. *Actinomycetes spp* was inoculated in ISP1 Broth. After fermentation, the growth of the *Actinomycetes* was observed in the broth (Figure 7). Filtration and centrifugation was carried out to remove the cells and debris and the fermented broth was taken in a separating funnel using solvent extraction method. Equal volume of chloroform solvent was added (Figures 8 and 9). The solvent phase and middle phase (pellet) was collected and evaporated in a dessicator (Figures 10 - 12). The completely dried residues were re-dissolved in dimethyl sulfoxide & then lyophilized (Figures 13 - 15).

Antibacterial activity: the antibacterial effect of the crude extract was evaluated against the organisms by agar well diffusion method. The solvent phase and middle phase crude extract showed zone of inhibition against *Bacillus subtilis*, *Escherichia coli, Klebsiella pneumoniae* and *Streptococcus spp.* The zone of inhibition was recorded at the concentration of 100µg/ml. The solvent phase (U1) crude extract had high zone of inhibition when compared to the middle phase (U2) (Table 2).

Degradation of biodegradable plastics: ISP 1 Broth was prepared and the *Actinomycetes spp* were inoculated. Biodegradable plastic cover was cut into small pieces and 0.6 gram of it was added into the broth.

Dry weight of the biodegradable plastic cover = 0.6 g

Wet weight of the biodegradable plastic cover = 0.4 g

Weight of biodegradable Plastics = Dry weight of the plastics – Wet weight of the plastics

= 0.6 - 0.4 = 0.2

The objective of the present study was to screen the antibacterial activity and degrade the biodegradable plastics cover. Biodegradable plastic cover, which has a wide range of application in day to day life is accumulated in the environment. Its inert properties to deteriorate and degrade are causing serious environmental problems. This biodegradable study reveals *Actinomycetes spp.* to be highly capable of disintegrating or degrading the biodegradable plastic covers.

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