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# RESEARCH ARTICLE

# ISOLATION, SCREENING AND IDENTIFICATION OF CELLULOLYTIC MARINE *ACTINOBACTERIA* FROM THE MANGROVE SEDIMENT OF BURMANALLAH, THE ANDAMAN

**Nandhini, C., *Ganesh, P., Sivagurunathan, P. and Sivakumar, K.**

Department of Microbiology, Faculty of science, Annamalai University, Annamalainagar, 608002, Tamil Nadu India

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## ABSTRACT

The actinobacterial colonies isolated from the mangrove sediment samples of the station of the Burmanallah using Kuster's agar medium. A total of ten morphologically distinct actinobacterial strains were selected. These strains were designated as AUBN-1, AUBN-2, AUBN-3, AUBN-4, AUBN-5, AUBN-6, AUBN-7, AUBN-8, AUBN-9 and AUBN-10. Morphologically distinct actinobacterial strains AUBN-1 to AUBN-10 were subjected to preliminary screening for cellulase enzymes activity. Screening for cellulase enzyme production was made using the Carboxy methyl cellulose agar medium. Higher cellulase enzyme production was found in the strain AUBN-9 with 18 mm of clear hydrolytic zone. Based on the performance of enzyme production, the potential strain AUBN-9 was selected for further conventional identification. The cultural and morphological characters were analyzed to identify the cellulase positive strain AUBN-9 and compared with the Streptomyces species given in the key of Nonomura 1974 and those described in the Bergy's Manual of Determinative Bacteriology. Based on the results, the cellulase positive strain AUBN-9 was tentatively identified as Streptomyces roseoluteus. The present investigation concludes that the mangrove sediment samples of the Burmanallah, the Andamans contain a good diversity of culturableactinobacterial strains of *Streptomyces*. These strains are able to produce cellulase enzymes. These strains can be further evaluated for the commercial scale production of enzymes and possess vast potential in varied biotechnological and industrial applications.

***Key words:*** *Streptomyces roseoluteus, Actinobacteria, Marine Actinobacteria*



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## INTRODUCTION

An ecosystem is a biological environment consisting of all the organisms living in a particular area, as well as all the nonliving, physical components of the environment with which the organisms interact, such as air, soil and water. Especially, marine ecosystems are found as the majority portion of the surface of the earth especially the deep sea sediments contain more than 1 billion cells/cm$^3$(Fenical*et al.,*2006). Oceans are the home to huge microbial populations and diversity (Stach*et al.,* 2005) and they live in every corner of the ocean and their habitats are diverse; they are distributed in open waters, sediments, associated with many organisms, estuaries, hydrothermal vents (Cevera*et al.,* 2005). They are always involved in the important processes of the sea in promoting organic material transformation and mineralization in the sediments and overlying waters (Das *et al.,* 2007).

***Corresponding author:* Ganesh, P.**
Department of Microbiology, Faculty of science, Annamalai University, Annamalainagar, 608002,
Tamil Nadu India.

Microbial communities are structured by temporal and spatial variability of physicochemical and biotic parameters (Hewson*et al.,* 2007).

### Actinobacteria

Actinobacteria (actinomycetes) are one of the largest taxonomic units within the Bacterial domain (Niva*et al.,* 2006; Jiang *et al.,* 2012). They are versatile aerobic gram-positive bacteria with a higher amount of guanine plus cytosine (>50mol% G+C) in their DNA (deoxyribo-nucleic acid). They possess a wide range of morphologically and physiologically diverse properties. They have been isolated from a wide variety of environmental sources, where they act as saprophytes, symbionts, chemo-organotrophs, parasites or even pathogens (Niva*et al.,* 2006; Trujillo, 2008). Their morphology ranges from coccoid (e.g. *Micrococcus*), rod-coccoid (e.g. *Arthrobacter*), fragmentinghyphal forms (e.g. *Nocardia*) to those with a highly differentiated branched mycelium (e.g. *Streptomyces*). In the case of filamentous actinobacteria, hyphae that branch repeatedly become attached on the surface

of the agar to form tough, leathery and velvety colonies, which highly resemble fungi (Trujillo, 2008)

**Marine Actinobacteria**

Marine environment represents a largely less tapped source for isolation of new microorganisms including actinobacteria (Bredholt*et al.,* 2008).. The first actinobacterium isolated from the oceanic sediments was not considered as a marine form. Scientists believed that it came from the spores of terrestrial bacteria that had simply blown into the oceans and remained dormant. But, further investigations showed that many actinobacteria isolated from the ocean sediments were true marine forms (Mincer *et al.,* 2002).Actinobacteria are ubiquitous in nature (Sethubathi*et al.,* 2013) and play important ecological roles and substantially impact the cycling of complex carbon substrates in the benthic and other ocean habitats (Mincer *et al.,* 2002). Given that actinobacteria living in the oceans experience a dramatically different set of environmental challenges compared to their terrestrial relatives, it is not surprising that speciation has occurred and unique marine taxa are now being recognized . Not only the full extent of marine actinobacterial diversity is yet to be determined, but also their adaptations to the environmental parameters in the sea are to be understood (Sethubathi*et al.,* 2013).

**Enzyme from marine actinobacteria**

The biological and chemical diversity of the marine environment has been the source of unique chemical compounds with the potential for industrial development as pharmaceutical, cosmetics, nutritional supplements, molecular probes, enzymes, fine chemicals and agrochemicals (Ireland *et al.,* 1993). Especially, marine actinobacteria are efficient producers of innovative secondary metabolites that show a range of biological activities including antibacterial, antifungal, anticancer, antioxidant and insecticidal substances as well as enzyme inhibitors and enzymes. Actinobacteria are capable of catalyzing various biochemical reactions with novel enzymes such as cellulase, dioxyribonuclease, lipase and protease (Sivakumar*et al.,* 2007), because they are metabolically active in the marine environment, producing various compounds that are not observed in terrestrial stains (Jensen *et al.,* 1991). Among them *Streptomyces* exhibit remarkable capacity for synthesis of secondary metabolites and use of numerous extra cellular hydrolytic enzymes to degrade organic material in their natural habitat (Morosoli*et al.,* 1997).Enzymes make ideal catalyst in food industries owing to their specificity, mild reaction condition and non-toxicity. Therefore, these have attracted the attention of the researcher all over the world due to their wide range of physiological, analytical and industrial application, because of their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation (Nalinisingh, 2012). Enzymes from marine microbes have unique protein molecule when compared with terrestrial microbes. Properties like high salt tolerance, thermostability and barophilisity of marine microbes made the scientist to consider these kinds of enzymes in large scale for commercial purpose (Sivakumar*et al.,* 2007).

**Cellulases**

Cellulose constitutes the major form of stocking glucose, obtained through photosynthesis and at the same time, it is the major component of solar energy conversion to biomass. It is also an important constituent of all the plant materials and that is why it is the most abundant organic material in nature, which is renewed every year (Devi *et al*, 2012). Cellulose has been used by man for centuries; however, its enormous potential as a renewable source of energy was recognized only after cellulose degrading enzymes or "cellulases" were identified (Bhat*et al*, 1997). Cellulases are the inducible bioactive compounds produced by microorganisms during their growth on cellulosic materials (Lee *et al*, 2001). Due to the increasing knowledge on the mode of action of cellulases, they have been used in enzymatic hydrolysis of cellulosic substances (Kubicek*et al.,* 1993). Cellulase has a wide range of applications in food, animal feed, textile, fuel and chemical industries, other areas include paper and pulp industry, waste management and medical treatment (Murugan*et al.,* 2007).

## MATERIALSAND METHODS

**sample Collection**

Sediment samples were collected from Burmanallah, South Andaman using a sterile spatula at a depth of 25 cm. The samples were placed in sterile polythene covers and brought to the field laboratory immediately and after arrival, necessary dilutions were made to carry out further microbiological analysis.

**Isolation of actinobacteria**

Isolation of actinobacteria was carried out in Kuster's agar medium (Appendix). The autoclaved Kuster's agar medium containing petriplates were prepared aseptically. To minimize the fungal and bacterial contamination, Kuster's agar medium was supplemented with cycloheximide(10 µg/ml) and nalidixic acid (10 µg/ml) respectively (Kathiresan*et al.,* 2005).One gram of pretreated sediment samples were serially diluted using sterile seawater and 0.1 ml of serially diluted samples were added to the petriplates containing Kuster's agar medium (Kuster*et al.*, 1964) and spreadusing a 'L' shaped glass spreader. The plateswere incubated at 37 °C for seven days in an inverted position. After the incubation period, morphologically distinct colonies were picked up from the petri dishes and re-streaked in appropriate media and pure cultures obtained and were maintained at 4°C for further studies. For screening of cellulase, Carboxy methyl cellulose agar medium was autoclaved and dispensed into petridishes and allowed to solidify. A loopful of culture was streaked on the medium and incubated at 37 °C for 5-7 days. After the growth for 5-7 days, the plates were flooded with iodine chemical solution (0.1 ml HCl + 5 ml of 1% iodine in 2% KI). Formation of clear zone around the colony against reddish-brown background indicated the cellulolytic activity of the strain (Radhakrishnan*et al.,* 2007). Ratio of the clear zone diameter to colony diameter was measured in order to select the highest cellulase activity producing strains.

**Identification of potentialactinobacteria**

Characterization and identification of the potential cellulase positive strain at genus level were made based on the criteria of (Cummins *et al.,*1956, Shirling*et al.,*1966), Lechevalier*et al* ., 1970) and (Nonomura.,1974).

**Conventional identification**

### Aerial mass colour

Colour of the mature sporulating aerial mycelium was recorded in a simple way (white, grey, black, red, blue and violet). When the aerial mass colour fell between two colour series, both the colours were recorded. If the aerial mass colour of a strain to be studied showed intermediate tints, then also, both the colour series were noted. The media used were Yeast Extract-Malt Extract Agar (ISP-2) and Inorganic-Salt Starch Agar (ISP-4) (Shirling*et al.,* 1966).

### Melanoid pigments

The grouping was made on the production of melanoid pigments (*i.e.* greenish brown, brownish black or distinct brown, pigment modified by other colours) on the medium. This test was carried out on the ISP-7 medium as recommended by International *Streptomyces* Project (Shirling*et al.,* 1966).

### Reverse side pigments

Strains were divided into two groups, according to their ability to produce characteristic pigments on the reverse side of the colony, namely, distinctive (+) and not distinctive or none (-). In case, a colour with low chroma as pale yellow, olive or yellowish brown occurred, it was included in the latter group (-). This test was carried out in the medium ISP7 as recommended by International Streptomyces Project (Shirling*et al.,* 1966).

### Soluble pigments

Strains were examined for their ability to produce soluble pigments other than melanin: namely, produced (+) and not produced (-). The colour was recorded (red, orange, yellow, blue, green and violet). This test was carried out on the media ISP-1 and ISP-7 as recommended by International *Streptomyces* Project (Shirling*et al.,* 1966).

### Spore chain morphology

Characteristics of the spore bearing hyphae and spore chains were determined using direct microscopic examination of the culture surface. Adequate magnification (400X) was used to establish the presence or absence of spore chains and to observe the nature of sporophores*viz*. rectiflexibiles (RF) and spirales (S).Spore morphological characters of the strains was studied by inoculating a loopful of one week old cultures into 1.5% agar medium contained in test tubes, at 37 °C. The actinobacteria was suspended and thoroughly mixed in the semisolid agar medium and 1 or 2 drops of the medium were aseptically pipetted on to a sterile glass slide. A drop of agar was spread well on the slide and allowed to solidify into a thin film so as to facilitate direct observation under the microscope. The cultures were incubated at 28±2 °C and examined periodically for the formation of aerial mycelium, sporophore structure and spore morphology.

## RESULTS AND DISCUSSION

### Isolation of actinobacteria

A total of 10 morphologically distinct actinobacterial strains were selected from the Kuster'sagar medium. These strains were labeled as AUBN-1, AUBN-2, AUBN-3, AUBN-4, AUBN-5, AUBN-6, AUBN-7, AUBN-8, AUBN-9 and AUBN-10 (Fig. 1 and Fig. 2). Kuster's agar supports the isolation of various types of actinobacteria especially from the mangrove sediments (Sivakumar *et al*.,2001). Similarly, Sahu*et al.* (2005) isolated maximum number of actinobacteria using this medium from Vellar estuary. (Raghavendrudu*et al*.,2007) studied that the distribution of actinobacteria in the Gaderu mangroves of Gautami Godavari estuarine system, east coast of India. They used five different agar media for isolation, among them, Kuster's agar was found to be more suitable for the isolation of the genus *Streptomyces*, which was observed very frequently on this medium. While, (Baskaran*et al.,* 2011) also reported that Kuster's agar was found to be the well supporting medium to marine actinobacterial population. Recently, (Sethubathi*et al.,* 2013) reported that Kuster's agar medium frequently yielded higher counts of actinobacterial colonies and (Mohseni*et al.*2013) isolated 44 actinobacterial strains from the sediments of the Caspian Sea using Kuster's agar also one of the medium.

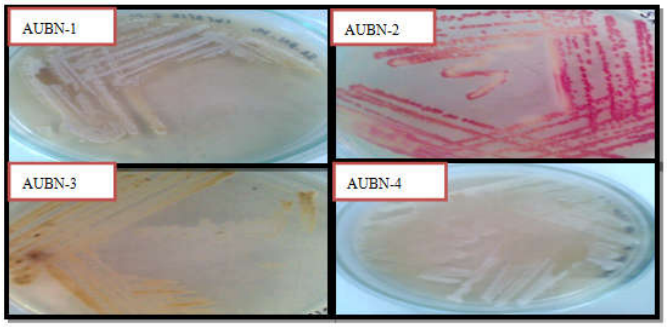


**Fig. 1. Agar plates shows the morphologically distinct actinobacterial strains**

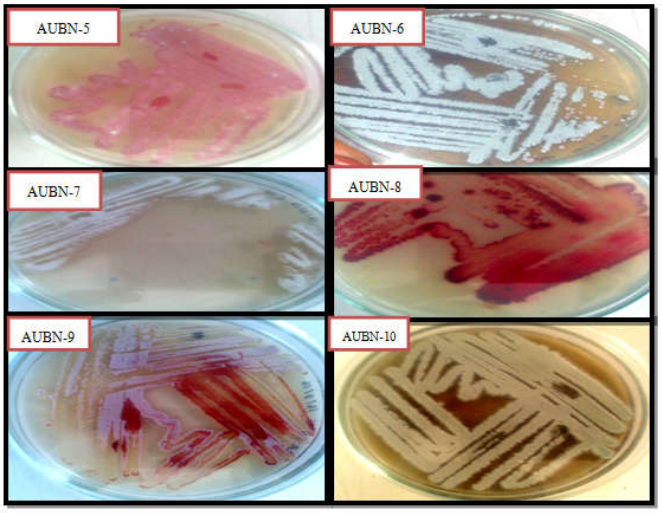


**Fig. 2. Agar plates shows the morphologically distinct Actinobacterial strains Cellulase enzyme screening**

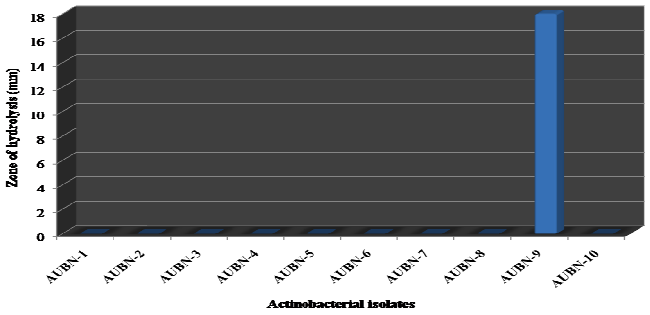


**Fig. 3. Cellulase enzyme activity in Carboxy methyl cellulose agar medium**

Screening for cellulase enzyme production was done for the ten isolated actinobacterial strains, using the Carboxy methyl cellulose agar medium. Higher cellulase enzyme production was found only in one strain AUBN-9 (Fig. 3) with 18 mm of clear zone (Actinobacteria, one of the known cellulase produces, has attracted considerable research interest due to the potential application (Jang *et al.,* 2003; Arunachalam*et al.,* 2010). *Streptomyces* are the largest and well studied group of actinobacteria. A wide variety of bacteria are known for their production of hydrolytic enzymes with *Streptomyces* being the best (Chellapandi*et al* ., 2008). Considering these, present study examines cellulase activity screening in ten actinobacterial isolates and among them, the strain AUBN-9 exhibited higher cellulase activity and identified as *Streptomyces* genus. (Murugan*et al.,*2007) isolated 35 actinobacterial strains from Vellar estuary, India and examined cellulase production. Among them, Starin CL-30 (*S. actuosus*) showed maximum cellulase activity. (Sirisha*et al.,*2013) reported bioactive compounds from marine actinobacteria isolated from the sediments of Bay of Bengal, and 24 % strains exhibited cellulase activity. (Meena*et al.*2013) reported marine sediments actinobacteria from Andaman and Nicobar islans and isolated 26 actinobacteria strains, among them two *Streptomyces* species (NIOT-VKKMA02 and NIOT-VKKMA26) have showed excellent activity of cellulase. Recently, Gobalakrishnan (2013) reported cellulase produced actinobacterial stains from Havelock island, the Andamans


**Fig. 4. Cellulase screening results in Carboxy methyl cellulose agar medium [A-Positive strain (AUBN-9), B-Negative strain (AUBN-10)]**

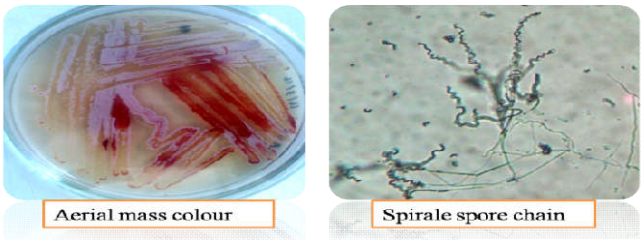


**Fig. 5. Morphological characteristics of the cellulase positive strain AUBN-9**

## Identification of cellulose positive strain

Strain AUBN-9 is a mesophilicactinobacterium, which forms an extensively branched substrate mycelium and grayish pink coloured aerial mycelium that differentiate into short, spiral spore chains (Fig. 5). Pink colour reverse side pigment and brown colour soluble pigments were produced on Peptone yeast extract iron agar. Melanin pigment was also absent on ISP7 agar .Results of the cultural and morphological characters were compared between the strain AUBN-9 and its closest match i.e., *Streptomycesroseoluteus* The strain AUBN-9 showed variation in only one character when compared to those of the reference species *S. roseoluteus* i.e. aerial mass colour. Except these, all the other characters were similar to those of *S. roseoluteus* (Table 1). Hence, the strain AUANI-1was tentatively identified as a species close to *S. roseoluteus*.

**Table 1. General characteristics of the strain AUBN-9 and the closed related Streptomyces species**

| Characters | AUBN-9 | *S. roseoluteus* |
|---|---|---|
| Aerial mass colour | Greyish pink | Red |
| Melanoid pigment | - | - |
| Reverse side pigment | + | + |
| Soluble pigment | + | + |
| Spore chain | Spiral | Spiral |

## Summary and conclusion

Present study was on the "Isolation, screening and identification of cellulolytic marine actinobacteria from the mangrove sediments of Burmanallah, the Andaman".

- The actinobacterial colonies were isolated from the mangrove sediment samples of the station of the Burmanallah using Kuster's agar medium.
- A total of ten morphologically distinct actinobacterial strains were selected. These strains were designated as AUBN-1, AUBN-2, AUBN-3, AUBN-4, AUBN-5, AUBN-6, AUBN-7, AUBN-8, AUBN-9 and AUBN-10.
- Morphologically distinct actinobacterial strains AUBN-1 to AUBN-10 were subjected to preliminary screening for cellulase enzymes activity.
- Screening for cellulase enzyme production was made using the Carboxy methyl cellulose agar medium. Higher cellulase enzyme production was found in the strain AUBN-9 with 18 mm of clear hydrolytic zone.
- Based on the performance of enzyme production, the potential strain AUBN-9 was selected for further conventional identification.
- The cultural and morphological characters were analyzed to identify the cellulase positive strain AUBN-9 and compared with the *Streptomyces* species given in the key of Nonomura (1974) and those described in the Bergy's Manual of Determinative Bacteriology.
- Based on the results, the cellulase positive strain AUBN-9 was tentatively identified as *Streptomyces roseoluteus*.
- The present investigation concludes that the mangrove sediment samples of the Burmanallah, the Andamans contain a good diversity of culturableactinobacterial strains of *Streptomyces*. These strains are able to produce cellulase enzymes. These strains can be further evaluated for the commercial scale production of enzymes and possess vast potential in varied biotechnological and industrial applications.

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