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Isolation of two thermophilic actinobacterial strains mud volcano of the Baratang Island, India

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Studies on actinobacteria of marine habitats are gaining international importance due to proven abilities of novel compound production. Information on actinobacteria of the marine environments is less and hence the present studies were optimum at higher temperature, which indicates that they are thermophilic in nature. It can be conducted that the two strains (TA-1 and TA-2) belong to two different genera *viz. Rhodococcus* (TA-1) and *Streptosporangium* (TA-2). Actinobacterial strains were isolated from the sediment samples collected from the mud volcano, of the Baratang Island andaman and Nicobar group of islands. Out of a total of six isolates, two different actinobacterial strains (TA-1 and TA-2) were selected based on the morphological distinct colour of spore mass, riverside colour, arial and mycelia format production of diffusible pigment sporophore and colony morphology. Morphological studies indicated that the strains belonged to two different genera *viz*. *Rhodococcus* (TA-1) and *Streptosporangium* (TA-2). The strain had no aerial mycelium and analysis of the strain TA-1 showed fragmented rods, filaments and hyphae, and the strain TA-2 showed that the formed globosa sporangia on the surface of the colony liberated non-motile spores.

Key words: Thermophilic actinobacteria, isolation and identification, Baratang Island.

INTRODUCTION

Many free living microorganisms including actinobacteria have a cosmopolitan distribution and have been detected in distant habitats (Cooper et al., 2001). Of these, thermophiles represent unique and important genetic resources as their macromolecules are stable at higher temperature (Brock, 1985). Although identification of thermophilic actinobacteria continues to pose challenges, branching patterns of actinobacteria help define species (Madigan, 2006). In some species, the terminal cells in a chain turn into spores and become specialized for airborne dispersal. Heat resistance of these spores varies depending on the species (Fergus, 1967). The *thermoactinomyces vulgaris* has spores that can withstand 100ºC for 4 h. It was already shown that the thermophilic actinomycetes can produce amylases, xylanases and cellulose digesting enzymes which retain their activity at high temperatures (50-65ºC) (Kuo and Hartman, 1966; Loginova et al., 1978; Stutzenberger, 1987).

The number of thermotolerant actinomycetes in strongly heated soils of deserts and volcanic regions is comparable to or exceeds the number of mesophilic actinomycetes (Zenova et al., 2009) and their habitats and methods for isolation and recovery of thermophilic actinomycetes, such as Streptomyces, Thermomonospora and Thermoactinomyces (Edwards, 1993). Thermophilic actinomycetes were isolated from 163 (48.95%) of 333 samples of vegetable substrates and soil from different sites in Anambra and Enugu States in Nigeria (Unaogu

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et al., 1994). The thermophilic actinomycete strain 21E isolated from saline Bulgarian soils produced a highly thermostable collagenase. Macro- and micromorphological characteristics of the strain were tested on 14 media. It was concluded that the strain 21E was a typical member of the genus *Thermoactinomyces* Petrova and Vlahov (2007).

Thermophilic forms are primarily aerobic and have an optimum growth temperature range between 40 and 80°C (Tortora et al., 2007). However, a range of actinobacteria likes that of *Streptomyces thermofuscus* and *Streptomyces thermophilus* have been reported to grow at 65ºC (Waksman et al., 1939). These thermophilic actinobacteria are effective decomposers, breaking down organic matter. In India, there are only very limited studies on the thermophilic actinobacteria. Considering this, the present study on isolation and identification of thermophilic actinobacteria from the mud volcanic sediment samples of the Baratang Island, Andaman and Nicobar group of islands, was undertaken during 2007.

MATERIALS AND METHODS

Sediment samples were collected from the molten lava of the mud volcano of Baratang Island, Andaman and Nicobar group of islands. For actinobacterial analysis, pre-cleaned materials were used for collection of samples and were stored in sterilized polythene bags.

Actinobacteria were isolated from the mud volcano sediment samples adopting the spread plate technique using ISP-2 medium for isolation and enumeration of actinobacteria Sivakumar *et al,* (2005) after suitable serial dilutions. Serially diluted sediment samples were inoculated on ISP-2 medium and incubated at 55 °C for 7 days. All colonies with tough, leathery nature were counted as actinobacteria and expressed as colony forming units per gram (CFU/g). Morphologically different colonies were isolated by streaking on nutrient agar for obtaining pure cultures.

Taxonomic investigation

Growth temperature optimum

The optimal growth temperature was determined with ISP- 2 medium in the range from 25 to 75°C.

Generic level identification

Hydrolysis

Hydrolysis was made for releasing amino acids. Harvested cells of each strain weighing 20 mg were placed in an ampo bottle and 1 ml of 6N HCl was added and sealed with alcohol blast burner. The samples were kept at 121°C for 20 h in a sand bath. The bottles were cooled by keeping them at a room temperature of 28±2°C.

Thin layer chromotography

Spotting of the whole cell hydrolysates was made carefully on TLC plates using a microlitre pipette. Spots were of 5-10 mm in diameter. This was done by multiple applications on the same spot of very small portions of the sample, which were dried by hand drier (Lechevalier and Lechevalier, 1970).

Amino acids and whole-cell sugars

Each sample (3) was applied on the base lines of cellulose TLC (20 cm x 20 cm); can authentic material mixture of Diaminopimelic acid (DAP) isomes) and 1 µl of amino acetic acid (glycine) and also 3 µl sugar (Galactose, Arabinose, Xylose, Mannose) solutions were spotted as standards. TLC plate was developed with the solvent system containing methanol: pyridine: glacial acetic acid: H_2O (5:0.5:0.125:2.5v/v) and ethyl acetate: pyridine: acetic acid: distilled water (8:5:1:1.5 v/v). It took approximately more than 4 h for development. The spots were visualized by spraying with 0.4% Ninhydrin solution in water-saturated n-butanol, followed by healing at 100°C for 5 min and aniline thalate reagent (2.5 g of pthalic acid dissolved in 2 ml of aniline and made up to 100 ml with water saturated n-butanol). The spot of amino acetic acid ran faster than DAP. The sample spots were immediately compared with the spots of the standards since spots gradually disappeared in few hours and the sprayed plate was heated at 100°C for 4 min. Hexoses appeared as yellowish brown spots and pentoses, as maroon coloured spots (Lechevalier et al., 1966).

Species level identification

Aerial mass colour

The colour of the mature sporulating aerial mycelium was recorded in a simple way (White, grey, 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 and Inorganic-Salt Starch Agar (Shirling and Gottlieb, 1996).

Melanoid pigments

The grouping was made on the production of melanoid pigments (greenish brown, brownish black or distinct brown, pigment modified by other colours) on the medium. The strains were grouped as melanoid pigments were delayed or weak, and therefore, it was not distinguishable. This is incubated as variable (V). This test was carried out on the media ISP-1 and ISP-7, as recommended by International Streptomyces Project (Shirling and Gottlieb, 1996).

Reverse side pigments

The 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 occurs, it was included in the latter groups (-) (Shirling and Gottlieb, 1996).

Soluble pigments

The strains were divided into two groups by 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) (Shirling and Gottlieb, 1996).

Spore chain morphology

The species belonging to the genus *Streptomyces* were divided into

Table 1. Cell wall amino acids and whole cell sugars of the two strains.

Strain Number	DAP		Glycine	Whole cell sugars		Wall type
	LL-DAP	Meso-DAP		Arabinose	Galactose	
TA-1	-	+	-	-		IV
TA-2	-	+	-	-	-	III

+ denotes presence; - denotes absence.

three section (Shirling and Gottlieb, 1966), namely rectiflexibiles (RF), rectinaculiaperti (RA) and spiral (S). A drop of agar was spread well on the slide and allowed to solidify into a thin film as to facilitate direct observation under microscope. The cultures were incubated at 28 ± 2°C and examined periodically for the formation of aerial mycelium, sporophore structure and spore morphology (Shirling and Gottlieb, 1996).

Assimilation of carbon source

The ability of different actinobacterial strains in utilizing various carbon compounds as source of energy was studied following the method recommended by International Streptomyces Project (ISP) (Shirling and Gottlieb, 1966). Carbon sources for this test were arabinose, xylose, inositol, mannitol, fructose, rhamnose, sucrose and raffinose. These carbon sources were sterilized by ether sterilization without heating. For each of the carbon sources, utilization is expressed as positive (+), negative (-), or doubtful (±). In the 'doubtful ' strains, only a trace of growth slightly greater than that of the control was noticed (Shirling and Gottlieb, 1996).

RESULTS AND DISCUSSION

Population density of thermophilic actinobacteria

During the present investigation, the actinobacteria were enumerated from the sediments collected from the mud volcano samples whose population density was $2.7x10^3$ CFU/g.

Taxonomic investigation

Two different actinobacterial strains (TA-1 and TA-2) were selected based on colony morphology and detailed taxonomic investigation was carried out. Results of the analysis of cell wall components of the two strains are given in Table 1.

The strain, TA-1 possesses meso -Diaminopimelic acid (DAP) along with arabinose and galactose sugar patterns. Presence of meso-DAP, arabinose and galactose sugar patterns indicates the cell wall chemo type - IV. The strains belonging to the wall type - IV are *Rhodococcus, Microbacterium, Nocardia* and *Actinopolyspora* (Lechevalier and Lechevalier, 1970). Similarly, TA-2 possesses meso-DAP (Diaminopimelic acid) and does not contain any glycine. It had no characteristic sugar pattern. Presence of meso-DAP without any sugar pattern indicates the cell wall type - III. The strains belonging to the wall type - III are *Actinomadura, Streptosporangium, Spirillospora, Dermatophilus, Thermoactinomyces, Microbispora* and *Nocardia* Lechevalier and Lechevalier (1970).

Micro morphological character studies

The strain had no aerial mycelium. Compound microscope (400X) analysis of the strain TA-1 showed fragmented rods, filaments and hyphae. Scanning electron microscopy (15,000X) revealed that the rods are non-motile (Figure 1). The rods were non-motile and road's varied in size and shape within a single sporophore. All rods showed a smooth surface and the rods diameter varied from 0.8 to 1.2 µm and length varied from 1.3 to 2.4 µm. A gap or small plug separated some rods. These charac-ters are the typical morphological features of the genus *Rhodococcus* (Cross and Goodfellow, 1973). Based on the cell wall type and micromorphological analyses, it was confirmed that the strain TA-1 belongs to the genus Rhodococcus.

The strain had aerial mycelium. Light microscope (400X) showed that the strain TA-2 formed globosa sporangia on the surface of the colony. The fine structure of the spore was studied using light microscope (400X) and scanning electron microscopy (5,000X). It showed that the strain TA-2 formed globosa sporangia and these sporangia liberated non-motile spores (Figure 2). The spores were square in shape and non-motile. The spores contained spines in the four corners and formed short chains with not more than 10 spores in each. Spores varied in size and shape within a single sporophore. All the spores showed a smooth surface and the spore diameter varied from 0.5 to 0.9 µm and length varied from 1.1 to 1.8 µm. A gap or small plug separated some spores (Figure 2). These characters are the typical morphological features of the genus *Streptosporangium* (Couch, 1955). Based on the cell wall type and micromorphological analyses, it was confirmed that the strain TA-2 belongs to the genus *Streptosporangium*. Few workers only reported the established segmentation of both the substrate and aerial mycelia, the formation of spores with the characteristics of bacterial endospores, and the biochemical properties determined strain 21E as a representative of the genus *Thermoactinomyces* (Petrova and Vlahov, 2007).

Detailed cultural, morphological and biochemical properties of the two strains, TA-1 and TA-2 were studied and they are presented in Tables 2 to 4. The strain TA-1

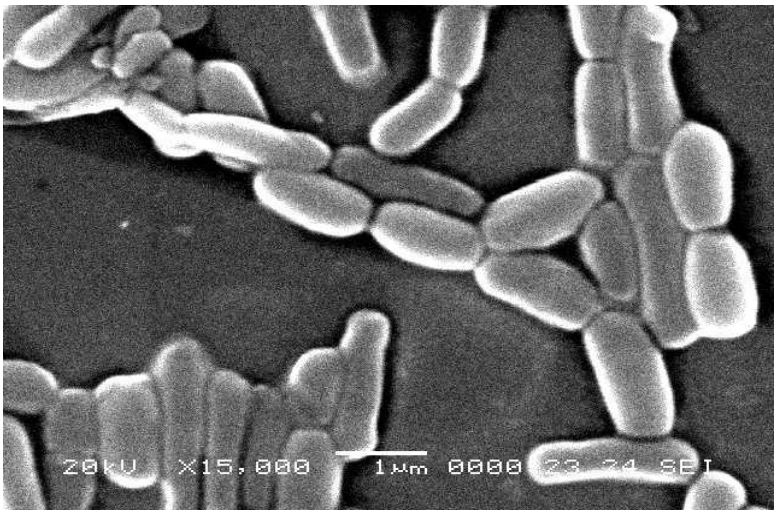

Figure 1. Scanning Electron Micrograph Non-motile rods formed by strain TA-1. (15,000X)

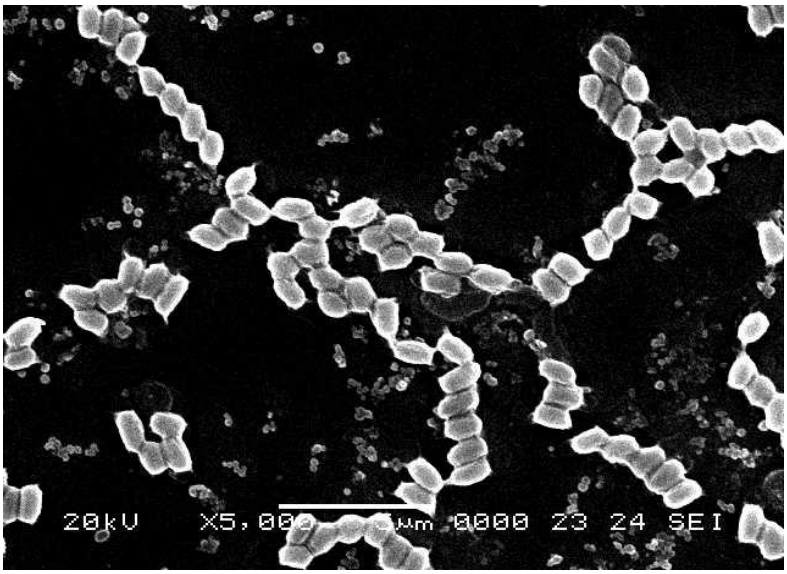

Figure 2. Scanning Electron Micrograph Non-motile spherical spores formed by the strain TA-2. (5,000X).

showed good growth on Yeast extract-malt extract agar medium after 5 days of incubation at 55°C temperatures and it showed poor growth on Starch casein agar and Glucose asparagines agarmedia. Strain TA-1 did not produced aerial mycelium but produced substrate mycelium. It did not produce any pigments such as melanoid, reverse side and soluble pigments. The spore mass of the strain TA-1 was found to be white in colour and apparent, particularly in Yeast extract-malt extract agar at 55°C temperature and 5 days incubation period. Substrate mycelia were well developed, branched and mostly unfragmented.

Cultural character studies

Like the strain TA-1, the strain TA-2 showed good growth on Yeast extract-malt extract agar medium after 5 days of incubation at 55°C and it showed poor growth on Starch casein agar and Glucose asparagines agar media. Strain TA-2 produced aerial mycelium as well as substrate mycelium. It did not produce any pigments such as melanoid, reverse side and soluble pigments. The spore mass of the strain TA-1 was found to be gray in colour and apparent, particularly in Yeast extract-malt extract agar at 55°C and 5 days of incubation period. The aerial mycelium

Table 2. Cultural and morphological characteristics of the strains TA-1 and TA-2.

Cultural and morphological characteristics	Strain TA-1	Strain TA-2
Yeast extract-malt extract agar medium	Good growth	Good growth
Starch Casein agar	Poor growth	Poor growth
Glucose asparagines agar	Poor growth	Poor growth
Aerial mycelium	Absent	Present
Substrate mycelium	Present	Present
Sporangia	Absent	present
Spore mass	White	Gray
Spore	Non-motile	Non-motile
Melanoid pigment	-	-
Reverse side pigment	-	-
Soluble pigment	-	-

Table 3. Biochemical characteristics of the strains TA-1 and TA-2.

Biochemical characters	Strain TA-1	Strain TA-2
Cellulose degradation	+	+
Hydrogen sulphide production	-	-
Nitrate reduction	+	-
Utilization of sole carbon sources		
Arabinose	+	+
Xylose	+	+
Inositol	+	+
Manitol	+	+
Fructose	+	+
Rhamnose	+	+
Sucrose	+	+
Raffinose	+	+

and substrate mycelia were well developed, branched and mostly unfragmented.

Physiological and biochemical properties

The strains TA-1 and TA-2 degraded the cellulose but they failed to produce hydrogen sulphide. The strain TA-1 reduced nitrate but strain TA-2 did not reduce nitrate. Both the strains utilized all the carbon sources *viz.* arabinose, xylose, inositol, manitol, frutose, rhamnose, sucrose and raffinose tested. The strains TA-1 and TA-2 showed good growth at 55°C and pH 7 and hence, this temperature and pH can be considered as the optimum range for these strains (Table 4). Sasagava et al. (1993) reported that the carbon metabolism is significant in taxonomy and the differences in the utilization of various carbon sources serve as additional criteria for species differentiation. International Streptomyces project (ISP) considers the utilization of nine sugars (Petrova and Vlahov, 2007).

The strain TA-1 and TA-2 showed good growth on Yeast extract -malt extract agar medium at temperature 55°C and pH 7. Below and above this temperature and pH, the strains showed poor growth. Hence, temperature 55°C and pH 7 can be detailed as, suitable for the optimum growth of the strains (TA-1 and TA-2) and these strains can be designated as thermophilic actinobacteria.

From the present study, it can be conducted that the two strains (TA-1 and TA-2) belong to two different genera *viz. Rhodococcus* (TA-1) and *Streptosporangium* (TA-2) whose growth was optimum at higher temperature which indicates that they are thermophilic in nature. There are few works on thermophilic actinobacteria from Aarthi (2007) who isolated actinobacterial strains from the mud volcanic samples and identified there as *Rhodococcus* like organisms which required higher temperature (50-60°C) and also reported the temperature range between 35 and 65°C, and did not grow at 33 and 67°C. The optimal growth temperature was from 55 to 60°C. Good growth occurred in the presence of 3-7% and it was weak at 10% NaCl (Petrova and Vlahov, 2007).

Further studies are needed to identify these two strains (TA-1 and TA-2) up to the species level through the conventional as well as molecular methods. Studies are also required to test the potential of the two strains (*Rhodococcus*

Table 4. Physiological characteristics of the strains TA-1 and TA-2.

Physiological characteristic	Strain-TA-1	Strain-TA2
Temperature range (°C)		
35	Poor growth	Poor growth
40	Poor growth	Poor growth
45	Moderate growth	Moderate growth
50	Good growth	Good growth
55	Good growth	Good growth
60	Good growth	Good growth
65	Moderate growth	Moderate growth
pH range		
5	No growth	No growth
6	Poor growth	Poor growth
7	Good growth	Good growth
8	Moderate growth	Moderate growth
9	Poor growth	Poor growth
10	No growth	No growth

and *Streptosporangium*) for the production of enzymes which could be stable at higher temperatures, as *Rhodococcus* and *Streptosporangium* were isolated from the mud volcanic samples where the minimum temperature was 50°C. If thermostable enzymes could be derived from these two strains, then they may be explored for this potential application in industries using enzymes at higher temperatures.

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