



Systematic characterization of potential cellulolytic marine actinobacteria *Actinoalloteichus* sp. MHA15



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ABSTRACT

Cellulose is the most abounding biopolymer in the world and there is a great interest in using this material as a substrate for various applications and it is the most important renewable resource for bioconversion. Therefore, it is necessary to screen the cellulolytic bioorganisms. In this context, actinobacteria are one of the most efficient prokaryotes, economically and biotechnologically, for their production of about half of the discovered bioactive secondary metabolites and they can metabolize many different compounds. Therefore, the present study was carried out to isolate and screen cellulase enzyme producing marine actinobacterial strains from the sediments of the Havelock island, the Andamans. Totally, 19 morphologically distinct actinobacterial strains were isolated and subjected to cellulose degradation assay. Out of the 19, four strains were found to possess good cellulose degradation activity and the strain MHA15 alone produced higher amount of cellulase enzyme (14.379 IU/ml) than the others. Taxonomical study of the strain MHA15 revealed that it belongs to the genus *Actinoalloteichus* and the molecular characters showed distinct difference in its phylogenetic relationship (8.4%) with *A. cyanogriseus*.

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1. Introduction

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 [1]. Annual production of cellulose has been estimated at 4.0×10^7 t [2]. Proportion of cellulose in plant tissues ranges from 20 to 45% of dry weight and to almost above 90% in cotton fiber [3]. Much of the cellulose in nature exists as waste paper [4].

Because of its highly ordered structure, cellulose is very hard to be degraded and so it is unusable and stocked in nature as waste. 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 [5]. Cellulases are the inducible bioactive compounds produced by microorganisms during their growth on cellulosic materials [6]. Due to the increasing knowledge on the

mode of action of cellulases, they have been used in enzymatic hydrolysis of cellulosic substances [7]. Although a large number of microorganisms are capable of degrading cellulose, only a few of them produce significant quantities of cell-free bioactive compounds, capable of completely hydrolyzing crystalline cellulose *in-vitro*. Though numerous investigations have reported the degradation of cellulosic materials, only a few studies have examined which microorganisms meet the industrial requirements [8].

Among the microbes, actinobacteria (actinomycetes) are one of the largest taxonomic units within the Bacterial domain [9,10] and they are the most efficient prokaryotes to be used economically and biotechnologically for their production of about half of the discovered bioactive secondary metabolites [11]. Actinobacteria can metabolize many different compounds including sugars, alcohols and amino acids. Additionally, many of them (e.g. *Streptomyces* and *Rhodococcus*) produce extracellular hydrolytic enzymes to obtain nutrients from cellulose, hemicellulose, proteins and fats [12]. Furthermore, some strains are degrading compounds of macromolecules (lignin, cellulose, chitin, in part starch and aromatic hydrocarbons). Therefore, actinobacteria often occur in materials where organic matter is degraded [13], such as soils, organic compost heaps and building materials. Their metabolic diversity is due to their extremely large genome which

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codes for a variety of transcription factors that in turn control gene expression, allowing them to respond to specific needs [12]. Having these in mind, present study was aimed at to isolate and screen cellulase enzyme producing marine actinobacterial strains from the Havelock island sediments.

2. Materials and methods

2.1. Collection of samples and isolation of actinobacterial strains

Sediment samples were collected during the month of November 2011 at a depth of 25 cm from six locations of the Havelock island using a sterile spatula. 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 was carried out in Kuster's agar medium (Glycerol – 10 g, Casein – 3 g, KNO_3 – 2 g, NaCl – 2 g, K_2HPO_4 – 2 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ – 0.05 g, CaCO_3 – 0.02 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ – 0.01 g, Agar – 15 g, 50% Sea water – 1000 ml, pH 7 ± 0.1). The autoclaved Kuster's agar medium containing petriplates were prepared aseptically. To minimize the fungal and bacterial contaminations, Kuster's agar medium was supplemented with cycloheximide (10 $\mu\text{g/ml}$) and nalidixic acid (10 $\mu\text{g/ml}$) respectively [14]. One gram of pretreated sediment samples was serially diluted using sterile seawater and 0.1 ml of serially diluted samples was added to the petriplates containing Kuster's agar medium [15] and spread using a 'L' shaped glass spreader. The plates were incubated at 37°C for seven days in an inverted position. The leathery colonies of actinobacteria that appeared on the petriplates were counted from the 5th day onwards upto 28th day. After the incubation period, morphologically distinct colonies were picked up from the petridishes and restreaked in appropriate media and pure cultures were obtained and the slants were maintained at 4°C for future.

2.2. Enzyme screening and preparation of crude enzyme cellulase

Morphologically distinct actinobacterial strains MHA10 to MHA19 were analyzed by the spot inoculation method using two different enzymatic agar media such as Cellulose Congo-Red agar medium (Cellulose powder – 1.88 g, KH_2PO_4 – 0.5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ – 0.25 g, Congo red – 0.2 g, Gelatin – 2.0 g, Agar – 16 g, 50% Seawater – 1000 ml) and Carboxy Methyl Cellulose agar medium (Carboxy methyl cellulose – 10 g, NaNO_3 – 2 g, KH_2PO_4 – 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ – 0.05 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ – 0.01 g, Yeast extract – 0.1 g, Agar – 16 g, 50% Seawater – 1000 ml, pH 7 ± 0.1) for the preliminary screening for cellulase enzyme activity [16,17,44]. The clear zone diameter was measured in order to select the highest cellulase activity producer.

A loopful of culture or the strain was incubated into carboxy methyl cellulose (CMC) medium in 100 ml (Carboxy methyl cellulose – 10 g, NaNO_3 – 2 g, KH_2PO_4 – 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ – 0.05 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ – 0.01 g, Yeast extract – 0.1 g, 50% Seawater – 1000 ml, pH 7 ± 0.1) in Erlenmeyer flasks (250 ml) in triplicate. Flasks were incubated at 37°C for 5–7 days in shaker incubator at 150 rpm. Part of samples (5 ml) was withdrawn from each flask after 7 days of fermentation and centrifuged at 2000 rpm for 10 min. The clear supernatant broth was collected aseptically to determine the enzyme yield.

2.3. Cellulase enzyme assay

Assay for the cellulolytic activities of cellulase was done by DNS (3,5-dinitrosalicylic acid) method [18]. Cellulase activity was

determined by incubating the crude enzyme (0.5 ml); 1% (w/v) CMC (0.5 ml) in 110 mM potassium phosphate buffer (pH 7) was mixed and incubated for 20 min at 70°C ; 1% DNS reagent (3 ml) (Dinitrosalicylic acid – 10 g, Phenol – 2 g, Sodium sulphate – 0.05 g, NaOH – 10 g, Distilled water – 1000 ml) was added in a capped test tube and the mixture was heated at 90°C for 5–15 min to develop red brown colour. Then, 1 ml of potassium sodium tartrate (Rochelle salt solution) was added to stabilize the colour. After cooling to room temperature in a cold water bath, absorbance was read in a UV-vis spectrophotometer (UV-2450, Shimadzu, Japan) at 575 nm. The reducing sugar formed was quantified as glucose using dinitrosalicylic acid.

2.4. Standard glucose solution (0.1 M)

A stock solution was prepared dissolving glucose (1.8 g) in potassium phosphate buffer (100 ml). Different concentrations of glucose standard solution were taken and diluted to final value of 1 ml. One ml of diluted standard solution and 1 ml of potassium phosphate buffer (pH 7) were mixed and incubated for 20 min at 70°C ; 3 ml of DNS reagent (1%) was added in a capped test tube and the mixture was heated at 90°C for 5–15 min to develop red brown colour. Then, 1 ml of potassium sodium tartrate (Rochelle salt solution) was added to stabilize the colour. After cooling to room temperature in a cold water bath, absorbance was read in a UV-vis spectrophotometer (UV-2450, Shimadzu, Japan) at 575 nm.

A standard curve was obtained with standard glucose solution without the enzyme. One unit (U) of CMCase activity is the amount of enzyme required to produce 1 μmol reducing sugar (glucose) per min in reaction mixture under specified conditions.

2.5. Taxonomical investigation

2.5.1. Genus level identification

Characterization and subsequent identification of the strain MHA15 to the genus level were made based on the chemical composition of the cell wall and micro-morphological studies [19–21]. Characterization of the strain MHA15 was made by following the method, described by Shirling and Gottlieb [22], using the standard ISP medium.

2.5.2. Species level identification

Species level identification of the strain was made based on the keys and by using the Bergey's Manual of Determinative Bacteriology. The status of the strain was also confirmed based on its molecular characters (16S rDNA gene sequences), with the following methodology.

2.5.3. Genomic DNA isolation and amplification of 16S rDNA

Total genomic DNA was extracted from the actinobacterial broth by phenol- chloroform isoamyl alcohol method, which removes the protein and their cellular components from the nucleic acid to obtain the pure DNA [23]. DNA sample (10 μl) was mixed with 2 μl of loading (6 \times) dye and loaded in 1% agarose gel. The separated DNA was visualized by UV transilluminator.

Each 50 μl amplification reaction contained 1 μl template DNA (50–200 ng), 5 μl 10 \times PCR buffer, 1 μl both forward [27F (5'-AGAGTTTGATCMTGGCTCAG-3')] and reverse [1492R (5'-GGY-TACCTTGTTACGACTT-3')] primers, 1 μl dNTP mix (10 mM), 6 μl MgCl_2 (25 mM), 2.5 U Taq DNA polymerase, 2.5 μl DMSO and 31.5 μl sterile water. The reaction conditions were initial denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 90 s. A final extension was performed at 72°C for 10 min [24]. Reaction products were electrophoresed on a 1% agarose gel

with 2 μ l ethidium bromide and visualized under UV transilluminator and then purified.

2.5.4. 16S rDNA sequencing and phylogenetic analysis

The purified fragment was directly sequenced using a Ampli Tag FS DNA sequencing Kit (Applied Biosystem). The data were analyzed using applied Biosystem DNA editing and assembly software and sequences comparisons were obtained using the Micro Seq Software.

Sequence similarity search was made for the 16S rDNA sequence of MHA15 by applying its sequence to BLAST search of the NCBI (National Centre for Biotechnological Information, USA). Final editing of sequence alignment was done using BioEdit [25] and phylogenetic analysis was performed with version 5 of the MEGA (Molecular Evolutionary Genetics Analysis) software package [26]. A phylogenetic tree was constructed by using the Neighbour-joining tree-making algorithm [27]. The topology of the phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein [28] with 1000 replicates.

3. Results

3.1. Isolation and cellulase enzyme screening of the actinobacteria

Actinobacterial colonies were isolated from the sediment samples of the six stations of the Havelock island, using Kuster's agar medium and a total of 19 morphologically distinct actinobacterial strains were obtained. All these morphologically distinct strains were subjected to enzyme screening using two different media. In both the Cellulose Congo-Red agar medium and Carboxy methyl cellulose agar medium, higher cellulase degradation zone, the maximum cellulase degradation characteristic, was found in MHA3, MHA10, MHA13 and MHA15 (Figs. 1 and 2).

3.2. Cellulase enzyme assay

Based on the performance, MHA3, MHA10, MHA13 and MHA15 were selected as potential cellulase enzyme producers and these strains were subjected to cellulase enzyme screening using the

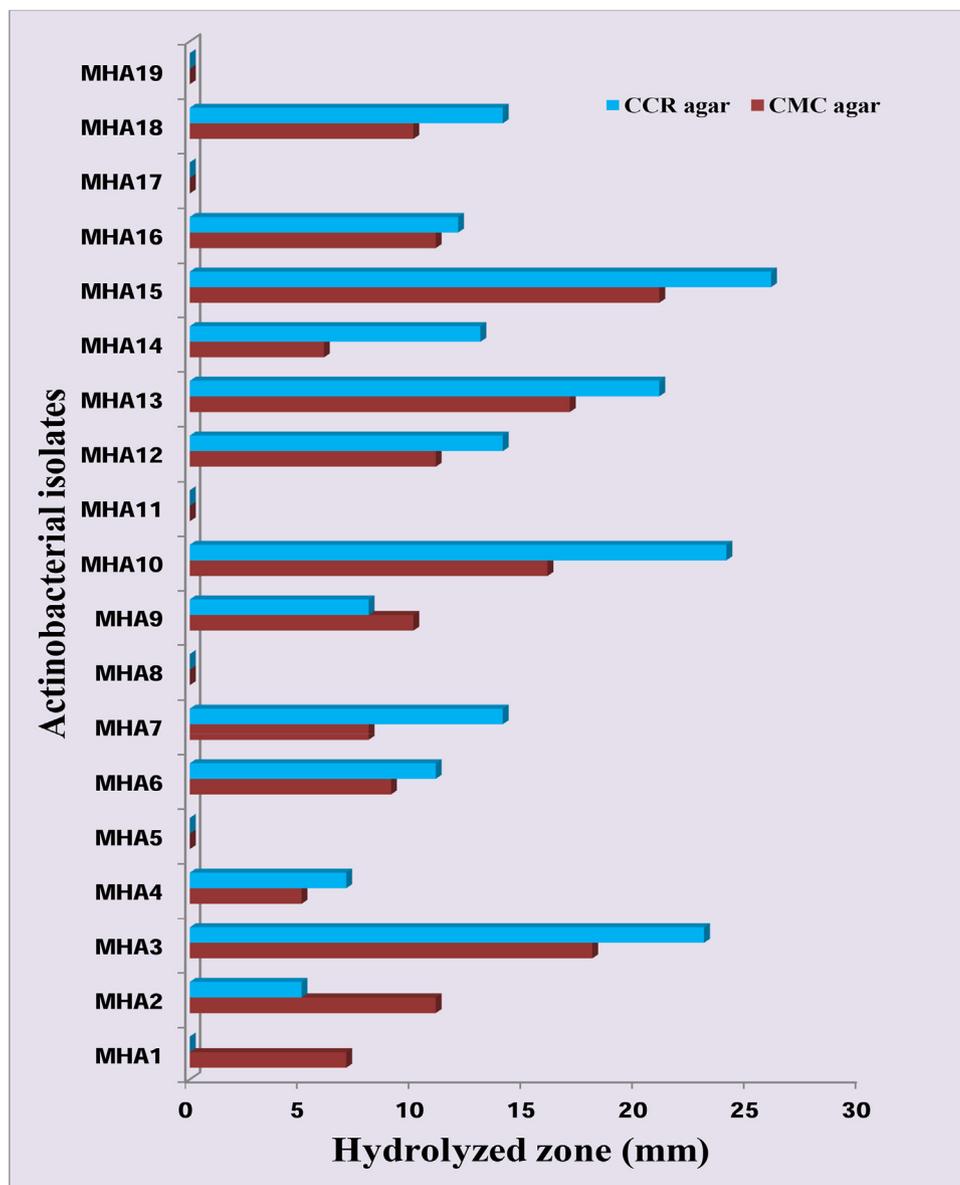


Fig. 1. Screening for the cellulase enzyme activity in CCR agar and CMC agar media.

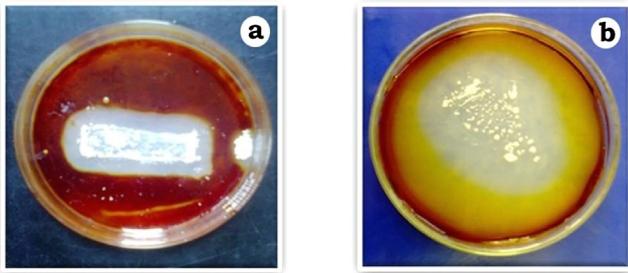


Fig. 2. Cellulolytic activity of strain MHA15 in CCR agar (a) and CMC agar (b) media.

DNS method and glucose was used as the standard. By comparing the glucose standard curve, cellulase enzyme production of the selected actinobacterial strains was measured. Higher amount of cellulase enzyme production was 14.379 IU/ml in MHA15, followed by MHA3 (14.213 IU/ml), MHA13 (13.356 IU/ml) and MHA10 (13.119 IU/ml). Among them, MHA15 alone produced more quantity of cellulase enzyme.

3.3. Taxonomical investigation of strain MHA15

Strain MHA15 formed extensively branched substrate mycelium and aerial hyphae that produced long spore chains (Fig. 3). In ISP2 agar, black with gray coloured aerial spores were formed (Fig. 3). In Peptone yeast extract iron agar, reverse side and soluble pigments were produced. Melanin pigment was also produced on ISP7 agar. The culture grew well when it was supplemented with

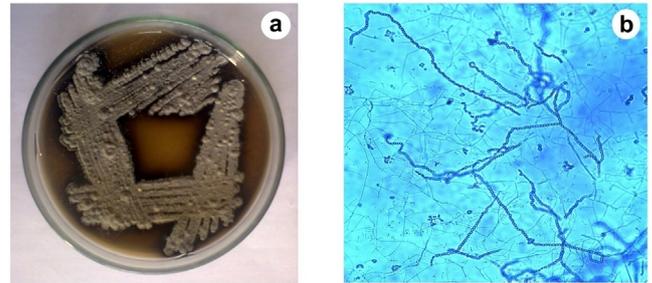


Fig. 3. Morphological characters (a-aerial mass colour; b- long spore chain) of strain MHA15.

the carbon sources viz. arabinose, xylose, inositol, mannitol, fructose, rhamnose and sucrose. No growth was observed in raffinose. In the strain MHA15, cell wall characteristics showed the presence of glucose, mannose, galactose and rhamnose as whole-cell sugars and *meso*-diaminopimelic acid as the amino acid in the cell wall and glycine was absent, indicating that this strain belongs to the cell wall chemo type III (Table 1).

3.4. Molecular identification

Further, for molecular identification, the strain MHA15 genomic DNA was extracted and purified and genomic DNA's 16S rDNA gene was amplified using Universal primers. Amplified product and genomic DNA were loaded in lanes 2 and 3 respectively and amplified 16S rDNA gene showed a distinct band pattern with a

Table 1
Comparative characteristics of the strain MHA15 with the *A. cyanogriseus*.

Characters studied	Strain MHA15	<i>A. cyanogriseus</i>
I. Cell wall amino acids		
LL-DAP	–	–
Meso- DAP	+	+
Glycine	–	–
II. Whole cell sugars		
Arabinose	–	–
Galactose	+	+
Rhamnose	+	+
Glucose	+	+
Mannose	+	+
III. Cell wall chemotype		
	III	III
IV. Characters studied (as per Tamura <i>et al.</i> , 2000)		
Colour of aerial mycelium	Blue-gray	Blue-gray
Melanoid pigment	Black	Black
Reverse side pigment	Black	Black
Soluble pigment	Black	Black
Spore chain	Long spore chain	Long spore chain
Carbon source assimilation		
Arabinose	+	±
Xylose	+	+
Inositol	+	–
Mannitol	+	+
Fructose	+	–
Rhamnose	+	+
Sucrose	+	±
Raffinose	–	–
V. Molecular characters		
Sequenced gene	16S rDNA	16S rDNA
NCBI Accession number	KF668663	NR_024650
No of bp.	873	1468
Similarity level with closest neighbour strain (%)	91.6	–

Positive (+); Negative (–); weakly utilized (±).



Fig. 4. Electrophoresis agarose gel results in strain MHA15.

molecular base pairs approximately near to 4361 bp (Fig. 4). A 873 bp of 16S rDNA sequences was determined for the strain MHA15, which was submitted to the GenBank (National Center for Biotechnology Information, USA) and an accession number (KF668663) was obtained. Based on the molecular data (16S rDNA sequences), phylogenetic tree was constructed on the comparison of 16S rRNA gene (873 bp) of the strain MHA15 with the previously reported sequence of *Actinoalloteichus* species (Accession number: NR_024650) deposited in the GenBank and closest species molecular data were also obtained from the GenBank. *Vibrio cholerae* rooted phylogenetic tree (Fig. 5) indicated that the strain MHA15 is related to the members of the genus, *Actinoalloteichus* and the strain MHA15 forms a branch with *A. cyanogriseus* with 91.6% similarity (Table 1).

Results of the cultural, cell wall chemotypical, morphological, physiological and molecular characters were compared between the strain MHA15 and its closest phylogenetic member. The strain MHA15 showed no variation in any of the characters when compared to those of the reference species, *A. cyanogriseus*. All the conventional characters were similar to those of *A. cyanogriseus*. But, the molecular characters showed distinct difference in the phylogenetic relationship (8.4%).

4. Discussion

Cellulose is the most important component of plant biomass and it acts as the renewable source for bioconversion to biofuels and by-products. In this respect, actinobacteria, one of the known cellulase producers, have evoked considerable research interest,

due to their potential application in the recovery of fermentable sugars from cellulose [29]. Further, these microbes are capable of producing an array of different extracellular enzymes including cellulase, chitinase and xylanase. In the present study, 19 actinobacterial isolates were screened for the cellulase activity.

Screening of actinobacterial strains was performed, using the cellulose Congo-Red agar and Carboxy Methyl cellulose agar media. In the cellulose Congo-Red agar medium, 74% of actinobacterial isolates (14 strains) showed the Zones of cellulose hydrolysis whereas, 29% of the actinobacterial isolates (5 strains) did not show the Zones of cellulose hydrolysis. In the Carboxy Methyl cellulose agar medium, 68% of the actinobacterial isolates (13 strains) showed Zones of cellulose hydrolysis whereas, 32% of actinobacterial isolates (6 strains) did not show the Zones of cellulose hydrolysis. In 1983, Veiga et al. [30] isolated 36 strains from marine sediments of about 10 m depth in Za corure Bay (Spain) and only 19 strains (>50%) showed enzymatic degradation of cellulose. Very recently, Gobalakrishnan et al. [44] have reported that the strain AUANI-5 isolated from the Neil island, produced higher cellulase enzyme and which was identified as *Streptomyces craterifer*.

In the present study, cellulose degradation zone was higher in Carboxy Methyl cellulose agar than that of the cellulose Congo-Red agar. This could be due to the fact that the cellulase is an inducible enzyme and it is affected by the nature of the substrate used for production, as explained by Haung and Monk [31] and due to the less complexity, leading to its easy assimilation by the isolated microbes [32].

Actinobacteria, one of the known cellulase producers have attracted considerable research interest in the recent times due to their potential application [33,34]. A wide variety of bacteria are known for their production of hydrolytic enzymes with *Streptomyces* species being the best known enzyme producers [35]. Present study also examined cellulase production in 19 marine antibacterial isolates; among them, four (MHA3, MHA10, MHA13 and MHA15) showed higher cellulase activity and the strain MHA15 was identified as belonging to the genus *Actinoalloteichus* and it formed a branch with *A. cyanogriseus* with 91.6% similarity, in the phylogenetic study. Recently, biologically active compounds have been discovered from species belonging to this rare genus. For instance, naturally active melanin and cytotoxic cyclic bipyridine glycosides have been reported from the marine isolated strains, *Actinoalloteichus* sp. MA-32 and *A. cyanogriseus* WH1-2216-6 [36,37]. In fact, Jose et al. [38] reported that the strain JAJ70 (isolated from hypersaline solar saltern) showed significant antimicrobial activity against the different clinical pathogens and the strain was identified as belonging to genus *Actinoalloteichus*.

Similarly, other researchers have also widely reported cellulase enzyme production by the marine actinobacteria: Veiga et al. [30] isolated 36 *Streptomyces* strains from 10 m depth of La Corufia Bay, Spain. Among them, 50% of isolates performed cellulolytic activity. Murugan et al. [39] isolated 35 actinobacterial strains from the Vellar estuary, India and examined their cellulase activity. Among them, strain CL-30 (*S. actuosus*) showed maximum cellulase activity. Stalin et al. [40] reported that 30 isolates (obtained from Tiruchendhur coastal area of Tamil Nadu, India) were screened for cellulase activity and *S. clavuligerus* alone produced maximum amount of cellulase. Recently, Sirisha et al. [41] reported bioactive compounds obtained from marine actinobacteria isolated from the sediments of the Bay of Bengal, and 24% of the strains exhibited cellulase activity. Meena et al. [42] isolated 26 strains of actinobacteria from the marine sediments of the Andaman and Nicobar islands and among them, two *Streptomyces* species (NIOT-VKKMA02 and NIOT-VKKMA26) showed excellent cellulase activity. Reyad [43] also reported the actinobacterial

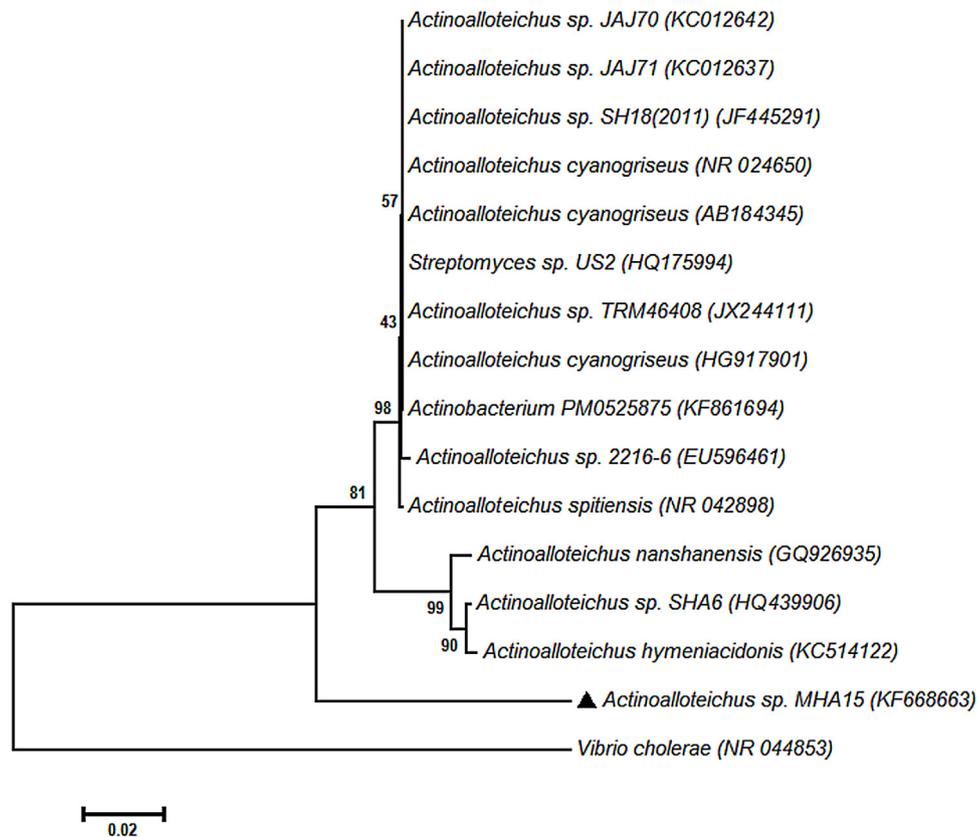


Fig. 5. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationship between the strain MHA15 and related and respective species of the genus *Actinoalloteichus*. Only bootstrap values (expressed as percentage of 1000 replications) >50% are given at nodes.

production of cellulase and ascertained that the halotolerant actinobacteria associated with mangroves are a good source of cellulase.

5. Conclusion

From the present study, it can be stated that the Havelock island coast of the Andamans is a significant source for isolation of potential actinobacteria for biotechnological purpose. Out of the 19 morphologically distinct strains, strain MHA15 showed higher cellulase activity (14.379 IU/ml), suggesting that it is an ideal organism for the bioconversion of cellulose. Detailed investigations revealed that the strain MHA15 belongs to the genus *Actinoalloteichus* and the molecular characters showed distinct difference in phylogenetic relationship (8.4%) with the closest reference species *A. cyanogriseus*. If detailed polyphasic taxonomic analysis of the strain is made, it could be a new species.

Conflict of interest statement

We declare that we have no conflict of interest.

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