

Amylolytic and Proteolytic Actinobacteria Isolated from Marine Sediments of Bay of Bengal

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Abstract: This study screened actinomycetes isolated from Pudimadaka Coast of the Bay of Bengal for enzymatic activities. A total of 98 actinomycete isolates were obtained from the 12 sediment samples collected at various depths and were then tested for amylase and protease activities. Results indicated that twenty one of the 98 isolates showed amylolytic activity and seventeen exhibited proteolytic activity, while 11 isolates exhibited both amylolytic and proteolytic activities. Cultural, morphological and chemotaxonomic characterization of the five most potent actinomycete isolates classified three isolates under the genus *Micromonospora* and two isolates under the genus *Streptomyces*. In conclusion, these results of enzymes production have increased the scope of finding industrially important marine actinomycetes from the Bay of Bengal.

Key words: Marine • Actinomycetes • Amylase • Protease

INTRODUCTION

Actinomycetes are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of half of the discovered bioactive secondary metabolites [1]. Recent findings have demonstrated that indigenous marine actinomycetes exist in the oceans and are widely distributed in different marine ecosystems [2-5]. They have attracted great attention since there is tremendous diversity and novelty among the marine actinomycetes present in marine environments. Marine actinomycetes are a prolific, but underexploited source for the various kinds of bioactive secondary metabolites [6-9].

With the advent of new frontiers in biotechnology, the spectrum of amylase and protease application has expanded beyond the traditional industries into many new fields, such as clinical, medicinal and analytical chemistry. As such the demand for microbial amylase and protease enzymes in industrial fields is increasing day by day. The present global market for these industrial enzymes is around \$ 2 billion and expected to rise at an average annual growth rate of 3.3% [10]. Although protease and amylase are mainly fungal and eubacterial products, the possibility of using actinomycetes for enzyme production has recently been investigated. Few members of the

actinomycetes that have been studied with reference to purification and characterization of amylase and protease enzymes include *Streptomyces*, *Nocardiopsis*, *Thermomonospora*, and *Thermoactinomyces* [11-19].

In the present study, one natural saline habitat along the Pudimadaka Coast of Bay of Bengal in Southern India was selected for the isolation followed by the assessment of enzymatic potential of marine actinomycetes.

MATERIALS AND METHODS

Sampling Procedure: In the course of screening for bioactive actinomycetes, altogether 12 marine sediment samples were collected from Bay of Bengal near Pudimadaka coast using a core sampler. The location and depths of these sampling stations are summarized in Table 1. The maximum depth of collection was 40m and all samples were transferred into sterile zipped polythene bags and transported to the laboratory for the isolation of actinomycetes.

Isolation of Actinomycetes Colonies from Marine Sediments: Isolation and enumeration of actinomycetes were performed by the serial dilution plate technique [20] using starch casein agar medium [21], Malt extract-yeast extract agar medium [22] and chitin agar medium [23].

Table 1: Location of sampling stations

Location	Depth(meters)	Latitude	Longitude
1.	1	+17.42462	+83.19234
2.	1	+17.46515	+83.23943
3.	10	+17.30500	+83.02300
4.	20	+17.30500	+83.04400
5.	30	+17.30500	+83.08000
6.	30	+17.29000	+83.00816
7.	20	+17.29000	+83.02205
8.	30	+17.29000	+83.05086
9.	40	+17.29015	+83.07480
10.	10	+17.27000	+82.59398
11.	20	+17.27000	+82.59295
12.	30	+17.27000	+83.01314

These media containing 50% seawater were supplemented with rifampicin 5µg/ml and nystatin 25µg/ml (Himedia Mumbai) to inhibit bacterial and fungal contamination, respectively. All plates were incubated at $28 \pm 2^\circ\text{C}$ for 2-3 weeks. After incubation, actinomycetes isolates were distinguished from other microbial colonies based on morphological features such as tough, leathery colonies which are partially submerged into the agar [2] and colours of pigmentation including diffusible pigments. Single separated actinomycetes colonies were selected and the subcultures were maintained on Starch casein slants at 4°C until further use.

Screening of Actinomycete Isolates for Enzymatic

Activities: Two stages of enzymatic screening were done. All isolates were subjected to primary screening, while secondary screening was performed for the total actinomycete isolates that showed enzymatic activities during the primary screening.

Preliminary Enzymatic Screening. All the isolates were tested for their amylolytic and proteolytic activities using starch agar and skim milk casein agar media respectively by conventional plate method [24]. Starch agar plates were flooded with Lugols iodine solution and hydrolysed zones were measured, while casein hydrolysis zones were measured directly.

Secondary Enzymatic Screening. Further studies for the production of enzymes were carried out by shake flask method. The production medium for amylase consisted of (g/l): 10.0 soluble starch, 2.0 Yeast extract, 5.0 Peptone, 0.5 MgSO_4 , 0.5 KH_2PO_4 , 1.5 NaCl and 0.15 CaCl_2 . The pH of the medium was adjusted to 7.0. The production medium for protease consisted of (g/l): 2.0 soluble starch, 0.5 peptone, 0.5 glucose, 0.5 yeast extract, 0.5 Casein, 0.2 soyabean meal, 0.6 $(\text{NH}_4)_2\text{SO}_4$, 0.8 CaCO_3 , 0.5 NaCl. The pH of the medium was adjusted to 7.0. The flasks were incubated at 28°C for 96 hrs on a rotary shaker at 180rpm.

Enzyme Assay: The enzyme assays were performed with the cell free supernatant of the fermented broth obtained by the centrifugation of the whole fermentation broth at 10,000 rpm at 4°C for 15 min as the crude enzyme source.

Assay of amylase: Amylase activity was determined by incubating a mixture of 0.5 ml of aliquote of each enzyme source and 0.5 ml of 1% soluble starch dissolved in 0.1 M phosphate buffer, pH=7, at 37°C for 15 min [25]. The reaction was stopped by adding 1 ml of 3, 5-dinitrosalicylic acid [26], and then followed by boiling for 10 min. The final volume was made up to 12 ml with distilled water and the reducing sugar released was measured at 540 nm. One unit (U) of amylase activity was defined as the amount of µg of maltose equivalents liberated per min per ml of enzyme under the conditions of assay. The amount of maltose was determined from the maltose standard curve.

Assay of protease: A 100 µl of aliquote of each enzyme source was incubated with 900 µl of 2% casein in NaOH- KH_2PO_4 standardization buffer (0.1 M, pH=7) at 37°C for 30 min. The reaction was arrested by the addition of 1.5 ml of 10% trichloroacetic acid. After 15 min, the mixture was filtrated and the protein concentration in 0.5 ml of the filtrate was measured by the method of Lowry *et al* [27]. One unit (U) of protease activity is equivalent to µg of tyrosine liberated per ml of enzyme under the assay conditions.

Characterization of the Five Most Potent Actinomycete

Isolates: The five most potent actinomycete isolates were identified up to the genera level by macroscopic and microscopic morphological methods as per Bergey's 2000 manual [28]. The macroscopic method was done by colony characterization on Yeast Malt extract Agar (YMA) medium. Color of colony and presence of

pigmentation were noted. The microscopic characterization was done by cover slip culture method observed after 3 days. The presence or absence of aerial and substrate mycelium, spore formation, and fragmentation of the vegetative or substrate mycelium were observed. Further characterization of selected isolates was done as per the ISP protocol [29]. The utilization of carbon sources was examined on Pridham and Gottlieb's medium [30] containing miscellaneous carbohydrates to a final concentration of 1%.

Chemotaxonomical Characteristics: Cell wall of the selected isolates was purified and analyzed by the methods of Lechevalier and Lechevalier [31]. Whole-cell sugars were analyzed according to the method of Becker *et al.* [32].

Sodium Chloride Tolerance: The selected isolates were screened for sodium chloride tolerance (0-10%) on Yeast-Malt agar slants according to Tresner *et al.* [33].

Statistical Analysis: All experiments were carried out in triplicates, and repeated three times. The samples collected from each replicate were tested for amylase and protease production and activity. Means of the amylolytic and proteolytic activities were calculated and significant differences were calculated by determining standard error.

RESULTS AND DISCUSSION

Isolation of Actinomycetes Colonies from Marine Sediments: Using the selective media, 98 actinomycete strains were isolated, of which maximum number of actinomycetes isolated was in Malt extract-Yeast extract agar medium (44) followed by, Starch casein agar medium (35) and Chitin agar medium (19). The entire twelve samples of Pudimadaka are found suitable for the isolation of actinomycetes and significant difference (at 10% level) is found between the counts of actinomycetes in different media, but not between samples. This suggests that the occurrence of actinomycetes in marine sediments of Pudimadaka might be persistent population of unexplored major new marine actinomycetes with potent enzymatic activities.

Only few reports of amylases and proteases from marine actinomycetes were made by various researchers although marine actinomycetes have excellent capacity to elaborate a wide diversity of enzymes [34-36].

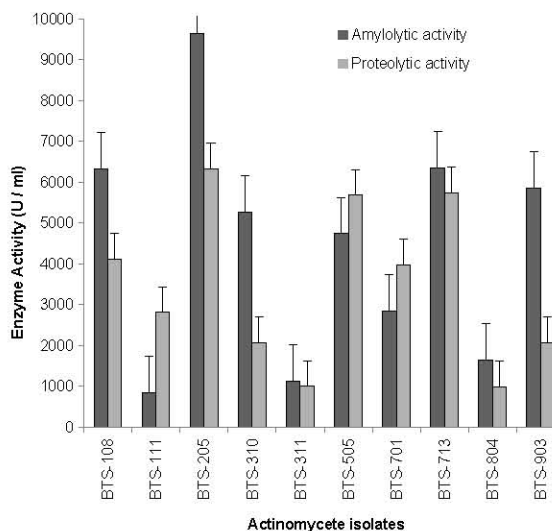


Fig. 1: Amylolytic and proteolytic activities of the select isolates under submerged fermentation conditions

Screening of Actinomycete Isolates for Enzymatic

Activities: Twenty one of the 98 isolates showed amylolytic activity and seventeen exhibited proteolytic activity while eleven isolates exhibited both amylolytic and proteolytic activities. This rather low estimate of the proportion of active strains may be due to the method of preliminary screening used. The small percentage of active strains might not indicate that the resource is not a good one, but rather that our method of screening was such that all isolates of actinomycetes could not grow well at the same incubation time. Moreover, there are so many factors which affect actinomycetes growth and enzyme production, including the chemical and biological environment. It is believed that the obtained results reveal that sediments of Pudimadaka are good sources for isolating actinomycetes. High or low numbers of active strains found depends on many factors: the sampling size, the probability of right sample to be inoculated, the medium and methods of screening. The enzymatic activities of 10 most promising isolates are shown in table 2. As indicated in the table, isolate BTS-205 showed maximum amylolytic activity ($R/r = 3.1$) and maximum proteolytic activity ($R/r = 2.8$) while the isolate BTS-310 showed the second highest amylolytic activity ($R/r = 2.9$) and isolate BTS-713 showed the second highest proteolytic activity ($R/r = 2.5$).

All the positive isolates were confirmed for their amylolytic and proteolytic activities by submerged fermentation conditions and the results of 10 most promising isolates are presented in Figure 1.

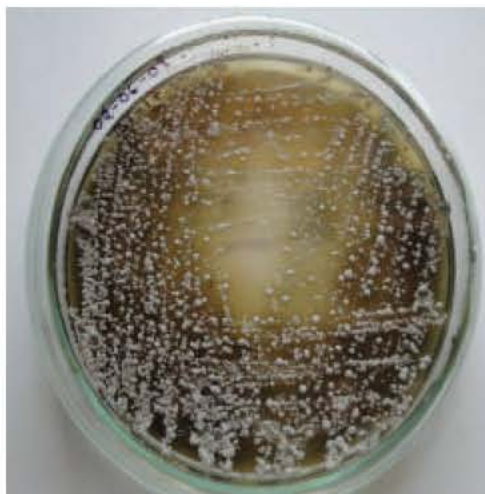


Fig. 2: Isolate No. BTS-205 identified as *Micromonospora* sp. on Starch casein media

The results indicated that the isolate BTS-205 (Figure 2.) exhibited maximum amylolytic activity (9629 U/ml) and maximum proteolytic activity (6326 U / ml) followed by the isolates BTS-310 (6572 U/ ml) and BTS-108 (6321 U/ ml) for amylolytic activity and the isolates BTS-713 (5743 U/ ml) and BTS-505 (5681 U / ml) for proteolytic activity.

Characterization of the Five Most Potent Actinomycete Isolates: Both macroscopic and microscopic methods were employed to describe the isolates BTS-108, BTS-205, BTS-310, BTS-505 and BTS-713, the five most potent actinomycete isolates. The cultural and morphological properties of the five isolates are shown in Table 3. The results indicate that among the five isolates, three isolates BTS-108, BTS-205, and BTS-713 were

characterized by single spores at the tips of sporophores and two isolates BTS-310 and BTS-505 by spiral polyspores. Out of five isolates, three isolates BTS-108, BTS-205, and BTS-713 showed black spore mass, while BTS-310 isolate showed yellow-brown spore mass color and BTS-505 isolate showed green-gray spore mass. The form of the colony of isolates BTS-108, BTS-205, and BTS-713 was described to be compact, folded, raised, and lichenoid to leathery and that of the isolates BTS-310 and BTS-505 was discrete, floccose and powdery. According to Waksman [37], such color and form are exhibited by colonies of both *Streptomyces* and *Micromonospora*. However, the color of the growth and the form of the colony could not serve as bases for pointing out the genus to which actinomycete isolates belongs to. Hence, its morphological properties must serve as the primary bases of characterization. One distinguishing morphological property possessed by the isolates BTS-108, BTS-205, and BTS-713 is the absence of an aerial mycelium, a characteristic possessed by all members of *Micromonospora* [28]. A well-developed substrate mycelium that partly penetrates the medium and the formation of single spores at the sporophore tips are two characteristics that may well qualify them as a species of *Micromonospora*. The formation of a dark-brown pigment by isolates BTS-205 and BTS-713 that dissolves into the medium has also been mentioned by Waksman [37] to be a possible characteristic of the genus, although not exclusively. Isolates BTS-310 and BTS-505 formed substrate mycelium and abundant aerial mycelium with powdery spore mass. Polysporic actinomycetes, forming characteristic aerial and substrate hyphae represent an important macroscopic criterion to identify the genus *Streptomyces* [28].

Table 2: Amylolytic and proteolytic activities of the selected isolates

Isolate No.	Amylolytic Zone (R / r)*	Proteolytic Zone (R / r)*
BTS-108	2.8	2.0
BTS-111	1.6	1.4
BTS-205	3.1	2.8
BTS-310	2.9	2.2
BTS-311	1.7	1.9
BTS-505	2.1	2.3
BTS-701	2.6	1.8
BTS-713	1.7	2.5
BTS-804	1.9	2.1
BTS-903	2.4	1.2

* R: Hydrolysed zone diameter; r: Growth zone diameter

Table 3: Cultural and Morphological characteristics of the selected isolates

Cultural / Morphological Characteristics	BTS-108	BTS-205	BTS-310	BTS-505	BTS-713
Spore formation	Singly at the tipsof sporophores	Singly at the tipsof sporophores	Spiral polyspores	Spiral polyspores	Singly at the tipsof sporophores
Spore mass colour	Black	Black	Yellow-brown	Green-gray	Black
Colour of the colony	White	White, then yellow	White	Pale yellow	White, then yellow
Form of the colony	Compact, folded, lichenoid then leathery,raised	Compact, folded,lichenoid then leathery,raised	Discrete,floccose and powdery	Discrete, floccose and powdery	Compact, folded,lichenoid then leathery,raised
Aerial mycelium	Absent	Absent	Present	Present	Absent
Substrate mycelium	Present	Present	Present	Present	Present
Pigmentation in the medium	Nil	Present (dak brown)	Nil	Nil	Present (dark brown)

Table 4: Physiological and biochemical characteristics of the selected isolates

Characteristics	Isolates				
	BTS-108	BTS-205	BTS-310	BTS-505	BTS-713
Grams staining	+	+	+	+	+
Melanin Pigmentation	--	+	--	+	+
H ₂ S production	+	+	--	+	--
Tyrosine reaction	--	+	--	+	+
Starch hydrolysis	+	+	+	+	+
Casein hydrolysis	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+
Nitrate reduction	+	--	+	+	--
Growth at different temperatures					
4°C	--	--	--	--	--
15°C	--	--	--	--	--
25°C	+	+	+	+	+
30°C	+	+	+	+	+
35°C	+	+	+	+	+
45°C	--	--	--	--	--
Maximum NaCl tolerance	7%	7%	7%	7%	7%
Utilization of:					
D-Glucose	+	+	+	+	+
D-Mannitol	--	--	+	--	w
D-Ribose	+	--	w	--	--
L-Rhamnose	--	--	+	+	w
D-Raffinose	w	--	+	+	--
Glycerol	--	--	+	--	w
Lactose	+	+	+	+	+
D-Galactose	+	w	--	+	--
L-Arabinose	+	--	w	--	--
Cellobiose	+	--	+	--	+
D-Fructose	+	+	+	+	+
D-Xylose	+	+	+	+	+

+, Positive; --, negative; w, weakly positive.

The physiological and biochemical characteristics of the isolates are shown in Table 4. Kampfer *et al.* [38] suggested the physiological tests as indispensable tools for classification and identification of actinomycetes. Isolates BTS-205, BTS-505 and BTS-713 exhibited positive reaction to melanin pigmentation and tyrosine reaction while these properties were found to be negative for isolates BTS-108 and BTS-310. Isolates BTS-108, BTS-205 and BTS-505 had the ability for H₂S production and nitrate reductase was secreted by isolates BTS-108, BTS-310 and BTS-505. All the isolates were positive for starch hydrolysis, casein hydrolysis and gelatin hydrolysis.

Carbohydrate utilization test plays a prominent role in the taxonomic characterization of actinomycete strains [30]. Abundant growth was attained with glucose, lactose, fructose and xylose by all the isolates used. However, little or no growth was attained when isolates BTS-108, BTS-205 and BTS-713 were grown on mannitol, rhamnose, raffinose and glycerol. Isolate BTS-310 efficiently utilized the carbon sources such as, mannitol, rhamnose, raffinose, glycerol and cellobiose. Rhamnose, raffinose and galactose were assimilated by the isolate BTS-505 as the good sources of carbon and energy while ribose, galactose, arabinose and cellobiose were well utilized by the isolate BTS-108.

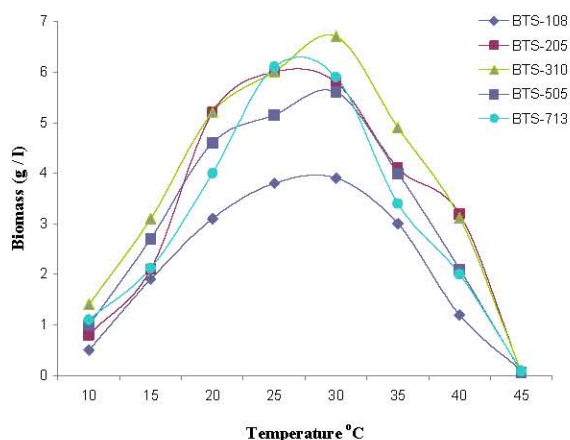


Fig. 3: Growth patterns of five actinomycete strains (BTS-108, BTS-205, BTS-310, BTS-505 and BTS-713) at different temperatures

Temperature is one of the physical factors that governs the distribution and activities of actinomycetes in natural habitats. In the present study, the five selected isolates were grown at different temperatures. None of them were able to grow at 4 and at 45°C. The optimum temperature for growth of most of the selected strain was between 25°C and 30°C (Figure 3.). This result showed similarities with the work of Goodfellow and Williams [39] who reported that most of the actinomycetes behave as mesophiles with an optimum growth at 30°C. Tolerance of the strains to NaCl concentration also serves as an important character for species identification. All the isolates exhibited salt tolerance up to 7%, indicating that the isolates are indigenous to marine environment.

Cummins and Harris [40] stated that actinomycetes have cell wall composition akin to that of gram-positive bacteria and indicated that the chemical composition of the cell wall might furnish practical methods of differentiating various types of actinomycetes. According to Lechevalier and Lechevalier [31] the sugar composition often provides valuable information on the classification and identification of actinomycetes. Considering the importance of chemotaxonomical characteristics in the identification of actinomycetes, an attempt was made to identify the actinomycetes, by analyzing their cell components. The cell-wall peptidoglycan of three isolates BTS-108, BTS-205, and BTS-713 contained Meso-diaminopimelic acid and glycine and the whole cell hydrolysates contained xylose and arabinose. This indicated that they belonged to cell-wall type II, which is a characteristic of the genus *Micromonospora* [41]. However, the cell-wall peptidoglycan of BTS-310 and BTS-505 isolates contained LL-diaminopimelic acid and glycine and the whole cell hydrolysates contained xylose.

This indicated that they belonged to cell-wall type I, which is a characteristic of the genus *Streptomyces* [41].

On the basis of morphological and chemotaxonomic characteristics, the isolates BTS-108, BTS-205 and BTS-713 were identified as belonging to the family Streptomycetaceae and the genus *Micromonospora* [42], while isolates BTS-310 and BTS-505 were assigned to the family of Streptomycetaceae and the genus *Streptomyces* [43, 44].

Based on the screening results, it has been shown that sediments of pudimadaka coast of Bay of Bengal possess industrially importance enzymes producing actinomycetes and may be tapped as one of the potential source of bioactive actinomycetes.

In conclusion, the present investigation has been directed towards exploring marine actinomycetes as a source for various compounds of industrial interest, such as enzymes. The results in general reflect on the potential of amylase and protease production among the relatively less explored group of marine actinomycetes. It is suggested that frequent and systematic screening for actinomycetes in the Bay of Bengal could provide novel species as well as potent enzymes.

ACKNOWLEDGEMENT

Financial assistance from University Grants Commission, New Delhi to R. Haritha is gratefully acknowledged.

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