Selection of Marine Actinomycetes with Bioactive potential isolated from sediments of Bay of Bengal and Characterization of promising isolate, ABT-103

ABSTRACT

Aim: Marine actinomycetes are a potential and untapped source for the production of novel bioactive compounds. The aim of the present work is to isolate actinomycetes strains from the marine sediments of Bay of Bengal for the production of bioactive compounds and characterization of the potential actinomycetes.

Place and Duration of Study: Department of Chemical Engineering and Biotechnology, ANITS, Visakhapatnam, between March, 2014 and April , 2015.

Materials and Methods: Marine sediment samples were collected along the coast of Bay of Bengal, Visakhapatnam, India. Actinomycetes were isolated on Starch Casein Agar plates by pour plate and spread plate methods. Morphologically distinct pure isolates were tested for antimicrobial activity by Cross-streak method in preliminary screening and by Cup-plate method in secondary screening. The marine isolates were also screened for enzyme activities of amylase, lipase, protease and L-asparaginase. The most potential isolate was characterized up to genus level based on morphological, chemotaxonomic, biochemical and physiological characteristics.

Results: A total of 74 bacterial strains were isolated from marine sediment samples of Bay of Bengal. Among them, 13 morphologically distinct pure isolates were screened for antimicrobial activity and also for enzyme activities. Five isolates exhibited antimicrobial activity among which ABT-205 isolate showed broad spectrum antimicrobial activity against both bacteria and fungi, and ABT-103 exhibited maximum antifungal activity. Screening for enzyme activities revealed that nine isolates exhibited enzyme activities of which, ABT-101 and ABT-201 are highly potential for the production of Protease, whereas ABT-103 and ABT-206 were best producers of Amylase and Lipase enzymes, respectively. Among all the isolates screened, ABT-103 was found to be a promising isolate as it produced red pigment, exopolysaccharide, amylase and exhibited antifungal activity. Hence, the isolate ABT-103 was characterized and identified as *Streptomyces* species.

Conclusion: The selected marine actinomycetes isolates, ABT-101, ABT-103, ABT-201, ABT-205 and ABT-206 could be useful or the production of novel antibiotics, enzymes with different physical and physiological properties, red pigment, exopolysaccharide and other compounds for various Biotechnological applications.

Keywords: Marine actinomycetes, Bioactive compounds, Antimicrobial activity, Enzyme activities, Streptomyces

1. INTRODUCTION

Actinomycetes are filamentous, gram-positive bacteria which represent a ubiquitous group of prokaryotes having economic and biotechnological significance. About half of the discovered bioactive compounds such as antibiotics [1, 2], enzymes [3], antitumor agents [4, 5,] immunosuppressive agents [6] have been produced from actinomycetes. Terrestrial soil was the most predominant and extensively exploited source for actinomycete compounds. As the frequency of novel bioactive compounds from terrestrial actinomycetes decreases with time, actinobacteria from diverse environments have been increasingly screened for production of novel bioactive compounds. During the last 20-30 years, interest in marine microflora increased as marine actinomycetes possess the ability to produce bioactive compounds due to their distinctive physiological properties [7, 8].However, the marine environment is not much exploited for the isolation of novel actinomycetes [9] and their derived novel metabolites [10, 11].

Recognizing the importance, potential marine actinomycetes are being isolated and identified by morphological, physiological, chemotaxonomic and molecular methods [12, 13, 14,15]. Several strains of marine actinomycetes isolated from different regions have been reported to possess antimicrobial activity [16, 17, 18, 19] and enzyme activities for amylase, protease, lipase, gelatinase, and L-asparaginase [20, 21, 22, 23, 24, 25]. Several bioactive compounds have been produced from marine actinomycetes since two decades [26, 27, 28]. *Streptomyces* species have been reported to contribute nearly 75% of the metabolites from actinomycetes and 25% from rare actinomycetes [1].

Stable enzymes with unique properties are required for various commercial biotechnological applications. In addition to antibacterial, antifungal, cytotoxic, neurotoxic, antimitotic and antiviral compounds, new drug targets have been added for AIDS, immunosuppression, anti-inflammation, Alzheimer's disease and aging processes. To accomplish these needs, the inexhaustible marine source is the potential source for isolation of novel species and therefore of novel drugs and enzymes. Keeping this in view, the present work is investigated with the objective of isolation and screening of actinomycetes species from the marine sediment samples of Visakhapatnam coast of Bay of Bengal for production of novel bioactive compounds.

2. MATRIALS AND METHODS

2.1 Sample collection and processing:

A total of five marine sediment samples were collected along the coast of Bay of Bengal from Visakhapatnam, AP, India at a depth of 1.5 meters in April, 2014. The samples were collected in sterile glass bottles, maintained with sea water and transported to the laboratory. The sediment samples were brown to black in colour with soft and sandy texture.

2.2 Isolation of Marine Actinomycetes from sediment samples:

Actinomycetes from marine sediment samples were isolated by pour plate and spread plate methods.

2.2.1 Pour plate method:

10 g each of the marine sediment samples were suspended in 100mL of distilled water and agitated at 150 rpm, 28^{o}C for 24h for the separation of filamentous actinomycetes and detachment of spores. After 24h, 15mL top suspensions of the samples were centrifuged at 5000 rpm for 10 minutes. 500µL of the supernatants were mixed with molten Starch Casein Agar medium supplemented with Rifampicin (5µg/mL) and Nystatin (25µg/mL) to inhibit bacterial and fungal contamination respectively, poured into petri plates and incubated at 28^{o}C for four weeks for the isolation of marine actinomycetes.

2.2.2 Spread plate method:

10 g each of the marine sediment samples were suspended in 100mL of distilled water and agitated at 150 rpm, 28^{o}C for 24h. The samples were centrifuged and supernatants were serially diluted to obtain10^{-1} to 10^{-10}dilutions with sterile sea water. 100µL of each dilution was spread on Starch Casein Agar plates and incubated at 28^{o}C for four weeks for the isolation of marine actinomycetes.

2.3 Maintenance of pure cultures:

Pure cultures were maintained on Yeast Extract Malt Extract medium (ISP-2) for better sporulation. The pure cultures were preserved at 4^{o}C and sub-cultured for every four weeks.

2.4 Screening of Marine Actinomycetes isolates for Antimicrobial activity:

Two stages of screening was performed namely preliminary screening by cross streak method and secondary screening using cup-plate method to test the isolates for their antimicrobial activity. Three

bacterial species, *Escherichia coli, Bacillus cereus, Pseudomonas aeruginosa* and three fungi, *Saccharomyces cereviseae, Yarrowia lipolytica* and *Aspergillus niger* were used as test organisms.

2.4.1 Preliminary screening by Cross-streak method:

Starch Casein Nutrient Agar medium was used for this method. The isolates were streaked in a straight line at the centre of the plate and incubated for 2 to 5 days at 28^{o}C to develop growth. After incubation, the test organisms were streaked perpendicular to the growth of the isolates, incubated for 48h and observed for the inhibition of growth of the test organisms near the isolates.

2.4.2 Secondary screening by Cup-plate method:

Isolates that showed positive results in preliminary screening were selected for secondary screening. Antimicrobial extracts were prepared by culturing isolates in production medium consisting of sucrose 20.0 g, malt extract 10.0 g, yeast extract 4.0 g, di-potassium hydrogen phosphate 5.0 g, sodium chloride 2.5 g, zinc sulphate 0.04 g, calcium carbonate 0.4 g, 1.0 L sterile distilled water with pH 7.0 for 5 days at 28^{o}C, 160 rpm. The bacterial and fungal test organisms were pour plated in Nutrient Agar medium and Potato Dextrose Agar medium respectively. Antimicrobial extract was added to the wells, the plates were incubated at 4^{o}C for 2h for diffusion of antimicrobial extract and at 28^{o}C for 48h to observe for the zones of inhibition.

2.5 Screening of Marine Actinomycetes isolates for enzyme activities:

The isolates were tested for their ability to produce the enzymes Amylase, Lipase, Protease and L-Asparaginase. The isolates were inoculated in plates containing Starch Agar medium for amylase, Tributyrin Agar medium for lipase, Skim Milk Agar medium for protease and M9 modified medium for L-Asparaginase by streaking in a single straight line. The plates were incubated at 28^{o}C for 2 to 5 days, observed for clear zone around the growth for amylase, lipase and protease and development of pink colour for L-asparaginase activities.

2.6 Characterization of Marine Actinomycetes isolates:

All the isolates were characterized morphologically and the potential isolate ABT-103 was further characterized to the genus level based on morphological, chemotaxonomic, physiological and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology (Edition 9).

3. RESULTS AND DISCUSSION

3.1 Isolation of Marine Actinomycetes from sediment samples:

A total of 74 isolates were obtained from the samples by both pour plate and spread plate methods. Of these, 34 isolates were obtained by pour plate method and 40 isolates by spread plate method. Among these isolates, morphologically distinct 13 pure gram-positive isolates were selected for further studies. Isolation of bioactive actinomycetes have also been reported from Point Calimere Coastal region, East Coast of India [12], Bay of Bengal Coast of Puducherry and Marrakkam [29], mangrove sediments of Andaman islands [30] and Pudimadaka coast of Bay of Bengal [13] as the marine actinomycetes are gaining importance in the search for novel bioactive compounds.

3.2 Screening of Marine Actinomycetes isolates for Antimicrobial activity:

Most of the secondary metabolites produced by actinomycetes till date are potential antibiotics and are widely exploited by pharmaceutical industry [31]. In the present study, all the 13 isolates were selected for preliminary screening. Of these, five isolates namely ABT-103, ABT-201, ABT-203, ABT-205 and ABT-206 showed antimicrobial activity against the selected test organisms.

In secondary screening, the isolate ABT-103 exhibited maximum anti-fungal activity against *A. niger* by inhibiting both sporulation and mycelium (Figure 1). The isolate ABT-201 showed antibacterial activity against *P. aeruginosa* and antifungal activity against *A. niger*. The isolate, ABT-203 exhibited antifungal activity against all the tested fungi. The isolate, ABT-205 is the only isolate exhibiting broad spectrum antimicrobial activity. It showed antibacterial activity against all the tested bacteria with maximum inhibition against *B. cereus* and antifungal activity against *A. niger*. Except ABT-103, all other active isolates inhibited only sporulation of *A. niger* but not mycelium growth. The isolate ABT-206 exhibited only antibacterial activity with the maximum zone of inhibition against *B. cereus* (Table 1).These findings suggest that ABT-103, ABT-205 and ABT-206 have scope in future studies for the production of novel antimicrobial compounds. Similar work on screening of marine actinomycetes isolated from Puducherry coast of Bay of Bengal for bioactivity showed that out of 50 strains, 12 strains have broad spectrum of activity [32]. Among 21 isolates obtained from coastal water samples of Thiruchendur, Thoothukudi and Kanyakumari, Tamilnadu, 6 isolates showed antimicrobial activity against the tested organisms [33]. Actinomycetes isolates obtained from Bay of Bengal [34] and Konkan Coast, Maharashtra [35] have also

shown antagonistic activity against bacteria and fungi. ~~In similar studies, marine actinomycetes isolated from different regions have been reported to possess antimicrobial activity [17, 18, 29~~].

Table 1: Antimicrobial activity of potential marine actinomycetes isolates by Cup-plate method showing zone of inhibition (mm)

Isolate No.	*E.coli*	*B.cereus*	*P.aeruginosa*	*S.cereviseae*	*Y.lipolytica*	*A.niger* (Zone of inhibition of sporulation)	*A.niger* (Zone of inhibition of mycelium)
ABT-103	-	-	-	-	-	12.5	12.5
ABT-201	-	-	8	-	-	11	-
ABT-203	-	-	-	8	10	11	-
ABT-205	4	11	4	-	-	12	-
ABT-206	5	13	8	-	-	-	-

~~**Figure 1: Agar plate showing zone of inhibition against *A. niger* by ABT-103**~~

3.3 Screening of Marine Actinomycetes isolates for enzyme activities:

All the 13 isolates were screened for the Amylase, Lipase, Protease and L-Asparaginase enzyme activities. The isolates ABT-103, ABT-201 and ABT-206 exhibited Amylase activity. The isolate, ABT-103 showed maximum zone of hydrolysis (Figure 1a). Four isolates, ABT-201, ABT-202, ABT-205 and ABT-206 showed lipase activity. Maximum zone of hydrolysis of 25 mm is exhibited by ABT-206 (Figure 1b). Protease activity was exhibited by five isolates, ABT-101, ABT-104, ABT-201, ABT-204 and ABT-207. Maximum zone of hydrolysis was shown by ABT-101 (Figure 1c) followed by ABT-201. None of the isolates exhibited L-Asparaginase activity. The present work revealed that ABT-101, ABT-103, ABT-201

and ABT-206 are promising isolates for the production of Protease, Amylase and Lipase enzymes (Table 2).

In marine environment, extracellular enzymes play a central role in the recycling of organic carbon and nitrogen compounds. High molecular weight organic compounds cannot be transported directly into bacteria. Thus, bacteria must hydrolyse these organic polymers to smaller molecules before they are incorporated into the cell for subsequent metabolism. This extracellular hydrolytic activity is performed by extracellular enzymes [36]. Thus, in this study, the marine actinomycetes have shown ability to produce extracellular hydrolytic enzymes, amylase, protease and lipase. Literature survey indicated that actinomycetes isolated from marine samples produced various extracellular hydrolytic enzymes. [37, 38, 39]. ~~Literature survey indicated that actinomycetes isolated from marine samples exhibited different enzyme activities [30, 31, 32, 33, 34].~~

Table 2: Enzyme activities of potential marine actinomycetes isolates

	Zone of hydrolysis in mm		
Isolate No	**Amylase**	**Lipase**	**Protease**
ABT-101	-	-	34
ABT-103	10	-	-
ABT-104	-	-	7.5
ABT-201	6	7	31
ABT-202	-	7	-
ABT-204	-	-	15
ABT-205	-	6	-
ABT-206	2	25	-
ABT-207	-	-	22

(a) (b) (c)

Figure 1: Agar plates showing clear zone of hydrolysis (a) Amylase activity by ABT-103, (b) Lipase activity by ABT-201 (c) Protease activity by ABT-101

3.4 Characterization of Marine Actinomycetes isolates:

The 13 isolates were characterized based on their morphological characteristics. The cultural characteristics are tabulated in Table 3.

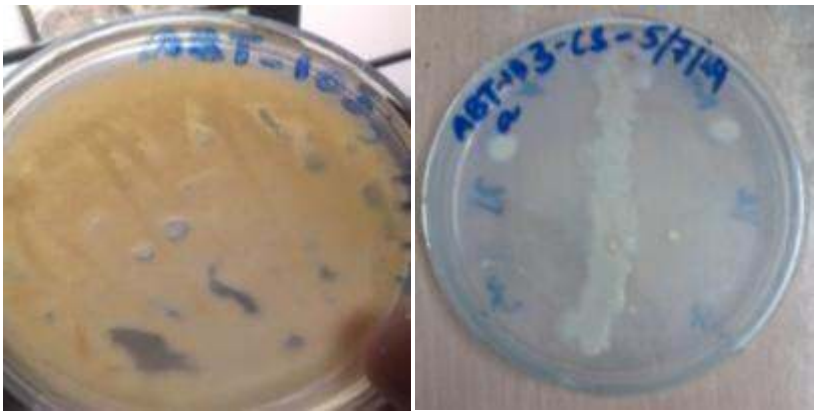
Table 3: Morphological characteristics of Marine isolates

Isolate No.	Aerial Mass Colour	Melanoid Pigmentation	Reverse side Pigmentation	Soluble Pigments
ABT-101	Red	Brown	Red	-
ABT-102	White	-	-	-
ABT-103	Grey	-	Red	Red
ABT-104	Cream	-	-	-
ABT-105	White	-	-	-
ABT-201	White	-	Brownish Black	-
ABT-202	Cream	-	-	-
ABT-203	Cream	-	-	-

ABT-204	White	-	-	-
ABT-205	White	-	Yellow	-
ABT-206	Grey	Brown	Yellowish Brown	Brown
ABT-207	Cream	-	-	-
ABT-208	Green	-	-	-

Among the 13 isolates, the marine actinomycetes strain ABT-103 was found to be a promising isolate for biotechnological applications as it showed amylase production (Figure 1a), red pigment production on SCA medium (Figure 2a&2b), exopolysaccharide production on YEME medium (Figure 2c), and antifungal activity (Figure 2d). Due to the above potential features, the isolate ABT-103 was characterized ~~up to genus level~~ based on morphological, chemotaxonomic, physiological and biochemical characteristics. These characteristics particularly chemotaxonomic can be used as a marker for the identification of actinomycetes up to generic level [18].

(a) **(b)**



(c) **(d)**

Figure 2: Agar plates showing (a & b) red pigment production (c) exopolysaccharide (d) antifungal activity against *A. niger* in primary screening by ABT-103

The substrate mycelium of the marine actinomycete strain, ABT-103 is thick and dry, producing grey colour aerial spore mass (Figure 2a) and reddish reverse side pigmentation (Figure 2b). Microscopic features are branching substrate and aerial mycelium, producing spore chains of Rectiflexible morphology ~~(Figure 3)~~.The cell wall composition studies revealed the presence of glycine and LL- DAP with no characteristic pattern for sugars. Hence, the strain, ABT-103 can be categorized under cell wall Type-I [40]. The biochemical and physiological characteristics are represented in tables 4 and 5. Based on ~~morphological, chemotaxonomic, physiological and biochemical~~ above features, the marine actinomycetes isolate ABT-103 was identified as *Streptomyces* species. The identification will be confirmed by Molecular analysis using 16S rRNA gene sequencing in future studies. As *Streptomyces* species are well known for valuable sources of new bioactive compounds, continuously being isolated from under-researched habitats and characterized based on morphological, physiological, biochemical and chemo-taxonomic methods [12, 41, 42].

~~**Figure 3: Spore chain structure of the Marine Actinomycete isolate, ABT-103 showing Rectiflexible morphology (400X Magnification)**~~

Table 4: Biochemical characteristics of the Marine Actinomycete isolate, ABT-103

S.No	TEST	RESULT
1	Indole	+
2	Methyl-Red	-
3	Voges - Proskauer	+
4	Citrate utilization	+
5	H_2S Production	-
6	Catalase	+
7	Starch hydrolysis	+
8	Casein hydrolysis	+
9	Urease	*±
10	Carbohydrate utilization	
	Glucose	+
	Lactose	+
	Maltose	+
	Sucrose	+
	Mannitol	-
	Xylose	+

* weakly positive

223 **Table 5: Physiological characteristics of the Marine Actinomycete isolate, ABT-103**

Characteristics	Growth
Tempetarure (^{o}C)	
25	Good
30	Good
35	Good
40	Moderate
45	Moderate
pH tolerance	
5	Poor
6	Moderate
7	Good
8	Good
9	Good
10	Moderate
NaCl tolerance (%)	
2	Good
5	Good
8	Poor
11	Nil
14	Nil
17	Nil

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4. CONCLUSION

Potential marine actinomycetes isolates were successfully isolated from the sediment samples of Visakhapatnam coast of Bay of Bengal. The isolate ABT-103 is the best isolate for antifungal activity against *Aspergillus niger*, ABT-205 is the most potential isolate for both antibacterial and antifungal activities, and ABT-206 for antibacterial activity. The isolates ABT-103 and ABT-206 are the best producers of Amylase and Lipase enzymes respectively while ABT-101 and ABT-201 are potential producers of Protease. The most promising isolate, ABT-103 was identified as *Streptomyces* species. The isolates ABT-101, ABT-103, ABT-201, ABT-205 and ABT-206 obtained from marine sediments of Visakhapatnam coast of Bay of Bengal are expected to be useful for the production of novel antibiotics, enzymes with different physical and physiological properties, red pigment, exopolysaccharide and other compounds for various Biotechnological applications in further studies.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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