

Screening of Marine Sediments from Bay of Bengal near Pudimadaka Coast of Andhra Pradesh for Isolation of Lipolytic Actinobacteria and Characterization of the Most Potent Isolates

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Abstract

Ten sediment samples collected at various depths of Bay of Bengal near Pudimadaka Coast of Andhra Pradesh were screened for the isolation of lipolytic Actinobacteria by tributyrin agar clearing method. The selected isolates were cultured under submerged fermentation conditions and assayed for their extra cellular lipase producing capabilities using natural triglyceride (triolein) as substrate. Results indicated that 38 of the 88 isolates from 10 sediment samples showed lipolytic activity after primary screening. These isolates were subjected to secondary screening and lipase activity is estimated quantitatively. After preliminary characterization studies the promising isolate BTS-713 was found to belong to genus *Streptomyces* and was designated as *Streptomyces* sp. BTS-713. In conclusion, these results increased the scope of finding industrially important marine actinomycetes from Pudimadaka Coast of Andhra Pradesh.

Keywords: Actinobacteria, Lipase, Marine

1. Introduction

Lipases have widespread occurrence through out the earth's flora and fauna, they are found in many species of animals, plants and microorganisms [Antonian, E., 1988]. Commercial lipases are obtained from microorganisms as they are more stable compared to plant or animal lipases and they can be obtained cheaply. In the field of biotechnology, much attention has been paid to the use of lipases of microbial origin. They constitute the most important group of biocatalysts having tremendous biotechnological potential because, firstly, they can be produced in large quantities from microbial sources, secondly, they display exquisite chemo selectivity, regioselectivity and stereo selectivity and thirdly, the crystal structures of many lipases have been solved, facilitating considerably the design of rational engineering strategies. These properties make lipases the most widely used group of biocatalysts in organic chemistry [Jaeger, K.E., 2002]. Important applications of lipases in biotechnology include their supplementation to detergents, in the manufacture of food ingredients, pitch control in the pulp and paper industry, and biocatalysis of stereo selective transformations.

Actinomycetes are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of half of the discovered bioactive secondary metabolites [Berdy, J., 1989]. Few members of the actinomycetes have been studied with reference to purification and characterization of lipase enzyme [Sharma, R., 2001]. The marine sediments as a source of bioactive actinomycetes was less exploited. In the present study, one natural saline habitat along the Pudimadaka Coast in the East coast of Southern India was selected for the isolation followed by assessment of lipolytic potential of marine actinomycetes.

2. Materials and methods

2.1 Sampling Procedure

Ten marine sediment samples were collected from Bay of Bengal near Pudimadaka coast using a core sampler. 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. The locations and depths of these sampling stations are summarized in Table 1.

2.2 Isolation of Actinomycetes colonies from Marine sediments

Isolation and enumeration of actinomycetes were performed by the serial dilution plate technique [Ellaiah, 1996] using starch casein agar medium [Kuster, 1964], malt extract-yeast extract agar medium [Pridham, T.G., 1956-57] and chitin agar medium [Hsu, S.C., 1975]. These media containing 50% sea water 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±2°C for 2-3 weeks. After incubation, actinomycetes isolates were distinguished from other microbial colonies which are partially submerged into the agar [Jensen, 1991] and colors of pigmentation including diffusible pigments. Single separated actinomycetes colonies were selected and the sub cultures were maintained on starch casein slants at 4 °C until further use.

2.3 Primary screening for lipolytic Actinobacteria

For the detection of lipolytic actinobacteria, tributyrin agar clearing method was followed [Jani, T.R., 1998]. Twenty ml of tributyrin agar medium was inoculated with a loopful of isolate and incubated at 28°C for five days. The composition of tributyrin agar medium is (g/l): (NH₄)₂ SO₄, 5; Na₂ HPO₄, 6; KH₂ PO₄, 2; MgSO₄, 3; CaCl₂, 3; agar 20 and tributyrin, 10ml with pH 6.0. Lipolytic zone of the isolates was measured and these isolates were subjected to secondary screening by submerged fermentation.

2.4 Secondary screening for lipase production

For the production of lipase, the selected isolates were cultivated in a synthetic medium containing olive oil (source of natural triglyceride, triolein) as the sole carbon source under submerged fermentation conditions and assayed for the lipolytic activity of the culture filtrates. 45ml of production medium is taken in 250ml Erlenmeyer flask and inoculated with a loopful culture of each isolate. The flasks were incubated at 28°C for 4 days on a rotary shaker (150 rpm). The culture broth was filtered and the clear filtrate was used as the source of crude enzyme. The composition of the production medium is (g/l): Olive oil, 10; (NH₄)₂ SO₄, 5; Na₂ HPO₄, 6; KH₂ PO₄, 2; MgSO₄, 3; CaCl₂, 3 with pH 6.0.

2.5 Lipase assay

The culture broth was filtered and the lipase activity in the culture filtrate was determined by titrimetry (olive oil substrate emulsion method) as described by Musantra [Musantra, 1992]. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1µmole equivalent fatty acid / ml/ min at 40 °C under the standard assay conditions. All the experiments were carried out in triplicate and the mean of the three values was presented.

2.6 Preliminary identification of the five most potent actinomycetes isolates

The five most promising actinomycetes isolates were identified up to the genus level by macroscopic and microscopic morphological methods as per Bergey's 2000 manual [Bergey, 2000]. The macroscopic method was done by colony characterization on yeast malt extract agar (YMA) medium. Colour of colony and presence of pigmentation were noted. The microscopic characterization was done by cover slip culture method observed after three days. The presence or absence of aerial and substrate mycelium, spore formation, and pigmentation of the vegetative or substrate mycelium were observed. Further characterization of selected isolates was done as per the ISP protocol [Shirling, 1966]. The utilization of carbon sources was examined on Pridham and Gottlieb's medium [Pridham, 1948] containing miscellaneous carbohydrates to a final concentration of 1%. Cell wall of the selected isolates was purified and analyzed by the methods of Lechevalier and Lechevalier [Lechevalier, 1980]. Whole cell sugars were analyzed according to the method of Becker *et al.* [Becker, B., 1964]. The selected isolates were screened for sodium chloride tolerance (1, 4, 7, 10 and 13% w/v) on Yeast-Malt agar slants according to Trenser *et al.* [Trenser, 1968]. Maximum sodium chloride concentration in the medium allowing any growth was recorded.

3. Results and discussion

3.1 Isolation of actinomycetes colonies from marine sediments

Using the selective media, 88 actinomycetes strains were isolated (Table 2), of which maximum number of actinomycetes isolated was in Malt extract - Yeast extract agar medium (44) followed by starch casein agar medium (25) and chitin agar medium (19). The entire 10 samples of Pudimadaka are found suitable for the isolation of actinomycetes. High or low number of active strains found depends on many factors like the medium

and methods of screening. Moreover, there are so many factors which affect actinomycetes growth and enzyme production, including the chemical and biological environment.

3.2 Primary screening for lipolytic actinobacteria

38 of the 88 isolates showed lipolytic activity (Table 3). This suggests that sediments of Pudimadaka are good sources for isolating lipolytic actinomycetes. Only few reports of lipases from marine actinomycetes were made by previous researchers although marine actinomycetes have excellent capacity to elaborate a wide diversity of enzymes [Sharma, 2001]. The lipolytic activities of all the stains are shown in Table 3. As indicated in the table, isolate BTS-713 showed maximum lipolytic activity after primary screening (Table 4a and 4b). Screening with the help of tributyrin is a convenient and presumptive test for the detection of lipolytic organisms; hence all the isolates were screened using tributyrin agar clearing method.

3.3 Secondary screening for lipase production

All the positive isolates were subjected to submerged fermentation conditions and assayed for lipolytic activity quantitatively. Since tributyrin is not a substrate for lipases alone, all the selected positive isolates are confirmed for their lipolytic activity by the hydrolysis of natural triglyceride (triolein) under submerged fermentation conditions and the results are presented in Table 5a and 5b. As shown in the Table 6, the isolate BTS-713 showed maximum lipolytic activity of 0.0514U among all the positive isolates. Optimization of nutritional and physical parameters for reduction of cost in industrial scale production and presence of other bioactive compounds influencing lipolytic activity of the isolate had to be determined to determine the potency of BTS-713 (Fig.1). But our study focuses on screening of marine sediments for lipolytic actinobacteria which has been accomplished.

3.4 Preliminary identification of the five most potent actinomycetes isolates

The five most potent lipolytic actinomycetes isolates were subjected to characterization up to genus level. The culture and morphological properties of the five isolates are shown in Table 7. The results indicate that among the five isolates, three isolates BTS-716, BTS-701, and BTS-711 were characterized by single spores at the tips of sporophores and two isolates BTS-713 and BTS-709 by spiral polyspores. Out of five isolates BTS-716, BTS-701, and BTS-711 showed black spore mass, while BTS-713 isolate showed green-grey spore mass. The form of the colony of isolates BTS-716, BTS-701 and BTS-711 was described to be compact, folded, raised and lichenoid to leathery and that of the isolates BTS-713 and BTS-709 was discrete, floccose and powdery. According to Waksman [1961] such colour and form is exhibited by colonies of both *Streptomyces* and *Micromonospora*. However, the colour of the growth and the form of the colony could not serve as basis of pointing out the genus to which actinomycetes isolates belongs to. Hence, its morphological properties must serve as the primary basis of characterization. One distinguish morphological property possessed by the isolates BTS-716, BTS-701, and BTS-711 is absence of an aerial mycelium, a characteristic possessed by all members of *Micromonospora* [Bergey, 2000]. 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-716 and BTS-701 that dissolves into the medium had also been mentioned by Waksman [1961] to be a possible characteristics of the genus, although not exclusively. Isolates BTS-713 and BTS-709 formed substrate mycelium and abundant aerial mycelium with powdery spore mass. Polysporic actinomycetes, forming characteristic aerial and substrate hyphae represent an important microscopic criterion to identify the genus *Streptomyces* [Bergey, 2000]. The physiological and biochemical characteristics of the isolates are shown in Table 8a and 8b. Kamfer *et al.* suggested the physiological tests as indispensable tools for classification and identification of actinomycetes. Isolates BTS-716, BTS-709, and BTS-701 exhibited positive reaction to melanin pigmentation and tyrosine reaction while these properties were found to be negative for isolates BTS-711 and BTS-713. Isolates BTS-711, BTS-716 and BTS-709 had the ability for H₂S production and nitrate reductase was secreted by isolate BTS-711, BTS-713, and BTS-709. 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 actinomycetes strains [Pridham, T.G., 1948]. 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-711, BTS-716 and BTS-701 were grown on mannitol, rhamnose, raffinose and glycerol. Isolate BTS-713 efficiently utilized the carbon sources such as, mannitol, rhamnose, raffinose, glycerol and cellobiose. Rhamnose, raffinose, galactose were assimilated by the isolate BTS-709 as the good source of carbon and energy while ribose, galactose, arabinose and cellobiose were well utilized by the isolate BTS-711. All the isolates could coagulate and peptonize milk. Tolerance of the strain 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. All the isolates were resistant to antibiotics used for identification studies and also grew well at 25-35°C. Isolates BTS-713 and BTS-709 showed growth at pH range of 6-8 and BTS-716, BTS-701 and BTS-711 at pH range 5-9. Cummins and Harris [Cummins, C.S., 1956] 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 [1980] the sugar composition often provides valuable information on the classification and identification of actinomycetes. Considering the above, an attempt was made to identify the actinomycetes, by analyzing their cell components. The cell wall peptidoglycan of three isolates BTS-716, BTS-701 and BTS-711 contained Mesodiaminopimelic 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* [Lechevalier, 1970]. However the cell wall peptidoglycan of BTS-713 and BTS-709 contained L-L-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* [Lechevalier, 1970]. Sequence based identification and further characterization studies were not performed due to lack of infrastructural facilities.

On the basis of morphological and chemo taxonomical characteristics, the isolates BTS-716, BTS-701 and BTS-711 were identified as belonging to the family Streptomycetaceae and the genus *Micromonospora* while isolates BTS-713 and BTS-709 were assigned to the family of Streptomycetaceae and the genus *Streptomyces* [Pridham, T.G., 1958; Williams, S.T., 1989]. This suggests that the natural substrates such as marine sediments are also good sources for isolation of lipolytic actinobacteria.

4. Conclusion

Based on the screening results it had been shown that sediments of Pudimadaka, coast of Bay of Bengal possess lipolytic actinomycetes and maybe tapped as one of the potential source for lipase production. The results in general reflect on the lipase production potentiality 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 promising lipolytic Actinobacteria.

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Table 1. Details of sampling stations

Station No:	Depth (meters)	Latitude	Longitude
BTS-1	10	+17.30500	+83.02300
BTS-2	20	+17.30500	+83.04400
BTS-3	30	+17.30500	+83.08000
BTS-4	10	+17.29000	+83.00816
BTS-5	20	+17.29000	+83.02205
BTS-6	30	+17.29000	+83.05086
BTS-7	40	+17.29015	+83.07480
BTS-8	10	+17.27000	+82.59398
BTS-9	20	+17.27000	+82.59295
BTS-10	30	+17.27000	+83.01314

Table 2. Number of Isolates from Sediment Samples

Station No:	Numbering of Isolates	Number of Isolates
BTS-1	BTS-101 to 104	4
BTS-2	BTS-201 to 208	8
BTS-3	BTS-301 to 312	12
BTS-4	BTS-401 to 405	5
BTS-5	BTS-501 to 509	9
BTS-6	BTS-601 to 613	13
BTS-7	BTS-701 to 716	16
BTS-8	BTS- 801 to 803	3
BTS-9	BTS- 901 to 907	7
BTS-10	BTS- 1001 to 1011	11

Table 3. Isolates having lipolytic activity (Primary Screening)

Station No.	Isolates having lipolytic activity	Number of lipolytic isolates
BTS-1	BTS-104	1
BTS-2	BTS-203,BTS-205,BTS-206	3
BTS-3	BTS-304,BTS-307,BTS-310,BTS-311, BTS-312	5
BTS-4	BTS-403	1
BTS-5	BTS-501,BTS-505,BTS-508,BTS-509	4
BTS-6	BTS-602,BTS-604,BTS-607,BTS-608,BTS-611,BTS-612	6
BTS-7	BTS-701,BTS-702,BTS-704,BTS-706,BTS-709,BTS-711,BTS-712,BTS-713,BTS-716	9
BTS-8	BTS-803	1
BTS-9	BTS-903,BTS-905,BTS-906	3
BTS-10	BTS-1003,BTS-1005,BTS-1006,BTS-1009,BTS-1010	5

Table 4. (a) Lipolytic activity of selected isolates on tributyrin agar

Station No.	Isolate No.	Lipolytic zone (R/r)
BTS-1	BTS-104	1.1
BTS-2	BTS-203	1.22
	BTS-205	1.35
	BTS-206	1.25
BTS-3	BTS-304	1.6
	BTS-307	1.89
	BTS-310	1.8
	BTS-311	1.625
	BTS-313	1.82
BTS-4	BTS-403	1.2
BTS-5	BTS-501	1.4
	BTS-505	1.416
	BTS-508	1.58
	BTS-509	1.38
BTS-6	BTS-602	2.27
	BTS-604	2.428
	BTS-607	2.33
	BTS-608	2.1
	BTS-611	2.13
	BTS-612	2.63

R: Hydrolyzed zone diameter (mm); r: Growth zone diameter (mm)

Table 4. (b) Lipolytic activity of selected isolates on tributyrin agar

Station No.	Isolate No.	Lipolytic zone (R/r)
BTS-7	BTS-701	3.7
	BTS-702	3.2
	BTS-704	3
	BTS-706	3.5
	BTS-709	3.9
	BTS-711	3.6
	BTS-712	3.4
	BTS-713	4
	BTS-716	3.8
BTS-8	BTS-803	1
BTS-9	BTS-903	1.26
	BTS-905	1.3
	BTS-906	1.28
BTS-10	BTS-1003	2
	BTS-1005	1.88
	BTS-1006	1.9
	BTS-1009	1.857
	BTS-1010	1.769

R: Hydrolyzed zone diameter (mm); r: Growth zone diameter (mm)

Table 5. (a) Lipolytic activity of the isolates under submerged fermentation conditions

Station No.	Isolate No.	Lipase activity (U/ml)
BTS-1	104	0.01428
BTS-2	203	0.01714
	205	0.01824
	206	0.01756
BTS-3	304	0.02788
	307	0.02832
	310	0.02805
	311	0.02791
	313	0.02812
BTS-4	403	0.01628
BTS-5	501	0.02
	505	0.02103
	508	0.02405
	509	0.01957
BTS-6	602	0.03132
	604	0.03205
	607	0.03142
	608	0.03
	611	0.0312
	612	0.03216

U=units of lipase activity were expressed in text

Table 5. (b) Lipolytic activity of the isolates under submerged fermentation conditions

Station No.	Isolate No.	Lipase activity (U/ml)
BTS-7	701	0.0471
	702	0.0405
	704	0.04
	706	0.0431
	709	0.0485
	711	0.0442
	712	0.0412
	713	0.0514
	716	0.0481
BTS-8	803	0.01328
BTS-9	903	0.01766
	905	0.01814
	906	0.01794
BTS-10	1003	0.02857
	1005	0.02826
	1006	0.02844
	1009	0.02813
	1010	0.02798

U=units of lipase activity were expressed in text

Table 6. Ten Most Promising Isolates having Lipase activity

Isolate No.	Lipase activity (U/ml)
BTS-713	0.0514
BTS-709	0.0485
BTS-716	0.0481
BTS-701	0.0471
BTS-711	0.0442
BTS-706	0.0431
BTS-712	0.0412
BTS-702	0.0405
BTS-704	0.0400
BTS-612	0.03216

Table 7. Cultural and Morphological characteristics of the selected Isolates

	BTS-713	BTS-709	BTS-716	BTS-701	BTS-711
Spore formation	spiral polyspores	spiral polyspores	singly at the tips of sporophores	singly at the tips of sporophores	singly at the tips of sporophores
Spore mass colour	yellow-brown	green-grey	black	black	black
Colour of the colony	white	pale yellow	white, then yellow	white, then yellow	white
Form of the colony	discrete, floccose and powdery	discrete, floccose and powdery	compact, folded, lichenoid then leathery, raised	compact, folded, lichenoid then leathery, raised	compact, folded, lichenoid then leathery, raised
Aerial mycelium	present	present	absent	absent	absent
Substrate mycelium	present	present	present	present	present
Soluble pigment	nil	nil	present(dark brown)	present(dark brown)	nil

Table 8. (a) Physiological and biochemical characteristics of the selected Isolates

Characteristics	BTS-713	BTS-709	BTS-716	BTS-701	BTS-711
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%
Antibiotic resistance (µg/ml)					
Erythromycin (10)	+	+	+	+	+
Kanamycin (12)	+	+	+	+	+
Rifampicin (5)	+	+	+	+	+
Streptomycin (10)	+	+	+	+	+

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

Table 8. (b) Physiological and biochemical characteristics of the selected Isolates

Characteristics	BTS-713	BTS-709	BTS-716	BTS-701	BTS-711
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	+	+	+	+	+
Milk coagulation and peptonization	+	+	+	+	+
pH tolerance	6-8	6-8	5-9	5-9	5-9
Cell wall type	I	I	II	II	II

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



Figure 1. Isolate No.BTS-713 identified as *Streptomyces sp.* on Starch casein agar