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 Bengalfor the production of bioactive compoundsand 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

Actinomycetesare filamentous, gram-positive bacteria whichrepresent 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 actinomycetespossess 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 actinomycetesisolated from different regions have been reported to possess antimicrobial activity [16, 17, 18, 19] andenzyme activitiesfor 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 targetshave 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 ofactinomycetes 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 collectedalong the coast of Bay of Bengal from Visakhapatnam, AP, Indiaat 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 plateand 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 wereserially 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 ofMarine 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, Pseudomonasaeruginosa* and three fungi, *Saccharomycescereviseae, Yarrowialipolytica*and*Aspergillusniger* 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 streakedin 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 forsecondary 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 inNutrient 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 ofMarine 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-Asparaginaseby 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-asparaginaseactivities.

2.6 Characterization ofMarine 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 isolatesby spread plate method.Among these isolates,morphologically distinct 13 pure gram-positive isolates were selected for further studies.

3.2 Screening ofMarine Actinomycetes isolates for Antimicrobial activity:

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. 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	-	-	-	-

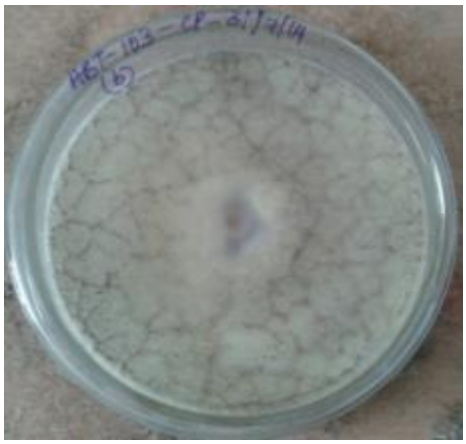

Figure 1: Agar plate showing zone of inhibition against *A. niger* by ABT-103

3.3 Screening ofMarine 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.Theisolate, ABT-103 showed maximum zone of hydrolysis (Figure 2a). 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 2b). 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 2c) 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).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 2: 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 MarineActinomycetes 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	-	-	-

174

175 Among the 13 isolates, the marine actinomycetes strain ABT-103 was found to be a promising isolate for
176 biotechnological applications as it showed amylase production (Figure 2a), red pigment productionon
177 SCA medium (Figure 3a& b), exopolysaccharide production on YEME medium (Figure 3c), and antifungal
178 activity (Figure 3d). Due to the above potential features, the isolate ABT-103 was characterizedupto
179 genus level based on morphological, chemotaxonomic, physiological and biochemical characteristics.

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182 **(a)** **(b)**

(c) **(d)**

Figure 3: 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 3a) and reddish reverse side pigmentation (Figure 3b). Microscopic features are branching substrate and aerial mycelium, producing spore chains of Rectiflexible morphology (Figure 4).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 [35]. The biochemical and physiological characteristics are represented in tables 4 and 5. Based on morphological, chemotaxonomic, physiological and biochemical features, the marine actinomycetes isolate ABT-103 was identified as *Streptomyces* species. The identification will be confirmed by Molecular analysis using 16S rRNAgene sequencing in future studies.

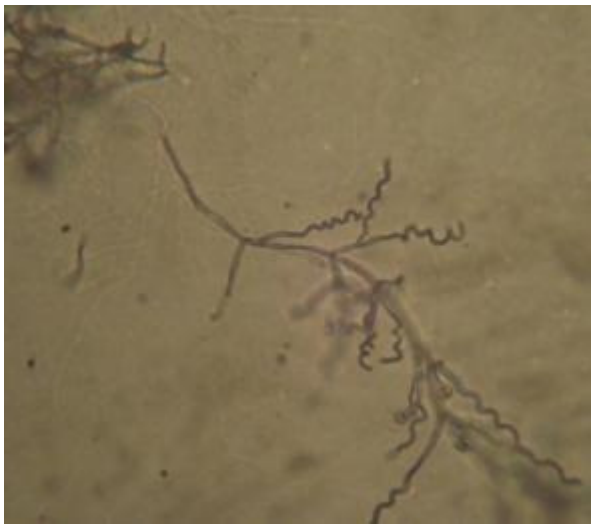

Figure 4: Spore chain structure of the Marine Actinomycete isolate, ABT-103 showing Rectiflexible morphology

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	+

202 * weakly positive

203

204 **Table 5: Physiological characteristicsof 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

4. CONCLUSION

Potential marineactinomycetes 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 *Aspergillusniger,* 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.

REFERENCES

1. Berdy J. Bioactive microbial metabolites, a personal view. J. Antibiotic. 2005; 58: 1-26.

2. Dasari VR., Muthyala MK, Nikku MY, Donthireddy SR. Novel Pyridinium compound from marine actinomycete, *Amycolatopsisalba*var. Nov. DVR D4 showing antimicrobial and cytotoxic activities in vitro. Microbiol. Res. 2012; 167:346-351.
3. Pecznska-Czoch W, Mordarski M. Actinomycete enzymes. In: Goodfellow M, Williams ST, Mordarski M, editors. Actinomycetes in Biotechnology. London, U.K. Academic Press. 1988; 219-283.
4. Zheng Z, Wei Z, Yaojian H, Zhiyuan Y, Jun L, Huirong C, Wenjin S. Detection of antitumor and antimicrobial activities in marine organism associated actinomycetes isolated from the Taiwan Strait, China. FEMS Microbiology Letters. 2000; 188:87-91.
5. Cragg GM, Kingston DGI, Newman DJ, Editors. Anticancer Agents from Natural Products. Taylor and Francis. 2005
6. Mann J. Natural products as immunosuppressive agents. Nat. Prod. Rep. 2001; 18:417-430.
7. Ventosa A, Nieto JJ, Oren A. Biology of Moderately Halophilic Aerobic Bacteria. Microbiol. Mol. Biol. Rev. 1998; 62:504-544.
8. Lam KS. Discovery of novel metabolites from marine actinomycetes. Curr. Opin. Microbiol. 2006; 9: 245-251.
9. Bull AT, Stach JEM, Ward AC, Goodfellow M. Marine actinobacteria: perspectives, challenges, future directions. Antonie Van Leeuwenhoek. 2005; 87:65-79.
10. Jensen PR, Gontang E, Mafnas C, Mincer TJ, Fenical W. Culturable marine actinomycete diversity from tropical Pacific Ocean sediments. Environ. Microbiol. 2005; 7: 1039-1048.
11. Fiedler HP, Bruntner C, Bull AT, Ward AC, Goodfellow M, Potterat O, Puder C, Mihm G. Marine actinomycetes as a source of novel secondary metabolites. Antonie Van Leeuwenhoek., 2005; 87:37-42.
12. Ramasamy V, Subbanand M, Annamalai P. Isolation, Characterization and Antimicrobial activity of Actinobacteria from Point Calimere Coastal region, East Coast of India, Int. Res. J. Pharm. 2010; 1(1):358-365.
13. Siva Kumar K, Haritha R, Jagan Mohan YSYV, Ramana T. Screening of Marine Actinobacteria for Antimicrobial Compounds. Res. J. Microbiol. 2011; 6(4): 385-393.

14. Usha KM, Vijayalakshmi M, Sudhakar P, Sreenivasulu K. Isolation and Identification of rare Actinomycetes from Mangrove Ecosystem of Nizampatnam. Malays. J. Microbiol. 2012; 8(2): 83-91.
15. Rao K V, T. Raghava Rao. Isolation and screening of antagonistic Actinomycetes from mangrove soil. Innovare Journal of Life Science, 2013; 1(3), 28-31.
16. Sirisha B, Haritha R, Jagan Mohan YSYV, Siva Kumar K, Ramana T. Bioactive Compounds from Marine Actinomycetes Isolated from the Sediments of Bay of Bengal. Int. J. Pharm. Chem. Biol. Sci. 2013; 3(2): 257-264
17. Dhanasekaran D. Rajkumar G, Sivamani P, Selvamani S, Panneerselvam A, Thajuddin N. Screening of salt pans Actinomycetes for antibacterial agents. The Internet. J. Microbiol. 2005; 1:6-12.
18. Sivakumar K, Sahu MK, Kathiresan K. Isolation and characterization of marine *Streptomycetes*, producing antibiotic from a mangrove environment. Asian J. Microbiol. Biotechnol. Environ. Sci. 2005; 7: 457-464.
19. Usama RA, Sheila MPE, Amro H, Mona R, Soad HAEE, Safwat A, Ute H. Isolation, Phylogenetic Analysis and Anti-infective Activity Screening of Marine Sponge-Associated Actinomycetes. Marine Drugs. 2010; 8(3):399-412.
20. Dhevagi P,Poorani E. Isolation and Characterizaion of L-asparginase from marine actinomycetes. Indian journal of Biotechnology. 2005; 5: 514-520.
21. Leon J, Liza L, Soto I, Cuadra D, Patino L, Zerpa R. Bioactives actinomycetes of marine sediment from the central coast of Peru. Revi Peru Boil. 2007; 14:259–270
22. Thumar JT, Singh SP. Secretion of an alkaline protease from a salt-tolerant and alkaliphilic, *Streptomyces clavuligerus* strain MIT-1. Brazil J Microbiol 2007; 38:766–772
23. Samrat C, Abhijit K, Chandrakant K, Kakasaheb M, Balasaheb C. Isolation and characterization of novel -amylase from marine Streptomyces sp. D1, Journal of Molecular Catalysis B: Enzymatic 2009;58: 17–23.

24. Subramani, R. and Narayanasamy, M. Screening of marine actinomycetes isolated from the Bay of Bengal, India for antimicrobial activity and industrial enzymes. World Journal of Microbiology and Biotechnology. 2009; 25(12): 2103-2111
25. Gulve RM, Deshmukh AM. Enzymatic activity of actinomycetes isolated from marine sedimentes. Recent Research in Science and Technology. 2011; 3(5): 80-83
26. Schumacher RW, Talmage SC, Miller SA, Sarris KE, Davidson BS, Goldberg A. Isolation and structure determination of an antimicrobial ester from a marine sediment derived bacterium. J. Nat.Prod. 2003; 66:1291-1293.
27. Asolkar RN. Schroder D, Hechmann R, Lang S, Doblerand IW, Laatsch H. Helquinoline: A new tetrahydroquinoline antibiotic from Janibacter limosus HELL. J. Antibiotic. 2004; 57:17-23.
28. Adinarayana G, Elliah P, Axel Z. Cytotoxic compounds from a marine actinomycete, *Streptomyces albivinaceus* var. baredar AUBN10/2. African J. Biotech. 2010; 9:7197-7202.
29. Asha Devi NK, Rajendran R, Karthik Sundaram S. Isolation and characterization of bioactive compounds from marine bacteria. Indian J. Nat. Prod. Resour. 2011; 2(1):59-64.
30. Selvam K, Vishnupriya B, Bose VSC. Screening and Quantification of Marine Actinomycetes Producing Industrial Enzymes Amylase, Cellulase and Lipase from South Coast of India. Int. J. Pharm. Biol. Arch. 2011; 2(5): 1481-1487.
31. Edilla RDS, Zozilene NST, Nuria MC, Diogo AJS. Aline SRB, Rodrigi PN. Production of α-Amylase from *Streptomyces* sp. SLBA-08 Strain Using Agro-Industrial By-Products. Braz. Arch. Biol. Technol. 2012; 55(5):793-800.
32. Vijayan N, Sagadevan E, Arumugam P, Hussain AJ, Jayaprakashavel MS. Screening of Marine Bacteria for multiple Biotechnological applications. J. Acad. Ind. Res. 2012; 1: 348-354.
33. Raghunathan R, Padhmadas R Production, purification and characterization of α-amylase using *Streptomyces* app. PDS1 and *Rhodococcus* spp. isolated from Western Ghats, Int.J.Curr. Microbiol. Appl. Sci. 2013; 2(8): 206-214.

34. Kadam A, Ruhi Rizvi S, Kamble LH. A report on extracellular enzyme production potential of actinomycetes isolated from sediments of river Godavari, India, Bioscience Discovery. 2014; 5(1): 121-122.
35. Lechevalier MP, Lechevalier H. Chemical composition as a criterion in the classification of aerobic actinomycetes. Int. J. Syst. Bacteriol. 1970; 20: 435-443.