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Biotechnological potential of marine actinobacteria: Screening for antimicrobials and industrial enzymes of manglicolous actinobacteria

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Abstract

Actinobacteria are widely distributed in the marine environment including the mangroves and the mangrove environment has been characterized as having abundant biological resources and a high level of endemism. Information on the actinobacteria of the mangrove environment is less and hence the present study was carried out in different mangrove areas of Bhiterkanika, Orissa. A total of 43 different actinobacterial strains were recorded from the mangrove sediment samples of Bhiterkanika. Among them, 19 isolates were morphologically distinct based on the colour of the spore mass, riverside colour, aerial and substrate mycelia, production of diffusible pigment and sporophore morphology. These isolates were identified as *Streptomyces* species. Actinobacterial strains were tested for amylase, cellulase, protease, lipase, L-asparginase, L-glutaminase, chitinase and pectinase activity and they were also screened for antimicrobial compounds; 10 isolates were found to be potential producers of these compounds. Results of the actinobacterial antimicrobial compounds and enzymes have indicated the scope for finding out more industrially important marine actinobacteria from the Bhiterkanika mangrove environment and they could be vital sources for the discovery of medicinally and industrially useful molecules and enzymes.

Key words: Mangrove, actinobacteria, industrial enzymes, antimicrobial compounds.

INTRODUCTION

Actinobacteria are aerobic, non-motile and gram-positive bacteria with a high G+C content in their DNA (70 to 80%) and are physiologically related to the bacteria based on the evidence of 16S ribosomal RNA cataloging studieds (Goodfellow and Williams, 1983). These microorganisms are phenotypically highly diverse and are found in most natural environments and are present in various ecological habitats such as soil, fresh water, back water, lake, compost, sewage and marine environment (Goodfellow and Williams, 1983). Actinobacteria are nutritionally versatile and produce a variety of high value metabolites (Gomathinayagam and Lakshmanaperumalswamy, 2001) which are continuously and routinely screened for new bioactive substances. Actinobacterial population has also been identified as one of the major groups of soil populations (Kuster, 1976), which may vary with the soil type. Isolation and characterization of novel actinobacteria from unexplored habitats are proved to be a valuable source of new bioactive metabolites (Ellaiya *et al*., 2004; Yi *et al*., 2004; Bull and Stach, 2007).

Hence, there is a need to explore the unexplored marine ecosystems such as mangroves that are a wealthy source of new and important bioactive compounds and commercially important industrial enzymes. In this context, mangroves which are unique intertidal ecosystems of the tropics and support genetically diverse groups of aquatic and terrestrial microorganisms (Surajit *et al*., 2006) can be explored. Further, studies on the diversity of mangrove actinobacteria are important not only in terms of basic research, but also for the biotechnological exploitation of such organisms (Chitti *et al*., 2008).

MATERIALS AND METHODS

Collection of soil sample

Actinobacteria were isolated from the sediment samples, collected from different (Dungmal, Bhiterkanika and Thakurdia) areas of the Bhiterkanika mangrove environment, situated along the east coast of Orrisa, India, using a core sampler. The collected samples were transferred to a sterile polythene bag and transported to the laboratory, amongst the samples stored and transported in cooling icebox were maintained.

Isolation of actinobacterial colonies

Isolation and enumeration of actinobacteria were performed by the serial dilution plate technique (Balagurunathan *et al*., 2002). Media containing 50% sea water were supplemented with streptomycin (5µg/ml) to inhibit bacterial and fungal contaminations and all the plates were incubated at 37°C for 2-3 weeks. After incubation, actinobacteria were isolated.

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Screening for antimicrobial activity of actinobacteria

Isolates were purified and screened for their antimicrobial activity against pathogenic bacteria. The 19 actinobacterial strains isolated were streaked as parallel lines in the Nutrient agar plates and incubatedat at 27^0C for 5 days. After observing a good ribbon- like growth of the actinobacteria in the petriplates, pathogens were streaked at right angles to the original streak of actinobacteria and incubated at 27^0C. The inhibition zone was measured after 24 and 48 hrs. A control plate was also maintained without inoculating the actinobacteria to assess the normal growth of the bacteria.

Screening for industrially important enzymes of actinobacteria

Amylase activity

Amylase activity of the strains was tested using Starch agar medium. Actinobacterial strains were streaked on Starch agar and incubated at 37°C for about 7 days. After incubation, iodine solution was flooded on to the plates and observed for zone of clearance (Ranjekar and Sridhar, 2002).

Cellulase activity

Cellulase activity of the strains was screened qualitatively in CMC (Carboxy Methyl Cellulose) agar medium. The plates were incubated at 37°C for 7 days. To visualize the hydrolysis zone, the plates were flooded with an aqueous solution of 0.1% Congo red for 15 min and washed with 1 M NaCl (Apun *et al.*, 2000).

Protease activity

Protease activity of the actinobacterial strains was determined using Gelatin agar medium. Actinobacterial strains were streaked on Gelatin agar and incubated at 37°C for 7 days. After incubation, all the plates were flooded with mercury chloride solution and observed for zone of clearance around the streak (Cruickshank et al., 1975),

Lipase activity

Actinobacterial strains were screened for lipase activity, using Tween's 80 agar medium. The strains were streaked on Tween's 80 agar and incubated at 37°C for 7 days. After incubation, lipase activity was determined by the formation of the characteristic opaque zone around the streak (Ranjekar and Sridhar, 2002).

L-Asparaginase activity

Asparaginase activity of the actinobacterial strains was determined, using Glycerol-Asparagine broth. The strains were suspended in distilled water and inoculated in 5 ml of Glycerol-Asparagine broth and incubated at 37°C for 7 days. After the 7th day, the broth was filtered through Whatman No. 1 filter paper. Activity of L-asparaginase was measured by adding 0.5ml of Nessler¢s reagent to the filter cultured broth (Dhevendaran *et al.,* 1999).

Glutaminase activity

Glutaminase activity of the actinobacterial strains was determined using Minimal glutamine broth. The strains were suspended in distilled water and this was inoculated in 5 ml of Minimal glutamine broth and incubated at 37°C for 7 days. After the 7th day, the broth was filtered through Whatman No. 1 filter paper. Activity of L-asparaginase was measured by adding 0.5ml of Nessler¢s reagent to the filter cultured broth (Dhevendaran *et al.,* 1999).

Pectinase activity

Pectinase activity of the actinobacterial strains was determined using Pectin agar. The strains were streaked on Pectin agar and incubated at 37°C for about 7 days. After incubation, iodine solution was flooded on to the plates and observed for the zone of clearance (Pereira *et al.,* 1999).

Chitinase activity

Chitinase activity of the actinobacterial strains was determined using chitin agar. Actinobacterial strains were streaked on Chitin agar and incubated at 37°C for about 7 days. After incubation, the plates were observed for the zone of clearance (Mukesh *et al*., 2009).

Taxonomic investigation

Cell wall of the selected isolates were purified and analyzed for (Lechevalier and Lechevalier, 1970) and the whole cell sugars were analyzed according to the method of Becker *et al.* (1964).

Species level identification

Aerial mass colour

Colour of the mature sporulating aerial mycelium was recorded in a simple way (White, grey, red, blue and violet). When the aerial mass colour fell between the two colour series, both the colours were recorded. If the aerial mass colour of a strain to be studied showed intermediate tints, then also, both the colour series were noted. This test was carried out on the ISP-1 medium (Shirling and Gottlieb, 1966).

Melanoid pigments

Grouping was made on the production of melanoid pigments (*i.e.* greenish brown, brownish black and distinct brown) on the medium. The strains were grouped as melanoid pigment produced (+) and not produced (-). This test was carried out on Tyrosine agar medium (ISP7), as recommended by International *Streptomyces* Project (Shirling and Gottlieb, 1966).

Reverse side pigments

Strains were divided into two groups, according to their ability to produce characteristic pigments on the reverse side of the colony, namely, distinctive (+) and not distinctive or none (-). In case, a colour with low chroma as pale yellow ,olive or yellowish brown occurred, it was included in the latter group (-).

Soluble pigments

Strains were divided into two groups based on their ability to produce soluble pigments other than melanin: namely, produced (+) and not produced (-). The colour was recorded (red, orange, yellow, blue, green and violet).

Spore chain morphology

A drop of agar was spread well on the slide and allowed to solidify into a thin film so as to facilitate direct observation under microscope. The cultures were incubated at 28 ± 2°C and examined periodically for the formation of aerial mycelium, sporophore structure, spore chain and spore morphology. Species belonging to the genus *Streptomyces* were divided into three sections (Shirling and Gottlieb, 1966), namely rectiflexibiles (RF), retinaculiaperti (RA) and spirals (S).

Assimilation of carbon source

Ability of the different actinobacterial strains in utilizing various carbon compounds as source of energy was studied following the method recommended by the International Streptomyces Project (ISP) (Shirling and Gottlieb, 1966). Carbon sources for this test were arabinose, xylose, inositol, mannitol, fructose, rhamnose, sucrose and raffinose. These carbon sources were sterilized by ether sterilization without heating. For each of the carbon sources, utilization is expressed as positive (+), negative (-), or doubtful (±). In the 'doubtful' strains, only a trace of growth slightly greater than that of the control was noticed.

RESULTS AND DISCUSSION

Isolation and identification of actinobacteria

In the present study, population density of actinobacteria was $5x10^4$ CFU/g. This is comparable with that of the earlier work by Sivakumar *et al.* (2005) who found the maximum density of $4x10^4$ CFU/g from the Phichavaram mangrove environment. Sahu *et al.* (2005) reported a maximum density of $3.15x10^3$ CFU/g from the Vellar estuary.

Actinobacteria were isolated from the mangrove sediments of the Andaman and Nicobar Islands, India. Here, the actinobacterial population density was recorded as 22 CFU-10^{-6}/gm in KUA medium followed by 12 CFU-10^{-6}/gm in AIA medium and 8 CFU-10^{-6}/gm in SCA medium (Baskaran *et al.,* 2011). Totally, 107 different actinobacterial strains were isolated from the marine sediments, collected from five coastal sites of Konkan coast of Maharashtra and 90 actinobacterial isolates were identified up to the genus level (Gulve and Deshmukh, 2011). A total of 38 different actinobacterial strains were obtained from mangrove soil samples collected from the Pichavaram mangroves, Tamil Nadu, India (Sweetline *et al.,* 2012). Recently, Mohanta (2014) reported nine isolates of cellulose-degrading actinobacteria from the sediment samples of the Bhiterkanika mangroves, and twenty five actinobacterial colonies were isolated from the sediment samples of Valapattanam mangrove swamp in Kerala, India (Das *et al.*, 2014).

Taxonomic investigation

Results of the analysis of cell wall components of the 19 strains (BKM 1 - BKM 19) are given in Table 1. The strains possess LL-DAP (Diaminopimelic acid) and all the strains tested contain glycine in their cell wall. Presence of LL-DAP along with glycine indicates the cell wall chemotype - I (Lechevalier *et al,* 1966; Lechevalier and Lechevalier, 1970). The strains belonging to the wall type - I are *Streptomyces*, *Streptoverticillium*, *Chainia, Actinopycnidium, Actinosporangium, Elyptrosporangium, Microellobosporia*, *Sporichthya* and *Intrasporangium* (Lechevalier *et al,* 1966; Lechevalier and Lechevalier, 1970). It is important to note that the presence of spores in a long chain occurring on the aerial mycelium eliminates all the genera having the cell wall type – I except *Streptomyces* (Lechevalier and Lechevalier, 1970). The branched nature of the substrate mycelium clearly indicated that the strains BKM 1 to BKM 19 belong to the genus *Streptomyces*.

Results (cultural and morphological characteristics) obtained for the actinobacterial strains are presented in Table 2. These results have been compared with those of the *Streptomyces* species given in the key of Nonomura (1974). These strains' cultural and morphological characters were similar to those of the *Streptomyces.* Hence, the strains were tentatively identified as species of *Streptomyces.*

They are as follows: *Streptomyces gedanensis, S. sclerotialus, S. orientalis, S. albofaciens, S. pseudogrisoelus, S. aureofasciculus, S. rangoon, S. mulabilis, S. gougeroti, S. alboniger, S. craterifer, S. candidus, S. griseus, S. spadicis, S. sindensis, S. citrus, S. puniceus, S. alni and S. nobilis.* Like that of the present investigation, other workers have reported the predominance of *Streptomyces* in their studies. Ramesh and Mathivannan (2009) reported that a total of 288 marine samples were collected from different locations of the Bay of Bengal starting from Pulicat lake to Kanyakumari and *Streptomyces* species were present in a large proportion (88%). Lakshmanaperumalsamy (1984) and Patil *et al.* (2001) have also earlier reported that *Streptomyces* is the

inhibited pathogenic strain. BKM 4, BKM 5, BKM 8, BKM 10 and BKM 16 showed moderate inhibition activity against it. *Pseudomonas* exhibited very least resistance to the actinobacterial strains. Many strains showed inhibition against *Klebsiella*, *E. coli* and *Pseudomonas.*

Thus, the study of the antimicrobial activities of the isolated strains from the mangrove soils of the Bhiterkanika revealed the good metabolic potential of the actinobacterial isolates as antimicrobial agent(s) producers. With the resistant pathogenic strains developing recently against the existing antibiotics, active compounds possessed by these manglicolous actinobacteria provide with new hopes for fighting against the drug-resistant pathogens. Further, new drugs, notably antibiotics, are urgently needed to halt and reverse the relentless spread of antibiotic resistant pathogens which cause life threatening infections and risks which are undertermining with the viability of the healthcare systems (Talbot *et al*., 2006).

Actinobacteria belonging to the order *Actinomycetales*, especially *Micromomospora* and *Streptomyces*, have a unique and proven capacity to produce novel antibiotics (Bentley *et al.,* 2002) and hence there is a continued interest in screening such organisms for novel bioactive substances. It is also becoming increasingly clear that un- and under-explored habitats, such as desert biomes and marine ecosystems, are a very rich source of novel actinobacteria which have the capacity to produce interesting new bioactive compounds, including antibiotics (Hong *et al*., 2009).

A total of 42 actinobacteria were isolated from the mangrove sediments of the Andaman and Nicobar Islands and were evaluated for their antibacterial activity against pathogenic bacteria on two different media (Baskaran *et al.,* 2011). Potential of mangrove actinobacteria to produce antimicrobial substances was studied in 38 strains isolated from different samples of the Pichavaram mangroves. Among them, seventeen (44.7%) showed antimicrobial activities against more than one genus of the test pathogens (Sweetline *et al.,* 2012). Likewise, twenty seven actinobacterial strains isolated from the Coringa mangroves exhibited antimicrobial activity against various test microorganisms (Deepthi *et al.,* 2012). Endophytic actinobacteria were also isolated from the root, stem and leaf parts of two species of mangroves, *Rhizopora apiculata* and *Avicennia marina* for their antimicrobial activity (Gayathri and Muralikrishnan, 2013). Recently, antimicrobial activity of four actinobacterial isolates was found to inhibit most of the gram positive and gram negative bacteria, indicating the broad antibacterial spectra of the isolates (Das *et al*., 2014). Actinobacterial genera isolated from the mangroves of Nizampatnam and Coringa regions of the Andhra Pradesh were capable of synthesizing several antimicrobial compounds (Mangamuri *et al*., 2014).

Screening of actinobacteria for industrially important enzymes.

All the 19 actinobacterial isolates were subjected to enzyme screening for extracellular amylase, cellulase, protease, lipase, L-asparginase, L-glutaminase, chitinase and pectinase activity (Fig. 1). Except BKM 3 and BKM 11, all the others showed positive and higher amylase cellulase, protease and lipase activity. Only 9 isolates showed L-asparaginase activity, while 6 isolates showed weak L-glutaminase activity. Although 10 isolates showed weak chitinase activity, 9 isolates did not show pectinase activity.

Amylolytic, cellulolytic, proteolytic and lipolytic activities are of quite common occurrence in bacteria and fungi; however, reports on extra-cellular enzymes producing actinobacteria are limited (Ellaiah *et al.,* 1997, 2004 and Kundu *et al.,* 2006). Sahu *et al.* (2007) have reported strong proteolytic and amylolytic activities of the genus *Streptomyces* and Ellaiah *et al.* (1997) have also reported strong proteolytic and amylolytic activities of 90% isolates. In the present study, 100% and 90% strains were found to possess amylolytic and proteolytic activity respectively, thus, conforming the study of Ellaiah *et al.* (1997).

Information on the distribution of lipase producing actinobacteria is almost nil except that of Gunalakshmi *et al.* (2008) who have isolated lipase producing actinobacteria from the sediment samples of a shrimp pond. In the present investigation, 100% of the strains have been found as lipase producers. This finding would pave way for further detailed investigation on the mass microbial production of lipase.

L-asparaginase is a medicinally valuable enzyme for cancer therapy. But none of the strains showed positive activity of L-glutaminase, chitinase and pectinase. It is worth mentioning here that, however cellulase and / or lipase producing marine actinobacteria have been isolated from the sediments of coastal areas and mangroves by many authors (Couto *et ali.,* 2010; Priya *et al*., 2012; Stalin *et al.,* 2012).

One hundred and seven actinobacterial strains isolated from marine sediments were collected from five coastal sites of the Konkan coast of Maharashtra and 90 actinobacterial isolates were identified up to the genus level. All of them possessed protease, gelatinase, amylase, lecithinase and cellulase activity (Gulve and Deshmukh, 2011). Fifty six actinobacterial strains were isolated from the marine sediments of the south Indian coastal region; nine isolates were selected on the basis of their amylolytic, cellulolytic and lipolytic activities (Selvam *et al.,* 2011). All the 27 actinobacterial strains isolated

Table.1. Chemotaxanomical charecters of the actinobacteriaal strains.

Strain	DAP		Glycine	Cell wall type	Spore chain	Spore surface	Presumed genus & species
	LL-DAP	Meso DAP					
BKM 1	+	-	+	I	Rectiflexibiles	Smooth	*S. gedanensis*
BKM 2	+	-	+	I	Spirales	Smooth	*S. sclerotialus*
BKM 3	+	-	+	I	Rectiflexibiles	Smooth	*S. orientalis*
BKM 4	+	-	+	I	Spirales	Smooth	*S. albofaciens*
BKM 5	+	-	+	I	Spirales	Smooth	*S. pseudogrisoelus*
BKM 6	+	-	+	I	Rectiflexibiles	Smooth	*S. aureofasciculus*
BKM 7	+	-	+	I	Spirales	Smooth	*S. Rangoon*
BKM 8	+	-	+	I	Rectiflexibiles	Smooth	*S. mulabilis*
BKM 9	+	-	+	I	Rectiflexibiles	Smooth	*S. gougeroti*
BKM 10	+	-	+	I	Rectiflexibiles	Smooth	*S. alboniger*
BKM 11	+	-	+	I	Rectiflexibiles	Smooth	*s. craterifer*
BKM 12	+	-	+	I	Rectiflexibiles	Smooth	*s. candidus*
BKM 13	+	-	+	I	Rectiflexibiles	Smooth	*s. griseus*
BKM 14	+	-	+	I	Rectiflexibiles	Smooth	*s. spadicis*
BKM 15	+	-	+	I	Rectiflexibiles	Smooth &warty	*s. sindensis*
BKM 16	+	-	+	I	Rectiflexibiles	Smooth	*s. citrus*
BKM 17	+	-	+	I	Rectiflexibiles	Smooth	*s. puniceus*
BKM 18	+	-	+	I	Rectiflexibiles	Smooth & Warty	*s. alni*
BKM 19	+	-	+	I	Rectiflexibiles	Smooth	*s. nobilis*

+ denotes presence; - denotes absence

Table 2. Comparison of cultural and micro morphological characteristics using the conventional keys (Nonomura, 1974).

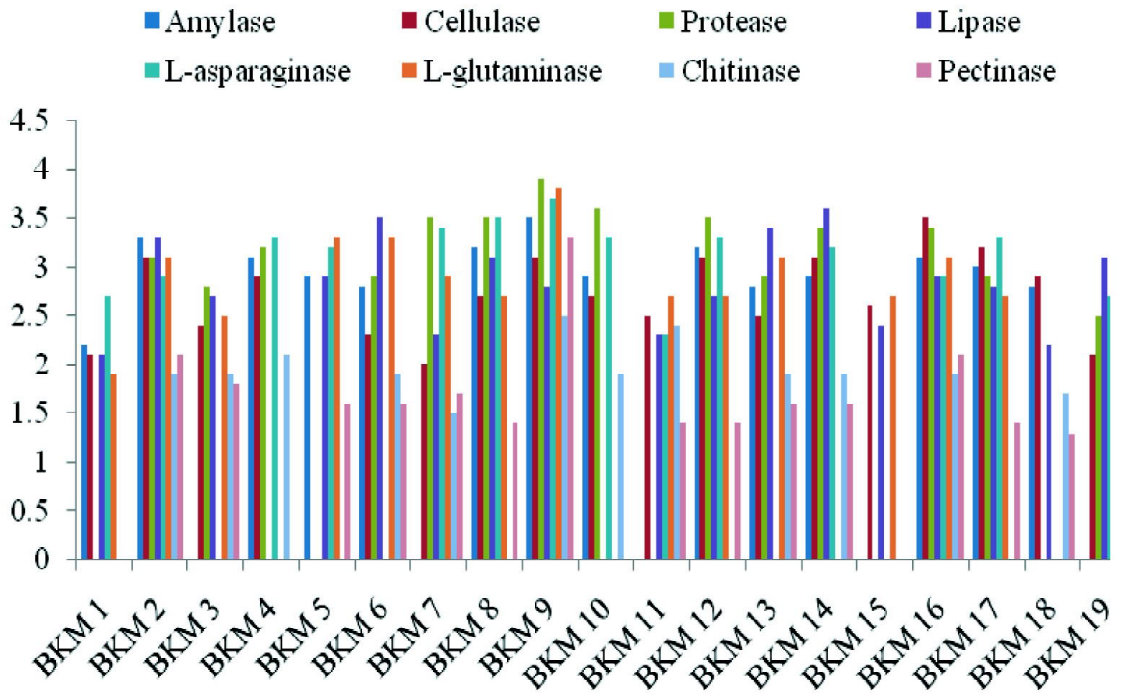
Cultural character							Carbon source Assimilation								
Strain No	Colour of aerial mycelium	Melanoid pigment	Reverse side pigment	Soluble	Spore chain	Spore surface	Arabinose	Xylose	Inositol	Mannitol	Fructose	Rhamnose	Sucrose	Raffinose	Species Name (As per the Nanomura key)
BKM 1	White	-	+	-	Rectiflexibiles	Smooth	+	-	+	+	+	+	+	-	*S. gedanensis*
BKM 2	White	-	-	-	Spirales	Smooth	+	+	+	+	+	+	+	+	*S. sclerotialus*
BKM 3	White	-	-	-	Rectiflexibiles	Smooth	+	+	+	+	+	+	+	+	*S. orientalis*
BKM 4	White gray	+	+	+	Spirales	Smooth	+	+	+	+	+	-	+	+	*S. albofaciens*
BKM 5	White	-	-	+	Spirales	Smooth	+	+	+	+	+	+	-	+	*S. pseudogrisoelus*
BKM 6	White	+	+	-	Rectiflexibiles	Smooth	+	+	+	+	+	+	+	+	*S. aureofasciculus*
BKM 7	White	-	-	-	Spirales	Smooth	+	+	-	+	+	-	+	-	*S. Rangoon*
BKM 8	White	-	-	-	Rectiflexibiles	Smooth	+	+	+	+	+	+	+	+	*S. mulabilis*
BKM 9	White	-	-	-	Rectiflexibiles	Smooth	+	+	+	+	+	+	-	+	*S. gougeroti*
BKM 10	White	+	-	+	Rectiflexibiles	Smooth	+	+	+	+	+	+	-	+	*S. alboniger*
BKM 11	Gray	-	-	-	Rectiflexibiles	Smooth	+	+	-	+	+	+	+	+	*s. craterifer*
BKM 12	White	-	-	-	Rectiflexibiles	Smooth	+	+	+	+	+	+	-	-	*s. candidus*
BKM 13	White	-	-	+	Rectiflexibiles	Smooth	+	+	+	+	-	-	-	+	*s. griseus*
BKM 14	Powdery white	-	-	-	Rectiflexibiles	Smooth	+	+	+	+	+	+	-	+	*s. spadicis*
BKM 15	White	-	-	-	Rectiflexibiles	Smooth &warty	+	+	-	+	+	-	+	-	*s. sindensis*
BKM 16	Yellow	-	+	-	Rectiflexibiles	Smooth	+	+	+	+	+	-	+	+	*s. citrus*
BKM 17	Yellow	-	+	-	Rectiflexibiles	Smooth	-	+	-	+	+	+	+	+	*s. puniceus*
BKM 18	Gray	-	-	+	Rectiflexibiles	Smooth & Warty	+	+	-	+	+	+	+	+	*s. alni*
BKM 19	Powdery white	+	+	-	Rectiflexibiles	Smooth	+	+	+	+	+	+	+	+	*s. nobilis*

Table 3. Antimicrobial activity of the antinobacterial strains

S. No.	Strain	*Pseudomonas*	***E. coli***	*Klebsiella*	***Bacillus***	***Proteus***
1	BKM 1	-	-	-	-	+
2	BKM 2	-	-	+	+	-
3	BKM 3	+	-	-	+	-
4	BKM 4	-	±	-	+	+
5	BKM 5	-	±	-	±	+
6	BKM 6	-	-	+	+	+
7	BKM 7	+	-	+	±	-
8	BKM 8	+	±	+	+	+
9	BKM 9	-	-	+	+	-
10	BKM 10	-	±	++	++	-
11	BKM 11	-	-	-	+	-
12	BKM 12	-	-	+	-	+
13	BKM 13	-	-	+	-	+
14	BKM 14	+	-	-	-	-
15	BKM 15	+	-	+	+	+
16	BKM 16	-	±	-	-	-
17	BKM 17	-	-	+	+	-
18	BKM 18	-	-	+	-	+
19	BKM 19	-	+	-	+	-

++: good +: Positive -: Negative ±: Moderate

Fig. 1. Enzyme activity (amylase, cellulase, protease, lipase, L-asparaginase, L- glutaminase, chitinase, pectinase) of the strains.



from the Coringa mangroves, and all those strains exhibited amylase, cellulase, L-asparaginase, chitinase and protease activity (Deepthi *et al.,* 2012). Cellulose-degrading actinobacteria were isolated from the Bhitarkanika mangrove environment and a total of nine actinobacterial isolates were found to produce cellulase enzyme activity (Mohanta, 2014).

In recent years, there has been an increasing interest in microbial enzymes as biocatalysts with novel features in many industrial processes. Microorganisms are the first choice as the source of enzymes because of their rapid growth, broad biochemical diversity and ease of genetic manipulation (Mangamuri *et al.,* 2014). To conclude, it can be stated that the isolation of culturable actinobacteria especially *Streptomyces* species from the mangrove environment of Bhiterkanika would contribute to our knowledge of manglicolous actinobacteria and offer an increased pool of actinobacteria for bioactive natural products screening.

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