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Antimicrobial potential of Actinobacteria against food borne microorganisms

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Abstract
Present study based on the search of alternative and active compounds from Actinobacteria that can be used as a bio preservative. Since Actinobacteria are more potent antimicrobial agent which has the potential to replace antimicrobial chemical additives. During the present study, 25 different Actinobacteria isolated from the different soil samples such as from Ganga river bed, Banana and Sugarcane soil from different localities of Haridwar and its adjoining places. These cultures were screened for antimicrobial activity against various food borne microorganisms including *Staphylococcus sp., Bacillus sp., Escherichia coli* and *Pseudomonas sp.* isolated from various food products in order to identify potential antibiotic producers. Among these isolates, 12 showed antimicrobial activity against test microorganisms and 6 (AC9, AC14, R3, R4, R8 & R10) exhibited promising broad spectrum activity with zone of inhibition more than 15mm against all the tested organisms. These cultures were further identified on the basis of cultural, morphological and biochemical characteristics. Further studies on the bioactive metabolites from these cultures which will be useful for discovering novel compounds of clinical industrial significance.

Keywords: Actinobacteria, *Escherichia coli, Staphylococcus sp., Bacillus sp.,* primary screening

Introduction
Bacteria have so far been the most promising resource for antibiotics in the past decades and will undoubtedly remain an important resource of innovative bioactive natural products in the future. Approximately 45% of bioactive compounds acquire from microbes were produced by Actinomycetes (Berdy, 2005) [4]. Actinobacteria remain the most economically and biotechnologically beneficial microbes, producing 80% of the world's antibiotics, mostly from the genera *Streptomyces* and *Micromonospora* (Singh and Sinha, 2012) [19]. Actinobacteria are aerobic, spore forming gram-positive bacteria, belonging to the order Actinomycetales characterized with substrate and aerial mycelium growth (Lechevalier and Lechevalier, 1981) [15]. It has a high (G+C) ratio of the DNA (>55mol %), which are phylogenetically related from the evidence of 16S ribosomal cataloguing and DNA: rRNA pairing studies (Good fellow and Williams, 1983; Korn-Wendisch and Kutzner, 1992) [7]. It shows one of the largest taxonomic units among the 18 major lineages currently recognized within the domain bacteria (Ventura *et al*., 2007) [22]. The name "Actinomycetes" was derived from Greek word "atkis" (a ray) and "mykes" (fungus), which have characteristics of both Bacteria and fungi (Das *et al.,* 2008) [6] but yet possess sufficient distinctive features to delimit them into 'Kingdom bacteria'. The Actinobacteria are potential producers of antibiotics and of other therapeutically useful compounds. The bioactive secondary metabolites produced by Actinobacteria include antibiotics, antitumor agents, immunosuppressive agents and enzymes. These metabolites are known to possess antibacterial, antifungal, antioxidant, Neuritogenic, anti-cancer, anti- algal, anti-helmintic, anti-malarial and anti-inflammatory (Kekuda *et al*., 2010; Ravikumar *et al*., 2011) [10, 17]. They exhibit a range of life cycles which are different among the prokaryotes and appear to play a major role in the cycling of organic matter in the soil ecosystem (Veiga *et al.,* 1983) [21]. Actinobacteria have proved their ability to produce a variety of bioactive secondary metabolites and for this reason, the discovery of novel antibiotic and non-antibiotic lead molecules through microbial secondary metabolite screening is becoming increasingly important.
Many vitamins, antibiotics, enzymes and Siderophores produced by Actinobacteria have pharmaceutical, veterinary, agricultural and clinical applications (Koehn and Carter, 2005; Kekuda *et al*., 2010; Naine *et al*., 2011) [12, 10, 16],

in addition to antitumor and wound healing properties (Janardhan *et al*., 2012; Jiao *et al*., 2013). Since the discovery of antibiotics, bacterial resistance to these drugs has continued to evolve. Thus, we are witnessing more and more multi resistant bacteria that have a serious public health problem. The present investigation aimed to study antimicrobial activity of Actinobacteria isolated from soil samples of different localities of Haridwar.

Materials and methods

Collection of soil samples: During the study. Three soil samples were collected aseptically from three different localities of Haridwar such as from Ganga river bed, Banana soil and sugar cane soil at different depth of 10 cm using standard methods and placed in clean polythene bags, sealed tightly and stored in a refrigerator at 4^0C.

Isolation of Actinobacteria: Actinobacteria were isolated by spread plate technique following the serial dilution of soil samples on starch casein agar (Williams & Davis 1965) [24] plates containing cyclohexamide and nystatin (each at concentration of 50 mg/ml of medium) and incubated at 28^0 C for 7 days. The following media were also used for the isolation of Actinobacteria: Starch casein agar, Starch casein nitrate agar, Actinomycetes isolation agar, Oat meal agar etc. Isolation plates were incubated at 28-30^0 C for 7-15 days for fast growing Actinobacteria or upto35 days for slow growing ones. Identification of Actinobacteria to genus level was conducted by first using morphological and chemical criteria according to Bergey's Manual of Determinative Bacteriology (Holt *et al*., 2000).

Characterization of Actinobacteria

The potent Actinobacteria isolates selected from primary screening were characterized by morphological, biochemical and physiological methods. The morphological method consists of macroscopic and microscopic characterization. Macroscopically the Actinobacteria isolates were differentiated by their colony characters, e.g. size, shape, color, consistency etc. For the microscopy, the isolates were grown by cover slip culture method (Kawato & Sinobu 1979) [9]. They were then observed for their mycelial structure, and conidiospore and arthrospore arrangements on the mycelia under microscope (1000X). The observed morphology of the isolates was compared with the Actinobacteria morphology provided in Bergey's Manual for the presumptive identification of the isolates. Various biochemical tests performed were catalase, oxidase, citrate utilization, nitrate reduction, starch hydrolysis, urea hydrolysis, gelatin hydrolysis, acid production from sugar, and the physiological test included motility, NaCl resistance, and temperature tolerance

Test Organisms: Various test organisms used in this study include: *Bacillus sp. Staphylococcus sp, Escherichia coli, Pseudomonas sp.* which were isolated from different food sources.

Sample Collection: Different food samples are collected from local market of Haridwar, Uttarakhand, India. All the samples were wrapped separately in sterile polyethylene bags and plastic bags and transported to the laboratory for microbial analysis.

Isolation of food borne pathogens: The samples are rinsed thoroughly with distilled water and used for isolation of bacteria on specific media Mannitol Salt agar, Mac Conkey agar and Cetrimide agar at 37^0C for 24 hours by serial dilution method (Waksman, 1961) [23].

Phenotypic characterization of Bacteria: Bacteria are identified by cultural characteristics such as abundance of growth, color change in media and morphological characteristics like form, size, margin and elevation are studied on culture plates. Identifying an isolates by Gram's reaction Gram's staining, Catalase, Oxidase, Nitrate Reduction, IMVIC test, Carbohydrate Utilization, Urease production, Gelatin Hydrolysis test were performed for the confirmation of the Bacterial isolates according to the Bergey's manual (Holt *et al*., 2000).

Screening of Actinobacteria for antibacterial activity

Primary screening was carried out using the modified method of Kirbey Bauer antibiotic susceptibility test (Bauer *et al*., 1966) [3]. Antibiotic activity was determined on Mueller Hinton agar media inoculated with Actinobacteria. The Actinobacteria isolates were lawn cultured by dense streaking on starch casein nitrate medium plates and incubated at 30^0 C for 7 days. Six mm agar discs were prepared using sterile cork borer from well- grown culture and placed on fresh lawn culture of test organisms. The plates were then kept at 4^0 C for 30 min for the diffusion of the culture broth, and then incubated at their respective optimum temperature (37^0 C). The zones of inhibition were determined after 18-24 h.

Result

Actinobacteria isolation

From all the soil samples collected from different places which varied in their texture, a total of 25 isolates of Actinobacteria were isolated. The distribution of Actinobacteria varied with the texture and cultivation status of the soil, with the highest number found in river soil. (Table 1). Each of the isolates were later categorized according to their morphology that includes colony colour ranging from dark grey, grey, dark brown, brown, whitish and yellowish white (figure 1) and also microscopic appearance of colonies after simple staining (Table 2).

Isolation of food borne pathogens

Pathogens are isolated by using specific media like Mannitol Salt agar was used for the identification of food borne *Staphylococcus sp* which ferment Mannitol, MacConkey Agar was used for the identification of food borne and Lactose fermenting organism like *E.coli*, Cetrimide agar was used for the identification of food borne *Pseudomonas sp* and Bacillus agar for the identification of *Bacillus sp* which is selective media for the bacterium. By using 4 different media totally different organism were identified based on color change in media and colony morphology as shown in (Table 4).

Biochemical characterization of Actinobacteria and Test Bacteria

Biochemical test were performed like Indole, MR, VP, Citrate, Nitrate Reduction, Urease, Catalase, Oxidase, Starch hydrolysis, Gelatin Hydrolysis, Lipid Hydrolysis and the results for Actinobacteria were tabulated in (Table3) and in (Table 5) for test bacteria. Based on the above biochemical

characteristics the isolated strains of Actinobacteria were tentatively identified as *Streptomyces sp, Micromonospora sp.*

Primary Screening

Four strains of pathogenic bacteria *Staphylococcus sp, Bacillus sp, Escherichia coli, Pseudomonas sp.* were chosen as the test strains for the study. Primary screening showed that 12 colonies were able to produce antibiotics. Out of those, six colonies (AC9, AC14, R3, R4, R8 &R10) had great potential for antibiotic production with large zone of inhibition (Table 5). The distribution of antibiotic producers varied with the texture and cultivation status of the soil, with the highest number found in river soil (figure 2).

Discussion

Actinobacteria are widely distributed in the nature and have the potentiality to produce many biologically active substances like antibacterial, antifungal, antiviral, anti-parasitic, herbicides, pesticides, antioxidant and antitumor. Prevalence of food borne diseases and food borne pathogens were increasing day to day life. Due to food products get contaminated while handling, harvesting and processing in equipments and transportation. Food products may become contaminated at different stages along the food chain, from growth or production until reach to the consumers. The microorganisms present in Fruits, Vegetables, Dairy products, Poultry products and Bakery products are a direct impression of the sanitary quality of the cultivation water, harvesting, transportation, storage, and processing of the product (Eni *et al.,* 2010). Keeping all this in our mind we made a little effort to adders this serious concerned. In present work different food samples like spoiled Fruits, Vegetables, Dairy products, Bakery products, Poultry products were selected to isolate food spoiling bacteria. Out of total 128 isolates, 31 *Pseudomonas* isolates (D3) (24.21%), 33 *Staphylococcus* isolates (B6) (25.78%), 30 *E.coli* (P10) (23.43%) and 20 *Bacillus* isolates (V4) (15.62%) were selected. Among the isolates many of them shows resistance or had reduced susceptibilities to multiple antimicrobial agents. The present study was performed to study the antimicrobial activity of Actinobacteria isolated from soil against different food borne pathogens. Actinobacteria have produce many industrially important bioactive compounds (Kumar and Jadeja, 2016) [14]. Three soil samples collected from three different localities of Haridwar. The samples were growing on starch casein agar for isolation and identification of Actinobacteria. All isolates (n = 25) were Gram positive aerobic slow growing bacteria (Anderson and Wellington, 2001) [1]. Out of the total 25 isolates, 12 isolates showed good activity in primary screening but six isolates exhibited a broad spectrum of antimicrobial activity against the test organism and the range of inhibition zones was b0etween 2.5 and 17.3 mm. Strain R10 showed the largest inhibition in *E. coli* (P10), while strain R3 was weakly active and showed the least inhibition zone in *Pseudomonas sp* (D3). (Singh and Pallavi, 2018) [18] reported the 2 strain of *Nocardia sp.* are active against *E. coli* (20mm) better as compared to *Bacillus (*13mm) strain. Similar reports on Actinobacteria was carried out by (Singh *et al.*, 2016) [20] in *Streptomyces.* (Chaudhary and Singh, 2015) [5] recorded fungicidal potency of *Cinnamomum tamala* leaves against common food borne pathogens. (Singh and khan, 2001) [11] isolated the Actinobacteria from the river bed of Ganga and GKV college garden resembles on the basis of culture characteristics with *Streptomyces sp*. Further investigations will be needed to identify the strain at molecular level and to determine the active metabolites of these isolates which will be useful against drug resistant bacteria.

Conclusion

We can conclude that the Soil samples are rich source of Actinobacteria which exhibit a broad spectrum antimicrobial activity. Most microorganisms have developed resistance to existing antibiotics. So it has provoked the need of consistent research like ours on the production of newer antibiotics to overcome the resistant microorganism. Further studies on the bioactive metabolites will be useful for discovering novel compounds of clinical and industrial significance.

Table 1: Total number of Actinobacteria isolated and antibiotic producing colonies

S. No.	Soil Source	No. of colonies isolated	Antibiotic producing colonies
1	Ganga river bed	15	AC2
			AC3
			AC4
			AC5
			AC8
			AC9
			AC14
			Nel1
2	Banana Soil	06	R3
			R4
			R8
3	Sugarcane Soil	04	R10

Table 2: Morphological Characterization of Actinobacteria

Isolates	Mycelium and nature of colony	Colour of colony	Types of spore	Gram stain
AC9	Smooth, granular Aerial & Substrate Mycelium	White	Monosporophore	+
AC14	Smooth, hairy, raised wrinkled Aerial mycelium	Creamish white	Long chain of spore	+
R3	Septate, branched, Coloured aerial mycelium	grayish white	Long chain of spore	+
R4	Aerial mycelium spiral	Grayish blue	Long chain of spore	+
R8	Aerial mycelia from white to grayish white to dark gray	Dark grey	Long chain of spore	+
R10	Smooth, granular Aerial & substrate Mycelium	Pinkish white	Long chain of spore	+

Table 3: Biochemical Characterization of Actinobacteria

Biochemical characterization	AC9	AC14	R3	R4	R8	R10
Indole	-	-	-	-	-	-
Methyl red	+	+	+	+	+	+
VP	-	-	-	-	-	-
Citrate utilization	+	+	+	+	+	
Nitrate Reduction	+	+	+	+	+	+
Urease	_	_	_	_	_	_
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Starch Hydrolysis	+	+	+	+	+	
Gelatin Hydrolysis	+	+	+	+	+	+
Lipid Hydrolysis	+	_	+	+	+	+
Caesin Hydrolysis	+	+	+	+	+	+

*(+) = Positive, (-) = Negative

Table 4: Number of Isolates in Food Samples

Source	Sample	Total no. of isolates	Total no. of *Bacillus* (V4) isolates	Total no. of *Staphylococcus* (B6) isolates	Total no. of *E. coli* (P10) isolates	Total no. of *Pseudomonas* (D3)isolates
Vegetables	Tomato	40	05	18	09	12
Dairy products	Raw milk	38	03	04	14	09
Bakery product	Cake	22	08	09	02	03
Poultry Product	Egg	28	04	02	05	07
		Total no. of isolates= 128	Total no. of *Bacillus* =20 (15.62%)	Total no. of *Staphylococcus*=33 (25.78%)	Total no. of *E. coli*=30 (23.43%)	Total no. of *Pseudomonas*=31 (24.21%)

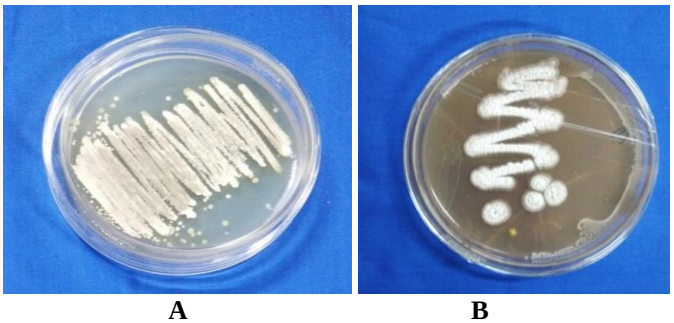
Table 5: Biochemical characterization of Test Bacteria

Biochemical characterization	*E. coli* (P10)	*Pseudomonas sp.* (D3)	*Staphylococcus sp.* (B6)	*Bacillus sp.* (V4)
Starch Hydrolysis	-	-	-	+
Fermentation				
(a)Lactose	AG	-	A	-
(b)Dextrose	AG	-	A	A
Indole	+	-	-	-
MR reaction	+	-	+	-
VP reaction	-	-	+	+
Citrate	-	+	-	-

***** (+) = Variable reaction, (+) = Positive, (-) = Negative, (AG) = Acid and gas

Table 6: Average inhibition zone of Actinobacteria isolated from soil samples against Test bacteria (Average of triplicates + SEM)

Isolates	Gram positive bacteria ZOI (in mm)		Gram negative bacteria ZOI (in mm)	
	Bacillus sp (V4)	*Staphylococcus sp* (B6)	*Escherichia coli* (P10)	*Pseudomonas sp* (D3)
AC2	10.6+0.3	0	0	0
AC3	8.6+ 0.8	0	0	0
AC4	7.6+ 0.8	0	0	0
AC5	9+ 0.0	0	0	0
AC8	12.3+ 0.3	0	0	0
AC9	0	16.3+0.8	9+ 0.5	6+ 0.3
AC14	0	17+ 0.5	4,6+ 0.5	8+ 0.8
Nel1	0	0	0	0
R3	11.6+ 0.3	13.3+ 0.6	15.6+ 0.3	2.5+ 0.3
R4	0	8.6+ 0.3	3.6+ 0.3	0
R8	0	10.6+ 0.3	8.3+ 0.3	0
R10	0	5.6+ 0.3	17.3+ 0.5	0



A **B**

Fig 1: Plate containing the purified colony of Actinobacteria (A) Isolate R3, (B) Isolate AC14

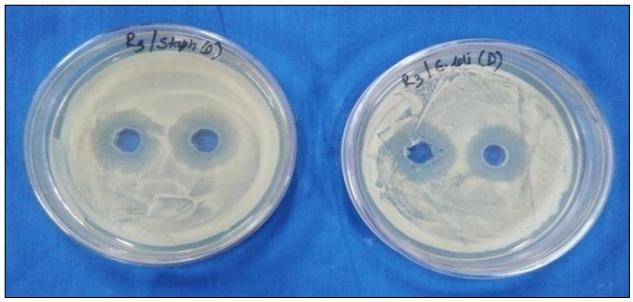


Fig 2: Plate Showing Primary Screening of Actinobacteria Isolate R3

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