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Diversity and antibacterial activity of actinomycetes from wetland soil

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Abstract

Diversity of soil actinomycetes isolated from various regions of a wetland was analysed. The strains were assigned under 11 genera according to their morphological and biochemical characteristics. They are: Nocardia, Pseudonocardia, Streptomyces, Micromonospora, Rhodococcus, Actinosynnema, Nocardiodes, Kitasatospora, Gordona, Intrasporangium and Streptoalloteichus. Around 55% of the identified strains are Nocardioform actinomycetes. Screening for their antibacterial activities revealed that 96% of the isolated strains showed different degrees of inhibition potential against 12 test pathogenic bacteria (including various serotypes of Salmonella, Vibrio cholerae, Bacillus subtilis and Escherichia coli). Of these about 41% of strains showed antagonism towards Bacillus subtilis. The Nocardioform actinomycetes exhibited antibacterial activity against 9 out of 12 test organisms. The least antibacterial potential was exhibited by Gordona, Rhodococcus, Micromonospora and Kitasatospora. The isolated strains differed among themselves in their ability to decompose proteins and amino acids and also in enzyme production potential.

Keywords: *Wetland, soil, actinomycetes, pathogenic bacteria, antibacterial activity*

1. Introduction

Soil microorganisms provide an excellent resource for the isolation and identification of therapeutically important products. Among them, actinomycetes are an important group producing antibiotics of agricultural and medicinal importance (Kavitha *et al*., 2010). Actinomycetes, the Gram-positive filamentous bacteria with true aerial hyphae, belonging to the phylum *Actinobateria* that represents one of the largest taxonomic units among the 18 major lineages currently recognized within the domain Bacteria (Olano *et al*., 2009). The majority of actinomycetes are free living, spore forming, saprophytic bacteria found widely distributed in soil, water and colonizing plants. Actinomycetes population has been identified as one of the major group of soil population, which may vary with the soil type. They are excellent elaborators of biotechnological products such as antibiotics, industrial enzymes and other bioactive compounds. The secondary metabolites obtained from the class actinobacteria are of special interest because of their diverse biological activities such as antibacterial, antifungal, antioxidant, antitumor and antiviral. They, especially *Streptomyces* species, account for more than 70% of the total antibiotic production. *Micromonospora* is the runner up with less than one-tenth as many as *Streptomyces* (Lam, 2006). However, the survey of *Streptomyces* and other common terrestrial actinomycetes is nearly exhausted. This and the rise of antibiotic-resistant pathogenic strains dictates an increasing need for the survey of unexplored and underexplored habitats for novel antibiotic-producing actinomycetes strains (Debananda *et al*., 2009).

Wetlands are regarded as important ecosystems which are transitional between open water and terrestrial ecosystems. They are endowed with specific structural and functional attributes performing major ecological roles in the biosphere (Udotong *et al*., 2008). They are considered as a key environment with a diverse range of biological resources to initiate ecological research into the habitat of actinomycetes that produces bioactive substances. However, reports on actinomycetes diversity from wetlands are rather sparse, though wetlands acts as nature's kidney and provide great range of natural nutrients and xenobiotics draining from the terrestrial environment, to support wide range of microorganisms. Hence the present study has been taken up with an objective of finding out the diversity of actinomycetes in wetland soil and to estimate their potential to provide novel antibiotics.

2. Methods

2.1 Sampling Procedure

Soil samples were collected monthly from the Kumarakom region (9^o 37' N lat. and 76^o25' E long., with an average temperature of 29^oC) of Vembanadu - Kol wetland of Kerala, along the southwest coast of India, during pre-monsoon and monsoon seasons of the year 2008. Three different habitats in this wetland area were selected for the study such as: mangrove, bird sanctuary and an area affected by anthropogenic activity. Composite soil samples (2-5 cm depth) were collected from each area in sterile plastic bags and transported to the laboratory under ambient conditions and were air dried at room temperature. Later the soil samples were subjected to dry-heat treatment at 50^oC for 1 hr to depress the number of other bacteria and for preferential isolation of actinomycetes.

2.2 Isolation and Enumeration of Actinomycetes from the Soil Sample

Isolation and enumeration of actinomycetes were done by applying standard serial dilution plate technique. Different aqueous dilutions (10^{-1} to 10^{-3}) of samples were prepared and spread plated on Kusters Agar (Glycerol 10g, Casein 0.3g, KNO_3 2g, K_2HPO_4 2g, $MgSO_4$ 0.05g, $CaCO_3$ 0.02g, $FeSO_4$ 0.01g, Agar 18g, Distillled water 1L, pH 7± 0.1) (Lakshmanaperumalsamy *et al*., 1986). After incubation of the plates at room temperature for 2-3 weeks typical actinomycete colonies were selected on morphological basis (Shirling and Gottlieb, 1966) and purified on Kusters Agar plate by restreaking and incubating at room temperature.

2.3 Characterization of Actinomycete Strains

Pure cultures of actinomycete strains were characterized by morphological tests as per Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 2000) and by physiological tests (Gordon, 1967).

2.3.1 Morphological characterization

Gross morphology is observed after 2-3 days of growth on Kusters agar plates. The morphology of actinomycete strains was examined using cover slip culture technique (Holt *et al*., 2000). The mycelium structure, arrangement of conidiospore and arthrospore on the mycelium was observed through the oil immersion (1000X). The observed structure was compared with Bergey's Manual of Determinative Bacteriology, 9th edition (Holt *et al.*, 2000) and the organism was identified.

2.3.2 Physiological characterization

The physiological and biochemical properties were tested for all actinomycetes isolates. These tests were performed as described by Gordon (1966; 1967), though with minor modifications. Physiological tests included decomposition of Casein, Tyrosine, Xanthine, Hypoxanthine, Urea and Esculin as well as evaluation of lysozyme resistance of the isolates. The isolates were also tested for their ability to produce acid from various carbohydrate sources such as arabinose, fructose, galactose, inositol, lactose, mannitol, mannose, rhamnose, sorbitol and xylose.

2.4 Evaluation of Antibacterial Activity

Antibacterial activity of the strains was determined by well diffusion method using agar wells in Glycerol-Yeast Extract Agar (Waksman, 1961). Lawn cultures of the test micro-organisms (*Salmonella bovis, Salmonella typhimurium, Salmonella senftenberg, Salmonella typhi, Salmonella mgulani, Salmonella enteritidis, Salmonella welteverden, Salmonella bareilly, Vibrio cholerae, Bacillus subtilis* and *E. coli*-78 (enterotoxigenic) and *E. coli*-12 (enteropathogenic), which were isolated from environmental samples) were prepared by swabbing young culture over the agar medium and 3mm diameter wells were punched. About 30 µl of four day old broth culture suspension of actinomycetes were pipetted into the wells and plates were incubated for 24 h at room temperature. Zone of inhibition around the wells were recorded in mm.

2.5 Soil Analysis

The soil samples collected for the isolation of actinomycetes were analysed for various physico-chemical parameters. pH of soil samples determined potentiometrically using pH meter (Systronics- model 361). Total Nitrogen in the soil samples was determined by microkjeldahl method, the available phosphorus was estimated by Bray and Kurtz method and organic carbon content was determined by Walkey and Black method (Jackson, 1973). The available potassium in the soil samples were extracted using neutral ammonium acetate as extractant and determined by using flame photometer (Systronics – model 128) (Maiti, 2003).

3. Results

3.1 Microbial and Pedological Characters of Soil Samples

In the present study an attempt has been made to study the diversity of actinomycetes in soils from various regions of the wetland (*i.e.* Mangrove region, bird sanctuary and an area affected by anthropogenic activity) and to evaluate their antimicrobial properties. Physico-chemical properties of the soil samples from the study area were also determined in order to test possible correlation with actinomycete microbiota. Soil characteristics and the load of actinomycetes are given in the Table 1. It was observed that soils in all the three sites were moderately acidic. Considerable variation in organic carbon content of the soil has been noticed among the samples from different sites during sampling period. Highest organic carbon was recorded in the site having anthropogenic activity (3.56%), during monsoon season. However, soil samples from the mangrove recorded an increase in the total nitrogen content during the onset of monsoon (2.57%). Soil samples from mangrove had the highest available potassium (30 ppm) during the study period. Highest actinomycete load was in the mangrove soil samples (75×10^3) (during monsoon period).

Table 1. Physico-chemical characteristics and load of actinomycetes in soil samples from wetland.

Sampling sites	Date of Collection	Tempe-rature (^oC)	pH	Total Nitrogen (%)	Available Phosphorus (ppm)	Available Potassium (ppm)	Organic Carbon (%)	Load of Actinomycetes (cfu/g)
Mangrove soil	01/05/08	28	4.87	1.43	0.05	30.00	1.50	17×10^3
	03/06/08	28	5.32	1.75	0.70	16.80	2.01	46×10^3
	06/07/08	30	4.31	2.57	0.15	20.40	2.47	75×10^3
Ornithogenic soil	01/05/08	28	4.28	0.92	0.25	15.00	2.50	12×10^3
	03/06/08	28	5.47	1.15	0.35	14.30	2.47	30×10^3
	06/07/08	30	4.80	0.90	0.63	13.60	1.15	40×10^3
Soil from an area affected by anthropogenic activity	01/05/08	30	4.68	0.41	0.02	17.00	0.92	9×10^3
	03/06/08	30	5.78	0.97	0.10	13.10	1.24	20×10^3
	06/07/08	30	6.76	0.92	1.25	19.80	3.56	16×10^3

A total of 22 actinomycete strains were recovered from the wetland soil samples during the study period. Isolated strains were identified based on their morphological and biochemical characteristics. The branching of the aerial and substrate mycelium and the arrangement of spores were observed for morphological identification. The percentage distribution of different genera is shown in Figure 1. Nocardioform actinomycetes was present in all the thee sampling sites. More diverse strains were obtained from the Mangrove soil. *N. caviae* showed the highest percentage (27.3%) of occurrence in the mangrove soil. While *N. asteroids* showed the highest percentage distribution (33.3%) in soil from bird sanctuary. *Intrasporangium*, *Nocardia*, *Rhodococcus* and *Streptoalloteichus* shared an equal percentage distribution (20%) in soil from the control site.

3.2 Physiological Properties of the Actinomycetes Isolates

Nearly 64% of the strains isolated from the Mangrove soil were able to decompose the milk protein casein and about 73% of the isolates from the Mangrove soil decomposed the amino acid hypoxanthine. While 50% of isolates from the soil of bird sanctuary (ornithogenic soil) were able to utilize casein and hypoxanthine, only 17% of the strains from this site were able to decompose xanthine. However, 40% in the isolates from the soil collected from the site affected by anthropogenic activity could decompose xanthine. Physiological capabilities of the isolates with regard to utilization of the many other substrates that are studied also showed variation, depending on the site. Most of the actinoycetes isolates from ornithogenic soil were able to decompose esculine and urea.

The utilization of various carbohydrates (such as lactose, galactose, xylose, inositol, sorbitol, mannitol, mannose, rhamnose and arabinose) by the isolated actinomycetes suggests a wide pattern of carbon source assimilation (Table 2). About 77% of isolates were able to utilize xylose as the source of carbon and only 55% of isolates were able to utilize inositol. Most isolates from ornithogenic soil (83%) were able to utilize mannitol, mannose, rhamnose and xylose.

3.3 Antibacterial Activity of the Actinomycete Isolates Against Pathogenic Bacteria

In the present study antagonistic action of 22 actinomycete isolates against 12 pathogenic bacteria were carried out. It was observed that 96% of actinomycete strains suppressed in different degrees the growth of the test organisms (Table 3). Among the test organisms, *Bacillus subtilis* was found to be affected by most of the actinomycetes (41%) isolated. Nocardioform actinomycetes (various species of *Nocardia* and *Pseudonocardia*) exhibited antimicrobial activity against 9 test organisms. Maximum inhibition zone of 35mm against *S. bareilly* was observed among the actinomycetes isolates from ornithogenic soil. In general the isolates from soil samples affected by anthropogenic activity and ornithogenic origin showed better antibiotic potential against all pathogenic strains used in this study.

4. Discussion

Actinomycetes are ubiquitous, though their population dynamics are often influenced by the available nutrients and the physico-chemical conditions of the ecosystem. Actinomycetes are more abundant in soils with high organic-matter and low moisture contents (Lee and Hwang, 2002). In the present study highest load of actinomycetes were obtained from the mangrove soil, having highest organic material (total nitrogen and potassium). All the soil samples show a pH in the acidic range. The existence of large diversity of acidophilic actinomycetes that differed morphologically and physiologically from neutrophilic species has been reported by Khan and Williams (1975).

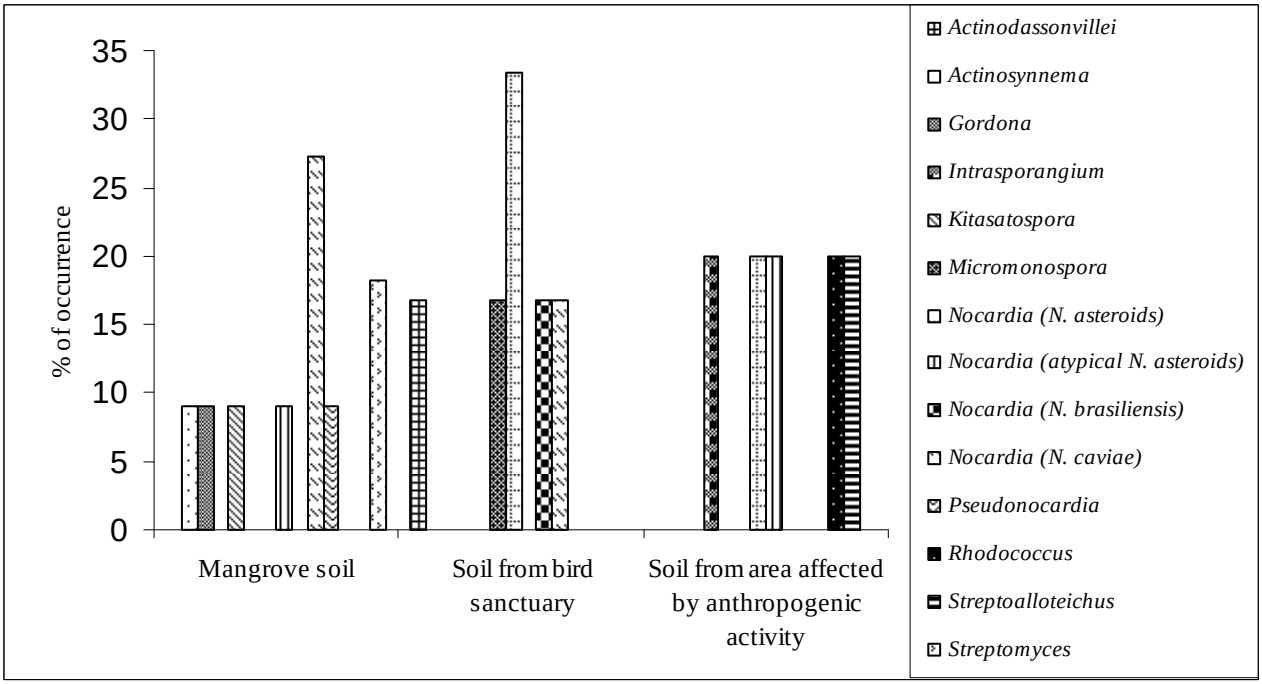

Figure 1. Percentage distribution of different genera of actinomycetes from different sampling sites of wetland.

Table 2. Physiological characteristics of actinomycetes isolates from different wetland soils.

Percentage of Isolates Capable of Utilizing	Mangrove soil	Bird sanctuary (ornithogenic soil)	Soil from an area affected by anthropogenic activity
Casein	63.6	50.0	20.0
Xanthine	36.3	16.6	40.0
Hypoxanthine	72.7	50.0	40
Tyrosine	45.4	16.6	40.0
Esculin	54.5	66.6	40.0
Urea	45.5	50.0	20.0
Lysozyme	81.8	66.6	100
Lactose	72.7	50.0	60.0
Inositol	54.5	50.0	60.0
Galactose	72.7	66.6	80.0
Mannitol	72.7	83.0	60.0
Mannose	82.0	83.0	40.0
Rhamnose	36.3	83.0	80.0
Arabinose	72.7	66.6	80.0
Sorbitol	72.7	66.6	80.0
Xylose	72.7	83.0	80.0

Table 3. Antagonistic activity of actinomycetes isolated from various wetland soil samples.

Sampling sites	% of activity of actinomycetes											
	S. b	Ec.78	S. t	S. s	*S. typhi*	S. m	V. c	Ec.12	S. e	B. s	S. w	*S. bareilly*
Mangrove	27	0	09	09	36	09	36	36	0	46	0	09
Bird sanctuary	33	0	17	0	0	33	0	33	17	33	17	17
Area affected by anthropogenic activity	60	20	40	0	0	20	0	20	20	40	40	20

S.b- *Salmonella bovis*, E.c- *Escherichia coli*, S.t- *Salmonella typhimurium*, S.s- *Salmonella senftenberg*, S.m- *Salmonella mgulani*, V.c- *Vibrio cholerae*, S.e- *Salmonella entritidis*, B.s- *Bacillus subtilis*, S.w- *Salmonella weltsverden*

All the 22 actinomycete isolates were identified based on their colony morphology and microscopic morphology. The mycelium structure, colour, and arrangement of spore were observed by cover slip technique and they were identified by using Bergey's manual of determinative Bacteriology, 9th edition (2000) along with the help of biochemical characters. Similar method has been followed by Berd (1973) and Mansour (2003). Macroscopically, some among the isolates had chalky appearance and some having smooth appearance. The colour of the substrate and aerial mycelia were varied. About 55% of the identified actinomycete strains belonging to the Nocardioform actinomycetes. Most *Nocardia* sp. develops well-formed, branched filaments with aerial mycelium which is sometimes visible grossly. They are aerobic, Gram-positive, non-motile actinomycete, which forms a branched substrate mycelium that fragments into irregular rod shaped elements (Hamid *et al.*, 2001). In the case of *Nocardia brasiliensis*, xanthine is not hydrolysed but it could hydrolyze casein. Those isolates that are casein negative and could not hydrolyze xanthine, hypoxanthine and tyrosine are assigned as atypical *Nocardia asteroids*. *Nocardia caviae* was found to hydrolyze xanthine, hypoxanthine and urea. They are resistant to lysozyme however were unable to decompose casein.

The *Rhodococcus* group microscopically consists of coccoid structures. They are mainly red pigmented ones. These isolates are positive for tyrosine decomposition, production of acid from sorbitol as reported by Berd (1973). *Micromonospora* bearing single spores in clusters also isolated in this study. Zig-zag hyphae of genus *Pesudonocardia* was identified in the present study.

Nearly 9% of isolates were belonging to the genus *Streptomyces*. *Streptomyces* are having short chains of large conidia formed on aerial and vegetative mycelium. Most of them do not exhibit urease activity as well as unable to resist lysozyme. They are able to utilize arabinose and galactose as the carbon source for energy and growth (Barbara, 2006). In the present study the frequency of occurrence of *Streptomyces* was low as they

favour alkaline condition which is an important characteristic feature of *Streptomycetes* sp. (Stackebrandt *et al.*, 1991). While, *Streptomyces pauciporeus*, *Streptomyces rubidus*, *Streptomyces guanduensis* and *Streptomyces yanglinensis* were isolated from acidic soil (Xu *et al.*, 2008).

Actinomycetes have been evaluated as a source of biocontrol agents and antibiotic compounds based on their distribution in various habitats (Ouhdouch *et al.*, 2001; Barakate *et al.*, 2002). The results were found to be interesting as more than 95% of the isolates had an inhibitory effect on pathogens used in this study. The antimicrobial activity among the isolates encountered in this study were considerably higher than those reported among sediment derived actinomycetes (Pisano *et al.*, 1985; Ellaiah *et al.*, 1996). The results also revealed that strains are having both broad spectrum and narrow spectrum of activity against the isolates. Out of 12 pathogenic strains used, 11 were gram negative organisms (*Salmonella* species, *Vibrio cholera* and *E. coli*). The only gram positive used was *Bacillus subtilis.* About 41% actinomycete isolates showed antibacterial activity against *Bacillus subtilis.*

The search for novel metabolites especially from actinomycetes requires a large number of isolates (over thousands) in order to discover a novel compound of interest. The search will be more promising if diverse actinomycetes are sampled and screened. However, the potential utility of these actinomycetes in screening programmes for bioactive natural products is confirmed. This ability is not restricted to one family or genus within actinomycetes, but rather, all of them offer opportunities to obtain bioactive compounds.

5. Conclusion

Results of the present study reveal good diversity of *Nocardioform* actinomycetes and *Streptomyces* in the wetland soils and many of them exhibited good antibacterial potential, which could be further explored for the development of new antibiotics.

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