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Diversity of *Bacillus* and *Actinomycetes* in the water and sediment samples from Kumarakom region of Vembanadu lake

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Diversity of different groups of *Bacillus* and *Actinomycetes* in the water and sediment samples from Kumarakom estuary was analyzed to find out potential strains for further application. *Bacillus* genera were identified and grouped into five phenogroups such as *Bacillus polymyxa, Bacillus subtilis*, *Bacillus sphaericus, Thermophiles* and *Alicyclobacillus*. Phenogroups show differences in the shape of the spore (oval or spherical), position of the spore (central or terminal) and swelling of the sporangium. Ability of the isolates to elaborate various hydrolytic enzymes and their ability to reduce nitrate and ferment various carbohydrate sources were also studied. Different plating media such as glycerol arginine agar, starch-casein agar and soil extract agar were used to isolate the *Actinomycetes*. Glycerol arginine agar found to be superior to the other two plating media. Diversity of *Actinomycetes* was less when compared to that of *Bacillus* genera. Potential of the *Actinomycetes* to produce antibiotics were evaluated by well diffusion method against 6 serotypes of *Salmonella*, *Vibrio parahaemolyticus* and *Escherichia coli.*

[**Keywords**: Wetlands, Microbial Diversity, *Bacillus*, Actinomycetes, Hydrolytic enzymes, Antibacterial activity]

Introduction

Genera *Bacillus* includes Gram-positive, rod-shaped bacteria that differentiate into heat-resistant endospores under aerobic conditions[1]. Spore formation is one of the major factors of ubiquity of bacilli; wind transfer ensures occurrence of these bacteria in practically every environment[2]. They can utilize plant and animal debris; a broad spectrum of substrates used, including cellulose, starch, agar, proteins, and carbohydrates[3]. These bacteria play an important role in the process of oil pollution removal in the ocean. Many bacilli produce antibiotics and biologically active compounds[4], they are therefore used as probiotics in aquaculture. In spite of their importance and ubiquity, these Gram-positive spore-forming bacteria, are more poorly studied than Gram-negative microflora. In addition to phenotypic heterogeneity, they also appear to be phylogenetically diverse[5], with five phylogenetically distinct clusters emerging from a comparative analysis of the small subunit rRNA. To date, the genus *Bacillus* has been subjected to numerous taxonomic reclassifications resulting in the proposal of new genera and species.

Actinomycetes, the Gram-positive filamentous, free-living saprophytic bacteria with true aerial hyphae widely distributed in soil and colonizing plant, are well known as a good source of microbial secondary metabolite producer in drug discovery programs. 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. Emergence of multiple drug resistant pathogens implies the need to search for new and novel antimicrobials[6,7] and the discovery of new molecules from actinomycetes has marked an epoch in antibiotic research and subsequent developments in antibiotic chemotherapy. The approaches considered in these research programmes include the isolation of new antibiotics from actinomycetes other than the genus *Streptomyces* and the exploration of new and particular ecological systems.

Present study was carried out in the Kumarakom region of Vembanadu lake which is virtually an unexplored wetland ecosystem. The aim of this investigation was to explore the diversity of *Bacillus* and antibiotic producing actinomycetes in the water and sediment samples from Kumarakom lake.

Materials and Methods

Study area

Water and sediment samples were collected from Kumarakom region (9°37'57" - 9°38'21"N, 76°25'06" - 76°25'11"W) of Vembanadu - Kol wetland of Kerala, along the southwest coast of India. Kumarakom lake is situated on the eastern banks of Vembanadu lake Fig. 1. Water and sediment samples

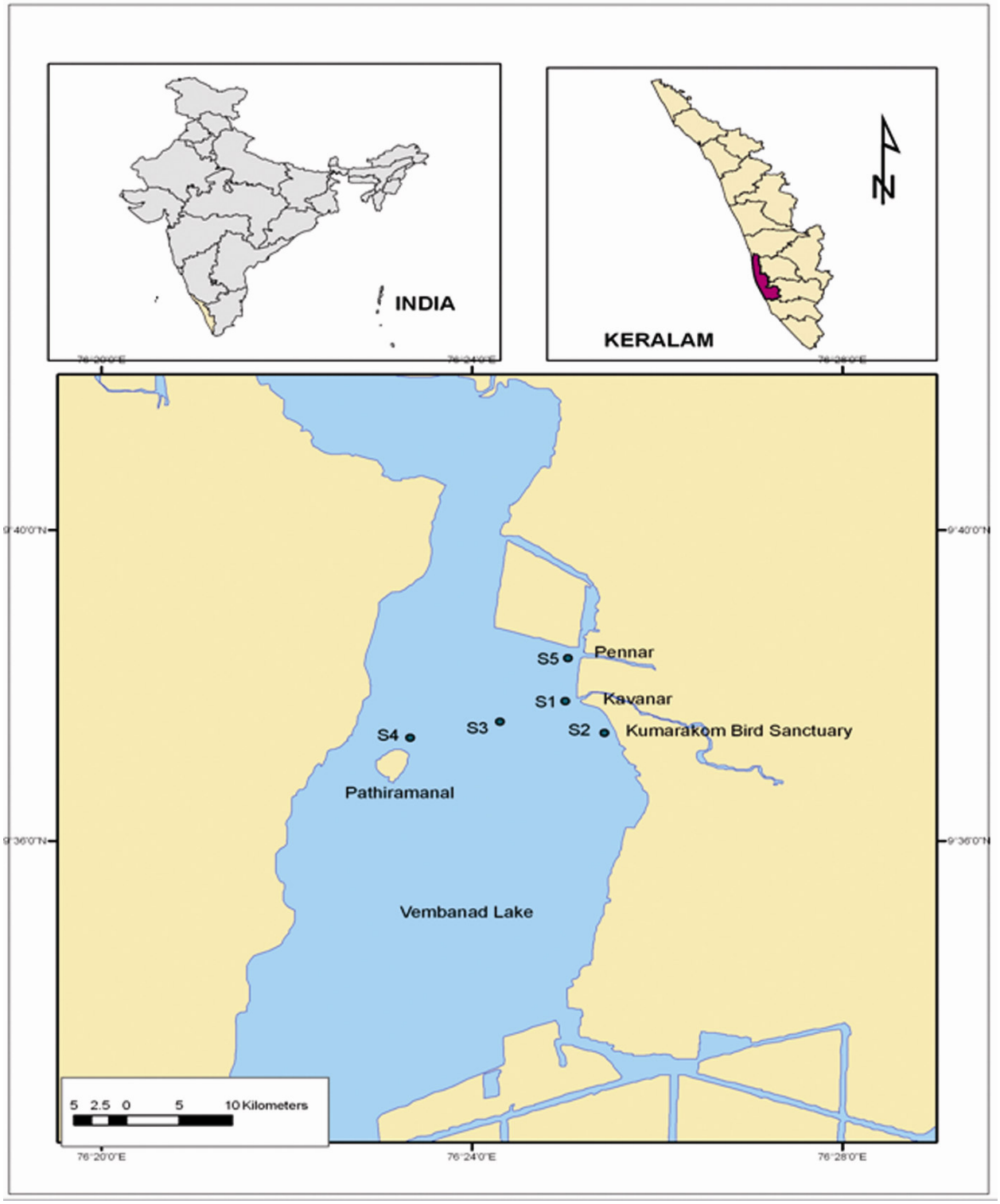

Fig. 1—Location of stations

for the present investigation were collected aseptically in sterile plastic bottles and polythene bags respectively and transported to the laboratory under ambient conditions.

Isolation of Bacillus and Actinomycetes from samples

Water and sediment samples were serially diluted and plated on tryptone soya agar (TSA, Himedia) for isolation of *Bacillus* strains. After incubation of the plates at 37°C for 48 hours, well separated colonies were isolated and restreaked to ensure purity and maintained on nutrient agar slants. Aseptic procedures were strictly followed during plating and isolation process. Generic level characterization of the *Bacillus* was carried out by Gram staining followed by spore staining. Grampositive, spore formers were identified as *Bacillus*.

Actinomycete strains were isolated from the air-dried sediment samples by applying serial dilution and spread plate technique on three different media such as Glycerol-Arginine Agar[8], Soil-Extract Agar and Starch-Casein Agar[9]. Different media were used in order to compare the recovery on different media as well as to obtain maximum diversity of actinomycete strains. Plates were incubated at room temperature for 2-3 weeks. After incubation typical actinomycete colonies were selected on morphological basis[10] and were transferred to Glycerol-Arginine Agar and maintained at room temperature.

Characterization of Bacillus isolates

The genus *Bacillus* were further checked for their motility using hanging drop method and characterized to species level by following various biochemical tests such as oxidation fermentation (O/F) test on OF Basal medium (Himedia), catalase test, nitrate reduction test, ability to ferment various carbohydrates such as glucose, lactose and sucrose and urea splitting ability (urease production) on Christiansen's urea agar (Hi-media). The ability of the *Bacillus* isolates to produce various hydrolytic enzymes such as amylase, lipase and gelatinase were evaluated by plate assay using nutrient agar supplemented with starch, tween 80 and gelatin respectively.

The ability of *Bacillus* isolates to grow at different temperatures such as 20°C, 37°C, 50°C and 55°C was also studied. The isolates were aseptically inoculated into sterile nutrient broth tubes and incubated at 37°C for 24 h. Growth was monitored by measuring the increase in optical density at 550 nm using a Spectronic 20 D spectrophotometer after 24 h of incubation.

Allocation of Bacillus species into groups

Grouping of Bacilli is based on the numerical classification of Priest[11] and they were classified into following groups:

Group I: All species are facultative anaerobes and grow strongly in the absence of oxygen. Acid is produced from a variety of sugars. Endospores are ellipsoidal and swell the mother cell.

Group II: All species produce acid from a variety of sugars including glucose. Most are able to grow at least weakly in the absence of oxygen, particularly if nitrate is present. Spores are ellipsoidal and do not swell the mother cell.

Group III: All species produce spherical spores which may swell the mother cell. All species are strictly aerobic but some have limited ability to produce acid from sugars.

Group IV: All these bacteria grow optimally at 50°C or above. Physiologically and morphologically they are heterogenous but most produce oval spores that swell the mother cell.

Group V: Thermophilic, acidophilic species with membraneous alicyclic fatty acids.

Identification and Evaluation of antibacterial activity of actinomycete isolates

Velvety actinomycete isolates were examined microscopically to determine if they have Gram-positive, filamentous or had long cells. Mycelium structure, arrangement of conidiospore and arthrospore on the mycelium was observed microscopically (Olympus 20i, Japan) using the oil immersion (100X) objective. Observed structure was compared with Bergey's Manual of Determinative Bacteriology, 9th edition (2000) and the organism was identified.

Antibacterial activity of actinomycete strains was determined by well diffusion method. Briefly, sterile glycerol-yeast extract agar plates were prepared and surface dried. Lawn cultures of pathogens such as *Salmonella* Typhi, *Salmonella* Paratyphi, *Salmonella* Enteritidis, *Salmonella* Senftenberg, *Salmonella* Bareily, *Salmonella* Mgulani, *Salmonella* Worthington, *Bacillus subtilis, Escherichia coli* O25 (enterotoxigenic), and *Vibrio cholera* were prepared on the above plates. Using a gel puncture, wells were punched (10 mm of diameter) in

fresh test microbial lawn cultures on glycerol-yeast extract agar and 100 µL of culture supernatant (5 day old culture incubated under shaking condition in Glycerol Arginine broth) of actinomycete isolates were then administered in each well and incubated at room temperature for 24 h. Bioactivity was determined by measuring the diameter of inhibition zones (mm) of test microorganisms around the well after incubation.

Results

Diversity of Bacillus

In the present investigation an attempt has been made to isolate *Bacillus* and actinomycete strains in the water and sediment samples from Kumarakom lake. A total of 50 strains belonging to the genus *Bacillus* and 56 actinomycetes were isolated from the samples. These *Bacillus* strains were classified into five pheno-groups based on their morphological, physiological and biochemical characters. These include *Bacillus polumyxa* (Group I), *Bacillus subtilis* (Group II), *Bacillus sphaericus* (Group IV), *Bacillus thermophiles* (Group V) and *Alicyclobacillus* (GroupVI). Their percentage distribution is given in the Table 1. It is found that most of the isolates were coming under the group *Bacillus subtilis* (68.75% from sediment sample and 55.56% from water sample).

Physiological characteristics of Bacillus isolates

Morphological and physiological characters of the *Bacillus* isolates is given in Table 2. Majority of the isolates are having oval spores (78.13% isolates from sediment sample and 83.3% isolates from water sample). Only 34.38% of isolates from sediment were able to reduce nitrate to nitrite, where as 50% of isolates from water were able to reduce nitrate. Most of the isolates were able to ferment one or more carbohydrates used in this study *viz*. glucose, lactose or sucrose. The preferred carbohydrate source was found to be glucose, followed by lactose and sucrose.

Table 1—Percentage distribution of different groups of *Bacillus* from the sediment and water samples of Kumarakom region of Vembanadu Lake

Bacillus Group	Percentage Distribution of *Bacillus* Groups	
	Sediment (n = 32)	Water (n = 18)
Bacillus polymyxa	9.375	11.1
Bacillus subtilis	68.75	55.56
Bacillus sphaericus	6.25	-
Bacillus thermophiles	3.125	-
Alicyclobacillus	3.125	16.6
Unidentified	9.37	16.7

Table 2—Morphological and Physiological characteristics of *Bacillus* from water and sediment samples from Kumarakom region of Vembanadu Lake

Morphological characteristics		Percentage of positives	
		Sediment	Water
Spore characteristics	Spherical spore	21.88	16.67
	Oval spore	78.13	83.3
	Swelling of mother cell	12.5	11.1
Location of the spore	Centrally located spore	50	22.22
	Terminally located spore	50	77.78
Physiological characteristics			
Utilization of carbon source	Glucose	100	94.45
	Lactose	46.88	83.33
	Sucrose	59.38	50
Motility		100	100
Oxidative		9.38	11.1
Fermentative		90.63	88.9
Catalase production		100	100
Nitrate reduction		34.38	50
Amylase production		100	100
Urease production		59.36	55.56
Lipase production		68.75	77.78
Gelatinase production		65.63	88.89
Growth at 20°C		28.13	22.22
Growth at 37°C		100	100
Growth at 50°C		15.63	27.78
Growth at 55°C		3.13	16.67

Growth response of *Bacillus* isolates at different temperatures (20°C, 37°C, 50°C and 55°C) reveal that they were able to survive at different temperatures and have an optimal growth at 37°C, whereas, 28.13% and 22.22% isolates respectively from sediment and water samples were able to grow at 20°C. Similarly 15.63% of isolates from sediment and 27.78% of isolates from water were able to grow at 50°C.

Hydrolytic enzyme production ability of different groups of *Bacillus* isolates from water and sediment samples in the Kumarakom lake is given in the Fig. 2. It revealed that all of them were capable of elaborating amylase. Besides several *Bacillus* isolates were also able to produce lipase, gelatinase and urease. However, the production level varied in different isolates. Only 55.56% of isolates from water and 59.36% of isolates from sediment sample of wetland are able to hydrolyze urea. Group I isolates from water and sediment and group VI isolates from water were the most efficient in the production of hydrolytic enzymes.

Diversity of Actinomycetes

Since actinomycetes show variation in their nutritional requirement, 3 plating media such as glycerol-arginine agar, starch-casein agar and soil extract agar were used to recover them from air dried sediments. The population obtained from different sediment samples on various plating media is presented in Table 3. Results revealed that the highest load of actinomycetes was obtained from the third sampling site. It was also found that glycerol-arginine agar was an efficient medium for the isolation of actinomycetes compared to starch-casein agar and soil-extract agar. The actinomycete strains were purified by visual, microscopic and cultivation methods and were maintained on glycerol-arginine agar at room temperature and were identified as actinomycetes as per Bergey's manual of determinative bacteriology (2000). These actinomycete isolates were classified into three morphological groups

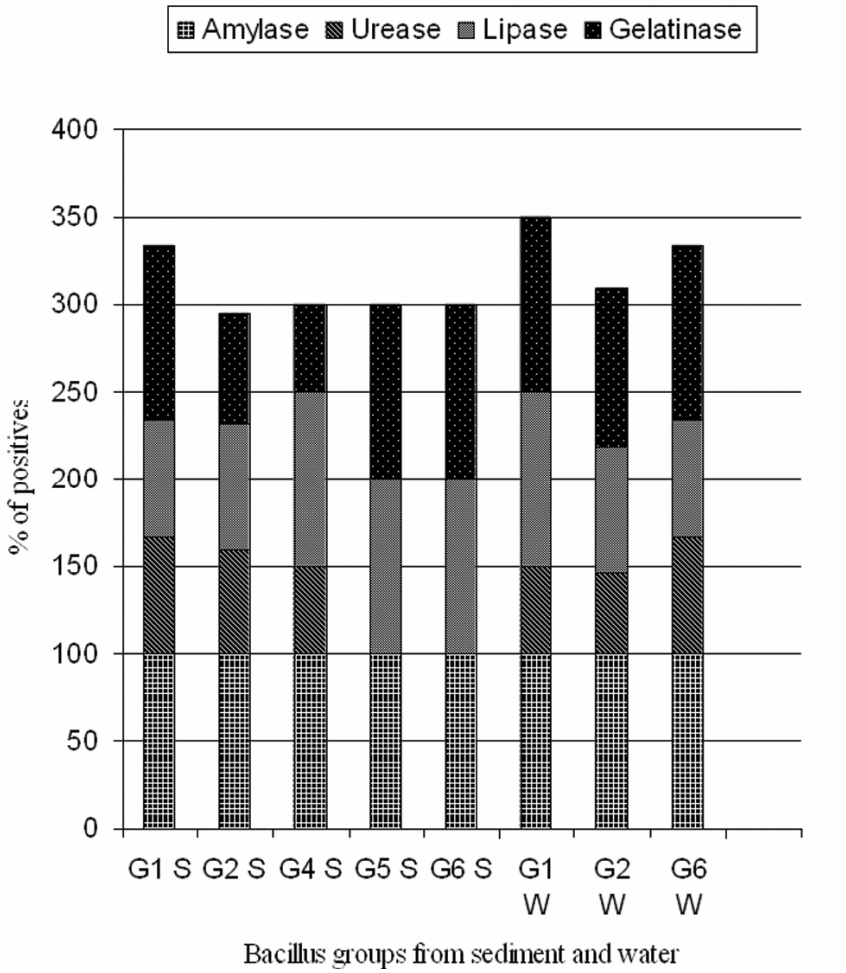

Fig. 2—Hydrolgtic enzyme production ability of different groups of Bacillus isolates from water and sediment

Table 3—Load of Actinomycetes in the sediment samples from Kumarakom estuary

Sample	Medium	Load of Actinomycetes (cfu/gm)
	Glycerol-arginine agar	-
Sediment sample 1	Starch-casien agar	13×10^7
	Soil-extract agar	-
	Glycerol- arginine agar	5×10^7
Sediment sample II	Starch casein agar	-
	Soil extract agar	-
	Glycerol arginine agar	16×10^7
Sediment sample III	Starch casein agar	2.1×10^7
	Soil extract agar	1×10^5

Table 4—Antibacterial activity of the actinomycete isolates against pathogenic strains

Pathogens tested	Zone of inhibition (mm)		
	A1	A2	A3
Salmonella paratyphi	25mm	25mm	22mm
Salmonella mgulani	18mm	12mm	-
Salmonella worthington	17mm	20mm	15mm
Vibrio cholera	21mm	15mm	18mm
Escherichia coli O25	13mm	20mm	20mm
Bacillus subtilis	25mm	25mm	24mm

based on their visual observation. Actinomycetes with green aerial mycelium and yellow substrate mycelium were placed under A1, those with white aerial mycelium and brown substrate mycelium as A2 and the actinomycetes with white aerial mycelim and yellow substrate mycelium were considered as A3. Percentage occurrence of different groups showed abundance of A2 (57.1%) in the sediment samples from Kumarakom lake, followed by A1 (41.7%). Occurrence of A3 type was very low (1.78%).

Antibacterial activity of Actinomycetes

The antibacterial activity of the *Actinomycete* isolates was found to vary. The result of the antibacterial activity of active *Actinomycete* isolates is given in Table 4. The results revealed that actinomycetes suppressed in different degrees the growth of six pathogenic strains of the total 10 bacterial strains tested. It is also observed that A1 and A2 strains showed the highest activity against *Salmonella* Paratyphi and *Bacillus subtilis* (25 mm). While A3 strain showed the highest activity against *Bacillus subtilis* (24 mm). The lowest activity was against *S.* Mgulani by A2 (12 mm).

Discussion

Bacilli are physiologically diverse and this can be grouped together based on the similarities in morphological, physiological and biochemical characters. Many studies have suggested that the strains of the genus *Bacillus* are more heterogenous than most other bacterial genera[11]. In the Bergey's manual of systemactic Bacteriology there are six genera of endospore forming bacteria featured. *Bacillus* is distinguished from the other endospore forming bacteria on the basis of being a strict or facultative aerobe, rod shaped and usually catalase positive. Here, 100% of the identified isolates are rod shaped and catalase positive. Genus *Bacillus* is generally motile with peritrichous flagella, the Anthrax bacillus being a notable exception[12]. All the *Bacillus* isolates encountered in the present study were motile and catalase positive.

The *Bacillus* isolates from the water and sediment samples of Kumarakom lake were grouped into 5 generic groups based on their morphological and biochemical studies. Group I include *Bacillus polymyxa* as a reference organism and comprise species such as *B. alvei, B. circulars* and *B. macrons* which produces oval spores that distend the mother cell. These ferment a variety of sugars and have reasonably fastidious growth requirements in the form of vitamins and amino acids. It was observed that 5.36% of isolates from sediment and 4.55% from the water were in the Group I.

B. subtilis and its relatives, *B. amyloliquefacience*, *B. licheniformis* and *B. pumilus* are included in group II. These bacteria differentiate into oval endospores that do not distend the mother cell. Most of these bacteria are regarded as strict aerobes but many, such as *B. subtilis,* have a limited ability to ferment sugars. However, they will grow readily under anaerobic conditions in the presence of glucose and nitrate as a terminal electron acceptor. Some species, such as *B. anthracis, B. cereus, B. licheniformis* and *B. thuringiensis* are true facultative anaerobes. These groups of Bacilli are reported to secrete several extracellular enzymes including many commercially important amylases[13]. Phosphate solubilizing *Bacillus amyloliquefacience* were isolated from arid mangrove ecosystem in Mexico[14]. It was found that 39.3% of isolates from sediment and 25% of isolates from water have the characteristics of Group II.

Bacilli which differentiate into spherical endospores are allocated to group IV. This is a phylogenetically homogenous group of species

including *B. sphaericus, B. psychrophiles, B. insolitus* and *B. psychrophilus* and some other species. These bacteria are strict aerobes[11]. Nearly 3.6% of isolates from sediment sample were categorized in group IV.

Nearly 1.8% of *Bacillus* isolates from sediment were found to be coming under the Group V (*Thermophile bacilli*). These bacteria grow optimally at 50°C or above. Physiologically and morphologically they are heterogeneous but most of them produce oval spores that swell the mother cell. They have various forms of energy metabolism ranging from strict aerobes to microaerophilic types. They are positive for amylase, catalase and motility tests. The *Thermophile bacilli* are phylogenetically diverse[3] and acidophilic thermopiles have recently been allocated to a new genus *Alicyclobacillus* (group VI). It was observed that 1.79% and 6.81% of isolates respectively from the wetland sediment and water sample have the characters similar to *Alicyclobacillus. Bacillus theromoamylovorans* were studied in detail for its physiological characters and it was found that they are moderately thermophilic, facultatively anaerobic, amylase positive, catalase positive, spore forming, rod shaped and are motile with peritrichous flagella[15].

All the *Bacillus* isolates encountered in this study had the ability to secrete amylase. There were several reports in yester years on amylase production in *Bacillus* species[16,17]. Bacterial amylases are very important with several commercial uses. These are used for preparation of sizing agents and removal of starch from woven cloth, preparation of starch pastes for use in paper coatings, liquefaction of heavy starch pastes which form during heating steps in manufacturing of corn and chocolate syrups, production of bread and removal of food spots in dry-cleaning industry where the amylase functions in conjunction with proteases[18].

The isolates were tested for the production of urease. It is observed that 55.56% of isolates from water and 59.36% of isolates from sediment sample of wetland are able to hydrolyze urea. *Thermobacilli* and *Alicyclobacillus* were not able to produce urease enzyme (Table 2). The isolates are also able to hydrolyze lipid and gelatin. About 77.78% of isolates from water and 68.75% isolates from sediment are able to produce lipase, while percentage of *Bacillus* capable of gelatinase production was 88.89 and 65.63 from water and sediment respectively. Lipase and gelatinase production by *Bacillus* isolates was reported[12].

Actinomycetes have been intensively studied for both theoretical and practical objectives; there is much scope for developing our basic knowledge of the means of detection and isolation of these microbes. This study also concentrated on methods for the isolation and determination of antimicrobial activities of actinomycetes. Studies of actinomycetes in soil are usually made with the dilution plate technique. The number of propagules of these organisms in most soils is intermediate between those of bacteria and fungi, so dilutions suitable for colony counting or isolation allow the development of large numbers of bacteria[19]. In the present work actinomycetes were isolated at dilutions 10^{-4} to 10^{-6}. Recovery of actinomycetes was significantly high in Glycerol-Arginine medium when compared to the other investigated media such as starch casein agar and soil extract agar. Preference for glycerol as carbon source by most actinomycetes and L-arginine as a selective nitrogen source favouring actinomycetes over bacteria was reported by earlier studies[20,8].

A total of 3 morphologically different actinomycete strains were isolated from the sediment samples studied. They were identified as actinomycetes as they were Gram-positive, branching, non-fragmenting hyphea and asexual spores[21]. Actinomycetes have been evaluated as a source of biocontrol agents and antibiotic compounds based on their distribution in various habitats[22,23,24]. In our study the isolated actinomycetes were tested for their activity against 10 pathogenic strains of bacteria and it was found that they were active against one Gram - positive (*Bacillus subtilis*) and five Gram - negative bacteria (*S.* Paratyphi*, S.* Mgulani*, S.* Worthington*, V. cholerae* and *E. coli* O25*).* The active compound from A1 and A2 strains showed highest activity against *Bacillus subtilis* (25 mm) and *Salmonella* Paratyphi (25 mm). The active compound from A3 showed maximum activity against *Bacillus subtilis* (24 mm).

Optimization of the conditions for antibiotic synthesis of the strains as well as establishing a suitable media for antibiotic production from stains is essential to determine about their activities. In the present work the antibacterial activity of actinomycete strains were checked by using three different medias (such as: Muller Hinton agar, Glycerol-Arginine agar and Glycerol-Yeast extract agar) and by three methods (such as: cross streak method, spectra-plak method and by well diffusion method). But the strains

showed antibacterial activity only by well diffusion method on Glycerol-Yeast extract agar. Thus, it is concluded that the substances repressing these bacterial strains were accumulated only in the mycelium and not in the medium[25].

Search for novel metabolites especially from actinomycetes requires a large number of isolates (over thousands) in order to discover a novel compound of pharmaceutical interest. The search will be more promising if diverse actinomycetes were sampled and screened[26]. Such an endeavor will undoubtedly lead to discoveries and new uses of secondary metabolites in other therapeutic areas such as cancer and immunosuppression, two areas where natural products from actinomycetes already have made substantial contributions.

Conclusion

Water and sediment from Kumarakom lake offers good diversity of different groups of *Bacillus* and Actinomycetes, with potential for further exploitation.

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