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Extraction, Purification and Antibacterial Activity of Bioactive Compounds from Marine *Bacillus* Species

M. Kannahi* and N.T. Eshwari

PG and Research Department of Microbiology,

S.T.E.T Women's College, Sundarakkottai, Mannargudi, Tamil Nadu, India

*Corresponding Author E-mail: kannahiamf@gmail.com

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ABSTRACT

Two coastal areas marine water and soil samples were collected from Vedharanyam and Muthupet of Thiruvarur and Nagapattinam District respectively. Marine Bacillus were isolated and identified according to morphology, biochemical test and colony characters on selective Marine agar medium. Marine Bacillus isolates were Bacillus licheniformis, Bacillus pumilus, Bacillus subtilis and Bacillus cereus. The TLC autobiographic overlay assay implied that the antibacterial activities produced by four strains with wide antibacterial spectrum. The isolates were tested for antibacterial activity against the terrestrial pathogens namely Salmonella typhi, Staphylococcus aureus, Escherichia coli and Enterobacter aerogenes. Bacillus licheniformis, Bacillus pumilus, Bacillus subtilis and Bacillus cereus were showed clear zone of inhibition against test organisms. The maximum antibacterial activity was noted in Bacillus subtilis (21.5mm) against Escherichia coli. The minimum antibacterial activity was recorded in Bacillus pumilus (13mm) against Escherichia coli.

Key words: *Marine Bacillus, Antibiotic, Antibacterial activities , Terrestrial Pathogens, Thin layer chromatography.*

INTRODUCTION

Earth is a biosphere sizzling with activities of all items of life. It is essentially a water planet, two thirds of which being covered with water especially marine water. The marine realm is an exceptional reservoir of biota and bioactive natural products, which has so far produced hundreds of novel structures with unique biological properties. The ocean environment is massively complex consisting of extreme variations in pressure, salinity, temperature and biological habitats. The potential of marine life as a source of novel molecules is immense and has been barely investigated. Because of their longer evolutionary history, marine organisms are likely to possess a greater molecular diversity than do their terrestrial counterparts.

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The rapid growth in the chemistry of marine organisms over the last 15 years has led to the discovery of a large number of new structures, many of which have no precedence among structures of terrestrial origin and possess previously unknown pharmacological and toxicological properties[1].

Marine microorganisms have unique properties since they have to adapt to extreme marine environment conditions such as high or low temperature, alkaline, or acidic water, high pressure and limited substrate in the deep sea water. The distinctive characteristics have attracted many researches to explore in depth since there is the potential of microorganisms used in biotechnological applications[2]

Marine life is a vast resource, providing food, medicine, and raw materials. Marine organisms contribute significantly to the oxygen cycle, and are involved in the regulation of the Earth's climate. Bacteria were isolated and cultivated from all possible regions of the earth's on the basis of their habitat, diversity, ecological functions, degree of pathogenicity and biotechnological applications. 70 % of the earth's surface is covered by oceans with rich microbial diversity.[3]

Thousands of marine *Bacilli* are known to contain antibiotic substance and less than 1% has been examined for their pharmaceutical activity. For example, *Bacillus silvestris*, *Bacillus cereus, Bacillus marinus, Bacillus subtilis, Bacillus licheniformis* , *Bacillus amyloliquefaciens* and *Bacillus pumilus* are known to produce bioactive substances in the marine environment even if they are specifically antibiotic producers. Bacteria exhibiting antibacterial activities have been isolated from various water and soil samples. In recent years, marine microorganisms have become important in the study of novel microbial products exhibiting antibacterial, antiviral, antitumour as well as anticoagulant and cardio active properties.

A number of biologically active compounds with varying degrees of action , such as antitumour, anticancer, antimicrotubule, antiproliferative, cytotoxic, photoprotective, as well as antibiotic and antifouling properties, have so far been isolated from marine sources[4,5,6].

The marine environment also represents a largely unexplored source of isolation of new microbes (Bacteria, Fungi, Microalgae, Cyanobacteria, and Diatoms) that are potent producers of bioactive secondary metabolites. Extensive research has been done to unveil the bioactive potential of marine microbes (free - living and symbiotic) and the results are amazingly diverse and productive[7,8] .

The present study was carried out to isolate the marine *Bacillus* from two coastal locations near Muthupet and Vedharanyam of Thiruvarur and Nagapattinam district respectively. The selected isolates were used to extract and purify bioactive compounds. The extracted bioactive compounds were subjected to antibacterial activity against terrestrial pathogens.

Experimental Section

Sampling Area

Marine soil samples and water samples were collected from Muthupet and Vedharanyam coastal areas of Thiruvarur and Nagappatinam district, Tamil Nadu, India.

Sample collection

In this study, the collected soil samples were placed in sterile polythene bags. The water samples were collected in clean, sanitized and sterilized glass water bottles.

Physico – Chemical parameter Analysis

Determination of parameter like p^H, Moisture, Temperature, Nitrogen, Pottasium, Phosphours, Calcium, Copper, Iron, Zinc, Carbon, Manganese were analysed in soil using standard methods[9]. P^H determination was carried out using p^H meter.

Enumeration of Total viable count

Total viable count (TVC) of aquatic samples were calculated by pour plate technique. Each sample was serially dilluted with dilutions ranging from 10^{-1} - 10^9 and 1ml of each dilution were poured on the nutrient agar plates. Plates were incubated at 37ºC for 48 hours in an inverted position and the colonies were

counted. The count were expressed as the number of colony forming units in 1 ml of the original sample. The colony count below 30 is indicated as TFTC (Too few to count) and above 300 as TNTC (Too number to count)[10].

Isolation of Marine *Bacillus*

The samples (0.5g) were triturated , suspended with sterile seawater and spreaded on the entire surface of 1/10 Marine Agar Medium. The medium were prepared by adding the components of peptone (0.12g) , Yeast extract (0.025 g) , $Fepo_4$ (0.025 g), Agar (3.75 g), Seawater (250 ml). After incubation at 25°c for 2 days, all colonies with different morphology were chosen for bacterial isolation[11.]

Identification of Marine *Bacillus*

The isolated marine *Bacillus* was identified based on the colony morphology, gram staining property and biochemical charactersitics. Finally, they were confirmed by their growth on Marine agar medium.

Preparation of *Bacillus* cultures and crude extracts

300 ml of marine broth was prepared. The medium were prepared by adding the components of Peptone (5g), Yeast extract (1g), $FePO_4$ (0.1g), Seawater (1 L). The marine *Bacillus* was cultured in 300 ml of marine broth in 500ml of conical flask for the production6.

Bioactive compound extraction and purification

For the extraction of crude bioactive compound, 100g of powdered material were exhaustively extracted with 200ml of ethyl acetate using soxhlet apparatus and evaporated under reduced pressure to yield viscous dark gum. The extract was stored at 4ºC in air - tight plastic vials for further studies[1]. Partial purification of bioactive compound was carried out using readymade silica gel coated TLC sheet. Crude extract were spotted at the bottom of TLC sheet using capillary tube and placed in a glass tank with solvent system. After running the chromatogram, the TLC plate was air dried and placed on closed chamber to clearly visualize the separated compounds as spots[12].

Antibacterial activity of bioactive compounds of marine *Bacillus* species

Antibacterial activity of bioactive compounds was tested for their activity against the terrestrial pathogens namely *Salmonella* typhi, *Staphylococcus aureus, Escherichia coli, Enterobacter aerogenes* and Streptococcus *mutans*. Antibacterial activity was assayed in duplicate using a standard paper disc assay[13]. The dried crude extracts were dissolved in EtOAc to a concentration of 100 mg ml^{-1}. The samples (20µl) were used to saturate the antibacterial assay paper disks (6mm) with a period of drying between each application. The disks were placed onto the agar surface containing the test microorganisms, and incubated at 37ºC for 24 hours after a diffusion process for 10 hours at 8ºC. The diameter of any inhibition zones formed around the paper disks were measured[14].

RESULT AND DISCUSSION

In the present study , marine water and soil samples were collected from Vedharanyam and Muthupet coastal areas of Nagapattinam and Thiruvarur District, Tamilnadu, India.

Analysis of Physico - chemical parameters of the marine samples

Analysis of the physico - chemical parameters of the marine water samples collected from two different sites. The parameters such as pH, temperature, electrical conductivity, dissolved solids, salinity, zinc, copper, iron, nickel, cobalt, total mercury, total cyanide, total lead, selenium, total silver, nitrate, nitrite, ammonia, inorganic sulphide and sulphate were analyzed using the standard methods[9].

The collected sediment sample were first air dried at room temperature, then crushed using a porcelain mortar and pestle and then sieved for further analysis. The p^H of the suspension was read using p^H meter (Systronics, India), to find out the soil P^H. Electrical conductivity of the soil was determined in the filtrate of the water extract using Conductivity Bridge and Caution Exchange Capacity (CEC) of the soil was

determined by using 1 N ammonium acetate solution. The reagents used for the analysis were AR grade and double distilled water was used for preparation of solutions. The Physico - chemical parameters were analysed and presented in Table – 1.

Enumeration of Total Viable Count

The most common procedure for the enumeration of bacteria is the viable plate count. In this method, serial dilutions of a sample containing viable microorganisms are plated onto a suitable growth medium. The suspension was either spreaded onto the surface of agar plates (Spread plate method), or mixed with molten agar, poured into plates, and allowed to solidify (pour plate method). The plates were then incubated under conditions that permit microbial reproduction so that colonies develop that can be seen without the aid of a microscope. It is assumed that each bacterial colony arises from an individual cell that has under gone cell division. Therfore, by counting the number of colonies and accounting for the dilution factor, the number of bacteria in the original sample can be determined [15]. The direct viable count, a microscopic method for enumeration of viable bacteria. The modified direct viable count will be growth and survival studies of bacterial cells in marine water samples[16].

The samples were subjected to serial dilution and the bacterial count was made in the plate counting techniques. The bacterial loads, obtained were enumerated from two coastal areas (Vedharanyam and Muthupet). Some colonies were counted particulary in the dilution rate of 10^{-4} – 10^{-7}. The numbers of colonies of Vedharanyam soil and water 118 x 10-4 cfu / ml, 120 x 10-5 CFU/ ml were recorded respectively. The total bacterial colonies of Muthupet soil and water were 125 x 10^{-5} cfu /ml, 152 x 10^{-6} CFU/ml recorded respectively. Total viable *Bacillus* species were enumerated and represented in Table-2 and plate 1.

Isolation and identification of Marine *Bacillus*

The samples (approximately 4 g each) were collected using some clean, dry and sterile polythene bag along with sterile spatula. All the samples were transferred to lab under sterile conditions, 1g of each soil samples was added to 5ml of nutrient broth and incubated at 35°C for 24 hours. After the incubation period, 0.1 ml of the supernatant of each tube containing suspension of soil and culture media were inoculated in nutrient agar plates by streaking at 30° C for 24 hours. After that, the plates were examined and the suspected colonies were stained by Gram staining method. The Gram positive, rod shaped, spore forming *Bacilli* were selected for additional identification tests. Subsequent identification tests including susceptibility test to penicillin, citrate hydrolysis, motility, Voges – proskauer, Indole production, catalase, nitrate reduction and production of H_2S were performed[17].

The marine *Bacillus* sps were isolated from Vedharanyam and Muthupet coastal areas. The marine *Bacillus* was isolated using Marine agar medium. The isolated *Bacillus* colonies were identified by cultural, morphological and biochemical characteristics and the results were showed in Table 3 and Plate 2.

The isolate 1 showed the result for Indole - negative, Methyl Red - positive, Voges Proskauer - positive, Citrate utilization test - negative, Catalase test - positive, Oxidase test - positive, Urease test - negative. Hence it was identified as *Bacillus licheniformis*. The isolate 2 showed result for Indole - negative, Methyl Red - positive, Voges Proskauer - negative, Citrate utilization test - positive, Catalase test - positive, Oxidase test - negative, Urease test - negative. Hence it was identified as *Bacillus pumilus*. The isolate 3 showed result for Indole - negative, Methyl Red - negative, Voges Proskauer - positive, Citrate utilization test - positive, Catalase test - positive, Oxidase test - negative, Urease test - negative. Hence it was identified as *Bacillus subtilis*. The isolate 4 showed result for Indole - negative, Methyl Red - positive, Voges Proskauer - positive, Citrate utilization test - positive, Catalase test - positive, Oxidase test - negative, Urease test - negative. Hence it was identified as *Bacillus*

cereus. The isolated marine *Bacillus* sps such as *Bacillus licheniformis, Bacillus pumilus, Bacillus subtilis, Bacillus cereus.* All the isolates showed positive results for gram staining and motility tests Table 4 and plate 3.

Table 1: Physico – Chemical Analysis of Soil Sample

S.No:	Name of the Parameters	Vedharanyam	Muthupet
1.	Moisture (%)	52.20	40.7
2	PH	7	7
3	Temperature(`c)	25	25
4	Nitrogen (kg)	0.04 ± 0.02	0.04 ± 0.02
5	Potassium (kg)	0.05 ± 0.04	0.06 ± 0.08
6	Phosphorus (kg)	0.02 ± 0.01	0.02 ± 0.01
7	Calcium (ppm)	4.5 ± 2.84	4.7 ± 3.08
8	Carbon (%)	4.2 ± 2.08	4.9 ± 3.10
9	Zinc (ppm)	2.7 ± 1.08	4.8 ± 3.120
10	Copper (ppm)	1.6 ± 0. 01	2.6 ± 1.010
11	Iron (ppm)	2.3 ± 1.05	4.1 ± 3.04
12	Manganese (ppm)	5.6 ± 3.08	5.2 ± 4.012

Values are expressed as Mean ± Standard deviation

Table 2: Enumeration of viable count of isolated *Bacillus* species

S. No:	Isolated Marine Bacillus species	Dilution factor	Vedharanyam		Muthupet	
			No. of Colonies in Soil (CFU/ml)	No. of Colonies in water (CFU/ml)	No. of Colonies in Soil (CFU/ml)	No. of Colonies in water (CFU/ml)
1	Bacillus licheniformis	10-4	30 x 10-4	30 x 10-4	36 x 10-4	40 x 10-4
2	Bacillus pumilus	10-5	35 x 10-5	30 x 10-5	24 x 10-5	35 x 10-5
3	Bacillus subtilis	10-6	28 x 10-6	24 x 10-6	30 x 10-6	42 x 10-6
4	Bacillus cereus	10-7	35 x 10-7	36 x 10-7	35 x 10-7	45 x 10-7

Table 3: Morphological & biochemical characteristics of Isolated Marine *Bacillus*

S. No:	Morphological & Biochemical Characteristics	*B. licheniformis*	*B. pumilus*	*B. subtilis*	*B. cereus*
1	Staining	+	+	+	+
2	Motility	+	+	+	+
3	Shape	*Rod*	*Rod*	*Rod*	*Rod*
4	Indole	-	-	-	-
5	Methyl Red	+	+	-	+
6	Voges Proskauer	+	-	+	+
7	Citrate utilization test	-	+	+	+
8	Catalase test	+	+	+	+
9	Oxidase test	+	-	-	-
10	Urease test	-	-	-	-

(+) Indicates Positive, (-) Indicates Negative.

Table 5: Antibacterial activity of isolated Marine *Bacillus species*

S.No:	Bacterial Strains	Zone of inhibition (mm in diameter)			
		Salmonella typhi	***Staphylococcus aureus***	***Escherichia coli***	***Enterobacter aerogenes***
1	*Bacillus licheniformis*	16 ±4.32	18.5±3.51	16±1.82	16.5±3.10
2	*Bacillus pumilus*	13.75±2.21	12.25±2.21	13±2.16	12 ±1.82
3	*Bacillus subtilis*	19±2.94	19±2.58	21.5±3.87	18.5±4.04
4	*Bacillus cereus*	14.5±5.08	12.25±2.21	14.25±6.02	16.5±1.29

Values are expressed as Mean ± Standard Deviation

Plate 1

Enumeration of total viable cells using Nutrient Agar medium

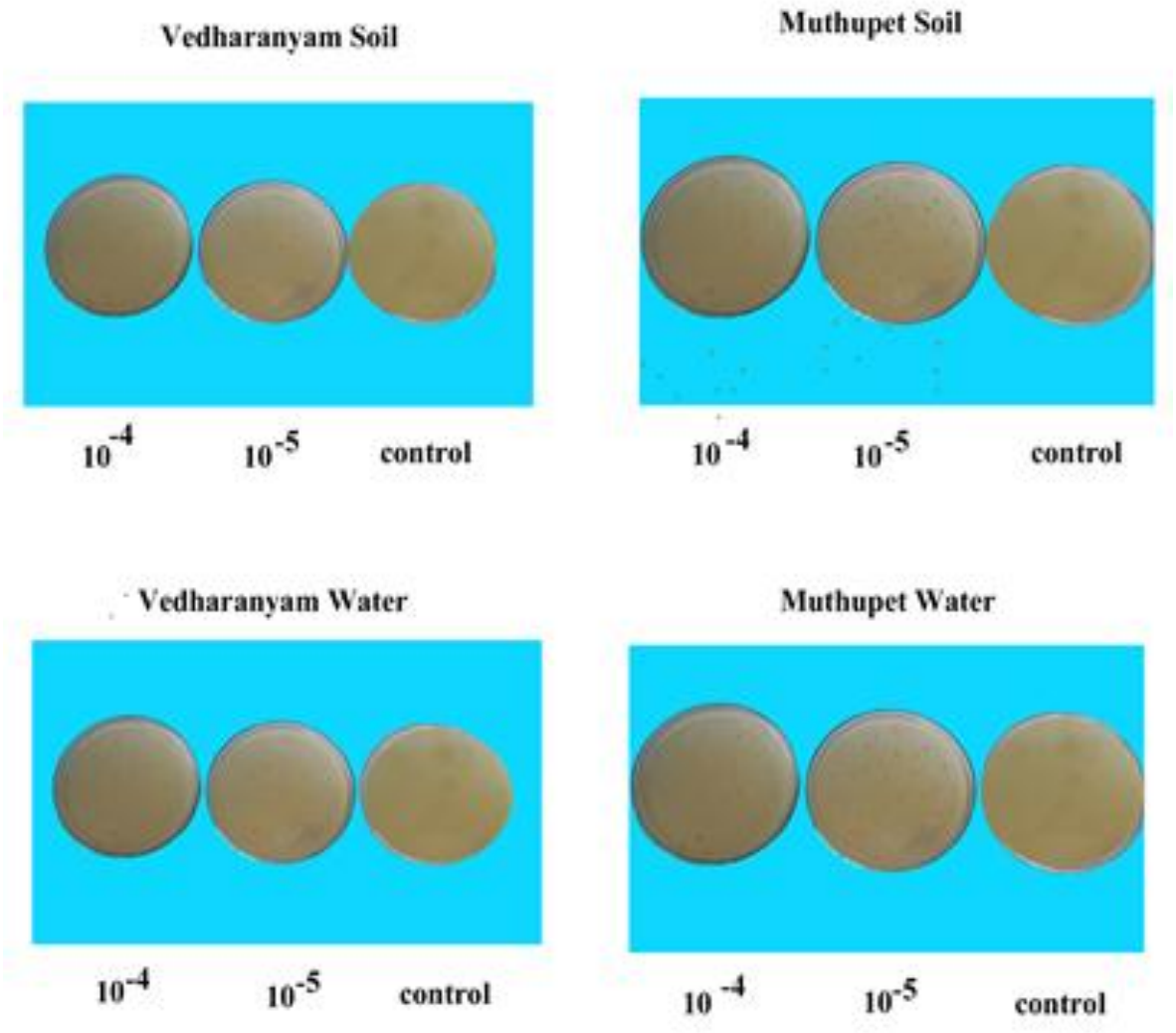


Plate - 4

Thin Layer Chromatography for Crude Extraction of Bioactive Compound

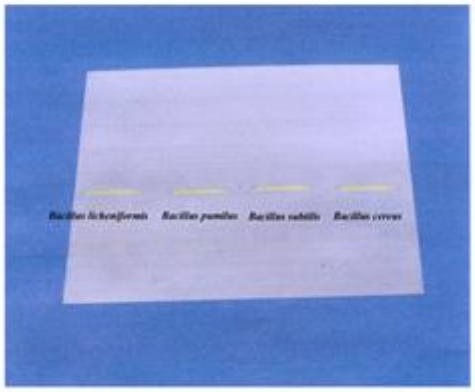
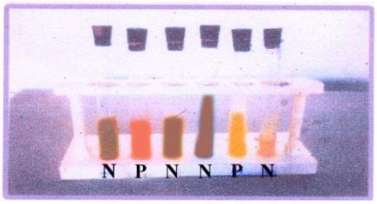
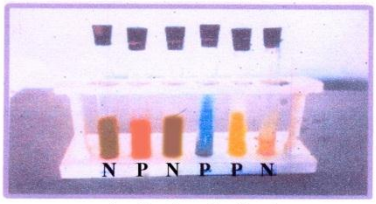


Plate - 3
Biochemical characteristics of Isolated Marine *Bacillus* species

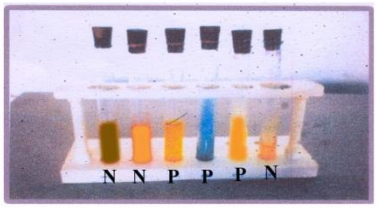
Bacillus licheniformis



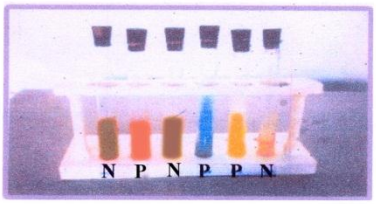
Bacillus pumilus



Bacillus subtilis



Bacillus cereus



Overall view of Biochemical Test

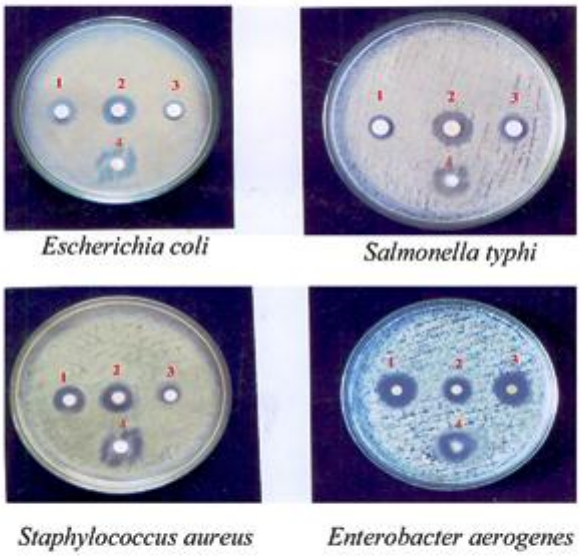
1. **Indole**
2. **Methyl Red**
3. **Voges Proskauer**
4. **Citrate utilization test**
5. **Catalase test**
6. **Urease test**

P - Positive

N - Negative

Plate - 5

Antibacterial activity of Marine Bacillus sps against selected pathogens



1. *Bacillus licheniformis*
2. *Bacillus pumilus*
3. *Bacillus subtilis*
4. *Bacillus cereus*

Preparation of *Bacillus* Culture

The identification of marine *Bacillus* sps such as *Bacillus licheniformis, Bacillus pumilus, Bacillus subtilis and Bacillus cereus* were cultured in the each 100 ml of marine broth in each 100ml of conical flask for the production.

Extraction and purification of bioactive compound

The crude extract, at 500 g µg / disc of srain 7B1 exhibited a promising anti-bacterial activity against the Gram positive test strains. It caused inhibition zones of 16,18 and 26 mm against *S.aureus, B.subtilis* and *M. luteus* respectively, Gram negative bacteria were resistant of the tested crude extract. This resistant may be attributed of the low permeability of the Gram negative bacteria, outer membrane and the lipopolysaccharide barrier for the hydrophobic compounds[18, 19].

The crude extract of bioactive compound was exhaustively extracted with 200ml of ethyl acetate using soxhlet apparatus. The extracted crude bioactive compound was carried out using silica gel coated TLC sheet. Crude extract were spotted at the bottom of TLC sheet using capillary tube and placed in a glass tank with solvent system. After running the chromatography, the TLC plate was air dried and placed on closed visualize the separated compounds as spots[12].

The crude extractions of bioactive compounds were exhaustively extracted with ethylacetate using soxhlet apparatus. The extract was stored at 4 °c in air-tight plastic vials for further studies[1]. The bioactive compounds were carried out using readymade silica gel coated TLC sheet. The crude extract was spotted at the bottom of TLC sheet using capillary tube and placed in a glass tank with solvent system. After running the chromatography, the TLC plate was air dried and placed on closed chamber to clearly visualize the separated compounds as spots. The visualized separated compounds were showed in the Plate – 4.

Antibacterial activity of bioactive compounds of marine *Bacillus* sps

Antimicrobial activity of *Streptomyces* species

by cross streak method in primary screening the activity of pure isolates were determined by cross streak method on nutrient agar[20]. The test organism used for this assay was *Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa.*

Antimicrobial activity was determined by using the purified extract which was eluted in ethyl acetate by agar diffusion method using 3 hours broth culture which was compared with MacFarland standard 0.5. 100µl of the crude extract was loaded in the sterile disc which was placed over the lawn culture. The plates were incubated at 37°C for 18-24 hours and examined. The diameter of the zones of complete inhibition was measured[21].

All the isolated marine bacteria were screened for antimicrobial activity against terrestrial microbes including *Salmonella typhi, Staphylococcus aureus, Escherichia coli, Enterobacter aerogenes and Streptococcus mutans* (Agricultural Culture Collection of Tamilnadu) as the test microorganisms. Antimicrobial activity was assayed in duplicate using a standard paper disc assay[13]. The dried crude extracts were dissolved in EtOAc to a concentration of 100 mg ml-1. The samples (20 µl) were used to saturate the antimicrobial assay paper disks (6 mm) with a period of drying between each application. The discs were placed onto the agar surface containing the test microorganisms and incubated at 37 °C for 24 h after a diffusion process for 10 h at 8°C. The diameters of any inhibition zones formed around the paper discs were then measured.

Antibacterial activity of bioactive compounds was tested for their activity against the terrestrial pathogens namely *Salmonella typhi, Staphylococcus aureus, Escherichia coli, Enterobacter aerogenes* and *Streptococcus mutans.* The crude extracts of bioactive compound tested for antibacterial activity by well diffusion method. Antibacterial activity of *Bacillus* species namely *Bacillus licheniformis, Bacillus subtilis, Bacillus pumilus and Bacillus cereus* against terrestrial pathogens were observed (Plate – 5).

In this study, the maximum antibacterial activity was noted in *Bacillus subtilis* (21.5 mm) against *Escherichia coli*, *Bacillus subtilis* (19 mm) against *Staphylococcus aureus, Bacillus licheniformis* (18.5mm) against *Staphylococcus aureus, Bacillus cereus* (16.5mm) against *Enterobacter aerogenes, Bacillus licheniformis* (16.5mm) against *Enterobacter aerogenes*: The minimum antibacterial activity were observed in *Bacillus cereus* (14.5mm) against *Salmonella typhi, Bacillus pumilus* (13.75mm) against *Salmonella typhi* and *Bacillus pumilus* (13mm) against *Escherichia coli*. The results were showed in Table 5.

From the above results, the maximum antibacterial activity was noted in *Bacillus subtilis* (21.5 mm) against *Escherichia coli.* The minimum antibacterial activity was recorded in *Bacillus pumilus* (13 mm) against *Escherichia coli*.

CONCLUSION

Screening the marine *Bacillus* sps from the two coastal regions were taken up to evaluate their antimicrobial potential against *Salmonella typhi, Staphylococcus aureus, Escherichia coli* and *Enterobacter aerogenes*. Marine *Bacillus* sps such as *Bacillus licheniformis, Bacillus pumilus, Bacillus subtilis* and *Bacillus cereus* isolated from two coastal regions of Vedharanyam and Muthupet soil and water sample showed antibacterial activity against terrestrial pathogens. Finally concluded that the isolated marine *Bacillus* species such as *Bacillus licheniformis, Bacillus pumilus, Bacillus subtilis* and *Bacillus cereus* were highly recommended for antibiotic production in the pharmaceutical field.

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