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ORIGINAL RESEARCH ARTICLE

Isolation and Characterization of Bacterial Strains from Sea Water of Mandaicad in Tamil Nadu, India

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ABSTRACT

The aim of present study was to isolate, characterize and identify bacteria from saline water. The bacterial isolates were isolated by serial dilution technique (Pour plate method). The isolated bacterial isolates were purified by frequent streaking in Nutrient agar slants and stored in the refrigerator at 4°C. The bacterial strains were identified by staining techniques, motility tests, platting on selective medium and biochemical tests. The isolated bacterial strain was Gram-positive bacilli, non-motile, catalase-positive and aerobic. Optimum growth was observed at 35°C and pH 8. The bacterial isolates were identified as *Bacillus laterosporous*. The macroscopic and microscopic observations were carried out on pure isolates. This study recommends that the bacteria isolated from Asian sea bass are having antimicrobial activities and could be used as a potential source for the development of marine drugs.

Key words: Bacteria**,** Serial dilution, Sea water and *Bacillus laterospoprous*.

1. INTRODUCTION

Moderately halophilic bacteria are widely distributed throughout hypersaline habitats and require from 3 to 15% w/v NaCl for satisfactory growth [1]. In recent years, it has been found that several products of these bacteria such as exopolysaccharides (EPSs), halophilic enzymes and compatible solutes, may have very useful applications in biotechnology [2, 3]. Nowadays, the study of bacteria from marine origin and their potential role in the production of bioactive compounds is becoming a new topic for research [4]. The number of natural products isolated from marine organisms increases rapidly and now exceeds 18,000 with hundreds of new compounds being discovered every year. The emergence of resistance of bacteria to antibiotics is a common phenomenon. Therefore, there has been a great concern from scientists to investigate marine microorganisms as new source of antibacterial compounds [5]. A number of bacteria present in aquatic ecosystems inhibit growth of other microorganisms by producing antimicrobial substances [6]. Several compounds such as alkyl amides, alkyl amines and phenolic compounds reported to have antimicrobial activities against both Gram positive and Gram negative organisms [7, 8, 9]. *Bacillus laterosporus* is widely used in plant and aquaculture productions. Modify soil texture, increase profitable soil microorganism, and improve soil organic matter.

2. MATERIALS AND METHODS

Site description and sample collection

Tamil Nadu is situated from11.00 north latitude and from 78.00 east longitude. Sea water samples were collected from sea beaches of Mandaicad, Kanyakumari districts. The samples were collected in 1 L sterile plastic bottles with proper level. The samples were immediately stored in a refrigerator for further microbial work. pH, water temperature and conductivity were measured the sampling site. The pH of the water sample was varied from 8.4 - 8.6, similarly the electrical conductance (Eh) of the samples varied from 24.9 - 61.8 (ms/ppt).

Isolation of bacteria

Nine millilitres of sterile water was poured aseptically into seven tubes each and 1 ml of the sea water sample was added to the first test tube and mixed thoroughly. Another 1 ml was taken from the first tube and added to the second test tube and mixed very well. From the second test tube, another 1 ml was taken and introduced into

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the third test tube and mixed very well. This procedure continued until the seventh test tube.

Inoculation of plates

Duplicate plates of Zobell Marine Agar were inoculated with 0.1 ml of the diluted solution (10^{-1} to 10^{-7}) using glass spreader technique. All plates were incubated at a temperature of 35°C for 72 hrs before colony enumeration and isolation [10].

Identification of isolate

The isolate were sub-cultured and Gram - staining was carried out. Identification of isolate was carried out based on the method described by Sakazaki and Shimad [11], Collins *et al*. [12] and Cheesbrough [13]. The Gram staining was aimed at differentiating Gram reactions, sizes, shapes and arrangement of cells of the isolates. For the Gram - staining of the various isolates, glass slides were washed and air dried. A drop of normal saline was placed on the slide. Using a flame inoculation wire loop, a small amount of inoculum was taken and smeared on the drop of normal saline on the slide. The smear was allowed to air dry and heat fixed by passing over flame three times. The fixed smear was flooded with crystal violet for a minute and then rinsed with clean water. Lugol's iodine was added for another one minute and this served as a mordant. This was later rinsed and cleaned with distilled water. Acetone-alcohol was added as decolouriser and rinsed immediately with clean water. A counter stain, safranin, was added and allowed to stand for a minute before being rinsed with clean water. This was allowed to dry before observing under oil immersion objective microscope.

Physiological and biochemical characters

The characters of the organisms were studied following the standard microbiological methods. Morphology, vegetative cell and spore characters were observed under a phase contrast microscope (100X objective) from 72 hrs old culture grown on a rotary shaker at 100 rpm, 30±1°C. The physiological and biochemical characters *via* growth on MacConkey, Indole test, Methyl red Test, Voges Proskauer test, Citrate utilization, H_2S production, Gas production from glucose, Casein hydrolysis, Starch hydrolysis, Nitrate reduction, Catalase test, Oxidase test, Arginine dihydrolase, Tween 80 hydrolysis, Acid production from dextrose, xylose, fructose, lactose, mannose, cellobiose were studied.

Growth Curve and optimization of the isolate

The growth curve of the isolate was studied in Zobell Marine Broth medium [14] modified with a balanced mixture of sea salts [15] at a concentration of 7.5 % (w⁄v). Incubation was at 35°C in a rotary shaker (100 rev /m).

To optimize the growth, we assayed the following variables in Zobell Marine Agar medium incubation time (1 – 8 days), temperature (4°C, 10°C, 25°C, 30°C, 35°C, 37°C, 42°C, 55°C and growth at anaerobic condition), sea - salt concentration (2, 4, 6, 8, 9, 10, 12) [w⁄v], glucose, lactose, sucrose, galactose, fructose, maltose as carbon sources at a concentration 0.02 mg to 0.1 mg, arginine, histidine, cysteine, tryptophan, tyrosine, proline as amino acid source at a concentration of 0.02mg to 0.1mg and incubated. Highly diluted suspensions of the organisms were spotted on the plates, incubated at 35 °C for 72 hrs and the growth of the organism was checked. The pH tolerance of the organisms was also checked on same medium maintained at different pH (5, 6, 8 and 9). The organisms were spotted on the plates by inoculating 0.1 ml of the diluted solution using glass spreader technique and incubated at 35°C for 72 hrs and growth was checked.

Antibiotic resistance

Response of the organisms to different antibiotics was tested on Zobell Marine Agar medium. Zobell Marine agar plates were surface seeded with 2 µl of 10^{-6} bacterial suspension/ml. Different antibiotic discs (Penicillin-G, Nalidixic acid, Erythromycin, Kanamycin, Neomycin, Rifampicin, Vancomycin, Gentamicin, Methicillin) with effective concentrations were placed over the plates. Inhibition of growth depicted by a clear zone formation around the discs indicated sensitive reaction otherwise the organism was resistant to the antibiotic. The diameter of the inhibition zone was measured with an antibiotic zone scale.

3. RESULTS AND DISCUSSION

The isolated bacterial strain was identified as *Bacillus laterosporus* using various biochemical tests as per the Bergey's manual of determinative bacteriology [16].

Based on the biochemical and morphological tests according to the Bergey's manual [17] the strain was identified as genus *Bacillus*. The isolated microorganism was stained Gram - positive. The strain was found to be rod shaped; circular configuration, entire, raised, smooth, opaque, scattered in arrangement with creamy yellow in pigmentation having a size from 1.0 - 1.5µm. They are non-motile (**Table 1**).

The physiological and biochemical characters *via* growth on MacConkey, Indole test, Methyl red Test, Voges Proskauer test, Citrate utilization,

H_2S production, Gas production from glucose, Casein hydrolysis, Starch hydrolysis, Nitrate reduction, catalase test, oxidase test, Arginine dihydrolase, Tween 80 hydrolysis, Acid production from Dextrose, Xylose, fructose, Lactose, Mannose, Cellobiose (**Table 2**) also confirm to the characters of *Bacillus* spp. Further, some confirmative tests were also done to identify the isolates up to species level. Taking all the above characters into consideration the isolates were tentatively identified as *Bacillus laterosporus*. Identification was made upto genus level.

Anaerobic and catalase test showed that the *Bacillus laterosporus* are not capable to grow under anaerobic condition but the strain is found to be catalase positive. These findings are in good agreement with previous studies which reported that aerobic organisms present in the saline environment are predominated by Gram - positive, facultative and spore forming rod bacteria [18]. The growth curve of the isolated bacteria was grown in Zobell Marine Broth medium containing 7.5% (w / v) total salts for 8 days at 35°C and 100 rev/min and read at 600 nm. Frequency of occurrence of the isolate *Bacillus laterosporus* was isolated from six out of seven inoculated plates. This represented 85.7% of the isolated sample .

The strain could grow at temperature ranging from 4°C to 42°C, however optimum temperature is 35°C (**Table 3**). The strain was tolerating upto 2% to 12% NaCl (w/v) respectively (**Table 4**), however optimum NaCl concentration was 9%. The isolate was able to grow only on a pH range 8 (Table - 4). The *Bacillus laterosporus* was capable of utilizing a wide range of carbon sources and amino acid sources. However, in the present study, maltose and tryptophan was found to be a relatively good carbon and amino acid source for bacterial production (**Table 5 & 6**).

The antibiotic sensitivity of the isolates showed that the isolate was sensitive to Rifampicin, Erythromycin, intermediate to Vancomycin and resistant to Penicillin-G, Nalidixic Acid, Kanamycin, Neomycin, Gentamicin and Methicillin (**Table 7**). Antibiotics are specific in nature, being effective against certain microorganism and not against others. Microorganisms vary in their susceptibility to different antibiotics basing on which they can be put into different groups.

It is known that *Bacillus* from saline environment has greater biotechnological potential compared to other group of bacteria [19]. Isolation of microbes from saline environment would also provide ample scope to assess their biotechnological potential. Attempt should be made for proper evaluation and exploration of these microbes for the biotechnological applications. *Bacillus laterosporus* helps to fight fungal infections such as Candida, athlete's foot, nail fungus and toenail fungus by establishing a healthy probiotic environment in the colon. These healthy organisms compete with the Candida or other fungal strains for essential nutrients. Further, by populating the colon with healthy bacteria, there is a reduced chance of intestinal inflammation which leads to "leaky gut syndrome". When proper digestion or absorption is impaired and the intestinal lining allows fungus, harmful bacteria, viruses, and parasites to enter the bloodstream the end result is systemic infections. These systemic infections present themselves as rashes, fatigue, headaches, nausea, toenail fungus, nail fungus, athlete's foot and thrush. *Bacillus laterosporus* has shown significant effectiveness in eliminating Candida, improving and, in many cases, eliminating gastrointestinal symptoms and food sensitivities while enhancing the patient's digestive capacities

Table 1: Morphological Test

Colony Morphology	Observations
Configuration	Circular
Margin	Entire
Elevation	Raised
Surface	Smooth
Pigment	Creamy yellow
Opacity	Opaque
Gram's reaction	+ve
Cell shape	Rods
Size(µm)	1.0-1.5
Arrangement	Scattered
Spore(s)	+ve (Bulged)
Motility	-ve

Table 2: Biochemical Tests

Tests	Results
Growth on MacConkey	Nlf
Indole test	-
Methyl red test	-
Voges Proskauer test	-
Citrate utilization	-
H2S Production	-
Gas production from	-
Glucose	-
Casein hydrolysis	-
Esculin hydrolysis	+
Gelatin Hydrolysis	+
Starch hydrolysis	-
Nitrate reduction	+
Catalase test	+
Oxidase test	(+)
Arginine dihydrolase	-
Tween 20 hydrolysis	+
Tween 40 hydrolysis	+

Tween 60 hydrolysis	(+)
Tween 80 hydrolysis	-
Acid production from	
Dextrose	+
Xylose	+
Fructose	+
Lactose	(+)
Mannose	+
Cellobiose	+

Table 3: Growth Curve at 35°C at 600nm

Day	Optical Density
1	0.26
2	0.5
3	0.55
4	0.92
5	0.92
6	0.8
7	0.76
8	0.74

Table 4: Optimization of growth

Growth at temperature	Results
4°C	+
10°C	+
25°C	+
30°C	+
35°C	+
37°C	+
42°C	+
55°C	-
Growth at pH	
pH5.0	-
pH6.0	-
pH8.0	+
pH9.0	-
Growth on NaCl (%)	
2	+
4	+
6	+
8	+
9	+
10	+
12	+
Growth under anaerobic	
condition	-

Table 5: Optimization of Carbon Sources

Carbon Source	Concentration	Day 2	Day 3	Day4	Day5	Day6	Day7
		Average number of colonies					
Sucrose	0.02	43	50	51	49	39	31
	0.04	1	2	2	2	2	1
	0.06	13	34	46	56	52	26
	0.08	31	37	86	55	65	64
	0.1	38	168	284	222	222	100
Glucose	0.02	0	26	45	45	45	35
	0.04	0	21	42	50	52	45
	0.06	0	27	52	58	63	45
	0.08	4	59	90	105	105	80
	0.1	0	90	96	95	88	52
Maltose	0.02	19	24	24	28	23	21
	0.04	47	75	80	78	71	40
	0.06	45	104	111	125	107	98
	0.08	37	97	108	127	107	98
	0.1	148	266	275	241	245	162
Galactose	0.02	39	45	41	40	37	26
	0.04	36	46	52	82	51	36
	0.06	34	49	54	55	48	38
	0.08	35	50	51	42	40	34
	0.1	62	100	102	101	97	70
Lactose	0.02	21	26	25	23	20	19
	0.04	13	14	15	19	19	14
	0.06	19	22	24	23	21	19
	0.08	20	24	26	27	25	16
	0.1	16	17	18	21	19	18
Fructose	0.02	0	25	26	24	23	20
	0.04	0	6	9	9	8	7
	0.06	0	13	14	16	16	12
	0.08	0	16	17	17	17	15
	0.1	0	5	6	6	3	2

Table 6: Optimization of amino acid sources

Amino acid Source	concentration	Day 2	Day 3	Day4	Day5	Day6	Day7
		Average number of colonies					
Cysteine	0.02	0	7	11	11	11	11
	0.04	0	0	0	0	2	1
	0.06	0	0	0	0	1	1
	0.08	0	0	0	0	3	2
	0.1	0	0	0	0	0	0
Tryptophan	0.02	0	21	27	25	24	22
	0.04	0	25	30	34	33	28
	0.06	0	15	21	22	21	20
	0.08	0	9	13	15	20	18

	0.1	0	0	0	0	0	0
Tyrosine	0.02	0	16	21	22	23	20
	0.04	0	13	18	24	25	18
	0.06	0	12	15	15	14	11
	0.08	0	15	14	14	13	11
	0.1	0	10	16	20	21	20
Arginine	0.02	0	47	49	45	40	23
	0.04	0	34	40	45	41	38
	0.06	0	37	42	44	40	28
	0.08	0	51	69	70	55	44
	0.1	0	29	37	38	35	33
Histidine	0.02	0	35	53	54	51	50
	0.04	0	15	60	61	40	32
	0.06	0	27	62	60	59	50
	0.08	0	37	47	51	45	30
	0.1	0	31	58	60	55	45
Proline	0.02	0	11	31	33	38	36
	0.04	0	37	43	55	51	50
	0.06	0	21	29	30	34	32
	0.08	0	41	66	65	60	58
	0.1	0	19	31	35	40	45

Table 7: Antibiotic susceptibility test for *Bacillus laterosporus*

Antibiotics	Zone of inhibition (mm)	Result
Penicillin-G	0	Resistant
Nalidixic Acid	0	Resistant
Erythromycin	21	Sensitive
Kanamycin	0	Resistant
Neomycin	0	Resistant
Rifampicin	32	Sensitive
Vancomycin	11	Intermediate
Gentamicin	0	Resistant
Methicillin	0	Resistant

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