Journal of Applied Biology & Biotechnology Vol. 4 (03), pp. 015-019, May-June, 2016
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2016.40303

Antimicrobial Activity Screening of Marine Bacteria Isolated from the Machilipatnam Sea Coast of Andhra Pradesh, India

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ARTICLE INFO

Article history:
Received on: 28/02/2016
Revised on: 09/04/2016
Accepted on: 22/04/2016
Available online: 21/06/2016

Key words:
Crude extracts, Antibacterial activity, Secondary metabolites, Carbon source utilization.

ABSTRACT

Bacterial colonies were obtained from water samples collected from sea-coast of Machilipatnam, Andhra Pradesh in South India. The isolates were cultured, named as M1, M2, M3 etc., and screened for antibacterial action. Among the 40 representative isolates obtained, three isolates showed substantial antibacterial activity with more than 10 mm of zone of inhibition against two test bacteria used in preliminary screening. These three isolates marked as M20, M22 and M23 showed most outstanding results against all the test bacteria used in further testing of antibacterial activity. The isolate M20 showed the highest activity with a zone of inhibition of 16mm against *Serratia marcescens*, 14 mm against *E.coli* and *Shigella*, and with 12 mm against *Salmonella paratyphi* and *Bacillus cereus*. The isolates M20, M22 and M23 were analyzed for their morphological, biochemical and physiological characteristics and tentatively identified as *Alcaligenes sp.* (M20), *Bacillus sp.* (M22) and *Bacillus sp.* (M23) from the performed biochemical tests.

1. INTRODUCTION

Microorganisms are ubiquitous in nature and inhabit an authoritative position in anthropomorphic eyeshot of life. Marine microbiota act as a potential source for commercially significant bioactive chemical compounds and their bioremediation potentiality is well remarkable [1, 2]. They also play an essential role in the putrefaction of organic substances and cycling of nutrients. Our cognition by marine microbial diverseness has, however, embodied gravely confined by relying on microorganisms that have been cultured [3]. After forty years of intensive research, biological products of marine microbiota have become an attractive field. Since 1995, there are signals of the diminished interest in the exploration of novel metabolites by conventional sources such as macroalgae and octocorals.

On the contrary, microbial metabolites are a rapidly developing field of study, anticipated, at least incoming part, to the suspiciousness that a number of metabolites obtained from algae and invertebrates can be acquired by associated microorganisms [4]. Studies are preoccupied with bacteria and fungus kingdom, stranded from salt water, deposits, algae, pisces and mainly from marine invertebrates. Although it is still too early to define tendencies, it may be explicit that the metabolites from microorganisms are in most cases quite different from those acquired from the invertebrate hosts. The marine microbiota surroundings are extremely diverse, with huge variations in pressure and temperature. Nevertheless, lifespan, particularly microbial life, flourishes throughout the marine biosphere and germs have adjusted completely to the diverging environments present.

Large scale DNA sequence based approaches have recently been practiced to investigate the marine environment and these studies have revealed that the oceans harbor unexampled microbial diverseness. The presence of several novel gene families from these uncultivated and highly diverse microbial populations exemplifies a challenge for the understanding and exploitation of the biological science and biochemistry from the sea surroundings [5].

The pharmacologically active chemical compound provides formerly unrecognized structures as tools for fundamental research. Microbial diversity plays a major role in isolation of new antibiotics. Marine bacteria are the most predominant group for the isolation of bioactive compounds [6]. Among the various microscopic organisms, bacteria are the most promising source of secondary metabolites [7]. A number of strains capable of producing antibiotic-like substances were isolated from the sea environment [8, 9]. The present study was aimed to isolate marine bacteria having potential antimicrobial activity.

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2. MATERIALS AND METHODS

2.1 Test Microorganisms and Growth Media

The test bacterial strains include *Serratia marcescens* (MTCC 4822), *Proteus mirabilis* (MTCC 425), *Salmonella paratyphi* (MTCC 3220), *Escherichia coli* (MTCC 41), *Shigella flexneri* (MTCC 1457), *Pseudomonas aeruginosa (MTCC 7926), Xanthomonas campestris* (MTCC 2286), *Bacillus cereus* (MTCC 430), *Proteus vulgaris* (MTCC 1771), *Bacillus subtilis* (MTCC 441). All these cultures were obtained from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India. Cultures were maintained on nutrient agar medium slants.

2.2 Isolation of bacteria from marine source

The water samples were collected from both on and off sea shores from different sites from sea coast of Machilipatnam, Andhra Pradesh, India at regular intervals of time. The samples were subjected to 10-fold serial dilutions, and placed on Zobell marine agar medium by the spread plate technique and incubated for 24 h. From the plates inoculated with samples, representative types of colonies were picked up and sub-cultured. The colony characteristics of the 40 representative colonies obtained were noted.

2.3 Antibacterial activity

Antibacterial activity of the isolates was studied by agar disc diffusion method [10]. Test cultures were individually spread on the agar medium and wells were made with 6mm sterile borer. The filtrate drawn from the broth cultures of the isolates was placed in the wells, incubated at 37 ^{0}C for 24h and determined the zone of inhibition.

Intially, all the 40 isolates were screened for antibacterial activity against two test bacterial namely *Bacillus cereus* and *Escherichia coli*. From this preliminary screening, three potential isolates were selected for further testing of their specific antibacterial activity against different Gram positive and Gram negative bacteria.

2.4 Biochemical Analyses

The selected three isolates (M20, M22 and M23) were subjected for specific biochemical analyses [11, 12]. The biochemical tests include IMVIC tests, H_2S production, Urease activity, Catalase activity, Oxidase test, Nitrate reduction test and Carbohydrate utilization.

2.4.1 *Indole test* (Tryptophan hydrolysis)

This test is performed to determine the ability of an organism to produce indole from the amino acid tryptophan using the enzyme tryptophanase. The isolates were incubated in the broth at optimum temp for 24-48 h. Kovac's Reagent (10-12 drops) was added to broth and observed for the formation of colored layer on the surface of the medium.

2.4.2 *Methyl Red test* (MR test)

This test was performed to determine the ability of an organism to produce mixed acid end products from glucose fermentation. The isolates were inoculated into MR-VP broth and incubated for 3-5 days at optimum temp. Three to four drops of methyl red reagent was added to interpret the MR positive and MR negative isolates, based on red or yellow colour development.

2.4.3 *Voges-Proskauer test* (VP Test)

This test was executed to determine the ability of an organism to produce acetoin; 2,3 butanediol; and ethanol. The isolates were inoculated into MR-VP broth and incubated for 3-5 days at optimum temp.

One milliliter of culture was then pipette out and to it Barritt's solution A (alpha-naphthol) and Barritt's solution B (KOH) were added in equal proportions. The solution was agitated vigorously and incubated for 15 to 20 minutes for the formation of red colour.

2.4.4 *Citrate utilization test*

This test was performed to determine whether the organism is capable of using citrate as the sole source of carbon with production of the enzyme citritase. The isolates were streaked onto Simmons citrate agar slants and incubated at optimum temperature for 24-48 hours and observed for any color change.

2.4.5 *Urease test*

Urease test was performed to determine the ability of an organism to split urea to form ammonia by the action of the enzyme urease. Urea broth was inoculated with the isolates and incubated at optimum temperature for 24-48 h for substantial color change.

2.4.6 *Catalase Test*

Catalase test was performed for the presence of enzyme catalase which decomposes H_2O_2 and enables the organism to survive. Nutrient agar slants were streaked with the isolates and incubated. Few drops of H_2O_2 (3%) was added on to the slant culture and observed for the effervescence.

2.4.7 *Oxidase test*

Oxidase test was carried out to determine the presence of oxidases, which are produced only by the obligate aerobes. The isolates were cultured on trypticase soy agar (TSA) plates and then incubated at 37 °C for 24-48 h to establish colonies. After the colonies were grown, 2-3 drops of TMPD (tetra-methyl-p-phenylenediamine dihydrochloride) reagent was added and substantial changes in colour was observed.

2.4.8 *Nitrate reductase test*

This test determines the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) or nitrogen gas (N_2) by the production

production of the enzyme nitrate reductase. Nitrate broth was inoculated with the isolates and incubated at the optimum temperature for 24-48 h. After incubation, 5 drops of Nitrate Reagent A (sulfanilic acid) and Nitrate Reagent B (dimethyl alpha naphtha amine) were added to the tube and observed for the colour development. For confirmation test, a pinch of zinc powder was added to the tubes.

2.4.9 *H_2S Production*

The ability of an isolate to produce H_2S (Hydrogen sulfide) was carried out by stabbing the isolate into KIA (Kligler's Iron Agar) medium and incubated. Gaps, cracks, or bubbles in the agar medium indicate the gas production [13].

2.5 Carbon Utilization Test

The ability of isolates to utilize an array of carbon sources including glucose, lactose, sucrose, maltose and fructose was tested by detecting the formation of acid or acid with gas production as end product due to fermentation of carbon source [14].

3. RESULTS

3.1 Isolation of bacteria and colony characteristics

In the present investigation, 40 marine bacterial isolates were obtained from the water samples drawn from seashore at Machilipatnam, Andhra Pradesh, India. The data on colony characteristics, Gram staining nature and preliminary antibacterial screening against two bacteria of all the 40 isolates is presented in Table-1. Of these 40 isolates, it was observed that 30 isolates were Gram +ve and rest 10 isolates were Gram –ve in nature. Majority of the isolates showed entire margin followed by undulate and crenated. Regarding the elevation, colour and shape of the colonies, the isolates exhibited considerable variation among themselves. Out of the 40 isolates, only M20, M22 and M23 isolates exhibited a promising antagonistic activity against tested bacteria namely *Bacillus cereus* and *Escherichia coli*.

Table 1: Colony characteristics and preliminary antibacterial screening of the isolates.

Sl. No.	Isolate	Margin	Elevation	Colour	Shape	Gram staining	Antibacterial activity (zone of inhibition in mm)	
							B.cereus	*E.coli*
1	M1	Entire	Flat	Golden yellow	Circular	G+ve	7	6
2	M2	Entire	Flat	Golden yellow	Circular	G+ve	6	7
3	M3	Entire	Flat	Orange	Circular	G-Ve	6	6
4	M4	Lobate	Flat	Milky white	Irregular	G-Ve	0	0
5	M5	Entire	Low convex	Cream	Circular	G+Ve	0	0
6	M6	Ciliate	Flat	Pale	Irregular	G+Ve	6	6
7	M7	Entire	Umbonate	Yellow	Irregular	G+Ve	0	0
8	M8	Crenated	Umbonate	White	Irregular	G+Ve	0	0
9	M9	Crenated	Low convex	Pale white	Circular	G-Ve	6	9
10	M10	Undulate	Low convex	Pale orange	Irregular	G+Ve	7	6
11	M11	Undulate	Umbonate	Cream	Circular	G+Ve	6	7
12	M12	Undulate	Raised	Brown	Circular	G+Ve	6	6
13	M13	Entire	Umbonate	White	Circular	G+Ve	6	7
14	M14	Entire	Convex	White	Circular	G+Ve	0	7
15	M15	Entire	Convex	Orange	Circular	G+Ve	0	0
16	M16	Crenated	Flat	Pale	Irregular	G+Ve	0	0
17	M17	Undulate	Flat	Creamy white	Irregular	G-Ve	0	0
18	M18	Entire	Flat	Colour less	Circular	G-Ve	0	0
19	M19	Entire	Low convex	Pale White	Circular	G-Ve	0	0
20	M20	Entire	Low convex	Pale yellow	Circular	G-Ve	12	14
21	M21	Entire	Flat	Pale white	Circular pinpoint	G+Ve	07	6
22	M22	Entire	Raised	Milky white	Circular	G+Ve	12	12
23	M23	Lobate	Flat	Pale brown	Irregular	G+ve	10	14
24	M24	Entire	Raised	Pale yellow	Circular	G+Ve	6	0
25	M25	Entire	Low convex	Pale yellow	Irregular	G+Ve	0	0
26	M26	Entire	Umbonate	Light brown	Irregular	G+Ve	7	6
27	M27	Entire	Flat	Colour less	Circular	G+Ve	6	6
28	M28	Undulate	Umbonate	Brown	Irregular	G+Ve	7	7
29	M29	Entire	Flat	Cream	Circular	G+Ve	6	7
30	M30	Rhizoid	Flat	Cream	Irregular	G+Ve	7	6
31	M31	Lobate	Umbonate	Pale yellow	Circular	G+Ve	7	0
32	M32	Undulate	Raised	Light	Irregular	G+Ve	7	0
33	M33	Undulate	Flat	Creamy white	Irregular	G-Ve	6	7
34	M34	Entire	Low convex	Pale White	Circular	G-Ve	7	7
35	M35	Undulate	Raised	Brown	Circular	G+Ve	0	7
36	M36	Undulate	Umbonate	Cream	Circular	G+Ve	0	0
37	M37	Entire	Umbonate	Light brown	Irregular	G+Ve	0	0
38	M38	Entire	Umbonate	Light brown	Irregular	G+Ve	0	7
39	M39	Entire	Flat	Golden yellow	Circular	G+ve	0	0
40	M40	Entire	Flat	Orange	Circular	G-Ve	0	0

3.2 Antibacterial activity

The three isolates viz., M20, M22 and M23 showed varied levels of antibacterial activity against the tested ten bacteria (Table-2). The isolates M20 and M22 were highly effective against *Serratia marcescens* with 16 mm and 14 mm zone of inhibitions, respectively. Whereas, M23 showed highest and equal effectiveness against *Serratia marcescens*, *Escherichia coli* and *Shigella* sp. with 14 mm zone of inhibition. Among the bacteria tested, *Proteus mirabilis* and *Bacillus subtilis* were found to be least sensitive to the three isolates. The three isolates also showed equipotent antagonistic activity against *E. coli* and *Shigella* sp.

Table 2: Antibacterial activity of marine bacterial isolates against test microorganisms.

Sl.No.	Test organisms	Zone of inhibition (mm)		
		M20	M22	M23
1.	*Serratia marcescens*	16	14	14
2.	*Proteus mirabilis*	10	10	10
3.	*Salmonella paratyphi*	12	10	10
4.	*Escherichia coli*	14	12	14
5.	*Shigella flexneri*	14	12	14
6.	*Pseudomonas aeruginosa*	10	12	10
7.	*Xanthomonas campestris*	12	12	10
8.	*Bacillus cereus*	12	12	10
9.	*Proteus vulgaris*	12	12	11
10.	*Bacillus subtilis*	10	10	10

3.3 Biochemical Analysis

For proper characterization of the selected three isolates, specific biochemical tests were performed and the results are tabulated (Table-3). All the three isolates were found to be positive for H_2S production, Urease test and Catalase activity, negative for Indole test. M20 and M22 isolates were positive for MR test and negative for VP test. Whereas, M23 is positive for VP and negative for MR tests.

Table 3: Results of biochemical tests of bacterial isolates.

		Isolates		
Sl. No.	Biochemical Tests	M20	M22	M23
1.	Indole Test	-	-	-
2.	Methyl Red Test	+	+	-
3.	Voges proskauer test	-	-	+
4.	Citrate Utilization Test	+	-	+
5.	H_2S Production	+	+	+
6.	Urease activity	+	+	+
7.	Catalase Test	+	+	+
8.	Oxidase Test	+	-	-
9.	Nitrate Reduction	-	+	+

'+ve': test positive; '-ve': test negative

3.4 Utilization of carbon source

The ability of the microorganisms to ferment a specific carbohydrate source with the end-product being an acid or acid with gas production was assessed by carbohydrate utilization analysis. From this analysis, it was observed that the three selected isolates have the ability to ferment specific carbohydrate source, except the lactose, with the production of only acid (Table 4). All the three isolates did not show the gas production with utilization of five carbon sources viz., glucose, sucrose, lactose, fructose and maltose. Based on the colony characteristics, Gram staining result, data on the biochemical tests and carbohydrate utilization capacity, the isolates were tentatively identified as *Alcaligenes sp.* (M20), *Bacillus sp.* (M22) and *Bacillus sp.* (M23).

Table 4: Carbohydrate Utilization Capacity of M20, M22 and M23 isolates.

Sl. No.	Carbohydrate source	Isolates		
		M20	M22	M23
1.	Acid from glucose	+	+	+
2.	Acid from sucrose	-	+	+
3.	Acid from lactose	-	-	-
4.	Acid from Fructose	+	+	+
5.	Acid from Maltose	+	+	+
6.	Gas from glucose	-	-	-
7.	Gas from sucrose	-	-	-
8.	Gas from Lactose	-	-	-
9.	Gas from Fructose	-	-	-
10.	Gas from Maltose	-	-	-

'+': Positive test, '-': Negative test

3.5 Scanning Electron Microscopy

The isolates M20, M22 and M23 were subjected to Scanning electron microscopy and the concerned images are given in Figures 1-3.

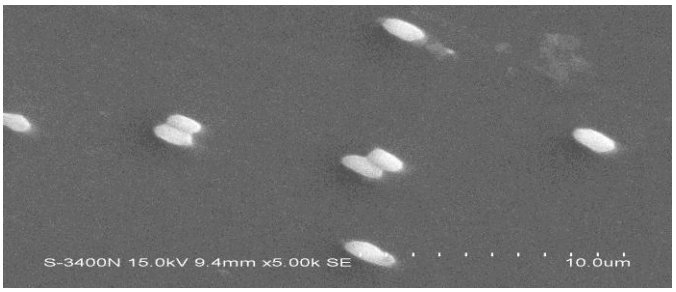

Fig.1: Scanning Electron Micro Photograph of M20 (10µm)

Alcaligenes faecalis is a Gram-negative, motile, oxidase-positive, catalase-positive organism. It is a rod shaped 0.4-1.0 µm wide and 0.6-2.5 µm long.

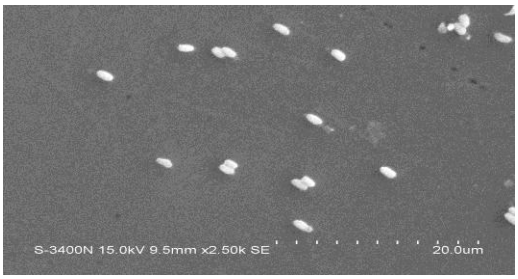

Fig. 2: Scanning Electron Micro Photograph of M22 (10µm)

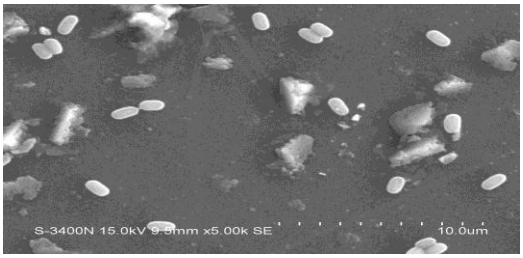

Fig.3: Scanning Electron Micro Photograph of M23 (10µm)

4. DISCUSSION

Marine microorganisms are one of the diverse sources of antimicrobial agents against an array of pathogenic bacteria. Since several decades, study of marine bacteria as potential antibacterial agents is of general practice [16]. However, the work on marine bacterial isolates from the seacoast of Andhra Pradesh, India against specific human pathogens has not been adequately reported. So, in the present study an attempt was made to isolate marine bacteria from seashore source and evaluated their potential antagonistic activity against some pathogenic bacteria. From the results, it was observed that 3 isolates namely M20, M22 and M23 showed the highest potential against potent pathogenic bacteria such as *S. marcescens* and *E.coli*. These prominent results suggested about their probable uses as possible therapeutic agents against multidrug resistant pathogenic bacteria [17]. From this preliminary screening of these isolates as antibacterial agents, we can predict their role as anti-infectives against a variety of pathogens and the probable source or genes responsible for such type of activity by further molecular characterization [18]. Based on the preliminary results the three metabolites possess biological activities and have potential to develop as therapeutic agents [19]. These three microorganisms can also be utilized as a potent source in the pharmaceutical industries as novel drugs in the future to avoid the multidrug resistance phenomenon.

5. CONCLUSION

Marine microbiota are still one of the most productive and prominent area of research as a source of natural products with potential therapeutic applications. In the present study also, investigation has been made to evaluate the antagonistic activity of marine bacterial isolates from the seacoast of Andhra Pradesh against a number of pathogens and found that three of the forty isolates obtained showed the most prominent antibacterial activity against some pathogenic bacteria. Therefore, these isolates can be further investigated for the presence of specific metabolites responsible for such potent antagonistic activity and their possible mechanism of action.

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How to cite this article:

Chandra KB, Rao VU, Pattnaik S, Busi S. Antimicrobial Activity Screening of Marine Bacteria Isolated from the Machilipatnam Sea Coast of Andhra Pradesh, India. J App Biol Biotech. 2016; 4 (03): 015-019. DOI: 10.7324/JABB.2016.40303