Research Article
Volume 7:7, 2021

Journal of Antimicrobial Agents

ISSN: 2472-1212 Open Access

Antibacterial Potential of Seaweed Associated Bacteria of Southeast Coast of India

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Abstract

The major groups of seaweed such as *Padina pavonica* and *Dictyota dichotoma* in Brown algae, *Halimeda macroloba* and *Chaetomorpha antennina* in green algae and the Red algae *Gracilaria parvispora* were examined for the presence of beneficial compounds and activity in symbiotically associated bacteria. Totally 40 isolated colonies were obtained and 10 physically similar strains were randomly selected and evaluate their antibacterial activity against human pathogen using the disc diffusion method. The strain A1 and D2 show the potential activity against all the pathogen, remarkably strain D2 had 15mm of inhibition zone against Proteus mirabilis, while A1 shows 13mm inhibition in Bacillus cereus and *Proteus mirabilis*. The GC-MS analysis on ethyl acetate extract of Strain A1 and D2 shows the presence of antimicrobial bioactive compounds. Based on the 16S *rRNA* gene sequences the isolates A1 and D2 were identical to *Vibrio harveyi* (KU197879.1) and *Photobacterium rosenbergii* (MN339950.1) respectively.

Keywords: Seaweed associated • Antimicrobial • GC-MS • Characterization

Introduction

The evolution of microbes with the resistance of antibacterial agents causing serious health issues. Most infectious bacteria are resistant to a minimum of one of the antibiotics that are generally used to eliminate the infection. This problem invigorates the study of new agents that can efficiently restrict the growth of microorganisms. Marine microorganisms are found in various environmental niches such as marine plants, animals, sediments and water. These microorganisms particularly bacteria have gained much importance for the production of novel antimicrobial compounds over the past three decades [1]. Antimicrobial secondary metabolites obtained from bacteria associated with marine organisms are known to produce unusual natural products not only for enabling the organism to compete with other species but also to protect the host from other macro and micro fouling community. Members of the genera Pseudoalteromonas and Bacillus associated with Ulva lactuca possess antibacterial and anti-diatom activity. However, they found a higher proportion of antimicrobial compound producing isolates from phylum Proteobacteria, with Vibrios being dominant. Isolates showing antimicrobial activity were found more in seaweeds [2]. During the past three decades, the search for the antimicrobial compound from marine bacteria associated with seaweeds and other marine invertebrates has increased many folds. Antimicrobial secondary metabolites produced by bacteria associated with seaweeds are found to control both clinical and fish pathogens and are useful. With this background, the present study has been undertaken to describe the isolation of epiphytic seaweed associated bacteria, screening, optimization, evaluation and identification of potential isolates and their antimicrobial activity against different pathogenic bacteria.

Materials and Methods

Sampling

Five different seaweeds representing the three major groups were handpicked from the intertidal shore of Manappadu, Thoothukudi, Tamil Nadu, Southeast coast of India. Among these, *Padina pavonica* (L) Thivy, *Dictyota dichotoma* (Hudson) J.V. Lamouroux are Brown algae, *Halimeda macroloba* Decaisne C, *Chaetomorpha antennina* (Bory) Kützing., belongs to green algae and the Red algae *Gracilaria parvispora* I.A.Abbott were found in benthic nature on rocks during the low tidal region. The collected samples were placed in sterile plastic bags and transported to the laboratory. The collected samples were washed thrice with autoclaved seawater to remove loosely bounded epiphytes, sand particles and other attached settlements on the surface of the thallus [3].

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Received: 06 September, 2021; **Accepted:** 20 September, 2021; **Published:** 27 September, 2021

Isolation of epiphytic bacteria

Associated bacteria were isolated by serial dilution to 10-7 using autoclaved seawater. From each dilution, 100 µl was spread-plated in triplicate Zobell Marine agar 2216 media (Hi-Media Laboratories Pvt. Limited, Mumbai, India). The plates were incubated at 37°C until colonies appeared or at least for 7 days. Visually distinct bacterial colonies were selected and further plated on Zobell agar medium until clonal cultures were obtained. The pure cultures were stored at 4° in Zobell marine broth supplemented with 20% glycerol (v/v).

Antimicrobial activity

10 ml of Mueller-Hinton (M173/M1084) broth in ten culture tubes were inoculated with Clinically isolated (Scudder Diagnostic Centre, Nagercoil, Tamil Nadu, India.), ATCC and MTCC provided human pathogens with each. The tubes were sealed with cotton plugs and cultured at 37ºC for 4-6 hrs until visible turbidity seen/0.5 McFarland's standard (1 × 108 CFU mL-1) was reached. Preliminary screening of isolated culture's broth in the exponential phase of growth (48 h) at 30°C, each culture was centrifuged (15000 × g, 4°C, 5 min) and the supernatant was tested against the pathogens using the Well-diffusion method Bauer et al., (1966). One hundred µL contained cell masses of each isolated bacteria in 0.9% NaCl solution for 24 h was inoculated into wells (8 mm in diameter) in Muller-Hinton agar plates containing the pathogenic bacteria. Sterile media without culture being adjusted to pH 7 were used as a control. The diameter of the inhibition zone was used as an index of antibacterial activity. Among the 10 distinct bacterial strain isolated, 2 strains which showed high activity against human pathogens, were selected for further antibacterial experiments.

Antibacterial activity of potential isolates

The potential isolates were cultured in the minimal medium for the extraction of secondary metabolites. Potential isolates were cultured on 100 ml marine broth (peptone 5 g, yeast extract 1 g,
MgSO4 0.1 g and KH_2PO_4 0.1 g, dissolved in 500mL distilled water and 500 mL seawater, pH 7.0-7.2) for 72 hrs, then the culture broth was centrifuged to cells free broth and extracted thrice with 100 ml of ethyl acetate and stored at -4°C. Sterile media without culture being adjusted to pH 7 were used as a control. The Stored ethyl acetate extract was characterized using GC-MS. Antibacterial activity was assayed by the disc diffusion method. Sterilized Whatman No. 1 filter paper of 5mm diameter was used. 200-500 µl Ethyl acetate extract of potential bacterial strains was added to the sterile disc incorporated individually with ethyl acetate extracts using a micropipette. Readymade Ampicillin 10 mcg/disc was used as Positive control. DMSO saturated disc is used as a negative control. Disc with Ethyl acetate solvent is used to making sure the antibacterial activity of marine bacterial strains. Mueller-Hinton (M173/M1084) agar plate is swabbed with the pathogen broth culture using sterilized cotton buds in a sterile condition. Then place the discs over the swab with appropriate spacing. Culture the plates inversely at 37°C for 18-24hrs. Antibacterial activity was evaluated by measuring the inhibition zone (in mm) from the edge of the disc [4].

Medium optimization

The current study was carried out in shaking cultures to determine the effect of physical and physiological condition over the production of antibacterial agents, (Peptone, Yeast extract, $(NH_4)2SO_4$ and $NaNO_3$) four various nitrogen sources at a concentration of 1% (w/v) to study the effects of different nitrogen sources on antibacterial agent production by isolate A1 and D2. Since yeast extract might be considered as a growth factor and nitrogen source. So, different concentrations of yeast extract were added to test their effect on the antibacterial agent production.

Results

Antibacterial activity

Symbiotic functions that have been attributed to microbial flora include processing of metabolic waste, secondary metabolite production and nutrient acquisition. For evaluating the biochemical characteristics to understand the eco-physiological and environmental functions of novel microbes with their potential applications, an isolation process is mandatory. In a study on antibiotic production in marine bacteria, have reported that 36% of the strains were Gram-negative rods. In our study, Gram-positive, as well as Gram-negative bacteria, were more or less equally represented in the producers encountered. It has also been suggested that some of these bacteria chemically defend the host against microbial infection. From the present study we have ascertained that the genera Vibrio, Pseudomonas/Marinobacter and Bacillus are dominantly represented. In the marine environment, the genus Vibrio has been reported from biofilms attached to surfaces, as pelagic bacteria.

Pathogens	A1	C1	C2	D1	D2	D3	E1	E2	E3
Escherichia coli	+++	-	-	-	+++	+	-	-	+
Enterococcus faecalis	++	++	-	-	-	+		-	-
Beta strepa	+++	-	-	-	+++	-	+	-	+
Serratia marcescens	++	-	+	-	-	-	-	-	-
Salmonella typhimurium	++	-	-	+	+++	-	-	+	-
Staphyloccus	+++	-	-	-	+++	-	-	-	-

aureus									
Proteus mirabilis	-	-	++	-	+++	+	-	-	-
Klebsiella pneumoniae	-	-	-	-	++	-	+++	-	++
Pseudomonas aeruginosa	+++	+	-	+	+++	-	-	-	-
Bacillus cereus	+++	-	-	-	++	-	-	-	++
No of Positive activity	8	2	2	2	8	3	2	1	4

Table 1. Preliminary antibacterial activity of isolated marine bacteria.

40 individual bacterial colonies isolated from seaweed by serial dilution were reduced to 10 by random selection based on colony morphology, and the 10 selected strains were subjected to preliminary screening for antibacterial activity (Table 1). Among them, 2 isolates (A1 and D2) exhibited distinct antibacterial activity against at least 8 tested pathogens. Therefore, they were selected as potential strains for secondary screening. A total of 10 tested pathogens were chosen for secondary screening of antibacterial activity using Ethyl acetate crude extracts of two selected isolates [5]. In the present study, the highest activity was exhibited from the strain A1, with an inhibition zone of 12 mm radius against Escherichia coli, Salmonella typhimurium, Staphylococcus aureus and Proteus mirabilis, and isolate D2 provide 13mm of inhibition zone against Staphylococcus aureus and Proteus mirabilis (Table 2).

S.No	Pathogens	Zone of inhibition (mm)		
		A1	D2	Positive Control
1	*Escherichia coli*	12	14	14
2	*Enterococcus faecalis*	10	13	12
3	*Beta strepa*	10	11	11
4	*Serratia marcescens*	10	10	14
5	*Salmonella typhimurium*	12	13	15
6	*Staphylococcus aureus*	10	14	15
7	*Proteus mirabilis*	13	15	15
8	*Klebsiella pneumoniae*	11	12	13
9	*Pseudomonas aeruginosa*	12	14	11
10	*Bacillus cereus*	13	13	15

Table 2. Antibacterial activity of potential isolates (A1 and D2).

Identification of isolated strains

The results of morphological, physiological and biochemical characteristics of all the 10 isolated strains were shown in Table 3. The selected strains A1 and D2 are Gram-Negative, Rod-shaped, motile bacteria. The strain A1 has positive results for Indole, Citrate. Oxidase, Catalase, Gelatinase and utilization of Sucrose, Glucose, Lactose and Mannitol as a source of carbon. According to investigated results on the morphological, physiological and biochemical characteristics of the strain, A1 preliminary classified to be Vibrio genus; while the strain D2 has a positive result for Voges Poskauer, Citrate, Oxidase, Catalase, Starch and Nitrate reduction. Sucrose, Maltose and Lactose are used as carbon source; indicates that the strain D2 is having a high affinity with Photobacterium sps.

The phylogenetic tree, which was constructed for comparison of the 16S rRNA gene sequences, indicated that strain A1 and D2 belonged to the genus of Vibrio and Photobacterium, respectively. The levels of similarity between the 16S rRNA gene of Vibrio A1, Photobacterium D2 and the 16S rRNA gene of other Vibrio and Photobacterium species are summarized in Figure 1. BLAST analysis in NCBI revealed that strain A1 has 99.60% similarity with Vibrio harveyi (NR042343.1); strain D2 has 99.74% similarity with Photobacterium rosenbergii (MN339950.1) with 100% query cover. The sequenced data is dumbed in NCBI Genbank with the accession number MZ098625 (Photobacterium rosenbergii) and MZ098228 (Vibrio harveyi).

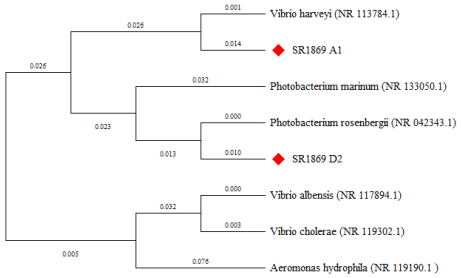

Figure 1. Phylogenetic tree based on 16S rRNA gene sequences and closely related members of the genus *Vibrio* and *Photobacterium*. Numbers at nodes are levels of bootstrap support based on neighbour-joining analyses of 1000 replications..

Medium optimization

The condition of incubation influenced quantitatively the biosynthesis of antibiotics as well as biomass. Production of antibiotics by biocontrol agents in liquid culture can be affected by several factors such as pH, temperature and composition of the

culture medium. It has been reported that nutritional requirement plays an important role during metabolite synthesis. The part of the work aims at the optimization of some culture conditions to attain maximum antibacterial agent production. The microorganism can utilize both inorganic and/or organic sources of nitrogen. The use of specific amino acids can increase productivity in some cases and conversely, unsuitable amino acids may inhibit the synthesis of secondary metabolites. Of all the tested nitrogen sources in the MB medium inoculated with A1 and D2 and incubated shaken for 24 h, yeast extract supported the highest level of antibacterial agent production (Table 3).

Test bacteria	Inorganic and organic nitrogen sources (zone of inhibition in mm)						
	Peptone		Yeast extract	$(NH_4)_2SO_4$	$NaNO_3$		
	A1	D2	A1	D2	A1	D2	A1
Escherichia coli	11	8	12	10	1	1	1
Enterococcus faecalis	8	9	10	10	2	1	2
Beta strepa	4	4	8	7	1	0	2
Serratia marcescens	10	5	10	11	0	1	1
Salmonella typhimurium	6	9	12	11	1	2	0
Staphylococcus aureus	8	7	12	13	1	1	3
Proteus mirabilis	10	8	12	13	2	1	0
Klebsiella pneumoniae	9	4	10	10	2	2	0
Pseudomonas aeruginosa	11	5	10	10	1	0	0
Bacillus cereus	7	4	9	11	2	2	2

Table 3. Effect of different inorganic and organic nitrogen sources for antibacterial agent production by Isolate A1 and D2.

The effects of different nitrogen sources on antibacterial agent production by Isolate A1 and D2 were also studied. 1.2 % (w/v) was the best concentration of yeast extract for optimum antibacterial agent production by Isolates A1 and D2. The specific nitrogen supplement is required to better differ from one microorganism to another. In most microorganisms, both inorganic and organic forms of nitrogen are metabolized to produce amino acids, nucleic acids, proteins and cell wall components. However, it was found that some nitrogen sources had an inhibitory effect on the antibacterial agent production and this may be due to organic acid accumulation, oxygen depletion or sugar catabolic repression. Depending on the biosynthetic pathways involved, nitrogen sources may significantly affect antibiotic formation. In addition, different carbon sources including starch, maltose, manitol, glucose, sucrose were used at the concentration of 0.4% (w/v) in the MB medium to study their effect on the antimicrobial activity of the strain. After shaking for 24 h, sucrose supported the highest level of antibacterial agent production. Antimicrobial metabolite production by *Vibrio sps*. for other studies was also optimally produced with sucrose in the medium.

Time course of the antibacterial agent production on the optimized medium

Time course from 12 to 48 h was followed in shaking incubated flask containing the optimized culture conditions inoculated with Isolates A1 and D2. The relationship between antibacterial activity and cell density is shown in Figure 2. Both the isolates could produce antibacterial substances only when the OD 660 value was above the threshold value of 1.0, at the beginning of the stationary phase. Antibacterial activity was highest when both strains were cultured in the marine broth after 30 h incubated shaken. It is reported that antibiotic production usually occurs in the stationary phase.. At the beginning of fermentation, the biomass content and pH was low. Then, along with the growth of the bacterium, after 12 h fermentation, the pH and OD values were increased quickly. When the strain was in the stationary phase, the pH value began to stabilize (pH 7.9) and unchanged the value during the

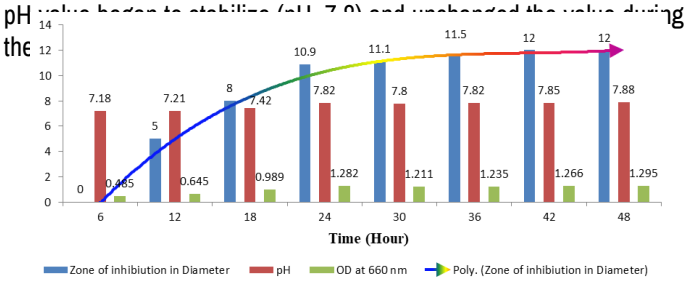

Figure 2. Time course of the antibacterial agent production against *S. aureus* and the growth by Isolate A1 (*Vibrio harveyi*)

Conclusion

The present study indicated that isolated bacteria from seaweeds remain an interesting source for new antibacterial metabolites and also suggested that isolate A1 (*Vibrio harveyi* - MZ098228) and D2 (*Photobacterium rosenbergii*-MZ098625) were produced secondary metabolites with antibacterial activity. The marine environment in the Manapaadu region of the southeast coast of India can be a potential source for natural products with biological activities to discover new compounds for the application of marine microbial sources in India.

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How to cite this article: M, Sathyananth, Raj Leon Stephan, and S Arokia Raj. "Antibacterial Potential of Seaweed Associated Bacteria of Southeast Coast of India." *J Antimicr Agent* 7 (2021) : 33443