World Journal of Agricultural Sciences 6 (3): 243-248, 2010
ISSN 1817-3047

Antifouling Potentials of Seaweeds Collected from the Southwest Coast of India

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Abstract: In the present study, a total of ten seaweed species collected from the Kollam coast (Indian ocean) were subjected to antifouling assays against the common fouling organisms such as *Balanus amphitrite*, *Mytilus edulis* and three biofilm forming bacteria, *Vibrio* sp., *Colwellia* sp. SW125 and *Pseudoalteromonas* sp. SW124. Of all the seaweeds tested, only one species, the red algae, *Laurencia brandenii* displayed broader spectrum of activity. The active principle of *L. brandenii* was purified in column chromatography and was identified by GC-MS. The GC-MS profile of *L. brandenii* suggested the purified fraction is primarily composed of octadecadienoic acid (49.75%) followed by "n-Hexadecanoic acid" (14.24%) which could have functional role in the chemical defense against fouling organisms and it could be utilized for the development of ideal antifoulants in future.

Key words: Biofouling · Seaweed extracts · Mussel · Barnacle

INTRODUCTION

Biofouling is a natural process of marine ecosystem caused by the surface colonization and development of micro and macrofoulers on submerged natural / man-made marine structures, leading to huge economic and environmental losses worldwide. The annual global loss of maritime domain is more than 6.5 million dollars [1]. Among the marine foulers, macrofoulers (invertebrates and algae) are the major fouling organisms creating extensive problems in marine technology like roughness in the ship's hull, reducing efficiency, speed and maneuverability [2, 3]. For defending epibiosis, marine organism deploys a variety of defenses such as growth of spines [4] mucus production, surface sloughing [5] and production of secondary metabolites [6]. The secondary metabolites exhibiting antifouling activity were already isolated from many marine organisms like sponges, seaweeds, sea grass, bryozoans, ascidians and gorgomans [7, 8]. The antifouling activity of sponges from peninsular coast of India is already reported [9]. Seaweeds are the major principle producers of oceanic plant community, distributed widely and indigenously and are recognized for their elaborate chemical defense characteristic against many biotic and abiotic factors. A variety of seaweed biogenic compounds exhibiting antifouling activity, which belongs to the group of fatty acids, lipopeptides, amides, alkaloids, terpenoids, lactones, pyrroles and sterols, were discovered [1]. As an alternative, the seaweeds can be utilized for the development of environmentally compatible antifouling agents. Therefore, the aim of this work is to investigate the antifouling activity of seaweeds collected from the Kollam coast against a range of fouling organisms.

MATERIALS AND METHODS

Collection of Seaweeds: Field collection of healthy and matured seaweeds were made from the rocky intertidal region of (08°54'N & 76°38'E) the Kollam coast (Table 1). Voucher specimens were deposited in the laboratory in 4% formaldehyde for future analysis. Immediately after the collection, the seaweed thallus was gently washed with filtered seawater to eliminate epibiota and other algal contaminants. The necrotic parts were also removed. The samples were washed with distilled water to remove salt and other associated debris. Cleaned samples were then surface dried by pressing it briefly between the sheets of filter papers and air dried under shade for one week at room temperature in order to prevent photolysis and thermal degradation of metabolites. Dried fronds of seaweed were powdered in a coffee grinder and packed in

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Table 1: Seaweeds collected from the southwest coast of India

Species
Acrosiphonia orientalis (J. Agardh) P. Silva.
Bryopsis plumosa (Hudson)
Grateloupia filicina (Lamouroux) C. Agardh
Dictyota dichotoma (Hudson) Lamouroux.
Lobophora variegata (Lamouroux) Womersley
Centroceras clavulatum (C. Agardh) Montagne
Cheilosporum spectabile Harvey ex Grunow
Portieria hornemannii
Portieria hornemannii (Lyngbye) P. Silva, Menez &Moe
Gelidium micropterum Kutzing
Laurencia brandenii Saito & Womersley

polyethylene bags and stored in moisture free place until extraction. Seaweeds samples were identified with the help of eminent Phycologist Dr. M.V.N Panikkar, Professor, Department of Botany, Sree Narayana College, Kollam, Kerala, India.

Extraction of Crude Bioactives: For the extraction of crude bioactives, 500 g of coarsely powdered algal material was vortexed and soaked in 5000 ml methanol in a ratio of 1: 10 for seven days at 35°C on a shaker at 120 rpm. After one week, algal material was collected and re-extracted with methanol in 3 L capacity round bottom flask in a water bath at 60°C for about 3 h. The individual crude extracts were pooled and filtered through whatman no 1 filter paper fitted with a Buchner funnel using suction pressure followed by the centrifugation (Eppendorf) at 5000 rpm for ten minutes. The supernatant was reduced to a dark green oily natured crude mass in a rotary vaccum evaporator (Yamato) at 40°C. The resultant extractives were collected in air-tight plastic vials and stored in the refrigerator for further activity studies.

Phycochemical Analysis of *L. brandenii*: The methanolic extracts of *L. brandenii* (200 gm) was applied in a silica gel (60-120 mesh) column developed with petroleum ether and eluted with petroleum ether and chloroform (9:1 to 1:9 and 100% chloroform) followed by chloroform and methanol (9:1 to 1:9 and 100% methanol) yielded eleven fractions. The individual fractions were screened for the biological activity (data not shown). The fraction that was eluted using petroleum ether:chloroform (6: 4) which exhibited activity was used for GC-MS (Hewlett Packard) analysis. Peak identification was carried out by comparison of the mass spectra with those available in the NIST Version 2 (2005).

Collection of Experimental Animals: Mussels and barnacles were collected from the infratidal and intertidal zones of the Kollam coast when they were in plenty (June to September 2 008) and were identified by Dr. M. Jayakumari and C.K. Thankachi, Professors, Faculty of Zoology, Sree Narayana College, Kollam, Kerala.

Isolation of Biofilm Forming Bacteria: The bacteria were isolated from the turbid water samples collected from intertidal pools of the Kollam coast were suspended in 10 ml of filter-sterilized aged seawater. A dispersed and diluted samples were serially diluted, plated in Zobell marine agar medium (HiMedia) and incubated at room temperature. The isolated biofilm forming bacteria were identified using biochemical tests and confirmed by reference to Bergey's Manual.

Antimicrobial Assays: Preliminary screening of seaweed extracts against biofilm forming bacteria was done by following Selvin and Lipton [10] agar diffusion method.

Biofilm Attachment Assay: Biofilm attachment assay was performed by quantifying the degree of attachment of bacteria to the cover slip coated with seaweed extracts of particular concentration to that of control. Briefly, three biofilm forming bacteria isolated from the Kollam coast were individually inoculated in a beaker containing 200 ml of aged seawater. Cover slip was pre-cleaned with 1 N HCl and washed with filtrated Milli-Q water. The seaweed extracts of different concentration (200, 300, 400 µg) were uniformly coated and was incubated for 48 h. Coverslip uncoated with seaweed extracts were used as control. During the experiment the average water temperature was about 21°C. After the 48 h incubation, cover slips were taken from test beaker and crushed using sterile mortar and pestle in distilled water, serially diluted and enumerated under a light microscope using haemocytometer.

Barnacle Bioassay (Antisettlement and Toxicity Assay): Barnacle bioassay was used to test the antisettlement activity of seaweed extracts against the cyprids of *Balanus amphitrite*. The adults were collected from the Kollam coast and acclimatized on laboratory in a thousand litre FRP tank filled with seawater at a constant temperature, salinity, aeration and feed for one week in order to produce nauplius. Six days after hatching, the young cyprids were collected by filtration and used for bioassay. Assay vials were inoculated with seaweed extracts of different concentration and solvent was

evaporated overnight in a sterile room. To the each assay vial 2 ml sterile seawater was added and ten young cyprids were introduced. Two other vials were kept as +ve and-ve control. Assay vial were incubated at room temperature for 12 hours. The settlement behavior of the cyprids were continuously examined and enumerated under a Zoom stereo microscope and recorded. The results were calculated according to the percentage of inhibition of settlement compared to the control and EC_{50} and LC_{50} was calculated using probit scale.

Mussel Bioassay (Foot Repulsive Assay): Foot repulsive activity was evaluated using the juvenile mussels of *Mytilus edulis*. The mussel bioassay was carried out according to Cho *et al* [11] Juvenile mussels were collected from the Kollam coast and maintained in a marine aquarium for three days. The healthy mussels with 3.3±0.2 cm shell length were used for the bioassay. The mussel foot was treated with test extracts by opening the shell and exposing the foot using forceps. Seaweed extracts of different concentration were dissolved in sterile seawater and used for bioassay. Appropriate amount of seaweed extracts were pipetted on the exposed foot and repulsive activity was recorded immediately after the treatment. Each test was repeated on twenty individuals. The experiment was performed at room temperature (30±2°C). ED_{50} value was also determined by probit scale analysis. The $CuSO_4$ solution diluted in sterile seawater was used as a liquid stimulant to study the reproducible foot repulsive reaction prior to the assay.

RESULTS AND DISCUSSION

Biodiversity of Kollam Coast: The coastal line is characterized by intertidal and subtidal rock out crops, pools, unvegetated sand beaches, bed rocks, boulders and partially buried cobbles support diverse macro fauna, including a large variety of crustaceans, sea anemone, sea cucumber, sea urchin and provide a habitat for both anadromous and catadromous fish species. The rocky habitat of supratidal and intertidal zone, an area wetted by spray and water splashing at high tide is dominated by gastropods. A distinct feature of this area is the barnacle which is particularly abundant along the stretch of shore. Moreover, Kollam coast is endowed with rich resource of marine algae. The most extensive algal flora is found in the Thirumullavaram coast [12]. Its seaweed floristic diversity is unmatched due to the presence of eighty genuses of algal species.

Table 2: Antibacterial activity of *L. brandenii*

		Colwellia	*Pseudoalteromonas* sp.
Seaweeds	*Vibrio* sp.	sp. SW125	SW124
L. brandenii	18±0.5	21±1.6	25±1.2
L. variegata	11±2.5	6±2.1	9±1.5

Mean±SD n = 6 experiments

Table 3: Biofilm attachment assay

	Concentration of *L. brandenii* (µg)			
Test organisms	100µg	200µg	400µg	Control
Vibrio sp.	5.3×10^5	4.8×10^3	5.8×10^2	6.3×10^8
Colwellia sp. SW125	5.6×10^5	5.1×10^3	2.8×10^2	6.0×10^8
Pseudoalteromonas sp. SW124	4.1×10^4	5.8×10^2	2.1×10^2	6.4×10^8

Overall Antifouling Activity: In the preliminary screening process, of the ten seaweed extracts, methanolic extracts of four algae showed considerable antifouling activity, such as *L. brandenii*, *D. dichotoma*, *L. variegata* and *A. orientalis* in the order of effectiveness. The following seaweed species were omitted from the tables as they showed only a meager activity. The extracts of *L. brandenii* and *L. variegata* recorded highest activity against biofilm forming bacteria. Besides, the extracts of *L. brandenii* and *D. dichotoma* showed significant antifouling efficacy against *M. edulis* and *B. amphitrite*. However, considering the broad spectrum activity and biomass availability, *L. brandenii* was chosen for further GC-MS analysis.

Antibacterial Activity Against Biofilm Forming Bacteria: Among the ten extract of seaweeds tested, methanolic extract of *L. brandenii* and *L. variegata* showed considerable antibacterial activity. *L. brandenii* showed mean zones of inhibition ranging between 18-25 mm. However, it exhibited the highest zone of inhibition (25 mm) against *Pseudoalteromonas* sp. (Table 2). This activity can be pertained to the antimicrobial compounds present in the *L. brandenii* extracts [13]. Reports are available on the use of active agents from seaweeds, in place of synthetic antifoulants, that are nontoxic, more systemic and easily biodegradable [14]. Bacteria isolated from the surface of seaweeds released compounds that repel other fouling bacteria, suggesting that they may protect the seaweed from fouling by other organisms [15, 16]. As far as our literature survey is concerned, there is no report on the antibacterial activity of *L. brandenii* from the Indian coast against biofilm forming bacteria.

Table 4: Anti settlement activity and mortality of *B. amphitrite*

Sl. No.	Seaweeds	EC_{50} (mgml^{-1})	LD_{50} (mgml^{-1})
1	*L. brandenii*	0.912±0.03	1.48±0.18
2	*D. dichotoma*	1.672±0.02	2.82±0.27
3	*A. orientalis*	2.491±0.07	3.76±0.13

Mean±SD *n* = 6 experiments

Table 5: Mussel foot repulsion assay

Sl. No.	Seaweeds	% of foot repulsion at 500µgml^{-1}
1	*L. brandenii*	90±2.06
2	*D. dichotoma*	65±3.16

Mean±SD *n* = 6 experiments

Biofilm Attachment Assay: A new finding of our study was the seaweed, *L. brandenii* showed significant decrease in the number of colonization /attachment of biofilm forming bacteria in comparison with the control. The colonization of bacteria was progressively inhibited as the concentration of the seaweed extracts increases to 400 µg whereas the extracts of other seaweeds showed no activity at the same concentrations (Table 3). The inhibitory activity of seaweeds was considered to be its ability to synthesize natural antifoulant metabolites. Many reports on antibacterial activity of marine organisms against biofilm forming bacteria are available [17, 18], but not much has been reported on the biofilm attachment assay. Therefore, the potential activities of *L. brandenii* against variety of microfoulers encourage developing a novel broad spectrum antifouling formulation in future.

Barnacle Bioassay: Out of ten seaweed extract tested, three species, *L. brandenii, D. dichotoma* and *A. orientalis* showed maximum activity against the larval settlement (Table 4). Of these, *L. brandenii* produced the highest activity and having EC_{50} and LD_{50} values of 0.412 and 1.48 mg/ml respectively, whereas the extracts of *D. dichotoma* and *A. orientalis* showed EC_{50} values of 0.472, 0.491 mg/ml and LD_{50} values of 1.62 and 1.76 mg/ml. Literature indicated that the antifouling property of seaweeds were associated with epiphytic microorganisms [15]. The results showed that the extracts from *L. brandenii* possessed good antifouling activity and are useful for developing new antifoulants with reducing environmental contamination and residual toxicity. There are many previous reports regarding the antifouling activity of seaweed extracts against *Balanus amphitrite* larvae [19, 20]. Steinberg *et al.* [21] has identified a bioactive metabolite, 5 B hydroxyaplysistatin, from *Laurensia obtuse* showing antisettlement activity. Halogenated monoterpenes from *Plocamium costatum* have previously been documented for exhibiting antifouling activity against the macrofouler barnacle [22]. Furanones from red alga *Delisea pulchra* were active against settlement of macrofouling larvae, bacterial colonization and spore of fouling algae [23, 24].

Mussel Bioassay: Data presented in the Table 5 showed the percentage of repulsive activity. The results of foot repulsive assays showed that crude extracts of *L. brandenii* and *D. dichotoma,* had activity.

Table 6: GC-MS profile of active fraction of *L. brandenii*

No	RT	Name of the Compounds	Molecular Formula	Molecular Weight	Peak Area %
1	5.01	Cyclohexasiloxane-dodecomethyl	$C_{12}H_{36}O_6Si_6$	444	1.07
2	8.20	Trisiloxane 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethyl silyl)oxy]-	$C_{12}H_{36}O_4Si_5$	384	0.89
3	9.36	3,5-Dimethyl-5-hexan-3-ol	$C_{18}H_6O$	128	0.11
4	11.64	3,5-Dimethyl-5-hexan-3-ol	$C_{18}H_6O$	128	0.11
5	13.59	4-Dodecanol	$C_{12}H_{26}O$	186	0.08
6	16.04	2-Decanone. 5,9-dimethyl-	$C_{12}H_{24}O$	184	0.13
7	18.65	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	14.24
8	20.90	9-Dodecanoic acid, methyl ester,(E)-	$C_{13}H_{24}O_2$	212	0.48
9	21.11	Oxalic acid, allyl hexadecyl ester	$C_{21}H_{38}O_4$	354	0.40
10	21.85	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280	49.75
11	24.33	Cyclohexanecarboxylic acid, decyl ester	$C_{17}H_{32}O_2$	268	2.70
12	25.96	9,12-Octadecadienoyl chloride (Z,Z)-	$C_{18}H_{31}ClO$	298	3.50
13	28.26	Oxalic acid, allyl pentadecyl ester	$C_{20}H_{36}O_4$	340	1.23
14	28.75	cis-9,10-Epoxyoctadecan-1-ol	$C_{18}H_{36}O_2$	284	0.61
15	29.79	Heptanoic acid, 9-decen-1-yl ester	$C_{17}H_{32}O_2$	268	0.65
16	30.63	9-Octadecenal	$C_{18}H_{34}O$	266	5.91
17	34.00	9,12-Octadecadienoic acid (Z,Z)-, phenylmethyl ester	$C_{25}H_{38}O_2$	370	0.44
18	34.51	Oxalic acid, allyl hexadecyl ester	$C_{21}H_{38}O_4$	354	1.11
19	37.64	Oxirane, tetradecyl-	$C_{16}H_{32}O$	240	3.49
20	38.61	1,2-15,16-Diepoxyhexadecane	$C_{16}H_{30}O_2$	254	3.01
21	40.00	7,11-Hexadecadienal	$C_{16}H_{28}O$	236	5.99
22	40.40	Thunbergol	$C_{20}H_{34}O$	290	4.11

Among these, most promising activity was displayed by *L. brandenii* causing 90% foot repulsion whereas *D. dichotoma* showed only 65% at 100 µg/ml and rest of the species activity was below 50%. A study by Cho *et. al.* [11] demonstrated the methanolic extracts of two seaweed, *Ishige sinicola* and *Ishige okamurae* can cause foot repellant activity. Katsuoka *et. al.* [25] acknowledged two compounds isolated from seaweed, *Costaria costata*, galactosyl and sulfo-quinovosyl-diacylglycerols had activity against blue mussel, *Mytilus edulis*. From these results it was understood that the seaweed, *L. brandenii* seemed to be a good candidate for the development of novel antifouling agents in future.

Spectrum of Active Fraction: A high resolution mass spectrum equipped with a data system in combination with Gas Chromatography (Hewlett Packard) was used for the chemical analysis of active fraction. Chemical characteristics of active fraction on the basis of spectral data by GC-MS were found to be a mixture of fatty acids. A total of 22 peaks were observed with retention times as presented in Table 6. It is possible that bioactive compounds primarily consisting of 9,12-Octadecadienoic acid (Z,Z)-(49.75 %) may be involved in biological activity. The fatty acid composition of active fraction revealed that the main acid was octadecadienoic acid (49.75 %) followed by "n-Hexadecanoic acid" (14.24 %). Seaweeds exhibit a high level of fatty acid diversity and many of which possess potential bioactivity. Therefore in the present study, biological activity of *L. brandenii* might be attributed due to the presence of fatty acid, octadecadienoic acid (49.75 %) in higher percentage.

CONCLUSION

The present study is successful in identifying potential candidate seaweed from the southwest coast of India, *L. brandenii* which can be exploited for the management of fouling organisms. The antifouling property of *L. brandenii* could be due to the presence of octadecadienoic acid (49.75%) followed by "n-Hexadecanoic acid" (14.24%). Considering its rich diversity of secondary metabolites, it is expected that this seaweed might be a promising antifouling agent. Therefore, the bioactive fatty acids of this seaweed together with knowledge of coating technology can be utilized for developing eco friendly antifouling alternatives in future.

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