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Biological activity of Seaweed extracts from *Cladophora clavuligera* (Kutzing, 1843) and *Sargassum wightii* (Greville, 1995) against marine fouling bacteria

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Present study was to assess the antifouling activity of seaweeds *Cladophora clavuligera* and *Sargassum wightii* collected from Tuticorin, Southeast coast of India. Crude methanol and dichloromethane extracts of the seaweeds were tested against five biofilm forming bacterial strains, *Bacillus* sp.1, *Bacillu*s sp.2, *Micrococcus* sp. *Pseudomonas* sp.1 and *Pseudomonas* sp.2, isolated from fouling test panels. Of these, MeOH extract of *Sargassum wightii* showed the activity against all the bacterial stains with significant activity (6-7 mm inhibition zone at 50µl/6mm disc) against *Pseudomonas* sp.1 and *Bacillu*s sp.2 whereas DCM extract showed the activity(3-4 mm inhibition zone at 50µl/6mm disc) against *Micrococcus* sp., *Pseudomonas* sp.1 and *Pseudomonas* sp.2. The MeOH and DCM extracts of *Cladophora clavuligera* (showed the activity 5 mm inhibition zone at 50µl/6mm disc) against *Bacillu*s sp.2 and 5 mm inhibition zone at 50µl/6mm disc against *Bacillus* sp.1 and *Pseudomonas* sp.2. The seaweeds were tested moderately toxic to *Artemia salina* as proved by brineshrimp lethality assay.

[Keywords: Seaweeds, Antifouling activity, Natural product, *Artemia salina*.]

Introduction

Marine biofouling can be defined as the undesirable accumulation of microorganisms, such as bacteria and microalgae, plants and invertebrates on artificial surfaces submerged in seawater[1]. Until recently most antifouling techniques have relied on organotins (tributyltin) or heavy metals (copper and zinc) based paints that act as broad spectrum toxins to target and non-target marine organisms[2]. However, these toxic organometal and heavy metal compounds lead to serious environmental problems at concentrations as low as sub-parts per billion[3], and their use is restricted due to their environmental damage[4]. One of the most promising alternative techniques to tributyltin is the development of naturally occurring antifouling compounds from marine organisms.

Large number of marine organisms produces a variety of chemicals for defense purposes, and they often serve as the basis for chemical ecological studies[5]. While some seaweed is heavily fouled, other species in the same habitat are rarely epiphytised, indicating the presence of antifouling or allelopathy mechanisms. Marine natural products or extracts with antifouling activities have been isolated from a wide number of seaweeds[6]. Compounds which have antifouling activity include: tannins extracted from *Sargassum natans*[7] a bromophenol extracted by the red alga *Rhodomela larix*[8] and diterpenes extracted from *Dictyota menstrualis*. The diterpenes inhibit settlement and development of a fouling bryozoan[9]. Halogenated furanones from *Delisea pulchra* have broad spectrum antifouling effect[10] and osteric acid from the seagrass *Zostera marina* inhibits settlement of *Ulva* spores[11]. Some waterborne algal compounds also prevent larval settlement[12], while several antifouling compounds from marine invertebrates have been identified using barnacle larvae[13] or mussel bioassay[14]. To find new antifouling substances from seaweeds, in the present study screened the antifouling activities of two seaweeds against major fouling bacteria.

Materials and Methods

Seaweeds *Cladophora clavuligera* and *Sargassum wightii* were collected from Tuticorin (Lat. 8° 48′ 36″ N; Long. 78° 8′ 24″ E) southeast of India. Seaweeds were shade dried completely for 3–7 days at room temperature and then ground to powder using an electrical blindere. For each 20 mg sample, 1 ml of

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100% methanol was used to extract the methanol soluble fraction at room temperature for a day[15]. Methanol extraction was repeated three times and combined. For a stock solution of the methanol-soluble fraction, 1ml of methanol was added for every 40 mg dried extract. After evaporating the methanol completely from the methanol-extract, 1 ml of distilled water was added to extract the water-soluble fraction for a day. The water-soluble stock solution was prepared by adding 1 ml of distilled water to every 40 mg dried extract. After dissolving methanol and water fractions, these solutions were diluted with seawater and passed through a 0.22 µm syringe filter before use.

Screening for antifouling activity was performed using seaweed extracts at a concentration of 30 µg/ml. All assays were run in triplicate. Negative controls with the solvent carrier (5% DMSO v/v) were performed in every assay and showed no inhibition of the biological activities. Five strains (*Bacillus* sp.1, *Bacillu*s sp.2, *Micrococcus* sp., *Pseudomonas* sp.1 and *Pseudomonas* sp.2) of marine biofilm bacteria were obtained from the test panels installed in the Vellar estuary.

Antibacterial assay of the extracts was performed with agar-plated petri dishes by a disc diffusion technique[16]. A sample consisting of 50 µl of product was loaded onto paper discs (6 mm diameter, Durieux, France). Microorganism cultures were grown in Zobell Marine Agar 2216 marine broth overnight, and 0.1 ml sample of the culture (10^6 cfu/ml) was spread over the agar. After incubation for 4 days at 20°C, the activity was evaluated by measuring the diameter (in millimeters) of the inhibition zones around the discs.

Brine shrimp lethality was performed using the freshly hatched free-swimming nauplii of *Artemia salina*. The assay system was prepared with 2 ml of filtered seawater containing different concentrations (1, 2, 4 and 6 mg/ml) of extract in cavity blocks (embryo cup) and 20 nauplii each was transferred in experimental, control and negative control wells. A survival was counted under the stereomicroscope after the 24 hours and the per cent death at each concentration was determined[17].

The major structure groups of the seaweed extracts were detected using Fourier transformed infrared (FTIR) spectroscopy (Bio Rad FTIR- 40 Modal USA spectrometer)[18].

Results

Antifouling activity

Two strains of *Bacillus* sp. two strains of *Pseudomonas* sp. and one *Micrococcus* sp. were isolated from the biofilm on wooden, fiber glass and aluminium test panels placed in the Vellar estuary at the depth of 1 meter by low tide level. The methanolic extract of *Sargassum wightii* was active against all five strains of fouling bacteria. But the DCM extract showed activity only against *Bacillus* sp.1, *Bacillus* sp.2, *Micrococcus* sp.1, *Pseudomonas* sp.1 and *Pseudomonas* sp.2

Methanolic extract of *Cladophora clavuligera* was active against *Bacillu*s sp.2, *Micrococcus* sp., and *Pseudomonas* sp.2 but did not exhibit activity against *Bacillu*s sp.1 and *Pseudomonas* sp.1. The DCM extract showed the activity against *Bacillu*s sp.1, *Bacillu*s sp.2 and *Pseudomonas* sp. 2 but the extract did not show any inhibition zone against *Micrococcus* sp. and *Pseudomonas* sp.1

The inhibition zone produced by methanolic extract of *Sargassum wightii* was maximum (7.3±0.9mm) against *Bacillus* sp.2 and minimum (1.0 mm) against *micrococcus* sp. while DCM extract exhibited maximum zone of inhibition (4.6±0.5mm) against *Bacillus* sp.2 and minimum (1.7±0.4mm) against *Pseudomonas* sp.1 and there was no inhibition zone formation in case of *Bacillus* sp.1.

Methanolic extract *Cladophora clavuligera* exhibited the maximum zone of inhibition (5±0.8 mm) against *Bacillus* sp.2 and no inhibition zone against *Bacillus* sp.1 and *Pseudomonas* sp.1 whereas the DCM extract exhibited maximum zone of inhibition (4.3±0.4 mm) in *Bacillus* sp.2 and no inhibition zone against *Micrococcus* sp. and *Pseudomonas* sp.1 (Table 1).

Brine shrimp lethality assay

Based on the brineshrimp lethality bioassay, the mortality was recorded at various concentrations (1, 2, 4 and 6 mg/ml). In *Sargassum wightii* the mortality recorded was 97.5% in 6 mg/ml concentration whereas *Cladophora clavuligera* showed 100% mortality in the same concentration (Table.3). The medium Lethal concentration was (LC_{50}) 2.3 mg/ml for *Sargassum* species and 1.93 mg/ml for *Cladophora clavuligera.* The toxicity profile of algal extracts considerably decreased at the lower concentrations.

Fourier transform infrared spectroscopy

A broad signals at around 1027.55 cm^{-1} to 1035.62 cm^{-1} in the FTIR spectrum (Fig.3&4) indicated the presence of sulphate (SO_4) group in large amounts which corroborated with high sulphate content obtained

in chemical analysis. The signal to 1123.81 cm^{-1} indicates the mannoronic units and 1072.55 cm^{-1} indicates the guluronic units. The C-O stretching COH is observed in the bands at 1100 cm^{-1} to 1025 cm^{-1} respectively.

The FTIR of the *Cladophora clavuligera* exhibited many peaks and the absorption in the range of (1)1044.91 cm^{-1} to 964.38 cm^{-1} assigned to SO_4 group, (2)1643.84 cm^{-1} to 1463.53 cm^{-1} assigned to CH_3CONH_3 group and (3) 2917.81 cm^{-1} to 2850.89 cm^{-1} assigned to hexose sugars.

Discussion

Macroalgae produce a wide range of secondary metabolites, many of which exhibit a broad spectrum of bioactivity[19] and could potentially be used to develop new antifouling agents[20]. In previous report the extracts of red algae from Brittany are prominent for their antifouling activity and 30 macroalgae exhibit antifouling activity[21]. In the present study *Sargassum wightii* showed the antifouling activity against five fouling bacteria. A broad signal at around 1123.82 to 1035.62 cm^{-1} in the FTIR spectrum indicated the presence of sulfate ester in large amounts, which corroborate with high sulfate content obtained in the chemical analysis. The spectrum 1100 cm^{-1} to 1025 cm^{-1} was assigned to C-O stretching the COH groups.

The present study, seaweed extracts of *Sargassum* species and *Cladophora clavuligera* were active against all five biofilm bacteria isolated from test panels. The FTIR spectrum of the *C. clavuligera* was assigned to SO_4 groups which were found active against biofouling bacteria. According to a previous

Table 1—Antifouling activity in terms of inhibition zone formation (mm)

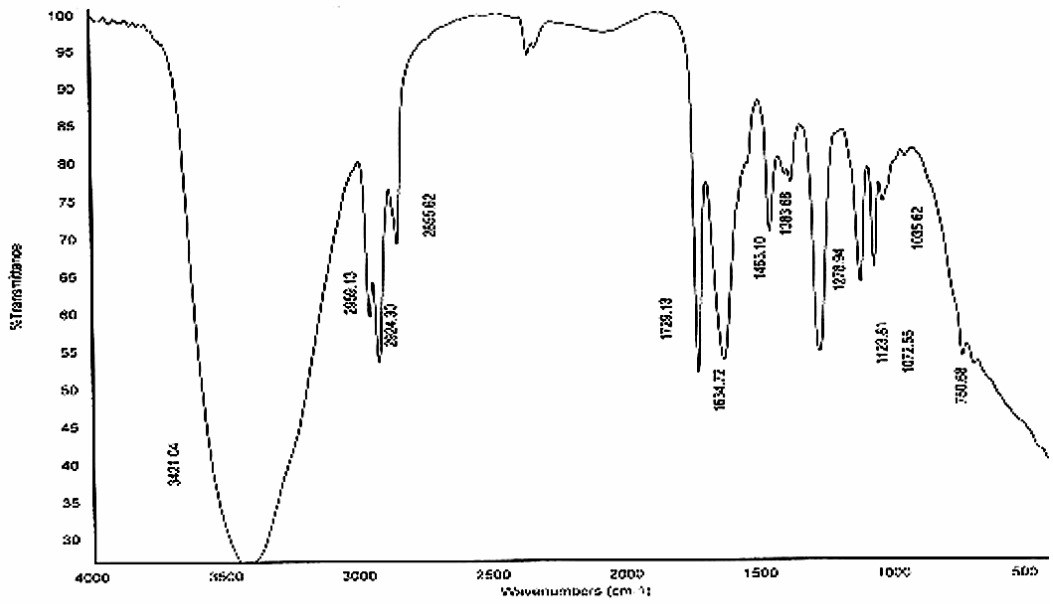
Fouling bacterial strains	Inhibition zone (mm) mean± SD				
	Sargassum wightii			*Cladophora clavuligera*	
	Control ($CuSO_4$)	Methanol	DCM	Methanol	DCM
Bacillus sp.1	11.3±0.9	3.0± 0.8	0± 0	0± 0	2.6± 0.5
Bacillus sp.2	15.6±0.4	7.3± 0.9	4.6± 0.5	5±0.8	4.3± 0.4
Micrococcus sp.	8.0±0.8	1.0± 0	3.3± 0.4	1.3± 0.4	0± 0
Pseudomonas sp.1	11.0±0.8	4.0± 0.8	1.7± 0.4	0± 0	0± 0
Pseudomonas sp.2	9.6 ± 0.4	6.6± 0.9	4.0± 0.8	3.3± 0.4	3.6± 0.4

Mean ± SD

Table 2—Brine shrimp lethality assay profile of algal extracts

Algal extracts	Concentration (mg/ml)	Mortality (%)
Sargassum wightii	1	10.2 ± 2.6
	2	50.0 ± 7.0
	4	91.6 ± 1.2
	6	97.5 ± 4.8
Clodophora clavuligera	1	20.2 ± 3.6
	2	60.0 ± 7.0
	4	90.6 ± 3.2
	6	100 ± 0.0

Mean ± SD n=10



Fig, 1—Fourier transform spectroscopy (FTIR) analysis of seaweed *Sargassum wightii*

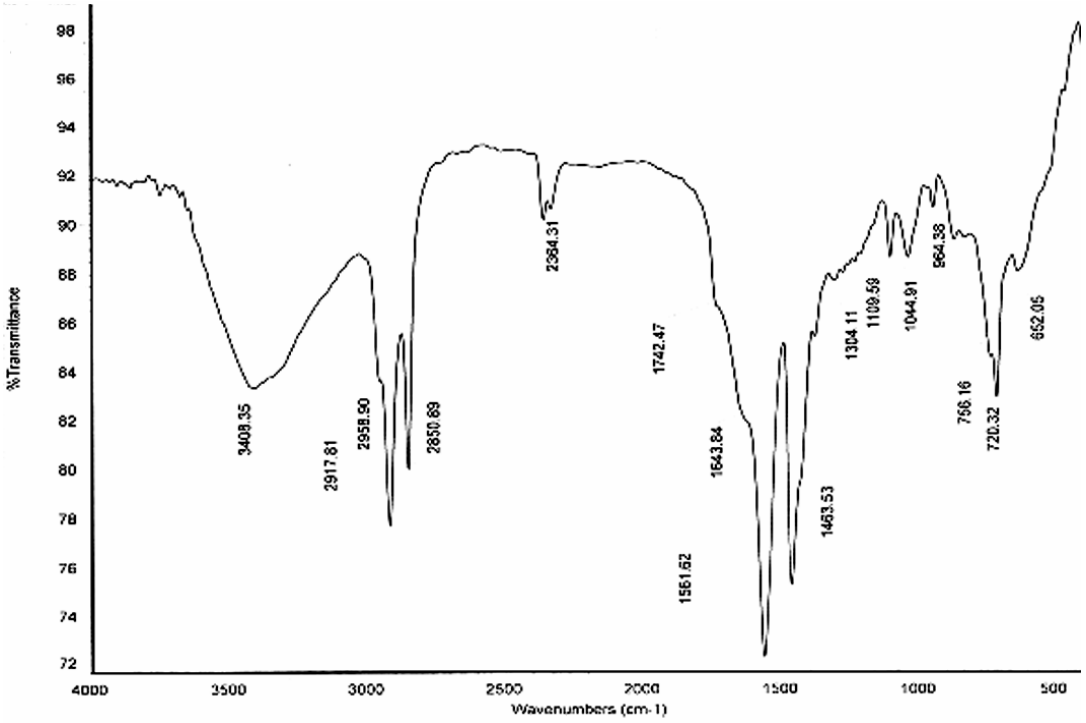


Fig. 2—Fourier transform infrared spectroscopy (FTIR) analysis of seaweed *Cladophora clavuligera*

report methanol extract of seaweed, *Ishige sinicola* shows strong inhibition of growth, spore settlement, zygote formation and germling of *Enteromorpha prolifera* and it also led to strong repulsive activity of the mussel foot and a strong inhibitor of mussel larval settlement in laboratory experiments[24]. The methanol extract of *Sargassum horneri* is shown to have antifouling activity with the green alga, not the mussel[6].

Sargassum wightii extract showed 97.5% mortality at 6 mg/ml, while at the same concentration the *Cladophora clavuligera* produced 100% mortality. This could be attributed cytotoxicity of seaweed secondary metabolities[25].

The results of the present study suggest that *Cladophora clavuligera* and *Sargassum wightii* have some potential as a source of natural antifoulant. Further bioassay-guided purification of the active extracts can lead to new alternative to the metal based antifouling paints currently in use.

Conclusion

Antifouling substance extracted from *Cladophora clavuligera* and *Sargassum wightii*, are soluble in methanol and dichloromethane. Isolation of the active alginate compound from *Sargassum wightii* is now in progress. Further panel tests are planned to elucidate possible antifouling activity in nature. A sulphate group with g antifouling activity from *C. clavuligera* is being characterized.

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