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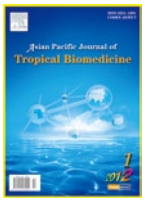

Document heading

Antifouling potentials of extracts from seaweeds, seagrasses and mangroves against primary biofilm forming bacteria

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ABSTRACT

Objective: To screen the antifouling potential of various extracts from seaweeds (*Ulva reticulate, Sargassum wightii, Halimeda macroloba*), sea grasseses (*Halodule pinifolia, Cymodocea serullata*) and mangrove plants (*Rhizophora apiculata, Rhizophora mucronata* and *Avicennia marina*) against some marine fouling bacteria. **Methods:** The different species of seaweeds, seagrasses and mangrove samples were collected, washed, air dried and fine powdered samples were subjected to solvent extraction by cold steep method. The extracts fraction was eluted with the ethanol and subjected to FTIR. The biofilm forming bacteria were scrapped from the marine environment by biofilm formed PVC sheet. Among these ten strains isolated, four isolates (*Flavobacterium* sp., *Bacillus* sp., *Cytophaga* sp., *Pseudomonas* sp.) were chosen for this study. **Results:** Among the tested extracts, *Avicennia marina* limited the growth of *Flavobacterium* sp. (16 mm) and *Bacillus* sp. (20 mm) and the extracts of *Rhizophora mucronata* limited the growth of *Flavobacterium* sp. (18 mm) and *Bacillus* sp. (18 mm). While comparing the inhibition activity of all the extracts, mangrove plants extracts had higher inhibiting activity against primary biofilm forming bacteria than seaweeds and seagrasses. The inhibition activity was mainly correlated with the major functional groups [hydroxyl, amino, carbonyl and phosphoryl functionalities, aliphatic (fatty acids), NH_2 (amide I & II)] of the extracts. **Conclusions:** The bioactive fractions from the above results indicates the occurrence of active constituents in the extracts of seaweeds, seagrasses and mangrove. It shows the improved antifouling activity against marine micro-fouling bacteria. These extracts can be used as the possible natural sources for anti-foulant.

1. Introduction

Biofouling is one of the major significant problems and ubiquitous in the marine environment. In aquatic environments, biofouling is a natural process of colonization of submerged surfaces, either living or artificial, involving a wide range of organisms from bacteria to invertebrates[1]. Biofouling simply refers to the undesired accumulation of an organisms like microbes, plants and animals to a surface of natural or any artificial structures in contact with water for a period of time, which are exposed to aquatic environments. It is one of the major unsolved problems currently affecting the shipping industry and industrial aquatic processes[2]. Commonly, fouling can occur in two types of organisms such as microfoulers (bacteria, algae and protozoa) and macrofoulers (barnacles, bryozoans and tube worms). Worldwide over 400 marine organisms are causing fouling problems. Biofilm formation is a key step during marine biofouling, the natural colonization of immersed substrata leading to major economic and ecological consequences[3]. Bacteria are among the first organisms to foul surfaces[4]. They form biofilms which is complex, clusters and three dimensional in nature and serve as a focus for the attachment and growth of other organisms, such as invertebrates, sessile plants, and animals[5,6]. Biofilms can enhance larval settlement of marine invertebrates and attachment of algal spores[7]. Mature marine biofouling communities are complex, highly dynamic ecosystems and, once established, are extremely difficult to eradicate[8]. Antifouling is generally defined as preventing the accumulation of fouling organisms[1]. In its broadest sense, it includes both the defensive biological processes used by macroorganisms to limit epibiosis, and technology applied to protect artificial submerged structures such as ships' hulls, aquaculture equipment[9] and optical

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devices[10]. Until recently most antifouling techniques have relied on organotin (tributyltin) or heavy metals (copper, zinc) based paints that act as broad spectrum toxins to target and non-target marine organisms[11]. However, these toxic organometal and heavy metal compounds lead to serious environmental problems at concentrations as low as sub-parts per billion[12], and their use is restricted due to their environmental damage[13]. Natural Product Antifoulants have been proposed as one of the best replacement options for the most successful antifouling agent, tri-n-butyl tin[14]. Marine organisms are a rich source of structurally novel and biologically active metabolites (primary and secondary). More than 100 species of marine organisms have exhibited antimicrobial activity as well as ability to prevent the settlement of the fouling organisms. Marine halophytes are the specialized group of plants adopted for high saline conditions which include mangroves, seaweeds and sea grasses. They are also proven to have rich source of structurally diverse bioactive compounds with valuable pharmaceutical and antifouling potential[15].

To date, a variety of natural products with antifouling activities have been isolated from lots of different marine organisms, including marine bacteria, algae, sponge, coral, bryozoa, ascidian, *etc*. In particular, seagrasses, seaweeds and mangrove plants have the efficient eco-friendly antifouling activity against the biofilm forming bacteria. Antifouling and antimicrobial potentials of marine origin have been extensively studied by many researchers in various species of mangroves[16-18], bacteria[19] sea grasses[20-22] seaweeds[23-25] and sea cucumber[26] *etc*. Infrared spectral study which helps to find out the active and major functional groups of organic materials in these extracts against biofouling. The FTIR spectrum of the seaweed extracts of *Cladophora clavuligera* assigned to SO_4 groups which were found active against biofouling bacteria[27,28]. There is scant information on isolation of antifouling compounds from mangrove, seaweeds and seagrass species, which are very important marine plants. The application of natural products from above marine organisms shows activity against microfouling organisms.

2. Materials and methods

2.1. Sample collection and extract preparation

Live and healthy samples of the sea weeds like *Ulva reticulate*, *Sargassum wightii*, *Halimeda macroloba* and seagrasses like *Halodule pinifolia*, *Cymodocea serullata* were collected by hand picking during low tide from Mandapam (Lat. 9028′N and Long. 79012′E) and Tuticorin coast (Lat. 8048′N and Long. 78011′E) of Gulf of Mannar, and mangrove samples *viz*. *Rhizophora apiculata*, *Rhizophora mucronata* and *Avicennia marina* from Pichavaram (Lat. 11° 26′ to 11° 30′N and Long. 79° 45′ to 79° 55′E) mangrove forest. These samples were thoroughly washed with seawater to remove all epiphytes, shells *etc*, and again washed with fresh water to remove the surface salts, sand particles if any and allowed to dry in the shady place for 3 to 4 days. The collected samples were identified by using standard books and manuals. The dried samples were then placed on blotting paper to remove the excess moisture before preparation of the seaweed, seagrass & mangrove extracts; the samples were ground to fine powder prior to solvent extraction. Each 20 g of seaweed, seagrass and mangrove powder was taken in 250 mL conical flask. About the same volume of solvents (v/v) like ethanol and methanol were added to get the natural concentrations of the seaweeds, seagrasses & mangroves; and they were extracted by cold steep method at - 10℃[29].

2.2. Biofilm development and bacterial characterization

Six PVC (Polyvinyl chloride) sheets were cut into the dimension of 8″× 6″, 4″× 2″, respectively and degreased using acetone. The sheets were mounted on a wooden rack having the total size of 75″×15″ using brass bolt and nut. The rack was immersed at 2 m depth from the mean surface seawater below the offshore platform of Central Electro Chemical Research Institute at Tuticorin unit during January 2009. Biofilm samplings were made for a period of seven days with the following time period intervals *viz*. 30 min, 1, 2, 4, 24, 48, 72, 96, 120 and 144 h, respectively. At every sampling period one PVC sheet was removed for biofilm collection. The biofilm was scrapped using sterile brush in a glass tube containing sterile seawater. Bacterial enumeration was done by pour plate method. Nutrient agar medium was used to enumerate the total heterotrophic bacteria. Average bacterial counts of the replicates were recorded. Morphologically dissimilar colonies were randomly selected, isolated and were maintained in slants at 4℃ for bacterial characterization. Gram staining, biochemical and motility tests were performed for preliminary identification of the bacterial isolate[30].

2.3. Bioassay

Antifouling activity was evaluated using the agar well method in petri dishes by using Muller Hinton Agar (MHA). 100 μL of each extract was loaded on agar well in MHA plates; biofilm bacterial isolates were spread on MHA plates with sterile effusion and the plates were placed on incubator at 37℃ for 24h. After incubation clear zone around a well was evidence of antimicrobial activity. Diameters of the zones of inhibition were measured in millimeters; each test was prepared in duplicate. The active fraction was eluted with the ethanol and subjected to FTIR (Instrument Model RXI).

3. Results

The primary biofilm forming bacterial strains were isolated from the Tuticorin coast and identified by various morphological, physiological and biochemical characteristics, gram staining and motility tests were performed for preliminary identification of the isolates (Table 1). The study revealed the antifouling activity of seaweed, seagrass and mangrove extracts against the four

Table 1
Biochemical characters of the isolated marine micro-fouling bacteria.

Organisms	Biochemical parameters							
	Gram staining	Motility	Indole	Catalase	Oxidase	TSI	Citrate	Pigment
Pseudomonas sp.	(−) ve	Motile	−	−	−	−	+	Bluish green
Bacillus sp.	(+) ve	Motile	−	+	+	+	+	−
Flavobacterium sp.	(−) ve	−	−	−	+	−	+	Orange
Cytophaga sp.	(−) ve	−	−	−	−	−	+	Yellow

Note: + Present; − Absent.

Table 2
Identification of functional groups through FTIR analysis.

Name of the species	Frequency (cm^{-1})	Bond	Functional group
Rhizophora apiculata	3 383.23 (s, b)	O-H stretch, H-bonded	Alcohols, phenols
	2 969.12 (m, n)	C-H stretch	Alkanes
	1 650.53 (m, sh)	-C=C- stretch	Alkenes
	1 057.04 (s, sh)	C-O stretch	Alcohols, carboxylic acids, esters & ethers
	879.61 (m, sh)	C-H "opp"	Aromatics
	653.51 (m, b)	C-Br stretch	Alkyl halides
Rhizophora mucronata	3 400.61 (s, b)	OH stretch, H-bonded	Alcohols, phenols
	2 934.69 (m, w)	C-H stretch	Alkenes
	1 617.48 (s, sh)	C-C stretch (in-ring)	Aromatics
	1 068.98 (m, n)	C-N stretch	Aliphatic amines
	628.59 (b, w)	-C=C-H : C-H bend	Alkynes
	1 439.69 (m, sh)	C-H bend	Alkanes
	1 368.33 (m, b)	C-H rock	Alkanes
	1 264.01 (w, b)	C-N stretch	Aromatic amines
	778.26 (m, w)	C-Cl stretch	Alkyl halides
Avicennia marina	3 492.97 (s, b)	O-H stretch, H-bonded	Alcohols, phenols
	2 968.35 (m, b)	O-H stretch	Carboxylic acid
	1 637.99 (m, n)	N-H bend	I^0 amines
	1 058.94 (s, n)	C-O stretch	Alcohols, carboxylic acids, esters & ethers
	880.81 (m, sh)	C-H "opp"	Aromatics
	660.92 (m, b)	C-Br stretch	Alkyl halides
	1 394.32 (m, b)	C-H rock	Alkanes
Halodule pinifolia	3 425.72 (s, b)	O-H stretch, H-bonded	Alcohols, phenols
	2 951.58 (m, sh)	C-H stretch	Alkanes
	1 642.61 (m, sh)	N-H bend	I^0 amines
	1 026.06 (s, sh)	C-O stretch	Alcohols, carboxylic acids, esters & ethers
	623.84 (m, b)	C-Br stretch	Alkyl halides
Sargassam wightii	3 391.96 (s, b)	OH stretch, H-bonded	Alcohols, phenols
	2 972.79 (m, sh)	C-H stretch	Carboxylic acid
	1 597.38 (m, n)	C-C stretch (in-ring)	Aromatics
	1 055.77 (s, sh)	C-O stretch	Alcohols, carboxylic acids, esters & ethers
	879.73 (m, sh)	C-H "opp"	Aromatics
	669.10 (m, b)	C-Br stretch	Alkyl halides
Ulva reticulata	3 398.34 (s, b)	OH stretch, H-bonded	Alcohols, phenols
	2 948.46 (m, sh)	C-H stretch	Alkenes
	1 598.04 (m, sh)	C-C stretch (in-ring)	Aromatics
	1 024.21 (s, sh)	C-O stretch	Alcohols, carboxylic acids, esters & ethers
	676.90 (s, b)	C-H rock	Alkanes
Cymodocea serrulata	3 415.52 (s, b)	O-H stretch, H-bonded	Alcohols, phenols
	2 954.55 (m, sh)	C-H stretch	Aromatics
	1 649.39 (m, sh)	N-H bend	I^0 amines
	1 408.18 (m, b)	C-C stretch (in-ring)	Aromatics
	1 025.48 (s, sh)	C-O stretch	Alcohols, carboxylic acids, esters & ethers
	659.64 (s, b)	-C=C-H : C-H bend	Alkynes
Halimeda macroloba	3 416.48 (s, n)	O-H stretch, H-bonded	Alcohols, phenols
	2 974.45 (m, sh)	C-H stretch	Aromatics
	1 599.12 (s, n)	N-H bend	I^0 amines
	1 054.68 (s, sh)	C-O stretch	Alcohols, carboxylic acids, esters & ethers
	679.87 (m, b)	C-Br stretch	Alkyl halides

m=medium, w=weak, s=strong, n=narrow, b=broad, sh=sharp.

primary biofilm bacterial strains *viz*., *Pseudomonas* sp., *Flavobacterium* sp. *Cytophaga* sp. and *Bacillus* sp.

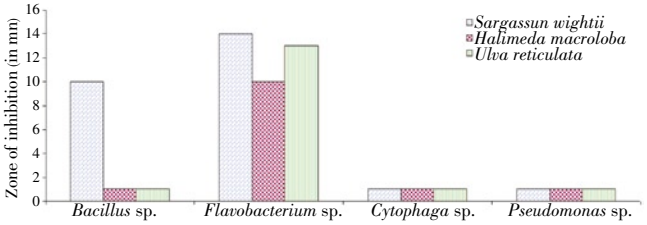

Figure 1. Effect of seaweed extracts against bio-film forming bacteria.

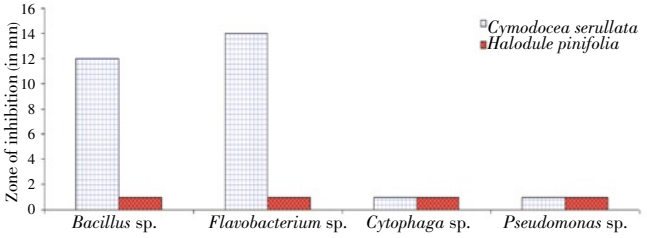

Figure 2. Effect of seagrass extracts against bio-film forming bacteria.

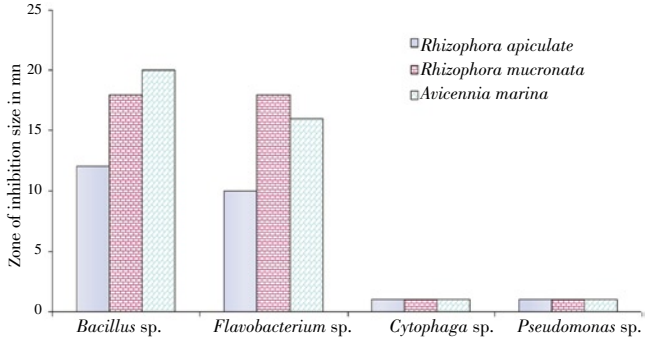

Figure 3. Effect of mangrove extracts against bio-film forming bacteria.

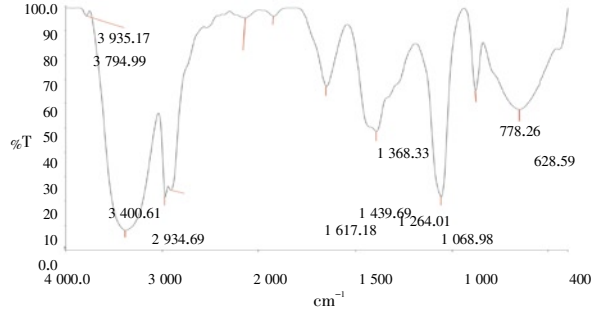

Figure 4. FTIR Spectrum of the bioactive fractions of *Rhizophora apiculata*.

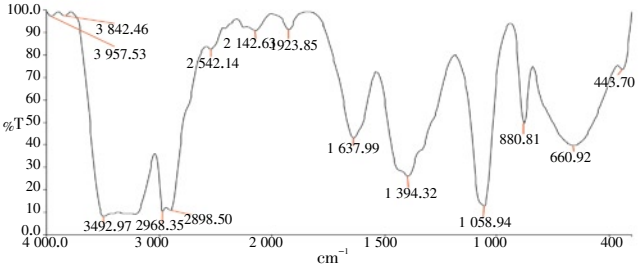

Figure 5. FTIR Spectrum of the bioactive fractions of *Rhizophora mucronata*.

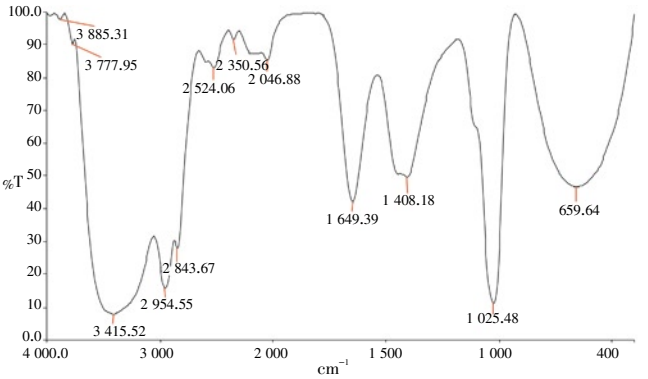

Figure 6. FTIR Spectrum of the bioactive fractions of *Avicennia marina*.

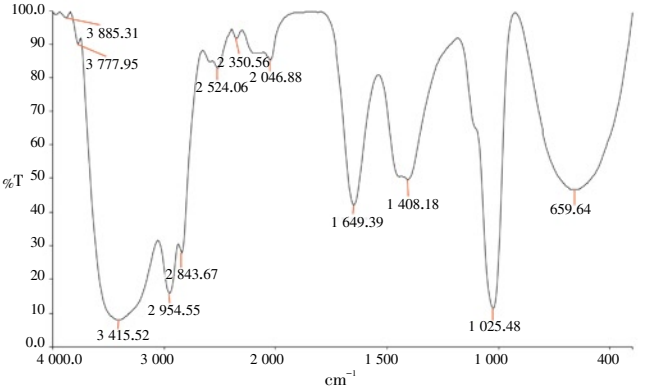

Figure 7. FTIR Spectrum of the bioactive fractions of *Halodule pinifolia*.

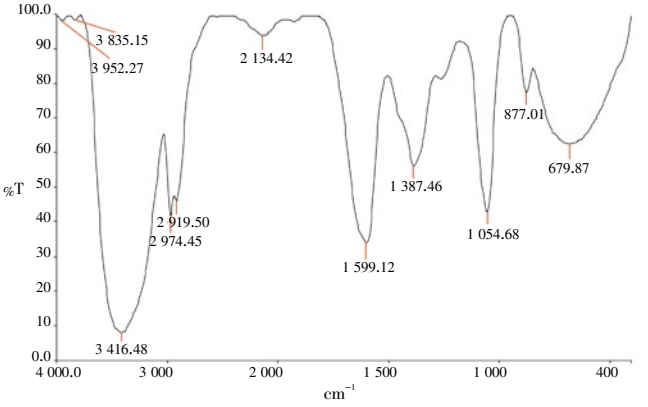

Figure 8. FTIR Spectrum of the bioactive fractions of *Cymodocea serrulata*.

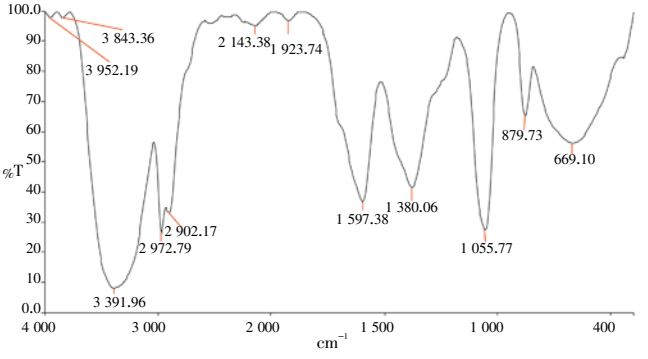

Figure 9. FTIR Spectrum of the bioactive fractions of *Halimeda macroloba*.

3.1. Bioassay –seaweeds, seagrass and mangrove extracts

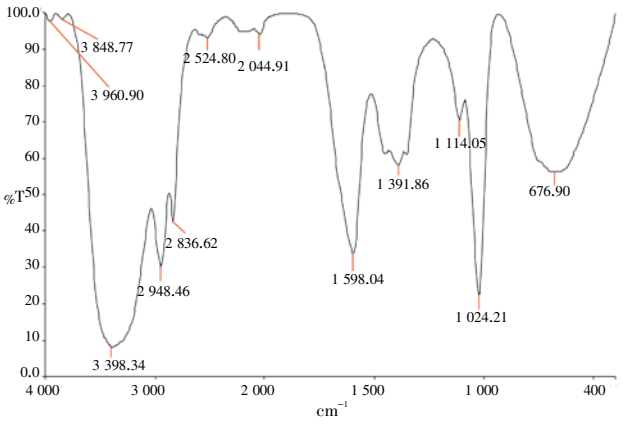

Figure 10. FTIR Spectrum of the bioactive fractions of *Sargassam wightii.*

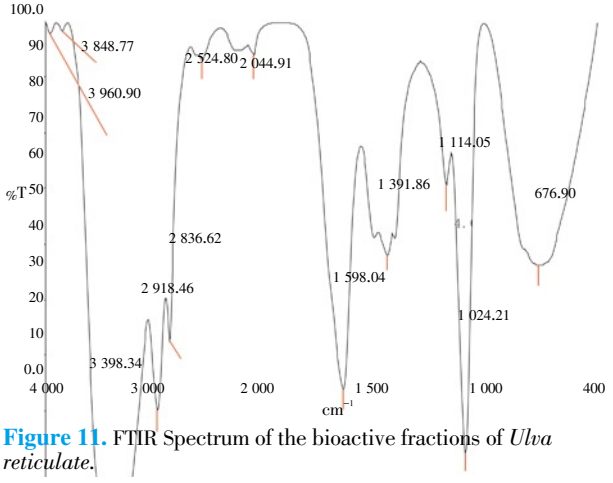

Figure 11. FTIR Spectrum of the bioactive fractions of *Ulva reticulate.*

The ethanol extract of seaweed *Sargassum wightii* showed maximal antibacterial activity against the *Flavobacterium* sp.(14 mm) & *Bacillus* sp.(10 mm) and *Ulva reticulata* showed maximal antibacterial activity against *Flavobacterium* sp. (13 mm) and minimum zone of inhibition was observed against remaining three biofilm bacteria. *Halimeda macroloba* showed maximal antibacterial activity against the *Flavobacterium* sp. (10 mm) and the minimum zone of inhibition (<1) was observed against remaining three biofilm bacteria (Figure 1). Whereas the ethanol extract of seagrass *Cymodocea serulata* showed maximal antibacterial activity against the *Flavobacterium* sp. (14 mm) & *Bacillus* sp. (12 mm) and *Halodule pinifolia* showed very trace activity against all the biofilm bacterial isolates (Figure 2). The ethanol extract of mangrove *Avicennia marina* showed maximal antibacterial activity against the *Bacillus* sp. (20 mm) & *Flavobacterium* sp. (16 mm). The minimal zone of clearance was observed for *Cytophaga* sp. and *Pseudomonas* sp. (<1 mm) where as *Rhizophora mucronata* showed maximal antibacterial activity against the *Bacillus* sp. (18 mm) & *Flavobacterium* sp. (1 mm) and the minimal zone of clearance was observed for *Cytophaga* sp. and *Pseudomonas* sp. (<1 mm). The ethanol extract of mangrove *Rhizophora apiculata* showed maximal antibacterial activity against the *Bacillus* sp. (12 mm) & *Flavobacterium* sp. (10 mm) and the minimal zone of clearance was observed for *Cytophaga* sp. and *Pseudomonas* sp.(<1 mm) (Figure 3).

3.2 FTIR analysis

The second derivative, IR spectrum in the mid-infrared region (400–4 000 cm^{-1}) was used for discriminating and identifying various function groups present in *Rhizophora apiculata*, *Rhizophora mucronata*, *Avicennia marina*, *Halodule pinifolia*, *Cymodocea serrulata*, *Halimeda macroloba*, *Sargassam wightii* and *Ulva reticulate*. The variation in spectral features of the IR band suggestions of the functional groups were given in the Table 2 (Figure 4 to 11).

4. Discussion

The bioassay study revealed that *Psuedomonas sp, Flavobacterium* sp., *Bacillus* sp., *Cytophaga* sp. were found to be sensitive to seaweed, seagrass and mangrove; while *Flavobacterium* sp. and *Cytophaga* sp. showed moderate sensitivity. However, in the present study, it is evident that all these 3 seaweeds, 2 seagrass and 3 mangroves possess anti-biofilm bacterial metabolites. The algae extracted in ethanol were found to show considerable antibacterial activity exhibited against biofilm bacteria. Similar observation was earlier made by Prem Anand *et al*[31] who reported that the hypobranchial glands of *Chicoreus virgineus* and egg capsules of *Rapana rapiformis* extracted with polar solvents like ethanol and methanol showed wide spectral antibacterial activities. Sastry *et al*[32] showed antibacterial activity against both gram positive and gram negative pathogenic bacteria after successive extraction with benzene, chloroform and methanol. Similarly, Marasneh *et al*[33] have shown antibacterial activity in organic extracts of six species of marine algae against multi-antibiotic resistant bacteria. It has been reported that biofilm bacteria may be 150–3 000 times more resistant to free chlorine than free floating bacteria is due to the excessive production of exo-polymers by biofilm bacteria. In the present study, almost all the extracts (seaweeds, sea grass, and mangroves) showed antibacterial activity against the most of the biofilm bacteria tested. However, mangrove extracts showed higher activity against biofilm forming bacteria compare to seaweed and sea grass extracts. Hence the present work suggests that these mangroves are potential sources of antibacterial compounds against biofilm bacteria and may be further investigated with various fractions of the extracts.

The study of carrageenans by FTIR and FT-Raman spectroscopy shows the presence of very strong absorption bands in the 1 210–1 260 cm^{-1} region (S–O of sulfate esters) and in 1 010–1 080 cm^{-1} region (glycosidic linkage) in all carrageenan types. The other chemical groups are characteristics of a given carrageenan type: 3,6-anhydro-D-galactose at 925/935 cm^{-1}, D-galactose- 4-sulfate at 840–850 cm^{-1}, D-galactose-2-sulfate at 820–830 cm^{-1}, D-galactose-6-sulfate at 810–820 cm^{-1}, and 3,6-anhydro-D-galactose-2-sulfate at 800–805 cm^{-1}[34]. In the FTIR spectra, both *k* and *i*-carrageenan present the 845–850 cm^{-1} band, but 800–805 cm^{-1} band is characteristic and distinctive of i-carrageenan.

The relative shape of the 820–830 cm^{-1} band allows us to distinguish the l (broad band) and j–variant (sharp band) [35]. In comparative studies of carrageenan types, the FTIR spectra provide enough information. However, FT–Raman is a more easily applied method and the correspondent spectra have a clear resolution. Discrimination between *k*– and *i*–carrageenan is based in the 805 cm^{-1} peak, which has a stronger signal in FTRaman spectra than in FTIR one. FT–Raman spectra have an 815–900 cm^{-1} band with additional information to distinguish the λ –family carrageenan variants when compared with FTIR spectra. The λ –variant spectrum shows the 825 and 900 cm^{-1} peak and j–variant spectrum shows the 815, 850 and 900 cm^{-1} peaks. This may be an advantage of FTRaman spectroscopy when compared with FTIR[36]. The overall antibiofilm metabolites assessed from the present results indicates the availability of active constituents in the extractions of seaweeds, seagrass and mangrove which showed better antimicrobial activity against micro–fouling bacteria. The mangrove extracts of *Avicennia marina* and *Rhizophora mucronata* showed better antibiofilm activity against *Bacillus* sp. and *Flavobacterium* sp. The inhibition activity is mainly correlated with the major active functional groups (hydroxyl, amino, carbonyl and phosphoryl functionalities, aliphatic (fatty acids), NH_2 (amide I & II)) of the extracts. The bonds such as O–H stretch, H–bonded, C–H stretch, –C=C– stretch, C–O stretch, C–Br stretch are principally involved in inhibition activity and mainly found in all the extracts. Hence, they can be considered as potential natural sources of bioactive metabolites acting as leading anti–biofilm molecules for the investigation of natural anti–foulant.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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