

Full Length Research Paper

Antifouling potential of seaweed, sponge and cashew nut oil extracts against biofilm bacteria and green mussel *Perna viridis* from Vellar estuary, Southeast coast of India

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Two species of common seaweeds and a single species of sponge were collected from Tuticorin coast and screened for antifouling activity. The seaweeds, *Sargassum wightii*, *Ulva lactuca*; sponge *Desmopsongiae* sp., and cashew nut oil extracts were tested *in vitro* against ten marine fouling bacteria isolated from test panels and the green mussel *Perna viridis*. The biofilm bacteria growth was inhibited by methanol extracts of the seaweeds *S. wightii*, *U. lactuca*, sponge *Desmospongae* sp., and the tropical cashew nut oil extracts. The bacterial growth was strongly inhibited by using extract concentrations as low as 30 µg mL⁻¹ with *S. wightii*, *U. lactuca*, *Desmospongiae* sp., and cashew nut oil. The byssus thread formation of the mussel was completely inhibited by methanol extracts of *S. wightii*, *U. lactuca* and cashew nut oil extracts at concentrations of 100 µg ml⁻¹. These extracts showed strong antifouling activities on green mussel attachment with 100 µg ml⁻¹ concentration. In this present study, there are exhibited preliminary evaluation of novel antifouling agents from marine macroalgae and tropical cashew nut oil.

Key words: *Perna viridis*, marine fouling bacteria, macroalgae, antifouling and tropical.

INTRODUCTION

All natural and man-made surfaces that are immersed in marine environments are potentially affected by the attachment of epibiotic and fouling organisms. The process of fouling begins as soon as a substratum is immersed with the adsorption of macromolecules, predominantly proteins, lipopolysaccharides and polysaccharides (Terlizzi et al., 2001). Subsequent microfouling and

macrofouling by microbial slimes, algae, and invertebrates can result in major economic lost through, for example, corrosion (Little et al., 1999) and a reduction in the fuel efficiency of ships underway due to increased drag. Biofouling control is a worldwide problem in marine systems, which costs the U.S. Navy, for example, an estimated \$1 billion per annum (Callow and Callow,

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2002). One of the most promising alternative techniques to tributyltin is the development of naturally occurring antifouling compounds from marine organisms. While some seaweed is heavily fouled, other species in the same habitat are rarely epiphytised, indicating the presence of antifouling mechanisms.

Marine natural products or extracts with antifouling activities have been isolated from a wide number of seaweeds. Compounds which have antifouling activity include, tannins extracted from *Sargassum natans* (Sieburth and Conover, 1965); a bromophenol exuded by the red alga *Rhodomela larix* (Phillips and Towers, 1982); diterpenes extracted from *Dictyota menstrualis*, which inhibit settlement and development of a fouling bryozoans (Schmitt et al., 1995); halogenated furanones from *Delisea pulchra* demonstrated broad spectrum antifouling effect (de Nys et al., 1995); and zosteric acid from the seagrass *Zostera marina* inhibited settlement of *Ulva* spores (Shin, 1998). Some waterborne algal compounds also deter larval settlement (Walters et al., 1996), while several antifouling compounds from marine invertebrates have been identified using barnacle larvae (Miki et al., 1996) or mussel bioassay (Devi et al., 1998). Thus, the aim of this study was to find new antifouling substances from seaweeds, sponge and cashew nut oil extracts against fouling bacteria.

MATERIALS AND METHODS

Collection site

Samples of seaweeds, *S. wighiti*, *Ulva lactate* and sponge *Desmospongiae* sp., were collected by hand picking during low tide near Tuticorin (Lat. 8° 48' 36" N; Long. 78° 8' 24" E), Southeast coast of India during January, 2008. Sampled material was transferred to dark recipients and stored in isothermal boxes to prevent photo and thermal degradation during the transport to the laboratory. Wet algae were cleaned from sediments and associated organisms (but no special treatment was employed to remove microorganisms) and divided into two aliquots: one for taxonomic identification (preserved in 4% formalin in seawater) and other to be used for extraction after weighing and dry the sample. This portion was dried at a room temperature of 17°C and in the dark until a steady weight was obtained (dry weight).

Extraction

Seaweed and Sponge were identified, washed with tap water and removed the surface associated materials before extraction. The surface microflora was removed by washing the algal samples for 10 min with 30% ethanol followed by Hellio et al. (2004).

Seaweeds *S. wighiti* and *U. lactate* were shade dried completely for 3-7 days at room temperature and then ground to powder using an electrical blinder. 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 vacuum evaporator (Yamato) at 40°C. The resultant extractives were collected in air-tight plastic vials and stored in the refrigerator for further activity studies (Hellio et al., 2004). Sponge sample (50 g wet weight) was cut into small pieces, homogenized and allowed to stand in a dark chamber with a combination of methanol, acetone, n-hexane (1:3:1 v/v/v), extracted for 48 h at room temperature and filtered through Whatman No.1. After that, each sponge extract was evaporated at reduced pressure. The crude extract was stored at 4°C for further analysis.

Antifouling assay

Screening for antifouling assay was performed using algal extracts at a concentration of 30 µg/ml. All assays were done 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. In all our different assays, we used the activities of cupric sulfate (CuSO₄), at the concentration of 1 mg/ml as the controls (Hellio et al., 2004).

Antifouling bacterial tests

Marine fouling bacteria were isolated from the culture collection, which had been collected from the three different test panels (Aluminium, Fibre and Wood) in Vellar estuary. Antifouling bacterial assay testing of the extracts were performed by modified standard disc diffusion method Devi et al. (1997) as previously described by Hellio et al. (2004). A sample consisting of 30 µl/disc of extract was loaded onto paper disks (6-mm diameter). The microorganism cultures were grown in Zobell Marine Agar 2216, Himedia, Mumbai and 0.1 ml samples of the culture (106 cfu/ml) were 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.

Mussel bioassay

Antifouling activity against *Perna viridis*

Juvenile mussels (*Perna viridis*, size 0.5 to 5 cm) were collected during low tide from the Vellar estuary, Parangipettai and kept in a 230 L recirculating laboratory aquarium at a constant temperature (20°C), salinity (35‰) and continued aeration for 12 h. Individuals were disaggregated by carefully cutting the byssus threads, and divided into size groups according to total shell length, ranging from 0.5 to 5 cm in a plastic tray with seawater. Individuals exhibiting substrate exploring behaviour (actively exposing their foot and crawling) were selected for experiments. Antifouling activity was measured by the procedure of Ina et al. (1989) and Goto et al. (1992) being modified. The water-resistant filter paper was cut into 9 cm diameter circles and soaked in solvent (control filter). Another 9 cm diameter set of filter papers (treatment filters) were cut in a chess board pattern (1 cm squares) and soaked in a natural concentration of extracts (determined as the extract equivalent to the DW of alga = DW of filter paper) or in a 15 mM solution of CuSO₄ (positive control). All filter paper circles were allowed to air dry. The entire filter circles were placed in the bottom of sterile polystyrene Petri dishes, over which treated chess board filters were placed. Dishes were filled with 80 ml of seawater and three mussel specimens (2.0 - 3.0 cm length) were added. In this way, mussels would have the same area of treated (superior and

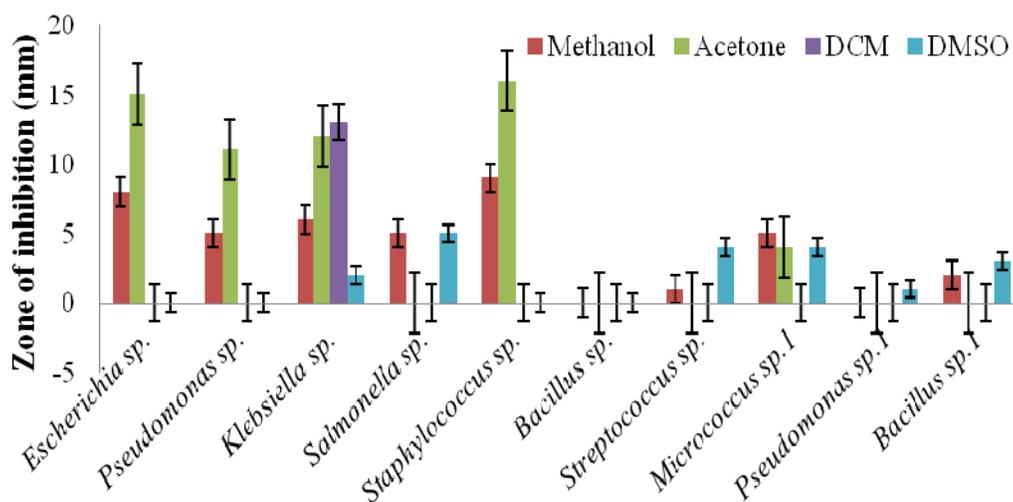


Figure 1. Antibacterial activity of Seaweed *U. lactuca* extracts against biofilm bacteria.

squared) and control (inferior and entire) filter paper to which to attach. Three replicates of each treatment (blank control, positive control- CuSO_4 , Seaweed and sponge extracts) were used.

Experimental dishes were kept in total darkness, as mussels have been shown to produce more byssal threads when held in the dark (Davis and Moreno, 1995). Experiments were allowed to run for 12 h. Mussel activities were recorded immediately after the start of the experiment, after 2 h and then after 12 h. The activities recorded were number of byssal threads attached to each substratum (control or treated filter paper, shell of another mussel or border of Petri dish). After the 12 h period, all records of attachment were checked, mussels were placed in plastic mesh bags tagged according to treatment, and suspended in a sea aquarium for 24 h to check for possible mortality due to exposure to the test substances.

Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) using statistical package of social science (SPSS) version 16.0 for windows. The values are mean \pm SE for three experiments in each group.

RESULTS

Antifouling assay

Ten biofilm bacterial strains were isolated from wood, fiber glass and aluminium test panels placed in vellar estuary. The methanol, acetone, dichloromethane (DCM) and DMSO extracts of seaweeds *S. wightii* and *U. lactuca* and cashew nut oil extracts showed antifouling activity against ten bacterial species.

Antibacterial (fouling) assay

Antibacterial activity of seaweed extracts

The sea weed *U. lactuca* extracts proved active against biofilm forming bacteria. The MeOH extract proved to be

active against eight species. The maximum inhibition zone formation was 9 mm in *Staphylococcus sp.* and minimum was 1 mm in *Streptococcus sp.* In Acetone extracts, the maximum inhibition zone 16 mm was formed in *Staphylococcus sp.* and minimum (4 mm) was shown in *Micrococcus sp.1*. In DCM extract, maximum of 13 mm was shown in *Klebsiella sp.* and no inhibition zone formation was observed in other strains. In DMSO extract, the maximum inhibition zone was formed against *Salmonella sp.* and minimum inhibition zone was found in *Pseudomonas sp.* (Figure 1).

Antifouling bacterial activity of *S. wightii* was active against all 10 fouling bacteria species. The MeOH extract showed activity against eight species. The maximum zone of inhibition showed against *Pseudomonas sp.* (8 mm) and minimum showed against *Staphylococcus sp.* (1 mm). With respect to acetone extracts, the maximum inhibition zone 13 mm was formed in *Salmonella sp.* and minimum of 1 mm was showed in *Pseudomonas sp.* In DCM extract, the maximum 14 mm showed in *Pseudomonas sp.* and 2 mm inhibition zone formation in *Pseudomonas sp.1* (Figure 2).

Antibacterial activity of sponge and cashew nut oil extracts

The crude extract of cashew nut oil was inhibited the growth of biofilm bacterial strains at a concentration of 30 $\mu\text{g}/\text{disc}$ concentration (Figure 3). The negative solvent control (MeOH) had no effect on bacterial growth, while all bacteria were susceptible to the positive controls (CuSO_4). The maximum inhibition zone exhibited 8 and 6 mm in methanol and DMSO extract against *Escherichia sp.* and minimum inhibition zone was 2 mm in both acetone and DMSO extracts against *Pseudomonas sp.*

In sponge, *Desmospongiae sp.* showed the maximum

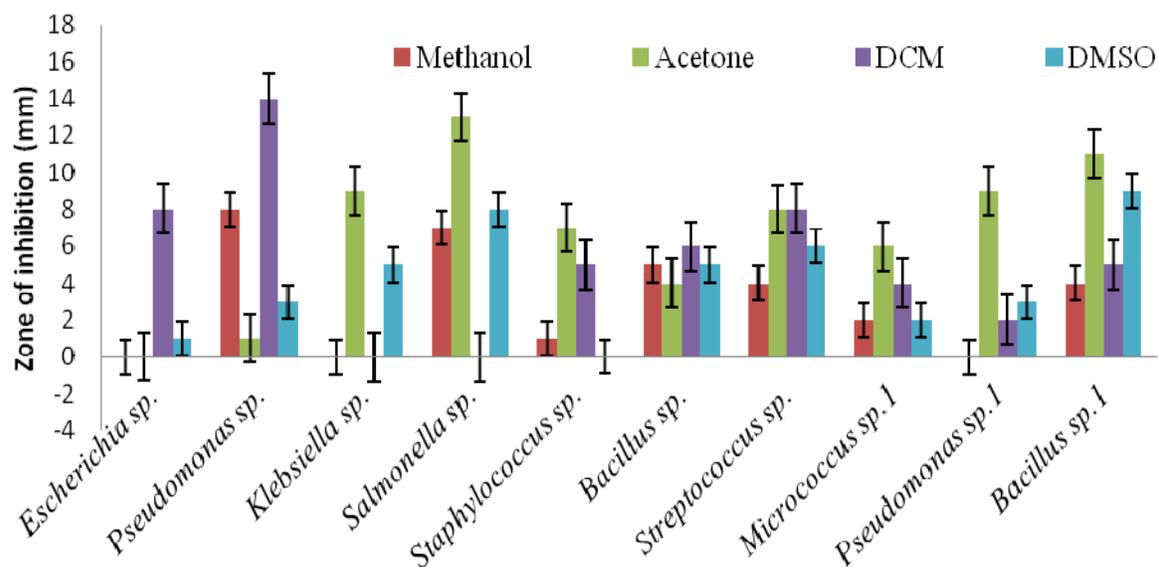


Figure 2. Antibacterial activity of Seaweed *S. wightii* extracts against biofilm bacteria.

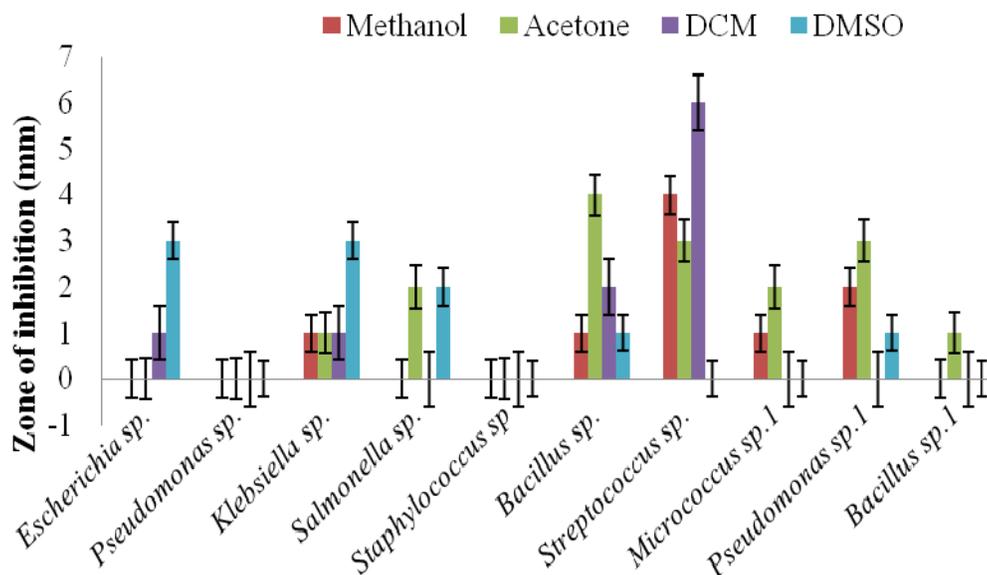


Figure 3. Antibacterial activity of sponge, *Desmosponge sp.* extracts against biofilm bacteria.

zone of inhibition against *Escherichia sp.*, (3 mm) and *Klebsiella sp.* (3 mm) in DMSO extracts. The minimum inhibition zone was found as 1 mm in all three extracts against *Klebsiella sp.* (Figure 4).

Mussel bioassay

Byssus thread bioassay

Juvenile mussels could possibly be attached to three different types of substrata within Petri dishes: filter paper

(control or treated), inner border of the dish or the shell of another mussel (generally the preferred substrate in this gregarious organism). Differences in attachment preferences within treatments were not considered for the purpose of this work, but differences among treatments were taken into account. The mean numbers of attached byssal threads per treatment are presented in Figure 5. Byssal threads were counted in all five treatments (control + two algal + 1 sponge and 1 plant extracts). Mussels attached a mean of 21.6 ± 1.2 byssal threads in controls, which was slight in *S. wightii* (15 ± 0.8), and *U.*

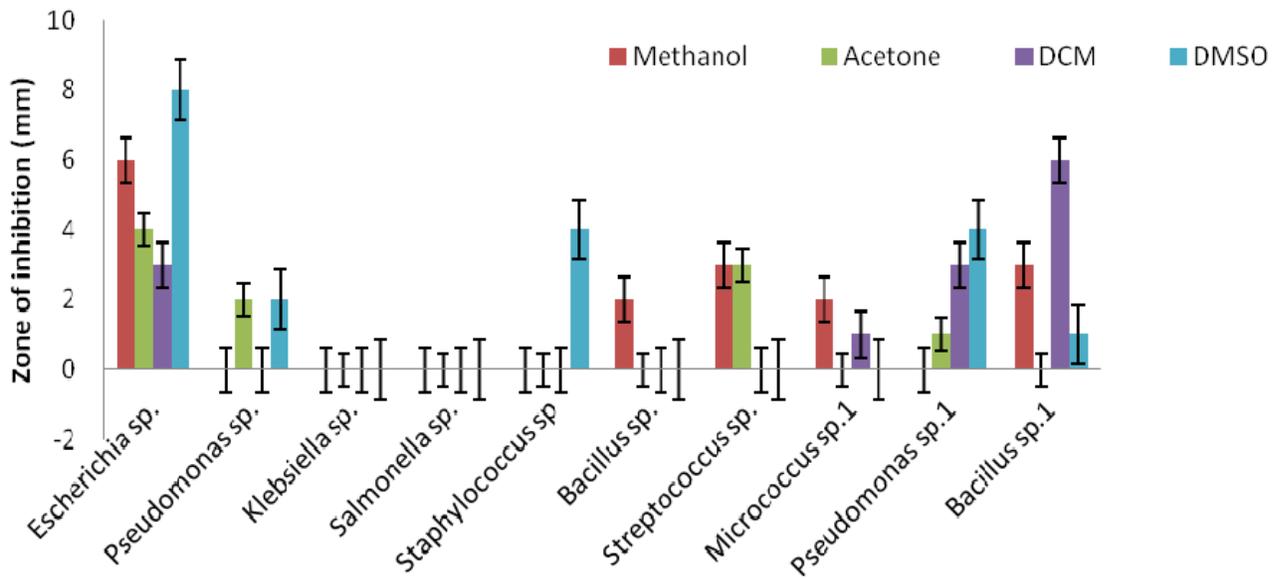


Figure 4. Antibacterial activity of cashew nut oil, *Anacardium occidentale* extracts against biofilm bacteria

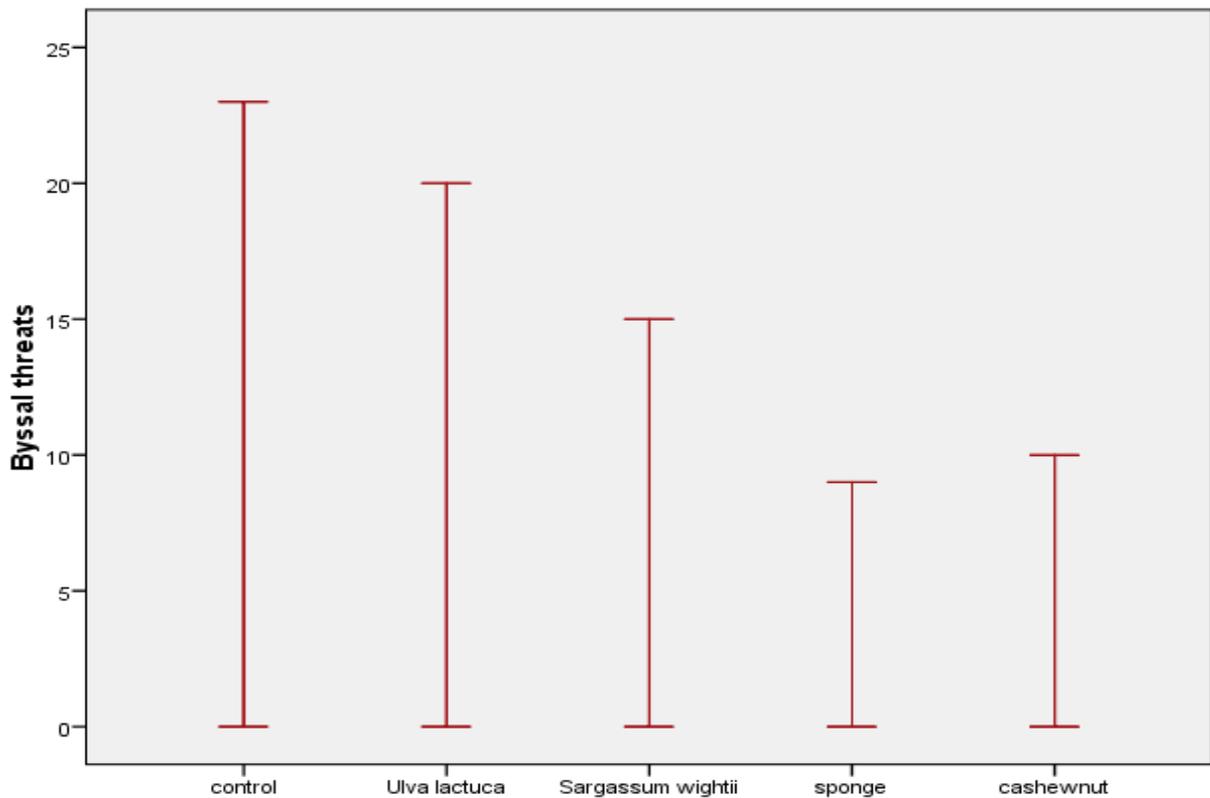


Figure 5. Mean number of attached byssal threads produced by the mussel, *Perna viridis* in response to extracts and compared to controls.

lactuca (20 ± 0.8). The extracts of sponge, *Desmosponge* sp. and cashew nut oil extracts significantly inhibited

mussel adhesion (6.0 ± 1.7 and 10.6 ± 0.9) respectively. As a general trend, all extracts inhibited mussel

attachment to some degree.

DISCUSSION

Studies on the antifouling mechanisms utilised by sessile aquatic organisms may provide valuable information for fouling control in marine biotechnology (Hellio et al., 2001a). Antifouling agents derived from natural products may be less environmentally harmful than the current toxins, having less activity against non-target species (Hellio et al., 2000). The results of the antifouling screening of the present study are on the extracts of *U. lactuca* activity against all species of fouling bacterial strains. The highest zone of inhibition was 16 mm against *Staphylococcus* sp. of acetone extract followed by 13 mm against *Klebsiella* sp. of dichloromethane extract. The methanol extracts showed 8 mm zone of inhibition against *Pseudomonas* sp. In the present investigation, *U. lactuca* and *Sargassum* sp. showed potential antifouling activity against biofilm forming bacteria. The present results were closely related to previous studies (Hellio et al., 2000a, b, 2001a, b, 2002; Hallio et al., 2004), which were based on the screening of antifouling activities among 30 macroalgae collected in Brittany coast which good activity against fouling diatoms and bacterial strains.

In India, 37 marine species including 16 species of flora and 21 species of marine fauna was screened as the antifouling potential against fouling organisms by Bhosale et al. (2002). Prabha Devi et al. (1997) reported that some marine plants possess antibacterial properties against the same bacterial strains. Macroalgae produce a wide range of secondary metabolites, many of which exhibit a broad spectrum of bioactivity (Da Gama et al., 2002) and could potentially be used to develop new antifouling agents (Rittschof, 2000; De Nys and Steinberg, 2002). Cho et al. (2001) have tested the antifouling activity of 27 species of common seaweeds which were collected from Korean coast. In previous study, it was reported that methanol extracts of *I. sinicola* at 200 $\mu\text{l ml}^{-1}$ showed a growth inhibition of 0.3 mm. Similar results were obtained in various screening programmes performed in different seas (Padamakumar and Ayyakannu, 1997; Devi et al., 1997; Pawlik, 2000; Bhosle et al., 2002; Da Gamma et al., 2002).

The sponge is a rich source for several alkaloids and peptides exhibiting diverse biological properties such as cytotoxic, antifungal, antimalarial, antituberculosis and antifouling properties (Orabi et al., 2002; Rittschof et al., 2003; Liu et al., 2004; Raveendran et al., 2008; Lima Mol et al., 2009). The soft coral *Dentronephthya* sp. can control macrofouling on its surface by the production of secondary metabolites (Wilsanand et al., 1999; Dobretsov et al., 2004). Dobretsov et al. (2005) have investigated the antifouling metabolites; demonstrated

the antifouling activity of micro and macrofouling communities *in situ*.

All over the world, efforts are oriented towards isolation of eco-friendly antifouling toxins from marine plants and organisms. Consequently few compounds having antifouling properties have been identified from marine plants and organisms by number of workers in the past. However, little attention is paid towards terrestrial plants. In light of this, the cashew nut oils are selected for screening of the antifouling activity. In the present study, different solvents extracts of methanol, acetone, DMSO and dichloromethane were used for test against the fouling bacteria. The methanol and ethanol extracts showed promising antifouling activity against the fouling strains. Previously, Swant and Wagh (1997) reported the terrestrial plants *Acacia pennata* and *Barringtonia acutangula* tested against the four fouling diatoms and barnacle larvae.

The other antifouling activity of mussel byssal threat bioassay has produced significant activity against green mussel *P. viridis*. Byssal threat bioassay was performed in two species of algae *S. wightii* and *U. lactuca*, one species of *Desmosponge* sp. and one species of cashew nut oil extracts. Mussels attached a mean of 21.6 ± 1.2 byssal threads in controls, with a slight one in *S. wightii* (15 ± 0.8), and *U. lactuca* (20 ± 0.8). The extracts of sponge, *Desmosponge* sp. and cashew nut oil extracts significantly inhibited mussel adhesion (6.0 ± 1.7 and 10.6 ± 0.9) respectively. In earlier studies, *I. sinicola*, which produced a reaction against the mussel assays, was soluble in acetonitrile, dimethylsulfoxide, ethylacetate, isopropanol and methanol, and isolation of the active blue alkaloid compound is in progress. *Scytosiphon lomentaria* showed antifouling activity only with the mussel. These seaweeds may have species-specific or reaction site-specific substances. The narrow spectral antifouling activity of natural nontoxic antifoulants may be overcome by employing mixtures of several compounds that could cover a range of antifoulant mechanisms (Cho et al., 2001).

Marine sponges contain a large number of compounds that show antibacterial (Walker et al., 1985) and antilarval (Becerro et al., 1997; Lee and Qian, 2003) activity in laboratory bioassays. In the present investigation, the antibacterial activity of sponge *Desmosponge* sp. showed promising activity against fouling bacteria which were isolated from test panels. The maximum inhibition zone showed 3 mm in DMSO extracts against *Escherichia* sp., and *Klebsiella* sp. The minimum inhibition zone was found in 1 mm in all three extracts against *Klebsiella* sp. In earlier studies, Lima Mol et al. (2009) guided purification of the acetone extract of the marine sponge, *Haliclona exigua*, (Gulf of Mannars, India) yielded a fraction rich in bis-1-oxaquinolizidine alkaloids, active against seven strains of fouling bacteria as well as cyprids of the cosmopolitan barnacle, *Balanus amphitrite*.

Raveendran and Limna Mol (2009) proposed Natural Product Antifoulants (NPA) as one of the best replacement options for the most successful antifouling agent, tri-*n*-butyl tin (TBT), which, due to its ecological incompatibility, is currently facing total global ban imposed by International Maritime Organization (IMO).

Conclusion

The strategy adopted in this study has identified a number of seaweed sponge that are capable of producing metabolites that, when incorporated into paint, retain their antifouling activity. Broad spectrum antifouling activity was achieved by combinations of these simple antimicrobial compounds. This work demonstrates the potential of marine algae, sponge and cashew nut oil in the production of antifouling coatings based on biodegradable natural products rather than the toxic compounds in current use.

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