ANTIBIOTIC ACTIVITY IN EPIBIOTIC BACTERIA OF SEAWEED, CAULERPA SCALPELLIFORMIS, SOUTHEASTERN INDIA

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Abstract- The surface associated seaweed epiphytic bacteria were isolated from *Caulerpa scalpelliformis* and screened for antibacterial activity. The epiphytic bacteria load on the surface of *C.scalpelliformis* was 30 x 10^2 CFU/cm^2. Only 10 morphologically distinct epiphytic bacterial strains were isolated and screened for antagonistic activity against human pathogenic bacteria. The epiphytic bacterial strains were given designated codes from CS1 to CS10. Out of 10 epiphytic bacterial strains, only two stains (CS4 and CS7) showed activity against 8 and 10 bacterial strains respectively. CS4 strain exhibited zone of inhibition range of 8 to 18 mm with maximum zone against *Bacillus subtilis* and minimum against *Salmonella typhimurium*, whereas CS7 strain showed inhibition between 7 and 20 mm with maximum against *Shigella sonnei.* The investigation has proved that seaweed *C. scalpelliformis* and its epiphytic bacterial strain could be the potential source of bioactive compounds of biomedical importance. However, further purification and characterization of the active compounds has to be done.

Keywords- *Caulerpa Scalpelliformis*, Epibiotic Bacteria, Antibiotic Activity, Southeastern India

I. INTRODUCTION

Seaweeds are found to be a good source of dissolved organic matter, which supports the growth of the associated microflora (Jones and Cannon, 1986) and also seaweeds provide their surface as suitable microniches where the conditions for specific microbial processes are more favorable than in the ambient water (Paerl and Pickney, 1996). The estimated number of epiphytic bacteria on the surface of algae is between 10^3 and 10^6 cells/cm^2 (Shiba and Taga, 1980). Bacteria and algae may compete for inorganic nutrients (Wheeler and Kirchman, 1986). However, the ecological significance of most such natural associations remains vague.

Marine bacteria often produce anticancer and antibacterial substances as a means of maintaining relationships between epiphytic micro-environments, inhibiting competing organisms and microbial pathogens (Avendano-Herrera et al., 2005; Chellaram *et al*, 2011; Prem Anand *et al* 2013a). The enhancement of antimicrobial compound production by bacteria when they are exposed to a different strain of bacteria suggests the existence of competition for space between epibiotic bacteria and their symbiotic role in reducing biofouling on their host (Shiba and Taga, 1980). Since the first antibiotic from a marine bacterium was described in 1966 (Burkholder *et al*., 1966; Prem Anand *et al*., 2013b; Chellaram *et al*., 2014), the antibiotic nature of marine bacteria got attention. The present investigation was carried out with the objective to isolate antibiotic producing bacteria from surface of green seaweed *Caulerpa scalpelliformis* and to study their antibacterial activity against human bacterial pathogens.

II. MATERIALS AND METHODS

2.1. Sample collection

The red algae *Caulerpa scalpelliformis* was collected fresh from Tuticorin (Lat 8 45' N; Long 78 10'E), South east coast of India. The seaweed sample was transported to the laboratory in sterile polythene bags immediately after collection. The seaweed was rinsed for a few seconds with sterile seawater to remove the loosely attached bacteria.

The surface associated seaweed epiphytic bacteria were isolated by the method outline by Wahl (1995). A sterile plastic film with 0.5 cm^2 hole was placed on the surface of the seaweed and the area within the hole was swabbed with a sterile cotton swab (Boyd *et al*., 1999). The swabs were then transferred to sterile glass tubes containing sterile seawater, which was then serially diluted, and pour plated into Zobell Marine Agar plates. The plates were incubated at room temperature for 24 hrs. The bacterial colonies obtained were counted and expressed as colony forming units (CFU) per cm^2. The sampling was done in triplicate and the average has been taken. Ten morphologically different strains of epiphytic bacteria were selected, isolated by repeated streaking and stored in ZMA slants at 4^oC until further study for antibacterial activity.

2.3. Screening of culture supernatants of epiphytic bacteria for antibacterial activity

Antibacterial assay was used as a preliminary screening to isolate antagonistic bacteria against human bacterial pathogens such as *Staphylococcus aureus, Micrococcus luteus, Streptococcus pyogenes, Bacillus subtilis, Enterobacter faecalis, Escherichia coli, Klebsiella pneumoniae, Shigella sonnei, Salmonella typhimurium* and *Vibrio cholera.*

The agar disc diffusion method of Ahmed *et al*. (2008) was used to assess the antibacterial activity of bacterial supernatants. The selected bacterial strains were inoculated into 25 ml of Zobell Marine Broth and incubated on a shaker at 120 rpm for 48 hrs. At the end of incubation period, the fermentation medium was centrifuged at 5000 rpm for 15 minutes to separate the cell mass. The collected supernatant was concentrated. The residue was diluted with 1 ml of distilled water and 100 µl of the supernatant was loaded on to the respective 6 mm sterile paper discs; after dried under room temperature, discs were placed over Mueller-hinton agar medium seeded with test pathogens. The plates were incubated at room temperature for 24 hrs and then observed. Diameter of the zone of inhibition across the disc was measured.

2.4. Identification of active epiphytic bacterial strain

The active epiphytic bacterial strains were identified up to genus level based on the morphological and biochemical characteristic features of the bacteria.

2.4.1. Morphological Characteristics Gram straining

Thin smear was prepared and gram stained. The slide was observed under 10, 45 and 100X magnification.

Motility determination

The isolated bacterial cultures were stab inoculated into the motility agar in a test tube. Then tubes were incubated at room temperature for 24 to 48 hrs. After incubation, the tubes were observed under the light source.

Biochemical Characteristics

The epiphytic bacterial isolate was subjected to various biochemical tests such as IMVIC, Oxidase, Catalase Carbohydrate utilization test, Urease and Antibiotic sensitive test. All the tests were done by standard biochemical procedures as follows.

Indole production test

One percent peptone broth was prepared, sterilized and inoculated with the isolates and incubated at room temperature for 48 hrs. After incubation, 1ml of Kovac's reagent was added and gently shaken. The results were observed after allowing the tubes to stand. A cherry red ring was taken as an indication of positive reaction.

Methyl red test

MR-VP broth was prepared, sterilized and inoculated with the isolates and incubated at room temperature for 48 hrs. After incubation, 5 drops of methyl red indicator was added and the tubes with red color indicated the positive reaction.

Voges proskauer test

MR-VP broth was prepared, sterilized and inoculated with the isolates and incubated at room temperature for 48 hrs. After incubation, 12 drops of α-naphthol and 2-3 drops of 40% Potassium hydroxide were added and the result was noted. Development of crimson to ruby-pink colour was considered a positive reaction.

Citrate utilization test

Simmons citrate agar medium was prepared, sterilized and inoculated with the isolates and incubated at room temperature for 48 hrs. A change in green color to Prussian blue was noted at the end of the incubation period.

Oxidase test

A piece of filter paper was soaked in the 1% N,N,N',N'- Tetramethyl-P-Phenylenediamine dihydrochloride in distilled water reagent solution. The fresh growth from the plate was scraped with a disposable loop and the colony was touched with the edge of the paper. The filter paper was examined for blue color within 10 seconds.

Carbohydrate fermentation test

Peptone broth containing 1% of the glucose sugar and pH indicator (Bromothymol blue) was prepared. After inoculating bacterium, test tubes were incubated for 24-48 hrs, observed for growth, acid and gas production.

Catalase test

Isolated bacterial strains were inoculated in the Zobell marine agar plate and incubated at 37°C for 24 hrs and 3% hydrogen peroxide was added on the inoculated and yet to be identified bacterial strains and formation of air bubbles was taken as positive reaction.

Urease test

An isolated bacterial stain was inoculated in the Urea Agar base slant and incubated at 37°C for 24 hrs. A change in the phenol red to deep pink was noted at the end of the incubation period.

Antibiotic Sensitive test

Disc diffusion or the Kirby-Bauer test is mostly used for the antibiotic sensitive test. Each isolated culture was inoculated in 5 ml marine broth and incubated at 37°C for 24 hrs. Sterile cotton swabs were swabbed with the culture, gently pressed against the wall of the test tubes to remove the excess bacterial inoculums and swabbed on the air-dried zobell marine agar plates aseptically. Using flamed forceps, the commercial penicillin antibiotic discs were transferred to the agar plates. After overnight incubation at 37°C, the antibiotic sensitivity of each culture was determined by measuring the zone of inhibition.

III. RESULTS

3.1. Epiphytic bacterial density

The *Caulerpa scalpelliformis* showed the presence of surface associated bacteria. The epiphytic bacteria

load on the surface of *C.scalpelliformis* was 30 x 10^2 CFU/cm^2. Only 10 morphologically distinct epiphytic bacterial strains were isolated and screened for antagonistic activity against human pathogenic bacteria. The epiphytic bacterial strains were given designated codes from CS1 to CS10. Out of 10 epiphytic bacterial strains, only two stains (CS4 and CS7) showed activity against 8 and 10 bacterial strains (Table 1).

3.2. Antibacterial activity epiphytic bacterial supernatants

The culture supernatants of both strains were subjected to antibacterial activity screening against pathogenic bacteria (Fig.1). Both CS4 and CS strains showed wide spectrum antibacterial activity. In CS4 strain, the zone of inhibition ranged from 8 to 18 mm with maximum zone against *Bacillus subtilis* and minimum against *Salmonella typhimurium.* In CS7 strain, zone of inhibition ranged between 7 and 20 mm with maximum activity against *Shigella sonnei* and lowest activity against *Enterobacter faecalis* and *Escherichia coli.*

3.3. Identification of epiphytic bacteria

The active epiphytic bacterial strains (UR4 and UR7) were identified up to genus level based on the morphological and biochemical characters (Table 2). The strains were identified as *Bacillus sp.* (UR7) and *Pseudomonas sp.* (UR4)

3.4. Identification of Pseudomonas sp. (CS4)

As the strain of *Pseudomonas* sp. showed pink colour for gram staining, they were identified as gram-negative rod. The stains were motile. When carbohydrate fermentation test was performed, they did not ferment the glucose or produced gas and acid. In oxidase test, the strain was positive as change of colour was observed. It showed negative result in indole, MR-VP and positive in citrate test by color change from green to Prussian blue. The stain was resistant to the penicillin antibiotic. But, they grew in cetrimide agar plate and produced blue green pigment.

3.5. Identification of Bacillus sp. (CS7)

As the strain of *Bacillus* sp. showed purple colour for gram staining, they were identified as gram-positive rod. The strains were motile. In oxidase test, the strain showed positive result. They showed negative in indole, MR test and positive in VP and Citrate tests.

DISCUSSION

The seaweed *Caulerpa scalpelliformis* was found to possess epiphytic bacteria on their surface. The average epiphytic bacterial population on the surface of the seaweed *C.scalpelliformis* was 30 X10^2 CFU/cm^2. But, when compared to the surrounding seawater bacterial load in Tuticorin water (110 x10^4 CFU/ml (Raja, 2008), the epiphytic load was low on this seaweed surface. It could be presumed that the surface characteristics of plants and animals play a key role in controlling epibacterial population density on their surface (Al-Ogily and Knight-Jones, 1977). The epiphytic bacterial load in the present investigation was lower than that of *Caulerpa scalpelliformis* (35 x 10^2 CFU/ cm^2) (Raja *et al*., 2008) and 28 x 10^2 CFU/cm^2 in *Gracilaria edulis.* In a similar study, Beleneva and Zhukova (2006), who have reported that surface associated bacterial density was 1.37 x 10^5 CFU / g on surface of *Camphylaephora hyphaeoides* and 1.71 x 10^5 CFU / g of *Gracilaria verrucosa.* The presence of epiphytic bacteria could be linked to the postulation of Tatewaki *et al.* (1983) that the surface bacteria may be required for the proper development of the seaweeds.

In the present study, moderate level wide spectrum antagonistic activity against human pathogens was observed in two out of 10 epiphytic bacterial strains isolated from the seaweed *C. scalpelliformis.* The epiphytic bacterial strains were randomly chosen based on colony morphology and hence, the contribution by non-prominent and non-cultivable bacteria is not ruled out. Also, in the present study, only standard media was used for isolation of epiphytic bacterial strains. It is to be noted that the media plays a key role in isolation process as observed by Jensen *et al.* (1996) who have reported a far greater recovery of marine bacteria from algal surfaces using low nutrient media compared to commonly used media rich in nutrients.

The percentage of active epiphytic strains from *C.scalpelliformis* was low when compared to Burgess *et al* (1999) who have shown that 35% of the 400 surface associated bacteria from seaweeds and invertebrates produced antimicrobial compounds. Also, Boyd *et al* (1999) found that 21% of epiphytic marine bacteria exhibited antimicrobial activity. The two epiphytic bacterial strains which showed wide spectrum antagonistic activity were cultured and tested for antibacterial extra-cellular metabolite production. The supernatants of the two strains showed wide spectrum activity against pathogenic bacteria at 100µl concentration. In a similar study, Kanagasabhapathy *et al* (2008) studied the antibacterial activity in the supernatants of epiphytic bacteria from red algae such as *Pachymeniopsis lauceolata, Plocamium telfairiae, Gelidium amansii, Chondrus oncellatus, Grateloupia filicina, Ceramium kondoi, Lomentaria catenata, Schizymenia dubyi* and *Porphyra yezoensis.* Thus, the seaweed associated epiphytic bacteria has shown that they could produce a high percentage of antimicrobial metabolites (Villarreal-Gómez *et al*., 2010). In some isolates of epiphytic bacterial strains, the activity was low

against the pathogens. It is possibly due to the fact that these pathogenic bacteria are not natural competitors for the epibiotic bacteria and therefore lack in secondary metabolite that would induce the antimicrobial activity (Kanagasabhapathy *et al.*, 2008).

In this investigation of the active strains were identified as *Pseudomonas sp*. (CS4) and *Bacillus sp*. (CS7). The results supported that a most active marine bacteria belongs to *Bacillus sp., Micrococcus sp., Pseudomonas sp., Vibrio sp., Flavobacterium sp., Alcaligenes sp., Xanthomonas sp.* and *Achromobacter* sp. (Bernan *et al.,* 1997). The present observation coincided with the earlier report of Raja (2008) that the *Pseudomonas* sp. from *Ulva lactuca* showed potent antagonistic activity and also produced extra-cellular metabolite that inhibited all biofilm bacterial strains.

The present study indicated the importance of epiphytic bacteria from seaweed as a potential source of antibiotics. This was presumed by Imamura *et al* (1997) who demonstrated that the marine epiphytic bacteria was shown to produce antimicrobial compounds such as phenazine produced by a strain of *Pelagiobacter variabilis* isolated from seaweed *Pocockiella variegata*. The antibacterial activity expressed by the epiphytic bacteria in the present study suggested that these algal associated bacteria have the potential to control the emerging antibiotic resistant strains. It was also proposed that epiphytic bacteria on macroalgae can produce antibacterial compounds that work in synergy with the seaweed derived compounds (Armstrong *et al.*, 2001; Boyd *et al*., 1999). These findings support the hypothesis that epiphytic bacterial strains offer a potential scope for antibacterial compounds of biomedical importance and further studies are needed to characterize the active compound.

Table 1: Antibacterial activity of supernatants of the epiphytic bacteria from *Caulerpa scalpelliformis*

S. No.	Isolated Epiphytic bacterial strains	Human bacterial pathogens									
		Staphylococcus aureus	*Micrococcus luteus*	*Streptococcus pyogenes*	*Bacillus subtilis*	*Enterobacter faecalis*	*Escherichia coli*	*Klebsiella pneumoniae*	*Shigella sonnei*	*Salmonella typhimurium*	*Vibrio cholerae*
		Zone of inhibition (mm) (100 µl/disc)									
1	CS1	-	-	-	-	-	-	-	-	-	-
2	CS2	-	-	-	-	-	-	-	-	-	-
3	CS3	-	-	-	-	-	-	-	-	-	-
4	CS4	14	12	12	18	-	-	16	10	8	12
5	CS5	8	-	-	-	-	7	-	-	-	-
6	CS6	-	-	-	-	-	-	-	-	-	-
7	CS7	16	10	10	10	7	7	12	20	8	8
8	CS8	-	-	-	-	-	-	-	-	-	-
9	CS9	-	-	-	-	-	-	-	-	-	-
10	CS10	-	7	-	-	7	-	-	-	-	-

- No activity

Table 2: Morphological and biochemical characters of active epiphytic bacteria from *Ulva reticulata*

Morphological and biochemical characters	*Pseudomonas sp.* (CS4)	*Bacillus sp.* (CS7)
Gram staining	Rod/negative	Rod/positive
Motility	Motile	Motile
Indole	-	-
Methyl red	-	-
Voges proskaur	-	+
Citrate	+	+
Oxidase	+	+
Carbohydrate fermentation of glucose	-	+
Sensitivity to penicillin disc	-	-

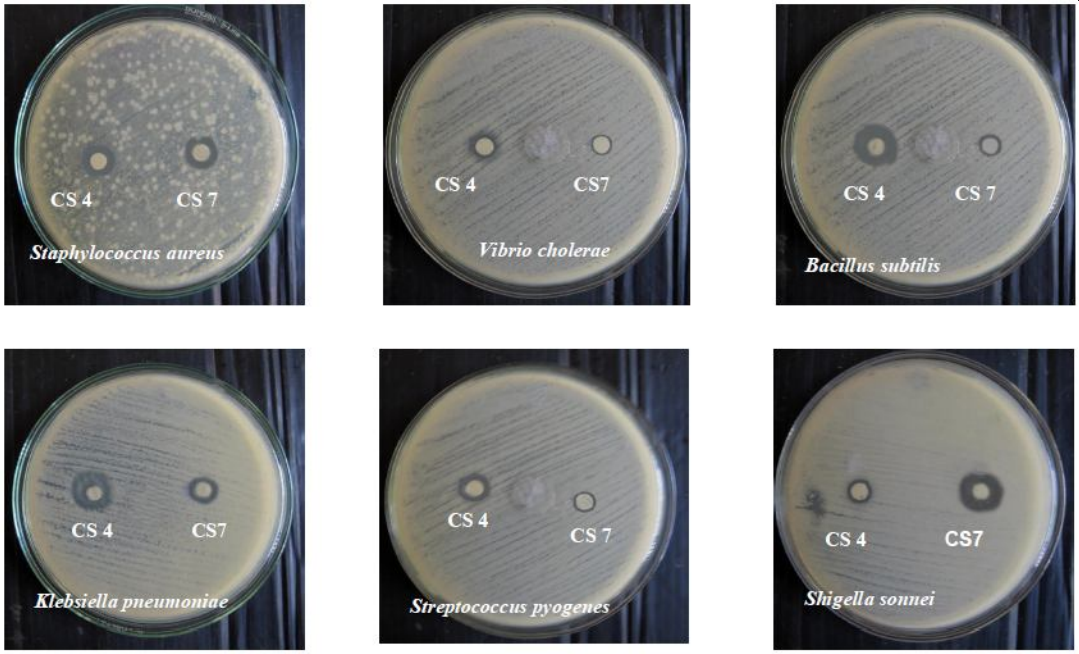


Fig. 1. Antibacterial activity of culture supernatants of epiphytic bacteria (CS4 and CS7) isolated from *Caulerpa scalpelliformis* against pathogenic bacteria

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