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Evaluating the Antagonistic Potential of Seaweed-associated Marine Bacteria Collected from the Southwest Coast of India

[1]Sujith Sugathan, [2]Aseer Manilal, [1]Joseph Selvin, [3]Akbar Idhayadhulla, [3]Radhakrishnan Surendra Kumar and [4]M.V.N. Panikkar

[1]Department of Bioinformatics, Bharathidasan University, Tiruchirappalli 620024, India
[2]Department of Biotechnology, Presentation College of Applied Sciences, Puthenvelikara 683594, Kerala, South India
[3]P.G and Research Department of Chemistry, Jamal Mohamed College, Tiruchirappalli-620020, India
[4]Department of Botany and Biotechnology, Sree Narayana College, Kollam-691001, Kerala, India

Corresponding Author: Sujith Sugathan, Department of Bioinformatics, Bharathidasan University, Tiruchirappalli 620024, India Tel: +919895530247

ABSTRACT

Seaweed-associated marine bacteria were recognized as a potential source of antimicrobial compounds. In the present study, a total of 27 epiphytic bacteria were isolated from four species of seaweeds collected from the Kollam coast (Indian Ocean) were investigated for antagonistic activity by cross streak method. Of the 27 bacterial isolates, 4 strains inhibited the growth of at least one shrimp vibrio pathogen tested. The active strains were further subjected to agar well diffusion assay. The result indicates that among the four seaweed isolates, SWI-24 strain exhibited highest spectrum of activity against all the tested shrimp pathogens. The molecular characterization based on partial 16S rRNA sequence revealed that the active isolate SWI-24 was *Pseudoalteromonas* sp. The efficient antagonistic potential exhibited by the SWI-24 against pathogenic shrimp bacteria may further reflect the potential use of seaweed-associated bacteria in managing the shrimp/fish disease.

Key words: Seaweed isolates, marine epiphytic bacteria, shrimp pathogen, antagonistic activity

INTRODUCTION

The marine flora and fauna are considered as a special "bio-reservoir" of bioactive metabolites (Manilal *et al*., 2010a). Among them, marine bacterial vegetation constitutes an eminent resource of untapped novel metabolites (Selvin *et al*., 2009; Manilal *et al*., 2010b; Reddy *et al*., 2011). The biosynthesis of antimicrobial metabolites by marine bacteria isolated from different marine environment has been known for a long time (Burkholder *et al*., 1966). The majority of these bacterial strains were sourced from seawater and marine sediments. Bacteria associated with marine organisms have reported to display significant bioactivity including antifouling, antibacterial, cytotoxic activities, anticoagulant and cardioactive potentials (Burgess *et al*., 1999; Sponga *et al*., 1999; Bernan *et al*., 1997; Imamura *et al*., 1997; Jensen and Fenical, 1994; Burgess *et al*., 1991; Austin, 1989; Marderosian, 1969). Moreover, deep studies of marine natural products envisages that many bioactive compounds previously found in marine animals and plants possess notable similarities to metabolites produced from their associated microorganisms

(Unson and Faulkner, 1993; Schupp *et al.*, 1999; Davidson *et al.*, 2001; Luesch *et al.*, 2001; Proksch *et al.*, 2002).

Seaweeds can host verdant varieties of heterotrophic bacteria and many of these bacteria play a cardinal role in maintaining the health of the host organism (Bolinches *et al.*, 1988) by producing unique bioactive secondary metabolites. Hence, epibiotic bacteria become an incredible source of new natural products (Holmstrom and Kjelleberg, 1999). These microbial bioactives could be utilized as a source of lead compounds for the biomedical and biopharmaceutical industry (Donia and Humann, 2003; Anand *et al.*, 2006). Isolation of these epibionts is relatively very easy as compared to other bacteria. Thus, seaweed-associated bacteria may be useful in the development and production of bioactive compounds and biocontrol agent.

Deployment of natural antibiotic and probiotic (biocontrol) offers a hopeful alternative to the synthetic antibiotics for fish and shrimp aquaculture system. The development of a suitable probiotics needs empirical and fundamental research, full scale trials, as well as the development of appropriate monitoring tools. The initial-step in the development of a probiotic involves isolation of appropriate bacteria from natural sources and optimization of its antagonistic activity. In this background, the present study is initiated to explore the antagonistic potential of marine culturable bacteria associated with seaweeds/seaweed habitat as a suitable source for the development of shrimp probiotics.

MATERIALS AND METHODS

Collection of seaweeds: Seaweeds were sourced from the Southwest coast of India (Kollam coast; 08°54'N and 76°38'E). Four species of seaweed specimens were handpicked during the lowest tide of the chart datum. Seaweed samples were identified by eminent algologist Dr. M.V.N. Panikkar, Director of Research, Department of Botany, Sree Narayana College, Kollam, Kerala, India.

Isolation of seaweed-associated microbes: The bacterial strains associated with seaweeds were obtained by using the following procedure. The seaweeds were rinsed repeatedly with sterile seawater in order to remove the freely attached bacteria. A small zone (1 cm^2) of the cleaned thallus of each seaweed species were separately scrubbed with a sterile cotton swab and plated on the entire surface of 180 mm Petri dishes containing ZoBell Marine Agar (ZMA) or Nutrient Agar (NA) in duplicate until the colonies were observed. The media were supplemented with nystatin and cyclohexamide at 25 and 10 μg mL^{-1}, respectively to minimize contamination with fungi and 10 μg mL^{-1} nalidixic acid was applied to minimize bacterial contaminant growth (Takizawa *et al.*, 1993; Ravel *et al.*, 1998). After 48 h at 28°C, different colonial morphologies of bacteria were chosen and purified by successive re-streaking. The pure bacterial cultures obtained were maintained on NA slants.

Screening of potential antagonistic bacteria against shrimp pathogens by cross streak method: The potential antagonistic bacteria were selected by screening the seaweed isolates against a battery of shrimp pathogens including six MTCC type culture and nine shrimp vibrio isolates (Table 1). The shrimp Vibrio isolates previously isolated from infected *P. monodon* collected from the shrimp ponds of Manroe Island, Kollam, South India were used for the inhibitory assay (Manilal *et al.*, 2010a). All the test organisms were maintained on nutrient agar slants at 4°C before being used in the inhibitory assays. Determinations of antimicrobial activity of the seaweed associated bacteria are performed by cross-streak method (Egorov, 1965). Mueller-Hinton agar plates were prepared and inoculated with seaweed isolates by a single perpendicular streak of

inoculum in the centre of the petridish and incubated at 27°C for 4 days. The plates were seeded with test organisms by a perpendicular streak at a 90° angle to the line of the seaweed isolates. Antagonism was observed based on the inhibitory interaction between the seaweed isolates and test organisms. The reference standard (Nalidixic acid) was used for the verification of strain sensitivity/resistance pattern. Inhibition activities and colonization effect were noticed at 24, 48, 72 and 96 h. All the tests were conducted in triplicates.

Screening of potential antagonistic bacteria against shrimp pathogens by agar well diffusion method: The bacterial strains which exhibiting the antagonistic activity in cross-streak method was further confirmed with the agar well-diffusion method (Manilal *et al*., 2010c). The seaweed associated bacterial strains were grown in a nutrient broth (Merck, Germany) incubated at 25°C for 18 h and adjusted to an approximate concentration of 10^8 cfu mL^{-1}. The Mueller Hinton agar plates were prepared and uniformly spread with appropriate shrimp pathogens. Thereafter, wells with 5 mm of diameter were prepared using a sterile cork borer. The resultant wells were carefully filled with 120 µL of the appropriate microbial cell suspension. The well with broth used for culture was considered as negative control. The assay was performed in triplicates of individual Petri dishes. The presence of antagonistic metabolites/inhibitory compounds produced by the isolates suppressed the growth of the test pathogen producing a zone of inhibition around the well. The diameter of the inhibition halo after 24 h of incubation at 37°C was considered to be indicative of bioactivity. Zones of inhibition were scaled when bacterial growth was visible. The net halo diameter was calculated after subtracting the diameter of the well (5 mm) (Manilal *et al*., 2010b).

Molecular identification of active seaweed isolates (SWI-24): Molecular identification of the active seaweed isolate was based on partial 16S ribosomal RNA gene sequencing. The active bacterial strain was cultured in marine broth at 25°C and the total genomic DNA was extracted by CTAB/NaCl method. PCR amplification of bacterial 16S ribosomal RNA gene was performed using the universal eubacterial 16S rRNA gene primers. The amplified 16S ribosomal RNA gene product was cloned by TA cloning method using a TOPO TA cloning kit as per the manufactures instruction (Invitrogen) for sequencing. The resultant 16S ribosomal RNA gene sequence from the active isolate SWI-24 was compared with other bacterial sequences by using BLAST (Basic Local Alignment Search Tool) (Altschul *et al*., 1990) to analyse pair wise homology. The sequence used in the analysis was deposited in GenBank, EMBL in Europe and the DNA Data Bank of Japan with an accession No. EU432052.

Statistical analysis: One-way ANOVA using SPSS was used to analyze the difference between treatments and controls. Values of $p<0.05$ were considered significantly different.

RESULTS

Description of seaweed collection site-Kollam coast: Kollam coast is situated at the lower portion of Southwest coast of India. The Kollam coast harbours a great diversity of species including micro and macroalgae, fishes, clams, crustaceans, sponges, sea anemones, sea cucumbers, sea urchins, soft corals and other sessile invertebrates (Manilal *et al*., 2010c). Bioactivity of marine organisms collected from the Kollam coast has been extensively reported (Manilal *et al*., 2009, Manilal *et al*., 2010a, 2010b, 2011). Hitherto, the antagonistic properties of seaweed-associated microbes from the Kollam coast have not been explored. The seaweed specimens collected from the study area are appended in the Table 2.

Table 1: Panel of shrimp pathogens nsed for antimicrobial assay

MTCC Cultures*	Shrimp *Vibrio* isolates**
V. alginolyticus (MTCC 4439)	*V. harveyi* (Vb15)
V. vulnificus (MTCC 1145)	*V. alginolyticus* (Vb11)
V. parahaemolyticus (MTCC 451)	*V. vulnificus* (Vb14)
V. alcaligenes (MTCC 4442)	*V. fischeri* (Vb17)
V. fischeri (MTCC 1738)	*V. parahaemolyticus* (Vb12)
V. harveyi (MTCC 3438)	*Ph. damsela* (Vb26)
	V. anguillarum (Vb10)
	V. campbellii (Vb 23)
	V. splendidus (Vb22)

*Microbial type culture collection, **Isolated from moribund shrimp

Table 2: Bacterial strains isolated from different species of seaweeds

Seaweeds	Isolated strains	Media used for the isolation
Padina tetrastromatica (Hauck)	SWI 1	ZMA
	SWI 2	NA
	SWI 3	ZMA
	SWI 4	ZMA
	SWI 5	ZMA
	SWI 6	NA
Sargassum wightii (Greville)	SWI 7	ZMA
	SWI 8	ZMA
	SWI 9	NA
	SWI 10	ZMA
	SWI 11	ZMA
	SWI 12	ZMA
	SWI 13	ZMA
Acrosiphonia orientalis (J. Agardh)	SWI 14	ZMA
	SWI 15	ZMA
	SWI 16	NA
	SWI 17	NA
	SWI 18	ZMA
	SWI 19	ZMA
	SWI 20	ZMA
	SWI 21	NA
	SWI 22	ZMA
Stœchospermum marginatum (C. Agardh)	SWI 23	ZMA
	SWI 24	NA
	SWI 25	NA
	SWI 26	NA
	SWI 27	ZMA

SWI: Seaweed isolates, ZMA: ZoBell marine agar, NA- Nutrient agar

Epiphytic microorganisms isolated from different species of seaweeds: According to the colony morphology, totally 27 epibiotic bacteria from the four species of seaweed are isolated and sub-cultured to obtain pure cultures. The isolates sourced from seaweed were abbreviated as SWI (Table 2). The highest number of bacterial strains was isolated from *A. orientalis* (33.3%), followed by *S. wightii* (25.9%), *P. tetrastromatica* (22.2%) and *S. marginatum* (18.5%). Among the 27

seaweed isolates, 8 were obtained on Nutrient agar and the remaining 19 were isolated on ZoBell Marine Agar (ZMA).

Antagonistic bacteria isolated from different species of seaweeds: In seaweed isolates, of 27 bacteria isolated, only four bacterial strains (SWI-8, SWI-17, SWI-24 and SWI-26) showed inhibitory effects against at least one of the tested strains. The activity of these strains was further re-confirmed with agar well-diffusion method. The results of agar well diffusion method showed that SWI-24 and SWI-26 exhibited 100% inhibitory activity against all the tested shrimp pathogens. Spectrum of activity was narrow and weak for other two isolates. The seaweed isolate SWI-24 showed a strongest inhibition range of 7 to 21 mm against the MTCC shrimp pathogens whereas, the activity was in the range of 7 to 14 mm against the shrimp vibrio isolates (Fig. 1 and 2). The Culture Suspension (CS) of SWI-26 produced a mean zone inhibition ranged between 8 to 12 mm against the MTCC whereas, 6 to 12 mm against the shrimp vibrio isolates (Fig. 1 and 2). None of the zones of inhibition exceeded 14 mm, indicating that shrimp vibrio isolates are resistant or only slightly susceptible to the action of seaweed isolates. Therefore, the strain SWI-24 was considered as the most active isolate of this group and therefore, subjected to further molecular examination.

The taxonomic affiliation of the 16SrRNA sequences of the SWI-24 was retrieved from classifier programme of Ribosomal Database Project II version 9.0. The 16S rRNA sequence of the isolate was blasted using megablast tool of GenBank (http://www.ncbi.nlm.nih.gov/). This revealed that the isolate was a *Pseudoalteromonas* sp. (Fig. 3).

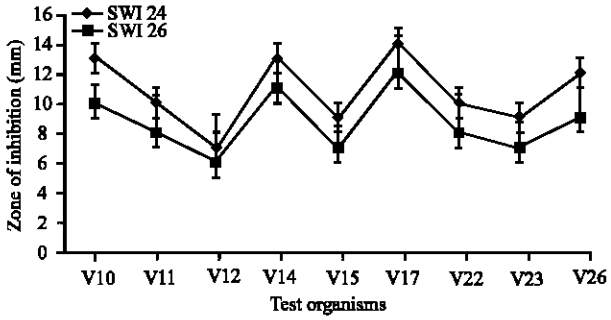

Fig. 1: Zone of inhibition of seaweed isolates against shrimp vibrio isolates

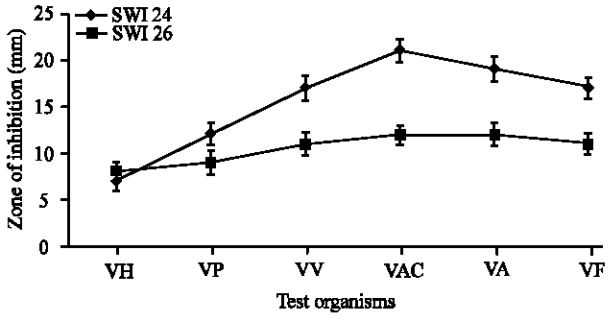

Fig. 2: Zone of inhibition of seaweed isolates against MTCC cultures

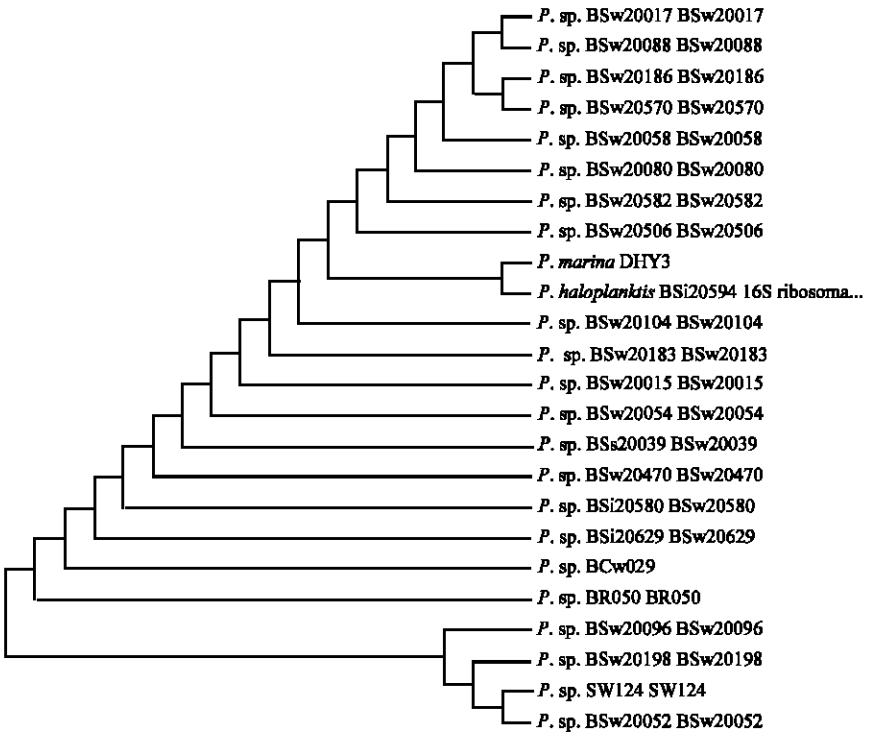

Fig. 3: Neighbor joining phylogenetic tree of the seaweed isolate SWI24 and their closest NCBI (megaBLAST) relatives based on the 16S rRNA gene sequences (Accession No. EU432052)

DISCUSSION

The first line of selection for a potential probiotics is to perform *in vitro* antagonism tests, in which pathogens are exposed to candidate probiotics in a liquid or solid medium (Balcazar *et al.*, 2006). Antibiotics are chemicals usually produced as secondary metabolites which, although created in small quantities, inhibit or kill other microorganisms (Brock and Madigan, 1997). The inhibitory compounds produced by these antagonistic bacteria could be extracted, characterised and utilized for the development of human and veterinary grade drugs. On the other hand, these antagonistic bacteria can be directly used as a live probiotic strains.

In the present study, totally 27 strains of associated bacteria were isolated from four species of seaweeds. Based on the stability of sub-culturing and bioactivity profile, four marine bacteria were obtained and deposited at the Marine Bio-prospecting Laboratory, Bharathidasan University. All the isolated strains were screened for their ability to suppress the growth of shrimp vibrio pathogens in cross streak and agar well diffusion assay. The results of the cross streak method showed that four species isolated from the seaweed could inhibit the growth of shrimp pathogens.

In the present study, antagonism by seaweed epiphytic bacteria was indicated by interruption in the growth of the tested shrimp pathogens. Of the 27 seaweed-associated strains, 14.8% (four strains) showed potential antagonistic activity against a battery of shrimp pathogens. Among the 4 isolates, SWI-24 exhibited the highest level of antimicrobial activity against all pathogenic shrimp pathogens tested. However, mild antagonistic activity was exhibited by other bacterial strains. Furthermore, inhibitory pattern of SWI-24 was more pronounced against the shrimp MTCC pathogens. Inhibition zones up to 21 mm were observed against three MTCC cultures such as

V. alginolyticus (19 mm), *V. vulnificus* (17 mm), *V. alcaligenes* (21 mm) and *V. fischeri* (17 mm). Molecular identification of the active isolates SWI-24 based on partial 16S rRNA sequencing revealed that the active strains belonged to the members of *Pseudoalteromonas* genus.

There is no previous report on the vibriocidal activity of seaweed associated bacterial strains isolated from the Kollam coast. It can be envisaged that the association of these antagonistic bacteria on the surface of this seaweed is to control biofouling. Antagonistic interactions among seaweed-associated bacteria that occur on the seaweed surface are of great interest to search for secondary metabolite-producing bacteria. Screening of marine bacteria isolated from the surface of seaweed and invertebrates has shown that a high percentage produce antimicrobial metabolites (Burgess *et al*., 1999). Lemos *et al*. (1985) acknowledged that the epiphytic bacterial strains isolated from the inter-tidal seaweeds demonstrated inhibitory activity against *V. harveyi, V. anguillarium* and *A. hydrpohila*. It was postulated that beneficial relationship exists between antagonistic bacteria and seaweeds/algae (Lemos *et al*., 1985) and animals that harbour them (Bernan *et al*., 1997). Marine *Pseudoalteromonas* species isolated from the surface of marine algae and invertebrates has shown that a high percentage produce anti-microbial metabolites (Holmstrom and Kjelleberg, 1999). The bacterium *Pelagiobacter variabilis* isolated from the seaweed, *Pocockiella variegata* produced phenazine antibiotics, pelagiomicins (Imamura *et al*., 1997). Jayanth *et al*. (2002) reported that *Alteromonas* strains isolated from seaweed exhibited a wide antibacterial activity against the shrimp bacterial pathogens viz., *V. alginolyticus, V. fischeri* and *V. harveyi*. The coral-associated bacterium, *Pseudoalteromonas luteoviolacea* was reported to inhibit the growth of shrimp pathogenic bacterium tested, *Vibrio harveyi* (Radjasa *et al*., 2005). Stelzer *et al*. (2006) showed that the green-yellow pigmented marine bacterium *P. tunicata* produces several target-specific compounds that act against a range of common fouling organisms, including bacteria, fungi, protozoa, invertebrate larvae and algal spores. Manilal *et al*. (2009) reported that the biological activity demonstrated by *A. orientalis* could be due to the presence of epiphytic bacteria. *Pseudoalteromonas* sp. associated with brown alga collected from Baltic Sea environment showed antagonistic property (Kennedy *et al*., 2009). Marine *Pseudoalteromonas* sp. displayed highest antibacterial activity against fish pathogens such as *V. alginolyticus*, *V. anguillarum*, *V. fluvialis*, *V. harveyi*, *V. metschnikovii*, *V. splendidus*, *V. ordalii*, *V. parahaemolyticus* and *V. vulnificus* (Isnansetyo *et al*., 2009). Marine bacteria, *Pseudoalteromonas tunicata* showed a very broad range of antagonistic activity against many pathogenic bacteria and fungi (Sivasubramanian *et al*., 2011).

The selection of biocontrol agent (probiotics) for aquaculture generally involves *in vitro* experiments whereby, the candidate microorganisms are screened for production of anti-microbial metabolites. Screening for organisms with antagonistic abilities towards pathogens may produce a large number of candidate probiotics. The efficient antagonistic potential exhibited by the SWI-24 against pathogenic shrimp bacteria may further reflect the potential use of seaweed-associated bacteria in managing the shrimp disease. Therefore, further *in vivo* efficacy validation is needed to confirm the effectiveness of this strain in the shrimp.

CONCLUSION

Four species of seaweeds were collected off the southwest cost of India were subjected to epiphytic microbial isolation. Totally, 27 strains of seaweed associated bacterial isolates were obtained and each screened for antagonistic activity by cross streak method. Of this, 4 strains exhibiting activity was subjected to agar well diffusion assay against shrimp pathogenic bacteria.

Among the four seaweed isolates, SWI-24 strains (*Pseudoalteromonas* sp.) exert significant antagonistic activity against all the tested vibrio pathogens. Therefore, the presence of these bacterial isolates could protect the aquatic animals against the infection by pathogenic vibrios and might be utilized as good source of probiotic in shrimp aquaculture.

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