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Studies on Novel bacteriocin like inhibitory substance (BLIS) from microalgal symbiotic *Vibrio* spp MMB2 and its activity against aquatic bacterial pathogens

Selvendran M* and Michael Babu M.
Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam-629 502, Tamil Nadu India.

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ABSTRACT

The present study was aimed to isolate the antagonistic bacteria from marine microalgal sample(*Chlorella salina*), the isolated bacterial strains were identified , screened for their bioactivity against the aquatic pathogens, to extract and characterize the compounds produced by antagonistic bacteria. Among the isolates *Vibrio* sp.MMB2 strain and *Pseudomonas* sp. had good antagonistic property against all tested pathogens. The maximum biogenic activity was occurred at the stationary phase culture. The metabolites extracted from *Vibrio* sp. MMB2 was showed highest zone of inhibition against *A.hydrophylla* .One active fraction was isolated by TLC. The functional group was identified through FTIR analysis ,the bacterial metabolites contains Nitrogenous compound as a functional groups. The Partially purified CFNS (bacteriocin) of *Vibrio* sp. MMB2 was showed two distinct bands on SDS-PAGE, its high molecular weight of antimicrobial peptides. The bacterial metabolites effectively controlled the pathogens at broad range, to compare commercial antibiotics.

INTRODUCTION

The marine environment harbours a wide range of microbes capable of exhibiting bacteriolytic and antibiotic activity. Marine organisms are a rich source of structurally novel and biologically active metabolites. So far, many chemically unique compounds of marine origin with different biological activity have been isolated and a number of them are under investigation and/or are being developed as new pharmaceuticals (Faulkner, 2000; Schwartsmann *et al*,.2001). Secondary or primary metabolites produced by these organisms may be potential bioactive compounds of interest in the pharmaceutical industry. However, the search for new chemotherapeutic agents needs to continue in order to discover compounds with effectiveness against antibiotic resistant strains of bacterial pathogens. In this respect, the marine environment may well be a source of new pharmaceuticals, including antibiotics (Austin, 2001). Consequently, this study focused on the recovery and characterization of compounds with effectiveness against.

** Corresponding Author*
E mail: selvendran008@gmail.com
Mobile: +919944932360

antibiotic-resistant strains of some common bacterial pathogens. The approach was based on the growth of marine bacteria in biofilms, insofar as earlier work (Austin and Billaud, 1990; Yan *et al*,.2003) demonstrated that the culture of marine bacteria at an interface could induce the production of antimicrobial compounds. Bacteriocins have extensively been studied with reference to microbiology, biochemistry and molecular biology, because of their applied importance in medicine, pharma-agro and food preservation industries. It has been evidenced that Bacteriocin Like Inhibitory Substances (BLIS) producing marine bacteria and their use in the control of undesirable bacterial infections with reference to their broad inhibitory spectrum against human, food spoilage and food borne pathogens. Certainly, these BLIS may prove useful alternatives to conventional chemotherapeutics and chemical additives (Zaid *et al*,.2004).

A deep sea pigmented *Brevibacterium* sp. has been shown to produce bacteriocin-like compound that could be used as probiotic in aquaculture feeds. The extracts of this bacteria have not only been suggested to be useful in prolonging shelf-life of dairy products but the culture *per se* could be used as probiotics and as feed additives in aquaculture (Loka Bharathi *et*

al., 2003). Antimicrobial substances are widespread produced among bacteria. Bacteriocins and bacteriocin-like inhibitory substances (BLIS) are antimicrobial peptides produced by a number of different bacteria that are often effective against closely related species (Tagg *et al.* 1976; Riley and Wertz 2002). Bacteriocin has received increasing attention due to their potential use as natural preservatives in food industry, as probiotic in the human health, and as therapeutic agents against pathogenic microorganisms (Riley and Wertz 2002).

Gradual increase in the antibiotic resistance among the bactericidal pathogens and intricacy in controlling them is of major concern (Ray 1992) so above the problems remedies to develop a novel antimicrobial compounds from novel organisms, Hence the present study was aimed the isolate antagonistic bacteria (antimicrobial peptide producing) from the marine micro algae of *Chlorella salina* .

MATERIALS AND METHODS

Isolation of marine microalgae associated bacteria

The marine microalgal sample (*Chlorella salina*) was collected from CMFRI at Tuticorin. The collected algal sample was aseptically transferred in a sterilized air tight container. The algae were subcultured in 500 ml conical flask containing Conway medium. The growth was estimated by cytometric counting. The micro algal associated bacteria were isolated from the growth phase of the algal culture which possessed the highest cell count. From this culture 1ml was taken and serially diluted by serial dilution technique. From the serially diluted samples (10^{-4} to 10^{-6}), 0.1 mL was aseptically transferred and spreader on Petri plates having Marine Zobell agar.

The plates were incubated at 37 ºC for 2- 3 days, then the colonies were picked up and transferred to Marine Zobell agar plate for the purpose of pure culture and the isolates were stored in Marine Zobell agar slant for further studies.

Identification of the isolates

The isolated bacterial strains were identified by the detailed procedure described by Holt *et al.* (1996) and are as follows:

Screening for bacteriocin producing isolates Double layer method (Tagg et al,.1976)

The isolated strains were centre streaked on Marine Zobell agar plates and incubated at 37°C for 48 – 72 hours. After incubation, the indicator organisms such as *Staphylococcus aureus, pseudomonas aurogenosa, Bacillus subtilis, Aeromonas hydrophila* and *Vibrio harveyi*, *Vibrio parahaemolyticus* were grown individually in nutrient broth for 16 – 18 hours at 37°C. The culture broths of indicator organisms, 5 ml were added individually into soft agar mixed uniformly and poured over into producer strain grown plates and the plates were incubated at 37^0C for 24 hours. After incubation, the plates were examined. The zone of clearance around the producer strain streaked area.

Agar well diffusion assay (Parente and Hill, 1992)

The bactericidal activity of the antimicrobial metabolites producing strains were again confirmed by agar well diffusion assay as described by Parente and Hill, 1992. Briefly, the isolated symbiotic bacterial strains were cultured aerobically in Marine Zobell broth for 48 – 72 hours at 37^0C. The culture broth (stationary phase culture of antimicrobial metabolites producing strains) was centrifuged at 8000 rpm for 10 min. Then the culture supernatant was evaluated for their antimicrobial activity by agar well diffusion method (Crupper *et al*,.1996) against *Staphylococcus aureus, Pseudomonas aurogenosa, Bacillus subtilis, Aeromonas hydrophila, Vibrio parahaemolyticus* and *Vibrio harveyi* as indicator organisms.

The diameter of zone of clearance was measured and expressed as, arbitrary units per ml (AU/ml) as per calculation given below

$$\text{AU/ml} = \frac{\text{Diameter of the zone of clearance (mm) x 1000}}{\text{Volume taken in well}}$$

Effect of different media for the production of bacteriogenic compounds

Different media like MRS broth, Zobell Marine broth, nutrient broth, TCBS broth, brain heart infusion broth were prepared for the production of bacteriogenic compound (Antimicrobial peptide compounds). The bacteriocin producing potent strain were grown in different production media. After that, centrifuge the culture broth and collect CFS for their antimicrobial activity. Their antagonistic activity was determined by agar well diffusion method.

Antimicrobial activity of secondary and primary metabolites of *Vibrio* sp. MMB2 Extraction of secondary metabolites

In 300 ml of MRS broth culture media was inoculated with 0.3 ml of *Vibrio* sp. MMB2 strain and incubated for 72 hours in rotary shaker incubator at (38.3±2ºC). After incubation period the culture broth was centrifuged at 8000 rpm for 10 minutes. Then the cell free supernatant was added with equal amount of ethyl acetate (1:1)for the extraction of secondary metabolites , and incubated for 24hours. After incubation two phases of solvent and lipid phases were formed, the lipid phases contains unwanted compound so that removed , by separating funnel. the solvent phases having a metabolites so that solvent phase was collected ,the extracted metabolites containing trace level of ethyl acetate evaporated by keeping at room temperature.

Assay of extra cellular metabolites activities

The Muller Hinton agar plates was prepared, and the indicator organisms were seeded. The extracted secondary metabolites was loaded on sterile paper disc(Hi-media)and then the disc were placed over the pathogen seeded Petri plates. Then the plates were kept in refrigerator at 4ºC for 30 minutes and incubated at 37ºC for 18-24 hours. After incubation the plates were observed for zone of inhibition.

Extraction and antibacterial assay of primary metabolites

The collected pellet was dried at 35ºC. After that 5ml of methanol and ethyl acetate were added individually and incubated for 48 hours. After that the mixture was grinded respective of solvent with the help of mortar and pestle and the mixture was centrifuged at 1000 rpm for 10 minutes and the supernatant was collected and the cell debris was removed . The collected supernatant was assayed against the aquatic pathogens by disc diffusion method.

Comparative analysis of commercial antibiotics and extra cellular metabolites against aquatic pathogens

Muller Hinton agar plates were prepared, and the indicator organisms were swabbed over the agar surface by using sterilized buds.

The sterilized paper discs were loaded with 75µl of partially purified antimicrobial compound and placed over the agar surface. Then the commercially available antibiotic discs (chloromphenicol, Streptomycin) placed over the agar surface, and incubate the plates at 37ºC for 18-24 hours After incubation zone of inhibition was observed and recorded.

Characterization of primary and secondary metabolites

Characterization of intracellular compounds by thin-layer Chromatography:

A clean glass dry plate was placed over flat surface. The slurry of the adsorbent was prepared using silica gel and distilled water in the ratio of 1:2. The prepared slurry was poured over the glass plate at a thickness of 0.15 nm uniformly from one end to the another using applicator. The plates were then allowed to dry at room temperature for 30 minutes. The plates were then heated in an oven at 80˚C for 1 hour to remove the moisture and adsorbent on the plate.

Sample application

2.5 cm was left from one end of the glass plate and at an equal distance from the edges. The sample was dropped at each point and allowed to dry.

The solvent used in this experiment were Methanol, Acetic acid, Benzene in the ratio of 3:1:1. Then plates were kept in a TLC chamber for the separation of compounds. The plates were removed from the chamber after the solvent reach the point. Plates were air dried and kept in a chamber with iodine crystals for the development of the spots. Rf value of the spots were calculated using the following standard formula.

$$\text{Rf value} = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

FTIR analysis

The bacterial primary metabolites was extracted using methanol and metabolites was analyzed qualitatively for the active compounds by Fourier Transform Infra Red (FTIR) method described by Kemp, 1991.

Partial purification of crude bacteriocin

The 0.5 ml of overnight culture (producer strain) of *Vibrio* sp. MMB2 was inoculated into 1000 ml of Marine Zobell broth and incubated at 37 ºC for 72 hours on rotary shaker at 90 rpm, the cells were removed by centrifugation. The cell free supernatant (bacteriocins) was precipitated by adding 80 ml of acetone per 100ml of supernatant, and kept for overnight incubation at 4 ºC to allow complete precipitation (Purification was carried out at 4 ºC). The resulting precipitate was collected by centrifugation (10,000 rpm, for 30 mins) and the pellet was air dried and re suspended in a minimal volume of 20 mM Tris HCl buffer, at pH 7.2. The precipitate was obtained at 80% saturation and was dialyzed against the same buffer using dialysis membrane.

Partial characterization of bacteriocin was studied through SDS – PAGE Electrophoresis (Laemmeli, 1970)

The molecular weight of the partially purified bacteriocin was determined by using 1.5% SDS-PAGE electrophoresis method (Laemmeli, 1970)

RESULTS

Isolation of microalgae associated bacteria

The microalgae sample was subjected for their associated bacteria by total plate count to enumerate the total bacterial load in term of Total Viable Count. Six different strains of associated bacteria were isolated. The results showed that the TVC in the dilution to $w10^{-3}$as 10^{-6}observed and recorded as 278.66 ± 13.52 CFU/ml in 10^{-3} 239.66 ± 17.55 CFU/ml in 10^{-4} 139.66 ± 37.50 CFU/ml in 10^{-5} and 45.33 ± 21.51 CFU/ml in 10^{-6} dilution respectively.

Identification of the isolates

The isolated bacterial strains were identified based on their biochemical characteristics and compared with Bergey's Manual of Determinative Bacteriology (Holt *et al*,.1998). The isolates were identified as *Pseudoaltermonas* sp, *Bacillus* sp , *Halomonas* sp, *Enterococcus* sp, *Vibrio* sp.MMB2, *Micrococcus* sp and *Streptococcus* sp. The results are shown in Table 1.

Screening of the isolates

Six different strains were isolated and subjected to screening for antagonistic compound production against five aquatic pathogens. From those isolates, two strains had broadest spectrum of antibacterial activity against all the aquatic pathogen. The screening was performed by double layer method. The zone of inhibition was measured. The screening results are shown in table 2.

Antimicrobial spectrum of bacteriocin

After screening, the bacteriocin producer strains were grown in marine Zobell's medium, the culture supernatants was evaluated for the antimicrobial activity. Among the 6 isolates, the potent bacteriogenic organisms were identified through agar well

diffusion method and the activity was recorded in table 3. The V*ibrio* sp., *Bacillus* sp., and *Pseudoaltermonas* sp. had broad spectrum of activity against all the pathogens. Among the isolates *Vibrio* sp. MMB2 had broadest spectrum of antimicrobial activity against all pathogens.

The activities are expressed in AU/mL (Arbitrary Unit) the results are summarized in table 3.

Antimicrobial activity of the potential strain by disc diffusion method.

The antimicrobial spectrum of the potential strain was studied. The maximum zone of inhibition was observed against *A.hydrophylla* with the range of 19.2 ± 0.16mm zone of inhibition.

The minimum biogenic activity was recorded against *P.auregenosa* it's range in zone of inhibition was 10.120 ± 0.25 mm, and *Pseudoaltermonas* sp also inhibit the pathogen range of broad spectrum activity table 4.

Effect of different culture media

The effect of various media were examined at different media such as MRS, Luria broth, Peptone enrichment media, Peptic soya broth, TCBS, Nutrient broth and Marine Zobell broth. The maximum bacteriocin activity of 14.8 ± 0.98 mm was recorded at MRS broth against *Vibrio harveyi.* The minimum bacteriocin activity of 4.3 ± 0.65 was recorded at nutrient broth against *V.harveyi* and no activity was found at peptone enrichment broth. The data were recorded in table 5.

Intracellular activity of *Vibrio* sp. MMB2strain

The extracted intracellular compound was assayed against aquatic pathogens. The maximum zone of inhibition of 20.2 ± 0.90 mm was recorded against *A.hydrophylla* and 20.3 ± 0.57 was recorded against *V.harveyi*. The minimum biogenic activity was recorded against *B.subtilis* and its range was 12.3 ± 0.98,where as *Pseudomonas* pathogens was resisted, and showing no activity in ethyl acetate fractions. The results are show in table 6.

Comparative analysis of commercial antibiotics and extra cellular metabolites

against aquatic pathogens

The activity of extra cellular metabolites were compared to commercial antibiotics. the commercial antibiotics were effectively controlled some aquatic pathogens and the *Vibrio harveyi* was resistant to Streptomycin and the *S.aureus* was also resistant to Chloromphenicol .Whereas the secondary metabolites of *Vibrio* sp.MMB2 strains effectively controlled all aquatic pathogens. Its biogenic activity is shown in Table 8. The maximum antibacterial activity was observed in *A.hydrophylla* and the zone of inhibition was 22.6 ± 0.96mm. The results are show in table 7.

Table. 1: Biochemical characteristics of symbiotic bacteria isolated from marine microalgae.

S. No.	Biochemical characteristics	*Pseudoaltermonas sp*	*Streptococcus sp*	*Bacillus sp*	*Micrococcus sp*	*Vibrio* sp.MMB2	*Enterococcus sp*
1	Gram's staining	–ve	+ve	+ve	+ve	–ve	+ve
2	Morphalogy Shape	Rods	Cocci	Rod	Cocci	Comma	Cocci
3	Motility test	++	++	++	++	++	++
5	Indole test	–	–	–	–	+	–
6	Methyl red test	–	–	+	–	+	–
7	Voges proskauer test	–	–	_	–	+	–
8	Citrate test	+	–	–	–	+	–
9	Urease test	–	–	–	+	–	–
10	Nitrate reduction test	+	–	+	±	_	_
11	Gelatinase test	+	–	+	+	+	+
12	Lipid	+	–	±	–	-	-
13	H_2S production	–	–	–	–	-	-
14	Lactose	–	Acid	–	–	+	–
15	Sucrose	–	Acid	Acid	–	–	+
18	Catalyse test	+	+	+	+	+	+
19	Oxidase test	+	–	–	-	+	+
20	Glucose	+	+	+	+	+	_
21	Maltose	+	_	+	+	_	_

Table. 2: Screening of the bacteriocin producing isolates by agar over lay method.

Isolates	*S.aureus*	*B.subtilis*	*V.harveyi*	*V.parahaemolyticus*	*A.hydrophylla*	*P.auregenosa*
***Vibrio* sp.MMB2**	+++	++	+++	+++	+++	++
***Pseudoaltermonas* sp.**	+++	++	+++	-	+++	++
***Halomonas* sp.**	-	-	++	-	++	-
***Streptococcus* sp.**	++	++	-	-	-	-
***Enterococcus* sp.**	++	++	-	-	-	++
***Micrococcus* sp.**	++	+++	-	-	-	-

Reference: +++ 10 to 15 mm zone of inhibition
++ <10 mm zone of inhibition
_ No activity

Table. 3: Antibacterial spectrum of the microalgal isolates against aquatic pathogens (zone of inhibition measured by AU/ml) .

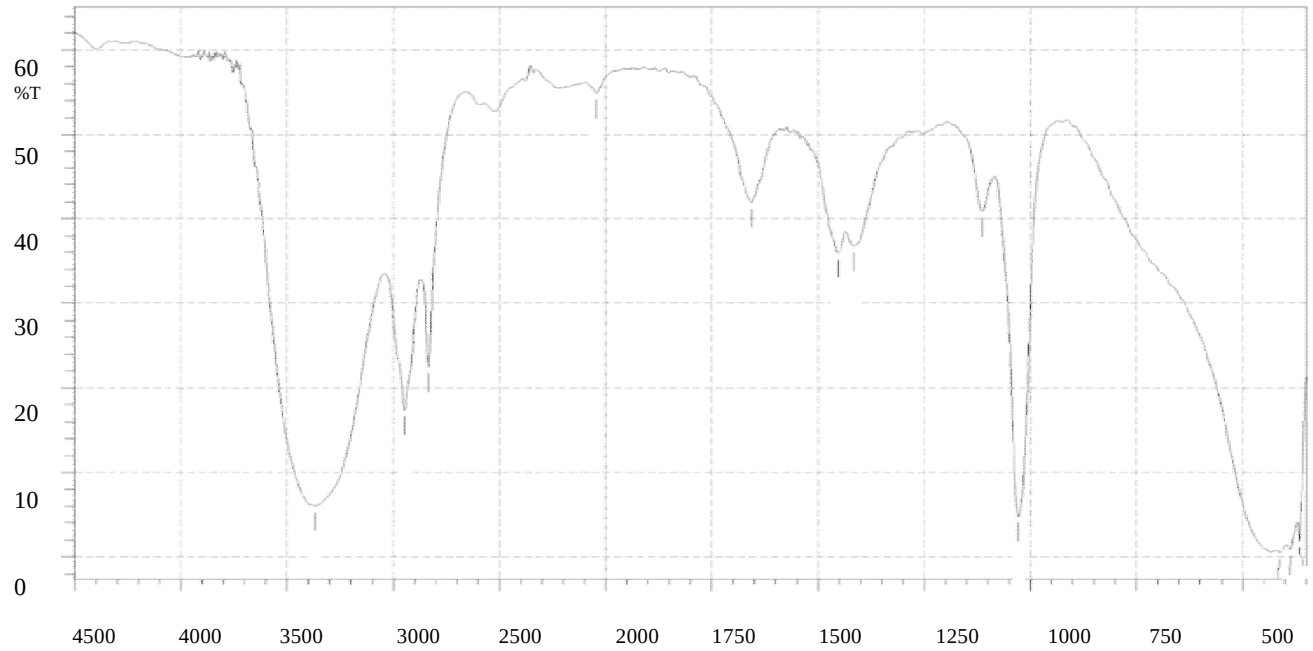
Isolates	*S. aureus*	*Pseudomonas aeuroginosa*	*V. parahaemolyticus*	*V. harveyi*	*A. hydrophila*
Pseudoaltermonas sp	1400	1200	600	600	1600
Streptococcus sp	800	-	-	-	-
Vibrio sp MMB2	1600	1000	1500	1600	1800
Halomonas sp	1000	800	-	-	1200
Bacillus sp	1600	1000	1400	1400	1600
Micrococcus sp	800	-	-	-	-
Pseudomonas sp	-	-	-	-	800
Enterococcus sp	1800	-	900	400	1000

Table. 4: Antimicrobial activity of the potent strains by Disc Diffusion Method.

Isolates	*S.aureus*	*B.subtilis*	*V.harveyi*	*V.parahaemolyticus*	*A.hydrophylla*	*P.auregenosa*
***Vibrio* sp.MMB2**	12.2 ± 1.31	10.3 ± 0.33	7.6 ± 0.57	12.6 ± 0.92	19.2 ± 0.16	10.12 ± 24
***Pseudoaltermonas* sp.**	13.4 ± 30	10.5 ± 29	13.4 ± 1.31	-	15.6 ± 0.16	13.5 ± 1.47

Table. 5: Effect of various media on Bacteriocin production and activity.

Media	Zone of inhibition				
	S. aureus	*Pseudomonas aeuroginosa*	*V. parahaemolyticus*	*V. harveyi*	*A. hydrophila*
Mz broth	14.67 ± 0.47	14.33 ± 3.09	17.67 ± 1.25	17.00 ± 0.82	16.00 ± 0.82
MRS	17.00 ± 0.82	13.00 ± 0.82	18.00 ± 0.82	16.67 ± 1.25	17.00 ± 0.82
LB	10.67± 1.25	7.33 ± 5.25	11.00 ± 0.82	13.00 ± 0.82	0
Peptone ± NaCl	0.00 ± 0.00	8.00 ± 5.72	11.00± 0.82	12.00 ± 0.82	12.67 ± 1.25
Peptic soya	10.33 ± 1.25	11.67 ± 2.05	13.00 ± 0.82	16.00 ± 0.82	15.00 ± 0.82
Brain heart infusion	9.33 ± 2.62	5.33 ± 3.77	10.00 ± 0.82	11.00 ± 0.82	9.00 ± 0.82
TCBS	11.33 ± 0.47	7.00 ± 4.97	12.00 ± 0.82	14.00 ± 0.82	12.67 ± 0.47
Nutrient broth	11.00 ± 0.82	11.00 ± 2.16	13.00 ± 0.82	12.33 ± 1.25	14.33 ± 1.25



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	366.45	1.905	5.244	370.31	349.09	22.372	1.826
2	387.67	0.825	1.571	397.31	372.24	43.616	3.321
3	412.74	0.465	0.516	422.38	399.24	49.426	3.237
4	1027.99	4.747	42.861	1083.92	937.34	82.221	35.927
5	1112.85	40.873	5.905	1195.78	1083.92	36.845	1.392
6	1415.65	36.716	3.637	1434.94	1326.93	40.932	1.701
7	1452.3	35.918	4.74	1514.02	1434.94	30.829	1.924
8	1656.74	41.923	1.856	1714.6	1649.02	22.264	0.589
9	2044.4	54.888	1.908	2094.55	1980.76	28.713	0.823
10	2835.16	22.449	13.795	2867.95	2661.58	77.408	5.571
11	2947.03	17.375	15.644	3041.53	2869.88	101.4	18.86
12	3369.41	6.035	34.292	3647.14	3043.46	503.947	258.921

Table. 6: Intracellular activity of *vibrio sp* MMB2 strain.

Extract	*S.aureus*	*B.subtilisi*	*V.harveyi*	*V.parahae molyticus*	*A.hydrophylla*	*P.auregenosa*
Methanol extracts	15.3 ± 0.89	12.0 ± 0.36	20.8 ± 0.20	16.8 ± 0.09	21.0 ± 0.16	-
Ethyl Acetate extracts	-	-	-	-	-	-

Table. 7: Comparative analysis of commercial antibiotics and extra cellular metabolites against aquatic pathogens.

Isolates	*S.aureus*	*B.subtilis*	*V.harveyi*	*V.parahaemolyticus*	*A.hydrophylla*	*P.auregenosa*
Streptomycin	-	21.6 ± 0.96	22.6 ± 0.42	20.4 ± 0.98	23.3 ± 0.60	20.6 ± 0.93
Chloromphenicol	21.6 ± 0.46	21.6 ± 0.46	-	-	23.6 ± 0.96	23.6 ± 0.96
Ethyl acetate extraction of intracellular metabolites of MMB2 strain	18.6 ± 0.96	16.2 ± 0.96	23.6 ± 0.63	21.6 ± 0.96	22.6 ± 0.96	16.6 ± 0.36

Characterization of intra cellular compounds by Thin-layer chromatography

The extracted metabolites was loaded on silica gel plates for the characterization of active principle compounds. Thin layer chromatogram showed the presence of single spot , one active fraction was obtained at the Rf value of 0.79 cm.

Fourier Transmission - Infra Red Analysis (FTIR) of intra cellular metabolites

The FT-IR spectral analysis, the intracellular metabolite extract of *Vibrio sp* MMB2 strain revealed that the spectral range of obtained functional group ranged was between 300 and 4000 cm^{-3}.

From the results, it was observed that. The peek signal recorded at 1027.99 and 1112.85 cm^{-} 1 may be due to C-F (Halogen molecules) and (C=N) carbon, nitrogen covalent bond. The sharp peek observed at 914.65 and 1452.3 $cm^{-}1$ may be due to nitro grouping (N^{-}). The peek at 1656.76 $cm^{-}1$ may be due to the presence of carbon, nitrogen (C=N) double bond. The vibration stretch recorded at 2835.16 and 2947.02 $cm^{-}1$ represents the possible presence of hybridized carbon atom ($Csp3^{-}H$). Finally a broad band at 3369.41 $cm^{-}1$ may be due to hydroxyl group (OH^{-}).

Molecular weight determination of antimicrobial peptides

The molecular weight of the peptide (antimicrobial peptides) was analyzed based on the Rf value of the markers and the Rf value of the peptide compounds in SDS – PAGE. After electrophoresis, it was recorded that the band 1 (AMP1) 16 kDa and the band 2 (AMP2) was having 32 kDa.

DISCUSSION

In the present study, the symbiotic bacteria were isolated from the micro algae *Chlorella salina*. The antimicrobial compound primary and secondary metabolites was isolated and characterized from the bacterial strains of *Vibrio* sp. MMB2. The symbiotic bacteria secretes antimicrobial metabolites that inhibit gram negative and gram positive pathogenic bacteria. There is growing concern over the development of antibiotic resistance in bacteria. For this reason, use of probiotic bacteria to prevent or reduce disease is receiving increasing attention as an alternative to antibiotics (Holzapfel and Schillinger, 2002; Irianto and Austin, 2002). Therefore, evaluation of probiotic bacteria capable of producing bacteriocin is an area of intensive research in several sectors of human nutrition, in animal husbandry and in fish farming (Hjelm, 2004).

In the present study the antimicrobial compounds were isolated from marine micro algal symbiotic bacterial strain . The marine bacterium *Vibrio* sp.MMB_2 strain had antimicrobial activity against shrimp pathogen. Which is isolated from marine microalgae. Similarly Longeon *et al*,. (2004) reported that the marine bacterium *Pseudoalteromonas sp* strain X153 had antimicrobial activity against human pathogens. Jorquera *et al.*, (1999) reports the marine *Vibrio* strain C33 having inhibitory effects on the growth of the pathogen *Vibrio anguilarum*-VAR was isolated from sea water. Recently Sethukkarasi *et al*.,(2012) reported the marine bacterium *Bacillus* spp. MB19 had antagonistic activity against human pathogens which was isolated from backwater of Arabian sea .

Mamdoh *et al*.,(2006) reported that the antimicrobial activity the bacteria *Bacillus licheniformis* isolated from sea weed. The strain produced a novel protein with antibacterial activity against Methicillin resistant *S.aureus* and Vancomycin resistant *Enterococci.*

The present study was concentrated to the comparison of the bacterial metabolite activity and commercial antibiotics like chloromphenicol and streptomycin. The 50 µl metabolites of *Vibrio* sp. MMB2 was produce higher zone of inhibition it was 22.6 ± 0.96 mm against *A.hydrophylla* and effectively control all pathogen. However, the *S.aureus* was resistant to chloromphenicol. Whereas all the pathogens were sensitive to bacterial metabolites. from our studies results revealed the bacterial metabolites effectively controlled maximum pathogen compare to commercial antibiotics. The similar study was reported by Rattanachuay *et al*,.2007). He reported that the bacterial metabolites activity was equal to commercial antibiotics. The zone produced by 70µl of W3 culture sulphamethoxale and 2µg oxolinic acid. However the inhibition zones produced by both tetracycline and norfloxine were bigger, but this was expected as these antibiotics are commonly used to treat shrimp diseases (Richards *et al*, 2005). However the results did show that the isolate W3 did inhibit the shrimp pathogenic bacterium, *V.harveyi*,

and it was therefore interesting to investigate its possible use as a biological control agent during shrimp cultivation. From our study it is inferred that the bacterial metabolites of *Vibrio* sp. MMB2 was inhibit shrimp pathogens. So this strain can been used as a biological control agent during shrimp cultivation. In the present study the metabolites were extracted from marine bacteria of vibrio spp MMB2 was subjected to FT-IR analysis the finding was The sharp peek observed at 914.65 and 1452.3 cm^{-1} may be due to nitro grouping and hydroxyl group .this may responsible for their antimicrobial activity. Similarly Sethukkarasi *et al.*,(2012) reported the marine bacterium bacillus sp MB19 metabolites subjected for FT-IR analysis its results the compound may be hydroxyl methyl group The earlier study of the molecular weight of bacteriocin was determined by SDS-PAGE analysis, Holck *et al,*.(1992) isolated a peptides from bacterial species the molecular weight of the Sakarin C2 was 5.5kDa, and Sakarin A was 4.3k Da. A similar finding reported by Chikhyouseuf *et al.*, (2004) also isolated the antimicrobial peptides in molecular range of 3kDa in size. In our study two peptides bands from the partially purified bacteriocin from *Vibrio* sp. MMB2 strain, which was 16kDa and 32kDa, in size.

CONCLUSIONS

This study has opened a new avenue to use bacteriocin like inhibitory substane instead of antibiotics .The antibiotics are being banned for its negative impact on animal such as biomagnifications and development of resistant strain in bacteria. More research in this area will help to identify the chemical constituent of this bacteriocin that lead to a discovery of novel antimicrobials which will not create problems in animals and bacteria.

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