ijlpr 2022; doi http://dx.doi.org/10.22376/ijpbs/lpr.2022.12.4.L14-22

International Journal of Life science and Pharma Research

ISSN 2250-0480

Research Article

Microbiology for health care

Antagonistic Potential Marine Bacteria Against Luminescent Vibriosis Pathogen Vibrio Harveyi Obtained from Shrimp Farm, Marakkanam, Tamil Nadu, India

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Abstract: Aquaculture farming practices in India and other countries are facing a lot of economic loss due to luminescent vibriosis. Ecological and cost-effective practices may have been warranted to combat this tricky disease. A total of 57 morphologically different marine bacterial isolates were isolated from samples collected at different aquaculture ponds in Marakkanam, Tamil Nadu, India and screened for their antibacterial potential against luminescent vibriosis causing bacteria *Vibrio harveyi*. All marine bacterial isolates were screened for antibacterial activity by cross streak method against two different isolates *V. harveyi* GNC01 and *V. harveyi* GNC03. Secondary screening of antagonistic isolates by dual culture plate method and agar well diffusion method leads to the identification of antagonistic isolates. The antagonistic isolates were characterized by molecular taxonomy (16S rRNA gene sequence) and phylogeny and identified as *Neobacillus niacini* (GNC04), *Bacillus halotolerans* (GNC06), *Bacillus amyloliquefaciens* (GNC16), *Lacticaseibacillus paracasei* (GNC23) and *Lactiplantibacillus plantarum* (GNC24). The minimum inhibitory concentration of all these selected bacterial crude secondary metabolites against both *Vibrio harveyi* isolates GNC01 and GNC03 ranged between 0.062 to 0.50 mg/mL. The GC-MS profiling of the ethyl acetate extract revealed the presence of antibacterial compounds such as Oxime-,methoxy-phenyl (26.63%), 2,3-Dihydroxybutane (9.52%), Phosphite, menthyldimethyl- (2.83%), Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (2.07%). The results of this study suggest that *Lactiplantibacillus plantarum* (GNC24) is a potential source for antagonistic secondary metabolites against luminescent vibriosis causing bacteria *Vibrio harveyi*. Further, biological control formulation with these isolates and their metabolites will be a reward for the integrated pest management in the aquaculture sector.

Keywords: Marine *Lactobacillus*, marine *Bacillus*, *Vibrio harveyi*, Luminescent vibriosis, GC-MS, 16S rRNA gene, Aquaculture

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Received On 1 February, 2022
Revised On 21 May, 2022
Accepted On 2 June, 2022
Published On 1 July, 2022

Funding This research did not receive any specific grant from any funding agencies in the public, commercial or not for profit sectors.

Citation M. Sakthivel,J. Jayanthi and M.G. Ragunathan , Antagonistic Potential Marine Bacteria Against Luminescent Vibriosis Pathogen Vibrio Harveyi Obtained from Shrimp Farm, Marakkanam, Tamil Nadu, India.(2022).Int. J. Life Sci. Pharma Res.12(4), L14-22 http://dx.doi.org/http://dx.doi.org/10.22376/ijpbs/lpr.2022.12.4.L14-22

1. INTRODUCTION

Marine shrimp farming became one of the most successful practices in the modern history of aquaculture considered to be the largest profitable aquaculture activity in the world. In farming practices, significant levels of chemical products are used, including antibiotics and other therapeutics, pesticides, conditioners, feed additives, etc. having a negative impact on environmental and human health.[1] Among the chemicals, excessive use of antibiotics poses a series of problems in residual content in commercialized aquatic products and the development of antibiotic-resistant in consumer as well as aquatic bacterial flora.[2] *Vibrio* spp. are the bacterial pathogens that are predominantly found in brackish aquaculture systems, affecting most of the aquaculture animals. In order to prevent aquaculture species, various antibiotic agents have been in use for years, through which several pathogenic *Vibrio* spp. have become drug-resistant and incurable, also the drug resistance gets transferred to pathogens of human concern.[3] *Vibrio harveyi* includes the species *V. carchariae* and *V. trachuri* as its least synonyms. The organism is well-known for the serious bacterial pathogen of marine invertebrates and fishes that also includes penaeid shrimp, in aquaculture.[4] More particularly shrimp, *V. harveyi* is the etiological agent of luminous vibriosis, where the affected animal glows in dark.[5] Although the intake seawater had *V. harveyi*, these strains were sensitive to antibiotics whereas many bacteria isolated were found that antibiotic-resistant *V. harveyi* had been colonizing larval tanks.[6] A study showed the virulence of the isolates from moribund larvae showed much higher IC_{50} values than isolates from natural seawater, thus indicating their higher virulence.[7] The indiscriminate usage of antibiotics in aquaculture farming practices leads to the rejection of produces in the export market due to the residual chemicals in the produce. In the concern with the economic losses to the farmer, an eco-friendly non-residual strategy can be adopted to mitigate this luminescent vibriosis in a sustainable manner. *Vibrio* spp. are very difficult to be controlled in an aquaculture pond ecosystem when the population of these bacteria is very high in nature.[8] Naturally antagonistic bacteria are capable of controlling them in a conditioned environment. Due to the usage of antibiotics, these beneficial antagonistic bacteria have also been killed from the natural pond ecosystem. Nevertheless, the lack of antibiotic resistance in antagonistic bacteria or the population of these bacteria is somehow minimized because of the various adverse conditions that arose from the pond practices. Hence, biological control of luminescent vibriosis is one of the promising tools to control the severity of the disease and thereby reduce economical losses.[9] Antagonistic bacteria from the marine environment to be used to combat luminescent vibriosis because marine bacterial species are rich in biologically active metabolites and structurally novel.[10] The marine bacteria that showed antagonistic activity mostly belong to the genus *Alteromonas, Bacillus, Micrococcus, Nocardiopsis, Pseudomonas,* and *Vibrio*.[11-14] However, marine bacteria obtained from the aquaculture ponds may be advantageous for the acclimatization and adaptation in the aquaculture farms as probiotics.[15] The augmentation of these probiotic strains in the farms during the infestation or before that may yield good disease control responses from the host animal. In the present study, we report the isolation of different marine bacteria associated with the aquaculture ponds near Marakkanam a well-known hotspot for shrimp aquaculture in the Southern part of India and checked their antagonistic potential against luminescent vibriosis causing pathogen *V. harveyi* for the ecologically adaptable biological control agent development.

2. MATERIALS AND METHODS

2.1. Collection of samples and marine bacterial isolation

Sediment, Infected prawn, and water samples were collected from different aquaculture farms in and around Marakkanam, Villupuram District, Tamil Nadu India (12.1899° N, 79.9249° E) in a sterile container. All the samples were brought to the laboratory within 3 h of collection. Zobell Marine Agar (ZMA) (Himedia Laboratories, Mumbai, India) was used for the isolation of heterotrophic bacteria from collected sediment, infected prawn, and water samples with appropriate serial dilution for the enumeration. In addition, Lactobacillus MRS agar was also prepared with little modification of which medium was prepared with 50% aged seawater for the isolation of marine *Lactobacillus* spp. The plates were kept for 24–72 h incubation at 37 °C for the colony appearance. The putative cultures were selected based on their distinct morphology and streaked in a ZMA and marine MRS agar. The marine bacteria from infected animals were tested for Gram's Staining and oxidase test. The Vibrio species isolated from infected animals were streaked on Thiosulphate Citrate Bile Salt (TCBS) agar (Himedia Laboratories, Mumbai, India) and selected only that showed bright luminescence streaked for further studies.

2.2. Antagonistic activity of the collected isolates

The marine bacteria isolated from ZMA and MRS agar were screened for their antagonistic activity using the cross streak[16] method against two distinct luminescent Vibrio isolates. Thus bioactive cultures were rescreened for their antimicrobial potential against two selected luminescent Vibrio isolates using ZMA agar double layer[12] method with little modification. Briefly, ZMA plates were spotted with 5 µl of 18 h grown cultures, each bacterial isolate selected from the cross streak method. Following incubation at 37 °C for 24 h, the well-developed colonies were killed using chloroform vapor for the period of 1 h. These plates were overlaid using 6 ml of Zobell marine broth supplemented with 0.9% agar, containing 100 µl of 1/10 dilution of 18 h cultures of two luminescent Vibrio isolates. After 24 h incubation, the antagonistic potential of these selected isolates was observed by measuring the zone of inhibition (ZOI), which appeared as a clearance zone around these colonies.

2.3. Anti-Vibrio activity of selected isolates secondary metabolites

The isolates which showed promising activity in the primary screening was inoculated into ZMB and marine MRS broth and incubated in a rotary shaker (150 rpm) at 30 ±0.5 °C for 48h. After incubation, the cell-free supernatant was obtained after centrifugation at 10000 rpm for 15 min at room temperature (Remi, Mumbai, India). The supernatant was successfully extracted twice with an equal volume of ethyl acetate, resulting in an organic layer was pooled together and concentrated using a rotary evaporator (Buchi, Switzerland). The resulting crude secondary metabolites were used for further anti-Vibrio activities. The antibacterial activity was determined using the well diffusion assay method of Holder and Boyce (1994). About 25 mL of sterilized

molten Mueller Hinton agar was prepared with aged seawater poured into a sterile Petri plate (Himedia, Mumbai, India). The plates were allowed to solidify, after which 18 h grown (OD adjusted to 0.6) 100 µl of two luminescent Vibrio isolates were transferred onto a plate and made culture lawn by using a sterile L-rod spreader. After five min setting of the aforesaid microbes, a sterile cork-borer was used to make 6 mm well on the agar. The test samples were dissolved in 5% dimethyl sulfoxide (DMSO) and loaded into wells with a definite concentration of 50 µg/mL. The 5% dimethyl sulfoxide-loaded well served as negative control and azithromycin (30 µg/mL) served as a positive control. The plates were incubated at 37 °C in a bacteriological incubator for 24 h. The antibacterial activity was determined by measuring the diameter of the zone of inhibition around the well using the antibiotic zone scale (Himedia, Mumbai, India). All the experiments were performed in triplicates.

2.4. Minimum inhibitory concentration (MIC)

MIC was determined by a standard macro-broth dilution test as recommended by the National Committee for Clinical Laboratory Standards, USA[17] against two different luminescent bacteria. A stock solution of 20 mg/mL of the test crude secondary metabolites was prepared, which was further diluted with 5% DMSO to give the required doubling dilution concentrations 2,000 to 1 µg/mL. The tubes were incubated at 35 °C for 24 h. The MIC value was defined as the lowest concentration of the crude metabolites showing no visible growth. Triplicate sets of tubes were maintained for each concentration of the test sample.

2.5 Gas Chromatography and Mass Spectrometry (GC-MS) profiling of selected isolate crude metabolites

The selected isolate based on their antibacterial potential was subjected to bioactive compounds identification using GC-MS profiling.[18] A Shimadzu GC-2010 Plus gas chromatograph was equipped with a straight deactivated 2 mm direct injector liner and a 15m Alltech EC-5 column (250µ I.D.,0.25µ film thickness). A split injection was used for sample introduction and the split ratio was set to 10:1. The oven temperature program was programmed to start at 35°C, hold for 2 minutes, then ramp at 20 °C per minute to 450 °C, and hold for 5 minutes. The helium carrier gas was set to 2 ml/minute flow rate (constant flow mode). A Direct connection with capillary column metal quadrupole mass filter prerod mass spectrometer operating in electron ionization (EI) mode with software GC-MS solution ver. 2.6 was used for all analyses. Low-resolution mass spectra were acquired at a resolving power of 1000 (20% height definition) and scanning from m/z 25 to m/z 1000 at 0.3 seconds per scan with a 0.2 second inter-scan delay. High-resolution mass spectra were acquired at a resolving power of 5000 (20% height definition) and scanning the magnet from m/z 65 to m/z 1000 at 1 second per scan. Identification of the components of the compound was matching their recorded spectra with the data bank mass spectra of NIST 11 GC Method / Retention Index MS Library provided by the instrument's software. GC/MS metabolomics Database was used for the similarity search with retention index.

2.6. Molecular identification of selected pathogens and antagonists

Genomic DNA was extracted from overnight grown cultures of the selected bacterial isolates using QIAGEN DNA isolation kit (Qiagen, Germantown, MD), suspended in 100 µ l of elution buffer (10 mM/L Tris-HCl, pH 8.5) and quantified by measuring OD at 260 nm. PCR amplification was performed using a 25 µ L reaction mixture consisting of 50 ng of template DNA, 12.5 µ L of 2X Taq DNA Polymerase Master Mix RED (Ampliqon, Odense, Denmark), 1 µ L of forward primer (10 µM), 1 µ L of reverse primer (10 µM) and makeup to 25 µ L with nuclease-free water. The sequences of 16S rRNA primers used were as follows: forward primer 27F- (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492R: (5′ - AAGGAGGTGATCCANCCRCA-3'). Amplification was carried out with an initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 45 sec, annealing at 56 °C for 45 sec, extension at 72 °C for 1 min and final extension at 72 °C for 5 min using a thermal cycler (iGPCR; Jayagen Biologics, Chennai, India). PCR products were analyzed on 1% agarose gel for 16S rRNA amplicons in a 1× TAE buffer at 100 V. The amplified product was sequenced using ABI PRISM 3730 Genetic Analyzer (Applied Biosystems). The sequences of these 16S rRNA genes were compared against the sequences available from NCBI using the BLASTN program[19] and RNA/ITS database was selected. Thus resulted from significantly aligned sequences were retrieved from the database and they were further aligned using CLUSTAL W software.[20] Distances were calculated according to Kimura's two-parameter correction.[21] Phylogenetic trees were constructed using the neighbor-joining method.[22] Bootstrap analysis was done based on 1000 replications.[23] The MEGA version X package[24]was used for all analyses.

3. RESULTS

3.1. Isolation and screening of antagonist marine bacteria against Vibrio sp.

Marine bacteria were isolated from different aquaculture pond samples. Different colony morphological featured marine bacterial colonies were observed in cultured plates at 10^{-5} and 10^{-6} dilutions. Marine bacterial isolates exhibited different colony morphology and characteristics (size, shape, and color) and the colonies were small to medium-sized. Both ZMA (30 isolates) and marine MRS agar (27 isolates) were found a good medium for the isolation of different marine bacteria yielded altogether 57 marine bacteria were recovered from sediments and water samples from aquaculture ponds and 24 different marine bacteria were isolated from infected animals using ZMA. Among the 24 isolates, 14 isolates were Gram's negative rod and only 5 of them were positive to oxidase were tested for luminescence and TCBS agar. Two distinct bacteria isolate GNC01 and GNC03 were selected owing to their best luminescence. The pathogenicity virulence analysis of these two bacteria was challenged against normal animals and found capable to infects them at assay conditions (Data not shown). All the isolates were obtained from sediment and water samples screened for antibacterial activity against the selected two *Vibrio* sp GNC01 and GNC03. About 44% of the isolates showed antibacterial activity in the primary cross streak method (Figure 1). Further, selected 24 isolates were screened using the dual-layer culture method and well diffusion method. Only selected isolates were given

alphanumeric isolate code starting from GNC01 to GNC27 (GNC indicates Guru Nanak College)

3.2. Antibacterial activity of crude secondary metabolites

The secondary screening of selected 24 isolates was checked its antimicrobial activity confirmation in both Dual culture method and crude secondary metabolites preparation showed only 5 isolates showed ZOI around the colony and 10 showed ZOI of above 9 mm around the well, respectively (Table 1; Figures 2A and 2B). The detailed antibacterial activity of crude secondary metabolites was given in table 1. Based on the antimicrobial activity spectrum of the isolates, only 5 different isolates showed both dual culture and secondary metabolites were selected for further culture characterization using molecular characterization and MIC determination.

3.3. Minimum inhibitory concentration

The MIC of the selected isolated crude secondary metabolites extracted using ethyl acetate was between 0.062 – 0.500 mg/mL. Table 2 shows the detailed MIC value against both the *Vibrio* sp. GNC01 and GNC03. It was confirmed that the isolated GNC24 crude metabolites had the lowest MIC value of 0.062 mg/mL against both *Vibrio* sp. The crude preparation of the extract from GNC24 was subjected to GC-MS profiling for compound identification.

3.4. Molecular identification of selected isolates

Selected five antagonistic bacterial isolates and two pathogenic *Vibrio* sp. were identified based on the 16S rRNA gene sequence analysis. PCR successfully amplified the 16S rRNA gene at the specified size of 1.5 kb amplicons were shown in Figure 3. The resulted PCR products were sequenced and sequence similarity was checked in the BLASTN program using the RNA/ITS database. The highest similarity bacterial species and its percentage along with NCBI-GenBank with accession numbers as given in Table 3. The phylogeny tree analysis based on the 16S rRNA gene sequence clearly revealed the species with closely related species from the NCBI database using the MEGA X program (Figure 4).

3.5. GC-MS profiling

The secondary metabolites profile of the selected isolate *L. plantarum* GNC24 extracted with ethyl acetate was analyzed using GC-MS and the total ion chromatogram is shown in Figure 5. The GC-MS spectrum of the crude extract showed the presence of 15 different molecules with different peak area percentages (Table 4). A few major peaks such as Oxime-, methoxy-phenyl (26.63%), 2,3-Dihydroxybutane (9.52%), Benzaldehyde, 2,4, dimethyl (5.38%), Phosphite, menthyldimethyl- (2.83%), 4-(3,5-Di-tert-butyl-4-hydroxyphenyl) butyl acrylate (2.52%) Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (2.07%) were seen in the metabolite profile of the ethyl acetate crude extract.

Table 1 Antibacterial activity of selected marine isolates against two different *Vibrio* sp. GNC01 and GNC03 using well diffusion assay

Isolate Code	Zone of inhibition (mm)	
	Vibrio sp. GNC01	*Vibrio* Sp. GNC03
GNC04	15.00 ± 1.00	13.00 ± 1.00
GNC05	8.33 ± 0.58	8.00 ± 1.00
GNC06	7.33 ± 0.58	7.33 ± 0.58
GNC07	7.67 ± 1.15	7.67 ± 1.15
GNC08	10.33 ± 0.58	9.67 ± 0.58
GNC09	8.33 ± 0.58	8.00 ± 1.00
GNC10	7.67 ± 1.15	7.67 ± 1.15
GNC11	9.33 ± 0.58	8.67 ± 0.58
GNC12	8.67 ± 1.15	8.33 ± 0.58
GNC13	8.33 ± 0.58	8.33 ± 0.58
GNC14	7.67 ± 0.58	7.67 ± 0.58
GNC15	10.33 ± 0.58	9.67 ± 0.58
GNC16	15.00 ± 1.00	13.67 ± 0.58
GNC17	11.00 ± 1.00	9.33 ± 0.58
GNC18	8.67 ± 0.58	8.67 ± 1.00
GNC19	8.33 ± 0.58	8.33 ± 0.58
GNC20	8.00 ± 1.00	7.67 ± 1.15
GNC21	8.33 ± 0.58	8.33 ± 0.58
GNC22	8.33 ± 0.58	8.33 ± 0.58
GNC23	14.00 ± 1.00	12.67 ± 0.58
GNC24	16.33 ± 0.58	14.33 ± 0.58
GNC25	8.00 ± 0.00	8.00 ± 0.00
GNC26	9.00 ± 1.00	8.67 ± 0.58
GNC27	8.67 ± 0.58	8.00 ± 1.00

Values are means of triplicate ±Standard deviations

Table 2 Determination of minimum inhibitory concentration of crude metabolites from selected 5 marine isolates against two different *Vibrio* sp. GNC01 and GNC03.

Isolate Code	Minimum Inhibitory Concentration (mg/mL)	
	Vibrio sp. GNC01	*Vibrio* Sp. GNC03
GNC04	0.125	0.250
GNC06	0.125	0.250
GNC16	0.250	0.500
GNC23	0.250	0.250
GNC24	0.062	0.062

Table 3 Selected isolates GenBank accession numbers of their 16S rRNA gene and its highest similarity percentage bacteria

Isolate Code	GenBank Accession No.	Database highest similarity %	GenBank Accession No.	Bacterial name as per NCBI GenBank
GNC01	OL824994	99.86%	NR_043165	*Vibrio harveyi*
GNC03	OL824995	99.79%	NR_119054	*Vibrio harveyi*
GNC04	OL824996	99.86%	NR_024695	*Neobacillus niacini*
GNC06	OL824997	99.72%	NR_115063	*Bacillus halotolerans*
GNC16	OL824998	99.72%	NR_117946	*Bacillus amyloliquefaciens*
GNC23	OL824999	99.51%	NR_113337	*Lacticaseibacillus paracasei*
GNC24	OL825000	99.73%	NR_115605	*Lactiplantibacillus plantarum*

Table 4 List of compounds from crude secondary metabolites of selected isolate *L. Plantarum* GNC24 using GC-MS profiling analysis

Peak#	R.Time	Name	Molecular Formula	MW	Area%
1	3.737	Oxime-, methoxy-phenyl	C8H9NO2	151.16	26.63
2	3.914	Butanoic acid	C4H8O2	88.11	6.24
3	5.339	2,3-Dihydroxybutane	C4H10O2	90.12	9.52
4	5.854	4-Ethylbenzoic acid	C9H10O2	150.17	5.21
5	6.806	2,5,8-Trioxatricyclo[4.2.1.03,7]nonan-9-ol	C6H8O4	144.12	2.95
6	7.552	Benzaldehyde, 2,4, dimethyl	C9H10O	134.17	5.38
7	8.287	Pyrazine	C4H4N2	80.09	3.69
8	8.932	Phenol, 2-methoxy-3-(2-propenyl)-	C10H12O2	164.20	5.52
9	10.612	1-Dodecanol	C12H26O	186.34	3.43
10	10.886	Phosphite, menthyldimethyl-	C12H25O3P	248.3	2.83
11	11.159	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	C11H18N2O2	210.27	2.07
12	11.504	4-(3,5-Di-tert-butyl-4-hydroxyphenyl)butyl acrylate	C21H32O3	332.47	2.52
13	11.864	Hexadecane, 2,6,11,15-tetramethyl-	C20H42	282.54	3.10
14	12.229	Diethyl benzene-1,2-dicarboxylate	C12H14O4	222.24	14.23
15	25.692	Bis(2-ethylhexyl) phthalate	C24H38O4	390.6	6.68

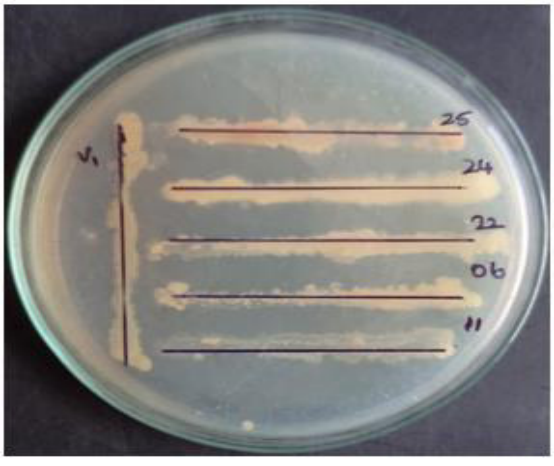

Fig 1 Antagonistic bacterial screening of marine bacteria against luminescent Vibrio sp. on cross streak method

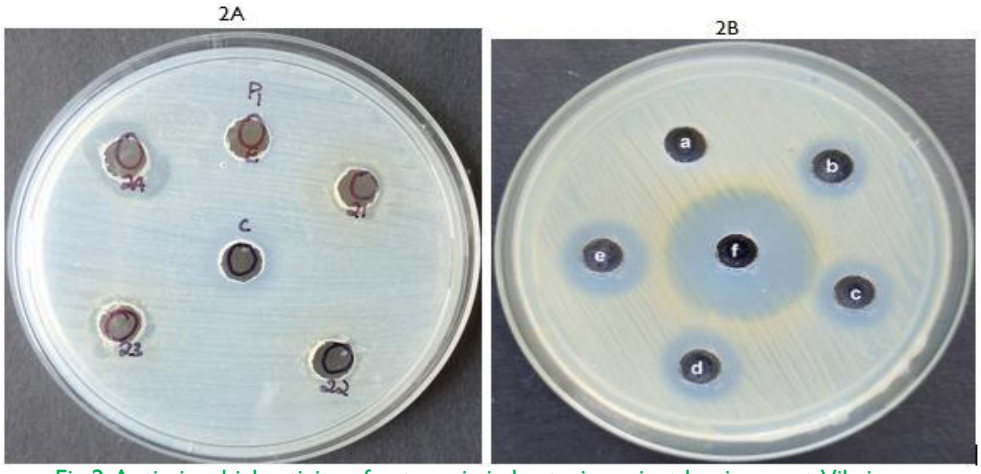

Fig 2 Antimicrobial activity of antagonistic bacteria against luminescent Vibrio sp.

A. Zone of inhibition showed by the marine bacteria on on well diffusion method

B. Zone of inhibition showed GNC24 crude metabolites added well on well diffusion assay

a:100 µl (5% DMSO); b:25 µg/mL; c:50 µg/mL; d:75 µg/mL; e:100 µg/mL; f: Azithromycin (30 µg/mL)

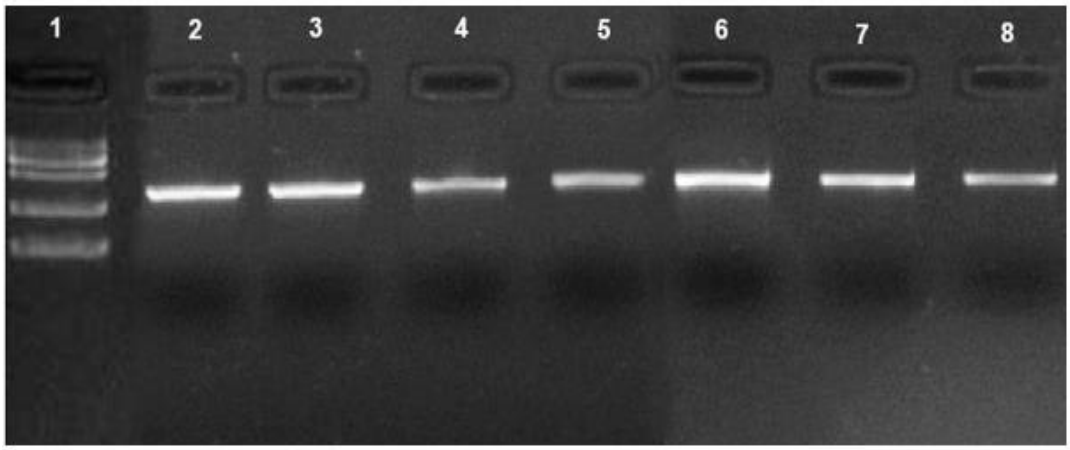

Fig 3 16S rRNA gene amplicons of 5 antagonist isolates and 2 pathogenic isolates showing with the specified 1.5 kb size resolved in 1% agarose gel electrophoresis. Lane 1 –marker (100 bp ladder), Lane 2 - GNC01, Lane 3 - GNC03, Lane 4 – GNC04, Lane 5 – GNC06, Lane 6 – GNC16, Lane 7 – GNC23, Lane 1 GNC24

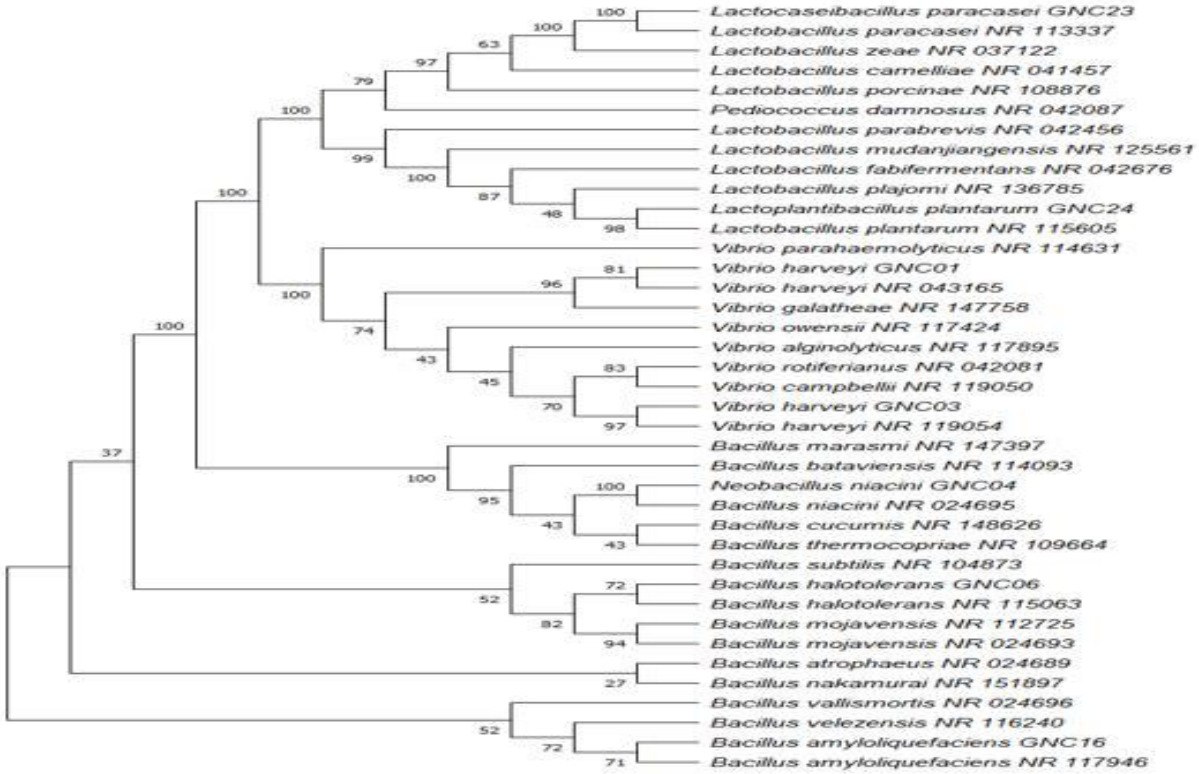

Fig. 4 Phylogenetic tree analysis of selected isolates based on 16S rRNA gene sequence as determined by neighbor-joining method

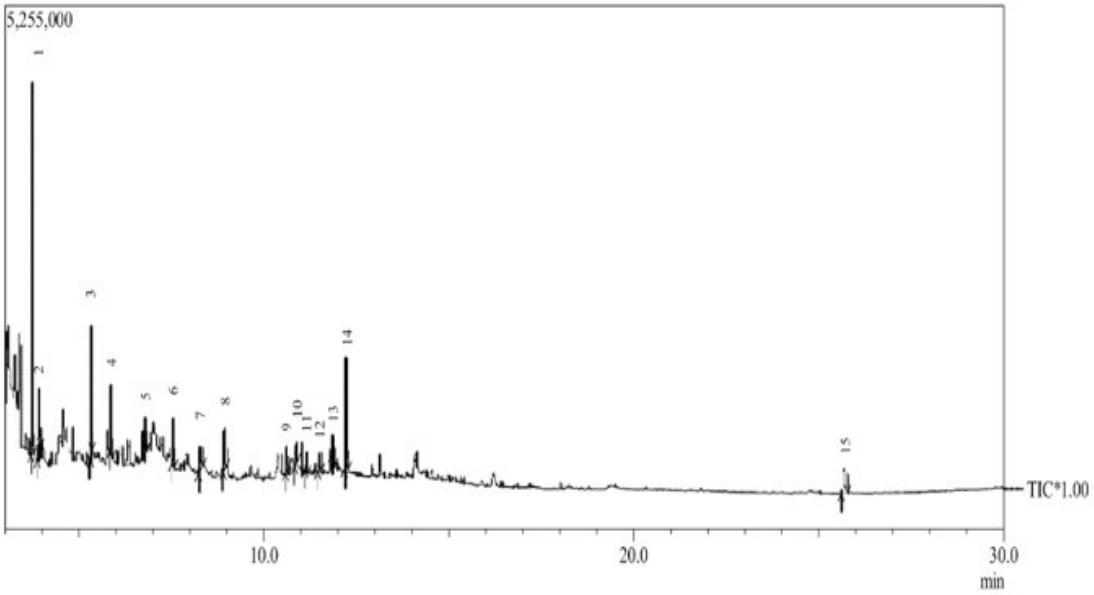

Fig 5 GC-MS total ion chromatogram of crude secondary metabolites obtained after ethyl acetate extraction from marine antagonistic isolate *L. Plantarum* GNC24

Numbers 1-15 refers the peak number and name of the peak is given in Table 4.

4. DISCUSSION

The marine ecosystem is one of the most complex environments on the earth having diversified living beings across the ocean realm. The various factors that influence this ecological niche resulted in multiple bioactive substances from marine microorganisms, especially bacteria. Isolation of marine-derived bacteria from various marine environments yielded many different kinds of bacterial populations with many biological properties25-26. In the present study, about 57 different marine bacterial isolates were isolated from sediment and water samples collected across aquaculture ponds. The isolates were tested for their antagonistic potential against two different luminescent vibriosis-causing pathogens V. harveyi GNC01 and V. harveyi GNC01. In the primary screening about 44% of the isolates showed antibacterial activity in the cross streak method, whilst in the secondary screening only 20% of the isolates showed a very good antibacterial activity against both the pathogens. Similarly, antagonistic potential against V. harveyi LS21 and LS22 strains were screened out of 173 isolates; only 36 showed antagonism12. In another study, there were 166 strains that were isolated from seawater sediments, etc. out of 166 strains only 10% of them showed antimicrobial against shrimp bacterial pathogens, including V. harveyi.6 Only 18% of the antagonistic actinomycetes were recorded out of 72 isolates were isolated.27 Recently, a study showed about 33% of the isolates were found to be antagonistic against newly isolated V. harveyi.28 However, out of 68 bacterial isolates, 15 isolates (22%) markedly exhibited antibacterial activity against three Vibrio species in various spectra.29 The antimicrobial activity of the marine antagonistic bacteria was exhibited remarkably due to its versatile nature of bioactive producing capacity. The marine environment harnesses these bacterial metabolites in a unique way with their terrestrial counterparts. The antibacterial compounds from marine bacteria showed antibacterial properties very effectively at lower concentrations even against drug-resistant pathogens.30 The MIC of the selected isolated crude secondary metabolites extracted using ethyl acetate in this study ranged between 0.062 – 0.500 mg/mL. The selected marine bacteria were more active towards both Vibrio harveri GNC01 and GNC03, indicating the potential application of these pathogens. In a study, a Streptomyces derived ethyl acetate extract was showed MIC against fish and shellfish pathogens at the range of 0.030 to 0.125 mg/mL.27 In another study, a purified anti-vibrio substance exhibited MIC against V. neptunius CZ-D1, V. sinaloensis QBSM3, V. campbellii AF5, V. harveyi LM2, V. fischeri CGMCC 1.1613, V. anguillarum XP and V. parahaemolyticus CGMCC 1.2164 was ranged between 0.016 to 0.064 mg/mL.30 Molecular identification of the antagonistic and pathogenic bacteria was determined using 16S rRNA gene sequencing technique revealing the species-level identification at high accuracy.3,14 In this study the best-selected isolates that showed antimicrobial potential in both dual culture and crude metabolites screening were subjected to molecular identification using the 16S rRNA gene sequencing method. The 16S rRNA sequences clearly revealed that the selected isolates belonged to the taxa Bacillus spp. (GNC04, GNC06 and GNC16) and Lactobacillus spp. (GNC23 and GNC24). Several studies indicated the presence of various antagonistic bacteria from marine especially from aquaculture ponds were belonging to the taxa such as Alteromonas, Bacillus, Lactobacillus, Micrococcus, Nocardiopsis, Pseudoalteromonas, Pseudomonas, and Streptomyces.3,6,11,14, 27-29 The metabolomics analysis based on the GC-MS profiling from the Lactobacillus spp. could be a useful tool to investigate and characterize the probiotic lactic acid bacteria.31 In this study a GC-MS profile of the secondary metabolites of the selected isolate L. plantarum GNC24 extracted with ethyl acetate was showed many antimicrobial, antioxidant, and stabilizer compounds. Among them, Oxime-, methoxy-phenyl, 2,3-Dihydroxybutane, Benzaldehyde, 2,4, dimethyl, Phosphite, menthyldimethyl-, 4-(3,5-Di-tert-butyl-4-hydroxyphenyl) butyl acrylate, and Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- were present in the ethyl acetate crude extract. Similarly, many compounds especially antimicrobial compounds were found in the crude metabolites of Lactobacillus and other marine bacteria.27,29 In conclusion, these antagonistic bacterial metabolites are needs to be further investigated as biological control agents especially controlling luminescent vibriosis caused by Vibrio harveyi.

5. CONCLUSION

The results from this study indicated that the anti-vibrio marine bacteria isolated from aquaculture ponds produced antibacterial molecules responsible for inhibiting the growth of luminescent vibriosis-causing pathogens. Further purification and spectral characterization of these anti-vibrio compounds and formulation of the molecule along with culture could be a biological lethal weapon to combat this tricky pathogen under aquaculture farms. However, the augmentation of native bacterial isolates is an added advantage to the pond ecosystem.

6. ACKNOWLEDGEMENTS

The authors sincerely thank the management of Guru Nanak College for providing the necessary facilities to carry out this research work.

7. AUTHORS CONTRIBUTION STATEMENT

Murugan Sakthivel carried out the experiment and wrote the manuscript with support from Jayaprakash Jayanthi. Manickavalli Gurunathan Ragunathan conceived the original idea and Jayaprakash Jayanthi designed the experiment and also supervised the project.

8. CONFLICT OF INTEREST

Conflict of interest declared none.

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