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Purification, characterization and production optimization of a vibriocin produced by mangrove associated *Vibrio parahaemolyticus*

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PEER REVIEW

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Comments

This is a valuable research in which authors have improved the vibriocin production with the molecular weight of approximate 18 kDa from *V. parahaemolyticus* and demostrated its antibacterial activity.
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ABSTRACT

Objective: To identify a potential bacterium which produces antimicrobial peptide (vibriocin), and its purification, characterization and production optimization. The bacteria subjected in the study were isolated from a highly competitive ecological niche of mangrove ecosystem.
Methods: The bacterium was characterized by phenotype besides 16S rRNA gene sequence analysis. The antibacterial activity was recognised by using agar well diffusion method. The vibriocin was purified using ammonium sulphate precipitation, butanol extraction, gel filtration chromatography, ion-exchange chromatography and subsequently, by HPLC. Molecular weight of the substance identified in SDS-PAGE. Production optimization performed according to Taguchi's mathematical model using 6 different nutritional parameters as variables.
Results: The objective bacterium was identified as *Vibrio parahaemolyticus*. The vibriocin showed 18 KDa of molecular mass with mono peptide in nature and highest activity against pathogenic *Vibrio harveyi*. The peptide act stable in a wide range of pH, temperature, UV radiation, solvents and chemicals utilized. An overall ~20% of vibriocin production was improved, and was noticed that NaCl and agitation speed played a vital role in secretion of vibriocin.
Conclusion: The vibriocin identified here would be an effective alternative for chemically synthesized drugs for the management of *Vibrio* infections in mariculture industry.

KEYWORDS

Vibriocin, *Vibrio parahaemolyticus*, *Vibrio harveyi*, Mangrove rhizosphere, Antimicrobial peptide

1. Introduction

Antimicrobial peptides have been isolated from many living forms that include microbes, plants, invertebrates and vertebrates. Bacteria can produce different kinds of antimicrobial peptides, especially ribosomally synthesized antimicrobial peptides like bacteriocins, and non-ribosomally synthesized peptides like gramicidins, polymixins and bacitracins[1,2]. Bacteriocin produced by lactic acid bacteria is the extensively studied ones with an established classification system[3].

Bacteriocins produced by the genus *Vibrio* are called vibriocins.They were reported from species such as *Vibrio cholerae* (*V. cholerae*), *Vibrio harveyi* (*V. harveyi*), *Vibrio mediterranei* (*V. mediterranei*), *Vibrio vulnificus* (*V. vulnificus*) and some *Vibrio* sp., possess good antibacterial activity against their own closely related *Vibrio* members such as *V. cholerae*, *V. harveyi*, *V. mediterranei*, *Vibrio parahaemolyticus* (*V. parahaemolyticus*) , *V. vulnificus* and *Vibrio fischeri*[3-9]. Though there are reports available on the bacteriocins produced by the genus *Vibrio*, such reports are only at latent stage of basic research. Being capable of producing bacteriocins against

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their own closely related species and having diverse ecological adaptations, the genus *Vibrio* could be an interesting candidate to search for bacteriocins, for the effective disease management in mariculture industry.

V. parahaemolyticus is a widely reported bacterium (both pathogenic and non-pathogenic) associated with the flora and fauna of the marine environments[10]. It is found even in rhizosphere of the mangrove plants as a heterotrophic diazotroph[11]. This organism is a major cause of acute gastroenteritis in humans through the contaminated sea foods, particularly in the areas of the world where sea food consumption is high[12]. Still the organism is widely studied for its harmfulness, and there are not too many reports available on its beneficial aspects.

Mangrove has a unique microbial diversity[13]. It is a highly challenging environment as it is nutritionally rich and dynamic due to its frequent change of physical and chemical conditions. Adaptable studies of these microbial communities would be helpful in understanding the nature of the habitat[14]. Only few works have been carried out on the rhizosphere microbiota of the mangrove plants[15]. Hence, the investigation of this microbiota will lead to the identification of novel, biotechnologically potent microorganisms having unambiguous variety of applications. Pichavaram mangrove is one of the important mangrove forests in India with *Avicennia marina* (*A. marina*) as the dominant plant flora[16].

In the present study, an effort has been made to purify, characterize and production optimization of vibriocin produced by the bacterium isolated from the rhizosphere of *A. marina* present in the Pichavaram mangrove ecosystem. This compound showed good antibacterial activity against the mariculture pathogenic members of *Vibrio*.

2. Materials and methods

2.1. Selection of potential isolate

A total of 66 isolates used in this study were isolated from the rhizosphere soil of Pichavaram (latitude 11°23′-11°30′N and longitude 79°45′-79°50′E) mangrove plant *Avicennia marina* and all the isolates were characterized using phenotype and 16S rRNA gene sequence analysis. All the isolates were cultivated in Zobell′s Marine broth for 24 h at 37 °C in an orbital shaker at 150 r/min. Culture broths were centrifuged to get cell free supernatant (CFS), which were sterilized through 0.22 μm filter paper (Millipore, Bedford, MA, USA) and subjected to agar well diffusion assay to check the cultures for the production of antimicrobial metabolites. Eighteen hour cultures of pathogenic type strains obtained from Microbial Culture Collection Centre (MTCC), India and environmental isolates (Table 1) were diluted with pre-sterilized normal saline and the turbidity of the culture was adjusted to 0.5 McFarland turbidity. Lawns of cultures were prepared by sterilized cotton swabs over the surface of the Brain Heart Infusion (BHI) agar. Wells were made in the inoculated plates using sterile metal borer. A total of 20 μL CFS were added in the wells, and the plates were incubated at 37 °C for 24 h. Then, the zone of inhibition was observed. The vibriocin titre and activity unit (AU) was determined with indicator type strain [*V. harveyi* (MTCC 3438)] in each experiment using the method described previously by Motta and Brandelli[17].

Table 1

Antibacterial activity of the culture free supernatant against type strains and environmental isolates of the genus *Vibrio*.

Strains		Zone of inhibition (mm)
MTCC strain	*V. alcaligenes* (MTCC 4 442)	6.1
	V. alginolyticus (MTCC 4 439)	–
	V. fluvialis (MTCC 4 432)	7.3
	V. harveyi (MTCC 3 438)	15.6
	V. mimicus (MTCC 4 434)	7.8
	V. parahaemolyticus (MTCC 451)	–
	V. vulnificus (MTCC 1 146)	8.0
Isolates isolated in present study	*V. natriegens* BPRIST034	7.2
	V. alginolyticus BPRIST033	–
	V. fischeri BPRIST032	7.1
	V. harveyi BPRIST031	15.8
	V. proteolyticus BPRIST030	7.1
	V. parahaemolyticus BPRIST002	–
	V. parahaemolyticus BPRIST056	–

Results were expressed in diameter of the zone of inhibition. –: No antibacterial activity.

2.2. Characterization of potential isolate

Phenotypic characterizations were performed as reported by Alsina and Blanch[18] and Farmer and Janda[19]. Further, phenotypic characters of the isolate BPRIST035 were compared with the type strain of *V. parahaemolyticus* (MTCC 451). For 16S rRNA gene sequencing and phylogenetic analysis, the genomic DNA of the potential isolate was extracted using the method described by Chen and Kuo[20]. PCR amplification and sequencing were performed using universal primers pA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and pH (5′-AAG GAG GTG ATC CAG CCG CA-3′). The partial 16S rRNA gene sequence of the isolate was compared with 16S rRNA gene sequences available with the BLASTN search of the NCBI, GenBank database (http://www.ncbi.nlm.nih.gov). Phylogenetic dendrogram was constructed by the neighbour-joining method using MEGA 4.1 (Molecular Evolutionary Genetic Analysis). The partial 16S rRNA gene sequence of the isolate BPRIST035 was submitted in GenBank data base and obtained the accession number (JF431424).

2.3. Purification of the vibriocin

The chosen potential strain was subjected to mass cultivation in Zobell′s Marine broth for 24 h at 37 °C in an orbital shaker at 150 r/min. The supernatant was separated by centrifugation and sterilized through 0.22 μm filter paper. The culture filtrate was precipitated with ammonium sulphate at 60% (w/v) saturation. The precipitate was recovered by centrifugation at 10000 *g* for 30 min at 4 °C and dissolved in phosphate buffer saline (PBS) and then extracted thrice with 1-butanol and evaporated with reduced pressure[21]. The resultant was suspended in 10 mL of 10 mmol/L phosphate buffer (pH 7.5) and designated as crude vibriocin.

The crude vibriocin was subjected to gel permeation chromatography using Sephadex G-100 column. Before elution, the column was pre-equilibrated with 20 mmol/L sodium phosphate pH 7.5, and then the sample was eluted with PBS and fractionated by 2 mL. Eluted fractions were tested for

the antibacterial activity against the indicator strain. The active fractions were pooled and subjected to ion-exchange chromatography using diethyl-aminoethanol cellulose anion exchange column, pre-equilibrated with 20 mmol/L sodium phosphate pH 7.5, then the sample was eluted with the gradient of 0 to 1.5 mol/L NaCl prepared in PBS and fractionated by 6 mL. Each fraction was subjected to antibacterial activity against the indicator strain. The fraction showing highest antibacterial activity was dialysed against double distilled water, subsequently freeze-dried and used for further analysis. Final purification of the sample was achieved with HPLC (Shimadzu, Japan) using a C18 column, UV detector absorbance at 280 nm and eluted with acetonitrile and PBS mixture (1:10), collected fractions tested for antibacterial activity against indicator strain by using above mentioned method. At each purification step, CFS, precipitation, extraction, gel filtration and ion exchange chromatography, protein concentration was evaluated (A280 nm).

2.4. Molecular weight determination of the vibriocin

To determine the molecular weight of purified vibriocin, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), (14% Polyacrylamide gel) was performed as described previously[22]. Medium range molecular weight markers (Sigma, USA) were used. Purity of the vibriocin was compared with crude and partially purified vibriocin. Band appeared on the gel was separated and washed with sterile double distilled water in order to remove the SDS, then placed in sterile Petri plate and flooded the BHI agar with the indicator strain *V. harveyi* over the gel, then the plate was incubated for 24 h at 37 °C and the zone of inhibition was observed. Remaining gel portion was subjected to Coomassie brilliant blue staining to visualise the markers and the target protein, the observed results were used for molecular weight identification of the vibriocin.

2.5. Effect of physiochemical factors on the vibriocin activity

Vibriocin was treated with various enzymes (proteinase, pepsin, trypsin, pronase E, lysosyme, lipase and α-amylase) as described by Prasad *et al*[7]. To analyze thermal stability, Purified vibriocin was treated to 50, 75, 100 and 125 °C for 15 min respectively. Sterile Petri plates containing 5 mL aliquots of vibriocin were exposed to UV irradiation for 15 to 90 min by placing at 30 cm from the UV bulb[23]. The activity of vibriocin at pH range (2-12) was tested at ambient temperature. The detergents, Tween 80 and Triton-×-100 were treated by preparing the final concentration at 2% (v/v) and for SDS at 2% (w/v), for metalloprotease inhibitor EDTA, 1% (w/v) was prepared with vibriocin. All these preparations were incubated for 15 min at ambient temperature. The effect of solvents (acetic acid, acetone, benzene, chloroform, dichloromethane, dichloromethane, dmso, ethanol, ethyl acetate, hexane, isopropanol and methanol) were tested by equal proportion of vibriocin and the solvent was mixed well and incubated for 1 h at ambient temperature. After the physiochemical treatments, the treated vibriocin was subjected to antibacterial activity assay against indicator strain.

2.6. Optimization of the vibriocin production

In order to improve vibriocin production by potential bacterium, seven critical parameters, *viz.*, NaCl concentration, temperature, agitation speed, pH, inoculum concentration, carbon and nitrogen source were optimized. A 32 runs orthogonal array (L32-OA) design was used to investigate the effect of selected parameters on vibriocin production. In Taguchi's method, quality is measured by the deviation of a characteristic from its target value and a loss function [L(y)]. The loss function was calculated by the Eq(1).

Eq (1): $L(y)=k\times(y-m)^2$

Where, k=The proportionality constant, m=Target value and y =Experimental value obtained for each trial.

In the present study, the goal is to achieve the higher vibriocin production. Such case "bigger-is-better" was chosen. In case of the "bigger-is-better" quality characteristics, the loss function can be written as

Eq (2): $L(y)=k\times(1/y^2)$

In such case the expected loss function can be represented by

Eq (3): $E[(Ly)]=k\times E\ (1/y^2)$

Where $E(1/y^2)$ can be estimated from a sample of n as

Eq (4): $\sum_i^n =1[1/y_i^2]/n$

To explore the obtained results, analysis of variance (ANOVA) test was performed. Further conformational experiments were performed in order to validate the optimum conditions. Qulitek-4® software, Nutek Inc. was used for designing and analysis of the experiments.

3. Results

3.1. Antibacterial activity

Among the 66 isolates screened, the isolate PBRIST035 showed good antibacterial activity against various *Vibrio* type strains, as well as environmental isolates except *V. parahaemolyticus* and *V. alginolyticus*. The maximum inhibitory activity was observed against *V. harveyi* and minimum inhibitory activity against type strain of *V. alcaligenes* (MTCC 4442). Table 1 shows the antibacterial activity of isolate PBRIST035 against the various *Vibrio* type and wild strains.

3.2. Characterization of potential isolate

16S rRNA gene sequence and phylogenetic analysis of the potential isolate showed 99.6% similarity with *V. parahaemolyticus* ATCC 17802T and 99.5% similarity with *V. alginolyticus* ATCC 17749T and the cluster formed by PRIST035 was evidenced by high bootstrap values (Figure 1). However, based on the results of phenotypic characterization the potential organism was identified as *V. parahaemolyticus* and the comparison study with the type strain, *V. parahaemolyticus* (MTCC 451) also confirmed the same (data not given), since *V. parahaemolyticus* cannot ferment sucrose and has a negative Voges Proskauer reaction, this was distinguished from *V. alginolyticus*[19,24]. As like other *Vibrio* members, it can withstand a wide range of pH and NaCl concentration. Tolerance up to 12 % NaCl concentration had confirmed its halotolerance.

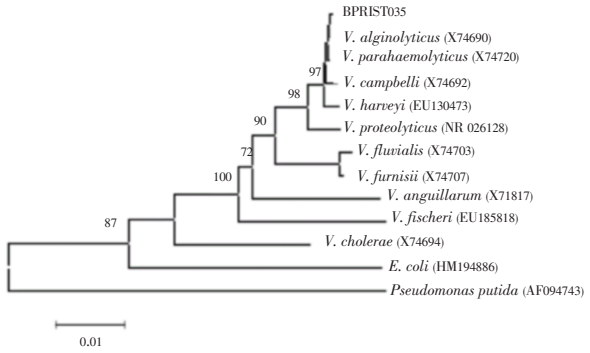

Figure 1. Neighbour-joining phylogenetic tree is based on 16S rRNA gene sequence of the potential isolate and their closest phylogenetic neighbours. Bootstrap values are indicated at nodes. Scale bar represents observed number of changes per nucleotide position.

3.3. Purification and molecular weight identification

Purification steps and recovery values of the peptide are given in Table 2. Where the total activity represents the activity given by the purified product and the specific activity represents activity with the amount of protein observed in each purification step. Extraction with 1-butanol showed the huge increase in the purification with 120.5 fold of increment. Gel permeation chromatography also showed 173.8 fold increases in the purification from the CFS. From the ion-exchange chromatography, the target protein was eluted at 1.1% of mol/L NaCl concentration and the final purification fold increased up to 260.4. The partially purified vibriocin showed 74.66% of purity, and pure vibriocin was eluted at the retention time of 2.92 min (Figure 2) in HPLC. The purified vibriocin showed 18 kDa of molecular weight in the SDS-PAGE (Figure 3).

Table 2
Purification and activity of the vibriocin.

Purification step	Total protein (mg)	Total activity (AU)	Specific activity (AU/mg)	Purification fold	Yield (%)
CFS	311.60	99 800	320	1.0	100.0
Precipitation	8.20	62 210	7 587	23.7	62.3
Extraction	1.30	50 120	38 554	120.5	50.2
Gel filtration	0.32	17 800	55 625	173.8	17.8
Ion exchange	0.18	15 000	83 333	260.4	15.0

All the values are the mean of three independent experiments.

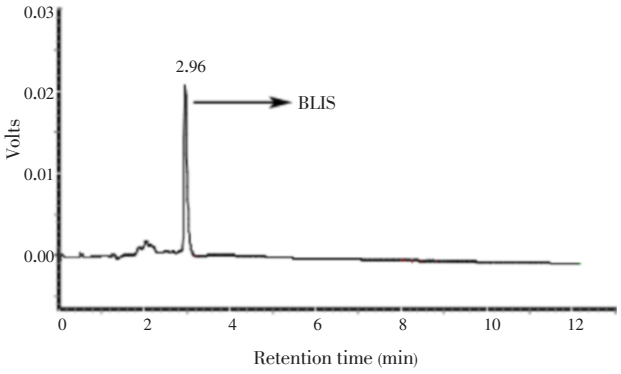

Figure 2. HPLC chromatogram showing a major peak corresponding to vibriocin.

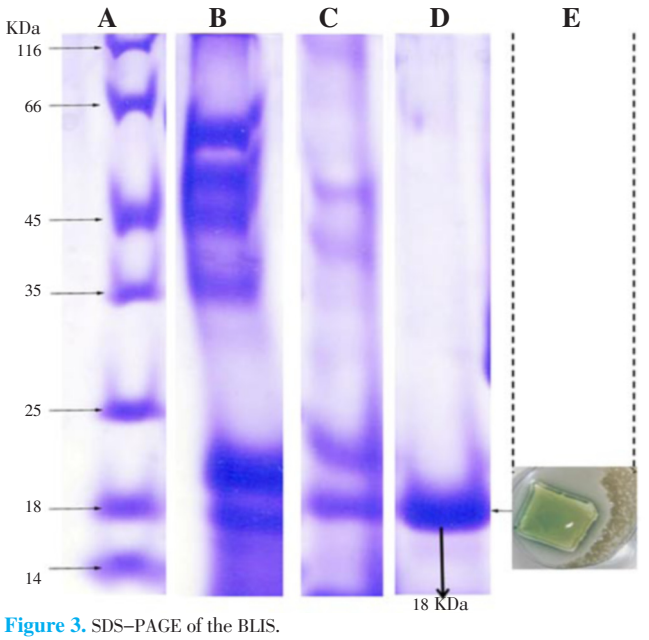

Figure 3. SDS-PAGE of the BLIS.
A: Molecular mass markers; B: Crude vibriocin; C: Partially purified vibriocin; D: Purified vibriocin; E: Vibriocin activity on the overlaid gel with a lawn of *V. harveyi* cells.

3.4. Effect of physiochemical parameters on the vibriocin activity

The vibriocin produced by *V. parahaemolyticus* PBRIST035 was stable in non proteolytic enzyme and unstable with proteolytic enzymes used. The vibriocin was stable in all the pH tested (pH 2-12) and resists the heat up to 75 °C for 15 min and resist the UV radiation up to 75 min exposure. Presence of residual activity were observed in detergents and polar solvents were used. The effect of physiochemical parameters on the vibriocin is summarised in Table 3.

Table 3
Effect of physiochemical parameters on the vibriocin activity.

Treatment		Residual activity
Enzymes	Proteinase	-
	Pepsin	-
	Trypsin	-
	Pronase E	-
	Lysosyme	+
	Lipase	+
	α-amylase	+
Chemicals	Tween 80	+
	Triton×-100	+
	SDS	+
	EDTA	+
Heat	Up to 75 °C	+
pH	2-12	+
UV radiation	Up to 75 min	+
Solvents	Acetic acid	+
	Acetone	-
	Benzene	-
	Chloroform	-
	Dichloromethane	-
	DMSO	-
	Ethanol	+
	Ethyl acetate	-
	Hexane	-
	Isopropanol	+
	Methanol	+

+: Presence of residual activity, -: No residual activity.

3.5. Production optimization

According to the experimental plan (L-32) the experiments were conducted, and the data revealed significant variation in the vibriocin production (Table 4). Minimum and maximum vibriocin production values were observed to be 490 and 2351 U/mL under the selected fermentation conditions. The average effect of the factors at the assigned levels on the vibriocin production by *V. parahaemolyticus* PBRIST035 is shown in Figure 4. At the individual level stage, the highest effect on vibriocin production was noticed with NaCl concentration at 4th level (1605.00) followed by inoculum concentration at 2nd

Table 4

L-32 orthogonal design along with the obtained vibriocin activity produced by *V. parahaemolyticus* BPRIST035.

NaCl concentration (% w/v)	Temperature (°C)	Agitation speed (r/min)	pH	Inoculum size (mL)	Glucose concentration (% w/v)	Peptone concentration (% w/v)	Vibriocin activity (U/mL)
1.0 (L1)	30 (L1)	80 (L1)	6.5 (L1)	1.0 (L1)	0.5 (L1)	0.5 (L1)	1438
1.0 (L1)	35 (L2)	100 (L2)	7.0 (L2)	2.0 (L2)	1.5 (L2)	1.5 (L2)	764
1.0 (L1)	37 (L3)	120 (L3)	7.5 (L3)	2.5 (L3)	2.0 (L3)	2.0 (L3)	1567
1.0 (L1)	39 (L4)	140 (L4)	8.0 (L4)	3.0 (L4)	3.0 (L4)	3.0 (L4)	876
2.0 (L2)	30 (L1)	80 (L1)	7.0 (L2)	2.0 (L2)	2.0 (L3)	2.0 (L3)	1695
2.0 (L2)	35 (L2)	100 (L2)	6.5 (L1)	1.0 (L1)	3.0 (L4)	3.0 (L4)	490
2.0 (L2)	37 (L3)	120 (L3)	8.0 (L4)	3.0 (L4)	0.5 (L1)	0.5 (L1)	616
2.0 (L2)	39 (L4)	140 (L4)	7.5 (L3)	2.5 (L3)	1.5 (L2)	1.5 (L2)	749
2.5 (L3)	30 (L1)	100 (L2)	7.5 (L3)	3.0 (L4)	0.5 (L1)	0.5 (L1)	578
2.5 (L3)	35 (L2)	80 (L1)	8.0 (L4)	2.5 (L3)	1.5 (L2)	1.5 (L2)	789
2.5 (L3)	37 (L3)	140 (L4)	6.5 (L1)	2.0 (L2)	2.0 (L3)	2.0 (L3)	980
2.5 (L3)	39 (L4)	120 (L3)	7.0 (L2)	1.0 (L1)	3.0 (L4)	3.0 (L4)	1300
3.0 (L4)	30 (L1)	100 (L2)	8.0 (L4)	2.5 (L3)	2.0 (L3)	2.0 (L3)	1745
3.0 (L4)	35 (L2)	80 (L1)	7.5 (L3)	3.0 (L4)	3.0 (L4)	3.0 (L4)	2100
3.0 (L4)	37 (L3)	140 (L4)	7.0 (L2)	1.0 (L1)	0.5 (L1)	0.5 (L1)	1784
3.0 (L4)	39 (L4)	120 (L3)	6.5 (L1)	2.0 (L2)	1.5 (L2)	1.5 (L2)	1657
1.0 (L1)	30 (L1)	140 (L4)	6.5 (L1)	3.0 (L4)	1.5 (L2)	1.5 (L2)	798
1.0 (L1)	35 (L2)	120 (L3)	7.0 (L2)	2.5 (L3)	0.5 (L1)	0.5 (L1)	1859
1.0 (L1)	37 (L3)	100 (L2)	7.5 (L3)	2.0 (L2)	3.0 (L4)	3.0 (L4)	2351
1.0 (L1)	39 (L4)	80 (L1)	8.0 (L4)	1.0 (L1)	2.0 (L3)	2.0 (L3)	721
2.0 (L2)	30 (L1)	140 (L4)	7.0 (L2)	2.5 (L3)	3.0 (L4)	3.0 (L4)	1300
2.0 (L2)	35 (L2)	120 (L3)	6.5 (L1)	3.0 (L4)	2.0 (L3)	2.0 (L3)	721
2.0 (L2)	37 (L3)	100 (L2)	8.0 (L4)	1.0 (L1)	1.5 (L2)	1.5 (L2)	1322
2.0 (L2)	39 (L4)	80 (L1)	7.5 (L3)	2.0 (L2)	0.5 (L1)	0.5 (L1)	2086
2.5 (L3)	30 (L1)	120 (L3)	7.5 (L3)	1.0 (L1)	1.5 (L2)	1.5 (L2)	984
2.5 (L3)	35 (L2)	140 (L4)	8.0 (L4)	2.0 (L2)	0.5 (L1)	0.5 (L1)	1864
2.5 (L3)	37 (L3)	80 (L1)	6.5 (L1)	2.5 (L3)	3.0 (L4)	3.0 (L4)	674
2.5 (L3)	39 (L4)	100 (L2)	7.0 (L2)	3.0 (L4)	2.0 (L3)	2.0 (L3)	823
3.0 (L4)	30 (L1)	120 (L3)	8.0 (L4)	2.0 (L2)	3.0 (L4)	3.0 (L4)	1429
3.0 (L4)	35 (L2)	140 (L4)	7.5 (L3)	1.0 (L1)	2.0 (L3)	2.0 (L3)	892
3.0 (L4)	37 (L3)	80 (L1)	7.0 (L2)	3.0 (L4)	1.5 (L2)	1.5 (L2)	2252
3.0 (L4)	39 (L4)	100 (L2)	6.5 (L1)	2.5 (L3)	0.5 (L1)	0.5 (L1)	981

Table 5

Interaction influence, ANOVA and optimized conditions for selected factors for vibriocin production by *V. parahaemolyticus* BPRIST035.

		NaCl concentration (% w/v)	Temperature (°C)	Agitation speed (r/min)	pH	Inoculum size (% v/v)	Glucose concentration (% w/v)	Peptone concentration (% w/v)	Others/error	Total
Interactions	NaCl concentration	0								
	Temperature	38.42	0							
	Agitation speed	48.50	17.05	0						
	pH	24.72	11.55	31.65	0					
	Inoculum Size	16.79	11.49	5.29	22.64	0				
	Glucose concentration	23.51	44.42	24.03	10.08	11.12	0			
	Peptone concentration	33.84	10.72	16.68	38.16	36.72	24.05	0		
ANOVA	DoF	3	3	3	3	3	3	3	10	31
	SS	1658928	413167.3	569640.3	1297073	1345134	364780.1	1366364	0.001	7015087
	Variance	552976	137722.4	189880.1	432357.8	448378	121593.4	455454.7	0.001	2338362
	F-ratio	2.368977	0.590010	0.813456	1.852242	1.920874	0.520912	1.951191		
	Percent contribution	23.648	5.889	8.12	18.4849	19.174	5.199	19.477	0.004	100
Optimum Conditions	Level	4	3	1	2	2	1	3		
	Level description	3	37	80	7	2	0.5	2		
	Contribution	349.218	187.468	213.593	216.343	347.468	144.968	197.593		1656.651
	Total contribution from all factors			1656.651						
	Current grand average of performance			1255.781						
	Expected results at optimum condition			2912.432						

DoF: Degree of freedom; SS: sum of squires.

level (1 603.25), pH at 2nd level (1 472.13), agitation speed at 1st level(1 469.38), peptone at 3rd level (1 453.38) temperature at 3rd level (1 443.25) and glucose at 1st level(1 400.75). The selected variables preferences have followed the order:

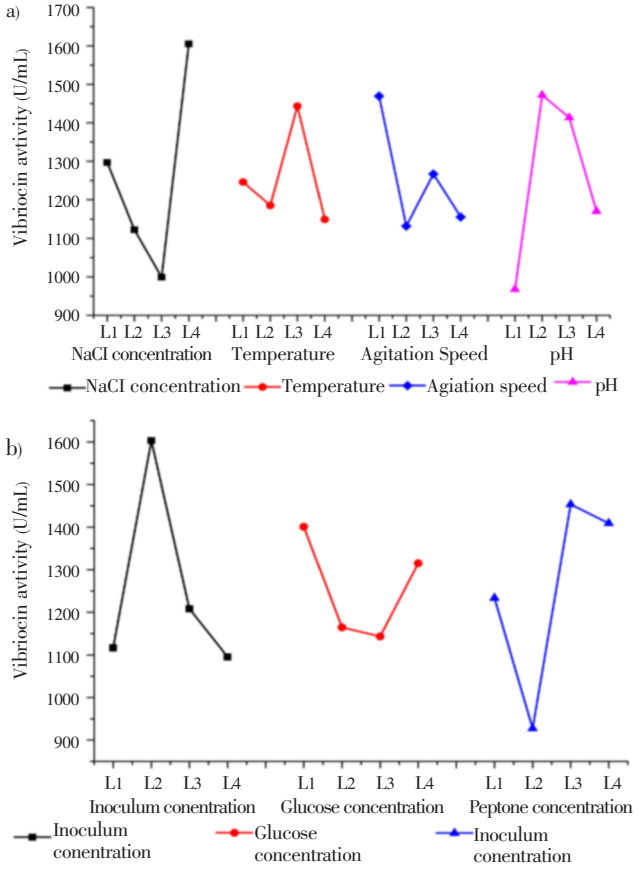

Figure 4. Main effects of the selected parameters at different levels on vibriocin production by *V. parahaemolyticus* BPRIST035.
a: NaCl, temperature, agitation speed and pH. b: Inoculum, glucose and peptone.

NaCl>Inoculum>pH>Agitation>Peptone>Temperature>Glucose

The levels of factors to produce the best results showed ANOVA with the percentage of contribution of each factor with interactions shown in Table 5. It was observed that incubation temperature has the major contribution (23.64%) on overall fermentation process for effective vibriocin production. After temperature, peptone concentration (19.477%) and inoculum concentration has the highest effect followed by agitation speed and NaCl concentration on vibriocin production by *V. parahaemolyticus* PBRIST035. Glucose concentration and pH has the least significance (5.199 & 5.899%) on antibiotic production. The observed very low error contribution (0.004%) indicates the accuracy of the experimental data.

The Taguchi DOE design, used in the present study for the maximizing the production of vibriocin from *V. parahaemolyticus* PBRIST035, revealed the effect of interaction of varying factors. Higher levels of vibriocin production were achieved with the software generated culture conditions: 3% NaCl, at the temperature of 37 °C, at the agitation speed of 80 r/min, pH 7, 2% inoculum concentration, 0.5% glucose concentration and 2% peptone concentration. The expected vibriocin yield at these optimum conditions is 2 912 U/mL with a total contribution from all the factors being 1 656 U/mL with grand average performance of 1 255 U/mL. Performing the validation experiments at the predicted conditions 2 187 U/mL activity was achieved. The obtained antibacterial activity is nearer to the predicted value. The validation experimental data at predicted optimum conditions revealed that 466 U/mL activity was improved.

The expected improvement on overall vibriocin production by this bacterial strain under submerged fermentation could be viewed from Figure 5. This figure explains the variation reduction achieved with optimized conditions. The improved S/N ratio at optimum condition depicts the reduction in the standard deviation before optimization.

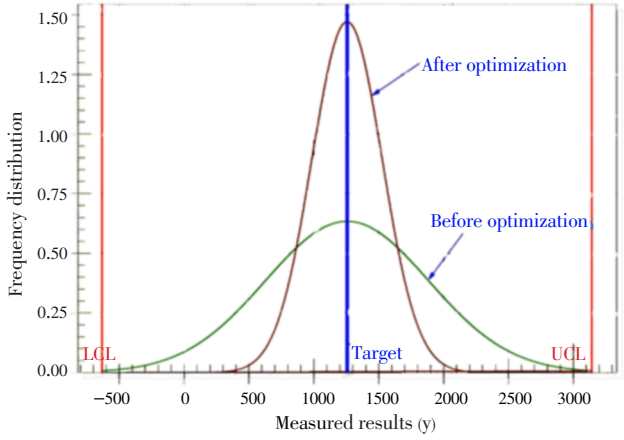

Figure 5. Variation reduction plot on vibriocin production by *V. parahaemolyticus* BPRIST035.

4. Discussion

This study revealed the identification of novel vibriocin producing *V. parahaemolyticus* BPRIST035, which is highly active against *V. harveyi*, a major mariculture pathogen, and the vibriocin was purified, characterized and increased production through optimizing the growth factors. Though *V. parahaemolyticus* is reported to be a diazotrophic rhizobacteria of mangrove plant[11], the ability of producing vibriocin in the competitive ecological niche is the first report ever made. CFS of the potential isolate did not exhibit intra species activity, an attribute unique to this strain. Prasad *et al.*[7] reported that the bacteriocin produced by *V. harveyi* VIB 571 shown activity against type strains, as well as environmental isolates of *V. harveyi*. Carraturo *et al.*[4] had reported that bacteriocin produced by *V. mediterranei*

1 showed good activity against environmental isolates of the same species. Same kind of results was obtained by Shehane and Sizemore[8] for *V. vulnificus* IW1, BC1 and BC2. Furthermore, the CFS did not exhibit any activity against *V. alginolyticus*, which may be due to its taxonomically close relatedness with *V. parahaemolyticus* species. Moreover, *V. alginolyticus* was previously known as biotype 2 of *V. parahaemolyticus*[24]. Indeed, the 16S rRNA gene sequence analysis of the potential strain also revealed the same. CFS showed excellent activity against *V. harveyi* MTCC 3438, which was twofold higher than activity observed with other type strains used. Same kind of activity was observed in the isolates of *Vibrio* from the same ecological niche. With these, it is clearly evident that the vibriocin produced by the *V. parahaemolyticus* BPRIST035 has good specificity against the *V. harveyi*. Hence, the strain, *V. harveyi* (MTCC 3438) was used as an indicator strain throughout the study. This feature contributes significant scope for the use of BLIS for the disease management against pathogenic *V. harveyi* in mariculture industries.

Vibriocin was released in the culture media in a relatively low concentration. In order to maintain the conditions of isolation procedure and strain's halotolerant nature, Zobell marine broth was used as the production medium. Though the quantity of vibriocin produced is low, it is good enough for the preliminary characterization. Accordingly, the vibriocin was concentrated by ammonium sulphate precipitation. Extractions with butanol showed a huge increase in the purification which is comparable to the Bizani *et al*.[21] results on the purification of cerein 8A and the same type of results were observed for gel permeation chromatography and ion-exchange chromatography. The purified vibriocin showed unique molecular mass (18 kDa) compared to the notable bacteriocins reported from the members of *Vibrio*, like 63-65 kDa Bacteriocin Like inhibitory substance (BLIS) produced by *V. mediterranei*[4], 24 kDa harveyicin produced by *V. harveyi* [5,6], 32 kDa BLIS from *V. harveyi*[7] and 1.35 kDa, 7.5 kDa and 9 kDa multiple peptides produced by *V. vulnificus*[8] and also well classified bacteriocins reported in other organisms till date[25].

As like other widely reported bacteriocin, the vibriocin produced by *V. parahaemolyticus* PBRIST035 was sensitive to all proteolytic enzymes used, but on the converse, it is resistant to other used enzymes, which indicated that the vibriocin is only proteinaceous in nature and not having any carbohydrate or lipid moiety in its structure. This is in contrast to the BLIS produced by *V. harveyi*, which exhibits lipid moiety in its structure[7] and BLIS was produced by *V. vulnificus* IW1 and BC1 which has carbohydrate moiety in it structure[8]. In the same way, BLIS produced by *V. mediterranei* 1 is resistant to trypsin[4]. Stability of the vibriocin in pH, heat and UV radiation, are useful for the application of the vibriocin as medicated feeds in mariculture industries. Because generally these characters will be considered for the storage and application of a feed, this is one of the easiest and economically important ways of drug delivery in the aquaculture[26]. The ability of the vibriocin existence in the marine medium and not losing its activity indicates that it can survives very well in the marine water conditions thus the application of the compound in mariculture industries holds lot of scopes. As like other bacteriocins, it is resistant to detergents like Tween 80, Triton ×-100 and SDS as also to EDTA. Vibriocin resistance to the SDS once again confirmed its mono peptide nature. In the same way, it is resistant to all polar solvents and sensitive to all non polar solvents used. On the whole, the vibriocin produced by *V. parahaemolyticus* is showed uniqueness and compatibility with other reported bacteriocins or BLIS in its character as well as in the structure[25].

Production of vibriocin by *V. parahaemolyticus* might be the sole defensive mechanism to survive in the particular ecological niche, when comparing with other *Vibrio* members isolated from the same niche, especially with *V. harveyi*, because population wise *V. harveyi* was the dominant member of the genus in the ecological niche by showing highest bacterial count. *V. parahaemolyticus* BPRIST035 did not possess any other rhizobacteria characters like N2 fixation, phosphate solubilization and/or IAA production to survive in the rhizosphere. Moreover, it is the only strain showed the production of vibriocin among the three strains of *V. parahaemolyticus* (BPRIST002, BPRIST034 and BPRIST056) isolated from different sites of the mangrove forest (results not given).

Characterization and optimization of each factor which influence the growth and yield of desired product are essential requirements before the selected strain is used for further investigation. In preliminary studies (data not given), it revealed that the secretion of vibriocin depends on the physiological and nutritional parameters as well as biochemical nature of the bacterium. Hence, further investigations were made to understand various requirements for vibriocin production by *V. parahaemolyticus* BPRIST035. A Taguchi experimental design was employed to investigate the interaction between the selected parameters and optimize the conditions for enhanced antibiotic production. The observed variation in vibriocin yield at various experiment certainly indicates the imperative role of optimization of all fermentation related factors in achieving the best possible yields.

It was observed that increased NaCl concentration played a vital role on vibriocin production. It is obvious that it could improve the bacterial growth as the bacterium

from marine origin and slightly halophilic in nature. There are several reports that showed increased production of bacteriocin produced by members of *Lactobacillus* by increased NaCl concentration[27,28]. It can also be discussed as the condition may mimic the environmental condition where the bacterium tend to produce vibriocin as a defensive product and the adopted expression mechanism of the bacterium to the particular salt concentration.

Next to NaCl inoculum size played an important role in the increased production of vibriocin. High inoculums will attain the late log phase quickly with increased biomass at this phase and may lead higher production of vibriocin. In the present study, it was observed that the vibriocin activity was first detected in late log phase and it confirms that the vibriocin is a secondary metabolite. Other parameters were not showed much effect in the increased vibriocin production.

In Taguchi method, the interaction between the two factors could be representing as interaction severity index (SI) which indicates the percent interaction between two factors. From Table 5, it was observed that interaction between NaCl concentration and agitation speed is the highest (48.5%) when compared with other factor interactions. Least SI was observed with the interaction between the inoculum concentration and agitation speed (5.29%). At the individual level, inoculum concentration has shown higher effect and the agitation speed showed moderate effect on vibriocin production whereas interaction of these two has least SI.

Though temperature and glucose have the least effect on the vibriocin production at individual levels their interaction has second highest SI (44.42%), which indicates that the production of antibiotic is depending on the individual as well as interaction effect of selected parameters. On the whole, using Taguchi experimentation enhanced the vibriocin yield from 2 351 to 2 187 U/mL (~20 % improvement) with the modified culture conditions.

To conclude, less effectiveness of chemically synthesized drugs in marine water leads to uncontrolled use in mariculture, and results in serious toxicological consequences on the living organisms[26]. The vibriocin obtained from the present study provided ample scope for the successful disease management of mariculture industry devastated by *V. harveyi*. In fact, this may be the first report on the production of vibriocin with novel characters by a halotolerant *V. parahaemolyticus*, which has substantial activity against both type strain and environmental isolate of *V. harveyi*. Extracellular production of vibriocin in the marine medium makes it easy to purify and scale up at industrial level. The obtained antibiotic has resistance to a wide range of pH, temperature, UV radiation and chemicals which designates it is best suitable for mariculture industry. This study also signifies the orthogonal method of optimization to improve the production of antibiotics with a minimum number of experiments.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Vibriocins are a group of bacteriocins produced by Gram-negative bacteria in the genus *Vibrio*. Like other bacteriocins, vibriocins are protein toxins. They can kill bacteria beyond the genus *Vibrio*, including other proteobacteria. Mariculture industry is drastically affected by the diseases caused by the *Vibrio*s which usually result in economic losses. The present study identified the potential candidate *V. parahaemolyticus* isolated from a highly competitive ecological niche of mangrove ecosystem, which produces vibriocin active against pathogenic *Vibrio*s.

Research frontiers

The present study performed a purification, characterization and production optimization of vibriocin produced by *V. parahaemolyticus* isolated from the rhizosphere of *Avicennia marina* present in the Pichavaram mangrove ecosystem. The vibriocin showed good antibacterial activity against the mariculture pathogenic members of *Vibrio*.

Related reports

There are many reports on bacteriocins production by the *Vibrio*s such as *V. cholerae*, *V. harveyi*, *V. mediterranei*, *V. vulnificus* and some *Vibrio* sp. The bacteriocins exhibited good antibacterial activity against their own closely related *Vibrio* members. However, according to our knowledge no work has been carried out on bacteriocin production from *V. parahaemolyticus*.

Innovations and breakthroughs

The authors used statistical tools and culture parameters to optimize the vibriocin production. An overall

approximately 20% of antibiotic production was improved, it was noticed that sodium chloride and agitation speed played a vital role in secretion of vibriocin.

Applications

The vibriocin produced from *V. parahaemolyticus* may be an effective alternative for chemically synthesized drugs.

Peer review

This is a valuable research in which authors have improved the vibriocin production with the molecular weight of approximate 18 kDa from *V. parahaemolyticus* and demostrated its antibacterial activity.

References

[1] Hancock RE, Chapple DS. Peptide antibiotics. *Antimicrob Agents Chemother* 1999; **43**(6): 1317-1323.

[2] Joerger RD. Alternatives to antibiotics: bacteriocins, antimicrobial peptides and bacteriophages. *Poult Sci* 2003; **82**(4): 640-647.

[3] Klaenhammer TR. Genetics of bacteriocin produced by lactic acid bacteria. *FEMS Microbiol Rev* 1993; **12**: 39-85.

[4] Carraturo A, Rajeta K, Ottaviani D, Russo GL. Inhibition of *Vibrio parahaemolyticus* by a bacteriocin-like inhibitory substances (BLIS) produced by *Vibrio* mediterranei 1. *J Appl Microbiol* 2006; **101**: 234-241.

[5] Hoyt PR, Sizemore RK. Competitive dominance by a bacteriocin producing *Vibrio harveyi* strain. *Appl Environ Microbiol* 1982; **44**(3): 653-658.

[6] McCall JO, Sizemore RK. Description of a bacteriocinogenic plasmid in *Beneckea harveyi*. *Appl Environ Microbiol* 1979; **38**(5): 974-979.

[7] Prasad S, Morris PC, Hansen R, Meaden PG, Austin B. A novel bacteriocin-like substance (BLIS) from a pathogenic strain of *Vibrio harveyi*. *Microbiol* 2005; **151**: 3051-3058.

[8] Shehane SD, Sizemore RK. Isolation and preliminary characterization of bacteriocins produced by *Vibrio vulnificus*. *J Appl Micrbiol* 2002; **92**(2): 322-328.

[9] Sugita H, Matsuo N, Hirose Y, Iwato M, Deguchi Y. *Vibrio* sp. strain NM 10, isolated from the intestine of a Japanese coastal fish, has an inhibitory effect against *Pasteurella piscicida*. *Appl Environ Microbiol* 1997; **63**(12): 4986-4989.

[10] Kaneko T, Cowell RR. Ecology of *Vibrio parahaemolyticus* in Chesapeake Bay. *J Bacteriol* 1973; **113**(1): 24-32.

[11] Criminger JD, Hazen TH, Sobecky PA, Lovell CR. Nitrogen fixation by *Vibrio parahaemolyticus* and its implications for a new ecological niche. *Appl Environ Microbiol* 2007; **73**(18): 5959-5961.

[12] Daniels NA, MacKinnon L, Bishop R, Altekruse S, Ray B, Hammond RM, et al. *Vibrio parahaemolyticus* infections in the United States, 1973-1998. *J Infect Dis* 2000; **181**(5): 1661-1666.

[13] Smith TJ, Boto KG, Frusher SD, Giddins RL. Keystone species and mangrove forest dynamics: the influence of burrowing by crabs on soil nutrient status and forest productivity. *Estuar Coast Shelf Sci* 1991; **33**: 419-432.

[14] Semenov AM, van Bruggen AHC, Zelenev VV. Moving waves of bacterial population and total organic carbon along roots of wheat. *Microbial Ecol* 1999; **37**(2): 116-128.

[15] Kathiresan K, Selvam MM. Evolution of beneficial bacteria from mangrove soil. *Bot Mar* 2006; **49**: 86-88.

[16] Kathiresan K. A Review of studies on Pichavaram mangrove, Southern India. *Hydrobiologia* 2000; **430**: 185-205.

[17] Motta AS, Brandelli A. Characterization of an antimicrobial peptide produced by *Brevibacterium linens*. *J Appl Microbiol* 2002; **92**(1): 63-70.

[18] Alsina M, Blanch AR. Improvement and update of a set of keys for biochemical identification of *Vibrio* species. *J Appl Bacteriol* 1994; **77**(6): 719-721.

[19] Farmer JJ, Janda JM, Family I. Vibrionaceae. In: Brenner DJ, Krieg NR, Staley JT, Garrity GM, editors. *Bergey's manual of systematic bacteriology*. 3rd ed, vol 2. New York: Springer; 2005, p. 491-555.

[20] Chen WP, Kuo TT. A simple and rapid method for the preparation of gram negative bacterial genomic DNA. *Nucleic Acids Res* 1993; **21**(9): 2260.

[21] Bizani D, Dominguez AP, Brandelli A. Purification and partial chemical characterization of the antimicrobial peptide cerein 8A. *Lett Appl Microbiol* 2005; **41**(3): 269-273.

[22] Hames BD. An introduction to polyacrylamide gel electrophoresis. In: Hames BD, Rickwood D, editors. *Gel electrophoresis of proteins*. UK: IRL Press; 1981, p. 1-92.

[23] Ogunbanwo ST, Sanni AI, Onilude AA. Characterization of bacteriocin produced by *Lactobacillus plantarum* F1 and *Lactobacillus brevis* OG1. *Afr J Biotechnol* 2003; **2**(8): 219-227.

[24] Pien F, Lee K, Higa H. *Vibrio alginolyticus* infections in Hawaii. *J Clin Microbiol* 1977; **5**(6): 670-672.

[25] Desriac F, Defer D, Bourgougnon N, Brillet B, Chevalier PL, Fleury Y. Bacteriocin as weapons in the marine animal-associated bacteria warfare: inventory and potential applications as an aquaculture probiotic. *Mar Drugs* 2010; **8**(4): 1153-1177.

[26] Rodgers CJ, Furones MD. Antimicrobial agents in aquaculture: Practice, needs and issues. In: Rodgers C, Basurco B, editors. *The use of veterinary drugs and vaccines in Mediterranean aquaculture. Series A: Mediterranean Seminars, No. 86*. Spain: CIHEAM; 2009, p. 41-59.

[27] Verluyten J, Messens W, De Vuyst L. Sodium chloride reduces production of curvacin A, a bacteriocin produced by *lactobacillus* curvatus strain LTH 1174, originating from fermented sausage. *Appl Environ Microbiol* 2004; **70**: 2271-2278.

[28] Rajaram G, Manivasagan P, Thilagavathi B, Saravanakumar A. Purification and characterization of a bacteriocin produced by *Lactobacillus lactis* Isolated from marine environment. *Adv J Food Sci Technol* 2010; **2**(2): 138-144.