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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

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