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Characterization of Vibriocin and Prospect of Co-Culture Method to Overcome the Diminishing Antibacterial Activity

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A B S T R A C T

The term 'Vibriocin' is specifically indicates the bacteriocin of Vibrio species. The present study reports the screening Vibriocin producing marine bacteria for food and aquaculture application. Out of 40 strains from Clam (*Meretrix meretrix*) species, screened against eight seafood borne pathogens, two Vibriocin producing bacteria were active against *Staphylococcus aureus*. Crude Vibriocin of these two isolates were subjected to salting-out for partial purification and exposed to different conditions *i.e.,* pH, temperature and detergent for its antibacterial activity. After few successive subcultures for characterization and bulk production, its antibacterial activity was decreased slowly and exhibited minimal antibacterial activity. Further, when the isolates were co-culture with *S. aureus* resulting in regain of antibacterial activity. This study revealed that Co-culture method is an appropriate approach to regain the Vibriocin production in Vibrio species and marine environment is a potential source of Vibriocin producing bacteria which can be exploited for various applications such as food preservation and as a probiotic for aquaculture ponds to control the pathogenic bacteria.

Introduction

Recent years, regulatory authorities are more stringent about incorporation of synthetic preservative in foods in order to control the spoilage and pathogenic bacteria. Hence, in future, the production of safety food is more difficult without a suitable alternative. Even though plant extracts, nanomaterials and oils are showing promising antibacterial properties (Viji *et al.,*2015; Visnuvinayagam *et al.,* 2019), each product has their own limitations;

moreover, it cannot be used for all processing methods. In recent year, recently, the application of bacteriocin in food as an emerging technique has gained considerable attention. Bacteriocin is secondary metabolite of bacteria and its application in the field of food preservation is termed as bio-preservation. Even though, it is an active metabolite of bacteria, it is safe and can be used as an antibacterial for food preservation. A novel bacteriocin *viz.,* Nisin (bacteriocin) has already been given the status of preservative by United States Food and Drug Administration (US-FDA) and is being used commercially in food industries (Sharma and Gautam, 2008). Hence, application of bacteriocin in fish would be a novel and promising platform to enhance the shelf life by eliminating the pathogenic and spoilage micro-flora in fish meat. Bacteriocins are substances having an essential biological protein moiety possess the bactericidal mode of action against other bacteria. It is considered as an immunity mechanism for the particular bacteria protects from other bacteria *i.e.,* Bacteriocins may serve as anti-competitor compounds to protect the own microbial community. Generally, bacteriocins are ribosomally synthesised substance active against closely related bacterial species; but, rarely some bacteriocin can target a broader range of bacterial species (Bakkal *et al.,* 2012). These bacteriocins sizes are varies from high molecular weight (like Colicin) to simple small peptides like Nisin (Sharma *et al.,* 1984). The advantages of bacteriocin are nontoxic and non-antigenic to animals including humans; moreover it is easily degraded by the proteolytic enzymes of the gastrointestinal tract; hence, it is safe to incorporate in food (Desriac *et al.,* 2010). Even though vast numbers of studies dealt bacteriocin, only limited studies are available on the Vibriocin. Hence, in the present study, Vibriocin was purified and characterized and its diminishing biological activity was effectively controlled by the modified co-culture method.

Materials and Methods

Isolation of Vibrios

Live Clam species were collected from retail fish market of Vashi, Navi Mumbai and brought to laboratory and were processed for isolation of *Vibrio* species by inoculating the Clam's gut content into alkaline peptone water (APW) containing 2% NaCl (Prasad *et al.,* 2005) for enrichment. After overnight incubation, a loop full culture was taken from surface pellicle and streaked over Thiosulfate-Citrate-Bile salts-Sucrose (TCBS) agar supplemented with 2% NaCl and incubated at 37°C. After overnight incubation, characteristic colonies were picked and inoculated into brain heat infusion (BHI) slant (with 2%) for further biochemical characterization.

Biochemical characterisation

Isolated Vibrio cultures were tested by gram stain and confirmed the Vibrio genus by various biochemical tests *viz.,* oxidase test, sensitivity against Vibrio-static agent O129,Arginine di-hydrolase, Ornithine de-carboxylase and Lysine decarboxylase.

Bio-screening

The selected 40 isolates were grown in Brain Heat Infusion (BHI) broth containing 2% NaCl and incubated at 37°C. Further, the overnight cultures were centrifuged at 10,000 rpm for 15 min and the cell free supernatant (CFS) was passed through 0.22 µ sterile syringe filter (Nowroozi *et al.,* 2004). These sterile filtrates were used for well diffusion assay to screen Vibriocin production against eight food borne bacteria *i.e., Aeromonas hydrophila, Bacillus cereus, Escherichia coli,*

Listeria monocytogenes, Salmonella, Staphylococcus aureus, Vibrio cholerae and Vibrio parahaemolyticus. For antibiogram assay, these pathogenic bacteria were grown in test tube and the concentrations of the cultures were adjusted to 0.5McFarland standard turbidity. In case of *S. aureus* and *V. parahaemolyticus* cultures, Mueller Hinton Agar with 2% NaCl was used (CLSI, 2014). Wells of 6 mm diameter were made on 200mm (diameter) agar plate by Cork-borer (HiMedia# LA737) and then the bottom of the wells was sealed with sterile 1% agar in order to avoid the leakage. Finally, all the wells were filled with 50µl of CFS and incubated at 37°C. These plates were examined after 24h for zone of inhibition (Fig. 1) with the aid of antibiotic zone scale.

Partial purification of vibriocin

CFS was precipitated by addition of ammonium sulphate into CFS fluid up to 60% saturation at 4°C for 6h (Seatovic *et al.,* 2011). The precipitates were collected by centrifugation at 10,000 rpm for 20 min. The pellet was re-suspended in 10 ml 0.1M Tris buffer, pH 7.0.

The pellet obtained from 60% fraction was dialysed against distilled water for 6 h using 12KDa dialysis membranes (Jini *et al.,* 2011). The partially purified Vibriocin was distributed in small quantity (aliquots) and stored in deep freezer (-20°C) for further usage.

Characterization of bacteriocin

Stability of the Vibriocin was checked as per Singh *et al.,* (2013) protocol by subjecting the bacteriocin with different proteolytic enzymes, temperature, pH and surfactant.

Effect of pH

The aliquots of Vibriocin were adjusted to different pH *i.e.,* from 2, 4, 6, 8, 10 and 12 then kept at room temperature for 30 minutes. The same aliquots were neutralised to pH 7 and antibiogram was carried out against *S. aureus.*

Effect of temperature

The effect of temperature on bacteriocin activity was tested by heating the Vibriocin aliquots at different temperatures from 40°C to 121°C. Simultaneously, one aliquot was maintained as control by incubating the test sample at 37°C for better comparison.

Aliquots of each sample were taken after 15, 30, 60 and 90 min, then the activity was assessed by well diffusion assay against *S. aureus*.

Effect of proteolytic enzymes

CFS was treated with Lysozyme, Papain and Proteinase K (each 1mg/ml) and incubated at 37°C for 1h then the antibacterial activity was assessed by antibiogram (Pilasombut *et al.,* 2006).

Effect of surfactants

Aliquots of Vibriocin was treated with surfactant *viz.,* SDS and urea, in different concentrations (0.25%, 0.5% and 1% w/v) and incubated at 37°C for 2h (Seatovic *et al.,* 2011) and the antibacterial activity was checked.

SDS-PAGE analysis

The partial purified Vibriocin samples were further subjected to SDS-PAGE with 10% running gel to determine the Molecular weight of the Vibriocin (Yamamuto *et al.,* 2003). Electrophoresis was performed at 100V for 1h and the gel was stained overnight with Coomassie Brilliant Blue, then de-stained and checked for specific band.

Plasmid isolation

Plasmid isolation was carried out as per Michael and Sambrook, (2012) to determine antimicrobial activity whether due to portion of bacterial genome or plasmid (Tolinacki *et al.,* 2010).

Co-culture method to overcome the diminishing antibacterial activity

In the present study, the decreased in the anti-bacterial activity was observed. Hence,100 µl of 1Mc Farland standard turbidity culture of VPB was inoculated into the 10ml containing 0.5Mc Farland standard culture of *S. aureus*. After overnight incubation VPB was isolated by streaking over TCBS supplemented with 2% NaCl. Similarly, three passages were carried out and found a considerable improvement in the production of Vibriocin.

Results and Discussion

In the present study, a total of 40 Vibrio species were isolated from clam samples and confirmed based on biochemical characters. Further, all isolates were screened for Vibriocin production and observed that only 2 strains *i.e.,* isolate number 7 and 8 were able produce Vibriocin, that could control the growth of *S. aureus* (Fig. 1). Between two strains, isolate Number 8 showed better inhibition than the isolate number 7. Hence, isolate number 8 was chosen for bulk production by inoculating into one litre MH Broth (supplemented with 2% NaCl) and CFS was subjected to partial purification by salting out using Ammonium sulphate. It was observed that the antimicrobial activity of the Vibriocin against *S. aureus* was enhanced after the purification (Fig. 2). Then the partially purified of Vibriocin producing strains were subjected to various biological conditions *i.e.,* treated with different pH, temperature, proteolytic enzymes and surfactant. While checking the activity at different level of pH4 to 8, it was observed that the maximum activity at neutral pH. Similarly, while subjecting to heat treatment, its activity was not lost even after 30 min at 60°C; but inactivation occurred when the incubation was continued for a longer period. However, total loss of activity was observed after incubation at 80°C. To study the stability with proteolytic enzymes, Vibriocin was treated with different proteolytic enzymes and found that complete loss of antimicrobial activity in agar plate. Addition of surfactant *viz.* Sodium dodecyl sulfate (SDS) with Vibriocin led to an enhanced zone of inhibition as compared to the SDS and untreated sample (Fig. 3). But, addition of urea with the Vibriocin leads to complete loss of the antimicrobial activity. The materials were again checked for SDS-PAGE analysis; but, no band could be observed. Isolate 8 was subjected to Alkaline lysine method a for plasmid isolation and found the there is no plasmid in the VPB. The antibacterial activity of the Vibriocin was diminished with frequent subculture in the MH agar. However with co-culture method its diminishing antibacterial activity was reverted back to normal level.

Emergence of multi drug resistant bacteria was frequently reported in seafood sector (Visnuvinayagam *et al.,* 2015, 2016, 2017, 2018). In the mean time the food controlling authority are more stringent in the addition of synthetic food substance in the food. So, this is the suitable period to find an alternative to the synthetic antibacterial substance. So in the present study the all Vibriocin was isolated and screened for the antibacterial activity. Bacteriocins in general, are mostly active against the closely related species. Ironically, in the present study, these two isolates were active against the *Staphylococcus* genus. Similarly, Balakrishnan *et al.,* (2014) isolated a Vibriocin from *Vibrio parahaemolyticus* against pathogenic *Vibrio harveyi* and recommended for the food application. Even though, the isolated metabolite obtained from

pathogenic *V. parahaemolyticus*, since it is only a metabolites (not bacteria as such), it is considered as safe for in food application. Even though Vibrio species are abundant in marine water as well as estuarine environment (Oliver and Kaper, 1997*)* reports are scant on Vibriocin production and characterization. However, voluminous data is available related to the other environments (Nowroozi *et al.,* 2004; Singh *et al.,* 2013; Olivera *et al.,* 2014). Sugita *et al.,*(1997) isolated a VPB from the marine fish intestine and reported activity against *Pasteurella piscicida.* Shehane and Sizemore (2002) isolated three VPB from marine waters and which exhibited antibacterial activity against *V. vulnificus*, *V. cholerae* and *V. parahaemolyticus*. Carraturo *et al.,* (2006) isolated Vibriocin from *V. mediterranei*-1 and found the antibacterial activity against *V. parahaemolyticus.*

The isolated Vibriocin was active between pH 4 to 8. Since, most of the fish and other food products are belong to same pH. It can be applied in food to control the *S. aureus*. In addition, it is fairly heat stable i.e., able to withstand 80°C similarly for short duration, it cannot be used for fresh fish.

It is suitable only after heat treatment or those foods not subjected to heat treatment. Sugita *et al.,* (1997) reported that Vibriocin production was observed while growing the culture only between 15 – 25°C; whereas, it did not produce the Vibriocin while growing 30 – 35°C. Antibacterial activity of the Vibriocin was assessed after treating with different enzymes and found that complete loss of antimicrobial activity. Similarly, Carraturo *et al.,* (2006) observed that Vibriocin antimicrobial was degraded by the Proteinase K; but it was resistant to trypsin and alpha-chymotrypsin. So, the isolates Vibriocin was highly sensitive to the proteolytic enzymes *viz.* lysozyme, papain and Proteinase K. In the continuation of the stability of the Vibriocin testing protocol, again the Vibriocin was treated with surfactant *viz.* Sodium dodecyl sulfate (SDS) and Urea. Interestingly, SDS treated Vibriocin exhibited drastic increase in zone of inhibition as compared to the SDS (alone) and untreated (Vibriocin alone) sample. Whereas urea treated Vibriocin did not exhibited any antibacterial activity, possibly due to the reason that urea cleaves the disulphide bonds present in the Vibriocin; so, thus it could be assumed that the Vibriocin compound contains a disulphide bond which has been cleaved by urea. The, presence of disulphide bond is highly essential for the action of the Vibriocin species.

Similarly, Elayaraja *et al.,* (2014) reported that vibriocin was moderately resistant to SDS and highly sensitive to Urea. In the present study, the Vibriocin was able to inhibit the only *S. aureus* species, which indicates the narrow killing ability of the Vibriocin towards *S. aureus* alone.

This narrow killing ability of particular pathogens would give an added advantage *i.e.*, it limits the ability of bacteria to evolve resistance to the antimicrobials and thus reduces the incidence of drug-resistant pathogens. Hence, it can be applied to foods by spray-drying as either dried bacteriocins or probiotic bacteriocinogenic strains (Bakkal *et al.,* 2012). For example, Brillet *et al.,* (2005) has shown that bacteriocin producer *Carnobacterium divergens* V41 can be used as a bio-preservative to inhibit the growth of *Listeria monocytogenes* in cold smoked salmon. Similarly, Schobitz *et al.,* (1999) directly applied a BLIS from *Carnobacterium piscicola* into vacuum-packed meat, which inhibited the growth of *L. monocytogenes* in the vacuum-packed meat after 14 days of storage at 4°C (Carraturo *et al.,* 1999).

Fig.1 Screening of the Vibriocin producing isolates by well diffusion assay in 200mm antibiogram plate; zone of clearance around isolate no. 8

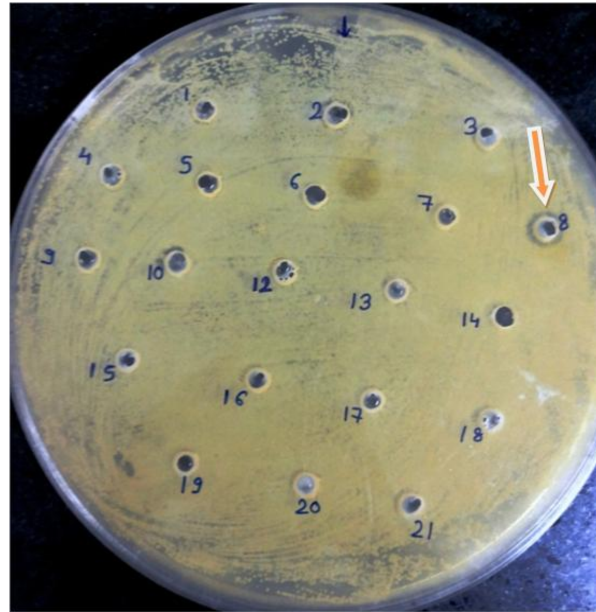


Fig.2 Partially purified Vibriocin exhibiting enhanced zone of inhibition in well diffusion assay

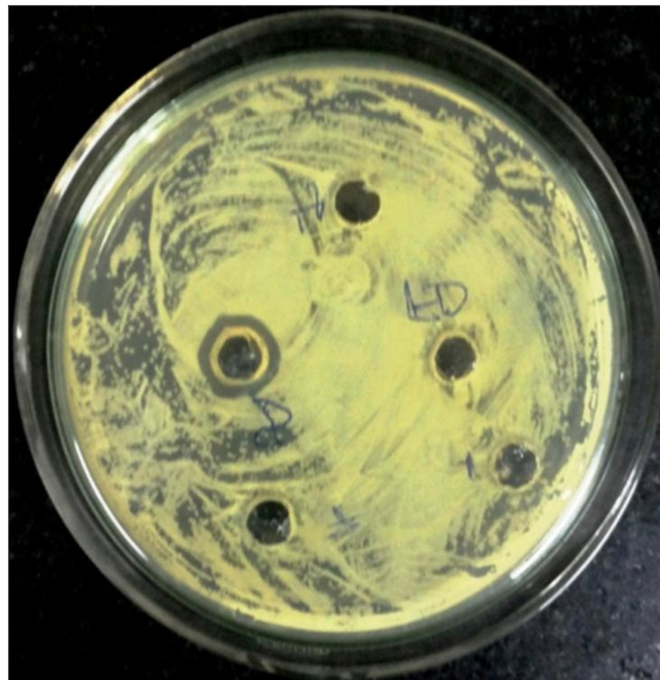
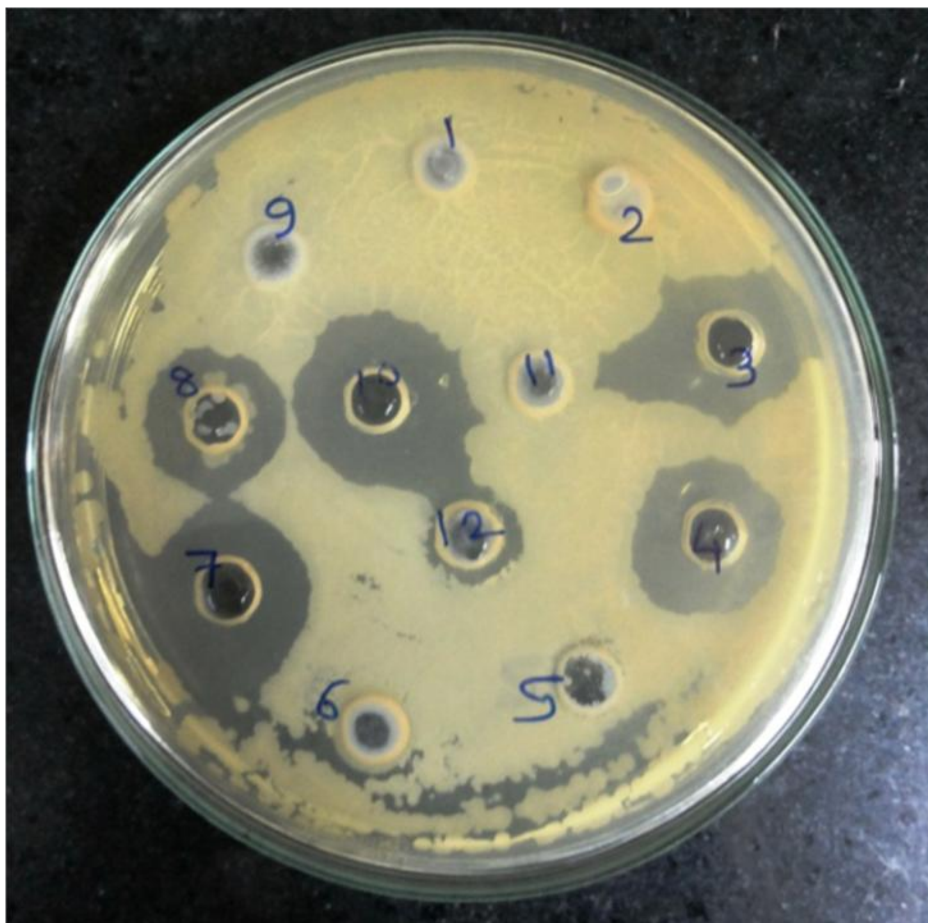


Fig.3 Enhanced activity of the Vibriocin after treatment with SDS different concentration; well no. 12 is untreated Vibriocin; well no. 8 is SDS control, Well no. 3,4 and 7 are the SDS treated Vibriocin with different concentration



These studies aid in the argument that bacteriocins should be used as a bio-preservation technique in the seafood industry. This technology has already emerged in the terrestrial food industry as we see with nisin (FDA approved food additive) and MicrogardTM (a milk-based BLIS). It has also been suggested that bacteriocins could be combined with current methods of antimicrobial treatment and preservation to produce synergistic effects, such as incorporating bacteriocins into bio-active packaging (Pilet *et al.,* 2011). For instance, bacteriocins can be impregnated into gel coatings and/or polyethylene films and can be applied to seafood during packaging (Neetoo *et al.,* 2008). In fact, immobilization of bacteriocins on coating materials for bio-preservation may actually reduce the cost of packaging due to the reduced amount and cost of the antibacterial materials needed to attach to the film (Galvez *et al.,* 2008). Creating combinations of bacteriocins and current methods used in the seafood industry has the potential to increase the guarantee of freshness by assuring the inhibition of spoilage causing microorganisms.

In the present study, the antibacterial activity was reduced with sub culturing. Similarly, Vriezen *et al.,* (2009) reported that reduction of inhibition activity with continued subculture in the non competitive media. The Colicin producing bacteria was subcultured upto 253 times (generation) and proved that, reduction in the killing ability of the bacteria while increasing the passaging/ subculturing/ generation and they identified that the change in gene expression (up regulation) and significant change in DNA repair mechanism

(down regulation). In the present study too. The decrease in antibacterial activity may be due to repeated subculture without any competitive medium. So, based on the suggestion, competition was created in the media *i.e.,* A MH broth containing fairly grown culture of the *S. aureus* was chosen as an inhibitory media for VPB. Similarly, Mearns-Spragg *et al.,* (1998) observed that the increased production of the antimicrobial compound while marine bacteria co-cultured with live bacteria (*Pseudomonas aeruginosa* and *Escherichia coli)* or inactivated (killed cells of *S. aureus)* bacteria.Reports published by Slattery *et al.,* (2001) also supported that in passaging the culture in the competitive media leads to increase in primary and secondary metabolites. Since, Vibriocin also one of the metabolites, competitive media facilitated increase in the production of Vibriocin.

Hitherto, most of the research studies on bacteriocin are based in pure cultures; but, in natural ecosystem it is too complex and significance in the gene responses can be observed In the present study, the VPB were isolated from Clams and after successive passages for characterization study, it's antibacterial activity was lost. Based on co-culture method its activity was reappeared. Similarly, Dusane *et al.,* (2011) isolated different bacteria from the Green mussel and found that these enhanced antibacterial activity after co-culture method.

The study indicates that marine environment is one of the excellent sources of VPB (Singh *et al.,* 2013). But, it was not elaborately dealt on revival of its lost antibacterial activity. Hence, the present study centred as overcoming the vanished antibacterial activity. Since, the use of prophylactic antibiotics is detrimental to aquatic and terrestrial environments, animal and human health bacteriocinogenic bacterial strains appear to be an excellent candidate for a friendly alternative, which will support the industrial scaling up of production. Based on the microbial sequencing, many of the researchers observed that the very high in biosynthesis activity its fermentation production (Udwary *et al.,* 2007). Especially, few bacterial and fungal species *i.e.,* Aspergilli and actinomycetes are carrying a huge genomic resources/backup for the alternative metabolic pathway (Knight *et al.,* 2003).

Hitherto, most of the studies followed a simultaneous co-culture method for the enhancement of biological activity; but, in the present study, a minor modification from the simultaneous co-culture method was followed. *i.e.,* After the sufficient grown culture of *S. aureus* in 5 mL of MH broth (1Mc Farland turbidity), 100 µl of 0.5Mc Farland turbidity concentration of VPB were inoculated and incubated at 37°C. After overnight incubation, the loop full culture was streaked over TCBS agar for isolation of VPB. The same procedure was repeated twice to regain its antimicrobial activity. Interaction, between the bacterial and fungus species also causes increased production of the active metabolite. Miao *et al.,*(2006) reported that most of the time, after co –culture with other bacteria, identified which is due to silence most of the time while in pure culture and also reported that the enhanced antibacterial activity of the while adding the cell free extract of the bacterial broth.

Based on the present study findings, it is concluded that marine water is a potent source of Vibriocin and the isolated Vibriocin can be used to control the *S. aureus* in the food. Antibacterial activity of the Vibriocin can be regained or increased by co-culture application.

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