BACTERIOCIN PRODUCTION BY INDIGENOUS MARINE CATFISH ASSOCIATED *VIBRIO* SPP.

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ABSTRACT

Fifty strains of genus *Vibrio* were isolated (identified) from healthy and diseased marine catfish(es). The isolates were screened for bacteriocin (vibriocin) production. About 32% isolates were found bacteriocin producers. The best producer was identified as *Vibrio anguillarum* AVP10. The maximum production of vibriocin AVP10 was manifested at 29°C at pH 7, after 18-20h of incubation. Vibriocin activity was enhanced in the presence of citrate-phosphate buffer. The vibriocin AVP10 withstands autoclaving temperature and showed activity even after prolonged chloroform treatment. Proteolytic enzymes inhibited its activity, while lipolytic enzyme had no effect. It was found bioactive only against intrageneric bacterial strains. Mode of action of vibriocin AVP10 varies with the indicator (sensitive) culture used *i.e*. bactericidal effects was exerted against *V. anguillarum* AVS9 while bacteriostatic effect was shown against entero-toxigenic *E. coli.*

Keywords: Vibrio, catfish, bacteriocin, intergeneric, toxicogenic.

INTRODUCTION

The genus *Vibrio* includes gram-negative bacteria that are obligate anaerobes. They are curved shaped motile organism with single polar flagella (Thompson *et al*., 2004). *Vibrios* constitute one of the most common bacteria in surface waters. They are ubiquitous in the aquatic environment and are commonly present in or on shellfish and other seafood (Morris, 2003). Vibrio are non-invasive pathogens, they cause some of the most serious cases of diarrhea in humans. These waterborne organisms are transmitted to humans via infected water or through fecal transmission. Pathogenic *Vibrio* include *V. Cholerae* (the causative agent of cholera), *V. parahaemolyticus* and *V. vulnificus* (Faruque and Nair, 2008). Several species of *Vibrio* have been associated with disease in fishes (Elhadi *et al.,* 2004; Ren and Su, 2006; Su and Liu, 2007). The bacteriocins of *Vibrio* are generally termed as "vibriocins" (Dastidar *et al.,* 1984). Vibriocins from different species of the genus *Vibrio* has been characterized e.g. *V. cholerae* (Telesmanich *et al,* 2000), *V. vulnificus* (Shehane and Sizemore, 2002), *V. harveyi* (Parsad *et al,* 2005), *V. mediterranei* (Carraturo *et a*l., 2006).

MATERIAL AND METHODS

Source of *Vibrio* strains: *Vibrio* strains used in this study were isolated from healthy and infected marine catfishes (*Arius thalassinus*) locally known as "Khaga". Fishes (collected from Fisheries of Karachi), had a visible infected area at the gut region and others had an obvious infection in the gills region. The infected regions of the fishes were dissected and suspended in 100mL of normal saline in separate flasks for further processing.

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Identification of *Vibrio* strains: Samples were taken from the suspension of the normal and infected fish parts serially diluted and plated on cholera medium named as thiosulfate citrate bile sucrose agar (TCBS) agar to get isolated colonies. The ability to grow on TCBS medium (pH. 8.6) is a presumptive identification of *Vibrio* species. Analyzing some renowned characters of V*ibrio* species did help in further confirmation (scheme No 1).

Growth on TCBS agar (8.6 pH)
↓
Growth on MacConkey agar
↓
Lactose negative
↓
Oxidase positive
↓
Glucose positive
↓
Growth on TCBS agar
(for cross checking)

Scheme 1: Identification of *Vibrio* spp.

Screening methods for vibriocin production

a. Stab-overlay method: Luria basal (LB) agar plates were stabbed with the *Vibrio* strains and incubated at 37°C for an overnight. Next day plates were exposed to chloroform vapours to kill the bacteria (Keeping the plates inverted and 9cm diameter piece of Whatman filter paper No.1 was inserted into the lid and impregnated with 1mL of chloroform for 20-30 min). Plates were then overlaid with 3mL soft agar containing $1x10^6$ cells of indicator organism. Plates were again incubated at 37°C for overnight to observe the clear zone around the producer culture (Rasool *et al.*, 1996).

b. Agar-well diffusion method: *Vibrio* isolates were grown in LB broth and incubated at 37°C for 24h. Next day cell-free neutralized supernatants (CFNS) were obtained by centrifugation (6000rpm, for 30min, 4°C). The cells were discarded and pH of the supernatant fluid was adjusted to 7.0 with 1N NaOH, plates were overlaid with 3mL soft agar containing $1x10^6$ cells of indicator organism. Wells (5mm diameter) were cut and 100µL of supernatant fluid of the test organism were poured into each well. Next day zone of inhibition of the indicator strain around the well was measured (Godic and Bogovic, 2003)

Choice of the best producer and sensitive strains: Fifty different *Vibrio* strains were screened for bacteriocin production against both intergeneric and intrageneric organisms. Since the strain *V. anguillarum* AVP10 was manifesting the largest zones of inhibition, hence selected for detailed studies. The sensitive strain *V. anguillarum* AVS9 was found to be the most susceptible, as all the (tested) vibriocin producing strains were bioactive against it. Hence the combination of *V. anguillarum*s AVP10 (producer) and *V. anguillarum*s AVS9 (sensitive) was selected for further studies.

Effect of various physical and chemical treatments on vibriocin AVP10 production: In order to evaluate the maximum vibriocin production, the producer strain AVP10 was stabbed on LB agar and incubated at 4°C, 29°C, 37°C and 40°C. Next day overlaid with 3.0mL sensitive culture. After an overnight incubation, zone size was measured. Similarly, vibriocin production was also checked on different culture media including: nutrient agar (NA), brain heart infusion agar (BHI), Luria basal agar (LB) and citrate-phosphate nutrient agar (CPN) and activity were checked by stab-overlay method (Saeed *et al,* 2006).

Physio-chemical characterization of vibriocin AVP10: To check the thermal stability, CFNS of vibriocin AVP10 was exposed to 60°C (60min), 80°C (40min), 100°C (20min) and 121°C (15min) and activity was checked by agar-well diffusion method. Similarly, the sensitivity of vibriocin to different enzymes (protease K, protenase, trypsin and lipase at the final concentration of 1mg/mL) was also examined. To observe the effect of chloroform on the vibriocin activity 24h old stabbed cultures were exposed to chloroform vapours for 10-30min, overlaid with sensitive cells and then next day zone size was measured (Ahmad and Rasool, 2003).

Activity spectrum: The activity spectrum of vibriocin AVP10 was determined against various gram-negative and gram-positive strains by agar-well diffusion method (Saeed *et al.*, 2006).

Mode of action: In order to ascertain the mode of action of vibriocin AVP10, modified method of Iqbal *et al* (2001) was followed. Vibriocin AVP10 was treated with sensitive cells of *V. anguillarum* AVS9 and enterotoxicogenic *E. coli* (at pH 7 and 9) incubated at 37°C, optical density (O.D.) at 600nm was measured and log CFU/mL was scored.

RESULTS AND DISCUSSION

Isolation and Identification of *Vibrio* Strains: All the *Vibrio* strains used in this study were isolated from healthy and infected marine catfishes (*Arius thalassinus*), locally known as "Khaga". Table 1 shows the total number of isolates from gills and gut region of normal and infected fishes. *Vibrio* strains grow on TCBS media and give green-yellow colour colonies (fig. 1, scheme 1). In order to identify the producer strain series of morphological, cultural and biochemical tests were done as given in table 3.

Table 1: Sources of *Vibrio* strains from healthy and diseased catfish(es)

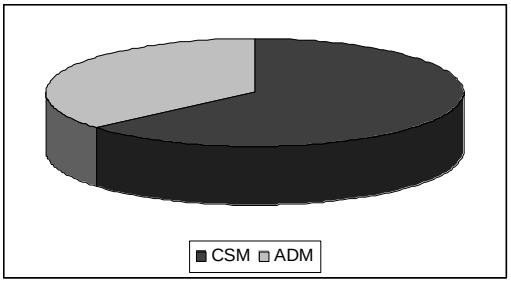
Fish (anatomical sites)	No. of *Vibrio* strains isolated
Healthy fish	
Gills region	06
Gut region	11
Diseased fish	
Gills region	13
Gut region	20

Frequency of vibriocin producers: Fifty v*ibrio* strains were screened for vibriocin production by stab-overlay and agar-well diffusion method. The highest number of bacteriocin producers was observed in stab-overlay method i.e., 40% and only 24% of the isolates were found to produce in agar-well diffusion (table 2 and graph 1). Carraturo *et al* (2006) reported that out of forty-five

Table 2: Percentage of vibriocin producers in different methods

Methods	Producers	Percentage (%) producers	Non-Producers	Percentage (%) non-producers
Stab-overlay method	20	40%	30	60%
Agar-well diffusion method	12	24%	38	76%

halophilic *Vibrio* spp. (screened for antimicrobial production) only one strain i.e., *V. mediterranei*, (a nonpathogenic strain), showed antimicrobial activity towards *V. parahaemolyticus*.



Graph 1: Percentage of bacteriocin (vibriocin) producers in different screening methods.

Key: CSM= cross-streak method; ADM= agar-well diffusion method

Factors affecting vibriocin AVP10 production: It is important to test the screening methods for inhibitory activity using a wide range of media and growth conditions, since the optimal conditions for growth of the test strain do not necessarily coincide with the one giving maximum bacteriocin production (Kang and Lee, 2005). Factors affecting optimal growth of a bacterium and optimal synthesis and release of bacteriocin include the composition of medium, incubation time, temperature and pH (Lucas *et al*, 2006). Effect of different temperatures on the production of vibriocin AVP10 was studied at 4°C, 29°C, 37°C and 40°C. No zone of inhibition was observed at 4°C. This could be due to killing or static nature of the culture at such low temperature or the culture does not produce bacteriocin at this temperature. There was no remarkable difference in the size of zone of inhibition observed at 37°C and 40°C. However, increase in zone size was observed at 29°C as shown in table 4. This may be due to the fact that 29°C is often recognized as the optimum temperature for the growth of marine bacteria. Earlier, Parasad *et al,* (2005) used 28°C for vibriocin production. Different culture media including, NA, BHI, LB and CPN agar were used for vibriocin production. Largest zone of inhibition was obtained in CPN agar while rest of the three media (tested) showed not much difference in zone of inhibition. Results indicate that citrate-phosphate buffer plays an important role in the synthesis of vibriocin and increased production of vibriocin took place on solid medium containing a citrate-phosphate buffer.

Factors affecting vibriocin activity: Vibriocin preparation was subjected to different heat treatments and consequently was found to be thermostable. Vibriocin AVP10 was found stable at 60°C (60min), 80°C (40min) and 100°C (20min). It was also stable at autoclaving temperature (121°C, 15psi for 15min) as shown in figure 2. Earlier, Parasad *et al*. (2005) worked on a novel BLIS from a pathogenic strain of *V. harveyi* and found it stable at 60°C for 10min.Vibriocin AVP10 was tested for sensitivity to various enzymes. It was susceptible to all the three protein-digesting enzymes, namely protease, proteinase K and trypsin, thus no zone of inhibition was observed after treatment with these enzymes (table 5). However, lipase had no effect on the vibriocin resulting in normal size of zone of inhibition. This shows that the vibriocin AVP10 is protein in nature. Parasad *et al.* (2005) obtained similar results, where vibriocin produced by *V. harveyi* was inhibited by treatment with proteolytic enzymes. Vibriocin AVP10 was found resistant to chloroform. Further, there was no increase or decrease in the activity of the vibriocin after chloroform treatment. Carraturo *et al.* (2006) reported BLIS from *V. mediterranei* was sensitive to proteinase K, stabled to pH ranged 5-9, resistant to organic solvents.

Table 3: Phenotypic characters used for the identification of *V. anguillarum* AVP10

Tests	Characteristics
MORPHOLOGICAL CHARACTRSTICS	
Gram-reaction	Gram-negatives
Shape	Comma
Arrangement	Scattered
Motility	Actively motile
CULTURAL CHARACTRSTICS	
Growth on TCBS	Green-yellow colonies
Growth at	
0°C	-
37°C	+
45°C	-
0% NaCl	+
3% NaCl	+
7% NaCl	-
BIOCHEMICAL CHARACTRSTICS	
Oxidase test	+
Utilization of	
Glucose	+
Lactose	-
Sucrose	+
Arabinose	+
Innositol	-
Mannitol	+
Xylose	-
Sorbitol	+
IMViC Test	
Indole	-
Methyl Red	+
Voges-Proskauer	-
Citrate	-

Table 4: Effect of different media and incubation temperature(s) on the production of Vibriocin AVP10

Treatments	Zone of inhibition (mm)
Media	
NA	25
BHI	27
LB	28
CPN	32
Incubation Temperature	
4°C	0
29°C	29
37°C	25
40°C	27

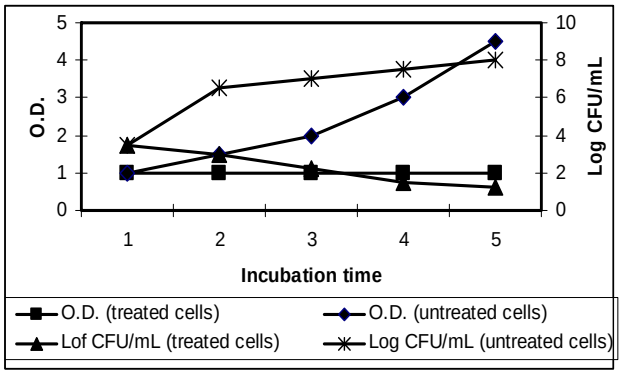
Key: NA= nutrient agar medium; BHI= brain heart infusion agar medium; LB= Luria basal agar medium; CPN= citrate phosphate nutrient agar medium; 0= no zone of inhibition

Table 5: Physio-chemical characterization of vibriocin AVP10**.**

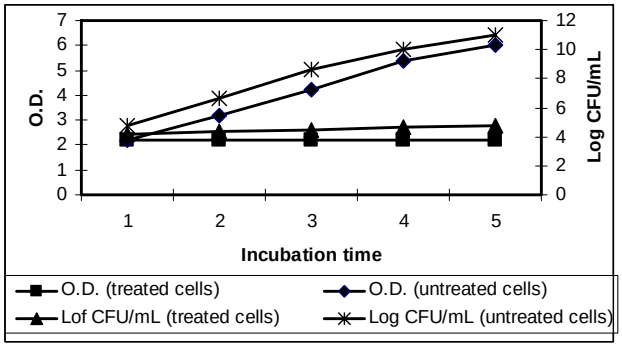
Treatments	Zone of inhibition (mm)
Temperatures	
60°C	28
80°C	26
100°C	25
121°C	26
Enzymes	
Protease K	0
Proteinase	0
Trypsin	0
Lipase	29
Chloroform	
Chloroform (10-30min)	27

Key: 0= no zone of inhibition.

Activity spectra of vibriocin AVP10: Bacteriocins produced by gram-negative bacteria usually have narrow-activity spectrum but those produced by gram-positive bacteria may have a wide range of action. In our study the vibriocin AVP10 behaved like a typical gram-negative bacteriocin with a narrow-spectrum of activity (showing bioactivity against other *Vibrio* strains, table 6). This means that vibriocin AVP10 has no intergeneric inhibitory action. However, when citrate-phosphate nutrient agar was used the growth of enterotoxicogenic *E. coli* was also inhibited. Earlier, Telesmanich *et al*, (2000) reported that vibriocin produced by *V. cholerae* non O1 strain P-11702 was inhibitory to many gram-negative bacteria including *S. flexneri*, *S. dysenteriae*, *S. sonnei, E. coli and V. cholerae.* While inter-strain and inter-species inhibition mediated by a bacteriocin-like inhibitory substance (BLIS) from a pathogenic *Vibrio harveyi* strain VIB 571 was demonstrated against *Vibrio* sp., including *V. fischeri*, *V. gazogenes* and *V. parahaemolyticus* (Prasad *et al*, 2005).



Graph 2a: Bactericidal effect of vibriocin AVP10 on growing cells of *V. anguillarum* AVS9 at 7 pH.



Graph 2b: Bacteriostatic effect of vibriocin AVP10 on growing cells of enterotoxicogenic *E. coli* at 7 pH.

Table 6: Inhibitory spectrum of vibriocin AVP10 produced by *V. anguillarim* AVP10 against gram-positive and gram-negative bacteria

Sensitive Cultures	MEDIA	
	LB	CPN
Gram-negative		
Enteropathogenic E. coli	0	0
Enteritoxcoigenic E. coli	0	30
Escherichia coli WT	0	0
Proteus mirabilis	0	0
Salmonella para typhi A	0	0
Salmonella typhi	0	0
Shigella dysenteriae	0	0
Pseudomonas aeruginosa	0	0
Klebsiella pneumoniae	0	0
Vibrio * (10 strains tested)	+ (4)	+ (4)
Gram-positive		
Staphylococcus aureus	0	0
Staphylococcus aureus (MRSA)	0	0
Staphylococcus epidermidis	0	0
Micrococcus luteus	0	0
Bacillus subtilis	0	0

Key: 0= no zone of inhibition; LB= Luria basal agar medium; CPN= citrate phosphate nutrient agar medium. *= out of 14 strains tested 4 were sensitive, zone size in mm

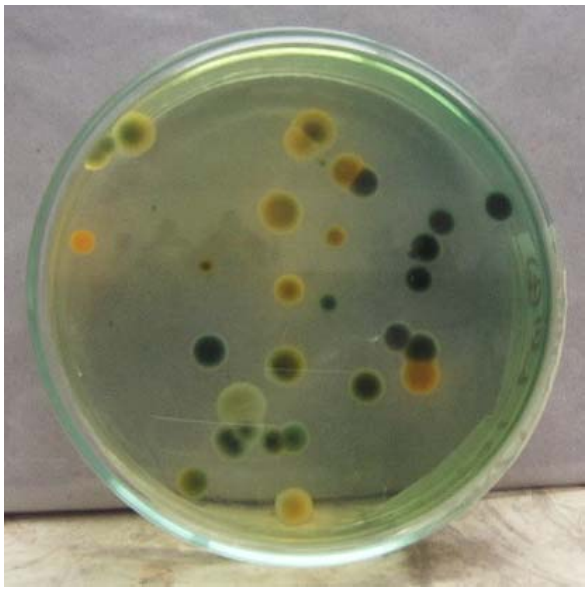


Fig. 1: Isolated colonies of *V. anguillarum* AVP10 on TCBS medium

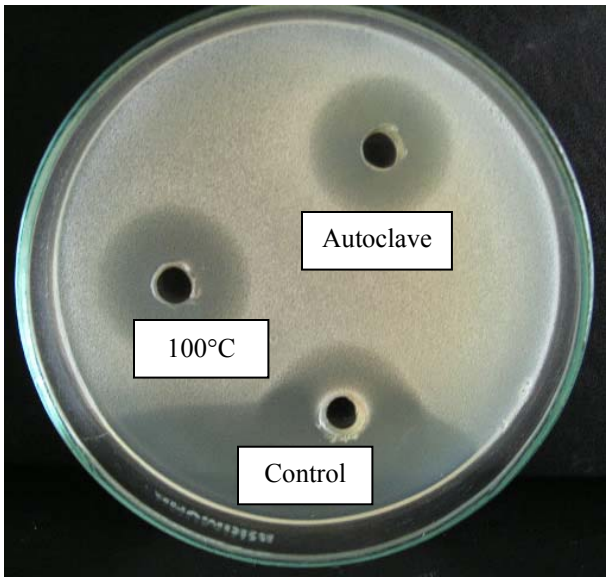


Fig. 2: Effect of temperature on the activity of vibriocin AVP10

Mode of action of vibriocin AVP10: The mode of action of vibriocin AVP10 was studied against two sensitive cultures *i.e. V. anguillarum* AVS9 and enterotoxicogenic *E. coli* by measuring the optical density (at 600nm) and viability of the sensitive cultures in terms of log CFU/mL (graph 2a and 2b). The effect of vibriocin AVP10 on growing cells of *V. anguillarum* AVS9 at pH 9 was found bactericidal since the optical density of sensitive cells remained static, showing that there is no lysis of the sensitive cells. However, the CFU/mL decreased rapidly and proportionally, indicating that the sensitive cells are being killed. Similar results were obtained at pH. 9. Telesmanich *et al*, (2000) reported bactericidal property of vibriocin from *V. cholerae* non 01 strain P-11702 against *Shigella flexneri, S. dysenteriae, S. sonnei*, *Escherichia coli* and *V. cholerae* strains.

CONCLUSION

Present study reports the ability of *Vibrio anguillarum* AVP10 to produce a bacteriocin *i.e*. vibriocin AVP10, inhibitory against the human and fish pathogens. The properties of resistance to wide range of temperatures, stability in the presence of chloroform vapors, and production in variety of different physiological conditions suggest, its potential application in food and medical microbiology.

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