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Screening and Evaluation of Marine Bacteriocins against Aquaculture pathogens

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Abstract: This study is aimed to isolate bacterial strains from marine environment and assess their bateriocin-producing activity. Among a total of 5 strains isolated from the marine sponge (*Tedania anhelans*), 2 strains (40%) were found to produce bacteriocins against the aquaculture pathogenic *Vibrio*s. The highest bacteriocin production activity was shown by two strains, namely S3 (*Bacillus thuringiensis*) and S4 (*Bacillus subtilis*). The antimicrobial activity of crude bacteriocin extracts from these strains was inactivated when treated with proteinase K and Trypsin. The growth curve and the bacteriocin production during different phases were studied. The bacteriocins produced by the isolates were partially purified by acetone precipitation. These substances were heat-sensitive and stable at a wide range of pH 3-5. This study explores the possibilty of marine bacteriocins as probiotics for sustainable aquaculture and for the biocontrol of *Vibrios* in particular.
Keywords : Marine Bacteriocins, *Bacillus*, Aquaculture pathogens.

Introduction

The Ocean is considered to be the mother of life that has given rise to many biological activities on the planet. It consists of a variety of microorganisms that are different in their physiology and adaptations. The marine environment is a largely unexplored source for the isolation of new microbes that are potent producers of bioactive secondary metabolites. A number of biologically active compounds such as anti-tumor, anti-cancer, anti-microtubule, anti-proliferative, cytotoxic, photo protective as well as antibiotic and antifouling properties has been isolated from the marine sources to date [1]. About 37% sponges, 21% coelenterates and 18% microorganisms are major sources of biomedical compounds, followed by 9% algae, 6% echinoderms, 6% tunicates, 2% molluscs, 1% bryozans, etc. in the marine environment [2].

Aquaculture is a growing production sector for high-protein animal food. The contribution of aquaculture to the total production of capture fisheries and aquaculture rose from 34.5% in 2006 to 36.9% in 2008.

A major setback in aquaculture is due to the sudden outbreak of diseases caused by *Vibrio* sp. The disease losses were globally estimated to be approximately US $3 Billion per year by the World Bank in 1997 [3]. Use of prophylactic antibiotics to cure the aquaculture diseases was proved to be detrimental. Hence, the scientific communities proposed a friendly alternative such as Bacteriocinogenic bacterial strains that appeared to be excellent for a friendly alternative since bacteriocin would be used as an antibiotic substitute [4].

Bacteriocins are proteinaceous compounds that are synthesized ribosomally and lethal to bacteria closely related to the producing bacteria, the latter being protected by an immunity phenomenon. In otherwords,

they are toxins produced by bacteria to inhibit the growth of closely related strains. One way to substitute antibiotics smartly and sustainably is by the selection of bacteriocinogenic and anti-pathogenic strains from animal-associated bacterial microorganisms for use as probiotics.

Materials and Methods

Collection of the Sample

The marine sponge (*Tedania anhelans*) was collected for the isolation of marine bacteria from Vizhinjam coast (10° 00' N, 76° 25' E), comprising the southern part of Kerala. *Tedania anhlans* belongs to the Phylum Porifera, Class Demospongiae, Order Poecilosclerida, Sub-order Myxillina, Family Tedaniidae and Genus *Tedania.*

Isolation of strains

The microorganisms associated with the sponges and sea weeds were isolated by culturing the crude in Zobell's marine agar 2216 (HiMedia) for the isolation of heterotrophic bacteria. The crude sample obtained from the sponges were serially diluted in normal saline (0.85% NaCl) and plated on prepared Zobell's marine agar plates to obtain the isolated bacterial colonies [5]. The plates were incubated at 20-25°C. The isolates were grown in Zobell medium, stored in refrigerator in Zobell agar slants and was used for all the experiments. The Zobell marine medium was used in all the experiments. Colonies showing different morphologies based on size shape and colour were picked. The isolated colonies were purified using streak plate technique and stored in Zobell marine agar slants at 4°C in a refrigerator. Glycerol stocks were made for preserving the cultures for further use. The antimicrobial activity was checked using pure cultures of aquaculture pathogens namely, *Vibrio vulnificans*, *Vibrio harveyi*, *Vibrio parahaemolyticus, Vibrio alginolyticus* obtained from National Centre for Aquatic Animal Health, Kochin.

Bacteriocin production

The isolates were grown in Zobell marine broth 2216 (HiMedia), incubated at 37°C for 24 hrs in environment shaker at 123 rev min^{-1}. The culture broth was centrifuged at 9400 *g* for 15 min at 4°C. The supernatant was collected and filtered through 0.22-µm membrane. This filtrate was used to evaluate antimicrobial activity without any heat treatment or change in pH.

Detection of anti-microbial activity

Antibacterial activity was determined by agar well diffusion assay [6]. Zobell marine agar plates were swab-inoculated with the test organisms grown in Luria Bertani broth (HiMedia) for 12 hrs and wells were cut. 20 µl of culture filtrate was added into the wells. Plates were incubated for 24 hrs at 37°C and the inhibition zones were measured. The assay was conducted in triplicate.

Enzyme treatment

To check the chemical nature of bacteriocin, the crude bacteriocin was treated with Proteinase K and Trypsin at a final concentration of 1 mg ml^{-1}. The fluids were incubated at 55°C for 3 hrs. The residual activity after enzyme treatment was determined by agar-well diffusion. Plates were incubated for 24 hrs at 37°C and the inhibition zones were measured.

Growth curve and bacteriocin production in Zobell's medium

The growth curve and bacteriocin production were investigated. The selected positive strains were cultured in Zobell medium. The temperature was maintained at 37°C. The agitation speed was 123 rpm and the pH was not controlled. Samples were taken at 3 hrs interval to measure the growth rate and bacteriocin activity. Growth rate was determined by measuring the optical density at 600nm (OD_{600}) using UV-visible spectrophotometer (VIS-Spectrophotometer-169, Systronics, India), and bacteriocin activity was tested by agar-well diffusion assay.

Effect of temperature

To check the thermal stability, samples of crude bacteriocin preparation were exposed to different temperatures (30°C, 45°C 60°C and 100°C) for 30 min and 121°C for 15 min. The samples were cooled and the residual activity was checked by agar-well diffusion method.

Effect of pH

The sensitivity to different pH was estimated by adjusting the pH of cell-free supernatant to pH 3, 4, 5, 6, 7, 8, 9 and 10 with 1N NaOH and 1N HCl. After 30 min of incubation at 30°C, the supernatant samples were tested by agar-well diffusion method against the aquaculture pathogenic strains.

Results and Discussion

Isolation of strains

The bacterial strains isolated from the marine sponge was identified using 16S rRNA gene sequence analysis. In 16S rRNA sequencing and phylogenetic analysis, the strains (S1, S2, S3, S4 and S6) isolated had closest match to *Bacillus* sp. Among the five isolates, strain S3 and S4 had 98% and 97% similarity to its nearest match in Gen bank respectively (Table 1).

Table 1: List of sponge associated microbes showing bacteriocin activity

Isolate	Strain	Accession no.	Source	Length [nt]	Nearest relative	Identity [%]	Phylogenetic affiliation (Phylum)
Sample 3	*Bacillus thuringiensis* KDRSS3	JX489609	*Tedania anhelans*	1436	*Bacillus thuringiensis* Bt407 (CP003889)	98	Firmicutes
Sample 4	*Bacillus subtilis* KDRSS4	KF726124	*Tedania anhelans*	1503	*Bacillus subtilis* DSM 10 (AJ276351)	97	Firmicutes

Screening of sponge associated bacteria for bacteriocin activity

In total, 5 strains were isolated from the marine sponge (*T. anhelans*). Of these, 2 strains (S3 and S4) exhibited inhibitory activity against *V. vulnificus*, *V. harveyi*, *V. alginolyticus* (Table 2). The antimicrobial activity was checked by agar well diffusion method. The zone of inhibition was measured in millimeters after 24 hrs of incubation period (Fig 1). The bacteriocin activity was inactivated by proteinase K and Trypsin, indicating a proteinaceous nature of the inhibitory compounds, called bacteriocins or bacteriocin like substances (BLIS). Proteinaceous substances are cleaved by enzymes such as trypsin and proteinase K.

Fig 1: Zone of Inhibition by sponge isolates

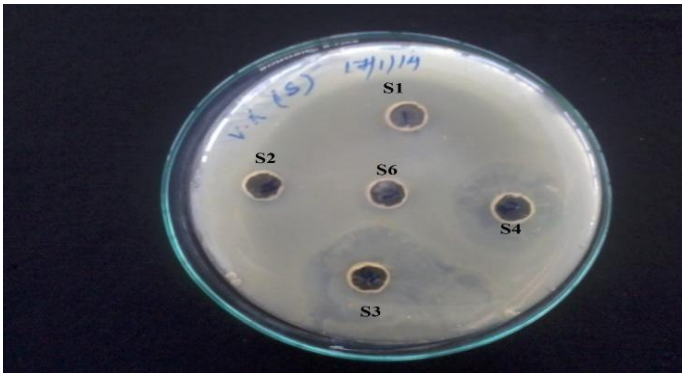

Table 2: Zone of Inhibition of the isolated strains against the aquaculture pathogens

Pathogens / Strains	Vibrio vulnificus	Vibrio harveyii	Vibrio parahaemo--lyticus	Vibrio alginolytic--us	Trypsin treatment	Proteinase K treatment
***Bacillus licheniformis* KDRSS1**	-	-	-	-	-	-
***Bacillus anthracis* KDRSS2**	-	-	-	-	0	0
***Bacillus thuringiensis* KDRSS3**	26 ± 1	15±1	-	-	0	0
***Bacillus subtilis* KDRSS4**	25 ± 1	25 ± 1	-	-	0	0
***Bacillus subtilis* KDRSS6**	-	-	-	-	0	0

Growth curve and bacteriocin production in Zobell's medium

The growth curve study was done for the positive isolates that showed the clear zone of inhibiton against the aquaculture pathogens by measuring the OD value of the culture at 600nm spectrophotometrically for every 3 hrs interval. The positive isolates were S3 and S4. The bacteriocin activity was checked for the positive strains at each time interval by agar-well diffusion method. The bacterial strains reached the exponential phase from 18th hour to 21st hour and attained the stationary phase during the 48th hour. The growth started to decline from 48th hour till the 72nd hour. The culture free supernatant was screened for bacteriocin activity at every three hours interval up to 72 hrs to find the optimum time of bacteriocin production. The bacteriocin activity was found to maximum during the exponential phase (Fig 2 and Fig 3).

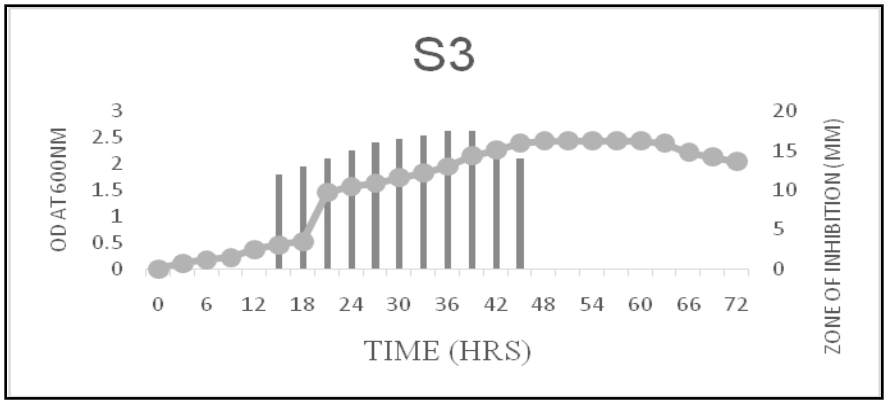

Fig 2: Growth curve and bacteriocin production at different phases for *Bacillus thuringiensis* KDRSS3

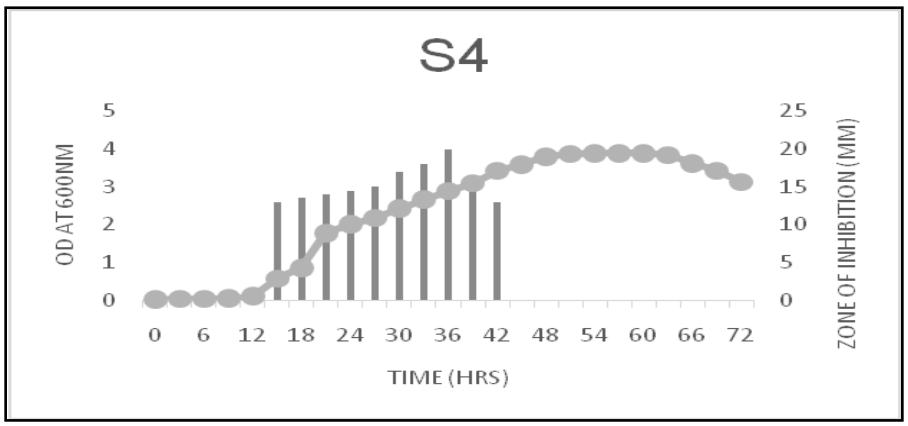

Fig 3: Growth curve and bacteriocin production at different phases for *Bacillus subtilis* KDRSS4

Effect of temperature

The effect of temperature was checked on the positive isolates by measuring the zone of inhibition against the respective pathogens. The bacteriocin activity was inhibited when the temperature was increased above 50°C (Fig 4 and Fig 5). Increase in temperature decreases the bacteriocin activity and it showed that the bacteriocin activity was completely inactivated by autoclave conditions.

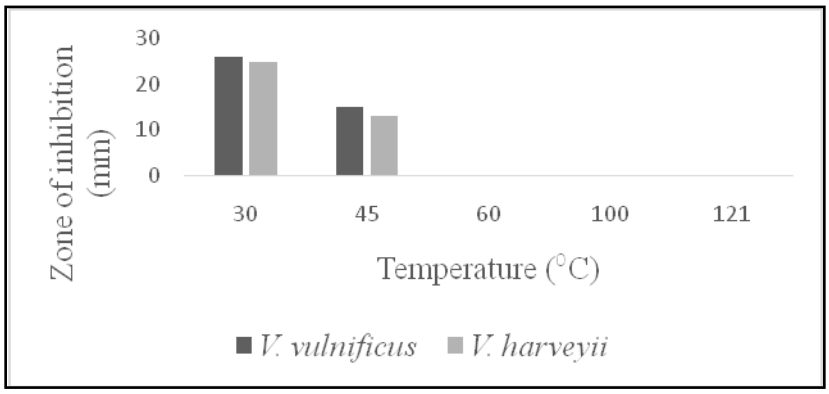

Fig 4: Effect of temperature on *Bacillus thuringiensis* KDRSS3 against *V. vulnificus* and *V. harveyii*

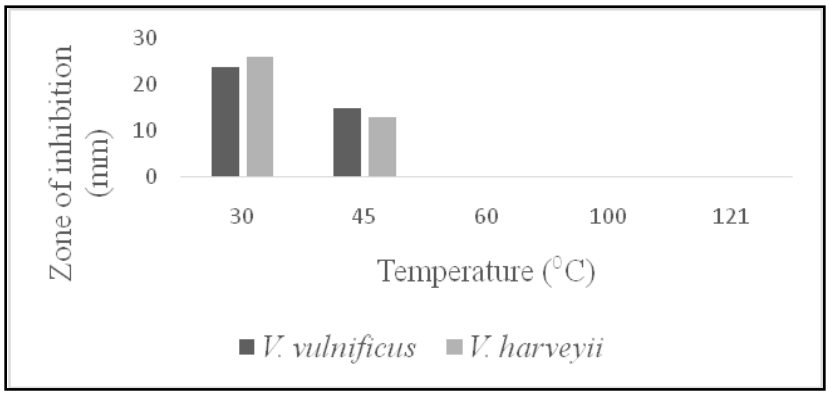

Fig 5: Effect of temperature on *Bacillus subtilis* KDRSS4 against *V. vulnificus* and *V. harveyii*

Effect of pH

The effect of pH was checked on all the positive isolates against the pathogen *V. vulnificus* by measuring the zone of inhibition. Experiments testing the effect of pH on bacteriocin activity showed that it was relatively stable at pH 3-5 for both the strains (Fig 6).

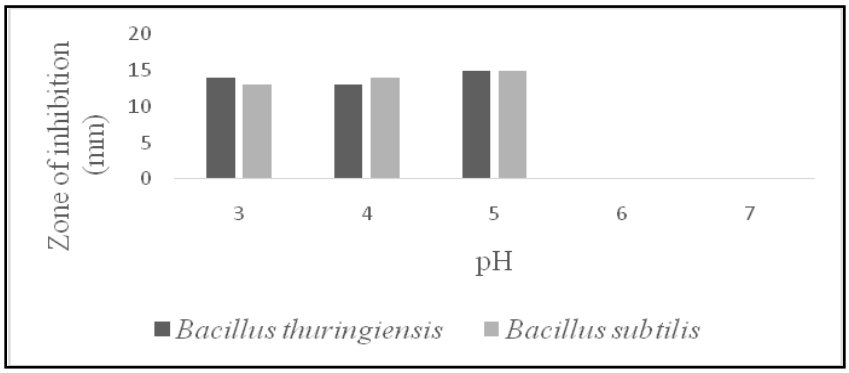

Fig 6: Effect of pH on the positive isolates

Conclusion

The Bacteriocins that are produced by the genus *Bacillus* are considered as significant as the bacteriocins produced by the lactic acid bacteria (LAB). The genus *Bacillus* produces a diverse array of antimicrobial peptides with different chemical structures [7, 8]. The few bacteriocins reported from *Bacillus* species have their antibacterial activity restricted to Gram-positive organisms. Bacteriocins active against Gram-negative organisms have been previously reported from other *Bacillus* sp.

The present study is on bacteriocin from marine origin. The activity of bacteriocin from *Bacillus* was stable between 30-50 ºC and over a wide pH range of 3-5. Bacillocin 490 from *B. licheniformis* 490/5 was stable at 4°C and 100°C over a wide pH range [9]. BLIS from *B. thuringiensis* showed stability at pH 3–5 and lost its activity on exposure to 121°C for 15 min [10]. Some bacteriocins lose their activity when subjected to high temperatures. Cerein 8A from *B. cereus* 8A had broad pH stability (pH 2–11) and was relatively thermostable, losing activity only at temperatures above 75°C for 30 min [11]. The major advantage on *Bacillus* strains for their survival in diverse habitats is the production of antimicrobial substances and its sporulating capacity. The presence of these bacteria in food does not always lead to spoilage or food poisoning. Several species of bacteria are used in human and animal food production. *B. subtilis* strains are used in the production of Natto, an East Asian fermented food [12]. It is also used as a starter culture for the fermentation of soya beans into the traditional West African condiment dawadawa [13] or African mesquite seeds for the Nigerian food condiment okpehe [14].

Bacteriocins from *Bacillus* are also used potential preservative in dairy products like milk and cheese [15]. Bacteriocin from *B. thuringiensis* strain with its antibacterial activity over a broad pH range can be used to address two important aspects: as a therapeutic agent and as a biopreservative in food processing industry considering its antibacterial activity against pathogenic Gram positive bacteria that cause foodborne illnesses.

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