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BIO-PHYSICO-CHEMICAL CHARACTERIZATION OF BLIS FROM AQUATIC BACTERIA

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ABSTRACT

Although more than 200 bacteriocins have been isolated yet numerous antimicrobial compounds or BLIS are still to be fully characterized. The aim of the current study was to characterize novel significant aquatic Bacteriocins like inhibitory substance (BLIS). The two most significant and thermostable bacteriocinogenic aquatic strains were chosen out of previously isolated 10 strains for bio-physico-chemical characterization and identified as *Bacillus coagulans* and *Bacillus* spp. (ZP-108). Our findings showed that the bacteriocin was sensitive to trypsin as partially losing activity after enzyme treatment indicating the inhibitory substance contains the proteinaceous nature. The arbitrary units of *Bacillus coagulans* were 12500/mL having two weeks shelf life, while *Bacillus* spp. (ZP-108) having three weeks shelf life and 1250 arbitraty units/mL. Bacteriocinogenesis was optimum in Nutrient agar medium having 0.5% concentration of NaCl. Cell free neutralized supernatant (CFNS) of aquatic bacteriocinogenic strains exhibited activity within a wide pH range of 1-11 for *Bacillus coagulans* and 1-9 for *Bacillus* spp. (ZP-108). *Bacillus* spp. (ZP-108) was stable against organic solvents like methanol, acetone and chloroform while *Bacillus coagulans* against ethanol. Both strains were also resistant to the surfactant SDS. Even against other organic solvents and surfactants tested none of the strains lost more than 25% of their residual activity. Altogether the findings of the present study strongly suggest that both the thermostable bacteriocins having high titer and quite resistant to wide range of pH, organic solvents and surfactants can have a great potential for the broad based practical applications.

Key Words: Bacteriocin, Bacillus, Antimicrobial compounds, drug resistance, antagonism

INTRODUCTION

The microorganisms from aquatic environment are believed to keep a lot of secrets in their genes regarding their survival in harsh conditions of the sea. These unicellular organisms contribute a lot in the ecosystem as the metabolites produced by the marine microbiota are more stable than those of animal and plant origins (Stach *et al.*, 2003) as they compete with each other for the nutrients and space developing unique properties within themselves (Zhang and Kim, 2010). However, in comparison with the terrestrial ecosystem the aquatic ecosystem still remains unexplored and underexploited (Querellou *et al.*, 2010).

The most of the bacteriocins are found to be produced by Eubacteria including both the gram positive (Jack *et al.*, 1995) and gram negative bacteria (Milind and Margaret, 2007) and the Archaebacteria (Webster *et al.*, 1991) called as Archaecins. Although, the bacteriocins from gram positive and negative are more or less similar, there is bit difference regarding their tolerance towards the heat and the spectra (Ingolf *et al.*, 2007). Moreover, the gram positive bacteriocins are mostly excreted into the environment. Up till now, gram positive bacteriocinogenic strains are subjected more to study and a lot is known because of their wider spectra (Jack *et al.*, 1995). Up till now, more than 200 bacteriocins have been isolated (Desriac *et al.*, 2010). On the other hand some bacterial species produce antimicrobial compounds that show numerous bacteriocin-like characteristics but are not fully characterized. These compounds are called bacteriocin like inhibitory substance (BLIS) (Messi *et al.*, 2003).

Aquatic bacteriocins or BLIS are diverse however; they do have some characteristics common to bacteriocin from terrestrial bacteria. For e.g. aquatic bacteriocins can be small peptides like class I and class II bacteriocins of Gram positive bacteria, they can be large in size like colicins of Gram negative bacteria, they can still remain active after cold treatment like colicins and colicins like bacteriocins or they can be inactivated after cold storage like phage-tail like bacteriocins, they can be resistant to treatment with organic solvents including ethanol, methanol, acetone, chloroform similar to some bacteriocins of Gram positive bacteria and Archaea. Moreover aquatic bacteriocins are produced during the stationary phase of growth similar to bacteriocins from Gram positive bacteria (Pinto *et al*., 2009).

Bacillus is Gram positive rod shaped, endospore forming, aerobic or facultative anaerobic bacterium. Many of the Bacillus sp. exhibit broad range of physiological abilities to cope with every natural environment (Claus and Barkeley, 1986). Members of genus *Bacillus* produce variety of antimicrobial compounds representing several different basic chemical structures (Von Dohren, 1995). Bacteriocins or BLIS production has been described in several species of Bacillus genus e.g. *Bacillus subtilis*, *Bacillus cereus* (Bizani and Brandelli, 2002), *Bacillus thuringiensis* (Paik *et al.*, 1997), *Bacillus coagulans*, *Bacillus brevis*, *Bacillus lichniformis*, *Bacillus amyloliquefaciens* and other *Bacillus* species (Risoen *et al.*, 2004).

Since last many years, significant research has been focused on bacteriocins from lactic acid bacteria (LAB) (Jack *et al*., 1995) while bacteriocins of *Bacillus* sp. have given relatively little attention even through some representatives of genus *Bacillus* such as *Bacillus subtilis* and *Bacillus licheniformis*, are generally regarded as safe bacteria (GRAS).

Considering the potential of bacteriocins regarding their activity against other bacteria, the scientists started ground-working on bacteriocins to be used as the therapeutic agent. In this aspect, Svetoch *et al.* (2011) checked the potential of enterocin S760 produced by *Enterococcus faecium* LWP760 against the *Bacillus anthracis* M-71 infection in the mice giving 90-100% results. Moreover, the efficacy of nisin, the GRAS bacteriocin was assayed in the bovine mastitis caused by *Staphylococcus aureus, versus* the gentamycin (GM) antibiotics (Cao *et al.,* 2007). Nisin therapy, according to the study was found to be effective to eliminate some drug resistant *S aureus.*

Scientists have delved into the task to overcome the pathogens that have developed the resistance against already explored antibiotics. As the matter of fact, few of the clinically important strains of pathogens have become resistant to almost all of the available agents generating MDRs (Multiple Drug Resistance). One of the significant threat among the MDR is MRSA (Methicillin Resistant *Staphylococcus aureus*) which is resistant not only to the Methicillin but also to the aminoglycosides, macrolides and other antibiotics and thus can be fatal to immunocompromised person.

At present, there are relatively few reports in the literature of antibacterial peptides produced by aquatic bacteria. In our previous study we had isolated 41 BLIS from aquatic environment of Sindh inhibiting various important clinical bacterial pathogens like MRSA, VRE and *S pyogenes*. Since these bacteriocinogenic compounds have variable bio-physico-chemical properties, in the proposed study we would like subjecting these compounds to various physical and biochemical parameters. Proposed research is necessary for characterizing these bacteriocinogenic compounds so that these may be used for broad based future practical applications.

MATERIALS AND METHODS

Bacteriocinogenic Strains and media: Significantly producing 10 BLIS were selected that have already been isolated from aquatic regions of Sindh. These strains have been preserved in glycerol stocks.

1) Thermostability of bacteriocin preparation: The 24 hours old bacterial suspension was centrifuged twice at 10,000 for 10 minutes. The cell free neutralized supernatant (CFNS) was passed to 0.22 µL millipore filters and then were exposed to 40˚C, 60˚C, 80˚C, 100˚C and 121˚C for 15 minutes. Subsequently, antimicrobial activity was checked in terms of residual activity against *Micrococcus luteus* using agar well method (Bilkova *et al.,* 2011).

2) pH stability: Bacteriocin preparation were adjusted to different pH levels between 2 to 12 with 10mM NaOH or 10mM. All the samples were readjusted to pH 7.0 with sterile 4.0 mM phosphate buffer and subsequently assayed for activity (Xie *et al.,* 2009, Singh *et al.,* 2012)**.**

3) Effect of NaCl concentrations on bacteriocinogenesis: Various concentrations of NaCl (0.5, 1.0, 2.5, 5.0%) were prepared in medium and were stabbed with the producer strain and maximum zone of inhibition was determined (Pirzada *et al.,* 2004).

4) Effect of various media on production of bacteriocins: Different media like Nutrient agar, Tryptone soy agar, Brain heart infusion agar, Iso sensitivity agar, Cysteine lactose electrolyte deficient medium (CLED) were tested to observe the effect of different media on bacteriocinogenesis (Saleem *et al.,* 2009).

5) Effect of organic solvents: Equal volumes of bacteriocin preparations were mixed with organic solvents including: methanol, ethanol, propanol, acetone and chloroform in a final concentration of 1.0%. Samples were incubated at 37^{o} C for 30 minutes, evaporated and subsequently assayed for antimicrobial activity agar well method (Xie *et al.,* 2009).

6) Effect of surfactants: Equal volumes of bacteriocin preparations were mixed with surfactants like SDS, EDTA, Tween 20 and Tween 80 in a final concentration of 1.0%. Samples were incubated at 37^{o} C for 6 hrs and subsequently assayed for antimicrobial activity (Xie *et al.,* 2009).

7) Bacteriocin titre: The 24 hours bacterial suspension was centrifuged twice (at 4,000 rpm for 30 minutes and at 10,000 rpm for 15 minutes). The CFNS of each strain was serially diluted by 10 folds in sterile nutrient broth.

Antimicrobial activity was assayed of all the dilutions and undiluted supernatant against *M. luteus* as an indicator (Irshad *et al.*, 2012).

$$AU/mL = \frac{\text{reciprocal of the highest dilution giving inhibition} \times 1000}{\text{Volume of bacteriocin}}$$

8) Shelf life of BLIS: The cell free neutralized supernatants of the producers was kept at 4˚C temperature and their antimicrobial activity was assayed for a month by agar well diffusion technique.

9) Effect of proteolytic enzyme: BLIS containing the proteolytic enzyme trypsin at a final concentration of 1.0 mg/mL was incubated at 37^{o} C for 1 hour. The residual activity was subsequently assayed (Saleem *et al.*, 2009; Xie *et al.*, 2009).

RESULTS AND DISCUSSION

The emergence of drug resistance among life threatening pathogens has brought the human life to a risk (Baylan, 2011; Okeke *et al.*, 2007). Here, bacteriocins have proved to be the best alternatives to already marketed antibiotics against which pathogens have developed resistance. Still, bacteriocins are needed to be studied in details to make the full use of the natural recourses instead of going for artificial ones.

To select the most significant BLIS 10 bacteriocinogenic strains that were previously isolated and preserved in the lab were re-examined for their antimicrobial activity. ZP-57, 61, 107, 108, 111 and 152 were selected for further characterization as all these strains gave significant zones of inhibitions in three activity monitoring methods applied (Table1).

Table1. Selection for the significant BLIS.

Producer Strain	Antimicrobial activity		
	Agar Well Method (mm)	Stab and Overlay (mm)	Spot Agar Method (mm)
ZP-57	25	-	30
ZP-73	24	30	-
ZP-152	30	-	-
ZP-75	-	31	30
ZP-107	25	-	-
ZP-61	28	-	-
ZP-77	-	32	30
ZP-82	-	-	25
ZP-108	27	30	32
ZP-111	27	30	33

Subsequently, the crude supernatant was exposed to different temperatures to check stability of BLIS ranging from 60˚C to 121˚C for 15 minutes. Strains ZP-57, 61 and 152 were stable till 60˚C and ZP-107 till 80˚C. Hence ZP-108 and ZP-111 were selected for further bio-physico-chemical characterization as these strains were the most stable and even resist the autoclaving temperature of 121˚C (Table 2).

ZP-111 was identified as *Bacillus coagulans* and ZP-108 belong to Bacillus spp. (Fig. 1).

CFNS of aquatic bacteriocinogenic strains exhibited activity within a wide pH range. *Bacillus coagulans* remained stable at the pH from 1-11 while *Bacillus* spp. ZP-108 in the range of 1-9 (Table 3). However the activity of both strains disappeared at the extreme pH 13.

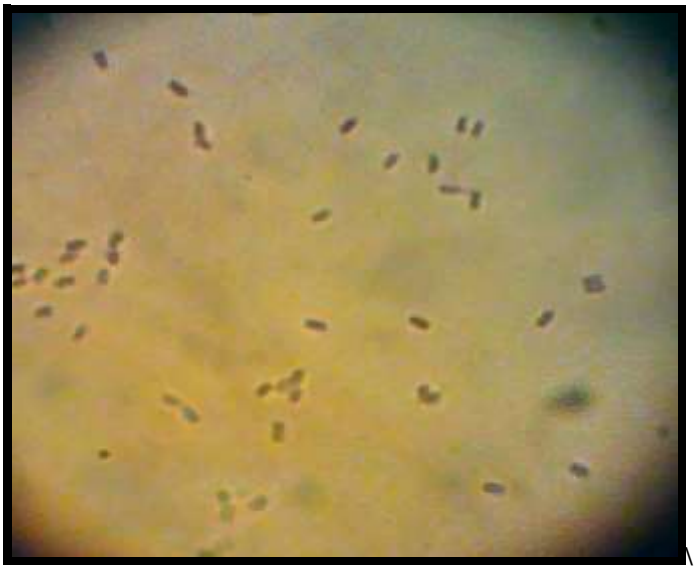

Fig. 1. Microscopy of bacteriocinogenic strain: *Bacillus* spp.

Table 2. Effect of Temperature on bacteriocinogenic activity

Strains	˚C	60˚C	80˚C	100˚C	121˚C	Interpretation
ZP 57	20	16	0	0	0	Stable till 60˚C
ZP 61	20	16	0	0	0	Stable till 60˚C
ZP 107	20	16	14	0	0	Stable till 80˚C
ZP 108	27	27	26	25	20	Stable till 121˚C
ZP 111	27	27	27	26	20	Stable till 121˚C
ZP 152	20	16	0	0	0	Stable till 60˚C

Table 3. Effect of pH on bacteriocinogenic activity

Bacillus spp. ZP-108		*Bacillus coagulans*	
pH	Residual activity	pH	Residual activity
1	87	1	90
3	78	3	90
5	100	5	100
7	100	7	100
9	87	9	86
11	00	11	72
13	00	13	00

Various media including Nutrient agar, Tryptone soy agar, Brain heart infusion agar, Iso sensitivity agar and CLED were used for monitoring the yield of bacteriocin production by stab-overlay method. However, the maximum zone of inhibition appeared in Nutrient agar medium.

Aquatic bacteriocinogenic strains were grown in Nutrient agar medium having different concentrations of NaCl. It was found that at 0.5% concentration of NaCl (Table 4) the zone of inhibition was maximum. More than 50% of the activity got lost when increasing more than 1% of NaCl concentration in the medium.

Table 4. Effect of NaCl on bacteriocinogenic activity

Strains	0.5%	1%	2.5%	5%
Bacillus spp. ZP 108	100	60	40	10
Bacillus coagulans	100	80	50	20

Different organic solvents such as acetone, ethanol, methanol, chloroform and butanol (at final concentration of 1.0%) were mixed with CFNS of aquatic bacterial isolates. *Bacillus* spp. ZP-108 was stable against methanol,

acetone and chloroform while *Bacillus coagulans* was against ethanol. Even against all other organic solvents tested none of the strains lost more than 25% of their activity suggesting that these BLIS are quite resistant to organic solvents (Table 5). All organic solvents (as control) had no activity on the indicator culture.

Table 5. Effect of Organic solvents on bacteriocinogenic activity

Strains	Methanol	Ethanol	Acetone	Butanol	Chloroform
Bacillus spp. ZP-108	100	92	100	100	80
Bacillus coagulans	94	100	76	76	80

Different surfactants like Tween 20 and 80, EDTA and SDS (at final concentration of 1.0%) were mixed with CFNS of bacteriocinogenic isolates. The residual antibacterial activity did not reduce significantly after surfactants treatment suggesting that the protein is resistant to the treatment with it. However *Bacillus* spp. ZP-108 strain was completely sensitive to Tween 20 treatment (Table 6).

Table 6. Effect of Surfactants on bacteriocinogenic activity

Strains	Tween 20	Tween 80	SDS	EDTA
Bacillus spp. ZP 108	00	93	100	80
Bacillus coagulans	65	84	100	80

To evaluate that minimum concentration of the bacteriocin that could be inhibitory to the sensitive organisms, 10 folds serial dilutions of the bacteriocins were made. Our findings showed the Arbitrary Units per mL of ZP-111 and ZP-108 to be 12500 and 1250 respectively (Table 7).

Table 7. BLIS titration

Strains	Undiluted	1:10	1:100	1:1000	1:10,000
Bacillus spp. ZP 108	+	+	+	-	-
Bacillus coagulans	+	+	+	+	-

The residual activity of the crude bacteriocins (*Bacillus coagulans* and *Bacillus* spp. ZP-108) stored at 0˚C remained 100% till the 14th day and started to decrease thereafter. The zone of inhibition of *Bacillus coagulans* disappeared by the 21st day and that of *Bacillus* spp. ZP-108 disappeared by the 28th day (Table 8).

Table 8. Shelf life of BLIS

Strains	Day 1	Day 7	Day 14	Day 21	Day 28
Bacillus spp. ZP 108	+	+	+	-	-
Bacillus coagulans	+	+	+	+	-

In order to confirm the protein nature of the BLIS, the both isolates were subjected to the proteolytic enzyme of trypsin (Table 9) by agar well diffusion method. Bacteriocin was sensitive to trypsin as the activity was partially lost after enzyme treatment. The results indicated the inhibitory substance contains also the proteinaceous nature and the preparation could be classified as bacteriocins or BLIS.

Table 9. Effect of enzyme on bacteriocinogenic activity

Treatment	Zone of Inhibition (mm)	Residual activity (%)
Bacillus spp. ZP 108		
Control	20 mm	
Trypsin	14 mm	70%
Bacillus coagulans		
Control	20 mm	
Trypsin	12 mm	60%

According to the findings of the present study both the thermostable bacteriocins having high titer and quite resistant to wide range of pH, organic solvents and surfactants can have a great potential for the broad based applications Further testing, purification and characterization at genetic level can make these compounds to be used at larger level.

Bacteriocins have a variety of applications and are widely used at industrial level (Margaret *et al* 2002). Previously chemicals were used to preserve food. In recent past, the usage of chemical food preservatives has been stopped and in many countries, it has been banned due to the side effects caused by the chemicals. Scientists are more interested in preserving the by natural means instead of the chemicals. In this aspect, the use of bacteriocins can be the best option (Settanni *et al* 2003). Nisin has been approved as food preservative from *Lactococcus* spp. From *Lactobacillus* spp., a number of bacteriocins are also been involved in food preservation (Parada *et al* 2007). Bacteriocins produced by marine bacteria have aroused great interest due to their potential to be used as antibiotics and probiotics in food industry. In addition, bacteriocins have a long list of qualities that make them best alternatives to antibiotics. Like they are non-toxic to eukaryotic cells and generally regarded as safe (GRAS) and they have relatively narrow spectrum activity in comparison to traditional antibiotics that reduces the incidence of development of drug resistant pathogens.

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