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PARTIAL CHARACTERIZATION OF BACTERIOCIN LIKE INHIBITORY SUBSTANCE FROM *BACILLUS SUBTILIS* BS15, A LOCAL SOIL ISOLATE

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Abstract

The aim of this study was to investigate the production of bacteriocin/bacteriocin-like inhibitory substances (BLIS) from *Bacillus subtilis* BS15, isolated from soil. The inhibitory substance was partially purified and characterized as BLIS with a molecular-weight of 3-5 kDa, as determined by SDS-PAGE. Its production was observed during the late exponential-phase or at the beginning of stationary-phase. It retained its activity up to 80°C and over a wide range of pH i.e., 3-9. It was found active against several clinically important bacterial species such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhi* and also against the food-spoilage causing microbes, and may be considered as future food preservative.

Introduction

Bacteria from various environmental habitats produce antimicrobial substances which are active against other bacteria (Risoen *et al*., 2004). Bacteriocins are ribosomally synthesized peptides with bactericidal activity towards species that are often closely related to the producer strain (Lisboa *et al*., 2006). These substances are produced by various gram-negative and gram-positive bacteria (Tagg *et al*., 1976). Bacteriocins are produced by the bacteria belonging to the family *Enterobacteriaceae* which are grouped into colicins and microcins while the pyocins produced by *Pseudomonas aeruginosa* were first reported by Jacob in1954. Nowadays, antimicrobial peptides and proteins produced by both gram-negative and positive bacteria are commonly referred as bacteriocins (Drider & Rebuffat, 2011). The term lantibiotics was introduced to some specific bacteriocins of gram-positive bacteria that contain an unusual amino-acid, lantithionine residues (Jack *et al*., 1995), while Klaenhammer (1993) proposed a classification for lactic acid bacteria (LAB) as lantibiotics and non-lantibiotics. Heng *et al*., (2007) grouped almost all bacteriocins into four classes. Class I bacteriocins are modified lantibiotics. Class II bacteriocins are small, heat-stable and non-modified peptides. Members of class III are large heat-labile proteins, and class IV comprises of cyclic peptides.

Lactic acid bacteria (LAB) have been a subject of intensive research as it is generally regarded as safe by the Food and Drug Administration (FDA). Until now, nisin is the unique bacteriocin produced by LAB. In comparison to antimicrobial peptide from LAB, little is known about bacteriocins from the genus *Bacillus* with the exception of BLIS produced by *Bacillus cereus* ATCC 4579 (Risoen *et al.,* 2004); megacin by *B. megaterium* (Tersch *et al.,* 1983); subtilosin by *B. subtilis* (Zheng & Slavic, 1999); BLIS by *B. amyloliquefaciens* (Lisboa *et al*., 2006); entomocin of *B. thuringiensis* (Cheriff *et al.,* 2008) and bacteriocin by *B. subtilis* LFB112 (Xie *et al*., 2009). Being capable of producing a large number of antimicrobial peptides, *Bacillus* is an interesting genus to investigate for inhibitory substances (Bizani *et al.,* 2005). The objective of the study was to evaluate potential antibacterial activity of BLIS produced by *Bacillus subtilis* BS15 isolated from an agricultural soil. The antibacterial spectrum and some properties of the BLIS are also described.

Material and Methods

Bacterial strains and growth conditions: The BLIS producing strain of *Bacillus subtilis* BS15 was isolated from an agricultural field, Malir, Karachi. All the indicator strains used during the study were grown in BHI agar or broth (Oxoid, Germany) at 37°C and maintained as 20% frozen glycerol stocks at -20°C. Identification of the isolate was performed according to the Bergey's Manual of Determinative Bacteriology (Williams & Wilkins, 1994).

Screening of BLIS and their spectrum of inhibition: The antimicrobial spectrum of the BLIS was performed by spot-overlay method with and without chloroform (Expert & Toussaint, 1985) and agar-well diffusion assay (Jack *et al*., 1995) against Gram-positive and Gram-negative bacteria.

Preparation of cell-free culture supernatant (CFCS): *Bacillus subtilis* BS15 was grown in BHI medium at 37ºC with continuous agitation at 120 rpm for 18-24 hrs. Cells were harvested and supernatant was collected, filter sterilized and quantified in terms of arbitrary unit (AU). One arbitrary unit (AU) was defined as a 50µL from the highest dilution of the bacteriocin preparation which inhibited growth of indicator strains. The activity of the bacteriocin preparation expressed in AU per milliliter was calculated with the formula (1,000/50) x (DF), where DF was the highest dilution that inhibited growth of indicator strains after 24 hrs incubation (Daba *et al*., 1991).

BLIS production kinetics: The kinetics of BLIS production was carried out as described by Bizani & Brandelli, (2002).

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Ammonium sulphate precipitation: Freshly prepared CFCS of *Bacillus subtilis* BS15 was subjected to 80% Ammonium sulfate precipitation. The resulting precipitate was separated by centrifugation at 10000rpm for 20 min at 4°C, suspended in 2.0 ml of 0.1 M Sodium phosphate buffer (pH 7.0) and used as bacteriocin like inhibitory substances (BLIS). BLIS activity was determined by agar-well diffusion assay (Jack *et al*., 1995). Protein content was determined by Bradford method (Bradford, 1976) using the Bio-Rad Quickstar ® assay kit (Bio-Rad, Germany) and bovine serum albumin was used as standard.

Physicochemical characterization of BLIS

Temperature and pH stability: Temperature stability was investigated by heating aliquots of BLIS at 60°C, 70°C, 80°C and 100°C for 30 min. and at 121°C (15 psi) for 15 min. The samples were then cooled in ice-bath and assayed for the residual BLIS activity using the agar-well diffusion assay. Similarly, effect of pH on BLIS activity was performed by adjusting the pH of CFCS with hydrochloric acid and sodium hydroxide (1N). Samples were incubated at different pH values (3-9) and neutralized after one hour (1 hr) incubation. Residual BLIS activity was detected against the indicator strain by agar-well diffusion assay (Saleem *et al*., 2009).

Effect of enzymes: BLIS was treated with proteinase K, papain, pepsin, α-chymotrypsin, lipase and lysozyme (Sigma) for 30 min. with a final concentration of 1 mg ml^{-1}. Untreated BLIS plus buffer, buffer alone and enzyme solutions served as controls (Nakamura *et al.,* 1983). After treatment bacteriocin activity was determined by AWDA.

Effect of organic solvents and EDTA on BLIS: BLIS preparations were treated with acetone, chloroform, methanol, and ethanol and EDTA, following incubation at 37°C for two hour (2 hrs) residual activity was assayed by agar-well diffusion assay. Untreated BLIS preparation and diluted solvents were served as control (Xie *et al*., 2009).

Effect of media composition on BLIS production: The following media were compared for BLIS production: brain-heart infusion (BHI), Mueller-Hinton (MH) and nutrient broth (NB). Overnight grown producer cells were inoculated 1% (v/v) and incubated for 48 hours at 37°C in shakobator. CFCS was prepared and subjected for antibacterial activity by agar-well diffusion assay (Todorov & Dicks, 2006).

Possibility of phages: Bacteriocin/BLIS, like traditional antibiotics, can diffuse tridimentionally while phages lack this ability. In order to confirm that the inhibitory activity was due to bacteriocin/BLIS, reverse side agar technique was employed as described by Kekessy & Piguet (1970).

Direct detection of BLIS on SDS-PAGE: To estimate the molecular weight of partially-purified BLIS of *Bacillus subtilis* BS15; Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 16% gel. Low molecular-weight peptide markers were used to compare the molecular mass of the BLIS (BioRad, USA). One part of gel was stained with commassie brilliant blue while the other was processed for BLIS detection. Briefly, gel was fixed in 10% acetic acid and 25% isopropanol followed by washing with sterile deionized water for one hour. After SDS concentration had dropped to nontoxic level, gel was then overlaid with soft agar (0.75%) seeded with indicator strains followed by overnight incubation at 37°C and examined for zones of inhibition (Risoen *et al*., 2004).

Results and Discussion

A total of 21 *Bacillus* strains were isolated from different environments like, *Tasman spirit* oil-spill site, agriculture soil, lab environment and clinical isolates. All the isolates were subjected to preliminary screening for bacteriocinogenesis by spot-overlay method against some selected indicator strains like *S. aureus*, *S. aureus* AB188, *S. epidermidis*, *B. cereus* 11778, *S. typhi* and *E. coli*. It was found that 10 isolates exhibited antibacterial activities in preliminary studies. These isolates were further screened by agar-well diffusion assay (Jack *et al*., 1995). Bacterial strain BS15 exhibiting strong and broad range antimicrobial activity was selected for further characterization. The *Bacillus* BS15 was identified as *Bacillus subtilis* on the basis of morphological, cultural and biochemical characteristics, as identified by others (Bizani & Brandelli, 2002; Saleem *et al*., 2009).

The production of bacteriocins or bacteriocin-like substances has already been described for *B. subtilis*, *B. cereus*, *B. stereothermophilus*, *B. megaterium* and other *Bacillus* species (Tagg *et al*., 1976). Hyung *et al*., (2001) reported a broad-spectrum bacteriocin produced by *Bacillus* sp., which is active against *B. anthracis* and *Shigella dysenteriae*. In yet another report, BLIS from *Bacillus amyloliquefaciens was* found inhibitory to several pathogenic and food-spoiling bacteria (Lisboa *et al*., 2006). In a recent study, Saleem *et al*., (2009) reported that *Bacillus brevis* bacteriocins are active against many of the gram-positive bacteria.

During this study it was revealed that BLIS from *Bacillus subtilis* BS15 showed the inhibitory activity against a wide-range of bacteria including both gram-positive and gram-negative like *B. cereus* ATCC 11778, *B. sublitis* ATCC 6633, *Corynebacterium hoffmanii*, *L. monocytogenes*, *S. epidermidis* and other *Bacillus* species locally isolated while it showed little activity against *Salmonella typhi*, and *S. dysentariae*. Similar results have been reported by others (Cherif *et al*., 2001 and 2008; Korenblum *et al*., 2005; Hyung *et al*., 2004; Lisboa *et al*., 2006; Saleem *et al*., 2009; Xie *et al*., 2009 BLIS produced by the *Bacillus* strain under study is active against pathogenic and food-spoilage microorganisms suggesting its possible future application in food-preservation and to control these pathogens and spoilage causing microorganisms.

Upon physicochemical characterization (Table 1), BLIS from *Bacillus subtilis* BS15 was found sensitive to proteinase-K and chymotrypsin resulting in the loss of activity; following the exposure to these proteolytic enzymes the residual activity was noted to 50% and 75%, respectively. However, other enzymes like papain, pepsin and lipase had no effect on antibacterial activity. It may be due to the fact that some *Bacillus* species produce cyclic peptides which are more resistant to proteases (Korenblum *et al*., 2005) while lipase and lysozyme reaction suggested that the inhibitory compound is protein in nature and require no carbohydrate moiety for its activity. Similar results have been reported from other researchers (Cherif *et al*., 2001 and 2008; Xie *et al*., 2009).

Table 1. Physicochemical characterization of BLIS from *Bacillus subtilis* BS 15 against *B. cereus* ATCC 11778 by agar-well diffusion assay.

Treatment	% Activity (AU/mL^{-1})
Untreated BLIS	100
Enzymes	
α-Chymotrypsin	75
Proteinase K	50
Pepsin	100
Papain	100
Lipase	100
Lysozyme	100
pH	
3	50
4-8	100
9	50
Temperature (°C)	
60 °C, 30 min.	100
70 °C, 30 min.	100
80 °C, 30 min.	100
100 °C, 30 min.	25
121°C, 15 min.	25
Chemicals	
Acetone (10% v/v)	100
Chloroform (10% v/v)	100
Methanol (10% v/v)	100
EDTA (1mM)	100
Ethylalcohol (10% v/v)	20

BLIS of *Bacillus subtilis* BS15 showed stability at a pH range 3-9 and found resistant to heat up to 80°C, as reported by many others (Cherif *et al*., 2001; Torkar & Matijasic, 2003; Ahern *et al*., 2003). Stability to a wide pH range and heat suggests its possible role in food preservation industry. BLIS produced by *B. subtilis* BS 15 was also resistant to many organic solvents tested, except ethyl alcohol. Stability of BLIS under study, to organic solvent suggests its probable hydrophobic nature, as the hydrophobic peptides are not affected by non-polar solvents. These results are in agreement with the findings of Hyung *et al.,* 2001; Risoen *et al*., 2004; Saleem *et al.,* 2009. Similarly, the protease inhibitors like EDTA treatment could not result in the loss of BLIS bioactivity indicating its nature other than metallo-proteins.

Optimal growth of the producer strain, synthesis and release of bacteriocins depend upon the components of media, incubation time and temperature. In the present study, maximum production of BLIS from *Bacillus subtilis* BS15 was noted when producer strain was grown in BHI medium at 37°C. Lisboa *et al*., (2006) used the BHI medium for the production of bacteriocin by *Bacillus amyloliquefaciens* at 37°C. Similar results were reported by Xie *et al*., (2009).

Generally, the production of bacteriocins by gram-positive bacteria is associated with the shift from log-phase to stationary-phase (Motta *et al*., 2007). In the present study, BLIS activity was not detected until the beginning of log-phase with an increase in anti-bacterial activity in late stationary phase (Fig. 1). Cherif *et al*., (2008) reported that the production of bacteriocin by *Bacillus thuringiensis* subsp. *entomicidus* started to appear at mid log-phase and reaches its maximum at early stationary-phase. A similar study was reported by Bizani & Brandelli (2002). Chen & Hoover (2003) also stated that in addition to the bacteriocins certain inhibitors may be produced by bacteria including enzymes, bacteriophages or other metabolic end-products. Antibacterial activity of the BLIS produced by *Bacillus subtilis* BS15 was confirmed by reverse-side agar technique.

BLIS from *Bacillus subtilis* BS15 was successfully precipitated by 80% Ammonium sulphate precipitation. The total protein was estimated before and after the precipitation and specific-activity determined which was found markedly increased after Ammonium sulphate precipitation. Cherif *et al*., (2008), Risoen *et al*., (2004) and Xie *et al*., (2009) also used 80% Ammonium sulphate saturation to precipitate bacteriocins by *Bacillus thuringiensis* subsp. entomocidus, *Bacillus cereus* and *Bacillus subtilis* LFB112, respectively.

In the present study, a single zone-of-inhibition was observed from the partially purified BLIS following non-denaturing SDS-PAGE (Fig. 2). On the basis of the position of zone of inhibition, the estimated size of BLIS peptide may be between 2.5-5 kDa. Cherif *et al*., (2001) reported that the thuricin of *Bacillus thuringiensis* BMG had an apparent molecular weight 11.6 kDa. In another research, Cherif *et al*., (2008) reported a 4.8 kDa molecular-weight bacteriocin produced by *Bacillus thuringiensis* subsp. entomocidus. Torkar & Matijasic (2003) identified 1-8 kDa while Lisboa *et al*., (2006) found 5 kDa antimicrobial protein from *Bacillus* spp. Recently, Xie *et al*., (2009) reported bacteriocin produced by *Bacillus subtilis* LFB112 with a apparent molecular-weight of 6.3 kDa determined by SDS-PAGE.

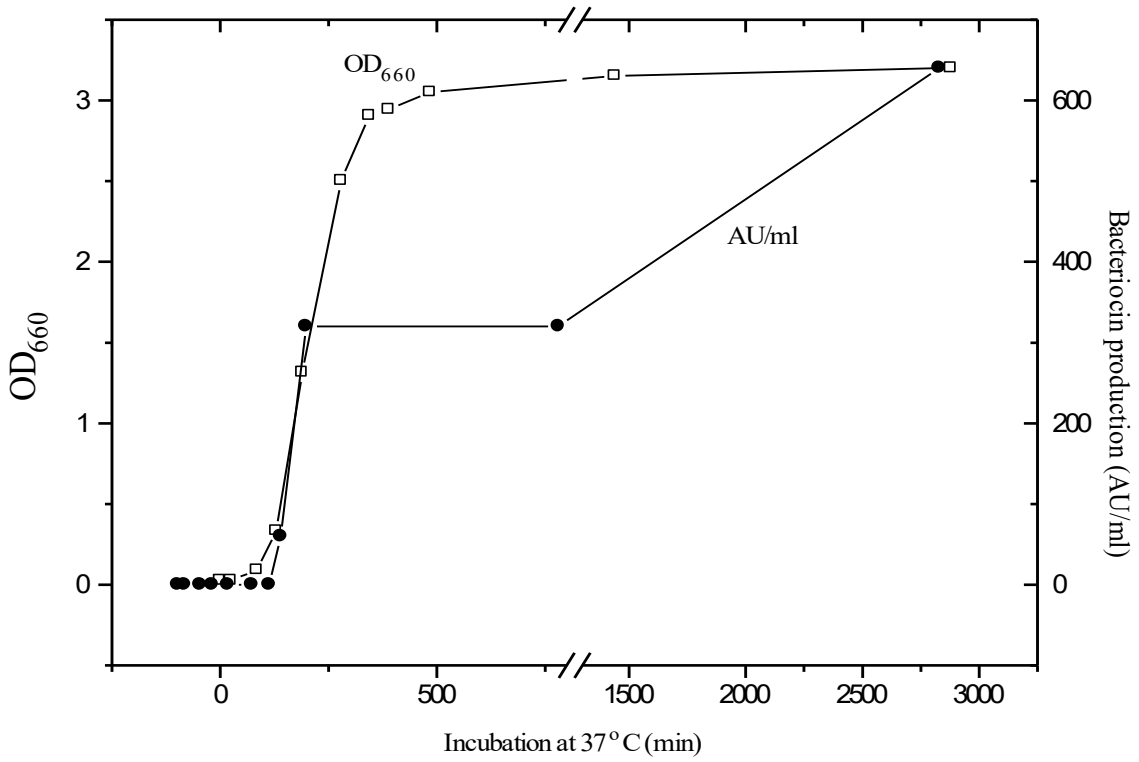

Fig. 1. Growth and production kinetics of *Bacillus subtilis* BS15. *Bacillus subtilis* BS15 was grown in BHI broth at 37°C and at various time intervals samples were taken for measurement of the optical density, OD_{600} nm (□) and for detection of bacteriocins activity (●) against *B. cereus* ATCC 11778.

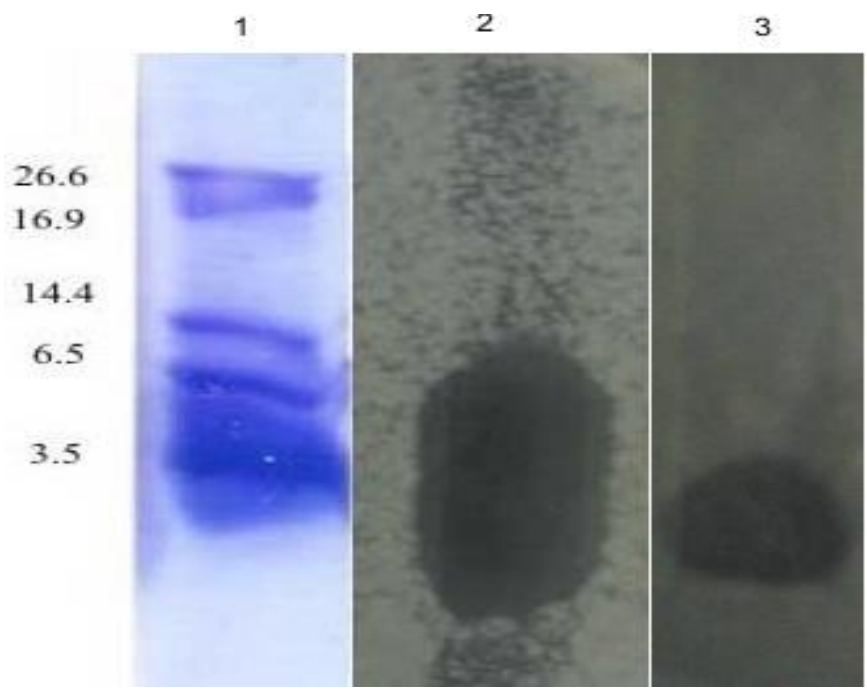

Fig. 2. SDS-PAGE and direct detection of partially purified BLIS from *Bacillus subtilis* BS15. Lane 1: Coomassie Brilliant blue-stained gel with low molecular weight of standard markers, lane 2: 80% ammonium sulphate concentrated BLIS overlaid with *B. cereus* ATCC 11778, lane 3: 80% ammonium sulphate concentrated BLIS overlaid with *S. aureus*. Sizes on the left are indicated in kDa.

Conclusion

During the present research work, the ability of *Bacillus subtilis* BS15 for the production of BLIS was investigated. This strain was isolated from the agricultural soil. On the basis of the studied characteristics of BLIS, it could be presumed that it may find applications in medical and food industry, to control the food-borne infections and food poisoning or as food-preservative that may be used while food packaging.

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