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RESEARCH ARTICLE

BIOCHEMICAL CHARACTERIZATION AND PARTIAL PURIFICATION OF A BACTERIOCIN LIKE-INHIBITORY SUBSTANCE PRODUCED FROM *Bacillus* sp. T68 STRAIN

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ABSTRACT

Bacteriocins are peptides produced by various types of bacteria. Members of the *Bacillus* genus are known to produce compounds with inhibitory activity in protein structure against pathogenic and non-pathogenic microorganisms. In this study, a bacteriocin-like inhibitory substance (BLIS) synthesized by *Bacillus* sp. T68 strain, which was previously isolated from soil, was characterized. T68 strain was grown on different media to produce bacteriocin. Crude BLIS obtained was tested by well diffusion method against indicator bacterium. It was investigated within the temperature range of 10-121 °C and pH range from 3.0 to 10.0. It was examined in terms of different organic solvents, enzymes and chemicals. Extracellularly produced BLIS was partially purified by ammonium sulphate precipitation method and analyzed on SDS-PAGE. Activity of partially purified BLIS was investigated. It was observed that BLIS produced in Luria Bertani Broth medium was higher as compared to the other media against indicator bacterium at 30 °C for 24 h. BLIS activity maintained at low temperatures (10-40 °C) and lost completely at high temperatures (> 60 °C). It was detected that BLIS exhibited activity in the pH range between 5.0 and 10.0. The effect of EDTA on BLIS activity was slightly positive. Proteinase K and trypsin inhibited BLIS activity. Among the detergents, sodium dodecyl sulphate and Triton X-100 reduced BLIS activity, while Tween 20 and Tween 80 retained it. Additionally, it was determined that application of Tween 20 at 30 °C for 5 hours increased the BLIS activity by 40%. It was found that the solvents used other than butan 1-ol preserved the BLIS activity over 80%. Chloroform and isopropanol increased the BLIS activity slightly. An inhibitory zone formed by the BLIS corresponding to a molecular weight of about 15 kDa was detected. This BLIS exhibited stability over wide pH and temperature ranges and in organic solvent treatments.

Keywords: *Bacillus*, BLIS, Antibacterial activity, *Bacillus thuringiensis* subsp. *indiana*, Soil

1. INTRODUCTION

Some bacteria produce many inhibitory compounds that can prevent the growth or development of certain microorganisms or pathogens that cause degradation in foods [1,2]. Bacteriocin, one of these compounds, was first discovered by Andre Gratia in 1925 and this inhibitor substance produced by *E. coli* was called as colisin [3]. Bacteriocins have recently received particular attention for the control of food-borne pathogens [4]. The use of bacteriocins in the food industry reduces the need for chemical preservatives and at the same time reduces the food damage caused by heat treatment [5,6].

Bacteriocins are ribosomally synthesized natural peptides produced by bacteria that kill or inhibit the growth of related bacteria [7]. Bacteriocin studies have gained great importance in the fight against numerous pathogens and the increasing number of antibiotic resistant pathogens. Most bacteriocins are produced by lactic acid bacteria (LAB) [8,9]. Some bacteriocins obtained from LAB are widely used in the food industry.

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The food industry faced a major challenge, opting for safer foods with longer shelf lives and minimally processed products without chemical preservatives [10]. The use of bacteriocins is critical for food preservation, because they extend the shelf life of foods, reduce the risk of contamination by foodborne pathogens, reduce economic losses, reduce the use of chemical preservatives, and bring to market "new" foods with lower acidity, lower salt content, and higher water content [11-13]. Nisin is the only bacteriocin commonly used as a food biopreservative. However, due to its low stability at neutral and alkaline pH and the emergence of nisin-resistant strains, new bacteriocins are needed for use in food biopreservation [14-18].

Unlike bacteriocins produced by LAB, which have a narrow antimicrobial spectrum [19], *Bacillus* bacteriocins attract attention [20]. *Bacillus* species are commonly found in almost all natural habitats and many other sources [21]. They produce important antimicrobial agents such as peptides, lipopeptides, antibiotics and bacteriocins [22]. After LAB, species in *Bacillus* genus have been extensively studied in terms of their bacteriocin and BLIS production. These studies identified many active molecules [23]. The *Bacillus* genus contains many species that have been found to be safe in the industry and food sector [24]. There are important species, such as LAB, registered as GRAS (generally recognized as safe) in the field of agriculture, industry and food. *Bacillus cereus* is one of the representatives of the genus *Bacillus*. The *Bacillus cereus* group consists of closely related species, including *B. anthracis*, *B. cereus*, *B. pseudomycoides* and *B. thuringiensis* [25,26]. In recent years, many bacteriocins have been identified in the *Bacillus cereus* group, such as lantibiotics, sactibiotics, circular bacteriocins and unmodified bacteriocins [26-29].

Bacillus sp. T68 strain was previously isolated from soil and found to have high antimicrobial activity against some tested bacteria [30]. Showing high antimicrobial activity against some pathogenic and non pathogenic bacteria make this *Bacillus* sp. T68 strain as a potential BLIS producer. In this study we aimed to identify the BLIS production ability of the *Bacillus* sp. T68 strain. Subsequently, the biochemical properties of the produced BLIS will be determined.

2. MATERIALS AND METHODS

In this study, the strain *Bacillus* sp. T68, which belongs to the *Bacillus cereus* group and has antibacterial activity [30], and *Bacillus thuringiensis* subsp. *indiana* HD521 (4S2, *Bacillus* Stock Center) was used as indicator.

2.1. BLIS Production

The BLIS production was carried out according to the methods described by Sensoy Karaoglu et al. [31] and Touraki et al. [32] with minor modifications. The preculture was prepared from the *Bacillus* sp. T68 strain in Nutrient Broth (NB) at 30 °C for about 18 h at 150 rpm (GFL 3031 incubator). To determine the effects of different media on BLIS production, Luria Bertani Broth (LBB), Triptic Soy Broth (TSB) and NB were inoculated from preculture. Samples were incubated at 30 °C for 5 days (NUVE EN120) and 2 ml of the cultures were taken every 24 hours. The cultures were centrifuged at 10,000 rpm for 30 min. The supernanant obtained was filtered through a sterile membrane filter (Whatman) with a pore diameter of 0.2 μm and used as crude BLIS for further characterization. The antibacterial activity of crude BLIS was determined using the well diffusion assay against indicator bacterium (*Bacillus thuringiensis* subsp. *indiana* HD521).

2.2. Well Diffusion Assay

To determine crude BLIS activity, a well diffusion assay was performed according to the method of Rajaram et al. [33] with slight modification. The nutrient agar (NA) medium containing 40 μl of an overnight culture (16-18 h) of the indicator bacterium was poured into the petri dish. The activity was

determined by pouring 100 µL of the crude BLIS into the hole (6 mm) generated on the NA plates. The plates were incubated at 30 °C for about 16-18 h and after incubation, the clear zones formed around the holes were measured.

2.3. Characterization of BLIS

The effects of temperature, pH, some proteolytic enzymes, organic solvents, chemicals and detergents on crude BLIS activity were identified. All experiments were performed in duplicate. The following formula was used to calculate the residual activities in % [34].

% = A * 100/K
Control zone diameter = K
Processed zone diameter = A

2.3.1. Effect of temperature on BLIS activity

To determine the effect of temperature on BLIS activity, the filtred supernatant was incubated at 10-90 °C for 30 min and at 121 °C for 15 min. Activity was then measured using the well diffusion assay.

2.3.2. Effect of pH on BLIS activity

Buffers (sodium acetate buffer, pH 3.0-5.0; potassium phosphate buffer, pH 6.0-7.0; Tris-HCl buffer, pH 8.0; and glycine NaOH buffer, pH 9.0-10.0) were prepared at a concentration of 10 mM for the pH experiments. The crude BLIS and pH buffer were mixed at a 1:1 ratio. The mixtures were treated at +4 °C for 24 h, and at 10 to 30 °C for 1 h. BLIS activity was measured using the well diffusion assay.

2.3.3. Effect of some organic solvents on BLIS activity

Organic solvents such as chloroform, acetonitrile, acetone, isopropanol, methanol, butan-1-ol, ethyl alcohol and hexane were used. Ethyl alcohol and hexane were added to the crude BLIS at a rate of 25% (v/v) and other solvents at a rate of 10% (v/v). Organic solvents without crude BLIS were used as negative controls, and crude BLIS without organic solvents were used as positive controls. BLIS activity was measured using the well diffusion assay.

2.3.4. Effect of some detergents and chemicals on BLIS activity

Sodium dodecyl sulfate (SDS), Tween 80, Tween 20 and Triton X-100 were used as detergents. The crude BLIS was incubated with different detergents at a final concentration of 1% (v/v) at 10 to 30 °C for 1 and 5 hours [35]. Untreated crude BLIS served as controls. BLIS activity was measured using the well diffusion assay.

The crude BLIS was incubated with ethylene diamine tetra-acetic acid (EDTA) at final concentrations of 0.1, 0.2, and 0.5 mM, at 10 to 30 °C for 1 and 5 h. At the end of this period, BLIS activity was measured using the well diffusion assay.

2.3.5. Effect of enzymes on BLIS activity

Proteinase K and trypsin enzymes were added to crude BLIS at a final concentration of 1 and 10 mg/ml, respectively. The mixtures were incubated at 37 °C for 1 and 2 h. Untreated crude BLIS were used as controls [36]. BLIS activity was measured using the well diffusion assay.

2.4. Partial Purification of BLIS

For the partial purification of the BLIS, ammonium sulfate was added to the crude BLIS to obtain 40%, 60%, and 80% saturation according to He et al. [37] with slight modifications. After each saturation, samples were centrifuged at 8,000 rpm for 10 min and the resulting pellets were dissolved in dH_2O and stored at -20 °C. BLIS activity was measured using the well diffusion assay.

2.5. Detection of BLIS Activity on Gel

The estimated molecular weight of the partially purified BLIS was determined by SDS-PAGE using 15% resolving and 5% stacking gels as described by Laemmli [38]. To detect the BLIS activity on gel, SDS and β-mercaptoethanol were not added to the sample buffer and the boiling step was also omitted. Electrophoresis was performed at 100 V. After electrophoresis, the gel was divided into two parts. To estimate the MW, one half, which contained the marker and samples, was placed in the staining solution prepared with Comassie Brilliant Blue R-250 (CBB R-250) for about 2.5 h and then placed in the decolorizing solution. To detect the BLIS activity, the other half of the gel was transferred to the sterile dH_2O at +4 °C, which was changed at regular intervals during about 16 and 18 h to remove SDS according to Lim et al. [39] with slight modifications. The gel was then covered with NA medium containing indicator bacterium. Plate was incubated at 30 °C for about 16-18 h and observed for the presence of an inhibition zone for the direct detection of BLIS. After incubation, the activity was examined by measuring the clear zone and compared with the stained part.

3. RESULTS

3.1. BLIS Production

The antibacterial activities of the crude BLIS of *Bacillus* sp. T68 strain grown in different media and at different time points are shown in Figure 1. Cultivation of strain T68 at 24 h proved to be best for BLIS production on LB medium.

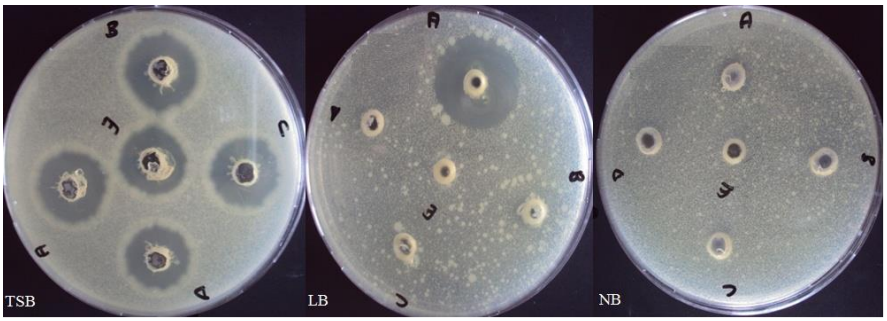

Figure 1. Antibacterial activities of crude BLIS by *Bacillus* sp. T68 in different media and at different time points. A) 24h, B) 48h, C) 72h, D) 96h, E) 120h.

3.2. Characterization of BLIS

Examination of the effect of temperature on BLIS activity identified good activity at between 10-40 °C, a decrease in activity between 50-60 °C and a loss of 100% of activity at temperatures above 60 °C. The highest BLIS activity was observed at 10 °C (Figure 2). The activity was 96% at 20 °C, 91% at 30 and 40 °C, 84% at 50 °C and 52% at 60 °C.

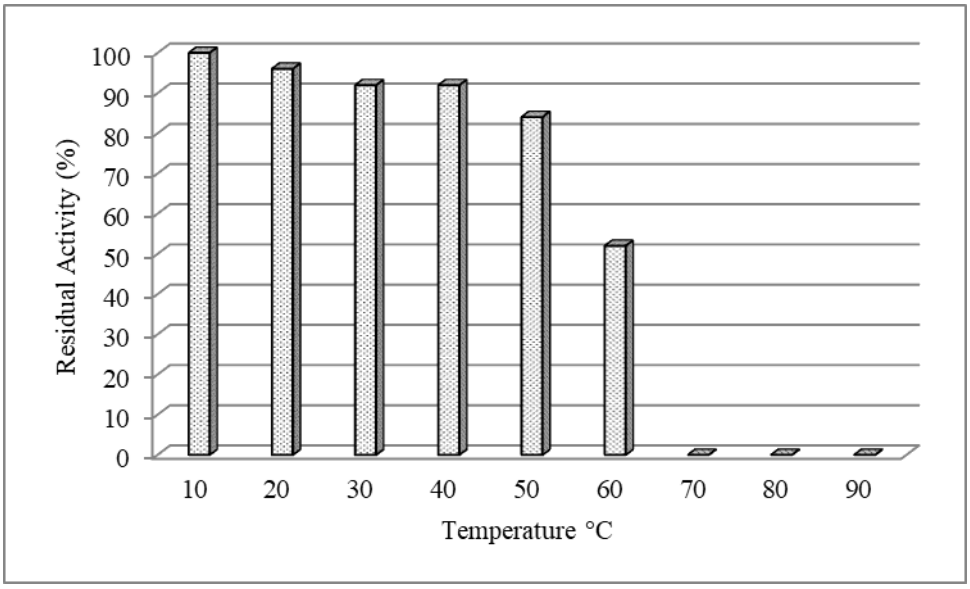

Figure 2. Effect of temperature on the activity of crude BLIS from strain *Bacillus* sp. T68.

When the effect of pH on BLIS activity was examined, it showed activity highest at pH 6 at 10 °C, highest at pH 10 at 30 °C. There was no loss of activity at pH 6, pH 9 and pH 10 at +4 °C (Figure 3).

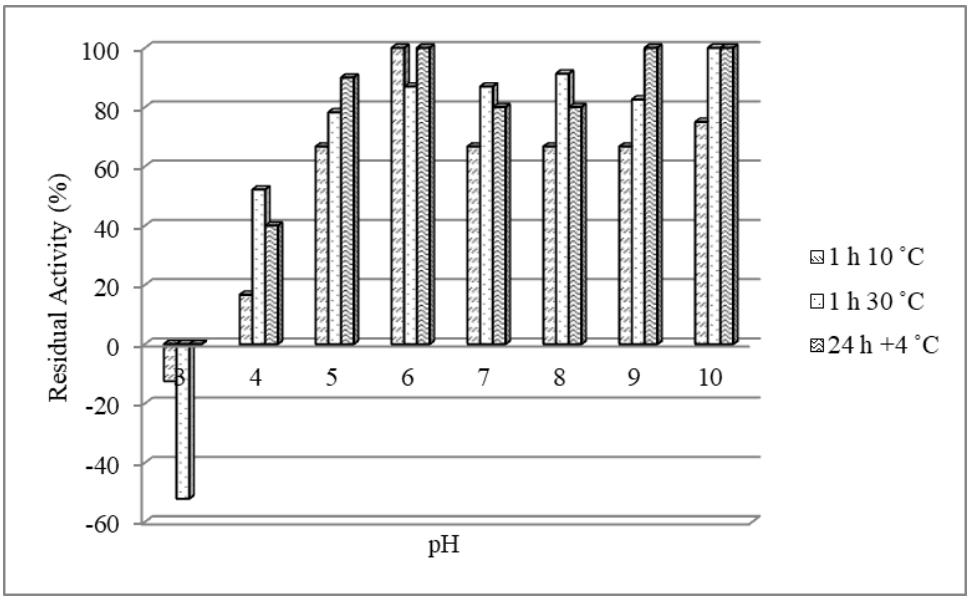

Figure 3. Effect of pH on crude BLIS obtained from *Bacillus* sp. T68 strain.

It was found that some organic solvents generally have a positive effect on crude BLIS activity. The activity of acetonitrile increased by 20%, that of ethyl alcohol by 12.5%, and that of hexane by 25% for 1 h at 10 °C, whereas the activity of methanol was reduced by 8.3%, that of butan-1-ol by 60% and that of chloroform by 10%. Isopropanol and acetone had no effect on the activity. The activity of acetonitrile and methanol for 1s at 30 °C increased by 13%, that butan-1-ol by 21%, that of isopropanol 31%, that of chloroform 33%, and that of ethyl alcohol 16%. Hexane and acetone had no effect on activity (Figure 4).

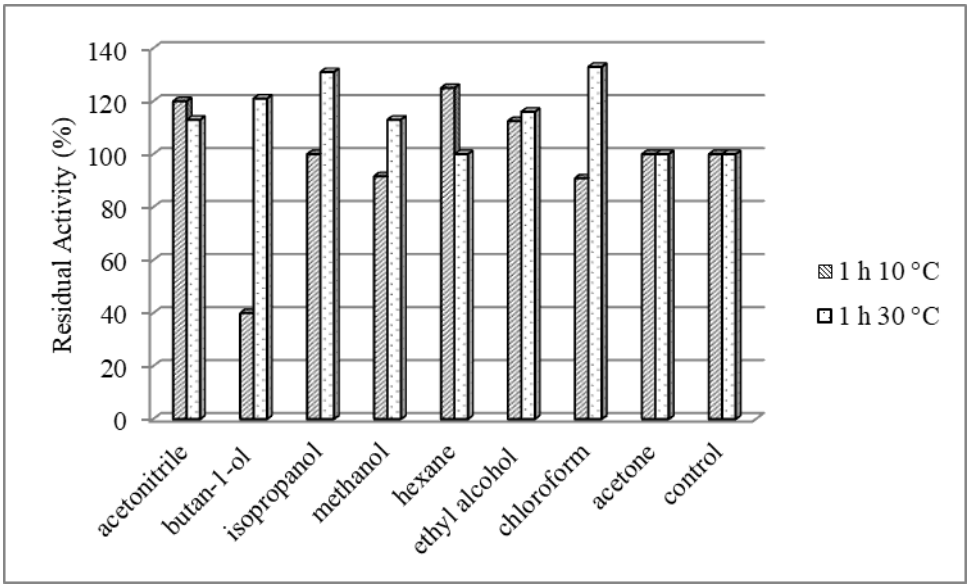

Figure 4. Effect of several organic solvents on crude BLIS obtained from *Bacillus* sp. T68 strain. 10% chloroform, acetonitrile, acetone, isopropanol, methanol, butan-1-ol, 25% ethyl alcohol and hexane sovents were used. Crude BLIS without organic solvents were used as positive controls.

When the effect of some detergents on crude BLIS was obtained, it was found that SDS and Triton X-100 were more effective in the activity for 1 and 5 h at 10 °C or 30 °C. For 1 h at 10 °C, Triton X-100 decreased activity by 91.7%, SDS decreased activity by 90.4% and Tween 80 decreased activity by 16.7%, while Tween 20 increased activity by 4.7%. For 5 h at 10 °C, Triton X-100 decreased activity by 83.3% and Tween 80 decreased activity by 16.6%, SDS inhibited activity 100%, and Tween 20 had no effect on activity. For 1 h at 30 °C, Triton X-100 decreased the activity by 75% while SDS inactivated it 100%, and activity increased by 16% in Tween 20 and Tween 80. For 5 h at 30 ˚C, Triton X-100 decreased activity by 80% while SDS inactivated it by 100%, and activity increased by 40% in Tween 20 and 25% in Tween 80 (Figure 5).

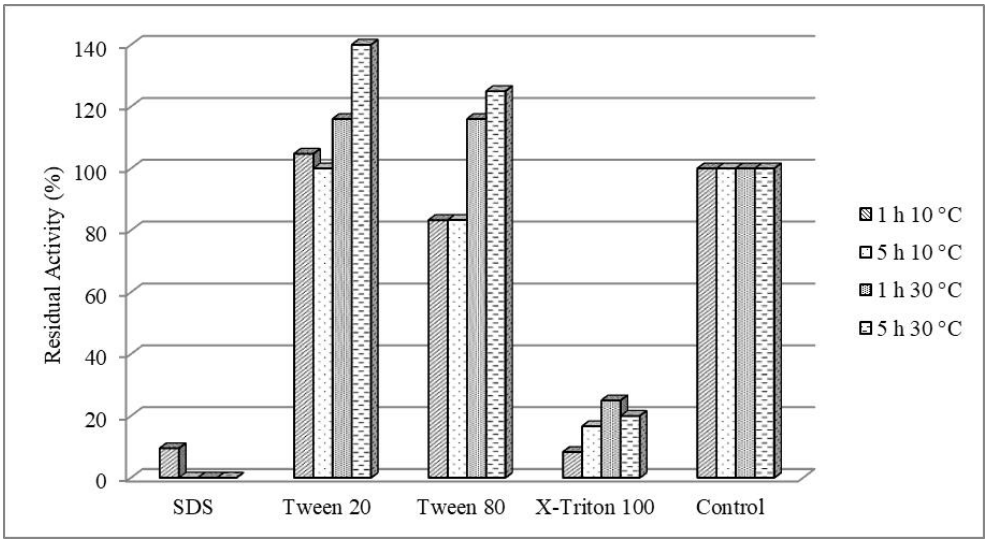

Figure 5. Effect of some detergents on crude BLIS from *Bacillus* sp. T68 strain.The crude BLIS was incubated with detergents at a final concentration of 1%. Untreated crude BLIS served as control.

For 1 h at 10 °C, EDTA increased BLIS activity by 4% at 0.1 mM, but by 8% at 0.2 mM and by 16% at 0.5 mM concentrations. For 5 h at 10 °C, activity decreased by 8% at 0.1 mM, increased by 12% at 0.2 mM and had no effect at 0.5 mM. At 1 h 30 °C, the activity increased by 10% at 0.1 mM, by 22% at 0.2 mM, andby 27% at 0.5 mM. While it did not affect the activity at 0.1 and 0.5 mM for 5 h at 30 °C, it increased by 9% at 0.2 mM.

Treatment with proteinase K resulted in 100% loss of activity. When treated with 1 and 10 mg/ml trypsin for 1 h at 37 °C, activity was reduced by 23% and 25%, respectively. For 2 h at 37 °C, activity was reduced by 53.5% and 76.4% upon treatment with 1 and 10 mg/ml trypsin, respectively.

3.3. Detection of BLIS Activity on Gel

Activity was observed in the ammonium sulfate precipitation of the crude BLIS of the T68 strain. The maximum antibacterial activity was found in the resolved precipitate with 40% saturation of ammonium sulfate. However, it still showed activity when 60 and 80 % saturation of ammonium sulfate was added. In the activity study, 40% saturation of ammonium sulfate precipitation, which showed the best activity, was used. Overlaying the gel with NA supplemented with the indicator strain revealed a single protein band in a region with antibacterial activity. The band had apparent molecular weight of about 15 kDa (Figure 6). The band corresponding to this region could not be seen on SDS-PAGE.

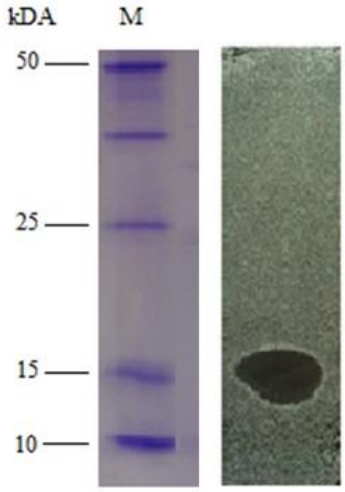

Figure 6. Activity of the BLIS (obtained through 40% ammonium sulfate precipitation). M: Marker (Promega)

4. DISCUSSION

Bacteriocins produced by the *Bacillus* genus are considered as important as those produced by LAB. Bacteriocins from *Bacillus* species, like those from LAB, have an important place for applications in human and animal health [39]. A wide variety of antimicrobial peptides with different chemical structures are produced by the genus *Bacillus* [40,41].

In the present study, isolation and characterization of a BLIS from *Bacillus* sp.T68 were performed.

The best antibacterial activity was found in LB medium for 24 h compared to other media. Different media have been used to produce bacteriocin from *Bacillus* species [31,37,42-44]. In a study using LAB isolates obtained from fish, it was reported that bacteriocin production increased to 72 h [45]. In

another study, the bacteriocin activity synthesized by *Bacillus thuringiensis* subsp. *entomocidus* was found to be higher in TSB and LBB medium compared to the other media [46]. Different media such as BHI and LB were used to produce bacteriocin by *Bacillus* species [37,42].

The antibacterial activity of BLIS in the present study was stable a wide temperature range and lost its activity at temperatures above 60 °C. Lee et al. [47] reported that a bacteriocin produced by *B. polyfermenticus* maintained its inhibitory activity up to 60 °C, and lost its activity after 30 min at 70 °C. The decreases in bacteriocin activity at high temperatures and denaturation or loss of total activity are due to the proteinaceous structure of bacteriocin [48]. Bacteriocin produced by *Brevibacterium linens* remained active for 30 minutes at 40-50 °C [49]. However, bacteriocin RX7 isolated from *Bacillus amyloliquefaciens* retained its activity at 100 °C for 30 minutes [39]. Bacthuricin F4 obtained from *Bacillus thuringiensis* subsp. *course* lost 80% of its activity at 90 °C within 30 min, but was protected at 40-70 °C [50].

The antibacterial activity of BLIS showed the best activity at pH 6-10 at different temperatures. It was observed that bacteriocin activity decreased or disappeared in acidic media at pH 9 and above [43,48,51]. In a study performed with Nisin, no activity was observed at pH 3, while the highest activity was observed at pH 6.5. Similarly, in a study performed with Sakasin A, the highest activity was observed at pH 5.5, while the activity decreased to 75% at pH 2 [52]. In other studies, bacteriocins from strains B10 [31] and LBM 5006 [43] were stable in a pH range of 3-9 and 3-8, respectively.

It has been found that BLIS generally has little or no effect on activity when treated with organic solvents such as acetonitrile, chloroform, ethyl alcohol, acetone, hexane, methanol, butan-1-ol and isopropanol. Many researchers have found that most bacteriocins produced by LABs are resistant to organic solvents [53-55]. In a study by Pirzada et al. [56], bacteriocin was found to remain active against propanol from organic solvents at a concentration of 1% and its activity was lost by acetone, methanol, heptane and chloroform.

In this study, SDS and Triton X-100 affected BLIS activity more than Tween 20 and Tween 80 and the complete disappearance of activity, especially when treated with SDS, suggests that the protein structure of bacteriocin is impaired. SDS and Triton X-100 treatment caused loss of activity and this effect became persistent. Another study investigated the effects of certain detergents on bacteriocin activity. The effect of detergents on bacteriocin activity provides information about the structure of the active molecule. Detergents form a complex with the hydrophobic center in the natural structure of proteins resulting in opening and deterioration of the three-dimensional structure of the protein. The decrease in activity after treatment with detergent is due to partial denaturation of bacteriocin or deterioration of its association with other molecules effective for stabilization of activity [35]. It was found that the BLIS activity of EDTA increased with increasing concentrations. However, the activity of bacteriocin incubated with EDTA and urea was completely lost [33]. EDTA can increase the antibacterial activity of bacteriocin by complex formation. It can damage the cell membrane and facilitate the antimicrobial effect of bacteriocin [57].

The antibacterial activity of BLIS was completely destroyed by proteinase K. Treatments with trypsin resulted in a reduction in activity of up to 76%. These results indicate that the antibacterial substance in the supernatant is a protein or peptide. It was concluded that the antimicrobial substance was a BLIS. It has been reported that protein-based bacteriocins are unable to stabilize and lose their antimicrobial activity after proteolytic enzyme treatment [58]. Many researchers have found that enterocins are sensitive to one or more proteolytic enzymes [59]. These results confirm that this bacteriocin has a proteinaceous structure and are consistent with this literature [36,60].

The best activity of the crude BLIS was obtained at 40% saturation in ammonium sulfate precipitate. Thuricin 439 was best obtained at 80% saturation [61]. In the activity study, a single inhibitory zone corresponding to about 15 kDa was found and this peptide was classified as an intermediate size (10-30 kDa) peptide. In a study by Tumbarski et al. [62], a peptide of approximately 19 kDa was reported as an intermediate size peptide. The molecular weight of the bacteriocin synthesized by *B. thuringiensis* entomocidus HD9 which is active against Gram (-), Gram (+) and some fungi was reported to be 12.4 kDa [7]. Polyfermenticin SCD obtained from a commercial probiotic strain of *B. polyfermenticus* has been reported to have a molecular weight of about 14.3 kDa [47]. Some other bacteriocins, produced by strains of the *Bacillus* genus, cerein [51], and thuricin 7 [63] have molecular weights of 11.6 and 9 kDa, respectively. The molecular weight of the bacteriocin obtained from *Bacillus subtilis* R75 was found to be 12 kDa [64].

Here we reported the biochemical characterization and partial purification of BLIS from *Bacillus* sp. T68. This BLIS showed stability over a wide range of pH values (5-10), and at heat and solvent treatments. Additionally, it was found sensitive to proteinase K, trypsin, SDS and triton X-100, and was generally unaffected by EDTA, and organic solvents. BLIS has a molecular weight of about 15 kDa.

CONFLICT OF INTEREST

The authors stated that there are no conflicts of interest regarding the publication of this article.

AUTHORSHIP CONTRIBUTIONS

Concept: H.K., Desing: H.K., S.K.Ş., Execution: S.K.Ş., Material supplying: H.K., Data acquisition: S.K.Ş., Data analysis/interpretation: H.K., S.K.Ş., Writing: H.K., S.K.Ş., Critical review: H.K., S.K.Ş.

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