Isolation and Characterization of a Bacteriocin-Like Substance Produced by *Geobacillus toebii* Strain HBB-247

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Abstract A total of 201 thermophilic bacteria isolated from various thermal spring, mud and soil were tested for their antibacterial activity. Among the mostly active isolates, *Geobacillus toebii* HBB-247 was further examined. Bacteriocin-like inhibitory substance (BLIS) produced by strain HBB-247 was found to be stable up to 60°C, sensitive to proteolytic enzymes and effective against *Enterococcus faecalis*, *Listeria* sp., *E. avium*, *Clostridium pasteurianum*, *Cellulomonas fimi* and some thermophilic strains isolated and identified in this study. As a result of Tricine-SDS-PAGE molecular weight of BLIS was estimated about 38 kDa. Production studies showed that *G. toebii* HBB-247 starts to produce antibacterial substance at early logarithmic phase of growth and maximum production was detected at the end of the logarithmic phase.

Keywords Antibacterial · *Geobacillus* · Characterization · Isolation · BLIS

Bacteriocins comprise a large and diverse group of ribosomally synthesised antimicrobial proteins or peptides, some of which undergo post-translational modifications [1]. According to Cotter's classification scheme bacteriocins divide into two distinct categories: lanthibiotics (Class I) and the non-lanthionine containing bacteriocins (Class II). Large, heat-labile murein hydrolases which were formerly known as Class III bacteriocins were termed as "Bacteriolysins". Class IV bacteriocins was not included

in these proposal because no members of this class convincingly demonstrated yet [2].

Bacteriocins are different from usual antibiotics in one critical way; they have a relatively narrow killing spectrum and are only toxic to bacteria closely related to the producing strain [3]. Despite of the reported bacteriocins are generally isolated from food-grade, mesophilic microorganisms, there is a few examples of bacteriocins produced by thermophilic bacteria and archaea [4–10].

In this study a total of 201 thermophilic bacteria were isolated from various thermal spring, mud and soil samples taken from Denizli, Aydın and Muğla (Turkey) provinces. *Thermus* medium used for enrichment and then strains isolated by streaking onto Caso Agar plates. The plates were incubated aerobically at 65°C. For screening, isolates were inoculated to Caso Agar plates with sterile toothpicks and antibacterial activities of strains were tested against *Geobacillus stearothermophilus* DSMZ 22 by method described by previously [9]. After 24 h of incubation, strains with inhibition halos evaluated and among them HBB-247 which was isolated from thermal pool in Gölemezli (Denizli) was chosen for studying in detail.

Strain HBB-247 identified based on 16S rDNA sequence analyses and carbohydrate fermentation reactions recorded with API 50 CH (BioMerieux, France). Genomic DNA of strain isolated as indicated by previously [11], 16S rRNA gene amplified via PCR and then amplicon was sequenced. The primers used for amplification were: 20F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1390R (5'-GAC GGG CGG TGT GTA CAA-3'). The partial 16S rRNA gene sequence of strain HBB-247 was submitted to GenBank Nucleotide Sequence Database and has been assigned accession number GQ487459. A neighbour-joining phylogenetic tree was constructed with the MEGA 5 according to p-distance method.

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To determine the antibacterial spectrum of BLIS, strain HBB-247 was cultured in BHI broth (Oxoid) for 24, 48, 72 and 96 h at 65°C. The cells were harvested ($8,000 \times g$, 10 min, 4°C), the cell-free supernatant sterilized through a milipore filtre (Sartorius) with 0.45 μm pore size. 350 μl bacterial suspension (0.5 MacFarland) was mixed with 35 ml agar media appropriate for each strain and then poured into plates. Wells 6 mm in diameter were cut into agar media by using cork borer and then 50 μl CFS were placed into each well. The plates were kept at 4°C for 2 h for BLIS diffusion then incubated at the appropriate temperatures for each bacterial strain and examined for zones of inhibition.

Cell-free supernatants from strain HBB-247 precipitated with 80% saturated ammonium sulphate. The pelleted ammonium sulphate precipitate was dissolved in 20 mmol l^{-1} sodium phosphate buffer (pH 7.0), and dialyzed against same buffer by using a 1,000 Da dialysis membrane (ZelluTrans-Roth E887.1). Dialysate was used to determine the molecular size of BLIS by tricine-SDS-PAGE [12]. Molecular weight standards were from Sigma (M3546). One half of the gel was stained with Coomassie Blue, and the other half was used for direct detection of bacteriocin activity. The gel, assayed for antibacterial activity was overlaid with soft (0.7%) BHI agar inoculated with the overnight culture of indicator strain *G. stearo-thermophilus* DSMZ 22 (1% v/v), then incubated at 55°C for 14 h, and observed for the formation of inhibition zone.

Cell-free culture supernatants obtained from 24 h old cultures of HBB-247 were used for characterization of antibacterial substance. To determine the thermal stability, BLIS samples were heated for 30 min and 2 h at 30, 45, 60 and 95°C, cooled and assayed for activity. Aliquots were also maintained at 4°C, for 1 month, then activity was tested. Untreated samples served as control [13]. The effects of various enzymes on BLIS activity were also tested. Aliquots were treated with pronase E, proteinase K, Rnase, Lipase, B-glucoronidase, lactase at final concentration of 1 and 10 mg/ml, catalase at final concentration of

300 IU/ml for 1 and 24 h. An untreated samples and the enzyme in the buffer alone served as controls [14]. In a separate experiment, the effect of the pH on the activity, was tested by adjusting each of the aliquots to pH 3.0–11.0 with sterile 1 mol l^{-1} NaOH or 1 mol l^{-1} HCl. After 24 h incubation at 4°C, the samples treated were adjusted to pH 7.0 with sterile 1 mol l^{-1} NaOH or 1 mol l^{-1} HCl and tested for activity [15]. Acetone, Chloroform, DMSO, Ethanol, Methanol and Xylol were added to 24 h-old cell-free culture supernatants at final concentration of 10% (v/v) and then incubated for 1 h at 30°C. Trichloroacetic acid was added at final concentration of 100 mg/ml, incubated for 1 h at 30°C. After treatment with TCA, samples were centrifuged at $10,000 \times g$ for 5 min and the supernatants were neutralized to pH 7.0 before testing for antibacterial activity. Tween 80, and urea were used at final concentration of 1 mg/ml, and EDTA was used at 0.1 mmol l^{-1} and then samples were incubated for 1 h at 30°C. Chemicals and BLIS aliquots diluted with sterile distilled water at same final concentration were used as control [16]. After each treatment, the samples were tested for antibacterial activity against *G. stearo-thermophilus* DSMZ 22 using well diffusion method.

HBB-247 was grown in BHI broth for 24 h and then two mililiter (2%, v/v, $\text{OD}_{600 \text{ nm}} = 0.1$) of culture was inoculated into BHI broth and incubated at 65°C without agitation. The growth was determined by measuring absorbance at 600 nm in a spectrophotometer (Shimadzu UV-1601). *G. stearo-thermophilus* DSMZ 22 was used as indicator strain and the titer was defined as the reciprocal of the highest dilution exhibiting inhibition of the sensitive strain and was expressed in arbitrary units (AU) per mililiter. Samples were taken every hour and absorbancies, and bacteriocin titres were recorded.

Although there are many examples of bacteriocins obtained from LAB, new strains able to produce antibacterial compounds are required. In this study we characterized and optimized the production of BLIS from newly isolated *G. toebii* strain HBB-247. The isolate is

Fig. 1 Phylogenetic tree showing the position of strain HBB-247 within the branches of the genus *Geobacillus*. Bar indicates 0.005 substitutions per nucleotide position. Bootstrap values (%) are based on 1,000 replicates and branches with 50% and more bootstrap support are shown

Table 1 Antibacterial spectrum of BLIS produced by *G. toebii* HBB-247

Indicator strain	Zone of inhibition (mm)			
	24 h CFS	48 h CFS	72 h CFS	96 h CFS
<i>G. stearothermophilus</i> DSMZ 22	20	20	20	20
<i>B. sphaericus</i> DSM 396	–	–	–	–
<i>M. luteus</i> ATCC 934	–	–	–	–
<i>L. plantarum</i> DSM 20174	–	–	–	–
<i>Listeria</i> sp. (food isolate)	9	9	9	9
<i>E. faecalis</i> ATCC 51299	9	9	9	9
<i>E. gallinarum</i> MÇ-3	–	–	–	–
<i>E. gallinarum</i> LC-M1	–	–	–	–
<i>E. faecium</i> MS-1	–	–	–	–
<i>E. faecium</i> KT-2	–	–	–	–
<i>E. durans</i> K-1	–	–	–	–
<i>E. durans</i> KT-1	–	–	–	–
<i>E. avium</i> AS-3	–	–	10	10
<i>Brochothrix thermosphacta</i> DSM 20171	–	–	–	–
<i>Cellulomonas fimi</i> DSM 20114	–	10	10	10
<i>Clostridium pasteurianum</i> DSM 525	20	20	20	20
<i>Anoxybacillus</i> sp. HBB-134	14	14	14	16
<i>Geobacillus</i> sp. HBB-234	–	–	–	–
<i>Geobacillus</i> sp. HBB-269	12	12	18	18
<i>Geobacillus</i> sp. HBB-270	14	14	18	20
<i>Anoxybacillus</i> sp. HBB-229	13	14	15	15
<i>Geobacillus</i> sp. HBB-301	–	–	–	–
<i>Geobacillus</i> sp. HBB-103	–	–	–	–
<i>Anoxybacillus</i> sp. HBB-225	–	–	–	–
<i>Anoxybacillus</i> sp. HBB-226	–	–	–	–
<i>Bacillus</i> sp. (soil isolate)	–	–	–	–
<i>B. mycoides</i> DSM 299	–	–	–	–
<i>B. cereus</i> ATCC 11778	–	–	–	–
<i>B. subtilis</i> ATCC 6633	–	–	–	–
<i>Serratia marcescens</i> (soil isolate)	–	–	–	–
<i>Pseudomonas fluorescence</i> DSM 50090	–	–	–	–
<i>Pectobacterium carotovorum</i> DSM 30168	–	–	–	–
<i>Staphylococcus aureus</i> ATCC 25923	–	–	–	–
<i>Streptococcus vestibularis</i> DSM 5636	–	–	–	–
<i>E. coli</i> ATCC 35218	–	–	–	–
<i>Listeria innocua</i> DSM 20649	–	–	–	–
<i>L. acidophilus</i> DSM 20079	–	–	–	–
<i>L. sakei</i> DSM 6333	–	–	–	–
<i>Leuconostoc mesenteroides</i> DSM 20343	–	–	–	–

+ inhibition zone observed, – no inhibition zone was observed

Gram-positive, aerobic, endospore forming thermophilic bacterium. According to biochemical test results HBB-247 was found to be catalase and oxidase positive, produced acid from glucose and hydrolyse esculin. The 16S rDNA sequence of the strain HBB-247 was 1311 nucleotides long

and showed highest similarity (99%) with *G. toebii* according to BLAST results. The phylogenetic tree was constructed using the neighbor-joining method (Fig. 1).

The BLIS produced by *G. toebii* HBB-247 showed inhibition of some tested Gram positive bacteria, whereas

none of the tested Gram negative strain was inhibited. Some researchers indicated that outer membrane of Gram negative bacteria may protect the cytoplasmic membrane against antibacterial substances [17]. BLIS was found to be active against *G. stearothermophilus* DSMZ 22, *Listeria* sp., *Enterococcus faecalis* ATCC 51299, *E. avium* AS-3, *Anoxybacillus* sp. HBB-134, *Geobacillus* sp. HBB-269, *Geobacillus* sp. HBB-270, *Anoxybacillus* sp. HBB-229, *Clostridium pasteurianum* DSM 525 and *C. fimi* DSM 20114 (Table 1). These findings are in consistent with the fact that bacteriocins inhibit strains that are in most cases closely related to the producer strain. Thermophilin 110 produced by *Streptococcus thermophilus* is effective against many Gram positive bacteria including *S. thermophilus*, *Lactococcus lactis*, *Lactobacillus delbruckei*, *Pediococcus* spp., *E. faecalis* and *Listeria monocytogenes* [18]. Similar results were reported for four different strains of *G. stearothermophilus* [10]. Strains 17, 30, 31 and 32A were recorded as active against not only thermophilic bacterial strains but also mesophilic ones. *T. rubens* [6], and two strains of *Bacillus thermoleovorans* [7] were reported as the other thermophilic bacteria that produce bacteriocin like antibacterial substances.

Cell-free supernatants obtained from 24 h culture of HBB-247 were assayed for their thermal stability and total loss of activity was recorded after treatment at 95°C for 30 min. Residual activity was found to be 100% at 60°C for 2 h and at 4°C for one month. Sensitivity of BLIS to various proteolytic enzymes tested and the residual activity was estimated. As a result of proteolytic enzyme treatment, slight reduction (30.8%) in activity was observed at higher final concentration (10 mg/ml) of proteinase K and (38.5%) for protease. Some researchers reported that a few examples of bacteriocins or BLISs especially those produced by *Bacillus* spp. are composed of unusual circular aminoacids and therefore they are resistant to proteolysis [14, 19, 20]. Treatment of the BLIS with catalase, RNAase, α -amylase and lipase did not change its activity. Among the tested enzymes glycosidases or lipase were not effect the activity, suggesting that BLIS is not a complex bacteriocin. Inhibitory activity of BLIS was completely lost with TCA treatment, which indicates its proteinaceous nature. BLIS remained stable after incubation for 24 h at pH values between 3 and 11.

Cell-free supernatant of HBB-247 was precipitated with ammonium sulphate and precipitant was applied to Tricin SDS-PAGE. Molecular weight of BLIS was estimated about 38 kDa (Fig. 2). Similar results have been reported for other bacteriocins with high (>10 kDa) molecular weight, thermoleovorins [7], thermophilin ST-1 [18], nigrin [21], cerein 8A [22], lactosporin [23].

Growth curve experiments in BHI broth showed that *G. toebii* HBB-247 started to produce BLIS at early

Fig. 2 Tricine-SDS-PAGE. *Left to right* molecular weight marker, protein profile of the ammonium sulphate precipitated culture supernatant of strain HBB-247 and direct overlay of gel with *G. stearothermophilus* DSMZ 22 indicating inhibition zone

Fig. 3 Production of BLIS during growth of *G. toebii* HBB-247. Symbols: filled square absorbance, filled diamond antibacterial activity of BLIS (AU/ml)

logarithmic phase, optimal production of BLIS was recorded at the end of the logarithmic phase (Fig. 3). According to curve different peaks in the BLIS activity were observed, indicating that BLIS was produced in distinct phases during growth. A non-linear relationship between biomass and BLIS production was observed during growth of HBB-247 in BHI broth.

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