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Medium Optimization for Bacteriocin Production and Bacterial Cell Growth of *Geobacillus sp*. 15 Strain

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Abstract

In this study we determined that *Geobacillus sp.* 15 strain secretes antibacterial compound-bacteriocin. Only few bacteriocin producing thermophilic bacteria of this genus have been identified to the date. Also, we optimized medium composition for the better cell growth of this strain and increased the yield of secreted bacteriocin. In this work we showed that some particular salts or their combinations may have impact on higher growth of *Geobacillus sp.* 15 strain bacteria or their produced bacteriocins. These results can facilitate research on this strain and its secreted bacteriocin. These antibacterial proteins and peptides are promising natural agents as an alternative to antibiotics in medicine or veterinary and to traditional preservatives in food industry. Optimized composition of the growth medium can be very useful for studies of other *Geobacillus spp.* strains and their produced bacteriocins. Furthermore, these data may be used to increase the biomass of *Geobacillus* bacteria and the yield of protein when it is dependent on bacterial cell yield.

Keywords *Geobacillus sp*; Bacteriocin; Medium; Optimization; Salts

Introduction

Geobacillus bacteria are rod-shaped Gram-positive, aerobic and spore-forming thermophiles. They are frequently isolated from hot environments like hot springs, oil wells, compost or desert soils. Most isolates grow in temperatures between about 45°C and 70°C, with optima between 50°C and 60°C. They are neutrophilic and grow within a relatively narrow pH between 5.0 and 9.0, and their optima lie within the range pH 6.2-7.5. Most strains will grow on routine media such as nutrient agar. For the species tested, growth factors, vitamins, NaCl, and KCl are not required. A wide range of substrates is utilized, including carbohydrates, organic acids, peptone, tryptone, and yeast extract; the ability to utilize hydrocarbons as carbon and energy sources is a widely distributed property in the genus [1,2].

Geobacillus spp. are attractive to the biotechnology industry as source of thermostable enzymes and natural products digesters of lignocellulose, bioremediators of hydrocarbons or as platforms for biofuel production [2,3]. Some *Geobacillus spp.* strains produce bacteriocins. It is ribosomally synthesised antimicrobial peptides secreted by various bacteria. They can be active against human and animal microbial pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *enterococci* (VRE) without showing toxicity. The advantages of bacteriocins are their physical stability and non-toxicity. Numerous bacteriocins have great potential as food preservatives or as therapeutic or bio-controlling agents [4-6].

To date, only few bacteriocin producing thermophilic bacterial strains of genus *Geobacillus* have been identified: *G. stearothermophilus* [7], *G. toebii* strain HBB-218 [8], *G. toebii* strain HBB-247 [9], *G. thermodenitrificans* [10] and in *Geobacillus sp.* strain ZGt-1 [11]. Studies of factors affecting the production of bacteriocins by *Geobacillus spp.* are relatively scarce.

The growth of bacteria and accumulation of their metabolites are strongly influenced by growth environment and medium composition [12,13]. The effects of medium composition for bacteriocin production have been studied for pediocin AcH [14], pediocin PD-1 [15], enterocin P [16], nisin [17], plantaricin 423 [18], and sakP [19]. Bacteriocin production may be strongly influenced by carbon sources, nitrogen sources, inorganic salts, temperature and pH [20-23]. Bacteriocin production is usually growth associated, i.e. the level at which the peptide is produced is linked to the biomass formed [19,24-27]. Some studies have indicated that bacteriocin production and activity levels do not always correlate with cell mass or growth rate of the producer strain [26,28]. Higher bacteriocin levels are often obtained at temperatures, nutrient sources and pH values lower than required for optimal growth [19,24,25,29-32].

It suggests that low growth rate, unfavourable growth conditions or other less optimal environmental conditions may also stimulate bacteriocin production [19,25,27]. Our lab experience with *Geobacillus* bacteria shows that some of the strains exhibit poor growth and sometimes it is hard to select appropriate medium for good growth. It complicates research on these bacteria and their produced compounds like bacteriocins. These reasons prompt us to optimize the growth medium for *Geobacillus sp.* 15 strain which produce antibacterial small proteins-bacteriocins. The aim of the present study was to optimize medium composition for a better cell growth of *Geobacillus sp.* 15 strain and for higher yield of the secreted bacteriocin in constituted growth media.

Materials and Methods

Strains used in this work

Geobacillus sp. 15 strain was previously isolated from oil well in Lithuania and was used as a bacteriocin producer in this work. *Geobacillus stearothermophilus* NUB36187 (BGSC No. 9A11) was selected as indicator strain for bacteriocin activity assays.

Strain identification

Genomic DNA of *Geobacillus sp.* 15 strain was extracted. Polymerase chain reaction (PCR) was used to amplify DNA of 16S rRNA coding region. Following PCR primers were used in this reaction: 27F (5′ GAG AGT TTG ATC CTG GCT CAG 3′) and 1495R (5′ CTA CGG CTA CCT TGT TAC GA 3′). DNA amplicon of 16S rRNA region was sequenced and the sequence was examined by comparing it with other sequences in database of DNA (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Growth media

Nutrient broth (NB) medium containing 10 g/l peptone from casein, 5 g/l meat extract and 5 g/l sodium chloride was used in this work. NB medium supplemented with 20% (v/w) glycerol was separately used for stock cultures stored in -70°C. Prior to use bacterial cultures in liquid media strains were cultivated on solid medium (NB supplemented with 1.5% v/w of agar). Liquid medium of NB was supplemented with following salts: KCl-0.07 µmol/l, $ZnSO_4$-0.91 µmol/l, $MnSO_4$-0.47 µmol/l, $CuSO_4$-0.6 µmol/l, $MgCl_2$-1.1 µmol/l, $CaCl_2$-2.3 µmol/l, $NaNO_3$-0.5 µmol/l, $NaHCO_3$-µmol/l in various variations (Table 1) for medium optimization experiments.

Additives	Max OD	Max AU/ml
Basic medium	3.2	40
$CuSO_4$, $NaHCO_3$,$NaNO_3$, $MnSO_4$, KCl, $CaCl_2$, $MgCl_2$, $ZnSO_4$	4.32	160
$CuSO_4$, $NaHCO_3$, $NaNO_3$, $MnSO_4$	0.62	80
KCl, $CaCl_2$, $MgCl_2$, $ZnSO_4$	3.2	160
KCl, $MgCl_2$	4.36	320
$CaCl_2$, $ZnSO_4$	2.76	320
$CuSO_4$, $NaNO_3$	2.48	80
$MnSO_4$, $NaHCO_3$	4.54	320
KCl	2.88	160
$MgCl_2$	3.76	160
$CaCl_2$	2.92	80
$ZnSO_4$	2.48	80
$NaNO_3$	2.88	40
$MnSO_4$	5.44	160
$CuSO_4$	2.88	40
$NaHCO_3$	2.56	80

Table 1: Additives, the highest bacteriocin activity (Max AU/ml) and the highest optical density (Max OD) during the growth.

These particular salts were chosen based on observations in our laboratory that *Geobacillus spp.* strains maintained better growth and bacteriocin production in NB medium that was prepared with a tap water. All previous salts and their concentrations used in this work were equivalent to the salts and their molarities that were found in the tap water of our laboratory. All media's components and chemicals were purchased from Merck.

Cultivation Conditions

50 ml of sterile NB medium was inoculated with of *Geobacillus sp.* 15 strain to the final optical density (OD) 0.06 measuring at 595 nm wavelength in 1 cm diameter cuvette. The pH of growth medium before use was adjusted to 6.85 with 5 M NaOH (Barta a Cihlar, spol. s. r. o.). Inoculated medium was cultivated for 12 hours at 55°C, 200 rpm. Every hour samples of bacterial culture were taken to measure OD and bacteriocin activity using agar well diffusion assay.

Selection of Bacteriocin Producing Strain

Melted NB medium supplemented with 1.5% agar was cooled down to 55°C and inoculated with the sensitive strain *Geobacillus stearothermophilus* to the final OD 0.32 measuring at 595 nm wavelength in 1 cm diameter cuvette. Inoculated medium was poured in a Petri plate. When the medium was solid bacteriocin producer strain *Geobacillus sp.* 15 was seeded in the center of the plate which was later incubated in a thermostat for 16 hour at 55°C. After that inhibition zone around the producer strain was inspected.

Agar Well Diffusion Assay

Melted NB medium supplemented with 1.5% agar was cooled down to 55˚C and inoculated with bacteriocin sensitive strain *Geobacillus stearothermophilus* to the final OD 0.32 measuring at 595 nm wavelength in 1 cm diameter cuvette. Inoculated medium was poured in a Petri plates. When the medium was solid 1 cm diameter wells were cut in it. Samples of bacterial culture were centrifuged at 10000 x g and supernatants were collected. Serial twofold dilutions of supernatant were made with buffer tris-HCl, 50 mM, pH 7.5. 100 µl of diluted supernatant samples were poured into the wells and incubated for 16 hour at 55°C. After that inhibition zones around the wells were inspected to determine antibacterial activity of bacteriocin which is expressed in arbitrary units (AU) per ml. AU/ml=(1 ml×V-1)/D-1, V-sample volume (ml), D-the reciprocal of the highest dilution showing a clear zone of growth inhibition around the well.

Bacteriocin Extraction and its Treatment With proteinase K

Geobacillus sp. 15 strain was grown in NB medium and when cells reached stationary phase the medium with bacteria was centrifuged at 10000 x G for 15 min at +4°C temperature. Supernatant was collected and saturated with ammonium sulphate up to 80%. Then it was centrifuged at 15000 x G for 30 min at +4°C temperature. Protein pallets were collected, dissolved in 50 mM tris-HCl (Sigma-Aldrich) pH 7.5 buffer and desalted in SnakeSkin 3.5 MWCO dialysis tubing (Thermo Scientific) which was immersed in the same buffer. Latter on this dialyzed crude protein extract (CPE) was treated with proteinase K (Sigma-Aldrich) enzyme. 100 µL of CPE mixed with 100 µL proteinase K (1 mg/ml in 50 mM tris-HCl pH 7.5) solution. For control experiment 100 µl of 50 mM tris-HCl pH 7.5 buffer was used instead of proteinase K solution. Mixtures incubated in 37°C temperature for 1 hour and then antibacterial activity was tested using agar well diffusion assay.

Results

Bacteriocin producer strain selection and its identification

Bacteriocin producing strain selection showed that *Geobacillus sp.* 15 strain secretes antibacterial substance that inhibits growth of *Geobacillus stearothermophilus* (Figure 1) and it was selected for further work in this study. DNA sequence coding 16S rRNA of strain 15 analysis showed 99% homology to *Geobacillus stearothermophilus* 16 rRNA coding sequence. Because of that bacteriocin producer strain were defined as *Geobacillus stearothermophilus* 15 strain and it is closely related to the sensitive strain.

Figure 1: *Geobacillus sp.* 15 strain antibacterial activity against *Geobacillus sp.* 9A11 strain.

Bacteriocin extraction and its treatment with proteinase K

CPE treated with proteinase K had decreased antibacterial activity against sensitive strain *G. stearothermophilus* (Figure 2). These results indicated that antibacterial activity is associated with protein or peptide.

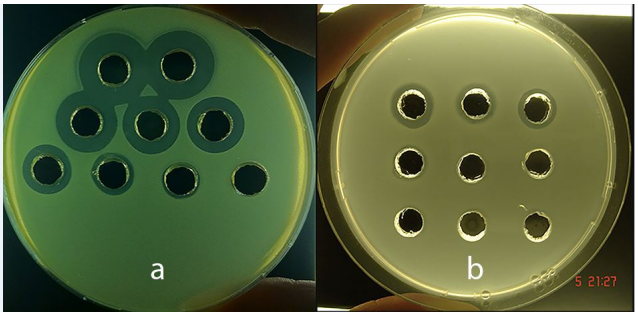

Figure 2: Antibacterial activity of CPE, (a)-not treated with proteinase K, (b)-treated with proteinase K.

Growth Medium Optimization for Higher Cell Growth and Bacteriocin Production

Bacterial strain 15 was grown in NB media supplemented with various variations of salts (Table 1). During the growth samples of bacterial culture were collected for OD determination and bacteriocin activity assays. Based on OD measurements growth curves were generated for all tested media which were supplemented with salts and compared to the control-growth in the medium without any salts added (Figure 4).

Testing every salt separately it was revealed that in the most cases the growth was reduced and only in the medium supplemented with $MgCl_2$ or $MnSO_4$ the cell growth was increased and higher OD was reached (Figure 4c and 4d).

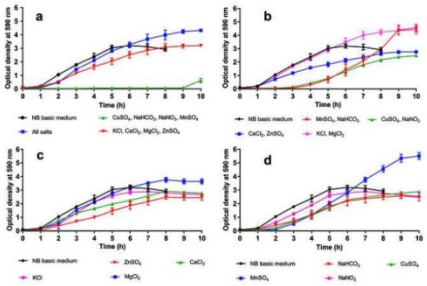

Figure 4: *Geobacillus sp.* 15 strain growth in the basic and modified media.

The growth in the media supplemented combining $CuSO_4$ and $NaNO_3$ or $CaCl_2$ and $ZnSO_4$ was decreased compared to that in the control NB medium without salts (Figure 3b). Meanwhile combining $CuSO_4$, $NaHCO_3$, $NaNO_3$ and $MnSO_4$ the growth was practically inhibited (Figure 3a).

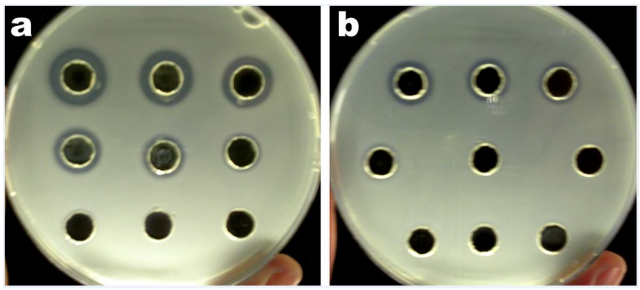

Figure 3: *Geobacillus sp.* 15 strain produced bacteriocin activity in supernatant: (a)-modified medium supplemented with KCl and $MgCl_2$; (b)-basic medium.

Combining all 8 tested salts or combinations of KCl and $MgCl_2$ or $MnSO_4$ and $NaHCO_3$ revealed higher cell growth and OD. Besides the growth in these media bacteriocin activities were also evaluated every hour. The highest activities observed during the growth in every modified media were compared between different media. In the control media where salts were not added the highest bacteriocin activity during the growth was determined 40 AU/ml. In the media where every salt was tested separately, the best results were achieved with KCl, $MgCl_2$ or $MnSO_4$, the activity in supernatant during the growth was increased up to 160 AU/ml. The same results were observed in the medium that was supplemented with all 8 salts or in

the medium supplemented with combining KCl, $CaCl_2$, $MgCl_2$ and $ZnSO_4$.

After testing all salts and combinations of it in this work, the highest bacteriocin activity was achieved in media supplemented combining KCl and $MgCl_2$ or $CaCl_2$ and $ZnSO_4$ or $MnSO_4$ and $NaHCO_3$. In this case the highest activity during the growth in these modified media was increased up to 320 AU/ml. All the rest medium variants did not increase or increased bacteriocin activity up to 80 AU/ml.

Discussion

Based on 16S rRNA sequence homology analysis it was revealed that 15 strain isolate may be defined to *Geobacillus stearothermophilus* species. Bacteriocin producer strain selection and agar well diffusion assay showed that this strain secretes antibacterial substance that can inhibit growth of closely related bacteria *Geobacillus stearothermophilus* NUB36187. Protein extraction from the supernatant and CPE treatment with protease results indicates that 15 strain secretes to the medium antibacterial compound-bacteriocin.

In this study growth conditions were determined for higher *Geobacillus stearothermophilus* 15 strain yield. Growth media optimization experiments showed that *Geobacillus stearothermophilus* strain 15 growth can be enhanced by $MgCl_2$. Combination of $MgCl_2$ with KCl can be even better that using $MgCl_2$ only. The best growth was achieved in NB medium supplemented only with $MnSO_4$. Growth media components were identified that may increase better bacteriocin production in *Geobacillus stearothermophilus* 15 strain. Evaluation of bacteriocin activity in media supplemented with every salt separately shows that activity may be enhanced using KCl, $MgCl_2$ or $MnSO_4$. The best results can be achieved combining two salts: KCl and $MgCl_2$, $CaCl_2$ and $ZnSO_4$ or $MnSO_4$ and $NaHCO_3$.

Results show that some particular salts or combinations may have impact on higher growth of bacteria but it not always has relation to higher bacteriocin activity in the supernatant. Conclusion may be done that bacteriocin secretion does not depend on better growth of bacterial culture. Determined salts for better bacterial growth and higher bacteriocin production will facilitate investigations of this strain and its secreted bacteriocin. These results may be very useful for studies of other *Geobacillus spp.* strains and their produced bacteriocins. Furthermore, these data may be used to increase the biomass and the yield of protein when it is dependent on bacterial cell yield.

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