

Characterization of Bacteriocin Produced by *Bacillus subtilis* 11A Isolated from the Gastrointestinal Tract of Indonesian Native Chicken (*Gallus domesticus*)

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ABSTRACT

The purpose of this study was to characterize bacteriocins produced by *Bacillus subtilis* 11A isolated from Indonesian native chicken's ileum. Characterization of bacteriocins included antimicrobial activity, the stability to temperature, pH, and storage time. Antimicrobial activity was tested against *Escherichia coli* FNCC 0091, *Salmonella typhimurium* FNCC 0134, and *Staphylococcus aureus* FNCC 0143. Stability to temperature was tested to 4, 30, 70, 80, 90, 100°C for an hour and 121°C for 15 min. Stability to pH was tested to pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 for an hour. Stability to storage was tested for 7, 14, 21, and 28 days at refrigerator (4°C) and room temperature (30°C). The result showed that bacteriocins produced by *Bacillus subtilis* 11A were more effective against pathogens Gram-negative (*Escherichia coli* and *Salmonella typhimurium*) than Gram-positive (*Staphylococcus aureus*) (P<0.05). The bacteriocins produced by *Bacillus subtilis* 11A were heat until 90°C, stable at pH 6–10 and stored at refrigerator up to 28 days (P<0.05). This study suggested that this bacteriocins might become potential candidate for use as biodegradable natural feed and alternative antimicrobial agents to solve the increasing trends of problems of antibiotic resistance.

Keywords: Antimicrobial activity, *Bacillus subtilis*, Bacteriocin, *Gallus domesticus*, Stability.

1. INTRODUCTION

Antibiotics in the field of animal health are used as prevention and treatment of diseases, as well as growth-promoting agents (AGPs) in chicken feed. Antibiotics added to the feed can improve feed efficiency and growth in chickens. The residues of amoxicillin as much as 16.92–152.62 µg/kg in liver and 45.38–60.55 µg/kg in breast meat in broiler chickens and layer chicken in Bangladesh [1]. In 2006, the European Union banned antibiotics as AGP in livestock production, but Indonesia applied these rules was in early 2018. Among alternatives to antibiotics are competitive exclusion products, probiotics, prebiotics, organic acids, plant extracts, and essential oils, feed enzymes, bacteriophages, and hen egg antibodies [2]. Probiotic is 'a live microbial feed supplement which beneficially

affects the host animal by improving its intestinal balance' [3]. By inhibiting pathogenic microorganisms in the gastrointestinal tract, probiotics should produce antimicrobial substances such as organic acids, diacetyl, hydrogen peroxide, and bacteriocins [4]. One of the known bacteria which produce bacteriocins genus *Bacillus*. Bacteriocins are ribosomally synthesized peptides or proteins with antimicrobial properties. Bacteriocins produced by *Bacillus* have broad-spectrum against Gram-positive and Gram-negative bacteria and could potentially be useful in the food and feed industries as natural preservatives for livestock [5]. Bacteriocins produced by *Bacillus* have an intense activity of inhibition against the growth of many pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, *Listeria monocytogenes*, *Pasteurella haemolytica*,

and *Clostridium perfringens* [6–9]. This study described the isolation of bacteriocins from the *Bacillus subtilis* 11A isolated from the gastrointestinal tract of Indonesian native chickens. It characterized the

bacteriocins produced by *Bacillus subtilis* 11A, which were evaluated by pH tolerance assay, temperature tolerance assay, and time of storage assay.

2. MATERIAL AND METHODS

2.1. Material

The tools used in this study were a set of tools to grow the bacteria, *Bacillus subtilis* 11A isolated from native chicken, three pathogen bacteria (*Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus*), spectrophotometer (Genesys 20™), centrifuge type 5810R (Eppendorf®), vortex mixer model VM-1000, 1.5 mL safe lock tube, laminar airflow, autoclave, calipers, and analytic scale.

2.2. Methods

2.2.1. Bacterial strains and culture condition.

Bacillus subtilis 11A was isolated from the native chicken intestines (*ileum*) and maintained at -20°C in nutrient broth (NB) no. 2 (Oxoid, UK) containing 20% glycerol (v/v). Indicator organism (*Escherichia coli* FNCC 0091, *Salmonella typhimurium* FNCC 0134 and *Staphylococcus aureus* FNCC 0143) were obtained from Food and Nutrition Culture Collection (FNCC) Universitas Gadjah Mada. Rejuvenation of all bacteria by inoculating an ose of bacteria to 10 mL NB media (Oxoid, UK) then incubated at 37°C for 24 hours.

2.2.2. Preparation of cell-free supernatants (CFS).

Bacteriocins extraction to obtain CFS refers to Sharma *et al.* [10]. *Bacillus subtilis* 11A was grown using Luria Bertani media (Oxoid, UK) under conditions pH 6.5 and temperature 39°C for 72 hours. Then, cell culture was centrifuged at 10,000 g for 15 min at 4°C , then CFS was filtered using a sterile 0.22 μm pore size membrane filters.

2.2.3. Partial purification of bacteriocins.

The crude bacteriocins were precipitated using 70% ammonium sulfate [10], and the obtained pellet was dissolved in 10 mM phosphate buffer saline (PBS), pH 7.0. The salt was removed using a 12 kDa ultra membrane filter overnight and washed once then harvested—the fraction containing bacteriocins will not pass through the filter.

2.2.4. Bacteriocins activity assay.

The antimicrobial activity assay using a well-diffused method refers to Balouiri *et al.* [11]. With modification in well diameter (5 mm). A total of 50 μL

indicator organisms (*Escherichia coli*, *Salmonella typhimurium*, and *Staphylococcus aureus*) dripped on to solid trypticase soy agar (TSA) media then swabbed evenly using a sterile cotton swab, and then, 5 mm well was made using a cork borer. Each well was dripped as much as 50 μL of bacteriocins. Then plates were incubated at 37°C for 24 hours. The presence of a clear zone around the well showed the bacteriocins activity of *Bacillus subtilis* 11A against indicator organisms. Then, clear zone diameter was measured by calipers and converted to an arbitrary unit (AU/ml) [12].

2.2.5. Effects of heat to bacteriocins stability.

The bacteriocins were exposed to various temperatures (4, 30, 70, 80, 90, 100°C) for 60 min and autoclaved at 121°C for 15 min to analyze its thermal stability. Then, the bacteriocins' antimicrobial activity were tested.

2.2.6. Effects of pH to bacteriocins stability.

Bacteriocins' pH was adjusted to various pH (2–12) for 60 min to examine the effect of pH on bacteriocins' stability, followed by neutralization of the bacteriocins. Then, the bacteriocins antimicrobial activity were tested.

2.2.7. Effects of storage time to bacteriocins stability.

The stability of bacteriocins was checked at a regular interval (7, 14, 21, and 28 days) at room (30°C) and refrigerator (4°C) temperature. Then, the bacteriocins antimicrobial activity were tested.

3. RESULT AND DISCUSSION

3.1. Effects of heat to bacteriocins stability

The results of the statistical analysis showed that heat treatment up to 100°C showed the results were not significantly different to the activity of bacteriocins on pathogenic Gram-negative (*Escherichia coli* and *Salmonella typhimurium*) ($P > 0.05$), instead showed real different results on pathogenic Gram-positive (*Staphylococcus aureus*) ($P < 0.05$). Bacteriocins produced by *Bacillus subtilis* 11A were stable up to a temperature treatment of 90°C because it could retain its activity against *Staphylococcus aureus*, *Salmonella typhimurium*, and *Escherichia coli*, respectively up to 60, 80 and 83%. Bacteriocins produced by *Bacillus subtilis* 11A stops its activity when given the autoclave treatment. Thus, the bacteriocins produced by *Bacillus* bacteria were stable for heat treatment [13]. The mechanism of bacteriocins stability to heat was associated with its molecular structure, which was simple peptides without tertiary structures [14].

The bacteriocins produced by *Bacillus* bacteria were stable to heat treatment up to 80°C [15]. Entomocin 9 and 110 produced by *Bacillus thuringiensis* subsp.

entomocidus HD110 could maintain its activity up to 72% and 53% after autoclaved [16]. The bacteriocins

treatment gave effect on the decreased activity of bacteriocins due to denaturation. Denaturation caused a

Table 1. The effect of heat to bacteriocins stability produced by *Bacillus subtilis* 11A

Temperature (°C)	<i>Staphylococcus aureus</i>	<i>Salmonella typhimurium</i> ^{ns}	<i>Escherichia coli</i> ^{ns}
4	3644.96 ± 189.40 ^b	3940.49 ± 417.03	4040.02 ± 189.42
30	3336.35 ± 345.58 ^b	3451.22 ± 060.15	3696.54 ± 056.86
70	2234.11 ± 084.24 ^a	3237.44 ± 238.65	3719.48 ± 467.18
80	2249.44 ± 189.99 ^a	3136.28 ± 512.77	3683.48 ± 200.26
90	2168.64 ± 187.08 ^a	3164.75 ± 433.29	3375.81 ± 541.20
100	2087.10 ± 060.16 ^a	3046.43 ± 000.00	3014.66 ± 536.49
121	-	-	-

^{a,b}: Different superscripts in the same column show significant differences (P<0.05)

^{ns}: non-significant (P>0.05)

Table 2. The effect of pH to bacteriocin stability produced by *Bacillus subtilis* 11A

pH	<i>Staphylococcus aureus</i>	<i>Salmonella typhimurium</i>	<i>Escherichia coli</i>
2	587.75 ± 049.61 ^a	682.11 ± 105.53 ^a	1385.00 ± 156.16 ^a
3	1647.97 ± 035.79 ^c	995.48 ± 078.62 ^b	1554.09 ± 081.21 ^a
4	1881.75 ± 108.31 ^d	1295.72 ± 099.90 ^c	1649.54 ± 143.19 ^a
5	2073.97 ± 060.23 ^e	1793.88 ± 063.90 ^d	2371.85 ± 133.07 ^b
6	2303.13 ± 147.52 ^f	3062.18 ± 071.26 ^g	3253.93 ± 263.88 ^d
7	2818.09 ± 044.90 ^g	3429.51 ± 168.59 ^h	4040.02 ± 189.42 ^e
8	2275.50 ± 132.39 ^f	3015.97 ± 096.01 ^g	3465.19 ± 316.07 ^d
9	2034.56 ± 022.57 ^e	2470.50 ± 169.56 ^f	2895.49 ± 211.74 ^c
10	1820.15 ± 153.96 ^d	2193.44 ± 061.66 ^e	2572.49 ± 265.32 ^{bc}
11	832.67 ± 031.90 ^b	927.18 ± 060.20 ^b	1342.29 ± 197.29 ^a
12	-	-	-

^{a,b,c,d,e,f,g,h}: Different superscripts in the same column show significant differences (P<0.05)

produced by *Bacillus subtilis* GAS101 were stable up to a temperature of 100°C for 60 minutes and 121°C for 20 minutes without losing its antimicrobial activity [10]. Bacteriocins produced by *Bacillus subtilis* 14B were heat stable up to 100°C for 120 minutes [17].

3.2. Effects of pH to bacteriocin stability

The results of the statistical analysis showed that there was significantly different on bacteriocins activity affected by the pH of bacteriocins produced by *Bacillus subtilis* 11A (P<0.05). The best pH treatment was 7. Bacteriocins produced by *Bacillus subtilis* 11A were stable in acidic conditions of pH 6 (82% against *Staphylococcus aureus*, 89% against *Salmonella typhimurium*, 81% against *Escherichia coli*). The bacteriocins were also stable in alkaline conditions up to pH 10 (65% against *Staphylococcus aureus*, 64% against *Salmonella typhimurium*, 72% against *Escherichia coli*). Compared to the bacteriocins produced by *Bacillus* bacteria, they were effective in conditions of pH 6-7 and decreasing its activity at pH 4 [15,18]. The bacteriocins produced by *Bacillus* bacteria worked effectively in a pH-neutral range [13]. It is different from bacteriocins produced by bacteria type of *Lactobacillus*, which is stable at acidic conditions (pH 2–6) [19–21]. The higher and the lower the pH in

change in the structure of the constituent, thereby lowering its biological function [22].

3.3. Effects of storage time to bacteriocin stability

The results of the statistical analysis showed that there was a significant difference in bacteriocins activity which affected by storage time at room temperature (P<0.05). Bacteriocins in the room temperature storage survived for 14 days. The 7-days storage bacteriocins had a high condition of antimicrobial activity. By the 21-days storage, bacteriocins had been inactive. There was a decreased activity of 13% (*Staphylococcus aureus*), 23% (*Salmonella typhimurium*), and 28% (*Escherichia coli*) (P<0.05) on the 14th day since the 7th day.

The results of the statistical analysis showed that there was no significant difference in bacteriocins activity affected by storage time at the refrigerator for 28 days (P>0.05). The activity of bacteriocins still showed the optimum activity after its storage for 28 days. The result indicated that the bacteriocins produced by *Bacillus subtilis* 11A was stable in 28 days storage in the refrigerator. The previous study showed that bacteriocins at a temperature of 4°C were able to

survive for 20 days, and its activity significantly decreased when stored at temperature 37°C on the 5th

Mada for financial support. The authors also gratefully acknowledge to Department of Feed and

Table 3. The effect of storage time at room temperature to bacteriocin stability produced by *Bacillus subtilis* 11A

Storage time (days)	<i>Staphylococcus aureus</i>	<i>Salmonella typhimurium</i>	<i>Escherichia coli</i>
7	2412.83 ± 063.99 ^b	2820.19 ± 204.74 ^b	3141.26 ± 164.24 ^b
14	2103.80 ± 237.41 ^a	2180.10 ± 069.89 ^a	2261.53 ± 107.47 ^a
21	-	-	-

^{a,b}: Different superscripts in the same column show significant differences (P<0.05)

Table 4. The effect of storage time at refrigerator temperature to bacteriocin stability produced by *Bacillus subtilis* 11A

Storage time (days)	<i>Staphylococcus aureus</i> ^{ns}	<i>Salmonella typhimurium</i> ^{ns}	<i>Escherichia coli</i> ^{ns}
7	3347.71 ± 122.15	3938.82 ± 356.77	3937.87 ± 328.16
14	3000.85 ± 122.66	3627.96 ± 175.12	3696.62 ± 254.21
21	3113.83 ± 328.28	3627.43 ± 133.45	3746.49 ± 207.48
28	3078.35 ± 135.52	3833.10 ± 270.36	3649.61 ± 058.01

^{ns}: non-significant (P>0.05)

day [20]. The bacteriocins produced by *Bacillus megaterium* 22 could survive up to 90 days at 4°C [9]. The best way to keep bacteriocin was at low temperatures (≤4°C) because it could maintain its stability up to 6 weeks, while bacteriocins in 37°C storage only last up to 4 weeks [23].

Bacteriocin's peptides were one of the nutrients that were needed by the microorganisms to grow. When bacteriocins stored at room temperature, many microorganisms may grow to utilize this peptide as nutrients source so that bacteriocins would be easily damaged. Therefore, its activity decreased due to the growth of microorganisms. Low temperatures storage inhibits the growth of microorganisms that use these peptides. Microorganisms are generally dormant at low temperatures (≤4°C), thus bacteriocins low-temperature storage could last longer, and the decrease of its activity was lower than storage at room temperature [24,25].

4. CONCLUSION

Based on the results of the research obtained, it is concluded that the bacteriocins produced by *Bacillus subtilis* 11A was heat-stable until 90°C, pH 6–10, and refrigerator storage up to 28 days.

ACKNOWLEDGMENTS

The authors would like to thank Rekognisi Tugas Akhir (RTA) program from Universitas Gadjah

Animal Nutrition, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta.

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