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Characterization of antibacterial activity produced by *Bacillus* spp. isolated from honey and bee-associated products against foodborne pathogens

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Abstract. *Magdalena S, Anggelia, Yogiara. 2020. Characterization of antibacterial activity produced by* Bacillus *spp. isolated from honey and bee-associated products against foodborne pathogens. Bioteknologi 17: 51-59.* Four potential isolates (*Bacillus velezensis* Y12, *Bacillus amyloliquefaciens* Y21, *Bacillus amyloliquefaciens* Y23, and *Bacillus velezensis* Y33) isolated from honey, propolis, and bee pollen from West Java, Indonesia showed antifungal and enzymatic activities. This study aimed to assay antibacterial activity against foodborne pathogens, determine the optimum condition for antibacterial compounds production, observe the effect of thermal and pH treatment on the antibacterial compounds, and detect the presence of antibacterial peptide biosynthesis gene. In this research, bacterial isolates showed antibacterial activity against *Bacillus cereus* ATCC 14579, *Salmonella enterica* 51741, and *Salmonella* Typhimurium ATCC 14028. The growth condition for antibacterial production of isolate *B. velezensis* Y12 was at the range of 25-60°C and pH 7-9. Meanwhile, the other three isolates showed the same pattern, also at the range of 37-60 °C. Antibacterial compounds against *B. cereus* were found heat stable at 4-90°C and active over pH 3-9. Cultivation of the isolates in BHIB and TSB did not significantly increase the antibacterial activity against all pathogens as compared to an LB medium. On the contrary, the antibacterial activity against *S. typhimurium* of the isolates cultivated in optimized medium two (yeast extract 32.5 g/L, glucose 33.4 g/L, $MnSO_4$ 0.042 g/L, $CaCl_2$ 0.031 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.5 g/L, $(NH_4)_2SO_4$ 1.0 g/L, and $MgSO_4$ 4.0 g/L) was significantly increased. Furthermore, seven antibacterial peptide biosynthesis genes primers were amplified from the genomic DNA of isolates *B. velezensis* Y12 and *B. velezensis* Y33 by PCR analysis. These genes were *fenD, srfAA, bacA, bmyB, ituC, ituD,* and *bmyD*. Meanwhile, there was no presence of *ituC* gene from the other isolates. This result suggests that isolate *B. velezensis*Y12 might be the most potential isolate for antibacterial compounds production.

Keywords: Antibacterial activity, *Bacillus*, foodborne pathogens, honey

INTRODUCTION

Microbes are potential resources of antimicrobial compounds that have novel mechanisms (Martinez-Klimova et al. 2017). Antimicrobial properties of honey and other bee-associated products such as a beehive, pollen, propolis, and royal jelly have been widely studied against pathogenic microorganisms, including bacteria and fungi. The inhibition of these microorganisms is due to the high osmolarity of sugar content, and high hydrogen peroxide compounds found in honey. The presence of some bacteria, mold, and yeast may also contribute to this activity in honey and other bee-associated products. The diversity of microorganisms found in honey can be expected because honey is not processed food. The microorganisms can originate from the environment like dust, dirt, air, pollen, flowers, other plants, and the digestive tracts of honeybee (Lee et al. 2008). Among all microorganisms, *Bacillus* spp. is one of the bacteria mostly found in honey samples. *Bacillus* has many potential antimicrobial compounds against bacteria or fungi plant pathogens, as reported by previous studies (Zhao et al. 2013). However, there has been no research on microorganisms in Indonesian honey and their potential microbial activities; even though Indonesia has various types of honey produced in different locations.

In our previous research, four potential isolates were isolated from honey, beehive, pollen, and propolis obtained from West Java, Indonesia (Purnawidjaja 2018). These bacterial isolates were identified as *Bacillus velezensis* and *Bacillus amyloliquefaciens*. The isolates had enzymatic and antifungal activities against *Candida albicans* ATCC 10231 and *Aspergillus fumigatus* ATCC 204305. However, there are still many potential antibacterial activities that could be assayed from these isolates. Characteristics of the antibacterial properties are considered to obtain information about the optimum growth conditions for antibacterial production. One of antimicrobial agents that recently emerged is an antimicrobial peptide (Mahlapuu et al. 2016). The antimicrobial peptide can potentially be used to combat the rapid increase in conventional antibiotics. In *Bacillus* strain, several antimicrobial peptides have been identified, such as fengycin, bacyllomycin, iturin, surfactin, bacilysin, subtilin (Mora et al. 2011; Sumi et al. 2015). The presence of those antimicrobial peptides can be detected by the presence of the gene *fenD, bmyB, ituC, srfA, bacA, spaS* (Mora et al. 2011).

This research aims to obtain potential antibacterial activity against foodborne pathogens, to obtain the optimum condition for cell growth and antibacterial production, to evaluate the stability of the extracellular antibacterial compounds, and to detect antibacterial peptide biosynthesis genes from genomic DNA of each isolate.

MATERIALS AND METHODS

Bacterial isolates and growth condition

Four *Bacillus* isolates used in this study were obtained from the culture collection of the Department of Food Technology, Atma Jaya Catholic University of Indonesia, Indonesia. These bacteria had been isolated from honey and other bee-associated products in previous research (Purnawidjaja 2018). The isolates were cultivated on Luria Agar (LA) and incubated for 24 h at 37°C. Gram-positive bacteria, *Bacillus cereus* ATCC 14579; and Gram-negative bacteria, *Salmonella enterica* subsp. *enterica* ATCC 51741 and *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 were used as bacterial indicators in antibacterial activity assay. All bacteria used as bacterial indicators were grown in Luria Broth (LB) for 24 h at 37°C.

Antibacterial activity assay

The antibacterial activity against bacteria was determined by the agar well diffusion method. Suspension of indicator bacteria was prepared according to the Clinical and Laboratory Standard Institute protocol (CLSI 2018) for the determination of antimicrobial activity. The cell suspensions were equivalent to 0.5 McFarland based on the absorbance value of 0.132 at 600 nm. The amount of 100 µL of bacterial suspensions was spread on the Mueller-Hinton Agar (MHA) plates. 6mm diameter wells were made by using a sterile cork borer.

Bacteria isolates were grown at 37ºC for 24 h in LB medium. The crude extract (cell-free supernatant) was obtained by centrifugation at 7800× g for 20 minutes. Each supernatant with the amount of 100 µL was loaded into the well and incubated at 37ºC for 24 h. The assay was carried out in triplicate. Streptomycin (25 µg) and Aztreonam (30 µg) antibiotic disc were used as a positive control. An uninoculated LB medium was used as a negative control. The inhibition was measured by the diameter of the clear zone (Lertcanawanichakul and Sawangnop 2008).

Optimization of cell growth for antibacterial compounds production

Bacteria isolates were cultivated in LB for 24 h and measured to reach 0.5 McFarland (OD_{600} = 0.132). For growth temperature optimization, the isolates were grown in LB medium at different temperatures ranging from 25°C, 37°C, and 60°C for 24 h. The antibacterial activity was determined by the agar well diffusion method, as previously mentioned. The growth of each isolate was verified by plating after 4 h, 8 h, and 24 h incubation. The optimum growth temperature was chosen based on the highest antibacterial production (Iqbal et al. 2018).

For pH optimization, bacterial isolates were grown in LB medium at different pH values (citrate buffer pH 4, phosphate buffer pH 7, and Tris buffer pH 9) and incubated at the optimum temperature. Antibacterial activity and plating of isolates were determined to evaluate the optimum pH value (Song et al. 2013).

Antibacterial compounds stability assay

The thermal stability of the crude extract of each bacterial isolate was determined by different temperatures ranging from 4, 37, 70, 90, and 121°C for 30 minutes. The pH stability was determined by adjusting the pH value with 0.1 M buffers: citrate buffer (pH 3 and 5), phosphate buffer (pH 7), and Tris buffer (pH 9) at 37°C for 2 h (Ramachandran et al. 2014). Enzymatic sensitivity was tested by treating the crude extract with the enzymes: papain (phosphate buffer pH 6.5), proteinase K (Tris buffer pH 8.5), and amylase (phosphate buffer pH 6.1) at final concentration 2 mg/mL for each enzyme. The crude extract was incubated for 2 h at different temperatures, depending on each enzyme treatment. Proteinase K and amylase were incubated at 37°C, bromelain at 55°C, and papain at 65°C. Each treatment was carried out in triplicate. The antibacterial activity was assayed after treatments by agar well diffusion methods against indicator strain (Alfonso et al. 2012).

Detection of antibacterial peptide biosynthesis genes

Genome isolation was carried out by using a DNA kit from SolGent™ Genomic DNA Prep Kit for Bacteria (Solution type) (Solgent Co., Ltd., South Korea). Isolated genomic DNA was used as a template for PCR reaction. Peptide biosynthesis gene was detected using PCR amplification method with specific primers (Table 1). The composition of PCR mixture was 1 µL DNA template, 1 µL of each forward and reverse primer, 22 µL of nuclease-free water, and 25 µL of 2X GoTaq Green master mix (Promega, USA). Amplification of no template control (NTC) was also included. PCR condition consisted of the initial denaturation step at 95°C for 4 minutes, 40 cycles of denaturation at 94°C for 1 minute, annealing step for 1 minute, elongation at 70°C for 1 minute, followed by final extension at 70°C for 5 minutes. The annealing temperature was set at 55°C for *bmyB*; 58°C for *ituC, bacA, fenD, srfAA,* and *bmyD*; and 50°C for *ituD*. PCR results were visualized by agarose gel electrophoresis at 1.5% agarose, 90 V for 60 minutes (Ramarathnam et al. 2007; Mora et al. 2011).

Media variation for antibacterial compounds production

Bacteria isolates were cultured in different liquid media such as Luria Broth (LB, 10 g/L tryptone, 5 g/L yeast extract, and 0.05 g/L NaCl), Brain Heart Infusion Broth (BHIB, 12.5 g/L brain infusion, 5 g/L beef heart infusion, 10 g/L peptone, 2 g/L glucose, 5 g/L NaCl, and 2.5 g/L Na_2HPO_4), Trypticase Soy Broth (TSB, 17 g/L pancreatic digest of casein, 3 g/L soybean meal, 5 g/L NaCl, 2.5 g/L dextrose, and 2.5 g/L K_2HPO_4), and two optimized media with different compositions.

Table 1. Gene primers for PCR amplification.

Primer	Sequence (5' → 3')	Gene	Product	Size (bp)	Source
FEND-F	GGCCCGTTCTCTAAATCCAT	*fenD*	Fengycin	269	Mora et al. 2011
FEND-R	GTCATGCTGACGAGAGCAAA				
SRFA-F	TCGGGACAGGAAGACATCAT	*srfAA*	Surfactin	201	Mora et al. 2011
SRFA-R	CCACTCAAACGGATAATCCTGA				
BAC-F	CAGCTCATGGGAATGCTTTT	*bacA*	Bacilysin	498	Mora et al. 2011
BAC-R	CTCGGTCCTGAAGGGACAAG				
BMYB-F	GAATCCCGTTGTTCTCCAAA	*bmyB*	Bacillomycin L	370	Mora et al. 2011
BMYB-R	GCGGGTATTGAATGCTTGTT				
ITUC-F	GGCTGCTGCAGATGCTTTAT	*ituC*	Iturin A	423	Mora et al. 2011
ITUC-R	TCGCAGATAATCGCAGTGAG				
ituD-F	ATGAACAATCTTGCCTTTTTA	*ituD*	Iturin A	1203	Hsieh et al. 2008
ituD-R	TTATTTTAAAATCCGCAATT				
BACC1-F	GAAGGACACGGCAGAGAGTC	*bmyD*	Bacillomycin D	875	Ramarathnam et al. 2007
BACC1-R	CGCTGATGACTGTTCATGCT				

Note: F-Forward Primer, R-Reverse Primer

Optimized medium 1 was adopted from Akpa et al. (2001). The medium was composed of sucrose 20 g/L, peptone 30 g/L, yeast extract 7 g/L, KH_2PO_4 1.9 g/L, $MgSO_4$ 0.45 g/L, and trace elements 9 mL/L. The composition of trace elements was 0.001 g of $CuSO_4$, 0.005 g of $FeCl_3$, 0.004 g of $NaMnO_4$, 0.002 g of KI, 0.014 g of $ZnSO_4$, 0.01 g of H_3BO_3, 0.0036 g of $MnSO_4$, 10 g of citric acid. Optimized medium 2 from Mosquera et al. (2014) was composed of yeast extract 32.5 g/L, glucose 33.4 g/L, $MnSO_4$ 0.042 g/L, $CaCl_2$ 0.031 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.5 g/L, $(NH_4)_2SO_4$ 1.0 g/L, and $MgSO_4$ 4.0 g/L.

All bacteria isolates were incubated at the optimum temperature for 24 h, and the antibacterial activity of the crude extract was assayed by the agar well diffusion method and carried in triplicate (Iqbal et al. 2018).

Statistical analysis

All data obtained from the research were analyzed by one way ANOVA with posthoc Tukey test using SPSS application to determine the significant differences between treatments ($P<0.05$).

RESULTS AND DISCUSSION

Antibacterial activity

Antibacterial activity assay showed that all isolates exhibited antibacterial activity against *B. cereus*, *S. enterica*, and *S. typhimurium* (Table 2). The highest activity was *B. amyloliquefaciens* Y21 against *B. cereus*, with a 10.3 mm zone of inhibition, among other tested strains.

Optimization of cell growth for antibacterial compounds production

Bacteria isolates were grown in various temperatures and pH levels to achieve the optimum condition for antibacterial compounds production. The selection of optimum range depended on cell growth and the inhibitory zones against pathogens.

It was shown that the optimum growth *B. velezensis* Y12 and *B. amyloliquefaciens* Y21,was achieved at 37°C, while *B. amyloliquefaciens* Y23 and *B. velezensis* Y33 was at 25°C. The highest antimicrobial activity at this optimum growth was achieved by *B. amyloliquefaciens* Y23, with a 13.0 mm of inhibition zone against *B. cereus*. All isolates could grow at 60°C even though the growth was lower than bacterial growth at 37°C.We can still observe antimicrobial activity against all pathogens, except for *B. amyloliquefaciens* Y21 (Figure 1). In *B. amyloliquefaciens* Y21, the antimicrobial compounds lose the activity against *S. enterica* and *S. typhimurium.*

Nevertheless, there is no significant difference between antimicrobial activity in various growth temperatures of *B. velezensis* Y12. In contrast, the antimicrobial compound that isolated from bacteria grown at 60°C showed significantly lower activity compared to other antimicrobial compounds from bacteria grown at 25 and 37°C. This might be due to the decrease in cell numbers that affected antimicrobial compound production.

As for pH study, the neutral condition (pH 7) exhibited the highest growth and antibacterial activity than the alkaline condition (pH 9). However, there was no significant difference between the results (Table 3). These conditions indicate that the pH condition was at the range of pH 7-9 for all isolates. Moreover, the acidic condition of pH 4 showed the lowest growth with no antibacterial activity was observed against all pathogenic bacteria.

Antibacterial compounds stability

Antibacterial compounds of each isolate remained stable over various heat treatments ranging from 4-90°C and various pH levels ranging from pH 3-9 against *B. cereus* (Figure 2). However, the antibacterial compounds showed no activity against *S. enterica* and *S. typhimurium* at heat treatment above 70°C and below pH 7. The antibacterial compound activity remained stable at 4-70°C and pH 7-9 for isolates *B. amyloliquefaciens* Y21 and *B. amyloliquefaciens* Y23. Antibacterial activity for the other two isolates significantly declined when exposed to higher pH values (Figure 3).

Table 2. Antibacterial activity of bacteria isolates against foodborne pathogens.

Isolates	Zone of inhibition (mm)		
	B. cereus **ATCC 14579**	***S. enterica*** **ATCC 51741**	***S. typhimurium*** **ATCC 14028**
B. velezensis Y12	9.3	6.5	6.5
B. amyloliquefaciens Y21	10.3	5.3	6.0
B. amyloliquefaciens Y23	9.3	4.7	5.3
B. velezensis Y33	8.0	5.3	7.5

Table 3. Antibacterial activity of cell culture grown in various pH levels.

Isolates	Growth condition	Zone of inhibition (mm)		
		B. cereus **ATCC 14579**	***S. enterica*** **ATCC 51741**	***S. typhimurium*** **ATCC 14028**
B. velezensis Y12	pH 4	-	-	-
	pH 7	11.5[a]	6.0[a]	7.0[a]
	pH 9	8.7[a]	5.0[a]	5.0[a]
B. amyloliquefaciens Y21	pH 4	-	-	-
	pH 7	10.3[a]	5.7[a]	7.0[a]
	pH 9	8.3[a]	4.7[a]	5.0[a]
B. amyloliquefaciens Y23	pH 4	-	-	-
	pH 7	13.7[a]	5.7[a]	6.3[a]
	pH 9	11.3[a]	4.7[a]	6.3[a]
B. velezensis Y33	pH 4	-	-	-
	pH 7	11.7[a]	7.0[a]	7.3[a]
	pH 9	10.0[a]	6.0[a]	6.3[a]

Note: [a,b] represent a significant difference between treatment (P<0.05)

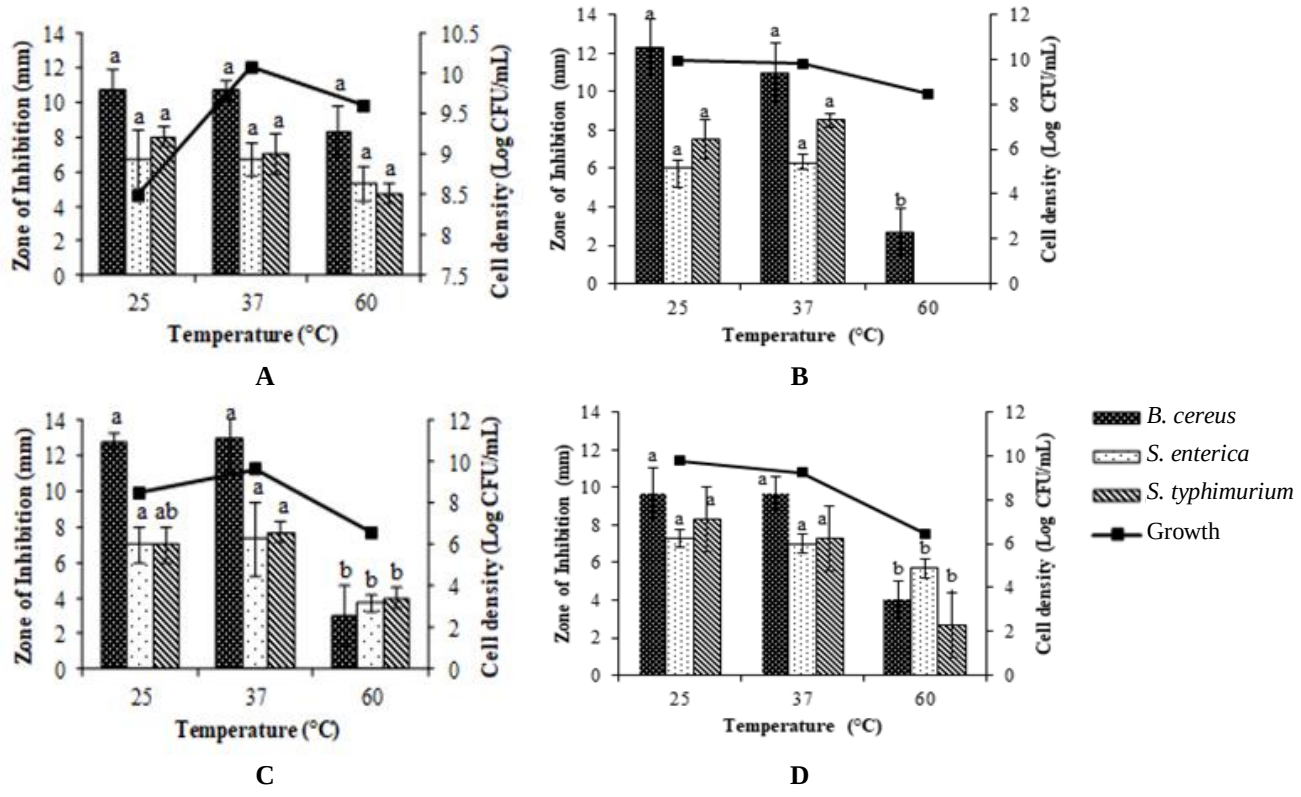

Figure 1. Effect of temperature variation on *Bacillus velezensis* Y12 (A), *B. amyloliquefaciens* Y21 (B), *B. amyloliquefaciens* Y23 (C), *B. velezensis* Y33 (D), growth (line) and antibacterial activity (column) against *B. cereus, S. enterica,* and *S. typhimurium.*

The significant decline of antibacterial activity was found after all antibacterial compounds being treated by papain and bromelain enzyme against *B. cereus* (Figure 4.A), except for *B. velezensis* Y33. The antibacterial compound isolated from *B. velezensis* Y33 lost its activity after being treated by papain and bromelain. Hence, these papain and bromelain treatment caused the loss of antibacterial activity of all antibacterial compounds against *S. enterica* and *S. typhimurium* (Figure 4.B). There was no effect after exposure to α-amylase and proteinase-K enzyme compared to control treatment without the addition of enzymes.

Detection of antibacterial peptide biosynthesis genes

As Figure 5 indicates, all DNA amplicon bands were visible, showing that *fenD, srfAA, bacA, bmyB, ituC, ituD,* and *bmyD* genes were present in genomic DNA of isolate *B. velenzensis* Y12. The same results were obtained for isolates *B. velezensis* Y33. Meanwhile, the other two isolates results showed no indication of *ituC* gene.

Media variation for antibacterial compounds production

All four isolates exhibited antibacterial activity on different media. The results showed that cultivation on BHIB and TSB media did not alter much the antibacterial activity against *B. cereus, S. enterica,* and *S. typhimurium* as compared to LB medium. Meanwhile, cultivation on optimized medium 1 (Akpa et al. 2001) lowered the activity of all isolates. Another optimized medium with different compositions adopted from Mosquera et al. (2014) study showed slightly increased activity against *S. typhimurium* (Figure 6).

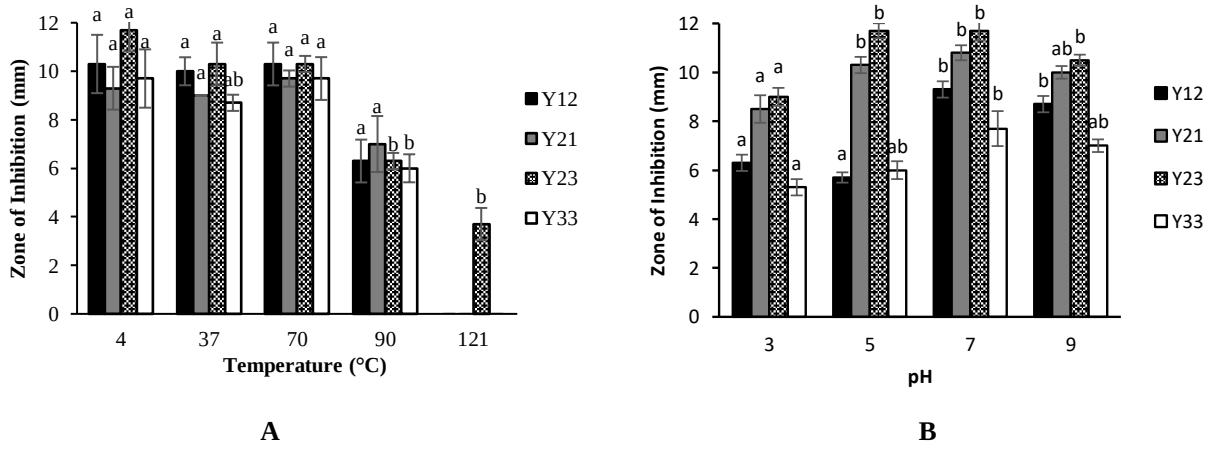

Figure 2. Heat (A) and pH (B) treatment on antibacterial activity against *Bacillus cereus.*

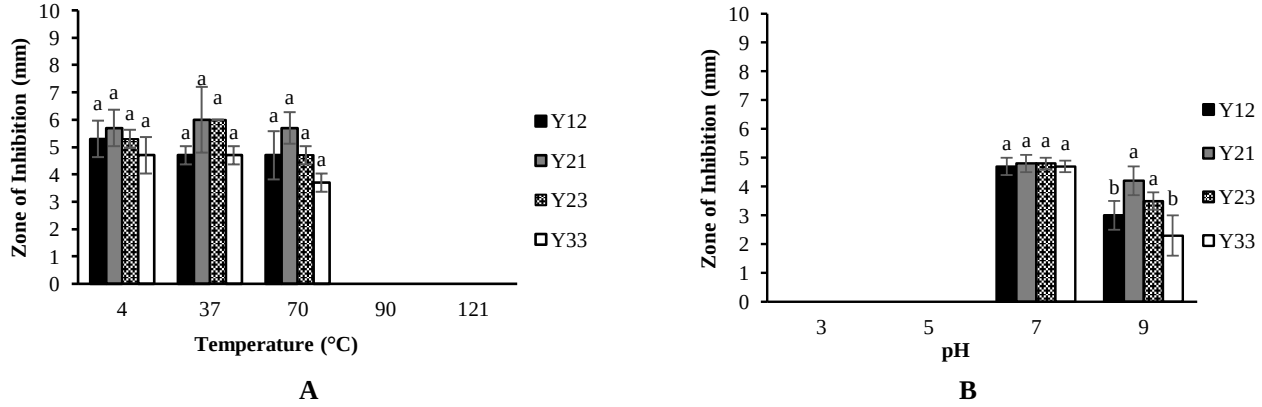

Figure 3. Heat (A) and pH (B) treatment on antibacterial activity against *Salmonella enterica.*

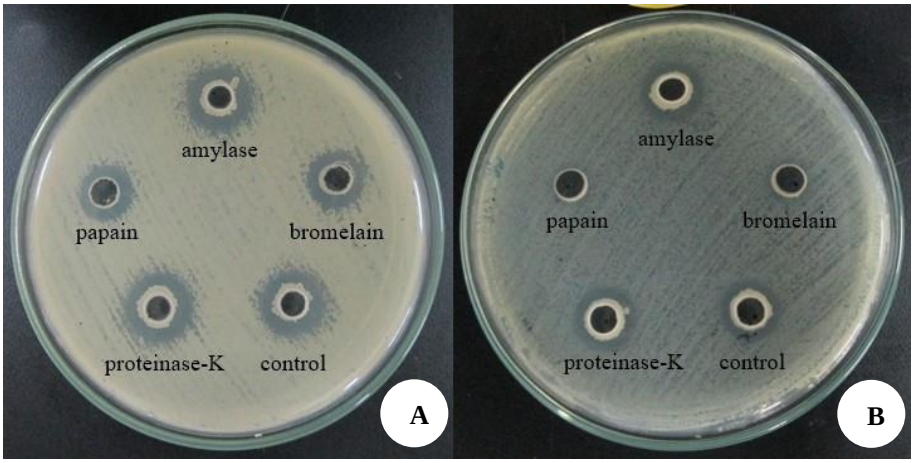

Figure 4. The results of enzymatic sensitivity assay of the antibacterial compound as represented by antibacterial compound isolated from *Bacillus amyloliquefaciens* Y23 against *B. cereus* (A) and *Salmonella enterica* (B).

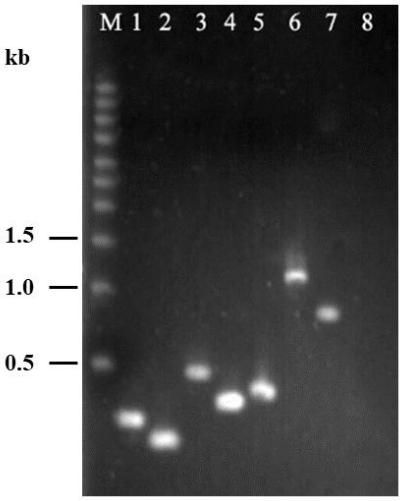

Figure 5. Representative gel image of PCR amplification results for isolate *Bacillus velezensis* Y12. Lane M is 1 kb DNA ladder, Lane 1-8, *fenD, srfAA, bacA, bmyB, ituC, ituD, bmyD,* and NTC, respectively.

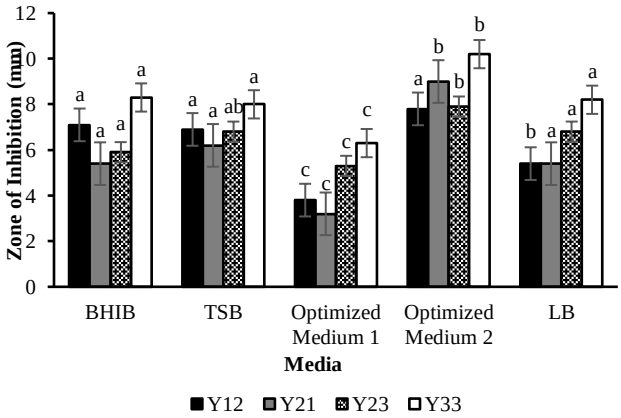

Figure 6. Antibacterial compounds production against *Salmonella typhimurium* in various media.

Discussion

The isolates of *B. velezensis* Y12, *B. velezensis* Y33, *B. amyloliquefaciens* Y21, and *B. amyloliquefaciens* Y23 exhibited antibacterial activity against all tested foodborne pathogens. *Bacillus* spp. is known as a cosmopolitan species due to its ability to survive in an adverse environment (Lee et al. 2008). This genus also produced beneficial compounds like antibacterial compounds with various action mechanisms. The activity of these compounds, especially antibacterial peptides, has been extensively explored against plant pathogens as one of the biocontrol agents (Li et al. 2016). *B. subtilis*, isolated from the honey sample and bee gut, showed activity against important honeybee pathogens, which were *Paenibacillus larvae* and *Ascophaeraapis* (Sabate et al. 2009). Several strains of *Bacillus* isolated from soil also produced compounds against pathogenic and food-spoilage bacteria such as *B. cereus* and *Listeria monocytogenes* (Lisboa et al. 2006).

Cell growth in various conditions, like temperature and pH medium, affected the production of antibacterial compounds. Various studies on incubation temperature revealed that *B. velezensis* Y12 showed the highest growth at 37°C yet showed no difference in terms of antibacterial compounds production at lower and higher temperatures. This indicates that production could be achieved regardless of optimum temperature. Meanwhile, the result of three isolates *B. amyloliquefaciens* Y21, *B. amyloliquefaciens* Y23, and *B. velezensis* Y33 showed the same pattern. As reported by Iqbal et al. (2018), temperature affected the antimicrobial activity in the way of denaturing the compounds to some extent. This might cause loss of antibacterial activity during heat exposure. Moreover, high temperature also decreased cell growth of isolate Y23 (Figure 1). It was indicated that antibacterial compounds were produced along with the cell growth of each isolate.

The condition of pH in the cultivation medium also affected the production of the antibacterial compound. The treatment of the acidic medium was not suitable for the growth of every isolate. There was no activity shown by isolate which was cultivated in pH 4 medium (Table 3). The cell growth was also inhibited, showing that the production of antibacterial compounds occurred in line with the bacterial cell growth. Both pH 7 and pH 9 of media were the conditions in which bacterial isolate could grow and produce antibacterial compounds better than acidic conditions. The result of each treatment indicated no significant difference. Thus, the medium of antibacterial compounds production could be adjusted to higher pH values.

In Moshafi et al.'s (2011) study, *Bacillus* sp. strain FAS_1 was isolated from soil produced antibacterial compounds at alkaline condition pH 8.4. There were changes in pH value as the biomass of cells increased. This condition shows that antibacterial compounds might not be associated with organic acids as the pH value continued to increase during cell growth. A similar finding was also found in Motta et al.'s (2004) study demonstrating that the inhibitory effect of *Bacillus* sp. isolated from the Amazon basin was not caused by organic acids, as the pH value increased during antibacterial production. The preferable condition was at 37°C with a neutral pH cultivation medium for further steps.

Antibacterial compounds were heat-stable, starting from 4°C until 70°C against all foodborne pathogens. Moreover, antibacterial compounds were stable until 90°C against *B. cereus* (Figure 2). In a study by Teixeira et al. (2013), antibacterial peptides produced by *Bacillus subtilis* could retain their activity until exposure at 80°C. The ability to retain antibacterial activity was also reported by Risoen et al. (2004), stating that the compounds had stable structures that could resist heat treatment. On the contrary, the present study suggests that exposure to extreme heat might cause the loss of antibacterial activity due to different characteristics of each compound produced by bacterial isolates. However, heat-stable antibacterial compounds were suitable for food applications which could be adapted to a harsh environment including those with higher temperatures (Sutyak et al. 2008).

Treatment of antibacterial compounds with various pH conditions showed no loss of activity against *B. cereus*. The compounds were stable over pH 3-9, with a slight decrease in activity for the treatment of pH 3 (Figure 2). A previous study stated that bacteriocin-like inhibitory substance (BLIS) from *B. amyloliquefaciens* LBM 5006 against *B. cereus* was stable over pH 3-8 (Lisboa et al. 2006). As for the activity against *S. enterica* and *S. typhimurium*, there was no loss of activity for the treatment of pH 7 and pH 9; however, there was loss of activity when the compounds were exposed to lower pH conditions, such as pH 3 and pH 5 (Figure 3). Another study by Ramachandran et al. (2014) reported that *B. subtilis* RLID 12.1 could retain 100% activity between pH 6 and pH 8. This ability indicates that bacteria isolates produced more than one compound to attack different kinds of pathogenic bacteria, which had different tolerance range for heat and pH treatment. Consequently, antibacterial compounds should be handled in suitable conditions to prevent loss of activity.

Proteolytic enzyme sensitivity test represented proteinaceous compounds of antibacterial compounds produced. The results of the papain and bromelain treatment show significant reduction against *B. cereus* (Figure 4). In the same treatment, antibacterial compounds showed no activity against *S. enterica* and *S. typhimurium*. Based on Sabate and Audisio (2013), antibacterial compounds were found active after treatment with proteinase-K. In agreement with that study, the other proteolytic enzyme which was proteinase-K did not eliminate or decrease the antibacterial activity of each isolate. This might happen due to the enzyme activity or the compounds itself. Proteolytic enzymes are divided based on their mode of action. Papain and bromelain actively cleave peptide bonds by the presence of cysteine at the active site. Proteinase-K is the group of serine protease whose activity is determined by the presence of serine (Walsh 2002).

Furthermore, *Bacillus* spp. produced cyclic peptides with unusual amino acids. This relatively rigid structure caused protease resistance due to the inaccessible cleavage site (Bizani and Brandelli 2002; Korenblum et al. 2005). The same results were obtained from glycolytic (α-amylase) enzyme treatment of antibacterial compounds that retained the antibacterial activity. This result indicates that there was no carbohydrate moiety involved in antibacterial activity. According to Zhao et al. (2013), inhibition of various pathogen bacteria and fungi is detected because *Bacillus* spp. can produce different kinds of antibiotics, enzymes, amino acids, or peptide.

A further step to detect the presence of antibacterial peptides biosynthetic genes was conducted using PCR analysis. The result showed that specific primer bands matched the sizes of PCR products conducted in previous research. Antimicrobial peptides, which are mostly cyclic lipopeptides, are non-ribosomally synthesized by enzymes called non-ribosomal peptide synthetases. Each gene encoded different products of peptides, including *fenD* for fengycin synthetase, *srfA* for surfactin synthetase subunit 1, *bacA* for bacilysin biosynthesis protein, *bmyB* for bacillomycin L synthetase B, *bmyD* for bacillomycin D synthetase C, *ituC* and *ituD* for iturin A synthetase (Ramarathnam et al. 2007; Hsieh et al. 2008; Mora et al. 2011).

Fengycin is cyclic decapeptide, whose biosynthesis consists of five genes (*fenA-E)*, including *fenD* conducted in this study. The mechanism of antagonistic activity was shown by the pore formation of the cell membrane (Gong et al. 2015). Surfactin is a cyclic lipoheptapeptide which alters membrane integrity and permeability by channel formation mechanism against several microorganisms (Meena and Kanwar 2015). Another lipoheptapeptide compound, iturin, consists of iturin A, C, D, and E, along with bacillomycin D, F, and L. Bacillomycin D has *bmy*gene clusters including *bmyD*, *bmyA*, *bmyB*, and *bmyC*. Iturin operon has four open reading frames, specifically, *ituA*, *ituB*, *ituC*, and *ituD*. Similar tofengycin, iturin also had pore formation activity against target bacteria (Maget-Dana and Peypoux 1994). The application of iturin has widely spread in food or pharmaceutical products. Meanwhile, bacilysin is a dipeptide compound composed of L-alanine and L-

anticapsin as an inhibitor of cell wall biosynthesis. It also has (*bacA-E*) gene clusters (Arguelles-Arias et al. 2009; Chen et al. 2009).

PCR amplification results of *B. velezensis* Y12 and *B. velezensis* Y33 align with a study by Palazzini et al. (2016), showing that fengycin, surfactin, iturin, bacillomycin, and bacilysin were identified in the genome sequence of *B. velezensis* RC 218 isolated from wheat anthers. As for *B. amyloliquefaciens* Y21 and *B. amyloliquefaciens* Y33, the lack of *ituC* gene shows that iturin A might not be present as one of the antibacterial peptides. Moreover, *ituD* gene is an essential gene in the biosynthesis of iturin A because its disruption caused iturin A deficiency (Tsuge et al. 2001).

Many factors influence the ability of bacteria to produce antibacterial compounds, such as the conditions of cultivation and nutrition of medium. These conditions include temperature, pH, and incubation period. Based on the results, the preferable condition was at 37°C with pH 7. The medium composition along with the metabolic capacity of each bacterium also played a significant role in the biosynthesis of antibacterial compounds (Kiranmayi et al. 2011). As reported in the study from Muhammad et al. (2015), nitrogen-rich medium (BHIB and TSB) enhanced both cell growth and antimicrobial compounds production than simple nutrient medium. In this study, cultivation in such media resulted in no significant enhancement of antibacterial activity. This might happen due to the complex genetic regulation of each bacterial isolates. Lisboa et al. (2006) also stated that the condition of cultivation might induce different peptide antibiotics production. Therefore, the selection of carbon and nitrogen source was critical, depending on bacteria strain (Ripa et al. 2009).

Besides, the incubation time influenced antibacterial compounds production. Jin et al. (2015) found that the production of iturin by *Bacillus subtilis* gradually achieved after the exponential growth phase and reached to maximum level during stationary phase after 24 h. Different lipopeptides, surfactin, and bacillomycin production were induced during stationary phase (Chen et al. 2009). A study by Coutte et al. (2010) showed that fengycin was produced at the end of exponential phase and continued until the next phase. Cyclic lipopeptides produced by *Bacillus* spp. showed the same tendency because those compounds are non-ribosomal peptides. The metabolites are more likely to be produced at the late phase of bacterial growth (Kumar et al. 2012).

Bacterial isolates cultivated in optimized medium 1 showed a reduction in activity (data not shown) as opposed to the previous study by Iqbal et al. (2018), showing that *Bacillus safensis* MK-12 grown in the optimized medium had the highest activity among other cultivation media. Akpa et al.'s (2001) study stated that cultivation of *B. subtilis* NT02 in the same optimized medium yielded homologous products identified as bacillomycin in vigorous intensity. These findings suggest that the production of antibacterial compounds depends on bacteria strain and its ability to produce different compounds by utilizing nutrients in the medium.

Another study by Mosquera et al. (2014) reported that higher cell density led to a higher activity of *B. subtilis*, which was achieved by optimizing the medium with a high dose of glucose and yeast extract. Glucose was the preferable source of carbon and energy. Meanwhile, yeast extract contained amino acids and nucleotides as precursors of cell biomass and antibacterial compounds. In this study, the results of antibacterial activity against *S. typhimurium* and *S. enterica* showed enhancement. Modification of nutritional factors in cultivation medium might promote the possibility of a new antibacterial compound biosynthesis. The selection of carbon source also played a significant role. As discussed in Liu et al. (2012), surfactin, a lipopeptide antimicrobial compound, was produced optimally by *Bacillus* sp. MB199 in medium with fructose as the carbon source and ammonium nitrate as the nitrogen source. The regulation of carbon and nitrogen source differed from each bacterial strain, as stated by previous studies.

Different sources of carbon (fructose, maltose, dextrose, mannitol, sucrose, and lactose) and sources of nitrogen (meat peptone, beef extract, yeast extract, tryptone, meat infusion extract, and malt extract) were also used in Kumar et al.'s (2012) study. The result showed that antimicrobial activity differed significantly when the sources were changed. In addition, the study also revealed that eliminating carbon source caused the reduction of antimicrobial compounds production. Therefore, further research is required to manipulate nutrients and determine the best source of carbon and nitrogen in the cultivation medium to achieve a higher activity in the inhibition of foodborne pathogens.

REFERENCES

Akpa E, Jacques P, Wathelet B, Paquot M, Fuchs R, Budzikiewicz H, Thonart P. 2001. Influence of culture conditions on lipopeptide production by *Bacillus subtilis*. Appl Biochem Biotechnol 91 (1): 551-561.

Alfonso A, Piccolo SL, Conigliaro G, Ventorino V, Burruano S, Moschetti G. 2012. Antifungal peptides produced by *Bacillus amyloliquefaciens* AG1 active against grapevine fungal pathogens. Ann Microbiol 62 (4): 1593-1599.

Arguelles-Arias A, Ongena M, Halimi B, Lara Y, Brans A, Joris B, Fickers P. 2009. *Bacillus amyloliquefaciens* GA1 as a source of potent antibiotics and other secondary metabolites for biocontrol of plant pathogens. Microb Cell Fact 8 (1): 63-75.

Bizani D, Brandelli A. 2002. Characterization of a bacteriocin produced by a newly isolated *Bacillus* sp. strain 8 A. J Appl Microbiol 93 (3): 512-519.

Chen XH, Koumoutsi A, Scholz R, Schneider K, Vater J, Sussmuth R, Piel J, Borris R. 2009. Genome analysis of *Bacillus amyloliquefaciens* FZB42 reveals its potential for biocontrol of plant pathogens. J Biotechnol 140 (1-2): 27-37.

CLSI. 2018. Performance Standards of Antimicrobial Susceptibility Testing. 28th Edition. CLSI Supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute.

Coutte F, Lecouturier D, Yahia SA, Leclere V, Bechet M, Jacques P, Dhulster P. 2010. Production of surfactin and fengycin by *Bacillus subtilis* in a bubbleless membrane bioreactor. Appl Microbiol Biotechnol 87 (2): 499-507.

Gong AD, Li HP, Yuan QS, Song XS, Yao W, He WJ, Zhang JB, Liao YC. 2015. Antagonistic mechanism of iturin A and plipastatin A from *Bacillus amyloliquefaciens* S76-3 from wheat spikes against *Fusarium graminearum*. PLoS One 10 (2): e0116871. DOI: 10.1371/journal.pone.0116871

Hsieh F, Lin T, Meng M, Kao S. 2008. Comparing methods for identifying *Bacillus* strains capable of producing the antifungal lipopeptide iturin A. Curr Microbiol 56 (1): 1-5.

Iqbal S, Qasim M, Begum F, Rahman H, Sajid I. 2018. Screening, characterization, and optimization of antibacterial peptides, produced by *Bacillus safensis* strain MK-12 isolated from waste dump soil KP, Pakistan. BioRXIV. 308205.

Jin H, Li K, Niu Y, Guo M, Hu C, Chen S, Huang F. 2015. Continuous enhancement of iturin A production by *Bacillus subtilis* with a stepwise two-stage glucose feeding strategy. BMC Biotechnol 15 (1): 53-62.

Kiranmayi MU, Sudhakar P, Sreenivasulu K, Vijayalakshmi M. 2011. Optimization of culturing conditions for improved production of bioactive metabolites by *Pseudonocardia* sp. VUK-10. Mycobiology 39 (3): 174-81.

Korenblum E, von der Weld I, Santos ALS, Rosado AS, Sebastian GV, Coutinho CMLM, Magalhaes FCM, de Paiva MM, Seldin L. 2005. Production of antimicrobial substances by *Bacillus subtilis* LFE-1, *B. firmus* H_2O-1 and *B. licheniformis* T6-5 isolated from an oil reservoir in Brazil. J Appl Microbiol 98 (3): 667-675.

Kumar SN, Siji JV, Ramya R, Nambisan B, Mohandas C. 2012. Improvement of antimicrobial activity of compounds produced by *Bacillus* sp. associated with a *Rhabditid* sp. (entomopathogenic nematode) by changing carbon and nitrogen sources in fermentation media. J Microbiol Biotechnol Food Sci 1 (6): 1424-1438.

Lee H, Churey JJ, Worobo RW. 2008. Antimicrobial activity of bacterial isolates from different floral sources of honey. Int J Food Microbiol 126: 240-244.

Lertcanawanichakul M, Sawangnop S. 2008. A comparison of two methods used for measuring the antagonistic activity of *Bacillus* species. Walailak J Sci Tech 5 (2): 161-171.

Lisboa MP, Bonatto D, Bizani D, Henriques JAP, Brandelli A. 2006. Characterization of a bacteriocin-like substance produced by *Bacillus amyloliquefaciens* isolated from the Brazilian Atlantic forest. Int Microbiol 9 (2): 111-118.

Li X, Zhang Y, Wei Z, Guan Z, Cai Y, Liao X. 2016. Antifungal activity of isolated *Bacillus amyloliquefaciens* SYBC H47 for the biocontrol of peach gummosis. PLoS One 11(9): e0162125. DOI: 10.1371/journal.pone.0162125

Liu X, Ren B, Gao H, Liu M, Dai H, Song F, Yu Z, Wang S, Hu J, Kokare CR, Zhang L. 2012. Optimization for the production of surfactin with a new synergistic antifungal activity. PLoS One 7 (5): e34430. DOI: 10.1371/journal.pone.0034430

Maget-Dana R, Peypoux F. 1994. Iturins, a special class of pore-forming lipopeptides: biological and physicochemical properties. Toxicology 87 (3): 151-74.

Mahlapuu M, Håkansson K, Ringstad L, Björn C. 2016. Antimicrobial peptides: An emerging category of therapeutics agents. Front Cell Infect Microbiol 6: 194.

Martinez-Klimova E, Rodríguez-Peña K, Sánchez, S. 2017. Endophytes as sources of antibiotics. Biochem Pharmacol 134: 1-17.

Meena KR, Kanwar SS. 2015. Lipopeptides as the antifungal and antibacterial agents: applications in food safety and therapeutics. Biomed Res Int 1 (1): 1-9.

Moshafi MH, Forootanfar H, Ameri A, Shakibaie M, Dehgan-Noudeh G, Razavi M. 2011. Antimicrobial activity of *Bacillus* sp. strain FAS_1 isolated from soil. Pak J Pharm Sci 24 (3): 269-275.

Mosquera S, Gonzalez-Jaramillo LM, Orduz S, Villegas-Escobar V. 2014. Multiple response optimization of *Bacillus subtilis* EA-CB0015 culture and identification of antifungal metabolites. Biocatal Agric Biotechnol 3 (4): 378-385.

Mora I, Cabrefiga J, Montesinos E. 2011. Antimicrobial peptide genes in *Bacillus* strains from plant environments. Int Microbiol 14 (4): 213-223.

Motta AS, Cladera-Olivera F, Brandelli A. 2004. Screening for antimicrobial activity among bacteria isolated from the Amazon Basin. Braz J Microbiol 35 (4): 307-310.

Muhammad N, Akbar A, Shah A, Abbas G, Hussain M, Khan TA. 2015. Isolation optimization and characterization of antimicrobial peptide producing bacteria from soil. J Anim Plant Sci 25 (4): 1107-1113.

Palazzini JM, Dunlap CA, Bowman MJ, Chulze SN. 2016. *Bacillus velezensis* RC 218 as a biocontrol agent to reduce *Fusarium* head blight and deoxynivalenol accumulation: Genome sequencing and secondary metabolite cluster profiles. Microbiol Res 192 (1): 30-36.

Purnawidjaja I. 2018. Isolation and Detection of Potential Microbial Activity from Honey and Other Bee-Associated Products in West Java [Thesis]. Atma Jaya Catholic University of Indonesia. [Indonesian]

Ramachandran R, Chalasani AG, Lai R, Roy U. 2014. A broad-spectrum antimicrobial activity of *Bacillus subtilis* RLID 12.1. Sci World J 968487.

Ramarathnam R, Bo S, Chen Y, Fernando WGD, Xuewen G, Kievit T. 2007. Molecular and biochemical detection of fengycin-and bacillomycin D-producing *Bacillus* spp., antagonistic to fungal pathogens of canola and wheat. Can J Microbiol 53 (7): 901-911.

Ripa FA, Nikkon F, Zaman S, Khondkar P. 2009. Optimal conditions for antimicrobial metabolites production from a new S*treptomyces* sp. RUPA-08PR isolated from Bangladeshi soil. Mycobiology 37 (3): 211-214.

Risoen PA, Ronning P, Hegna IK, Kolsto AB. 2004. Characterization of a broad range antimicrobial substances from *B. cereus*. J Appl Microbiol 96 (4): 648-655.

Sabate DC, Carillo L, Audisio MC. 2009. Inhibition of *Paenibacillus larvae* and *Ascosphaeraapis* by *Bacillus subtilis* isolated from honeybee gut and honey samples. Res Microbiol 160 (3): 193-199.

Sabate DC, Audisio MC. 2013. Inhibitory activity of surfactin, produced by different *Bacillus subtilis* subsp. *subtilis* strains, against *Listeria monocytogenes* sensitive and bacteriocin-resistant strains. Microbiol Res 168 (3): 125-129.

Song B, Rong YJ, Zhao MX, Chi ZM. 2013. Antifungal activity of the lipopeptides produced by *Bacillus amyloliquefaciens* anti-CA against *Candida albicans* isolated from clinic. Appl Microbiol Biotechnol 97 (16): 141-150.

Sutyak KE, Wirawan RE, Aroutcheva AA, Chikindas ML. 2008. Isolation of the *Bacillus subtilis* antimicrobial peptide subtilisin from the dairy product derived *Bacillus amyloliquefaciens*. J Appl Microbiol 104 (4): 1067-1074.

Sumi CD, Yang BW, Yeo IC, Hahm YT. 2015. Antimicrobial peptides of the genus *Bacillus*: a new era for antibiotics. Can J Microbiol 61(2):93-103.

Teixeira ML, Rosa AD, Brandelli A. 2013. Characterization of an antimicrobial peptide produced by *Bacillus subtilis* subsp. *spizezinii* showing inhibitory activity towards *Haemophilus parasuis*. Microbiology 159 (5): 980-988.

Tsuge K, Akiyama T, Shoda M. 2001. Cloning, sequencing, and characterization of the iturin A operon. J Bacteriol 183 (21): 6265-6273.

Walsh G. 2002. Proteins: Biochemistry and Biotechnology. John Wiley & Sons, USA.

Zhao X, Zhou ZJ, Han Y, Wang ZZ, Fan J, Xiao HZ. 2013. Isolation and identification of antifungal peptides from *Bacillus* BH072, a novel bacterium isolated from honey. Microbiol Res 168 (9): 598-606.