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Antibacterial Activity of Genus *Bacillus* Isolated from Fresh Fruits and Vegetables against Some Foodborne Pathogens

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ABSTRACT

Genus *Bacillus* were isolated from fresh fruits and vegetables and its ability to inhibit the growth of some foodborne pathogens, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella* Typhimurium, *Escherichia coli* and *Serratia marcescens* was screened an antagonistic capacity using Agar spot assay and Agar well diffusion assay. One hundred and sixty-four isolates of genus *Bacillus* showed successfully an antibacterial activity. The isolated 16ST4 and 17ST3 revealed a strong inhibition activity against *L. monocytogenes* at 208.8 and 169.75 AU/ml, respectively. While the best potential against *S.* Typhimurium was isolated 17ST3 which showed inhibition activity at 146.12 AU/ml. Moreover, CFS of both isolates was stable over a wide temperature range and pH value from 2 to 9. The antibacterial substance was sensitive to all proteolytic enzymes which were properties of protein characteristics. The isolated 16ST4 and 17ST3 were identified by using phenotypic characterization and API 50 CHB indicated that these isolates have high similarity with *B. firmus* and *B. subtilis/amyloliquefaciens*, respectively.

Keywords: Genus *Bacillus*; Antibacterial activity; *Listeria monocytogenes*; Foodborne pathogens; Fresh fruits and vegetables

1. Introduction

Fresh fruits and vegetable products have continuously increased in the last decades as a result of the consumer's interest in healthy foods that are easy to prepare. However, these food compositions can provide conditions for microbial growth including foodborne pathogens [1]. In fact, raw fruits and vegetables have been identified as a vehicle of transmission of foodborne diseases outbreak, such as *E. coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and *Clostridium botulinum* which were found

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0.6 %, 0.8 % 1.4 % 50.3 % and 13.3 %, respectively [2-5]. Thailand has been informed by the United States Food and Drug Administration to investigate and monitor imported apples potentially contaminated with *L. monocytogenes* from the United States. These bacteria are considered ubiquitous in soil and vegetables and play an important role in listeriosis epidemiology particularly harmful serotype 4b which is found in worldwide; moreover, *L. monocytogenes* has the ability to grow well at refrigerated temperature [6-8]. In addition, foodborne pathogens have been reported, such as *S.* Typhimurium which is one of the leading serovars responsible for salmonellosis in human and the contaminated source with vegetables has been increasing in the last few years [9, 10] Moreover, the increasing rate of antibiotic resistance in *S.* Typhimurium is considered to be a global public health concern [8, 9].

Hurdle technology and bacteriocin have been introduced to preserve and control foodborne pathogens in the food industry [10]. Nisin is a commercial food grade preservative which is produced by lactic acid bacteria, *Lactococcus lactis*, However, nisin usually inhibits the growth of microorganisms of the closely related strains. Many microorganisms are known to produce bacteriocins, one of them is genus *Bacillus* which is widely studied due to their capability of bioactive substance such as lipopeptides, polypeptides, macrolactones, fatty acids, polyketides, lipoamides, and isocoumarins. These complex substances have an efficiency for antimicrobial, anticancer and antialgal [11] including inhibit growth of both gram positive, negative bacteria and pathogenic fungi. There is some commercial bacteriocin report of produced by *B. subtilis* such as Rhio-plus KFZB; Biotechnic LTD, Germany and Serenade; Agro- Guess Inc., USA can control plant pathogenic fungi, *B. amyloliquefaciens* can retard growth of *L. monocytogenes* [12, 13]. Indeed, some genus *Bacillus* is used in the food and agriculture industries such as *B. subtilis, B. lichenisformis* and *B. coagulans* as well as lactic acid bacteria [14, 15].

Therefore, genus *Bacillus* has exhibited the potential to produce bacteriocins and be applied as a food preservative in the future. Combining it with other food science and technology will help to develop a better protocol of food safety. This research is to isolate genus *Bacillus* from fruits and vegetables to produce bacteriocin or other bioactive compounds to inhibit foodborne pathogens. The results can help the readily consumable fruits and vegetables product industries comply with the exporting standard and consumer safety.

2. Materials and Methods

2.1 Indicator microorganisms

Indicator microorganisms usually found contaminating fruits and vegetables which cause foodborne infection and illness in humans include gram positive as *Listeria monocytogenes* DMST1327, *Staphylococcus aureus* TISTR1466 and *Bacillus cereus* TISTR687 and gram negative as *Salmonella* Typhimurium DMST562, *Escherichia coli* TISTR780 and *Serratia marcescens*.

2.2 Sample preparation and isolation of genus *Bacillus*

Twenty-three samples of fresh fruits and vegetables including 9 types of fruits and 14 types of vegetables were kept in sterile polyethylene bags and used for isolation of genus *Bacillus*. By using aseptic technique, pieces or slices of fresh samples were cut and weighted to 25 g and homogenized in 225 ml of 0.1% peptone water for 2 min in a laboratory blender and placed in water bath at 80^{o}C for 10 min due to heat treatment possibly destroying vegetative cells in samples. Suspension were ten-fold serially dilution and plated on Nutrient agar (NA) added with $MgSO_4$. $7H_2O$ 20 mg/l. Plates were incubated at

30ºC for 24 h. Colonies of genus *Bacillus* were selected and purified by cross streak plate method. Each isolated colony was tested for gram staining and endospore staining. Isolated cultures were maintained at -80ºC in nutrient broth (NB) supplemented with 20% (w/v) sterile glycerol.

2.3 Screening of inhibition by isolated genus *Bacillus*

Screening for antibacterial activity was done by using the agar spot assay. Isolated genus *Bacillus* were spotted on NA plate and incubated at 30ºC for 24 h. One ml of each indicator microorganism suspension containing 10^6 cfu/ml was mixed in 7 ml of soft Tryptic soy agar (TSA) by adding 0.7% agar and overlaid on the NA plate containing previously isolated colonies of genus *Bacillus.* Agar plates were incubated at 30 ºC for 24 h. After the incubation, the diameter of the inhibition zone was measured.

2.4 Detection of antibacterial activity by Agar well diffusion assay [16]

2.4.1 Preparation of cell free supernatant (CFS)

CFS of each isolated genus *Bacillus* was prepared by inoculating in NB and then incubated at 30ºC for 24 h. CFS was collected by centrifugation at 8,500×g for 15 min at 4ºC and then filtered through a 0.45 µm membrane (Millipore Corp., USA) and stored in a sterile vial at 4°C until used for the antibacterial activity assay.

2.4.2 Antibacterial activity assay

The agar well diffusion method is widely used to determine antibacterial activity. This procedure was performed by TSA agar being added with 1 ml of indicator microorganism suspension containing 10^6 cfu/ml and poured into a sterile plate. Then, a hole with a diameter of 8 mm was punched aseptically with a sterile cork borer and 80 µL of CFS was introduced into the well. Then, agar plates were incubated at 30ºC for 24 h. After incubation, plates were examined for inhibiting zones around wells, expressed in arbitrary units per milliliter (AU/ml) and calculated as below [17].

$$\text{AU/ml} = \frac{\text{Diameter of the inhibition zone(mm)} \times 1000}{\text{Volume added in the well (µl)}}$$

2.5 Effect of heat stability, pH and proteolytic enzymes on antibacterial activity

Bacteriocin produced from genus *Bacillus* was a proteinaceous compound. CFS was measured following the modified method of Lisboa et al. [12]

2.5.1 Effect of heat stability

CFS was exposed to a range of temperatures by dipping in water bath at 10, 40, 60, and 80ºC for 30 min, at 100ºC for 10, 20, 30 and 60 min, and at 121ºC for 15 min. Antibacterial activity was assessed by an agar well diffusion assay in which CFS without heat treatment served as a control.

2.5.2 Effect of pH stability

CFS pH was adjusted between the range of 2, 5, 7, 9 and 10 by 1N HCl or 1N NaOH and left at room temperature for 2 h. After that CFS was adjusted back to pH 7. The CFS was tested for antibacterial activity by the agar well diffusion assay in which CFS without any adjusted pH served as a control.

2.5.3 Sensitivity to proteolytic enzyme

Proteinase K, chymotrypsin and trypsin in CFS were added to the final concentration of enzyme at 1 mg/ml. Both CFS with and without enzyme were incubated at 37ºC for 1 h. The CFS was tested for inhibiting growth capability of indicator microorganisms by an agar well diffusion assay in which the negative control was CFS without adding any enzyme.

2.6 Identification of the best isolated genus *Bacillus*

Carbohydrate fermentation profiles of the best isolated genus *Bacillus* were determined using API 5 0 CHB strips and API CHB medium according to the manufacturer's instructions (BioMerieux®sa, France)

3. Results and Discussions

3.1 Isolations of genus *Bacillus*

The results obtained were one hundred and sixty-four of genus *Bacillus* isolated from 23 samples, including 9 samples of fruits and 14 samples of vegetables. Most of genus *Bacillus* was isolated from lettuce, iceberg lettuce, green oak lettuce and beans. For preliminary characterization, each of isolates was sub-cultured on NA medium to observe colony morphology, gram and endospore staining which are the basic features of primary bacterial identification. One hundred and sixty-four of these isolates proved to be gram positive, endospore-forming producing, motile, catalase positive rod shape including entire, undulate, smooth-wrinkle, mucoid and rough colony form. Therefore, they were characterized as memberd of the genus *Bacillus***.**

3.2 Screening of inhibition by isolated genus *Bacillus*

The preliminary screening of the 164 isolates of genus *Bacillus* for inhibiting indicator microorganisms by using agar spot assay revealed that 17 isolates showed effectively against indicator microorganisms (Table 1). Interestingly, isolated 16ST4 and 17ST3 showed inhibition capability on both gram positive and negative bacteria. Thus, the antibacterial substance from these isolates were considered as broad spectrum. Similarly, the research of Lim et al. [13] found *B. sonorensis* could produce sonorensin which inhibited both gram positive and negative bacteria. Notably, this sonoresin had a structure similar to putative thiazole-containing heterocyclic bacteriocin which was produced by *B. licheniformis* ATCC 14580 [18] and *B. coagulans* [19].

Table 1. Inhibitory spectrum of 17 isolates of genus *Bacillus.*

Isolated strains	*Bacillus cereus*	*Listeria monocytogenes*	*Staphylococcus aureus*	*Salmonella* Typhimurium	*Serratia marcescens*	*Escherichia coli*
15LS2	++	++	++	++	+	+
15ST1	-	+	-	+	++	+
15ST2	+	-	-	+	++	+
15ST3	+	+	-	+	-	-
16ST2	+	+	-	-	-	-
16ST3	-	+	-	+	-	+
16ST4	++	+++	++	++	++	+
17ST1	-	+	-	+	+	+
17ST2	+	-	-	+	+	+
17ST3	++	++	++	+++	++	+
17ST4	-	-	-	+	-	+
17ST5	+	-	-	++	++	+
16LS2	+	-	-	-	-	-
16LS3	+	+	+	-	-	-
16LS4	+	+	+	-	-	-
16LS5	+	+	+	-	-	-
16LS6	+	+	+	-	-	-

Note: The symbols of inhibitory zone means +++ strong inhibition (>14 mm), ++ moderate inhibition (10-14 mm), + less inhibition (<10 mm) and - no inhibition respectively.

3.3 Detection of antibacterial activity by agar well diffusion assay

CFS from genus *Bacillus* isolated 1 6 ST4 and 1 7 ST3 were tested for the effective capability inhibition of antibacterial activity. Eighty microliters of CFS was dropped into the well. The results showed CFS of isolated 16ST4 and 17ST3

could inhibit growth of indicator microorganisms (Fig. 1). CFS of isolated 16ST4 provided the antibacterial activity order of *L. monocytogenes*, *S. aureus* and *B. cereus* value as 208. 88, 195.8 and 154.87 AU/ml, respectively. Isolated 17ST3 could inhibit *L. monocytogenes* and *B. cereus* values as 169.75 and 162.25 AU/ml, and inhibit *S.* Typhimurium value as 146.12 AU/ml. Similar to the study of nisin was commercial food preservative which could inhibit both gram positive and negative foodborne bacteria. Generally, gram negative bacteria are resistant to the bacteriocin produced by gram positive bacteria due to their outer membrane, which acts as an effective barrier structure [20]. Moreover, the genus *Bacillus* produced antibacterial substance which was peptide and active alike antibiotic or bacteriocin and basically synthesized from ribosome such as lipopeptides, polypeptides, macrolactones, fatty acids, polyketides, lipoamides, and isocoumarins. This complex substance had an efficiency for antimicrobial, anticancer and antialgal [11].

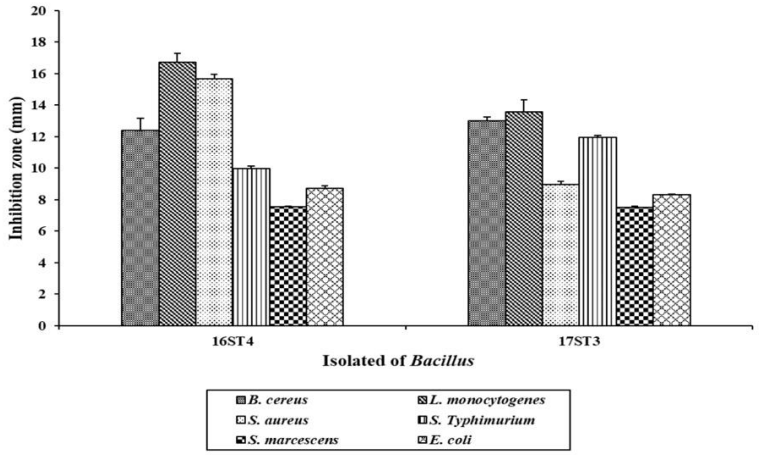

Fig. 1. Antibacterial activity of isolated 16ST4 and 17ST3 inhibit indicator microorganisms; Negative control was fresh NB medium without isolated strains; no inhibition zone.

3.4 Effect of temperature stability, pH and proteolytic enzymes on antibacterial activity of isolated *Bacillus*

3.4.1 Effect of temperature stability

The results showed CFS of isolated 16ST4 and 17ST3 were stable at a wide range of temperature treatment when compared with negative controls. In addition, the activity of both isolates remained almost completely at a lower temperature. However, the best antibacterial activity against *L. monocytogenes* occurred at 40°C for 3 0 min. Then, antibacterial activity was decreased after treated at higher temperature until activity was destroyed completely at 121°C and 15 min (Fig. 2). Accordingly, an inhibiting test on *S.* Typhimurium at different temperatures found that isolated 16ST4 and 17ST3 showed antibacterial activity was stable under different heat treatments. At low temperature was a lost inhibition zone. The best inhibition activity was at 40°C for 30 min, the same as inhibiting *L. monocytogenes*. Nevertheless, the temperature resistance of antibacterial substance produced by isolated 16ST4 and 17ST3 against *S.* Typhimurium gradually declined when either increasing the temperature and time until reaching either 100°C for 60 minutes or at 121°C for 15 min, at which point inhibitory activity was destroyed completely (Fig. 2). Notably,

antibacterial substance from both isolates provided a wide range of temperature tolerance but could not exceed at sterilization level [21]. This result was similar to the research of Chalasani et al. [21] which found *B. subtilis* URID 12.1 could inhibit *S. aureus, S. epidermidis, Streptococcus pyogenes* and *Enterococcus faecalis.* Even after incubating at 80ºC for 1 h, 100ºC for 30 min and 121ºC for 20 min, the remain inhibiting capability was still at 80, 75 and 60 %, respectively. The efficiency of the bioactive compound produced from *B. atrophaeus* was reduced to 50 % when it was incubated at 121ºC for 15 min [22]. However, the temperature resistant property of the antibacterial substance which was produced by microorganisms would be able to apply to the food industries such as pasteurization and sterilization process [23].

3.4.2 Effect of pH stability

The effect of pH on antibacterial activity which was produced from isolated 16ST4 and 17ST3 could inhibit *L. monocytogenes* and *S.* Typhimurium within pH 5-9 range and antibacterial activity was completely destroyed at pH 2. However, isolated 16ST4 more stable to pH ranges than isolated 17ST3. At pH 10, CFS of isolated 16ST4 was stable and showed effectiveness to only *L. monocytogenes*. (Fig. 3). According to the pH stability test, the result revealed that the antibacterial substance produced from genus *Bacillus* could remain effective in weak acidity and alkalinity pH range 5-9 and inhibit both *L. monocytogenes* and *S.* Typhimurium, but could not tolerate stronger levels. Kindoli et al. [24] also found *B. subtilis* W42 produced bacteriocin performed very well at pH 7 while declining at pH 3 -6 and 8 -9. A bacteriocin-like substance from *B. amyloliquefaciens* could inhibit *L. monocytogenes* at pH ranges 3-8 and its efficiency declined at pH 9-10 [12]. Bacocin was a novel bacteriocin produced by *B. amyloliquefaciens* that had a broad antibacterial spectrum, inhibiting gram-positive and negative bacteria. It was stable at high temperatures for more than 30 min to 1 h and stable at extreme pH. [25] The wide range pH stability of an antibacterial compound would be considered useful in the preservative process of food industries.

3.4.3 Sensitivity to proteolytic enzyme

The effect of proteolytic enzyme on the activity of antibacterial substance found that CFS of both isolated 16ST4 and 17ST3 was inactivated completely after treatment with proteinase K, while enzyme chymotrypsin and trypsin caused partial efficiency of antibacterial substances (Fig. 4). According to the results, this substance was identified as a protein-like structure or bacteriocin-like substance. Generally, bacteriocin is a peptide structural substance which is synthesized in ribosome and digested by enzyme proteinase. It could provide inhibition ability on specific bacteria. Furthermore, chymotrypsin and trypsin act as catalytic enzymes to increase digestion of protein at peptide bond which had direct effect on the structure of bacteriocin as well [12, 24].

3.5 Identification of the best isolated *Bacillus*

The identification of the best isolated *Bacillus* which could be highest producing bacteriocin was isolated 16ST4 particularly inhibiting *L. monocytogenes*. On the other hand, isolated 17ST3 was very effective with gram negative bacteria specific to *S.* Typhimurium. Both isolates were gram positive, rod shaped and endospore forming bacteria.

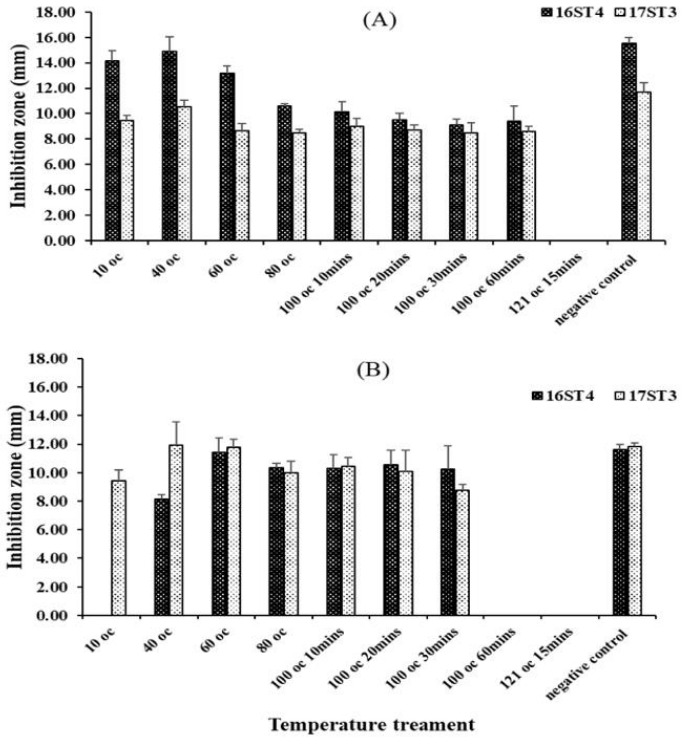

Fig. 2. Effects of temperature on antibacterial activity against (A) *L. monocytogenes* and (B) *S.* Typhimurium; Negative control means CFS without heat treatment.

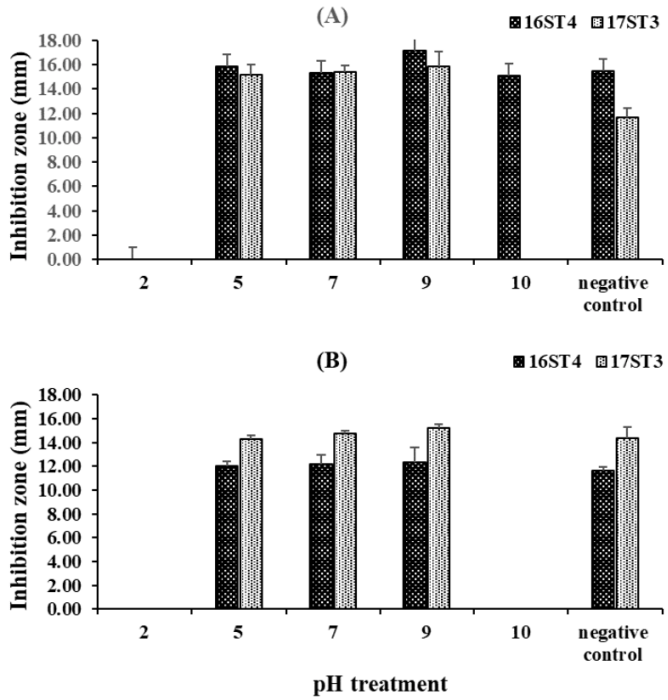

Fig. 3. Effects of pH on antibacterial activity against (A) *L. monocytogenes* and (B) *S.* Typhimurium; Negative control means CFS without adjusted pH.

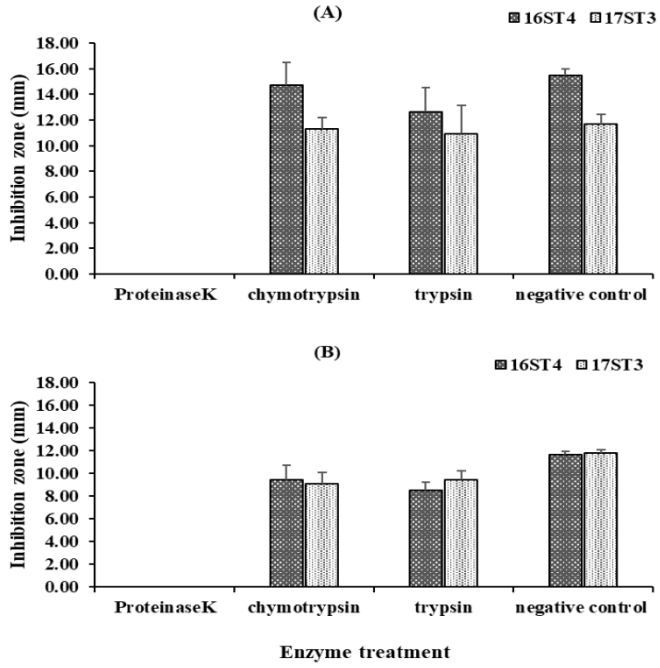

Fig. 4. Effects of proteolytic enzyme on antibacterial activity against (A) *L. monocytogenes* and (B) *S.* Typhimurium; Negative control means CFS without adding any enzyme.

A carbohydrate fermentation profile test done by using API 50 CHB strips indicated that isolate 16ST4 and 17ST3 were identified as *B. firmus* with % ID 99.8 and *B. subtilis/amyloliquefaciens* with % ID 99.7, respectively (Table 2). There were several reports of both isolates producing antibiotic or bacteriocin which were used for inhibiting gram positive and negative bacteria, fungi and pests. For instance, Geng et al. [26] found that *B. firmus* DS-1 could produce some toxic compound to control *Meloidogyne incognita* dangerous root-knot nematode. The toxic compound was identified as peptidase S8 or a protein called Sep1. Presently, Agro-Green Company, Israel has commercialized this product with the trading name of BioNem-WP. Furthermore, *B. subtilis/amyloliquefaciens* was well known microorganisms as ribosome lipopeptides (LPs) producer such as surfectin, iturin and fengycin which control plant pathogenic fungi. Also *B. amyloliquefaciens* ZJHD3-06 could produce CAMT2, a novel bacteriocin which destroys cell membranes of *L. monocytogenes*. This compound had temperature resistance up to 100 ^{o}C and inhibits several pathogenic bacteria such as *L. monocytogenes, S. aureus, E. coli* and *Vibrio parahaemolyticus* [27,28]. Moreover, *B. amyloliquefaciens* LBM 5006, which was isolated from forest soil in Brazil, could produce a substance like bacteriocin. This substance was characterized as the peptide bond having molecular weight as 5 kDa, pH tolerance range 3-8, temperature resistance at 80 ^{o}C for 30 min and was inhibited by proteolytic enzyme. Indeed, its inhibition ability was effective on several types of bacteria such as *L. monocytogenes, B. cereus, S. marcescens* and *Pasteurella haemolytica* [29].

4. Conclusion

Bacillus firmus 16ST4 and *B. subtilis/amyloliquefaciens* 17ST3 were isolated from fresh fruits and vegetables, produced bacteriocin- like substance that sensitive to proteolytic enzyme. The screening antibacterial activity showed that it could inhibit both gram positive and negative bacteria. This bacteriocin was heat and pH stable; therefore, it has the potential to be used as bio-preservative for processed food.

Acknowledgement

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Table 2. Carbohydrate fermentation profile of isolated 16ST4 and 17ST3 by using API 50 CHB strips.

Type of test	16ST4	17ST3	Type of test	16ST4	17ST3
Control	-	-	Esculin	-	+
Glycerol	-	+	Salicin	-	-
Erythritol	-	-	D-Cellobiose	+	+
D-Arobinose	-	-	D-Maltose	-	+
L-Arabinose	-	+	D-Lactose	-	-
D-Ribose	-	-	D-Melobiose	+	-
D-Xylose	-	-	D-Sucrose	-	+
L-Xylose	-	-	D-Trehalose	-	+
D-Adonitol	-	-	Inulin	-	+
methyl-D-Xylopyranoside	-	-	D-Melezitose	-	-
D-Galactose	+	-	D-Raffinose	-	+
D-Glucose	+	-	Starch	-	-
D-Fructose	+	-	Glycogen	-	+
D-Mannose	-	+	Xylitol	-	-
L-Sorbose	-	-	Gentiobiose	-	-
L-Rhamnose	-	-	D-Turanose	-	+
Dulcitol	-	-	D-Lyxose	-	-
Inositol	+	-	D-Tagatose	-	-
D-Mannitol	-	+	D-Fucose	-	-
D-Sorbitol	-	+	L-Fucose	-	-
Methyl-D-Mannopyranoside	-	-	D-Arabitol	-	-
Methyl-D-Glucopyranoside	-	+	L-Arabitol	-	-
N-Acetylglucosamine	-	-	Potassium Gluconate	-	-
Amygdalin	-	+	Potassium 2-Keto-Gluconate	-	-
Arbutin	+	+	Potassium 5-Keto-Gluconate	-	-

Note: The symbols of test means + is positive reaction and – is negative reaction.

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