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Application of Antimicrobial *Bacillus subtilis* Strain as a Starter Culture to Improve Qualities and Safety of Fermented Soybean (*SIENG)* Produced in Cambodia

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Abstract: Fermented foods play a very important role in Cambodian health and nutrition, as well as other developing countries where food preservation methods may be limited. *SIENG*, a Khmer fermented soybean product, naturally contains both beneficial and pathogenic microorganisms. Traditional fermentation that relies on natural microbial flora and environmental conditions results in variable product quality and can lead to spoilage. A starter culture such as *Bacillus subtilis* can ensure the safety and stability of the products. The objective of this study is to control the growth of Gram positive pathogens contaminated into traditional fermented soybean (*SEING*) by using antimicrobial *Bacillus subtilis* isolated from the same kind of food. Out of 120 *SIENG* samples, 49 *B. cereus* strains were isolated, and 12 of *B. cereus* were positively synthesized compared with the lyophilized control enterotoxin. Two of these strains (*BTM8-7* and *BTM8-8*) produced high levels of enterotoxin. We identified five *Bacillus* strains with the ability to fight against indicator pathogenic microorganisms. Among the five strains, *B. CeM6-2* had the highest activity level against *Lactobacillus plantrum ATCC 8014* and the largest diameter. *B. CeM6-2* tolerated up to 20 h at 30 °C and 22 h at 37 °C. In testing the strains with PK and PK-PMSF enzymes, bacteriocin produced by the strain *B. CeM6PK* untreated and *B. CeM6-2PK-PMSF* had a significantly stronger ability to suppress all the pathogenic indicators from 0 h to 47 h compared to the *B. CeM6-2PK*. Moreover, *CeM6-2* outperformed the *Miyagino* strains, as it actively produced bacteriocin that fought against all four indicator strains of Gram positive and lactic acid groups, especially against *Listeria monocytogenes*, *Streptococcus pyrogene*, *Leuconostoc mesenterids* and *L. plantarum*, from 0 h-58 h and 0 h-40 h at 35 °C. *CeM6-2* (1%) strain had the highest ability to fight against *B. cereus* at 24 h and at 34 h to 44 h incubation as well. *CeM6-2* (10:0 mL) and *CeM6-2* (9:1 mL) have the strongest ability to fight against *B. cereus* at room temperature (48 h and 72 h). The longer incubation and time at room temperature produce the highest level of bacteriocin. Thus, bacteriocins produced by *B. CeM6-2* can be used as a preservative in food processing industries to avoid food spoilage even in higher temperatures and time.

Key words: *SIENG* (Khmer traditional fermented soybean), soybean, fermentation, bacteriocin, *Bacillus subtilis*, *Bacillus cereus*, pathogens, spoilage, microorganism.

1. Introduction

Food is the most important basic need of all living on earth. In recent year's health-consciousness consumers prefer for natural foods without chemical preservatives to fit in their healthy lifestyle. According to health and food safety experts more than 200 known diseases are transmitted through contaminated food and masses of illness cases annually by food-borne pathogens [1]. *Bacillus cereus* has been detected and implicated in contaminated foods containing fermented soybeans, and numerous outbreaks of food poisoning have been caused by *B. cereus* [2]. *B. cereus* toxins in these products, and the symptoms of *B. cereus* diarrheal type food poisoning, including abdominal pain, cramps, nausea, and rarely

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vomiting and watery diarrhea occur at 8 h to 16 h after ingestion of contaminated food [3]. Though Cambodia is the third world country, Royal Government of Cambodia is also considering to reduce foodborne disease and improve public health status by introducing food safety standards and market regulation into food industries [4].

Fermented foods are commonly found in Cambodia and in other Asia countries since they play a very important role for health. In fact, soybean has been produced into fermented soybean, commonly called *SIENG*, and there are two kinds of fermented *SIENG* in Cambodia, we called *SIENG PRAI* and *SIENG PA-AEM*. They are very famous and healthy foods among other Cambodian's soybean processed products [5]. *Bacillus subtilis* may be present on the soybean seed during cultivation or contaminate in *SIENG* during process according to Cambodia production context where *SIENG* is fermented using a pure starter strain of *B. subtilis*, naturally occurring microorganisms or seeds (a portion of the product) used to produce fermented soybeans in other Asian countries [6]. *B. subtilis* has been reported to produce bacteriocins which suppress the growth of Gram positive spoilage and pathogenic bacteria [7, 8]. The bacteriocins produced by these strains are thought to be potent food preservatives that are applicable for Cambodian food. Bacteriocins produced by industrial important *B. subtilis*, have a history of safe use in food and industry [9].

Recent studies have recommended that the antibacterial substances produced by isolated bacterial strains may be applied to industrial-scale fermented soybean foods. *B. subtilis* has extensively been used to inhibit the growth of foodborne pathogens, particularly in the production of fermented and non-fermented foods. Nevertheless, the preservation of foods by natural and microbiological methods may be a satisfactory approach solving economic losses due to microbial spoilage of raw materials and food products, to reduce the incidence of food borne illnesses, and to meet the food requirements of the growing world population. The bacteriocins produced by these strains are thought to be potent food preservatives that are applicable and useful for Cambodian food industry.

2. Material and Methods

2.1 Bacillus cereus Isolation and Checking the Contamination Rate of Toxin

One hundred and twenty fermented soybeans samples were purchased from local market in Cambodia. Each of samples of *SEING* was one loop inoculated in NB medium and incubated for 2 days, 35 °C. Then, culture was streaked on NGKG (NaCl glycine Kim and Goepfert) agar plate to isolate typical colonies of *Bacillus cereus* and suspected colonies were confirmed by Gram staining and biochemical test and finally were confirmed by analytical profile index (API 50CHB V4.1). Identify *Bacillus cereus* toxin genes by PCR method: there were 2 steps conducted in PCR and well diffusion assay. The first will use exactly the CRET-RPLA for enterotoxin and second PCR reaction testing method [10].

2.2 Isolation and Screening Bacteriocin Producing Bacillus subtilis Strains

One hundred and twenty fermented soybeans samples were purchased from local market in Cambodia. Each of samples was mixed with 10 times volume of nutrient broth and incubated at 35 °C for 18 h. The culture was streaked on nutrient agar plate to isolate typical colonies of *B. subtilis*. All *Bacillus* strains that have ability to inhibit the grow of indicators from the previous step were streaked into GSP and Mannitol salt agar plate, incubated at 35 °C for 24-48 h, and isolated bacteria were identified by Gram staining and biochemical tests using catalase/OF test and finally were confirmed by API.

Antimicrobial activity against indicator organisms was determined by using a well diffusion assay. There were 3 steps conducted in well diffusion assay. The

first and second were used exactly the same testing method. However, either the first or second testing is of minor difference with the third one specifically on (1) with filtration, and (2) without filtration. One loop of each of the spore suspension of isolated *B. subtilis* strains was inoculated into 5 mL of LB broth and incubated at 35 °C for 18 h. Filtered culture supernatant was subjected to agar well diffusion assay. *Lactobacillus plantarum* ATCC 8014 was used for indicating bacteria and *B. subtilis*, *Miyagino* strains (used for *Natto* production) were used for negative control, respectively.

2.3 Characterize the Bacteriocin Produced by Isolated Strain

2.3.1 Incubator Temperature and Timing Treatment

The spore culture of bacteriocin produced by *B. subtilis* strain was determined. One loop of the spore suspension of isolated *B. subtilis* strain was transferred to 5 mL of LB broth and kept on incubator shaker at 35 °C, 72 h, and then was heated and cooled down. There were 2 steps conducted in well diffusion assay as follows:

A total of 0.5 mL was transferred into 100 mL aliquots flasks of sterile composed LB broth. Then, the flasks were incubated in 2 deference incubator shakers. In both started at 7:00 AM, and 17:00 PM the flasks were incubated at 30 °C and 35 °C, then take 1 mL of individual sample respectively at each different timing 6, 8, 10, and 12 h and 12, 14, 16, 18, 20, and 22 h to transfer into micro-centrifuge tubes.

L. plantarum was inoculated into the mixture (1:1 volume) of MRS broth contained supplement agar and keep it till become solid and make wells, and then filtered culture supernatant of *B. subtilis* or *Miyagino* strain and incubated 24 h, 30 °C. Growth of inoculated *L. plantarum* in this MRS broth was measured and observed length of wells by mm/mL.

2.3.2 Enzyme Treatment (Indicator Strain *LP*)

The sensitivity of the bacteriocin to enzymes was checked by using a well diffusion assay. *B. subtilis* and *Miyagino* strain for control were inoculated into a 30 mL of LB broth and placed on the incubator shaker at 30 °C on 250 rpm for overnight.

Five (5) mL *L. plantum* (*LP*) was transferred to MRS and LB broth and incubated at 30 °C and 35 °C, overnight and after shaker measure turbidness at OD 650 nm and dilute until OD 0.1 by PBS all of strains, into the mixture (1:1 volume) of PK and PK-PMSF and filtered culture supernatant of *B. subtilis* or *Miyagino* strains, is kept in bioplotter and incubated 24 hours at 35 °C. Following the incubation, length of wells was observed. The antagonistic activity in OD 650 nm was calculated as a measure of bacteriocin production.

2.4 Apply the Bacteriocin to Control the Growth of Gram Positive Pathogenic or Spoilage Bacteria in Foods

Bacteriocin to control the growth of Gram positive pathogenic or spoilage bacteria in foods was applied by using a well diffusion assay. There were 2 kinds of *Bacillus subtilis* strains, (1) *B. subtilis* (Cambodia) and (2) *Miyagino* for control (Japan). Each strain was inoculated into a 20 mL of LB broth centrifuge tubes and placed on the incubator shaker at 35 °C, 16 h. Gram positive group (*Enterococcus faecium*, *Listeria monocytogenes*, *Bacillus cereus*, *Streptococcus pyrogene*, *Micrococcus luteu* and *S. aureus*) and lactic acid group (*Lactobacillus brevis*, *Leuconostoc mesenterides*, *L. curvatus*, *Lactobacillus plantnum*, *Lc. lactis* (*NinA*+) and *Lactobacillus lactis*) were inoculated separately to BHI and MRS in incubator shaker at 35 °C for 18 to 24 h. After shaker measure turbidness at OD 650 nm and dilute until OD 0.1 by PBS all of strains.

Filtered culture supernatant of antimicrobial *B. subtilis* or *Miyagino* strains was mixed with same volume of MRS or BHI broth for lactic acid bacteria or other Gram-positive bacteria, respectively. Each of the pre-cultures of indicator strains was inoculated into this mixture and cultivated at 35 °C. Change of

the optical density at 650 nm was recorded automatically.

2.5 Co-cultivate Bacillus cereus and Bacillus subtilis in a Tripticase Soy Broth or Soybean and Check the Suppression of the Growth of Bacillus cereus

This test will be designed by using broth dilution and/or counting methods which have percentage and timing to record the time as desired and follows:

For co-culture experiments, *Bacillus cereuses* and *Bacillus subtilis* (*B. CeM6-2*) were grown in nutrient broth and MRS broth for 24 h at 35 °C. And following the incubation time, both of *Bacillus* do the dilution with normal saline and adjust the density to reach 10^6 to 10^8 colony forming units (CFU)/mL by using spectrophotometer with the optical density at 600 nm (OD600) of the bacterial cells that reached approximately 0.1 of optical density.

Then, *Bacillus cereus* 10^6 to 10^8 CFU/mL with different concentrations of potential *Bacillus subtilis* (*B. CeM6-2*) isolates (0.1%, 0.2%, 0.3%, 0.4%, 0.5%, and 1%), were co-inoculated in tripticase soy broth containing *B. cereus* 10^6 to 10^8 CFU/mL and then incubated for 24 h, 34 h and 44 h at 35 °C. After that, the cells remained in contact with the substance for different timing and the presence of growth and/or counts colonies by NGKG agar were considered a positive result.

2.6 Check the Quality of SEING Produced by Antimicrobial Bacillus subtilis Strain

Soybeans (500 g) were washed and soaked in clean or sterile potable water for 16 h at room temperature (RT). After draining the water, soaked soybean weight will be increased by approximately twofold. The drained soybeans were steamed at 100 °C for 3 h and cooled at 40 °C. Then, *B. cereus* and potential *B. CeM6-2* cultures were inoculated to a density of 10^6 to 10^8 CFU/mL (OD 600 = 0.1) after 24 h incubation in NB and MRS broth at 30 °C to 35 °C (the same process as the objective 3).

After cooling, the surfaces of the cooked soybean will be inoculated with 1% (*v*/*w*), (10^6 to 10^8 CFU/g) inoculum in the different radio:

(1) Mixed culture *B. CeM6-2* and *B. cereus* in ratio 10:0 for control;

(2) Mixed culture *B. CeM6-2* and *B. cereus* in ratio 0:10 for control;

(3) Mixed culture *B. CeM6-2* and *B. cereus* in ratio 9:1 for treatment;

(4) Mixed culture *B. CeM6-2* and *B. cereus* in ratio 1:9 for treatment;

(5) Mixed culture *B. CeM6-2* and *B. cereus* in ratio 5:5 for treatment;

(6) Mixed culture *B. CeM6-2* and *B. cereus* in ratio 7:3 for treatment;

(7) Mixed culture *B. CeM6-2* and *B. cereus* in ratio 3:7 for treatment.

After that the mixtures were fermented at 35 °C for 24 h, 48 h and 72 h, and then the *B. cereus* was isolated from each treatment, the cells remained in contact with the substance for different timing and the presence of growth and/or counting colonies by NGKG agar will be considered a positive result to observe the suppression of the growth of *Bacillus cereus* on the fermented soybean.

2.7 Data Processing and Analysis

The results were analyzed by Excel, CFU/mL and OD. In analyzing data, both quantitative and qualitative methods were used, and other appropriate methods such as Preference Ranking and Indexing were also used.

3. Results and Discussion

3.1 Bacillus cereus Isolation and Checking the Contamination Rate of Toxin

One hundred and twenty (120) raw *SIENG* were isolated for *Bacillus cereus* strains. As a result, only a total of 49 samples (41%) of *Bacillus cereus* strains were presented on the NGKG agar, whose colonies were white and slightly thick, as the medium around

colonies presents red. *Bacillus cereus* strains are potential of toxin production by *B. cereus s*trains. To test with Gram reaction, oxidase, catalase, aerobic growth, anaerobic growth, VP are resulted positive. In starch hydrolyze test there is only thirty-seven (31%) *Bacillus cereus* that presented negative, yet. *Bacillus cereus*, which was compared with positive indicator of *Bacillus cereus* presented positive. Also, as *B. cereus* has been detected and implicated in contaminated foods containing fermented soybeans, numerous outbreaks of food poisoning have been caused by *B. cereus* and it is an important foodborne pathogen with a distribution similar to that of *Salmonella, Escherichia coli*, and *Listeria monocytogenes* in food industry [2].

3.1.1 *Bacillus cereus* Toxin Genes by PCR Method

Bacillus cereus toxin genes were determined by using a PCR and well diffusion assay methods. There were 2 steps conducted in PCR and well diffusion assay. The first will use exactly the CRET-RPLA and second PCR reaction testing method. The cell-free culture supernatants of *Bacillus cereus* isolates were subjected to detect for enterotoxin production by *B. cereus*. Enterotoxin-Reverse Passive Latex sensitized (CRET-RPLA) test kit was used. The results as reported in Fig. 1 showed that 12 of 120 *Bacillus cereus* isolates gave positive sensitivity (25%) compared with control enterotoxin by lyophilization and then 2 (*BTM8-7* and *BTM8-8*) of these 12 isolated *Bacillus cereus* strains produced the highest level of enterotoxin, which may cause diarrhea respectively.

As shown in Fig. 2, 49 of *Bacillus cereus* isolates, which did not give multiplex PCR positive, showed correlation with RPLA test. So that, all *Bacillus cereus* isolate strains do not have multiplex PCR positive with 227 bp genes positive control *Bacillus cereus* (C = Control Cereus rid Toxin produce) and similar negative control still 134 bp (L = Internal Control), but also difference showed negative Latex sensitized (CRET-RPLA) test. In general, the emetic type of *Bacillus cereus* foodborne illness is caused by a small cyclic heat-stable peptide toxin known as cereulide, which causes vomiting and nausea for a few hours after consumption of contaminated food. Though Cambodia is the third world country, Royal Government of Cambodia is also considering to reduce food borne disease and improve public health status by introducing food safety standards and market regulation into food industries [4].

3.2 Isolating Bacillus Strains from Cambodian Fermented Soybean (SIENG) Samples

Bacillus subtilis strains are important for seed fermentation because of their enzymatic activities contributing to desirable texture, flavor and pH development [11]. And it was resistant to heat which showed growth in 70 $g \cdot dm^{-3}$ NaCl, nitrate reductase positive reaction [12]. As shown in Table 1, these isolated strains among 120 *Bacillus* strains, were found only 5 *Bacillus* strains that have ability to fight against the indicator microorganisms. Furthermore, among 5 samples, only one strain (*Bacillus CeM6-2*) has shown active zone. *Bacillus CeM6-2* strain had the highest level antimicrobial compound of *Lactobacillus plantarum ATCC 8014* at 28% of total zone activity compared to others. One of the main characteristics shared among *Bacillus* strains is the ability to produce a wide range of antimicrobial compounds active against bacteria. The rank based on per mm/mL and percentage of total area of level antimicrobial compound of *Lactobacillus plantarum ATCC 8014* showed the trend of all type *Bacillus* strains.

3.2.1 Identification from Isolated Bacteriocin Producing by *Bacillus subtilis* Strains

The samples were spread plated and streaked on GSP agar plate to get the pure culture and the morphology of the colony is observed. As a result, colonies were found to be circular and irregular in shape, white to creamish in colour and measured to be 1-3 mm in size mostly, and the optimum temperature of *Bacillus* strains in order to grow was between 30-37 °C [13]. In opposite, in anaerobe production, there

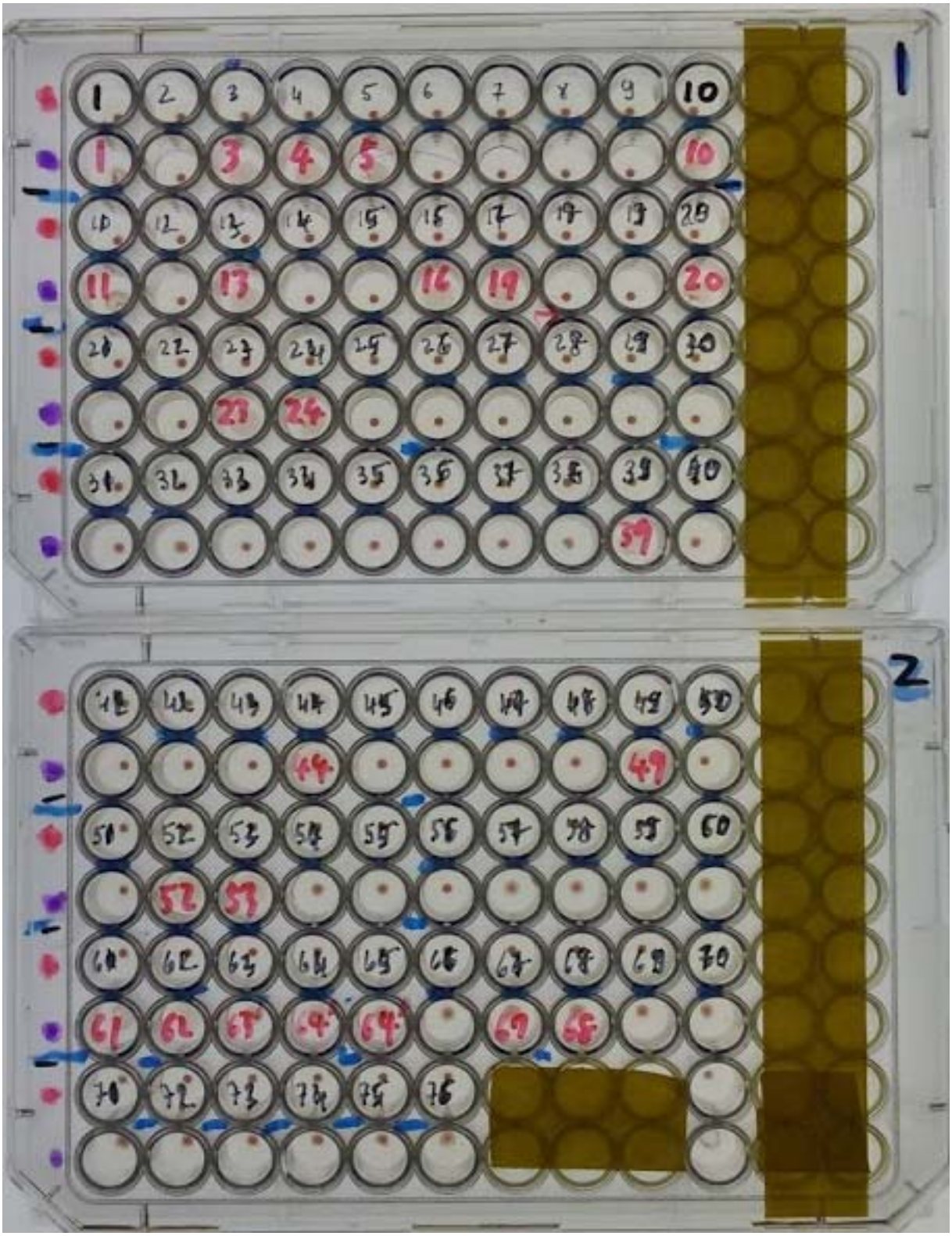

Fig. 1 ***Bacillus cereus*** **produced enterotoxin by CRET-RPLA method.**
Numbers 28 to 76 of *Bacillus cereus* were isolated from 120 raw *SIENG*.

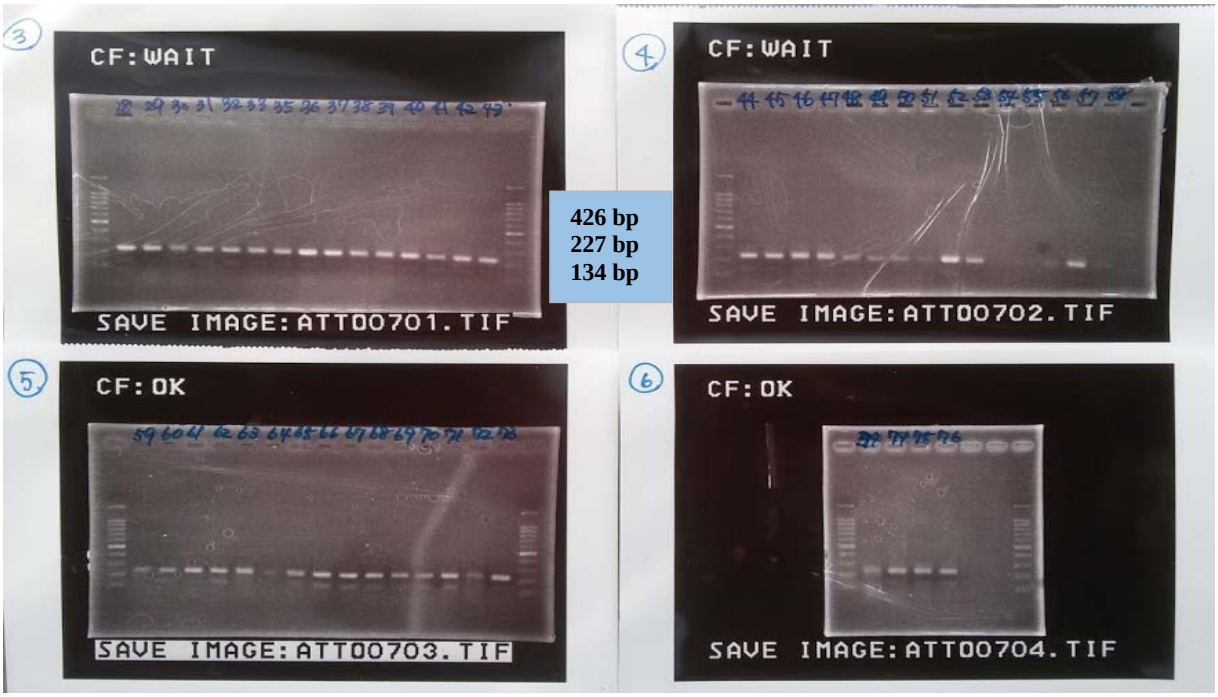

Fig. 2 ***Bacillus cereus*** **toxin genes by multiplex PCR method.**

Table 1 Growth of isolated strains and the zone activities of the isolated strains.

No.	*Bacillus* strains	Incubator time	Incubator temperature	Diameter of zone activity (mm/mL) for *Lactobacillus plantarum ATCC 8014*		
				Total	Average	Percentage
1	*CeM6-2*			52.00	13.00	28.00
2	*CeM6-8*			48.00	12.00	26.00
3	*CeM6-7*	Overnights	35 °C	40.00	10.00	21.00
4	*BTM8-4*			34.00	8.50	18.00
5	*TSM5-9*			14.00	3.50	7.00
Total				188.00	47.00	100.00

Size of well (5 mL) was excluded from calculation of all the samples.

was only one *Bacillus* strain growing and taking a very longer time for growing but staying for only a short life compared to aerobe production because *Bacillus* species including *Bacillus subtilis* can be obligated in both conditions—aerobes (mostly) and anaerobes (rarely and stay in short life) according to the existing publications [7]. Nonetheless, according to another publication [12], a good condition that *Bacillus* strain can grow well is in aerobes production and its typical characteristics of the *Bacillus subtilis* are a facultative aerobic, Gram-positive rod, and spore formation. There are three kinds of test for biological counting VPOF test, Manito Salt, and Catalase test. *Bacillus* species will be positive for the enzyme catalase when there has been oxygen. The result has shown that all *Bacillus* strains are fermented in OF test, where only one of *BTM8-4* was negative in VP and all positive in VP, Manito Salt and Catalase test, including *Bacillus subtilis*, and they were catalase-positive reaction, mannitol, and maltose [12].

3.2.2 Subject to Assay of *Bacillus subtilis* Strains

Five (5) selected strains of *Bacillus* have been taken to determine antimicrobial activity against indicator organisms and were determined by using a well diffusion assay method. There was a significant difference among the zone activities of *Bacillus subtilis* strains classifications against indicator organisms of *Lactobacillus plantarum* bacteria, and they mostly had

activity at 35 °C much more than at 30 °C.

From the past step, after receiving results of bacteriocin producing *Bacillus* strains at 17 and 24 hours, those 5 strains were gone for further test to isolate the strongest *Bacillus subtilis* strain which could produce bacteriocin the best in comparison with other strains. The total diameter of zone activity (mm/mL) for *Lactobacilus plantarum* and API50 CH was used in the context of "Measure of the Rank and Classify Bacteriocin Productions on the *Bacillus subtilis* Strain" for final selection of bacteriocin. The categories, rank and selection of bacteriocin production analysis of all types of *Bacillus* are presented in Table 2.

Table 2 revealed that mean of *CeM6-2* strain was 13.50 mm/mL, 53%, which was the highest proportion of total area of zone activity for *Lactobacilus plantarum* linked to other strains that were measured and the following order of *Bacillus* strain was *BTM8-4* (0.75 mm/mL, 3%), *CeM6-8* (1.50 mm/mL, 6%), *TSM5-9* (3.00 mm/mL, 12%) and *CeM6-7* (7.50 mm/mL, 27%) strains respectively. The rank and selection of *Bacillus* strain were based on its size (mm/mL) and percentage of zone activity that showed the trend for all types of *Bacillus subtilis* strains.

Accordingly, only one strain *CeM6-2* which had very strong zone activities was selected for further test since it was able to suppress the growth of *Lactobacillus plantarum* bacteria, which was in parallel with existing researches stating that, some *Bacillus subtilis* have been reported to produce bacteriocins which suppress the growth of Gram positive spoilage and pathogenic bacteria [2, 14]. In addition, we also found that antimicrobial activity against indicator organism of *CeM6-2* was more active at 30 °C compared to 35 °C, overnight. There are also some strains such as *CeM6-2* strains which showed antimicrobial activity against indicator organism. However, these strains are half-strong compared to other strains. The strain *CeM6-2* was identified as *Bacillus subtilis* by 96.2% and a *t*-value at 0.76 homology correspondingly counting on API 50 CH V4.1 (BioMérieux) profiles (not shown) and their physiological characteristics. Finally, only 1 *Bacillus* strain that had strong activity was selected for the next experiment.

3.2.3 Characterize the Bacteriocin Produced by Isolated Strain

The result obtained from our test showed *B. CeM6-2* strain had ability resistant to heat. Similarly, *B. subtilis* strains may produce other antimicrobial substances, which have been characterized to a much lesser extent [7].

(1) Incubator Temperature and Timing

In this test, we used two variables of temperature (30 °C and 37 °C) and different timing (0 h, 6 h, 8 h and 12 h for first day and 0 h, 14 h, 16 h, 18 h, 20 h and 22 h for second day), and the *Lactobacillus plantarum* bacteria were used as the indicator strain. The test was conducted for 2 days.

Table 2 Rank and selection of bacteriocin produced by *Bacillus subtilis* strain category by total diameter of zone activity (mm/mL) for *Lactobacilus plantarum.*

S.N.	Type of *Bacillus subtilis* strains	Incubator time (h)	Incubator temperature (°C)	Total No. zone activity (mm/mL) at 35 °C	Incubator Temperature (°C)	Total No. zone activity (mm/mL) at 30 °C	No. respondents of *B. subtilis* activates	Diameter of zone activity (mm/mL) of *Bacillus subtilis* strain for *Lactobacilus plantarum* bacteria		
								Total	Mean	Percentage
1	*CeM6-2*			26		28	4	54.00	13.50	53.00
2	*CeM6-7*			14		14	4	27.00	7.00	27.00
3	*TSM5-9*	Overnight	35	7	30	5	3	12.00	3.00	12.00
4	*CeM6-8*			6		ND	2	6.00	1.50	6.00
5	*BTM8-4*			3		ND	2	3.00	0.75	3.00
	Total	-	-	-	-	-	-	103	-	100.00

ND: not detected.

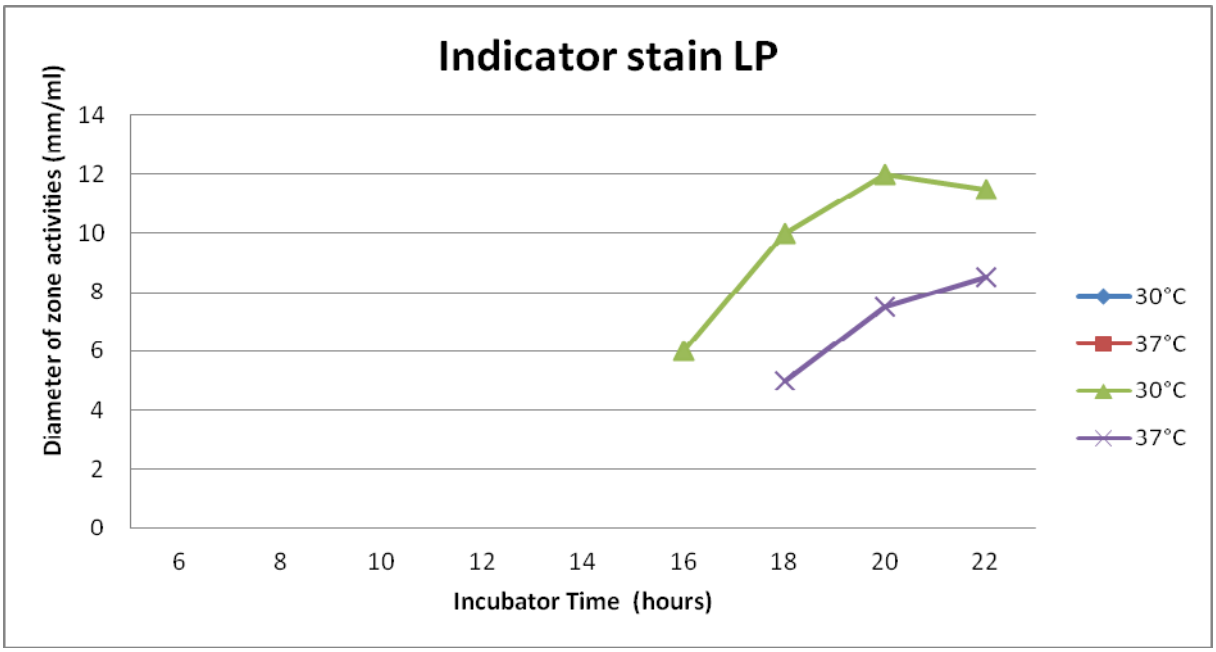

Fig. 3 Effect of difference incubator temperature and timing on the activity of bacteriocin produced by *Bacillus CeM6-2* strain.

As seen in Fig. 3, in the first day of test, the antimicrobial activities of bacteriocin had no antimicrobial activities at all timing and temperatures. The antimicrobial activities actively happened in second day of the test. The same figure and table revealed that the antimicrobial activities of bacteriocin started to be active after 16 h of incubation at 30 °C. The antimicrobial activities kept increasing consistently for four hours, and gradually decreased after 20 h of incubation at 30 °C. At 37 °C, the bacteriocin started increasing its antimicrobial activities after 18 h of incubation which was later compared with 30 °C of incubation temperature. Though, its antimicrobial activity was kept increasing slightly for just only one hour, and started decreasing sharply for the next four hours until became inactivated after 22 h of incubation at 37 °C. It demonstrated that bacteriocin produced by *CeM6-2* strain is more likely to be active at 37 °C for a longer period compared with its activities at 30 °C for two tested days. The finding was similar to previous researches that indicated the behavior of bacteriocin towards heat-resistant pathogens also varies [14].

(2) Enzyme Treatment (Indicator Strain *LP*)

In this test, we used the enzyme contained in *PK and PK-MSF* as the treatment, and used one type of indicators *Lactobacillus plantarum ATCC 8014* (*LP*). We also used other three of bacteriocin produced by *Bacillus subtilis* strains including *B. CeM6-2PK*, *B. CeM6-2PK-MSF* and *B. CeM6-2* untreated, for comparing the result. The effects of heat, nutritional composition and incubation time at 35 °C and the antibacterial substances were stable within OD, 650 nm of all *Bacillas sutilis* strains.

Fig. 4 presented the characteristics of antimicrobial activity against *LP* by *Bacillus subtilis* strains such as *B. CeM6-2PK*, *B. CeM6-2PK-PMSF* (*SEING*) and *B. CeM6-2* untreated, after been cultured in MRS broth contained against *LP*. It was indicated that, at different timing (0 h to 47 h), the result showed that the strain *B. CeM6-2* untreated and *B. CeM6-2PK-PMSF* produced the strongest antimicrobial activity against MRS broth contained *LP* in comparison with *B. CeM6-2PK*, but the other remaining strains *B. CeM6-2PK* were found to have no antimicrobial activity at all. At 0 h, all strains were calculated 0 OD equally. At 0 h to 47 h, the *B. CeM6-2* untreated and *B. CeM6-2PK-PMSF* were calculated to be 0 OD respectively, which in comparison

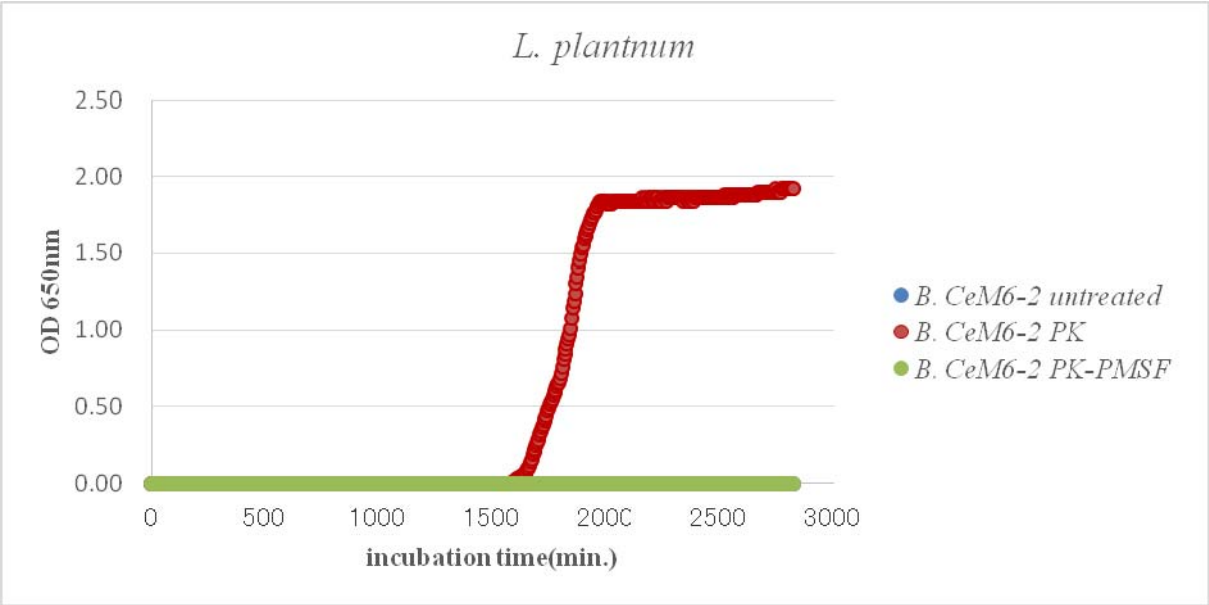

Fig. 4 Enzymes of *PK* and *PK-PMSF* resistant for strain *B. CeM6-2* compared with *B. CeM6-2* untreated strains against indicator *LP* at different time in MRS broth at 35 °C, 47 h.

were less than *B. CeM6-2PK* that presented at 0.01 OD to 1.92 OD at 26 h to 47 h , in correspondingly.

3.2.4 Apply the Bacteriocin to Control the Growth of Gram Positive Pathogenic or Spoilage Bacteria in Foods

Applying bacteriocin to control the growth of Gram positive pathogenic or spoilage bacteria and lactic acid group in foods was conducted against three indicator strains such as *Enterococcus faecium*, *Listeria monocytogenes*, *Bacillus cereus*, *Streptococcus pyrogene*, *Micrococcus luteu* and *S. aureus* (Gram positive pathogenic or spoilage bacteria) and lactic acid group (*Lactobacillus brevis*, *Leuconostoc mesenterides*, *L. curvatus*, *Lactobacillus plantnum*, *Lc. lactis* (*NinA+*) and *Lactobacillus lactis*) contained *B. CeM6-2* comparing with *Miyagino* strain after culturing in HBI contained against Gram positive; and MRS containing against lactic acid group (OD = 0.1, 650 nm), and volume measurement culture prior to start experiment. Similarity searches with sequences in the bacteriocin produced by *Bacillus subtilis* strain *LFB112* from Chinese herbs, were effective against both Gram-positive and Gram-negative bacteria involved in domestic animal diseases [15].

(1) Gram Positive Group

In order to study the growth controlling of Gram positive pathogenic or spoilage bacteria in foods of antimicrobial compound of the two kinds of *B. subtilis* group *B. CeM6-2* (Cambodia) and *Miyagino* (Japan) growth, the inhibitory activity present in cell-free samples taken at different time intervals was measured. Antibacterial activity could be detected at the mid-log growth phase and quickly extended a maximum at the early inactive phase, subsequently, the antagonistic activity declined.

- Indicator Strain *Enterococcus faecium*

The control of antimicrobial activity against *Enterococcus faecium* of two strains *B. subtilis* and *Miyagino* after being cultured in BHI broth contained against *Enterococcus faecium* (OD = 0.1, 650 nm) at 35 °C for 58 h, was discussed in Fig. 5.

Fig. 5 illustrated that strains *B. CeM6-2* did not have antimicrobial activity at 0 min as its OD is above *Miyagino* (0.03 compared to 0.02 OD). The OD of *B. CeM6-2* started to be extremely lower than *Miyagino* from 100 min (0.04 to 0.05) to 2,000 min (0.14 to 0.97) which resulted in strong power of antimicrobial activity of *B. CeM6-2* against *Enterococcus faecium*

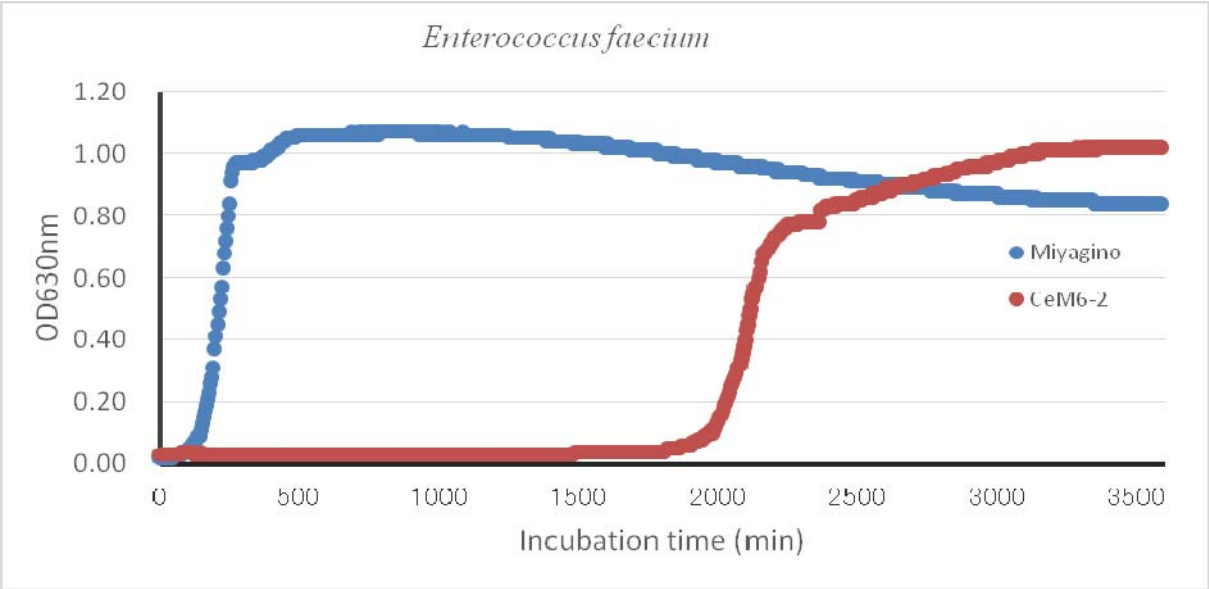

Fig. 5 Effect of bacteriocin produced by *Bacillus CeM6-2* strain on the growth of *Enterococcus faecium* compared with *Miyagino* at different time in HBI broth at 35 °C for 58 h.

compared with *Miyagino* strain during this timing. From 2,005 to 2,645 min, *B. CeM6-2* has less antimicrobial activity comparing to previous timing but its OD is still far lower than *Miyagino.* From 2,650 to 3,500 min, *B. CeM6-2* has 0.90 to 1.02 OD higher than *Miyagino* that has 0.89 to 0.84 OD which resulted in no inhibition within this specific timing.

- Indicator Strain *Listeria monocytogenes*

The control of antimicrobial activity against *Listeria* of two strains *B. subtilis* and *Miyagino* after being cultured in BHI broth contained against *Listeria* OD = 0.1, 650 nm) at 35 °C for 30 h. As seen in Fig. 6, both strains have the same 0.03 OD at 0 min. However, *B. CeM6-2* commencing falls at 90 min to 0 OD stably until 3,500 min. Differently, OD of *Miyagino* is above *B. CeM6-2* at all entire experiment timing. *Miyagino* maintains almost stable of OD from 0 min (0.03 OD) to 1,145 min (0.07 OD) and sharply increases afterward until 1,510 min (1.07 OD). It is gradually reducing later until 3,500 min to 0.72 OD. This result interpreted that *B. CeM6-2* has a strong antimicrobial activity against *Listeria monocytogenes* as its amount of OD is lower than *Miyagino* for entire experiment timing.

- Indicator Strain *Bacillus cereus*

Fig. 7 indicated that from 0 to 1,310 min, *Bacillus CeM6-2* has inhibited against *Bacillus cereus* compared with *Miyagino* since its OD is 0.02 to 0.71 comparing to 0.04 to 0.72. From 1,315 to 2,050 min, *Bacillus CeM6-2* slowly fluctuated with above OD amount comparing with *Miyagono* strain from (0.72 OD to 0.67) to (0.71 to 0.66 OD) which means that it has no antimicrobial power against *Bacillus cereus* within this specific timing. At 2,085 min onward, the OD amount of *Bacillus CeM6-2* resumed its antimicrobial activity as its OD amount started to decrease below *Miyagino* from (0.66 to 0.39 OD) comparing to (0.67 to 0.77 OD). This result concluded that *Bacillus CeM6-2* has specific antimicrobial activity against *Bacillus cereus* within certain timing from 0 to 1,310 min; and from 2,085 to 3,500 min only.

- Indicator Strain *Streptococcus pyrogene*

According to Fig. 8, *Bacillus CeM6-2* strain has a perfect antimicrobial activity against *Streptococcus pyrogene* compared with *Miyagino* as it has stable amount of OD starting from 0 min (0.01 OD) to 3,500 min (0.02 OD). In contract, *Miyagino* strain has slowly increased amount of OD starting from 0 min at 0.04 OD to 1,255 min at 0.08 OD and it sharply increased

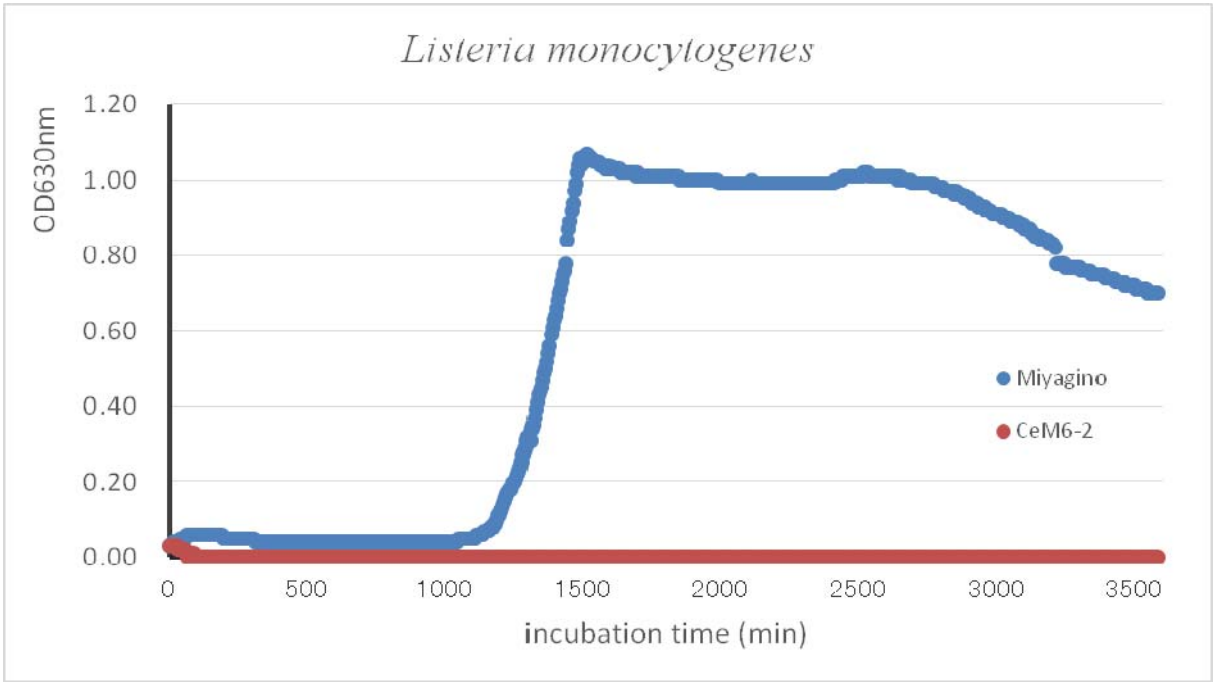

Fig. 6 Effect of bacteriocin produced by *Bacillus CeM6-7* strain on the growth of *Listeria monocytogenes* compared with *Miyagino* at different time in BHI broth at 35 °C for 58 h.

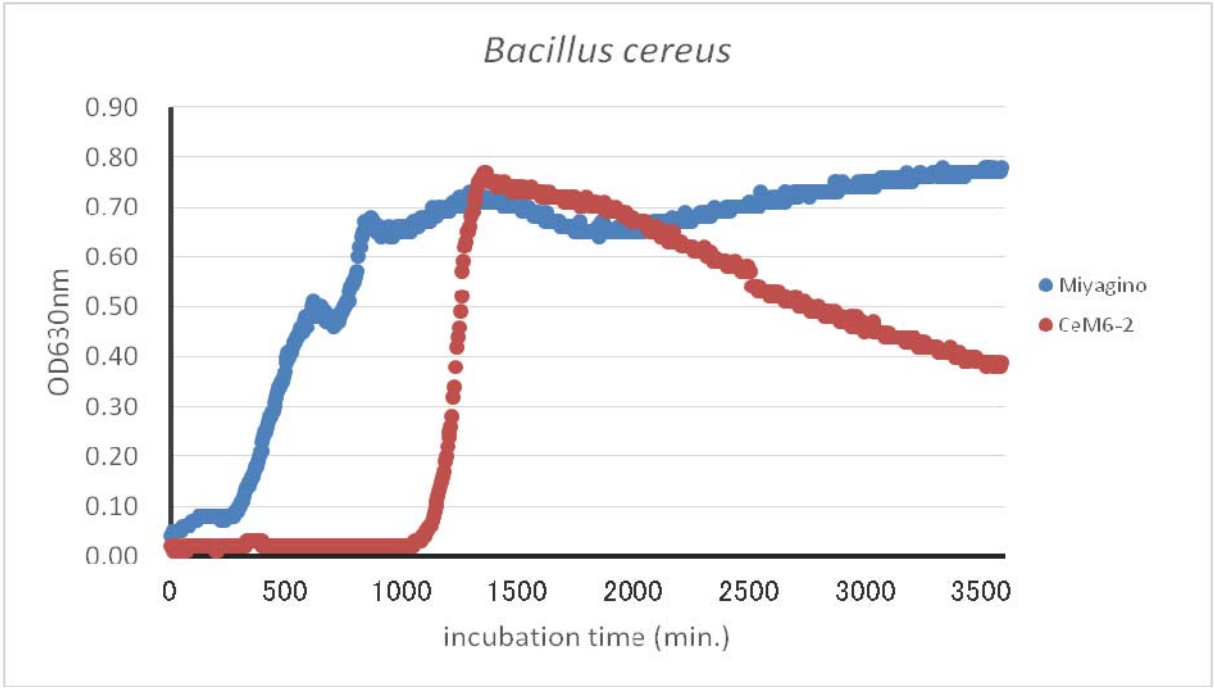

Fig. 7 Effect of bacteriocin produced by *Bacillus CeM6-2* strain on the growth of *Bacillus cereus* compared with *Miyagino* at different time in BHI broth at 35 °C for 58 h.

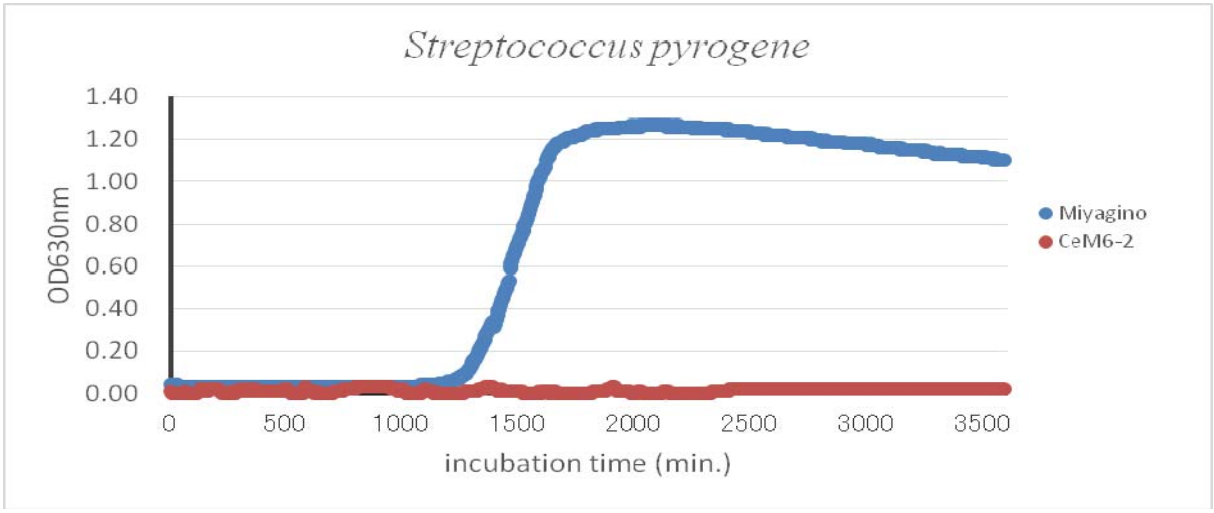

Fig. 8 Effect of bacteriocin produced by *Bacillus CeM6-2* strain on the growth of *Streptococcus pyrogene* compared with *Miyagino* at different time in BHI broth at 35 °C for 58 h.

afterward to 3,500 min at 0.78 OD.

- Indicator Strain *Micrococcus luteus*

Fig. 9 indicated that both strains, *Miyagino* and *Bacillus CeM6-2* strains, have same amount of OD starting from 0 to 35 min at 0.03 OD. From 40 to 575 min, *B. CeM6-2* has higher amount of OD compared with *Miyagino*; 0.03 and 0.02 OD comparing to 0.02 and 0.01 OD of *Miyagino* strain. In 580 to 1,925 min, *B. CeM6-2* and *Miyagino* have same amount of OD (0.02). In 1,935 to 2,465 min OD of *B. CeM6-2* and *Miyagino* are in stable amount (0.02 OD) and (0.01 OD). From 2,470 to 3,500 min, both strains have the same amount of OD, 0.02 equally. This can be concluded that *B. CeM6-2* strain has no antimicrobial activity against *Micrococcus luteus.*

- Indicator Strain *S. aureus*

Fig. 10 showed the result of comparison on effect of bacteriocin produced by *Bacillus CeM6-2* strain on the growth of *Staphylococcus aureus* comparing to *Miyagino* at different time in BHI broth at 35 °C for 41 h. In according with the same figure, in 0 to 2,500 min, strain *Bacillus CeM6-2* has suddenly increased OD amount from 0.04 to 1.46 OD higher than *Miyagino* strain which is increasing from 0.04 OD to 0.83 OD. Consequently, the result demonstrated there is no antimicrobial activity of *B. CeM6-2* strains against *Staphylococcus aureus.*

In short, the *Bacillus CeM6-2* strain performed well when treated with Gram positive group; especially it has strong antimicrobial activity against *Listeria monocytogenes*, and *Streptococcus pyrogene.* Following from this, *Bacillus CeM6-2* has partially antimicrobial activity, during specific timing, against *Bacillus cereus* (0 to 1,310 min and 2,085 to 3,500 min), and *Enterococcus faecium* (100 to 2,000 min and 2,005 to 2,645 min), but it was found to have no power to suppress against *Micrococcus luteus* and *Staphylococcus aureus.* This result was in parallel with existing researches stating that, some *Bacillus subtilis* have been reported to produce bacteriocins which suppress the growth of Gram positive spoilage and pathogenic bacteria [2, 14].

(2) Lactic Acid Groups

In this test, we used two variables of temperature 35 °C and incubator timing 40 h, and one kind of antibacterial produced by *B. CeM6-2* (Cambodia) of the tested strains compared with *Miyagino* strain after being cultured in MRS containing against lactic acid group (*Lactobacillus brevis*, *Leuconostoc mesenterides*, *L. curvatus*, *Lactobacillus plantnum*, *Lc. Lactis* (*NinA+*) and *Lactobacillus lactis* with OD = 0.1, 650 nm), bacteria were used as the indicator strain.

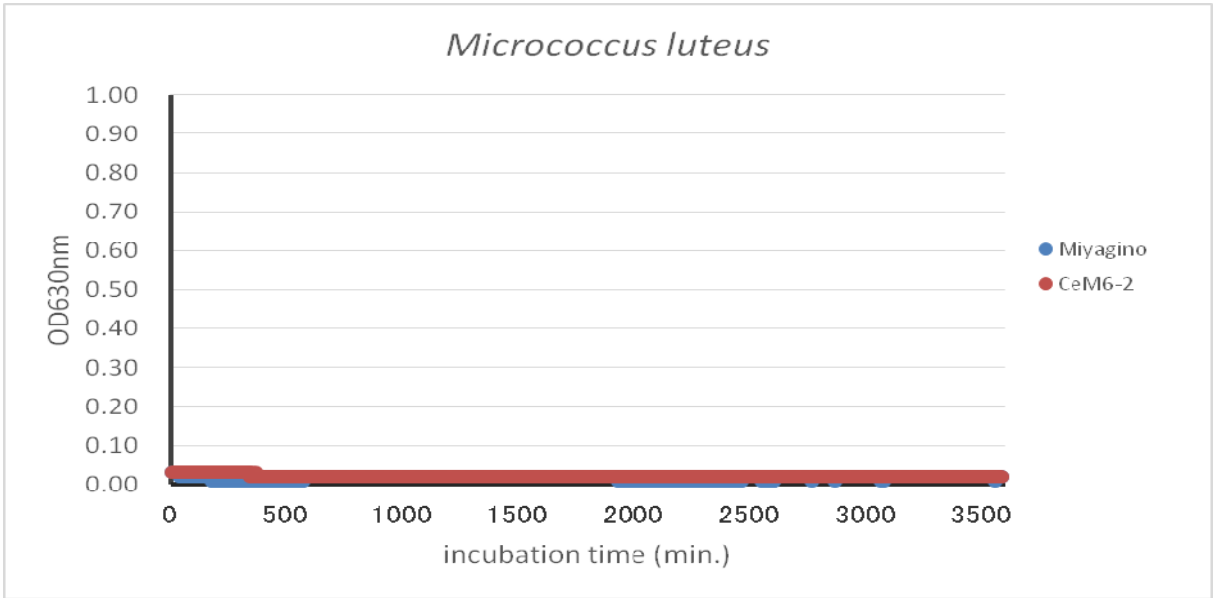

Fig. 9 Effect of bacteriocin produced by *Bacillus CeM6-2* strain on the growth of *Micrococcus luteus* compared with *Miyagino* at different time in BHI broth at 35 °C for 58 h.

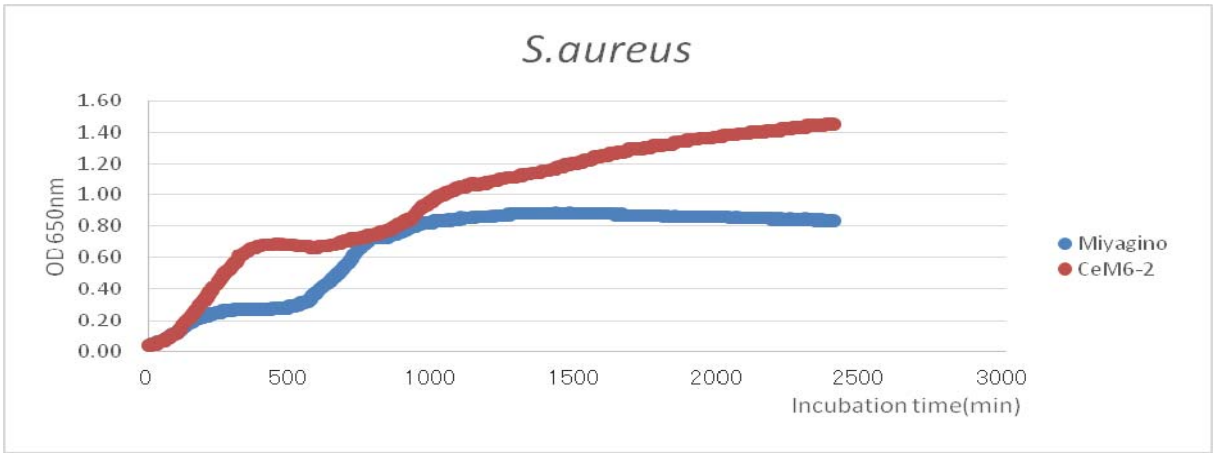

Fig. 10 Effect of bacteriocin produced by *Bacillus CeM6-2* strain on the growth of *Staphylococcus aureus* compared to *Miyagino* at different time in BHI broth at 35 °C for 41 h.

However, the test was conducted to measure pH value of each indicator of bacteriocin produced by *Bacillus subtilis* strains culture prior to starting experiment and the effects of heat, pH, nutritional composition and incubation time at 35 °C and the antibacterial substances were stable within OD, 650 nm and wide range pH value from 3.858 to 3.923 as shown by the isolates strain against on inhibitory by the bacteriocin produced by *B. CeM6-2* strains compared with *Miyagino* strain to be using control strain. In the cell-free samples taken at different time intervals were measured. Antibacterial activity could be detected at the mid-log growth phase and quickly extended a maximum at the early inactive phase, subsequently, the antagonistic activity declined.

- Indicator Strain *L. brevis*

Fig. 11 expressed the strong antimicrobial activity of *B. CeM6-2* strain against *L. brevis* compared with *Miyagino* starting from 0 min (0.04 OD) to 1,605 min (0.09 OD). It continued its antimicrobial activity afterward until 2,065 min (0.93 OD). In 2,070 to 2,500 min (0.97 OD to 1.62 OD), its power was reduced and not suppressed against *L. brevis* if

compared with *Miyagino* (0.96 OD to 0.97 OD). Consequently, *B. CeM6*-2 has strong power to fight against *L. brevis* starting from 0 to 1,605 min only.

- Indicator Strain *Leuconostoc mesenterids*

The comparison of effect of bacteriocin produced by *B. CeM6-7* strain on the growth of *Leuconostoc mesenterids* compared with *Miyagino* was argued in Fig. 12. In 0 to 50 min, the OD amount of *B. CeM6-2* is between 0.05 to 0.07 OD which is higher than *Miyagino* 0.04 to 0.07 OD. From 55 to 2,400 min, OD of *B. CeM6-2* starts falling down from 0.07 to 1.60 lower than *Miyagino* stated at 0.08 to 1.84 OD.

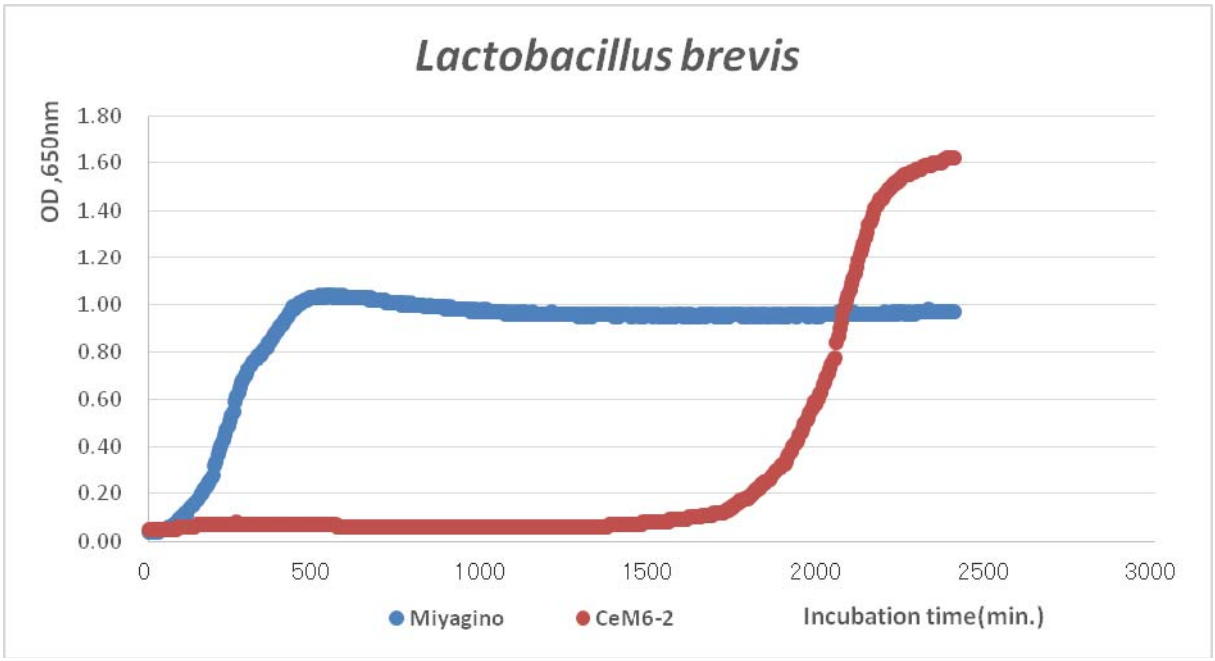

Fig. 11 Effect of bacteriocin produced by *B. CeM6-2* strain on the growth of *L. brevis* compared with *Miyagino* at different time in MRS broth at 35 °C for 40 h.

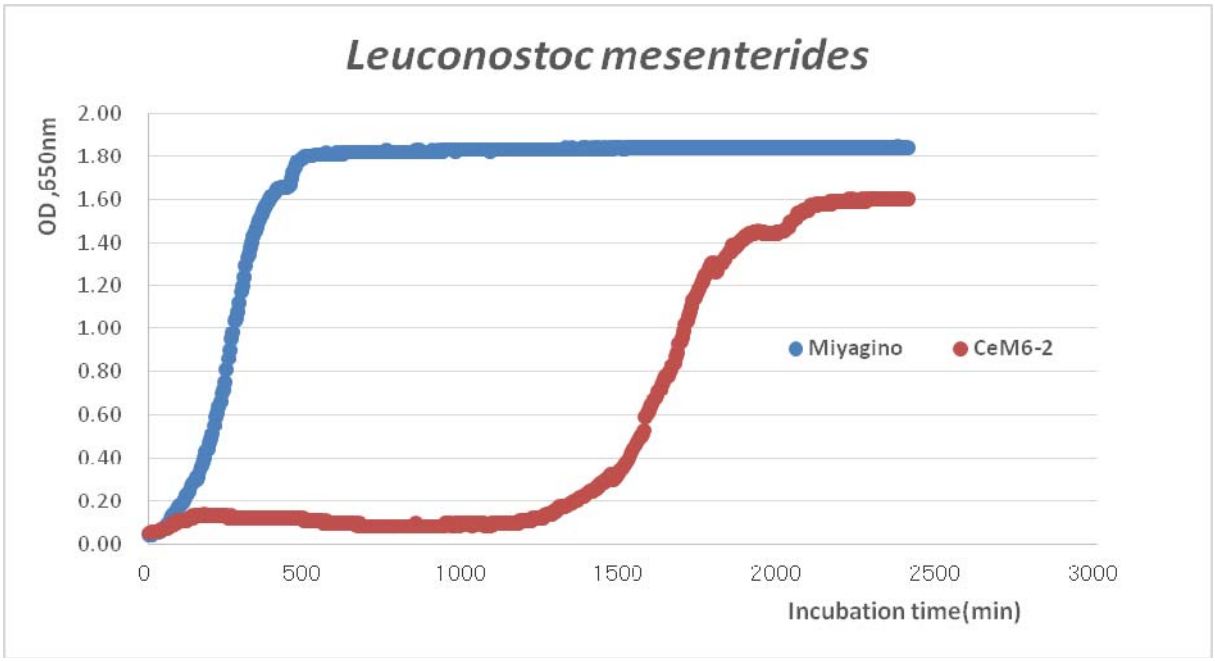

Fig. 12 Effect of bacteriocin produced by *B. CeM6-2* strain on the growth of *Leuconostoc mesenterids* compared with *Miyagino* at different time in MRS broth at 35 °C for 40 h.

This result can be summarized that from the beginning to 50 min, there is absence of antimicrobial activity of *B. CeM6-2* against *Leuconostoc mesenterids.* The presence of antimicrobial activity has started from 55 to 2,400 min.

- Indicator Strain *L. curvatus*

Fig. 13 discussed on the effect of bacteriocin produced by *B. CeM6-2* strain on the growth of *L. curvatus* compared with *Miyagino*. The result indicated that from 0 to 895 min, *B. CeM6-2* has OD between 0.06 and 0.10 OD lower than *Miyagino* 0.06 to 0.11 OD which showed the presence of antimicrobial activity in this period of time. From 905 to 2,400 min the amount of OD of *B. CeM6-2* sharply increased from 0.12 and 1.94 OD, higher than *Miyagino* presented between 0.11 to 1.94 OD which indicated no antimicrobial activity from 905 min to the end of experiment timing.

In brief, the effect of bacteriocin produced by *B. CeM6-2* strain on the growth of *L.* curvatus inhibited during minute 0 to 895 only.

- Indicator Strain *L. plantarum*

Fig. 14 demonstrated that *B. CeM6-2* strain has a very strong antimicrobial activity against *L. plantarum* compared with *Miyagino.* From 0 to 2,500 min OD of *B. CeM6-2* decreased from 0.06 to 0.03 OD, lower than *Miyagino* that increased from 0.06 to 2.00 OD within same period of time.

- Indicator Strain *Lactococcus lactis* subsp. *lactis* (Produce Nisin A)

The discussion of effect of bacteriocin produced by *Bacillus CeM6-2* strain on the growth of *Lactococcus lactis* subsp. *lactis* (produce Nisin A) was presented in Fig. 15. From the experiment result, the OD of *Bacillus CeM6-7* increased higher than *Miyagino* strain over the period of time from the beginning of experiment till the end. In 0 to 355 min the OD amount of both strains dramatically increased. OD of *Bacillus CeM6-2* presented at 0.03 to 1.54 much more than *Miyagino* that stood between 0.02 and 1.46 OD. From 360 to 2,400 min the OD amount of both strains is in slow increasing level. OD of *Bacillus CeM6-2* stood at 1.55 to 1.76 while the *Miyagino* stated at 1.47 to 1.67 OD. From the finding above we can conclude that *Bacillus CeM6-2* strain has no power to fight against *Lactococcus lactis* subsp. *lactis* (produce Nisin A) comparing to *Miyagino.*

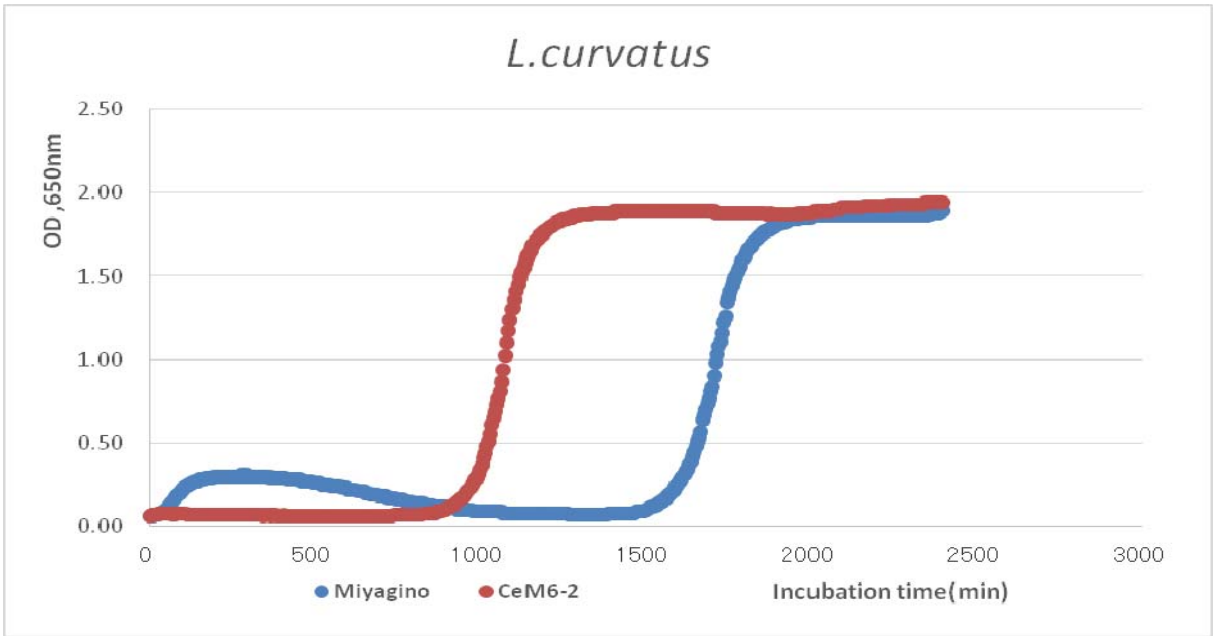

Fig. 13 Effect of bacteriocin produced by *B. CeM6-2* strain on the growth of *L. curvatus* compared with *Miyagino* at different time in MRS broth at 35 °C for 40 h.

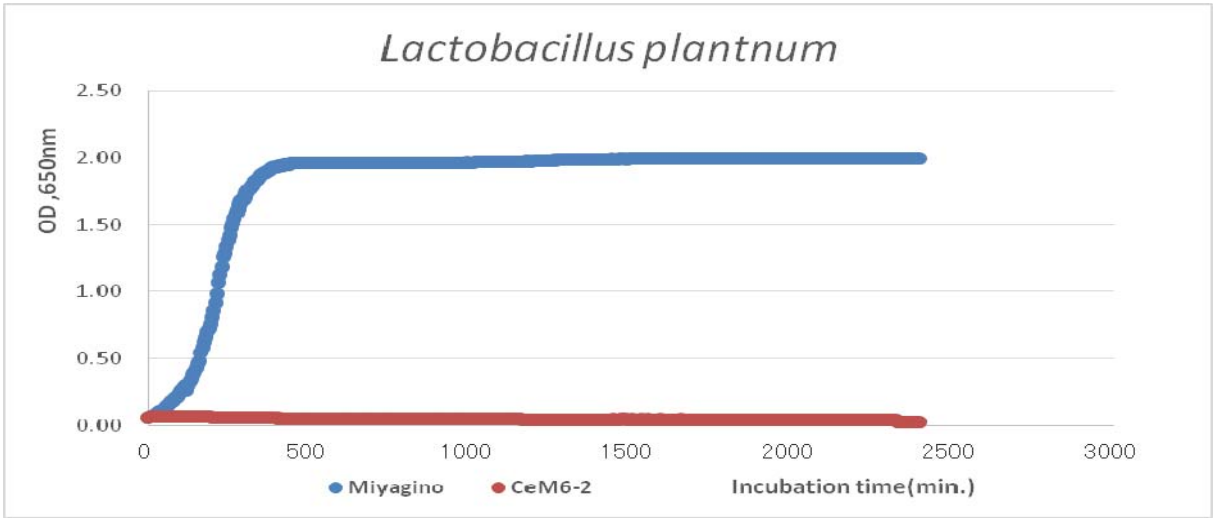

Fig. 14 Effect of bacteriocin produced by *B. CeM6-2* strain on the growth of *L. plantarum* compared with *Miyagino* at different time in MRS broth at 35 °C for 40 h.

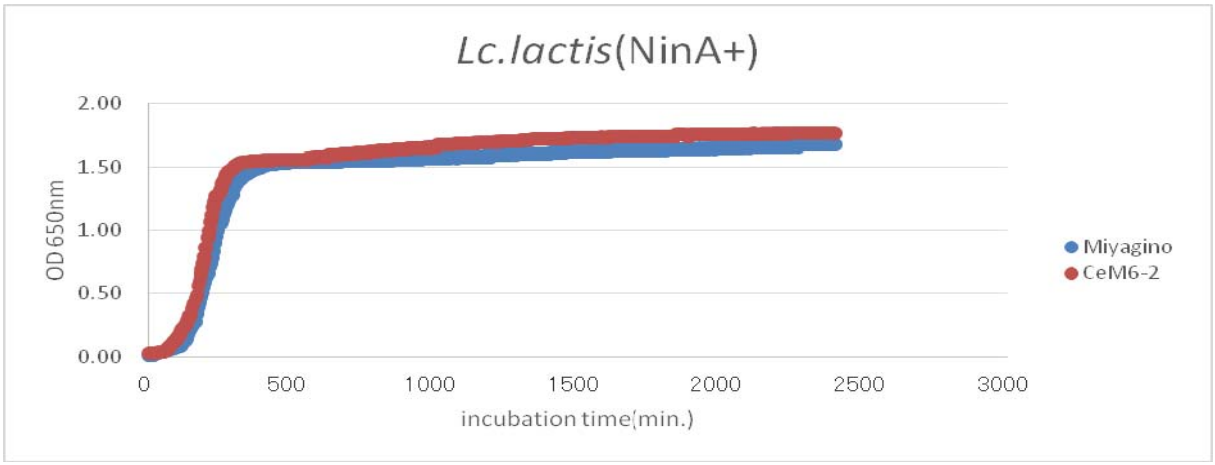

Fig. 15 Effect of bacteriocin produced by *Bacillus CeM6-2* strain on the growth of *Lactococcus lactis* subsp. *lactis* (produce Nisin A) compared with *Miyagino* at different time in MRS broth at 35 °C for 40 h.

- Indicator Strain *Lb. lactis*

Fig. 16 described finding of effect of bacteriocin produced by *B. CeM6-2* strain on the growth of *Lb. lactis* compared with *Miyagino* strains. At starting point, 0 to 40 min, the OD of *B. CeM6-2* stood stably at 0.04 OD higher than *Miyagino* that presented at 0.03 OD. From 45 to 65 min, OD of both strains stated 0.05 equally. From 70 to 1,080 min both strains increased amount of OD dramatically; *B. CeM6-2* was observed with 0.05 to 1.59 OD lower than *Miyagino* observed with 0.06 to 1.60 OD. However, from 1090 to 3,500 min, OD of *B. CeM6-2* rose slowly above *Miyagino* starting from 1.60 to 1.63 OD which is over *Miyagino* starting from 1.59 to 1.62 OD.

In brief, strain *B. CeM6-2* showed antimicrobial activity against *Lb. lactis* at a specific timing from 70 to 1080 min only. From all above results, *Bacillus CeM6-2* strain performed well when treated with lactic acid group. It demonstrated very strong inhibitation against *Leuconostoc mesenterids* and *L. plantarum* for entire experiment timing, but it partially showed high ability to fight against *L. brevis* only during 0 to 1,605 min, *L. curvatus* from 0 to 895 min, and *Lb. lactis* from 70 to 1,080 min. Nevertheless, *Bacillus CeM6-2* is not inhibited against *Lactococcus lactis* subsp. *lactis* (produce Nisin A) in the whole experiment timing.

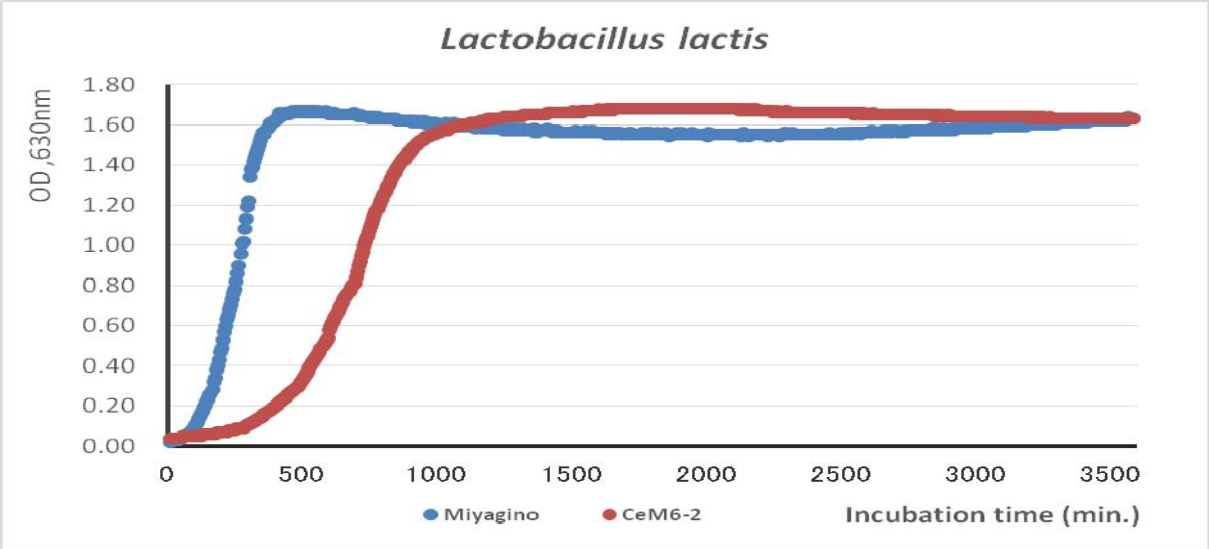

Fig. 16 Effect of bacteriocin produced by *B. CeM6-2* strain on the growth of *Lb. lactis* compared with *Miyagino* strains at different time in MRS broth at 35 °C for 40 h.

Thus, this property of bacteriocins produced by *B. CeM6-2* strain can be used as additive in food processing industries to avoid food spoilage even in 35 °C temperatures and longer time. Many researches on *Bacillus* bacteriocins are becoming more intensive and important due to their inhibition activity, which may include Gram-negative bacteria, yeasts or fungi, in addition to Gram-positive species, some of which are known to be pathogenic to humans and animal [15].

3.3 Co-cultivate Bacillus cereus and Bacillus subtilis in a Tripticase Soy Broth or Soybean and Check the Suppression of the Growth of Bacillus cereus

In this test, we used the tripticase soy broth (TSB) as the treatment, and used one type of indicator *Bacillus cereus*. We also used bacteriocin produced by *Bacillus subtilis* (*B. CeM6-2*) strains and different timing from 0 h, 24 h, 34 h and 44 h (first day and 0 h, 24 h, and 34 h and 44 h for second day). The test was conducted for 2 days.

Fig. 17 presented the characteristics of antimicrobial activity against *B. cereus* by *B. subtilis* strains such as *B. CeM6-2* (isolated from traditional fermented soybean (*SEING*)) after being cultured in *Bacilli's. CeM6-2*-TSB contained is against *B. cereus* (OD = 0.1). The result showed that the concentration strain *B. CeM6-2* (1%) produced the strongest antimicrobial activity against *Bacillus CeM6-2*-TSB contained concentration against *B. cereus* compared with all different concentrations strain *B. subtillis* (0.1%, 0.2%, 0.3%, 0.4% and 0.5%) and indicator strain *B. cereus* non-contained against *Bacilli's. CeM6-2*, but the other remaining strains *B. subtillis* (0.1%, 0.2%, 0.3%, 0.4% and 0.5%), was found to have similar antimicrobial activity with *B. cereus* at all different timing. At 0 h, all strains were counted to be 6.00 log CFU/mL equally. At 24 h, 34 h and 44 h, the concentration strain *B. CeM6-2* (1%) was calculated to be 8.21 log CFU/mL, 8.16 log CFU/mL and 8.14 log CFU/mL respectively which in comparison were less than all different concentrations strain *B. subtillis* (0.1%, 0.2%, 0.3%, 0.4% and 0.5%) that presented at 9.49 log CFU/mL, 9.27 log CFU/mL and 8.72 log CFU/mL and indicator strain *B. cereus* non-contained against *Bacilli's. CeM6-2*, which presented at 12.26 log CFU/mL, 9.67 log CFU/mL and 11.03 log CFU/mL correspondingly. Concentration strains *B. CeM6-2* (1%) were found to have high significant protection from *Bacilli's. CeM6-2*-TSB contained is against *B. cereus* (OD = 0.1). Basically, to control *B. cereus* and other foodborne

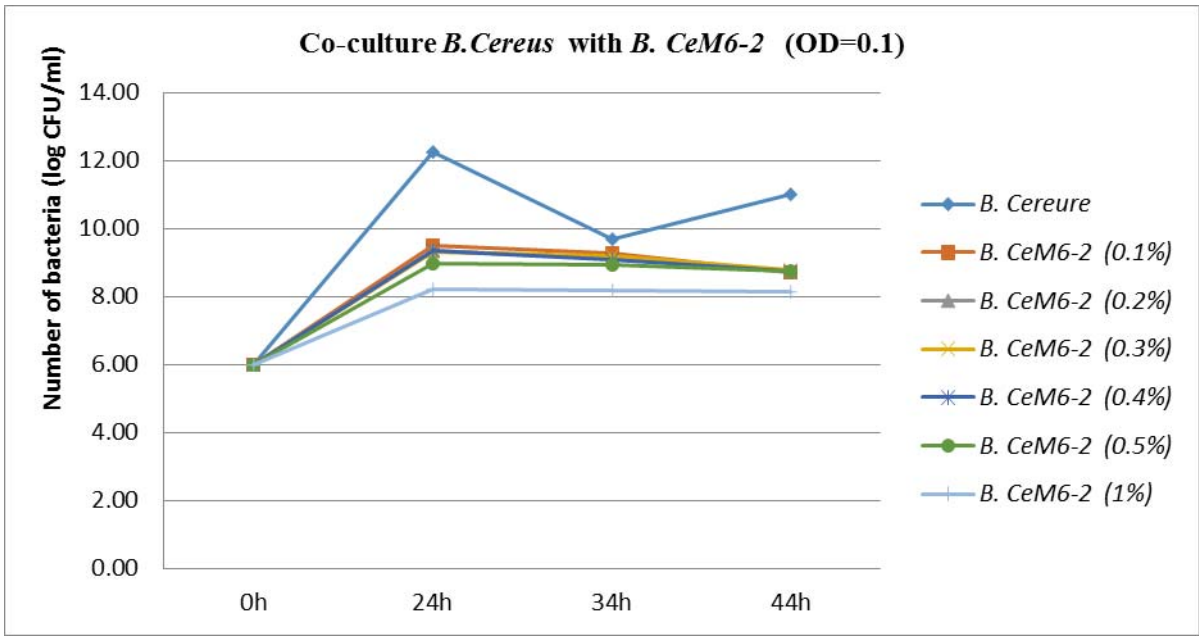

Fig. 17 Effect of bacteriocin produced by *B. CeM6-2* strain on the growth of *B. cereus* strains at different time in NGKG agar at 35 °C for 44 h.

pathogens, a variety of techniques have been evaluated including Nisin like antibiotic substances and chemical, heat, enzymatic, and acid treatments [2]. In general, *Bacillus subtilus* is the most dominant bacterium in fermented soybean [9]. Some *Bacillus subtilis* have been reported to produce bacteriocins which suppress the growth of Gram positive spoilage and pathogenic bacteria [7, 8].

3.4 Check the Quality of SEING Produced by Antimicrobial Bacillus subtilis Strain

The time and temperature play a vital role in bacteriocin production. Therefore, the modification test was conducted using cooked soybean mixed with culture *B. CeM6-2* and *B. cereus* in ratio, the mixtures were fermented at room temperature (RT) with different timing (0 h, 24 h, 48 h and 72 h). Within the expectation, *B. CeM6-2* is capable to produce bacteriocin to fight against *B. cereus* indicator strains finally in fermented soybean in Table 3.

The characteristics of antimicrobial activity against *B. cereus* of seven different concentrations ratio with *B. CeM6-2* strain, after cultured in *Bacillus*-fermented soybean contained against *B. cereus* (OD = 0.1), were discussed in Table 3. The table indicated that at 24 h all of strains, except *B. CeM6-2 + B. cereus* in ratio 10:0 mL strain, had antimicrobial activity. Nevertheless, at 48 h and 72 h, only two concentrations ratio strains *B. CeM6-2 + B. cereus* (10:0 mL) and *B. CeM6-2 + B. cereus* (9:1 mL) were able to inactivate *Bacillus*-fermented soybean contained against *B. cereus.* At these two timing, 48 h and 72 h, *B. CeM6-2 + B. cereus* (10:0 mL) strain showed the most powerful antimicrobial action compared to other concentrations ratio strains. The seven different concentration ratio strains *B. CeM6-2* cultured growing between 6.00 log10 CFU/g at 0 h. At 24 h, the five concentration ratio antimicrobial strains were developed similarly between 4.55 and 4.95 log10 CFU/g which is less than concentration ratio *B. CeM6-2 + B. cereus* (0:10 mL) and *B. CeM6-2 + B. cereus* (3:7 mL) strain in fermented soybean calculated to be 5.09 log10 CFU/g to 5.41 log10 CFU/g. At 48 h and 72 h striking in NGKG agar plate, two concentrations ratio strains *B. CeM6-2 + B. cereus* (10:0 mL) and *B. CeM6-2 + B. cereus* (9:1 mL) were counted at 2.71-3.63 log10 CFU/g and 3.05-3.37 log10 CFU/g that are higher suppression than the other

Table 3 Application of bacteriocin produced by *B. CeM6-2* strain to control the growth of *B. cereus* in fermented soybean by log CFU/g at different time and room temperature.

Number of mix of both *Bacillus* strains	0 h		24 h		48 h		72 h	
	CFU/g	log CFU/g	CFU/g	log CFU/g	CFU/g	log CFU/g	CFU/g	log CFU/g
B. CeM6-2 + *B. cereus* (10:0 mL)	1×10^6	6.00	3.52×10^4	4.55	5.17×10^2	2.71	1.13×10^3	3.05
B. CeM6-2 + *B. cereus* (0:10 mL)	1×10^6	6.00	2.59×10^5	5.41	3.08×10^5	5.49	4.93×10^5	5.69
B. CeM6-2 + *B. cereus* (9:1 mL)	1×10^6	6.00	5.48×10^4	4.74	4.23×10^3	3.63	2.35×10^3	3.37
B. CeM6-2 + *B. cereus* (1:9 mL)	1×10^6	6.00	8.95×10^4	4.95	3.93×10^5	5.59	2.57×10^5	5.41
B. CeM6-2 + *B. cereus* (5:5 mL)	1×10^6	6.00	7.85×10^4	4.89	3.93×10^5	5.59	4.39×10^5	5.64
B. CeM6-2 + *B. cereus* (7:3 mL)	1×10^6	6.00	5.69×10^4	4.76	3.35×10^5	5.52	1.44×10^5	5.16
B. CeM6-2 + *B. cereus* (3:7 mL)	1×10^6	6.00	1.24×10^5	5.09	4.17×10^5	5.62	4.46×10^5	5.65

five concentration ratio strains existing between 5.52-5.62 log10 CFU/g and 5.16-5.66 log10 CFU/g. Together with report of *B. subtilis HJ18-4*, which also exhibited strong antibacterial activity against *Bacillus cereus*, and also found that water extracts of soy product fermented with *B. subtilis HJ18-4* significantly inhibited the growth of *B. cereus* and toxin expression [15]. The bacteriocins produced by these strains are thought to be potent food preservatives that are applicable for Cambodian food.

4. Conclusion

Fermented foods are commonly found in Cambodia and in other Asia countries since they play a very important role for health, especially in developing countries. In the processing procedure, *SIENG* naturally contains various kinds of microorganisms including useful microorganism and pathogenic microorganism. So, the use of good starter culture like *B. subtilis* can ensure the safety and stability of the products.

In this work, we demonstrated the diversity of *B. cereus* in *SIENG*, a Cambodian's traditional fermented soybean food. Out of 120 *SIENG* samples, 49 samples of *B. cereus* strains were isolated and there were only 12 of *B. cereus* that gave positive sensitivity compared with control enterotoxin by lyophilization and then 2 (*BTM8-7* and *BTM8-8*) of these 12 isolated *Bacillus cereus* strain produced the highest level enterotoxin, which may cause diarrhea, vomiting and nausea a few hours after consumption of contaminated food.

One hundred and twenty (120) samples *SIENG* were found only 5 of *Bacillus* strains that have ability to fight against the indicator microorganisms *Lactobacillus plantarum ATCC 8014* by agar well diffusion assay and among 5 samples, only one strain (*Bacillus CeM6-2*) has shown a great active zone against *Lactobacillus plantarum. B. CeM6-2* strain could tolerate with heat up to 20 h at 30 °C temperature and 22 h at 37 °C. Besides, bacteriocin produced by *B. CeM6-2* untreated and *B. CeM6-2PK-PMSF* has more ability to suppress all the indicators starting from 0 h to 47 h compared with *B. CeM6-2PK* strain at a very significant level. Moreover, *Bacillus CeM6-2* strain performed very well when treated with Gram positive group, against *Listeria monocytogenes*, and *Streptococcus pyrogene* and lactic acid groups, against *Leuconostoc mesenterids* and *L. plantarum* at a very extensive level. *Bacillus CeM6-2* strain has higher strength unlike Japanese strains (*Miyagino*) from 0-58 h and 0-40 h incubation time.

In similar finding to the prior test of co-cultivate *Bacillus cereus* and *Bacillus subtilis* at 35 °C, 44 h *CeM6-2* (1%) strain had the highest ability to fight against *B. cereus* indicator strain beginning from 24 h incubation and it was best to suppress *B. cereus* indicator at 34 h to 44 h incubation as well. However, to check the quality of *SEING* produced by antimicrobial *Bacillus CeM6-2* strain, two concentrations ratio strains *B. CeM6-2* + *B. cereus* (10:0 mL) and *B. CeM6-2* + *B. cereus* (9:1 mL) have

the strongest power to fight against *B. cereus* indicator strain at both room temperature time (48 h and 72 h). In the meantime, *B. CeM6-2* strain produced the largest amount of bateriocin to inactivate *B. cereus* indicator at 24 h to 72 h incubation and room temperature as well. It means that, the longer incubation and room temperature time (in specific timing), the higher bacteriocin. Thus, this property of bacteriocins produced by *Bacillus CeM6-2* can be used as a preservative in food processing industries to avoid food spoilage even in higher temperature and time.

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