

Isolation, Characterization and Bio-Control Activities of *Bacillus Subtilis* from the Fermented Soybean in Cambodia

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Abstract: The objective of this study was to isolate *Bacillus subtilis* produced *Bacteriocin* from Khmer traditional fermented soybean to control the growth of pathogenic bacteria from 120 *SIENG* samples from 9 different local open markets in Phnom Penh, 1 local open market and 2 local producers in Kandal province in Cambodia. 119 out of 120 samples were positive with *Bacillus subtilis* strains. Fifteen of these isolated strains exhibited antimicrobial activity to *Lactobacillus plantarum* by agar well diffusion assay and one of them (*CeM6-7* strain) exhibited strongest antimicrobial activity. The filtered supernatant of this strain also suppressed the growth of several Gram-positive pathogens (such as *Staphylococcus aureus*) or lactic acid bacteria in broth. The filtrate could also suppress the growth of *L. plantarum* inoculated into soymilk. Antimicrobial *B. subtilis* strain *CeM6-7* is thought to be applicable for making a starter culture to produce much safe fermented soybean foods by suppressing the growth of naturally contaminated *B. cereus* or *S. aureus* during production. In addition, the use of this starter culture may contribute to prevent spoilage or quality loss of soybean products caused by the over growth lactic acid bacteria or other Gram-positive bacteria.

Key words: *B. subtilis*, fermented soybean, *bacteriocin*, food poisoning, spoilage bacterial.

1. Introduction

Fermented foods are commonly produced in Cambodia and other South-Eastern countries since they are cheap, high nutrient values and benefit for health. In Cambodia, soybean is categorized in the fourth main agricultural commodities list which is actively generated yearly income for rural farmers [1] and certain amount of soybean are used for fermented soybean production, commonly called *SIENG* in Cambodian which famous and healthy foods among other Cambodian's soybean processed products. There are 2 kinds of fermented *SIENG* in Cambodia, *SIENG PRAI* (salted Soybean), and *SIENG PA-AEM* (Soybean which made from salts and sugar). Not only

in Cambodia but also Japan, Korea, Taiwan, and China are also produced similar fermented soybean products [2].

Bacillus subtilis is a gram-positive bacterium in genus *Bacillus* which is naturally found in upper layer of the soil [3]. *Bacillus subtilis* may present on the soybean seed during cultivation or contaminate in *SIENG* during process according to Cambodia production context which *SIENG* is fermented using starter *Bacillus subtilis*, naturally on soybeans in other Asian countries [4]. Up to date research report that antibacterial substance produced by an isolated *Bacillus subtilis* strain LFB112 from Chinese herbs, was identified as *bacteriocins* and effective against both Gram-positive and Gram-negative bacteria involved in domestic animal diseases, including *Escherichia coli*, *Salmonella pullorum*, *Pseudomonas*

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aeruginosa, *Pasteurella multocida*, *Clostridium perfringens*, *Micrococcus luteus*, *Streptococcus bovis* and *Staphylococcus aureus* [5]. In recent years, considerable effort has been made to develop food applications for many different *bacteriocins* using *bacteriocinogenic* strains [6-9]. *Bacteriocins* produced by industrially important *Bacillus subtilis*, which have a history of safe use in food and industry [10]. The *bacteriocins* produced by these strains are thought to be potent food preservative that are applicable and useful for Cambodian food industry.

However, limited research has been done concerning with *bacteriocins* produced by *Bacillus subtilis* compared to LAB species as food preservatives. Hence, the objective of this study was to isolate, characterize and optimize the condition antimicrobial *Bacillus subtilis* strains from fermented soybeans to control Gram-positive pathogenic bacteria (*Bacillus cereus*, *Staphylococcus aureus*, etc.) or spoilage lactic acid bacteria in foods.

2. Material and Methods

2.1 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 hr. 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 was identified by gram staining and biochemical tests using catalase/OF test.

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

minor different 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* strain (used for *Natto* production) were used for negative control, respectively.

2.2 Characterize the Bacteriocin Produced by Isolated Strain

- **Incubator temperature and timing treatment:**

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

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 were 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, 22 and 24 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.

- **Soy milk treatment:**

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.

5 mL *L. plantum*, *Levconostoc mesentanides* Jen 6129, *Micrococcus luteus* Spi-flams IFo 13867 and *Staphylococcus aureus*. IFo 13276 were transferred to MRS and LB broth and incubated at 30 °C and 35 °C, overnight. After dilution all strains were inoculated shaker from 3, 6 and 9 h at 35 °C into the mixture (1: 1 volume) of soymilk and filtered culture supernatant of *B. subtilis* or *Miyagino* strains.

L. plantum, *Lev. mesentanides*, *M. luteus* and *S. aureus* plates, the MRS broth contained agar purified powder and TSA agar, which aimed at assess viable bacteria count over time used by spiral plate systems, are kept in incubator at 35 °C for 24 hrs. Growth of inoculated all indicator strains in this soymilk was measured by plate counting method and calculated the number of CFU/g or mL of test sample.

Eventually, to evaluate whether all incubated plates are appropriate for the organism sought, we compared by using inoculating culture supernatant against indicator organism. For this purpose, four kinds of bacterial inoculate were prepared in two groups such as MRS broth and LB broth contains 5 mL *L. plantum*, *Lev. mesentanides*, *M. luteus* and *S. aureus*, incubated at 30 °C and 35 °C. Two groups of bacterial inoculate were inoculated into the mixture (1: 1 volume) of MRS broth. After that make wells, and filtered culture supernatant of *B. subtilis* or *Miyagino* strain and incubated 24 h, at 30 °C. Growth of inoculated *L. plantarum* in this MRS broth was measured and observed length of wells by arbitrary unit/mL (AU/mL) was calculated [11].

2.3 Optimize the Bacteriocin Production of the *Bacillus Subtilis* Strain by Cultivating in Different Media and Under Different Growth Condition

The optimize bacteriocin production of the *B. subtilis* strain by cultivating in different media and under different growth condition were determined by using the spore culture and well diffusion assay. *B. subtilis* and *Miyagino* strain are for control. Each strain was separately inoculated into a 5 mL of LB

broth and placed in the incubator shaker at 37 °C, 24-72 h, heated and cool down, and then store at -20 °C, 24 h.

Next step, spore culture from two strains was series dilution (10^2 , 10^4 , and 10^6) and then spread on TSA agar plats, incubated at 35 °C for 24 h. Growth of inoculated all indicator strains in this TSA was measured by plate counting method and calculated the number of CFU/g or mL of test sample. Following counting colonies, 10^4 dilutions were used spore culture into 30 mL LB broth in flasks incubator shaker at 30 °C for 16, 20 and 23 h.

Four kinds of bacterial inoculate were prepared in two groups such as MRS broth and LB broth contains 5 mL *L. plantum*, *Lev. mesentanides*, *M. luteus* and *S. aureus*, incubated at 30 °C and 35 °C. Two groups of bacterial inoculate were inoculated into the mixture (1: 1 volume) of MRS broth contained supplement agar and kept it till become solid. After that make wells, and filtered culture supernatant of *B. subtilis* and *Miyagino* strains and incubated 24 h at 30 °C. Growth of inoculated *L. plantarum* in this MRS broth was measured and observed length of wells by arbitrary unit/mL (AU/mL) [11].

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 were inoculated into a 20 mL of LB broth centrifuge tubes and placed on the incubator shaker at 35 °C, 16 h. Gram positive group (*S. aureus*, *Listeria*, and *Lactbacillus*) and Lactic acid group (*L. plantum*, *L. brevis*, and *Lb. lactis*) were inoculated separately to BHI and MRS in incubator shaker at 35 °C for 18 to 24 h.

Filtered culture supernatant of antimicrobial *B.*

subtilis or *Miyagino* strains was mixed with same volume of MRS or BHI broth for lactic acid bacteria (*L. plantum*, *L. brevis*, and *Lb. lactis*) 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 after shaker measure turbidness at OD 650 nm and dilute until OD 0.1 by PBS all of strains.

2.5 Data Processing and Analysis

The results were analyzed by Excel, AU, ANOVA 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 Isolating *Bacillus* Strains from Cambodian Fermented Soybean (SIENG) Samples

120 raw *SIENG* were isolated for *Bacillus* strains. As the result, only a total of 119 samples of *Bacillus* strains were isolated from 120 raw *SIENG*. *Bacillus subtilis* strains are important for seed fermentation because of their enzymatic activities contributing to desirable texture, flavor and pH development [12]. In general, *Bacillus Subtilis* is the most dominant bacterium in fermented soybean [13]. And it was resistant to heat which showed growth in 70 g·dm⁻³ NaCl, nitrate reductase positive reaction [2]. These isolated among 119 *Bacillus* strains, there were found only 15 *Bacillus* strains that had ability to fight against the indicator microorganisms. Furthermore, among 15 samples, only one stain (*Bacillus CeM6-7*) has shown highest level antimicrobial compound of *L. plantum* at 12% of 15.00 mm/mL 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. Even though microbial control exerted by these metabolites was demonstrated in plant environments,

few reports focused their attention on how these compounds can interact with food microbiota [14]. The rank based on per mm/mL and percentage total area of level antimicrobial compound of *L. plantum* showed the trend of all type *Bacillus* strains.

3.2 Identification from Isolated Bacteriocin Producing by *Bacillus Subtilis* Strains

From the result of previous step, isolating *Bacillus* strains, the 15 colonies of *Bacillus* activities strains were developed to have another four additionally. We therefore obtained 19 purified of *Bacillus* activities strains finally and they were used in this step.

3.2.1 Biochemical and Morphological of *Bacillus* Strains

Bacillus is a genus of Gram-positive, rod-shaped bacteria and a member of the phylum *Firmicutes* [15]. 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 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 growth was between 30-37 °C [16], but, a well growth condition is 35 °C.

The result revealed that in aerobe production *Bacillus* strains were growth all strains. In opposite, in anaerobe production, there were only four *Bacillus* strains grown and had taken a very longer time for growing but stay for only a short life compared to aerobe production because of *Bacillus* species including *B. subtilis* can be obligated in both condition aerobes (mostly) and anaerobes (rarely and stay in short life) according to the existing publications [15]. Nonetheless, according to another publication [2], a good condition that *Bacillus* strain can growth well is in aerobes production and its typical characteristics of the *B. subtilis* is 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 and all positive in Manito Salt, VP and Catalase test, including *B. subtilis*, and they were catalase-positive reaction, mannitol, and maltose [2].

3.2.2 Subject to Assay of *Bacillus Subtilis* Strains

Selected 19 strains of *B. subtilis* 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 *B. subtilis* strain classifications. As an overview on the result, *B. subtilis* strains had antimicrobial activity against indicator organisms of *L. plantum* bacteria and they mostly had activity at 35 °C much more than at 30 °C. Further, the *B. subtilis* strains most likely had antimicrobial activity when using un-filtration method. As detail in the result, 6 strains named *B. BTM8-9*, *B. TaM2-4(1)*, *B. BTM8-10*, *B. TSM5-10*, *B. TSM5-3*, *B. CKM9-2* did not show any antimicrobial activity, meanwhile, other 4 strains had total zone activities between 6-11 mm/mL, including *B. BTM8-4(2)*, *B. TaM2-4(2)*, *B. TSM5-8(1)*, *B. TSM5-7*, shown less antimicrobial activity, and other 9 remaining between 12-64 mm/mL, *B. CeM6-7*, *B. BTM8-4(1)*, *BTM8-1*, *CKM9-1*, *DM1-14(2)*, *DM1-14(1)*, *CKM9-7* and *DM1-17* strains have strong activities against indicator organisms in both temperature condition, and both methods (filtration, and without filtration). These 9

Bacillus strains that had strong activities are predicted to be able to suppress the growth of *L. plantum* bacteria, and they were the samples (culture supernatant) in MRS broth with supplement 1% agar mixed. Finally, only 9 *Bacillus* strains that had strong activity were selected for the next experiment.

Table 1 exposed that in average size, *Bacillus CeM6-7* strain had the largest zone activities presented for 10.67 mm/mL *L. planum* bacteria and the *Bacillus BTM8-4(1)*, *TSM5-8(2)*, *CKM9-1*, *DM1-14(2)*, *DM1-14(1)*, *CKM9-7* and *DM1-17* strains had the average zone activities 6.00, 5.83, 4.17, 3.67, 2.83, 2.67, 2.50 and 2.00 mm/mL, correspondingly.

3.2.3 Bacteriocin Producing *Bacillus Subtilis* Strain

From the previous step, subject to assay of *Bacillus* strains, one of *Bacillus* activity strain namely *CKM9-7(1)* was developed to have one additional strain. We finally obtained purified 10 of *Bacillus* activities strains.

After receiving high bacteriocin producing *Bacillus* strains, those 10 strains were gone for further test to isolate the strongest *Bacillus subtilis* strain which could produce bacteriocin the best in comparing with other strains. The total diameter of zone activity (mm/mL) for *Lactobacillus planum* and API50 CH were 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

Table 1 Average zone activity (mm/ml) for *lactobacillus planum* by type of *bacillus* strain.

S.N	Type of <i>Bacillus</i> strains	Incubator time (h)	Incubator temperature	Incubator time (hrs)	Incubator temperature	N ⁰ respondents of <i>B. activates</i>	Diameter of zone activity (mm/ml) for <i>Lactobacillus planum</i> bacteria			
							Min	Max	Total	Average
1	<i>CeM6-7</i>	17	35 °C	24	30 °C	6	5	15	64	10.67
2	<i>BTM8-4(1)</i>	17	35 °C	24	30 °C	4	5	10	36	6.00
3	<i>BTM8-1</i>	17	35 °C	24	30 °C	5	5	10	35	5.83
4	<i>TSM5-8(2)</i>	17	35 °C	24	30 °C	4	5	8	25	4.17
5	<i>CKM9-1</i>	17	35 °C	24	30 °C	3	6	9	22	3.67
6	<i>DM1-14(2)</i>	17	35 °C	24	30 °C	3	3	7	17	2.83
7	<i>DM1-14(1)</i>	17	35 °C	24	30 °C	2	0	8	16	2.67
8	<i>CKM9-7</i>	17	35 °C	24	30 °C	3	0	8	15	2.50
9	<i>DM1-17</i>	17	35 °C	24	30 °C	3	3	9	12	2.00
Total		-	-	-	-	33	32	242	391	97.75

analysis of all types of *Bacillus* are presented in Table 2.

Table 2 revealed that mean of *CeM6-7* strain was 8.50 mm/mL, 71%, which was the highest proportion of total area of zone activity for *Lactobacillus planum* likened to other strains that we were measured and the following order of *Bacillus* strain was *DM1-14(2)*, *DM1-14(1)*, *CKM9-7(2)*, *CKM9-7(1)*, *BTM8-1*, *BTM8-4(1)*, *TSM5-8(2)*, *CKM9-1* and *DM1-17* strains respectively. The rank and selection of *B. subtilis* strain were based on its size (mm/mL) and percentage of zone activity that shown the trend for all type *B. subtilis* strains.

Accordingly, only one strain *CeM6-7* which had very strong zone activities was selected for further test since it was able to suppress the growth of *L. plantum* bacteria which it was in parallel with existing researches stated that, some *B. Subtilis* have reported to produce *bacteriocins* which suppress the growth of Gram positive spoilage and pathogenic bacteria [17, 18]. In addition, we also found that antimicrobial activity against indicator organism of *CeM6-7* was more actively at 35 °C compared to 30 °C. There are also some strains such as *DM1-14(1)*, *DM1-14(2)*, and *CKM9-7(2)* which shown antimicrobial activity

against indicator organism. However, these strains are half-strong compared to *B. CeM6-7* strain. The strains *CeM6-7* was identified as *B. subtilis* by 96.2% and a T-value (0.76) homology correspondingly counted on API 50 CH V4.1 (BioMérieux) profiles (not shown) and their physiological characteristics.

3.3 Characterize the Bacteriocin Produced by Isolated Strain

The result obtained from our test shown *B. CeM6-7* strain had ability resistant to heat. Similarly, *B. subtilis* strains may produce other antimicrobial substance, which have been characterized to a much lesser extent [15].

3.3.1 Incubator Temperature and Timing

In this test, we used two variables of temperatures (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 *L. plantum (LP)* bacteria was used as the indicator strain. The test was conducted for 2 days.

As seen in Fig. 1, in the first day of test, the antimicrobial activities of bacteriocin were activated at 12 h (10 mm/mL) at 37 °C only, beside there was no antimicrobial activities at all. The antimicrobial

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

S.N	Type of Bacillus strains	Incubator time (hrs)	Incubator temperature (°C)	Total N ⁰ zone activity (mm/mL) at 30 °C	Incubator temperature (°C)	Total N ⁰ zone activity (mm/mL) at 30 °C	Total N ⁰ respondents of B. activates	Diameter of zone activity (mm/mL) for <i>Lactobacillus planum</i>		
								Total	Mean	Percentage
1	<i>CeM6-7</i>	overnight	35	28	30	6	4	34	8.50	71
2	<i>DM1-14(2)</i>			ND		ND	2	5	1.25	10
3	<i>DM1-14(1)</i>			4		ND	2	5	1.25	10
4	<i>CKM9-7(2)</i>			ND		ND	2	4	1.00	9
5	<i>CKM9-7(1)</i>			ND		ND	ND	ND	ND	ND
6	<i>BTM8-1</i>			ND		ND	ND	ND	ND	ND
7	<i>BTM8-4(1)</i>			ND		ND	ND	ND	ND	ND
8	<i>TSM5-8(2)</i>			3		2	ND	ND	ND	ND
9	<i>CKM9-1</i>			3		2	ND	ND	ND	ND
10	<i>DM1-17</i>			ND		ND	ND	ND	ND	ND
Total		-	-	38	-	10	10	48	-	100.00

Note: Not detected (ND).

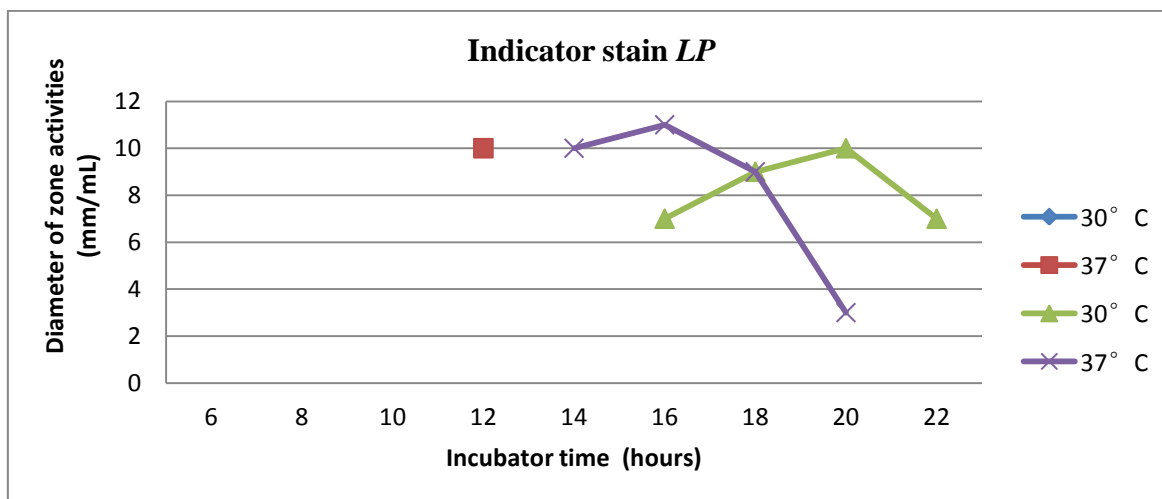


Fig. 1 Effect of difference incubator temperature and timing on the activity of bacteriocin produced by *B. subtilis* CeM6-7 strain.

activities were actively happened in the second day of test. In 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 decrease after 20 h of incubation at 30 °C. At 37 °C, the *bacteriocin* started increasing its antimicrobial activities after 14 h of incubation which was faster compared with 30 °C of incubation temperature. Though, its antimicrobial activity was kept increasing slightly for just only two hours, and started decreasing sharply for the next four hours until became inactivated after 20 h of incubation at 37 °C. It demonstrated that bacteriocin produced by CeM6-7 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 [8]. Additional finding, at 37 °C, *Bacillus* CeM6-7 strain could tolerate with heat up to 16 h, which was higher toleration than at 30 °C, and it still had ability to fight against indicator strain LP.

3.3.2 Soybean Milk Treatment

In this test, we used the enzyme contained in soybean milk as the treatment, and used four types of indicators including *L. plantum* (LP), *Lev.*

mesentanides (LM), *S. aureus*. (SA), and *M. luceus* *Spi-flams* (ML). We also used other five of bacteriocin producing by *Bacillus subtil* strains including, *B. CeM6-7*, and Miyagino (control strain) for comparing the result.

A. Indicator Strain LP

In Fig. 2, presented the characteristics of antimicrobial activity against LP by *B. subtilis* stains such as *B. CeM6-7* (isolated from traditional fermented soybean (SEING)), and *Miyagino* after cultured in *Bacillus-Soybean milk* broth contained against LP (dilution of 103 times). The result shown that the stain *B. CeM6-7* produced the strongest antimicrobial activity against *Bacillus-Soybean milk* broth contained LP compared with *Miyagino*, but the other remaining strains *B. CeM6-7* and *Miyagino*, were found to be no antimicrobial activity at all. At 0 h, all strains were counted to be 3.33 log₁₀ CFU/mL equally. At 3 h and 6 h, the *B. CeM6-7* was calculated to be 2.643 log₁₀ CFU/mL to 3.204 log₁₀ CFU/mL and at 9 h was calculated 2.623 log₁₀ CFU/mL respectively, which in comparison were less than *Miyagino* that presented at 3.794 log₁₀ CFU/mL to 4.549 log₁₀ CFU/mL and 4.853 log₁₀ CFU/mL at 9 h correspondingly.

B. Indicator Strain LM

In Fig. 3 introduced the characteristics of antimicrobial activity of *Bacillus subtilis* CeM6-7 strains,

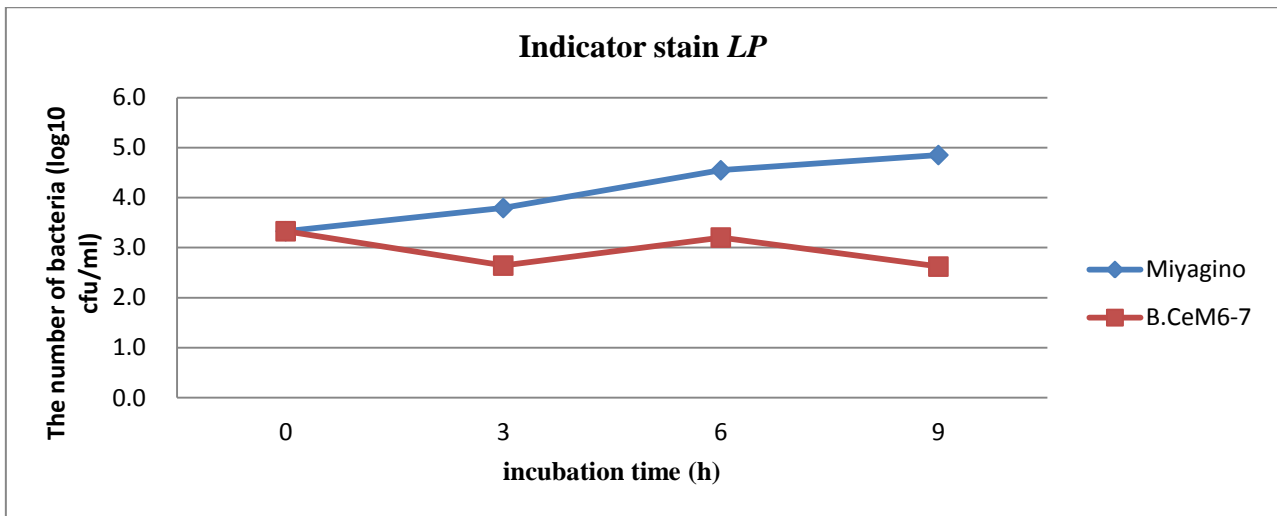


Fig. 2 Enzymes of soybean milk resistant for strain *bacillus CeM6-7* compare with Miyagino strains against indicator *LP*.

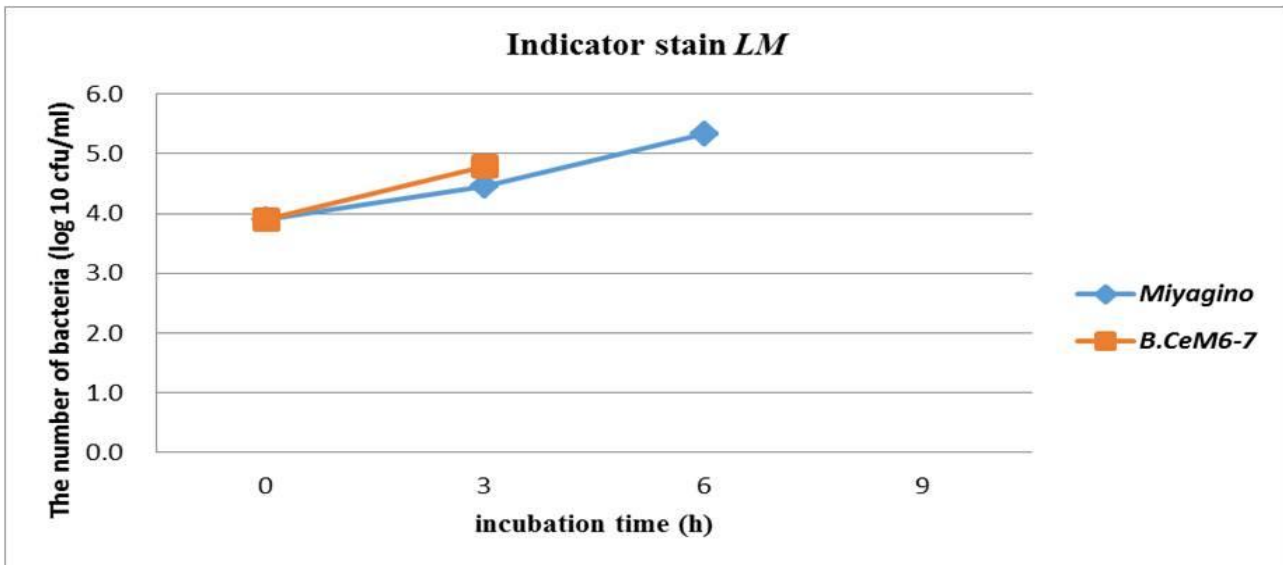


Fig. 3 Enzymes of soybean milk resistant for strain *bacillus CeM6-7* compare with Miyagino strains against indicator *LM*.

and *Miyagino* against *LM* indicator strain after cultured in *Bacillus-Soybean milk* broth contained against *LM* (dilution of 103 times). As the result, the *B. Ce6-7* stains no powerful antimicrobial activity with against *LM* at 3 h to 9 h incubation timing, since it was counted to be 4.795 log₁₀ CFU/mL to MPN, which were less than *Miyagino* (4.461 log₁₀ CFU/mL to 5.336 log₁₀ CFU/mL) at 3 h to 6 h.

C. Indicator Strain *SA*

The characteristics of antimicrobial activity against *SA* of five strains *B. subtilis* and *Miyagino* after cultured in *Bacillus-Soybean milk* broth contained

against *SA* (dilution of 103 times) were discussed in Fig. 4.

The figure indicated that at 3 h to 6 h *B. CeM6-7* strains had antimicrobial activity able to inactivate *Bacillus-Soybean milk* broth contained against *SA*. At these two incubation timing, 3 h and 6 h, *B. CeM6-7* strain showed the most powerful antimicrobial action compared to *Miyagino* strains. At 9 h, there was no antimicrobial activity shown. The *B. CeM6-7* and *Miyagino* strains cultured growing between 3.785 log₁₀ CFU/mL at 0 h. At 3 h, the antimicrobial produced by *B. CeM6-7* strains were developed between

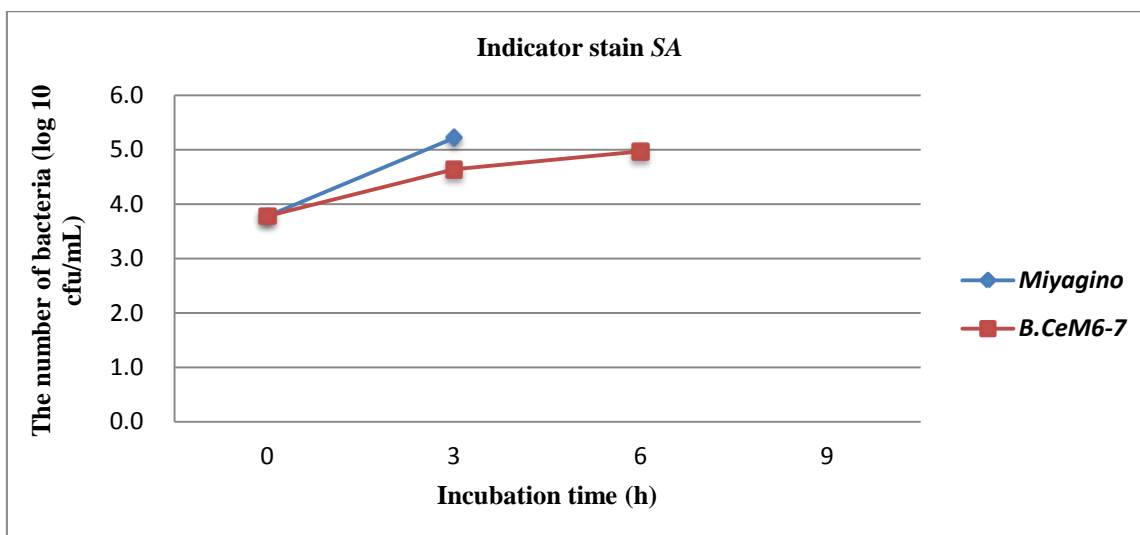


Fig. 4 Enzymes of soybean milk resistant for *bacillus CeM6-7* strain compare with other strains against indicator SA.

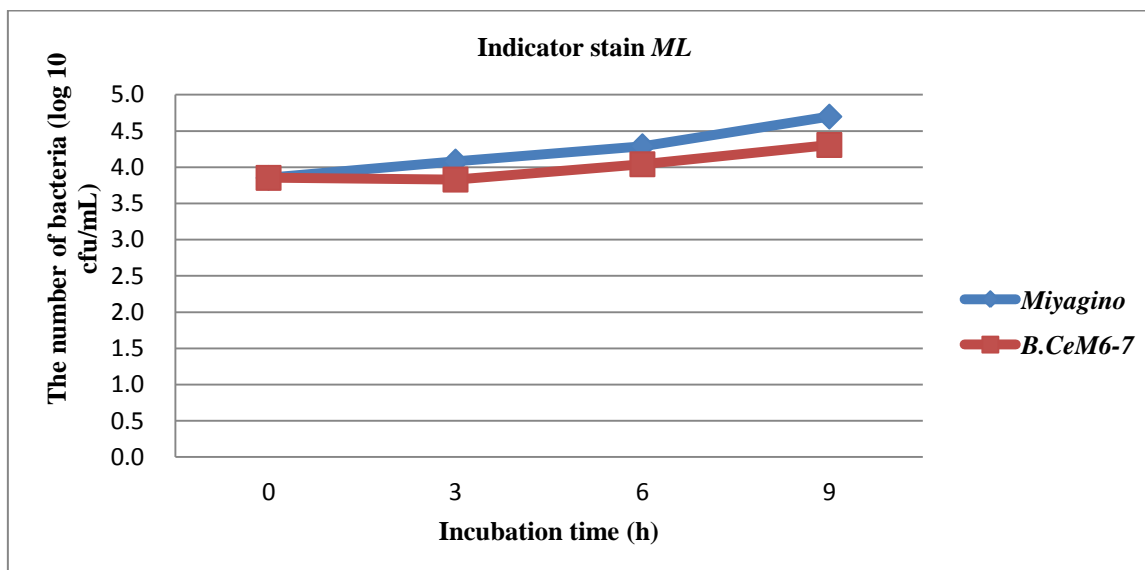


Fig. 5 Enzymes of soybean milk resistant for *bacillus CeM6-7* strain compare with Miyagino strains against indicator ML.

4.641 and 4.641 log₁₀ CFU/mL which less than Miyagino strain was calculated to be 5.220 log₁₀ CFU/mL. At 6 h inoculation, Miyagino strain was counted at MPN that higher than the *B. CeM6-7* strains existed 4.973 log₁₀ CFU/mL.

D. Indicator Strain ML

In Fig. 5, the characteristics of antimicrobial activity against ML of *B. CeM6-7* and *Miyagino* strains after culturing in *Bacillus-Soybean milk* broth contained against ML (dilution of 10³ times) were presented. It was indicated that, at different timing (3 h, 6 h, and 9 h), the *B. CeM6-7* strain had enough

ability to fight against *Bacillus-Soybean milk* broth contained ML compared to strain Miyagino, but it's more likely be less action. The *B. CeM6-7* strain cultured and grew the same amount at 3.854 log₁₀ CFU/mL at 0 h inoculation and it was increased 3.830-4.407 log₁₀ CFU/mL at 3 h to 9 h incubation time. The amount of *B. CeM6-7* strain cultured and grew in each timing, 3 h, 6 h and 9 h were automatically smaller than Miyagino strain that was 4.079, 4.290, and 4.699 log₁₀ CFU/mL respectively.

In short, the *B. CeM6-7* strain performed very well when treated with enzyme, especially for

Bacillus-Soybean milk broth contained against *SA*, while they were the most powerful to fight against *Bacillus-Soybean milk* broth contained against *LP*, but the antimicrobial activity of *B. CeM6-7* strain against *ML* indicator were found to be in less power (not much different), although they were significant different from *Miyagino* strain.

3.3.3 Evaluation of Characteristic of the *Bacteriocin* Producing by *Bacillus Subtilis*

Table 3 revealed that *B. CeM6-7* strains had powerful antimicrobial activities zone which presented for 375 AU/mL with against *SA*, but they were very higher activities zone than indicator against *PL* and *ML* and *LM*, which *B. CeM6-7* strain that was existed 275 AU/mL and 75 AU/ml and for *LM* indication, *B. CeM6-7* strain shown none bio-control activity, correspondingly. Meanwhile, in *SA* indication *B. CeM6-7* was most powerful to fight against indicator. Nonetheless, other remaining strains did not show any action with indicator against *LM*. Eventually, in *ML* indicator, *B. CeM6-7* had a weak positive antimicrobial activity against the indicator strain comparing of the *Miyagino* strain (not detected). Therefore, *B. CeM6-7* had its powerful antimicrobial activities against uniquely *SA* indicator strain at a very significant level.

On the other hand, the existed results also show the complexity of microbiological flora in the products

which was reflected wisely biological diversity of the natural environments used in the fermentation process, suggesting the potential value of traditional fermented foods as natural resources of bacteria and enzymes for industrial application. Likewise, Cambodian fermented soybean (*SIENG*) is similar to Thua nao in Thailand that make from fermented soybean. In fact, strains in the Thua nao (*Bacillus strains*) produced in northern Thailand exhibited complex microflora and molecular diversity [2].

3.4 Optimize the Condition of the *Bacteriocin* Production

The optimization the condition of the *bacteriocin* production was conducted to be fully understand about the whole process of antimicrobial activity against four indicator strains (*LP*, *LM*, *SA* and *ML*) of all our five tested strains comparing with *Miyagino* strain after culturing in MRS broth with 1% agar purified powder contained against *LP* and *LM*; and TSA agar containing against *SA* and *ML* (dilution of 104 times). All five tested strains and *Miyagino* strain were diluted in three differences times (102, 104, and 106) and counted spore culture prior to start experiment.

3.4.1 Counting the Colony of Two *B. Subtilis*

Table 4 indicated that there was non-significant different between the colony of spore culture of *B.*

Table 3 Evaluation of characteristic of isolated *bacteriocin* activity by using *bacteriocin* bioassay by AU (arbitrary unit).

Type of <i>Bacillus</i> strains	Incubator temperature	Incubator time	Diameter of the inhibition-zone for <i>LP</i>		Diameter of the inhibition-zone for <i>LM</i>		Diameter of the inhibition-zone for <i>SA</i>		Diameter of the inhibition-zone for <i>ML</i>	
			mm/mL	AU/mL	mm/mL	AU/mL	mm/mL	AU/mL	mm/mL	AU/mL
Miyagino	35 °C	Overnight	ND	ND	ND	ND	ND	ND	ND	ND
<i>B.CeM6-7</i>			11.00	275.00	0.00	0.00	15.00	375.00	3.00	75.00

Note: Not detected (ND).

Table 4 Comparison of average colony counts obtained from Petri films between 2 kinds of *Bacillus subtilis* using one way ANOVA.

Type of <i>bacillus</i> strains	Parameters of <i>bacillus subtilis</i>					
	Dilution of 10 ² times (log10 CFU/mL)	Dilution of 10 ⁴ times (log10 CFU/mL)	Dilution of 10 ⁶ times (log10 CFU/mL)	n	\bar{X}	S ±
Miyagino	5.556	5.292	5.230	3	5.359	0.173
<i>B.CeM6-7</i>	5.497	4.870	4.630	3	4.999	0.447
F-value						1.694
Sig						0.263

CeM6-7 and *Miyagino* strain (Sig = 0.263) in the three different timing of dilution (102, 104, and 106) on its *bacillus subtilis* growth.

3.4.2 Production of *Bacteriocin* at Different Incubation Timing

Incubation time and temperature play a vital role in bacteriocin production. Therefore, the modification test was conducted using LB broth and incubation at 30 °C with different timing (0 h, 16 h, 20 h and 23 h) and diluted 104 times only. Within the expectation, *Bacillus subtilis* is capable to produce bacteriocin to fight against four indicator strains finally. The details are presented in Fig. 6. The results were shown the antimicrobial production was begun at 16 h incubation with *B. CeM6-7* strain that was able to fight against MRS broth contained *LP* indicator strain. The *bacteriocin* production of *B. CeM6-7* strains, were very active to kill all four indicator strains at 20 h counted between 175-325 AU/mL. At 23 h incubation, the antimicrobial production of tested strains had the most power to kill four indicator strains and its *bacteriocin* productive volume was listed respectively from strong to low, *B. CeM6-7*, and calculated between 163-275 AU/mL. Controlled strain, *Miyagino*, was found no action in both MRS broths and TSA

agar containing *LP*, *LM*, *SA* and *ML* against indicator.

In similar finding to the prior test, at 30 °C, *B. CeM6-7* stain had highest ability to fight again *LP* indicator strain beginning from 20 h incubation to 23h incubation and it was the best to kill *LM* and *ML* indicator at 20 h incubation as well. However, *B. CeM6-7* stain had a similar powerful to fight against *SA* and *ML* indicator strain at the incubated time (23 h). At the meantime, *B. CeM6-7* stain produced the largest amount of *bacteriocin* to be inactivating *SA* and *ML* indicator at 23 h incubation as well. We therefore found that, the productivity of antimicrobial production of tested strains including *B. CeM6-7*, seem to be in parallel direction with incubation timing. It means that, the longer incubation time (in specific incubation timing) produce the higher *bacteriocin*.

3.5 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 *S. aureus*, *Listeria* and *L. brevis*, and *Lactic acid* group (*L. plantum*, *Lb. lactis*

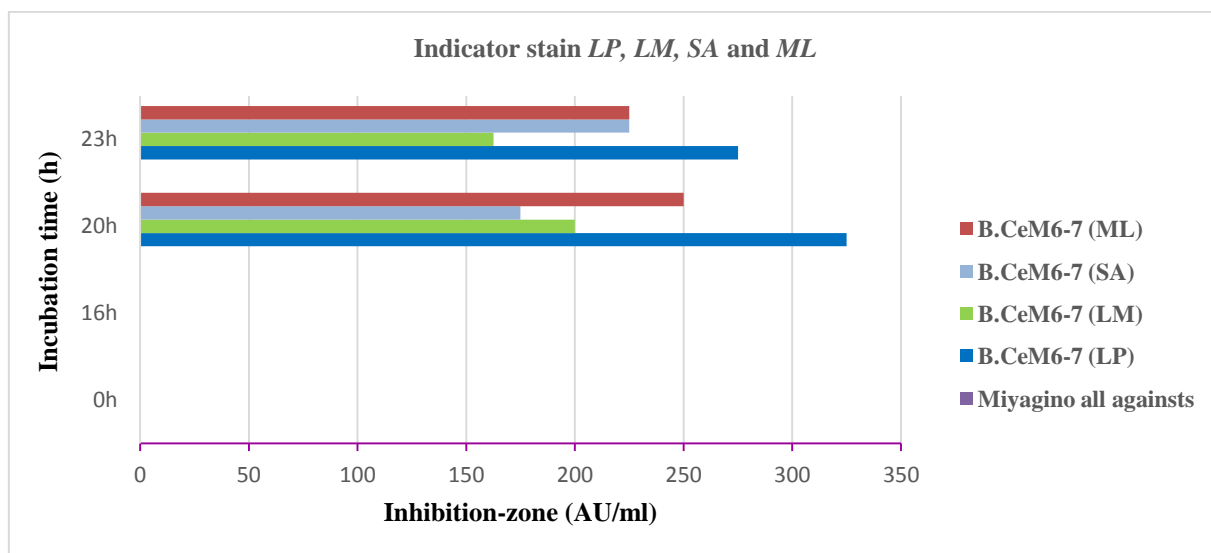


Fig. 6 Production of *bacteriocin* at different incubation time for *Bacillus CeM6-7* strain compare with *Miyagino* strains non-producing *bacteriocin* against different indicators.

and *Lactococcus lactis subsp. lactis* (produced Nisin A)) contained *B. CeM6-7* comparing with *Miyagino* strain after culturing in HBI contained against Gram positive; and MRS containing against *Lactic acid* group (O.D = 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, was effective against both Gram-positive and Gram-negative bacteria involved in domestic animal diseases [14].

3.5.1 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. CeM6-7* and *Miyagino* 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.

A. Indicator strain *Staphylococcus aureus*

As shown in Fig. 7, presented the control bacteriocin activity against *S. aureus* by *B. CeM6-7* (reference strains producing bacteriocin) and

Miyagino after cultured in HBI broth contained against *S. aureus* (O.D = 0.1, 650 nm) at 35 °C for 30 h. At 0 h, *B. CeM6-7* was calculated to be 0.06 O.D. higher than *Miyagino* (0.05 O.D). Until 160 min (2: 40 h) and 1,800 min (30 h), *B. CeM6-7* was calculated to be 0.27 and 0.62 O.D respectively, which were less than *Miyagino* that presented at 0.48 and 0.99 O.D correspondingly. In general, from 160 mn (2: 40 h) to 1,800mn (30 h), the result shown that the stain *B. CeM6-7* produced the strongest antimicrobial activity against HBI broth contained *S. aureus* in compared with *Miyagino* strain.

B. Indicator strain *Listeria*

The control of antimicrobial activity against *Listeria* of two strains *B. subtilis* and *Miyagino* after cultured in BHI broth contained against *Listeria* O.D = 0.1, 650 nm) at 35 °C for 30 h, were discussed in Fig. 8. The figure indicated that at 0 min (0 h), both strains did not have antimicrobial activity and their O.D was equally 0.03. Nevertheless, at 185 min (3 h), the strains *B. CeM6-7* was able to inactivate BHI broth contained against *Listeria* and it was developed to 0.13 O.D less than *Miyagino* strain at 0.15 O.D. At 1,150 min (19 h) and 1,800 min (30 h), the *B. CeM6-7* was calculated 0.07 O.D and 0.03 O.D respectively, which were less than *Miyagino* that presented for 0.09

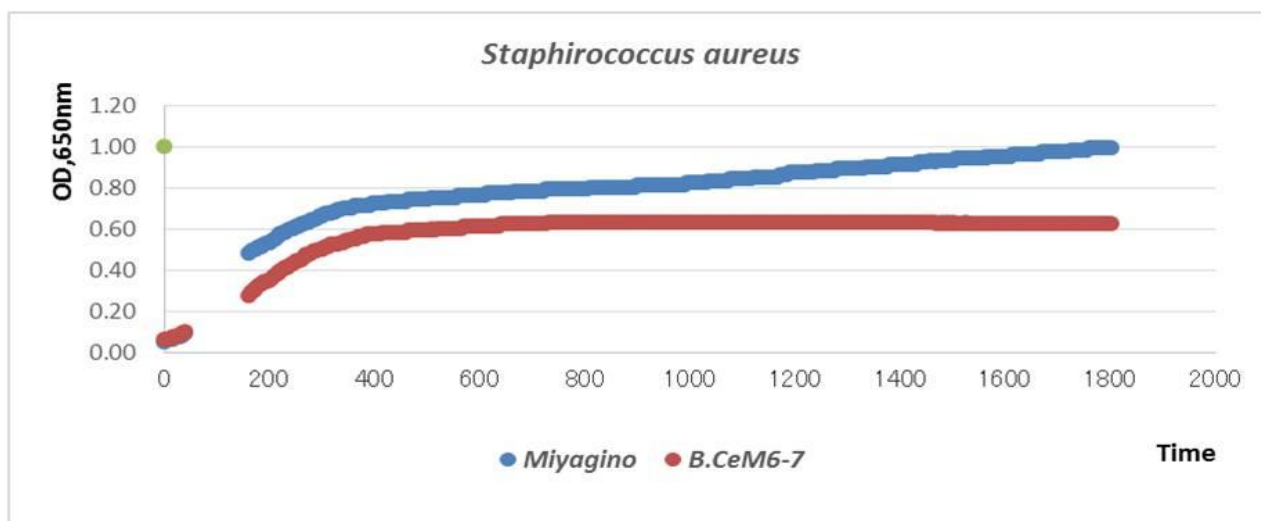


Fig. 7 Effect of bacteriocin produced by *B. CeM6-7* strain on the growth of *S. aureus* compare to *Miyagino* at different time in BHI broth at 35 °C for 30 h.

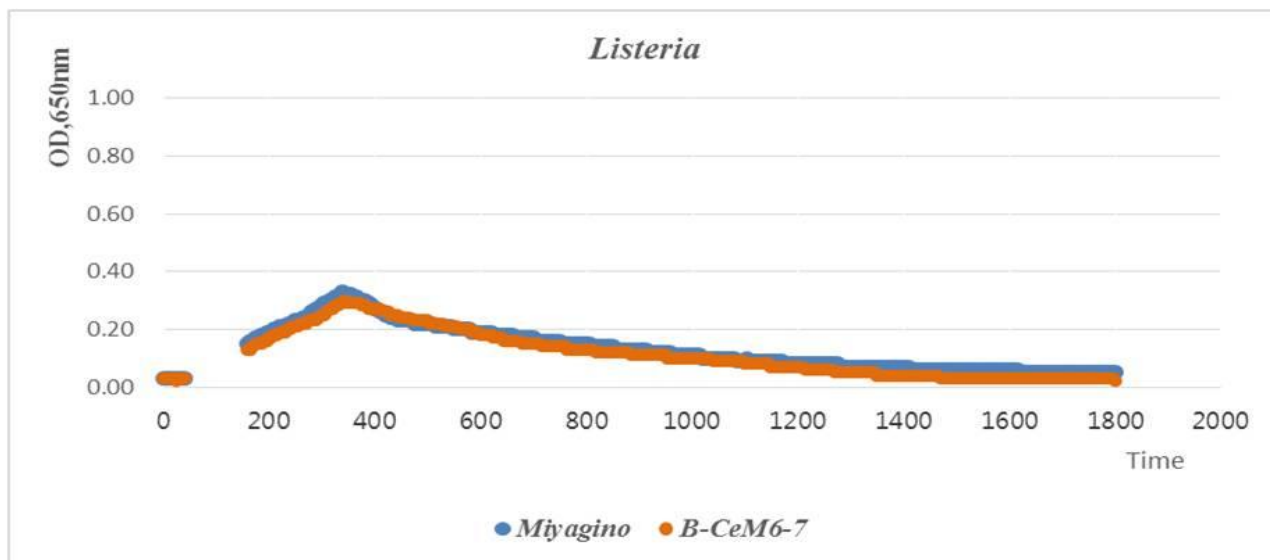


Fig. 8 Effect of bacteriocin produced by *B. CeM6-7* strain on the growth of *Listeria* compare with Miyagino at different time in BHI broth at 35 °C for 30 h.

to 0.05 O.D correspondingly. In short, from 160 min (2: 40 h) to 1,800 min (30 h), *B. CeM6-7* strain shown the most powerful antimicrobial action compare to Miyagino (Fig. 8).

C. Indicator Strain *L. brevis*

In commonly, the bacteriocin produced by *Bacillus strains* against were stable within wide range of pH values from 3.923 to 4.058, and non-significant different between the pH values. The effects of heat, pH, nutritional composition and incubation time at 35 °C and the antibacterial substances were stable within OD, 650 nm (0), the *B. CeM6-7* against. There were exceptions in the case of the *Miyagino* strain. This strain did not inhibit *L. brevis* at a temperature of 35 °C, 25 h and it was using as control strain.

In Fig. 9 indicated that at 0 min (0 h) to 60 min (1 h) all of strains, did not had antimicrobial activity. Nevertheless, at 60 min (1 h) to 800 min (13: 33 h), *B. CeM6-7* cultured growing between 0.03 to 0.91 O.D higher than *Miyagino* 0.03 to 0.88 O.D. After that, *CeM6-7* strain was powerless in antibacterial against *L. brevis* at 800 min (13: 33 h) to 1,500 min (25 h) the antimicrobial activity was almost the same with *Miyagino* (0.91-0.90 O.D) compared to (0.87-0.90 O.D). Consequently, *B. CeM6-7* did not inhibit *L. brevis* at a temperature of 35 °C, 25 h.

In short, the *Bacillus CeM6-7* strain performed very well when treated with Gram positive group, especially for HBI broth contained against *S. aureus* was the most powerful to fight against MRS broth, but the antimicrobial activity of *B. subtilis* strains against *Listeria* indicator were found to be no power to suppress and against *L. brevis* indicator was impossible, although it was significant different from *Miyagino* strain, which it was in parallel with existing researches stated that, some *Bacillus Subtilis* have reported to produce bacteriocins which suppress the growth of Gram positive spoilage and pathogenic bacteria [17, 18].

3.5.2 Lactic Acid Groups

In this test, we used two variables of temperature 35 °C and incubator timing 25 h, and the three kinds of antibacterial produced by *B. subtilis CeM6-7* of the tested strains comparing with *Miyagino* strain after culturing in MRS containing against Lactic acid group (*L. plantum*, *Lb. lactis* and *Lactococcus lactis subsp. lactis* (produced Nisin A) with O.D = 0.1, 650 nm), bacteria was used as the indicator strain. However, the test was conducted to measure pH value of each indicators of bacteriocin produced by *B. subtilis* strains culture prior to start experiment and the effects of heat, pH, nutritional composition and incubation

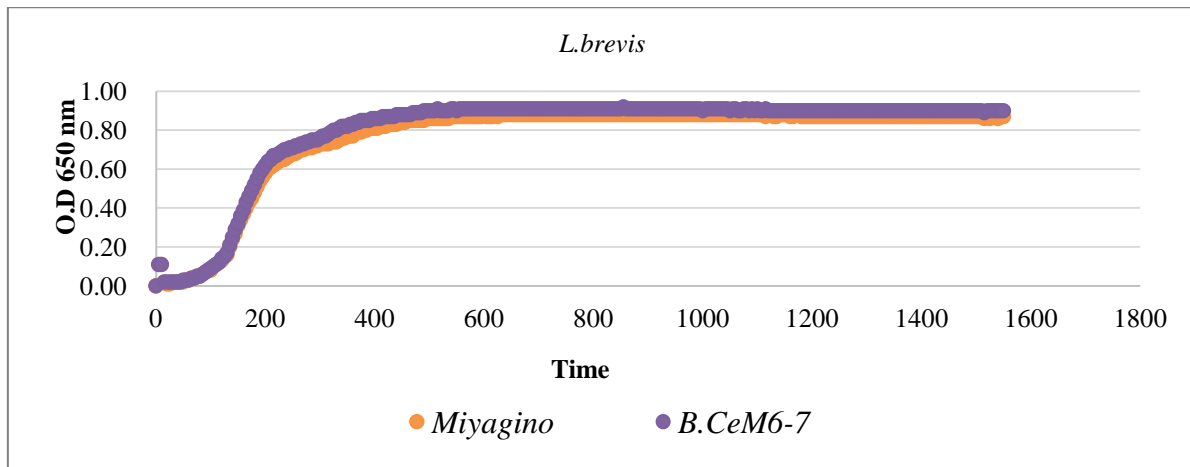


Fig. 9 Effect of bacteriocin produced by *B. CeM6-7* strain on the growth of *L. brevis* compare between Miyagino at different time in MRS broth at 35 °C for 25 h.

time at 35 °C and the antibacterial substances were stable within O.D, 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 producing form *B. CeM6-7* strains was compared with *Miyagino* strain to be using control strain 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.

A. Indicator Strain *L. plantum*

In generally, the antibacterial produced by *B. CeM6-7* strains agents were stable within wide range of pH values from 3.923 to 4.058. The effects of heat, pH, nutritional composition and incubation time at 35 °C and the antibacterial substances were stable within O.D, 650 nm of *B. CeM6-7*. There were exceptions in the case of the *Miyagino* strain. This strain did not inhibit *L. plantum* at a temperature of 35 °C, 25 h and it was using as control strain.

In Fig. 10, shows that the control of antibacterial activity against *L. plantum* of *B. CeM6-7* and *Miyagino* strains *L. plantum* were presented. It was indicated that, at different timing (0 h to 25 h), *B. CeM6-7* had enough ability to fight against MRS broth contained *L. plantum* compared to strain *Miyagino*. *B. CeM6-7* were found to be the most powerful antimicrobial

activity at 800 to 860 min (12 h to 13 h). At 0 h, all strains were counted to be 0 O.D equally. At 5 min to 800 min (12 h), the *B. CeM6-7* was calculated to be 0.02 and 0.06 O.D. Subsequently, 800 min (12 h) to 1,500 min (25 h), the *B. CeM6-7* was calculated to be 0.06 to 1.80 O.D, which in comparison were less than *Miyagino* from 300 min (5 h) to 1,500 min (25 h) which presented at 0.53 to 2.02 O.D. At 1,500 min (25 h), the result has shown so much different turbines of *B. CeM6-7* from starting point.

B. Indicator Strain *Lb. lactis*

In normally, the bacteriocin produced by three *Bacillus* strains agents were stable within wide range of pH values from 4.05 to 4.17, and nonsignificant different between the pH values. The effects of heat, pH, nutritional composition and incubation time at 35 °C and the antibacterial substances were stable within O.D, 650 nm (0), *B. CeM6-7* against. There were exceptions in the case of the *Miyagino* strain. This strain did not inhibit *Lb. lactis* at a temperature of 35 °C, 25 h and it was using as control strain.

The control of antimicrobial activity against *Lb. lactis* of three strains *B. subtilis* and *Miyagino* after cultured in MRS broth contained against *Lb. lactis* (O.D = 0.1) were discussed in Fig. 11. The figure indicated that at 0 h all of strains were started at 0.00 O.D, and at 180 min (3 h) they both were almost had equal O.D (*CeM6-7*, 0.24 O.D) and (*Miyagino*, 0.25 O.D).

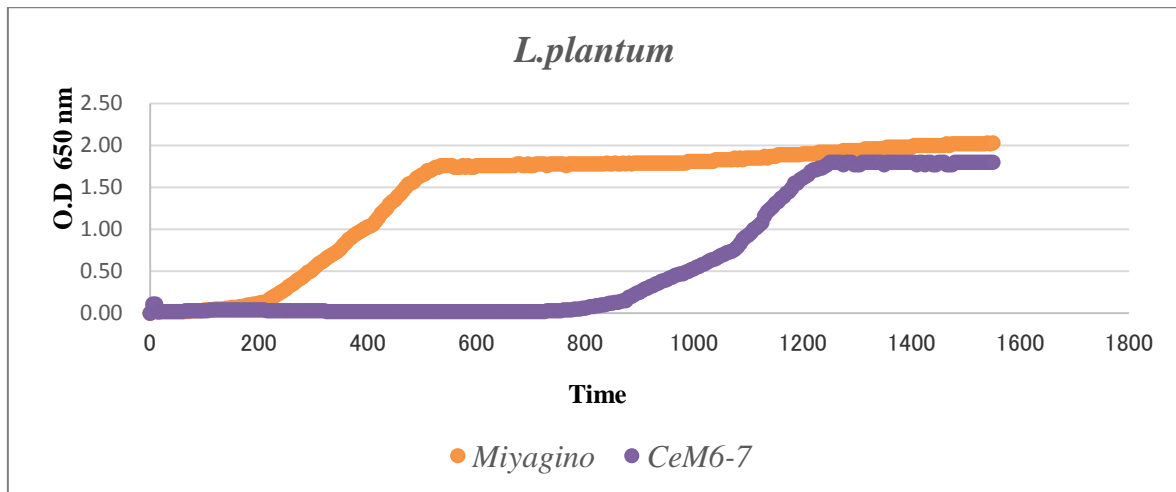


Fig. 10 Effect of bacteriocin produced by *B. CeM6-7* strain on the growth of *L. plantum* compare with *Miyagino* at different time in MRS broth at 35 °C for 25 h.

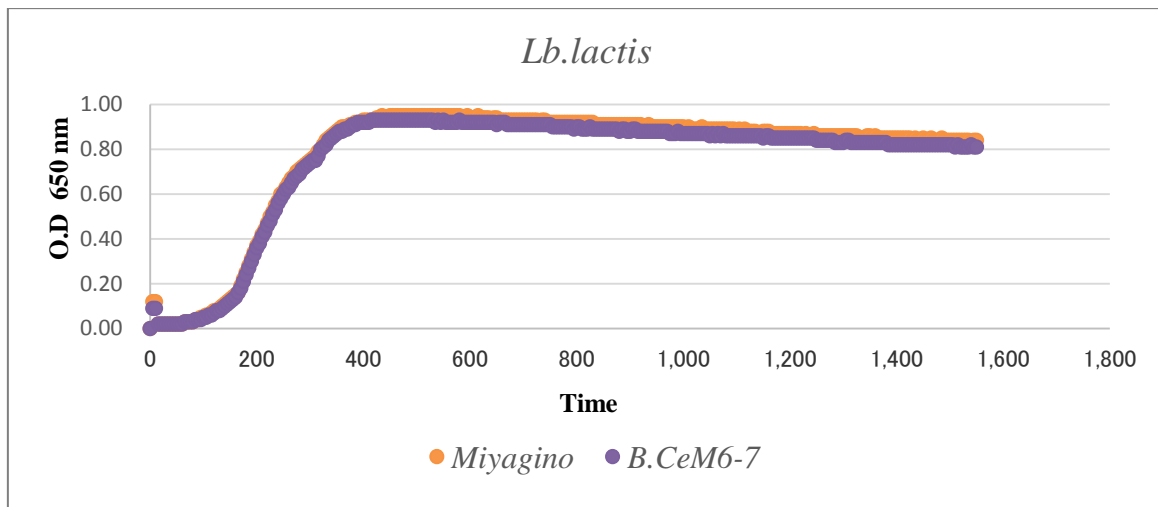


Fig. 11 Effect of bacteriocin produced by *B. CeM6-7* strain on the growth of *Lb. lactis* compare with *Miyagino* strains at different time in MRS broth at 35 °C for 25 h.

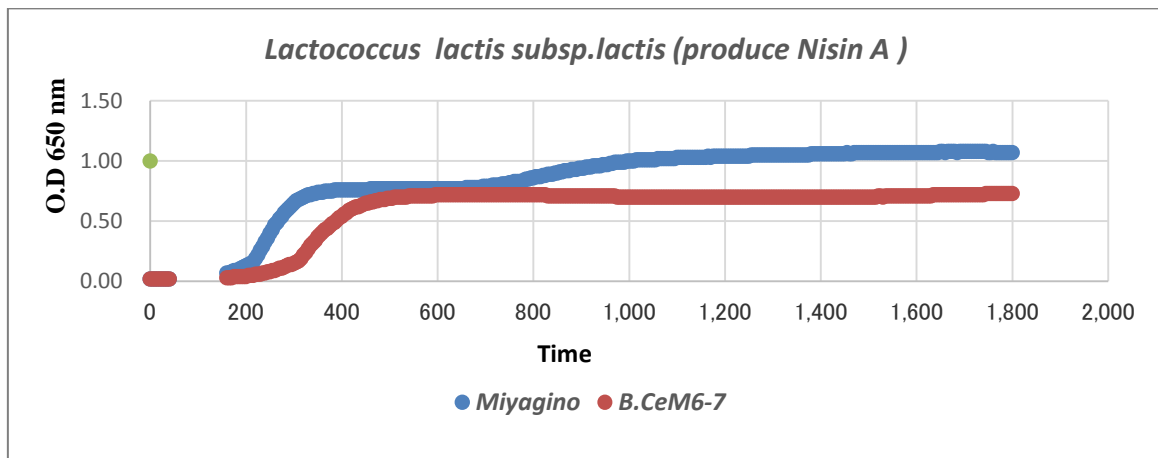


Fig. 12 Effect of bacteriocin produced by *B. CeM6-7* strain on the growth of *L. lactis subsp. lactis* (produce Nisin A) compare with *Miyagino* at different time in MRS broth at 35 °C for 30 h.

Nevertheless, at 600 min (10 h) *B. CeM6-7* strains were counted to be 0.92 O.D and be able to inactivate MRS broth contained against *Lb. lactis*, compared with *Miyagino* (0.94 O.D). From 720 min (12 h) to 1,500 min (25 h), *B. CeM6-7* strain (0.91-0.82 O.D) shown the most powerful antimicrobial action compared to *Miyagino* strains. Therefore, *B. CeM6-7* strain had higher antibacterial action against *Lb. lactis* from 12 h to 25 h, at 35 °C unlike *Miyagino*.

C. Indicator Strain *Lactococcuslactis subsp. lactis* (produce Nisin A)

The control bacteriocin activity against *L. lactis subsp. lactis* (produce Nisin A) by *B.CeM6-7* and *Miyagino* after cultured in MRS broth contained against *Lactococcus lactis subsp. lactis* (produce M6-7 Nisin A), O.D = 0.1, 650 nm at 35 °C for 30 h. The result shown that the stain *B.CeM6-7* produced the strongest antimicrobial activity against MRS broth contained *L. lactis subsp. lactis* (produce Nisin A) in compared with *Miyagino* strain.

In Fig. 12, at 0 h, all strains were calculated to be 0.02 O.D equally. In 170 min (2: 50 h), *B. CeM6-7* was presented 0.03 O.D which was less than *Miyagino* that presented at 0.08 O.D. At 600 min (10 h) to 825 min (14: 15 h) the *bacteriocin* of *B. CeM6-7* increased turbidity (*Miyagino* was counted at 0.77-0.88 O.D and *B. CeM6-7* presented at 0.72-0.72 O.D). At 840 min (14 h) to 1,530 min (25: 50 h) the antibacterial activity produced by *B. CeM6-7* was 0.71 and 0.70 O.D respectively less than *Miyagino* which counted for 0.89 and 1.07 correspondingly. After that, at 1,560 min (26 h) to 1,800 min (30 h) bacteriocin of *B. CeM6-7* increase turbidity because of the O.D of *Miyagino* was stable at 1.07 higher than *B. CeM6-7* counted for 0.71 and 0.73 O.D. This was indicated that *B. CeM6-7* shown higher ability to fight against *L. lactis subsp. lactis* (produce Nisin A) compared with *Miyagino*.

In short, the *B. CeM6-7* strain performed very well when treated with *Lb. lactis*, especially for MRS broth contained against *Lb. lactis*, and were the most

powerful to fight against MRS broth contained against *L. planum* and *L. lactis subsp. lactis* (produce Nisin A), but it was somewhat higher antimicrobial activity compare to *Miyagino* to fight against *Lb. lactis*, though the action is limited. This result was aligned with existing researches stated that, the effect of different pH on the antimicrobial potentials of the *Lactobacillus* strains indicates highest inhibitory activities between pH 5.5 to pH 7.5 and the survival rates of the pathogenic indicator bacteria in the fermented food sources were between 8 and 14 days [6].

Applying *bacteriocin* to control the growth of Gram positive pathogenic or spoilage bacteria of Cambodian strain *CeM6-7* indicated that Cambodian strain has similar strength with *Miyagino* strains (considered to be the strongest one of Gram positive and Lactic acid groups) since it actively produced *bacteriocin* fighting against all three indicator strains of Gram positive and Lactic acid groups from 0-30 h and 0-25 h incubation times. Thus, this property of *bacteriocins* produced by *B. CeM6-7* 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 in addition to Gram-positive species, some of which are known to be pathogenic to humans and animal [19].

4. Conclusion

In this work, we demonstrated the diversity of *B. subtilis* in *SIENG*, a Cambodian's traditional fermented after culturing in MRS soybean food. 119 samples of *Bacillus* strains were isolated and there were found only 15 of *Bacillus* strains that have ability to fight against the indicator microorganisms *Lactobacillus plantum ATCC 8014* by agar well diffusion assay. Furthermore, among 15 samples, only one stain (*Bacillus CeM6-7*) has shown a great active zone against *Lactobacillus plantum ATCC 8014*.

Additional finding, *Bacillus CeM6-7* strain could tolerate with heat up to 20 h at 30 °C temperature and 16 h at 37 °C. Besides, bacteriocin produced by *Bacillus CeM6-7* strain has more ability to suppress all the indicators starts from 20 hours compared with *Miyagino* stains. Further, it has strength compare with *Miyagino* originated in Japan, but it has a special unique ability to fight against indicator strain SA at a very significant level.

Moreover, *Bacillus CeM6-7* strain performed very well when treated with Gram positive group, against *S. aureus* and Lactic acid groups, against *Lactobacillus plantum* at a very extensive level. *Bacillus CeM6-7* strain has higher strength unlike Japanese strains (*Miyagino*), since it actively produced bacteriocin fighting against all three indicator strains of Gram positive and Lactic acid groups from 0-30 h and 0-25 h incubation times.

So, *Bacillus CeM6-7* strain can be used as a biological control agent in fermented soybean products to exclude growth of pathogenic or spoilage bacteria during manufacturing without inhibiting the fermentation of *Bacillus spp.* and may have further uses in the food, agricultural, and pharmaceutical industries.

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