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Identification and characterization of membrane receptor protein YueB in *Bacillus subtilis* isolated from Thua Nao (Thai fermented soybean)

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Abstract

Thua nao (Thai fermented soybean) is a conventional and spontaneously fermented soybean in northern Thailand. The fermentation process relies on certain microbial activities, especially the *Bacillus* species. Bacteriophage contamination on Thua nao can rapidly decrease the *Bacillus* growth and the quality of any related products. The bacteriophage infection occurred through the YueB membrane receptor protein (coded by *yueB* gene) on the bacterial surface. Twenty isolates including *Bacillus* spp. were isolated from Thua nao. Phylogenetic analysis of *Bacillus* was determined using 16S-23S rRNAs nucleotide sequencing and compared with those obtained from the GenBank® database. Twenty *Bacillus* spp. isolated were classified into six clusters, which included *Bacillus amyloliquefaciens*, *B. aryabhattai*, *B. cereus*, *B. licheniformis*, *B. sonorensis*, *B. subtilis* and *B. vallismortis*. *B. subtilis* isolate TM4, TM5, TK7 and MR4 were selected to determine the variation of nucleotide sequences of *yueB* gene. Approximately 3.2 kb of *yueB* gene were amplified by Polymerase chain reaction (PCR) and directly sequencing. The translation of proteins retrieved 1,059 amino acid residues in all the isolate strains. The similarity alignment protein sequence of *B. subtilis* 168 as a reference strain revealed maximum identity scores of 81% and 1% of the gaps on the protein BLASTX alignment tools. The secondary structure of YueB protein indicated that the six transmembrane domain regions at one *N*-terminal and five *C*-terminal from *B. subtilis* isolate MR4, TK7, TM4 and TM5 were similar to that of *B. subtilis* 168. At the position between 200 to 600 amino acid residues, four regions were folded as coiled coil structures.

Keywords: Thua nao, 16S-23S rRNAs, *Bacillus subtilis*, *yueB* gene, YueB membrane receptor protein.

1. Introduction

This fermented soybean product is traditionally produced from soybeans and widely consumed as a major ingredient in condiments in the northern provinces of Thailand [1-2]. Thua nao is not only an important food and

condiment, but is also considered a low-cost protein dietary supplement [3]. Thua nao is brownish in color and it has a unique flavor. It is layered as a slightly sticky mass and gives off a strong ammonia-like odor. *B. subtilis* is the predominant bacteria that is used from the beginning to the end of the soybean fermentation process. However, fermented soybean products are generally prepared on a household scale and depend on natural microflora, which are then mixed with the culture in the environment and at the level of localized production. Thua nao contains a mix of cultures of *Bacillus* strains derived from the environment including *B. subtilis*, *B. cereus*, *B. megaterium* and *B. pumilus*. However, Japanese fermented soybeans (or Japanese natto) use a pure culture strain of *B. subtilis* to produce fermented soybean products yielding unique aromas and flavors [4-5]. Verities of soybean fermentation products are found in Asia, such as *natto* from Japan, *kinema* from India, *schuidouchi* from China, *dawadawa* from Nigeria, and *chungkukjang* from Korea [6-7]. However, the process is at risk from both spoilage and contamination by bacteriophage microorganisms during the course of the fermentation process such as machines, and facilities including the floor, wall, and windows [8-9].

Bacteriophages are prokaryote viruses that infect the host bacteria by recognizing a membrane specific receptor of the host cell for viral transfection and multiplication [10]. Bacteriophage infection is initiated by a specific interaction of the virion with receptors at the host cell surface. Bacteriophage SPP1 is a *Siphovirus* that specifically infects soil bacterium such as *B. subtilis* [11]. Bacteriophage infection is initiated by the recognition and reversible binding to the host cell membrane surface. Then, it is attached irreversibly to the receptor-binding protein (RBP) and ejected from the bacteriophage DNA from the capsid through a portal protein. The tail spike (gp21 protein) of SPP1 binds to the *Bacillus subtilis* receptor membrane YueB protein. The SPP1-YueB interaction is transmitted through signals along to the tail tube and the connector channel is opened that is controlled by gp7 protein. The DNAs are released from the phage capsid to the host cell bacterial cytoplasm. With regard to membrane receptor proteins, YueB protein families are the main proteins that are involved in the phage binding process. The *YueB* gene is coded for the YueB protein, which is found in Gram-positive bacteria. The YueB is predicted to insert in the membrane through one N-terminal and five C-terminal transmembrane domains (TMDs). It contains specific binding sites for bacteriophage infection that occurs only for Gram-positive bacteria [12-13]. During Thua nao fermentation, contamination by other microbes and bacteriophages from the air and environment can be observed. These can directly affect the growth of *Bacillus* strains and the quality of Thua nao products. Based on the mechanism of bacteriophage infection *via* the YueB membrane receptor protein, disruption of the *yueB* gene in *B. subtilis* can prevent the bacteriophage transmission into the bacterial membrane. This also can be used as a model study for the purposes of developing a bacteriophage biocontrol model and can be applied with other bacteria in further research.

In this research study, the screening and identification of twenty *Bacillus* isolates in the production of Thua nao was done using full-length 16S rRNAs and partial 23S rRNAs. The *yueB* gene from *B. subtilis* isolates was investigated for the nucleotide sequences and amino acid sequences. The disruption *yueB* gene strain can be used in Thua nao fermentation to improve the quality of Thua nao products, such as in terms of colour, odor and taste. These three factors of quality are the unique features of Thua nao that can be affected during the production process. The necessary *Bacillus* strains are only grown in Thailand and are used in Thua nao production. Consequently, the risk of spoilage was decreased resulting in natto products that can be stored for longer periods of time and better preserved than in the household spontaneous fermentation process. The findings of this research study could be applied in other fermentation processes and could possibly be used as a bacteriophage biocontrol for other bacteria in further analysis.

2. Materials and methods

2.1 Bacterial Strains and Growth Conditions

Bacillus spp. isolates were isolated from Thua nao which were kindly provided from the Program in Biotechnology, Faculty of Science, Maejo University, Chiang Mai, Thailand. Twenty *Bacillus* spp. isolates were listed in Table 1. All isolates were cultivated in Luria-Bertani (LB) agar plates (2.0% [w/v] LB agar). A single colony was inoculated in LB medium [14] and incubated at 37 °C with incubator shaking (160 rpm) for 24 h. The cell pellets were then harvested by centrifugation for 5 min at 8,000 rpm and the supernatant was removed. The cell pellets were stored at -80 °C until analysis. *Escherichia coli* DH5α cells that were transformed with pTA2 cloning vector (Toyobo, Japan) were selected and medium supplemented with ampicillin (at a final concentration of 100 µg/ml).

Genomic DNA was extracted using NucleoSpin® Tissue kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. The quality of the genomic DNA was determined by 0.8% (w/v) agarose gel electrophoresis and the genomic DNA was stored at -20 °C until further analysis.

Table 1 Bacterial strains, bacteriophage and plasmid vector that were used in this study.

Bacterial/plasmid	Isolate/strain	Source/reference
Bacillus spp.	BP1-10, BP5-11, BP6-1, BP7-3, BP8-23, BP9-12, BP10-5, TK2, TK5, TK6, TK7, TM2, TM3, TM4, TM5, MR4, MR6, MR9, MT3, PW10	Program in Biotechnology, Faculty of Science, Maejo University, Chiang Mai, Thailand
Bacillus subtilis	168	Applied Microbiology Unit, Food Research Institute, National Agriculture and Food Research Organization, Japan
Escherichia coli	DH5α	Toyobo, Japan
Bacteriophage	SPP1 (ATCC® 27689-B1™)	American Type Culture Collection (ATCC®), USA
Plasmid	pTA2	Toyobo, Japan

2.2 PCR Amplification of 16S rRNAs and 23S rRNAs

The DNA of the bacterial isolates and reference strains were used as DNA templates to amplify 16S rRNAs and 23S rRNAs using universal primers. The primers used for 16S rRNAs and 23S rRNAs amplification and sequencing were listed in Table 2. The 16S rRNAs derived from *Bacillus* spp. was amplified using 20F/1500R primers set and tested for PCR reaction at gradient annealing temperatures (from 48 °C to 59 °C) in order to optimize the annealing temperature. For the 23S rRNAs, the 118V/1037R universal primer that was complementary to the conserved regions was used, and the annealing temperature was optimized between 50 °C and 61 °C. PCR amplification was conducted in a total reaction volume of 50 µl containing 2.5 µl genomic DNA, 1.25 µl 10 µM of each primer, 20 µl sterile deionized water, and 25 µl 2X PCR master mix solution (i-*Taq*™) (iNtRON Biotechnology, Korea). The PCR conditions were applied in automating thermal cycling (Biometra, Germany): initial denaturation at 94 °C for 2 min, followed by 30 cycles at 94 °C denaturation for 30 sec, and annealing temperature for 30 sec, and extension at 72 °C for 2 min, with a final extension step at 72 °C for 7 min and then cooled and held at 10 °C.

The PCR products were determined by 1.2% (w/v) agarose gel (Bio Basic Inc., Canada) stained with RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, Korea) in 1X TAE electrophoresis buffer and visualized under UV transilluminator. The 16S rRNAs and 23S rRNAs PCR products of *Bacillus* spp. isolated from Thua nao were then purified using NucleoSpin® Gel and PCR clean-up kit (Macherey-Nagel, Germany) according to the manufacturer's instruction.

Table 2 Nucleotide sequences of oligonucleotide primers were used for amplification and sequencing of 16S rRNAs, 23S rRNAs and *yueB* gene.

Primer name	Sequence (5' to 3')	Position	Target	References
20F	GAGTTTGATCCTGGCTCAG	9-27	16S rRNAs	[15]
1500R	GTTACCTTGTTACGACTT	1509-1492	16S rRNAs	[15]
1500R/520F	CAGCAGCCGCGGTAATAC	519-536	16S rRNAs	[15]
20F/520R	GTATTACCGCGGCTGCTG	536-519	16S rRNAs	[15]
920F	AAACTCAAATGAATTGACGG	907-926	16S rRNAs	[15]
920R	CCGTCAATTCATTTGAGTTT	926-907	16S rRNAs	[15]
118V	CCCAATGGGGAAACCCA	114-130	23S rRNAs	[16]
1037R	CGACAAGGAATTTCGCTAC	1953-1971	23S rRNAs	[16]
11a	GGAACTGAAACATCTAAGTA	190-209	23S rRNAs	[17]
62ar	GGGGCCATTTTGCCGAGTTC	1736-1717	23S rRNAs	[17]
M13F(-20)	GTAAAACGACGGCCAGT	600-616	*yueB* gene	This study
M13R(-24)	GGAAACAGCTATGACCATG	828-846	*yueB* gene	This study
yueB765F	CCATCCAAGCACAGCAAAAGGCG	764-786	*yueB* gene	This study
yueB2570R	GCACGAGTTTCGTGGAGGCG	2592-2573	*yueB* gene	This study
yueBFBamHI	AAGATCGATgaatccATGACAGAACA ACGAAAAAGCTTG	1-24	*yueB* gene	This study
yueBRSmaI	AAGATCGATcccgggTCACGATTCAT ACGTTTCATCGC	3209-3231	*yueB* gene	This study

2.3 Nucleotide Sequencing of 16S rRNAs and 23S rRNAs

Full-length 16S rRNAs and partial 23S rRNAs were amplified using genomic DNA obtained from twenty strains of *Bacillus* spp. that were used as templates, and then all PCR products were purified by NucleoSpin® Gel and PCR clean-up kit. The purified 16S rRNAs and 23S rRNAs PCR products underwent nucleotide sequencing using 1500R/520F, 20F/520R, 920F and 920R primers for full-length sequencing of the 16S rRNAs and the 11a and 62ar primers were used for 23S rRNAs partial sequences. DNA sequencing was performed by the Big Dye terminator technique (First BASE Laboratories Sdn Bhd, Malaysia).

2.4 Nucleotide Sequences and Phylogenetic Analysis

The sequencing data of twenty complete full-length nucleotide sequences of 16S rRNAs and partial sequences of 23S rRNAs derived from *Bacillus* spp. were retrieved and aligned by ClustalW tools [18] using Biological sequence alignment editor (BioEdit) program (version 7.2.5). The 16S rRNAs and 23S rRNAs nucleotide sequences were then carried out by nucleotide BLAST search program analysis of the NCBI database. The 16S rRNAs and 23S rRNAs sequences data were submitted to the GenBank® public database and published with accession numbers at the closest sequence. Aligned sequences were analyzed using the Molecular Evolutionary Genetics Analysis (MEGA) program (version 6.06). A phylogenetic tree was generated using the Neighbor-joining method to classify the 16S rRNAs and 23S rRNAs of *Bacillus* spp. isolated from Thua nao. Bootstrap confidence values were generated using 1,000 replications of the data set for the 16S rRNAs and 23S rRNAs to derive the nucleotide sequence similarities.

2.5 Molecular Cloning of YueB Gene

The *Bacillus* spp. were classified using phylogenetic analysis from 16S rRNAs and 23S rRNAs. The *B. subtilis*, which were obtained from phylogenetic tree identification including *B. subtilis* isolate MR4, TK7, TM4 and TM5, were selected to be investigated and were then used to identify the *yueB* gene. The conserved regions present in the *yueB* gene of *B. subtilis* 168 (GenBank® accession no. NR102783.1) that were obtained from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST) database were then used to design a set of primers to amplify the *yueB* gene. Two sets of primers for PCR amplification, which included yueBFBamHI and yueBRSmaI primers, contained a unique restriction site for *Bam*HI and *Sma*I, respectively. The PCR conditions were applied as follows: suspension in a final volume of 50 µl that consisted of 2.5 µl genomic DNA isolated from *B. subtilis*, 1.5 µl, 1.25 µl 10 µM of each primer, 20 µl sterile deionized water, and 25 µl 2X PCR master mix solution (i-*Taq*™). The PCR conditions included an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing for 30 sec, and extension at 72 °C for 1 min. A final step at 72 °C for 7 min was performed to end DNA synthesis and the holding temperature was 10 °C. The efficiency of *yueB* gene amplification was determined by 1.0% (w/v) agarose gel electrophoresis.

The *yueB* gene was amplified by PCR and directly purified from agarose gel using NucleoSpin® Gel and PCR clean-up kit. DNA fragments of yueB genes from *B. subtilis* isolate MR4, TK7, TM4 and TM5, which contained 3'−A overhang were ligated to a pTA2 cloning vector. The transformation of pTA2-yueB recombinant DNA into *E. coli* DH5α as the competent cells was performed. *E. coli* carrying recombinant plasmid was grown in the LB agar plates that were supplemented with ampicillin. The transformants expressing β-galactosidase were selected in X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and induced by IPTG (isopropyl-beta-D-thiogalactopyranoside) and supplemented on the LB agar plates. Recombinant DNA purification was carried out using TIANprep Rapid Mini Plasmid Kit (Tiangen Biotech, Beijing) and the orientation of the *yueB* gene was confirmed by endonuclease restriction enzyme analysis.

A total of four *B. subtilis* isolates of the *yueB* gene were amplified by PCR as has been described previously. Purified plasmid of pTA2-yueB recombinant DNA was used for nucleotide sequencing. The recombinant plasmids were then sequenced using the sequencing primers yueBFBamHI, yueBRSmaI, yueB765F, yueB2570R, M13F (-20) and M13R (-24). The pTA2-yueB recombinant DNA was submitted to First BASE Laboratories Sdn Bhd, Malaysia for nucleotide sequencing. The nucleotide sequences were aligned and translated to amino acid residues using BLASTX program analysis on the NCBI database.

2.6 Bacteriophage sensitivity assay

The bacteriophage SPP1 was propagated in *B. subtilis* isolate MR4, TK7, TM4, TM5 and *B. subtilis* 168 was used as the reference strain. Bacteriophage SPP1 infection was performed by the soft-agar overlay and plague-counting method. All *B. subtilis* strains were inoculated into LB medium and incubated at 37 °C and shaking at 160 rpm for 18 h. Then, 2.0% (w/v) LB agar plate contained sterile 10 mM $CaCl_2$ solution for underlay were

prepared. The serial 10-fold dilutions (10^{-1}-10^{-10}) of bacteriophage SPP1 stock were prepared using SM (Saline-magnesium) buffer with gelatin. Then, one hundred microliter of each bacteriophage dilution was transferred to each microcentrifuge tube containing 100 µl of *B. subtilis* cell suspension and mixed well. Then, 200 µl of bacteriophage SPP1- *B. subtilis* suspension was incubated for 30 min at 37 °C. After incubation, 200 µl of bacteriophage- *B. subtilis* suspension was added into a test tube containing 3 µl 0.5% (w/v) LB semi solid medium which contained sterilized 10 mM $CaCl_2$ solution for overlay and gently mixed. The overlay medium was immediately poured into the underlay surface and allowed the overlays for 10 min at room temperature to solidify before incubation. The culture plates were incubated at 37 °C for 24 h. Then, the plaques were visualized as zones of clearing in the bacteria lawn. The plaques forming unit (pfu/ml) was calculated using serial dilutions of phage The equation is "(*Plaques forming unit (pfu/ml)= Number of plagues/(D×V)*"; Whereas, D is serial dilutions and V is the volume of diluted virus added to the plate.

3. Results and discussion

3.1 16S rRNAs and 23S rRNAs Sequence Analysis

The 16S rRNAs and 23S rRNAs have been widely used for the identification and classification of microorganism nucleotide sequences analysis, which is a simple and commonly used method. In this study, 16S rRNAs and 23S rRNAs which were obtained from twenty *Bacillus* spp. isolates were sequenced. The nucleotide sequences were published and were available on the NCBI database. Complete 16S rRNAs and partial 23S rRNAs sequences were determined using universal primers. It was found that 55.5 °C was the optimal annealing temperature to amplify 1.5 kb of the 16S rRNAs without non-specific bands and high PCR product yield. The amplification of the 23S rRNAs was done at 50.3 °C to obtain the annealing temperature and the PCR product was found to be approximately 900 bp in size. The complete full- length nucleotide sequences of 16S rRNAs and the partial nucleotide sequences of 23S rRNAs were aligned and used to construct a phylogenetic tree. The position differences of the nucleotide sequences of 16S rRNAs and 23S rRNAs are shown in figure 1 and 2, respectively. The phylogenetic tree of 16S rRNAs obtained from twenty *Bacillus* spp. isolates using bootstrap values based on 1,000 replications was shown in Figure 3A. The 16S rRNAs sequences of *Bacillus* spp. revealed more than 97 to 98% similarity when compared with the *B. subtilis* 168 reference strain. The twenty *Bacillus* spp. isolated from Thua nao were separated into six clusters, which included *B. amyloliquefaciens*, *B. aryabhattai*, *B. cereus*, *B. licheniformis*, *B. sonorensis*, *B. subtilis* and *B. vallismortis*. Moreover, *Bacillus* spp. isolate TK6, TK2, TM2 and MT3 were found to be closely related to *B. licheniformis* and *B. sonorensis*. These strains could not be identified in the related *Bacillus* spp. group. The partial 23S rRNAs was then used to confirm the identification of twenty *Bacillus* spp. isolates from Thua nao. Approximately 900 bp of the 23S rRNAs sequences were amplified using universal primers. Sequencing of the twenty *Bacillus* spp. strains was obtained from partial nucleotide sequences. Genetic relationship analysis of partial 23S rRNAs was used to verify that *B. amyloliquefaciens*, *B. anthracis*, *B. aryabhattai*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. sonorensis*, *B. subtilis* and *B. vallismortis* were separated (Figure 3B). From the results, *Bacillus* spp. isolate TK6 and MT3 were confirmed as being similar to *B. sonorensis* and *Bacillus* spp.; isolate TK2 and TM2 were included in the *B. licheniformis* group with 99% similarity. Analysis of all *Bacillus* spp. revealed more than 97 to 99% similarity to the 23S rRNAs sequences as has been referenced with the GenBank® database.

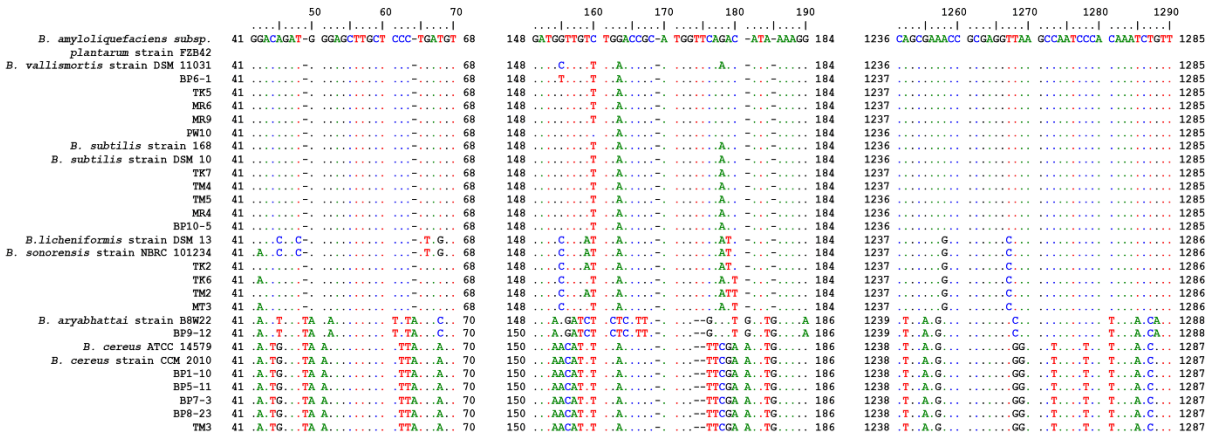

Figure 1 Difference positions in full-length 16S rRNAs nucleotide sequences using ClustalW alignment analysis of the *Bacillus* spp. and twenty isolates from Thua nao.

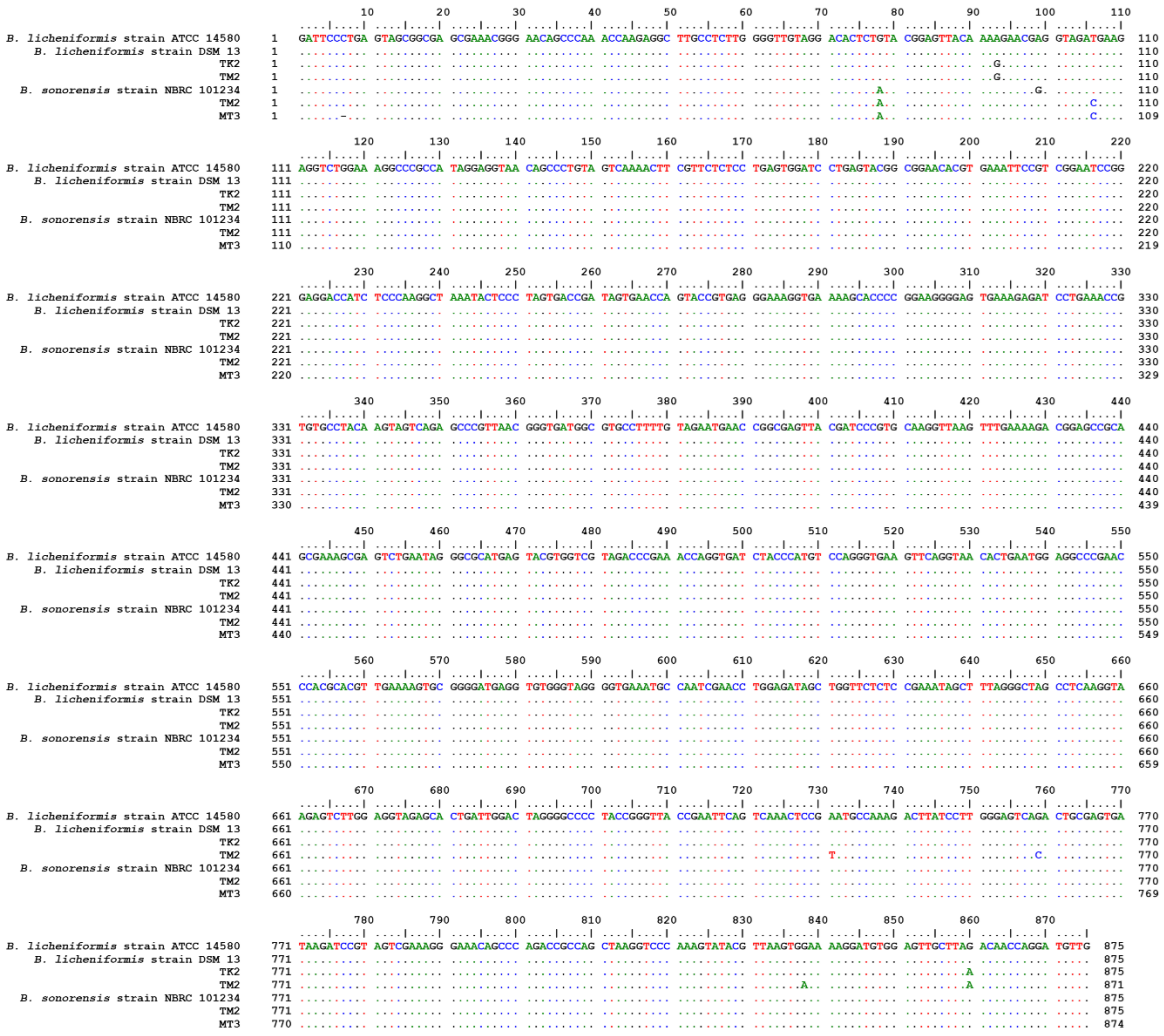

Figure 2 Difference positions in partial 23S rRNAs nucleotide sequences using ClustalW alignment analysis of the *B. licheniformis*, *B. sonorensis* and *Bacillus* spp. isolated from Thua nao.

In the previous study, the taxonomy of the members of the *Bacillus* species group was determined using 16S rRNAs and 23S rRNAs sequence analysis. Analysis of the *Bacillus* group strains indicated that the three groups consisted of *B. cereus*, *B. thuringiensis*, and *B. mycoides*. Based on 16S rRNAs and 23S rRNAs nucleotide sequences, the *B. cereus* group was divided into seven sub-groups including *B. anthracis*, *B. cereus* A and B, *B. mycoides* A and B and *B. thuringiensis* A and B [19]. In addition, the *B. subtilis* was used to generate a phylogenetic tree from the 16S rRNAs gene. Phylogenetic analysis revealed the division of the *Bacillus* group into four clusters: cluster I contained *B. subtilis*, *B. vallismortis* and *B. mojavensis* strains; clusters II and III contained strains of *B. atrophaeus* and *B. amyloliquefaciens*, respectively; and cluster IV contained *B. sonorensis* and *B. licheniformis*. All *Bacillus* strains were recorded at more than 98% similarity [20].

The Thua nao fermentation process was similar to the process s which was used in Japanese natto production. The process employed the *Bacillus* species as microorganisms in the fermentation process. In Thailand, Thua nao was produced using a traditional fermentation method that used natural microflora, whereas Japanese natto was fermented using a pure culture of *B. subtilis*. Leejeerajumnean and co-workers reported that Thua nao fermentation produced at 0 to 36 h involved natural microflora of *B. subtilis*, *B. megaterium* and *B. cereus* that were produced over a period of 72 h. The *B. subtilis* was the predominant microorganism from the beginning to the end of the fermentation process employing the traditional method [21]. In the fermentation process of soybeans, many researchers had reported that the *Bacillus* species were predominant strains and it was contaminated by bacteriophages and other microorganisms. However, contamination of soybean fermented with bacteriophages could rapidly decrease the *Bacillus* species growth rate and affected the quality of the products. In 2008, Suppadit and co-workers revealed the preliminary data of Thua nao production, processing, and the quality from provinces in northern Thailand for improving the production process and quality. The fermentation process of Thua nao differed in the different locations and the quality of Thua nao was inconsistent which was affected by the season. Moreover,

the fermented soybean process not only contained predominant of the *Bacillus* species and fungi (*Rhizopus* spp.) but also *Aspergillus flavus* and *Aspergillus niger* were found and caused to lower the quality of the products [5]. In this research, we found *Bacillus* isolates MR4, TK7, TM4 and TM5 were closely related to those of *B. subtilis* recorded in the GenBank® database.

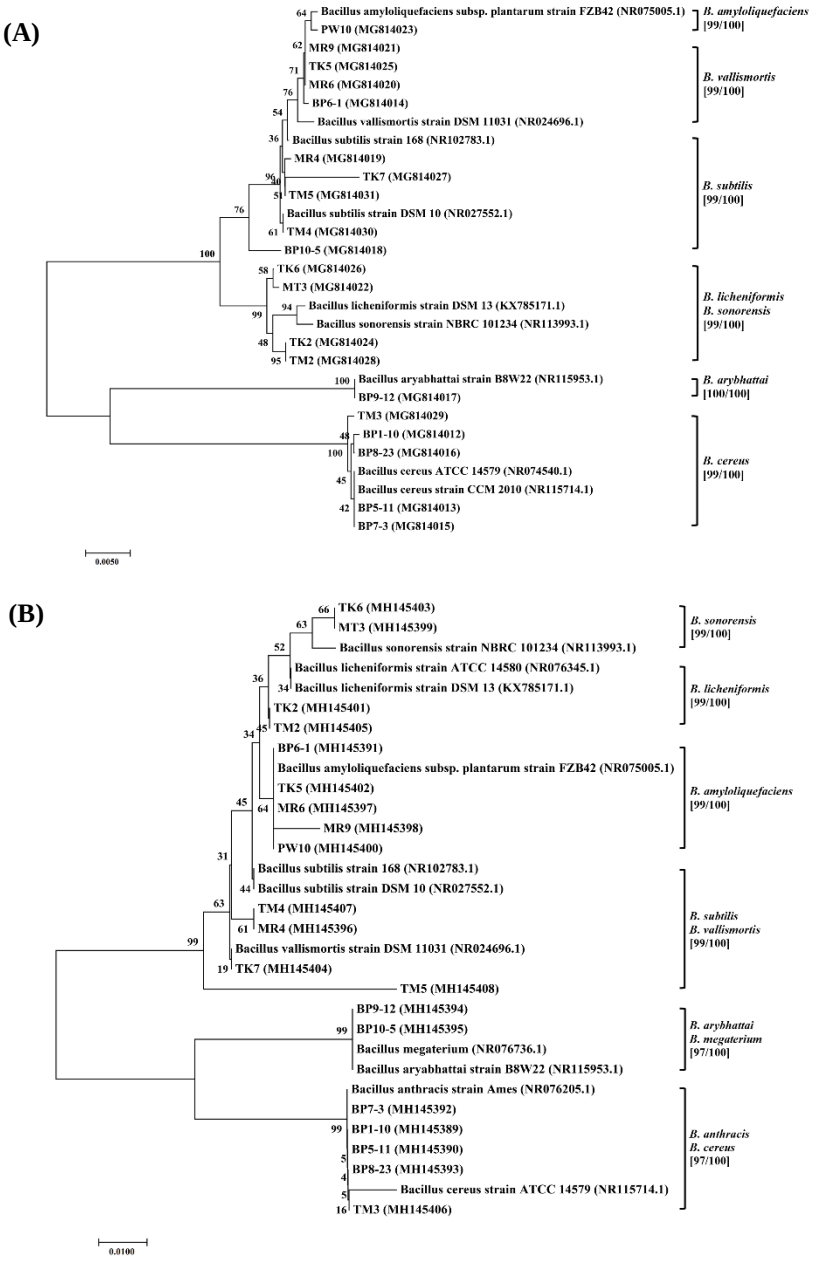

Figure 3 The genetic relationship among the twenty *Bacillus* spp. isolates based on (A) 16S rRNAs and (B) 23S rRNAs. The phylogenetic tree was constructed with the Neighbour joining method to demonstrate the classification of *Bacillus* spp. (based on 1000 bootstrap replications).

3.2 Nucleotide Sequences and Amino Acid Sequences of YueB Gene

The YueB membrane receptor protein was coded by the *yueB* gene, which was the essential gene involved in the specific irreversible binding of bacteriophage SPP1 to *B. subtilis. B. subtilis* isolate MR4, TK7, TM4 and TM5 were selected to investigate the *yueB* gene. As a result, *yueB*-analysis full-length nucleotide sequences obtained from *B. subtilis* isolate MR4, TK7, TM4 and TM5 were related to *B. subtilis* 168 with a maximum identity score of 86% according to the nucleotide BLASTN search program analysis. The nucleotide deletion of *B. subtilis* isolate MR4, TK7, TM4 and TM5 were observed at the position 1,160-1,165 (ΔGAGAAGA), 1,351-1,356

(ΔTCTCCTAATA), 1,369-1,376 (ΔCCGTCGAA), 1,444-1,447 (ΔGATA), 1,481-1,489 (ΔGAAACGGGAC), 1,567-1,624 (ΔAATCAAAGTGATGGGCTA). Moreover, only three strains containing *B. subtilis* isolate MR4, TM4 and TM5 were observed to display the nucleotide deletion at position 1,363 (ΔG) and position 176 (ΔA) of *B. subtilis* isolate MR4. The nucleotide insertions were found in *B. subtilis* isolate TM5 at positions 2,232 (C), 2,264 (C) and 2,273 (T), while *B. subtilis* isolate TK7 revealed the insertion at position 1,363 (C) (Supplemental data). Nucleotide sequences were then translated to amino acid sequences using the BLASTX program on the NCBI GenBank® database. The complete protein translation retrieved 1,059 amino acid residues in all isolate strains, while the nucleotide sequence obtained from the *B. subtilis* 168 encoded 1,076 amino acid residues (as shown in Figure 4). Similarly, the alignment protein sequence of *B. subtilis* 168 as the reference strain revealed a maximum identity score of 81% and only 1% of the gaps on the protein alignment tools. The protein alignment containing four regions of 387-388, 458-462, 488-491 and 546-541 gap position were observed.

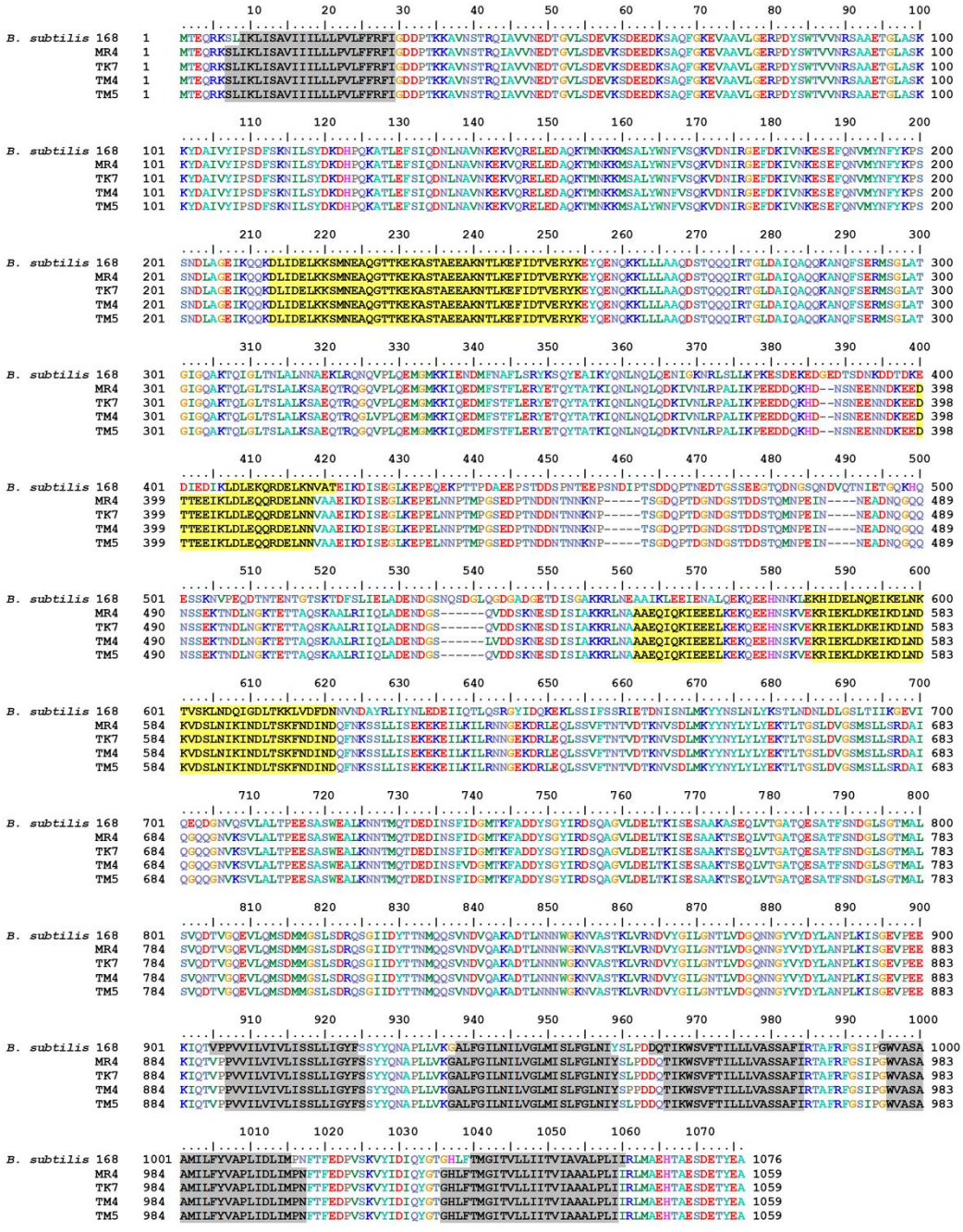

Figure 4 *Yue*B gene amino acid sequence alignment of *B. subtilis* isolate MR4, TK7, TM5 and TM5. *B. subtilis* 168 was used as the reference strain. Subcellular transmembrane regions (dark grey) and coiled coil structures (yellow).

In YueB protein, the transmembrane domain regions (black) of *B. subtilis* isolate MR4, TK7, TM4 and TM5 were predicted using UniProt BLAST alignment tools and the MARcoil prediction program on the ExPASy bioinformatics resource portal. The transmembrane domain regions were comprised of six regions at N-terminus and C-terminus that the positions were similar to *B. subtilis* 168. The amino acid sequence BLASTX alignment of *B. subtilis* isolate MR4, TK7, TM4 and TM5 were analyzed with maximum identities score 99% similarity. Furthermore, the position between 200 to 600 amino acid residues, four regions were folded as coiled coil structures (yellow) in *B. subtilis* isolate MR4, TK7, TM4 and TM5 while three regions of 213-254, 407-421 and 585-621 in *B. subtilis* 168 were found. The amino acid deletion of *B. subtilis* isolate MR4, TK7, TM4 and TM5 were observed at position 387-388 (ΔGE), 458-462 (ΔSNDIP), 488-491 (ΔDVQTN) and 536-541 (ΔNQSDGL).

In the previous study, the secondary structure of YueB membrane receptor protein was investigated. The YueB protein was predicted to insert in the extracellular space of *B. subtilis* membrane which was composed of one N-terminal and five C-terminal transmembrane domains in range 1 to 30 and more of 900 residues. The coiled coil structure between 30 and 901 amino acid residues was bound with bacteriophage SPP1 [12]. In bacteriophage infection, the tail proteins gp21 was bound to coiled coil structures of the *B. subtilis* membrane receptor YueB protein that was located on extracellular. In our study, the YueB membrane receptor protein presented in *B. subtilis* isolate MR4, TK7, TM4 and TM5 was expected to serve as a bacteriophage SPP1 receptor protein. It should be noted that the different positions of coiled coil structures and the deletion of amino acid in four isolates of *B. subtilis* might affect the interaction between YueB-bacteriophage during infection. The bacteriophage SPP1 tail was an essential for the recognition site of the membrane receptor. In this study, the 1,059 amino acid residues of YueB protein in *B. subtilis* isolate MR4, TK7, TM4 and TM5 were shorter than *B. subtilis* 168 as a reference strain that contained 1,076 amino acid residues. The efficiency of the tail proteins gp21 interact with the YueB membrane receptor protein might be decreased. The YueB membrane receptor protein in the *B. subtilis* strain was an essential target for bacteriophages SPP1 infection. The bacteriophages adsorption to the microorganism was involved in the specific interaction and the binding in a reversible or irreversible manner. Thua nao is a fermented soybean product that utilizes the *Bacillus* spp. as the predominant microorganism in the fermentation process. With regard to the bacteriophage infection of the YueB membrane receptor protein, information of the *Bacillus* species in Thua nao fermentation has not been fully reported. In this research study, bacteriophage SPP1 biocontrol was used in Thua nao fermentation using *yueB* gene disruption from *B. subtilis*. The integrant *yueB* gene can be used to improve the quality of Thua nao and may be applied in other fermentation processes.

3.3 Bacteriophage sensitivity assay

To investigate the infection of bacteriophage SPP1 on *B. subtilis* isolate MR4, TK7, TM4 and TM5, bacteriophage sensitivity assay was performed and *B. subtilis* 168 used as positive control. The bacteriophage sensitivity was determined by counting the plaques (zone of clearing) and plaque forming unit were evaluated. As in the literature reviews the YueB protein was an essential receptor protein for bacteriophage SPP1 infection [12]. In this work, only the *B. subtilis* isolate MR4, TM4 and TM5 were specifically infected by bacteriophage SPP1. The results showed that *B. subtilis* isolate MR4, TM4 and TM5 were infected by bacteriophage SPP1 with a titer 2.5×10^{9}, 5.8×10^{3} and 4.2×10^{2} pfu/ml, and compared with *B. subtilis* 168 was 3.7×10^{8} pfu/ml (as shown in Table 3 and Figure 5). In addition, only *B. subtilis* isolate TK7 was not infected by bacteriophage SPP1. This indicated that the differences of nucleotide sequences and amino acid sequences of YueB protein in all four strains might affect the bacteriophage SPP1 infection.

Table 3 Bacteriophage SPP1 sensitivity of *yueB* gene in the *B. subtilis* 168, *B. subtilis* isolate MR4, TK7, TM4 and TM5.

B. subtilis isolate	Bacteriophage SPP1 infection	Dilution	pfu/ml
BS168	+	10^{-9}	3.7×10^{8}
MR4	+	10^{-8}	2.5×10^{9}
TK7	-	-	-
TM4	+	10^{-2}	5.8×10^{3}
TM5	+	10^{-1}	4.2×10^{2}

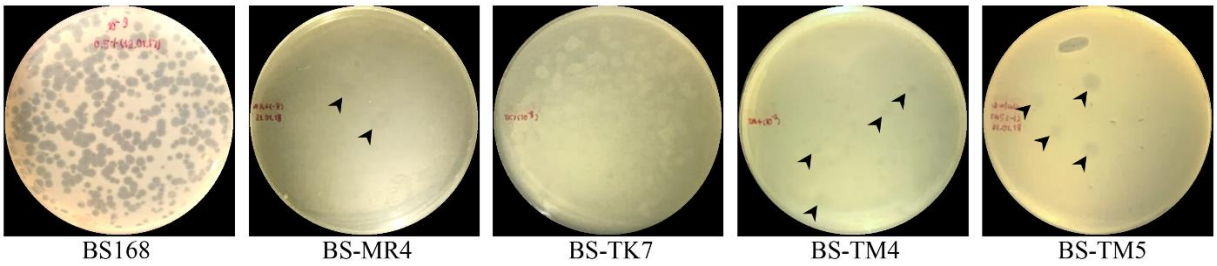

Figure 5 Bacteriophage SPP1 infected in the *B. subtilis* 168, *B. subtilis* isolate MR4, TK7, TM4 and TM5.

In our finding, the YueB primary sequences of *B. subtilis* isolate MR4, TK7, TM4 and TM5 consisted of 1,059 amino acid residues when compared with *B. subtilis* 168 (1,076 amino acid residues). The secondary predicted structures of YueB revealed that the ectodomain cellular contained putative N-terminal signal peptide and five transmembrane segments were localized C-terminus which was retrieved from all isolates. The four regions of coiled coil structure in *yueB* gene from *B. subtilis B. subtilis* isolate MR4, TK7, TM4 and TM5 were located in different positions from *B. subtilis* 168 which were comprised of three coiled coil regions. The efficiency of bacteriophage SPP1 infection in *B. subtilis* isolate MR4, TK7, TM4 and TM5 revealed that the small phage plaques were produced. In bacteriophage SPP1 infection, viral DNA ejection was required for the receptor binding for interaction with the tail of bacteriophage so that the signal was transmitted to the tail helical structure leading to the opening of the portal protein gp16 channel and the tail tip was essential for the recognition site of the membrane receptor and degradation of the cell wall during phage infection. This result indicated that the coiled coils structure of the YueB membrane receptor protein and deletion of amino acid residues in *B. subtilis* isolate MR4, TK7, TM4 and TM5 could be affected to interact with bacteriophage conformation, specific recognition on the cell surface site might be changed for initiate infection [12]. However, the *B. subtilis* isolates should be observed an OD_{600} before the determining of bacteriophage infection with the initial of *B. subtilis* culture in each isolate. In addition, it should be noted that the three-dimension structure of the YueB membrane receptor protein in *B. subtilis* was not fully investigated. The YueB protein structure could be providing more information using X-ray crystallography which is the current technique for structure determination of proteins and biological macromolecules such as proteins, nucleic acids, or viral particles. The three-dimensional structure provided information on the atom positions and specific atomic interactions. Crystallography could provide reliable answers to many structure-related questions from global folds to atomic [10]. This leads to a lack of evidence to understand the pathway of bacteriophage SPP1 infection on *B. subtilis*.

4. Conclusions

In summary, twenty isolates of *Bacillus* spp. isolated from Thua nao were successfully investigated, classified and presented in a phylogenetic tree. Nucleotide sequences of full-length 16S rRNAs and partial 23S rRNAs were published in terms of the sequences available *via* the GenBank® database. The results of the phylogenetic analysis indicated that twenty *Bacillus* spp. isolated from Thua nao were closely related to the six clusters of *B. amyloliquefaciens*, *B. aryabhattai*, *B. cereus*, *B. licheniformis*, *B. sonorensis*, *B. subtilis* and *B. vallismortis*. *Bacillus* spp. isolate MR4, TK7, TM4 and TM5 were similar to *B. subtilis*. The nucleotide sequences of the *yueB* gene obtained from *B. subtilis* isolate MR4, TK7, TM4 and TM5 were found to be approximately 3.2 kb in size. The complete protein translation was performed and 1,059 amino acid residues were retrieved in all the isolates, whereas *B. subtilis* 168 encoded 1,076 amino acid residues. A similar alignment protein sequence with a maximum identity score of 81% was observed and 1% of gaps on protein alignment tools were found. The predicted secondary structure of the YueB membrane receptor protein comprised of six transmembrane domains presented in *B. subtilis* isolate MR4, TK7, TM4 and TM5. Moreover, the four regions were folded as coiled coil structures in *B. subtilis* isolate MR4, TK7, TM4 and TM5 while three regions in *B. subtilis* 168 were found. In addition, based on the mechanism of bacteriophage infection *via* YueB membrane receptor protein, the disruption of the *yue*B gene in *Bacillus* species can prevent the bacteriophage transmission into the bacterial membrane. Therefore, the bacteriophage biocontrol model was used in Thua nao fermentation using *yue*B gene knock out strains from *Bacillus* spp. and could provide benefits for the Thua nao fermentation process in the future.

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6. References

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