CHARACTERIZATION OF BACTERIOPHAGES INFECTING *Bacillus subtilis* TN3

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MASTER OF SCIENCE

IN

BIOLOGICAL SCIENCE

SCHOOL OF SCIENCE

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ABSTRACT

Infection of bacteriophage is of great concern in food industry as this can result in complete fermentation failure. In this work, a virulent bacteriophage, named BasuTN3 was isolated from *thua nao*, a traditional fermented soybean in Thailand. The stability of phage was evaluated under various ranges of temperature, pH, chloroform, UV, and disinfectants. Our results showed that this phage appeared to be quite specific to its bacterial host, which was further identified as *Bacillus subtilis* strain TN3 based on the 16S rRNA gene sequence analysis. Under TEM, the BasuTN3 phage belonged to the family Myoviridae member. This phage could withstand a wide temperature range (4 – 45 ^{o}C) and pH conditions (5 - 11). The BasuTN3 phage was sensitive to chloroform, UV, and commonly used disinfectants. The results obtained expand the knowledge of the *Bacillus* phage diversity in the fermented soybean product, and provide a basis of information for the phage and its bacterial starter culture which can be useful for phage control.

Keywords: *Bacillus subtilis*, Bacteriophage, Fermented Soybean, *Thua Nao*

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Bacteriophage

Bacteriophages (Phages) were recognized as a virus infecting bacteria and it is the most abundant biological entities on earth, which outnumber bacteria by an estimated 10-fold (Brüssow & Hendrix, 2002; de Melo et al., 2018). The structure of bacteriophages mostly consists of head (capsid), which contains phage genetic material (either DNA or RNA), and tail. At least 5,360 tailed and 179 cubic, filamentous, and pleomorphic bacterial viruses have been examined under the electron microscope since the introduction of negative staining in 1959 (Ackermann, 2011). Bradley (1967) reported that the three obvious taxonomic criteria, which could divide bacteriophages into groups, were nucleic acid type, morphology, and host range, the first two being generally acceptable. International Committee on Taxonomy of Viruses (ICTV) classified bacteriophages into *Caudovirales* order, and it was divided into 13 families and 30 genus. The morphological of bacteriophages could considered into four major groups: (I) tailed phage (with contractile tails, long and non-contractile tails, and short tails); (II) polyhedral phage; (III) filamentous phage; (IV) pleomorphic phage (Figure 1.1). The nucleic acid of bacteriophages have four types, it is consist of single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA). Over 96% of phages are tailed and contain dsDNA. This phage are cubic symmetry head (polyhedral or icosahedral), helical tail and contained dsDNA. Tailed phages were divided into 3 families: (I) *Myoviridae*, it has got central tube surrounding by sheath and found 25% out of phages, and it was divided into 6 genus; T4, P1, P2, Mu, SPO1 and ΦH; (II) *Siphoviridae*, it is a long tail phage and found 61% out of phages, and it was divided into 6 genus; λ, T1, T5, L5, c2 and

ΨM; (III) *Podoviridae*, it is short tail phage and found 14% out of phages, and it was divided into 3 genus; T7, P22 and Φ29.

Bacteriophages are interrupting bacteria with their pathways either lytic or lysogenic pathways. Lytic phage (or lytic pathway) is replicating inside the bacterial host by using host's biosynthesis, and it produces endolysin enzyme to destroying host's cell wall, then their progeny were released (Clokie et al., 2011). On the other hand, lysogenic phage (or lysogenic pathway) is integrating their genome into the host genome, and then it replicates together with their host (Orlova, 2012). Most phages, the whole processes (from attachment to lysis) were showed in figure 1.2. The first step is attachment or adsorption (bacteriophage attaches to a protein or polysaccharide molecule (receptor) on the surface of the bacterial host). Second step is penetration (it injects its genome into the bacterial cell). Third step is biosynthesis (Phage genes are expressed resulting in the production of phage DNA and phage proteins). Fourth step is assembly (pieces or parts of phage are assembled to create complete new phage particles). Last step is release (bacteriophages escape from the bacterial cell by lysis of the cell).

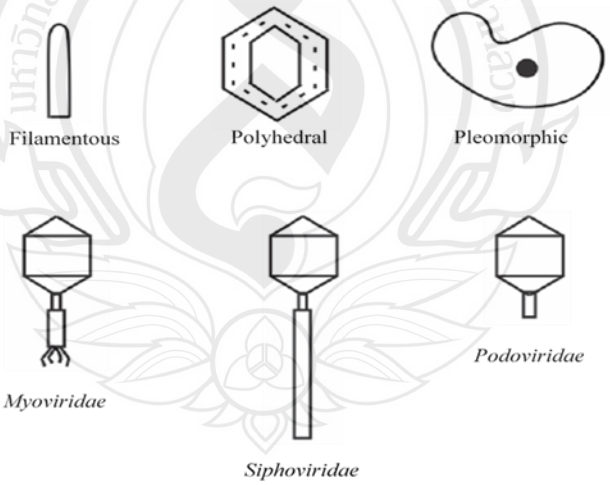
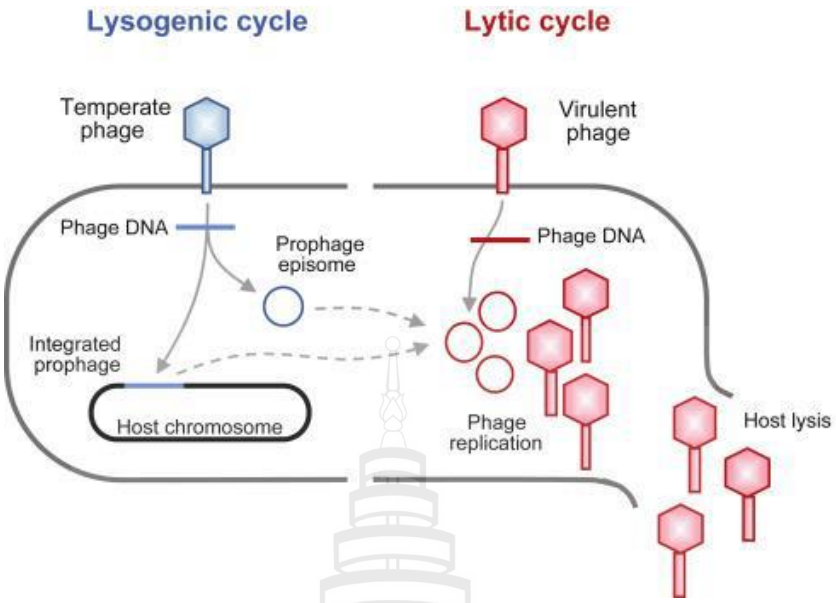


Figure 1.1 Bacteriophages structures



Source Fortier (2017)

Figure 1.2 Lytic and lysogenic cycles

1.2 Prevalence of Bacteriophages in Fermented Food Products

Bacteriophage ecology has raised an increasing attention over the past few years. Fermented foods have got host dense and diverse microbial communities. Therefore, it represented an ideal biotope for bacteriophages (Paillet & Dugat-Bony, 2021). The diversity of microbial is according to the nutrients available in the raw material, abiotic parameters such as temperature, humidity, oxygen, and osmotic pressure, and microbial interactions. Phage-bacteria interactions can potentially affect the balance between the different functional groups of microbes and then (re-)shape microbial communities (Paillet & Dugat-Bony, 2021). Up to date, industries such as foods and medicals are using bacteriophage to be a biocontrol agent pathogenic bacteria and bacteria antibiotic resistance. In the other hand, some products of fermented foods used bacteria to fermentation, but its manufacturing was contaminated by bacteriophage. Hence, it was affecting to the quality of product and leading to the loss of manufacturing business. Many products were interfered by phages such as daily

based foods (cheese and yogurt), vegetable-based foods (fermenting cucumber, kimchi, kefir, soybean and wine), and animal-based foods (fermented sausages and fermented pork) were showed in table 1.1. These products were not completely fermentation and low quality than normal. Moreover, the screening of bacteriophages in fermented soybeans are extremely reported, especially in Japanese *natto*. Fujii et al. (1967) reported that manufacturing of Japanese *natto* had been contaminated by PM1 phage resulting in non-normal production of the viscous polymer polyglutamic acid (PGA). Umene and Shiraishi (2013) reported that one ORF of PM1 phage could be producing an enzyme poly-glutamate hydrolase. So, this phage was assumed to degradation the capsular barrier of host cells for increasing the efficiently of infection (adsorption step). Therefore, *natto* products were affected by bacteriophage, were considered spoilage, and led to a tremendous loss to the *natto* manufacturing business (Chukeatirote et al., 2018). Besides, Kim et al. (2011) also reported that the phage Bp-K2 was also found to be active against many *B. subtilis* starter culture strains of the Korean *chungkookjang*, albeit less active against to Japanese *natto* bacterial strains. In addition, Chukeatirote et al. (2018) also reviewed the characteristic of related bacteriophage in fermented soybean products (Table 1.2). Currently, there are many studies of phage contamination and prevention in food products and manufacturing, but still limited, because of the diversity and environmental of bacteria.

Table 1.1 Fermented food products and their associated bacteriophages

Fermented food	Phage type	Bacterial host	References
Cheese	Virulent	*Propionibacterium freudenreichii*	Cheng et al. (2018)
	Virulent	*Enterococcus faecalis*	Del Rio et al. (2019)
	Virulent	*Brevibacterium aurantiacum*	de Melo et al. (2020)
	Temperate and filamentous	*Propionibacterium freudenreichii*	Gautier et al. (1999)

Table 1.1 (continued)

Fermented food	Phage type	Bacterial host	References
Fermented pork	Virulent	*Weissella cibaria*	Pringsulaka et al. (2011)
Yoghurt	Virulent and temperate	*S. thermophilus L. delbrueckii*	Auad et al. (1997)
Fermented cucumber	Virulent	*Lactobacillus plantarum*	Plengvidhya et al. (2003)
	Virulent	*Pediococcus* sp.	Yoon et al. (2007)
Fermented soybean	Virulent and temperate	*Bacillus subtilis*	Nagai and Yamasaki (2009)
	N.D	*Bacillus cereus*	Oh and Park (2017)
	N.D	*Pediococcus halophilus*	Uchida and Kanbe (1993)
Kefir	N.D	*Lactobacillus plantarum*	De Antoni et al. (2010)
Kimchi	N.D	*Weissella cibaria, Leuconostoc citreum*	Kong and Park (2019)
Sauerkraut	N.D	*Leuconostoc fallax*	Barrangou et al. (2002)
	Virulent	*Leuconostoc mesenteroides*	Lu et al. (2010)
Salami	N.D	*Staphylococcus carnosus*	Bruttin et al. (1992)
Sourdough bread	Virulent and temperate	*Lactobacillus fermentum*	Foschino et al. (2001)
Wine	Virulent	*Oenococcus oeni*	Jaomanjaka et al. (2016)
	Temperate	*Oenococcus oeni*	Santos et al. (1996)
	Temperate	*Gluconobacter cerinus*	Philippe et al. (2018)

Table 1.2 Fermented soybean (FSB) products related bacteriophages

FSB-related phages	Host	Family	Morphology	References
Natto				
JNDMP	BS	S	Hexagonal head Non-contractile tail	Nagai and Yamazaki (2009)
ϕNIT1	BS	M	Isometric head Contractile tail	Ozaki et al. (2017)
PM1	BS	S	A long, non-contracted tail virus	Umene et al. (2009)
BN100	BS	S	Hexagonal head Non-contractile tail	Nagai and Itoh (1997)
PBND8	BS	M	Hexagonal head Contractile tail	Tsutsumi et al. (1990)
ONPA	BS	M	Hexagonal head Contractile tail with sheath	Nagai and Yamazaki (2009)
Cheonggukjang				
816 phage	BS	ND	Hexagonal head	Lee (1978)
BCP8-2	BC	M	Similar to BCP1-1, with long contractile tail	Bandara et al. (2012) and Asare et al. (2015)
BCP1-1	BC	M	Icosahedral head Contractile tail	Bandara et al. (2012)
Bp-K2	BS	M	Icosahedral head Contractile tail with a basal plate	Kim et al. (2011)

Table 1.2 (continued)

FSB-related phages	Host	Family	Morphology	References
Cheonggukjang				
BSP18	BS	M	Isometric head Contractile tail	Ghosh et al. (2018)
JBP901	BC	M	Hexagonal head Contractile tail	Shin et al. (2011) and Asare et al. (2015)
Soy sauce				
ϕD10	TH	S	Hexagonal head Non-contractile tail	Higuchi et al. (1999)

Note BS for *Bacillus subtilis*, BC for *B. cereus*, TH for *Tetragenococcus halophile*, S for *Siphoviridae*, M for *Myoviridae*

1.3 *Thua Nao*

Thua nao is a traditional fermented soybean food widely consumed in northern Thailand (i.e., Chiang Mai, Chiang Rai, Lampang, and Mae Hong Son) for flavor enhancer (Chukeatirote, 2015; Dajanta et al., 2011; Leejeerajumnean, 2003). *Thua nao* is usually produced conventionally by spontaneous fermentation of soybeans using naturally occurring microbes, and it consists of two forms are fresh and dry forms (Figure 1.3). This results in vastly different properties of *thua nao* in different areas. The manufacturing of *thua nao* can be divided into 3 major steps: the cleaning and soaking of raw soybeans, cooking and preparing soybeans by heat, and wrapping by banana leaf. Similar fermented soybean products to *thua nao* include *tempeh* in Indonesia (Astuti et al., 2000) *dou chi* in China, *kinema* in India and Nepal, *cheonggukjang* in Korea, and *natto* in Japan (Hosoi & Kiuchi, 2003). Mostly, the soybean fermented food products were fermented by *Bacillus* group as predominant,

especially *B. subtilis* (Chukeatirote & Thakang, 2006). Steinkraus (2002) and Chukeatirote (2015) also reviewed the fermented soybeans are mainly produced by the activity of the bacteria belonging to *Bacillus* genus. As the result, the *Bacillus* genus can form spores that are even less susceptible to UV, heat, desiccation, and the lack of nutrients, which can enhance the survival and consequently augment the dispersal of microorganisms belonging to this genus (Polonca, 2020). Besides, the final product of the other fermented soybean products were received scientific attention due to their potential health benefits such as anticancer, antioxidant, fibrinolytic activities, anti-diabetic, anti-inflammatory, anti-hyperlipidemic, immunostimulatory activity, and neurostimulatory (Ghosh et al., 2018; Jayachandran & Xu, 2019; Jung et al., 2006; Kwak et al., 2007; Park et al., 2003; Petchkongkaew et al., 2008; Shin & Jeong, 2015). For these, the soybean has protein-rich, and it was hydrolyzed into peptides, amino acids, and ammonia by proteolysis and other enzymatic activities such as amylases, phytases, and lipases from bacterium, especially *B. subtilis* which have been reported during fermentation of *thua nao* (Chukeatirote & Thakang, 2006). Hence, these fermented soybean products have different textures, flavors, aromas, and nutrition. Therefore, the quality of fermented soybean products depends on soybean variety, fermentation conditions, and indigenous microflora. Furthermore, the ammonia releasing are a cause for the alkaline conditions. This alkaline condition, the pH can reach as high as eight, so this condition is selective for some certain bacteria such as *Bacillus* species (Chukeatirote, 2015).

Bacteriophages are ubiquitous in nature and have significantly affected to food industries as they can kill the bacterial starter, thus delaying the manufacturing and yield low-quality products, leading to economic losses (Garneau & Moineau, 2011; Nagai, 2012). Bacteriophage infecting bacteria on the manufacturing of soybeans and fermented soybean products are mostly studies in Japan, Korea, and only a few studies in Thailand. The aim of this study was characterization BasuTN3 bacteriophage isolated from *thua nao*. This phage was infecting *B. subtilis* strain TN3, which was also isolated from *thua nao* and it could be a potential bacteria starter culture for soybean fermentation based on its protease activity. This phage might have an effect to fermentation failure and infecting to other *Bacillus* species. Therefore, the efficient

control to this phage contamination for bacteria starter of *thua nao* product or others bacterial starter was studied.

Fresh Dry

Figure 1.3 *Thua nao* forms

CHAPTER 2

MATERIALS AND METHODS

2.1 *Thua Nao* Sample and Bacterial Strains

Sixteen *thua nao* samples were collected from Mueang Chiang Rai, Chiang Rai, Thailand. The bacterial strains (*Bacillus* and non-*bacillus*) were used in this study are listed in table 2.1. All of these bacteria were kept as 30% glycerol stock at -70 °C. These bacterial strains were routinely cultured in trypticase soy broth (TSB) or plated on trypticase soy agar (TSA) medium (Difco Laboratory, Detroit, MI, USA). The broth cultures were grown with aeration at 37 °C in a shaking incubator (200 rpm), and the agar plates were incubated at 37 °C for 24 h.

Bacterial strains (*Bacillus* strains TN3, DM1-1, DM1-3 and FM2-3) isolated from *thua nao* were screened for their proteolytic activity. Their protease activity of these bacterial strains were compared to *B. subtilis* strains TN51, ASA, BEST195, ATCC6633 and TISTR1248. Activity of the protease enzyme was evaluated by using casein agar assay according to Dajanta et al. (2009) with some modifications. For this, a single colony of these bacteria was inoculated into a test tube containing 5 ml trypticase soy broth. The culture was then grown at 37 °C for 24 h with aeration in a shaking incubator (200 rpm). After incubation, the bacterial cells were harvested by centrifugation at 14,000 rpm for 15 min at 4 °C, and then the supernatant was transferred into new 1.5 ml tube and centrifugation one more round. The supernatant was collected for further use as the source of the protease enzymes, which was referred to the “crude extract”. Twenty microliter of the crude extract were used to spot on a trypticase soy agar plate (containing 20% skim milk). For the colony, a single colony of these bacterial strains were also spotted on a trypticase soy agar plate (containing 20% skim milk). The inoculated plates were then incubated at 37 °C for 24 h.

The presence of a clear zone was recorded and used for indicate the bacterial ability to produce proteases.

Table 2.1 List of bacterial strains used in this study

Species	Strains	Source	Reference
***Bacillus* species**			
B. cereus	ATCC 11778	ATCC	-
B. cereus	TISTR 687	TISTR	-
B. cereus	TISTR 1527	TISTR	-
B. cereus	TN69	*Thua Nao*	Dajanta et al. (2009)
B. licheniformis	TISTR 1109	TISTR	-
B. subtilis	ASA	*Natto*	Dajanta et al. (2009)
B. subtilis	ATCC 6633	ATCC	-
B. subtilis	BEST 195	*Natto*	Qiu et al. (2004)
B. subtilis	DM1-1	*Thua Nao*	This study
B. subtilis	DM1-2	*Thua Nao*	This study
B. subtilis	DM1-3	*Thua Nao*	This study
B. subtilis	FM2-1	*Thua Nao*	This study
B. subtilis	FM2-2	*Thua Nao*	This study
B. subtilis	FM2-3	*Thua Nao*	This study
B. subtilis	FM2-4	*Thua Nao*	This study
B. subtilis	S1-13	*Terasi*	Chukeatirote et al. (2016)
B. subtilis	S4-5	*Terasi*	Chukeatirote et al. (2015)
B. subtilis	TISTR 1248	TISTR	-
B. subtilis	TN3	*Thua Nao*	This study
B. subtilis	TN51	*Thua Nao*	Dajanta et al. (2009)
B. thuringiensis	S2-3	*Terasi*	Chukeatirote et al. (2015)

Table 2.1 (continued)

Species	Strains	Source	Reference
Non-*Bacillus* species			
E. aerogenes	TISTR 1540	TISTR	-
E. coli	TISTR 527	TISTR	-
M. luteus	TISTR 884	TISTR	-
P. aeruginosa	TISTR 781	TISTR	-
S. aureus	TISTR 746	TISTR	-

Note ATCC for American Type Culture Collection, TISTR for Thailand Institute of Scientific and Technological Research

2.2 Screening and Isolation of Bacteriophage Specific for *B. subtilis*

The initial preparation of *thua nao* samples, twenty grams of *thua nao* sample were mixed with 80 ml of trypticase soy broth (TSB) (Difco Laboratory, Detroit, MI, USA), and then incubated at 37 °C with shaking (200 rpm) for 72 h to increase the number of bacteriophages. After incubation, the mixture was centrifuged at 11000 g for 10 minutes and filtered through a 0.2 µm filter (Whatman, Little Chalfont, UK) to remove any food matrix and bacterial lysates. The filtrate was tested with a spotting technique to detect any presence of bacteriophage, and the phage filtrate was further propagated and purified according to Sambrook and Russel (2001) with some modifications. For the spotting technique, briefly, the fresh culture of the bacterial host was mixed with the molten soft TSA containing 0.5% of agar, and the mixture was poured onto the TSA plate to form a bacterial lawn layer (or an upper layer). After the upper layer solidifies, 10 µl of filtrate was then applied (or spotted) and left to dry. After that, the plates were incubated at 37 °C for 24 h, and the presence of clear zones, or plaque formation, indicated the presence of bacteriophages specific to the bacterial host. For the enumeration of phage in a suspension, the double layer agar (DLA) technique was used. Similarly, the fresh culture of the bacterial host was mixed with the serially

diluted phage suspension in molten soft TSA, and poured onto TSA plates and left to solidify prior to incubation. After the incubation, the number of clear dots (or plaques) were used to calculate the number of phage particles, which was expressed as plaque forming unit per milliliter (PFU/ml).

2.3 Bacterial Identification

Bacillus bacterial strains sensitive to the bacteriophage were further identified as follows: morphological, biochemical, 16S rRNA gene sequence analysis and antibiotic susceptibility tests according to MacFaddin (2000) and Kanghae et al. (2016).

2.3.1 Morphological Characterisation

The bacterial strains were susceptible to bacteriophage, were characterized based on morphological characteristics. Initial typing was based upon colony and cell morphology, presence of endospore, and Gram-staining after growth on trypticase soy agar (TSA) (Difco) for 24 h. For this, a single colony of the bacteria was spotted on TSA plate. The plate was then incubated at 37 °C for 24 h for observe the colony morphology, and the Gram-straining was examined for cell morphology.

2.3.2 Biochemical Test

The bacterial strains were characterised to observe their biochemical properties. These included Gram-staining, presence of spore, oxygen requirement, catalase test, IMViC test, nitrate reduction, fermentation of glucose, arabinose, xylose, and sucrose, and starch hydrolysis (MacFaddin, 2000). Additionally, the bacterial species confirmation was performed using the API-50 CHB kit (bioMerieux, Inc.).

2.3.3 16S rRNA Gene Sequence Analysis

The 16S rRNA gene was amplified using the universal primers 518F and 800R (Ghyselinck et al., 2013). For this, the total genomic DNA was extracted using a G-spin genomic DNA extraction kit (iNtRON Biotechnology, Inc.). The 16S rRNA gene was then PCR-amplified according to the method described previously by Chukeatirote et al. (2015). The purified PCR products were sent to U2Bio (Thailand) for sequencing. The accession number of the *Bacillus* sp. TN3, DM1-2, FM2-3 and ASA were

OL677200, OL677201, OL677202 and ON358102 respectively. The sequence was then compared to those of the related *Bacillus* species in the GenBank database. The phylogenetic tree was constructed using MEGA 11 (Tamura et al., 2021).

2.3.4 Antibiotic Susceptibility Tests

Antimicrobial susceptibility was performed using the disc agar diffusion method (National Committee for Clinical Laboratory Standards [NCCLS], 1997). A bacterial culture was prepared in 5 ml of tryptic soy broth and incubated at 37 °C for 24 h with shaking at 180 rpm. A sterile cotton swab was dipped into the inoculum and applied evenly onto the surface of trypticase soy agar plate. After drying for 5 min, the antibiotic discs (Oxoid, England) were placed and the plates were incubated at 37 °C for 24 h. The zones were measured and expressed as sensitive, intermediate, and resistant as described by Hong et al. (2008). Antibiotic discs were used consist of ampicillin (10 μg), bacitracin (10 U), chloramphenical (30 μg), clindamycin (10 μg), cefpodoxime (10 μg), erythromycin (15 μg), kanamycin (30 μg), streptomycin (10 μg), tetracycline (30 μg), polymyxin (300 U), and vancomycin (30 μg).

2.4 Characterization of Bacteriophage

2.4.1 Phage Purification and Propagation

The phage sample (e.g., filtrate or a plaque) was used for bacteriophage propagation. One-hundred microliters of the filtrate (or phage suspension), or one loop-full of agar plaque, was mixed with 100 μl of suitable bacterial host strain in 10 ml of TSB. The mixture was incubated for 24 h at 37 °C with shaking (180 rpm). After incubation, the mixture was then centrifuged at 11000 g for 10 min to remove bacterial lysates. The supernatant was filtered using 0.2 μm pore size filters, and the concentration of the bacteriophage was calculated according to 2.2 by the double layer agar (DLA) technique. The phage suspension was then kept at 4 °C and wrapped with aluminum foil to avoid contacts with light.

The bacteriophage was isolated and purified using DLA technique. The serially diluted (20 - 100 PFU/ml) phage suspension was used with DLA technique to obtain a single, isolated plaque formation. After the incubation, the single, clear lytic plaque was

selected and repeated for further rounds of the bacteriophage purification. The purification of bacteriophage was repeated at least 3 times to ensure the purity of bacteriophage. After the purification, the lytic activity and the purity of the bacteriophage isolates was tested using the spotting and DLA techniques.

The phage suspension of high titer was prepared using the plate elution method. Briefly, serially diluted phage suspension (10^5 - 10^6 PFU/ml) was used for DLA technique. After incubation, the upper layer, which was fully filled with the clear plaque formation, was added with two milliliters of 10 mM Tris-HCl buffer solution, and removed into 50 ml tubes. The content was then centrifuged at 11000 g for 10 minutes, and the supernatant was filtered using 0.2 µm pore size filters to remove bacterial lysates. The suspension was then determined for phage concentration by DLA techniques and kept at 4 °C.

2.4.2 Host Range Determination

The infectivity of bacteriophage isolates were determined on multiple bacterial hosts using the spotting technique. The phage suspension was serially diluted to obtain pre-determined concentration of phage (10^2-10^7 PFU/ml) and used in the spotting technique. Each dilution of phage suspension was applied on the bacterial lawn to determine the specificity and the minimum concentration required for lytic activity on different bacterial hosts. The bacterial hosts used listed in table 2.1.

2.4.3 Efficiency of Plating (EOP)

Relative efficiency of plating (EOP) of BasuTN3 bacteriophage on *B. subtilis* strains TN3 and ASA were determined. It was performed by the double layer agar technique of the diluted phage (10-10^3 PFU/ml) and differences hosts. Percent EOP was calculated by the formula (phage titer on target strain divided by phage titer on the host), and the values for EOP of >0.5 were ranked as ‘high’ efficiency; 0.2 - 0.5 as ‘medium’ efficiency; 0.001 - 0.2 as ‘low’ efficiency; <0.001 was considered as not effective against the target strain (Vongkamjan et al., 2017).

2.4.4 Optimal Multiplicity of Infection (MOI) Determination

The optimal MOI for each bacteriophage was determined according to Ji et al. (2015) with some modifications. Briefly, the bacteriophage suspension with known

concentration was prepared. One-hundred microliter of bacteriophage suspension with known concentration (10^4-10^7 PFU/ml) and 100 µl of fresh bacterial culture with known concentration (10^5 CFU/ml) were added into 1.5 ml tubes, containing 800 µl of TSB. For the control, the sterile distilled water was used instead of phage suspension. The mixture was then incubated at 37 °C for 3 hours. The number of colonies forming unit (CFU) of any surviving bacteria was determined. To evaluate the optimal MOI, a reduction rate (log Nt/Nc) was calculated by comparing the number of the host cells in the control (Nc) with the number of the host cells treated with the bacteriophage (Nt) after incubation (Phongtang & Chukeatirote, 2021).

2.4.5 Effects of Divalent Cations on Phage - Host Interaction

The effects of divalent cations on adsorption rate of bacteriophage isolate was determined by incubating bacteriophage with a specific host in TSB with and without salt (10mM of $CaCl_2$ and 10mM of $MgCl_2$) supplement. Similar to the 2.4.3, the bacteriophage suspension and the fresh bacterial culture were mixed in TSB (with and without salt supplement) to achieve the MOI of 0.1. After that, the mixture was incubated at 37 °C for 2 h. After the incubation, the culture was centrifuged at 11000g for 10 minutes to precipitate the bacteria, and the supernatant was collected to determine the number of free phage particles in the solution by DLA technique.

2.4.6 Adsorption Assays

The influence of temperature and pH on phage-host interaction was determined according to Chen et al. (2019) with some modifications. Briefly, one-hundred microliter of phages (10^6 PFU/ml) and host culture (10^6 CFU/ml) were suspended in the 1.5 ml tubes at the MOI 1, and then incubated in different conditions.

The influence of temperature on phage adsorption, the suspended between phage and host at the MOI 1 was incubated at 4, 37, and 45 °C for 1 h. Another experiment for the influence of temperature on phage adsorption was determined by incubate the suspended at 37 °C for 20, 40, 60, and 65 min. The influence of pH on phage adsorption, the suspended between phage and host at the MOI 1 was incubated at pH values of 5, 6, 7, 8, and 9 at 37 °C for 1 h. After the incubation, the samples were centrifuged at 11000g for 5 min to precipitate the phage attachment with bacteria, and the supernatant (containing free phage particles) was collected to determine the number

of free phage particles by DLA technique. For the control, the TSB was used instead of bacteria suspension. Results were expressed as percentages of adsorption. Percent adsorption of the phage was calculated as [(control titre – residual titre)/ control titre] x 100% (Fleming et al., 2003).

2.4.7 Stability Assays

The stability of bacteriophage was tested in terms of temperature, pH, ultraviolet (UV) light exposure and chloroform. For the temperature stability, 200 µl of each phage suspension was aliquoted into 1.5 ml tubes, and the tubes were incubated at -25, 4, 37, 45, 55, 60 and 65 °C for 2 h. For the pH stability, 100 µl of each phage suspension was mixed with 900 µl of pH adjusted TSB (pH 3 - 13) and incubated at 37 °C for 2 h. For UV stability, 500 µl of phage suspension was applied on a sterile petri-dish and exposed to the UV light in BSC (15, 30, 45, 60, and 120 min). Chloroform sensitivity of the bacteriophage was also determined by adding 50 µl of chloroform to the 1000 µl of phage suspension in 1.5 ml tubes. The tubes were sealed with para-film and kept at 4 °C for 24 h. In addition, to investigate the presence of lipid in the viral capsid, 200 µl of phage sample was mixed with 200 µl of chloroform, and then incubated for 30 min at room temperature (Seaman & Day, 2007). The phage concentration used in the aforementioned experiments were in the range of 10^6 to 10^8 PFU/ml. After all these experiments, the bacteriophage residual was determined using the DLA technique.

2.4.8 Effects of Disinfectants on Bacteriophage Viability

The stability of bacteriophage in various commercial disinfectants were also determined. The commercial disinfectants were used in this study were consists of 70% v/v Ethyl alcohol, 0.06% v/v NaOCl (Haiter^{TM}), 5% v/v Dettol^{TM} and 5% v/v Dishwashing solution (Lipon F^{TM}). Briefly, 100 µl of phage was added into 900 µl of already prepared disinfectant. Immediately after mixing, 100 µl of the mixture was serially diluted and determined for the bacteriophage residual by DLA technique.

CHAPTER 3

CHARACTERISATION OF BACILLUS STRAINS SUSCEPTIBLE TO BACTERIOPHAGE BasuTN3

3.1 Introduction

Thua nao is an ethnic fermented soybean of Thailand. Its manufacturing was cooking soybeans with naturally occurring microbes (Chukeatirote, 2015). It is similar to Japanese *natto* (Kiuchi & Watanabe, 2004), Indian *kinema* (Sarkar & Tamang, 1995), and Korean *chongkukjang* (Lee et al., 2005). These fermented soybean foods were also processed by microbial activity. In general, fermented food products could be classified base on the raw materials (i.e. beverages, dairy, fish or meat, fruit, and vegetable) (Steinkraus, 2002). For *thua nao*, it is a fermented soybean product. The soy proteins are hydrolyzed to be peptides, amino acids, and ammonia by enzymatic activities of bacterium, especially *B. subtilis* (Chukeatirote & Thakang, 2006). Many *Bacillus* species have been isolated during the production process as well as from the final products, these are suggesting their abundance (Kanghae et al., 2016). Besides, the potential health benefits of these products received scientific attention.

Bacteriophages are prevalent and have been reported in many food products. Their presence is important as interacting with their bacterial host in food matrices (Chukeatirote et al., 2018). This includes the fermentation of soybeans, which provides a unique niche for the *Bacillus* phages (Chukeatirote et al., 2018). Therefore, the presence of bacteriophage, It can destroy bacterial hosts, and makes the diversity of bacterial complex species by its life cycles. However, there are a few studies the bacteriophage in *thua nao*. This study were screening for bacteriophage infecting *B. subtilis* in *thua nao,* and characterization of *B. subtilis* susceptible to BasuTN3 bacteriophage.

3.2 Prevalence of *Bacillus subtilis*-infecting Bacteriophage

Eight filtrates from *thua nao* samples were screened for the presence of the lytic bacteriophage specific to *B. subtilis*. Initial screening was performed by applying *thua nao* filtrate samples on the bacterial lawn plates of seven *B. subtilis* strains (*B. subtilis* strains TN3, TN51, ASA, BEST 195, ATCC 6633, TISTR 1248, and S1-13). The isolation rate of *B. subtilis* bacteriophage obtained from this present study was 12.5%. The host range examination was quite low in each samples, whereas the prevalence of the *B. subtilis* bacteriophage in Japanese *natto* was 17.5% (Kubo et al., 2018), and 100% for Korean soybean-based fermented foods (Ghosh et al., 2018). It should be noted, however, that information on the existence of the bacteriophage in *thua nao* is limited due to the areas. Nevertheless, it could be assumed that the phage population would be plentiful considering the natural state of the fermented food products related to bacterial-associated fermentation. In addition, other eight filtrate samples were tested on the bacterial lawn plates of forty *Bacillus* strains isolated from *thua nao*. Interestingly, only one sample occurred the plaque morphology on *B. subtilis* strain TN3 (Figure 3.1). This bacterium has highest proteolytic activity. Subsequently, this filtrate was collected to purification and propagation for testing.

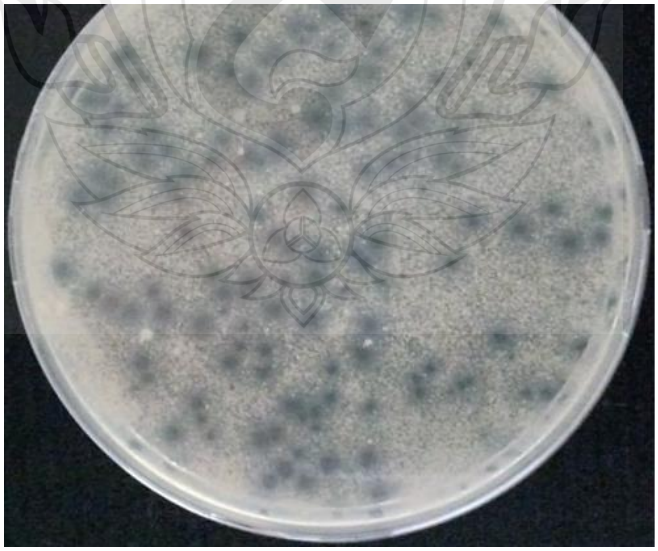


Figure 3.1 Plaque formation of BasuTN3 bacteriophages on *B. subtilis* strains TN3

3.3 Proteolytic Activity of *Bacillus subtilis* Strains

The *Bacillus* strains were calculated their proteolytic activity by using casein agar. In this study, the bacterial strains were isolated from *thua nao* (*Bacillus* strains TN3, DM1-1, DM1-3 and FM2-3), and *Bacillus* strain TN51 (previous studied) (Dajanta et al., 2009), the type strains (*B. subtilis* strains ATCC6633 and TISTR1248), and *B. subtilis* strains BEST195 and ASA (it was isolated from Japanese *natto*) were determined their proteolytic activity. The presence of clear zone was observed, the proteolytic activity was calculated and expressed as the relative index value (RI, a ratio between the diameter of the clear zone and that of the bacterial colony) (Kanghae et al., 2016). The RI values of colony of *Bacillus* strains BEST195, TN3, FM2-3 and TN51 were 1.69, 1.50, 1.48 and 1.47, respectively. For the supernatant of *Bacillus* strains TN3 and FM2-3 were 1.47 and 1.39, respectively (Table 3.2). *B. subtilis* strain TN3 exhibited its proteolytic activity, was higher than those of other *Bacillus* strains suggesting superior proteolytic activity. It is also expected that *B. subtilis* strain TN3 could be used as a pure starter culture based on its proteolytic activity, and this thus helps improving the manufacturing process and fermented soybean products.

Table 3.1 Proteolytic activity of *B. subtilis* strains

***Bacillus* sp.**	**RI value**	
	Colony	**Supernatant**
TN3	1.50±0.15	1.47±0.34
TN51	1.47±0.69	1.05±0.51
DM1-1	1.02±0.00	0.67±0.09
DM1-2	0.00±0.00	0.00±0.00
DM1-3	1.17±0.00	0.75±0.11
FM2-3	1.48±0.24	1.39±0.04
ASA	1.20±0.06	1.03±0.24

Table 3.1 (continued)

Bacillus sp.	RI value	
	Colony	Supernatant
BEST195	1.69±0.96	1.19±0.69
ATCC6633	1.00±0.63	0.63±0.57
TISTR1248	1.19±0.52	0.84±0.55

Note ATCC for American Type Culture Collection, TISTR for Thailand Institute of Scientific and Technological Research

3.4 Taxonomic Identification of Bacteriophage-susceptible *B. subtilis* Strains

3.4.1 Morphological Determination

The *B. subtilis* strains ASA, TN3, FM2-3 and DM1-2 were susceptible to BasuTN3 bacteriophage. These bacterial strains were grown on tryptic soy agar. The colony patterns of these *Bacillus* strains were very diverse, and their color were white. The strains TN3 and FM2-3 had mucilage (biofilm formation) around the circumference of the colony, and ASA had mucilage over the colony. For the DM1-2, it was quite not occurring mucilage (Figure 3.1). Tasaki et al. (2017) reviewed that, the colony morphology takes the shape of concentric rings, which result from a biofilm life cycle, fast expansion due to swarming motility and consolidation by proliferating matrix producers, alternate in the colony development. The cell morphology of these bacterial strains were gram-positive and endospore-forming bacilli (Figure 3.1). Several studies had showed that the predominant bacterial group of fermented soybean products are *Bacillus* species (Chukeatirote et al., 2018). Chantawannakul et al. (2002) and Chukeatirote and Thakang (2006) also reported that many of gram-positive, endospore-forming bacilli were also detected in *thua nao* from the beginning throughout the fermentation period. For these, this bacteriophage seems specific to its bacterial cognate.

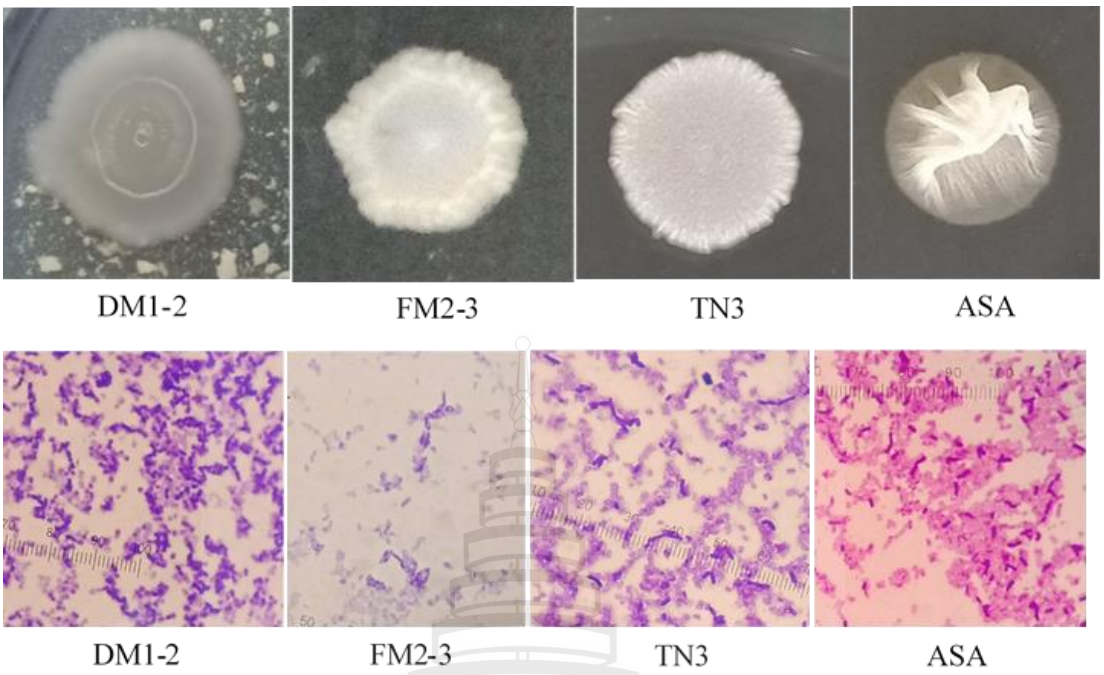


Figure 3.2 Colony and cell morphology of *B. subtilis* strains were susceptible to Basu TN3 phage

3.4.2 Biochemical Assays

The characterization of *B. subtilis* hosts were infected by BasuTN3 bacteriophage, the bacterial hosts were consist of *B. subtilis* strains TN3, DM1-2, FM2-3 and ASA. It was carried out using biochemical assay for bacterial identification suggested by Norris et al. (1981). All strains were classified as the *B. subtilis* results for biochemical tests as seen in table 3.2, according to the following taxonomic criteria: gram-positive bacilli, endospore forming, facultative anaerobic, catalase, positive reactions production of acetylmethylcarbinol and acid from glucose. Besides, the biochemical profiles of these strains were also similar to those of *B. subtilis* strains BEST195, which were obtained from *natto* products (Kanghae et al., 2016) and *Bacillus* sp. strain S1-13 isolated from Terasi products (Chukeatirote et al., 2016). Chantawannakul et al. (2002) also reported that the key identification of *Bacillus* species included catalase assay, Voges Proskaur (VP) test, growth under anaerobic condition, and starch hydrolysis. In this study, *B. subtilis* strains TN3, FM2-3 and ASA also exhibited strong proteolytic activity (Table 3.1).

In addition, the evaluation of carbohydrates utilization patterns of these bacterial strains were also determined using the API-50 CHB kit (Table 3.3). These bacterial strains were classified as *B. subtilis*/*B. amyloliquefaciens* for strains TN3 and DM1-2, *Paenibacillus validus* for strain ASA, and *Geobacillus thermoglucosidasius* for strain FM2-3, because its percentage of identification (Table 3.4). Bacterial strains TN3, ASA, and DM1-2 are *Bacillus* genus, and strain FM2-3 is *Geobacillus* genus. Analytical Profile Index (API) kit method was used in order to make suitable determine for complex species. Most *Bacillus* species differ in only one biochemical property makes classical biochemical identification at the species level very difficult (Aruwa & Olatope, 2015). Logan and Berkeley (1984) reported that, it was easy to identify the typical strains of common species by the dichotomous key but difficulty was encountered with atypical or intermediate strains. For these, Collins et al. (1991) noted that the API identification kits were more reliable in these circumstances. Therefore, API identification kits was used as a confirmation for bacterial species level.

Table 3.2 Biochemical test of *B. subtilis* strains were susceptible to BasuTN3 phage

Biochemical test	***B. subtilis* TN3**	***B. subtilis* ASA**	***B. subtilis* DM1-2**	***B. subtilis* FM2-3**	***B. subtilis* TN51**
Gram-staining	+	+	+	+	+
Shape	Rod	Rod	Rod	Rod	Rod
Starch hydrolysis	+	+	+	+	-
Methyl red test	-	-	-	-	-
Formation of Indole	-	+	-	-	+
Catalase test	+	+	+	+	+
Voges Proskauer	+	+	+	+	+
Citrate utilization	+	+	NA	NA	+
Nitrate reduction	-	+	NA	NA	+
Oxygen requirement	+	+	+	+	+

Table 3.2 (continued)

Biochemical test	***B. subtilis* TN3**	***B. subtilis* ASA**	***B. subtilis* DM1-2**	***B. subtilis* FM2-3**	***B. subtilis* TN51**
Acid from glucose	+	+	+	+	+
Gas in glucose	-	-	-	-	-
Fermentation of glucose	+	+	+	+	+
Lactose fermentation	+	NA	+	+	NA
Maltose fermentation	+	NA	+	+	NA
Protease production	+	+	+	+	+
Oxidase test	+	NA	+	+	NA
Growth in 7% NaCl	-	-	NA	NA	-
NB 50˚C	-	-	NA	NA	-
NB 65˚C	-	-	NA	NA	-

Note + for Positive reaction, - for Negative reaction, NA for Non-available

Table 3.3 Fermentation of carbohydrates of *B. subtilis* strains were susceptible to Basu TN3 phage

Biochemical test	***B. subtilis* TN3**	***B. subtilis* ASA**	***B. subtilis* DM1-2**	***B. subtilis* FM2-3**	***B. subtilis* TN51**
Glycerol	+	+	+	+	+
Erythirol	-	-	-	-	-
L-Arabinose	+	-	+	+	-
D-Ribose	+	+	+	+	+
D-Xylose	W	+	+	+	-
L-Xylose	-	-	-	-	-

Table 3.3 (continued)

Biochemical test	*B. subtilis* TN3	*B. subtilis* ASA	*B. subtilis* DM1-2	*B. subtilis* FM2-3	*B. subtilis* TN51
Dulcitol	-	-	-	-	-
L-Fucose	-	-	-	-	W
D-Arabitol	-	-	-	-	-
L-Arabitol	-	-	-	-	W
Xylitol	-	-	-	-	-
D-Mannitol	+	+	+	+	-
D-Sorbitol	-	-	-	-	-
Methyl α-D-mannopyranoside	-	-	-	-	-
Methyl α-D-glucopyranoside	W	+	+	+	-
N-Acetylglucosamine	-	-	-	-	+
Amygdalin	-	-	-	-	-
Aubutin	-	-	-	-	+
Esculin ferric citrate	+	+	+	+	+
Salicin	-	-	-	-	-
D-Cellobiose	-	-	-	-	-
D-Galactose	-	-	-	-	-
D-Glucose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Mannose	+	+	+	+	-
D-Sorbose	-	-	-	-	-
D-Arabinose	-	-	-	-	-
D-Turanose	-	-	+	-	-
D-Maltose	+	+	+	+	+

Table 3.3 (continued)

Biochemical test	*B. subtilis* TN3	*B. subtilis* ASA	*B. subtilis* DM1-2	*B. subtilis* FM2-3	*B. subtilis* TN51
Gentiobiose	-	-	-	-	-
D-Lyxose	-	-	-	-	-
D-Tagatose	-	-	-	-	-
D-Fuctose	-	-	-	-	-
D-Lactose (bovine origin)	-	-	-	-	-
D-Melibiose	-	-	+	+	-
D-Saccharose (sucrose)	+	+	+	+	+
D-Trehalose	+	+	-	-	+
Inulin	+	+	+	-	-
D-Melezitose	-	-	-	-	-
D-Raffinose	+	+	+	+	-
Amidon (Starch)	+	+	+	+	-
Potassium 2-ketogluconate	-	-	-	-	-
Potassium gluconate	-	-	-	-	W
D-Adonitol	-	-	-	-	-
Methyl β-D-xylopyranoside	-	-	-	-	-
Inositol	+	+	-	-	-
L-Rhamnose	-	-	-	-	-
Potassium 5-ketogluconate	-	-	-	-	W
Glycogen	+	-	+	-	-

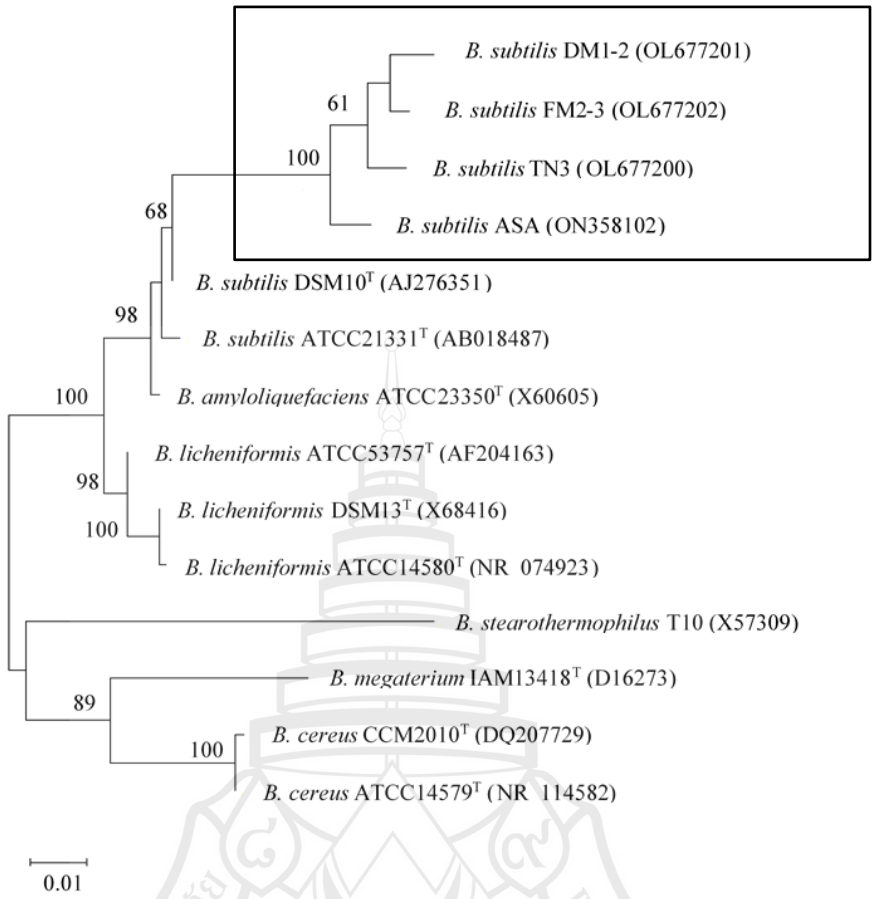
Note - for Negative (no change), + for Positive (yellow), W for weak reaction

Table 3.4 Percentage identification of *Bacillus* strains were susceptible to BasuTN3 phage

Bacillus **strains**	**Significant taxa**	**% identification**
TN3	*B. subtilis*/*B. amyloliquefaciens*	98.9
ASA	*Paenibacillus validus*	95.2
	B. subtilis/*B. amyloliquefaciens*	4.7
DM1-2	*B. subtilis*/*B. amyloliquefaciens*	97.7
FM2-3	*Geobacillus thermoglucosidasius*	41.9
	B. subtilis/*B. amyloliquefaciens*	41.1

3.4.3 Molecular Analysis of 16S rRNA Gene

The 16S rRNA gene sequences of the *B. subtilis* strains TN3, ASA, DM1-2 and FM2-3 have been deposited in GenBank with accession numbers OL677200, ON358102, OL677201 and OL677202, respectively. BLASTN results of the partial sequence of 16S rRNA gene (about 967-994 nucleotides) showed high similarity with *Bacillus* spp. with maximum identities for each isolate was more than 99%. The phylogenetic tree showed that four *Bacillus* strains were very closely related strains and grouping in the *B. subtilis* (Figure 3.2). Krasowska et al. (2015) reported that bacteriophages have a narrow host range may be useful for the phagotyping of bacterial species are closely related strains. This application depend on phage specificity (Hagens & Loessner, 2010), which is the phage can only attach to bacterial cells that possess the appropriate receptor (a protein or polysaccharide molecule on the surface of the bacteria cell).



Note The tree was generated by maximum likelihood method. Bootstrap values are based on 100 replicates and only the values greater than 50% are shown. Bar indicates 0.01 substitutions per 100 nucleotilde position. *B. cereus* was used as the outgroup taxon to root the tree.

Figure 3.3 A phylogenetic tree based on the 16S rRNA gene sequences of the *B.subtilis* species complex

3.4.4 Antibiotics Assay

These *Bacillus* strains were also examined for their antimicrobial susceptibility by using the agar disc-diffusion assay as recommended by the NCCLS (1997). The antimicrobial susceptibility test was performed to detect possible drug resistance in common pathogens (Jorgensen & Ferraro, 2009). Our data showed that all *B. subtilis* (TN3, ASA, DM1-2 and FM2-3) were sensitive to ampicillin, chloramphenicol and tetracycline. *B. subtilis* TN3 was resistant to bacitracin, cefpodoxime, polymyxin and

vancomycin, and moderately sensitive to erythromycin. *B. subtilis* ASA was resistant to bacitracin and moderately sensitive to erythromycin, kanamycin and streptomycin. *B. subtilis* DM1-2 and FM2-3 were not resistant to these antibiotics, except *B. subtilis* FM2-3 was moderately sensitive to erythromycin and streptomycin. All of these results were showed in table 3.5. Antibiotic susceptibility of *Bacillus* species, especially for *B. subtilis* has generally received little attention. It might be possible because *B. subtilis* is generally regarded as safe (GRAS) status. Besides, several *B. subtilis* strains are being used for producing fermented soybean products and industrial enzymes (Schallmey et al., 2014). In general, it is recommended that *B. subtilis* is safe and could be used in food industry. Our data also indicated that using of these strains are possible.

Table 3.5 Antibiotic susceptibility of *B. subtilis* strains TN3, DM1-2, FM2-3 and ASA

Antibiotic discs	***B. subtilis* TN3**	***B. subtilis* ASA**	***B. subtilis* DM1-2**	***B. subtilis* FM2-3**
Ampicillin (10 μg)	23±0.0 (S)	30±0.6(S)	25±1.0(S)	28±0.6(S)
Bacitracin (10 U)	7±0.0 (R)	10±0.6(R)	31±0.6(S)	31±0.6(S)
Chloramphenical (30 μg)	18±0.0 (S)	25±0.6(S)	41±1.2(S)	27±1.0(S)
Clindamycin (10 μg)	20±0.0 (S)	ND	ND	ND
Cefpodoxime (10 μg)	17±0.0 (R)	ND	ND	ND
Tetracycline (30 μg)	14±0.0 (S)	26±0.6(S)	24±2.3(S)	29±0.6(S)
Polymyxin (300 U)	8±0.0 (R)	ND	ND	ND
Kanamycin (30 μg)	20±0.0 (S)	17±0.6(I)	26±1.0(S)	18±1.0(S)
Streptomycin (10 μg)	19±0.0 (S)	14±0.6(I)	17±0.1(S)	13±0.0(I)
Vancomycin (30 μg)	13±0.0 (R)	13+0.0(S)	21±0.6(S)	21±1.2(S)
Erythromycin (15 μg)	18±0.0 (I)	22±0.6(I)	36±1.2(S)	22±2.0(I)

Note Data were diameters of inhibition zones obtained by three individual experiments. R for resistance, I for intermediate, S for sensitive, ND for Not determined.

3.5 Conclusion

The prevalence of bacteriophages infecting *B. subtilis* strains found the lytic phages isolate for 12.5%, and the host range examination was quite low in each sample. Unfortunately, these *thua nao* samples were only collected in Chiang Rai, Thailand. Therefore, the information on the existence of the bacteriophage in *thua nao* is still limited, and this study is the first report for screening of bacteriophage infecting *B. subtilis* existence in *thua nao*. The *B. subtilis* strains TN3, DM1-2 and FM2-3 were isolated from Thai *thua nao*, and strain ASA was isolated from Japanese *natto*. These bacterial strains were infected by BasuTN3 bacteriophage. It was studied in terms of its phenotypic and biochemical properties, and their 16S rRNA gene also studied. Our results indicated that these bacterial strains were identified as *B. subtilis* species. The phylogenetic tree indicated that these bacterial strains were closely related strains. For these, the BasuTN3 bacteriophage could be used for phagotyping to identification the bacterium complex species of these bacterial strains, because of it seems quite specificity to its bacteria cognate especially, *B. subtilis* strains. Furthermore, the *B. subtilis* strains TN3, FM2-3 and ASA exhibited high proteolytic activity. These three bacterial strains can be a potential starter culture as part of the research program in development of the fermentation process of *thua nao* as their protease enzyme. This study, provided some information of the lytic bacteriophage infecting *B. subtilis* in *thua nao*, and provided some key characteristics for *B. subtilis* strains in comparison with those isolated from similar fermented products.

CHAPTER 4

CHARACTERIZATION OF BasuTN3 PHAGE

4.1 Introduction

Bacteriophages (or phages), are viruses that can infect bacteria. They are at present the most abundant biological entities in the biosphere, with an estimated total abundance of 10^{31} (Hendrix et al., 1999; Mushegian, 2020). Since their discovery, phages have found their way of use ranging from a key model in molecular biology study to various biotechnological applications (Salmond & Fineran, 2015). Phages have also gained much attention in food industry. In fact, many phages have been isolated and studied from various food products, including dairy foods (Binetti et al., 2008), fermented vegetables (Fleming et al., 2003) salami (Bruttin et al., 1992), and wine (Jaomanjaka et al., 2016). It should be noted, however, that the study of the LAB (lactic acid bacteria) bacteriophages have been extensively studied and possibly the most documented (Mahony et al., 2014). The phages derived from these fermented foods seem to be diverse, however only a few studies have described their possible ecological roles and their impact on the food microbiota (Paillet & Dugat, 2021). In general, the bacteriophage existence in food industry is mostly concerned as the phages have been responsible for fermentation failure. However, an increasing attention to use these isolated phages for bio-controlling the (foodborne) pathogenic bacteria has been proposed and reviewed by Endersen and Coffey (2020).

Thua Nao is an ethnic fermented soybean food of Thailand. Similar products are found in many Asian countries including *natto* in Japan, *kinema* in India, and *chongkukjang* in Korea. Traditionally, it is manufactured by fermenting cooked soybeans with naturally occurring microbes which mainly are *Bacillus* species. *Bacillus subtilis*, a Gram-positive, nonpathogenic, spore-forming bacterium, has been described as a major fermenting microorganism in these fermented soybean food products (Chantawannakul et al., 2002; Hosoi & Kiuchi, 2003; Inatsu et al., 2006; Ghosh et al.,

2018). Most of these products (except *natto*) are prepared traditionally (i.e., the fermentation occurs by naturally occurring microbes). It should be noted, however that an attempt to screen for specific *B. subtilis* strain with desired features, has been undertaken in order to use as a pure starter culture (Dajanta et al., 2012; Lee & Chang, 2017). Besides, these fermented soybean foods have recently been of interest to scientists based on their health benefits (Jayachandran & Xu, 2019).

Although microbial community of these soybean-based fermented foods has been studied extensively, existence and diversity of the phages are scarce. Compared to the LAB phages which has been well documented, the fermented soybeans may represent an underexplored source of the phages. Besides, the predominant bacterial group of these products is *Bacillus* species, and not LAB. To the best of our knowledge, there are a few studies related to the *B. subtilis* phages, and these are limited to *natto* and *cheonggukjang* (Chukeatirote et al., 2018). Therefore, the purpose of this study was characterisation the bacteriophage BasuTN3. To study their taxonomy and viability, different conditions were used including (1) morphology; (2) effects of divalent cations; and (3) phage stability (Optimal Multiplicity of Infection (MOI), host range specificity, efficiency of plating (EOP), adsorption assay, temperature, pH, ultraviolet, chloroform and disinfectants).

4.2 Host Range Specificity

The host range of the BasuTN3 bacteriophage was determined by using spotting technique. The bacterial strains used listed in table 2.1. BasuTN3 bacteriophage showed lytic activity (Table 4.1) on four strains of *B. subtilis* (strains TN3, DM1-2 and FM2-3 were isolated from *thua nao*, and ASA was isolated from *natto*). This phage did not lyse other *Bacillus* species nor other bacterial species. It should also be noted, however that the BasuTN3 bacteriophage was active against *B. subtilis* ASA, but inactive for *B. subtilis* strain BEST195, considering that both bacterial starter cultures were isolated from the Japanese *natto*. Hence, the data obtained suggested that BasuTN3 bacteriophage was quite specific to its cognate *B. subtilis* strain TN3, and it thus might have a narrow host range. In contrast, previous work described a broad spectrum of the

B. subtilis lytic phages isolated from the fermented soybean food products. For example, several *B. subtilis* (*natto*) strains were sensitive to the isolated phages (Nagai & Yamasaki, 2009). The phage Bp-K2 was also found to be active against many *B. subtilis* starter culture strains of the Korean *chungkookjang*, albeit less active against to Japanese *natto* bacterial strain (Kim et al., 2011). This high specificity of the BasuTN3 bacteriophage is of great importance and should be further studied. Considering that the bacterium strain TN3 exhibits strong proteolytic activity and thus can be used as a potential starter culture for the soybean fermentation, the BasuTN3 bacteriophage contamination must be controlled to prevent the fermentation failure. However, this would not affect another potential *B. subtilis* starter culture strains (e.g., strain TN51, also isolated from *thua nao* product) for the fermentation purpose (Dajanta et al., 2012). Besides, an application of the phage typing to differentiate the closely related *B. subtilis* strains may be introduced (Ackermann et al., 1995).

Table 4.1 The infectivity of bacteriophages on different bacterial hosts

Species	Strains	Infectivity
***Bacillus* species**		
B. cereus	ATCC 11778	-
B. cereus	TISTR 687	-
B. cereus	TISTR 1527	-
B. cereus	TN69	-
B. licheniformis	TISTR 1109	-
B. subtilis	ASA	+ (10)
B. subtilis	ATCC 6633	-
B. subtilis	BEST 195	-
B. subtilis	DM1-1	-
B. subtilis	DM1-2	+ (10^5)
B. subtilis	DM1-3	-
B. subtilis	FM2-1	-

Table 4.1 (continued)

Species	Strains	Infectivity
***Bacillus* species**		
B. subtilis	FM2-2	-
B. subtilis	FM2-3	+ (10^4)
B. subtilis	FM2-4	-
B. subtilis	S1-13	-
B. subtilis	S4-5	-
B. subtilis	TISTR 1248	-
B. subtilis	TN3	+ (10)
B. subtilis	TN51	-
B. thuringiensis	S2-3	-
Non-*Bacillus* species		
E. aerogenes	TISTR 1540	-
E. coli	TISTR 527	-
M. luteus	TISTR 884	-
P. aeruginosa	TISTR 781	-
S. aureus	TISTR 746	-

Note ATCC for American Type Culture Collection, TISTR for Thailand Institute of Scientific and Technological Research

+ for presence of plaque, - for absence of plaque, the number in the parentheses indicate the phage titre

4.3 Efficiency of Plating (EOP)

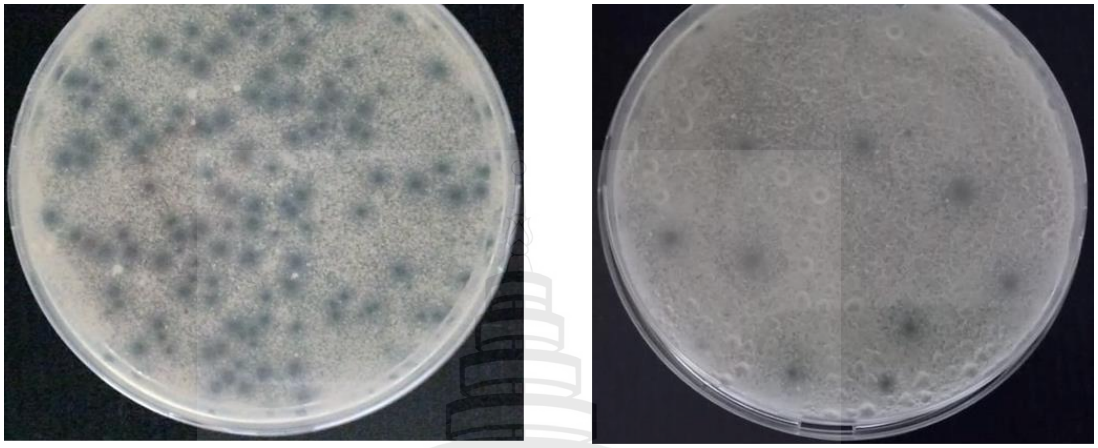
The relative of EOP of this phage revealed that only two *B. subtilis* strains ASA and TN3 were sensitive to this phage by forming plaques when using the double-layer agar technique (Figure 4.1). The EOP values revealed that *B. subtilis* strain TN3 was greatest to be host than strain ASA. The EOP values of *B. subtilis* strain TN3 was 1.00

and strain ASA was 0.88, these results showed that, both of these bacterial strain were ranked as high efficiency (Vongkamjan et al., 2017). For the remaining two *B. subtilis* strains (DM1-2 and FM2-3), the plaque formation (clear zones) was only observed when the high phage titre ($>10^4$ PFU/ml for FM2-3 and $>10^5$ PFU/ml for DM1-2) by using spotting technique. Besides, the BasuTN3 bacteriophage may have lysed the two *B. subtilis* strains (DM1-2 and FM2-3) 'lysis from without' or 'abortive infection' (Abedon, 2011). This phenomenon can occur when the bacterial hosts are exposed to an overload of the phages that simultaneously infects a bacterium resulting in the cell lysis without production of new phage (Abedon, 2011). For these results, *B. subtilis* strain TN3 was used for phage propagation.

4.4 Plaque Morphology

The morphology of bacteriophage plaque formation is used as one of the criteria for bacteriophage characterization, and it is also used for phage nomenclature in terms of their original host. Kropinski et al. (2009) proposed the nomenclature of the viruses based on their host, morphology and origin. The plaque morphology was observed using the DLA technique. After the incubation at 37 °C for 24 h, the plaque formation of bacteriophage BasuTN3 was observed (Figure 4.1). As shown in figure 4.1, the phage formed plaques 1-2 mm in diameter with a halo and slightly turbid at the periphery on *B. subtilis* TN3 lawn, and 1-1.5 mm in diameter with a halo and slightly turbid at the periphery on *B. subtilis* ASA lawn. The halo formation of bacteriophage BasuTN3 could be an indication of incomplete cell lysis. Also, phages that exhibit the halo formation could potentially possess the ability to target or degrade bacterial capsules, and the halo zone is the result of newly released virion activity or free enzymes (e.g., tail spike proteins) targeting bacterial capsules (Knecht et al., 2020). Willms and Hertel (2016) reported that bacteriophage vB_BsuP-Goe1 was infecting *B. subtilis* 168, NCIB3610 and Δ6, its morphology and size of plaques were differentiating. These plaques morphology seems to indicate the *B. subtilis* bacteriophages have various features. In addition, the plaque formation of a particular

bacteriophage should be consistent under normalized conditions, and the change in plaque morphology could indicate mutation(s) of bacteriophages (Gallet et al., 2011).



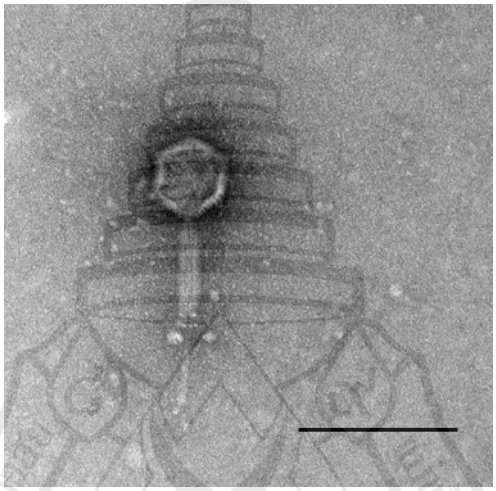
B. subtilis TN3 *B. subtilis* ASA

Figure 4.1 Plaque formation of BasuTN3 bacteriophages on *B. subtilis* strains TN3 and ASA by double layer agar technique

4.5 Morphology Examination by TEM

The phage morphology was then determined using transmission electron microscope (Figure 4.4). The TEM observation revealed the structural features of the bacteriophage (n=20), with an icosahedral head 103.16 ± 11.04 nm in diameter, and a long contractile tail 159.24 ± 48.16 nm in length. According to the TEM result, this *B. subtilis* lytic bacteriophage was classified as Myoviridae because of its tail. Currently, nearly all extent bacteriophages are in the order Caudovirales (tailed phage group) (Ackermann, 2007). Based on the distinct tail feature, this order is classified into three families of Myoviridae (long contractile tail), Siphoviridae (long non-contractile tail), and Podoviridae (short non-contractile tail). Bacteriophage BasuTN3 was designated as vB_BsuM-TN3 (in short, BasuTN3). The naming of the phage was based on the systematic scheme suggested by Kropinski et al. (2009). Which is the phage name should consist of v(a)_(b)(c)_(d). Section (a) is named based on the type of the virus such as bacterial virus (vB) and archaeal virus (vA), and section (b) is based on the

abbreviation of the genus and species of host infected (the abbreviation is according to REBASE database). Section (c) and (d) refers to the first letter of viral family name (e.g., *Myoviridae*: M; *Siphoviridae*: S) and the designation by the specific laboratory (e.g., Phage origins, laboratory numerical orders etc.), respectively. This finding is consistent with previous studied, especially when considering that the *B. subtilis* bacteriophages isolated from the fermented soybean-based food products, belonged to the members of the Myoviridae and Siphoviridae families (Chukeatirote et al., 2018).



Note The scale bars shown are 200 nm

Figure 4.2 TEM micrograph of BasuTN3 bacteriophage

4.6 Effects of Divalent Cations on Phage-Host Interaction

The first step in the life cycle of bacteriophage is attachment (adsorption) of the phage to the surface of the bacterial cell. Most of bacteria and phage have strong anion on the surface. Therefore, the divalent cation is a factor to phage-host interaction. The comparison between phage titer with the addition and absence of divalent cations carried out by using the double-layer plaque technique. The result showed that the numbers of phage titer were not significantly different between both conditions. This indicated that the presence of mixed 10 mM $CaCl_2$ and $MgCl_2$ had no effects to this bacteriophage on the bacterial hosts, in terms of adsorption. *Thua nao*, classified as an

alkaline fermented food (Chukeatirote, 2015). This indicated that the condition of *thua nao* fermentation is not influent to phage-host interaction. Albeit, many reports were showed the addition of divalent cations could improve bacteriophage infectivity or the adsorption rate (Bandara et al., 2012; Suárez et al., 2008; Wang et al., 2010).

4.7 Influence of Temperature and pH on Phage Adsorption

Bacteriophage infection of bacteria constitutes one of the major problems in the dairy industry, causing economic losses and a constant risk of low quality and/or unsafe foods. The first step in the phage biology is the adsorption on the host cell surface (Marcó et al., 2010). Interaction between phage and bacterial host involves many receptor; for Gram-positive bacteria, these receptors are mainly associated with either peptidoglycan or teichoic acid (Dowah & Clokie, 2018). Besides, several moieties present in the Gram-positive bacterial cell wall can contribute to or interfere with the phage adsorption (Quiberoni et al., 2000). Caso et al. (1995) reported that the effect of temperature on phage propagation could be due to temperature-dependent changes in the bacterial cell wall affecting the adsorption ability of the phages, and temperatures of 30 and 37 °C may be more effective. For these, we evaluated the effect of temperature and pH on the adsorption ability of BasuTN3 bacteriophage on its host (*B. subtilis* strain TN3). The result in figure 4.3 was showed that, the adsorption rate at 60 and 65 min were 97.40 and 99.06% respectively, while at the 20 and 40 min were 84.61 and 86.34% respectively. The result in figure 4.4 was showed that the adsorption rate at 4, 37, and 45 °C for 60 min were 97.45, 95.88 and 86.75% respectively. Subsequently, the adsorption rate at 37 °C for 20, 40, 60 and 65 min were determined. For the effect of pH were showed in figure 4.5, the highest adsorption values on the host cells were at pH ranging from 6 to 9 (more than 90%), while the pH 5 showed that more than 80%. Ghosh et al. (2018) reported that BSP18 bacteriophage effectively inhibited the growth of *B. subtilis* ATCC 15245 by gradually increasing the time up to 7 hour at MOI 0.1 and 1, the result was showed that bacteria decreased and no bacterial regrown after 24 h. This might be explained that the time has influenced to phage-host interaction. Effect of temperature and pH were considered as denature of protein receptor and addition of

the ion. Knowledge on factors that influence the binding of phages to the sensitive cells allows developing strategies to enhance the performance of the strains in industrial environments (Marcó et al., 2010). Our results showed that this phage adsorbed effectively on the sensitive cells by temperature and pH factors. Thus, the thermal treatments or changes in environmental pH values could be considered when designing *B. subtilis* TN3 as a starter culture in fermentation processes.

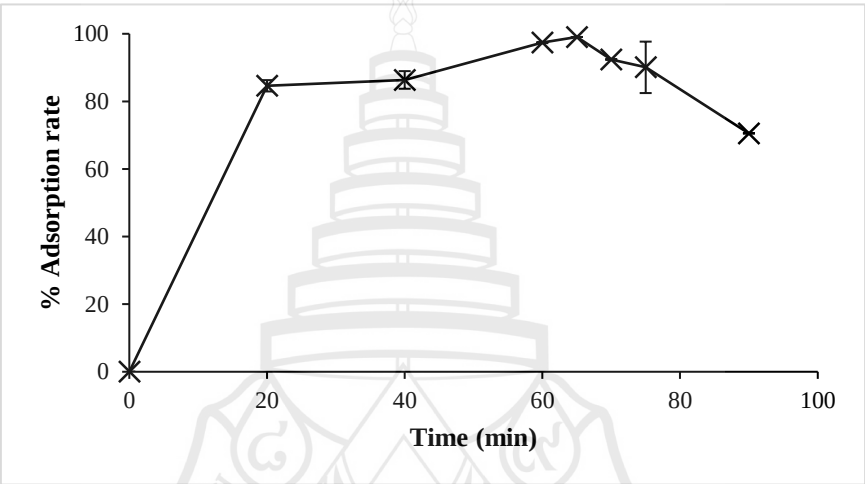


Figure 4.3 The numbers of free phage particles after an incubation in TSB media at 37 ˚C in difference timing

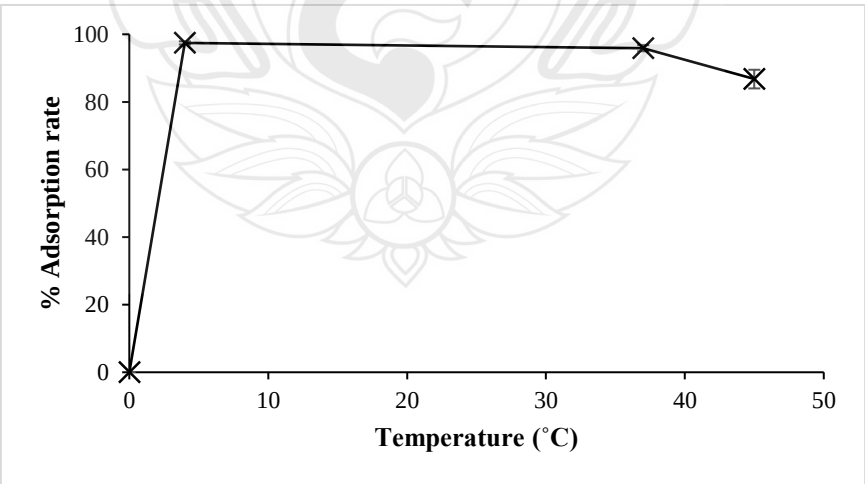


Figure 4.4 The numbers of free phage particles after an incubation in TSB media at 4, 37 and 45 ˚C for 60 min

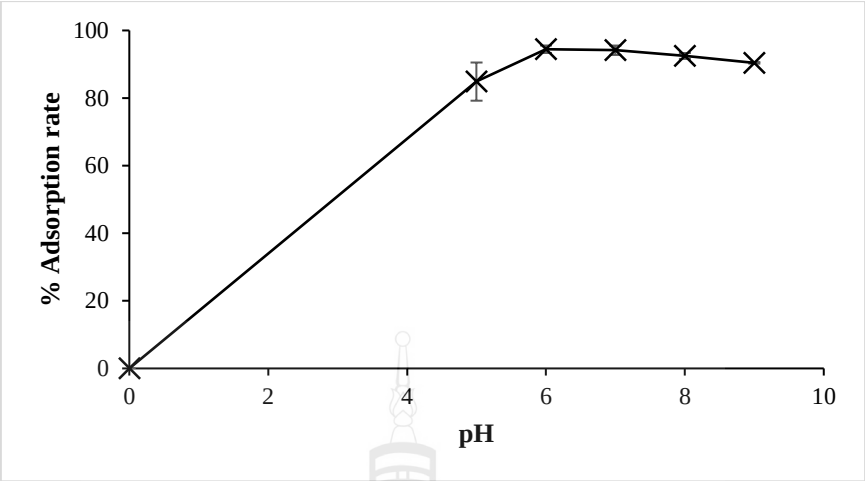


Figure 4.5 The numbers of free phage particles after an incubation at 37 ˚C in TSB media adjusted with pH range 5-9 for 60 min

4.8 Optimal Multiplicity of Infection (MOI) Determination

The optimal ratio of bacteriophage to bacteria or MOI is one of the crucial criteria to determine the successful infection. BasuTN3 bacteriophage showed increased effectiveness of bacterial reduction as MOI increased (Figure 4.6). The MOI of 0.1, 1 and 10 showed minimum effectiveness (Log reduction of -1.12 ± 0.63, -1.44 ± 0.51 and -1.64±0.70 respectively), but the MOI of 100 and 1000, the log reduction increased drastically to -2.32 ± 0.55 and -3.01 ± 0.37 respectively. The increasing of log reduction as MOI increasing can be explained by the higher number of bacteriophage particles having a higher chance of encountering bacteria before they can replicate. Abedon (2011) said the higher MOI would ensure the destruction of host cells, it may also cause the "lysis from without" and result in no viral progeny generation. Previous study by Viazis et al. (2011) also reported the greatest reduction of *E. coli* at the maximum MOI is 100. Especially in *in vivo* setting, a high number of MOI is more desirable for successful biocontrol, and an extremely high MOI of 10^6 have also been reported to be used for bacteriophage biocontrol against *E. coli* in meat (O'flynn et al., 2011). Ghosh et al. (2018) reported that BSP18 bacteriophage effectively inhibited the growth of *B. subtilis* ATCC 15245 by gradually increasing the

time up to 7 hour at MOI 0.1 and 1, the result showed the bacteria decreased and no bacterial regrown was detected even after 24 h. This was explained the time has influent to host eradication.

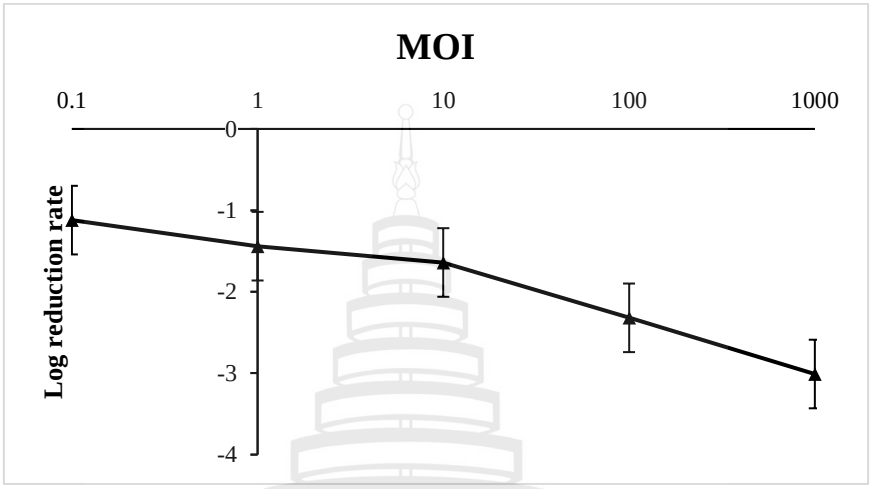


Figure 4.6 Log reduction rate of *B. subtilis* strain TN3 treated with BasuTN3 bacteriophage at different multiplicity of infections

4.9 Phage Stability Analysis

4.9.1 Effect of Temperature

Temperature is one of the important criteria to determine bacteriophage survival (Olson et al., 2004). The effecting of the temperature associates the mechanism of attachment, penetration, and multiplication. Low temperature could result in the delay in failed phage infection, while the high temperature could simply disintegrate the phage particles by losing the ability to attach to the host cells. The BasuTN3 phage was exposed to different treatments, following by detecting its residual titre. The isolated phage was found to be stable between 4 and 45 ˚C (>60% survival rate) for at least 2 h. The phage titre dropped significantly after incubating at 55 ˚C. It was showed that the phage was susceptible and could not survive at -25 and 65 ˚C (Figure 4.7). Temperature susceptibility of phage in this present finding is in conformity to those reported by Fleming et al. (2003) who found that phages get inactivated at 70 ˚C and above, while

Krasowska et al. (2015) reported that phages of *Bacillus* were resistant to high temperatures (80 ˚C). For these, it was showed that the *Bacillus* bacteriophage have diversity in the effecting by the temperature. High temperature is known to cause protein and genetic material denaturation, and enough temperature can cause complete degradation of biomolecules. When bacteriophage is exposed to high temperature, the denaturation of proteins on phage structure can impair its ability to attach and infect bacteria. Therefore, bacteriophage simply degrades under high temperature (Jończyk et al., 2011).

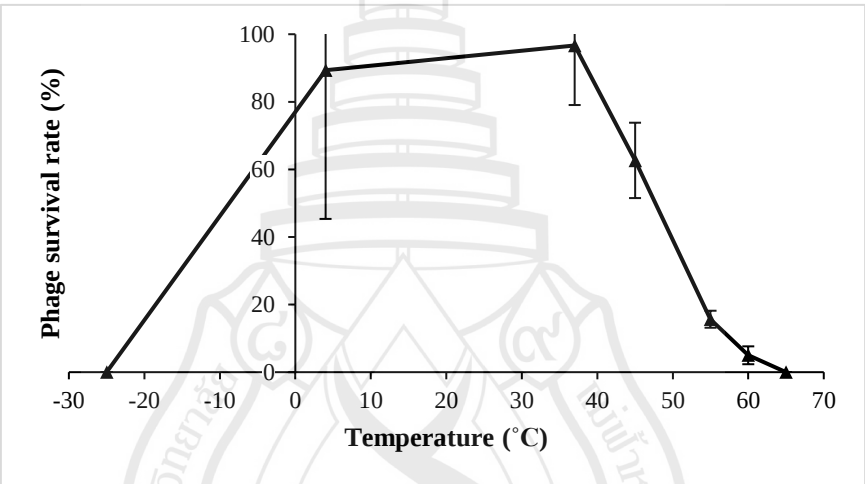


Figure 4.7 Ability of the BasuTN3 phage to survive under different conditions of temperature

4.9.2 Effect of pH

The pH value is an important factor for bacteriophage stability. The alteration of pH value could result in precipitation or change in the conformational of proteins on phage structure. Low pH reportedly affects to phage aggregation and reduces their abilities to penetrate the host cells (Shende et al., 2017). The stability of bacteriophage under different alkaline and acid conditions after 2 h of treatment in pH-adjusted TSB were determined. A pH stability analysis revealed that the BasuTN3 phage was stable in a wide range of pH (between 5 and 11) with more than 50% survivability (Figure 4.8). The phage could survive to some extent at pH 4 (28.89%) and pH 12 (4.71%); however, this phage isolate was undetectable when incubated at pH 3 and 13. The

previously studied found that phage viability was maximal between 5 and 9, and many phages were completely inactivated at pH 3 and 11 (Shukla & Hirpurkar, 2011; Tiwari et al., 2010). On the contrary, Krasowska et al. (2015) reported that phages of *B. subtilis* phages resistant to the acidic (4.0) and alkaline (9.0 - 10.0).

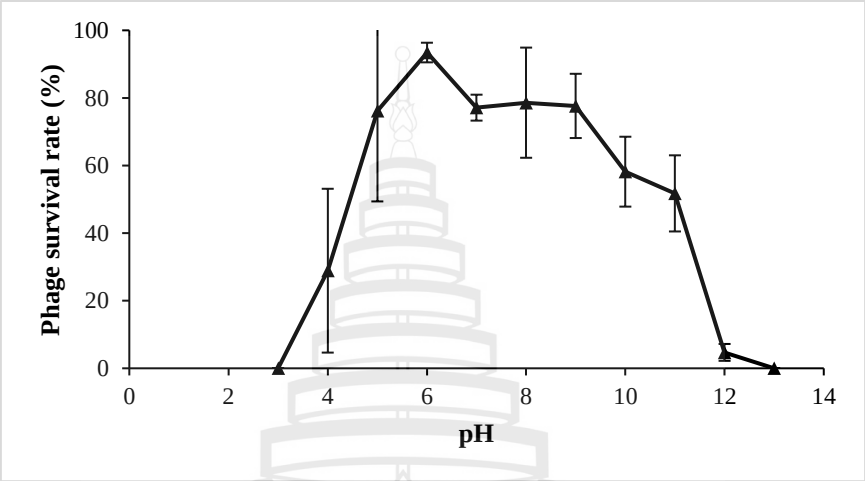


Figure 4.8 Ability of the BasuTN3 phage to survive under different conditions of pH

4.9.3 Effect of UV

UV light is known to cause DNA damage and peptide bond alteration, and it is considered one of the main causes of phage death in nature (Tom et al., 2018). Therefore, the stability of bacteriophage after an exposure to UV light for up to 2 h was determined. The BasuTN3 bacteriophage was showed drastically reduced viability to 8.00 ± 1.62 percentage of survival after 15 min-exposure under the UV irradiation (UVC, $\lambda = 254$ nm). The viability of this bacteriophage isolate continued to decrease as the exposure time increased, the phage was then almost died out under UV treatment for 1 h, and no phage survival was detected after 2 h of UV light exposure (Figure 4.9). Numerous studies of UV-induced inactivation focused on non-enveloped viruses (Ma et al., 2021). Brodetsky and Romig (1965) reported that group of six phages, SP5, SP6, SP7, SP8, SP9, and SP13, which used the *B. subtilis* strain Marburg as host were inactivated by UV light exposure after 30 to 45 sec. Phongtang and Chukeatirote (2021) also reported the *B. cereus* phages (BaceFT01 and BaceCM02) were not survival after 2 h of UV light exposure. Ultraviolet (UV) irradiation (200-280 nm) has proven to be

effective for virus disinfection, especially on the surfaces and in the air, due to its rapid effectiveness and limited to no material corrosion.

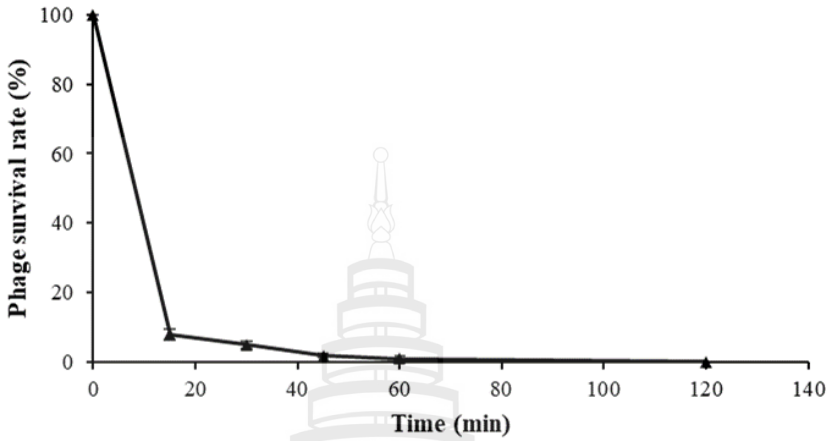
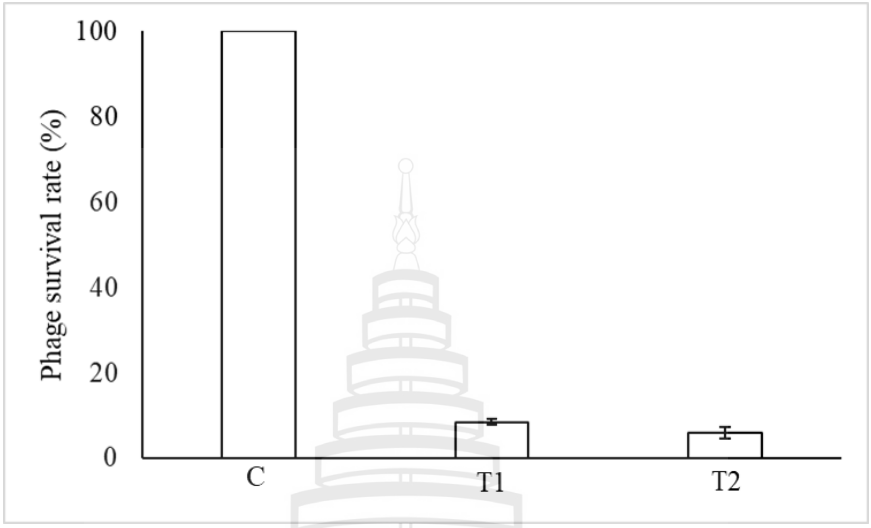


Figure 4.9 Ability of the BasuTN3 phage to survive under UV irradiation in different time

4.9.4 Effect of Chloroform

Chloroform could be used to prolong the shelf-life of bacteriophage stock by preventing the contamination of other microorganisms. Therefore, the chloroform sensitivity determination can be beneficial in terms of storage optimization. The bacteriophage isolate was tested for their tolerance to chloroform in terms of storage stability. For this, the phage suspension with 5% of chloroform and storage at 4 ˚C for 24 h. Its remained viability was quite low (<9%). This phage was also tested in terms of equal volume between phage and chloroform for 30 minutes and it quite sensitive to chloroform by the phage residual was 5.95 ± 1.43. Romig and Brodetsky (1961) also reported that several of the bacteriophages for *B. subtilis* isolated from soil were unusually sensitive to chloroform and all of them were relatively unstable when stored at refrigerator temperatures. Nichihara and Romig (1964) reported that *B. subtilis* bacteriophage SP3 was also lose their titre after treated with chloroform. Beside, in some cases, the use of chloroform caused a decrease in phage titer; in others, there was essentially no change (Nichihara & Romig, 1964). The phage sensitivity to the

chloroform assay indicated a presence of lipids in the phage structure (Seaman & Day, 2007).



Note C for control, T1 for 5% chloroform at 24 h, T2 for Equal volume at 30 min

Figure 4.10 Ability of the BasuTN3 phage to survive in chloroform

4.9.5 Effect of Disinfectants

Bacteriophages had been contaminated in the food manufacturing industries, especially the fermented foods, and led to the production fails. For instance, the phages that infecting to *B. subtilis* starter culture resulted in the spoilage of *natto* products (Nagai & Yamasaki, 2009). Therefore, disinfectants commonly used (e.g., 70% ethanol, 0.06% sodium hypochlorite, 5% Dettol, and 5% commercial dishwashing liquid) were tested on BasuTN3 phage. The BasuTN3 phage as the phage titres were undetected after all these chemical treatments. Halfhide et al. (2008) studied treated the bacteriophages were three closely related strains of *Myoviridae* (namely PVP3A, PVP4C, and PVP5A) and three strains of *Siphoviridae* (namely lambda gt11, PVP38, and PVP39), the most effective of these bacteriophages were Virkon (1%), which inactivated all six phages rapidly. Ethanol (75%) was completely effective against to the *Myoviridae* after 10 - 30 min, but it had a little effected on the *Siphoviridae*, was reduced by only 1 log reduction after 1 h. Ethanol can denature proteins and interfere with protein-protein interactions, and disrupt membranes of the viruses (Kundu et al.,

2016). Dettol™ was also completely against with BasuTN3 bacteriophage to inactivating at 5% v/v concentration. The ingredient in Dettol™ is chloroxylenol. It is a disinfectant commonly used for household and wound cleaning, and it was found to be effective against human viruses such as herpes simplex 1 and HIV (Lin et al., 2020). In addition, Haiter™ is one of household cleaning, and its active ingredient is sodium hypochlorite (NaClO), a strong oxidizing agent. Clevenger et al. (2007) reported that bacteriophage MS2 (ATCC 15597-B1) was effectively inactivated by four disinfectants (sodium hypochlorite, ClorTech, MIOX and MIOXa). Lipon F™ is one of household cleaning, which it is consists of linear alkylbenzene sulfonates (LAS) and sodium lauryl ether sulfate (SLES), both are anionic surfactants. Chattopadhyay et al. (2002) demonstrated the sorption of hydrophobic viruses was favored by hydrophobic sorbents, while the sorption of hydrophilic viruses was favored by hydrophilic sorbents. The bacteriophages nowadays become an important tool in modern biotechnology, and find their way of application including phage biocontrol (including phage therapy), phage display systems, vaccine delivery vehicles and even for diagnostic use. Whereas some bacteriophages are disadvantages to the manufacturing of industries. Therefore, it is important that information is available on the effect of chemicals (and other) that can inactivate them.

4.10 Conclusion

In the present study, it was provided the fundamental and inforation characteristics of bacteriophage isolated from *thua nao*, namely BasuTN3 phage. It could lyse *B. subtilis* strain TN3, a potential bacterial starter culture based on its proteolytic activity. This bacteriophage was classified as *Myoviridae* phage with hexagonal heads and contractile tails. Its host range determination indicated that BasuTN3 phage was infected to *B. subtilis* strains TN3 and ASA, both have high proteolytic activity. In addition, BasuTN3 phage was infected to *B. subtilis* strains DM1-2 and FM2-3 in terms of “lysis from without”. The MOI was showed that increased effectiveness of bacterial reduction as MOI increased. It can be explained by the higher number of bacteriophage particles having a higher chance of encountering

bacteria before they can replicate. In addition, the effect of temperature and pH were examined based on adsorption, which is it seems to be influenced. Generally, temperature and pH are the key factors to phage-host interaction. The various condition consists of thermal, pH, UV radiation, chemical disinfectant and chloroform stabilities were examined on this bacteriophage. Knowledge on factors that influence on this phage allows developing strategies to enhance the performance of the strains in industrial environments.

CHAPTER 5

CONCLUSION

This study described the fundamental information and characterization of bacteriophage infecting *B. subtilis* strain TN3 (named BasuTN3), which could lyse *B. subtilis* strain TN3, a potential bacterial starter culture based on its proteolytic activity. Including screening for the prevalence of bacteriophage infecting *B. subtilis* in *thua nao*. It was isolated from *thua nao*, a Thai traditional fermented soybean product. The bacteriophage infecting *B. subtilis* strain TN3 was named as vB_BsuM-TN3 (in short, BasuTN3), it was designated by their morphology and original source isolated. According to the TEM result, the BasuTN3 bacteriophage was classified as Myoviridae because of its tail. The host range specificity of BasuTN3 bacteriophage showed that it was quite specific to its cognate *B. subtilis* strain TN3, because it was infecting only *B. subtilis* strains TN3, ASA, DM1-2 and FM2-3, which were classified as *B. subtilis* species based on their 16S rRNA gene. Therefore, BasuTN3 bacteriophage might have a narrow host range and it can be an application of the phage typing to differentiate the closely related to these *B. subtilis* strains. Furthermore, it might have the relationship related host range to *Bacillus* genus, especially *B. subtilis*. For these, this harmful bacteriophage should be concerned.

In addition, *B. subtilis* strains TN3, FM2-3 and ASA exhibits strong proteolytic activity and suggested to be a pure bacteria starter culture for fermentation or enzymatic application based on their protease. However, a contamination of phage specific to th potential starter cultures can be detrimental resulting in a failure of fermentation. Besides, the multiplicity of infection (MOI) showed that increasing of bacteriophage could efficiently inhibit the bacterial growth, and the adsorption assay revealed that temperature and pH were efficient to phage-host interaction (adsorption). As know that *thua nao* is an alkaline condition (base condition). Hence, the manufacturing condition

of *thua nao* has to be a concerned for BasuTN3 bacteriophage contamination because of its pH. This issue is not surprising, because this harmful bacteriophage could destroyed by under various inappropriate conditions. This bacteriophage was sensitive to extreme temperatures more than 55 ˚C, and could not survive at -25 and 65 ˚C and pH at 3 and 13 for 2 h. It was also sensitive to ultraviolet light, chloroform, and disinfectants. These various conditions inactivated this phage by damage to its structure (envelope, head, and tail), lipid loss, and/or nucleic acid structural changes. Unfortunately, those conditions are not commonly applied during fermentation, thus limiting greatly the application of strategies available to prevent phage infections in fermented food industrial environments.

In addition, eight *thua nao* samples were obtained from Mueang Chiang Rai, Chiang Rai, Thailand. It was screened for presence of bacteriophage infecting *B. subtilis*. The isolation rate of the *B. subtilis* lytic bacteriophage obtained from our study was quite low, when compared with others traditional fermented soybean products such as *chongkokjang* and *natto*. It should be noted, however that the incidence of bacteriophage infecting *B. subtilis* was detected in *thua nao* still limit in terms of the areas. Therefore, this study revealed fundamental and important characteristics of the BasuTN3 bacteriophage, which could lyse *B. subtilis* strains TN3, ASA and FM2-3 a potential bacterial starter culture based on its proteolytic activity, and it might be lyse other *B. subtilis* strains. To the best of our knowledge, this is the first report describing the *B. subtilis* lytic bacteriophage isolated from *thua nao*.

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