Properties of Proteinaceous Antimicrobial Compound Produced by *Bacillus thuringiensis* strain SS01 Isolated from Mangrove Forest in Thailand

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ABSTRACT

The mangrove forest is a dynamic ecosystem, with a diverse ecology containing plants, animals and microorganisms. This ecosystem represents an interesting natural source of novel and useful biological substances. The aims of this study were to determine the optimum conditions for antimicrobial agent production of target bacterial strain SS01 isolated from a mangrove ecosystem as well as some significant factors affecting its antimicrobial activity. According to biochemical test results and 16S rDNA gene sequence, strain SS01, which was isolated from soil from a mangrove forest in central Thailand, was identified as *Bacillus thuringiensis*. The optimum conditions for antimicrobial production of *B. thuringiensis* strain SS01 was observed when culturing in M17 broth without NaCl at 35 °C. The highest antimicrobial activity for inhibiting *Micrococcus luteus* IFO 12708 (800 $AU \cdot mL^{-1}$) was found during culture for 36-48 h, with pH 6.77-7.06. Moreover, the secondary metabolite in CFNS exhibited a proteinaceous nature, which is the most important characteristic of a bacteriocin. The broad-spectrum antimicrobial compound in the cell-free neutralized supernatant (CFNS) produced from *B. thuringiensis* strain SS01 was heat tolerant, stable from pH 2-8, and displayed bacteriostatic mode of action against *M. luteus* IFO 12708. Its antimicrobial activity was partially reduced under 3-11% (w/v) NaCl. Consequently, the antimicrobial compound possessing unique and interesting properties produced by *B. thuringiensis* strain SS01 shows high potential for applications in food products, animal feed and related industries.

Keywords: Antimicrobial compound, *Bacillus thuringiensis*, Bacteriocin, Mangrove ecosystem, Thermotolerant

INTRODUCTION

Mangroves, the intertidal forests along tropical and subtropical coasts, are among the most interesting sources of productivity and biodiversity. These ecosystems yield valuable services for humanity, including cultural and religious value, as well as habitat for aquatic species (Walters *et al*., 2008). Microorganisms are important members of the mangrove forest ecosystem, and bacteria are the dominant group among all mangrove microorganisms (Kathiresan and Qasim, 2005). Moreover, mangrove microorganisms can adapt to constant variations of their environment (e.g., temperature, salinity, and UV rays.); therefore, these factors are the driving forces for microbial diversity (Rigonato *et al*., 2018). Several studies have explored the unique identity of mangrove sediments with respect to their microbial composition (Thatoi *et al*., 2012; Saravanakumar *et al*., 2016; Azman *et al*., 2017). Mangrove microorganisms play an important role not only in degrading organic

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and water-soluble inorganic substances accumulated in the mangrove forest ecosystem, but also are a major source of antimicrobial agents and produce a wide range of important medicinal compounds (Thatoi *et al*., 2012).

Bioactive compounds are natural components that can replace synthetic chemicals as natural preservatives, flavoring agents, antioxidants or antimicrobial agents to control the growth of microorganisms. Thus, some of these compounds are used in food preservation for extending shelf-life and inhibiting the growth of other related species of bacteria or spoilage bacteria, molds and yeasts in the food (Pandey and Upadhyay, 2012; Reis *et al*., 2012). One of the current research areas in microbiology focuses on exploring new microorganism strains from mangrove forests that can produce bioactive compounds for further development and application (Narendran and Kathiresan, 2016; Sangkanu *et al*., 2017). Several studies have applied intensive effort to develop a new class of antimicrobial agents that are produced by mangrove microorganisms with broad antimicrobial spectrum and high density threshold (Azman *et al*., 2017; Khan *et al*., 2017; Haryani *et al*., 2020). Consequently, mangrove forests are a meaningful potential source for the discovery of new bacterial strains due to their special environment; the constant change may create a high diversity of microorganisms.

From our previous work, a highly applicable antimicrobial-producing bacterial strain (strain SS01) was isolated from soil samples collected in a mangrove forest in central Thailand. Preliminary studies suggested that this strain had a broad antimicrobial spectrum against various target microorganisms involved in food quality and safety; moreover, its antimicrobial compound seemed to possess a thermotolerant property. The aims of this study were to identify the antimicrobial-producing strain, determine the optimum conditions for antimicrobial compound production of the target strain, and identify critical factors affecting the antimicrobial compound's stability. Meanwhile, we also determined the mode of action of the target antimicrobial compound against significant fish-spoilage bacteria. All of the information gained from this study is necessary for future large-scale production, purification and in-depth characterization of purified antimicrobial peptide from the target strain before moving up to industrial applications.

MATERIALS AND METHODS

Antimicrobial-producing bacterial strain

Bacterial strain SS01, an antimicrobial-producing bacterial strain, was isolated from a mangrove ecosystem in central Thailand. This strain showed broad antimicrobial spectrum against target indicator bacteria including *Bacillus cereus* JCM2152, *Enterococcus faecalis* ATCC 19433, *Micrococcus luteus* IFO 12708, *Staphylococcus aureus* ATCC 25923 and *Streptococcus agalactiae* (isolated from Tilapia fish sample) when preliminarily determined by direct method described by Marshall *et al*. (2010). Meanwhile, *Micrococcus luteus* IFO 12708 (a significant fish spoilage bacterium) was the most sensitive target indicator strain to antimicrobial compound produced by strain SS01. Strain SS01 was kept on M17 agar slants at 4 °C and also in M17 broth with 20% glycerol (v/v) at -20 °C during this experiment.

Bacterial strain identification

Bacterial strain SS01 was identified by using *in vitro* biochemical tests (Gram stain, motility, catalase production, starch hydrolysis, nitrate reduction, citrate utilization) and the 16S rDNA gene sequence. Strain SS01 was grown in M17 broth and incubated at 35 °C for 48 h, and then centrifuged at 8,000×g for 10 min at 4 °C. DNA was extracted from bacterial cells using a DNA extraction kit (DNAzol®, USA). Target DNA was amplified by using polymerase chain reaction (PCR). The reaction mixture consisted of (1) target DNA, 1 µL; (2) One PCR Master mix (OnePCR™), 25 µL; forward primer (5'-GCGGCGTGCCTAATACATGC-3'), 1 µL; reverse primer (5'-CACCTTCCGATACGGCTACC-3'), 1 µL; and (4) ddH2O, 22 µL. The PCR reaction was carried out by first incubating for 5 min at 95 °C. Then, 30 cycles were performed as follows: denaturation for 40 s at 95 ºC, 40 s at the annealing temperature of 60 ºC, and 90 s at 72 ºC for

target DNA extension. Reaction was then incubated at 72 ºC for another 10 min. PCR product was analyzed by gel electrophoresis at the concentration of 1.5% agarose in tris-acetate buffer followed by purification of the target DNA fragments using PCR Purification Kit (FavorPrepTM.GEL (Favorgen Biotech Corp.), USA). The purified DNA fragment was cloned with vector (pGEM-T) to recombinant DNA using pGEM®-T Easy Vector (Favorgen Biotech Corp., USA). The transformation vector containing insert (PCR product after gel purifies) into the host (*Escherichia coli* JM109) followed by spreading 100 µL of transformed competent cells on LB agar plate with 0.01% Ampicillin and incubating at 35 °C for 16-18 h. After incubation, the white colony that formed was extracted for pure plasmid with Plasmid Extraction Mini Kit (FavorPrepTM. (Favorgen Biotech Corp.), USA) and target nucleotide sequences in the purified plasmid were determined by accredited private laboratory. The target DNA sequence of 16S rDNA was compared to bacterial 16S rDNA sequences compiled in the GenBank database using Blast search.

Production of antimicrobial compound under optimal conditions

Culture media

Strain SS01 was cultured in five different culture media including Brain Heart infusion broth, Lactobacilli MRS broth, M17 broth, Mueller Hinton broth and Nutrient broth. All culture media were incubated at 35 °C for 48 h. After incubation, the growth of bacteria in each culture media was determined using optical density measurement at 600 nm (OD_{600}). The cell-free neutralized supernatant (CFNS) was prepared as described by Aarti *et al*. (2016). Briefly, the culture broth was centrifuged at 8,000 g for 10 min at 4 °C to remove bacterial cells. The culture supernatant was adjusted to pH 7.0 with 2.5 M NaOH in order to exclude the antibacterial effect of organic acids in the culture media. The pH-neutralized culture supernatant was sterilized using filtration through a syringe filter membrane with pore size of 0.45 µm. Antimicrobial activity against target indicator strain in CFNS was assessed by critical dilution method described by Mayr-Harting *et al*. (1972). Briefly, the CFNS were two-fold serially diluted with sterile distilled water, and aliquots (20 µL) of each dilution were spotted onto the dried surface of TSBYE soft agar plate (10 mL) inoculated with 100 µL of log phase *Micrococcus luteus* IFO 12708 (the most sensitive indicator bacteria) and incubated at 35 ºC for 24 h. After incubation, antimicrobial activity was calculated as arbitrary activity units ($AU \cdot mL^{-1}$). The arbitrary activity unit (AU) was defined as the reciprocal of the highest dilution producing a distinct inhibition of the indicator lawn, and was multiplied by a factor of 50 to obtain the $AU \cdot mL^{-1}$ of the original sample.

Temperature

Strain SS01 was cultured in M17 broth and incubated at 25, 30, 35, 40 or 45 °C for 48 h. After incubation, the growth under each designed temperature was determined using optical density measurement at 600 nm. The CFNS was prepared and antimicrobial activity against target indicator strain in CFNS was assessed by critical dilution method.

NaCl concentration

Strain SS01 was cultured in M17 broth containing 0, 2, 4 or 6% (w/v) NaCl and incubated at 35 ºC for 48 h. After incubation, the growth under each NaCl concentration was determined using optical density measurement at 600 nm. The CFNS was prepared and antimicrobial activity against target indicator strain in CFNS was assessed by critical dilution method.

Growth and antimicrobial activity at different incubation time intervals

Strain SS01 was cultured in M17 broth and incubated at 35 ºC. At each incubation time interval (0, 12, 24, 36 and 48 h), the growth was determined using optical density measurement at 600 nm. The CFNS was prepared and antimicrobial activity against target indicator strain in CFNS was assessed by critical dilution method.

Stability of antimicrobial activity in CFNS under designed critical conditions

CFNS prepared from culture broth of strain SS01 was treated with various proteolytic enzymes, high temperature, acid-base environment and NaCl concentrations. After being treated with each set of designed critical conditions, antimicrobial activity in CFNS was assessed by critical dilution method described by Mayr-Harting *et al*. (1972). (1) The stability to different proteolytic enzymes (Flavourzyme, Neutrase, Papain, Protamex and Protease M) was tested. CFNS was adjusted to the pH suitable for each enzyme's functioning. Proteolytic enzyme was added into CFNS to give a final concentration of 1 $mg \cdot mL^{-1}$ and incubated at optimum temperature for 1 h. After incubation, the enzyme activity of all treated CFNS samples was neutralized by heating in boiling water for 5 min. CFNS without proteolytic enzyme heated in boiling water for 5 min was used as a control. (2) The heat stability was tested by holding CFNS samples at 100 °C for 15, 30, 45 and 60 min, and by autoclaving at 121 °C for 15 min, compared to CFNS sample without heating (control). (3) The pH stability of antimicrobial activity was tested by adjusting pH of CFNS with 2.5 M of HCl or NaOH to pH 2, 4, 6, 8, 10 and 12, and incubating for 1 h at 4 ºC. After incubation, all CFNS samples were readjusted to pH 6.5-7.0 with 2.5 M of HCl or NaOH and lastly with sterile distilled water to maintain the same volume. CFNS without adjustment of pH (adding sterile distilled water to keep the same volume of treatments) was used as a control. (4) The NaCl stability was assessed by adding NaCl to CFNS at the final concentrations of 0, 3, 5, 7, 9 and 11% (w/v), while CFNS without NaCl and M17 broth containing the same level of NaCl were used as controls.

Mode of action of antimicrobial compound in CFNS against target indicator strain

CFNS from strain SS01 was evaporated in rotary evaporator to increase the concentration of antimicrobial compound in CFNS. Then, two-fold serial dilutions of concentrated CFNS diluted with Tryptic soy broth containing 0.6% (w/v) yeast extract (TSBYE) from 1:2 to 1:2048 were prepared. Each dilution (2 mL) was pipetted to a sterile culture tube. A volume of 200 µL of log phase *Micrococcus luteus* IFO 12708 grown in TSBYE was inoculated into each culture tube and incubated at 35 °C for 36 h. After incubation, growth of *M. luteus* IFO 12708 in each culture tube was determined by using optical density measurement at 600 nm. Culture media from each culture tube with no apparent growth was transferred by sterile 3 mm loop to streak on Tryptic soy agar containing 0.6% (w/v) yeast extract (TSAYE) and incubated at 35 °C for 24 h. Growth of *M. luteus* IFO 12708 indicated by colony forming on each TSAYE plate was recorded.

Statistical analysis

Analysis of variance was performed, and means were compared using Duncan's multiple range tests. The tests were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Bacterial strain identification

Based on its cell morphology, formation of endospore, parasporal crystals, and biochemical tests, strain SS01 was classified to genus *Bacillus* (data not shown). The sequence of 1,548 bp of the 16S rDNA gene from strain SS01 was compared to the 16S rDNA gene library of the National Center for Biotechnology Information (NCBI) database, National Library of Medicine, USA. It was shown that the studied strain shared 98.58 % similarity with *B. thuringiensis* HER1410 (accession number CP050183.1). Consequently, this study defines the name of bacterial strain SS01 as *B. thuringiensis* strain SS01. In general, *B. thuringiensis* is a group of Gram-positive, spore-forming bacteria producing parasporal crystals. They are distributed in the natural environment from the Arctic to the Tropics, and are widely used for biocontrol purposes (Bravo *et al*., 2010; Ujváry, 2010). *Bacillus thuringiensis* is a member of the *B. cereus* group, which also includes *B. cereus, B. anthracis* and *B. mycoides*. *Bacillus thuringiensis* can be readily distinguished from the other members based on its entomopathogenic properties and its capability of producing parasporal

crystals or insecticidal proteins (Cry and Cyt toxins) during the sporulation phase. These crystals are predominantly comprised of one or more proteins, also called δ-endotoxins or Cry proteins (Bravo *et al*., 2010). Similar to our finding, various species of antimicrobial-producing *Bacillus* have been isolated from mangrove-derived samples (Hu *et al*., 2010; Ramasubburayan *et al*., 2015; Haryani *et al*., 2020). Similarly, there are several reports on antimicrobial compound produced by *B. thuringiensis* isolated from various sources other than from mangrove ecosystems (Abriouel *et al*., 2011; Salazar-Marroquín *et. al*, 2016). Even though strains of *B. thuringiensis* have been isolated from mangroves in different locations (Maeda *et al*., 2001; Alarfaj *et al*., 2015; Chatterjee *et al*., 2016), most of the studies did not focus on antimicrobial-producing properties. Furthermore, other antimicrobial-producing Gram-positive bacteria such as lactic acid bacteria have also been isolated from soil, water and leaf samples collected from mangrove forests (Hwanhlem *et al*., 2014).

Production of antimicrobial compound under optimal conditions

Determination of optimal conditions for antimicrobial compound production of *Bacillus thuringiensis* strain SS01 are necessary for further studies and for up-scaling production. *B. thuringiensis* strain SS01 was cultured in five different culture media and incubated at 35 °C. After 48 h of incubation, *B. thuringiensis* strain SS01 cultured in M17 broth showed the highest ($p<0.05$) growth (OD_{600} = 1.251) and antimicrobial activity (800 $AU \cdot mL^{-1}$) against *Micrococcus luteus* IFO 12708 (Table 1). In this study, M17 broth contained more diversified organic nitrogen sources (in the digested form of beef, casein and soybean) than the other experimental culture media used. Moreover, it also contains growth enhancers such as yeast extract and ascorbic acid, as well as a large amount of disodium beta-glycerophosphate, which increases the buffering capacity of media (Wilaipun *et al*., 2002; Zimbro *et al*., 2009). Therefore, M17 broth was selected for use in the subsequent experiments.

Bacillus thuringiensis strain SS01 was cultured in M17 broth under various temperatures, and the results (Table 2) indicated that the most suitable temperature for growth of this strain was 30 °C, whereas maximum antimicrobial activity against *M. luteus* IFO 12708 was displayed at 35 °C (400 $AU \cdot mL^{-1}$). Bacterial stress factors caused by incubation temperatures lower or higher than is optimal for growth of *B. thuringiensis* strain SS01 might cause cell stress and induce more secondary metabolite production. Based on this result, an incubation temperature of 35 °C was used in the next experiment, because this study focused on antimicrobial production of the target strain. This finding is similar to a study using marine-sourced *Bacillus* DK1-SA11, which produced maximum antimicrobial activity at a temperature different from the optimum temperature for growth (Khan *et al*., 2017). Meanwhile, some strains of *B. thuringiensis* were reported to have maximum growth and antimicrobial activity (in terms of primary metabolite) at the same temperature (Ugras *et al*., 2013; Huang *et al*., 2016).

Table 1. Growth (OD_{600}) of *Bacillus thuringiensis* strain SS01 in different culture media and incubated at 35 °C for 48 h, and its antimicrobial activity against *Micrococcus luteus* IFO 12708.

Culture media	pH of culture media before incubation	pH of culture media after incubation	Growth (OD_{600})	Antimicrobial activity ($AU \cdot mL^{-1}$)
Brain Heart infusion broth	7.413 ± 0.010^{a}	6.837 ± 0.021^{b}	0.984 ± 0.010^{b}	0.000 ± 0.000^{c}
Lactobacilli MRS broth	6.537 ± 0.015^{e}	5.250 ± 0.017^{e}	0.888 ± 0.011^{c}	50.000 ± 0.000^{b}
M17 broth	6.952 ± 0.010^{c}	6.937 ± 0.015^{a}	1.251 ± 0.014^{a}	800.000 ± 0.000^{a}
Mueller Hinton broth	7.281 ± 0.010^{b}	6.510 ± 0.010^{d}	0.481 ± 0.013^{d}	0.000 ± 0.000^{c}
Nutrient broth	6.873 ± 0.015^{d}	6.580 ± 0.010^{c}	0.186 ± 0.014^{e}	0.000 ± 0.000^{c}

Note: Means within a column superscripted with different lowercase letters indicate significant ($p<0.05$) difference.

Table 2. Growth (OD_{600}) of *Bacillus thuringiensis* strain SS01 grown in M17 broth under different incubation temperatures and NaCl concentrations and its antimicrobial activity against *Micrococcus luteus* IFO 12708.

Growth conditions	Growth (OD_{600})	Antimicrobial activity ($AU{\cdot}mL^{-1}$)
Temperature (°C)		
25	0.343 ± 0.010^{d}	0.000 ± 0.000^{d}
30	0.865 ± 0.010^{a}	200.000 ± 0.000^{b}
35	0.827 ± 0.011^{b}	400.000 ± 0.000^{a}
40	0.586 ± 0.011^{c}	100.000 ± 0.000^{c}
45	0.147 ± 0.011^{e}	0.000 ± 0.000^{d}
NaCl concentration (% w/v) in culture media		
0	1.129 ± 0.012^{a}	800.000 ± 0.000^{a}
2	0.622 ± 0.010^{b}	200.000 ± 0.000^{b}
4	0.396 ± 0.011^{c}	0.000 ± 0.000^{c}
6	0.073 ± 0.011^{d}	0.000 ± 0.000^{c}

Note: Means within a column superscripted with different lowercase letters indicate significant ($p<0.05$) difference.

After finding the optimum temperature for antimicrobial production in culture broth, *B. thuringiensis* strain SS01 was cultured in M17 broth with different final concentrations of NaCl (0, 2, 4, 6% w/v). The highest growth (OD_{600} = 1.1285) and antimicrobial activity (800 $AU{\cdot}mL^{-1}$) was found in M17 broth without adding NaCl, as shown in Table 2. Meanwhile, growth and antimicrobial activity decreased to 200 $AU{\cdot}mL^{-1}$ at a concentration of 2% NaCl, and was 0 $AU{\cdot}mL^{-1}$ for the higher levels of NaCl. By this result, it was apparent that the NaCl concentration adversely affected growth of *B. thuringiensis* strain SS01 as well as antimicrobial production. The underlying reason could be that NaCl caused cell plasmolysis, release of intracellular substances and inhibition of enzyme function, as well as metabolite production in non-salt-resistant bacteria under high osmotic pressure (Li *et al*., 2020).

Antimicrobial metabolite production of *B. thuringiensis* strain SS01 in M17 broth was established in an early stationary growth phase during 12-24 h of incubation. The highest antimicrobial activity in CFNS (800 $AU{\cdot}mL^{-1}$) was recorded after 36 h of the incubation period and hence implied that large amounts of antimicrobial compound in terms of secondary metabolites were produced after 36 h of inoculation. In addition, the pH value of the media showed slight change throughout the incubation period (Figure 1). This finding is consistent with *Bacillus* sp. URID12.1 isolated from soil samples and *B. subtilis* subsp. subtilis R isolated from a mangrove area. Both produced antimicrobial compound during stationary growth phase, which is one of the key characteristics of secondary metabolite produced by microorganisms (Ramachandran *et al*., 2014; Chalasani *et al*., 2015). Similarly, *B. thuringiensis* Bt BRC-ZYR2, *B. thuringiensis* NEB17 and *B. thuringiensis* 4AJ1 also produced peptide antimicrobial compound (Thuricin BtCspB, Thuricin 17 and Thuricin 4AJ1) in the manner of secondary metabolite (Huang *et al*., 2016; Nazari and Smith, 2020; Su *et al*., 2020).

Consequently, it was concluded that *B. thuringiensis* strain SS01 could be cultured optimally in M17 broth without NaCl, at 35 °C for 36-48 h, with pH 6.77-7.06 in order to produce maximum secondary metabolites in the antimicrobial compound.

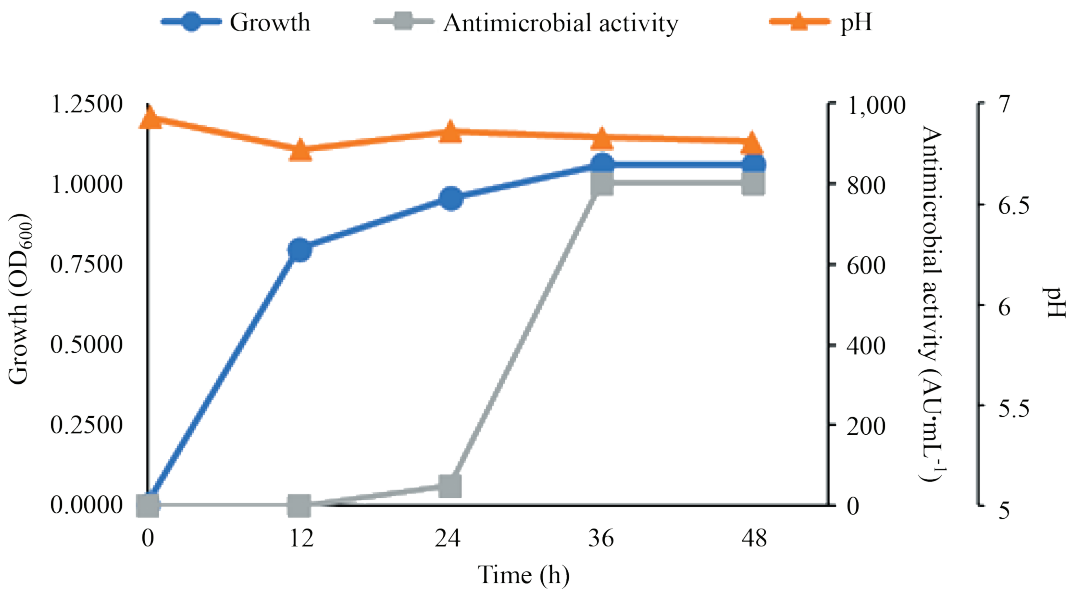

Figure 1. Profiles of growth (OD_{600}), antimicrobial activity and pH of culture broth of *Bacillus thuringiensis* strain SS01 grown in M17 broth at selected time intervals.

Stability of antimicrobial activity in CFNS of Bacillus thuringiensis strain SS01 under designed critical conditions

Information on stability of antimicrobial compound produced by *Bacillus thuringiensis* strain SS01 under some critical conditions are necessary for its application in related fields. Sensitivity of antimicrobial compound in CFNS of *B. thuringiensis* strain SS01 to the selected proteolytic enzymes is summarized in Table 3. Antimicrobial activity was completely eliminated after treatment with Neutrase and Protease M, while flavourzyme, papain, and protamex dramatically reduced antimicrobial activity without completely stopping it. By this finding, antimicrobial compound produced by *B. thuringiensis* strain SS01 was confirmed to be a naturally occurring protein compound that possesses important characteristics of bacteriocin. In brief, bacteriocins are ribosomally synthesized antibacterial peptide/protein produced by bacteria which either kill or inhibit the growth of closely related bacteria (Klaenhammer, 1993; Nes *et al*., 2013). Reduction of bacteriocin antimicrobial activity after treatment with proteolytic enzymes is related to the degradation of peptide bonds at a specific cleavage site in target bacteriocin by each proteolytic enzyme, causing a change of active structure configuration and some functional properties of bacteriocin (Ennahar *et al*., 2000). Previously, *B. thuringiensis* strains were reported to produce different types of bacteriocin, and some of them resemble lantibiotics and other small linear antimicrobial peptides produced by other antimicrobial-producing bacteria (Salazar-Marroquín *et al*., 2016). Interestingly, crude bacteriocin of some *B. thuringiensis* had broad antimicrobial spectrum against significant foodborne microorganisms (De la Fuente-Salcido *et al*., 2008) as it does in *B. thuringiensis* strain SS01, suggesting its benefits for application in food and related industries.

In Table 4, relative antimicrobial activity resulting from various heat treatments of the CFNS is shown. Once the CFNS was heated to 100 °C, the antimicrobial activity was sharply reduced to 50 %, regardless of exposure time. Even when the temperature was raised to 121 °C (treated for 15 min), the same relative antimicrobial activity was observed.

Table 3. Stability of antimicrobial activity in CFNS of *Bacillus thuringiensis* strain SS01 after treatment with different proteolytic enzymes against *Micrococcus luteus* IFO 12708.

Proteolytic enzymes	Relative antimicrobial activity (%)
Control	100
Endopeptidase	
Neutrase	0
Papain	12.50
Protease M	0.00
Protamex	12.50
Exopeptidase-Endopeptidase	
Flavourzyme	6.25

Table 4. Relative antimicrobial activity in CFNS from *Bacillus thuringiensis* strain SS01 against *Micrococcus luteus* IFO 12708 after heat treatment.

Heat treatment	Relative antimicrobial activity (%)
Untreated (Control)	100
15 min at 100 °C	50
30 min at 100 °C	50
45 min at 100 °C	50
60 min at 100 °C	50
15 min at 121 °C	50

According to the results in Table 5, one-hour treatments of CFNS produced from *B. thuringiensis* strain SS01 at pH 2, 4, 6, and 8 did not reduce antimicrobial activity in CFNS. On the other hand, at higher pH (10, 12), relative antimicrobial activity was reduced to 50 % compared with the control.

Heat tolerance and stability of proteinaceous antimicrobial compound produced by *B. thuringiensis* strain SS01 under a wide pH range was similar to many other antimicrobial peptides from *B. thuringiensis*, such as Thuricin S (Chehimi *et al*., 2007), Bacthuricin F103 (Kamoun *et al*., 2011), Thuricin Bn1 (Ugras *et al*., 2013) and Thuricin 4AJ1 (Su *et al*., 2020). Formation of small globules, presence of stable cross-links (salt bridges, hydrogen bonds, disulfide bonds) and strong hydrophobic regions in some antimicrobial peptide molecules can reduce the risk of instability under certain heating conditions (De Vuyst and Vandamme, 1992; Kumar *et al*., 2000; Simons *et al*., 2020). Therefore these similar factors might be involved in providing partial heat tolerance properties of antimicrobial compound produced by *B. thuringiensis* strain SS01. The exact mechanism for stability of bacteriocin under acid conditions, as observed in this experiment, is still unclear. However, positively charged amino acids, hydrophobicity and small molecule size were reported to be involved with the stability of peptide antimicrobial compound (Duarte *et al*., 2011).

According to Table 6, the effects of salinity were also apparent, whereby addition of 3-7% NaCl dramatically reduced relative antimicrobial activity of CFNS to 25 %. Interestingly, further increases of salinity (9 and 11 %) resulted in enhancement

of the antibacterial activity (from 25 to 50 %). A certain NaCl concentration leads to an increasing surface tension of water and generates a competition between protein and salt ions for hydration. NaCl strips off the essential layer of water molecules from the protein surface, eventually denaturing the protein (Sinha and Khare, 2014). This might be involved in the partial change of active structures and loss in some of the inhibition activity of antimicrobial compound produced by *B. thuringiensis* strain SS01. From a microbiological perspective, NaCl easily binds with free water molecules and leads to the reduction of free water molecules needed for growth of all microorganisms. In case of water containing 10% (w/v) NaCl, water activity was reduced to 0.92, which adversely affects bacterial cells (Lampel *et al*., 2012). The higher values of relative antimicrobial activity in 9 and 11% NaCl environments compared to 3-7% NaCl (Table 6) can be understood given the assumption that reduction of water activity in 9-11% NaCl caused *M. luteus* IFO 12708 to be more sensitive to peptide antimicrobial compound than in the lower NaCl environment. Until now, there have been few reports on the effect of NaCl on antimicrobial peptide produced by *B. thuringiensis*. This finding might reveal increased opportunities for application of antimicrobial compound produced by *B. thuringiensis* strain SS01 in food products containing mild and medium levels of salt.

In summary, antimicrobial compound in CFNS from *B. thuringiensis* strain SS01 is proteinaceous in nature, heat tolerant, stable under pH 2-8, and its antimicrobial activity is partially reduced in environments of 3-11% (w/v) NaCl. These properties are useful for their applications for the inhibition of some target contaminating bacteria, either as a single antimicrobial agent or in combination with another factors under hurdle food preservation technology.

Table 5. Relative antimicrobial activity in CFNS from *Bacillus thuringiensis* strain SS01 against *Micrococcus luteus* IFO 12708 when treated under different pH values.

pH Treatment	Relative antimicrobial activity (%)
Control (pH = 6.92)	100
2	100
4	100
6	100
8	100
10	50
12	50

Table 6. Relative antimicrobial activity in CFNS from *Bacillus thuringiensis* strain SS01 against *Micrococcus luteus* IFO 12708 under different NaCl concentrations.

NaCl concentration (%)	Relative antimicrobial activity (%)
0 (control)	100
3	25
5	25
7	25
9	50
11	50

Mode of action of antimicrobial compound in CFNS against target indicator strain

After incubating all diluted culture tubes inoculated with log phase *Micrococcus luteus* IFO 12708, only the control tube (without CFNS) and the culture tubes with dilutions of 1:1024 and higher showed evidence of *M. luteus* IFO 12708 growth. Meanwhile no growth was observed in the culture tubes with dilutions lower than 1:1024. In addition, subsequent experimental results showed that all TSAYE plates streaked with culture media from culture tubes with no apparent growth had colonies forming on the surface. This indicated that proteinaceous antimicrobial compound in CFNS of *Bacillus thuringiensis* strain SS01 possessed a bacteriostatic mode of action against *M. luteus* IFO 12708 but not a bactericidal mode of action. Thus, it only inhibited growth but could not kill *M. luteus* IFO 12708 in the culture broth, even at high concentrations. A similar result was reported on antimicrobial peptide produced by *B. thuringiensis* NEB17, which had bacteriostatic effects against *B. cereus* ATCC (Gray *et al*., 2006). Meanwhile, *B. thuringiensis* HD198 and *B. thuringiensis* BUPM103 produced antimicrobial peptide with a bactericidal effect toward target strains (Chehimi *et al*., 2007; Kamoun *et al*., 2011). The significant inhibition mechanism of antimicrobial peptide (bacteriocin) on target cells is pore formation caused by binding of cationic antimicrobial peptide to negatively charged phospholipid, which increases the permeability of the cytoplasmic membrane, leading to an efflux of intracellular components (Ennahar *et al*., 2000; Kumariya *et al*., 2019). Meanwhile, some types of bacteriocin inhibit cell wall and nucleic acid synthesis as well as specific enzyme reactions that are essential for the targeted bacteria (Simons *et al*., 2020). The variation in mode of action of each bacteriocin depends on the nature, structure and concentration of bacteriocin as well as growth stage, cell stress, genetic profile and the outer membrane structure of target bacterial strains (Gray *et al*., 2006; Bharti *et al.*, 2015; Nazari and Smith, 2020). Some strains of *B. thuringiensis* produced antimicrobial peptide with both bacteriocidal and bacteriolytic effects, depending on the applied concentration level (Ugras *et al*., 2013). Mode of action in each bacteriocin is one of the significant criteria for finding a suitable approach for controlling target bacteria in each application.

CONCLUSION

Bacillus thuringiensis strain SS01, identified by biochemical tests and 16S rDNA homology, was isolated from a soil sample collected from a mangrove forest located in central Thailand. The CFNS of *B. thuringiensis* strain SS01 strongly inhibited the growth of target Gram-positive bacteria and *Micrococcus luteus* IFO 12708 to the most sensitive indicator bacteria. The optimum conditions for growth and antimicrobial production of this strain were culturing in M17 broth without NaCl at 35 °C for 36-48 h. The secondary metabolite antimicrobial compound in CFNS was proteinaceous in nature, tolerant to heat, and stable under pH 2-8. It exhibited a bacteriostatic mode of action against a target indicator strain. Meanwhile, its antimicrobial activity was partially reduced to 25-50 % in environments with 3-11% (w/v) NaCl. These specific properties are beneficial for applications in food products, animal feed and related industries. Finally, the data we have presented here can serve as a platform for more detailed studies on large-scale production, purification and in-depth characterization of purified antimicrobial peptide from *B. thuringiensis* strain SS01.

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