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The Antimicrobial Effects of *Bacillus subtilis* B-1 isolated from a Fish Culture Pond

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Abstract

Bacillus subtilis B-1, isolated from fish culture pond water, produced antimicrobial substances that inhibited an array of pathogenic bacteria, including *Streptococcus agalactiae* ABRCS-1, *Aeromonas hydrophila* ABRCA-1, *Staphylococcus aureus* ATCC 12600, *Listeria innocua* ATCC33090, *Micrococcus luteus* IFO 12708, *Bacillus circulans* JCM 2504 and *Bacillus coagulans* JCM 2257. Particularly, *L. innocua* ATCC 33090 showed the highest sensitivity to *B. subtilis* B-1 out of all indicator strains tested. The cell-free neutralized supernatant from *B. subtilis* B-1 was stable when tested at 100 °C for 50 min across a range of pH values (2-10) and had a maximum activity of 320 $AU \cdot mL^{-1}$. When tested after 24 h at 4 °C the stability results were the same.However, the maximum activities decreased to 80 $AU \cdot mL^{-1}$ at pH values of 4, 5 and 6.The activity of B-1 also decreased when subjected to a temperature of 100 °C for 60 min. The antimicrobial substances in *B. subtilis* B-1 supernatants were sensitive to the proteolysis by α–chymotrypsin, trypsin, proteinase K and a serine type protease from *Bacillus* sp. They were stable in the presence of sodium chloride (NaCl) up to a solution of 15%. The high stability of antimicrobial substances in *B. subtilis* B-1 makes it suitable for the inhibition of food-borne spoilage bacteria and pathogenic bacteria in aquaculture. Purifying the antimicrobial substances by amberlite adsorption and reverse-phase chromatography resulted in a single active peak, which was designated B1-1. The molecular weight of this fraction by mass spectrometry was 3,398.05 m/z.

Introduction

Nile tilapia farming has been affected by the outbreak of streptococcosis that has resulted in huge production losses and has negatively impacted the aquaculture industry. The indiscriminate use of antibiotics and their residual effects has produced resistant pathogenic bacteria. Recent research suggests that probiotic organisms such as *Bacillus*, *Lactobacillus, Carnobacterium* and *Vibrio* could be used to control pathogenic bacteria. *Bacillus* produces an array of antimicrobial peptides with varying structures and specificities. For example, bacteriocins are synthesized by ribosomes and are post-translationally modified by sublancin, subtilosin and subtilin (Brownyn and Helmann, 2006; Paik et al. 1998; Zheng and Slavik, 1999). Bacteriocins are resilient against a variety of bacteria,

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including pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus* and *Streptococcus* sp. In this study, *B. subtilis* B-1was isolated from fish culture ponds. By bathing fingerling fish, the pathogenic bacteria, *Streptococcus agalactiae in vivo* could be controlled. The aim of this study was to characterize the antimicrobial substances secreted by *B. subtilis* B-1 and to measure their sensitivity to proteolysis, high temperature, pH and sodium chloride (NaCl). The antimicrobial substance from *B. subtilis* B-1 was partially purified by high performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer (MALDI-TOF). This data were used to implement an effective testing methodology to combat detrimental bacteria found in the aquaculture industry.

Materials and Methods

Bacterial Strains and Culture Condition

A bacterial strain isolated from fish culture pond water in Kamphangsaen Fisheries Research Station (Kasetsart University, Nakornprathom Province, Thailand) was identified as *B. subtilis* B-1 and used for further studies. Eight different species of pathogenic bacteria were used as indicator strainsto study the antimicrobial properties of *B. subtilis* B-1 (Table 1). The indicator are *S. aureus* ATCC 12600, *L.innocua* ATCC 33090, *E. coli* JM 109, *A. hydrophila* ABRCA-1, *S. agalactiae* ABRCS-1, *B. circulans* JCM 2504, *B. coagulans* JCM 2257 and *M. luteus* IFO12708. *Bacillus subtilis* B-1 was cultured in tryptic soy broth (TSB) medium at 37 °C and was stored in 20% glycerol at -20 °C. The indicator strains were grown in TSB medium at temperatures between 30 and 37 °C.

Table 1. Antimicrobial activity of *B. subtilis* B-1 against spectrum bacterial strains by spot method (clear zone) and antimicrobial activity of CFNS *B. subtilis* B-1 against bacterial strains.

Indicator strains	Clear zone	Antimicrobial activity($AU \cdot ml^{-1}$)
*S. aureus*ATCC 12600	+ve	20
E. coli JM 109	-ve	0
*L. innocua*ATCC 33090	+ve	320
*S. agalactiae*ABRCS-1	+ve	20
*A. hydrophila*ABRCA-1	+ve	20
*M. luteus*IFO 12708	+ve	80
*B. circulans*JCM 2504	+ve	40
*B. coagulans*JCM 2257	+ve	20

+ve = clear zone
-ve = no clear zone

Preliminary Study of Antimicrobial Activity

The spectrum of antimicrobial activity was tested against the indicator strains using the spot method. A total of 5 mL of 0.7% tryptic soy agar (TSA) containing 100 µL of an indicator strain was overlaid onto 1.5% agar plate. The plate was then spotted with B-1 using a needle. The

appearance of inhibition zone or clear zone was determined after incubating the cultures for 24 h at 37 °C.

Antimicrobial Activity

Antimicrobial activity was determined by the critical dilution method and the bacteriocin activity assay, described by Schillinger et al. (1993) and Cintas et al. (1995), respectively. *Bacillus subtilis* B-1 cultures were grown in TSB medium for 5 days at 37 °C. Cell-free neutralized supernatant (CFNS) was obtained by centrifuging *B. subtilis* B-1 cultures at 14,000 rpm for 15 min at 4 °C. The samples were sequentially diluted two times with 50 µL of distilled water. Each dilution was added to a well on TSA plates and was overlaid with 5 mL of TSA soft agar medium containing 10^7 CFU·mL^{-1}of an indicator bacteria strain. The plates were then cultured for 24 h. The arbitrary activity unit (AU) was defined as the reciprocal of the highest dilution thatyielded a clear inhibition zone on the indicator strain and multiplied by the dilution factor of 20 to obtain the AU·mL^{-1}of the original sample.

Effect of Enzyme Digestion, Heat Treatment, pH and NaCl on Antimicrobial Activity

Enzyme Digestion

The CFNS of *B. subtilis* B-1 was grown in TSB at 37 °C for 5 days and was treated with the following enzymes at a final concentration of 1 mg·mL^{-1}: α-chymotrypsin pH 7: trypsin pH 7: proteinase K pH 7: protease pH 5-9; and lipase pH 7. All chemicals were obtained from Sigma Fine Chemicals (St. Louis, Mo, USA) unless otherwise noted. After incubation at 37 °C for 3 h, the CFNS was exposed to heat (100 °C) for 5 min to inactivate the enzymes, and the antimicrobial activity was determined. An untreated sample was used as a control, and the residual antimicrobial activity was assayed by the critical dilution method, using *L. innocua* ATCC 33090 as the indicator strain.

Heat Treatment

The effect of high temperature on the antimicrobial activity was investigated by heating the CFNS from *B. subtilis* B-1 at 100 °C for 5, 10, 15, 20, 30, 40 or 50 min, or at 121°C for 15 min in an autoclave. The CFNS was then inactivated on ice for 5 min before determining the residual antimicrobial activity.

pH

The effect of different pH levels on the antimicrobial activity of B-1 was investigated. *Bacillus subtilis* B-1 was grown in TSB at 37 °C for 5 days and two samples of CFNS were collected. Both samples were adjusted to a pH level between 2 and 10 using 5 N HCl or NaOH. The first sample was heated to 100 °C for 60 min and then cooled on ice. The second sample was left to

stand at 4 °C for 24 h. All samples were adjusted to a pH of 6.5 before determining the residual antimicrobial activity.

NaCl

The effect of different concentrations of NaCl on the antimicrobial activity was studied. Sodium chloride was added to samples of CFNS of *B. subtilis* B-1 at final concentrations of 0-15%, and then the samples were divided and treated at two different conditions. The first sample was incubated at 4 °C while the second waskept at room temperature (approximately 30 °C) for 24 h before determining the residual antimicrobial activity.

Partial Purification of Antimicrobial Substances

Concentration of Antimicrobial Substances using Amberlite XAD-16

Bacillus subtilis B-1 was grown in 1 L TSB broth at 30°C for 5 days. The culture was centrifuged at 14,000 rpm for 20 min at 4°C to obtain the CFNS. Purification was carried out as described by Pilasombut et al. (2006). Antimicrobial substance adsorption was achieved by adding 20 g of amberlite XAD-16 (Sigma, St.Louis, Mo) to 1 L of CFNS and shaking at room temperature for 2 h. The hydrophobic amberlite adsorbing substances were collectedand washed with 100 mL of distilled water, followed by 100 mL of 40% (v/v) ethanolin distilled water. Finally, the bacteriocin was eluted with 100 mL of 70% isopropanol solution in distilled water. The activity of the various bacteriocin antimicrobial fractions was compared to *L. innocua* ATCC 33090.

Purification of Antimicrobial Substances by Reversed-Phase HPLC

The antimicrobial substance that adsorbed amberlite XAD-16 was purified by reversed phase-HPLC usinga BDS hypersil C_{18} reverse-phase column (Thermo Scientific, Thermo Fisher Scientific Inc., USA). Elution was carried out using a linear gradient from 100% A/0% B to 20%A/80% B, where solvent A was 0.1% trifluoroacetic acid (TFA) in water and solvent B was acetonitrile containing 0% TFA. Elution was carried out at a flow rate 1 $mL \cdot min^{-1}$ for 30 min. Fractions were detected by their absorbance at 220 nm. These fractions were evaluated for their antimicrobial activity against the indicator strain *L. innocua* ATCC 33090. Acetonitrile containing 0.1% TFA was used as negative control.

Results

Preliminary Study of Antimicrobial Activity

Bacillus subtilis B-1 was tested for antimicrobial activity against several microorganisms including Gram-positive and Gram-negative bacteria. The results from those tests are shown in Table 1. All of the indicator strains except *E.coli* JM109 showed sensitivity to *B. subtilis* B-1.

Bacillus subtilis B-1 showed antimicrobial activity towardpathogenic bacteria found in freshwater aquacultures, namely *S. agalactiae* and *A. hydrophila*.

Antimicrobial Activity

The CFNS was tested for antimicrobial activity against several Gram-positive and Gram-negative microorganisms by the critical dilution assay and the agar well diffusion method. The results were reported as a function of the highest dilution of CFNS that yielded a clear zone on the indicator strain multiplied by a dilution factor of 20. The highest activity was detected in *L. innocua* ATCC 33090 at 320 $AU \cdot mL^{-1}$. The lowest activity was found in *S. aureus*, *S. agalactiae*, *A. hydrophila* and *M. luteus*, with activities of 20 $AU \cdot mL^{-1}$. Thus, *L. innocua* was used as the indicator strain for residual CFNS antimicrobial activity after either physical or chemical treatment.

Effect of Enzyme Digestion, Heat Treatment, pH and NaCl on Antimicrobial Activity

Enzyme Digestion

Bacillus subtilis B-1 antimicrobial substances were tested for enzymatic sensitivity by digesting the CFNS with the following enzymes: α–chymotrypsin, trypsin, proteinase K, protease and lipase. The residual antimicrobial activity of B-1 against *L. innocua* ATCC 33090 was measured using the agar well diffusion assay. α–chymotrypsin produced the largest decrease in the antimicrobial activity of the B-1 substances while lipase treatment did not affect antimicrobial substances (Table 2).

Table 2. Effects of enzymes digestion and heat treatment on antimicrobial activity of the CFNS of *B. subtilis* B-1.

Enzyme/ Heat Treatment	**Residual activity ($AU \cdot mL^{-1}$)**
Untreated (control)	320
α–Chymotrypsin	40
Trypsin	80
Proteinase K	80
Protease	80
Lipase	320
5 min/100 °C	320
10 min/100 °C	320
15 min/100 °C	320
20 min/100 °C	320
30 min/100 °C	320
40 min/100 °C	320
50 min/100 °C	320
15 min/121 °C	0

Heat Treatment

The heat sensitivity of the B-1 antimicrobial substances was determined by measuring the residual antimicrobial activity of the CFNS after incubation at 100 °C for different durations (5, 10, 15, 20, 30, 40 and 50 min) and at 121 °C for 15 min in an autoclave. These antimicrobial substances showed high temperature tolerance. The antimicrobial activity was still retained after heating at 100 °C for 50 min, with a residual activity of 320 $AU \cdot mL^{-1}$ (Table 2).

pH

Antimicrobial substances were adjusted to obtain pH values between 2 and 10. The samples were either heated to 100 °C for 60 min or cooled to 4 °C for 24 h and the residual antimicrobial activity was measured using the agar well diffusion assay against *L. innocua* ATCC 33090. At 4 °C for 24 h, the antimicrobial activity was mostly retained for all pH values, while at 100 °C for 60 min, the activity was clearly reduced for pH values between 2 and 8 and was completely lost at 9 and 10 (Table 3).

Table 3. Antimicrobial activities of the CFNS from *B. subtilis* B-1 at different pH values andheating/cooling treatments.

pH	Residual activity ($AU \cdot mL^{-1}$)	
	4 °C for 24 h	100 °C for 60 min
2	320	20
3	320	20
4	320	80
5	320	80
6	320	80
7	320	40
8	320	40
9	320	0
10	320	0

NaCl

The antimicrobial activity of *B. subtilis* B-1 was not affected by NaCl at any concentration when incubated at 4 °C for 24 h (320 $AU \cdot mL^{-1}$). However, incubation at 30 °C for 24 h clearly reduced the antimicrobial activity of the CFNS (80 $AU \cdot mL^{-1}$).

Partial Purification of Antimicrobial Substances

One single active peak, designated B1-1, was obtained from the purification of the B-1 antimicrobial substances by amberlite adsorption and 30 min reverse-phase chromatography purification by HPLC (Fig 1). This fraction was rerun on the same column using the following

gradient: 75 % A/ 25% B to 35% A/ 65% B. This product inhibited *L. innocua* ATCC 33090 and had a molecular weight of 3,398.05 m/z, as determined by mass spectroscopy.

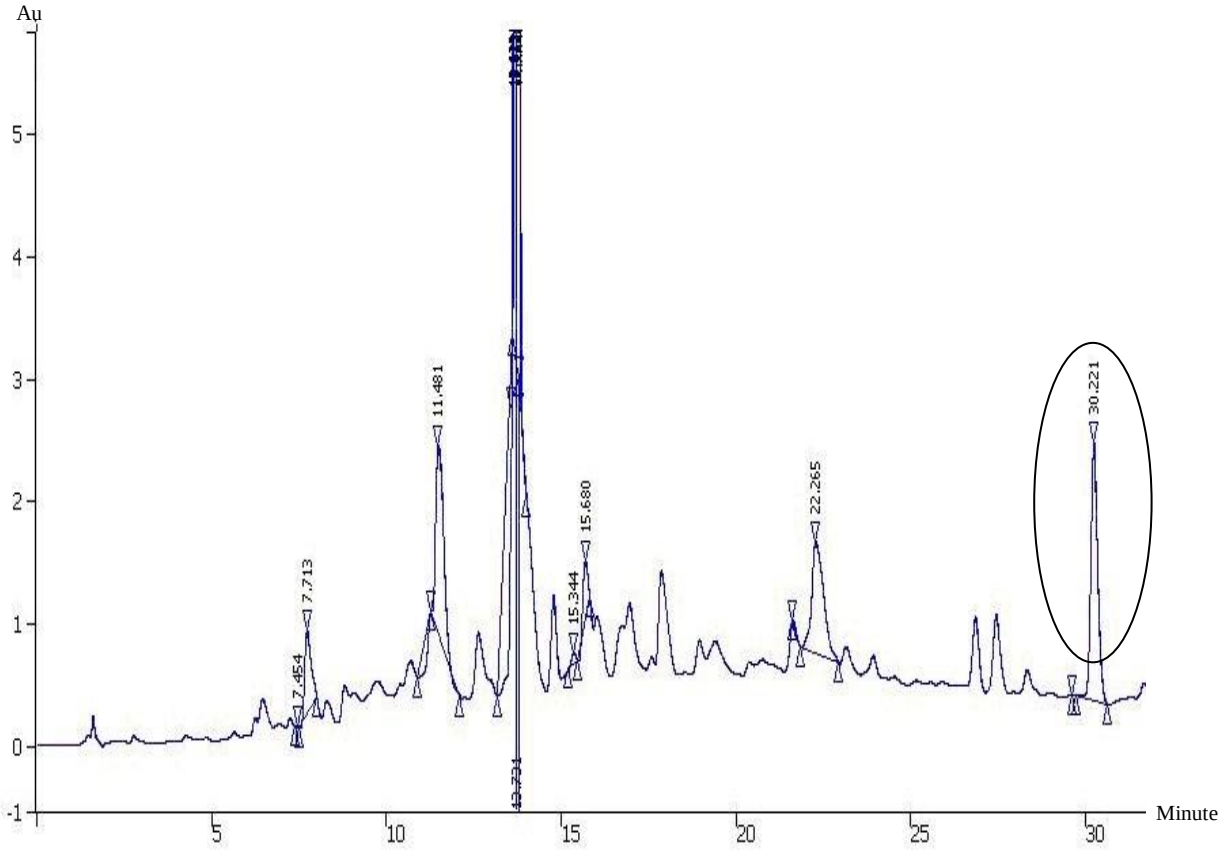

Fig. 1. Reverse-phase chromatography of the antimicrobial substance by amberlite adsorption showing one single active peak.

Discussion

Bacillus subtilis B-1, isolated from the water of a Nile tilapia culture pond, showed significant inhibitory activity against many indicator strains of bacteria, as determined by the spot and agar well diffusion methods. Indicator strains that were used included the following: *L. innocua* ATCC 33090, *S. aureus* ATCC 12600, *A. hydrophila* ABRCA-1, *S. agalactiae* ABRCS-1, *B. coagulans* JCM 2257, *M. luteus* IFO 12708 and *B. circulans* JCM 2504. This inhibitory activity indicated that *B. subtilis* B-1 could produce the substance with antimicrobial activity. The antimicrobial activity of *Bacillus* spp. has been studied extensively in previous literatures and similar results have been found. Zheng and Slavik (1999) reported that the crude antibacterial preparation from *B. subtilis* could inhibit the growth of *B. cereus* ATCC 14893, *S. aureus* ATCC 6538, *Salmonella typhimurium* ATCC 14028, *E. coli* O157: H7 ATCC 43888, *Yersinia enterocolitica* ATCC 27729 and *L. innocua* in agar well diffusion. A similar report by Ouoba et al.

(2006) indicated that antimicrobial substances from *B. subtilis* isolated from African locust bean could inhibit the growth of *M. luteus* A1, *B. cereus* A4, *B. cereus* A10, *E. coli* A13, *S. aureus* A11 and *S. aureus* A12. Cherif et al. (2003) reported that antimicrobial peptides of *B. thuringieusis* ssp. *entomocidus* HD 9 "Entomocin 9" inhibited the growth of many Gram-positive bacteria, including *L. monocytogenes*, *Pseudomonas aeruginosa* and fungi such as *Aspergillus nidulans*, *Fusarium oxysporum*, *F. graminis* and *Botrytis cinerea*. Martirani et al. (2002) reported that the antimicrobial substance "Bacillocin 490" from *B. licheniformis* had a high temperature tolerance and could inhibit the growth of *B. anthracis*, *B. stearothermophilus* and *B. smithii*.

Many Gram-positive bacteria produce antimicrobial peptides called "bacteriocins". Thesebacteriocins are proteinaceous compounds that are synthesized by ribosomes and post-translationally modified. These proteins are widely classified according to the nature of the compoundand can be divided into three main groups: modified bacteriocins known as lantibiotics (class I), heat-stable, unmodified bacteriocins (class II), and larger, heat-labile bacteriocins (class III) (Joerger, 2003; Papagianni, 2003; Boman, 1995). Generally, bacteriocins are extremely active against Gram-positive bacteria. Bacteriocin commonly act by forming a pore in the cell membrane which dissipates the membrane function by inducing ion leakage and/or interfering with cellular ATP production (Sang and Blecha, 2008; Jack et al. 1995; Ralph et al.1995). The antimicrobial activity of CFNS from *Bacillus* sp. B-1 in this study was significantly reduced by proteolytic enzymes, which was consistent with the finding of Oguntoyinbo et al. (2007) and Qi-qin et al. (2006), who found that the antimicrobial substances from *B. subtilis* were sensitive to proteolytic enzymes. This sensitivity was also reported for compounds isolated from other species of *Bacillus* such as *B. amyloliquefaciens* (Lisboa et al. 2006; Sutyak et al. 2008) and *B. cereus* 8A (Bizani et al. 2005). The antimicrobial peptides produced by the genus *Bacillus* are highly rigid, hydrophobic structure with cyclic component and are generally resistant to hydrolysis by peptidases and proteases (Qi-qin et al. 2006; Stein, 2005; Katz and Demain, 1977). Aunpad and Na-Bangchang (2007) reported that *Bacillus pumilus* strain WAPB4 produced antimicrobial peptides (Pumilicin) that were not active following treatment with the proteolytic enzymes trypsin, chymotrypsin and pronase E. The sensitivity of the antimicrobial substances from *B. subtilis* B-1 to certain proteolysis enzymes suggests that these substances may have proteinaceous properties. The antimicrobial substances of *B. subtilis* B-1 showed remarkably stable behavior under extreme temperatures, pH values and high concentration of NaCl. Teixeira et al. (2009) reported that antimicrobial substances from *B. licheniformis* strain P 40 were resistant to temperatures of 100 °C for 30 min at pH values ranging from 3-10. A similarly report by Riazi et al. (2009) found that *B. coagulans* ATCC 7050 produced the antimicrobial substance "Lactosporin", which retained activity at pH values between 3-7 but lost all activity at pH values above 7. Lactosporin remained active following exposure to high temperature (100 °C) for 30 min. Similarly, *Bacillus* sp. MTCC 43 produced bacteriocins that tolerated a wide range of pH values (4-10) and were heat-stable at temperatures between 40-90 °C for 10 min, but lost activity at 100 °C (Sharma et al. 2009). Kamoun et al. (2005) identified Bacthuricin F, produced by *B. thuringiensis*, which retained its antimicrobial activity when subjected to a range of temperature conditions (40-90 °C) for 30 min, and also showed stable

activity for a range of pH values (3-9) when incubated at 4 °C for 4 h. However, Bacthuricin F lost all activity at 100 °C. Xie et al. (2009) reported that *B. subtilis* could produce bacteriocin that retained activity following exposure to high temperature (100 °C) for 15 min and pH 3-10. The partial purification of *B. subtilis* B-1 resulted in only a single active peak (B1-1) at 30 min. The active antimicrobial compound in B1-1 had a value of 3,398.05 m/z, as determined by MALDI-TOF mass spectrometry. In previous literature, Stein et al. (2004) found that *B. subtilis* 168 produced a compound with a mass of 3,400.7±0.2 m/z. As measured by MALDI-TOF mass spectrometry, which corresponds to Subtilosin A while *B. subtilis* ATCC 6633 produced a peptide with a mass of 3,3319.4 m/z. Shelburne et al. (2007) reported that *B. subtilis* ATCC 6633 produced an antimicrobial substance (bacteriocin) with a MALDI-TOF signal at 3,400.7 m/z, which confirmed the identity of the peptide as Subtilosin A.

Conclusion

Bacillus subtilis B-1, isolated from the water of a Nile tilapia culture pond and its CFNS, showed good inhibition activity against pathogenic diseases and food-borne bacteria. The antimicrobial substance from *B. subtilis* B-1 had a proteinaceous property in response to many proteolytic enzymes. It showed remarkable tolerance to high temperature (100 °C) for 50 min, pH values and various concentrations of NaCl. The partial purification of *B. subtilis* B-1 had a single active peak at 30 min and a molecular weight of 3,398.05 m/z, as determined by MALDI-TOF. The data obtained from this study indicated that some characteristics of *B. subtilis* B-1 are ideal for the implementation in health and quality management for aquaculture and the food industry.

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