

## Bioactivities of black soldier fly larvae protein hydrolysate

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### Abstract

One of the potential insects for feed and food sources is the Black Soldier Fly (BSF) (*Hermetia illucens*). The BSF originated in South America and has now spread to many tropical countries. The BSF larvae (BSFL) had attracted much attention due to their good nutrient content and were recently reported as having useful bioactivities. BSFL has been widely used as feed and has been found to reduce bad smells associated with the growth of pathogenic microbes. The high protein content in BSFL is advantageous in the production of bioactive peptides. This research aimed to extract and hydrolyze the protein from BSFL with protease enzyme to produce protein hydrolysate or peptides with antioxidant and antibacterial activities. The antioxidant activity was measured using DPPH and FRAP, while the antibacterial was analyzed using the agar well diffusion method. BSFL was hydrolyzed with bromelain extracted from pineapple or commercial enzyme (Enzyplex). The IC<sub>50</sub> shown by protein hydrolysate was 9.43±0.84% (v/v) or 0.72 mg/mL. The FRAP value was 521.21±264.53 µM for BSFL protein without enzyme addition, 792.14±291.02 for BSFL with the addition of bromelain, and 5,352.114±861.99 µM for BSFL with the addition of Enzyplex. The addition of bromelain increased the FRAP value 1.2 times, while the addition of Enzyplex increased the FRAP value 10.2 times. The antibacterial activity indicated that protein hydrolysate with phosphate buffer pH 8 at 50°C and 24 hrs incubation showed the highest antibacterial activity to the lowest are *Vibrio cholerae*, *Bacillus cereus* and *Staphylococcus aureus*, respectively. The results suggest that BSFL has antibacterial and antioxidant bioactivities using bromelain extracted from pineapple and Enzyplex, a commercial enzyme that contains protease, lipase and amylase. Using Enzyplex as an enzyme to hydrolyze BSFL shows a very high antioxidant capacity compared to bromelain as an enzyme. This suggests that more protein and other macromolecules contribute to the antioxidant capacity. Using bromelain as an enzyme to hydrolyze BSFL, shows that BSFL peptide has higher antibacterial activity in gram-negative bacteria.

## 1. Introduction

Insects have been recognized as one of the potential sources of future food with more than 1,900 species already documented as suitable for food consumption due to their good nutrition content. Insect farming has been proven as environmentally friendly with low and efficient investment costs (Food and Agriculture Organization of the United Nations, 2012).

One of the insects, which has been the research focus

presently, the Black Soldier Fly (*Hermetia illucens*) is known to live in a warm climate and is mostly found in environments such as organic waste (Lievens *et al.*, 2021). Black Soldier Fly Larvae (BSFL) are rich in protein and essential amino acids and usually are harmless to humans and livestock. BSFL is getting popular in its use as feed, while for food applications, BSFL still needs to be developed from its present form. Studies have pointed application of BSF as a future food ingredient and health application. This is related to a

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report on the antioxidant and inhibitor of microorganism activities shown by BSFL extracts (Choi *et al.*, 2012; Park *et al.*, 2015).

Antioxidant substances function to overcome or neutralize reactive oxidative species (ROS) produced by our daily activities. An excessive amount of ROS will be detrimental to various cell components and molecules of our cells, DNA, membrane, and proteins which lead to denaturation of these molecules and result in fast cellular aging. Denaturation of DNA mitochondria especially reduces much of the cellular ability to divide (Suh *et al.*, 2010). BSFL hydrolysate or peptide was reported to have antioxidant activity. Amino acids that have imidazole groups, aromatic residues, and sulfur-containing groups can inhibit free radicals by electron transfer to the free radicals. It is reported that BSFL has high hydrophobic and aromatic amino acids (Suh *et al.*, 2010; Firmansyah and Abduh, 2019).

BSFL extract was also reported to have antimicrobial activity against Gram-negative pathogens (Choi *et al.*, 2012; Xia *et al.*, 2021) and Gram-positive bacteria (Park *et al.*, 2015). Various pathogens exhibit resistance to antibiotics, and even worse become multidrug resistance which makes infectious diseases hard to treat. Furthermore, antibiotics resistances are found commonly in communities and hospitals (Choi *et al.*, 2012; Park *et al.*, 2015). Therefore, the development of a new antibacterial source can offer a good alternative. Maggot is used as a medicine and has been utilized for medical treatment (Park *et al.*, 2015). Secretion from maggots produced on the surface or within the digestive tract has been reported to have antibacterial activity (Choi *et al.*, 2012).

Protein hydrolysate can be produced by applying protease enzymes such as bromelain, papain, subtilisin, or other proteases to animal, vegetable, or insect protein sources. Many proteins hydrolysates show biological activities such as antioxidant, antimicrobial, anticancer, or immune enhancer (Firmansyah and Abduh, 2019). BSFL products seemed to be a good alternative to be developed into high-value bioactive substances useful for health applications.

This research aimed to produce protein hydrolysates with antioxidant and bacterial inhibiting activities from Black Soldier Fly Larvae (BSFL) using two types of proteases which are bromelain extracted from pineapple and Enzyplex which contain protease, lipase, and amylase.

## 2. Materials and methods

### 2.1 Materials

Frozen BSFL was collected from Maggot Farm Ciburial, Sumedang. The crude bromelain was extracted from Honey Pineapple, Sunter, Jakarta. The commercial enzyme tablet -Enzyplex- (Darya Varia Laboratoria, Bogor, Indonesia) is used as a commercial enzyme. Chloramphenicol 250 mg capsule (Hufamycetin, Gratia Husada Farma, Semarang, Indonesia) antibiotic was collected from Jakarta. The bacteria used in this research are *Bacillus cereus* ATCC 10876 (Gram-positive), *Staphylococcus aureus* ATCC 25923 (Gram-positive), and *Vibrio cholerae* ATCC 14035 (Gram-negative), which were collected from Atma Jaya Culture Collection Jakarta.

### 2.2 Crude bromelain extraction

The pineapple was washed with running water, then the pineapple was peeled, and the flesh of the fruit was chopped into small chunks and then blended until smooth (without adding water). The extract was filtered using a sieve and the supernatant was kept in the refrigerator at 4°C and freezer at -20°C.

### 2.3 Hydrolysis Black Soldier Fly larvae protein

The hydrolysate of BSFL was produced using the method from Firmansyah and Abduh (2019) with several modifications. The frozen BSFL was thawed and washed, then crushed with mortar and pestle. The BSFL was dissolved in phosphate buffer 50 mM pH 8 (1 g /5 mL). After being dissolved, the sample was incubated in a water bath shaker with various temperatures (Table 1) and enzyme.

Table 1. Hydrolysis temperature treatments on various samples.

Samples	Temperature
Extracted bromelain enzyme 10% (v/v)	50°C
Commercial enzyme (Enzyplex) 10% (w/v)	37°C
BSFL	50°C
BSFL + Extracted bromelain enzyme 10% (v/v)	50°C
BSFL + Commercial enzyme (Enzyplex) 10% (w/v)	37°C

The hydrolysis reaction was done in a water bath shaker at 150 rpm. The enzyme concentration used for antioxidant activity analysis was 10%. The temperature used was based on the optimal temperature of each enzyme, 50°C for bromelain and 37°C for Enzyplex (Dubey *et al.*, 2012; Xia *et al.*, 2021).

The hydrolysis was terminated by incubating the samples in a water bath shaker at temperature 90°C, with the agitation 150 rpm for 10 mins. The samples were

then centrifuged at 4,000×g for 30 mins. The supernatant was collected and stored at -20°C.

#### 2.4 Protease activity

The enzyme activity was analyzed following Bergmeyer and Grassl (1983) method. The protease activity was analyzed using casein as substrate and tyrosine as standard and quantified by using the following equation:

$$UA = ([\text{tyrosine}] \times \text{total volume assay}) / (T \times \text{Venzyme})$$

where UA is the unit of protease activity or amount of tyrosine produced/mL/min (U/mL), [tyrosine] is the concentration of tyrosine (μmol), total volume assay (mL), Venzyme is the volume of enzyme used (mL), and T is incubation time (10 mins).

#### 2.5 Protein concentration

The concentration of protein was measured by using the Bradford method. Around 100 μL of sample and blank were mixed with 2 mL of Bradford reagent then homogenized and incubated for 5 mins at room temperature. The absorbance of the extract was measured at 595 nm by a microplate reader. A standard curve was used using Bovine Serum Albumin (BSA) in the 0.1 to 1.0 mg/mL range (Bradford, 1976). This test was replicated 3 times.

#### 2.6 DPPH radical scavenging calorimetric assay

DPPH assay was conducted by the method described by Olugbami et al. (2015). As much as 0.1 mM DPPH was dissolved in methanol. The positive control used was L-ascorbic acid with several concentrations of 0, 50, 100 and 200 μg/mL. For BSFL hydrolysate, the dilutions were 2.5, 5, 10, 20, 40%. As much as 100 μL samples were reacted with 1 mL DPPH. After the reaction, the samples were incubated in the darkroom for 30 mins. After incubation, the samples were read at 517 nm. The free radical inhibition concentration was calculated by the following equation:

$$\text{Radical scavenging activity (\%RSA)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\%$$

To calculate IC<sub>50</sub>, the curve that shows a relationship between sample concentration and radical scavenging activity was made to get regression and equation of the relationship between sample concentration and radical scavenging activity. To determine IC<sub>50</sub>, 50% was inserted in the equation to determine the concentration that inhibits 50% of the DPPH.

#### 2.7 Ferric reducing antioxidant power assay

The ferric reducing antioxidant power (FRAP) assay

was done using the method from Szeto et al. (2002) and Youn et al. (2019). The FRAP reagent contained 300 mM acetate buffer pH 3.6, 10 mM 2,4,6-Tris(2-Pyridyl)-S-Triazine (TPTZ) in 40 mM HCl and 20 mM FeCl<sub>3</sub>·6H<sub>2</sub>O. These three solutions were mixed with the ratio of acetate buffer, TPTZ, and FeCl<sub>3</sub>·6H<sub>2</sub>O 10:1:1. The standard used was FeSO<sub>4</sub>·7H<sub>2</sub>O at 1000 μM. The FRAP reagent and samples were reacted with the ratio of 30:1, and incubated at 37°C for 4 mins. After the incubation, the samples were read at 593 nm. The following equation was used to calculate the FRAP value.

$$\text{FRAP value} = \frac{\text{Absorbance sample}}{\text{Absorbance standard}} \times \text{standard FRAP value (1000 } \mu\text{M)}$$

#### 2.8 Antibacterial assay

The antibacterial assay was conducted based on the agar well diffusion method to detect inhibition activity from protein hydrolysate samples against *Bacillus cereus*, *Staphylococcus aureus* and *Vibrio cholerae*. All pathogens were inoculated in Luria Bertani (LB) Broth and then incubated in a water bath shaker at 37°C with shaking (120 rpm) overnight. A 100 μL of each suspension (OD<sub>600</sub> = 0.132 = 1.5×10<sup>8</sup> CFU/mL) was spread onto nutrient agar (NA). The agar plate was punched aseptically with a sterile cork borer (8 mm). For positive control, 250 mg of Hufamycin chloramphenicol in 300 ml of aquadest, inactive bromelain 20% (v/v), and phosphate buffer were used and pipetted into each well. The agar plates were incubated at 37°C for 24 and 48 hrs. The diameter of the clear zone was measured in mm by a ruler. This test was performed in duplicate (Valgas et al., 2007).

The extract with the highest zone of inhibition was retested in various dilutions (100%, 50%, and 25% v/v) and compared to the extract with Enzyplex treated in various dilutions (100%, 50% and 25% v/v). All extract dilution used phosphate buffer pH 8 as solvent. This test was performed at least three times.

### 3. Results and discussion

Protein hydrolysate or peptides show diverse biological activity, such as antihypertensive, antioxidant, antibacterial, immune enhancer, and anticancer. The substrate used in the production of the protein hydrolysate is dominated by animal protein (Firmansyah and Abduh, 2019).

In this research, BSFL is used as the substrate for the protein hydrolysate. BSFL is harmless to humans and livestock and contains high protein and essential amino acids (Choi et al., 2012). Protein hydrolysis can be produced chemically or enzymatically. Enzymatic

hydrolysis is preferred because this can be performed without extreme conditions, has no side reactions, and does not reduce the nutritional value of the protein sources.

The protein concentration for various samples treated with phosphate buffer pH 8, 50°C, and 24 hrs incubation was shown in Table 2. This result indicates that the protein concentration varies in treatment with a different enzyme. Table 3 shows the protease activity of crude bromelain extract from pineapple and The New Enzyplex (commercial enzyme). The BSFL hydrolysate made with bromelain 20% (2.04 U/mL) in phosphate buffer pH 8, incubated at 50°C 24 hrs showed the highest protein concentration of 9.680 mg/mL. The optimum hydrolysis condition is in agreement with that reported by Firmansyah and Abduh (2019). In another report, the optimal conditions for producing BSFL protein hydrolysate were also in water at pH 8, and 50°C with 1% Alcalase (protease 2.972 U/mL) (Ravi *et al.*, 2020). Effective hydrolysis of protein during extraction is related to environmental conditions, such as temperature, pH (acids or alkalis), and time of hydrolysis (Bahri *et al.*, 2021). The optimum activity of crude bromelain in ripe pineapple was reported at a broad pH range (5 – 9) and temperature of 37°C to 63°C (Vallés *et al.*, 2007; Martin *et al.*, 2014).

Table 2. Protein concentration on various samples.

Samples	Concentration (mg/mL)
Extracted bromelain enzyme 10% (v/v)	1.91±0.24
Commercial enzyme (Enzyplex) 10% (w/v)	2.28±0.05
BSFL	7.19±0.46
BSFL + Extracted bromelain enzyme 10% (v/v)	7.71±0.33
BSFL + Commercial enzyme (Enzyplex) 10% (w/v)	5.79±0.35

Values are presented as mean±SD of triplicate determinations.

Table 3. The protease activity of bromelain and commercial enzyme (Enzyplex).

Sample	UA (U/mL)
Crude bromelain 20% (v/v)	2.04±0.38
Commercial enzyme (Enzyplex) 20% (w/v)	2.22±0.94

Values are presented as mean±SD of triplicate determinations. P-value >0.05.

One of the methods used to measure antioxidant activity is to calculate the ability of BSFL's protein hydrolysate to scavenge free radical DPPH. DPPH can scavenge free radicals by hydrogen atom transfer (HAT) and single electron transfer (SET). The HAT mechanism in the antioxidant compound enables hydrogen atom donors in the compound to move to the free radical. This is especially supported by the hydrogen bonding between O, N and S. Antioxidant compounds donate their

electrons to DPPH and hence stabilize the DPPH (Foti and Amorati, 2009).

Antioxidant activity with DPPH was expressed as IC<sub>50</sub>. The IC<sub>50</sub> of BSFL protein hydrolysate was calculated to measure the ability of antioxidative capacity in the hydrolysate. The regression and equation for L-ascorbic acid were R<sup>2</sup> = 0.992 and y = 28.886x - 31.1, which makes the IC<sub>50</sub> for L-ascorbic acid was 3.11±0.2 µg/mL. The regression and equation for BSFL protein hydrolysate with the addition of Bromelain can be seen in Figure 1. The IC<sub>50</sub> for BSFL with the addition of bromelain was 9.43±0.84% (v/v) or 0.72 mg/mL. This is comparable to and even slightly better than that reported previously, that is, BSFL hydrolysate with a molecular weight below 3 kDa showed its IC<sub>50</sub> being 0.76 mg/mL (Zhu *et al.*, 2020). Thus, using bromelain from pineapple suggests slightly better antioxidant capacity in BSFL. DPPH method to measure the antioxidant activity of BSFL hydrolysate produced with Enzyplex 10% (w/v) was not suitable. The problem lies in the color of the sample, which became thick green colour. The green colour during the reaction interferes with the yellow indicator of the scavenging activity in DPPH (Kedare and Singh, 2011).

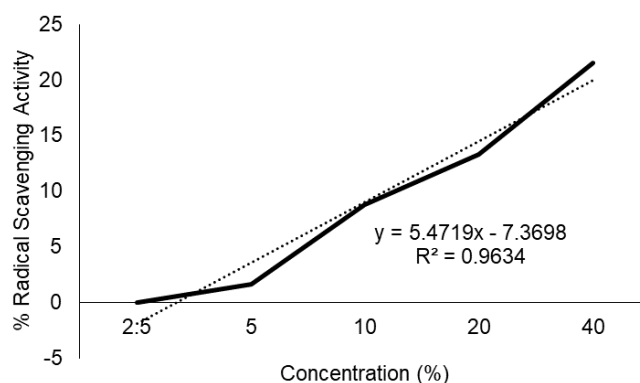


Figure 1. Relationship between %RSA with concentration in BSFL hydrolyzed with bromelain.

On the other hand, the FRAP method measured the antioxidant capacity, specifically the reductive antioxidant. The antioxidant will reduce the FRAP molecule, which is Fe<sup>3+</sup> with its ligand tripyridyltriazine. FRAP is used to measure the total antioxidant in a sample (Benzie and Strain, 1999; Szeto *et al.*, 2002). The capacity of a total antioxidant in both extracts was compared to BSFL extracts without enzyme addition, while the enzyme was tested by the FRAP method to determine whether the enzyme gave a false-positive result. As shown in Figure 2, all the BSFL protein hydrolysates show high antioxidant capacity, whereas the enzyme alone has no antioxidant capacity.

The antioxidant activity of BSFL hydrolysate produced by the addition of 10% Enzyplex (w/v) has a FRAP value 10.2 fold higher than that before enzyme

addition, while BSFL extracts with the addition of 10% bromelain extracts (v/v) have FRAP value of only just 1.5 fold higher (Figure 2). The Enzyplex/commercial enzyme contains 10,000 units of amylases, 9,000 units of proteases, and 240 units of lipases which may hydrolyze the carbohydrate and lipid that are still bound to the protein in BSFL. As a result, the BSFL protein was more available for proteolytic hydrolysis by the protease in the Enzyplex and increased more significantly the FRAP value of the BSFL hydrolysate. For the bromelain FRAP value, this enzyme has a high antioxidant capacity. Therefore, the enzyme must be inactivated at the end of the reaction as well as the sample. The purpose of this reaction termination was to avoid false-positive results (Firmansyah and Abduh, 2019).

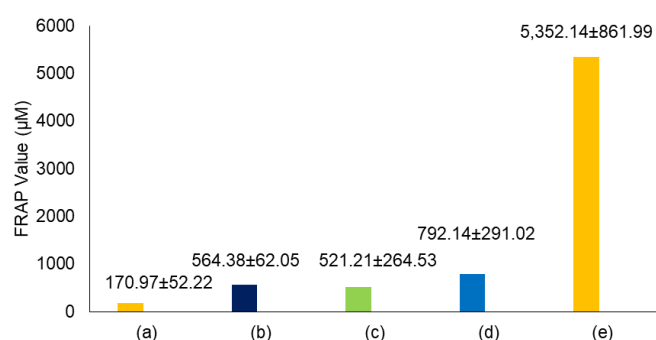


Figure 2. Antioxidants were calculated as FRAP values of various samples. (a) Bromelain extract 10% (v/v), (b) Commercial enzyme at 10% (w/v), (c) BSFL not hydrolysed, (d) BSFL with the addition of bromelain extract's 10% (v/v), (e) BSFL with the addition of Commercial Enzyme (Enzyplex) 10% (w/v). Values are presented as mean±SD of triplicate determinations.

The antioxidant capacity of the silk moth pupae powder (*Bombyx mori*) extracted with 50 mM phosphate buffer pH 8 and mixed with protease showed a FRAP value of  $1.826 \pm 131.9 \mu\text{mol/g}$  Trolox (Felix et al., 2020). BSFL hydrolysate produced by the addition of Enzyplex 10% (w/v) (Figure 2) showed a FRAP value of 2.9 higher than that of the silk moth. There might be a synergistic mechanism of enzymes present in the Enzyplex, these enzymes hydrolyzed macromolecule in the larvae matrix, that is protein, lipid, and amyllum or starch. Protein is usually associated with lipids and other macromolecules, joined by noncovalent bonds. When the macromolecules were hydrolyzed by proteases, lipases, and amylases, the noncovalent bonds were denatured and then produced more bioactive peptides (Agrawal et al., 2018). For the information, BSFL contains protein of ~40% and lipid of ~30% (Diener et al., 2011). BSFL is also known to contain chitin from 7.61% to 18.62% (Chung et al., 2019).

The antibacterial activity assay in this research used the agar well diffusion method. The method is widely used to detect the antibacterial activity of natural

products because the sample can be directly added and diffused in the agar. The antibacterial activity was indicated by the appearance of the inhibition zone (clear zone) around the well. The well diffusion method is more sensitive and convenient than the disc diffusion method (Valgas et al., 2007). The pathogens used in this research were *Bacillus cereus*, *Staphylococcus aureus*, and *Vibrio cholerae*.

*Bacillus cereus* is a Gram-positive bacteria associated with food poisoning. *Bacillus cereus* is a spore-forming bacteria known to contaminate food due to the adhesive effect of its endospores. *Bacillus cereus* can survive in extreme environmental conditions because of this spore which causes problems in the food industry and hospitals (Bottone, 2010). *Vibrio cholerae* is the Gram-negative bacteria that causes cholera. *Vibrio cholerae* contaminates food and produces enterotoxin. *Vibrio cholerae* have pili and outer membrane protein (OMP) for adhesion to the host cell and produce lipopolysaccharide (LPS) endotoxin that triggers immune responses in the host (Wilson et al., 2002). *Staphylococcus aureus* is a Gram-positive opportunistic pathogen. *Staphylococcus aureus* has three types of plasmids. Class I plasmid carries single antibiotic resistance, Class II plasmid codes the  $\beta$ -lactamase and confers resistance to inorganic ions, and Class III plasmid carries multiple resistance determinants. *Staphylococcus aureus* has a golden pigment that acts as a virulence factor to protect the pathogen against oxidant activities from the immune system (Plata et al., 2009). *Staphylococcus aureus* also secretes several enzymes that degrade host cell molecules (Wilson et al., 2002).

One of the hydrolysate samples was BSFL protein hydrolysate with the addition of bromelain. Bromelain is a cysteine endopeptidase that hydrolyzes the peptide bond within the protein substrate (Bahri et al., 2021). Bromelain itself has antibacterial activity (Indumathy et al., 2017). Thus, after hydrolysis of the BSFL protein, the mixture was incubated in a water bath shaker at 90°C for 10 mins to inactivate this enzyme (Firmansyah and Abduh, 2019) to avoid false-positive results in the antibacterial assay.

From this research, the highest inhibition zone was shown by BSFL hydrolysate extracted with buffer phosphate pH 8 at 50°C and 24 hrs incubation with the enzyme. The protein hydrolysate with the highest activity was retested with various dilutions (100%, 50% and 25% v/v). This result showed that the BSFL protein hydrolysate with the addition of crude bromelain showed higher antibacterial activity than the hydrolysate treated with a commercial enzyme as exhibited by *V. cholerae* and *S. aureus*, but lower in *B. cereus* (Table 4) after 24



Table 4. Diameter of inhibition zone (mm) from protein hydrolyzed with bromelain and commercial enzyme (Enzyplex) after 24 hrs incubation of hydrolysate and bacteria.

Pathogens	24 hrs treated with					
	BSFL with bromelain at several dilutions			BSFL with commercial enzyme (Enzyplex) at several dilutions		
	100%	50%	25%	100%	50%	25%
<i>Bacillus cereus</i>	9±1.4	6.4±2.1	5±2.1	8±2.4	7.7±1.7	6.75±1.5
<i>Staphylococcus aureus</i>	7.8±1.7	5.5±3.7	0.0±0.0	5.7±2.8	4±3.6	0.0±0.0
<i>Vibrio cholerae</i>	11.2±3.8	7.8±0.8	3.6±3.3	7.7±1.7	6±2.8	4.25±2.6

Values are presented as mean±SD of triplicate determinations. P-value <0.05.

hrs incubation of the pathogen sample. Furthermore, the incubation time was increased to 48 hrs for all pathogens, the results can be seen in Table 5. The inhibition zone of hydrolysate on 3 different bacteria can be seen in Figure 3, while Figure 4 shows the inhibiting activity of hydrolysate with an incubation time of 24 hrs on the growth of *V. cholerae* only.

Davis and Stout (1971) have classified the zone of inhibition (ZOI) in four intensities: no response (< 5 mm), medium (5 – 10 mm), strong (10 – 20 mm), and very strong (> 20 mm). Based on these criteria, the highest antibacterial activity which was from the BSFL protein hydrolysate (100%) with the addition of bromelain was effective against *Vibrio cholerae* (strong), *Bacillus cereus* (medium), and *Staphylococcus aureus* (medium). Both protein hydrolysate with various dilutions (100%, 50% and 25% v/v) showed antibacterial activity for all tested pathogens, except the 25% of diluted extracts for *S. aureus* (0 mm) and 25% of diluted extracts for *V. cholerae* (3.6 mm). This indicates the different responses of the pathogen bacteria to the concentration of BSFL protein hydrolysate.

The BSFL protein extracted with methanol was reported as effectively inhibiting the Gram-negative pathogens (*Klebsiella pneumoniae*, *Neisseria gonorrhoeae* and *Shigella sonnei*) at 20 – 80 mg/mL concentration after 12 hrs incubation, but the effect was decreased after 24 hrs and did not show any activity against Gram-positive pathogens (*Bacillus subtilis*, *Streptococcus mutans* and *Sarcina lutea*) (Choi et al., 2012). In another report, the water-soluble antimicrobial peptides (AMPs) extracted from immunized BSFL showed a broad-spectrum antimicrobial activity which

effectively inhibits Gram-negative and strongly inhibits Gram-positive bacteria, including MRSA (Park et al., 2015). Bromelain appeared to be effective in the production of antimicrobial peptides. Lestari and Suyata (2019) reported that protein hydrolysate of goat milk hydrolyzed with crude bromelain was effective against *Escherichia coli* and *S. aureus*. In the other report, protein hydrolysate from white-bottomed sea cucumber (*Actinopyga lecanora*) hydrolyzed by bromelain incubated for 1 hr was most effective for both gram-positive (*S. aureus*) and gram-negative (*Pseudomonas aeruginosa* and *E. coli*) (Ghanbari and Ebrahimpour, 2017).

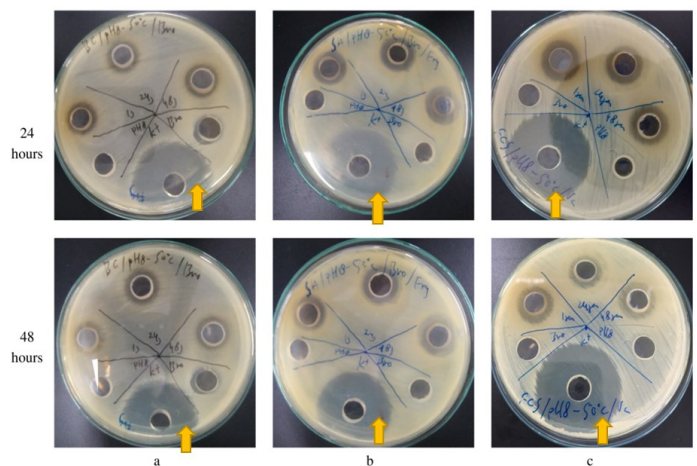


Figure 3. Inhibiting activity of BSFL hydrolysate with phosphate buffer pH 8, at 50°C, and variation of time incubation up to 48 hrs on 3 different bacteria: (a) *Bacillus cereus*, (b) *Staphylococcus aureus*, and (c) *Vibrio cholerae*. The yellow arrow indicated bacterial inhibition in the presence of antibiotic 250 mg of chloramphenicol.

BSFL was able to live in a harsh and adverse environment. Because of this environmental challenge,

Table 5. Diameter of inhibition zone (mm) from protein hydrolysed with bromelain and commercial enzyme (Enzyplex) after 48 hrs incubation of hydrolysate and bacteria.

Pathogens	48 hrs treated with					
	BSFL with bromelain			BSFL with Commercial Enzyme (Enzyplex)		
	100%	50%	25%	100%	50%	25%
<i>Bacillus cereus</i>	9.2±1.7	6.6±2.1	5.4±1.8	8.6±2.6	7.75±1.7	7±2.9
<i>Staphylococcus aureus</i>	8.2±1.8	6±4.3	0.0±0.0	7.7±2.5	4.25±3.5	0.0±0.0
<i>Vibrio cholerae</i>	11.8±3	8.4±1.3	3.6±3.5	7±2.1	6.5±1.9	3.5±3.4

Values are presented as mean±SD of triplicate determinations. P-value <0.05.

BSFL produces various bioactive substances, such as antimicrobial peptides (AMPs) as innate immune systems for protection against bacteria, fungi and viruses. The AMPs have various types of structures. However, the biological mechanism of the AMPs is related to the destruction of the bacterial cell membrane and permeabilization (Xia *et al.*, 2021). The presence of positively charged and hydrophobic amino acid residues plays a role in interacting with the negative charge in lipopolysaccharide (LPS) for Gram-negative bacteria and teichoic acids on the outer surface of the peptidoglycan (Ghanbari and Ebrahimpour, 2017). Further research on the characterization and purification of protein hydrolysate of BSFL is required for its development as a new antibacterial source.

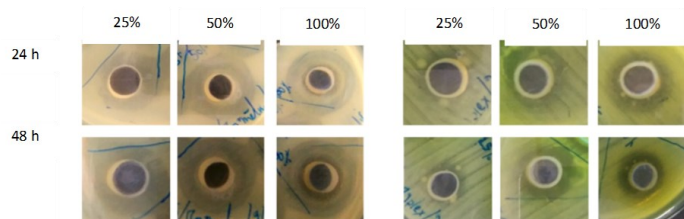


Figure 4. Inhibiting activity of BSFL hydrolysate treated with bromelain 20% (v/v) (left) and commercial enzyme (Enzyplex) 20% (w/v) (right) on the growth of *Vibrio cholerae* with various incubation times (24 hrs and 48 hrs) and various BSFL hydrolysate dilution (25%, 50% and 100%). Both BSFL hydrolysates treated with bromelain and commercial enzyme were using phosphate buffer pH 8 and incubated for 24 hrs at 50°C.

#### 4. Conclusion

Enzyme hydrolysis of BSFL protein produced high antioxidant activity which can be further developed into a highly economically valued medical product. The BSFL protein hydrolysate also potential to be developed as a novel broad-spectrum antibacterial. Further research on the purification and characterization of peptides from BSFL protein hydrolysate is needed for health applications.

#### Conflict of interest

The authors declare no conflict of interest.

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#### References

Agrawal, R., Senwal, S., Kumar, R., Mathur, A., Gupta, R.P., Tuli, D.K. and Setlewal, A. (2018). Synergistic enzyme cocktail to enhance hydrolysis of steam exploded wheat straw at pilot scale. *Frontiers in*

*Energy Research*, 6, 122. <https://doi.org/10.3389/fenrg.2018.00122>

Bahri, S., Hadati, K.S. and Satrimafitrah, P. (2021). Production of protein hydrolysate from tofu dregs using the crude extract of bromelain from pineapple core (*Ananas comosus* L). *Journal of Physics: Conference Series*, 1763(1), 012008. <https://doi.org/10.1088/1742-6596/1763/1/012008>

Benzie, I.F.F. and Strain, J.J. (1999). Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods in Enzymology*, 299, 15-27. [https://doi.org/10.1016/S0076-6879\(99\)99005-5](https://doi.org/10.1016/S0076-6879(99)99005-5)

Bergmeyer, H.U. and Grassl, M. (1983). *Methods of Enzymatic Analysis*. Vol. 2. Weinheim, Germany: Verlag Chemie.

Bottone, E.J. (2010). *Bacillus cereus*, a volatile human pathogen. *Clinical Microbiology Reviews*, 23(2), 382-398. <https://doi.org/10.1128/CMR.00073-09>

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1-2), 248-254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3)

Choi, W.H., Yun, J.H., Chu, J.P. and Chu, K.B. (2012). Antibacterial effect of extracts of *Hermetia illucens* (Diptera: *Stratiomyidae*) larvae against Gram-negative bacteria. *Entomological Research*, 42(5), 219-226. <https://doi.org/10.1111/j.1748-5967.2012.00465.x>

Chung, Y.W., Rosli, S.S., Uemura, Y., Yeek, C.H., Leejeerajumnean, A., Kiatkittipong, W., Chin, C.K., Man, K.L. and Jun, W.L. (2019). Potential protein and biodiesel sources from black soldier fly larvae: insights of larval harvesting instar and fermented feeding medium. *Energies*, 12(8), 1570. <https://doi.org/10.3390/en12081570>

Davis, W.W. and Stout, T.R. (1971). Disc plate method of microbiological antibiotic assay, I: Factors influencing variability and error. *Journal of Applied Microbiology*, 22(4), 659-665. <https://doi.org/10.1128/am.22.4.659-665.1971>

Diener, S., Zurbrugg, C., Roa Gutiérrez, F., Nguyen, H.D., Morel, A., Koottatep, T. and Tockner, K. (2011). Black soldier fly larvae for organic waste treatment - prospects and constraints, presented at the 2nd International Conference on Solid Waste Management in Developing Countries, p. 1-8. Khulna, Bangladesh.

Dubey, R., Reddy, S. and Murthy, N.Y.S. (2012).

- Extraction and purification of bromelain. *Asian Journal of Chemistry*, 24(4), 1435-1438.
- Felix, M., Bascon, C., Cermeno, M., FitzGerald, R.J., de la Fuente, J. and Carrera-Sanchez, C. (2020). Interfacial/foaming properties and antioxidant activity of a silkworm (*Bombyx mori*) pupae protein concentrate. *Food Hydrocolloids*, 103, 105645. <https://doi.org/10.1016/j.foodhyd.2020.105645>
- Firmansyah, M. and Abduh, M.Y. (2019). Production of protein hydrolysate containing antioxidant activity from *Hermetia illucens*. *Heliyon*, 5(6), E02005. <https://doi.org/10.1016/j.heliyon.2019.e02005>
- Food and Agriculture Organization of the United Nations. (2012). The State of Food and Agriculture: Investing in agriculture for a better future. Retrieved on March 29, 2022 from FAO Website: <https://www.fao.org/3/i3028e/i3028e.pdf>
- Foti, M.C. and Amorati, R. (2009). Non-phenolic radical-trapping antioxidants. *Journal of Pharmacy and Pharmacology*, 61(11), 1435-1448. <https://doi.org/10.1211/jpp/61.11.0002>
- Ghanbari, R. and Ebrahimpour, A. (2017). Separation and identification of bromelain-generated antibacterial peptides from *Actinopyga lecanora*. *Food Science and Biotechnology*, 27(2), 591-598. <https://doi.org/10.1007/s10068-017-0267-z>
- Indumathy, S., Kiruthiga, K., Saraswathi, K. and Arumugam, P. (2017). Extraction, partial purification and characterization of bromelain enzyme from pineapple (*Ananas comosus*). *Indo American Journal of Pharmaceutical Research*, 7(8), 566-579.
- Kedare, S.B. and Singh, R.P. (2011). Genesis and development of DPPH method of antioxidant assay. *Journal of Food Science and Technology*, 48(4), 412-422. <https://doi.org/10.1007/s13197-011-0251-1>
- Lestari, P. and Suyata. (2019). Antibacterial activity of hydrolysate protein from Etawa goat milk hydrolysed by crude extract bromelain. *IOP Conference Series: Materials Science and Engineering*, 509, 012111. <https://doi.org/10.1088/1757-899X/509/1/012111>
- Lievens, S., Poma, G., De Smet, J., Van Campenhout, L., Covaci, A. and Van Der Borght. (2021). Chemical safety of black soldier fly larvae (*Hermetia illucens*), knowledge gaps and recommendations for future research: A critical review. *Journal of Insects as Food and Feed*, 7(4), 383-396. <https://doi.org/10.3920/JIFF2020.0081>
- Martin, B.C., Rescolino, R., Coelho, D.F., Zanchetta, B., Tambourgi, E.B. and Silveira, E. (2014). Characterization of bromelain from *Ananas Comosus* agroindustrial residues purified by ethanol fractional precipitation. *Associazione Italiana Di Ingegneria Chimica*, 37, 781-786. <https://doi.org/10.3303/CET1437131>
- Olugbami, J., Gbadegesin, M. and Odunola, O. (2015). In vitro free radical scavenging and antioxidant properties of ethanol extract of *Terminalia glaucescens*. *Pharmacognosy Research*, 7(1), 49-56. <https://doi.org/10.4103/0974-8490.147200>
- Park, S.I., Kim, J.W. and Yoe, S.M. (2015). Purification and characterization of a novel antibacterial peptide from black soldier fly (*Hermetia illucens*) larvae. *Developmental and Comparative Immunology*, 52(1), 98-106. <https://doi.org/10.1016/j.dci.2015.04.018>
- Plata, K., Rosato, A.E. and Wegrzyn, G. (2009). Review: *Staphylococcus aureus* as an infectious agent: Overview of biochemistry and molecular genetics of its pathogenicity. *The Journal of the Polish Biochemical Society and of the Committee of Biochemistry and Biophysics Polish Academy of Sciences*, 56(4), 597-612. [https://doi.org/10.18388/abp.2009\\_2491](https://doi.org/10.18388/abp.2009_2491)
- Ravi, H.K., Degrou, A., Costil, J., Trespeuch, C., Chemat, F. and Vian, M.A. (2020). Effect of devitalization techniques on the lipid, protein, antioxidant, and chitin fractions of black soldier fly (*Hermetia illucens*) larvae. *European Food Research and Technology*, 246, 254-2568. <https://doi.org/10.1007/s00217-020-03596-8>
- Suh, H.J., Kim, S.R., Lee, K.S., Park, S. and Kang, S.C. (2010). Antioxidant activity of various solvent extracts from *Allomyrina dichotoma* (Arthropoda: Insecta) larvae. *Journal of Photochemistry and Photobiology Biology*, 99(2), 67-73. <https://doi.org/10.1016/j.jphotobiol.2010.02.005>
- Szeto, Y.T., Tomlinson, B. and Benzie, I.F.F. (2002). Total antioxidant and ascorbic acid content of fresh fruits and vegetables: Implications for dietary planning and food preservation. *British Journal of Nutrition*, 87(1), 55-59. <https://doi.org/10.1079/BJN2001483>
- Valgas, C., De Souza, S.M. and Smânia, E.F.A. (2007). Screening methods to determine antibacterial activity of natural products. *The Brazilian Journal of Microbiology*, 38(2), 369-380. <https://doi.org/10.1590/S1517-83822007000200034>
- Vallés, D., Furtado, S. and Cantera, A.M.B. (2007). Characterization of news proteolytic enzymes from ripe fruits of *Bromelia antiacantha* Bertol. (*Bromeliaceae*). *Enzyme and Microbial Technology*, 40(3), 409-413. <https://doi.org/10.1016/j.enzmictec.2006.07.011>



- Wilson, J.W., Schurr, M.J., LeBlanc, C.L., Ramamurthy, R., Buchanan, K.L. and Nickerson, C.A. (2002). Mechanisms of bacterial pathogenicity. *Postgraduate Medical Journal*, 78(918), 216-224. <https://doi.org/10.1136/pmj.78.918.216>
- Xia, J., Chaorong, G. and Huaiying, Y. (2021). Antimicrobial peptides from black soldier fly (*Hermetia illucens*) as potential antimicrobial factors representing an alternative to antibiotics in livestock farming. *Animals*, 11(7), 1937. <https://doi.org/10.3390/ani11071937>
- Youn, J.S., Kim, Y.J., Na, H.J., Jung, H.R., Song, C.K., Kang, S.Y. and Kim, J.Y. (2019). Antioxidant activity and contents of leaf extracts obtained from *Dendropanax morbifera* LEV are dependent on the collecting season and extraction conditions. *Food Science and Biotechnology*, 28(1), 201–207. <https://doi.org/10.1007/s10068-018-0352-y>
- Zhu, D., Huang, X., Tu, F., Wang, C. and Yang, F. (2020). Preparation, antioxidant activity evaluation, and identification of antioxidant peptide from black soldier fly (*Hermetia illucens* L.) larvae. *Journal of Food Biochemistry*, 44(5), e13186. <https://doi.org/10.1111/jfbc.13186>