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by Siska Zafrida

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Optimization of Crude Protease Production from *Bacillus thuringiensis* HSFI-12 and Thrombolytic Activity Its Enzyme Dialysate

Siska Zafrida[1], Stalis Norma Ethica[1,*], Aditya Rahman Ernanto[2] and Wijanarka Wijanarka[3]

[1]*Magister of Clinical Laboratory Science, Postgraduate Program, Universitas Muhammadiyah Semarang, Central Java 50273, Indonesia*
[2]*Diploma Study Program of Medical Laboratory Technology, Universitas Muhammadiyah Semarang, Central Java 50273, Indonesia*
[3]*Faculty of Biology, Universitas Diponegoro, Semarang, Central Java 50272, Indonesia*

([*]Corresponding author's e-mail: norma@unimus.ac.id)

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Abstract

Cardiovascular disease (CVD) is among the leading causes of death in the world caused by thrombosis. Thrombosis is the formation of excessive blood clots on the walls of blood vessels called thrombus. This could lead to fatal blockage of the heart muscle or brain. Thrombosis can be treated using enzyme-type drugs such as thrombolytic proteases. There have been many studies of bacteria producing thrombolytic enzymes isolated from fermented foodstuffs. However, commercial production of bacterial enzymes to fulfill the need of thrombolytic agents is still limited, causing high price of CVD therapy. *Bacillus* sp. HSFI-12 (*Holothuria scabra Fermented Intestine*-12) isolated from the fermented intestine of sea cucumber *Holothuria scabra* had been reported to have a competitive thrombolytic activity to the commercial Nattokinase. This study aimed to determine bacterial optimum incubation time based on enzyme activity after bacterium molecular identification was done. It also aimed to obtain the dialysate of bacterial crude protease and then determine the thrombolytic activity of the obtained dialysate. Thrombolytic activity was tested on 4 different types of blood (A, B, AB and O). Results of molecular identification showed that strain HSFI-12 shared similarity level of 99.80 %, with *Bacillus thuringiensis*. Activity of crude protease from the incubated cultures peaked at 48 h of incubation with activity of 191.5 U/mL The activity of concentrated protease after precipitation and dialysis process (dialysate) was 2-times higher by 355,7 U/mL. The percentage of lysis of blood clots produced by crude blood groups A, B, AB and O showed a range of values of 66.423 - 67.656 % while those that produce dialysate are 77.564 - 78.861 %. As conclusion, the best (optimized) incubation time to produce crude enzyme from *B. thuringiensis* HSFI-12 with the highest activity was 48 h. The process of concentrating the crude thrombolytic protease of *B. thuringiensis* HSFI-12 increases both activity and thrombolytic ability of the bacterial enzyme.

Keywords: *Bacillus thuringiensis*, Cardiovascular disease, Thrombolytic agent, Protease activity, Molecular identification

Introduction

Cardiovascular Disease (CVD) is one of major health problems worldwide resulting in 30 % of all deaths each year. CVD is a type of disease involving the heart or blood vessels [1]. According to the World Health Organization (WHO), mortality rate reaches 17.9 million people per year and is the 31 % of the causes of death worldwide [2]. In Indonesia, based on Basic Health Research Data (Riset Kesehatan Dasar, Riskesdas) in 2018, it was stated that the incidence of heart disease was 2.78 million people [3]. WHO predicted that by 2030, the number of people who die from CVD will continue to increase to more than 23.3 million people [2]. Recently, hospitals in Indonesia reported that 16.3 % of patients treated from the COVID-19 isolation room had CVD [3].

Chronic CVD causes acute myocardial infarction and stroke. One of the reasons is marked with the process of formation of blood clots (thrombus) in excessive blood vessels known as thrombosis [4,5]. Thrombosis consists of fibrin, red blood cells, and some components of platelets and leukocytes. Massive

thrombus will cause blockage of blood vessels which can lead to blockage of the heart muscle or brain, so that it can end in death [6,7].

Anticoagulants, antiplatelet drugs, surgery and thrombolytic agents can used to treat thrombosis. The commercial thrombolytic agents currently in use are t-PA, Nattokinase and Streptokinase. However, these thrombolytic agents are expensive and can cause side effects such as gastrointestinal bleeding and allergic reactions. Therefore, other thrombolytic agents are needed which are cheaper, safer and do not cause side effects [8-10]. Enzyme protease is a thrombolytic agent that has the potential to be developed. One of the protease-producing microorganisms is proteolytic bacteria, namely bacteria that are able to degrade proteins and produce extracellular proteases. Sources of protease enzymes include plants (43.85 %), bacteria (18.09 %), fungi (15.08 %), animals (11.15 %), algae (7.42 %) and viruses (4.41 %) [11,12]. Isolation of bacteria from the digestive organs of sea cucumbers to obtain bacterial protease to be used as a thrombolytic agent had been conducted by Hidayati *et al*. [13]. One the most promising bacterial isolates producing thrombolytic protease obtained was strain HSFI-12. However, the identification of strain HSFI-12 was limited to only on bacterial morphology. Thus, molecular identification to determine the species of strains HSFI-12 is carried out in this study by analyzing bacterial 16S rRNA gene sequence amplified using PCR/polymerase chain reaction.

Crude enzyme *Holothuria scabra* Fermented Intestine-12 (HSFI-12) isolate had shown higher thrombolytic ability than that of Nattokinase as positive control [13]. Meanwhile, incubation time greatly affect the growth of enzyme producing bacteria [14]. Thus, this study aims to produce crude protease of strain HSFI-12 at an optimized incubation time based on the highest enzyme activity. Next, the bacterial crude protease will be partially purified through precipitation using ammonium sulphate followed by salting out/dialysis. Enzyme activity and thrombolytic ability of both crude enzyme and enzyme dialysate were then measured and compared. Finally, to support the future application of bacterial protease as thrombolytic agent, thrombolysis activity of protease dialysate was performed on A, B, AB and O types of human blood samples.

Materials and methods

Bacterial subculture

Bacterial strain HSFI-12 was obtained from Ms. Nur Hidayati, which previously stored at Microbiology Laboratory, Universitas Muhammadiyah Semarang, Indonesia. Subculture of isolates was carried out by cultivating a loopful of bacterial colony of HSFI-12 isolate on BHIB (Brain Heart Infusion Broth, growth provider media), followed by NA (Nutrient Agar, provider media) for colony purification and BAP (Blood Plate Agar) under aseptic conditions. The media was then incubated at 37 °C for 24 h [14].

Bacterial identification based on 16S rRNA gene sequence

HSFI-12 cells grown in a mL BHIB media after incubated at 37 °C for 2×24 h was centrifuged for 1 min at 12,000 rpm. Bacterial genomic DNA was extracted with a DNA extraction kit (Presto™ Mini gDNA Bacteria Kit, Geneaid). Amplification process was carried out using Go Tag Green Master Mix (Promega) with universal specific primers for 16S rRNA 27-F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492-R (5'-GGTTACCTTGTTACGACTT-3'). PCR was carried out in a total volume of 25 μL using a Thermocycle (Biometra) [15]. The PCR process consisted of initial denaturation at 95 for 4 min, then followed by 40 cycles of denaturation at 95 °C for 30 s each cycle, annealing at 55 °C for 35 s, extension at 72 °C for 45 s. The PCR results were examined using 2 % agarose gel electrophoresis based on the appearance of a single band at 1,500 bp. Amplicon visualization using Major Science UV Transluminator [16,17]. The cloning of the 16s rRNA gene using pTA2 vector and *E. coli* Zymo 5α was carried out in several steps as reported by Gao *et al*. [18]. Sequencing of amplicon and clone of 16S rRNA gene was conducted with a PRISM 377 DNA Analyzer (Applied Biosystems, Carlsbad, CA) using primer T3 and T7 [19]. DNA sequencing was carried out on the ABI PrismTM 310 Genetic Analyzer, while the resulted sequences were assembled and analyzed using DNA-Baser (Heracle BioSoft, Romania) program [19,20]. Sequencing aimed to determine the sequence of nucleotides in DNA fragments so that they can be used to determine the identity and function of genes by comparing their sequences with other known DNA sequences in the Genbank database using BLAST [21,22].

Isolation of protease-producing bacteria

After obtaining a single isolate resulting from bacterial purification on NA media, the next step was to test the production of protease enzymes in Skim Milk Agar (SMA) producer media. Bacteria growing

on NA media were scratched on SMA media and incubated for 24 h at 37 °C and then observed the formation of a clear zone around the colony growth [13,16,23].

Optimization of HSFI-12 bacterial isolate incubation time based on protease activity

Colonies of HSFI-12 isolate were inoculated on Minimal Synthetic Medium (MSM) containing NaCl 0.1 %, K_2HPO_4 0.1 %, ammonium sulfate 0.7 %, $MgSO_4.7H_2O$ 0.01 %, yeast extract 0.05 %, Skim milk 1 %) and incubated at 37 °C and the enzyme activity test was carried out every 24 h interval, namely 24, 48 and 72 h [24-26]. The extraction of thrombolytic enzymes was carried out by centrifugation of bacterial culture at 3,000 rpm for 15 min at a temperature of 4 °C, the supernatant was regarded as crude protease. Furthermore, the measurement of enzyme activity using the Bergmeyer method (1984) on a spectrophotometer with a wavelength of 600 nm [27]. HSFI-12 bacterial isolates were inoculated on MSM media. Isolate in incubation at 37 °C and the enzyme activity test was carried out with 24 h interval, i.e. 24, 48 and 72 h. Protease production using 250 mL of MSM media and carried out at 37 °C. Extraction of protease enzymes was carried out by centrifugation of the bacterial growth medium at a speed of 3,000 rpm for 15 min at 4 °C. With this technique, the cells will settle under the force of gravity while the enzymes remain in the supernatant. The supernatant as a sample was tested for its protease activity and protein content.

Enzyme precipitation using ammonium sulfate

Ammonium sulfate 70 % is slowly added to the crude enzyme, stirrer slowly under cold conditions until dissolved for 2 h. Store the enzyme solution in the refrigerator to allow the enzymes to settle overnight. The enzyme solution was centrifuged at 4 °C at a speed of 15,000 rpm for 15 min and the pellets formed were taken. The pellet was dissolved with 15 mL of 0.05 M phosphate buffer pH 7 until clean [25].

Salting out/dialysis of crude enzyme

Cellophane bags need to be prepared before use. Cellophane bags are immersed in hot water at 60 °C for 2 min, the soaking water is replaced with 0.2 % sulfuric acid, after that the soaking water is replaced with hot water for 2 min. The enzyme obtained from the precipitation of ammonium sulfate was put in a cellophane bag. The 2 ends of the cellophane bag were tied, then the cellophane bag was soaked with 500 - 700 mL of 0.05 M phosphate buffer pH 7 at ± 4 °C for 2 h. Phosphate buffer solution of 0.05 M pH 7 was replaced with 0.025 M phosphate buffer pH 7. The soaking buffer was replaced every 2 h until all salts were removed [28]. Dialysis was ended when all the ammonium sulfate salt had come out of the membrane, by testing it using a solution of $BaCl_2$ and HCl. Three drops of 0.1 M BaCl2 and 3 drops of 0.1 M HCl were added to the buffer solution outside the cellophane bag. Sulfate ions (SO_4^{2-}) will form a white precipitate of $BaSO_4$. The semi-pure enzyme solution was then tested for its activity using the method of Bergmeyer (1984) on a spectrophotometer at 600 nm [27,28].

Activity measurement of crude enzyme and enzyme dialysate

Enzyme activity was measured using the modified Bergmeyer (1984) method [27]. Four test tubes were coded 1, 2, 3 and 4. In each of tubes 1 and 2, 500 L of 1 % casein was added to phosphate buffer pH 7 and 500 L of enzyme solution, while 500 L of 1 % and 500 L of casein were added to each of tubes 3 and 4. Liter of the inactivated enzyme solution was incubated at 37 °C for 10 min and 1,000 L of 10 % TCA was added. Then it was centrifuged at 4 °C at 10,000 rpm for 5 min. A total of 500 L of the supernatant was reacted with 1.25 mL of 0.4 M sodium carbonate and 200 L of Folin's reagent with a vortex, then incubated again at room temperature for 30 min. The absorbance was then measured at 600 nm [25,27,28].

Measurement of *In vitro* blood clot lysis activity

In vitro blood clot lysis activity test was carried out on blood taken from 4 volunteers each with blood groups A, B, AB and O. The 4 types of human blood were obtained from the blood bank of Blood Donor Unit of Universitas Muhammadiyah Semarang, Indonesia collected in August 2021. Six 1.5 mL microtube tubes that had been weighed were prepared. Each tube is coded 1 - 6, tube 1 (negative control), tube 2 (positive control), tube 3 (blood type A), tube 4 (blood type B), tube 5 (blood type AB), tube 5 (blood type AB) and tube 6 (blood type O). Six hundred L of blood was added to each tube and allowed to stand until clots form for 30 min. Serum was completely removed after clot formation by centrifugation. The tube containing the clot was then weighed again. The weight of the clot was determined by subtracting the weight of each tube (the weight of the clot = the weight of the clot

containing the tubes the weight of the empty tubes). Tube 1 was added to 100 L of PBS (negative control), tube 2 was added to 100 L of Nattokinase (positive control), tubes 3 - 6 were added to 100 L of pure enzyme. The samples were then incubated at 37 °C overnight [13,23,29].

The lysed liquid was completely absorbed from each tube with filter paper and the tube was then reweighed. The difference in weight of each tube before and after incubation was calculated and calculated the percentage of blood clots in the sample into Eq. (1) [29]:

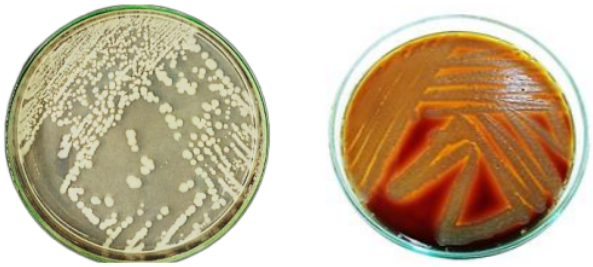
$$\% \text{ Clot lysis} = \frac{\text{weight of initial blood clot} - \text{weight of final blood clot}}{\text{weight of initial blood clot}} \times 100\ \% \quad (1)$$

The effect of lysis of blood clots can also be seen microscopically by using the Eustrek (removal) technique. This method is used to see the condition of blood cells microscopically, according to the May Grunwald-Giemsa mixture method [30]. Prepared 6 pieces of glass slides that are clean and not greasy and each labeled number 1 to number 6. Blood from test tubes numbered 1 to 6, each taken as much as 20 L. The blood is smeared on top of each slide number 1 - 6 in sequence. The drops of blood on the slide are touched with a cover slip so that it expands and the layer is thin to the edge of the slide. The preparations were fixed in ethanol solution to cover the surface for 15 min and aerated to dry. The preparations were then immersed in Giemsa's solution for 30 min and rinsed with water, then allowed to air dry. The results were observed under a light microscope with 400× magnification and documented with a camera [31].

Results and discussion

Bacterial characterization of HSFI-12

Macroscopic characterization can be seen from the shape, color, size, edge and elevation of the colonies on NA media (**Figure 1**). Morphological characteristics on the Blood Agar Plate (BAP) media are displayed in **Figure 2**. Bacterial hemolysis on BAP is indicated by the presence of clear zone area around the colony.



(a) (b)

Figure 1 Colony characteristics of bacterial strain HSFI-12 on (a) Nutrient agar media and (b) Blood agar plate media showing β-hemolysis patterns.

Bacterial colonies obtained on average were small round and large jagged. The colony shape was *circular*, while the edge of the colony was *entire* sized 3 mm with yellow color, convex elevation and smooth consistency. Based on the Gram staining that has been carried out, it was found that the bacteria were Gram-negative, rod-shaped (bacilli), and had spores. The macroscopic characteristics of bacterial cells after Gram staining can be seen in **Figure 2**.

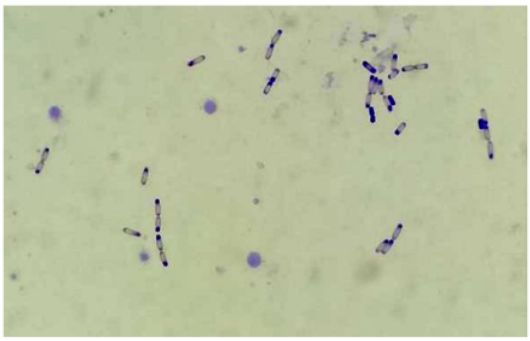


Figure 2 Macroscopic characteristics of bacterial cells of strain HSFI-12 based on Gram-staining.

Bacteria identification using PCR method

Genomic DNA was extracted from HSFI-12. It was then used as a template in the process of amplification of the 16S rRNA gene by PCR method. The results of PCR amplification were electrophoresed using 2 % agarose gel and visualized under UV light produced a single band with a size of about 1,500 bp (**Figure 3**) corresponding to the value indicated by the DNA marker. Amplification of HSFI-12 genomic DNA using 47F - 1492R primers produced a single band with a size of ~1,500 bp according to the value indicated by DNA marker. The obtained size is in accordance with the expected size of bacterial 16S rRNA genes, which is 1,500 bp [19,32].

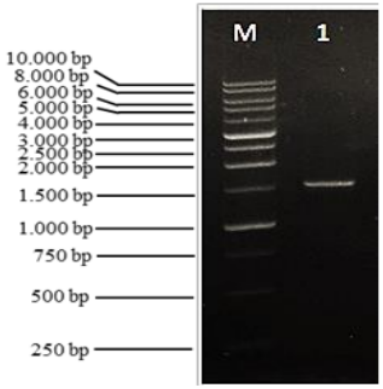


Figure 3 Electrophoresis visualization of 16S rRNA gene of bacterial strain HSFI-12 with size of ~1,500 bp amplified by Polymerase Chain Reaction. M = Ladder DNA Marker 1 kb; 1 = Amplified gene of bacterial strain.

Cloning of the 16S rRNA gene with the pTA-2 vector

The cloning process was carried out by ligation, transformation and isolation of recombinant DNA from transformants [33,34]. Ligation reaction is a combination of target DNA molecules with vectors to produce recombinant DNA. The target gene is the 16S rRNA gene isolated by PCR method and the vector is pTA2 (**Figure 3**). Recombinant DNA was transformed in bacterial cells of *E. coli* DH5α and grown on solid medium, the success of cloning can be seen through white colonies (clones carrying recombinant DNA) [33,34]. The recombinant DNA was PCR before sequencing, using 2 primers, namely T7 (5'-TAATACGACTCACTATAGGG-3') and T3 (5'-CCCTTTAGTGAGGGTTAATT-3'). The positions of the T7 and T3 primers on the pTA2 T-Vector are shown in **Figure 4**.

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>Bacillus thrungiensis 16S rRNA HSFI-12
TACGGTTACCTTGTTACGACTTCACCCCAATCATCTGTCCCACCTTAGGCGGCTG
GCTCCAAAAAGGTTACCCCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGAC
GGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGAT
TACTAGCGATTCCAGCTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGA
ACGGTTTTATGAGATTAGCTCCACCTCGCGGTCTTGCAGCTCTTTGTACCGTCCA
TTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCC
CACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGATG
GCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGAC
ACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCTCCCGAAGGAGAAGCCC
TATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTT
CGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGT
TTCAGCCTTGCGGCCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAACTTCAGCA
CTAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTAC
CAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGTGTCAGTTACA
GACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTCCATATCTCTACGCATTTCACC
GCTACACATGGAATTCCACTTTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAAT
GACCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCACCTGCG
CGCGCTTTACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGG
CTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCCAGC
TTATTCAACTAGCACTTGTTCTTCCCTAACAACAGAGTTTTACGACCCGAAAGCC
TTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCC
CTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGAT
CACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGTGAGCCGTTACCTCACCAAC
TAGCTAATGCGACGCGGGTCCATCCATAAGTGACAGCCGAAGCCGCCTTTCAATT
TCGAACCATGCGGTTCAAAATGTTATCCGGTATTAGCCCCGGTTTCCCGGAGTTA
TCCCAGTCTTATAGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGCTAACTT
CATAAGAGCAAGCTCTTAATCCATTCGCTCGACTTGCATGTATTAGGCACGCCGC
CAGCGTTCATCCTGAGCCATGATCAAACTCTA
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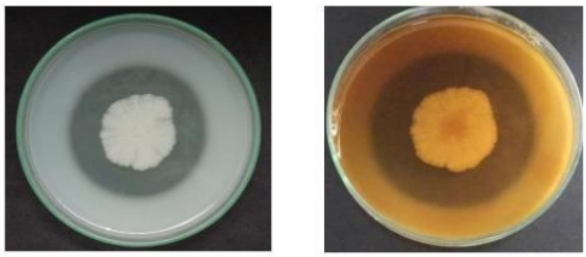
Figure 4 Sequencing results of 16S rRNA gene sequence of *Bacillus thrungiensis* HSFI-12.

Bioinformatics analysis with BLAST

Consensus was made on the forward and reverse sequences of the 16S rRNA gene using the DNA Baser Assembler Program. The results of bacterial DNA sequencing were analyzed and matched with the data available in the Gen Bank Basic Local Alignment Search Tool (BLAST). BLAST analysis was performed on the nucleotide sequence of the 16S rRNA gene online via https://blast.ncbi.nlm.nih.gov/Blast.cgi [22,35]. The results of the BLAST analysis of the HSFI-12 isolate bacteria obtained the sequence of the 16s rRNA gene fragment which showed a homology level of 99.80 % with that of the bacterial isolate *Bacillus thuringiensis* FDAARGOS_791 (Acc. No. CP054568.1). Based on the results of the overall bioinformatics analysis carried out in this study, the HSFI-12 bacterial isolate was named *Bacillus thuringiensis* HSFI-12.

Proteolytic test

The pure bacterial isolates obtained are then carried out proteolytic tests on SMA media. The aim was to determine whether bacteria have the potential to produce proteases or not. The presence of proteolytic activity is indicated by the appearance of a clear zone around the bacteria [13,16] as shown in **Figure 6**.



(a) (b)

Figure 6 Proteolytic activity shown by B. *thuringiensis* HSFI-12 on SMA media: (a) No staining and (b) With 0.5 % lugol stain.

The clear zone formed around the colony was due to the ability of bacterial protease to degrade the casein substrate contained in the *skim milk* agar media. Casein is hydrolyzed by bacterial protease to produce soluble peptides and amino acids [36,37]. hydrolysis activity of bacterial protease could be measured by calculating the ratio between the diameter of the clear zone around the bacterial colony and the diameter of the bacterial colony [38]. The value of the HC index shown by *B. thuringiensis* HSFI-12 was 1.75, with a clear zone diameter of 70 mm and a colony diameter of 40 mm.

Optimization of bacterial incubation time

The enzymes used are enzymes produced from the results of culture incubation for 48 h on MSM media. The concentration of ammonium sulfate at the enzyme concentration stage was used at a concentration of 70 %, to precipitate proteases with maximum activity. In a solution containing a high salt concentration, the number of water molecules in the solution will decrease so that the free salt molecules will attract water that is bound to the non-polar group of the enzyme protein. This causes the opening of the protein structure so that the nonpolar protein groups will gather and precipitate, resulting in a decrease in the solubility of the protein in solution. Ammonium sulfate is used in this activity because it is highly soluble, precipitates proteins effectively, can be used at various pH and temperature, and is inexpensive [39].

Enzyme activity test was conducted to determine both the optimized bacterial incubation time and to compare activity of crude enzyme and enzyme dialysate. The activity of the protease enzyme was determined by interpolating the absorbance value into the regression equation from the obtained tyrosine standard curve. Blanks are made in the same way, but the enzymes are first inactivated. One unit of protease enzyme activity is defined as the amount of enzyme capable of hydrolyzing casein to produce a peptide equivalent to 1 mol of tyrosine product per minute at optimum measurement conditions [38].

Extracellular proteolytic activity of *B. thuringiensis* HSFI-12 can be calculated using the following equation (Eq. (2)):

$$Y = 0.0019\,X + 0.0092, \text{ where } Y = \text{absorbance, while } X = \text{enzyme activity (U/mL)} \quad (2)$$

Table 1 Enzyme activity of crude protease from *B. thuringiensis* HSFI-12 at varied incubation time.

Incubation time (h)	Enzyme activity (U/mL)
24	126,210
48	191,473
72	138,315

Table 1 showed data of activity of crude enzymes produced by *B. thuringiensis* HSFI-12 at varied incubation time (24, 48 and 72 h). Based on **Table 1**, the best (optimized) incubation time to produce crude enzyme from *B. thuringiensis* HSFI-12 with the highest activity was 48 h. This result is in line with that reported by Sridhara *et al*. [40] and Harer *et al*. [41] demonstrating that the optimized incubation time for *B. thuringiensis* isolated from paddy field in producing protease is 48 h. Harer *et al*. [41] stated that factors affecting activities of bacterial protease from *B. thuringiensis*-SH-II-1A isolated from soil include pH, temperature, as well as metal ion and inhibitor presence.

Meanwhile, the value of the enzyme dialysate activity was 355,688 U/mL showing a double increase from the activity value of its crude enzyme (191,473 U/mL). This showed that in general, precipitation of bacterial crude protease using ammonium sulfate followed by salting out with dialysis could significantly improve its activity. Such increase is likely due to more concentrated enzyme level with less contaminants as result of partial purification. Similar outcomes had been previously reported by several studies related with bacterial proteases [41,42]

Clot lysis activity of crude protease and its dialysate

Crude and dialysate enzymes were tested for its clot lysis activity to determine if they had thrombolytic abilities. The results showed that both crude and the dialysate enzymes from *B. thuringiensis* HSFI-12 were able to lyse clots of A, AB, B and O blood group samples better than commercial Nattokinase as control. The thrombolytic ability was characterized by a higher percentage of clot lysis of 4 samples after the addition of both crude and dialysate enzymes (**Figure 7** and **Table 2**).

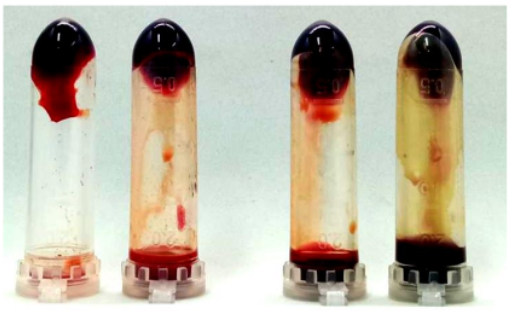


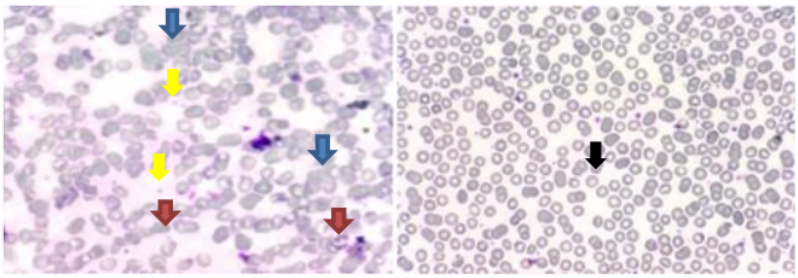
Figure 7 Results of the blood clot lysis activity test of 4 blood samples representing ABO group.

Table 2 Thrombolytic activity test results.

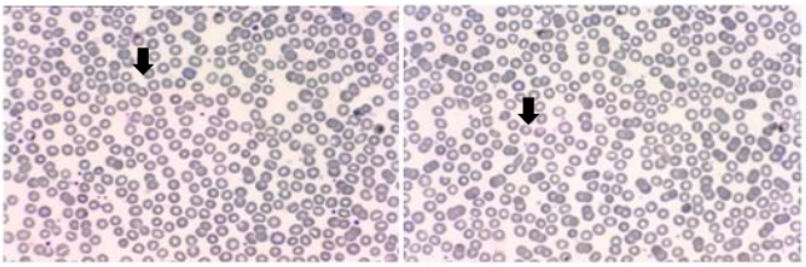
Blood group	Percentage of clot lysis (%)			
	Control (-)	Control (+) Nattokinase	*Crude* enzyme	Dialysate enzyme
A	0	58,275	66,423	77.564
B	0	57,755	67.021	78,414
AB	0	57,587	67.576	77,862
O	0	57,692	67,656	78.861

The contents of the clot produced by crude in all blood groups of A, B, AB and O showed a lysis percentage (range of value) of 66.423 - 67.656 % while those produced by dialysate of 77.564 - 78.861 % (**Table 2**). Results shown in **Table 2** showed that clot lysis ability of dialysate protease towards 4 blood group samples of A, B, AB and O does not show significant differences.

Next, the lysed blood clots of 4 blood group (A, B, AB and O) before and after the addition of crude and dialysate enzymes were then observed under a microscope with a magnification of 400× (**Figure 8**). The aim was to see the abnormality on shapes of blood cells. As seen in **Figure 8**, The negative control smear (**Figure 8**(**a**)) showed the presence crenation (red arrows) on erythrocytes, while platelet aggregation (yellow arrows) and erythrocyte clots were also seen (blue arrows). Meanwhile, smears of positive control (Nattokinase, **Figure 8**(**b**)) as well as samples (**Figures 8**(**c**) and **8**(**d**)) show there was no crenation presence on erythrocytes, and there was also no platelet aggregation. The erythrocytes were evenly distributed on the smears of positive controls and all samples. This result infers that crude enzyme and dialysate did not cause crenation, platelet aggregation, nor erythrocyte clot. The same had been reported for Nattokinase, which was used as a positive control in this study [43].



(a) Negative control (b) Positive control (Nattokinase)



(c) Enzyme dialysate (d) Crude enzyme

Figure 8 Microscopic observation on clot lysis product after administration of crude enzyme and enzyme dialysate on human blood with 400× magnification: The blue arrows indicate erythrocytes clotting; red arrows indicate crenation of erythrocytes; Yellow arrows indicate platelet aggregation; Black arrows indicate erythrocytes are evenly distributed, do not show any crenation and there is no platelet aggregation.

Partial purification of bacterial crude protease had supported the product to have better characteristics in dissolving blood clot without causing crenation or aggregation of other blood components. This is in line with results reported by Fuad *et al*. [23] reporting fibrinolytic protease from *Bacillus aryabatthai*. It is important to note that clot lysis ability of both crude and dialysate protease of strain HSFI-12 was found to be higher, not only from that of thrombolytic protease from *B*. *aryabatthai*, but also from that of Nattokinase, a commercial thrombolytic protease produced by *B*. *natto*. These enhance the potential of thrombolytic protease produced by *B*. *thuringiensis* HSFI-12 as a competitive alternative antithrombosis agents.

Conclusions

As conclusion, the optimized incubation time for *B*. *thuringiensis* HSFI-12 in producing protease is 48 h. Partial purification of crude protease of strain HSFI-12 using ammonium sulphate precipitation followed by salting out or dialysis resulted in increased specific activity and thrombolytic ability of the enzyme. Further enzyme purification of enzyme dialysate using chromatography could be done to improve specific activity of bacterial protease. Also, *In vivo* study is suggested to evaluate thrombolytic ability of the purified protease using testing animals to develop it as antithrombosis agent.

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