PRODUCTION OPTIMIZATION, PARTIAL PURIFICATION, AND THROMBOLYTIC ACTIVITY EVALUATION OF PROTEASE OF *Bacillus cereus* HSFI-10

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ABSTRACT

Cardiovascular disease is the primary cause of mortality in the world due to the formation of blood clots or thrombi in blood vessels. Bacterial proteases commonly function as thrombus dissolver agents in the pharmaceutical industry. Bacterial isolate HSFI-10 (*Holothuria scabra* Fermented Intestine-10) previously isolated from Rusip fermented sea cucumber had demonstrated thrombolytic activity. This study aimed to produce crude protease of HSFI-10 strain at an optimized incubation time and determine the thrombolytic activity of crude and dialysate proteases on A, B, AB, and O blood types. Isolate HSFI-10 was first molecularly identified and found to be *Bacillus cereus* with a homology level of 99.80% with *Bacillus cereus* strain ST06. The optimum crude enzyme was obtained after 48-h incubation with an activity of 222.52 U/mL, which increased to 438.84 U/mL after ammonium sulfate precipitation and dialysis. Clot lysis activity of crude enzymes was measured based on the gravimetry method on blood in the ABO system, showing results that ranged from 68.99% to 69.76%, while the dialysate ranged from 81.16% to 82.52%. In conclusion, partial purification of bacterial protease could increase both its specific and thrombolytic activities on human blood in the ABO system, with only 1% activity variability between A, B, AB, and O blood types.

Keywords: *Bacillus cereus* HSFI-10, blood system, clot lysis, partial purification, thrombosis

INTRODUCTION

Thrombosis is the leading cause of global death in cardiovascular disease (CVD) (Scheres *et al*. 2018). Cardiovascular is a group of diseases related to the heart and blood vessels. According to the World Health Organization (WHO), this disease causes 31% of death worldwide. WHO predicts that by 2030, cardiovascular disease will continue to increase to more than 23.6 million people. The number is two times higher than the death rate from cancer (WHO 2017).

Thrombosis causes blood to clot (forming a thrombus), so blood vessels in the heart and brain will be blocked (Martina *et al*. 2019). Thrombus occurs in the area of the injured vascular wall, thereby stimulating platelet adhesion in that direction. The attached platelets are activated and release Adenosine Diphosphate (ADP) and thromboxane A2 (TxA2), which causes other platelets to stick to the activated platelets. Platelet aggregation is strengthened with the help of clotting factors in the form of fibrin threads to form a hemostatic plug (Bartinelli *et al*. 2013; Durachim and Astuti, 2018). Thrombosis therapy can use anti-coagulant agents, anti-platelet drugs, fibrinolytic/thrombolytic enzymes, and surgery. However, these drugs have side effects such as headaches, urticaria (allergic reactions), increased clotting time, nausea, vomiting, bleeding complications, low fibrin specificity, and

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relatively high price (Saxena *et al.* 2015; Krishnamurthy *et al.* 2018; Sharma *et al.* 2019).

Research conducted by Hidayati *et al.* (2021) successfully isolated protease-producing bacteria from the digestive organs of the sand sea cucumber coded HSFI-10 to -12 (*Holothuria scabra* Fermented Intestine-10). The crude enzyme of bacterial isolate HSFI-10 showed higher thrombolytic ability than the positive control Nattokinase (a commercial antithrombosis agent). However, the bacterial isolates were only known for their microscopic and macroscopic characteristics. In addition, the thrombolysis test does not explicitly mention blood groups A, B, AB, and O. In contrast, antigens A and B in the ABO system blood group impact hemostatic balance and respond differently to thrombolytic therapy (Separham *et al.* 2020; Mehic *et al.* 2020). Identification was done to determine bacterial species by amplifying the 16S rRNA gene using Polymerase Chain Reaction (PCR). This molecular diagnostic method is more sensitive, specific, efficient, and faster than manual identification (Shahi *et al.* 2018).

Bacteria secrete proteases in the production medium. Each bacterium has a different enzyme production time depending on the type of bacteria (Sharma *et al.* 2015)—partial purification of the protease enzyme *Bacillus* sp. HSFI-10 can be done by precipitating enzymes using ammonium sulfate. This salt has a fairly high ionic strength, does not cause damage to proteins, and has a high solubility in water (Mukherjee, 2019). The remaining ammonium sulfate salt from the enzyme precipitate is removed by dialysis (Razzaq *et al.* 2019). This study aims to determine the type of bacteria based on the 16S rRNA gene sequence using the PCR method, to determine differences in protease enzyme activity based on the optimization of production time for 24, 48, and 72 Hours and to determine the description of A, B, AB, and O blood smears after the addition thrombolytic protease enzymes of *Bacillus* sp. HSFI-10.

MATERIALS AND METHODS

Bacterial Origin

Bacterial strain HSFI-10 was previously isolated in an earlier study from sand sea cucumber *H. scabra* captured from its captivity at Marine Bio Industry Office (BBIL), The Indonesian Institute of Sciences (LIPI), Kodek Gulf Village, Lombok, West Nusa Tenggara (Figure 1). The isolate was then subcultured, and the purified colonies were further analyzed to identify and confirm its fundamental characteristics.

Figure 1 Location of the captivity of sand sea cucumber *H. scabra*, which intestine was the source of HSFI bacteria including HSFI-10 strain: (red dot, coordinates 8°24'15.1"S 116°04'47.2"E) (Google Map 2022)

Procedures

Bacterial subculture and crude protease production

Bacterial subculture was done by cultivating a loop-full of HSFI-10 bacterial single colony Nutrient Agar (NA) medium and then incubated at 37℃ for 24 hours. A single colony of isolate HSFI-10 obtained was then tested to confirm its proteolytic activities by streaking it on skim milk agar (SMA) media and re-incubated for 24 hours at 37°C. The clear proteolytic zone formed around the colony's growth was observed (Fuad *et al.* 2020; Hidayati *et al.* 2021).

Bacterial Molecular Identification by Cloning 16S rDNA

Bacterial colonies of HSFI-10 were inoculated into the BHIB 1 ml medium. After incubation at 37°C for 2 × 24 hours. BHIB media was centrifuged for 1 min at 12000 rpm. DNA genome was extracted with a DNA extraction kit from Geneaid, namely Presto™ Mini gDNA Bacteria Kit. The amplification process used Go Tag Green Master Mix (Promega). The universal primers used were 27-F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492-R (5'-GGT TAC CTT GTT ACG ACT T-3'). Amplicon was visualized using the Major Science UV Transilluminator (Darmawati *et al.* 2015).

Cloning of the 16S rRNA gene was done as previously reported by Darmawati (2015) using Plasmid Vector pTA2 transformed in *E. coli* Zymo 5α competent cells. Recombinants were screened using the blue-white method (Green & Sambrook 2021). Plasmids were isolated according to the previously reported method, with the results of recombinant DNA checked with electrophoresis on 0.8% agarose gel. Sequencing was carried out using ABI sequence programs with primers T7 and T3 5'-TAATACGACTCACTATAGGG-3' and 5'-CCCTTTAGTGAGGGTTAATT-3' as previously reported (Darmawati 2015). Next, bioinformatics DNA sequencing results are analyzed using bioinformatics devices, then processed manually and matched with data in www.ncbi.nih.gov through the BLAST program (Ethica *et al.* 2018).

Optimization of Bacterial Crude Protease Production

Colony of *Bacillus* sp. HSFI-10, which has proteolytic abilities inoculated in minimally synthetic medium (MSM) (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 for 24,48 and 72 h (Farooq *et al.* 2021). The extraction of thrombolytic protease enzymes is done by bacterial culture centrifugation at 10,000 rpm, where supernatant was regarded as a crude enzyme. It further measured the enzyme activity of the Bergmeyer and Grab method (1983) using a spectrophotometer at λ 600 nm.

Partial Purification of Bacterial Protease

Partial purification was initiated by precipitation using 70% ammonium sulfate, as much as 400 g added slowly in 1 L of crude enzymes as previously described (Natsir *et al.* 2015). Cellophane bags were initially soaked in hot water at 60°C for 2 mins, then replaced with 0.2% sulfuric acid as previously reported. Enzymes obtained from the deposition of ammonium sulfate were put in cellophane bags. Both ends of the cellophane bag are tied, then the cellophane bag is soaked with a 500-700 ml phosphate buffer of 0.05 M pH 7 at ± 4°C for 2 h. The soaking buffer is replaced every 2 hours until all salts are separated (Abbas *et al.* 2018).

Specific Enzyme and Clot Lysis Activity Assay

The protease-specific activity was measured using the modified Bergmeyer & Grab (1983) (Natsir *et al.* 2015; Si & Jang 2018). In vitro, blood clot lysis tests were conducted on blood from 4 volunteers, each with blood types A, B, AB, and O, in 6 of the 1.5 mL microtubes previously weighed. Each tube was 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), and Tube 6 (blood type O), and filled with 600 µL blood of each. As previously reported, gravimetry determined clot lysis percentage (Fuad *et al.* 2020; Hidayati *et al.* 2021; Prasad *et al.* 2006).

Proteolytic Activity Test

A single colony of *Bacillus* sp. bacteria. HSFI-10 obtained from the results of bacterial purification in NA media tested its proteolytic abilities. Bacteria that grow on NA media were streaked on skim milk agar (SMA) media and incubated for 24 h at 37°C then the clear zone

formed around the growth of the colony was measured (Fuad *et al.* 2020; Hidayati *et al.* 2021).

Optimization of Crude Enzyme Protease Production Time from Bacillus cereus Culture

Colony of *Bacillus* sp. HSFI-10 with proteolytic abilities was inoculated in minimal synthetic medium (MSM) incubated at 37°C for 24, 48, and 72 h. Extraction of thrombolytic protease enzymes was done by centrifugation at 10,000 rpm for 20 mins at 4°C. The obtained supernatant was regarded as a crude enzyme, and its activity was further measured by Bergmeyer & Grab method (1983) using a spectrophotometer at λ=600 nm (Farooq *et al.* 2021).

Partial Purification of Crude Protease

70% of ammonium sulfate (400 g) was inserted slowly in 1 L of crude enzymes in cold conditions until dissolved for 2 hours. The enzyme solution was kept in the refrigerator to precipitate the enzymes overnight. The enzyme solution was concentrated at 4°C at 10,000 rpm for 30 mins, and the formed pellet was kept. The pellet was then rushed with a 15 ml Tris-Cl buffer of 0.05 M pH 8 (Natsir *et al.* 2015). Cellophane bags were prepared by soaking in hot water and 0.2% sulfuric acid solution before use. Enzymes obtained from the deposition of ammonium sulfate are put in cellophane bags. Dialysis was conducted as previously described until all salts were separated (Abbas *et al.* 2018).

Enzyme-Specific Activity Assay

The protease-specific activity was measured using the modified Bergmeyer & Grab (1983) Folin reagent method (Natsir *et al.* 2015; Si and Jang 2018).

Clot Lysis (Thrombolysis) Activity In Vitro Assay

In vitro blood clot lysis tests before and after enzyme purification were conducted on blood taken from 4 volunteers, each with blood types A, B, AB, and O. Six 1.5-mL-microtube tubes that had been weighed were prepared. Each tube was 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), and tube 6 (blood type O). The percentage of % blood clots was determined following the previously reported method and was conducted in duplicates (Prasad *et al.* 2006; Fuad *et al.* 2020; Hidayati *et al.* 2021). The effect of blood clot lysis was also observed microscopically by the Eustrek (removal) technique according to the May Grunwald-Giemsa mixed method (Geneser 1994). Results were observed with magnification under a Dino-Lite digital microscope and documented with a camera (Ethica *et al.* 2018).

RESULTS AND DISCUSSION

Bacterial Subculture and Morphology Characteristics

Bacillus sp. HSFI-10 has a circular shape, edge (entire), size (3 mm), milk-white color, convex elevation, and smooth consistency (Figure 2A)—*Bacillus* sp. HSFI-10 has a short life in NA media (Figure 2B), a colony life of approximately 4 days of storage at cold temperatures, characterized by colony colors beginning to fade and cannot grow back on the new NA medium.

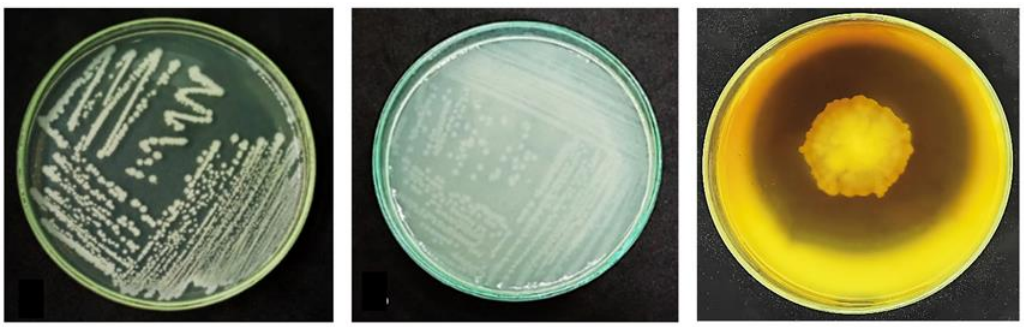


Figure 2 Characteristics of *Bacillus* sp. HSFI-10 in Agar Nutrient (NA) (A) Colonies after 24-h incubation, (B) after stored for 4 days. C. on skim milk agar media with Lugol-staining (clear zone showing proteolytic activity)

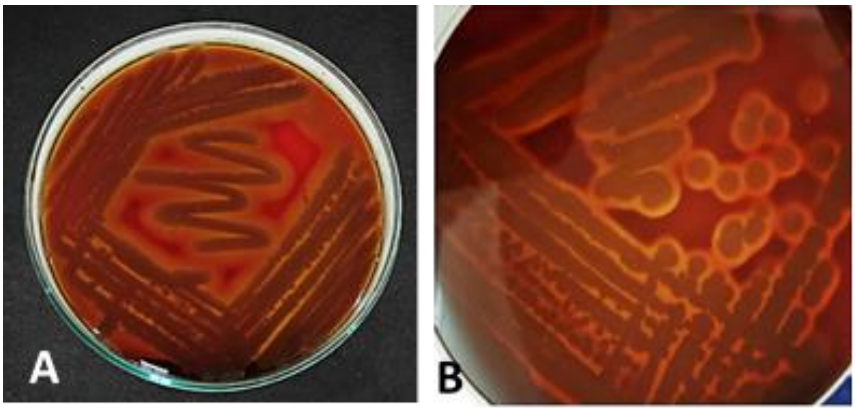


Figure 3 Similar characteristics of *Bacillus* sp. HSFI-10 on Blood Agar Plate media. (A) compared to (B) *B. cereus* indicates β-hemolysis (Milojevic *et al.* 2019)

Gram staining showed the rod shape was Gram-positive, lined, and had spores. *B. cereus* could produce a clear zone on the 7th day of cultivation on Skim Milk Agar (SMA) media with a diameter of 36 mm. SMA contains casein as a protease enzyme substrate. Bacterial growth in blood agar plate (BAP) media indicates a β-hemolysis pattern, characterized by an area of clear zone around the colony (Figure 3) (Bottone 2010; Lu *et al.* 2018).

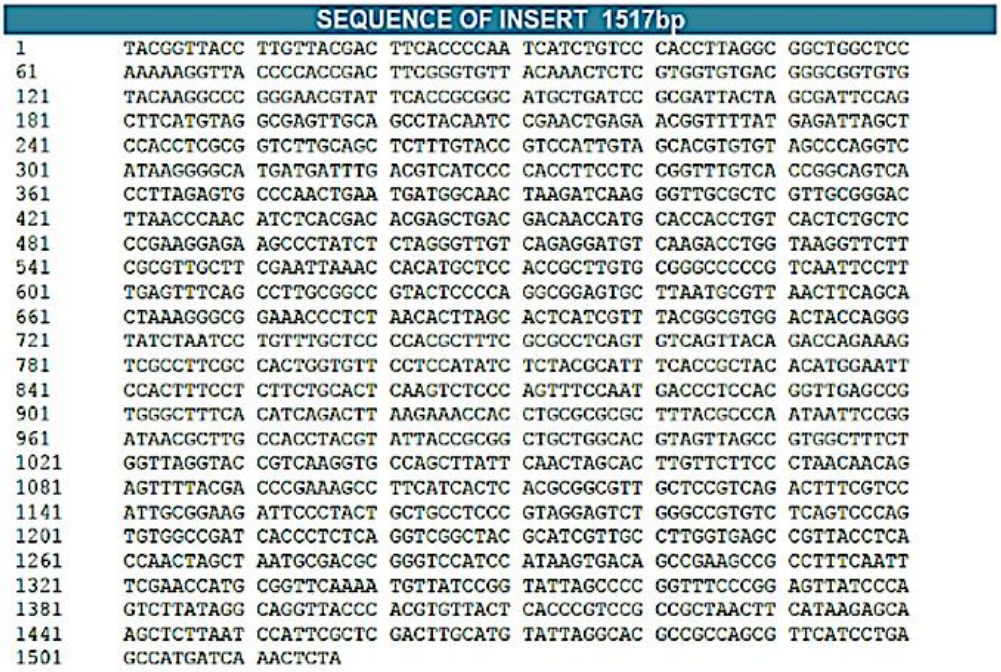
As seen in Figure 3, the macroscopic characteristics of bacteria on BAP media were evaluated by the shape, color, size, edge, and elevation of the colony as well as discoloration in the media (e. g. hemolysis in the medium for blood) (Pitt *et al.* 2012). The result aligned with the Mogrovejo *et al.* (2020) study reporting that *Bacillus cereus* can disintegrate red blood cells (forming a β-hemolysis pattern on BAP).

B. cereus HSFI-10 morphology and properties reported in this study following the results reported by Hidayati *et al.* (2021). The proteolytic ability test aims to determine bacteria that have the potential to produce proteases, characterized by the formation of a clear zone around the bacterial colony (Assaf *et al.* 2020). The clear zone produced by proteolytic bacteria occurs due to protease activity which breaks the peptide bonds of casein in skim milk medium by breaking the CO-NH peptide bond with the entry of water into the molecule, thereby releasing amino acids (Baehaki *et al.* 2011; Ethica *et al.* 2018; Artha *et al.* 2019).

Bacterial Molecular Identification

DNA extract of *Bacillus* sp. HSFI-10 has a concentration of 232.6 ng/µl and a purity of 1.82. The purity of extracted DNA was high if the absorbance ratio (λ260/λ280) was 1.83. If the absorbance ratio is less than 1.8, proteins still contaminate DNA. If greater than 1.8, the DNA is contaminated with RNA (Gupta 2019).

The genomic 16S rRNA gene cloning technique obtained the full-length 16S rRNA gene sequence (Green & Sambrook 2021). The cloning process was done by ligating, transforming, and isolating recombinant DNA from transformants (Green & Sambrook 2021). Recombinant DNA was then transformed in *Escherichia coli* Zymo 5α bacterial cells and grown on a solid medium. The success of cloning could be seen through white colonies (clones carrying recombinant DNA). The white colony indicates the LacZ region on the plasmid is inactive because it has been inserted by the inserted gene so that the galactose present in the media cannot be hydrolyzed by the -galactosidase enzyme which, if active, will form blue colonies (Sambrook *et al.* 1989; Darmawati 2015)—results of the 16S rRNA gene cloning of *Bacillus* sp. HSFI-10 is displayed in Figure 4, with an amplicon size of 1517 bp.



SEQUENCE OF INSERT 1517bp

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1     TACGGTTACC TTGTTACGAC TTCACCCCAA TCATCTGTCC CACCTTAGGC GGCTGGCTCC
61    AAAAAGGTTA CCCCACCGAC TTCGGGTGTT ACAAACTCTC GTGGTGTGAC GGGCGGTGTG
121   TACAAGGCCC GGGAACGTAT TCACCGCGGC ATGCTGATCC GCGATTACTA GCGATTCCAG
181   CTTCATGTAG GCGAGTTGCA GCCTACAATC CGAACTGAGA ACGGTTTTAT GAGATTAGCT
241   CCACCTCGCG GTCTTGCAGC TCTTTGTACC GTCCATTGTA GCACGTGTGT AGCCCAGGTC
301   ATAAGGGGCA TGATGATTTG ACGTCATCCC CACCTTCCTC CGGTTTGTCA CCGGCAGTCA
361   CCTTAGAGTG CCCAACTGAA TGATGGCAAC TAAGATCAAG GGTTGCGCTC GTTGCGGGAC
421   TTAACCCAAC ATCTCACGAC ACGAGCTGAC GACAACCATG CACCACCTGT CACTCTGCTC
481   CCGAAGGAGA AGCCCTATCT CTAGGGTTGT CAGAGGATGT CAAGACCTGG TAAGGTTCTT
541   CGCGTTGCTT CGAATTAAAC CACATGCTCC ACCGCTTGTG CGGGCCCCCG TCAATTCCTT
601   TGAGTTTCAG CCTTGCGGCC GTACTCCCCA GGCGGAGTGC TTAATGCGTT AACTTCAGCA
661   CTAAAGGGCG GAAACCCTCT AACACTTAGC ACTCATCGTT TACGGCGTGG ACTACCAGGG
721   TATCTAATCC TGTTTGCTCC CCACGCTTTC GCGCCTCAGT GTCAGTTACA GACCAGAAAG
781   TCGCCTTCGC CACTGGTGTT CCTCCATATC TCTACGCATT TCACCGCTAC ACATGGAATT
841   CCACTTTCCT CTTCTGCACT CAAGTCTCCC AGTTTCCAAT GACCCTCCAC GGTTGAGCCG
901   TGGGCTTTCA CATCAGACTT AAGAAACCAC CTGCGCGCGC TTTACGCCCA ATAATTCCGG
961   ATAACGCTTG CCACCTACGT ATTACCGCGG CTGCTGGCAC GTAGTTAGCC GTGGCTTTCT
1021  GGTTAGGTAC CGTCAAGGTG CCAGCTTATT CAACTAGCAC TTGTTCTTCC CTAACAACAG
1081  AGTTTTACGA CCCGAAAGCC TTCATCACTC ACGCGGCGTT GCTCCGTCAG ACTTTCGTCC
1141  ATTGCGGAAG ATTCCCTACT GCTGCCTCCC GTAGGAGTCT GGGCCGTGTC TCAGTCCCAG
1201  TGTGGCCGAT CACCCTCTCA GGTCGGCTAC GCATCGTTGC CTTGGTGAGC CGTTACCTCA
1261  CCAACTAGCT AATGCGACGC GGGTCCATCC ATAAGTGACA GCCGAAGCCG CCTTTCAATT
1321  TCGAACCATG CGGTTCAAAA TGTTATCCGG TATTAGCCCC GGTTTCCCGG AGTTATCCCA
1381  GTCTTATAGG CAGGTTACCC ACGTGTTACT CACCCGTCCG CCGCTAACTT CATAAGAGCA
1441  AGCTCTTAAT CCATTCGCTC GACTTGCATG TATTAGGCAC GCCGCCAGCG TTCATCCTGA
1501  GCCATGATCA AACTCTA
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Figure 4 The complete sequence of the 16S rRNA *Bacillus* sp. HSFI-10 obtained from cloning and sequencing 16S rRNA gene

The consensus was made on the forward and reversed 16S rDNA sequences using the Bio Edit program (Hall 2004). The 16S rRNA gene sequence data saved in FASTA format were then analyzed and matched with the data available in the Gene Bank Basic Local Alignment Search Tool (BLAST). The results of BLAST analysis of *Bacillus* sp. HSFI-10 16S rRNA gene fragments showed a homology level of 99.80% with *Bacillus cereus* strain ST06 (Acc. No.: MH475925.1).

Optimization of Crude Production Time for Protease Enzymes from Bacterial Culture

Crude enzyme production was optimized with various incubation times of 24, 48, and 72 hours. The results of this study indicate that *Bacillus cereus* HSFI-10 can produce the most optimum crude enzyme at 48 hours with an enzyme activity of 222.52 U/mL. It has been reported that protease production was proportional to bacterial growth. In the early stages of the growth curve, bacteria produced few proteases and optimum production in the stationary phase (Ahmadpour and Yakhchali 2017; Pagarra *et al.* 2020; Suleiman *et al.* 2020).

Partial Purification of Protease and Specific Activity Assay

The protease activity of *B. cereus* HSFI-10 was calculated using the equation from Hidayati *et al.* (2021): Y = 0.0019 X + 0.0092. Y = absorbance, while X = enzyme activity (U/mL). Enzyme activity was expressed as the number of tyrosine amino acids released by the casein substrate per unit of time under test conditions (Zainuddin *et al.* 2020). Results of specific activity assay on crude and dialysate protease of *B. cereus* HSFI-10 are shown in Table 1.

Table 1 Absorbance and specific activity data of crude and dialysate protease of *B. cereus* HSFI-10

Protease extract	Absorbance at λ=600 nm at incubation time (h)*:			Enzyme activity (U/mL) at incubation time (h)*:		
	24	48	72	24	48	72
Crude	0,264	0,433	0,346	134,105	222,526	146,000
Dialysate		0,843			438,842	

*All tests were conducted in duplicate

Based on data in Table 3, it could be inferred that ammonium sulfate precipitation followed by dialysis could increase the enzyme-specific activity by almost doubling from 222.526 U/m to 438.842 U/mL. These results are to the research of Mothe and Sultanpuram (2016), reporting that enzymes purified by dialysis had high activity compared to crude enzymes, where the level of enzyme purity could reach 2.23 times higher.

Clot Lysis Activity Assay on Crude and Partially Purified Protease

Crude and dialysate enzyme *B. cereus* HSFI-10 could lyse ABO blood clot better than Nattokinase as control (Figure 5). A higher percentage of clot lysis characterized it. The % blood clot lysis of each sample is shown in Table 2.

Nattokinase is a serine protease enzyme with fibrinolytic and antithrombotic activity. This ability can be used for cardiovascular treatment. Several studies have stated that Nattokinase can thin the blood and dissolve blood clots in experimental animals and humans (Gallelli *et al.* 2021; Chen *et al.* 2022). This study shows that crude enzymes and dialysate can lyse blood clots better than Nattokinase (Figure 5). Hence, the protease enzyme from *B. cereus* HSFI-10 bacteria can be used as a substitute for Nattokinase.

As seen in Table 2, part of the limitation of this preliminary study is that the thrombolysis ability test was conducted only in duplicates with the objective of screening. One of the reasons is that the gravimetry test, first described by Prasad *et al.* 2006, is semi-quantitative. The qualitative part of the assessment lies in whether the lyse blood is still present before weighing the step of the method. Thus, the more accurate thrombolysis test should be confirmed with the *in vivo* one involving precise crude and dialysate bacterial protease dosage followed by statistical analysis (Dewi *et al.* 2022).

The effect of clot lysis on O blood cells before and after adding crude and dialysate enzymes was also observed under a microscope with 400× magnification, with results displayed in Figure 6.

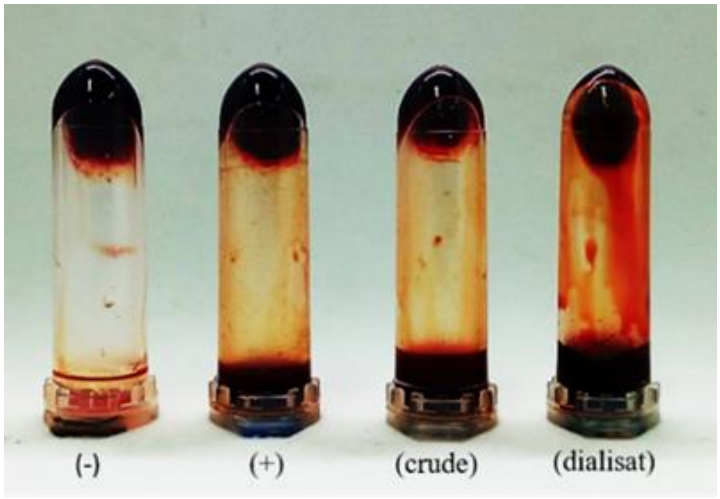

Figure 5 Crude protease enzyme thrombolysis test and dialysate on blood type O

Table 2 Results of the Thrombolysis Ability Test for crude protease enzymes and dialysates in ABO blood type

Blood Type	Initial clot weight (g)*			Final clot weight (g)*			% Clot lysis*		
	NK	Crude	Dialysate	NK	Crude	Dialysate	NK	Crude	Dialysate
A	0.358	0.358	0.299	0.141	0.111	0.055	60.614	68.994	81.605
B	0.333	0.334	0.282	0.133	0.101	0.051	60.060	69.760	81.914
AB	0.290	0.290	0.276	0.116	0.088	0.052	60.000	69.655	81.159
O	0.369	0.368	0.349	0.145	0.113	0.061	60.704	69.293	82.521

*All measurement was conducted in duplicates
NK: Nattokinase

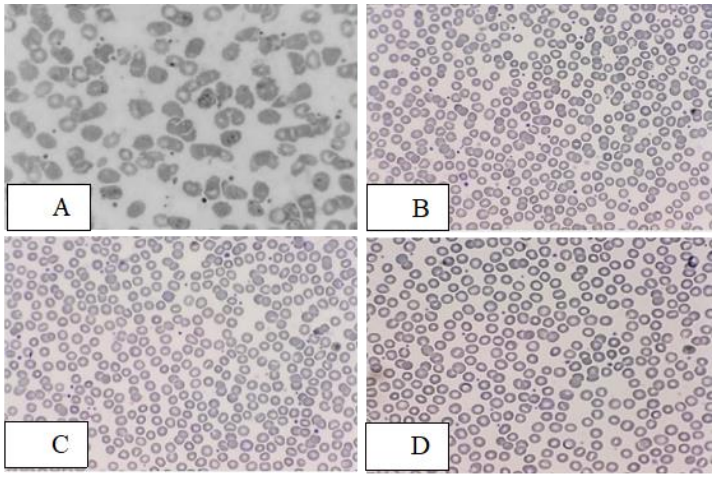

Figure 6 The observation results on protease clot lysis activity on O blood cells using a Dino-Lite Digital Microscope. (A) negative control, (B) positive control, (C) crude enzyme, (D) dialysate enzyme

As seen in Figure 6, platelets undergoing aggregation and erythrocytes experience changes in shape and overlap (Figure 6A), indicating the occurrence of blood clots. While Figures 6B, C, and D show that platelets did not undergo aggregation, erythrocytes did not change in shape and are evenly distributed. The platelet condition was due to favorable control treatment, crude, and dialysate, which can lyse blood clots. These results align with the research of Fuad *et al.*, 2020, where crude protease enzymes produced by *Staphylococcus hominis* HSFT-2, *S. saprophyticus* HSFT-11, and *Bacillus aryabhattai* HSFT-5 caused erythrocytes not to create, spread evenly and platelets did not aggregate.

Our results showed that partial purification by ammonium sulfate precipitation and dialysis on produced bacterial crude protease is very beneficial. The partial purification could increase bacterial protease-specific activity from 222.52 U/mL to 438.84 U/mL. It could also increase clot lysis activity based on the gravimetry method on blood in the ABO system from 68.99% - 69.76% (crude protease) to 81.16% (crude 82.52% (dialysate protease). The next step will be enzyme purification techniques using various chromatography-based methods to maximize protease's specific and clot lysis activity from *B. cereus* HSFI-10 (Westphal & van Berkel 2021).

In this study, bacterial proteases had a similar effect on the lysis of ABO blood clots regardless of their different types of agglutination. Despite the previously reported data that blood type did affect the formation of blood clots, it has been known that individuals with non-O blood type have been shown to have a higher risk of thrombus formation than individuals with blood type O. This was associated with levels of coagulation factors, especially von Willebrand factor (vWF), vWF levels 30% higher in individuals with non-O blood type compared with blood group O (Holle *et al.* 2020; Mohamed *et al.* 2020; Separham *et al.* 2020; Pendu *et al.* 2021).

CONCLUSION

The optimum production of crude enzyme of thrombolytic protease-producing bacterium, *B. cereus* HSFI-10, resulting in the highest specific activity, was at 48-h incubation. Partial purification of bacterial protease increased both its specific and thrombolytic activities in human blood of the ABO group system with only 1% activity variability between A, B, AB, and O blood types.

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