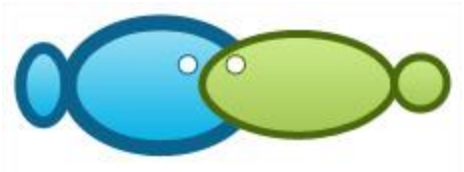

Exploration of bacteria isolated from "rusip" fermented tissue of sand sea cucumber *Holothuria scabra* with fibrinolytic, anticoagulant and antiplatelet activities

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Abstract. Sand sea cucumber (*Holothuria scabra*) is an important sea commodity containing high levels of protein. Fibrinolytic proteases are extracellularly produced from certain bacteria isolated from fermented, protein-rich substrates. The importance of fibrinolytic proteases is largely attributed to their applicability as anticoagulants and antiplatelets, mainly used in therapy for cardiovascular disease, the number one cause of death in the world. This study aimed to obtain new sources of fibrinolytic protease from bacteria isolated from the fermented tissue of *H. scabra* captured in Kodek Bay of North Lombok Island, Indonesia. To stimulate the growth of proteolytic bacteria prior to the isolation process, the tissue of the sand sea cucumber was first fermented as "rusip", a fish-based traditional fermentation product from Kalimantan. The selection of fibrinolytic protease producing bacteria was carried out using Skim Milk Agar (SMA) and Fibrin Agar (FA) media followed by crude protease extraction using Skim Milk Broth (SMB). The obtained crude protease extracts were then subjected to anticoagulant and antiplatelet activity tests using Lee-White, Eustrek and Clot-lysis methods, respectively. Bacterial isolates showing fibrinolytic, anticoagulant and antiplatelet activities were identified using Polymerase Chain Reaction (PCR) targeting their 16S rRNA gene. As results, 3 out of 17 bacterial isolates from the fermented tissue of *H. scabra* identified as *Staphylococcus hominis*, *Bacillus aryabhattai* and *Staphylococcus saprophyticus* could show proteolytic and fibrinolytic activities. Further test results demonstrated specific characteristics of bacterial crude extracts as both antiplatelet and anticoagulant agents. In conclusion, the fermented tissue of *H. scabra* is a valuable source of bacteria producing fibrinolytic proteases with anticoagulant and antiplatelet activities.
Key Words: fibrinolytic protease, fish-based fermentation, proteolytic bacteria, thrombosis.

Introduction. Sand sea cucumbers belonging to the class Holothuroidea are known as an important sea commodity because they are rich in bioactive compounds. Holothurians including *Holothuria scabra* contain high amounts of protein around 22.5% in the body, and 55.18% in the body wall. They have a very low lipid content of about 1.55% in the whole body and 1.02% in the body wall. Having been widely used as anticoagulant and antiplatelet agents, the group of marine invertebrates are also known to have protease enzyme activity (Bordbar et al 2011; Siahaan et al 2017; Hernández-Sámano et al 2017; Sroyraya et al 2017).

Cardiovascular diseases (CVD) are the number one cause of death in the world and are triggered by disorders of the heart and blood vessels. CVD groups include coronary heart disease, heart rhythm disorders (arrhythmias), heart failure, hypertension, eye disorders and strokes (Afifah et al 2014; Anh et al 2015; Bhatnagar et al 2015; Hicks et al 2018). Based on data from the World Health Organization (WHO) in 2016, premature deaths worldwide due to CVD reached more than 17.9 million (31%) and are expected to increase, reaching 23.6 million by 2030. In addition, more than 80% of CVD deaths occur in low-middle income countries and occur equally in men and women (Ali et al 2019).

The basic pathophysiology of the CVD process is the formation of thrombus (thrombosis). Thrombosis is characterized by the development of blood or fibrin clots attached to the walls of blood vessels (intravascular). Excessive accumulation of fibrin in blood vessels can disrupt blood flow and damage tissue in the heart, causing irregular heartbeats, heart attacks, and death. Fibrin is a major protein component of blood clotting formed from fibrinogen by thrombin (Kumaran et al 2011; Anh et al 2015; Stephani et al 2017).

Therapies to treat thrombosis including surgery are carried out to removing blockages (Haxhibeqiri-Karabdić et al 2015) and/or by administering oral drugs or injection of anti-thrombosis drugs that lyse blood clots (Devaraj et al 2018). Generally, thrombolysis drugs are divided in two groups based on the mode of action. The first group is the group of fibrinolytic enzymes in the form of plasminogen activators (PA) such as tissue plasminogen activators (t-PA), streptokinase (SK, 3.4.99.22) and urokinase (UK, EC 3.4.21.31) (Anh et al 2015; Devaraj et al 2018). Fibrinolytic enzymes in the PA group could lyse fibrin through the formation of plasmin from plasminogen. The second group is a group of fibrinolytic enzymes that can directly degrade fibrin (the main component of thrombus) such as plasmin. PA and plasmin enzymes have been widely used as thrombolysis agents supported by intensive research that has been done so far. The prices of both are still high, and other issues include thermos-lability, low specificity, and some have side effects in the form of gastrointestinal bleeding, allergic reactions, acute coronary disease and resistance to replication (Kumaran et al 2011; Afifah et al 2014). On the other hand, anticoagulants are drugs also used in the therapy of CVD that work by preventing the occurrence of blood clots. Fibrinolytic protease enzymes are also known to have abilities in preventing blood clots, which work directly to lyse thrombin *in vivo* and oral administration can increase fibrino-plasma activity and t-PA22-24 production (Chen et al 2018).

Fibrinolytic enzymes are known to be produced by animals, plants and also microorganisms. Organisms which have potential to produce fibrinolytic enzymes include bacteria, actinomycetes, snakes, earthworms, sea animals, insects and fungi (Kotb 2014; Anh et al 2015; Susanti & Fibriana 2017). Bacteria have the potential to produce economically valuable enzymes with several advantages, including: faster growth, the scale of cell production can be more easily increased, production conditions do not depend on the season, the time required is relatively short, low cost, high activity, safe, non-toxic and they are easily manipulated genetically (Akhtar et al 2017). Fibrinolytic enzymes can also be produced from various types of bacteria isolated from traditional fermented foods. These bacteria are *Stenotrophomonas* sp., *Bacillus licheniformis*, *B. cereus* and *Stenotrophomonas* sp. isolated from oncom, *B. pumilus* 2.g isolated from gembus, *B. subtilis* LD-8547 from douche, and *B. amyloliquefaciens* RSB34 from doenjang products (Yuan et al 2012; Afifah et al 2014; Nailufar et al 2016; Yao et al 2017).

The enzyme nattokinase (NK) from the fermented natto product is known to be able to directly lyse thrombin *in vivo* and its administration could increase fibrino-plasma activity and t-PA22-24 production. Similar fibrinolytic enzymes from douchi, doufuru, oncom and jeotgal saeu (small shrimp) were also confirmed to be able to degrade fibrin directly and efficiently *in vitro* and *in vivo* (Chen et al 2013; Anh et al 2015; Hu et al 2019; Chen et al 2018). Fibrinolytic proteinase has been isolated from fermented food products from Indonesia, but it is still limited to fish, gembus and oncom (Afifah et al 2014; Prihanto et al 2016; Stephani et al 2017; Purwaeni et al 2018). Protease-

producing bacteria in shrimp paste and rusip have been reported, but have not been specific to the fibrinolytic group (Sulthoniyah et al 2015; Japri et al 2019; Fazri et al 2019; Islami 2019). This research was conducted on bacteria that produce fibrinolytic protease enzymes with different abilities, including as an anticoagulant and antiplatelet agent from rusip fermentation products of sand sea cucumber.

Bacterial relationships from fermented products (rusip) of sand sea cucumber (*H. scabra*) with the potential to produce a protease enzyme with fibrinolytic activity are a subject of interest. The virtues of bacteria as fibrinolytic enzyme producers, as well as the opportunities for novelty that exist provide the prospects of developing new antithrombotic agents from bacteria. This research seeks to explore the potential of *H. scabra* regarding problems related to CVD.

Material and Method

Materials used. The sample used in this study was a 1-year-old *H. scabra*, with 17 cm length, 4 cm width, and 5 cm thickness. Its weight was 144.01 g. It was obtained from the LIPI Lombok Marine Bio Industry Center (from captivity). The intestine of the organism was removed and washed with sterile water. The clean muscle tissue sample was collected from the white flesh, cut into pieces, and made into rusip (Figure 1).

Figure 1. Muscle tissue sample of *Holothuria scabra.*

Bacterial isolation. For the rusip fermentation process, sea cucumber pieces weighing 11.36 g were added and mixed with 2.84 g kosher salt and 1.14 g palm sugar (Fazri et al 2019). After 7 days of rusip fermentation, 1 mL of *H. scabra* sample was diluted and then cultivated on Nutrient Agar (NA) media at 37°C for 24 h. Colony morphology observation was conducted on every single isolated colony showing distinct appearance. Each distinct isolate was individually purified on NA media in triplicates (Japri et al 2019).

Bacterial proteolytic enzyme production test. The proteolytic activity of single isolates resulted from bacterial colony purification on NA media was tested on SMA after incubation for 24 h at 37°C. The formation of a clear zone around the growth of the colony was observed as an indication of protease production by bacteria (Harun et al 2018).

Isolation of crude proteolytic enzymes from bacteria. Each morphologically identified proteolytic isolate was inoculated on Skim Milk Broth (SMB) medium containing peptone 5 g L^{-1}, beef extract 1.5 g L^{-1}, yeast extract 1.5 g L^{-1}, sodium chloride 35 g L^{-1}, and casein 10 g L^{-1} with incubation for 72 h at 37°C (Paik et al 2004). The fibrinolytic protease enzyme was extracted by centrifuging the bacterial culture at a speed of 3000 rpm for 15 min at 4°C. Supernatants were then subjected to blood clot-lysis test (Baehaki et al 2011).

Anti-thrombosis activity test of proteolytic bacterial enzymes. *In vitro* blood clot lysis activity test was conducted using fresh blood from the blood bank (without anticoagulant addition) Each individual in this study gave written informed consent prior to the blood sample collection. Each of 5 microtubes were weighed and coded 1-5. 600 µL of blood was added in each tube, and left until the clot formed after approximately 30 min. Serum was completely removed after clot formation by centrifugation. The tube containing the clots was then weighed again. Clot weight was determined by subtracting the weight of each tube (clot weight = weight of lump containing tubes - weight of empty tubes). 100 µL of water was added to tube 1 (negative control); tube 2 had 100 µl of nattokinase added (positive control); tube 3 had 100 µL of crude enzyme added; tube 4 had 200 µL of crude enzyme added; tube 5 had 300 µL of crude enzyme added. The samples were incubated at 37°C overnight (Prasad et al 2006).

The lysis fluid from each tube was completely absorbed with filter paper and the tubes were then weighed again. The difference in weight before and after incubation was calculated and the percentage of blood clots in the sample was calculated with the formula:

Lysis percentage of blood clot = [(weight of the initial blood clot - weight of the final blood clot)/weight of the initial blood clot] x 100

Protease enzyme activity test as anticoagulant. Two methods were used to conduct anticoagulant tests on bacterial crude extracts. The first was the modified Lee-White method (Gandasoebrata 1992). This method was used to determine the visually observed blood clotting period. Normal blood clotting generally occurs between 3-18 minutes in humans. The second was the blood smear Eustrek method (Lukens 1993).

The procedure of the modified Lee-White method was as follows: three clean and labelled test tubes with a diameter of 8 mm of each were prepared. The tubes were then placed in the tube rack. The blood used was the blood of 2 volunteers. 1 mL of blood was inserted into each test tube coded 1-3. Then tube 1 had 100 µl of EDTA added, tube 2 was not treated, and tube 3 had 100 µL of crude enzyme added using a micropipette and was mixed using a vortex. At the same time as mixing, a stopwatch was used to determine the period of clot formation that occurred. After 3 min, the tubes were removed and each test tube was tilted to see if there has been freezing or not. If there had been no clotting, the tube was placed back on the test tube rack and the procedure was repeated every 30 seconds (Tangkery et al 2013).

The effect of blood clotting can also be seen microscopically using the Eustrek technique (smear). This method was used to see the state of blood cells microscopically, according to the May Grunwald-Giemsa mixed method (Geneser 1994). Three pieces of clean and non-greasy slides were prepared and labeled with a number from 1 to 3. Slide number 1 was for the blood sample with EDTA taken from test tube 1; slide number 2 was for the blood sample without treatment taken from test tube 2; slide number 3 was for the blood sample with crude enzymes (100 µL) from test tube 3. 20 µL of blood was collected from each test tube. The blood was then smeared on top of each respective slide. The blood droplets on the slides were touched with the cover glass so that the blood drops would widen, producing a thin layer 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 soaked in Giemsa solution for 30 min, rinsed with water, then aerated until dry. The results were observed under a light microscope at 400x magnification and documented with a camera (Tangkery et al 2013).

Fibrinolytic activity test. The fibrinolytic activity test was performed on FA medium. In FA, 6 holes were made for the enzyme and 1 hole for the standard Nattokinase. A total of 50 µL of rejuvenated proteolytic bacteria inoculum was inserted into 6 holes and 1 hole had the standard of natto. The FA medium was then incubated at 32°C for 24 h, at pH 7. The fibrinolytic activity was indicated by the presence of a clear zone around the hole and was determined by measuring the fibrinolytic enzyme activity index (IAE), by calculating the clear zone diameter divided by the colony diameter. Furthermore, one

bacterial isolate that had the highest fibrinolytic activity index was selected to be identified (Kumaran et al 2011; Khikmah & Sulistyani 2018).

Fibrin agar media was prepared by mixing 1.7% agar and 3% fibrin, in a 0.1 M borate buffer solution (pH 7.8). After addition to dissolve the agar by heating, the medium (12 mL) was poured into a test tube and covered with a cotton swab. Media was sterilized at 121°C for 30 min. 200 µL of methylene blue were added to the plate so that the clear fibrinolytic zone could be seen better. The media was poured into a petri dish and allowed to solidify (Kumaran et al 2011).

Molecular identification of bacteria. Bacterial cells demonstrating fibrinolytic protease activity were diluted 1×10^9, then the DNA was extracted from them using Presto™ Mini gDNA Bacteria Kit from Geneaid. Next, amplification of the 16S rRNA gene fragment was carried out following the previous method (Ethica et al 2018a). The primer used was: forward - 5'-AGAGTTTGATCMTGGCTCAG-3', positions 8-27; and 1500 reverse primer - 5'-GGTTACCTTGTTAC GACTT-3', positions 1510-1492, based on the 16S rRNA *E. coli* numbering (Weisburg et al 1991).

16S rRNA DNA sequencing and bioinformatics analysis. The amplified DNA sequencing was carried out at PT Genetics Science Indonesia with the Sanger sequencing method. The sequencing results were analyzed using bioinformatics tools. Consensus generation was carried out on forward and reverse sequences of the 16S rRNA gene with the Baser Assembler DNA Program. The results of bacterial DNA sequencing were analyzed and matched with data available in the Gen Bank Basic Local Alignment Search Tool (BLAST). BLAST analysis was carried out on the nucleotide sequence of the 16S rRNA gene online via https://blast.ncbi.nlm.nih.gov/Blast.cgi (Ethica 2019).

The phylogenetic tree of the 3 obtained bacteria was designed using the MEGA X program. The algorithm used to design the phylogenic tree was the Neighbor-Joining Tree (Tamura et al 2004; Ethica 2019).

Results and Discussion

Sample overview. The fermentation container of sand sea cucumber rusip opened on the 7th day had a distinctive smell of rusip, and there were bubbles in the fermentation liquid product. These are among the signs of successful rusip fermentation. The appearance of an odor (fermented acid) is caused by volatile substances. The odor is related to the excretion of bacteria that produces organic acids such as lactic acid (Yuliana et al 2018).

Isolation and characterization of bacteria. There were 17 bacterial isolates with different or unique colony characteristics (Table 1) obtained from fermented rusip of *H. scabra* tissue. Macroscopic characteristics can be seen from the colony shape, colony color, colony size, edge and elevation of the colony. Each colony with a unique morphology was purified at least 3 times with the final result in the form of pure isolates. All of these bacterial isolates obtained from the colony purification process were identified for their morphology and characteristics based on Gram stain under microscope observation through 100× magnification. Gram staining aims to determine the group of bacteria: Gram-positive if bacteria are purple, and Gram-negative if bacteria are red (Holderman et al 2017).

Table 1

Morphology of bacterial colonies isolated from fermented tissue of *Holothuria scabra* on nutrient agar (NA) medium

No	*Isolate code (strain)*	*Colony morphology*						*Gram staining*	
		Shape	*Edge*	*Size (mm)*	*Color*	*Elevation*	*Consistency*	*Cell shape*	*+/-*
1	HSFT-1	Circular	Entire	2	Yellow	Convex	Smooth	Bacilli	-
2	HSFT-2	Circular	Entire	3	Cream	Raised	Smooth	Coccus	+
3	HSFT-3	Irregular	Undulate	1	White	Umbonate	Smooth	Coccus	-
4	HSFT-4	Circular	Entire	1	White	Convex	Smooth	Coccus	
5	HSFT-5	Circular	Entire	5	White	Crateriform	Smooth	Bacilli	+
6	HSFT-6	Circular	Entire	1	Cream	Convex	Smooth	Coccus	-
7	HSFT-7	Circular	Entire	2	Cream	Convex	Smooth	Coccus	+
8	HSFT-8	Circular	Entire	3	Cream	Raised	Smooth	Coccus	-
9	HSFT-9	Circular	Entire	1	Cream	Covex	Smooth	Bacilli	-
10	HSFT-10	Circular	Entire	1	Yellow	Convex	Smooth	Coccus	+
11	HSFT-11	Circular	Entire	1	Cream	Umbonate	Smooth	Coccus	+
12	HSFT-12	Circular	Entire	3	Yellow	Convex	Smooth	Coccus	-
13	HSFT-13	Circular	Entire	2	Cream	Convex	Smooth	Coccus	-
14	HSFT-14	Circular	Entire	1	Cream	Convex	Smooth	Coccus	+
15	HSFT-15	Circular	Entire	3	Cream	Flat	Smooth	Coccus	+
16	HSFT-16	Circular	Entire	2	White	Convex	Smooth	Coccus	+
17	HSFT-17	Circular	Entire	2	Cream	Raised	Smooth	Coccus	-

Note: HSFT - *Holothuria scabra* fermentation tissue; (+) - Gram-positive; (-) - Gram-negative.

Based on Table 1, 8 of 17 (total) isolates were Gram-positive and the rest were Gram-negative. Based on cell shape, the majority are coccus. All of these colonies were subjected to the protease enzyme production test on SMA.

Isolation and selection of proteolytic bacteria. The proteolytic activity of the 17 isolates resulted from colony purification was tested (Table 2). From the data in Table 2, there were 14 bacterial isolates that produced a clear zone around the colony on SMA media. The bacterial isolate with the largest hydrolysis index was HSFT-10, with 3.6. The presence of a clear zone around the colony in the SMA media indicated that the bacteria remodel the protein, the magnitude of the proteolytic hydrolysis ability being indicated by the larger diameter of the resulting clear zone.

Table 2

The proteolytic index test on proteolytic bacterial isolates

No	*Isolate code*	*Diameter of clear zone (mm)*	*Diameter of colony (mm)*	*Hydrolysis capacity index*
1	HSFT-1	30	9	3.33
2	HSFT-2	28	10	2.80
3	HSFT-3	2	8	0.25
4	HSFT-4	10	5	2.00
5	HSFT-5	24	10	2.40
6	HSFT-6	0	9	0.00
7	HSFT-7	20	10	2.00
8	HSFT-8	10	8	1.25
9	HSFT-9	0	5	0.00
10	HSFT-10	18	5	3.60
11	HSFT-11	22	8	2.75
12	HSFT-12	6	9	0.67
13	HSFT-13	22	9	2.44
14	HSFT-14	20	8	2.50
15	HSFT-15	22	10	2.20
16	HSFT-16	18	10	1.80
17	HSFT-17	0	5	0.00

According to Naiola & Widhyastuti (2002), the results of protein polymer overhaul are indicated by the presence of a clear zone, which indicates that the protein has been successfully converted into short chain peptide compounds and amino acids, which are soluble in media.

In the protease activity tests, 5% Lugol solution was used as an indicator on SMA for the proteolytic zone (Figure 2). After a drop, a yellow color was formed on the bacterial colony, a light color in the clear zone and the medium was grayish brown. The clear zone of the formed bacterial isolates helped in determining the proteolytic index by calculating the ratio between the diameter of the clear zone around the bacterial colony and the diameter of the bacterial colony (Baehaki et al 2011).

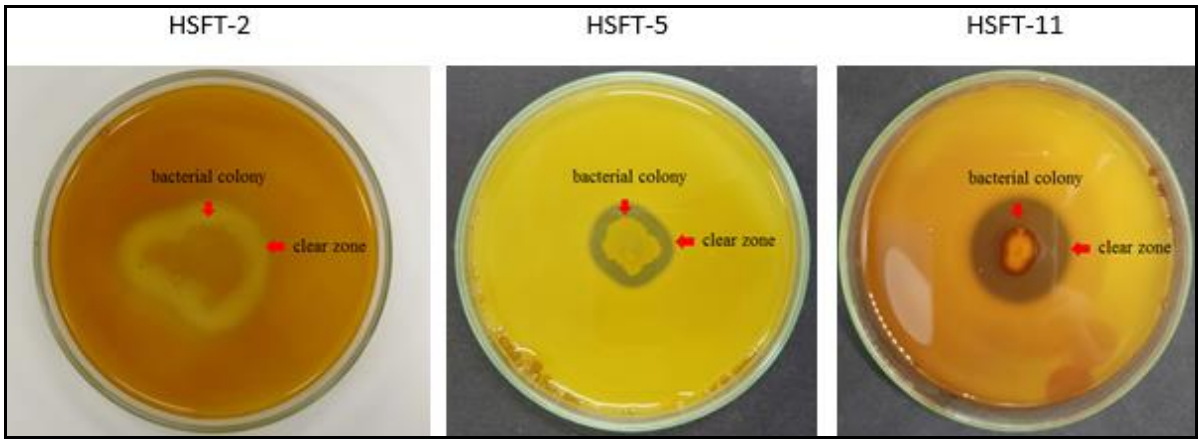

Figure 2. The proteolytic activity test of HSFT-2, HSFT-5 and HSFT-11 isolates on skim milk agar media with 0.5% Lugol staining. Clear zone shows proteolytic activity of bacterial colony on skim milk agar media containing casein, a substrate of protease.

Anti-thrombosis test. Cultivation of bacteria in Eppendorf tubes was carried out to obtain crude enzymes in the SMB media. The crude enzyme obtained was tested for its activity in lysis of blood clots (thrombolysis or anti-thrombosis test using the gravimetric method) (Prasad et al 2006). The crude enzyme from the proteolytic selected bacteria was tested for antithrombotic activity to determine whether all proteolytic bacteria obtained in this study had antithrombotic ability, regardless of whether it is present through the fibrinolytic mechanism or not. The results are displayed in Table 3. The highest activity was in HSFT-2, HSFT-5, HSFT-10, HSFT-11, HSFT-12, and HSFT-14 bacterial isolates. Crude enzymes of these isolates were then subjected to anticoagulation tests.

Table 3

Antithrombotic activity of fibrinolytic bacterial isolates

No	*Isolate code*	*Percentage of clot lysis*
1	HSFT-2	54.97
2	HSFT-5	47.92
3	HSFT-10	51.00
4	HSFT-11	52.66
5	HSFT-12	55.68
6	HSFT-14	55.46
7	Aquadest (negative control)	0.24
8	Nattokinase (positive control)	73.38

Figure 3 presents the results of the clot-lysis activity tests on all fibrinolytic bacterial isolates obtained in previous step.

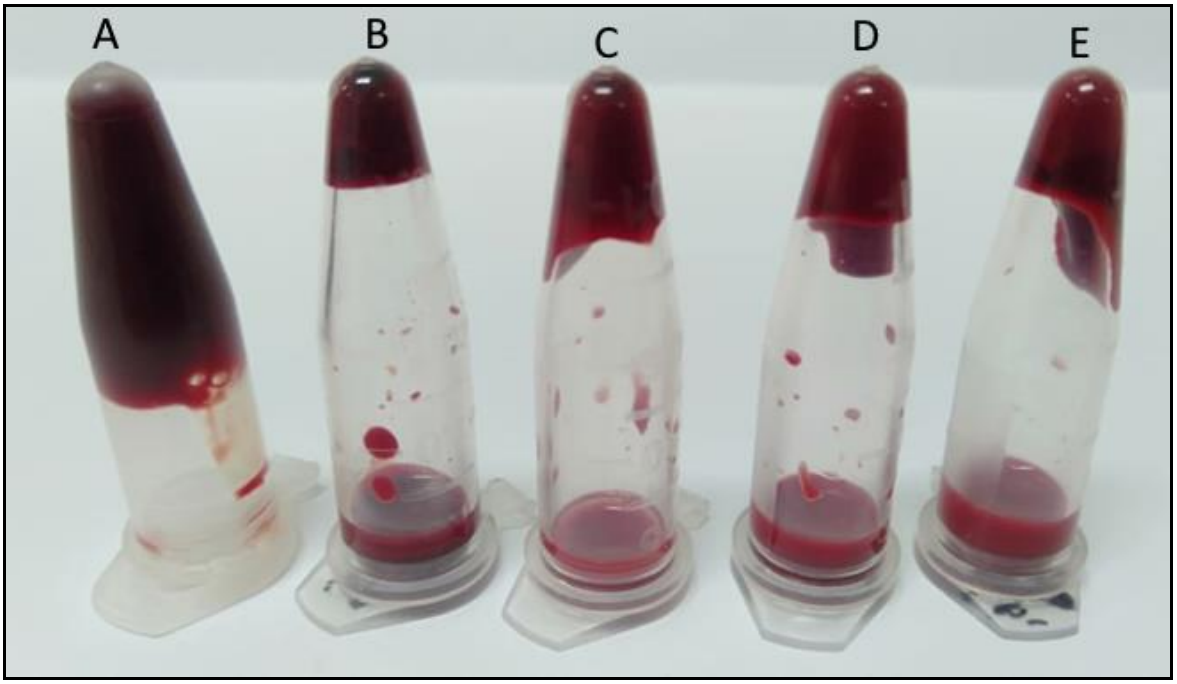

Figure 3. Blood clot-lysis test results using bacterial crude enzyme; A - blood clot without addition of enzymes (control); B - blood clot with addition of 100 µL nattokinase; C - blood clot with addition of 100 µL crude 100% enzyme; D - blood clot with addition of 200 µL crude 100% enzyme; E - blood clot with addition of 300 µL 100% crude enzyme.

Anticoagulant test. The results of the crude enzyme anticoagulant activity test from 6 bacterial isolates using the Lee-White method and blood smears, obtained 3 isolates that have the potential to be anticoagulants, i.e. isolates code HSFT-2, HSFT-5, and HSFT-11. In the HSFT-2, HSFT-5, and HSFT-11 tubes, the blood did not coagulate (Figure 4).

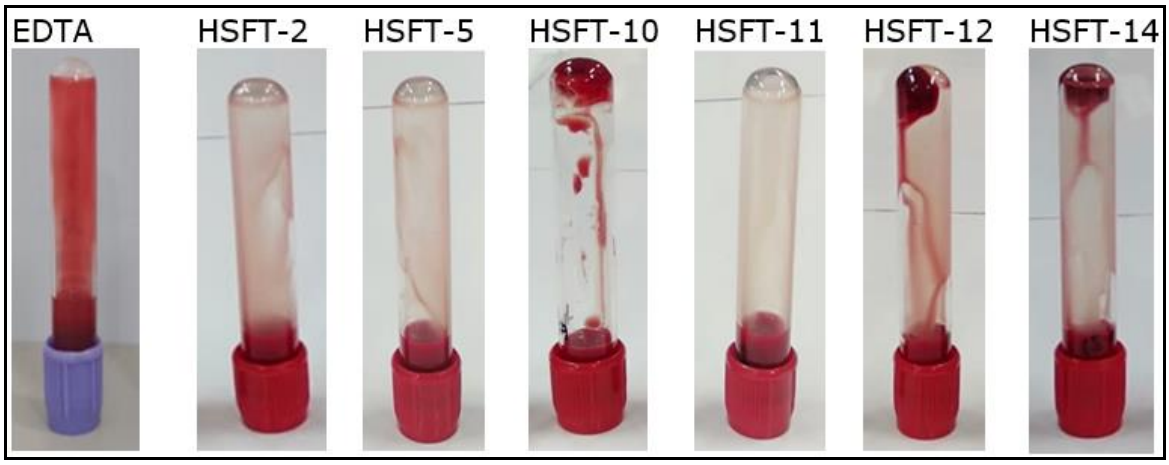

Figure 4. Anticoagulant test results using the Lee-White method. Anticoagulant activity is proved by the absence of any blood clot in test tubes after crude enzyme treatment (HSFT-2, HSFT-5, and HSFT-11 tubes).

Blood clotting was observed microscopically to see the state of the blood cells. In the EDTA, HSFT-2, HSFT-5 and HSFT-11 smear preparations, the erythrocytes looked good, the erythrocytes were not crenated, there was no platelet aggregation. This inferred that HSFT-2, HSFT-5 and HSFT-11 showed antiplatelet activities. On the other hand, the HSFT-10, HSFT-12 and HSFT-14 preparations were poor, the erythrocytes were crenated, there was platelet aggregation and visible blood clotting (Figure 5).

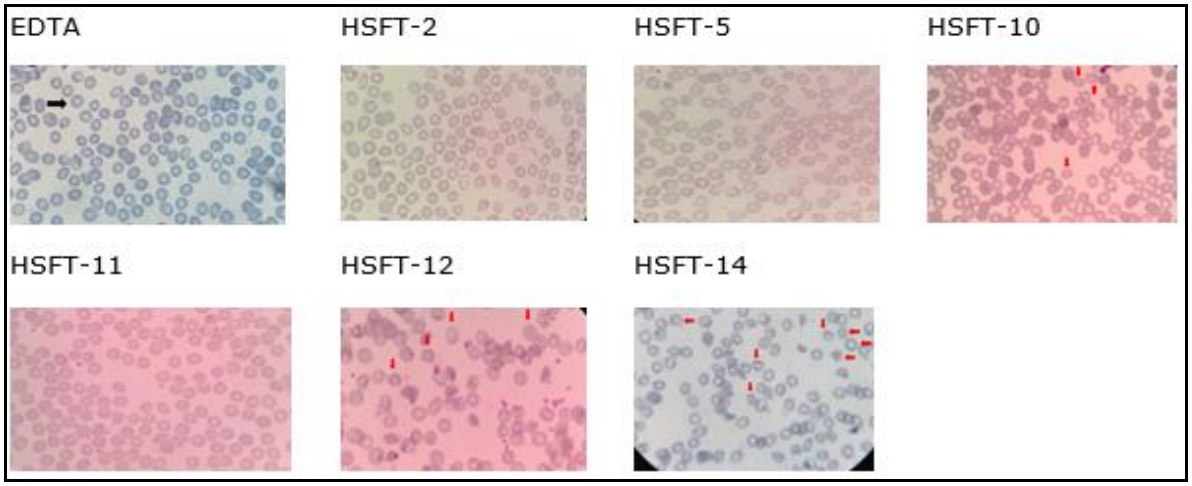

Figure 5. The results of the anticoagulant test using the blood smear method. Red arrows show crenated (negative result), while black arrow shows non-crenated cell (positive result).

Fibrinolytic test. 3 bacterial isolates with antithrombotic and anticoagulant activities were tested for their fibrinolytic abilities (Figure 6). Their fibrinolytic indexes are displayed in Table 4. The tests were carried out on selective media containing 3% w/v fibrin. Fibrinolytic activity was determined by the ability of the bacterial isolate to hydrolyze the fibrin substrate (thrombus binding component) characterized by the formation of a clear zone around the bacterial wall (Figure 6).

Milner & Makise (2002) stated that the formation of a clear zone, larger and clearer, shows that more fibrin is hydrolyzed by fibrinolytic enzymes. This is due to the capacity of extracellular enzymes from bacteria to lysis the thrombus (Anh et al 2015).

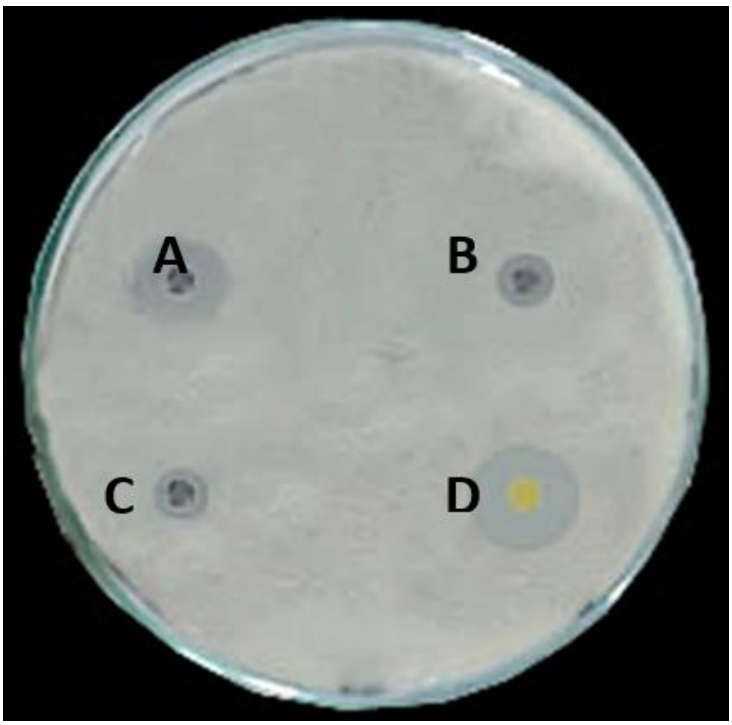

Figure 6. Fibrinolytic activity test on fibrin media; A - clear zone from HSFT-2 isolate; B - clear zone from HSFT-5 isolate; C - clear zone from HSFT-11 isolate; D - clear zone from the nattokinase control.

Table 4

Fibrinolytic index of 3 fibrinolytic bacterial isolates

Sample no	*Isolate code*	*Clear zone of on fibrin plate (mm) after incubation period of 1 and 5 days*		*Fibrinolytic index*
		1 day	*5 day*	
2	HSFT-2	5	9	3.6
5	HSFT-5	-	7	2.8
9	HSFT-11	-	5	2.0
15	Nattokinase	7	15	6.0

Note: (–) - not available.

Nattokinase was used as a positive control in fibrin medium and blood clot lysis tests. According to Jang et al (2013), nattokinase increases blood flow by inhibiting platelet aggregation and thrombus formation through the action of fibrin degradation. The proteolytic activity of bacterial isolates also has different activities against fibrin. The comparison of proteolytic activity index (Table 2) and fibrinolytic index (Table 4) showed that the HSFT-2, HSFT-5 and HSFT-11 bacterial isolates had a high proteolytic and fibrinolytic index. The fibrinolytic index of 2 isolates (HSFT-2 and HSFT-5) was higher than the proteolytic index of the SMA media. These results indicate that the fibrinolytic enzymes produced by the two bacterial isolates have specific inducible properties on fibrin substrates.

Lehninger et al (2021) stated that under normal conditions the amount of enzymes produced is small, but will increase rapidly if the substrate is present in the media, especially if the substrate is the only source of carbon for the cell. HSFT-2, HSFT-5, HSFT-11 bacterial isolates are different from the rest of the isolates obtained in this work, which have relatively high proteolytic indices, but did not show fibrinolytic activity. Proteolytic bacteria that have no fibrinolytic activities show that they are likely able to degrade proteins other than fibrin.

Amplification of 16S rRNA gene. The PCR amplification results were visualized by electrophoresis using 2% agarose gel and visualized with UV light to produce a single band with a size of about 1500 bp according to the value indicated by the DNA marker (Figure 7). This size is in accordance with the expected size of the bacterial 16S rRNA genes, which is 1500 bp. The sequencing process was carried out after the amplification results obtained to see the bacterial species (Ethica et al 2018b).

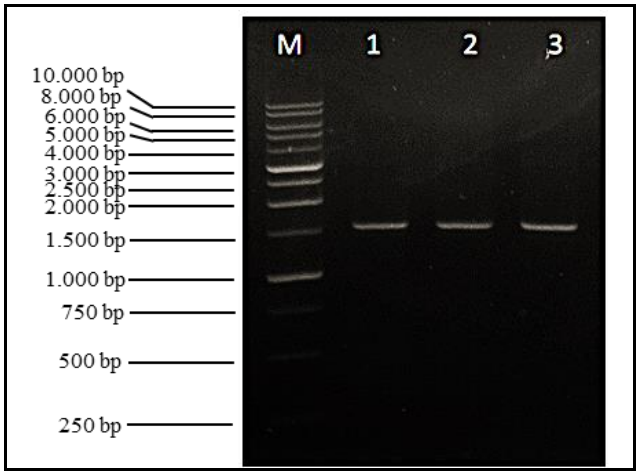

Figure 7. Electrophoresis visualization result of 16S rRNA PCR gene products with UV transluminator. M - marker; 1 - HSFT-2 bacterial isolate; 2 - HSFT-5 bacterial isolate; 3 - HSFT-3 bacterial isolate.

Figure 7 shows that the HSFT-2, HSFT-5 and HSFT-11 isolates produced a single band with a size of about 1500 bp according to the value indicated on the DNA marker.

Bioinformatics analysis. The results of BLAST analysis of the fibrinolytic bacteria HSFT-2, HSFT-5 and HSFT-11 were obtained. The sequence of the 16S rRNA gene fragment of the HSFT-2 isolate showed a homology level of 99.93% with that of the *Staphylococcus hominis* isolate. The sequence of the 16S rRNA gene fragment of the HSFT-5 isolate showed a 99.93% homology level with that of the *Bacillus aryabhattai* bacterial isolate. The sequence of 16S rRNA gene fragments of the HSFT-11 isolate showed a 100% homology level with that of the *Staphylococcus saprophyticus* isolate (Table 6).

Table 6

BLAST results of 16S rRNA DNA sequences of 3 fibrinolytic bacterial isolates

Strain	*Amplicon size (bp)*	*Species*	*Similarity level (%)*	*Closest related strains Acc. no.*
HSFT-2	1396	*Staphylococcus hominis*	99.93	MK743937.1
HSFT-5	1395	*Bacillus aryabhattai*	99.93	MK743937.1
HSFT-11	1401	*Staphylococcus saprophyticus*	100	

Based on molecular identification results obtained in this study, the 3 bacterial isolates producing crude proteases with *in vitro* thrombolysis, anticoagulant and fibrinolytic activities were *Staphylococcus hominis* HSFT-2, *B. aryabhattai* HSFT-5 and *S. saprophyticus* HSFT-11.

S. hominis was first discovered by Kloos & Schleifer (1975), when it was isolated from skin samples from people living in New Jersey in 1975. Mohanasrinivasan et al (2015) reported that *S. hominis* can produce staphylokinase. *S. saprophyticus* was first discovered by Kloos & Schleifer (1975), who isolated it from skin samples from people living in Raleigh in 1975. Staphylokinase is one of the potent clot-destroying fibrinolytic enzymes (Lack 1948; Gerheim 1948; Banerjee et al 2003; Chandrappa et al 2017). In addition, staphylokinase is relatively inexpensive when compared to other thrombolytic agents such as streptokinase and tissue plasminogen (t-PA). Even though streptokinase is inexpensive, it is immunogenic and relatively inefficient for early coronary artery recanalization (Srinivasan et al 2013). Staphylokinase also has a role in anti-clotting. The staphylococcal extracellular enzyme is considered to be a thrombolytic agent, which helps in dissolving plasminogen into inactive plasmin proenzymes (Collen & Lijnen 1996; Bokarewa et al 2006). Thereby, it acts as clot breaker. Staphylokinase also helps lyse clots through its proteolytic action on fibrin, the main constituent of thrombus (Pulicherla et al 2011). Compared to chemical agents such as EDTA or heparin, staphylokinases can be used as anticoagulants that are more economical and safer (Srinivasan et al 2013).

B. aryabhattai was first reported by Shivaji et al (2009), being isolated and identified from cryotubes used to collect air samples at an altitude of 27-41 km in India in 2009. The bacterium has the ability to produce useful extracellular enzymes such as α-amylase, cellulase, β-glucosidase, lipase, laccase, pectinase and protease (Shivaji et al 2009; Paz et al 2016).

The phylogenetic tree of the 3 obtained bacteria is presented in Figure 8. Figure 8 shows the relationship between one bacterium and another. From the phylogenetic tree (Figure 8), HSFT-2 and HSFT-11 isolates are in the same phylogenic branch, one node (genus), but different clade (species). Meanwhile, HSFT-5 and HSFT-2 and HSFT-11 are not in the same phylogenetic branch, one clade (species), or one node (genus). This indicates that the HSFT-2 and HSFT-11 isolates are similar. The HSFT-2 and HSFT-11 isolates did not have similarities with HSFT-5 in the sequence of nucleotide bases or phylogenic proximity. The 3 isolates had the same root (ancestor), but underwent different changes when they evolved.

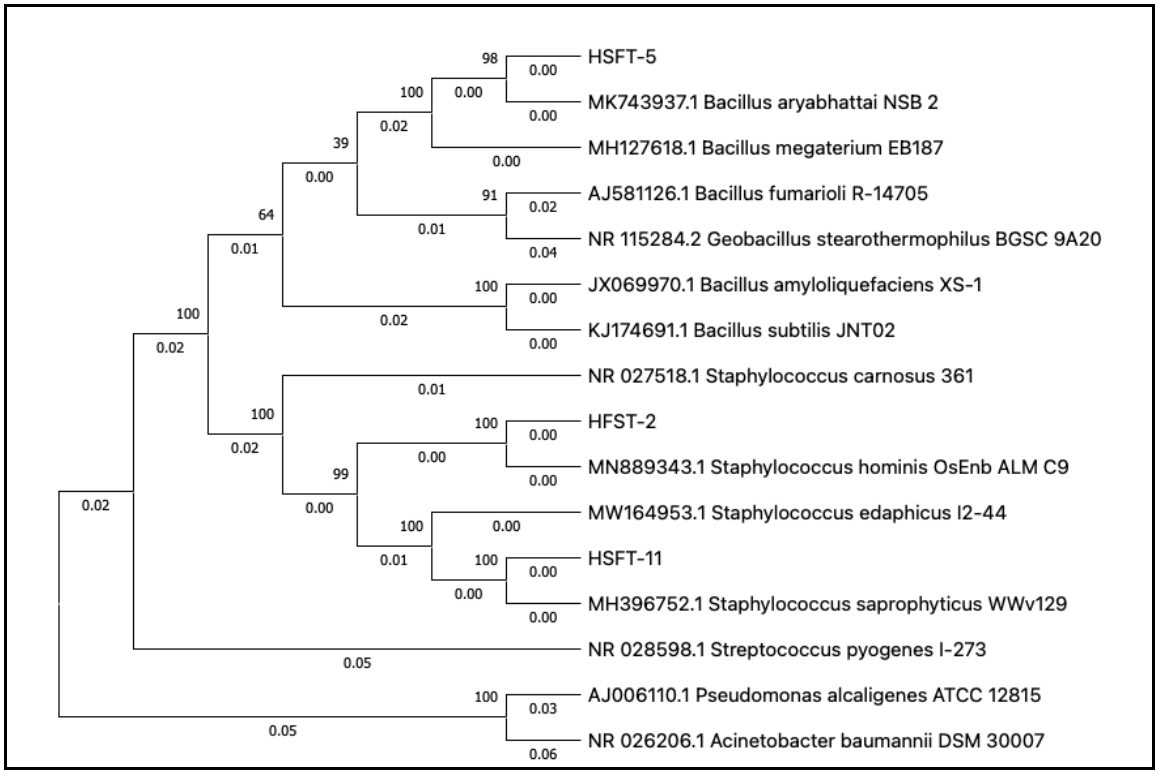

Figure 8. Phylogenetic tree of 3 fibrinolytic bacterial isolates obtained in this study (HSFT-2, HSFT-5, and HSFT-11 isolates) with bootstrap value of 500.

Based on all the results obtained in this study, efforts to obtain a new source of fibrinolytic protease enzymes from bacteria in *H. scabra* muscle tissue from the marine waters of Mataram Indonesia showed positive results. Three bacteria producing fribrinolytic protease enzymes with early indications of their antithrombotic ability were obtained, namely *S. hominis* HSFT-2, *B. aryabhattai* HSFT-5 and *S. saprophyticus* HSFT-11. This result is in line with that reported by Lopetcharat & Park (2002), where proteolytic bacteria *Staphylococci* with strong activity, along with *Pseudomonads* and *Enterobacteriaceae* were found in fermented Pacific whiting (*Merluccius productus*). However, further research needs to be done to test whether the enzymes produced by the 3 isolates actually belong to the staphylokinase and subtilisin groups, which are widely reported to have anti-thrombotic abilities, with a lower risk of allergy than streptokinase. In addition, it is also interesting to test how the 3 enzymes perform in different pH or temperature conditions.

Conclusions. From fermented rusip of *H. scabra* tissue, 3 bacterial isolates could be obtained as potential antithrombotic agents, namely *Staphylococcus hominis* HSFT-2, *Basillus aryabhattai* HSFT-5 and *S. saprophyticus* HSFT-11. Crude proteases of these isolates demonstrated fibrinolytic, anticoagulant and antiplatelet activities.

Acknowledgements. This work was financially supported by Program Hibah Penelitian Pascasarjana (PPS) Magister Tesis 2020 from Kemenristek/BRIN. The authors cordially thank Balai Bio Industri Laut LIPI (Lembaga Ilmu Pengetahuan Indonesia) Mataram for providing sand sea cucumber samples and facilitating the bacterial screening work.

Ethical Clearance Statement. The study was approved by the Research Ethics Committee of the Public Health Faculty of Universitas Muhammadiyah Semarang (Document number: 377/KEPK-FKM/UNIMUS/2020).

Conflict of Interest. The authors declare that there is no conflict if interest.

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Received: 05 December 2020. Accepted: 25 February 2021. Published online: 11 May 2021.
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How to cite this article:
Fuad H., Hidayati N., Darmawati S., Munandar H., Sulistyaningtyas A. R., Ernanto A. R., Muchlissin S. I., Zilda D. S., Nurrahman N., Ethica S. N., 2021 Exploration of bacteria isolated from "rusip" fermented tissue of sand sea cucumber *Holothuria scabra* with fibrinolytic, anticoagulant and antiplatelet activities. AACL Bioflux 14(3):1242-1257.