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***Bacillus tequilensis* Isolated from Fermented Intestine of *Holothuria Scabra* Produces Fibrinolytic Protease with Thrombolysis Activity**

Nur Hidayati¹, Nurrahman Nurrahman², Hayatun Fuad¹, Hendra Munandar³, Dewi Seswita Zilda⁴, Aditya Rahman Ernanto⁵, Amin Samiasih⁵, Oedjijono Oedjijono⁶, Stalis Norma Ethica^{1*}

¹Magister Study Program of Medical Laboratory Science, Universitas Muhammadiyah Semarang, 50273, Indonesia

²Food Technology Study Program, Faculty of Nursing and Health Sciences, Universitas Muhammadiyah Semarang, 50273 Indonesia

³Balai Bio Industri Laut - LIPI (Lembaga Ilmu Penelitian Indonesia) Lombok, Nusa Tenggara Barat, 83352, Indonesia

⁴Research and Development Center for Marine and Fishery Product Processing and Biotechnology, Ministry of Maritime Affairs and Fisheries of Indonesia, Jakarta, 10260, Indonesia

⁵Faculty of Nursing and Health Sciences, Universitas Muhammadiyah Semarang, 50273 Indonesia

⁶Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, 53122, Indonesia

*Corresponding author: norma@unimus.ac.id

Abstract. Among essential treatment of cardiovascular disorders are fibrinolytic proteases. Most thrombolysis agents are fibrinolytic enzymes from group of bacterial proteases. This work reports a potent bacterium isolated from fermented intestine of *H. scabra*, which could produce fibrinolytic protease with high thrombolysis activity. Bacterial selection was conducted based on proteolytic and fibrinolytic activities indicated as clear zone on skim milk and fibrin agar media, respectively. Crude proteases from the selected bacterial isolates were subjected to thrombolytic activity test based on gravimetric method, which results were confirmed after 7 repetitions. As result, 4 fibrinolytic protease-producing bacterial isolates HFSI-3, HFSI-4, HFSI-5 and HFSI-8 were obtained. Among them, HFSI-5 isolate identified as *Bacillus tequilensis* on the basis of the 16S rRNA gene sequencing and morphological properties produced crude protease with the highest thrombolytic activity. The thrombolytic activity of crude protease produced by *B. tequilensis* HFSI-5 is worthy of comparing to that of standard fibrinolytic enzyme Nattokinase showing its potential as thrombolysis agent.

1. Introduction

Cardiovascular diseases (CVDs) have been known as one of the main causes of deaths, which account for 31% of mortality all over the world [1]. It has been predicted by The American Heart Association that by 2030, mortality by CVDs shall go over 23.6 million [2]. The imbalance fibrin formation and fibrinolysis in the body is the underlining problem in CVDs [3]. Such imbalance leads to thrombosis or intravascular clotting in blood vessels [4-5]. Thrombus limits the circulation of blood by blocking it



from passing through veins and arteries. This medical condition could lead to cardiac abnormalities such as heart stroke, myocardial infarction, embolism, etc. [6-9]. For both the prevention and treatment of thrombosis, thrombolytic agents play important role [8]. In fact, thrombolysis is the only therapy that can re-establish blood flow quickly and easily [7].

Among essential treatment of cardiovascular disorders functioning as thrombolysis agent are fibrinolytic enzymes, mostly from group of proteases of bacteria [4-6]. Since ancient times microorganism has contributed to the use and production the fibrinolytic enzymes. In the last decades, researchers have intensively paid reported production of fibrinolytic enzymes by bacteria, particularly those from the food sources. A number of fibrinolytic enzymes have been reported to have anti-thrombosis activities, particularly from the genus *Bacillus* [10-13].

Holothuria scabra is among sand sea cucumbers commonly found in Indonesia and has been favourite sea invertebrate captured by local fishermen. The holothurian had been reported to have high protein content giving it a possibility to be a rich substrate for protease producing bacteria [14]. Some of bacterial proteases, particularly those with serine active site have been reported to be able to degrade fibrins. Degradation of fibrins by these fibrinolytic proteases is among the key step of thrombolysis. This makes serine proteases from bacteria are potential to be used as thrombolysis agent.

To date, the applications of fibrinolytic enzymes to manage heart diseases are still costly. Therefore, the search for new sources of fibrinolytic proteases is essential allowing the use of large quantities of the enzymes as the could be produced conveniently and efficiently [11]. This work aimed to report identification of bacterium isolated from fermented intestine of *H. scabra* capable of producing fibrinolytic protease with thrombolysis activity.

1.1. Material

Samples used in this study were 12 bacterial isolates from sea cucumber (*H. scabra*) intestine obtained from its captivity located at Lembaga Ilmu Pengetahuan Indonesia Institute for Bio Industry (BBIL LIPI), Kodek Gulf Village, Lombok, West Nusa Tenggara. Culture media included Nutrient Agar (NA) and Skim Milk Agar (SMA) (both from Oxoid, UK), fresh blood from volunteers, fibrin agar media (freshly prepared from the fresh blood), (Oxoid, UK), and Gram-staining reagent. Identification of bacterium required optical microscope, Presto™ gDNA Bacteria Kit (Geneaid, Taiwan). The equipment used were analytical balance (Mettler Toledo), autoclave (Hirayama HVE-50, Japan), Laminar Cabinet (Dalton, Japan), spectrophotometer Maestro Nano-Pro (Maestro-Gen, Taiwan), Micro-centrifuge (Bechman Counter, USA), incubator (Mammert, Germany), micropipette (Socorex Calibra 822, French), Vortex (Genie 2TM 12-82, USA), calliper, microtubes, and pipette tips.

2. Method

2.1. Isolation and Selection of Proteolytic Bacteria

According to previous work, after colony selection and purification on NA media, 12 bacterial isolates were obtained on bacteria isolated from fermented intestine of *H. scabra*. After sub-culturing and colony purification, Gram staining was performed on bacterial cells and the result was observed under optic microscope. Next, each of these bacterial isolates was tested for proteolytic activity on SMA and the presence of clear (proteolytic) zone was observed within a-week of cultivation [15]. Bacterial isolates which show growing proteolytic zones within 7 days were then screened for fibrinolytic activity using fibrin media [16].

2.2. Selection of Fibrinolytic Protease Producing Bacteria on Fibrin Media

Fibrinolytic activity test on proteolytic bacterial isolates was performed on fibrin agar medium. Fibrin solid medium was prepared from fresh human blood plasma following previously reported method by Zhang et al. (2012) [17]. Well diffusion method was applied on solid fibrin medium to cultivate bacterial colonies. A total of 50 µL of rejuvenated proteolytic bacterial inoculum for each sample and another 50 µL Nattokinase for control was inserted into each of well. The culture was then incubated at 37°C at pH =7 for 5 days. Every day within 5 days the plates were observed for the presence of a clear zone around the well as indication of fibrinolytic activity [18-19].

2.3. Clot Lysis Activities of Bacterial Crude Proteases

For the gravimetric thrombolysis test, the empty micro-centrifuge tube was weighed and each weight recorded. Blood was taken from healthy volunteers as much as 500 μ l and put in a micro centrifugation tube and incubated at 37 °C for 45 min. After clotting, serum was taken so as not to interfere with the blood clot. Weight of the clot was determined as follows: weight of clot = weight of tube with clot - weight of empty tube [20].

2.4. Bacterial DNA Isolation

The DNA isolation process was carried out according to the steps instructed by the DNA kit manufacturer. Sample preparation for Gram-positive was started by dilution bacterial sample 1×10^{-9} then following kit procedures including extraction, lysis, DNA binding to the column filter, washing and elution. Measurement of DNA concentration and purity was done using spectrophotometer Maestro Nano-Pro.

2.5. Amplification 16S rRNA and Gel Agarose Electrophoresis

The amplification process used Go Tag Green Master Mix (Promega). Primers used are universal primers 27-F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492-R (5'- GGT TAC CTT GTT ACG ACT T-3'). The PCR steps were initial denaturation at 95 °C for 4 minutes, then followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 35 s, extension at 72 °C for 45 s. Electrophoresis used 2% agarose in 100 ml TAE and used Cybersafe as DNA dye. Vivantis 1 Kbp marker was used to check the amplicon size [21].

2.6. DNA Sequencing

The 16S rRNA DNA fragment of bacterium obtained as PCR product or amplicon was then sequenced. The DNA sequencing was based on Sanger method [22] was carried out by PT. Genetika Science Indonesia.

2.7. Bioinformatics Analysis

The results of 16S rRNA DNA sequencing were analysed using bioinformatics tools. Assembly consensus sequence was carried out on forward and reverse sequences of the 16S rRNA gene with the DNABaser Assembler Program v3x [23]. The consensus was then processed and matched with data at www.ncbi.nih.gov through the BLAST (Basic Local Alignment, Search Tool - for nucleotide) program to determine the species of isolate from the Genbank database [24-26].

3. Results and Discussion

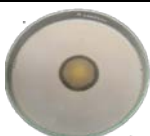



















This study aimed to obtain a potent bacterium isolated from fermented intestine of *H. scabra*, which could produce fibrinolytic protease with high thrombolysis activity and to identify it using PCR method. Several tests were conducted to analyze the capacity of bacterial isolates to produce protease after 7-day incubation and ability of the isolates to degrade fibrin and blood clot as model of thrombus. The selection of proteolytic bacteria isolates was carried out based on ability to produce protease using Skim Milk Agar (SMA) media. SMA media contains casein, a protease substrate. Activity of bacterial protease may break the peptide bonds of casein in skim milk [15]. Formation of a clear zone indicates the occurrence of casein hydrolysis by bacterial protease.

Table 1 showed the capacity of bacterial isolates to produce protein on SMA media observed on the 1st and 7th day of incubation. As seen on Table 1, proteolytic zones of the colonies of Isolate HSFI-2 to -6 and HSFI-8 to -12 (HSFI refers to *Holothuria scabra* Fermented Intestine) were all greater after 7-day incubation.

Expanded clear zone on SMA medium confirmed capacity of these isolates to produce proteases. Such positive results allow the next selection process to determine which proteolytic bacteria produces fibrinolytic proteases. Fibrinolytic test was then conducted on proteolytic isolates, and the results are displayed by Figure 1. Nattokinase was used as standard positive control. Nattokinase is well established as promising enzyme produced by *Bacillus natto* in fermented soybean in the Japanese diet

providing them with the lowest rate of thrombosis disorders all over the world. Each fibrinolytic enzyme usually has a special structure and mechanism of action [27].

Table 1. Proteolytic zone of bacteria isolates from fermented intestine of *H. scabra* after 7-day incubation on SMA media.

| Isolate code | 1-Day Proteolytic (clear) zone | 7-Day Proteolytic (clear) zone | Isolate code | 1-Day Proteolytic (clear) zone | 7-Day Proteolytic (clear) zone |
|--------------|---|---|--------------|---|---|
| HSFI-2 |  |  | HFSI-8 |  |  |
| HSFI-3 |  |  | HFSI-9 |  |  |
| HSFI-4 |  |  | HFSI-10 |  |  |
| HSFI-5 |  |  | HFSI-11 |  |  |
| HSFI-6 |  |  | HFSI-12 |  |  |

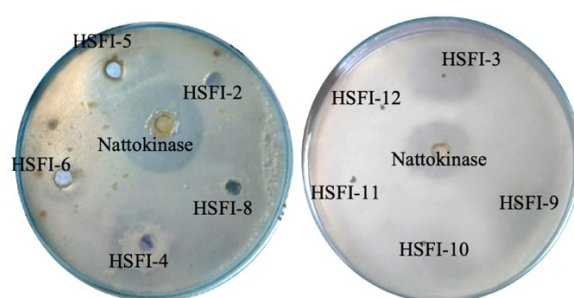


Figure 1. Clear zones indicating positive fibrinolytic test results of isolate HSFI-3, HSFI-4, HSFI-5 and HSFI-6 on fibrin agar media using Nattokinase as positive control

Fibrinolytic activity was determined by the ability of the isolate's enzyme to hydrolyze the fibrin substrate, which is indicated by the formation of a clear zone around the bacterial colony. The greater the fibrinolytic activity, the larger the clear zone formed, indicating the more fibrin is hydrolyzed by fibrinolytic enzymes [28]. Fibrinolytic ability test was performed on selective media containing 3% w/v fibrin. The fibrin used was obtained from healthy human plasma [29]. Results in **Figure 1** also showed that only 4 of 10 proteolytic bacterial isolates, HSFI-3, HSFI-4, HSFI-5 and HSFI-6, could degrade fibrin substrates indicated by their ability to form clear zone around the colony on fibrin agar

media. Based on this, only crude fibrinolytic proteases of the 4 isolates were subjected to thrombolysis test.

In this study, crude fibrinolytic protease from bacteria was tested for its thrombolysis activity. The crude enzyme was isolated by centrifuging the bacterial culture in broth media to obtain supernatant. The supernatant containing crude enzyme could be then tested for its activity in lysing blood clots *in vitro* by gravimetry method as previously reported [30]. Figure 2 and 3 showed centrifugation step and the obtained crude enzyme from the thrombolysis test, respectively, based on gravimetric method.



Figure 2. Centrifugation of bacterial culture in skim milk broth to obtain supernatant considered as crude protease



Figure 3. Crude protease from HSFI-3, -4, -5, and -6 isolates obtained after centrifugation

Results of thrombolytic activity screening test conducted on crude proteases of HSFI-3 to -6 against fresh blood clot using Gravimetric method were displayed in Table 2.

Table 2. Results of the thrombolysis activity test using the Gravimetric method using fresh blood clot

| Isolate Code | Average weight of initial blood clot (g)* | Average weight of final blood clot (g)* | Percentage (%) |
|--------------|---|---|----------------|
| HSFI-3 | 0.2326 | 0.2204 | 26.06 |
| HSFI-4 | 0.2388 | 0.1927 | 5.24 |
| HSFI-5 | 0.4128 | 0.1567 | 62.03 |
| HSFI-6 | 0.2486 | 0.1667 | 32.94 |
| Aquadest | 0.2668 | 0.2650 | 0.29 |
| Nattokinase | 0.2462 | 0.1082 | 56.05 |

Note: Measurement in duplo

Table 3. Thrombolysis activity test results using the Gravimetric method on crude fibrinolytic protease of HSFI-5 and Nattokinase as standard.

| Measurement | HSFI-5 crude enzyme | Nattokinase (standard) |
|-------------|---------------------|------------------------|
| 1 | 60.44 | 48.39 |
| 2 | 81.13 | 63.16 |
| 3 | 74.44 | 78.39 |
| 4 | 70.32 | 64.41 |
| 5 | 64.41 | 63.15 |
| 6 | 64.93 | 60.36 |
| 7 | 62.04 | 64.26 |
| Average | 68.24 | 63.16 |

Based on data of Table 2, crude fibrinolytic protease from HSFI-5 isolate showed the highest thrombolytic activity. To confirm this, the gravimetric measurement was carried out with 7 repetitions

in particular for crude fibrinolytic enzyme of HSFI-5 isolate and Nattokinase as standard of thrombolysis agent. The result is shown by Table 3.

The crude enzyme from bacteria previously selected based on proteolytic and fibrinolytic activities were tested for thrombolysis activity to determine whether they had the ability to lyse blood clots. *In vitro* thrombolysis test used Gravimetric method, the weight of clots before lysis and after lysis is considered the right determinant for calculating the percentage of clot lysis [20]. As seen on **Table 3**, the gravimetric test results showed that in average crude protease of isolate HFSI-5 could lyse 68.24% clot lysis isolate HSFI-5. As comparison, commercial fibrinolytic enzyme Nattokinase in average could lyse clot in slightly lower percentage by 63.16%. This shows that crude protease from HFSI-5 has potential to be used as a competitive thrombolysis agent. It is therefore important to identify the species of HFSI-5 isolate to determine optimum condition for larger production scale of the bacterial crude protease.

Part of bacterial identification was determining bacterial morphologic characteristics. The morphological characteristics of colonies and cells of bacterial isolate HSFI-5 are described in Figure 2 and Table 4. Based on Figure 2 and Table 4, colonies and cells of HFSI-5 are uniform showing colony purity of the bacterium. Next molecular identification on HFSI-5 isolate could be conducted based on DNA sequence of 16S rRNA gene of the bacterium [31].



Figure 4. Colony morphology of HSFI-5 isolate



Figure 5. Cell morphology of isolate HSFI-5

Table 4. Colony characteristics (A) and Gram staining and cell characteristics (B) of isolate HSFI-5

| Isolate Code | Colony Characteristics | | | | |
|--------------|------------------------|----------------------|--------------|------------------|--------------------|
| | <i>Form</i> | <i>Margin</i> | <i>Color</i> | <i>Elevation</i> | <i>Consistency</i> |
| HSFI-5 | filamentous | filiform | white | crateriform | rough |
| | Cell Characteristics | | | | |
| | <i>Shape</i> | <i>Gram staining</i> | | | |
| | Bacilli | Gram-Positive | | | |

Molecular identification of bacterial isolate HFSI-5 was based on sequence 16S rRNA gene and compared to the database in Genbank [32]. PCR product using bacterial genomic as template was visualized as 16S rRNA DNA band sized about 1500 bp on electrophoresis agarose gel (Figure 6).

The results of BLAST analysis of the HSFI-5 isolate based on the sequence of 16S rRNA gene fragment showed a homology level of 100% with that of *Bacillus tequilensis* JLS-12 (Figure 7). Based on the result, HFSI- bacterial isolate was named *Bacillus tequilensis* HSFI-5. This molecular identification result was confirmed by morphology of colonies and cells of HSFI-5 isolate, which resemble those of *B. tequilensis* previously reported by Li et al. in 2018 [33].

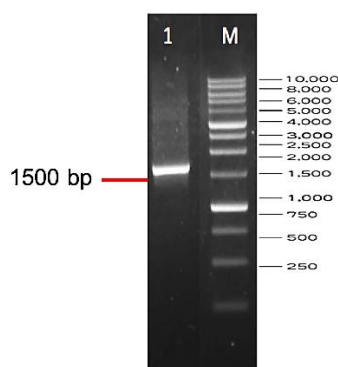


Figure 6. Visualization DNA fragment of 16S rRNA from PCR products

| | Description | Max Score | Total Score | Query Cover | E value | Per. Ident | Accession |
|---|---|-----------|-------------|-------------|---------|------------|----------------------------|
| ✓ | Bacillus tequilensis strain JLS12 16S ribosomal RNA gene, partial sequence | 2535 | 2535 | 100% | 0.0 | 100.00% | MT501810.1 |
| ✓ | Bacillus subtilis subsp. subtilis str. 168 chromosome, complete genome | 2535 | 25264 | 100% | 0.0 | 100.00% | CP053102.1 |
| ✓ | Bacillus subtilis subsp. subtilis str. 168 chromosome, complete genome | 2535 | 25264 | 100% | 0.0 | 100.00% | CP052842.1 |
| ✓ | Bacillus subtilis subsp. subtilis str. 168 chromosome, complete genome | 2535 | 25264 | 100% | 0.0 | 100.00% | CP051860.1 |
| ✓ | Bacillus subtilis subsp. subtilis strain UCMB5121 chromosome, complete genome | 2535 | 25273 | 100% | 0.0 | 100.00% | CP051465.1 |
| ✓ | Bacillus subtilis subsp. subtilis str. SMY chromosome, complete genome | 2535 | 25256 | 100% | 0.0 | 100.00% | CP050532.1 |
| ✓ | Bacillus subtilis strain NFAA 16S ribosomal RNA gene, partial sequence | 2535 | 2535 | 100% | 0.0 | 100.00% | MT192659.1 |
| ✓ | Bacillus subtilis strain H1 chromosome, complete genome | 2535 | 25209 | 100% | 0.0 | 100.00% | CP026662.1 |
| ✓ | Bacillus subtilis strain LSRBMoFPIKRGCFTRI33 16S ribosomal RNA gene, partial sequence | 2535 | 2535 | 100% | 0.0 | 100.00% | MT133340.1 |
| ✓ | Bacillus subtilis strain LSRBMoFPIKRGCFTRI32 16S ribosomal RNA gene, partial sequence | 2535 | 2535 | 100% | 0.0 | 100.00% | MT133339.1 |

Figure 7. Result of 16S rRNA gene fragment sequence alignment from BLAST analysis tool [24]

Bacillus tequilensis was first reported by Gatson *et al.* 2006 from a two-thousand-year-old Mexican shaft tomb near the city of Tequila [34]. *B. tequilensis*, first identified and described in 2006, has the ability to degrade ammonia nitrogen (NH₃-N) in its cells [35]. *B. tequilensis*, Gram-positive spore forming bacteria was reported as a new species [34]. *B. tequilensis* can grow in anaerobic, motile, positive oxidase and catalase tests, has the ability to degrade tryptophan and starch but does not degrade urea, and can utilize citrate as a carbon source, physiologically, bacteria are able to withstand salt concentrations as high as 8% [35].

B. tequilensis isolated from marine sediment samples could produce surfactin, while *B. tequilensis* from the intestinal content of earthworms that have high collagenase activity [36-37]. The bacterium has also previously been reported to have the ability as a starch hydrolyzer [38]. Based on the 16S rRNA gene sequence, there is a 99% similarity between *B. tequilensis* and *B. subtilis* [34;36]. This explains BLAST results shown in Figure 7 displaying very high similarity (100%) shared between *B. tequilensis* *B. subtilis*.

Based on literature study, *B. tequilensis* with the ability to produce fibrinolytic protease enzymes capable of lysing blood clots (thrombus) has not been reported. Thus, this study is the first to report potential application of *B. tequilensis* in the health sector, particularly in relation to its ability to lyse thrombus competing with a commercial thrombolysis agent, Nattokinase.

4. Conclusion

Bacillus tequilensis HFSI-5, a potent fibrinolytic protease-producing bacterium from fermented intestine of *H. scabra*. The thrombolytic activity of the bacterium's fibrinolytic protease is worthy of comparing to that of standard fibrinolytic enzyme Nattokinase.

5. Statement

This study was approved by the Ethics Committee of Komisi Etik Penelitian Kesehatan (KEPK) of Faculty of Public Health, Universitas Muhammadiyah Semarang, Indonesia (approval no. 377/2020)

6. Acknowledgments

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