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Fibrinolytic Protease-Producing Bacteria with Varied Hemolysis Pattern Associated with Marine Algae *Dictyota* sp.

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Abstract: The main death factor of cardiovascular disease (CVD) is the formation of a blood clot (thrombus). Thrombus is formed by the action of fibrin, playing a role as a blood coagulation agent. Administration of fibrinolytic enzymes can degrade fibrin through the fibrinolysis process. Therefore, searching for new sources of fibrinolytic enzymes becomes critical in eradicating diseases by fibrinolysis of thrombus. This study aims to isolate fibrinolytic protease-producing bacteria associated with fermented brown algae products *Dictyota* sp, of Awur Bay, Jepara, Indonesia, and to observe their hemolysis pattern. As many as 14 unique bacterial colonies previously isolated from fermented *Dictyota* sp. were sub-cultured using Zobell Agar (ZA) medium. Skim Milk Agar (SMA) and Fibrin Agar (FA) were then used as selective media to detect the presence of fibrinolytic protease-producing bacteria, which was indicated by their ability to form a clear proteolytic and fibrinolytic zone simultaneously around bacterial colonies. Hemolysis characteristics of fibrinolytic bacteria were determined using Blood Agar Plate (BAP) to test their ability to produce hemolysin toxin. As a result, of these 14 isolates, 3 of them, namely FD-09, FD-13, and FD-14 (FD= Fermented Dictyota), could produce both proteolytic and fibrinolytic zone with a fibrinolytic index range of 2.0–2.9. Isolate FD-09 is the least pathogenic (γ-hemolytic) compared to other fibrinolytic isolates, FD-13 (β-hemolytic) and FD-14 (α-hemolytic), in terms of hemolysin toxicity. In conclusion, fermented *Dictyota* sp. is a potential source of bacteria-producing fibrin-degrading protease with varied hemolysis patterns. It is necessary to identify bacteria-producing fibrinolytic protease isolates Dictyota sp. and further characterization regarding the specificity and activity of the resulting protease to develop its potential as an antithrombotic agent.
Keywords: Antithrombosis; cardiovascular disease; *Dictyota* sp.; fibrinolytic enzyme.

INTRODUCTION

Cardiovascular disease (CVD) is one of the deadliest degenerative diseases in the world. The World Health Organization (WHO) has predicted that from 2016 to 2025, there will be an increase in the mortality rate of up to 35% due to CVD (World Health Organization, 2018). In 2014 the death rate reached 1.8 million cases due to CVD in the Southeast Asian region. According to the Ministry of Health data in 2014, the death rate in Indonesia due to CVD is fairly high, namely 1.25 million people (Ferdiani et al., 2023).

Most cases of death in CVD are caused by the formation of a blood clot (thrombus). It occurs due to the formation of blood clots from the release of thrombus from the blood vessels, which move freely in the blood circulation towards the body's

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organs (Kartal, 2014). The thrombus in blood vessels is due to instability in the hemostasis system involving coagulation and fibrinolytic factors. Currently, the therapy for thrombosis is in the form of anticoagulation and surgery, which poses a dangerous risk (Bi et al., 2013).

The current use of antithrombotic drugs is known to have various side effects, including allergies (Altaf et al., 2021). Thus, the search for sources of fibrinolytic enzymes should be carried out to obtain antithrombotic agents that are safe to use. Brown algae *Dictyota* sp. is a potential source of numerous bioactive and bio-functional components (Pradhan et al., 2020).

Marine biota such as algae has been extensively researched to find new and safe sources of antithrombotic agents. Algae are rich in functionality and bioactivity. Many new thrombolytic components have been reported by researchers globally (Safitri et al., 2018). In Indonesia, several potential sources of bacteria that produce new thrombolytic agents have been reported from marine organisms, as reported by Fuad et al., 2020 and Hidayati et al., 2021; the isolates of *Staphylococcus hominis*, *Bacillus aryabhattai*, *S. saprophyticus*, and *B. tequilensis* producing fibrinolytic proteases isolated from muscle tissue and digestion of sea cucumber (*Holothuria scabra*) obtained from the waters of Lombok, West Nusa Tenggara.

The use of fibrinolytic enzyme products produced from Dictyota sp. as an antithrombotic drug agent requires further research to determine whether the bacteria have the potential to become pathogens. This must be done while taking into consideration the potential for negative effects when applied to the human body in order to develop a safe antithrombotic drug (Hidayati et al., 2021). The ability of bacteria to produce hemolysin, or the ability of bacteria to lyse blood cells, is one of the characteristics of pathogenicity. As a result, isolates of bacteria that produce fibrinolytic proteases must be examined for hemolysis characteristics to identify prospective non-hemolytic isolates with lower pathogenicity risk. A blood agar plate medium can be used to test for hemolysis (Sabrina & Ethica, 2018).

Dictyota sp. is a family of *Dictyotaceae* and belongs to the seaweed genus. This species of algae is usually found in tropical. Furthermore, subtropical seas and has important value in the development of medicines in the world, given their promising potential. *Dictyota* was identified and classified for the first time by Lamouroux, 1809. The latest taxonomic data reported by Silberfeld et al., 2014 on *Dictyota* were: Eukaryota Empire, Chromista Kingdom, Ochrophyta Phylum, Phaeophyta Class, Dictyotales Order, Dictyotaceae Family, and Dictyota Genus.

Dictyota contains enough protein to serve as a substrate for bacteria that produce proteases or enzymes that degrade proteases. Proteases that degrade fibrin play a critical role in antithrombotic therapy (Fuad et al., 2020). Zobell Agar (ZA) media must be utilized in the process of extracting bacteria from *Dictyota* sp. because it is suited for the growth of marine algal symbiont bacteria since its composition is made to mimic marine ecosystems, allowing bacteria from *Dictyota* sp. to grow and develop optimally (Fuad et al., 2021). The selection process of protein-degrading bacterial isolates can be conducted on skim milk agar (SMA) medium, and then fibrin agar (FA) media must be chosen to determine fibrin-degrading bacteria (Hidayati et al., 2021).

Marine algae have biological components important for drug development in the pharmaceutical industry. Several studies that have been conducted have succeeded in isolating the secondary metabolite components of diterpenes in the brown algae *Dictyota* sp., which are known to have antithrombotic, antimicrobial, antiviral, and anti-inflammatory activities (Chen et al., 2018). The presence of symbiotic microorganisms in algae makes it a potential source of alternative raw

materials. Marine algae can be used in medicine, considering the potential of the bioactive components contained therein and their adequate functionality (Safitri et al., 2018).

Components of diterpenes, pachidictyol, and isopachidictyol in the Genus *Dictyota* have been investigated for their antithrombotic properties and showed an inhibitory activity of 50% against thrombin which is a pro-coagulation molecule causing the formation of blood clots (thrombus). The presence of this component in the *Dictyota* genus makes it a promising source of producing antithrombotic agents (Cristina et al., 2017). Previous studies reported antithrombotic proteases from marine bacteria isolated from the genus Laminaria of brown algae by Zhao et al., 2016. Exploration of genus *Dictyota* as the source of fibrinolytic protease-producing bacteria is barely reported. This study, *Dictyota* sp. was used as a source of bacteria-producing fibrinolytic enzymes as candidates for antithrombotic agents, referring to their secondary metabolite components, bioactivity, and potential bio-functionality to be developed. The research aims to isolate fibrinolytic protease-producing bacteria associated with fermented brown algae products *Dictyota* sp. of Awur Bay, Jepara, Indonesia and to observe their hemolysis pattern to determine the pathogenicity indication.

MATERIALS AND METHODS

Bacterial Samples

Fourteen bacterial isolates coded FD-1 to -14 (Figure 2) as samples in this study were sub-cultured from those stored in the Microbiology Laboratory of Universitas Muhammadiyah Semarang, Indonesia. The original isolates were previously isolated by Afriansyah et al., 2021 from the fermented product of brown algae *Dictyota* sp. (Figure 1) samples taken from Awur Bay, Jepara, Indonesia, in January 2020 (Afriansyah et al., 2021). The morphology characteristics of the sample were checked based on the description of the genus *Dictyota* sp. from literature (Lamouroux, 1809).

Figure 1. Brown Algae (*Dictyota* sp.) Samples Were Collected in December 2020 from the Seas of Awur Bay, Jepara, Central Java, Indonesia.

Morphology Identification of Bacterial Colonies

Before screening for fibrinolytic protease activity, each sub-cultured isolate was purified on *Nutrient Agar* (NA) medium (cat. no. CM000, Oxoid) 3 times. Next, bacterial colony morphology characteristics, including shape, colour, edge, elevation, and consistency, were observed (Fuad et al., 2020).

Selection of Proteolytic Bacteria

To qualitatively determine the protease activity of bacteria, the purified isolates were grown in 5% (w/v) Skim Milk Agar (SMA) media. This SMA media was prepared from 5g skim milk and 100 mL distilled water. Distilled water was first autoclaved at 121ºC 1 atm for 5 min. After cooled to warm, 5g of skim milk powder was dissolved and then re-autoclaved at 121ºC 1 atm for 5 min (Fuad et al., 2020).

For proteolytic bacteria selection, each of the 14 bacterial isolates was cultivated on plates containing SMA media and then incubated for 24-48 h at 37ºC. The clear zone that appears around the colony indicates the presence of proteolytic activity. The diameter of the clear zone and the diameter of the colony were measured. The proteolytic activity is expressed as a proteolytic index which is the ratio between the diameter of the clear zone divided by the diameter of the bacterial colony (Hidayati et al., 2021).

Bacterial Cell Morphology Analysis on Proteolytic Isolates

Observation of bacterial cell characteristics under the optic microscope was carried out on the selected proteolytic isolates. After Gram-staining, the Gram-coloration characteristics, bacterial cell shape, and spores were also analyzed (Cahyaningrum & Tri, 2021).

Selection of Fibrinolytic Bacteria

Screening of fibrinolytic bacteria was conducted on proteolytic isolates. To qualitatively determine the presence of fibrinolytic enzyme activity, the positively selected proteolytic isolates were grown on 3% (w/v) Fibrin Agar (FA) media containing 1.7g agar in 20 mL distilled water; 3g fibrin in 20 mL 0.1 M buffer pH 7-8. The mixture of all media components was autoclaved at 121ºC for 30 mins. Each proteolytic bacterial isolate was grown on plates containing FA media and then incubated for 24-48 h at 37ºC. The clear zone that appeared around the colony indicates the presence of fibrinolytic activity. The diameter of the clear fibrinolytic zone and the diameter of the colony were measured. Fibrinolytic activity is expressed as a fibrinolytic index which is the ratio between the diameter of the clear zone divided by the diameter of the bacterial colony (Fuad et al., 2020).

Determination of Proteolytic dan Fibrinolytic Indexes

The diameter of the clear zone formed around the colony on the medium was measured, including the diameter of the colony and the diameter of the clear zone, using a ruler or calliper. Hydrolysis Capacity (HC) formula was used for calculating the proteolytic and fibrinolytic index; the formula is as follows (Cahyaningrum & Tri, 2021):

$$HC = \frac{\text{Clear Zone Diameter} - \text{Bacterial Colony Diameter}}{\text{Clear Zone Diameter}}$$

The values of the hydrolysis capacity index can be a reference for the presence of enzyme activity or the ability of bacteria to produce enzymes (Cahyaningrum & Tri, 2021).

Hemolysis Pattern Analysis of Fibrinolytic Protease-Producing Bacteria

All of the selected isolates of fibrinolytic protease-producing bacteria were tested for their hemolysis activity. An active hemolysis activity indicates that certain bacteria could produce hemolysin toxin, which indicates pathogenicity (Dayu et al., 2021). To determine the hemolysis activity of fibrinolytic protease-producing bacteria obtained in this study, all selected isolates were grown on Blood Agar Plate (BAP) media (cat.no. CM0271, Oxoid). This media contained 5% sheep blood (v/w) and then incubated at 37ºC for 18-24 h. The appearance of a hemolysis zone around the bacterial colonies on the medium indicates the presence of hemolysin activity. The hemolysis zone around the colony emits a greenish colour indicating the type of α hemolysis. The β hemolysis shows a bright hemolysis zone around the colony, while the γ hemolysis type does not show a hemolysis zone around the colony (Manu et al., 2019).

RESULTS AND DISCUSSION

Bacterial Colony Morphology and Proteolytic Screening Results

The algae samples were obtained from the waters of Awur Bay, Jepara. The algae obtained had similar morphological characteristics as described on literature (Lamouroux, 1809), such as ribbons, flat thallus with smooth edges, branching dichotomous, and musty blunt. Of the 14 isolates shown in Figure 2, 8 isolates were positive as protease producers, as indicated by the presence of a clear zone around the colonies on SMA media. The colony of these eight isolates and their proteolytic index values , ranging from 0.30 to 1.00, are shown in Table 2. As seen in Table 2, isolates FD-02, FD-05, and FD-09 had the highest proteolytic index of 1.00, while the isolates with the lowest proteolytic index were FD-13 of 0.30. Isolates FD-01, FD-04, FD-06, FD-10, FD-11, and FD-12 showed negative results. Thus, they were grouped as non-protease producers. In this study, 3 of 8 isolates were positive as protease producers, as shown in Figure 4, were identified to produce fibrinolytic enzymes.

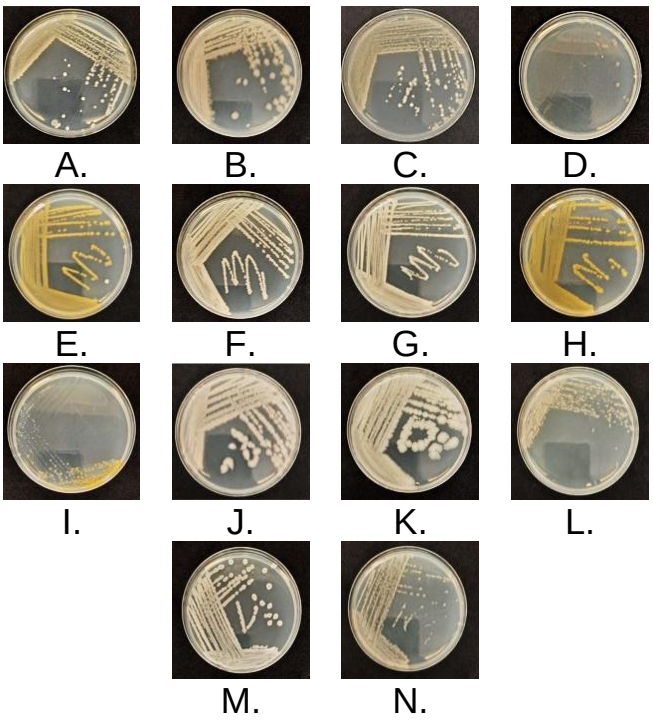


Figure 2. Fourteen Pure Isolates Obtained From the Fermentation of *Dictyota* sp. in Nutrient Agar (NA) Medium. A. FD-01, B. FD-02, C. FD-03, D. FD-04, E. FD-05, F. FD-06, G. FD-07, H. FD-08, I. FD-09, J. FD-10, K. FD-11, L. FD-12, M. FD-13, N. FD-14

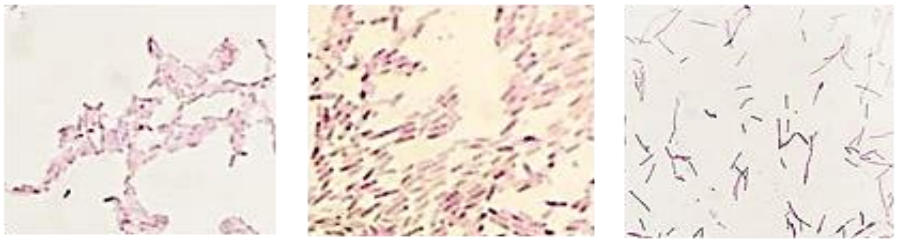
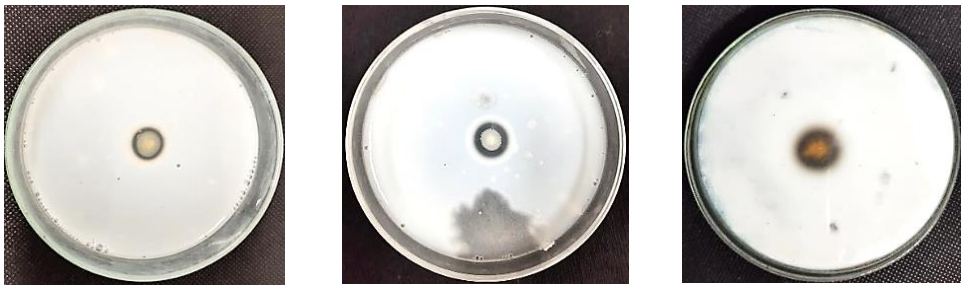


Figure 3. Gram Staining Results On Bacterial Isolates Producing Fibrinolytic Proteases Under a Microscope with 100X magnitude. A. FD-09, B. FD-13, C. FD-14 Isolates.

As seen in Figure 3, these three positive isolates showed typical cell characteristics. The cells of FD-13 and FD-14 isolates have rod shapes (bacilli) while those of FD-09 isolate have coccobacillus shapes. All of these bacteria did not form any spores. Their Gram characteristics are summarized in Table 1.

Table 1. Gram-Staining Analysis Result of Fibrinolytic Protease-Producing Bacteria

Isolates code	Shape	Spore	Gram type
FD-09	Coccobacillus	-	Gram Negative
FD-13	Bacillus	-	Gram Negative
FD-14	Bacillus	-	Gram Negative



A B C

Figure 4. Skim Milk Agar Plate Showing Clear Zone of Bacterial İsolates Producing Protease Enzyme with Fibrinolytic Activity. A. FD-09; B. FD-13; C. FD-14.

Table 2. Colony Morphology and Proteolytic Index Data of Subculture Isolates

Bacterial isolate	Shape	Edge	Color	Elevation	Consistency	Proteolytic index
FD-01	round	entire	cream	convex	rough	0,00
FD-02	irregular	undulate	cream	umbonate	rough	1,00
FD-03	circular	Entire	cream	convex	rough	0,45
FD-04	circular	entire	cream	convex	smooth	0,00
FD-05	circular	entire	cream	umbonate	smooth	1,00
FD-06	irregular	undulate	cream	convex	smooth	0,00
FD-07	circular	entire	cream	convex	rough	0,58
FD-08	circular	entire	yellow	flat	rough	0,50
FD-09	circular	entire	yellow	convex	smooth	1,00
FD-10	irregular	undulate	white	convex	smooth	0,00
FD-11	irregular	undulate	cream	flat	rough	0,00
FD-12	circular	entire	white	convex	smooth	0,00
FD-13	circular	undulate	cream	umbonate	rough	0,30
FD-14	circular	entire	cream	convex	smooth	0,42

Fibrinolytic Activity of the Selected Proteolytic Bacteria

The 3 of 8 isolates that were positive for protease production (Figure 4), only 3 isolates, including FD-09, FD-13, and FD-14 were positively identified to produce fibrinolytic enzymes (Figure 5). This was indicated by the presence of a clear zone around the colony on FA media using Nattokinase as control. The appearance of a clear zone indicated that the bacteria were able to degrade the fibrin substrate contained in the medium.

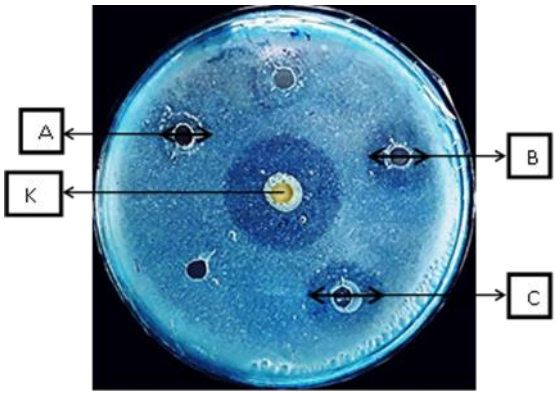


Figure 5. Fibrin Agar Plate Showing a Clear Zone of Bacterial Isolates Producing Fibrinolytic Enzymes. K = Nattokinase, A = FD-09, B = FD-13, C= FD-14 isolates.

The fibrinolytic index values of these three isolates ranged from 2.0 to 2.9, as shown in Table 3. FD-13 isolate had the highest fibrinolytic index value compared to other isolates, namely 2.9, followed by FD-14 with a fibrinolytic index value of 2.6 and FD-09 with a fibrinolytic index of 2.0. Results of Gram stain observations of bacteria-producing fibrinolytic proteases are shown in Figure 3. The three bacterial isolates are in a group of Gram-positive rod-shaped bacteria (bacilli), as shown in Figure 3.

Table 3**.** Fibrinolytic Index (FI) of Selected Isolates

Bacterial isolate	Fibrinolytic index
FD-09	2.0
FD-13	2.9
FD-14	2.6

The value of the fibrinolytic index can be used as a reference for the activity of fibrin-splitting or fibrinolytic enzymes or the amounts of enzymes that bacteria can produce. The high and low values of the fibrinolytic index indicate the amount of enzyme production that bacteria can produce, but this can be influenced by many factors, such as the age of the bacteria, storage conditions, living environment, and the availability of sufficient nutrients to carry out enzyme production (Afriansyah et al., 2021). Fuad et al., 2020. reported that three isolates producing fibrinolytic enzymes were derived from the fermentation of *H. scabra* muscle tissue with a fibrinolytic index ranging from 2.0 to 3.6. Fibrinolytic enzymes or plasmin are a group of protease enzymes that can degrade or dissolves fibrin or fibrinogen. In the body, fibrinolytic enzymes are produced by endothelial cells in the pancreatic duct. The production of fibrinolytic enzymes in the body can be influenced by many factors, such as age and diet; this can cause reduced plasmin production so that the performance of the fibrinolytic system in the body is disrupted (Hu et al., 2019).

The mechanism of action of antithrombotic agents is divided into four main points, namely plasmin activation, fibrin degradation, fibrinogen degradation, and preventing the activation of fibrinogen into fibrin. In the case of thrombosis, it occurs due to the formation of a blood clot (thrombus) in a blood vessel (Kartal, 2014). Under normal conditions, a thrombus is formed to prevent bleeding, but under abnormal conditions, a thrombus will form even though there is no trigger. In this case, fibrin plays a role in thrombus formation in blood vessels (Kumar Arun, 2014). The accumulation of thrombus causes blockage of blood vessels resulting in ischemia and infarction that can trigger cardiovascular disease (CVD) (Xin et al., 2018).

Administration of fibrinolytic enzymes can degrade fibrin which plays a role in the process of thrombus formation or the so-called fibrinolysis process. Fibrinolysis is an enzymatic degradation of fibrin and will be activated automatically along with the blood clotting process. The fibrinolysis process can be seen in vitro using the fibrin plate assay method; if bacteria produce fibrinolytic enzymes, the fibrin substrate contained in the medium will be degraded, which is characterized by the appearance of a clear zone around the colony (Devaraj et al., 2018).

Isolate FD-09 did not show hemolysin production activity, indicated by the absence of a hemolysis zone or identified as producing γ-hemolysis, while isolate FD-13 showed a bright hemolysis zone around the colony on the medium or identified as β-hemolytic (Table 4). As shown in Figure 6, The FD-14 shows only partial hemolysis (see arrow), and therefore categorized as α-hemolytic. By showing β and α-hemolysis patterns, respectively, both FD-13 and FD-14 isolates are hemolysin producers indicated by their blood lysis activity, which could be an indication of pathogenicity (Asril & Leksikowati, 2019).

Figure 6. Hemolysin Activity Test Results on Blood Agar Media (BAP) of 3 Isolates of Fibrinolytic Proteases-Producing Bacteria. Note: A = FD-09, B = FD-13, C = FD-14 isolates.

Table 4. Hemolysis Patterns of Fibrinolytic Protease-Producing Isolates

Isolates code	Hemolysis zone	Hemolysis type
FD-09	-	γ-hemolysis
FD-13	+	β-hemolysis
FD-14	+	α-hemolysis

In this study, the three fibrinolytic protease isolates showed varying abilities to produce hemolysins (Figure 6), including the FD-13 isolate, which produced a hemolytic toxin, indicating high pathogenicity, while the FD-14 isolate, which produced a hemolytic toxin, showed moderate pathogenicity. The isolate FD-09 did not produce a hemolytic toxin, which indicates very low pathogenicity, so it can be said that it is safe to be used as a candidate for antithrombotic therapy because the health risk is very low. It has the potential for large-scale enzyme production. For the fibrinolytic protease isolates in this study, future testing needs to be done, such as anticoagulant, antiplatelet, and thrombolysis, regarding their potential as antithrombotic agents for CVD therapy.

The application of fibrinolytic enzymes in the pharmaceutical industry can be used as an antithrombotic agent. There are many antithrombotic agents in circulation, such as nattokinase, streptokinase, urokinase, anistreplase, and tissue plasminogen activator. However, in some cases, the existing antithrombotic agents are known to have good side effects to be considered by their users (Sri Pananjung et al., 2016).

Side effects that may appear can be caused by the ability of bacteria to produce enzymatic toxins, one of which is hemolysin. The presence of hemolysin causes bacteria to be able to lyse blood cells. The level of toxicity is distinguished based on the type of hemolysis, but not limited to, hemolysis (Dayu et al., 2021).

Fibrinolytic enzyme activity can be tested in vitro through the fibrin plate assay method, anti-thrombolytic effect using the Lee and White method, relative partial thromboplastin time (RPTT), PT, aPTT, and can also be tested by clot retraction examination with slight modifications. It is important to test the enzyme activity to determine the functional ability of the enzyme (Umar & Sujud, 2020).

Factors affecting enzyme activity include temperature, acidity, inhibitors, activators, and substrate concentration. The presence of inhibitors and activators causes the working mechanism of the enzyme to be not optimal, or it can be said to reduce its activity. Therefore, optimization needs to be done. Inhibitors and activators can be removed through enzyme purification, which aims to remove ions, compounds, and residues, as well as other elements that are not needed containing in enzymes so that pure enzymes are obtained (Islamiyah et al., 2022; Wolberg et al., 2004). Several enzyme purification methods have been successfully applied, such as precipitation using EDTA and/or (NH4)2SO4, dialysis or ion exchange, chromatography, and to measure molecular weight and visualize enzyme activity using the zymographic method (Sri Pananjung et al., 2016).

The search for new and safe sources of fibrinolytic enzymes still needs to be done because the need for antithrombotic agents will continue to increase. Applying fibrinolytic enzymes as antithrombotic agents can be the right solution in dealing with the problem of thrombus formation, in addition to the many advantages behind applying fibrinolytic enzymes from natural sources. Marine organisms such as *Dictyot*a sp. are a potential source of fibrinolytic proteases and have many advantages, including being able to be produced on a large scale, productivity and quality can be increased, and terms lower prices (Barzkar et al., 2022).

Limitations of this study, as other bacterial screening research, are the simplified bacterial models, single-target focus (on bacterial enzyme) and limited physiological relevance. Our bacterial screening assay was conducted on the laboratory-adapted strains as the simplified model, which may not accurately represent the bacterial populations in real-world testing. Our study focused on evaluating the activity of the bacterial protease. While this approach provides valuable information about the potential enzyme activity, it may not capture the complexity of bacterial protease production mechanisms. Bacteria can employ various strategies in producing proteases, which may not be fully captured in single-target assays (Knyphausen et al., 2023). Also, our tests were conducted under controlled laboratory conditions, and factors such as temperature, nutrient availability, and pH may differ from the conditions encountered by bacteria in natural environments (Jin & Kirk, 2018). As a result, the activity of compounds observed in vitro may not fully reflect their efficacy in vivo. Further research still needs to be done for this research, such as testing the optimal work activity of fibrinolytic enzymes, production of fibrinolytic enzymes, zymography analysis, and activity test of fibrinolytic enzymes for antithrombotic drug development. Furthermore, the fibrinolytic protease reported from this study needs further confirmation of its antithrombotic characteristics in vivo before it can be used as a candidate antithrombotic agent in clinical trials.

CONCLUSION

The results of this study indicate that *Dictyota* sp. is a potential source of fibrinolytic protease-producing bacteria. Three of 14 sub-cultured bacterial isolates, the FD-09, FD-13, and FD-14, originated from the brown algae fermentation product and were able to produce degradation zones both on skim milk and fibrin agar plates. Regarding hemolysin toxin production, the three protease-producing bacterial isolates have varied hemolysis patterns on blood agar plates, indicating different pathogenicity levels.

CONFLICT OF INTEREST

The author declares no conflict of interest.

REFERENCES

Altaf, F., Wu, S., & Kasim, V. (2021). *Role of Fibrinolytic Enzymes in Anti-Thrombosis Therapy*. *8*(May), 1–17. https://doi.org/10.3389/fmolb.2021.680397

Afriansyah, M, A., M., Kamaruddin, M., Norma Ethica, S., & Fitria Aprianti, N. (2021). Aktıvıtas Antı-Bıofılm Bakterı Darı Produk Alga Coklat Dictyota sp. *Medika Alkhairaat : Jurnal Penelitian Kedokteran Dan Kesehatan*, *3*(3), 89–93. https://doi.org/10.31970/ma.v3i3.82

Asril, M., & Leksikowati, S. S. (2019). Isolasi dan Seleksi Bakteri Proteolitik Asal Limbah Cair Tahu Sebagai Dasar Penentuan Agen Pembuatan Biofertilizer. *Elkawnie*, *5*(2), 86. https://doi.org/10.22373/ekw.v5i2.4356

Barzkar, N., Jahromi, S. T., & Vianello, F. (2022). Marine Microbial Fibrinolytic Enzymes: An Overview of Source, Production, Biochemical Properties and Thrombolytic Activity. *Marine Drugs*, 20(1), 2-13. https://doi.org/10.3390/md20010046

Bi, Q., Han, B., Feng, Y., Jiang, Z., Yang, Y., & Liu, W. (2013). Antithrombotic effects of a newly puri fi ed fi brinolytic protease from Urechis unicinctus. *Thrombosis Research*, *132*(2), e135–e144. https://doi.org/10.1016/j.thromres.2013.07.001

Cahyaningrum, E., & Tri, A. (2021). Isolasi dan Pengaruh Monosodium Glutamat terhadap Pertumbuhan Bakteri Proteolitik Limbah Cair Tahu Isolation and Effect of Monosodium Glutamate on Growth Tofu Liquid Waste Proteolytic Bacteria. *Berkala Ilmiah Biologi, 23*(2), 84-90.

Chen, J., Li, H., Zhao, Z., Xia, X., Li, B., Zhang, J., & Yan, X. (2018). Diterpenes from the Marine Algae of the Genus Dictyota. *Mar. Drugs*. 2018, 16(5), 159; https://doi.org/10.3390/md16050159

Cristina, R., Pereira, C., Lourenço, L., Terra, L., Abreu, P. A., & Castro, H. C. (n.d.). Marine Diterpenes : Molecular Modeling of Thrombin Inhibitors with Potential Biotechnological Application as an Antithrombotic. *Mar. Drugs*, 15(3):79, 1–13. https://doi.org/10.3390/md15030079

Dayu, D., Turista, R., Puspitasari, E., & Kurnanda, F. (2021). *T*he Potential Use of EDTA as an Alternative to Defibrination in Preparing Blood Agar Plates with Human AB Blood Type on Staphylococcus aureus Culture. *Ina. J. Med. Lab. Sci. Tech*. 2019; 1(1), 64–71. https://doi.org/10.33086/ijmlst.v3i1.1923

Devaraj, Y., Rajender, S. K., & Halami, P. M. (2018). Purification and characterization of fibrinolytic protease from Bacillus amyloliquefaciens MCC2606 and analysis of fibrin degradation product by MS/MS. *Preparative Biochemistry and Biotechnology*, *48*(2), 172–180. https://doi.org/10.1080/10826068.2017.1421964

Ferdiani, D., Zilda, D. S., Afriansyah, M. A., & Ethica, S. N. (2023). Characteristics and Substrate Specificity of Semi-Purified Bacterial Protease of Bacillus thuringiensis

HSFI-12 with Potential as Antithrombotic Agent. *Science and Technology Indonesia*, *8*(1), 9–16. https://doi.org/10.26554/sti.2023.8.1.9-16

Fuad, H., Hidayati, N., Darmawati, S., Munandar, H., Rahmawati Sulistyaningtyas, A., Nurrahman, N., Rahman Ernanto, A., Seswita Zilda, D., Widjanarka, W., & Norma Ethica, S. (2020). Prospects of fibrinolytic proteases of bacteria from sea cucumber fermentation products as antithrombotic agent. *BIO Web of Conferences*, *28*(December), 02006. https://doi.org/10.1051/bioconf/20202802006

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), 1242–1258.

Hidayati, N., Fuad, H., Munandar, H., Zilda, D. S., Sulistyaningtyas, A. R., Nurrahman, N., Darmawati, S., & Ethica, S. N. (2021). Potential of fibrinolytic protease enzyme from tissue of sand sea cucumber (Holothuria scabra) as thrombolysis agent. *IOP Conference Series: Earth and Environmental Science*, *743*(1). https://doi.org/10.1088/1755-1315/743/1/012007

Hu, Y., Yu, D., Wang, Z., Hou, J., Tyagi, R., Liang, Y., & Hu, Y. (2019). Purification and characterization of a novel, highly potent fibrinolytic enzyme from Bacillus subtilis DC27 screened from Douchi, a traditional Chinese fermented soybean food. *Scientific Reports*, *9*(1), 3–12. https://doi.org/10.1038/s41598-019-45686-y

Islamiyah, N., Ethica, S. N., Afriansyah, M. A., Mukaromah, A. H., & Zilda, D. S. (2022). The Importance of Purification and Activity Analysis of the Purified Product of Thrombolytic Protease from Bacillus sp. HSFI-12– A Review . *Proceedings of the 7th International Conference on Biological Science (ICBS 2021)*, *22*(May). https://doi.org/10.2991/absr.k.220406.052

Jin, Q., & Kirk, M. F. (2018). pH as a primary control in environmental microbiology: 1. thermodynamic perspective. *Frontiers in Environmental Science*, *6*(May), 1–15. https://doi.org/10.3389/fenvs.2018.00021

Kartal, V. (2014). A trombosis story and PRES. *Northern Clinics of Istanbul*, *1*(1), 49–52. https://doi.org/10.14744/nci.2014.25744

Knyphausen, P., Rangel Pereira, M., Brear, P., Hyvönen, M., Jermutus, L., & Hollfelder, F. (2023). Evolution of protease activation and specificity via alpha-2-macroglobulin-mediated covalent capture. *Nature Communications*, *14*(1), 1-15. https://doi.org/10.1038/s41467-023-36099-7

Kumar Arun. (2014). Cardiovascular diseases: Are we overlooking some cardiovascular disease risk factors/ markers? *Journal of Biomedical Sciences*, *3*(1), 1–4. https://doi.org/10.3823/1021

Lamouroux, J.V.F. (1809). Exposition des charactères du genre *Dictyota*, et tableu des espèces qu'il referme. *Journal de Botanique (Desvaux)* 2: 38-44.

Manu, K. R., Tangkonda, E., & Gelolodo, M. A. (n.d.). Isolasi dan identifikasi terhadap bakteri penyebab mastitis pada sapi perah di Desa Benlutu Kecamatan Batu Putih Kabupaten Timor Tengah Selatan. *Jurnal Veteriner Nusantara*, *2*(2), 10-19.

Hidayati, N., Nurrahman, N., Fuad, H., Semarang, U. M., & Munandar, H. (2021). Bacillus tequilensis Isolated from Fermented Intestine of Holothuria scabra Produces Fibrinolytic Protease with Thrombolysis Activity Bacillus tequilensis Isolated from Fermented Intestine of Holothuria Scabra Produces Fibrinolytic Protease with Thrombolytuc Activity. *March*. *IOP Conf. Ser.: Earth Environ. OP Conf. Series: Earth and Environmental Science 707 (2021) 012008*, 1-9.

https://doi.org/10.1088/1755-1315/707/1/012008

Pradhan, B., Patra, S., Nayak, R., Behera, C., Ranjan, S., Nayak, S., Bihari, B., Bhutia, S. K., & Jena, M. (2020). Macromolecules Multifunctional role of fucoidan , sulfated polysaccharides in human health and disease : A journey under the sea in pursuit of potent therapeutic agents. *International Journal of Biological Macromolecules*, *164*, 4263–4278. https://doi.org/10.1016/j.ijbiomac.2020.09.019

Sabrina, A. N., & Ethica, S. N. (2018). Potensi Bakteri Indigen Penghasil Enzim Protease dan Lipase sebagai Agen Bioremediasi Limbah Biomedis Puskesmas Tlogosari Kulon Potential of Indigenous Bacteria Producing Protease and Lipase Enzymes as Bioremediation Agents of Biomedical Waste of Puskesmas. *Prosiding Seminar Nasional Mahasiswa Unimus, 1*, 276–282.

Safitri, R., Muchlissin, S. I., Mukaromah, A. H., Darmawati, S., & Ethica, S. N. (2018). Isolasi Bakteri Penghasil Enzim Protease Bacillus Thuringiensis Pada Oncom Merah Pasca Fermentasi 24 Jam dan Identifikasi Molekuler Bakteri Berbasis Gen 16S rRNA. *Seminar Nasional Edusainstek*, *October*, 31–39.

Silberfeld, T., Rousseau, F. & Reviers, B. de (2014). An updated classification of brown algae (Ochrophyta, Phaeophyceae). *Cryptogamie Algologie* 35(2): 117-156.

Sri Pananjung, A. M., Ulfa, E. U., Senjarini, K., & Arimurti, S. (2016). Karakterisasi Isolat Bakteri Fibrinolitik Wu 021055* Asal Perairan Pantai Papuma, Jember. *Jurnal Bioteknologi & Biosains Indonesia (JBBI)*, *2*(1), 1. https://doi.org/10.29122/jbbi.v2i1.528

Umar, I., & Sujud, R. W. (2020). Hemostasis dan Disseminated Intravascular Coagulation (DIC). *Journal of Anaesthesia and Pain*, *1*(2), 19–32.

Wolberg, A. S., Meng, Z. H., Iii, D. M. M., & Hoffman, M. (n.d.). A Systematic Evaluation of the Effect of Temperature on Coagulation Enzyme Activity and Platelet Function. *The Journal of Trauma*, *56*(6), 1221-8. https://doi.org/10.1097/01.TA.0000064328.97941.FC

WorldHealthOrganization. (2018). *NCDs Country Profiles 2018 WHO*. 224. accessed on 22-06-2023. https://apps.who.int/iris/handle/10665/274512

Xin, X., Ambati, R. R., Cai, Z., & Lei, B. (2018). Purification and characterization of fibrinolytic enzyme from a bacterium isolated from soil. *3 Biotech*, *8*(2), 1–8. https://doi.org/10.1007/s13205-018-1115-4

Zhao, X., Guo, F., Hu, J., Zhang, L., Xue, C., Zhang, Z., & Li, B. (2016). Antithrombotic activity of oral administered low molecular weight fucoidan from Laminaria Japonica. *Thrombosis Research*, *144*, 46-52. https://doi.org/10.1016/j.thromres.2016.03.008