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The fibrinolytic potential of *Bacillus amyloliquefaciens* isolates from salt-fermented shrimp paste *terasi*

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Abstract. *Pinontoan R, City S, Widjaja AN, Purnomo JS, Dikson. 2024. The fibrinolytic potential of* Bacillus amyloliquefaciens *isolates from salt-fermented shrimp paste* terasi. *Biodiversitas 25: 3193-3199.* Thrombosis, a major pathology in Cardiovascular Diseases (CVDs), significantly contributes to global mortality. Although medicinal prevention and management of recurrent thromboses do exist, alternative means using natural sources are actively sought because of their lower costs, better compatibility, and lower risks of side effects. Due to their beneficial microorganisms, fermented foods offer a potential thrombolytic source for managing CVDs. In this study, we aimed to isolate and identify bacteria with thrombolytic activity from fermented shrimp paste *terasi*. Potential protease-producing bacteria from *terasi* were determined via cell and colony morphology, biochemical properties, and 16S rRNA sequence analyses. Subsequently, the thrombolytic and fibrinolytic activities of the bacteria were assessed by performing whole-blood clot tests and fibrin degradation assays; two protease-producing bacteria, designated as TJU5 and TMAD4 isolates were identified as *Bacillus amyloliquefaciens.* The isolates demonstrated thrombolytic activity by significantly reducing whole-blood clot mass after 2 h of incubation. The thrombolytic mechanism involves fibrinolysis indicated by the rapid degradation of Aα, Bβ, and γ fibrin chains observed within 1 min of incubation. These findings highlight the beneficial bacteria from fermented shrimp paste *terasi*, identified as *B. amyloliquefaciens* TJU5 and TMAD4, with high thrombolytic and fibrinolytic activities, underscoring their potential role in bolstering cardiovascular health.

Keywords: *Bacillus amyloliquefaciens*, cardiovascular health, fibrinolytic, *terasi*, thrombolytic

Abbreviations: BLAST: Basic Local Alignment Search Tool, FU: Fibrinolytic Units, NB: Nutrient Broth, PBS: Phosphate-Buffered Saline, WBLT: Whole-Blood Clot Lysis Tests

INTRODUCTION

Cardiovascular Diseases (CVDs) are a prominent cause of death worldwide, with thrombosis being a common manifestation of their pathogenesis. Thrombosis, characterized by the formation of unwanted blood clots (thrombi) within blood vessels, arises from imbalances between normal coagulation and thrombolytic processes (Lisman et al. 2021). While thrombi are typically produced by a healthy human body to reduce bleeding in response to vascular injuries, thrombosis occurs when thrombi undesirably form within vessels (Tang et al. 2023). Thrombosis causes tissue ischemia and can lead to life-threatening organ damage if not treated swiftly (Tsujimoto and Kaijo 2019; Gall et al. 2021). It is considered either the prime complication or part of the pathogenesis of various CVDs, such as stroke, heart failure, and deep vein thrombosis. Therefore, early treatment and prevention of thrombosis are imperative to improve patient survival and quality of life. Existing medications for resolving and preventing thrombosis are fast-acting, but they can be expensive. Moreover, if not properly implemented, they may cause severe hemorrhage (Wang et al. 2023). Therefore, maintaining a delicate balance of hemostatic homeostasis requires constant surveillance of the patient's condition and responses to these medications, which also requires careful administration.

Preventive measures should be prioritized over curative interventions to manage thrombosis. For example, improving lifestyle and diet are known to prevent thrombosis. Therefore, thrombolytic agents from natural sources are actively sought to avoid recurring thromboses without upsetting hemostatic homeostasis. These natural sources include fermented foods, which provide nourishment and various health benefits due to the fermenting microorganisms and their active compounds (Estruch and Lamuela-Raventós 2023).

Several soybean-based fermented foods from various Asian countries, such as natto, cheonggukjang, and douchi, have been shown to help prevent CVDs (Dwivedi et al. 2023). These fermented foods share the same ability to restore blood clotting balance via fibrinolysis. Their primary fermenting agent, *Bacillus subtilis*, has been extensively studied for its ability to produce extracellular proteases that effectively prevent thrombosis. Other *Bacillus* species have also been found to be capable of fibrinolysis, including *B. velezensis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. halotolerans*, *B. cereus*, *B. altitudinis*, and *B. pumilus* (Yao et al. 2018; Yao et al. 2019; Syahbanu et al. 2020; Meng et al. 2021; Rajaselvam et al. 2021; Salunke et al. 2022).

As an archipelago, Indonesia boasts of highly biodiverse marine resources that produce various foods that can be fermented for preservation. These fermented marine products also offer a variety of health benefits, such as improving gut microbiota, reducing oxidative stress diseases, and reducing CVDs and cancer risks (Helmi et al. 2022; Chan et al. 2023). Indonesian fish-based fermented products, such as *terasi*, kecalok, bakasang, petis, and rusip, contain fermenting bacteria that may provide health benefits. *Terasi* is a fermented shrimp/fish paste produced via spontaneous fermentation of milled, sun-dried, and salted shrimp and fish combination that is compressed into cuboids and easily crumbled.

As one of many specialties of Indonesian cuisine, *terasi* is extensively used as a pungent flavoring agent to enhance the savory flavor and aroma of all types of food (similar to anchovies in Western countries). *Terasi* fermentation takes 7-28 days (Surono 2016), during which certain beneficial microorganisms, such as several *Bacillus* and lactic acid bacteria species, have been reported (Prihanto and Muyasyaroh 2021). Exploring the fibrinolytic capability of microorganisms in Indonesian *terasi* offers a pathway for discovering naturally occurring fibrinolytic bacteria in foods. It increases current knowledge regarding the health benefits of *terasi* consumption. Therefore, this study aimed to explore the beneficial thrombolytic-capable bacteria isolated from fermented shrimp paste *terasi* as an alternative strategy to manage CVDs.

MATERIALS AND METHODS

Procedures

Research sample

Terasi samples were purchased from the cities of Indramayu, Madura, and Medan, as well as from the districts of Asahan, Bangka, and Cirebon of Indonesia.

Screening of proteolytic bacteria

One gram of each sample was crushed and mixed with saline water (0.9% NaCl) into a colloid and centrifuged for 5 min at 2,500 × g. The supernatant was cultured on skim milk agar medium at 37°C for 24 h. Potential proteolytic activity was confirmed by clear zones around the colonies, followed by the purification of these potential colonies for further analysis.

Morphological characteristics of bacteria

The morphological characteristics of the colonies were observed for their shape, elevation, margin, color, and surface. Cellular morphology was analyzed by cell shape, Gram staining, and endospore formation under a light microscope at 1000× total magnification.

Biochemical characteristics of bacteria

The biochemistry of the bacterial isolates was partially analyzed using the Voges-Proskauer test to determine their acetoin production in Methyl Red-Voges-Proskauer medium when supplemented with alpha-naphthol and sodium hydroxide. The indole test assessed their tryptophan degradation into indole using the Kovacs reagent. The gelatinase test was carried out to determine the liquefaction of solid gelatin agar medium.

Molecular identification of the bacterium

The purified culture was incubated into nutrient broth (NB) liquid media at 37°C overnight, followed by centrifugation to obtain the bacterial pellet for genomic DNA (gDNA) extraction using the Zymo Research Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, United States of America), following the manufacturer's protocols. Next, to identify the bacterium by molecular means, the gene encoding 16S ribosomal RNA (rRNA) was amplified from gDNA via polymerase chain reaction (PCR) involving a master mix of Kapa HiFi Taq Polymerase (Roche, United States of America) and bidirectional primers 27F (5′-AGA-GTT-TGA-TCM-TGG-CTC-AG-3′) and 1492R (5′-GGT-TAC-CTT-GTT-ACG-ACT T-3′) on a thermal cycler (Techne, Minnesota, United States of America) programmed to run the first denaturation at 95°C for 180 s, 25 cycles of 20 s long 98°C denaturation, 15 s long 50°C annealing, and 60 s long 72°C extension, and a final extension at 72°C for 120 s. The amplicons were sent to the Malaysian company's 1st BASE for Sanger dideoxy sequencing. The resulting chromatograms were analyzed and base called, and their ambiguous signals were pruned using the GEAR Genomics TEAL (https://www.gear-genomics.com/teal/). The clean sequences were overlapped into a clean, contiguous 16S rRNA gene sequence.

Crude enzyme extraction

The isolate was incubated in NB liquid media for 24 h at 37°C. After incubation, the culture was centrifuged, and the supernatant was processed using the methods described in our previous study (Pinontoan et al. 2021). The crude enzyme was extracted by performing a 1:3 (supernatant: acetone) cold acetone precipitation with a subsequent 60 min incubation at -30°C. The crude enzyme was centrifuged for 5 min at 9,000 × g, and the resulting pellet was air-dried. The dried pellet was resuspended in 20 µL of Phosphate-Buffered Saline (PBS) solution. The protein concentration of the suspension was estimated by measuring at an A^{280} value. This suspension will be used in the whole-blood clot lysis and fibrin degradation tests.

Analysis of whole-blood clot lysis

Previous studies by Pinontoan et al. (2021) performed the Whole-Blood Clot Lysis Test (WBLT) using 0.15 gr pieces of chicken whole-blood clots obtained from a local abattoir. The clot pieces were mixed with the crude enzyme extract (1 mg protein/ml), incubated at 37°C, and observed after 2 h of incubation. Negative and positive controls contained 1 mL of PBS and 50 Fibrinolytic Units (FU) of nattokinase (Doctor's Best, Tustin, California, United States of America), respectively. The degradation percentage was calculated as the difference between the blood clot mass before and after all treatments.

Fibrin degradation assay

Previous studies on fibrinolytic enzymes by Pinontoan et al. (2024a) described the preparation steps for generating fibrin subunit chains by mixing 1 mg of fibrinogen (Sigma-Aldrich, Saint Louis, Missouri, United States of America) and 10 µL of thrombin (100 NIH units/mL; Merck, Rahway, New Jersey, United States of America) in PBS. The mixture was then incubated at 37°C for 60 min. The fibrin suspension was incubated with the crude enzyme extract at 37°C for different time points (1, 3, 5, 10, 15, and 30 min). Positive and negative controls contained 50 FU nattokinase and PBS, respectively. Fibrin degradation was visualized on a 12% polyacrylamide resolving gel via 60 min 150mV SDS-PAGE, as described by Pinontoan et al. (2024a,b). The Precision Plus Protein Dual Xtra protein marker (Bio-Rad, Hercules, California, United States of America) was used to measure the molecular weight of the protein bands.

Data analysis

The species identities of the isolates were determined using the NCBI Nucleotide BLAST search, followed by constructing a phylogenetic tree using neighboring 16 rRNA gene sequences using MEGA11 (Tamura et al. 2021). On MEGA 11, the phylogenetic tree structure was constructed with the maximum likelihood method, implemented using the General Time Reversible substitution model whose substitution rates were modeled using gamma distribution with invariant sites (G+I) with five discrete gamma categories inferred utilizing the level 5 SPR Heuristic method. The initial tree template was established using Maximum Parsimony, with confidence values measured from 500 bootstrap replications. The 16S rDNA gene sequences of isolates TJU5 and TMAD4 were uploaded to the NCBI GenBank database under the accession numbers PP188688.1 and PP188692.1, respectively.

The WBLT whole-blood clot mass data from six replicates (n=6) were averaged and statistically analyzed. One-way analysis of Variance was used to calculate any significant differences in the data, and post-hoc Bonferroni and Hold tests were used to determine statistical differences between the data.

The fibrin degradation SDS-PAGE gel was stained and photographed, and the protein band molecular weights were estimated by computing the bands' retention factors calibrated against the protein marker bands using the Gel Analyzer 23.1.1 (Lazar and Lazar 2023).

RESULTS AND DISCUSSION

Isolation of proteolytic bacteria

Protease-secreting microorganisms were screened on skim milk agar. The use of casein in skim milk as the primary screening step for thrombolytic enzymes allows for the screening of serine proteases (Ouertani et al. 2018), such as nattokinase, which is commonly associated with thrombolytic capacity (Zhou et al. 2021). This approach is supported by previous research identifying microorganisms producing thrombolytic enzymes from *tempoyak* and natto (Lucy et al. 2019; Priskila et al. 2022). Based on the proteolytic screening using skim milk, two bacterial isolates were yielded, tentatively identified as isolates TJU5 and TMAD4.

Morphological, biochemical, and molecular species identification

After screening and subsequent purification, the isolates were identified based on their morphological and biochemical features and the sequences of the 16S rRNA gene. Table 1 shows the morphological and biochemical features of TJU5 and TMAD4.

The cell and colony morphologies of TJU5 and TMAD4 are nearly identical, whereas their biochemical characteristics are identical (Table 1). The isolates differed only in the margination of their colonies (Table 1). Cell and colony morphology and biochemical characteristics show that TJU5 and TMAD4 belong to the *Bacillus* genus. Molecular identification of the isolates through 16S rRNA gene sequencing was then further employed to validate the isolates' identities on the species level.

The initial PCR amplification yielded amplicons of approximately 1.4 kbp, sequenced and trimmed of ambiguous bases before species identification via BLAST homology search. The final sequences for isolates TJU5 and TMAD4 were 1382 and 1346 bp long, respectively. BLAST results indicated that the 16S rRNA gene sequences of TMAD4 and TJU5 are 98.61 and 98.44% similar to those of the type strain *B. amyloliquefaciens* DSM7, respectively.

To reinforce the classification of *B. amyloliquefaciens* isolates, we constructed a phylogenetic tree based on 16S rRNA gene sequences from various fibrinolytic Bacillus species. The analysis showed that isolates TJU5 and TMAD4 are in the same cluster as *B. amyloliquefaciens* EGE-B-2d.1 and the type strain DSM7, indicating that TJU5 and TMAD4 are most closely related to B. amyloliquefaciens among the fibrinolytic Bacillus species (Figure 1).

Table 1. Morphological and biochemical characteristics of bacteria TJU5 and TMAD4

	Isolate	
	TJU5	**TMAD4**
Cell morphology		
Shape	Rod	Rod
Gram staining	Positive	Positive
Spore staining	Positive	Positive
Colony morphology		
Form	Irregular	Irregular
Elevation	Raised	Raised
Margin	Entire	Undulate
Surface	Glistening	Glistening
Color	White	White
Consistency	Mucoid	Mucoid
Biochemical characteristics		
Starch hydrolysis	+	+
Catalase	+	+
Gelatin hydrolysis	+	+
Indole test	-	-
MR-VP	+	+

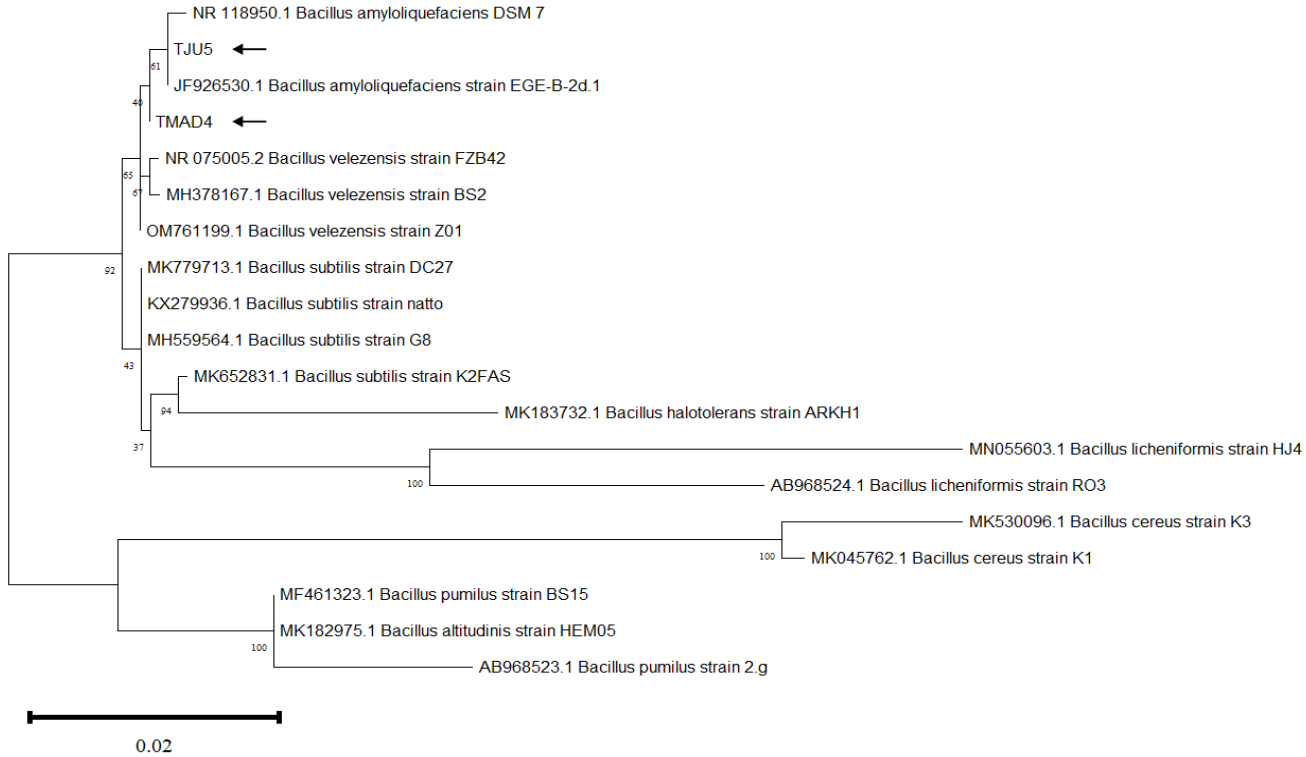

Figure 1. Phylogenetic relationship of isolates TJU5 and TMAD4 with other *Bacillus* species with fibrinolytic abilities. The maximum likelihood tree comparing 16S rRNA gene sequences was constructed using MEGA-11 software with 500× bootstrapping. The bootstrap values near the nodes indicate confidence in the tree's topology, and black arrows indicate the positions of isolates TJU5 and TMAD4. The scale bar indicates a phylogenetic distance of 0.02 nucleotide substitutions per site

Bacillus amyloliquefaciens is a versatile gram-positive bacteria found in various fermented foods, particularly fermented marine foods such as Korean jeotgal (Kim et al. 2017). It is generally recognized as a safe fermenting agent in food and beverages. In addition to its culinary use, *B. amyloliquefaciens* has been extensively used in industrial, agricultural, and medical applications because of its highly adaptable metabolism, rapid growth rate, robust expression systems, excellent genetic stability, and low cultivation costs. Examples of such uses include as a bioindicator for sterilization, in genetic engineering and the bulk production of desired proteins or secondary metabolites, for bolstering agriculture by protecting crops from phytopathogens such as fungi, and promoting crop growth in an ecologically friendly manner (Zalila-Kolsi et al. 2023). Furthermore, *B. amyloliquefaciens* has also been used in aquaculture to improve the immune system of aquatic livestock, such as whiteleg shrimp (Llario et al. 2018). In industrial applications, *B. amyloliquefaciens* is prized for its various carbohydrate-active and proteolytic enzymes, such as cellulase, acid-stable α-amylase, β-glucanase, mesophilic α-amylase, alkaline protease, and keratinase. Particularly in medical applications, *B. amyloliquefaciens* has been used as a probiotic to bolster the gut microbiota, improve the line of defense against infectious diseases, and facilitate efficient nutrient absorption (Llario et al. 2018; Zhou et al. 2022a; Zalila-Kolsi et al. 2023).

Whole-blood clot lysis tests

A whole-blood clot lysis assessed the isolates' thrombolytic activities through their extracellular enzymes. Initially, protein precipitation was performed by mixing the isolates' liquid culture with cold acetone to extract the extracellular enzymes. The crude enzyme extracts were incubated with whole-blood clots at 37°C for 2 h. The color of the liquid medium and the final whole-blood clot masses were observed (Figure 2).

Bacillus amyloliquefaciens TJU5 and TMAD4 crude enzyme extracts decreased the whole-blood clot mass by 83.1 and 19.2%, respectively (Figure 2). In comparison, the positive and negative controls of 50 FU nattokinase and PBS decreased the whole-blood clot mass by 43.0 and 4.2%, respectively (Figure 2.C). Interestingly, the crude enzyme extract of TJU5 led to a more significant reduction in blood clot mass ($p<0.01$) compared with that achieved by 50 FU/mL nattokinase ($p<0.05$). Blood clot degradation by TMAD4 was much lower than that by TJU5 and nattokinase; however, it was significantly higher than that of the negative control ($p<0.05$). These WBLT results indicate that TJU5 and TMAD4 isolates produced extracellular proteases with thrombolytic activities of varying strengths. Studies involving thrombolytic bacterial isolates from fermented foods have a similarly high degree of thrombolysis based on blood clot lysis assessment (Pinontoan et al. 2021; Priskila et al. 2022). In addition, the whole-blood clot lysis test results showed that the extracted enzyme of TJU5 performs better than nattokinase. A fibrin degradation assay was performed to verify the thrombolysis mechanism, which was visualized using SDS-PAGE.

Fibrin degradation assay

The thrombolytic mechanism employed by *B. amyloliquefaciens* TMAD4 and TJU5 was further studied using their crude enzyme extracts to degrade human fibrin directly. The enzyme samples' potential substrate specificity and reaction speed were assessed by incubating the mixtures at six different time points (1, 3, 5, 10, 15, and 30 min) and visualizing the resulting by-products by SDS-PAGE (Figure 3).

The fibrin protein (Figure 3, lane -) is composed of three different chains—Aα (83 kDa), Bβ (59 kDa), and γ (51 kDa)—consistent with other reports (McDonagh et al. 1972). Under physiological conditions, these meshed chains are typically crosslinked by Factor XIII to form stable fibrin clots (Weisel and Litvinov 2017). Thus, in the absence of Factor XIII, no crosslinked fibrin chains were detected in the assay (Vasilyeva et al. 2020). Upon incubation with the crude extracts of *B. amyloliquefaciens* TJU5 and TMAD4 (Figure 3), the intensity of these bands diminished, indicating proteolytic activity. Most fibrin chains were cleaved within the first minute of incubation, and complete degradation of the remaining fibrin chains occurred within 3 min. The positive control using nattokinase fully degraded fibrin chains without producing any residues, fibrin cleavage by the enzymes from both isolates produced bands with lower mass residues; most were observed at 44, 39, and 33 kDa, indicating that the enzymes may have specific cleavage sites. The degradation of the Aα, Bβ, and γ chains indicated that the thrombolytic activity of both isolates was mediated by fibrinolysis. These results verified that *B. amyloliquefaciens* TJU5 and TMAD4 secrete extracellular fibrinolytic enzymes with similar substrate preferences, as indicated by the similar fragmentation of fibrin (Yogesh and Halami 2015).

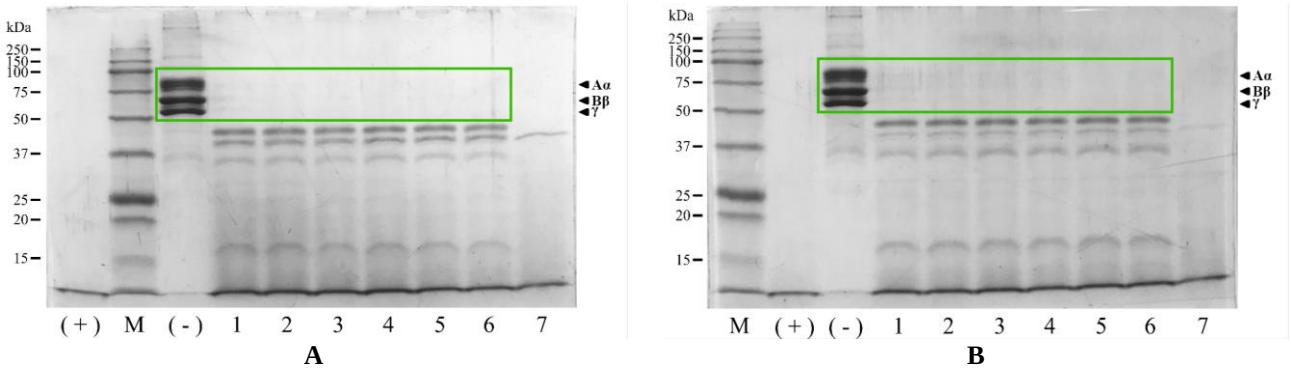

Figure 3. Fibrin degradation patterns by crude enzyme extracts of *B. amyloliquefaciens* TMAD4 and TJU5 on SDS-PAGE. (A) TJU5 and (B) TMAD4. Lane M contained protein marker; lane (+) contained fibrin incubated with nattokinase (50 FU/mL) for 30 min; lane (-) contained fibrin incubated with phosphate-buffered saline; lanes 1-6 contained fibrin and crude enzyme extract incubated for 1, 3, 5, 10, 15, and 30 min, respectively; and lane 7 contained crude enzyme extract. Arrowheads indicate the positions of the three fibrin chains. The green boxes indicate the complete degradation of fibrin chains

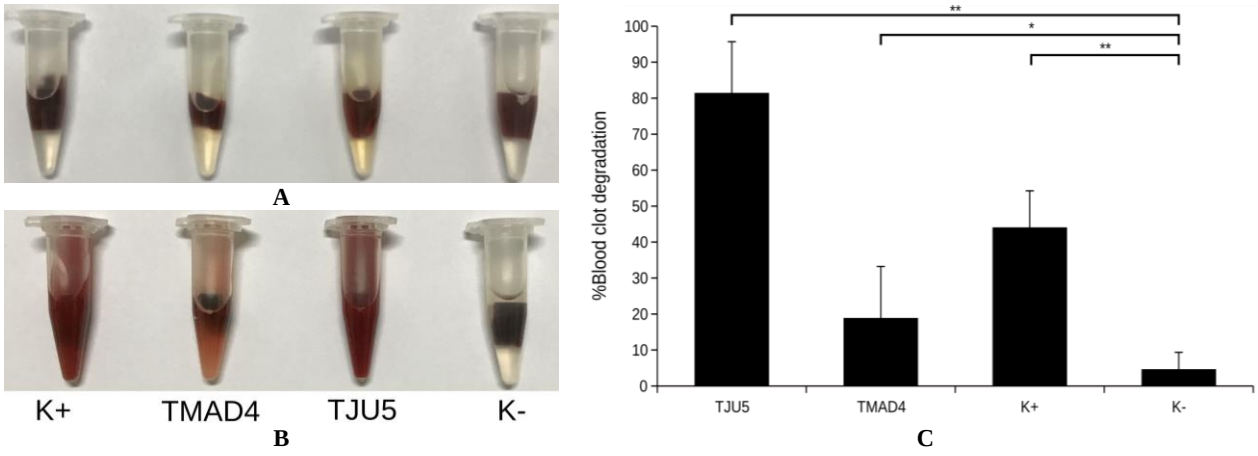

Figure 2. Whole-blood clot lysis test of isolates TJU5 and TMAD4. A. Whole-blood clots before incubation (0 h), B. Degraded whole-blood clots after 2 h of incubation, C. Whole-blood clot degradation (%) weighted after 2 h of incubation. K+, positive control (nattokinase); TMAD4: bacterial isolate TMAD4; TJU5: bacterial isolate TJU5; K-: negative control (phosphate-buffered saline). The standard deviation bar represents the variability of the data. * indicates significance at $p<0.05$; ** indicates significance at $p<0.01$

While fibrin is an essential factor in the process of sequestering erythrocytes, macrophages, and fibroblasts around wounds to achieve hemostasis, degrading fibrin is paramount to resolve and prevent unwanted thrombus formation from causing organ damage (Vilar et al. 2020; Risman et al. 2023). Successful thrombolytics are agents that effectively degrade fibrin proteins; both isolates displayed this characteristic, as the visualization of fibrin degradation rate using SDS-PAGE (Figure 3) indicated rapid fibrin degradation, with most of the fibrin protein degraded within 1 minute. To the best of our knowledge, the complete and rapid degradation of fibrin chains is unprecedented, as the results of fibrin degradation studies on *B. amyloliquefaciens* Jxnuwx-1 isolated from Chinese douchi showed staggered fibrin chain degradation, with the purified enzymes degrading the fibrin chains in the following order: Aα, Bβ, then γ chains (Yang et al. 2020). This characteristic appears to be shared across multiple *Bacillus* species, such as marine *B. velezensis* Z01 (Zhou et al. 2022b) and *B. subtilis* (Yao et al. 2017).

Several studies have reported that extracellular proteases secreted by *B. amyloliquefaciens* possess fibrinolytic capabilities. Several strains of *B. amyloliquefaciens* and *B. velezensis*, such as *B. velezensis* BS2 (Yao et al. 2019), *B. velezensis* Z01 (Zhou et al. 2022a), *B. velezensis* SW5 (Ning et al. 2021), *B. amyloliquefaciens* KJ10 (Rajaselvam et al. 2021), *B. amyloliquefaciens* GUTU06 (Ning et al. 2021), and *B. amyloliquefaciens* EGE-B-2d.1 (Slem et al. 2016) have also been reported to have fibrinolytic capabilities. In addition, other rarely reported *Bacillus* species with fibrinolytic enzymes include *B. licheniformis* RO3 (Afifah et al. 2015), *B. licheniformis* HJ4 (Meng et al. 2021), *B. halotolerans* ARKH1 (Salunke et al. 2022), *B. cereus* K1 and K3 (Syahbanu et al. 2020), *B. altitudinis* HEM05 (Salunke et al. 2022), *B. pumilus* 2. g (Afifah et al. 2014), and *B. pumilus* BS15 (Yao et al. 2018).

In conclusion, *Bacillus amyloliquefaciens* TJU5 and TMAD4 isolated from Indonesian *terasi* were found to have good thrombolytic and fibrinolytic potential. In WBLT and fibrin lysis tests, the isolates' secreted extracellular enzymes could significantly degrade whole-blood clots and fibrin chains. These findings lay the groundwork for future studies on these isolates, such as whole-genome analysis of their genes encoding said extracellular enzymes. They also highlight their potential role as alternative means of bolstering cardiovascular health and reducing CVDs risks.

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