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RESEARCH ARTICLE

Fibrinolytic and coagulation activities of crude extracellular preparation from *Bacillus cereus* group strain SF-4

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Abstract

Fibrinolytic enzymes have attracted considerable attention as potential alternatives to conventional thrombolytic agents because they degrade fibrin, a major structural component of blood clots. Fermented seafood products are promising sources of protease-producing microorganisms; however, fermented bamboo clam (*Solen* sp.) *rusip* remains underexplored as a source of bacteria with fibrinolytic potential. This study aimed to isolate and screen protease-producing bacteria from this product and to evaluate the fibrinolytic, thrombolytic, and whole-blood clotting-time effects of a crude extracellular preparation from the selected isolate. Eight bacterial isolates were recovered, and isolate SF-4 exhibited the highest proteolytic activity, was selected for further evaluation. Based on 16S rRNA gene sequencing, SF-4 was assigned to the *Bacillus cereus* group. The crude extracellular preparation was obtained after cultivation at 37°C for 72 h and was assessed using fibrin agar, a blood clot-lysis assay, and a whole-blood clotting-time assay. The preparation showed a fibrinolytic index of 1.33 ± 0.17, produced 13.51 ± 2.72% clot lysis, and prolonged whole-blood clotting time to 9.30 ± 0.26 min (mean ± SD, n = 3). Differences among treatments were performed using one-way ANOVA. These findings provide preliminary functional evidence of fibrinolysis-related activity in a bacterium from fermented bamboo clam *rusip*. However, toxin-gene screening, quantitative haemolysis testing, activity normalisation, and pathway-specific coagulation assays were not performed.

1. INTRODUCTION

Thrombotic disorders, including arterial thrombosis and venous thromboembolism (VTE), remain an important global health problem because thrombus formation can obstruct blood vessels and lead to severe cardiovascular and cerebrovascular complications. Recent global assessments continue to show a substantial burden of VTE and its associated morbidity and mortality (1). Fibrinolysis is an essential physiological process that maintains vascular patency through the degradation of fibrin, the structural component of blood clots (2,3). Clinically used thrombolytic agents, including streptokinase, urokinase, and tissue plasminogen activator, act through different mechanisms but are limited by bleeding

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risk, treatment complexity, and other pharmacological constraints (2-4). These limitations have encouraged continued investigation of microbial fibrinolytic enzymes as potential sources of bioactive molecules for further development.

Fibrinolytic enzymes can promote fibrin degradation through different mechanisms, including indirect fibrinolysis mediated by plasminogen activation and direct proteolytic degradation of fibrin (2,3). Microorganisms are important sources of extracellular proteases because they can grow under diverse conditions and secrete enzymes with broad substrate specificity. *Bacillus* species, in particular, are well recognized for producing extracellular proteases, including enzymes with reported fibrinolytic activity (4,5). Traditional fermented foods have therefore received considerable attention as microbial sources of fibrinolytic enzymes, and fibrinolysis-related activities have been reported from microorganisms associated with diverse fermented food matrices (6,7).

Fermented seafood is an important but comparatively less characterised group of fermented food matrices for microbial enzyme bioprospecting. Recent reviews have documented proteolytic and fibrinolysis-related activities associated with salt-fermented fish and other fermented seafood products (7). In Indonesia, *terasi* is a traditional salt-fermented shrimp paste in which microbial and endogenous proteolysis contributes substantially to the biochemical characteristics of the product (8). Recent experimental work has also demonstrated fibrinolytic and thrombolytic activities in *Bacillus amyloliquefaciens* isolates recovered from *terasi*, providing further evidence that fermented seafood can serve as a source of microorganisms with fibrinolysis-related activity (9).

Rusip is another traditional Indonesian fermented seafood product that has been investigated as a source of proteolytic microorganisms. Amatullah et al. (10) reported proteolytic bacteria from fish-based *rusip*, including members of the *Bacillus cereus* group, and a subsequent study reported a proteolytic bacterium from Pacific oyster-based *rusip* (10,11). These findings indicate that *rusip* fermentation matrices can support bacteria with extracellular proteolytic activity. However, available studies have focused primarily on fish- and oyster-based *rusip*, whereas *rusip* made from fermented bamboo clam (*Solen* sp.) remains comparatively underexplored as a source for microbial enzyme bioprospecting.

In the present study, bacterial isolates recovered from bamboo clam *rusip* were initially screened for extracellular proteolytic activity, and the isolate with the highest proteolytic index was selected for further characterisation. The isolate was identified by 16S rRNA gene sequencing, and its crude extracellular preparation was assessed using a fibrin agar assay, an in vitro blood clot-lysis assay, and a whole-blood clotting-time assay. Because proteolytic activity detected by casein-based screening does not establish fibrinolytic specificity, protease screening and fibrinolytic assays were treated as distinct stages of characterisation. This exploratory bioprospecting study aimed to provide preliminary functional evidence of fibrinolysis-related activity from an underexplored fermented seafood matrix. The molecular mechanism, active component, biosafety, and therapeutic efficacy of the crude extracellular preparation were not established.

2. MATERIALS AND METHODS

2.1. Sample Collection and Preparation

Fresh bamboo clams (*Solen* sp.) were purchased from a local seafood supplier (CV. Fos Jaya, Mojokerto, East Java, Indonesia) (Figure 1). Whole clams were cleaned, aseptically opened, and the edible muscle tissue was separated, rinsed with sterile distilled water, and cut into small pieces for laboratory-scale *rusip* fermentation.

2.2. *Rusip* Fermentation and Bacterial Isolation

Five grams of muscle tissue was mixed with traditional coarse salt, locally known as *krosok salt* (3%, w/w), and palm sugar (1%, w/w). The mixture was transferred into a sterile airtight container and allowed to ferment spontaneously at 28 ± 2°C for 7 days.

After fermentation, 1 mL of fermented *rusip* was aseptically transferred into 9 mL of sterile physiological saline (0.85% NaCl) to obtain a 10^{-1} dilution. Ten-fold serial dilutions were prepared up to 10^{-5}, and 100 µL aliquots of appropriate dilutions were spread onto Zobell Marine Agar (ZMA; Cat. No. M384, HiMedia Laboratories, Maharashtra, India) and incubated at 37°C for 24 h. Colonies with distinct morphologies were selected and re-streaked on fresh ZMA plates until pure cultures were obtained. The isolation procedure was adapted from previously published methods with minor modifications (8,10).



Figure 1. Bamboo clam (*Solen* sp.) used in this study

2.3. Phenotypic and Biochemical Characterisation of Isolate SF-4

The selected isolate was characterised based on colony morphology (colour, shape, margin, elevation, and surface) and conventional phenotypic and biochemical tests. Colony morphology was observed on ZMA medium based. Gram staining was performed to determine the Gram reaction and cell morphology. Growth and haemolytic activity were assessed on blood agar plates (BAP), and growth on Mannitol Salt Agar (MSA). Catalase activity was determined by observing bubble formation after exposing the culture to hydrogen peroxide, and oxidase activity was assessed using a conventional oxidase test. These results served as preliminary phenotypic characterisation of isolate SF-4 and were interpreted together with the 16S rRNA gene sequencing results.

2.4. Protease Screening

Pure isolates were screened for extracellular protease production on Skim Milk Agar (SMA; Cat. No. M763, HiMedia Laboratories, Maharashtra, India) supplemented with 5% (w/v) skim milk. Skim milk powder (5 g) was dissolved in 100 mL of sterile distilled water, sterilized separately, and aseptically added to the sterilized basal medium. Each isolate was spot-inoculated onto SMA plates and incubated aerobically at 37°C for 24 h. Protease production was indicated by a clear hydrolysis zone around the colony (8,13).

The proteolytic index (PI) was calculated as the ratio of the diameter of the clear hydrolysis zone, including the bacterial colony, to the bacterial colony diameter (Equation 1), where $D_{\text{clear zone}}$ is the diameter of the clear zone, including the colony, and D_{colony} is the colony diameter. Both were measured in millimetres with a digital calliper. Measurements were performed in triplicate and expressed as mean ± standard deviation (SD).

$$PI = \frac{D_{\text{clear zone}}}{D_{\text{colony}}} \quad (1)$$

2.5. Production of The Crude Extracellular Preparation

The selected isolate SF-4 was inoculated into Skim Milk Broth (Cat. No. M609S, HiMedia Laboratories, Maharashtra, India) containing peptone (5 g/L), beef extract (1.5 g/L), sodium chloride (35 g/L), and casein (10 g/L), and incubated aerobically at 37°C for 72 h. The culture was centrifuged at 3,000 rpm (approximately 1,000 × g) for 15 min at 4°C to remove cells and the cell-free supernatant was collected and used directly as the crude extracellular preparation in the fibrinolytic, thrombolytic, and whole-blood clotting assays. Because no purification or separation of individual components was performed, the preparation was not considered a purified single protease.

2.6. Molecular Identification of Isolate SF-4 by 16S rRNA Gene Sequencing

Molecular identification of isolate SF-4 was performed at PT. Genetika Science Indonesia (Tangerang, Indonesia) by 16S rRNA gene sequencing. Genomic DNA was extracted using the Quick-DNA Fungal/Bacterial Miniprep Kit (Cat. No. D6005, Zymo Research, California, USA) according to the manufacturer's instructions. DNA concentration and purity were assessed with a NanoDrop spectrophotometer from absorbance at 260 and 280 nm.

The 16S rRNA gene was amplified with the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). Polymerase chain reaction (PCR) was performed in a 25 µL reaction containing 12.5 µL MyTaq HS Red Mix (2×; Cat. No. BIO-25048, Bioline, Tennessee, USA), 1 µL of each primer (10 µM), 5–100 ng genomic DNA, and nuclease-free water. Cycling consisted of initial denaturation at 95°C for 1 min, followed by 35 cycles of 95°C for 10 s, 52°C for 15 s, and 72°C for 15 s. Amplicons of approximately 1,500 bp were verified by electrophoresis on a 0.8% agarose gel in 1× Tris–Borate–EDTA (TBE) buffer, visualized under UV illumination (312 nm), and subjected to bidirectional Sanger sequencing.

The sequence was compared with the National Centre for Biotechnology Information (NCBI) GenBank database using Basic Local Alignment Search Tool for nucleotide sequences (BLASTn) (14) and deposited in GenBank under accession number PZ789830. A phylogenetic tree was constructed in MEGA version 11 using the Neighbour-Joining (NJ) method with 1,000 bootstrap replications (15). Because 16S rRNA sequences have limited discriminatory power within the *Bacillus cereus* group, SF-4 was assigned to the *B. cereus* group rather than to a resolved species (16,17).

2.7. Fibrinolytic Activity Assay

Fibrinolytic activity was assessed by the fibrin agar plate method with minor modifications. The medium contained 1.7% (w/v) agar and 3% (w/v) fibrin in 0.1 M borate buffer (pH 7.4). After solidification, three wells (6 mm diameter) were punched into each plate. The wells received 50 µL of SF-4 crude extracellular preparation; 50 µL of nattokinase solution (Natto-10, Lapi Laboratories, Jakarta, Indonesia; BPOM Reg. No. SD111541911) as the positive control. No negative control was included. Plates were incubated at 37°C for 24 h. Lytic zone diameters were measured in two perpendicular directions with a digital calliper, and the mean was used to calculate the Fibrinolytic Index (FI) according to the Equation 2.

$$FI = \frac{D_{\text{lytic zone}}}{D_{\text{well}}} \quad (2)$$

Where $D_{\text{lytic zone}}$ is the diameter of the clear lytic zone and D_{well} is the diameter of the well (6 mm). All experiments were performed in triplicate and expressed as mean ± SD.

2.8. Thrombolytic Activity Assay

Thrombolytic activity of the SF-4 crude extracellular preparation was evaluated by an in vitro blood clot lysis assay with minor modifications (18). Sterile 1.5-mL microcentrifuge tubes were pre-weighed, and 1,000 µL of freshly collected human whole blood was added to each. Tubes were left at room temperature for 30 min to allow clotting. The serum was carefully removed without disturbing the clot, and the tubes were weighed again. Initial clot weight was obtained by subtracting the empty tube weight.

The commercial nattokinase supplement Natto-10 (Reg. No. SD111541911, Lapi Laboratories, Jakarta, Indonesia) was dissolved at 0.01 g in 1 mL phosphate-buffered saline (PBS), resulting in a nominal concentration of 10 mg/mL, and 100 µL (1 mg of the commercial product) served as the positive control. The experimental groups were an untreated clot, 100 µL of Natto-10 solution, and 100 µL of SF-4 crude extracellular preparation.

After treatment, tubes were kept at room temperature and observed for visible clot lysis. The released fluid was removed without disturbing the remaining clot, and the tubes were reweighed. Clot lysis (%) was calculated using the Equation 3. Where W_i is the initial clot weight and W_f is the final clot weight after treatment. Experiments were performed in triplicate and expressed as mean ± SD.

$$\text{Clot lysis (\%)} = \frac{W_i - W_f}{W_i} \times 100 \quad (3)$$

2.9. Whole-blood Clotting Time Assay

The effect of the SF-4 crude extracellular preparation on clotting time was evaluated by the Lee–White method with minor modifications (19). One millilitre of freshly collected human whole blood was placed in sterile glass tubes (8 mm internal diameter). The experimental groups were untreated blood, 10 µL of 10% ethylenediaminetetraacetic acid (EDTA) as the positive control, and 100 µL of SF-4 crude extracellular preparation. Tubes were kept at approximately 22°C and gently tilted at 30 s intervals beginning 3 min after blood collection. Clotting time was recorded when a stable clot first formed. Experiments were performed in triplicate and expressed as mean ± SD.

2.10. Blood Smear Examination

Immediately after the clotting-time assay, thin blood smears were prepared, air-dried, and stained with Wright stain according to the manufacturer's instructions. Smears were examined with an Olympus CX23 light microscope (Olympus Corporation, Tokyo, Japan) using a 100× oil-immersion objective (1000× total magnification). Erythrocytes were assessed for crenation, echinocyte formation, rouleaux, membrane abnormalities, and other alterations, and representative images were captured.

2.11. Human Blood Samples and Experimental Replication

Fresh human whole blood was obtained from healthy adult volunteers after written informed consent, in accordance with the Declaration of Helsinki. The study was approved by the Health Research Ethics Committee of Faculty of Nursing and Health Science, Universitas Muhammadiyah Semarang (Ethical Clearance No. 551/KE/08/2024). Blood samples were not selected according to ABO blood group, as this was not a study variable. The clot lysis and clotting-time assays used blood from different individual donors. Within each assay, blood from a single donor was distributed among the experimental groups, and each condition was tested in triplicate. The three measurements therefore represent experimental replicates from one donor sample rather than independent biological replicates, and the limited number of donors should be considered when interpreting the findings and their generalizability.

2.12. Statistical Analysis

Data are presented as mean ± SD. Normality was tested with the Shapiro–Wilk test and homogeneity of variance with Levene's test. Data meeting these assumptions were analysed by one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) post hoc test when ANOVA indicated significant differences. A p-value < 0.05 was considered statistically significant. Analyses were performed using IBM SPSS Statistics version 25.0 (IBM Corp., Armonk, NY, USA).

3. RESULTS AND DISCUSSION

3.1. Isolation and Screening of Protease-Producing Bacteria

A total of eight bacterial isolates were recovered from fermented bamboo clam *rusip* and screened for protease production on skim milk agar. Isolate SF-4 showed the highest proteolytic activity, with a clear hydrolysis zone of 12.00 ± 0.06 mm, a colony diameter of 7.00 ± 0.10 mm, and a proteolytic index (PI) of 1.71 ± 0.02 (Table 1). SF-4 was therefore selected for phenotypic characterisation, molecular identification, and evaluation of fibrinolytic, thrombolytic, and whole-blood clotting activities.

The clear hydrolysis zone indicated that SF-4 produced extracellular protease capable of degrading casein. Skim milk agar is widely used as a preliminary screening medium for extracellular protease production because casein serves as a general protein substrate (20). However, casein hydrolysis does not necessarily predict fibrinolytic activity, as casein and fibrin differ in structure and substrate characteristics (21). Selection of SF-4 was therefore a practical step to identify a candidate for subsequent functional testing, not evidence of fibrinolytic potential in itself.

Recovery of eight isolates, of which only SF-4 was carried forward, illustrates a systematic screening approach for bacteria associated with *rusip*. This is relevant because the substrate remains relatively underexplored as a source of microorganisms producing extracellular proteases with fibrinolytic and blood clot-lysis activities. The contribution of this study is the isolation and preliminary functional evaluation of a protease-producing isolate from this substrate, not the discovery of a new bacterial species or a fully characterised fibrinolytic enzyme.

Colonies of SF-4 on Zobell Marine Agar were yellow, rhizoid, with irregular margins, flat elevation, and a rough surface (Figure 4B; Table 2). Gram staining revealed Gram-positive rods arranged predominantly as single bacilli (Figure 4A; Table 2). SF-4 was catalase-positive and oxidase-negative. On Mannitol Salt Agar (MSA), it formed convex colonies with a flat edge, and the medium remained red, indicating no apparent mannitol fermentation (Figure 4C; Table 2).

These characteristics are consistent with previous reports of protease-producing *Bacillus* spp., including *Bacillus cereus*, isolated from fermented foods (5,10-12). Gram-positive rod morphology and catalase activity are typical of the genus (5). Phenotypic identification alone is insufficient for definitive species assignment because biochemical characteristics overlap considerably among *Bacillus* species, but it provides useful preliminary information prior to 16S rRNA gene sequencing (17,22).

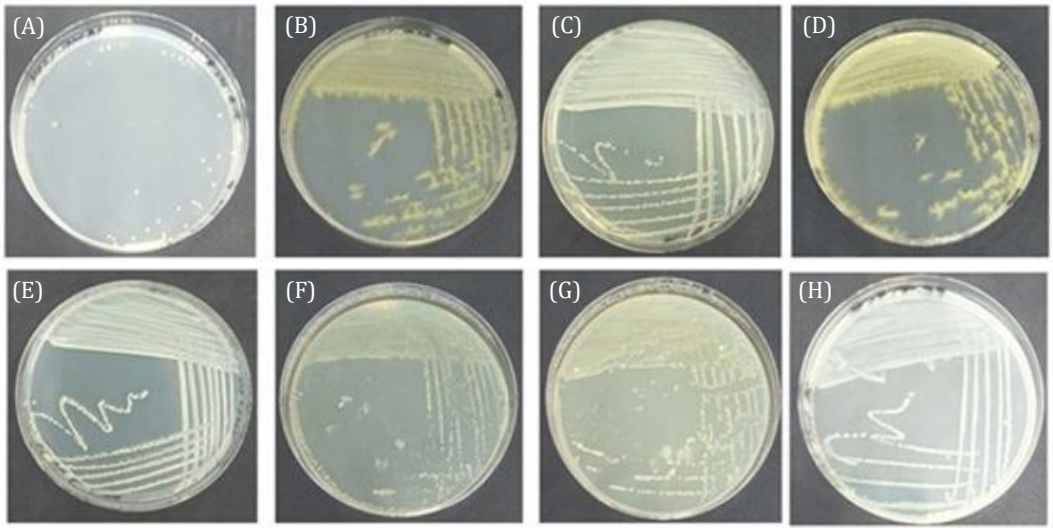


Figure 2. Morphologically distinct bacterial isolates recovered from fermented bamboo clam (*Solen* sp.) *rusip*. (A) SF-1. (B) SF-2. (C) SF-3. (D) SF-4. (E) SF-5. (F) SF-6. (G) SF-7. and (H) SF-8

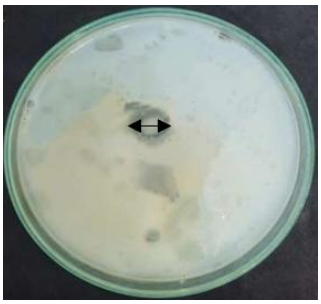


Figure 3. Proteolytic activity of isolate SF-4 on skim milk agar after 24 h of incubation at 37°C

Table 1. Proteolytic index of isolate SF-4

Isolate	Clear Zone Diameter (mm)	Colony Diameter (mm)	Proteolytic Index
SF-4	12.00 ± 0.06	7.00 ± 0.10	1.71 ± 0.02

Note: Values are presented as mean ± SD (n = 3)

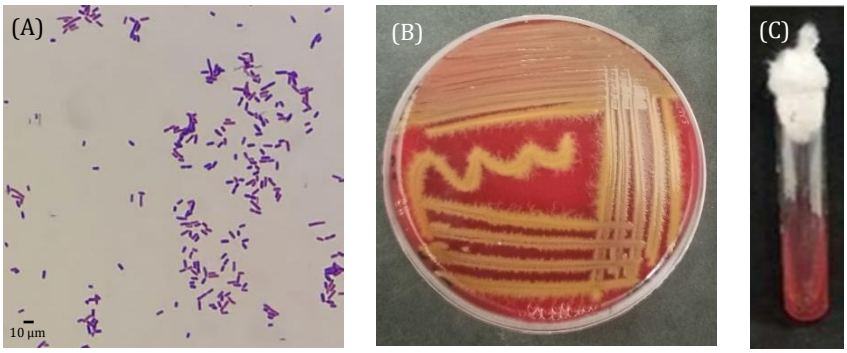


Figure 4. Phenotypic characterization of *Bacillus cereus* group strain SF-4. (A) Gram staining showing Gram-positive rod-shaped cells. (B) Haemolytic test on blood agar showing no visible haemolytic zone. (C) Growth of SF-4 on Mannitol Salt Agar (MSA), showing red medium without apparent mannitol fermentation

Table 2. Characteristics of proteolytic bacterial isolates from *rusip* bamboo clam

Characteristics		Isolate code
		SF-4
ZMA Medium	Form	Rhizoid
	Colour	Yellow
	Edge	Uneven
	Elevation	Flat
	Consistency	Rough
BAP Medium	Haemolysis type	γ
MSA Medium	Colour	Red
	Elevation	Convex
	Edge	Flat
	Catalase	Positive
	Oxidase	Negative
Gram Stain	Colour	Purple
	Form	Rod
	Arrangement	Solitary
	Gram type	Gram Positive

The absence of a visible haemolytic zone on blood agar is only a preliminary observation and must not be interpreted as evidence of biosafety. Toxin-gene screening and quantitative haemolysis assays were not performed. This is an important limitation because members of the *B. cereus* group may carry virulence-associated genes, and phenotypic haemolysis alone is insufficient to assess the safety of a strain or its extracellular preparation (22,23). Toxin-gene screening, quantitative haemolysis assessment, and broader hemocompatibility and toxicological evaluation would be required before any biomedical application is considered.

3.2. Molecular Identification Places Isolate SF-4 within the *Bacillus cereus* Group

PCR amplification yielded a 16S rRNA gene fragment of approximately 1,500-bp (Figure 5A), with no amplification in the no-template control (NTC). The sequence was deposited in the NCBI GenBank database under accession number PZ789830. BLASTn analysis showed 100% sequence similarity to several members of the *B. cereus* group, including *B. cereus* strain SRS-1 (PQ797086.1), *B. cereus* strain FG104 (PQ764047.1), *Bacillus anthracis* strain AML5 (PQ459417.1), and *Bacillus* sp. strain FM1 (JN206613.1). A similarity of 99.93% was observed with *Bacillus thuringiensis* strain JC-3 (MT492022.1) and other closely related strains (Table 3). Neighbour-Joining phylogenetic analysis placed SF-4 within the *B. cereus* group cluster (Figure 5B).

Isolate SF-4 was assigned to the *B. cereus* group. However, the 16S rRNA gene lacks the resolution to distinguish species within this group, and the phenotypic characteristics (Gram-positive rods, catalase-positive) likewise do not allow

species-level identification. More definitive taxonomic assignment would discriminatory markers such as *gyrB*, *rpoB*, or *panC*, multi locus sequence analysis; or whole-genome sequencing, which were beyond the scope of this study.

The molecular identification nevertheless provides a taxonomic framework for interpreting the functional assays. Members of the *B. cereus* group are known to produce diverse extracellular hydrolytic enzymes, including proteases, and several *Bacillus* strains have been investigated as sources of fibrinolytic enzymes (10-12). The placement of SF-4 is thus consistent with its extracellular proteolytic activity, although the functional findings do not depend on species-level assignment.

3.3. The SF-4 Crude Extracellular Preparation Exhibits Fibrinolytic Activity

Fibrinolytic activity of the SF-4 crude extracellular preparation was assessed by the fibrin agar plate assay, with nattokinase as the positive control. Clear lytic zones formed around both treatment wells (Figure 6), indicating fibrin degradation under the assay conditions.

The fibrinolytic index (FI) was 1.33 ± 0.17 for SF-4 and 1.60 ± 0.02 for nattokinase. One-way ANOVA showed no statistically significant difference between the two ($F(1,4) = 7.34$, p-value = 0.054) (Table 4). The assay therefore provides evidence of fibrin-degrading activity in the crude SF-4 preparation, but it does not establish equivalence to, or superiority over, nattokinase reference.

Although the FI of SF-4 was modest relative to the nattokinase, the magnitude of activity in a preliminary agar-based assay is not the only criterion for evaluating an underexplored microbial source. SF-4 was obtained from fermented bamboo clam *rusip*, a relatively underexplored fermentation matrix, and its preparation showed activity in both the fibrin agar and the blood clot-lysis assays. These findings offer preliminary functional evidence that this substrate can yield extracellular preparations with fibrinolysis-related activity. Further purification and optimisation are needed to identify the active component and determine its specific activity.

The comparison was based on equal sample volumes, not equivalent protein concentrations or standardised fibrinolytic activity. Because SF-4 preparation was a crude culture supernatant, the measured activity may reflect extracellular proteins together with other supernatant components. FI values should therefore be interpreted as assay-specific activity, not as a specific catalytic potency.

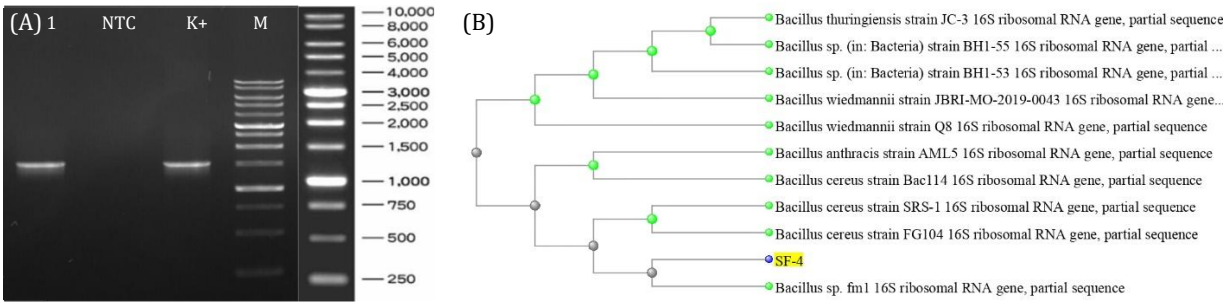


Figure 5. Molecular analysis of isolate SF-4 based on 16S rRNA gene amplification and sequence analysis. (A) Agarose gel electrophoresis of the 16S rRNA gene PCR product showing an approximately 1,500-bp amplicon. Lane 1, SF-4; NTC, no-template control; K+, positive control; M, 1-kb DNA ladder. (B) Neighbour-joining phylogenetic tree of isolate SF-4 based on the 16S rRNA gene sequence

Table 3. Representative top BLASTn matches of the 16S rRNA gene sequence of isolate SF-4

Query Isolate	Closest Database Match	Similarity (%)	Reference GenBank Accession
SF-4	*Bacillus cereus* strain SRS-1	100.00	PQ797086.1
	Bacillus cereus strain FG104	100.00	PQ764047.1
	Bacillus anthracis strain AML5	100.00	PQ459417.1
	Bacillus sp. strain FM1	100.00	JN206613.1
	Bacillus thuringiensis strain JC-3	99.93	MT492022.1
	Bacillus sp. strain BH1-55	99.93	MN410700.1
	Bacillus sp. strain BH1-53	99.93	MN410698.1
	Bacillus wiedmannii strain JBRI-MO-2019-0043	99.93	MN865955.1
	Bacillus cereus strain Bac114	99.93	PQ813848.1
	Bacillus wiedmannii strain Q8	99.93	MK719881.1

Note: Accession numbers represent reference sequences retrieved from GenBank for BLASTn comparison; isolate SF-4 was deposited under accession number PZ789830.

Table 4. Fibrinolytic index of isolate SF-4

Group	Fibrinolytic Index
Nattokinase	1.60 ± 0.02
SF-4 crude extracellular	1.33 ± 0.17

Values are presented as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA, F(1,4) = 7.34, p-value = 0.054.

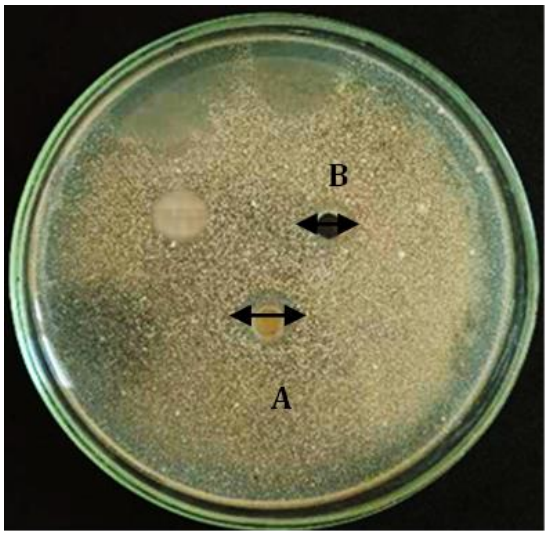


Figure 6. Fibrinolytic activity of the SF-4 crude extracellular preparation. A: Nattokinase (positive control); B: SF-4 crude extracellular preparation

Fibrinolytic activity has been reported for extracellular proteases from several *Bacillus* species, but it varies with strain, culture conditions, enzyme composition, and downstream processing (24-26). Purification and biochemical characterisation of the SF-4 preparation, including molecular characteristics, optimal reaction conditions, and substrate specificity, are needed to identify the component.

3.4. The SF-4 Crude Extracellular Preparation Exhibits Thrombolytic Activity

Representative clot images are shown in Figure 7. The SF-4 crude extracellular preparation produced a mean clot lysis of 13.51 ± 2.72%, compared with 10.00 ± 2.50% for nattokinase and 5.88 ± 2.94% for the untreated clot (Table 5). One-way ANOVA showed a significant difference among the three groups (F = 5.90, p-value = 0.038). Post hoc analysis indicated that SF-4 produced significantly greater clot lysis than the untreated clot, whereas the difference between SF-4 and nattokinase was not significant. These findings indicate that the SF-4 crude extracellular preparation was capable of promoting measurable clot degradation under the assay conditions.

This result is consistent with the fibrin agar assay and supports the ability of the SF-4 preparation to act on fibrin-containing substrates. The thrombolytic activity observed in the present study should be interpreted within the limitations of the experimental design. A single applied volume was used and no dose-response relationship was evaluated. No uninoculated-medium or vehicle control was included, and the preparation was not normalised to protein concentration or standardised enzymatic activity. The observed lysis cannot be attributed exclusively to a defined protease or used to infer dose-dependent activity or catalytic potency. The comparison with nattokinase was likewise made on an applied-volume basis, and although SF-4 gave numerically greater lysis, the difference was not statistically significant. Finally, all blood-based assay used blood from a single donor, with three technical replicates from the same sample. The results are considered preliminary and cannot be generalised.

3.5. The SF-4 Crude Extracellular Preparation Prolongs Clotting Time Without Detectable Erythrocyte Morphological Changes

The untreated whole-blood sample clotted in 8.00 ± 0.00 min, whereas addition of the SF-4 preparation prolonged clotting time to 9.30 ± 0.26 min (Table 6). The 10% EDTA control did not form a measurable clot within the observation period. Untreated blood served as the baseline, and EDTA served as a positive control for the assay. Because EDTA acts through calcium chelation, a mechanism distinct from proteolytic modulation of coagulation, it was not used as a mechanistic comparator for SF-4.

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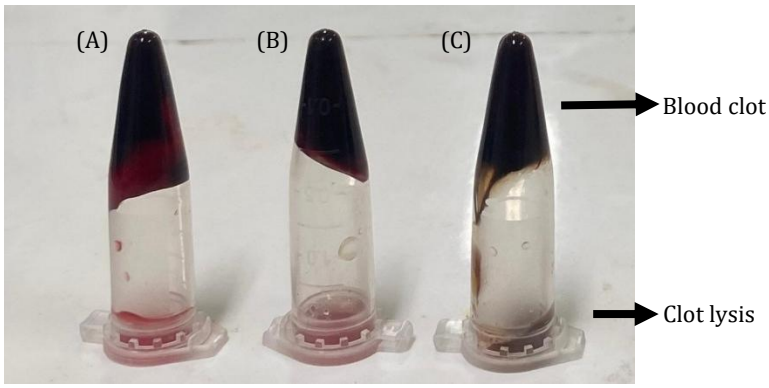


Figure 7. Representative images of thrombolytic activity under the experimental conditions. (A) Untreated clot. (B) nattokinase. (C) SF-4 crude extracellular preparation.

Table 5. Thrombolytic activity of crude extracellular preparation of *Bacillus cereus* group strain SF-4

Sample Code	Clot lysis (%)
SF-4 crude extracellular	13.51 ± 2.72[a]
Untreated clot	5.88 ± 2.94[b]
Nattokinase	10.00 ± 2.50[a,b]

Note: Values are presented as mean ± SD (n = 3). Different superscript letters (a and b) indicate statistically significant differences between groups, whereas groups sharing the same superscript letter are not significantly different, according to one-way ANOVA followed by Fisher's least significant difference (LSD) post hoc test (p-value < 0.05)

The prolongation with SF-4 should be interpreted cautiously, as delayed clot formation rather than definitive evidence of anticoagulant activity. The assay gives a global measure of clot formation and does not identify the coagulation pathway involved. Adding 100 µL of crude preparation to 1 mL of whole blood may also introduce a dilution effect, particularly as no uninoculated-medium or vehicle control was included. In addition, the assay was performed at approximately 22°C rather than at physiological temperature. The prolongation therefore cannot be attributed to a specific anticoagulant mechanism. Further investigation at 37°C using standardised assays, including prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombin time (TT), is needed to determine whether the effect persists under physiological conditions and which pathway is affected.

Wright-stained blood smears showed no detectable erythrocyte morphological abnormalities after treatment with the SF-4 preparation (Figure 8). Black circles mark representative areas showing erythrocyte distribution in each group. Erythrocytes retained their general morphology, with no apparent fragmentation, membrane disruption, crenation, or haemolysis. These are preliminary morphological observations only and do not establish haemocompatibility or biological safety. Haemolysis, cytotoxicity, and haemocompatibility studies would be required before any biomedical application is considered.

Taken together, the fibrinolytic, clot-lysis, and clotting-time findings indicate that the SF-4 crude extracellular preparation has measurable in vitro activities relevant to thrombosis research. These were observed with a crude preparation and a single-donor design, and no uninoculated-medium control was included. Because the preparation was the cell-free culture supernatant, contributions from residual medium components cannot be excluded and the effects cannot be attributed to a single extracellular protease.

3.6. The SF-4 Crude Extracellular Preparation Exhibits Fibrinolytic Activity

The fibrinolytic activity of the SF-4 crude extracellular preparation was evaluated using the fibrin agar plate assay, with nattokinase used as the positive control. Clear lytic zones were observed around both treatment wells (Figure 6), indicating that the SF-4 preparation was capable of degrading fibrin under the assay conditions.

Table 6. Whole-blood clotting time assessed by the Lee-White method

	Sample Code		
	Negative control	Positive control	SF-4
Clotting Time (min)	08.00 ± 0.00	Not measurable	09.30 ± 0.26

Note: Values are presented as mean ± SD (n = 3); the three experimental replicates were performed using blood obtained from the same donor. "Not measurable" indicates that no clot was observed during the observation period

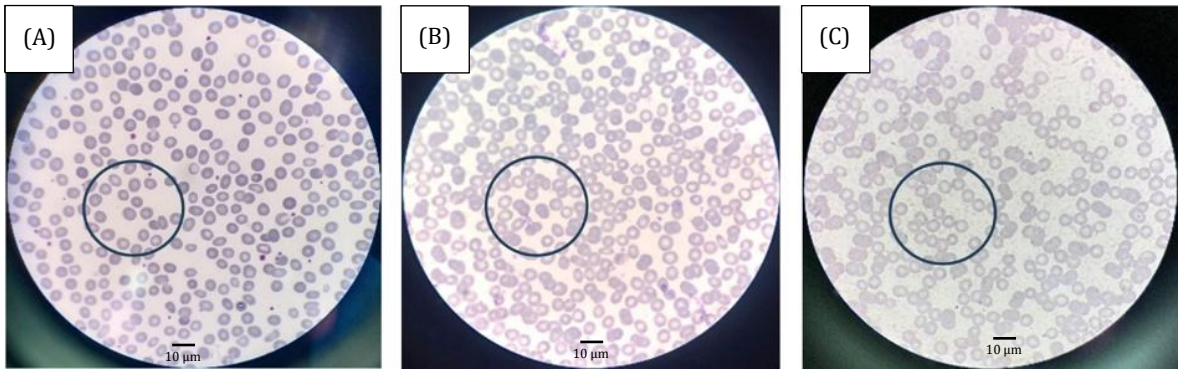


Figure 8. Wright-stained blood smears after treatment under the experimental conditions. (A) 10% EDTA. (B) Untreated blood. (C) SF-4 crude extracellular preparation. Black circles indicate representative areas showing the distribution of erythrocytes in each treatment group

4. CONCLUSIONS

This study demonstrated that a protease-producing bacterial isolate, SF-4, recovered from fermented bamboo clam (*Solen* sp.) *rusip* yielded a crude extracellular preparation with measurable fibrinolytic and blood clot-lysis activities and prolonged whole-blood clotting time under the experimental conditions. Based on 16S rRNA gene sequencing, SF-4 was assigned to the *Bacillus cereus* group, although species-level identification was not resolved. These findings provide preliminary functional evidence that fermented bamboo clam *rusip* is an underexplored microbial source of extracellular preparations with fibrinolysis-related activity. Because the preparation was crude and the active component and molecular mechanism, and dose relationship were not determined, the findings should be regarded as preliminary. Further purification, biochemical characterization, pathway-specific coagulation assays, and appropriate safety evaluation are required to clarify the biological properties of the preparation and its potential biomedical relevance.

Author contributions: MAA, SSD: Conceptualization, Methodology. MASS, CERP: Investigation. MAA: Formal analysis, Writing—original draft. MAAT, MAA, SSD: Data analysis. CERP: Sample collection and documentation. SSD, SNE, AHM: Supervision. MAAT, SSD, SNE, HF: Writing—review and editing. All authors read and approved the final manuscript.

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Ethics statement: This study was approved by the Health Research Ethics Committee under Ethical Clearance No. 551/KE/08/2024. All human blood samples were obtained from healthy adult volunteers after written informed consent had been obtained prior to sample collection. All procedures involving human participants were conducted in accordance with the ethical principles of the Declaration of Helsinki.

Conflict of interest: The authors declare no competing conflicts of interest.

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