

Purification and evaluating *in vitro* activity of a fibrinolytic protease produced by a mangrove isolate *Bacillus subtilis* AIBL_AMSB2_M7E32

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ABSTRACT

One of the main causes of heart-related problems globally is intravascular thrombosis. Microbial fibrinolytic proteases play a very crucial role in the management of thrombosis. Plasminogen activators and plasmin-like proteins, which are fibrinolytic enzymes of microbial origin, hydrolyze thrombin with great efficacy and without causing noticeable side effects. These fibrinolytic enzymes can also be manufactured efficiently and economically in huge quantities within a short period. However, the search for novel fibrinolytic enzymes demands difficult purification procedures, as well as physicochemical and structural-functional properties that provide information about their mode of action. In this study, the strain named *Bacillus subtilis* AIBL_AMSB2_M7E32, isolated from the Coringa mangrove soils, was shown to produce a fibrinolytic enzyme. The enzyme was purified by a four-step purification process that resulted in a 43.33-fold purification with a specific activity of 6368.20 (U/mg). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the purified enzyme was a protein of about 28 kDa and showed fibrinolytic activity on a zymogram. *In vitro* assay on blood clots revealed that the purified enzyme could lyse the blood effectively by a percentage of $72.96 \pm 0.16\%$, indicating that the enzyme could be used as a fibrinolytic agent. Furthermore, an observation for enzyme stability and its *in vivo* activity studies would be necessary for its application in the market.

1. INTRODUCTION

Cardiovascular diseases are the primary cause for deaths worldwide. It is believed that the production, accumulation, and clotting of fibrin in blood arteries, which results in intravascular thrombosis, is the cause of cardiovascular illnesses such as ischemic heart disease, ischemic stroke, and venous thromboembolism [1]. Furthermore, a functioning hemostatic system creates a thrombus to prevent excessive blood loss. Uncontrolled hemostasis causes unsuppressed thrombosis, which can lead to several cardiovascular problems (deep vein thrombosis, vascular congestion, embolism in the pulmonary veins, acute myocardial infarction, etc.), as fibrin clots accumulate inside blood vessels and eventually obstruct blood flow [2]. Plasminogen activators, which convert inactive plasminogen (zymogen) into active plasmin, are the only fibrinolytic drugs currently available in the market. In the blood, plasmin, a serine protease, dissolves fibrin clots and prevents thrombosis [3]. Tissue-type plasminogen activators, urokinase-type plasminogen activators, and bacterial plasminogen

activators are different types of plasminogen activators and have been used as thrombolytic drugs. Additionally, it has been noted that the commonly used plasminogen activators in fibrinolytic treatment have very limited fibrin specificity, have unfavorable side effects such as internal bleeding, and are neurotoxic at high doses [4]. In the past decade, various potent thrombolytic agents have been discovered and characterized from microorganisms [5-8], earthworms [9], snake venoms [10], insects [11], and leeches [12]. These enzymes are potential as both therapeutic agents and valuable tools for figuring out the fibrinolytic process. To avoid unwanted contamination and a huge cost of purification, these sources must be recommended after numerous stages of screening and feasibility studies [13]. Members of the *Bacillus* species are among the most studied microbes due to their innate ability to create fibrinolytic proteases without endotoxins and short fermentation cycles, with certain species being regarded as safe for medication administration [14,15]. The identification of microbial proteases that are plasmin-like and have a high level of fibrin selectivity has sparked recent interest in fibrinolytic drugs. The price of biomedical products like therapeutic enzymes depends on the quantity and kind of downstream processing processes [16]. Therefore, a simple and cost-effective purification technique is required to separate the desired bioproducts. The purification of a protein is crucial for examining its physical and biological features from the viewpoint of improving the knowledge on how enzymes work. Additionally, a substantial portion of the biochemical exams requires purifying the

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substances being examined because they need to be generally free of “contaminants” to be adequately characterized [17].

In this study, a novel fibrinolytic enzyme produced by *Bacillus subtilis* AIBL_AMSB2_M7E32 was purified by a four-step purification process. As previously reported, the strain was discovered in the soils of Coringa mangrove forests. The study also reports the *in vitro* activity of the fibrinolytic enzyme on blood clots, thus showing evidence of its thrombolytic potential.

2. MATERIALS AND METHODS

2.1. Chemicals

Chemicals and culture medium for this study including both thrombin and fibrinogen were purchased from Himedia Pvt. Ltd. in Mumbai. DEAE Cellulose and DEAE Sephadex-G50 were purchased from Sigma Pvt. Ltd. The agarose was purchased from Lonza Rockland, ME, USA. Trichloroacetic acid was supplied by Fisher Scientific, while Hammarsten Grade Casein was purchased from MP Biochemicals. Solvents used in this study were purchased from Merck. Fibrinogen was purchased from Sigma-Aldrich, USA. All the chemicals used for the downstream process were purchased from Sigma Pvt. Ltd. and Himedia Pvt. Ltd. in Mumbai.

2.2. Bacterial Strain

Bacillus subtilis AIBL_AMSB2_M7E32, a producer of the fibrinolytic enzyme, was isolated from Coringa Mangroves soils in the Kakinada District of Andhra Pradesh (Latitude 16°49'18.5"N, 82°17'53.2"E, Longitude 16.821803, 82.298114). By using ultraviolet mutagenesis and ethyl methyl sulfonate mutagenesis, the *B. subtilis* AIBL_AMSB2_M7E32 (accession number: OQ600796) strain was obtained, which has better fibrinolytic activity when compared with the wild strain. The variant was kept at -20°C as glycerol stocks and 4°C on an agar plate and was used for further studies.

2.3. Purification of Fibrinolytic Protease

After 12 h fermentation of *B. subtilis* AIBL_AMSB2_M7E32 in the optimized skim milk broth consisting of 3% sucrose, 3% yeast extract, 0.3% MgSO₄, 2% inoculum, and 1% skim milk with a pH of 7.2, the cell-free supernatant was obtained by centrifuging the whole broth at 10,000 rpm for 20 min at 4°C. Ammonium sulfate (AS) was added to the cell-free supernatant until 60% saturation was attained and the mixture was kept in equilibrium at 4°C for 16 h. The precipitate was obtained by further centrifugation of the mixture at 10,000 rpm for 15 min at 4°C. The precipitate was dissolved in a 10 mM Tris-HCl buffer of pH 7.2 and dialyzed overnight at 4°C against the same buffer using a 30 kDa MWCO membrane. After the completion of dialysis, the dialyzed enzyme was further purified by anion exchange chromatography on DEAE Cellulose (diethylaminoethyl) (Sigma Chemical Co.). The dialyzed fraction was passed through 10 ml of Sephadex column (13 cm × 30 cm), equilibrated against 25 mM Tris-HCl and 25 mM NaCl, and washed with the same buffer. The desired protein was eluted at an elution rate of 1 ml/min with different elution buffers of 25, 50, 75, 100, 125, and 150 mM of Tris-HCl. Fractions showing higher protein absorbance at 280 nm were pooled, concentrated, and further loaded in Sephadex-G 50 column and eluted against 0.1 M phosphate buffer (pH 7.2) at an elution rate of 1 ml per 2 min. All fractions were collected separately and the absorbance of each fraction was measured at 280 nm with a UV spectrophotometer (UV-1900 Shimadzu). All the purification experiments were carried out at 4°C.

2.4. Enzyme Activity Assay

Enzyme activity was assessed by measuring the total protein content (TPC) using Bradford's method for protein estimation. This was followed by conducting protease and fibrin assays. For the determination of the TPC, 2.5 ml of Bradford's reagent was added to the 0.5 ml of the enzyme-purified fractions, the reaction mixture was incubated in the dark for 10 min, and the absorbance was read at 595 nm. The total protein was calculated from the standard plot made by using different concentrations of standard protein bovine serum albumin [18]. For protease assay, 0.25 ml of the purified fractions of the enzyme was mixed with 1.25 ml of 0.6% casein in 50 mM potassium phosphate buffer having pH 7.5 and 0.25 ml and was incubated at 37°C for 15 min. The reaction was then stopped by the addition of 1.25 ml of 110 mM trichloroacetic acid and incubated at room temperature for 30 min. The reaction mixtures were centrifuged at 10,000 rpm for 15 min, and the absorbance of the supernatant was read at 280 nm. A tyrosine standard curve was used as a reference for protease activity. A unit of enzyme activity is defined as the amount of tyrosine (mg) released per minute per milliliter at 37°C [19].

For the fibrin assay, 0.1 ml of enzyme supernatant and 2.5 ml of Tris-HCl buffer (0.1 M, pH 7.8) containing calcium chloride (0.01 M) were added. To this mixture, 2.5 ml of fibrin (1%) was added and incubated at 37°C for 30 min. The reaction was then terminated by adding 5.0 ml of trichloroacetic acid (0.11 M) containing sodium acetate (0.22 M) and acetic acid (0.33 M). Furthermore, the reaction mixture was centrifuged at 10,000 rpm for 10 min, and the supernatant was collected to read absorbance at 275 nm. One unit of fibrinolytic activity was referred to in terms of the amount of enzyme per 1 mg of L-tyrosine per minute under standard assay conditions [20].

2.5. SDS and Zymography

To determine the molecular weight of the purified enzyme, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% polyacrylamide separating gel with 5% stacking gel. Following electrophoresis, the gel was incubated in a fixing solution consisting of methanol: glacial acetic acid: distilled water solution (5:1:4). To the gel, silver staining was performed [21]. A wide-range molecular marker, pre-stained protein ladder (Himedia) with a range of (11–100 kDa) was used for calibration. Fibrin zymography was carried out to determine the fibrinolytic activity of the samples on SDS-PAGE. SDS-PAGE of 12% was polymerized with 0.2% fibrinogen and 50 µl (10 NIH Units). The samples to be loaded were unheated, and the loading buffer was devoid of the reducing agent. Fibrin zymography was carried out at 4°C for 1.5 h at 100 V. After zymography, the gel was soaked in Triton X-100 containing 50 mM Tris (pH 7.4) for 30 min and further washed with distilled water. After this, the gel was incubated in 30 mM Tris buffer (pH 7.4) containing 200 mM NaCl, 10 mM CaCl₂, and 0.02% NaN₃ (0.1 M, pH 7.2) at 37°C for 16 h. The following day, the gel was stained with Coomassie Blue-R-250 for 1 h and de-stained [22].

2.6. Assessment of Fibrinolytic Activity of the Enzyme *In Vitro*

To evaluate the *in vitro* activity of the purified fibrinolytic enzyme, a modified protocol by D Souza et al. (2020) was followed, wherein 0.5 ml of blood was transferred in a pre-weighed sterile 1.5-ml centrifuge tube and incubated at 37°C for 45 min for the formation of a clot [23]. After clot formation, the serum was removed without disturbing the clot formed. Each 1.5-ml centrifuge tube with the clot was weighed and labeled W₁. To determine the clot weight, the following formula was used: (weight of clot containing the 1.5-ml centrifuge tube – the weight of the 1.5-ml centrifuge tube alone). Furthermore, different

volumes of 5, 10, 50, and 100 μ l of the purified enzyme samples were added to the 1.5-ml centrifuge tubes. To the control tube, 100 μ l of saline was added. All the 1.5-ml centrifuge tubes were incubated at 37°C for 24 and 48 h and observed for clot lysis. After incubation, the dissolved blood was removed and the 1.5-ml centrifuge tube was weighed again to observe the difference in clot lysis. The difference in the weight taken before and after lysis is given as the percentage of clot lysis. The experiment was carried out in triplicates. The formula of clot lysis is given as follows:

$$\text{Lysis percentage} = 100 - \{(W3 - W1)/(W2 - W1)\} \times 100$$

where $W1$ is the weight of the empty 1.5-ml centrifuge tube, $W2$ is the weight of the 1.5-ml centrifuge tube with the blood clot, and $W3$ is the weight of the 1.5-ml centrifuge tube after clot lysis.

2.7. Statistical Analysis

IBM SPSS Statistics Version 24.0, one-way analysis of variance (ANOVA) was combined with Duncan multiple range test. With a statistical difference of $p < 0.05$, the results were presented as means SE (variable).

3. RESULTS

3.1. Purification of Fibrinolytic Protease

The fibrinolytic enzyme was extracted from the supernatant of *B. subtilis* AIBL_AMSB2_M7E32 using AS precipitation, and dialysis was followed by DEAE-Cellulose and Sephadex G-50 chromatography. The TPC (mg/ml) of the purified enzymes revealed that the final purified TPC of the enzyme sample after gel filtration (GF1) was found to be $0.05 \pm 0e$ mg/ml as illustrated in Figure 1. Subsequently, the enzyme activity in each purification step was determined, and it was found that the final fraction after gel filtration (GF1) had an enzyme activity (U) of $0.42 \pm 0.008e$ as shown in Figure 2. The purified sample exhibited fibrinolytic activity (U/ml) at the rates of $564.28 \pm 0.04a$, $453.18 \pm 12.87b$, $362.65 \pm 0.00c$, $332.48 \pm 0.00d$, and $320.82 \pm 0.00d$ after AS, Dialyzed, DEAE-Cellulose (IE), and Sephadex G 50 (GF1), respectively, as shown in Figure 3. The effectiveness of filtration at each stage has had a major effect. Ion exchange chromatography and gel filtration chromatography achieved a higher degree of purification compared with the previous stage. The final purification degree was

43.33-fold with a specific activity of 6368.20 (U/mg). The purification steps are summarized in Table 1. The active fraction was evaluated at 280 nm and the majority of contaminants were removed by Sephadex G 50 yielding the desired protein using SDS-PAGE.

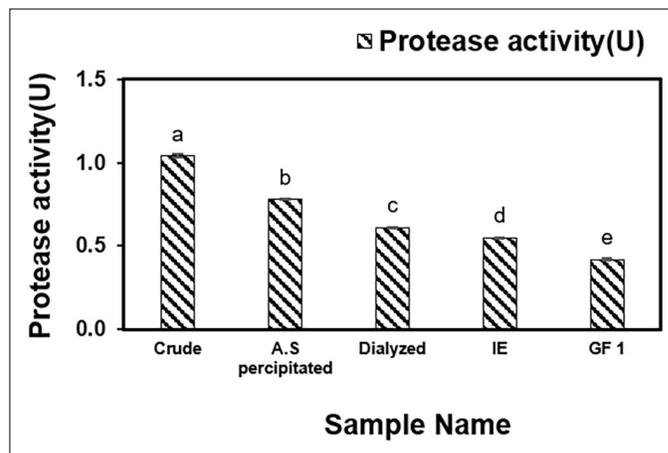


Figure 2: Protease activity (U) after four-step purification of *B. subtilis* AIBL_AMSB2_M7 isolated from *Coringa* mangroves soil samples. The values indicate the mean \pm SE of three replicates. Duncan's multiple range test ANOVA revealed that values without a matching alphabet were substantially different ($p < 0.05$).

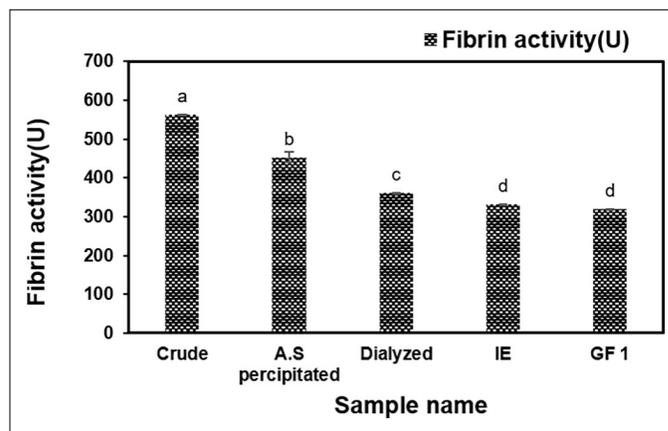


Figure 3: Fibrin activity (U/ml) after four-step purification of *B. subtilis* AIBL_AMSB2_M7 isolated from *Coringa* mangroves soil samples. The values indicate the mean \pm SE of three replicates. Duncan's multiple range test ANOVA revealed that values without a matching alphabet were substantially different ($p < 0.05$).

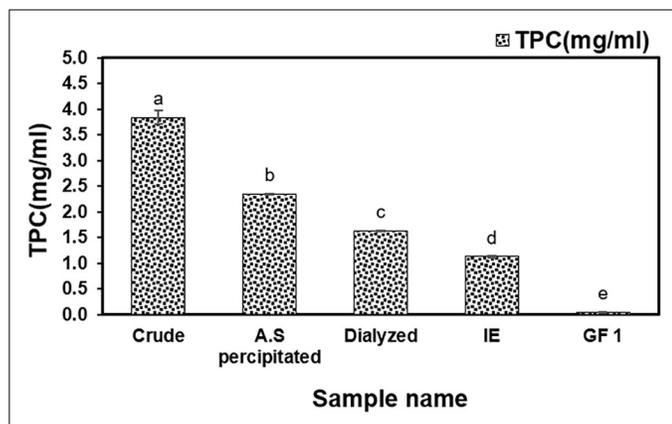


Figure 1: TPC (mg/ml) after four-step purification of *B. subtilis* AIBL_AMSB2_M7 isolated from *Coringa* mangroves soil samples. The values indicate the mean \pm SE of three replicates. Duncan's multiple range test ANOVA revealed that values without a matching alphabet were substantially different ($p < 0.05$).

Table 1: A stepwise purification of fibrinolytic enzyme from *B. subtilis* AIBL_AMSB2_M7E32.

Sample Name	Total Protein (mg/ml)	Total Fibrinolytic Activity (U/ml)	Specific Activity (U/mg)	Purification Fold (\times fold)
Crude	3.84 \pm 0.15a	564.28 \pm 0.04a	146.94	1
AS precipitation	2.35 \pm 0.00b	453.18 \pm 12.87b	192.51	1.31
Dialyzed	1.63 \pm 0.01c	362.65 \pm 0.00c	222.48	1.51
DEAE Cellulose 52	1.13 \pm 0.01d	332.48 \pm 0.00d	293.87	2.00
DEAE G-50	0.05 \pm 0e	320.82 \pm 0.00d	6368.20	43.33

The data are presented as mean \pm SD ($n = 3$). ANOVA revealed that values without a matching alphabet in the same column were substantially different ($p < 0.05$).

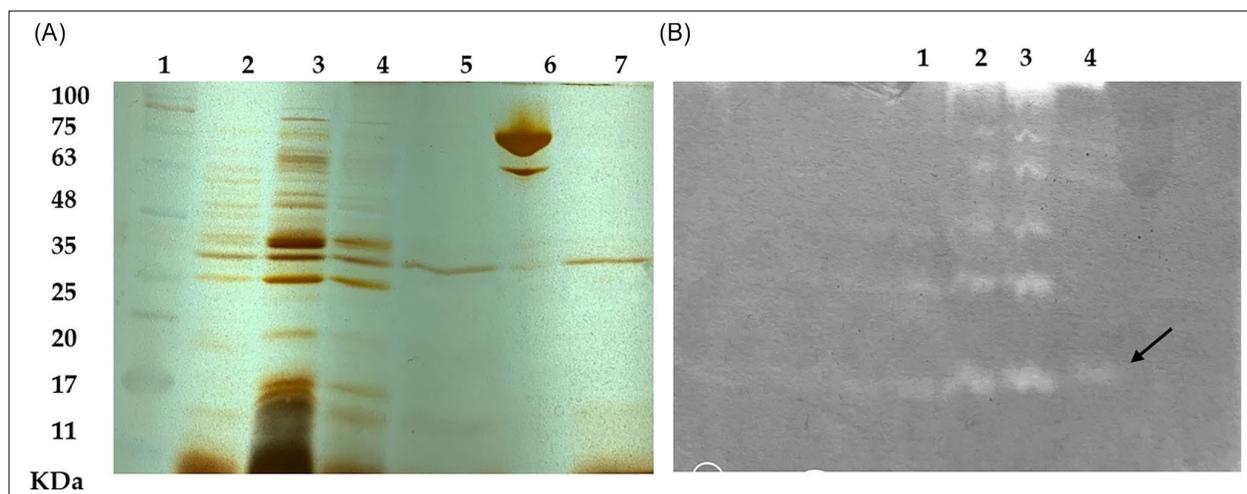


Figure 4: (A) SDS-PAGE of the purified fibrinolytic enzyme from *B. subtilis* AIBL_AMSB2_M7E32. Lane 1: molecular weight markers; Lane 2: crude enzyme; Lane 3: enzyme dialysate; Lane 4: DEAE cellulose anion exchange active fraction; Lane 5: purified enzyme after DEAE Sephadex-G50 Gel filtration; Lane 6: streptokinase marker; Lane 7: purified enzyme after DEAE Sephadex-G50 Gel filtration corresponding to 28 kDa. (B) Fibrin zymography (12%) of the purified fibrinolytic enzyme from *B. subtilis* AIBL_AMSB2_M7E32 as indicated by the zone of clearance (as indicated by the arrow).

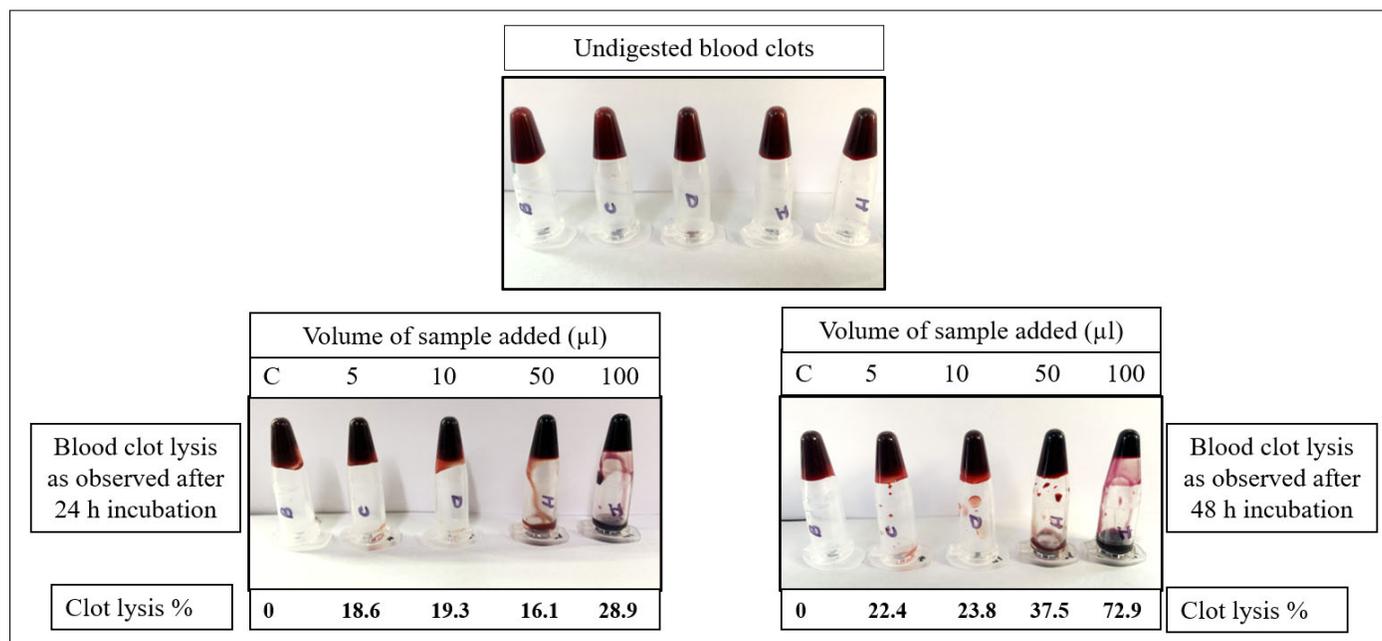


Figure 5: Blood clot lysis experiment with purified fibrinolytic enzyme from *B. subtilis* AIBL_AMSB2_M7E32 for a total of 48 h of incubation.

3.2. SDS and Zymography

To verify the molecular weight and purity of the purified enzyme, SDS-PAGE and zymography were carried out. The molecular weight of the enzyme when estimated by SDS-PAGE was approximately 28 kDa when compared with a standard protein marker (Himedia). Fibrin zymography results indicated that purified protein from the culture *B. subtilis* AIBL_AMSB2_M7E32 showed fibrinolytic activity by the formation of a thin clear band of fibrinolysis on the separating gel (non-denaturing) [Figure 4]. The result of zymography correlated with the molecular weight of the purified protein in SDS-PAGE.

3.3. Assessment of Fibrinolytic Activity of the Enzyme *In Vitro*

The purified fibrinolytic enzyme isolated from *B. subtilis* AIBL_AMSB2_M7E32 was evaluated for lysis of the blood clot after 24 and 48 h,

respectively. Different volumes of purified enzyme samples 5, 10, 50, and 100 µl were used. The percentage of clot lysis after the treatment of the enzyme is shown in Figure 5. Clot digested with 100 µl of the enzyme showed a maximum lysis percentage of $72.96 \pm 0.16a$ after incubation at 37°C for 48 h and $28.982 \pm 0.33a$ after incubation at 37°C for 24 h. It is well acknowledged that examining the thrombolytic activity of fibrinolytic enzymes using *in vitro* blood clot lysis is an accurate approach [24].

4. DISCUSSION

In recent decades, several thrombolytic drugs have been discovered and characterized in the past few decades from a variety of sources, of which the most prominent ones are fermented foods [25]. Therefore, increasing attention is being paid to fibrinolytic enzymes from non-food sources.

However, the majority of findings demonstrated that a range of extracellular and intracellular fibrinolytic enzymes are produced by *Bacillus* species. The fibrinolytic enzyme produced by *B. subtilis* was reportedly employed as a fibrinolytic treatment as early as the 1990s [16]. The use of an enzyme ultimately determines the degree of purity it achieves. Numerous methods for purifying enzymes are designed to provide better purity and strong catalytic activity [26]. This study reported that a Coringa mangrove isolate, namely, *B. subtilis* AIBL_AMSB2_M7E32, showed fibrinolytic activity, with a 43.33-fold purification and a molecular weight of 28 kDa. To the best of our knowledge, there are no reports of a purified fibrinolytic enzyme of 28 kDa produced by *B. subtilis* AIBL_AMSB2_M7E32 from Coringa mangrove soils. Furthermore, the fibrinolytic enzyme showed a specific activity of 6368.20 (U/mg), which was higher than the previously reported studies where a study reported that a purified fibrinolytic enzyme from *Bacillus* sp. like *Bacillus tequilensis* showed an activity of 2373.59 ± 54.81^a with a purification fold of $329.76 \pm 12.34a$ [27]. Another fibrinolytic enzyme from *Bacillus velezensis* Z01 showed a specific activity of 76.46 U/ μ g with a purification of 7.77-fold [28]. Also, the purification of BSF 1 Protease from *B. subtilis* A26 resulted in a specific activity of 3211 U/mg with a purification of 4.97-fold [29]. A five-step purification of fibrinolytic enzyme-producing *B. subtilis* DC33 resulted in a 34.6-fold purification with a specific activity of 15495.9 U/mg [30]. This molecular weight was similar to the previously reported nattokinase of approximately 27.7 kDa [31]. Some studies have reported a subtilisin DFE of 28 kDa that was isolated from *Bacillus amyloliquefaciens* DC-4 [32]. However, the molecular weight of the purified enzyme of our study was lower than the commonly used thrombolytic agents such as streptokinase (47 kDa) and urokinase (54 kDa), thus showing lesser chances for antigenicity and being a safer thrombolytic drug [33]. Some fibrinolytic enzymes so far reported by *Bacillus* sp and from fermented food sources are a 27.7 kDa enzyme from *B. subtilis* DC 33 isolated from *Douchi* a fermented food [30], and 27 or 29.60 kDa enzyme from *Bacillus* sp. JS2 isolated from Jeotgal [34], a 31 kDa enzyme BK17 from *Bacillus* BK-17 [35], and a 45 kDa enzyme from *Bacillus* sp. KDO13; in contrast, we report a purified 28 kDa fibrinolytic enzyme from a non-food source, i.e., Coringa soils for the very first time. Furthermore, in our study, clot dissolution of $72.96 \pm 0.16\%$ was observed after 48 h of incubation, which was consistent with other reports showing that *B. amyloliquefaciens* strain KJ10 isolated from soya bean paste had a clot dissolution of $28 \pm 1.8\%$ [37]. *B. subtilis* LD-8547 isolated from *Douchi* only showed a clot resolution of 38.4% [38]. The use of commercially available streptokinase showed clot lysing activity between 62.2% and 70.8% [24]. *B. cereus* SRM-001, isolated from the blood-containing soil of a chicken coop, showed that almost 70–80% of the blood clot was dissolved by the fibrinolytic enzyme [39]. The strong thrombolytic activity of the protease demonstrated by the *in vitro* blood clot lysis experiment supports its potential application as a bioactive molecule for thrombolytic treatment, but the stability of the enzyme, its amino acid composition, and some *in vivo* studies need to be further investigated.

5. CONCLUSION

The fibrinolytic drugs available on the market generally have low selectivity and undesirable side effects such as internal hemorrhage and neurotoxicity. Microbial-based fibrinolytic enzymes have proven to be safe in this aspect. Therefore, the search for microbial fibrinolytic enzymes has been intense over the past decade. Therefore, current research is focused on the development of new fibrinolytic drugs with improved therapeutic properties and selectivity. In this study, we report the purification of a fibrinolytic enzyme from *B. subtilis* AIBL_AMSB2_M7E32 isolated from Coringa mangrove soils. The enzyme was purified

in a four-step purification process that yielded a purification of 43.33-fold with a specific activity of 6368.20 (U/mg). The molecular weight of the isolated fibrinolytic enzyme was calculated to be 28 kDa, and it appeared homogeneous on SDS-PAGE. The zymographic analysis also showed the fibrinolytic activity of the purified enzyme. When its activity to lyse blood clots in blood samples was evaluated, the purified enzyme showed a lysis percentage of $72.96 \pm 0.16a\%$ after 48 h of incubation. Further studies need to be conducted to confirm the performance of the enzyme in terms of stability and *in vivo* activity.

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7. AUTHOR CONTRIBUTIONS

SB participated in the concept and research design, data collection, data analysis, interpretation of results, and writing the manuscript. AKM contributed to the idea and design of the experimental work and participated in the critical revision of the article. Both authors approved the manuscript.

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

12. PUBLISHER'S NOTE

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