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Production, Partial Purification and Preliminary Characterization of a Fibrinolytic Enzyme-Active Preparation from a Soil-Derived Bacterial Isolate

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Abstract

Nattokinase (NK) is a fibrinolytic serine protease associated with fermented soybean products and has attracted considerable research interest because of its ability to hydrolyze fibrin and related protein substrates. The present study investigated the isolation of a natto-kinase-producing bacterial strain, optimization of production conditions, recovery and partial purification of the enzyme, biochemical characterization, and its qualitative fibrinolytic activity against a pre-formed blood clot. Bacteria were isolated from two soil samples collected in Lucknow by serial dilution and spread plating. Three colony morphotypes were evaluated, and screening on skim milk agar identified the isolate from streak plate 2 as the strongest casein-hydrolyzing culture. The selected isolate was reported as Bacillus Pumulis. One-factor-at-a-time optimization showed that the production medium containing tryptone (10 g/L), yeast extract (5 g/L), dextrose (5 g/L), and NaCl (10 g/L), adjusted to pH 9 and incubated at 37 °C, produced the highest reported activity of 0.0477 U/mL/min. The optimized fermentation was performed in 200 mL medium at 37 °C and 120 rpm for 7 days. The culture supernatant was recovered by centrifugation and subjected to 40% ammonium sulfate precipitation followed by dialysis. Protein concentration was 34.765 µg/mL in the crude enzyme and 24.00 µg/mL after purification, while caseinolytic activity was 0.0477 and 0.0376 U/mL/min, respectively. The purified preparation showed its highest activity at pH 9 (0.0378 U/mL/min) and 37 °C (0.039 U/mL/min). CaCl2 increased activity to 0.0504 U/mL/min, whereas MgCl2 produced 0.0334 U/mL/min. EDTA reduced activity to 0.0070 U/mL/min. SDS-PAGE analysis showed reported bands at approximately 35 and 29 kDa. In the blood-clot assay, addition of 100 µL purified enzyme to a pre-formed clot resulted in visible hydrolysis over the observation period, whereas distilled water served as the control. These findings demonstrate measurable extracellular proteolytic and fibrinolytic activity in the selected bacterial isolate under the reported culture conditions and provide a basis for further quantitative purification, molecular identification, and fibrinolytic validation.

Keywords: *Nattokinase, fibrinolytic enzyme; Bacillus; caseinolytic activity; fermentation; ammonium sulfate precipitation; dialysis; blood clot hydrolysis.*

1.0 Introduction

Thrombotic disorders are associated with the formation and persistence of blood clots within the vascular system and represent an important area of biomedical research. Although coagulation is essential for preventing excessive blood loss following vascular injury, uncontrolled clot formation can interfere with normal blood circulation and contribute to pathological conditions (Seré & Hackeng, 2003) . Fibrin is a major structural component of blood clots and forms an insoluble network that stabilizes the clot. The controlled degradation of fibrin through fibrinolysis is therefore essential for maintaining vascular homeostasis. Fibrinolytic enzymes capable of degrading fibrin and other protein substrates have consequently attracted considerable interest because of their potential biomedical and biotechnological applications (Nascimento et al., 2017).

Microorganisms represent important sources of extracellular proteolytic enzymes because they can be cultivated under controlled conditions and may secrete enzymes into the surrounding culture medium. Microbial enzyme production can be influenced by nutritional and physicochemical parameters, including carbon and nitrogen sources, pH, temperature and the concentration of individual medium components (VADLAMANI & PARCHA, 2011a) . Optimization of these parameters is therefore an important step in developing an efficient microbial enzyme-production process. Soil microorganisms, in particular, represent a diverse source of bacteria with potentially useful extracellular enzymatic activities. Screening environmental bacterial isolates for proteolytic activity provides an initial strategy for identifying promising enzyme-producing microorganisms (VADLAMANI & PARCHA, 2011b) .

Among microbial fibrinolytic enzymes, nattokinase has received considerable research attention. Nattokinase is associated with natto, a traditional Japanese fermented soybean food, and has been investigated because of its reported fibrinolytic activity. The literature describes nattokinase as a serine protease with fibrinolytic properties and includes investigations of fibrinolytic enzymes produced by Bacillus species, including organisms associated with Bacillus subtilis and Bacillus natto (Mohanasrinivasan et al., 2017) . Previous studies have examined different aspects of nattokinase production, purification, biochemical characterization and potential thrombolytic applications. The association of fibrinolytic enzyme production with Bacillus species is particularly relevant because members of this genus are recognized producers of extracellular proteases and can therefore serve as potential sources of industrially and biomedically relevant enzymes (Coutinho et al., 2024a) .

The initial identification of a promising enzyme-producing microorganism generally requires an appropriate screening strategy. Protein-containing solid media, such as skim milk or casein-containing agar, can be used for preliminary detection of extracellular protease production. Hydrolysis of casein produces a clear zone around bacterial growth, providing a visual indication of extracellular proteolytic activity (Sahadat Ali et al., 2025a). The extent of hydrolysis can consequently be used as an initial criterion for selecting bacterial isolates for further investigation. However, casein hydrolysis represents general proteolytic activity and does not by itself establish fibrinolytic specificity. Further enzymatic characterization and functional assessment are therefore necessary to determine the potential of a selected bacterial isolate as a source of a fibrinolytic enzyme (Khikmah et al., 2024) .

The production of extracellular enzymes is strongly influenced by culture conditions. Carbon and nitrogen sources provide essential nutrients for microbial metabolism and protein synthesis, whereas pH and temperature can influence cellular growth, enzyme secretion and enzyme stability. The concentration of specific nutritional components may also affect enzyme production. Accordingly, optimization of culture conditions is an important component of microbial enzyme-production studies (Morabandza et al., 2021) . In the present work, selected nutritional and physicochemical parameters were evaluated using a one-factor-at-a-time approach. Nitrogen source, carbon source, pH, tryptone concentration and incubation temperature were investigated individually, and the selected conditions were subsequently used for fermentation and enzyme production.

Following fermentation, recovery and partial purification are important for obtaining an enzyme preparation suitable for biochemical characterization. Separation of bacterial cells from the fermented broth provides a cell-free extracellular enzyme preparation. Protein precipitation using ammonium sulfate can then be employed as an initial concentration and partial purification step, followed by dialysis to remove excess salts and other small molecules (Khadka et al., 2024). In the present study, the extracellular enzyme was recovered from the culture supernatant and subjected to ammonium sulfate precipitation

followed by dialysis. The crude and processed preparations were evaluated for protein concentration and caseinolytic activity to document their biochemical characteristics.

Biochemical characterization provides additional information regarding the functional properties of an enzyme preparation. pH and temperature can substantially influence enzymatic activity, while metal ions and chemical inhibitors may affect catalytic performance. Evaluation of these parameters therefore provides preliminary information about the conditions under which the enzyme preparation exhibits measurable activity. SDS-PAGE can additionally be used to determine the apparent molecular-weight profile of proteins present in a partially purified preparation. However, molecular-weight bands alone cannot establish the identity of a particular protein as nattokinase, and further molecular identification and purification would be required for definitive confirmation.

Assessment of fibrinolytic potential represents an important component of nattokinase research. A preliminary blood-clot hydrolysis assay can provide qualitative evidence of clot degradation by comparing an enzyme-treated clot with an untreated or control clot. Although such an assay can indicate potential fibrinolytic activity, quantitative fibrin-degradation assays and molecular characterization would provide stronger evidence for establishing fibrinolytic specificity. Therefore, observations from a preliminary clot-hydrolysis assay should be interpreted as initial functional evidence rather than definitive proof of nattokinase identity (Sahoo & Sahoo, 2020) .

The main objective of the present study was to isolate and screen a bacterial strain capable of producing nattokinase from soil and to establish suitable conditions for its production. The selected bacterial isolate was evaluated for extracellular proteolytic activity and subjected to optimization of nutritional and physicochemical parameters, including carbon source, nitrogen source, pH, tryptone concentration and incubation temperature. The optimized conditions were subsequently employed for fermentation and enzyme production. The produced enzyme was recovered from the culture supernatant and partially purified by ammonium sulfate precipitation followed by dialysis (Ju et al., 2019) . The partially purified enzyme was then characterized with respect to pH and temperature effects and the influence of selected activators and inhibitors. Its apparent protein profile was examined by SDS-PAGE, and its ability to hydrolyze a pre-formed blood clot was assessed as a preliminary indication of fibrinolytic activity. Overall, the study was designed to evaluate a soil-derived bacterial isolate as a potential source of nattokinase through a sequential workflow encompassing bacterial isolation, proteolytic screening, production optimization, fermentation, partial purification, biochemical characterization and preliminary fibrinolytic assessment (Xin et al., 2018) .

2. Materials and Methods

2.1 Sample collection

Two soil samples were collected and designated as Sample 1 and Sample 2. Sample 1 was dark brown and moist with a pH of 7.5, whereas Sample 2 was light brown and dry with a pH of 5.8. The experimental work was carried out at MRD LifeSciences, Lucknow.

2.2 Isolation and purification of bacterial cultures

For bacterial isolation, 1 g of each sample was suspended separately in 10 mL of sterile 0.85% normal saline and vortexed thoroughly. A 1 mL aliquot of each suspension was transferred to 9.0 mL sterile normal saline and mixed to prepare serial decimal dilutions. From each of the last three dilution tubes, 20 µL was spread aseptically onto solidified agar plates. The plates were incubated at 37 °C for 24 h, and distinct colonies were selected for purification. Selected colonies were purified by repeated streaking on nutrient agar. A sterile inoculating loop or needle was flame-sterilized, cooled and used to transfer bacterial growth to the agar surface. After streaking, the plates were incubated at 37 °C for 24 h. Representative colonies were examined for shape, margin, elevation, pigmentation, surface, texture and opacity (Coskun et al., 2025) .

2.3 Screening for extracellular proteolytic activity

Proteolytic activity was screened on skim milk agar containing casein as the substrate. The purified isolates were inoculated onto the medium and incubated at approximately 37 °C for 24-48 h. A clear zone around the bacterial growth was considered indicative of casein hydrolysis. The screening response was recorded qualitatively as low, medium or high. The culture from streak plate 2 showed the strongest secondary response (+++, high) and was selected for subsequent experiments. The selected isolate was reported as *Bacillus Pumulis* in the original experimental work; this designation is

retained as reported because molecular identification data were not available ("EVALUATION OF PROTEOLYTIC Bacillus Spp. ISOLATED FROM SOIL AND CHARACTERISTION OF THEIR GROWTH AND ACTIVITY OF PROTEASES," 2017; Sahadat Ali et al., 2025b) .

2.4 Growth-curve analysis

Growth kinetics of the selected culture were monitored in 100 mL broth containing NaCl, tryptone, yeast extract and dextrose. The culture was incubated in a shaker incubator at 120 rpm, and optical density was measured at 600 nm at approximately 1 h intervals. Uninoculated broth was used as the control (Sezonov et al., 2007) .

2.5 One-factor-at-a-time optimization of enzyme production

The production conditions were optimized using a one-factor-at-a-time approach, in which individual nutritional and physicochemical parameters were varied while the other production conditions were maintained. The parameters evaluated included nitrogen source, carbon source, initial pH, tryptone concentration and incubation temperature. Peptone (10 g/L) and tryptone (10 g/L) were evaluated as nitrogen sources, while dextrose (5 g/L) and maltose (5 g/L) were evaluated as carbon sources (Chavan et al., 2025) . The initial pH was evaluated at 5, 7 and 9, and tryptone concentration was varied between 5, 10 and 15 g/L. Incubation temperatures of 4, 37 and 50 °C were also evaluated. The basal production medium consisted of tryptone (10 g/L), yeast extract (5 g/L), dextrose (5 g/L) and NaCl (10 g/L), adjusted to pH 9 and incubated at 37 °C. The cultures were incubated for 72 h, after which protein concentration and caseinolytic activity were determined (Park & Kim, 2015) .

Table 1. Experimental conditions evaluated for optimization of enzyme production using the one-factor-at-a-time approach.

Modified Media (MM)	**Factors**	**Composition**
PM	Production medium	Tryptone 10 g/L + yeast extract 5 g/L + dextrose 5 g/L + NaCl 10 g/L; pH 9; 37 °C
MM1	Nitrogen source	Peptone, 10 g/L
MM2	Nitrogen source	Tryptone, 10 g/L
MM3	Carbon source	Dextrose, 5 g/L
MM4	Carbon source	Maltose, 5 g/L
MM5	Initial pH	pH 5
MM6	Initial pH	pH 7
MM7	Initial pH	pH 9
MM8	Tryptone concentration	5 g/L
MM9	Tryptone concentration	10 g/L
MM10	Tryptone concentration	15 g/L
MM11	Incubation temperature	4 °C

2.6 Calibration curves

A bovine serum albumin (BSA) calibration curve was prepared for protein estimation using the Bradford assay, with absorbance measured at 595 nm. The reported regression equation was y = 0.0158x − 0.0007 (R^2 = 0.9925). A tyrosine calibration curve was also prepared for estimation of enzyme activity at 560 nm, with the reported regression equation y = 0.0394x + 0.0569 (R^2 = 0.9854) (Srivastava et al., 2025) .

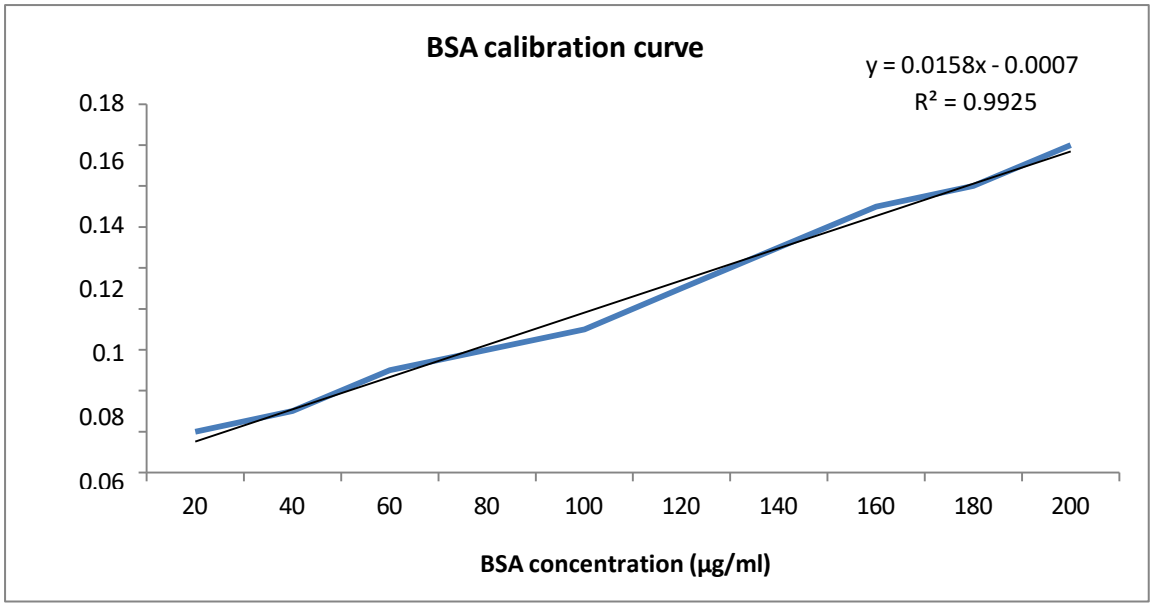


Figure 1: BSA Calibration curve for Bradford method

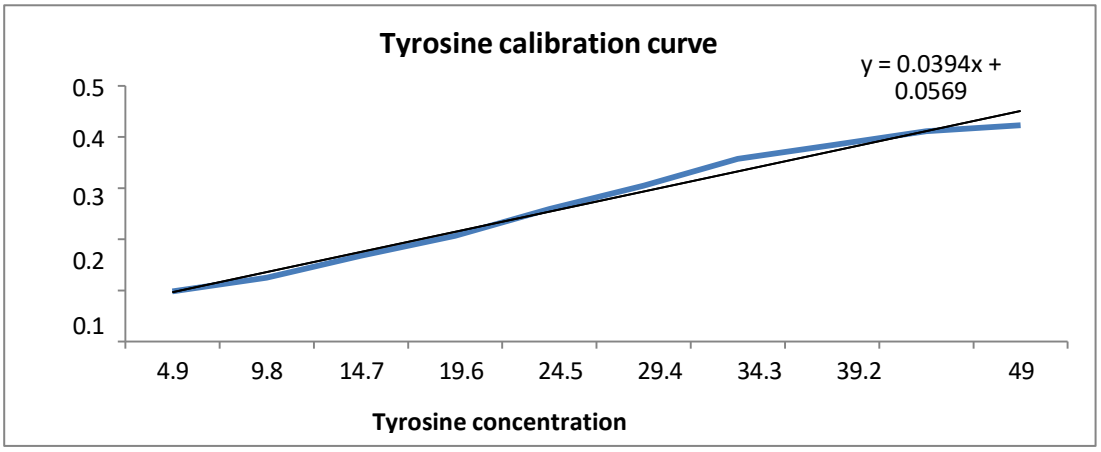


Figure 2: Standard Curve for Tyrosine at 560 nm

2.7 Fermentation and recovery of the extracellular enzyme

The selected bacterial culture was inoculated into 200 mL of optimized production medium containing tryptone (10 g/L), yeast extract (5 g/L), dextrose (5 g/L) and NaCl (10 g/L), adjusted to pH 9.0. The culture was incubated at 37 °C with shaking at 120 rpm for 7 days. The fermented broth was centrifuged at 10,000 rpm for 10 min at 4 °C, and the cell-free supernatant was collected as the crude extracellular enzyme preparation (Deb et al., 2013; Gu et al., 2015) .

2.8 Protein estimation by Bradford assay

For protein estimation, 1000 µL distilled water was used as the control. For the test, 500 µL distilled water was mixed with 500 µL of the centrifuged enzyme sample. Bradford reagent (2.5 mL) was added to each tube, followed by incubation in the dark for 30 min. Absorbance was measured at 595 nm, and protein concentration was obtained from the BSA calibration curve (Kielkopf et al., 2020a) .

2.9 Caseinolytic activity assay

Caseinolytic activity was determined using 2% casein as substrate. Potassium phosphate buffer (700 µL), 2% casein (100 µL) and enzyme solution (100 µL) were mixed and incubated at 37 °C for 5 min. Trichloroacetic acid (TCA; 100 µL) was then added, and the mixture was incubated at 4 °C for 30 min (Kielkopf et al., 2020b) . The samples were centrifuged at 5,000 rpm for 5 min, and the supernatant was collected. Absorbance was measured at 560 nm. Enzyme activity was expressed as U/mL/min. Formula for Enzyme activity Test: (Janssen & Boyle, 2019)

E.A = $\mu\, mol\ of\ Tyrosine\ release \times Total\ volume\ of\ assay\ /\ volume\ of\ Enzyme \times Time \times Volume\ used\ in\ colorimeter$

2.10 Partial purification by ammonium sulfate precipitation and dialysis

The cell-free supernatant was subjected to salting-out precipitation using solid ammonium sulfate at the reported 40% condition. After 24 h, the precipitated protein was collected by centrifugation at 10,000 rpm for 10 min, dissolved in Tris buffer and dialyzed overnight against the same buffer with buffer changes. The dialyzed preparation was used for subsequent protein estimation and enzyme-activity assays (Page & Thorpe, 2009) .

2.11 Biochemical characterization

The effect of pH was evaluated using 2% casein prepared in appropriate buffers at pH 5, 7 and 9. The enzyme-substrate mixtures were incubated for 10 min at 37 °C before activity measurement. Temperature dependence was evaluated using 2% casein in 100 mM Tris buffer (pH 7) at 4, 37 and 50 °C. Calcium chloride (CaCl2) and magnesium chloride (MgCl2) were tested as activators, whereas EDTA and SDS were tested as inhibitors. Each compound was used at 1 mg/mL, and activity was determined using the caseinolytic assay (Fromm, 1975) .

2.12 SDS-PAGE

The partially purified enzyme preparation was analyzed by SDS-PAGE to determine the apparent molecular-weight profile. The reported preparation showed bands at approximately 35 and 29 kDa (Kumar et al., 2004) .

2.13 Blood-clot hydrolysis assay

Blood from a healthy donor was transferred into two microcentrifuge tubes and allowed to clot for 48 h at room temperature. After clot formation, the tubes were centrifuged at 10,000 rpm for 10 min and the serum was discarded. Partially purified enzyme preparation (100 µL) was added to one clot, while 100 µL distilled water was added to the control clot. Both tubes were incubated for 24 h, and clot hydrolysis was observed at the reported observation times (Vijayaraghavan et al., 2016) .

3. Results

3.1 Sample characteristics

The two collected soils differed in physical appearance and pH. Sample 1 was dark brown and moist with a pH of 7.5, whereas Sample 2 was light brown and dry with a pH of 5.8.

Table 2. Physical characteristics and pH of the collected soil samples

Sample	Colour	Texture	pH
Sample 1	Dark brown	Moist	7.5
Sample 2	Light brown	Dry	5.8

3.2 Isolation, purification and screening

Serial dilution and spread plating produced bacterial colonies that were subsequently purified by streaking. Three representative colony types were characterized. All three colonies were circular and non-pigmented with smooth surfaces, while differences were observed in margin, elevation, texture and opacity. Among the screened cultures, streak plate 2 showed the strongest casein hydrolysis response, with a secondary-screening score of +++ (High), compared with ++ (Low) for plate 1 and ++ (Medium) for plate 3. The selected culture was subsequently reported as *Bacillus Pumulis.*

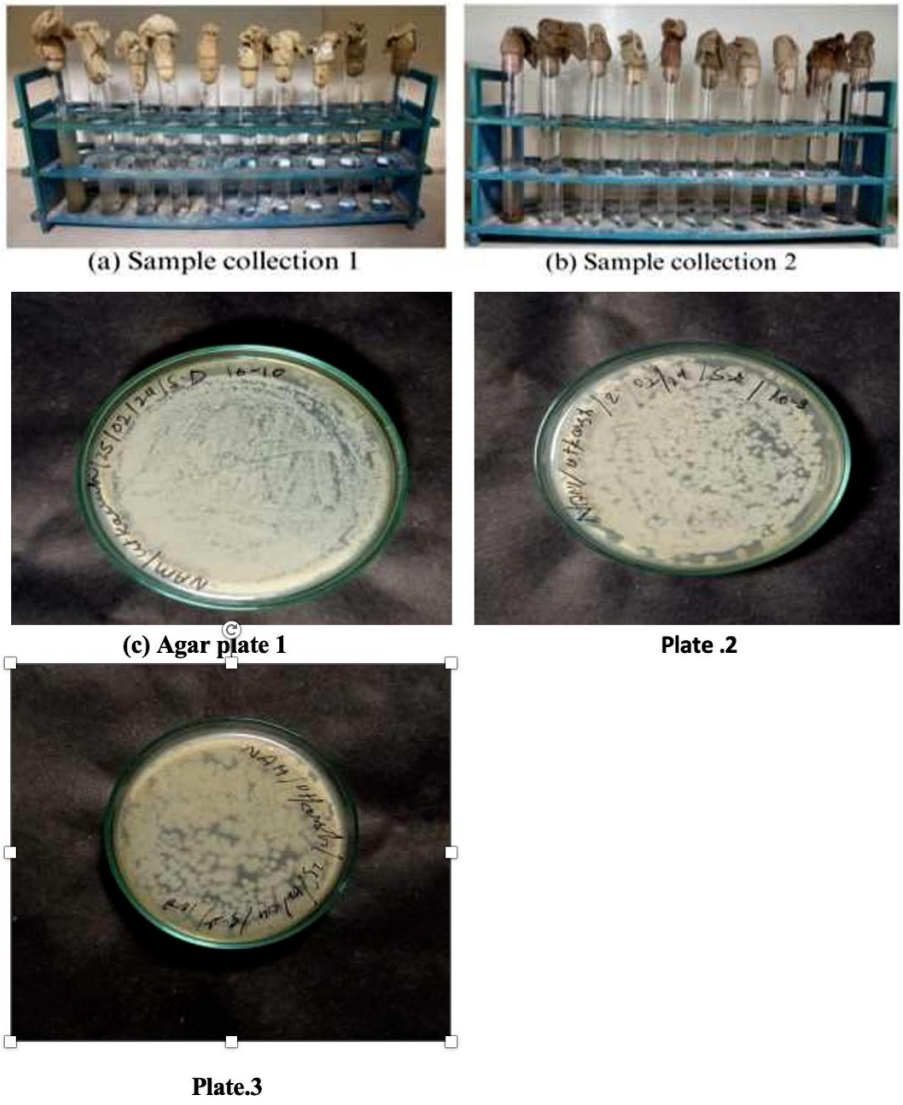


Figure 3 : Isolation and purification of bacteria from collected sample where **(a)** Serial dilution of sample 1, **(b)** Serial dilution of sample 2, **(c)** Inoculation of sample on agar plates and purification of selected colony

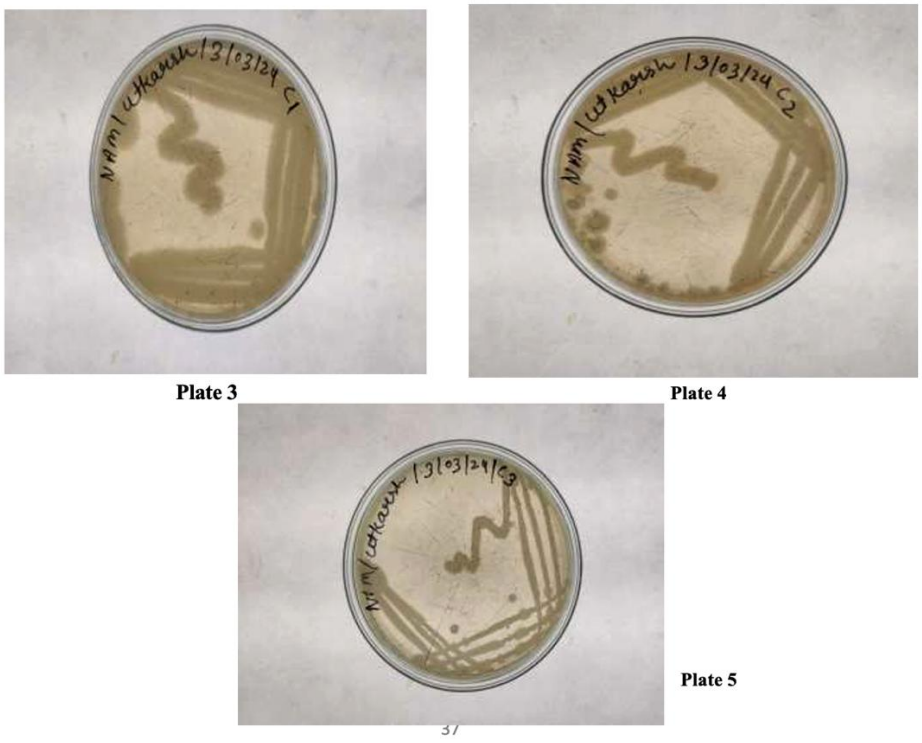


Table 3: Colony morphology 42of Colony 1, 2 and Colony 2 was observed.

Parameters	Observation		
	Colony 1	**Colony 2**	**Colony 3**
Shape	Circular	Circular	Circular
Margin	Undulate	Undulate	Entire
Elevation	Flat	Flat	Raised
Pigmentation	No pigmentation	No pigmentation	No pigmentation
Surface	Smooth	Smooth	Smooth
Texture	Soft	Soft	Hard
Opacity	Translucent	Translucent	Opaque

3.3 Growth kinetics

Optical density measurements were obtained at 600 nm at one-hour intervals. The reported data reports that the culture entered the stationary phase on approximately days 3-4 and that maximum growth was observed at 37 °C and pH 9.0. The reported growth curve therefore supported the use of these conditions during subsequent production experiments.

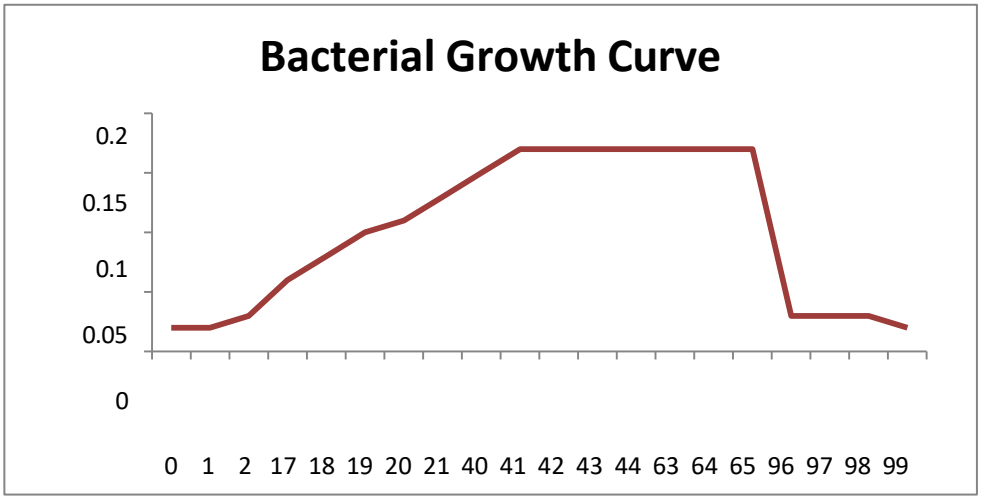


Figure 4: Showing growth curve of the culture plate 2.

3.4 Optimization of enzyme production

The production medium and individual medium modifications produced measurable differences in caseinolytic activity. The production medium (PM) containing tryptone, yeast extract, dextrose and NaCl at pH 9 and 37 °C produced the highest reported activity, 0.0477 U/mL/min (G&#65533;mez et al., 1992) .

Table 4. Enzyme activity under the reported production-medium modifications.

Factors	OD at 560 nm	Enzyme activity (U/mL/min)
Production medium (PM)	0.398	0.0477
Peptone, 10 g/L	0.296	0.0334
Tryptone, 10 g/L	0.358	0.0421
Dextrose, 5 g/L	0.260	0.0284
Maltose, 5 g/L	0.108	0.0071
pH 5	0.078	0.0029
pH 7	0.224	0.0234
pH 9	0.365	0.0431
Tryptone, 5 g/L	0.124	0.0093
Tryptone, 10 g/L	0.288	0.0323
Tryptone, 15 g/L	0.137	0.0112
4 °C	0.090	0.0046
37 °C	0.370	0.0438
50 °C	0.155	0.0137

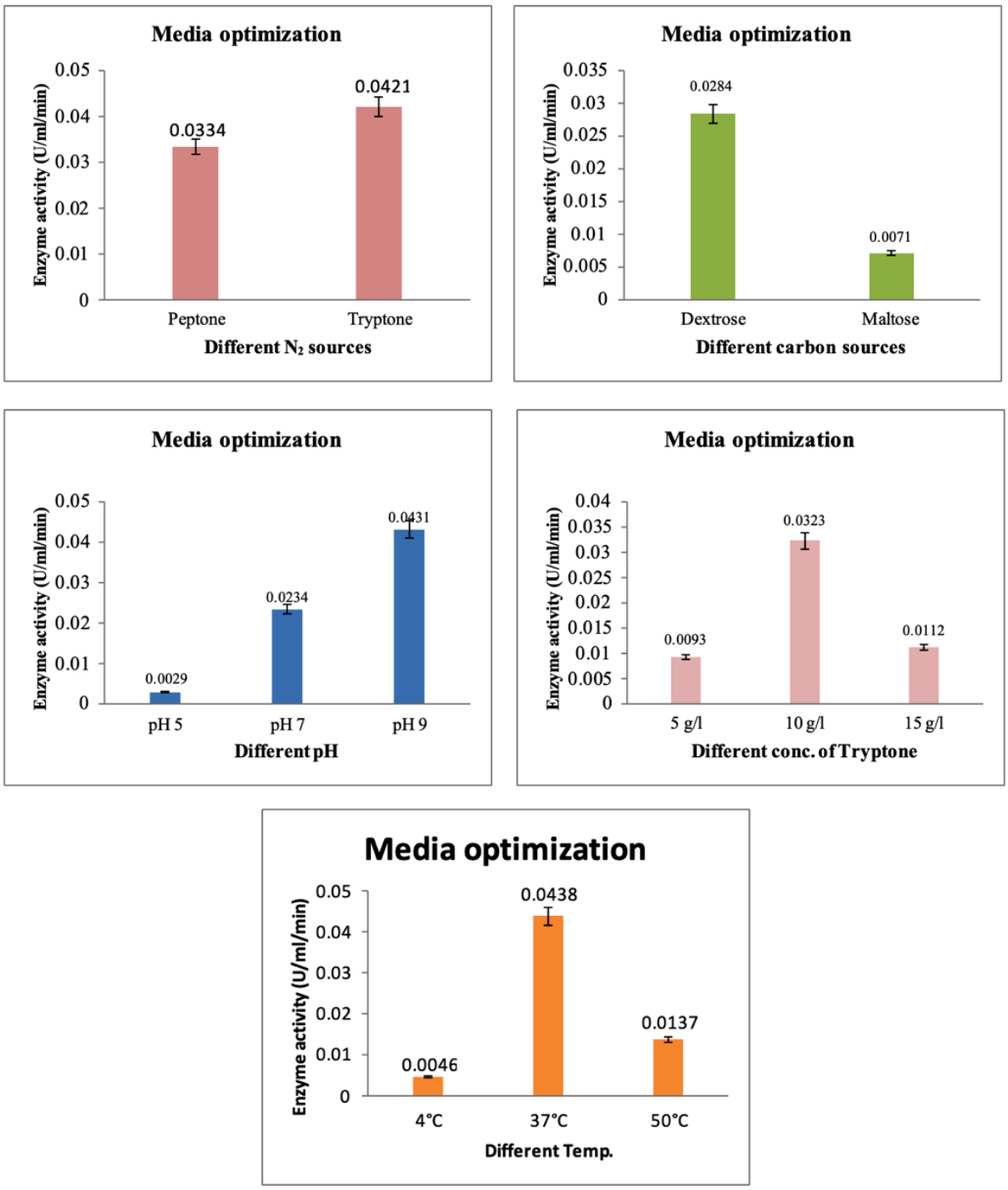


Figure 5: Different media modification for fermentation through the one factor at a time method

When nitrogen sources were compared, tryptone gave an activity of 0.0421 U/mL/min compared with 0.0334 U/mL/min for peptone. For carbon sources, dextrose produced 0.0284 U/mL/min, whereas maltose produced 0.0071 U/mL/min. Among the tested pH values, pH 9 produced the highest activity (0.0431 U/mL/min), followed by pH 7 (0.0234 U/mL/min) and pH 5 (0.0029 U/mL/min). For tryptone concentration, the reported activities were 0.0093, 0.0323 and 0.0112 U/mL/min at 5, 10 and 15 g/L, respectively. Temperature also strongly affected activity, with 37 °C giving 0.0438 U/mL/min compared with 0.0046 U/mL/min at 4 °C and 0.0137 U/mL/min at 50 °C.

3.5 Protein concentration and enzyme activity before and after purification

The Bradford assay showed a protein concentration of 34.765 µg/mL in the crude enzyme preparation and 24.00 µg/mL after ammonium sulfate precipitation and dialysis. Caseinolytic activity was 0.0477 U/mL/min in the crude preparation and 0.0376 U/mL/min in the purified preparation.

Table 5. Protein concentration and caseinolytic activity of crude and purified enzyme preparations.

Enzyme preparation	OD at 595 nm	Protein concentration (µg/mL)	OD at 560 nm	Activity (U/mL/min)
Crude enzyme	0.55	34.765	0.398	0.0477
Purified enzyme	0.38	24.00	0.326	0.0376

3.6 Effect of pH

The purified enzyme showed increasing activity across the tested pH range. Activity was 0.0084 U/mL/min at pH 5, 0.0234 U/mL/min at pH 7 and 0.0378 U/mL/min at pH 9. Thus, pH 9 represented the highest tested activity condition.

Table 6. Effect of pH on purified-enzyme activity.

pH	OD at 560 nm	Enzyme activity (U/mL/min)
5	**0.117**	**0.0084**
7	**0.244**	**0.0234**
9	**0.327**	**0.0378**

3.7 Effect of temperature

The highest activity among the tested temperatures was recorded at 37 °C (0.039 U/mL/min). Activity at 4 °C was 0.017 U/mL/min, while activity at 50 °C was 0.023 U/mL/min (King & Morgan, 1970) .

Table 7. Effect of temperature on purified-enzyme activity.

Temperature	OD at 560 nm	Enzyme activity (U/mL/min)
4 °C	**0.181**	**0.017**
37 °C	**0.340**	**0.039**
50 °C	**0.227**	**0.023**

3.8 Effect of activators

Calcium ions increased the measured enzyme activity relative to the preparation without added activator. CaCl2 gave an activity of 0.0504 U/mL/min, while MgCl2 gave 0.0334 U/mL/min compared with 0.0376 U/mL/min in the preparation without activator.

Table 8. Effect of selected activators on purified-enzyme activity.

Activator	OD at 560 nm	Enzyme activity (U/mL/min)
None	0.326	0.0376
CaCl2	0.417	0.0504
MgCl2	0.296	0.0334

3.9 Effect of inhibitor

EDTA markedly reduced the measured activity from 0.0376 U/mL/min in the uninhibited preparation to 0.0070 U/mL/min in its presence. The corresponding absorbance values were 0.326 and 0.107, respectively.

Table 9. Effect of EDTA on purified-enzyme activity.

Inhibitor	OD at 560 nm	Enzyme activity (U/mL/min)
None	**0.326**	**0.0376**
EDTA	**0.107**	**0.0070**

3.10 Apparent molecular-weight profile

SDS-PAGE analysis of the purified preparation showed two reported bands at approximately 35 kDa and 29 kDa. Because the reported data does not provide a sequencing-based identification of the individual bands or a purified single-band preparation, these values are presented as the observed molecular-weight bands rather than as definitive molecular-weight assignment of a single nattokinase species.

3.11 Blood-clot hydrolysis

The purified enzyme was evaluated against a pre-formed blood clot using distilled water as the control. The reported data reports visible clot hydrolysis after addition of 100 µL purified enzyme, with dissolution observed during the observation period and documented after 1 h and 24 h. The control received 100 µL distilled water. No quantitative percentage clot-lysis value was reported; therefore, the result is presented as a qualitative fibrinolytic observation.

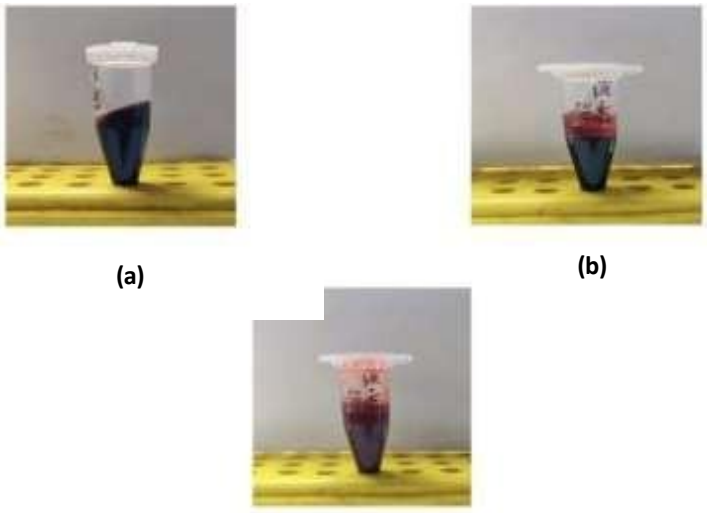


Figure 6: (a) micro-centrifuge tube containing 48 hrs clotted blood sample, (b)Showing hydrolysis of blood sample by the enzyme after 1 hrs, (c) Showing hydrolysis of blood sample by the enzyme after 24 hrs.

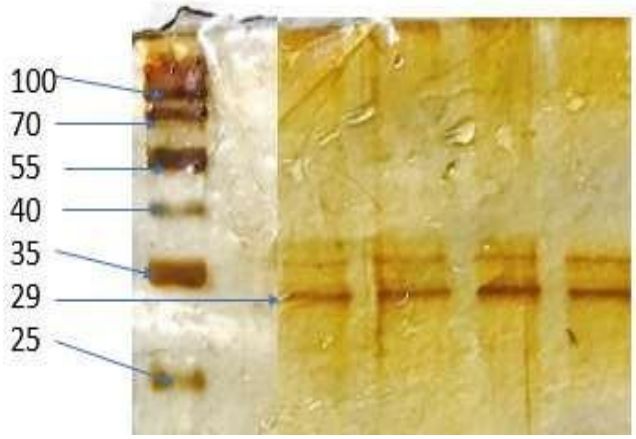


Figure 7: SDS-PAGE analysis of Purified natto kinase. M: protein molecular weight marker (the molecular weight of each band is indicated on the left); lanes 1: Band was achieved at 35 KDa and 29 KDa.

4. Discussion

The present study demonstrates a sequential workflow for isolation, screening, production and partial purification of a bacterial fibrinolytic enzyme. The strongest casein-hydrolyzing isolate was selected through skim milk agar screening, providing an initial functional indication of extracellular proteolytic activity. The selected culture was reported as *Bacillus Pumulis*, and its subsequent performance was evaluated under controlled fermentation conditions (Desai et al., 2015) .

The one-factor-at-a-time experiments showed that enzyme activity was sensitive to both nutritional composition and physicochemical conditions. Tryptone was the better of the tested nitrogen sources, producing 0.0421 U/mL/min compared with 0.0334 U/mL/min for peptone. Dextrose gave substantially higher activity than maltose (0.0284 versus 0.0071 U/mL/min). The pH comparison showed a clear increase in activity from pH 5 to pH 9, while temperature testing identified 37 °C as the most favorable condition among those examined (Thanh, 2023) .

The optimized production medium consisted of tryptone (10 g/L), yeast extract (5 g/L), dextrose (5 g/L) and NaCl (10 g/L) at pH 9 and 37 °C. Under this production-medium condition, the reported activity was 0.0477 U/mL/min. The optimized

fermentation was then carried out in 200 mL medium for 7 days at 120 rpm. These results indicate that both medium composition and culture conditions influenced the measured extracellular proteolytic activity (Paredes et al., 2017) .

Following fermentation, ammonium sulfate precipitation and dialysis reduced the measured total protein concentration from 34.765 to 24.00 μg/mL. The corresponding measured caseinolytic activity decreased from 0.0477 to 0.0376 U/mL/min. The reduction in activity after processing does not by itself establish purification efficiency because work does not report total enzyme volume, total activity, specific activity, recovery percentage or purification fold. Accordingly, the present study reports the observed activity and protein values without calculating unsupported purification parameters (Junaidi et al., 2017) .

The characterization experiments provided additional information about the biochemical behavior of the preparation. The highest activity occurred at pH 9 and 37 °C. Addition of CaCl2 increased activity to 0.0504 U/mL/min, whereas MgCl2 produced 0.0334 U/mL/min. EDTA reduced activity to 0.0070 U/mL/min. These observations are consistent with a preparation whose activity is influenced by pH, temperature and the presence of selected metal-chelating or metal-ion conditions, although the present data do not establish the molecular mechanism responsible for these effects (Lal et al., 1981).

The SDS-PAGE result showed bands at approximately 35 and 29 kDa. The presence of two bands indicates that the preparation was not demonstrated to be electrophoretically homogeneous. Therefore, further purification and molecular identification would be required before assigning a specific band exclusively to nattokinase. The blood-clot assay nevertheless provided a functional observation: the purified preparation visibly hydrolyzed the pre-formed clot during incubation, whereas distilled water was used as the control (Wang et al., 2009) .

Overall, the findings support the feasibility of obtaining a fibrinolytic enzyme-active preparation from the selected bacterial isolate under the reported fermentation conditions. However, the current dataset is primarily a preliminary production and characterization study. Quantitative fibrinolytic assays, replicate-based statistical analysis, molecular identification and a complete purification profile would strengthen the evidence for a defined nattokinase preparation (Coutinho et al., 2024b).

5. Conclusion

A bacterial isolate reported as *Bacillus Pumulis* was selected on the basis of strong casein-hydrolyzing activity and evaluated for nattokinase production. The highest reported production activity was 0.0477 U/mL/min in a medium containing tryptone (10 g/L), yeast extract (5 g/L), dextrose (5 g/L) and NaCl (10 g/L) at pH 9 and 37 °C. The enzyme preparation was recovered from the fermentation supernatant, precipitated with 40% ammonium sulfate and dialyzed. The purified preparation showed 0.0376 U/mL/min caseinolytic activity, maximum tested activity at pH 9 and 37 °C, enhanced activity in the presence of CaCl2, and inhibition by EDTA. SDS-PAGE showed reported bands at 35 and 29 kDa, while the blood-clot assay demonstrated qualitative hydrolysis of a pre-formed clot. These results provide a basis for further purification and rigorous validation of the fibrinolytic enzyme.

References

[1]. Seré KM, Hackeng TM, 2003. Basic Mechanisms of Hemostasis. Semin Vasc Med 03, 003–012. https://doi.org/10.1055/s-2003-38328

[2]. Nascimento TP, Sales AE, Porto TS, Costa RMPB, Breydo L, Uversky VN, Porto ALF, Converti A, 2017. Purification, biochemical, and structural characterization of a novel fibrinolytic enzyme from Mucor subtilissimus UCP 1262. Bioprocess Biosyst Eng 40, 1209–1219. https://doi.org/10.1007/s00449-017-1781-3

[3]. VADLAMANI S, PARCHA SR, 2011. STUDIES ON INDUSTRIALLY IMPORTANT ALKALINE PROTEASE PRODUCTION FROM LOCALLY ISOLATED SUPERIOR MICROBIAL STRAIN FROM SOIL MICROORGANISMS. International Journal of Biotechnology Applications 3, 101–105. https://doi.org/10.9735/0975-2943.3.3.102-105

[4]. VADLAMANI S, PARCHA SR, 2011. STUDIES ON INDUSTRIALLY IMPORTANT ALKALINE PROTEASE PRODUCTION FROM LOCALLY ISOLATED SUPERIOR MICROBIAL STRAIN FROM SOIL MICROORGANISMS. International Journal of Biotechnology Applications 3, 101–105. https://doi.org/10.9735/0975-2943.3.3.102-105

[5]. Mohanasrinivasan V, Mohanapriya A, Potdar S, Chatterji S, Konne S, Kumari S, Keziah SM, Subathra Devi C, 2017. In vitro and in silico studies on fibrinolytic activity of nattokinase: A clot buster from Bacillus sp. Front Biol (Beijing) 12, 219–225. https://doi.org/10.1007/s11515-017-1453-3

[6]. Coutinho SB, Shirodkar RR, Kanjirakkandy A, Arulmozhi V, Vaithilingam M, Chandrasekaran SD, 2024. Production of Nattokinase from Bacillus amyloliquefaciens MRS18: A Bacterial Strain Isolated from Fermented Beans. Cardiovasc Hematol Agents Med Chem 22, 223–229. https://doi.org/10.2174/0118715257275639231219054353

[7]. Sahadat Ali M, D. Eisenback J, Tuz Zohora Mony F, 2025. Protease Activity Assay by Skim Milk Agar Method v1

[8]. Khikmah N, Astrika NR, Widaryanti B, 2024. Seleksi Bakteri dengan Aktivitas Fibrinolitik yang Diisolasi dari Tanah Rumah Potong Ayam. SCISCITATIO 5, 1–9. https://doi.org/10.21460/sciscitatio.2024.51.159

[9]. Morabandza CJ, Dibangou V, Mabika FAS, Gatse EV, Ngoulou TB, Itsouhou N, Nguimbi E, 2021. Optimization of Culture Conditions for Protease Production using Three Strains of Bacillus. J Pure Appl Microbiol 15, 621–629. https://doi.org/10.22207/JPAM.15.2.05

[10]. Khadka DB, Pahadi T, Aryal S, Karki DB, 2024. Partial purification and characterization of protease extracted from kinema. Heliyon 10, e27173. https://doi.org/10.1016/j.heliyon.2024.e27173

[11]. Sahoo SK, Sahoo S, 2020. Exploitation of Fibrinolytic Enzymes in Combating Blood Clotting Disorders – Recent Advances and Strategies: A Comprehensive Review. In: Advances in Pharmaceutical Biotechnology. Springer Singapore, Singapore, pp 383–395

[12]. Ju S, Cao Z, Wong C, Liu Y, Foda MF, Zhang Z, Li J, 2019. Isolation and Optimal Fermentation Condition of the Bacillus subtilis Subsp. natto Strain WTC016 for Nattokinase Production. Fermentation 5, 92. https://doi.org/10.3390/fermentation5040092

[13]. Xin X, Ambati RR, Cai Z, Lei B, 2018. Purification and characterization of fibrinolytic enzyme from a bacterium isolated from soil. 3 Biotech 8, 90. https://doi.org/10.1007/s13205-018-1115-4

[14]. Coskun FS, Quick J, Toprak E, 2025. A Simple, Low-Cost, and Efficient Protocol for Rapid Isolation of Pathogenic Bacteria from Human Blood

[15]. EVALUATION OF PROTEOLYTIC Bacillus spp. ISOLATED FROM SOIL AND CHARACTERISTION OF THEIR GROWTH AND ACTIVITY OF PROTEASES, 2017. Zagazig Journal of Agricultural Research 44, 2061–2077. https://doi.org/10.21608/zjar.2017.51236

[16]. Sahadat Ali M, D. Eisenback J, Tuz Zohora Mony F, 2025. Protease Activity Assay by Skim Milk Agar Method v1

[17]. Sezonov G, Joseleau-Petit D, D'Ari R, 2007. *Escherichia coli* Physiology in Luria-Bertani Broth. J Bacteriol 189, 8746–8749. https://doi.org/10.1128/JB.01368-07

[18]. Chavan A, Mavlankar G, Khan A, Mourya N, Salve A, Kakde U, 2025. Response Surface Methodology-Based Optimization of Natural Pigment Production from Penicillium purpurogenum. GSC Biological and Pharmaceutical Sciences 33, 245–255. https://doi.org/10.30574/gscbps.2025.33.3.0512

[19]. Park E, Kim J, 2015. Characteristics of Culture Conditions for the Production of Biosurfactant by Bacillus pumilus IJ-1. J Appl Biol Chem 58, 81–88. https://doi.org/10.3839/jabc.2015.014

[20]. Srivastava Y, Tripathi K, Kumar N, 2025. Quantitative protein estimation using the Bradford assay: Principles, protocols, and applications. In: Biotechnology Lab Techniques: Culture Media, Microscopy, and Microbial Analysis. Deep Science Publishing, pp 157–159

[21]. Deb P, Talukdar SA, Mohsina K, Sarker PK, Sayem SA, 2013. Production and partial characterization of extracellular amylase enzyme from Bacillus amyloliquefaciens P-001. Springerplus 2, 154. https://doi.org/10.1186/2193-1801-2-154

[22]. Gu W, Chen SW, Chen GP, Ji ZX, 2015. Enhancement of *Haemophilus parasuis* serovar 5 yields by medium optimization. Lett Appl Microbiol 61, 44–49. https://doi.org/10.1111/lam.12421

[23]. Kielkopf CL, Bauer W, Urbatsch IL, 2020. Bradford Assay for Determining Protein Concentration. Cold Spring Harb Protoc 2020, pdb.prot102269. https://doi.org/10.1101/pdb.prot102269

[24]. Kielkopf CL, Bauer W, Urbatsch IL, 2020. Bradford Assay for Determining Protein Concentration. Cold Spring Harb Protoc 2020, pdb.prot102269. https://doi.org/10.1101/pdb.prot102269

[25]. Janssen RC, Boyle KE, 2019. Microplate Assays for Spectrophotometric Measurement of Mitochondrial Enzyme Activity. pp 355–368

[26]. Page M, Thorpe R, 2009. Purification of IgG by Precipitation with Sodium Sulfate or Ammonium Sulfate. pp 1749–1751

[27]. Fromm HJ, 1975. The Effect of Temperature and pH on Enzyme Activity. pp 201–235

[28]. Kumar S, Dhillon S, Singh D, Singh R, 2004. Partial Purification and Characterization of Superoxide Dismutase from Tomato (Lycopersicon esculentum) Fruit. Prev Nutr Food Sci 9, 283–288. https://doi.org/10.3746/jfn.2004.9.3.283

[29]. Vijayaraghavan P, Vincent SGP, Arasu MV, 2016. Purification, Characterization of a Novel Fibrinolytic Enzyme from <I>Paenibacillus</I> sp. IND8, and its in Vitro Thrombolytic Activity. South Indian Journal of Biological Sciences 2, 434. https://doi.org/10.22205/sijbs/2016/v2/i4/103450

[30]. Gómez R, López R, Peláez C, 1992. Caseinolytic activity ofLactococcus lactis subsp. lactis: study of different culture media. Zeitschrift für Lebensmittel-Untersuchung und Forschung 195, 569–571. https://doi.org/10.1007/BF01204566

[31]. King J, Morgan HG, 1970. The temperature activity relationships of serum cholinesterases. J Clin Pathol 23, 730–732. https://doi.org/10.1136/jcp.23.8.730

[32]. Desai SA, Patel VP, Patel D V., 2015. PRODUCTION AND PURIFICATION OF PHARMACEUTICALLY IMPORTANT FIBRINOLYTIC ENZYME FROM BACILLUS SPECIES. International Journal of Pharmaceutical Sciences and Drug Research 493–497. https://doi.org/10.25004/IJPSDR.2015.070610

[33]. Thanh BT, 2023. OPTIMIZATION OF CULTURE CONDITIONS AND NUTRIENT SOURCES FOR Bacillus sp. ES4 SYNTHESIZES FIBRINOLYTIC ENZYME. Tạp chí Khoa học 20. https://doi.org/10.54607/hcmue.js.20.3.3756(2023)

[34]. Paredes LA, Flores Fernández CN, Zavaleta AI, 2017. OPTIMIZACIÓN DEL MEDIO PARA LA PRODUCCIÓN DE PROTEASAS EXTRACELULARES POR Pseudomonas sp. M211 EN FERMENTACIÓN SUMERGIDA. Revista de la Sociedad Química del Perú 83, 449–462. https://doi.org/10.37761/rsqp.v83i4.217

[35]. Junaidi Y, Pertiwiningrum A, Erwanto Y, Fitriyanto NA, 2017. Semi Purification and Identifications Molecule Protein Weigh of Alkaline Protease Enzyme from Bacillus cereus LS2B. International Journal of Bio-Science and Bio-Technology 9, 89–100. https://doi.org/10.14257/ijbsbt.2017.9.3.08

[36]. Lal DM, Calton GJ, Neeman I, Burnett JW, 1981. Characterization of Chrysaora quinquecirrha (sea nettle) nematocyst venom collagenase. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 69, 529–533. https://doi.org/10.1016/0305-0491(81)90346-1

[37]. Wang C, Du M, Zheng D, Kong F, Zu G, Feng Y, 2009. Purification and Characterization of Nattokinase from Bacillus subtilis Natto B-12. J Agric Food Chem 57, 9722–9729. https://doi.org/10.1021/jf901861v

[38]. Coutinho SB, Shirodkar RR, Kanjirakkandy A, Arulmozhi V, Vaithilingam M, Chandrasekaran SD, 2024. Production of Nattokinase from Bacillus amyloliquefaciens MRS18: A Bacterial Strain Isolated from Fermented Beans. Cardiovasc Hematol Agents Med Chem 22, 223–229. https://doi.org/10.2174/0118715257275639231219054353

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