ABSTRACT

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In accordance with the World Health Organisation (WHO), cardiovascular diseases (CVDs) are a major cause of death worldwide, accounting for 17.9 million deaths per year as of 2021. Conditions including hypertension, myocardial infarction, diabetes, hyperlipidemia, coronary heart disease, and stenocardia increase the risk of CVDs. Thrombosis, the most prevalent cardiovascular condition and a significant cause of mortality worldwide, is characterized by intravascular coagulation due to the aggregation of fibrin, the principal protein in thrombi. This condition can lead to partial or total blood flow obstruction, resulting in ischemia and tissue necrosis. Managing thrombosis involves surgical interventions, anticoagulants, antiplatelet drugs, and fibrinolytic agents, which dissolve acute thrombi in arteries. However, the use of these agents is often limited by drawbacks such as allergic reactions, hemorrhagic side effects, inflammation, expensive costs, short half-lives, and poor fibrin selectivity. These challenges underscore the need for improved therapeutic approaches to effectively and safely manage thrombosis and its associated complications.

Fibrinolytic enzymes, capable of breaking down fibrin clots, have been identified from diverse sources, including earthworms, algae, insects, and snake venom. Microbial sources are preferred due to their diversity and industrial scalability. Microorganisms including bacteria, actinomycetes, and fungi have yielded numerous fibrinolytic enzymes, many of which originate from traditional fermented foods deemed safe (GRAS category). Examples include enzymes derived from fermented foods like Chinese Douchi, Korean Chung kook-jang, Tofuyo, and fermented shrimp paste, highlighting the role of fermentation in enzyme discovery. The fibrinolytic enzyme Nattokinase from *Bacillus natto*, discovered in 1987, exemplifies the therapeutic potential of such enzymes in treating thrombosis-linked cardiovascular diseases. The genus *Bacillus*, particularly those associated with fermented foods, has been recognized for its prolific enzyme production, including fibrinolytic enzymes, which have been widely purified and characterized for possible use in thrombolytic drug development. This exploration underscores the significance of microbial enzymes in industrial biotechnology, offering a diverse and efficient resource for developing therapeutic enzymes.

The structure-function relationships and characteristics of fibrinolytic enzymes obtained from the *Bacillus* genus were investigated in this study, focusing on their active sites, substrate selectivity, and catalytic residues to elucidate the mechanisms of fibrin clot degradation. Insights gained can guide enzyme engineering to improve activity, stability, and

specificity, with applications in therapeutic and industrial processes. Sequence analyses revealed conserved domains and regions essential for enzyme function and stability. Proteins demonstrated high solubility and thermostability, evidenced by low GRAVY scores and high aliphatic indices, supporting their functionality under diverse environmental conditions. Structural models generated via in-silico tools validated through QMEAN z-scores and ERRAT analyses, confirmed the stability and accuracy of the predicted 3D structures. Signal peptides in the proteins indicate their extracellular nature, which is crucial for industrial and therapeutic applications. High serine content and specific amino acid properties contribute to the enzyme's adaptability and efficiency in alkaline conditions. Transmembrane helices and conserved motifs further underline their role in secretion and function.

Key sequences, such as from *Bacillus amyloliquefaciens* and *Bacillus subtilis*, demonstrated superior thermostability and fibrinogenase activity, making them promising candidates for pharmaceutical applications. Their mid-molecular weights and low GRAVY indices suggest enhanced tissue penetration, reduced immunogenicity, and compatibility with simple expression systems. These enzymes degrade fibrinogen's α- and β-chains efficiently, reinforcing their potential as potent fibrinolytic agents. The findings lay a theoretical foundation for designing mutant enzymes with enhanced properties for industrial and clinical use.

Further, in this study, *Bacillus megaterium* strain SC_G1, isolated from alcohol starter cultures in Northeastern India, exhibited remarkable fibrinolytic activity. Initial screening of six bacterial strains resulted in four isolates showing activity, with SC_G1 demonstrating the highest. It efficiently degraded all three fibrinogen chains (Aα, Bβ, and Cγ) and exhibited plasmin-like activity without requiring endogenous activators. The strain was identified as gram-positive *Bacillus megaterium* using molecular techniques, including 16s rRNA sequencing and phylogenetic analysis. The findings underscore the potential of microbes as bio-factories for fibrinolytic enzyme production, highlighting the enzyme's specificity for fibrin as a promising therapeutic application for hyperfibrinogenemia-related disorders.

The study also focused on producing and characterizing a recombinant fibrinolytic protease from *Bacillus subtilis*. The process involved codon optimization, gene cloning into the pET26b(+) expression vector, expression in *E. coli* under the T7 promoter, optimization of refolding conditions, purification using affinity chromatography, and validation of enzyme activity. The recombinant protease exhibited specificity for fibrin and degraded fibrinogen's

Aα-chain, categorizing it as an α-fibrinogenase. Additionally, the recombinant protease displayed anticoagulant and thrombolytic effects in vivo, prolonging clotting times by inhibiting thrombin activity, a critical step in the blood coagulation cascade. These characteristics position it as a potential anticoagulant therapy for thrombotic conditions. The absence of toxicity in preclinical safety assessments further supported the enzyme's therapeutic promise. The recombinant protease showed no adverse behavioural or biochemical effects in Wistar rats, and histological analyses of vital organs revealed no abnormalities. These results underscore its clinical safety for cardiovascular treatment.

Despite the promising findings, improvements in the expression system, promoter selection, and refolding techniques are necessary for enhanced recovery and industrial-scale production. Advanced genetic tools, such as metabolic engineering and error-prone PCR, offer potential avenues for optimizing enzyme activity, stability, and specificity. Before advancing to clinical trials, further preclinical studies are essential to establish the recombinant protease's therapeutic potential fully.

This thesis has been organized into the following six chapters for easy understanding.

Chapter I: Cardiovascular diseases (CVDs), the primary cause of the ensuing rise in death worldwide, are discussed in this chapter. It also highlights the process of blood clotting and the molecular mechanism of fibrin formation. This chapter also briefly describes the various thrombolytic agents being used to treat CVDs and their limitations. The aim and objectives of the present study are also described in this chapter.

Chapter-II: This chapter describes fibrinolytic enzymes and the sources from which they are obtained. It also reviews the published literature on the production of the fibrinolytic enzyme by recombinant process and their characterization, particularly the fibrinolytic enzymes derived from *Bacillus* spp. isolated from fermented food. There has also been a discussion of the pharmacological, thrombolytic, and toxicological assessment data, along with the structural-function characteristics of *Bacillus* spp. derived fibrinolytic enzyme.

Chapter-III: Outlines the study's chemicals and consumables and the procedures and methods utilized to carry out the different experiments.

Chapters-IV, V, and VI: These chapters provide results and discussions, and a brief overview of each chapter's content is provided below.

Chapter-IV: 60 nucleotide sequences of fibrinolytic enzyme from the *Bacillus* genus were investigated for their structure-function properties. Protein sequences ranged from 126 to 810 amino acids, and their alignment identified a conserved catalytic triad (Asp, His, Ser). The predicted isoelectric points indicated stability across a wide pH range, aligning with physiological conditions. The extinction coefficients varied, while stability analyses demonstrated that most enzymes were stable in vivo. High aliphatic indices suggested robust thermostability, which benefits pharmaceutical and industrial applications. Additionally, low GRAVY indices indicated excellent water solubility, enhancing drug bioavailability by improving absorption and distribution.

The retrieved enzyme sequences underwent comprehensive structural analysis, beginning with primary sequence characterization. A heat map of amino acid distribution revealed alanine as the most widespread amino acid, while cysteine was the least prevalent. Hydrophilic amino acids, particularly serine, indicated an extracellular nature facilitating secretion, with significant pharmaceutical implications. Motif analysis via the MEME suite identified six conserved motifs, particularly motif 3, which featured prominently in 56 sequences and was associated with the peptidase S8/S53 domain, a critical domain for peptide bond catalysis. Secondary structure analysis highlighted a dominance of random coils across most sequences, with α-helices prevailing in specific cases. Seven groups with distinct motif maps and topology diagrams were identified, revealing varying structural features such as helix-helix interactions and β-sheet motifs. Disulfide linkages, observed in a few sequences, were noted for their role in protein stability and potential applications in drug development, including improving pharmacokinetics in therapeutic proteins.

Tertiary structure analysis employed QMEAN4, Verify3D, and ERRAT evaluations to assess protein model quality. The QMEAN4 z-scores for the sequences were close to zero, indicating high structural reliability. Verify3D and ERRAT analyses confirmed that most models achieved high-quality scores, affirming their stability and dependability. GlobPlot analysis showed varying numbers of disordered regions and ordered domains across sequences, influencing protein functions like signal transduction and binding specificity. Peptide cutter software identified numerous cleavage sites for digestive enzymes across the sequences, with implications for therapeutic protein engineering.

Experimental validation supported the in-silico findings, with molecular weight consistency for 16 sequences. The optimum pH for fibrinolytic activity ranged from 3.0-9.0,

and thermal stability was observed between 40-60°C. Certain enzymes, including those derived from *Bacillus amyloliquefacien* and *Bacillus subtilis,* showed efficient degradation of fibrinogen α- and β-chains and N-succinyl-Ala-Ala-Pro-Phe-pNA substrates, confirming their potential as potent fibrinolytic agents.

Chapter-V: Bacterial strains were isolated from three fermented food/ starter culture samples collected from Northeastern India. Six bacterial cultures were obtained and screened for fibrinolytic activity. Among them, four strains exhibited zones of hydrolysis on fibrin agar plates, with strain SC_G1 showing the highest fibrinolytic activity by degrading all fibrin chains within 90 minutes during SDS-PAGE analysis. The SC_G1 strain was isolated as a pure culture and identified as *Bacillus megaterium* based on morphological, biochemical, and genetic analyses. Genomic DNA was successfully isolated, and the 16S rRNA gene was amplified and sequenced. Phylogenetic analysis revealed 99.93% similarity with *Bacillus* species, clustering closely with *Bacillus megaterium.*

Additionally, PCR amplification confirmed the presence of a protease gene (~1003 bp), highlighting the strain's potential as a fibrinolytic enzyme producer. Based on the physiochemical parameter values ascertained by the ExPASy-ProtParam tool, it was inferred that the protease gene sequence was thermostable and soluble in hydrophilic solvents. However, it was found that the sequence lacked the serine protease-specific catalytic domain when its amino acid sequence was compared to other homologous fibrinolytic protein sequences. Consequently, the sequence was not pursued for additional research.

Chapter-VI: The fibrinolytic gene derived from *Bacillus subtilis* was codon-optimized for expression in *E. coli* BL21 (DE3) and cloned into the pET-26b(+) vector with a His-tag for purification. The recombinant protease was expressed under the T7 promoter and purified using Ni-NTA chromatography. SDS-PAGE analysis showed a ~40 kDa protein, with renaturation by dialysis yielding the highest fibrinolytic activity, selectively degrading the Aα-chain of fibrin without fibrinogenolytic activity. The recombinant protease exhibited anticoagulant properties, prolonging clotting times (PT, APTT) dose-dependent manner and interacting with thrombin to inhibit thrombin's catalytic activity. It demonstrated in vitro thrombolytic activity and antiplatelet effects, with minimal hemolytic activity and no cytotoxicity against NRK-52E cells. In vivo studies confirmed its anticoagulant efficacy and safety, with no adverse effects on biochemical parameters or histopathological changes in recombinant protease-treated rats

at 4 mg/kg. These findings establish the recombinant protease as a potent, safe thrombolytic and anticoagulant candidate.

Chapter VII: This chapter provides the study's conclusion and illustrates how the results could be implemented.

The current study's findings emphasize the need for extensive pharmacokinetic and pharmacodynamic evaluations before the recombinant protease is put through a clinical trial. It is also recommended that more bioavailability research be conducted using thrombus-targeting drug delivery systems in order to create efficient cardiovascular medicines.