CHAPTER V

Isolation and characterization of fibrinolytic enzyme producing bacteria from few fermented food/ starter culture samples of Northeastern, India

5.1 Brief introduction

The leading causes of the subsequent global increase in mortality are cardiovascular diseases (CVDs) for individuals with hypertension, myocardial infarction, diabetes mellitus, hyperlipidemia, coronary heart disease, or stenocardia. The World Health Organisation (WHO) estimates that 17.9 million deaths globally are caused by cardiovascular diseases (CVDs) each year [1]. The most common cardiovascular disease and the foremost reason for mortality globally is thrombosis, an accumulation of fibrin inside blood arteries [2-4]. Correspondingly, elevated blood fibrinogen levels (clinically defined as hyperfibrinogemia) enhance myocardial infarction risk by accelerating platelet aggregations, raising blood viscosity, encouraging fibrin formation, and stimulating thrombotic events [5-7]. Hyperfibrinogenemia causes lipid propagation of the blood vessels, which starts atherosclerosis and leads to ischemic pathology [8].

Numerous fibrinolytic enzymes have been identified from a variety of sources, including earthworms [9], polychaetes [10], algae [11], insects [12], and snake venom [13]. The vast diversity and ease of industrial production make fibrinolytic enzymes derived from microbial sources preferred. Numerous fibrinolytic enzymes from bacteria [14], actinomycetes [15], and fungus [16] have recently been found. Traditional fermented foods that have been consumed for generations worldwide include microbial resources classified as safe (GRAS) category [17]. Foods and beverages that undergo fermentation are rich in bacteria, and the variety of these bacteria enables researchers to identify enzymes with fibrinolytic activity [18].

The fibrinolytic enzyme Nattokinase, discovered from *Bacillus* natto in 1987, established the potential for treating cardiovascular illnesses linked to thrombosis [19-21]. Since then, research into isolating and characterizing novel fibrinolytic enzymes from fermented edible foods, including Chinese Douchi [22], Tofuyo [23], fermented shrimp paste [24], edible honey mushroom [25], and Korean Chung kook-jang soy sauce [26] has gained increasing attention on a global scale. The exceptional competencies of microorganisms and their enzymes exhibit a wealth of variety, efficacy, and specificity. They are regarded as a gold mine for the exploration of economically feasible and superior goods in this contemporary age of industrial biotechnology. The best examples of these items are therapeutic enzymes. In particular, the genus *Bacillus* has earned recognition for its contribution to producing numerous enzymes with economic

significance; the fibrinolytic enzyme is one such industrially important enzyme. Several such bacterial enzymes have been isolated, purified, and biochemically characterized, revealing their potential for developing thrombolytic drugs [27-30].

In this study, serial dilution screened bacteria from few fermented food/ starter culture samples (fermented fish and alcohol starter culture) for strains with protease activity, phenotypically characterized and identified using 16s rRNA sequencing, analysed the fibrinolytic activity on plasminogen-free fibrin-agar plates and by SDS-PAGE analysis, and its genomic DNA was used to amplify protease-encoding genes with specific primer sets.

5.2 Results

5.2.1 Screening of fibrinolytic enzyme-producing bacteria

Three fermented food/ starter culture samples were collected in sterile tubes from different places in North-eastern India in the initial stage of the screening procedure. The fermented food samples were serially diluted to 10^{-4} in sterile saline and plated on a nutrient agar plate supplemented with casein. The plates were incubated at 37°C for 24 h to allow the growth of bacterial colonies as mentioned in the experimental approach outlined in Materials and Methods section 3.2.2.2 (Figure 5.1). From the three fermented food samples, six bacterial cultures were obtained (Table 5.1).

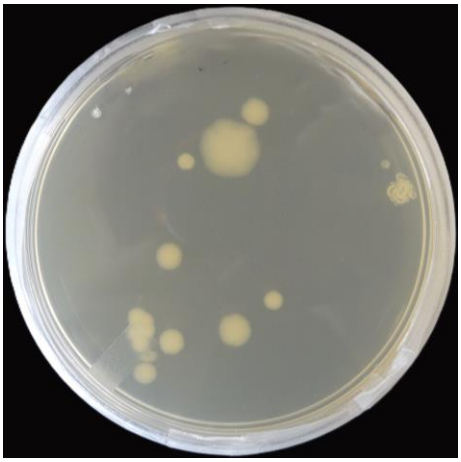

Figure 5.1: Screening of protease-producing bacteria on Nutrient agar plate supplemented with casein

Table 5.1 List of selected protease producing bacteria from few fermented food/ starter culture samples of Northeast India

Sl. No.	Place of collection of sample	Sample code	Isolated Bacterial Code
1.	Napaam (Assam)	SC_N	SC_N4
			SC_N5
2.	Guwahati (Assam)	SC_G	SC_G1
			SC_G3
3.	Dimapur (Nagaland)	FF_D	FF_D1
			DD_D1

5.2.2 Fibrinolytic protease production

The isolated bacterial colonies were additionally sub-cultured in M9 media, and cell-free supernatants were used to determine their fibrinolytic activity and to select the potential bacterial strain for further investigation.

5.2.2.1 Zone of hydrolysis

Out of the six screened bacterial isolates, only four bacterial strains exhibited zone of hydrolysis on plasminogen-free fibrin agar plate (Figure 5.2). The measurement of the visible zone of clearance around the well is tabulated in Table 5.2.

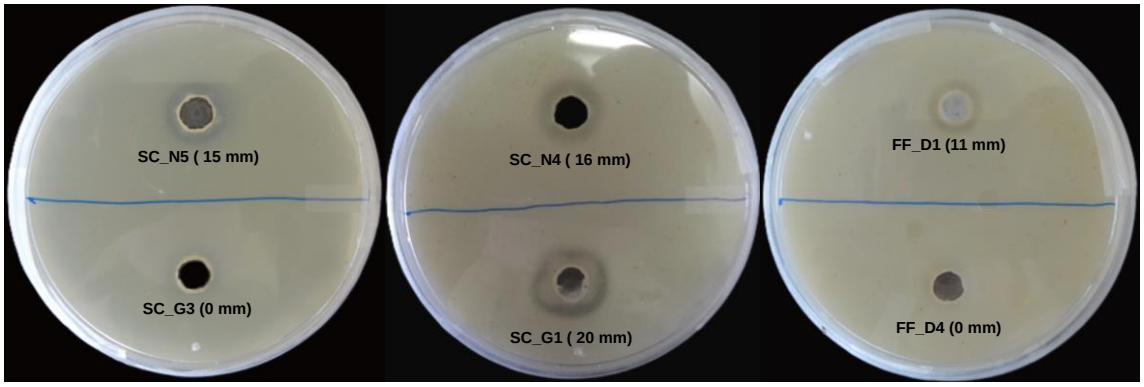

Figure 5.2: Zone of hydrolysis around the well containing the bacterial isolate on fibrin agar plate.

Table 5.2: Zone of hydrolysis on fibrin agar plate

Sl. No.	Sample Code	Zone of hydrolysis (mm)
1.	SC_N4	16
2.	SC_N5	15
3.	SC_G1	20
4.	SC_G3	0
5.	FF_D1	11
6.	FF_D4	0

5.2.2.2 Fibrinolytic assay:

To elucidate the fibrinolytic activity of the screened bacterial isolates, 12.5% SDS-PAGE separated digested fibrin fragments. The fibrin degradation pattern demonstrated that all the samples (SC_N4, SC_N5, SC_G1 and FF_D1) exhibit fibrinolytic activity. However, sample SC_G1 demonstrated the highest activity by preferentially degrading all the chains of fibrin within 90 min of incubation (Figure 5.3). This outcome suggests that sample SC_G1 can be considered a potent fibrinolytic-producing bacteria because of its highest fibrin degradation competency. The fibrin degradation percentage of the selected bacterial isolates which was analysed using ImageQuant TL 8.1 software are presented in Figure 5.4.

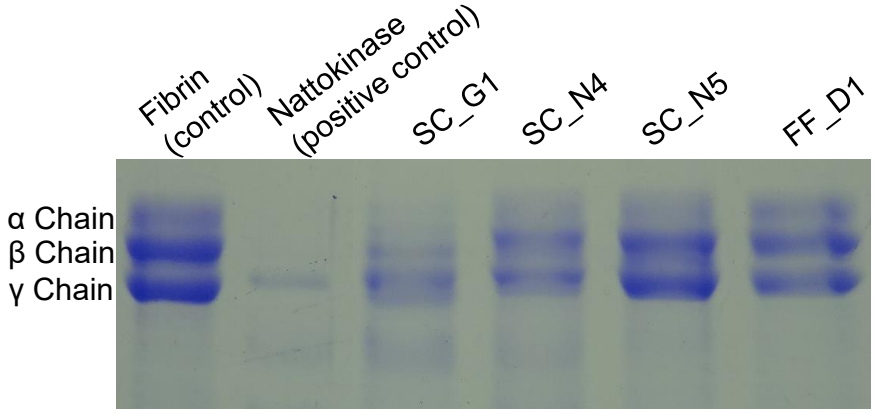

Figure 5.3: A SDS-PAGE analysis of fibrin degradation by selected bacterial isolates within 90 min of incubation at 37°C.

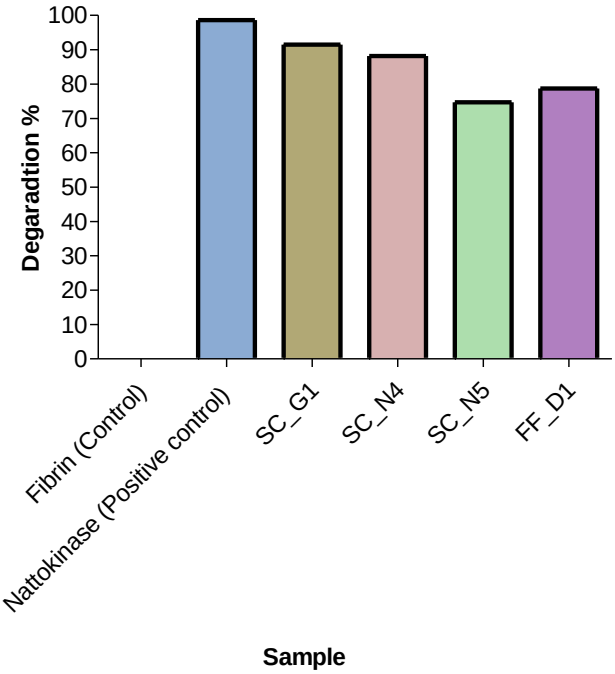

Figure 5.4: Percentage of fibrin degaradtion by the selected bacterial isolates

5.2.3 Pure culture of fibrinolytic-producing bacterial isolates

To obtain the pure culture of the bacterial colony, the extracellular protease-producing viable bacterial colony (SC_G1 strain) was serially diluted up to 10^{-4} in sterile saline and plated on the nutrient-agar plate, which was incubated for 24 h at 37°C. The bacterial culture from 10^{-4} dilution plate was then streaked on a nutrient-agar plate to isolate a single pure colony of the bacteria following the experimental protocol described in Materials and Methods section 3.2.2.4 (Figure 5.5).

Figure 5.5: Pure culture colony of SC_G1 bacterial isolate on a nutrient-agar media plate

5.2.4 Identification of bacterial strains

5.2.4.1 Morphological and biochemical identification

The findings of the biochemical and morphological analyses performed on the specific bacterial isolate are presented in Table 5.3. Based on bacterial morphology and biochemical test results, the strain SC_G1 has been suggested to belong to the *Bacillus sp.* genus (Bergey's manual of systemic bacteriology, 1999, 2001, 2005)

Table 5.3: Biochemical and morphological tests of bacterial strains

Characteristics		**SC_G1**
Morphology		Rod-shaped, motile, Gram-positive, circular border, creamy white color
Catalase		Negative
Voges-Proskaurer Test		Negative
Methyl Red Test		Negative
Acid from:	D-Glucose	Positive
	Sucrose	Positive
	Lactose	Negative
	D-Mannitol	Positive

Gas from Glucose	Positive
Utilization of Citrate	Positive
Formation of Indole	Positive
Oxidase test	Negative
Triple sugar iron agar test	Negative
Urease test	Positive

5.2.4.2 Genomic DNA isolation and PCR amplification of 16s rDNA gene

Genomic DNA was successfully isolated from the SC_G1 strain using the GeneJet Genomic DNA Purification Kit (Figure 5.6 A) and served as a template for amplifying the 16s rRNA gene with gene-specific primers. When examined on a 1% agarose gel, the amplified product showed up as a single band close to 1500 base pairs (bp). This result is in consistent with the 16s rRNA gene's size (Figure 5.6 B).

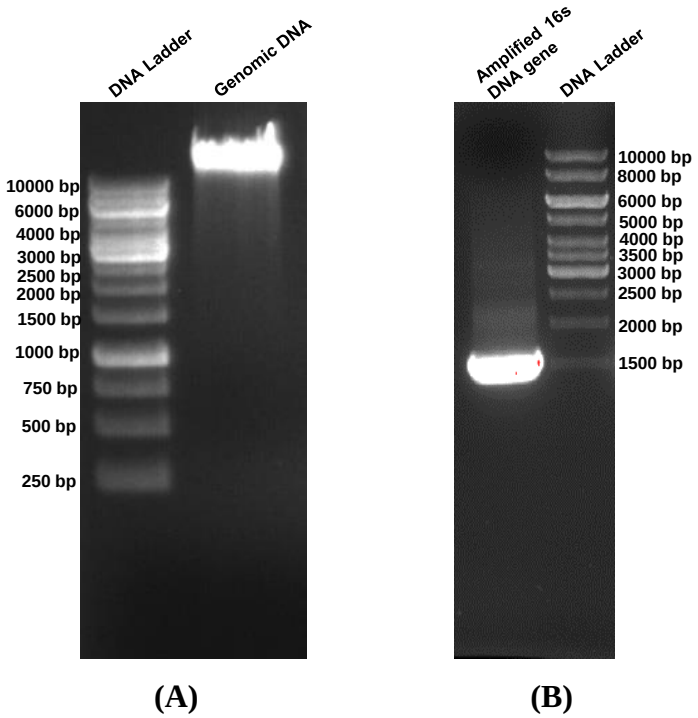

Figure 5.6: 0.8% Agarose gel run. **(A)** Agarose gel run of isolated genomic DNA. **(B)** Agarose gel run of PCR amplification of 16s rDNA gene.

5.2.4.2.1 Phylogenetic tree analysis of bacterial isolates based on 16s rDNA sequencing

The amplified 16s rRNA gene was sequenced, and the sequence is shown in Table 5.4. The SC_G1 strain's homologous search result for the 16s rRNA sequence showed 99.93% similarity with other *Bacillus* species (Table 5.5). The neighbor-joining method generated the phylogenetic tree from the sequence data, and the Escherichia coli strain (accession no. NR024570.1) sequence was considered out-group. The constructed phylogenetic tree revealed that the SC_G1 strain shared the closest clad with the *Priestia aryabhattai* (accession no. MG905882.1), which is formerly known as *Bacillus megaterium* [31] (Figure 5.7). Based on phenotypic characteristics, homologous sequence search result by nBLAST and neighbor-joining phylogenetic tree, the bacterial strain SC_G1 was identified as *Bacillus megaterium*.

Table 5.4: DNA sequence of conserved region of 16s rDNA gene of SC_G1 bacterial strain

Bacterial isolate	**Amplified 16s rDNA sequence**
Strain SC_G1	TGCAAGTCGAGCGAACTGATTAGAAGCTTGCTTCTATGACGTTAGCGGCG GACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTC GGGAAACCGAAGCTAATACCGGATAGGATCTTCTCCTTCATGGGAGATGA TTGAAAGATGGTTTCGGCTATCACTTACAGATGGGCCCGCGGTGCATTAG CTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCATAGCCGACCTG AGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG GGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAG CAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTA GGGAAGAACAAGTACGAGAGTAACTGYTCGTACCTTGACGGTACCTAACC AGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG CAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTA AGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGG GGAACTTGAGTGCAGAAGAGAAAAGCGGAATTCCACGTGTAGCGGTGAAAT GCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTTTGGTCTGTA ACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT GGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCC TTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCA AGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG TGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTG ACAACTCTAGAGATAGAGCGTTCCCCTTCGGGGGACAGAGTGACAGGTGGT GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACG AGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGA CTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCC CCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCTGCA

	AGACCGCGAGGTCAAGCCAATCCCATAAAACCATTCTCAGTTCGGATTGTAG GCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCAT GCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGA GAGTTTGTAACACCCGAAGTCGGTGGAGTAACCGTAAGGAGCTAGCCGCCTTAA

Table 5.5: Homologous search results of 16s rDNA gene partial sequence of the isolated bacteria using Basic Local Alignment Tool (BLASTN) tool from National Centre Biotechnology Information (NCBI).

Sl. No.	**Accession**	**Description of bacterium**	**Query coverage**	**Max identity**
1.	MT453994.1	*Bacillus megaterium* strain S1 16S ribosomal RNA gene, partial sequence [*Priestia megaterium*]	99%	99.93%
2.	MN620500.1	*Bacillus aryabhattai* strain CN13-1 16s ribosomal RNA gene, partial sequence [*Priestia megaterium*]	99%	99.93%
3.	CP032527.2	*Priestia megaterium* NCT-2 chromosome, complete genome [*Priestia megaterium* NCT-2]	99%	99.93%
4.	CP041519.1	*Priestia aryabhattai* starin KNU10 chromosome, complete genome [*Priestia aryabhattai*]	99%	99.93%
5.	CP041066.1	*Priestia megaterium* strain KNU-01 chromosome, complete genome [*Priestia megaterium*]	99%	99.93%
6.	MK825199.1	Bacterium strain BS2011 16S ribosomal RNA gene, partial sequence [bacterium]	99%	99.93%
7.	MK825195.1	Bacterium strain BS2007 16S ribosomal RNA gene, partial sequence [bacterium]	99%	99.93%
8.	MK824872.1	Bacterial strain BS1684 16S ribosomal RNA gene, partial sequence [bacterium]	99%	99.93%
9.	MK823803.1	Bacterium strain BS0615 16S ribosomal RNA gene, partial sequence [bacterium]	99%	99.93%
10.	MG309386.1	*Bacillus sp.* (in: Bacteria) strain 201705CJKOP-73 16S ribosomal RNA gene, partial sequence [*Bacillus sp.* (in: firmicutes)]	100%	99.86%
11.	MH144225.1	*Bacillus aryabhattai* strain PgBE2 16S ribosomal RNA gene, partial sequence [*Priestia aryabhattai*]	99%	99.93%

12.	MG822729.1	*Bacillus horikoshii* strain 2 16S ribosomal RNA gene, partial sequence [*Sutcliffiella horikoshii*]	99%	99.93%
13.	MH683133.1	*Bacillus sp.* (in: Bacteria) strain Firmi-44 16S ribosomal RNA gene, partial sequence [*Bacillus sp.* (in; firmicutes)]	99%	99.93%
14.	MH683131.1	*Bacillus sp.* (in: Bacteria) strain Firmi-42 16S ribosomal RNA gene, partial sequence [*Bacillus sp.* (in: firmicutes)]	99%	99.93%
15.	MH553938.1	*Bacillus aryabhattai* strain PgKB11 16S ribosomal RNA gene, partial sequence [*Priestia aryabhattai*]	99%	99.93%
16.	CP024794.1	*Bacillus sp.* Y-01 chromosome complete sequence	99%	99.93%
17.	KY860719.1	*Bacillus sp.* (in: Bacteria) strain DE024 16S ribosomal RNA gene, partial sequence	99%	99.93%
18.	KY860718.1	*Bacillus sp.* (in: Bacteria) strain DE023 16S ribosomal RNA gene, partial sequence	99%	99.93%
19.	MG905882.1	*Priestia aryabhattai* strain FJAT-40010 16S ribosomal RNA gene, partial sequence	99%	99.93%
20.	MG266360.1	*Bacillus sp.* (in: Bacteria) strain MRF-28 16S ribosomal RNA gene, partial sequence	99%	99.93%

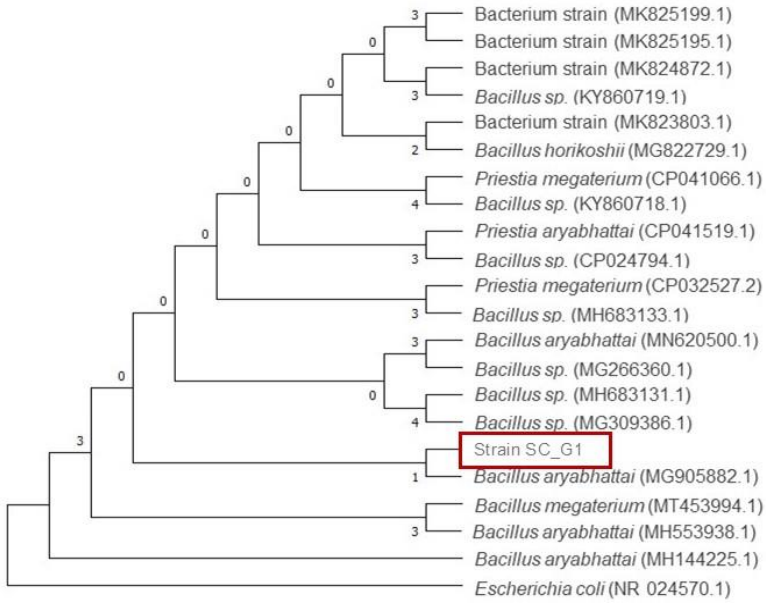

Figure 5.7: Phylogenetic tree constructed based on the 16S rDNA sequences of the isolated bacterial strain SC_G1 and other related species.

5.2.5 PCR amplification of protease gene

The isolated genomic DNA from the SC_G1 strain was used for the PCR amplification of the protease gene, which resulted in the amplification of ~1003 bp product (Figure 5.8 A & B).

(A) **(B)**

Figure 5.8: (A) 0.8% agarose gel run of PCR amplification of protease gene. **(B)** Nucleotide sequence of the PCR amplified fibrinolytic encoding gene isolated from Strain SC_G1

5.2.6 Computational analysis of amplified fibrinolytic encoding gene sequence

The corresponding amino acids of the amplified fibrinolytic encoding gene were deduced using Expasy translate tool. Using the deduced amino acid sequence, its in-silico characterization was done. The details of various physiochemical properties that were computed using the Protparam server are in Table 5.6. From the computed values it can be predicted that the sequence was thermostable and soluble in hydrophilic solvent. However, when its amino acid sequence was aligned with other homologous fibrinolytic serine protease sequences, it was found that the catalytic domain (Ser, His, and Asp) which is an important aspect for a fibrinolytic serine protease to exert their catalytic activity was absent in the sequence (Figure 5.9). So therefore, the sequence was not carried forward for further investigation.

Table 5.6: Physiochemical characteristics of the amplified fibrinolytic encoding gene determined by computational analysis

Sl. No.	Components	Value
1	Total amino acid	270
2	Molecular weight	28279.29
3	Theoritical pI	5.11
4	Extinction coefficient	16960
5	Instability index	27.61
6	Aliphatix index	103.22
7	GRAVY	-0.074

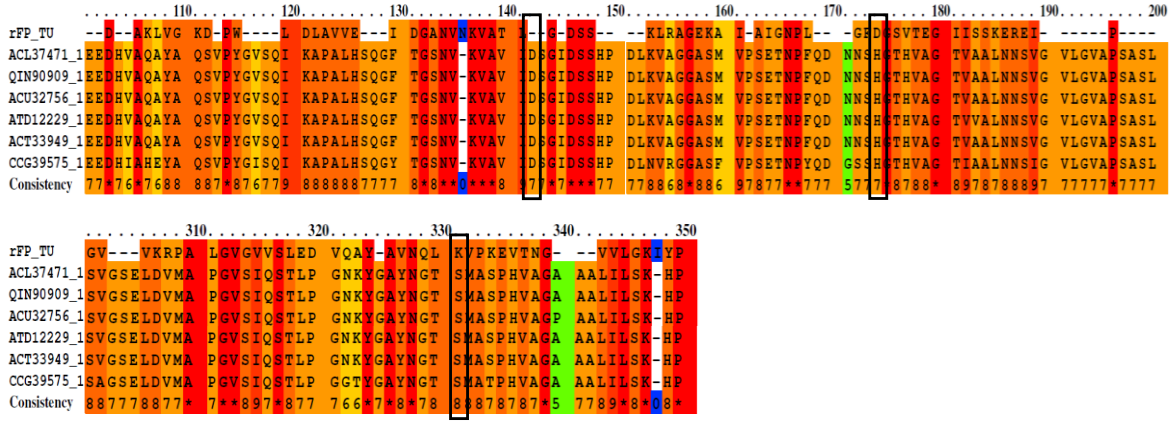

Figure 5.9: Amino acid sequence alignment of the fibrinolytic encoding gene with few other fibrinolytic serine protease sequences

5.3 Discussion

Enzymes are essential catalysts in biological processes and are used extensively in industry, chemistry, medicine, food processing, and agriculture. Their significant catalytic activity is primarily higher than synthetic or organic catalysts. Additionally, they exhibit substrate selectivity, significantly speed up chemical reactions, and perform well in aqueous solutions with mild pH and temperature changes. Even yet, only a few other catalysts have each of these characteristics. Enzymes now serve specialized purposes in various sectors [32]. Bacteria have garnered greater interest among the several natural sources of enzyme synthesis due to their quick growth, minimal growth requirements, and potential for genetic engineering to optimize their production [33]. One of the main

causes of death in the globe is thromboembolic illnesses. The few fibrinolytic drugs currently available on the market, including urokinase, streptokinase, alteplase, and tenecteplase, have serious side effects, including a high production cost, a propensity for bleeding, and poor specificity. In order to circumvent these issues, new sources of fibrinolytic enzymes must be found.

Therapeutic industries prefer fibrinolytic enzymes with high specificity towards fibrin because they have fewer side effects and allergic reactions. *Bacillus* species' fibrinolytic alkaline proteases are well known for having a comparatively high substrate selectivity to fibrin [22,34]. *Bacillus* species with fermented food origins have been recommended as a possible source for the medicinal sectors due to their relatively high sensitivity towards fibrin and their vast diversity of fibrinolytic enzymes, as evidenced by zymograms.

In contrast to plasmin, which is a naturally occurring fibrin lysis enzyme that is converted from plasminogen by plasminogen activators such as streptokinase and urokinase [35], the SC_G1 strain (*Bacillus megaterium*) is a bacterial fibrinolytic enzyme that was studied in this work. Even though the majority of bacterial fibrinolytic enzymes have been found and isolated from bacteria linked with soy fermentation [22,26,36], the protease enzyme produced by *Bacillus megaterium* is a fibrinolytic enzyme derived from alcohol starter culture. A total of 3 fermented food samples were collected from random places in Northeastern India. Initial Screening by serial dilution resulted in isolating six bacterial isolates on a nutrient agar media plate. Cell-free supernatant harvested through spinning has been utilized as a crude sample for quantitative assessment of protease activity. Four samples among the six isolates exhibited fibrinolytic activity, out of which the SC_G1 strain showed the highest activity.

Fibrinogen, which consists of three pairs of polypeptide chains bound by disulfide (Aα, Bβ, and Cγ), is the precursor to fibrin. Out of the six isolates, the SC_G1 strain demonstrated intense fibrinolytic activity as it broke down all three chains of fibrin in 90 min of incubation. The fibrinogen degradation pattern was investigated for the screened bacterial isolates using the fibrin plate method and SDS-PAGE analysis. It is reasonable to assume that the screened bacterial isolates have a fibrinolytic activity because the transparent area is directly proportional to the enzymatic activity. The addition of the screened bacterial isolates produced a visible void on the plasminogen-free plate,

suggesting that they were plasmin-like, direct-acting fibrinolytic protease that lysed the thrombin without the need for endogenous fibrinolytic factors. Therefore, it might be possible to prevent side effects such as plasminogen activators-induced plasmin formation linked to platelet activation [37].

Biochemical characterization identified the strain SC_G1 to be gram-positive and belonged to the *Bacillus* sp. Several investigations have documented the existence of bacteria belonging to the genus *Bacillus* on fermented foods [21,38,39]. It ought to be clear that using molecular identification techniques provides additional benefits. These techniques are more dependable than the conventional ones [40]. The SC_G1 strain was further identified by molecular biological technique (ribotyping), which was made possible by amplifying and sequencing the gene encoding the 16s rRNA. The agarose gel electrophoresis of the PCR amplified products revealed approximately 1500 bp bands, corresponding with the amplicons' size found in other investigations. A phylogenetic tree was built for the SC_G1 strain by aligning the nucleotide sequences of the genes that encode 16s rRNA sequence and several other well-characterized and published *Bacillus sp.* that were recovered from the NCBI database to get a more accurate categorization. The results indicate a strong relationship among the various *Bacillus* species. The SC_G1 bacterial strain belonged to the genus *Bacillus*, according to the results of the BLAST analysis and matching with other sequences of bacteria in NCBI databases. It is evident from the branch arrangement that the SC_G1 strain and *Bacillus megaterium* belonged to the same clan.

Microbes might be regarded as a potential biofactory for producing fibrinolytic enzymes because they possess the natural ability to manufacture primary metabolites. Since many natural microorganisms are still unknown, efforts to find such microbes should continue. The SC_G1 strain protease enzyme's strong substrate specificity for fibrin could elucidate its crucial application in treating disorders associated with hyperfibrinogenemia. Consequently, it expands the possibilities for protein engineering, its expression, characterizing the protease enzyme produced by the SC_G1 strain, and evaluating the pre-clinical safety of the purified enzyme in an animal model.

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