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Original Article

FIBRINOLYTIC ENZYME FROM BACILLUS AMYLOLIQUEFACIENS: OPTIMISATION AND SCALE UP STUDIES

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ABSTRACT

Objective: This research work was carried out to identify a potent microorganism, which produced the fibrinolytic enzyme and to optimise the media and growth parameters to achieve the maximal enzyme production for commercial application.

Methods: Microorganisms were isolated from different sources and assayed for fibrinolytic activity. The shortlisted cultures after preliminary screening (casein hydrolysis, blood plate assay and blood clot dissolution) were identified using 16S rRNA amplification method. The media and growth parameters were optimized to achieve the maximal enzyme activity. In-silico studies were carried out to identify the activators and inhibitors of the enzyme.

Results: Two species of Bacillus, namely, *Bacillus amyloliquefaciens* and *Bacillus licheniformis*, isolated from spoilt milk and soy flour, respectively, exhibited fibrinolytic activity. In the laboratory scale studies, of these two cultures, *B. amyloliquefaciens* produced the Fibrinolytic enzyme in higher quantities, 28.98 FU/mL, compared to 26.63 FU/mL in *B. licheniformis*. The maximal activities were obtained after 72 h. The optimum conditions at laboratory scale for the maximal production of the fibrinolytic enzyme were: pH 7.2, temperature 37 C and agitation 200 rpm. When scale up studies with *B. amyloliquefaciens* in a 7 L Fermentor were undertaken. The maximal activity obtained was 55.60 FU/mL in 72 h, compared to that of 28.98 FU/mL in shake flask studies. The molecular weight of the enzyme was estimated to be about 38 kDa. In in-silico studies, it was observed that PMSF inhibited the fibrinolytic activity, thereby, confirming this fibrinolytic enzyme is a serine protease (Nattokinase).

Conclusion: The enzyme had exhibited excellent blood clot dissolving property and therefore may be considered for further scale up and commercial exploitation.

Keywords: Fibrinolytic enzyme, *Bacillus* sp, Optimisation, Scale up studies, Commercial prospects.

INTRODUCTION

Medical science has synthesised various compounds to help thin blood (from Aspirin to Warfarin and urokinase to streptokinase) and each has their own role. The use of blood thinners, either drugs or natural compounds, such as, garlic, ginseng, bromelain etc, may provide temporary relief by increasing the blood flow through blocked areas. However, this is only a quick-fix approach. To really address fibrin accumulation at its root, blood thinning is not enough. These blockages must be dissolved/removed and optimally prevented. Injectable drugs, such as, urokinase, streptokinase, Activase etc are used for this purpose in emergency situations [1]. While these medications certainly do the job, they are extremely expensive and short acting (4 – 20 min). Thus, fibrinolytic enzymes from microbial sources, like, Nattokinase, offer great promise in the management (7 – 8 h) and support of healthy blood circulation.

It was Sumi *et al* [2], who first isolated and identified Nattokinase, a potent fibrinolytic enzyme, derived from *Bacillus subtilis* var *natto* from traditional Japanese soybean food, Natto. It is considered as a potent cardiovascular drug. Nattokinase is one of the most powerful new dietary supplements introduced in the market in recent years. This fibrinolytic enzyme (Nattokinase) is unique in profoundly lowering fibrinogen levels and degrading branched fibrin [3]. It has 3 different mechanisms of action. It lyses fibrin directly, changes prourokinase to urokinase and increase tissue plasminogen activator, which increases our own plasmin. Nattokinase lessens excessive coagulation and thus improves circulation, increasing oxygen flow to tissues. Nattokinase is actually superior to conventional clot dissolving drugs [4] with many benefits, including oral administration, known efficacy, and the prolonged effects, cost effectiveness and prevention of clot formation. Nattokinase has been demonstrated to have pH and temperature stability and so can be found in the gastrointestinal tract [5]. Nattokinase belongs to the alkaline serine protease family, the catalytic centre which contains three conserved residues asp32, his64, and ser221 [6,7]. Nattokinase not only dissolves blood clots [8] but also degrades amyloid fibrils [9]. The major objective of this research work was (a) to identify a potent microorganism, which produced the fibrinolytic enzyme, comparable to Nattokinase (b) to optimise the media and growth parameters in order to achieve the maximal enzyme production and (c) to study its potential for commercial application.

MATERIALS AND METHODS

Bacteria and fungi, isolated from the samples of Soya flour, Soil from an abattoir in Chennai, spoilt milk, Rinds of Water Melon, Soybean and Shouyu (a Japanese soybean sauce) were subjected to Casein Hydrolysis Plate Assay method [10]. Twelve bacterial cultures, which exhibited clear zone in the plates (proteolytic activity), were further evaluated for their proteolytic enzyme producing capacity. The bacterial cultures were grown in a minimal medium prescribed by Deepak *et al* [11], but slightly modified, containing: Glucose 1.0%, Peptone 3.0%, $MgSO_4$ 0.20% and $CaCl_2$ 0.50%, pH adjusted to 7.0, at 37 C at 200 rpm. After 72 h of growth, the biomass was separated by centrifugation (10,000 rpm, 15 min, 4 C) and the clear supernatant was taken for proteolytic enzyme assay as prescribed by Sigma-Aldrich. The cultures were also grown on blood plates to observe the clear zone. Later, the crude enzyme (culture filtrate) preparations were tested for their property in dissolving the blood clot.

Two bacterial cultures, which were shortlisted based on the above screening procedures were identified using microscopic and biochemical tests [12]. The genomic DNA was isolated and quantified [13] and identification of the cultures was done by amplification of 16S rRNA analysis. Amplification for gene, specific for serine protease from *B. amyloliquefaciens*, was carried out in a gradient PCR unit [14].

For the optimal production of proteolytic enzyme, various media, viz, glucose medium, yeast extract medium, glucose and soybean meal medium, maltose and wheat bran medium and green gram dhal (our own recipe) were tried. Effect of different carbon (glucose, fructose, xylose, sucrose, maltose and corn starch) and nitrogen (ammonium nitrate, ammonium sulphate, potassium nitrate, peptone, soybean meal and casein) sources were studied. The culture filtrates were periodically assayed for proteolytic activity (Sigma-Aldrich) and total protein content [15]. Experiments were also carried out to study the optimum pH (4.0 to 9.0), temperature (20 to 50 C) of the medium and the agitation (150 to 225 rpm). Based on the above studies, a production medium containing (%): Wheat bran 4.0, maltose 2.5, NH_4NO_3 0.3, Sodium chloride 0.5, KH_2PO_4 0.1, K_2HPO_4 0.4 and Magnesium sulphate 0.05 (pH 7.2), was optimized. The optimum pH for the enzyme activity was studied by varying the pH levels, ie, from 4.0 to 9.0. Similarly, the optimum temperature for the enzyme activity was estimated by studying the enzyme activity at different temperatures, from 20 - 50 C. The Fibrin Assay Method was carried out according to the protocol of Yin *et al* [16].

Ammonium sulphate precipitation (20 – 80%) was tried for the partial purification of the enzyme. Dialysis was done until the protein concentrate was obtained. The ammonium sulphate fraction was further purified through Gel filtration using Sephadex G-75 gel (GE Health Care Life Science). SDS-PAGE electrophoresis was carried out with the protein concentrates [17].

Scale up studies were carried out in a laboratory 7 L Fermentor (Scigenics. India). The composition of the seed medium (%) was: Glucose 2.0, Peptone 1.0, yeast extracts 0.6 at pH 7.2. The Production Medium (%) contained: Wheat bran 4.0, maltose 2.5, NH_4NO_3 0.3, Sodium chloride 0.5, KH_2PO_4 0.1, K_2HPO_4 0.4, magnesium sulphate 0.05 at pH 7.2. The culture filtrate was harvested after 72 h, filtered through a cloth filter and centrifuged (Beckman, 10,000 rpm, 15 min). The clear supernatant was concentrated through ultrafiltration (Millipore, 10 KDa cartridge membrane). The concentrated enzyme was then made into powder (sodium chloride and Dextrin were used as fillers) by Spray drying (Labultima). The test enzyme was compared with other fibrinolytic enzymes using the blood clot dissolution method (time of dissolution).

As crystal structure of serine protease is unavailable, 3D structure was built using the homology modeling. The protein sequence of Nattokinase (Accession No: WP_017417394.1, length: 382 amino acid) was retrieved from NCBI GenBank. The template protein was identified using BLASTp tool. The crystallographic structure of 1SBN was downloaded from Protein Data Bank (PDB) and used as a template (resolution 2.10A). The possible binding pockets for the target protein were searched using Computed Atlas of Surface Topography of Proteins (CASTP) [7]. The binding pockets residues were then compared with active site residues of the template protein. The drug ligands was retrieved from NCBI-PubChem compound database [3,18] for molecular docking studies. The ligands were Serpin, Lupeol, PMSF, α-amyrin, ß-amyrin, Curcumin, IPTG, Serpin_Zn^{2+}, Lupeol_Zn^{2+}, α-amyrin_Zn^{2+}, ß-amyrin_Zn^{2+}, Curcumin_Zn^{2+} and dipicolinic acid were selected [19,20]. Pharmacodynamics and Pharmacokinetics properties were determined for retrieved ligands using Qikprop tool of Schrodinger suite. In order to acquire a thorough understanding of binding affinity of each activator and inhibitor with modeled serine protease protein, a molecular docking study of retrieved ligands with modeled serine protease was performed employing Glide application of Schrodinger suite [21]. To gain further detailed knowledge of the modeled protein and ligand complex interaction and stability a molecular dynamics simulation of the complex obtained from docking was performed using Schrodinger suite.

RESULTS AND DISCUSSION

Forty five bacterial and thirty fungal cultures were isolated from the above mentioned sources. From these cultures, twelve microorganisms were shortlisted based on the clear zone in Casein Plates (Casein hydrolysis). They were grown in a minimal medium and the proteolytic enzyme activity was estimated in the culture filtrate. The results are presented in Table 1. It is clear from the results that two bacterial isolates, namely, MB and SF2, gave maximal protease activities. Incidentally, these two bacterial isolates exhibited bigger clear zones in the Casein plate assay. Both the cultures exhibited very clear zones in the blood plate assay and dissolved the blood clots.

Table 1: Protease enzyme activity

S. No.	Culture	Enzyme Activity (U/mL)
1	SF1	1.45
2	SF2	5.76
3	SF3	1.24
4	MB	6.70
5	MB1	2.41
6	MB2	1.36
7	SB1	2.19
8	SB2	3.21
9	SH1	4.01
10	SH2	2.90
11	WM1	1.45
12	WM2	3.67

The final shortlisted two bacterial cultures were identified as gram positive rods. The bacterial cells were arranged in pairs or chains with single elliptical endospore, typical of *Bacillus* species [22]. Based on the results obtained from the biochemical tests, the cultures were confirmed as *Bacillus* species. The genomic DNA isolated from both the cultures was amplified and 16S rRNA was sequenced. The sequence of the primers used were:

Bac 16s-F: 5’ AGAGTTGATCATGGCTCAG 3’

Bac 16s-R: 5’ TACGGCTACCTTGTTACGACTT 3’

16S rRNA Sequence Analysis

>V250_16S_F

ACGGAGATGGGGGGGCTTCCCTAAAAATTGCAAGTCGGAGCGGACAGATG
GGAGCTTGCTCCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGT
AACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGG
ATGGTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACC
ACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTC
ACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG
GACTGAGACACGGCCCATACTCCTACGGGAGGCACCGTAGGGAATCTTCC
GCATTGGACGAAAGTCTGACGGAGCAACGCCGCGTGGGAGTGATGAAGGTTT
TCGGATCGTAAAGCTCTGTTGTTAGGGAAAAACAAGTGCCGTTCAAATAG
GGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAATTACGTGCC
GCAGCCGCGGTAATACGTAGGTGGCAATCGTTGTCCAGAAATTATTGGGC
GTAAAAGGGCTCGCAGGCGGTTTCTTAATTCTGATGTGAAAAGCCCCCGG
CTCAACCGGGGAGGGTCATTGGAAACTGGGGGAACTTGAGTGCAAAAAGG
AGAGTGGAATTCCACGTGTATCGGTGAAATGCGTAAAATGTGGAGGAACA
CCAGTGGCAAAGGCGATTCTCTGGTCTGTAACTGACGCTCAGGAGCGAAA
TCGTGGGGAACGAACCGGATTAGATACCCTGGGTATCCCCCCCTAAACGA
TGATTGCCCAGTGTTAGGGGGTTTCCCCCCCCTTATGCTGCCA

Fig. 1: Sequence of amplified of 16S rRNA (MB isolate)

>BRTPL_RG_16s

AGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACG
GGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGG
GAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAGAC
ATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTA
GCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACC

TGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTA
CGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGG
AGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTT
GTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTA
CCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAC
GTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAG
GCGGTTTCTTAAGTCTGATGTGAAAGCCCCC

Fig. 2: Sequence of amplified of 16S rRNA (SF2 isolate)

Based on the sequencing, the bacterial culture (MB) was identified as *Bacillus amyloliquefaciens* (Figure 1) and SF2 was identified as *Bacillus licheniformis* (Figure 2). The sequences were registered with NCBI GenBank (*B. Amyloliquefaciens* - KF186621.1; *B. licheniformis* - KF186622.1). Through NCBI Blast Analysis and Phylogenetic Dendrogram, it was confirmed that our isolates were indeed *B. amyloliquefaciens* and *B. licheniformis* (Figure 3 and Figure 4).

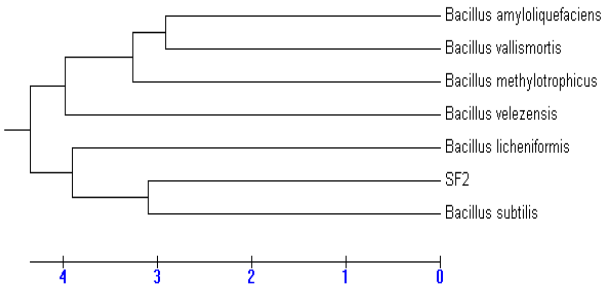

Fig. 3: Phylogenetic Tree of *B. amyloliquefaciens*

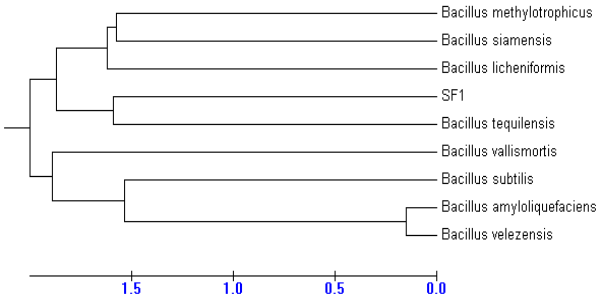

Fig. 4: Phylogenetic Tree of *B. licheniformis*

Based on the primers: **Fsp F: 5' TCACAGCTTTTCTCGGTC '3**; **Fsp R: 5' TGATCCGATTACGAATGC '3**, the Gene specific for Serine Protease (fibrinolytic enzyme) was amplified (Figure 5).

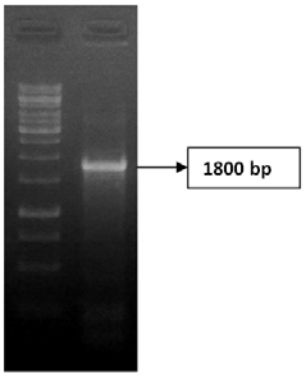

Fig. 5: Amplification of fibrinolytic serine protease gene

Lane 1: 1Kb Ladder (10,000 bp, 8000 bp, 6000 bp, 5000 bp, 4000 bp, 3000 bp, 2500 bp, 2000 bp, 1500 bp, 1000 bp, 750 bp, 500 bp, 250 bp

Lane 2: PCR Amplicon of fibrinolytic serine protease gene (*B. amyloliquefaciens*)

Sequence Analysis of *B. amyloliquefaciens*

>054-1212_153_001_RAN_FSP-F-F08. .ab1

CAAGAGAGCGATCGCGGCTGTGTACAAATACTCATGTCCTTCCATCGGT
TTTTTCCATTA

AAATTTAAATATTTCGGGTTCCTATTAAACGAAAGAGAGATGATATAC
CTAAATAGAAAT

AAAACAAACTGAAAAAAATTGGGTCTACTAAAATATTATTCCATGCTA
TACAATTAATCC

ACAGAATAATCTGTCTATTGGTTGTTCTGCAAATGAAAAAAAGGAGAG
GATAAAGAGTGA

GAGGCAAAAAGGTATGGATCAGTTTGCTGTTTGCTTTAGCGTTAATCT
TTACGATGGCGT

TCGGCAGCACGTCTCCTGCCCAGGCGGCAGGGAAATCAAACGGGGAAA
AGAAATACATTG

TCGGATTTAAACAGACAATGAGCACGATGAGCGCCGCTAAGAAAAAAG
ATGTCATTTCTG

AAAAAGGCGGGAAAGTGCAAAAGCAATTCAAATATGTAGACGCAGCTT
CAGCTACATTAA

ATGAAAAAGCCGTAAAAGAGCTGAAAAAAGACCCTAGCGTCGCTTACG
TTGAAAGAAGAT

CACGTTGCACAGGCGTACGCGCAGTCCGTGCCTTTACGGCGTATCACAG
ATTAAAGCCCC

TGCTCTGCACTCTCAAGGCTTCACCGGATCAAATGTTAAAGTAGCGGTT
ATCGACAGCGG

TATCGATTCTTCTCATCCTGGATTTAAGGGTAGCAGGCGGAGCCAGCAT
GGTTCCCTTCT

GAAAACAAATCCTTTCCAAGAACAACACTCTCACGGAACTTCACGTTGC
CGGTCAGTTGC

CGCTTCTTAATTAACCTCAGTCGGTGTATTTAGGCGTTGCGCCAAGCGC
ATCTTCTTTAC

GCTTGTAAAAGTTCTCGCGCCTGAACGGGTCGCCCATTACAGCTTGGAA
TCATATCGAAT

Both *B. amyloliquefaciens* and *B. licheniformis* were grown in several media for the evaluation of their proteolytic enzyme production capability. Both the cultures produced maximal activities in Maltose+WheatBran medium; the proteolytic activity in *B. .amyloliquefaciens* was 15.85 U/mL (Figure 6) and in case of *B. .licheniformis*, the activity was 14.65 U/mL (Figure 7).

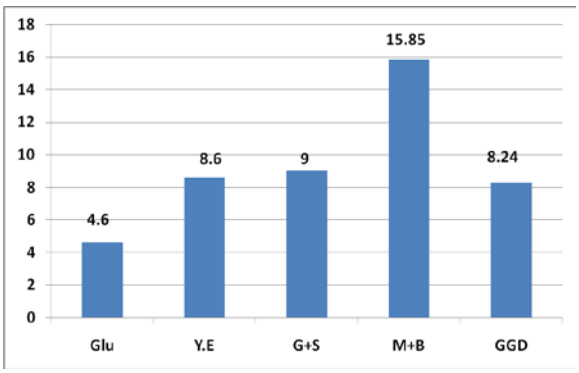

Fig. 6: Proteolytic Activity of *B. amyloliquefaciens* in Different Media

When different carbon sources were tried at different dosages, the medium with maltose at 2.5% yielded higher activities; the activity was 18.21 U/mL in *B. .amyloliquefaciens* and 16.21 U/mL in *B. .licheniformis*. Similarly, when different nitrogen sources were used in the media in different dosages, ammonium nitrate at 0.3% gave maximal activity, 10.0 U/mL in *B. .amyloliquefaciens*; in case of *B. .licheniformis*, the activity was 8.76 U/mL.

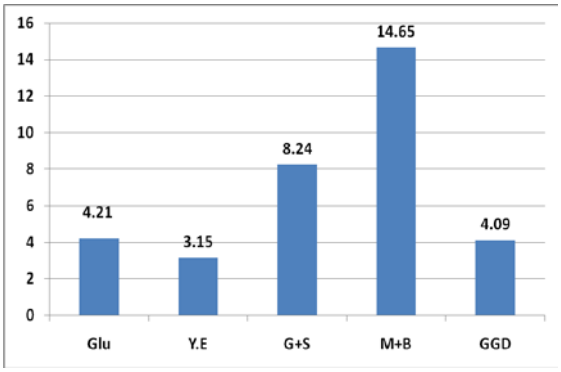

Fig. 7: Proteolytic activity of *B. licheniformis* in different media

When the proteolytic activity was estimated in the medium with different dosages of Wheatbran, at 4% Wheatbran, *B. .amyloliquefaciens* produced 17.42 U/mL of enzyme, while *B. .licheniformis* produced an activity of 15.92 U/mL. The activity was maximal (16.28 U/mL) at 37 C in *B. .amyloliquefaciens*, whereas the activity was 15.78 U/mL in *B. .licheniformis* at 37 C. The proteolytic activity in *B. .amyloliquefaciens* was 15.52 U/mL in pH 7.0 and 15.38 U/mL in pH 8.0. In case of *B. .licheniformis* the proteolytic activity was 14.86 U/mL at pH 7.0 and 15.01 U/mL at pH 8.0. The next batch of experiments was carried out to find out the exact pH of the media (7.0 – 8.0). At pH 7.2, the proteolytic activity was maximal at 15.56 U/mL in *B. .amyloliquefaciens*. In case of *B. .licheniformis*, the activity was 15.13 U/mL at pH 7.4.

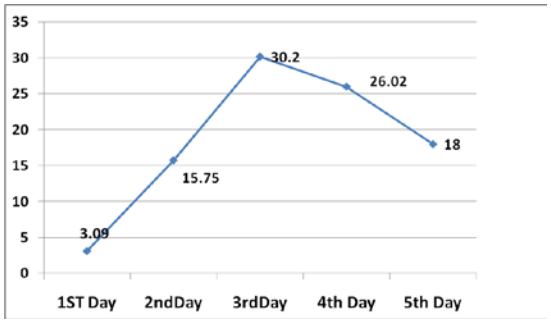

Fig. 8: Proteolytic Activity of *B. amyloliquefaciens*

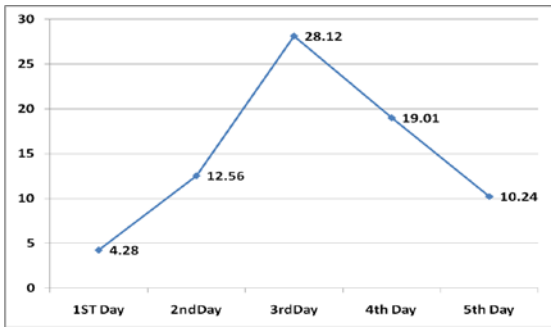

Fig. 9: Proteolytic Activity of *B. licheniformis*

In the optimized medium, Wheatbran at 4%, maltose at 2.5% and ammonium nitrate 0.3% (other components remaining the same), a maximal activity of 30.2 U/mL was obtained in *B. .amyloliquefaciens* at 72 h (Figure 8). In *B. .licheniformis* also the activity was maximal (28.12 U/mL) at 72 h (Figure 9). When the same culture filtrate was evaluated for fibrinolytic activity using Fibrin assay method, the activity was 28.98 FU/mL in *B. .amyloliquefaciens* and 26.63 FU/mL in *B. .licheniformis* (Table 2). The fibrinolytic activity of *B. .amyloliquefaciens* was 28.5 FU/mL and 28.21 FU/mL, when the agitation was adjusted to 200 and 225 RPM, respectively. In case of *B. .licheniformis* the fibrinolytic activity was 27.2 FU/mL at 200 RPM. There was hardly any increase (27.28 FU/mL) when the agitation was increased to 225 RPM. Stability of the enzyme was evaluated at different pH (4.0 - 9.0) and temperature (35 C – 70 C) levels. The enzyme of *B. .amyloliquefaciens* was active at pH 7.8 and temperature of 37 C. The enzyme was stable up to a temperature of 55 C. In *B. .licheniformis*, the enzyme was active at pH 8.0 and temperature 37 C and it was stable up to 50 C.

Table 2: Fibrinolytic Activity

Description	State	Activity (FU/mL)
Nattokinase (Commercial)	Powder	370 (FU/g)
Fibrinolytic Enzyme *B. .amyloliquefaciens*	Liquid	28.98
Fibrinolytic Enzyme *B. .licheniformis*	Liquid	26.63

According to Al-Juamily *et al* [23], the optimal conditions for fibrinolytic enzyme production were determined, using a solid Lentils medium (activity 25.25 U/mL) at pH 7.2 (65.381 U/mL), 48 h incubation time (15.766 U/mL) and shaking incubator (95.992 U/mL). The optimal carbon and nitrogen sources were mannitol and peptone or soya peptone with activity 44.0 and 50.0 unit/mL, respectively. Wang *et al* [3] reported that maltose was the best carbon and soy peptone was the best nitrogen sources for nattokinase production, however, Bhunia *et al* [24] inferred that lactose gave the maximal activity. In our study, it was observed that maltose and NH_4NO_3 were the best carbon and nitrogen sources for optimal enzyme production. In our study, we had experienced that the maximal activity was obtained at 200 rpm in shake flask studies. Kumar and Takagi [25] had tested different agitations (0, 50, 100 and 200 RPM) for the maximal production of fibrinolytic enzyme and according to them, the optimum agitation speed was 200 RPM, which gave the maximal activity. The ability of the *Bacillus licheniformis* to produce fibrinolytic enzyme at different pH (6.2, 6.8, 7.2, 7.5, 8) was studied [26]. According to Siraj [26], the productivity increased and the maximal activity was at the pH level of 7.2, which corresponds with our results. We obtained the maximal activity on 72 h, whereas, Ellaiah and Srinivasulu [27] had mentioned that the optimum incubation period for serine protease activity was 24 h. However, Wang *et al* [3] mentioned that the maximal enzyme activity was achieved at 60 h. The studies on the optimum pH and temperature on the enzyme activity revealed that the enzyme was active at a pH level of 7.0 – 7.5 and at temperatures of 45 – 50 C. In a similar study, Babu *et al* [28] observed that the optimum pH for enzyme activity was 7.0 and the temperature was 37 C.

The crude culture filtrate was purified partially with ammonium sulphate and Gel filtration methods. When the media components were optimized, the fibrinolytic enzyme activity of *B. amyloliquefaciens* in the culture filtrate was 28.98 FU/mL, which increased to 79.85 FU/mL in ammonium sulphate precipitate. After the Gel filtration the activity was 110.5 FU/mL. The specific activity increased from 1.38 in the crude enzyme to 11.41 in ammonium sulphate precipitate, to 210.47 after the gel filtration (Table 3). The purity of the enzyme improved by 152 folds after the gel filtration.

In case of *B. licheniformis*, the fibrinolytic enzyme activity was 26.63 FU/mL in the crude enzyme filtrate, 64.11 FU/mL in ammonium sulphate and 75.5 FU/mL after the Gel filteration (Table 4). The specific activity of 1.33 in the crude enzyme increased to 10.49 after ammonium sulphate precipitation and to 172.57 after the gel filtration. Simultaneously, the purity improved by 129.75 folds after passing through Sephadex gel.

Table 3: Purification Table of *B. .amyloliquefaciens*

Purification Step	Volume (mL)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification Fold
Crude Extract	100	2100	2898	1.38	1.0
$(NH_4)_2SO_4$	20	140	1597	11.41	8.26
Sephadex G-75	4	2.1	442	210.47	152.51

Table 4: Purification Table of *B. icheniformis*

Purification Step	Volume (mL)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification Fold
Crude Extract	100	2000	2663	1.33	1.0
$(NH_4)_2SO_4$	18	110	1154	10.49	7.88
Sephadex G-75	4	1.75	302	172.57	129.75

Scale up trials in a 7 L Laboratory fermentor were undertaken with *B. amyloliquefaciens*. In the first batch, the maximal activity was only 24.9 FU/mL after 68 h. In the second batch, after some fine-tuning of the fermentor, an activity of 42.6 FU/mL was achieved. In the third batch, a new seed medium containing soybean meal was tried. However, the activity was only 35.0 FU/mL after 70 h. Therefore, the seed medium used for the first two batches was tried in the fourth batch with some modifications in the operating parameters of the fermentor. This batch, after 72 h, yielded an activity of 55.6 FU/mL (compared to that of 28.98 mg/mL in shake flask studies). The enzyme activity in the harvested broth after the filtration and centrifugation was 52.0 FU/mL. When concentrated through ultrafiltration, the activity increased to 210.0 FU/mL (Table 5). The concentrated enzyme was then made into a powder by Spray Drying methods and the fibrinolytic activity in the powder was 745.0 FU/g (Table 6).

Table 5: Concentration of Enzyme

Parameter	Volume (g)	Activity (FU/mL)
Harvested Broth	1000	52.0
Prefiltration (100µ)	1000	51.5
Microfiltration (0.45µ)	985	50.25
Ultrafiltration (10kDa)	200	210.0

Table 6: Formulation of the Enzyme

Parameter	Volume (g)	Activity
Concentrated Enzyme	200	210.0 (FU/mL)
Filler Materials:	10	
Dextrin	30	
Sodium chloride	0.4	
Precipitated Silica		
Spray dried powder	40	745.0 (FU/g)

Table 7: Comparison of Enzymes based on Blood Clot Dissolution

Fibrinolytic Enzyme	FU/mL	Blood clot dissolution time (min)
Streptokinase	100	20
t -PA	100	25
Commercial Nattokinase	100	45
Test Enzyme	100	35

The Fibrinolytic activity of the test enzyme was higher than the other commercial protease / fibrinolytic enzymes. The time taken by the test enzyme for dissolving the blood clot was nearly the same as that of other available fibrinolytic enzymes (Table 7). As crystal structure of serine protease is unavailable, the 3-Dimensional structure (Figure 10) was built using homology modeling tool of Schrodinger suite. The protein sequence of Nattokinase (Accession No. WP_017417394.1, length: 382 amino acid) was retrieved from NCBI GenBank [4]. The template protein was identified using BLASTp tool [5]:

>tr|E5LCP0|E5LCP0_BACAM Fibrinolytic enzyme OS=*Bacillus amyloliquefaciens* PE=3 SV=1

MRGKKVWISLLFALALIFTMAFGSTSPAQAAGKSNGEKKYIVGFKQTMS TMSAAKKKDVI

SEKGGKVQKQFKYVDAASATLNEKAVKELKKDPSVAYVEEDHVAQAYA QSVPYGVSQIKA

PALHSQGFTGSNVKVAVIDSGIDSSHPDLKVAGGASMVPSETNPFQDNN SHGTHVAGTVA

ALNNSVGVLGVAPSASLYAVKVLGADGSGQYSWIINGIEWAIANNMDVI NMSLGGPSGSA

ALKAAVDKAVASGVVVVAAAGNEGTSGGSSTVGYPGKYPSVIAVGAVNS SNQRASFSSVG

SELDVMAPGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILSKHPNWT NTQVRSSLENT

TTKLGDAFYYGKGLINVQAAAQ

The crystallographic structure of 1SBN [29] was downloaded from Protein Data Bank (PDB) [30] and used as a template (resolution 2.10 Å). The template and the target sequences showed 97% identity and 71% similarity with query coverage of 503 total score and E value of 7e-179. Thus, the sequence similarity between the

target and 1SBN is reasonable for selecting 1SBN as a suitable template for modeling of our sequence (Figure 11).

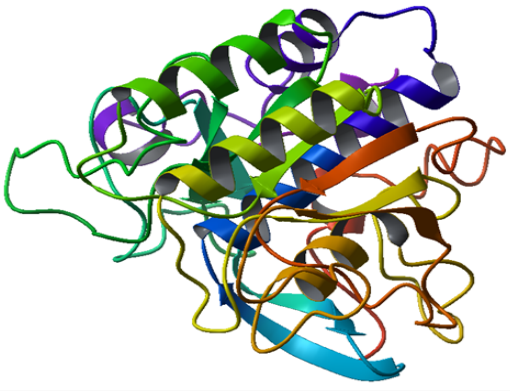

Fig. 10: 3D structure of the modeled protein

Fig. 11: Alignment between Template and Target Sequence

The initial model was developed using Prime Module software [31]. The bumps were removed and missing side chain atoms were added using the WHAT IF Web Interface (http: //swift. .cmbi. .ru. .nl/servers/html/ index. .html). The prerefined model was examined through Ramachandran plot, among total 382 residues the phi-psi distribution for 293 residues (90.4%) constituting of core alpha (A), core beta (B) and core left-handed alpha were found in most favoured region [32]. The loop refinement was performed using Prime Module. The modeled structure was evaluated for its stereochemical quality using PROCHECK server [6]. The structural superimposition of 1SBN chain A and target protein was performed using PyMOL molecular viewer (http: //www. .pymol. .org) and the structural alignment was done using Genious Pro (http: //www. .geneious. .com/). Results obtained from the above analysis were used for defining the initial docking sites for protein and drug ligands for further docking and molecular simulation studies.

The ligands were prepossessed using LigPrep tool of Schrodinger suite wherein the stereoisomer, tautomer, ionisation states were generated by Epik module [8]. The modeled protein was prepossessed using PrepWizard tool of Schrodinger suite which performs adding hydrogen, assigning bond-order, deleting heteroatoms and optimizing the protein. A grid for receptor centred at all residues was generated of 20, 20 and 20 Å (x, y and z) dimension. An extra precision, flexible ligand docking was performed using the generated grid. The in depth information of the binding site residue of the modeled protein and ligand residue was gained by visualising the docked protein-ligand binded complex structure at the atomistic basis. Based on the GlideScore obtained from post dock analysis, the highest interacting protein-ligand complex; protein-Curcumin Zn^{2+}complex was subjected for 100 picoseconds molecular dynamics simulation run employing MacroModel application of Schrodinger Suite. The atomic forces were defined by OPLS_2005 force field [33]. Trajectories were generated for 100 samples applying no constraints to bonds and angles. Simulation was performed in water solvent at 300K temperature using PRCG minimisation method for 500 iterations and 0.05 convergence threshold. The root mean squared deviation (RMSD) of the atomic position of the modeled protein-ligand complex with time was observed to understand whether the binding of ligand with the protein causes any structural deformation of the modeled serine protease protein. The superimposed configuration of all 100 samples at each picosecond is shown in Figure 12 and the RMSD plot of the protein-Curcumin Zn^{2+}complex for 100 picoseconds simulation run confirmed the stability of the protein structure.

Fig. 12: Superimposed Configuration of Modeled Serine Protease-Curcumin Zn^{2+}Complex for 100 Ps MD Simulation Run

Vasantha *et al* [34] had designed the Forward (5_-ATGGCGCAGTCCG TGCCTTAC-3_) and reverse (5_-TTACTG-AGCTGCCGCCTGTAC-3_) primers from the nucleotide sequence corresponding to the Nattokinase peptide, in order to amplify the sequence. Sequencing of the amplified fragment revealed 100% similarity between the peptides of the fibrinolytic (Nattokinase) from *B. amyloliquefaciens* and the published literature. Homology among these and other bacterial serine proteases was checked using the BLAST algorithm (NCBI, http: //www. .ncbi. .nlm. .nih. .gov). *B. subtilis* K-54, which originates from traditional Korean food, produces the fibrinolytic enzyme subtilisin K-54. The fibrinolytic (Nattokinase) enzyme produced by our strain, *B. amyloliquefaciens* isolated from spoilt milk, and subtilisin K-54 displayed the highest similarity and may therefore have a very similar three-dimensional structure, especially around the substrate binding sites. The crystal structure is unavailable for serine protease so the similarity observed between 1SBN and serine protease showed that 1SBN can be used as template sequence for 3-Dimensional structure modeling of target protein. Ligands were retrieved from PubChem compound database and their pharmacodynamics and pharmacokinetics properties were studied. The selected ligands were interacted with the modeled protein sequence. The detailed various energy contribution to the glide scoring function combined by *Emodel* score shows that the GlideScore for protein-Curcumin Zn^{2+} complex possess highest interaction energy of -5.49 kcal/mol as compared to other drug ligands. The ChemScore of lipophilic pair term and the fraction of the total protein-ligand van der Waals energy were found to have -3.72 kcal/mol of contribution and was dominant as compared to other interaction energy factors like hydrogen bond ChemScore (-1.83 kcal/mol) electrostatic rewards (-0.52 kcal/mol) and penalty for low molecular weight (-0.27 kcal/mol). This can be attributed to the involvement of lipophilic or hydrophobic protein residue leucine (LEU 82) in binding of Curcumin with the serine protease moreover the minimal electrostatic reward is due to polar but uncharged asparagine (ASN 77) residue interaction with the ligand. The hydrogen bonding interaction (Figure 13 and Figure 14) of leucine O-atom and asparagine H-atom with Curcumin Zn^{2+} at bond distance

of 2.136 Å and 2.053 Å respectively (Table 4.8) and leucine being one of the oxidatively stable residue provides stability to the protein-ligand complex. This essentially indicates that the nonbonded force of -3.72 kcal/mol makes Curcumin conformationally fit to the binding site via non-bonded interaction. Tailoring of protein residues may enhance stability and binding affinity of protein-Curcumin Zn^{2+} complex. A 100 ps molecular dynamics simulation run further strengthened the view that the molecular interaction maintains the stability of protein and curcumin after binding to protein does not deform or vary the protein conformation. The root means squared deviation analysis clearly implies that the protein-ligand complex is quite stable.

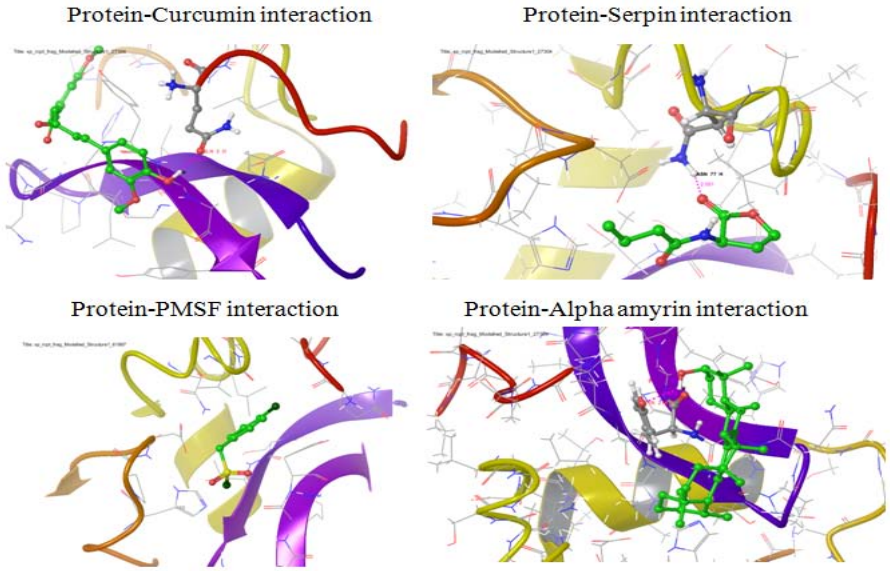

Fig. 13: Interaction profile of docked protein-inhibitor ligand complex binding site (hydrogen bond is shown by red dotted)

Protein-ligand binding affinity being one of the major keys for understanding therapeutic application of a drug was studied of modeled serine protease and selective ligands. The molecular docking study implied serine protease and Curcumin Zn^{2+} complex possesses highest interaction profile with GlideScore of -5.49 kcal/mol.

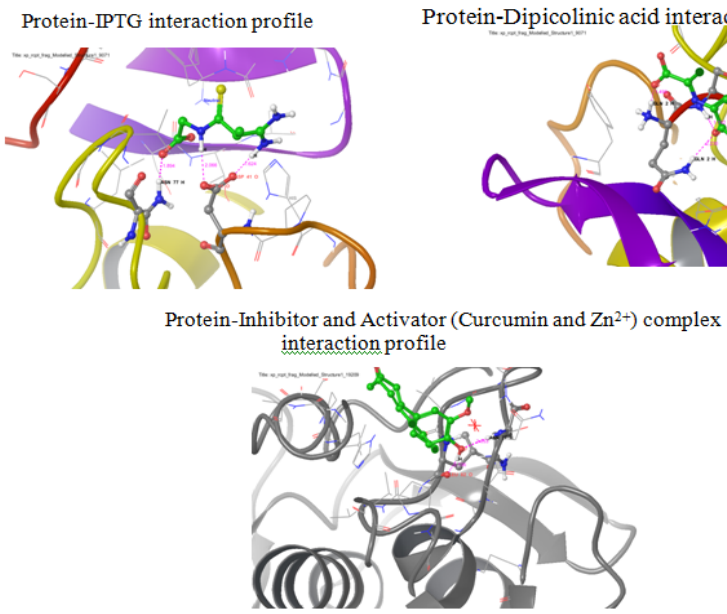

Fig. 14: Interaction profile of docked protein-activator ligand complex binding site and docked protein-activator and inhibitor (zn^{2+}) complex b (hydrogen bond is shown by red dotted line)

The protein residues, leucine and asparagine, were the active site of interaction of Curcumin Zn^{2+} complex with the protein.

Molecular dynamics simulation of modeled serine protease and Curcumin Zn^{2+}complex for 100 ps showed protein-ligand complex stability during simulation and at 100^{th} ps simulation run.

CONCLUSION

In this research work, two species of Bacillus, namely, *Bacillus amyloliquefaciens* and *Bacillus licheniformis*, were isolated from spoilt milk and soy flour, respectively, which exhibited fibrinolytic (Nattokinase) activity. In the laboratory scale studies, of these two

cultures, *B. amyloliquefaciens* produced the Fibrinolytic enzyme in higher quantities, 28.98 FU/mL, compared to 26.63 FU/mL in *B. licheniformis*. The maximal activities were obtained after 72 h. The optimum conditions at laboratory for maximal production of the fibrinolytic (Nattokinase) enzyme were: pH 7.2, temperature 37 C and agitation 200 rpm. When scale up studies with *B. amyloliquefaciens* in a laboratory 7 L Fermentor were undertaken, the maximal activity obtained was 55.6 FU/mL in 72 h, compared to that of 28.98 FU/mL in shake flask studies. The increase in activity in the Fermentor by about 2 times more than the shake flasks clearly indicates that the activity of 55.6 FU/mL is positively expected to increase to about 100.0 FU/mL in the large fermentors during commercial operation. After ultrafiltration, the activity improved to 210 FU/mL and in the spray dried powder, the activity as 745 FU/g. The molecular weight of the enzyme was estimated to be about 38 kDa. The enzyme had exhibited excellent blood clot dissolving property and therefore may be considered for further scale up and commercial exploitation.

An *in-silico* study has been performed to investigate the activators and inhibitors of serine protease. A 3-D structure of serine protease has been modeled by homology modeling. Binding affinity between protein and ligand plays a major role in enhancing or inhibiting the biological activity of a protein which can be understood by molecular docking studies. In in-silico studies, it was observed that PMSF acts as an inhibitor, thereby, confirming this fibrinolytic enzyme is a serine protease (Nattokinase). The future prospects of this study are to scale up the production trials in fermentors of 50 L and above capacities. The downstream processing has to be standardized in order to reduce the loss in activity and to formulate the enzyme as a powder for commercial exploitation as a Nutraceutical.

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CONFLICT OF INTERESTS

Rajani Gopal Gad (RGG), is a Senior Research Fellow and Prof S Nirmala, is her Research supervisor. Dr S Narendar Sivaswamy, is RGG's mentor and his company, SynkroMax Biotech Pvt Ltd, has supported the research work by providing the necessary facilities, financial assistance etc. As such, there is no conflict of interest among the three authors.

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