CLOT DISSOLVING ENZYME FROM A NEWLY ISOLATED BACTERIUM FROM SLAUGHTER HOUSE SOIL AND ITS FIBRINOLYTIC GENE EXPRESSION

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Abstract

A potent strain of fibrinolytic enzyme producing bacterium isolated from slaughter house soil was identified on the basis of its phylogenetic analysis using 16SrRNA sequence. The partial 16SrRNA sequence of the isolated strain was deposited in Gen Bank under accession number KF275670. The genomic DNA of the *Bacillus cereus* SPL3 was isolated and a primer for fibrinolytic enzyme gene was designed on the basis of already reported fibrinolytic enzyme gene of *Bacillus subtilis.* This primer is used for PCR amplification of fibrinolytic enzyme gene of *Bacillus cereus* SPL3. The vector and PCR product were digested by Bam H1 and Xho 1 resriction enzymes. After purification, the digested products were ligated by T4DNA ligase and constructed the recombinant plasmid. Recombinant transformants were produced using JM109 competent cells. To confirm the fibrinolytic activity of the expressed protein Fibrin zymography and fibrinolytic enzyme assays were done. This study provides ample evidence that the fibrinolytic enzyme can be actively expressed in *E. coli* using recombinant DNA technology.

Keywords*: Bacillus cereus***,** fibrin zymogram, fibrinolytic assay.

1. INTRODUCTION

We all are aware about the importance of maintaining a healthy life style. So we follow a balanced diet with nutritional supplements and exercise regularly. If our circulatory system is not working properly not only our cells will not get adequate nutrients but also results in imbalanced hemostasis mechanism. In a healthy person fibrinolytic system, is always in balance with the system that forms clot. Formation of blood clot, in a blood vessel is one of the main causes of cardiovascular diseases (CVDs). CVDs are the number one cause of death globally. More people die annually from CVDs than from any other cause [12]. Awareness about a heart healthy diet, including food additives containing fibrinolytic enzyme is very much important to reduce the risk of cardio vascular diseases. Fibrinolytic enzymes are therapeutic enzymes given after a heart attack to dissolve the blood clot blocking the coronary artery [12]. Although plasminogen activators including SK, t-PA and UK are widely used currently, these enzymes exhibit several weak points such as high price, low fibrin specificity, allergic reactions, and undesirable side effects like internal hemorrhaging. Therefore, new types of safer and less expensive fibrinolytic enzymes search are going on all over the world. Microorganisms are important resources for thrombolytic agents due to their rapid growth and limited space required for cultivation [5]. Several investigations are being done to enhance the efficacy and specificity of fibrinolytic therapy. In this regard, microbial fibrinolytic enzymes have much more medical interests during these decades. The most attracted among these is the genus *Bacillus species* from traditional fermented foods [3].

Fibrinolytic enzymes, specifically those from food-grade microorganisms, have the potential to be developed as functional food additives and drugs to prevent or cure thrombosis and other diseases related to thrombo-embolism. Nattokinase is such a food based dietary supplement, discovered in 1980 by Dr Hiroyuki Sumi, a researcher at Chicago University after testing over 173 natural foods as potential thrombolytic agents, searching for a natural agent that could effectively dissolve thrombus allied with cardiac and cerebral infarction [3]. The microbial source for nattokinase is *Bacillus subtilis* subsp. *Natto* and the fibrinolytic enzyme extracted is subtilisin NAT [4]. Subtilisin, a member of subtilases is a fibrinolytic enzyme very much common in *Bacillus* species. Among the microbial fibrinolytic enzymes, subtilisin attracted medical interests during these decades and regarded as a gift for thrombolytic therapy, due to its safety, convenient oral administration, confirmed efficacy, long term effects, protection, low-cost, and stability in the gastrointestinal tract [29]. Subtilisin (EC 3.4.21.14), an alkaline serine protease, is the most important extracellular protease of Bacillus species [30].Subtilisin belongs to the second largest family of serine proteases and in contrast to metallo proteases, which are widely distributed in nature, subtilisin has been found so far only among Gram-positive *Bacillus* spp. indicating a narrow functional diversity [Fuka et al., 2008]. Several subtilisins have been produced by different Bacillus species members. Subtilisin Carlsberg from Bacillus licheniformis, subtilisins BPN0 and DJ-4 from Bacillus amyloliquefaciens, subtilisin E, NAT, and J from B. Subtilis, Choggokkinase (CK) from Korean Chonggok-Jang, and subtilisin DFE from Chinese Dou-chi were the major subtilisin studies reported [31].

Subtilisins are monomeric proteins secreted extracellularly by numerous microorganisms, including *Bacillus* strains [32]. There are also several reports about the production of subtilisins by Flavobacterium [33]. Subtilisins are globular proteins, with the active site located in a shallow groove on the surface. The mechanism is the same with that of trypsin-like family enzymes but is evolutionary and structurally different [34]. All subtilisins have the same amino acid residues at the active site (conserved sequences especially around the three amino acids, serine 221, histidine 64, and aspartic acid 32, and therefore, basically the same mechanism of catalysis [35]. Moreover, for decades, subtilisins have also been a great model protein for studying protein engineering and intra-molecular chaperone-mediated protein folding. Because subtilisins are commercially important enzymes and are widely used for industrial purposes, they are regarded not only as a good model to analyze structure–function relationships of proteins, but also as potential candidates for biotechnological applications. For these reasons, along with the appropriate cloning of the gene, ease of expression and purification and availability of atomic resolution structures, subtilisin became a model system for protein engineering studies [36].

In this paper we describe about the fibrinolytic gene of the newly isolated *Bacillus cereus SPL3* cloned into a cloning vector and expressed in *Escherichia coli* JM109 cells, in order to makeover it into a fibrinolytic enzyme producing food grade microorganism which can produce the specific enzyme in large quantities. To make the therapeutic enzyme more safe, non-pathogenic *Escherichia coli* is selected as host for cloning as it has a short generation time, an established fermentation procedure, ability for high production of foreign protein, and is the most widely used prokaryotic organism for recombinant protein production. Moreover, *Escherichiia coli* belong to GRAS (generally regarded as safe) microorganism. The primer is selected from already reported subtilisin gene primer of *Bacillus subtilis*, which is also a food grade microorganism and is well known for its fibrinolytic enzyme, Nattokinase, which is an oral therapeutic enzyme [2].

2. MATERIALS AND METHODS

2.1 Screening of fibrinolytic enzyme producing bacterium and its identification by Phylogenetic analysis

For screening of the fibrinolytic enzyme producing bacterium soil samples were collected from slaughter house and isolated the bacterium by spread plate technique. The medium used for screening was minimal fibrin agar medium. Fibrin was purchased from Hi media laboratories, India and the components used for minimal fibrin agar medium were fibrin 10g, $MgSO_4$ 0.5g, KCl 0.5g, K_2HPO_4 1g, $FeSO_4$ 0.04g, $ZnSO_4$ 0.025g, $MnSO_4$ 0.025g, Na_2B_4O7 0.025g, NH_4MoO_2 0.025g, $CuSO_4$ 0.025g, Agar agar 3g and 1000 ml distilled water. The fibrinolytic enzyme production was indicated by a halo zone of clearance around the microbial colony, due to fibrin degradation. After confirming the fibrinolytic enzyme production, the bacterium was identified by phylogenetic analysis using bioinformatics tools

2.2 Isolation of genomic DNA from the *Bacillus species* and spectrophotometric quantification of DNA

The *Bacillus cereus* SPL3 was grown in LB broth and the cells were harvested for extraction of DNA according to phenol chloroform method by Sambrook et al (15). The concentrations of DNA in the sample and the purity of the DNA were estimated with the help of a spectrophotometer (Shimadzu). After the isolation of nucleic acids, the solution may be contaminated with proteins. The purity determination of DNA is performed based on different absorption characteristics of the proteins and nucleic acids. In spectrophotometric analysis, nucleic acids have an absorption maximum at 260 nm, whereas proteins absorb at 280nm. Therefore the purity of nucleic acids is assessed by determining the quotient of absorption measured at 260 nm and 280 nm. If the quotient is between 1.8 and 2, the purity is 70 to 90%.

2.3 Detection of genomic DNA by agarose gel electrophoresis

In 100 ml 1X TBE buffer 0.8 agarose was dissolved, by heating the agarose and then cooled the solution to 60^0C. Then added ethidium bromide to a final concentration of 0.5 mg/ml and mixed well by shaking the flask gently. The agarose solution was poured in to the gel casting tray and the solution was kept for 40 minutes to solidify into a gel. After solidification of the gel, the gel along with the tray was placed into the electrophoretic tank. The sufficient amount of TBE buffer was poured in to the tanks so that the gel was immersed in the buffer. 5µl of loading buffer was added into genomic DNA sample which has to be electrophoresed and mixed uniformly. The sample was loaded in to the wells in the gel electrophoresis unit and was run till the bromophenol blue dye reached the other end of the gel. The gel was analyzed and photographed using a U.V-transilluminator.

2.4 Selection of the primer

A primer for fibrinolytic enzyme gene was designed on the basis of already reported fibrinolytic enzyme gene of *Bacillus subtilis* [2]. The primers were synthesized and supplied by Bangalore Genei Biotech. The primers selected were *XhoI*-linked forward primer, 5'-CCG CTC GAG ATG AGA AGC AAA AAA TTG TGG-3' and *BamHI*-linked reverse primer, 5'- CGC GGA TCC TTA TTG TGC AGCTGC TTG TAC-3'(*XhoI, BamHI* sites were underlined).

2.5 PCR amplification of the fibrinolytic gene

The PCR amplification of Fibrinolytic gene was done using Taq DNA polymerase and the specific primers synthesized for the expression of subtilisin gene. The PCR reaction mixture consisted of 5 µL of 10XPCR reaction buffer (100mM Tris-HCl pH 8.3, 500 mM KCl, 11 mM MgCl2, and 0.1% gelatin), 3 µL of 2.5 mM dNTP mix, 1 µL each of forward and reverse primers (250µg / ml), 1µL of genomic DNA template (200µg/ml), and 3 units of Taq DNA polymerase prepared in a final 75µL reaction volume. Amplification was carried out in PCR setting the thermal cycling program as initial denaturation at 94°C for 2 minutes, which was followed by 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 60°C for 30 seconds, extension at 72°C for 60 seconds, and a final extension at 72°C for 2 minutes. The amplified PCR products were analyzed by 0.8% (w/v) agarose gel to confirm the amplification.

2.6 **Restriction Digestion**

The enzymes selected for restriction digestion were *Xho*1 and *Bam*H1 restriction enzymes. 7µl of amplified DNA as well as plasmid were taken in eppendorf tubes. To each tube 4µl of 10X assay buffer was added. To one eppendorf 2 µl of *xho*1 restriction enzyme was added. To the second eppendorf of 2 µl of *Bam*H1 restriction enzyme was added. To the third eppendorf 1µL each of *xho*1 and *Bam*H1 resriction enzymes were added. To all these tubes 7µl of sterile water was added to make up the volume to 20 µl. To the 4th tube 7µl of sterile water was added which was taken as control. Then eppondorfs were given a shot spin and mixture was incubated at 37^{0}C overnight.

2.7 Ligation

7µl of DNA and 7µl of plasmid were added to the eppendorf tube. To this, 2.5 µl of buffer and 2µl of ligase enzyme were added. Finally it was made up to 25µl by adding 6.5µl water to the reaction mixture and was incubated overnight at 4^{0}C.

2.8 Detection of restriction digestion and ligation by agarose gel electrophoresis

Agarose gel (0.8%) was prepared in TBE buffer and gel was casted. The sufficient amount of TBE butter was poured in to the tanks and the gel was immersed in the buffer. To the plasmid and genomic DNA sample, 5µl of loading buffer was added and mixed uniformly and loaded the samples to the wells and the electrophoresis was carried out. The gel was viewed and photographed using a U.V-transilluminator.

2.9 Transformation of *Esherichia coli* with a plasmid containing ampicillin resistant gene

Transformation involves two main steps, preparation of competent cells and transformation. Ampicillin was added to LB agar to get a final concentration of 100mg/ml and the LB agar plates are prepared. X-Gal (20mg dissolved in 1 ml dimethylformamide) and IPTG (100mg dissolved in 1 ml sterile milli Q water) were prepared. 40 µl X-Gal and 5 µl IPTG were spread over the surface of agar plates and incubated at 37 ^{0}C for 2 hours before use. The bacterium used was *E. coil* (JM 109) cells in calcium chloride solution were competent for transformation. For transformation pUC18 vector was used. All the steps for transformation were carried out in aseptic condition in a laminar air flow cabinet and were done according to the protocol by Sambrook et al [15].

2.10 Recombinant- Plasmid DNA isolation

The protocol followed for plasmid isolation was the alkaline lysis method, which was also from Sambrook et al., (1989). The isolated DNA was collected by centrifugation at 12000 rpm for 5 minutes. The pellet was washed with 70% of ethanol and dried till the alcohol evaporated completely. The DNA pellet was dissolved in 50 µl TE buffer and stored the plasmid DNA at –20^{0}C.

2.11 Construction of genomic DNA library

After transformation, the transformed cells are inoculated in to an ampicillin containing LB agar plate. White colonies, which were the transformed cells, have been aseptically transferred to fresh LB agar plates containing ampicillin (100mg/ml) using sterile tooth pick in an arranged manner.

2.12 Preparation of fibrinolytic protease from *E. coli*

E. coli cells containing the recombinant plasmids, pUC18-BFE were cultured overnight on LB agar plate containing ampicillin (100 mg/ml), and a single colony was then transferred into 20 ml of LB broth and allowed to grow overnight at 37^{0}C and with 120 rpm shaking. The seed cultures obtained were then inoculated into 500 ml of LB broth containing ampicillin (100µg/ml) and allowed to grow at 37^{0}C until reaching an optical density of 0.8 at 600 nm. At this point, 1mM of IPTG (isopropyl-α-thio-galactopyranoside) was added, and the cells were re-incubated for 4 hours at 20^{0}C and centrifuged at 12,000

rpm for 30 minutes at 4^0C. The supernatants and pellets were obtained by centrifugation at 12000 rpm for 20 minutes for further analysis of fibrinolytic activity.

2.13 Fibrinolytic Activity confirmation

In vitro fibrinolytic activity of the protease from *E. coli* was determined by fibrin plate method by Astrup and Mullertz (1952), with slight modification.10 µl each of IPTG induced culture supernatant, JM 109 competent cell supernatant and LB broth as control were dropped on the fibrin plates and incubated for 15 hours at 37^0C. The fibrinolytic enzyme production was further confirmed by minimal fibrin agar plate and by fibrin zymogram analysis.

3. RESULTS AND DISCUSSION

3.1 Isolation and identification of fibrinolytic enzyme producing bacterium

A potent strain of fibrinolytic enzyme producing bacterium was isolated from slaughter house soil using spread plate method in minimal fibrin agar medium (fig.3.1), confirmed its fibrinolytic activity by fibrin plate assay by Astrup & Mullertz method [1] .The newly isolated bacterium was identified on the basis of its phylogenetic analysis using 16S r RNA sequence and named as *Bacillus cereus* SPL3 (Gen Bank accession number KF275670).

Fig No. 3.1 Fibrin agar plate showing zone of clearance

3.2 DNA Isolation and PCR amplification of fibrinolytic enzyme gene

The genomic DNA of *Bacillus cereus* SPL3 was isolated by phenol chloroform method. Genomic DNA subjected to agarose gel electrophoresis and was photographed in a U.V-transilluminator. It was found that the DNA isolated was of good quality and free of RNA and protein contamination (Fig 3.2). The PCR amplification of fibrinolytic gene was done using the subtilisin specific primer as template for the genomic DNA of *Bacillus cereus* SPL3. To check the concentration of genomic DNA, UV spectrophotometric analysis was done. Concentration of genomic DNA was found to be 615 mg/ml. The quality of DNA obtained was of good quality as it was found that the A [260]/A [280] ratio observed by spectrophotometric analysis was 1.93.

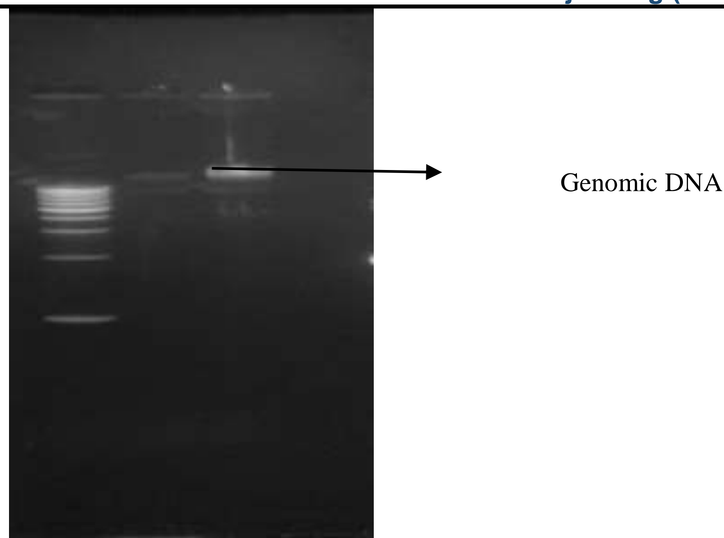

Fig No: 3.2 Genomic DNA. Lane 1 shows 1 kb DNA ladder and lane 2 and 3 show genomic DNA

3.3 Restriction digestion and Ligation of plasmid DNA and genomic DNA

The vector pUC18 and PCR product of gene BFE (Bacillus Fibrinolytic Enzyme) were digested by BamHl and Xhol restriction enzymes. The digested products were ligated by T4DNA ligase and constructed the recombinant plasmid pUC18-BFE (Fig 3.3). From the agarose gel electrophoresis it was found that the plasmid and genomic DNA were digested by restriction enzymes, Xho1 and Bam H1 and its combination. New bands were observed in agarose gel after ligation of genomic DNA.

Figure No: 3.3 Restriction digestion and ligation Vector pUC18 and gene BFE were digested by BamHl and Xhol restriction enzymes and the digested products were ligated by T4DNA ligase.

3.4 Transformation of the recombinant plasmid in E.coli

Recombinant transformants were produced using JM109 competent cells. The transformants were identified and cultured in LB medium at 37^{0}C. When the optical density of the culture reached 0.8 at 600nm, 0.7 mmol per litre, isopropyl-beta-D-thiogalactopyranoside (IPTG) was added and incubated at 20^{0}C for 16 hours.

After 16 hours of incubation, colonies were observed on LB agar plate. Growth of JM109 cells were observed on Amp- plates whereas no growth was observed on Amp+ plates. Transformed colonies were observed on plates containing ampicillin (100mg/ml), x-gal and IPTG. Blue and white colonies were clearly developed in agar plates incubated at 4 ^{0}C.

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Figure No: 3.4 Transformation of recombinant plasmid in *Ecoli* Recombinant plasnid was cloned into JM109 competent cells**.** Blue colored colonies with non-recombinant plasmid and white colonies with recombinant plasmids were observed in LB agar plates.

Blue colored colonies with non-recombinant plasmid and white colonies with recombinant plasmids were developed (Fig 3.4). If a substrate X-gal is present in the medium, the hybrid beta-galactosidase hydrolyses it and produces a blue product. Therefore, colonies containing non recombinant pUC18 appeared blue in colour. White coloured colonies were positive which contained inserted DNA fragments. Since lacZ operon become inactivated (Insertional inactivation), colonies containing recombinant vector fails to produce beta galactosidase and appeared as white colonies [15].

3.5 Construction of genomic DNA library

Genomic library was constructed by inoculating white colored colonies observed on LB agar plates after transformation and recombinant plasmid was isolated (fig.3.5).

Figure No: 3.5 Genomic DNA library plates. White colored colonies which were positive contained inserted DNA fragments and a genomic library was constructed using these transformed colonies.

3.6 Confirmation of fibrinolytic enzyme activity in expressed protein

Fibrinolytic activity was confirmed by fibrin plate assay, minimal fibrin plate as well fibrin zymogram. Fibrinolytic enzyme assay showed that the extracellular protein produced by recombinant clone had activity after IPTG induction and incubation at a lower temperature. The supernatant of the recombinant clone showed activity in minimal fibrin agar plate (Fig No: 3.6) and in the fibrin assay plate (Fig. 3.7). The activity was further confirmed by zymogram analysis. Fibrin zymogram analysis showed a single band of activity of subtilisin protein of approximate molecular weight 29kDa (Fig No: 3.8). All these results confirmed that the subtilisin gene of *B. cereus* SPL3 was cloned and the protein is actively expressed in *Ecoli.*

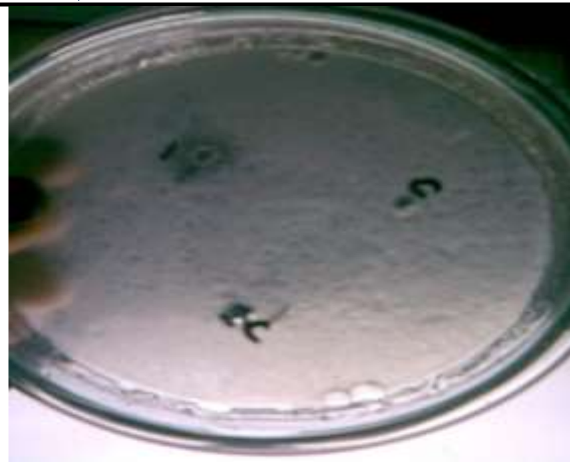

Figure No: 3.6 Minimal fibrin agar plate assay IPTG induced culture supernatant (1) showing zone of clearance, JM 109 competent cell supernatant (2c), LB broth as control (c)

Figure No: 3.7 Fibrin assay plate showing zone of clearance (Fibrinolytic assay by Astrup and Mullert) indicating that the IPTG induced *E. coli* culture supernatant has the ability to degrade fibrin.

Figure No: 3.8 Fibrin Zymogram of culture supernatant of clone *Escherichia coli.* The clear transparent band visualized on fibrin zymogram gel confirmed the fibrinolytic activity. The recombinant enzyme visualized in fibrin zymogram had approximately the same molecular weight (29 Kda) as other reported subtilisins.

4. CONCLUSION

This study provides evidence that the fibrinolytic enzyme BFE can be actively expressed in *E. coli* using recombinant DNA technology. PCR amplification of the genomic DNA of *Bacillus cereus* SPL3 by means of the subtilisin specific primers resulted in successful cloning and expression of fibrinolytic gene in *Escherichia coli*. The short growth cycle and simple culture medium of *Escherichia coli* make this strategy a potential way in producing this enzyme. The expression and recovery levels of recombinant enzyme can be enhanced by optimizing culture conditions and fermentation medium, which facilitate the development of this enzyme into an oral therapeutic product.

Several investigations are being pursued to enhance the efficacy and specificity of fibrinolytic therapy. In this regard, microbial cloning and expression of fibrinolytic enzymes in *Escherichia coli* attracted much more medical interests as this bacterium belongs to GRAS category. The Fibrinolytic enzyme gene from *Bacillus cereus* SPL3 was cloned into the vector and expressed in

Escherichia coli strain JM109. Recombinant plasmid was isolated and the recombinant clone showed extracellular fibrinolytic activity in IPTG induced culture supernatant. So the supernatant was subjected to ammonium sulphate precipitation and dialysis. Then it was concentrated by ultracentrifugation and analyzed by fibrin zymogram.

The recombinant enzyme visualized in fibrin zymogram had approximately the same molecular weight (29 Kda) as other reported subtilisins and the *E.coli* transformants showed subtilisin activity as the crude enzyme was inhibited by PMSF, which is a serine protease inhibitor. This study provides proof that subtilisin can be actively expressed in *E.coli*. The commercial availability of subtilisin is of great significance for industrial applications and also pharmaceutical purposes as thrombolytic agent. Thus, the new recombinant subtilisin is not only of academic interest, but also of practical importance.

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