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# Confirmation of FibrinolyticEnzyme present in Extracellular Proteins of *Bacillus cereus* SPL3 Screened from Slaughter House Soil

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**ABSTRACT:** A fibrinolytic enzyme producing bacterium was isolated from slaughter house soil and identified that it belongs to *Bacillus cereus* species and hence named as *Bacillus cereus* SPL3. Minimal fibrin agar was used for primary screening. Fibrinolytic spectrophotometric assay revealed that fibrinolytic enzyme production started at 72 hours of cultivation, reaching maximal values at 144 hours, $35^0$C and 120 rpm culture conditions. Its fibrinolytic enzyme activity was 15.5 U/ml in tyrosine-equivalent units. Fibrin plate assay method confirmed that it does not require any activation by plasminogen and it directly acts on fibrin and lysed the enzyme as it is not a plasminogen activator. Zymography also showed clear bands in fibrin zymogram gel. As fibrinolytic enzyme production was confirmed by spectrophotometric assay, fibrin plate method, minimal fibrin agar method and fibrin zymogram, this potent bacterial strain *Bacillus cereus* SPL3 was selected for further studies.

**KEYWORDS***:* Minimal fibrin agar medium, fibrinolyticassay, fibrin plate method, fibrin zymography, *Bacillus cereus*

## I.INTRODUCTION

The primary stage in the development of an industrial fermentation process is to isolate a specific strain capable of producing the target product and to confirm the specific enzyme production by different qualitative and quantitative methods. The next step is to identify the strain using morphological, physiological, biochemical as well as phylogenetic analysis. Bacterial fibrinolytic enzyme production is the most stable and economic way to produce protein with fibrinolytic activity. Proteolytic enzymes are the most important industrial enzymes that execute a wide variety of functions and have several important biotechnological applications (Prakash*et al*., 2005). The genus *Bacillus* contains a variety of industrially important species and approximately half of the present commercial production of enzymes is producing from the strains of *Bacillus* sp. (Beg and Gupta, 2003).

*Bacillus* strains are specific producers of extracellular proteases (Singh *et al*., 2001) and may be cultivated under extreme temperature and pH conditions to offer products that are, in turn, stable in an extensive range of unfavorable environments (Han and Damodaran, 1997). The enhancement of protease production by genetic manipulation has been studied in *Bacillus cereus*, *Bacillus subtilis*, *Bacillus stearothermophilus*etc by a number of researchers, which additionally highlights the importance of the enzymes from Bacillus species (Rao*et al*., 1998).*Bacillus cereus* group at present consists of six closely related species *Bacillus pseudomycoides* (Nakamura, 1998), *Bacillus mycoides* (Lechner*et al.,* 1998; Nakamura, 1998; Nakamura & Jackson, 1995), *Bacillus weihenstephanensis* (Lechner*et al.,* 1998), *Bacillus cereus* (Smith *et al.,* 1952; Somerville & Jones, 1972), *Bacillus thuringiensis* (Nakamura, 1994; Smith *et al.,* 1952; Somerville & Jones, 1972) and *Bacillus anthracis* (Logan et al., 1985; Smith et al., 1952; Somerville & Jones, 1972). The most common and well-studied of these members are *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis.* Genomic sequencing data has revealed that these three members are very closely related (Rasko *et al*. 2004) with their 16S rRNA gene sequence sharing over and above 99% similarity. Although they have similar

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characteristics, they are distinguishable as *Bacillus cereus* is most motile, *Bacillus thuringiensis*produces toxins (CRY crystal protein) and has plasmid encode, while *Bacillus anthracis*is not hemolytic.

In this paper we describeabout screening, isolation, identification and characterization of a *Bacillus* strain which showedverystrong fibrinolytic activity.To isolate this fibrin specific fibrinolytic enzyme producing bacterium we used minimal fibrin agar medium with some slight modification for preliminary screening. Here we also discuss about the various assay methods used for the further demonstration of the fibrinolytic activityas well as fibrin zymographywhich is another confirmation technique and why we selected this particular bacterium for further studies.

## II. MATERIALS AND METHODS

### A.SAMPLE COLLECTION FOR THE SCREENING OF MICROORGANISMS

The soil samples were collected from Western Ghats as well as slaughter houses in and around Thiruvananthapuram district of Kerala. Water sample also was collected from Hymavathy pond in Kerala University, Karyavattom campus, Thiruvananthapuram, India. The samples were taken to the laboratory, stored in refrigerator and screened for microorganisms following the standard procedures.

### B. MEDIUM USED FOR THE SCREENING PURPOSE, MINIMAL FIBRIN AGAR MEDIUM

The minimal medium with fibrin as the sole source of nitrogen was used for the screening of fibrinolytic enzyme producing microorganisms. This medium was described by Crabill and Reed in 1915, as a technique for the preliminary testing of the ability of microorganisms to synthesize fibrinolytic enzymes. 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 halozone of clearance around the microbial colony, due to fibrin degradation.

### C. FIBRINOLYTIC ENZYME PRODUCTION BY SUBMERGED FERMENTATION

Pre-inoculum of selected isolates were prepared from stock culture. The cultures (5 ml) were then inoculated in 50 ml of minimal fibrin liquid medium and incubated for 24 hours at 120 rpm and $37^0$C in a shaker incubator (New Brunswick shaker incubator) for 7 days. Samples were periodically withdrawn, centrifuged for 15 minutes at 10,000 rpm, and used as crude enzyme extracts for determining fibrinolytic activity.

### D. FIBRINOLYTIC ENZYME ASSAY BY SPECTROPHOTOMETER

Fibrinolytic activity was spectrophotometrically(Shimadzu) assayed according to the method described by Anson (1938) with some slight modification. The incubation mixture contained 0.625ml of 1.2% fibrin (1.2 g of fibrin was dissolved in 80 ml of 0.2 N NaOH and adjusted to pH 7.8 with 6 N HCl), 0.875 ml of 0.1 MTris-HCl (0.01 M $CaCl_2$, pH 7.8), and 0.25ml of crude enzyme. The incubation was carried out at $37^0$C for 30 minutes, and the reaction was stopped by adding 1.25 ml of 0.11 Mtrichloroacetic acid containing 0.22 M sodium acetate and 0.33 M acetic acid followed by 10 minutes incubation at room temperature and centrifugation. The absorbance of the supernatant was measured at 275 nm. All experiments were performed in triplicate. A fibrinolytic unit is defined as the amount of enzyme that gave an increase in absorbance at 275 nm is equivalent to 1 microgram of tyrosine per minute under the assay conditions of pH 7.8 and temperature $37^0$C. The total protein content determination was performed according to the method described by Lowry *et al*, 1951.

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### E. DIRECT AGAR PLATE ASSAY WITH MINIMAL FIBRIN AGAR MEDIUM

After confirming that considerable amount of enzyme production starts only from third day by spec assay, to visualize this in a petri plate the selected *Bacillus* strain was again inoculated in minimal fibrin agar medium and incubated for three days which was described as screening medium in this paper (Crabill and Reed technique, a qualitiative assay method for the qualitative estimation of the fibrinolytic enzyme).

### F. FIBRIN PLATE BY ASTRUP AND MULLERTZ METHOD

Fibrin plate method provides visual evidence of the presence of fibrinolytic enzyme. The original fibrin plate method of Astrup and Mullertz (1952) with slight modification was used for measuring the fibrinolytic activity of the test preparation. In Petri dishes, 9ml of 0.5% w/v fibrinogen (from bovine plasma, Sigma) in 50 mMTris–HCl buffer (pH 7.8) were taken. To each of these petridishes, 0.2 ml of plasminogen (10 units) was added and mixed well. In the next step clotting was induced by addition of 0.2 ml of thrombin solution (20 units). Plasminogen free fibrin plates were also prepared to detect whether the enzyme is a plasminogen activator or not. In order to speed up the clotting process, that is, for the rapid formation of fibrin clot, the plates were incubated at $37^0$C for 30 minutes. The plates after loading with extracellular protein of the bacterium were then again incubated at $37^0$C for 16 hours and visually inspected for liquefaction of fibrin clot.

### G. FIBRIN ZYMOGRAM

Specifically, fibrin zymography was performed out according to Kim *et al* (1998) with slight modifications. To cast the fibrin gel 0.12% fibrinogen (w/v) and 0.1 ml thrombin (10NIHU/ml) were mixed with 12% polyacrylamide gel solution. Following electrophoresis of the enzyme on the fibrin gel, the gel was soaked in 2.5% Triton X-100 containing 50 mMTrisHCl buffer (pH7.8) and then in distilled water for 30 minutes at room temperature, respectively. Subsequently, the gel was incubated in30 mMTrisHCl buffer (pH 7.8) containing 200 mMNaCl, 10 mM CaCl2, and 0.02% NaN3 at $37^0$C for 16 hours. The gel was stained with 0.5% Coomassie brilliant blue for 2 hours and then destained in destaining solution (methanol: acetic acid: water, 5:10:40).

### H. IDENTIFICATION AND CHARACTERIZATION OF THE SELECTED BACTERIUM

Biochemical characterization of the isolated bacterium with the largest transparent zone in minimal fibrin agar medium as well as highest fibrinolytic activity detected by assay was carried out. After the preliminary identification of the selected bacterium in our lab, it was sent to Institute of microbial technology, Chandigarh (IMTECH). Colony characteristics, Gram's staining, physiological tests like growth at different temperatures, pH and growth in different concentrations of NaCl were carried out. The selected biochemical tests included casein hydrolysis, gelatin hydrolysis, starch hydrolysis, esculin hydrolysis, MR, VP, citrate, nitrate, arginine, ornithin, lysine, indole degradation, urea production, catalase test, oxidase test, lactose fermentation growth on Mac Conkey agar, hemolysis, hydrolysis and fermentation of sorbitol, xylose, mannose, cellobiose, salicin, fructose, melibiose, sorbitol, adonitol, dulcitol, galactose, and raffinose. Phylogenetic analysis was also conducted using bioinformatics tools which have been discussed in another paper (PriyaLakshmi.S and A. Jayakumaran Nair, 2021).

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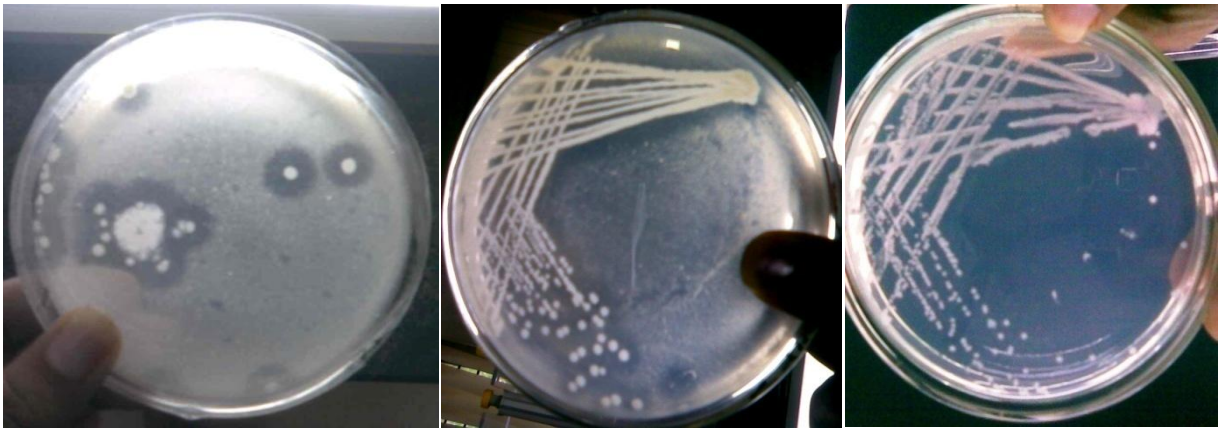
## III. RESULTS AND DISCUSSION

### A. ISOLATION OF THE MOST POTENT FIBRINOLYTIC ENZYME PRODUCING MICROORGANISM

Among the three samples collected from three sources of origin, microbial isolates from slaughter house soil showed highest fibrinolytic enzyme production in minimal fibrin agar plates. Among the 33 bacterial strains screened from the slaughter house soil, the strains which showed higher zones of clearances were subjected for further studies regarding confirmation of enzyme production. Moreover, pour plating as well as streak plating for isolating pure culture colonies of the bacteria strain SPL3 showed higher zone of clearances in minimal fibrin agar plate, after 24 hours of incubation. However, it did not show any zone of clearance in nutrient agar plate, which served as the control (Fig No.3.1).



(a)Pour plate (b) Streak plate (c) Control nutrient agar plate

Figure No: 3.1Minimal fibrin agar plate showing zone of clearances

### B. FIBRINOLYTIC ASSAY FOR THE SELECTION OF THE MOST POTENT STRAIN

Following the minimal fibrin-agar plate screening, the isolates with demonstrated fibrinolytic activity which were named as SPL1, SPL2, SPL3, SPL4 and SPL5 were selected for the fibrinolytic assay using spectrophotometer. For the strain SPL3, fibrinolytic enzyme production started at 72 hours of cultivation, reaching maximal values at 144 hours, $35^0$C and 120 rpm culture conditions. The bacterium was able to release a protein content of 2.2 mg/ml and its fibrinolytic enzyme activity was15.5 U/ml in tyrosine-equivalent units (one unit of the enzyme activity was defined as the amount of enzyme which releases 1 microgram of tyrosine per min under the assay conditions).The specific activity estimated was 7.05 U/mg.

### C. CONFIRMATION OF FIBRINOLYTIC ENZYME PRODUCTION

The selected strain of bacterium SPL3, which showed the strongestfibrinolytic activity after doing spectrophotometric assay was investigated for further confirmation by (1) minimal fibrin plate technique (Crabill and Reed, 1915), (2) fibrin plate method (Astrup and Mullertz, 1952) and (3) fibrin zymogram analysis (Kim *et al* 1998).

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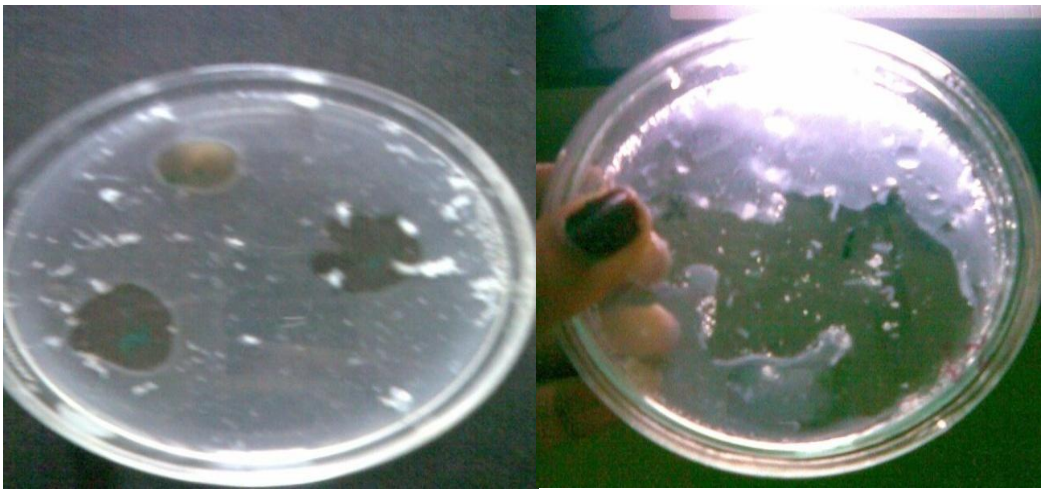
**D. MINIMAL FIBRIN AGAR PLATE DIRECT ASSAY METHOD**

According to the fibrinoytic enzyme assay using spectrophotometer, a detectable amount of enzyme was observed after three days of incubation in submerged fermentation medium. Therefore the minimal fibrin plate inoculated with the strain SPL3 was incubated for 3 days in an incubatorset at $37^0$C and strong fibrinolytic enzyme production was visualized by halo zone of clearance (Fig No.3.2).

Figure No: 3.2 Zone of clearance of Bacillus strain after three days of incubation.

**E. FIBRIN PLATE ASSAY METHOD**

The enzyme started degrading the fibrin clot after 30 minutes of incubation at $37^0$C when subjected to the fibrin plate assay method which is evident in fig. 3.3 (a). After 16 hours of incubation, due to strong fibrinolytic activity, it was not able to measure the diameter length of zone of clearance as it is observed in figure 3.3 (b).



(a)After 6 hours of incubation (b) After 16 hours of incubation

Figure No: 3.3 Fibrin plate method (Astrup's and Muellertz) showing zone of clearances

**F. FIBRIN ZYMOGRAM**

The prominent activity bands observed in the zymogram gel confirmed the presence of fibrinolytic enzyme and its structural isoforms. Area of fibrinolytic activity appeared as clear bands against dark blue back ground, where the enzyme has digested the fibrin. According to the fibrin zymography different protein bands identified corresponds to fibrinolytic activity of supernatant obtained. When the zymogram was developed, more than three highly active clear

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bands were observed when different fractions of crude enzymes from ammonium sulphate precipitation were applied to zymogram gel (Fig 3.4).

FigNo.3.4 Fibrin Zymogram

## G. IDENTIFICATION AND CHARACTERIZATION OF THE SELECTED BACTERIUM

IMTEC, Chandigarh, India reported that the selected strain SPL3 is closely related to *Bacillus cereus*.Gram's staining, showed rod shaped cells with ellipsoidal spores that are subterminal. The colony morphology details are irregular configuration, entire margin, flat elevation, rough surface, moist texture, off white pigmentation and opaque colonies. The strain grew at temperatures between $25^0$C to $42^0$C with a pH range between 5–12 and NaCl concentration between 2% - 8% (w/v). The strain is facultative anaerobic, nitrate positive, ornithin positive, lysine positive and catalase positive while it gave negative results to oxidase, MRVP, citrate, indole, arginine tests. Acid production was observed from xylose, mannose, galactose and raffinose while it gave negative results to substrates like sorbitol, cellobiose, salicilin, fructose, melibiose, adonitol and dulcitol. The strain gave negative results to casein hydrolysis and starch hydrolysis while it gave positive result to gelatin hydrolysis and fibrin hydrolysis. The strain is beta hemolytic. Phylogenetic analysis was also done and it showed that the bacterium is closely related to *Bacillus cereus* strains. All these results suggest that the bacterium belongs to *Bacillus* species and is closely related to *Bacillus cereus* and named as *Bacillus cereus* SPL3.

## IV. CONCLUSION

The fibrinolytic enzyme producing microorganisms have been extensively investigated from several natural sources and preliminary screening was being usually done using casein agar plates or skimmed milk agar plates, but here we used minimal fibrin agar plate for primary screening and selected a bacterial strain which does not degrade casein. Fibrinolytic activity is calculated by taking the ratio of FA (Fibrinolytic Activity) and CA (Caseinolytic Activity) and if this is below 1, indicates proteolysis occurrence than fibrinolysis (Hong *et al*., 2004). As this *Bacillus*, degrades only fibrin, but does not degrade casein it can be considered as a promising alternative source of a fibrin specific clinical therapeutic agent that cures thrombosis and related dysfunctions. Quantitative analysis of fibrinolytic activity by spectrophotometric assay revealed the enzyme units obtained was 15.5 U/ml, when minimal fibrin production medium was used and it became definite that the procedure followed for the production of enzyme are suitable for the industrial enzyme production and its scale up can be easily achievable by using suitable optimization of medium components as well as other conditions like temperature, pH, shaking speed etc. *In-vitro* blood clot lysis by crude enzyme gives the major finding of this study as the enzyme lysed the clot within 30 minutes. To investigate the action mode of the fibrinolytic enzyme from the bacterial strain SPL3, the plasminogen-rich and plasminogen-free fibrin plates were used. The enzyme solutions formed a clear halo zone on both plate types indicating that the enzymes are able to degrade the fibrin clot. However because the size of the clearing zone was same, it can be confirmed that the enzymes lacked the ability to convert plasminogen to plasmin (Jeong*et al*., 2001, Lu *et al*., 2007). Furthermore, in contrast with already described Tissue-plasminogen activator, Urokinase-plasminogen activator and Streptokinase plasminogen activator type enzymes, direct fibrinolytic activity would represent an advantage since undesirable side effects such as platelet activation due to plasmin (Kim *et al*., 2006) and systemic bleeding (Park*et al*., 1998) could be avoided.As our experiment became successful in isolation of a potent fibrinolytic protease producer from slaughter house soil, it could be concluded that fibrinolytic enzyme producing microbes are present in the areas where blood is present in large

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quantities and primary screening using minimal fibrin agar plates instead of casein agar or skimmed milk agar plates will help in isolation of more fibrin specific fibrinolytic enzyme producing microorganisms.

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