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Production of fibrinolytic protease from *Streptomyces lusitanus* isolated from marine sediments

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Abstract This study aim was to isolate, screen, characterize and optimize marine *Streptomyces* for fibrinolytic enzyme production. The potent actinomycete isolate was subjected to optimization. The parameters for optimization included pH, temperature, carbon, nitrogen sources. The crude supernatant produced was purified using size exclusion gel filtration chromatography. The optimized parameters for maximum productivity were found to be pH 7, 37°C, maltose and peptone respectively. The molecular weight of the purified enzyme was found to be 21kDa.

1. Introduction

Acute myocardial infarction was one of the prevalent cardiovascular diseases. [1] In the biological system formation of fibrin and fibrinolysis are maintained in balanced state. [2]. A statistical survey done by World Health Organization in 2005, reported that 17.5 million people were expired from cardiovascular diseases in 2005. Most of the global deaths are by CVDs only. [3] Throughout this period the leading causes of death remains to be caused due to heart disease and stroke [4] Different microbes serve as an ample source for fibrinolytic enzymes. *Streptomyces* are gram positive, aerobic members of the order *Actinomycetales*. A potent fibrinolytic protease to treat myocardial infarction has been produced from the marine isolate *Streptomyces lusitanus*. This study targets the characterization of fibrinolytic enzyme producing *Streptomyces* sp.

2. Materials and Methods

2.1. Isolation of Actinomycetes

Soil sample was collected from 5 meter depth beneath the upper earth surface from Kovalam beach, Chennai, Tamil Nadu. Isolation was done with starch casein agar (SCA) medium. The cultures were grown for 7 days at 30 °C. [5]

2.2 Primary screening of the isolates

The isolated strains were screened for proteolytic activity. Protease production was detected by casein hydrolysis in the skim milk agar media [6]

2.3 Morphological and biochemical characterization

The potent actinomycete isolates were selected based on screening results and subjected to biochemical and molecular characterization. [7] Biochemical characterization was done using a standard KB001 HilMVIC™ biochemical Test kit.



2.3.1. 16S r-RNA gene sequencing

The PCR amplification of 16Sr-RNA sequencing was carried out and the homology search for the sequence was done using BLAST analysis.

2.4. Protease production

The strain showed potent activity was cultured in starch casein broth production medium. The enzyme production was facilitated by incubating the strain at room temperature, (120 rpm) for 7 days [8].

2.5. Enzyme purification

The culture supernatant was precipitated at 60 % ammonium sulfate saturation, and dialysed against TE buffer (pH 7) overnight at 4 °C. Ion exchange chromatography was done with the agarose column against the same dialysis buffer. Elution was carried out by liner gradient of NaCl (0.1M - 0.6M). Fractions collected were checked for activity. [9]

2.6. Casein hydrolysis method

Tyrosine has been taken as standard for this test. The enzyme precipitated was mixed with 0.1M of sodium phosphate buffer 1.5M of TCA and incubated for 30 min . Then the optical density was measured at 690nm [10].

2.7. Clot lysis activity

Blood clot lysis was carried using modified Holmstrom's method [11].

2.8. Fibrinolytic activity

Fibrin plate assay was carried out according to Astrup and Mullertz. [12]

2.9. HPLC

High-performance liquid chromatography was done to determine the retention time of the purified enzyme. [13]

2.10. FTIR Analysis

The presence of different functional groups was determined by FTIR analysis. [14]

2.11. Optimization

The small scale production of fibrinolytic enzyme was determined by various parameters. The pH optimization was done from 6,7, 8 and 9 . It was incubated for 7 days at 37⁰C Different temperature ranging from 35°C, 40°C; 45°C were studied . Carbon sources like dextrose, sucrose, maltose and fructose; nitrogen sources like casein, yeast, peptone and L-asparagine were studied . The inoculated production medium was incubated for a week at 37°C. [15]

3. Results and Discussion

3.1. Isolation of marine soil actinomycete

Four actinomycetes were isolated from marine sediments (Fig.1)

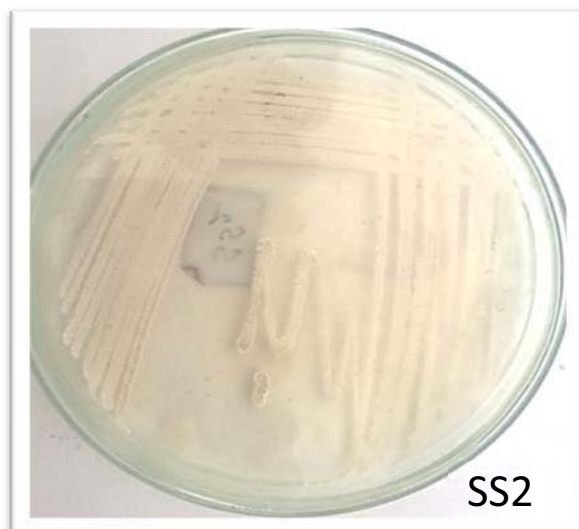


Figure 1 .Pure culture of potent actinomycete isolate

3.2. Screening for protease producing strain

Protease production was screened using casein plasminogen overlay method where the isolate SS2 demonstrated potent activity. (Fig.2)



Figure 2. Zone of hydrolysis on casein Plasminogen agar

3.3. Morphological and Cultural Characterization

The organisms were gram positive. Spores and hyphae of the actinomycete isolate was observed under 100x magnification of light microscopy and scanning electron microscopy. (fig.3, fig.4)The cultural characteristics were determined by aerial and substrate mycelium. Light pink, yellow and grey color colonies were observed.

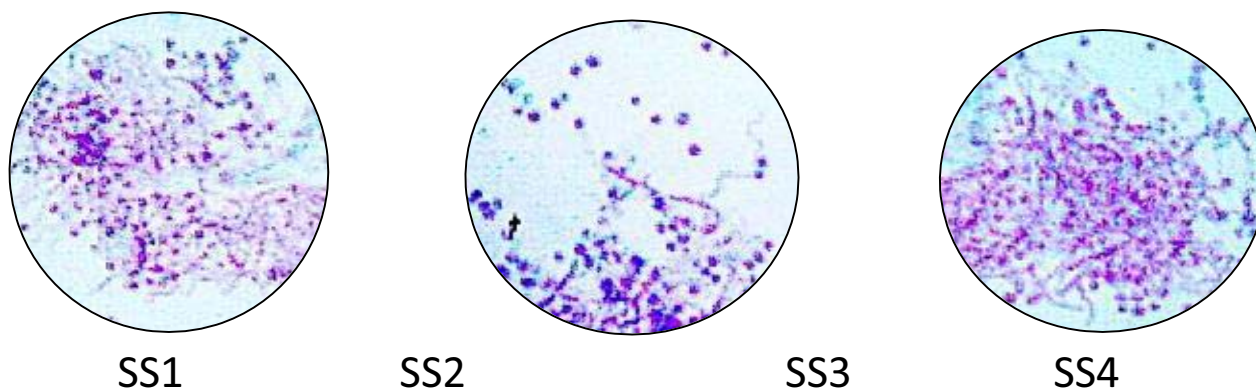


Figure 3: Gram staining-100X view of the isolates

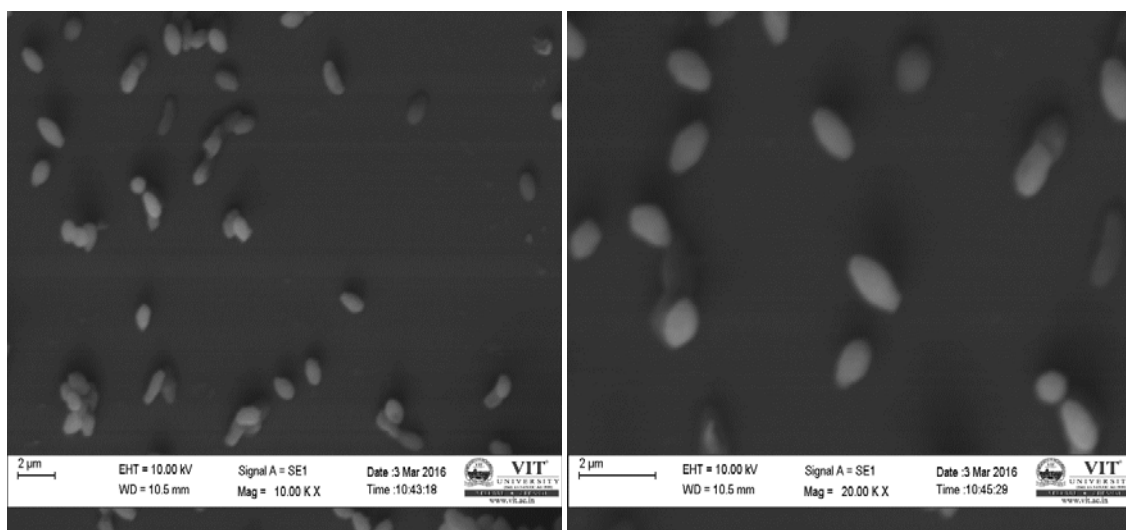


Figure 4. SEM analysis of actinomycete spores

3.4 Biochemical characterization

The organism showed positive for citrate, oxidase, urease, glucose, arabinose and negative for indole, methyl red and voges proskauer test. The results are compared with the Bergey's manual; the isolate was identified as *Streptomyces* sp.

3.5 Molecular characterization

16S r-RNA sequencing was done for molecular characterization. BLAST results of VITSAS was 99% similar to *Streptomyces lusitanus* (Fig5).

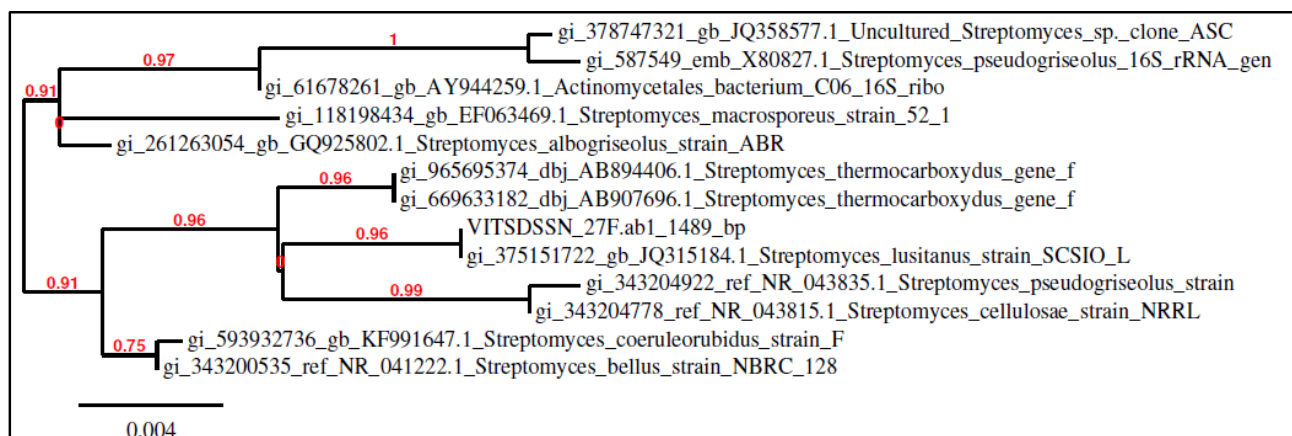


Figure 5.Phylogenetic tree of *Streptomyces lusitanus*

3.6 Purification of enzyme

3.6.1. Ammonium sulphate precipitation

Varying concentrations of ammonium sulphate salt concentrations was used to precipitate the protein. Precipitated samples were used for further analysis.

3.7. partially purified enzyme assay

3.7.1. Casein Hydrolysis

Maximum hydrolysis was observed for samples from 50% and 60% salt concentration representing maximum activity (Fig.6)

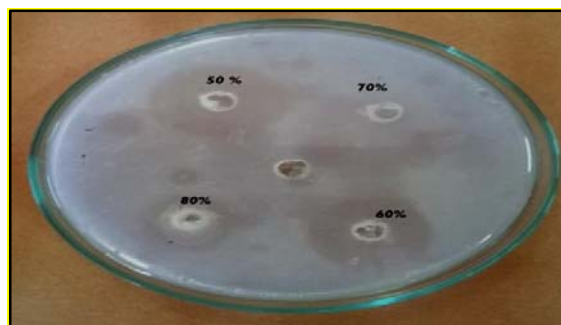


Figure 6.Casein plate method

3.7.2. Gel filtration chromatography

Elution flow rate was 2 mL/min. The eluted fractions were then assayed for protease activity. Fraction 9 showed maximum protease activity. (Fig.7)

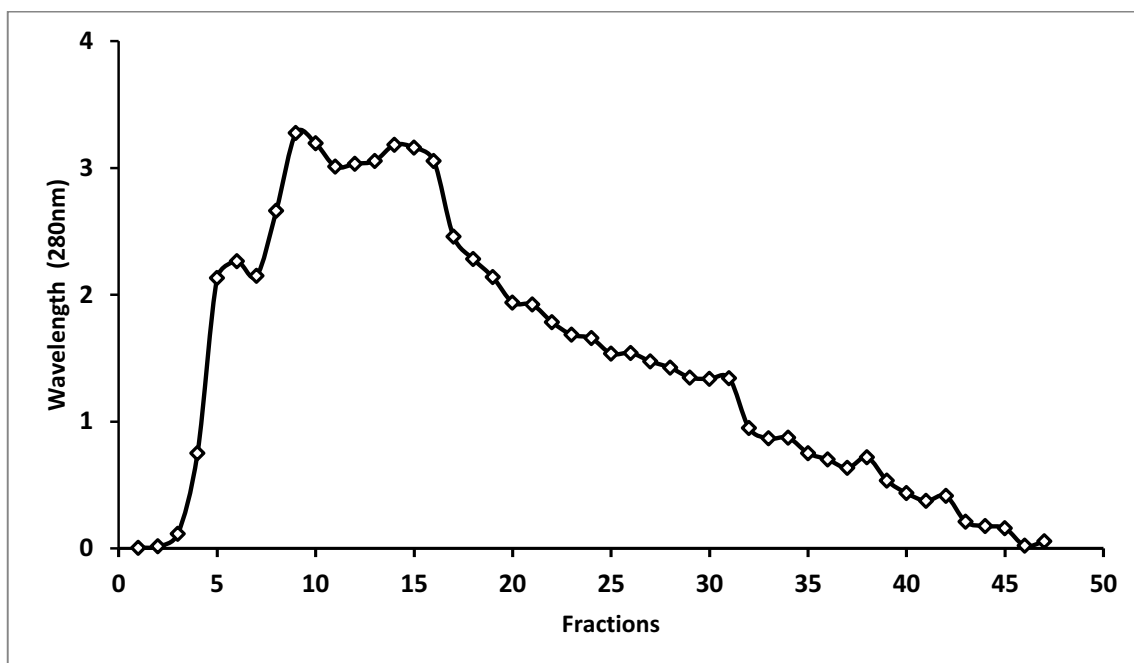


Fig 7 : Elution profile of fractions

3.7.3 Clot lysis assay: partially purified enzyme

Samples of 60% precipitation, dialyzed, partially purified showed clot lysis activity. (Fig.8). Purified enzyme showed maximum activity.

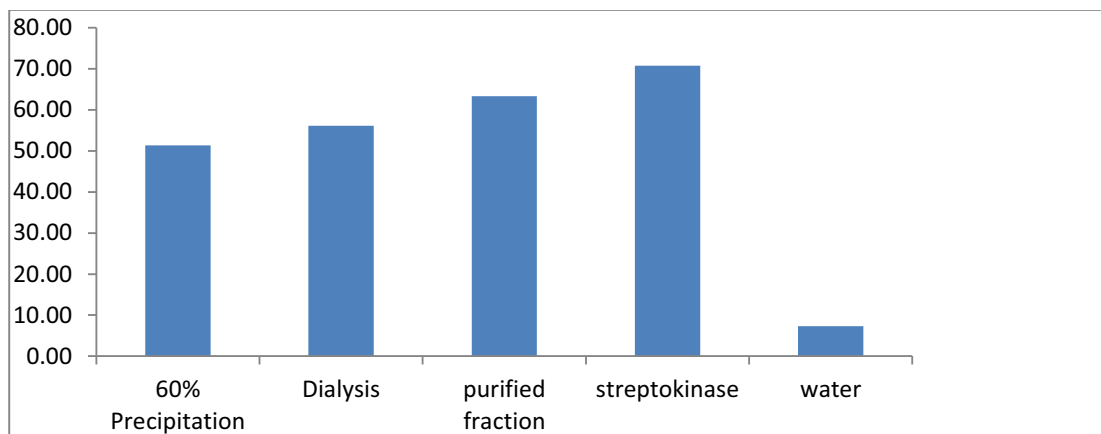


Figure 8. Clot lysis activity of partially purified enzyme

3.8. Fibrin plate assay

The crude enzyme was added on fibrin plate and incubated for 12h at 37°C. A clear zone of lysis around the well indicated degradation of fibrin. Purified enzyme showed the maximum fibrinolytic activity with clear zone of 8mm (Fig.9)

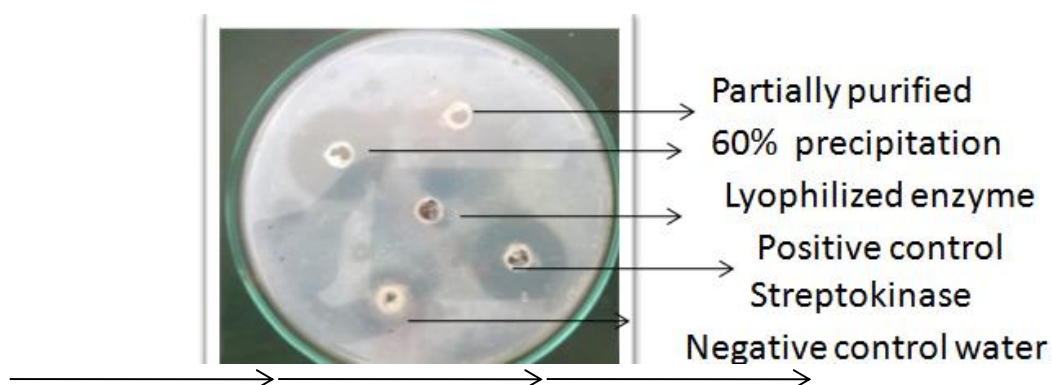
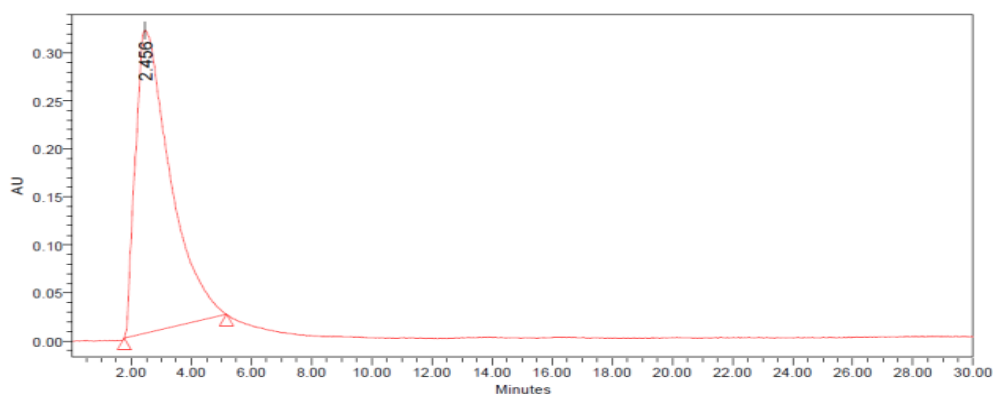


Figure 9. Fibrinolytic activity of enzyme from actinomycetes by fibrin plate method.

3.9 HPLC

The HPLC analysis of purified sample showed, the retention time was 2.4 min with 83.5% purity. (Fig.10)



10.

Figure HPLC

Peak Results					
Name	RT	Area	Height	Amount	Units
1	2.456	25365885	315823		

chromatogram.

3.10. FTIR -Fourier Transform Infrared spectroscopy analysis

In FTIR analysis, two absorption peaks were obtained at 1645.28 and 1145.72 cm^{-1} . It has C=C- (alkene) and C-N (aliphatic amine). Absorption peaks showed in the region of 989.48 and 935.48 (alkenes and carboxylic group). absorption peak at 495.71 cm^{-1} (C-Br) group. (fig.11)

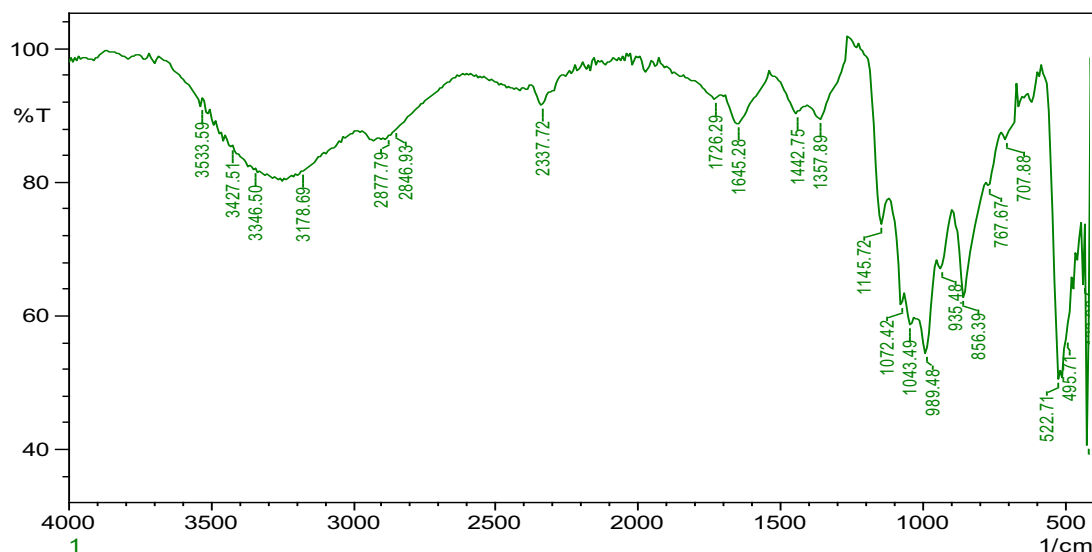


Figure 11. FTIR absorption peaks

3.11. SDS-PAGE

The molecular weight of fibrinolytic protease was 21kDa. (Fig.12)



Figure 12.SDS-PAGE

3.12. Optimization

The pH 7 was identified as optimum for the growth of the actinomycete isolate . It showed a maximum enzyme activity of 0.0062 U/mL. At very low and high pH (8 or 9), the activity of the enzyme decreased considerably. Growth rate was decreased at low temperature and no growth was observed at higher temperatures (45°C). Actinomycete showed efficient growth and maximum enzyme activity of 0.0114U/mL at 37°C. Among all the carbon sources, sucrose was the optimal since enzyme activity was 0.0174 U/ml. Altering the nitrogen source affects the growth of the organism. The organism showed maximal growth when starch casein broth was used as the optimal culture media with an enzyme activity of 0.0102 U/ml.

4. Conclusion

Naturally occurring protease may be the interesting candidate for the medical and industrial application. Marine microorganisms serve as new sources of therapeutics. The present study was focused on production and purification of fibrinolytic protease from marine *Streptomyces*. This study further encourages research in *Streptomyces* sp. because of its pharmacological value.

Acknowledgements

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