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Screening, molecular characterization and assessment of profound protease activity from *Streptomyces glomeroauranticus* VITSDVM6

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Abstract : In the present study, actinomycetes were isolated from soil samples collected from the plant nursery at VIT University; eight different strains were isolated and screened for proteolytic property. The isolate exhibited profuse hydrolytic activity on casein media were considered as potent strain and was subjected to 16srDNA analysis. The BLAST search revealed the highest similarity with *Streptomyces glomeroaurantiacus* strain NBRC 15418, further the study strain was coined as *Streptomyces glomeroaurantiacus* VITSDVM6. The total protein contents of the crude enzyme extract, precipitated, dialysed and purified enzyme were determined by Lowry's method. The specific activity was determined by casein hydrolysis assay which was found to have for the crude enzyme (897U/mg), Precipitated (1082 U/mg), dialysed (1285 U/mg) and purified (1336 U/mg) respectively. Hence the results showcase the productivity of *Streptomyces glomeroaurantiacus* VITSDVM6 as efficient producer of extra cellular protease, which can be beneficial for industries applications.
Keywords: Actinomycetes, *Streptomyces glomeroauranticus*, protease, clot lysis.

Introduction

Actinomycetes are a heterogenous group of gram positive, mycelium-forming bacteria that are found to be abundant in both soil and marine habitats[1]. They are known for the presence of high G+C content in their DNA. They have proven to be a valuable source of antibiotics and other important metabolites that act as antifungals, antivirals, antithrombotics, immunomodifiers, anti-tumor drugs and enzyme inhibitors; and in agriculture, including insecticides, herbicides, fungicides and growth promoting substances for plants and animals. Protease is an important enzyme from the commercial point of view. There are various types of proteases that include alkaline protease, aspartic protease, serine protease and metalloprotease. Proteases have proven to be useful in a number of industries including the food industry, detergent industry, pharmaceuticals and in leather industry. Due to their wide range of applications, the production of protease enzyme using microbial sources like bacteria and fungi has gained a lot of importance in the recent past. Although actinomycetes are primarily known for its antibiotic production, recently efforts are being made to produce industrially important enzymes like protease among many others using actinomycetes. Protease secretion is dependent on the availability of nutrient sources as well as the growth rate. It also depends on environmental factors like pH and temperature [2]. Proteases have potential applications as stain-removers that can be used as

additives to detergents. In the food industry, certain types of proteases like papain are utilized for meat tenderization[3]. Certain proteases like Tissue Plasminogen Activator also find medical applications as in treatment of blood clots[4]. Bromelain has proven to have anti-inflammatory activity[5]. Also, some proteases have been shown to exhibit cytotoxic activity on selected cancer cell lines[6]. Hence the use of *Streptomyces* for proteases production is justified by their ability with potential applications.

Materials and methods

Sample collection

Soil samples were collected in sterile plastic bags from the VIT nursery, located in VIT University, Vellore.

Isolation of actinomycetes from the soil sample

Diluted soil samples were plated separately on actinomycetes isolation agar obtained from HiMedia laboratories with pH adjusted to 7.2. The plates were incubated for 7 days at room temperature. The plate containing well-isolated colonies was selected for further studies. The different colonies were then cultured on actinomycetes isolation agar and incubated for 7 days at room temperature. The colonies were designated as VITSDVM-1 to VITSDVM8.

Screening for protease activity

Primary screening was carried out by streaking the cultures on casein agar followed by incubation at 27˚C for 5 days[7]. For the secondary screening, the various cultures showing zone of clearance from the primary screening were inoculated into starch casein broth containing starch, casein, KNO_3, NaCl, K_2HPO_4, $MgSO_4$, $CaCO_3$ and $FeSO_4$. pH of the broth was adjusted to 7.2. The inoculated broths were incubated for 7 days at room temperature on a mechanical shaker and the broths were centrifuged at 10,000 rpm for 10 minutes. For the secondary screening, 100µl of the supernatants were used for diffusion assay on casein agar and skim milk agar.

Morphological and biochemical characterization and microscopic characteristics

The culture VITSDVM6 showing effective hydrolysis of casein in the secondary screening was subjected to microscopic and biochemical characterization. It was also streaked on various International Streptomyces Project (ISP) media (ISP-1 to ISP-7) and the aerial and surface morphologies on these media were studied. The VITSDVM6 culture was also used for protease production. The biochemical characterization by testing for citrate utilization, lysine utilization, ornithine utilization, urease production, phenylalanine deamination, nitrate reduction, H_2S production and utilization of sugars like glucose, lactose, arabinose, sorbitol and adonitol.

Molecular characterization and Phylogenetic analysis of the culture VITSDVM6

Total genomic DNA was isolated using the phenol Chloroform method. PCR amplification of 16SrDNA was carried out using the primers FC27 (5_to3_ AGAGTTTGATCCTGGCTCAG) and RC1492 (5_to3_ TACGGCTACCTTGTTACGACTT**)** [8]. The PCR product was detected by agarose gel electrophoresis. Sequencing was performed using big dye terminator cycle sequencing kit (Applied BioSystems, USA). The sequence was subjected to homology search using BLAST programme of the National Center for Biotechnology Information (NCBI) and the sequence data has been submitted to the GenBank database under the accession number KJ725088 . The acquired sequences were used for a gene homology search, with the 16S rRNA sequences available in the public databases from BLAST and were identified to the generic level. Using the CLUSTAL-W Multiple sequence alignment program (Strasburg, France), the 16SrDNA sequences of the strains were aligned with sequences of related organisms obtained from GenBank and a phylogenetic tree was constructed by neighbor-joining method using the Evol View program[9]. To validate the reproducibility of the branching pattern, a bootstrap analysis was performed

Production, extraction and purification of protease enzyme

The production strain VITSDVM6 was inoculated into starch casein broth [10] and incubated for 7 days at room temperature on a mechanical shaker. 50ml of this broth-culture was subjected to centrifugation at 10,000 rpm for 10 minutes. Ammonium sulphate was added to the culture supernatant in small quantities with constant

stirring in order to achieve saturation. The supernatant was then concentrated by precipitation with ammonium sulphate to 60%–70% levels. The precipitates were dissolved in 25mM phosphate buffered saline and dialyzed over night against the same buffer. The resultant dialysates were regarded as partially purified and were used for further studies. Gel filtration chromatography was carried out with a self-packed Sephadex G-100 column (1.5-2.0 cm in diameter; 25 cm gel bed height; 0.8 ml sample volume) equilibrated with sterile column buffer phosphate buffered saline (pH 7.0). 0.8 ml dialyzed sample was loaded on the prepared column with a flow rate of 1ml/min. The fractions containing 2 ml were eluted and analyzed.

Determination of total protein content

The total protein contents of the crude enzyme extract, precipitated sample and the dialysed were determined by Lowry's method. 200µl of each of the samples were mixed with 1ml of Mixed reagent and incubated for 10 minutes at room temperature. This was followed by addition of 300µl of Folin Ciocalteau(FC) reagent and incubation for 60 minutes at room temperature. The absorbance was read at 660nm. Bovine serum albumin was used as the standard.

Protease Assay

The specific enzyme activity was determined by casein digestion method. 0.5 g of casein was dissolved in 25ml of PBS and kept in water bath for 5 minutes. 1ml of this casein was added to 200µl of each of the crude, precipitated, dialysed and purified samples and kept in water bath for 30 minutes. 1ml of trichloroacetic acid was added to each of the tubes and further incubated for 30 minutes in a water bath[11]. The absorbance was read at 560nm. Tyrosine was used as the standard. One unit of protease activity (AU) was defined as the amount of enzyme releasing 1 µmole of tyrosine equivalent/min.

Results and discussion

The potential use of *Streptomyces* for producing proteases has various applications. *Streptomyces* that produce protease include *S. clavuligerus, S. griseus, S. rimosus, S. thermoviolaceus* and *S. thermovulgaris*[16]. They produce variety of extracellular proteases that have been related to aerial mycelium formation and sporulation[15, 17]. Bacterial proteases are most significant compared with animal and fungal proteases and have wide range of industrial application[14] Some of these proteases, like the serine proteases of *Streptomyces griseus*[19,20] have been characterized structurally and enzymatically. There have also been many descriptions of isolation and partial characterization of alkaline protease activities from other members of the genus *Streptomyces* like *Streptomycesgulbargensis, Streptomycesviridifaceins,Streptomyces* sp.[18].The screening of protease producing actinomycetes was isolated from soil sample. Out of the eight colonies screened one isolate showed prominent proteolytic activity with maximum zone of clearance on casein agar. The supernatants from the centrifuged broth-cultures were tested for proteolytic activity by diffusion test. The degrading property on skim milk agar of VITSDVM6 was observed by zone of clearance for crude enzyme (8mm), dialysed (9mm) and purified (9mm) respectively. The casein agar plates revealed the maximum hydrolysis by forming translucent zone upon qualitative screening (Fig1). The potent isolate VITSDVM6 was subjected to morphological, biochemical and molecular characterization. The morphology of the VITSDVM6 colony was determined in the selective media. Isolates showed no pigmentation, produced mycelium, aerial hyphae and spores with filamentous colony margin and Physiological and biochemical properties have been studied. The strains differ by their ability to take up different sources of carbon and intensively utilize glucose. The culture VITSDVM6 showed mostly cream surface morphology on International Streptomyces Project (ISP) media 2, 3, 4, 5, 6, 7 whereas it showed black surface morphology on ISP1. The aerial morphological characteristics were grey, creamy, white, white, grey, white and milky white respectively on the ISP media. The culture designated VITSDVM6 showed good growth on all the ISP media. (Table 1,2). The protease producing VITSDVM6 resembling *Streptomyces* were identified following 16srDNA sequence analysis which showed similarity to *Streptomyces glomeroaurantiacus* strain NBRC 15418 and were subsequently designated as *Streptomyces glomeroaurantiacus* VITSDVM6 and were submitted to NCBI under accession numbers KJ725088. (Fig 2). The isolate VITSDVM6 revealed the maximum protein content and showed maximum productivity with protease activity in production media. The similar reports on maximum protease production were also reported by [21] on *Streptomyces globisporus* 203A. Studies on *Streptomyces clavuligerus* cultures for protease production showed that the amount of enzyme produced varies greatly with the culture media used[13]. As a result of the investigations carried out it has been established that protease producers are more efficient from the genera *Streptomyces*. The elution profile yielded a well-resolved single peak showing protease enzyme after activity measurement (Fig 3). The absorbance values have been found to be the maximum at the fractions number 13,14

and 15. These fractions thus contain the purified enzyme in maximal amounts. The total activity of the crude enzyme was found to be 45951 U, precipitated 11380 U, dialysed 8620 U and purified8148 U respectively. The purified enzyme showed the 17.8 % recovery with the specific activity of 1336 U/mg and the purification fold of 1.5(Table 3).Similar study on time course production of protease enzyme from *Streptomyces Pseudogrisiolus* NRC-15 was found to have specific activity of 270 U/mg proteins at 96 h^{12}. The results indicate that gel filtration chromatography yielded a pure enzyme and the activity of enzyme is attributed by the removal of other unwanted proteins by purification. Hence the study strain has shown the potential productivity of protease enzymes.

(A) (B) (C) (D)

Fig 1 (A) Plate showing zone of clearance around the streaked colony on starch casein agar. (B) Plate showing zone of clearance around the skim milk agar (C). Plate showing zone of clearance produced by the supernatant from the centrifuged broth-culture on skim milk agar (D) Plate showing zone of hydrolysis on casein agar

Table 1: Biochemical characterization of *Streptomyces glomeroaurantiacus* VITSDVM6

Test	**Reaction**
Citrate utilization	Negative
Lysine utilization	Negative
Ornithine utilization	Positive
Urease	Positive
Phenylalanine deamination	Negative
Nitrate reduction	Negative
H_2S production	Positive
Glucose utilization	Positive
Adonitol utilization	Negative
Lactose utilization	Negative
Arabinose utilization	Positive
Sorbitol utilization	negative

Table 2: Morphology of *Streptomyces glomeroaurantiacus* VITSDVM6 on the various ISP media

Isp Medium	**Aerial Morphology**	**Surface Morphology**	**Growth**
1	Grey	Black	Good
2	Creamy	Creamy	Good
3	White	Cream	Good
4	White	Cream	Good
5	Grey	Cream	Good
6	White	Cream	Good
7	Milky white	Cream	Good

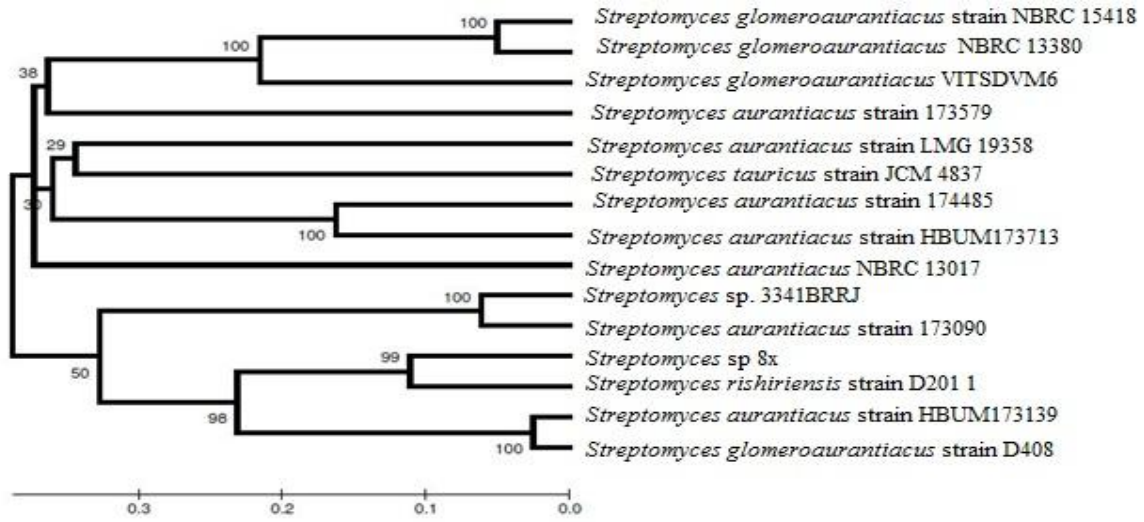

Fig 2 Phylogenetic tree of the strain *Streptomyces glomeroaurantiacus* VITSDVM6

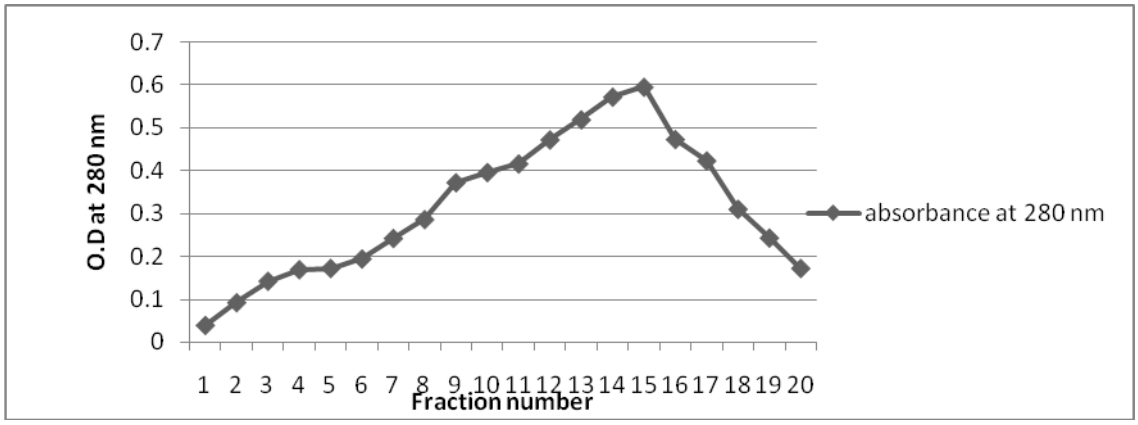

Fig 3 Elution profile of *Streptomyces glomeroaurantiacus* VITSDVM6 after gel filtration chromatography

Table 3: Depicting the enzyme activity of the crude, precipitated and dialysed samples

Purification step	Total Volume	Total activity(U)	Total Protein(mg)	Specific activity(U/mg)	Recovery (%)	Purification (fold)
Crude	50	45951	51.2	897	100	1
60-70% Precipitate	9	11380	10.5	1082	24.8	1.2
Dialysis	5	8620	6.7	1285	18.8	1.4
Sephadex G-100	4	8148	6.1	1336	17.8	1.5

Conclusion

It is clear fact that utilization of casein substrate showed with the maximum outcome of enzyme yield and highest productivity. Hence the study evidences the protease enzyme to exhibit potential property. Futher application studies are likely to possess several advantages of protease including the applications ranges from industrial, bioremediation process and pharmaceutical medicines.

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