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PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR PROTEASE FROM SOIL ISOLATE *Stenotrophomonas maltophilia* AS NOVEL TARGET FOR FIBRINOLYSIS

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ABSTRACT

Fibrinolytic protease has a potential role as thrombolytic agent and useful in cardiovascular disease (CVD) treatment. In this study, a potent fibrinolytic protease-producing bacterium was isolated from casein growth medium and identified as *Stenotrophomonas maltophilia* by characterizing biochemical tests and 16 s rRNA sequencing. The protease production was carried out by submerged fermentation and further purified by ammonium sulphate precipitation, dialysis and ion-exchange chromatography methods. The specific activity of protease significantly increased with step by step of purification process and finally became 1.87 U/mg protein with a purification fold of 1.68 and yield of 59.52%. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed molecular weight of purified protease as ~47kDa, respectively. To the authors' knowledge, this is the first report of ~47 kDa protease from the bacterial strain *Stenotrophomonas maltophilia*. The characterized enzyme exhibits maximal enzyme activity at pH 8 and temperature 40°C. The activity of enzyme is activated by cations Na^{+}, K^{+}, Ca^{++} and Mg^{++}, whereas significant loss of activity was observed with EDTA, Zn^{++}, Cu^{++} and Hg^{++}. The Lineweaver-Burk plot analysis showed a k_m value of 0.303 mg/ml and V_{max} as 0.00714. The present study indicates that fibrinolytic protease produced from the *Stenotrophomonas maltophilia* KJ801664 has an antioxidant property by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) method.

Keywords: Fibrinolytic, Caseinolytic, SDS-PAGE, Amino acid composition, Antioxidant activity

INTRODUCTION

Proteases are proteolytic enzymes which catalyze the hydrolysis of proteins based upon their structures or properties of the active site. Cardiovascular (CDV) disease is one of the major reasons of death globally. Cardiovascular disease is caused due to abnormal fibrin accumulation in the blood vessels or a fibrin clot adhering to unbroken vessel walls of the endoepithelium. Thrombosis can stop blood circulation in vessels (arteries or veins) and may cause acute myocardial infarction, high blood pressure, ischemic heart and stroke (Mine et al 2005). Fibrin is an insoluble protein which formed from fibrinogen by thrombin (EC 3.4.21.5) and are lysed by plasmin (EC 3.4.21.7), which is activated from plasminogen by tissue plasminogen activator. In blood clotting cascade, a soluble protein precursors forms fibrin as end product which lay down inside blood vessels and forms minuscule strands that harden blood vessel. Today, enzymes are used as Anticoagulants, Oncolytics, Thrombolytics, Anti-inflammatories, Fibrinolytics, Mucolytics, Antimicrobials and Digestive aids (E.kotb 2012). Currently, Several thrombolytic agents such as Streptokinase, Urokinase, Prourokinase, Retephase (r-PA), Altephase (t-PA), Reptilase, Brinase and Anisoylated purified streptokinase activator complex (APSAC) are available for clinical use. All these thrombolytic agents still suffer significant shortcomings, including requirement of large therapeutic dose, short plasma half-life, modest fibrin specificity and bleeding complications. Fibrinolytic protease has an ability to degrade fibrin. Fibrinolytic protease are found in plant, animal and microbial sources. Fibrinolytic protease from microbial sources have been reported from various species of *Aspergillus* (Larcher G et al. 1992), *Fusarium* (Abdel-Fattah A et al. 1993, El-Aassar SA 1995) and *Streptomyces* (Chitte R 2000). Lumbrokinase from earthworm and fibrolase from snake venom (Peng, Y et al. 2005) are well-known plasmin-like proteins. Fibrinolytic enzymes are classified into three types: Serine protease, Metalloprotease and mixture of both serine and metalloprotease. Genetic studies and enzyme purification studies has always been simpler with regards to bacteria hence bacteria are mostly preferred source for fibrinolytic protease production. Thrombolytic agents such as plasminogen activators and urokinase are still widely used in thrombolytic therapy. But their expensive prices and undesirable side-effects have incited to scientific community to search for cheaper and safer resources. Microbial fibrinolytic protease is good option to enhance the efficacy and specificity of fibrinolytic. The physiochemical parameters of protease have been characterized, it's effectiveness in thrombosis in vitro has been studied. Therefore, microbial fibrinolytic protease especially apart from food-grade microorganisms a *Stenotrophomonas maltophilia*, a soil isolate also have potential to be developed as drug thrombosis related diseases. This is first report of a fibrinolytic protease from *Stenotrophomonas maltophilia*,a soil isolate but need of preliminary toxicology studies for safety of mammals.

MATERIAL AND METHODS

Materials

All the chemicals were procured from Himedia Laboratories Pvt. Ltd. and Fisher Inorganic and Aromatics Ltd. Other fine chemicals and reagents used in this study were commercial grade.

Isolation of fibrinolytic enzyme producing strain

Samples collected were plated onto agar plates containing casein 0.5%, peptone 0.5% and yeast extract 0.1%, (pH 7.0). These plates were incubated for 24–48 h at 37°C and a clear zone gave an indication of protease producing strains. These protease producing strains were subjected to fibrinolytic enzymes screening. Fibrinolytic enzymes production was carried out in the culture medium composed of casein 0.5%, peptone 0.5% and yeast extract 0.1%, (pH 7.0). Medium was autoclaved at 121°C for 20min and a loopful culture of the selected organism was inoculated. Submerged fermentation was performed on a rotary shaker (150 rpm) for 48 h at 37°C, in 250mL Erlenmeyer flasks. The cultures were centrifuged and the supernatants were used for determination of fibrinolytic activity using a fibrin plate. The fibrin plate was composed of 1% (w/v) agarose, 0.5% (w/v) fibrinogen, 0.2%(v/v), and thrombin (100 NIH units/mL) (pH 7.4) (T. Astrup and S.M¨ullertz 1952). The fibrin plate was allowed to stand for 1 h at room temperature to form a fibrin clot layer. Ten microliters of crude enzyme was dropped into holes and incubated for 5 h at 37°C, fibrinolytic enzymes exhibited a clear zone of degradation of fibrin around the well indicating its activity. The

single-strain CNK-7 showing the largest halo zone on the fibrin plate was selected and further identified.

Protease assay

The protease activity was measured by a Lowry method (Whooley, M. et al.1983) with little modification. A reaction mixture of 2ml of 0.5% casein dissolved in 0.1M carbonate buffer (pH 9.3) and 0.1 ml of enzyme solution was incubated for 5 mins at 37°C and then reaction was stopped by adding 0.5 ml 15% (w/v) trichloro-acetic acid, centrifuged (34,000 × g) and the solubilized proteins in the supernatant assayed at 660nm. A unit of enzyme activity is defined as the amount of enzyme which releases 1.0 µmole of tyrosine equivalent per min.
Protein of all enzymatic preparations was determined according to Lowry *et al.* using bovine serum albumin as the standard. Readings were carried out in a spectrophotometer at 660 nm.

Identification of strain by 16S rRNA sequencing

For DNA sequencing, the genomic DNA was extracted from the bacterial culture. The bacterial culture was suspended in extraction buffer (10mM Tris HCl, pH 8; 1mM EDTA, pH 8) and proteinase K solution was added at the final concentration of 100 µg/mL and incubated at 55°C for 2h with continuous shaking. NaCl (0.5 M) was added and incubated at 72°C for 30 min. DNA was extracted with phenol-chloroform, washed with 70% ethanol, dissolved in Tris-EDTA buffer (p^H 8) electrophoresed on 1% agarose gel and visualized by ethidium bromide staining (Yates C et al 1997). PCR analysis was done utilizing 16sF, 16sR primers and MyGene Thermocycler 25+ (Long Gene technologies) in 0.2 ml PCR tubes. The PCR products were purified using Qiagen PCR Product Purification kit. Purified PCR products were taken for cycle sequencing with ABI Big Dye Terminator chemistry using either forward primer or reverse primer in Gene AMP PCR System 9700. The cycle sequencing products were purified according to Applied Biosystems protocol. Purified products were sequenced in ABI 3730XL Sequencer. The 16s rDNA sequence was initially analyzed using the BLAST search facility (www.ncbi.nlm.nih.gov/blast/blast.cgi). Multiple sequence alignments were performed using CLUSTAL W version 1.8 (Thompson J et al. 1994). Phylogenetic tree was constructed from evolutionary distances using the neighbor joining method (Saitou N and Nei M 1987). Tree files generated by PHYLIP were further viewed by TREE VIEW program. Bootstrap analysis was applied.

Antibiotics sensitivity Test (Williams et al., 1989)

The ability of the isolates to resist various antibiotics was one of the criteria for identification of new isolates. The isolates were streaked on the agar surface. Antibiotic disc were placed and incubated at 37°C for 24 h and observed for the presence or absence of growth. The resistance was scored as (+).The following five different antibiotic discs were tested: Penicillin G (10 IU/disc), Streptomycin (10 IU/disc), Tetracycline (50 IU/disc), Nystatin (100 IU/disc) and Gentamicin (100 IU/disc).

Purification of the fibrinolytic protease from *S. maltophilia* strain CNK-7R

The culture broth was centrifuged (5000×g, 20 mins) to remove residue, bacterial cells and other particles. The filtrate was adjusted to 40%-80% precipitation with saturated ammonium sulfate and centrifuged at 10,000×g, 10 mins. The pellet was dissolved in 0.1M sodium phosphate buffer, pH 7.2 and dialyzed in 10mM sodium phosphate buffer. The dialyzed enzyme was applied on a (2.5cm ×15cm) DEAE-Cellulose column pre-equilibrated with 25 mM HCl and 25mM Tris buffer at the flow rate of 0.4ml/min. The column was washed with the same buffer initially and eluted with a 0.25 to 0.5M NaCl gradient. Fractions (2ml) containing fibrinolytic protease was dialyzed again in sodium phosphate buffer.

Effect of Physico-chemical Factors on fibrinolytic protease production

Effect of Various Carbon and Nitrogen Sources on Fibrinolytic Protease Production

The fibrinolytic protease production by the selected bacterium was also optimized by supplementing different nitrogen sources individually at the concentration of 0.5% such as beef extract, peptone, yeast extract, casein and gelatin (T. Jayalakshmi and et al. 2012). To identify the suitable carbon sources for fibrinolytic protease production by the *Stenotrophomonas maltophilia* the following different carbon sources were tested such as glucose, starch, fructose, maltose and sucrose with sample concentration of 0.5% in the optimized production medium at 37°C (M.G. Sher and et al. 2012).

Effect of age of inoculum, level of inoculum (%) and volume of culture media on fibrinolytic protease production

The age of inoculum is important to achieve optimum yield of fibrinolytic protease. To study the effect of age of inoculum, 6,12,18,24 and 30 h old inocula were added to 10% v/v level to the production medium. The fermentations were carried out and assays were taken. A higher concentration of cells in inoculum was reported to be resulting in longer lag phase in the growth profile. To study the effect of level of inoculum, different levels of inoculum (5%, 7.5%, 10%, 12.5% and 15% v/v) were added to the production medium. The fermentations were conducted, samples were assayed. To study the effect of aeration on protease production, different volumes (25, 50, 75 and 100 ml) of production medium were taken and fermentation was conducted and tested for enzyme activity.

Effect of Various p^H on Fibrinolytic Protease Production

The effect of pH for fibrinolytic protease production was determined by culturing the bacterium in the production media with different pH. The experiment was carried out individually at various p^H 3, 4, 5, 6, 7, 8, 9 and 10. The fermentations were carried out, samples were assayed. [F. Essam and et al. 2012).

Partial characterization of the fibrinolytic protease Enzyme

Effect of pH and temperature on fibrinolytic protease activity

The activity of enzyme was evaluated at different pH values. The partially purified enzyme was incubated using 0.1 M of four buffers in the range between pH 3-10, under assay conditions and the amount of tyrosine liberated was determined. Buffers used were citrate-phosphate (pH3.0-7.0), sodium-phosphate (pH6.0-8.0), Tris-HCl (pH8.0-9.0), and glycine-NaOH (9.0-10). Optimum temperature for enzyme activity was determined by incubating the standard reaction mixture at temperature ranging from 10-90°C.

Effect of different metallic salts and various compounds on fibrinolytic protease activity

The effect of metal ions of several mineral salts (i.e. Na^+, K^+, Zn^{2+}, $Hg^{2+}$and Cu^{2+},Zn^{2+},Mg^{2+}) EDTA (Ethlene diamine-tetraacetate) at two different concentrations (1mM,5mM) of the salts that were incubated with the partially purified enzyme for 30 mins. After the exposure time, enzyme activity in each sample was measured and expressed IU/ml.

Thermodynamics of casein hydrolysis by protease of Stenotrophomonas maltophilia Catalytic constants for casein hydrolysis

The Michaelis constant (Km) value of the purified enzyme was estimated in a range of casein concentrations of 0.1 to 1 g. The apparent Km value of partially purified caseinolytic enzyme was calculated from the Lineweaver Burk plots (Lineweaver and Burk 1934) relating 1 / [V] to 1 / [S]. The Michaelis constant (Km) and maximal velocity (Vmax) of the enzyme were determined using casein as substrate in the range of 0.1–1.0 gm with the help of Lineweaver-Burk plot.

Determination of molecular weight

SDS PAGE was performed according to the method of (Laemmli 1970), with a separating acrylamide gel of 10% and stacking gel of 5% containing 0.1% SDS. The gel was stained with coomassie brilliant blue R-250 and destained with a solution of methanol, acetic acid and water in the ratio of 4:1:5. The molecular weight of the purified fibrinolytic protease was determined in comparison with standard molecular weight markers phosphorylase b (97.4 kDa), Bovine serum albumin (66 kDa), Ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soyabean trypsininhibitor (20.1kDa).

Antioxidant activity

The antioxidant capacity of the partially purified enzyme samples was studied through their scavenging activity against 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals (Van Amsterdam et al. 1992). DPPH is a stable deep violet radical due to its unpaired electron. In the presence of an antioxidant radical scavenger, which can donate an electron to DPPH, the deep violet color decolorize to the pale yellow non-radical. The bleaching of DPPH was monitored at absorbance of 517 nm. The percentage of DPPH bleaching utilized for IC_{50} (half maximal scavenging concentration) was calculated.

Amino Acid Composition

Amino acid sequences of *Stenotrophomonas maltophilia* Protease protein was retrieved from uniprot database (http://www.uniprot.org) in FASTA format.

Amino acid composition of retrieved sequences was done using BIOEDIT software.

RESULTS AND DISCUSSION

Bacterial selection and identification

A total of twelve protease producing bacteria were screened for fibrinolytic properties from soil samples. It was found that CNK-7R strain was efficient caseinolytic protease producer and capable of dissolving fibrin clot within 15 hrs of incubation at 37°C among all isolates. The CNK-7R strain which appeared curved rod, gram negative and non-endospore forming organism. It was aerobic, motile and strong oxidase, lipase and protease positive. Additional morphological, physiological and biochemical test were conducted as shown in (Table-1). The feature agreed with description of Bergey's Manual of Systematic Bacteriology. It was identified to be *Stenotrophomonas maltophilia.* On the basis of morphological characteristics and 16s rRNA studies the strain was found to be *Stenotrophomonas sp.*, having 98% similarity with *Stenotrophomonas maltophilia*. From this result, the CNK-7R strain was identified as *Stenotrophomonas maltophilia.* A 16 s rRNA gene sequence was submitted to NCBI GenBank (Accession No.KJ801664).

Table 1 Morphological and biochemical characterization of isolated strain CNK-7R

Characters	Strain
Morphology	Curved rods
Gram nature	-ve
Motility	Motile
Oxidase	+ve
Catalase	-ve
Nitrate reduction	-ve
Lipase	+ve
Protease	+ve
Hydrolysis of:	
Gelatin	+ve
Utilization of:	
D-Glucose	+ve
Lactose	+ve
D-mannose	+ve

Figure 1 Phylogenetic tree of isolate CNK-7R to other *Stenotrophomonas sp.*

Antibiotics sensitivity Test

The isolates were tested for resistance to Penicillin, Streptomycin, Tetracycline, Nystatin and Gentamicin (100 IU/disc). Identified *S. maltophilia* from a soil, was susceptible to two out of five broad spectrum antibiotics and showed resistance to Penicillin, Nystatin and Gentamicin.

Purification and characterization of *S. maltophilia* protease

We attempted to purify the extracellular protease from *S. maltophilia* strain CNK-7R. The sequential multi-steps purification procedure was summarized in Table 2. Fig. 2 shows the elution profile of purification of the partial purified fibrinolytic protease on DEAE-cellulose column.

The purified enzyme appeared as a single band on SDS-PAGE, corresponding to a molecular mass of approximately 47 kDa (Figure 3). These results indicated that the purified protease is a monomeric enzyme.

Table 2 Purification and recovery of fibrinolytic protease

Sample	Enzyme Activity (U/ml)	Protein concentration (mg/ml)	Specific activity (Umg-1)	Purification fold	% Recovery
Crude enzyme	0.068	0.061	1.11	1	100
Ammonium sulphate precipitation	0.079	0.056	1.41	1.27	78.74
Dialysis	0.058	0.038	1.52	1.36	73.52
DEAE-Cellulose	0.052	0.031	1.87	1.68	59.52

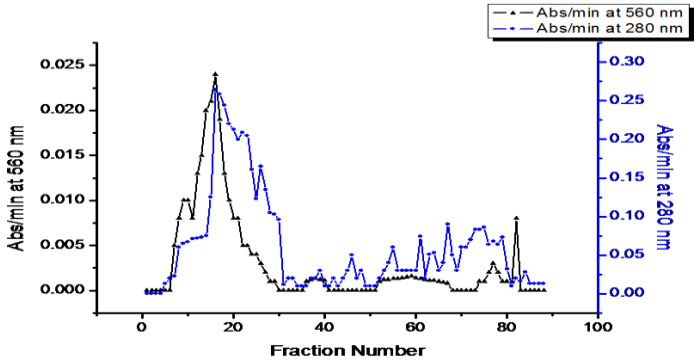

Figure 2 Elution diagram of fibrinolytic protease from DEAE-cellulose.

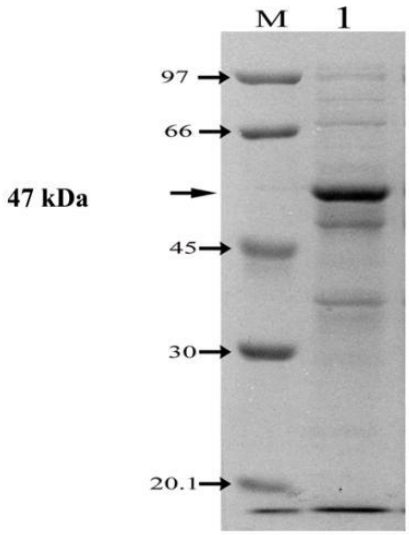

Figure 3 SDS-PAGE of the purified Fibrinolytic protease (Lane 1) and marker (M). Protein molecular weight markers are phosphorylase b- 97kDa, Bovine serum albumin- 66 kDa, Ovalbumin- 45 kDa, Carbonic anhydrous-30 kDa, Soyabean trypsininhibitor- 20.1 kDa.

Effect of Physico-chemical factors on fibrinolytic protease production

Effect of pH on fibrinolytic protease production

The important characteristic of most microorganisms is their strong dependence on the extracellular pH for cell growth and enzyme production due to pH of culture strongly affects enzymatic processes and transport of various components across the cell membrane. It was observed that the fibrinolytic enzymatic activity increased from pH 7(0.48 IU/ml) (figure 8) and reached maximum at pH 8 (0.62 IU/ml) and again there was a decrease in fibrinolytic activity to pH 10 (0.12IU/ml). Maximum activity of the fibrinolytic enzyme was observed at pH 8. The optimum pH5-8 reported for maximum fibrinolytic protease production by *Bacillus subtillis, ß-hemolytic* (Mander P., Rym Agrebi et al. 2009), *Schizophyllum commune* (Patcharaporn P et al. 2008), *Pseudomonas aeruginosa* (Amrita Raja et al. 2012), *Ganoderma lucidum* VK12 (Sekar K et al. 2011), *Escherichia coli* (Dong genu Lee et al. 2007), *Candida guilliermondii* (Mona M et al. 2012),

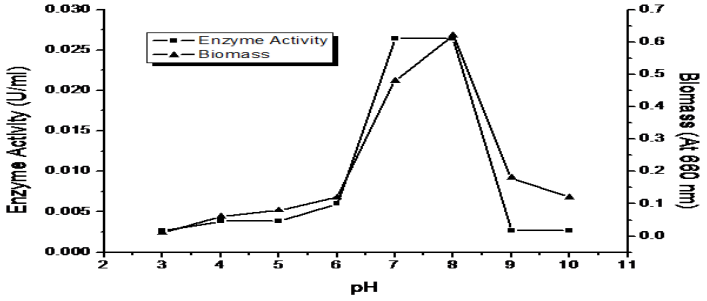

Figure 4 Effect of pH on fibrinolytic enzyme production

Effect of carbon and nitrogen sources on fibrinolytic protease production

The fibrinolytic protease production is dependent on the availability of both carbon and nitrogen sources in the medium. Studies have indicated a reduction in fibrinolytic protease production due to catabolic repression by glucose (Amrita Raja et al. 2012, Tingwei K et al. 2009). Increased yields of fibrinolytic protease were reported by several workers who used different sugars such as (Amrita Raja et al. 2012), glucose (Tingwei K et al. 2009 , Usama F et al. 2008) and fructose . However, a repression in enzyme synthesis was observed with these ingredients at high concentrations and starch, polysaccharides at 0.5 % concentration has given maximum fibrinolytic enzyme yield.

Low levels of fibrinolytic protease production were reported with the use of inorganic nitrogen sources in the production medium (Amrita R et al. 2012, Jaya R et al. 2010). However, *Stenotrophomonas maltophilia* fibrinolytic protease successfully utilized complex low-cost nitrogen source gelatin and starch carbon source and produced appreciable titres of fibrinolytic protease (figure 9 and 10).

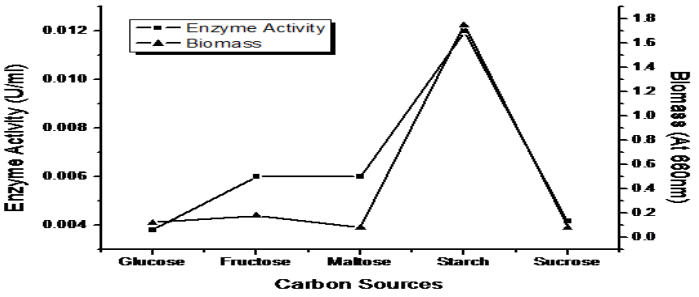

Figure 5 Effect of carbon sources on fibrinolytic enzyme production

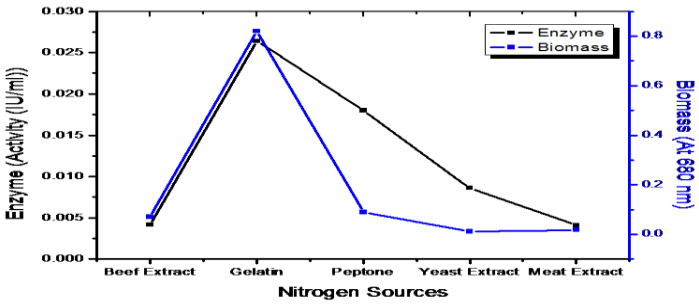

Figure 6 Effect of nitrogen sources on fibrinolytic enzyme production

Effect of age of inoculum, level of inoculum (%) and volume of culture media on fibrinolytic protease production

Inocula are generally transferred at the logarithmic phase of growth, just before confluence is reached. The age of inoculum is important to achieve optimum yield of the metabolites. The maximum enzyme activity was seen with 48 hrs of incubation (figure 11).

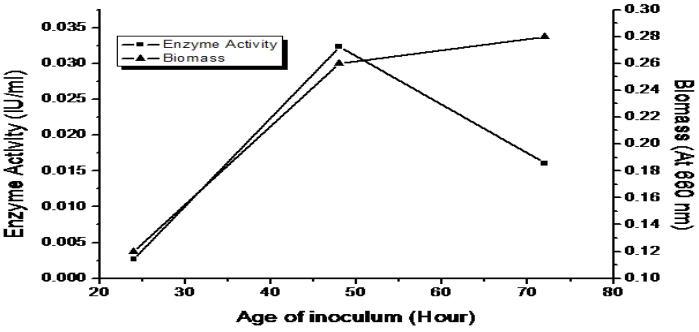

Figure 7 Effect of age of inoculum on fibrinolytic enzyme production

The growth profile of cells is affected by the size of inoculum and its physiological condition. Hence, it is proposed to study the effect of level of inoculum on fibrinolytic enzyme yield. The maximum activity was seen with 15% of inoculum (figure 12). Renganathan *et al.* (2011) have studied the effect of inoculum concentration on protease production by Bacillus sp. RRM1 and reported that 15% inoculum supported maximum protease production, whereas further increase or decrease in concentration resulted in the decline of protease production.

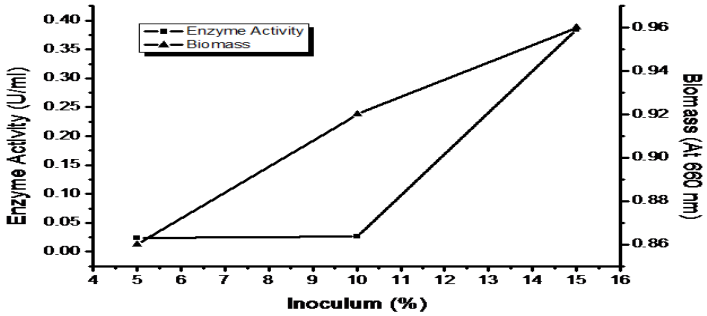

Figure 8 Effect of level of inoculums on fibrinolytic protease production

During fermentation, the aeration rate directly correlate with the dissolved oxygen level in the fermentation broth. *Stenotrophomonas maltophilia* exhibited maximum yield of fibrinolytic protease at 60 ml volume of media (figure 13).

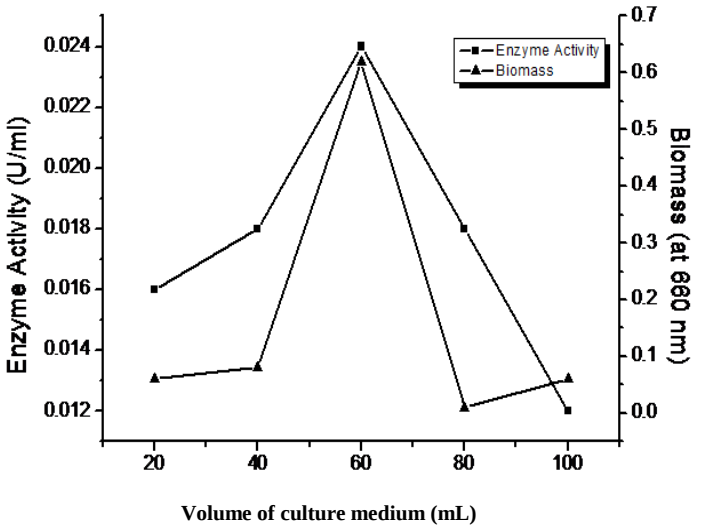

Figure 9 Effect of volume of culture medium on fibrinolytic enzyme production

Characterization of Fibrinolytic protease

Effect of pH and temperature on fibrinolytic protease activity

The enzyme activity of the purified fibrinolytic protease was measured in the pH range 3-10 (figure 3) and showed a typical bell shape. The optimum pH was 8.0 and stability at pH 8.0-10, classifying the enzyme as an alkaline protease. The fibrinolytic enzyme from different *Bacillus* sp. exhibited maximum activity at pH8.0-9.0. (Vishalakshi N et al. 2009, Agrebi R et al. 2010, Mahajan P et al. 2012 and Bajaj B et al. 2013). *Streptomyces* sp. fibrinolytic enzyme showed considerable loss of activity at acidic and basic pH(Simkhada J et al. 2010). However, fibrinolytic proteases from *B.subtilis* A26 (Agrebi R 2009) and *B.amyloliquefaciens* An6 showed stability over a broad range of acidic and alkaline pH. *Stenotrophomonas maltophilia* fibrinolytic protease showed maximum activity at 40°C and stability upto 50°C (figure 4). Several *Bacillus spp.* fibrinolytic proteases have shown maximum activity at 35-40°C. In contrast, optimum temperature for fibrinolytic proteases from *B.subtilis* A26 and *Streptomyces* sp. (Mander P et.al. 2011) was 60°C.

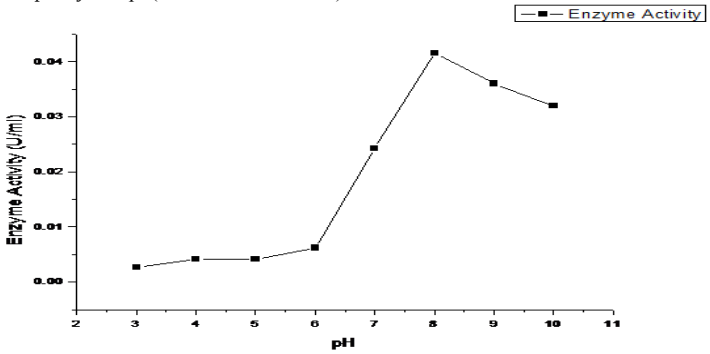

Figure 10 Effect of pH on fibrinolytic protease activity

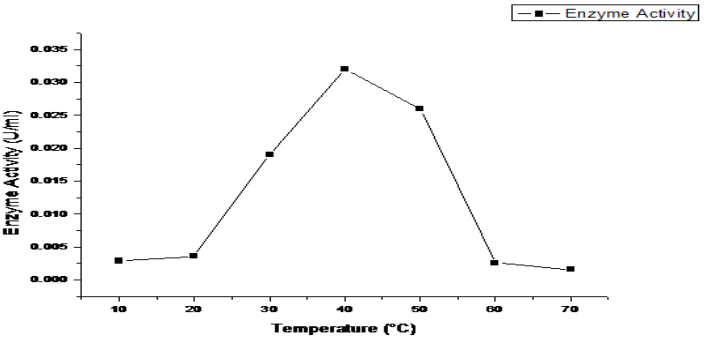

Figure 11 Effect of temperature on fibrinolytic protease activity

Thermodynemics of casein hydrolysis by fibrinolytic protease of Stenotrophomonas sp.

Catalytic constants for casein hydrolysis

The data indicated that the enzyme extracted from *Stenotrophomonas maltophilia* had apparent affinity towards substrate casein (figure 6). Our results are in agreement with what has been reported by other studies. The Km and Vmax values as determined by double reciprocal Line weaver-Burk plot for hydrolysis of casein at 40°C and pH 8.0 were 0.303 mg/ml and 0.00714 IU/ml respectively (Figure 7).

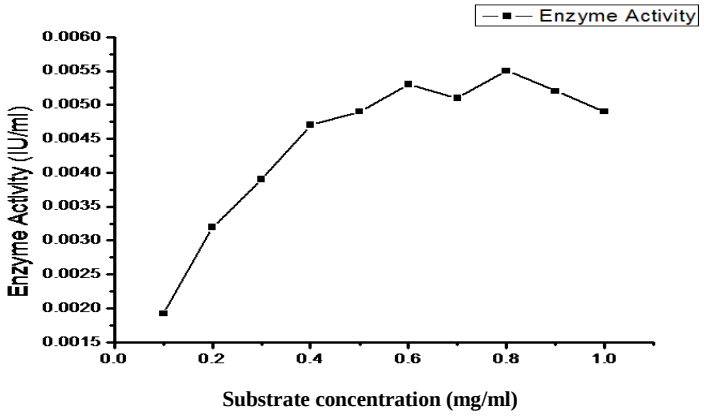

Figure 12 Effect of casein concentration (mg/ml) on fibrinolytic protease activity

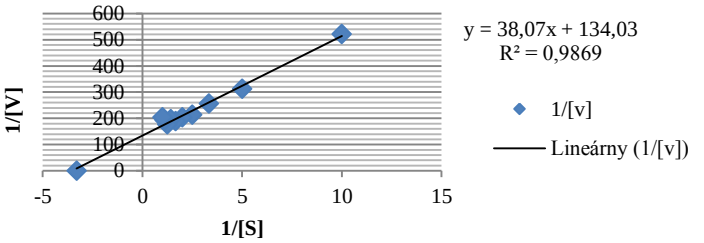

Figure 13 Lineweaver - Burk plot of caseinolytic (fibrinolytic protease) activity with casein as a substrate

Effect of different metallic salts and compounds at various concentrations

Among the salts tested, considerable loss of activity was observed only with EDTA, Zn^{++}, while Na^{+}, K^{+}, Ca^{++} and Mg^{++} acting somehow as an enhancer (Table 3). In contrast, *B.cereus* NS-2 fibrinolytic protease was activated by Fe^{2+} but inhibited by Pb^{2+} and Hg^{2+} (Bajaj et al. 2013) . *B. subtilis* A26 fibrinolytic protease remained unaffected in the presence of Ca^{2+}, got slightly influenced by Na^{+}, K^{+}, Ba^{2+}, Cu^{2+},Mg^{2+} and Mn^{2+}, but significantly inhibited by Hg^{2+} and Zn^{2+} (Agrebi R et al. 2009). *B.subtilis* ICTF-1 protease was activated in presence of Ca^{2+} and shear loss in activity in presence of Zn^{2+}, Fe^{3+} and Hg^{2+} (Mahajan P et al. 2012). EDTA caused inhibition to the activity of *Stenotrophomonas maltophilia* protease. In the same way, fibrinolytic proteases from *B.amyloliquefaciens* An6 (Agrebi et al. 2010) and *Streptomyces* sp. CS684(Simkhada J. et al .2010) have been reported to be inhibited by EDTA.

Table 3 Effect of different salts on fibrinolytic protease activity

Activator or Inhibitor	Relative Activity (%)	
	10^{-3}M	5×10^{-3}M
Control	100	100
NaCl	50±0.12	112±0.28
KCl	75±0.21	114±0.28
$CuSO_4$	25±0.31	5±0.36
$ZnSO_4$	41±0.22	11±0.23
$HgCl_2$	45±0.13	17±0.16
EDTA	37±0.28	6.25±0.22
$MgSO_4$	101±0.11	112±0.06
$CaCl_2$	68±0.24	105±0.27

(Values are mean ± Standard deviation, n=3)

Fibrinolytic assay

The maximum enzyme activity was seen in crude enzyme with a zone of 8mm.The zones by enzymes of different stages during purification and column are given below (Table-4 and figure 14).

Table 4 Zones in fibrinolytic assay

Enzymes of different stages	Zone(mm)
Plasmin (Positive Control)	8
Crude enzyme	4
Dialysis enzyme	2
Column fraction 1	1

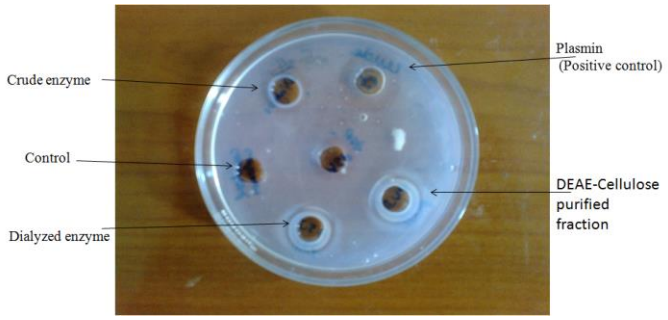

Figure 14 Fibrinolytic assay

Antioxidant activity

The antioxidant capacity of the enzyme was investigated using DPPH assay. DPPH is a stable radical, which could provide a relative radical scavenging activity of a tested probe. The DPPH assay showed that *Stenotrophomonas maltophilia* possessed low scavenging activity with IC_{50} values of 295.4µg/mL compared to the scavenging activity of the well-known antioxidant (ascorbic acid, IC_{50} 14.87 µg/mL) (figure 15). Antioxidant is very important due to the deleterious role of free radicals in foods & biological system.

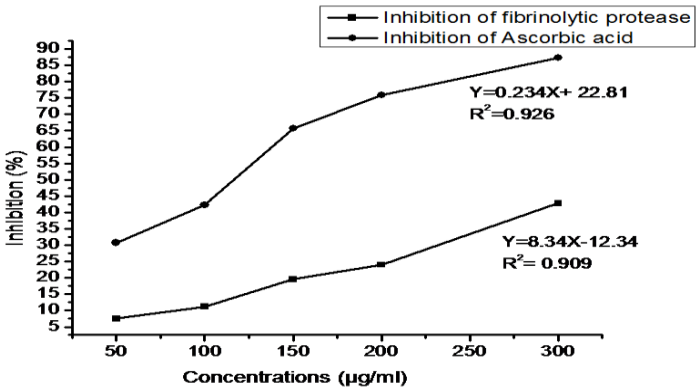

Figure 15 DPPH scavenging activity

Amino Acid composition

The primary sequence of the enzyme was retrieved from uniprot database (http://www.uniprot.org) in FASTA format having accession No: A0A0H2R6Y2. The sequence obtained was given below.

MARSPSETLDLPVLPLRDVVVFPHMVIPLFVGRDKSMHALEQAMEADKR
ILLLAQKSAETDDPHAADLYQVGTLAQVLQLLKLPDGTIKVLVEGLSRV
QVTHVDERNGSLHGQAVEIDASDEREAREVEAIARSLMSLFEQYVKTNR
KLPPELLQTLSGIDEPARLADTIAAHISVRLADKQRLLETLAVGDRLEMLV
GLVDGEIDVQQMEKRIRGRVKSQMEKSQREYYLNEQMKAIQKELGDLD
DAPGELEELARKIAEAGMPKAVEAKARNELNKLKQMSPMSAEAAVVRN
YLEWLLGVPWKKRSKVRKDLKVAQDTLDADHYGLEKVKERILEYLAVQ
SRVKQMKGPILCLVGPPGVGKTSLGQSIAKATNRKFVRMSLGGVRDEAEI
RGHRRTYVGSMPGRIVQNLNKVGSKNPLFVLDEIDKMSMDFRGDPSSAL
LEVLDPEQNNAFNDHYLEVDLDLSEVMFVATSNSLNIPGPLLDRMEVIRI
PGYTEDEKLNIATRYLVPKQVKANGLQPEELEIGSDAIQDIVRYYTRESG
VRNLEREIAKICRKVVKEIALAGPQPVKAKKGAKKKALVSVSSKNLDKY
LGVRRFDFGRAEEENEIGLVTGLAWTEVGGDLLQIESTLVPGKGQLILTG
QLGNVMKESASAALSVVRSRAVGFGIDSDFLQKHDVHLHVPDGATPKD
GPSAGAAMVTSLVSMLTKVPVRADVAMTGEITLRGRVTAIGGLKEKLLA
ALRGGIRTVIIPEENRKDLADIPANVTRDLEIVPVKYIEEVLDLALERPLAP
KKARKSAQRVTVRSKAKPSGNARVKH

The molecular weight obtained from BIOEDIT data (815 amino acids) was 89,920 Da and the amino acid composition of fibrinolytic enzyme (protease) was mentioned in (Table 5**).** The data shows, enzyme was rich in leucine (Figure-16)

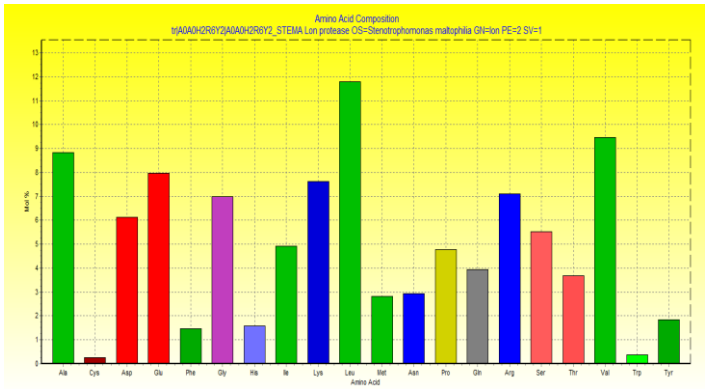

Figure 16 Amino Acid Composition Of protease from *Stenotrophomonas maltophilia*

Table 5 Amino acid contents (mole %) of *Stenotrophomonas maltophilia* Protease protein

Protein: Protease
Length = 815 amino acids
Molecular Weight = 89,920 Daltons

Amino Acid	Number	Mole (%)
Ala A	72	**8.83**
Cys C	2	**0.25**
Asp D	50	**6.13**
Glu E	65	**7.98**
Phe F	12	**1.47**
Gly G	57	**6.99**
His H	13	**1.60**
Ile I	40	**4.91**
Lys K	62	**7.61**
Leu L	96	**11.78**
Met M	23	**2.82**
Asn N	24	**2.94**
Pro P	39	**4.79**
Gln Q	32	**3.93**
Arg R	58	**7.12**
Ser S	45	**5.52**
Thr T	30	**3.68**
Val V	77	**9.45**
Trp W	3	**0.37**
Tyr Y	**15**	**1.84**

CONCLUSION

The purified fibrinolytic protease from *Stenotrophomonas maltophilia* has a favorable activity over wide ranges of pH and temperature, high affinity towards substrate casein and fibrin, and Thermostability, which worth further investigations of its proper utilization. In addition, fibrinolytic activity of the enzyme could be used to develop therapy of thrombolytic diseases.

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CONFLICT OF INTEREST STATEMENT

No conflict of interest statement

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