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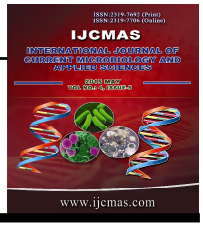

Original Research Article

Production and characterization of keratinolytic protease from *Streptomyces* sp

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Keywords

Keratinase, *Streptomyces* sp, Keratinolytic protease, Feather degradation

A B S T R A C T

Alkaline keratinolytic protease from *Streptomyces sp* has been partially purified and characterized in detail. The proteases are characterized on the basis of biochemical properties like optimum pH and stability, optimum temperature and stability, metal ion requirement, inhibitors, substrate specificity and its kinetic behavior. Enzyme production was done by using feathers as sole source of carbon and nitrogen and enzyme produced after 96h was showing activity 96.25 units/ml. partially purified enzyme was characterized and it was found that: The best enzyme-substrate reaction time found was in the range of 20-25 minutes.Maximum enzyme activity was observed at 55^{o}C temperature.Optimum pH for enzyme activity was 9 indicating alkaline nature of the keratinolytic protease. Keratinolytic protease showed maximum activity against casein followed by ABS(albumin bovine serume) and `feathers Highest enzyme activity was noticed for metal ions like Mg^{+2},followed by Co^{+2},Ca^{+2}, K^{+}, Fe^{+3} and Ni^{+2}. Enzyme was inhibited by Ba^{++} ions (50% activity loss). The keratinolytic protease was strongly inhibited by 5mM EDTA (Ethylene diamine tetraacetic acid) indicating that enzyme is a mettalo protease. Enzyme was stable for 10min at 55˚C and 90min at room temperature (31˚C). Enzyme was stable for 3h at pH 8.5 and remains stable for one hour at pH 9.0, and for two hours at pH 10 at room temperature. K_m and Vmax values of Enzyme were 4.4 mg and 2000U/ml respectively.

Introduction

Keratin-containing materials are abundant in, nature but have limited uses in practice since they are insoluble and resistant to degradation by the common proteolytic enzymes. Keratinous wastes represent a source of valuable proteins and amino acids and could find application as a fodder additive for animals or source of nitrogen

for plants. Keratinolytic enzymes that may have potential roles in biotechnological processes involving keratin containing wastes from poultry and leather industries. The potential use of keratinase is in different application where keratins should be hydrolysed such as the leather and detergent industries, textiles waste bioconversion, medicine and cosmetics for drug delivery through nails and degradation of keratinized skin, besides it involves in the hydrolysis of prion proteins that arise as novel outstanding applications of the enzyme. Actinomycetes genera are becoming increasingly important as a source of novel products (Nord *et al.*, 1998).So in this study we characterized keratinolytic protease from S*treptomyces sp*.

Materials and Methods

A well characterized and properly identified Culture of *Streptomyces sp.* (A_1) collected (Shilpa A jani et al, 2014) for our study and cultivated on skim Milk agar medium. The culture was a potent producer of keratinase using feathers as sole source of carbon and nitrogen.Culture was allowed to streak on Skim milk agar medium of pH 8.5 and incubated at 37±2^{o}c for 1-2 days to allow the colonies to grow. Cultures were preserved at 4 ºC on casein agar slants. Sub culturing was carried out every 30 day's interval.

Culture *(Streptomyces sp*A_1) was inoculated in optimized production medium (Table: 1). 3 ml fresh culture suspension of optical density 0.75 at 680nm was inoculated in production medium containing feathers (feathers were the sole source of carbon and nitrogen) (Adriano Brandelli 2008).Then the flasks were put on an environmental shaker at 100 rpm at 37±2˚C for 96h .The supernatant was collected after centrifugation at 10,000 rpm at 4±2˚C temperature for 10 minutes and used as crude enzyme source. Keratinolytic protease activity in the supernatant was determine by using spectrophotometer method, given by Anson – Hagihara method(1958) with minor modification.

Extraction and dialysis of enzyme

Enzyme concentrated by ammonium sulphate precipitation.250 ml of the cell free filtrate was subjected for precipitation by concentrations of (0-80%)concentration of ammonium sulphate according to (Khalil and Gupt,2003) solid ammonium sulfate (enzyme grade) (Gomori 1955 ; Dixon and Webb 1964) . The precipitated protein was obtained by centrifugation at 10,000 rpm for 15 minutes at 4ºC.The concentrated enzyme was collected in glycine NaOH buffer and dialysis overnight against the same buffer till the ammonium sulphate was completely removed. The caseinolytickeratinase activity of this partial purified enzyme preparation was determined by Hagihara(1958) method.

Determination of optimum reaction time for enzyme activity

The optimum enzyme reaction time was determined by assaying the caseinolytic activity of enzyme by incubating the reaction system for varying period of time (10 to 35 minutes) at 55°C, by Anson and Hagihara method.

Determination of optimum temperature for enzyme activity

The optimum temperature for enzyme activity was determined by assaying the caseinolytic activity by incubating the enzyme-substrate reaction system at different temperature range (35,40,45,50, 55,60, 65,70,75,and 80°C) for 25 minutes, and then the activity was determined by Anson and Hagihara method (1958). Temperature at which enzyme gave maximum activity was considered as optimum temperature for activity.

Determination of optimum pH for enzyme activity

The optimum pH for enzyme activity was determined by assaying the activity of partial purified enzyme at various pH values from 7 to 12. Substrates (1% casein) were prepared in Glycine-NaOH buffer. The Caseinolytic activity was determined using these substrates in the reaction system at 55°C for 25 min. Maximum activity yielding pH was considered as optimum pH for activity.

Substrate specificity

The activity of partial purified enzyme in different substrates was studied. The partial purified protease was incubated with different natural substrates like casein, keratin(chicken Feather) , bovine serum albumin, and hydrolytic activity of protease for each substrate was determined by a modification in the method of Hagihara *et al*. (1958). Substrate buffer solution (1.5 ml) (1% substrate in 2.0 mM Glycine NaOH buffer pH 9.0) was pre-incubated for 5 min. The reaction was started with addition of 0.5ml of enzyme. After 25 min of incubation at 55°C, the reaction was terminated by adding 3 ml of chilled 10% (w/v) trichloroacetic acid (TCA). The tubes were centrifuged at 10,000 rpm for 10 min and the released tyrosine is measured by Folin's and Lowry method (Lowry *et al* 1951). For blank tubes, the enzyme was added after TCA.

Effect of metal ions on protease activity

Effect of various metal ions on the enzyme activity was studied by pre incubating partial purified enzyme with different metal ions in 2.5 mM concentration for 10 minutes before the addition of substrate at room temperature. Metal ions used were : , Mn^{2+}, Mg^{2+}, Ca^{2+} and ,Fe^{2+}, Ba^{2+}, Co^{2+}, Ni^{2+}, K^{+}, Hg^{2+}, Mn^{2+} in the form of chlorides. The caseinolytic activity was determined and relative activity was calculated with respect to the control without treatment with metal ions.

Effect of inhibitors on enzyme activity to determine protease type

The partial purified enzyme was pre incubated with different inhibitors at 5 mM concentrations for 10 min before the addition of substrate at room temperature (Adinarayana *et al*, 2003). The inhibitors used were: phenyl methyl sulfonyl fluoride (PMSF)serine inhibitor, dithiothreitol (DTT) a reducer of disulfide bonds , pera-chloro mercuric benzoate (PCMB) thiol group containing amino acid inhibitor and ethylene diamine tetra acetic acid (EDTA) chelator of divalent metal ions; The caseinolytic activity was determined at 55°C using Glycine NaOH buffer (pH 9) by Anson and Hagihara method.. The relative activity was calculated with respect to the control without treatment with inhibitors.

Temperature stability

The partial purified enzyme was incubated with 50 mM Glycine NaOH buffer pH 9.0 at different temperatures room temperature and 55°C.The caseinolytic activity was determined at intervals of 30min, 60min, 90min, 2hrs, 24hrs at room temperature (31˚C) and 10min,20min,30min,40min,50min,60min,90 min at 55˚C incubation of protease for all the mentioned temperatures. The residual activity was expressed as % of the initial activity.

pH stability of protease

pH stability of partial purified enzyme was determined by pre incubating the enzyme with glycine NaOH buffers of varying pH

values(8.5,9,10) at room temperature(34°C) temperature for 1 h to 5 h. The caseinolytic activity was determined at varying time intervals to determine the stability of the protease at different pH values.

Thermodynamics of casein hydrolysis by keratinolytic protease of *Streptomyces sp.*: Catalytic constants for casein hydrolysis

Kinetic constants (V_{max}, K_m,) were determined using Line weaver-Burk double reciprocal (1/v versus 1/S) plot (Dixon and Web, 1979) by assaying fixed amount of protease with substrate concentrations (casein in 50 Mm Glycine NaOH buffer pH 9.0) 0.1-2.0 mg/ml for 20 min at 55°C, and then caseinolytic activity was determined.

Results and Discussion

We successfully cultivated keratinolytic alkaline protease producing bacteria *Streptomyces sp* using alkaline skim milk agar medium. After 48h incubation at 37˚C typical growth observed along with pure isolated colonies. (Figure:1)

Measure of keratinolytic protease production (Enzyme assay)

Results indicated that *Streptomyces* sp. (A1) produced keratinolytic protease maximally after 96h which is 96.25 unit/ml. After 96h the enzyme production was found nearly stable (Figure:2.) , Our results matches with our previous dissertation batch which was 92.81 units/ml. (Shilpa Ashok Jani et al,2014)

Extraction and dialysis of enzyme

The cell free filtrate was subjected for precipitation by (0-80 %) concentration of ammonium sulphate (Khalil and Gupt2003). Precipitated protein was obtained by centrifugation at10,000 rpm for 15 min at 4^0C .The concentrated enzyme was collected in glycine NaOH buffer(pH 9) and dialysis overnight against the same buffer till the ammonium sulphate is completely removed .Keratinolytic protease activity of this partially purified enzyme is determined by Hagihara method.Enzyme activity before enzyme extraction is 96.25 U/ml And after concentration and dialysis was 1037.50 U/ml which shows concentration increased activity nearly 18.86 fold. (Table:2) The dialysed and concentrated enzyme was partially purified which we used for the characterization of the enzyme.

Characterization of Karetinolytic protease Enzyme

Determination of optimum reaction time for enzyme activity

The optimum enzyme reaction time was determined by assaying the caseinolytic activity of enzyme by incubating the reaction system for varying period of time at 55°C**.** The results indicated that the best reaction time found was in the range of 20-25 minutes. (Figure: 3).

Determination of optimum temperature for enzyme activity

The optimum temperature for enzyme activity was determined by assaying the caseinolytic activity by incubating reaction mixture at different temperatures and it was found that, the enzyme was most active at 55°C. (Figure: 4)

Our results are exactly similar with keratinase of *Streptomyces thermoviolaceus* (Chitte *et al.* 1999) which has optimum temp

55˚C. Other similar results are also noticed for optimum temperature *B. subtilis* KD-N2 (Cai *et al.* 2008b) showed high activity at 55˚C.similar range were reported for *Clostridium sporogenes* ,55˚C (Ionata *et al.* 2008): *B. licheniformis* K-508, 52˚C Rozs *et al*. 2001.

Determination of optimum pH for enzyme activity

The optimum pH for enzyme activity was determined by assaying the activity of partial purified enzyme at various pH values from 7 to 12 with the use of glycine NaOH buffer and the results revealed that the maximum activity was obtained at pH 9.0 with glycine NaOH buffer. More over it was found that enzyme is more stable at pH 9. The enzyme gave significant activity in a broader range of 8 to 9.5.(Figure : 5)

One of the most significant features of the present enzyme is its alkaline nature as it has a pH optimum of 9.0. Our results are exactly similar with *keratinase of Streptomyces gulbagensis* DAS 131 (Syed *et al*. 2009) which has optimum pH 9.0. *Streptomyces albidoflavus* alkaline protease enzyme exhibited optimum activity at pH 9 (Hanaa *et al*, 2010.) This results were similar to that deduced from several *Streptomyces* strains (Hames-Kocabas and Uzel, 2007; Vishalakshi *et al.*, 2009). The protease of *B. licheniformis* NH1 has also been reported to have similar properties (Hmidet *et al*, 2009). A similar trend has also been reported for the protease obtained from *B. cereus* MCM B-326 at pH 9.0 (Nilegaokar *et al*, 2007).

Substrate specificity

The important feature of alkaline protease is their ability to discriminate among competing substrates and utility of these enzymes often depends on their substrate specificity. In general alkaline proteases have broad substrate specificity and hydrolyze variety of natural as well as synthetic substrates. The activity of protease towards substrates like casein, albumin bovine serum and feather meal was determined. Figure 6 represents the activity of enzyme obtained by action on these substrates. The purified enzyme showed maximum activity in presence of casein followed by BSA as substrates. Comparatively less activity was observed observed with feather meal as substrate may be because of time factor. Hence it can be concluded that enzyme can degrade ABS and feathers but is having highest specificity for casein looking to the time factor.

These results are in agreement with other reports where the proteases are more active towards casein compared to albumin bovine serum. The proteases from *Conidiobolus* sp (Sutar *et al*., 1991); *Cephalosporium* sp. KM388 (Tsuchiya *et al*., 1987) and *Beauveria bassiana* (Bidocka and Khachatourians, 1987) have been reported to be more active towards casein compared to albumin bovine serum. Protease Ds1 from the nematode-trapping fungus *Dactylella shizishanna* was particularly effective at degrading casein and skimmed milk, while being less effective at hydrolyzing ABS (Wang *et al*., 2006).

Effect of metal ions on protease activity

Enzyme activity was checked by pre incubating the enzyme with various metal ions (2.5mM) for 10 min. Significant increase in enzyme activity was noticed for metal ions like Mg^{+2}, followed by Co^{+2}, Ca^{+2}, K^{+}, Fe^{+3} and Ni^{+2}. (Figure:7) We found in literature that Mg^{+2} and Mn^{+2} ions are increasing activity of enzyme

(Manachinin *et.al*, 1998). Enzyme was inhibited by Ba^{+2} ions (50% activity loss).

Table.1 Composition of production medium [Rao and Narasu 2007]

Solution	K_2HPO_4	KH_2PO_4	$MgSO_4$	$CaCl_2$	Feather meal	pH	$NaNO_3$	Distilled water
Amount	0.2g	0.2g	0.1g	0.1g	2.5g	9.0	1g	1000ml

Table.2 Purification profile of keratinolytic protease from Streptomyces sp

Purification steps	**Total activity (U/ml)**	**Total protein (mg/ml)**	**Specific activity (U/mg)**	**Purification (fold)**
Crude enzyme	96.25	1.75	55	1.75
Concentrated and dialyzed enzyme	1037.5	0.1	10375	18.86

Figure.1 Growth characteristics of *Streptomyces* sp on skim milk agar plate after 48 h

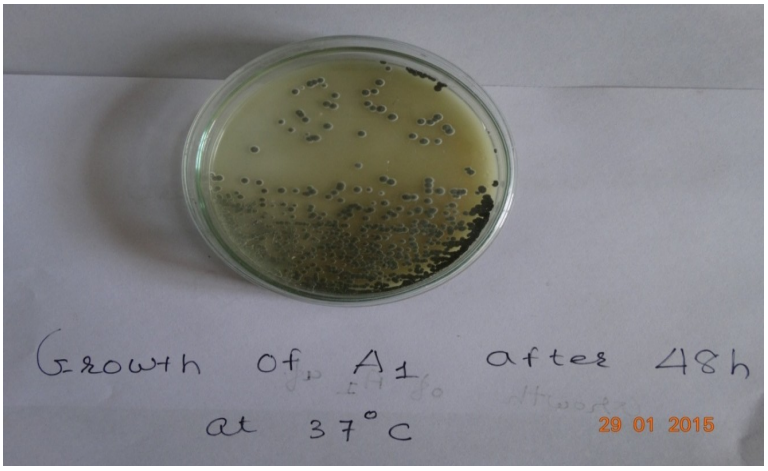


Figure.2 Keratinase production by Streptomyces sp

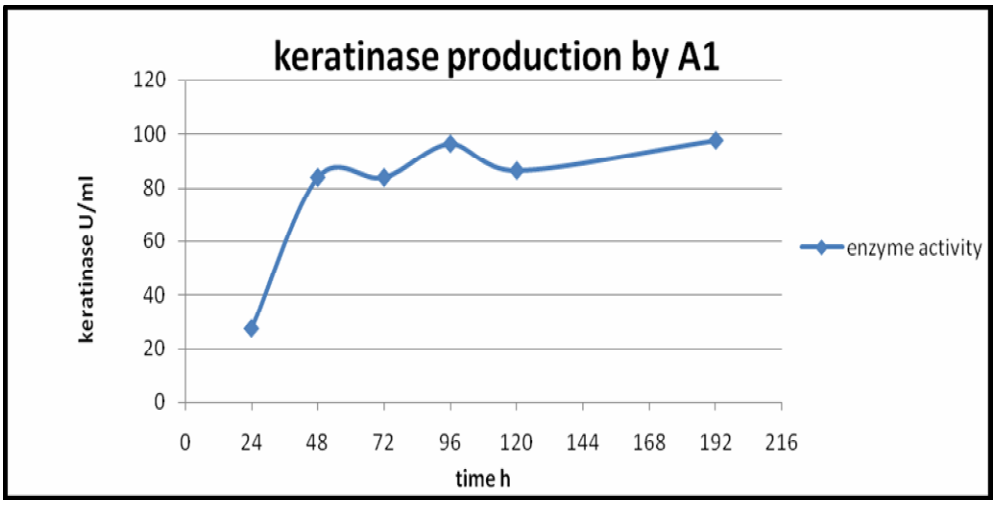


Figure.3 Determination of reaction time for caseinolytic keratinase activity

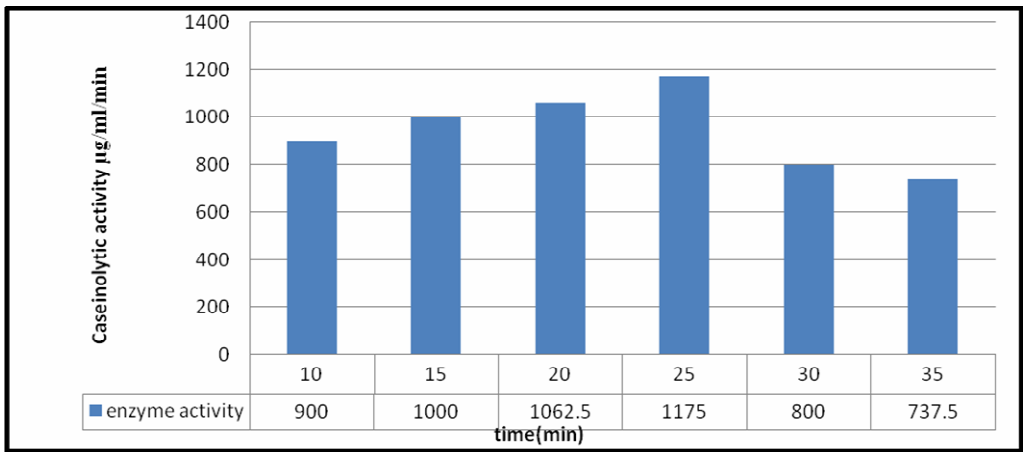


Figure.4 Determination of optimum temperature for casenolytic keratinase activity

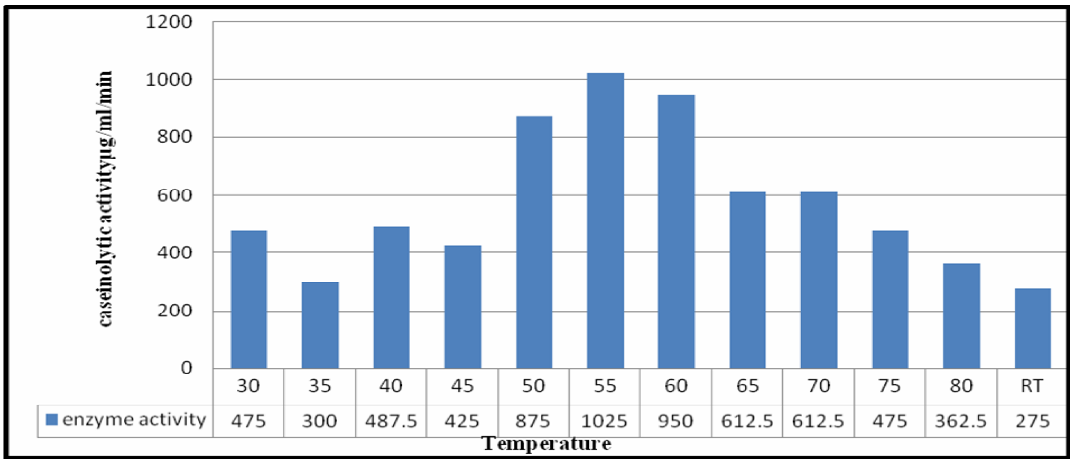


Figure.5 Determination of optimum pH for caseinolytic keratinase activity

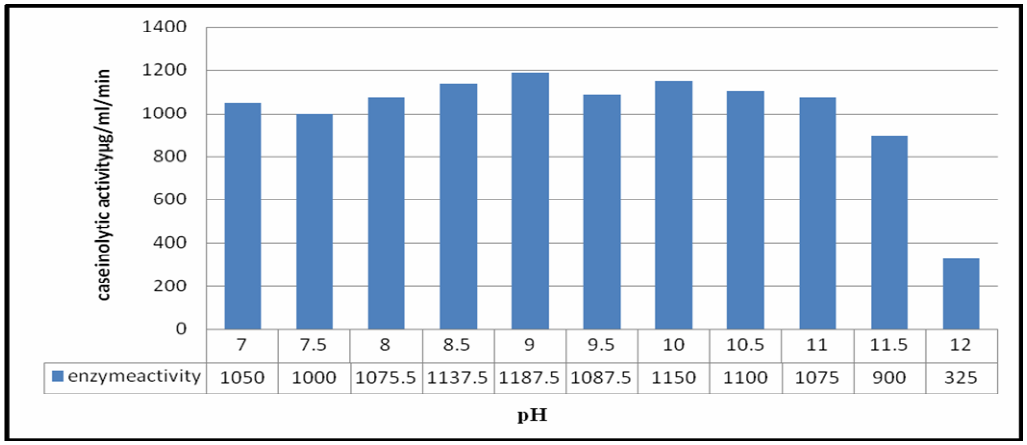


Figure.6 Substrate specificity of partial purified caseinolytic keratinase protease

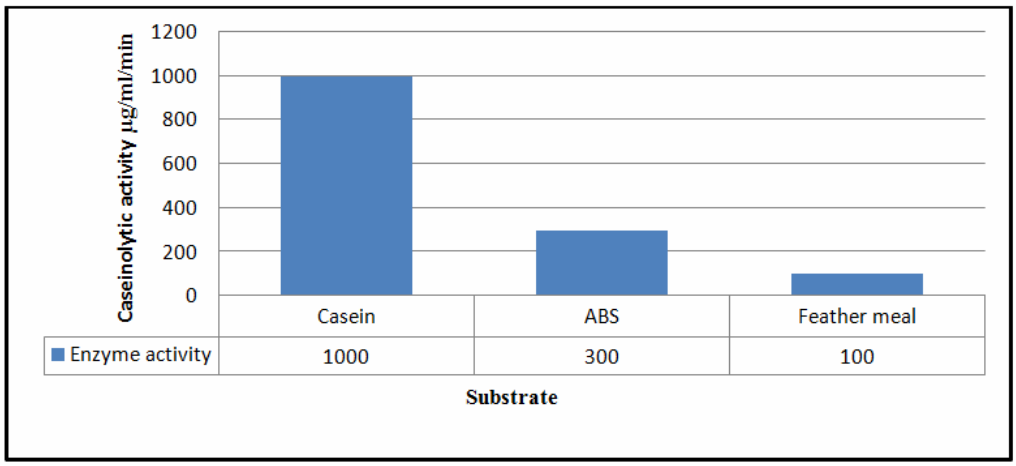


Figure.7 Effect of Metal ions on enzyme activity

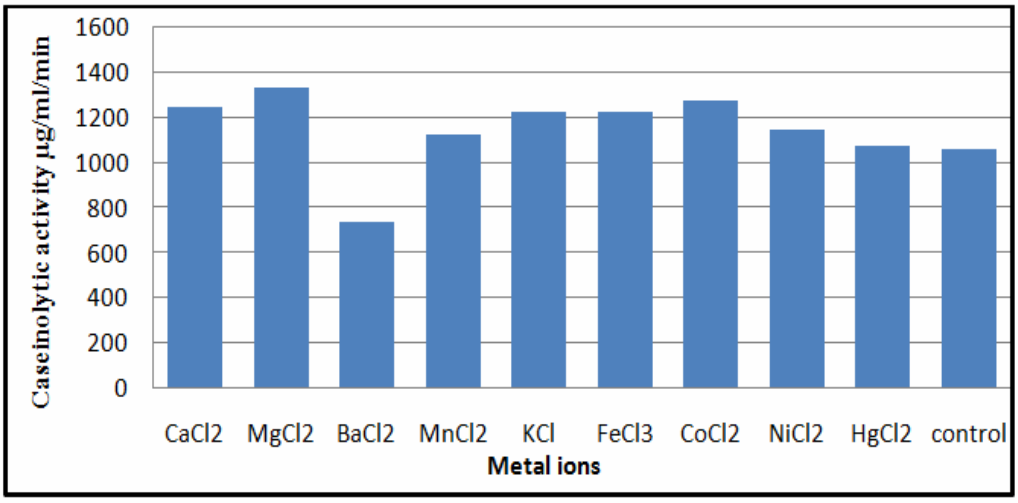


Figure.8 Effect of Inhibitors on caseinolytic keratinase protease activity

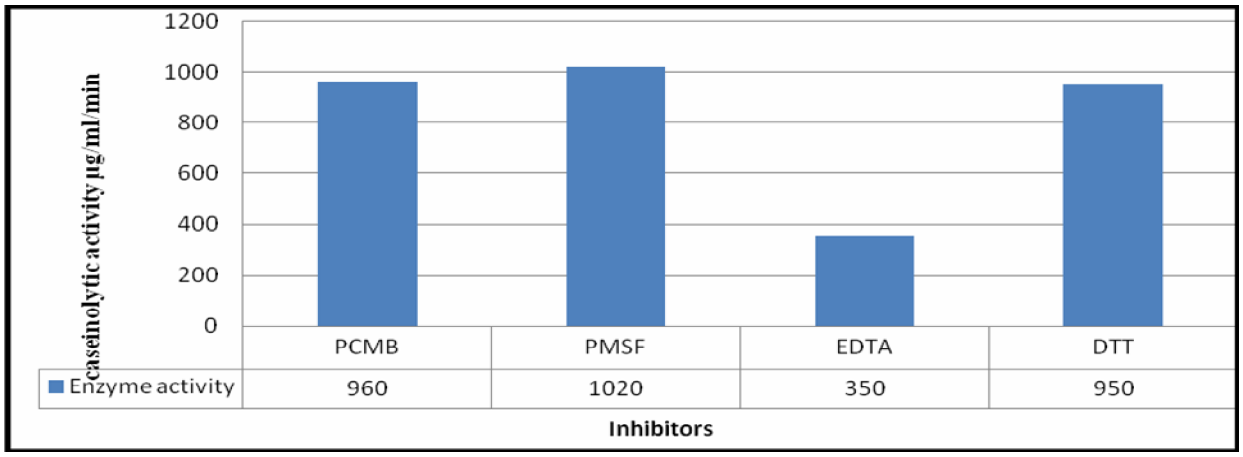


Figure.9 Stability of caseinolytic keratinase protease at 55°C

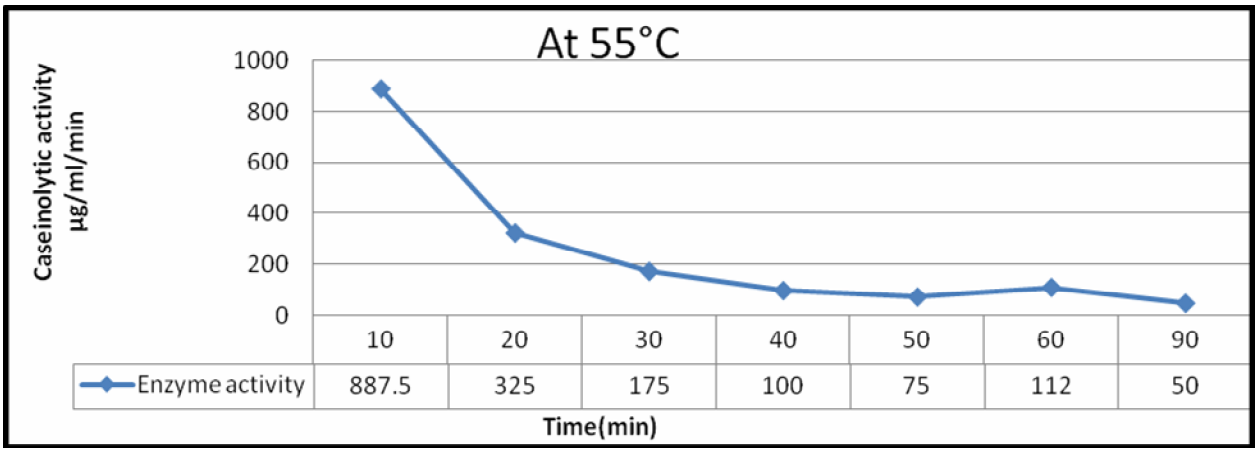


Figure.10 Stability of caseinolytic keratinase protease at room temperature (31˚C)

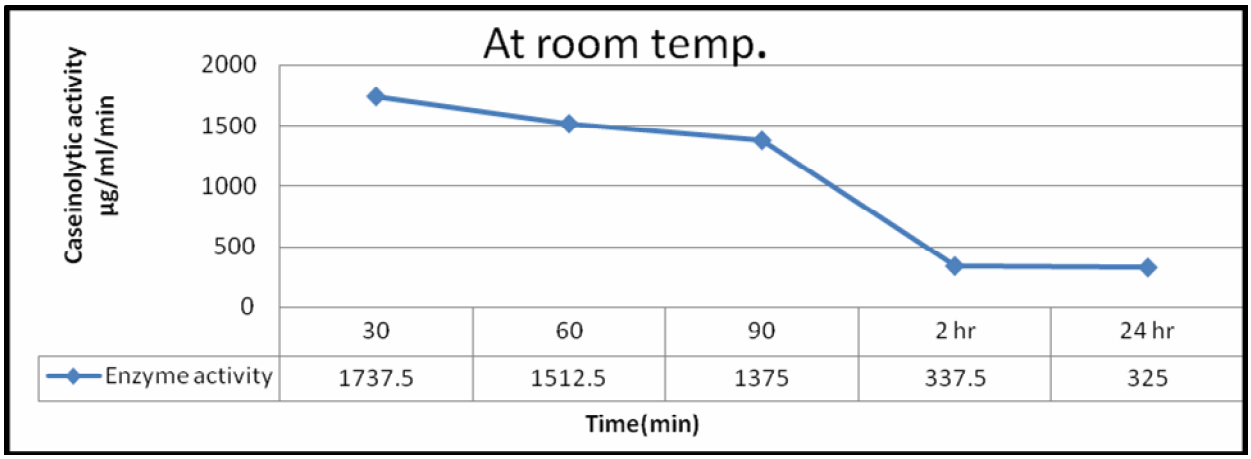


Figure.11 pH stability of keratinolytic protease at pH 8.5

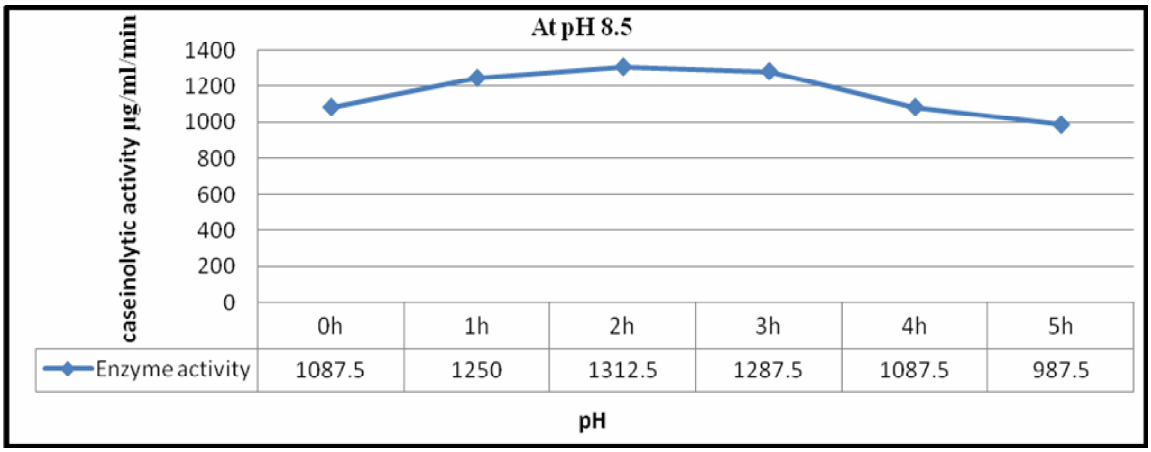


Figure.12 pH stability of caseinolytic keratinase protease at pH 9

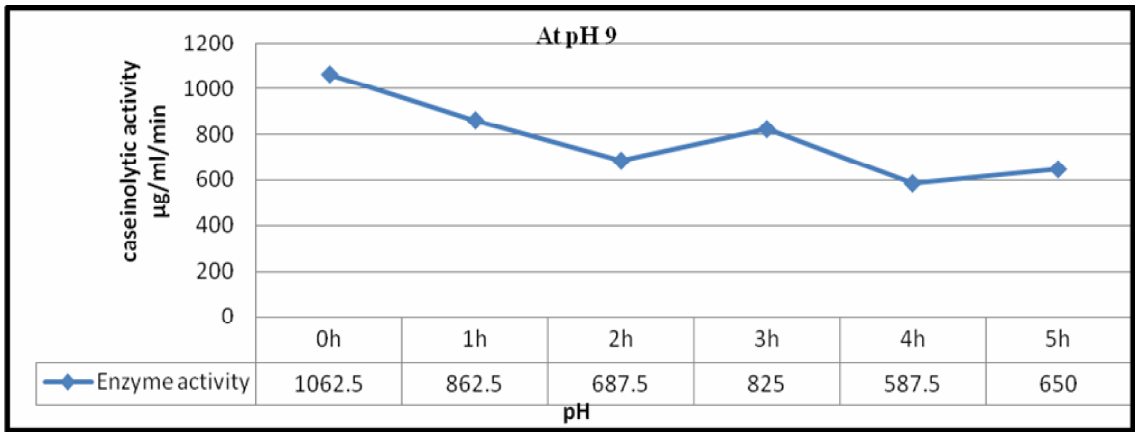


Figure.13 pH stability of caseinolytic keratinase protease at pH 10

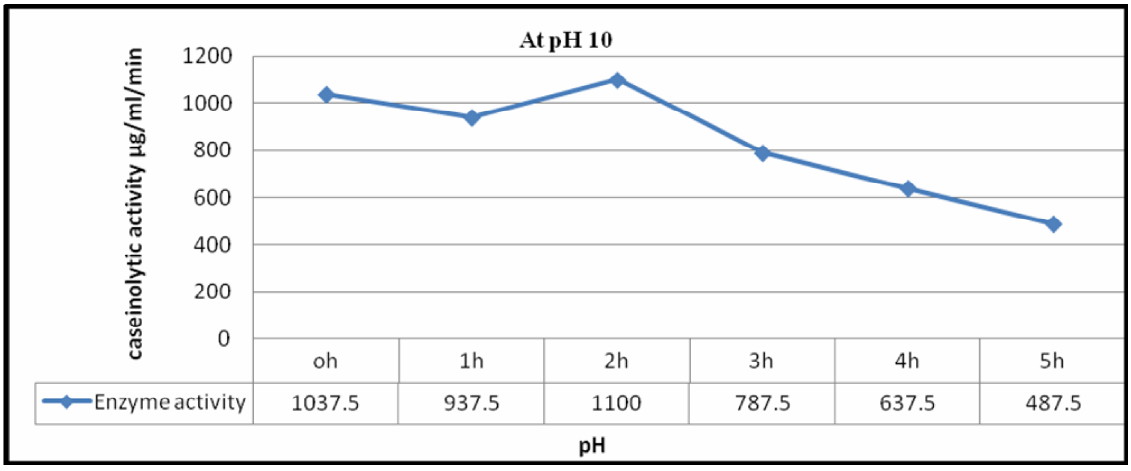


Figure.14 Casein hydrolysis by caseinolytic keratinase protease of Streptomyces sp

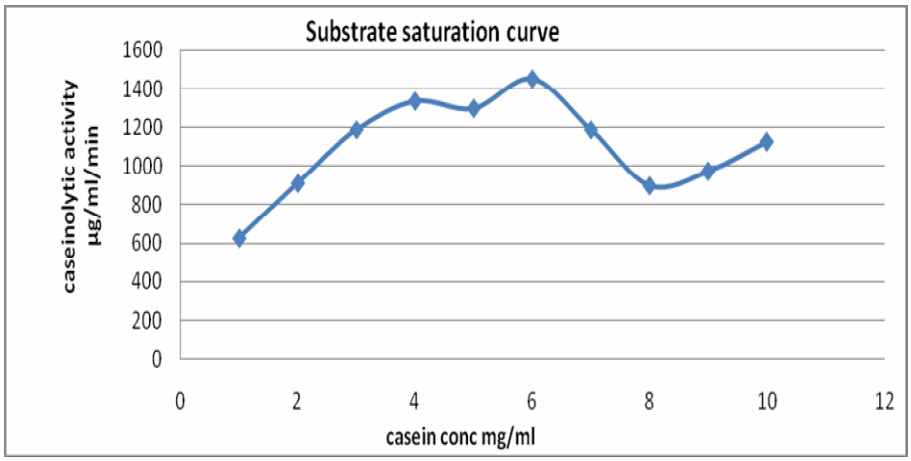
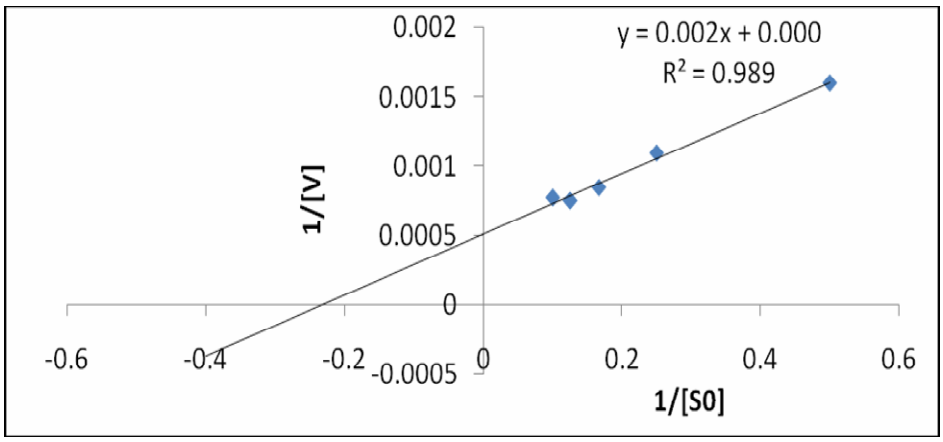


Figure.15 Line weaver-Burk plot



Km= 4.4 mg Vmax=2000 U/ml

Our results are similar with keratinase *of Bacillus sp* JB(99) especially with, Ca^{2+}, Mg^{2+},Co^{2+} increased enzyme activity.

Effect of inhibitors on enzyme activity to determine protease type

The protease was strongly inhibited by 5 mM EDTA indicating that the enzyme to be a Mettalo protease (Figure:8). Keratinolytic protease was also inhibited by 5 mM DTT. Negligible inhibition was observed in presence of 5 mM PCMB and 5 mM PMSF, indicating that it is neither a serine protease nor a cystein ptotease.

Our results are similar with keratinase of *Streptomyces* sp. S7 (Tatineni *et al*. 2008); *Bacillus* sp. SCB-3(Lee *et al*. 2002) and *B. Subtilis* MTCC (9102) (Balaji *et al*. 2008) both the keratinases were mettalo proteases.

Temperature stability

The partially purified enzyme was pre-incubated at 55°C and room temperature with different time interval. The results indicated that our enzyme was stable for 10min at 55°C and it was quite stable for about 90 min at room temperature .(Figure:9 and 10) This exercise proves that enzyme is not thermo stable but can with stand room temperature for 90 min.

pH stability of protease: Purified enzyme was pre incubated with Glycine NaOH buffers of varying pH values at room temperature(34˚C) for 1,2,3,4,and 5, hours to determine the stability of the protease at different pH values(8.5,9.0,and,10) .The results indicated (Figure :11) that the protease found stable for three hours at pH 8.5 and remained stable for one hour at pH 9, and for two hours at pH 10 at such a room temperature (Figure:12, 13).

Thermodynemics of casein hydrolysis by keratinolytic proteas of *Streptomyces sp.* Catalytic constants for casein hydrolysis

The K_m and V_{max} values as determined by double reciprocal Line weaver-Burk plot for hydrolysis of casein at 55°C and pH 9.0 were 4.4mg/ml and 2000 U/ml respectively.(Figure14, and 15)

Km= 4.4 mg Vmax=2000 U/ml

our results are nearly similar with protease of haloalkaliphilic bacterium sp. AH-6 (Km of 2.5mg/ml and Vmax of 625 U/min) (Dodia *et al*, 2008) and *Pseudomonas aeruginosa* PseA (Km of 2.69 mg/ ml and Vmax of 3.03 mmol/ min) (Gupta *et al*,2005).

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