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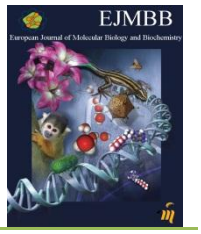

BIOCHEMICAL CHARACTERIZATION OF A PARTIALLY PURIFIED PROTEASE FROM *BACILLUS SUBTILIS*

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ABSTRACT
Living beings are organized by varieties of biochemical reactions, most of which are mediated by a series of remarkable biological catalyst known as enzymes. Proteases are enzymes that selectively conduct cleavage of peptide bonds of proteins to release amino acids. Nearly all plants, animals and microorganisms have protease, up-to 2% of the genes for enzymes in higher organisms. The protease was extracted with 0.1M phosphate buffer of pH 7 and the precipitated with ammonium sulfate. The protease has maximum activity at pH 7 and temperature of 37^{o}C. The optimum time of incubation was 30 minutes. The straight line was obtained for the effect of enzyme concentration with optimum enzyme concentration 96.42µg. The K_m and V_{max} value were 50.91µM and 833.33 pmol/min respectively.

INTRODUCTION

Living beings are shaped by an enormous variety of biochemical reactions, nearly all of which are mediated by a series of remarkable biological catalyst known as enzymes. A protease (also termed as peptidases or proteinases) are enzymes that selectively conduct proteolysis, that is, being protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein [1-4].

Proteases regulate the fate, localization and activity of many proteins, modulate protein- protein interactions, create new bioactive molecules, contribute to the processing of cellular information and generate, transduce, and amplify molecular signals. As a direct result of these multiple actions, proteases influence DNA replication and transcription, cell proliferation and differentiation, tissue morphogenesis and remodeling, heat shock and unfolded protein responses, angiogenesis, neurogenesis, ovulation, fertilization, wound repair, stem cell mobilization, hemostasis, blood coagulation [5], inflammation, immunity, autophagy, senescence, necrosis, and apoptosis. Consistent with these essential roles of proteases in cell behavior and survival and death of all organisms, alterations in proteolytic systems underlie multiple pathological conditions such as cancer, neurodegenerative disorders, and inflammatory and cardiovascular diseases [6]. Accordingly many proteases are a major focus of attention for the pharmaceutical industry as potential drug targets or as diagnostic and prognostic biomarkers. These enzymes are widely distributed in nearly all plants, animals and microorganisms [7]. In higher organisms, nearly 2% of the genes code for these enzymes.

The proteases are useful in various ways and their applications are increasing at a fantastic rate. Proteases are used in the degumming of silk goods, in the manufacture of liquid glue, in the preparation of cosmetics, in the preparation of detergents, in the meat tenderization, in the preparation of cheese, in medicine preparation and in agriculture as growth promoters. The major sources of these proteases are microorganisms. Bacillus species produce a large variety of extracellular enzymes such as protease, which have significant industrials importance. In the same vein, bacterial enzymes are known to possess

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more thermo stability than fungal proteases. Members of the genus Bacillus are heterogeneous and they are very versatile in their adaptability to the environment. There are various factors that influence the nature their metabolic processes and enzymes produced. A great deal of attention given to thermophilic and extremely thermophilic microorganisms and their enzymes. *Bacillus subtilis* is a Gram-positive, rod shaped, endospore-forming bacteria. It is catalase positive and commonly found in soil including low nutrient soil. Unlike several other well –known species, *B. subtilis* has historically been classified as an obligate aerobe, though in 1998 research demonstrated that this is not strictly correct [8]. Due to its association with soil particles, it is also inevitably transferred to plants, foods, animals and even marine and fresh water habitats. Main Objectives of this research is to produce and purify protease from *Bacillus subtilis*, to precipitate protease by ammonium sulfate, to estimate total protein, to determine protease activity and to characterize protease for the effect of pH, temperature, time of incubation, enzyme concentration, and substrate concentration

MATERIALS AND METHODS

Collection of *Bacillus subtilis*

The culture of *Bacillus subtilis* was collected from Kantipur College of Science and Technology, Sitapaila, Kathmandu, Nepal.

Isolation of *Bacillus subtilis*

a. Inoculation and incubation of sample on NA media plates:

The selective isolation of Bacillus subtilis was done by streak plate technique, on NA plates. The bacteria were streaked uniformly over the NA media, which was then incubated at 370C for 24 hours in incubator. The colonies forming clear zones were picked up and streaked on nutrient agar plates to get pure culture. Pure isolates were maintained at 40C in refrigerator for further studies.

b. Screening of potent protease producing bacteria

Skim milk agar was used for screening of purified culture for protease production. The isolated pure strains were screened for the production of extracellular protease using skim milk agar plates. The pure cultures were streaked as a line on the skim milk agar plates and plates were incubated at 37^0Cfor 24 hours. The isolates producing clear zones of hydrolysis were considered as protease producer and further investigated.

Identification of proteases producing bacteria

A. cultural identification

The isolates were observed under the microscope the colony morphology was noted with respect to color, shape, size, nature of colony and pigmentation.

B. Microscopic observation

The bacterial isolation was gram stained and observed under high power magnifying lens in light microscope.

C. **Biochemical characterization**

The bacterial isolates were characterized biochemically by MR, VP test, sugar fermentation test and endospore forming test.

Screening of microorganism producing protease

The sample was just streaked in a skim milk agar and the plate was incubated at 37^oC for 24 hours. A clear zone of hydrolysis gave an indication of protease production by *Bacillus subtilis*.

Production of protease from *Bacillus subtilis*

10 ml of overnight culture of *Bacillus subtilis* was used to inoculated in 500ml Erlenmyer flask containing 490 ml aliquots of liquid production medium consist of glucose 0.5% (wt/vol), peptone 0.75% (wt/vol), $KH_2PO_4$0.5% (wt/vol), $MgSO4_4.7H_2O$ 0.05% (wt/vol), $FeSO_4.5H_2O$ 0.01% (wt/vol), pH–7.0 and maintained at 37^oC for 24 hours in a shaker incubator. The pH of the medium was adjusted by 1N NaOH or 1N HCl. After the completion of fermentation, the whole fermentation broth was centrifuged at 15,000 rpm at 4^oC for 15 minutes and the clear supernatant was recovered. The crude enzyme supernatant was subjected to further studies.

Bradford assay of crude extract

Working solution of BSA was made by diluting standard BSA solution of 1mg/ml to 0.1 mg/ml. in 5 test tubes 200µl, 400µl, 600µl, 800µl and 1 ml of BSA working solution was taken and named 1-5. The volume was maintained to 1 ml by adding distilled water. A blank was set with only distilled water. 500µl of crude extract was taken for Bradford assay and 500µl was added to make final volume of 1ml. then 5 ml of Bradford reagent was added and after 5 min of incubation absorbance was taken with UV-ray spectrophotometer at 595 nm.

Proteolytic activity of crude extract

To calculate activity, 50µl of BSA (1mg/ml) standard solution and around 20µl was taken. The BSA and enzyme were mixed and volume was maintained to 200µl by addition of phosphate buffer and the mixture were incubated for 30 min and the 2.3 ml of Bradford reagent was added. After 5 min, absorbance was measured with UV-ray spectrophotometer at 595 nm against blank with reference to two controls: one way of enzyme only and another was of BSA only.

Ammonium sulfate salt precipitation of crude extract

400 ml of crude extract was taken in a beaker and then 176.8g (70%) of ammonium sulfate was added slowly with stirring and then stirred on magnetic stirrer for one hour then left over on refrigerator for one hour then

centrifuged at 15,000 rpm for 15 min at 4°C. The pellet obtained was dissolved on 15 ml 0.1M phosphate buffer.

Dialysis

Activation of dialysis tube

Dialysis tube was cut to fragments of appropriate length and then boiled on 100 ml of 2% (wt/vol) sodium bicarbonate solution in 1mM EDTA solution of pH 8 for 5 min. Then after the dialysis tubes were rinsed with distilled water and again boiled in 1mM EDTA solution of pH 8 for 10 min. Tubes then allowed to cool and then used for dialysis. Before use dialysis tubes were washed with distilled water and whole procedure were handled with gloves [9].

Dialysis of ammonium sulfate precipitated extract

Fraction was loaded into dialysis tube, both side was made tight and placed in a beaker containing phosphate buffer of pH 7, 0.1M. Then it was dialyzed with stirring by placing the beaker on magnetic stirrer against phosphate buffer of pH 7, 0.1M.

Bradford assay of 70% ammonium sulfate precipitated extract

Working solution of BSA was made by diluting standard BSA solution of 1mg/ml to 0.1mg/ml. In five test tubes 200µl, 400µl, 600µl, 800µl and 1ml of working solution was taken and named 1-5. The volume was maintained to 1 ml by adding distilled water. A blank was set with only distilled water. 20µl of 70% fraction was taken for Bradford assay and mixed with 980µl distilled water to maintain final volume as 1ml. Then 5 ml of Bradford reagent was added and after 5 min of incubation absorbance was taken with UV-ray spectrophotometer at 595nm.

Protease activity of 70% ammonium sulfate precipitated extract

To calculate protease activity 50 µl BSA (1mg/ml) standard solution and 30µl of 70% ammonium sulfate precipitated extract was taken. The BSA and enzyme were mixed and volume was maintained 200µl by adding phosphate buffer and the mixture were incubated for 30 min and the 2.3 ml of Bradford reagent added. After 5 min absorbance was measured with UV-ray spectrophotometer at 595 nm against blank with reference to 2 controls: one was of enzyme only and another was of BSA only.

Characterization of protease of 70% ammonium sulfate precipitated extract

Effect of pH

The effect of pH on proteolytic activity of 70% ammonium sulfate salt precipitated extract was characterized. For which different pH from 1 to 10 were prepared for the use as buffer. 30µl of enzyme extract was mixed with 50 µl of BSA standard solution (1mg/ml) and the volume was maintained to 200 µl by adding buffer of different pH and after incubation of 30 min, 2.3ml of Bradford reagent was added. The absorbance was noted after 5 min of extra incubation with UV-ray spectrophotometer at 595 nm. The activity was calculated with reference to two controls one is enzyme control having 30 µl of enzyme only and the next one is BSA control having 50µl BSA only.

Effect of Temperature

The effect of temperature on protease activity of 70% ammonium sulfate precipitated extracts was characterized on different temperature. For different temperature fridge (4°C), room temperature (20°C), incubator (40°C), oven of 60°C, 80°C, 100°C were used.

30 µl of enzyme extract was mixed with 50µl of BSA standard solution and the volume was maintained to 200 µl by adding phosphate buffer and after incubation of 30 min, 2.3ml Bradford reagent was added and the absorbance was noted after 5 min of extra incubation with UV-ray spectrophotometer at 595nm. The activity was calculated with reference to two controls one is enzyme control having 30 µl of enzyme only and the next one is BSA control having 50µl BSA only.

Effect of time of incubation

The effect of time of incubation on protease activity of 70% ammonium sulfate precipitated extract was characterizes at incubation time of 0, 15, 30, 60, 120, 180min.

30µl of enzyme extract was mixed with 50 µl of BSA standard solution and the volume was maintained to 200 µl by adding buffer and after incubati0n of 30 min, 2.3ml of Bradford reagent was added and the absorbance was noted after 5 min of extra incubation with UV-ray spectrophotometer at 595nm. The activity was calculated with reference to two controls one is enzyme control having 30 µl of enzyme only and the next one is BSA control having 50µl BSA only.

Effect of enzyme concentration

The effect of enzyme concentration on protease activity of 70% ammonium sulfate precipitated extracts were characterizes.

16.07, 32.14, 48.21, 64.28, 80.35, and 96.42µg of enzyme were used for determining the effect of enzyme concentration on protease activity of 70% ammonium sulfate precipitated. The different concentration of enzymes were mixed with 50 µg of BSA standard solution and the volumes were made to 200µl and after incubation of 30 min the Bradford reagent of 2.3ml were added in each. After again incubation for 5 min and the absorbance was noted with UV- ray spectrophotometer at 595nm.

Effect of substrate concentration

The effect of substrate concentration on protease activity of 70% ammonium sulfate precipitated extracts

were characterized using 20, 40, 60, 80, and 100µg of BSA as a substrate.

48.21µg of enzyme extract was mixed with different amount of BSA (20, 40, 60, 80, and 100µg) and phosphate buffer (pH 7, 0.1M) was added to make final volume of 200µl. The reaction mixture was incubated for 30 min and then 2.3ml of Bradford reagent was added. Then after 5 min of incubation time absorbance was taken by UV-ray spectrophotometer at 595nm.

Effect of Metal ions on protease activity

The effect of metal ions on protease activity was studied by pre-incubating the enzyme in the presence of 5mM of metal ion (Ca+2, Mg+2, Zn+2, Ba+2 and Mn+2) for 1 hour at 37oC, and then performing the assay as in above process.

RESULTS

Identification of the bacterial isolate:

Bacillus species were isolated and screened for the production of protease in skim milk agar. Maximum growing microorganism was selected for further studies. Table 1 shows some physical and biochemical characteristics of the bacterial isolate.

Bacteria were found to be Gram-positive, MR-negative, VP-positive, Catalase-positive and possessed the ability to ferment sugar. On the basis of this characterization the bacterial isolate has been identified as *Bacillus subtilis.*

Calculation of protein concentration and determination of protease activity of crude extract

From equation of Bradford assay graph (figure 2), y = .0056x + 0.04 and absorbance of crude extract of 200 µl = 0.403, total protein in 420 ml of crude extract was found to be 136.125 mg.

The activity of protease was calculated with the following equation.

$$\text{Enzyme activity (pmol per min per ml)} = \frac{\text{amount of BSA chopped in µg}}{\text{incubation time in min} \times \text{mol.wt of BSA in g} \times \text{vol.of enzyme in ml}} \times 10^6$$

As shown in table 2, the activity of crude enzyme was found to be 71.03 pmole/min/ml.

Calculation of protein concentration and determination of protease activity of 70% ammonium sulfate precipitated extract

From equation of Bradford assay graph y = 0.0054+0.0064(as shown in figure 3) and absorbance of ammonium sulfate ppt. extract of 20µl = 0.180 total protein in 20 ml of ammonium sulfate precipitated extract was found to be 32.14 mg.

As shown in table 3 protease activity of 70% ammonium sulfate precipitated extract was found to be 440.83pmol/min/ml

Summary of protein and protease purification

The summary of protein and protease purification is shown in the table 4;

Characterization of protease of 70% ammonium sulfate precipitated extracts

Effect of Ph

As shown in Fig. 4, the highest proteolytic activity was obtained at pH 9.

Effect of temperature

As shown in figure number 5, the optimum temperature of extract was found to be 37^{o}C, while protease was still active at 80^{o}C too.

Effect of time of incubation

The proteolytic activity of extract was maximum at 30 min (Figure 6) and then decreased gradually.

Effect of enzyme concentration

The effect of enzyme concentration on protease activity was found to increasing linearly up to 96.42µg. (Shown in Figure 7)

Effect of substrate concentration

Lineweaver-Burk plot (figure 8) was used to determine the K_m and V_{max} of 70% ammonium sulfate precipitated extract and is shown in table 5.

Effect of Metal ions

As shown in figure 9, the metal ions Ca^{+2} and Mn^{+2} increase the protease activity by 23% and 30% respectively. The metal ions as $Mg^{+2,}$ $Ba^{+2}$and Zn^{+2} decrease the protease activity by 5.15%, 3% and 22% respectively.

Table 1. Morphological and biochemical characteristics of bacterial isolate

Characters	*Bacillus subtilis*
Shape	Irregular
Size	Large
Color	White
Consistency	Dry
Margin	Filamentous
Elevation	Flats
Gram staining	+, rods
Voges-Proskauer test	+
Methyl Red test	-
Catalase	+

Sugar fermentation 1. Glucose 2. Mannitol	 + +

Table 2. Activity of protease of crude extract

Remained BSA in µg	Chopped BSA in µg	Activity in pmole/min/ml
45.57	4.43	71.03

Table 3. Protease activity of 70% ammonium sulfate precipitated extract

Remained BSA in µg	Chopped BSA in µg	Activity in pmol/min/ml
22.50	27.50	440.83

Table 4. summary of protein and protease purification

Purification step	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Activity ($pmolmin^{-1}ml^{-1}$)	Total activity ($pmolmin^{-1}ml^{-1}$)	Specific activity ($pmolmin^{-1}ml^{-1}$)	Purification factor[a]	Overall yield (%)[b]
Crude extract	420	0.321	135.17	71.8	30156	223.10	1	100
70% ammonium sulfate ppt. extract	20	1.607	32.14	440.83	8816.6	274.3	1.23	29.23

Table 5. K_m and V_{Max} of 70% ammonium sulfate precipitated extract

Sample	K_m (µM)	V_{max} (pmol/min)
70% $(NH_4)_2SO_4$ precipitated extract	50.91	833.33

Figure 1. Proteolysis: how protease breakdown a peptide to amino acids.

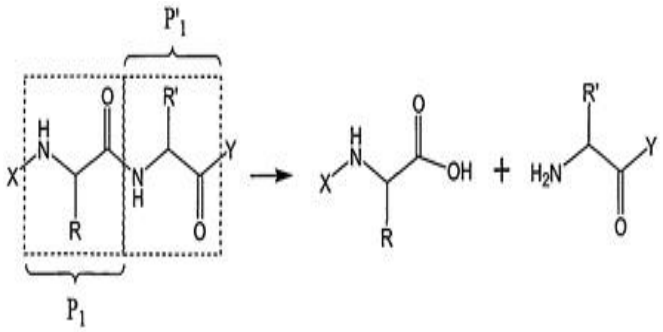


Figure 2. Standard curve of Bradford assay for crude extract

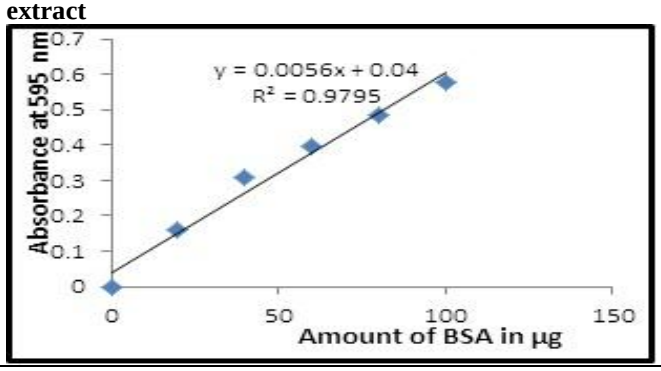


Figure 3. Standard curve of Bradford assay for 70% ammonium sulfate precipitated extract

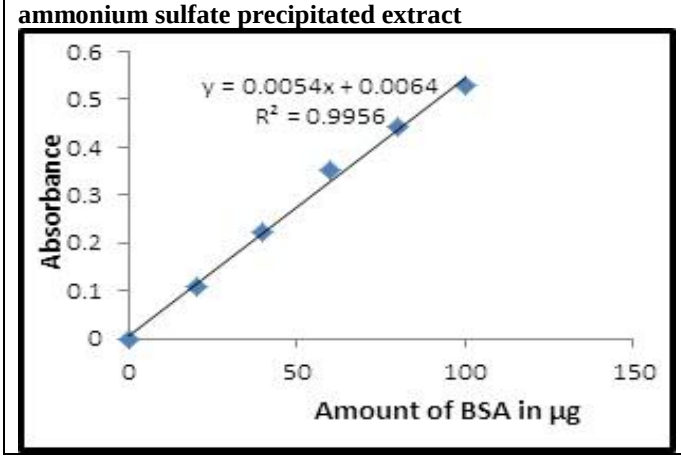


Figure 4. Effect of pH on proteolytic activity of 70% ammonium sulfate precipitated extract

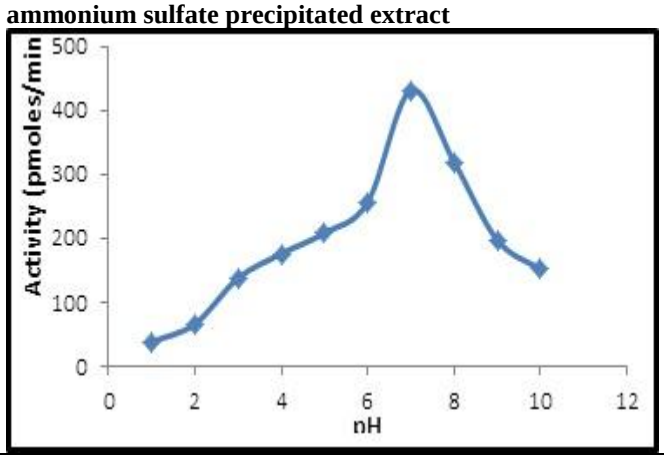


Figure 5. Effect of temperature on protease activity of 70% ammonium sulfate precipitated extract

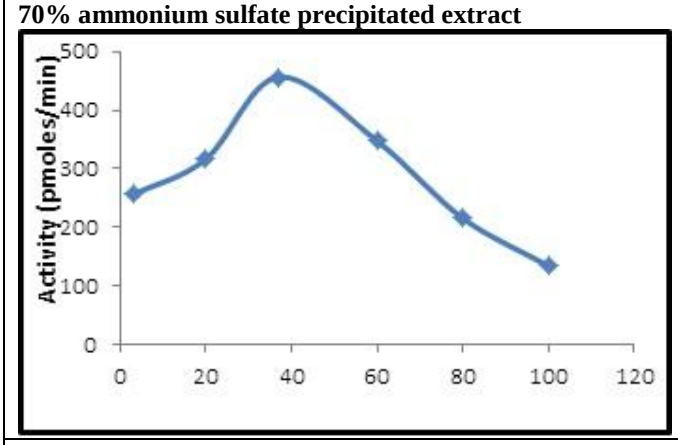


Figure 6. Effect of time of incubation on protease activity of 70% ammonium sulfate precipitated extract

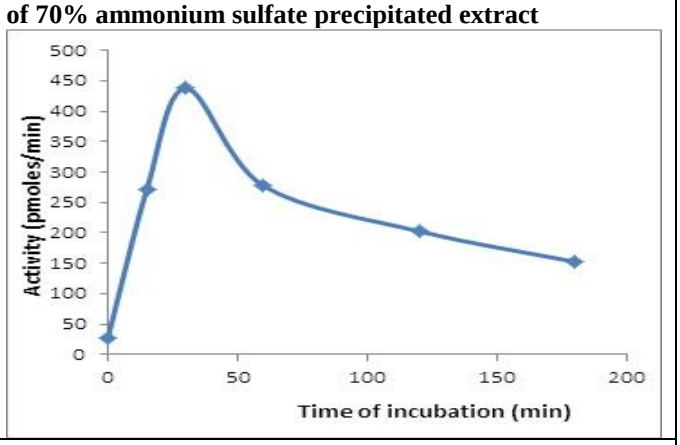


Figure 7. Effect of enzyme concentration on protease activity of 70% ammonium sulfate precipitated extract

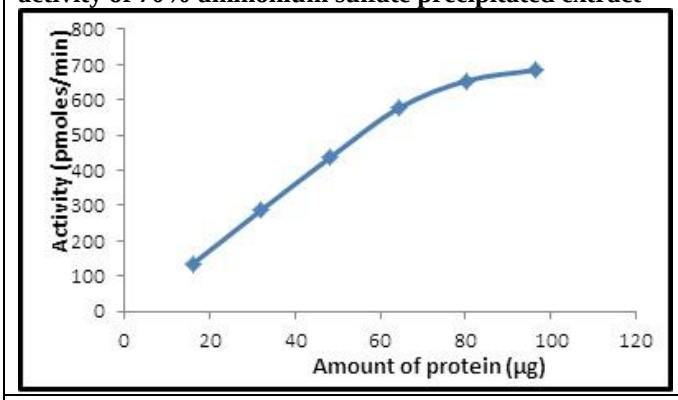


Figure 8. Effect of substrate concentration on 70% ammonium sulfate precipitated extract (Lineweaver-Burk plot)

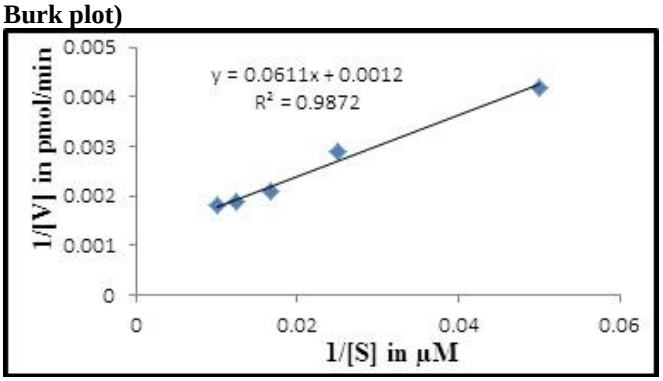
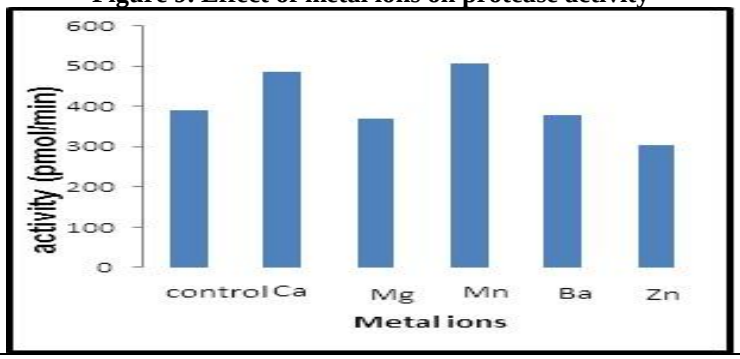


Figure 9. Effect of metal ions on protease activity



DISCUSSION

The specific activity of 70% ammonium sulfate precipitated fraction was found to be significantly high as compared to that of crude extract indicating purification of protease by 1.23 fold. The optimal pH of protease was 7 that suggest it can be classified as neutral protease. However protease activity was significantly active at pH 8 compared to pH 7. The optimum temperature of protease was found to be 37^{o}C. The activity remained at 60^{o}C, so protease is quite thermostable, this is may be due to presence of higher amount of cysteine residues. The initial increase in protease activity with temperature is due to collision between enzyme and substrate. The increase in temperature causes increase in collision between enzymes and substrates. Beyound these optimal temperatures the activities were decreased due to denaturation of proteases due to heat. The incubation time was found to be most effective only for 30 minutes. The activities beyond 30 minutes were gradually fell indicating inactivation of enzyme with time. Slightly linear line was obtained for effect of enzyme concentration on protease activity indicated that the enzyme concentration enhances the rate of reaction. The linear line obtained for the effect of substrate concentration on protease activity indicated that the rate of reaction increases with the increase in substrate concentration. The low K_m value of proteases indicated that the substrate is tightly bound to enzume. The metal

ions Ca^{+2} and Mn^{+2} increase the protease activity by 23% and 30% respectively. The metal ions as $Mg^{+2,}$ Ba^{+2} and Zn^{+2} decrease the protease activity by 5.15%, 3% and 22% respectively.

CONCLUSION

The protease from *Bacillus subtilis* have been successively purified by $(NH_4)_2SO_4$ salt and characterizes. Protease was actively working for upto 30 minutes. The optimum pH of proteolytic activity was 7 and optimum temperature was 37°C.Proteolytic activity was also seen significant in 80°C.

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