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Kinetic study of free and immobilized protease from Aspergillus sp.

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Abstract: *In the present investigation partially purified alkaline protease from Aspergillus sp. As#6 and As#7 strains were entrapped in calcium alginate beads and characterized using casein as a substrate. Temperature and pH maxima of protease from As#6 strain showed no changes before and after immobilization and remained stable at 45^0C and pH 9, respectively. However km value was slightly shifted from 4.5mg/ml to 5 mg/ml. Proteases from As#7 strain showed shifting in pH optima to a more alkaline range (10.0) as compared with free enzyme (9.0). Optimum temperature for protease from As#7 strain showed changes after immobilization and shifted from 65^0C to 85^0C. However there was no significant effect on Km value but Vmax of immobilized protease from As#7 strain was also shifted from 200U/ml to 370U/ml. Immobilized protease from As#6 strain was reused for 3 cycles with 22% loss in its activity whereas immobilize protease from As#7 strain was reused for 3 cycles with 17% loss in its activity. Protease from As#7 strain has a higher affinity for the substrate and higher proteolysis activity than protease from As#6 strain. The present work concludes that Aspergillus As#7 strain may be a good source of industrial protease.*

Key Words: *Alkaline protease, Aspergillus sp, Immobilized protease, Km, pH, Temperature, Vmax.*

I. Introduction

The continuous requirement of proteases has made them industrially important enzyme. They account for 65% of total world wide enzyme sale [1]. They have major applications in industrial process such as laundry detergents, leather, silk, pharmaceutical, food and degradation of gelatin on X-ray films [1, 2, 3, 4]. They are also used in bioremediation process [5].

Proteases are generally distributed in nature. But proteases of microbial origin possess industrial potential due to their biochemical diversity. A variety of microorganisms such as bacteria, fungi, yeast and actinomycetes are known to produce these enzymes [6]. It would be beneficial to use a fungal protease as fungal expression systems are capable of producing larger quantities of enzymes than bacterial expression system [7]. Proteases are produced by many species of fungi such as *Aspergillus*[8], *Mucor* [9], *Fusarium* [10], *Cephalosporium* [11] *and Rhizopus* [12]. Filamentous fungi, such as *Aspergillus* have been the organism of choice for large scale production of bulk industrial enzymes. [13].

For commercial application of enzyme it must be produced at low cost and should be reused, reproduce result with consistent efficiency. Thus different methods have been used to reduce the cost and increase the utilization of proteases, one of which is immobilization. Immobilized enzymes offer advantages which include high productivity, automation, continuous processing, precise control of the extent of reaction, easy product recovery and the enzyme does not contaminate the final product [14]. Additional benefits arise from stabilization against harsh conditions which are deleterious to soluble enzyme preparations. The properties of immobilized enzyme preparations are governed by the properties of both enzyme and the carrier material. The specific interaction between the latter provides an immobilized enzyme with distinct chemical, biochemical, mechanical and kinetic properties. Calcium alginate is the most widely used matrix for entrapment of enzyme. Entrapment within insoluble calcium alginate is recognized as a rapid, nontoxic, inexpensive and versatile method for immobilization of enzyme as well as cells [15].

The present study reports the kinetic study of extracellular free and immobilized protease from local soil isolate of *Aspergillus sp,* AS#6 and AS#7 .These protease were partially purified and were immobilized in calcium alginate beads. Then their kinetic properties were studied.

II. Materials And Methods

Micro organism and maintenance of culture -

The fungal strains used throughout this study were isolated from soil samples of Jabalpur area. These isolates were identified as of *Aspergillus sp,* on the basis of their morphological and microscopic identification. The *Aspergillus* Strains AS#6 and AS#7 were maintained on potato dextrose agar plates at 28 $\pm 2^0$C. Spores for inoculums were prepared from 5-7 days old cultures by sterile cork borer in Tween - 80 solution.

Growth Conditions -

The medium used for protease production by *Aspergillus sp,* was Yeast Extract Broth, composed of Yeast Extract, 0.5%, KCl 2%, Peptone, 2%, Sucrose 2%, Casein, 1.0%, pH 7.5. YE broth was autoclaved at 121^0C for 15 mins. Broth was inoculated with two *Aspergillus* strains AS#6 and AS#7 and incubated in a rotary shaker at 150 rpm for 96h at 28 $\pm 2^0$C, in separate 250 ml Erlenmeyer flasks with working volume of 100 ml. The cultures were centrifuged at 10,000 rpm for 10 min at 4^0C to remove fungal mycelia and supernatants were used as the crude enzyme solution.

Assay of protease activity -

Protease activity was measured by the method of Anson [16], using casein as a substrate. A control lacking the enzyme was included in each assay.

One unit of protease hydrolyzed casein to produce color equivalent to 1.0µmol (181µg) of tyrosine per minute at pH 7.5 at 37^0C. The enzyme activity was expressed as U/ml.

Determination of Protein content -

The protein content of fraction, obtained after ammonium sulphate precipitation was determined by the method of Lowry [17] using BSA as a substrate.

Ammonium Sulphate fractionation -

Solid ammonium sulphate was added to the crude extract to 0-50% saturation. The precipitate was collected by centrifugation, dissolved in minimal volume of Tris-HCl buffer (pH 7.8) and desalted by using prepacked desalting column.

Protease Kinetics -

1. **Effect of different substrates on hydrolytic action of protease -**

To study the effect of various substrates, 1 ml of the partially purified enzyme solution of the *Aspergillus* strains were incubated with 5 ml of each of these substrate (casein, gelatin, BSA and Hemoglobin 0.65% w/v) for 10 min at 37^0C, separately. After incubation the enzyme activities were determined.

2. **Effect of initial enzyme concentration on casein hydrolysis.**

For this, the reaction mixtures were prepared varying the enzyme concentration from 12U/ml to 110U/ml. 5 ml of substrate solutions was added to each of the above enzyme concentrations, then these were incubated at 37^0C for 10 min for protease obtained from both the strains AS#6 and AS#7. Then enzyme assay was done under standard conditions.

3. **Effect of initial substrate concentration on casein, hydrolysis**

In this experiment reaction mixtures were designed varying the substrate concentration from 1mg/ml to 7 mg/ml to which 1ml of partially purified protease solution from the two *Aspergillus* strains AS#6 and AS#7 were added in two separate set of experiments. The reaction mixutres were then incubated at 37^0C for 10 min and then protease activity was determined. Kinetic parameters Km and Vmax were calculated from Line weaver Burk Plots [8].

4. **Effect of pH on casein hydrolysis**

To evaluate casein hydrolysis at different pH (ranging from 5-10), the 1 ml of enzyme solution was added to the 0.65% casein solution by adjusting the pH from 5-10. For pH 5 acetate buffer, pH 6-7 phosphate buffer, pH 8-9 tris buffer, pH-10 glycine - NaOH buffer were used. Protease activity was determined under standard assay conditions.

5. **Effect of reaction temperature on casein hydrolysis -**

Optimum temperature for activity of the protease was determined by carrying out the reactions at temperatures ranging from 0^OC to 65^0C. At each temperature the substrate was pre incubated at the required temperature before the addition of the enzyme.

Immobilization method -

Entrapment -

The partially purified protease from AS#6 and AS#7 strains were immobilized in the calcium alginate beads through entrapment by the method of Banerjee et al., [19]. Bovine serum albumin (5mg) was added to 5.0 ml of enzyme solution. To this 1.5% of sodium alginate was added and stirred gently. The entrapment was

carried out by dropping the mixture through a glass pipette into 50 ml of 2.0% (W/v) $CaCl_2$ solution. The beads so formed were left for 1h in calcium chloride solution and then stored in 0.1 M Tris HCl buffer, pH 9.0 at 4^0C.

Calculation for protease from AS#6 strain [20]

Initial activity of the free enzyme = 297U/ml
Volume of enzyme solution = 5 ml
Weight of beads fromed after immobilization of enzyme solution = 2.5 gm
Enzyme solution enterapped in 0.5 g beads = 0.98 ml
Therefore enzyme enterapped = 199U/0.5g beads
Activity of immobilized enzyme obtained in 0.5 g beads = 192U/0.5g beads
Therefore total enzyme activity after entrapment = 96%

Protease from AS#7 strain

Initial activity of the free enzyme = 203.5U/ml
Volume of enzyme solution = 5 ml
Weight of beads formed after immobilization of enzyme solution = 1.63 gm
Enzyme solution entrapped in 0.5 g beads = 1.53 ml
Activity of immobilized enzyme obtained in 0.5 g beads = 324.36U/0.5g beads
Therefore enzyme entrapped = 228.25U/0.5g beads
Therefore total enzyme activity after entrapment = 76%

Kinetic properties of immobilized protease –

1. Effect of pH and temperature on the immobilized protease-

Effect of pH and temperature on the immobilized protease was determined under standard assay conditions using casein as a substrate. Protease activity was studied in the pH range from 5-10 for immobilized form of enzymes and then their activity was measured at various temperatures (0^0C to 65^0C). Per assay tube 5 beads of immobilized enzyme were added.

2. Effect of substrates concentration on immobilized protease -

The Km and Vmax value were determined for the hydrolysis of casein by immobilized protease. A Lineweaver Burk plot was drawn between the inverse of different concentration of casein and reaction velocity to determine the Michaelis constant Km and Vmax for immobilized protease.

III. Results and Discussion

Partial Purification -

A better understanding of the functions of enzymes could be determined by purification of enzyme [21]. Partial purification of the enzyme by ammonium sulphate precipitation followed by desalting through prepacked desalting column of dextrin, resulted in nearly 7.5 fold increase in the specific activity of the enzyme (Table- 1) from AS#6 strain and 19.2 fold increase in the specific activity of the protease from AS#7 strain (Table - 2).

Protease Kinetics (Free Form) -

Enzyme Kinetics is the most fundamental aspect of enzymology. For the commercial exploitation of enzyme it is desirable to choose an enzyme, which will have the fastest reaction rate per unit amount of enzyme. Reaction rate indicates the maximum effect for minimum amount of added catalyst [22].

Effect of initial substrate concentration on casein hydrolysis -

The assay substrate, casein, when used in increasing concentrations resulted in the saturation of the enzyme (Fig -1). There was an increase in substrate hydrolysis upto 6 mg/ml in both the *Aspergillus s*trains AS#6 and AS#7 and thereafter there was saturation of the enzyme. From Michaelis-Menten equation it is seen that the rate of enzyme reaction is directly proportional to the substrate concentration when the concentration of substrate is low. But at higher magnitude the reaction rate is not influenced by substrate concentration [23].

From Lineweaver Burk Plot, the Km and Vmax of the reaction was found to be 4.5 mg/ml and 142.8 U/ml, respectively for protease obtained from AS#6 strain (Fig 3) whereas protease from AS#7 strain showed Km and Vmax value 2.5 mg/ml and 200 U/ml, respectively (Fig : 4) Since protease from AS#7 strain has a higher afinity for the substrate and highest proteolytic activity than protease from AS#6 strain therefore it suggests that partially purified protease from AS#7 strain may have more industrial applications.

Effect of enzyme concentration on casein hydrolysis-

The study of effect of initial enzyme concentration in Fig:5,6 showed that at low concentration the rate of hydrolysis increased to linearly. It was observed from figure that 89.3 U/ml and 69.5 U/ml enzyme concentration was optimum for protease from AS#6 and AS#7 strains, respectively. This may be due to the limitation of availability of substrate for the active sites of enzyme and for which further addition of the enzyme could not enhance the rate of product formation [24].

Effect of different substrates on hydrolytic action of protease -

Among the various substrates studied, casein was found to be the best substrate for protease obtained from both the strain AS#6 and AS#7 (Fig : 7, 8). When gelatin and BSA were used as the substrates, protease from AS#7 strain showed no proteolysis activity. Protease obtained from AS#6 strain showed highest proteolysis activity in casein followed by hemoglobin, gelatin and BSA.

Effect of pH on casein hydrolysis -

Enzymes, being proteinaceous in nature, have properties that are quite pH sensitive. pH can affect activity by changing the charges on an amino acid residue which is functional in substrate binding or catalysis.

Partially purified protease from AS#6 and AS#7 strains both showed maximum activity at pH 9 (Fig 9, 10). A fall in the hydrolysis rate on either side of the optimum value is due to decrease in affinity of enzyme for the substrate [23]. Similar observation was reported in a new strain of *Aspergillus Oryzae* AWT 20. The optimum pH of the free enzyme of this strain was 9.0 [25].

Alkaline protease from *Bacillus circulans* also show activity over broad pH range (8-11) with optimum pH at 9.0 [26].

Effect of reaction temperature on casein hydrolysis -

Partially purified protease from *Aspergillus* AS#6 strain showed increased rate of enzyme reaction with increase in temperature up to 45^0C but thereafter activity decreased whereas partially purified protease from AS#7 strain showed optimum activity at 65^0C (Fig : 11, 12). Increase in temperature above optimum level affects important factors like protein denaturation, protein ionization state and solubility of species in solution reducing enzyme activity [27]. The optimum temperature of protease obtained from AS#7 strain was slightly higher than those from other fungal proteases such as *Rhizopus oryzae* [28] and *Aspergillus niger 1* [29] which exhibited optimum temperature at 60^0C. *Aspergillus niger* NRRL 1785 exhibited optimum temperature at 50^0C [30] which is slightly higher than from protease of AS#6 strain which showed optimum activity at 45^0C.

Immobilized protease Kinetics -

Effect of substrate concentration on activity of immobilized protease

Michaelis - Menten (Km) value of entrapped protease was determined by studying the hydrolysis of different concentration of substrate (casein), ranging from 1mg/ml to 7 mg/ml. Line weaver Burk Plot was drawn between the inverse of different concentrations of casein and reaction velocity to determine the constant for immobilized protease. The Km and Vmax value for immobilize protease from AS#6 strain was found 5 mg/ml and 167 U/ml, respectively, (Fig :13). Protease from AS#7 strain showed 2.6 mg/ml Km and 370 U/ml Vmax value, (Fig : 14) respectively for immobilized enzyme while Km and Vmax for free enzyme was 2.5 mg/ml and 200 U/ml/min, respectively. This increase in substrate concentration might be due to the inability of high molecular weight substrate casein to diffuse rapidly into the Ca-alginate matrix [31]. AS#7 strain showed 85% increase in Vmax value of the immobilized enzyme as compared to free enzyme. This may be due to the rate of diffusion of substrate to the enzyme may rise to a limiting value as the substrate concentration in the bulk solution is increased. If this limiting value is reached after an immobilized enzyme is completely saturated with substrate, then its apparent Vmax value will high than that in free solution [32].

Effect of temperature and pH on activity of immobilized protease -

Alginate entrapped enzyme was assayed at different temperature and pH ranging from 30-85^0C and pH 5-10, respectively, The optimum temperature of entrapped enzyme from AS#6 and AS#7 strain was 45^0C and 85^0C respectively (Fig: 15, 16). In the present case for protease from AS#6 strain the optimum temperature (45^0C) and pH (9) (fig- 17) of free and immobilized enzyme remain the same. Consistently, Arya and Srivastava [33] and Anwar et al, [34] reported that no change occurred in the optimum temperature and pH of CGtase and protease, respectively, before and after entrapment in calcium alginate beads.

The optimum pH value of the immobilized protease form AS#7 strain shifted to pH 10 from 9.0 which was the optimum pH of the free enzyme (Fig 10). This may be due to the anionic nature of the alginate support used for immobilization and change in microenvironment of the immobilized enzyme. Similar observation reported in protease immobilization studies [25]. At 85^0C, the immobilized protease from AS#7 strain retained

~121% of its activity while free enzyme could retain 70% activity. The thermostability of enzyme increased very significantly after entrapment. This probably reflects the fact that entrapped enzyme is not chemically modified but remain in its native form in the gel matrix. Another reason, for this high optimum temperature of immobilized protease from AS#7 strain may be high reaction rate of immobilized enzyme due to which reaction is completed within a few minutes and the risk of denaturation was minimal [23]. Similar result was also reported by Sharma et al, [25] with protease immobilized in Ca-alginate capsules.

Reusability of immobilized protease -

The activity of entrapped enzyme was assayed for the three cycles with casein as a substrate, in order to find out the reusability of the entrapped enzyme. The entrapped enzyme showed 78% and 83% activity during the second reuse and 78% and 80% activity on its third use, respectivety for protease from AS#6 and AS#7 strain. This decrease in activity was due to leakage of enzyme from the beads. In another study of entrapped enzyme during forth cycle activity is completely lost. [20].

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Table -1Partial purification of crude protease from *Aspergillus* As # 6 strain by ammonium sulphate precipitation.

Purification steps	Total enzyme activity (U)	Total protein (mg)	Specific activity U/mg	Fold Purification	Yield (%)
Crude enzyme	298	99.4	3.0	1	100
Ammonium sulphate fractionation	153	6.8	22.5	7.5	51

Table-2Partial purification of crude protease from *Aspergillus* As # 7 strain by ammonium sulphate precipitation.

Purification steps	Total enzyme activity (U)	Total protein (mg)	Specific activity U/mg	Fold Purification	Yield (%)
Crude enzyme	385	98.2	3.9	1	100
Ammonium sulphate fractionation	165	2.2	75	19.2	42.9

Fig.-1 Effect of substrate concentration on protease activity from As # 6 strain

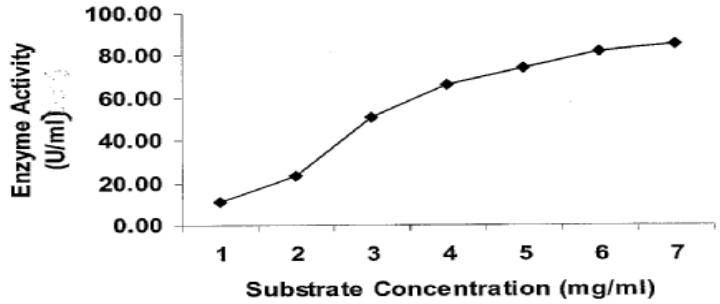

Fig.-2 Effect of substrate concentration on protease activity from As # 7 strain

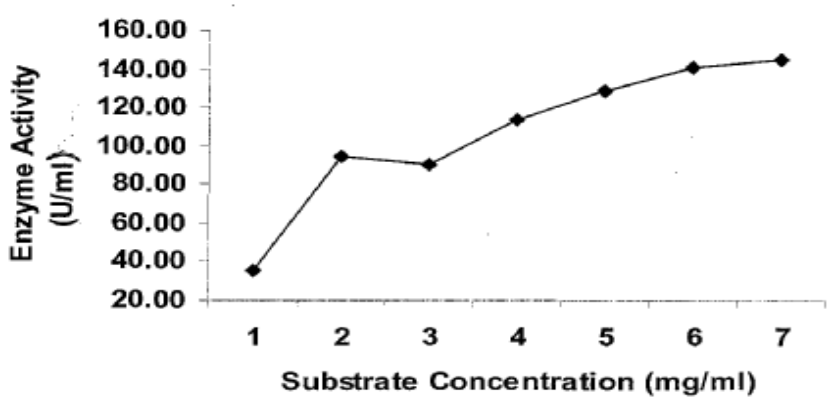

Fig. 3 Lineweaver Burk plot of extracellular protease from As # 6 strain

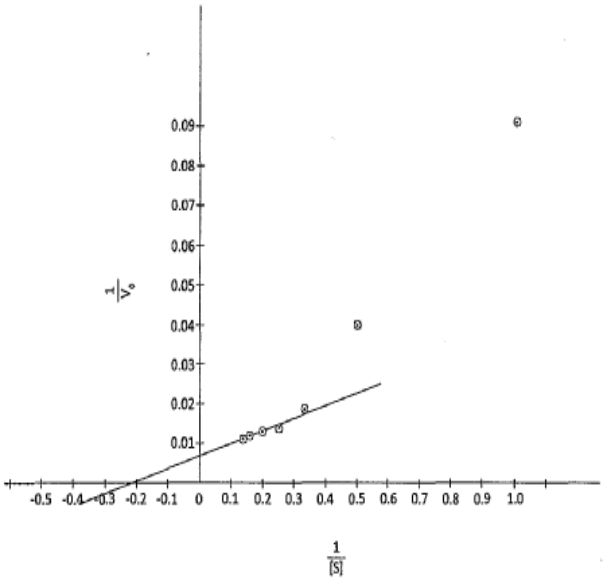

Fig. 4 Lineweaver Burk plot of extracellular protease from As # 7 strain

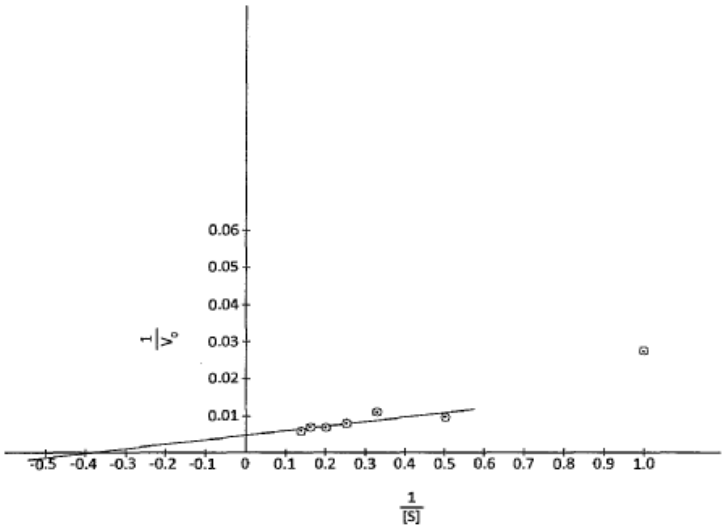

Fig.-5 Effect of enzyme concentration on protease activity from As # 6 strain

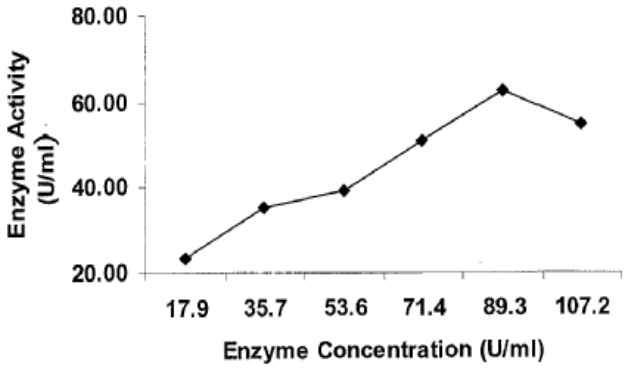

Fig.-6 Effect of enzyme concentration on protease activity from As # 7 strain

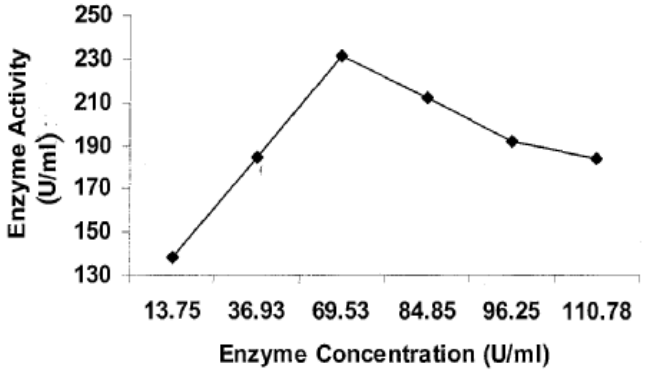

Fig.-7 Effect of different substrates on protease activity from As # 6 strain

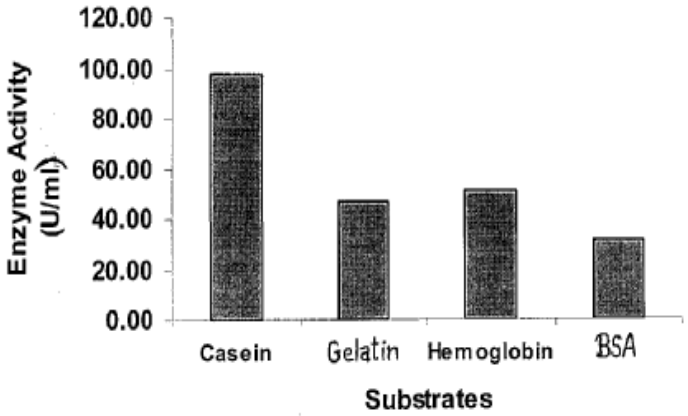

Fig.- 8 Effect of different substrates on protease activity from As # 7 strain

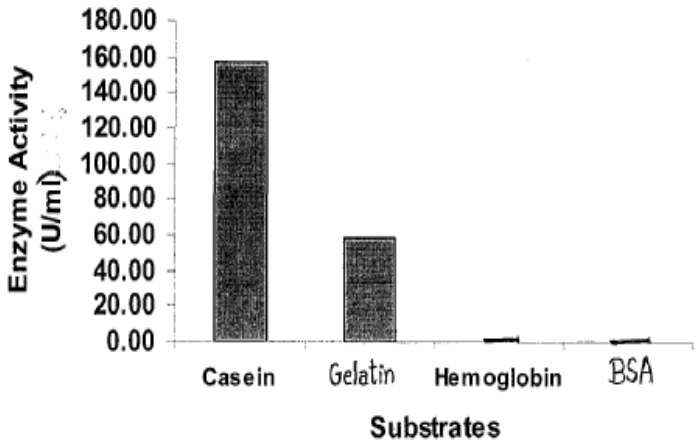

Fig.-9 Effect of pH on protease activity from As # 6 strain

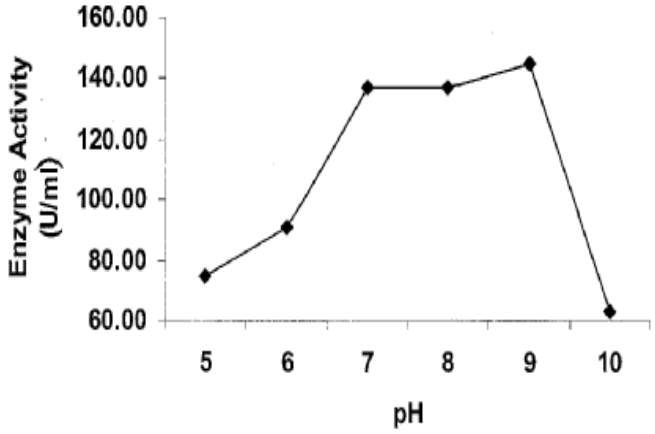

Fig.-10 Effect of different pH on protease activity from As # 7 strain

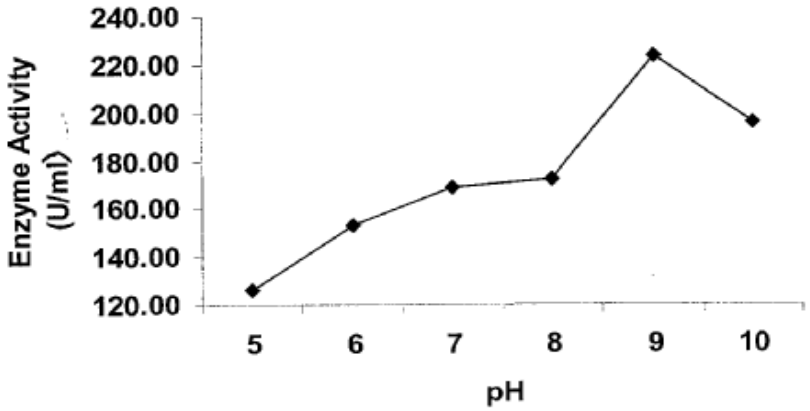

Fig: 11 Effect of different temperature on protease activity from as#6 strain

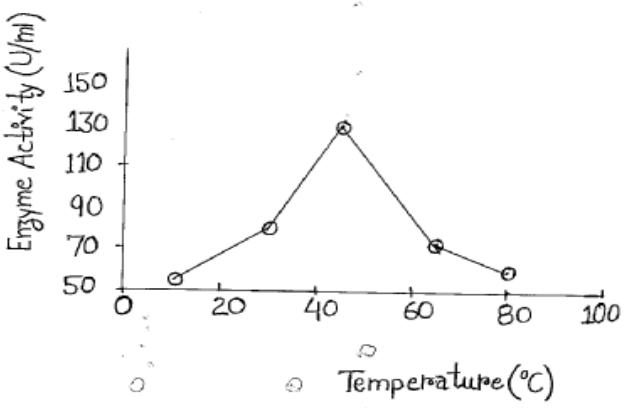

Fig : 12 Effect of different temperature on protease activity from As#7

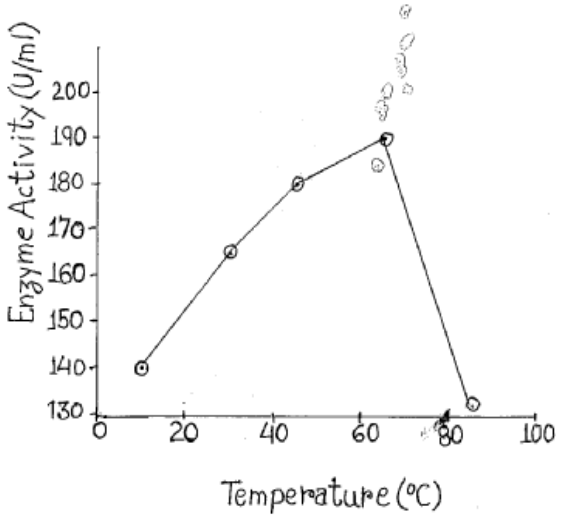

Fig. 13 Lineweaver Burk plot of extracellular immobilized protease from As # 6 strain

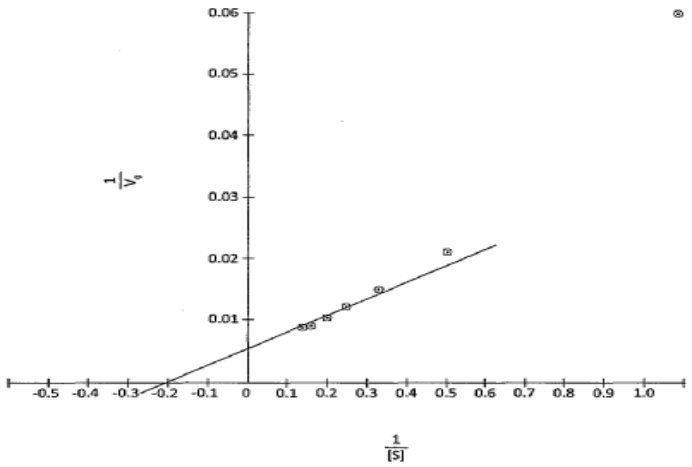

Fig. 14 Lineweaver Burk plot of extracellular Immobilized protease from As # 7 strain

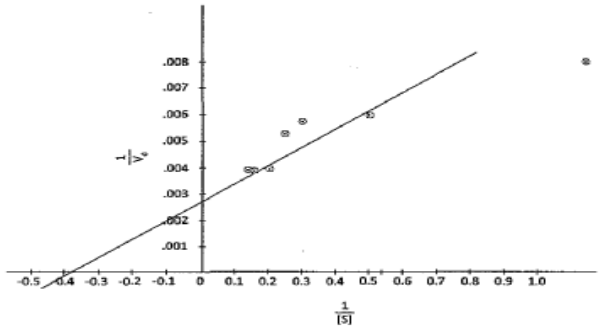

Fig : 15 Effect of different temperature on immobilized protease activity from As#6 strain

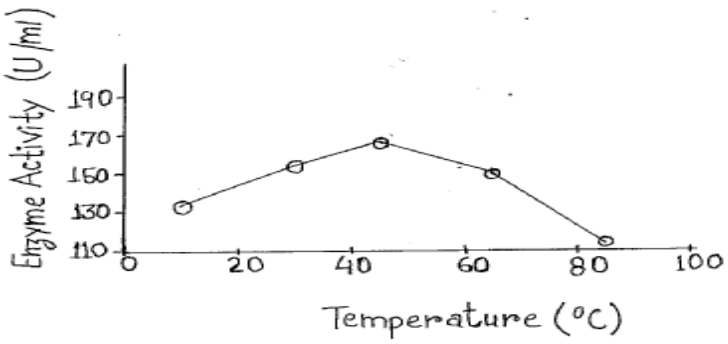

Fig : 16 Effect of different temperature on immobilized protease activity from As#7 strain

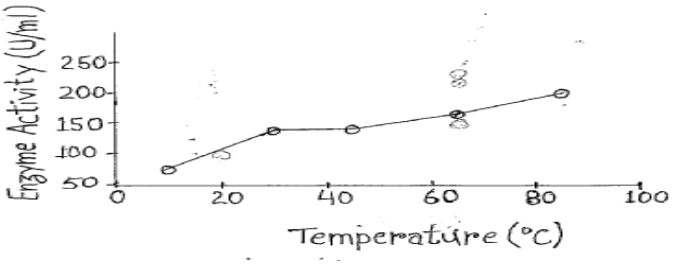

Fig.-17Effect of pH on immobilized protease activity from As # 6 strain

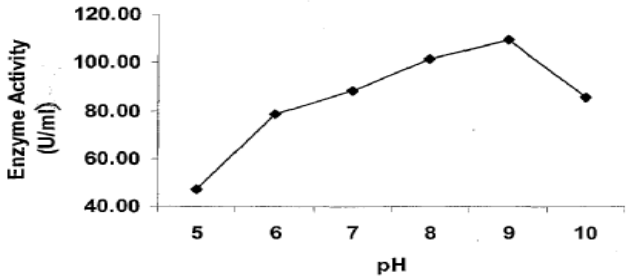

Fig.-18 Effect of different pH on immobilized protease activity from As # 7 strain