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Immobilization and Characterization of an alkaline Protease from *Aspergillus niger*

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Abstract

A protease is an enzyme that performs proteolysis that begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein. Proteases are the single class of enzyme which occupies a pivotal position with respect to their applications in both physiological and commercial fields. Aspergillus niger was selected for production, partial purification and characterization with various process parameters. In specific media containing Casein (1%) maximum production of free enzyme was observed at pH of 8.5 and temperature of 50°C. The optimum pH and temperature for the assay of immobilized protease was 9.0 and 55°C. The enzyme was partially purified using 40% ammonium sulphate precipitation followed by dialysis. A gradual decrease in the activity of the free and the immobilized protease was observed when the temperature was increased beyond their optimum temperature. A gradual decrease in the activity of the free protease was observed when the pH was increased beyond their optimum pH. The maximum activity range of immobilized enzyme over pH 8.5-9.0 revealed its resistance to alkaline changes in the medium as compared to free enzyme. There was increase in activity of the free and the immobilized protease as the concentration of casein was increasing at certain point after that, it reaches the saturation point and remains constant. Immobilized enzyme preparation had retained almost 33.6% activity after 4th Cycle.

Keywords: *Alkaline Protease, Ammonium sulphate precipitation, Immobilization.*

Introduction

A protease is an enzyme that performs proteolysis, that is, begins protein catabolism by hydrolysis of peptide bond that link amino acid together in polypeptide chain forming protein. Proteases are the single class of enzymes which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Proteases occur in all organisms from prokaryotes to eukaryotes to viruses. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated cascades (e.g., blood clotting cascade, the compliment system, apoptosis pathway, and the invertebrate prophenoloxidase-activating cascade). Some snake venoms are also proteases, such as pit viper haemotoxin and interfere with the victim's blood clotting cascade. Bacterial and fungal proteases are important to the global carbon and nitrogen cycles in the recycling of proteins, and such activity tends to be regulated by nutritional signals in these organisms. Microbial proteases represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Microbial proteases account for approximately 60% of the total worldwide enzyme sales (Godfrey and West, 1996).

In industrial products, the classification relates to the pH optimum of the respective enzyme. There are acidic, neutral and alkaline proteases. Most commercial proteases, mainly neutral and alkaline are produced by the organisms belonging to the genus *Bacillus.* Fungi elaborate a wider variety of enzymes than do bacteria. *Aspergillus niger, Aspergillus oryzae*, play a dominant role in the production of protease enzymes. Protease has a long history of application in food and detergent industries. Their applications in the leather industry for dehairing and bating hides to substitute currently used toxic chemicals is a relatively new development and has conferred added biotechnological importance. (Fogarthy, 1996). This continuous requirement of protease has made them important industrial enzyme which is about the 60% of the total commercial enzymes involved in the industries. Thus different measures have been taken to reduce the cost and increase the utilization of protease, one of which is enzyme immobilization. Immobilized enzymes are widely used in different industries especially in food and pharmaceutical and offer several advantages over bulk or free enzymes. Advantages include high productivity, automation, continuous processing, precise

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control of the extent of the reaction, easy product recovery and enzyme does not contaminate the final product. Entrapment of the enzyme in calcium alginate is one of the important methods of the immobilization. Alginates are commercially available as water soluble sodium alginate and they have been used for more than 65years in the food and pharmaceutical industries as thickening, emulsifying and film forming agents.

Alkaline proteases useful for detergent applications were mostly active in the pH range 8 - 12 and at temperatures between 50˚C - 70˚C. The optimum pH range of alkaline proteases is generally between pH 9 and 11, with a few exceptions exhibiting higher pH optima, up to a pH range 12 - 13. The optimum temperature of alkaline proteases ranges from 50˚C to 70˚C. Interestingly, the enzyme from an alkalophilic Bacillus sp. B189 showed an exceptionally high optimum temperature of 85˚C. Alkaline proteases from *Bacillus sp., Streptomyces sp. and Thermus sp.* are quite stable at high temperatures, and the addition of $CaCl_2$ further enhances enzyme thermostability.

In general, alkaline proteases require metal ions for their maximum activity. The most commonly used metal ions are Ca^{2+}, Mg^{2+} and Mn^{2+}. Ca^{2+} ion is also known to play a major role in enzyme stabilization by increasing the activity and thermal stability of alkaline proteases at higher temperatures. Other metal ions such as Ba^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, Fe^{3+} and Zn^{2+} are also used for stabilizing proteases. These metal ions protect the enzyme against thermal denaturation and play a vital role in maintaining the active confirmation of the enzyme at higher temperatures. Presence of Ca^{2+} is known to activate proteases by increasing thermostability. Metal ions like Hg^{2+}; Cu^{2+}, Ag^{2+}, Fe^{2+} and Zn were found inhibitory to majority of proteases.

Material & Method

The protease enzyme from *Aspergillus niger* (MTCC No. 281) was grown on the Czapek yeast extract broth medium. *Aspergillus niger* (MTCC No. 281) was collected from Institute Of Microbial Technology, Sector – 39, Chandigarh.

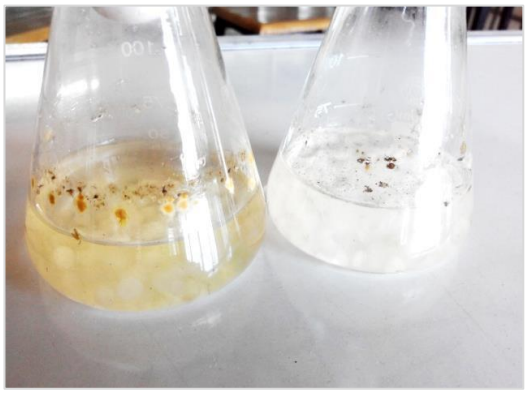
Czapek Yeast Extract Broth

K_2HPO_4-1g,Yeast extract-5g, Sucrose-30g,Casein-1%, Distilled water- 1liter

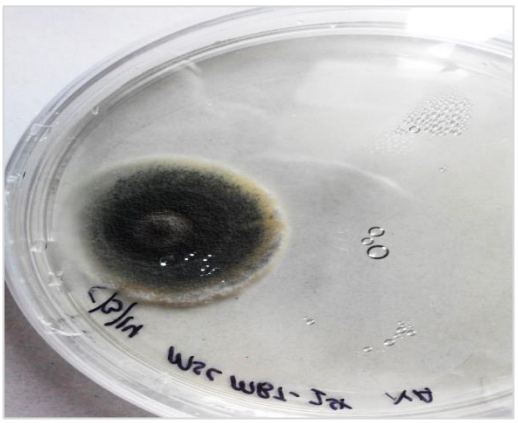
*Czapek concentrate- 10ml [*Czapek concentrate ($NaNO_3$ -30g, KCl-5g, $MgSO_4.7H_2O$- 5g, $FeSO_4.7H_2O$- 0.1g, Distilled water- 100ml)]

Culture Conditions

Growth conditions	aerobic
Temperature	25-30^{o}C
Incubation time	7days
Subculture	30days
Special feature	Production of gluconic acid and citric acid.



(a)



(b)

Fig 1. (a) Growth of *Aspergillus niger* in production media containing casein, (b) Growth of *Aspergillus niger* on Czapek's Dox Yeast Extract Agar medium

Mode of harvest

The culture was transferred to 6 flasks each having 50ml of production medium (250ml Erlenmeyer flask) for 7 days under shaking conditions at 25^{o}C (Fig 1)

Precipitation of protease

The supernatant obtained after 7days of production was used for purification of protease. Ammonium sulfate was added to the supernatant to achieve 40% saturation. Required amount of ammonium sulfate was added to the cell free culture broth, the contents were mixed thoroughly and kept at 4^{o}C overnight. Thereafter the precipitates was sedimented by centrifugation at 5000 rpm for 20 min. The supernatant was discarded and precipitates were dissolved in minimal volume of 0.08M Tris- HCl (pH 8) and extensively dialyzed against same buffer at 4^{o}C. (Fig.3). Dialyzing membrane -60 was used

for dialysis having average width of 25.27mm, average diameter of 15.9mm, average capacity of 1.99ml/cm. Finally, protease activity was assayed.

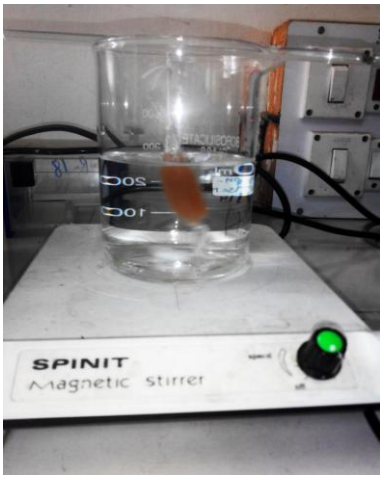


Fig.3 Dialysis of Crude Enzyme

Protease assay

3ml of reaction mixture containing 0.5% casein in 2.95ml of 0.1M Tris-HCl buffer, pH 8.5 and 0.1ml of enzyme was incubated at 50°C for 10 min; the reaction was stopped by adding 3 ml of cold 10% TCA (Trichloroacetic acid). After 1 hour, the culture filtrate was centrifuged at 8000 rpm for 5 min to remove the precipitates and absorbance of supernatant was read spectrometrically at 280nm.

Immobilization of protease

The protease from *Aspergillus niger* (MTCC No. 128) was immobilized on agarose beads. Agarose gel was prepared by making a clear solution with TAE buffer (3g agarose in 100ml 1X TAE). 50ml of 3% agarose was prepared in flask and heated for 2min for clear solution of agarose. Cooled to room temperature and 2ml of enzyme was added into it and mixed properly. Poured into petriplates and circular disc type beads were formed. Each disc of the matrix measures 0.4cm in radius. Each circular disc weigh about 0.11g (approx) which contain 100µl of enzyme per beads (Fig.4). Beads were first washed into distilled water and then kept in 1X TAE buffer at 4°C until further use.

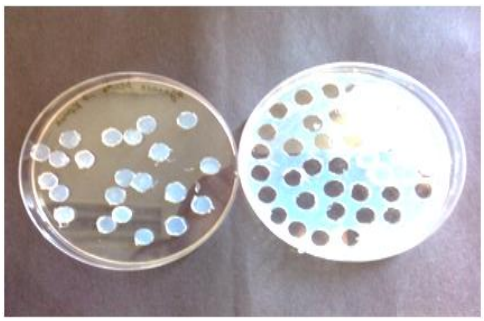


Fig.4 Immobilized Agarose Beads

Characterization of free and immobilized protease

Effect of pH on Protease activity

The free and immobilized enzyme matrix (0.44g/4beads) was taken and assayed for protease activity in Tris-HCl (0.1M) at different pH (6.5, 7.0, 7.5, 8.0, 8.5, 9.0)

Effect of temperature on Protease activity

The free and immobilized enzyme matrix (0.44g/4beads) was taken and assayed for protease activity in Tris-HCl (0.1M) at different temperatures (45, 50, 55, 60, 65°C).

Effect of substrate concentration on Protease activity

The free and immobilized enzyme matrix (0.44g/4beads) was taken and assayed for protease activity in Tris- HCl (0.1M) at different concentrations (0.3, 0.4, 0.5, 0.6, 0.7%).

Reusability of immobilized enzyme

To determine the reusability the immobilized enzyme was washed and enzyme activity was assayed at 50°C for 10min repeatedly till the activity ceases. The initial activity was measured and conjugate was then subjected to 4 cycles of repeated use.

Result & Discussion

Enzyme Purification

The initial activity of protease produced by *Aspergillus niger* in the harvested production medium in total was found to be 71U with a total protein content of 706mg/ml The specific activity was calculated to be 4.6 IU/mg (Table 4).

The culture broth was saturated with 40% of ammonium sulfate. The precipitate was reconstituted in Tris-HCl buffer (pH 8) and subjected to extensive dialysis against Tris- HCl buffer (pH 8). After ammonium sulphate precipitation the protease activity was calculated to be 1.15U and the protein content was 12mg/ml. The specific activity was calculated to be 10 IU/mg. The dialysate showed protease activity of 0.23IU. The specific activity of the concentrated enzyme was calculated to be 35.3IU/mg

Table 4 Which indicated purification of enzyme

Fraction	Volume (ml)	Protease activity (iu)	Specific activity (iu/mg)	Yield %
Crude	200	71	4.6	100
Ammonium Sulphate Precipitation	87	1.15	10	16
Dialysis	5	.023	35.3	3

In the present study, the protease produced by *Aspergillus niger* (MTCC No. 128) was immobilized on

agarose beads having 0.4cm radius by dissolving 2ml of the enzyme in 50ml of 3% agarose (in 1X TAE Buffer) gel. Kept for solidifying and cut into uniform circular disc type beads.). Recently, in the study the agarose beads were used which were cut into blocks of equal size and weight and added to sterile 0.1 M phosphate buffer(pH-6.9) and kept in refrigerator for curing and washed with sterile distilled water for further use (Abhijit Poddar *et al.*,2011). Previously many methods have been used to immobilize proteases including calcium alginate bead method (Johnsen and Flink, 1986), immobilization in polyacrylamide gel, by encapsulation in agar (Veelkan and Pape, 1982).

Effect of temperature on the activity of Protease

Protease activity at different temperature was examined keeping other conditions constant. The maximum activity of free enzyme i.e 31.2IU was obtained at 50^{o}C and maximum activity of immobilized enzyme i.e 29IU was obtained at 55^{o}C (Fig 5). After immobilization a shift in such temperature was observed and the immobilized enzyme exhibited the highest activity at 55^{o}C (Fig 5). After further increase in temperature, the enzyme activity ceases may be due to denaturation of the enzyme. Lower activity of immobilized enzyme was seen in assay as compared to the free enzyme. It might be due to the decreased affinity of the enzyme for the substrate because of the immobilization.

In recent study, the Maximum activity of protease was obtained at 45°C. Growth and protease production ceased at higher temperature (50°C) similar observation were shown by Morimura *et al.*, (1994) for *Aspergillus usami.*

Effect of pH on the activity of protease

The optimum pH of free and immobilized protease was studied at various pH values (6.5-9.0) (fig 6). The results indicate that the optimum pH values for free and immobilized protease were 8.5 and 9.0 respectively (Fig 6). The pH shifts towards alkaline value upon immobilization are suggested to be because of secondary interaction between the enzyme and the matrix (Keerti *et al.*, 2014). The maximum activity range of immobilized enzyme over pH 8.5-9.0 revealed its resistance to alkaline changes in the medium as compared to free enzyme.

Effect of substrate concentration on the activity of protease

Different substrate concentrations were used ranging from 0.3% to 0.7%. The results indicate the gradual increase in the enzyme activity of both free and immobilized enzyme upto certain concentration of casein. After certain concentration it reaches the saturation point and becomes constant (Fig 7). Recently, same results were derived from the strain *Bacillus subtilis* DKMNR (Kezia *et al.*, 2011)

Reusability of immobilized enzyme

A continuous assay of residual enzyme activity of the protease immobilized on agarose gel was performed to find out the leaching of bound protease. Immobilized enzyme preparation had retained almost 33.6% activity after 4th cycle (Fig 8).The reusability of the alkaline protease immobilized on Eudragit S-100(a commercial protease) has also been studied. There was decline in activity on repeated use of immobilized Esperase, conjugate retained 72% of its original activity after five cycles of repeated uses, showing a high reusability (Carla J.S.M. Silva *et al.*, 2006).

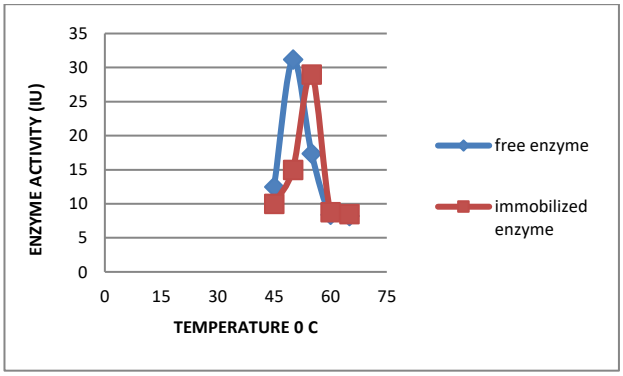


Fig.5 Optimization of temperature for free and immobilized enzyme

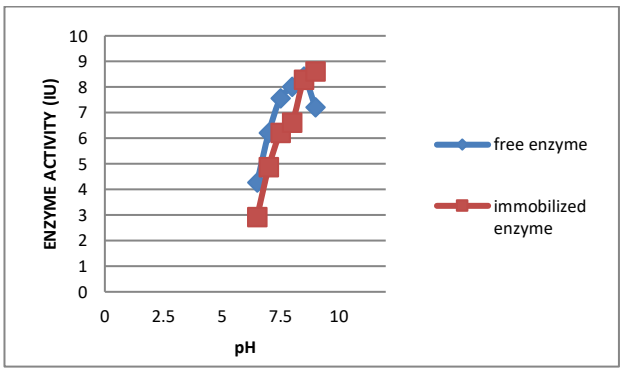


Fig.6 Optimization of pH for free and immobilized enzyme

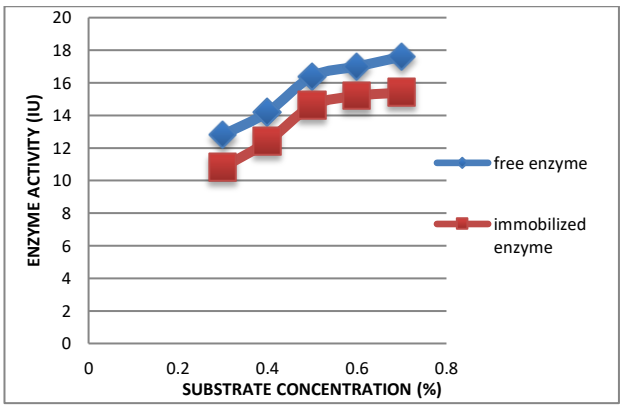


Fig.7 Optimization of substrate concentration for free and immobilized enzyme

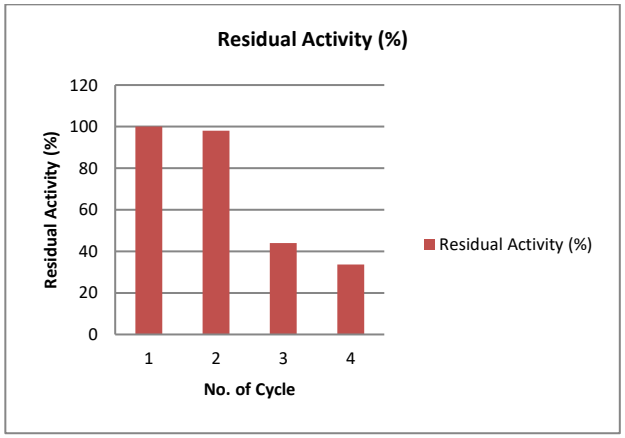


Fig.8 Reusability of immobilized enzyme

Conclusion

The protease enzyme was successfully immobilized on the agarose beads. The optimum temperature for free enzyme is 50^{o}C and for immobilized enzyme is 55^{o}C, from results we concluded that the immobilized enzyme can withstand higher temperature and shows the activity of 29IU. In case of pH the optimum pH for free enzyme is 8.5 and that for immobilized is 9. So the protease enzyme can efficiently work in alkaline conditions. On the basis of this, it could be concluded that agarose gel immobilized protease can withstand higher temperature and pH and shows greater stability and reusability. From these characteristics, application of immobilized protease in wastewater treatment, detergent industry, leather industry and other industries may be envisaged.

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