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Study of Protease Enzyme from Bacillus Species and its Application as a Contact Lens Cleanser

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ABSTRACT

Aims: To study protease production from four Bacillus species. To optimize the production of extracellular protease by testing various environmental and nutritional factors and its application as a contact lens cleanser.
Methodology: The assay was carried out in duplicates. Cultures of *Bacillus subtilis, Batcillus licheniformis, Bacillus thermophilus,* and *Bacillus cereus* were used for Protease production and assay. Sterile Bushnell – Hass medium containing 1% casein was used. The clear zone of casein hydrolysis indicated protease secretion. Protease assay was done using modified sigma protocol for enzymatic assay of protease. Effect of incubation time, pH, temperature and aeration on protease production was studied. Diameter of colony and zone of clearance was measured and Cx ratio was calculated. The best protease producer was selected. Extracted and crude protease enzymes were added to artificial tear solution prepared with 0.2% lysozyme. Enzyme treatment was done for 10, 30, 60 and 90 min at optimum temperatures of respective enzymes. Light transmission readings were recorded using visible range spectrophotometer at 285 nm.
Results: All four species of Bacillus produced protease enzyme. *Bacillus subtilis* (Cx ratio=6) was the best protease producer. 37°C temperature, 48 hrs of incubation and pH 7.5 was optimum for maximum production of protease from *Bacillus subtilis*. Optimum temperature and pH for the crude enzyme activity was 40ºC and 8. The optimum ammonium sulfate fractionation (40% (w/v) saturation) showed 4.76 fold increase in the specific activity of the crude extract. Protein solution was degraded by purified enzyme in 30 min whereas crude enzyme required 60 min.
Conclusion: Protease enzyme extracted from *Bacillus subtilis* showed good activity against artificial tear solution.
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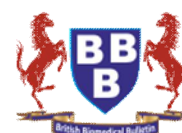

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Introduction

Microorganisms are the most preferred source of Protease enzymes in fermentation bioprocesses because of their fast growth rate, their ability to engineer genetically to generate new enzymes with desirable abilities and enzyme overproduction.[1] Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology.[2] The major uses of free proteases occur in dry cleaning, detergents, meat processing, cheese making, silver recovery from photographic films and certain medical treatments of inflammation and wounds.[3] Proteases are important components in biopharmaceutical products such as contact-lens enzyme cleaners and enzymatic debriders.[2,4]

It is well known that the deposits on contact lenses consist of proteins, lipids and mucin as tear components.[5] Protein deposits are natural deposits on contact lenses which are unavoidable as they are formed by the interaction of the protein in our natural tears and the contact lenses. Contact lens cleansing solutions have been prepared using plant (papain) and animal (pancreatin, trypsin and chemotrypsin) proteases. However, in most instances they impart an unpleasant odor to the cleansing bath or develop an odor after a few hours of use. Several microbial proteases from Bacillus species, Streptomyces sp., and Aspergillus sp. were reported to clean tear films and debris of contact lenses.[6] In order to overcome these drawbacks and to make cleaning composition odorless and safe, i.e., not producing an allergic response or causing irritation to the eyes, bacterial proteases are gaining importance. Several reports are available on production of proteases from bacterial cultures with Bacillus sp. as the dominating organism.[7-9]

In the present study we report the isolation of protease from Bacillus species, optimizing the production of extracellular protease by testing various environmental and nutritional factors and its application as contact lens cleanser.

Materials and Methods

Micro-organism

Cultures of *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus thermophilus* and *Bacillus cereus* were obtained from the Department of Microbiology, Changu Kana Thakur College, Panvel, Mumbai, India.

Protease production and assay

Sterile Bushnell- Hass medium containing 1% casein was used for screening of isolates for protease production. The isolates used for screening were *Bacillus subtilis, Bacillus licheniformis, Bacillus thermophilus,* and *Bacillus cereus.* 24 hrs old cultures of these isolates were streaked on above plates and incubated at 37°C for 24 hrs. The method was performed in duplicates. The clear zone of casein hydrolysis was an indication of protease secretion. Protease assay was done using modified sigma protocol for enzymatic assay of protease.[10,11]

Selection of best protease producer

The cell count of each organism was calculated using standard plate count. For this, 0.1 O.D adjusted saline suspensions of the above cultures were used according to Miles and Misra's method.[12] Results were seen after 8 hrs. The experiment was performed in duplicates. Based on results of this experiment, agar spot experiment was performed.[13] In this experiment, Sterile Bushnell - Hass medium containing 1% casein agar plates were used. Saline suspensions adjusted to 0.1 O.D of the cultures were prepared. 10µL of each suspension was spotted on above agar plates and the plates were incubated at 37°C for 24hrs. After incubation, diameter of colony

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and zone of clearance was measured and Cx ratio was calculated.

Cx ratio = diameter of zone of clearance / Diameter of colony.

Effect of Incubation Time

2 ml of culture suspensions grown overnight on sterile Nutrient agar slants were used to inoculate into 100 ml of production medium. The flasks were incubated at 37°C on a shaker. Aliquots were withdrawn every 24 hrs from the flasks until 96 hrs and were assayed for protease activity.[3,14]

Effect of pH of the medium

Four different pH values were employed. The pH values used were 6.0, 7.5, 8.0, and 9.0. 2 ml of culture suspensions grown overnight on N.A slants were used to inoculate into 100 ml of production medium. The flasks were incubated at 37°C on a shaker. Aliquots were withdrawn after 48 hrs and were assayed for protease activity.[3,14]

Effect of Temperature of fermentation

The pH of the production medium was adjusted to 7.5. It was incubated at R.T and 37°C to find optimum temperature of incubation. 2 ml of culture suspensions grown overnight on N.A slants were used to inoculate into 100 ml of production medium. The flasks were incubated on a shaker. Aliquots were withdrawn after 48 hrs and were assayed for protease activity.[3,14]

Effect of Aeration conditions

To determine the effect of aeration, one production flask was incubated on a shaker and another one was incubated at stationary conditions at room temperature. The production medium was adjusted to pH 7.5. 2 ml of culture suspensions grown overnight on N.A slants were used to inoculate into 100 ml of production medium. Aliquots were withdrawn after 48 hrs and were assayed for protease activity.[3,14]

Extraction and purification of enzyme

St. Bushnell - Hass broth containing 1% casein (pH 7.4) was used for the production of protease. *Bacillus subtilis* was used for the production. Three 500 ml conical flasks containing 100 ml aliquots of the medium were incubated at 37°C for 48 hrs on shaker conditions. After 48 hrs, broth was centrifuged at 5000 rpm for 20 min. The supernatant collected was used as crude extract and was filter sterilized to remove the spores. Protease activity of crude extract was determined.[3] Partial purification was carried out using Ammonium sulphate fractionation and dialysis method. Continuous Ammonium sulphate saturation was done.[15]

Effect of pH and temperature on activity of enzyme

Effect of pH and temperature on activity of both crude enzyme and partially purified protease was studied. Effect of pH and temperature on activity of crude enzyme was studied before purification. Both the values were compared with that of partially purified enzyme. In order to study the effect of pH on the activity of the enzyme, 0.65% casein (substrate) was prepared in phosphate buffer having pH values 7, 8, 9 and 10. In order to study the effect of temperature on the activity of the enzyme, 0.65% casein (substrate) was prepared in phosphate buffer with pH 8.0.[3]

Application of protease in contact lens cleansing

Partially purified proteases and crude extract were used for the application. A filter sterilized artificial tear solution was prepared with 0.2% lysozyme in electrolyte solution (0.22g Na2CO3 and 0.7 g NaCl, pH 8). This solution was heated at 50°C for 20

min to denature lysozyme protein. 2ml of filter sterilized tear solution was taken in sterile test tube and 1 ml of enzyme was added to the tube. Similarly, a control set containing artificial tear solution but treated with phosphate buffer instead of enzyme was used. Enzyme treatment was done for 10, 30, 60 and 90 min at optimum temperatures of respective enzymes. Light transmission readings were recorded using visible range spectrophotometer at 285 nm.[6]

Results and Discussion

In the present study, all 4 species of Bacillus (*Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus thermophilus* and *Bacillus cereus*) produced protease which was observed as a zone of clearance around the colony as shown in figures 1, 2, 3 and 4. By Miles and Misra method, cell count was calculated which was around 10^{10} cells of each organism at 0.1 O.D.[12] Results of agar spot method showed that *B. subtilis* (Cx ratio=6) was the best protease producer amongst the above 4 organisms.

Effect of incubation time on protease production

Since micro-organisms show considerable variation at different incubation period, it was very essential to detect the optimum incubation time at which the organism showed highest enzyme activity.[16] Figure 5 shows that 48 hrs of incubation for *B. subtilis* was suitable time for maximum production of protease. Maximum production of proteases with 48 to 72 hrs of incubation by bacteria as reported by Hoshino *et al*.[17] The results of Safey *et al* indicted that *B. subtilis* exhibited their maximum ability to biosynthesize protease within 30 hrs of incubation period.[3] Ammar *et al*, (2003) reported that the optimum incubation period for thermostable purified protease enzyme was ranging from 60 to 72 hrs.[18]

Effect of pH on protease production

Microorganisms are sensitive to the changes in the hydrogen ion concentration of their environment.[16] El- Hawry concluded that the optimum pH must meet the requirements of the protease producing gene. Bacteria were more sensitive to pH when used for the production of enzymes.[19] Figure 6 shows that pH 7.5 was optimum for the production of protease from *B. subtilis*. Sharmin *et al* found that *B. licheniformis* showed high protease production at pH 8.5.[20] Safey *et al* found pH 7.0 was the optimum pH for the protease production from *B. subtilis*.[3] Shehri *et al* reported maximum protease production was obtained at alkaline pH 8 reaching 255 U/ml.[14]

Effect of temperature on protease production

The growth and enzyme activity of micro-organisms is greatly influenced by different incubation temperatures. The growth of micro-organisms can be inhibited at one temperature but it can be activated at another temperature.[16] Figure 7 shows that protease production at 37°C was more than at RT. So, 37°C was the suitable temperature for protease production. It was previously reported that 40°C was the optimum temperature for the protease production from *B. subtilis*.[3]

Effect of aeration conditions

Oxygen shows diverse effects on product formation in aerobic fermentation process by influencing metabolic pathway and changing metabolic fluxes. Figure 8 shows that protease production was more when the medium was in shaker condition than in static condition.

Effect of pH and temperature on activity of crude enzyme

Effect of pH and temperature on activity of crude enzyme was checked to determine the optimum pH and temperature.

As shown in Figure 9 and 10, the optimum pH was found to be 8 and the optimum temperature was found to be 40°C.

Extraction and purification of enzyme

The optimum ammonium sulfate fractionation (40% (w/v) saturation) showed 4.76 fold increase in the specific activity of the purified extract as compared to the crude extract (Table 1). The specific activity was 106.9(units/mg prot/ml-1). Safey *et al* reported that the purification of protease resulted in an enzyme with specific activity of 6381.75 (units/mg prot/ml^{-1}) with purification folds 7.87 times.[3]

Effect of temperature and pH on partially purified enzyme

The effect of temperature on the activity of the purified protease is shown in Figure 12. The optimum temperature for activity of partially purified protease enzyme was 40ºC, while the temperature below or above 40ºC exhibited lower activities of protease. 45°C was the optimum temperature of the extracellular proteinase (PSCP) produced by *Pseudomonas cepacia* as reported earlier.[21] In addition to that, Lee *et al* reported that the optimum temperature of purified protease was within the range of 40ºC to 50ºC.[22]

Application of protease in contact lens cleansing

In order to study the effectiveness of *B. subtilis* protease in removing proteinaceous deposits and debris from contact lenses, artificial tear solution was prepared. It was treated with crude enzyme and purified enzyme separately. The spectrophotometric analysis indicated that before treatment % transmittance was 75% and after addition of crude and purified enzyme it was 92 and 98% respectively (Table 2).

The increased transmittance indicated that the enzyme had potential in the removal of protein deposits from contact lens. Similarly, a post treatment transmittance of control (untreated solution) using phosphate buffer was 75%, indicating no protein degradation. The optimal time for the degradation was 30 and 60 min. It was previously reported that the optimal time for protease from Bacillus sp. 158, for contact lens cleansing was 60 min.[6] Greene *et al* reported that the enzyme from marine bacterium degraded lysozyme, the major protein contaminant of contact lens.[23]

Conclusion

- It was found that *Bacillus subtilis* produced maximum protease when incubated at 37°C under shaker conditions.
- The optimum pH of the medium for maximum production of protease was found to be 7.5.
- The optimum ammonium sulfate fractionation (40% (w/v) saturation) showed 4.76 fold increase in specific activity of the purified enzyme. The specific activity of the purified enzyme was 106.9 (units/mg protease/ml^{-1}).
- The optimum pH and temperature for both enzymes was found to be 8 and 4^{0o}C respectively.
- The crude and partially purified enzymes were tested for the application in treatment of protein deposits on contact lenses. Protein solution was degraded by purified enzyme in 30 min where as crude enzyme required 60 min.
- In this work, the activity of protease on artificial tear solution was checked. Further studies can be directly carried out on used contact lenses.
- The bacterial protease enzyme can be used along with chemical disinfectants to clean the contact lenses.

- The present study achieves reducing the production cost, increasing the products quantity and overall profit.
- Although, *Bacillus subtilis* shows a very high potential for commercial production of protease, a lot of research and development studies have to be carried out on used contact lenses.

Authors Contributions

All the authors take full responsibility of the content of the paper and contributed equally to the work. All the authors read and approved the final manuscript.

Acknowledgement

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Table 1. Extraction and purification of protease enzyme

Stage of purification		Total protease units	Total protein (mg)	Specific activity (mg^{-1})	Fold of purification	% yield
Crude (0%)		2798.10	125.664	22.26	1	100%
20%	*Ppt*	190.74	7.075	26.8	1.2	6.8%
	Sup.	2096.6	100.632	20.8	0.93	74.9%
40% ppt		1657	15.527	106.9	4.76	59.22%

Table 2. Data of transmission (%) and protein degradation

Protein solution	Transmittance (%)				
	0 min	10 min	30 min	60 min	90min
Phosphate buffer as blank	100	100	100	100	100
Untreated solution	75	75	75	76	75
Treated solution with crude enzyme	75	80	88	92	92
Treated solution with purified enzyme	76	89	95	96	98

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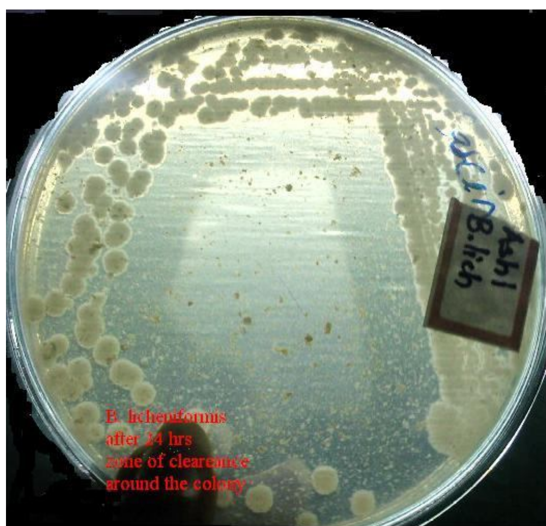

Figure 1. *Bacillus licheniformis* after 24 hrs on BH agar containing 1% casein

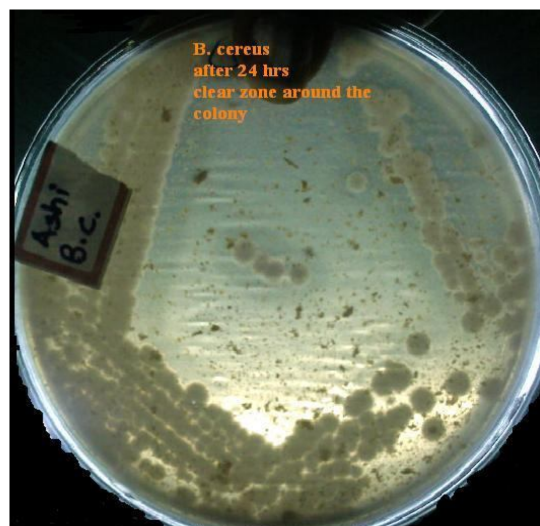

Figure 2. *Bacillus cerus* after 24 hrs on BH agar containing 1% casein

Figure 3. *Bacillus subtilis* after 24 hrs on BH agar containing 1% casein

Figure 4. *Bacillus thermophilus* after 24 hrs on BH agar containing 1% casein

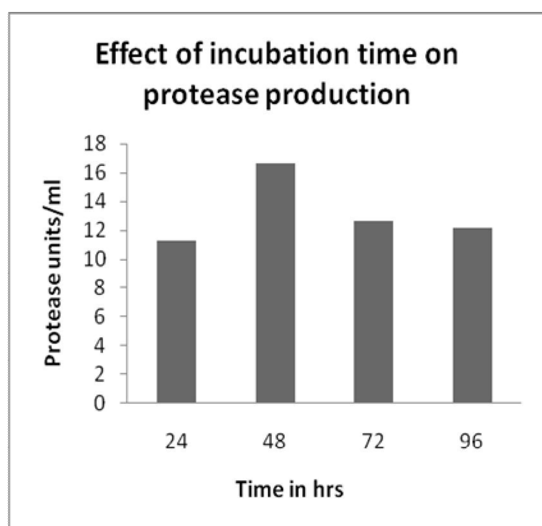

Figure 5. Effect of incubation time on protease production

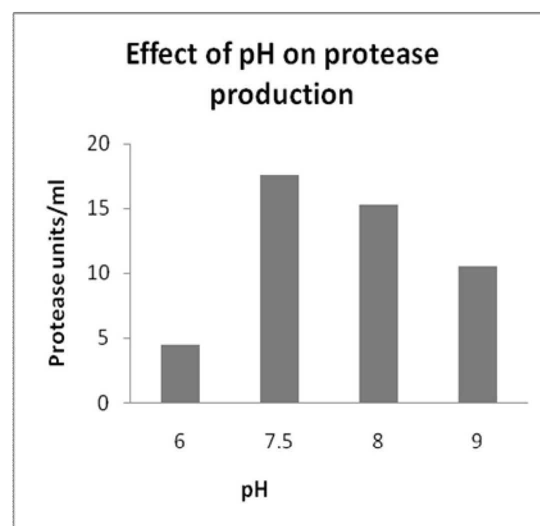

Figure 6. Effect of pH on protease production

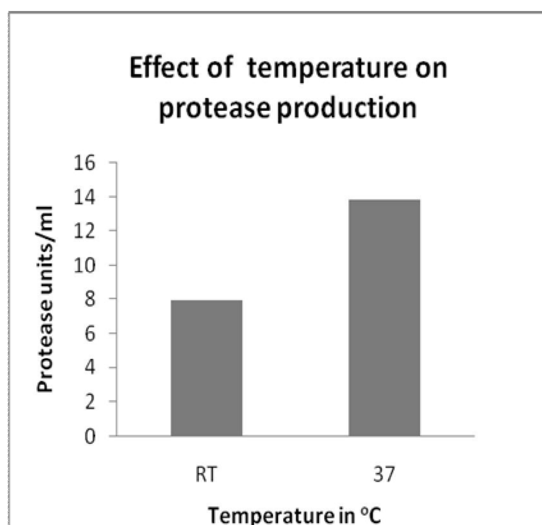

Figure 7. Effect of temperature in protease production

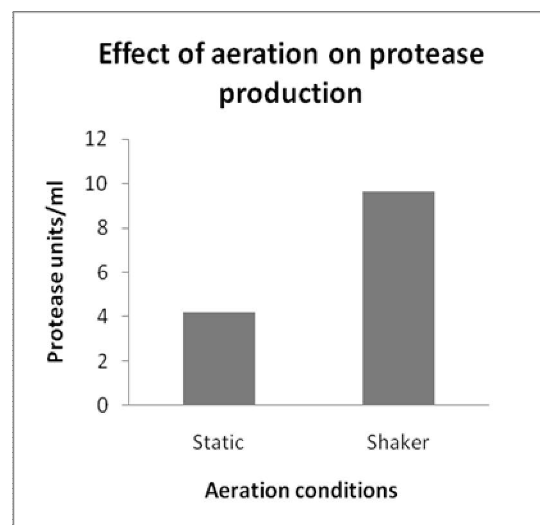

Figure 8. Effect of aeration conditions on protease production

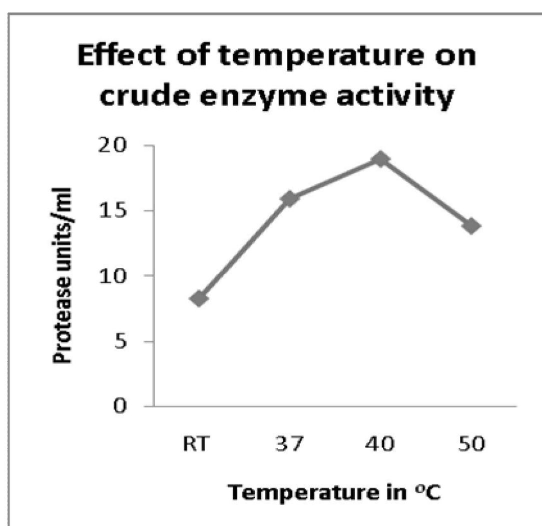

Figure 9. Effect of temperature on Crude enzyme activity

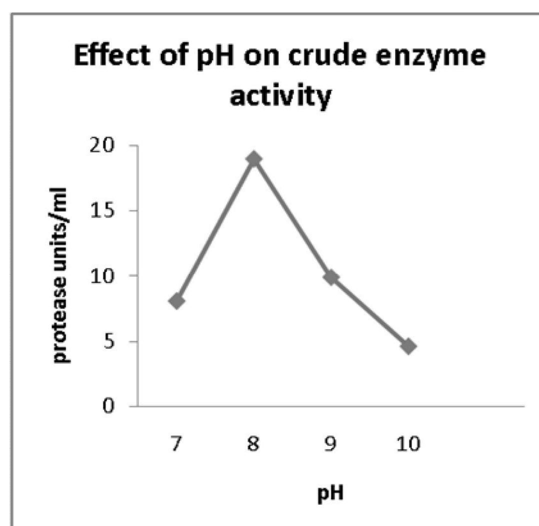

Figure 10. Effect of pH on crude enzyme activity

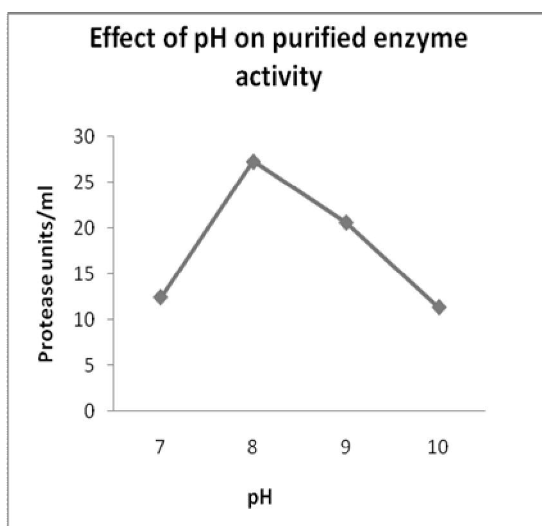

Figure 11. Effect of pH on partially purified enzyme

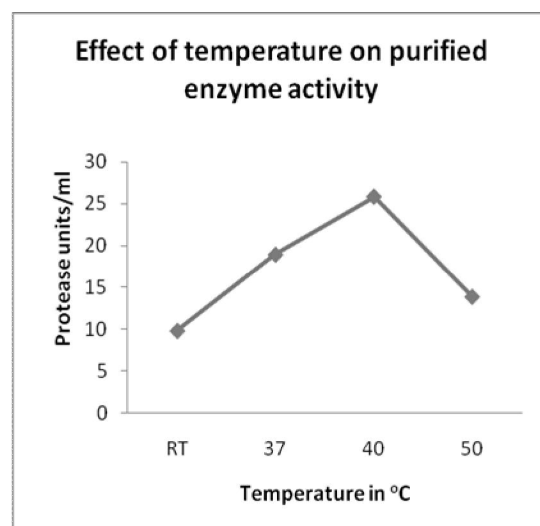

Figure 12. Effect of temperature on partially purified enzyme