Purification and Characterization of Protease extracted from *Bacillus licheniformis* (B1)

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Abstract

Protease was purified by two steps included precipitation with 70% saturation ammonium sulphate and ion exchange chromatography by (DEAE-Sephadex). The enzyme specific activity was 86.6 U/mg. and fold of purification was 2.72 with 45 % enzyme recovery. Characterization of enzyme showed that the optimum pH for enzyme activity and stability was 9.0 (86 U/ml). Maximum enzyme activity appeared at 50°C. (88U/ml.). The enzyme activity remained was 87% at 50°C for 30 min. Effect of some metal ions, reducing agents and chelating on purified protease was studied, the remaining activity was 110% when it incubated with 10 mM of Mn^{2+}, whereas the remaining activity was 95% and 92% when enzyme incubated with 10mM of Ca^{2+} and Mg^{2+}, respectively. There was little effect for 10 mM of PMSF on enzyme activity, whereas the activity declined to 18% when enzyme was treated with 10 mM of 10 mM EDTA .

Keywords: Protease, Purification, Characterization, *Bacillus licheniformis*.

تنقية وخصائص انزيم البروتييز من بكتريا *Bacillus licheniformis* B1

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الخلاصة :

نقي الانزيم بخطوتين تضمنت الترسيب بكبريتات الامونيوم بنسبة تشبع 70% ثم كروموتوغرافيا التبادل الايوني بأستخدام عمود DEAE–Sephadex، وكانت الفعالية النوعية للانزيم المنقى (86.6U/ml.) و بعدد مرات تنقية 2.72 وبحصيلة انزيمية مقدارها 45%. تمت دراسة توصيف الانزيم المنقى، وأظهرت النتائج بان افضل فعالية انزيمية وثباتية كانت عند الرقم الهيدروجيني 9 (86U/ml.)، واظهرت اقصى فعالية للانزيم عند درجة حرارة 50°م (88U/ml.) واحتفظ الانزيم بـ(87%) من فعاليته عند نفس درجة الحرارة لمدة 30 دقيقة. درس تأثير بعض الايونات الفلزية والمواد المختزلة والكلابية على فعالية الانزيم المنقى، ولوحظ زيادة في في فعالية الانزيم بحدود 110% عند حضنه مع ايون Mn^{2+} بتركيز 10 ملي مولار بينما كانت الفعالية المتبقية للانزيم عند حضنه مع نفس التركيز من ايوني Ca^{2+} و Mg^{2+} هي 95% و 92% على التوالي، كما لوحظ تأثير قليل لمادة PMSF بتركيز 10 ملي مولار في حين انخفضت النسبة المؤية للفعالية الى 18% عند معاملة الانزيم بمادة EDTA بتركيز 10ملي مولار.

الكلمات المفتاحية: انزيم بروتييز ، تنقية، توصيف، *Bacillus licheniformis*

INTRODUCTION

Proteases or Peptidases are enzymes that catalyze hydrolytic reactions (hydrolysis peptide bond) in which protein molecules were degraded to peptides and amino acids. According to the Enzyme Commission (EC) classification, proteases belong to group 3 (hydrolases), and sub-group 4 (which hydrolyse peptide bonds) (1).They are necessary for the survival of all living creatures, and they are encoded by about 2% of genes in all kinds of organisms (2). They are important for many biological processes and the versatility of proteases ranging from being the major armour of protein degrading saprophytes to the signal sequence cleaving peptidase enzymes of higher organisms, clearly illustrates their influence in the biosphere (3).Proteases have found a wide range of applications in various industries such as food, pharmaceutical, cosmetic, *etc.* and have been widely commercialized by various companies throughout the world (4).The metabolic diversity of *Bacillus licheniformis* has led to its exploitation in a variety of bio-industrial processes, including production of enzymes (5). It is considered as one of the examples of thermophilic bacteria belongs to *Bacillus* genus (6). Its optimal growth temperature is around 50-55°C (7). Researches interested in these organisms has increased due to their biotechnological potential, especially as sources of thermostable enzymes (8). The advantage of using enzymes from thermophilic microorganisms is that the thermostability of such proteins at high temperatures is an intrinsic property (9). The alkaline proteases resulting mainly from *Bacillus licheniformis* were used in detergents and occupy a large portion of the market (10). This study was aimed to isolate locally highest protease producer *Bacillus* and purified to used in many applications and economical advantages

MATERIALS AND METHODS

All experiments achieved in duplicate.

Extraction of enzyme

B.licheniformis cultured under optimum conditions composed (soluble casein 0.5 g, yeast extract 0.5g, glucose 0.1g, KH_2PO_4 0.02g, K_2HPO_4 0.02g, $MgSO_4.7H_2O$ 0.01g and D.W, and incubated for 48h. at 37 ˚C (11). The enzyme was extracted by extracted by cooling centrifuge at 3000rpm for 45min, the enzyme activity and protein concentration were assayed in the supernatant (crude enzyme) and specific activity was measured.

Assay of Protease Activity

Protease activity was determined spectrophotometrically according to method of Anson (12) with some modification. Enzyme extract solution 0.2 ml was incubated with 1.8 ml casein solution at 40°C for 15min. The blank, consisted of 1.8 ml casein solution and 3.0ml 5% TCA (trichloroacetic acid) and 0.2 enzyme solution. The reaction was stopped by the addition of 3.0ml 5 % trichloroacetic acid and incubated at 25°C for 10 min. The mixture was centrifuged for 10min. then supernatant was separated. Quantity 2.5ml of 0.5M Na_2CO_3 solution was added to 1 ml of the supernatant, and 1ml Folin–Ciocalteus reagent was added and Incubated at 37°C for 20 min. The absorbance (O.D.) was measured at 600 nm for solution.One unit of protease activity was defined as the amount of enzyme required to liberate one µg tyrosine per minute per ml under assay conditions.

Purification of enzyme

1. Precipitation of enzyme with ammonium sulfate

The crude protease solution was precipitated with different concentration of ammonium sulfate (40% - 80%) saturation under cooling condition, the precipitates were separated by centrifugation at 3000 rpm for 45 min and dissolved in small amount of buffer 0.2M Tris- HCl. The final volume of solution, activity of enzyme and protein concentration were measured and specific activity was calculated.

2. Dialysis of enzyme

The precipitated protein solution was dialyzed against 0.2M of Tris- HCl buffer over night at 4°C. The dialyzed enzyme was concentrated with sucrose and kept at 4°C for the next step of purification.

3. Ion exchange chromatography by DEAE-Sephadex

The enzyme solution (3ml.) was loaded on DEAE-Sephadex column (2.5x20cm). The column was washed with 0.2M Tris-HCl buffer pH 8.0 and eluted with gradient (0.1-03M) NaCl solution at flow rate of 30ml/h. Fractions of 5ml./tube were collected and the optical density at 280nm. was measured. The fractions with high protease activity were collected, volume, enzyme activity and protein concentration were estimated..

Characterization of purified protease.

Determination of optimum temperature for protease activity.

Quantity 1.8 ml of reaction solution was incubated at different temperature (30, 37, 40, 50, 60 and 70 °C) 0.2 ml of enzyme was added to reaction solution at each temperature and incubated for 15 min and then the enzyme activity was assayed.

Determination of protease stability at different temperatures.

One ml of purified protease was incubated in water bath at 30, 40, 50, 60, 70, 80, 90, and 100°C for 30min, and immediately transferred into an ice bath. The enzymatic activity was measured and the remaining activity was calculated percentage and plotted against the temperature. The remaining activity was estimated according to the following equation:

$$\text{Remaining activity (\%)} = \frac{\text{Activity of enzyme after treatment}}{\text{Activity of enzyme before treatment}} \times ١٠٠$$

Determination of optimum pH of protease activity.

Reaction solution was prepared at different pH values (6.0-11.0). 0.2 ml enzyme solution was mixed with 1.8 ml reaction solution and incubated at 50 °C for 15 min then enzyme activity was assayed.

Determination of protease stability at different pHs.

Equal volumes of purified enzyme and buffer solutions with pH range (6 to 11) were incubated at a 25ºC for 30 min. and cooled in ice bath. The enzymatic activity for each treatment was measured and the remaining activity (%) for protease was calculated.

Determination of metal ions and inhibitor effects on protease activity.

One ml. of purified enzyme was mixed with 1ml of 20 mMmetal ions solution $CaCl_2$, $MnCl_2$ and $MgCl_2$. EDTA solution and PMSF, then incubated at 25ºC. for 30 min. The final concentration of each one was 10 mM 0.2ml. of each enzyme solution mentioned earlier was mixed with 1.8ml. of reaction solution and incubated at 50°C for 15min. Then enzyme activity was assayed and remaining activity was calculated.

Statistical Analysis.

The Statistical Analysis System- SAS (13) was used to determine the significant differences between the different parameters. LSD test (Least Significant Difference). $P \leq 0.05$ was applied to the compare between means.

RESULTS AND DISCUSSION

Purification of protease.

Ammonium sulphate precipitation was achieved using different percentages of saturation ratio

ranging between (40% -80%) to concentrate the protease produced by *B. licheniformis*B1. Result in Figure (1) showed that specific activity of protease increased gradually with increasing of saturated percentage up to (10.77U/mg) at 70% concentration. Fold of purification was 1.76 with 52.9% recovery. Salting out using ammonium sulfate is one of the classical methods in protein biochemistry. Formerly it was widely used for the fractionation of proteins, it rather used as an inexpensive way of concentrating a protein extract (14).

Ammonium sulfate is favored in precipitation step due to its high solubility, availability, being cheap and it does not damage most enzymes (15).Ahmed, *et al*., (16) isolated protease from *Bacillus subtilis* and precipitated at 70% saturation with specific activity of 55.71 U/mg and 1.11 fold purification.

Enzyme solution was applied to DEAE- Sephadex column and washed with 0.2M trisHCl buffer pH8.0 to remove uncharged and positively charged proteins in enzyme solution. The bound proteins were eluted using gradient concentration of (0.1-0.3) M. sodium chloride. The result showed two protein peaks in the eluted fractions, only the first peak represented protease, located at fractions 11-17 which eluted with 0.2M sodium chloride solution (Figure 2). The result indicated that protease has a negative net charge since it bounds with anionic ion-exchange. The specific activity increased in this step to 16.6 U/mg proteins, with 2.72 fold of purification and 45% recovery (Table 1).Chromatography has become an essential tool in biochemestry laboratory as, protein purification is widlyneeded (17). Ion exchange chromatography is prefer in protein purification, it can distinguish between two proteins different with one amino acid (18).This technique uses materials such as Sephadex or cellulose which have high capacity for bioseparation, easy to prepare, multiple use, in addition to simplicity to separate different biomolecules, principle which depending on charge difference (19).Akel, *et al*., (20) used DEAE-Sepharose as a final step for purification of thermostable protease from *Bacillus* strain HUTBS71, with 59 fold of purification and 1.7% recovery. Banik and Prakash, (21) purified laundry detergent compatible alkaline protease from *Bacillus cereus* by anion exchange chromatography with 5.32 fold of purification and 53.61% recovery.

Characterization ofpurified protease

Effect of temperature on protease activity.

Protease activity was assayed at different temperatures ranging from 30°C to70°C (Figure 3). The results showed that enzyme activity increases with temperature increasing within the range of 30 °Cup to 50 °C the maximum enzyme activity (88u/ml) at 50 °C, a reduction in enzyme activity was observed above 50°C. The decrease in enzyme activity at high temperatures due to the destruction of enzyme or changes in its tertiary structure (22). The lowoptimal temperature for protease activities is desirable for detergent formulations for washing at normal temperatures (23). Similar reports were obtained for Bacillus sp. MIG (24) and Bacillus sp. CEMB10370 (25). Sangeetha, et al. (26) reported that protease from B. licheniformis VSG1 exhibited maximum activity at 45°C. While, alkaline protease from B. mojavensis was optimally active at 60°C.with rapid loss of activity above 65°C (27). However, this result gives the protease an economic property since it can be used as

catalyst in a wide range of temperature.

Effect of temperature on protease stability.

The thermostability of the protease was examined. The results indicated that the enzyme was nearly stable at a temperature 30, 40 and 50°C for 30min. the remaining activity was 95%, 95% and 87% respectively, while a significant reduction in enzyme stability was observed above 50°C.(Figure4). This indicating that *B. licheniformis* B1 protease is moderately stable at hightemperature and hence it remains active at temperature above the normal or physiological

temperature, this property make the enzyme at high temperature. Relative activity of protease enzyme produced by *B.clausii* was reported to be 100% after incubation the enzyme at temperature ranging from 30°C to 65°C for 60 min. (28). Akel, *et al*. (20) found that protease from *Bacillus* strain HUTBS71was stable after incubation at 50ºC. and 60ºC. for 2h. and retained 84% of its original activity.

Effect of pH on protease activity

The pH range from 6.0 to 11.0 was used to study the effect of pH purified protease activity (Figure 5). The results showed that protease is active in a wide range of pH (6.0-11.0) but it is more active at pH 9.0 than other value. The activity was (86U/ml.). The pH effect on enzyme activity in different ways; on the ionization of groups in the enzyme's active site, on the ionization of groups of substrate, or by affecting the conformation of either the enzyme or the substrate (14), which could explain the decrease in the activity value of pH 6.0 and 11.0.The present result was in line with the findings obtained for the optimum pH for enzymatic activity of other *Bacillus* species: for *B. cereus* KCTC 3674 (29). Olajuyigbe and Ajele.(30) reported protease from *B.licheniformis* Lbbl-11 shows the optimum pH 8.0. However,different finding reported by Chantawannakul*et al*. (31) who isolated *Bacillus* from fermented soybean they found the optimum pH for protease was 6.

Effect of pH on protease stability.

The purified *B. licheniformis*B1 protease was incubated in different pH (6.0-11.0) for 30min, the results showed that protease has good stability in pHs 8 - 10 with highest remaining activity at pH 9 (83%) (Figure 6). The activity decreased slightly at pHs 10 and 11 the remaining activity were 71% and 61% respectively, whereas more than half of enzyme activity lost at pH6.

This result may give a conclusion that the protease of *B. licheniformis*B1 is more stable in alkaline pH than neutral and acidic pH. The effect of pH on the enzyme stability could be explained in the formation of improper ionic form of enzyme or the active sites and irreversible inactivation (32). Generally, most of the commercial available alkaline proteases are active in the pH and temperature range between 9.0–12.0 and 50–60°C, respectively (27). The effect of pH on stability of alkaline protease from *B.licheniformis* LBBL-11 showed that the enzyme had optimum pH for stability at 7.0 it retained 86% of its activity at pH 11.0 (30).

Metal ions and inhibitor effects on protease activity.

Results mentioned in table (2) showed that the protease activity increased when incubated with 10mM. Mn^{2+} while Ca^{2+} and Mg^{2+} slightly decreased protease activity at the same concentration. From these results it can be concluded Mn^{2+} was the most effective ion since it increase the remaining activity to (110%) Ca^{2+} and Mg^{2+} nearly have no effect on enzyme they gave 95% and 92% respectively. Suggesting that metal ions had a capability to protect enzyme against denaturation.Where, Akel*et al*., (20).mentioned that these metal ions protected the enzyme from thermal denaturation and maintained its active conformation at the high temperature. Some protease required a divalent cation like Ca^{+2} and Mn^{+2} or combination of these cations for its maximum activity (33).

Furthermore, these cations may enhance the stability of a *Bacillus* protease (34). Shaheen*et al*., (22) found that strong inhibition or stimulation by metal ions in case of protease activity, was not observed. The results revealed a slight decrease in protease activity occurred after incubation with 10mM. phenyl methyl sulfonylfloride, while it lost most of its activity after incubation with 10mM. EDTA (chelating agent). This mean that the protease produced by *B. licheniformis* B1 is

not serine protease but it is metallo-protease which require metal ions for its activity removal of metal ions from enzyme structure leads to entire loss enzyme activity (35). *Bacillus* are known to secrete two major types of proteases, serine and metallo-protease, both having application in industries (25). The results of Akel*et al*., (20) in their study on protease from *Bacillus* strain HUTBS71 indicated that the presence of 1mM EDTA had slight inhibitory effect on protease activity. According to the report of Arulmani*et al*. (4) EDTA mild inhibitory effect was observed on serine protease from thermostablealkalophilic*Bacillus laterosporus*-AK1.

CONCLUSION

Ammonium sulphate at 70% saturation was used to precipitated the protease enzyme producedfrom *B. licheniformis*B1. Enzyme was purified by Ion exchange chromatography by DEAE-Sephadex. The net charge of enzyme was negative. The enzyme was active with broad rang of pH an temperature. This enzyme mostly, metallo-protease and Mn^{+2} enhance its activity.

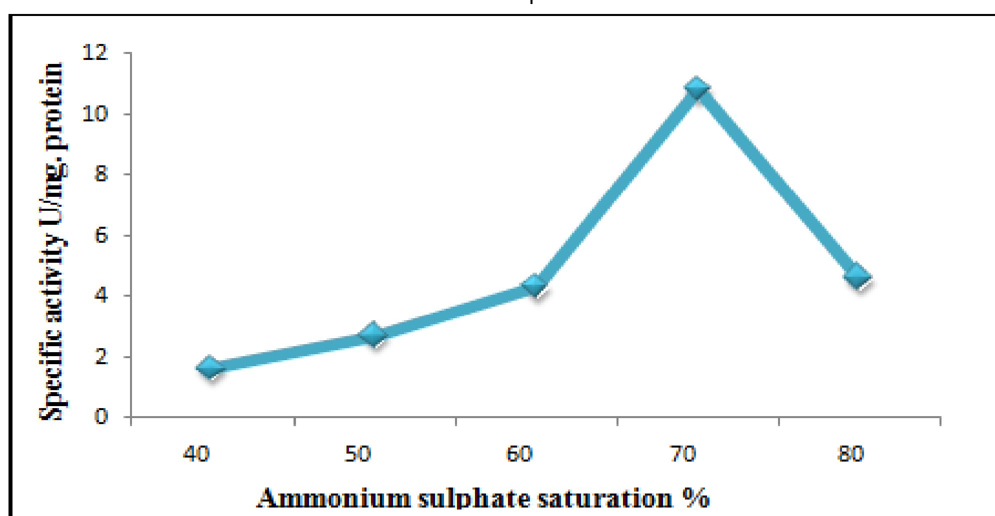

Figure 1: Specific activity of *B. licheniformis* B1 protease after precipitation with ammonium sulphate.

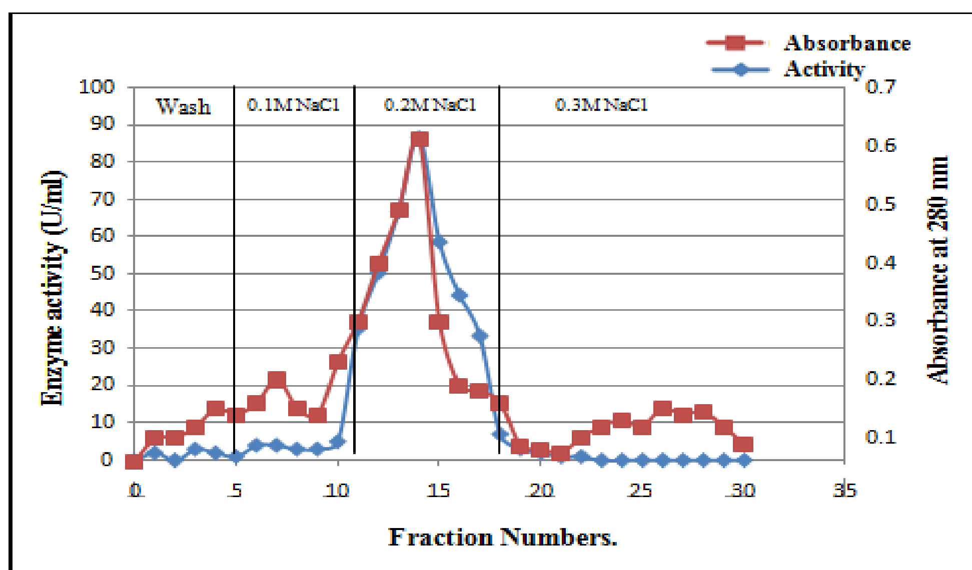

Figure2: Purification of protease produced by *B. licheniformis* (B1) using ion exchange chromatography DEAE-Sephadex column (2.5x20)cm. the fraction were collected with 5ml/tube at flow rate 30ml/hr and eluted with (0.1-0.3)M NaCl solution.

Table (1): Purification steps of protease produced from *B. licheniformis (B1)*.

Steps	Volume (ml)	Enzyme Activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Total activity (U)	Fold of purification	Yield %
Crude	**80**	**43.3**	**7.1**	**6.1**	**3464**	**1**	**100**
70% $(NH4)_2SO_4$ precipitation	**25**	**73.3**	**6.8**	**10.77**	**1832**	**1.76**	**52.9**
Ion-exchange DEAE .Sephadex	**18**	**86.6**	**5.2**	**16.6**	**1559**	**2.72**	**45**

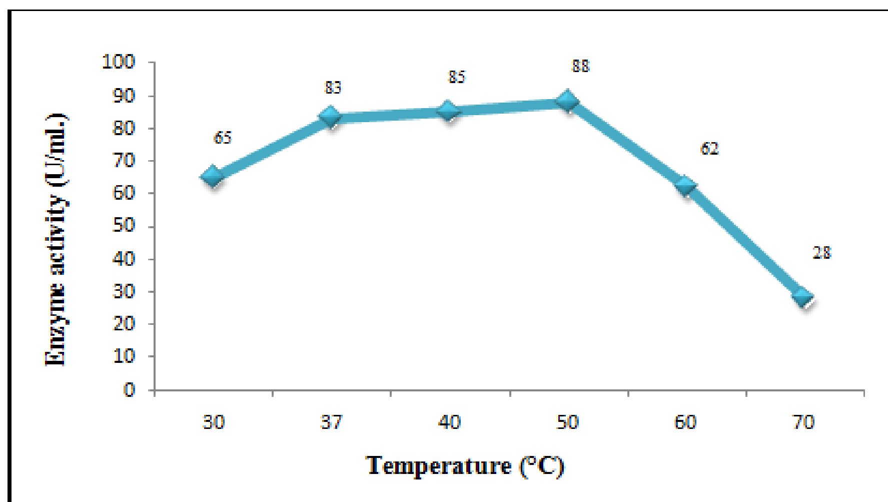

Figure 3: Effect of temperature on purified *B. licheniformis* B1 protease activity. [LSD Value: 0.05 = 12.64]

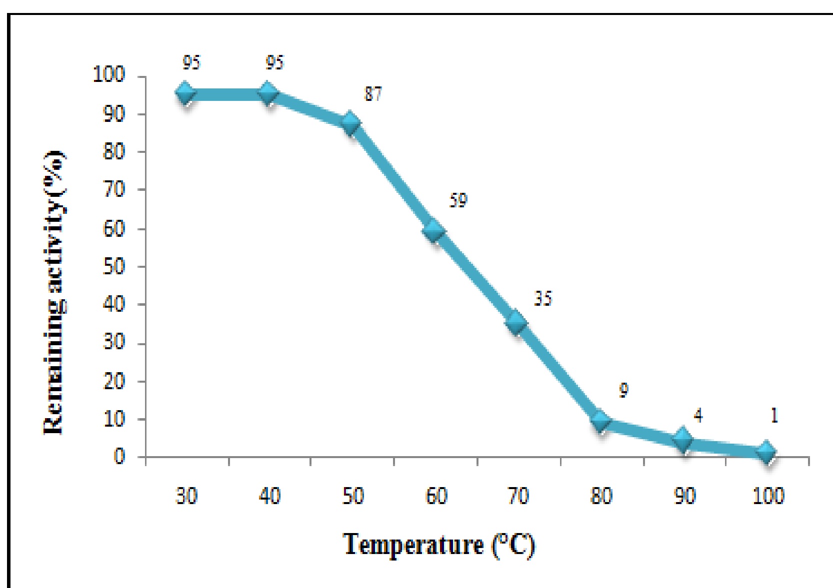

Figure 4: Effect of temperature on purified *B. licheniformis* B1 protease stability. [LSD Value: 0.05 = 13.75]

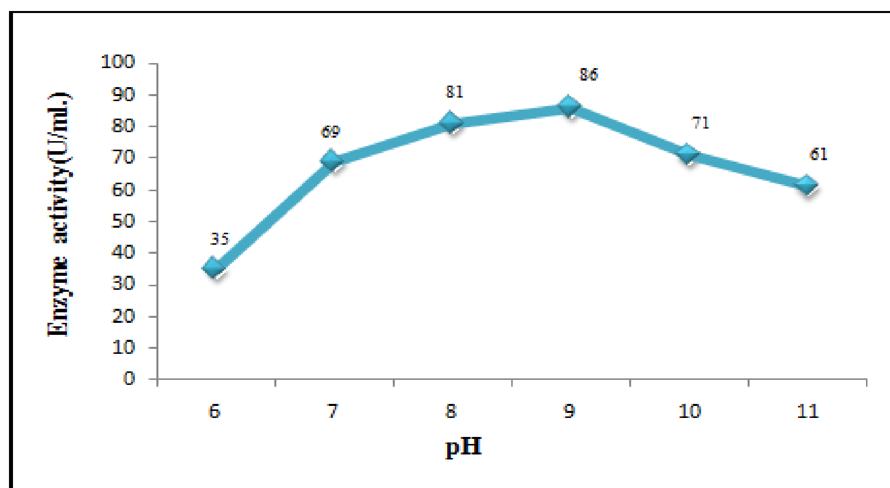

Figure 5: Effect of pH on purified *B. licheniformis* B1 protease activity. [LSD Value: 0.05 = 8.592]

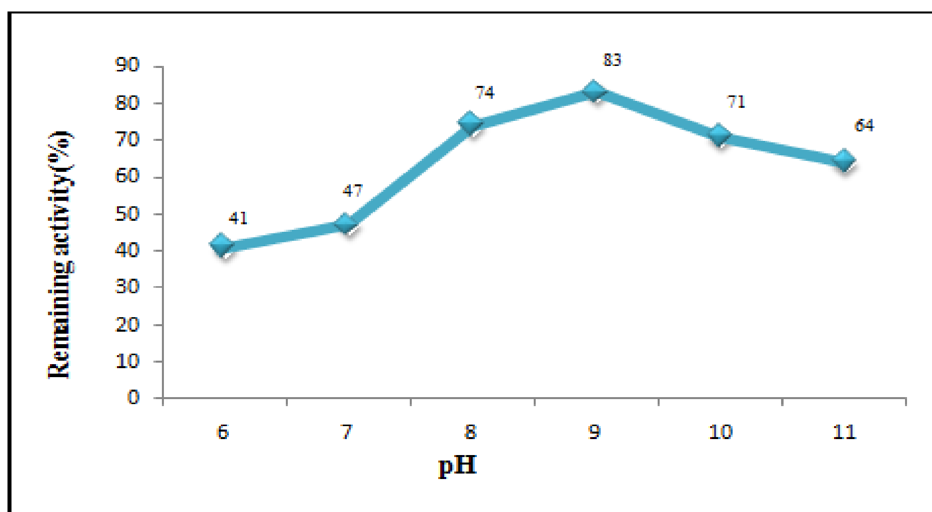

Figure 6: Effect of pH on stability of purified *B. licheniformis* B1 protease. [LSD Value: 0.05 = 6.334]

Table (2): Effect of metal ions and inhibitors on protease activity produced by *B. licheniformis* B1.

Reagent	Concentration (mM)	Remaining activity (%)
$CaCl_2$	10	95
$MnCl_2$	10	110
$MgCl_2$	10	92
PMSF	10	78
EDTA	10	18
Control	---	100
LSD Value : 0.05	---	5.292 *

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