Research Article

Purification and Characterization of Fibrinolytic Enzyme Produced from *Bacillus lichniformis* B4

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Abstract

Forty –five local isolates belongs to the genus Bacillus were selected for production of fibrinolytic enzyme (E.C. 3.4.). The isolate *Bacillus lichniformis* B4 was selected due to its high productivity of fibrinolytic enzyme. The protease enzyme was purified by ammonium sulfate precipitation, ionic exchange with DEAE-cellulose and Sephacryl S-200 filtration. A trial for the purification of protease resulted in an enzyme with specific activity of 32.52 unit/mg protein with purification folds 30.11times. An optimum incubation temperature was 37 ºC. Purified protease enzyme had a maximum activity at pH 7.0 of phosphate buffer. The fibrinolytic enzyme produced by *B. lichniformis* B4 keep it's total activity when preserve it in (20-40) ^{0} C for 1 hour. The molecular weight of the fibrinolytic enzyme was 50118 Dalton, Km and Vmax values of purified fibrinolytic enzyme were (100 mM) and (200 mM/min^{-1}) respectively.

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Introduction

A protease enzyme hydrolyses the peptide bonds that link amino acids together in the polypeptide chain forming a protein molecule. Proteases are essential constituents of all forms of life on earth, including prokaryotes, fungi, plants and animals. It can be cultured in large quantities in a relatively short time by established methods of fermentation and they also produce an abundant, regular supply of the desired product. Alkaline proteases are defined as those proteases that are active in a neutral to alkaline pH range. They are either a serine protease or a metalloprotease (Bhunia *et al*., 2010).

Alkaline proteases enjoy a big share of the enzyme market with two thirds of share in detergent industry alone (Anwar and Saleemuddin, 2000). Besides they are also used in leather, food and textile industries; organic synthesis and waste water treatment (Kumar and Takagi, 1999). Microbial alkaline proteases occupy nearly 40% of the total worldwide enzyme sales and use of protease in detergent industry accounts 25% of the total worldwide sales of enzyme (Rao *et al.*, 1998). A micro-organism is excellent source of enzyme, better than plants or animal due to their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation (Patel *et al.*, 2005). Alkaline proteases can be produced from bacteria, fungi and yeast using fermentation technique (Bhunia *et al*., 2010). Although there are several microbial alkaline proteases producers, only a few are considered industrially useful (Gupta *et al.,* 2002). Gram-positive bacteria, especially the genus *Bacillus* are considered an important commercial enzyme producer of proteases. Of them, *Bacillus lichniformis, Bacillus subtilis, Bacillus alcalophilus, Bacillus lentus,* for instance, are industrial protease producers (Gupta *et al.,* 2002).

The human body produces several types of enzymes for making thrombus, but only one main enzyme for breaking it down and dissolving it - plasmin. The properties of nattokinase closely resemble plasmin. According to Dr. Martin Milner, from the Center for Natural Medicine in Portland, Oregon, what makes nattokinase a particularly potent treatment, is that it enhances the body's natural ability to fight blood clots in several different ways; Because it so closely resembles plasmin, it dissolves fibrin directly. In addition, it also enhances the body's production of both plasmin and other clot-dissolving agents, including urokinase (endogenous) (Sumi *et al.*,1990).The aim of this study is to purification and characterization of the fibrinolytic enzyme from local strain *Bacillus lichniformis* B4.

Materials and Methods

Bacterial culture

Bacillus lichniformis B4 was cultured in nutrient broth medium and incubated at 37°C for 24 hr.

Preparation of the production medium

Lentils were moisture with sodium phosphate buffer of pH 7 and inculcated with *Bacillus lichniformis* B4 for 48 hr at 37°C in shaking incubator then centrifuged in cooling centrifuge at 5000xg for 10 min. and determined enzyme activity and protein concentration.

Measurement of Fibrinolytic enzyme activity

Test was conducted in two tubes, one for the test and the other for control, The test was conducted by mixing 0.2ml of human plasma and 0.8ml of normal saline with 0.25 ml of 0.25% of calcium chloride, 0.5 ml of liquid bacterial culture or enzyme were added to the test tube and 0.5 ml of normal saline to the control tube then mixed and incubated for 30 min. The positive result is hemolytic the clot formed. One unit of fibrinolytic activity (U) was defined as the amount of enzyme releasing 1 µg of soluble protein equivalent per 30 min. (Al Shalji, 2001; Hassan, 2005).

Extraction of the fibrinolytic enzyme

Sodium phosphate solution 0.2M and pH 7 used to moisten lentils medium. 50 ml of this solution added to the lentils medium then centrifuged in cooling centrifuge at 5000xg for 10 minute and determined enzyme activity and protein concentration.

Purification of fibrinolytic enzyme:-

fibrinolytic enzyme was purified from crude extraction of *Bacillis lichniformis* as in the following steps:

Precipitation by ammonium sulfate:-

A specific weight of ammonium sulfate crystals was added to the crude enzyme gradually in an ice bath with continuous stiriling for 30 min to get saturation percentage of 0-25 and 25-50%, then centrifuged at 10000 rpm at 4ºC for 20 min, then the precipitate was taken and dissolved in minimal amount of 0.2M phosphate buffer, finally it had dialyzed against the same buffer for 24 hour.

Separation with ionic exchange by using diethyl Aminoethyl- Cellulose (DEAE- cellulose)

Ion exchange chromatography (DEAE-Cellulose) column was prepared according to previous reports (Whitaker and Bernhard, 1972). A column with a diameter 15 x1.5 cm was washed with equilibration 0.05M sodium phosphate buffer pH 7.2, partially purified fibrinolytic enzyme (10ml) was separately passed after loaded onto the column carefully. Then (100ml) of 0.05M sodium phosphate buffer pH 7.2 was added. Proteins were eluted by using (200ml) of a gradient NaCl concentration from (0.05-1.5M) phosphate buffer (pH 7.2). Fractions of (5ml) were collected and absorbance was read at 280nm. The presence of the fibrinolytic enzyme was estimated from each fraction of the major peaks, and then activity was determined for the collected active fractions.

Gel filteration Sephacryl S-200

Sephacryl S-200 column (54×1.5cm) was prepared and packed according to the instruction of the manufacturing company (Pharmica Sweden). The column was equilibrated with (0.1M) phosphate buffer (pH 7.2) at a flow rate of (60ml/hr).

A (4ml) sample of each concentrated partially purified fibrinolytic enzyme was added to the column. Elution of proteins was done with the application of (200ml) of (0.1M) phosphate buffer pH (7.2). A (5ml) fraction was collected of each enzyme then protein contents were estimated by measuring the absorbance at (280nm), the major peaks for each fibrinolytic enzyme were determined by plotting the absorbency of protein fractions versus the elution volumes.

Fibrinolytoc enzyme activity was determined for each fraction of the major peaks. Protein concentrations and specific activity was also determined for the collected fraction of the major peaks of the different enzyme.

Characterization of fibrinolytic enzyme

Determination of the molecular weight

Molecular weight was determined by gel filtration chromatography and the standard proteins for the standard curve and their relevant molecular weights were (Alkaline phospahate, 80000; Bovine serum albulmin, 67000; endoglucanase, 28000; catalase 232000 Dalton).

Determination of optimum pH for fibrinolytic activity

Buffers of different pH were prepared according to Deutscher (1990). To measurement of optimal pH for enzyme activity, 25 µl of the purified enzyme solution was mixed with 100 µl buffer of different pH, from 5-10 (each in a single test tube) separately. Tubes were incubated tubes at 37º C for 30 minutes and the activity of fibrinolytic activity was measured as described above.

Determination of optimum pH for the stability of fibrinolytic enzyme

Tested the stability of the pure enzyme at consistent temperature 37ºC using different pH as follows:
Prepare different buffer solutions with different pH values (5 to10). Add 25 µl of purified enzyme for each test tube, and incubate tubes in buffer solutions at different pH 5- 10 for 30 min at a temperature of 37º C. The residual fibrinolytic enzyme activity was determined according to the assay procedure.

Effect of temperature on activity and Stability of fibrinolytic enzyme

The effect of temperature on the fibrinolytic enzyme activity was determined by incubating the reaction mixtures at different temperatures ranging (20, 25, 30, 37, 40, 45, and 50) ºC. After incubation, fibrinolytic enzyme activity was assayed. The effect of temperature on the fibrinolytic enzyme stability was determined by pre-incubating the enzyme without substrate at different temperatures (20, 25, 30, 37, 40, 45, and 50) ºC. The residual fibrinolytic enzyme activity was determined according to the assay procedure.

Measurement of fibrinolytic enzyme kinetic constants

Different volumes of blood clot (0.25-200) ml prepared to calculate the initial velocity of enzyme reaction toward the substrate for each constant of substrate concentrations.

Estimate the value of K_m and V_{max} of the enzymatic reaction from the plot the relationship between initial velocity and substrate concentration according to Lineweaver-Burk Reciprocal plot and Hans-Woolf Plot (Segal, 1976).

Chemical mutation by using nitrosoguanidine Mutagenesis procedure:

According to Gerhardt *et al*., (1981) chemical mutation was done using nitrosoguanidine mutagenesis by growth *B. lichniformis* B4 bacterial strain to the mid logarithmic stage (ca. $5x10^8$ cell/ml) in nutrient broth. Centrifuge the pellet of cells, and resuspend the cell pellet in an equal volume of Tris-maleic buffer at pH 6.0. Added a freshly prepared solution of nitrosoguanidine (NTG) (1mg/ml) in sterile water) to the culture prepared to a final concentration of 0.02 µg/ml. incubate at 37 ºC for 30 min. Centrifuge the treated culture and resuspend the pellets in an equal volume of M56 minimal medium. The mutagenic *B lichniformis* isolate culture on solid lentils medium with pH 7.2 at 37 ºC and bacterial inoculum concentration 3.6×10^5 for 48 hour in shaking incubator. The enzyme extracted and estimate enzymatic activity.

Results and discussion

Extraction and purification of fibrinolytic enzyme

Produce the fibrinolytic enzyme, an analyst for the fibrin clot from bacillus *lichniformis* bacteria cultured on Solid lentils medium with pH of 7.2, temperature 37^0C and inoculums amount of 3.6×10^5 cell/g wet weights for 48 hour in shaking incubator. Phosphate buffer with pH of 7.2 was used for enzyme extraction then the extraction complete with cooling centrifuge, was obtained enzymatic activity of (18.766 U/ ml). The enzyme extraction from the solid media washing with appropriate buffer which decrease the enzymatic activity, and then get the crude extract by using centrifuge one of common methods used in many studies (Dubey *et al*., 2011).

Fibrinolytic enzyme purification steps

Ammonium sulphate precipitation

Ammonium sulfate used at different saturation rates (0-25 and 25-50%), was appointed rate of saturation of (25-50%) to precipitate the enzymatic crude extracted, as it specific activity was (4.64 unit / mg protein) with (4.29) folds and yield of (57.7) Table (1). It is noted that ammonium sulfate salts is the most widely used in the concentration of enzymes due to high solubility and low cost, compared with the other organic solvents and no effect on pH or the stability of the enzyme (Whitaker, 1972).

In other study the activity of precipitates in the crude enzyme supernatants with 30-80% saturations $(NH_4)_2SO_4$ was contrasted to preserve the fibrinolytic activity of fibrinolytic enzyme as much as possible, according to the activity assay, the precipitate formed in 30.60% saturation of $(NH_4)_2SO_4$ was collected (Wang *et al.*, 2009).The fibrinolytic enzyme were also purified by ammonium sulfate saturation. The protein fraction was precipitate with 85% ammonium sulfate (Dubey *et al.*, 2011).

Table 1 The ammonium sulphate precipitation of fibrinolytic enzyme produce by *B. lichniformis B4.*
Ionic Exchange Chromatography

Ammonium sulphate (%)	Activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg of protein)
0-25	30.59	8.29	3.69
25-50	95.90	20.66	4.64

Ionic Exchange Chromatography

The method of ion exchanger is one of appropriate separation method, with which can distinguish two types of proteins the difference between them a single amino acid. Concentrated protein from ammonium sulphate precipitation step passed to the ion exchanger column (DEAE cellulose) a negative exchange (Anion exchange). which preceded budget with solution phosphate buffer (0.05 Molar and pH 7.0) absorbance was measured for washing portions at 280 nm and upon the arrival of absorbance to the line of zero (line base), after that recovery of proteins operation associated with the ionic exchanger conducted (proteins that carry a negative charge).The recovery was by using a phosphate buffer solution 0.05 Molar pH 7.0 saline gradient NaCl (0-1.5M). Ionic exchange chromatography results show that two peaks appeared when reading absorbance at wavelength of 280nm **Figure 1**, when tested enzyme efficiency in the resulting parts enzyme effectiveness recorded in parts (45-85) enzymatic activity reaching 96.1 U/ml in parts (50-60) .

The proteins that carry charges on the surface increases the ability to link with opposite charged surfaces may be so severe that it is difficult separate it, Therefore, increase the concentration of salt in a recovery buffer solution helps to separate the protein (Lee *et al.*, 2002).

The obtained ammonium sulfate precipitate (in solution) was introduced into special plastic bag for dialysis against crystals of sucrose until it reach to volume of (1 ml) and kept in the refrigerator at 4ºC for further purification as mention by Ellaiah and Srinivasulu (1996).

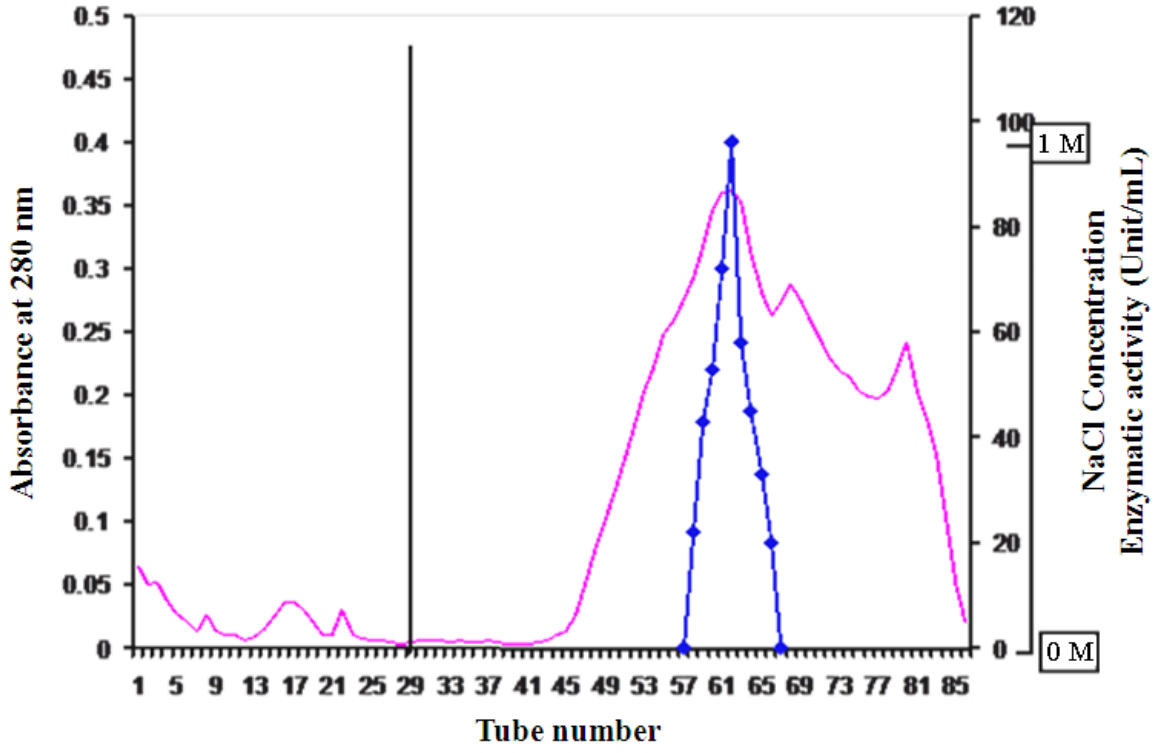

Figure 1 Ionic Exchange Chromatography for fibrinolytic enzyme from *B. lichniformis* through DEAE-cellulose column (15×1.5) cm. the column was calibrated with 0.05M phosphate buffer pH 7.0; flow rate 60ml/h and 5 ml fraction.

Gel Filtration

Protein resulting from the previous step passed in the gel filtration column using, two peaks where appeared when reading absorbance at wavelength of 280 nm and when testing for enzymatic activity in resulting parts, enzymatic activity recorded in (23, 27), the specific activity reached 32.52 unit/ mg as mentioned in **Table 2** and **Figure 2.** Ali and Ibrahim (2008) considered that ionic exchange chromatography and gel filtration were main steps in fibrinolytic enzyme purification. In other study a novel fibrinolytic enzyme from *Rhizopus chinensis* was purified through ammonium sulfate precipitation, hydrophobic interaction, ionic exchange, and gel filtration chromatography.

Table 2 Purification of fibrinolytic product from *B. lichniformis* B4.

purification steps	Volume / ml	Activity U/ml	Total activity (Unit)	Protein concentration (mg/ ml)	Specific activity (U/ mg protein)	folds	Yield (%)
Crude extract	200	18.77	3153.2	17.45	1.08	1	100
Ammonium sulfate	19	95.9	1822.1	20.656	4.64	4.29	57.7
DEAE-Cellulose	15	96.1	1441.5	7.33	13.11	12.14	45.72
Sephacryl S-200	15	94.322	1414.83	2.9	32.52	30.11	44.86

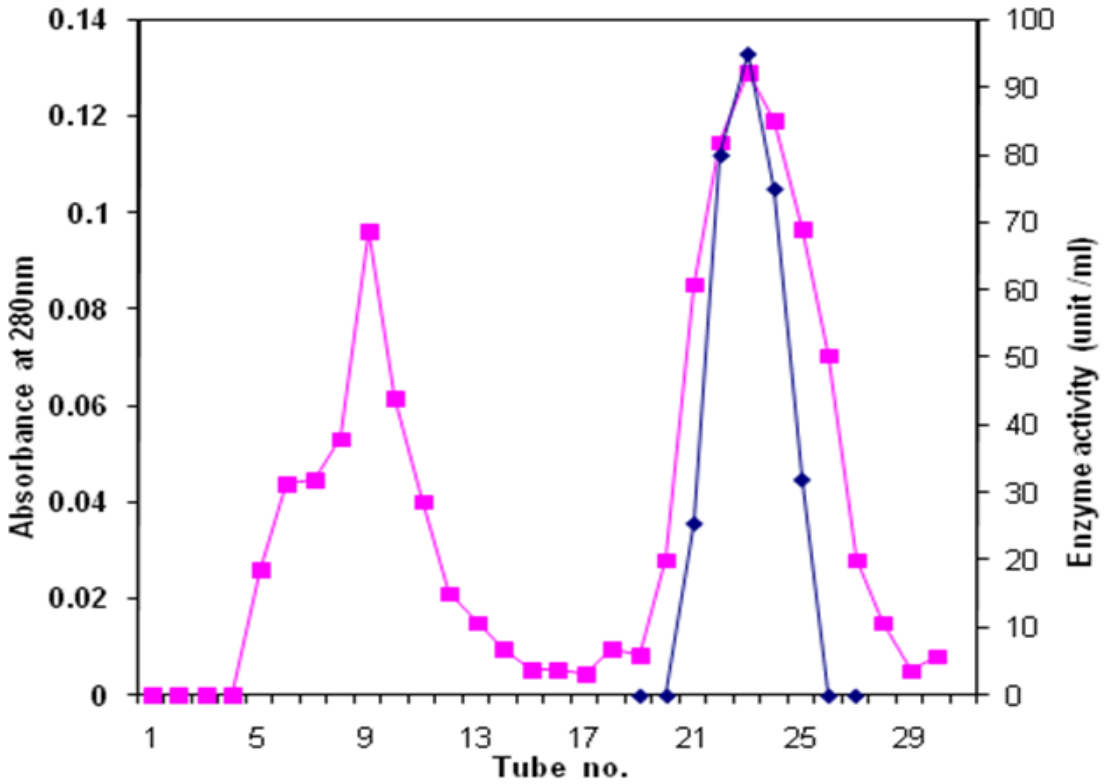

Figure 2 Gel filtration chromatography for purified fibrinolytic enzyme from *B. lichniformis* by using Sephacryl S-200 column (1.5x54) cm. The column was calibrated with 0.1 M phosphate buffer pH 7.0; flow rate 30 ml/hrs and 5 ml/fraction.

Enzyme characterization

Determination of molecular weight

Gel filtration method using column Sephacryl S200 fowled in estimating the molecular weight of fibrinolytic enzyme under study. **Figure (3)** illustrates molecular weight logarithm standard curve versus against elution volume / void volume (Ve/ V_0) of standard proteins and by using this relationship the molecular weight of the enzyme estimated with (50118Dalton). Molecular weight different from species to other, the metalloprotease of *Bacillus sp. KA38* (41 kDalton) (Kim *et al.*, 1997), *Bacillus sp*. *KDO-13* (45 kDalton) (Li *et al*., 2009),

The optimum pH for fibrinolytic enzyme activity

The effect of pH on the activity of the partially purified fibrinolytic enzyme at pH values ranged between (5-10) with a difference of half a degree and found that the optimum pH for the activity of the fibrinolytic enzyme is 7.5. It was also observed a decrease in the enzymatic activity at basal values (8.5-10.0) (**Figure 4**) and this result compatible with that mentioned by El-Safey and Abdul Raouf (2004), who mentioned that the pH values for maximal activity is 7.0 with 851.13units/ml. Although fibrinolytic enzyme showed high activity around pH 10 to 12 in the presence of substrate, its stability was decreased during incubation in the absence of substrate at a pH above 11. The optimum activity as affected by different pH values was obtained at (pH 7.0) giving (2.29 Units/mg protein) (Ali and Ibrahim, 2008).

The optimum pH for fibrinolytic enzyme stability

The results show that the optimum pH for fibrinolytic enzyme stability is 7 as the enzyme keeps 100% of its activity which means the enzyme stay at its optimum state in this pH compared with other. The enzyme keep approximately 13.9% of its activity at alkaline hydrogen number pH 10 while it keep 26% of its activity at acidic hydrogen number pH 5 **figure (5).** This result refers to decrease the enzyme stability at hydrogen number which far of pH 7 this decline due to the pH effect on the enzymatic protein structure or irreversible denaturation may occur in high acidic or basic solution (Whitaker, 1972). Kim *et al*., (2006) found the enzyme was highly stable in conditions with a pH range of 7.0-8.0 at37°C for 1 h, and more than 80% of its activity was sustained to a pH of 10.0. The enzyme activities remained over 89.22% at pH 5.10 for 1 hour.

The optimum temperature for fibrinolytic enzyme activity

The effect of temperature on the purified enzyme activity has been studied and observed increase in the enzyme activity with the increase of temperature until 37 ^{0}C **Figure (6)**. When the activity reach its maximum value at 37 ^{0} C degree, then it decrease with the continuous increasing of temperature until it lose most of it's activity which refers to that the enzyme is heat liable. This due to that the velocity of the enzymatic reaction increase with the increase of temperature within limited range because of the increase of kinetic energy of molecules until it reach to the degree of maximum reaction velocity, but the increase of temperature at ranges which lead to disruption of the three dimensional structure of the enzyme and then decrease it's activity (Segal, 1976).

The optimum temperature of enzyme activity and the human body temperature is 37 ^{0}C and that noticed the suitability of the body temperature to the enzyme work. Ability of the fibrinolytic enzyme to hydrolyze the blood clot help in the clinical application of the enzyme activity as mentioned previously. This result agreement with Siraj (2011) who mentioned that characterization studies showed an optimum temperature 37°C. Ellaiah and Srinivasulu (1996) also mentioned that the optimum incubation temperature for purified protease enzyme was 35 ºC. The purified protease activity reached up to 1168.15 units/ml. While the temperature below or above 35 ºC exhibited lower activities of protease.

The influence of temperature on the fibrinolytic activity from medicinal mushroom *Cordyceps militaris* showed that it was active between 20 and 40°C with an optimum activity at 37°C (Kim *et al.*, 2006).

The Optimum Temperature for fibrinolytic Enzyme Stability

The result refer to that the fibrinolytic enzyme produced by *B. lichniformis B4* keep with it's total activity when preserve it in (20-40) ^{0}C for 1 hour, then the activity begun to decrease with the increase of temperature **Figure (7).** The most enzymes were more stable at low temperature, and in order to maintain the activity of enzymes preferably saved it in low temperatures. The reduced activity of the enzyme under study when his lap at temperatures higher than 40 ^{0}C comes as a result of the effect of heat in the tripartite structure of the protein and may lead to denaturation of protein and loss it's

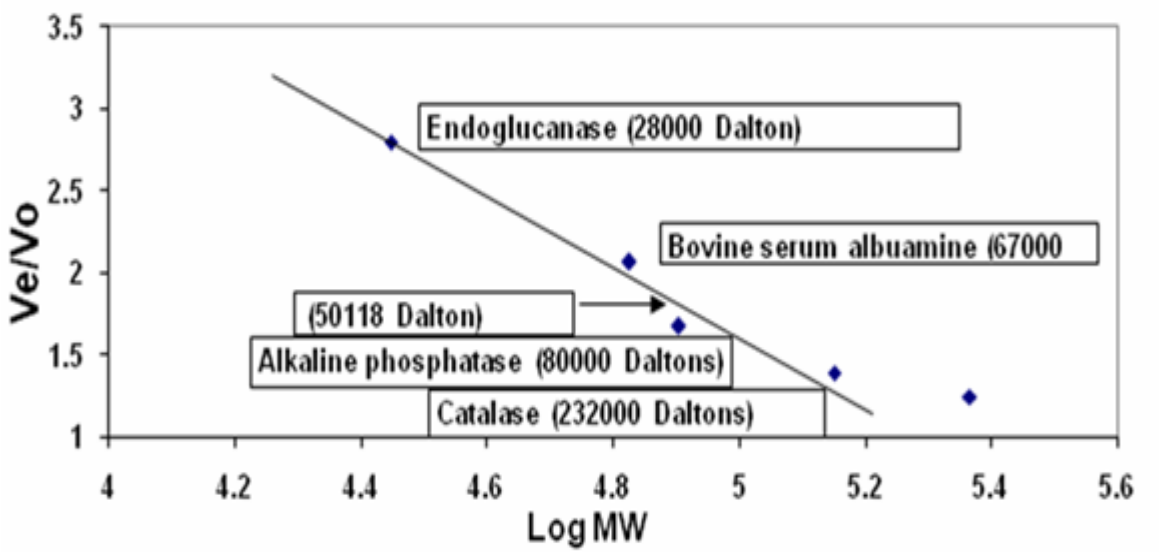

Figure 3 Standard curve to estimate molecular weight of fibrinolytic enzyme produced by *Bacillus lichniformis* B4 using gel filteration Sephacryl S-200 column.

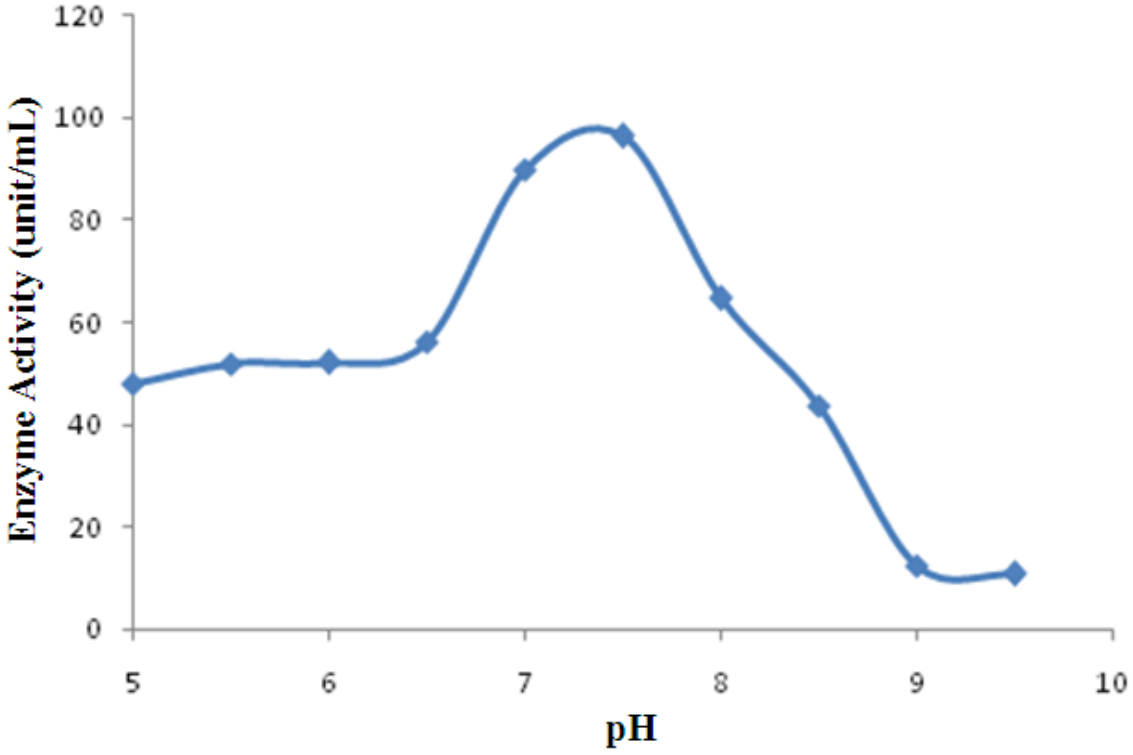

Figure 4 Efficiency of different pH on purified fibrinolytic enzyme production from *B. lichniformis B4.*

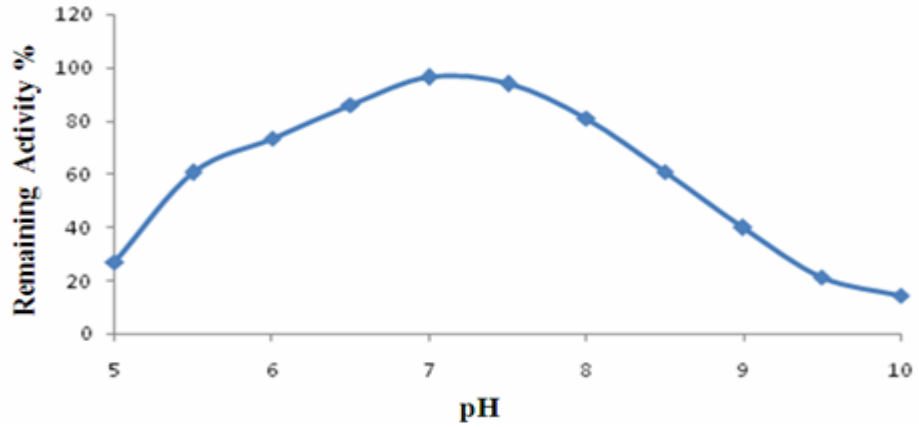

Figure 5 Effect of different pH on purified fibrinolytic enzyme stability from *B. lichniformis* B4.

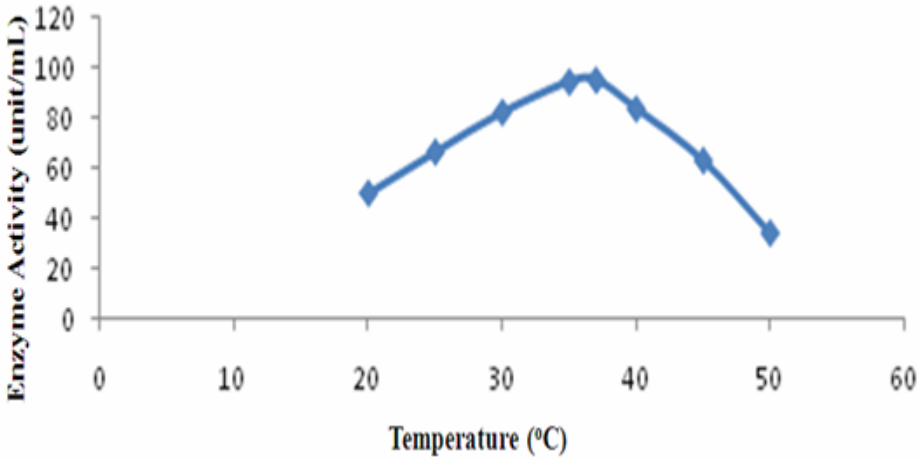

Figure 6 Effect of different temperature on activity of purified fibrinolytic enzyme from *B. lichniformis* B4.

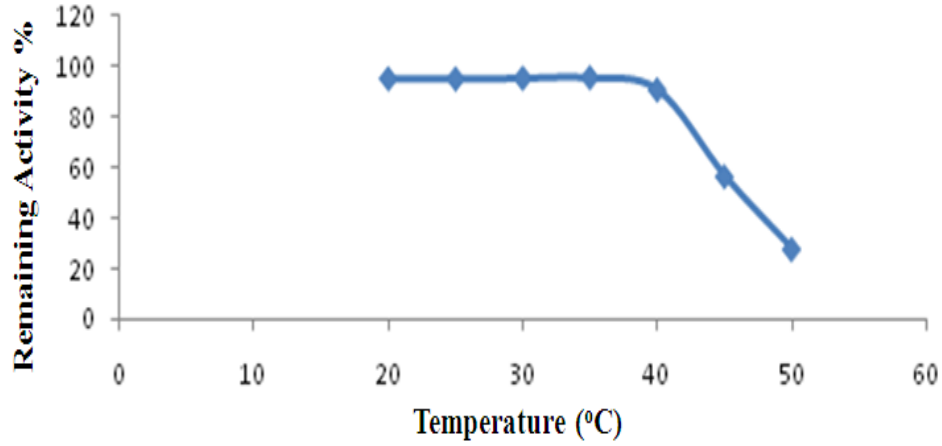

Figure 7 Effect of different temperature on stability of purified fibrinolytic enzyme.

activity which noticed when keep the enzyme at 50 ^{0}C as it lose high percentage of its activity as the remaining enzymatic activity arrived 28% of it's of the origin activity. (**Figure 7**)

This result agreement with Wang *et al.,* (2009) the optimum temperature of fibrinolytic was 40°C. The protease was relatively stable below 50°C. However, remarkable loss of activity was observed at 60°C. The enzyme was relatively stable between 30 and 50°C, and the enzyme activity decreased slowly with time. As the temperature was raised to 60°C, the activity of fibrinolytic enzyme showed a rapid decline, nearly 70% of the enzyme activity was retained at 60°C for 40 min, 80% activity was retained at 50°C for 1 h, and the overall initial activity after 60°min of treatment at 60°C was lost (Wang *et al*., 2009). Some previous studies on the most ability of fibrinolytic showed that the fibrinolytic enzyme was relatively stable at temperatures lower than 40°C and lost all initial activity after 10 min at 60°C (Chang *et al*., 2000) or 1 h at 50°C (Ko *et al*., 2004).

The measurement of fibrinolytic enzyme kinetic constant

The determination of the Mechalis Menton Kinetic constant (K_m) value and the maximum velocity (V_{max}) of the purified concentrated fibrinolytic enzyme using blood clot as described basis material. After the calculation of 1/[υ] and 1/[S] values, result shown in **figure (1-8)** indicate that the K_m and V_{max} values of purified fibrinolytic enzyme were (100 mM) and (200 mM/min^{-1}) respectively (Segel, 1976).

Screening the effect of chemical mutagenesis nitrosoguanidine concentration of 1mg/ml

The effect of chemical mutagenesis (Nitrosoguanidine) concentration of 1mg/ml for 1 hour (Gerhardt *et al.,* 1981), killing rate of the cells was 90% we obtain mutant cells under these conditions which gave bacterial mutant subjected to qualitative screening process to screen the ability of the isolate for producing of fibrinolytic enzyme. The result showd that superiority the mutant isolate in its high production of fibrinolytic enzyme compared with the original isolate (wild type) as the activity value was (68.177U/ml) while the wild type was (45.131U/ml).

Ali (2006) used nitrosoguanidine as chemical mutagenes which increase the production of thermostable α- amylase from Bacillus species.

Conclusion:

In conclusion, we observed that the fibrinolytic enzyme which isolated from *B.lichniformis* B4 by several steps included ammonium sulphate salt precipitation, ionic exchange using DEAE-Cellulose , then gel filtration in Sephacryl S-200. We also noted that the characterization of fibrinolytic were the optimum pH activity 7.5 and kept its fully activity at pH 7 for 1 hour. The optimum temperature for fibrinolytic enzyme activity was 37C and kept it's fully activity at 20-40C for 1 hour. The enzymatic activity developed for Bacillus lichniformis B4 for fibrinolytic enzyme production by using chemical mutangenesis (nitrosoguaidine) depending on killing percentage of 90% and the mutant isolate characterized with its high productivity to the fibrinolytic enzyme.

Figure 8.1

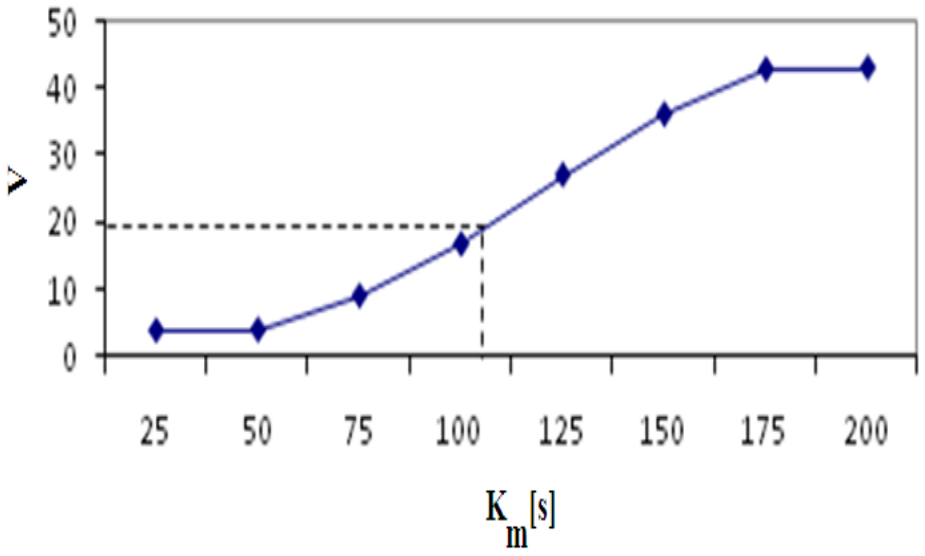

Figure 8.2

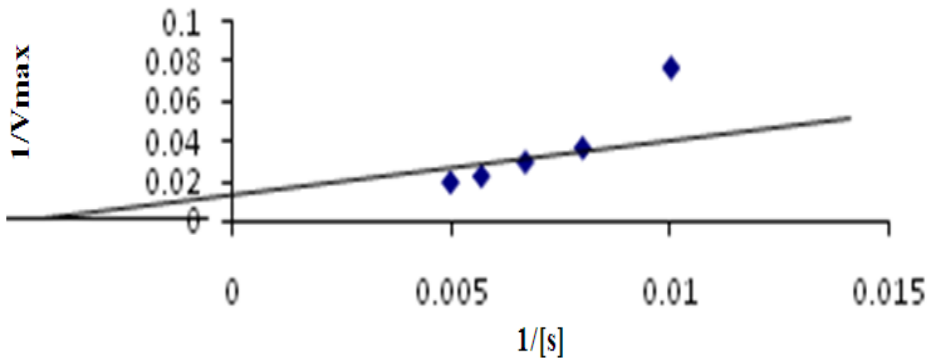

Figure 8 Kinetic Constant of fibrinolytic enzyme isolated and purified from *B. lichniformis* B4.

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