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Original Article

Isolation, Extraction, Purification, and Characterization of Fibrinolytic Enzyme from *Pseudomonas aeruginosa* and Estimation of the Molecular Weight of the Enzyme

Jasim, B. H[1 *], Ali, E. H[1]

1. Biotechnology Branch, Departments of Applied Sciences, University of Technology, Baghdad, Iraq

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Corresponding Author: bilalmaster20@gmail.com

Abstract

Pseudomonas aeruginosa was isolated from injuries of patients' wounds and burns, and to ensure that the isolate was belonging to *P. aeruginosa*, several tests were performed, such as staining techniques, a biochemical test, morphological test, Vitek 2 system, and sensitivity test. The results of the gram stain test showed rod pink gram-negative bacteria, demonstrating that the isolate belonged to *P. aeruginosa*. Growth optimization of bacterial was performed by assessing different combinations of pH and temperatures. It is revealed that the best conditions for increasing the number of bacteria were achieved at 37°C with the bacterial number of 5.53×10^8 and pH 6 with the bacterial number of 5.87×10^8. Fibrinolytic enzyme is an agent that lysis fibrin clots. This fibrinolytic factor has prospective use to treat cardiovascular diseases, such as stroke and heart attack. Cardiovascular diseases have attracted worldwide attention for their elevation morbidity and mortality. Fibrinolytic enzyme was extracted by centrifugation at 10000 × g at 4°C for 10 min, the supernatant was kept and the pellet having bacterial cells was discarded. Purification of the fibrinolytic enzyme was achieved using salt precipitation, ion exchange, and gel filtration chromatographic techniques. The results showed that the gel filtration chromatography had optimal specific activity and purification fold at 562.6 U/ml, and the final specific activity of the purified enzyme increased 4.1 times. The molecular weight of the fibrinolytic enzyme was determined at26 kDa by gel filtration chromatography. The purified fibrinolytic enzyme had optimum activity atpH 7 and40°C.The pH stability for the enzyme activity was found in pH 6-7 and the range of 10-40°C.

Keywords: Characterization, Isolation, Identification, *Pseudomonas Aeruginosa*, Fibrinolytic enzyme

1. Introduction

Fibrinolytic enzymes are a sub-family of the proteases EC 3.4. The importance of these enzymes is related to their applications in medicine and their various beneficial effects. One of the most considerable medical applications of these enzymes is their enzymatic action on the fibrin protein (insoluble protein) that contributes to blood clotting. Fibrin deposits inside the blood vessels, which makes an individual vulnerable to disease or injury (1). The molecular weight ranges from 14-97 kDa, and as it contributes to dissolving blood clots (thrombi), maintains a regular flow in the blood vessels. Thrombosis is a major risk factor for myocardial infarction, deep venous thrombosis, and a group of cardiovascular diseases (2). These enzymes have the ability to degrade fibrin as fibrin is usually composed of fibrinogen with the action of thrombin (EC 3.4.21.5). Thrombin, which is hydrolyzed by plasmin (EC 3.4.21.7), is activated from plasminogen by the tissue plasminogen activator.

Fibrin clots are hydrolyzed by plasmin to avoid blood clots in the blood vessels. In the pathological cases that result from physiological disorders, the fibrin sheets

formed inside the blood vessels are not dissolved, leading to their deposition, blood clots, and other cardiovascular diseases (3). Thrombotic disturbances are the main cause of death worldwide manifested in the form of stroke, myocardial infarction, and embolism. The formation of intravascular thrombus leads to the improvement of various cardiovascular disorders (CVDs), which eventually results in death. In 2011, the statistics of the American Heart Association showed that 31.3% of deaths were caused by thrombosis, and according to the report of the World Health Organization, about 17 million individuals are succumbing to thrombotic disorders annually (4). This study aimed to investigate:

1. Isolation and diagnosis of *Pseudomonas aeruginosa* and confirm the production of fibrinolytic enzyme;
2. Extraction and purification of the molecular enzyme by salting out using ammonium sulphate deposition, ion exchange chromatography, and gel filtration chromatography;
3. Estimation of the molecular weight of the enzyme; and
4. Characterization of the enzyme during the determination of the optimal pH and temperature of fibrinolytic activity and stability.

2. Material and Methods

2.1. Patients, Specimens, Collection

The specimens (n=107) were collected from injuries of wounds and burns within September-December 2020. The samples were collected by transport swabs with the transporting medium from the injured patients hospitalized in the hospital of Al-Rusafa (Medical City Hospital), Baghdad, Iraq, and were diagnosed externally, microscopically, and by Vitek 2 system (5).

2.2. Samples Identification

All the isolates of bacteria were examined for gramstain ability (6). The structure and color of the cells were observed by light microscope using oil emersion. *Pseudomonas aeruginosa* was streaked over the agar surface. Some singular bacterial cells were separated and well-spaced from each other. As the original specimen was diluted by being streaked over successive quadrants and was then incubated at 37°C for 24h, the number of organisms reduced and showed the bacterial morphology (7).

2.3. Optimal Temperature and pH for the Production of Bacteria

The bacterial suspension was cultured once at a constant pH, however, at different temperatures (32, 35, and 37°C) and another time at a constant temperature and different pH (5.5, 7, and 9) and measures the absorbance at 600 nm (8).

2.4. Extraction of Enzyme

The amount of 75 ml of the produced broth containing the bacterial cells, in which the extracellular fibrinolytic enzyme was found, was carried into centrifuge tubes, and the bacterial cells were cooled by centrifugation at 10,000 × g at 4°Cfor 1، min. The supernatant, containing extracellular protein, was kept and the pellet having bacterial cells was discarded (9).

2.5. Determination of Protein Concentration

Protein concentration was determined according to the Bradford technique (1976). The protein concentration was determined on the following method:20µl of the crude enzyme was mixed with 50µl of 1 M NaOH with shaking for 2-3 min; afterward, 1 ml of Bradford solution was added with shaking, and the absorbance was measured at 595 nm by spectrophotometer (10).

2.6. Measurement of Crude Enzyme Activity

The estimation of fibrinolysis activity was accomplished according to the Chang method (11). Accordingly, 1.4 mL of 50 mMTris-HCl (pH 8.0) and 0.4 mL of 0.72% fibrinogen solution (w/v) were taken in a sterile tube and incubated in a water bath at 37°C for 5 min. Subsequently, 0.1 ml of thrombin was added and the tubes were incubated in a water bath at 37°C for 10 min. Following, 0.1 ml of crude extract was added and the tube was incubated for 60 min. After that, 0.2 M of trichloroacetic acid was added to it. The mixture was centrifuged at 10,000 rpm for 10 min, where the precipitate was discarded and the filtrate was

taken and the efficacy was estimated based on measuring the absorbance at a wavelength of 275 nm. The same steps were followed forpreparing the blank sample by adding the suspension solution before adding the raw enzymatic extract. One unit of fibrinolytic activity (fibrin degradation unit) is described as the amount of enzyme required to increase an absorbance equal to 0.01 in 1 min at 275 nm.

2.7. Ammonium Sulfate Precipitation

Ammonium sulfate was added at more than one saturation ratio (20-80%), and to reach the best ratio of ammonium sulfate, salt was added progressively to each 10 ml enzyme solution in the ice bath and magneticstirrer for 1 h. The final solution was centrifuged at 10,000 rpm/min for 10 min. The precipitate was kept and the supernatant was discarded, 25ml phosphate buffer saline pH 7.2 was dissolved in the precipitate, and the activity of enzyme and protein concentration were calculated (12).

2.8. Purification by Dialysis Tube

The dialysis process of the enzyme was carried out from the ammonium sulfate sedimentation step; where4 mL of the enzyme was placed in a dialysis tube with a diameter of 2.5 cm, which allowed the passage of materials with a molecular weight of 8,000-14,000 Dalton. The dialysis tube was placed in a container containing potassium phosphate, which led to the separation of the brine solution. It was placed at a temperature of 4°C for a day and the solution was changed twice. After the completion of the dialysis process, the absorbance was measured using a spectrophotometer with a wavelength of 275 nm (13).

2.9. Separation of the Enzyme through Ion Exchange Resin (Diethylaminoethyl Cellulose)

Diethylaminoethyl cellulose was prepared according to a method conducted by Whitaker and Bernard (1972).The enzyme, by adding 35 mL of the crude enzyme purified by dialysis tube, was separated slowly and diagonally to the walls of the ion exchanger column containing the exchanger material using the Dropper. Subsequently, the separated fraction was collected in appropriate and sterilized tubes at a flow rate of 36 mL/h at a volume of 3 mL for each part. A step wash was then performed with phosphate buffer saline with a pH of 7.2. The elution retrieval step was conducted using different concentrations of NaCl (0.1, 0.3,0.5, 0.7,0.9,and 1 molar), after which the absorbance of each retrieval fraction was measured at a wavelength of 280 nm for each of the washing and retrieval steps, followed by the calculation ofthe enzyme activity in the fractions. The enzyme activity was calculated by collecting the parts of the ion exchanger to determine the fractions containing the enzyme activity by calculating the enzyme activity, concentration, and protein volume (14).

2.10. Enzyme Separation through Sephadex G-150 Column

Sephadex G-150 gel filtration was prepared according to the manufacturer's guidelines (i.e., Pharmacia Fine). An amount of27mlof the enzyme purified by an ion-exchanger step was added slowly into the column walls and the enzyme was retrieval led with the same solution used for calibration at a flow rate of 30 mL/h per fraction. Afterward, the absorbance of the protein fraction was measured at a wavelength of 280 nm. The enzymatic activity of the absorbance peaks was measured, the enzyme activity was measured for all protein peaks, and the protein concentration was measured (15).

2.11. Estimation of the Molecular Weight of the Enzyme

The molecular weight of the enzyme was estimated by gel filtration chromatography. A Sephadex G-150 (2×35 cm) column was used and titrated with 50 mM of phosphate buffer solution, and the recovery step was performed using the same phosphate buffer solution. The following standard crystal proteins act as molecular weight markers (150,000 Dalton), Albumin (66,000 Dalton), Carbonic anhydrase (29,000 Dalton), and lysozyme (14,300 Dalton). The void volume was assessed by Blue Dextran at a wavelength of 600 nm, and the recovery volume of each standard protein was

measured at 280 nm using a spectrophotometer (UltravioletVis BioRad system). From the recovery volume of the enzyme, the molecular weight was determined depending on the molecular weight of the known standard protein (16).

2.12. Characterization of Fibrinolytic Enzyme

2.12.1 OptimumpH for Fibrinolytic Enzyme Activity

The optimum pH for the fibrinolytic enzyme activity was determined using sodium acetate $C_2H_3NaO_2$, which was prepared with a concentration of 0.1 M and pH range of5-6, phosphate buffer saline with 0.1 M concentration and pH range of7-8, and Tris-HCl 0.1 M concentration and pH range of 9-10. Afterward, equal volumes of these buffers were mixed with the substrate fibrinogenat 0.1 M concentrations (1:1). At the next stage, 0.1 ml of the purified enzyme was added to 0.9 ml substrate (17).

2.12.2. Optimum Temperature for Fibrinolytic Enzyme Activity

The optimum temperature for fibrinolytic enzyme activity was determined using0.9 mL of the substrate fibrinogen with 0.1Mconcentration added to 0.1 mL of purified enzyme solution and then incubated for 10 min in a water bath at different temperatures (i.e., 30, 35, 40,and 45°C), followed by the determination of the enzyme activity for each temperature. Subsequently, the relationship between enzymatic activity and temperature was plotted to determine the optimum temperature for enzyme activity (18).

2.12.3. Optimum pH for Fibrinolytic Enzyme Stability

The optimal pH for fibrinolytic enzyme stability was determined using equal volumes of purified enzyme (i.e., 0.4 ml), which were mixed with each buffer with a pH range of 5-10 at 0.1 M concentration and substrate. The solutions were incubated in a water bath at 37°C for 30 min and then transferred to an ice bath. Afterward, the absorption was measured with the optical spectrometer at a wavelength of 275 nm, and the relationship between the percentage of residual activity and optimal pH for enzyme stability was plotted (19).

2.12.4. Optimum Temperature for Fibrinolytic Enzyme Stability

The optimum temperature for fibrinolytic enzyme stability was determined by 0.5 ml of the purified fibrinolytic enzyme with 0.1, which was incubated in a water bath at different temperatures (i.e., 30, 35, 40, and 45°C) for 30 min. The enzyme-containing tubes were then moved directly to an ice bath. The residual activity and the relationship between temperature and percentage of residual activity were evaluated to determine the optimal temperature for enzyme stability (20).

3. Results and Discussion

3.1. Identification of bacteria

Identification of bacteria initiated primarily by culturing the specimens on nutrient agar and incubating at 48°C.Finally,Vitek 2 system was performed to ensure that the isolate belonged to *P. aeruginosa* (21).

3.2. Optimal pH and Temperature for Bacterial Growth

The bacterial suspension was tested after being grown in the nutrient medium to find the best temperature and pH for the growth of the bacteria, where it was incubated at different temperatures (32,35,and 37°C). The best growth temperature was obtained at 37°C, at which the numbers of bacteria reached 5.53×10^8 cells/ml, compared to 35°C, at which the number of bacteria was 3.46×10^8 cells/ml, and 32°C, at which the number of bacteria was 2.63×10^8 cells/ml (Figure 1). After that, the bacteria were grown in different culture media with pH 6, 7,and 9 to determine the optimum incubation temperature; accordingly, the best growth was revealed to be at pH 6, in which the number of bacteria was 5.87×10^8 cells/ml, compared to pH 7 and 9, in which the numbers of bacteria were$4.33 \times 10^8$and 2.91×10^8 cells/ml, respectively (Figure 2). The results of the current study were in line with those of a study (22) reporting that the best temperature and pH were obtained at 37°C and 5.5, respectively.

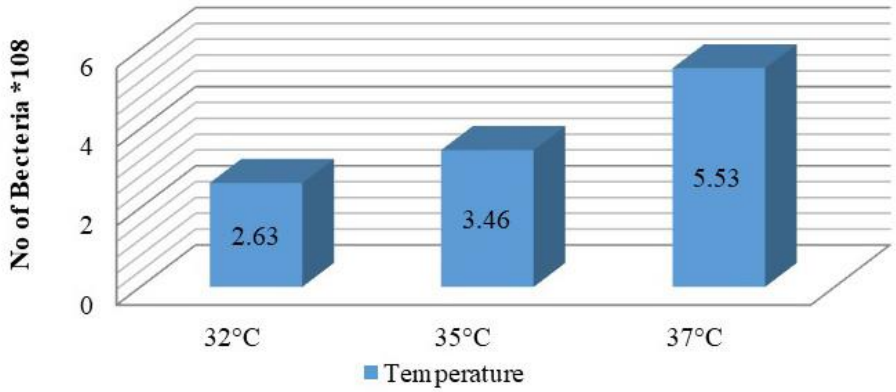

Figure 1. Optimum temperature of *Pseudomonas aeruginosa*

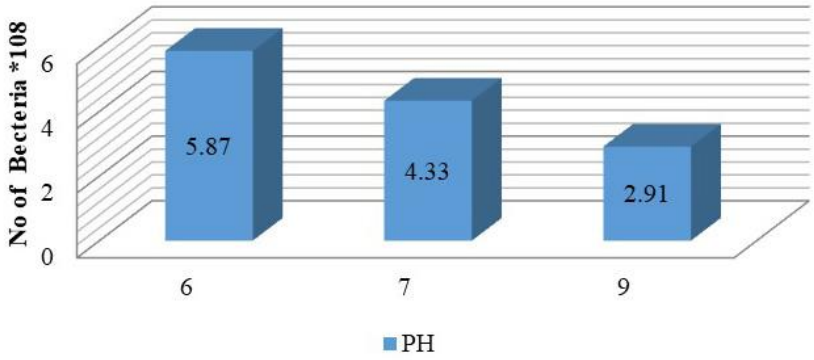

Figure 2. Optimum pH of *Pseudomonas aeruginosa*

3.3. Extraction and Purification of Fibrinolytic Enzyme

In this study, the fibrinolytic enzyme produced by *P. aeruginosa* in the culture broth was subjected to a purification protocol. After that, the crude enzyme activity was estimated from the supernatant. The enzyme activity and specific activity of crude enzyme were calculated at 27.3 unit/ml and 136.5 unit/mg, respectively. The purification involved ammonium sulphate precipitation and dialysis tube, followed by ion exchange and gel filtration.

3.4. Ammonium Sulfate

The greatest ratio for the crude extract precipitation of the enzyme was estimated at 80%, when the specific activity reached151.5 U/mg, with the purification folds of 1.1 times and the yield of 73.9%. The results of this study were in agreement with those of a study (23) indicating that the best saturation percentage was at a concentration of 75%.

3.5. Dialysis

In this step, ammonium sulfate salts were eliminated and the purification results showed an increase in the specific activity of the enzyme, reaching 275.3U/mg, compared to the specific efficacy after the sedimentation step with ammonium sulfate, which reached 150 U/mg and the purification fold and enzyme yield were 2 and 70.6 %, respectively.

3.6. Ionic Exchange Chromatography

Ionic exchange chromatographyis one of the most useful methods for protein purification. In this process, depending on the surface molecule charge, the protein, and the buffer conditions, the protein will have a net positive or negative charge. Fibrinolytic enzyme was obtained using phosphate buffer solution

(pH=7.2). The absorbance of eluted fractions was measured at 280 nm upon the arrival of absorbance to the line of zero. Afterward, the same buffer with the NaCl gradient (0.1-1M) was used to elude the bounded protein. Ionic exchange patterns showed two protein peaks, including one in elution and one in the wash, and one enzyme peak in gradient elution, representing enzyme activity (tubes 66-77). Those fractions were pooled and tested for the specific activity of 499 U/mg, fold purification of 3.6 times, and enzyme yield of 65.8% in different parts (Figure 3).

3.7. Gel Filtration Chromatography

The purification process was carried out by gel filtration using Sephadex G-150. Enzymes fractions from the ionic exchange were pooled and passed through the gel filtration column. The fractionation yielded two protein peaks, absorbance reading at the wave length of 280 nm, only one peak appearing when reading absorbance was at the wavelength of 280 nm and when was determined for enzyme activity in resulting parts enzyme activity recorded in 20-29, the specific activity reached to 563.7U/mg, fold of 4.1, and a yield of 52.8% (Table 1 and Figure 4).

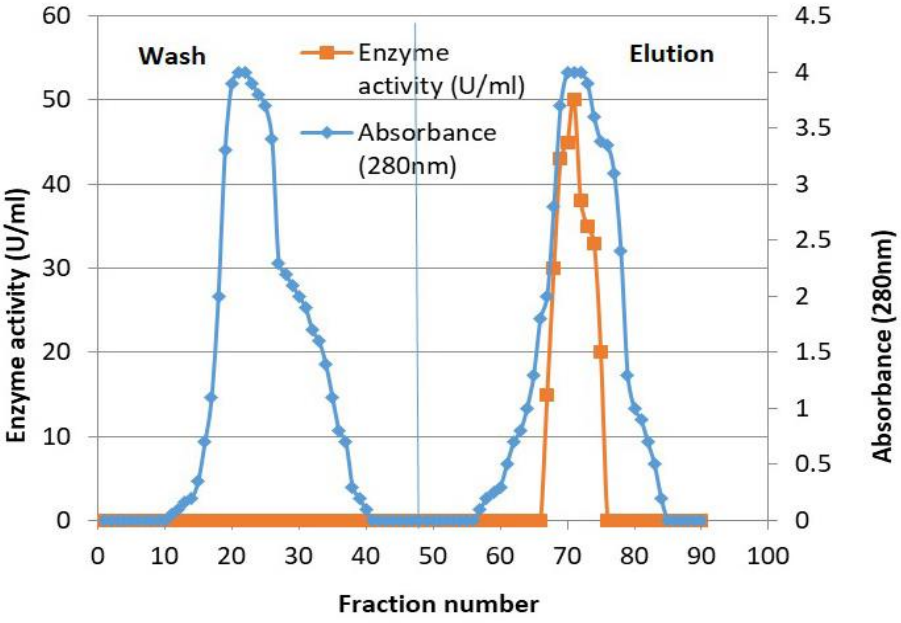

Figure 3. Ion exchanger chromatography of fibrinolytic produced by the *Pseudomonas aeruginosa*

Table 1. Purification steps of the fibrinolytic enzyme

Purification step	Volume (ml)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (folds)	Yield (%)
Crude enzyme	75	27.3	0.2	136.5	2047.5	1	100
Ammonium sulphate precipitation 80%	50	30.3	0.2	151.5	1515	1.1	73.9
Dialysis	35	41.3	0.15	275.3	1445.5	2	70.6
Diethylaminoethyl cellulose	27	49.9	0.1	499	1347.3	3.6	65.8
Sephadex G-150	24	45.1	0.08	563.7	1082.4	4.1	52.8

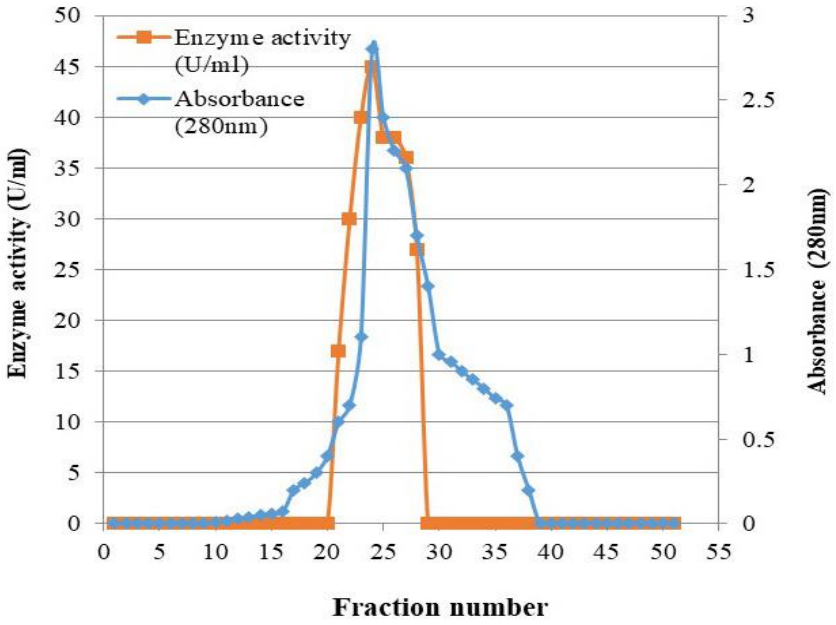

Figure 4. Gel filtration for the purification of a fibrinolytic enzyme produced from local isolation *Pseudomonas aeruginosa*

3.8. Molecular Weight of Fibrinolytic Enzyme

The molecular weight of the enzyme was determined by gel filtration chromatography. A Sephadex G-150(1.5×55cm) column was used for the enzyme purification from *P. aeruginosa* depending on the size and charge of the separated particles. As in figure 5, according to the algorithm of molecular weight Ve/Vo, the molecular weight of an enzyme was found to be 26,000 Dalton. This result was consistent with that reported in a study conducted by Devi, Mohanasrinivasan (24), according to which, the molecular weight produced by *P. aeruginosa* was estimated at 27,000 Dalton using the gel filtration method.

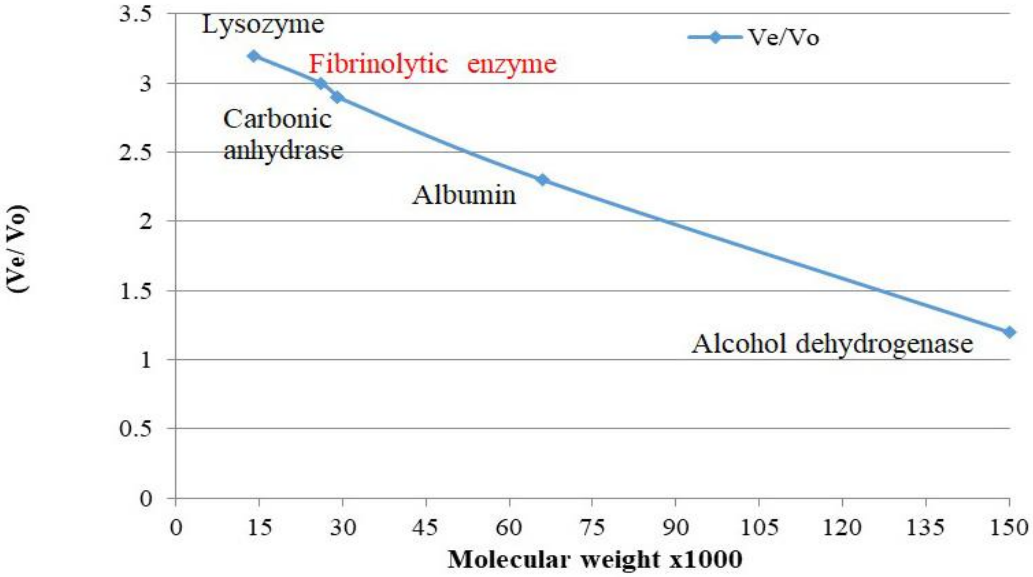

Figure 5. Molecular weight of purified fibrinolytic enzyme from *Pseudomonas aeruginosa*

3.9. Characterization of Fibrinolytic Enzyme

3.9.1. pH and Temperature for Optimum Activity

The results of this study showed that the maximum fibrinolytic activity was detected at 40°C and a decreased activityat45°C (Figure 6). This finding was in line with that of a study carried out by Taneja, Bajaj (23), in which they reported the maximum fibrinolytic activity at 40°C.The pH activity curve showed that the maximum fibrinolytic enzyme and a decrease at pH 7 and pH 8, respectively (Figure7).These results were in agreement with that reported in research conducted by Lee, Kim (25) regarding the low activity curve of the fibrinolytic enzyme at pH 8.

3.9.2. pH and Temperature Stability

The pH stability curve showed that the fibrinolytic enzyme was stable at pH 6 and 7. The stability data showed a decline in the fibrinolytic activity below 6 and above 7.However, 93%and 95% relative activity were retained at this pH (Figures 8 and 9).This result was consistent with that reported in the study performed by Taneja, Bajaj (23) explaining the purified enzyme and showing higher stability between pH 6 and 8 retaining more than 90% of its activity, while it retained 87.22% and 74.23% of its activity at pH 5 and 9, respectively. Maximal temperature stability of the fibrinolytic enzyme was observed in the temperature range of 30-40°C. At 80°C, the enzyme maintained 20% of enzyme activity.

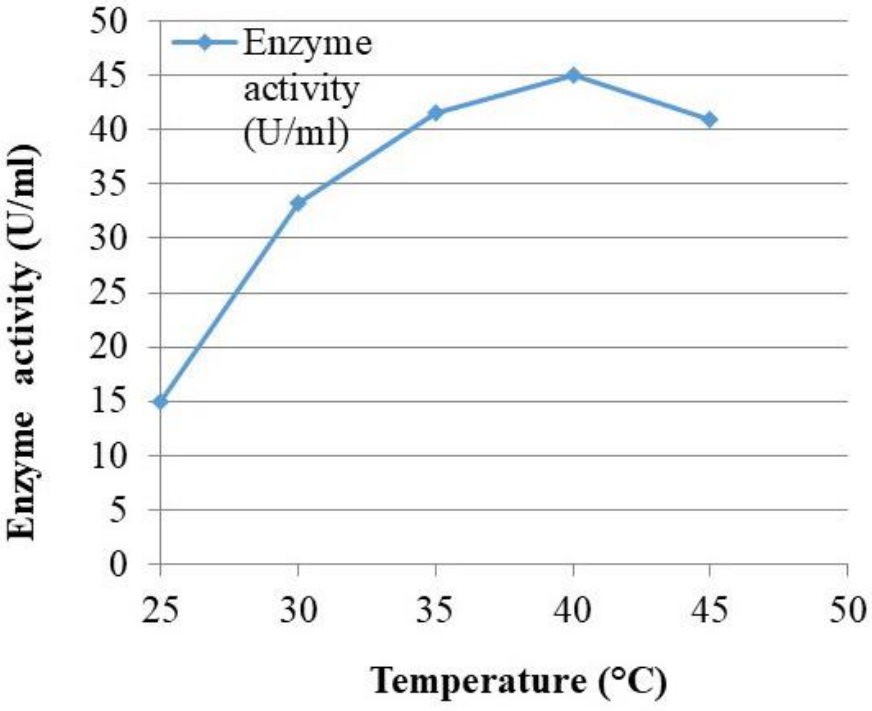

Figure 6. Optimum temperature of fibrinolytic activity

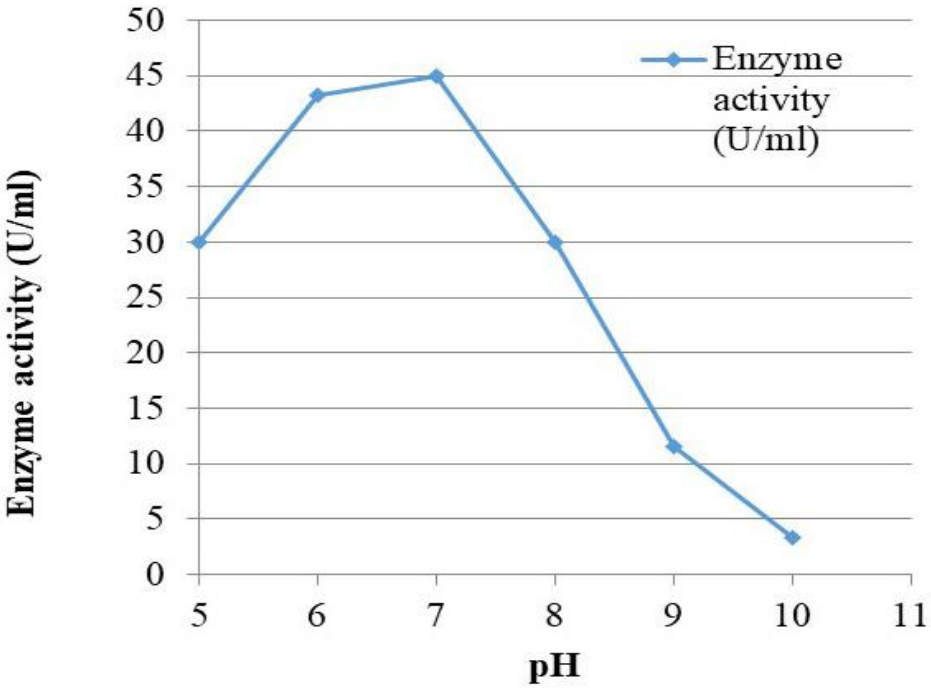

Figure 7. Optimum pH of fibrinolytic activity

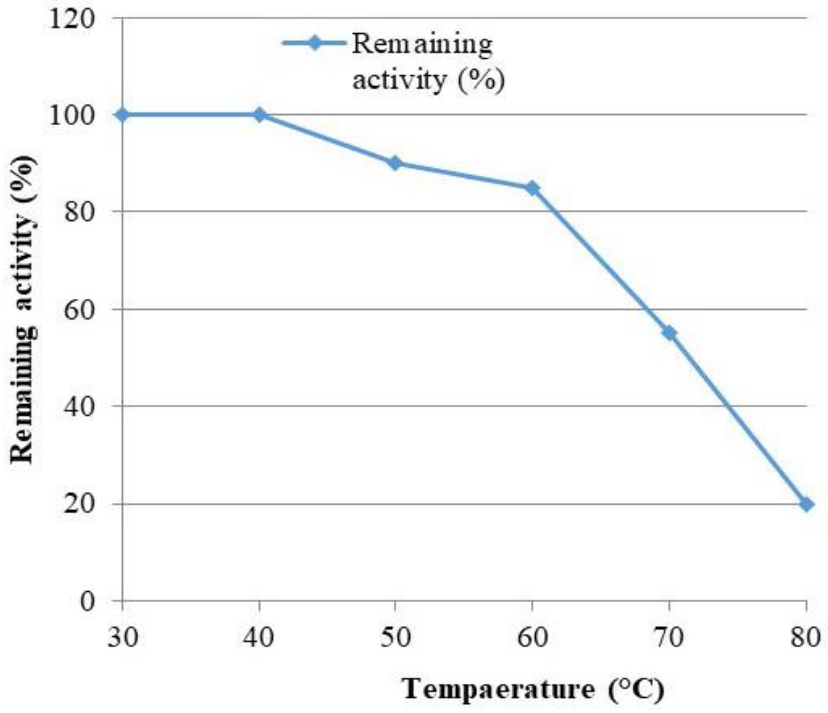

Figure 8. Temperature stability of Fibrinolytic enzyme

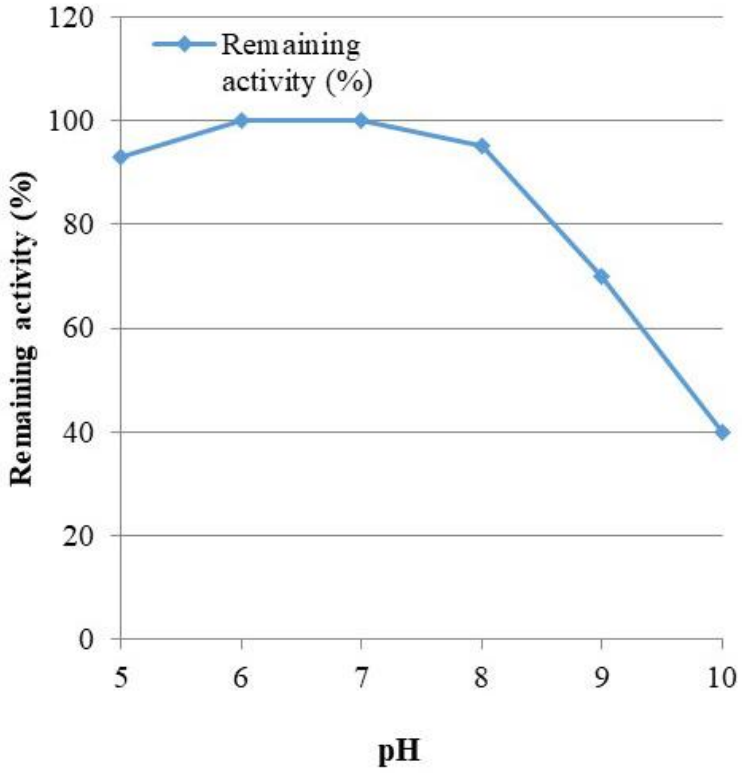

Figure 9. pH stability of the fibrinolytic enzyme

In conclusion, in this study, the fibrinolytic enzyme was extracted from *P. aeruginosa* bacteria, and it was possible to obtain pure fibrinolytic enzyme from bacteria using purification steps with high purity and good quality efficacy. The optimum conditions for the fibrinolytic enzyme were the optimum temperature of 40°C and stability enzyme range of 30-40°C.The optimum pH for the performance of the fibrinolytic enzyme was reported at 7 and its stability was found at the range of 6-7. Molecular weight of purified fibrinolytic enzyme from *P. aeruginosa* is was 26,000 Dalton.

Authors' Contribution

Study concept and design: B. H. J.
Acquisition of data: E. H. A.
Analysis and interpretation of data: B. H. J.
Drafting of the manuscript: E. H. A.
Critical revision of the manuscript for important intellectual content: E. H. A.
Statistical analysis: B. H. J.
Administrative, technical, and material support: B. H. J. and E. H. A.

Ethics

All procedures performed in this study involving human participants were in accordance with the ethical standards of the University of Technology, Baghdad, Iraq Under the project number 2020-658-789-7.

Conflict of Interest

The authors declare that they have no conflict of interest.

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