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Purification and optimization of gelatinase of local x*Pseudomonas aeruginosa* isolates from clinical specimens

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Abstract:

A total of 100 clinical samples were collected from patients with wound, burn, and urinary tract infections at various hospitals in Baghdad from September to December 2025. Using standard microbiological methods (culture on MacConkey and Cetrimide agar, Gram staining, biochemical tests) followed by confirmation with the VITEK 2 compact system, forty isolates (40%) of *Pseudomonas aeruginosa* were identified. Screening for gelatinase production on gelatin agar medium revealed that 28 isolates (70%) were positive, and the isolate showing the highest enzymatic production was selected for purification and characterization. The enzyme was purified through ammonium sulfate precipitation, ion-exchange chromatography, and gel filtration, yielding a specific activity of 11.4 U/mg protein, a purification fold of 4.6, and a final yield of 45.71%. The purified gelatinase had an estimated molecular weight of approximately 34 kDa, as determined by SDS-PAGE. The enzyme exhibited optimal activity and stability at 37°C and pH 7.0, while remaining stable across a broad pH range (4–9). These findings support the physiological relevance of gelatinase as a potential virulence factor contributing to *P. aeruginosa* pathogenicity during host infection.

Keywords: *Pseudomonas aeruginosa*, Gelatinase enzyme, Purification, Clinical isolates, Virulence factor

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Introduction

Pseudomonas aeruginosa is a rod-shaped, blue-green, non-fermenting, motile, Gram-negative bacterium of the Pseudomonadaceae family. It has remarkable genetic variation, allowing it to grow in diverse environments, from soil and water to the human body. This adaptability is largely due to its large genome, which contains many regulatory genes and genes for transport and metabolism of various nutrients (Ambreetha *et al.*, 2024). As a result, *P. aeruginosa* can survive extreme conditions, such as low oxygen, limited nutrients, and the presence of antimicrobial agents. This metabolic and genetic flexibility also enables the bacterium to produce a wide range of virulence factors, including exotoxins, proteases, and biofilm-associated proteins, all of which contribute to its pathogenicity (Sendra *et al.*, 2024). Furthermore, *P. aeruginosa* shows both intrinsic and acquired resistance to most clinically available antibiotics, making it a formidable opportunistic pathogen frequently isolated from wound, burn, and urinary tract infections. Its ability to adapt to hostile environments, including hospital settings, has led to its widespread distribution and persistent colonisation in immunocompromised patients (Moradali *et al.*, 2017).

Gelatinase is a virulence factor produced by several pathogenic bacteria, including *P. aeruginosa* (Stehling *et al.*, 2008). This enzyme belongs to the metalloprotease family and requires zinc ions for its activity. Its main function is to break down gelatin, which is denatured collagen, and other extracellular matrix components (Klein & Bischoff, 2011). By degrading these structural barriers, gelatinase helps bacteria avoid the host immune system and spread into deeper tissues. Because of this, gelatinase is viewed as a possible target for treating biofilm-related and chronic wound infections (Okojie & Okotie, 2021). In *P. aeruginosa* strains taken from chronic wounds, gelatinase is often reported as a common soluble virulence factor. Several studies have linked gelatinase production to tissue damage and slower wound healing. It has also been observed that many biofilm-forming isolates produce gelatinase, suggesting that this enzyme contributes to the persistence of infection (Georgescu *et al.*, 2016). Therefore, the present study was aimed to isolate and identify *P. aeruginosa* from clinical samples, screen local isolates for gelatinase-

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producing ability, and purify and biochemically characterize the enzyme _including its optimal temperature, pH, and stability profile _ in order to better understand its potential role as a virulence factor contributing to *P. aeruginosa* pathogenicity.

Materials & Methods

Collection of specimens

From September to December 2025, one hundred different clinical specimens included wound and burn swabs and urine specimens' patients were collected from different hospitals in Baghdad/Iraq. Specimens were taken from patients using sterile cotton swabs and sent directly to the microbiology laboratory under cooling conditions for bacterial isolation. The wound swabs were cultured on Blood agar, MacConkey agar, and Cetrimide agar medium followed by morphological and biochemical assays.

Identification of isolates

The identification of bacterial isolates was carried out using standard morphological and biochemical procedures, following the guidelines outlined in Bergey's Manual of Systematic Bacteriology, 2nd edition (Garrity *et al*., 2005). Isolates that were presumptively identified as *P. aeruginosa* based on these methods were further confirmed using the automated VITEK 2 compact system (Ossman *et al.*, 2025). Screening the ability of *P. aeruginosa* isolates for gelatinase production

Qualitative detection of gelatinase using the tube method

Gelatinase production was detected using the nutrient gelatin stab method. Briefly, nutrient gelatin deep tubes (containing 12% gelatin) were stab‑inoculated with a fresh bacterial colony using a sterile needle. The tubes were then incubated at 37 °C for 24–48 hours. After incubation, all tubes were placed in a refrigerator at 4 °C for about 30 minutes to allow the medium to solidify. A positive result was recorded when the inoculated tube remained liquid after chilling, indicating that gelatin had been hydrolysed. The uninoculated control tube always solidified completely (Microbe Notes, 2022)

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Semi-quantitative screening of gelatinase production

Semi-quantitative primary screening for gelatinase production is conducted as mentioned by Pathade *et al.,(*2024). using gelatinase plate medium. A single colony from a 24-hour-grown bacterial culture was spotted in the center of the gelatinase plate medium, and it was subsequently incubated for 48 hours at 37°C. The diameter of clear hydrolysis zone around the colony was measured as an indicator of gelatinase production. To visualize hydrolysis, plates were flooded with 10% trichloroacetic acid (TCA), which precipitates undigested protein and leaves a clear halo around gelatinase‑positive colonies (Medina & Baresi, 2007).

Quantitative screening of gelatinase production

A quantitative secondary screening of gelatinase-producing isolates was performed as reported by. Pitt & Dey, (1970) The isolates with the highest productivity based on primary screening were chosen and grown on submerged fermentation medium by inoculating 100 ml of this medium with 1% of the overnight bacterial isolate. After that, the flasks were incubated in a rotary shaker incubator to evenly spread the inoculum throughout the media at 37°C for 48 hours at 120 rpm. Following a 48-hour incubation period, the fermentation medium was centrifuged at 10,000 rpm for 10 min at 4°C, and the clear supernatant was utilized as a crude enzyme solution to estimate gelatinase activity and protein concentration.

Estimation of gelatinase activity

According to Moore and Stein (1948) gelatinase activity was estimated by Preparation of Standard Curve for leucine, A stock solution of leucine was made at 10 mg/ml.Serial concentrations of leucine (1, 3, 5, 7, 9 mg/ml) were prepared from stock solution by dilution with distilled water. A 0.5 ml of acetate cyanide and 0.5 ml of 3% Ninhydrin were added to 1 ml of each concentration and heated in a water bath at 100 ºC for 15 min. After removal from the water bath, 5 ml of isopropyl alcohol water diluent was added, and the combination was shaken by vortex. The absorbance of every concentration was estimated after cooling at room temperature by a spectrophotometer at 570 nm. The absorbency was

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plotted against the corresponding concentrations of leucine as exhibited in Figure (1).

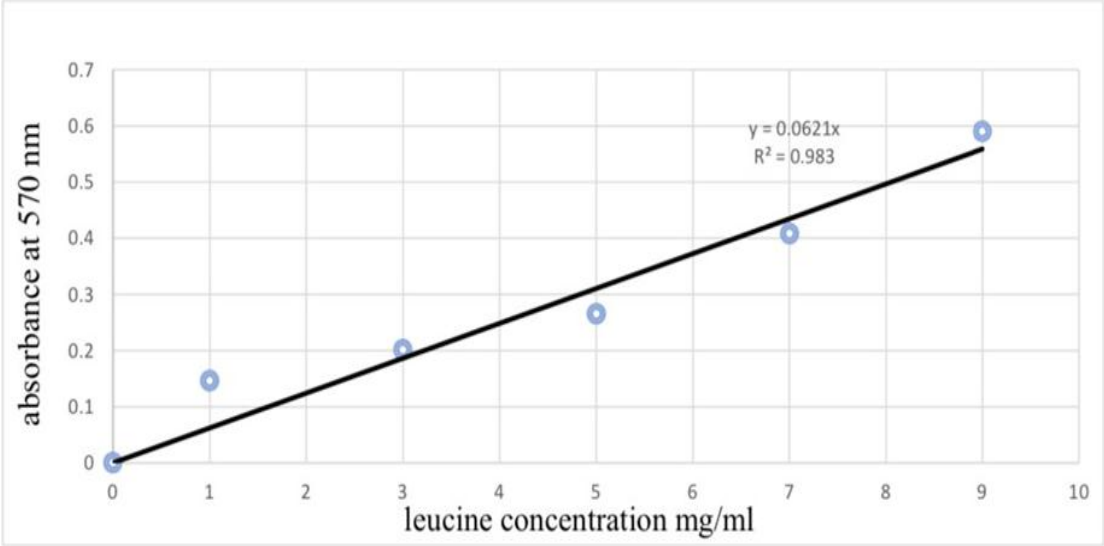

Figure (1) Standard curve of leucine for estimation of gelatinase activity

The measurement of the gelatinase activity relied on the amount of free amino groups released from gelatin by the Rosen method. A 10 mg of gelatin was liquefied in 800 µl of 0.1M Tris-HCl buffer and 50 mM CaC12 (pH 7.5) and incubated with 200 µl of the enzyme at 37 °C for 30 min. The reaction was stopped by adding 1 ml of 0.1M acetic acid (Nagano & To, 2000). The released free amino acid was estimated by a meta procedure stated by (Rosen, 1957), by adding 0.5 ml of acetate cyanide and 0.5 ml of 3% Ninhydrin to the mixture and heating for 15 min at 100 °C in a water bath. After being removed from the water bath, 5 ml of isopropyl alcohol-water diluent was added, the mixture was shaken by vortex and permitted to cool at room temperature, then the mixture was read using a spectrophotometer at 570 nm. Two types of control solutions were employed: Gelatin solution with all the additives and with the same steps without the enzyme, and the enzyme solution with all the additives and with the same steps without gelatin. The amount of liberated free amino acid was determined by using standard leucine curves. One unit of gelatinenolytic activity was expressed as 1 µmol of leucine equivalents released per minute per ml of enzyme (Bhagwat *et al*., 2016).

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Enzyme activity (U/ml) = umol of liberated leucine / V x T
µmol of liberated leucine: was taken from the standard curve.
V: The volume of enzyme sample. T: The hydrolysis time.

Estimation of protein concentration

The protein content was calculated employing the Bradford (1976) technique, which is based on the binding of Coomassie brilliant blue G-250 stain with protein to generate the appearance of blue color, by consulting a standard curve of bovine serum albumin. Protein concentration was estimated by mixing 0.4 ml of 0.05M phosphate buffer pH 7, 0.1 ml of the sample, and 2.5 ml of Coomassie brilliant blue G-250 stain, the mixture was left for 5 min. 0.5 ml of 0.05M phosphate buffer pH 7 and 2.5 ml of Coomassie brilliant blue G-250 stain were added to the preparation of blank. Absorbency was measured at wavelength of 595 nm using spectrophotometer Figure (2). The protein concentration standard curve was schemed using the relationship between absorbance (595 nm) and the concentration of bovine serum albumin (mg/ml).

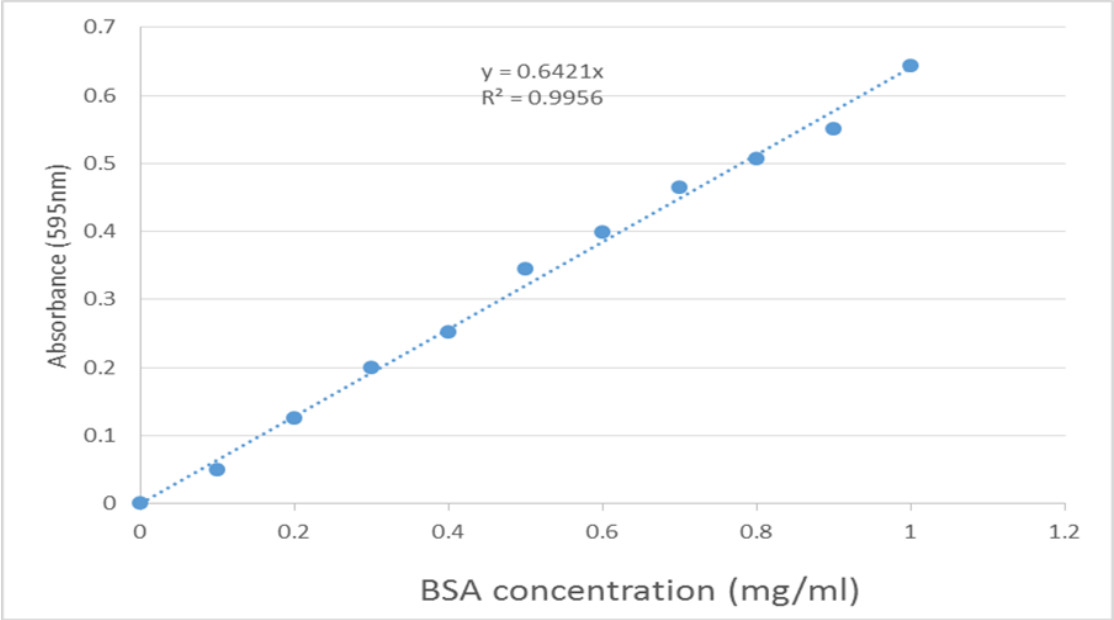

Figure (2): Standard curve of bovine serum albumin

Determination of specific activity

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Specific activity of the enzyme was calculated using the following formula (Whitaker & Bernard, 1972):

$$Specific\ activity\left(\frac{unit}{mg}\right)=\frac{activity(unit/ml)}{Protein\ concentration(mg/ml)}$$

Extraction of gelatinase from *P. aeruginosa*

Extraction of gelatinase enzyme from selected isolate was performed, the selected isolate was grown in the production medium, the flask was incubated in rotary shaker incubator at 37°C for 48 hours at 120 rpm. After that, the bacterial growth was centrifuged at 10,000 rpm for 10 min at 4°C. The clear supernatant was used to measure enzyme activity, protein concentration, and purification experiments.

Purification of gelatinase from *P. aeruginosa*

The gelatinase enzyme was purified according to Sharma *et al.*, (2006) through the following steps:

Precipitation of enzyme by ammonium sulfate

The ammonium sulfate was added to the crude enzyme with gradual saturation ratios ranging between 40% to 80% under cooled conditions with continuous stirring, then it kept at 4°C overnight and then centrifuged at 10,000 rpm for 10 min. The resulting precipitate was liquefied in a minimum volume of 0.05 M phosphate buffer (pH 7.0). Enzyme activity and protein content were measured after every step of ammonium sulfate precipitation.

Dialysis

Enzyme solution acquired after ammonium sulfate precipitation at a suitable saturation ratio was loaded inside a dialysis bag and placed inside a sterile large container at 4°C for dialysis against 0.05 M phosphate buffer (pH 7.0) overnight, then enzyme efficacy and protein content were measured.

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Purification by ion exchange chromatography.

This step was accomplished using a DEAE-Cellulose column which was prepared based on Whitaker and Bernard, (1972). A DEAE-Cellulose column size (2x20cm) was pre-equilibrated and charged with sodium chloride solution and followed by distilled water before it was equilibrated with hydrochloric acid solution. The dialyzed enzyme was loaded onto the ion exchange chromatography column and the column was washed with 0.05 M phosphate buffer (pH 7.0) and the attached proteins were eluted step wise from the column with increasing concentration of sodium chloride ranging from 0.1 to 1 M and the absorbance of each fraction was measured by a spectrophotometer at 280 nm. The activity of enzymes and protein concentration were determined.

Purification by gel filtration chromatography

Gelatinase enzyme was purified by gel filtration chromatography using Sephadex G-150 column and Sephadex G-150 was prepared according to the instructions given by Pharmacia Fine Chemicals Company. An aliquot of highly concentrated fractions obtained after the ion exchange purification step was poured onto the Sephadex G-150 column size (1.5x80cm), pre equilibrated with 0.05 M phosphate buffer (pH 7.0). Elution was done at a flow rate of 3 ml/ fraction using the same buffer for equilibration. The absorbance at 280 nm of each fraction was measured, then the fractions of the peaks were collected and the enzyme activity and protein concentration were measured.

Molecular Weight Determination

SDS-PAGE was performed using the Laemmli procedure described by Garfin, (1990). A 30% acrylamide-bisacrylamide gel in Tris-glycine buffer (pH 8.3) was used for SDS-PAGE. The enzyme was run on a denaturing polyacrylamide gel along with a protein marker. (Proteins with different known molecular weights were used, ranging between10-180 KD). Protein bands were identified using Coomassie blue staining Ibrahim et al., (2024). The molecular weight of gelatinase was calculated using relative mobility (Rm) as given below:

$$Rm = \frac{Distance\ of\ protein\ band\ traveled\ (mm)}{Distance\ of\ Bromomphenol\ blue\ dye\ front\ (mm)}$$

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Effect of temperature on gelatinase activity

The optimal temperature for gelatinase activity was determined by measuring enzyme activity across a temperature range of 25–47°C, (25, 30, 37, 42, 47°C). The reaction mixture was incubated at each designated temperature, and enzyme activity was measured as previously described. The relationship between enzyme activity and temperature was plotted to define the optimal temperature (Abood *et al.*, 2018). All experiments were performed in triplicate (n = 3) independent trials, and results were expressed as Mean ± SD.

Effect of temperature on gelatinase stability

The thermal stability of the purified gelatinase was assessed by pre-incubating the enzyme in a water bath at temperatures ranging from 25 to 47°C for 30 min, followed by immediate transfer to an ice bath to halt further denaturation. Residual enzymatic activity was then measured and expressed as a percentage of the initial (untreated) activity, which was plotted against temperature (Abood *et al.*, 2018). Experiments were conducted in triplicate (n = 3), and data are presented as Mean ± SD. (Abood *et al*,. 2018).

Effect of pH on gelatinase activity

The effect of pH on purified gelatinase activity was evaluated using gelatin as substrate, prepared in the following buffer systems: 0.02 M sodium acetate buffer (pH 4.0, 5.0, 6.0), 0.02 M sodium phosphate buffer (pH 7.0), and 0.02 M Tris-base buffer (pH 7.0, 8.0, 9.0). Gelatinase activity was measured under each pH condition to determine the optimal pH for enzyme activity (Sakurai *et al.*, 2009). All assays were performed in triplicate (n = 3), and results are expressed as Mean ± SD.

Effect of pH on gelatinase stability

The effect of pH on gelatinase stability was determined by mixing equal volumes (1:1) of purified enzyme with buffers of varying pH (4.0–9.0), followed by incubation in a water bath at 37°C for 30 min. Samples were then immediately transferred to an ice bath to stop the reaction. Residual enzymatic activity (%) was assayed and plotted against pH to define the optimal pH for gelatinase stability (Yasuko *et al.*, 2014). Experiments were performed in triplicate (n = 3), and data are presented as Mean ± SD.

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Statistical Analysis

All experiments were performed in triplicate (n = 3), and data are expressed as Mean ± Standard Deviation (SD). Statistical differences among groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons, using SPSS software (version 20.0). Means bearing different superscript letters within the same column are significantly different at $P < 0.05$.

Results & Discussion

Identification of *P. aeruginosa* isolates

A total of 100 different clinical specimens (urine, burn and wound swabs) were obtained from several hospitals in Baghdad city from September to December-2025 .Forty isolates (40%)belonged to *P. aeruginosa*, of these 17 (42.5%) were recovered from wound infections, 13 (32.5%) from urinary tract infections (UTIs), and 10 (25%) from burn wounds .While 40(40%) belonged to other bacteria genera and 20(20%) were reported as negative growth.Table(1). All isolates were identified according to culture, microscopic examination, biochemical tests and VITEK® 2 Compact system. Isolates of Pseudomonas aeruginosa were screened and isolated that produce gelatinase. These strains grow in MacConkey agar producing a light blue, non-fluorescent pigment (pyocyanin) and form smooth, spherical colonies on the media, due to the lack of lactose. *P.aeruginosa* is the only bacteria that produces pyocyanin pigment which diffuses in the culture media and allows smooth growth of yellow-green colonies on Cetrimide agar. Pseudomonas spp. are straight or slightly curved red (Gram-negative) bacteria that can be seen singly, in pairs, or in short chains on a Gram stained smear prepared from previous culture. The biochemical properties of the *P.aeruginosa* isolates are listed in Table (1).

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Table (1): Distribution of *P.aeruginosa* according the types of specimens

Type of specimens	number of isolates	%
Wound	**17**	**42.5**
Urine	**13**	**32.5**
Burn	**10**	**25**
Total	**40**	**100**

Table (2): Identification of *P. aeruginosa* isolates by Biochemical, gram staining, growth conditions, and pyocyanin pigment production tests.

No.	Test	Result
1	Gram stain	-ve
2	Catalase enzyme	+
3	Oxidase enzyme	+
4	gelatinase enzyme	+/-
5	Indole	-
6	Methyl red	-
7	Voges Proskauer	-
8	Citrate	+
9	Hemolysin	Beta hemolysis
10	Growth at 42 °C	+

+ Positive; -: Negative; +/-: Variable result.

For urinary tract infections, the proportion found in this study (32.5%) is higher than figures from Port Sudan, where *P. aeruginosa* made up 9.5% of all positive UTI isolates (Ibrahim *et al.*, 2025). This difference may reflect geographic variation or differences in patient populations. Regarding burn wounds, the 25% isolation rate observed here closely aligns with a study from Duhok, Iraq, which reported that *P. aeruginosa* represented 27.0% of burn isolates (Ali & Assafi, 2024).

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Evaluation of gelatinase production by *P.aeruginosa* isolates

Three screening methods were used to evaluate the ability of *P. aeruginosa* isolates to produce gelatinase. these included qualitative, semi-quantitative, and quantitative assays.

Qualitative detection of gelatinase production

Among the forty *P. aeruginosa* clinical isolates subjected to the nutrient gelatin stab procedure, gelatin liquefaction persisted in 28 isolates (70%) upon refrigeration at 4°C, indicating active gelatinase production, whereas the remaining 12 isolates (30%) showed no evidence of hydrolytic activity, as the medium retained its gel-like consistency throughout the observation period. When compared with findings from recent investigations, the proportion recorded here appears modest. A gelatinase positivity rate of 84.26% was noted by Saha *et al.* (2025) among a comparable clinical series, and an even higher figure of 93.3% emerged from the work of Al-Daghistani *et al.* (2025).

Semi-quantitative screening of gelatinase production

The forty clinical *P. aeruginosa* using the gelatin agar plate method. The results revealed that 28 isolates (70**%)** isolates were screened for gelatinase production were positive, and indicated by the formation of clear zones of varying diameters surrounding the colonies on the gelatin agar medium, while the remaining 12 isolates (30%) showed no hydrolytic activity, as depicted in Figure (3). These findings were fully consistent with those obtained by the nutrient gelatin stab method, in which the same 28 isolates (70%) showed gelatin liquefaction, thereby supporting the reliability of both qualitative assays. The results obtained in this study are in close agreement with those reported by Pramodhini *et al*. (2016), who found that 71.4% of *Pseudomonas aeruginosa* isolates were positive for gelatinase production.

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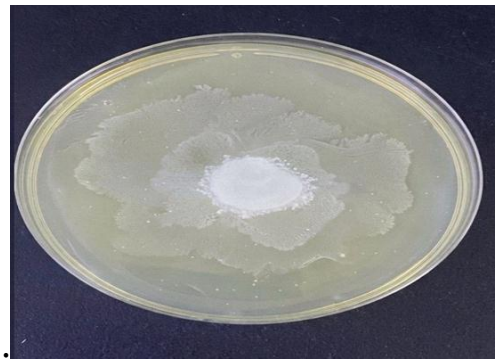

Figure (3): Gelatinase detection in *P. aeruginosa* on nutrient gelatin agar plates.

Quantitative screening of gelatinase production

For further selection of the most productive isolates, the 28 *P. aeruginosa* isolates that tested positive in primary screening were subjected to quantitative assessment of their gelatinolytic activity using submerged fermentation medium.

The results revealed that all tested isolates were gelatinase producers with variable degrees, and the specific activity in culture filtrates ranged from 0.004 to 0.496 U/mg protein .This variability in gelatinase production among clinical isolates is consistent with previously reported findings; Aqel *et al.* (2023) demonstrated that production levels differed according to the infection site of isolation, with wound and urinary tract isolates yielding the highest enzymatic activity. Notably, Edward *et al.* (2023) linked this inter-isolate variation to the differential carriage of the lasB gene, which encodes the primary gelatinolytic enzyme in *P. aeruginosa*, thereby implicating genotypic diversity as a key determinant of gelatinase output among clinical strains.

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This technique constitutes a quantitative test of gelatinase activity based on gelatin substrate hydrolysis. When gelatinase cleaves gelatin, free amino groups are released; these are subsequently measured colorimetrically using the ninhydrin reagent, producing a purple color proportional to amino acid concentration. Zhang *et al.* (2013) established and validated this ninhydrin-based approach as a reliable spectrophotometric method for quantifying gelatinolytic activity, expressed as micromoles of leucine equivalent per minute per milliliter of culture filtrate. According to these findings, *P. aeruginosa* isolate Ps.32 was selected for subsequent gelatinase extraction, purification, and characterization, as it demonstrated the highest specific gelatinase activity among all tested isolates.

Purification of gelatinase from *P. aeruginosa*

Ammonium sulfate precipitation

The crude gelatinase extracted from selected isolate (Ps.32) was precipitated using ammonium sulphate at different saturation levels. Among the tested levels, 60% saturation gave the highest enzyme activity (1 U/ml) and specific activity (3.3 U/mg protein), with a purification fold of 1.36 and a yield of 74.1%, as shown in Table (3), and was selected for the next purification steps. Proteins dissolve in water because their amino acid side chains form hydrophilic bonds with water molecules. Ammonium sulphate breaks these bonds by reducing the water available, which lowers protein solubility and causes it to precipitate. The saturation level needed differs from one protein to another based on its hydrophobicity, where more hydrophobic proteins need higher concentrations to precipitate (Khwen, 2021). After precipitation, the enzyme solution was dialyzed against 0.1 M Tris-HCl buffer with 50 mM $CaCl_2$ at pH 7.5 for 24 hours at 4°C with continuous stirring, then concentrated by sucrose to 15 ml, which is an important step to remove excess salts before chromatographic purification.

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Ion exchange chromatography (DEAE-Cellulose)

The dialyzed gelatinase sample was concentrated with sucrose to 10 ml and loaded onto a DEAE-cellulose column. The column was first washed with 0.05 M phosphate buffer (pH 7.0) to remove uncharged and positively charged proteins. Then, negatively charged proteins were eluted using a gradient of NaCl from 0.1 to 1 M. Absorbance was measured at 280 nm for each fraction. Figure(4), one protein peak appeared during the washing step, while two protein peaks appeared after elution with the NaCl gradient. The two peaks from the elution step were combined and tested for gelatinase activity. The results showed that the eluted proteins (fractions 58to 65) had the highest activity, reaching 0.9 U/ml. The specific activity of gelatinase in this step was 9 U/mg protein. The purification fold was 3.67, and the enzyme yield was 58.77%. These findings indicate that the gelatinase produced by P. aeruginosa has a negative net charge because it bound to the anionic DEAE-cellulose column. Previous studies have used similar methods. Samaan *et al.* (2012) purified a protease from *P. aeruginosa* isolated from wound and burn infections using DEAE-cellulose and Sephadex G-100. Hussein et al. (2024) purified a protease from UV-mutated *P. aeruginosa* using ammonium sulfate precipitation DEAE-cellulose, and Sephadex G-200.

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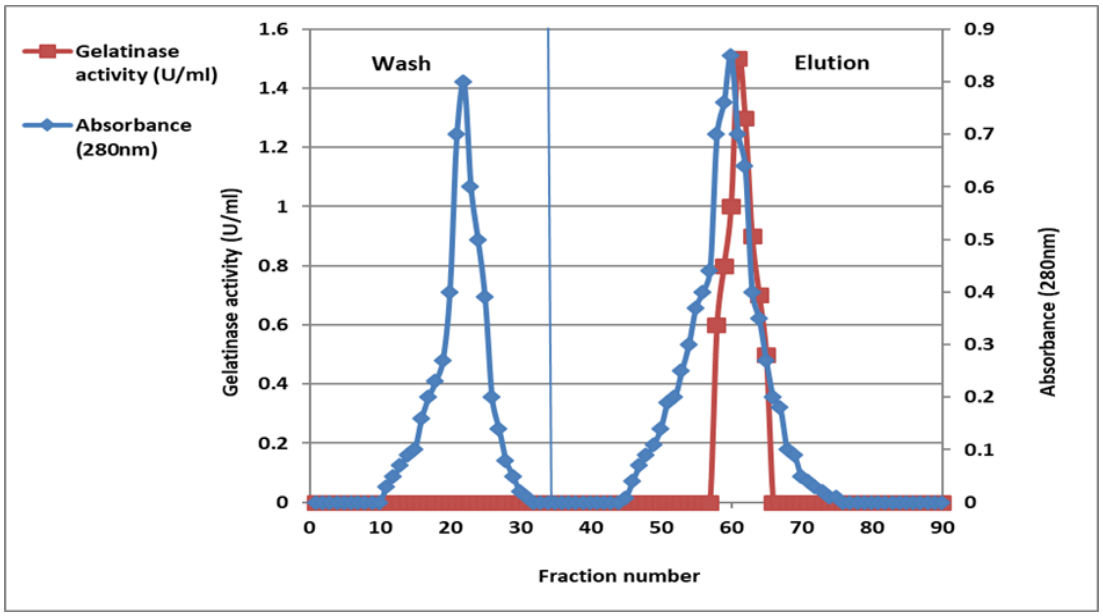

Figure (4): Ion exchange chromatography of gelatinase on DEAE-cellulose column.

Gel filtration chromatography

To complete the purification, the most active fractions collected from the DEAE-cellulose step (fractions 58-65) were pooled and concentrated with sucrose to a final volume of 10 ml. This concentrate was then loaded onto a Sephadex G-150 column that had been pre-equilibrated with 0.05 M phosphate buffer (pH 7.0). Elution was performed using the same buffer. As illustrated in Figure (5), a single, large protein peak emerged after elution. When the collected fractions were assayed for gelatinase activity, only one active peak was detected, which corresponded to fractions (25-31). From the data summarized in figure (4), the specific activity of the purified gelatinase reached 11.4 U/mg protein. The overall purification fold was calculated to be 4.6, and the final enzyme yield was 45.71%.

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Gel filtration has also been successfully applied to purify proteases from P. *aeruginosa* by other research groups. For instance, Obady and Rasheed (2025) used a Sephadex G‑150 column to purify a protease from a clinical isolate of P. *aeruginosa* and achieved a considerable increase in specific activity. In another study, Abdulwahhab *et al.* (2024) reported a marked rise in specific activity following gel filtration for a protease obtained from the same bacterial species. These reports collectively confirm that Sephadex G‑150 chromatography is an effective tool for recovering active gelatinase from *P. aeruginosa*.

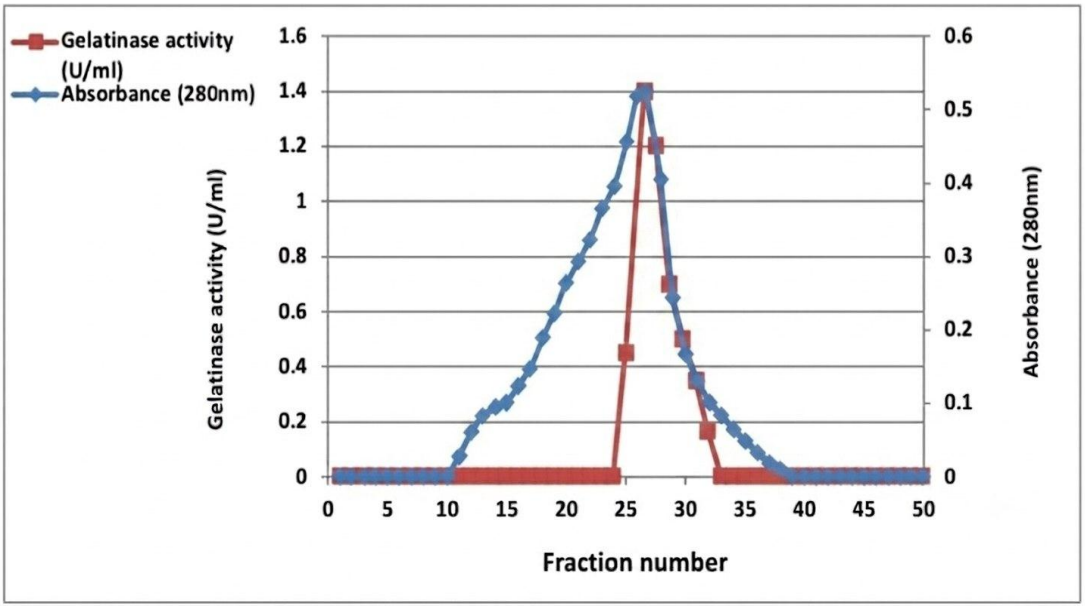

Figure (5).Gel filtration chromatography of gelatinase purified from; *P. aeruginosa* by using Sephadex G-150 column (1.5x80) cm.

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Table (3): Purification steps of gelatinase produced by *P. aeruginosa*

Purification step	Volume (mL)	Enzyme activity (U/mL)	Protein concentration (mg/mL)	Total protein (mg)	Specific activity (U/mg protein)	Total activity (U)	Purification (folds)	Yield (%)
Crude enzyme	75	0.49	0.2	15.0	2.45	36.75	1	100
Ammonium sulphate precipitation	25	1.0	0.3	7.5	3.3	25.0	1.34	68.02
Ion exchange on DEAE-cellulose	24	0.9	0.1	2.4	9.0	21.6	3.67	58.77
Gel filtration	21	0.8	0.07	1.47	11.4	16.8	4.65	45.71

U/mL: Units per milliliter; mg/mL: milligrams per milliliter; U/mg protein: Units per milligram protein; U: Units.

The purification fold obtained in the present study (4.6) is relatively modest compared with some previously reported enzyme purifications employing more extensive multi-step protocols, yet comparable to studies using similar three- to four-step purification schemes. For instance, a *Bacillus amyloliquefaciens* alkaline protease purified through a four-step protocol reached a purification fold of 6.48 (Guleria *et al.*, 2016), a value in the same general range as that obtained in the present study. In contrast, *Pseudomonas aeruginosa* A2 elastase, purified from culture supernatant using ultrafiltration, gel filtration, and anion-exchange chromatography, showed a comparatively lower purification fold of 2.23 with 64.3% recovery (Ghorbel-Bellaaj *et al.*, 2012), indicating that the extent of fold-increase during purification varies substantially even among proteases from the same bacterial species, depending on the enzyme's initial purity in the crude extract and the specific purification protocol employed. Taken together, these comparisons suggest that the moderate purification fold achieved in the present study (4.6) falls within the range commonly reported for extracellular bacterial enzymes purified using multi-step chromatographic protocols, and likely reflects the relatively high initial purity of gelatinase in the crude extract combined with the expected progressive loss of activity across purification steps, as reflected in the yield declining from 100% to 45.71%.

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Estimation of the molecular weight of purified gelatinase

The gelatinase purified from *P. aeruginosa* was electrophoresed using SDS-PAGE alongside standard protein markers. The results revealed a single protein band, indicating the homogeneity of the enzyme preparation Figure (6). By comparing its migration distance with those of the marker proteins, the molecular weight of the purified gelatinase was estimated to be approximately 34 kDa.

Similar molecular masses have been reported for related proteases from *P. aeruginosa* and other bacteria. Ghorbel‐Bellaaj *et al.* (2012) purified an elastase from *P. aeruginosa* A2 with an estimated molecular mass of 34 kDa. Jellouli *et al.* (2008) also characterized a 34 kDa metalloprotease (elastase) from *P. aeruginosa* .Furthermore, the gelatinase (GelE) from *E. faecalis* has a calculated molecular weight of 34,582 Da (Su *et al.*, 1991). The molecular weight of the gelatinase obtained in this study (34 kDa) is in close agreement with these reported values, particularly those of the elastase enzymes from different *P. aeruginosa* strains (Ghorbel‐Bellaaj *et al.*, 2012; Jellouli *et al.*, 2008).

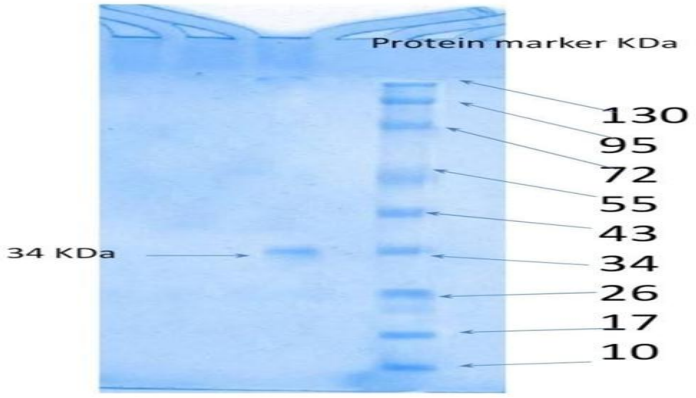

Figure (6): SDS-PAGE chromatograph of gelatinase purified from *P. aeruginosa*

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Log	Rf	KDa
5.11	0.15	130
4.98	0.3	95
4.85	0.4	72
4.74	0.45	55
4.63	0.54	43
4.53	0.63	34
4.41	0.7	26
4.23	0.74	17
4.00	0.8	10

Figure (4): Log molecular weight of gelatinase produced by *P. aeruginosa* after electrophoresis.

Effect of temperature on gelatinase activity

The influence of temperature on gelatinase activity was assayed across a range of 30–47°C. The mean activity values (U/mL) were 1.74 at 30°C, 2.50 at 37°C, 2.18 at 42°C, and 2.09 at 47°C (Figure 8). Maximum activity was observed at 37°C, while a clear decline in activity was noted above 42°C. The overall variation was statistically significant ($P < 0.05$). The optimal activity at 37°C likely reflects an evolutionary adaptation of *P. aeruginosa*, an opportunistic human pathogen, to the physiological temperature of its host environment, allowing its extracellular gelatinase to function most efficiently during colonization and tissue invasion. The subsequent decline in activity above 42°C is consistent with thermal denaturation, whereby elevated temperatures progressively disrupt the enzyme's tertiary structure and reduce its catalytic efficiency — a typical feature of mesophilic bacterial enzymes.

This thermal profile aligns with findings from other gelatinase-producing microorganisms, though the degree of similarity appears closely tied to each organism's ecological niche. Sai-Ut *et al.* (2014) reported an optimal

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activity temperature of 37°C for gelatinolytic enzymes from *Bacillus amyloliquefaciens* H11, consistent with the present findings. In contrast, Masurkar *et al.*
(2025) reported a considerably lower optimum of 20°C for a gelatinase from *Serratia marcescens* isolated from organic waste, likely reflecting adaptation to ambient environmental conditions rather than a host-associated niche. As Podar *et al.* (2020) noted, microbial thermal optima generally mirror each organism's predominant ecological habitat. The 37°C optimum observed here therefore appears functionally significant rather than coincidental, supporting the role of this gelatinase as a virulence factor that enables *P. aeruginosa* to efficiently degrade host collagen and gelatin-containing tissues during infection, contributing to tissue invasion and persistence within the host.

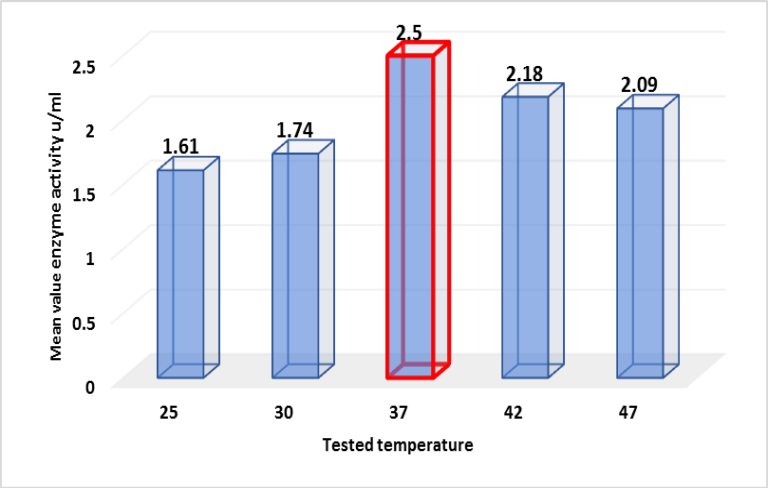

Figure 8): Activity of gelatinase at different Temperature values
Values are expressed as Mean ± SD (n = 3). ($P < 0.05$).

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Table (4) :Effect of temperature on gelatinase activity

Tested temperature	enzyme activity u/ml Mean± SD
25	**1.61±0.1**
30	**1.74±0.3**
37	**2.5±0.2**
42	**2.18±0.2**
47	**2.09±0.1**
P value	**< 0.05**

Effect of Temperature on Gelatinase Stability

Purified gelatinase was incubated at various temperatures ranging from 25 to 47 °C for 30 minutes to assess the influence of temperature on enzyme stability. The enzyme was most stable at 37 °C, with a recorded stability of 2.39 U/ml. At 30 °C, the stability was 1.29 U/ml, while at 42 °C and 47 °C, the values were 1.72 U/ml and 1.62 U/ml, respectively Figure (9) Table (5) .A comparison with previously reported data reveals close agreement. Pathade *et al.* (2024) characterized gelatinase-producing microorganisms from soil, including *P. aeruginosa* and *B. subtilis*, and reported that the *P. aeruginosa* enzyme retained 75% stability at 37°C, while the *B. subtilis* enzyme retained 80% under the same conditions. This close correspondence, despite the two species occupying different ecological habitats, suggests that thermal stability at 37°C may be a broadly conserved feature among bacterial gelatinases rather than a trait unique to pathogenic strains. This thermal robustness is further supported by Saha (2025), who demonstrated that clinical isolates of *P. aeruginosa* consistently produce gelatinase across different colonial morphotypes, indicating the enzyme's inherent stability under physiological conditions.

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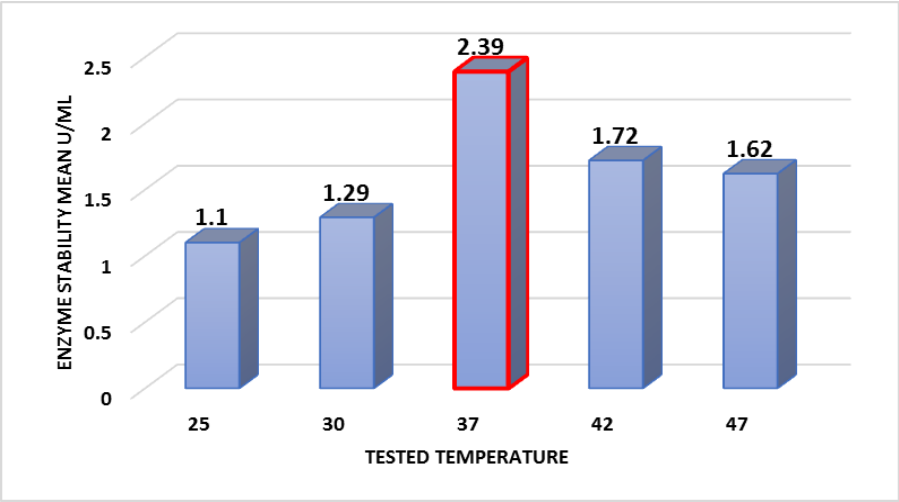

Figure (9): Stability of gelatinase at different Temperature values.

Effect of pH on gelatinase activity

Table (5): Effect of temperature on gelatinase stability

Tested temperature	enzyme stability Mean±SD u/ml
25	1.1±0.1
30	1.29±0.1
37	2.39±0.4
42	1.72±0.1
47	1.62±0.2
P value	<0.001

Values are expressed as Mean ± SD (n = 3). ($P < 0.001$).

The effect of pH on the gelatinase activity was tested at pH 4–9. The purified gelatinase was found to be active over a wide range of pH from 4 to 9 with optimum activity at pH 7.0 (2.91 U/ml) as shown in the results given in figure (10) Table (6). While the enzyme retained high activity at pH 8.0 (2.80 U/ml) and pH 9.0 (2.70 U/ml), a clear decline in activity was noted at pH 4.0–6.0, with the lowest values recorded at pH 4.0 (2.33 U/ml) and pH 5.0 (2.35 U/ml). These findings indicate that the gelatinase is most active at neutral pH where it prefers slightly alkaline pH rather than strongly acidic pH. These findings agree with Pathade *et al*. (2024), who reported an optimal pH of 7.0 for *P. aeruginosa* gelatinase,

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reinforcing pH 7.0 as a characteristic optimum across strains of this species. Similarly, Abdulwahhab *et al.* (2024) found maximal protease activity at pH 8.0 in clinical *P. aeruginosa* isolates, remaining stable across neutral-to-alkaline conditions — together indicating that extracellular proteolytic enzymes of this pathogen are consistently adapted to the neutral-to-mildly-alkaline, host-like environment rather than to acidic niches.

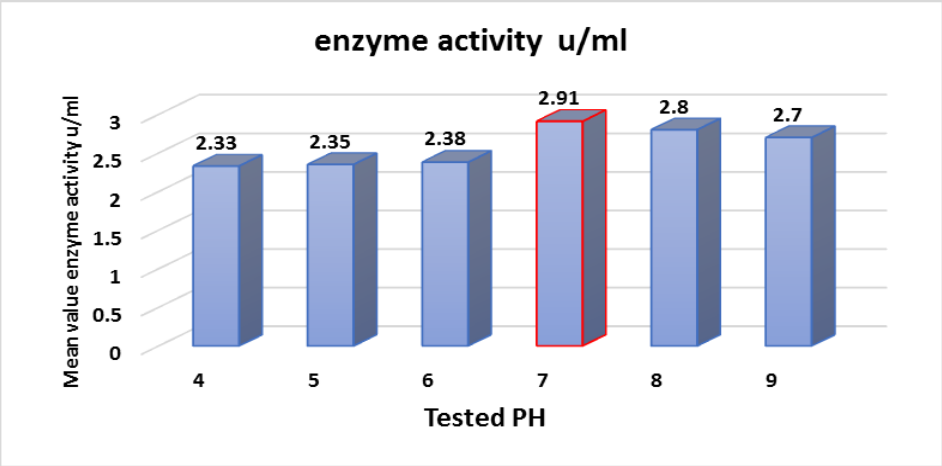

Figure (10): Activity of gelatinase at different pH values

Table (6): Effect of pH on gelatinase activity

Tested pH	enzyme activity u/ml Mean±SD
4	2.33±0.4
5	2.35±0.5
6	2.38±0.3
7	2.91±0.2
8	2.8±0.1
9	2.7±0.21
P value	0.05 sig

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Effect of pH on Gelatinase Stability

The stability of gelatinase was assessed across a pH range of 4 to 9. The results obtained indicated that the enzyme remained stable over a broad pH range from 5 to 9. The highest stability was recorded at pH 7.0 (3.20 U/ml). At pH 6.0 and pH 8.0, the enzyme showed stability values of 2.82 U/ml and 2.90 U/ml, respectively. However, enzyme stability decreased at pH 4.0, giving a value of 2.58 U/ml Figure (11) Table (7). the overall trend indicates that the enzyme retains greater structural stability under neutral-to-slightly-alkaline conditions than under acidic conditions, mirroring the physiological pH of host tissues (≈7.35–7.45) encountered by *P. aeruginosa* during infection. This broad pH stability, together with the neutral-pH optimum, suggests that the enzyme is well-suited to remain functionally intact within the pH range typically encountered in host extracellular environments, supporting its potential role in sustained tissue degradation throughout the course of infection. These findings are consistent with Pathade *et al.* (2024), who reported that gelatinase from *P. aeruginosa* retained 70% stability at pH 7.0, reinforcing neutral pH as a favorable condition for both the activity and structural stability of this enzyme across different *P. aeruginosa* strains.

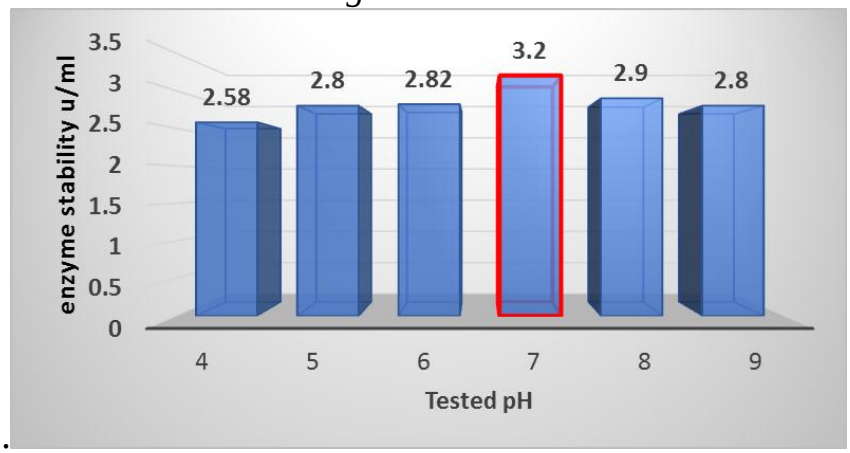

Figure (11): Stability of gelatinase at different pH values

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Table (7); Effect of pH on gelatinase stability

Tested pH	enzyme stability Mean±SD u/ml
4	2.58±0.3
5	2.8±0.1
6	2.82±0.2
7	3.2±0.5
8	2.9±0.3
9	2.8±0.2
P value	0.04 sig

Study Limitations

While the present study provides valuable insight into the purification, biochemical characterization, and physicochemical properties of gelatinase from *P. aeruginosa*, several limitations should be acknowledged. First, the purification and characterization were conducted on a single bacterial isolate; consequently, the findings may not fully capture the extent of biochemical variability that could exist among different *P. aeruginosa* strains, and broader conclusions regarding species-wide enzymatic behavior should be drawn with appropriate caution. Second, the molecular identity of the purified enzyme was inferred from its biochemical and physicochemical properties, without genetic characterization such as sequencing of the corresponding gene; molecular confirmation would strengthen the identification of the enzyme and enable more precise comparisons with previously characterized gelatinases at the genetic level. Third, the kinetic parameters of the enzyme, including the Michaelis constant (Km) and maximum reaction velocity (Vmax), were not determined in this study; these parameters are essential for a complete understanding of the enzyme's catalytic efficiency and substrate affinity, and their absence limits direct comparison with the kinetic behavior of gelatinases reported in other studies. Future studies incorporating multiple bacterial isolates, molecular

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and genetic characterization, and detailed kinetic analysis are recommended to build upon and extend the findings presented here.

Potential Biomedical and Industrial Applications

Beyond its role as a virulence factor, the physicochemical properties of the purified gelatinase — including its stability and optimal activity near physiological temperature and neutral pH — suggest potential relevance in both medical and industrial contexts. From a medical perspective, extracellular proteases such as LasB elastase have been proposed as promising targets for anti-virulence therapeutic strategies aimed at inhibiting bacterial proteolytic activity without directly targeting bacterial viability, thereby limiting tissue damage during infection while potentially reducing selective pressure for antibiotic resistance (Everett & Davies, 2021). From an industrial standpoint, bacterial gelatinases have recently gained attention as sustainable, eco-friendly alternatives to conventional chemical agents in leather processing, particularly in enzymatic dehairing, owing to their ability to degrade gelatin- and collagen-containing substrates while reducing chemical usage and wastewater toxicity (Bizualem *et al.*, 2026). Further studies evaluating the enzyme characterized in the present study under industrially relevant conditions would be needed to assess its practical applicability in such settings.

Conclusion

Gelatinase production was detected in a high proportion of the tested isolates, supporting its role as a common virulence factor associated with wound, burn, and urinary tract infections caused by *P. aeruginosa*. The purified enzyme (34 kDa) exhibited a purification fold of 4.6, with optimal activity and stability at 37°C and pH 7.0, consistent with a mesophilic metalloprotease adapted to host physiological conditions and supporting its role in tissue invasion during infection. These findings enhance our understanding of *P. aeruginosa* pathogenicity and highlight the enzyme's potential relevance for future anti-virulence therapeutic strategies and sustainable industrial applications, such as eco-friendly leather processing.

Ethics Approval

This study was approved by the ethical committee of Mustansiriyah University, Baghdad, Iraq and the ethical approval code is BCSMU/10025/00083M, Date 15/9/2025. Written consents of the participants were provided.

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تنقية وتوصيف إنزيم الجيلاتينيز من عزلات بكتيريا الزائفة الزنجارية *Pseudomonas aeruginosa* المحلية المعزولة من عينات سريرية

مستخلص البحث:

جمعت مائة عينة سريرية من مرضى يعانون من خمج (التهابات) الجروح، الحروق، والمسالك البولية في مستشفيات مختلفة في بغداد خلال الفترة من أيلول/سبتمبر إلى كانون الأول/ديسمبر 2025. وباستخدام الطرق الميكروبيولوجية القياسية (الاستنبات على أوساط ماكونكي وسيتريميد آغار، صبغة غرام، والاختبارات الكيموحيوية) متبوعة بفحص تأكيدي باستخدام نظام "VITEK 2 compact" ، تم تشخيص أربعين عزلة (40%) تعود لبكتيريا الزوائف الزنجارية(*Pseudomonas aeruginosa*) . تم اختبار قدرة عزلات *P. aeruginosa*على إنتاج إنزيم الجيلاتينيز مظهرياً على وسط آغار الجيلاتين المناسب، وكذلك بواسطة الفحص الكمي الذي أكد قدرة البكتيريا على إنتاج الإنزيم. وأظهرت نتائج الاختبار أن 28 عزلة (70%) كانت موجبة لإنتاج الجيلاتينيز على وسط الجيلاتين. وقد تم اختيار العزلة رقم 32، والتي أظهرت أعلى إنتاجية للإنزيم، لغرض التنقية وتحديد الخصائص .تم استخلاص إنزيم الجيلاتينيز الخام، وجرت عملية تنقيته عن طريق الترسيب بكبريتات الأمونيوم عند نسبة إشباع 60%، ثم الديلزة (الفرز الغشائي)، يليه كروماتوغرافيا التبادل الأيوني على عمود "DEAE-Cellulose" ، وأخيراً كروماتوغرافيا الترشيح الهلامي باستخدام "Sephadex G-150". وقد أسفرت خطوة التنقية النهائية عن الحصول على فعالية نوعية بلغت 11.4 وحدة/ملغم بروتين، وبمعدل تنقية قدره 4.6 مرات، وحصيلة إنزيمية بلغت 45.71%. كما قدّر الوزن الجزيئي للجيلاتينيز المنقى بنحو 34 كيلودالتون (kDa) باستخدام تقنية الهجرة الكهربائية (SDS-PAGE). وأظهرت الدراسة أن الرقم الهيدروجيني (pH) الأمثل لنشاط الإنزيم كان 7.0، مع إظهاره استقراراً وثباتاً عبر مدى واسع من الأرقام الهيدروجينية (4.0، 5.0، 6.0، و8.0، 9.0). وعلاوة على ذلك، تبين أن درجة الحرارة المثلى لنشاط إنزيم الجيلاتينيز واستقراره هي 37 درجة مئوية.

الكلمات المفتاحية: الزائفة الزنجارية ، انزيم الجلاتينيز ، التنقية ، العزلات السريرية ، عوامل الامراضية.

ملاحظة : البحث مستل من رسالة ماجستير.