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Purification and characterization of collagenase produced by *Enterobacter cloacae* using environmental wastes

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Abstract:

This study focuses on the purification of collagenase EC.3.4.24, which was produced using the bacteria *Enterobacter cloacae* isolated from the local environment, utilizing chicken foot meal as a source of carbon and energy. The enzyme was partially purified through precipitation using ammonium sulfate at 70% saturation, followed by a dialysis process. The purification continued using a DEAE-Cellulose ion exchange column, and finally, gel filtration was performed through a Sephadex G-100 column. The purification steps resulted in a specific activity of 54.63 units/mg, with a purification factor of 3.325 times and an enzyme yield of 4.830%. Additionally, optimal conditions for enzyme activity, including pH, temperature, time for its activity, and enzyme concentration were studied. The results showed that the ideal pH for collagenase activity is 8.0, while the optimal temperature for its activity is 40 degrees Celsius, and the ideal time for its activity is 60 minutes. Additionally, the optimal concentration of the substrate (collagen) was 1.5%.

Keywords: Collagenase, Environmental waste, *Enterobacter cloacae.*

تنقية وتوصيف الكولاجينيز المنتج من بكتريا *Enterobacter cloacae* وبأستعمال مخلفات بيئية

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مستخلص:

تضمنت هذه الدراسة تنقية أنزيم الكولاجينيز EC.3.4.24. المنتج باستخدام بكتريا *Enterobacter cloacae* والمعزولة من البيئة المحلية والتي تستهلك مسحوق أرجل الدجاج كمصدر للكاربون والطاقة. تمت التنقية الجزئية للانزيم بخطوات اشتملت على الترسيب باستخدام أملاح كبريتات الأمونيوم نسبة تشبع 70٪، ثم تبعتها عملية الديلزة ، ثم استمرت عملية التنقية باستعمال المبادل الأيوني DEAE-Cellulose ، واخيراً كان الترشيح الهلامي باستعمال عمود Sephadex G-100 . كانت حصيلة خطوات التنقية هي الحصول على فعالية نوعية بلغت 54.63 وحدة/ ملغم وبعدد مرات تنقية كانت 3.325 مرة وبحصيلة انزيمية بلغت 4.830 ٪. تم توصيف الانزيم المنقى جزئيا لتحديد الظروف المثلى لفعاليته ، اذ اظهرت النتائج ان الرقم الهيدروجيني الامثل لفعالية الانزيم هو 8.0 ودرجة الحرارة المثلى لفعاليته 40 مْ ، بينما بلغ الزمن الامثل لفعاليته 60 دقيقة في حين كان التركيز الامثل للمادة الاساس (الكولاجين) 1,5 ٪ .

الكلمات المفتاحية : الكولاجينيز ، المخلفات البيئية ، *Enterobacter cloacae*.

Introduction

Collagenase EC.3.4.24 belongs to the metalloprotease group, along with many other proteolytic enzymes. Collagenase is a specialized enzyme that acts on the substrate collagen (Wu *et al*., 2023). Collagenase was first discovered in 1949 by Mandl, after being isolated from the mammalian pancreas (Luchian *et al.,* 2022).

Collagenase is an important enzyme that plays a crucial role in breaking down collagen, the main protein in connective tissues of living organisms. This enzyme is considered highly significant in the fields of medicine, industry, and scientific research, as it is used in wound treatment, scar tissue removal, and promoting skin healing. It is also utilized in the pharmaceutical and cosmetic industries due to its role in enhancing tissue regeneration and scar treatment. Additionally, collagenase is used in several industrial applications, such as gelatin production and leather processing, highlighting its importance across various sectors (Herman *et al.,* 2020).

The collagenase family, a class of enzymes specifically responsible for collagen degradation, has received widespread attention due to its important roles in tissue repair, remodeling, and medical fields (Wang *et al.,* 2024). Collagen is the major fibrous component of animal extracellular connective tissue and is found in skin, bones, tendons, teeth, and blood vessels (Alam *et al.,* 2022).

Collagenases can be classified into two groups of applications: the first group includes those used directly in clinical therapy and as experimental reagents in laboratory work, while the second group consists of those in which the reaction products are used as active agents in disease treatment (Oslan *et al.,* 2022).

This study aimed to purify and characterise the collagenase enzyme produced by Enterobacter cloacae using chicken foot meal as a source of carbon and energy, and evaluate enzyme activity in terms of specific activity and recovery rate.

Materials and Methods

Thirty different samples were collected from soil, poultry, and sheep

waste. A series of decimal dilutions were then performed and the isolates were cast onto solid gelatin medium. Primary screening was performed on the same medium using a schematic method, selecting 17 well-grown samples on solid gelatin medium. These isolates were then re-cultivated on the same medium by making a circle in the center of the plate (Wollum, 1982). Six isolates were collected. Secondary screening was performed on pure collagen medium, and by measuring colony diameters, the two most efficient isolates were selected. These isolates were then screened onto three environmental wastes (chicken feet, bedbug skin, and fish skin). The bacteria were identified using culture and biochemical tests, and the diagnosis was confirmed using a Vitek2 device. The bacteria were found to belong to the genus *Enterobacter cloacae*. Then the bacteria were cultured on the liquid medium of the waste.

Estimation of enzyme Concentration

The method described by (Lowry *et al*.,1951) was used to estimate protein. The required concentration was prepared from the original concentration of bovine serum albumin (100 µg/ml), then 4 ml of sodium potassium tartrate solution and Na_2CO_3 solution were added to each tube and left for 10 minutes. 400 µl of folin reagent was added to each tube, shaken well, and left for 30 minutes. The absorbance value was read at a wavelength of 600 nm.

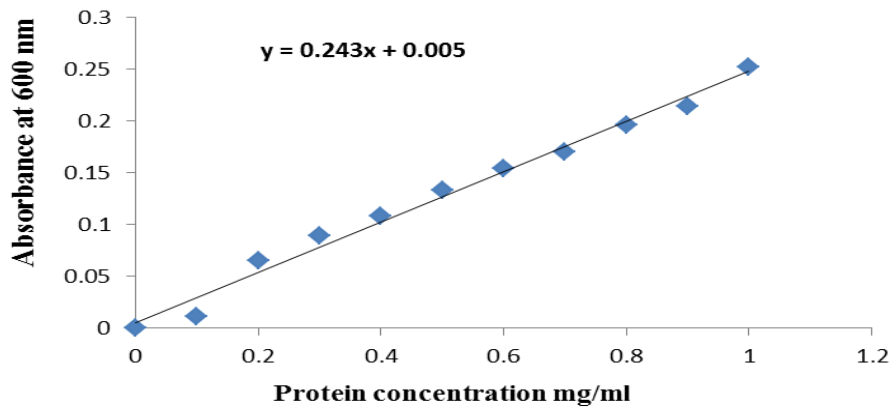

Figure 1: Standard curve for estimating protein concentration

Estimation of collagenase enzyme activity

Enzyme activity was estimated according to the method of (Moore and Stain, 1954).

Method

1. After centrifugation at 4,000 rpm for 10 minutes, 150 microliters of culture filtrate were added to tubes containing 750 microliters of matrix solution, which was prepared by mixing 0.5 g of pure collagen powder with 100 ml of 50 mM Tris-HCl buffer at pH 7.5.

2. The tubes were incubated at 37°C for 10 minutes.

3. 900 microliters of 0.2 M TCA solution were added to the tubes to stop the reaction.

4- The tubes were incubated for 10 minutes at 37°C.

5- The blank solution was prepared in the same manner as above, except that the culture filtrate was not added. Centrifugation was then performed at 4000 rpm for 10 minutes.

6- 1 ml of the filtrate with the above solutions was added to 1 ml of Folin-Ciocalteau reagent and incubated for 10 minutes at 37°C.

7- The absorbance of the filtrate with the reagent was measured at a wavelength of 578 nm using a spectrophotometer.

Analytical activity is defined as: the amount of enzyme that causes an increase in absorbance of 0.01 at a wavelength of 578 nm under experimental conditions.

Enzyme purification

Purification with Ammonium Sulfate

The enzyme was precipitated by gradually adding ammonium sulfate to the crude enzyme extract, stirring continuously on a magnetic stirrer until dissolution at 4°C for 2 hours, until saturation (20-80%) was reached. Centrifugation was then performed at 15,000 rpm for 20 minutes, and the enzyme's analytical activity was determined for each precipitation stage. The precipitate was then collected and dissolved in a small volume of 0.05 M sodium phosphate buffer solution at pH 6.5. Dialysis was performed against distilled water using membrane osmosis bags at 4°C for 24 hours, with water replaced every 6 hours. The resulting solution volume, enzyme activity, and

protein concentration were then determined.

Ion Exchange Chromatography

A - Column Preparation and Exchanger Activation

DEAE Cellulose was prepared according to Whitaker (2018). Twenty grams of DEAE Cellulose were suspended in 500 ml of distilled water and left to stand for 24 hours at room temperature. The material was then washed several times with distilled water until ready for use. It was placed in a filter funnel lined with Whatman No. 1 filter paper. The water column was slowly raised until the material was submerged in water to remove air. Then, it was loaded into a chromatography column (80 x 2.5) and covered with a layer of distilled water. Next, the DEAE Cellulose was washed with a 0.05 M Tris-HCl solution at pH 8 until the filtrate became clear. After that, the air removal process (degassing) was carried out to avoid the formation of bubbles inside the column. The column was balanced with three times its volume of the equilibrium solution (20 mM potassium phosphate buffer solution at pH 7.0) at a flow rate of 30 ml/hour.

B- Method

After dialysis, the enzyme was carefully placed on a 2.5 x 15 cm ion-exchange chromatography (DEAE-Cellulose) column, pre-equilibrated with 20 mM potassium phosphate buffer at pH 7.0 using a syringe. The separated fractions were then collected into appropriate tubes at a flow rate of 1 mL/min, 5 mL per tube, at 25°C. A washing step was then performed using 20 mM potassium phosphate buffer at pH 7.0, while the extraction was performed using gradually varying concentrations of sodium chloride (0-1 M). The absorbance of each fraction was then measured at a wavelength of 280 nm using a spectrophotometer. The enzyme activity and protein concentration in each fraction were also determined.

Gel Filtration Chromatography

A -Gel Preparation

The gel was prepared by suspending 20 grams of Sephadex G100 in 500 ml of distilled water according to the manufacturer's instructions. The mixture was then gently stirred and heated

at 90°C for 5 hours to allow the granules to swell and absorb. The mixture was then allowed to cool, and 0.2% sodium azide was added to prevent microbial growth. Air was removed by degassing, and the 80 x 2.5 cm column was immediately filled and left to stand for approximately 7 hours to stabilize. The column was then equilibrated using a buffer solution (0.05 M sodium phosphate, pH 6.5), equivalent to three times the volume of the gel in the column. The flow rate was then regulated at 30 ml/hour.

B- Method

The enzyme extracted from the previous purification steps was passed over the inner sides of the column near the surface of the gel. Subsequently, a recovery process was conducted using a sodium phosphate solution. The resulting fractions from the column were collected in test tubes at a rate of 3 ml per tube, with a flow rate of 30 ml/hour. The absorbance of each fraction was then measured at a wavelength of 280 nanometers. Enzyme activity was measured in the separated peaks after plotting the relationship between the number of separated fractions and their absorbance at 280 nanometers. The fractions containing the enzyme were collected, and the activity and protein concentration were estimated.

Characterization of the Collagenase Enzyme

Determination of the Optimal pH for Collagenase Activity

The substrate (collagen) was prepared at a concentration of 0.5 g/100 ml using a 0.5 M Tris-HCL solution. The pHs were adjusted to (4,5,6,7,8,9,10). Then, 150 microliters of partially purified enzyme were added to 750 microliters of the substrate at a concentration of 0.5 g/100 ml of a 0.5 M Tris-HCL solution. The solution was incubated for half an hour, after which the enzyme activity was determined at all the aforementioned pH values.

Determining the optimum temperature for collagenase activity

The activity of partially purified collagenase was determined after incubation at 20°C, 30°C, 40°C, and 50°C for 30 minutes. This was achieved by adding 150 microliters of partially purified enzyme to 750 microliters of 0.5 g/100 ml of 0.5 M Tris-HCL at the optimum pH. The enzyme activity of each was

determined.

Determining the optimal time for collagenase activity

The activity of the partially purified enzyme was determined by adding 150 microliters of enzyme to 750 microliters of 0.5 g/100 ml of 0.5 M Tris-HCl substrate at the optimal pH and temperature, and for various incubation periods of 5, 10, 20, 30, 60, 90, and 120 minutes to determine the optimal time for enzyme activity.

Estimating the Optimal Enzyme Concentration

Various concentrations of collagen were prepared (0.5, 1, 1.5, 2, 2.5, and 3%) using 0.5 M Tris-HCl solution at the optimal pH, temperature, and reaction time. Then, 150 microliters of partially purified enzyme were added to 750 microliters of the substrate at the above concentrations. The enzyme activity was estimated for each of the above concentrations.

Results and Discussion

Enzyme Purification

Successive steps were followed to purify the enzyme to achieve the optimal purification level and remove the largest possible amount of protein and impurities present in the enzyme extract, thus achieving high enzyme purity.

Enzyme purification using ammonium sulfate

The enzyme precipitation process with ammonium sulfate showed that the best saturation rate was 70%, with a specific activity of 21.499 units/mg and an enzyme yield of 12.849%, with a purification rate of 1.308 times, as shown in Table 1. The high activity in this step is attributed to the role of ammonium sulfate salts in the activity of collagenase enzyme, as a result of reducing the size of the enzyme model and eliminating impurities. Liu and his collogues (2010) obtained the best enzyme yield of 66.5%, a specific activity of 441 units/mg, and a purification rate of 3.7 times, when using a saturation rate of 75% ammonium sulfate.

Dialysis

To remove ammonium sulfate salts, dialysis bag purification was employed. The results of this step indicated an increase in the specific activity of the enzyme, reaching 35.256 units/mg, compared to the specific activity after

the ammonium sulfate precipitation step (21.499 units/mg). The purification factor was 2.146, and the enzyme yield was 8.276%, as shown in Table 1. This increase was attributed to the role of the dialysis process in concentrating the enzyme after the removal of salts resulting from the ammonium sulfate precipitation process, as well as the removal of some proteins present in the solution. These results are consistent with those reported by Nagano and To (2000), which showed an increase in the specific activity of the enzyme produced by *Bacillus Subtilis* FS-2 bacteria after using dialysis bags. The specific activity of the enzyme increased from 26.91 units/mg to 105.98 units/mg.

Ion Exchange Chromatogram

The results shown in Figure 2 indicate the appearance of protein peaks in the fractions recovered from the ion exchanger. However, the enzyme activity was concentrated in only one peak in fractions (34-40). This is because during the recovery step, as the enzyme passes through, its negative charges are bound to the positive charges of the column material, while proteins with a charge opposite to that of the enzyme pass through the column. The enzyme was recovered by adding a recovery buffer containing a salt gradient of (0.15-1) M sodium chloride. This step demonstrated an increase in the specific activity of the enzyme, reaching 54,860 units/mg protein. The number of purifications was 3,339, and the enzyme yield was 7.963%, as shown in Table (1). Setyahadi and Meinhardt (2012) were able to purify collagenase from *Bacillus licheniformis* F 11.4 using the DEAE-Cellulose ion exchanger, with an enzyme yield of 2.6% and a purification rate of 26.3 times.

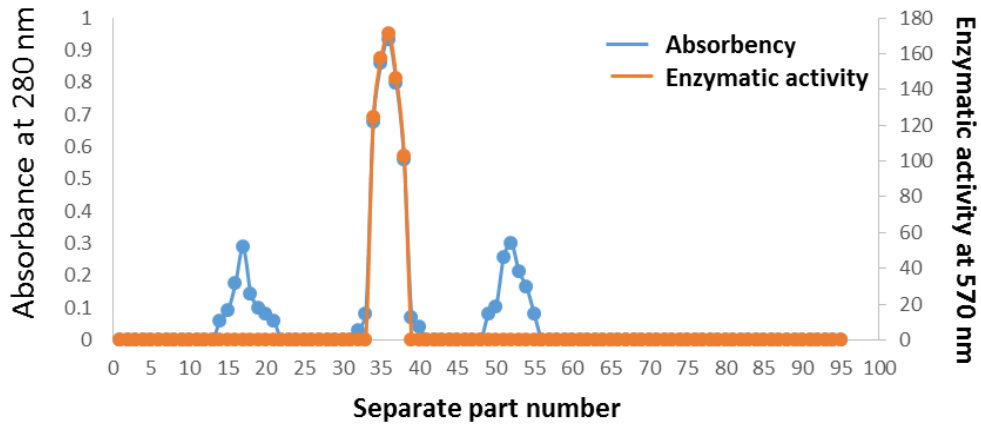

Figure 2: Ion exchange chromatography for purification of collagenase produced **from *Enterobacter cloacae* using the DEAE-Cellulose ion exchanger.**

Gel Filtration Chromatography of Collagenase

The results shown in Figure 3 indicate an increase in enzyme purity when using the gel filtration column. Protein peaks were observed, and the enzyme activities were concentrated in a single peak within fractions (6-14). At this stage of the purification steps, the specific activity reached 54.63 units/mg of protein, with a purification factor of 3.325 times and an enzyme yield of 4.830%, as detailed in Table (1). Liu and his collogues (2010) used gel filtration techniques to purify collagenase from Bacillus cereus LMB13, achieving a specific activity of 2443 units/mg, with a purification factor of 20.4 times.

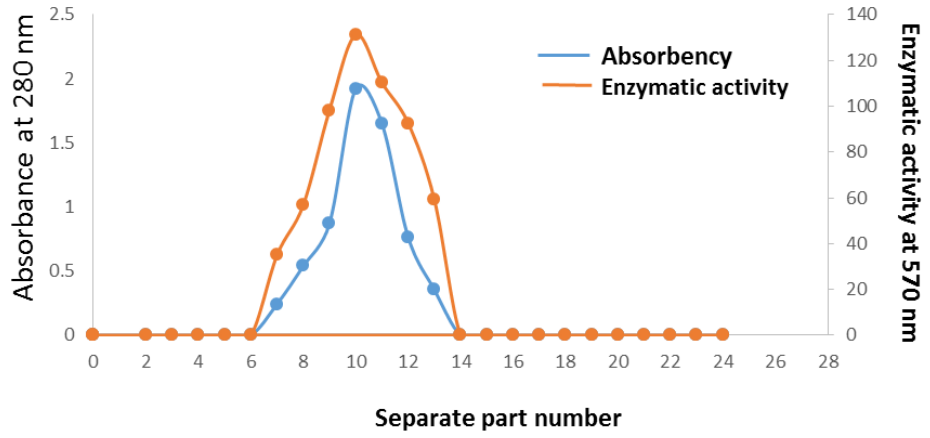

Figure 3: Gel filtration chromatography for purification of collagenase enzyme **produced from *Enterobacter cloacae* using Sephadex G-100**

Table 1: Purification of collagenase enzyme produced from *Enterobacter cloacae* bacterial isolate

Purification steps	Size ml	Activity unit/ml	Protein concentration ml/mg	Specific activity mg/unit	Total Effectiveness Unit	Number of purifications	Enzyme % yield
Raw	500	108.57	6.609	16.427	54285	1	100
Ammonium sulfate precipitation	40	174.38	8.111	21.499	6975.2	1.308	12.849
Dialysis	35	128.37	3.641	35.256	4492.95	2.146	8.276
ion exchanger DEAE Cellules	25	172.92	3.152	54.860	4323	3.339	7.963
gel filtration Sephadex G100	20	131.12	2.4	54.63	2622.4	3.325	4.830

Characterization of Collagenase Enzyme

Determining the Optimal pH for Enzyme Activity

Figure 4 shows the optimal pH for collagenase activity. The results showed that the optimal pH was 8.0, with the optimal activity reaching 6.100 units/ml, while the lowest activity was 1.334 units/ml at pH 4. This may be due to the reaction medium, the binding of the reactant to the enzyme, and the ionic state of the amino acids involved in enzyme catalysis (Chesworth *et al.*, 2012). The reason for the decreased activity at highly acidic and basic pHs is the ionic groups present in the active site of the enzyme, the substrate, or both, due to a change in the ionic state of these groups, which affects the enzyme's ability to bind to the substrate (Segel, 1991).

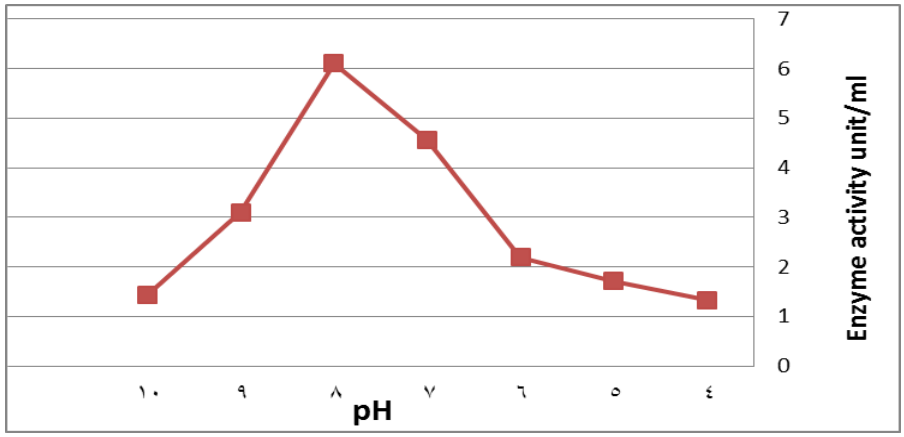

Figure 4: Effect of pH on the activity of partially purified collagenase enzyme.

Determining the optimum temperature for collagenase enzyme activity

Figure 5 shows the effect of temperature on the activity of partially purified collagenase enzyme at pH 8.0. It shows a clear increase in enzyme activity at 40°C, reaching 7.251 units/ml, then decreasing at 50°C to 4.637 units/ml. This may be due to the fact that the rate of the enzyme reaction increases with increasing temperature, but within a certain range due to the increase in the kinetic energy of the molecules. However, temperatures that exceed certain limits lead to denaturation of the enzyme and reduce its activity (Segel, 1991). This result is consistent with (Nagano and To, 2000), who found that the optimum temperature for the activity of collagenase enzyme from *Bacillus Subtilis* FS-2 is 40°C. Meanwhile, (Zhu *et al.,* 2022) found that the optimum temperature for the activity of the enzyme isolated from *Bacillus Subtilis* bacteria was 50°C.

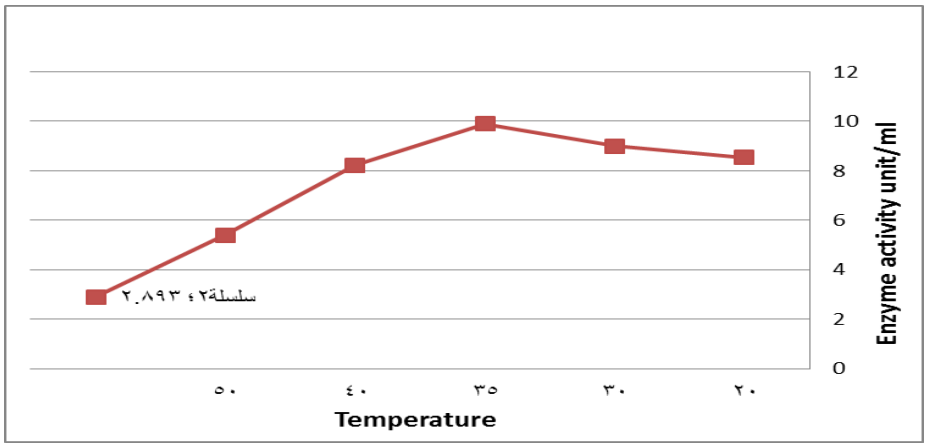

Figure 5: Effect of temperature on the activity of partially purified collagenase enzyme

Determining the optimal time for collagenase enzyme activity

The reaction time of the partially purified collagenase enzyme was studied at 40°C and pH 8.0 for periods ranging from 5 to 120 minutes. Figure 6 shows that the highest enzyme activity occurred after a 60-minute incubation period, reaching 8.545 units/ml. The enzyme activity then began to decline, reaching its lowest level after a 120-minute incubation period, at 6.384 units/ml. The reason for the increased enzyme activity after a 60-minute in-

cubation period is that all active sites of the enzyme were occupied by the substrate molecules, which led to increased activity. This result is consistent with (Lima *et al.,* 2015), who found that the optimal time for the activity of collagenase isolated from *Bacillus sp*. was 60 minutes. Meanwhile, Tran and Nagano (2000) showed that the optimal time for the activity of collagenase isolated from *Bacillus Subtilis* CN2 was 8 hours.

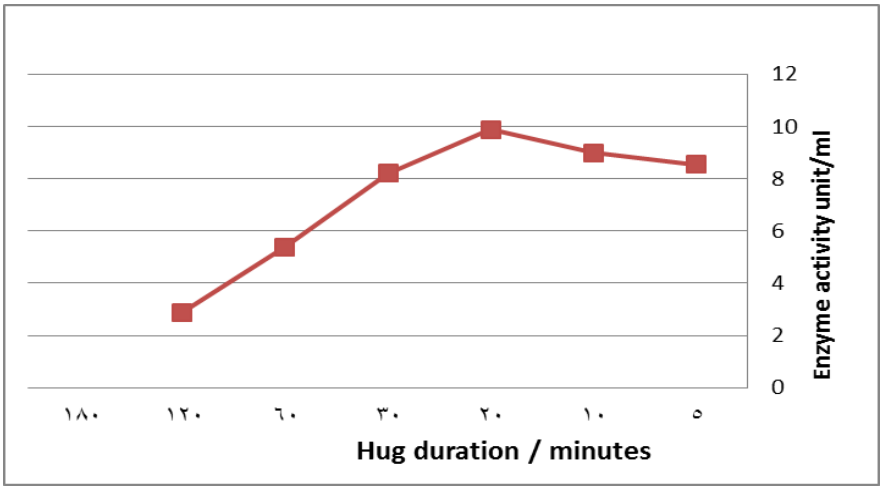

Figure 6: Effect of reaction time on the activity of partially purified collagenase enzyme.

Determining the effect of the optimum concentration of the reactant on the activity of collagenase enzyme

The effect of the substrate concentration (collagen) for the enzyme reaction solution was studied at 40°C, pH 8.0, and an incubation period of 60 minutes. Figure 7 shows that the enzyme activity increased with increasing substrate concentration. The maximum enzyme activity reached 9.892 units/ml at a 1.5% substrate concentration, after which the activity decreased to 2.893 units/ml at a 3% substrate concentration. The decrease in activity may be due to saturation of the enzyme's active sites with the substrate and the occupancy of all active sites, or inhibition by the substrate due to the substrate binding to inactive sites of the enzyme, which reduces its activity (Rodrigues *et al*., 2013).

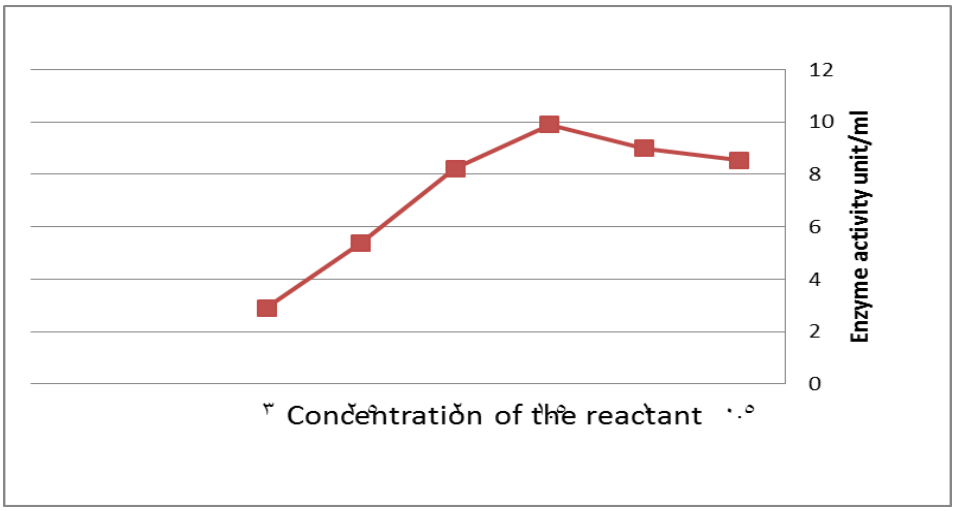

Figure 7: The effect of different concentrations of the reactant on the activity of partially purified collagenase enzyme.

Conclusions

Enterobacter cloacae demonstrated its ability to produce collagenase using organic waste. The enzyme was efficiently purified, with a specific activity of 54.63 units/mg, with a recovery rate of 4.83%. The optimal conditions for enzyme activity were: 40°C, pH 8.0, incubation time of 60 minutes, and a collagen concentration of 1.5%.

References

Alam, M. R., Shahid, M. A., Alimuzzaman, S., and Khan, A. N. (2022). Sources, extractions and applications of bio-maker collagen–A review. Biomedical Engineering Advances, 4, 100064.

Chesworth, J. M., Stuchbury, T., and Scaife, J. R. (2012). An introduction to agricultural biochemistry. Springer Science & Business Media.

Herman, I. M., Niranjan, P., and Grover, K. (2020). Protease technology in wound repair. In Wound Healing, Tissue Repair, and Regeneration in Diabetes (pp. 357-391). Academic Press.

Lima, L. A., Cruz Filho, R. F., Santos, J. G. D., and Silva, W. C. (2015). Protease with collagenolytic activity produced by *Bacillus sp*. DPUA 1728 from Amazonian soil. Brazilian Journal of Microbiology, 46(4), 1217-1223.

Liu , L. ; Meihu, M. ; Zhaoxia, C. ; Xieli, Y. and Wentao, W. (2010). Purification and properties of a collagenolytic protease produced by Bacillus cereus MBL13 Strain .J. Food Technol. Biotech. 48 (2) : 151 – 60 .

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L. and Randall, R. J. (1951). Protein measurement with folin phenol reagent. J. Biol. Chem., 193(1): 265-275.

Luchian, I., Goriuc, A., Sandu, D., and Covasa, M. (2022). The role of matrix metalloproteinases (MMP-8, MMP-9, MMP-13) in periodontal and peri-implant pathological processes. International Journal of Molecular Sciences, 23(3), 1806.

Moore, S., and Stein, W. H. (1954). A modified ninhydrin reagent for the photometric determination of amino acids and related compounds : 907-913.

Nagano, H., and To, K. A. (2000). Purification of collagenase and specificity of its related enzyme from *Bacillus subtilis* FS-2. Bioscience, biotechnology, and biochemistry, 64(1), 181-183.

Oslan, S. N. H., Shapawi, R., Mokhtar, R. A. M., Noordin, W. N. M., and Huda, N. (2022). Characterization of acid-and pepsin-soluble collagen extracted from the skin of purple-spotted bigeye snapper. Gels, 8(10), 665.

Rodrigues, R. C., Ortiz, C., Berenguer-Murcia, Á., Torres, R., and Fernández-Lafuente, R. (2013). Modifying enzyme activity and selectivity by immobilization. Chemical society reviews, 42(15), 6290-6307.

Segel, L. A. (Ed.). (1991). *Biological kinetics* (Vol. 12). Cambridge University Press.

Setyahadi, S., and Meinhardt, F. (2012). Purification and characterization of collagenase from *Bacillus licheniformis* F11. 4. African Journal of Microbiology Research, 6(10), 2373-2379.

Tran, L. H., and Nagano, H. (2002). Isolation and characteristics of *Bacillus subtilis* CN2 and its collagenase production. Journal of food science, 67(3), 1184-1187.

Wang, Z. Z., Wang, K., Xu, L. F., Su, C., Gong, J. S., Shi, J. S., ... and Qian, J. Y. (2024). Unlocking the potential of collagenases: Structures,

functions, and emerging therapeutic horizons. Biodesign Research, 6, 0050.

Whitaker, J. R. (2018). Principles of enzymology for the food sciences. Routledge.

Wollum, A. G. (1982). Cultural methods for soil microorganisms. Methods of soil analysis: part 2 chemical and microbiological properties, 9, 781-802.

Wu, S., Zhou, X., Jin, Z., and Cheng, H. (2023). Collagenases and their inhibitors: a review. Collagen and Leather, 5(1), 19.

Zhu, Y., Wang, L., Zheng, K., Liu, P., Li, W., Lin, J., ... and Zhang, H. (2022). Optimized recombinant expression and characterization of collagenase in *Bacillus subtilis* WB600. Fermentation, 8(9), 449.