IJMPR 2025, 9(4), 1-6

International Journal of Modern Pharmaceutical Research
www.ijmpronline.com

ISSN: 2319-5878
IJMPR
Research Article
SJIF Impact Factor: 6.669

SCREENING OF COLLAGENASE FROM BACTERIAL ISOLATES PRESENT IN CHICKEN BONES

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Article Received on: 06/02/2025
Article Revised on: 26/02/2025
Article Accepted on: 16/03/2025

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ABSTRACT
This study investigated the isolation and characterization of collagenase from chicken bone. Collagenases have widespread applications in food industry and clinical therapies. This enzyme hydrolyses collagen by breaking down peptide bonds at different sites. In this research, collagenase producing bacteria were isolated by repeated streaking method. Morphological examination confirmed the classification of the bacterial isolates as Bacillus sp. The parameters such as time of incubation, pH, substrate concentration, metal ions, carbon and nitrogen sources were also optimized for maximum production of the enzyme. The optimal time of incubation for enzyme activity was 48 hours. The maximal enzyme activity was obtained at pH 8.0 and optimum substrate concentration was 2% gelatin. The results revealed that MgSO4, CaCl2, glucose, and beef extract are best sources of metal ions, carbon, and nitrogen for the maximum production of enzyme. Our research work has investigated those bacteria that are producing collagenase enzyme with greater activity.

KEYWORDS: Collagenase, Isolation, Characterization, Bacillus sp., screening.

INTRODUCTION

Collagenase, a metalloproteinase, is a key enzyme in collagen metabolism in mammalian tissues. Given that skin contains roughly 70-80% collagen, the importance of collagenase is clear. It specifically targets native collagen, assisting in the breakdown of necrotic tissue in healthy wounds and promoting healing. However, in non-healing wounds, conditions such as diabetes or age-related disorders can reduce collagenase activity, resulting in the accumulation of necrotic tissue that impedes the healing process (Sherman, Yang, & Meyers, 2015).

These enzymes are big multi-domain proteins with a molecular weight of around 115 kDa. They are made up of an N terminal collagenase unit, which is further separated into two domains: activator and peptidase. Following the collagenase unit, there is a varied array of accessory domains that participate in substrate recognition and collagen swelling. The catalytic zinc is kept in place in the domain by two histidine residues, as well as a downstream glutamate (Rich & Crick, 1955).

Microbial collagenases offer a wide range of uses in industries such as food, tannery, meat, cosmetics, and pharmaceutical manufacturing. They also play an important function in the medical field, making it easier to isolate and cultivate mammalian cells. Their medical applications include the treatment of burns, wounds, scar tissue, organ transplantation, Peyronie's disease, fibrosis, and cirrhosis, as well as improving blood purification for better medical diagnostic screening (Bhagwat & Dandge, 2018).

Microbial collagenases are common experimental reagents in lab-scale experiments. Cell culture is widely used in biotechnology, molecular biology, and toxicology to address major biological, technical, and scientific difficulties. The use of microbial collagenase has been successful in separating cells from bone, endothelium, neurons, and the islets of Langerhans. One of the most significant applications of microbial collagenase is in the medical field. It has been used directly in therapeutic therapies (Gaurav and Suresh, 2016).

MATERIALS AND METHODS

Collection of Sample

A piece of chicken bone was obtained from a nearby butchery to identify bacteria that generate collagenase. The cartilage-rich area of the bone was used as a primary substrate for collagenase. The sample was weighed and taken to prepare a stock solution for further isolation of collagenase producing bacteria. Subsequently, serial dilutions were performed while maintaining a constant dilution factor.

Isolation of Bacterial Colonies

The collagenase producing bacterial colonies were isolated from chicken bones. 20µL of each dilution was uniformly distributed across the surface of the gelatin agar medium containing 1g of gelatin, 0.5g of glucose,

0.1g of yeast extract, 0.02g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 0.02g of calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), and 1.5g of agar in 100ml of distilled water. Streak plate method was used to obtain the growth of the single isolated colony (Medina and Baresi, 2007).

Screening of Collagenase

The isolated bacteria were screened using a 35% trichloroacetic acid solution. It was based on the ability of collagenase to hydrolyze gelatin, resulting in the formation of clear zones around the bacterial colonies (Medina and Baresi, 2007).

Preparation of Crude Enzyme Extract

5ml gelatin media was taken in a test tube and was inoculated with bacterial colony. The test tube was incubated for 24 hours facilitating bacterial growth. Further assays were performed using prepared inoculum media containing bacteria and gelatin broth, enabling the determination of enzymatic activity of the bacteria (Suphatharaprateep et al., 2011).

Assay of Collagenase Activity

For extracellular enzyme assay, a reaction mixture of 0.3ml of 0.2% gelatin solution, 0.2ml of 150mM Tris HCl, 0.1ml of 12mM CaCl2, and 0.1ml enzyme was added in a test tube and incubated at 30°C for 30 minutes. Then 0.6ml of 0.1N HCl, and 2ml of ninhydrin solution was added after incubation. Further incubation was performed at 80°C for 10 minutes (Tran and Nagano, 2002).

For intracellular enzyme assay, the centrifugation of the culture was performed at 6000rpm for 15 minutes to separate the pellet and then 1ml chloroform was added. The pellet was subjected to vortex for 5 minutes and centrifuged at 10000rpm for 15 minutes to separate the supernatant and pellet, then 0.5ml pellet was taken in a test tube and 0.5ml of gelatin solution was added as a substrate. Incubation was done for 10 minutes at 50°C in a water-bath. After incubation, 2ml of ninhydrin solution was added to it and placed in a water bath at 92°C. Change in colour was observed (Tran and Nagano, 2002).

Characterization of Collagenase

Several different parameters were optimized in order to determine the maximum activity of extracted collagenase (Suphatharaprateep et al., 2011).

Effect of pH

The effect of pH on collagenase production was examined by growing the isolates in assay medium with different pH ranging from pH 4 to pH 8.

Effect of Time of Incubation

To determine the effect of time of incubation on the production of collagenase, bacterial culture was incubated for 24, 48, 72, and 96 hours.

Effect of Metal Ions

1% of various metal ions including sodium chloride, calcium chloride, magnesium sulphate, and iron sulphate were added in 5ml gelatin medium with inoculated culture. Bacterial culture was incubated for 24 hours and the effect of metal ions on the activity of collagenase was examined.

Effect of Carbon Sources

The effect of various carbon sources such as glucose, sucrose, lactose, maltose, and starch was examined by adding 1% of carbon sources in 5ml of gelatin medium containing bacterial culture. The media was then incubated for 24 hours to determine maximum activity of enzyme.

Effect of Nitrogen Sources

The effect of several nitrogen sources such as peptone, casein, yeast extract, beef extract, and ammonium hydrogen citrate was optimized and examined by adding 1% of nitrogen sources into 5ml of gelatin medium containing inoculum. The media was then incubated for 24 hours to determine maximum activity of enzyme.

Effect of Substrate Concentration

The effect of different gelatin concentrations on the activity of collagenase was examined by adding 0.5%, 1%, 1.5%, and 2% concentrations of gelatin into 5ml of medium containing inoculum. This allowed the determination of collagenase activity in response of diverse gelatin concentrations.

RESULTS AND DISCUSSION

The isolation of bacteria on the gelatin agar plate resulted in the formation of distinct colonies, which were characterized by their morphological characteristics and enzymatic activities. After adding 35% trichloroacetic acid solution to the media, the amino acids remained, while the undegraded collagen and other proteins precipitate out. The TCA solution served as a precipitating agent, enhancing the visibility of the hydrolysis zones by precipitating the undigested gelatin.

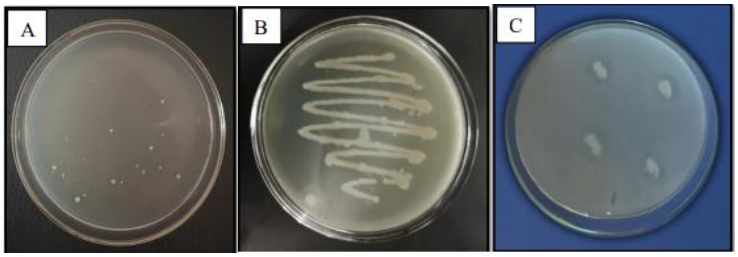

Figure 1: A. Isolation of collagenase producing bacteria on gelatin agar plate; B. Purification of bacterial colonies; C. Collagenase producing bacteria showed clear zones of hydrolysis on gelatin agar plate.

Table 1: Colony morphology of isolated bacteria.

Features	Size	Shape	Color	Surface	Elevation	Opacity	Consistency	Odor
Bacteria	0.1mm	Round	White	Smooth	Slightly raised	Opaque	Sticky and thick	Null

Biochemical Testing

Gram staining technique resulted in the identification of bacteria. Upon microscopic examination, the bacterial isolates exhibited a distinct purple coloration, indicating that they belonged to gram positive category.

Characterization of Collagenase

Effect of pH

The enzymatic activity of the bacterial isolates was characterized across a range of pH values to determine the optimal conditions for extracellular and intracellular enzyme activity. The highest collagenase activity was observed at pH 8 (2.888U/ml). Emma et al. (2016) reported that higher collagenase activity was observed in the pH range between pH 7 and 9, peaking at pH 8. Lili et al. (2010) also reported that the optimum pH for collagenase activity was 8.

Effect of Metal Ions

Characterization was done by using different metal ions and the highest collagenase activity was observed in the presence of magnesium sulphate (2.203U/ml). According to Danial et al., 2019, collagenase was activated by Mg2+ and Ca2+. Similarly, Wu et al. (2010) also reported higher collagenase activity due to Mg2+ and Ca2+.

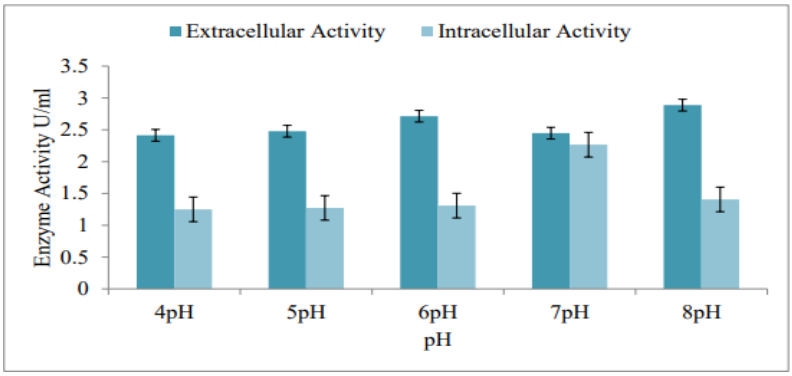

Figure 2: Graphical representation of enzyme activity at different pH. Among these pH, enzyme activity was highest at pH 8 (2.888 U/ml), followed by pH 6 (2.713 U/ml), pH 5 (2.478 U/ml), pH 7 (2.447 U/ml), and the lowest activity at pH 4 (2.413 U/ml).

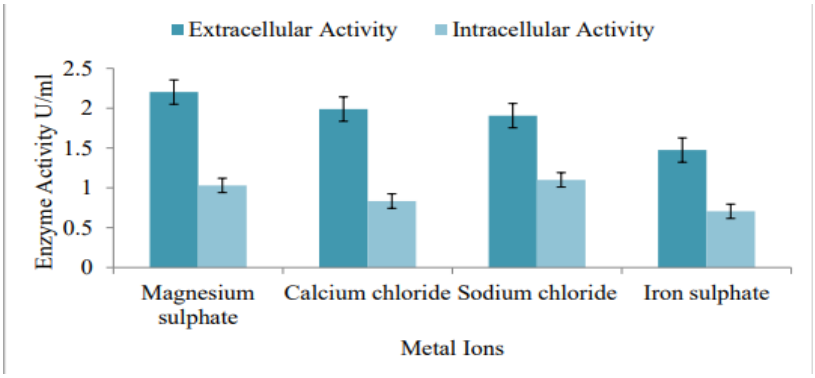

Figure 3: Graphical representation of enzyme activity in the presence of various metal ions. Among these metal ions, enzyme activity was highest in the presence of MgSO4 (2.203 U/ml), followed by CaCl2 (1.988 U/ml), NaCl (1.906 U/ml), and the lowest activity in the presence of FeSO4 (1.475 U/ml).

Effect of Carbon Sources

Characterization of collagenase producing bacteria was done by using different carbon sources and the highest collagenase activity was obtained in the presence of glucose (2.216U/ml). Xingshuo et al. (2016) reported that an increased yield of collagenase was observed by the addition of 2% glucose as a carbon source to the medium.

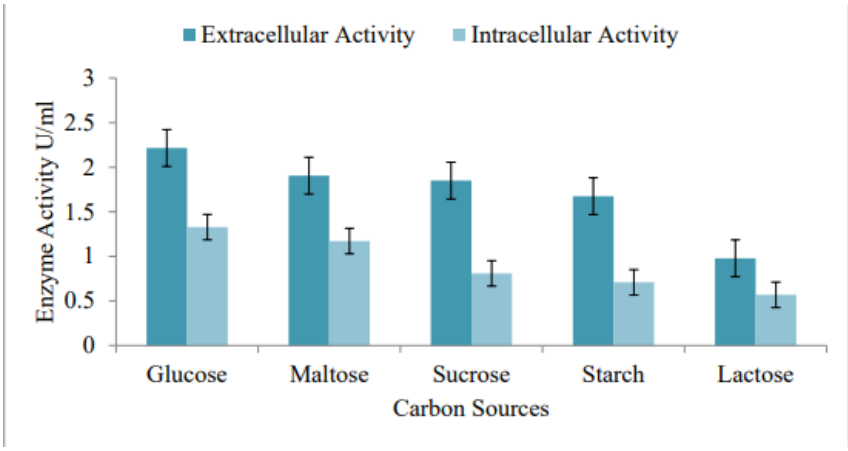

Figure 4: Graphical representation of enzyme activity in the presence of various carbon sources. Among these carbon sources, enzyme activity was highest in the presence of glucose (2.216 U/ml), followed by maltose (1.906 U/ml), sucrose (1.850 U/ml), starch (1.675 U/ml) and the lowest activity in the presence of lactose (0.978 U/ml).

Effect of Nitrogen Sources

Characterization of collagenase producing bacteria was done by using different nitrogen sources and the highest collagenase activity was observed in the presence of beef extract (2.888U/ml). Hanaa and Hussein, (2022) reported yeast extract as the best nitrogen source for the highest activity of collagenase.

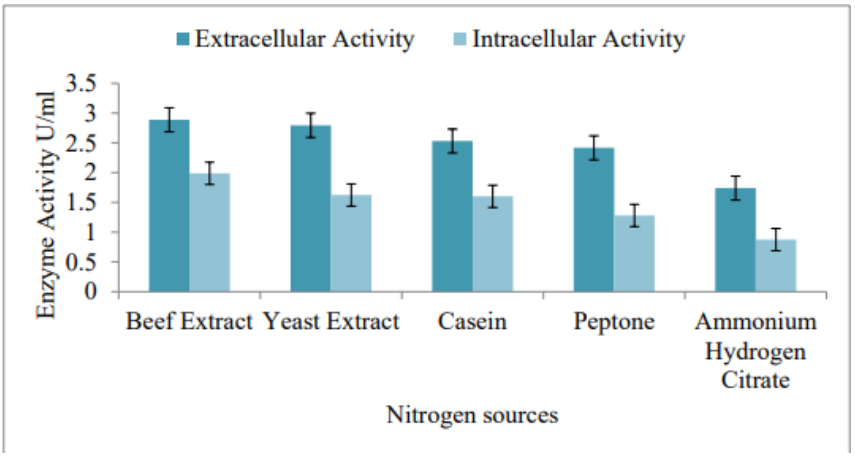

Figure 5: Graphical representation of enzyme activity in the presence of various nitrogen sources. Among these nitrogen sources, enzyme activity was highest in the presence of beef extract (2.888 U/ml), followed by yeast extract (2.794 U/ml), casein (2.531 U/ml), peptone (2.419 U/ml) and the lowest activity in the presence of ammonium hydrogen citrate (1.741 U/ml).

Effect of Substrate Concentration

Characterization was done at different gelatin concentrations and the highest collagenase activity was observed at 2% gelatin concentration (2.778U/ml). Savita and Pethe, (2015) reported that 1.5% substrate concentration was the optimum condition for the growth of the enzyme.

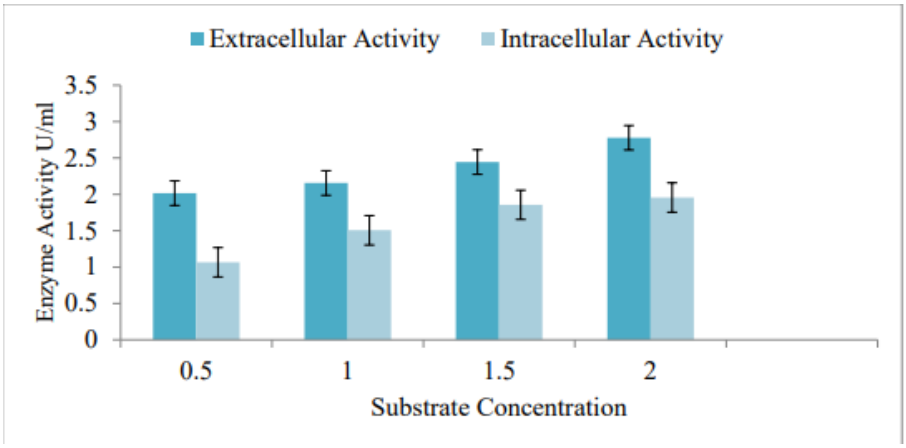

Figure 6: Graphical representation of enzyme activity at different substrate concentrations. Among these substrate concentrations, enzyme activity was highest at the concentration of 2% gelatin (2.778 U/ml), followed by 1.5% (2.444 U/ml), 1% (2.156 U/ml), and the lowest activity at the substrate concentration 0.5% (2.016 U/ml).

Effect of Time of Incubation

Characterization was done at different time of incubation and the highest extracellular activity was observed after 48h (2.747U/ml). Hanaa and Hussein, (2022) reported that the optimum time of incubation for the specific collagenase activity was after 48 hours of incubation.

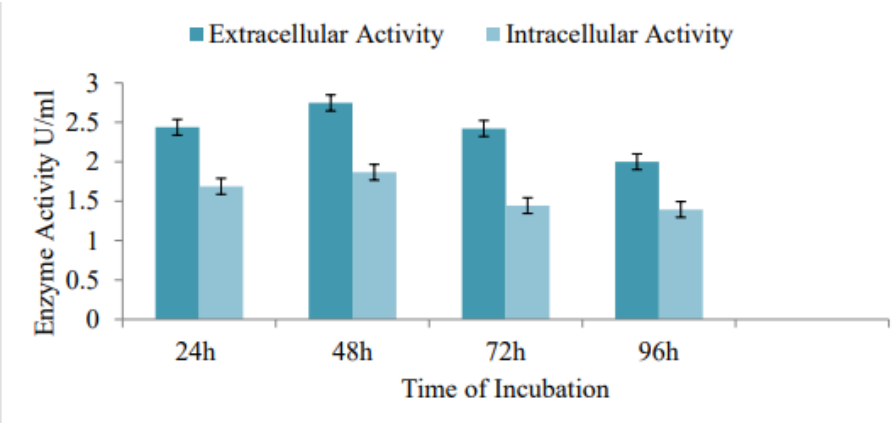

Figure 7: Graphical representation of enzyme activity at different incubation times. Among these incubation times, enzyme activity was highest after the period of 48 hours (2.747 U/ml), followed by 24 hours (2.438 U/ml), 72 hours (2.422 U/ml), and the lowest activity at the incubation time of 96 hours (2.000 U/ml).

CONCLUSION

This research successfully isolated and identified collagenase producing bacteria from chicken bones, with potential applications in various industries. The bacteria belonged to Bacillus sp. Bacteria were grown on gelatin agar plate and their pure isolates were used for morphological tests and characterization. The enzyme activity was observed under different parameters including pH, time of incubation, metal ions, carbon sources, nitrogen sources, and substrate concentrations. 8pH, 48 hours of incubation, MgSO4 as metal ion, glucose as carbon source, beef extract as nitrogen source, and 2% gelatin as substrate concentration were the optimum conditions that showed higher collagenase activity.

ACKNOWLEDGEMENTS

We acknowledge Lahore College for Women University for providing all the necessary support for this study.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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