

## ISOLATION AND CHARACTERIZATION OF COLLAGENASE PRODUCING BACTERIA FROM SOIL SAMPLE

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### ABSTRACT

Collagenases play a crucial role in treating multiple diseases such as Dupuytren's disease and Peyronie's disease and medical problems. Collagenases are responsible for the degradation of native collagen to small peptide fragments. It has wide applications in the food industry as meat tenderization, cosmetics, wound healing, diabetic ulcers, arterial ulcers, etc. Collagenases are mostly obtained from *Clostridium histolyticum*. In this research, the source used was soil from which the enzyme was extracted, and the culture was purified by the streaking method, further TCA solution was used to produce zones around colonies which showed the hydrolysis of gelatin, a substrate for collagenase. Biochemical testing confirmed the presence of *Bacillus sp.* and it is a good source of collagenase. The bacteria was confirmed by bioinformatics analysis by predicting its structure. The enzyme activity exhibited the highest incubation period of 24 hours with other parameters such as ammonium hydrogen citrate as a nitrogen source, glucose as a carbon source, sodium chloride as a metal ion, and a maximum substrate concentration of 2 %. This research shows an optimization of characterization for maximum production of collagenase.

**KEYWORDS:** Collagenase, isolation, characterization, *Bacillus sp.*, screening.

### INTRODUCTION

Enzymes are important for many industrial processes, such as cosmetics, food production, nutraceuticals, medicines, and more.<sup>[1]</sup> Collagen plays an essential role in the food industry such as tenderization of red meats, as a tenderizer. Among the different forms of protease sources, microbial proteases are particularly significant because they are the ones that are produced rapidly and inexpensively on a large scale, which is why they are being produced broadly in several different fields.<sup>[2]</sup> Collagenases are particularly crucial because they are the only ones that can hydrolyze fibrous collagen, collagen is the necessary component of skin, tendons, bones, cartilage, and blood vessels.<sup>[3]</sup>

Collagenases isolated from *Clostridium histolyticum* and *Clostridium welchii* are considered the only enzymes that meet many of these requirements for a significant amount of time. microbial collagenases are mostly obtained from *Clostridium histolyticum*, a pathogenic microorganism.<sup>[4]</sup> Those enzymes function as exotoxins, meddle with the connective tissue metabolism, and instruct the host cells to start hydrolyzing collagen.<sup>[5]</sup> When talking about proteases, those that were secreted by *Bacillus* species are microbial proteases, which are reported for most of the market shares of commercial enzymes.<sup>[6]</sup>

*Bacillus subtilis* is used because they are generally regarded as safe and high amounts of extracellular enzymes are being excreted by it without causing toxins or any undesired byproducts in the culture medium, and it is broadly used in biotechnological applications.<sup>[7]</sup>

The optimization and enhancement of the physicochemical parameters for the extraction of novel collagenolytic-producing microorganisms, which will allow for the characterization of collagenase for manufacturing purposes.<sup>[8]</sup>

Therefore, in this study previously produced strain that produces collagenase from *Bacillus subtilis* subsp. *subtilis* S13<sup>[9]</sup>, was subjected to the characterization of various parameters, incubation time, pH, carbon source, nitrogen source, and substrate (gelatin) concentration had a substantial impact on collagenase synthesis. The collagenase's ideal activity that was attained points to its possible use as a meat-tenderizing enzyme, among other things.

### MATERIALS AND METHODS

#### Collection of Soil Sample

The soil sample was collected from the Lahore College for Women University for the isolation of collagenase-producing bacteria. The layer under the topmost solid layer was taken of soil because it was refined and didn't

contain any huge solid particles that would disrupt the research work.

### Isolation of Collagenase producing Bacteria

The serially diluted soil sample was spread over the gelatin agar media plate which was prepared by adding the following chemicals: Gelatin, glucose, yeast extract, 0.02 g of magnesium sulfate heptahydrate ( $MgSO_4 \cdot 7 H_2O$ ), calcium chloride dihydrate ( $CaCl_2 \cdot 2 H_2O$ ) and adjusting the pH at 7.5. Plates were incubated for 24 hours at 37°C (10). To obtain the purified bacterial colonies, the streaking was done three times. First by the quadrant method and then by zig-zag method.

### Screening of Bacterial Colonies

Screening was performed to obtain the collagenase producing bacteria. 35% TCA (Trichloroacetic Acid) was used to flood the plates and produced zones around the collagenase producing bacterial colonies.<sup>[11]</sup>

### Preparation of Inoculum

Gelatin Broth media or LB of 50ml containing yeast extract, peptone, NaCl, and gelatin at pH 7 (NaOH was used to bring the pH down to 7). The LB media was autoclaved at 121°C at 15psi for 15 minutes.<sup>[12]</sup> Test tube filled with 5ml broth and inoculated with bacterial culture. Placed in a shaking incubator for 24 hours for growth. And use it for the characterization of enzyme assay.

### Assay of Enzyme Activity

For extracellular enzyme assay, 0.3 ml of gelatin solution, 0.2 ml of 150 mM Tris-HCl, 0.1 ml of 12 mM  $CaCl_2$ , and 0.1 ml of enzyme were added into a test tube. For 30 minutes, the mixture was incubated at 30°C in the water bath. 0.6 ml of 0.1 N HCl and 2 ml of ninhydrin solution were added after the mixture had been incubated. Then the test tube was incubated for 10 minutes at 80°C in a water bath again.

For intracellular enzyme assay, the test tube was incubated at 80°C for 10 minutes, and the incubated culture was centrifuged for 15 minutes so that the pellet and supernatant would be separated, 1ml chloroform was added in the separated pellet. Shake it well on vortex for 5 minutes, then centrifuge it at 10000 rpm for 15 minutes again. Take 0.5 ml pellet in a test tube and add the 0.5 ml of gelatin solution as a substrate. In the water bath, incubate the test tube for 10 minutes at 50°C. After 10 minutes, 2ml ninhydrin solution was included. The test tubes were placed for 10 minutes in a water bath set at 92°C. The color change was examined.<sup>[13]</sup>

### Identification of Collagenase producing Bacteria

The collagenase-producing bacteria was identified by using different biochemical tests like gram staining and endospore staining. These tests were used for the morphological characterization of selected isolates.

### Characterization of Collagenase producing Bacteria

The maximum activity of collagenase, obtained from the soil sample was determined by optimizing different parameters.<sup>[14]</sup>

### Effect of pH

pH of the culture media was observed by optimizing the different pH ranges at 4, 5, 6, 7, and 8. 0.1M NaOH and 0.1M HCl were added to adjust the pH and restore the acidity and alkalinity to the desired levels.

### Time of Incubation

The incubation time was studied at 24, 48, 72, and 96 hours and examined its effect on the growth of bacterial production. The production of bacterial growth rates and enzyme activity was examined at different incubation periods in 5ml of broth and the results were recorded.

### Effect of Metal Ions

Different metal ions including calcium chloride, sodium chloride, magnesium sulphate, and iron sulphate were used for the optimization and were studied by adding 1% of metal ions to 5ml of broth. The mixture was autoclaved at 121°C for 15 minutes and after autoclave, the bacterial culture was inoculated in the media and placed in the shaking incubator for 24 hours at 120 rpm.

### Effect of Carbon Sources

5 ml of broth was mixed with 1% of each of the following carbon sources: glucose, maltose, lactose, sucrose, and starch, to maximize their respective effects. The media was autoclaved and placed in a shaking incubator for 24 hours at 120 rpm and 37°C after being inoculated with the culture.

### Effect of Nitrogen Sources

5 ml of broth was mixed with 1% of the nitrogen sources: peptone, yeast extract, casein, ammonium hydrogen citrate, and beef extract. This was done to optimize the effects of each nitrogen source. After the media was autoclaved at 121°C, inoculated with the culture, and kept in a shaking incubator for 24 hours at 120 rpm and 37°C.

### Effect of Substrate Concentration

Collagenase enzyme growth was observed in the effect of varying amounts of gelatin 121°C, inoculated with the culture, and kept in a shaking incubator for 24 hours at 120 rpm and 37°C.

### Effect of Substrate Concentration

Collagenase enzyme growth was observed in the effect of varying amounts of gelatin concentration, which was evaluated by adding 0.5%, 1%, 1.5%, and 2% gelatin to 5 ml of broth. The enzyme activity values were analyzed.

### 3D Structure Analysis

For retrieving the enzyme's three-dimensional structure, Phyre 2 was used. It uses detection methods to construct the three-dimensional models, predicts the ligand binding

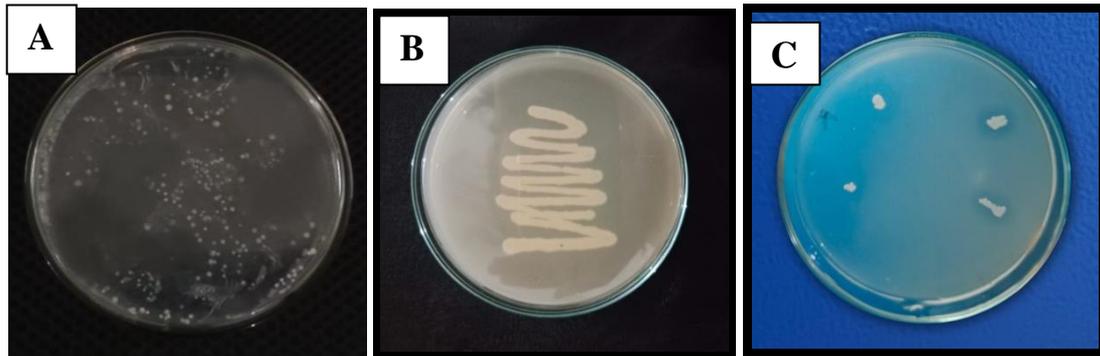
sites, and the amino acid variants of the required protein, were observed.

**RESULTS AND DISCUSSION**

**Isolation of Bacterial Colonies**

Bacterial colonies were isolated on gelatin agar media, after 24 hours of incubation and resulted in rounded and cream-colored colonies which were then purified by streaking method for further screening. The streaking of colonies gave the isolated pure strain of bacteria.

TCA was applied to the plates following incubation. Due to the synthesis of gelatinase enzymes, four isolates created distinct zones surrounding their colonies, showing that they hydrolyzed gelatin (showed in figure 1). After adding TCA, the hydrolyzed gelatin did not precipitate, leaving a clear area in the agar, revealing the clear zones. The same method was used by.<sup>[14]</sup>



**Fig 1: Plates showing (a) Streaking (b) Bacterial inoculum (c) Isolates showing zones.**

**Identification of Bacteria**

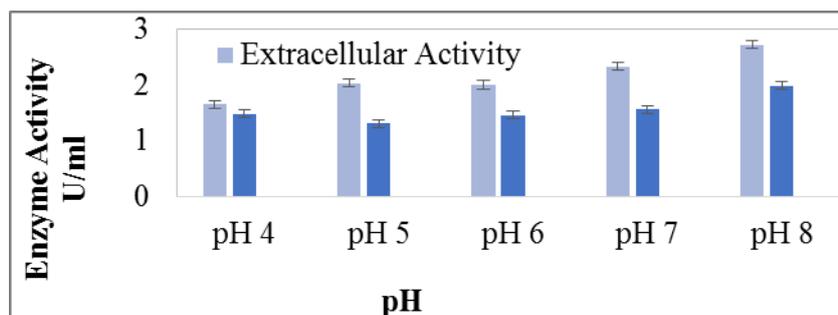
Bacteria was identified using morphological and biochemical characteristics (Table 1).

**Table 1: Morphological and biochemical results of bacterial colonies.**

S No.	Morphological Characteristics	Observation	
		Bacteria 1	Bacteria 2
1	Size	0.1 mm	0.3mm
2	Shape	Round	Round
3	Color	Milky	Creamy
4	Elevation	Raised	Raised
5	Consistency	Non-sticky	Sticky
6	Odor	Null	Null
7	Opacity	Opaque	Translucent
8	Pigmentation	Null	Null
9	Gram character	+ve rods	+ve rods
10	Endospore	+ve	+ve

**Effect of pH:** The highest collagenase activity was observed at pH 8. (15) investigated the impact of pH on the synthesis of collagenase in a pH range of 5 to 8.5. Collagenase synthesis was seen in isolate *Bacillus megaterium* KM 369985 across a wide pH range of 5 to

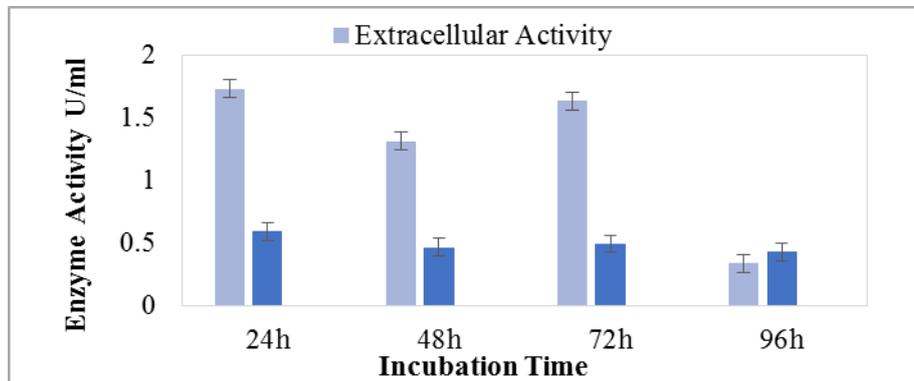
8.5. The highest activity of *Bacillus licheniformis* F11 was observed at pH 7, whereas the highest collagenase activity was observed by *Bacillus cereus* between pH 7 and pH 7.5.



**Figure 2: Bacteria show the highest activity at pH 8 (2.731 ± 0.0064 U/ml), then pH 7 (2.334 ± 0.0020 U/ml), pH 5 (2.036 ± 0.1184 U/ml), pH 6 (2.005 ± 0.0042 U/ml) and lowest at pH 4 (1.656 ± 0.0019 U/ml).**

**Effect of time of incubation:** The highest collagenase activity was observed after 24 hours of incubation.<sup>[16]</sup> reported that the isolate AD18 showed the maximum collagenase activity at 72 hours of incubation. The same thing was also noted in isolates Ad 7 and 4 which

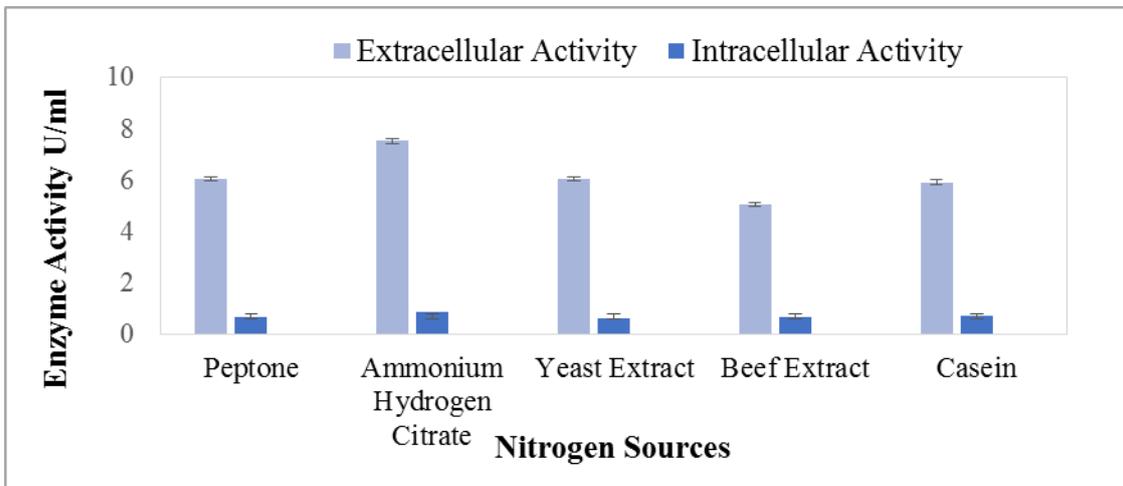
estimated the maximum production of enzymes at 72 hours of incubation. However, in the case of isolate Ad 11, the highest enzyme activity was observed at 48 hours of incubation.



**Figure 3:** Bacteria show the highest activity after 24 hours (1.733 ± 0.0015 U/ml), then after 72 hours (1.634 ± 0.0014 U/ml), 48 hours (1.315 ± 0.0014 U/ml) and lowest after 96 hours (0.336 ± 0.0015 U/ml).

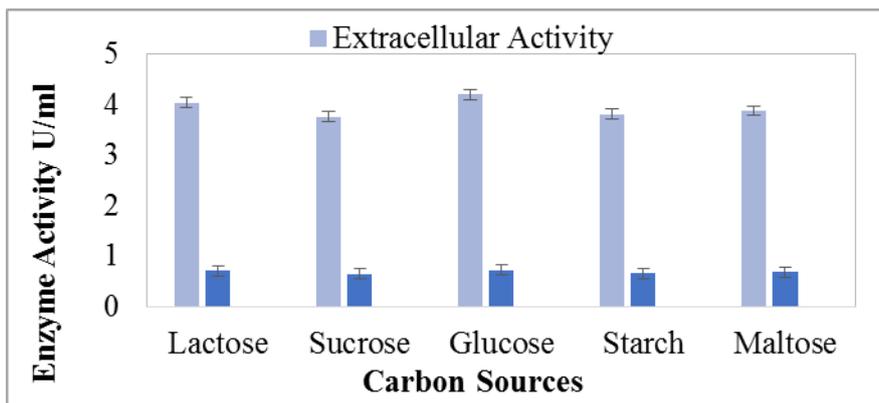
**Effect of nitrogen sources:** The highest collagenase activity was observed with Ammonium Hydrogen Citrate.<sup>[17]</sup> stated that for the maximum production of the enzyme extracellularly, nitrogen sources played a vital part in the media for the best utilization of carbohydrates. The effect of nitrogen sources like ammonium ferrous

sulphate (AFS), yeast extract, ammonium sulphate (AS), peptone, beef extract, urea ammonium nitrate (AN), and ammonium hydrogen carbonate (AHC) on the production of collagenase after 72 hours of incubation and the maximum collagenase activity was observed with minimum Ammonium ferrous sulphate.



**Figure 4:** Bacteria show the highest activity with ammonium hydrogen citrate (7.531 ± 0.0021 U/ml), then with peptone (6.041 ± 0.0100 U/ml), and yeast extract (6.041 ± 0.0100 U/ml), casein (5.918 ± 0.0014 U/ml), and lowest value with beef extract (5.062 ± 0.0456 U/ml).

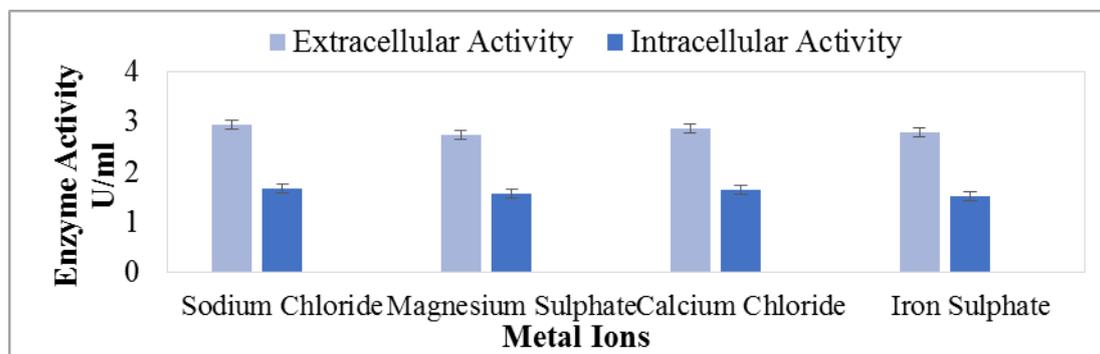
**Effect of carbon sources:** The highest collagenase activity was observed with glucose. For bacteria, glucose is the perfect carbon source since it provides the energy and natural resources needed for strain development.



**Figure 5:** Bacteria show the highest activity with glucose ( $4.206 \pm 0.0019$  U/ml), then with lactose ( $4.043 \pm 0.0014$  U/ml), maltose ( $3.880 \pm 0.0015$  U/ml), starch ( $3.808 \pm 0.0014$  U/ml) and lowest with sucrose ( $3.758 \pm 0.0015$  U/ml).

**Effect of metal ions:** The highest collagenase activity was observed with sodium chloride. Sodium and chloride ions create an optimal ionic environment for collagenase production. Metal ions like calcium, zinc, and magnesium estimated the increased production of

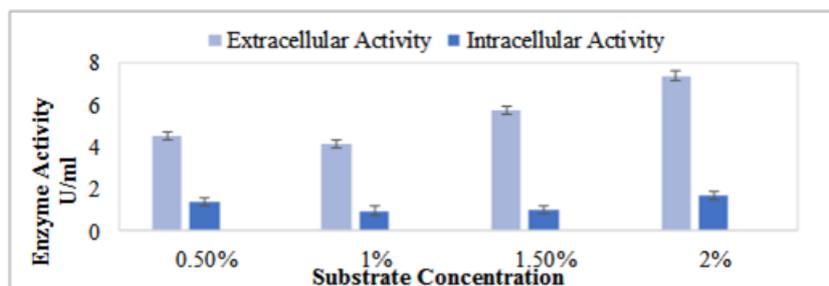
collagenase activity of purified collagenase enzyme, according to.<sup>[18]</sup> The article also reported that collagenase requires calcium for its optimal activity as they are stabilizer and protects them from conformational changes.



**Figure 6:** Bacteria show the highest activity with sodium chloride ( $2.945 \pm 0.0140$  U/ml), then with calcium chloride ( $2.875 \pm 0.0009$  U/ml), iron sulphate ( $2.797 \pm 0.0158$  U/ml), and lowest with magnesium sulphate ( $2.754 \pm 0.0147$  U/ml).

**Effect of substrate concentration:** The highest collagenase activity was observed at 2%. The same reporting was also claimed by<sup>[19]</sup> in their articles where

the maximum activity was observed when the enzyme was supplemented with 1.5% gelatin as a substrate concentration.



**Figure 7:** Bacteria show the highest activity at 2% substrate concentration ( $7.381 \pm 0.0020$  U/ml), then at 1.5% ( $5.731 \pm 0.0010$  U/ml), 0.5% ( $4.506 \pm 0.0010$  U/ml), and lowest with 1%.

**Structure Analysis:** The enzyme's three-dimensional structure was predicted through Phyre2. 3D structure shows 670 residues (69% of the sequence) have been modeled with 100.0% confidence. Detailed structure predicted the percentage of alpha helix and beta strands.

The alpha helix is **24%** and the beta strands are **18%** in sequence.



Figure 8. 3D image of collagenase from Phyre2.

## CONCLUSION

In this study soil was used as a sample as one of the sources of collagenase enzyme. Collagenase producing bacteria were isolated and screened using TCA. Then biochemical tests like gram staining and endospore test were done which gave the purple-colored bacteria and endospores under a light microscope. Characterization of the enzyme was done using different parameters and the enzyme showed the maximum activity on pH 8, sodium chloride as a metal ion, glucose used as a carbon source, and ammonium hydrogen citrate used as a nitrogen source. The collagenase activity was maximum at 24 hours of incubation period and worked best at 2% substrate concentration. Further analysis using bioinformatics techniques confirmed the identity of the bacteria as belonging to the *Bacillus* genus.

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