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Hydrolysis of Gelatin from Animal Hoof Using Bacterial Gelatinase

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Abstract: Enzymatic hydrolysis of gelatin increases its functional, textural, and nutritional characteristics. Gelatinases are enzymes used to hydrolyze gelatin into peptides and amino acids. In recent years gelatin hydrolysates have gained a considerable indication in the applications of food and non-food products. This study aimed to isolate the gelatinase enzyme from bacteria and optimize the reaction condition for gelatin hydrolysis. A total of 100 bacterial isolates were isolated from soil and water samples. All isolates were screened for the production of gelatinase on gelatin-containing media. One isolate was selected for further analysis based on the eminent properties of the enzymatic reaction. The organism can grow under solid-state fermentation and produce gelatinase enzyme with a high activity using wheat bran as a carbon source. Gelatinase was optimally active at a temperature of 50°C and at pH 8. The optimum enzyme production under solid-state fermentation includes an incubation period of 120 h and a moisture content of 66.7%. The isolate produced more enzymes with the addition of peptone as a nitrogen source. The enzyme was used to produce gelatin hydrolysate by hydrolyzing animal hooves. The production process for the gelatin hydrolysis was optimized by varying enzyme concentration and incubation time. The hydrolysis has maximum activity at 4 h incubation period and at a high amount of enzyme concentration. This study indicated that animal waste like hoofs can be converted to useful gelatin hydrolysate using microbial gelatinase and used for various applications.

Keywords: Gelatinase, Hooves, Hydrolysis, Enzymes

1. Introduction

Ethiopia has a large number of cattle that serve as sources of meat and milk. In addition, animal skin and hide are used as raw materials by the leather industries. Oftentimes, animal organs such as bones, blood, and other offal were considered to have little or no use rather than being damped elsewhere causing environmental pollution. Recent research works have shown that organs such as bones are known to be potential sources of gelatin [1].

Gelatin is a protein derived from animals such as cows, pigs, and fish by boiling skin, ligaments, hoofs, and bones. Chemically it is composed of glycine (21%), proline (12%), arginate (8%), alanine (9%), aspartate (6%), and the remaining 44% from other types of amino acids [2]. In the food industry, gelatin has different applications which include the preparation of confectionary and different dessert foods [3]. It is also used in the production of tablet coating and suppositories by the pharmaceutical industry and the production of X-ray films [4].

Gelatin is hydrolyzed by the enzyme gelatinase into smaller peptides and amino acids. Thus, gelatinase can be used for the production of gelatin hydrolysates that have a wide array of applications in several industries. Gelatinase enzymes are produced by bacteria such as *Bacillus species, Pseudomonase aeraginosa, Staphylococcus aureus, and*

Clostridium perifergens. Enzymatic hydrolysis of gelatin increases its functional, textural, and nutritional characteristics. In recent years, hydrolysates of gelatin demonstrated for their values in a range of applications in food formulations [5]. This is because such hydrolysates have a neutral taste, low bloom, and contain a high concentration of purified protein. It can also be used for the production of protein bars; protein drinks, smoothies, joint health nutritional products, emulsifiers, candies, and wider application in the cosmetics industry [6].

Considering the availability of raw material and the potential applications of gelatin hydrolysate, Ethiopia can gain a huge benefit from such resource utilizing the appropriate technology for the production of gelatin hydrolysates. To the best of our knowledge, no studies have been generated so far showing this potential use. In the production of hydrolysates, the availability of an efficient gelatinase enzyme is considered very critical. Therefore, the aim of this study was, to isolate bacteria with the potential of gelatinase production, characterize the enzyme, and optimize the conditions of enzyme hydrolysis.

2. Materials and Methods

2.1. Isolation and Screening of Gelatinase Producing Bacteria

Soil samples were collected from Addis Ababa University, Arat Kilo Campus, while spring water and mud samples were collected from Lake Afdera, Afar region, Ethiopia. Both samples were transferred to sterile containers and then transported to the laboratory using an icebox. One gram of soil sample was suspended in 1ml of sterile distilled water and serially diluted in sterilized water from 10^{-1}-10^{-7}. The water sample from Lake Afdera was diluted using 20 ml of sterile distilled water and 5 ml of it was aseptically transferred into a 250 ml Erlenmeyer flask containing 50 ml sterile enrichment liquid medium. The enrichment medium was composed of gelatin (1.5%), yeast extract (0.1%), KH_2PO_4 (0.1%), NaCl (0.5%), $MgSO_4 \cdot 7H_2O$ (0.02%), and $CaCl_2 \cdot 7H_2O$ (0.01%). The flasks were incubated on shaker (120 rpm) at 45°C-55°C for 24 h. Following incubation, 1 ml of culture was taken and serially diluted (10^{-1}-10^{-7}) with sterile distilled water. Thereafter 100 µl of the diluted samples were inoculated on gelatin agar plates containing gelatin (15g), peptone (4 g), yeast extract (1 g), agar (15 g), and water (1000 ml), and the plates were incubated for 48 h at 37°C [7, 8].

2.2. Characterization of Bacterial Isolate

The identification of bacteria was performed using morphological, microscopic and biochemical analysis. Gram-stained slides were microscopically examined. Catalase activity and gelatin hydrolysis test were utilized for biochemical analysis and colony morphology was also done on fresh bacterial isolates grown on culture. In addition, proteolytic activity was tested by inoculating the isolate in to skim milk agar medium [9, 10].

2.3. Enzyme Production

2.3.1. Submerged Fermentation (SMF)

Sterile gelatin broth (25 ml) was added in 250 ml conical flask and inoculated with a loop full of the fresh culture then incubated at 37°C for 48 h on a rotary shaker at 150 rpm. Following incubation, 1ml of the culture was transferred to 1.5 ml sterilized eppendorf tubes and centrifuged at 10,000 rpm for 5 min. After centrifugation, the cell free supernatant which contains the produced enzyme was collected and used as a crude enzyme preparation [11].

2.3.2. Solid-State Fermentation (SSF)

SSF media was used for enzyme production from the selected isolates using wheat bran as a sole carbon source with the addition of the following nutrients per 13ml final volume: NaCl (0.5 g), casein (0.5 g), $CaCl_2 \cdot 7H_2O$ (0.01 g), $MgSO_4 \cdot 7H_2O$ (0.02 g), KH_2PO_4 (1 g) and 1;10 g wheat bran was moistened with above nutrient solution to a moisture level of 60% (v/w ratio) in 250 ml Erlenmeyer flasks for cultivation of the bacterial isolates. After sterilization, each SSF medium was inoculated with 2 ml of overnight-cultured inoculum of bacterial isolate and incubated at 37°C for 120 h. The crude enzyme from SSF media was extracted by adding 100ml of sterile distilled water and shaken at 120 rpm for 1h on the shaker and filtered through a muslin cloth. Finally the enzyme activity was measured by enzyme assay methods [12, 13].

2.3.3. Enzyme Assay

The reaction mixture containing 450 µl 1% (w/v) of casein in 100 mM Tris buffer at pH 8 was mixed with 50 µl enzyme extract and incubated for 30 min at 50°C. The reaction was stopped by adding 450 µl of 10% Trichloroacetic acid (TCA) solution (w/v). Following the termination of the reaction, the mixture was centrifuged at 10,000 rpm for 5 min, then 150 µl supernatant was mixed with 750 µl 0.5 M Na_2CO_3 solution and 150 µl 0.2 N Folin solution reagents was added to the test tube for color detection. The reaction mixture was incubated in dark at room temperature for 30 min. Finally, the optical density (OD) was measured against a sample blank at 660nm using spectrophotometer [14].

2.3.4. Gelatinase Assay

In gelatinase assay, 250 µl gelatin and 250 µl buffer solutions were added to test tubes and the reaction was started by adding 100 µl diluted crude enzyme extract solution. The reaction mixture was then incubated at 50°C for 30 min. After incubation, 400 µl HCl solution (0.1M) was used to stop the reaction and then 10 µl 4 M NaOH, 15 µl acetate buffer, and 100 µl Ninhydrin reagents were added for color detection. The reaction mixture was incubated in a water bath for 15 min. After removing from the water bath, the test tubes were cooled and 1 mL diluents were added. The OD was measured against a sample blank at 570 nm using spectrophotometer [15].

2.4. Enzyme Characterization

2.4.1. Effect of pH on Activity of Gelatinase

To determine the effect of pH on the activity of gelatinase, three different buffer systems i.e., phosphate buffer (pH 6-8), Tris-buffer (pH 7-9) and Glycin buffer (pH 8-10.5) were used. The three buffers were mixed with 450 µl 1% casein solution and 50 µl of enzyme extract. Following incubation of the reaction mixtures at 50°C for 30 min, the residual activity was measured using enzyme assay methods as described in section 2.3.3 [16].

2.4.2. Effect of Temperature and Thermo-Stability on Gelatinase Activity

To determine the effect of temperature on the activity of gelatinase, the crud enzyme diluted (1:20) mixed with 100 mM Tris–buffer and 1% casein substrate. The reaction mixtures were incubated at different temperatures ranging from 40°C to 65°C for 30 min. Determination of Ca^{2+} requirement for gelatinase activity was also done by another similar setup of experiment without the addition of Ca^{2+}. The substrate used contains 5 mM of $CaCl_2$ solution at pH 8. The residual activity was measured by using enzyme assay methods [17].

The thermostability was determined by incubating the enzyme at 50°C, 55°C, and 60°Cfor 0-60 min in a water bath. First 100ul enzyme extract was added into labeled eppendorf tubes and incubated in a water bath at respective temperatures. Each eppendorf tube was taken out and stored in refrigerator (+4°C) at 10 min interval. Then each of the incubated enzymes was assayed and residual activity was determined [18].

2.5. Culture Conditions Optimization for Enzyme Production

2.5.1. Time Course of Enzyme Production

To determine the period of maximum enzyme production, the organism was grown in liquid medium and incubated at room temperature on rotary shaker at 120 rpm. Two ml of the culture broth containing the bacteria was added onto solid medium (wheat bran) and incubated at 37°C for 24, 48, 72, 96, 120 and 144 hours.

Finally enzyme production of the culture filtrate was determined by assaying the activity of the enzyme at each incubation time [19].

2.5.2. Effect of Moisture Level on Production of Gelatinase Enzyme

Optimum moisture content required for the growth of bacteria and production of gelatinase was determined by growing the organism at 37°C in the SSF media at a moisture level (v/w) of 33.3%, 50%, 60%, 66.7%, and 75%. The enzyme was harvested after 120 h and the maximum enzyme production was determined by using enzyme assay methods [20].

2.5.3. Effect of Nitrogen Source on Enzyme Production

To determine the appropriate nitrogen source, the organism was grown in a liquid medium at room temperature. After the organism grew, 2 ml of culture broth was added into solid-state medium that contained different nitrogen substrates such as casein, ammonium sulfide, peptone, sodium nitrate and yeast extract. The inoculated solid-state medium was incubated at 37°C for 120 h and the maximum enzyme production was determined using enzyme assay methods [21].

2.6. Isolation of Hoof Gel

The hooves were chopped into small pieces and washed using tap water. The chopped hooves were boiled for a minimum of 10 h. After boiling, it was kept in beaker and stored at 4°C for 2 h. After refrigeration, the oil found at the top of the sample was removed by using spatula. The gel portion was transferred to a new flask and stored at +4°C for further analysis [22, 23].

2.7. Hydrolysis of Gelatin and Hoof Gel

Five grams of commercial gelatin (BD, United States of America) powder was dissolved in a test tube containing 20 ml of distilled water while the hoof sample was added in to a flask and then heated until it becomes liquefied. Then, 20 ml of liquid hoof gel was transferred in to a test tube and 500 µL enzyme extract was added in both test tubes and incubated at 50°C for 3 h. After the end of the incubation time the samples were incubated in to a refrigerator (+4°C) for 1h. Finally, the control (Commercial gelatin), was compared with hydrolyzed samples [24].

2.8. Optimization of Condition for Hydrolysis; Enzyme Concentration, Incubation Time and Viscosity of Hoof Gel

To determine the optimal enzyme concentration of hydrolysis, first commercial gelatin powder was dissolved in distilled water and hoof samples were boiled, then the two samples (commercial gelatin powder and hoof) were added into separate test tubes. Different amount of concentrated enzymes (50 ul-200 ul) were added into the test tubes and incubated at 50°C for 3 h, then the degree of hydrolyzed was measured [25]. In this research the degree of hydrolysis (DH) value was measured by using titration. The hydrolyzed samples were added into a cylinder and one drop of phenophitaline reagent was added into the reaction mixture. Then a droplet of sodium hydroxide was added until light pink color appeared. Finally, the amount of sodium hydroxide was recorded and the DH value was calculated using the formula;

$$\text{Concentration of NaOH} = \frac{\text{mole of NaOH}}{\text{Titrated volume (ml)}}$$

To determine the optimal incubation time, the reaction mixtures were incubated at 1 h, 2 h, 3 h and 4 h at 50°C, then the incubated samples were titrated as described in section 2.10 then the degree of hydrolysis was calculated.

In this study, fluidity was assessed by adding 10 ml of

hoof gel and 150 µl of enzymes to a test tube, and then incubated at 50°C for 1 to 4 hours. After incubation, the volume was measured after the hydrolyzed sample had gone through a funnel as comparison to a water sample (3 ml of water was taken in to the funnel, sealed at the bottom, then released to follow downward and measure the time interval) that against the hydrolyzed hoof gel.

2.9. Production of Gelatin Hydrolysate in Powder Form

For the production of hydrolysate powder, the hoof sample and enzyme extract were added in to beaker, and then incubated at 50°C for 3 hr. After incubation, the hydrolyzed hoof gel was poured in to eppendorf tube and centrifuged at 1300 rpm for 5 min. The supernatant was added to a Petri dish and dried using an oven. The dried sample was grinded manually using a mortar and pestle then the powder was kept at room temperature.

2.10. Data Analysis

The enzyme optimal density was measured and its concentration was calculated by using standard Tyrosin curve. The enzyme concentration was converted in to unit per gram and finally, the data was processed by using Microsoft excel.

3. Results

3.1. Isolation of Gelatinase Producing Bacteria and Enzyme Production

In this study, fifty bacterial organisms were isolated from soil sample from Addis Ababa University and the remaining fifty was isolated from muddy water sample collected in Afdera, Afar region, Ethiopia. Among the entire isolates, only 24 of them were positive and used for gelatinase hydrolysis test. Of the 24 isolates, only one isolate was selected for its high enzyme activity based on the highest value of optical density. The isolated bacteria were gram-positive, rod-shaped, slightly yellow in color and positive on catalase and gelatin hydrolysis test. Based on morphological and biochemical characteristics, the isolate was categorized to the genus Bacillus (Figure 1).

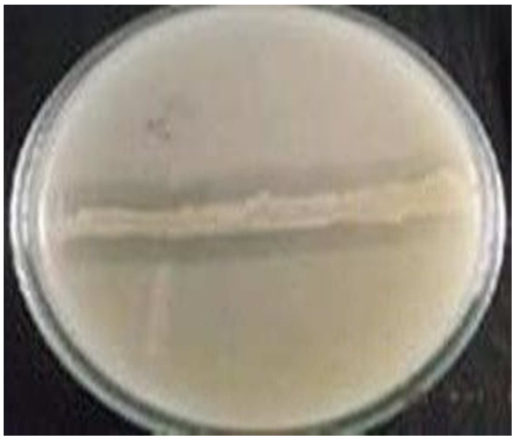

Figure 1. *Proteolytic activity of gelatinase producing bacteria.*

In this study, the enzyme was produced in solid-state fermentation using 10 g, 50 g and 100 g of wheat bran. Maximum enzyme production and activity was obtained in SSF with 100 g of what bran (Figure 2).

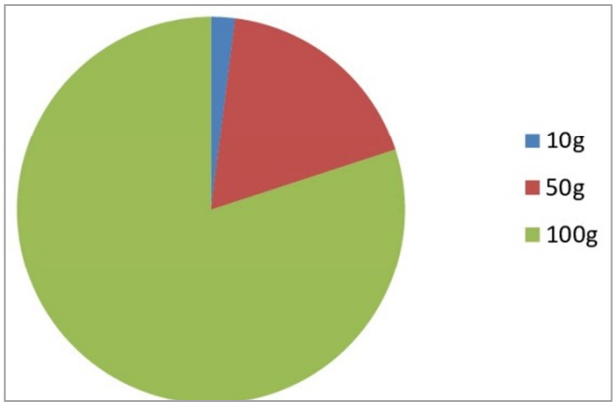

Figure 2. *Enzyme production at different amount of wheat bran.*

3.2. Effect of Temperature and pH on the Enzyme Activities

The maximum enzyme activity was recorded at 50°C. However, the enzyme activity was reduced above the optimal temperature. The enzyme showed relatively higher activity in the presence of Ca^{2+} (Figure 3).

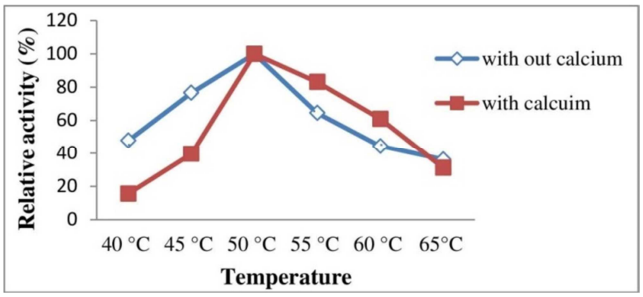

Figure 3. *The effect of temperature on the activity of gelatinase in the presence and absence of 5mM$CaCl_2$.*

The effect of pH on the enzyme activity was determined in the pH range from 6 to 10.5 and maximum gelatinase activity observed at pH 8. When it's beyond pH 8, the enzyme activity was decrease because the pH becomes more alkaline (Figure 4).

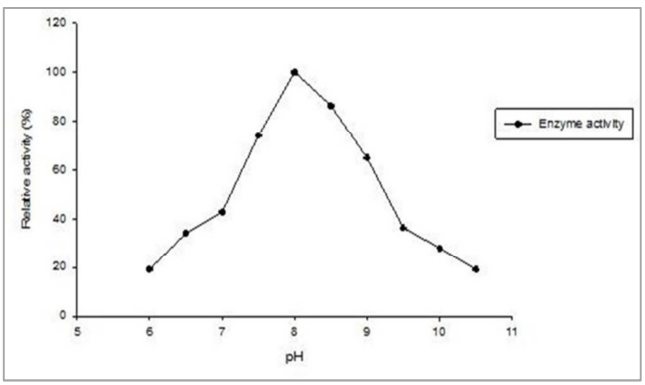

Figure 4. *Activity of Gelatinase enzyme at different pH.*

3.3. Thermostability and Effect of Incubation Time on Gelatinase Production

The thermo-stability of gelatinase enzyme was relatively stable at 55°C (Figure 5). This result indicates that relative activity of the enzyme was decreased with increasing incubation time.

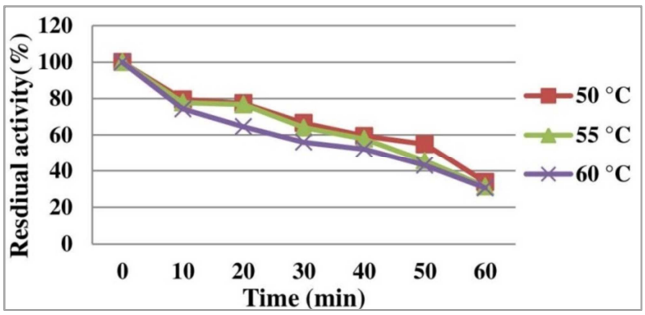

Figure 5. *Thermo-stability of gelatinase enzyme without Ca^{2+}.*

The maximum enzyme production was recorded at 120 h of incubation time. When it is incubated beyond 120h, the enzyme production was decreased (Figure 6).

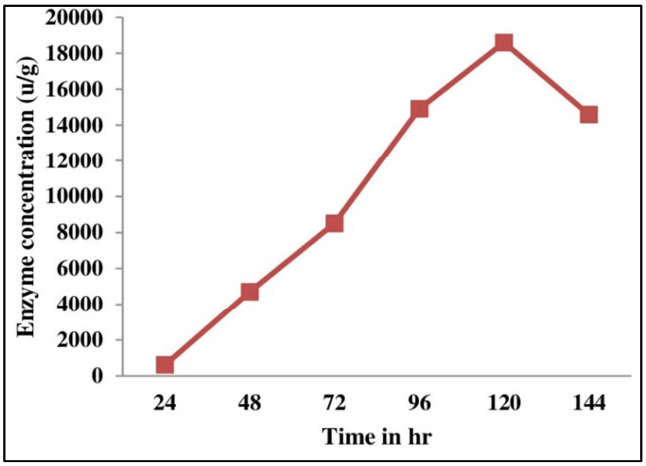

Figure 6. *The effect of time course on gelatinase production.*

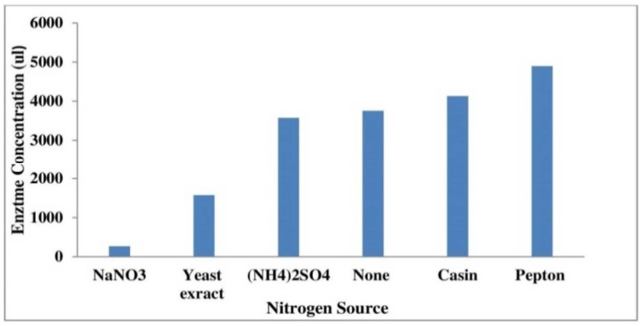

Figure 7. *The effect of nitrogen source on gelatinase enzyme.*

3.4. Effect of Nitrogen Source on Enzyme Production

Enzymes production was done on five nitrogen sources. The maximum enzyme production was recorded in solid material supplemented with peptone (Figure 7). However, gelatinase production was decreased in the presence of sodium nitrate.

3.5. Effect of Moisture Level and Optimum Incubation Time of Hydrolysis

To determine the optimal moisture level, the enzymes was produced at different amount of moisture level. Maximum enzyme production was recorded at 66.7% and less enzyme production was observed at 33.3% ratio of moisture level (Figure 8).

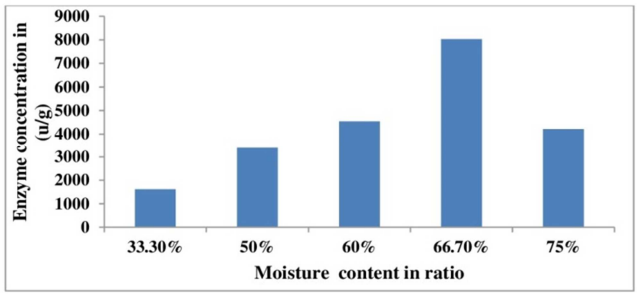

Figure 8. *Effect of moisture content on gelatinase enzyme production.*

The optimization of hydrolysis condition was done by hydrolyzing two samples (gelatin powder and hoof gel). The two samples were hydrolyzed by varying the incubation time and at a constant amount of enzyme. The incubation time was varied from 1 h up to 4 h at 50°C. The maximum degree of hydrolysis occurred at 4 h. Generally, when incubation time increased, degree of hydrolysis was also increased (Figure 9).

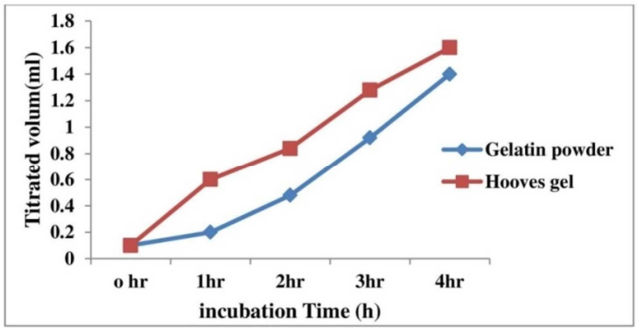

Figure 9. *Commercial gelatin and hooves gel hydrolysis against incubation time.*

3.6. Optimization of Hydrolysis Conditions and Hoof Gel Fluidity

Commercial gelatin powder and hoof gel were hydrolyzed by varying enzyme concentration at constant temperature (50°C) and incubation time. Maximum titrated volume was recorded at high amount of enzyme concentration. Generally, when enzyme concentration increased degree of hydrolysis also increased (Figure 10).

In this study, it has shown that when incubation time increased, the fluidity of hoof gel was also increased (Figure 11).

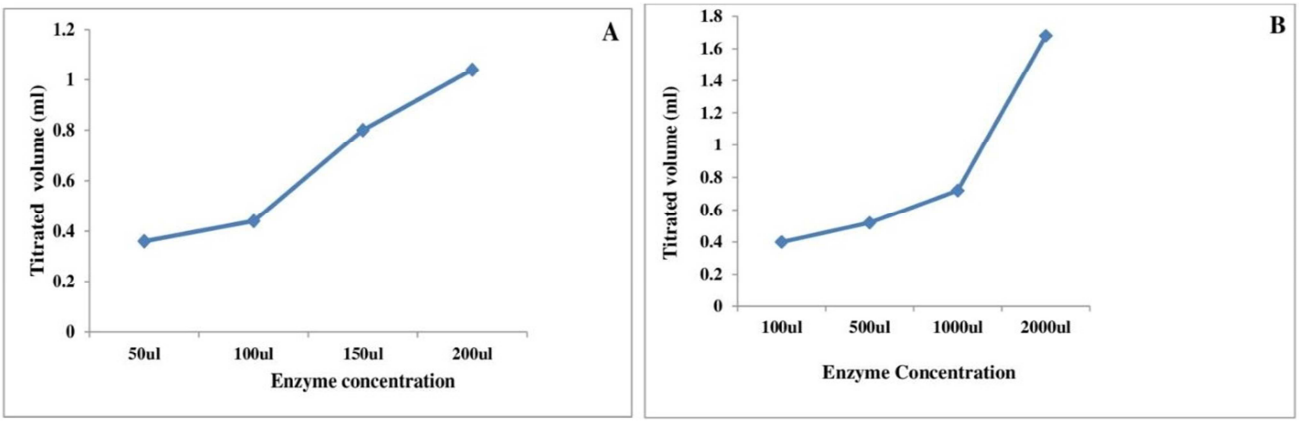

Figure 10. *A and B Commercial gelatin powder and Hoof gel hydrolysis in different enzyme concentration.*

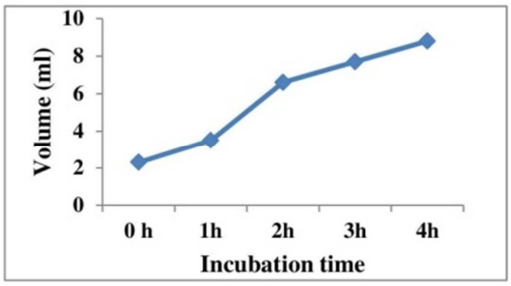

Figure 11. *A profile of viscosity of Hoof.*

3.7. Production of Hydrolysate Powder

Hydrolysate powder was produced when hoof gel was hydrolyzed and dried. Once dried, its appearance will become light yellow color, ability to become sticky when moistened with water and it can also be stored at room temperature. These properties are similar as compare to commercial gelatin powder (Figure 12).

Figure 12. *Hydrolysate powder produce from hoof of animal.*

4. Discussion

Gelatinases are enzymes that hydrolyze gelatin into smaller oligopeptides and amino acids that are important for industries engaged in the production of hydrolysates. A gelatinase was optimally active at 50°C. Similarly previous studies also reported microbial gelatinases as optimally active at 50°C [26]. The optimum pH for activity was set at pH 8, but the enzyme was active in the pH range of 6 to 10.5. Similarly, a collagenase enzyme with optimum activity at pH 7.5 was also reported from other microbial sources [27]. This indicates that the enzyme identified in this study has properties similar to those of other enzymes and indicates its potential application for gelatin hydrolysis.

For many industrial enzymes, the cost is often a major factor, greatly limiting their wider applications. About 40% of the production cost of microbial enzymes is accounted by the cost of growth substrate [28]. Organisms capable of growing under solid-state fermentation using cheap agro-industrial wastes allow a substantial reduction of the production cost of enzymes [29]. As a means of reducing production costs, isolate was grown under solid-state fermentation using wheat bran as a substrate. The highest enzyme production was observed at a substrate to moisture ratio of 66.6%. When the moisture content was low, the enzyme production decreased, most likely because the substrate's solubility in the medium was reduced. On the other hand, above the optimum moisture content, enzyme production decreases because water molecules occupy the space in the wheat bran, thus decreasing the availability of oxygen [30].

The time at which the culture grows is an important factor in the production of enzymes. Maximum enzyme production in microbial strains was captured in the late early stationary and exponential phases [30]. In this study gelatinase enzyme was optimality active at 120 h of incubation time, when the incubation period increased beyond 120 h the activities of enzyme were decrease. This was probably because of the depletion of essential nutrients and the accumulation of nutrients possibly producing toxic substances.

The availability of nitrogen sources that can be readily assimilated by the organism is known to enhance enzyme production. In this study, out of the five different nitrogen sources, peptone supported the highest enzyme production. This shows that the protein available in the wheat bran is not sufficient to support maximum enzyme production. Thus, supplementing the culture medium with organic nitrogen sources such as peptone, which is rich in peptides, vitamins, and amino acids, could lead to better growth and enzyme production [31].

Scaling up of SSF by isolating bacteria from 10 g, 50 g, and 100 g led to a progressive increase in enzyme production. Compared to submerged fermentation, SSF appeared to be relatively cheap. However, one drawback of SSF is the difficulty associated with scaling up. As with the increasing volume of SSF substrate, the amount of heat generated correspondingly increases and this poses challenges in temperature control. In this study, maximum enzyme production was observed at the highest substrate amount used (100 g). This is probably because at a high substrate amount, the heat generated might be optimal for the growth of the isolate.

The maximum gelatin hydrolysis using enzyme was recorded after a 4 h incubation period because the longer hydrolysis time allowed the enzyme to solubilize (degrade) more gelatin and a greater amount of peptide bond was broken, resulting in a large number of H^+ ions being released [32]. When the amount of enzyme level varies, the maximum activity was recorded at a high amount of enzyme concentration because enzymes affect active sites and degrade more hydrogen bonds, so it can increase peptide bond cleavage and degree of hydrolysis or DH values [25].

In this study, the viscosity of the raw hoof sample was measured by comparing the viscosity of hydrolysates produced by gelatinase to the viscosity of water. The maximum activity was recorded in the presence of enzyme and at high incubation time because, in the presence of enzyme, the viscosity of the hoof starts to hydrolyze (degrade) and converted to its lowest form unit.

5. Conclusion

A gelatinase producing bacteria were isolated from soil and water samples in Addis Ababa and Afdera, Ethiopia. This shows the potential of Ethiopia's unique microbial diversity as a source of enzymes for different industrial applications. Currently Ethiopia slaughters a large number of cattle and release huge quantities of animal bones, pieces of skin and other offal thus causing serious environmental pollution. On the other hand, if properly handled these wastes could be used as sources of valuable products. The result reported in this study showed that enzymatic hydrolysis of collagen contained in animal waste could be enzymatically hydrolyzed using microbial gelatinases and the resulting gelatin hydrolysate can be used for a variety of food and non-food applications such as making special desert foods, candies, and cosmetics.

6. Recommendation

From the result obtained in this study we recommend that before initiating large-scale gelatin hydrolysis, the process should be evaluated first in small pilot-scale production and The potential application of the resulting hydrolysate in the food industry or in the cosmetics industry should be tested.

Conflict of Interest

The authors declare that they have no competing interests.

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