

**ADDIS ABABA UNIVERSITY SCHOOL OF GRADUATE STUDIES, DEPARTMENT OF
MICROBIAL, CELLULAR AND MOLECULAR BIOLOGY**



Hydrolysis of Gelatin from Animal Hoof using Bacterial Gelatinase

By

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ABBREVIATIONS

AAU.....Addis Ababa University

DH.....Degree of hydrolysis

ODOptical density

Rpm.....Revolution per minute

SSF.....solid state fermentation

SMF.....Submerged fermentation

TCATrichloro acetic acid

ABSTRACT

Gelatinases are enzymes used to hydrolyze gelatin into peptides and amino acids. Enzymatic hydrolysis of gelatin increase its functional, textural and nutritional characteristics. The hydrolysates have neutral taste, low bloom and contain high amount of purified protein. In recent years gelatin hydrolysates find a number of increasing applications in food and non food products. The main objective of this study was to isolate gelatinase enzyme from soil bacteria and optimize the reaction condition for gelatin hydrolysis. A total of 100 bacterial isolates were isolated from soil samples collected around Addis Ababa University (Arat Kilo Campus) and water samples collected from Lake Afdera. All isolates were screened for the production of gelatinase on gelatin containing media. Based on the properties of the enzyme, one isolate designated as **aau17** was selected for further study. The organism can grow under solid-state fermentation and produce gelatinase enzyme with high activity using wheat bran as a carbon source. The enzyme 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 up on addition of peptone as a nitrogen source. The enzyme was used to produce gelatin hydrolysate by hydrolyzing animal bone, animal skin, animal hooves and commercial gelatin. 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 high amount of enzyme concentration. This study indicated that by using product of animal waste can be converted to useful gelatin hydrolysates using microbial gelatinases for various applications.

Key words: Gelatinase, Hooves, Hydrolysis

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. Other by products of the meat industry currently finds little or no use including animal bones, pieces of skin, blood and other offal often discarded thus causing environmental pollution. On the other hand bone and animal skin are known sources of gelatin (Mariod and Adam, 2013).

Gelatin is a protein derived from animals such as cows, pig and fish by boiling skin, ligament hoof and bone. Chemically it is composed of 21% of glycine, 12% proline, 8% arginate, 9% alanine, 6% aspartate and the remaining containing other types of amino acids (Steavens, 1992). Gelatin has different applications in the food industry, for example in the preparation of confectionary and different dessert foods (Karim and Bhat, 2008), production of tablet coating and suppositories by the pharmaceutical industry and production of X-ray films in other industries (Cavello *et al.*, 2013).

Gelatin is hydrolyzed by the enzyme gelatinase to smaller peptides and amino acids. Thus gelatinase can be used for the production of gelatin hydrolysates that find several interesting applications in different industries. Gelatinase enzymes are produced by bacteria such as *Bacillus sp.*, *Pseudomonase aeruginosa*, *Staphylococcus aureas* and *Clostridium perifergens*. Enzymatic hydrolysis of gelatin increase its functional, textural and nutritional characteristics. In recent years gelatin hydrolysates find a number of increasing applications in food formulations (Wei and Zhimin, 2006). This is because such hydrolysates have neutral taste, low bloom and contain high amount of purified protein. It can thus be used for the production of protein bars, protein drinks, smoothies and joint health nutritional products, emulsifiers, candies and even fit in wider use in the cosmetics industry (Liu *et al.*,2010).

Considering the availability of gelatin raw material and the potential applications of gelatin hydrolysate, Ethiopia can gain huge benefit from this resource by developing appropriate technology for the production of gelatin hydrolysates. But today there has not been any attempt at utilizing this resource for the production of gelatin hydrolysates. In the production of hydrolysates availability of efficient gelatinase enzyme is considered very critical.

The aim of this study was, therefore, to isolate gelatinase enzyme producing bacteria, characterize the enzyme and optimize the reaction condition of enzyme hydrolysis.

2. OBJECTIVES

2.1. General objective

The general objective of this study was to isolate gelatinase producing microbial strains and optimize the reactions for enzyme hydrolysis

2.2. Specific objectives

- To isolate gelatinase producing bacteria and characterize the enzyme
- Optimize the reaction condition for the hydrolysis of gelatin derived from animal hooves
- To formulate gelatin hydrolysate as a powder form.

3. LITERATURE REVIEW

3.1 Proteins

Proteins are large macromolecules made up of amino acids that use in different metabolic processes. Proteins differs from one another by their sequence of amino acid and gene sequence which usually results in protein folding into a specific three-dimensional structure that determines their activity (Gutteridge and Thornton, 2005). There are many amino acids but 20 of them are only utilized by human body, these amino acids are obtained during consumptions of food such as animal tissue and animal product (Wu *et al.*, 2016). These food products are the main source of proteins. The protein products are hydrolyzed by using either chemical or enzymatic processes. In many industries chemical hydrolysis is common but biological processes that use the addition of enzyme are more promising when product with high nutritional value and improved functionality are required.

3.2 Proteases

Microbial protease is a large class of industrial enzymes accounting 40% of the total worldwide scale of enzymes (Rao *et al.*, 1998) and digests protein from sources such as milk, meat and plant source. Proteases have different industrial application such as detergent, tannery, food industries and for the production of different media. Proteases are classified into different clans and families depending on their amino acid sequences, evolutionary relationships and pH optimal. Based on the optimal pH protease are classified as acidic, neutral, or alkaline proteases (Jisha *et al.*, 2013). It can also be classified in to different groups; these are serine protease, cysteine protease, asparatic protease and metalloprotease (Rawlings and Barrett, 1993).

3.3 Collagen

Collagen is the most abundant fibrous protein that can be extracted by products of animals slaughter such as bones, skin, cartilage and connective tissues (Muyonga *et al.*, 2004; Cheng *et al.*, 2009). It is composed of 35% glycine, 11% alanine, 21% proline and hydroxyprolin. These amino acids are the most important amino acids in collagen accounting for 50% of the total protein content (Mat maroh *et al.*, 2011). The proline and hydroxyproline content is particularly important for the gelling effect.

Collagen has different properties related with gelling behavior such as texturizing, thickening, gel formation and water binding capacity. It has also a property related to their surface behavior, which include emulsion, foam formation, stabilization, adhesion and cohesion, protective colloid function and film-forming capacity (Gomez-Guillen *et al.*, 2011).

The structure of collagen contains 1000 amino acids, (Liu *et al.*, 2001; Cheng *et al.*, 2009).The chains became entangled, forming a stable triple helix which is varied in size. The triple helix molecule has terminal globular domains called procollagen. These globular regions are cleaved and form polymerized structure (tropocollagen). The polymerized structure molecules stabilized by hydrophobic and electrostatic interaction.

3.3.1 Application of collagen in food and pharmacological industries

Collagen is used in different industries due to its functional property such as ether absorbance capacity, gel formation and stabilize emulsion that are responsible for various industrial applications (Lafarga and Hayes, 2014). In food industries it improves color, texture, flavor or qualities and in confectionary products it is used as emulsifier, thickener and for preservation (Hashim *et al.*, 2015). It is also used in beverage industries to make collagen-drinks that is used in global markets. These products used in different Asian countries especially in Malaysia. According to Tree (2012) an energy drink infused with collagen promotes the body's natural capacity to generate the fatty tissues .In pharmacological industries the application of collagen is related to its natural properties, including hemostatic activity, biodegradability, low allergenicity with high antigenicity and biocompatibility (Helena *et al.*, 2013). It is used mainly for the production of different drugs, proteins, genes substitute for human skin and wound healing (Kim and Mendis, 2006; Gómez-Guillén *et al.*, 2011).

3.3.2 Collagen hydrolyzing enzyme

Collagen is hydrolyzed by enzymes known as collagenases. Collagenase enzymes are one group of metalloprotease enzyme that degrades native collagen into useful product (gelatin). These enzymes are produced by bacterial isolates such as thermophilic *Bacillus sp.* Collagenase enzyme produced by a *Bacillus sp* has a maximum activity at neutral pH which ranges from (7.0-8.0) (Harrington, 1996) and optimum temperature between 45 up to 50°C.

3.4 Gelatin

Gelatin is a pure protein that can be obtained from the thermal denaturation of collagen derived from animal skin, white connective tissue and bones of animals (Morrison *et al.*, 1999). Gelatin contains all the essential amino acids except tryptophan. There are two types of gelatins based on their source; gelatin A and gelatin B. Gelatin A is obtained from porcine while gelatin B is derived from bovine. Both sources contain components with different molecular weights with wide distribution ranging from 10 – 400 kda. Most commercial gelatin is currently sourced from beef bone, hide, pigskin and more recently, pig bone (Raja *et al.*, 2011). It was reported that 41% of the gelatin produced in the world is obtained from pigskin followed by bovine bones (29.5%) and bovine hides (28.5%). Gelatin from pigskin is preferred because it required low cost and has high gel strength.

3.4.1 Structural and Chemical property of gelatin

Gelatin has high molecular weight polypeptides ranging from 20,000 to 250,000kda. It is tasteless, odorless and with whitish-yellow color (Cole and Roberts, 1997). Commercially available gelatin generally contains 9–10% moisture. It is not only soluble in cold water, but also in aqueous solutions of polyhydric alcohols such as glycerol and propylene glycol. Gelatin contains large amount of glycine and proline (Schgens *et al.*, 1995).

The structure of gelatin contains primary and secondary structure. The primary structure is related to collagen with little difference related to the source of raw material in combination with the pretreatment and extraction on procedure. Secondary structure of gelatin has polypeptide chain including alpha chain, beta chain and gamma timer of alpha chain component (Fernandez-Diaz *et al.*, 2010) and have high gel strength that determined by using Bloom Strength, which ranges from 50-325 g.

3.4.2 Application of gelatin

Gelatin has been widely used in different industries such as food, pharmacological and other industries. Due to its content of proteins and amino acids gelatin has been used in food industries. Edible gelatin is made from animal hide and bone through the processing techniques such as defatting, pulverization, freezing and drying (Fu *et al.*, 2000).

Edible gelatin contains confectionery products (Karim and Bhat, 2009). In Confectionery products, gelatin is used to make gummy products and candies that made from sugar, glucose syrup and gelatins with some gel strength varying from 50-300 bloom grams. Gelatin is also used in beverage and juice industries for clarification purpose. It is also used as a stabilizer, particularly in dairy industry for making special dessert foods such a sour cream, yogurt and cheese (Gimenez *et al.*, 2005).

In pharmacological industries gelatin is used mainly for hard and soft gelatin capsule, tablet coating, encapsulation and granulation. Hard gelatin capsule is produced using high bloom gelatin, 200-260 bloom by a dipping process. The capsule has body and cap used for protection of the active ingredient from unnecessary exposure to moisture, heat or other extreme conditions thus increasing stability and make it easy to swallow (Gibbs *et al.*, 1999). Soft gelatin capsules are special coatings that are used to protect the drug or active substance from gastric enzyme, make it easy to swallow and increase the bioavailability of active substance. It is also used as suppositories for insertion to rectum and vagina, because it is nontoxic and nonirritating to mucous membranes.

Gelatin is used for preparation of bacterial medium that is used for detection and counting of various bacteria strains. A medium prepared from gelatin is used for research and diagnostics purposes. In the preparation of oil emulsion gelatin is used as tropical allowing the oil to be stored, maintained droplet distribution and droplet size over extended period (Dickinson and Lopez, 2001).

3.5 Methods of hydrolysis of gelatin

There are two types of hydrolysis methods namely chemical and enzymatic methods. Chemical methods consist of acidic and alkaline treatment (Sinha *et al.*, 2007).

3.5.2 Chemical methods

Chemical methods of protein hydrolysis are techniques used for hydrolysis of different organic compounds by using alkaline and acidic substances (Schaafsma, 2009). Acid hydrolysis is a chemical method of hydrolysis carried out by adding strong acids such as hydrochloric acid and sulfuric acid on the protein to be hydrolyzed.

Acid treatment break peptide bond and sometimes it may destroy some amino acids such as tryptophan, methionine and cysteine. Alkaline hydrolysis is carried out with the addition of strong alkaline chemicals such as sodium hydroxide and potassium hydroxide with the appropriate temperature treatment for facilitation of the process (Dieterich *et al.*, 2014).

3.5.3 Enzymatic methods

Enzymatic hydrolysis is breaking down of compounds using enzymes. During hydrolysis, the bonds that link amino acids in protein molecules are cleaved to produce peptides at optimum temperature and pH. This can be used to develop new functional properties by directing and managing the hydrolysis process (Khalil *et al.*, 2006). Enzymatic hydrolysis does not destroy any amino acids and up on the completion of the hydrolysis process the enzymes can be easily deactivated to facilitate the isolation of the protein hydrolysates. The disadvantages of enzymatic hydrolysis of proteins include the relatively high cost and the potential presence of enzyme inhibitors in the raw protein materials (Hou *et al.*, 2017).

3.5.3.1 Degree of hydrolysis

The degree of hydrolysis is the percentage of cleaved peptide bonds in a protein. It is used to quantify the range of proteolysis, the degree to which a protein source has been hydrolyzed. It is a reflection of the number of peptide bonds broken (Wang *et al.*, 2013). Degree of hydrolysis is used to evaluate the released amount of nitrogen from the hydrolysis of protein in the presence of TCA which is the precipitating agent (Haslanize *et al.*, 2010) and it can be done using titration. These methods are also useful to establish the relationships between the proteolysis and the improvement of functional bioactive and sensory properties of these bimolecular. Even though several techniques are currently used to determine the degree of hydrolysis there is no standard method for determining DH (Rutherford, 2010).

3.6 Gelatin hydrolyzing enzymes

Gelatinase enzymes are exoenzymes that are used to degrade gelatin, casein and fibers. These enzymes are produced by microorganisms such as bacteria and fungi. The bacteria that are used for production of gelatinases include *Bacillus sp*, *Pseudomonase aeruginosa*, *Staphylococcus aureas* and *Clostridium perifergens*. The presence of gelatinase producing bacteria can be detected by gelatinase hydrolysis test. Some *Bacillus* strains secrete gelatinolytic enzymes that are useful for production of gelatin and collagen derived hydrolysates and peptides (Rao *et al.*, 1998; Gómez-Guillén *et al.*, 2011). Some fungi such as *ascomycets* also produce gelatinase. The gelatinase from fungal species isolated from marine environment are optimally active at neutral pH and temperature range of 30-50°C.

3.6.1 Classification of gelatinase enzyme

There are two types of gelatinase enzymes; gelatinase A and gelatinase B. Both gelatinase A and B enzymes are used for degradation of gelatins and collagens. Regarding its structure, gelatinase B has domains which can bind with gelatin type II, laminin and collagens type I and IV-collagenases (Van *et al.*, 2002). Gelatinase A degrades gelatin type I and collagenase type IV and V (Murphy *et al.*, 1985). Both enzymes have polypeptide domain, an N-terminal domain, amino acid domain homolog and zinc binding active sites. Gelatinase B plays several important functions in neutrophilic action, such as degrading extracellular matrix, activation of IL-1 and cleavage of several chemokines (Opdenakker *et al.*, 2001). After gelatin is hydrolyzed by using gelatinase enzyme, hydrolysate is formed. This hydrolysate has different physical property, nutritional value and industrial applications.

3.7 Gelatin hydrolysate

Gelatin hydrolysates are produced from enzymatic hydrolysis of gelatin powder that is isolated from animal products. Depending on the hydrolysis condition and the starting material, the hydrolysates have different characteristics including improved solubility, thermostability, enhancing and reducing emulsifying property (Mahmoud *et al.*, 1992). Gelatin hydrolysate powder contains high amount of purified protein or amino acids. It has neutral taste which cannot affect sensory property and low amount of gelling property. The hydrolysates are used to improve food and non-food products (Liu *et al.*, 2010).

4. MATERIALS AND METHODS

4.1 Sample collection

Soil samples were collected from Addis Ababa University, Arat Kilo Campus. The soil samples were aseptically transferred to sterile bags and transported to the laboratory using an icebox maintained at (+4°C). Spring water and mud samples were collected from Lake Afdera, Afar region and the samples were aseptically transferred into bottles and transported using an icebox.

4.2 Isolation of gelatin hydrolyzing bacteria

One gram soil sample was suspended in 1 ml of 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 (1.5%) of gelatin, yeast extract (0.1%), KH_2PO_4 (0.1%), NaCl (0.5%), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.02%) and $\text{CaCl}_2 \cdot 7\text{H}_2\text{O}$ (0.01%). Then the flasks were incubated on shaker at 45 °C-55 °C for 24 h and at 120 RPM. After 24 h incubation 1 ml of culture was taken and serially diluted (10^{-1} - 10^{-7}) with sterile distilled water. Thereafter 100 µl of the diluted culture was inoculated and spreaded on gelatin agar plates. Gelatin agar medium containing gelatin (15 g), peptone (4 g), yeast extract (1 g), agar (15 g) and water (1000 ml). The plates were incubated for 48 h at 37 °C.

4.3 Detection of gelatin hydrolyzing bacteria (primary screening)

A total of 100 distinct bacterial colonies were picked from the gelatin agar plates. These, 100 bacterial colonies were transferred onto fresh gelatin agar plates and incubated at 37 °C for 48 h to purify and check their purity. Gelatinase producing bacteria isolates were detected by using skim milk agar medium and gelatin hydrolysis test.

4.3.1 Gelatin Hydrolysis Test

Gelatinase producing bacteria were detected using gelatin hydrolysis test. First (12 g) nutrient gelatin powder were dissolved in 100 ml of distilled water. About 5 ml gelatin medium was distributed into culture tubes and sterilized. The pure bacteria isolates were inoculated into the gelatin medium and incubated at 37 °C for 24 h. After 24 h incubation the test tubes were cooled in a refrigerator for 20 min. The test tubes that contained liquified medium after this treatment were considered positive for gelatinase production (Clarke, 1953).

4.3.2 Hydrolysis test on Skim milk agar

Skim milk agar media containing (g/l) skim milk powder (2.5 g), agar (3.5 g), yeast extract (2 g), casein (2.5 g) peptone (2.5 g), $\text{CaCl}_2 \cdot 7\text{H}_2\text{O}$ (0.1 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g) and KH_2PO_4 (2 g) was prepared and inoculated with selected bacterial isolates. After 48 h incubation at 37 °C, colonies were checked for the formation of a clear halo zone around colonies.

4.4 Enzyme production

4.4.1 Submerged fermentation (SMF)

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

4.4.2 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 (g/13ml): (0.5 g) NaCl, 0.5 g casein, $\text{CaCl}_2 \cdot 7\text{H}_2\text{O}$ (0.01 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (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 distilled water and shaken for 1h at 120 RPM on the shaker, then filtered through a muslin cloth. Finally the enzyme activity was measured by enzyme assay methods.

4.5 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 crude enzyme extract and incubated for 30 min at 50 °C. The reaction was stopped by adding 450 µl of 10% TCA solution.

After the reaction stopped the supernatant and the pellet was separated by centrifugation at 10,000 rpm for 5 min. Then 150 μ l supernatant was mixed with 750 μ l 0.5 M Na_2CO_3 solution and 150 μ l 1/5 N Folin reagent was added to the test tube for color detection and the reaction mixture was incubated at room temperature for 30 min. Finally, optical density (OD) was measured against a sample blank at 660nm using spectrophotometer (Amare and Berhanu, 1997).

4.5.1 Gelatinase assay

In gelatinase assay, the reaction mixture containing 250 μ l gelatin solution and 250 μ l buffer solutions were added to test tubes. The reaction was started by adding 100 μ l diluted enzyme solution and incubated at 50 °C for 30 min. After incubation 400 μ l HCl solution (0.1M) was used to stop the reaction and then 100 μ l of the above reaction mixture was taken into new test tube and then 10 μ l 4 M NaOH, acetate buffer and 100 μ l Ninhydrin reagents were added for color detection. The reaction mixture was boiled in a water bath for 15 min in boiling water. Finally the test tubes were cooled and 1,000 μ l diluents were added, after a few min. The optical density (OD) was measured at 570 nm (Rosen, 1957).

4.6 Enzyme characterization

4.6.1 Effect of pH on activity of gelatinase

To determine the effect of pH on the activity of gelatinase, three different buffer systems were used. These buffers were phosphate buffer (pH 6.0, 6.5, 7.0, 7.5 and 8), Tris-buffer (pH 7.5, 8.0, 8.5 and 9.0) and glycine buffer (pH 8.5, 9.0, 9.5, 10.0 and 10.5). The three buffers solutions were mixed with 1% casein solution, enzyme diluted (1:20) and incubated at 50 °C for 30 min and then the enzyme activity was measured as described in section 4.5.

4.6.2 Effect of temperature on the activity of gelatinase enzyme with and without Ca^{2+}

The effect of temperature on the gelatinase activity was determined by taking 1% casein substrate, 100 mM Tris–buffer and the enzyme (1:20 dilution) and incubated at 40 °C – 65 °C for 30 min. Determination of Ca^{2+} requirement for gelatinase was done by incubating the enzyme at 40-60 °C for 30 min. The substrate contained 5 mM of CaCl_2 solution at pH 8. The enzyme activity was determined as described in section 4.5.

4.6.3 The thermostability of gelatinase enzyme

The thermostability of gelatinase enzyme was determined by incubating the enzyme from 50 °C - 60°C for 0-60 min in a water bath. First 100 ul crude enzymes 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 following the standard assay procedure described in section 4.5 at 50 °C. Finally residual enzyme activity was determined.

4.6.4 Optimization of culture conditions for enzyme production

4.6.5 Time course of enzyme production

To determine the period of maximum enzyme production, first, the organism was grown in liquid medium incubated at room temperature on rotary shaker at 120 rpm. After the organism grew, 2 ml of the culture broth was added into solid medium and incubated at 37°C for 24 h, 48 h, 72 h, 96 h, 120 h and 144 h. Finally enzyme production of the culture filtrate was determined by assaying the activity of the enzyme at each incubation time following the standard enzyme assay as described in 4.5.

4.6.6 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.

4.6.7 Effect of nitrogen source on enzyme production

To determine the appropriate nitrogen source, the organism was grown in liquid medium at room temperature. After the organism grew, 2 ml of culture broth was added into solid state medium that contained different nitrogen substrate 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 then the culture grew and the maximum enzyme production was determined.

4.7 Isolation of hoof gel

Gelatin was isolated from animal hooves. The hooves were chopped into small pieces by using grinder and then stored in freezer at -20 °C to prevent contamination. The samples were washed three or four times by using tap water to remove the blood or any debris and unwanted materials. The samples were added into aluminum extractor. The aluminum extractor was filled with distilled water and hoof sample was boiled for minimum of 10 h. After boiling the oil was removed and the water part added into a flask and stored at 4 °C.

4.8 Gelatin hydrolysis

Gelatin hydrolysis was done by using commercial gelatin powder and hoof gel. Five grams of commercial gelatin powder was dissolved in 20 ml of distilled water while the hoof sample was added in to a flask and then heated until it becomes liquid following this ,10 ml liquid hoof sample was added in to a test tube and then 500 ul enzyme was added in both samples and incubated at 50 °C for 3 h. After 3 h the incubated samples were taken in to a refrigerator (+4°C) for about 1h. Finally, the control remains solid, the hydrolyzed samples become liquids.

4.9 Optimization of condition for hydrolysis

4.9.1 Enzyme concentration

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 were added into test tubes. Different amount of concentrated enzymes were added into the test tubes and incubated at 50 °C for 3 h.

4.9.2 Incubation time

To determine the optimal incubation time the reaction mixtures were incubated at 1 h, 2 h, 3 h and 4 h at 50 °C. Finally, degree of hydrolysis was measured (Samart *et al.*, 2013). In this research DH value was measured by using titration. The hydrolysates 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 below.

DH calculated as:- concentration of NaOH = $\frac{\text{mole of NaOH}}{\text{Titrated volume (ml)}}$.

4.10 Viscosity of hooves gel

Viscosity measures the amount of fluidity by using viscometer. In this study fluidity was determined on hoof samples. To determine the fluidity, 10 ml of hoof gel was added into a test tube and then 150 ul of enzyme was added into the test tube containing the hoof gel. The reaction mixture was incubated at 50 °C for 4 h and the hydrolyzed hoof sample passing through a panel was collected into a cylinder and the volume was recorded at 1 h interval.

4.11 Production of gelatin hydrolysate in powder form

After hydrolysis, the hoof sample was centrifuged for 5 min and for separation of the supernatant and pellet. The supernatant was added in to a plate and dried using an oven. The dried sample was grinded using mortar and pestle to make powder.

4.12 Biochemical and morphological characterization of aau17

The bacteria isolate was identified by morphological and biochemical tests. The biochemical tests were Gram staining and catalase activity; colony morphology was also done for pure culture of the isolate.

4.12.1 Gram staining: - For Gram staining, pure bacteria culture was taken in to slide and heat fixed. After heat fixation the Gram staining procedure was followed. The bacteria isolate stained with crystal violet, iodine, ethanol and safranin. During the staining the slide was stayed for one min and the slide was washed with tap water. Finally, the bacteria isolate was observed under a microscope (Bartholomew and Mittwer, 1952).

4.12.2 Catalase test:- Catalase test was done according to the method of Clarke and Cowan(1952). In this method pure bacterial colony was taken, one drops of 3% hydrogen peroxide was added to slide and mixed with the bacterial isolate, presence of bubble formation was considered as catalase positive and absence of bubble formation was considered catalase negative.

5. RESULTS

5.1 Isolation of gelatinase producing bacteria

Out of a total of 100 bacterial isolates tested (50 from Addis Ababa University and 50 from Lake Afdera), 24 isolates were positive for gelatinase production and three of the isolates is indicated in (Fig 1).

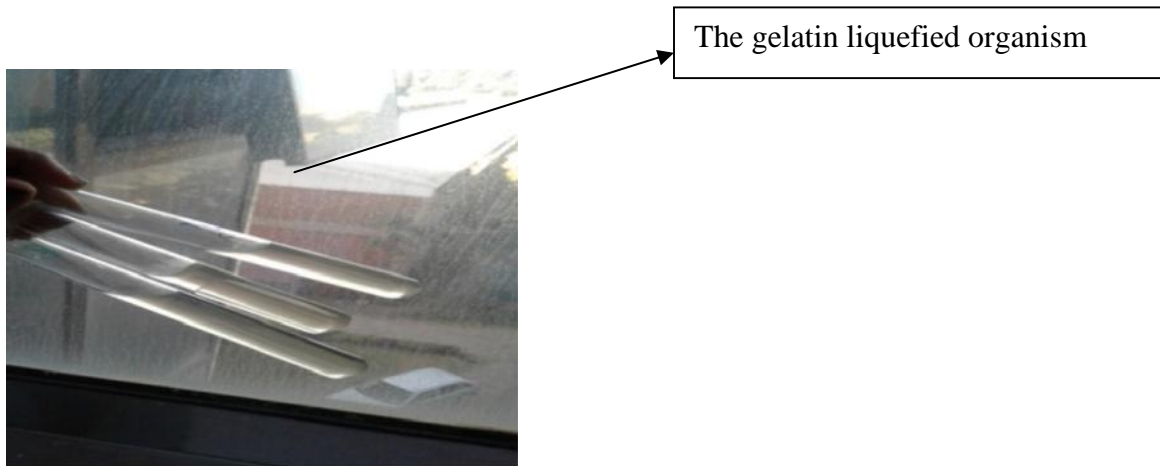


Fig. 1:-Gelatin hydrolysis test positive organism

5.2 Identification of bacteria isolates

The bacterial isolate used in this study was gram positive, rod shape, slightly yellow color and catalase positive. It formed clear zone on skim milk medium, this indicated that the bacterium has the ability to hydrolyze protein and positive on gelatin hydrolysis test. This bacterial isolate belongs to *Bacillus* species.



Fig. 2:-Proteolytic activity of **aau17** showing clear zone around the colony

5.3 Enzyme production

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 a SSF with 100 g of wheat bran (Fig. 3).

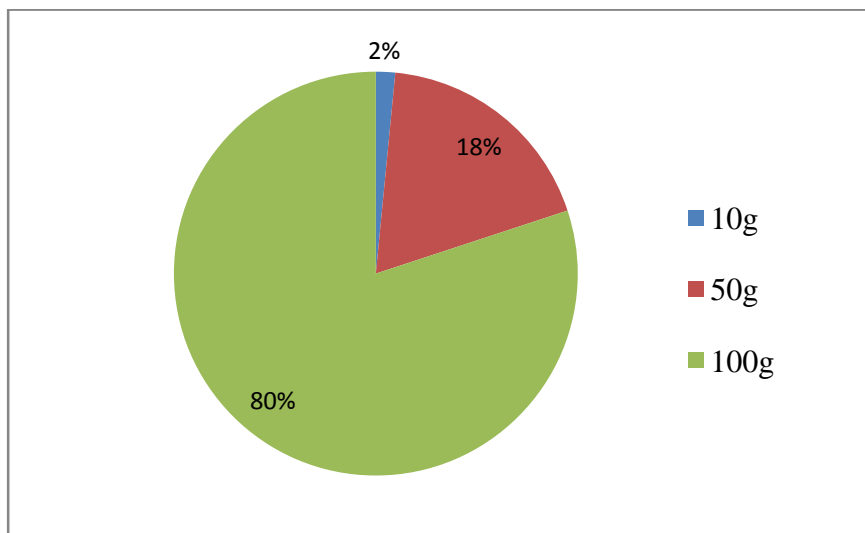


Fig. 3:-Enzyme activity at different concentration of wheat bran

5.4 Enzyme characterization

5.4.1 Effect of temperature on the activities of gelatinase enzyme with or without Ca^{2+}

The temperature profile was determined in the range of 40 °C to 65 °C (with 5 intervals) (Fig. 4). 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} above its optimum temperature.

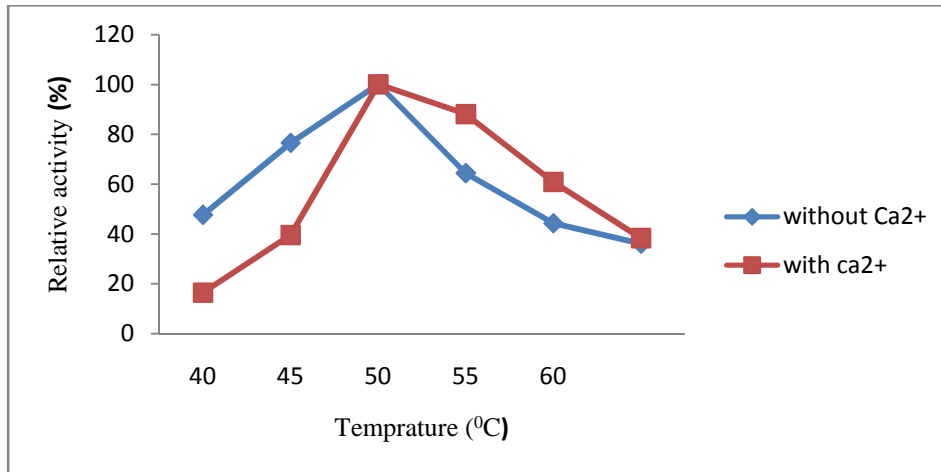


Fig.4:-The effect of temperature on the activity of gelatinase in the presence and absence of 5m M CaCl₂

5.4.2 Effect of pH on gelatinase enzyme

The effect of pH on the enzyme activity was determined in the pH range of 6 to 10.5. The maximum gelatinase activity was recorded at pH 8.0 (Fig. 5).

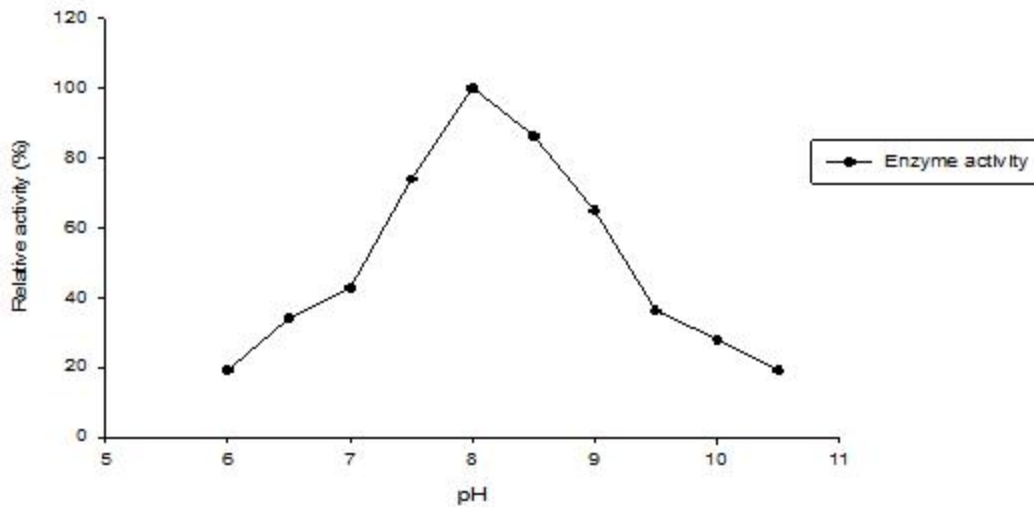


Fig. 5:-Activity of gelatinase enzyme at different pH value

5.4.3 The thermostability of gelatinase enzyme

The temperature stability of the enzyme was determined by incubating at 50 °C, 55 °C and 60 °C and assaying the pre-inoculated enzyme (Fig. 6). The relative activity of the enzyme was decreased with increasing incubation time. The enzyme was relatively stable at its optimal temperature and 55 °C.

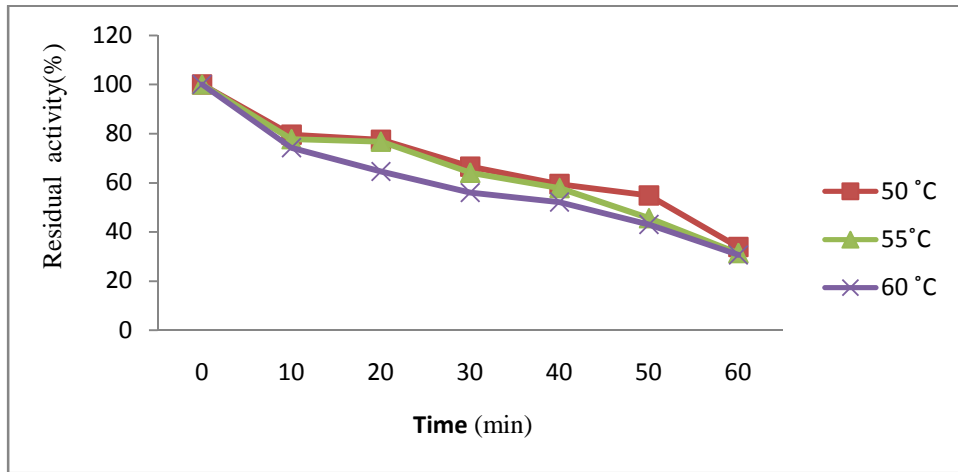


Fig. 6:- Thermo stability of gelatinase enzyme with out Ca^{2+}

5.4.4 Effect of incubation time on gelatinase production

The maximum enzyme production was recorded at 120 h incubation time at 50 °C (Fig. 7). The enzyme production was increased as the incubation time increased but reduced at 144 h.

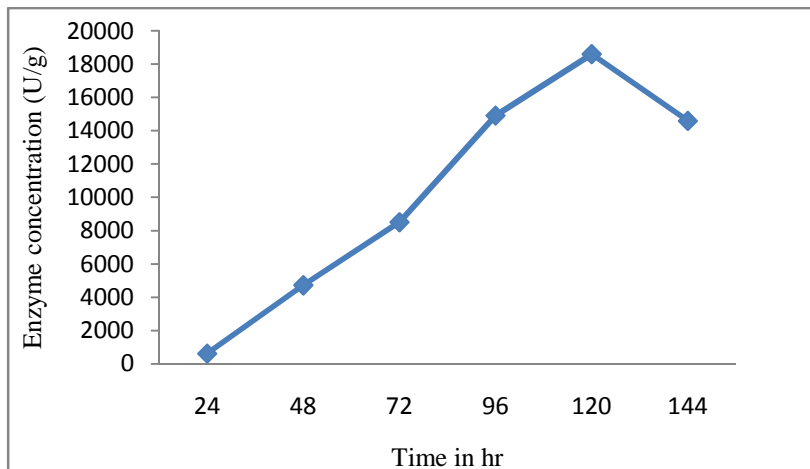


Fig. 7:-Time course of gelatinase production by **aau17**

5.4.5 Effect of moisture level on gelatinase production

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

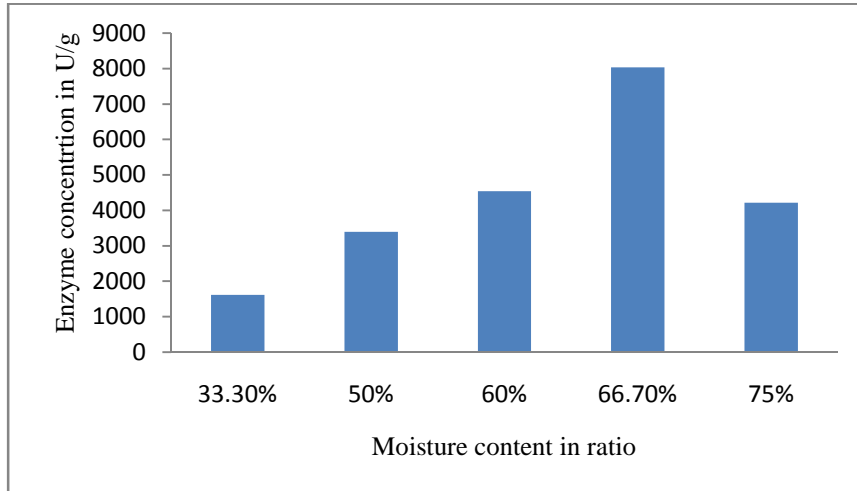


Fig. 8:-Effect of moisture content on production of gelatinase

5.4.6 Effect of nitrogen source on enzyme production by *aau17*

Enzymes production was done on four nitrogen sources. The maximum enzyme production was record in solid material supplemented with peptone (Fig. 9).

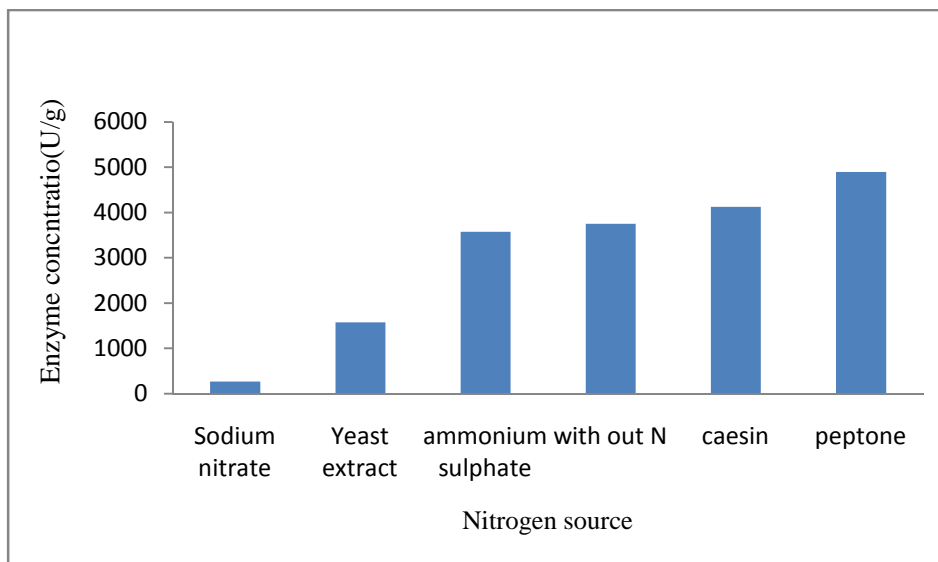


Fig. 9:-Effect of nitrogen source on enzyme production

5.5 Optimization hydrolysis condition

5.5.1 Incubation time

The optimization was done by hydrolyzing two samples (gelatin powder and hooves gel). The two samples were hydrolyzed by varying the incubation time (Fig. 10). 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, increase in the degree of hydrolysis was observed.

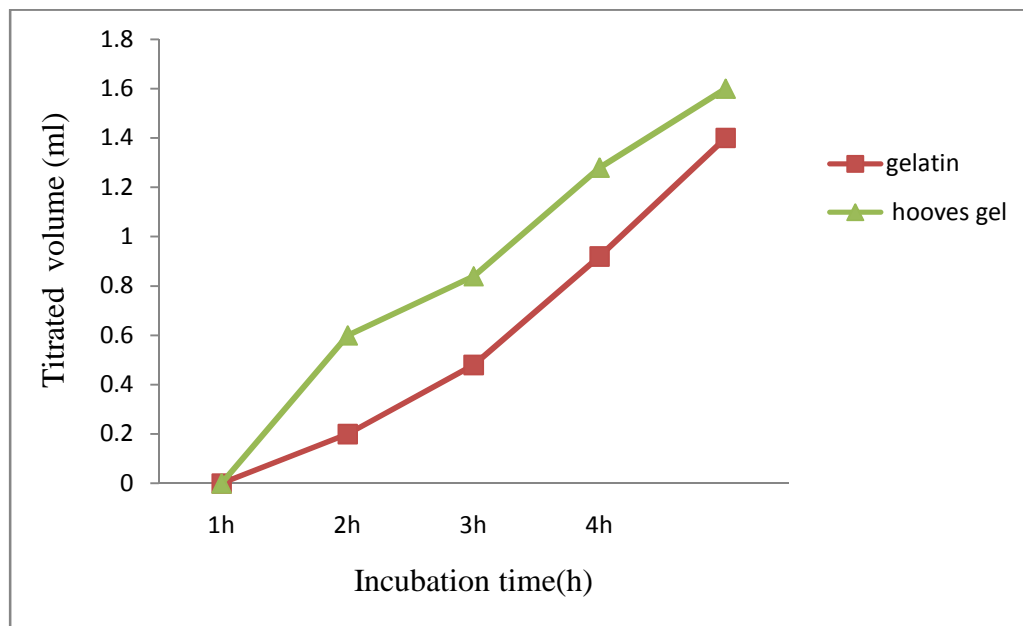


Fig. 10:-Commercial gelatin and hooves gel hydrolysis by *aau17* against incubation time

5.5.2 Optimization of enzymatic hydrolysis conditions

Commercial gelatin powder and hoof gel were hydrolyzed by varying enzyme concentration at 50 °C and at a constant incubation time (Fig. 11). In both samples as enzyme concentration increased, increase in the degree of hydrolysis was observed.

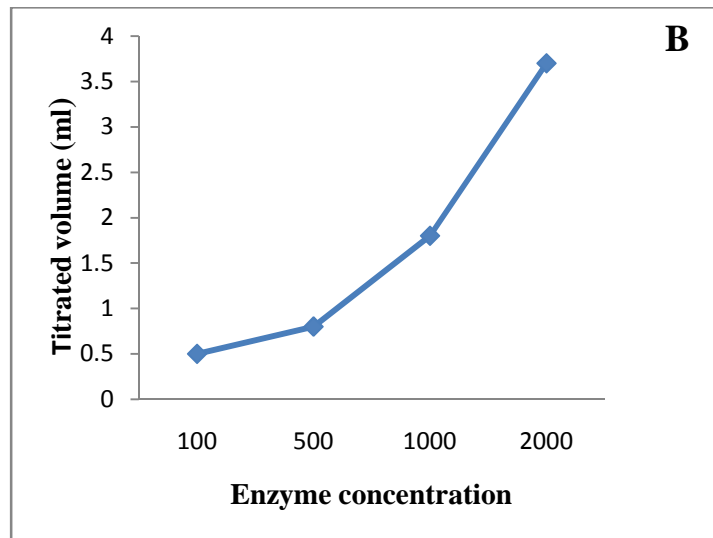
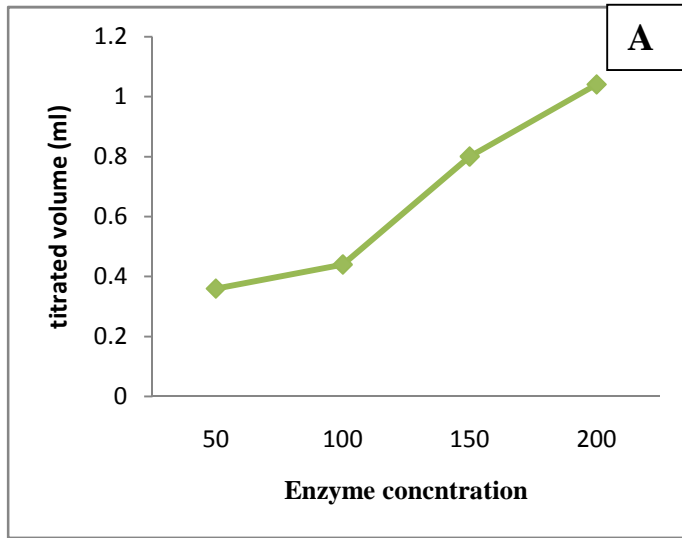


Fig.11:-Figure A and B shows that Commercial gelatin and Hoof gel hydrolysis in different enzyme concentration

5.6 Fluidity (viscosity) of hooves gel

Fluidity was determined on hoof sample (Fig. 12). The incubation time varies from 1 h to 4 h. When incubation time increased, the fluidity of hoof gel was also increased. The maximum fluidity was recorded at 4 h of incubation.

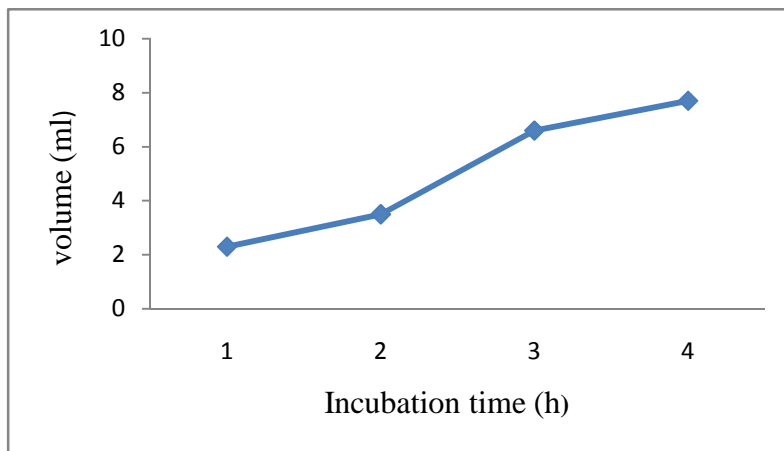


Fig. 12:-A profile of viscosity of hoof hydrolysis by **aau17**

5.7 production of hydrolysate powder

Hydrolysate powder is produced when hoof gel was hydrolyzed using enzyme and incubated for 3 h at 50°C then dried by using oven. After that it's able to be identified as light yellow color and when moistened with water it became sticky similar to gelatin, but gelatin is more jelly.



Fig. 13:-Hydrolysate powder produce from hoof animal

6. DISCUSSION

Gelatinases are enzymes that hydrolyze gelatin into smaller oligopeptides and amino acids that are important industrially for the production of hydrolysates. The aim of this study was to isolate gelatinase producing bacterial strains that can be used as sources of enzymes for industrial scale application. A gelatinase optimally active at 50°C temperature was isolated from soil. Previous studies also reported microbial gelatinases that are optimally active at 50°C (Nagano and To, 1999).

The optimum pH for activity was recorded at pH 8 but the enzyme was active in the pH range of 6 to 10.5. Similarly collagenase enzyme with optimum activity at pH 7.5 was also reported from other microbial sources (Petrova *et al.*, 2006) This indicates that the enzyme identified in this study have properties similar to other enzymes and indicate its potential for application for gelatin hydrolysis.

For many industrial enzymes cost is often a major factor greatly limiting their wider applications. In relation to microbial enzymes up to 40% of the production cost of enzymes is accounted for by the cost of growth substrate (Joo and Chang, 2005). Organisms capable of growing under solid state fermentation using cheap agroindustrial wastes allow substantial reduction in production cost of enzymes (Pandey, 2003). As a means of reducing production cost isolate **aau17** 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 below the optimum enzyme decreased probably because solubility of the substrate in the medium is reduced. On the other hand above the optimum moisture content enzyme production decreased because water molecule occupies the space in the wheat bran thus decreasing availability of oxygen (Gupta *et al.*, 2002).

The time at which enzyme harvested from the culture is important factor in the enzyme production. In most microbial strains maximum enzyme production was recorded at the late exponential and early stationary phases (Gupta *et al.*, 2003). For isolate **aau17** the highest enzyme production was observed at 120 h incubation. Extending the incubation period beyond 120 h led to a decrease in enzyme production probably because of depletion of essential nutrients or due to accumulation of toxic substance produce.

Availability of nitrogen source that can be readily assimilated by the organism is known to enhance enzyme production. In this study, out of the four different nitrogen source, 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 with organic nitrogen sources such as peptone which is rich in peptides, vitamins and amino acids (Nehete *et al.*, 2013) could lead to a better growth and enzyme production.

Scaling up of SSF by isolate **aau17** from 10 g, 50 g and 100 g led to progressive increase in enzyme production. Compared to submerged fermentation SSF is considered to be cheap. However, one drawback of SSF is the difficulty associated with scale up (Bashir *et al.*, 2011). As with increasing volume of SSF substrate the amount of heat generated correspondingly increase and this poses challenges in temperature control. In this study maximum enzyme production was observed at the highest substrate amount used (100 g). Probably this is because at high substrate amount the heat generated might be optimal for the growth of the Isolate.

The maximum gelatin hydrolysis using enzyme aau17 was archived at 4h incubation period, because the longer hydrolysis time allowed enzyme to solubilized (degrade) more gelatin and larger amount of peptide bond were broken so large number of H⁺ ions released (Khantaphant and Bengkulu, 2008). When the amount of enzyme level vary, the maximum activity was record at high amount of enzyme concentration because enzymes affect active sites and degrade more hydrogen bonds so it can increase peptide bond cleavage and DH values (Samart *et al.*, 2013).

In this research the viscosity of the raw hoof sample was measured by comparing the viscosity of hydrolsates that produced by gelatinase to the viscosity of a water. The maximum activity was record 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) gelatin and will converted in to lowest form unit .

7. CONCLUSION AND RECOMMENDATION

A new gelatinase producing bacteria were isolated from soil and water samples in Addis Ababa and Afdera and in this study only one isolates was selected for further characterization. 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 waste could be used as sources of valuable products. The result reported in this study shows that enzymatic hydrolysis of collagen containing 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. However, before initiating large-scale gelatin hydrolysis it is recommended that the process be scaled up to a pilot scale. It is also recommended that the potential application of the resulting hydrolysate in the food industry, such as for making special dessert foods and candies or its potential application in the cosmetics industry is tested.

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