ADDIS ABABA UNIVERSITY COLLEGE OF NATURAL SCEINCES SCHOOL OF GRADUATE STUDIES DEPARTMENT OF MICROBIAL, CELLULAR AND MOLECULARBIOLOGY

"PRODUCTION OF MICROBIOLOGICAL PEPTONE FROM HYDROLYSIS OF SLAUGHTERHOUSE OFFAL USING BACTERIAL PROTEASE"

By

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Abbreviation

AAs ..... amino acids

BPA ..... bone peptone agar

CFU ..... colony forming unit

CPA ..... commercial peptone agar

EA ..... Enzyme activity

EB ..... Enzyme blank

EPHI ..... Ethiopia public health institute

hr ..... hour

LPA ..... lung peptone agar

OD ..... Optical density

RA ..... Relative activity

Rpm ..... Revolution per minute

RRA ..... Relative residual activity

SMF ..... Submerged fermentation

SPA ..... soya bean peptone agar

SSF ..... Solid state fermentation

TCA ..... Trichloro acetic acid

ABSTRACT

Proteases are the most important class of industrial enzymes accounting for 60% of the global industrial enzyme market. Microorganisms are the major source of these enzymes. Production of hyrolysates from different protein sources is among the different application of proteases. Protein hydrolysates have a variety of food and non-food applications. Although different proteases are available in the market, there is always a need for the development of new enzymes from bacterialsources. This is especially important in countries like Ethiopia where there are no local enzyme producers. The aim of this study was, therefore, to isolate new protease producing bacterial isolates to be used for the hydrolysis of slaughterhouse offal, optimize enzyme production and hydrolysis conditions, and test hydrolysates as a microbiological growth media. Based on screening data on solid and liquid media, one bacterial isolate designated as ***aau*$_5$** was selected for further study. The isolate grew under solid-state fermentation (SSF) and produced up to 5,773 U/g of enzyme. Enzyme production was optimal when the solid to moisture ratiowas 1:2(66.7 % moisture content) and in the presence of organic nitrogen sources. Protease ***aau*$_5$** was optimally active at pH 7.5 and temperature of 55°C. After one hour incubation, the enzyme retained up to 66% and 41% of its original activity at 50°C and 55°C, respectively. Protease ***aau*$_5$** was used for the hydrolysis of slaughter house offal (lung and bone) and soybean protein. The hydrolysate (peptone) was then tested as a microbiological media for the growth of different bacterial species. Compared to commercial peptone, hydrolysate obtained from lung (LPA) and bone extracted protein (BPA) supported better growth of the test organisms. So, by using waste and by products of slaughter houses, beneficial hydrolysate like peptone can be produced through enzymatic hydrolysis.

1. INTRODUCTION

Proteases are the most important class of commercial enzymes accounting for 60% of the global industrial enzyme market (Gupta *et al*., 2005). They find huge application in detergent, food, pharmaceutical, silk and leather industries.

Although all organisms produce proteases, from a commercial perspective microorganisms are the most preferred sources of these enzymes. This is because microorganisms have fast growth rate, require limited space for their cultivation and the ease at which their enzymes can be genetically manipulated to generate new enzyme variants for specific applications (Harmsen and De Haard, 2007).

Protease production by microorganisms is affected by different parameters, such as growth temperature, quantity of inoculum, medium pH, and type and composition of medium (Puri *et al*., 2002). Once produced the activities of microbial protease can also be influenced by temperature and pH of the reaction medium. Thus based on the optimum reaction proteases are classified as acid, neutral or alkaline proteases (Narasimha *et al*., 2011).

One important application for microbial proteases is in the hydrolysis of proteins from different wastes, such as offal from slaughterhouses, feather from poultry processing industries and fish processing wastes (Dalev, 1994). Protein rich wastes released by slaughterhouses include meat attached to bone and animal viscera. The protein in these wastes can be enzymaticaly hydrolyzed in to peptides or amino acids and can be used for different applications. Some of the applications include use as food or feed supplements, leather tanning supplements, microbiological media and cosmetics (Cerqueira *et al.,* 1999; Rebah and Miled, 2013)

Currently the non edible part of the cattle like, lung, trachea, kidney, brains, spleen, intestine, bone and other Slaughterhouse offal are mainly used for wild and domestic animals feed (Irshad and Sharma, 2015). Considering the high protein content of these wastes, such practices are wasteful, especially for developing countries like, Ethiopia where the hydrolysate could find huge application in different sectors of the economy. For example in Ethiopia, the only known use for cattle lung is as a feed to cats. But in the majority of cases it is disposed as waste at the slaughterhouse. Similarly meat attached to bones and the intestine parts of cattle is also discarded to the garbage. This in

addition to losing valuable resource, it potentially causes environmental pollution. Development of methods for the enzymatic hydrolysis of such offal is expected to have significant economic returns and that requires development of appropriate enzymes for the hydrolysis and optimization of the reaction conditions.

The aim of this study was, therefore, to isolate a new protease producing bacteria, characterize the enzyme, optimize reaction conditions for the hydrolysis of slaughterhouse offal and test the hydrolysates as a microbiological media.

1.2. OBJECTIVE OF THE STUDY

1.2.1. General objective

The main objective of this study was to identify a new protease locally that can be used for the production of protein hydrolysates from slaughterhouse offal and evaluation of the hydrolysates for different applications.

1.2.2. Specific objectives

- Isolate microbes that produce an extracellular protease, optimize cultivation conditions and characterize the enzyme
- Optimize the enzymatic hydrolysis of slaughterhouse offal
- Test the resulting protein hydrolysates as a component of microbiological media

2. Literature review

2.1 Protein

Protein is very complex macromolecules that are made up of different amino acids (AAs), these AAs are linked by peptide bond. They have a very diverse function that is related to their chemical structure. Protein is essential for all living organism. For example, animal tissue and products like meat, milk, and egg are composed of proteins as a major component (Wu *et al*., 2016). For the production of hydrolysate either through chemical or enzymatic method the plant and animal source of proteins is preferable. Hydrolysis of protein can improve their nutritional quality and reduce any associated anti-nutritional factors (Dieterich *et al*., 2014).

2.2. Source of proteins

- **Protein ofanimal Source**

The major protein source of animal includes; blood, meat, casein, whey, and intestine-mucosa. According to Jayathilakan *et al*. (2012), many of meat processing industry wastes such as bones, tendons, skin, gastro-intestinal tract, blood and internal organs have high protein content. Their protein content is varying with each type of animal.

The byproducts (including organs, fat or lard, skin, feet, abdominal and intestinal contents, bone and blood) of cattle, pigs and lams represents 66.0, 52.0 and 68.0% of the live weight, respectively (Jayathilakan *et al*., 2012). Among the byproducts more than 50% are not suitable for normal consumption and this leads to an increasing cost of disposing this product. However, using the waste and byproduct of those meat processing industry for the production of hydrolysate have an advantage by sustain profitability of the industry.

- **Protein of plant source**

Plant is the other preferred source of protein for the production of hydrolysate. Those plant sources include cereals, legumes, oilseeds and vegetable source such as sorghum, Soybean, Pea, Chickpea, Lupin, and Canola etc. Plant proteins are classified in to four major classes: albumins, globulins, prolamins and glutelins based on their solubility and extractability in various solvents and these four proteins are known as “Osborne fractions” (Osborne, 1924). Albumins and globulins are concentrated in the aleurone layer, bran, and germ, whereas their concentration in the

starchyendosperm is relatively low. Predominantly, prolamins and glutelins are the main proteins storage of cereal grains (Koehler and Wieser, 2013).

2.3. Proteolytic enzyme (proteases)

Proteases are a group of enzyme, which hydrolyze peptide bonds in aqueous environment that means can hydrolyze proteins via the addition of water across peptide bonds (Beg *et al*., 2003). Proteases are essential constitutes of all forms of life on earth, including prokaryotes, fungi, plants and animals. They comprise one of the most important groups of commercial enzymes. These enzymes have ample utilization in industrial process such as detergent (a major consumer of protease) as well as food and leather industries (Kumar and Takagi, 1999; Gupta*et al*., 2002).

2.4. General classification of proteases

The classifications of protease do not comply easily with the general system of enzyme nomenclature due to their huge diversity of action and structure. On the basis of their site of action on protein substrates, proteases are broadly classified as endo- or exo-enzymes. They are further categorized as serine proteases, aspartic proteases, cysteine proteases and metalloproteases depending on their catalytic mechanism. Proteases are also classified into different clans and families depending on their amino acid sequences and evolutionary relationships. Based on the pH optima, they are referred to as acidic, neutral, or alkaline proteases (Jisha *et al*., 2013). Each family of peptidases has been assigned a code letter denoting the type of catalysis, i.e., S, C, A, M, or U for serine, cysteine, aspartic, metallo, or unknown type, respectively (Rawlings and Barrett, 1993).

2.5. Major Sources of protease

Proteases are necessary for physiological processes of living organisms, they are found in a wide diversity of sources such as animal, plant and microorganism (including, bacteria, fungi and virus) (Rao *et al.,* 1998).

2.5.1. Animal Proteases

Pancreatic trypsin, chymotrypsin, pepsin and rennin are the most familiar proteases from animal origin. These enzymes are responsible for different application like for the hydrolysis of protein, diagnostic, analytical application and in the dairy industry to produce stable curd with good flavor. But, the production of animal protease depends on the availability of livestock for slaughter, which in turn is governed by political and agricultural policies (Boyer, 1971).

2.5.2. Plant Proteases

Recently, the interest received by plant proteases has increased significantly as they are widely used in medicine and food industry (Gonzalez *et al.*,2011). As plant-derived products, they are perceived as safe and "natural" ingredients for use in the food applications and may offer unique benefits and functionality. However, the problem associated with the production of plant proteases lies in the selection of suitable climatic conditions and land for cultivation. As the concentration of enzyme in plant tissue is generally low, processing of large amounts of plant material is necessary and protease production from plant sources will be a time consuming process. Papain, bromelin, keratinases, and ficin are some of the well-known proteases of plant origin (Rao *et al.*, 1998).

2.5.3. Microbial proteases

Microorganisms are the best preferable sources of protease as compared to plants and animals. This is because of their short cultivation time, required small area for cultivation, easily genetically manipulated, due to their fast growth and simplicity of life for the generation of new recombinant enzymes with desired properties. Furthermore, most of enzyme in microorganism are extracellular, no need of cellular disruption (Rao *et al*., 1998).

Microorganisms account for over a two-third share of commercial protease production in the enzyme market across the world (Kumar and Takagi, 1999). Proteases play a decisive role in detergent, pharmaceutical, leather, food and agricultural industries (Gupta *et al.*, 2002). A variety of proteases are produced by different microbial species. The protease from those microbes can vary depending on the species of the producer or the strains eventhey belong to the same species. For example from *Bacillus* species *B. subtilis* and *B. cereus* produce neutral and alkaline protease, respectively. Among other types of protease alkaline serine proteases are the most dominant group of proteases produced by bacteria, fungi, yeast and actinomycetes (Gupta *et al*., 2002).

- **Fungal proteases**

The high diversity of fungal protease with broad substrate specificity and stability under extreme conditions can attract many researches and can offer an advantage of separation of mycelium by simple filtration. Fungal proteases can conveniently be produced in solid-state fermentation process and it can also use for modifying food proteins (Jisha *et al*., 2013). Fungi elaborate a wider variety of enzymes than do bacteria. For example, *Aspergillus oryzae* produces acid, neutral, and alkaline proteases.

- **Bacterial proteases**

From the total number of industrial enzyme more than 70% of the enzyme is produced from a bacterial source. Bacterial protease contributes to a major extent for the industrial applications. For example, proteases from *Bacillus* find applications at various steps of leather processing; neutral proteases in soaking, alkaline proteases in dehairing and acid proteases in batting (Hindhumathi *et at*., 2011)

Based on their pH optima, proteases are referred to as acidic, neutral, or alkaline proteases (Narasimha *et al*., 2011). Alkaline and neutral bacterial proteases are more commercially important in laundry, food, leather and silk industries due to their catalytic activity and high production capacity. *B. subtilis, Lactococcus, Serratia, Pseudomonas, Aeromonas, Vibrio, E.coli* are some of the microorganisms for this application.

Bacterial neutral proteases are active in pH range of (pH 5 to 8) and have relatively low thermo tolerance. Due to their intermediate rate of reaction, neutral proteases generate less bitterness in hydrolyzed food proteins than do the animal proteases and hence are valuable for use in the food industry. A bacterial neutral protease is insensitive to the natural plant protease inhibitors and is therefore useful in the brewing industry. In addition, low thermotolerance bacterial proteases are advantageous for controlling their reactivity during the production of food hydrolysates with a low degree of hydrolysis (Rani *et al*., 2012).

2.6. Effect of growth conditions on protease production

Proteases that are useful for industrial application can be influenced by different parameters like, temperature, incubation period, quantity of inoculum, medium pH, NaCl concentration and the composition of media.The majority of industrial processes are performed under harsh conditions; it would be of great importance to have microbial enzymes that demonstrate optimal activities at wide ranges of pH, temperature and salt concentration (Han and Damodaran, 1997).

There are different nutritional and physicochemical factors that affect the production of extracellular protease which is predominantly produced by microorganisms. The nutritional factor includes the requirement of organic compounds as carbon and energy sources, the availability of simple sugars which are rapidly taken by microbes that can support most growth. In order to have a cost effective method of enzyme production, optimization of various media components is needed.

Otherwise, the media for enzyme production can greatly affect the enzyme production cost. Importance of this step is to achieve the fact that 30% - 40% of the production cost of industrial enzymes is estimated to be the cost of the growth medium (Joo *et al*., 2003). The changes in C/N ratio, presence or absence of sugars like glucose and sucrose that can easily be metabolized than other carbon sources can also affect the protease production (Wang *et al*., 2008).

The pH of the culture medium is related to the transport of compounds across the cell membrane and it can strongly affect many enzymatic processes (Kumar *et al.,* 2002). This includes the total net charge of the enzymes and the distribution of charges on their exterior surfaces, in addition to the reactivity of the catalytically active groups. These effects are especially important in the neighborhood of the active sites, which will overall affect the activity, structural stability and solubility of the enzyme (Chaplin and Bucke, 1990). Therefore, controlling the optimum pH of the culture condition can favor the high yield of protease production by microorganisms (Mao *et al*., 1992).

A balance between biomass and available material is expressed by size of the inoculums that enhance optimum enzyme production (Sandhya *et al*., 2005). As many research reports, a good yield of inoculum is at 0.5-8% level to be used (Sinha and Satyanarayana 1991; Sen and Satynarayana, 1993; Gajju *et al.,* 1996). However, a higher inoculum level, 5-10% has been reported to be optimum for the enzyme production by thermophilic *Streptomyces*spp. (Mizusawa *et al.,* 1969).

Depending on the microorganisms the incubation period required for maximum production of the enzyme is also different. According to many researches the incubation period of bacteria other than actinomycetes is usually in the range of 24-48 hr (Na and Yu, 1988; Sinha and Satyanarayana, 1991). However, Gajju *et al.* (1996), reported a longer incubation period of 96hr for maximum alkaline protease production by *Bacillus* sp. An optimum incubation period of five days has been reported for the alkaline protease production by actinomycetes such as *Streptomyces moderatus* (Chandrasekaran and Dhar, 1983) and *Nocardiopsis dassonvillei* (Tsujibo *et al.,* 1990).

2.7. Methods for protein hydrolysis

Depending on the source of protein, the methods used to hydrolyze protein are different. For example, proteins from feathers, bristles, horns, beaks or wool contain the keratin structure and

therefore, usually hydrolyzed by acidic or alkaline treatment, or by bacterial keratinases (Pasupuleki *et al.*, 2010). On the other hand, animal products (e.g, casein, whey, intestine, and meat) and plant ingredients (e.g., soy, wheat, rice, pea, and cottonseed proteins) are hydrolyzed through enzymatic or microbial hydrolysis (Dieterich *et al.*, 2014).

2.7.1. Acid hydrolysis

Acid hydrolysis of a protein takes place at a high temperature with a much shorter period of time (2 to 6 hr) to produce hydrolysate. The method of acid hydrolysis of a protein offers the advantage of low cost. But this process can lead to a partial or complete destruction of some amino acid like, tryptophan, methionine and the conversion of glutamine into glutamate and asparagine into aspartate (Pasupuleki and Braun, 2010).

2.7.2.Alkaline hydrolysis

According to Dai *et al*. (2014), alkaline chemical like Calcium, Sodium or Potassium hydroxide requires high temperature (up to 105°C) in order to complete the hydrolysis process. However, some food processing industries use low temperature (27-55°C) within short period of time to produce hydrolysate (Pasupuleki and Braun, 2010). With regard to the cost alkaline hydrolysis has the same advantage as that of acid hydrolysis. But this process results in the complete destruction of most amino acids.

2.7.3. Enzymatic hydrolysis of proteins

Enzymatic hydrolysis of proteins is the hydrolysis of proteins by the action of protease enzymes of different source. During hydrolysis, the bonds that linked amino acids in protein molecule are cleaved to produce peptides. Then the catalytic action of the enzyme is reflected by the degree of hydrolysis (DH) which is the percentage of the peptide bonds cleaved (Adler-Nissen, 1986).

Relative to the above two methods, the enzyme hydrolysis of proteins have the following advantages: (1) it has mild hydrolysis conditions like temperature and pH; (2) do not result in destroying any of amino acids; (3) proteases are more specific and precise to control the degree of peptide-bond hydrolysis; and (4) after the completion of the hydrolysis the enzymes can be easily deactivated to facilitate the isolation of the protein hydrolysates. The disadvantages of enzymatic hydrolysis of protein include the relatively high cost and the potential presence of enzyme inhibitors in the raw protein materials (Hou *et al*., 2017).

2.8. Protein hydrolysate

After the end process of protein hydrolysis the next step is known as post hydrolysis treatment. This include heat inactivation, ultra filtration, hydrolysis by exoproteases and treatment with specific enzymes those have a function of inactivation of proteolytic enzymes, removal of high molecular weight proteins and peptides, hydrolysis and reduction of bitterness, reduce content of specific amino acids respectively (McCarthy *et al*., 2013).

protein hydrolysate from plant and animal source have a variety of application such as food supplement, for microbiological industry, in medicine, for weed control, as a natural pesticide and herbicides (Christians *et al*., 1994; Neklyudov *et al.,*2000). Based on the hydrolysis process and the starting material condition those hydrolysates have a character of improving solubility, thermal stability, enhancing and reducing emulsifying property (Mahmoud *et al*., 1992).

2.8.1 Use of hydrolysate as microbiological media

The use of hydrolysate in industrial fermentation for cell culture and microbiological media is due to the reason of using hydrolysate as a nitrogen source. Furthermore, hydrolysate can improve the bio-performance of the culture medium by providing peptides, carbohydrates, lipids, vitamins and minerals as a medium supplement (Neklyudov *et al.,* 2000). For example, tryptone (a digest of casein) in Luria-Bertani broth supplies essential growth factors for *E. coli* (Vasileva*et al*., 2007).

The microbiological industry increasingly uses hydrolysates of wastes from the food industry or byproducts that are unsuitable for food production (Neklyudov *et al.,* 2000). Raw materials used in the production of hydrolysates include organs of slaughtered animals (liver, kidneys, spleen) or their mixtures, chicken embryos, hen eggs, gastric or duodenal mucosa, internal organs of fowl, and wastes of fish and marine product processing (Batista and Nunes, 1997). The hydrolysates of concentrated-protein and vitamin containing products can serve as a good basis for nutrient media designed for growing a wide range of pathogenic and nonpathogenic microorganisms (Neklyudov *et al.,* 2000).

3. MATERIALS AND METHODS

3.1. Isolation and screening of protease producing bacteria

Soil samples were collected from different areas of Addis Ababa University Arat Killo Campus plant gardens and the collected soil samples were air dried. To isolate bacterial isolates 1 gram of soil was suspended in 9 ml of sterile distilled water and serially diluted in the range of 10^{-2} to 10^{-6}. Then 0.1 ml aliquot from each dilution was spread on skim milk agar plates containing (g/l): skim milk powder, 10; peptone, 3; yeast extract, 3; NaCl, 5; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.1; and K_2HPO_4, 0.2, finally the pHwas adjusted by 0.1% of KH_2PO_4 at pH 7. After 48 h incubation at 37 ℃, isolates that form clear hallo zone were considered protease positive. All positive isolates were purified through repeated streaking on agar plates. The pure isolates were preserved at 4 ^{o}C for further study.

3.2. Characterization of selected isolate

The identification of bacteria was carried out by morphological and biochemical studies. The biochemical tests were Gram staining and catalase activity. Culture characterization on agar plate like colony morphology was also done. In addition, starch and gelatin hydrolysis were tested.

- **Gram staining**

  a loopful bacterial colonies were taken and spreaded uniformly on the middle of the clear glass slide and fixed by passing it over the gentle flame for two or three times. Following to heat fixation, the slide was flooded with crystal violet solution and allowed to react for 1 min and then washed with gentle tap water. The slide was then flooded with iodine solution for 1 min and washed thoroughly with 95% alcohol for 10 sec. After drained off the alcohol, it was rinsed and safranin was applied for 1 min. Then after the final rinse, it was examined under microscope.

- **catalase test**

  The test was done by applying one loopful of the fresh bacterial culture on a sterile slide and adding a drop of hydrogen peroxide to the slide. Bubble production indicated positive result.

3.3. Enzyme production

3.3.1 Submerged fermentation

Sterile skim milk broth (25 ml) in a 250 ml conical flask was inoculated with a loop full of the culture from fresh slants and incubated at 30°C on a rotary shaker at 150 rpm. After 48 h of incubation, 1 ml 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.

3.3.2. Solid state fermentation

Solid substrate (10g wheat bran) was transferred to a 250 ml conical flask; 13 ml of salt solution with the composition of NaCl, 0.5; $MgSO_4.7H_2O$, 0.02; $CaCl_2$, 0.01; and K_2HPO_4, 0.02, was added and autoclaved at 121°C for 30 min. Each flask was inoculated with 2ml of the selected bacterial broth culture grown at 37°C for 48 hr. At the end of fermentation period, the enzyme was extracted by adding 100ml of distilled water followed by filtration through a muslin cloth.

3.4. Protease activity assay

Proteolytic activity was measured using casein as the substrate. To 450µl of 1% (w/v) of casein in 50 mM phosphate buffer, pH 7 was mixed with 50µl crude enzyme extract and incubated for 20 min in a water bath at 50 ºC. The reaction was stopped by adding 450 µl of 10% (w/v) trichloroacetic acid (TCA) and was kept at room temperature for 10 min. After centrifugation at 10,000 rpm for 5 min, 150 µl supernatant was mixed with 750 µl of 0.5 M Na_2CO_3 and 150µl of 1 N Follin Ciocalteus phenol reagent. The mixture was incubated in the dark for 30 min and absorbance was measured at 660 nm against a reagent blank. One unit of protease activity was defined as the amount of enzyme that resulted in the release of 1 µg of amino acid equivalent to tyrosine per min.

3.4.1 Preparation of Tyrosine standard curve

To prepare the standard curve 0.5M Na_2CO_3, 50 mM Glycine buffer, pH 10.0, 1:4 diluted 2N Folin reagent and 200 µg/ml of tyrosine stock solution with dilution 1:2 were used. A required amount of buffer and Tyrosine were added in each test tube except the blank. Then 150 µl of 0.5M Na_2CO_3 was added in each test tube including the blank and the mixtures were kept at room temperature for 10 min. After 150µl of 2N Folin reagent was added in each test tube including the blank, the solution was mixed immediately and kept for 30min at room temperature. Finally, the optical

density (OD) was measured at 660 nm. Within this procedure the amount of amino acid released due to the action of protease was calculated by the equation obtained from tyrosine standard curve with a correlation coefficient (r^2) of 0.995 (Fig.1).

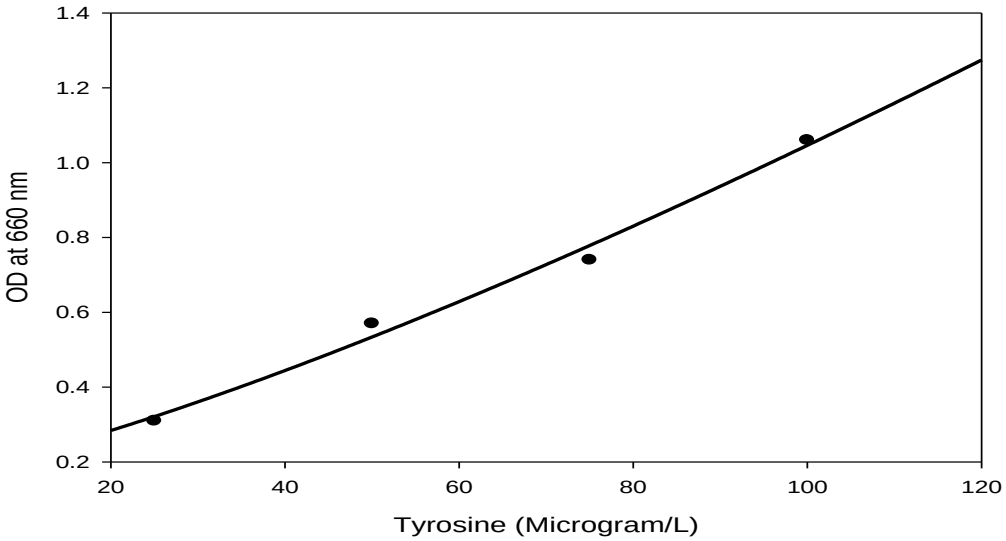

Fig. 1 Tyrosine standard curve

$$EU = [(116.8102 * \text{OD} * \text{DF} - 15.72)]\mu mol/ml$$

Where EU: Enzyme unit; OD: Optical density; DF: Dilution factor

3.5. Characterization and optimization of reaction conditions

3.5.1. Effect of pH on enzyme activity

The effect of pH on activity of the enzyme was assayed in the pH range of 6.0 – 10.5. The buffers used include phosphate (pH 6.0 – 8.0), Tris HCl (pH 7.5 – 9.0), and glycine-NaOH buffer (pH 8.5 – 10.5).

3.5.2. Effect of temperature on enzyme activity

The effect of temperature on enzyme activity was determined by performing the standard assay procedure at pH 7 within a temperature range from 40 ºC to 65 ºC in a 5 ºC interval.

3.5.3. Temperature stability

Thermostability of enzyme was measured by incubating the protease alone in different eppendorf tubes at different incubation time of 0, 10, 20, 30, 40, 50 and 60 min in a water bath at 50 and 55

°C. Then each of the incubated enzymes was assayed in the standard assay procedure at 50°C. Finally the residual activity of protease enzyme was calculated as follow.

$$\text{Relative residual activity (RRA)} = \frac{individual\ \ residual\ \ activity}{residual\ \ activity\ \ at\ time\ \ 0} \times 100\%$$

3.6. Optimization of culture conditions for enzyme production

The protease activity was determined by varying the pH, temperature and fermentation period to optimize the reaction condition in order to scale up the target product.

3.6.1. Time course of enzyme production

The culture medium was incubated for varying periods of time, 24, 48, 72, 96 and 120hr to find the optimum time required for maximum enzyme production by SSF media at 37°C. At the end of the incubation time, the enzyme was harvested and activities were determined by standard enzyme assay procedure.

3.6.2. Effect of moisture level

Optimum moisture content required for the growth of bacteria and for protease production 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 72hr and activity determined.

3.6.3. Effect of nitrogen sources on protease production

The effect of nitrogen sources for protease production was determined by organic and inorganic nitrogen source. This includes peptone, ammonium nitrate, yeast extract, casein and sodium nitrate. Each source was used at a concentration of 0.5% (w/v) to replace nitrogen sources. Protease yield was determined after 72hr of incubation at 37 °C in a SSF with the moisture content of 66.7%. After production, the yield was expressed as U/g.

3.7. Collection and preparation of protein

3.7.1. Offal collection (animal source)

Samples of slaughterhouse offal (lung and raw meaty bone waste) was collected from the local retail butcher shop in sterilized container and transported to the laboratory. The lung was chopped and the meat on the bone was detached by using NaOH to dissolve proteins on the meat. Then the dissolved protein was precipitated by HCl (Nolsoeand Undeland, 2009)

3.7.2. Soya bean protein isolation

Isolation of soya protein was carried out following the methods of Bogracheva *et al.*, (1996). A 10% (w/v) of defatted soya flour was dissolved in distilled water and pH adjusted between pH 10-11 using1N NaOH. After removing the insoluble fraction, the solution was precipitated by adjusting the pH in the range of 5 and 6 using 1N HCl. The protein precipitate was recovered, dried in oven over night at 40°C, and pulverized to a powder passing through a 250µm sieve.

3.7.3. Deffatting process

- **Soya flour defatting**

  Soya flour from plant source was defatted by using hexane. The flour was soaked for 2hr and for this time solvent to flour ratio was 1:3. Then the defatted portion was separated through filtration and it was dried by atmospheric temperature (air- dried overnight).

- **offal defatting**

  First lung of the cattle was chopped by knife and soaked in n-hexane for 2hr. the lung defatting process was repeated depending on the fat content with n-hexane for better defatting and the defatted portion was separated through filtration. Then it was dried overnight in oven at 50 °C Finally it was milled to a powder form and then sieved through 250µm sieves to remove all fibers and it was stored at 4°C for further analysis (Amare Gessesse and Berhanu Andualem, 2013).

3.8. Proteins hydrolysis

Defatted lung and bone protein was dissolved with distilled waterby adjusting the pH to 7.5 using 1N NaOH or 1N HCl, mixed the enzyme and incubated at 55°C for 3hr.

Followed to protein isolation (section 3.7.2.) soya bean hydrolysis is done by the same process to offal hydrolysis. Then, after 3hr of incubation the inactivation of the enzyme took place by boiling the solution for 5min. Finally, the hydrolysate solution was dried at 40°C over night in oven. After drying, the hydrolysate was mashed until it became powder and sieved through 250µm sieves to use as a peptone for microbiological media.

3.9. Test of protein hydrolysate as microbiological media components

3.9.1. Media preparation

Microbial media were prepared from the hydrolysate of defatted flour of soya bean, lung, bone and commercial peptone agar that has a composition of (0.5% peptone, 0.1% dextrose, 0.1% yeast extract and 1% agar) in distilled water. The pH was adjusted to 7.4 ± 0.2 at 25°C by 1N NaOH. Finally the media were sterilized by autoclaving at 121 °C for 15 min (Uzeh *et al*., 2006). From each culture broth dilution, 0.1 ml was spreaded on the agar medium. The plates were incubated at 37 °C for 24 – 48 hr. For each culture duplicate plates were prepared (Amare Gessesse and Berhanu Andualem, 2013).

3.9.2. Inoculum preparation

From Ethiopian health and nutrition research institution (EHNRI) a pathogenic bacteria of *E. coli*, *Streptococcus agalaetiae*, *Staphylococcus aureus*, *Shigellaflexneri*, *Salmonella thyphimerium* and *Klebsiella pneumonia* were obtained. Each bacterium was separately cultured on nutrient agar for 24 hr at 37°C to refresh the pathogens and for isolation of pure colony. Single colony was taken and inoculated to nutrient broth and incubated for 24hr. Then 1 ml of cultured broth was serially diluted in sterile distilled water (10^4-10^9 dilution) (Amare Gessesse and Berhanu Andualem, 2013).

3.9.3. Growth measurement

The numbers of colonies were directly counted from the culture. It was done in duplicate to increase the validity of number of colony counting.

4. RESULTS

4.1. Isolation and screening of protease producing bacteria

Based on the formation of a clear zone around the colony on skim milk agar media (Fig. 2) a total of 17 bacterial isolates were selected as protease producers.

Fig.2 Protease producing isolates on skim milk agar media

On the basis of enzyme production in liquid culture and their ability to grow in SSF, 4 isolates were selected for further screening. Out of the four isolates, one isolate that has relatively high enzyme production designated as aau_5 based on the site where it isolated (Addis Ababa University) was selected for further study.

4.2. Characterization of selected isolate

Isolate aau_5 was a Gram-negative, rod shaped, catalase positive, and white creamy with irregular shape of colonies on skim milk agar medium (Table 1). This isolate was not able to hydrolyze starch and gelatin but can hydrolyze skim milk. Based on these morphological and biochemical features this isolate was tentatively grouped under the genus *Pseudomonas*.

Table 1.Morphological and biochemical features of the bacterial isolate (aau_5)

No.	Characterization	Result
1	Shape	Rod
2	Gram staining	-ve
3	Catalase	+ve
4	Casein hydrolysis	+ve
5	Starch hydrolysis	-ve
6	Gelatin hydrolysis	-ve
7	Skim milk hydrolysis	+ve

4.3. Enzyme production through solid state fermentation

4.3.1. Effect of moisture level on protease production

To determine the optimum moisture level for enzyme production isolate aau_5 was grown on wheat bran containing different moisture levels. Maximum enzyme production (5,031.93U/g) was observed at moisture level of 66.7%. With increasing moisture level above its optimum enzyme production sharply decreased (Fig. 3).

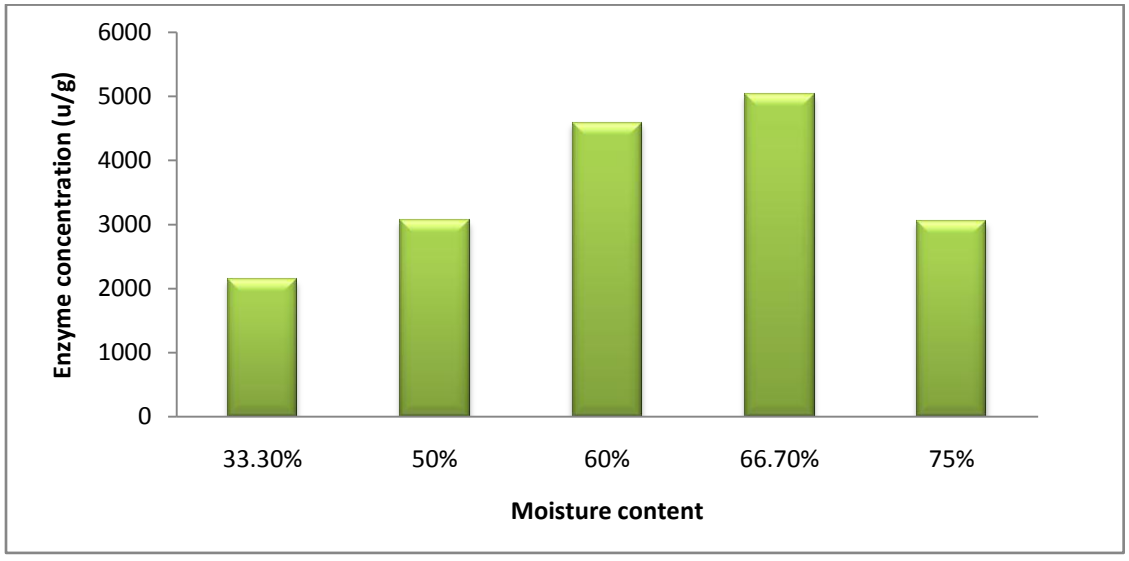

Fig. 3 Moisture levels of SSF medium and enzyme production by isolate aau_5.

4.3.2. Effect of incubation period on protease production

Enzyme production increased as time of incubation increased from 24 hr – 72 hr (Fig. 4) in the solid state fermentation. Then maximum protease production (5773 U/g) of *aau*$_5$ was observed at 72 hr of incubation. After 72hr of incubation period a gradual reduction in the relative enzyme production was observed.

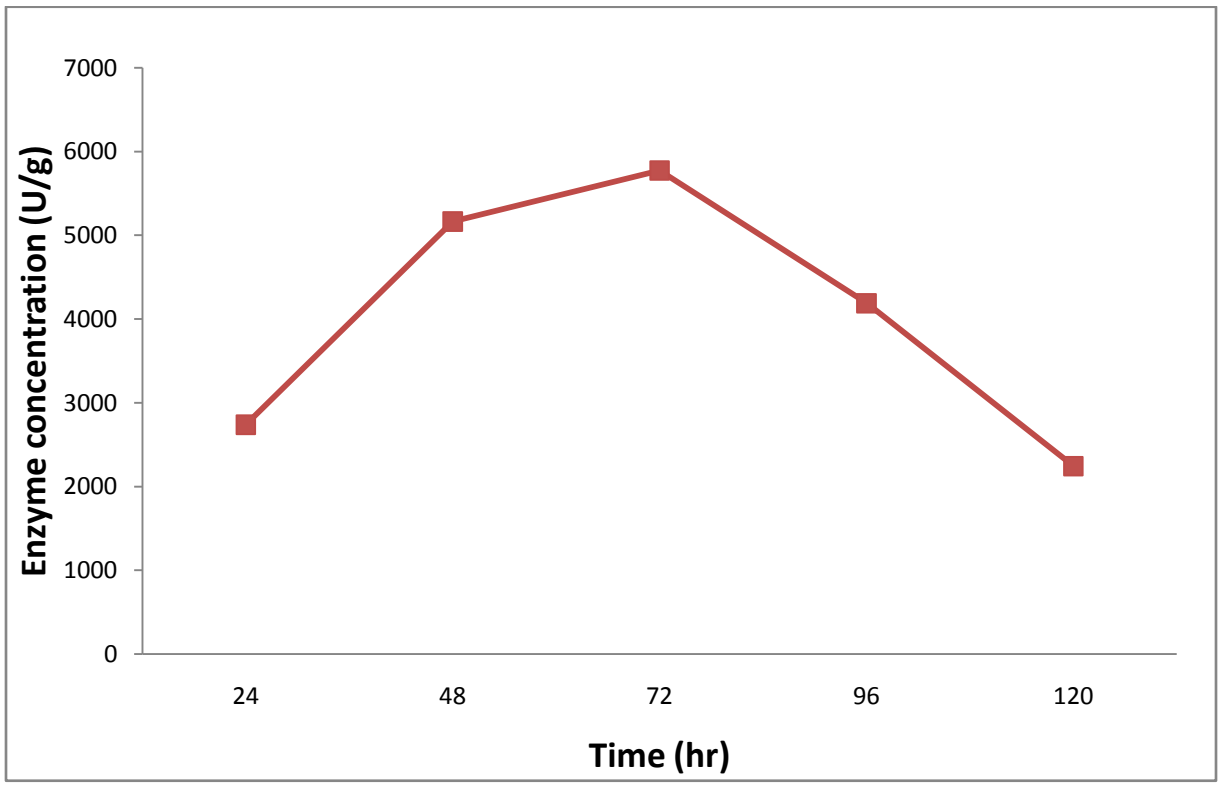

Fig. 4 Time course of protease production by isolate aau$_5$

4.3.3. Effect of Nitrogen source on Enzyme production by *aau*$_5$

Best nitrogen source was determined in the presence and absence of different nitrogen sources under a solid state fermentation in a wheat bran medium. Of the different nitrogen supplements tested enzyme production was the highest (4870 U/g) in the presence of casein. On the other hand, using Sodium nitrate for ***aau***$_5$growth showedalmost equal amount ofprotease compared to protease production in the absence of nitrogen supplement (Fig. 5). Relatively, addition of organic nitrogen source (yeast extract, peptone and casein) supported higher enzyme production.

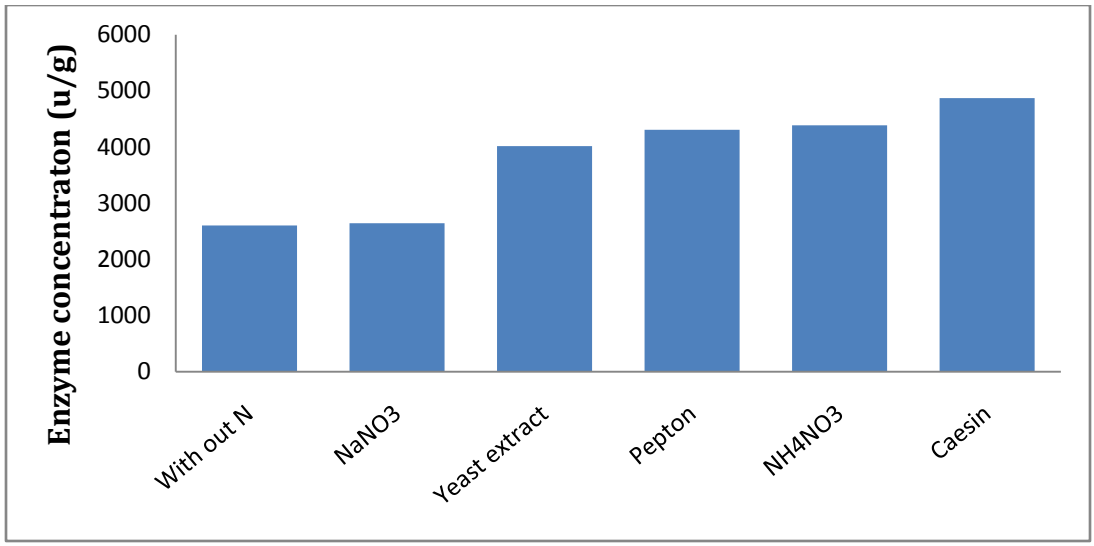

Fig. 5 Effects of different nitrogen sources on protease production by isolate aau_5

4.4. Characteristics of the protease enzyme

4.4.1. Effect of pH on the activity of *aau_5* protease

The ***aau_5*** protease has higher activity in wider pH range and the maximum activity was observed at pH 7.5. The enzyme maintained more than 75% of its activity in the pH range of 6 to 10. Relatively, the lowest activity was observed at pH 10.5 (Fig. 6).

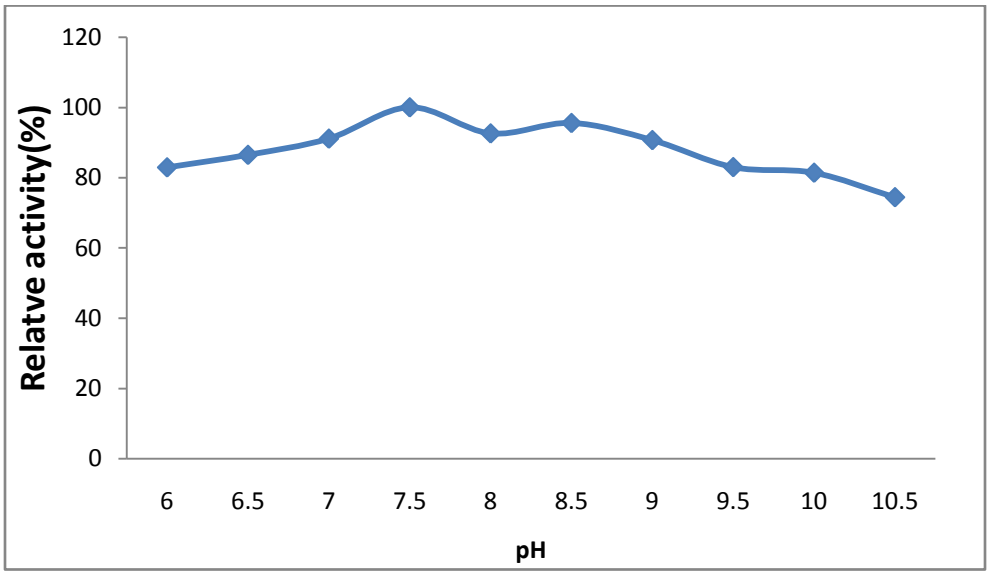

Fig. 6 Relative activity of aau_5 protease at different pH value

4.4.2. Effect of temperature on the activity of protease

The optimum temperature for ***aau$_5$****protease* was at 55 °C and retained 90% of its maximum activity at 60 °C (Fig. 7).

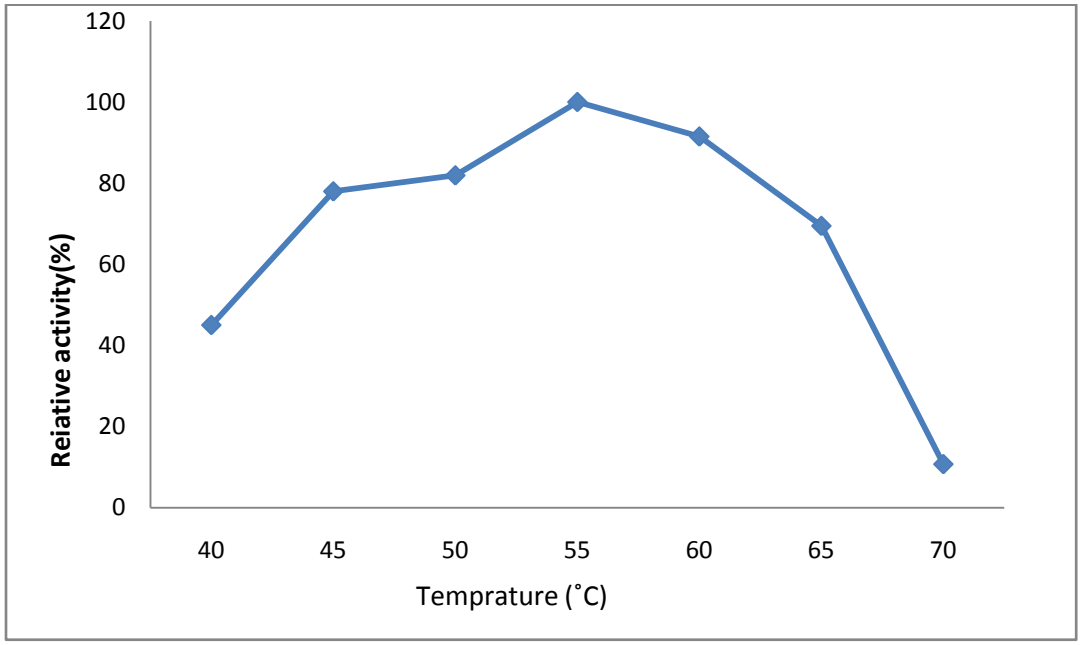

Fig. 7 The relative activity of aau$_5$ protease at different temperature values

4.4.3. Effect of temperature on the stability of protease

The temperature stability of *aau$_5$*protease was determined by assaying the pre-incubated enzyme. The relative activity of this enzyme decreased with increasing incubation temperature. At 55 ˚C about 41% of its activity was retained after 60 min. The enzyme also retained 60% of its activity after 1 hr incubation at 50 ˚C (Fig .8).

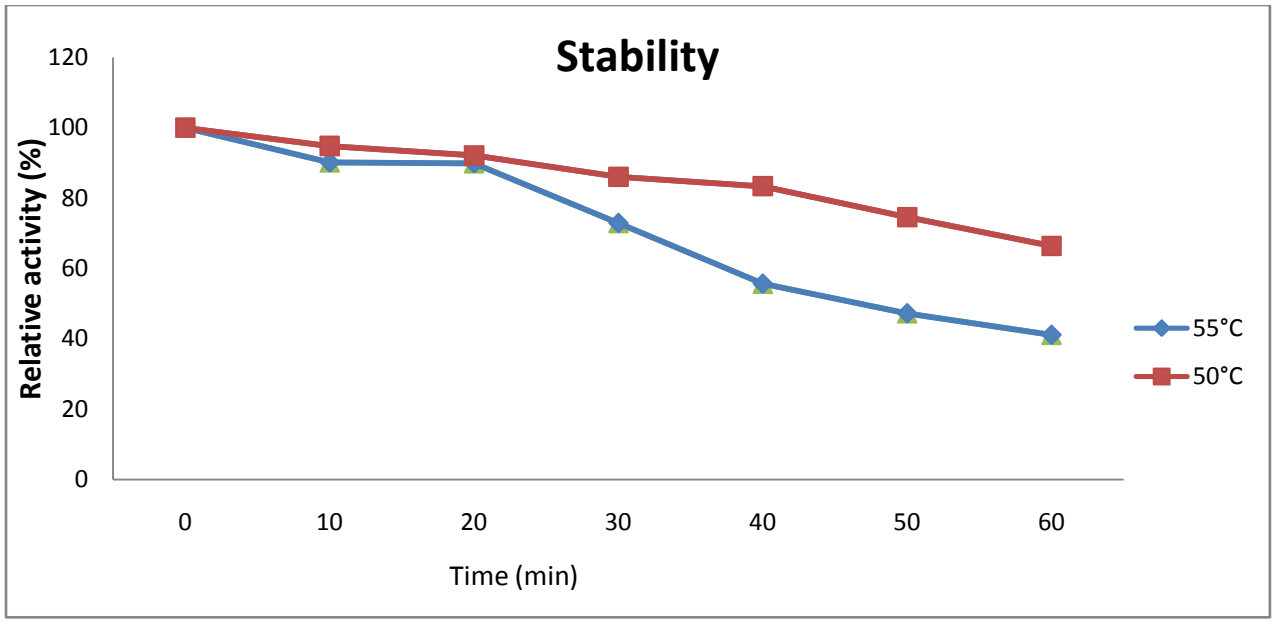

Fig. 8 Temperature stability of aau$_5$protease at 50 °C and 55 °C

4.5. Test the hydrolysate as a microbiological media

4.5.1. Bacterial growth on the laboratory produced peptone

The peptone produced from lung and bone was compared with the soya bean and commercial peptone as a microbial media for the growth of different pathogenic microorganisms. Media prepared from laboratory prepared peptone supported a good growth of the test bacteria and was better than media prepared from commercial peptone (Table 2).

Table 2. Comparison of pathogenic bacterial colony count on lung and bone peptone agar against soya bean and commercial peptone agar.

Test Bactria	*Cfu/ml*			
	LPA	BPA	SPA	CPA
E.coli	87×10^7	89×10^7	80.14×10^7	70.28×10^7
Shigellaflexneri	37.14×10^7	42.14×10^7	29.57×10^7	32.21×10^7
Streptococcus agalactiae	11.14×10^7	13.43×10^7	7.57×10^7	7.86×10^7
Staphylococcus aureus	4.36×10^7	6.21×10^7	0.29×10^7	1.07×10^7
Salmonella thyphimerium	22.21×10^7	20.71×10^7	21.14×10^7	13.57×10^7
Klebsiellapneumoniae	42.57×10^7	39.35×10^7	40.21×10^7	41.21×10^7

Note: - BPA,bone peptone agar; CPA, Commercial peptone agar;LPA, lung peptone agar and SPA, soya bean peptone agar.

Compared to the commercial peptone agar, *E.coli* and *Klebsiella pneumonia* showed better growth in media prepared from lung, bone, and soya peptone (Fig 9). LPA and BPA were better for microbial growth in almost all organisms except *Klebsiella pneumoniae* which was slightly higher CFU in commercial peptone agar than BPA and SPA. Relatively, *Staphylococcus aureus* showed the least growth in all media. In general the peptone from animal source (lung and bone) was better for bacterial growth than soya bean and commercial peptone agar media.

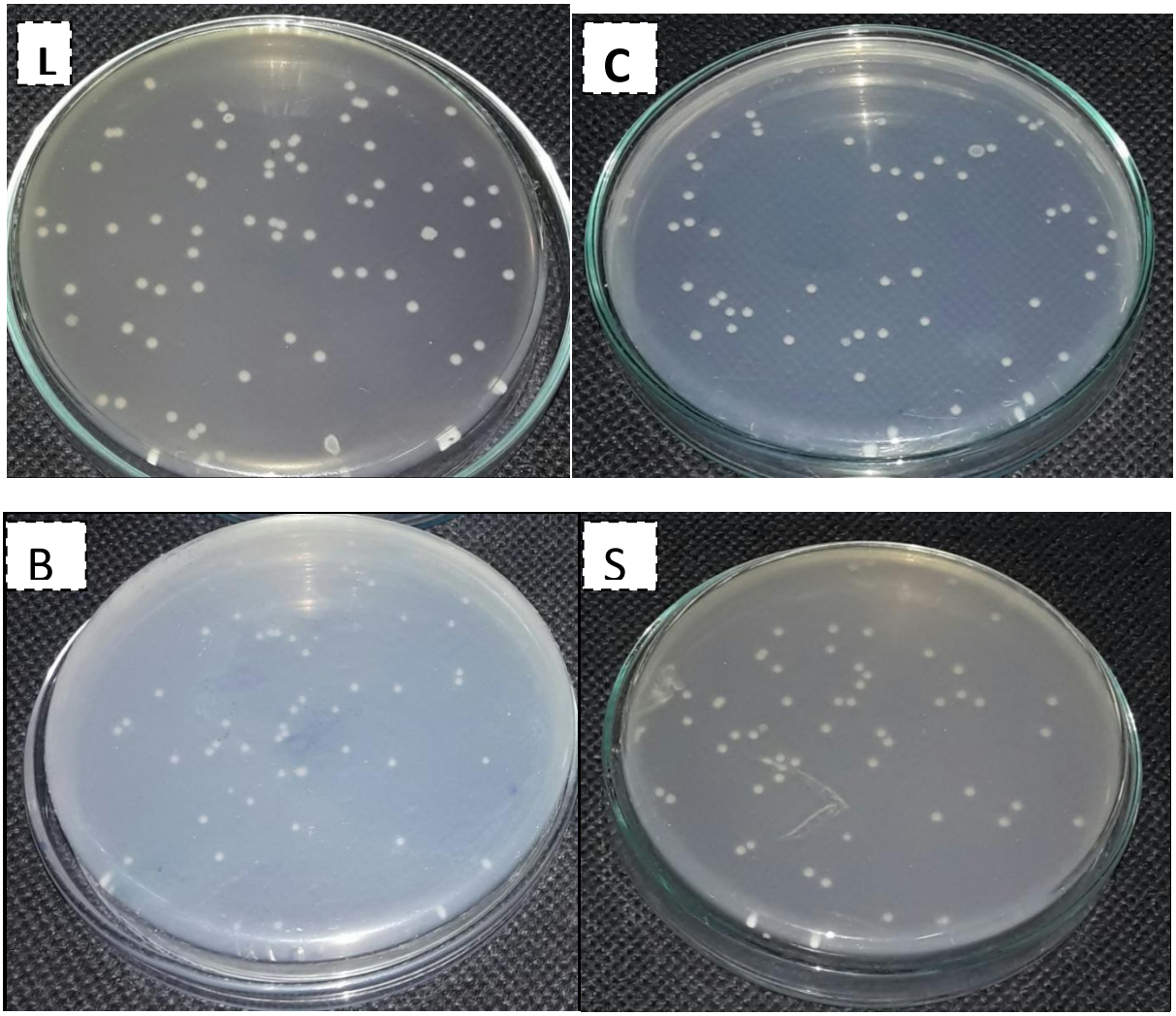

Fig.9 Colony of Salmonella agalaetiae on lung peptone agar (LPA), bone peptone agar (BPA), soya peptone agar (SPA) and commercial peptone agar (CPA).

Note: - B, BPA; C, CPA; L, LPA and S, SPA

5. DISCUSSION

Soil contains many nutrients and it is often considered as a major source of different useful microbes, including those producing proteases (Alves *et al*., 2014). In this study the 75% of the bacterial isolates were shown a proteolysis activity on skim milk agar and they were isolated from soil sample. For the initial isolation of protease producers a simple and efficient screening procedure could have huge advantages. Based on its simplicity, rapidity, range of detection and sensitivity, the clear zone formation due to proteolytic activity of the microbe on the skim milk agar plate was used for the initial screening of protease producing microbes (Rajamani and Hilda, 1987). However, in this study the size of clear zone on skim milk agar was given ambiguous result in some selected isolate. Therefore, use of other screening method like enzyme production on SMF and SSF was necessary to select best protease producing isolate.

The selected isolate (isolate aau_5)grow under SSF using wheat bran shows its potential as a source of commercially important enzyme with low production cost. For the large-scale application of proteases reduction in the production cost of the enzyme(s) is very important. Currently large-scale production of proteases in developed countries is carried out in submerged fermentation. However, in developing countries enzyme production through solid state fermentation (SSF) offer several advantages. First, it uses cheap agro processing wastes that can help to greatly reduce the production cost. Secondly, growth under SSF is often associated with higher product yield (Pandey, 2003).Third, most of bacteria require higher water activity for growth, but SSF has a lower risk of contamination.

The growth and enzyme production of microorganisms under a SSF can be influenced by the moisture level (Kim *et al.*, 1985). The highest enzyme production for isolate $\boldsymbol{aau_5}$ (reaching up to 5031 U/g) was observed in the presence of 66.7% moisture. Increasing the moisture content to75% resulted in reduction of enzyme production by more than 40%. Similarly, enzyme production below the optimum moisture level was also lower. Most bacterial species optimally grow in the presence of high water activity. But in SSF increasing moisture content above a critical value could affect porosity of the medium and limit gas transfer (Lonsane *et al*., 1992). On the other hand low moisture content leads to reduction in nutrient diffusion and that can also affect the enzyme production (panday, 2003). The optimum moisture content required for high enzyme production is

different based on the strains. For example maximum protease enzyme production by Penicillium sp. was observed at moisture content of 50% (Agrawal *et al.*, 2004).

In most organisms, maximum extracellular enzyme production is often observed at or around the end of the exponential or beginning of the stationary phase where the concentration of one or more nutrients in the medium is depleted (Gupta *et al.*, 2002). Isolate ***aau*$_5$** showed fast enzyme production on wheat bran under SSF (up to 2,740 U/g) after 24hr of incubation. But, maximum enzyme production was observed after 72hr incubation. After that enzyme production showed a sharp decrease. The end of the stationary phase of microbial growth cycle is associated with depletion of essential nutrients in the culture medium and accumulation of waste product in the medium that inhibit growth and enzyme production (Maier *et al*., 2000).

For maximum enzyme production the culture medium must contain the required carbon and nitrogen sources. For organisms that grow under SSF using wheat bran, the solid substrate could supply up to 75% of the required carbohydrate (Stone and Morell, 2009). This shows that there is no need to supplement wheat bran with additional carbon sources. On the other hand the nitrogen content of wheat bran may not be sufficient to support microbial growth. Therefore, there is a need to add nitrogen supplements to bring about optimal growth under SSF. For isolate ***aau*$_5$**protease enzyme production under SSF was higher in cultures supplemented with organic nitrogen than cultures grown without any nitrogen supplement and cultures supplemented with $NaNO_3$ as inorganic nitrogen source. Other reports also showed that supplementing wheat bran with organic nitrogen sources lead to better growth and protease production (Phadatare *et al*., 1993). This shows wheat bran may not contain the required amount of nitrogen for the optimum growth of the organism. Some reports showed that supplementation of some strains with inorganic nitrogen source resulted in better growth and protease production (El-Safey and Abdul-Raouf, 2004). This shows the requirement of specific nitrogen source differs from organism to organism (Kumar *et al*., 1999).

Protease ***aau*$_5$**was active in the pH range of 6.5-9.0, with an optimum at pH 7.5. Protease optimally active around neutrality are considered ideal for application in foods processing industries (Rao *et al.,* 1998). Moreover, for industrial application in food processing proteases active in the temperature range of 50- 60°C are considered ideal (Racheal *et al.,* 2015). ***aau*$_5$**protease showed very good stability and activity in the temperature range of 50°C to 55°C. This shows that the

enzyme has a good potential for application in the protein hydrolysis and other food processing industries.

Hydrolysis by protease ***aau*$_5$** was used for the production of peptone from slaughter house offal (lung and meat extracted from bone) and soybean protein used for the formulation of microbiological growth media. Gray *et al.* (2008) reported that peptone prepared from different sources show significant differences in supporting good microbial growth. In this study, compared to the commercial peptone, peptone produced in the laboratory from slaughterhouse offal (lung and bone) and from soybean protein supported better growth of the test bacterial species (Table 2).The difference in the different peptone preparations might be a result of differences in composition of essential amino acids, vitamins, and/or other growth factors (Dufosse *et al*., 1997). To date media used for microbial growth in Ethiopia is imported from abroad. Given the large quantity of offal released each day in the country, the results of this study suggest the existence of a good potential to produce effective microbiological media from cheap local resources and help the country save in foreign currency.

In this study the growth of tested bacteria favorably well grow in animal source compared to that of plant and commercial source of peptone. Based on the source and hydrolysis process of proteins, the essential amino acids in the peptone are also different. For example the amino acids composition of plant and animal proteins is quite different (Nehete *et al*., 2013). In addition to that peptones that are produced from plants source have an antibacterial property over that of animal based peptone. Soya bean is one of the plant sources of protein that have an anti microbial effect against *Staphylococcus aureus* (Wang *et al*., 2010).

Peptones derived from animal source have also an advantage by reducing the cost of raw material for peptone production. According to Durrani *et al*. (2010), most peptone from plant source is derived from edible source of cereal and leguminous plants like soya bean. In contrast, peptone from slaughterhouse offal can be produced from inedible parts such as lung, tendons, intestine and bone allowing waste valorization. And these ultimately greatly reduce the production cost of the peptone while at the same time avoiding environmental pollution from the release of offal.

6. Conclusion and recommendation

Protein hydrolysates prepared from slaughterhouse offal through enzymatic hydrolysis were tested as potential microbiological media. Peptone prepared in the lab supported the growth of different test microorganisms better than or equal to commercial peptone. Given the large number of animals slaughtered each year in Ethiopia and the amount of offal disposed as waste, protein recovery and enzymatic hydrolysis could lead to production of protein hydrolysates with huge economic benefit. This requires availability of efficient enzymes with affordable cost. The result of this study shows the potential of Ethiopia's bacterial diversity as a source of new enzymes for protein hydrolysis. The fact that the organism grew under SSF fermentation using cheap agricultural waste help to greatly reduces the cost of enzyme production.

For large-scale application it is recommended that enzyme production and hydrolysis reaction is scaled up. It is also recommended that the resulting protein hydrolysate to be tested or used in the food, animal feed, and cosmetics industries is studied.

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Declaration

I, the under signed, declare that this thesis is my original work. It has never been submitted in any institution and that all sources of materials used for thesis have been acknowledged.

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