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Optimization of hyaluronidase enzyme conditions produced from *pseudomonas aerigenosa* isolated from diabetic foot ulcer patients

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Abstract

Using clinically isolated *pseudomonas aerigenosa*, the best conditions for making hyaluronidase were studied. The production medium pH7 with 0.5% lactose and yeast extract, a novel bacterial culture (106Cell/ml), and 37°C for 24 hours produced the maximum hyaluronidase. The specific activity of the hyaluronidase that was generated in the culture medium rose to 130 U/mg of protein as a result of these circumstances.

Keywords: hyaluronidase, *pseudomonas aerigenosa*, culture medium.

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1. Introduction

The hyaluronidase enzyme is among the most investigated enzymes in several species. Hyaluronidases can be produced by organisms with either a Gram-positive or Gram-negative bacterial morphology. However, the hyaluronidases produced by Gram-negative organisms were discovered to be periplasmic rather than extracellular, indicating that they are less likely to contribute to pathogenesis (Spellerberg, 2000; Bailey *et al*.,2014), whereas, in Gram-positive bacteria, the enzymes were frequently released extracellularly and were assumed to play a function in disease (Hynes and Walton, 2000; DeLorenzi, 2017). As the enzyme was isolated from bacteria found in the pus, which is an essential indicator of the potential role of the enzyme in the dissociation of tissue as well as its role in diseases of the teeth and gums, also it was found that the continued production of the enzyme leads to break down connective tissue (Cavallini *et al.*,2013). There have been reports of HA accumulation in several human malignancies, including those of the prostate, colon, breast, stomach, ovary, and pancreatic (DeLorenzi, 2017). These enzymes break the glycosidase bond (β 1–4) and make disaccharide sugar the main product. Also used as a spreading factor in several medical fields, such as orthopedics, surgery, ophthalmology (Meyer and Palmer, 1990), dermatology and dentistry (Tam and Chan, 1985), oncology, gynecology, and obstetrics to speed up the changes to the uterine cervix needed for childbirth (Kavanagh *et al*., 2006).

Generally, the enzyme from phages has a molecular weight of 36–40 kDa, which is higher than the molecular weight of enzymes from other sources (Hynes and Walton, 2000) To determine the enzyme's molecular weight, researchers employed a number of techniques, including ultracentrifugation (Aronson and Davidson,1967) and gel filtration by using different columns such as Sephadex G-150 (Hill, 1976) Sephacryl S-300 column or by electrophoresis(Tam & Chan, 1983). One of the essential components of the ground

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material of the subcutaneous tissues, hyaluronan or hyaluronic acid, also plays important functions in tissue growth, development, and repair. It is well understood that hyaluronan levels rise during embryological development, wound healing, and cancer (Laurent and Fraser, 1992). Also called acid (Hyaluronate) and (Hyaluronan). First isolated by Meyer and Karl in 1934 from vitreous (vitreous humor) eyes of cows (Botzki, 2004), and this explains it termed (Hyaluronic acid) as the (hyalos) mean glassy (Laurent, 2007).

2. Materials and Methods

2.1. The Samples: Twenty samples were gathered from persons of diverse ages and genders who had diabetic foot ulcer infections (A unique form was designed for each patient's name, gender, age, sampling date, and previous treatments). Cotton swabs were used to capture the samples, which were then cultured independently in nutrient broth and incubated at 37°C for 24 hours. After incubation, they were subcultured on blood agar and McConkey's agar to provide a clearer picture of what they were.

2.2. Identifying isolates of pathogenic bacteria:

2.2.1. MacConkey agar Medium(Atlas *et al.*, 1995)

Following the manufacturer's instructions, this medium was produced and autoclaved at 121°C (15lb/in^2) for 15 minutes.

2.2.2.1. Blood Agar Medium (Atlas *et al.*, 1995)

Blood base agar with a pH of 7.0 was put in an autoclave and cooled to 45°C. Then, 5% blood plasma was added and mixed well. The appearance of translucent or green zones in the region around the colonies was a sign of blood hemolysis.

2.2.3. Gram stain

Atlas *et al.* (1995) recommended that stains and reagents be produced (1995).

2.2.4. Vetic 2Identification:

2.3 Optimum conditions for hyaluronidase production

To achieve a higher rate of hyaluronidase production, creating growth conditions were optimized as follows:

2.3.1. To produce hyaluronidase, the medium has to be prepared with varied pH levels, ranging from (4-10). After *Pseudomonas aeruginosa* log phase culture was inoculated into the production medium and incubated at 37°C, the supernatant was tested for hyaluronidase activity (Brunish and Mozersky,1958).

2.3.2. *Pseudomonas aeruginosa* was cultivated in the production medium and incubated at temperatures ranging from (20 to 55) C. The hyaluronidase activity was measured in the supernatants following 20 minutes of centrifugation at 8000 rpm (Brunish and Mozersky,1958).

2.3.3. *Pseudomonas aeruginosa* was raised in various HA concentrations (0, 10, 20, 40, 80, 160, and 320 mg/ml) and incubated at 37°C for 24 hours. The same action was then identified(Brunish and Mozersky,1958).

2.3.4. For this experiment, we added carbon (glucose, starch, sucrose, lactose, and mannitol) and nitrogen (urea) to the minimal medium (ammonium chloride, ammonium acetate, ammonium sulphate, sodium nitrate, and yeast extract). The medium's final carbon and nitrogen concentrations were both 0.5% (w/v) from all sources combined. Initial acclimatization was performed with the medium's pH set at 7.0. Following inoculation of the medium with 1% of a *Pseudomonas aeruginosa* log phase culture and incubation at 37°C, hyaluronidase activity was assessed in the supernatants (Brunish and Mozersky,1958).

2.3.5. The effect of various *Pseudomonas aeruginosa* inoculum sizes on hyaluronidase generation was investigated. This was performed by inoculating the production medium individually with inoculum sizes varied from (10^{2-}×10^9cell/ml),

and the hyaluronidase activity was assessed as a consequence (Brunish and Mozersky,1958).

3. Results and Discussion

3.1 Quantitative detection of hyaluronidase enzyme

Inhibitory zones were studied using brain heart serum albumin medium (1% bovine serum albumin and 400 g/ml hyaluronidase enzyme) to test *Pseudomonas aeruginosa* isolates' hyaluronidase enzyme production. *Pseudomonas aeruginosa* isolates were found to generate hyaluronidase enzymes with a zone of hydrolysis ranging from 4 to 18 mm. Pseudomonas aeruginosa 9 was the most successful at generating hyaluronidase because of its largest hydrolysis diameter (18 mm) on a medium containing brain heart serum albumin. The greatest technique, which researchers frequently employ, was utilized to look into how both gram-positive and gram-negative bacteria can identify the enzyme hyaluronidase. This approach measured the activity of the hyaluronidase enzyme generated by the H4489A phage after it infected *Streptococcus pyogenes* and transferred the genes to *E.coli* bacterium (Baker et al.,2002). A similar method was used to identify the enzyme in *Streptococcus suis* bacteria(King *et al*., 2004).

3.2 Optimum conditions for hyaluronidase production

Several parameters were studied to find the best circumstances for hyaluronidase production, and they are as follows:

3.2.1 Optimum pH

To get the most hyaluronidase enzyme out of *Pseudomonas aeruginosa*, the pH levels were changed from 4 to 10. Figure 1 shows that when the production medium pH was 7.0, the enzyme's specific activity reached 120 U/mg of protein. At other pH levels, the specific activity of the hyaluronidase enzyme decreased in different ways. Since it affects media properties, material solubility, and the ionic state of material interaction HA in the growth of bacteria and many metabolic processes, medium pH optimization is essential for optimum production (Purohit, and Marthur 1996, Sahoo *et al*.,2007). A change in the pH of the culture medium, which can lead to complicated alterations in the three-dimensional structure of proteins, can be brought on by a change in the concentration of hydrogen ions (H+). This is because an enzyme is destroyed when H+ and/or OH- compete with its ionic and hydrogen bonds (Tung *et al*., 1994).

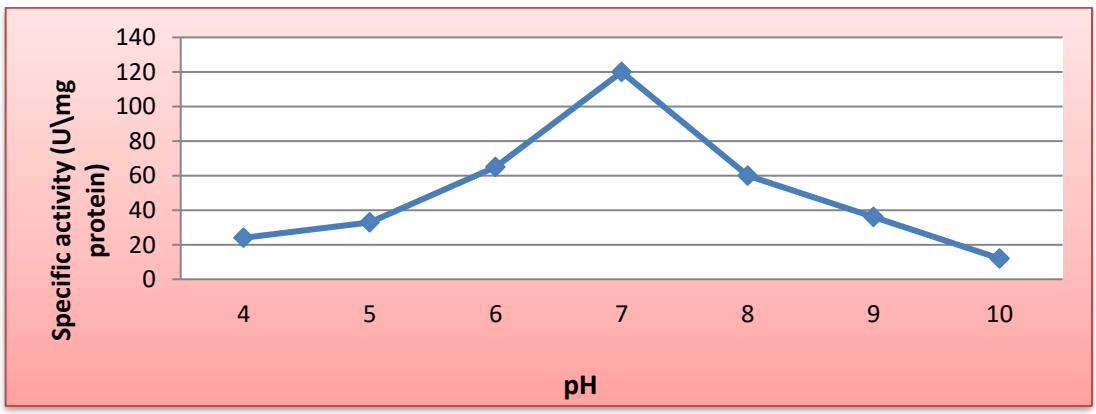


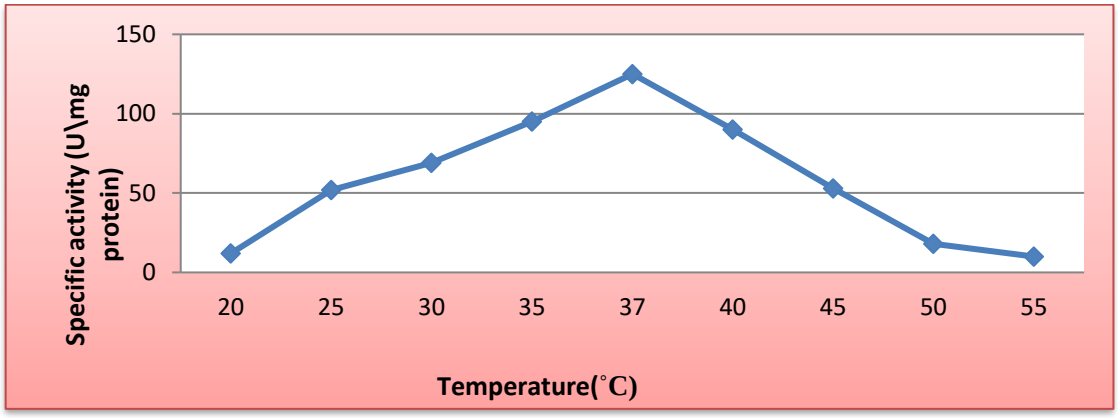
Figure (1): The impact of pH on hyaluronidase production by *Pseudomonas aeruginosa*

3.2.2Optimum incubation temperatures

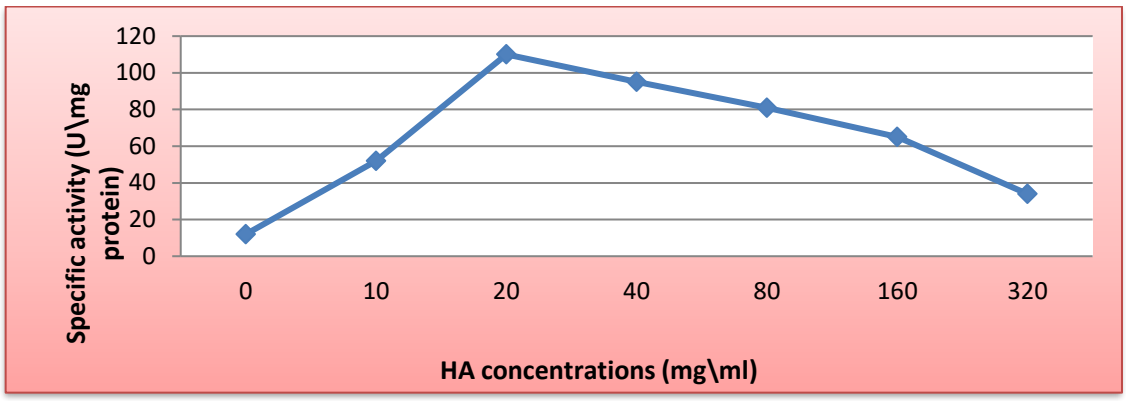
The optimal temperature for hyaluronidase synthesis by *Pseudomonas aeruginosa* was determined by incubating the bacteria at temperatures ranging from 20 to 55° C. Figure (2) shows that when the culture medium was incubated at 37°C, the maximal generation of hyaluronidase was attained, and the specific activity of hyaluronidase was 125 U/mg protein. Enzymatic denaturation results from an elevation of temperatures beyond or below ideal temperature and causes the breakdown of hydrogen and other non-covalent bonds, which causes the loss of its three-dimensional structure (Tortora *et al*., 2004). The solubility of O_2 in the medium, the molecules' kinetic energy, and the molecules' reaction speed all impact plant and animal development. Consequently, these variables may impact hyaluronidase production (Bull, A. T. and Bushnell, M. E. (1976) and Kandela, 2006). The dynamic energy of molecules and the rate of cell responses and metabolic activities may also be affected by temperature, slowing bacterium development at low and high temperatures (Frost, 2007).

3.2.3 Optimum HA concentration

In certain types of microorganisms, adding HA substrate leads to stimulating enzyme production in a specific concentration. Hence different concentrations of HA ranging from(10-320) mg\ml were used to measure the specific activity of the enzyme of *Pseudomonas aeruginosa* isolate. The result shows that specific activity reached 110 U/mg protein when HA concentration is 20 mg\ml (Figure 3). The need to add 0.5 mg/ml of HA to the production medium to produce hyaluronidase enzyme from *Citrobacter freundii*. In contrast, 0.6 mg/ml was needed to stimulate the production of the enzyme in *Streptococcus pyogenes*. On the other hand, the absence of the substrate (HA) in the production medium led to producing the enzyme in some bacterial species due to the nature of the genes encoding for the enzyme, as maybe some of them have a synthetic nature (constitutive enzyme) and can secrete the enzyme without the need for the substrate. In contrast, other types of genes (induced genes) cannot be expressed without a substrate in the production medium (Hill, 1976, Brunish and Mozersky, 1958).



Figure(2): The impact of optimum temperature on hyaluronidase production by *Pseudomonas aeruginosa* isolate



Figure(3): The impact of optimum HA concentration on hyaluronidase production by *Pseudomonas aeruginosa* isolates

3.2.4 Optimum culture conditions

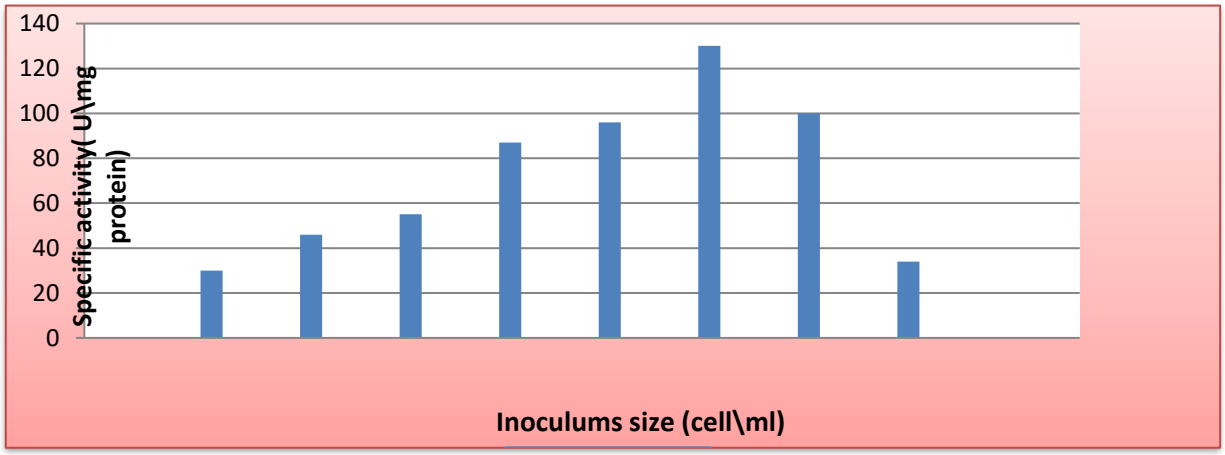
The type of energy source was also investigated using different sources of energy such as starch, glucose, mannitol, and sucrose as carbon sources and yeast extract, ammonium acetate, ammonium sulfate, sodium nitrate, and ammonium chloride as nitrogen sources. The results suggest that starch and lactose were the best for hyaluronidase synthesis since the enzyme's specific activity reached 120 and 100 mg protein, respectively. In contrast, glucose, mannitol, and sucrose had the least specific activity. The kind of nitrogen supply also has an impact on enzyme synthesis. Yeast extract and ammonium acetate had the highest hyaluronidase-specific activity (123 and 90 U/mg, respectively), whereas ammonium sulfate, sodium nitrate, and ammonium chloride had the lowest specific activity (Table 1). Microbiology enzyme production varies with medium components such as carbohydrates, proteins, and inorganic substances. Enriching media with nitrogen sources like yeast extract provides amino acids and peptides for cell growth. Enzyme production follows (Mikiashvili *et al*., 2008). After 8 days of submerged cultivation, nitrogen supply dramatically boosted final biomasses compared to the control medium, which in turn enhanced enzyme production and activity (Galhaup *et al*., 2007). In *Pleurotus ostreatus*, the addition of mannitol, glucose, and sodium gluconate increases hyaluronidase activity (King *et al*., 2004). The beneficial effects of carbohydrates on hyaluronidase production are probably due to the availability of water-soluble aromatic chemicals (flavones and flavonols) that encourage enzyme biosynthesis (Mikiashvili *et al*., 2008).

Table (1): The impact of the carbon and nitrogen sources on hyaluronidase production by *Pseudomonas aeruginosa*

Carbon source	Specific activity (U/mg protein)	Nitrogen source	Specific activity (U/mg protein)
Starch	100	**Yeast extract**	123
Glucose	55	**Ammonium acetate**	90
Mannitol	76	**Ammonium sulfate**	55
Lactose	120	**Sodium nitrate**	34
Sucrose	94	**Amonium chloride**	51

3.2.5 Optimum inoculums size for hyaluronidase production

The optimal inoculum size for *Pseudomonas aeruginosa* hyaluronidase production was determined using several inoculum sizes. These inoculums ranged in size from (10^{2}-10^{9}) Cells/ml and were utilized to inoculate the production medium separately. The generation of hyaluronidase by *Pseudomonas aeruginosa* was regulated by the microorganism's inoculum size, as shown in Figure 4. Moreover, it was shown that increasing the inoculum size raised hyaluronidase production slightly to 10^{7} Cells/ml, then reduced with increasing the inoculum size. Maximum hyaluronidase synthesis, however, was observed at 10^{7} Cells/ml, with a specific activity of 130 U/mg. Enzyme synthesis in sufficient quantities to use the substrate in the production medium required a sufficient quantity of cell inoculum size since smaller inoculum sizes required more time for the cells to grow to adequate numbers to create enzymes. Proliferation and biomass synthesis would accelerate with more inoculum. However, increasing biomass can deplete nutrients, lowering enzyme synthesis and metabolic activity (Kashyap, 2002). The enzyme is produced at its highest level when a balance between the production medium's available nutrients and the biomass that is growing is achieved (Ramachandran, 2004).



Figure(4): The impact of inoculum size on hyaluronidase production by *Pseudomonas aeruginosa* isolate

Conflict of interest

There is no conflict of interest.

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