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Research Article
Studies on Hyaluronidase Extracted from *Staphylococcus aureus* Isolated in Khartoum/Sudan

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Abstract

Background and Objective: Hyaluronidase is an enzyme that breaks down primarily hyaluronic acid and it splits it into polysaccharide and used in medicine in conjunction with other drugs to speed their dispersion and delivery. The aim of this research was to isolate, screen and characterize Hyaluronidase producing *Staphylococcus aureus* isolated from infected diabetic wounds. **Materials and Methods:** Thirty samples were randomly isolated from infected wounds from Zenam Centre for Diabetes and Diabetic Wounds/Sudan. The isolates showing high hydrolyzed zones were identified. Cell growths as well as hyaluronidase activity is measured spectrophotometrically by Turbidity Reduction Assay. **Results:** 15 out of 30 (50%) isolates were detected as *staphylococcus aureus*. Primary screening revealed that 6 out of 15(60%) could be enzyme producers with inhibition zone of 4.5 cm. Growth profile was increasing with time. **Conclusion:** The study reveals that *Staphylococcus aureus* is a promising source with higher yield compared with similar study. Enzyme production is growth associated and the highest yield was obtained at the log phase of bacterial growth.

Key words: Hyaluronidase , *Staphylococcus aureus*, infected diabetic wounds, turbidity reduction assay (TRA)

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hyaluronidase is an enzymes that are able to break down primarily hyaluronic acid (HA)[1]. It acts on two substances called hyaluronate and chondroitin and it splits them into polysaccharide product by catalyzing the hydrolysis of hyaluronan, a constituent of the extracellular matrix (ECM), hyaluronidase lowers the viscosity of hyaluronan, thereby increasing tissue permeability. Hyaluronidase has got other names, diffusing factor, lyase, spreading factor, mucinase and hyaluronic. It is inhibited by formalin. It is of low antigenicity and has no role to play in pathogenicity[2]. Hyaluronidase could be used in medicine in conjunction with other drugs to speed their dispersion and delivery. Common applications are in ophthalmic surgery, in combination with local anesthetics[3]. It also increases the absorption rate of parenteral fluids given by hypodermoclysis and is an adjunct in subcutaneous urography for improving resorption of radiopaque agents. Hyaluronidase is also used for extra vasations of hyperosmolar solutions. The addition of Hyaluronidase to local anesthetics in vitreoretinal surgery promotes the dispersion of the local anesthetics within the orbit by increasing the surrounding tissues permeability. Moreover, Hyaluronidase minimizes the increasing orbital pressure associated with the volume of injected anesthetics and enhances the quality of globe akinesia which reduces the incidence of transient postoperative extra ocular muscle paresis as well as, the frequency of postoperative pain[4]. Hyaluronidase is a recommended antidote for vinca alkaloid overdose or extravasation[5]. On December 2, 2005, the FDA approved a synthetic (recombinant or rDNA) human hyaluronidase, Hylenex (Halozyme Therapeutics) Hylenex[6]. The FDA also approved HyQvia in late[7] 2014 a form of subcutaneous immunoglobulin (SCIG) that uses Hylenex to allow for a far greater volume of SCIG to be administered than would normally be possible to administer subcutaneously, providing a form of SCIG that can be dosed on a monthly basis, a longer period of time than other forms of SCIG allow. HyQvia had a rate of systemic adverse effects higher than traditional subcutaneous forms of immunoglobulin injection but lower than those typical in IVIG patients[8]. Also it is used in epidural lysis of adhesions for pain management. Hyaluronic acid (HA)-based fillers are the most commonly used fillers. Furthermore, Hyaluronidase has also been suggested for the treatment of the rare adverse reactions to HA fillers as by hypothesis some of the HA might still be present in the skin which might be targeted by Hyaluronidase[9,10]. The role of Hyaluronidase in cancer has been historically controversial due to contradictory observations, namely that levels of Hyaluronidase (HYAL1/2) are increased in some cancers[11] whereas low expression of HYAL1 is correlated with a decrease in survival of pancreatic adenocarcinoma patients[12,13]. Hyaluronidase helps cancer cells escape from primary tumor masses. However, studies show that removal of hyaluronan from tumors prevents tumor invasion. Hyaluronidases are also thought to play a role in the process of angiogenesis, although most Hyaluronidase preparations are contaminated with large amounts of angiogenic growth factors[14]. The role of Hyaluronidase in fertilization is released by the acrosome of the sperm cell after it has reached the oocyte, by digesting hyaluronan in the corona radiata[4]. Certain species of bacteria, especially streptococci, produce Hyaluronidase to dissolve the polysaccharide that binds the cells together. The dissolving action is believed to be associated in the blackening of infected wounds and to help the bacteria spread from the initial infection site towards other body parts. It could be added with a drug to promote the spread of the drug in a target body tissue[15]. Hyaluronidases has a panoramic use in biotechnology and since much of preparations of Hyaluronidase are of animal source, with limited sources of microbial origin and are found in the venom of certain lizards and snakes as well as honeybees, where they are referred to as spreading factors, having a function akin to bacterial Hyaluronidase[16]. This prompted researchers to screen and isolate a new promising bacterial species with higher yield.

MATERIALS AND METHODS

Study design: This is descriptive laboratory based study.

Sampling: A total of 30 samples (swab) were collected from diabetic's wounds taken from Zenam Medical Centre for Diabetics and Diabetic Wounds (Khartoum/Sudan). The samples were taken into sterile cotton swabs and transferred to Central Laboratory, Khartoum, Sudan. The study conducted from June-November, 2018.

Data analysis: Data was analyzed statistically through the program of excel. The experiment was conducted in triplicate and the average values were taken into consideration.

Isolation of micro-organism: Isolation of micro-organism was performed by serial dilution plate technique[17]. In this technique, 1 g of each soil sample was taken in 9 mL of sterilized distill water in pre- sterilized test tube. Serial aqueous dilutions were prepared by transferring 1 mL of the soil

suspension into 9 mL of sterilized distill water in sterilized test tubes. Different aqueous dilutions (10^{-4}-10^{-7}) of the soil suspension were applied separately and transferred and cultured into a selective media (Manitol Agar) to allow only *Staphylococcus* growth. positive samples were those giving distinguishing golden yellowish colors[18].

Primary screening of hyaluronidase: 25 mL of each isolates were aseptically added into sterile wells of different molten nutrient agar plates containing 1 mL of substrate hyaluronic acid (Hyaluronic acid sodium salt purified from Streptococcus equi was purchased from Sigma-Aldrich) to test its ability to produce Hyaluronidase, at a concentration of 10 mg mL^{-1}. The plates were then incubated at 37°C for 24 h. The hydrolyzed zone (diameter in cm) of isolates was recorded. Upon such result, isolates showing high hydrolyzed zones were subjected to characterization and selected to be used for qualitative determination of the enzyme.

Extraction and isolation of hyaluronidase: The isolates showing high hyase production were further screened by shake flask fermentation method. The selected isolates were sub-cultured onto nutrient agar slants, incubated at 37°C for 24 h. Content of each slant was transferred into 50 mL of nutrient broth in 250 mL Erlenmeyer flask. The flasks were incubated at 37°C for 48 h on rotary shaker at 150 rpm. The fermentation broth of each flask was centrifuged at 8000 rpm and at 4°C then the clear supernatant was used for the crude hyaluronidase assay. The experiment was conducted in triplicate and the average values were taken into consideration[19].

Hyaluronidase activity assay: Hyaluronidase activity is measured spectrophotometrically by turbidity reduction assay using HA sodium salt as substrate [20]. in which the enzymatic reduction in turbidity resulting when 1 mL of HA at 70 mg mL^{-1} was incubated with 1 mL of enzyme sample in the presence of 0.05 M sodium phosphate buffer with 0.05 M NaCl of pH 7.0. After incubation of the mixture for 30 min, 2.5 mL of acidified protein solution (1% w/v) bovine serum albumin fraction (BSA) in 0.5 M sodium acetate buffer of pH 3.1 was added and incubated at 37°C for 10 min and reduction in turbidity was read by measuring the absorbance at 600 nm. The un-inoculated culture broth was used as a blank. One unit of hyaluronidase activity was defined as the amount of enzyme that causes a reduction in turbidity at 600 nm (A_{600}) under specified assay conditions.

Time course of enzyme production: The colonies that showed large zone on Manitol Agar Medium were used of inoculums preparation. About 10% inoculum was added to 100 mL of production medium into 250 mL cultures then incubated at 37°C and 150 rpm on rotary shaker incubator for 60 h. Sample were removed periodically every 6 h and cell growths as well as hyaluronidase activity were determined.

RESULTS

Sampling results showed that all of the isolates (30 samples) were reveal the turbidity on nutrient broth (Fig. 1), while 15 out of 30 isolates (50%) were positive and gave the distinguishing golden yellowish color on Manitol Agar Media indicating the presence of *Staphyloccus aures* and confirmed by morphological test of gram positive (+), cocci, regular and forming an aggregates or tetramers. Such characteristics are similar to those of *Staphyloccus aures* as described[21].

Primary screening of the isolates: Figure 2 showed that isolates no. 2, 5, 12 and 7 showed highest reduction in

Fig. 1: Turbidity on nutrient broth

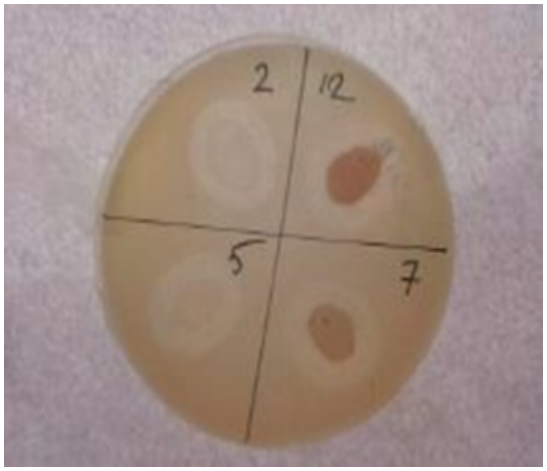

Fig. 2: Zone of inhibition on molten nutrient agar plates containing 1 mL of substrate hyaluronic acid

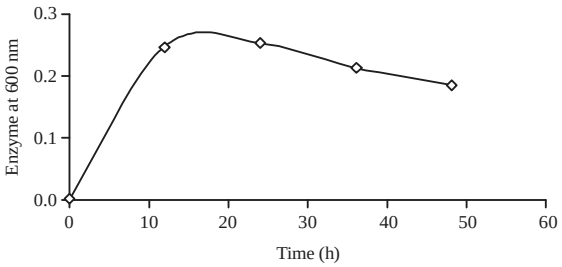

Fig. 3: Enzyme activity (U mL^{-1}) at 600 nm vs. time (h)

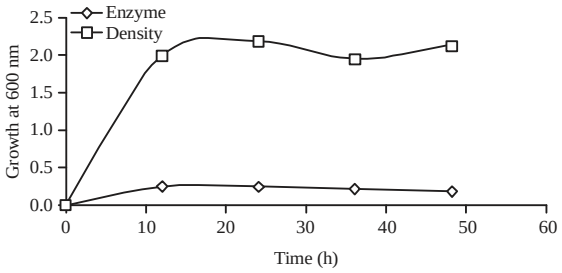

Fig. 4: Time course of hyaluronidase activity (U mL^{-1}) and bacterial growth curve (at 590 nm)

turbidity and larger inhibition zone (cm) of 2.0, 4.5, 3.00 and 2.5, respectively upon treated with hyaluronic acid as a substrate solution around colony, then selected for secondary screening by shake flask fermentation method and further for hyaluronidase activity.

Enzyme extraction and activity: After 24 h cultivation of *Staphyloccus aures* in a 250 mL shake-flask, the fermentation broth was centrifuged at 8,000 rpm at 4°C for 30 min to remove cells. The clear supernatant was used as a crude hyaluronidase. The data recorded in Fig. 3 indicated that the isolate exhibited maximum hyaluronidase activity of 0.253 U mL^{-1} at 24 h noticed that the activity of the enzyme was increased with time then after, decreased to 0.18 U mL^{-1}. at 48 h incubation.

Time course of hyaluronidase production: It was found that hyaluronidase activity was increasing with time of 0.253 U mL^{-1} at 24 h incubation and decreasing thereafter. The highest yield of the enzyme was attained at the log phase of bacterial growth at 24 h as showed in Fig. 4. The optimum temperature for its production is 30°C although *Staphylococcus aureus* is mesophilic 37°C.

DISCUSSION

The application of enzyme technologies to pharmaceutical research, development and manufacture is a growing and an emerging field which is subject to many articles reviews and books[22]. The action of hyaluronidase has promising medical application. In this study all of the samples isolated were reveal the turbidity on nutrient broth and showed good hyase producing activity in comparison to the reference strain of *Staphylococcus aureus.* Similar results for screening of hyaluronidase producing isolates employing turbidity reduction assay were reported by many workers, after subjecting the 30 suspected bacterial isolates to the related biochemical tests, results showed that 15 of them were *Staphylococcus aureus*. Pervious study[23] showed that four isolates showing marked reduction in turbidity (A600 nm) and hydrolyzed zones. This study gives new insights into that Sudanese habitats are excellent for hyaluronidase producing micro-organism. Morphological, cultural and biochemical tests is proposed that the isolate can be characterized as a strain of *S. equi*[23]. Other study[24] reported that *Clostridium perfringens* growing in tissues produced hyaluronidase employing Turbidity Reduction Assay. Group B *Streptococci* with good hyaluronidase activity was isolated by many workers some workers also reported the production of hyaluronidase (Hyaluronate lyase) by *Corynebacterium acnes*[25]. Only 3 out of 135 showed ability to produce hyaluronidase by using Turbidity Reduction Assay method with highest hydrolyzed zone was recorded by isolate of *S. pyogenes* reached 12 mm. This study showed sample (13) showed highest inhibition zone of 4.5 cm. Hyaluronidase production by *Streptococcus pyogenes* and other pathogenic *streptococci* (groups B, C and G and *Streptococcus pneumonia*) has been a subject of interest since early reports of Ahmed *et al.*[26].

The principle of using Turbidity Reduction Assay for detection of hyaluronidase was depended on reduction turbidity of the medium when inoculated with hyaluronidase producing micro-organism The highest yield of hyaluronidase was attained at the log phase of bacterial growth of 24 h. Our data disagree with Nicoll *et al.*[27], who found that the optimum temperature for production of *Staphylococcus aureus* is detected in 3-8 h.

The study reveals that *staphylococcus aureus* is a promising source for hyaluronidase production with higher yield compared with similar studies. Hyaluronidase production is growth associated and the highest yield was obtained at the log phase of bacterial growth. A detailed comparison was done to establish the novelty of our isolate.

CONCLUSION

It was found that 100% of the isolates were revealing the turbidity on nutrient broth. Fifty percent of the detected

Staphylococcus aureus were hyaluronidase producers. The hyaluronidase is produced at the log phase of bacterial growth. Hyaluronidase production is growth associated.

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