Production and optimisation of hyaluronic acid extracted from *Streptococcus pyogenes*

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Objectives Because of the less studies in this field in Iraq, this study aimed to use local *Streptococcus pyogenes* isolates to produce hyluronic acid.
Methods The quantitative estimation of hyaluronic acid (HA) produced from eight local *S. pyogenes* isolates at different pH (6.3, 6.6, 6.9, 7.2, and 7.5) and glucose concentration (4%, 6%, 8%, and 1%) were done using the HA ELISA kit.
Results This study showed that the maximum yield of HA was obtained at pH 7.5, and it was found that the differences in pH of the production media of HA enhanced the HA production, but the glucose concentration has no beneficial effect for HA production.
Conclusion There were differences in HA production among local isolates at the same pH.
Keywords *Streptococcus pyogenes*, hyaluronic acid, HA ELISA kit, pH, glucose concentration

Introduction

Hyaluronic acid (HA), also known as hyaluronan, is a linear polysaccharide (up to 10 MDa) consisting of alternating units of (1,4)-glucuronic acid and (1,3)-N-acetyl-D-glucosamine (GlcNAc).[1] Additionally to retaining and modulating the flow of water, it also helps as the backbone for proteoglycan gathering by binding aggrecan monomers via link protein.[2] Furthermore, HA carries numerous functions in altering cellular functions during the development of human, specifically during the development of diarthrodial joints. In developed cartilage, HA has a great significance of ligand for cell-matrix interactions with pericellular matrix via the CD44 cell receptor.[3] The physical, chemical, and biological attribution of the HA includes lubricity, visco-elasticity, holding of water, biocompatibility, cell multiplication, morphogenesis, inflammation, and wound repair as well as specific signal transduction and cellular interactions through cell surface receptors.[4,5]

HA is highly hygroscopic, biocompatible, and decomposable biopolymer, real attractive for biomaterials fabrication. It is intensively used in cosmetics, surgery, and delivery of drugs.[6]

HA has been normally extracted from rooster combs and bovine vitreous humor. However, it is difficult to isolate high molecular weight HA at industrially practicable rate from these sources, because it makes a complex with proteoglycans exist in animal tissue. It is presently impractical to manipulate the molecular weight of the biopolymer while it is synthesized in animal tissue. Moreover, the use of animal-derived biochemicals for human therapeutics has embossed ethical outcomes, and is met with growing resistance. To overcome these disadvantages, the recent tendency involves the usage of Lancefield's group A and group C Streptococci, which naturally produce a mucoid capsule of HA.[7]

The requests for HA products from bacterial fermentation have fundamentally expanded because of both their increased use as medical devices and the immune issues that happened from the use of animal-based HA[5]. Because of both the high prices of HA and the high standard requirements of its applications in medical products, high-quality HA products rather than high quantity have been the essential criteria utilized when selecting the bacterial strains utilized for HA generation. Streptococci are ideally meant and adapted for studying the biosynthesis of HA due to the abundant availability of hyaluronate and since in this organism the hyaluronate is the only polymer into which glucuronic acid is comprised.[8]

In Streptococci, HA is created as a secondary metabolite and the production is affected by different agents that involve genetic as well as nutritional. Streptococci produces HA both under aerobic and anaerobic condition.[9]

Materials and Methods

Bacterial Isolates

Eight local isolates of *Streptococcus pyogenes* were isolated from ENT infectious, and identified depending on traditional methods as described by Macfaddin,[10] in addition to use the strepto-system 9R according to the manufacture's instructions and molecular methods by amplification of universal and specific species genes. These isolates are given numbers 1, 2, 3, 4, 5, 6, 7, and 8.

Isolation of HA

HA creating bacteria were chosen based upon their hemolytic character. Best hemolysis producing colonies were picked from every blood agar plate and streaked on Todd Hewitt agar plates. The plates were incubated at 37°C in a 5% CO_2 atmosphere for 24 hours.[9]

pH Effect on the Production of HA

The effect of pH on the production of HA was measured by the following technique. Ten milliliters of Tood Hewitt broth (THB) medium kept up at five different pH (6.3, 6.6, 6.9, 7.2, and 7.5) were inoculated with a loopful of *S. pyogenes* isolates, and incubated at 37°C for16 hours. The overnight cultures were inoculated into 10 ml of fresh THB medium and inoculated at 37°C for 24 hours under shaking condition. The resulting suspensions were centrifuged, washed with 10 ml of 10 mM Tris HCl (pH 7.5) and vortexed for 10 seconds.[9]

Glucose Concentration Effect in the Production of HA

The effect of glucose concentration in the production of HA was measured by the following technique. Four conical flasks

containing 10 ml of THB medium, each was supplemented with different glucose concentration (0.4, 0.6, 0.8, and 1%) were inoculated with 15–30 colonies of *S. pyogenes* isolates, and incubated at 37°C for 16 hours. The overnight culture were sub-cultured in to 10 ml of fresh THB media and incubated at 37°C for 24 hours under shaking condition. The resulting suspensions were centrifuged, washed with 10 ml of 10 mM Tris HCl (pH 7.5), and vortexed for 10 seconds.[9]

HA Extraction

Bacterial cells cultivated under different condition were pelleted out and the cell pellets were resuspended in 1.5 ml of water and vortexed for 10 seconds. This washing sequence was repeated twice and the cell pellets were resuspended in water to a final volume of 1.5 ml. The HA capsule was extracted by adding 1.5 ml of chloroform and shaking for 1 minute. Cell remained at room temperature for 1 hour and then was pelleted and the aqueous phase was used for estimation.[9]

Quantitative Estimation of HA by HA ELISA Kit

The quantitative estimation of HA according to the manufacture's instructions of the HA ELISA kit (Elabscience/China) was carried out.

Results

Quantitative Estimation of HA

Eight different isolates of *S. pyogenes* with pathogenic characters were used for HA production. After the cultivation of *S. pyogenes* isolates in Todd Hewitt agar plates, the large white mucoid colonies were selected for HA production. The HA content of these isolates, which were incubated at different pH, were analyzed according to HA ELSA kit protocol. Based on the results obtained in Fig. 1 the maximum HA yield was observed with isolated numerated one at pH 7.5 (67.9 ng/ml). While the same pH (7.5) shows no HA production in isolated number 5, 6, 7, and 8.

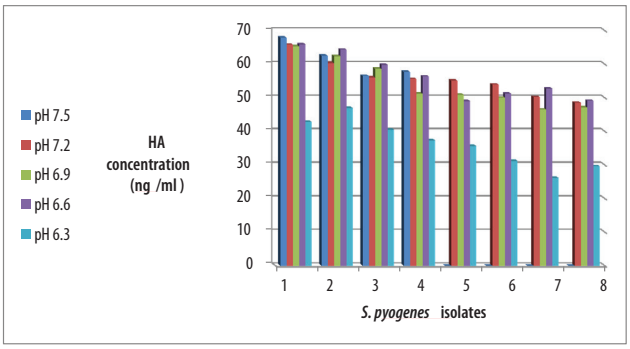

Fig. 1 **HA yield (ng/ml) generated from *S. pyogenes* under different pH.**

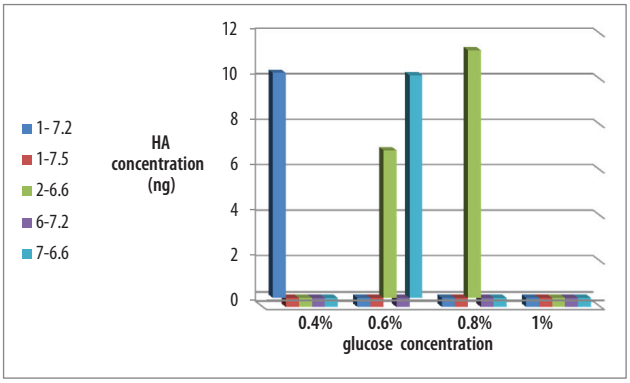

Fig. 2 **HA yield (ng/ml) generated from some S. pyogenes isolates under different glucose concentration.**

Discussion

The ELISA procedure is sensitive, simple, and is based on a microtitre plate format. The assay involves competition between HA absorbed to the plate and HA free in solution for binding to biotinylated cartilage proteoglycan binding region. The range of the assay is 10–2500 ng/ml with 50% inhibition at about 200 ng/ml. This technique involves fewer experimental steps and is simpler to perform than other methods.[11]

Production media were maintained at an optimised pH of some isolates estimated at four different glucose concentrations (0.4, 0.6, 0.8, and 1%) according to the ELSA kit protocol. Based on the results shown in Fig. 2, all of selected isolates undergo an acute decline in HA production.

The study by Saranraj et al.[9] showed gradually increase of HA yields with an increase of glucose concentration.

The decline of HA yields may be due to the decrease of the pH of the medium resulted from the high consumption of the carbon sources, which led to the production of organic acid and reduce the pH of the medium.[12] The other reason may be its agitation speed. The increase of agitation speed led to a significant decrease of cell growth and HA production.[12]

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