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REVIEW ARTICLE

Hyaluronic Acid Production and its Applications - A Review

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ABSTRACT

Hyaluronic acid is a naturally derived, non - immunogenic, non - adhesive glycosaminoglycan that plays a prominent role in various wound - healing processes, as it as it is naturally angiogenic when degraded to small fragments. Hyaluronic acid promotes early inflammation which is critical for initiating wound healing, but then moderates later stages of the process, allowing matrix stabilization and reduction of long term inflammation. Hyaluronic acid is widely distributed in mammalian cells and tissue but is primarily found in synovial fluid, vitreous humor of the eye and loose connective tissue such as rooster comb, umbilical cord, dermis and arterial wall. It is also found in the capsular component of certain bacterial such as *Streptococcus* sp. and *Staphylococcus* sp. These have been biotechnologically developed and are now a main source of commercial Hyaluronic acid for pharmaceutical, medical and cosmetic application. The present review was based on Hyaluronic acid production and application. This review assesses the following topics: Structural features and properties of Hyaluronic acid, Rheological properties of Hyaluronic acid, Lubricity of Hyaluronic acid, Hydrophilicity of Hyaluronic acid, Hyaluronic acid production by bacterial fermentation, Biosynthesis of Hyaluronic acid in *Streptococcus*, Enzymes involved in Hyaluronic acid biosynthesis, Optimization and extraction of Hyaluronic acid, Analysis of Hyaluronic acid and Applications of Hyaluronic acid.

Key words: Hyaluronic acid, Bacterial fermentation, Optimization, Extraction, Enzymes, *Streptococcus* sp., *Staphylococcus* sp. and Application.

1. INTRODUCTION

Hyaluronic acid (HA), a naturally occurring glycosamino glycan, offers many unique advantages as a building block for biomaterials. For e.g., Hyaluronic acid is non - immunogenic, enzymatically degradable and relatively non-adhesive to cells and protein [1]. Physiologically, Hyaluronic acid has a role in several process including angiogenesis, extra cellular matrix, homeostasis, wound healing and the mediation of long-term inflammation. Hyaluronic acid is a high molecular weight, linear polysaccharide that is produced commercially for a wide range of use, principally as an ophthalmic surgery aid. The traditional method of production involves extraction of Hyaluronic acid from rooster combs [2]. Chemically classified as a glycosamino glycan and is composed of the repeating disaccharide units of (1 – 4 – β – D – glycospyranosyl uronic acid), (1 - 3) N acetyl 2 – amino – 2 deoxy – β – D glycopyranosyl acid. The polysaccharide contains between 500 and 50,000 monosaccharide residues per molecules. Thus, its molecular weight ranges from 10^4 to 10^7 Da [3, 4]. Hyaluronic acid is ubiquitous in the organisms with the highest concentration found in soft connective tissue. It plays an important role for both mechanical and transport purposes in the body. Hyaluronic acid is very important application for different field such as comfort eye drops, osteoartitis, wound healing, anti ageing periodontitis, etc. Hyaluronic acid has been traditionally extracted from rooster combs and bovine vitreous humour. However, it is difficult to isolate high molecular weight Hyaluronic acid economically from these sources because it forms a complex with proteoglycans [5]. In *Streptococcus*, unlike other sources is an exclusive synthesis of only one glycosaminlglycan. Effectively, Hyaluronic acid is isolated at relatively low cost and in high yields.

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As a mimic of the embryonic environment, exogenous Hyaluronic acid supplementation promotes faster and more extensive regeneration in adult injures. This cumulative evidence suggests that Hyaluronic acid is an ideal candidate material for modulating wound healing. The cost of high quality Hyaluronic acid is about $500 per 1000mg in injectable form (used in face lifts and joint therapy) and $12 per 1000 mg in pills form. Our body produces 1000s of dollars worth of Hyaluronic acid every day. However, our cell does not always produce Hyaluronic acid efficiently. When Hyaluronic acid production dwindles, it results in joint pain, mycofacial rigidity, skin aging, dryness and wrinkles [6].

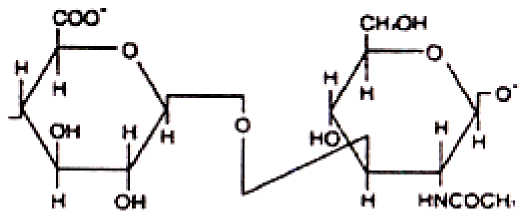
Hyaluronic acid has substantial medical and biological importance, owing to its non – immunogenic nature [7]. A highly viscous solution of Hyaluronic acid can effectively retain moisture and exhibit wound healing property and also act as effective lubricant [8] and as a fluid replacement, in pathological conditions. In ophthalmic surgery such as cataract, glaucoma, corneal transplant, retinal and vitreous surgery, Hyaluronic acid is extensively used as a supportive medium. Usage of high Molecular weight @ Hyaluronic acid (1%) created the conceit if viscosurgery [9]. The hydrophilic nature makes it an ideal constituent in moisturization lotions for cosmetic use [10].

Hyaluronic acid is a naturally occurring Biopolymer, which serves important biological functions in bacteria and higher animals including humans. It was first isolated from the vitreous body of the eye in 1934 by Karl Meyer, who called it Hyaluronic acid. The term hyaluronan is attributed Endre Balazs, who coined it to encompass the different form the molecule can take for example the acid form, Hyaluronic acid and the salts such as sodium hyaluronate, which form at physiological pH.

After 70 years, quite a lot is known about the appearance of the hyaluronan molecule, its behavior, its occurrence in different tissues and body fluids, the manner in which it is synthesized by the cells, metabolized and cleared from the body, and the nature of some of the functions it performs. The main purpose of this review is to make readers aware of this increasingly important biomaterial and to survey the techniques for improving the production of Hyaluronic acid from *Streptococcus pyogenes*, thereby modifying synthetic material surfaces with hyaluronan.

2.STRUCTURAL FEATURES AND PROPERTIES OF HYALURONIC ACID

Hyaluronic acid is comprised of linear, unbranching, polyanionic disaccharide units consisting of glucuronic acid (Glc UA) an N – acetyl – glycosamine (Glc NAC) joined alternatively by β 1-3 and β 1-4 glycoside bonds. It is a member of the glycosaminoglycan family, which includes chondroitin sulphate, dermatin sulphate and heparin sulphate. Unlike other members of this family, it is not found covalently bound to proteins. Each repeating disaccharide unit has one carboxyate group, four hydroxyl groups and an acetamido group. Hyaluraonan differs from the other major groups of glycosaminoglycans in that it does not have sulfate group [11].



Structure of Hyaluronic acid

Hyaluronic acid exists in a random coil configuration, which is polyanionic at physiological pH. At high molecular weights, these random coils become entangled to form a viscoelastic gel. Hyaluronan possesses a unique set of characterestics: its solution manifests very unusual rheological properties and is exceedingly lubricious and it is very hydrophilic.

3.RHEOLOGICAL PROPERTIES OF HYALURONIC ACID

Hyaluronic acid solutions are characteristically viscoelastic and pseudoplastic. This rheology is found even in very dilute solutions of the polymer where very viscous gels are formed. In solutions, the hyaluronan polymer chain takes on the form of an expanded, random coil. These chains entangle with each other at very low concentrations, which may contribute to the usual rheological properties. At higher concentrations, solutions have an extremely high but shear dependent viscosity. A 1% solution is like jelly, but when it is put under pressure it moves easily and can be administered through a small bore needle. It has therefore been called a “Pseudo-plastic” material [12].

4. LUBRICITY OF HYALURONIC ACID

The extraordinary rheological properties of hyaluronan solutions make them ideal as

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lubricants. There is evidence that hyaluronan separates most tissue surface that slide along each other. This viscoelastic property of Hyaluronic acid solution is controlled by the concentration and molecular weight of the Hyaluronic acid chains. The molecular weight of Hyaluronic acid from different sources is polydisperse and highly variable ranging from 10^4 to 10^7 Da. Hyaluronic acid solution with this molecular weight is extremely lubricious [13].

5. HYDROPHILICITY OF HYALURONIC ACID

When incorporated in to a neutral aqueous solution hydrogen bond formation occurs between water molecules and adjacent carboxyl and N-acetyl groups. This imparts a conformational stiffness to the polymer, which limits its flexibility. The hydrogen bond formation results in the unique water bonding and retention capacity of the polymer. It also follows that the water binding capacity is directly related to the molecular weight of the molecule. Upto 6L of water may be bound per gram of Hyaluronic acid [14]. This imparts the property that Hyaluronic acid can interact preferentially with certain proteins and cells.

Analytical data sheet of 1% Hyaluronic Acid

Organoleptic Analysis	
Appearance	Fine Powder
Color	White
Physical & Chemical Properties	
pH (in solution at 1%)	6.5 ± 1.0
Solubility in water	Soluble
Solubility in Organic solvent	Insoluble
Average molecular weight	1000 kDa
Microbiological Analysis	
Total plate count	<100/g
Yeast mold	0/g
Escherichia coli	0/g

6. HYALURONIC ACID PRODUCTION BY BACTERIAL FERMENTATION

Hyaluronic acid has been traditionally extracted from rooster combs and bovine vitreous humour. However, it is difficult to isolate high molecular weight Hyaluronic acid economically from these sources because it forms a complex with proteoglycans. Subsequent extraction and purification processes result in an inherent, molecular weight reduction [15]. The use of animal derived biochemicals for human therapeutics is being met with growing resistance, besides ethic arguments, because of the risk of viral infection. Industries have turned to bacterial fermentation processes with the hope of obtaining commercially viable polymer. In bacterial fermentation, extra cellular polysaccharide is released in to the growth medium and control of polymer characteristics and product yields are feasible. The amount of biopolymer that can be produced by this route is theoretically unlimited.

7. BIOSYNTHESIS OF HYALURONIC ACID IN *Streptococcus*

Streptococci are nutritionally fastidious anaerobes, which produce lactic acid as a by-product of glucose metabolism. The Hyaluronic acid capsule is a biocompatibility factor, formed as a mucoid capsule around the cell, which enables the gram positive bacteria to evade host immune defenses and hence accounts for its characteristically high virulence level.

In *Streptococcus*, Hyaluronic acid is produced as a secondary metabolite and the production is influenced by various factors that include genetic as well as nutritional. *Streptococcus* produces Hyaluronic acid both under aerobic and anaerobic condition [16]. Certain strains of S*treptococcus* produce Hyaluronic acid at a particular stage in their life cycle and the same organism secrete enzyme hyaluronidase at a later time, which degrades the Hyaluronic acid produced earlier, Hence, the strain selected for Hyaluronic acid production were negative for hyaluronidase activity and non pathogenic [17].

8. ENZYMES INVOLVED IN HYALURONIC ACID BIOSYNTHESIS

- Phosphoglucomutase
- Pyrophosphorylase
- UDP – Glucose dehydrogenase
- Hyaluronate synthase
- Pyrophosphorylase
- Acetyl transferase
- Mutase
- Amidotransferase

9. OPTIMIZATION AND EXTRACTION OF HYALURONIC ACID

Streptococcus fermentation was able to produce Hyaluronic acid within average molecular weight in the range of 1 - 4 MDa. The energy recovered by these bacteria during anaerobic fermentation is relatively low. Hence, the yield of Hyaluronic acid from bacterial fermentation has been characteristically low (0.1g/g glucose) [18]. However, high yield of high molecular weight Hyaluronic acid has been shown under aerobic condition [19].

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Several strategies were undertaken to increase the life span of the synthase enzyme thereby increasing the production of Hyaluronic acid from *Streptococcus pyogenes.*

- Increasing the energy resource for the cell (glucose concentration) in the production media.
- Optimizing the pH of the medium and
- Mutation – mutating the genes having the negative effect on has gene (Hyaluronate synthase producing gene).

There are two biosynthetic processes complete for the limited resources namely carbon, nitrogen and energy. At low specific growth rates the cell directs more glucose – derived activated precursors to Hyaluronic acid synthesis rather than cell wall synthesis. The higher ATP yields from aerobic glucose catabolism favors the formation of UTP, which is required for the formation of the two activated precursors of Hyaluronic acid synthesis, The optimized pH favors the energy yield from glucose, which may be used to synthesis Hyaluronic acid [20]. The Hyaluronic acid produced under optimized condition was extracted by chloroform treatment at room temperature [21].

10. ANALYSIS OF HYALURONIC ACID

Spectrophotometric method was used to estimate the concentration of Hyaluronic acid present in the sample. Hyaluronic acid in the sample react with stains all reagents giving purple color. The color developed was read at 640 nm [22].

Chromatographic techniques like higher performances liquid chromatography (HPLC), size exclusion chromatography (SEC) coupled with multi angle laser light scattering photometry (Malls) were the other methods used to quantify hyaluronic acid [23]. H^1-NMR spectroscopy was used to qualitatively verify the Hyaluronic acid. The samples were dissolved in D_2O and the spectra were record using a varian inova 500 spectrophotometer [24].

Saranraj *et al*. [25] carried out optimization study for Hyaluronic acid production at different pH and different glucose concentration. Mutation studies were also carried as a mean for improving the Hyaluronic acid production. Hyaluronic acid compounds were qualitatively analyzed through HNMR spectroscopy. The Hyaluronic acid capsule of the two strains were quantified spectrophotometrically using stains all assay method. Based on the optimization study at different pH the maximum yield of Hyaluronic acid was obtained at pH 6.9. The yields of Hyaluronic acid from were *Streptococcus pyogenes* parallelly increases with increase in glucose concentration. The maximum Hyaluronic acid production was observed at 1% glucose concentration from *Streptococcus pyogenes* marked under optimized pH 6.9. The production gradually increases due to improved energy yield. Mutation, with UV rays improves Hyaluronic acid production from *Streptococcus pyogenes* after 10 minutes of exposure. UV mutation produces positive effect on HAS gene.

11. APPLICARIONS OF HYALURONIC ACID

Naturally occurring hyaluronic acid was found in the tissues of higher animals, in particular as intercellular space filler [26]. In the body hyaluronic acid is synthesized by many types of cells and extruded in to the extra cellular space where it interacts with the other constituents of the extra cellular matrix to create the supportive and protective structure around the cells. It is present as a constituent in all body fluids and tissues and is found in higher concentration in the vitreous humour of the eye and synovial fluids in the joints. In mammals, the highest reported concentration is found in umbilical cord [27]. The commercial source of hyaluronic acid is rooster combs, which contain the polymer at a higher concentration with respect to other animal tissues. Another important source of Hyaluronic acid is from microorganism, such as Gram - positive *Streptococci*, where Hyaluronic acid appears as a mucoid capsule surrounding the bacterium.

Over the past few years Hyaluronic acid has become a buzzword synonymous with youth, beauty, and pain relief and accelerated healing. Our body produces thousands of dollars worth of hyaluronic acid every day. However, our cells don't always produce Hyaluronic acid efficiently, when Hyaluronic acid production dwindles, it results in joint pain, myofascial rigidity, skin ageing, dryness and wrinkles, In addition, slow healing of wounds, muscles, tendons etc [28].

Hyaluronic acid and its derivatives formulated through *Streptococcal* fermentation (both aerobically and anaerobically) has been largely studied and applied in the biomedical arena. Its high level of biocompatible has accentuated the appeal of this polymer. It has been used in viscosurgery to allow surgeons to safely create space between tissues. As a microcapsule it can be used for targeted drug delivery. Viscosurgical implants are constructed from hyaluronic acid [29]. Its viscoelastic character has been used to supplement the lubrication in arthritic joints. Because of its high water retention capacity this extra cellular polysaccharide also occupies a niche in the lucrative cosmetic market.

11.1. Ophthalmology

A field in which the purely physico - chemical properties of high molecular weight Hyaluronic acid, play a predominant role is ocular microsurgery, specifically; it is extremely useful in anterior or posterior segment eye surgery; cataract extraction, with or without intraocular lens implantation, kertoplasty and vitreous – retinal surgery [30].

During eye surgery, Hyaluronic acid provides effective protection to exposed tissues, such as the corneal endothelium and permits the reconstruction of anatomical form of the operation site [31]. Exogenous Hyaluronic acid introduced into the eye does not exert any negative effect on post surgical intraocular pressure, nor does it trigger any inflammatory sequelae in the intraocular environment. In addition Hyaluronic acid may leave in the eye as it is rapidly eliminated by physiological mechanism, this property is very useful. Hyaluronic acid solutions are very similar to natural tears that are viscous enough to adhere to the corneal epithelium when the eyelids are open [32]. Hence, Hyaluronic acid products have received the approval as drugs in some countries and as surgical soft instruments in others.

11.2. Osteoarthritis

Numerous clinical investigations have demonstrated the efficacy and safety of Hyaluronic acid in the treatment of Osteoarthritis of the knee and other large joints. Intraarticular injection of Hyaluronic acid restores the viscoelasticity of synovial fluid, normalize the synthesis and inhibit the degradation of endogenous Hyaluronic acid and relieve joint pain [33]. It also keeps swelling and swallowing at a minimum, allowing cells to continually produce Hyaluronic acid thereby increasing mobility and strength.

11.3. Wound Repair

Many of the biological processes mediated by Hyaluronic acid are central to the wound healing process. Following injury, wound healing relies on a series of tightly regulated sequential events like inflammation, formation of granulation tissue, reepithelization and remodeling. Hyaluronic acid is likely to have a multifaced role in the mediation of these cellular and matrix events [34]. Hence, Hyaluronic acid is used for the treatment of acute and chronic wounds such as abrasions, donarsites and postoperative incisions, first and second-degree burns, metabolic ulcers and pressure sores.

11.4. Dermatology and Cosmetics

The ageing of human skin is accompanied by changes in Hyaluronic acid content and metabolism with most dramatic decline being observed after age 50. Low molecular weight Hyaluronic acid can penetrate easily through the skin and re-establish the Hyaluronic acid content. When Hyaluronic acids are applied to the surface of the skin, a viscoelastic film is formed. This film inhibits penetration of foreign substances and maintains the skin moisture [35]. Hence, the commercial value of Hyaluronic acid far exceeds that of other microbial extra cellular polysaccharides. With an estimated world market value of $US500 million, it is sold for upto $US 100.000 per Kilogram. Compare this with another leading microbial extra cellular polysaccharides, xanthan gum derived from *Xanthomonas campestris* which sells for upto $US 11 per Kilogram.

CONCLUSION

Hyaluronic acid is a substance that is naturally present in the human body. It is found in the highest concentrations in fluids in the eyes and joints. The hyaluronic acid that is used as medicine is extracted from rooster combs or made by bacteria in the laboratory. People take hyaluronic acid for various joint disorders, including osteoarthritis. It can be taken by mouth or injected into the affected joint by a healthcare professional. The FDA has approved the use of hyaluronic acid during certain eye surgeries including cataract removal, corneal transplantation, and repair of a detached retina and other eye injuries. It is injected into the eye during the procedure to help replace natural fluids. Hyaluronic acid is also used as a lip filler in plastic surgery. Some people apply hyaluronic acid to the skin for healing wounds, burns, skin ulcers, and as a moisturizer. There is also a lot of interest in using hyaluronic acid to prevent the effects of aging. In fact, hyaluronic acid has been promoted as a "fountain of youth." However, there is no evidence to support the claim that taking it by mouth or applying it to the skin can prevent changes associated with aging.

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