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**Production methods for hyaluronic acid**

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ملخص :

حمض الهيالورونيك (HA) هو بوليمر طبيعي معروف بخصائصه اللزوجة والامتصاص العالية، مما يجعله ذا قيمة كبيرة في التطبيقات الطبية والتجميلية والصيدلانية. تشمل طرق إنتاج HA التخمير الميكروبي، استخلاص الأنسجة الحيوانية، التخليق الكيميائي والإنتاج النباتي. يوفر التخمير الميكروبي، الذي يستخدم بكتيريا مثل *Streptococcus zooepidemicus* وسلالات *Escherichia coli* المؤتلفة، عائدًا ونقاءً وقابلية للتوسع عالية، ولكنه يتطلب مفاعلات حيوية متطورة. يوفر استخلاص الأنسجة الحيوانية، الذي كان شائعًا تاريخيًا، HA بوزن جزيئي مرتفع ولكنه يتضمن مخاطر التلوث ومخاوف أخلاقية. يسمح التخليق الكيميائي بالتحكم الدقيق في هيكل HA ولكنه معقد ومكلف. الطرق النباتية الناشئة، على الرغم من أنها أخلاقية وصديقة للبيئة، لا تزال قيد التطوير بعوائد أقل. يتأثر اختيار طريقة الإنتاج على نطاق صناعي بعوامل مثل العائد والنقاء والقابلية للتوسع والتكلفة والمتطلبات التنظيمية والمخاوف الأخلاقية. اقتصاديًا، HA له تأثير كبير بسبب نطاق تطبيقاته الواسع والطلب العالي، مما يدفع التقدم في طرق الإنتاج لتقليل التكاليف وتحسين الكفاءة. بشكل عام، يُفضل التخمير الميكروبي لإنتاج HA على نطاق صناعي، بينما تظهر الطرق النباتية وعودًا للتقدم المستقبلي.

الكلمات المفتاحية: حمض الهيالورونيك (HA) ; بوليمر حيوي ; تطبيقات ; تخمير ميكروبي ; استخلاص الأنسجة الحيوانية ; العائد.

## Résumé

L'acide hyaluronique (AH) est un biopolymère naturellement présent, connu pour ses propriétés viscoélastiques et hygroscopiques exceptionnelles, ce qui le rend très précieux dans les applications médicales, cosmétiques et pharmaceutiques. Les méthodes de production de l'AH incluent la fermentation microbienne, l'extraction des tissus animaux, la synthèse chimique et la production à base de plantes. La fermentation microbienne, utilisant des bactéries telles que *Streptococcus zooepidemicus* et des souches recombinantes d'*Escherichia coli*, offre un rendement, une pureté et une évolutivité élevés, mais nécessite des bioréacteurs sophistiqués. L'extraction des tissus animaux, historiquement courante, fournit de l'AH de poids moléculaire élevé mais implique des risques de contamination et des préoccupations éthiques. La synthèse chimique permet un contrôle précis de la structure de l'AH mais est complexe et coûteuse. Les méthodes émergentes à base de plantes, bien qu'éthiques et respectueuses de l'environnement, sont encore en développement avec des rendements plus faibles. Le choix de la méthode de production à l'échelle industrielle est influencé par des facteurs tels que le rendement, la pureté, l'évolutivité, le coût, les exigences réglementaires et les considérations éthiques. Économiquement, l'AH a un impact significatif en raison de sa large gamme d'applications et de la forte demande, ce qui stimule les avancées dans les méthodes de production pour réduire les coûts et améliorer l'efficacité. Globalement, la fermentation microbienne est privilégiée pour la production d'AH à l'échelle industrielle, tandis que les méthodes à base de plantes montrent un potentiel pour les progrès futurs.

**Mots-clés :** Acide Hyaluronique (AH) ; Biopolymère ; Applications ; Fermentation Microbienne ; Extraction des Tissus Animaux ; Rendement.

## Abstract

Hyaluronic acid (HA) is a naturally occurring biopolymer known for its exceptional viscoelastic and hygroscopic properties, making it highly valuable in medical, cosmetic, and pharmaceutical applications. HA production methods include microbial fermentation, extraction from animal tissues, chemical synthesis, and plant-based production. Microbial fermentation, utilizing bacteria such as *Streptococcus zooepidemicus* and recombinant *Escherichia coli*, offers high yield, purity, and scalability but necessitates sophisticated bioreactors. Animal tissue extraction, historically common, provides high molecular weight HA but involves contamination risks and ethical concerns. Chemical synthesis allows precise control over HA structure but is complex and costly. Emerging plant-based methods, while ethical and environmentally friendly, are still developing with lower yields. The choice of production method at an industrial scale is influenced by factors such as yield, purity, scalability, cost, regulatory requirements, and ethical considerations. Economically, HA has a significant impact due to its wide range of applications and high demand, driving advancements in production methods to reduce costs and improve efficiency. Overall, microbial fermentation is favored for industrial-scale HA production, while plant-based methods hold promise for future advancements.

**Keywords:** Hyaluronic Acid (HA); Biopolymer; Applications; Microbial Fermentation; Animal Tissue Extraction; Yield.

## List of abbreviations

**HA** : Hyaluronic acid

**GlcUA** : Glucuronic Acid

**GlcNAc** : N-Acetylglucosamine

**OA**: Osteoarthritis

**MDa** : Millions of Daltons

**HAS** : Hyaluronan Synthase

**UDP-GlcUA** : Uridine Diphosphate Glucuronic Acid

**UDP-GlcNAc** : Uridine Diphosphate N-Acetylglucosamine

**O-GlcNAc** : O-Linked N-Acetylglucosamine

**ECM** : Extracellular Matrix

**UDP**: Uridine Diphosphate

**DNA**: Deoxyribonucleic acid.

**ATCC**: American type Culture Collection

**KFCC**: Korean type Culture Collection

**PH**: Potential hydrogen.

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# Introduction

### Introduction

Polysaccharides, complex carbohydrates composed of long chains of monosaccharide units, are fundamental to a myriad of biological processes and structural functions in living organisms (Bhatia, 2016). They exhibit either linear or branched structures, with their complexity dictating various functions such as energy storage, structural support, cellular protection, and surface adherence. (Chakraborty et al., 2019). Among the diverse family of polysaccharides, hyaluronic acid (HA) stands out due to its unique structure and significant biological roles. Unlike other polysaccharides that are primarily involved in energy storage or structural support, such as starch and cellulose, hyaluronic acid serves as a critical component of the extracellular matrix in connective tissues. Its distinctive repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine confer remarkable viscoelastic properties, enabling it to retain water and maintain tissue hydration. The study of hyaluronic acid, therefore, offers valuable insights into the broader understanding of polysaccharides and their diverse functional roles within biological systems. This connection highlights the importance of polysaccharides not only in energy storage and structural integrity but also in facilitating complex physiological functions that are essential for health and disease management.

Hyaluronic acid (HA), a naturally occurring glycosaminoglycan, is a critical component of the extracellular matrix in connective tissues throughout the human body. Known for its remarkable capacity to retain water, HA plays a pivotal role in maintaining tissue hydration, elasticity, and overall homeostasis. This polysaccharide is predominantly found in the skin, joints, and eyes, where it contributes to lubrication, structural integrity, and repair processes.

The unique physicochemical properties of hyaluronic acid, such as its high molecular weight and viscoelasticity, have spurred extensive research and application across various fields, including dermatology, orthopedics, and ophthalmology. In the medical and cosmetic industries, HA is celebrated for its versatility and efficacy, manifesting in products ranging from dermal fillers to visco-supplements for osteoarthritis management.

Despite its widespread use, the mechanisms underlying the synthesis, degradation, and precise functions of hyaluronic acid in different tissues remain an active area of investigation. Recent advancements in biotechnology and pharmaceutical sciences have paved the way for innovative HA-based therapies and enhanced delivery systems, aiming to optimize its therapeutic potential and minimize adverse effects.

The objective of this research is to conduct a comprehensive literature review on hyaluronic acid (HA), focusing on its structure, applications, and production pathways. This study

aims to delve into the molecular architecture of HA, examining its unique properties and how they contribute to its diverse uses in medical and cosmetic fields. Furthermore, the research will explore various methods of HA production, including both natural extraction and synthetic approaches, comparing their efficiencies, advantages, and limitations. By synthesizing current knowledge and recent advancements, this work seeks to provide a detailed understanding of hyaluronic acid, highlighting the comparative benefits and drawbacks of different synthesis routes to inform future research and industrial applications.

# **Chapter01.hyaluronic acid**

## **1.1. History and discovery**

The discovery of hyaluronic acid dates back to 1934 when Karl Meyer and John Palmer isolated an unknown substance from the vitreous body of cow eyes. They identified two sugar molecules within the substance, one of which was uronic acid. Naming it "hyaluronic acid" from the Greek word "hyalos" for glass plus uronic acid, they did not anticipate its future significance. Hyaluronic acid's first commercial use came in 1942 when Endre Balazs patented its application as an egg white substitute in bakery products. Its medical debut in human applications occurred in the late 1950s, initially as a vitreous replacement during eye surgeries, sourced from human umbilical cords and later from rooster combs in purified, high molecular weight forms. The chemical structure was elucidated by Karl Meyer and his associates in the 1950s, recognizing its behavior as a salt (sodium hyaluronate) under physiological conditions. In the early 1980s, HA was used to create plastic intraocular lenses for implantation, and it became a major material in ophthalmic surgery. A variety of other applications have since been proposed and developed (Liu et al., 2011). The term "hyaluronan" was coined in 1986 by Endre Balazs to encompass various forms of the molecule, including hyaluronic acid and its salts like sodium hyaluronate. Subsequent studies isolated hyaluronic acid from various sources, leading to comprehensive investigations into its physicochemical properties and biological roles (Necas et al., 2008).

## **1.2. Overview of hyaluronic acid**

Hyaluronic acid (HA), or hyaluronan, is a linear polysaccharide composed of repeating disaccharide units of N-acetyl-d-glucosamine and glucuronic acid. It typically has a high molecular weight, often reaching millions of Daltons, and exhibits unique viscoelastic properties due to its polymeric and polyelectrolyte nature. It is widely present in the connective tissues of vertebrates and offers exceptional physicochemical properties such as biodegradability, biocompatibility, no toxicity, and no immunogenicity. In clinical medicine, it serves as a diagnostic marker for various conditions, including cancer, rheumatoid arthritis, and liver diseases. Additionally, it is used to supplement impaired synovial fluid in arthritic patients through intra-articular injections. HA also finds biomedical applications, including osteoarthritis surgery, ocular surgery, plastic surgery, tissue engineering and drug delivery. Hyaluronic acid plays a crucial role in cushioning and lubricating the body and is abundant in the eyes, joints, and heart valves (Kogan et al., 2007).

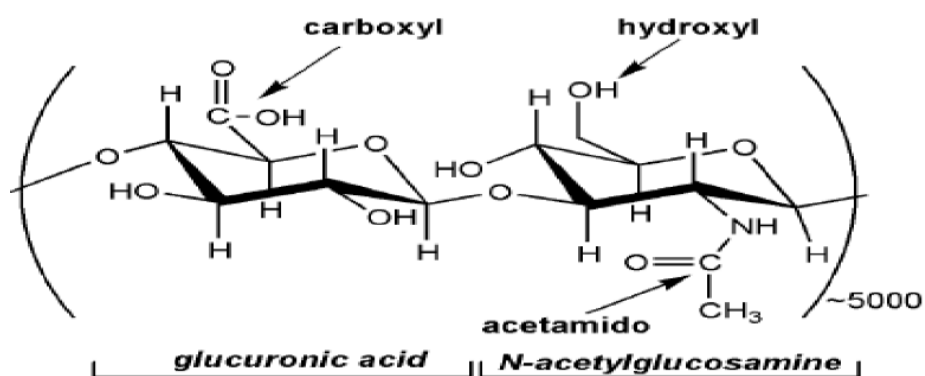
Hyaluronan, a versatile polysaccharide, plays multiple roles in the human body, contributing to the formation of flexible and protective tissue layers. It is involved in various signaling pathways

essential for embryonic development, wound healing, inflammation, and cancer processes. With significant pharmaceutical applications, particularly in treating arthritis and osteoarthritis, its commercial value surpasses that of other microbial extracellular polysaccharides. Traditionally sourced from animal waste, hyaluronan extraction remains a well-established method (Boeriu et al., 2013).

### 1.3. Chemical structure and properties of HA

#### 1.3.1. Molecular structure

Hyaluronic acid, is a linear poly-saccharide composed of a repeating disaccharide unit of  $\beta$  (1,4)-glucuronic acid (GlcUA)- $\beta$ (1,3)-N-acetylglucosamine (GlcNAc) (Figure 1). Both individual carbohydrate residues in hyaluronan adopt the stable chair conformation which determines the conformation of the polymer in solution that is described as an overall random coil structure that may have also highly flexible regions. Nevertheless, in terms of chemical structure, hyaluronan is a simple linear polymer with high molecular mass and exceptional rheological properties. Hyaluronan is a member of the glycosaminoglycans family that includes chondroitin/dermatan sulfate, keratan sulfate and heparin/heparan sulfate each with a characteristic disaccharide-repeating structure of an amino sugar, either glucosamine or galactosamine, and a hexose, either galactose, glucuronic acid, or iduronic acid which can be carboxylated or sulfated. Hyaluronan is the only glycosaminoglycan member that is not sulfated and is not covalently bound to a proteoglycan core protein (Boeriu et al., 2013).



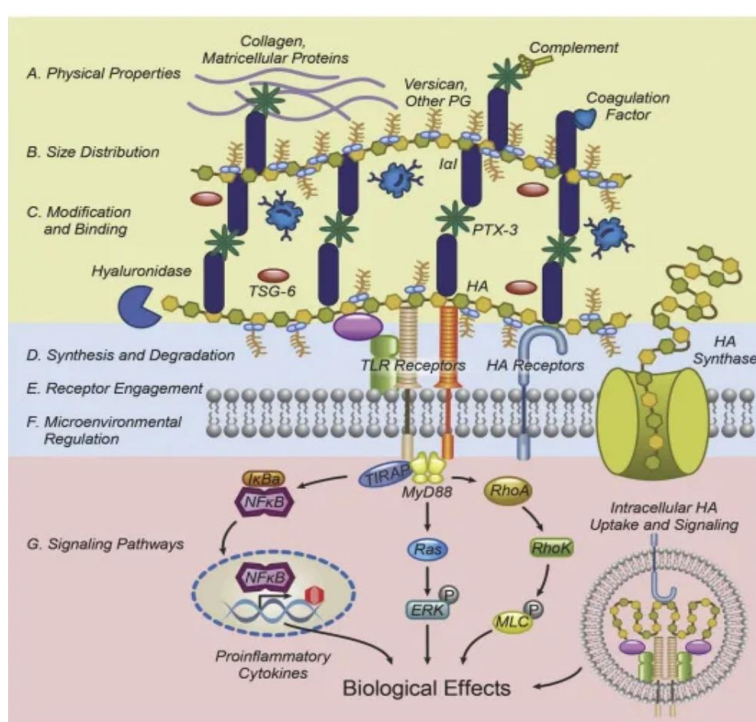
**Figure 1:** Structure of disaccharide repeating unit of HA. (Liu et al., 2011).

#### 1.3.2. Physical and chemical properties

Hyaluronic acid (HA) is a widely distributed polysaccharide found in humans, animals, bacteria, algae, and mollusks. Its simple structure, composed of repeating disaccharide units of N-acetyl-d-glucosamine and glucuronic acid, imparts several distinctive physical and chemical properties that are crucial for its diverse biological and medical applications (Figure 2). In aqueous solutions, HA behaves as a stiffened random coil, leading to its unique rheological properties. Even at low

concentrations, it can form highly viscous solutions, which are useful in medical and cosmetic formulations (Fallacara et al., 2018). These rheological characteristics are largely influenced by the molecular weight of HA, which explains why many of its biological functions depend on its molecular size (Marcellin et al., 2014). HA typically exhibits a high molecular weight, which can range from thousands to several million Daltons. The molecular weight significantly influences its viscosity, viscoelasticity, and biological activity. Higher molecular weight HA tends to form more viscous and elastic solutions, which are beneficial in applications requiring lubrication and space-filling properties (Bukhari et al., 2018).

Hyaluronic acid is a polyanion at physiological pH due to the carboxyl groups on the glucuronic acid residues. This charge density allows HA to interact with a variety of cations, proteins, and other molecules, facilitating its role in cellular signaling and matrix interactions. While HA is generally stable under physiological conditions, it is susceptible to degradation by extreme pH, high temperatures, and oxidative environments. This sensitivity necessitates careful handling and storage conditions to maintain its efficacy (Chang et al., 2021).



**Figure 2.** Function and activity of HA depends on several key mechanisms (Garantziotis et al., 2019)

## **I. 4. Application and uses of HA**

The multifaceted biological roles of HA have sparked renewed interest among biologists and clinicians alike, leading to new avenues of research across various medical fields. These include ophthalmology, joint disorders, wound healing, skin rejuvenation, vascular surgery, adipose tissue engineering, nerve repair and cancer treatment. Pharmaceutical companies are also investing in HA research, leveraging advanced technologies to enhance its properties and prolong its presence in human tissues while boosting its anti-inflammatory effects. Through minor chemical modifications like esterification with benzyl alcohol, water-insoluble polymers of HA have been developed, available in diverse forms such as membranes, gauzes, meshes, gels and tubes. These biomaterials serve as effective wound dressings, anti-adhesive barriers and scaffolds for tissue regeneration, including epidermis, dermis, vascularized skin, cartilage and bone (Abatangelo et al., 2020).

### **I.4.1. Arthritis and osteoarthritis treatment**

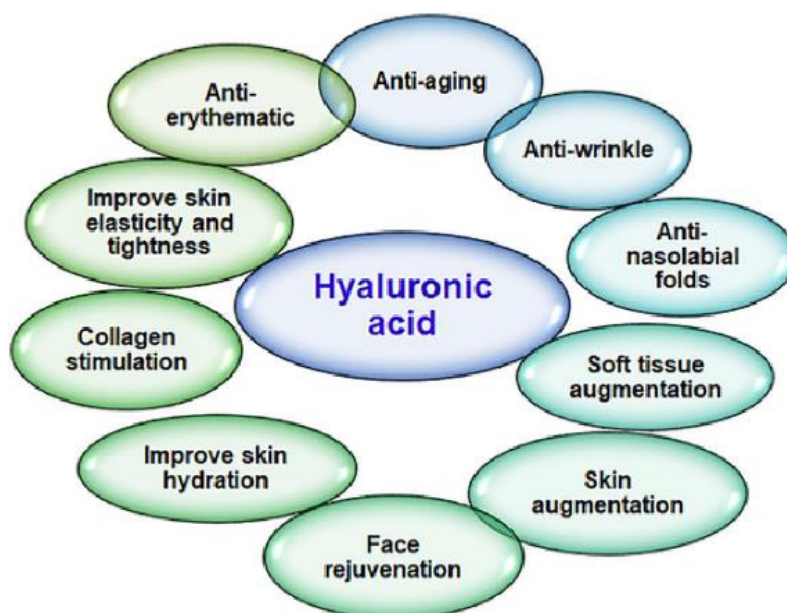
Hyaluronic acid is a natural compound that plays a significant role in treating arthritis by enhancing the health and functions of cartilage in damaged joints. According to research, arthritis patients often experience a deficiency in hyaluronic acid, leading to a loss of flexibility and comfort in movement. Hyaluronan plays a crucial role in regulating inflammation and pain in the joints and understanding how particle size affects these processes could pave the way for targeted and efficient treatments to address this health problem (Cyphert et al., 2015). Studies also indicate the importance of developing delivery systems based on hyaluronic acid to improve drug delivery and reduce side effects enhancing the quality of care for arthritis patients (Walvekar et al., 2024).

Osteoarthritis (OA) is a persistent degenerative condition affecting the joints, leading to pain and gradual loss of function. The use of intra-articular hyaluronic acid, known as visco-supplementation, is

recommended in professional guidelines for knee OA treatment, with potential application to all synovial joints to alleviate pain and enhance joint lubrication. Hyaluronic acid supplementation can stimulate chondrocyte synthesis, protect cartilage from degradation and facilitate its regeneration. Additionally, it can suppress the production of inflammatory mediators and matrix metalloproteinases involved in OA progression (Migliore and Procopio., 2015). Intra-articular hyaluronic acid preparations are recommended for managing pain in knee osteoarthritis patients who haven't responded well to conservative non-pharmacological treatments and basic pain relievers. Clinical studies demonstrate that this therapy leads to notable enhancements in knee pain and functionality, surpassing placebo effects and rivaling the efficacy of non-steroidal anti-inflammatory drugs (Hochberg, 2000).

### 1.4.2 Skincare and cosmetics

Hyaluronic acid (HA) is abundant in the intercellular spaces of the epidermis and connective tissues of the dermis, offering numerous biological benefits (Figure 3). These include retaining water, supporting cellular functions, collagen stimulation, moisturizing anti-aging benefits, regulating skin balance.... (Kuo-Ching Wen et al., 2010; Juncan et al., 2021).

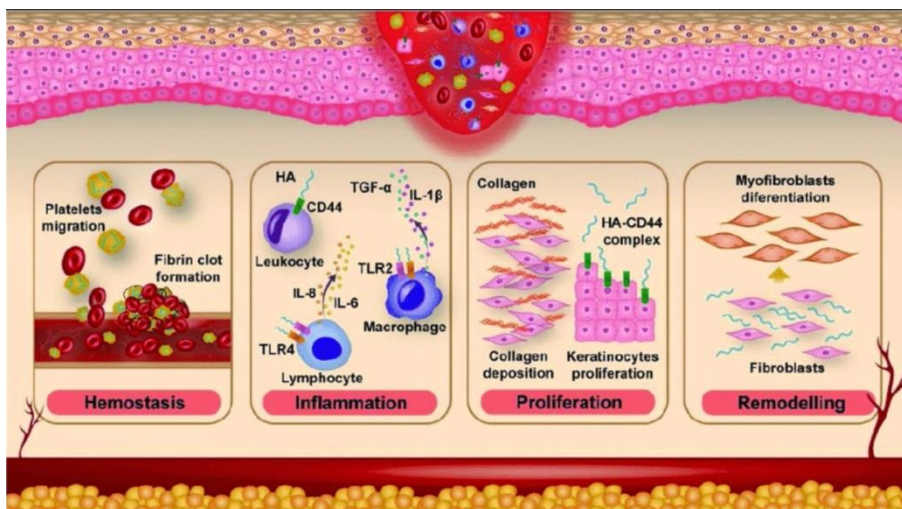


**Figure 3.** Uses of HA and its effects (Bukhari et al., 2018).

### 1.4.3. Tissue regeneration

The natural process of wound healing, which unfolds in four distinct phases: hemostasis, inflammation, proliferation, and remodeling. Successful wound healing relies on the precise orchestration of these phases within the correct timeframe. Various factors can disrupt this process, leading to improper or delayed wound healing (Dipietro, 2010). Enhancing skin repair poses a significant clinical hurdle due to the intricate nature of wound healing. Hydrogels offer promise in wound dressing applications owing to their resemblance to living tissue and advantageous traits like high water content, permeability to oxygen and flexibility. Hence, natural polymers like chitosan, alginate and hyaluronic acid, known for their biocompatibility and non-toxicity, are being explored either individually or in combination with other polymers. Additionally, they are being loaded with drugs, bioactive compounds or nanomaterials to improve their efficacy in wound healing applications (Yuan et al., 2023). The utilization of industrial HA products, such as HA hydrogels, has demonstrated enhanced wound healing outcomes (Figure 4) (Valachova et Soltes, 2021). With its distinctive properties and notable efficacy in treating wounds, particularly

second and third-degree burns and external skin damage, the demand for HA is on the rise in the market (Longinotti, 2014).



**Figure 4.** Effect of Hyaluronic Acid in Different Stages of Wound Treatment (Valachova et Soltes, 2021).

#### I.4.4. Ophthalmology

Hyaluronic acid has emerged as a widely used substance in eye surgeries and the management of eye ailments. HA-based materials are renowned for their high tolerability and exceptional biocompatibility, making HA a highly sought-after ingredient in ophthalmic treatments. Nevertheless, achieving optimal performance and efficacy requires fine-tuning various parameters, given the intricate nature of HA properties, which poses significant challenges for biological and structural characterization (Angeles et Nesporova, 2021). HA is a biocompatible polysaccharide found naturally, known for its distinctive viscoelasticity and ability to retain moisture. Its natural lubricating properties and remarkable water retention make it highly suitable for incorporation into ophthalmic products (Marjori, 2011).

### I.5. The dynamic metabolic and critical roles of HA in the body

Hyaluronic acid, Hyaluronan, a polysaccharide found in vertebrate tissues and body fluids, is also present in certain bacteria. This high molecular weight linear polymer is particularly abundant in loose connective tissue. Synthesized at the cellular plasma membrane, hyaluronan exists in multiple forms: associated with cell surfaces, bound to other matrix components, and as a mobile pool. Specific proteins, known as hyaladherins, recognize its structure, facilitating interactions with proteoglycans to stabilize the matrix structure and with cell surfaces to influence cell behavior. Due to its unique physicochemical properties, hyaluronan serves various physiological functions including lubrication, water regulation, filtration effects, and

plasma protein distribution regulation. In animals and humans, its tissue half-life varies from less than a day to several days, undergoing catabolism through receptor-mediated endocytosis and lysosomal degradation, primarily in lymph nodes. The remaining hyaluronan enters the bloodstream, where it is swiftly removed by the endothelial cells of the liver sinusoids, with a half-life of 2-5 minutes (Fraser et al., 1997).

HA, a key polysaccharide in the extracellular matrix, is crucial for tissue structure organization and the regulation of cellular functions such as proliferation and migration. These roles are facilitated by its interactions with cell-surface receptors and binding molecules. The biosynthesis and degradation of hyaluronan are tightly regulated, which controls its turnover rate, concentration, and molecular size in tissues. Despite its simple chemical structure, hyaluronan's diverse functions are significantly influenced by its varying molecular weights, which determine its specific roles based on tissue concentration.

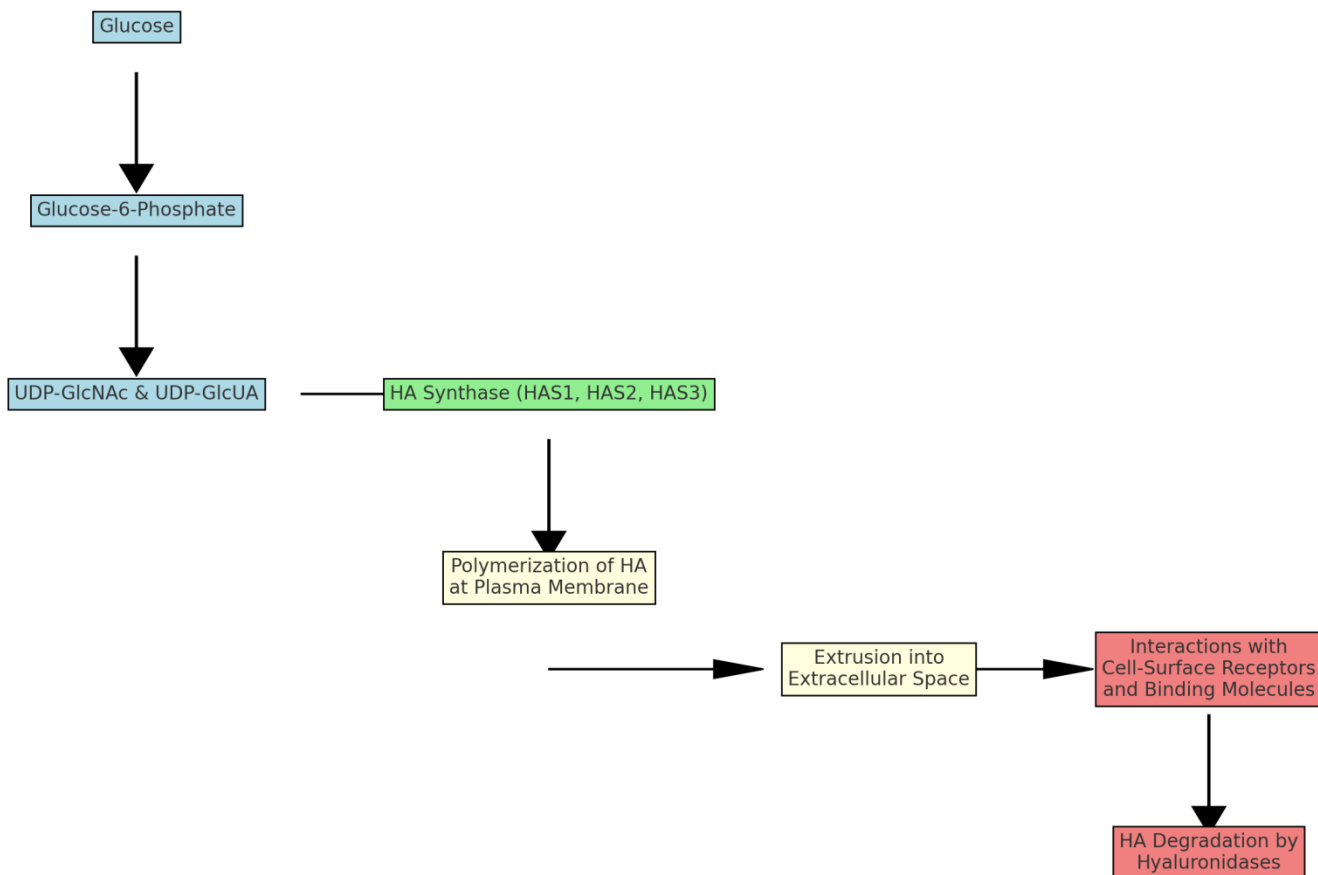
Research involving genetic engineering and pharmacological interventions has demonstrated a close relationship between hyaluronan metabolism and various physiological and pathological processes, including morphogenesis, wound healing, and inflammation. Recent studies indicate that dysregulated expression of hyaluronan synthases and hyaluronidases leads to the accumulation of hyaluronan and its fragments in the extracellular matrix. This accumulation alters the tumor microenvironment, contributing to cancer development and progression.

Moreover, recent metabolomic studies have uncovered that hyaluronan synthesis also plays a role in regulating cellular functions by reprogramming cellular metabolism. This review highlights the current understanding of hyaluronan biosynthesis and catabolism, as well as the diverse functions associated with hyaluronan metabolism (Kobayashi et al., 2020).

# **Chapter II : Production of hyaluronic acid**

### I.1. Biosynthesis pathway of HA

Hyaluronan, a large glycosaminoglycan molecule, is typically produced by enzymes called hyaluronan synthases located on the cell membrane. These enzymes, HA Synthase (HAS1, HAS2, HAS3), sequentially add UDP-GlcNAc and UDP-GlcUA, which are present in the cytoplasm, to elongate the HA chain. The hyaluronan chains are then released into the extracellular space. The metabolism of hyaluronan within cells is constantly active, involving both synthesis and breakdown processes. Interestingly, UDP-GlcNAc, one of the substrates for hyaluronan synthesis, is also utilized by O-GlcNAc transferase, a key regulator of various cellular pathways (Hascall et al., 2014). The HA is found extensively within the extracellular matrix of various mammalian tissues. It plays a pivotal role in both the normal functioning and disorders of these tissues. HA provides an ideal environment for cell migration and proliferation, and it interacts with numerous cellular receptors initiating diverse signal transduction pathways. Precise control over HA synthesis is crucial for ECM organization and cellular processes (Figure 5) (Vigetti et al., 2014).



**Figure 5.** The proposed biosynthetic pathway for hyaluronic acid (O'Regan et al., 1994).

### II.2. Exploring the Diverse Methods of Hyaluronic Acid Production

Hyaluronic acid (HA) is a versatile compound with various production methods tailored to diverse applications. One common method involves microbial fermentation, where specific strains of bacteria or yeast are engineered to produce HA through biotechnological processes. Another approach is through enzymatic synthesis, which utilizes enzymes to catalyze the formation of HA from simpler precursor molecules. Extraction from natural sources such as rooster combs or other animal tissues was once common but is now less favored due to ethical and regulatory concerns. Enzymatic synthesis is still utilized in various applications. This method involves employing enzymes, typically hyaluronan synthases, to catalyze the formation of HA from precursor molecules such as glucuronic acid and N-acetylglucosamine. Additionally, chemical synthesis is also employed, albeit less frequently due to its complexity and higher costs compared to biological methods. Each method offers distinct advantages and limitations, allowing for flexibility in HA production to meet specific quality, quantity, purity, scalability, cost-effectiveness and cost requirements for various industries ranging from cosmetics to pharmaceuticals.

#### II .2.1. Microbial fermentation

Microbial fermentation stands as a pivotal method in the production of hyaluronic acid (HA), harnessing the capabilities of microorganisms like bacteria or yeast to synthesize this valuable compound. In this process, genetically modified strains of bacteria or yeast (*Streptococcus zooepidemicus*, *Escherichia coli*, *Corynebacterium* and *Agrobacterium* sp.) are cultivated in bioreactors under controlled conditions, where they metabolize substrates such as glucose or other carbon sources to produce HA as a byproduct. Microorganisms such as *Streptococcus zooepidemicus*, a Gram-positive bacteria, is one of the most widely used organisms for the production of HA due to its high hyaluronic acid production rate and ease of cultivation (Serra et al., 2023). The table below (table 1), summary some of the bacteria used with the substrat and mode of fermentation. The fermentation process involves precise manipulation of factors like pH, temperature, oxygen levels, and nutrient concentrations to optimize HA yield and quality. This method offers several advantages, including scalability, high purity of the final product, and relatively low production costs compared to other techniques. Moreover, microbial fermentation allows for customization of HA properties by adjusting the genetic makeup of the microorganisms or modifying fermentation conditions, catering to the specific requirements of various industries, from skincare to medical applications (Ferreira et al., 2021a).

**Table 1.** Types of bacteria, substrate and mode of fermentation used to produce hyaluronic acid.

Microorganisms	Substrate	Fermentation mode	Reference
<i>Escherichia coli</i> K12 HA03GlcA	Glucose	Batch fermentation	(Woo et al., 2019)
<i>Agrobacterium</i> sp. ATCC 31749	Glucose	-	(Mao et Chen, 2007)
<i>Streptococcus Zooepidemicus</i> ATCC 39920	Glucose	Batch fermentation	(Chen et al., 2009)
<i>Streptococcus Zooepidemicus</i> KFCC 10830	Glucose	Batch fermentation	(Kim et al., 1996)
<i>Bacillus subtilis</i> A164Δ5	Glucose	-	(Widner et al., 2005)

### II.2.2. Extraction of hyaluronic acid from natural sources

Initial laboratory experiments aimed at exploring the biomedical potential of hyaluronic acid (HA) involved extracting HA from various animal tissues, including pigskin, joint fluid, human and animal umbilical cords, rabbit pericardial fluid, bovine vitreous humor, and shark cartilage. Among these sources, rooster combs, human umbilical cords, and bovine vitreous bodies emerged as promising candidates for large-scale HA production due to their abundant availability and capacity to yield HA with a high molecular weight. Presently, industrial production of HA for medical and pharmaceutical applications predominantly relies on animal-derived sources. However, isolating high molecular weight and pure forms of HA poses challenges due to its coexistence with other polymers in living organisms. Nevertheless, HA's water solubility facilitates its extraction process, rendering it more manageable (O'Regan et al., 1994).

Various methods have been established to disrupt complexes and liberate hyaluronic acid (HA) molecules, including the use of proteases such as papaya proteinase, pepsin, and Actinase E, as well as precipitation techniques involving ion-pairing, carbon-based solvents, or immersion precipitation, among others (Schiraldi et al., 2010). Subsequent to purification, degraded materials and undesirable by-products are removed utilizing ultrafiltration and chromatography methods, while sterile filtration ensures the elimination of microorganisms from the final product (Shiedlin et al., 2004).

Despite meticulous purification processes, hyaluronic acid isolated from animal tissues may still harbor unwanted proteins and DNA contaminants, with the extent of contamination varying depending on the source animal. Research suggests that HA extracted from human umbilical cords or bovine eye vitreous

humor may exhibit a higher concentration of undesired biological macromolecules compared to HA derived from rooster combs or bacterial fermentation (Shiedlin et al., 2004).

### **II.2.3. Enzymatic synthesis using isolated hyaluronan synthases**

Hyaluronic acid (HA) synthases fall into two distinct groups, Class I and Class II, differentiated by their primary sequences and structural characteristics. Class I enzymes, primarily found in Group A and C Streptococci, dominate HA synthase enzymes. These enzymes operate by incorporating UDP substrates into HA biopolymers at the reducing end, closely associated with lipid molecules for synthesis and extrusion of HA outside the cell membrane (Weigel, 2002; Weigel and DeAngelis, 2007). In contrast, Class II enzymes, known as pm HAs, are exclusive to *Pasteurella multocida*, although their genome can be expressed in other microorganisms like *Escherichia coli*. Class II HA synthases, larger in size than Class I, function as amphitropic proteins, not relying on phospholipid bilayers or membrane transport proteins. Class II HAs possess dual catalytic domains for GlcNAc and GlcUA transferase activity, attaching UDP substrates to HA at the non-reducing end (Jing and DeAngelis, 2000). The extraction and purification processes of Class I HAs are intricate due to their lipid dependence, necessitating the use of surface-active agents for solubilization. While feasible, isolation of Class I HAs is not efficiently scalable for industrial application (Tlapak-Simmons et al., 2005). Conversely, Class II HAs, being peripheral membrane proteins, hold promise for commercial-scale HA production. However, optimization is required to enhance final product quantity. Soluble enzymes can be generated by removing membrane regions without affecting enzyme function, enabling in vitro production yielding high molecular mass hyaluronan (1-2 MDa) but with a relatively low final HA yield (Sze et al., 2016).

### **II.2.4. Chemical Synthesis**

Chemical synthesis of hyaluronic acid (HA) typically involves the utilization of monomeric precursors, glucuronic acid, and N-acetylglucosamine. These precursors undergo various chemical reactions to form the glycosidic bonds that constitute the HA polymer chain. Additionally, protecting groups are employed to control the regioselectivity and stereochemistry of these reactions, ensuring the desired structure of the synthesized HA. Catalysts, solvents, and reagents specific to the chosen synthetic route are also used to facilitate the synthesis process and optimize yields. Overall, chemical synthesis of HA requires a combination of precise chemical reactions, protecting group strategies, and suitable reaction conditions to achieve the desired product. (Kogan;Stern.,2007)

### II.2.5. Recombinant DNA Technology

Recombinant DNA technology is another method that is still utilized for the production of hyaluronic acid (HA). This approach involves the use of genetic engineering techniques to introduce genes responsible for HA production into host organisms such as bacteria or yeast. These host organisms then serve as factories to produce HA through the expression of the introduced genes.

Recombinant DNA technology offers several advantages for HA production. Firstly, it allows for the production of HA in large quantities, as the host organisms can be grown in bioreactor systems on a large scale. Secondly, it enables the production of HA with specific properties by modifying the genes or optimizing the fermentation conditions. Additionally, this method can be more cost-effective and environmentally friendly compared to traditional extraction methods from animal tissues. Overall, recombinant DNA technology is a valuable tool for HA production, offering scalability, versatility, and the ability to tailor HA properties to meet specific industrial and medical needs. (Ucm et al.,2022).

### II.3. Problems and challenges of hyaluronic acid production

While significant advancements have been made in the microbial bioproduction of hyaluronan, certain challenges must be addressed. Producers face the task of discovering cost-effective production methods and sourcing raw materials, as the prices of substrates continue to rise. This issue could potentially deter investor interest in commercial HA production. Hence, further research is necessary to identify more economical alternatives to the current raw materials.

The extraction process of hyaluronic acid (HA) from rooster combs is facing increasing scrutiny due to concerns regarding animal-derived components in biomedical and pharmaceutical applications. Consequently, microbial production of HA has emerged as a viable alternative. Molecular weight serves as a crucial quality parameter for commercial HA products, influencing rheological properties, physiological responses, and application suitability. However, the escalating cost of raw materials poses challenges to the competitiveness of microbial HA production, necessitating the exploration of cost-effective substrate alternatives. Additionally, obtaining HA with specific molecular weights or uniform sizes is imperative to broaden its applications and enhance the quality of HA-containing biomedical products. While a model for HA polymerization has been proposed and some key intracellular metabolites influencing molecular weight have been identified, further research is needed to elucidate the mechanisms of molecular weight control (Zhen et al., 2017).

The production of hyaluronic acid faces several challenges, including viscosity constraints that affect process efficiency and the final product volume. Additionally, research and development focus on improving quality standards such as purity and molecular weight of the product. Despite progress in enhancing current strains and processes, the need for innovative methods remains crucial. Metabolic engineering provides new opportunities, as the feasibility of producing hyaluronic acid in unconventional hosts is being explored. With this technology nearing market entry, a deep understanding of internal mechanisms becomes essential to fully realize the potential of metabolic engineering (Chong et al., 2005).

### **I.4. Comparative study of the production methods of hyaluronic acid**

Hyaluronic acid (HA) is produced using several methods, each with distinct advantages and disadvantages. Traditional extraction from animal tissues, such as rooster combs, involves mechanical and chemical processes to isolate HA. This method is well-established and relatively simple but faces ethical concerns, risk of disease transmission, and batch variability.

Microbial fermentation, using bacteria such as *Streptococcus* species or *Bacillus subtilis*, has become popular due to its high yield and scalability. This method offers consistent quality and high purity of HA but requires stringent controls to prevent contamination, particularly with endotoxins.

Genetic engineering leverages genetically modified bacteria or yeast to produce HA through fermentation. This method is noted for its very high purity and reduced risk of contamination. It is customizable for different HA molecular weights, although it comes with high initial setup costs and requires complex technology. Here is a summary table comparing these methods based on recent research findings. (G Boeriu et al.,2013).

## Chapter 02: Production of hyaluronic acid

**Table 2.** Comparative analyse of the production methods of hyaluronic acid

<b>Method</b>	<b>Microbial Fermentation</b>	<b>Animal Tissue Extraction</b>	<b>Recombinant Production</b>
<b>Microorganisms/Source</b>	<i>Streptococcus zooepidemicus</i> , <i>Bacillus subtilis</i>	Rooster combs, bovine vitreous humor	<i>E. coli</i> , yeast (e.g., <i>Pichia pastoris</i> )
<b>Yield</b>	Moderate to high (5-6.7 g/L)	Low to moderate	High (varies with genetic optimization)
<b>Cost</b>	Moderate (can be reduced using by-products)	High (due to processing of animal tissues)	Moderate to high (depends on scale and optimization)
<b>Purity</b>	High (depends on downstream processing)	Lower (requires extensive purification)	Very high (minimal contamination)
<b>Sustainability</b>	High (utilizes renewable resources, waste products)	Low (animal welfare concerns)	High (renewable, can be scaled efficiently)
<b>Scalability</b>	High (industrial fermentation processes)	Low (limited by animal tissue availability)	Very high (bioreactor scalability)
<b>Regulatory Acceptance</b>	High (GRAS status for many microbial sources)	Variable (concerns over animal-derived products)	High (recombinant DNA technology acceptance)
<b>Applications</b>	Medical, cosmetic, food industry	Primarily cosmetic and medical	Medical, cosmetic, food industry

# **Conclusion**

### Conclusion

Hyaluronic acid (HA) finds applications across diverse fields including pharmaceuticals, medicine, aesthetics, dentistry, environmental science, chemistry, and food industries. Its high added value drives the increasing commercial demand for this biopolymer annually. Research into various microorganisms aims to maximize HA production, offering advantages over animal-derived HA by enabling industrial-scale production and eliminating animal proteins, thus reducing the risk of allergic reactions. Consequently, microbial production is the most widely used method.

Bacterial systems are particularly notable for optimizing yield and product quality through controlled growth conditions. Among these, *Streptococcus zooepidemicus* is distinguished for its high HA production capacity, making it a primary focus in research. Producing HA using microorganisms involves a complex process that includes selecting suitable substrates, supplements, and culture conditions. Alternative substrates such as agricultural waste, industrial waste, and synthetic materials can be utilized, with the choice depending on the microorganisms and production scale.

Nutrient sources (carbon, nitrogen and salts) must be provided in appropriate quantities to optimize bioproduction. Additionally, environmental factors such as pH, temperature, agitation, and aeration are crucial for maintaining the microorganism's physiological needs. Supplementing the culture medium with various nutrients and growth factors can significantly influence the HA production rate. Optimal culture conditions, which vary based on the microorganisms used, include specific temperature, pH, aeration, agitation, and substrate concentration parameters.

The fermented configuration also plays a vital role, with its design depending on production scale and the microorganisms involved. Establishing the culture mode is crucial as each mode has unique advantages and disadvantages. Researchers often employ a combination of different culture modes to identify conditions that yield the highest HA production.

This study provides comprehensive information on HA, detailing its history, applications, biosynthesis, and production methods.

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