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Microbial Hyaluronic Acid Production: A Comprehensive Review of Strategies, Challenges and Sustainable Approaches

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Abstract
Medical-grade hyaluronic acid (HA), typically extracted from animal sources or produced by microbial fermentation, offers a wide range of benefits, including wound healing and joint pain relief. However, current methods of extraction from animal tissues, such as eye corneas, umbilical cords and rooster combs, are limited in terms of future raw material availability. Microbial HA production offers several advantages over animal extraction: increased reproducibility and scalability, shorter production times, and yield optimisation through genetic engineering and mutations. This review provides a comprehensive literature survey on microbial HA production, including a comparative analysis of production methods, key challenges and future prospects. Key challenges such as fermentation optimisation, HA purification and contamination are highlighted along with potential solutions. Recent advances and applications of HA in various fields are analysed to understand future opportunities and growth potential. Techno-economic analyses highlight the importance of balancing production costs with desired HA properties. This review aims to provide valuable insights for the development of efficient, sustainable and economical microbial HA production methods and to drive progress in the field. Continued research and development efforts focused on improving fermentation efficiency, downstream processing techniques and host strain engineering are essential to maintain cost effectiveness and scalability in the face of an ever-growing HA market.
Keywords: *hyaluronate acid (HA), fermentation, glukosa, streptococcus zooepidemicus*

Abstrak
HA (Asam Hyaluronat) kelas medis, biasanya diekstraksi dari sumber hewani atau diproduksi melalui fermentasi mikroba, menawarkan berbagai manfaat, termasuk mempercepat penyembuhan luka dan mengatasi nyeri sendi. Namun, metode ekstraksi saat ini dari jaringan hewan seperti kornea mata, tali pusat, dan jengger ayam menghadapi keterbatasan ketersediaan bahan baku di masa depan. Produksi HA mikroba menawarkan beberapa keunggulan dibandingkan ekstraksi dari hewan: peningkatan reproduktifitas dan skalabilitas, waktu produksi lebih singkat dan optimalisasi hasil melalui rekayasa genetika dan mutasi. Tinjauan ini melakukan kajian pustaka komprehensif tentang produksi HA mikroba, meliputi analisis perbandingan metode produksi, tantangan utama dan prospek masa depan. Tantangan utama seperti optimasi fermentasi, pemurnian HA, dan kontaminasi disorot, bersama dengan solusi potensial. Perkembangan terbaru dan aplikasi HA dalam berbagai bidang dianalisis untuk memahami peluang dan potensi pertumbuhan di masa depan. Analisis tekno-ekonomi menekankan pentingnya menyeimbangkan biaya produksi dengan sifat HA yang diinginkan. Tinjauan ini bertujuan untuk memberikan wawasan berharga untuk pengembangan metode produksi HA mikroba yang efisien, berkelanjutan, dan ekonomis, serta mendorong kemajuan di bidang ini. Upaya penelitian dan pengembangan berkelanjutan yang berfokus pada peningkatan efisiensi fermentasi, teknik pemrosesan hilir, dan rekayasa strain inang sangat penting untuk mempertahankan efektivitas biaya dan skalabilitas dalam menghadapi pasar HA yang terus berkembang.
Kata Kunci: *asam hyaluronate (HA), fermentasi, glukosa, streptococcus zooepidemicus*

1. Introduction

Biopolymers are one of the most exciting biotechnology products and a promising class of biomaterials, occurring naturally or produced by microorganisms. These versatile materials are making waves in medicine, food, and even cosmetics. Examples include surgical devices, implants, drug delivery systems; enzyme/cell immobilization carriers; biosensors; diagnostic assay components; bio adhesive devices; and orthopedic materials. Biopolymers can be synthesized with specific chemical, physical, and interfacial biomimetic characteristics, enabling diverse applications and tailoring for specific end-use

requirements [1]. Microbial Hyaluronic Acid (HA) is a product of biopolymers. Biopolymers are essentially long chains of sugar molecules produced by living organisms. Biopolymers are large molecules found in living organisms, constructed from repeating sugar units like beads on a string. HA boasts a unique structure, built upon a repeating pattern of negatively charged sugar units. Remarkably, its molecular weight can reach into the millions, creating a large and impactful molecule. What truly sets HA apart is its three-dimensional shape. Imagine a double helix, the iconic structure of DNA. These distinct regions create a fascinating duality, allowing HA to interact with both water and other molecules within our tissues. However, the shape of HA isn't static. This dynamic characteristic allows HA to play a versatile role in various tissues throughout the body [2].

Hyaluronic Acid (HA) is currently obtained from rooster combs or fermented by microbes. It plays a crucial role in keeping tissues flexible due to its water-loving nature. HA also interacts with cells and influences their behavior. Modified versions of HA, like sulfated HA, show promise in wound healing, bone regeneration, and as implant coatings. Studies suggest oral HA can improve knee pain and function, while cosmetically it acts as a moisturizer and wrinkle reducer. Two techniques exist for producing hyaluronic acid (HA): chemical extraction from animal tissue and microbial fermentation. Each faces unique challenges. Extracting HA from animal tissue requires extensive processing to remove contaminated proteins and other polysaccharides [3]. Additionally, animal-derived HA carries the risk of infection from viruses and other agents. Bypassing animal extraction, microbial fermentation emerges as a promising alternative for hyaluronic acid (HA) production. Leading companies leverage Group C Streptococci, specifically S. equi and S. zooepidemicus, in this bioengineering feat. This five-step bioprocess mimics controlled microbial cultivation: (1) meticulous strain selection and cryopreservation ensure genetic stability; (2) optimized fermentation media provide essential nutrients for robust bacterial growth; (3) rigorous sterilization protocols mitigate contamination risks; (4) the chosen bacterial strain efficiently bioconverts sugars into HA through fermentation; and (5) downstream processing meticulously extracts and purifies the target molecule. This biomanufacturing approach offers a sustainable and potentially safer alternative to animal-derived HA, maximizing purity and minimizing potential zoonotic risks [4]. **Figure 1** illustrates the biomedical applications of hyaluronic acid.

Fig. 1: Biomedical applications of hyaluronic acid [1]

While the future of HA production remains dynamic, microbial fermentation holds immense promise for its scalability, cost-effectiveness, and regulatory compliance, solidifying its position as a frontrunner in the ever-evolving landscape of HA bioengineering. While microbial fermentation offers a promising approach to producing hyaluronic acid (HA), several challenges persist across different stages. Optimizing bacterial strains for efficient HA production might require genetic engineering, raising ethical and regulatory questions. Efficient extraction and purification of HA from fermentation broth while maintaining quality and minimizing losses requires further development. Accurate and sensitive techniques for monitoring and quantifying HA yield and purity throughout the process are essential. This review tackles these challenges head-on, exploring the impact of various factors like substrate concentration, nitrogen content, C/N ratio, aeration, agitation, and bioreactor mode on HA yield and efficiency. By analyzing these

influences, the review seeks to illuminate potential optimization strategies for a more robust and efficient fermentation process [5].

Production history and market

Hyaluronic acid (HA) gets its name from the Greek words "hyalos," meaning glass, and "uronic acid." This term originated in 1934 when Karl Meyer and John Palmer isolated a chemical substance from bovine vitreous humor. Isolation of HA flourished in the 1930s-40s, utilizing diverse sources like umbilical cord, vitreous humor, streptococci, and chicken combs. This groundwork paved the way for future biotechnological advancements in HA production and applications. Notably, Levene and Lopez-Suarez had already been studying polysaccharides from the vitreous body and cord blood since 1918, naming the substance "mucoidin-sulfuric acid. Woven from glucuronic acid and N-acetylglucosamine, HA's intricate biopolymer architecture grants it center stage in the extracellular matrix. This remarkable structure empowers HA to act as a maestro, orchestrating cellular interactions and guiding tissue repair and wound healing. There, it acts as a maestro of cell communication, expertly facilitating complex signaling pathways that guide tissue repair and accelerate wound healing. Its unique properties inspire scientists to unlock its full potential and revolutionize various life science fields [6]. HA's versatility extends beyond its vital roles in the body, with diverse applications in medicine and cosmetics.

Research during the 1950s-60s focused on the physicochemical characterization of HA, leading to the adoption of the modern name "hyaluronan." By the early 1980s, Endre Balazs successfully developed HA from umbilical cord and chicken combs for use as health products in clinical medicine. One significant invention of this era was a plastic intraocular lens made from HA for ophthalmic surgery [7]. **Figure 2** illustrates the hyaluronic acid market size & trends, 2020-2030.

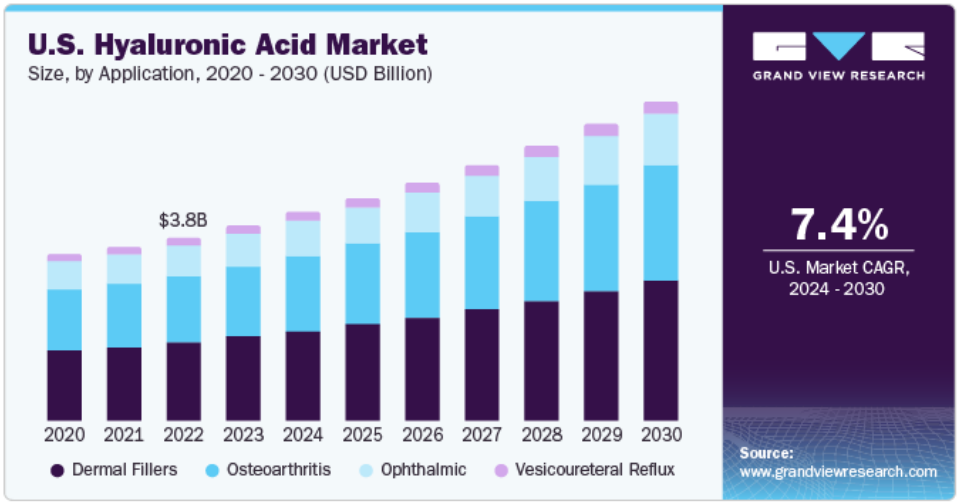

Fig. 2: Hyaluronic Acid Market Size & Trends, 2020-2030 [6]

The journey of hyaluronic acid (HA) began in 1937 when F. Kendall discovered its production potential by isolating it from a streptococcal capsule. In a moment, in 1943, E.A. Balazs and L. Pille unveiled its presence in the knee joints of dogs, highlighting its significance in connective tissues. C. Ragan and K. Mayer further extended this understanding in 1949 by finding HA in rheumatoid synovial fluid, hinting at its role in inflammation. Interestingly, 1942 saw the first commercial application of HA when Endre Balazs proposed it as an alternative to egg whites in bread. Meanwhile, between 1948 and 1951, multiple researchers like A. Dorfman, A.G. Ogston & J.E. Stanier, and E.A. Balazs himself, delved into the structure and properties of HA. They utilized various techniques like fermentation analysis, solution observation, UV light, and even X-ray diffraction to unravel its mysteries. This early groundwork continues to fuel modern research on HA, especially in areas like genetic engineering, which promises exciting future developments in this field [8].

The dependence on animal components in HA production raises ethical and sustainability red flags for biotechnologists. This reliance necessitates exploring alternative, bioderived approaches for a more responsible and sustainable future of HA production. This pressing issue arises due to ethical and sustainability concerns, highlighting the need for alternative methods. Microbial fermentation technology emerges as a promising solution, offering a plethora of advantages. The fermentation process itself boasts a simplified flow, minimizing complexity and ensuring cost-effectiveness. Additionally, the readily

available raw materials and environmentally friendly nature further solidify its appeal. Furthermore, fermented HA achieves both impressive quality and high yields, making it a potent alternative. Research on HA production using microbial fermentation began in the 1980s and continues to see rapid growth (Huang et al., 2006; Johns et al., 1994). While Streptococcus currently serves as the most common microbial host, several challenges impede progress. These hurdles include the presence of interfering pathogenic genes within the host, increased broth viscosity hindering oxygen transport, decreased dissolved oxygen limiting microbial growth, and the presence of unwanted by-products requiring additional purification steps. Addressing these challenges represents the next frontier in unlocking the full potential of microbial fermentation for HA production [9].

Material-based engineering products such as HA have played an important role in the global market. Viscos supplementation, an injectable HA treatment, has received positive reception since its U.S. debut in 2009, fueling a yearly demand increase exceeding 15%. After entering the European market in 2013, viscos supplementation gained significant traction in Asia-Pacific, with Japan exceeding US$300 million in market value. Notably, Seikagaku (Tokyo) treated 2 million patients with 14 million injections annually in Japan. While Bio-Technology General Corporation and Genzyme offer HA products in the U.S., Q-Med's microbial-based HA holds an advantage over Genzyme's animal-derived options [10].

2. Production pathway and structural characteristics

Hyaluronic Acid (HA) possesses a fundamentally modular structure built upon repeating subunits called disaccharide units. We can visualize these disaccharides as identical building blocks that interconnect to form a long chain, ultimately constituting the entire HA structure. This specific architecture endows HA with unique properties that are highly valuable in various engineering applications [13]. **Figure 3**(a) depicts the foundational unit, the disaccharide. **Figure 3**(b) offers a magnified view of a slightly larger unit, the tetrasaccharide. As illustrated in **Figure 3**, the key to HA's remarkable properties, such as lubrication and hydration, lies within its intricate, modular design. These strategically positioned groups act as microscopic water magnets, scientifically termed hydrophilic groups. Their affinity for water molecules allows HA to attract and retain them effectively. This remarkable capability underpins HA's exceptional lubrication and hydration abilities, making it a valuable material in various engineering applications. By leveraging this profound understanding of HA's molecular structure, engineers can unlock a world of potential. Tailored biomaterials with diverse properties can be designed.

The water-filled gel matrix can act as a reservoir, gradually releasing the drug molecules over time. Beyond these specific applications, HA's unique structure with its remarkable water-attracting properties allows it to function in various ways. Joint Lubrication HA forms a cushion-like gel within joints, reducing friction and wear during movement. It's important to note that the length of HA chains plays a significant role. These chains vary in size, influencing their physical properties and specific functions within different tissues. Shorter HA chains are typically more mobile and contribute to lubrication, while longer chains form more viscous gels suitable for scaffolding applications [11].

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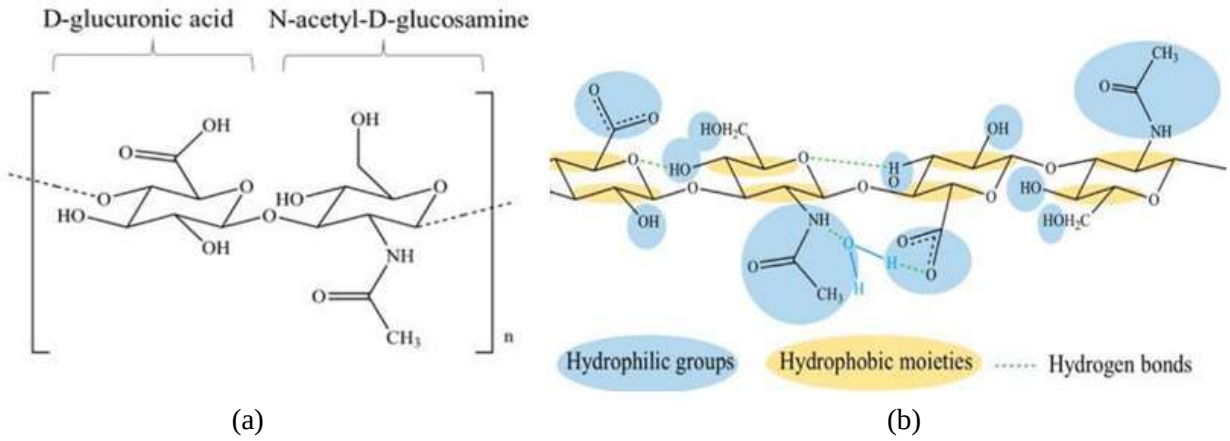

Fig. 3: (a) Chemical structure of the disaccharide unit HA and (b) tetrasaccharide unit HA, which are hydrophilic, hydrophobic, and hydrogen bond functional groups [16].

However, to achieve consistently high HA yields, high-molecular-weight HA without animal-derived impurities remains a key challenge. Researchers are actively addressing this through improved purification and standardization techniques. Additionally, utilizing diverse lignocellulosic feedstocks as a sustainable food source for HA-producing microbes holds significant potential. The scalability and efficiency of microbial production compared to traditional sources like animal tissues is a major advantage. Complex separation steps required for animal-derived HA are eliminated, streamlining the process. Current microbial HA production heavily relies on the bacterium Streptococcus zooepidemicus. This microbe employs a fascinating two-pathway strategy to synthesize the precursor molecule for HA.

Pathway one begins with the readily available sugar molecule, glucose. We can visualize UDP-glucuronic acid as a specialized building block, prepared for assembly with its partner, N-acetyl glucosamine, to form the foundation of the HA polymer. The vast diversity within the Streptococci genus offers exciting possibilities. By investigating alternative species, researchers might unlock strains with potentially optimized HA production capabilities or tailored properties. This exploration could lead to the discovery of microbes that produce HA with specific molecular weight distributions or enhanced biocompatibility, catering to specific engineering applications[13].

Microbial Engineering of Hyaluronic Acid

To achieve large-scale (mass) production remains a significant challenge due to critical limitations associated with the chosen microorganisms. By introducing essential genes responsible for HA synthesis directly into the microbial genome, this technique empowers the microorganisms to produce HA more efficiently. Studies have demonstrated remarkable improvements in HA yield using these techniques In some instances, researchers have achieved a sevenfold increase in HA production. This approach essentially diverts the building blocks utilized for cellular energy production towards the synthesis of HA. Despite these advancements, a key obstacle persists: achieving consistent HA molecule length distribution. The current methods struggle to ensure that the produced HA molecules possess a relatively uniform length. Another significant challenge arises from the high viscosity of the culture medium when HA concentration surpasses 4 grams per liter (g/L). This shift in metabolism diverts the carbon flux away from HA production and towards biomass generation, resulting in the formation of undesirable byproducts like lactate [14].

Despite progress in microbial HA production, further advancements are needed for commercial viability. A key challenge is achieving high HA titers (concentrations) while maintaining the desired, molecular weight comparable to animal-derived sources. This is important because molecular weight affects functionality. Focusing on standardization is also important. Here, researchers can explore using diverse, cost-effective lignocellulosic feedstocks like agricultural residues for microbial growth. These abundant, renewable resources require pretreatment to break down their structure and make accessible sugars for HA biosynthesis. Developing such pretreatment technologies is crucial for sustainable production. Fortunately, microbial HA production offers distinct advantages. Microorganisms can rapidly proliferate under controlled conditions, enabling large-scale, consistent production. This contrasts with animal sources like bovine vitreous humor or rooster combs, where complex separation of HA from proteoglycans limits scalability. Furthermore, microbial fermentation offers a relatively simple and controllable process flow compared to animal-derived HA, facilitating optimization for yield, quality, and

consistency [15]. **Figure 4** explains the schematic biosynthesis of hyaluronic acid with different molecular weights and its biomedical applications.

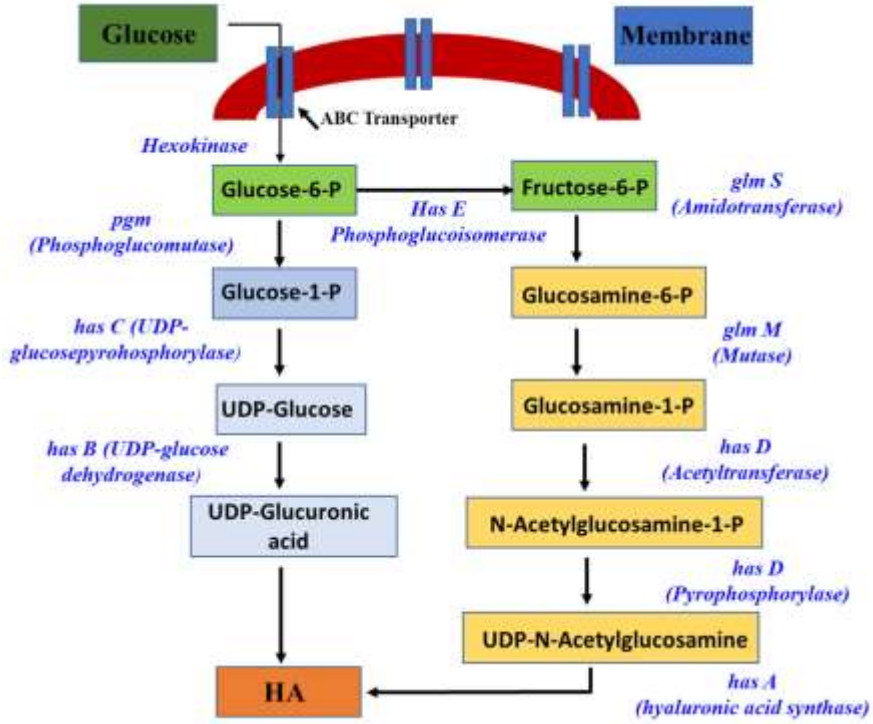

Fig. 4: The schematic biosynthesis of hyaluronic acid, pathways and reproduced [23]

Hyaluronic acid (HA) production primarily relies on specific bacterial strains, particularly Streptococcus equi subsp. Zooepidemicus. This gram-positive bacterium is remarkably diverse, encompassing 49 species and eight subspecies. Notably, classification within Streptococci relies on the Lancefield grouping system, differentiating based on cell wall carbohydrate antigens. Research on prokaryotic HA synthesis heavily focuses on S. zooepidemicus. This microorganism employs two distinct pathways to generate the HA precursor. Glc-6-P serves as the critical starting point for HA biosynthesis. The first pathway involves glucuronic acid production. Finally, UDP-glucose dehydrogenase (hasB) oxidizes UDP-glucose, generating the first HA precursor, UDP-glucuronic acid. This explanation highlights the key role of S. zooepidemicus and its specific metabolic pathways in HA production. Understanding these mechanisms is crucial for optimizing and manipulating microbial HA production for various industrial and biomedical applications [16].

Zooepidemicus utilizes a second pathway alongside the glucuronic acid route to generate the essential building block for HA: N-acetyl glucosamine. Next, the enzyme amidotransferase (glmS) modifies Fru-6-P by attaching an amido group. The final step involves the transformation of GlcN-1-P into the second HA precursor, UDP-N-acetyl glucosamine. This crucial enzyme polymerizes the two building blocks in an alternating fashion, ultimately forming the hyaluronic acid polymer. Many of the intermediates generated during HA production are also utilized for constructing biomass, cell walls, and lactate through glycolysis. A thorough understanding of these microbial biosynthetic pathways is vital for optimizing fermentation technologies. By understanding the metabolic pathways, engineers can determine the exact nutrients required for efficient HA production and optimize their addition to the fermentation medium. Real-time monitoring of specific metabolites and intermediates within the pathway provides valuable insights into the fermentation process. This allows for adjustments to optimize HA yield and ensure consistent production that meets market demands [17].

3. The Evolution of Fermentation Technology

Microbial fermentation plays a critical role in HA production using Streptococcus equi subsp. Zooepidemicus. Researchers have explored various fermentation modes, including batch, fed-batch, and continuous cultures, to optimize HA yield and quality. A key challenge in batch fermentation is the influence of specific growth rate on metabolic products. Continuous and fed-batch modes offer greater

control over this parameter, allowing for targeted manipulation to achieve higher HA production. This approach reduces fermentation time while simultaneously enhancing HA yield. Continuous fermentation offers distinct advantages for HA production. It facilitates the extension of the cell growth cycle, minimizing waste generation due to faster fermenter response times. Additionally, continuous cultures often result in a narrower distribution of HA molecular weight (MW polydispersity). HA chain elongation primarily occurs in the first half of fermentation. Recognizing this, researchers have adopted a two-stage fermentation strategy with segmented control to optimize HA production. This approach involves maintaining specific temperature (31°C) and pH (8.0) conditions during the initial stage to promote high MW HA formation. This two-stage approach has demonstrated success in achieving high HA titers (concentrations) [18, 27].

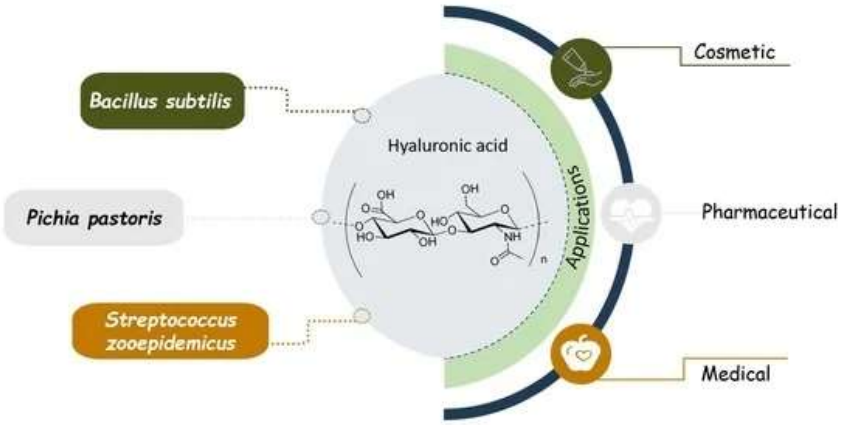

Fig. 5: Microbial Hyaluronic Acid Production [27]

Figure 4 depicts the metabolic pathways involved in bacterial HA biosynthesis meaning it has all the genes necessary for HA synthesis naturally encoded within its genome. Current industrial-scale HA production, exceeding 1 million Daltons (MDa) in molecular weight, relies on either animal tissue extraction or genetically modified bacterial fermentation. The HA obtained from both methods finds applications in cosmetics and biomedicine. However, animal tissue extraction presents drawbacks. Firstly, it carries the risk of contamination with viruses, potentially leading to compatibility issues in downstream processing (DSP). Microbial fermentation for HA production initially employed Streptococcus groups C and A. However, these strains posed challenges due to the co-production of toxins as byproducts. The next generation of bacterial HA production utilized genetically modified Gram-positive bacteria with inserted HA biosynthetic pathway genes. Currently, Bacillus subtilis serves as the preferred chassis organism for fermentative HA production. While promising at small scale, genetically modified bacterial systems for HA production face limitations when scaled up to large fermenters. Further increasing production encounters a bottleneck: high media viscosity. As HA concentration increases, the culture medium becomes increasingly viscous, hindering mass transfer and mixing efficiency within the fermenter, ultimately limiting production capacity. An additional challenge lies in achieving monodisperse (uniform molecular weight) HA in bacterial fermentation. This characteristic is highly dependent on maintaining optimal culture conditions throughout the production process [19].

Current limitations associated with mass production of HA, from both animal and bacterial sources, have spurred interest in cell-free production systems (in vitro) utilizing hyaluronan synthase enzymes (HAS). However, Class-I HAS enzymes present challenges. Being integral membrane proteins, they require complex isolation procedures and lose functionality when separated from the phospholipid layer. Therefore, researchers are focusing on Class-II HAS enzymes from P. multocida, which are peripherally bound and more suitable for in vitro HA production at a commercial scale. By deleting specific residues corresponding to the membrane anchoring domain (residues 704-972) of Class-II HAS, scientists have engineered a soluble enzyme, pmHAS1-703, capable of HA synthesis. This cell-free approach using Class-II HAS enzymes offers significant advantages. Furthermore, by adding HA oligomers to the reaction mixture, processability can be further tuned. These oligomers act as primers, bypassing the rate-limiting step of initial glycosidic linkage formation. This approach facilitates rapid chain elongation and high MW HA production, but may limit overall yield. To address yield limitations, researchers are exploring the cloning of Class-I or Class-II HAS enzyme-encoding genes into non-pathogenic host bacteria like E. coli. These efforts aim to leverage the potential synergy between Class-I and Class-II HAS enzymes within a bacterial

expression system, potentially leading to more efficient HA polymer elongation and improved yields [20]. **Figure 6** illustrates schematic models of exploring the cloning of Class-I or Class-II HAS enzyme-encoding genes.

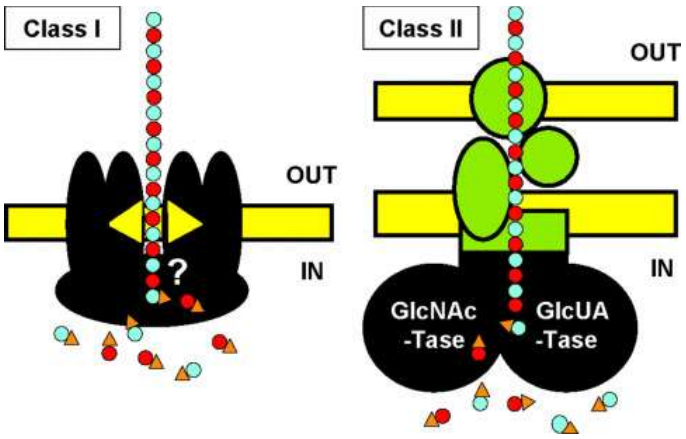

Fig. 6: Schematic models of class I and II hass [29]

The emergence of synthetic biology offers a greener approach to producing high-value HA. This approach involves constructing HA biosynthesis pathways within recombinant microbes using fundamental molecular components. Researchers have utilized genome-scale models (GEMs) to analyze the entire metabolic network of HA production in recombinant Lactococcus lactis. This approach, combined with the consideration of inosine-based HA synthesis pathways, has led to a threefold enhancement in HA titers. These findings highlight the crucial role of well-constructed GEMs in further optimizing HA biosynthesis. A recent advancement in synthetic biology employs rational design to create biosynthetic pathways. This technique, implemented in Bacillus subtilis, has achieved the production of uniform HA with a titer of 6.8 g/L. This method relies on a two-stage induction strategy, where two artificial operons are introduced: one carrying the P. multocida hasA gene and the other containing precursor gene (hasB and hasC) expression cassettes. Another synthetic biology approach focuses on enhancing the flux of UDP-GlcNAc, a crucial precursor for HA production. By upregulating the glucose-6-phosphate isomerase (pgi) enzyme in S. zooepidemicus, researchers were able to achieve a two-fold increase in HA yields, highlighting the potential of this strategy.

Carbon flux redirection represents another powerful tool in the synthetic biology toolbox. By strategically inhibiting other metabolic pathways competing for resources, researchers can reroute carbon flux towards HA production. For example, successful HA production has been achieved by reducing the expression of glycolytic pathway enzymes while ensuring the basic physiological needs of the bacteria are met. The most successful synthetic biology approaches combine multiple genetic strategies. These may include targeted overexpression of pathway enzymes, attenuation using antisense RNA, and pathway knockouts, along with additional promoter inclusions. Such strategies have been reported to achieve impressive HA titers of up to 28.7 g/L. It's important to note that most successful reports using synthetic biology approaches have been conducted at the lab-scale. The next crucial step is to scale-up these processes and develop robust microbial strains equipped with synthetic biology machinery for efficient HA production. Furthermore, integrating HA production into a biorefinery context presents exciting possibilities. Additionally, advancements in fermentation technologies and downstream processing hold promise for further increasing HA yields and expanding its potential industrial applications [21, 29].

4. Downstream Strategies for HA Recovery

Downstream processing and purification are critical bottlenecks in achieving high molecular weight (MW) and purity for commercial-grade HA. While most reported purification methods have been demonstrated at the lab scale, a few studies have explored large-scale simulations using isopropanol as the primary precipitating agent [22]. The precipitation of HA shares similarities with protein precipitation using organic solvents. This phenomenon leads to increased HA chain aggregation and subsequent precipitation. Laboratory-scale protocols often involve a three-step approach: Capsular HA liberation: This typically employs a detergent, such as sodium dodecyl sulfate (SDS), to release HA from the bacterial capsule. HA precipitation: Cold ethanol is commonly used for this purpose. HA fractionation: Gel filtration

chromatography is frequently employed to separate HA molecules based on their varying molecular weights. This complex is then precipitated by centrifugation, followed by redissolution and treatment with cold ethanol for final HA purification [23]. **Figure 7** illustrates downstream process intensification for biotechnologically generated hyaluronic acid.

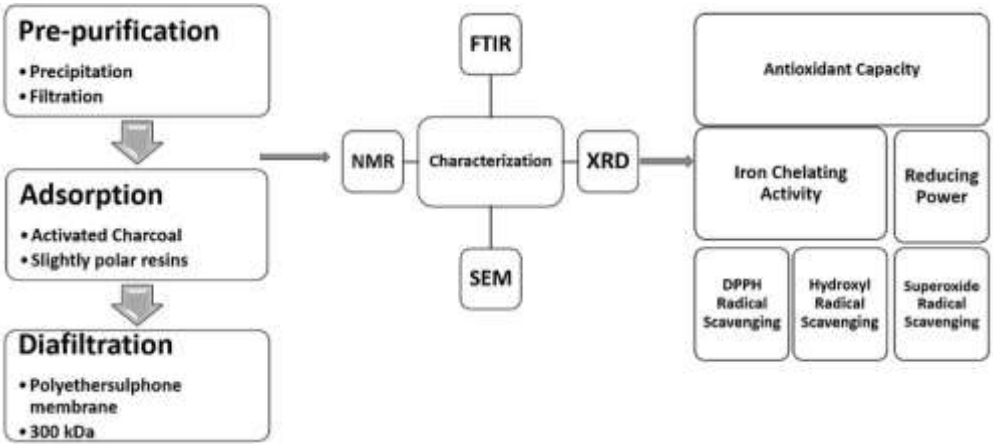

Fig. 7: Schematic process intensification for generated hyaluronic acid [28]

While traditional purification methods often rely on expensive chromatographic techniques, alternative approaches hold promise for large-scale HA production. A study proposed a novel strategy that integrates fed-batch fermentation with a simplified recovery/purification process. The UF-DF step utilizes a 100 kDa membrane to effectively remove impurities like salts, sugars, and peptides from the extracellular broth containing the HA. This diafiltration mode allows for efficient separation of these low molecular weight components from the high molecular weight HA. Following UF-DF, TFF serves a critical role in product concentration. By reducing the overall process volume, TFF facilitates further purification steps and simplifies downstream processing. This combined approach, utilizing fed-batch fermentation and a streamlined UF-DF/TFF purification process, offers a promising path towards achieving high-quality and high molecular weight HA at an industrial scale. The resulting purified HA serves as a valuable commodity with diverse applications across various sectors, including pharmaceuticals, medicine, biomaterials, and cosmetics [24].

5. Industrial scenario of HA

In industrial HA production, the key challenge lies in balancing production cost, product yield, and environmental impact of the fermentation process. While high yields are desirable, achieving the stringent purity levels required for consumer markets necessitates robust purification techniques like ultrafiltration, diafiltration, and activated carbon adsorption. These methods, as evidenced by recent studies, significantly contribute to overall production costs. Furthermore, the removal of endogenous toxins produced by Streptococci strains during fermentation is crucial to ensure both human health and environmental safety. Hyaluronic acid (HA) production is driven by market demands, with the primary application dictated by its molecular weight. To cater to the needs of the biomedical and cosmetic industries, HA formulations are engineered to enhance product efficacy and user experience. These engineered HA products, characterized by their ability to impart viscosity to human tissues and ease of use, are experiencing significant growth in the healthcare sector due to their potential applications in treating osteoarthritis, managing cancer progression, and even tissue regeneration for a perceived anti-aging effect [25].

Market-driven engineering tailors HA production for specific healthcare applications. Hyaluronic acid (HA) production is strategically targeted based on its molecular weight, a key factor determining its primary function. To meet the demands of the biomedical and cosmetic industries, HA formulations are engineered to optimize product efficacy and user experience. These engineered HA products offer several advantages: they impart essential viscosity to human tissues and boast ease of use for desired effectiveness. This focus on targeted engineering has fueled significant growth in HA's healthcare applications, including treatment for osteoarthritis, potential management of cancer progression, and even exploration in tissue regeneration for its perceived anti-aging properties. China stands out as a major consumer, accounting for a staggering 430 Metric Tons (MT) in 2018, exceeding 80% of the global market share. Furthermore,

application projections in the United States suggest a potential market size of USD 2.18 billion for HA within the dermatological sector by 2024, highlighting a significant area of growth [26].

Traditional HA production methods pose challenges for large-scale, controlled manufacturing. While this method focuses on increasing concentration, purity, and yield, it presents limitations for industrial applications. The official adoption of animal-derived HA in 1979, as evidenced by Balazs' patent, relied on a multi-step process with grinding, acid treatment, and organic solvent extractions. This approach suffers from a lack of control over key parameters, leading to potential losses in size homogeneity and yield. Additionally, the use of animal tissues introduces contamination risks, including HA-binding proteins, genetic material, and even disease vectors. These limitations necessitate the exploration of alternative production methods for a more robust and controlled industrial-scale HA manufacturing process [27].

The microbiological route leverages microorganisms for HA biosynthesis, with Streptococci groups A and C being well-acknowledged for their inherent HA production abilities. Shiseido achieved a breakthrough in industrial-scale HA production using this method in 1980. S. zooepidemicus strains were commonly employed, achieving productivities of up to 7 grams per liter (g/L) under optimal fermentation conditions. However, these strains present a challenge due to their production of various toxins. Recent advancements involve genetically modified microorganisms engineered to express the pmHAS gene, offering a potentially safer and more targeted approach for HA production. Chemical synthesis of HA typically employs a preactivation-based chemoselective glycosylation strategy to produce disaccharide, hexasaccharide, and decasaccharide units. This intricate process utilizes two key building blocks with specific functional groups to facilitate bond formation and control reactivity. However, to achieve precise control over glycosylation, glucuronic acid introduction, and protective group management for glucosamine moieties remains paramount to avoid undesired modifications during sugar sequence elongation [28].

Current research emphasizes simulation and techno-economic analysis to optimize industrial HA production, as evidenced by various publications. One study investigated the influence of raw material sources and extraction methods on HA yield, highlighting the potential of animal byproducts and waste streams as cost-effective feedstocks for glycosaminoglycan (GAG) extraction. Another study identified downstream processing, such as tangential-flow filtration and polishing steps, as a significant contributor to production costs. Interestingly, research by a third group suggests that genetically modified microorganisms engineered for HA production via batch fermentation offer a potentially more economical approach within the microbiological route. This implies that genetic engineering holds promise for streamlining production processes and improving the overall economic feasibility of HA manufacturing [29].

Techno-economic aspects of HA production

In medical contexts, injectable HA for conditions like osteoarthritis necessitates extremely high purity. This purity requirement drives prices up to 30 times higher compared to HA intended for topical or dietary use. Data from the US International Trade Commission and scientific literature suggests an average selling price of $2,000 per kilogram for "topical" HA and a staggering $50,000 per kilogram for "injectable" HA. This significant price disparity highlights the critical link between HA purity and its designated function within the medical and cosmetic engineering industries. Techno-economic analyses of HA production primarily focused on simulations using wild-type Streptococci sp. Some studies, however, explored recombinant microorganisms (scenarios BS1 and BS2 in Table 4). Notably, specific scenarios (B, S3, S4) aimed to simulate production of high-purity injectable HA, reflecting the economic impact of product quality on process design [30].

Techno-economic analyses of HA production processes revealed positive net present value (NPV) for all evaluated scenarios, with BS2, B, and SE1 being particularly attractive (reference sources mentioned). Interestingly, scenario BS2, despite boasting the highest NPV, also required the greatest initial investment due to its reliance on recombinant strains. This highlights the current economic disadvantage of recombinant technology for HA production. While all analyzed processes, except S1, demonstrated high internal rates of return, many studies recommend prioritizing fed-batch fermentation with wild-type Streptococci strains. Notably, the Bacillus subtilis 3NA recombinant strain displayed a promising economic profile, suggesting its potential future viability. Additionally, incorporating reagent recycling strategies is highly encouraged wherever feasible. In conclusion, the market value of HA and the efficiency of its recovery process remain the primary factors influencing the overall economic viability of HA production [31].

This versatile biomolecule's growing presence in cosmetics, pharmaceuticals, and regenerative medicine underscores its significance in biomedical engineering. However, this key component of the Streptococcus group A capsule (potentially linked to virulence) presents production challenges. To address

this and meet growing demand, research is exploring renewable carbon feedstocks as a sustainable and cost-effective carbon/nitrogen source for fermentation. This approach would require careful selection of lignocellulosic biomass, methods for obtaining second-generation sugars (2G sugars), and appropriate microbial cultivation and downstream processing strategies to achieve desired HA properties and optimal recovery. At the industrial scale, cost reduction strategies involve using isopropanol as a solvent. This is followed by ultrafiltration (with a 100 kDa cutoff) and activated carbon treatment to obtain HA with a lower molecular weight. Techno-economic analyses emphasize fed-batch fermentation using wild-type strains with solvent recycling as the most cost-effective and potentially sustainable approach for large-scale HA production [32].

Future perspectives

Traditional methods for producing HA involve extracting it from animal tissues, such as rooster combs and bovine eyes. Microbial production of HA offers a sustainable and scalable alternative to traditional methods. Microbial production methods can be tightly controlled to ensure the production of high-quality HA. The cost of producing HA by microbial methods is still too high to be competitive with traditional methods. Fermentation processes for HA production need to be optimized to improve yields and reduce costs. Despite these challenges, the future of microbial HA production is bright. As research continues, these challenges are likely to be overcome, and microbial production will become the dominant method for producing HA. Metabolic engineering can be used to improve the yields of HA from microbial production. Bioprocess engineering can be used to optimize fermentation processes for HA production. These advances will make microbial HA production even more sustainable, scalable, and versatile. The potential applications of HA are vast and still being explored [31-32].

The economic viability of HA production hinges on both its market value and the efficiency of downstream processing for recovery. Although the use of existing methods continues, progress is needed to meet increasing market demand. Creating robust, industrial host strains through a combination of genomics and metabolic engineering holds promise for tailoring HA production to diverse industrial applications. Synthetic biology offers a potentially more sustainable and economical alternative to traditional Streptococci fermentation and animal-derived extraction. Firstly, engineering robust host strains optimized for HA yield and specific functionalities is crucial. Secondly, manipulating bacterial cell size via synthetic biology presents an intriguing solution. Overexpression of genes encoding cell division inhibitor proteins can lead to larger intracellular volumes, facilitating greater HA accumulation, which simplifies downstream processing and recovery.

Furthermore, synthetic biology can be harnessed to engineer specific molecular weight (MW) HA oligosaccharides, catering to targeted industrial needs. Since downstream processing efficiency significantly impacts HA MW and purity, traditional methods require integration with advanced physical, chemical, and membrane technologies. This is essential for handling the highly viscous, non-Newtonian broths and overcoming deproteinization challenges associated with obtaining high MW, pure HA. Although mature industrial production technologies exist, the burgeoning market, projected sales volume increase, and expanding application landscape necessitate more efficient processes and enhanced HA productivity. In the future, perspectives of microbial HA production include the development of safe hyaluronic acid producer microorganisms, optimization of fermentation processes, and the use of alternative culture media to maintain low production costs [32-33].

6. Conclusion

This review has comprehensively explored various aspects of HA production, highlighting the need for innovative engineering approaches to address these challenges and secure a sustainable future for HA. Microbial fermentation, particularly using genetically modified organisms, offers a promising alternative to traditional extraction methods from animal tissues. Metabolic and genomic engineering strategies can be employed to create robust industrial host strains optimized for HA yield and tailored for specific functionalities. Synthetic biology presents a powerful tool for further advancements. By manipulating bacterial cell size through overexpression of cell division inhibitor proteins, larger intracellular volumes can be achieved, facilitating greater HA accumulation and simplifying downstream processing. Additionally, synthetic biology can be harnessed to engineer specific MW HA oligosaccharides, catering to targeted industrial needs.

The evolution of fermentation technology plays a crucial role in optimizing HA production. Fed-batch cultivation strategies offer significant advantages over traditional batch processes, leading to improved HA titers and reduced production costs. Downstream processing remains a critical bottleneck, with the efficiency of HA recovery directly impacting its MW and purity. Techno-economic analyses

emphasize the importance of balancing production costs with desired HA properties. While wild-type Streptococci strains remain the workhorse for industrial HA production, recombinant strains and alternative feedstocks derived from renewable carbon sources hold promise for future sustainability and cost reduction. Continuous research and development efforts focused on improving fermentation efficiency, downstream processing techniques, and host strain engineering are vital for maintaining cost-effectiveness and scalability in the face of the ever-growing HA market. In conclusion, engineering a sustainable future for HA production necessitates a multi-pronged approach. Leveraging advancements in microbial engineering, synthetic biology, fermentation technology, and efficient downstream processing holds the key to overcoming current limitations in HA production.

7. References

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