Biointerface Research in Applied Chemistry
Open-Access Journal (ISSN: 2069-5837)

Article
Volume 13, Issue 3, 2023, 211
https://doi.org/10.33263/BRIAC133.211

Hyaluronic Acid – from Production to Application: A Review

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Scopus Author ID 35323468700
Received: 16.04.2022; Accepted: 16.05.2022; Published: 5.06.2022

Abstract: Hyaluronic acid (HA) was traditionally obtained by extraction from animal sources, specifically from rooster combs. However, this method of obtaining it has several disadvantages. Therefore, the number of researches has increased, and several microbial strains have been studied to synthesize HA. In this review, the characteristics of hearing aids were addressed; the microorganisms involved in the production of HA, operations for HA recovery and purification, and HA applications. Several species of microorganisms are capable of producing HA, mainly *Streptococcus zooepidemicus*, which can produce up to 7 g/L under ideal growing conditions. The culture medium, as well as the environmental conditions (pH, temperature, aeration, agitation), are factors that directly influence the production of HA, which can reduce or maximize it. Regarding recovery and purification methods, several techniques in sequence are used involving the precipitation of HA from the fermentation broth, usually using repeatedly organic solvents, surfactants, centrifugation, and membrane separation, among others. The functions of the HA cover several areas such as pharmaceutical, medical, and aesthetics, among others, causing the commercial demand for this biopolymer to increase every year, justifying research involving its bioproduction.

Keywords: biopolymer; hyaluronan; bioproduction; *Streptococcus zooepidemicus*; metabolic engineering.

1. Introduction

Hyaluronic acid (HA) is a natural polymer that belongs to a group of heteropolysaccharides found in various human and animal tissues and through microbial sources [1]. Its structural characteristics and physical, chemical, and biological properties allow its use to be wide and cover several areas, mainly pharmaceutical, medical, dental, and biomedical [2,3].

Traditionally, HA is still obtained from extractions from animal sources, more specifically from rooster combs. However, due to the complexity of the matrix, which consists of a tangle of glycosaminoglycans linked to proteins, the process necessary for its extraction and purification is characterized by the complexity and severity of the steps involved, discouraging its production [4]. Thus, the production of HA by microorganisms appears as an alternative to replacing the traditional route, which can also increase production through metabolic engineering and synthetic biology approaches. Initially, it was found that wild strains of *Streptococcus* sp, such as *S. zooepidemicus*, *S. thermophilus*, *S. pyrogenes*, and *S. equisimilis*

synthesize HA precursors. Of these, in the last 3 decades, *S. zooepidemicus* has been at the center of the HA production industry due to its excellent results [5]. In recent years, through genetic modifications, other strains, such as *Escherichia coli*, *Lactococcus lactis*, *Bacillus subtilis*, *Agrobacterium* sp., and *Corynebacterium glutamicum* also began to be studied as possible HA producers [6].

In addition, the microbial synthesis of HA allows the use of a wide variety of media sources for bioproduction, which must present a balance between the contents of carbon, nitrogen, and salts, so that there is no deficiency or excess of them, harming the microbiota development and consequently the production of HA. Other factors such as pH, temperature, agitation, and aeration must also be taken into account, as each microorganism has its nutritional needs and environmental conditions to keep its physiological needs functioning properly [7]. Regarding the production process, the separation and purification steps, normally performed through the sequential combination of different methods, must be considered, thus ensuring the safety and final quality of the biopolymer [8].

In this context, the objective of this review is to serve as a theoretical basis for future studies on the production of hyaluronic acid by microorganisms, and the topics covered are HA characteristics; microorganisms used in the production of HA; operations for HA recovery and purification, as well as HA applications, through available online databases.

2. Materials and Methods

A literature review was carried out in the main open databases (Scielo, ScienceDirect, (describe others used using), having as descriptors hyaluronic acids; physical, chemical, and biological properties; HA production, including animal source and microbial source; recovery and purification of HA, in Portuguese and English, considering the articles published in the last 15 years.

3. Results and Discussion

3.1. Hyaluronic acid: structure and chemical constitution.

The first study concerning HA dates back to 1880 when the French scientist Portes observed that the vitreous body mucin was different from other mucoids in the cornea and cartilage and called it “hyalomucin”. In 1934, Karl Meyer and John Palmer first isolated a glycosaminoglycan (GAG) from the vitreous humor of the bovine eye, calling it “hyaluronic acid” (derived from hyaloid [vitreous] and uronic acid). The first pharmaceutical-grade HA was produced in 1979 by Balazs through successive steps of extraction and purification of rooster combs and human umbilical cords, thus establishing a basis for the industrial production of HA. The nomenclature was changed in 1986 to “hyaluronan”, confirming the nomenclature of this polysaccharide, described as a unique biomolecule with different biological functions, attributed to its physicochemical properties and its specific interactions with cells and extracellular matrix. Subsequently, the presence of hyaluronic acid was confirmed in other organs (joints, skin, rooster comb, umbilical cord, among others) and tissues (connective, epithelial, and nervous) [2, 9].

Hyaluronan is a linear polymer of glycosaminoglycan (GAG) with repeating units of β-1,3-N-acetyl glucosamine and β-1,4-D-glucuronic acid linked together (Figure. 1) [10]. When both sugars are in the β configuration, the hydroxyls, the carboxylate group, and the anomeric carbon on the adjacent sugar are in sterically favorable equatorial positions. At the

same time, all the hydrogen atoms occupy the less favorable axial positions. This makes the structure of HA energetically very stable, and due to the anionic character of the molecule, it becomes highly hydrophilic [11].

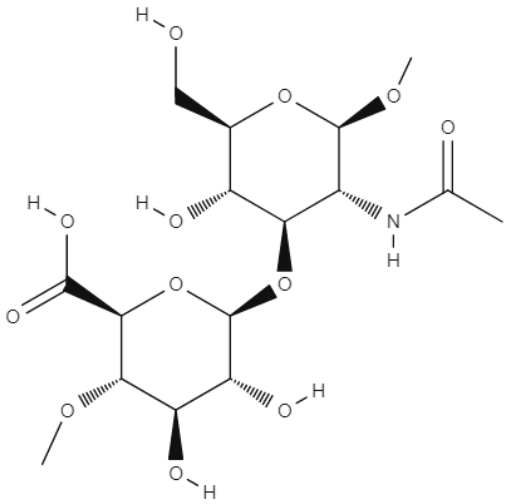

Figure 1. Chemical structure of hyaluronic acid.

HA is the only GAG that is not sulfated and bound to proteins. It is usually composed of 100 to 20,000 repeating units and has a molecular weight between 10^5 and 10^8 Da, unlike other GAGs, which are smaller [12]. Depending on the source, the molecular weight of HA can vary. For example, animal-derived hyaluronic acid tends to have high molecular weights (up to 20,000 kDa). On the other hand, bacterial HAs tend to have a lower molecular weight, between 1000 and 4000 kDa.

HA is a ubiquitous component of all tissues and fluids in the human body. However, it is more abundant on the skin (50%), where it participates in wound healing. In addition, HA is essential to maintain moisture and regulate the skin's osmotic pressure, retain large amounts of water, and reduce wrinkles [13]. HA is synthesized extracellularly by six glycosyltransferases known as hyaluronan synthases (HASs). In mammalian cells, HASs can appear in three isoforms (HAS1, HAS2, and HAS3) that differ in catalytic activity or cell type. The most expressive isoform in keratinocytes is SAH2, characterized by its production decreasing with the aging of the skin [14].

Due to its physicochemical and biological properties, in 1950, HA started to be commercialized on an industrial scale as an input/additive for several products, mainly linked to formulations destined for the medical and cosmetic areas. The main precursor sources used to obtain it were umbilical cords, bovine vitreous, and, mainly, rooster combs, which stand out for their current concentration, with an amount of approximately 7.5 mg / g [10].

The high consumption of HA, associated with the lack of its main raw material (cockscomb), in addition to the possible contamination with animal endotoxins that cause undesirable effects in the form of immune responses and allergies, are factors that trigger the increase of the search for sources and, mainly, for alternative ways of obtaining HA. In recent years, one of these alternatives studied has been the microbiological route [4].

In this context, the production by bacterial fermentation from *S. zooepidemicus* stands out, a bacterium known to cause infection in many animals, such as horses, cows, rabbits, pigs, dogs, and cats, but rarely in humans. The interest of the scientific community for this bacterium, and mainly for its application in the production of HA, has been growing in recent years (Figure

2) and is mainly due to the production process (production, extraction/purification), which is easily demonstrated scalability, and the quality of HA obtained [10].

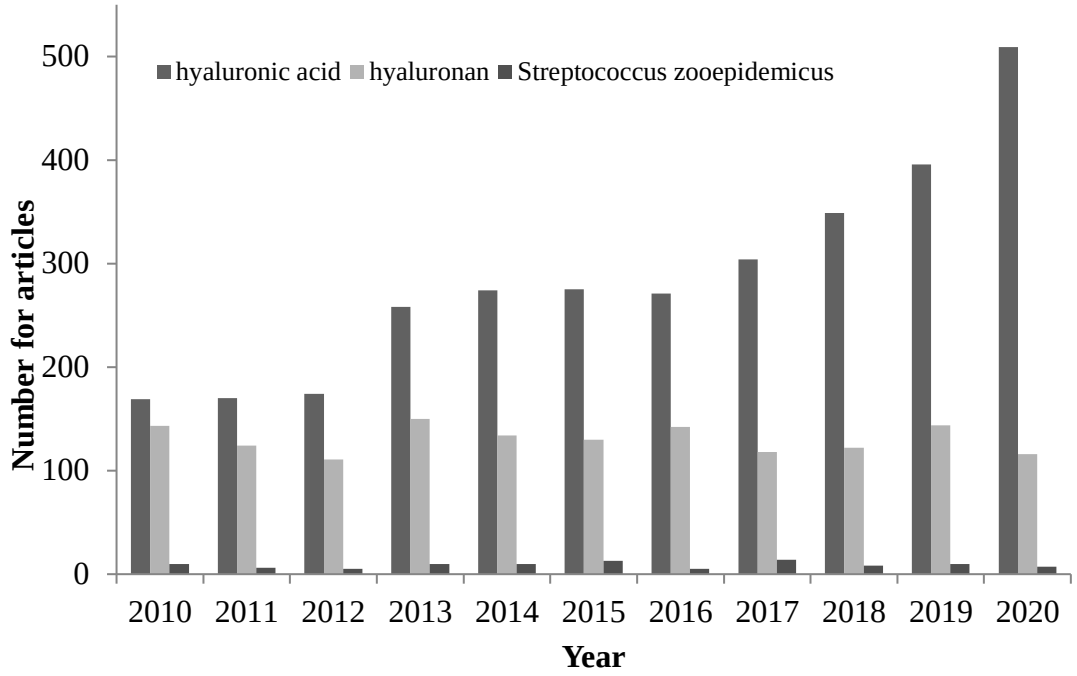

Figure 2. Publications of research articles and bibliographic reviews in Science Direct referring to the keywords "hyaluronic acid", "hyaluronan" and "*Streptococcus zooepidemicus*" in the period from 2010 to 2020.

It is possible to observe an increase in the number of articles approximately 3 times in a period of 10 years in relation to "hyaluronic acid". However, in relation to the keywords "hyaluronan" and "*Streptococcus zooepidemicus*", the number of articles showed few changes in this same period.

3.2. Properties: physical, chemical, and biological.

The physical, physicochemical, and biological properties of HA are directly linked to its molecular weight. Molecular weights from 0.4 to 4.0 kDa allow it to act as an inducer of heat shock proteins and as a non-apoptotic property. Polysaccharides with a 6–20 kDa molecular weight have immunostimulatory, angiogenic, and phlogotic activities. Hyaluronic acid with a molecular weight of 20–200 kDa participates in biological processes such as embryonic development, wound healing, and ovulation. In contrast, high molecular weight (>500 kDa) hyaluronic acid has antiangiogenic activity and can function as a natural space filler and immunosuppressant. The increase in molecular weight and concentration in polymer solutions leads to the reinforcement of the three-dimensional network of the polymer and, consequently, results in an increase in the solution's viscosity and viscoelasticity. In some cases, for example, in the electrospinning process, molecular weight, concentration, and viscosity are the main parameters considered in obtaining nanofibers [15]. In addition, HA can have high moisture retention capacity, high biocompatibility, and hygroscopic properties, providing HA with the ability to act as a lubricant, dampener, joint structure stabilizer, and water balance flow resistance regulator [2]. Due to its chemical structure, HA is easily modifiable and malleable in terms of handling, which allows the creation of different physical forms, making it extremely versatile in terms of applications.

Each of these forms has its niche and application, such as:

• Formation of hydrogels: when used as a base for hydrogels, they form networks of interconnected chains. To improve crosslinking functions, covalent bonds must be made. Hyaluronic acid has two functional groups (COOH and OH) that influence the type of crosslinker used to modify matter. The carboxylic group can be modified, for example, by the addition of N-hydroxysuccinimide. The hydroxyl group can be crosslinked by glutaraldehyde, for example. However, the covalent crosslinking method requires biologically hostile reagents that are not suitable for cells and tissues. Compared to the original hyaluronic acid, the crosslinked type, used mainly in tissue engineering, exhibits more robust mechanics and is less susceptible to enzymatic degradation. The gelation kinetics must be fast enough for tissue engineering purposes to allow encapsulation into the cell/gel material [16]. These materials can be considered favorable for use both in the pharmaceutical industry and in biomedical applications and can act as drug carriers [17].

• Viscoelastic solutions: due to their high molecular weights, their solutions can be extremely viscous and present non-newtonian flow properties [18]. The high volume occupied by its polymeric chains, most of them linked to water, gives flexibility to hyaluronic acid, contributing to constant structural changes [19]. Viscoelastic HA solutions are excellent for simulating the synovial fluid found in joints, whose natural viscoelastic properties can be attributed to the concentration of hyaluronic acid present in them. However, these solutions do not have lasting mechanical integrity [20].

• HA-based scaffolds are temporary support structures that help promote the ingrowth of cells and tissues through biodegradable structures such as hydrogels. They are being widely used in the medical industry for various therapeutic purposes, such as applications in bone tissue, space-filling, nerve and brain tissue repair, and cell distribution and muscle regeneration [21]. Certain criteria must be met by these structures, such as the scaffold surface must allow adhesion and cell growth; it must be biocompatible, it must not promote inflammation or immune response, and the degradation of the scaffold surface must not produce toxic products; scaffolding must have the physical and mechanical properties of the original fabric it is trying to imitate; the porosity of the structure must be sufficient to allow cell growth and nutrient diffusion [22].

• AH nanoparticles (NPs-AH): in medicine, nanoparticles are nanotechnological innovations that aim to diagnose, treat or prevent diseases [23]. HA and its derivatives have been extensively investigated in the design of drug carriers by the pharmaceutical industry [24]. Reports show that this biopolymer can bind to CD44 receptors, which are considered to be overexpressed in malignant cells with high mobility and invasive capacity through hydrogen bonds. Thus, HA-based nanoparticles can have a special target tumor tissue, being able to self-assemble containing several hydrophobic nuclei and a hydrophilic outer layer of HA. Furthermore, NPs-HA demonstrates better accumulation at the tumor site compared to water-soluble HA derivatives [25].

In addition to providing interesting mechanical and physicochemical properties for tissues, HA also performs important biological functions as a signaling molecule and is easily degraded into bioactive fragments [11]. In this context, various chemical modifications can be carried out to increase its structural stability. These modifications target the carboxylic acid functional groups of the glucuronic acid portion of the hydroxyl of the N-acetylglucosamine portion. Carboxylic acid groups can be modified by amidation, Ugi condensation, or ester formation, while hydroxyl groups can be modified by ether formation, hemiacetal formation using glutaraldehyde, ester formation, carbamate formation, or oxidation.

These HA derivatives can be chemically crosslinked by covalent bonds, physically crosslinked using non-covalent interactions, or combined with both methods. The crosslinking process leads to the formation of a 3D network capable of retaining large amounts of water; that is, they form a hydrogel with properties similar to those of biological tissues, which will result in excellent biocompatibility [26].

3.3. Applications.

The in vivo functions and industrial applications of HA are directly related to its viscoelastic and pseudoplastic properties based on the molecular weight of the polymer [27]. For example, low molecular weight HA, also called hyaluronic oligo-acid, has antioxidant activity superior to conventional molar mass HA.

Considering that it can be obtained with different molecular weights, this product is being marketed both for use in pharmaceutical products (treatment of dry eye, osteoarthritis, osteogenesis, wound healing) and in cosmetics (dermal fillers) [10]. Its antioxidant capacity suggests that it can be used as a supplement in juices and yogurts, aiming to reduce the number of free radicals present in the body [28].

3.3.1. HA in the extracellular matrix.

When in physiological solutions, mutual repulsions occur between their carboxylic groups, causing the HA to swell, forming a hydrated network with excellent plasticity. When introduced into the cellular matrix of soft connective tissues, HA plays an important role in minimizing the effects of aging through its lubricating action, osmotic tamponade, and space-filling, such as orthopedic disorders, dry eye syndrome, and, especially, flaccidity of the skin [26].

Of these, the aesthetic result provided by filling soft tissue with reticulated or stabilized HA, which leads to the elimination of wrinkles, in addition to allowing the modeling of the facial contour, is responsible for the greater commercial demand for HA, especially in low molecular weight (≈105 Da). It is used in several cosmetic formulations for topical use to improve tissue hydration [11].

3.3.2. HA in the healing process.

According to Dovedytis, Liu, and Bartlett [20], the healing process is divided into four main phases: hemostasis, inflammatory, proliferation, and maturation. Therefore, the application of hyaluronic acid is mainly due to its role during these processes.

HA provides two very important functions during wound healing as part of cell proliferation and migration. First, this biopolymer provides a temporary structure in the early stages of the wound. This structure helps to facilitate the diffusion of nutritional supplies and helps to rid the wound of cellular metabolism waste. In the background, HA is intimately involved in the proliferation and migration of keratinocytes (type of cell in the epidermis or outermost layer of the skin). This temporary structure is replaced as the wound matures by the addition of proteoglycan proteins and collagen molecules. In addition to these functions, being a hygroscopic macromolecule, it is highly osmotic, allowing hydration control during periods of wound repair and associated inflammatory processes. Its presence at high levels during this process is relevant to cell proliferation and migration. As the granulation tissue matures, HA is degraded, and as levels drop, more protein molecules are produced. Proteins bind to HA to

become proteoglycans and continue the healing process to increase tissue resilience. These molecules are able to absorb up to 3.000 times their weight in water and are, therefore, an important wetting agent for tissue [29].

Foglarová *et al*. [30] developed HA-based films for biomedical applications. The films produced had a smooth and homogeneous surface and were prepared based on palmitoyl esters, a material insoluble in water. Based on the characterization, the film maintained the molecular weight of the polymer used and proved not to be cytotoxic, having a great potential for application in several areas of biomedicine, including its use in tissue engineering for wound healing.

HA gels have good absorption capacity and histocompatibility, so they are often used to prevent postoperative tissue adhesions by separating the tissue surface during tissue wound repair, such as the endometrium, preventing adhesions and formation of fibrous tissue, in addition to inhibiting the migration and phagocytosis of granulocytes and the deposition of platelets [31].

3.3.3. HA in inflammation and tumor metastasis processes.

Its functions include mediating migration, adhesion, proliferation, cell signaling and differentiation, immunoregulatory, anti-inflammatory, antioxidant, and anti-aging activity. It can also be used as the main raw material for building biomaterials bases in tissue engineering and promoting the differentiation of adipose tissue-derived mesenchymal stem cells into insulin-producing cells. Furthermore, studies have shown that HA can increase insulin secretion by the HIT-T15 pancreatic β cell line, suggesting that hyaluronic acid may be a potential regulator of stem cell differentiation that may treat type 1 diabetes [32].

There are two common cell surface receptors that interact with HA molecules. They are called CD44 and RMMAHA (receptor for hyaluronic acid-mediated motility). The length of the polysaccharide chain plays a crucial role in the effect; in particular, small HA oligomers, emerging due to hyaluronidase (HYAL) activity, have an extraordinarily high biological activity. For example, high molecular weight HA molecules suppress angiogenesis, while small fragments stimulate the same process. There are many other examples of such opposite impacts, depending on the length of the chain. It is also known that small HA fragments participate in inflammation and tumor metastasis processes through interaction with the aforementioned receptors, generating an attractive starting point for the structural chemical modification of HA molecules to investigate changes in biological activity or generation of new properties due to a change in the secondary/tertiary structure and behavior of the polyelectrolyte [33]. This biocompatibility, biodegradability, active targeting characteristics, and easily modified chemical structure of HA make it widely used in various cancer treatment methods [34].

Table 1 presents some studies with applications of HA in different areas and with different functions.

Table 1. HA applications.

Area	AH application	Reference
Medicine; Pharmacists; Dentistry; Biomedical	HA-loaded electrotrophied fibers for wound healing and dressing applications	[28, 35, 36]
	HA-based nano drugs for cancer treatment	[37, 38]
	Hyaluronic acid application in bone regeneration	[39]
	HA injection for papilla regeneration; Dental implant coverage with HA to improve its osseointegration; Mixture of HA with synthetic bone graft material;	[40, 41]

Area	AH application	Reference
	Coverage of surgical area (inside and outside) by HA to improve and accelerate the tissue healing process; HA injection for papilla regeneration; Dental implant coverage with HA to improve its osseointegration; Mixture of HA with synthetic bone graft material; Coverage of surgical area (inside and outside) by HA to improve and accelerate the tissue healing process; Use of HA after scaling and root planning as an adjacent therapy for periodontitis; Topical application of HA to treat oral ulcers; Use of HA as an adjunct to gingivitis and peri-implantitis treatments; Use of HA as an adjunct to non-surgical treatment of gingival recession; Mix HA with platelet-rich fibrin, plasma, and growth factors to improve overall results; Used as a nano-sized drug carrier; HA as a matrix to encapsulate stem cells and signaling molecules for the reconstruction of the temporomandibular joint, salivary glands, dental pulp, dental bone, enamel, root canal, and mucosa. Hyaluronic acid-based microneedle matrix	
	Use as eye drops; Intra-articular HA injections as a treatment option for osteoarthritis; Filling of gums, lips and wound healing	[42]
	Thin film made from a mixture of collagen, hyaluronic acid, and chitosan crosslinked with starch dialdehyde	[43]
	Formation of hydrogels for biomedical and tissue engineering applications	[44-48]
	HA for dermal filling in the periocular area	[49]
	Applications of hyaluronic acid in ophthalmology and contact lenses	[50]
	Application of HA as a drug carrier for sustained release	[51, 52]
	Facial rejuvenation	[53]
Environmental and Chemistry	Synthesis of fluorescent carbon dots (CDs) using hyaluronic acid as carbon source and application for selective detection of Fe 3+ and folic acid ions	[54]
Food	Film-forming and gelling properties for edible packaging	[55]

Source: The Author (2022).

As shown in Table 3, HA applications are numerous in various areas (dental, medical, food, cosmetic, etc.). However, the use of this glycosaminoglycan is notorious, mainly in dental, medical, and aesthetic procedures.

3.4. HA production/obtainment.

The global market for HA-based products, estimated to be around $8.3 billion in 2018, is expected to exceed $15 billion by 2026. Depending on the application, the value of HA and its derivatives range from $2.000 to 60.000/kg [10]. This huge price difference is mainly due to the high purity required, particularly for medical applications, and, to a lesser extent, the molecular weight required for these applications. HA production is growing, mainly in China, where sales are expected to reach 613 tons by the end of 2022. By comparison, HA production in early 2000 was estimated at 10-20 tons/year for ophthalmic applications, cosmetic and dietary, and less than 1 ton/year for medical-grade applications [56].

This great commercial demand requires that HA production processes are optimized to obtain products that meet high-quality standards associated with affordable yields and costs. Both the source and the purification process collaborate to determine the characteristics of the HA produced in terms of purity, molecular weight, yield, and cost, which represents a major challenge in the field of applied research for high-quality, high-yield hyaluronans using smaller methods, expensive [9].

3.4.1. Animal source.

According to Yao *et al*. [57], during the 1930s, HA was successively isolated from a wide variety of different animal tissues. The first time that HA was obtained with a high degree

of purification, in addition to high molecular weight, was from umbilical cord and rooster comb, extraction performed by Endre Balazs, being one of the most applied products in ophthalmic surgery.

Obtaining HA of animal origin, mainly rooster comb and bovine vitreous humor, is based on solvent extraction, with subsequent precipitation and washing as final extraction treatments. Previously, extraction solvents were composed of mixtures of chloroform and water. However, they have been replaced by more environmentally friendly water and ethanol or acetone mixtures. After the extraction process, the solutions containing HA are subjected to subsequent treatments, involving bacterial removal and subsequent separation of the protein chains linked to the HA structure, using bactericides and proteolytic enzymes, respectively [8].

The disadvantages of animal tissue extraction methods can be summarized as follows: limited availability of animal tissue; the extraction process is extremely complicated; it can generate environmental impact; the extraction rate of AH production is low, and the quality control is unreliable. According to Badri *et al*. [58], all of these factors contribute to limiting the use of the animal tissue extraction method for large-scale commercial production of HA.

3.4.2. Microbial source.

Microorganisms play an important economic role in metabolic and biochemical processes that include reactions ranging from enzyme production to biopolymers [59]. Investigations into the production of HA by microbial fermentation began to appear in the 1980s and have evolved rapidly in recent decades [57]. This production increase is due to some advantages, such as avoiding the risk of cross-species viral infection, more efficient purification, lower production costs, and higher yields compared to animal sources [60].

Some microbial production techniques were developed with the objective of obtaining high molecular weight through the isolation of the capsule, which is composed of HA polymers, in addition to suitable microbial sources [27].

Microbial HA production can be considerably affected by culture conditions, including temperature, pH, aeration rate, agitation speed, dissolved oxygen, shear stress, and type of bioreactor. Therefore, optimal parameters in microbial production were investigated [61].

3.4.2.1 Wild microbial strains.

According to Rohit *et al*. [62], several wild microbial strains naturally produce HA capsules as part of their self-defense against invading hosts. Examples of these species are *Streptococcus zooepidemicus*, *Streptococcus pyogenes*, *Streptococcus equisimilis*, *Streptococcus thermophilus* and other organisms such as *Pasteurella multocida* and *Cryptococcus neoformans*. However, the current industrial production of HA is carried out by *Streptococcus species*, mainly *S. zooepidemicus*, due to their high production of HA (6 - 7 g/L) (Table 2).

Table 2. Microbial hyaluronic acid production.

Microorganism	Medium of production	pH	Temperature (C°)	Agitation (rpm)	Aeration (vvm)	HA (g/L)	Reference
Streptococcus zooepidemicus ATCC 35246	Whey and whey hydrolyzate (various concentrations); glucose (50 g/L); lactose (50 g/L); yeast extract (5 g/L); tryptone (15 g/L)	6.7	37	500	1	4	[4]
	Sugarcane molasses and corn steeping water (50 g/L); yeast extract (5 g/L); Tryptone (15 g/L)	6.7	37	500	1	3.8	[62]
Streptococcus zooepidemicus MTCC 3523	Palm sugar (10,0–50 g/L); various sources of nitrogen (17,5 g/L)	7	37	200	1	1.22	[60]
	Glucose at varying concentrations (10–60 g/L); yeast extract (10 g/L)	7	37	400	DI[1]	3.345	[56]
Streptococcus zooepidemicus ATCC 39920	Cashew juice; yeast extract (60 g/L)	6.5	37	150	2	0.0070	[63]
	Concentrated soy hydrolyzate; whey; cashew juice; steep corn liquor	7.5	37	150	DI[1]	0.89	[64]
	Maltodextrin (30 g/L); yeast extract (5 g/L)	6.4	37	90	natural aeration	0.92	[65]
	Sucrose (20 g/L); hydrolyzed casein (25 g/L); yeast extract (3,5 g/L)	7	37	400	2	5.10	[66]
	Glucose (30 g/L); yeast extract (10 g/L)	7.2	37	200	1	5	[67]
	Glucose (50 g/L); extrato de levedura (20 g/L); solução com oligoelementos	7	37	200	DI[1]	0.226	[68]
	Yeast extract (ranging from 14,65 to 85,35 g/L; cane molasses (ranging from 14,65 to 85,35 g/L)	8	37	150	0,5	2.825	[69]
	Glucose (25 g/L); yeast extract (60 g/L)	7	37	DI[1]	2	2.5	[57]
	Glycerol (40 g/L)	7	37	1200	0.1 a 1	7	[70]
Synechococcus sp. PCC 7002	Air enriched with CO2 (1% (v/v)) under continuous illumination (300 µmol photons m-2 s-1)	DI[1]	38	DI[1]	DI[1]	0.08	[71]
Streptococcus zooepidemicus WSH-24	Yeast extract (25 g/L); sucrose (70 g/L)	7	37	294	1.5	6.2	[72]
Streptococcus zooepidemicus NJUST01	Starch (5%); glucose (0,5%); yeast extract (0,5%)	7	37	220	natural aeration	6.74	[73]
Streptococcus zooepidemicus 3523-7	Glucose (different concentrations); yeast extract (different concentrations)	7	37	DI[1]	DI[1]	1.38	[74]
Streptococcus thermophilus YI T 2084	10% skimmed milk	DI[1]	42	DI[1]	No agitation	0.8	[75]
Bacillus subtilis 3NA	Glucose (30 g/L); yeast extract (10 g/L)	7	37	300	DI[1]	0.096	[76]
Streptococcus equisimilus (MK156140)	Sucrose; yeast extract; beef extract (different concentrations)	7	37	180	DI[1]	7.16	[77]

[1]Data not informed.

The different results obtained (0.007 to 6.74 g/L) may be related to the use of different mediums of production (synthetic and/or agro-industrial residues), the species of the microorganism, or even the variation of experimental conditions (pH, temperature, agitation, and aeration).

However, among the works found in the literature concerning *Streptococcus zooepidemicus*, the pH and temperature range are factors that do not vary (between 6 and 8 and 37°C, respectively), unlike agitation, which can vary from 90 to 500 rpm. The aeration used in the studies is usually 1 vvm, but it can still vary from 0.1 to 2 vvm.

3.4.2.2. *Streptococcus zooepidemicus.*

Investigations on the production of HA by microbial fermentation began to appear in the 1980s with *Streptococcus zooepidemicus* and evolved rapidly over the decades; this microorganism was used for the first time for commercial production during this period and continues to this day. However, some challenges persist in the fermentation process of *S. zooepidemicus*: the viscosity of the fermentation broth increases dramatically when the HA content reaches above 4 g/L, and the dissolved oxygen (DO) is drastically reduced, making it difficult for biomass accumulation and HA yield; during carbon consumption, the concentration of DO drops rapidly, which can lead to the accumulation of byproducts of the anaerobic pathway, such as lactate [57].

In addition to reducing the risk of viral contamination, microbial HA production requires simpler processing. It is devoid of instabilities, as it does not depend on seasonality and has fewer batch variations. Given these considerations, with regulatory acceptance in the US and the UK, the literature on the production of HA by *S. zooepidemicus* presents numerous attempts to increase the amount produced, including conventional techniques (e.g., optimizing the extraction process, adapting the medium of culture and select strains with high HA productivity) and metabolic engineering methods [78].

How these bacteria acquired the ability to synthesize hyaluronan is still a matter of debate, but the *Streptococcus bacterium* needs three different genes to produce the HA capsule [79]. The genes hasA, hasB, and hasC are transcribed in an operon and encode hyaluronic acid synthase, UDP-glucose dehydrogenase, and UDP-glucose pyrophosphorylase, respectively. The microorganism *S. zooepidemicus* contains two additional genes (hasD and hasE) that encode acetyltransferase, pyrophosphatase, and phosphoglucose isomerase, activating additional metabolic pathways to promote HA synthesis. Initially, glucose-6-phosphate is converted to glucose-1-phosphate by glucosophosphatase. Then, UDP-glucose pyrophosphorylase (hasC) transfers the UDP group to glucose-1-phosphate to produce UDP-glucose. From this, UDP-glucose is oxidized by UDP-glucose dehydrogenase to UDP-glucuronic acid, obtaining one of the precursors of HA. In the UDP-N-acetyl-glucosamine biosynthesis pathway, glutamine fructose-6-phosphate aminotransferase converts fructose-6-phosphate to glucosamine-6-phosphate through the transfer of amino groups from glutamine, thus obtaining N-acetyl- glucosamine-1-phosphate by phosphoglucosamine mutase. Subsequently, acetyltransferases convert N-acetyl-glucosamine-1-phosphate to UDP-N-acetyl-glucosamine, another precursor of HA [57].

Table 3. Produção de ácido hialurônico por microrganismos geneticamente modificados.

Microorganism	Medium of production	pH	Temperature (C°)	Agitation (rpm)	Aeration (vvm)	HA (g/L)	Reference
Escherichia coli K12 W3110	LB supplemented with 3 g/L glucose and 3 g/L lactose	DI[1]	37	200	DI[1]	0.299	[80]
Lactococcus lactis	M17 broth supplemented with 0,5% (weight/vol) glucose	DI[1]	37	-	-	6.09	[6]
	M17 medium containing 10 g/L glucose	DI[1]	30	200	DI[1]	0.68	[81]
	M17 supplemented with 1% (w/v) glucose	DI[1]	30	170	DI[1]	0.65	[82]
Bacillus subtilis	Sucrose (20 g/L); yeast extract (10 g/L)	DI[1]	37	280	DI[1]	0.97	[22]
	LB	DI[1]	37	200	DI[1]	6.8	[83]
Ogataea polymorpha NCYC495	1% yeast extract, 2% peptone and 2% glucose	DI[1]	37	200	DI[1]	0.197	[84]
Kluyveromyces lactis	YNB medium and glucose (40 g/L)	6.0	30	200	2	1.89	[85]
Pichia pastoris	Glucose (40 g/L); yeast extract (7,5 g/L); peptone (10 g/L)	7.0	30	500	0,7	1.7	[86]
Streptococcus thermophilus	M17 with 1% lactose	6.8	40	100	No agitation	1.2	[87]
Agrobacterium sp.	LB	DI[1]	30	250	DI[1]	0.3	[88]

[1]Data not informed

3.4.3. Recombinant microbial strains.

The production of HA by recombinant bacteria presents itself as an alternative source due to safety concerns about the pathogenicity of natural HA-producing strains. In this sense, several attempts to obtain this biopolymer with Gram (+) and Gram (-) bacterial hosts have been investigated, using, for example, *Escherichia coli*, *Lactococcus lactis*, *Bacillus subtilis*, *Agrobacterium* sp. and *Corynebacterium glutamicum* (Table 3) [6].

The increase in microbial strains is probably one of the most important methods to solve problems of low substrate conversion efficiency, byproduct formation, and low-stress tolerance through the use of random mutagenesis, site-directed mutagenesis, target gene overexpression, evolution laboratory, and high-throughput screening. Other approaches to improve phenotypic expression are also reported, such as genome scrambling and artificial transcription factor engineering [57].

Several strategies can be adopted to build these HA producers, with the selection of HA synthases and host strains representing the most critical step, as variations in the HA synthase sequence, structural conformation**,** or metabolic capabilities of the host cell make a difference in the production yield or on the molecular weight of the HA. The first time that the cloning of HA synthase and related gene clusters from *S. zooepidemicus* occurred in 1993, and it performed heterologous HA synthesis. The functions of HA synthase have been enhanced by protein engineering or by modifying the microenvironment of the enzymatic reaction, including the lipid composition of the membrane. In addition, metabolic engineering strategies such as overexpression of enzymes from intermediate metabolic pathways (e.g., UDP-glucose 6-dehydrogenase or glucosamine-1-phosphate N-acetyltransferase) or blocking the synthesis of unwanted metabolites (e.g., L-lactate) have been adopted to drive the generation of intermediate metabolites necessary for the synthesis of HA [57,69].

3.5. Factors that interfere with the microbial production of HA.

According to Chahuki *et al*. [4], the microbial synthesis of HA is an energy and carbon-intensive process, where approximately 5% of the carbon source is responsible for the synthesis of HA, while cell growth and the production of lactate and acetate consume about 10 and 80 % of carbon source, respectively. UDP glucuronic acid and UDP N acetyl glucosamine are precursors of both HA and cell wall biosynthesis. Thus, a competition for energy and carbon sources ends between HA biosynthesis and cell growth. HA biosynthesis also faces competition between cell wall biosynthesis and the glycolytic pathway. In this sense, reducing cell growth and the glycolytic pathway may be alternatives to obtaining a greater production of HA.

The influence of the composition of the culture medium on the production of HA by fermentation has been extensively investigated. Some of the main parameters of the culture medium, such as pH, presence of mineral ions, and carbon/nitrogen ratio, were studied in order to improve production [7]. However, the effects of these factors on the production of HA will depend on the experimental conditions and the deformation used [89].

3.5.1. Carbon.

Carbon is not only essential for microbial growth but also the backbone synthesis of the HA sugar chain. However, there is a cellular tolerance to nutrient concentrations that need to be taken into account. Different glucose concentrations ranging from 10 to 60 g/L have already

been tested and the results found were that the maximum molecular weight of HA obtained was at a glucose concentration of 40 g/L. When increasing the glucose concentration above 40 g/L, bacterial growth is inhibited, and the molecular weight of HA also decreases. This indicates that microorganisms have specific cutoffs regarding the use of carbon sources and can also modulate metabolism by detecting environmental changes. Carbon can result in excessive osmotic pressure at high concentrations, which can be detrimental to HA production, thus converting more carbon fluxes into other competitive pathways [57].

In general, culture media used for the production of microbial HA contain glucose as a carbon source [65]. However, studies indicate that the use of sucrose in the fermentation medium can increase the molecular weight of HA by 800 kDa more than the use of glucose as a carbon source [61].

Some of the carbon sources can also include agro-industrial substrates, such as molasses, sugarcane juice, and steep corn liquor, among others. These relatively low-cost byproducts can be used in the culture medium, thus reducing the cost of producing high-value polymers such as HA [70].

3.5.2. Nitrogen.

Growth and HA production potential by *S. zooepidemicus* is highly dependent on organic nitrogen. The first medium consisted of animal sources such as brain and heart infusion (BHI) and sheep blood. However, other alternative sources of culture medium have been studied, such as microbial sources. Yeast extract and/or peptone are extensively added as nitrogen sources in the culture medium. The literature states that this bacterium may require very high concentrations (up to 20 g/L) of peptone and/or yeast extract for the production of HA [7]. Other sources that include organic substrates are soy protein and whey protein, which can reduce HA production costs [70].

3.5.3. Reaction medium supplementation.

Although many studies have been carried out to optimize the production of HA, few works have been published related to the use of supplements to increase the production of HA. The result of supplementation with some compounds in the production medium is described by Aroskar; Kamat; Kamat [66]:

- Magnesium sulfate ($MgSO_4$): This compound is a necessary cofactor for the HAS enzyme to become a holoenzyme. The addition of this magnesium can increase the molecular weight of the HA formed.
- Phosphates: their addition can increase the molecular weight of HA. However, they do not increase the yield of the polymer.
- L-arginine: the role of this amino acid is related to the donation of carbon and nitrogen in the synthesis of purine and pyrimidine, thus saving energy consumption by the microorganism.
- Glutamine: is a component that is directly involved in the HA synthesis route, donating the amine group for the conversion of fructose 6 phosphate to glucosamine 6 phosphate, which is an important precursor for HA synthesis, which, in turn, will produce N-acetylglucosamine in others, thus increasing the yield of HA.
- Uridine: may increase HA production due to the involvement of molecules in the HA synthesis pathway.

- Sodium glucuronate: activates HA synthesis and acts on the lengthening of the HA molecular chain.

Other mineral elements such as sodium (Na) and manganese (Mn) also participate in several reactions and functions:

- Manganese (Mn): acts as a cofactor for glycosyltransferases that are involved in the synthesis of disaccharides in polymer chains.
- Sodium (Na): may contribute to the microbial production of HA, due to its role in lactate excretion by bacteria of the genus Streptococcus [65].

3.5.4. Temperature.

Temperature is also considered a fundamental parameter for fermentation, as it can modify both the maximum concentration of HA and its average molecular weight [8]. Temperature affects the rate of biochemical reactions, intracellular enzyme catalytic activity of microorganisms, generation time, and activity of the microorganism involved. The microorganism's reaction rates increase with temperature until a threshold temperature is reached, after which the growth rate decreases. The ideal temperature for producing polysaccharides such as HA will depend on the type of microorganism involved in bioproduction [90,91].

3.5.5. pH.

The pH of the medium is one of the essential parameters to be controlled in most fermentation processes. The effect of pH is shown to influence polysaccharide production than on cell growth [91]. In particular, HA is sensitive to changes in pH. At relatively acidic (< 4.0) or basic (>11) pHs, HA is degraded by hydrolysis. Of these, under alkaline conditions, this effect is more pronounced due to the disruption of the H bonds, which participate in the structural organization of the AH chains [9].

3.5.6. Dissolved oxygen (OD).

Dissolved oxygen (DO) levels affect intracellular redox potentials, energy charges, and oxygen mass transfer coefficients, which interfere with microbial metabolism. The availability of DO in the fermentation process for the production of fermented HA is an important factor, and DO concentrations have been shown to significantly affect synthesis efficiency. The viscosity provided by macromolecular HA causes a decrease in the DO of the fermentation broth, making it difficult to maintain normal cell metabolism and the effective accumulation of HA, which forms a capsule-like layer at the end of fermentation, inhibiting bacterial growth metabolism [57].

3.5.7. Agitation.

According to [92], the main function of the function is to homogenize the broth. Vigorous weather also favors oxygenation but does not directly aid in the production of HA.

Shoparwe *et al*. [91] observed for *Streptococcus zooepidemicus* that cell biomass and HA production increased significantly with increasing agitation speed up to 300 rpm, obtaining maximum cell biomass of 2.20 g/L and a HA concentration of 1.23 g/L. According to the authors, the increase in speed up to 300 rpm provides a greater subdivision of the bubbles, resulting in a greater surface area for the occurrence of gas-liquid mass transfer, reducing the

thickness of the gas and liquid films responsible for the resistance to transport of dough. It was also observed that the HA yield and cell growth decreased with increasing agitation speed from 400 to 500 rpm. In this case, there was a more drastic decrease in HA production compared to cell growth, indicating that HA production is more sensitive to stirrer speed than cell growth. Thus, a higher shear rate is needed to release the microorganism's HA capsule into the medium. However, this agitation speed should not be much higher because it tends to be detrimental to the quality of the HA and because it can damage the biopolymer [91].

3.6. HA recovery and purification operations.

The production of hyaluronic acid occurs intracellularly, as the enzyme HasA (hyaluronan synthase) acts on the cell plasma membrane, polymerizing the units of UDP-glucuronic acid and UDP-N-acetylglucosamine. After polymerization, a large part of the HA is excreted outside the cell medium, in the form of a capsule, which will allow the polymer to elongate and ensure a high molecular weight, increasing interest in the product [93].

According to Güngör *et al*. [94], the different extraction methods in the literature include SDS lysis (sodium dodecyl sulfate), sonication, centrifugation, ethanol precipitation, dialysis, membrane filtration, salting in and salting-out strategies. In the work carried out by these authors, sequential filtration with an 8–0.45–0.2 µm membrane was performed, followed by lyophilization of the HA after precipitation with ethanol. The use of this sequential method enabled the recovery of 12 g/L HA.

The separation and purification of HA involve repeatedly precipitating the fermentation broth using organic solvents such as ethanol, acetone, isopropanol, etc. These processes involve large amounts of solvents and are time-consuming, which can lead to increased costs. Although membrane technology has been used in some purification processes, the process only concentrates the HA solution or removes small soluble molecules [95].

Many authors use sodium dodecyl sulfate (SDS) to release hyaluronic HA produced from within the excreted capsule or which has remained within the cell [60,63,92,96,97]. Other studies go directly to the precipitation and purification of the HA present in the broth, without the addition of SDS or any other surfactant to break the capsule [64-66,93,98].

According to Souza *et al*. [99], the use of surfactants for HA recoveries, such as sodium dodecyl sulfate, lauryl sulfate, or sodium deoxycholate, is not very suitable because it introduces a new contaminant to the fermentation broth, which must later be removed in the purification step. It also claims that only steps of centrifugation of the broth at low speeds for a long time are necessary for the release of HA to occur. According to Cavalcanti *et al*. (2020), the use of quaternary salts (cetylpyridinium chloride – CPC or cetyltrimethylammonium bromide – CTAB) can be used in broths for the formation of complexes with HA, facilitating its recovery.

According to Cavalcanti *et al*. [8], the degree of purity of HA is a determining factor for successful clinical applications. For example, when using unpurified HA, inflammation can develop in intraocular surgery. This is due to its molecular structure, as HA retains contaminants in its structure, which is highly hydrated and negatively charged when at physiological pH. Therefore, precipitation, filtration, adsorption, electrophoresis, and ion exchange are the most widely studied separation and purification processes. The combination of purification operations can lead to optimized processes in terms of effectiveness and efficiency for removing impurities, as shown in Table 4**.**

Table 4. Methods of recovery and purification of HA produced by microorganisms.

Microorganism	The sequence of steps applied in the recovery and purification of HA	Produçion (g/L)	Reference
Streptococcus zooepidemicus ATCC 35246	SDS; Ethanol; Precipitation by centrifugation; NaCl solution;	2.47	[9]
Streptococcus zooepidemicus ATCC 39920	Centrifugation; Ethanol precipitation; NaCl solution; Carbazole	0.3	[3]
	SDS; Centrifugation; Ethanol precipitation; Carbazole	2.4	[92]
	Centrifugation; Ethanol; Carbazole	0.219	[70]
	Centrifugation; Membrane filtration; Activated charcoal; Precipitation with isopropanol; NaCl solution	2.5	[57]
	SDS; Centrifugation; NaCl solution; Modified carbazole	5	[67]
	High Pressure Liquid Chromatography (HPLC))	0.111	[76]
Streptococcus zooepidemicus RSKK 677	SDS; Centrifugation; NaCl solution; dialysis column	1.2	[94]
Streptococcus zooepidemicus 3523-7	Precipitation with isopropyl alcohol; NaCl solution; Activated charcoal; Centrifugation; Filtration; Carbazole	1.386	[74]
Streptococcus thermophilus YIT 2084	Trichloroacetic acid; Ethanol precipitation; Freeze-drying; Gel chromatography	0.208	[100]
Streptococcus zooepidemicus MTCC3523	SDS; Centrifugation; Ethanol precipitation; NaCl solution; CTAB	0.54	[60]

It can be observed that in all the studies covered in Table 4, several techniques were used for the recovery and purification of HA, suggesting, therefore, that there is no single technique that is more efficient for these processes and that further research in this area is necessary to develop an efficient method so that it can be used, if not in all, but most microbial productions of HA.

4. Conclusions

Hyaluronic acid has several purposes/applications in numerous areas such as pharmaceutical, medical, aesthetic, dental, environmental, chemical, and food. Its high added value justifies the increase in commercial demand for this biopolymer every year. In view of this, several microorganisms are being studied seeking to maximize the production of HA, presenting an advantage to that obtained from animals, as it allows its production on an industrial scale and, as it does not have animal proteins, it does not cause allergic reactions, being, therefore, the most used form.

The bacterial production system has stood out for allowing the optimization of yield and product quality by controlling growing conditions. *Streptococcus zooepidemicus* stands out in relation to other microorganisms due to its high capacity to produce HA, which makes this strain one of the most used for research in this area.

The sources of nutrients (carbon, nitrogen, and salts) that can be used to feed this microorganism are diverse. However, they must be offered in adequate quantities so that bioproduction can take place in an optimized way. Other environmental factors (such as pH, temperature, agitation, and aeration) must be considered in order to maintain the physiological needs of the microorganism.

Therefore, this work provides information about HA, describing its structure, applications, production, separation, and purification processes. This literature review seeks to contribute to the scientific community, supporting future and current studies on the bioproduction of HA by microorganisms.

Funding

This research received no external funding.

Acknowledgments

The authors would like to thank URI Campus Erechim and Universidade Tiradentes for providing the laboratories and materials needed to carry out the work.

Conflicts of Interest

The authors declare no conflict of interest.

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