Chapter 2

Literature Review

2. Literature review

2.1. History of hyaluronic acid

Karl Meyer and John Palmer described a peculiar polymer with an exceptionally substantial molecular weight obtained from the vitreous bovine eyes in 1934 (Meyer & Palmer, 1934). Since they were the first to refer to it, they named the newly discovered compound hyaluronic acid (HA), which originated from the words "hyaloid" (which looks like glassy glass) and "uronic acid."

Although Levene and Lopez-Suarez identified a novel polysaccharide from the vitreous body and cord blood called "mucositis-sulfuric acid" in 1918, Meyer and Palmer are generally credited with discovering hyaluronic acid (Selyanin et al., 2015). It contained glucuronic acid, glucosamine, and a trace amount of sulfate ions.

This material has been identified as hyaluronic acid, so it can be extracted using a combination of sulfated glycosaminoglycans. The polysaccharides comprising most of our planet's biological matter were already well recognized when hyaluronan was discovered. There had already been the discovery of several so-called mucopolysaccharides, today known as glycosaminoglycans.

It is commonly recognized that hyaluronic acid falls into this category as well. Mucopolysaccharides were isolated from mucus, which give mucus its viscous lubricating qualities. These characteristics, in turn, are associated with the high water-binding capacity of glycosaminoglycan.

Meyer and other researchers extracted hyaluronan from numerous animal organs for ten years. For instance, polysaccharide was discovered in joint fluid and the umbilical cord. It is

also now possible to extract HA from practically all vertebrate tissues. HA was extracted from the capsules of *Streptococci* groups A and C by F. Kendall in 1937. Since *Streptococci* groups are now the most reliable and cost-effective source for the industrial synthesis of hyaluronic acid, this research was of significant scientific and practical value (Kendall et al., 1937).

The establishment of the molecular structure of the polysaccharide molecule was made possible by identifying enzymes that could specifically degrade hyaluronan. Nuclear magnetic resonance spectroscopy (NMR), a potent method for studying the structure of polysaccharides, was not yet widely used. Currently, monosaccharide biopolymer residue composition, centers for substitution reactions, sequencing, and three-dimensional structure can all be determined using NMR.

In a 1943 publication, E.A. Balazs and L. Piller detailed research on the function of hyaluronan in canine knee joints. They discovered enough viscous mucin in the synovium's intercellular matrix to replenish the mucin taken from the knee (Selyanin et al., 2015).

These findings paved the way for additional research on the function of hyaluronan in healthy and damaged joints. C. Ragan and K. Mayer described the discovery of hyaluronan in the synovial fluid of rheumatoid arthritis in a highly significant paper published in 1949. It was the first study to assess the content and viscosity of hyaluronan in average and diseased synovial fluids (Ragan & Meyer, 1949).

In the early part of the 20^{th} century, hyaluronic acid research took on some significant directions. They have recently grown into branches within various scientific disciplines, such

as polymer chemistry, radiochemistry, biochemistry, molecular biology, medicine, and glycobiology.

The various observed viscosities of the hyaluronan solutions in the presence of the different inorganic salts intrigued researchers and left them in awe of science. The solution in distilled water had the highest viscosity. It was suggested that the pH and ionic strength of the solution might influence the viscosity. Although R. Fuoss first exclusively described this phenomenon for solutions of synthetic polyelectrolytes, it is now widely known (Fuoss, 1948).

When E.A. Balazs' study about the physicochemical properties of HA was published in 1951, it was regarded as the beginning of fundamental research on these properties (Balazs & Laurent, 1951). The solution's viscosity completely vanished when HA was initially sterilized using UV light.

A. Caputo's 1957 X-ray exposure of the hyaluronan solution produced a comparable result (Caputo, 1957). Later, it was discovered that even at low initial doses of ionizing radiation absorbed, HA degrades when subjected to gamma radiation or electron beams.

The radiochemistry of biomolecules is undergoing extensive research into the polysaccharide radiolysis processes, which are connected to polymer degradation and involve free radicals. The discovery that hyaluronan speeds up cell growth provided some of the first evidence that HA can interact with living cells, unlike sulfated polysaccharides.

Hyaluronan has also been seen to start some cell aggregation. It was the first proof that the polysaccharide explicitly bound to the cell surface. Many receptor proteins attached to the

HA cytoplasmic membrane have been identified, including the high-affinity receptor CD44 and RHAMM (receptor for hyaluronan-mediated motility).

2.2. Production Strategies

Different HA production processes have been well-researched and employed for mass production (Figure 2.1, Table 2.1).

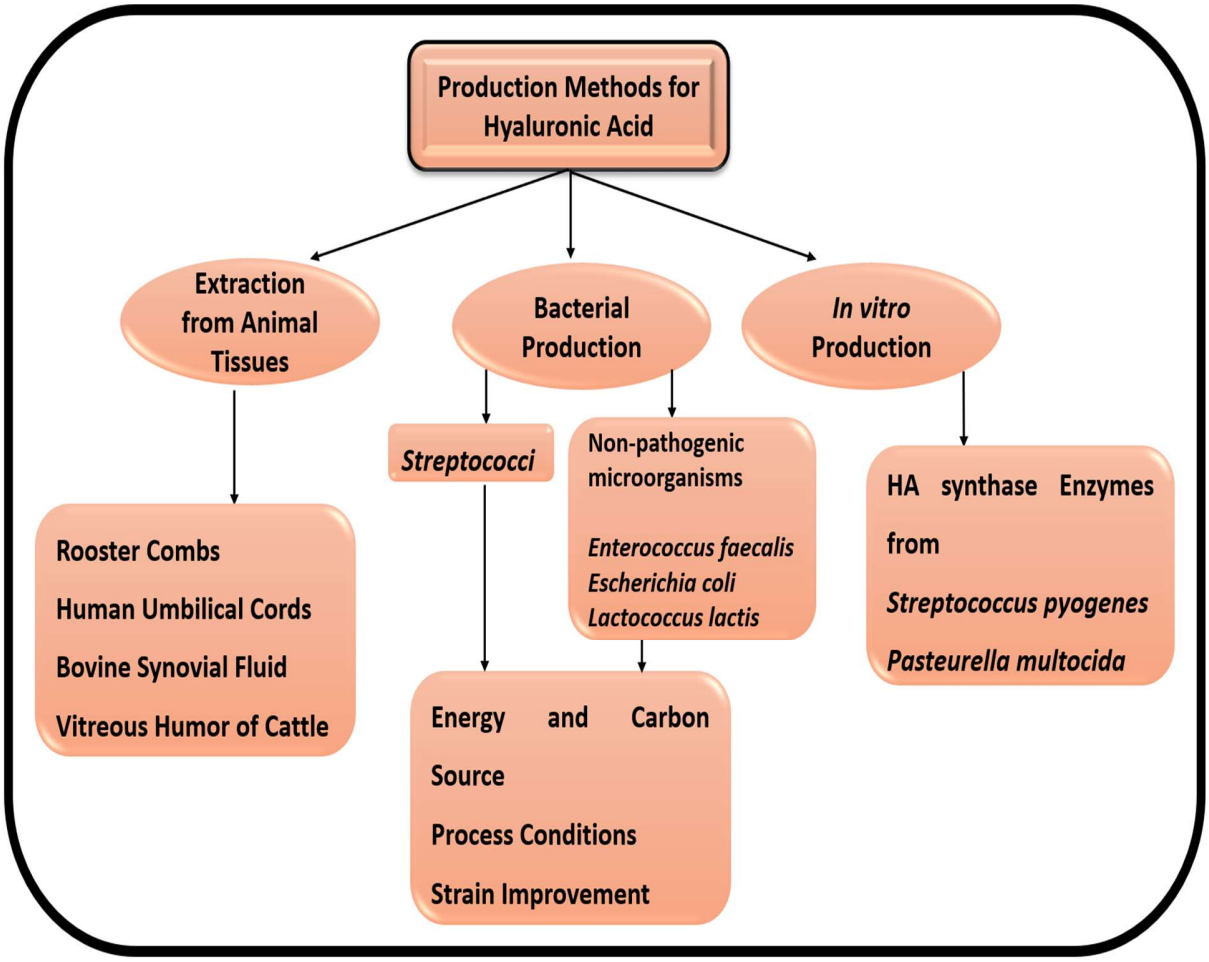

Figure 2.1. The schematic representation of commonly used methods utilized for Hyaluronic acid production includes extraction from animal tissues and microbial and *in vitro* production (Boeriu et al., 2013).

Table 2.1. Comparison of different technologies of HA production (Boeriu et al., 2013), highlighting their advantages and disadvantages

Process	Advantages	Disadvantages
Extraction	➢ Cheaper raw materials ➢ Naturally extracted product ➢ Well-developed technique ➢ Higher Molecular weight products up to 20MDa	➢ Lower yield ➢ Chances of degradation of the polymer ➢ Ambient purification required ➢ Chances of contamination are high
Fermentation	➢ Discreet technology ➢ Higher yield ➢ Higher Molecular weight products, 1-4Mda	➢ Genetically modified organisms are used ➢ Chances of contamination are high with bacterial protein, nucleic acids, etc.
Enzymatic Synthesis	➢ Skilled technology ➢ The quality of the product is constantly maintained ➢ Desired molecular weight, 0.55-2.5 MDa	➢ Technology is still emerging ➢ Demonstration of economic viability must be done

	➢ Contamination chances are negligible	

Initially, the procedure of removing HA from animal tissues was used for laboratory tests to characterize and identify the polymer and learn more about its biological potential and applications.

HA was extracted from all the vertebrate tissues and described, such as the cartilage of sharks, the pericardial fluid of the rabbit, pigskin, the umbilical cord, synovial fluid, and vitreous humor of the eye (Boeriu et al., 2013). As an alternative source of animal husbandry, HA extraction from fish's eyes was reported (Amagai et al., 2009b).

Pharmaceutical-grade HA was achieved in 1979 despite the several extraction techniques used in the past. Balazs found an effective way to extract and purify HA from the human umbilical cords and rooster combs, which became the base for the commercial fabrication of HA (Balazs, 1979).

HA is water-soluble. Water soluble components are primarily stored in compartmentalization or binding to cell membranes. For their purification, polar organic solvents like methanol or ethanol are used in the extraction process (Shimizu & Li, 2006).

Mostly HA, when extracted, is found in a complex form with different biopolymers; for instance, members of the lectican family, such as aggrecan and versican, as well as other proteoglycans, can combine to form a complex with HA outside the cell (Kakehi et al., 2003;

Reddy & Karunakaran, 2013) which is non-desirable; therefore, extracting pure and high molecular weight HA from animal tissues is a challenging procedure (Boeriu et al., 2013).

The first challenge is the problematic extraction processes brought on by grinding, acid treatment, and repeated extraction with organic solvents. Thus, the extraction processes have always had technological limits. This degrading process negatively impacts HA's yield and polydispersity (Boeriu et al., 2013). Secondly, all the undesirable contaminants that form complexes with HA outside the cell during its isolation process are removed (Fraser et al., 1997).

Isolating HA from these complexes is a complex process that involves various purification steps, including the use of detergents, nonsolvent precipitation, precipitation with organic solvents, HA ion-pair precipitation, a proteolytic enzyme, and so forth (Boeriu et al., 2013). Other contaminants and degradation products were removed by using ultrafiltration and chromatography techniques.

Despite the thorough purification, the chances of contamination in HA extracted from animal tissues with nucleic acid and proteins are high. The nature and quantity of the contaminants can vary with the source of the extraction, as the content of proteins and nucleic acid was high in the HA extracted from bovine vitreous humor and human umbilical cord as compared to the one isolated from the bacterial capsule (Shiedlin et al., 2004).

These impurities can lead to different diseases, so the purification process was constantly improved over the years to accomplish the desired high standards of the product for various medical applications.

An essential source for the industrial synthesis of HA is animal waste, which offers up to multiple tones of pharmaceutical-grade HA annually. Diosynth of the Netherlands, Genzyme, Pfizer of the USA, and Pharmacia of Sweden are the primitive corporations that manufactured HA from animal tissue waste at the commercial level.

High MW HA varying from a few thousand to 2.5 MDa is obtained from the extraction process and is available in the markets (Kogan et al., 2007). Extraction of HA from animals was the first technique implied. Biotechnological production of HA was an alternate process as it was cost-effective, less contamination-prone, and environmentally friendly (Chien & Lee, 2007a; Chien & Lee, 2007b; Chong & Nielsen, 2003; Liu et al., 2008b).

Bacterial fermentation has evolved over the past two decades as an outstanding procedure for producing HA, as shown in Table 2.2. Understanding their biosynthetic pathways is essential to the fermentative production of secondary metabolites (Figure 2.2.).

Table 2.2. List of microorganisms producing HA with their respective production medium, yield, and molecular weight

S.No.	**Microorganism**	**Production Method**	**Production Medium**	**HA Yield**	**Molecular weight**	**References**
1	*Streptococcus thermophillus*	Genetic modification	Streptococcus–*Escherichia coli* shuttle vector, pBE31, was transfected	1.2g/L	1.0×10^{6} Da	(Izawa et al., 2011)

			in *S. thermophillus*			
2	*Streptococcus zooepidemicus*	Fermentation	Glucose concentration was studied at 10-60 g/L	0.589g/L	NA	(Don & Shoparwe, 2010)
3	*Lactobacillus acidophilus*	Genetic modification	Vector pJH181.3 containing *hasA* and *hasB* was transformed into *L. acidophilus*	1.7g/L	<27 KDa	(Chahuki et al., 2019)
4	*Streptococcus zooepidemicus*	Fermentation	Mussel processing wastewater and tuna peptone	3.67g/L	2500KDa	(Vázquez et al., 2010)
5	*Streptococcus*	Fermentatio	Optimized	0.87g/L	NA	(Gedikli et

	equi	n	different carbon and nitrogen source			al., 2018)
6	*Lactococcus lactis*	Recombinant Technology and Fermentation	Sucrose, Nisin induction	6.09g/L	NA	(Sunguroğlu et al., 2018)
7	*Pichia pastoris*	Recombinant Technology and Fermentation	Yeast-extract-peptone dextrose medium with zeocin at 100 µg/mL	1.7g/L	2.5 MDa	(Jeong et al., 2014)
8	*Escherichia coli*	Recombinant Technology and Fermentation	Glucose and Galactose	29.98 mg/mL	1386.5Da	(Woo et al., 2019)

9	*Streptococcus thermophilus*	Fermentation	Soybean peptide supplementation in milk	100 mg/L	2000KDa	(Izawa et al., 2010)
10	*Streptococcus zooepidemicus*	Fermentation	Cheese whey	4.0g/L	>3000KDa	(Amado et al., 2016)
11	*Streptococcus zooepidemicus*	Fermentation	Sugarcane Molasses	2.825g/L	1.3.5 × 10^3	(Pan et al., 2017)
12	*Streptococcus zooepidemicus*	Fermentation	Molasses and Sheep wool protein hydrolysate	3.54g/L	NA	(Arslan & Aydogan, 2021)
13	*Streptococcus zooepidemicus*	Fermentation	Palmyra palm based medium	0.54 ± 0.08 g/L	0.96 MDa	(Rohit et al., 2018)
14	*Streptococcus zooepidemicus*	Fermentation	Bored coffee beans based medium	2.7g/L	NA	(Flores-Méndez et al., 2021)
15	*Streptococcus zooepidemicus*	Fermentation	Sucrose-modified iron nanoparticles	0.226g/L	1.37 × 10^6 Da	(Wang et al., 2021)

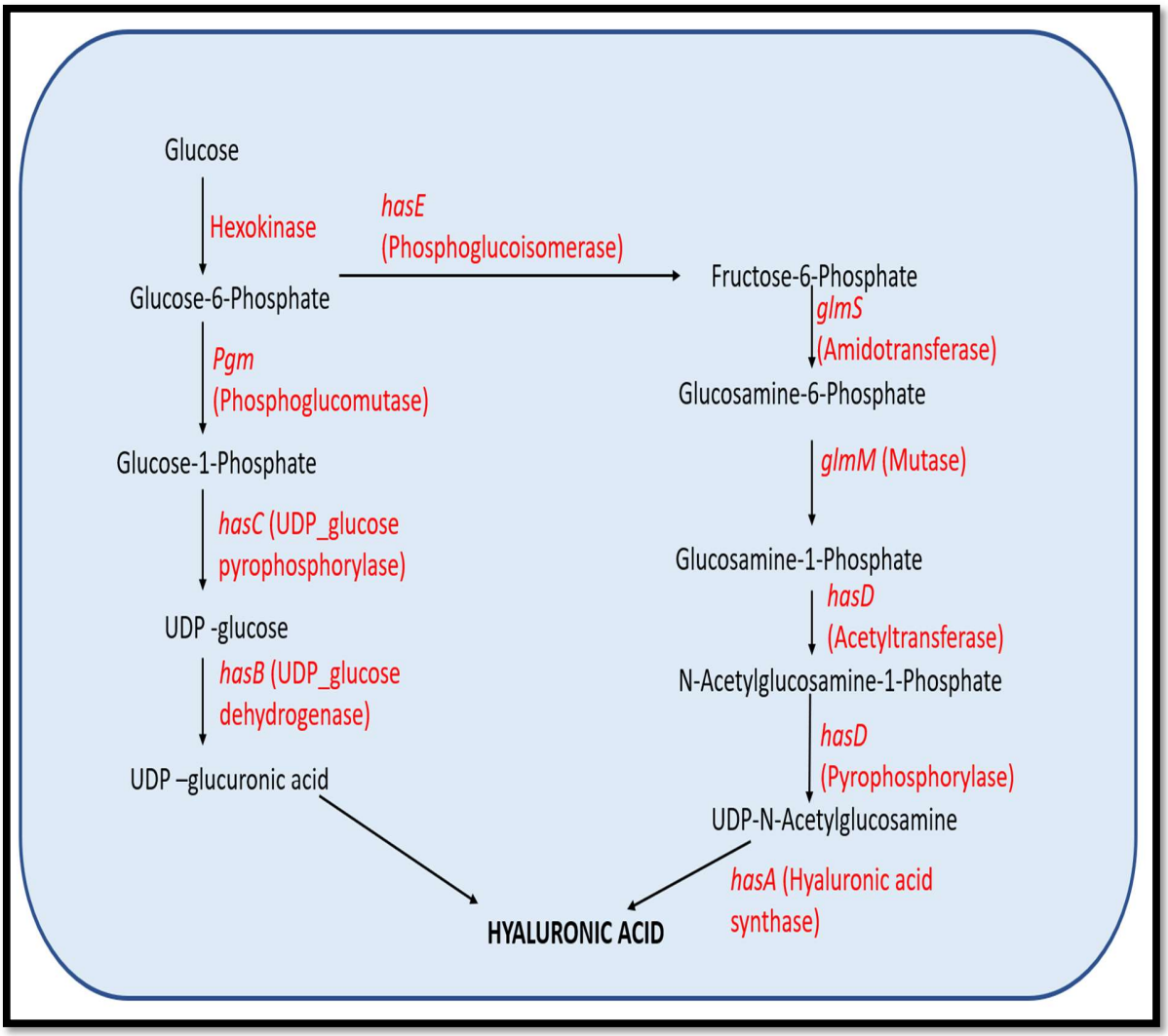

Figure 2.2. Biosynthetic pathway for HA in *S. zooepidemicus*. With the help of hexokinase, glucose is first converted into glucose-6-phosphate, which indistinctly follows two different routes to form UDP-glucuronic acid and UDP-N-acetylglucosamine. With the use of HA synthase, both bound together to form hyaluronic acid.

HA has a sugar backbone that is made up of fructose-6-phosphate and glucose-6-phosphate. Two different sets of reactions lead to the formation of HA. In the initial set, glucose-6-

phosphate is transformed into UDP-glucuronic acid in a series of reactions governed by various enzymes at each step; this is the first precursor of HA.

While in the second assortment, to form the second precursor of HA, fructose-6-phosphate is transformed into UDP-N-acetylglucosamine. Therefore, the higher growth rate of cells is unsuitable for HA synthesis (Figure 2.3.) (Armstrong et al., 1997).

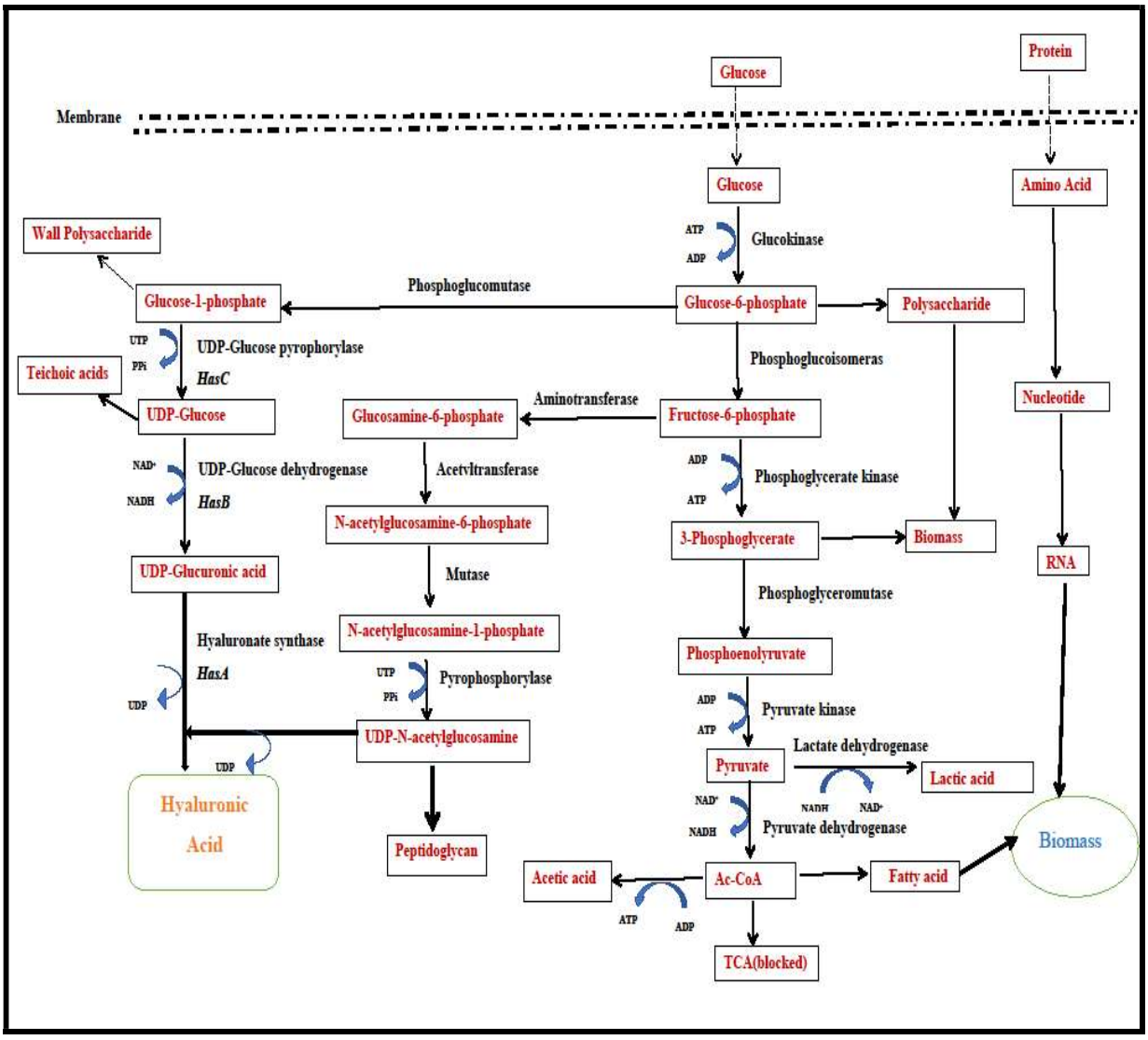

Figure 2.3. Biosynthetic pathway of hyaluronic acid in *S. zooepidemicus*. This figure shows

the conversion of glucose into hyaluronic acid and the microbial biomass production running parallelly (Liu et al., 2011a).

Microorganism-based HA synthesis has been the subject of extensive research. Nevertheless, many limitations of organisms may prevent its mass manufacturing, which can be avoided by using enzymes from the Class I HAS and Class II HAS family members in *in vitro* synthesis. Furthermore, these cell-free systems are still not optimal due to their meager yields and inability to serve as a substitute for industrial production (Chong et al., 2005; Sze et al., 2016).

The production of HA has been increased using specific nanoparticles and genetic engineering, which involves introducing crucial genes (such as *hasA, hasB, hasS,* and *glmU*) (Sze et al., 2016) associated with HA synthesis. The HA synthase genes from the *Streptococci* are incorporated into various microbial hosts, which include *E. coli* (Yu & Stephanopoulos, 2008), *L. lactis* (Chien & Lee, 2007a), *Bacillus sp*. (Widner et al., 2005), and *Agrobacterium sp* (Mao & Chen, 2007) through different plasmid vectors such as pSJR3 (co-expressing *hasA*, *hasB,* and *hasC* genes).

HA production could sometimes be boosted sevenfold, or its molecular weight could be increased by partially blocking the glycolytic route and deflecting carbon flux toward HA formation (Shah et al., 2013). The main obstacle of generating HA with a comparatively uniform length has not yet been overcome.

Consequently, the medium's viscosity at HA concentration greater than 4g/L restricts oxygen transfer, resulting in an anaerobic surrounding that regulates carbon flow more toward

biomass formation than HA, resulting in polluting byproducts such as lactate (Yao et al., 2021).

Streptococci sp., peculiarly *Streptococcus equi* sub sp. *zooepidemicus*, are typically the leading producers of HA. *Streptococci*, a gram-positive bacteria with 49 species and eight subspecies, is exceptionally diverse and heterogeneous. It is based on serological responses to various polysaccharide compositions of cell walls to categorize the Lancefield *Streptococci* group.

The *S. zooepidemicus* has been significantly explored by many research groups for HA production. Various fermentation modes have been studied for *S. zooepidemicus*, including continuous, fed-batch, and repeated batch fermentation (Liu et al., 2009a; Liu et al., 2008a; Liu et al., 2009b). Regulating the bacterial growth rate utilizing continuous or fed-batch mechanisms to achieve increased metabolite yield can counteract the specific growth rate's intrusive effect on metabolic products (Liu et al., 2008a). The traditional method of producing HA in batches (Chen et al., 2009a; Marcellin et al., 2009) was changed to a fed-batch module; a decrease in the fermentation time was observed with increased output (Liu et al., 2008a).

Since the fermenter responds quickly, fermentation in a continuous mode for HA generation helps extend the growth cycle, minimize waste, and reduce MW polydispersity (Huang et al., 2008; Wang et al., 2016b). A two-stage fermentation method with a fragmented control approach was used to synthesize HA since HA chain extension happens in the primary fermentation stage, and HA accumulation proceeds in the latter. The initial fermentation stage (31°C, pH 8.0) and the accumulation stage (37°C, pH 7.0) were designed to increase

the MW of HA. The suggested two-stage fermentation produced an ideal outcome with high HA titers (Liu et al., 2018).

The HA (>1MDa) produced on an industrial scale, either by the fermentation of genetically engineered bacteria or through the extraction of animal tissue, is suitable for use in aesthetic and biological applications (Liu et al., 2011a). The potential of contamination with other viruses, which have compatibility difficulties and require time-consuming, expensive DSP removal techniques, affects the extraction of HA from animal tissue.

The *Streptococci* C and A groups started the bacterial fermentation process that produced HA, but the toxic byproducts hampered them. The primary by-product of HA fermentation is lactic acid, and as it accumulates, cell growth and HA synthesis are severely inhibited. Acetic acid production also hampers HA production (Liu et al., 2011a). The HA biosynthesis pathway genes were inserted into the genetically engineered bacterium (Gram +ve bacteria), which began producing HA. The microbial biotechnological HA is manufactured using a *B. subtilis*-dependent synthesizing system that combines three overexpressed native *B. subtilis* precursor genes with expression constructs of the *hasA* gene from *S. equisimilis.*

The HA manufactured by genetically engineered *B. subtilis* is recognized as GRAS because there are no exo-endo-toxins in the output streams (Widner et al., 2005). Different plasmid vectors have been designed to express HA synthase genes and produce HA from various host bacteria. Artificial operons were introduced into the *B. subtilis* genome using the plasmids pNNBT20 and pNNBT21 to express HA synthase proteins (Widner et al., 2005). *L. lactis* has introduced the plasmids pEIrkA, pEIrkB, and pEIrkAB, each containing the *hasA, hasB,* and *hasA* and *hasB* genes from *S. equi* subsp. *zooepidemicus* (Chien & Lee, 2007a).

Small-scale fermenters successfully synthesize HA with genetically engineered bacterial cultures up to 6-7g/L yields. Intense media viscosity of the large-scale fermenters causes impoverished mass-transfer rates and inadequate mixing for better results (Liu et al., 2011a). Additionally, obtaining a monodisperse-HA in the microbial biotechnological production process is troublesome because it relies on the growth circumstances.

Due to the disadvantages of mass manufacturing, either through bacterial or animal origins, utilizing hyaluronan synthase (has), a cell-free synthesis technique has been created. The Class I-has of *P. multocida* are integral membrane proteins requiring time-consuming obstructions. They have impeded functions without being intimately associated with the phospholipid layer, making them the most practical options for commercial cell-free HA propagation.

After the membrane motifs (residues 704–972) were deleted, Class II– created a soluble enzyme that catalyzes "pmhas1–703" that could manufacture the HA (Jing & DeAngelis, 2000). Class II-has-based *in vitro* (cell-free) production methods can combine HA oligomers to produce 1-2 MDa HA with tuned processability and low polydispersity (Jing & DeAngelis, 2004). By forbearing the accelerated accumulation of sugars at the oligomeric ends, the inclusion of HA oligomers skips the first glycosidic linkage development, which is frequently a rate-limiting step and results in the acquisition of a high MW-HA but falls short of creating larger quantities of HA. Since then, numerous researchers have experimented with cloning the genes of host bacteria (non-pathogenic), such as *E. coli*, that code for Class-I or -II enzymes (Mao et al., 2009). Enzymes from classes I or II work together in bacterial expression systems to lengthen the HA polymer (Yu & Stephanopoulos, 2008).

One of the more environmentally friendly methods for manufacturing high-value HA is the creation of HA biosynthesis pathways employing fundamental molecular building blocks in recombinant microorganisms (de Oliveira et al., 2016). Understanding how HA's biosynthesis quality is primarily based on the 'has' enzyme activity is essential to building a cell factory that produces HA using a synthetic biology technique. A nucleoside inosine-based synthesis approach was suggested using a genome-scale model (GEM), which highlighted the need for carefully designed GEMs for further development of HA biogenesis and led to a treble enhancement in HA titer ratio (Badri et al., 2019).

Using a two-stage induction technique and two synthetic operons harboring the *P. multocida hasA* gene and *B. subtilis hasB* and *hasC* precursor genes, the rational design-based biogenesis, a neoteric development in synthetic biology, offers the foundation for static HA yield with 6.8 g/L. A new synthetic biology technique produces twice as substantial HA outputs by keeping up with the glucose-6-phosphate isomerase in *S. zooepidemicus* (Chen et al., 2009b).

Carbon flow rerouting is one technique for increasing HA yields; as part of the technique, numerous HA syntheses are accomplished by reducing the expression of glycolytic pathway enzymes and enabling the basic physiological requirements of bacteria to be satisfied (Zhang et al., 2016a). The expression of the driven pathway enzymes, knock-out routes, antisense RNA-mediated attenuation, and additional promoter inclusions have all been shown to work together genetically to shift the carbon flux to HA generation, culminating in a 28.7 g/L HA concentration (Cheng et al., 2019).

The recommended efficient synthetic biology techniques were lab-scale results that urgently needed scaling by applying synthetic biology machinery to create dominant HA-producing microbial strains (Pourzardosht & Rasaee, 2017). *Streptomyces* sp. might be utilized in a biorefinery context to establish a microbial conversion strategy for the synthesis of HA as an enhancement to the lignocellulosic biorefinery.

The fermentation technical advancements toward larger HA productivity can be further expedited by creating a flawless downstream approach for expanded commercial uses of HA.

2.3. Purification Strategies

Downstream and purification operations are crucial to overcoming obstacles and creating HA with a high purity index and MW (Yao et al., 2021). Most papers on HA downstream procedures depend on laboratory-scale techniques, whereas only a few rely on industrial-scale methods (Rodriguez-Marquez et al., 2022). When under physiological pH, HA's molecular structure causes it to hold onto pollutants in its highly hydrated and negatively charged form.

Since the publication of the research, HA from different origins has been used to study the purification and separation procedures involving numerous downstream processes (Figure 2.4). The HA downstream process operations that have received the most attention are the filtration, adsorption, precipitation, and ion exchange procedures.

Rangaswami *et al.* (Rangaswamy & Jain, 2008b) improvised a single solvent precipitation system for the purification procedure. Single-solvent precipitation was utilized to reduce the solvent needed in other unit operations. The ability to produce pure and uniform material is the principal benefit of the precipitation process.

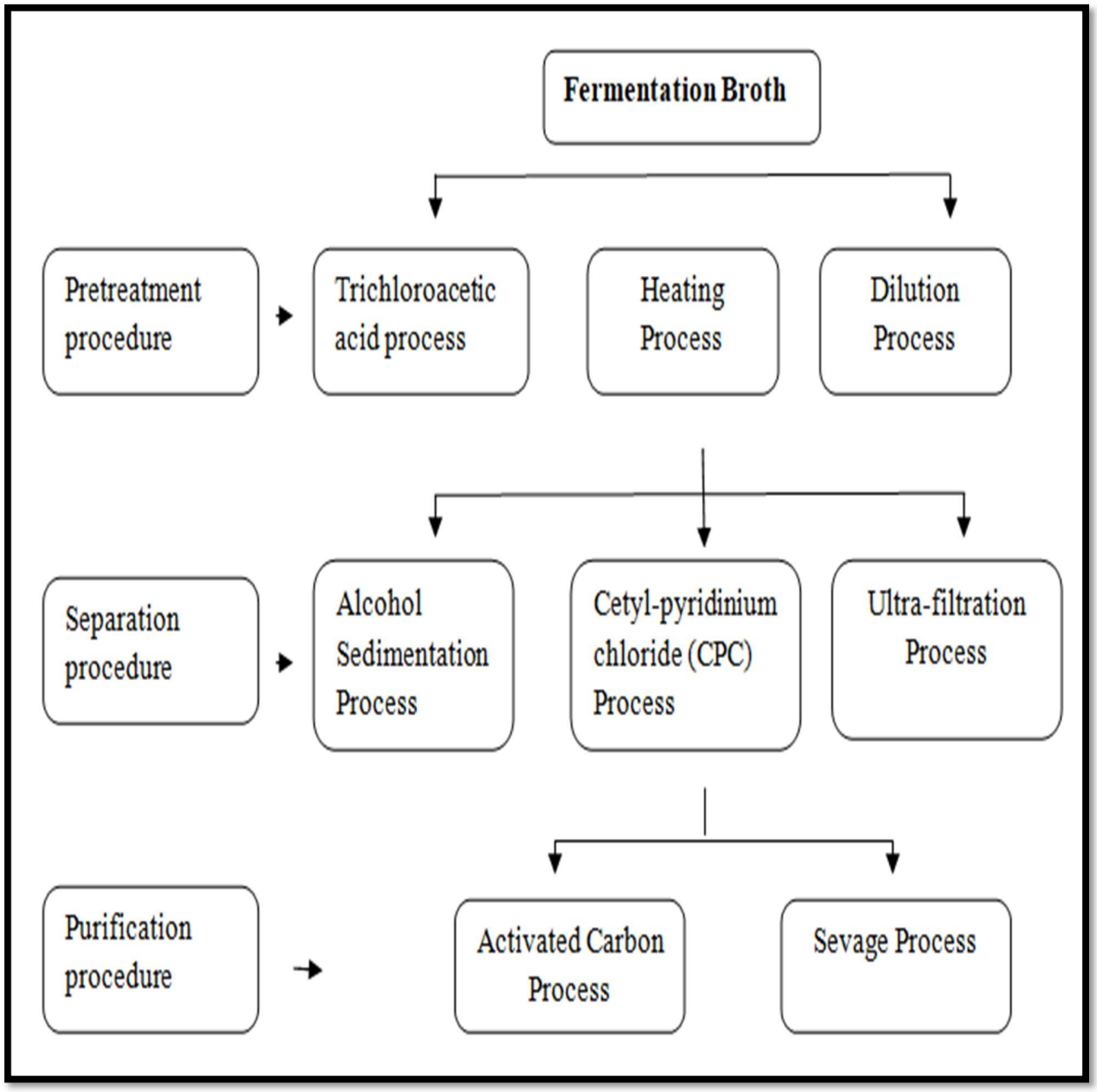

Figure 2.4. Flow sheet diagram of purification strategies for extraction of HA from the fermented broth. The purification process is divided into three main steps pre-purification, separation, and final purification (Wang et al., 2014).

However, the main drawback is separating salts following the precipitation procedure. If the precipitation occurs intermittently, it is incredibly challenging to maintain stable product

quality throughout the entire precipitation process. Utilizing a single solvent precipitation system decreases the proportion of salts in the solution and reduces solvent usage. Specifically, the solvent was used to dilute the HA in diafiltration, typically done at low concentrations to enhance the HA's quality. It resulted in 65% of the HA recovery (M_w~ 4 × 10^6 Da) with less than 0.1% protein contamination (Rangaswamy & Jain, 2008b).

Cleland and Sherblom (Cleland & Sherblom, 1977) isolated HA from the bovine nasal septum through precipitation utilizing cetylpyridinium chloride, and 96% glucosamines were obtained (Cleland & Sherblom, 1977).

Similarly, Amagai *et al.* utilized the cetylpyridinium chloride precipitation technique for HA extraction from fish eyeballs, yielding 10.5 mg of hyaluronan from a single tuna eye (Amagai et al., 2009b). The method of precipitating human umbilical cord remains using ammonium quaternary salt solution after treating it with sodium chloride solution was developed by Lago *et al.* The hyaluronan ammonium quaternary salt complex is then dissociated from the solid using a calcium chloride solution, and ethanol precipitates it (Lago et al., 2005).

Yang and Lee used co-precipitation to aid HA recovery by conjugating chitosan with magnetic nanoparticles. Here, a pH lower than a point of zero charges favored capturing HA; about 39 mg per gram of particle was captured at pH 6 (Yang & Lee, 2007).

Organic solvent-based purification techniques were once considered expensive for large-scale downstream operations. As precipitation results are very effective, organic solvents are frequently used in laboratory-level HA production studies. However, the cost is unsuitable for abundant quantities; many research reports suggest membrane technologies like

membrane filtration (MF), ultrafiltration (UF), or diafiltration (DF) are the best for large-scale HA purification instead of using organic solvents.

It has been found that tangential flow MF and UF effectively separate HA from *S. zooepidemicus* broth medium. At this stage, a serial procedure using microfiltration and ultrafiltration membranes produced a high yield (89%) and less water (Zhou et al., 2006).

In a different method, DF was used to extract HA produced by microbes. The highest purity grade (about 90%), with a yield greater than 90%, was reached after seven diavolumes. For example, HA purity stayed unchanged with six diavolumes, whereas HA production declined by 20% after ten diavolumes.

The number of diavolumes explicitly characterized HA's evolution in yield and purity. Controlling the concentration of salts in the solution was essential for the DF because salts could change the structure of hyaluronan due to the electrostatic shielding of the carboxyl groups. (Oueslati et al., 2015).

In a study, purification of HA was carried out by passing the HA solution through a filter (0.22mm). Then, UF and DF were carried out utilizing a 300 kDa membrane to sterilize HA after dilution with pyrogen-free water further (Kanala et al., 2011). The relevant impact of electrostatic interactions between membrane materials and the solutes must be considered when working with the purification and recovery of molecules via membrane processes (Castro-Muñoz et al., 2021; Hadidi et al., 2016).

A polyethersulfone UF membrane cassette with a nominal molecular weight cut-off (NMWCO) of 300 kDa was used for HA separation under a pressure range of 1-0-1.5 bar. After that, a 0.22 m cellulose acetate filter was used to filter the diafiltered broth after being

subjected to an adsorbent treatment of 1% activated charcoal for 2-3 hours while continuously stirring. The end outcomes revealed a high-purity HA with an MW ranging from 0.6 to 1.8 MDa and an overall HA yield of roughly 0.8-1.0 g/L (Rajendran et al., 2016).

Using DF methodology, a low-cost fish eyeball procedure produces HA with a clinical grade purity (more than 99.5%) (Murado et al., 2012). In order to improve the accumulation of the final yield and lessen environmental problems associated with conventional methods, electrofiltration has been investigated as a post-synthesis step for HA.

Comparing electro-filtration-based HA extraction to filtration tests without an electrical field while maintaining the same molecular weight and structure has increased concentration factors. (Gözke et al., 2017). The capacity of chemicals to be selectively retained on the surface of porous substances serves as the foundation for the adsorption process. Adsorption is typically utilized in batch mode to purify HA, followed by precipitation and filtration.

Activated charcoal, silica gel, alumina, and resins are the most common adsorbents used for HA purification (Choi et al., 2014; Han et al., 2009; Rangaswamy & Jain, 2008b). With a yield of 2.3 g/L, the glucuronic acid imprinted particles can be reused repeatedly without significantly decreasing adsorption capabilities (Akdamar et al., 2009).

In a different method, a stiff and durable substance was made for the separation and isolation of HA by fusing the mechanical properties of cryogel with the discriminating of glucuronic acid imprinted polymer particles. HA was isolated from the fish eye and microbially fermented broth (Ünlüer et al., 2013).

Wibowo and Lee carried out the HA adsorption with maximum adsorption capacities of 184 mg/g and 351 mg/g for Si-Quaternary ammonium-containing compounds and choline

surface-functionalized cotton fibers, respectively (Wibowo & Lee, 2010). HA could be efficiently retrieved from a *B. subtilis* culture with a 15 mg/g capacity utilizing Si-QAC-modified antimicrobial cotton fiber in situations of high contamination levels.

The effectiveness of electrophoresis, a technique frequently used for protein separation and identification, depends on the gel employed and the target molecules' density, size, and purity. Because electrophoresis has a poor protein removal capability compared to other processes, it is uncommon to employ it for HA purification.

Hong *et al.* achieved HA quantification through capillary electrophoresis, and hyaluronate oligomers (80 kDa) were accomplished using columns packed with a highly viscous polyacrylamide matrix (Hong et al., 1998). Grundmann *et al.* demonstrated that strong electrical field strengths, short-length, small-ID capillaries, and an adjusted buffer composition may achieve high separation efficiency in conjunction with quick migration times.

Additional tests revealed no worsening effects when injecting protein samples, and the approach was effectively used on hyaluronan digest samples (Grundmann et al., 2012). At the commencement of the processing stage, Murado et al. documented employing protein electrodeposition in conjunction with diafiltration to purify and recover HA (Murado et al., 2012).

2.4. HA Applications

The elemental composition and physical attributes determine the end-use of HA and characteristics like viscoelasticity, lubricity, biocompatibility, immunostimulation, and many more properties (Figure 2.5, Table 2.3). OA treatment, joint injections, eyes, and plastic

surgery, components for skin burns, and anti-aging therapies all contain HA (Abdallah et al., 2020; De Bartolo et al., 2012; de Oliveira et al., 2016; Dovedytis et al., 2020; Robert, 2015).

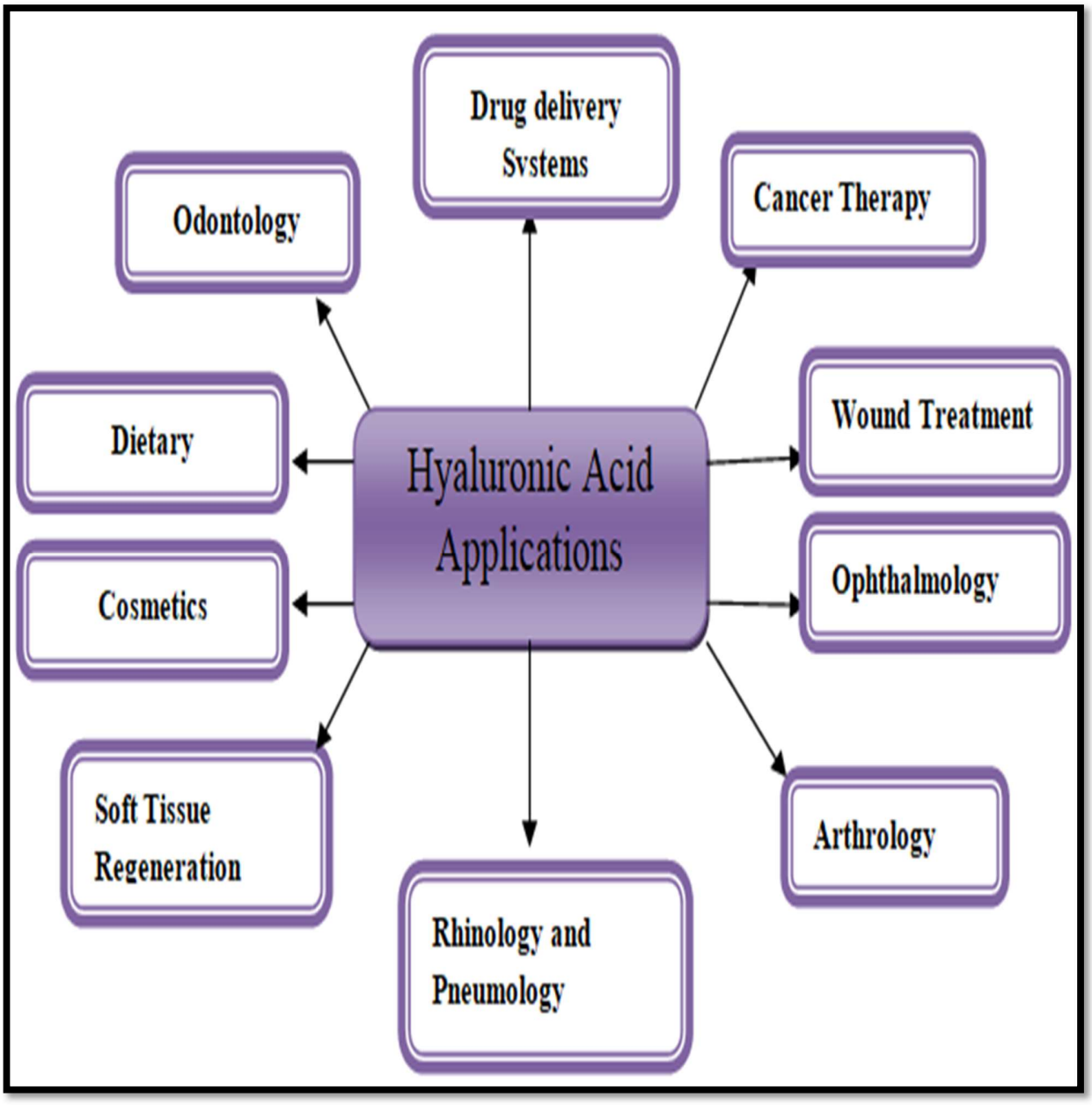

Figure 2.5 Applications of Hyaluronic Acid in different biomedical aspects (Boeriu et al., 2013).

Table 2.3. Application of hyaluronic acid in different fields

Drug Delivery System	
Hyaluronic acid gel systems	Delivers siRNA (Lee et al., 2007)
Amphipathic vector hyaluronic acid-PEI (HAP)	It was less toxic, effectively separated DNA from the complex, and prevented nuclease degradation (Yao et al., 2010).
Hyaluronic acid-spermine conjugate	It increases the effectiveness of encapsulated DNA transfection (Shelma, 2022)
Cancer Therapy	
Hyaluronan–doxorubicin nanoconjugate	The conjugate significantly slows the spread of breast cancer *in vivo*, increasing survival rates (Cai et al., 2010).
Hyaluronan- paclitaxel hydrogel	It shows an anti-tumor effect on human ovarian cancer (Bajaj et al., 2012).
Paclitaxel loaded hyaluronan nanoparticles micelles.	A focused and effective chemotherapy regimen for cancer cells that overexpress CD44 (Thomas et al., 2015)
Osteoarthritis	
Durolane HA	It is a single-injection method that is quick, effective, and secure for treating osteoarthritis (Leighton et al., 2018).
Supartz (Sodium hyaluronate)	It improves gait patterns, strength of muscles, and balance (Bronstone et al., 2019).
Hyaluronic acid-Chitlac,	It reduces inflammatory conditions caused by osteoarthritis (Tarricone et

a lactose-modified chitosan	al., 2021).
Tissue Healing	
Sodium hyaluronate (Healon®)	It gives maximum endothelium protection and evident postoperative manifestations (Pape & Balazs, 1980).
hyaluronan-enhanced expanded polytetrafluoroethylene	It has excellent potential for cardiovascular transplant material (Bui et al., 2018).
chitosan conduit combined with HA	It prevented sciatic nerve extraneural scarring and adherence to some degrees and encouraged neural regeneration and recovery (Li et al., 2018).

Due to its role in the extracellular matrix (ECM) and the variety of derivatization scenarios it can undergo, HA is frequently employed in drug delivery through various channels, including cutaneous, topical, and ocular (intravitreal, periocular, subretinal), oral, and nasal. HA can be integrated into multiple molecular architectures or coupled with therapeutic molecules (as prodrugs) (microparticles, nanoparticles, gels, microspheres, polymersomes, polyplexes, micelles, liposomes, implants, and many more).

Higher therapeutic efficacy and improved physicochemical characteristics are found in the HA constructs. Targeting for skin disorders, the regulated release of proteins, cancer therapy, antibiotics, and antiseptics are just a few examples of how HA is used in drug delivery (Bayer, 2020; Dubashynskaya et al., 2019; Fallacara et al., 2018; How et al., 2020; Huang & Huang, 2018; Trombino et al., 2019; Vasvani et al., 2020).

2.4.1. Applications in oncology

The realm of oncology is another area where HA may see more application. The stroma of many tumors and the surrounding tissue matrix exhibit an elevated HA concentration, which poses a challenge. Tumors of epithelial origin have a particularly notable rise in HA levels. A dismal prognosis typically accompanies this process.

Apoptosis, drug resistance, and invasiveness are all caused by an increase in HA production. Increased interstitial pressure is linked to increased HA in the tumor environment; the blood vessels can narrow due to this condition. This condition causes drug resistance and hypoxia.

In addition to the aforementioned physicochemical characteristics, HA plays a significant part in the physiology of tumors, particularly concerning its impact on the receptors of tumor cells (Jacobetz et al., 2013; Jiang et al., 2012; Kultti et al., 2012). This information can result in the development of several HA-based cancer therapeutic strategies.

First, mentioning the conjugation of paclitaxel (PXT) and docetaxel (DOX) is essential. Paclitaxel (PXT) and docetaxel (DXT) are anti-cancer chemotherapy drugs. Due to its hydrophobicity and undesirable side effects, PXT alone is unsuited for intravenous administration (Auzenne et al., 2007; Lee et al., 2008; Wu et al., 2017).

The PXT-HA combination appears to overcome restrictions and is sufficiently hydrophilic. Hydrophobic drug molecules can be added to HA micelles for targeted drug delivery to cancer cells.

Drugs that are hydrophilic and lipophilic can both be put into polymersomes. The primary benefits of the previously described structural modulations are the improvement in solubility

and the ability to target CD44 receptors on tumor cells. When HA alters mesoporous silica nanoparticles, cells overexpressing CD44 are more likely to take them up. Dendrimers and liposomes are different nanomaterials with the potential to be effective in cancer treatment. Moreover, HA-coated nanoparticles are highly desirable for cancer treatment.

NIR-loaded nanoparticles, oxide nanoparticles, gold nanoparticles, Prussian Blue nanoparticles, functionalized graphene, and other particles are employed in hyperthermia, which increases the temperature of tumor cells to around 42–46°C (related to magnetic hyperthermia treatment). Immunotherapy, photodynamic, and sonodynamic therapy also used HA-based nanoparticles (Chis et al., 2020; Kim et al., 2018; Kim et al., 2019a; Kim et al., 2019b; Lee et al., 2020; Li et al., 2021; Wickens et al., 2017).

2.4.2. Applications in osteoarthritis

As HA occurs naturally in the joint capsule, synovial fluid, and articular cartilage, orthopedics frequently uses it. This substance mainly treats joint conditions like OA or rheumatoid arthritis. The most prevalent joint disease, OA, causes significant impairment and reduces the caliber of living. In this disorganization, there is an imbalance in the middle of the formation and degradation of articular cartilage, with the latter occurring more frequently (Gupta et al., 2005). In this state, intra-articular modifications such as a reduction in GAG, a growth in proteoglycans and collagen-degrading enzymes, and a rise in deposited water may be observed.

Endogenous HA changes depend on variations in its molecular weight and amount (Barbucci et al., 2002; Moreland, 2003). Reactive oxygen species are produced in more significant quantities as a result of inflammation, and they are what cause collagen, laminin, and HA to

break down (Bates et al., 1984). HA is a high molecular mass molecule naturally occurring in synovial fluid and can neutralize free radicals (Saari & Konttinen, 1989). By reducing chemotaxis and migration of inflammatory cells, high molecular mass HA serves as an excellent barrier to the inflammatory process.

The crucial issue about OA's etiopathogenesis relates to HA's inhibitory and stimulatory effects on chondrocyte death and proteoglycan production. HA directly contributes to the analgesic action (Balazs & Laurent, 1998; Barbucci et al., 2002; Ghosh et al., 1995; Moore & Willoughby, 1995; Moreland, 2003). The therapeutic effects (pain reduction) of intra-articular HA preparations are well tolerated and supported by randomized trials (Altman, 1998; Moreland, 2003).

The absence of systemic side effects from intra-articular injections is a crucial benefit (Szabó et al., 2011). Patients are increasingly interested in oral HA administration in addition to HA injections. On the other hand, oral formulations do not have any proven therapeutic benefits for OA. According to the research that is now available on oral HA formulations, oral supplementation may reduce pain and improve quality of life.

2.4.3. Applications in ophthalmology

Numerous uses for HA exist in ophthalmology, both from a conservative and practical standpoint. Due to its viscoelastic characteristics, it is widely employed as the "lubricant" component and frequently makes up most artificial tear formulations used to treat dry eyes. It soothes discomfort, hydrates the eye, and makes up for any sodium hyaluronate deficiency in the tear film.

The substance is frequently offered in an unpreserved form. People who wear contact lenses utilize eye drops. The symptoms of dry eye are significantly lessened by its noteworthy qualities, which include securing the tear film, reducing friction while blinking, and preventing dangerous particles from adhering to the eye. More than 50% of respondents say they no longer want to wear contact lenses due to dry eye.

Because most ophthalmic solutions contain artificial ingredients and preservatives, they leave residues on the eye's surface, making distinguishing treatments containing HA from other eye drops easy. Additionally, the fluid frequently does not disperse evenly on the eye's surface, resulting in visible blurring and decreased vision.

Because HA is hydrophilic and viscoelastic, it reduces friction and slows the evaporation of tears. HA replaces water in these medications because they do not dilate conjunctival blood vessels, making them safe during winter (Kogan et al., 2007; Maltese et al., 2006).

2.4.4. Applications in orthokeratology

Orthokeratology treats refractive errors in patients by having them wear a specific lens at night. Viscous artificial tears (established upon HA) were superior to the saline solution when used to fit orthokeratology lenses. Amido bonds were used to bind nisin to HA. This modified polysaccharide's biocidal capacity (added in gels or solutions) was testified on Gram-positive microorganisms with encouraging outcomes. Eye surgery was performed using HA conjugated with ciprofloxacin and vancomycin to prevent infections.

HA has an essential role in the rapid restoration of healthy ocular epithelium, and it also improves the amount of moisture retention and hence relieves dry eye syndrome after

surgery; it can also be added to artificial tears (Beck et al., 2019; Pinto-Fraga et al., 2017; Salzillo et al., 2016). Since HA is found in the vitreous humor of the eye, it can be utilized in the artificial vitreous humor (Raia et al., 2020; Schramm et al., 2012). Because of its biocompatibility and biodegradability, HA can substitute silicone oil in vitrectomy, preventing adverse effects, cytotoxicity, silicone oil emulsification, and second surgery (Barth et al., 2016).

Because of their many advantages, it is common for cataract surgeries to use ophthalmic viscoelastic devices (OVDs). On the other hand, prolonged OVD retention durations may increase intraocular pressure (IOP). IOP did not significantly increase using two OVDs (Healon 5, 2.3 percent sodium hyaluronate Healon GV, and 1.8 percent sodium hyaluronate).

The most popular treatment for dry eye conditions is artificial tears. Comparing HA and carmellose (carboxymethylcellulose)-based tears to regular saline solution, researchers found that the latter was superior in terms of tear film stability and visual clarity (Bayer, 2020; Carracedo et al., 2018; Lequeux et al., 2014; Malvankar-Mehta et al., 2020; Vandermeer et al., 2018; Zhang et al., 2020).

2.4.5. Applications in cosmetics

HA is currently among the active components most frequently used in cosmetic drafting. Both industry professionals and consumers are constantly interested in the general perception of skin regeneration. It is obvious that HA is one of the critical components of good skin and serves as a health indicator for individuals (Baumann & Baumann, 2009).

Today, research is being done to produce biopolymers with the proper molecular weight. Studies suggest that this particular element depends precisely on biological processes. Even though HA was created long ago, its physical, chemical, and biological characteristics still need to be studied (Witting et al., 2015); the nasolabial folds and wrinkles can be removed, the horizontal forehead lines can be reduced, the eyebrows can be raised, the nose can be positioned, the lips' shape and volume can be changed, the cheeks and chin can be modeled, and the body contouring (enlarging and modeling the thighs, breasts, buttocks, and calves) can all result in beautiful results.

More recently, boosting the shape of the labia has also been effective (labiaplasty). The full effect lasts around six months after intradermal or subcutaneous injections of small amounts of HA. Products for the eyes, face, neck, and body, as well as in anticellulite and anti-stretch mark cosmetics, use a high molecular mass HA composition to build a protective layer that makes skin texture smoother and feel softer to the touch (Kanchwala et al., 2005; Tezel & Fredrickson, 2008).

Numerous *in vitro* and *in vivo* studies have demonstrated the efficacy of HA therapy, including its skin regeneration, chondroprotective, anti-aging, anti-inflammatory, and immunosuppressive properties. Hyaluronan has many uses, but further study and technical advancement are still required to understand some current problems fully.

In order to understand the numerous biological functions and predict the consequences that can fluctuate with the molecular mass of HA, additional thought must first be given to features of HA metabolism and receptor clustering analysis. Diverse molecular weights of HA can be included in some medications and cosmetic products.

Therefore, research must determine molecular weight's relevance to HA's effects. The primary goal is to develop next-generation products with high biocompatibility, a prolonged half-life, and permanent in situ performance using HA-conjugated polymers. In order to properly designate the efficacy profile and safety of these drugs, a clinical investigation is essential. So far, the safety and effectiveness of these exciting and innovative substances have been the subject of encouraging *in vitro* studies (Fallacara et al., 2018; Vasvani et al., 2020).