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Innovative strategies for biotechnological production of hyaluronic acid from different sources

Safa Said, Rania Ahmed, Gamal Enan[1] , and Ahmed Askora *
1Department of Botany and Microbiology, Faculty of Science, Zagazig University 44511, Zagazig Egypt.
*Corresponding author: Email: ahmedaskora@gmail.com

ABSTRACT* : *Hyaluronic acid (HA) is a biological macromolecule belongs to a class of polysaccharides called glycosaminoglycans/mucopolysaccharides, a linear polysaccharide consist of repeating disaccharide unites. In the healthcare and pharmaceutical industries, HA has a wide range of uses. It is used in a variety of medical applications such as osteoarthritis (OA) treatment, cancer treatment, ophthalmology (aiding in eye surgery), wound healing, ageing processes, cosmetics and tissue engineering applications. HA has also recently been investigated as a drug delivery agent for a variety of routes including nasal, oral, pulmonary, ocular, topical and parenteral. HA can be produced by different methods such as physical, chemical and biological methods. Microorganisms have been used to produce HA, and this process has long been researched. The production of HA is enhanced by genetic engineering technique and optimizing the nutritional and environmental parameters of the fermentation medium. The aim of this review is to high light on the different strategies for HA production.

KEYWORDS: HA, Microorganisms, Medical, Application.

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1. Discovery, Biosynthetic pathway, Structure and Distribution of HA

1.1. HA Discovery:

In 1934, Karl Meyer and John Palmer Discovered an unusual extreme high molecular weight polysaccharide isolated from the vitreous humor of bovine eyes **(Meyer and Palmer, 1934)** and **(Buckley *et al.*, 2022)**. They were the first to give the new substance the name hyaluronic acid, abbreviated as HA and its name derived from "hyaloid=vitreous" which is a Greek word means glass (glassy appearance) and "uronic acid". While Meyer and Palmer are generally considered the discoverers of HA, it should be mentioned that Levene and Lopez-Suarez had isolated a new polysaccharide from vitreous humor and umbilical cord blood as early as 1918, which they called " mucoitin-sulfuric acid" **(Levene & López-Suárez, 1918)** and (**Selyanin *et al.*, 2015)**. It consisted of glucosamine, glucuronic acid and a small amount of sulfate ions. It is now clear that this substance was in fact HA extracted with a mixture of sulfated glycosaminoglycans. The first study on HA was performed in 1880 by the French scientist Portes, who observed that the vitreous body mucin was different from other mucoids found in the cornea and cartilage and gave it the name "hyalomucin." **(Gupta *et al.*, 2019)**. According to **Buckley *et al.*, (2022),** The nomenclature of HA was changed in 1986 by Endre Balazs to "hyaluronan since HA was first isolated as an acid but it is found that HA can produced as sodium hyaluronate salt at physiological pH where the carboxyl groups of the molecule are dissociated and thus attract cations, such as Na+ **(Balazs *et al.*, 1986**) and **(Fallacara *et al.*, 2018)**.

1.2. HA Structure:

HA is a linear polysaccharide consisting of repeating disaccharides unites. These disaccharide unites consist of uronic acid (d-glucuronic acid) and aminosugar (d-N-acetylglucosamine) which joined by alternate beta-1,4 and beta-1,3 glycosidic bonds **(Radaeva *et al.*, 1997)** and (**Gallo *et al.*, 2019).** Each repeating disaccharide unit has one carboxylate group, four hydroxyl groups and an acetamido group. These groups are responsible for the hydrophilic characteristics of the HA molecule. HA synthase enzymes produce large and linear polymers of the repeating disaccharide unites of HA and (**Selyanin *et al.*, 2015).** A produced HA molecule may have 10,000 or more repeat disaccharides, giving it a molecular mass of around 4 million Daltons (each disaccharide weighs 400 Daltons) **(Cowman and Matsuoka, 2005).** The repeating disaccharide units are twisted through 180 degrees to form the secondary structure, which is a tape-like twofold helix **Figure (1)**, **(Blundell *et al.*, 2006)** and **(Sasaki**

& Miyata, 2019). The tertiary structure of HA is formed of B-sheets based on twofold antiparallel helices **(Snetkov *et al*., 2020).**

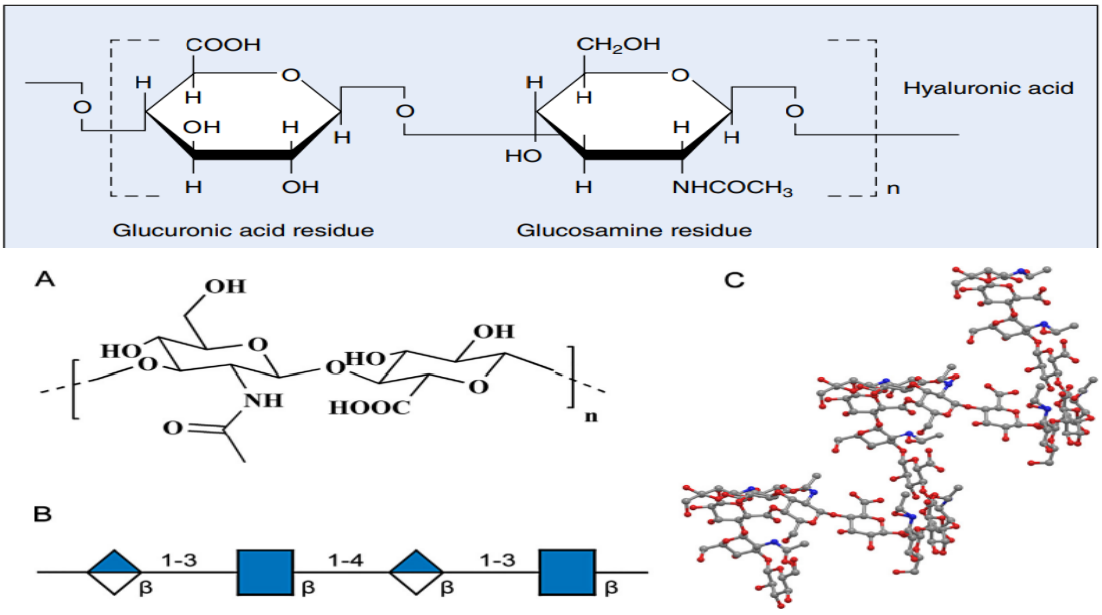

Figure (1) (Shikina *et al*., 2022): (A) Fragment of the HA primary structure. Linear chains of HA range from 20 to 15,000 repeats **(Owen *et al*., 2017)**; (B) two repeating units of HA structure **(Neelamegham *et al*., 2019)**; (C) 3D model of a fragment of the HA secondary twofold twisted helix built of repeating disaccharide units **(Haxaire *et al*., 2000).**

1.3. HA Biosynthetic pathway:

S. zooepidemicus, which produces HA precursors via two different routes, has been the focus of the majority of research on HA production in prokaryotes **(DeAngelis, 1996), (Chong *et al*. 2005)** and (**Sze *et al*., 2016)**. Hexokinase phosphorylates glucose to create glucose-6-phosphate, which is the primary precursor in the biosynthesis of HA. From here, the HA synthesis route can be separated into two different pathways that synthesize glucuronic acid and N-acetylglucosamine, the two components that make up HA presented in **Figure (2)**. In the first series of events, a-phosphoglucomutase (pgm) turns glucose-6-phosphate into glucose-1-phosphate before UDP-glucose pyrophosphorylase (hasC) adds a phosphate group from UTP to glucose-1-phosphate to create UDP-glucose. UDP-glucose dehydrogenase (hasB) then oxidizes UDP-glucose to produce the first HA precursor, UDP-glucuronic acid (UDPGlcUA). In the second set, phosphoglucoisomerase transforms glucose-6-phosphate into fructose-6-phosphate (hasE). Following conversion, glucosamine-6-phosphate is changed by mutase (glmM) to form fructose-1-phosphate by adding an amido group that had previously attached to a glutamine residue via amidotransferase (glmS). The second HA precursor, UDP-N-acetylglucosamine (UDP-GlcNAc), is produced by successively acetylating and phosphorylating this intermediate by acetyltransferase and pyrophosphorylase (hasD), respectively. After the two precursors are created, HA synthase (hasA) alternately polymerizes the two parts to create the HA polymer **(Sze *et al*., 2016)**.

Table (1): Proteins involved in HA biosynthesis by *S. zooepidemicus*, and their functions (Shikina *et al.*, 2022).

Gene	Enzyme	Function	Reference
Has A	Hyaluronic acid synthase	HA synthesis and transport	**(Crater & Van de Rijn, 1995)**
Has B	UDP-glucose dehydrogenase	UDP-GlcA biosynthesis	**Dougherty & Van de Rijn, 1993)(Chen *et al.*, 2019)**
Has C	UDP-glucose pyrophosphorylase		**(Crater *et al.*, 1995)**
Has D	Acetyltransferase and pyrophosphorylase (bifunctional)	UDP-GlcNAc biosynthesis	**(Blank *et al.*, 2008)**
Has E	Phosphoglucoisomerase		

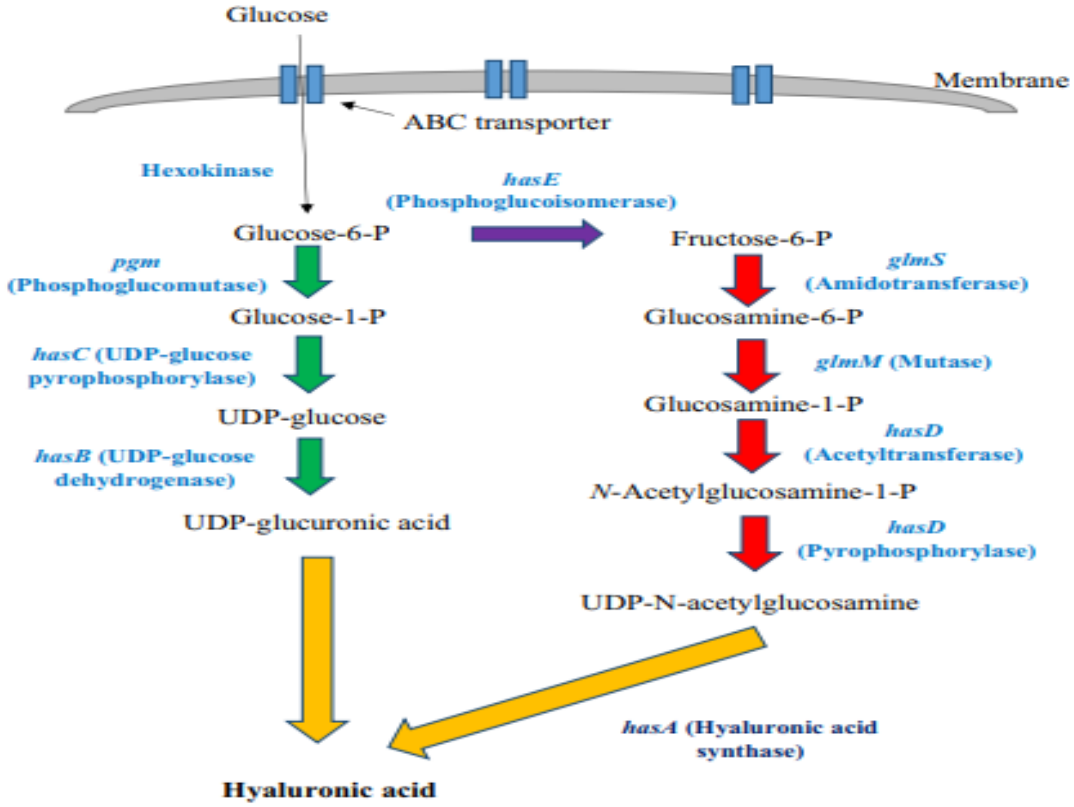

Figure (2): **Biosynthetic pathways of HA production by *S. zooepidemicus* (Sze *et al.*, 2016).**

1.4. HA Distribution:

HA is widely distributed in all body where it is discovered that all vertebrates have HA, which one of the crucial component of extracellular matrices (ECMs) is found in the majority of adult tissues. It is found that daily, nearly 5-15 g of dry HA can be produced and cleaved in the body of an adult man weighting 70-kg **(Fraser *et al.*,1981)** and **(Gupta *et al.*, 2019)**. In the body, HA occurs in the salt form (hyaluronate) and is found in high concentrations in many tissues such as connective, epithelial, and nervous tissue **(Fallacara *et al.*, 2018)** and **(Gupta *et al.*, 2019)**.

Subsequently, the presence of HA was established in other organs such as joints where it is found in the extracellular matrix of the joints, skin, rooster comb and umbilical cord **(Weissmann & Meyer, 1954)** and **(Shiedlin *et al*., 2004)** but the greatest amount of the HA is present in the skin, about half of the total HA (50%**) (Salwowska *et al*., 2016)**. Significant amounts of HA are also found in lung, kidney, brain, and muscle tissues. It is also reported that HA was produced in intracellular fluids, including the aqueous and vitreous humour of the eye (0.1-0.4 mg/g wet weight in humans) **(Meyer & Palmer, 1934)** and synovial joint fluid (3-4 mg/ml) **(Hamerman & Schuster, 1958)** and **(Raio *et al*., 2005)**. It is also found in the pathological matrix that close off the artery in coronary restenosis, and the matrix generated by cumulus cells around the egg before to ovulation **(Hascall *et al*., 2002)** and **(Raio *et al*., 2005)**. It is found that HA half-life is 1 to 30 weeks in the joints, up to 1 to 2 days in the skin and derma, and just 2 to 5 minutes in the blood **(Fraser *et al*., 1997)**, **(Papakonstantinou *et al*., 2012)** and **(Fakhari & Berkland, 2013)**. HA was initially identified as an animal polysaccharide; however, it was quickly discovered that bacteria also can produce the HA where it is found in its capsule to help it to escape from human immune system and protect it from phagocytosis. in 1937, Kendall, Heidelberger and Dawson extracted a polysaccharide from the broth culture of hemolytic streptococcus which precipitated with acetic acid and ethanol it was found that the isolated polysaccharide is identical to HA isolated from mammalian tissues and the hypothesis was later confirmed **(Kendall *et al*., 1937)** and **(Selyanin *et al*., 2015)**.

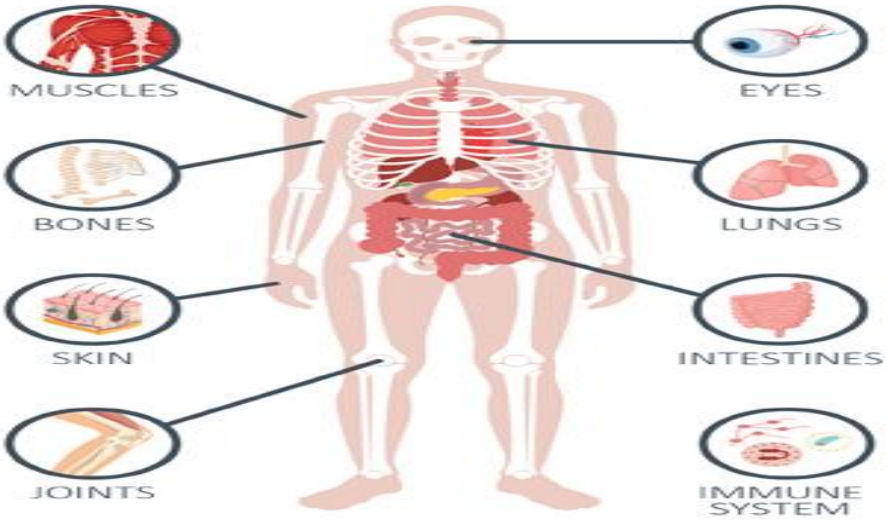

Figure (3): HA distribution in the body. **(https://www.shutterstock.com/image-vector/hyaluronic-acid-human-body-600w-543128473.jpg).**

I. THE CHARACTERS OF HA

HA has an identical chemical structure when it examined in all known species so it is regarded as a fairly conservative polysaccharide. The conservatism of its chemical structure demonstrates the significance of its biological roles. The characters of HA were summarized as following, it is linear, non-branched, non-sulfated and water soluble polysaccharide which consists of 5,000–30,000 repeated disaccharide units consisted of N-acetyl-d-glucosamine and D-glucuronic acid connected with beta-1-3 glycoside bond. These disaccharides are connected with beta-1-4-glycosidic bonds. It was found that HA not bound to proteins but it may bound to other components, it also may present as a free molecule. It is not chemically modified after synthesis and it has high molecular weight reach to 8×106 Da. The amino group of the amino sugars is usually acetylated, which leads to the disappearance of the positive charge of HA. At the pH 7, the HA carboxylic groups are dissociated and the polymer molecules contain high-density negative charges, they draw osmotically active cations such sodium, potassium, magnesium, calcium, and others. As a result, HA can bind up to 1000 times more water than the weight of HA

itself. HA and its salts are readily soluble in water and can generate very viscous aqueous solutions even at low concentrations while in high concentrations (1-4% W/V) it produces pseudo-gels. The presence of the large amount of HA-bound water creates a swelling pressure (turgor) that resists stress forces. The viscoelastic properties of HA in the synovial liquid of joints allow the biopolymer to act as a lubricant and shock absorber. It provides protection from penetration of high molecular weight (HMW) toxins and microbiological invasions (**Selyanin *et al.*, 2015)**.

II. IMPORTANCE OF HA

Due to the distinct characteristics of HA, such as high hygroscopicity; viscoelasticity; high biocompatibility; non-immunogenicity, anti-inflammatory, antiseptic, wound healing properties and it does not generate toxic products when degraded, that makes it a very attractive molecule and used in a variety of biological applications, such as osteoarthritis (OA) treatment, cancer treatment, Ophthalmology (aiding in eye surgery), wound healing, ageing processes, cosmetics and tissue engineering applications **(De Oliveira *et al.*, 2016)**. HA has also recently been investigated as a drug delivery agent for a variety of routes, including nasal, oral, pulmonary, ocular, topical and parenteral **(Fakhari & Berkland, 2013)**. HA improves epithelial regeneration, inhibits the growth of granulation tissue, adhesions, and scars, decreases swelling and itching, improves blood circulation, improves venous ulcer scarring, and maintains internal eye tissue **(Radaeva *et al.*, 1997)**.

3.1. Tissue Engineering:

Tissue engineering (TE) is the replacement and improvement of injured and diseased tissues by creating functional constructions that preserve, repair, or enhance damaged tissues. many skin constructs have been developed employing components like HA in the form of hydrogels **(Valachová & Šoltés, 2021)**. HA hydrogels serve as scaffolds or cell carriers for cell loading, growth and differentiation in tissue regeneration **(Zhai *et al.*, 2020).** Scaffolds are just short-term supports that can allow the ingrowth of cells and tissues through biodegradable structures like hydrogels. Most scaffolds must meet a number of requirements, including:

- The scaffold's surface should promote cell adhesion and growth.
- The scaffold should not trigger an immunological or inflammatory response.
- The scaffold should be biocompatible and not produce toxic products when degraded.
- The scaffold should have the same physical and mechanical characteristics as the native tissue.
- The scaffold should have enough porosity to promote cell growth and nutrient diffusion.

HA hydrogels have the capacity to alter the three-dimensional architectures of the natural ECM to improve the therapeutic efficiency of the matrix or scaffold **(Chircov *et al.*, 2018).** A class of HA hydrogel called bulking agents, working as a biological glue, which also improves anti-aging process. Transplanting cells into the body for tissue repair and regeneration, including bone, cartilage, and smooth muscle, is another use for HA hydrogels **(Nosenko *et al.*, 2020)**. There are several applications of TE such as lung, skin, bone, cartilage **(Dovedytis *et al.*, 2020)**, dermal, brain and heart that have been researched by scientists to use in burns and tissue transplantation **(Sudha & Rose, 2014)**.

3.2. Cosmetics:

HA is distinguished by its capacity to bind to a great amount of water so, it used as a cosmeceutical to treat skin problems such as wrinkles, skin hydration and skin ageing **(Caló and Khutoryanskiy, 2015)**. It acts as collagen stimulator and anti-aging factor. HA has been used into a variety of cosmetic compositions to keep skin flexible, moist, and turgid **(Dong *et al.*, 2017)**. A study was done on 76 females with periocular wrinkles who were between the ages of 30 and 60. They were instructed to use the HA cream formulation twice daily for two months around the wrinkled area. The HA-based cream produced amazing effects for skin hydration and flexibility. HA has been utilized as a targeting ligand for the delivery of targeted drugs by increasing their ability to penetrate the biological membranes and enhance their targeting effectiveness **(Gomes *et al.*, 2020)**. HA has been used in a variety of forms, including creams, serums, gels, lotions, intra-dermal filler injections, and facial fillers, to study its nutricosmetic and cosmetic benefits which allow collagen stimulation, tissue augmentation, and face rejuvenation **(Salwowska *et al.*, 2016)**.

3.3. Ophthalmology:

The vitreous fluid of the eye naturally contains HA, which serves as a space-filling matrix, therefore it is injected intraocularly to maintain the frontal chamber's shape **(Salzillo *et al.*, 2016)**. Due to the viscoelastic properties of HA, it is used to lubricate the optical surface, reduce friction and treat eye dryness infections. HA solution can be utilized as an additional agent in eye drops for eye tissue repair **(Huynh and Priefer, 2020)**. HA is utilized to

improve the eye health and regenerating eye parts by protecting the visible tissue including the corneal endothelium. HA plays a crucial role in treating eye dryness, a disease which is associated with the ocular surface and tear infection causing symptoms including visual disturbance, discomfort, and volatile tear film, burning, stinging, redness and photophobia. The most common treatment at the moment is the use of artificial tears made from cellulose derivatives, hydroxypropyl guar, HA and polyvinyl alcohol **(Salwowska *et al.*, 2016)**.

3.4. Cancer Treatment:

The most common cancer treatment is chemotherapy which has a number of disadvantages **(Cho *et al.*, 2008)**, where the multiple intravenous administrations of a cancer treatment cause drug resistance. It is also cytotoxic, which causes patient off-target cell death **(Oh *et al.*, 2010)**. As a result, research on targeted medicine delivery has increased. The CD44 (Cluster of Differentiation 44), RHAMM (receptor for hyaluronic acid-mediated motility), TSG6 (Tumor necrosis factor- (TNF) stimulated gene-6), GHAP ((glial hyaluronic acid -binding protein), ICAM-1 (intracellular adhesion molecule-1) and LYVE-1 (Lymphatic-Vessel Edothelial hyaluronan receptor 1) are the targetable receptors of HA that found on the cell components **(Trombino *et al.*, 2019)**. It was found that the CD44 and RHAMM are overexpressed on the surfaces of a variety of tumor cell types, including lung cancer, acute leukemia, colon cancer, and human breast epithelial cells and can be targeted with intravenous doses of HA **(Zamboni *et al.*, 2017)**. Drug delivery to target tumor cells can be facilitated by modifying HA, because it has the characteristics that make it an effective anti-cancer drug vector due to its biocompatibility, allows controlled release of the drug and also protects it from being degraded and can enter the cell through endocytosis, where it was found that the increase in CD44 and the aggregation of HA around tumor cells promotes the endocytosis **(Huang & Huang, 2018)**. HA conjugates included anticancer medications showed enhanced cancer targeting capability and increased therapeutic efficiency compared to free anticancer drugs. One such medication is the anticancer medicine paclitaxel, which is poorly soluble in water and cannot penetrating tumor cells. These drawbacks can be overcome by HA, which thanks to its hydrophobic backbone and hydrophilic functional groups can protect the medication from the aqueous environment. Another chemotherapeutic agent called cisplatin (also known as CDDP, or cis-diaminedichloroplatinum) is a commonly used for the treatment of a wide range of solid cancers **(Wiercińska *et al.*, 2020)**. Cisplatin (HA-Pt) conjugates administrated into a lady with breast cancer, the results showed a successful drainage of HA-cisplatin (HA-Pt) conjugates into the auxiliary lymph lumps with condensed systemic toxicities. HA-Pt conjugate administration into the lungs may help treat lung cancer by reducing overall toxicity and increasing CDDP deposition and retention inside lung tumors, surrounding lung tissues, and in the meditational lymph **(Cho, 2020)**. Using hyaluronic acid as a cationic gene carrier is another way to use it as a drug delivery system such as the delivery of Small interfering RNA (siRNA) which cleavage mRNA and inhibit gene expression. This can be used in cancer treatments to stop the expression of proteins needed for proliferation or interactions between the tumor and its host **(Dana *et al.*, 2017)**. siRNA can be quickly broken down before entering the cell, so thiolated HA can be utilized to encapsulate siRNA to improve its absorption. thiolated HA contain SH group which can form disulfide bonds enabling the encapsulation of siRNA. After endocytosis, the siRNA can be released when glutathione in the cells breaks the disulfide bonds **(Huang & Huang, 2018)**. In conclusion, HA is essential for the treatment of cancer cell metastasis, stopping it, and directing medications to the tumorous cells as well as the surrounding lymph nodes **(Lee *et al.*, 2020)**, also it is employed in some cancer types to track the disease's progression due to its mucoadhesive abilities **(Luo *et al.*, 2019)**.

3.5. Wound Healing:

HA plays a vital role in the diverse biological processes required for wound healing including, Hemostasis, Inflammatory, Proliferation and Maturation phase through the production of granular tissue, inflammation and the development of the wounded epithelium and its remodeling **(Figure 4) (Su *et al.*, 2021)**. HA has numerous advantages that other products don't have that make it useful for treating wounds, including it doesn't cause allergic reactions and promotes excessive bio-stimulating and inflammatory effects throughout the regeneration process **(Vasvani *et al.*, 2020).** HA can bind to a great amount of water that maintains a moist environment necessary for the formulation of granular tissue **(Taddeucci *et al.*, 2004)**. It was observed that HA increases at the wound site after the wound occurs, where it was found that HA serves as a temporary structure due to its large molecular size. This structure allows the diffusion of nutrients supplies and waste products removal from the injury site. As the wound heals, collagen molecules and proteoglycan proteins replace this temporary structure. It also serves as a mediator during the wound healing processes through tissue formation and remodeling so, it is utilized to treat sores, scars, external skin wounds and ulcers in the stomach and duodenum, where HA possess anti-ulcerant properties by inhibiting H2 histamine receptors and trypsin action **(Vasvani *et al.*, 2020)**. It is also utilized to treat both chronic and acute wounds, including surgical incisions, abrasions, and burns since it also plays a variety of roles in cellular and matrix events **(Brown and Jones, 2005)**. Due to HA's high biocompatibility, it can be

combined with various medications for all areas of wound healing, including surgery, nutritional ulcer healing, and burn healing. It is particularly helpful during the first three days of wound healing **(Jin *et al.*, 2010)**. Once HA bound to CD44, it directly affects the proliferation and migration of Keratinocytes cells which produce keratin and form tight junctions **(Voigt & Driver, 2012)**. CD44 HA receptor recruitment of fibroblasts into the wound area from surrounding tissue that synthesizes the extracellular matrix and collagen. CD44 then stimulates a signaling cascade which initiates the cell growth and cell motility process **(Johnson *et al.*, 2018)**. RHAMM HA receptor also triggers numerous protein kinases which activates cellular movement and fibroblast proliferation *in vivo* **(Dovedytis *et al.*, 2020)**. CD44 and RHAMM interaction regulate the signal transduction, cell movement, and wound healing **(Leng *et al.*, 2019)**. lower molecular weight (LMW) HA induce inflammation **(Altman *et al.*, 2019)** by the activation of several macrophages, pro-inflammatory cytokines, and chemokines **(Filion & Phillips, 2001)**. HA also can possess anti-oxidative properties where HMW HA can protect against the effects of reactive oxygen species (ROS) **(Moseley *et al.*, 2002)**. It is supposed that hydroxyl functional groups on HA can absorb ROS **(Mendoza *et al.*, 2009)**. During angiogenesis, LMW HA stimulates vascular endothelial cell proliferation and migration which is a necessary phase of the healing process.it is also found that LMW HA also stimulates the expression of Ezrin, a vital protein for cellular adhesion **(Pardue *et al.*, 2008)**. When tissue is wounded, the amount of HA already present is degraded causing the appearance of a LMW oligosaccharide debris that is responsible for the formation of a temporary ECM and act as a transporter of bioactive substances **(Bowman *et al.*, 2018)**. Injectable multifunctional hydrogel made with HA, dopamine, and carboxymethyl chitosan (CMC/HA-DA) was successfully used to promote the healing of skin wounds due to its excellent adhesive, anti-inflammatory, and hemostatic characteristics **(Cui *et al.*, 2022).** In order to achieve anti-biofouling and antioxidant properties, the drug delivery hydrogel consists of alginate, HA, and polylysine (PLL) was created with anti-biofouling and antioxidant properties and carried with curcumin and epigallocatechin gallate (EGCG), to lessen the development of irradiation-induced skin injury **(Zhang *et al.*, 2021)**. By sufficiently saturating the lesion of skin-abraded rats with saline, chlorhexidine digluconate, and 0.2% HA. The results showed that 0.2% HA demonstrated higher healing efficacy of skin abrasions **(Leite and Frade, 2021)**.

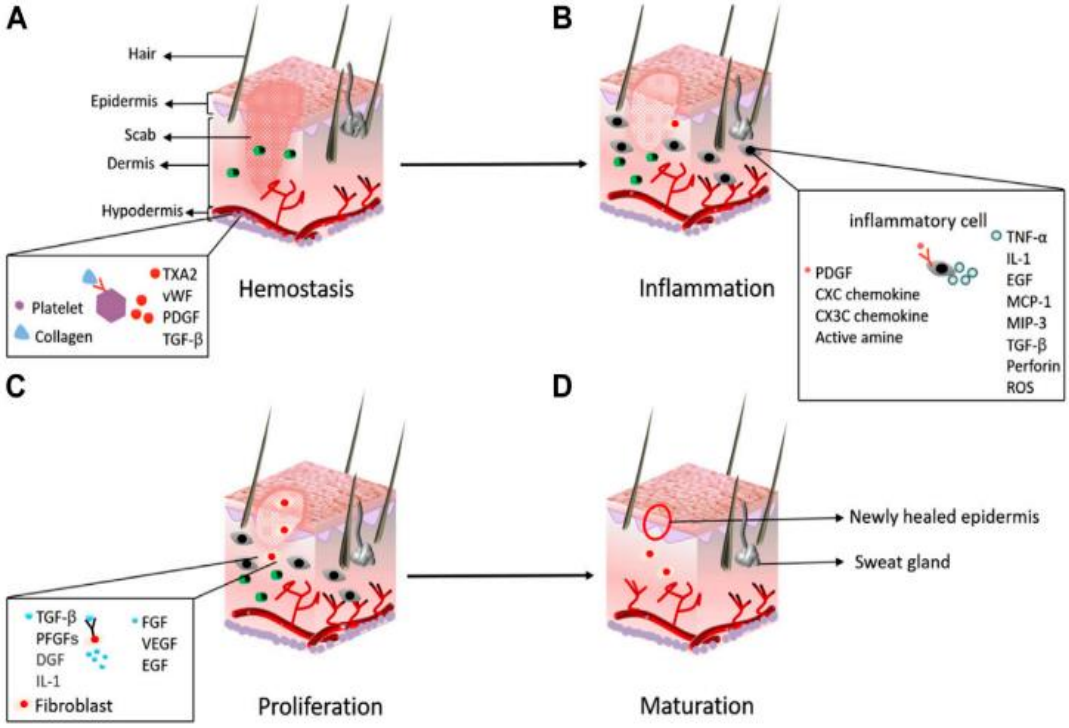

Figure (4): Major stages in the process of wound healing: (A) hemostasis; (B) inflammation; (C) proliferation; and (D) maturation **(Su *et al.*, 2021).**

3.6. Osteoarthritis treatment:

HA occurs naturally in the synovial fluid, joints, and articular cartilage so, it is extensively utilized in OA treatment. OA is the most prevalent condition affecting joints, which causes articular cartilage to break down

(Gupta *et al.*, 2005). This break down occurs due to the production of more collagen-degrading enzymes, ROS and water accumulation and all of which accelerate the breakdown of collagen, laminin, and HA **(Salwowska *et al.*, 2016)**. The OA disease leads to the appearance of several symptoms such as, the loss of cartilage, discomfort, and escalating weakness. HMW HA occurs naturally in the synovial fluid and has the ability to neutralize free radicals. Other studies on HA have revealed that it stimulates the production of cartilage matrix, stops its degradation, decreases inflammation, stimulates the production of endogenous HA, and increases the cartilage's resilience and moisture content **(Barbucci *et al.*, 2002)**. According **to Moreland (2003),** intra-articular HA preparations have good tolerability and therapeutic efficacy, reduce joint pain, restore the viscoelasticity of synovial fluid, don't cause any systemic side effects, allowing cells to continuously manufacture HA and enhancing flexibility and strength **(Szabó *et al.*, 2011)** and **(Saranraj & Naidu, 2013)**. All of intra-articular HA characteristics allow the need for nonsteroidal anti-inflammatory drugs is reduced so, HA products such as Orthovisc and Healon are used for joint lubrication, stress absorption, reduce pain and inflammation in OA **(Brown & Jones, 2005)**.

III. METHODS of HA production

a. Chemical production:

Pre-glycosylation oxidation strategy and post-glycosylation oxidation strategy are the two main chemical methods used to synthesize HA oligosaccharides, and they are distinguished by how glucuronic acid (GlcA) is connected **(Mende *et al.*, 2016)**. Due to the low reactivity of GlcA, the pre-glycosylation oxidation method introduces GlcA residues before glycosylation, resulting in low to moderate glycosylation yields. Because of this, researchers developed the post-glycosylation oxidation method, in which glucose units are converted to GlcA after glycosylation. However, the oxidation of these intermediates is a difficult and expensive phase in the synthesis process. Even though numerous studies on the synthesis of HA oligosaccharides have been showed, the sector still faces a number of obstacles. The chemical production of HA is still difficult and expensive because of the stereo- and regio-selectivity of sugars **(Fu *et al.*, 2017)**. It is still difficult to install the exact GlcA residue quickly or to obtain affordable monosaccharide building blocks or disaccharidic repeating units. Another obstacle to the large-scale chemical production of HA oligosaccharides is the liberation of the target compounds and the purification of the polyanionic target molecules.

b. Physical production:

Isolation of HA from animal by grinding animal tissues considered the most traditional way for HA production where it was successively isolated in 1930 from a wide range of various animal tissues. HA was first produced from the umbilical cord and rooster comb by Endre Balazs. These HA have a high molecular weight but needs a high degree of purification **(Yao *et al.*, 2021)**. Solvent extraction is used to get HA of animal origin, primarily from rooster comb and bovine vitreous humor, with subsequent precipitation and washing serving as the final extraction steps. After the extraction process, the HA-containing solutions are exposed to further treatments using bactericides to remove the bacteria and proteolytic enzymes for the subsequent separation of the protein contaminants extracted with the HA **(Cavalcanti *et al.*, 2020)**. The HA isolated from animal source has some drawbacks include limited availability of animal tissue; severe, complex and harsh extraction method frequently leads to poor yield and molecular weight polydispersity **(Lee *et al.*, 2021)**; potential for environmental damage; poor extraction rate of HA production; poor quality control, high purification costs, and the loss of HA by degradation by the activity of the natural endogenous hyaluronidase enzyme. Animal-derived HA may also contain contaminants like viruses, proteins and nucleic acid **(Manfrão-Netto *et al.*, 2022)**. All of these disadvantages leads to restrict the utilization of the animal tissue extraction method for the manufacture of HA on a wide scale for commercial purposes (**Badri *et al.*, 2018)**.

c. Microbial production:

Microorganisms are vital for metabolic and biochemical activities, which range from the creation of enzymes to the generation of biopolymers **(Delangiz *et al.*, 2022)**. Due to the drawbacks of HA production from the physical and chemical methods, another biological, alternative method was found by using microorganisms where HA first produced in the 1980 by the microbial fermentation process **(Yao *et al.*, 2021)**. This rise in production is brought on by a number of benefits, including a reduced risk of viral infection between species, improved purification, lower production costs, and higher yields compared to other production methods **(Amado *et al.*, 2016**). In order to obtain high molecular weight, some microbial production procedures were created that combine suitable microbial sources with the isolation of the capsule, which is made up of HA polymers **(Attia *et al.*, 2018)**. HA can have produced naturally by native / wild strains as a form of defense against invading hosts, certain wild microbial strains naturally manufacture HA capsules such as *Streptococcus zooepidemicus, Streptococcus equi,*

Streptococcus pyogenes, Streptococcus equisimilis, Streptococcus thermophiles, Pasteurella multocida and *Cryptococcus neoformans* fungus. *Streptococcus* species particularly *S. zooepidemicus*, produce the majority of the current industrial HA production (6–7 g/L) **(Rohit *et al.*, 2018)**. Researchers have been looking for other strains that are generally regarded as safe (GRAS) and modifying them for HA synthesis to get rid of *Streptococcus* endotoxin problems during the production of HA **(Sheng *et al.*, 2015)**.

IV. How to enhance HA production?

5.1. Genetic engineering technique:

Genetically engineered / Recombinant strains such as *Escherichia coli, Lactococcus lactis, Bacillus subtilis, Agrobacterium sp., Enterococcus faecalis, Streptomyces albulus, Corynebacterium glutamicum, Yeasts, Saccharomyces cerevisiae, Pichia pastori fungus and Kluyveromyces lactis fungus* can produce HA and considered an alternative source for the pathogenic naturally occurring HA-producing strains **(Sunguroğlu *et al.*, 2018)**. In 1993, *S. zooepidemicus's* HA synthase and associated gene clusters were cloned for the first time, and this new strain produced heterologous HA. By altering the microenvironment of the enzymatic reaction, the functionalities of HA synthase have improved. Additionally, metabolic engineering techniques have been used to promote the production of intermediate metabolites required for the synthesis of HA, such as overexpressing enzymes from intermediate metabolic pathways (such as UDP-glucose 6-dehydrogenase or glucosamine-1-phosphate N-acetyltransferase) or blocking the synthesis of undesirable metabolites (such as L-lactate) **(Wang *et al.*, 2020)** and **(Yao *et al.*, 2021)**.

5.2. Optimization of fermentation medium:

It has been thoroughly studied how the culture medium's components and culture medium parameters, such as temperature, pH, aeration rate, agitation speed, dissolved oxygen, shear stress, the presence of mineral ions, the carbon/nitrogen ratio and type of bioreactor can have a significant impact on the production of microbial HA by fermentation. Consequently, researchers investigated the best microbial production factors **(Gedikli *et al.*, 2018)**. In order to increase production, some of this parameters have been researched **(Arslan & Aydogan, 2021)**.

5.2.1. Carbon:

Carbon is necessary for the synthesis of the HA chain's backbone as well as for microbial development where, Chahuki claim that the microbial synthesis of HA is an energy and carbon-intensive process. The precursors of HA and cell wall biosynthesis include UDP glucuronic acid and UDP N acetyl glucosamine. Thus, the glycolytic process and cell wall biosynthesis compete with HA biosynthesis. In this regard, inhibiting cell proliferation and using the glycolytic pathway may be substitutes for increasing HA synthesis **(Chahuki *et al.*, 2019)**. This suggests that microbes can adjust metabolism and diverting more carbon fluxes into other competitive routes **(Yao *et al.*, 2021)**. Typically, glucose is employed as a carbon source in culture media for the formation of microbial HA where, the maximal molecular weight of HA was attained at a glucose concentration of 40 g/L **(Pires *et al.*, 2010)**. However, studies show that using sucrose rather of glucose as a carbon source can result in an 800 kDa greater increase in HA's molecular weight **(Gedikli *et al.*, 2018)**.

5.2.2. Nitrogen:

Organic nitrogen is crucial for *S. zooepidemicus*' ability to grow and produce HA. The original medium was composed of animal sources like sheep blood and brain and heart infusion (BHI). Recently, Peptone and yeast extract are frequently added to the growth medium as nitrogen sources, where It is found that the organic nitrogen is more effective compared to the inorganic nitrogen in HA production **(Gedikli *et al.*, 2018)**. According to Arslan & Aydogan, this bacterium may need very high concentrations of peptone and yeast extract (up to 20 g/L) in order to produce HA **(Arslan & Aydogan, 2021)**. Soy protein and whey protein are other sources that contain organic substrates and can lower the cost of HA synthesis **(Pan *et al.*, 2015)**.

5.2.3. Temperature:

Temperature is one of the most effective parameters on the HA production where, it was found that the maximal concentration of HA and its average molecular weight can both be altered by temperature, which is another crucial factor in fermentation **(Cavalcanti *et al.*, 2020)**. The rate of biochemical reactions, microorganisms' intracellular enzyme catalytic activity, production times, and activity all depend on temperature. The reaction rates of the microorganism rise with temperature up to a threshold temperature, after which the growth rate falls. The type of microbe used in bio-production will determine the optimal temperature for generating polysaccharides like HA **(Liu *et al.*, 2018)** and **(Shoparwe *et al.*, 2020)**.

5.2.4. pH:

One of the most crucial factors that must be managed in the majority of fermentation processes is the medium's pH. It has been demonstrated that pH has a greater impact on polysaccharide formation than on cell proliferation

(Shoparwe *et al*., 2020). HA is particularly sensitive to pH fluctuations, where it was found that HA is broken down by hydrolysis at relatively basic (>11) or acidic (4.0) pH levels. Due to the breakdown of the H bonds, which are involved in the structural organization of the HA chains, this effect is more obvious in alkaline conditions **(Fallacara *et al*., 2018)**.

5.2.5. Agitation:

According to **(Huang *et al*., 2007)**, The purpose of agitation is to homogenize the broth. Although it does not directly help in the formation of HA, but provides favorable aeration and oxygenation for the cultivated microorganism. Shoparwe found that *Streptococcus zooepidemicus* produce maximum HA (1.23 g/L) and maximum cell biomass (2.20 g/L) when the agitation speed was raised up to 300 rpm **(Shoparwe *et al*., 2020)**. It was also shown that as agitation speed increased from 400 to 500 rpm, HA yield and cell proliferation dropped. the fact that HA synthesis decreased more dramatically than cell growth suggests that HA production is more sensitive to agitation speed than cell growth, So The agitation speed shouldn't be much greater because it can degrade the biopolymer and leads to reduce the quality of the HA **(Shoparwe *et al*., 2020)**.

CONCLUSION

HA is an endogenous substance detected and isolated from various tissues and biological fluids. In addition to its anti-inflammatory and antioxidant actions, HA's physicochemical properties enable it to play a role in numerous biological processes at the intra- and extracellular levels, including skin hydration, joint lubrication, wound healing, cancer treatment, Ophthalmology, ageing processes, cosmetics and tissue engineering applications. It also found it has a great effect on the condition of the hair, nails, and general health. HA supports a number of methods of treatment, including those for osteoarthritis, gingivitis, stomatitis, and ulceration. Moreover, thanks to its anti-inflammatory and antioxidant characteristics, it is useful in dentistry.

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