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Review Article

Production Methods for Hyaluronan

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Hyaluronan is a polysaccharide with multiple functions in the human body being involved in creating flexible and protective layers in tissues and in many signalling pathways during embryonic development, wound healing, inflammation, and cancer. Hyaluronan is an important component of active pharmaceutical ingredients for treatment of, for example, arthritis and osteoarthritis, and its commercial value far exceeds that of other microbial extracellular polysaccharides. Traditionally hyaluronan is extracted from animal waste which is a well-established process now. However, biotechnological synthesis of biopolymers provides a wealth of new possibilities. Therefore, genetic/metabolic engineering has been applied in the area of tailor-made hyaluronan synthesis. Another approach is the controlled artificial (*in vitro*) synthesis of hyaluronan by enzymes. Advantage of using microbial and enzymatic synthesis for hyaluronan production is the simpler downstream processing and a reduced risk of viral contamination. In this paper an overview of the different methods used to produce hyaluronan is presented. Emphasis is on the advancements made in the field of the synthesis of bioengineered hyaluronan.

1. Introduction

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

Research on hyaluronan expands over more than one century (Table 1). The first report that can be linked to hyaluronan dates from 1880, when the French chemist Portes observed that the mucin in the vitreous body, which he named "hyalomucine," behaved differently from other mucoids in cornea and cartilage. Fifty-four years later Meyer and Palmer described a procedure for isolating a novel glycosaminoglycan from the vitreous of bovine eyes, which they named hyaluronic acid based on hyaloid (vitreous) and uronic acid [3]. Since *in vivo* the polymer exists in an ionized form as polyanion, it is generally referred to as hyaluronan.

In living organisms, hyaluronan is produced by hyaluronan synthase enzymes, which synthesize large, linear polymers of the repeating disaccharide β(1,4)-GlcUA-β(1,3)-GlcNAc by alternate addition of GlcUA and GlcNAc to the growing chain using their activated nucleotide sugars (UDP-glucuronic acid and UDP-*N*-acetylglucosamine) as

Table 1: Important events in research on hyaluronan products.

Time	Event
1880	Portes reported that mucin from the vitreous body differs from other mucoids in cornea and cartilage and named it hyalomucine [2].
1934	Meyer and Palmer isolated and identified the polysaccharide from the vitreous body and named it hyaluronic acid [3].
30s–50s	Hyaluronan from many different tissues of vertebrates was isolated, identified, and characterized. A few pathogenic bacteria were found that produce hyaluronan and use it to encapsulate their cells.
50s	The chemical structure of hyaluronan was elucidated by Karl Meyer and his team. They used hyaluronidase to produce overlapping oligosaccharides that were structurally analyzed by conventional techniques [4]. Interest emerged to use hyaluronan in eye surgery as a substitute of the vitreous body.
40s–70s	Extraction processes from animal tissues were optimized to remove protein and to minimize hyaluronan degradation. First studies on hyaluronan production through bacterial fermentation and chemical synthesis were initiated.
1979	First patent on ultrapure hyaluronan isolated from rooster combs [5]. This was the starting of the industrial manufacturing of hyaluronan from animal sources for human applications. In 1980, using the methods of Balasz Pharmacia (Sweden) introduced Healon, a product used in cataract surgery.
90s–00s	Revival of studies on bacterial fermentation to produce hyaluronan of high molecular weight. Emphasis on controlling polymer size and polydispersity.
1993	The gene encoding for a single enzyme that polymerizes UDP-GlcNAc and UDP-GlcUA into hyaluronan is isolated by DeAngelis and coworkers from *Streptococcus pyogenes*. Hyaluronan synthases from other microorganisms were identified and characterized [6, 7].
1996	The largest hyaluronan fragment, an octamer, was chemically synthesized through controlled addition of disaccharide units [8].
2003	Research on the enzymatic synthesis of hyaluronan and monodisperse hyaluronan oligosaccharides with defined length [9, 10].

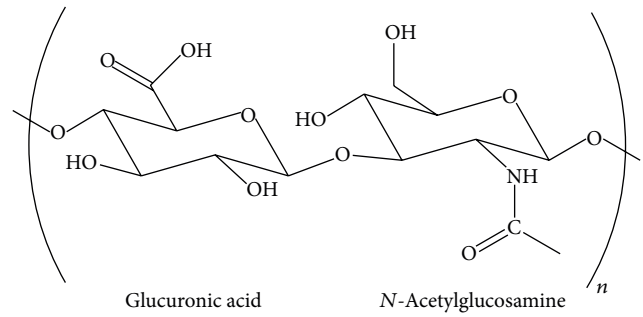

Figure 1: Repeating unit of hyaluronan.

substrates. The number of repeat disaccharides, n, in a completed hyaluronan molecule is approximately 10,000 but chains of hyaluronan with up to 25,000 disaccharide units have been reported. The size of the polymers (M_w from 5,000 Da to 20 million Da) *in vivo* varies with the type of tissue. For example, hyaluronan in the human umbilical cord has a molecular mass of 3-4 million Da, whilst the molecular mass of hyaluronan from human synovial fluid is 6 million Da. However, the reported molecular weight of isolated hyaluronan polymers may be underestimated, depending on the isolation and analysis method used. Hyaluronan is not monodisperse in molecular weight and the experimentally determined polydispersity (M_w/M_n) of the polymer depends as well on both the method of extraction and the method of analysis used. For example, the values of the polydispersity of a hyaluronan sample determined by high-performance liquid chromatography varied with the type of column, the detection method, and the flow rate used [11–13]. This results in a big variation of the values reported for the M_w and polydispersity of hyaluronan between laboratories and shows the importance for standardization of the methodology for characterization and reporting of hyaluronan molecular properties.

Hyaluronan is present in all vertebrates. High concentrations are found in the umbilical cord, the synovial fluid between joints, skin, and the vitreous body of the eye. It was estimated that in the body of a person of 70 kg, about 15 g hyaluronan is found in different tissues, of which one third is turned over every day. The human skin contains over 50% of the hyaluronan in the body [14, 15]. Rooster combs contain the highest concentrations of hyaluronan (7.5 mg g^{-1}) ever reported for animal tissues [16]. Hyaluronan is also present in the capsule of a small number of microbial pathogens such as *Pasteurella multocida* and group A and C streptococci among which *Streptococcus pyogenes* (a human pathogen) and the animal pathogens *Streptococcus equi* and *Streptococcus uberis*, that quite likely pirated the enzymatic machinery from vertebrate hosts for its synthesis. These microorganisms use hyaluronan to encapsulate their cells, forming a perfect disguise against the animal defense system and facilitating the adhesion and colonization of the bacterial cells [17]. Since the hyaluronan polymer produced in animals and the aforementioned bacteria is identical, the host immune defense is not triggered to repel the pathogenic bacteria contrary to other bacteria with a different extracellular capsule. Intriguingly, the nonimmunogenic and noninflammatory properties of bacterial hyaluronan benefit mammals currently too, since bacterial hyaluronan forms an excellent source for medical grade hyaluronan. Initially hyaluronan was believed to be an inert compound having no specific interaction with other macromolecules. However,

Table 2: Biomedical and pharmacologic applications of hyaluronan.

Area	Application	Example
Biomedical	Orthopedic surgery	Arthritis
	Rheumatology	Osteoarthritis
	Ophthamology	Eye surgery
	Otolaryngology	Treatment of the vocal fold
	Dermatology and plastic surgery	Dermal filler
	Wound healing and dressing	Diabetic ulcer, skin burns
	Tissue engineering	
Pharmacology	Drug delivery	

from the discovery of the interaction of hyaluronan with cartilage proteoglycans by Hardingham and Muir [18] a large number of reports have been published on the role of hyaluronan in cellular activity, migration, mitosis, inflammation, cancer, angiogenesis, fertilization, and so on (see for more information [19, 20]).

In addition, hyaluronan and its derivatives have been largely studied and applied in the biomedical field (Table 2) [21–23]. Due to the high level of biocompatibility, hyaluronan is extensively used in viscosurgery, in the treatment of arthritis to supplement the lubrication of arthritic joints, as microcapsule for targeted drug delivery and in cosmetics as a hydrating and antiaging material.

The biological functions of hyaluronan are strongly depending upon its size. High molecular weight hyaluronan polymers ($M_w > 5\times10^5$ Da) are space filling, antiangiogenic, and immunosuppressive; medium size hyaluronan chains (M_w between 2×10^4–10^5 Da) are involved in ovulation, embryogenesis, and wound repair; oligosaccharides with 15–50 repeating disaccharide units (M_w between 6×10^3–2×10^4 Da) are inflammatory, immuno-stimulatory, and angiogenic while small hyaluronan oligomers (M_w from 400 to 4000 Da) are anti-apoptotic and inducers of heat shock proteins [24]. Lower molecular weight hyaluronan and small oligosaccharides are produced by controlled depolymerization of high molecular weight hyaluronan using physical treatment (thermal treatment, pressure), irradiation (electron beam, gamma ray, microwave), acid treatment, ozonolysis, metal catalyzed radical oxidation, and enzymatic hydrolysis with hyaluronidase (EC 3.2.1.35).

The commercial value of hyaluronan far exceeds that of other microbial extracellular polysaccharides. With an estimated world market value of $US 500 million, it is sold for up to $US 100,000 per kilogram [25]. On top of this, several groups have reported effects of hyaluronan oligosaccharides on cellular behavior that could imply the use of these molecules in cancer treatment or in wound healing [26]. It is clear that hyaluronan is much more than a space-filling inert component of the extracellular matrix and will become more and more important as a pharmaceutical component and indeed is a multifunctional megadalton stealth molecule [27].

In this paper the different methods to produce hyaluronan will be discussed as depicted in Figure 2.

2. Biosynthetic Pathways for Hyaluronan in Living Organisms

The overall reaction of hyaluronan synthesis is as follows:

$$n\,\text{UDP-GlcUA} + n\,\text{UDP-GlcNAc} \longrightarrow 2n\,\text{UDP} + [\text{GlcUA} + \text{GlcNAc}]_n. \quad (1)$$

The reaction is catalyzed by a single enzyme utilizing both sugar substrates to synthesize hyaluronan [28]. In contrast to other glycoaminoglycans which are synthesized in the Golgi network, hyaluronan is synthesized at the plasma membrane [29, 30]. Hyaluronan synthases (EC 2.4.1.212) are predicted to have multiple membrane spanning domains with a large intracellular loop on the plasma membrane's inner face. The only known exception is the *P. multocida* hyaluronan synthase which has a membrane attachment domain near the carboxyl terminus. Hyaluronan molecules are extruded into the extracellular matrix in coordination with their synthesis [31], but they also exist intracellularly. In the extracellular matrix hyaluronan exists in a number of different forms. For example, in vertebrates it can be intercalated within a proteoglycan complex, referred to glycocalyx if it is a more delicate pericellular matrix, or it can be bound to membrane receptors of the cell surface. It can interact with binding proteins so-called hyaladherins, which is also the case for intracellular hyaluronan [15]. The mechanisms regulating extracellular versus intracellular location of hyaluronan are not known yet. Recently, the presence of hyaluronan cables has been reported that was the result of the incorporation of the heavy chain of interalpha-trypsin inhibitor with hyaluronan [32]. Depending on the type of tissue, different hyaluronan concentrations and molecular weight evoke different cell responses, for example, during embryonic development, healing processes, inflammation, and cancer. How the cell response is influenced by the different molecular weight of hyaluronan is still an intriguing question [15, 24].

In 1996 the first reports were published concerning the cloning of mammalian hyaluronan synthases [33, 34]. Since then in human cells three hyaluronan synthase genes have been found encoding enzymes having distinct enzymatic properties with respect to hyaluronan size and size distribution [35] which are differentially inducible by growth factors and cytokines [36, 37].

In 1993 the first gene encoding the enzyme responsible for hyaluronan synthesis, denoted HA synthase or HAS, was identified from group A streptococci [6, 7]. This gene is part of an operon containing the *has*A gene encoding hyaluronan synthase, the *has*B gene encoding UDP-Glucose dehydrogenase, and the *has*C gene encoding UDP-Glucose-pyrophosphorylase [38–40]. A hydropathy plot of the *has*A gene predicts three transmembrane segments which is in accordance with the early findings of Markovitz and coworkers who showed that the hyaluronan synthase activity is located in the cell membrane of streptococci [41]. Cloning of

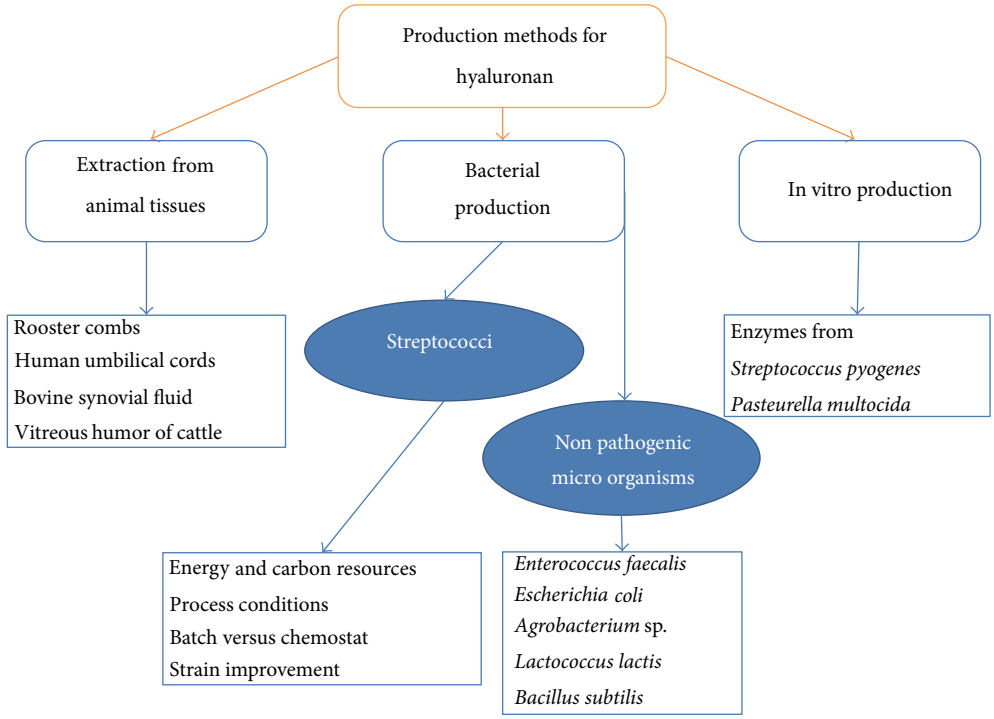

Figure 2: Production methods for hyaluronan.

the group A and later the group C streptococcal hyaluronan synthase genes [42] allowed researchers to confirm that only one gene product (the HA-synthase protein) is required for hyaluronan biosynthesis.

The first vertebrate gene identified as being HA synthase was the *Xenopus laevis* gene DG42 [43]. Since then more vertebrate HA synthase genes were identified [44] and in addition an HA synthase from an algal virus was discovered [17, 45]. The streptococcal, vertebrate, and viral HA synthases show protein sequence similarity and appear to have a single glycosyl transferase 2 (GT2) family module [46] with some similarities to chitin synthase. The last extension of organisms in which a HA synthase gene was identified is *Cryptococcus neoformans*. The CPS1 gene from this pathogenic yeast encodes a HA synthase with high homology to the previously mentioned HA synthase proteins [47]. An HA synthase that differs in protein sequence and in predicted topology from all other HA synthases was identified and cloned from type A *P. multocida*, an animal pathogen [48].

Unlike the other HA synthases (Table 3; grouped as Class I HA synthases) which are integral membrane proteins and elongate hyaluronan chains at the reducing end (Class I-R) or at the nonreducing end [50], the *P. multocida* HA synthase has a membrane attachment domain near the carboxyl terminus, has two GT2 modules, and elongates the HA chains at the nonreducing end. It is therefore called a Class II HA synthase [17]. So far the *P. multocida* HA synthase is the only known member of the Class II HA synthases [49, 51, 52].

The regulation of hyaluronan synthesis is much more complex in vertebrates than in bacteria because hyaluronan fulfills various functions in the mammalian body depending on the tissue involved and the hyaluronan chain length required. For the three mammalian HA synthase isozymes different expression patterns are observed between adult and embryonic tissues [54, 55]. Several regulatory factors are known to be involved in controlling HA synthase transcription, such as morphogenesis, cytokines, growth factors, and antisense mRNA [56, 57]. Furthermore, hyaluronan synthesis seems to be controlled through translation regulation, since a latent pool of HA synthase exits within the cell interior, in the endoplasmic reticulum-Golgi compartments, that upon insertion in the cell membrane becomes activated [53]. HA synthase activity can be modulated by posttranslational modification, such as phosphorylation and *N*-glycosylation [58]. Intermediate and small oligosaccharides are probably provided through hyaluronan cleavage by hyaluronidases, since mammalian HA synthase isozymes synthesize hyaluronan

Table 3: Hyaluronan synthase classification system as proposed by Weigel and DeAngelis [49].

	Class I		Class II
	Reducing	Nonreducing	
Members	*Streptococcus pyogenes*, *S. equisimilis*, *S. uberis*, humans, and mice	*Xenopus laevis*, algal virus	*Pasteurella multocida*
Hyaluronan chain growth	Reducing end	Nonreducing end	Nonreducing end

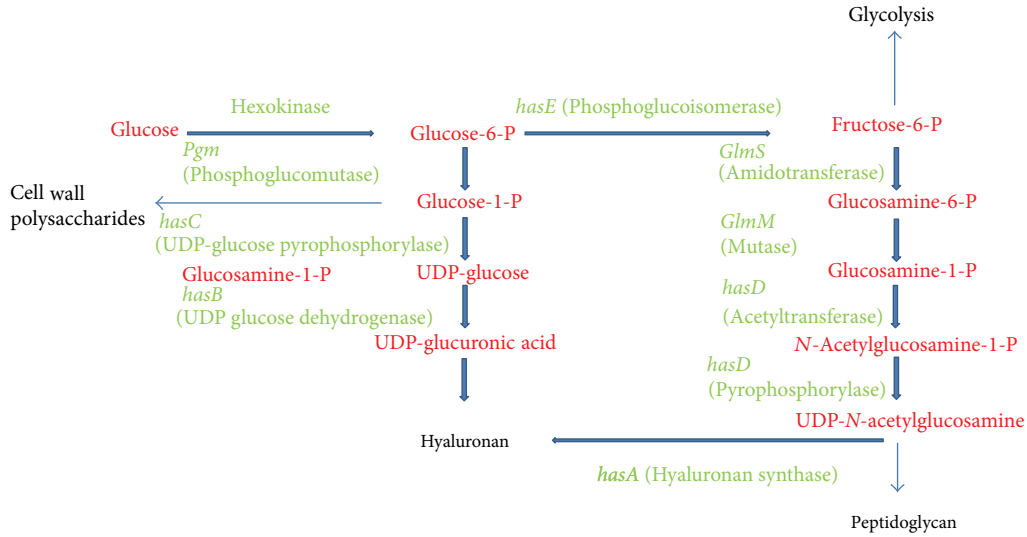

FIGURE 3: Schematic overview of the hyaluronan synthesis pathway in *S. zooepidemicus* adapted from [53] 2013 The American Society for Biochemistry and Molecular Biology.

higher than 10^5 Da [14]. Due to the high complexity of the regulation of hyaluronan synthesis in vertebrates a cohesive view on the relation between the regulatory components is not available yet.

Recently, the metabolic route of hyaluronan formation in *S. zooepidemicus* was elucidated. The streptococcal HA synthase is found in operons also encoding one or more enzymes involved in biosynthesis of the activated sugars [59]. The *has* operon in *S. zooepidemicus* encodes for five genes (Figure 3): hyaluronan synthase (*has*A), UDP-glucose dehydrogenase (*has*B), UDP-glucose pyrophosphorylase (*has*C), a *glm*U paralog encoding for a dual function enzyme acetyltransferase and pyrophosphorylase activity (*has*D), and a pgi paralog encoding for phosphoglucoisomerase (*has*E) [60]. *Has*B and *has*C are involved in UDP-GlcUA synthesis, while *has*D and *has*E are involved in the synthesis of UDP-GlcNAc. The hyaluronan biosynthesis is a costly process for bacteria in terms of carbon and energy resources and competes with cell growth because of the large pathway overlap with the cell wall biosynthesis (Figure 3). Other streptococci strains have *has* operons that contain besides hyaluronan synthase, only the *has*B and *has*C genes. This suggests that the superior hyaluronan synthesis observed for *S. zooepidemicus* relates to the availability of UDP-sugar precursors. Understanding the metabolic routes for hyaluronan synthesis had a significant role in the optimization of the microbial production of hyaluronan, allowing the control of the polymer chain length and increasing the product yield.

3. Production of Hyaluronan

Industrial manufacturing of hyaluronan is based on two main processes, the extraction from animal tissues and microbial fermentation using bacterial strains. Both technologies produce polydisperse high molecular weight hyaluronan ($M_w \geq 1 \times 10^6$ Da, polydispersity ranging from 1.2 to 2.3) for biomedical and cosmetic applications [12, 61, 62]. The first process, to be applied at industrial scale, was the extraction of hyaluronan from animal waste which is still an important technology for commercial products, but is hampered by several technical limitations. One drawback in the extraction process is the inevitable degradation of hyaluronan, caused by (a) the endogenous hyaluronidase activity in animal tissues, breaking down the polymer chain through enzymatic hydrolysis, and (b) the harsh conditions of extraction. Extraction protocols have been improved over the years, but still suffer from low yields, due to the intrinsic low concentration of hyaluronan in the tissue, and from high polydispersity of polymer products due to both the natural polydispersity of hyaluronan and to the uncontrolled degradation during extraction. As in any process for the production of therapeutic compounds from animal sources, there is a potential risk of contamination with proteins and viruses, but this can be minimized by using tissues from healthy animals and extensive purification. Nevertheless, concerns on viral (particularly avian) and protein (particularly bovine) contamination increased the interest in the biotechnological production of hyaluronan.

Production of hyaluronan by bacterial fermentation evolved to a mature technology in the last two decades of the 20th century. In the early developmental stages of bacterial fermentation using group A and C streptococci, optimization of culture media and cultivation conditions along with strain improvement were used to increase hyaluronan yield and quality. Hyaluronan yields reached 6-7 g L^{-1}, which is the upper technical limit of the process caused by mass transfer limitation due to the high viscosity of the fermentation broth. Bacterial fermentation produces hyaluronan with high molecular weight and purity, but risk of contamination with bacterial endotoxins, proteins, nucleic acids, and heavy metals exists. The identification of the genes involved in the biosynthesis of hyaluronan and of the sugar nucleotide

Table 4: A comparative view of technologies for manufacturing hyaluronan.

	Advantages	Disadvantages
Extraction from animal materials	(i) Well-established technology (ii) Available raw material at low costs (iii) Product with very high M_w up to 20 MDa (iv) Natural product	(i) Variation in product quality (ii) Risk of polymer degradation (iii) Risk of contamination with protein, nucleic acids, and viruses (iv) Extensive purification needed (v) Low yield
Bacterial production	(i) Mature technology (ii) High yield (iii) Product with high M_w (1–4 MDa)	(i) Use of genetically modified organism (GMO) (ii) Risk of contamination with bacterial endotoxins, proteins, nucleic acids, and heavy metals
Enzymatic synthesis	(i) Versatile technology (ii) Control of the M_w of the products. Products up to 0.55–2.5 MDa and also defined oligosaccharides can be obtained (iii) No risks of contamination (iv) Constant product quality	(i) Emerging technology in development stage (ii) Technological and economic viability must still be demonstrated

precursors in the 90s opened the way towards hyaluronan production using safe, nonpathogenic recombinant strains.

In the past decade a new technology emerged using isolated HA synthase to catalyze the polymerization of the UDP-sugar monomers. This novel enzymatic technology for hyaluronan synthesis is very versatile allowing producing both high molecular weight hyaluronan and hyaluronan oligosaccharides with defined chain length and low polydispersity. Production of monodisperse hyaluronan oligosaccharides on mg-scale using two single-action mutants of the *P. multocida* HA synthase was demonstrated by the group of DeAngelis [9], but large scale production was not achieved yet. A comparative analysis of established and emerging technologies for high molecular weight hyaluronan production is given in Table 4.

3.1. Extraction from Animal Tissues. Extraction of hyaluronan from animal tissues was initially used for laboratory investigations in order to identify and characterize the polymer and to elucidate its biological potential and biomedical application. Hyaluronan from almost all tissues of vertebrates including the vitreous body of the eye, umbilical cord, synovial fluid, pig skin, the pericardial fluid of the rabbit, and the cartilage of sharks was isolated and described [63]. Recently, the extraction of hyaluronan from fish eyes as an alternative resource outside animal husbandry was reported [64]. Nevertheless, the most accessible sources for large scale production high molecular weight hyaluronan are rooster combs (1.2×10^6 Da), the human umbilical cords (3.4×10^6 Da), the vitreous humor of cattle (7.7×10^4–1.7×10^6 Da), and the bovine synovial fluid (14×10^6 Da).

Despite the numerous methods for isolation and purification of hyaluronan reported in the early literature, it was only in 1979 that a method became available for the production of pharmaceutical grade hyaluronan by extraction from animal tissues. Balazs [5] developed an efficient procedure to isolate and purify hyaluronic acid from rooster combs and human umbilical cords that set the basis of the industrial production of hyaluronan from rooster combs for medical application.

Hyaluronan is soluble in water, but extraction of highly pure, high molecular weight hyaluronan from animal tissues is difficult, since in biological materials hyaluronan is usually present in a complex with other biopolymers including proteoglycans [65]. Methods to release hyaluronan from these complexes comprise the use of proteolytic enzymes (i.e., papain, pepsin, pronase, and trypsin), hyaluronan ion-pair precipitation (with e.g., cetylpyridinium chloride), precipitation with organic solvents, nonsolvent precipitation, detergents, and so forth [21]. Ultrafiltration and chromatography are used to remove the degradation products and other contaminants. Sterile filtration is used to remove all microbial cells prior to alcohol precipitation, drying, and conditioning of the end product.

Despite extensive purification, animal hyaluronan can be contaminated with proteins and nucleic acids. The quantity and nature of contaminants differ with the source, as was shown for hyaluronan isolates from human umbilical cord and bovine vitreous humor, containing elevated protein and nucleic acid levels compared to those from rooster comb and bacterial capsule isolates [12]. Diseases as bovine spongiform encephalopathy (BSE) made us aware of the risk of cross-species viral infection. Consequently, hyaluronan from animal sources has to be extensively purified to get rid of these contaminants and extraction and purification processes are continuously improved to fulfill the high quality standards for medical applications. To date, animal waste is still the most important source for the industrial manufacturing of hyaluronan for medical application and provides up to several tones of medical-grade hyaluronan preparations per year. Pharmacia (Sweden), Pfizer (USA), Genzyme (USA), and

Diosynth (The Netherlands) were among the first companies that produced hyaluronan from animal waste at industrial scale. Commercially available hyaluronan preparations from animal sources have a molecular weight ranging from several hundred thousand up to 2.5 million Da [66].

3.2. Bacterial Production. The development of hyaluronan production through bacterial fermentation started in the 60s, when it was acknowledged that animal derived hyaluronan sources can contain undesired proteins causing possible allergic inflammation responses [67]. Since the hyaluronan polymer produced in animals and bacteria is identical, bacterial hyaluronan is not immunogenic and therefore is an excellent source for medical grade hyaluronan. Extracting hyaluronan from microbial fermentation broth is a relatively simple process with high yields. An additional and important advantage of microbial hyaluronan production is that microbial cells can be physiologically and/or metabolically adapted to produce more hyaluronan of high molecular weight. Therefore, microbial hyaluronan production using either pathogenic streptococci or safe recombinant hosts, containing the necessary hyaluronan synthase, is nowadays more and more preferred.

3.2.1. Production of Hyaluronan with Streptococci Strains. The first reported hyaluronic acid isolation from group A hemolytic streptococci resulted in 60–140 mg L^{-1} hyaluronan [68]. Since then, many different attempts have been made to increase the amount of hyaluronan, including traditional techniques as optimizing the extraction process, adapting the culture media, and selecting strains with high hyaluronan productivity. In thirty years, the hyaluronan yields using batch fermentation increased from 300–400 mg L^{-1} [69] to 6-7 g L^{-1} [70], which is the practical limit due to mass transfer limitations [25]. Group C streptococci which are not human pathogens and have superior hyaluronan productivity are frequently used for hyaluronan synthesis instead of Group A streptococci or instead the animal pathogenic bacterium *P. multocida*. The most used strains are *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus*. From dairy food products *S. thermophilus* strains with high productivity of useful exopolysaccharides including hyaluronan were isolated [71], offering a safe hyaluronan producing organism. Recently, a recombinant *S. thermophilus* was constructed that was able to produce 1.2 g L^{-1} hyaluronan and the average molecular weight was comparable with the wild type strain [72].

Streptococci strains for hyaluronan production generally use glucose as carbon source. The yield of hyaluronan on glucose under aerobic fermentation conditions varies between 5 and 10% [25, 73, 74], which is significantly higher than typical yields for complex polysaccharides in lactic acid bacteria. Other carbon sources like starch [75], lactose, sucrose, and dextrin [76] can be used for hyaluronan productivity similar to glucose. The use of carbon sources like starch and lactose which are largely available at lower costs is important in view of the process economics.

Research on optimization of hyaluronan production through fermentation of streptococci strains addressed the improvement of the hyaluronan yield, the increase of the molecular weight, and the reduction of the polymer polydispersity, parameters which are essential for hyaluronan applications. The main question was if the molecular weight could be further regulated either through strain improvements, optimized process conditions, or through metabolic engineering. These topics will be discussed below.

Regulation within Bacterial Cells. Energy and Carbon Resources. Hyaluronan biosynthesis in streptococci requires a large amount of energy and competes with the bacterial cell growth for glucose as energy provider or as UDP-sugar precursor. Indeed, when unlimited glucose is available, the highest bacterial growth was observed at optimal cultivation conditions (pH 7 and 37°C) whilst the highest hyaluronan productivity and molecular weight were obtained at suboptimal growth conditions, since when cells are growing slowly, the carbon and energy resources are available for other processes [77]. Under glucose-limiting conditions first hyaluronan productivity and then molecular weight declines [25]. Decrease of the initial glucose concentrations from 60 to 20 g L^{-1} decreased the hyaluronan concentration and molecular weight from 4.20 g L^{-1} and 3.0 million Dalton, to 1.65 g L^{-1} and 2.65 million Dalton, respectively [77].

Aerobic culturing conditions increased the hyaluronan productivity by 50% as well as hyaluronan molecular weight whereas cell growth was unaffected [77–79]. Under aerobic fermentation conditions streptococci change their metabolism from producing lactate into producing acetate, formate, and ethanol. This generates four ATP molecules per hexose for the acetate production instead of two ATP molecules formed in lactate production [80]. It was assumed that the increase of hyaluronan production is due to the increased levels of ATP or to increased levels of NADH oxidase which removes the excess of NADH in the presence of oxygen. However, studies designed to improve ATP formation by increased acetate production through maltose metabolism [80] or increasing NADH oxidase levels by metabolic engineering [81] revealed that hyaluronan synthesis was not limited by energy resources.

Studies have shown that only a critical level of dissolved oxygen of 5% is needed to increase the hyaluronan yield during cell growth, above this value the yield is constant [79]. Interestingly, the expression of HA synthase in *S. zooepidemicus* is nine times higher under aerobic conditions than under anaerobic conditions [74], explaining the increase of hyaluronan synthesis. Furthermore, oxygen induces enzymes involved in the production of UDP-GlcNAc (*has*D) and acetoin recycling, which offers an additional ATP molecule and acetyl-CoA that can be used in hyaluronan production [82].

Based on these results, we can conclude that elevated HA synthase concentrations and UDP-GlcNAc availability seem to be the main reason for the increase in hyaluronan synthesis under aerobic conditions.

Influence of Process Conditions on Hyaluronan Yield and Molecular Weight. Streptococcal fermentation to produce hyaluronan is, like other fermentations, influenced by

medium composition, pH, temperature, aeration, and agitation. Especially aerobic conditions and the initial glucose concentration had a large influence on hyaluronan yield and molecular weight, as was discussed above. Both agitation and fermentation modus have a rather complex effect on hyaluronan production and are, therefore, in greater detail described, below.

The effect of agitation on the yield and the molecular weight of hyaluronan in *Streptococcus* fermentation is complex. Increasing agitation increases the hyaluronan yield under both anaerobic and aerobic conditions, most probably due to an enhanced mass transfer induced by the reduced viscosity of the broth [77, 79, 83, 84]. However, hyaluronan molecular weight increases at moderate impeller speed due to mass transfer enhancement, but decreases at high impeller speed most probably due to degradation by the reactive oxygen species that are formed under aerobic conditions at high impeller speed [85]. Oxidative degradation of hyaluronan can be prevented by the addition of oxygen scavengers such as salicylic acid in the culture media resulting in an increase of the hyaluronan molecular weight [85, 86].

Batch versus Chemostat. In batch fermentation, the hyaluronan production is limited to 6-7 g L^{-1} due to high viscosity of the broth and mass transfer constraints. Further improvement of the hyaluronan yield is therefore not possible through high-cell-density fermentation or through a high-yield strain. A continuous culture is the best strategy to improve the volumetric productivity of hyaluronan synthesis for four reasons. First, cell growth of streptococci can be maintained at the exponential phase. Extension of the exponential phase could lead to an increased amount of hyaluronan, since it was shown that hyaluronan synthesis stops in the stationary phase [40, 87] and the excretion of the HA synthase and other cell wall proteins which takes place in the stationary phase is prevented [88]. Second, suboptimal growth conditions can be imposed by nutrient limitation in order to decrease the resource competition between cell growth and hyaluronan synthesis [77, 89]. Third, a turnaround phase is avoided by using a continuous culture [90]. Finally, the viscosity of the broth can be controlled by controlling the hyaluronan concentration, thus enhancing the mass transfer in the reactor. Chemostat cultivation of *S. zooepidemicus* by controlling the medium conditions has been reported [75, 90, 91] and this opens the way for further improvement of the hyaluronan production process.

Strain Improvement. Improved hyaluronan producing strains have been obtained by random mutagenesis and selection of mutant strains oriented to the reduction of the hyaluronan-depolymerising enzyme responsible for the decrease of molecular weight of hyaluronan during fermentation (hyaluronidase-negative strains) and to the reduction of streptolysin, the exotoxin responsible for the β-hemolysis (nonhemolytic strains). Fermentation of nonhemolytic and hyaluronidase-free mutants produced hyaluronan with a molecular weight varying from 3.5 to 5.9 million Da and yields around 6-7 g L^{-1} [70, 76, 92]. Random mutagenesis has also resulted in Streptococci strains that produce hyaluronan of extraordinary high molecular weight ranging from 6 to 9 million Da and with moderate yields of 100 to 410 mg L^{-1} [93].

Further improvement of strains requires genetic engineering of the producer microorganisms to engineer the hyaluronan metabolic pathway. Metabolic engineering in *S. zooepidemicus* and in recombinant hosts has demonstrated that the ratio between UDP-GlcNAc and UDP-GlcUA and the ratio between HA synthase and substrates are the most important factors to influence hyaluronan molecular weight. Chen et al. individually overexpressed each of the five genes in the *has* operon in *S. zooepidemicus*. They discovered that overexpression of *has*A, involved in hyaluronan biosynthesis, and *has*B and *has*C, involved in UDP-GlcUA biosynthesis, decreased molecular weight, while overexpression of *has*E, involved in UDP-GlcNAc biosynthesis, greatly enhanced molecular weight. Overexpression of *has*D had no effect, but molecular weight was further increased when combined with *has*E overexpression [60]. Upregulation of *has*D in *S. zooepidemicus* has been observed at aerobic conditions [82] but also in the presence of an empty plasmid [94], both accompanied by a production of higher M_w hyaluronan. Marcellin et al. [94] suggested that regulation of *has*D is at the translational or posttranslational level and that the plasmid burden is sufficient to remove the *has*D encoded step as a limiting step. In summary, these results indicate that, with a proper balance between the synthesis pathways to UDP-GlcNAc and UDP-GlcUA, the hyaluronan molecular weight can be further increased and controlled.

In addition to the ratio between the two precursors, the importance of the ratio between precursor UDP-GlcUA and HA synthase also influences hyaluronan molecular weight. This was demonstrated for the heterologous host *L. lactis*, two plasmids were constructed containing either *has*A or *has*B from *S. zooepidemicus* with the inducible expression promoters NICE and lacA. Hyaluronan molecular weight increased significantly at *has*A/*has*B ratios below 2, indicating that higher UDP-GlcUA availability per HA synthase enhances hyaluronan molecular weight [95].

To conclude, production of hyaluronan by fermentation of streptococci strains is a mature technology, affording high molecular weight, highly pure polymers suitable for medical, pharmaceutical and cosmetic applications. Several tons per year of bacterial hyaluronan are currently produced for medical and cosmetic applications. Companies that produce hyaluronan through streptococcal fermentation are, for instance, Q-Med (Uppsala, Sweden), Lifecore Biomedical (Chaska, USA), and Genzyme (Cambridge, USA).

3.2.2. Hyaluronan from Recombinant Nonpathogenic Microorganisms. To avoid the risk of exotoxin contamination in hyaluronan products from pathogenic streptococci strains, safe organisms have been genetically engineered into hyaluronan producers, by introducing HA synthase enzymes from either streptococci or *P. multocida*. Using this approach, hyaluronan producing strains of *Enterococcus faecalis* [7], *E. coli* [7, 48, 96, 97], *Bacillus subtilis* [98], *Agrobacterium* sp. [99], and *L. lactis* [95, 100–102] were obtained and exploited for hyaluronan production. Heterologous expression of HA synthase (*has*A gene in streptococci) was sufficient

to induce production of hyaluronan. Since the hyaluronan biosynthesis has partially a common pathway with cell wall biosynthesis (Figure 3), production of hyaluronan by the host goes, however, on the expense of cell growth, due to the depletion of sugar precursors. Better hyaluronan producing heterologous strains with improved intracellular availability of sugar precursors were obtained by coexpression of the HA synthase *has*A gene derived from *S. equi* or *P. multocida* with *has*B homologue (UDP-glucose dehydrogenase) or *has*B + *has*C homologues (UDP-glucose dehydrogenase + UDP-glucose pyrophosphorylase) from *E. coli*. A recombinant *E. coli* strain obtained using this strategy produced 2 g L^{-1} hyaluronan, and the yield was increased to 3.8 g L^{-1} when the culture media was supplemented with glucosamine [99].

Using a similar strategy, namely the coexpression of the HA synthase *has*A gene from *P. multocida* with *has*B homologue (UDP-glucose dehydrogenase) from *E. coli*, into the food-grade microorganisms *Agrobacterium* sp. [99] and *L. lactis* [101], these strains were able to produce hyaluronan at levels up to 0.3 g L^{-1} for engineered *Agrobacterium* sp. and 0.65 g L^{-1} for the recombinant *L. lactis*. Although hyaluronan production yields were low, the hyaluronan production from these food-grade microorganisms has high potential for food and biomedical applications.

Without overexpression of *has*B or an analog encoding for UDP-glucose dehydrogenase, UDP-GlcUA levels are often limiting in heterologous hosts [7, 97, 98, 101], whereas UDP-GlcNAc seems to be adequately available in the hosts since it is a main component for bacterial cell wall synthesis. Attempts to overexpress both synthetic pathways to UDP-GlcNAc and UDP-GlcUA in heterologous hosts have been so far unsuccessful, but overexpression of *has*A, *has*B, and *has*C has led to considerable increases in hyaluronan synthesis varying between 200 and 800% depending on the host [96, 100].

A process for the production of ultrapure sodium hyaluronate by the fermentation of a novel, nonpathogenic recombinant strain of *B. subtilis* was developed by the biotech company Novozymes [103]. A major advantage of using *B. subtilis* as host microorganism is that it is cultivable at large scale, does not produce exo- and endotoxins, and does not produce hyaluronidase. To build up the recombinant *Bacillus* strain that produces hyaluronan, expression constructs utilizing the *has*A gene from *S. equisimilis* in combination with overexpression of one or more of the three native *B. subtilis* precursors genes—*tua*D (*has*B homologue, encoding UDP-glucose dehydrogenase), *gta*B (*has*C, encoding UDP-glucose pyrophosphorylase), and *gca*D (*has*D, encoding UDP-*N*-acetyl glucosamine pyrophosphorylase). The recombinant *B. subtilis* strain was able to produce up to 5 g L^{-1} of hyaluronan with a molecular weight of 1–1.2 million Da, when cultivated on a minimal medium based on sucrose at pH 7 and 37°C [94]. The hyaluronan is secreted into the medium and is not cell-associated, which simplifies the downstream processing significantly, making the use of water-based solvents possible for hyaluronan recovery. The *Bacillus* hyaluronan shows very low levels of contaminants (i.e., proteins, nucleic acids, metal ions, and exo- and endotoxins) and thus has a high safety profile, including no risks of viral contamination or of transmission of animal spongiform encephalopathy.

As an alternative to prokaryotic HA production, hyaluronan can be produced by infecting green algae cells of the genus Chlorella with a virus [45, 104], although the reported yields were low (0.5–1 g L^{-1}).

3.3. In Vitro Production of Hyaluronan Using Isolated HA Synthase. Synthesis of hyaluronan using isolated HA synthase becomes relevant when hyaluronan polymers of defined molecular weight and narrow polydispersity are needed. Isolated HA synthase is able to catalyze *in vitro* at well-defined conditions the same reaction as it catalyzes *in vivo*, namely, the synthesis of hyaluronan from the nucleotide sugars UDP-GlcNAc and UDP-GlcUA. Preparative enzymatic synthesis of hyaluronan using the crude membrane-bound HA synthase from *S. pyogenes* was demonstrated, although the yield was low, around 20% [105]. The hyaluronan yield was increased to 90% when the enzymatic hyaluronan synthesis was coupled with *in situ* enzymatic regeneration of the sugar nucleotides using UDP and relatively inexpensive substrates, Glc-1-P and GlcNAc-1-P in a one-pot reaction. The average molecular weight of the synthetic hyaluronan was around 5.5×10^5 Da, corresponding to a degree of polymerization of 1500.

High molecular weight monodisperse hyaluronan polymers with M_w up to 2.500 kDa (~12,000 sugar units) and polydispersity (M_w/M_n) of 1.01–1.20 were obtained by enzymatic polymerization using the recombinant *P. multocida* HA synthase, PmHAS, overexpressed in *E. coli* [106]. PmHAS uses two separate glycosyl transferase sites to add GlcNAc and GlcUA monosaccharides to the nascent polysaccharide chain. Hyaluronan synthesis with PmHAS was achieved either by *de novo* synthesis from the two UDP-sugars precursors (1) and by elongation of an hyaluronan-like acceptor oligosaccharide chain by alternating, repetitive addition of the UDP-sugars as follows:

$$n\,\text{UDP-GlcUA} + n\,\text{UDP-GlcNAc} + \text{z}[\text{GlcUA-GlcNAc}]_x \longrightarrow 2n\,\text{UDP} + [\text{GlcUA} + \text{GlcNAc}]_{x+n}. \quad (2)$$

The control of the chain length and polydispersity of the hyaluronan polymer is determined by the intrinsic enzymological properties of the recombinant PmHAS, (i) the rate limiting step of the *in vitro* polymerization appears to be the chain initiation, and (ii) *in vitro* enzymatic polymerization is a fast nonprocessive reaction. Therefore, the concentration of the hyaluronan acceptor controls the size and the polydispersity of the hyaluronan polymer in the presence of a finite amount of UDP-sugar monomers [106]. Using this synchronized, stoichiometrically-controlled enzymatic polymerization reaction, low molecular weight hyaluronan (~8 kDa) with narrow size distribution was synthesized. One important feature of the PmHAS is that chain elongation occurs at the nonreducing end of the growing chain and this makes the use of modified acceptors as substrates possible and consequently the synthesis of hyaluronan polymers with various end-moieties. The elongation of a hyaluronan tetrasaccharide labeled at the reducing end with the fluorophore 2-aminobenzoic acid

using PmHAS and the quantitative formation of fluorescent hyaluronan oligomers and polymers was demonstrated [10, 107].

These studies have shown the high potential of the *in vitro* enzymatic synthesis of hyaluronan polymers and oligomers with low polydispersity, but for the large scale application further developments are needed. Technological bottlenecks that must be resolved are (a) production of robust enzymes for hyaluronan synthesis, (b) selective separation of the UDP byproduct from the reaction mixture, to prevent enzyme inactivation and to allow UDP recycling for the synthesis of UDP-sugar substrates, (c) development of efficient processes for the production of the hyaluronan-like template for elongation, and (d) the development of simple, low cost technologies for the synthesis of sugar nucleotide substrates starting from bulk carbohydrates. Since sugar nucleotides are expensive substrates their regeneration is a crucial step for the development of an economic process for the production of hyaluronan.

For *in vitro* production of hyaluronan the Class I HA synthases are less suitable due to the fact that they are integral membrane proteins. Research with these HA synthases is mostly performed on membrane fractions but this system is less suitable for larger scale production. An alternative could be the immobilization of the enzyme in the cell wall of for instance yeast. Production of various human oligosaccharides has shown to be feasible with yeast cells in which glycosyl transferases are expressed and anchored in the cell wall glucan [108].

A far more promising enzyme for *in vitro* production of hyaluronan is the *P. multocida* Class II HA synthase. This enzyme is not an integral membrane protein and the analysis of the subcellular location and enzyme activity has shown that a recombinant truncated version of the protein lacking a 216 carboxy terminal amino acids retained HA synthase activity but was located in the cytoplasm when expressed in *E. coli* [109]. Furthermore, it was shown that two distinct transferase activities are located on the *P. multocida* HA synthase each of which can be inactivated by mutations in the DXD amino acid motif present in the active site while retaining the other transferase activity [110].

UDP-Recycling and Sugar Nucleotide Synthesis (Enzymatic Cascade, Bacterial Production). The general metabolic routes for incorporation of sugar units into glycosaminoglycans are their prior conversion into sugar nucleotides such as UDP-GlcA and UDP-GlcNAc for hyaluronan synthesis. The biosynthetic reactions are known as LeLoir pathways and these pathways can be different in microorganisms [111]. For example, the pathways for the synthesis of UDP-GlcNAc in eukaryotes and prokaryotes are different. Production of sugar nucleotides needs also cofactor regeneration for preparative applications, because the cofactors are too expensive to be used as stoichiometric agents. Nowadays, several cofactors can be effectively regenerated using enzymes, for example, NAD^+ by glutamate dehydrogenase with α-ketoglutarate, or whole cell based methods, for example, *Corynebacterium ammoniagenes* that converts orotic acid into UTP [112–115].

4. Future Perspectives

Hyaluronan, with its exceptional properties and multiple and diverse applications, has proved to be an exceptional material for medical, pharmaceutical, and cosmetic applications. Tremendous developments have been achieved in the past decades in all hyaluronan-related fields, covering the whole chain from the production of hyaluronan polymers and oligomers, to the development of advanced materials for clinical applications. Latest accomplishments in hyaluronan production and in particular the elucidation of the biosynthetic pathways in hyaluronan producing microorganisms opens new premises for the further optimization of biotechnological process for hyaluronan production with safe hosts. Further understanding of the *in vivo* regulation of hyaluronan molecular weight will benefit the biotechnological production of defined hyaluronan products with narrow polydispersity. We believe that bacterial fermentation using nonpathogenic, safe heterologous hosts will become the main source of high molecular weight hyaluronan, and metabolic engineering will be used to improve and control molecular weight. Stimulated by the developments of production techniques and the growing knowledge on the biological function of hyaluronan, research to enhancing existing medical products and to validating new concepts in medical therapies will be set forth.

Abbreviations

BSE:	Bovine spongiform encephalopathy
HA synthase or HAS:	Hyaluronan synthase
M_w:	Weight average molecular mass
M_n:	Number average molecular mass
GlcAU:	Glucuronic acid
GT:	Glycosyl transferase
UDP:	Uridine diphosphate
GlcNac:	*N*-acetyl glucosamine
GMO:	Genetically modified organism.

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