Perspective

Biofpr Biofuels Bioproducts & Biorefining

Microbial production of hyaluronic acid: the case of an emergent technology in the bioeconomy

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Abstract: In 1988 a novel fermentation route to high molecular weight hyaluronan mediated by *Streptococcus zooepidemicus* was first reported. In a few years, following rapidly expanding demand for hyaluronan as a key ingredient of facial dermal fillers, streptococcal production was commercialized, first in Western Europe and North America, and subsequently in China. Outlining the case for the microbial production of hyaluronan, an emergent technology in the bioeconomy, the purpose of this study is to further advance the field of sustainable, high-added-value, biobased production. The study recounts how this bioproduction actually developed, its advantages, and its current limitations. The research and educational outcomes of the study offer useful lessons to bioeconomy scholars and practitioners. © 2021 The Authors. *Biofuels, Bioproducts and Biorefining* published by Society of Industrial Chemistry and John Wiley & Sons Ltd

Key words: bioeconomy; hyaluronan; cell factory; bioproduction; hyaluronic acid

Introduction

First isolated in 1934, the mucopolysaccharide found in the vitreous humor of the bovine eye called 'hyaluronic acid' by Meyer and Palmer (from the Greek 'hyaloid' for 'vitreous' and uronic acid)[1] today has numerous biomedical and cosmetic applications.[2] Remarkably, most applications of this glycosaminoglycan, which is also abundant in the human body, evolved directly from the early discoveries of Balazs, whose 1942 doctoral thesis in medicine at the University of Budapest reported that the polysaccharide extracted from chicken combs was an effective treatment for patients with osteoarthritis.[3] Today, intra-articular injections of sodium hyaluronate, a component of synovial fluid, are the main treatment for osteoarthritis of the knee, typically by injection into the knee joint.[4]

In 1947 Balazs moved to the Karolinska Institutet in Stockholm, and three years later to Massachusetts, where he developed the application of hyaluronic acid (HA) in cataract surgery, during which the viscoelastic hydrogel formed by HA in aqueous solution provided exceptional protection to the eye tissues.[5] By 2015 some 300 million patients were estimated to have undergone cataract surgery using eye protection with HA-based hydrogels,[6] often using the first hyaluronic acid-based ophthalmic-surgical aid (trade named *Healon*) developed by Balazs in collaboration with a pharmaceutical company in Sweden.

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Today, beyond application in osteoarthritis and ophthalmology, the use of HA is much broader in routine clinical practice, spanning from stem cell therapy and tissue engineering,[7] through wound dressings.[8] Cosmetic and medical uses of hyaluronan also include the application of hyaluronan as dermal filler treatment for facial and neck wrinkles,[9] adhesion prevention after abdominal surgery, use as a dietary supplement ingredient, use in eye drops and as a coating for contact lenses, and use in the treatment of periodontal lesions.[10]

A linear, unbranched polysaccharide made from alternating *N*-acetyl-d-glucosamine and d-glucuronic acid, hyaluronan is ubiquitous in human tissues, including skin and cartilage, and it is particularly abundant in the umbilical cord. At physiological pH, HA is present in the form of hyaluronate, providing structure to tissues by forming viscoelastic hydrogels.[11] In 1986, in agreement with the IUPAC recommendations on polysaccharide nomenclature, Jeanloz, Balazs and his former colleague, Laurent in Sweden, introduced the term 'hyaluronan' as alternative name for HA, irrespective of the dissociation degree of the molecule.[12]

The rapidly increasing global demand and the low yield extraction of hylauronan from animal and human sources such as rooster combs and umbilical cords created the conditions for the commercial introduction of microbial production. Outlining the case for an important production technology in the emerging bioeconomy, the microbial production of hyaluronan, the purpose of this study is to further advance the field of sustainable biobased production of high added value. The study recounts how such bioproduction actually developed, its advantages, and its current limitations. The research and educational outcomes of the study offer useful lessons of relevance to bioeconomy scholars and practitioners.

Key aspects in hyaluronan microbial synthesis

In 1988 Nimrod and co-workers developed a novel fermentation route to hyaluronan utilizing *Streptococcus zooepidemicus* treated with mutagen nitrosoguanidine.[13] The process affords an enhanced amount of HA of high molecular weight (MW) (exceeding 3.5 MDa).

The molecular weight of hyaluronan in the human body is very high. For example, in normal human synovial fluid, the average molecular mass is approximately 6000–7000 kDa,[14] corresponding to chain lengths between 15 000 and 17 500 disaccharide units of d-glucuronic acid and d-*N*-acetylglucosamine bound through alternating β-1,4 and β-1,3

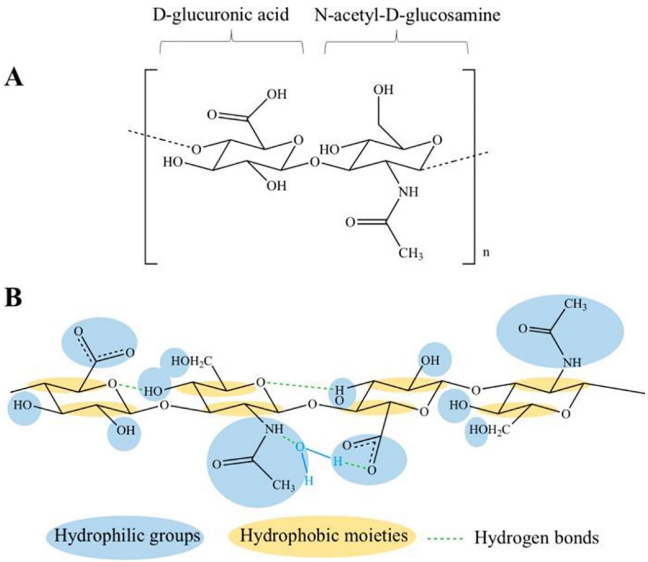

Figure 1. Structure of disaccharide repeating unit of hyaluronic acid. Chemical structures of the HA disaccharide unit (A) and HA tetrasaccharide units where the hydrophilic functional groups and the hydrophobic moieties are respectively evidenced in blue and yellow, while the hydrogen bonds are represented by green dashed lines (B). Reproduced from Ref. 15 Creative Commons Attribution License.

glycosidic bonds (8000 kDa), since the molecular weight of each disaccharide (Fig. 1(A)) is ~400 Da (401 Da).

Rotation around the glycosidic bonds is limited because both monosaccharides are in the energetically stable β configuration, in which the bulky functional groups (hydroxyl, carboxyl, acetamido, anomeric carbon) are in the sterically favorable equatorial position, resulting in a rigid conformation where hydrophobic CH alternates with polar groups linked by intra- and inter-molecular hydrogen bonds (Fig. 1(B)).[15]

In a few years, following the rapidly expanding demand for hyaluronan as a key ingredient of facial dermal fillers, the streptococcal production was commercialized, first in Western Europe and North America, and subsequently in China. By mid-2012, a European company started the production of pharmaceutical grades of the biopolymer over recombinant *Bacillus subtilis* at a new factory in Tianjin (China).[16]

From the application viewpoint, HA with MW >1800 kDa is considered to have a high molecular weight, whereas HA with a MW between 1000 and 1800 kDa is considered to have a medium MW.[17] For pharmaceutical and cosmetic applications it is important to enhance the degree of polymerization of microbially synthesized HA, whereas other applications require either medium or low MW hyaluronan.

Today's state-of-the-art production plants achieve yields of HA fermentation of 10–11 g L^{-1},[18] although this requires continuous removal of HA because solutions with titers higher than 5–6 g L^{-1} become too viscous. Along with enhanced productivity, two key developments that enabled the economic viability of HA microbial manufacturing were the ability to reproducibly target production of HA of different and narrowly distributed molecular weights, depending on the needs of a specific application (Fig. 2), and the ability to isolate HA of medical grade via economical and effective purification routes. For example, in the microbial fermentation over genetically modified *B. subtilis* the molecular weight of HA ranges from 6937 kDa at 47 °C to 392 kDa at 32 °C.[19]

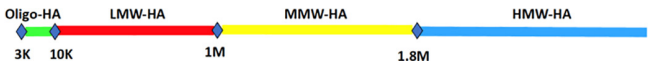

Figure 2. Conventional division of oligomeric, low, medium, and high molecular weight hyaluronic acid. Reproduced from Ref. 17 with kind permission.

In brief, the faster the growth of bacteria fed with sucrose (20 g L^{-1}) and $(NH_4)_2SO_4$ (3 g L^{-1}) is, the lower the degree of polymerization of HA produced and the higher the titer. At 32 °C, the HA titer in a 5 L fermenter reached 3.65 ± 0.13 g L^{-1} (Fig. 3(b)). The highest molecular weight HA was obtained at 47 °C, although the titer was low (1.5 g L^{-1}).

In the case of HA produced based on gram-positive *Streptococci* (Lancefield Groups A and C) bacterial strains, control over the molecular weight is achieved due to hyaluronidases enzymes, which are used to depolymerize HA in a controlled fashion to obtain low molecular weight

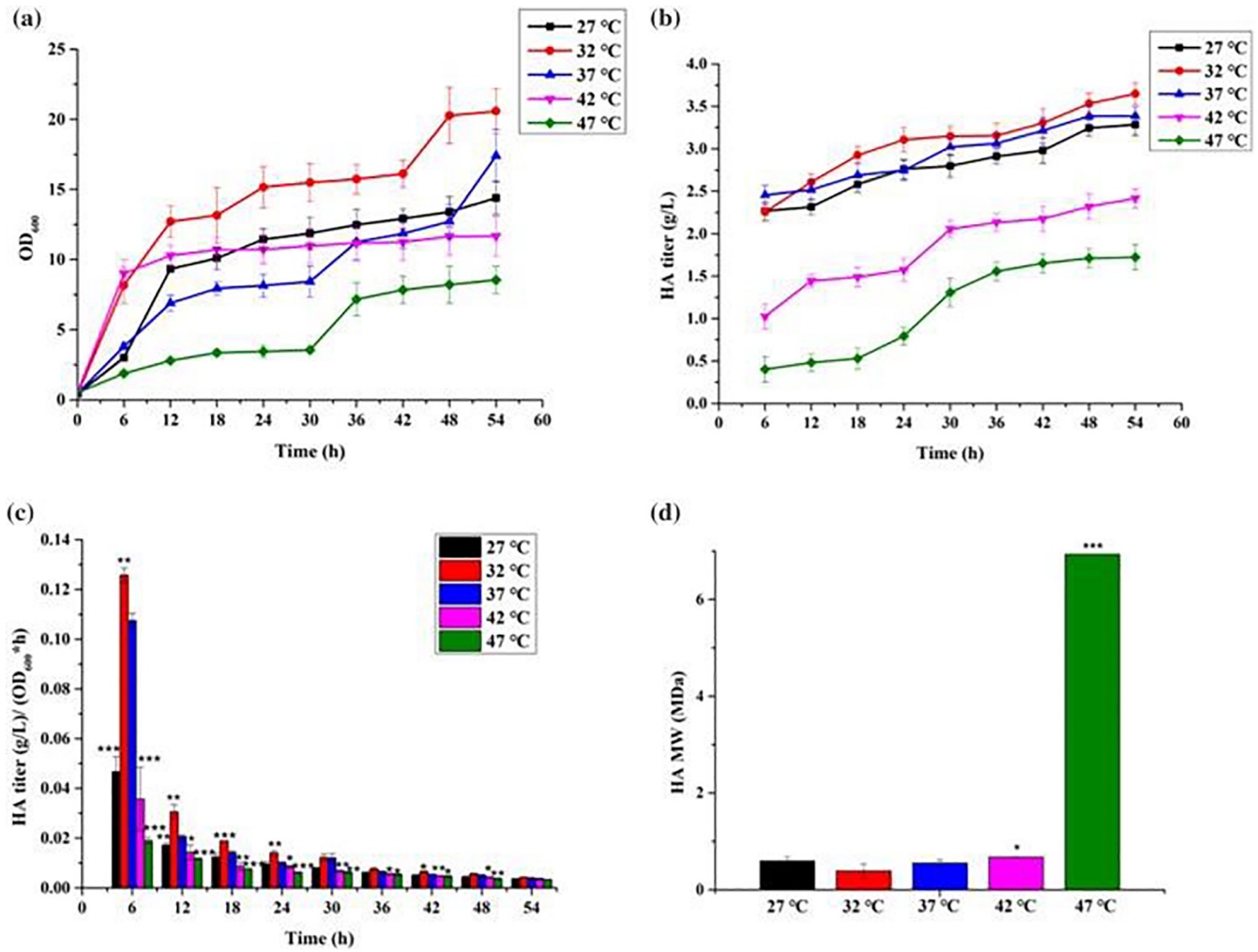

Figure 3. (a) Growth curves, (b) HA titers, (c) average HA titer measured every 6 h, and (d) HA MWs of WmB in 5 L fermenters at different temperatures (27, 32, 37, 42, and 47 °C); 37 °C was used as a control temperature. Reproduced from Ref. 19 with kind permission.

Table 1. Properties of the hyaluronic acid from a typical batch. Reproduced from Ref. 20 with kind permission.

Test	British Pharmacopeia specifications*	Sample
Appearance of solution	Clear; $A_{600\ nm}$ = ≤0.01	Clear; $A_{600\ nm}$ = 0.004
IR spectra**		Complies
pH	5.0–8.5	6.65
Nucleic acids	$A_{260\ nm}$ = ≤0.5	$A_{260\ nm}$ = 0.033
Protein	≤0.1%	0.056%
Loss on drying	≤20% by weight	18.2%
Molecular weight		

*British Pharmacopeia 2003.
**The spectrum of the test substance corresponds to the reference spectrum of sodium hyaluronate.

HA (LMW-HA) with high selectivity under mild reaction conditions. Table 1 shows that the quality of HA obtained from *Streptococcus equi* subsp. *Zooepidemicus* in a 10 L bioreactor with 50 g sucrose L^{-1} and 10 g casein hydrolysate/L affording 5–6 g hyaluronic acid/L after 24–28 h exceeds the specifications of the British Pharmacopeia for medical grade HA.[20]

Following centrifugation to separate the HA molecules from the bacterial strains, a series of filtration steps through silica gel and active carbon followed by diafiltration to remove the remaining impurities (including harmful components such as proteins and endotoxins) yield HA with 0.06% protein. A final 0.22 µm filtration step sterilizes the product isolated as pure HA with a MW of ca. 4000 kDa by precipitation with ethanol (reducing solvent use when compared to older isolation processes).[20]

In hyaluronan production over recombinant *B. subtilis*, the polysaccharide is secreted into the medium. This simplifies downstream processing, making the direct extraction of HA from the aqueous solution by spray drying possible, by spraying fine droplets of the HA solution through the top of a large cylinder vessel at high temperature. The process evaporates water, affording microbially pure (steam-sterilized) HA in powder form, ready for packaging and delivery.[21]

Industrial and economic aspects

According to a joint industry-academy research team reporting the successful synthesis of HA with molecular weight in the 1000 kDa range from genetically modified *B. subtilis* (in which the *has* A gene from *Streptococcus equisimilis* encoding the enzyme hyaluronan synthase had been expressed) the worldwide market for HA in 2005 was estimated at over $1 billion.[22] Driven by hyaluronan effectiveness, lack of toxicity, and booming global demand in 2020 the market for hyaluronan increased to $9.1 billion, and was expected to reach $17 billion by 2027, growing at a compound annual growth rate of 8.1%.[23] In China, where most of the bioproduct is currently manufactured, the export price of pharmaceutical-grade hyaluronan in 2020 varied between $2700 and $50 000 kg^{-1}, depending on purity and molecular weight.[24]

The key chemistry and biochemistry advances that enabled the replacement of hyaluronan animal sourcing with the microbial route in about a decade were the control achieved over the molecular weight, and the high purity of the microbially obtained HA. Owing to biological functions such as stimulation of fibroblast proliferation,[25] wound healing properties,[26] ease of dissolution and low viscosity, low molecular weight HA for instance is used in tissue engineering, as well as in eye drops and skin ointments.

With the exception of the proprietary bacillus-based production technology of hyaluronan (tradenamed *HyaCare*) commercialized in China in 2012 by the aforementioned Denmark-based biotechnology company, most HA currently on the marketplace is obtained via streptococcal fermentation. The bacillus-based production technology was subsequently sold by the Danish company to a large chemical company, which currently supplies bacillus-produced medium molecular weight (800 kDa) hyaluronan (tradenamed *HyaCare*) for cosmetic applications as a 'skin-identical' HA anti-aging moisturizer.[27]

In 2014, a China-based company opened a fermentation plant in Jinan with a hyaluronan capacity of about 200 t a^{-1}.[28] Chiefly commercialized as pharmaceutical-grade sodium hyaluronate, the product has the *Biohyalux* tradename. Large investment in an advanced microbial production process allowing careful control of the molecular weight and purity of the microbially produced HA quickly gained the company about 40% of the hyaluronan global market, with production volume going from 300 kg to 180 t annually.[28]

In China alone, the amount of hyaluronic acid commercialized increased from 125 t in 2013 to 308 t in 2017.[29] Jinan is also home to the Shandong University's School of Pharmaceutical Sciences where Chinese scholars advanced the microbial fermentation route including the molecular weight tailoring of microbially obtained HA based on hyaluronidases.[30] Perhaps not surprisingly, the bio-fermentation route to HA in China was first industrialized successfully by a pharmaceutical company based in Jinan.

Like the European and North American pharmaceutical and cosmetic companies that had entered China's market years before, this company does not manufacture only sodium hyaluronate sold as ingredient but also manufactures 'injection-grade' sodium hyaluronate manufactured according to the strict manufacturing practice requirements for the manufacturing of active pharmaceutical ingredients (ICH Q7).[24] The product is sold in the form of (Class-III) medical devices to be used as dermal fillers to remove facial neck wrinkles and for lip augmentation.

Showing evidence of the economic viability of the microbial route to hyaluronan, the same company in 2018 reported a 424 million yuan net profit from a 1.26 billion yuan revenue,[29] namely a 34% profit rate, which is typical of companies operating in the most profitable segments of today's marketplace.

Driven by rapidly expanding demand for high-quality hyaluronan that is not of animal origin, several other companies in China currently manufacture HA based on the microbial route. Prior to the quick emergence of China's hyaluronan industry, the main producers were based in Sweden, the country where Balazs had worked in the 1940s at the Karolinska Institute. Even today, the main injectable hyaluronan commercialized in China (with the trade name Restylane) is produced by a company based in Uppsala.[31] Thanks to innovative precipitation and isolation technology, another manufacturer based in the same city in Sweden provides the steam-sterilized biopolymer in the form of spherical particles, affording smoother results in cosmetic surgery, commercialized with the Decoria trade name.[32]

Outlook and perspectives

In the bioeconomy, functional substances and chemical products, currently mostly obtained from chemicals derived from oil feedstocks, are synthesized starting from renewable biological resources,[33] particularly from agro-food and forest by-products. Accordingly, the development of the microbial production of this glycosaminoglycan from sugar and ammonia provides several instructive lessons of relevance to bioeconomy scholars.

'Until recently', wrote Khabarov and co-authors describing the methods of hyaluronic acid production in 2015, 'the most economically viable way of obtaining HA was by its extraction from chicken combs'.[34] In the subsequent five years, driven by exceptional growth in demand, the microbial production route became largely predominant. In brief, it took about a decade to shift the production of hyaluronan from extraction at 0.1% yield from chicken combs to microbial cell factories, expanding production by two orders of magnitude. This, *inter alia*, also demonstrates the practical viability of microbial cell factories for a high-value bioproduct with multiple medical uses.

Bioeconomy and chemistry educators using recent research achievements to foster student creativity,[35] find in the microbial production of hyaluronan an exemplary case of advanced bioproduction in which accelerated technical progress in synthesis and isolation[36] created the conditions for closing plants carrying out microbial productions of technical grade hyaluronan.[37] The old plants were replaced by new plants relying on fermentation routes using safe bacterial strains such as *B. subtilis* in which the biopolymer is released smoothly from the bacterial cells into the fermentation media and is easily recovered without the need to use organic solvent.[21]

Ubiquitous in soil,[38] and present also in the gastrointestinal tract of humans,[39] the hay or grass bacillus, *B. subtilis*, is widely used by the biotechnology industry to produce most of today's industrial enzymes, with no exo- or endotoxins.[40]

Five main bioeconomy guidelines emerge from this study, which will be useful both to chemistry and biochemistry scholars and for companies and countries willing to replicate China's success with this bioproduction.

First, policy makers should be aware of the relevance of investments in higher education and biotechnology research. For instance, the world's largest HA manufacturer today found the knowledge and competences required to industrialize the new bio-fermentation route at Jinan's Shandong University.[30]

Second, China's new hyaluronan manufacturers, rather than focusing on selling the ingredient to pharmaceutical and medical device companies, focused on the production of their own medical devices such as dermal fillers and osteoarthritis injectable hyaluronan. In this way, they emulated several of Germany's fine chemical companies that became pharmaceutical companies between the second half of the 19th century and the early 1900s.[41]

Third, the technical infrastructure for hyaluronan biobased production consists of relatively small and digitally controlled bioreactors in plants operated by qualified personnel, producing little or no waste, in line with recent forecasts concerning the new chemical industry increasingly using biotechnology-based productions and starting from renewable raw biological resources.[42]

Fourth, and linked to the previous point, similar bio-based production of highly valued bioproducts such as HA also allows the concomitant presence in the marketplace of competing producers in high wage countries, as shown by the fact that manufacturers of microbially obtained HA based in Sweden, Germany, or in North America continue to produce and commercialize their own HA-based products successfully.

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Fifth, the new availability of high quality hyaluronan at lower production cost, allows its applications to be expanded to fields such as stem cell therapy, tissue engineering,[7,8] and nutraceutics, thereby creating further room for new firms willing to focus on the production of high-quality microbial HA.[33]

Hyaluronan, for example, is also a key ingredient of nutraceutical formulations such as one combining low molecular weight hyaluronic acid and natural astaxanthin to alleviate joint pain.[43]

In conclusion, we anticipate the microbial production of HA using bacteria that are not human pathogens,[44] including the 'generally recognized as safe' (GRAS) *B. subtilis*, will remain of primary industrial relevance for two major reasons. The first is that this technology produces hyaluronan of controlled and narrowly distributed polymer size.[21] The second is the level of immunogenic effect of protein and toxin residuals. As suggested by Khabarov and co-workers in their seminal book on hyaluronan,[1] this can be greater in streptococcal hyaluronic acid than in animal hyaluronic acid regardless of the low overall protein content.

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