



## Review

## Prospective bacterial and fungal sources of hyaluronic acid: A review

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## ABSTRACT

The unique biological and rheological properties make hyaluronic acid a sought-after material for medicine and cosmetology. Due to very high purity requirements for hyaluronic acid in medical applications, the profitability of streptococcal fermentation is reduced. Production of hyaluronic acid by recombinant systems is considered a promising alternative. Variations in combinations of expressed genes and fermentation conditions alter the yield and molecular weight of produced hyaluronic acid. This review is devoted to the current state of hyaluronic acid production by recombinant bacterial and fungal organisms.

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**Abbreviations:** amyE,  $\alpha$ -amylase (*B. subtilis*); aprE, subtilisin E (*B. subtilis*); araR, repressor of arabinose operons; ATP, adenosine triphosphate; cat, acetyl-CoA:CoA transferase; CSDB, Carbohydrate Structure Database; dcas9, dead Cas9 (derived from *S. pyogenes*); DO, dissolved oxygen; eglS, endoglucanase (*B. subtilis*); epr, minor extracellular protease (*B. subtilis*); FDA, Food and Drug administration; GAG, glycosaminoglycan; galR, HTH-type transcriptional regulator GalR (*E. coli*); galS, HTH-type transcriptional regulator GalS (*E. coli*); galU, UTP-glucose-1-phosphate uridylyltransferase; Glc, glucose; GlcP, glucuronic acid; GlcPNAc, N-acetylglucosamine; glmM, phosphoglucosamine mutase; glmS, L-glutamine-D-fructose-6-phosphate aminotransferase; glmU, bifunctional UDP-N-acetylglucosamine diphosphorylase/glucosamine-1-phosphate N-acetyltransferase (*Corynebacterium glutamicum*); GRAS, generally recognized as safe; HA, hyaluronic acid; HAS, hyaluronic acid synthase; HW HA, high-molecular weight hyaluronic acid; HYBID, hyaluronan-binding protein involved in HA depolymerization; kfiD, UDP-glucose 6-dehydrogenase; lacA (ganA),  $\beta$ -galactosidase (*B. subtilis*); Idh, lactate dehydrogenase; LW HA, low-molecular weight hyaluronic acid; mazF, endoribonuclease (*E. coli*); MMP-2, matrix metalloproteinase 2; mpr, extracellular metalloprotease (*B. subtilis*); MurNAc, N-acetylmuramic acid; MW, molecular weight; ND, no data; nprB, neutral protease B (*B. subtilis*); nprE, bacillolysin (*B. subtilis*); pfkA, ATP-dependent 6-phosphofructokinase (*E. coli*); pgcA, phosphoglucomutase; poxB, pyruvate:quinone oxidoreductase; pta-ackA operon, phosphate acetyltransferase and acetate kinase; RHAMM, receptor for hyaluronic acid mediated motility; sigF, RNA polymerase sigma-F factor (*B. subtilis*); SNFG, symbol nomenclature for glycans; thrC, threonine synthase (*B. subtilis*); tuaD, UDP-glucose 6-dehydrogenase (1); udgA, UDP-glucose 6-dehydrogenase (2); UDP, uridine 5'-triphosphate; UMP, 5'-uridylic acid; upp, uracil phosphoribosyltransferase (*B. subtilis*); UTP, uridine-5'-triphosphate; vHMW HA, very-high-molecular weight hyaluronic acid; wprA, cell wall associated protease (*B. subtilis*); xylR, xylose operon repressor (*B. megaterium*); zwf, glucose-6-phosphate 1-dehydrogenase.

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**1. Introduction**

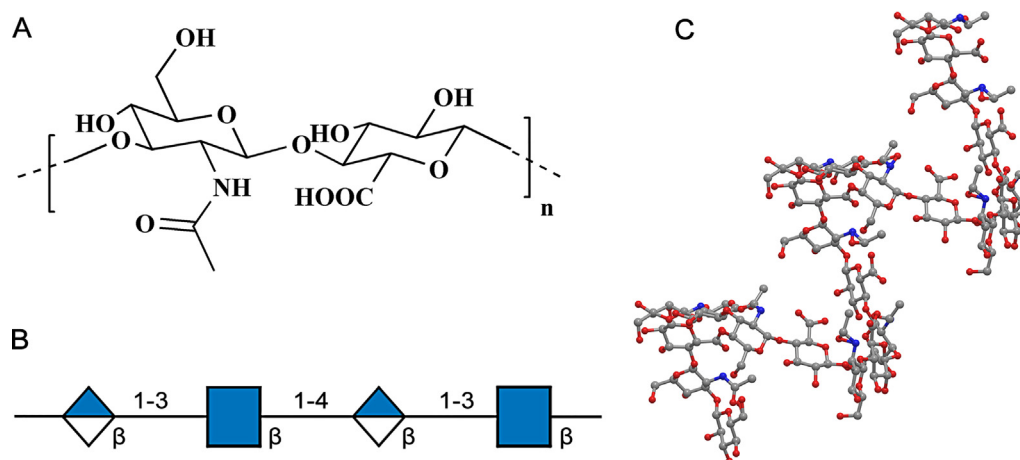
Glycosaminoglycans (GAGs), also referred to as mucopolysaccharides, are a class of biomolecules consisting of linear negatively-charged polysaccharides composed of repeating disaccharide units. GAGs are involved in various biological processes, such as cell signaling [1] and extracellular matrix assembly. Due to their unique biological properties, there has been a burgeoning interest in glycosaminoglycans for medical use as coating materials for anti-inflammatory agents [2,3] and anticoagulants [4,5]. A review about the development of GAG applications in medicine and pharmacy has been published recently [6].

GAGs are divided into two types - sulphated and non-sulphated polysaccharides. Sulfated GAGs include heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate and keratan sulfate [7]. Hyaluronic acid (HA) or hyaluronan is the only reported non-sulfated natural glycosaminoglycan [8,9]. HA (Fig. 1(A)) is a biodegradable linear unbranched polyanionic heteropolymer consisting of covalently linked interleaving residues of *N*-acetyl- $\beta$ -D-glucosamine and  $\beta$ -D-glucuronic acid (  $\rightarrow 4$  ) $\beta$ -D-GlcA(1 $\rightarrow 3$ ) $\beta$ -D-GlcNAc(1 $\rightarrow$  ). It was

predicted theoretically and subsequently confirmed experimentally that HA properties depend on the polymer molecular weight [10,11].

Extraction from animal sources has been considered a traditional method for obtaining hyaluronic acid for years, however it is not associated with large scale production anymore [12]. Currently, the most common industrial production process employed to obtain HA is streptococcal fermentation [13]. Fortunately, the risk of contamination of HA with endotoxins in both approaches is much lower than the one allowed by modern high requirements for biological safety in medical and cosmetic applications. Recombinant organisms that have been granted the Generally recognized as safe (GRAS) status from the Food and Drug Administration (FDA) of the USA have become prospective alternative sources of hyaluronic acid. The rising interest in recombinant producers can be seen in the increasing number of research articles over the last years [14–16].

This paper reviews the characteristics, biological activity and biosynthesis of HA from various origins. The use of fungal and microbial sources for the production of HA is summarized.



**Fig. 1.** (A) Fragment of the HA primary structure. Linear chains of HA range from n = 20 to n = 15,000 repeats [21]; (B) two repeating units of HA structure in SNFG [22] format; (C) 3D model of a fragment of the HA secondary twofold twisted helix built of repeating disaccharide units (the model was generated from a PDB file provided in [19]).

## 2. HA structure

As a biopolymer, HA retains the tendency to fold into highly organized structures. The primary structure of HA is presented in Fig. 1(A). The ability to adopt a secondary structure depends on the molecular weight of the polymer. The secondary structure is a tape-like twofold helix formed by twisting of repeating disaccharide units through  $180^\circ$  (Fig. 1(B)) and facilitated by five hydrogen bonds [17,18]. According to calculations, the formation of a double helix is possible only if the anti-parallel duplexes differ from each other by a maximum of  $10^\circ$  in relative rotation [19,20].

HA tertiary structure is formed of  $\beta$ -sheets based on twofold antiparallel helices [11,23]. Both construction and destruction of the tertiary structure are observed under physiological conditions ( $37^\circ\text{C}$  and  $0.15\text{ M NaCl}$ ) [24], while the secondary structure is conserved. The spatial structure of HA varies depending on temperature, solvent, and pH level.

The rupture of the hydrogen bond between acetamido and carboxylate groups causes reversible dissociation of the tertiary structure of high-molecular weight HA (HW HA) in the aqueous solution when heated [25,26]. Increasing the concentration of HA results in a change of its conformation from a rigid rod structure towards a random coil with a greater density of chain segments near the center. Interchain interactions of these segments increase respectively.

Conformational transition of HA also depends on the solvent. Software such as NAMD [27], which has a high performance in the simulation of large biomolecular systems, is used to estimate more precisely the influence of the solvent on secondary and tertiary structures. [28]. A detailed overview of models for calculation of solvent effects on reaction rate in various systems is given by Aumond in his doctoral thesis [29].

By virtue of the nucleophilic nature of acidic hydrolysis, the depolymerization of HA occurs at pH above 4 on a glucuronic acid residue because the lowest unoccupied molecular orbital is localized on this residue. HA decomposition at pH below 11 is a result of the destruction of the *N*-acetylglucosamine residue, on which the highest occupied molecular orbital is located [30]. HA depolymerization occurs faster under alkaline than acidic conditions. In basic medium, the viscosity of the HA solution is low due to the cleavage of hydrogen bonds, increased chain flexibility, and destruction of sheet and tubular structures formed in aqueous solutions [11,31,32].

Knowledge of the dependence of physico-chemical properties of the HA solution on the pH allows the prediction of HA action in the human organism and the specification of possible HA applications. The effect of conformation on physical properties of hyaluronan has been recently reviewed [11,19,32].

## 3. HA bioactivity

The biological role of HA depends on the interaction mechanism between HA and HA-specific receptors such as the membrane glycoprotein CD44 [40], or the receptor for hyaluronic acid mediated motility (RHAMM), also known as CD168 [33], which regulate the motility and cell proliferation of endothelial cells [34,35]. CD44 is a cell-adhesion molecule and a cell surface receptor that implements the binding of hyaluronate. Its degree of affinity is regulated by granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (L3) and kit ligand (KL) [36,37]. CD44 can form complexes with RHAMM [38]. RHAMM is located on the centrosome and interphase microtubules [39,40], and is responsible for the proliferation of cancer cells [41,42]. The CD44 clustering depends on the HA molecular weight and affects the degree of HA binding to immune cells [43].

Unlike other GAGs that bind to core proteins with high selectivity and specificity [44], HA does not form covalently linked proteoglycans [45,46]. HA is found in the extracellular matrix (ECM) of almost all vertebrate tissues [47], most abundantly in the soft connective tissues, such as articular cartilage and synovial fluid [48]. In the human body, synovial fluid contains HA with a molecular weight of  $7 \times 10^6$  Da; the aquatic and vitreous humors of the eye-ball consist of macromolecules with a molecular weight of  $5\text{--}6 \times 10^6$  Da [49]. Cowman [50] reviewed the distribution and biological function of HA in tissues and fluids depending on its molecular weight.

High-molecular weight HA (HW HA) is a class of polymers with a molecular weight ranging from  $1 \times 10^6$  to  $6 \times 10^6$  Da, whereas low molecular weight HA (LW HA) are samples with a molecular mass lower than  $0.25 \times 10^6$  Da [51]. The main difference between their biological activity *in situ* is that LW HA shows angiogenic activity while HW HA is anti-angiogenic [52]. During the LW HA interaction with CD44, the production of the matrix metalloproteinase 2 (MMP-2) protein is stimulated. This protein induces cell invasion through extracellular matrix barriers, which leads to an increase in the angiogenic response. HM HA interaction with cellular CD44 receptors, in turn, inhibits MMP-2 production, induces cell cycle arrest, and interrupts the process of cell proliferation [53]. Although it was previously thought that only LW HA activates the immune cells [51], recent studies have shown that the immunostimulant activity of both LW HA and HW HA is comparable [11].

Notwithstanding the lack of a strict classification, polymers with a molecular weight greater than  $6.1 \times 10^6$  Da are commonly called very-high-molecular weight HA (vHMW HA). vHMW HA, obtained from naked mole-rats, demonstrates suppression of CD44 protein-protein interactions [54], which explains its unique cytoprotective activity [11].

## 4. HA application

Hyaluronic acid has applications in various aspects of the pharmaceutical, medical, and cosmetological industry, implying strict requirements for the purity of the final product [55]. Due to this requirement, optimization of biotechnological production and improving methods of obtaining hyaluronic acid with the desired properties is a major challenge.

The unique viscosity and high rheological affinity for natural fluids has rendered a high interest in cross-linked HA in a wide range of biomedical applications, such as destruction preventer and restoration initiator of cartilage during osteoarthritis [56,57], eye drops for dry eye syndrome [58,59], and dermal fillers [60,61].

Osteoarthritis is associated with the excess hyaluronan-binding protein involved in HA depolymerization (HYBID) in cartilage [62]. HYBID causes degradation of very-high-molecular-weight endogenous HA in joints [63]. The HA, formed as a result of this process, has a lower molecular weight, and poorer mechanical and viscoelastic properties [67]. Intra-articular injection of HA promotes viscoelasticity maintenance and inhibits inflammatory cytokine production [64]. As a result, this induces a short-term pain relief [65]. Over the last decades, HA has been a leading drug against osteoarthritis [66]. The meta-analysis of the recent data on the treatment of osteoarthritis with hyaluronic acid shows controversial results [67–69]. Recently published articles point to the ambiguity of the effectiveness of osteoarthritis treatment with platelet-rich plasma and hyaluronic acid [70,71]. However, all the articles stated that HA injections can reduce pain caused by osteoarthritis.

Dry eye syndrome is characterized by a loss of tear film homeostasis and ocular surface inflammation [72]. Due to high mucoadhesion, HW HA is efficient in precorneal water retention and

protection of the ocular surface [73]. Meta-analyses have shown no clinical difference in the application of HA-based versus non-HA-based medications [74] but other medical studies, nevertheless, report that a HW HA lacrimal substitute has the potential to become an alternative to autologous serum eye drops in the treatment of dry eye syndrome [59,75,76].

The main challenge of cosmetic dermatology is resisting face aging, and one of the possible solutions is a soft tissue augmentation by injection. The use of a filler based on poly-L-lactic acid [77] and polymethylmethacrylate [78,79] is efficient against face aging, but is associated with potential adverse events. The effect of dermal fillers based on HA, on the other hand, is temporary, safer and rarely leads to side effects [80,81]. HW HA injections stimulate the collagenization process and reduce the rate of atrophic scars [82,83].

On account of the rising interest in HA for cosmetic and medical applications, the volume of the global hyaluronic acid market amounted to USD 3.5 billion in 2021 and is expected to grow with a rate of 6.8 % during 2022–2030 [84]. The major area consuming the hyaluronic acid is Asia, in particular, PRC. In 2018, the Chinese sale of hyaluronic acid amounted to 430 million ton, which accounted for more than 80 % of the global market [85]. This part is higher than indicated by an earlier analysis as reviewed in [86], which confirms the faster relative growth of the Asian HA market. The use of HA in pharmacology and the preparation of HA-based biologically active agents have been reviewed in detail by Bayer [87].

## 5. HA synthesis

In industry, HA is obtained mainly by biological methods such as extraction from animal sources with application of cetylpyridinium chloride precipitation [12] or microbial fermentation [13]. Besides that, a wide range of synthetic approaches are available nowadays, such as chemical, chemoenzymatic and enzymatic methods. In the recently published reviews by the Qiu Yibin [88] and Yao Zhi-Yuan [89] groups, special attention is paid to regulating the molecular weight of the produced HA.

### 5.1. Chemical synthesis and modification

The most common synthetic approaches include click chemistry reactions [90] and irradiation with UV [91] or visible light [92]. The diversity of approaches to chemical synthesis of HA has been reviewed in detail by Jingmin [93].

The uniqueness of click reactions stems from a high thermodynamic driving force and high yields of reaction product. This has caused a rising interest in such reactions, in particular in bio-orthogonal methods for obtaining a cross-linked hydrogel [94]. One of the most common methods, the copper-catalyzed click cyclization, results in azide-alkyne cycloaddition [95]. The main disadvantage of this reaction is the high contamination of the target product with copper [96] and the difficulties in achieving a high water content. An alternative to copper click reactions is a reaction between azide and dibenzyl cyclooctyne [97] or between thiol and alkene derivatives for radical-initiated thiol-ene click reactions [98,99] which are now actively used. Ya Li summarized the data on recent advances in click-chemistry with an emphasis on the variety of obtained biopolymers, HA in particular [100].

The main problem of obtaining a cross-linked HA is the contamination by residual chemical crosslinking agents. The best methods free of toxic chemical crosslinking agents are self-oxidation by catechol groups [101] and UV or visible light irradiation [92]. Due to the tyramine self-crosslinking initiated by the irradiation in the

presence of riboflavin as photoinitiator [102], tyramine-substituted hyaluronate can be obtained [92].

Detailed discussion of the chemical synthesis and modification of the hyaluronic acid is out of the scope of this review, and has been reviewed elsewhere [103].

### 5.2. Chemoenzymatic synthesis

Chemoenzymatic synthesis combines the high specificity of enzyme-catalyzed reactions and the flexibility of chemical derivatization [104]. In recent years, enzyme cascades, reported as efficient tools for multi-stage synthesis, have increasingly drawn attention [105]. This approach is especially useful for obtaining non-natural chemicals [106,107]. Recent studies have shown a high efficiency of using enzyme cascades immobilized by magnetic beads for obtaining HW HA [108]. Several reviews on strategies for chemoenzymatic synthesis with an emphasis on the use of enzyme cascades have been published recently [106,109].

### 5.3. Enzymatic synthesis

Enzymatic methods can be used for obtaining a monodisperse HA through degradation by hyaluronidase [110]. Various sources of hyaluronidase were reported [111]. Bacterial hyaluronidases from the  $\beta$ -endoglycosidase family degrade hyaluronic acid to a disaccharide with a 4,5-unsaturated glucuronic acid residue by  $\beta$ -elimination, while bacterial  $\beta$ -exoglycosidases, such as  $\beta$ -glucuronidase and  $\beta$ -N-acetyl-hexosaminidase, remove a single monosaccharide unit [112]. Enzymatic crosslinking by regenerated silk-fibroin is used to produce hyaluronic acid-tyramine hydrogels with high values of compressive modulus [113]. The use of mammalian enzymes makes it possible to work without a pH restriction due to the wide pH range of the enzymatic activity, but these enzymes lack absolute substrate specificity [114]. Reviews on HA enzymatic synthesis have been recently published [106].

### 5.4. Extraction from animal sources

The general approach for HA extraction from animal sources is tissue hydrolysis with subsequent removal of proteins and HA purification [115]. The most widespread animal source of high molecular weight HA is the rooster comb [116]. The isolation of HA from animal sources is complicated by formation of stable complexes of HA with proteoglycans [117]. The problems with animal sources are difficult purification, risk of cross-viral infection, and seasonal source variability. Over the past decade, several reviews have been devoted to the description of different extraction and purification methods of HA from marine animals [118]. Currently, for the industrial production of hyaluronic acid microbial fermentation is preferred more often than extraction from animal sources [16,119].

### 5.5. Fermentation

For economic reasons, microbial fermentation has been used for commercial production of HA for decades. The major problems in industrial production of HA include 1) the inhibition of HA synthesis as a result of the accumulation of lactic acid, which is one of the coproducts of enzymatic synthesis [120]; and 2) the decrease of HA yield due to loss of mixture saturation with oxygen upon growing substrate viscosity [121]. The former problem can be solved by expression of the bacterial hemoglobin gene *Vhb* in the organism that produces HA [122].

In a recently published review, the economic benefits of the production of HW HA by native and recombinant organisms were analyzed based on costs associated with production, fermentation



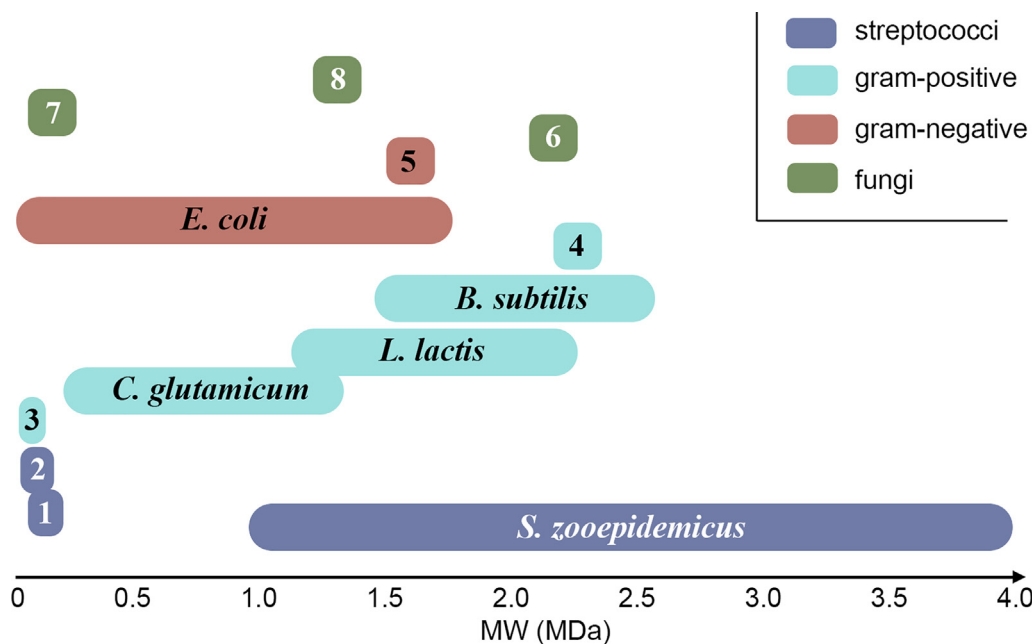


Fig. 2. Ranges of molecular weights of hyaluronic acid from various sources. 1 - *S. equi* ssp. equi, 2 - *S. iniae*, 3 - *Lactobacillus acidophilus*, 4 - *Streptomyces albus*, 5 - *Agrobacterium*, 6 - *Saccharomyces cerevisiae*, 7 - *Kluyveromyces lactis*, 8 - *Pichia pastoris*.

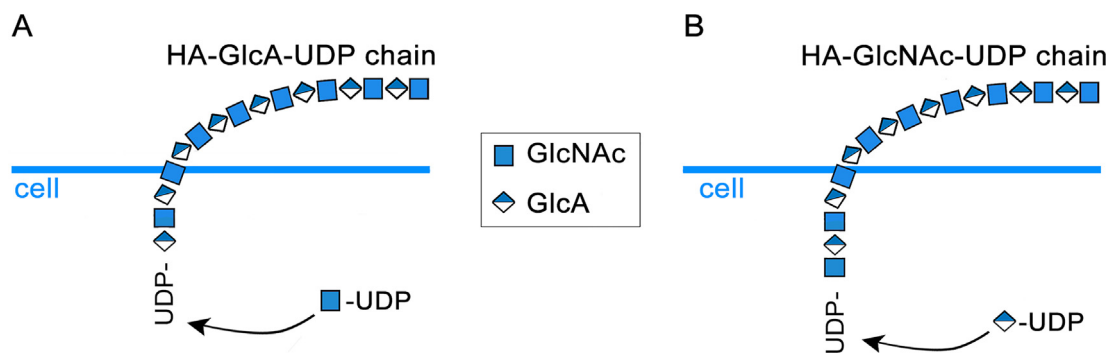


Fig. 3. Scheme of the *in vitro* HA translocation and chain growth at the reducing end by class I hyaluronan synthases. HA is synthesized on the inner surface of the plasma membrane [141]. Residue icons are given in accordance with SNFG standard. The blue line stands for the cell wall. Two simultaneous pathways are shown - adding GlcNAc-UDP (A) or GlcA-UDP (B) to the polysaccharide chain [139]. The chain growth is sequentially performed by HAS in a continuously alternating manner. For pathway A, HAS functions such as GlcNAc-UDP acceptor binding, HA-GlcA-UDP donor binding, and HA-GlcA-UDP:GlcNAc-UDP,  $\beta$ 1-3(HA-GlcA-)-transfer are required; for pathway B, GlcA-UDP acceptor binding, HA-GlcNAc-UDP donor binding, and HA-GlcNAc-UDP:GlcA-UDP,  $\beta$ 1-4(HA-GlcNAc-)-transfer are required; both pathways end with HA translocation [124].

time, and product yield [123]. According to this review, the product yields and molecular weight (Fig. 2) achieved by native sources always exceeded those from the recombinant organisms. However, the use of recombinant organisms is not economically inefficient, since this analysis did not take into account the costs for biosafety equipment in large-scale production.

### 6. HA biosynthesis

Enzymes of the Hyaluronic Acid Synthase (HAS) family synthesize HA in both eukaryotes and prokaryotes, and are located in the inner plasma membrane [124]. HA in mammals is produced by fibroblasts [125] and is located on the cell surface or in the extracellular matrix. In vertebrates, the HAS family consists of three isoenzymes, namely HAS1, HAS2, and HAS3. The functional catalytic glycosyltransferase unit in the HAS sequence is a polypeptide monomer that simultaneously transfers two distinct sugars [126]. Hyaluronan synthases are classified into two groups: Class I HASs,

shorter than 280 amino acids, and Class II HASs, longer than 280 amino acids [127]. The only known representative of Class II HASs is a HAS from *Pasteurella multocida* [128]. According to the Carbohydrate Structure Database (CSDB) [129], the abovementioned disaccharide unit is a part of multiple products of prokaryotes (68 glycans were reported to contain a  $\beta$ -D-GlcNAc-(1→3)- $\beta$ -D-GlcNAc fragment; 116 glycans were reported to contain a  $\beta$ -D-GlcNAc-(1→4)- $\beta$ -D-GlcNAc fragment), and 24 articles reported pure hyaluronic acid polymers in association with various, mainly bacterial, taxa [130]. Inasmuch as HA is a natural component in a host organism, the presence of hyaluronic acid in the polysaccharide cell capsule of *Streptococcus* and *Pasteurella* is a virulence factor for these organisms [131,132]. According to CSDB glycosyltransferase database [133], transferase activity to synthesize these bonds was predicted for seven enzymes of five serogroups of *E. coli* (Uniprot IDs A0A0B1DH13, A0A0A8J741, A0A0A8J775, Q8GMK1, Q8GMJ5, B5L396, D8WNA7 [134]).

**Table 1**  
Proteins involved in HA biosynthesis by *S. zooepidemicus*, and their functions.

Gene	Enzyme	Function	Reference
hasA	Hyaluronic acid synthase	HA synthesis and transport	[176]
hasB	UDP-glucose dehydrogenase	UDP-GlcA biosynthesis	[177,178]
hasC	UDP-glucose pyrophosphorylase	UDP-GlcA biosynthesis	[179]
hasD	Acetyltransferase and pyrophosphorylase (bifunctional)	UDP-GlcNAc biosynthesis	[180]
hasE	Phosphoglucoisomerase	UDP-GlcNAc biosynthesis	[180]

The direction of chain elongation and the mechanism of HA biosynthesis has been a subject of discussion for decades. Earlier theory suggested that all HASs attach new sugar residues to the non-reducing end of HA [135]. However, the latest research indicates that the attachment by a native bacterial synthase occurs at the reducing end [136]. HASs from vertebrates [137] and *P. multocida* [126], as well as recombinant synthases, add new monosaccharides to the non-reducing end [138]. The translocation of HA is mediated via HAS, and combines HA extrusion across the cell membrane with HA synthesis [124,139]. The scheme of HA translocation, chain growth at the reducing end, and multifunctionality of class I HAS is shown in (Fig. 3). Owing to transcriptional control, HA is produced during the growth phase rather than during the stationary phase in wild type strains [140].

Metabolic engineering is used to obtain recombinant organisms capable of HA production. That requires the reconstruction of the HA biosynthetic pathway in the chosen organism by expression of genes from natural producers that are responsible for the production of enzymes involved in the HA biosynthesis. The genus *Streptococcus*, specifically *Streptococcus zooepidemicus* [142–144], and *P. multocida* [145,146] are primarily used as gene origin for expression. Currently, HA synthase can also be cloned from eukaryotes, notably *Xenopus laevis* [147,148], small rodents (rats, hamsters and rabbits) [149,150], pigs [151], cows [152], and humans [153,154]. *Chlorovirus* PBCV-1, whose open reading frame A98R is an equally authentic hyaluronan synthase, is also of interest as gene origin for expression [155]. The key advantage of recombinant organisms is the opportunity to prevent contamination with toxins in the target product, resulting in a lesser pathogenicity. This causes a product cost depreciation due to the absence of the need for further purification. The main disadvantage in the case of recombinant bacteria is a decrease in yield due to plasmid instability [16].

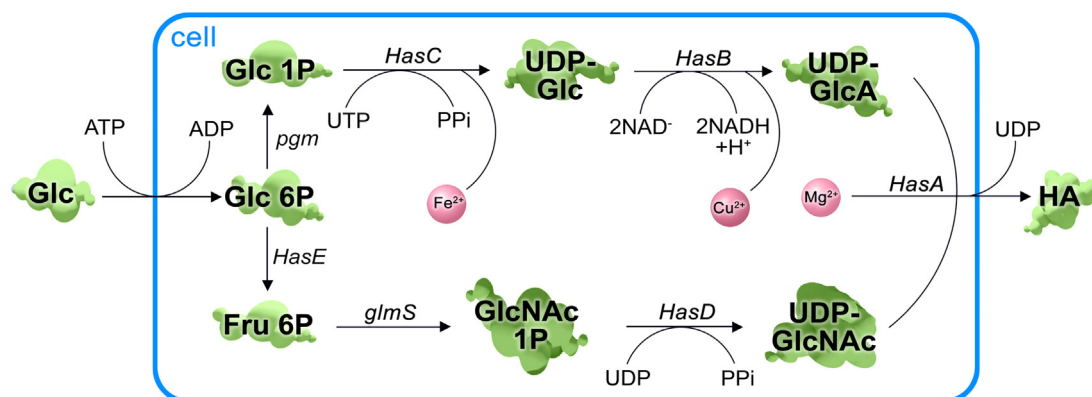
UDP-GlcNAc and UDP-GlcA are indispensable precursors for HA biosynthesis. In pure form, UDP-GlcA is found as a component within the cell wall in native HA-producing bacteria. Usually, these bacteria (e.g. *E. coli* [156]) are pathogenic or conditionally pathogenic. In HA non-producing bacteria, UDP-GlcA can be obtained by using UDP-glucose dehydrogenase for catalytic oxidation of UDP-Glc, which is naturally synthesized as one of the cell wall precursors [157]. UDP-GlcNAc is present in almost all bacteria since it is a precursor of UDP-MurNAc, which in turn is a precursor of peptidoglycans [158–160]. A noteworthy feature of hyaluronic acid biosynthesis in comparison with other GAGs is the transportation of the UDP-GlcNAc and UDP-GlcA from the cytoplasm to the plasma membrane instead of sulfation in the Golgi apparatus [7,161].

Insomuch as HA biosynthesis is an energy demanding process, consuming three moles of ATP and two moles of UTP per mole of HA [162], consideration of the metabolic cost is necessary to create a recombinant organism, whose HA productivity could surpass the yields of HA in a native source. Co-expression of *Vitreoscilla* hemoglobin and HA-expressing genes has been proposed as one of the ways to compensate for the energy costs of HA biosynthesis [163,164].

## 7. Production of HA with streptococci

The genus *Streptococcus* includes over 50 species [165]. Streptococci are classified into four groups based on criteria proposed by Sherman – growth initiation temperature, growth medium, stability at 60 °C, reducing properties, and capability of obtaining ammonia from peptones [166]. *Streptococcus* groups A and C are human pathogens [167,168]. Group A *Streptococcus* causes diseases of varying severity, such as meningitis [169], pharyngitis [170], streptococcal toxic shock syndrome [167], and poststreptococcal glomerulonephritis [171]. The pathogenesis of *Streptococcus* group A is reviewed in detail by Cunningham [172] and Carapetis [173], and the pathogenesis of group C by Kłos [174]. *S. zooepidemicus* presents the most interest for industry [175] and has been actively used for fermentative production of HA since the 1980s [13].

The genes *hasA*, *hasB*, *hasC*, *hasD* and *hasE* are responsible for the synthesis of HA in *S. zooepidemicus* [181]. The enzyme products encoded by these genes and their functions in *S. zooepidemicus* are listed in (Table 1). The scheme of HA biosynthesis in *S. zooepidemicus* is presented in (Fig. 4). The main by-products in the fermentative production of HA are lactic acid, small amounts of ethanol, carbon dioxide, acetic and formic acids [182]. Expression of the polyhydroxybutyrate synthase operon is an efficient way to reduce the concentration of free lactic acid, which prevents the formation of HA [120]. Since streptococci HAS enzymes use  $Mg^{2+}$  and  $Mn^{2+}$



**Fig. 4.** Biosynthetic pathways of HA production by *S. zooepidemicus*. Italicized enzyme names are shown beside the arrows.

**Table 2**  
Overview of HA from *Streptococci* sp.: fermentation conditions, yield and molecular weight.

Strain	Fermentation conditions	HA yield (g/l)	HA MW ( $\times 10^6$ Da)	Reference
<i>S. zooepidemicus</i> ATCC 39,920	<b>Medium:</b> 50 g/l sucrose; 3,5 g/l yeast extract; 10 g/l casein enzyme hydrolysate; 2 g/l $K_2HPO_4$ ; 1.5 g/l NaCl; 0.4 g/l $MgSO_4 \cdot 7H_2O$ <b>Aeration:</b> 2 vvm <b>Temperature:</b> 37 °C <b>Duration:</b> 28 h <b>pH:</b> 7 <b>Impeller speed:</b> 400 rpm	5.00	3.50–3.90	[175]
<i>S. zooepidemicus</i> 3523	<b>Medium:</b> 50 g/l glucose; 10 g/l yeast extract; 3 g/l $MgSO_4 \cdot 7H_2O$ ; 1.5 g/l $K_2HPO_4$ ; 3 g/l $KH_2PO_4$ ; 5 g/l $NH_4Cl$ <b>Aeration:</b> ND <b>Temperature:</b> 37 °C <b>Duration:</b> 12–14 h <b>pH:</b> 7 <b>Impeller speed:</b> 400 rpm	2.80	1.59	[242]
<i>S. zooepidemicus</i> HA-13-06	<b>Medium:</b> 70 g/l glucose; 10 g/l yeast extract; 5 g/l tryptone; 0.4 g/l $MgSO_4 \cdot 7H_2O$ ; 1.8 g/l $K_2HPO_4$ <b>Aeration:</b> 1 vvm <b>Temperature:</b> from 31 °C to 37 °C <b>Duration:</b> 24 h <b>pH:</b> from 8.0 to 7.0 <b>Impeller speed:</b> 250 rpm	4.75	2.36	[12]
<i>S. zooepidemicus</i> MTCC 3523	<b>Medium:</b> 20.0 g/l palmyra palm jaggery; 2.5 g/l yeast extract; 2.5 g/l peptic digest of animal tissue; 2.5 g/l casein enzyme hydrolysate; 5.0 g/l peptic digest of soybean meal; 5 g/l beef extract; 0.5 g/l ascorbic acid; 0.25 g/l $MgSO_4$ ; 19.0 g/l disodium $\beta$ glycerophosphate <b>Aeration:</b> 1 vvm <b>Temperature:</b> 37 °C <b>Duration:</b> overnight <b>pH:</b> 7 <b>Impeller speed:</b> 180 rpm	5.96	0.93–0.96	[243]
<i>S. zooepidemicus</i> ATCC 39,920	<b>Medium:</b> 85.35 g/l sugarcane molasses, 50 g/l yeast extract; 2.5 g/l $K_2HPO_4$ ; 2.0 g/l NaCl; 1.5 g/l $MgSO_4$ <b>Aeration:</b> 0.5 vvm <b>Temperature:</b> 37 °C <b>Duration:</b> 24 h <b>pH:</b> 8 <b>Impeller speed:</b> 100 rpm	2.83	1.35	[244]
<i>S. zooepidemicus</i> ATCC 35,246	<b>Medium:</b> 50 g/l glucose; 5 g/l yeast extract; 5.00 g/l cheese whey protein (Lowry); 2.00 g/l $KH_2PO_4$ ; 0.50 g/l $K_2HPO_4$ ; 0.50 g/l $MgSO_4$ ; 0.50 g/l $(NH_4)_2SO_4$ <b>Aeration:</b> 1 vvm <b>Temperature:</b> 37 °C <b>Duration:</b> 16 h <b>pH:</b> 6.7 <b>Impeller speed:</b> 500 rpm	4.02	3.71	[245]
<i>S. zooepidemicus</i> ATCC 35,246	<b>Medium:</b> 50 g/l glucose; 5 g/l yeast extract; 8.5 g/l cheese whey protein (Lowry); 2.00 g/l $KH_2PO_4$ ; 0.50 g/l $K_2HPO_4$ ; 0.50 g/l $MgSO_4$ ; 0.50 g/l $(NH_4)_2SO_4$ <b>Aeration:</b> 1 vvm <b>Temperature:</b> 37 °C <b>Duration:</b> 16 h <b>pH:</b> 6.7 <b>Impeller speed:</b> 500 rpm	3.19	3.32	[245]
<i>S. zooepidemicus</i> WSH-24	<b>Medium:</b> 70 g/l sucrose; 25 g/l yeast extract; 1.3 g/l $K_2SO_4$ ; 2.0 g/l $MgSO_4 \cdot 7H_2O$ ; 6.2 g/l $Na_2HPO_4 \cdot 12H_2O$ ; 0.005 g/l $FeSO_4 \cdot 7H_2O$ ; 2.5 ml trace element solution (2.0 g/l $CaCl_2$ , 0.046 g/l $ZnCl_2$ and 0.019 g/l $CuSO_4 \cdot 5H_2O$ ) <b>Aeration:</b> ND <b>Temperature:</b> 37 °C <b>Duration:</b> 16 h <b>pH:</b> from 7 to 8.5 for 1 h <b>Impeller speed:</b> 200 rpm	6.5	ND	[121]
<i>S. zooepidemicus</i> ATCC 35,246	<b>Medium:</b> 50 g/l glucose, 5 g/l yeast extract, 15 g/l viscera without hydrolysis (autoclaved at 101 °C for 1 h) from <i>Scyliorhinus canicula</i> ; 2.0 g/l $K_2HPO_4$ ; 2.0 g/l $KH_2PO_4$ ; 0.5 g/l $MgSO_4$ ; 0.5 g/l $(NH_4)_2SO_4$ <b>Aeration:</b> without aeration <b>Temperature:</b> 37 °C <b>Duration:</b> 18 h <b>pH:</b> 6.7 <b>Impeller speed:</b> 500 rpm	2.53	ND	[246]
<i>S. equi</i> ssp. <i>equi</i> RSKK 677	<b>Medium:</b> 9.9 g/l peptone, 79.86 g/l sucrose, 28 g/l yeast extract; 0.51 g/l $K_2SO_4$ ; 1.25 g/l $MgSO_4$ ; 10.38 g/l $Na_2HPO_4$ ; 0.008 g/l $FeSO_4$ ; 1.23 g/l NaCl <b>Aeration:</b> ND <b>Temperature:</b> 37 °C <b>Duration:</b> 24 h <b>pH:</b> 7.8 <b>Impeller speed:</b> 187 rpm	12.01	0.0794	[247]

Table 2 (continued)

Strain	Fermentation conditions	HA yield (g/l)	HA MW ( $\times 10^6$ Da)	Reference
<i>S. iniae</i>	<b>Medium:</b> 10 g/l peptone, 20 g/l glucose, 20 g/l yeast extract; 20 g/l beef extract; 2 g/l MgSO <sub>4</sub> ·5H <sub>2</sub> O; 2 g/l KH <sub>2</sub> PO <sub>4</sub> <b>Aeration:</b> ND <b>Temperature:</b> ND <b>Duration:</b> 15 h <b>pH:</b> 7.4 <b>Impeller speed:</b> ND	0.12	0.30	[248]

ND – no data.

ions as cofactors [183], the addition of MgCl<sub>2</sub> to the cultivation medium for recombinant organisms, in which *Streptococcus* genes are being expressed, allows achievement of a greater yield of HA [184]. Presumably, this is due to the ability of the divalent ion to stabilize the intermediate compounds of the pyrophosphate group, and to form a complex with a sugar nucleotide that increases the activity of the glycosyltransferases [185,186]. Fermentation conditions, yield and molecular weight of HA obtained from different streptococci species are presented in (Table 2).

#### 7.1. *Streptococcus equi* subsp. *zooepidemicus* (formerly, *S. zooepidemicus*)

*S. zooepidemicus* is one of the most commonly used biological sources of HA in commercial production [187]. Its main drawback is its high pathogenicity. *S. zooepidemicus* is an opportunistic pathogen that causes both respiratory [188] and neurological [189,190] diseases of varying severity, namely pneumonia [191,192], meningitis [193,194], purulent pericarditis [195], bacterial arthritis [196] and endometritis [197], affecting multiple mammal species, in particular, dogs [198,199], horses [200,201], sheep [202], pigs [203], cats [204,205], rodents [206] and humans [207,208].

The impact of varying conditions on the yield of the final product was determined as follows (listed in significance-decreasing order): temperature > oxygen level > mixing rate > pH during fermentation [209]. It was experimentally shown that the molecular weight of HA obtained under anaerobic conditions ( $1.22 \times 10^6$  Da) is almost half the size of the molecular weight of HA obtained under aerobic conditions ( $2.19 \times 10^6$  Da) at a level of dissolved oxygen (DO) of 50 % [210]. Initially, the critical DO level for *S. zooepidemicus* was defined as <2 % [210], but further studies indicated that the critical level was 5 % [211]. If the level of dissolved oxygen is above the critical level, the impact of aeration rate and agitation speed on the HA yield is significantly reduced [187]. An efficient strategy to increase the yield of HA is a two-stage fermentation. Changes in yield and molecular weight of HA in *S. zooepidemicus* HA-13-06 can be observed by varying the fermentation conditions at the stages of accumulation and optimization of molecular weight [12]. The maximum molecular weight and yield of HA were  $2.36 \times 10^6$  Da and 4.75 g/l, respectively [12].

Generally, a mixture of sugars, yeast, various peptones and sera is used as a nutrient source. With an increase in sucrose concentration and a decrease in casein concentration, the density of the mixture and the HA yield increase [175]. But an increase in the primary glucose concentration results in a non-linear increase of molecular weight of synthesized HA and a decrease in product yield [27]. Peptones are a nutrient source necessary for fermentation by Gram-positive bacteria because of the limited ability of the latter to produce B-vitamins and some amino acids [212–214]. It has been suggested that molasses as the main carbon source, and sheep wool

peptone after alkaline hydrolysis by KOH as main peptone source could be efficiently used as a cheap complex substrate for the fermentative production of HA [215]. Despite the economic benefits of sheep wool peptones, the use of plant peptones in the production of HA for pharmaceutical and cosmetic applications is preferable due to the purity requirements [216]. The introduction of amino acids such as lysine, cysteine, glutamic acid and arginine into the nutrient medium also increases the HA yield [217]. The presence of microelements and mineral nutrients, such as Mg and P in the nutrient medium significantly increases the yield of HA in *S. zooepidemicus* strains [215]. A study has been undertaken to clarify the possibility of obtaining HA with predetermined characteristics by adding ions of various metals. It has been demonstrated that the presence of Na<sup>+</sup> ions in the cultivation medium significantly reduced the concentration of produced HA [218], which was associated with the ability of monovalent ions to inhibit hyaluronan synthase, thereby reducing the reaction yield.

The overexpression of genes involved in UDP-Glc6P biosynthesis makes it possible to obtain a LW HA and increase the yield [219]. Overexpression of genes implementing the biosynthesis of UDP-Glc6PNAc, in contrast, increases the molecular weight and reduces the yield. Overexpression of genes for production of HA synthase contributes to an increase in yield and a decrease in molecular weight [220]. The elimination of genes encoding hyaluronidase leads to an increase in the yield of high-molecular HA [221].

#### 7.2. *Streptococcus equi* subsp. *equi*

*Streptococcus equi* subsp. *equi* is a  $\beta$ -hemolytic Lancefield group C streptococcus, whose distinctive feature among other  $\beta$ -hemolytic streptococci is the ability to evade phagocytosis [222]. Consideration of DNA hybridization shows that *S. equi* subsp. *equi* is a subtype of *S. zooepidemicus* [223], although it was previously mistakenly classified as an archetype of *S. zooepidemicus*. *S. equi* subsp. *equi* is known as an opportunistic pathogen that causes strangles abscesses in horses [224–226], local tissue necrosis in fish [227], and invasive infections in immunocompromised patients [228].

It was experimentally shown on *S. equi* subsp. *equi* that 4-methylumbelliferone reduces the yield of HA due to inhibition of enzymatic activity of HAS in intact cells [229].

#### 7.3. *Streptococcus pyogenes*

*Streptococcus pyogenes* is a  $\beta$ -hemolytic group A streptococcus [230], a human pathogen [165,231], which causes puerperal sepsis [232], tonsillitis [233], toxic shock syndrome [234,235], and necrotizing fasciitis [236,237].

SpHAS (HAS gene from *S. pyogenes*) encodes two enzymes, HASB and HASC [179]. SpHAS has been shown to be 30 % identical to the



**Table 3**  
Overview of HA fermentation conditions, yield and molecular weight of HA obtained from gram-positive bacteria except *Streptococci*.

Host organism	Gene and origin	Fermentation conditions	HA yield (g/l)	HA MW ( $\times 10^6$ Da)	Ref.
<i>L. acidophilus</i> PTCC1643	hasA and hasC co-expression assembled with plasmid pJH181.3	<b>Medium:</b> 20 g/l lactose, De Man, Rogosa and Sharpe broth, 12.26 g/l pyruvate, 1.27 ml/l trace elements, 3 g/l urea, 3 g/l $(\text{NH}_4)_2\text{SO}_4$ <b>Aeration:</b> ND <b>Temperature:</b> 38 °C <b>Duration:</b> 48 <b>pH:</b> ND <b>Impeller speed:</b> ND	1.7	< 0.027	[327]
<i>C. glutamicum</i> ATCC13032	hasA (from <i>S. equisimilis</i> ) and hasB induced by $P_{\text{tac}}$ promoter	<b>Medium:</b> 40 g/l glucose, 11 g/l yeast extract, 30 g/l $(\text{NH}_4)_2\text{SO}_4$ , 1 g/l $\text{KH}_2\text{PO}_4$ , 0.5 g/l $\text{K}_2\text{HPO}_4$ , 5 g/l $\text{MgSO}_4$ , 0.01 g/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01 g/l $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ <b>Aeration:</b> 1 vvm <b>Temperature:</b> 28 °C <b>Duration:</b> 48 <b>pH:</b> 7.2 <b>Impeller speed:</b> 600 rpm	8.3	1.30	[249]
<i>C. glutamicum</i> ATCC13032	hasA, hasB and aceE induced by $P_{\text{tac}}$ promoter, $\Delta\text{ldh}$ , $\Delta\text{ackA-pta}$ , $\Delta\text{cat}$ , $\Delta\text{poxB}$ , $\Delta\text{zwf}$	<b>Medium:</b> 20 g/l corn syrup powder, 40 g/l glucose, 1 g/l $\text{KH}_2\text{PO}_4$ , 0.5 g/l $\text{K}_2\text{HPO}_4$ , 30 g/l $(\text{NH}_4)_2\text{SO}_4$ , 10 mg/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 10 mg/l $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ , 50 $\mu\text{g/ml}$ kanamycin <b>Aeration:</b> 1 vvm <b>Temperature:</b> 28 °C <b>Duration:</b> 24 h <b>pH:</b> 7.2 <b>Impeller speed:</b> 600 rpm	28.7	0.21	[279]
<i>C. glutamicum</i> ATCC13032	hasA (from <i>S. equisimilis</i> ) and hasB induced by $P_{\text{tac}}$ promoter, $\Delta\text{ldh}$	<b>Medium:</b> 40 g/l corn syrup powder, 30 g/l $(\text{NH}_4)_2\text{SO}_4$ , 1 g/l $\text{KH}_2\text{PO}_4$ , 0.5 g/l $\text{K}_2\text{HPO}_4$ , 5 g/l $\text{MgSO}_4$ , 0.01 g/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01 g/l $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ , 5 $\mu\text{g/ml}$ chloramphenicol <b>Aeration:</b> 1 vvm <b>Temperature:</b> 28 °C <b>Duration:</b> 48 h <b>pH:</b> 7.2 <b>Impeller speed:</b> 600 rpm	21.6	1.28	[142]
<i>S. albulus</i> CRM003	hasA (from <i>S. zooepidemicus</i> FERM BP878), <i>udgA</i> , <i>glmU</i> , <i>gtaB</i> (from <i>S. avermitilis</i> NBRC14893) induced by $P_{\text{pls}}$ promoter	<b>Medium:</b> 50 g/l glucose, 5 g/l yeast extract, 1 g/l $(\text{NH}_4)_2\text{SO}_4$ , 1.6 g/l $\text{Na}_2\text{HPO}_4$ , 1.4 g/l $\text{KH}_2\text{PO}_4$ , 0.5 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.04 g/l $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03 g/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ <b>Aeration:</b> 3.5 vvm <b>Temperature:</b> 30 °C <b>Duration:</b> 30 <b>pH:</b> 6.8 (first-stage fermentation), 4.2 (second-stage fermentation) <b>Impeller speed:</b> 150 rpm (first-stage fermentation), 500 rpm (second-stage fermentation))	3.60	2.20	[143]
<i>B. subtilis</i> 168	hasA (from <i>S. zooepidemicus</i> ) induced by $P_{\text{xyIA}}$ promoter, co-overexpression of <i>tuaD</i> , <i>glmU</i> and <i>gtaB-glmM-glmS</i> (induced by $P_{\text{veg}}$ promoter), $\Delta\text{lacA}$	<b>Medium:</b> 2 g/l tryptone, 20 g/l yeast extract, 20 g/l xylose, 15 g/l sucrose, 1.5 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ <b>Aeration:</b> 2.0 vvm <b>Temperature:</b> 37 °C <b>Duration:</b> 24 h <b>pH:</b> 7.0 <b>Impeller speed:</b> 600 rpm (first-stage fermentation), 800 rpm (second-stage fermentation))	3.16	1.40–1.83	[314]
<i>B. subtilis</i> WB600 ( $\Delta\text{nprE}$ , $\Delta\text{aprE}$ , $\Delta\text{nprB}$ , $\Delta\text{bpr}$ , $\Delta\text{mpr}$ , and $\Delta\text{epr}$ )	hasA (from <i>S. ubris</i> ), hasB and hasC co-expression, $\Delta\text{upp}$ , $\Delta\text{sigF}$	<b>Medium:</b> 20 g/l sucrose, 3 g/l $(\text{NH}_4)_2\text{SO}_4$ , 6.5 g/l $\text{KH}_2\text{PO}_4$ , 4.5 g/l $\text{Na}_2\text{HPO}_4$ , 2 g/l sodium citrate, 3 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 6 ml/l of a trace metallic elements solution (100 g/l citric acid, 20 g/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 5 g/l $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 2 g/l $\text{Cu}_2\text{O}_4 \cdot 5\text{H}_2\text{O}$ , 2 g/l $\text{ZnCl}_2$ ) <b>Aeration:</b> ND <b>Temperature:</b> 32 °C <b>Duration:</b> 54 <b>pH:</b> ND <b>Impeller speed:</b> ND	3.65	< 0.39	[317]
<i>B. subtilis</i> 1A751 ( $\Delta\text{aprA3}$ )	seHas (from <i>S. equisimilis</i> ) and <i>tuaD</i> induced by $P_{\text{grac}}$ promoter at the <i>amyE</i> locus, <i>dcas9</i> (derived from <i>S. pyogenes</i> ) and <i>xyIR</i> (from <i>Bacillus</i> )	<b>Medium:</b> 20 g/l sucrose, 10 g/l yeast extract, 1 g/l $(\text{NH}_4)_2\text{SO}_4$ , 9.15 g/l $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ ; 3 g/l $\text{KH}_2\text{PO}_4$ , 1 g/l trisodium citrate- $2\text{H}_2\text{O}$ , 2.5 g/l casamino acids, 5.5 mg/l $\text{CaCl}_2$ , 13.5 mg/l	1.46	1.67	[328,329]

Table 3 (continued)

Host organism	Gene and origin	Fermentation conditions	HA yield (g/l)	HA MW ( $\times 10^6$ Da)	Ref.
$\Delta$ eglS102 $\Delta$ bgIT)	<i>megaterium</i> ) induced by $P_{xyIA,Bm}$ promoter at the lacA locus, ftsZ-gRNA. P79NT(15C-U) cassette induced by $P_{xyIA,SphI+1}$ promoter at the wprA locus	FeCl <sub>2</sub> ·6H <sub>2</sub> O, 1 mg/l MnCl <sub>2</sub> ·4H <sub>2</sub> O, 1.7 mg/l ZnCl <sub>2</sub> , 0.43 mg/l CuCl <sub>2</sub> ·2H <sub>2</sub> O, 0.6 mg/l CoCl <sub>2</sub> ·6H <sub>2</sub> O, 0.6 mg/l Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O <b>Aeration:</b> ND <b>Temperature:</b> 37 <b>Duration:</b> overnight <b>pH:</b> ND <b>Impeller speed:</b> 280 rpm	1.25	2.10	[328,329]
<i>B. subtilis</i> 1A751 ( $\Delta$ aprA3 $\Delta$ eglS102 $\Delta$ bgIT)	seHas (from <i>S. equisimilis</i> ) and tuaD induced by $P_{grac}$ promoter at the amyE locus, dcas9 (derived from <i>S. pyogenes</i> ) and xylR (from <i>Bacillus megaterium</i> ) induced by $P_{xyIA,Bm}$ promoter at the lacA locus, pgsA and clsA induced by $P_{grac}$ promoter at the thrC locus, ftsZ-gRNA.P533NT cassette induced by $P_{xyIA,SphI+1}$ promoter at the wprA locus	<b>Medium:</b> 20 g/l sucrose, 10 g/l yeast extract, 1 g/l (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 9.15 g/l K <sub>2</sub> HPO <sub>4</sub> ·3H <sub>2</sub> O; 3 g/l KH <sub>2</sub> PO <sub>4</sub> , 1 g/l trisodium citrate·2H <sub>2</sub> O, 2.5 g/l casamino acids, 5.5 mg/l CaCl <sub>2</sub> , 13.5 mg/l FeCl <sub>2</sub> ·6H <sub>2</sub> O, 1 mg/l MnCl <sub>2</sub> ·4H <sub>2</sub> O, 1.7 mg/l ZnCl <sub>2</sub> , 0.43 mg/l CuCl <sub>2</sub> ·2H <sub>2</sub> O, 0.6 mg/l CoCl <sub>2</sub> ·6H <sub>2</sub> O, 0.6 mg/l Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O <b>Aeration:</b> ND <b>Temperature:</b> 37 <b>Duration:</b> overnight <b>pH:</b> ND <b>Impeller speed:</b> 280 rpm	~1.3	1.64	[14]
<i>B. subtilis</i> 1A751	seHas ( <i>S. equisimilis</i> ) and tuaD induced by $P_{grac,UPmod}$ promoter at the amyE locus, dcas9 (derived from <i>S. pyogenes</i> ) and xylR (from <i>B. megaterium</i> ) induced by $P_{xyIA,Bm}$ promoter at the lacA locus, araE induced by $P_{grac,\Delta UP}$ promoter at the vpr locus, araR and zwf-gRNA.P92NT(15C-A cassette induced by $P_{xyIA,SphI+1}$ promoter and mazF (from <i>E. coli</i> ) induced by $P_{araE}$ promoter at the wprA locus	<b>Medium:</b> 20 g/l sucrose, 1 g/l (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 9.15 g/l K <sub>2</sub> HPO <sub>4</sub> ·3H <sub>2</sub> O, 3 g/l KH <sub>2</sub> PO <sub>4</sub> , 1 g/l trisodium citrate·2H <sub>2</sub> O, 10 g/l yeast extract, 2.5 g/l casamino acids, 5.5 mg/l CaCl <sub>2</sub> , 13.5 mg/l FeCl <sub>2</sub> ·6H <sub>2</sub> O, 1 mg/l MnCl <sub>2</sub> ·4H <sub>2</sub> O, 1.7 mg/l ZnCl <sub>2</sub> , 0.43 mg/l CuCl <sub>2</sub> ·2H <sub>2</sub> O, 0.6 mg/l CoCl <sub>2</sub> ·6H <sub>2</sub> O, 0.6 mg/l Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O <b>Aeration:</b> ND <b>Temperature:</b> 37 °C <b>Duration:</b> 10 h <b>pH:</b> ND <b>Impeller speed:</b> 280 rpm	~0.9	1.77	[14]
<i>B. subtilis</i> 1A751	seHas ( <i>S. equisimilis</i> ) and tuaD induced by $P_{grac,UPmod}$ promoter at the amyE locus, dcas9 (derived from <i>S. pyogenes</i> ) and xylR (from <i>B. megaterium</i> ) induced by $P_{xyIA,Bm}$ promoter at the lacA locus, araE induced by $P_{grac,\Delta UP}$ promoter at the vpr locus, araR and zwf-gRNA.P603NT(15G-U) cassette induced by $P_{xyIA,SphI+1}$ promoter and mazF (from <i>E. coli</i> ) induced by $P_{araE}$ promoter at the wprA locus	<b>Medium:</b> 20 g/l sucrose, 1 g/l (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 9.15 g/l K <sub>2</sub> HPO <sub>4</sub> ·3H <sub>2</sub> O, 3 g/l KH <sub>2</sub> PO <sub>4</sub> , 1 g/l trisodium citrate·2H <sub>2</sub> O, 10 g/l yeast extract, 2.5 g/l casamino acids, 5.5 mg/l CaCl <sub>2</sub> , 13.5 mg/l FeCl <sub>2</sub> ·6H <sub>2</sub> O, 1 mg/l MnCl <sub>2</sub> ·4H <sub>2</sub> O, 1.7 mg/l ZnCl <sub>2</sub> , 0.43 mg/l CuCl <sub>2</sub> ·2H <sub>2</sub> O, 0.6 mg/l CoCl <sub>2</sub> ·6H <sub>2</sub> O, 0.6 mg/l Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O <b>Aeration:</b> ND <b>Temperature:</b> 37 °C <b>Duration:</b> 10 h <b>pH:</b> ND <b>Impeller speed:</b> 280 rpm	~0.2	~2.20	[330]
<i>L. lactis</i> NZ9000	hasA (from <i>S. uberis</i> 0140 J ATCC BAA-854) and hasB (from <i>S. zooepidemicus</i> ATCC 35246) co-expression under control of nisin-inducible promoter pNZ8148	<b>Medium:</b> M17 medium with 1 % glucose and 10 mg/L chloramphenicol <b>Aeration:</b> ND <b>Temperature:</b> 30 °C <b>Duration:</b> 24 h <b>pH:</b> ND <b>Impeller speed:</b> 200 rpm	~0.7	~1.18	[331]
<i>L. lactis</i> NZ9000	hasA and hasB (from <i>S. equi</i> subsp. <i>zooepidemicus</i> ATCC 35246) co-expression under control of nisin-inducible promoter pNZ8148	<b>Medium:</b> 5 g/l brain heart infusion, 5 g/l yeast extract, 30 g/l glucose, 0.5 g/l MgSO <sub>4</sub> ·7H <sub>2</sub> O, 0.5 g/l ascorbic acid, 0.5 g/l KH <sub>2</sub> PO <sub>4</sub> , 1.5 g/l K <sub>2</sub> HPO <sub>4</sub> <b>Aeration:</b> ND <b>Temperature:</b> 30 °C <b>Duration:</b> 30 <b>pH:</b> 7 <b>Impeller speed:</b> 200 rpm	~1.3	~1.10	[331]
<i>L. lactis</i> NZ9000	hasA, hasB and hasE (from <i>S. equi</i> subsp. <i>zooepidemicus</i> ATCC 35246) co-expression under control of nisin-inducible promoter pNZ8148	<b>Medium:</b> 5 g/l brain heart infusion, 5 g/l yeast extract, 30 g/l glucose, 0.5 g/l MgSO <sub>4</sub> ·7H <sub>2</sub> O, 0.5 g/l ascorbic acid, 0.5 g/l KH <sub>2</sub> PO <sub>4</sub> , 1.5 g/l K <sub>2</sub> HPO <sub>4</sub> <b>Aeration:</b> ND <b>Temperature:</b> 30 °C <b>Duration:</b> 30 <b>pH:</b> 7 <b>Impeller speed:</b> 200 rpm			

DG42 gene known as xIHAS from *X. laevis* [238,239]. SPhAS expression in *Escherichia coli* with the availability of UDP-GlcA and UDP-GlcNAc afforded *in vitro* synthesis of HW HA [240]. Though encapsulated *S. pyogenes* is a natural source of HA [241], it is highly pathogenic, and thus is neither used as a native producer nor as a gene origin for expression in commercial HA production.

## 8. Production of HA with other gram-positive bacteria

The main drawback of the implementation of streptococci is their high pathogenicity. This makes it necessary to explore new organisms that could be used in the industrial production of HA. Insofar as there are no native HA producers among Gram-positive bacteria except streptococci [143,162] all potential HA-producing strains are recombinant organisms obtained with the aid of gene expression. To create a recombinant organism that could synthesize HA, the expression of genes encoding the HasA enzyme from exogenous sources is enough [249]. Fermentation conditions, yield and molecular weight of HA obtained from gram-positive bacteria except streptococci are presented in (Table 3).

### 8.1. *Lactobacillus*

The genus *Lactobacillus* is widely used in food industry for the production of yeast bread [250,251] and dairy products [252–254]. Extensive medical research has confirmed that lactobacilli are efficient as probiotics against enteropathogenic bacteria [255,256]. Oral administration and application on damaged tissues using heat-killed *Lactobacillus plantarum* L-137, inter alia, initiates HA biosynthesis in mammals [257]. Currently, the genus *Lactobacillus* includes over 40 species according to the NCBI taxonomy database [258]. Twelve species, namely *L. acidophilus*, *L. bulgaricus*, *L. casei*, *L. delbrueckii*, *L. fermentum*, *L. gasseri*, *L. helveticus*, *L. johnsonii*, *L. plantarum*, *L. reuteri*, *L. sakei* and *L. salivarius*, which lack *pgdA* and *GlcNAc* deacetylase genes, represent potential recombinant organisms for HA biosynthesis. *LacA* promoter from *Lactococcus lactis*' 8-kb lactose operon can be used to efficiently regulate gene expression in lactobacilli [259].

As exemplified on *L. acidophilus* FTDC 1231, the addition of  $Fe^{2+}$  and  $Cu^{2+}$  ions to the cultivation medium leads to a higher HA yield due to an increase of free binding energy between substrates towards UDP-glucose pyrophosphorylase and UDP-glucose dehydrogenase [260]. Subsequent examination of the effects of the addition of various metal ions to the nutrient medium for the growth of *Lactobacillus rhamnosus* FTDC 8313 have proven that the addition of  $Mn^{2+}$  and  $Mg^{2+}$  synergistically increases the binding of substrates with glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, phosphopyruvate hydratase, and pyruvate kinase, which facilitates an increase in HA yield [261].

### 8.2. *Corynebacterium glutamicum*

*Corynebacterium glutamicum* is a non-pathogenic and non-sporulating bacteria classified as GRAS. It is widely used for large-scale production of various organic compounds, such as organic acids [262,263], alcohols [264–266] and pyrazine [267–269], and has a special significance in the industrial production of amino acids [270–274]. In a detailed survey, the variety of applications of this organism in industry was analyzed by Becker [275]. Rapid growth, high yields and absence of capsules makes *C. glutamicum* a promising recombinant organism for the production of heterologous proteins [276]. The issue of the recombinant protein

expression in *C. glutamicum* is narrowly reviewed by Liu [277] and Lee [278].

*C. glutamicum* encapsulates itself by the produced HA, which leads to hyper hyaluronan production. The analysis of the final product yield allows proposing the theory that overexpression of *hasD* or *hasE* causes metabolic stress in recombinant organisms. The expression of *glmM*, which encodes a phosphoglucosamine mutase, has no benefit in HA production [279]. Depending on the gene origin, *hasA* expression is implemented to varying extents; *sphasA* expression from *S. pyogenes* is the most efficient strategy [280] along with the combination of *hasAB* operons allowing a HA yield of 8.4 g/l [142]. An examination of the effect of operon co-expression on the output indicates that the co-expression of *hasABC* operons leads to yields of  $2.15 \pm 0.10$  g/l, which is twice the values obtained during co-expression of *hasAB* operons. Moreover, if *glmU* expression and induction are sequentially carried out during fermentation, a dramatic decline is observed in the amount of HA obtained [281]. The overexpression of the *ftsZ* gene increases 13.5-fold the ability of the organism to produce HA due to proliferation of single-cell HASs on the cell surface [282].

### 8.3. *Streptomyces*

*Streptomyces* is widely used to obtain antibiotics [283,284]. *Streptomyces lividans* and *Streptomyces albus* are of particular importance for industry because of their high throughput [285–287]. The potential of using *Streptomyces* for the production of secondary metabolites is reviewed in detail by Baltz [288].

The main problem of the biosynthesis of HA by recombinant *Streptomyces* is its production of hyaluronidase, which digests the produced HA [289]. The co-expression of *udgA* and *hasA* genes from *S. zooepidemicus* results in a sharp decrease in the amount of product [143]. Unlike the hyaluronidase from streptococci, the activity of the hyaluronidase from *Streptomyces roseofulvus* S10 (LC314796) rises markedly in the presence of  $Mg^{2+}$ , but decreases in the presence of  $Mn^{2+}$  and  $Zn^{2+}$  ions [290].

### 8.4. *Synechococcus*

Thanks to its capacity to adapt to changing conditions [291] and low requirements to the cultivation medium [292], *Synechococcus* can be found in various aquatic biomes around the world [293]. The ability to implement oxygen photosynthesis in adverse environmental conditions turns it into a key participant in Earth's carbon cycle [294]. *Synechococcus* has already attracted attention as a glycogen producer [295–297], which can be applied in advanced biofuel production [298,299]. Depending on the amount of ambient  $CO_2$ , the affinity of this organism to external inorganic carbon varies, reflecting its potential to accumulate  $CO_2$  inside the cell [300,301].

It has been shown that by elimination of the gene encoding the endogenous cellulose synthase and by optimization of fermentation conditions, *Synechococcus* sp. PCC 7002 gained the potential to become a new economically acceptable and safe source of HA from carbon dioxide by photosynthetic transformation [302].

### 8.5. *Bacillus*

Recognized as having GRAS status and possessing a well-developed biosynthetic capability associated with high product yields, the genus *Bacillus* is a valuable model for laboratory and industrial applications [303]. This includes production of D-form amino acids [304,305], antibiotics [306,307], vitamins B2 and K2 [308–311] as well as economically important enzymes such as alpha-amylases [312,313]. Its main advantages, compared to streptococci strains, are low requirements for nutrient medium and

**Table 4**

Overview of HA fermentation conditions, yield and molecular weight of HA obtained from gram-negative bacteria.

Host organism	Gene and origin	Fermentation conditions	HA yield (g/l)	HA MW ( $\times 10^6$ Da)	Reference
<i>E. coli</i> BL21	hasA (from <i>S. zooepidemicus</i> ATCC 39920) induced by TAC promoter	<b>Medium:</b> 50 g/l glucose, 15 g/l tryptone, 5 g/l yeast extract, 2 g/l $\text{KH}_2\text{PO}_4$ , 2 g/l $\text{K}_2\text{HPO}_4$ , 0.5 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.0 mM of IPTG <b>Aeration:</b> ND <b>Temperature:</b> 37 °C <b>Duration:</b> 12 h <b>pH:</b> 7 <b>Impeller speed:</b> 200 rpm	0.53	0.0346	[371]
<i>E. coli</i> K12 W3110	hasA (from <i>S. zooepidemicus</i> ATCC 35246) assembled with plasmid pTac15k under the control of the TAC promoter, galU and udgA overexpression assembled with plasmid pTrc99a under the control of the TAC promoter, $\Delta\text{galR}$ , $\Delta\text{galS}$ , $\Delta\text{zwf}$ , $\Delta\text{pfkA}$	<b>Medium:</b> 3 g/l galactose, 3 g/l glucose, 5 g/l yeast extract, 10 g/l peptone, 10 g/l NaCl, 50 $\mu\text{g/ml}$ ampicillin, 25 $\mu\text{g/ml}$ kanamycin, 17.5 $\mu\text{g/ml}$ chloramphenicol, 1 mM of IPTG <b>Aeration:</b> ND <b>Temperature:</b> 37 °C <b>Duration:</b> ND <b>pH:</b> ND <b>Impeller speed:</b> 200 rpm	0.03	0.00139	[15]
<i>E. coli</i> Top10	hasA (from <i>Streptococcus equisimilis</i> ) and <i>ugd</i> and <i>galF</i> (from <i>E. coli</i> K12 MG1655) expression assembled with arabinose inducible plasmid pMBAD	<b>Medium:</b> LB liquid medium. 100 g/l ampicillin, 10 mM $\text{MgCl}_2$ , 0.1 g/l <i>l</i> -arabinose, 2.5 g/l $\text{K}_2\text{HPO}_4$ , 1.0 g/l sorbitol, 10 g/l glucose (added at 5 h) <b>Aeration:</b> ND <b>Temperature:</b> room temperature <b>Duration:</b> 48 h <b>pH:</b> ND <b>Impeller speed:</b> ND	0.26	0.51	[184]
<i>E. coli</i> Top10	hasA (from <i>Streptococcus equisimilis</i> ) and <i>ugd</i> and <i>galF</i> (from <i>E. coli</i> K12 MG1655) expression assembled with arabinose inducible plasmid pMBAD	<b>Medium:</b> LB liquid medium. 100 g/l ampicillin, 10 mM $\text{MgCl}_2$ , 0.1 g/l <i>l</i> -arabinose <b>Aeration:</b> ND <b>Temperature:</b> room temperature <b>Duration:</b> 72 h <b>pH:</b> ND <b>Impeller speed:</b> ND	0.20	1.70	[184]
<i>Agrobacterium</i> ATCC31749	has (from <i>P. multocida</i> subsp. <i>multocida</i> ATCC 15742) assembled with plasmid pBQ and <i>kfiD</i> gene induced by T5 promoter expression assembled with plasmid pBQHas	<b>Medium:</b> 125 mM sucrose, 50 mM <i>N</i> -acetylglucosamine, 10 mM lactose, 30 mM $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ , 20 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 17 mM sodium citrate, 2 mM $\text{MnCl}_2$ , 5 g/L casamino acid. <b>Aeration:</b> ND <b>Temperature:</b> 30 °C <b>Duration:</b> 70 h <b>pH:</b> ND <b>Impeller speed:</b> 250 rpm	0.30	1.56	[375]

ND – no data;

absence of exotoxins [162]. Despite its commercial availability and the number of its advantages, attempts to create a recombinant *Bacillus* strain, whose productivity would be higher than that of streptococci, have so far failed.

The expression of the heterologous HA synthase gene leads to the accumulation of HA, in particular the co-overexpression of *tuaD* and *gtaB* genes increases HA production to 2.48 g/L [314]. Simultaneous overexpression of the *hasA*, *hasB* and *hasC* genes leads to an increase in molecular weight of the produced HA compared to overexpression of the *hasD* gene only [315,316]. *N*-terminal engineering causes the HA yield to rise dramatically from 5.96 g/L to 19.38 g/L [314]. The fermentation temperature affects the molecular weight of HA; in particular, during the fermentation at 48 °C *B. subtilis* WmB strains produce HA with a molecular weight of  $6.97 \times 10^6$  Da [317].

### 8.6. *Lactococcus lactis*

*Lactococcus lactis* has a number of commercial applications, in particular, in the production of dairy products [318,319]. *L. lactis* has GRAS status, which qualifies it as a metabolically engineered organism [320,321]. When all five genes encoding enzymes involved in HA biosynthesis, namely *hasA*, *hasB*, *hasC*, *hasD* and *hasE*, are co-expressed, a segregation instability of the recombinant

organism occurs [322]. The degree of *nisA* promoter induction is associated with the presence of *NisR* and *NisK* proteins [323]. The application of nisin as an inducer of gene expression is not correlated with the rising molecular weight, but multiplies the product yield [324]. The initial concentration of nisin affects the HA yield, the optimal concentration for recombinant *L. lactis* CES15 being 7.5 ng/ml. At this level, a yield of 6.09 g/l was achieved (*hasA* was expressed from *Streptococcus equi* subsp. *zooepidemicus* RSKK 677) [325]. Lactose-induced UDP-glucose dehydrogenase has a significantly increased activity, which allows the achievement of higher concentrations of the synthesized HA [326].

## 9. Production of HA with gram-negative bacteria

Among gram-negative bacteria, only *P. multocida* natively synthesizes HA. Due to its high pathogenicity, it is used as pmHas gene origin only, but not as a HA source [332,333]. *Escherichia coli* belongs to endotoxin-free organisms, thus it is a promising recombinant organism for the HA production [184]. Useful means for obtaining recombinant *E. coli* are PCR-based homologous Lambda Red recombineering [334,335] and seamless modification using a negative selection marker system based on *kil* counter-selection [336,337]. Due to its well-characterized genetics, the *E. coli* lac operon promoter is a successful genomic tool for control of recom-



binant protein production in bacteria [338,339]. Relevant strategies for production optimization of recombinant proteins using *E. coli* as bacterial host for industrial applications were considered by Bhatwa [340]. A list of HA fermentation conditions, yield and molecular weight of HA obtained from gram-negative bacteria is presented in (Table 4).

### 9.1. *Pasteurella multocida*

*P. multocida* is a widespread causative agent of pasteurellosis [341,342] and haemorrhagic septicaemia [343,344] in animals. *P. multocida* is classified as serogroup A, B, D, E, or F based on the cell wall composition [345]. Hyaluronan and related compounds such as heparin and chondroitin are components of the extracellular capsules of *P. multocida* serogroups A, D and F [346,347]. Extrusion of HA into the extracellular matrix is *P. multocida* serogroup A distinctive feature [346,348–350].

The gene responsible for the biosynthesis of HA in *P. multocida* serogroup A can be divided into three regions – HA export, synthesis, and phospholipid substitution regions [351–353]. In serogroup A, the locus proteins responsible for the synthesis of HA are encoded by five genes (*hyaA*, *hyaB*, *hyaC*, *hyaD* and *hyaE*) [345]. The *hyaA* and *hyaC* (*PmuHasB*) genes encode enzymes whose functions are similar to a glycosyltransferase and UDP-glycodehydrogenase, while *hyaD* (*PmuHasA*) is similar to HAS from *P. multocida* and *EpsI* from *S. thermophilus* [354,355]. The *pmCS* isolated from *P. multocida* Type F is 90 % identical to *pmHas* [356] and is active in the pH range from 6.5 to 8.6 [357]. Like in streptococci, the co-factors for *pmHas* are  $Mg^{2+}$  and  $Mn^{2+}$ , with  $Mn^{2+}$  having a greater influence [350], which indicates a difference in the kinetic optima of these organisms. When the terminal part of *pmHas* is replaced, binding to the GlcA-transferase domain increases sharply due to increased flexibility [358]. The transfer of GlcNAc and GlcA is catalyzed by two different glycosyltransferase sites [332], which are assumed to coexist in a single polypeptide [359].

**Table 5**

Overview of HA from fungi: fermentation conditions, yield and molecular weight.

Host organism	Gene and origin	Fermentation conditions	HA yield (g/l)	HA MW ( $\times 10^6$ Da)	Ref.
<i>S. cerevisiae</i> INSc1	DG42 gene (from <i>X. laevis</i> ) expression assembled with plasmid pYES2	<b>Medium:</b> uracil-deficient synthetic media with 1.5 % raffinose and 5 % glycerol <b>Aeration:</b> ND <b>Temperature:</b> ND <b>Duration:</b> ND <b>pH:</b> ND <b>Impeller speed:</b> ND	ND	~0.067	[399]
<i>K. lactis</i> GG799	<i>hasB</i> (from <i>X. laevis</i> ) and <i>hasA</i> gene (from <i>P. multocida</i> ) expression induced by $P_{LAC4}$ -inductive promoter	<b>Medium:</b> 7.5 g/l yeast extract, 10 g/l peptone, 40 g/l glucose, 2.5 g/l $K_2HPO_4$ , 0.9 g/l $MgSO_4 \cdot 7H_2O$ , 5 g/l NaCl, 0.4 g/l glutamine, 0.6 g/l glutamate. <b>Aeration:</b> 2 vvm <b>Temperature:</b> 30 <b>Duration:</b> 24 <b>pH:</b> 6 <b>Impeller speed:</b> 200 rpm	1.89	2.097	[148]
<i>P. pastoris</i> GS115	<i>xhasA2</i> (from <i>X. laevis</i> ) and <i>hasC</i> , <i>hasD</i> , (from <i>P. pastoris</i> GS115) genes assembled with plasmid pAO815 and <i>xhasB</i> (from <i>X. laevis</i> ), <i>hasC</i> , <i>hasD</i> , <i>hasE</i> (from <i>P. pastoris</i> GS115) genes assembled with plasmid pGAPZB overexpression	<b>Medium:</b> YPD medium (1 % yeast extract, 2 % peptone, 2 % glucose), 40 g/l glucose, 7.5 g/l yeast extract, 10 g/l peptone, 2.5 g/l $K_2HPO_4$ , 0.5 g/l $MgSO_4$ , 5 g/l NaCl, 0.4 g/l glutamine, 0.6 g/l glutamic acid, 0.2 g/l oxalic acid <b>Aeration:</b> 0.7 vvm <b>Temperature:</b> 30 <b>Duration:</b> 48 <b>pH:</b> 7 <b>Impeller speed:</b> 500 rpm	1.70	1.20	[408]

ND – no data.

### 9.2. *Escherichia coli*

*E. coli* is a well-documented genus [360] widely used in biochemical industry [361,362]. Most *E. coli* strains are harmless to humans, however, there are pathogenic strains that are the prominent cause of enteritis [363], diarrhea [364], septicemia [365], and other symptoms.

In *E. coli*, the *ugd* gene encodes an enzyme with a function analogous to that of *hasB* in streptococci; for their part, *galF* is similar to *hasC*, and *glmU* is akin to *hasD*, with sequence similarity of 54.2 %, 36.9 % and 39.4 %, respectively, compared to genes from *S. pyogenes* M1 GAS [184]. The substrates UDP-GlcA and UDP-GlcNAc, necessary for HA production, are not synthesized by *E. coli*. Therefore exogenous polysaccharide is used as an acceptor substrate [366]. The central part, also known as region 2 in the capsular family of genes of *E. coli* strain K4, contains a gene encoding a bifunctional enzyme that includes two conserved glycosyltransferase sites responsible for the synthesis of a specific K-antigen [367–369]. The functions of *KfoC* are identical to those of the hyaluronan synthase from *P. multocida* [370]. The addition of phosphorus and sorbitol salts such as isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) [371] leads to an increase of the HA molecular weight.

### 9.3. *Agrobacterium*

*Agrobacterium* is classified as a Gram-negative  $\alpha$ -proteobacterium, commonly used as a genome-editing tool to generate transgenic plants [372]. The variety of applications of *Agrobacterium* in plant biotechnology was studied by Sardesai and Subramanyam [373].

Curdlan (1,3- $\beta$ -D-glucan) producing *Agrobacterium* species are of interest for the oligosaccharide synthesis on account of their propensity for highly efficient regeneration of UDP-glucose [374], however, the use of curdlan-deficient mutants for the production

of HA does not contribute to an increase in product yield [375]. The concentration of UDP-glucose is proportional to that of uridine monophosphate (UMP) [376]. As demonstrated by Mao *et al.*, co-expression of the HA synthase gene from *P. multocida* and *kfiD* gene encoding UDP-glucose dehydrogenase from *E. coli* K5 enables *Agrobacterium* to produce HA [375], albeit the use of two T5 promoters is recommended for a successful expression of the *kfiD* gene [375].

## 10. Production of HA with fungi

In biotechnology, fungi have a number of undeniable advantages over bacteria – fast growth, easy genetic manipulation, and absence of endotoxins and viral DNA. They are capable of producing a variety of substances, including HA and its derivatives [377]. However, the main disadvantage of using yeast is the high risk of hyperglycosylation of recombinant proteins [378–380]. The most promising hosts for the production of heterologous proteins among yeasts are *Saccharomyces cerevisiae* [381–383] and *Pichia pastoris* [384–386]. The HA yield by recombinant yeasts in which mammalian genes were expressed does not exceed that from recombinant organisms with bacterial gene expression [148]. Their low activity is presumably associated with post-translational regulation [387]. An overview of HA fermentation conditions, yield and molecular weight of HA obtained from fungi is presented in (Table 5).

### 10.1. *Cryptococcus neoformans*

*Cryptococcus neoformans* is a pathogenic yeast that causes meningitis [388] and fungal meningoencephalitis [389]. The latter is especially harmful to immunocompromised patients. Due to the pathogenicity of *C. neoformans*, it has not been previously used as a HA producer.

The *cps-1* gene from *C. neoformans* encodes a protein analogous to the hyaluronic acid synthase of higher eukaryotes [390]. The presence of HA as a capsule component provides virulence to *C. neoformans* [391]. *C. neoformans* can be used as an origin of the *cps-1* gene for heterogeneous expression [392].

### 10.2. *Saccharomyces cerevisiae*

*Saccharomyces cerevisiae* is a fully sequenced [393,394] non-methylotrophic yeast recognized as a GRAS organism. The FDA has approved the use of *S. cerevisiae* for the production of pharmaceuticals, e.g. artemisinin [395] and emodin [396]. A detailed review on biologically active secondary metabolites of *S. cerevisiae* used in pharmacology has been published [397].

The activity of glycosyltransferases of *S. cerevisiae* has been painstakingly explored [133]. Due to the fact that *S. cerevisiae* contains intrinsic UDP-glucose, the reconstruction of the UDP-Glc6P synthase pathway is possible with the genes *UGD1* and *UXS3* from *Arabidopsis thaliana* [398]. It was demonstrated that the presence of  $Mg^{2+}$  ions is required for HA synthesis *in vitro* [399]. The co-expression of UDP-dehydrogenase and *CPS1p* genes from *C. neoformans* in *S. cerevisiae* allows attainment of a high level of hyaluronic acid [392].

### 10.3. *Kluyveromyces lactis*

*Kluyveromyces lactis* is a non-methylotrophic yeast with GRAS status that is involved in industrial scale production of secreted and intracellular enzymes, such as lactase [400,401], bovine prochymosin [401] and inulinase [402]. The prospects of using *K. lactis* for applications in food industry and biotechnology were

reviewed by Karim [403]. The highest HA titers were achieved by co-expression of *hasA* gene from *P. multocida* with *hasB* gene from *X. leavis* [148].

### 10.4. *Pichia pastoris*

*Pichia pastoris* is a methylotrophic yeast that is in contrast to *H. polymorpha* not thermotolerant. *P. pastoris*, as well as *S. cerevisiae*, is extensively used as an expression system for recombinant protein production [404], in particular for biosynthesis of intricate bioactive molecules such as parasin I peptide [405] and streptavidin [406]. It was previously reported by Farinha that *P. pastoris* could be used as a source of valuable polysaccharides, e.g. a chitin-glucan copolymer complex with a molar ratio of chitin to  $\beta$ -glucan of 12:88 [407].

In recombinant *P. pastoris* strains, UDP-GlcA has a more significant influence on HA synthesis than UDP-GlcNAc. This was experimentally confirmed by the finding that the HA amount produced by organisms with a higher *xhasB* copy number did not significantly exceed the HA yield in organisms with a lower number of *xhasB* [408]. Lowering of the temperature at the cultivation stage leads to a decrease in the concentration of produced HA [408].

## 11. Conclusion and perspective

Due to the variety of applications, the commercial interest in large-scale and cost-effective production of HA remains strong and is growing steadily. *S. zooepidemicus* has been studied the most among all reported biological sources of HA because it was historically used for HA manufacturing. In this review, we summarized the key factors limiting HA production by *S. zooepidemicus*, however, these factors need to be further clarified.

Recombinant organisms are considered safe and cost-effective alternative sources of HA due to the absence of the need for purification from toxins. Among gram-positive and gram-negative bacteria, *Bacillus* sp. and *Escherichia coli*, respectively, have the greatest potential because of their commercial availability, however, their productivity still cannot be compared with that of native producers due to the instability of plasmids used for gene expression. Fungi are superior to bacteria in growth rate and are less demanding on the nutrient medium, moreover they are simpler to genetically manipulate. These factors make them one of the most promising organisms for modern HA production.

Obtaining HA via microbial and fungal fermentation is still highly efficient on an industrial scale due to the simplicity of implementation and minimal economic costs.

### CRedit authorship contribution statement

**E.V. Shikina:** Writing – original draft, Visualization. **R.A. Kovalevsky:** Writing – original draft, Formal analysis. **A.I. Shirkovskaya:** Writing – review & editing. **Ph.V. Toukach:** Supervision, Conceptualization, Data curation, Writing – review & editing.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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