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Microbial engineering for natural and unnatural glycosaminoglycans biosynthesis

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Microbial synthesis of glycosaminoglycans (GAGs) facilitates sustainable biomanufacturing using cost-effective carbon feedstocks. This transformative framework is driven by three core innovations: *de novo* GAGs biosynthesis, sulfation engineering, and new-to-nature GAGs analogs creation. Despite these advances, critical challenges hinder industrial-scale efficiency, such as suboptimal distribution of metabolic flux, insufficient sulfation environments, and host incompatibility with unnatural analogs. In this review, we present a systematic analysis of microbial hosts, biosynthetic pathways, and microbial engineering strategies for GAGs production. We first describe how strategic host optimization and pathway manipulation can tap the full potential of microorganisms for efficient GAGs biosynthesis. Then, we analyze the development of microbial cell factories (MCFs) for GAGs biosynthesis from the simple pathway transplantation to systemic *de novo* construction of metabolic systems, thereby establishing programmable platforms to surpass natural biosynthesis limits. Next, we present a tripartite engineering framework for GAGs sulfation that integrates precursor synthesis modules, sulfate donor accumulation systems, and sulfotransferase networks, thereby progressing sulfation control from biomimetic mechanisms to programmable artificial systems. Further, we discuss the microbial synthesis of new-to-nature GAGs analogs through the incorporation of unnatural precursors or the reprogramming of natural precursors, thereby enabling MCFs to construct non-canonical glycopolymers with designed function. Finally, we prospect the development of multifunctional customized MCFs to drive breakthroughs in industrial-scale GAGs bioproduction.

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1. Introduction

Glycosaminoglycans (GAGs), primarily including hyaluronic acid (HA), heparin/heparan sulfate (HP/HS), chondroitin/dermatan sulfate (CS/DS), and keratan sulfate (KS), are a ubiquitous class of linear polysaccharides composed of repeating disaccharide units featuring hexosamine and uronic acid or galactose residues.[1] Owing to their structural diversity and functional specificity, GAGs exhibit significant application potential across diverse fields such as human health maintenance, disease therapeutics, and biomedical development, making them essential bioactive molecules.[2–4] As a clinically established treatment for osteoarthritis (OA), HA can mediate its therapeutic effects through intra-articular viscosupplementation to restore synovial fluid viscoelasticity.[5,6] Recent advancements in nano-based delivery systems have further enhanced HA therapy by improving its sustained-release characteristics and joint-targeting efficacy.[7,8] CS also has been demonstrated clinically significant benefits in managing symptomatic OA.[9,10] Further, preclinical studies have revealed its anti-inflammatory properties and potential antitumor effects.[11–14] Meanwhile, HP can exert its anticoagulant activity through allosteric activation of antithrombin III, resulting in selective inhibition of coagulation factors Xa and IIa.[15,16] Given the immense application potential of GAGs and the limitations of traditional production methods, research on their synthetic approaches has attracted growing attention.[17,18] However, the structural complexity of GAGs poses substantial challenges to the development of efficient production and modification strategies.[19]

Recent advances in metabolic engineering, synthetic biology, chemical engineering, and enzyme engineering have expanded GAGs production into two main categories: natural and unnatural GAGs. Natural GAGs represent the classical linear polysaccharides and ubiquitously present in animal tissues including humans as well as certain microbial species. Natural

GAGs consist of non-sulfated forms such as HA and their sulfation precursors non-sulfated chondroitin and heparosan, and various sulfated forms such as HP/HS, CS/DS, and KS. In contrast, unnatural GAGs are a new-to-nature class of polysaccharide analogs created through strategic structural modifications with chemical synthesis, enzymatic remodeling or metabolic engineering. Based on these strategies, natural GAGs architectures can be precisely changed for developing customized glycan structures with enhanced or novel biological functions through targeted functional group modifications and complete molecular scaffold redesigns.

GAGs production and modification strategies encompass physical extraction, chemical synthesis, multi-enzyme cascades, and biological synthesis.[20–23] Physical extraction isolates native GAGs directly from animal tissues.[24] For instance, chondroitin sulfate can be obtained from steam-exploded chicken sternal cartilage with a reported total yield of 18.55%.[25] While this method benefits from mature industrial protocols and preserves the native bioactive structure of GAGs, it suffers from low sustainability, and potential contamination risks.[26] Chemical synthesis of GAGs relies on stepwise organic reactions, including glycosylation and sulfation, to assemble GAGs chains.[27] The β-selective glucuronylation reaction has been developed using phenyl 2,4-di-*O*-acetyl-1-thio-β-D-glucopyranosidurono-6, 3-lactone as the glycosyl donor. Glycosylation of this donor with hexosamine derivatives proceeds with excellent yield and β-stereoselectivity, enabling efficient synthesis of GAGs-type disaccharides.[28] While this approach enables precise control over polymer length, it suffers from multistep synthetic complexity, and environmental concerns associated with chemical waste.[29] The multi-enzyme cascade approach enables *in vitro* GAGs production and modification by employing synthases, sulfotransferases, and other enzymes, typically implemented *via* one-pot synthesis or immobilized enzyme catalysis.[30] For example, in a HA synthesis system, a one-pot reaction comprising 7 enzymes utilized GlcA and GlcNAc as substrates, with UTP and ATP as cofactors and polyphosphate as an energy carrier for cofactor regeneration, driving HA production.[31] This system achieved a titer of 0.81 g per L HA with 1.17 MDa molecular weight. While the multi-enzyme cascade offers advantages such as mild reaction conditions and environmental sustainability, its industrial application remains constrained by high enzyme/cofactor costs and the complexity of reaction system design.[32] Animal cells can use two distinct biosynthetic pathways for GAGs production. The first pathway involves core protein-dependent pathways that generate CS, HS, and KS. The initial step for CS biosynthesis is in the endoplasmic reticulum where xylosyltransferase (XylT) facilitates the attachment of UDP-xylose to serine residues on core proteins *via O*-xylosidic linkages. Subsequent processing in the Golgi apparatus entails the sequential addition of two galactose units by galactosyltransferase (GalT-I/GalT-II), along with the addition of one GlcA unit by glucuronosyltransferase I (GlcAT-I), thereby completing the tetrasaccharide linker region. Chain elongation then occurs through the alternating actions of GlcAT-II and *N*-acetylgalactosaminyltransferase II (GalNAcT-II), thus forming the characteristic backbone. The final sulfation modifications are mediated by sulfotransferases using PAPS as a donor molecule. Similarly, HS biosynthesis also depends on core proteins, but it assembles a GlcA-β1,4-GlcNAc-α1,4 backbone with subsequent epimerization of GlcA to IdoA. Similarly, KS biosynthesis requires core proteins, but it polymerizes a Gal-β1,4-GlcNAc-β1,3 repeating backbone. KS chains initiate *via* N-linkages between GlcNAc and asparagine (KS-I), O-linkages between GalNAc and serine/threonine (KS-II: GalNAc-Ser/Thr), or O-linkages between mannose and serine (KS-III: Mannose-Ser), and then elongate through alternating β1,3 and β1,4 linkages between the disaccharide units.[33] The second pathway is core protein-independent pathway that generates GAGs such as HA. HA synthesis is achieved by directly polymerizing GlcA-β1,3-GlcNAc-β1,4 chains with UDP-GlcA and UDP-GlcNAc as substrates at the inner membrane surface catalyzed by plasma membrane-integrated HA synthases. After that, the completed polymers are extruded into the extracellular space without the involvement of Golgi apparatus.[34] Microbial synthesis employs synthetic biology tools to construct and optimize GAGs biosynthetic pathways, enabling microbial production of GAGs.[35] Significant breakthroughs have been achieved in microbial production of HA, chondroitin, and heparosan. This approach utilizes low-cost, sustainable carbon sources for scalable fermentation, achieving animal-free production.[36] Further, metabolic engineering strategies allow for precise molecular weight control and sulfation patterning of GAGs.[37] For example, to achieve the *de novo* microbial synthesis of chondroitin sulfate A (CS-A), the chondroitin synthesis module, chondroitin-4-*O*-sulfotransferase gene expression module, and enhanced PAPS supply module were reconstructed and assembled in *Pichia pastoris* to construct an engineered microbial cell factories (MCFs) for CS-A production.[38] As a result, the engineered strain *P. pastoris* Pp008 achieved a titer of 2.1 g per L CS-A using methanol as carbon source. Therefore, microbial GAGs synthesis has emerged as a transformative manufacturing platform that overcomes the limitations of traditional production methods.

This review summarizes recent advances in the microbial synthesis of natural and unnatural GAGs from three perspectives (Fig. 1). First, natural and unnatural GAGs-producing microorganisms are introduced, and their metabolic engineering and synthetic biology strategies for GAGs production are discussed. Second, many useful strategies for GAGs sulfation are analyzed, including the enhancement of sulfotransferase activity and the pathway engineering of sulfonate donor and its integration with UDP-sugar polymerization. Finally, new-to-nature GAGs analogs are summarized, and the corresponding strategies for constructing its MCFs are discussed. From the perspective of GAGs biosynthesis, the design, construction and optimizationof GAGs and its analogs high-producing MCFs will provide a transformative roadmap for advancing scalable and resource-efficient GAGs biomanufacturing.

2. Microbial synthesis of GAGs

Microbial synthesis of GAGs mainly contains three categories: non-sulfated GAGs, sulfated GAGs, and new-to-nature GAGs analogs. The development of MCFs for these GAGs represents

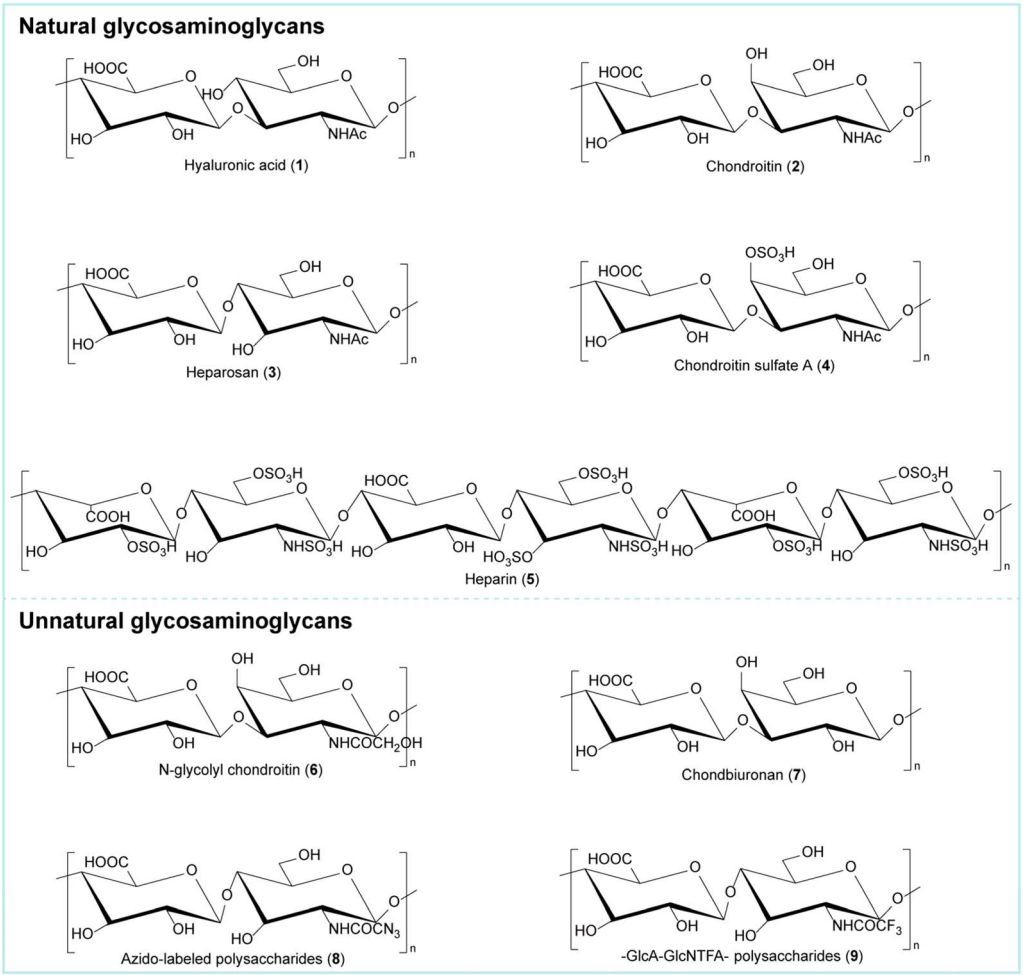

Fig. 1 Structural diversity of both natural and unnatural GAGs produced by microbial synthesis. Natural GAGs mainly consist of non-sulfated GAGs including hyaluronic acid (**1**), chondroitin (**2**), and heparosan (**3**), and their sulfated counterparts chondroitin sulfate A (**4**) and heparin (**5**). Unnatural GAGs mainly consist of structurally modified GAGs analogs such as *N*-glycolyl chondroitin (**6**), chondbiuronan (**7**), azido-labeled polysaccharides (**8**), and -GlcA-GlcNTFA- polysaccharides (**9**).

a significant advancement beyond simple pathway transplantation. It exemplifies the successful implementation of rational design to engineer customized biosynthetic pathways, thereby enhancing the structural and functional diversity of this important class of polysaccharides.

2.1. Non-sulfated GAGs

Non-sulfated GAGs, including HA, chondroitin and heparosan, can be naturally biosynthesized by certain microorganisms. HA typically exists in its non-sulfated form, whereas chondroitin and heparosan primarily serve as precursor substrates for the biosynthesis of sulfated GAGs in biological systems.

2.1.1. HA. HA is a linear polysaccharide composed of repeating disaccharide units of GlcA and GlcNAc linked by alternating β-1,3 and β-1,4 glycosidic bonds.[39] Unlike other GAGs, HA retains its biological activity without sulfation modifications. In mammals, HA is a fundamental structural component of the extracellular matrix (ECM), crucial for tissue hydration and osmotic protection.[40] Pathogenic microbes, particularly *Streptococcus* species, have evolved to produce HA capsules to evade the immune system.[41] Biotechnological methods have largely replaced animal-derived HA with microbial production, offering a more sustainable manufacturing alternative.[42] Recent advancements have expanded HA applications in cutting-edge fields, such as tissue engineering scaffolds, regenerative therapies, and precision nanomedicine delivery systems.[43,44]

2.1.2. Chondroitin. Chondroitin is a linear polysaccharide consisting of repeating disaccharide units of GalNAc and GlcA linked by alternating β-1,3 and β-1,4 glycosidic bonds. *Escherichia coli* K4 naturally produces fructosylated chondroitin, necessitating enzymatic or chemical defructosylation to obtain chondroitin. To circumvent the challenges associated with fructose removal, heterologous production systems have been developed for direct chondroitin biosynthesis. Although the direct applications of unsulfated chondroitin are not extensively studied, initial studies suggest potential benefits for osteoarthritis management.[45,46] Currently, chondroitin is primarily used as a substrate for the enzymatic synthesis of CS through *in vitro* catalytic strategies, thereby increasing its utility.

2.1.3. Heparosan. Heparosan is a linear polysaccharide composed of repeating disaccharide units of GlcA and GlcNAc linked by alternating β-1,4 and α-1,4 glycosidic bonds. *E. coli* K5 naturally produces heparosan as a capsular polysaccharide. Furthermore, microbial hosts have been successfully employed for the heterologous biosynthesis of heparosan, thereby significantly promoting the development of microbial production. Heparosan has demonstrated promising potential for various biomedical applications, including injectable dermal fillers, nanomedicines, and anticancer drug delivery systems.[47–50] Most importantly, heparosan serves as the essential biosynthetic precursor for heparin and heparan sulfate, which are crucial for the production of anticoagulant drugs.[51]

2.2. Sulfated GAGs

Sulfated GAGs, particularly heparin and CS-A, are crucial sulfated polysaccharides with significant pharmacological applications. Recent development in MCFs have facilitated the biosynthesis of chondroitin and heparosan core structures. Coupled with the successful heterologous expression of sulfotransferases, these engineered systems now enable the complete *de novo* microbial production of structurally defined sulfated GAGs.

2.2.1. CS-A. CS-A is a linear sulfated GAGs composed of repeating disaccharide units of GlcA and GalNAc4S linked by alternating β-1,3 and β-1,4 glycosidic bonds, with sulfation at the 4-O position of GalNAc. As a dominant subtype of CS, CS-A is defined by its signature C4 sulfation pattern on GalNAc residues and is abundantly distributed in cartilaginous and connective tissues. Current industrial production relies predominantly on animal tissue extraction, as microbial strains lack the requisite sulfotransferases for CS-A biosynthesis. However, synthetic biology approaches have enabled recombinant CS-A production in engineered *E. coli* and *P. pastoris* through the heterologous expression of chondroitin synthase and sulfotransferases.[52,53] CS-A demonstrates significant therapeutic potential in various medical applications, including the prevention of kidney stone formation, glioma therapy, and regenerative treatments for neurological disorders.[54–56] Furthermore, CS-A serves as a crucial enzymatic substrate for the synthesis of chondroitin sulfate E (CS-E), a derivative that has shown promising antiviral properties in preclinical studies.[57,58]

2.2.2. Heparin. Heparin, a highly sulfated GAGs, is a linear polysaccharide composed of repeating disaccharide units of uronic acid (IdoA or GlcA) and GlcN linked by alternating α-1,4 glycosidic bonds.[59] The structural complexity of heparin stems from heterogeneous modifications including *N*-deacetylation and differential *O*-sulfation at multiple positions. This biologically active polymer is endogenously synthesized in the Golgi apparatus of mast cells, predominantly localized in the liver, intestinal mucosa, and pulmonary tissues.[60] Its unique sulfation patterns not only confer potent anticoagulant and antithrombotic activities but also contribute to diverse pharmacological properties, including antitumor, anti-inflammatory, and antiviral effects.[61,62] Although commercial production still relies on extraction from porcine intestines, advances in synthetic biology have enabled the *de novo* biosynthesis of bioengineered heparin in *P. pastoris*, allowing for precise control over sulfation patterns.[63] These developments are critical for the industrial-scale microbial synthesis of heparin.

2.3. New-to-nature GAGs analogs

Innovative microbial biosynthesis has facilitated the creation of new-to-nature GAGs analogs, including *N*-glycolyl chondroitin, azido-labeled polysaccharides, -GlcA-GlcNTFA- polysaccharides, and chondbiuronan. These synthetic biology strategies have significantly advanced the design of new-to-nature GAGs analogs, allowing for precise structural modifications that enhance the functional repertoire of these biologically important polysaccharides beyond their natural forms.

2.3.1. Chondbiuronan. Chondbiuronan is a chondroitin-like polysaccharide characterized by repeating disaccharide units of β3-galactose (Gal)-β4-GlcA, where Gal replaces the GalNAc residue found in canonical chondroitin structures.[64] Through metabolic engineering of *E. coli*, microbial synthesis of chondbiuronan has been achieved.[65] Due to its unique structure, chondbiuronan shows potential applications in chemical conjugation, material coating, and drug delivery systems.

2.3.2. Azido-labeled polysaccharides. Azido-labeled polysaccharides are produced by replacing *N*-acetylhexosamines with *N*-azidoacetylhexosamines in the repeating units of non-sulfated GAGs. Currently, microbial synthesis has enabled the production of various azido-modified polysaccharides including azido-heparosan, azido-chondroitin, and azido-HA.[66] These azido-labeled polysaccharides serve as versatile chemical tools that retain biological properties similar to their natural analogs while enabling bioorthogonal conjugation with probes for imaging, detection, and metabolic analysis of GAGs in both *in vitro* and *in vivo* systems. The site-specific introduction of azido groups into the disaccharide repeating units allows for predictable functionalization while maintaining the structural integrity and biological activity of the native polysaccharides.

2.3.3. -GlcA-GlcNTFA- polysaccharides. The -GlcA-GlcNTFA- polysaccharides are biosynthesized through the precursor-directed fermentation using metabolically engineered *E. coli* K5, where GlcNAc in the repeating units of heparosan is systematically replaced with GlcNTFA when cultured in the presence of the synthetic precursor Ac_4GlcNTFA. These unnatural GAGs serve as superior substrates for the chemoenzymatic synthesis of heparin, effectively overcoming the inherent limitation of *N*-deacetylase activity in conventional methods for heparin production. This innovative approach enables the development of a novel and efficient route for producing anticoagulant heparin by precisely controlling sulfation patterns and improving pharmacological properties, representing a significant advancement in the field of therapeutic glycan manufacturing.

2.3.4. *N*-Glycolyl chondroitin. *N*-Glycolyl chondroitin is a linear polysaccharide composed of repeating disaccharide units of GalNGc and GlcA linked by alternating β-1,3 and β-1,4 glycosidic bonds, with glycolyl substitution at the C2 amino group to replace the conventional acetyl group in standard

chondroitin.[67] This unnatural GAGs have been successfully biosynthesized through the precursor-directed fermentation using metabolically engineered *E. coli* by feeding *N*-glycolylglucosamine as the biochemical precursors. *N*-glycolyl chondroitin has potential applications as a cancer biomarker.[68]

3. Microbial hosts for GAGs biosynthesis

Exploring GAGs-producing microorganisms offers a sustainable and controllable microbial production platform, enabling large-scale fermentation of diverse GAGs. Microbial hosts for GAGs biosynthesis can be categorized into natural hosts for synthesizing GAGs and engineered hosts for heterologous GAGs biosynthesis. Natural hosts for synthesizing GAGs have evolved complete GAGs biosynthesis systems, allowing them to synthesize specific GAGs without introducing heterologous metabolic pathways. However, these natural strains are typically limited to producing a single type of GAGs and often suffer from insufficient metabolic flux for GAGs production. Engineered hosts for heterologous GAGs biosynthesis, despite lacking natural GAGs biosynthesis pathways, exhibit many advantages such as rapid growth, well-characterized metabolism, and high amenability to metabolic engineering for large-scale production. These engineered strains can produce GAGs, which are inaccessible to natural producers. However, due to the complexity of GAGs biosynthetic pathways, the introduction of multiple heterologous genes may impose metabolic burden, leading to growth inhibition and suboptimal expression balancing. Thus, the industrial-scale production of GAGs can be advanced by leveraging the complementary advantages of natural and unnatural GAGs-producing microorganisms, paving the way for tailored biotechnological applications.

3.1. Natural hosts for GAGs biosynthesis

Natural hosts for GAGs biosynthesis are defined as microbial species endowed with the biosynthetic pathways for specific GAGs. However, the limited diversity of such wild-type producers constrains microbial GAGs biosynthesis. *Streptococcus* species possess natural HA-producing systems, serving as model microorganisms for elucidating the enzymatic mechanisms underlying microbial HA biosynthesis (Table 1). *E. coli* K4 and K5 can naturally synthesize fructosylated chondroitin and heparosan, respectively, paving a new pathway for the microbial production of sulfated GAGs.

3.1.1. Streptococcus. *Streptococcus*, a Gram-positive bacterium, serves as the most widely studied microbial platform for HA biosynthesis due to its innate capacity to produce high-molecular-weight HA.[69] Several *streptococcal* species, including *Streptococcus equisimilis*, *S. pyogenes*, and *S. uberis*, can naturally produce HA through microbial fermentation attributed to its genomic integration of a dedicated HA synthesis operon (HasA-E).[70,71] This operon encodes the enzymatic machinery required for precursors generation (UDP-GlcNAc and UDP-GlcA) and their subsequent polymerization into HA. Notably, *S. equi* subsp. *zooepidemicus* (synonymously designated *S. zooepidemicus*) exhibits exceptional HA productivity.[72] To deeply explore the potential of *S. zooepidemicus* for HA production, atmospheric and room temperature plasma (ARTP) was employed for mutagenesis to construct a high-throughput mutant library.[73] As a result, the mutant strain *S. zooepidemicus* mut-A17 achieved a titer of 0.813 g per L HA, representing a 42.8% increase compared to that of the wild-type *S. zooepidemicus*. Thus, ARTP mutagenesis serves as a high-throughput tool for directed evolution, enabling rapid screening of natural high-yield HA-producing strains while enhancing their metabolic robustness and fermentation stability. This approach facilitates the optimized GAGs biosynthesis, improving both titer and molecular weight control in microbial production systems.

3.1.2. Escherichia coli. *E. coli*, a Gram-negative bacterium, serves as a natural producer of non-sulfated GAGs.[74–76] Among wild-type strains, *E. coli* K4 and *E. coli* K5 are particularly notable for their ability to synthesize fructosylated chondroitin and heparosan, respectively. *E. coli* K4 harbors a dedicated chondroitin biosynthesis operon (KfoA-G), which orchestrates the generation of UDP-sugar precursors (UDP-GalNAc and UDP-GlcA), their glycosidic polymerization and fructosylation modification, resulting in the production of fructosylated chondroitin.[77] To obtain mutant phenotypes with the enhanced production of fructosylated chondroitin, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) was used to generate random mutations.[78] As a result, mutant strain *E. coli* VZ15 achieved a titer of 0.214 g per L fructosylated chondroitin, representing a 82% increase compared to wild-type *E. coli* strain. Thus, NTG mutagenesis serves as an effective tool for directed evolution,

Table 1 Natural hosts for GAGs biosynthesis

Host	Substrate	Product	Titer	Molecular weight	Ref.
S. zooepidemicus	Glucose	HA	29.38 g L^{-1}	—	72
S. equi ssp. *equi*	Sucrose	HA	12 g L^{-1}	79.4 kDa	105
S. sp. ID9102	Glucose	HA	6.94 g L^{-1}	5.9 MDa	106
S. equisimilus MK156140	Beef extract	HA	7.16 g L^{-1}	—	107
S. thermophilus	Whey permeate	HA	0.34 g L^{-1}	9.22–9.46 kDa	108
S. equi	Glucose	HA	0.992 g L^{-1}	—	109
S. iniae	Glucose	HA	0.12 g L^{-1}	300 kDa	110
E. coli K4	Glucose	Fructosylated chondroitin	5.3 g L^{-1}	—	111
E. coli K5	Glucose	Heparosan	15 g L^{-1}	84 kDa	81
E. coli Nissle 1917	Glucose	Heparosan	3 g L^{-1}	68 kDa	112

enabling rapid isolation of high-yield fructosylated chondroitin-producer. However, the fructose moiety in fructosylated chondroitin prevents its direct application in chondroitin sulfate biosynthesis.[79] To overcome this limitation, targeted deletion of fructosyltransferase (KfoE) gene enables the production of non-fructosylated chondroitin, which serves as a direct precursor for sulfation.[80] Similarly, the genome of *E. coli* K5 harbors a heparosan biosynthesis operon (KfiA-D) that orchestrates the production of UDP-sugar precursors (UDP-GlcNAc and UDP-GlcA) and their subsequent polymerization into heparosan. To enhance heparosan production in *E. coli* K5, a fed-batch fermentation strategy was employed, thereby maximizing heparosan biosynthesis. After optimization, *E. coli* K5 achieved a titer of 15 g per L heparosan, demonstrating the critical impact of fermentation optimization on microbial production of heparosan.[81] This fermentation strategy not only validates the potential of process intensification but also provides a foundation for further metabolic engineering and scalable bioproduction of GAGs.

3.2. Engineered hosts for heterologous GAGs biosynthesis

Engineered hosts for heterologous GAGs biosynthesis are genetically engineered by introducing exogenous biosynthetic pathways into host strains to enable GAGs production. Yeasts, with their eukaryotic protein modification machinery, are ideal chassis for producing complex GAGs requiring sulfation. *E. coli* has been extensively utilized for industrial-scale biosynthesis of non-sulfated GAGs, capitalizing on its minimal metabolic complexity and established high-density fermentation protocols. GRAS (Generally Recognized as Safe) hosts such as *Bacillus*, *Corynebacterium glutamicum*, and *Lactococcus lactis* serve as safe hosts for GAGs production, particularly through their endotoxin-free cellular machinery.

3.2.1. Yeasts. Yeasts, recognized as a GRAS microorganism, is widely utilized for recombinant protein production and biosynthesis of high-value chemicals due to its high cell density growth and eukaryotic post-translational modification capabilities.[82] Among yeast species, *P. pastoris* and *Kluyveromyces lactis* have been successfully engineered for the heterologous biosynthesis of GAGs.[83,84] However, wild-type yeasts lack the GAGs synthesis operon, rendering them incapable of producing GAGs without genetic modification. To address this limitation, metabolic engineering strategies have been employed to introduce heterologous GAGs biosynthesis pathways. *K. lactis* has been engineered for HA production, and *P. pastoris* has been successfully utilized to synthesize HA, bioengineered heparin, and chondroitin sulfate.[38,63,83,84] To construct complete HA biosynthetic pathway in *P. pastoris*, heterologous HA synthase (HasA) and UDP-glucose dehydrogenase (HasB) genes from *Xenopus laevis* were introduced along with endogenous UDP-glucose pyrophosphorylase (HasC), UDP-*N*-acetylglucosamine pyrophosphorylase (HasD), and phosphoglucose isomerase (HasE) genes to produce HA. The resulting strain *P. pastoris* EJP-D achieved a titer of 1.6 g per L HA.[84] Although metabolic engineering of yeasts has enabled *de novo* biosynthesis of GAGs, production efficiency remains suboptimal due to insufficient fermentation optimization. Systematic refinement of cultivation parameters combined with predictive fermentation modeling will be essential for establishing precise control of GAGs biosynthesis.

3.2.2. Escherichia coli. *E. coli* is well-established model microorganisms in synthetic biology due to their well-characterized metabolic pathways and extensive genetic engineering toolkit. These features make *E. coli* K-12 and BL21 ideal chassis microorganisms for the heterologous production of GAGs, including HA, heparosan, and chondroitin.[85–88] Since wild-type *E. coli* K-12 and BL21 strains lack the GAGs biosynthesis operon, they cannot naturally synthesize these high-value polysaccharides. However, by reconstructing the metabolic pathway using heterologous enzyme genes, including HA synthase (HasA), glucose-1-P uridyltransferase (GalF) and UDP-glucose 6-dehygrogenase (Ugd) genes, the engineered *E. coli* strains could be reprogrammed for HA production. To further enhance HA biosynthesis, a random mutant library of the RNA polymerase sigma factor RpoD and RNA polymerase sigma factor RpoS genes was constructed and subjected to high-throughput screening for GAGs-producing strains.[89] As a result, mutant strain *E. coli* D72 achieved a titer of 0.56 g per L HA, representing a 10.1% increase compared to that of the parental strain *E. coli* C1. Thus, the construction of metabolic pathway for GAGs biosynthesis, coupled with high-throughput screening in *E. coli*, represents an effective strategy for enhancing GAGs production. Future efforts should focus on the systematic rewiring of metabolic networks to further refine production efficiency.

3.2.3. Bacillus. *Bacillus* species, renowned for their robust capacity for protein secretion and remarkable resistance to environmental stress, have emerged as efficient microbial chassis for the production of industrial enzymes and the biosynthesis of food-grade chemicals owing to its strong promoter systems and high production potential. Several *Bacillus* strains, including *Bacillus subtilis*, *B. megaterium*, and *B. amyloliquefaciens*, have been successfully engineered for GAGs biosynthesis.[90–92] However, wild-type *Bacillus* strains lack specific GAGs biosynthesis operons, rendering them incapable of natural GAGs production. Through metabolic engineering strategies, various *Bacillus* species have been repurposed for heterologous synthesis of HA, chondroitin, and heparosan.[93–95] For instance, to construct the chondroitin and heparosan biosynthetic pathways, UDP-glucose 4-epimerase (KfoA) and chondroitin synthase (KfoC) genes from *E. coli* K4, along with α-UDP-GlcNAc glycosyltransferase (KfiA) and UDP-GlcA glucuronosyltransferase (KfiC) genes from *E. coli* K5, were introduced into *B. subtilis* for chondroitin and heparosan production, respectively.[96] Consequently, the engineered strains *B. subtilis* E168C and E168H achieved a titer of 1.83 g per L chondroitin and 1.71 g per L heparosan, respectively. While *Bacillus* has exhibited versatility in GAGs production, current applications typically focus on single-GAG biosynthesis. To fully exploit the production potential of *Bacillus* platforms, future metabolic engineering efforts should investigate the co-synthesis of multiple GAGs through pathway optimization, alongside the development of modular genetic circuits to balance precursor flux while minimizing metabolic burden.[97]

3.2.4. Corynebacterium glutamicum. *C. glutamicum*, a GRAS-certified industrial workhorse, is widely recognized for its robust capability in amino acid biosynthesis and metabolic engineering, attributed to its efficient carbon flux and minimal byproduct formation. However, wild-type *C. glutamicum* is devoid of endogenous GAGs biosynthetic operons, necessitating the reconstruction of heterologous pathways for GAGs production. Through metabolic engineering strategies, *C. glutamicum* has been successfully repurposed to synthesize HA, chondroitin, and heparosan.[98,99] To establish the biosynthetic pathway for chondroitin production, the *kfoA* and *kfoC* genes from *E. coli* K4 were heterologously expressed in *C. glutamicum*, enabling the biosynthesis of the non-sulfated chondroitin. The engineered strain *C. glutamicum* CG02 achieved a titer of 0.38 g per L chondroitin.[99] Furthermore, metabolic engineering of *C. glutamicum* has demonstrated the feasibility of chondroitin sulfate production, thereby establishing this organism as a promising microbial chassis for industrial-scale GAGs biosynthesis.[100] However, the current efficiency of chondroitin sulfate production remains suboptimal for commercial applications. To enhance the production of sulfated GAGs, it is essential to address the critical metabolic bottlenecks by augmenting sulfonate donor pools *via* pathway engineering and by developing highly efficient sulfotransferases with robust activity and specificity for GAGs sulfation.

3.2.5. Lactococcus lactis. *Lactococcus lactis*, a GRAS-certified, Gram-positive homofermentative bacterium, serves as an industrially validated chassis organism for dairy fermentations. It has been successfully engineered as a MCF for the production of recombinant nutraceuticals, leveraging its nisin-inducible expression system, inherent probiotic characteristics, and well-established biosafety profile.[101,102] Although *L. lactis* possesses endogenous pathways for UDP-GlcNAc and UDP-GlcA biosynthesis, it lacks the necessary GAGs synthases, rendering it incapable of naturally producing GAGs production. Through metabolic engineering strategies, this limitation has been overcome for HA production by introducing heterologous HA synthase.[103] For instance, to engineer the HA biosynthesis pathway, the *hasA* gene from *S. zooepidemicus* was heterologously expressed in *L. lactis*, enabling HA production.[104] Consequently, the recombinant strain *L. lactis* CES15 achieved a titer of 6.09 g per L HA, showing its potential as a MCF for industrial-scale GAGs biosynthesis. However, current metabolic engineering efforts predominantly focus on HA, while the biosynthetic pathways for chondroitin and heparosan remain insufficiently explored in this host. To fully exploit *L. lactis* as a platform for sulfated GAGs production, pathway engineering efforts are essential to establish functional chondroitin and heparosan biosynthetic modules.

4. Microbial pathways for GAGs biosynthesis

The elucidation of GAGs biosynthetic pathways marks a pivotal advancement in microbial polysaccharide production, thereby providing critical insights into GAGs assembly mechanisms. These pathways can be categorized into two main types: natural pathways for GAGs biosynthesis and unnatural pathways for GAGs biosynthesis. A comprehensive understanding of these interconnected metabolic pathways has greatly facilitated the rational design of MCFs. This enables precise manipulation of both natural and unnatural products through targeted genetic modifications and precursor-directed biosynthesis strategies.

4.1. Natural GAGs biosynthetic pathways

The biosynthetic pathways of natural GAGs constitute well-defined metabolic routes that are ubiquitously present in biological systems and have been thoroughly characterized through extensive research. These pathways encompass the pathways for non-sulfated GAGs and sulfated GAGs biosynthesis. The mechanistic elucidation of these natural biosynthetic pathways has facilitated their precise reconstruction in microbial chassis, enabling scalable industrial production of natural GAGs.

The biosynthesis of natural non-sulfated GAGs shares the conserved pathways for generating essential nucleotide sugar precursors, particularly UDP-GlcA and UDP-GlcNAc (Fig. 2). This metabolic pathway begins with fructose-6-phosphate. One branch involves sequential actions of fructose-6-phosphate aminotransferase (GlmS), phosphoglucosamine mutase (GlmM), and GlcNAc-1-phosphate uridyltransferase/glucosamine-1-phosphate acetyltransferase (GlmU), producing UDP-GlcNAc, while another branch utilizes glucose-6-phosphate isomerase (Pgi), phosphoglucomutase (Pgm), UTP-glucose-1-phosphate uridylyltransferase (GalU), and UDP-glucose 6-dehydrogenase (KfoF) to generate UDP-GlcA. These activated sugar donors then serve as substrates for GAGs-specific synthases that determine polysaccharide structure. HasA converts UDP-GlcA and UDP-GlcNAc into HA chains, whereas heparosan synthesis requires the coordinated activity of KfiA and KfiC to link these same nucleotide sugars.[113,114] In contrast, chondroitin biosynthesis involves an additional epimerization step where KfoA first converts UDP-GlcNAc to UDP-GalNAc, followed by KfoC-catalyzed polymerization with UDP-GlcA to form the characteristic chondroitin backbone.[87] This conserved yet diversified biosynthetic logic enables cells to produce structurally distinct GAGs from common metabolic precursors through enzyme-specific regulation of glycosidic linkages and sugar composition.

Microbial synthesis of sulfated GAGs requires additional processes for enzymatic sulfation of the basic polysaccharide chain in non-sulfated GAGs such as HA, chondroitin, and heparosan. For CS-A production, the biosynthetic pathway necessitates PAPS as an essential cofactor, with chondroitin-4-*O*-sulfotransferase catalyzing the position-specific sulfation of the galactosamine residues at the C4 position.[38] Similarly, heparin biosynthesis involves a more complex, multi-step sulfation cascade where heparosan first undergoes *N*-deacetylation and *N*-sulfation catalyzed by *N*-deacetylase/*N*-sulfotransferase (NDST), followed by C5 epimerization of GlcA to IdoA *via* C5-epimerase, and subsequent *O*-sulfation at the C2, C6, and C3 positions through the coordinated action of 2-*O*-

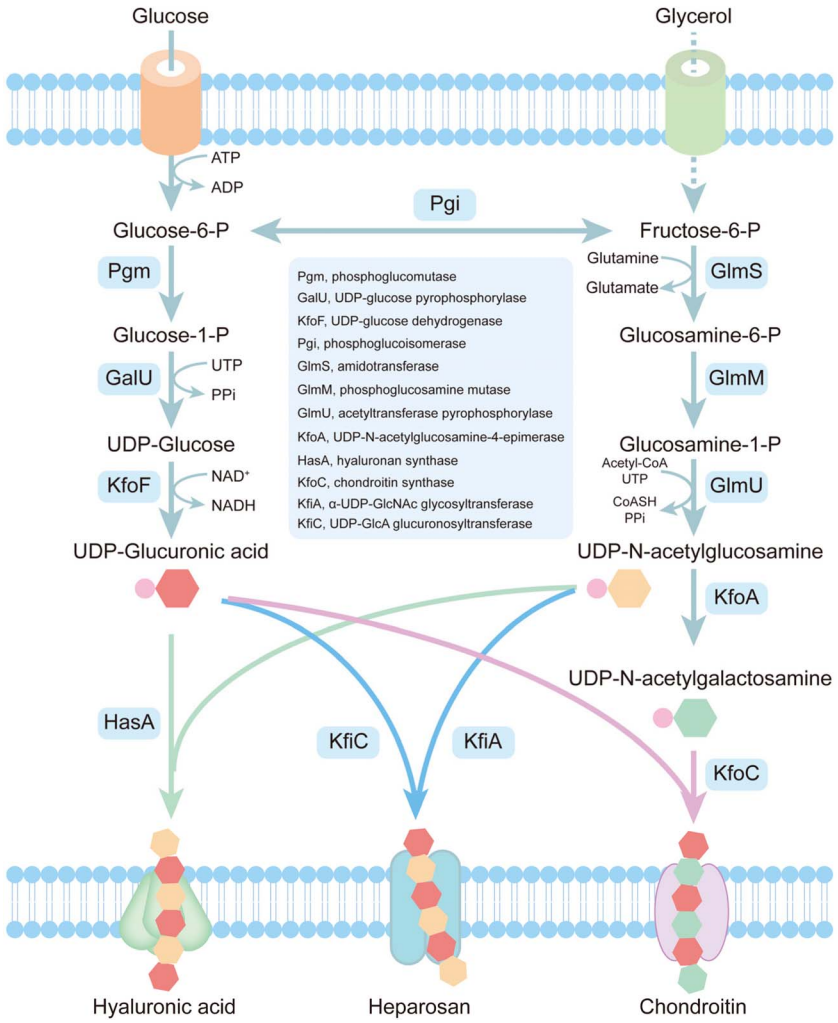

Fig. 2 Microbial pathways of hyaluronic acid, chondroitin, and heparosan biosynthesis. Abbreviations: Glucose-6-P, glucose-6-phosphate; Glucose-1-P, glucose-1-phosphate; Fructose-6-P, fructose-6-phosphate; Glucosamine-6-P, glucosamine-6-phosphate; Glucosamine-1-P, glucosamine-1-phosphate. Pgm, phosphoglucomutase; GalU, UDP-glucose pyrophosphorylase; KfoF, UDP-glucose dehydrogenase; Pgi, phosphoglucoisomerase; GlmS, amidotransferase; GlmM, phosphoglucosamine mutase; GlmU, acetyltransferase pyrophosphorylase; KfoA, UDP-*N*-acetylglucosamine-4-epimerase; HasA, hyaluronan synthase; KfoC, chondroitin synthase; KfiA, α-UDP-GlcNAc glycosyltransferase; KfiC, UDP-GlcA glucuronosyltransferase.

sulfotransferase (2-OST), 6-*O*-sulfotransferase (6-OST), and 3-*O*-sulfotransferase (3-OST), respectively, with each modification step requiring PAPS as the sulfate donor to progressively transform heparosan backbone into the highly sulfated heparin structure.[63] This sophisticated sulfation machinery distinguishes sulfated GAGs biosynthesis from their non-sulfated counterparts and presents unique challenges for microbial production attempting to recapitulate these complex post-polymerization modifications.

4.2. Unnatural GAGs biosynthetic pathways

Unlike natural pathways for GAGs biosynthesis, the biosynthetic pathways for unnatural GAGs do not exist in nature and must be deliberately engineered through rational metabolic design. These synthetic pathways enable the production of structurally modified GAGs analogs including *N*-glycolyl chondroitin, azido-labeled polysaccharides, -GlcA-GlcNTFA-polysaccharides, and chondbiuronan (Fig. 3). Systematic characterization of these biosynthetic routes has permitted their successful reconstitution in microbial chassis, significantly expanding the diversity of obtainable GAGs derivatives with precisely controlled structural features. This technological breakthrough has created new paradigms for designing GAGs-based biomaterials with programmable biological activities and physicochemical properties.

The biosynthetic pathway of *N*-glycolyl chondroitin closely resembles that of conventional chondroitin, with the critical distinction in inherent inability of microbial systems to produce UDP-*N*-glycolylgalactosamine endogenously. This limitation necessitates exogenous supplementation of synthetic *N*-glycolylglucosamine, which undergoes phosphorylation by *N*-acetyl-D-glucosamine kinase (NagK) to form *N*-glycolylglucosamine-6-phosphate, followed by sequential conversion through GlmM,

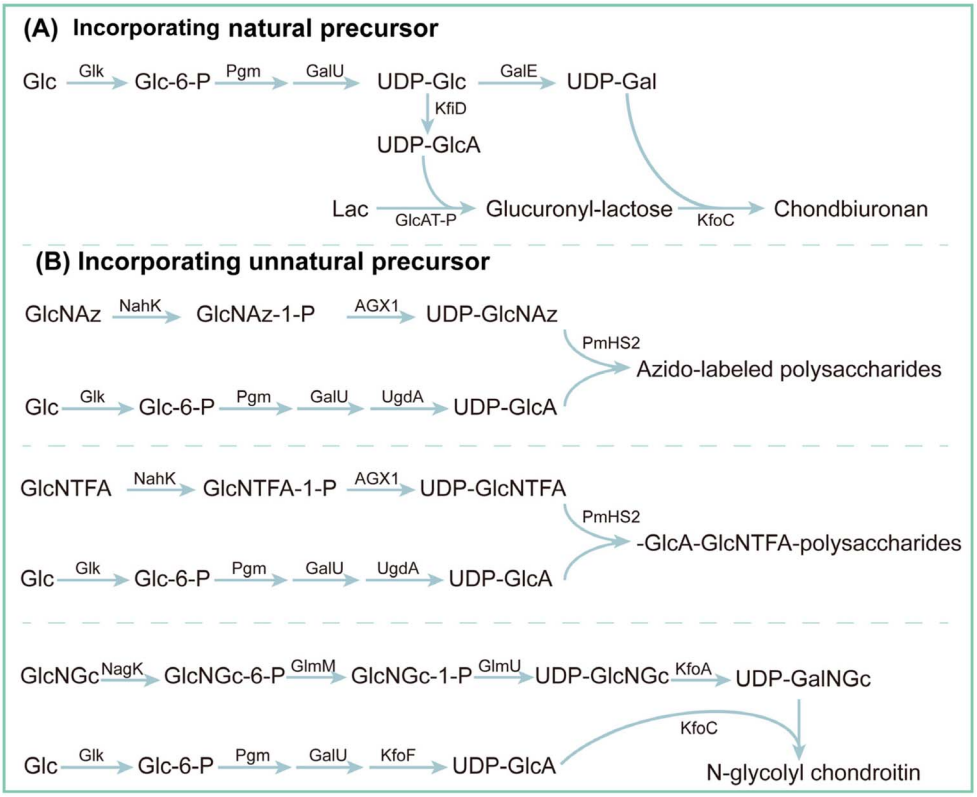

Fig. 3 Designing the biosynthetic pathways of new-to-nature GAGs analogs. (A) The incorporation of natural precursors enables the biosynthesis of chondbiuronan. (B) The incorporation of unnatural precursors enables the synthesis of azido-labeled polysaccharides, -GlcA-GlcNTFA- polysaccharides, and *N*-glycolyl chondroitin. Abbreviations: NahK, *N*-acetylhexosamine 1-kinase; AGX1, UDP-*N*-acetyl hexosamine pyrophosphorylase 1; PmHS2, heparosan synthase 2; Glk, glucokinase; Pgm, phosphoglucomutase; GalU, UTP-glucose-1-phosphate uridylyltransferase; UgdA/KfiD/KfoF, UDP-glucose 6-dehydrogenase; NagK, *N*-acetyl-D-glucosamine kinase; GlmM, phosphoglucosamine mutase; GlmU, bifunctional GlcNAc-1-phosphate uridyltransferase/glucosamine-1-phosphate acetyltransferase; KfoA, UDP-glucose 4-epimerase; GlcAT-P, β-1,3-glucuronyltransferase; KfoC, chondroitin synthase; GalE, UDP-glucose 4-epimerase.

GlmU, and KfoA to generate UDP-GalNGc, which is subsequently polymerized with UDP-GlcA into *N*-glycolyl chondroitin catalyzed by KfoC.[67] Similarly, the production of azido-labeled polysaccharides such as azido-heparosan requires external provision of GlcNAz, because microorganisms cannot naturally synthesize UDP-GlcNAz. This precursor (GlcNAz) is then converted to UDP-GlcNAz through the coordinated action of *N*-acetylhexosamine 1-kinase (NahK) and UDP-*N*-acetyl hexosamine pyrophosphorylase (AGX1). The resulting UDP-GlcNAz, along with UDP-GlcA, is incorporated into azido-heparosan polysaccharides by heparosan synthase 2 (PmHS2).[66] The biosynthetic route for -GlcA-GlcNTFA- polysaccharides mirrors this process exactly, differing only in the use of Ac_4GlcNTFA as an exogenous substrate that NahK and AGX1 similarly convert to UDP-GlcNTFA for subsequent polymerization with UDP-GlcA by PmHS2. In contrast, chondbiuronan biosynthesis initiates with β-1,3-glucuronyltransferase (GlcAT-P) for catalyzing the transfer of GlcA from UDP-GlcA to lactose, forming glucuronyl-lactose (GlcA-Lac), while UDP-glucose 4-epimerase (GalE) concurrently generates UDP-galactose from UDP-glucose, ultimately allowing KfoC to polymerize these intermediates into the characteristic repeating β3-Gal-β4-GlcA units that define chondbiuronan unique structure.[65]

5. Microbial engineering strategies for GAGs production

Microbial engineering strategies have tapped the potential of GAGs production with microbial strains. These strategies have been used for producing different GAGs: the production of non-sulfated GAGs through chassis optimization and carbon flux redirection to overcome natural microbial production limitations; the production of sulfated GAGs by addressing challenges in sulfotransferase functional expression and PAPS supply constraints to achieve *de novo* microbial synthesis; and the production of new-to-nature GAGs analogs through artificial metabolic route creation that significantly expands the GAGs product spectrum. Together, these systematic engineering strategies have transformed microbial systems into versatile platforms for both natural and unnatural GAGs production.

5.1. Microbial engineering strategies for the production of non-sulfated GAGs

Microbial engineering strategies for the production of non-sulfated GAGs leverage synthetic biology approaches to systemically optimize carbon flux distribution, maximizing its

diversion toward GAGs biosynthetic pathways for enhancing production efficiency. Microbial engineering strategies for the production of non-sulfated GAGs include rewiring metabolic networks of chassis strain, enhancing the supply and polymerization of precursors, and promoting GAGs secretion. Rewiring metabolic networks of chassis strain is conducted by restructuring central metabolic pathways to optimize carbon flux toward GAGs synthesis, thereby improving carbon utilization efficiency and increasing metabolic flux. However, this approach may elevate metabolic burden and cause genetic instability. Enhancing the supply and polymerization of precursors is conducted by overexpressing key enzymes or regulating precursor synthesis pathways to increase GAGs precursor concentration and polymerization efficiency, thereby directly boosting precursor accumulation. However, this may result in excessive accumulation of intermediates and metabolic imbalance. Promoting GAGs secretion is achieved by engineering host secretory systems to facilitate the translocation of intracellularly synthesized GAGs to the extracellular environment. This strategy promotes GAGs efflux and reduces potential metabolic inhibition of host cells.[115] However, the process of GAGs chain elongation requires sufficient reaction time, and the excessive promotion of GAGs secretion may create conflicts between these two processes. Consequently, microbial engineering strategies for the production of non-sulfated GAGs not only represents a technological revolution surpassing conventional production paradigms, but more importantly inaugurates a new era of GAGs biosynthesis characterized by the design–construction–evaluation–optimization (DCEO) biotechnology.[116]

5.1.1. Rewiring metabolic networks of chassis strain. Rewiring metabolic networks of chassis strain enables targeted redirection of carbon flux toward UDP-sugar metabolic nodes, thereby optimizing substrate availability for GAGs production. However, over-attenuation of central metabolic pathways during chassis network rewiring may inadvertently induce metabolic incompatibilities. Predicting metabolic flux based on GEMs can identify keys nodes in the competing pathways for carbon flux competition, thereby facilitating the calculation of optimal yield solutions and yield space.[117] Redirecting metabolic flux based on rational engineering enables the precise rebuilding of carbon flux through targeted modification of key metabolic nodes, thereby preventing systemic metabolic dysregulation in MCFs (Fig. 4A).

5.1.1.1. Predicting metabolic flux based on GEMs. Predicting metabolic flux based on genome-scale metabolic models (GEMs) represents a sophisticated computational framework for systematically reconstructing and characterizing entire metabolic networks.[118] Through the integration of constraint-based modeling methodologies such as flux balance analysis and advanced optimization algorithms, GEMs facilitate the precise prediction of metabolic flux distributions and GAGs biosynthesis capacities across diverse environmental conditions.[119] The principal strategies employing GEMs can be classified into two distinct approaches: GEMs-guided carbon flux redirection and GEMs-guided cofactor supply optimization.

GEMs-guided carbon flux redirection is a precision optimization strategy based on systematic metabolic network analysis. By integrating metabolic flux distribution calculations and pathway competition analysis, it achieves targeted regulation of central carbon metabolic pathways, thereby minimizing metabolic shunt effects, optimizing carbon source utilization efficiency, and ultimately maximizing the biosynthetic flux of GAGs. To identify potential metabolic engineering targets for enhancing chondroitin biosynthesis in *E. coli*, lytic murein transglycosylase (MltB) gene was computationally predicted to catalyze the degradation of peptidoglycan into GlcNAc, which serves as a precursor for UDP-GalNAc biosynthesis, thereby augmenting chondroitin accumulation.[120] The engineered strain *E. coli* (overexpressing *kfoC*, *kfoA*, *ugd*, and *mltB* genes) demonstrated a significant improvement in chondroitin production, achieving a titer of 0.091 g L^{-1}, corresponding to a 46.8% enhancement compared to that of the parental strain *E. coli* (overexpressing *kfoC*, *kfoA*, and *ugd* genes). GEMs-guided carbon flux redirection has been demonstrated to enhance GAGs biosynthesis, but the development of comprehensive carbon flux optimization strategies remains imperative. To systematically engineer a superior *C. glutamicum* cell factory for HA production, *in silico* predictions suggested that attenuating the glycolysis and pentose phosphate pathways, coupled with the knockout of lactate and acetate biosynthetic pathways, would significantly improve HA synthesis.[113] By the down-regulation of fructose-1,6-diphosphate aldolase (Fba) gene and the knockout of glucose-6-phosphate 1-dehydrogenase (Zwf), lactate dehydrogenase (Ldh), phosphate acetyltransferase (AckA), acetate kinase (Pta), acetyl-CoA:CoA transferase (Cat), and pyruvate:quinone oxidoreductase (PoxB) genes, the engineered strain *C. glutamicum* CgHA21 exhibited a remarkable increase in HA titer up to 24.5 g L^{-1}, marking a 71.3% enhancement over the parental strain *C. glutamicum* CgHA00. Consequently, the integration of GEMs-based predictions with experimental validation has successfully resulted in a high-yield HA-producing strain.

GEMs-guided cofactor supply optimization represents a systematic metabolic engineering approach that leverages cofactor metabolic network analysis and redox potential optimization to dynamically balance key cofactor pools. This strategy ensures the precise regulation of energy metabolism and redox homeostasis in GAGs biosynthetic pathways, ultimately enhancing the production efficiency of target metabolites. To identify potential targets for improving HA biosynthesis in *L. lactis*, computational predictions indicated that the inosine utilization pathway could augment UTP biosynthesis, thereby enhancing the production of UDP-GlcNAc and UDP-GlcA.[121] Experimental validation demonstrated that supplementation with 4 g per L inosine significantly increased HA accumulation in the engineered strain *L. lactis* SJR6 to 1.1 g L^{-1}, reflecting a 197.3% enhancement compared to the non-supplementation. These findings not only validate the role of inosine in promoting HA biosynthesis but also provide novel targets for further metabolic engineering efforts aimed at optimizing HA production. GEMs enable the prediction of potential metabolic engineering targets, thereby offering

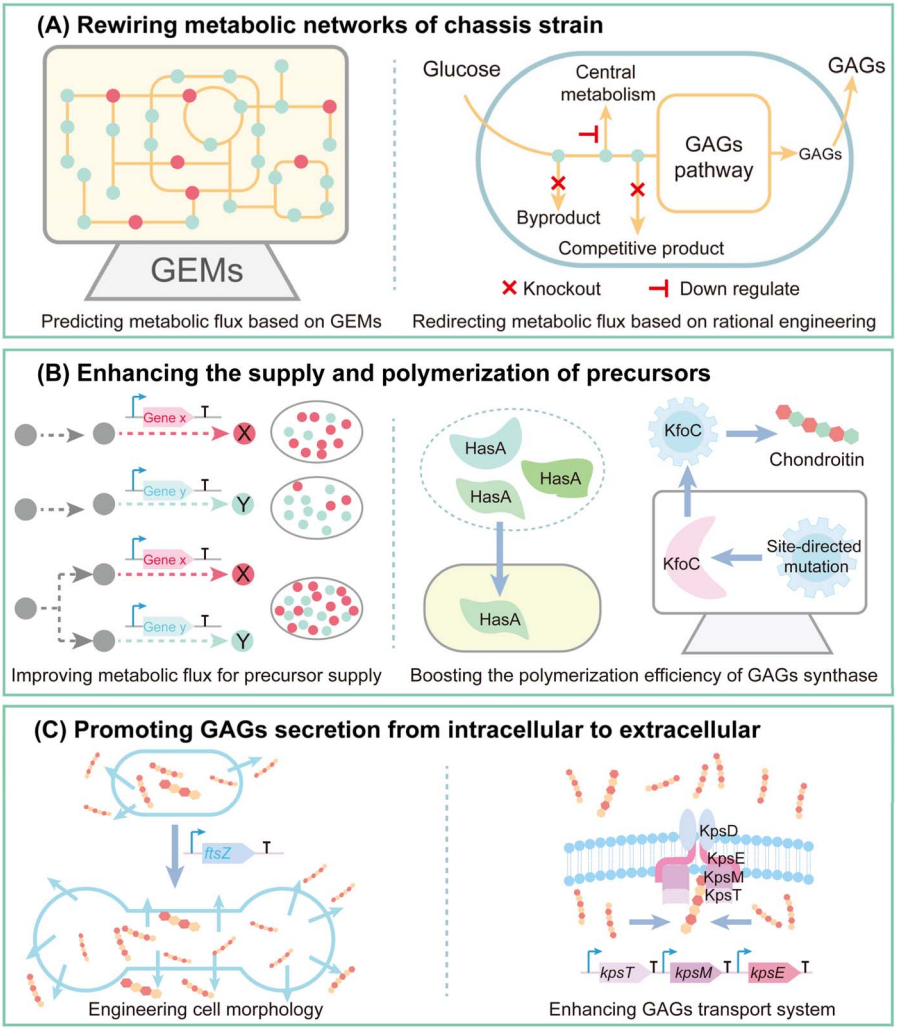

Fig. 4 Strategies for reconstructing the biosynthetic pathways of non-sulfated GAGs. (A) The efficiency of metabolic network rewiring in chassis strains can be maximized by predicting metabolic flux based on GEMs or by redirecting metabolic flux based on rational engineering. (B) The performance of precursor supply and polymerization systems can be enhanced by improving metabolic flux for precursor supply or by boosting the polymerization efficiency of GAGs synthase. (C) Promoting GAGs secretion from intracellular to extracellular. The secretion of GAGs can be significantly improved by engineering cell morphology or by enhancing GAGs transport system. Abbreviations: HasA, hyaluronic acid synthase; KfoC, chondroitin synthase; KpsT/KpsM/KpsE/KpsD, components of an ABC transporter.

a theoretical foundation for the rational design of high-yield GAGs-producing cell factories. However, the inherent variability in metabolic network architectures and GAGs-producing regulatory mechanisms across different microorganisms often limits the applicability of conventional GEMs to specific hosts.[122] Thus, a plug-and-play strategy integrating universal core metabolic modules with host-specific metabolic pathways can be developed, enhancing the model adaptability to diverse GAGs-producing microbial hosts and enabling more precise and efficient metabolic engineering strategies.[123]

5.1.1.2. Redirecting metabolic flux based on rational engineering. Redirecting metabolic flux based on rational engineering can enhances GAGs production efficiency by precisely redirecting carbon flux at key network nodes, thereby channeling more substrates into target biosynthetic pathways.[87] Currently, rational carbon flux redirection strategies can be divided into three main approaches: reconstructing central carbon metabolism pathways, engineering competitive product pathways, and suppressing byproduct pathways.

Reconstruction of central carbon metabolism pathways focuses on redirecting carbon flux from core metabolic pathways, such as glycolysis and TCA cycle, toward the synthesis of target products.[124] To redirect carbon flux from central metabolic pathways toward HA biosynthesis, the expression of 6-phosphofructokinase (PfkA) and *zwf* genes was suppressed using CRISPR interference (CRISPRi), thereby enhancing HA synthesis.[125] The engineered strain *B. subtilis* AW019-3 achieved a titer of 2.26 g per L HA, representing a 108% increase compared to that of the parental strain *B. subtilis* AW009. Engineering competitive product pathways focuses on optimizing or reconstructing the distribution of carbon flux in metabolic pathways that compete with the target products for

carbon sources, energy, or precursors, thereby enhancing carbon flux toward the biosynthetic pathway of target products. To reduce carbon flux diversion toward competitive polysaccharide synthesis, the polysaccharide operon (EpsA-O) and levansucrase (SacB) genes were knocked out in *B. amyloliquefaciens* using the CRISPR-Cas9n system, promoting HA accumulation.[92] As a result, the engineered strain *B. amyloliquefaciens* NFΔSEL-AB achieved a titer of 1.89 g per L HA, representing an 18.1% increase compared to that of the parental strain *B. amyloliquefaciens* NF-AB. Suppression of byproduct pathways involves the downregulation or elimination of metabolic branches to reduce carbon flux diversion, thereby increasing the availability of precursors for the biosynthesis of GAGs.[126] To minimize carbon flux loss due to lactate production, *ldh* gene was knocked out in *C. glutamicum* to promote carbon flux redistribution for chondroitin synthesis.[127] As a result, the engineered strain *C. glutamicum*-Δldh-CgKfoCA achieved a titer of 0.88 g per L chondroitin, representing a 252% increase compared to that of the parental strain *C. glutamicum* CgKfoCA. Thus, rational carbon flux redirection enables the optimization of carbon flux redistribution in microbial metabolic networks, significantly enhancing the synthesis efficiency of GAGs. While rational carbon flux redirection has been widely applied in GAGs production, it may unpredictably inhibit cell growth and weaken metabolic robustness, thereby compromising the performance of MCFs. By leveraging synthetic biology strategies, the design of orthogonal metabolic systems fully decoupled from the native metabolic network can eliminate metabolic interference and improve the controllability of carbon flux allocation.[128]

5.1.2. Enhancing the supply and polymerization of precursors. Enhancing the supply and polymerization of precursors is achieved through direct modulation of UDP-sugar accumulation and rational engineering of synthase properties, thereby significantly improving GAGs biosynthesis (Fig. 4B). Dysregulation of precursor supply and polymerization enhancement may lead to excessive UDP-sugar accumulation, causing a metabolic bottleneck that impedes efficient GAGs polymerization. Improving metabolic flux for precursor supply by engineering key metabolic nodes increases UDP-sugar flux, enabling high-yield GAGs production. Boosting the polymerization efficiency of GAGs synthase drives UDP-sugar conversion to GAGs, providing a core functional element for GAGs production.

5.1.2.1. Improving metabolic flux for precursor supply. The precursor supply for GAGs biosynthesis is enhanced through regulating the expression of key enzymes in UDP-sugar metabolic pathways, ensuring efficient carbon flux toward UDP-sugar formation and optimal GAGs production. UDP-sugars, such as UDP-GlcA, UDP-GlcNAc, and UDP-GalNAc, are critical precursors for GAGs biosynthesis, and their availability directly impacts GAGs synthesis efficiency. Current metabolic engineering strategies for enhancing precursor supply primarily focus on increasing carbon flux toward UDP-sugar synthesis and balancing metabolic flux distribution among UDP-sugars. Insufficient supply of precursor molecules can lead to the lack of substrates for key enzymes in the biosynthetic pathway of GAGs, significantly reducing the production of GAGs. To improve the efficiency of chondroitin production, the overexpression of UDP-glucose 6-dehydrogenase (TuaD) gene was employed to enhance the accumulation of the precursor UDP-GlcA.[96] As a result, the engineered strain *B. subtilis* E168C/pP43-D achieved a titer of 2.54 g per L chondroitin, representing a 39% increase compared to that of the parental strain *B. subtilis* E168C. Although the accumulation of UDP-GlcA improves chondroitin synthesis, the insufficient supply of the precursor UDP-GalNAc may become a critical limiting factor for further chondroitin accumulation. To further improve precursor supply for chondroitin synthesis, *glmM* and *kfoA* genes were co-expressed to enhance carbon flux toward UDP-GalNAc production.[129] Consequently, the engineered strain *B. subtilis* E168C-DMA achieved a titer of 2.66 g per L chondroitin, representing a 12.7% increase compared to that of the parental strain *B. subtilis* E168C-D. While increasing carbon flux toward UDP-sugar biosynthesis enhances GAGs synthesis, the imbalance between UDP-sugar precursors may limit the overall efficiency of target product production. To balance carbon flux between the precursors UDP-GalNAc and UDP-GlcA, the expression of *glmM–glmS*, *galU–pgm*, and *kfoC* genes was modularly optimized using RBS engineering to enhance the biosynthesis of fructosylated chondroitin.[130] As a result, the engineered strain *E. coli* ZQ25 achieved a titer of 0.52 g per L fructosylated chondroitin, representing a 46.3% increase compared to that of the parental strain *E. coli* ZQ14. Therefore, enhancing precursor supply by redirecting carbon flux toward GAGs biosynthesis significantly increases precursor metabolic flux, thereby improving the overall efficiency of the GAGs biosynthetic pathway. However, the oversupply of precursors may result in the accumulation of metabolic intermediates, potentially inducing feedback inhibition mechanisms in the pathway. Directed evolution or rational design can be employed to engineer key enzymes, enhancing their substrate specificity and resistance to feedback inhibition, ultimately improving the efficiency of precursor utilization.[131,132]

5.1.2.2. Boosting the polymerization efficiency of GAGs synthase. Boosting the polymerization efficiency of GAGs synthase can enhance the synthesis efficiency of GAGs by engineering its activity for polymerization toward precursors.[84] The primary strategies for boosting the polymerization efficiency of GAGs synthase include synthase expression optimization, heterologous synthase screening, and rational design of synthases. Synthase expression optimization focuses on regulating the protein expression levels of synthases to improve their catalytic efficiency toward UDP-sugars.[133] To enhance the synthesis of fructosylated chondroitin, the expression level of *kfoC* gene was regulated using a combined strategy of promoter engineering and plasmid copy number optimization, thereby improving its polymerization activity toward precursors.[134] As a result, the engineered strain *E. coli* BK4062 achieved a titer of 0.245 g per L fructosylated chondroitin, representing a 113.7% increase compared to wild-type *E. coli* K4 strain. Heterologous synthase screening can improve the synthesis efficiency of GAGs by identifying potential synthases from other microorganisms that exhibit higher catalytic efficiency, stronger substrate specificity, or greater stability. To enhance HA accumulation, HA synthase

genes from *S. parauberis* and *S. pyogenes* were screened to improve the polymerization activity toward the precursors UDP-GlcA and UDP-GlcNAc.[88] As a result, the engineered strain *E. coli* SPA01 was capable of achieving a titer of 0.17 g per L HA, representing a 17.2% increase compared to that of the parental strain *E. coli* SPY01. Rational design of synthases is a precision engineering strategy based on the relationship between protein structure and function.[135] Through rational design, the active site structure of synthases can be optimized to enhance substrate binding affinity, thereby improving its catalytic efficiency. To enhance the polymerization activity toward UDP-GalNAc and UDP-GlcA, the synthase mutant *kfoC*^{R268Q} gene was identified using molecular docking technology. This mutant significantly reduced the interaction energy with UDP-sugars, leading to improved substrate binding efficiency and catalytic activity.[130] As a result, the engineered strain *E. coli* ZQ14 was able to achieve a titer of 0.356 g per L fructosylated chondroitin, representing a 18.7% increase compared to that of the parental strain *E. coli* ZQ13. Therefore, boosting the polymerization efficiency of GAGs synthase can enhance their catalytic efficiency, accelerate precursor polymerization, and provide robust technical support for the efficient production of GAGs. However, despite its significant role in GAGs synthesis, synthase engineering faces several challenges, including the trade-off between enzyme activity and stability, metabolic burden on host cells, and soluble expression in heterologous hosts. By integrating multi-omics data, developing artificial intelligence-assisted design tools, and constructing high-throughput screening platforms, more efficient synthases can be obtained for advancing the industrial-scale production of GAGs.[136]

5.1.3. Promoting GAGs secretion from intracellular to extracellular. Promoting GAGs secretion from intracellular to extracellular helps overcome feedback inhibition caused by intracellular accumulation, thereby improving the efficiency of GAGs production in MCFs (Fig. 4C). The availability of advanced technologies for enhancing GAGs secretion limits the development of efficient secretion systems. GAGs secretion can be improved by engineering cell morphology to increase GAGs secretion surface area and enhancing GAGs transport system to enable specific recognition and active efflux of GAGs.

5.1.3.1. Engineering cell morphology. Engineering cell morphology can increase the surface area of microbial cells to enhance the secretion capacity of GAGs. Currently, the primary strategy for engineering cell morphology is direct rational modification by targeted regulation of genes related to cell shape. To improve HA production, cell division protein FtsZ gene was overexpressed to significantly enlarge the single-cell surface area of *C. glutamicum.*[137] As a result, the engineered strain *C. glutamicum*/pEC-AB-FtsZ achieved single-cell HA production capacity up to 7.12 ng per cell, representing a 13.5-fold increase compared to that of the parental strain *C. glutamicum*/pEC-AB. Therefore, increasing the surface-area-to-volume ratio enhances HAS expression on the cell membrane, thereby promoting GAGs secretion and improving single-cell HA production capacity. While rational cell morphology engineering may enhance GAGs secretion, it can also reduce the rate of cell growth and the efficiency of target product synthesis, potentially offsetting the benefits of morphological modification. The development of dynamic regulation systems based on metabolite sensors or cell growth-responsive elements can ensure that morphological changes occur only during the product synthesis phase, avoiding negative impacts on cell growth and maximizing the benefits of morphology engineering.

5.1.3.2. Enhancing GAGs transport system. The GAGs transport system is enhanced through engineering efflux proteins to enhance polysaccharide transport for reducing intracellular accumulation, thereby improving secretion efficiency and GAGs production. Current strategies for enhancing GAGs transport system primarily include transcription factor engineering and ABC transporter modification.

Transcription factor engineering can be used to modify transcription factors to regulate the expression of genes associated with polysaccharide transport systems, indirectly enhancing GAGs efflux efficiency. To enhance the efflux of fructosylated chondroitin, transcriptional regulator SlyA gene was overexpressed to indirectly upregulate the expression of capsule polysaccharide export inner-membrane protein KpsE and polysialic acid transport protein KpsM genes.[138] As a result, the engineered strain *E. coli* THslyA achieved a titer of 1.0 g per L fructosylated chondroitin, representing a 85% increase compared to that of the wild-type *E. coli*. Transcription factor engineering promotes the efficiency of GAGs efflux by regulating the expression of genes associated with polysaccharide transport systems. Although transcription factors can coordinately regulate the expression of multiple pathway genes through simultaneous activation and repression mechanisms, they also potentially cause metabolic burden due to imbalanced resource allocation.

GAGs efflux efficiency is directly modulated through ABC transporter engineering, which can be enhanced by the targeted regulation of key proteins in the transporter gene cluster. To improve the intracellular accumulation of chondroitin, the expression of polysialic acid transport ATP-binding protein KpsT and *kpsM* genes was downregulated using CRISPRi to reduce chondroitin efflux capacity.[139] As a result, the engineered strain *E. coli* K4ΔkfoEΔcysH(DE3)-M1 exhibited a 60% reduction in extracellular chondroitin concentration (79.79 mg per g DCW) compared to that of *E. coli* K4ΔkfoEΔcysH(DE3). These findings demonstrate that ABC transporter engineering significantly impacts the efficiency of polysaccharide transport systems. To promote heparosan secretion, the ABC transporters *kpsT*, *kpsM*, and *kpsE* genes were overexpressed to enhance transport capacity.[140] As a result, the engineered strain *E. coli* EcN/pET-kfiACB3/pCDF-kpsTME achieved a titer of 1.03 g per L heparosan, representing a 83.9% increase compared to that of *E. coli* EcN/pET-kfiACB3.

Therefore, enhancing GAGs transport system can significantly enhance the secretion efficiency of GAGs, reduce intracellular metabolic stress caused by product accumulation, and avoid the inhibition of cell growth and metabolic pathways, thereby improving the efficiency of GAGs synthesis. However, since transport systems rely on ATP hydrolysis for energy supply, overexpressing these systems may increase the

metabolic burden on cells, leading to the competition for energy resources and the inhibition of cell growth and product synthesis.[141] Modular optimization of energy metabolism, design of dynamic regulation systems for energy metabolism, and introduction of synthetic energy pathways can be employed to enhance metabolic capacity, providing sufficient driving force for the efficient production of GAGs.

5.2. Microbial engineering strategies for the production of sulfated GAGs

Microbial engineering strategies for the production of sulfated GAGs were enabled through modular integration of UDP-sugar polymerization pathways, PAPS donor pools, and functional sulfotransferase systems. Engineering donor pools of sulfonate group can enhance PAPS accumulation, thereby providing sufficient driving force for sulfated GAGs biosynthesis. However, excessive PAPS synthesis consumes substantial ATP, creating energetic competition in metabolism. Expressing biologically active sulfotransferases is conducted by synthetic biology and protein engineering strategies to provide key enzyme components for GAGs sulfation. Nevertheless, improving the enzyme activity of sulfotransferases remains challenging. Producing sulfated GAGs is conducted by constituting of the complete pathway (encompassing precursor supply, sulfotransferase activity, and PAPS supply) in microbial hosts established a non-animal platform for sulfated GAGs production. Current challenges primarily include fine-tuning the coordination between these engineered modules. This systematic framework for GAGs sulfation establishes a good foundation for developing next-generation biosynthesis platforms for structurally defined sulfated GAGs.

5.2.1. Engineering donor pools of sulfonate group. Engineering donor pools of sulfonate group supplies critical cofactors for sulfotransferase-mediated reactions (Fig. 5A). Current technological limitations in significantly boosting intracellular PAPS levels remain a major bottleneck for efficient biological sulfation. Enhancing PAPS supply generates the necessary thermodynamic driving force for robust sulfation, enabling the improved microbial production of sulfated glycosaminoglycans. Exploring alternative donor of sulfonate group presents an attractive approach to circumvent the substantial ATP demand of conventional sulfation pathways, potentially reducing metabolic stress in engineered microbial systems.

5.2.1.1. Enhancing PAPS supply. PAPS supply is enhanced through systematic optimization of PAPS biosynthesis, utilization, and regeneration processes, addressing inherent microbial metabolic limitations to meet GAGs sulfation demands. These technological breakthroughs significantly advance the

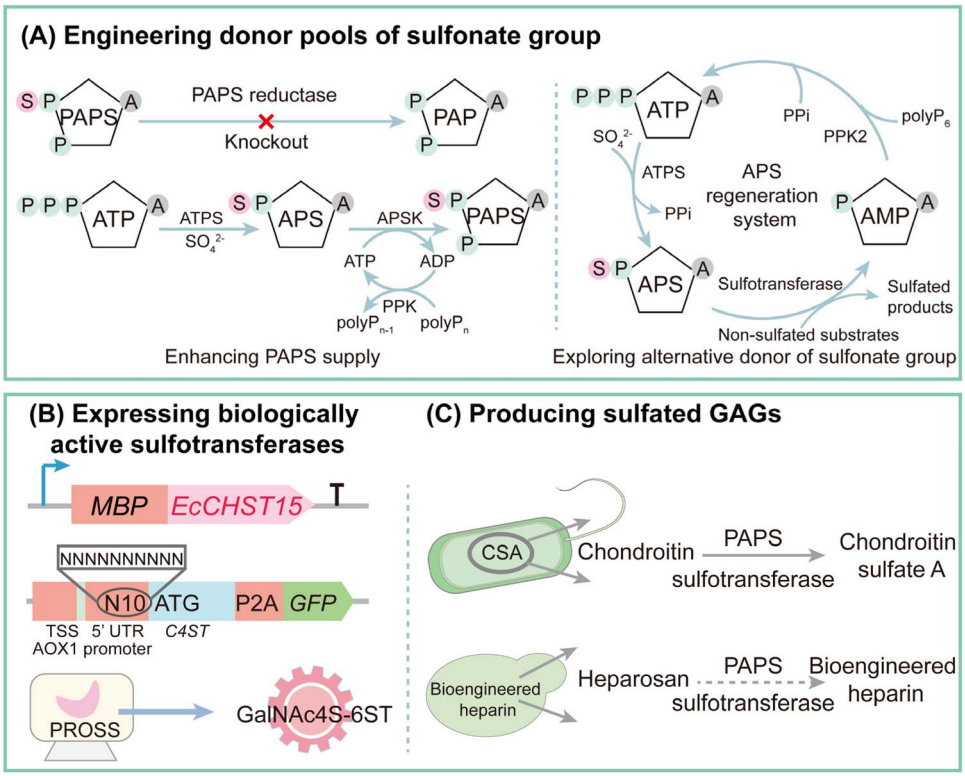

Fig. 5 Developing the biosynthetic pathways of sulfated GAGs. (A) The sulfonation efficiency of GAGs can be significantly improved by enhancing PAPS supply or by exploring alternative sulfonate group donors. (B) The biological activity of sulfotransferases can be effectively enhanced by modifying protein tags to promote solubility, rationally designing enzymes for improved stability, or implementing high-throughput screening to optimize gene expression. (C) The production of sulfated GAGs can be obtained by engineering *E. coli* or *P. pastoris*. Abbreviations: ATPS, ATP sulfurylase; APSK, adenosine-5′-phosphosulfate kinase; PPK, polyphosphate kinase; polyP, polyphosphate; MBP, maltose-binding protein; CSA, chondroitin sulfate A.

biosynthesis of sulfated GAGs while simultaneously establishing a platform for microbial synthesis of diverse sulfated natural compounds.

As the high-energy bioactive sulfonate group donor, the supply of PAPS directly determines the catalytic efficiency of sulfotransferases.[142] The approaches to augment PAPS availability emphasize reconstructing the PAPS biosynthetic pathway, reducing PAPS metabolic flux diversion, and implementing ATP regeneration systems. The PAPS biosynthetic pathway is reconstructed through systematic metabolic reprogramming, establishing an efficient anabolic network for PAPS production.[143] To enhance the ATP-to-PAPS conversion efficiency, adenosine-5′-phosphosulfate kinase from *Penicillium chrysogenum* (PcAPSK) gene was rationally engineered, yielding an L7 variant with improved catalytic efficiency for the APS-to-PAPS conversion step.[144] The engineered strain *E. coli* 11 achieved a titer of 73.59 mM PAPS, representing a 94% increase compared to the parental strain *E. coli* 04. These results demonstrate that rational design of high-efficiency enzyme variants for key steps in the PAPS biosynthetic pathway can significantly enhance overall pathway performance. Reducing PAPS flux diversion aims to minimize unproductive PAPS consumption by genetically knocking out or inhibiting either the PAPS degradation pathway or competing pathways, thereby optimizing substrate supply for sulfation reactions.[145] To enhance PAPS biosynthesis in *E. coli*, the PAPS reductase (CysH) gene was deleted to block PAPS catabolism.[146] The engineered strain *E. coli* ΔH-DNCQ achieved a 1000-fold increase in PAPS accumulation compared to the parental strain *E. coli* DNCQ. An ATP regeneration system is implemented through heterologous expression of ATP-regenerating enzymes in the host strain, establishing enhanced ATP recycling capacity to alleviate energy constraints during PAPS biosynthesis. To optimize ATP utilization efficiency, polyphosphate kinase from *Rhodobacter sphaeroides* (PPK) gene was introduced into *E. coli* to catalyze ATP regeneration from ADP and exogenous polyphosphate (polyP).[147] Subsequently, the engineered *E. coli* P1 strain, engineered for enhanced PAPS accumulation, was employed as a whole-cell biocatalyst for the conversion of chondroitin into CS-A, achieving a conversion yield of 89.5%. The critical rate-limiting role of PAPS supply in microbial sulfated GAGs biosynthesis makes its enhancement crucial for optimizing production efficiency. The ATP-intensive nature of PAPS biosynthesis imposes significant metabolic burden, potentially disrupting energy homeostasis and consequently inhibiting microbial proliferation.[148,149] Consequently, spatial decoupling strategies employing engineered PAPS-synthesizing microcompartments can create localized high-concentration PAPS microenvironments that drive efficient GAGs sulfation while maintaining cellular energy economy.

5.2.1.2. Exploring alternative donor of sulfonate group. Exploring alternative sulfonate group donors may circumvent the metabolic burden of ATP-intensive PAPS biosynthesis, thereby overcoming a key bottleneck in microbial GAGs sulfation. This strategy could establish an eco-efficient platform for producing structurally diverse sulfated GAGs while reducing cellular energy demands.

To evaluate APS as a sulfonate group donor for sulfation reactions, heparan sulfate *N*-sulfotransferase and chondroitin 4-*O*-sulfotransferase were employed as catalysts to mediate the sulfation of heparosan and chondroitin.[150] The reaction system utilizing APS as the sulfonate group donor successfully produced both *N*-sulfated heparin disaccharide and CSA disaccharide, confirming APS as an effective sulfonate donor for enzymatic sulfation. To establish a short APS regeneration cycle, ATP sulfurylase (ATPS) gene from *Saccharomyces cerevisiae* was employed to convert ATP to APS. The resulting APS was then transformed to AMP under sulfotransferase catalysis, followed by final conversion of AMP back to ATP mediated by polyphosphate kinase family 2 (PPK2) from *Staphylococcus epidermidis*. The biosynthesis of PAPS requires two ATP molecules per synthesis cycle, whereas direct utilization of APS as a sulfonate donor reduces ATP demand by 50%, thereby alleviating cellular energy stress. Consequently, employing APS as an alternative sulfonate group donor circumvents the metabolic burden associated with the high ATP consumption in PAPS synthesis, enabling more efficient sulfation modifications in MCFs. Moreover, the APS pathway may be beneficial to sulfation reactions that are challenging to catalyze through the conventional PAPS-dependent route, thereby facilitating the synthesis of novel functional compounds. While most sulfotransferases exhibit strict dependence on PAPS as the sulfonate group donor and minimal catalytic efficiency with APS, protein engineering approaches such as directed evolution or rational design can be employed to modify their active sites and screen mutants with enhanced APS affinity. Native microbial metabolic networks exhibit an inherent preference for APS conversion to PAPS, creating substrate competition that limits APS-dependent sulfation efficiency. Therefore, precise regulation of the metabolic flux between APS and PAPS pathways is required to enhance the intracellular accumulation of APS.

5.2.2. Expressing biologically active sulfotransferases. Functional expression of biologically active sulfotransferases is achieved through integrating synthetic biology design with protein engineering, enabling soluble, stable, and catalytically active expression in heterologous systems.[151,152] These studies mark a pivotal breakthrough in the large-scale production of sulfated GAGs, establishing an eco-friendly platform that circumvents the ethical and supply limitations associated with traditional animal-derived sources.

The engineering strategies for achieving functional sulfotransferase expression include protein tag modification for promoting solubility, rational design for enhancing enzyme solubility and stability, and high-throughput screening for improving gene expression (Fig. 5B). Protein tag modification focuses on the incorporation of functional protein domains at specific sites of target proteins to enhance their expression characteristics or functional activity. To achieve active expression of sulfotransferase for chondroitin sulfate E synthesis, *Erpetoichthys calabaricus* sulfotransferase (EcCHST15) gene was identified as capable of active expression in *E. coli* and fused with maltose-binding protein (MBP) to improve solubility.[153] The optimized MBP-EcCHST15 exhibited an enzyme activity of 125 100 U L^{-1}, representing a 2.8-fold increase compared to the

untagged EcCHST15. Rational protein engineering employs integrated structure prediction algorithms and computational design to systematically optimize target protein stability, catalytic activity, and solubility. To engineer a high-activity chondroitin 4-sulfate 6-*O*-sulfotransferase (GalNAc4S-6ST) in *E. coli*, *Homo sapiens* GalNAc4S-6ST gene was engineered through stability enhancement *via* mutation sites predicted by the PROSS server and solubility improvement by replacing transmembrane domains with MBP.[57] The resulting mutant M9 E133 exhibited an enzyme activity of 35 U L^{-1}, representing a 2.2-fold increase compared to the parental WT E133. High-throughput screening represents an integrated technological platform leveraging automated systems to process large-scale biological libraries through standardized workflows, combining multimodal detection and intelligent data analysis for targeted optimization of protein expression activity. To enhance active expression of chondroitin 4-*O*-sulfotransferase in *Komagataella phaffii*, a high-throughput screening system based on 5′UTR mutagenesis was developed.[154] The selected variant AE-1 exhibited an enzyme activity of 999.1 U L^{-1}, demonstrating a 16.1-fold increase compared to the Δ37 variant. The development of strategies for expressing biologically active sulfotransferases is expected to accelerate the large-scale biosynthesis of sulfated GAGs with customizable sulfonation degrees. Although biologically active sulfotransferases have been successfully expressed in *E. coli* and yeast, most of the recombinant proteins form inclusion bodies, resulting in insufficient enzyme activity and expression levels to meet industrial-scale requirements. Therefore, more effective solubilizing tags and molecular chaperones systems need to be engineered to enhance soluble expression of sulfotransferases, thereby accelerating the industrialization of MCFs for sulfated GAGs production.

5.2.3. Introducing heterogenous sulfation pathways for sulfated GAGs biosynthesis. Biosynthetic production of sulfated GAGs employs synthetic biology strategies to reconstruct eukaryotic sulfation pathways in microbial hosts, facilitating the efficient production of bioactive sulfated GAGs. This approach establishes an environmentally sustainable platform for generating structurally defined sulfated GAGs that are inaccessible *via* conventional animal tissue extraction.

Current microbial platforms for sulfated GAGs production primarily utilize *E. coli* and *P. pastoris* (Fig. 5C). *E. coli* has been successfully engineered as an efficient microbial host for chondroitin production, PAPS accumulation, and functional sulfotransferase active expression, demonstrating superior capability for sulfated GAGs biosynthesis. To establish a chondroitin sulfation pathway, *cysH* gene was knocked out to enhance PAPS accumulation, while PROSS-predicted mutant sulfotransferases were employed to improve sulfation efficiency.[139] To increase intracellular chondroitin concentration, ABC transporters *kpsT* and *kpsM* genes were downregulated *via* CRISPRi to reduce chondroitin secretion. The engineered strain *E. coli* K4ΔkfoEΔcysH (DE3) pETM6-Sw-M1 achieved a 55% chondroitin sulfation rate, representing a 189.5% increase over the parental strain *E. coli* K4ΔkfoEΔcysH (DE3) pETM6-Sw. Finally, to further optimize sulfation, strain *E. coli* MG1655ΔcysH (DE3) pETM6-PCAFSw was constructed, yielding CS-A with 96.12% sulfation levels and a titer of 27 μg per g DCW. The native post-translational modification capacity of *P. pastoris* provides inherent advantages for producing sulfated GAGs. To establish a CS-A biosynthetic pathway in *P. pastoris*, *kfoC*, *kfoA*, *tuaD*, and chondroitin-4-*O*-sulfotransferase genes were introduced to assemble both the chondroitin synthesis and sulfation modules.[38] To further enhance CS-A sulfation, *ATPS* gene from *S. cerevisiae* and *APSK* gene from *P. pastoris* were overexpressed to boost PAPS accumulation. The engineered strain *P. pastoris* Pp008 achieved a titer of 2.1 g per L CS-A with 4.0% sulfation. In another study, the biosynthetic pathway for bioengineered heparin was established in *P. pastoris* through co-expression of sulfation module enzyme genes (*H. sapiens* NDST/C5 epi, *Gallus gallus* 2-OST/6-OST, and *Mus* 3-OST) with heparosan backbone synthesis genes (*kfiA*, *kfiC*, *tuaD*).[63] The engineered strain *P. pastoris* Pp28 achieved a titer of 2.08 g L^{-1} bioengineered heparin with 4.37% sulfation. MCFs have successfully reconstructed complete biosynthetic pathways to produce structurally uniform sulfated GAGs, establishing robust production platforms. Nevertheless, the industrial-scale manufacturing of sulfated GAGs encounters substantial obstacles stemming from the intricate biosynthetic pathways, constrained PAPS cofactor availability, and suboptimal catalytic performance of sulfotransferases. Implementing synthetic biology principles, sophisticated multi-module spatiotemporal programming strategies are developing to combine CRISPR-dCas9-mediated metabolic flux control with dynamic regulation systems to achieve precise temporal separation between GAGs chain elongation and sulfation modification processes.

5.3. Microbial engineering strategies for the production of new-to-nature GAGs analogs

Microbial engineering strategies for the production of new-to-nature GAGs analogs leverage synthetic biology tools to enable the design and synthesis of novel GAGs analogs with unnatural modifications or backbone architectures. Microbial engineering strategies for the production of new-to-nature GAGs analogs include incorporating natural and unnatural precursors (Table 2). Incorporating unnatural precursor enables the introduction of chemically synthesized functional groups into GAGs structures, but efficient incorporation methods for these precursors remain lacking. Incorporating natural precursors (*e.g.*, UDP-galactose) into GAGs structures can expand their functional diversity. However, GAGs biosynthetic pathways often lack the capacity to utilize such precursors. Creating new-to-nature GAGs analogs not only broadens the structural repertoire of GAGs, but also establishes a synthetic paradigm for these biomolecules.

5.3.1. Incorporating natural precursors. Incorporation of natural precursors facilitates the development of new-to-nature GAGs analogs. By achieving UDP-sugars and incorporating them into GAGs backbones *via* GAGs synthases, structurally diverse GAGs analogs can be generated.

Rational engineering of natural metabolic pathways of precursors enables the efficient synthesis of new-to-nature

Table 2 Microbial engineering strategies for natural and unnatural GAGs production

Type	Host	Product	Strategies	Titer	Sulfation degree	Ref.
Natural hosts	*S. zooepidemicus*	HA	ARTP mutagenesis	4.56 g L^{-1}	—	73
	E. coli K4	Fructosylated chondroitin	RBS engineering Protein engineering	8.43 g L^{-1}	—	130
	E. coli Nissle 1917	Heparosan	RBS engineering Transporter engineering	11.2 g L^{-1}	—	140
Engineered hosts	*E. coli* K12 W3110	HA	Pathway construction Medium optimization	2.28 g L^{-1}	—	88
	E. coli BL21	Heparosan	Pathway construction	1.88 g L^{-1}	—	156
	E. coli BL21	Chondroitin	Pathway construction	2.4 g L^{-1}	—	87
	C. glutamicum	Chondroitin	Pathway construction	7.4 g L^{-1}	—	99
		HA	Precursors supply enhancement	8.9 g L^{-1}		
		Heparosan	Medium optimization	5.6 g L^{-1}		
	P. pastoris	HA	Pathway construction	1.7 g L^{-1}	—	84
	K. lactis	HA	Pathway construction Enzyme screening	1.89 g L^{-1}	—	83
	L. lactis	HA	Pathway construction	6.09 g L^{-1}	—	104
	B. amyloliquefaciens	HA	Pathway construction The competing pathway blocking Medium optimization	2.89 g L^{-1}	—	92
	B. megaterium	Heparosan	Pathway construction	1.96 g L^{-1}	—	90
	B. subtilis	Chondroitin	Pathway construction	5.22 g L^{-1}	—	96
		Heparosan	Precursors supply enhancement	5.82 g L^{-1}		
	B. subtilis	HA	Pathway construction Precursors supply enhancement The competing pathway blocking	1.39 g L^{-1}	—	93
	E. coli MG1655	CS-A	Protein engineering Pathway construction Cofactor engineering	27 μg per g DCW	96.12%	139
	E. coli BL21	CS-A	Protein engineering Pathway construction Cofactor engineering	1.89 g L^{-1}	76%	53
	P. pastoris	CS-A	Pathway construction Cofactor engineering	2.1 g L^{-1}	4.0%	38
	P. pastoris	CS-A	Pathway construction Cofactor engineering Medium optimization	1.15 g L^{-1}	96%	52
	C. glutamicum	CS-A	Pathway construction The competing pathway blocking	0.291 g L^{-1}	—	100
	P. pastoris	Bioengineered heparin	Protein engineering Pathway construction	2.08 g L^{-1}	4.37%	63
	E. coli K5	Azido-labeled polysaccharides	Pathway construction The competing pathway blocking	0.025 g L^{-1}	—	66
	E. coli K5	-GlcA-GlcNTFA-polysaccharides	Medium optimization	—	—	155
	E. coli K4	*N*-glycolyl chondroitin	The completing pathway blocking	—	—	67
	E. coli DH1	Chondbiuronan	Pathway construction	—	—	65

GAGs analogs. Representative natural precursor-reprogrammed GAGs analogs are chondbiuronan. Chondbiuronan is synthesized through the microbial polymerization of UDP-GlcA and UDP-Galactose catalyzed by KfoC, forming repeating Gal-GlcA structure. To achieve the polymerization between Gal and GlcA, GlcAT-P, along with *kfiD* and *kfoC* genes, were introduced to realize the biosynthesis of chondbiuronan.[65] Consequently, under lactose and glucose supplementation, the engineered strain *E. coli* EcDGCø successfully accomplished the *de novo* synthesis of chondbiuronan. Consequently, the biosynthesis of new-to-nature GAGs can be achieved by reprogramming natural metabolic pathways to generate UDP-sugar precursors. Nevertheless, the narrow substrate specificity of native GAGs synthases constrains the structural diversity of GAGs analogs. Directed evolution of GAGs synthases to expand their substrate promiscuity represents a promising strategy to improve the synthetic efficiency of non-canonical GAGs architectures.

5.3.2. Incorporating unnatural precursors. Incorporating unnatural precursors enables structural diversification of GAGs analogs by introducing chemically synthesized unnatural monosaccharides, which are metabolically activated into UDP-sugar forms and subsequently incorporated into GAGs

backbones *via* GAGs synthases, generating functionalized GAGs analogs.

By employing a chemo-bacterial synthesis strategy, the incorporation of unnatural precursors can significantly improve microbial metabolic capabilities for these chemically synthesized compounds.[3] Representative unnatural precursor-incorporated GAGs analogs include azido-labeled polysaccharides, -GlcA-GlcNTFA- polysaccharides and *N*-glycolyl chondroitin. Azido-labeled polysaccharides are synthesized through the microbial polymerization of unnatural UDP-GlcNAz and UDP-GlcA, forming repeating GlcNAz-GlcA structure. To enable the polymerization of GlcNAz and GlcA, *kfiA* and *glmS* genes were knocked out to block heparosan synthesis, while *nahK* gene from *Bifidobacterium longum*, *AGX1* gene from *H. sapiens*, and *PmHS2* gene from *Pasteurella multocida* were introduced to drive the utilization of GlcNAz.[66] As a result, the engineered strain *E. coli* K5ASSH achieved the synthesis of azido-labeled polysaccharides under exogenous GlcNAz supplementation. -GlcA-GlcNTFA- polysaccharides are synthesized through microbial polymerization of unnatural UDP-GlcNTFA and UDP-GlcA, forming repeating GlcNTFA-GlcA disaccharide structure. To enable polymerization of GlcNTFA with GlcA, the engineered strain *E. coli* K5ASSH, originally developed for azido-labeled polysaccharides production, was adapted to incorporate GlcNTFA.[66,155] The engineered strain *E. coli* K5ASSH successfully produced -GlcA-GlcNTFA- polysaccharides, achieving 74% substitution of GlcNAc by GlcNTFA under exogenous Ac_4GlcNTFA supplementation. *N*-glycolyl chondroitin is synthesized through the polymerization of UDP-GlcA and UDP-GalNGc catalyzed by KfoC, forming repeating GalNGc-GlcA structure incorporated into chondroitin. To facilitate the polymerization between GalNGc and GlcA, chemically synthesized *N*-glycolylglucosamine was supplemented and subsequently converted to UDP-GalNGc, thereby enabling the engineered *E. coli* K4 ΔkfoE strain to achieve the biosynthesis of *N*-glycolyl chondroitin.[67] Therefore, by designing a chemo-bacterial synthesis strategy, microbial systems can be engineered to incorporate unnatural precursors for synthesizing new-to-nature GAGs analogs. However, the low utilization efficiency of unnatural precursors in microorganisms significantly hinders the production of new-to-nature GAGs analogs. Developing novel utilization pathways through metabolic engineering and synthetic biology strategies can enhance the incorporation efficiency of unnatural precursors, thereby enabling the establishment of a transformative platform for next-generation new-to-nature GAGs analogs biomanufacturing.

6. Concluding remarks and future perspectives

MCFs serve as powerful platforms for producing GAGs, such as HA, chondroitin, and heparosan, sulfated GAGs, and new-to-nature GAGs analogs. To date, significant breakthroughs have been achieved through the construction of MCFs capable of synthesizing HA, chondroitin, and heparosan, demonstrating remarkable progress in microbial GAGs biosynthesis. The establishment of precise metabolic flux prediction models will further elucidate the intricate metabolic networks of GAGs, providing rational guidance for strain engineering to enhance production efficiency. Advances in multi-omics technologies will enable the discovery of novel GAGs biosynthetic pathways, while the integration of synthetic biology and protein engineering will facilitate the design of optimized enzymatic cascades for synthesizing GAGs.[157] For molecular weight customization, coupling precursor flux modulation and GAGs synthase engineering allows microbial systems to produce GAGs with tunable molecular weight distributions. Targeted evolution of GAGs synthases offers a robust approach to expand the molecular weight customization range. In sulfated GAGs production, modular optimization strategies that balance GAGs biosynthesis, supply PAPS precursor, and improve sulfotransferase activity are critical to enhance sulfation efficiency. Orthogonal GAGs metabolic networks and sulfation modules can further enable simultaneous biosynthesis of multiple sulfated GAGs. For new-to-nature GAGs analogs, metabolic pathway rewiring minimizes competition with native GAGs synthesis, thereby maximizing the diversion of carbon flux from native GAGs synthesis toward the production of new-to-nature GAGs analogs. Although high-throughput screening and directed evolution have enhanced microbial GAGs production, the inherent complexity of GAGs biosynthetic networks remains a bottleneck for hyperproduction. Future endeavors in engineered MCFs will focus on enhancing GAGs titers and productivity, refining GAGs molecular weight regulation paradigm, achieving precise sulfation patterning, and expanding the structural diversity of new-to-nature GAGs analogs through innovative pathway design and enzyme engineering.

For constructing GAGs-producing cell factories, the regulatory mechanisms of microbial GAGs metabolism must be artificially engineered. First, feedback inhibition of key rate-limiting steps by intermediate metabolites needs to be eliminated to enhance the flux efficiency of precursor metabolism.[158] To improve the conversion of Fru-6P to GlcN-6P, a *glmS* mutant ($glmS^{E14K/D386V/S449P/E524G}$) gene was introduced in *E. coli* to relieve GlcN-6P-mediated feedback inhibition, thereby significantly enhancing *N*-acetylneuraminic acid biosynthesis.[159] Second, dynamic regulation systems need to be designed to fine-tune UDP-sugar interconversion for optimal precursor allocation. For instance, to precisely control UDP-GlcA and UDP-GalNAc accumulation, a bifunctional molecular switch incorporating growth-phase-responsive promoters and degradation tags was implemented in *E. coli* to redistribute UDP-sugar carbon flux, thereby significantly enhancing chondroitin biosynthesis.[160] Finally, an optimized substrate uptake environment needs to be established to accelerate global metabolic flux storage. For instance, to eliminate the glucose uptake inhibition caused by HA in *C. glutamicum*, leech hyaluronidase was supplemented to degrade the capsule-like layer, thereby significantly enhancing HA production.[161]

For customizing GAGs molecular weight, microbial GAGs molecular weight tailoring strategies need to be expanded. First, convenient GAGs molecular weight regulation tools should be

developed to facilitate gradient customization of GAGs sizes. For instance, to achieve customized HA molecular weights in *B. subtilis*, leech hyaluronidase gene was introduced and precisely expressed through an artificial expression cassette based on an RBS mutant library, enabling the production of HA with variable specific molecular weights.[162] Second, GAGs depolymerization inducer should be explored to enable artificial induction of GAGs molecular weight variation. For instance, to obtain low molecular weight HA, hydrogen peroxide and ascorbate were supplemented to drive redox-mediated HA depolymerization, thereby achieving controlled HA size reduction.[163] Finally, novel fermentation processes should be developed to establish scalable molecular weight customization strategies for GAGs production. For instance, to produce high-molecular-weight HA, a two-stage fermentation process was developed to achieve high-level synthesis in both product titer and molecular weight.[164]

For modifying GAGs with sulfonate group, microbial engineering is required to overcome the current limitation of insufficient sulfation driving force. First, low-energy-demand sulfonate group donors should be developed to reduce the metabolic burden on MCFs. For instance, to reduce ATP consumption during the sulfation of trehalose and *p*-coumaric acid, an APS regeneration system was developed, enabling sulfation with the expenditure of just one ATP molecule per reaction.[150] Second, high-efficiency sulfotransferases should be explored to overcome the activity limitations of existing enzymes. For instance, to develop effective tools for polyphenol sulfation, sulfotransferase genes from *Desulfofalx alkaliphile* (DalAST) and *Campylobacter fetus* (CfAST) demonstrated superior polyphenol sulfation efficiency.[165] Finally, compartmentalization strategies should be developed to coordinate UDP-sugar polymerization with sulfation processes. For instance, to achieve dynamic cofactor release, the protein phase-separation element A-IDP was engineered to construct an energy adapter that dynamically couples light-dependent reactions with dark reactions.[166]

For creating new-to-nature GAGs analogs, microbial precursor incorporation patterns should be fundamentally redesigned. First, natural GAGs synthase should be engineered to enhance their affinity for natural precursors. For instance, to alter cofactor specificity, rational protein engineering was applied to modify glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and malate dehydrogenase (Mdh) genes in *C. glutamicum* S9114, successfully switching the native cofactor specificity from NAD^+ to $NADP^+$, thereby improving GlcNAc biosynthesis.[167] Second, new-to-nature GAGs synthase should be screened to develop strategies for incorporating unnatural precursors. For instance, to enable non-canonical amino acid incorporation, the pEVOL-based ncAA/*p*-acetyl-L-phenylalanine (pAcF) system was introduced into *E. coli* to incorporate pAcF at amber stop codons, thereby facilitating efficient biosynthesis of *N*-acetylglucosamine and *N*-acetylneuraminic acid.[168] Finally, new-to-nature GAG synthases must be engineered to enhance the incorporation efficiency of unnatural precursors. For instance, to enhance nonstandard amino acid incorporation efficiency, an *in vivo* evolution platform was implemented in a genomically recoded *E. coli* strain, enabling high-yield and high-fidelity biosynthesis of elastin-like polypeptides.[169]

7. Author contributions

Chunlei Zhao: writing – review & editing, writing – original draft. Jinyi Qian: writing – review & editing, validation. Xiulai Chen: writing – review & editing, writing – original draft, supervision, funding acquisition, conceptualization.

8. Conflicts of 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.

9. Abbreviations

Glc	Glucose
Lac	Lactose
GlcNAc	*N*-acetylglucosamine
Glc-6-P	Glucose-6-phosphate
UDP	Uridine diphosphate
UDP-Glc	UDP-glucose
UDP-GlcA	UDP-glucuronic acid
UDP-Gal	UDP-galactose
UDP-GlcNAc	UDP-*N*-acetylglucosamine
UDP-GalNAc	UDP-*N*-acetylgalactosamine
GlcNTFA	*N*-trifluoroacetylglucosamine
UDP-GlcNTFA	UDP-*N*-trifluoroacetylglucosamine
Ac_4GlcNTFA	1,3,4,6-Tetra-*O*-acetyl-2-deoxy-2-trifluoroacetylamino-D-glucopyranose
GlcNAz	*N*-azidoacetylglucosamine
GlcNGc	*N*-glycolylglucosamine
UDP-GlcNGc	UDP-*N*-glycolylglucosamine
UDP-GalNGc	UDP-*N*-glycolylgalactosamine
PAPS	3′-Phosphoadenosine-5′-phosphosulfate
APS	Adenosine 5′-phosphosulfate

10. Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

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