



Synthetic biology strategies for improving microbial synthesis of “green” biopolymers

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Polysaccharide-based biopolymers have many material properties relevant to industrial and medical uses, including as drug delivery agents, wound-healing adhesives, and food additives and stabilizers. Traditionally, polysaccharides are obtained from natural sources. Microbial synthesis offers an attractive alternative for sustainable production of tailored biopolymers. Here, we review synthetic biology strategies for select “green” biopolymers: cellulose, alginate, chitin, chitosan, and hyaluronan. Microbial production pathways, opportunities for pathway yield improvements, and advances in microbial engineering of biopolymers in various hosts are discussed. Taken together, microbial engineering has expanded the repertoire of green biological chemistry by increasing the diversity of biobased materials.

Context for polysaccharide-based biopolymers

Polysaccharide-based scaffolds have applications in medicine, as agricultural and food products, and as biomaterials with bioactive, biocompatible, and biodegradable properties (Table 1). Biopolymers, such as the chitin derivative chitosan, have been shown to accelerate wound healing (1) and offer opportunities for scalable manufacturing in the bioprinting industry (2). Traditionally, biopolymers are obtained from natural sources by extraction from the environment and require further downstream processing, including, in many cases, the use of harsh chemicals to obtain desired material properties. For example, chitin, which is traditionally sourced from shellfish, is a waste product resulting from the seafood industry that requires chemical protection and deprotection steps of various hydroxyl groups to impart desired functional properties (3).

Biobased production of chemicals from sugars and biomass is more sustainable than traditional non-renewable petrochemical routes (4). The cell factory approach, where a chemical is synthesized *in vivo*, utilizes simple and inexpensive starting materials like glucose. Metabolic pathways can be overex-

pressed and optimized in native organisms or reconstructed into heterologous hosts for improved yields. In the past 30 years, notable advances have been made in the microbial biosynthesis of building block chemicals, such as dicarboxylic acids (e.g. glucaric acid (5)), diamines (e.g. putrescine (6)), hydroxyacids (e.g. 3-hydroxybutyrate (7)), and diols (e.g. butanediol (8)). Whereas microbial production of bioplastics from hydroxyacid monomers is a keystone example of industrially relevant biopolymerization (9), this Minireview will not cover this area as other reviews sufficiently discuss this topic (10).

By harnessing nature’s toolbox of diverse biochemistry, microbial production of building block monomers can be extended to in-cell functionalization and polymerization. One-step microbial production of biopolymers is a sustainable alternative to avoid the use of environmentally damaging chemicals and catalysts and offers a scalable process that does not depend on harvesting from fragile ocean ecosystems, as is the case for biopolymers chitin and alginate; competing for valuable land as is the case for cellulose; or interfering with ethics of animal-based products as is the case for hyaluronan. Synthesizing biopolymers through enzymatic or whole-cell biocatalysis allows for higher regio- and stereoselectivity for in-cell composition-tailoring of polymers, which can reduce downstream processing. Useful objectives for metabolic engineering are polymer chain length by molecular weight control, sequence of saccharide units for composition control, and yield improvements for increased economic feasibility. Although greener methods do exist for biopolymer extraction from natural sources, such as utilization of ionic liquids for extracting chitin from crustacean shells (11), the economic competitiveness *versus* synthetic biology strategies has not yet been demonstrated.

This Minireview highlights strategies for cellular biosynthesis of select industrially and medically relevant polysaccharides: cellulose, alginate, chitin, chitosan, and hyaluronan. These biopolymers are examples of polysaccharides that are synthesized by the synthase-dependent pathway where polymerization and translocation processes are performed by a single synthase protein complex (12). Native biosynthetic mechanisms, such as microbial exopolysaccharide (EPS)³ biosynthesis, serve

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³ The abbreviations used are: EPS, exopolysaccharide; BC, bacterial cellulose; BCS, bacterial cellulose synthase; DO, dissolved oxygen; GFA1, glutamine-fructose-6-phosphate transaminase; GlcN-6-P, glucosamine 6-phosphate; GlcNAc-6-P, N-acetylglucosamine-6-phosphate; HA, hyaluronic acid; HAD, haloacid dehalogenase-like; HAS, hyaluronan synthase; USD, United States dollars; CRISPR, clustered regularly interspaced short palindromic repeat.

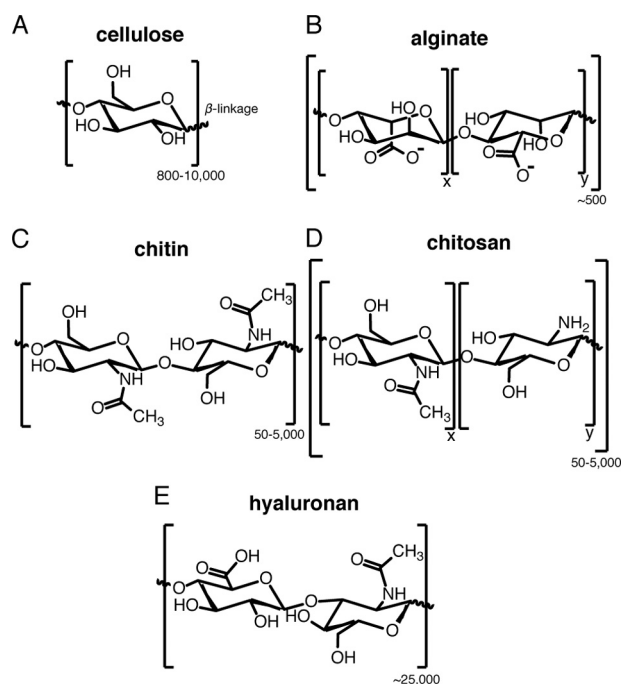


Figure 1. Chemical structures of biopolymers highlighted in this Minireview. A, cellulose; B, alginate; C, chitin; D, chitosan; E, hyaluronan. The number of units is indicated on the bottom right of each bracket. X and Y designate different monomeric units.

as a template for biotechnological production of biomaterials. Typically, synthase-dependent pathways favor homopolymer formation, and the polymers are released into the extracellular environment as non-covalently associated EPS fibers. These fibers are secreted into the surrounding environment at high molecular weights and can be harvested from cell cultures in a cost-effective manner by filtration. Several studies have demonstrated the synthesis of natural or novel variants of biopolymers from engineered organisms (13). General production strategies for microbial biosynthesis of biopolymers, such as increasing the pool of metabolite precursor supply and carbon flux toward the end product (14), are discussed within. Because of the diversity of material design options, microbial production of biopolymers also offers an attractive opportunity toward the production of new, custom-made materials beyond those from natural sources, such as engineered biosynthesis of non-natural fluorinated polyhydroxyalkanoates for bioplastics (15).

Synthetic biology strategies for polysaccharide biosynthesis

Cellulose

Cellulose is the most abundant polymer on the planet and is one of the most widely used natural materials in products such as papers and textiles. The value of the global market size for cellulose fiber was USD 20.61 billion in 2015 and is projected to reach USD 48.37 billion by 2025 (16). Cellulose is a monomeric polymer of $\beta(1\rightarrow4)$ D-glucose units (Fig. 1A) forming chains that twist into higher crystalline structures of cellulose I (triclinic structures I α and I β) and cellulose II. Cellulose crystals are formed by aggregation of nearby chains secreted from collections of cellulose synthase complexes arranged in the cell

membrane, typically forming rosettes in plant cells and axially aligned lines in bacteria (17).

Bacterial cellulose (BC) production offers unique advantages over plant fiber processing by reducing the chemical and power input during purification and offering access to the cell surface through the media to modulate crystal formation during synthesis. Manual addition of hydrogen bonding molecules to the culture media can control I α /I β ratios and molecular weights in resulting cellulose particles (18, 19). BC crystals interweave in random patterns according to bacterial movement and form a pellicle at the oxygen–media interface that grows to take the shape of the bioreactor. Pellicles (also called tea mushroom or Symbiotic Culture of Bacteria and Yeast (SCOBY)) occur naturally on the surface of Kombucha fermented tea cultures and have been used as materials for thousands of years (20). When grown in controlled conditions, regular cellulose films with complex structure can incorporate functional additives to achieve optical activity, conductivity, magnetism, and photocatalytic degradation (21). The genetic tractability of bacteria makes cellulose material synthesis an attractive target for synthetic biology.

Bacterial cellulose synthase (BCS) requires cyclic di-GMP for activation and is internally regulated by accumulation of UDP, which is released from UDP-glucose during polymerization (Fig. 2) (22). Heterologous expression of *Gluconacetobacter xylinus* cellulose synthase genes from plasmids in *Escherichia coli* led to amorphous synthesis and a non-native cellulose II structure indicating the importance of membrane organization to crystallization (23). Bacteria that produce crystalline fibrils include a fourth gene *bcsD* that is likely to facilitate the formation of multienzyme structures in the membrane, as evidenced by knockouts that produce cellulose with reduced crystallinity (24). BCS operons are known to occasionally include a gene encoding endoglucanase, BcsZ, which is capable of specifically degrading amorphous improperly formed cellulose chains. Inclusion of BcsZ protein after fibrils are formed increases crystallinity in reconstituted *in vitro* systems (24). Induction by quorum-sensing molecule *N*-acyl homoserine lactone can be used to penetrate the pellicle layer and lead to effective control of engineered expression in a newly isolated strain of *Komagataeibacter rhaeticus* (25). Maximum titers of 15.3 g/liter at production rates of up to 3.1 g/liter/h have been achieved in bioreactor fermentations with *Acetobacter* (Table 1) (26).

Alginate

Alginates are natural biopolymers that are abundant in marine brown algae (Phaeophyceae) (27) and in *Pseudomonas* and *Azotobacter* genera of bacteria (28). These EPS are the major structural component of algal cell walls, comprising up to 40–45% of the total algal dry matter (29), whereas in *Pseudomonas* and *Azotobacter*, alginates contribute to highly-structured biofilm-matrix and cyst-wall formations, respectively (30). Commercially, alginates have widespread use as stabilizers, viscosifiers, and gelling agents in food, cosmetics, beverage, paper, printing, and pharmaceutical industries (28). Globally, the demand for alginates was valued at USD 624 million in 2016, and the demand is projected to reach USD 923.8 million by 2025 with a consumption volume of 21,516 tons (31).

Table 1
Overview of microbial production of carbohydrate biopolymers: cellulose, alginate, chitin, chitosan, and hyaluronan
 Mana is mannuronic acid.

	Primary chemical structure	Metabolic precursor	Polymerizing enzyme	Natural/Industrial source	Select examples; host(s), titer, scale	Industrial application
Cellulose	β -(1,4)-Linked homopolymer of D-Glc	UDP-Glc	Cellulose synthase (BcsA)	Green plants; some algae; oomycetes	<i>Acetobacter xylinum</i> BRC5, 15.3 g/liter, 10-liter batch (26)	Electronics (diaphragms of acoustic transducers); food (nata de coco); medicine (scaffolds for tissue engineering, wound dressing, soft tissue replacement)
Alginate	β -(1,4)-Linked non-repeating heteropolymer of Mana and UDP-guluronic acid	GDP-ManA	Glycosyltransferase (Alg8)	Seaweed; some bacteria	<i>Azotobacter vinelandii</i> , 6.6 g/liter, 1.5-liter batch reactors (99)	Feed; food (gelling agents, stabilizers); medicine (tissue scaffolds, drug delivery; research)
Chitin	β -(1,4)-Homopolymer of GlcNAc	UDP-GlcNAc	Chitin synthase (NodC)	Fungi; crustaceans; insects; beaks of cephalopods; some fish and amphibians	GlcN: <i>E. coli</i> , 17 g/liter, 1-liter fed batch (43) GlcNAc: <i>E. coli</i> , 110 g/liter, 1-liter two-phase fed batch (43) Mixture of chitin oligosaccharides penta- <i>N</i> -acetylchitopentaose and tetra- <i>N</i> -acetylchitopentaose: <i>E. coli</i> , 2.5 g/liter, 2-liter fed batch (64)	Agriculture (pesticides); manufacturing (bioprinting); medicine (wound healing, drug delivery)
Chitosan	β -(1,4)-Linked heteropolymer of GlcNAc and GlcN					
Hyaluronan	β -(1,4)-Linked repeating heteropolymer of disaccharide units of GlcUA and GlcNAc	UDP-GlcUA + UDP-GlcNAc	Hyaluronan synthase (HasA)	Vertebrates (e.g. rooster combs); some bacteria	<i>C. glutamicum</i> , 21.6 g/liter, 5-liter fed batch (77)	Cosmetics; medicine (visco-supplementation, tissue repair, drug delivery)

Structurally, alginates are a family of linear, non-repeating block copolymers consisting of variable ratios of β -D-mannuronic acid and its C5 epimer α -L-guluronic acid linked by β -(1,4)-glycosidic bonds (Fig. 1B) (32). Variation in the molar ratios of β -D-mannuronic acid to α -L-guluronic acid residues controls the molecular weight and material properties of alginates. Because of their unique water-retaining capability, biocompatibility, low toxicity, relatively low cost of production, and temperature-independent mild gelation (sol-gel transition) ability in the presence of multivalent cations (e.g. Ca^{2+}), alginates are excellent biomaterials for use in biomedical applications, including wound healing, dental implants, drug delivery systems, tissue engineering, and regenerative medicine (33).

The alginate biosynthesis pathway has been extensively investigated in *Pseudomonas aeruginosa* (34) and *Azotobacter vinelandii* (35), but the complex polymerization, transport, and secretion system, as well as regulatory mechanisms controlling the pathway, are not fully understood. Metabolic engineering efforts for increasing alginate production are still in their infancy. Alginate production is tightly regulated in bacteria, and thus efforts have been made to characterize and engineer the regulatory system in *Pseudomonas fluorescens* (36) to identify the correlation between precursor availability and alginate production. Maleki *et al.* (37) showed increased alginate production (2.2 g/liter) from glycerol in an engineered *P. fluorescens* strain, in which deletion of glucose-6-phosphate dehydrogenase redirected more carbon flux through the Entner-Doudoroff pathway to produce alginate precursor fructose 6-phosphate (Fig. 2). A similar correlation between alginate production and precursor availability was observed in another recent study with *P. fluorescens* (38). Studies with *A. vinelandii* (39) utilized a different strategy, in which increased carbon fluxes were channeled toward alginate production by disrupting the competing polyhydroxybutyrate pathway. Implementing this strategy resulted in an alginate titer of up to 6.6 g/liter with a yield of 1.9 g/g sucrose. Single gene-deletion studies in *A. vinelandii* (39, 40) also increased alginate yield (0.66 g/g on sucrose) (Table 1) and lowered the degree of acetylation for altered molecular composition. More recently, alginate overproduction was reported during biofilm formation in a newly discovered strain *Pseudomonas mandelii* 6A1 (41) that was isolated from Antarctica (41). Biofilm formation by the strain was increased at lower temperatures due to increased alginate productivity, which in turn was correlated to the down-regulation of the regulatory protein MucA, which acts as a repressor in the alginate operon (41).

Synthetic biology strategies for aminopolysaccharide biosynthesis

Chitin and chitosan

The aminopolysaccharide monomer glucosamine (GlcN) and its derivative *N*-acetylglucosamine (GlcNAc) are attractive candidates for microbial biosynthesis because of the facility of the amine group for functionalization and the utility for subsequent polymerization into chitin and chitosan (Fig. 2). GlcN and GlcNAc are glucose moieties with C2 hydroxyl substitution

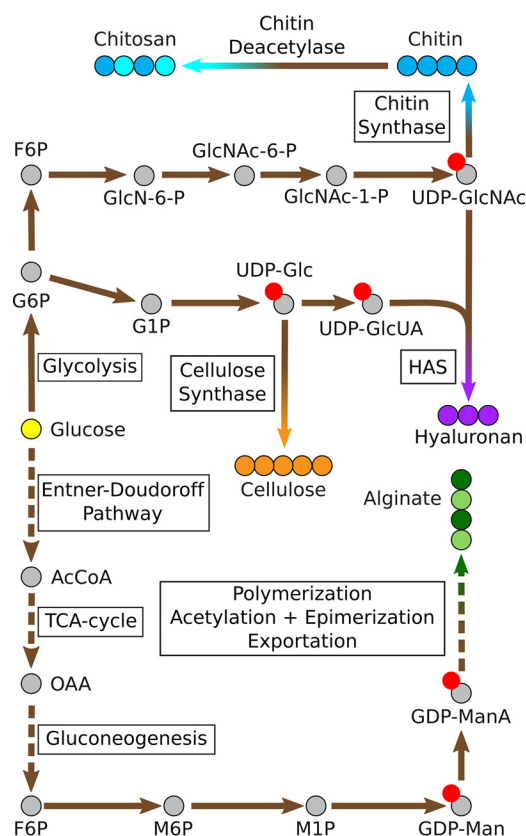


Figure 2. Overview of biosynthetic routes to biopolymers. The main steps in the microbial biosynthetic routes for cellulose, alginate, chitin, chitosan, and hyaluronan from glucose are briefly depicted. The *solid arrow* represents an enzymatic step, and the *broken arrow* represents a multistep pathway that includes a number of enzymatic steps. The *yellow circle* represents the starting material, glucose; *gray circles* represent intermediate metabolites; *red circles* represent a sugar nucleotide; and *blue, orange, purple, and green circles* represent product biopolymer molecules. Abbreviations used are as follows: AcCoA, acetyl coenzyme-A; BC, bacterial cellulose; F6P, fructose 6-phosphate; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; GDP-Man, GDP-mannose; GDP-ManA, GDP-mannuronic acid; GlcN-6-P, glucosamine 6-phosphate; GlcNAc-1-P, N-acetylglucosamine 1-phosphate; GlcNAc-6-P, N-acetylglucosamine 6-phosphate; M1P, mannose 1-phosphate; M6P, mannose 6-phosphate; OAA, oxaloacetate; TCA, tricarboxylic acid; UDP-Glc, UDP-glucose; UDP-GlcNAc, UDP-N-acetylglucosamine; UDP-GlcUA, UDP-glucuronic acid.

by an amino group and acetylated amino group, respectively. Aminopolysaccharides and subsequent biopolymers have traditionally been obtained through strong acid hydrolysis of chitin from shellfish. In recent years, microbial production of GlcN and derivatives has been demonstrated in a variety of hosts including *E. coli*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* (42).

In *E. coli*, expression of GlcNAc transferase and deletion of *nagE*, a GlcNAc transporter, increased GlcN titer to 17 g/liter (43). The GlcNAc synthesis module has been strengthened at the transcriptional level by increasing enzyme expression through testing a range of promoters for two key enzymes, glucosamine synthase and glucosamine acetyltransferase (44). Elimination of acidic by-products was accomplished by knocking out *ldh* and *pta* of the lactate and acetate synthetic pathways, respectively. By overexpressing glucosamine synthase (GlmS), inactivating catabolic genes, and utilizing a two-stage fed-batch fermentation, Deng *et al.* (43) achieved GlcN titers of up to 110 g/liter in *E. coli* in a fed-batch fermentation (Table 1).

In *B. subtilis*, expression of various combinations of synthetic small regulatory RNAs and the Hfq protein, designed to repress glycolysis by targeting *pfk* (encoding phosphofruktokinase) and peptidoglycan synthesis by targeting *glmM* (encoding phosphoglucosamine mutase), improved GlcNAc titers to 31.65 g/liter in a 3-liter fed-batch bioreactor (45). With a dynamic metabolomics approach, Liu *et al.* (46) found that a futile cycle between N-acetylglucosamine-6-phosphate (GlcNAc-6-P) and GlcNAc is the primary challenge for pathway productivity, due to high energy demands of ATP phosphorylation-dephosphorylation. Deletion of the responsible glucokinase doubles GlcNAc productivity through a dual effect of increasing ATP and restoring healthy growth to the cell.

In *S. cerevisiae*, a synthetic suicide riboswitch that regulated growth in response to the precursor GlcN-6-P was applied to screen for overproducers of GlcNAc. The growth-coupled circuit allowed for screening of an effective glutamine-fructose-6-phosphate transaminase (GFA1) mutant and haloacid dehalogenase-like (HAD) phosphatase (47). The mutant contained changes in GFA1 expression, which is the first and rate-limiting step of chitin biosynthesis, along with overexpression of HAD phosphatase YqaB, specific for conversion of GlcNAc-6-P to GlcNAc. Subsequently, GlcNAc production was further improved by reducing glycolytic flux by the disruption of *pfk-2*, achieving titers of 1.2 g/liter when fed glucose and 1.8 g/liter when fed galactose, in shake flask fermentation (48). Under galactose feeding, deletion of *pfk-2* allowed for enough reduction of glycolysis to activate gluconeogenesis thus allowing for galactose to be used as a sole carbon source.

Microbial production challenges in the biosynthesis of GlcN and GlcNAc include feedback inhibition effects where GlcN-6-P is a strong inhibitor of GlcN synthase and GlcN degradation, thus limiting the accumulation of GlcN inside the cell (Fig. 2) (49). Additionally, aminosugars can serve as alternative carbon and nitrogen sources, so it is difficult to achieve high titers in culture broth, unless a recovery strategy is incorporated in the fermentation (43). UDP-N-acetylglucosamine (UDP-GlcNAc) is normally maintained at high intracellular concentrations in growing bacterial cells to balance growth and production as the sugar donor for the synthesis of N-acetylated chitoooligosaccharide, the precursor for the biosynthesis of peptidoglycan. Aminosugars containing free amino groups are unstable in aqueous solution at neutral pH where GlcN can undergo spontaneous rearrangement and dimerization to form fructosazine, D-arabinose, and pyrazine derivatives, among others (50). Thus, biopolymerization is advantageous to circumvent degradation issues. Opportunities exist for combining strategies for GlcN and GlcNAc overproduction for subsequent biopolymerization.

Chitin is the second most abundant biopolymer on the planet and is found in almost all fungi, many animals (invertebrates), several protists, and a few algae, playing an essential role in structure. Over 800 putative chitin synthases associated with 130 genomes have been identified (51). Chitin is a hexosamine biopolymer composed of as many as 5,000 β -(1,4)-glycosidically linked GlcNAc units cross-linked by hydrogen bonding (Fig. 1C). Chitosans are deacetylated chitin, as heteropolymers of GlcNAc and GlcN units (Fig. 1D). Chitosans are variable

mixtures of molecules depending on the degree of polymerization and the degree of acetylation and are valuable functional biopolymers due to their physicochemical and biological compatibility. Chitosans have many agricultural, industrial, and biomedical applications, including use as an agricultural agent for plant defense and yield increase (52), drug delivery (53), wound healing (54), water filtration (55), and bio-printing (2). Chitosan trisaccharide is a valuable precursor for synthesis of epitopes such as type II blood group antigens (56). The global market size for chitin and chitosan was valued at USD 3.19 billion in 2015 and is projected to reach USD 17.84 billion by 2025 (57, 58). Microbial production of chitin and chitosan offers a green alternative to shellfish harvesting and allows for control of degree of polymerization and acetylation (59). van Montagu and co-workers (60) pioneered a cell factory approach for chitin oligosaccharide biosynthesis in *E. coli* by functional expression of the *nod* gene cluster from *Rhizobium*. NodC is a chitin oligomer synthase (Fig. 2) producing fully acetylated chitin oligomers of 2–5 saccharide residues (61), where the C-terminal domain of transmembrane NodC controls chain length (62). *In vitro* exposure of chitin oligosaccharides to enzymes NodB from *Rhizobium* sp. GRH2 and COD from *Vibrio cholerae* allowed for specific patterning of deacetylated chitosan oligomers (63). In 1997, Samain *et al.* (64) demonstrated the production of gram amounts of a perfectly defined, mono-deacetylated chito-pentose with a GlcN unit at the non-reducing end in *E. coli* (Table 1). Novel chitosan oligomers have been obtained, such as *N*-acetyl-lactosamine (65) and thio-chito-oligosaccharide analogs, where the oxygen glycosidic linkage is replaced with sulfur for improved stability against hydrolysis by chitinases (66).

Hyaluronan

Hyaluronic acid (HA), also known as hyaluronan, is known for its structural role in the extracellular matrix of vertebrate epithelial, neural, and connective tissues like cartilage. HA is a linear copolymer of disaccharide units of $\beta(1\rightarrow3)$ -GlcNAc and $\beta(1\rightarrow4)$ -glucuronic acid (Fig. 1E), produced in varying molecular weights, by vertebrates and prokaryotes. The repeating carboxylate groups from glucuronic acid moieties are highly hydrophilic and HA polymers have been incorporated for water retention and viscosity properties in cosmetics for more than 100 years. Purified high molecular weight preparations of HA elicit no detectable inflammatory response in mammalian cells, making HA a key functional material in designs for surgical biomaterials and cell scaffolds (67) such as HA-based hydrogels enabling the successful culture of rod photoreceptors *in vitro* (68). The global demand for HA was estimated to be worth USD 7.2 billion in 2016; thanks to the steady increase in demand, the global market size is projected to reach USD 15.4 billion by 2025 (69).

Traditionally, HA is purified from animal tissues such as rooster combs and umbilical cords. A lower cost bacterial fermentation method has been developed by leveraging the natural producer *Streptococcus zooepidemicus*. However, natural microbial HA pathways have typically evolved as a masking technique to hide invading cells within the human body, and *S. zooepidemicus* is itself a recognized human pathogen. Endo-

toxins and viral contaminants from animal and pathogen sources are a source of concern and have driven development of sustainable Generally Recognized as Safe (GRAS) alternatives. Synthesis of HA has been demonstrated in *B. subtilis* (70), *Lactococcus lactis* (71), and *Pichia pastoris* (72). In bacteria, HA is synthesized from UDP-glucuronic acid (UDP-GlcUA) and UDP-GlcNAc by a single enzyme complex hyaluronan synthase (HAS) (Fig. 2). UDP-GlcUA and UDP-GlcNAc occur naturally as part of cell wall synthesis, which directly competes with HA synthesis. The relative abundance of precursors and HAS has a definitive effect on chain length and average molecular weight of resulting HA polymers (73).

A key challenge for HA and most polysaccharide syntheses stems from the tradeoff between a high ATP and NAD^+ requirements leading to high dissolved oxygen (DO) requirements for electron cycling *versus* low molecular diffusion in high-viscosity cultures as the concentration of high-molecular-weight polymer increases. DO has a critical effect on molecular weight, possibly by affecting the abundance of precursors (74). The desired outcome of high titer leads to poor mixing and an increasingly anaerobic environment. Countering this effect mechanically with increased aeration and mixing is energy-intensive and impractical at higher viscosities. In strains capable of anaerobic fermentation, this leads to elevated levels of fermentation products such as lactic acid, which limits HA production (*S. zooepidemicus*) (75). In strains that are sensitive to low DO, such as *B. subtilis*, the anaerobic environment leads to early cessation of production at 3 g/liter (76). This limitation in *B. subtilis* was overcome by controlled expression of hyaluronidase to reduce molecular weight and viscosity of the culture with the tradeoff that smaller chains are produced (6×10^3 Da *versus* 6×10^6 Da) (76). In anaerobic-tolerant *Corynebacterium glutamicum*, heterologous expression of the HA pathway with knockout of lactate dehydrogenase allowed for accumulation of 21 g/liter HA with a mid-range mass of 2×10^5 – 8×10^5 Da (77) (Table 1). In *S. zooepidemicus*, a recombinant suicide plasmid added to prevent natural expression of hyaluronidase, yielded 9 g/liter of a higher molecular weight product (78).

Summary and future outlook

Metabolic engineering and synthetic biology strategies have advanced the techniques for microbial production of biopolymers and promise sustainable and reliable alternatives to current production from natural sources. Synthetic biology strategies highlighted include the implementation of a riboswitch to balance glycolytic flux in the biosynthesis of chitin and HA precursor GlcNAc (47), expression balancing with promoter replacement of synthase and acetyltransferase genes in the biosynthesis of GlcNAc (45), redirection of carbon flux by deleting glycolytic genes in the biosynthesis of alginate (37), and preventing expression of degradative enzymes like hyaluronidase for HA biosynthesis (78).

Although efforts have successfully demonstrated microbial biopolymer production, there are still challenges to address: 1) understanding competition for endogenous cellular resources, such as precursor sugar nucleotide pools and energy requirements (*e.g.* ATP, NAD^+); 2) transcriptional regulation, where synthesis is tightly regulated and controlled by complex regu-

latory machinery, which functions when cells need to construct structural components like EPS that relate to pathogenicity and defense mechanisms; 3) *in vivo* biopolymerization, where there is a need for better characterization of polymerization enzymes (79) and the steps in EPS biosynthesis, even though gene clusters have been known for several years (80); and finally, extracellular secretion, which poses process engineering challenges where titer is limited by viscosity leading to mass transfer issues (81). Host compatibility should also be considered, for example, the robustness of model organisms like *E. coli* to industrial conditions.

To address these challenges, the advent of new technologies and approaches is critical. Although dynamic regulation of carbon fluxes has been implemented in monomeric carbohydrate biosynthesis like glucaric acid (82), application of similar strategies for biopolymer synthesis has yet to be explored. Precursor supply by regenerating sugar nucleotides can balance cellular resources (42), and genome-level metabolic modeling of microbial cell factories is instrumental for optimizing the performance of heterologous biopolymer-producing pathways (83). For example, model-guided metabolic engineering followed by experimental validation of growth-coupled glycan-overproducing strains identified metabolic imbalances that rerouted flux toward glycan precursor synthesis (83). ¹³C metabolic flux analysis is another powerful tool for identifying pathway bottlenecks (84) in the optimization of microbial biopolymer synthesis (84). DNA sequencing (85) and synthesis technologies coupled to machine learning (86), along with the development of CRISPR–Cas9 gene-editing technologies, have allowed for increased engineering efficiency (87). Bioprospecting for new sequences and functions (88) can help characterize polymerization protein complexes and also help identify novel molecular targets for the potential of tailor-made mixed biopolymers of varying material properties made from functionalized block copolymers.

Copolymer formulations provide further opportunities for tailoring, where strategies of metabolic engineering and growth medium modifications can help control biopolymer compositions (89). Yadav *et al.* (90, 91) demonstrated bacterial production of a hybrid cellulose-chitin copolymer for biomedical applications where lysozyme susceptibility allowed for *in vivo* biodegradation. Structurally, the presence of GlcNAc in bacterial cellulose disrupts the highly ordered cellulose crystalline structure, thus transforming the cellulose type I α structure to cellulose-chitin type II due to alterations in fibril–fibril interactions (92). Further functionalization of modified bacterial cellulose through deacetylation can generate materials with a reactive amine surface that allows for various applications, such as engineering novel biocomposites, tissue engineering scaffolding, biosensor small molecule detection, and drug delivery vehicles (90).

For microbial production of biopolymers to be considered a green technology, important criteria, including energy efficiency, material efficiency, land use, and costs metrics need to be assessed. Whereas building block chemicals such as lactic acid and isoprene have been assessed by green metrics (93), an opportunity exists for assessing other valuable biosynthesized materials. Microbially-produced lactic acid and the polymer

polylactic acid have higher economic efficiency over chemically-produced similar materials due to increased energy efficiency and fermentation-driven stereoselection of D(–) or L(+)-lactic acid (94). Moving beyond cellular control, more opportunities for green processing exist, including valorization with CO₂ and utilization of feedstocks from biodegradation of waste products such as bioplastics (95). Other metrics such as life cycle assessment, the E-factor, and principles of green chemistry should be implemented to drive a circular and sustainable economy forward with reduced waste and conservation of resources. The environmental and health risk of biopolymers, for instance fluorine-containing bioplastics (15), need to be addressed. Biopolymer microbial synthesis could also be suitable in sustainable systems such as space stations or intergalactic habitats because of its renewability and replicability. There is an overlap between closed systems designed for space habitation and “green” technology on Earth (96).

Emerging technologies for *in silico* design and predictions of material properties will help advance the cell factory approach to biopolymer production such as in the creation of biomimetic scaffolds composed of 3D cell culture polysaccharide hydrogels (97). Opportunities also exist for combination of the cell factory approach with manufacturing such as in the controlled biofilm layering by EPS secreting microbes biofabricated by 3D printing, along with advances in 4D printed biomaterials with integrated “smart” diagnostics (98). Imagine a world where microbes are full cell factories, not just making single molecules but assembling entire functional materials.

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