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Perspective

Enzymatic Synthesis of Artificial Polysaccharides

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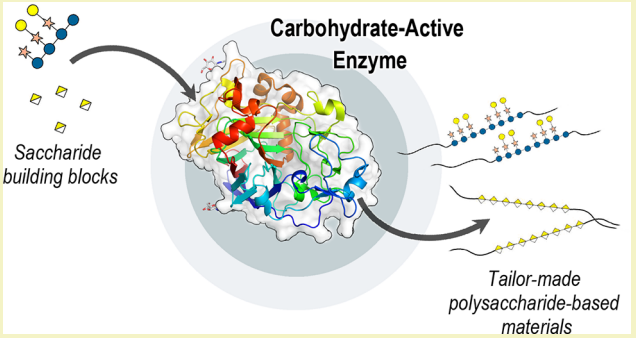
ABSTRACT: Polysaccharides are the most important renewable polymers on Earth and hold an enormous potential for the production of ecofriendly functional materials. In addition to being sustainable, they have superior properties to synthetic polymers, particularly in the biomedical field where biocompatibility and biodegradability are vital. Derivatization of polysaccharides obtained from plant biomass paves the path forward for the design and manufacturing of advanced materials with specific properties adapted to meet definitive needs. However, these advances have been severely limited due to issues with establishing structure−property relationships, which are hampered by the heterogeneity of target polysaccharides and the random distribution of functional groups obtained after their chemical modification. An accurate correlation of structure−property relationships at multiple length scales requires substrates with defined sizes, sequences, and substitution patterns. Such tailor-made polysaccharides may be obtained by implementing a bottom-up approach, starting from monosaccharide or oligosaccharide building blocks followed by their polymerization and substitution through catalysis by different carbohydrate-active enzymes such as glycosynthases, phosphorylases, sucrases, and glycosyltransferases. Recent progress in the enzymatic synthesis of artificial polysaccharides is reviewed, with an emphasis on the potential of the synthesized products, either as new materials or as tools to study structure−property relationships. The obtained information will guide future developments of rationally designed biobased materials for industrial and biomedical applications.



KEYWORDS: *Carbohydrate-active enzymes, Bioinspired materials, Polymerization, Biomass, Polysaccharide, Glycan*

INTRODUCTION

Carbohydrates are essential molecules of life.[1] They form the major component of plant biomass, are responsible for numerous key biological functions in animals, and mediate animal−microbe, plant−microbe, and intermicrobial and intramicrobial interactions. Furthermore, carbohydrates are an important source of energy in nearly all organisms. On the basis of their size, carbohydrates are classified into monosaccharides, oligosaccharides, and polysaccharides. Polysaccharides are linear or branched chains with a degree of polymerization of typically more than 20 monosaccharide backbone units. The biosynthesis of polysaccharides is performed by a myriad of glycosyltransferases (GTs) that add monosaccharides to specific saccharide acceptors from activated nucleotide sugar donors.[2] During or after assembly of the glycans, further modifications such as sulfation, phosphorylation, acetylation, methylation, and other modifications can be introduced and removed. The synthesis of structurally defined polysaccharides becomes possible when, after addition of a monosaccharide donor to an acceptor glycan, the product cannot serve as an acceptor substrate again. In plants and fungi, the backbones of the polysaccharides are often homooligomeric, branched, and furnished with heterogeneous substitution patterns, resulting from a less controlled biosynthetic process (Figure 1).[3] In other types of types of polysaccharides, the backbone is composed of more than one type of sugar, including glycosaminoglycans in the extracellular matrix of animal cells, capsular polysaccharides or lipopolysaccharides in the cell walls of bacteria, and some plant pectic polysaccharides (Figure 1). The high structural complexity of polysaccharides makes it difficult to determine their structures and to identify the molecular pattern responsible for certain properties or biological functions.

Polysaccharides do not only have essential structural and biological functions, they can also be explored as materials.[4] Being advantageous due to their carbon neutrality, renewability, and biodegradability, biosourced polymers such as polysacchar-

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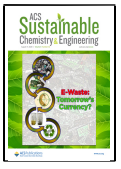

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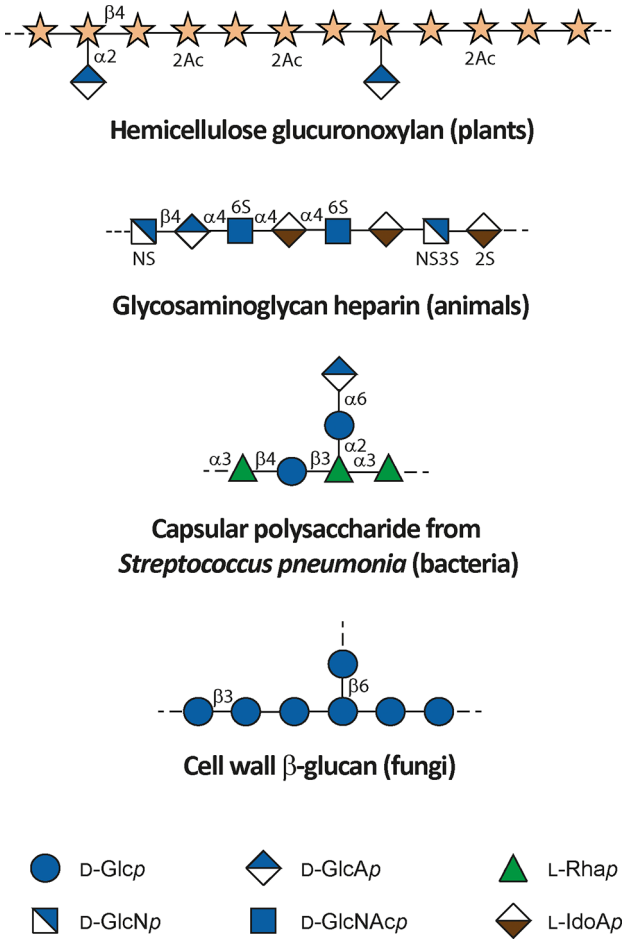

Figure 1. Molecular structures of exemplary polysaccharides from plants, animals, bacteria, and fungi. NS = *N*-sulfated, 2S = 2-sulfated, 3S = 3-sulfated, and 6S = 6-sulfated.

ides have the potential to replace a significant fraction of synthetic polymers derived from fossil resources in the future. Besides being sustainable and ecofriendly, materials from renewable resources often have superior properties to synthetic polymers, particularly in the biomedical field where biocompatibility and biodegradability is vital. In particular, cell wall polysaccharides from plant biomass represent a diverse and abundant source of polymer building blocks whose broad exploration for the production of various types of materials has just begun.[5]

As the major component of plant biomass, cellulose has been explored for the production of materials to the greatest extent.[6] Native cellulose and cellulose fibers are widely used to produce tissue and paperboard and textiles and to control rheology in food. Derivatized celluloses, such as ethers and esters of cellulose, are used in cosmetics, food, and pharmaceuticals. The hemicelluloses xylan and mannan, which represent the second most abundant polysaccharides in plant biomass, have been considered as promising materials for wound dressing, drug carriers, and edible coatings.[7] In contrast to cellulose, they occur in many structural variations, differing in the amount, localization, and constitution of their side chains.[8] Starch and algal polysaccharides have very good abilities to form membranes and coatings and have been commercialized in the form of plastic bags, food packaging, and food additives. Chitosan, produced from chitin that is isolated from exoskeletons of insects and the shells of crustaceans through deacetylation, is frequently used as a food additive and in filtration and packaging materials and has antibacterial properties. Bacterial exopolysaccharides are among other things heavily explored as high-value biomaterials for medical applications.[9]

Improved functional and biomedical materials based on biomass are being continuously developed. However, their rational design suffers from a lack of control over the length of the polysaccharides combined with modulating the abundance and patterning of the substituents along the polymer backbone. The physicochemical properties of polysaccharides are directly derived from their molecular structures and supramolecular organizations, which are determined by the sequence of their monosaccharide units. Compared to isolation from natural sources, bottom-up synthesis can provide polysaccharides with more defined structures that hold promise as novel materials, or at minimum, model compounds to investigate the influence of structural factors on functional properties.[10] Stepwise chemical syntheses benefit from a tremendous amount of control over the individual reactions and can provide glycans of almost any structure. However, although automation can significantly accelerate this process,[11] the size of routinely producible glycans by total synthesis remains limited and requires enormous quantities of resources.[12] Chemical polymerization of protected and activated saccharide monomers can provide larger glycans in a single reaction step, but the accessible structures are rather primitive and of limited diversity. Furthermore, a final deprotection step is required that may be challenging to perform with very large molecules. Chemical approaches toward polysaccharide synthesis have been reviewed elsewhere and will not be discussed here.[13]

Another approach to glycan synthesis involves the utilization of enzymes.[14] A variety of biocatalysts have been identified that can form glycosidic bonds between saccharides in a highly selective and efficient manner without the need for tedious protection and deprotection steps, as required in chemical synthesis. Enzymatic polymerizations can provide large amounts of well-defined polysaccharides, as long as the required substrates and enzymes are available in sufficient amounts.[15] There are three classes of enzymes that can be employed in cell free systems for polysaccharide synthesis: (i) GTs that transfer monosaccharides from activated sugar nucleotides to suitable acceptor substrates, (ii) glycoside hydrolases and glycosynthases, which are engineered glycoside hydrolases that make use of the reverse reaction of a glycoside hydrolysis, and (iii) phosphorylases and sucrases that use sugar-1-phosphates or sucrose as glycosyl donors (Figure 2).

In this perspective, we provide an overview of recent advancements in enzymatic polysaccharide synthesis and give a perspective on the potential of *in vitro* polysaccharide synthesis for materials science.

ENZYMATIC POLYSACCHARIDE SYNTHESIS WITH GLYCOSYNTHASES

Concept and Mechanisms. Glycoside hydrolases (GHs) are carbohydrate-active enzymes (CAZymes; www.cazy.org) that catalyze the hydrolytic cleavage of glycosidic bonds.[16,17] By degrading polysaccharides, including cellulose, hemicellulose, and starch, these enzymes are essential for the efficient valorization of plant biomass.[18] They are typically very stable and can be produced in large scale in bacterial expression systems. GHs are classified as either exoglycosidases that act on terminal monosaccharides or as endoglycosidases (also named

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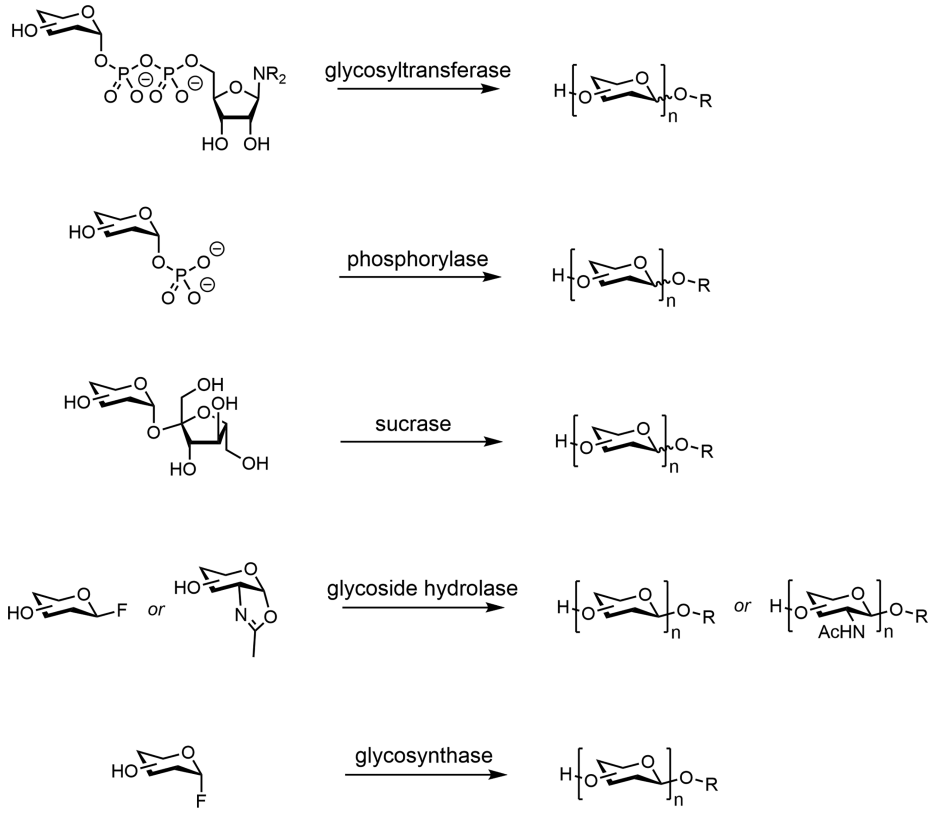

Figure 2. Typical classes of enzymes explored for preparation of polysaccharides through enzymatic polymerizations and the reactions they catalyze.

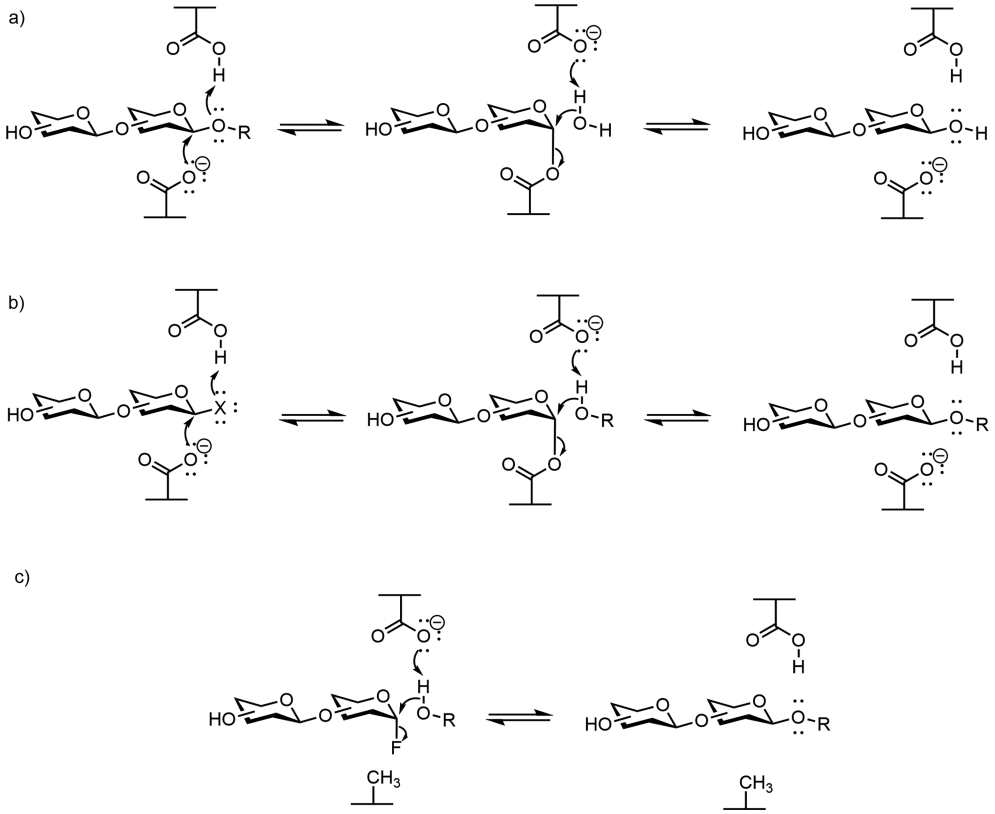

Figure 3. Reaction mechanisms for (a) saccharide hydrolysis catalyzed by retaining glycosidases, (b) retaining glycosidase-catalyzed glycosidic bond formation, and (c) glycosynthase-catalyzed glycosidic bond formation.

endoglycanases) that hydrolyze internal glycosidic bonds. Furthermore, they can be classified according to their catalytic mechanism as either retaining or inverting (Figure 3).[19] In retaining glycoside hydrolases, the configuration at the anomeric

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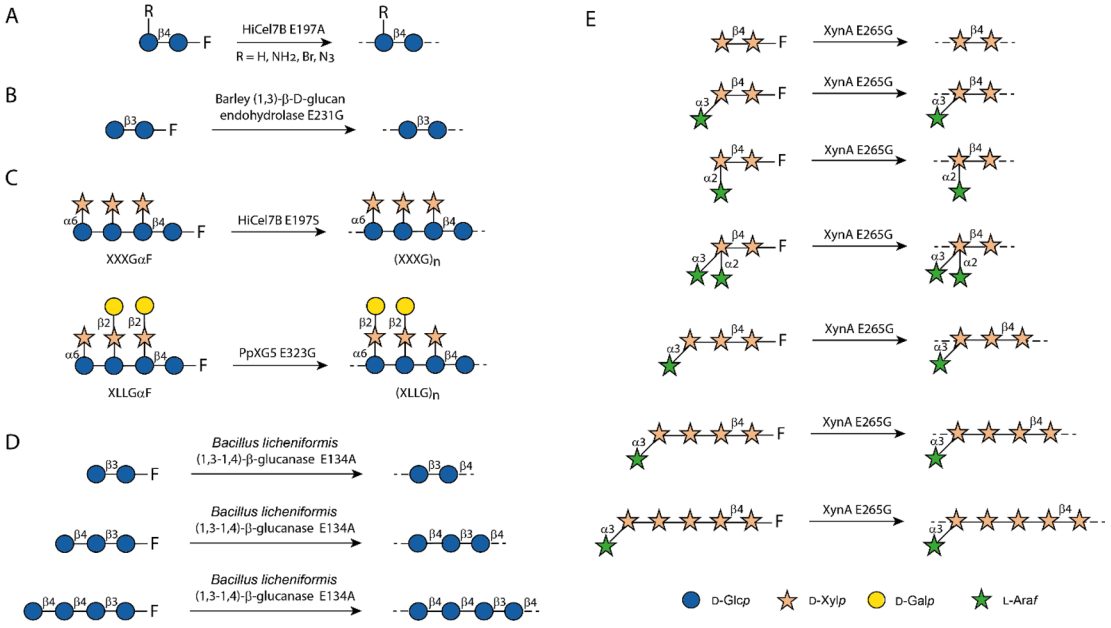

Figure 4. Collection of reported glycosynthase-catalyzed polymerization reactions.

carbon is retained through a double-displacement mechanism involving the formation of an inverted covalent intermediate with the active site nucleophile. Inverting glycoside hydrolases, on the other hand, use a single displacement mechanism, involving a carbenium ion transition state, leading to a net inversion of the anomeric center. GHs are also able to catalyze the reverse reaction to form a glycosidic bond, referred to as transglycosylation. The ability to catalyze transglycosylation is more often found in retaining enzymes. In the presence of a reactive donor saccharide, such as a glycosyl fluoride or saccharide oxazoline, high concentrations of glycosyl−enzyme intermediates are formed that can preferentially react with suitable acceptor substrates. In this way, many different polysaccharides, including derivatized celluloses and glycosaminoglycans, have been prepared.[15] However, the chain lengths and yields of polysaccharides produced by this method are inherently limited by the competing hydrolytic activity of the enzymes.

The Withers group introduced mutated glycoside hydrolases now referred to as "glycosynthases", in which the catalytic nucleophile has been replaced by a non-nucleophilic residue, abolishing background hydrolysis activity and improving yields.[20−23] When activated donors are used in conjunction with glycosynthases that (i) include a suitable leaving group at the anomeric carbon and (ii) have the opposite configuration to that of the native substrate, glycosidic bonds are very efficiently formed. While the first and most efficient glycosynthases were derived from retaining glycoside hydrolases, inverting glycoside hydrolases have recently been converted into glycosynthases as well.[24] Endoacting GHs and glycosynthases are unique among all enzymes capable of polysaccharide synthesis in their potential to transfer activated oligosaccharides rather than only activated monosaccharides. This provides greater control over the structure of the synthesized glycans, as particular features can be preinstalled in the oligosaccharide building blocks. In this section, we exclusively cover glycosynthase-catalyzed polymerizations. Traditional kinetically controlled syntheses catalyzed by unmodified glycoside hydrolases have been extensively described previously.[15,25]

Glycosynthase-Mediated Synthesis of Cellulose. Cellulose is the world's most abundant biopolymer and consists of long, linear chains of β-1,4-linked glucose residues.[6] In the plant cell wall, 18−36 individual glucan chains form crystalline microfibers of cellulose I that provide tensile strength to the cell matrix. Algae and bacteria also produce cellulose, albeit with different macromolecular properties and characteristics. The first glycosynthase reported to efficiently promote the self-condensation of oligosaccharide donors into polysaccharides was the E197A mutant of the endocellulase *Hi*Cel7B.[26] The synthase catalyzed the polymerization of α-cellobiosyl fluoride into insoluble, low molecular weight cellulose II which consists of a different hydrogen bonding network than natural cellulose (Figure 4). Also, an amino-functionalized cellobiose building block with fluoride at C6 of the nonreducing glucose polymerized when incubated with *Hi*Cel7B E197A. The resulting aminocellulose polysaccharide, which can be regarded as a chitosan mimetic, was fully water soluble with a weight-average molecular weight of 5 kDa. Bromo- and thioxylosyl-substituted cellobiose fluorides were polymerized into insoluble polysaccharides with undetermined degrees of polymerization. More recently, the Planas laboratory has prepared cellulose polysaccharides with every second glucose substituted at C6 with an azide moiety.[27] Water-insoluble azidocellulose was obtained in high yield, and the weight-average molecular weight was determined to be 5.8 kDa. Analysis of polysaccharide morphology by scanning electron microscopy (SEM) revealed that porous spherulites were formed, similar to enzymatically synthesized unsubstituted cellulose II. This morphology differs from the microcrystalline morphology found in natural cellulose. In contrast to chemical modification of natural cellulose by substitution of leaving groups installed at the C6 positions of the glucose units, enzymatic polymerization enables the synthesis of artificial cellulose with regular functionalization patterns.

Synthesis of Hemicelluloses. Cellulose fibers in the plant cell wall interact with a diverse group of polysaccharides named hemicelluloses.[8] The most prominent hemicelluloses are xyloglucans, xylans, and mannans, which occur in many structural variations. Xylan is the second most abundant

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polysaccharide in plant biomass but is underexplored as a resource for the production of materials and fuels.[28] Xylan backbones consist of β-1,4-linked xylopyranoses that are, depending on the plant species, substituted at C2 and/or C3 of the xylose units with glucuronic acids and/or arabinofuranoses and are often highly *O*-acetylated. Several glycosynthases were developed based on endoxylanases that have been shown to catalyze the polymerization of xylobiose fluoride into linear xylan polysaccharides of varying lengths.[29−31] Particularly powerful among these is the glycosynthase XynA E265G from *Geobacillus stearothermophilus*, which is capable of producing chains with a degree of polymerization (DP) of more than 100 monosaccharides.[31] The potential of this glycosynthase to prepare xylans with regular substitution patterns was further explored. Seven different glycosyl fluorides were polymerized using XynA E265G into arabinoxylan polysaccharides with regular substitution patterns.[32] Water-insoluble and water-soluble arabinoxylans could be readily separated and ranged in size from 4.7 to 29.5 kDa. These artificial arabinoxylans proved to be valuable tools to correlate properties with molecular structures, and it was shown that water-insoluble polysaccharides were formed when every third xylose residue in the backbone was substituted with an arabinofuranose either at the C2 or C3 position. For these polysaccharides, significant crystallinity was observed, while the soluble arabinoxylans were completely amorphous. Furthermore, the ability of the synthetic arabinoxylans to associate with cellulose was investigated by quartz crystal microbalance experiments. Interestingly, the synthetic xylan where every second backbone residue is substituted with arabinose adsorbed more strongly onto cellulose than xylan with a decoration on every third xylose. These findings are in line with extensive solid-state NMR studies which suggest that there are distinct domains of xylan in plant cell walls and that only xylan domains with even-numbered substitution patterns associate with cellulose microfibrils.[33] In these domains, xylan adopts a 2-fold helical screw conformation, with all substituents pointing in the same direction in order to enable close contacts with the cellulose surface.

Xyloglucans are heavily substituted β-1,4-glucans, with repeating units composed of two or three consecutive glucose units substituted at the C6 position with a xylopyranose, which are then separated by two, or more commonly one, unsubstituted glucose residues.[34] The xylose residues may be further substituted with galactosyl and fucosyl residues, generating the molecular diversity observed in different tissues and plant species. The substitution pattern of xyloglycans can be described by a one letter code: G = unsubstituted glucose, X = α-1,6-xylosyl substituted G, L = β-1,2-galactosyl substituted X, and F = α-1,2-fucosyl substituted L.[35] High molecular weight xyloglucan polysaccharides of the type (XXXG)n were obtained when the oligosaccharide fluoride XXXGαF was incubated with *Humicola insolens* Cel7B E197S, the serine version of the *Hi*Cel7B E197A glycosynthase, which had allowed the preparation of various cellulose derivatives.[36] The serine mutant exhibited much higher reaction rates than the parent synthase, and polysaccharides with a molecular weight of mostly 15−40 kDa were obtained. Similar to the glycosynthase-catalyzed formation of artificial xylan polysaccharides, water-soluble and water-insoluble fractions were formed. While *Hi*Cel7B E197S was able to catalyze the polymerization of XXXGαF, galactosylated xyloglucans could not be generated, as XLLGαF was unable to serve as a substrate. The synthesis of high molecular weight galactosylated xyloglucans became possible when the highly xyloglucan-specific *endo*-glucanase *Paenibacillus pabuli* XG5 (*Pp*XG5) was mutated to function as a glycosynthase.[37] The *Pp*XG5 E323G mutant accepted XLLGαF both as a glycosyl donor and acceptor, catalyzing its polymerization into artificial polymers with a weight-average molecular weight of 12 kDa. The product was then enzymatically fucosylated to generate F side chains using *Arabidopsis thaliana* fucosyltransferase 1 (*At*FUT1). Whether the glycosynthase would also be able to accept XLFGαF as a substrate is unknown, as no XLFGαF-monomer could be prepared.

Mixed-linkage glucans are major hemicellulosic polysaccharides in cereals and grasses.[38,39] These linear glucans are composed of short heterogeneous stretches of β-1,4-linked oligosaccharides that are connected through β-1,3-linkages. The E134A glycosynthase version of *Bacillus licheniformis* (1→3, 1→4)-D-glucanase was shown to catalyze the polymerization of α-laminaribiosyl fluoride (G3GαF) into unique artificial glucans with regularly alternating β-1,3- and β-1,4-linkages.[40] The reaction products had an average DP of 12 according to methylation analysis. Analysis with light and electron microscopy indicated that products aggregated into porous spherulites. Later, the glycosynthase-catalyzed polymerization of α-laminaribiosyl fluoride was optimized to produce higher molecular weight products, and trisaccharidyl and tetrasaccharidyl fluorides, consisting of two or three β-1,4-linkages, respectively, and a β-1,3-linkage at the reducing end (G4G3GαF and G4G4G3GαF), were employed as starting materials.[41] In all cases, regular mixed-linkage glucan polysaccharides, with a β-1,3-linkage in every second, third, or fourth position of an otherwise β-1,4-linked glucan backbone were produced. The weight-average molecular weight of the products ranged from 9.5 kDa (dp 29) for (4G3G)n to 14.5 kDa (dp 30) for (4G4G3G)n to 12.9 kDa for (4G4G4G3G)n. Recently, the Planas laboratory reported that, in some cases, polymers with a slightly higher DP are obtained when a carbohydrate-binding module (CBM), recognizing the respective polymer, is added to the polymerization reaction.[42] The presence of a CBM may reduce precipitation of the higher molecular weight polysaccharides, thus promoting the temporary generation of an oversaturated solution. Similar effects were observed when a CBM was covalently attached to a glycosynthase via a short peptide linker. However, the observed effect was not very pronounced, probably reflecting the distrubutive mode of action by glycosynthases. Scanning electron microscopy (SEM) revealed the same spherulite morphology for (4G4G4G3G)n polymers as previously found for (4G3G)n. In contrast, a heterogeneous amorphous morphology was observed for (4G4G3G)n polymers, independent of sample preparation. The morphology of (4G4G3G)n was thus more similar to a natural barley mixed-linkage glucan, which contains mostly oligosaccharide stretches of 4G4G3G. It seems that higher molecular weight fibrillar structures, as found in nature, cannot be obtained by glycosynthase-catalyzed polymerizations.

Synthesis of β-1,3-Glucan. β-1,3-Glucans are major cell wall polysaccharides in algae, fungi, and some bacteria and act as potent immunomodulators with antibacterial and antiviral effects.[43] Depending on their origin, β-1,3-glucans are branched to different degrees with β-1,6-glucan side chains. Biological activity of these glucans strongly depends on the fine structures that can differ with respect to DP, degree of substitution, extent of branching, and conformation. However, β-glucans isolated from biological sources are usually heterogeneous mixtures, making it difficult to correlate structure with function. The

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E231G glycosynthase mutant of a barley endo-(1,3)-β-D-glucan endohydrolase was able to catalyze the polymerization of α-laminaribiosyl fluoride into a white flocculant precipitate of pure β-1,3-glucan with an average DP of 30.[44] Replacement of the interglycosidic oxygen in α-laminaribiosyl fluoride by sulfur still led to polymer formation, albeit much shorter polymers. SEM investigations of the insoluble polymerization products revealed mostly the formation of disks with diameters up to 5 μm. These disks were found to be hexagonally shaped platelets using TEM. When the TEM was run in the diffraction mode, electron diffraction patterns were recorded for selected areas of hexagonal platelets. The obtained diffraction pattern matched the crystal structure of anhydrous, triple helical β-1,3-glucan, as found in Curdlan, a bacterial β-1,3-glucan. The triple helices formed lamellar single crystallites while being packed perpendicularly to the plane of the respective hexagonal crystallite. The average thickness of the crystallites was found to be about 8 nm, which matches the expected lengths of a β-1,3-glucan triple helix with DP 28. A particular feature of the glycosynthase-catalyzed β-1,3-glucans was that a relatively narrow range of DPs was obtained, which could be explained by the processive mechanism exhibited by this particular glycosynthase. In this scenario, the glycosynthase only dissociates from the polysaccharide chain when a DP is reached where the β-1,3-glucans form triple helices and precipitate.

Synthesis of Chitin and β-Mannan Oligosaccharides. While impressive achievements have been made in the enzymatic synthesis of various glucans and xylans, most natural polysaccharides are still not available through glycosynthase technology. For chitin and β-mannans, glycosynthases have been reported to catalyze the formation of oligosaccharides but not polysaccharides. Mannan glycosynthase Man26A E320G derived from a *Cellvibrio japonicus* endomannanase efficiently catalyzed the reaction between α-mannobiosyl fluoride and *p*-nitrophenyl glucoside, but subsequent glycosylations of the resulting trisaccharide with further equivalents of α-mannobiosyl fluoride were found to be slow.[45] Unusually, the inverting chitin hydrolase from *Bryum coronatum* BcChi-A could be converted in a synthase by mutation of S102 to alanine.[46] However, this glycosynthase was only able to produce limited amounts of a chitin tetrasaccharide starting from chitobiosyl fluoride and chitobiose. In this case, chitin hydrolases are still superior in synthesizing long-chain chitooligosaccharides and artificial chitin derivatives.[47−49]

Generation of New Glycosynthases. While a great number of glycosynthases acting on different types of substrates is known, it is still highly desirable to generate further glycosynthases with different substrate scope and product profile. This task may be realized by converting new glycoside hydrolases into the respective glycosynthases by reengineering existing glycosynthases or by directed evolution.

Reengineering glycosynthases using site directed mutagenesis is most useful, if there is detailed knowledge on structure−function relationships of the parent hydrolase. With detailed knowledge of the catalytic machinery and residues involved in substrate binding, critical residues can be identified and subsequently changed by targeted mutagenesis to improve transglycosylation activity. Recently, it was shown that the site-directed mutagenesis of Endo-S, a GH able to remove the complex branched *N*-glycans from antibodies, can improve the transglycosylation activity and influence the affinity toward the targeted glycan.[50] However, systematic mutagenesis to modify substrate specificities is challenging, and respective studies are limited. Davis and co-workers have targeted residues forming the −1 subsite in *Sulfolobus solfactarius* β-glycosidase glycosidase to broaden the substrate scope.[51] Through structure-based site-directed mutagenesis, in combination with knowledge of the proposed transition state geometry, it was possible to generate a synthase that is not only able to efficiently synthesize glucosides but also mannosides and xylosides.

A combination of structure-based mutagenesis with enzymatic characterization of the generated mutants was used to unravel the role of selected substrate binding sites in the GH18 chitinases A from *Serratia macerans* and D from *S. proteamacula,* showing that manipulation of the transition state geometry as well as the hydrophobicity of the acceptor subsites have a particularly strong effect on the transglycosylation to the hydrolysis ratio.[52] An impressive reengineering approach was applied by Iglesias-Fernandez et al. to change the catalytic mechanism of an GH1 hydrolase from SN_2 to SN_i, thereby avoiding the need to use α-configured fluorides for the respective synthase reaction.[53] Inexpensive, often commercially available β-configured *p*NP donors were used, leading to β-configured products due to the engineered front face SN_i-like mechanism.

Though impressive progress has been made to understand the underlying determinants for creation of efficient glycosynthases with desired selectivity and specificity, the challenge remains to paint a comprehensive picture for each enzyme and the specific roles of selected residues in the catalytic cycle. In particular, transition state stabilization, which determines substrate specificity, is of fundamental importance. Furthermore, the role of second and higher shell residues around the immediate active site in the catalytic cycle is often discovered serendipitously and poorly understood. For the efficient synthesis of larger oligosaccharides and polysaccharides, striking the right balance between affinity and specificity for the carbohydrate acceptor, while keeping enough flexibility for efficient product release, remains a major challenge.

A more common method to create and redesign glycosynthases is directed evolution. Key to this method is the high-throughput screening of hundreds of mutants to identify variants with improved activity. The advantage of this method is that detailed knowledge of the parent hydrolase is not required. Initially, success was limited due the need for specific activity assays for every glycosynthase. For example, a coupled enzyme assay was developed for the directed evolution of a β-glucosidase from *Agrobacterium sp.*, coupling the glucosidase with a cellulase (Cel5a). If the glucosidase shows synthetic activity, a chromogenic substrate for Cel5a is produced and subsequently cleaved to release the chromophore. The glycosynthase library was generated with error prone PCR, and initially, 10,000 clones were tested, with the best variants undergoing further rounds of error prone PCR, leading to a glycosynthase variant with 500% increased activity and a broader substrate scope.[54] Chemical complementation was used to improve a glycosynthase based on *Humicola insolens* Cel7B.[55] The authors developed a system to couple glycosynthase activity to a phenotypic outcome via a genetic trigger. In that case, bacteria harboring an active glycosynthase are able to grow on leucine deficient media. The drawback of this method is that every complementation substrate has to be synthesized and optimized for each glycosynthase under investigation. To overcome the need for specific assays for every newly developed glycosynthase, efforts have been made to detect the release of fluoride in a high throughput assay. Ben-David et al. used this approach to screen

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for improved variants of the xylan glycosynthase XynB2 (E335G).[56] A change in pH, resulting from the formed hydrofluoric acid during consumption of the fluorinated donor, was used to screen a library of more than 10,000 mutants. The Planas group developed a fluorescence-based method to detect the release of fluoride using 4-methylumbelliferyl t-butyldimethylsilylether (MUTBS) as a detection agent.[57] Upon fluoride release, the sensor is cleaved, and 4-methylumbelliferone (4-MU) is released. This method was used to identify and characterize a glycosynthase based on the β-1,3- and β-1,4-glucanase from *Bacillus licheniformis*. New fluoride-sensitive probes or dyes may allow one to directly screen colonies more efficiently without current limitations, which would greatly increase the number of potential variants that could be screened.

The number of enzymes and enzyme families populating the Carbohydrate-Active enZYmes Database (CAZy) continues to grow at a rapid rate. This offers an opportunity to select new GHs for conversion into glycosynthases that allow the synthesis of new glycans. However, mechanisms of the parental hydrolases must be characterized in detail before the catalytic nucleophile can be replaced by a non-nucleophilic residue. Complex multilinkage substrates, such as glycogen, are degraded by enzymes composed of multiple catalytic domains. These are interesting engineering targets to produce complex carbohydrates with natural branching patterns in one-pot reactions. Furthermore, a system based on cellulosome-type multiactivity enzyme systems, might lead to an artificial "synthasosome" for complex plant cell wall polymers. In general, hydrolases with inherent transglycosylation activity are expected to provide better synthases, due to naturally primed acceptor subsites with higher affinity for carbohydrates compared to water. To overcome solubility issues, often observed with polymeric substrates, hybrid constructs with carbohydrate binding modules might help to keep longer structures in solution and increase the contact time of the enzyme with the newly synthesized polymeric substrate for efficient catalysis.[40] Acidic carbohydrate polymers, such as pectin, can be cleaved by polysaccharide lyases using a β-elimination mechanism. It remains to be seen if the glycosynthase concept can be expanded to this class of carbohydrate active enzymes

ENZYMATIC POLYSACCHARIDE SYNTHESIS WITH PHOSPHORYLASES AND SUCRASES

Concept and Mechanisms. Owing to the growing demand for green synthetic processes based on renewable resources such as starch, cellulose, and sucrose, research on the synthesis of carbohydrate-based natural and nature-inspired materials has attracted considerable attention during the last decades.[58] Starch is the most diversified raw material in industry. The starch component that draws the most interest due to its biotechnological potential is amylose, with its linear backbone composed of α-1,4-linked glucopyranosyl units able to adopt double helical organizations and higher hierarchical structures.[59] Furthermore, the presence of suitable hydrophobic "guest molecules" such as fatty acids, alcohols, and synthetic polymers triggers the formation of single-stranded helical inclusion complexes, a property exploited in the formulation of biocompatible amylose-based delivery carriers and self-assembly structures.[59,60] The precise *in vitro* synthesis of amylose and its derivatives is feasible by means of enzymatic and chemoenzymatic routes often including α-glucan-phosphorylases from the glycosyltransferase family 35 (GT35) coupled to disaccharide phosphorylases from the glycoside hydrolase families 13 and 94 (GH13 and GH94), which provide the donor α-D-glucose 1-phosphate (Glc-1-P).[61] Phosphorylases occupy a central role in the metabolism of storage polysaccharides and are ubiquitous in all kingdoms of life. α-Glucan phosphorylases catalyze the release of Glc-1-P from glycogen, starch, and maltodextrins, as well as the reversible phosphorolysis of α-1,4-linked polysaccharides.[62] Both reactions are at equilibrium as the free energy released by the cleavage of the glycosidic bond in the saccharide chain is similar to the energy liberated by the cleavage of the phosphate ester product.[63] The reaction involves the covalently bound cofactor, pyridoxal 5′-phosphate, and depending on the direction of the reaction, the presence of either inorganic phosphate (Pi) or Glc-1-P.[62] Under these conditions, GT35 enzymes perform the breakdown of glycosidic linkages at the nonreducing end of the saccharide or the reverse phosphorolysis, leading to glucan synthesis in a primer-dependent polymerization reaction that requires maltooligosaccharide acceptors with dp $\geq$ 3.[64] The feasibility of synthesizing amylose with phosphorylases was recognized decades ago; however, the role of starch phosphorylases in higher plants remained unclear until recently when the starch plastidial phosphorylase (Pho1) was found to play a crucial role in the initiation of starch synthesis in developing rice seeds.[65] In bacteria, α-glucan phosphorylases facilitate the degradation of glycogen and maltodextrins formed in the course of maltose metabolism.[66]

Some microorganisms and plants utilize nonreducing fructose polysaccharides (fructans) and other α-glucans instead of starch or glycogen as carbohydrate reserves. For example, the fructans inulin and levan have backbone chains linked through β-2,1 and β-2,6 bonds, respectively, and serve as carbohydrate reserves in roughly 15% of flowering plant species.[67] In addition to their role as storage carbohydrates, fructans potentially play a role in stress tolerance, participate in biofilm formation, and act as virulence factors or signaling molecules.[68,69] Fructan synthesizing and/or degrading enzymes are found in all three domains of life and include invertases/fructofuranosidases and fructansucrases from GH32 and GH68 (clan GH-J).[67,68,70] In a similar manner to fructansucrases, glucansucrases (GH70 and some members of GH13) employ sucrose to synthesize branched and linear α-glucans with various linkage types and diverse physicochemical properties. GH70 sucrases are exclusively expressed by some biofilm-forming genera of lactic acid bacteria.[71]

Sucrases catalyze hydrolysis and transglycosylation through a double displacement reaction and employ an aspartate as the catalytic nucleophile/base, a glutamic acid as the proton donor and transition state stabilizing residue. Upon sucrose binding in the subsites -1 and $+1$, the cleavage of the glycosidic linkage results in a covalent β-glucosyl- or α-fructosyl-enzyme intermediate, with the stereogenic center of the monosaccharides inverted with respect to their configuration in the donor. The retaining mechanism further proceeds through a second transition state and the nucleophilic attack of the acceptor substrate onto the covalent glycosyl-enzyme intermediate to yield a glycosylated product.[72,73] Sucrases are naturally promiscuous in relation to the type of acceptor molecules that they recognize, permitting the glycosylation of various saccharides and aglycone substrates.[74−76]

Synthesis of Amylose, Derivatives, and Copolymers. In most crops, starch granules are composed of approximately 20%−30% amylose, which is embedded in the lamellae of amylopectin, a highly branched α-1,4-linked glucan with α-1,6-

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branching points that constitutes about 70%−80% of the saccharide content.[77] Amylose-rich resistant starch escapes digestion in the small intestine owing to its packed structure, and therefore, much work is focused on the development of high-amylose crops with potentially unique functional properties such as protection against diabetes, colon cancer, and hepatic diseases.[78] Biosynthesis of starch is a complex process in terms of the enzymatic and regulatory elements involved.[79] The plastidial pathway of starch synthesis is conserved among higher plants, and therefore, the participating enzymes are proposed to share a common origin.[80]

Extraction of amylose from starch via aqueous leaching[81] or fractionation[82] produces heterogeneous amylose chains. Application of multienzymatic processes that utilize potato or thermostable GT35 phosphorylases, Glc-1-P, and auxiliary carbohydrate processing enzymes permits the practical preparation of customizable amylose from various glucosyl donors, such as sucrose, cellobiose, and solid cellulose.[61,83,84] By this means, size-defined amylose with a low polydispersity index (a ratio of Mw to Mn close to 1.0) is attainable in reactions containing a controlled ratio of Glc-1-P to primer.[61]

The design of block and graft copolymers as well as heteropolysaccharides is a frequent strategy to bypass the limitations imposed by the intrinsic physicochemical properties and/or supramolecular organization of natural polymers.[85−87] The possibilities to generate amylose derivatives are quite extensive and rely on the appropriate choice of primers, auxiliary enzymes, hybrid counterpart, and set of chemical tools. For example, functionalized maltooligosaccharide primers have been used in the design of polytetrahydrofuran-*b*-amylose or poly(2-vinylpyridine)-*b*-amylose block copolymers with the capability of self-organizing,[86,87] and maltopentaose-based functional PEG derivatives synthesized via copper(I)-catalyzed Huisgen cycloaddition were used to produce spherical branched ("star") polymers with glucose or glucosamine at the nonreducing ends.[59] Star polymers act as supramolecular multivalent hosts for guests of varied functionality such as chromophores, dyes, and lipids and function as chaperones to assist DNA strand exchange. As amylose-based surfactants can be internalized into living cells they are proposed as an alternative to often toxic and unstable carriers, i.e., liposomes, inorganic nanoparticles, and nanogels.[59,83] Block copolymers are also attainable by multienzymatic synthesis combining various polymerizing activities. Flexible block polymers alternan-*b*-amylose and dextran-*b*-alternan-*b*-amylose obtained by the joint action of glucansucrases and phosphorylases are examples of such syntheses.[85]

Synthesis of α-Glucans and Fructans with Sucrases. Sucrases are employed for the synthesis of industrially relevant biodegradable and biocompatible oligosaccharides and polysaccharides. As such, fructans and glucans can be used as substrates for tissue engineering and for the formulation of functional nanomaterials.

All fructans are produced by structurally and mechanistically similar clan GH-J enzymes. Their structure consists of a catalytic N-terminal 5-fold β-propeller, with some members displaying an additional β-sandwich domain in the C-terminal region that serves carbohydrate binding and/or folding, solubility, and stability functions.[73,88,89] In spite of intensive investigation, molecular determinants responsible for the regioselectivity, transglycosylation/hydrolysis partition, and molecular weight of products by GH-J enzymes remain elusive. Microbial enzymes from clan GH-J undertake the production of extracellular fructooligosaccharides with a DP of 2−20 and/or large fructans with a DP > 50,000 and variable degree of branching.[90] On the basis of structural and mutagenesis studies on microbial fructansucrases, the synthesis of β-2,1- and β-2,6-linked fructans (levan and inulin, respectively) is anticipated to follow distinct elongation pathways, with specific structural elements being able to preferentially stabilize the binding of inulin- or levan-type oligosaccharides.[91−93] Three factors are proposed to contribute to higher transglycosylation activities in GH-J enzymes: (i) a long-lived covalent enzyme-fructose intermediate which could be better disrupted by a saccharide acceptor than by water, (ii) an impaired positioning of the hydrolytic water with respect to the enzyme's covalently linked fructose, and (iii) an increased affinity for saccharide acceptors in the positive subsites.[72,94]

In the course of fructans synthesis, various oligosaccharide and polysaccharide series are generated, as revealed for the levansucrase from *Bacillus subtilis*.[95] Protein topology in combination with fine-tuned enzyme-carbohydrate contacts are likely responsible for the capacity of bacterial fructansucrases to synthesize either oligosaccharides or polymers.[90,96] This knowledge has been used in the design of enzyme variants with diverse synthetic properties. Position-dependent tyrosine modification of the levansucrase from *B. megaterium* either caused the enrichment of short oligosaccharides—up to 800% in some cases—or triggered the formation of high molecular weight polymers by preventing premature enzyme-oligosaccharide disengagement events.[97,98]

Linear and branched α-glucans containing all possible glycosidic linkages are multifunctional sucrose-derived biopolymers that are easily accessed by the combined action of extracellular glucansucrases from families GH70 and GH13. GH70 glucansucrases have been identified in the genera *Leuconostoc*, *Streptococcus*, *Lactobacillus*, and *Weissella*, and on the basis of their product specificity, they are categorized as glucan and branching sucrases, as well as α-4,6 and α-4,3 glucanotransferases. Glucansucrases are further classified in accordance to the backbone linkage of their products, which are known as dextran (α-1,6), mutan (α-1,3), alternan (alternating α-1,3/α-1,6), reuteran (α-1,4/α-1,6), and amylose (α-1,4). Amylosucrases are glucansucrases belonging to GH13 that generate linear amylose-like polymers without the need of carbohydrate primers. They display a broad acceptor promiscuity that includes various saccharides as glycogen and starch, as well as alcohols and flavonoids.[99,100] Amylosucrases are proposed to participate in energy storage and possibly in sucrose catabolism.[101] As glucan-phosphorylases, amylosucrases have been used to prepare self-assembled amylose-based systems, as amylose-single-walled carbon nanotubes (SWCNTs)[102,103] or matrices for immobilization of antibodies.[104]

Lactobacilli "classical" GH70 enzymes likely evolved from GH13 α-amylases in a diet-driven process via the newly discovered GH70 subfamilies that use α-1,4-glucans as substrates instead of sucrose.[105] The peptide chain of GH70 enzymes follows a U-shaped path creating domains A, B, C, IV, and V, which are formed by two discontinuous segments from the N- and C-termini, except for domain C. Domains A, B, and C are common to GH13 enzymes, while domains IV and V are only found in family GH70. Catalytic domain A in GH70 sucrases contains an α-amylase like (β/α)8 barrel that is circularly permuted compared to GH13 and GH77 enzymes[71,106] (Figure 5). The linkage specificity of glucansucrases—in particular from *Lactobacillus reuteri*, *Streptococcus*

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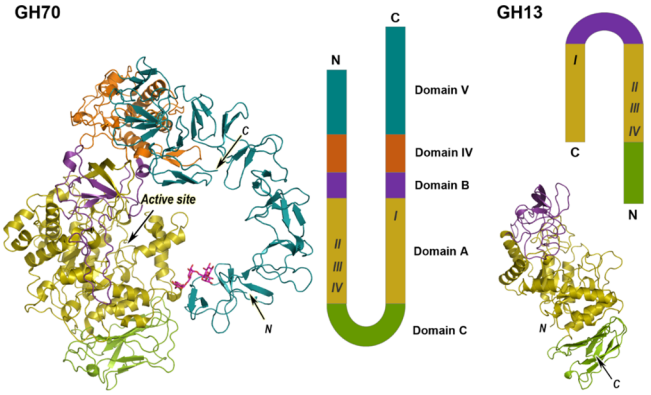

Figure 5. Structural organization of two representative enzymes from families GH70 and GH13. Crystal structures of the dextransucrase DSR-M from *L. citreum* (GH70, PDB ID 5ngy) and the α-amylase from *Bacillus licheniformis* (GH13, PDB ID 1bli). The domain organization of each enzyme is also displayed, showing the circular permutation that results in a different order of the conserved motifs I–IV localized in domain A. These motifs contain the catalytic triad and other substrate binding amino acids.

oralis, and *Leuconostoc citreum*—has been a matter of intense study, discovering that the interplay of acceptor substrates between the acceptor binding sites +2 and +3 defines the alternating α-1,3/α-1,6 specificity.[107−110] The regioselectivity of glucansucrases has also been guided by substrate engineering approaches.[111] Although it is known that the highly flexible domain V participates in polymer elongation, its total or partial deletion affects the molecular weight of α-glucans and glucansucrases' activity in an enzyme-dependent fashion, and thus, general conclusions cannot be drawn.[106,112] Aside from domain V, other structural elements have been found to influence the size of α-glucans. In the distributive glucansucrase from *L. citreum*, which elongates oligodextrans regardless of their length, the mutation of a residue serving as an anchoring point for polymer elongation located between domains A and B influences the dynamics of the active site and water accessibility, resulting in the synthesis of shorter products.[112] Because of the strong potential of α-glucans for industrial applications, the accelerated pace of research concerning the missing aspects of the catalysis of glucansucrases is not expected to slow down in the near future.

ENZYMATIC POLYSACCHARIDE SYNTHESIS WITH GLYCOSYLTRASFERASES

Concept and Mechanisms. Understanding the molecular machinery diverse organisms utilize to synthesize glycopolymers brings us closer to designing polymers with tailored functionalities for the intended use both *in vivo* and *in vitro*. In nature, polysaccharides are almost exclusively formed through the action of Leloir GTs, which catalyze the transfer of a sugar moiety from an activated nucleotide sugar donor (NDP-sugar) to a wide range of saccharide and nonsaccharide acceptors.[2,113] Similar to GHs, glycosyltransfer reactions occur through the action of inverting or retaining GTs. For example, inverting enzymes, such as the GT2 β-1,4 glucan synthases involved in cellulose synthesis (CES), transfer Glc from UDP-α-D-glucose onto a glucan acceptor forming a β-linked product, β-1,4-glucan. The majority of inverting GTs characterized to date operate via an S_N2 reaction mechanism through a single displacement reaction, where the nucleophile is one of the hydroxyl groups of the saccharide acceptor and the leaving group is the nucleotide, and often but not always employ an Asp, Glu, or His as a catalytic base.[113,114] In contrast to that of inverting GTs, the mechanism(s) used by retaining enzymes remains enigmatic and has been recently reviewed.[115] Currently, it is thought that these reactions proceed through a front-face or S_Ni (substitution nucleophilic internal)-like mechanism where the action takes place in the "front face" of the sugar for retaining GTs lacking a nucleophile.[114,115]

Like GHs, GTs are classified on the basis of sequence similarity in the CAZy database.[17] At the time of publication, there were 107 GT families (GT1-GT107), comprising 560,939 classified enzyme modules with more than 11,000 additional modules that are annotated as "Non Classified" (GTnc). Structural analysis has shown that their catalytic domains can be categorized into three general three-dimensional (3-D) fold classes: GT-A, GT-B, and GT-C. The smallest of the fold families is represented by GT-C enzymes, integral membrane proteins which often use lipid-linked sugar donors. However, GT-Cs have not been widely characterized, so they are not discussed herein. The majority of GTs with GT-A and GT-B folds are Leloir GTs and almost exclusively use activated nucleotide sugars as donor substrates, with a few exceptions.[2] GT-A enzymes have a single Rossman-like domain, are almost always metal-ion dependent, and have been found to possess a consensus DXD motif that functions to coordinate divalent cations, often Mg^{2+} or Mn^{2+}. In contrast, the GT-B glycosyltransferases are composed of two Rossman-like domains, do not possess a DXD motif, and are therefore almost always metal-ion independent.

Synthesis of Cellulose and Callose. The potential of cellulose as a biomaterial has long been realized due to its unique structural properties such as high tensile strength and rigidity. Notably, intrachain and interchain hydrogen bonding forms stable two-dimensional sheets of aligned glucans, and van der Waal interactions promote intersheet coalescence.[116,117] Synthetically produced cellulose, while likely unable to compete on a cost basis with the abundance of available natural cellulose, may find uses in specialty applications due to the potential for controlling crystallinity or the addition of specific side chains or functional groups/custom sugars that can influence material properties or be used as chemical handles, for example, to produce high-value products such as biocompatible, organic drug delivery chassis. Furthermore, the chemical processes required for the purification of cellulose from composite plant materials present limitations for producing cellulose with desired variations in structure and size. Cellulose produced by bacteria and plants is referred to as cellulose I based on spectroscopic and scattering characteristics that distinguish this highly crystalline, recalcitrant form of cellulose from other allomorphs that result from modification by mercerization and/or regeneration (cellulose II), ammonia treatment (cellulose III), and thermal treatment (cellulose IV).[118] A major obstacle in utilizing cellulose I for materials is purification of native cellulose from complex matrices while retaining a native allomorph. Currently, the two most prevalent cellulose I-derived materials are cellulose nanocrystals (CNCs) and cellulose nanofibrils (CNFs).[119] CNCs can be isolated from plant cell walls by strong acid hydrolysis to produce needle-like crystals several hundred nanometers in length. In contrast, CNFs are longer fibrillar networks that can be produced through various chemical, physical, and enzymatic means but are highly variable in morphology and less crystalline than CNCs.[119] Studies of

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these two types of nanocellulose in poly(ethylene oxide) nanocomposites have shown that CNCs have lower strength and modulus than CNFs but higher strain-at-failure, differences that were attributed to the morphology of the nanocelluloses.[119] Although nanocelluloses are proving to be a useful tool for materials research and applications, the ability to tailor the properties of cellulose would greatly expand the utility of cellulosic resources.

Biological restrictions including processivity and entanglement in complex matrices limit the morphology and accessibility of cellulosic resources from biological sources. For these reasons, a great deal of effort has been dedicated to understanding cellulose biosynthesis using various model organisms. Early studies of cellulose biosynthesis were focused on *in vivo* synthesis of ribbons of cellulose I in the bacterium *Komagataeibacter xylinus*,[120] formally known as *Acetobacter xylinum*.[121] In general, cellulose synthases are GT2 family integral membrane proteins that utilize UDP-glucose as the substrate to processively produce a glucan chain that is extruded through a proteinaceous pore before coalescence with other glucan chains.[122] Bacterial cellulose synthases are embedded in the cytoplasmic plasma membrane,[123] and extrude glucan chains through the periplasmic space and outer membrane into the extracellular space where ribbons of approximately 46 glucan chains coalesce to form cellulose I.[120,124] Interestingly, when the cytoplasmic membranes of *K. xylinus* are isolated and used for *ex vivo* cellulose synthesis, cellulose II is produced, indicating that components other than cellulose synthases *per se* are required for production of cellulose I.[123,125,126]

Biocatalytically synthesized cellulose has wide-ranging potential applications. *In vitro* cellulose production was first demonstrated by expressing *Rhodobacter sphaeroides* bacterial cellulose synthase (Bcs) complex proteins, BcsA and BcsB, in *E. coli*.[127] However, the dp of the cellulose produced by BcsA-B was limited to 200−300 glucose molecules, potentially limiting use for material production.[127] Structural characterization of the BcsA-B complex has proven to be instrumental in understanding the molecular-level controls of bacterial cellulose biosynthesis, including activation of BcsA by cyclic di-GMP,[128] glucose transfer onto the growing glucan chain,[129] membrane translocation by the glucan chain,[129,130] and transition states during cellulose biosynthesis.[128−130] Furthermore, these works provide the foundation for understanding structure−function relationships relevant to β-1,4-glucan synthesis by GT2 family enzymes with the potential to optimize reaction conditions to increase cellulose I yields. Biocatalytic modification of cellulose is also a promising avenue for materials research, as exemplified by the recent discovery of phosphoethanolamine cellulose made by uropathogenic *E. coli* by the BcsG enzyme.[131] Phosphoethanolamine cellulose is a component of biofilms that confers adhesive properties to bladder epithelial cells and is a primary determinant of pathogenicity,[132] demonstrating how cellulose modifications can influence properties of biologically active composites, such as biofilms.

Plant cellulose synthesis can also be achieved *ex vivo*. To date, *ex vivo* cellulose biosynthesis has been demonstrated with detergent-solubilized extracts from tobacco suspension culture cells,[133] cotton (*Gossypium hirsutum*),[134,135] Italian ryegrass (*Lolium multiflorum*),[136] mung bean (*Vigna radiata*),[137] blackberry (*Rubus fruticosus*),[138] and hybrid aspen (*Populus tremula* X *tremuloides*).[139] However, GT2 plant cellulose synthase (CESA) activity was always accompanied by relatively higher levels of callose (β-1,3-glucopyranose) synthesis. Callose synthases are multimembrane-spanning GT48 family proteins that also use UDP-glucose as a substrate.[140] Together, these studies indicate that enzyme purity is a limiting factor in plant-derived *ex vivo* cellulose production, much like how the presence of matrix polysaccharides complicates cellulose isolation form native plant cell walls. *In vitro* cellulose biosynthesis by heterologously expressed and purified plant CESAs has been achieved with single CESAs from hybrid aspen (PttCESA8),[141] the moss *Physcomitrella patens* (PpCESA5),[142] and six CESAs from bamboo (*Bambusa oldhamii*).[143] Production of functional plant CESAs requires expression in a eukaryotic organism such as yeast, as opposed to bacterial expression.[141−143] Eukaryotic expression systems are favorable for plant protein expression likely due to compatible translational mechanisms and post-translational modifications.[144] However, purification of active CESAs is challenging, requiring detergent solubilization, affinity purification, chromatographic separation, and reconstitution into proteoliposomes,[141−143] thereby limiting the scale at which pure CESAs are isolated. Additionally, the physical and mechanical properties of cellulosic products from recombinant plant CESAs are not well characterized, although the products from PttCESA8 are partially resistant to acid hydrolysis suggesting coalescence of glucans into crystals.[141] Thus, *in vitro* cellulose synthesis is possible with bacterial and plant enzymes, but there is a need to increase scale, efficiency of enzyme purification, and yield before heterologous expression and *in vitro* systems ameliorate capabilities of cellulose I isolation.

Synthesis of Plant Matrix Polysaccharides. Matrix polysaccharides present an attractive target for enzymatic synthesis and modification due to the intrinsic nature of these polymers. For one, plant cell wall matrix polysaccharides are more easily extracted from cellulosic plant cell wall components and upon extraction are typically soluble in aqueous solutions. This enables the use of enzymatic technologies to create or modify these large and often heterogeneous polysaccharides *in vitro*. Furthermore, unlike cellulosic materials, matrix polysaccharides are almost always decorated by both glycosyl and nonglycosyl substituents. The structure, abundance, and patterning of these substituents often dictate the formation and nature of polymer networks and thus influence the material properties of polymer solutions and composites. At present, many of the enzymes responsible for synthesizing the backbone and side chain components of matrix polysaccharides have been discovered and characterized, some as purified recombinant proteins with demonstrated *in vitro* activity as reviewed by Amos and Mohnen.[145] Here, we discuss the potential application of matrix polysaccharide biosynthetic enzymes toward the synthesis of inspired materials with favorable properties. For organizational purposes, we arbitrarily group these enzymes into two categories: main chain synthases and side chain transferases.

It comes perhaps as no surprise that many of the matrix polysaccharides which bear a backbone similar to that of cellulose, such as xyloglucan, mannans, and mixed-linkage glucans, are synthesized by enzymes which structurally resemble the CESAs from GT2. Members of this family are classical type 1 integral transmembrane proteins and as a result are currently difficult to use *in vitro*, similar to cellulose synthases. However, recent work has endeavored to synthesize polysaccharides using GT2 enzymes. Xyloglucan backbone synthesis has been demonstrated using recombinant enzyme expression in yeast cells, and recent work has also demonstrated a yeast-based system for the successful production of both mannan and

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glucomannan chains.[146] Mixed-linkage glucans are also synthesized by GT2 family enzymes and are of particular interest due to their ability to synthesize long, water-soluble polysaccharide chains that do not require side groups to maintain solubility, due to the presence of regularly spaced β-1,3-glucan linkages within the main chain. The processive nature of GT2 enzymes makes them an alluring target for the production of long polysaccharide chains which may be necessary in materials pursuits but face many challenges due to their more complex *in vitro* requirements.

Perhaps currently of more utility for the *in vitro* synthesis of polysaccharide main chains are those which are synthesized by type II transmembrane enzymes found within the lumen of golgi and ER bodies and are responsible for the synthesis of polymers such as xylans and pectic polysaccharides. Backbone synthesis for homogalacturonan (galacturonosyltransferases, GAUTs),[147] galactan (galactan synthase, GalS),[148] and xylan (xylan synthase, XYS)[149] have been characterized using heterologously expressed enzymes. Intriguingly, these enzymes can all function in a distributive mechanism to extend short oligosaccharide acceptors and form polymeric products, though in some cases such as for GAUT1 the enzymes may have an affinity for longer acceptors.[150] The potential uses of these enzymes in material science are substantial, as one could envision applying systems in which oligosaccharides derived from what are often considered waste streams of industrial processes can be used as low cost material or acceptors that can be biocatalytically polymerized in a controlled manner to reform usable, structurally defined polymers.

For many applications, the best use of enzymatic technologies for glycopolymer production may not be reliant on the synthesis of a main linear backbone chain but instead as a way to site selectively decorate the backbone with glycosyl and nonglycosyl substituents. The biosynthetic pathway of the complex hemicellulose xyloglucan is one of the most well characterized. Nearly the entire suite of enzymes responsible for the synthesis of the side chains of xyloglucan have been determined either through genetic or recombinant approaches (Figure 6), with some enzymes such as XYLOGLUCAN XYLOSYLTRANSFERASE1 (XXT1) and FUCOSYLTRANSFERASE1 (FUT1) prompting in depth structural studies of crystal structures.[151,152] Strikingly, many of these enzymes display a strict regiospecificity for particular glycan structures, suggesting that they may be useful tools for the controlled synthesis of fine structural features which can be difficult to achieve by conventional chemical synthesis methods.

Much like for the xyloglucan biosynthetic pathway, most of the enzymatic players involved in xylan decoration have emerged in recent years. Groups have now successfully demonstrated the recombinant *in vitro* activity of xylan glucuronosyltransferases (GUXs; named for GlcA sUbstitution of Xylan).[153] The spacing and abundance of these glycosyl modifications have been suggested to influence xylan morphology and ability to interact with cellulose, considerations with great importance for future use in composite materials.[154,155] Aside from those enzymes responsible for the glycosidic modifications, enzymes responsible for the methylation, acetylation, and sulfation of polysaccharides have also been characterized for many polymers. Methyltransferases belonging to the Domain of Unknown Function (DUF) 579 family have been shown to catalyze the formation of 4-O-methyl ethers on glucuronic acid side chains of xylans and arabinogalactan polysaccharides.[156,157] Systems for the *in vitro* acetylation of

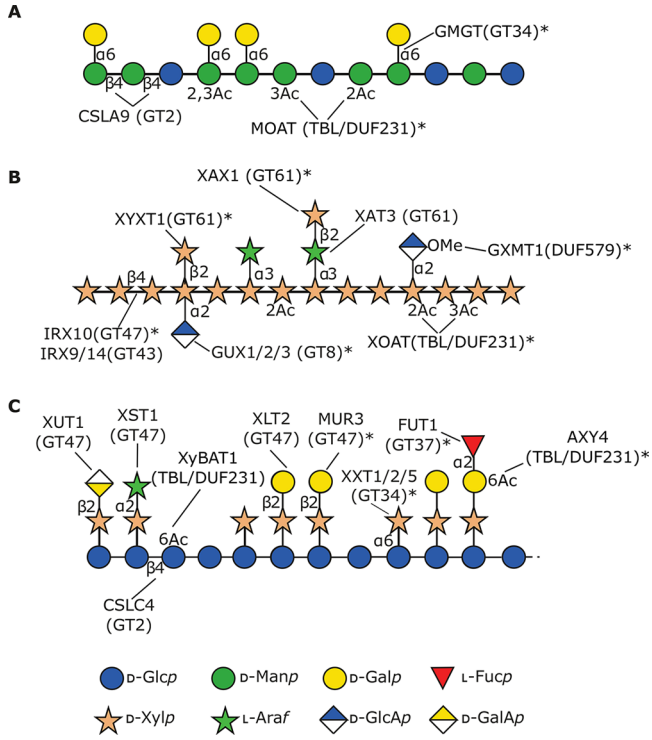

Figure 6. Enzymes with known roles in the biosynthesis of galactoglucomannan (A), xylan (B), and xyloglucan (C). The presence of an asterisk next to the enzyme name denotes that the enzyme has demonstrated *in vitro* activity. The structures used here are meant as models and do not necessarily represent the full extent of enzymes involved in polymer synthesis or accurate structures found within plant biomass.

xylans,[149] mannans,[158] and xyloglucans[159] have been described and may hold great promise for the site specific acetylation of polysaccharides. In addition, acetylation has been suggested to influence the spacing and abundance of other polymer modifications, such as the patterning of xyloglucan xylosyl residues[160] and the spacing of glucuronosyl residues in xylan.[155] Recently, the first structure of a plant polysaccharide acetyltransferase has been solved, lending insight into their specificity and mechanism of action.[161] These results suggest that the enzymatic synthesis of structurally well-defined polymers may rely on the use of multiple enzymes in a stepwise fashion to maintain correct patterning.

Synthesis of Glycosaminoglycans. Other carbohydrate-based polymers, such as glycosaminoglycans (GAGs), are also experiencing a renaissance in strategies for the chemoenzymatic synthesis of the glycan backbone. Main chain elongation of most GAG polysaccharides *in vivo* is performed by a bifunctional enzyme capable of transferring both the acidic and *N*-acetyl component of the alternating disaccharide backbone. Many groups have pursued bioengineering strategies for the enzymatic production of these valuable polysaccharides, and progress in this field has recently been reviewed.[162,163]

Sulfotransferases responsible for the modification of GAGs have been the subject of intense interest due to the importance of these groups on GAG bioactivity and pharmacology. The enzymes responsible for this modification, referred to as *N*- or *O*-sulfotransferases (NST and OST, respectively), are often highly specific and therefore very useful in creating defined structures while avoiding unwanted side products. Many of these enzymes have been successfully expressed as recombinant proteins and

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applied to synthetic GAG production.[162] In addition to sulfation, some GAGs such as heparan, heparin sulfate, and dermatan sulfate require epimerases for the conversion of backbone GlcA residues to the C5 epimer, L-iduronic acid. The enzyme responsible for this conversion has been successfully cloned and characterized in bacterial expression systems,[164] with results suggesting that structural features of the substrate, such as the sulfation profile of neighboring residues, play important parts in substrate recognition.[165] Taken together, these studies again suggest that systems employing tightly controlled enzymatic cascades can be used for the creation of polysaccharides with well-defined decoration patterns which can confer biological function as bioactive components of materials and medical devices.

PERSPECTIVES FOR MATERIALS SCIENCE

Plastic production at a large scale only began in the 1950s and continues to grow globally. The disposal and persistence of nonbiodegradable polymers and single-use plastics generate huge environmental problems.[166] It was estimated that only 9% of plastic is recycled globally as of 2015, while landfill disposal (~79% of plastic) results in the retention of large amounts of materials in the environment that are harmful to human and animal health, and their incineration (~12% of plastic) produces airborne particulates and greenhouse gas emissions.[166] Waste mismanagement, typically on land, has also led to extensive release and persistence of microplastics, mesoplastics, and macroplastics into the marine environment.[167] The use of biodegradable biopolymers, such as biomass-derived polysaccharides, is an attractive alternative to fossil fuel-derived plastics, but issues associated with their mechanical performance and their price have to be overcome. Furthermore, our limited knowledge of the heterogeneous structures and the properties of biomass-derived polymers hampers improvements in their processing and a more rational design of final products. Polymer production in microorganisms or by *in vitro* synthesis with expressed enzymes allows full control over their structure and ultimately their physicochemical properties. A prerequisite, however, is scalable and efficient enzyme production. The rapid advancements in our ability to gain molecular-level insights into their mechanisms of action and substrate interactions facilitate computational approaches to engineer them for improved or novel function and versatility as biocatalysts. However, the successful application of these enzymes to biomaterials pursuits still relies on the advancement of a number of factors for affordable and scalable production.

Glycosyltransferases hold great potential for biocatalytic synthesis of specifically structured polymers; however, perhaps of most concern at present for enzymatically catalyzed synthesis of biomaterials is the high costs of donor nucleotide sugars. Even the most affordable of these sugars, UDP-glucose, typically retails for greater than $100 per gram, making it cost prohibitive for any form of large-scale synthesis, while other more specialty sugar nucleotides such as UDP-xylose, UDP-galactose, and UDP-GlcA can be orders of magnitude more expensive. Efforts of many groups have sought to provide solutions for these cost barriers by designing enzymatic cascade systems or semisynthetic methods which mimic *in vivo* metabolic pathways for the *in vitro* or fermentative production of large quantities of sugar nucleotides. However, given these current cost constraints, it is likely that only high value products, such as medical or specialty items, will have economic viability in a free market system. It may also be a better strategy to utilize enzymes for the site-specific modification of polysaccharides derived from affordable sources, such as from agricultural or industrial waste streams. Numerous studies have enumerated the effects of small structural modifications on polymer characteristics such as rheology and polymer−polymer interactions. Perhaps targeting these special modifications with the enzymes described above, in concert with other synthetic techniques, can deliver well-defined, cost-effective products not achievable by either method alone.

The possibilities for carbohydrate biotransformation involving phosphorylases and sucrases are extraordinarily vast. Recent advances in protein and substrate engineering have greatly contributed to widening their scope of application, enabling the bioprocessing of non-native donors/acceptors into biofuels or carbohydrate products with customized functionalities.[168] When applied to the breakdown of glycosidic linkages, phosphorylases are able to provide a constant supply of Glc-1-P from cheap glucan substrates, which can, for instance, be used as the carbon source in multienzymatic systems for sugar-powered biobatteries with high energy densities and much lower environmental impact than lithium-ion batteries.[169] Reverse phosphorolysis (buildup of glycosidic linkages) is also a powerful biotechnological tool that can be utilized for the cost-effective transformation of nonfood biomass such as cellulose to amylose-rich resistant starch.[84] Cellulosic biorefineries consisting of a cascade of coupled enzymatic reactions may help to alleviate food demands and the ecological impact of agriculture imposed by the continuing world population growth. Moreover, phosphorylases could be employed in the preparation of amylose-based affordable materials with application in the biological and pharmaceutical research fields. Thermostable phosphorylases often display a broader use of substrates than mesophilic enzymes, and potato phosphorylase has a lower activation energy for synthesis and a high activation energy for degradation.[84] Therefore, the selection of suitable enzymatic components is of the utmost importance in the design of synthetic biotransformation systems.

While polymerization reactions with GTs and phosphorylases can offer good control over the synthesized structures, they cannot produce branched molecules with perfectly regular structures. This is the result of using monosaccharides as monomers, which do not allow preinstallment of branching points. Glycosynthases, on the other hand, offer full control of the structures, as more complex oligosaccharides can be employed as starting monomers. Due to the challenging preparation of the respective oligosaccharide fluoride monomers, production of such defined materials in bulk might not be feasible. However, enzymatically synthesized polysaccharides are not only interesting for direct use as high-end materials but also as model systems for structure−property relationship studies and promoting a better understanding of biomass-derived polysaccharides.

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Notes

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Peter J. Smith received his B.S. in Biology from Northeastern University in 2012. He then went on to complete his Ph.D. in biochemistry and molecular Biology at the University of Georgia under the direction of Dr. William York and Dr. Breeanna Urbanowicz. Peter's research is focused in the field of glycobiology, with specialty in applying plant enzymes toward the biocatalytic synthesis of polysaccharide-based materials.

Maria Elena Ortiz-Soto received her Ph. D. in biochemical sciences from Universidad Nacional Autónoma de México (UNAM) under the supervision of Prof. Agustín López-Munguía. During this time, she worked on protein engineering and biocatalyst design. She obtained a scholarship from the German Academic Exchange Service (DAAD) in 2011 to pursue postdoctoral research in Germany. She is currently working in the group of Prof. Jürgen Seibel at the University of Würzburg as a research associate. Her research focuses on structural and biochemical studies of bacterial and eukaryotic glycoside hydrolases and glycosyltransferases, as well as on protein modification by genetic and chemical approaches.

Christian Roth received his Diploma from the Technical University "Bergakademie" Freiberg and his Ph.D. from the University Leipzig. He moved to York to join the Davies Group at the York Structural Biology Laboratory (YSBL). Now, he is a group leader at the Max Planck Institute of Colloids and Interfaces in Potsdam. His research is focused on the structural and functional characterization of carbohydrate active enzymes.

William J. Barnes is currently a Postdoctoral Research Associate at the Complex Carbohydrate Research Center at the University of Georgia studying under the supervision of Dr. Breeanna Urbanowicz. Will's research is primarily focused on biochemically characterizing plant glycosyltransferases involved in cellulose biosynthesis and elucidating complex pectin structures. During his Ph.D. at Penn State, Will used microscopy-based techniques to study plant cell wall dynamics with Dr. Charles Anderson.

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Jürgen Seibel studied chemistry at the Georg August University of Göttingen. During his Ph.D. studies under the guidance of Professor Dr. Dr. h.c. Lutz F. Tietze, he was involved in the synthesis of vitamin E. He moved to the University of Oxford to work with Professor Dr. Chris Schofield in the Dyson Perrins Laboratory (Department of Organic Chemistry) on serine proteases and hypoxia-inducible factors (HIF) on the mechanisms and as therapeutic targets. Back in Germany, he started his independent research at the University of Braunschweig, where he received his habilitation and moved to Helmholtz Centre for Infection Research (HZI). In 200, he became a professor at the University of Würzburg. His research interests are focused on bioorganic chemistry and chemical biology including the development of chemical and enzymatic syntheses, biocatalysis, protein engineering, drug delivery, and glycosciences.

Breeanna R. Urbanowicz received her Bachelor's degree from Purdue University and her Ph.D. from Cornell University. She is an Assistant Professor in the Department of Biochemistry and Molecular Biology at the University of Georgia and a member of UGA's Complex Carbohydrate Research Center and New Materials Institute. Her core research is centered on the structure, biosynthesis, and biological function of the plant cell wall, a cellular component that is integral to many facets of plant biology. The tools and approaches developed are paving the path forward toward deeper analysis of glycopolymer synthesis and deconstruction pathways used by plants and other organisms and are now being applied toward the development of new biobased plastics and coatings.

Fabian Pfrengle studied chemistry at the Free University Berlin, Germany, and received his Ph.D. in 2010 after working with Prof. Hans-Ulrich Reißig. Subsequently, he was a postdoctoral research associate at The Scripps Research Institute in La Jolla, CA, USA. In 2013, he started his independent career as a group leader in the Max Planck Institute of Colloids and Interfaces in Potsdam, Germany, supported by the Emmy Noether program of the German Research Foundation. Since 2020, he has been a professor of organic chemistry at the University of Natural Resources and Life Sciences, Vienna, Austria. His research interests are the chemical and enzymatic synthesis of carbohydrates and their functions in biology, with a particular focus on plant glycans.

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