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Preparation and Analysis of Cello- and Xylooligosaccharides

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Abstract This review provides a general overview of preparation, separation, and analytical methods for cello- and xylooligosaccharides. Arising as side-stream products of different biorefinery processes, these compounds have increasingly gained the interest of researchers and engineers in the last few decades. Beside their application as additives in the food, feed, and pharmaceutical industries, these oligomeric carbohydrates are of key importance as model compounds for studying the dependence of physicochemical properties on the degree of polymerization (DP). First, different preparation methods for mixtures of oligosaccharides with DPs between 1 and 30 are discussed. These methods include acetolysis, acid and enzymatic hydrolysis, and glycoside synthesis. Then, separation techniques, including size exclusion chromatography, normal phase and hydrophilic interaction chromatography, and chromatography on cation exchange resins, are presented. Analysis of oligosaccharides by different techniques is described.

Keywords Cellooligosaccharides • Cellulose • Cellulose hydrolysis • Chromatography of cellooligosaccharides • Synthesis of cellooligosaccharides • Xylan • Xylan hydrolysis • Xylooligosaccharides

Contents

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1 Introduction

Celluloses and heteropolysaccharides (or hemicelluloses) are among the most abundant natural materials on earth. Together they form the polysaccharide fraction of the plant cell wall, where they provide structural integrity and act as a barrier between the inside and the outside of the cell. In addition, hemicelluloses can be used as a seed storage carbon source and as a mobile carbon source in the non-reproductive tissues of some plants [1]. The practically inexhaustible nature and unique properties of these polysaccharides from the perspective of a strongly increasing demand for sustainable, re-growing resources make them a raw material of great interest for researchers and engineers. The history of the industrial utilization of cellulose in particular is long and diverse, reaching from its application as a raw material in the pulp, paper, and fiber industry to a source of carbon and chemical energy in biotechnological processes, to its use in (anti-nutritional) food additives and in high-tech applications (e.g., as the stationary phase in column chromatography). Furthermore, the chemical replacement of hydroxyl groups of the polymer chain with different substituents provides the possibility to generate materials with new characteristics. In this context, cellulose ethers, esters, nitrates, and acetates are the most prominent types of derivatives that give the polymer different, interesting new features, such as film- and gel-forming properties [2, 3]. Hemicelluloses, although less known, are also of commercial significance because they can impart important properties to many food and feed products [4]. Figure 1 depicts the structures of cellulose and xylan, a certain type of hemicellulose.

"Biorefinery" is a general term for the conversion of natural (plant) feed stock materials to products of higher value. Many process strategies for the degradation and/or transformation of cellulose and hemicellulose to desired compounds have been developed and successfully applied [5]. In this regard, the hydrolysis of polysaccharides to their monomeric building units can be subjected to fermentation processes with a large variety of potential end products (e.g., bioethanol, biogas, propanol, acetic acid), or can be chemically treated to produce platform chemicals such as furfural and furan. However, the oligomeric degradation products, which can be seen as intermediates of a total hydrolysis of the polymers, are not given similar consideration, in spite of their potentially great importance in present and future technologies.

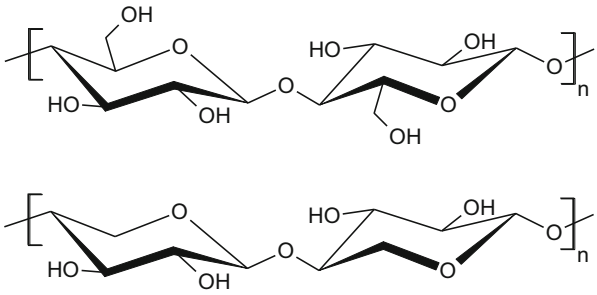

Fig. 1 Molecular structure of cellulose (*top*) and xylan (*bottom*)

With respect to the degree of polymerization (DP), oligosaccharides fall between the monosaccharides and the corresponding polysaccharides. There is no exact definition, that is, no DP limit above which they are referred to as a polymer and below which as oligomers. However, a very common distinctive feature is the solubility in water, which means that with reference to cellulose, the water-soluble saccharides ($DP \leq 8$) are referred to as cellooligosaccharides, and the insoluble saccharides having a higher DP are called polysaccharides. This strict demarcation, justified solely by the solubility in water, is very often not useful, because other DP-dependent physicochemical properties do not change as suddenly. In this review, the term "oligosaccharide" is regarded in a broader sense, meaning saccharides having DP values up to about 30–35. These higher oligosaccharides and short-chain celluloses are of central significance for the elucidation of physicochemical properties in relation to the DP [6, 7]. Studies of homologous series of cellooligosaccharides (also referred to as cellodextrins) that asymptotically approach a polymeric structure provide insight into the macromolecule character of celluloses with increasing chain length [6, 8]. Oligosaccharides with a defined DP can be used as a simple model for cellulose in structural investigations [8, 9]. In addition, cellodextrins are considered to be useful substrates for the study of cellulose hydrolysis and can also be used in the screening of cell cultures for specific cellulase activities and in induction studies of microbial cellulase expression [10]. Oligosaccharides originating from cellulose are used in the food industry as anti-nutritional additives and have potential application in the pharmaceutical industry as coating agents for the controlled release of active ingredients. There is also a broad area of application for xylooligosaccharides, reaching from the pharmaceutical to the food and feed industries [11].

Several methods for the generation of cellooligosaccharides have been developed during the past century, and can principally be divided into two basic strategies: (1) fragmentation of polymers to shorter chain lengths by partial hydrolysis, and (2) synthesis of oligomers by selective condensation of smaller saccharides. The latter procedures inherit difficulties with regard to the stereo- and regioselectivity of the reactions, because the synthesis of natural polymers such as polysaccharides necessitates a precise steric control of the polymerization [12]. Section 2 of this review is dedicated to the variety of preparation methods for cello- and xylooligosaccharides.

Unfortunately, the methods for analysis of these compounds are still not well developed. Although mono- and disaccharides as well as polysaccharides can be

analyzed by different means relatively easily (e.g., gas chromatography for the small sugars and gel permeation chromatography for the polymers) proper techniques for saccharides with a DP range between 10 and 50 do not exist. The separation of cellooligosaccharides according to their DP to obtain monodisperse fractions (or at least fractions with a very narrow molecular weight distribution) of oligosaccharide mixtures is currently still a subject of research. The reason for this is that the physicochemical properties of cellodextrins, including molecular dimensions, molecular weight, and melting points, alter very slowly with a change in chain length, leaving effective separation with respect to the DP as challenge for scientists. Therefore, Sect. 3 of this review is dedicated to the different chromatographic and other separation methods that have the potential to solve this problem and to other analytical procedures for the characterization of these compounds.

In order to overcome these difficulties, strategies have to be worked out for the preparation of cello- and xylooligosaccharides in the 10–50 DP range that are homogeneous with regard to molecular weight. These can then be used as standard

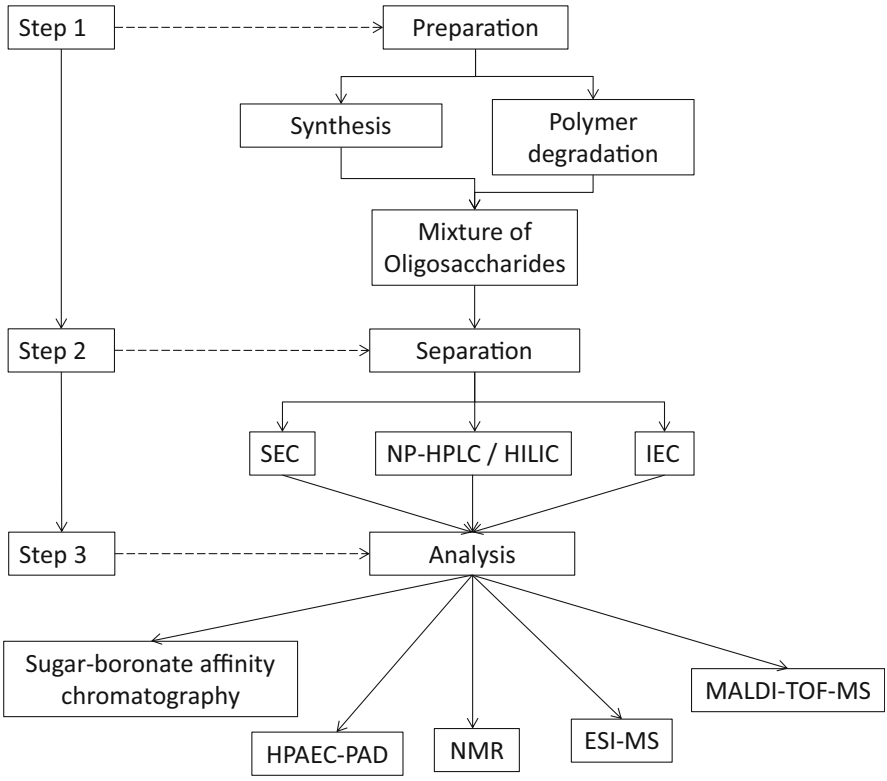

Fig. 2 Principal scheme for the generation of homogeneous oligosaccharides. *SEC* size-exclusion chromatography, *NP-HPLC* normal phase high-performance liquid chromatography, *HILIC* hydrophilic interaction chromatography, *IEC* ion exchange chromatography, *HPAEC-PAD* high-performance anion exchange chromatography coupled with pulsed amperometric detection, *NMR* nuclear magnetic resonance, *ESI-MS* electrospray ionization mass spectrometry, *MALDI-TOF-MS* matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

compounds for the development of analytical methods. The principal scheme for such an effort essentially consists of three steps, as illustrated in Fig. 2. Step 1 is preparation of an oligosaccharide mixture, either by hydrolysis of the parent polymers or polymerization of the corresponding monomers. Step 2 is separation according to the DP. Step 3 is analysis of the separated fractions in terms of structure and purity. Although several publications, including some review papers, describe one of these three steps, to the best knowledge of the authors there is no general account fully covering this topic. The intention of this review is therefore to provide the reader with a broad comprehensive overview of the variety of techniques that exist for the production of cello- and xylooligosaccharides, particularly at a preparative scale; to provide a summary of the different methods for their separation according to molecular weight; and to survey the different possibilities for analysis.

2 Preparation of Oligosaccharides from Celluloses and Hemicelluloses

2.1 Synthesis of Oligosaccharides

One possibility for the generation of oligosaccharides from cellulose or hemicellulose is to synthesize them from their monomeric or, in the case of cellulose, dimeric building units. This requires the formation of a new glycosidic bond between a glycosyl donor and a glycosyl acceptor (i.e., the growing oligosaccharide). In order to synthesize a desired oligomer, it has to be ensured that the linkage is formed regio- and diastereoselectively. In this context, the high number of glycosidic links that can be possibly created is a major complexity of the problem. Because the electrophilic attack of the anomeric carbon of the donor molecule at a hydroxyl oxygen of the (non-protected) acceptor can principally take place at any of the free OH groups, the number of potential oligosaccharide products rises drastically with an increase in DP. In order to force the coupling reaction exclusively toward a specific glycosidic bond (e.g., in the case of cellooligomers, the reaction of the anomeric carbon of the donor with the terminal 4-OH of the acceptor) it was necessary to develop strategies to make the reaction regioselective (i.e., to avoid the formation of "wrong" bonds). In addition to this problem of regioselectivity, the stereochemistry of the hydroxyl group at C1 raises another difficulty. In solution, pyranoses display an equilibrium between their α- and β-configurations, which consequently leads to the formation of both the α- and β-forms of the glycosidic bond. Efforts to synthesize (pure) cellooligosaccharides, which require generation of the desired β-(1,4)-glycosidic linkage only, therefore have to include routes that help to make the reaction both regio- and stereoselective. In this regard, several strategies have been developed, with different degrees of success. They can be divided into two groups: those that use an enzyme as a catalyst, and those that

do not. Historically, the latter approach appeared earlier and is therefore discussed first.

2.1.1 Chemical Synthesis

The targeted chemical synthesis of cellooligosaccharides (or any except "non-random" oligosaccharides) necessitates the introduction of blocking groups that prevent the formation of undesired glycosidic bonds. This means that those OH groups that should not participate in the glycosidic linkage must be substituted in such a manner that electrophilic attack at the hydroxyl oxygen cannot take place. In addition to this, the anomeric C atom of the glycosyl donor must be activated in order to favor coupling reactions (see Fig. 3). The classical Koenigs–Knorr method, developed in 1901 [13] and continuously improved later, achieves this activation by the formation of glycosyl halides (mainly bromides and chlorides), and the reaction with the acceptor takes place in the presence of heavy-metal salts (preferably Ag salts). Pfaeffli et al. [14], for example, successfully synthesized the disaccharide isomaltose by coupling the glucose derivatives 2,3,4-tri-*O*-benzyl-1-thio-β-D-glucopyranoside as acceptors and 6-*O*-acetyl-2,3,4-tri-*O*-benzyl-α-D-glucopyranosyl chloride as donors, resulting in a disaccharide product containing 72% of the desired α-(1,6)-configuration, but also 28% of the β-anomeric gentiobioside. The halide at C1 of the donor works as an activator for the anomeric carbon and is the leaving group during formation of the glycosidic bond, whereas the benzyl groups and the acetyl group at C6 of the donor act as protecting groups for the positions that should not be involved in the linkage.

In 1973, Schuerch [15] reviewed approaches to the chemical synthesis of polysaccharides and divided the problem into two main areas. On the one hand was the stepwise synthesis of complex oligo- and polysaccharides by a consecutive series of reactions, forming one new regio- and stereoselectively correct bond at a time. On the other hand were the ring-opening propagation (see Fig. 4) and condensation reactions, which are more suitable for the generation of homooligosaccharides (e.g.,

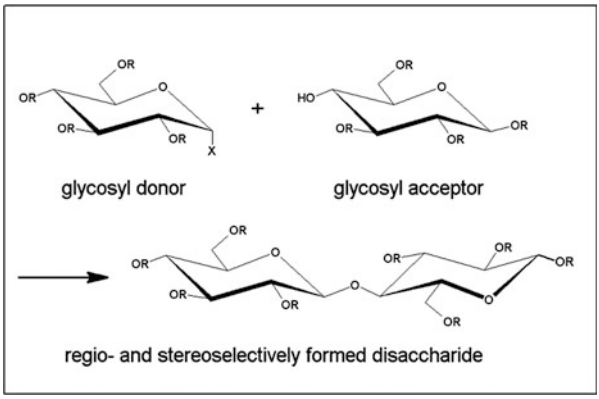

Fig. 3 Glycosidation is formation of a glycosidic bond between glycosyl donor and glycosyl acceptor with the aid of protecting groups (*R*) and an activating group (*X*)

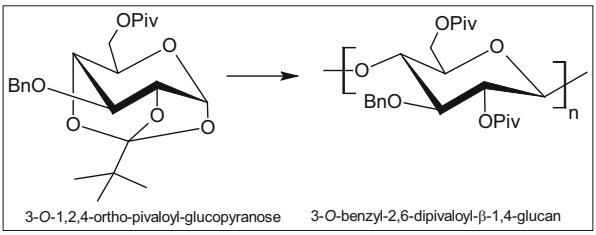

Fig. 4 Typical ring-opening polymerization starting from a glucose 1,2,4-orthopivalate as the precursor

cellooligosaccharides) and polymers of simple repeating sugar units (as well as polysaccharides with a random sequence of monomers). The latter type of approach is discussed in more detail next.

In the 1950s and 1960s some efforts were made to generate stereoregular oligosaccharides and polysaccharides through self-condensation of carbohydrate derivatives with ester functions as blocking agents. Haq et al. [16], for example, published in 1956 the first chemical synthesis of an α-(1,2)-linked glucoside out of 1,2-anhydro-3,4,6-tri-*O*-acetyl-α-D-glucopyranose. The reaction, however, displayed a lack of stereoselectivity, leading to a variety of di- and oligosaccharide side products. In the same year, the authors reported [17] the chemical synthesis of a homologous series of β-(1,6)-D-glucopyranans up to a DP of 9 using 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide as the monomeric glycosyl donor and Ag_2O as the catalyst. However, isomer formation occurred and the yields were very low (<1%). More than a decade later, McGrath et al. [18] made similar attempts at regio- and stereoselective preparation of oligo- and polysaccharides, showing again that the drawbacks were poor stereoselectivity and a very low yield in general. Thus, there are obviously two main disadvantages [15] in attempts at polymerization via self-condensation using ester derivatives of the carbohydrates as building blocks: (1) The ester groups tend to migrate, which leads to formation of isomeric structures within the oligo- or polymer. (2) The achieved yields and degrees of polymerization are generally low as a result of side reactions, which cause chain termination. A more promising approach, especially for the generation of cellooligosaccharides, was shown by Husemann et al. [19] in 1966, using glucose 2,3,6-tri-carbanilate as the monomer with P_2O_5 as the catalyst. The study demonstrated the formation of unbranched polysaccharides with a DP of up to 60, displaying β-(1,4)-glycosidic linkages between the glucopyranosyl monomers only. However, the major drawback to this effort was the extremely long time (over 10 days) required for the preparation.

A different approach to stereo- and regioselective polymerization of carbohydrate derivatives to obtain desired oligo- and polysaccharides was introduced by Kochetko et al. [20, 21] using a different mechanism, ring-opening polymerization. The use of cyclic orthoester derivatives of different sugar monomers without free OH groups, in the presence of $HgBr_2$ as a catalyst and an alcohol initiator, led to the formation of polysaccharides of comparatively high DPs of between 23 and 60 at high yields of 20–50%. The achieved molecular weight was dependent on the ratio of monomer to initiator, and the reaction rate of polymerization was dependent on

the amount of catalyst used. However, these methods still showed weaknesses in terms of regioselectivity, resulting in the occurrence of some random links and branches in the final product mixture. In 1970, Masura et al. [22] investigated the propagation of a polysaccharide using the cellobiose derivative 1,6-anhydro-2,3-di-*O*-benzyl-4-*O*-(2,3,4,6-tetra-*O*-benzyl-β-D-glucopyranosyl)-β-D-glucopyranose as a dimeric building unit with the aid of a Lewis acid as the catalyst. The ring-opening polymerization of 1,6-anhydro-2,3,4-tri-*O*-benzyl derivatives of monomeric β-D-pyranosides had been studied in the same laboratory in earlier years [23–28], and investigators managed to stereoselectively synthesize oligo- and polysaccharides of relatively high DPs (150–350). In this context, the temperature at which polymerization took place as well as the choice of Lewis acid had a strong impact on the DP finally reached and the stereoselectivity of the prolongation reaction [24, 28]. With this experience, the authors managed to build derivatized product polymers out of the 1,6-anhydro-cellobiose benzyl ether derivative, with number average molecular weights of $6–7 \times 10^3$ g mol^{-1}. Again, the choice and concentration of Lewis base (PF_5 was the most suitable), as well as the temperature and the initial substrate concentration, had significant impact on the stereoselectivity and DP. A final debenzylation resulted in stereoisomerically pure 4-β-D-glucopyranosyl-(1, 6)-α-D-glucopyranan containing between 14 and 16 D-glucose units at yields of up to 70%.

In his review, Schmidt [12] offered an overview of different alternatives to the classical Koenigs–Knorr method that poses some major disadvantages [12], including harsh conditions during formation of the glycosyl halide, low thermal stability and tendency for hydrolysis of the glycosyl halides, and the potential hazard of heavy-metal salts. Two methods for monomer derivatization were presented as being most suitable for the stereo-controlled formation of glycosidic bonds: (1) direct 1-*O*-alkylation of sugars, which eases the generation of saccharides by using a comparatively simple method [29], and (2) the trichloroacetimidate method, resulting in stable *O*-glycosyl trichloroacetimidates with high glycosylation potential in a stereo-controlled manner.

As a consequence of the enormous number of possible ways that carbohydrates can be linked to one another by glycosidic bonds, every chemical oligosaccharide synthesis requires deep knowledge of reaction mechanisms and experimental methods. Different reactivities and stereoselectivities of glycosyl donors and acceptors, induced by altering the through-space steric interactions and amphiphilic properties of protecting groups and neighboring groups, render the development of generalized methods almost impossible [12]. Therefore, it is no wonder that it took until the early 1980s before reports of the first synthesis of pure β-(1,4)-linked cellooligosaccharides were published by Schmidt [30] and Takeo [31], although yields were not very high. In the first case, cellotetraose was synthesized using the α-trichloroacetimidate of 2,3,4,6-tetra-*O*-acetylglucopyranose as donor and 1,6-anhydro-β-D-glucopyranose carrying benzyl protecting groups at the 2-*O*- and 3-*O* positions as initial acceptor in a stepwise procedure. In the latter procedure, cellobiose, cellotriose, and cellotetraose were synthesized via the Koenigs–Knorr method using benzyl ethers as protecting groups and bromide as the activating

reagent. In their "Synthetic studies of cellulose" series of articles, Takano et al. systematically investigated the impact of substituting groups on the stereoselectivity of the glycosylation reaction [32–34] and evaluated different starting materials for the convergent synthesis of cellooligosaccharides [35, 36]. The authors primarily focused on the use of α-imidates as donors, testing different substitution patterns (acetyl or benzyl groups) on both the donor and the acceptor, and were finally able to establish some general principles for the effect of substituents on linkage formation: (1) The substituent at position 3-*O* is crucial, which was in agreement with an earlier publication by Sina [37]. A benzyl group there leads to β-glycosylation in an extremely high yield, whereas an acetyl function at this position results in the predominant formation of the α-glycoside in significantly lower yields. (2) The character of the protective group at 4-*O* of the α-imidate (donor) also has a significant impact on bond formation. Electron-withdrawing functions (e.g., acyl groups) there lead to an increase in the stability of the donor, but on the other hand to a lower β-glycoside yield. In contrast, electron-donating ether groups (in particular benzyl and *p*-methoxybenzyl) result in a high yield of the β-glycoside. In conclusion, 2,6-di-*O*-acetyl-3-*O*-benzyl-4-*O*-*p*-methoxybenzyl-α-D-glucopyranoside was named as the most suitable α-imidate glycosyl donor for the stereoselective formation of β-(1,4)-glycosidic linkages between glucose derivatives [34].

In an effort to gain information about the impact of the type of derivatization on the yield of cellooligosaccharides, Nishimura et al. [38] compared different glycosyl donors for the synthesis of cellotetraose. The highest yield of more than 70% was found when working with the 4-*O*-acetyl trichloroacetimidate form as the glycosyl donor. The authors found that the reactivity of donor and acceptor decreases with an increase in chain length [38]. When trying to overcome this problem with a higher amount of catalyst (BF_3 etherate) or higher reaction temperatures, side reactions such as glycosyl fluoride formation and cleavage of the *p*-methoxy-benzyl groups (temporary *O*-4′ protective group on the donor) occurred. The use of acetyl groups instead of *p*-methoxy-benzyl groups significantly enhanced the outcome, so that the aforementioned high yields could be reached [38]. In a subsequent publication, Nishimura [7] and his team presented a high-yield β-glycosylation using a convergent synthetic method between a cellotetraosyl donor and acceptor, resulting in the formation of a cellooctaose derivative. The one-step anhydrous glycosylation was performed under high vacuum to minimize imidate side reactions (e.g., hydrolysis, glycosyl fluoride formation), using pivaloyl, allyl, and benzyl functions as protecting groups. After the reaction, these groups were replaced by acetyl groups, which were removed in a last step to give pure cellooctaose with a yield of 87%. In 1996 Nakatsubo et al. [39] succeeded in performing the first cellooligosaccharide synthesis via cationic ring-opening polymerization. The reaction, performed with 3,6-di-*O*-benzyl-α-D-glucose 1,2,4-orthopivalate and the aid of a triphenylcarbenium tetrafluoroborate initiator, was shown to be highly stereoselective. The number-average molecular weight of the product was 8.3×10^3 g mol^{-1}, which corresponds to a DP of approximately 20. Complete removal of the protective group via temporary

acetylation resulted in the underivatized cellooligosaccharide product. Later, it was again Nishimura who presented the first stepwise synthesis of a homologous series of cellulose analogs [8, 40]. The authors conducted sugar chain prolongation via stepwise additions of cellotetraosyl units, thus investigating the same pattern of protective and activating groups as in their previous work [7]. In this way, a DP of up to 20 for the acetylated end product was reached, with an overall yield of 37%.

In 2009, Adelwöhrer et al. [41] reported the successful synthesis of ^{13}C-perlabeled cellulose through an approach involving cationic ring-opening polymerization. As precursor, the authors used 3-*O*-benzyl-$^{13}C_6$-glucopyranose 1,2,4-orthopivalate and obtained fully labeled ^{13}C-cellulose as the cellulose II allomorph, with a DP of 40 and an overall yield of 28%. In conclusion, despite great advances in chemosynthetic methods during recent decades, the strategies of conventional chemical synthesis of cellooligosaccharides have not yet produced fully satisfying results in terms of either time and work intensity or the regio- and stereospecificity of the products. In order to find ways to produce these compounds by polymerization of smaller saccharides in useful amounts within reasonable preparation times, different approaches applying enzymatic catalysis in combination with conventional chemical approaches may be the answer.

2.1.2 Chemo-Enzymatic Synthesis

Another way to produce cellooligo- and cellopolysaccharides is to synthesize them with the aid of an enzyme that can catalyze formation of the glycosidic bond. The main advantage of these procedures is that enzymes in general work in an extremely specific manner, which means that, for the generation of polysaccharides, the glycosides are produced with high regio- and stereoselectivities. This major characteristic of enzymes is of great advantage in oligo- and polysaccharide synthesis, making chemo-enzymatic approaches superior to conventional chemical approaches for three main reasons: (1) The laborious introduction of protective groups and subsequent deprotection after the product is formed becomes obsolete because of the high regioselectivity of the biocatalyst, so that an underivatized polymer can be created directly [42–44]. (2) The reactions can be performed under mild conditions of temperature, pH, and salt concentration and the conversion rates are comparatively fast [43, 44]. (3) The use of potentially harmful catalysts (e.g., heavy metals) can be avoided, and undesired side reactions generally do not occur [43, 44]. Since the early 1990s, the application of enzymes in polysaccharide synthesis has undergone a massive upsurge [45]. Many of these efforts have in common that the design of an activated donor molecule is required. According to Pauling [46], enzymatic reactions can happen under very mild conditions because of the formation of an intermediate enzyme–substrate complex, which is energetically favored over the free substrate. This so-called stabilization of the transition state allows an increase in reaction rate of several orders of magnitude by decreasing the activation energy of the reaction. Another convenient characteristic of enzymes is that they are able to catalyze their reaction not only on the natural

substrate, but also on artificial substrates that closely resemble their natural relative. In this context, Kobayashi et al. [44, 47–49] suggested that, for an effective polymerization to polysaccharides, it is possible to design such an altered substrate, a so-called transition-state analog substrate (TSAS), that is readily incorporated into the active site of the enzyme and, later, rapidly attached to the growing polymer chain. The enzymes involved in these efforts can be divided into two main classes [43]: (1) glycoside hydrolases, which catalyze hydrolytic cleavage of the glycosidic bond and the back reaction (i.e., formation of such a linkage), and (2) transferases or, specifically, glycosyltransferases, which catalyze the transfer of a carbohydrate monomer moiety (glycoside donor) to a glycoside acceptor. In the following sections, these two types of enzymes are discussed in more detail with regard to their applicability in oligo- and polysaccharide synthesis.

Glycoside Hydrolases

Glycoside hydrolases (EC 3.2.1) are enzymes designed by nature for the catalytic degradation of oligo- and polysaccharides. However, as for any enzyme their reactions are principally reversible, so that, under appropriate conditions (especially regarding water activity), they can also catalyze the reverse reaction (i.e., the formation of a glycosidic bond between a glycosyl donor and an acceptor). As extracellular (secreted) enzymes, they display some major technical advantages, including good stability in aqueous solution, easy accessibility in terms of purification, and a relatively low price [44]. With regard to the substrate, these hydrolases can be categorized into two major groups: those that attack the polymer chain at the end, releasing one monomer at a time (referred to as "exo-types"), and those that cleave the chain at a random position somewhere in the middle, leading to fragments of the original polysaccharide chain (called "endo-types") [50]. The latter have proved to be far more suitable for enzymatic polymerization to polysaccharides [49], a result of the different topology of their catalytic domain, which is shaped like a cleft rather than a tunnel. Two common ways of activating the glycosyl donor have been described in the literature. They not only differ in the type of derivatization but also in the character of the polymerization reaction. The first method involves activation by a fluoride atom to give glycosyl fluorides, which lead to a polycondensation type of polymerization. The second method involves activation of C1 by introduction of an oxazoline group, resulting in a ring-opening polymerization [44]. Although both of these artificial substrates are readily recognized by the cellulase, the former displays some important advantages [42–44, 51]: (1) The size of the fluoride atom closely resembles the size of an OH group, minimizing interfering steric effects; (2) Glycosyl fluorides are the only glycosyl halides that are stable in an unprotected form, allowing the reaction to be performed in aqueous media. (3) Fluoride is a very good leaving group, widely used in chemical synthesis. Because glycosyl fluorides are the main substrate used for the generation of cello- and xylooligosaccharides, the focus of this review is on activation by fluoride, whereas the other type of activation is not discussed further.

Kobayashi et al. [52, 53] were the first to publish the successful synthesis of cellulose by polycondensation, using an endocellulase as catalyst and β-D-cellobiosyl fluoride as the activated donor molecule. The reason for the use of the disaccharide donor (rather than monomeric β-D-glucopyranoside) was that this substrate is more readily recognized by the enzyme, resulting in faster polymerization. A mixture of an organic solvent and an aqueous buffer was selected as reaction medium in order to avoid excessive water activity, which would favor hydrolytic cleavage of the glycosidic links. In this regard, a 5:1 mixture of acetonitrile and acetate buffer (pH 5) was found to result in the best polymer yields, up to 54% for water-insoluble fractions (i.e., DP > 8). The highest DP achieved during these efforts was 22. The suggested reaction mechanism for this polymerization is as follows [52]: In a first step, a cellobiosyl–enzyme intermediate or, alternatively, a glycosyloxocarbenium ion is formed under elimination of the fluoride anion at the active site of the enzyme. In a second step, this highly reactive intermediary compound is attacked by the terminal 4-OH oxygen of the growing polymer chain (carrying a fluoride group at its C1 end), which is located at a sub-site of the catalyst. The stereoselectivity of the reaction is achieved by a "double inversion" of the anomeric site of the donor, and thus a "net retention" of the β-conformation, leading to exclusively β-(1,4)-glycosidic linkages.

Another huge benefit of using these enzymes in polysaccharide synthesis is that it is also possible to produce functionalized polymers with exactly defined structures, not only in terms of stereo- and regioselectivity of the glycosidic bonds, but also regarding the regioselectivity and distribution of the substituents [43], which makes these methods superior to conventional chemical modification techniques. For example, a modified cellooligomer carrying methyl groups exclusively at C6 was synthesized by Okamoto et al. [54] using 6,6′-di-*O*-methyl-β-cellobiosyl fluoride derivatives as substrates for a cellulase from *Trichodermaviride*. The resulting cellulose derivate displayed a unique structure that is not achievable by the conventional chemical modification of cellulose polymers. Similarly, Izumi et al. [55] reported the successful synthesis of a 2-*O*-methylated derivative of a cellooligosaccharide. Furthermore, the application of hydrolases in polysaccharide synthesis is not restricted to the generation of homopolymers. Shoda et al. [56] used an endoglucanase for the enzymatic polymerization of α-(1,6)-xylopyranosyl-β-cellobiosyl fluoride as monomer to an artificial xyloglucan oligomer, with α-(1,6)-xylopyranosyl residues linked to the alternating glucose residues in the main chain. Fujita et al. [57] presented a xylanase-catalyzed polymerization of the unnatural monomer 4-*O*-β-D-xylopyranosyl-β-D-glucopyranosyl fluoride, resulting in a novel polysaccharide having a glucose–xylose repeating unit (i.e., a cellulose–xylan hybrid polymer), again demonstrating the great potential of these enzymes in polysaccharide synthesis.

At this point it should be mentioned that the use of glycoside hydrolases for these efforts has one major disadvantage: they are actually designed by nature to catalyze the opposite reaction (i.e., the hydrolytic cleavage of the glycosidic bond). To suppress this undesired reverse reaction, the water activity in the media has to be kept at a low value. Thus, the choice of solvent mixture is of crucial importance. As

demonstrated by Kobayashi et al. [52] and several publications thereafter, a combination of acetonitrile and aqueous buffer is the most suitable system in this regard. However, a more effective way of overcoming this problem can be found by means of genetic engineering, using mutant cellulases [58] that are less prone to cleave the glycosidic bonds. A common strategy in this regard is to produce a cellulase that is lacking the so-called cellulose binding domain, which is required to perform the hydrolysis reaction on a solid substrate [44]. However, these methods are not discussed in detail in this review. A very useful publication for starting a literature research on the topic of genetically engineered cellulases is chapter 4 of Kadokawa's review [43] on enzymatic polysaccharide synthesis.

A somewhat different approach to cellulose synthesis using a hydrolytic enzyme for the formation of the β-(1,4)-glycosidic bond was published recently by Egusa et al. [59, 60]. In contrast to the aforementioned efforts, a non-aqueous solvent was used as the reaction media, namely a solution of LiCl in *N*,*N*-dimethylacetamide (DMAc). This solvent system has been known for a long time and is commonly used for the dissolution of cellulose [61]. Most enzymes, including cellulases, are usually not stable and therefore not able to catalyze their reaction in this environment. In order to preserve catalytic activity, the enzyme was treated with a non-ionic surfactant (dioleyl-*N*-D-glucono-L-glutamate), which kept it stable in this aprotic medium. With the aid of this so-called surfactant-enveloped enzyme (SEE) and a protic acid co-catalyst, the investigators were able to generate artificial cellulose with chain lengths of up to 120 anhydroglucose monomers. A great virtue of this method is that the "reversed hydrolysis" works without any pre-activation of the glycoside donor (or acceptor), that is, natural, untreated cellobiose can be used directly for the polycondensation.

Glycosyltransferases

In nature, polysaccharides are synthesized via catalytic action of glycosyltransferases, which catalyze the formation of a glycosidic bond using an activated glycosyl donor in which the OH group at C1 is substituted by a phosphate function [62]. According to the nature of the substitution to be recognized by the enzyme, there are two main types of glycosyltransferases: (1) those that are dependent on sugar mono- or diphosphonucleotides as donor substrates, referred to as Leloir-glycosyltransferases or glycoside synthases; and (2) those that utilize sugar-1-phosphates, sugar-1-pyrophosphates, or sugars linked to a lipid via phosphoester or phosphodiester linkage, referred to as non-Leloir-glycosyltransferases or phosphorylases [63]. In both cases, the anomer configuration of the activated donor displays the α-isomeric form. In the following sections, the two types of enzymes are discussed in more detail and examples of their application in oligosaccharide synthesis are given.

Leloir-Glycosyltransferases

Leloir-glycosyltransferases (very often referred to as glycoside synthases) employ the high energy bond of the glycosyl nucleotide donors (usually UDP–monosaccharides) to provide the free energy needed for formation of the glycosidic bond [42, 63]. The highly negative ΔG of the substrate phosphorolysis renders the reaction practically irreversible in the synthesis direction. Plant cell wall cellulose is synthesized by the enzyme cellulose synthase situated in the cell membrane [64]. The catalytically active enzyme exists as a complex of six subunits of six single enzymes, together shaping a rosette-like structure [65]. Their location in the membrane highlights a major disadvantage of employing these enzymes for in vitro oligosaccharide synthesis. The location complicates purification of the active enzyme, making these biological catalysts quite expensive compared with, for example, hydrolases [44]. Another drawback, arising from their existence as *trans*-membrane proteins, is their decreased stability in solution [44, 66]. Additionally, nucleoside diphosphates act as inhibitors of these enzymes, which has to be overcome either by the exploitation of phosphatases to degrade the nucleotides [67] or, alternatively, by in situ regeneration of sugar nucleotides with the aid of pyrophosphorylases [68]. In spite of these hindrances, some successful applications of Leloir-glycosyltransferases have been reported. Rosette-like particles corresponding to the rosettes of the plasma membrane were isolated from mixtures of synthesizing complexes from mung beans by means of gel electrophoresis [64] and used for the synthesis of cellulose with UDP–glucose as a substrate [69]. Futaki and Mizumo [70] reported the preparation of high molecular weight complexes with β-(1,4)- and β-(1,3)-synthase activity from azuki bean epicotyls. A further purification by affinity chromatography with anti-tubuline as a ligand [71] resulted in the isolation of a pure β-(1,4)-glycan synthase (i.e., cellulose synthase), that could be used for in vitro synthesis experiments. A mechanism of the cellulose synthase reaction was suggested by Saxena et al. [72]: nucleophilic attack of the C4-OH group of the non-reducing chain end at the α-C1 position of the UDP–glucose substrate takes place via a single displacement mechanism with inversion of the anomer configuration, resulting in the formation of β-(1,4)-linkages. A consecutive polymerization is achieved by the so-called two-residue addition model, whereby simultaneous coupling of two monosaccharide monomers occurs successively during chain propagation.

Non-Leloir Glycosyltransferases (Phosphorylases)

In general, sugar-nucleotide-independent glycosyltransferases, often referred to as phosphorylases, catalyze the transfer of a monosaccharide moiety from a poly- or oligosaccharide, or from a nucleoside to an orthophosphate ion, in other words, phosphorolysis of the glycosidic bond. The bonding energy of the resulting sugar-1 phosphate is low enough to make the reaction practically reversible [63]. Consequently, in nature these enzymes are involved in both the degradation and synthesis

of polysaccharides. They all have in common that they catalyze an exo-wise phosphorolysis at the non-reducing end [42, 43, 63] and work in a very strict regiospecific manner, cleaving only "their" type of glycosidic bond [63]. They can be classified either according to the anomeric form of the glycosidic bond they cleave (the anomeric form of the glycosyl-1-phosphate product [63]) or by the reaction mechanism, that is, whether an anomeric retention or an inversion occurs during the catalysis [63]. Phosphorylases are usually named after the substrate to be degraded and, since the first was found almost 100 years ago [73], many phosphorylases have been discovered in a huge number of different organisms. In the field of cellooligomer synthesis, the so-called cellobiose-phosphorylase (EC 2.4.1.20) and, even more so, cellodextrin-phosphorylase (EC2.4.1.49) are of interest. The former catalyzes the reversible cleavage of cellobiose yielding α-glucose-1-phosphate (inversion mechanism) and glucose and can be found in bacteria capable of metabolizing cellulose [74]. The enzyme recognizes the β-anomeric OH group at the reducing end, but only of oligos with a maximum DP of 3 [63]. Thus, the enzyme is not suitable for the generation of higher cellooligosaccharides; nevertheless, it has been successfully used for the generation of trimers [63]. Cellodextrin-phosphorylases, on the other hand, are adequate for the synthesis of cellooligos larger than this, because of their ability to recognize longer chains. Similarly, they catalyze the cleavage of a monosaccharide moiety by an inversion mechanism, releasing α-glucose-1-phosphate and a cellodextrin chain shortened by one monomer [75]. They have only been found in *Clostridia*, which also express cellobiose-phosphorylase [76]. With regard to the back reaction (i.e., glycosidic bond synthesis), they cannot recognize glucose as a substrate, but different types of aryl-β-glucosides and β-glucosyl-disaccharides are properly transferred to the elongating oligosaccharide chain [75]. With their aid, different cellooligosaccharide analogs have been synthesized that can be used as artificial inhibitors for cellulases [77]. Samain et al. [66] reported the phosphorylase-mediated synthesis of crystalline non-substituted cellodextrins as well as cellodextrins substituted at their reducing end (depending on the primer used; see below). The enzyme employed has been isolated from *Clostridium thermocellum* grown on cellulose-based media, inducing the expression of cellobiose-phosphorylase as well as cellodextrin-phosphorylase. The authors exploited an interesting feature of cellodextrin-phosphorylases, namely their ability to synthesize cellooligomers when the enzymes are incubated with a primer ($Glc_{n\ (n\geq 2)}$; e.g., cellobiose) and glucose-1-phosphate to produce Glc_{n+1} and pyrophosphate [78]. In order to remove cellulase activity, the enzyme was purified from cell extracts by precipitation with protamine sulfate and subsequent fractionation with ammonium sulfate. The non-substituted crystalline cellodextrins produced with the aid of this isolate were shown to have an average DP of 8, with crystal structures closely resembling those of low molecular weight cellulose II. It was suggested that the chain elongation does not proceed beyond a DP value of 8 as a result of immediate dissociation of enzyme and oligosaccharide chain after every monomer addition. Therefore, the enzyme requires its substrate to be in aqueous solution [66], which is not possible for non-substituted cellooligomers above a chain length of eight. A similar observation was made earlier by Ziegast et al. [79]

for a potato amylose phosphorylase, additionally supporting this suggestion of an immediate dissociation.

2.2 *Generation of Oligosaccharides by Degradation of Polymers*

For the generation of cellooligosaccharides through degradation of cellulose, a variety of methods was developed during the last century [80, 81]. The breakdown of long polysaccharide chains into smaller fragments requires hydrolytic cleavage of the glycosidic bonds, which can be achieved using different chemical catalysts (usually acids) or specific hydrolytic enzymes (cellulases). The two most prominent methods in this regard are fragmentation of cellulose by acetolysis [82], applying a mixture of acetic acid, acetic anhydride, and concentrated sulfuric acid, and direct acid hydrolysis using hydrochloric acid [83]. The methods, especially those employing halogen acids, rely on the reduction of cellulose crystallinity to render it more amorphous and thus easier to hydrolyze at temperatures where sugar degradation plays a very minor role [84]. Furthermore, a number of direct hydrolysis techniques has been reported that exploit different acids (including sulfuric acid [10], mixtures of hydrochloric and sulfuric acid [85], and weak acids such as pivaloyl acid (pivaloylysis) [86]) and methods that apply water under supercritical conditions [87] without using any chemical catalyst. For many of these methods, a thorough control of process parameters, especially acid concentration, temperature, reaction time, and the nature of the acids and solvents is crucial in order to avoid the formation of unwanted side products [88]. These approaches are discussed in more detail in the following sections.

2.2.1 Acetolysis

The degradation of cellulose by applying a mixture of glacial acetic acid, acetic anhydride, and concentrated sulfuric acid was originally developed by Hess et al. [82] in 1935 and then further explored in several publications, for example by Miller et al. [83], Dickey and Wolfrom [89], Wolfrom and Dacons [90], and Wolfrom and Thompson [91]. The main product compounds of the hydrolysis are peracetylated cellooligosaccharides. In the original form, the hydrolysis mix consists of the three compounds in a ratio of 10:10:1 (acetic acid:acetic anhydride: sulfuric acid) containing about 10–12% (w/w) cellulose. Because contact of the acid with the cellulose substrate is strongly exothermic, the reaction mixture has to be kept below a temperature of 40°C by external cooling. The hydrolysis reaction is allowed to proceed for 60 h before it is quenched by transferring the now pale yellowish cellulose solution into ice-cold water, which precipitates the mixed acetylated oligosaccharides. The cellooligosaccharide acetates are then washed

with H_2O, the excess acid neutralized with $NaHCO_3$, the precipitate washed again with H_2O, dried, and then suspended in anhydrous methanol. The suspension is then filtered, the filtrate evaporated until dry, and the gummy white residue dissolved in a small amount of hot chloroform. In a final step, this solution is transferred into an excess of ice-cold hexane in order to re-precipitate the acetylated cellooligosaccharides, which are dried in a vacuum oven to obtain a solid, pure form. Using this method, the DP of the isolated oligomers ranged from 1 to 6 [80, 89]; a value of 7 has also been reported [90]. Oligomers of higher chain length are present in the product in very low amounts, if at all. The yield of acetylated oligosaccharides is around 32% (w/w), related to the amount of cellulose used [80].

In order to address the preparation of cellooligosaccharides with higher degrees of polymerization, Kaustinen et al. [92] presented a method for selective acetolysis of cellulose to DPs ranging from 18 to 100. The study was inspired by a method originally developed by Frith [93] in an effort to determine the kinetics of the acid-catalyzed acetylation of cellulose. The reaction mixture contains glacial acetic acid, acetic anhydride, and dichloromethane in a ratio of 1:4:6 and either sulfuric or perchloric acid as the catalyst. The amount of cellulose used should be around 3% (w/w). Through variation of the reaction parameters (i.e., type and concentration of catalyst, temperature, and time), different product compounds with regard to the average DP of the peracetylated polysaccharides can be achieved, ranging from DP 18, when hypochloric acid at the highest concentration is used, to DP 100, when sulfuric acid at the lowest concentration is used [92]. The products are isolated by increasing the pH with sodium acetate, which precipitates the oligosaccharides. The grainy, yellowish cellulose triacetate is subsequently washed first with water and then with methanol to remove smaller (water-soluble) saccharides and the yellow color. The yield with regard to the amount of cellulose powder employed was as high as 90% (w/w) [92].

2.2.2 Direct Acid Hydrolysis

Hydrochloric Acid

The preparation of cellooligosaccharides from cellulose by applying hydrochloric acid was first reported by Zechmeister et al. [94] in 1931, later explored by Jermyn [95], and published with modifications by Miller et al. [83, 96, 97], Hamacher et al. [88], and Huebner et al. [98]. The procedure starts with a pre-wetting of cellulose powder in saturated HCl (37% w/w) solution at room temperature, which favors homogeneity in the subsequent stages. The suspension is then treated with fuming HCl at 0°C, resulting in a homogeneous, viscous, yellowish solution containing about 10% cellulose. The HCl concentration needed for complete dissolution is about 40% (w/w) [81], which is achieved by bubbling HCl gas through the saturated solution. The hydrolysis reaction is usually performed for

1–3 h. Again, an efficient cooling system is required in order to avoid formation of unwanted side products. The reaction parameters (time, temperature, and HCl concentration) have a strong impact on the relative yield for different (with regard to their chain length) oligomers [81]. However, rigorous control of those parameters is difficult because of the strong exothermic character of the acid–cellulose contact and the use of an oversaturated HCl solution. In 1960, Miller [83] had already shown that the rate of hydrolysis is linear and that the yield of cellodextrins attains a maximum after 2 h of reaction time. The optimal time and temperature for the preparation of cellodextrins has been determined empirically by several authors, and mathematical models for the degradation kinetics of acid-catalyzed hydrolysis have been proposed [99]. However, the reproducibility of these is rather limited as a result of the above-mentioned complexity of parameter control. After the degradation, the solution has to be neutralized, which is required for subsequent separation procedures and also increases the stability of the cellodextrins [81].

The most common method for increasing the pH is direct neutralization with $NaHCO_3$ [83, 96, 97], which has the disadvantage of producing huge amounts of NaCl. Two alternatives have therefore been developed: (1) preliminary HCl removal by vacuum suction before the neutralizing agent is added [98], necessitating trapping of the evaporated hydrochloric acid gas, and (2) a rather laborious but effective washing procedure with 1-propanol and ethanol [88], which allows the simultaneous removal of excess acid and the main hydrolysis products, glucose and cellobiose. Another possibility would be the application of an anion exchange resin [80], which has the drawback of temperature gradients in the resin bed as a result of the high acid concentration employed. The DP of the isolated cellooligosaccharides does not exceed a value of 7; saccharides of a higher chain length are only present, if at all, in insignificant amounts. The relative yields of the different cellooligosaccharide species are about 13–23% (w/w) [80] with respect to the cellulose amount employed and are also dependent on the method of neutralization after hydrolysis.

With the above-described methods of direct acid hydrolysis using highly concentrated HCl, it is not possible to prepare cellooligosaccharides with a DP above 8 in reasonable amounts, because these fractions are usually removed together with the larger fractions during the procedures. According to Isogai et al. [100], higher cellooligomers can be isolated from cellulosic starting materials by performing the hydrolysis reaction in a heterogeneous state, exploiting the fact that when celluloses are hydrolyzed in dilute acids at high temperatures they display a rapid and drastic decrease in chain length until they reach a constant value, referred to as the level-off degree of polymerization (LODP) [101]. This LODP behavior is thought to be related to the size of the crystalline zones along the cellulose fiber and is therefore dependent on the species and tissue from which the cellulose originates [102]. When alkali-treated native and regenerated celluloses were subject to hydrolysis with 1 M HCl solutions at 105°C for 3 h, the degraded samples (regardless of their origin) showed bimodal size exclusion chromatography

(SEC) elution patterns, indicating the presence of a predominant high molecular mass fraction and a minor low molecular mass fraction [100]. The DP values of the former ranged from 35 to about 100; those of the minor fraction from 18 to 24.

Sulfuric Acid

The fact that cellodextrins are formed as intermediates by the action of concentrated sulfuric acid has been known for a long time [10, 103], but it took until 1984 that Voloch et al. [10] presented a method for the production of cellooligosaccharides by direct acid hydrolysis employing concentrated sulfuric acid. An 80% (w/w) H_2SO_4 solution is added to crystalline cellulose to a comparatively high final concentration of 2 g cellulose per milliliter of hydrolysis solution. Again, because of the exothermic contact of the acid and the polysaccharide, both have to be pre-cooled, and this is performed in an ice-water bath. After stirring for a few minutes, the acid is diluted with water to a H_2SO_4 concentration of 33% (w/w) and the reaction mixture is transferred to a water bath at 70°C where the hydrolysis reaction is allowed to proceed for 14 min. The reaction is quenched by the addition of pre-cooled absolute ethanol and the hydrolyzate, having a dark brown color and containing some unreacted solids, is transferred to an ice-water bath. The color can be removed by adsorption on activated charcoal (pre-wetted with ethanol) and subsequent filtration, resulting in a clear, yellowish solution. The ethanol concentration is then increased to 93–95%, which results in precipitation of the cellodextrins. Excess acid is removed by washing the white precipitate with ethanol. The solids are then dried. The merit of the precipitation with ethanol lies in the fact that glucose and cellobiose, as the main degradation products, stay in solution to a predominant extent whereas oligosaccharides with a $DP \geq 3$ are precipitated. The DP range of the oligomers isolated in this way is from 3 to about 8, and the yield related to the amount of cellulose used around 1.5% (w/w), which is significantly below [10, 81] the value reached by the method employing fuming HCl.

As reported (amongst others) by Kim et al. [104], cellulosic starting materials can be degraded under so-called extremely low acid conditions at elevated temperatures above 200°C. The degradation reactions are performed in different types of reactors with sulfuric acid concentrations as low as 0.07%(w/w), resulting in glucose yields of up to 91% (w/w) with regard to the amount of cellulose used. However, the study focused on the maximization of fermentable monomer yield. A separate investigation focusing on the impact of reaction parameters (especially time and temperature) on the molar mass distribution of the product compound could provide valuable information for the development of new strategies for cellooligosaccharide preparation.

Mixed Acid Hydrolysis

The method of cellodextrin preparation employing a mixture of concentrated hydrochloric acid and concentrated sulfuric acid was first reported by Zhang et al. [85] in 2003. The method somewhat circumvents the disadvantages of the above-described methods, such as the application of fuming HCl in Miller's method [83], the rather time-consuming procedure when cellulose is degraded by acetolysis [82], and the comparatively low yields when concentrated H_2SO_4 is applied [10]. It was shown that the optimal ratio of HCl (37% w/w) to H_2SO_4 (98% w/w) for the production of cellodextrins is 4:1 [85]. Higher amounts of the latter lead to fast formation of by-products by oxidation, as indicated by the occurrence of a black color in the hydrolyzate. Lower amounts significantly increase the time needed to obtain a clear hydrolyzate, to over 12 h. The acids are added in a pre-cooled state, and the hydrolysis reaction is then performed at room temperature. The optimal reaction time was determined to be between 3 and 5.5 h, after which the amount of glucose formed is gradually increasing, while the already formed cellodextrins are successively degraded. The hydrolyzate develops a yellowish color during the reaction, which is stopped by transferring the solution into an excess amount of acetone at –20°C, resulting in abundant formation of a white precipitate consisting of water-soluble cellodextrins as well as cellodextrins with a higher DP. The smaller compounds are extracted by centrifuging, washing the pellet with water, and re-centrifuging, resulting in a clear supernatant containing components with a DP of 1–6 (only very low amounts of DP 7 and 8) and a pellet containing the larger fractions. Because the study focused on the preparation of water-soluble cellodextrins, the higher cellooligosaccharides were not discussed further. Analysis of the remaining pellet, especially with regard to the molar mass distribution, could provide useful information on the interdependency of reaction parameters (acid concentrations, time, and temperature) and the formation of cellooligosaccharides with a higher DP. However, the study revealed that, for the smaller fractions (DP 3–6), yields of about 23% related to the cellulose amount employed were reached after a reaction time of 5.5 h. The individual yields for certain species are strongly dependent on the reaction time, favoring lower DPs after longer reaction times. Table 1 summarizes and compares the different hydrolytic preparation methods discussed.

Other Acids

In addition to the above-described methods using the common strong acids HCl and H_2SO_4, which are the most frequently mentioned in the literature, some other strategies for the degradation of cellulose by acid hydrolysis have been developed. However, most of these comparatively recent investigations focus on the yield maximization of fermentable sugars, and consequently parameter optimization for

Table 1 Preparation of cellooligosaccharides by polymer degradation with the aid of acids

	Acetolysis	Selective acetolysis	Concentrated HCl	Diluted HCl	H_2SO_4	Mixed HCl/H_2SO_4
DP range	1–7	18–100	1–7	18–24/35–100[a]	3–8	1–6
Yield (%)[b]	~32	90	100	–	~1.5	23[c]
Reaction time (h)[d]	>60	1–11	1–3	3	<0.5	3–5.5
Comments	Peracetylated product	Peracetylated product	Difficult reaction control	Heterogeneous reaction	–	–
References	[81, 90, 91]	[92]	[84, 89, 95–99]	[101]	[10]	[85]

[a]Molecular weight distribution of the two fractions is dependent on the type of cellulose used
[b]Yields are given as the ratio of mass of the isolated oligomers over the mass of the employed cellulose/cellulose acetate
[c]Value for the fraction DP 3–6
[d]Net hydrolysis reaction time; subsequent precipitation, neutralization, or purification steps are not included

the preparation of cellooligosaccharides is not part of these publications. Nevertheless, as these methods could potentially be useful for the production of oligomers, for the sake of completeness the most important ones are included in this review. Harmer et al. [105], for example, reported a process giving monosaccharides in high yields from biomass that employed a combination of sulfuric and phosphoric acid at elevated temperatures of about 200°C. The two-step strategy is characterized by a preliminary decrystallization and subsequent hydrolysis of the biomass to glucose and xylose at a conversion ratio of about 90% (w/w). The application of formic acid for the hydrolysis of organosolv-derived pulp at temperatures of 180–220°C was published by Kupiainen et al. [106]. As expected, they found the glucose yields from fibrous cellulose (pulp) to be significantly higher than those obtained from microcrystalline cellulose, which is often used as a model compound for cellulose hydrolysis. A comparison of different dicarboxylic acids with regard to their ability to hydrolytically degrade cellulose was presented by Mosier et al. [107]. It was shown that maleic acid is an especially suitable catalyst for the rupture of the glycosidic bond, because the degradation of microcrystalline cellulose was as effective as with dilute sulfuric acid but only a very small amount of glucose degradation was seen. The lower pK_a value of dicarboxylic acids compared with their monocarboxylic relatives is thought to be the explanation for their better performance as catalysts in polysaccharide degradation [108].

The application of maleic acid and oxalic acid at high salt concentrations was tested by vom Stein et al. [108]. Introducing a high ionic strength by addition of 30% (w/w) NaCl to the catalyst solution allowed the hydrolysis reaction to be performed under comparatively mild conditions (100–125°C), which advantageously results in less thermal decomposition of the sugar. The action of the salt is presumably similar to the action of ionic liquids, helping to break the dense H-bond network of cellulose fibers and thus making the glycosidic bonds better accessible to the catalyst molecules. Production of soluble cellooligomers with yields of up to 5% (w/w) with respect to the amount of cellulose initially employed, at concentrations of 0.25 and 1 g L^{-1}, depending on the type of cellulose, temperature, reaction time, and type and amount of catalyst used, can be reached. Amarasekara et al. [109] presented a study comparing different alkyl/aryl sulfonic acids, especially with sulfuric acid of the same acid strength. They were able to reach yields of up to 30.3% of total reducing sugars (i.e., glucose + soluble oligosaccharides) using 4-biphenylsulfonic acid as a catalyst (160°C, reaction time of 3 h), which is significantly above the value of 21.7% when aqueous sulfuric acid is used. A somewhat radically different approach to the preparation of cellulosic oligosaccharides from cellulose was reported by Redlich et al. [86, 110]. The process, referred to as pivaloylysis, was developed with the aim of generating oligosaccharides under mild conditions with regard to temperature as well as type of catalyst in order to prevent the formation of undesired side products. The hydrolysis is conducted on fully acetylated cellulose with pivalic anhydride and boron trifluoride etherate in dichloromethane at 40°C. Variation of the reaction time allows the specific yield for certain oligomers to be controlled, favoring smaller DPs after longer times and vice versa. Yields for the total amount of acetylated

cellooligosaccharides with a DP range of 1–8 are typically very high (~0.83 g g^{-1} cellulose acetate). However, the method has some major disadvantages, first and foremost the fact that the reaction has to be conducted in the strict absence of water, severely complicating the experimental work, and with long reaction times of about 40–50 h.

2.2.3 Hydrothermal Treatment

As reported by several authors (see, for example, [87, 111–114]), it is possible to hydrolytically degrade cellulose in pure H_2O without using any catalytic agents such as acids or enzymes. Zhao et al. [87] reported a procedure for oligosaccharide generation by supercritical hydrolysis of cellulose and lignocellulose. The authors developed a combined supercritical/subcritical technology that they used as a method for pretreatment and hydrolysis of the starting material. The supercritical step yields mainly oligosaccharides (in addition to some monosaccharides and their degradation products), whereas in the second, subcritical step these oligomers are further hydrolyzed to glucose. That subcritical conditions are more effective than supercritical conditions with regard to glucose production concurs with a publication by Ehara et al. [115]. However, as reported by Jin et al. [116], the decomposition rate of glucose under subcritical conditions is significantly higher than the rate of cellulose hydrolysis, rendering the production of glucose under these conditions complicated. The combined method used by Zhao et al. circumvents these difficulties by "pre-degrading" the cellulose to oligomers under supercritical conditions.

With regard to the production of cellooligosaccharides, the best reaction conditions were found to be a temperature of 380°C and a reaction time of 16 s, with an initial concentration of microcrystalline cellulose of 2.4% (w/w) in deionized water. Using these conditions, 40% (w/w) of the employed cellulose can be converted to cellooligosaccharides with a DP range of 2–6; 24% (w/w) are converted to glucose. The residual amount consists to a large extent of glucose decomposition products. Whether these methods applying supercritical conditions for cellulose degradation can be adjusted for the production of cellooligosaccharides having higher DPs is highly questionable, because the hydrolysis in the supercritical environment is extremely fast [87] and thus thorough control seems almost impossible. Griebl et al. [117] presented a procedure for the formation of xylooligosaccharides through the hydrothermolysis of xylan derived from the steeping-lye of the viscose process. The hydrothermal treatment, conducted at varying temperatures between 120°C and 180°C, resulted in a soluble fraction containing mainly neutral and acidic xylooligosaccharides, and an insoluble fraction that was predominantly highly crystalline cellulose. The DP of the neutral xylooligosaccharides could be varied in a wide range from approximately 1 to 15 by altering the reaction conditions (time and temperature). The isolated acidic fraction displayed a DP range of 3–17.

2.2.4 Enzymatic Degradation

The application of enzymes that are designed by nature to rupture glycosidic bonds for the production of mono- and oligosaccharides from polysaccharides seems obvious at a first glance. However, their employment is not as simple as the use of chemical catalysts, because enzymes need to be purified from cell extracts in laborious procedures and are sensitive to environmental conditions (pH, temperature, and presence of inhibitors) that can alter their activity or even denature them. Moreover, in nature cellulose is not degraded by just one enzyme, but by a combination of three classes of enzymes working together in a synergistic manner [118] to produce sugars that can be metabolized by the corresponding microorganisms [65]. An effective process requires enzyme preparations with the highest possible activity and a cellulosic substrate with sufficiently high reactivity. The commercial availability of purified cellulases with reasonably high activity was practicably negligible before the 1980s and the crude products contained high amounts of impurities in the form of other proteins and had a high price [119]. Therefore, it is no wonder that methods exploiting these biocatalysts for the degradation of polysaccharides had been developed long after the conventional chemical procedures.

A pioneering work in the field of enzymatic hydrolysis of cellulose was published by Reese et al. [119, 120], who made the requirement of a complex of enzymes for the depolymerization of cellulose to glucose commonly accepted [121]. In 1963, the authors described a procedure for the production of cellobiose and cellotriose, employing a cellulolytic filtrate isolated from the supernatant of a *Trichoderma viride* culture grown on cellulosic media [119]. The advantage of the application of enzymes is that the hydrolysis reaction can be performed under very mild conditions with respect to temperature and pH, which avoids the formation of unwanted side products and leaves other components unaltered. Because the generation of sugar decomposition compounds (which often have an inhibiting effect on fermentation processes) is avoided, glucose solutions resulting from enzymatic digestions are very well applicable as substrates for biotechnological processes. For that reason, the vast majority of methods developed focused on the maximization of glucose formation, leaving the formation of oligomeric intermediates relatively untouched. Recent and by no means exhaustive examples of the rare exceptions in this regard are given in the following paragraph. For a deeper insight into the state-of-the-art production of cellulolytic enzymes and their potential in technical applications, the reader is referred to a review by Wang et al. [122] and, for a more biological viewpoint on the topic, to a review by Lynd et al. [65].

Andersen et al. [121] studied the synergy and the interactions of the three enzyme classes with respect to the impact of individual concentrations on the hydrolysis pattern of the product mixture (i.e., the relative amounts of glucose and oligosaccharides formed). The authors performed assays with binary and ternary enzyme cocktails on two different cellulosic substrates, a microcrystalline cellulose and a cellulose pretreated by swelling in phosphoric acid. As expected, the

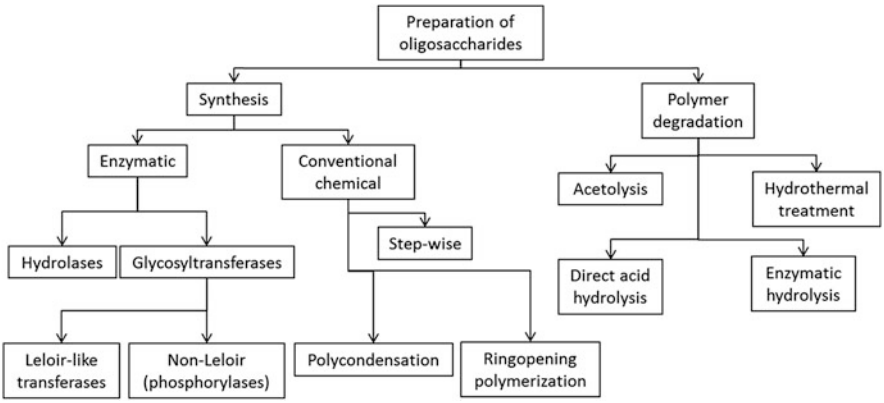

Fig. 5 Preparation of oligosaccharides from celluloses and hemicelluloses

highest yield of soluble oligosaccharides, with a DP range of 1–6, was achieved when working with a relatively low enzyme concentration of 0.1 μM. Interestingly, the relative amount of individual oligomers formed was dependent on the type of cellulose substrate used. A procedure for the coproduction of oligosaccharides and glucose from corncobs was published by Garrote et al. [11]. In a first step, the raw material containing mainly cellulose and hemicellulose (xylan) is subject to autohydrolysis at elevated temperatures of 202–216°C, during which most of the xylan is degraded to xylooligosaccharides. After this hydrothermal step, the reaction mixture consists of a liquid part (referred to as liquor) containing the soluble xylooligosaccharides as well as soluble compounds not originating from the hemicellulose fraction and side products, and a solid fraction consisting predominantly of cellulose. This insoluble part is then subject to enzymatic hydrolysis by cellulases in order to produce fermentable glucose. Under optimal conditions, a maximum xylooligosaccharide yield of 32.2% (w/w) of the dry substance of the raw material can be achieved, while the conversion of cellulose to glucose is almost quantitative, resulting in glucose solutions with concentrations of up to 97.2 g L^{-1}. Rydlund et al. [123] reported the preparation of neutral and acidic oligosaccharides derived from the hemicellulosic fraction of an unbleached birch Kraft pulp with the aid of an endoxylanase from *Trichoderma reesei*. They were able to show that the mixture of hydrolysis products after 24 h at 40°C consists of a neutral fraction (mainly xylose, xylobiose, and xylotriose) and an acidic fraction, bearing α-(1,2)-linked uronic acid groups attached to the xylose unit adjacent to the non-reducing chain end, with a DP of up to 5.

An overview of the different strategies for the preparation of cello- and xylooligosaccharides is given in Fig. 5, summarizing both the synthetic and the degradation approaches.

3 Separation and Analysis of Oligosaccharides

The separation of cello- and xylooligosaccharides according to their DP is a challenging task. Nevertheless, several methods exploiting different separation mechanisms have been developed during the last few decades. On a preparative scale, the most commonly used techniques, according to the literature, are: (1) SEC on particulate polyacrylamide or crosslinked dextran gels [80, 81, 85, 88, 124–129]; (2) partition/adsorption chromatography on charcoal, untreated charcoal–celite, or stearic-acid treated charcoal–celite columns [80, 83, 96] and on cellulose-based stationary phases [80, 130]; (3) hydrophilic interaction chromatography (HILIC) and normal phase high-performance liquid chromatography (NP-HPLC) on silica gels, amino-bonded silica columns, or matrices with copolymer-bonded cyclodextrins [80, 89, 131, 132]; and (4) ion exchange chromatography on cation exchange resins with sulfo-groups coupled with metal counter ions (e.g., Ca^{2+}, Li^{+}, Ag^{+}, Na^{+}, Pb^{2+}) or hydronium ions [10, 80, 98, 133]. For analytical purposes, especially for purity investigations on isolated fractions homogenous with respect to the DP, a variety of methods exist. In this regard, metal-loaded cation exchangers (e.g., sulfonated styrene-divinylbenzene copolymers with Ca^{2+}, Li^{+}, Ag^{+}, Na^{+}, or Pb^{2+}) are widely used stationary phases that can be used for the analysis of non-derivatized oligosaccharides in different operation modes, such as ion exchange, ion exclusion, and ligand exchange [134]. Furthermore, separation of saccharides as their borate complexes on anion exchange resins has been shown to be a powerful technique for the detection and quantification of impurities [81, 88, 124].

High-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD), where the sugars are transformed into their oxyanionic forms at high pH, is another potent method for the analysis of closely related oligosaccharides, which was reviewed recently by Corradini et al. [134]. As reported by Weith et al. [135] and later by Liu et al. [136], sugar boronate affinity chromatography can also be applied as a useful separation method for carbohydrates, exploiting the formation of cyclic diesters between *cis*-diols (sugars) with borate bound to the stationary phase. In principle, oligosaccharides can also be separated by means of classical reversed phase chromatography employing alkylated silica gels [137–139]. However, the chromatograms are often difficult to interpret because anomers of each oligosaccharide are separated. Because these methods have not been much used for the separation of cello- or xylooligosaccharides, reversed phase HPLC techniques are not discussed further in this review.

Especially for analytical purposes, capillary zone electrophoresis (CZE) is another possibility for the study of oligosaccharides derived from xylan or cellulose, provided that they can be dissolved in the used background electrolyte. Rydlund et al. [140], for example, demonstrated the separation efficiency of CZE on xylooligosaccharides. The separation was performed in a concentrated alkaline borate buffer in a fused-silica capillary column at constant power supply (1,200 mW), with on-column UV-detection at 245 nm. A pre-column derivatization was carried out by reductive amination with 6-aminoquinoline (6-AQ) and the

saccharides were separated as their borate complexes. The method was found to have a relatively low minimum concentration limit in the micromolar range, which corresponds to the limit of detection in the femtomolar range. Xylooligomers up to the hexaose were separated with baseline resolution. Sartori et al. [141] applied CZE for the separation of xylo- and cellooligosaccharides derived from alkaline degradation of the parental polymers after pre-column derivatization with *p*-aminobenzonitrile (UV tag). Cellooligomers up to the heptaose were efficiently separated by this method. A high borate concentration was needed in both cases, because electrophoretic mobility is a function of the net negative charge and thus of the extent of borate complex formation. On the other hand, the mobility decreases with the size of the analyte molecules; thus smaller molecules elute first followed by the larger molecules. Furthermore, CZE was demonstrated to be a useful technique for the structural elucidation of wood-derived polysaccharides with respect to their degradation products after hydrolysis, especially when combined with mass spectrometry [123, 142].

Once fractions are obtained that are homogeneous with respect to the DP, or at least have a very narrow molecular weight distribution, they can be further analyzed with regard to structural homogeneity and the presence of impurities having the same molecular weight by means of mass spectrometry (see, for example, [6, 123, 128, 142]) and nuclear magnetic resonance (NMR) spectroscopy (see, for example, [6, 86, 123, 142–144]). For a detailed overview of the mass spectroscopic characterization of oligo- and polysaccharides and their derivatives, the reader is referred to the comprehensive review by Mischnick [145]. In the following sections, some of the above-mentioned separation methods are discussed in more detail, with a slight emphasis on those that can be applied in preparative efforts.

3.1 Size Exclusion Chromatography

Size exclusion chromatography (SEC), also referred to as gel permeation chromatography (GPC), is a technique that allows the separation of analytes according to their hydrodynamic volume. The method is widely used in polymer analysis for the determination of molar mass distributions, in biochemical laboratories for protein or nucleic acid purification, and it is also applied for the investigation of oligosaccharides. In contrast to other chromatographic methods, no enthalpic interaction between analytes and the column material should occur. The stationary phase usually consists of a porous particulate or continuous gel with clearly defined pore sizes. Depending on their hydrodynamic volume, which is an expression of their size in solution, the molecules to be analyzed have different abilities to enter these pores. Small molecules are able to penetrate the pores of the stationary phase, whereas larger molecules (with a higher hydrodynamic radius) leave the column without entering the pores. In other words, the extent to which a molecule can freely diffuse into the pore volume determines its duration in the column. Molecules that are too large to enter the pores elute when the void volume of the column is reached.

Those that are small enough to diffuse in a completely free manner elute with the total elution volume of the column. According to Churms [126], the optimal column length is between 50 and 100 cm (4–8 mm internal diameter), which can be achieved by connecting two or more shorter columns in series. The method has been applied to the separation of cellooligosaccharides in several studies [126].

Polyacrylamide (PAA) gels are the most frequently used stationary phases in these efforts, because they are advantageous in terms of selectivity, resolution, low band broadening, and linearity between the logarithm of the distribution coefficients and the DP [81, 126]. The main drawback of using PAA gels (e.g., BioGel P-2, P-4, or P-6; Bio-Rad, Richmond, CA, USA) lies in the lack of resistance to high pressures, resulting in long separation times of 24 h and more [81, 126]. To overcome this problem, gels have been developed that are capable of withstanding higher pressures, including Trisacryl GF05 (LKB, Bromma, Sweden), which is a crosslinked polymer of *N*-acryloyl-2-amino-2-hydroxymethyl-1,3-propanediol [146], and Toyopearl HW-40S (Toyo Soda, Tokyo, Japan), a hydroxylated-methacrylic polymer [147]. Furthermore, the successful application of diol-modified silica for the separation of oligogalacturonic acids with a DP range of 2–19 was reported by Naohara et al. [148]. However, these high-performance techniques are inferior to conventional SEC with regard to resolution, as reviewed by Churms [149].

Hamacher et al. [88] reported the separation of cellooligosaccharides (DP 1–8), obtained by hydrochloric acid hydrolysis and acetolysis, at a preparative scale using PAA (BioGel P-4; Bio-Rad, Richmond, CA, USA) columns with a total length of 210 cm (5 cm internal diameter) and double-distilled water at 65°C as the eluent. The dry gel had to be especially wind-sieved in advance to give the desired narrow range of particle sizes. Detection was performed with a differential refractive index (RI) detector. Additionally, the system was calibrated with D-glucose eluting at the inner volume of the column and dextran 70 (molecular weight $\sim 7 \times 10^4$ g mol^{-1}) eluting at the void volume. With this set-up, separation up to the cellooctaose was possible with a good resolution, and re-chromatography of the fractions indicated products of uniform molecular weight. However, the time needed for this procedure was more than 22 h. In addition, it was shown that the fractions, although apparently homogeneous according to SEC, contained some side products arising from the harsh conditions during cellulose degradation and possibly also during SEC [124], which was shown by sugar borate chromatography (see Sect. 3.3.2). Similar procedures have been reported by Schmid et al. [124, 125] and Pereira et al. [81]. The procedure can be somewhat advanced by the introduction of the "recycle-SEC" technique [124], which allows a separation of cellooligosaccharides up to a DP of 12. Zhang et al. [85] combined a PAA column (100×5 cm) with a cation exchange column (see Sect. 3.3.1) and efficiently separated water-soluble cellooligomers, obtained through mixed acid hydrolysis, up to a DP of 8 in gram quantities within less than 30 min, demonstrating the good performance of this method in terms of productivity on a preparative scale. In a comparative study, Akpinar et al. [80, 81] tested different chromatographic techniques for the separation of cellodextrins, including SEC on the polyacrylamide gel BioGel P-2. In this

case, SEC was shown to be inferior to other methods, especially adsorption chromatography on charcoal–celite. An effective separation was only possible up to the hexaose and the preparation times were longer than in the case of other methods. However, separation of cellooligosaccharides with a higher DP (10–50) was not addressed by the aforementioned efforts, which is a consequence of the preparation methods, during which these compounds are removed together with the polymer fraction.

Kaustinen et al. [92], who developed the method of selective acetolysis, allowing the preparation of cellooligosaccharides with a defined DP of 18–100 by altering the reaction conditions, used SEC on a silica-based material to determine the molecular weight of their product hydrolysates. Elution was performed with 1:1 (v/v) 1,4-dioxane and 1,2-dichloroethane. Fractions with a molecular weight ranging from 8,000 to 24,000 g mol^{-1} (peracetylated saccharides), corresponding to DP values of approximately 30–100, were isolated. However, as can be demonstrated, the separation performance decreases with a decline in DP, indicating the particular difficulty of analyzing cellooligosaccharides with a DP between 10 and 50. Isogai et al. [100] prepared cellooligomers by dilute HCl hydrolysis that had DPs of 18–24 and 35–100 (depending on the DP of the cellulose used). The authors also used SEC to characterize the hydrolyzates. The water-insoluble oligosaccharides were dissolved in 8% LiCl in *N*,*N*-dimethylacetamide (DMAc) and analyzed on a styrene-divinylbenzene copolymer gel (KD-803; Shodex, Japan) with 1% LiCl/DMAc as the mobile phase. Detection was performed with a combination of a differential refractive index detector and multi-angle laser light scattering (MALLS) to obtain the molecular weight distributions of the products. Fraction collection, which would allow obtaining solutions of cellooligosaccharides with a very narrow molecular weight distribution, however, was not performed.

The SEC analysis of xylan and xylooligosaccharides was reported by several authors, for example, Rasmussen et al. [150] and Deery et al. [128]. Rasmussen [150] used a polymer-based aqueous SEC column (300 × 8 mm, Shodex SB-806 HQ; Showa Denko K.K., Tokyo, Japan) for monitoring the enzyme-catalyzed hydrolysis of xylan substrates. Quantitative profiling of the reaction mixture was performed with 0.1 M sodium acetate as the eluent and an RI detector, using standard compounds (pullanans, xylohexaose, xylose, and dextrans) as molecular weight markers for column calibration. Deery [128] reported the combination of SEC and different mass spectrometry techniques for the characterization of arabinoxylan fragments derived by either acid or enzyme-catalyzed hydrolysis. The polymer-based aqueous SEC columns (PL Aquagel-OH 30 8 μm, 300 × 7.5 mm; Polymer Laboratories, Church Stretton, UK) were calibrated with dextran standards. The mass spectrometry detection methods applied were on-line electrospray ionization mass spectrometry (ESI-MS) and off-line matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

3.2 Normal Phase HPLC and Hydrophilic Interaction Chromatography

Normal phase high-performance liquid chromatography (NP-HPLC) and hydrophilic interaction chromatography (HILIC) are operation modes in which a polar stationary phase is used in combination with a less polar mobile phase for the chromatography of apolar substances. As reviewed by Churms [149], the stationary phases predominantly used for the separation of carbohydrates by partition and/or adsorption in normal phase chromatography can be divided into three groups: (1) silica gels having their surface covered with hydrated hydroxyl groups, (2) matrices in which polar phases are covalently bound to silica gels, and (3) methods based on polymers with polar functional groups. The application of microparticulate silica gels with spherical particles of an average size of 3–5 μm in a packing of homogeneous beads results in good chromatographic efficiency. The material can be used in its unmodified form or with polar groups attached to the gel network. In this regard, aminopropyl silica packings were widely used [149] for the separation of carbohydrates, in spite of the major drawback that reducing sugars react with the amino groups, which leads to loss of analytes and deactivation of the column.

As alternative bonded-phase packings, bonded amide, cyano, diol, and polyol phases have to be mentioned. Furthermore, Alpert [151] suggested the application of a novel stationary phase, in which ethanolamine is incorporated into a coating of polysuccinimide covalently bound to silica. Unmodified silica gel was successfully used for the separation of acetylated cellooligosaccharides in the middle of the past century [89]. In their comparative study of different cellooligosaccharide preparation and separation methods, Akpinar et al. [80] also tested separation by normal phase chromatography on silica gel. Using a 1:1 (v/v) mixture of ethyl acetate and toluene as the eluent, they were able to effectively purify (on a preparative scale) peracetylated cellooligomers (obtained by acetolysis) up to hexaose, and also successfully applied the system for the separation of both acetylated and deacetylated cellooligomers on an analytical scale. Armstrong et al. [152] introduced silica packings bearing cyclodextrins for the analytical separation of carbohydrates, especially for the distinction of isomers. Simms et al. [132] applied β-cyclodextrin columns for the separation of neutral oligosaccharides derived from cellulose, xylan, and other polysaccharides. They used a Cyclobond I column (250×4.6 mm; Rainin Instrument, Woburn, MA, USA) with a matrix of β-cellodextrin molecules coupled to 5 μm spherical silica gel particles via a 10-atom spacer arm, acetonitrile–water mixtures as eluents, and a differential refractometer as detection system. The authors found a clear dependence of the retention behavior on the monosaccharide composition and the types of glycosidic linkages present in the saccharides. The cellodextrin separation material was shown to be similar in selectivity to aminoalkyl-bonded silica gels but superior in durability. Berthod et al. [131] investigated cyclodextrin columns with regard to the separation mechanism of oligosaccharides from different origins including cello-

and xylooligosaccharides. They used commercial cellooligomer standards up to a DP of 5 and xylooligomer standards up to a DP of 6. It was shown that partitioning between the mobile (different acetonitrile–water mixtures) and the stationary phase and hydrogen bonding are the two possible mechanisms responsible for carbohydrate retention in these columns. For detection, an RI detector as well as a UV detector operated at 190 nm were employed.

Because the solubility of silica in aqueous solutions increases rapidly above a pH of 8 and below a pH of 2 and is, moreover, dependent on the concentration of water and buffer [149], the application of the aforementioned materials is somewhat restricted. To overcome this problem, polymer-based sorbents that are stable in environments of very high or very low pH have been developed [149]. The materials must be able to withstand the high pressures and flow rates applied in HPLC methods without deformation, shrinking, or swelling with the solvent. Important examples of such macroporous polymers carrying polar functional groups used for HILIC of carbohydrates are highly crosslinked sulfonated polystyrene cation exchange resins and vinyl polymers. Mobile phases for partition chromatography on silica materials include different organic solvents (very often acetonitrile) mixed with water in different ratios for adsorption chromatography of carbohydrate derivatives on silica [149].

A widely used detection system for HILIC of sugars is the RI detector. However, although the detection limits have improved in recent years, RI detectors display a major drawback, namely their sensitivity to changes in solvent composition. Thus the elution of higher oligosaccharides, which usually requires a gradient elution mode, cannot be followed with this detection system. Evaporative light-scattering (ELS) detection, which is compatible with changing solvent compositions, has therefore emerged as a good alternative [153], extending the upper limit of the resolution of oligosaccharides to a higher DP. ELS detectors are able to detect sugars and alditols with much higher sensitivity than RI detectors. They have great baseline stability and are independent of changes in temperature [153]. The authors recently tested the application of an ELS detector for the detection of fully acetylated cellooligosaccharides with a higher DP obtained by acetolysis of

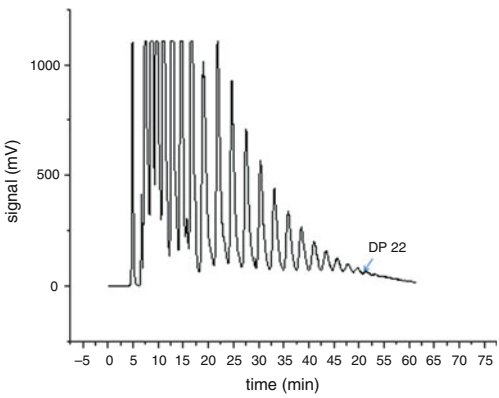

Fig. 6 Evaporative light-scattering detection of acetylated cellooligosaccharides after normal phase separation on a silica-based column

microcrystalline cellulose. Separation was realized with a normal phase silica column up to a DP of 22. Eluents were ethyl acetate and toluene. A linear gradient starting with 70% of the former to 100% within an hour was used. The results can be seen in Fig. 6.

It was demonstrated that detection of even very low amounts of cellooligomers is possible using an ELS detector, and that a good separation of compounds having a DP of up to 22 is easily feasible using NP-HPLC in gradient elution mode with common solvents.

Other possibilities for effective detection of the analytes after silica or silica-based chromatography is pre-column derivatization introducing chromophoric or fluorescent groups into the analytes, and post-column derivatization allowing fluorescence, UV, or electrochemical detection systems [149].

Adsorption chromatography on charcoal–celite columns is a widely used and effective technique for the preparative separation of cellooligosaccharides. The method, displaying the benefit of an inexpensive column material and easily available elution solvents, has been known for a long time and was used in early attempts of oligosaccharide separation by, for example, Miller et al. [83]. Separation of analyte molecules is achieved by adsorption on the charcoal surface, while the celite is used to improve the flow characteristics of the charcoal, which is necessary because of the granulation of the charcoal [80]. For a given series of homologous oligosaccharides, retention is a function of the molecular weight, because the extent of adsorption increases strongly and regularly with an increase in molecular weight. Once oligosaccharides are adsorbed in the stationary phase, they can be desorbed by using a water → ethanol gradient. In order to improve desorption and eliminate re-adsorption of oligomers with a higher DP, the charcoal is often treated with stearic acid [80]. It was demonstrated [80, 83] that, with this method, oligomers of DP 1–7 can be separated with good resolution. However, adsorption chromatography with charcoal–celite columns displays some major drawbacks. First of all, the time needed for a separation run is extremely long (several days), because the packing is not able to resist high pressures. Once used, the separation capability of the material is diminished, so that a new adsorbent material has to be prepared before each run [98].

A comparatively newly developed stationary phase for the fractionation of cellooligosaccharides was developed by Akpinar et al. [130] using a cellulose-based material for adsorption chromatography with water–ethanol mixtures as eluents. This method exploits the affinity of the oligomers to their parent polymers. The main advantage lies in the fact that cellulose is a relatively inexpensive material that is readily available at cellooligosaccharide processing facilities, and that these columns can easily be regenerated by purging with water. However, with regard to the separation performance, the cellulose-based columns are considerably inferior to other systems, allowing an effective fractionation only in terms of separating oligomers above DP 4 from smaller oligomers.

3.3 Ion Exchange Columns

3.3.1 Cation Exchange Resins

The use of cation exchange resins, such as sulfonated polystyrene-divinylbenzene copolymer matrixes, has been shown by several authors to be a powerful technique for oligosaccharide separation at both analytical and preparative scales [10, 80, 81, 124, 125, 133]. The mode of action of these columns combines a variety of separation mechanisms, including ion exclusion, ion exchange, ligand exchange, size exclusion, reversed phase, and normal phase partitioning, and is referred to as ion-moderated partitioning [154]. The application of such stationary phases for the HPLC of cellooligosaccharides was introduced by Ladisch et al. [155] using Ca^{2+} as a fixed counter ion, and later by Bonn et al. [156] employing Ag^{+} counter ions. Both methods were developed for analytical scale separation and were found to have a good scale-up potential for the production of cellooligosaccharides in larger amounts [157]. Since then, these materials have been widely used for the chromatographic separation of oligosaccharides. Ladisch et al. [133], for example, used a cation exchange resin in its Ca^{2+} form to separate cellodextrins from DP 1 to DP 7 effectively within 30 min using water as the eluent and a differential RI detection system. The same authors combined a strong cation exchange resin (Ca^{2+}) column with a SEC column for a one-step desalting and separation procedure for the preparation of pure component solutions of cellooligosaccharides of DP 2–7 [98]. Pereira et al. [158] employed a styrene-divinylbenzene cation exchange matrix with Ca^{2+} as counter ion and H_2SO_4 as the eluent for the separation of cellodextrins obtained according to Voloch method [10]. The analytical column combined with an RI detector was shown to be suitable for the analysis of submicrogram quantities of oligosaccharides. Zhang et al. [85] also combined a strong cation exchange column (29×5 cm internal diameter) in its Ca^{2+} form with a SEC column (100×5 cm internal diameter) and water as the mobile phase. Through their procedure, a separation of cellodextrins of DP 1–8 at a preparative scale was possible; however, a major drawback of the method was the long run time of about 24 h. In a comparative study by Akpinar et al. [80], cation exchange resins were also tested regarding their cellooligosaccharide separation capabilities. A polystyrene-divinylbenzene stationary phase in its Ag^{+} form was used with water as the eluent and was able to resolve cellooligosaccharides up to the hexaose in less than 1 h.

Table 2 gives an overview of the most important chromatography procedures used for the separation of cellooligosaccharides at a preparative scale.

3.3.2 Anion Exchange Chromatography

Anion exchange chromatography of sugar-borate complexes is one procedure used for analysis of oligosaccharides. For analytical purposes carbohydrates can be

Table 2 Preparative separation techniques for cellooligosaccharides

Chromatography mode	Stationary phase	Mobile phase	Separation range	Comments	References
SEC	Polyacrylamide	Water	1–8	Long run times	[88]
	Polyacrylamide	Water	1–12[a]	Long run times	[124]
	Polyacrylamide	Water	1–8	Connected in series with a CIEX column	[85]
	Silica-based	1:1 (v/v) *p*-diosane: 1,2-dichloroethane	30–100[b]	–	[92]
	Styrene-divinylbenzene copolymer gel	1% LiCl in DMAc	18–100	No fractionation performed	[100]
NP-HPLC/ HILIC	Silica based	1:1 (v/v) ethylacetate: toluene	1–6	–	[80]
		Acetonitrile–water mixtures	1–5	–	[131, 132]
Adsorption	Charcoal/celite-based	Water–ethanol gradient	1–7	Long run times, column material not reusable	[80, 83]
	Cellulose-based	Water–ethanol mixtures	≤4 from larger oligomers	–	[130]
Ion moderated	Styrene-divinylbenzene copolymer-based	Water	1–7	–	[133]
	Styrene-divinylbenzene copolymer- and Polyacrylamide-based	Water	1–8	Connected in series with a SEC-column	[85]

[a]Recycle-SEC mode (rechromatography of eluted solution)
[b]Fractions were not monodisperse but displayed a narrow molecular weight distribution

separated in anion exchange columns after transformation to their corresponding borate complexes. Oligosaccharides that have been purified by other methods can be further investigated as potential side components of the mixture by these means. Hamacher et al. [88] used an anion exchange resin for the detection of structural heterogeneities of cellooligomers that were homogeneous according to SEC. They used a strong-base anion exchange resin (DURRUM DA-X4-20) at 60°C with dimension of 0.6×30 cm and applied a two-step borate buffer elution followed by a regeneration and equilibration procedure to separate the borate complexes of the oligosaccharides. As a detection system, a post-column derivatization strategy with orcinol–sulfuric acid reagent was used, followed by measurement of the absorbance at 420 nm. The authors reported the presence of several other oligomers in the sample solutions. These secondary components were found to contain at least one monomeric unit that was structurally different from glucose. Similar results were published by several other authors, for example, Schmid et al. [124, 125] and Pereira [81].

HPAEC-PAD is another powerful method of sugar analysis and exploits the fact that carbohydrates are weak acids with pK_a values between 12 and 14 and can thus be transformed into their oxyanion form under strongly alkaline conditions and then readily separated in anion exchange columns. After passing through the column, the anions can be detected directly by pulsed amperometric detection, typically using platinum electrodes operated in a three-step potential wave form, which provides the ability for effective detection and simultaneous prevention of electrode fouling [159]. However, strongly alkaline solutions in combination with sugar analysis are often problematic because of possible degradation and β-fragmentation reactions, which can alter the product distribution of the sample considerably; this also applies to HPAEC, as discussed below. Corradini et al. [134] reviewed the application of HPAEC-PAD for the analysis of carbohydrates of interest in food science. The method, which is applied in a variety of routine monitoring and research applications, offers the possibility to separate all classes of alditols, aminosugars, mono-, oligo-, and polysaccharides according to structural features including size, composition, anomericity, and type of glycosidic bonds. A major requirement for the stationary phases is the ability to maintain stability at very high pH values, which is, for example, the case for quaternary ammonium-bonded pellicular anion exchange materials. The method inherits the advantage of good performance in terms of selectivity and efficiency. Furthermore, the method avoids common detection problems, such the sensitivity of RI detectors to the changing eluent composition and absorption of UV light by the solvent when using a UV detector. The main separation parameters with regard to the analyte molecules are DP and linkage position, which means that, for a series of homologous oligosaccharides, retention is directly proportional to the DP and indirectly proportional to the pK_a in a regular and predictable manner.

For the separation of oligosaccharides, CarboPac PA100 and PA200 (Dionex) are widely used columns, usually operated in a sodium acetate gradient elution mode. Griebl et al. [117], for example, used HPAEC-PAD as an analysis method for the characterization of the SEC fractions of the hydrolyzate obtained by

hydrothermolysis of xylan. The column was a DionexCarboPac PA100 with dimensions of 4×250 mm. The system was calibrated with xylooligosaccharide standards having DPs of up to 6 and fucose as internal standard. Higher xylooligosaccharides had to be quantified by extrapolation because of the non-availability of standards. Elution was performed in gradient mode starting with pure 0.15 M NaOH, to 0.15 M NaOH plus 0.5 M NaOAc.

3.4 Sugar Boronate Affinity Chromatography

Sugar boronate affinity chromatography was first introduced by Weith et al. [135] in 1970. The stationary phase usually consists of phenyl-boronate-agarose with immobilized boronate ligands, which display great specificity for a wide variety of compounds containing *cis*-diols (e.g., nucleosides, nucleotides, and carbohydrates). The separation principle of these columns is an esterification reaction between the boronate ligands and *cis*-diols [136]. Boronate, usually having a trigonal planar geometry, can be hydroxylated under alkaline conditions, resulting in a tetrahedral boronate anion that is able to react with the *cis*-diol analytes. The product diester can then be hydrolyzed by decreasing the pH, reversing the reaction. The method has been used in many publications for the separation of different mono- and oligosaccharides and for the analysis of purified cellodextrin fractions. Schmid et al. [124, 125], for example, used sugar boronate affinity chromatography for the purification of cellooligomers obtained by acetolysis or direct acid hydrolysis that were homogenous with regard to their DP according to SEC. The authors used preparative phenyl boronate-agarose columns (PBA 60; Amicon, Danvers, MA, USA) with dimensions of 100×0.9 cm internal diameter and 100 mM $(NH_4)_2CO_2$ buffer (pH 10.5) as the mobile phase. The method was shown to be very effective for detecting impurities and for preparative purification procedures. The impurities, often characterized by having at least one monomer different to glucose, could not be separated or even detected by other methods such as SEC or HPLC on cation exchange resins, demonstrating the unique power of sugar boronate affinity chromatography.

4 Summary and Outlook

Cello- and xylooligosaccharides with a DP between 2 and approximately 30 offer a wide field of potential applications. In addition to their use as anti-nutritional additives in the food industry and their employment as coating agents in the pharmaceutical industry, oligomeric compounds originating from cellulose in particular are of greatest interest for research on physicochemical properties as a function of DP, for structural and macromolecular investigations, as well as for studies of (enzymatic) cellulose hydrolysis. They are also gaining increasing

importance as intermediates in current biorefinery scenarios. In this regard, it is of central significance to have procedures available that allow the production of cello- and xylooligosaccharides with a defined DP that can be used as standard compounds in analytical efforts. Nevertheless, targeted techniques to generate these compounds at a preparative scale and, even more demanding, to separate them according to their DP and analyze the obtained fractions have not been fully developed. Principal strategies for the preparation of oligosaccharides are either enzymatic or conventional syntheses using the respective monomers as starting material or partial hydrolysis of the parent polymers, which can be achieved with the aid of different acids or enzymes. For the separation of the mixtures of oligomers obtained in this way, different chromatography modes have been evaluated; SEC and NP-HILIC have turned out to be the most promising techniques. Remarkably, most publications dealing with the degradation of cellulose and hemicellulose focus on the production of monomeric sugars or very short-chained oligosaccharides that can be subjected to fermentation processes. This is probably the reason why the preparation, separation, and analytical methods for oligomers having a DP between 8 and 30 are still in the early stage of development. Future studies should elucidate which methods are the most suitable for isolation of cello- and xylooligosaccharides and how they can be advanced.

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