Soluble cello-oligosaccharides produced by carbon catalyzed hydrolysis of cellulose

Pengru Chen,[a],[b] Abhijit Shrotri,[a] and Atsushi Fukuoka*[a]

Abstract: Cello-oligosaccharides are biologically important molecules that can elicit a defensive immune response in plants and improve the health of animals. Cellulose, a polymer of glucose linked by β-1,4-glycosidic bonds, is an ideal feedstock for synthesis of cello-oligosaccharides. However, cello-oligosaccharides rapidly degrade under the conditions used for cellulose hydrolysis. Here, we report the hydrolysis of cellulose over carbon catalyst in a semi-flow reactor to achieve high yield of cello-oligosaccharides (72%). The excellent activity of oxidized carbon catalyst, adsorption of cellulose on the catalyst and high space velocity of products in the reactor were essential. We also developed a method for quantification of individual cello-oligosaccharides, which suggested a reduction in the rate of hydrolysis with a reduction in chain length.

Cello-oligosaccharides are short chain linear polymers of glucose linked by β-1,4-glycosidic bonds. They are biologically active molecules with physiological properties that can benefit the health and growth of plants, animals and humans.[1] A low concentration dose of cello-oligosaccharides to plant elicits an immune response and increases resistance towards diseases.[2] Animal studies have shown that adding cello-oligosaccharides to the feed of calves causes fewer incidences of gastrointestinal infections and reduces the use of antibiotics.[3] Cello-oligosaccharides also have the potential to act as a non-digestible prebiotic that can reduce the risk of lifestyle diseases in humans by promoting the growth of beneficial bacteria and inhibiting pathogenic bacterial species in the gastrointestinal tract.[1b,4] Thus, cello-oligosaccharides can provide multiple benefits to the agricultural and healthcare industries.

Research for studying the benefit and application of cello-oligosaccharides is hindered by their limited availability. Cellobiose, containing two glucose units, is the only commercially available cello-oligosaccharide. Higher cello-oligosaccharides are produced in small amounts and are up to 500 times more expensive.[4] Commercial application of cello-oligosaccharides is not feasible until a cost-effective process is developed for their synthesis.

Cello-oligosaccharides can be produced by partial hydrolysis of cellulose, an abundant polysaccharide made up of β-1,4 linked glucose units.[5] However, the packed crystalline structure of cellulose and the presence of hydrogen bonds make it resistant to chemical attack.[6] Severe reaction conditions are required to overcome these barriers and cleave the β-1,4-glycosidic bonds by hydrolysis.[7] As a result, cello-oligosaccharides are only obtained as intermediates that rapidly hydrolyze to glucose during the reaction (Figure 1).

Previously, the enzymatic hydrolysis of cellulose was modified by removing β-glucosidase from the enzyme mixture to produce cellobiose from cellulose.[8] However, higher oligosaccharides cannot be obtained by this method. Homogeneous catalysts, such as 3.8% HF/SbF_5[9] and 85% H_3PO_4[10] could dissolve and hydrolyze cellulose, resulting in cello-oligosaccharides with high degree of polymerization (DP). Issues such as catalyst separation and toxicity limited the application of these methods in food and agricultural industries. Recently, depolymerization of cellulose was achieved by solvent-free mechanocatalysis.[11] Although cellulose was completely converted to water-soluble oligosaccharides, 70% of them contained α-1,6 linkages formed by glycosylation of small sugar molecules.[12] These reports also highlight the importance of characterization of oligosaccharides to detect undesired functionalization.

Carbon materials containing oxygenated functional groups are potential heterogeneous catalysts for the synthesis of cello-oligosaccharides from cellulose.[6a, 13] These functional groups are stable under the ball milling and hydrothermal conditions required for cellulose hydrolysis.[14] During the reaction, cellulose adsorbs on the carbon surface by CH-π and hydrophobic interactions[15] and then the β-1,4-glycosidic bonds are hydrolyzed by the acidic functional group present on the catalyst surface.[16] Here, we report the use of carbon catalyst for hydrolysis of cellulose in a semi-flow reactor to obtain high yield of cello-oligosaccharides that can be readily used in food and agricultural industry.

Activated carbon (AC) was oxidized under air at 698 K for 10 h to prepare the carbon catalyst (AC-Air). The oxidation treatment reduced the surface area of AC from 1143 $m^2\ g^{-1}$ to 877 $m^2\ g^{-1}$ without altering the microporous structure (Figure S1A). A peak for C=O stretching of carboxyl groups appeared in the Fourier transform infrared spectroscopy (FT-IR) spectrum at 1710 cm^{-1} after oxidation, suggesting incorporation of weakly acidic functional groups (Figure S1B).[17] The total amount of acidic functional groups was measured as 2560 μmol g^{-1} by titration.[18]

Cello-oligosaccharides was obtained in 71% yield by hydrolysis of cellulose using AC-Air in the semi-flow reactor (Figure S2) at 453 K with a space velocity (SV) of 70 h^{-1} (Figure 2, MMC AC-Air). Glucose (6.8%) and cellobiose (5.0%) were obtained as minor products. A trace amount of levoglucosan (0.2%), a glucose degradation product, was also detected. The presence of acidic functional groups and the adsorption of cellulose on carbon by mix-milling were both crucial for high cello-oligosaccharide yield.

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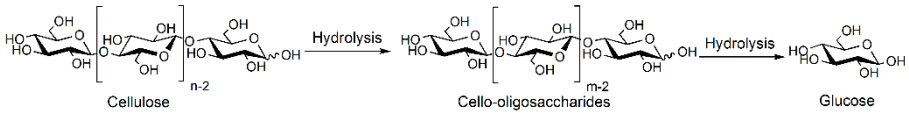

Figure 1. Cello-oligosaccharides are formed as intermediates in the cellulose hydrolysis reaction

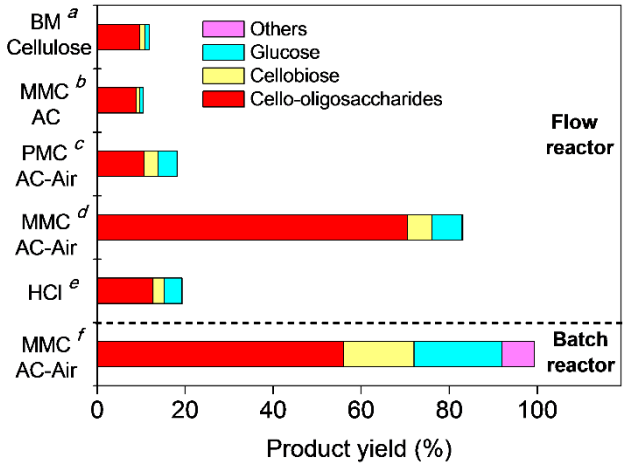

Figure 2. Yield of products after hydrolysis of cellulose. Reaction conditions for flow reactor: 453 K, 40 min, 0.75 mL min^{-1} distilled water. [a] 0.150 g ball milled cellulose. [b] 0.175 g cellulose mix-milled with AC (substrate to catalyst ratio S/C= 6.5). [c] 0.175 g cellulose physically mixed with AC-Air (S/C = 6.5). [d] 0.175 g cellulose mix-milled with AC-Air (S/C = 6.5). [e] 0.150 g ball milled cellulose, 0.012 wt. % HCl aqueous solution instead of water. [f] Reaction conditions for batch reactor: 0.374 g cellulose mix milled with AC-Air (S/C = 6.5), 40 mL distilled water, 453 K, 20 min.

In the absence of a catalyst when ball-milled cellulose was used as substrate, the yield was 9.7% along with 2.2% of glucose and cellobiose (Figure 2, BM cellulose). A similar result was obtained when cellulose was adsorbed on AC by mix-milling. In addition, a homogeneous acid catalyst (0.012 wt. % HCl, pH 2.5) only contributed 13% yield of cello-oligosaccharides. When cellulose was physically mixed with AC-Air, the selectivity for cello-oligosaccharides was reduced. These results suggest that the catalytic activity is markedly increased by adsorbing the cellulose on oxidized carbon catalyst via mix-milling.[19] In this context, we evaluated the effect of mix-milling on the yield of cello-oligosaccharides (Table S1). With an increase in mix-milling duration, the hydrolysis rate of cellulose enhanced, which contributed to higher yield. X-ray powder diffraction (XRD) of all mix-milled samples showed that the cellulose was amorphous, and the degree of crystallinity did not influence the rate of hydrolysis (Figure S3). Consequently, we concluded that the reactivity increased owing better adsorption of cellulose on the carbon surface resulting from longer milling time. Adsorption resulting from 1 h of mix-milling was enough and further increase in milling duration did not affect the yield or selectivity of cello-oligosaccharides. The semi flow reactor, operating at an SV of 70 h^{-1}, prevented the secondary hydrolysis of cello-oligosaccharides. In the case of batch reactor, a large amount of dimer, monomer and degradation compounds were obtained even under optimized condition (Figure 2).

The advantage of semi-flow reactor was further established by varying the hydrolysis temperature. At lower temperatures below 443 K, the total yield after 40 min was low as the rate of hydrolysis was slow (Table S1). Plotting the evolution of products showed that the hydrolysis was not complete within 40 min at lower temperature (Figure S4). The highest yield of cello-oligosaccharides was obtained as 72% at 473 K (Table S1). Increase in temperature did not cause over hydrolysis and the yield of glucose was 6.4% even at 523 K. Furthermore, replacing pure cellulose with recycled paper or eucalyptus biomass also produced cello-oligosaccharides as the main product (Figure S5). To explore the distribution of products in the hydrolysis mixture, we developed a method for qualitative and quantitative analysis of individual cello-oligosaccharides using matrix assisted laser desorption / ionization – time of flight mass spectroscopy (MALDI-TOF MS). The spectrum of freshly obtained product solution (Figure 3A) revealed the peaks were separated by m/z of 162, indicative of a polymer containing anhydroglucose monomer units. Cello-oligosaccharides with DP up to 13 were observed, which involved multiple peaks corresponding to $[Mw - H_2O + Na]^+$, $[Mw + Na]^+$ and its isotopes, and $[Mw + K]^+$ (e.g., cellopentaose, Figure 3B). The total area of all these peaks was combined during analysis. For quantification, internal standards with different molecular weights such as β-cyclodextrin[20] and xylo-oligosaccharides did not work. However, cello-oligosaccharides showed an excellent correlation between concentration and peak area among themselves. Therefore, here we used cellotriose as a reference for the quantification. Firstly, the absolute concentration of cellotriose in the sample was measured. Secondly, the concentration of other cello-oligosaccharides was evaluated relative to cellotriose. For determination of cellotriose, the diluted sample (10 times) was spiked with increasing amount of cellotriose and then analyzed. The normalized area for cellotriose peaks was then plotted against the concentration of added cellotriose (Figure 3C). The concentration of cellotriose in the unadulterated sample was determined as 0.038 mg mL^{-1} by extrapolating the plot to the point where the value of ordinate became zero.[21] Based on this concentration, the carbon yield of cellotriose was calculated as 8.4%, which was similar to the value calculated by high performance liquid chromatography (HPLC) (7.9%).

The concentration of other cello-oligosaccharides was calculated using equation 1.

$$[G_x] = R_{fx} \times [G_3] \times \frac{AG_x}{AG_3} \qquad (1)$$

Where $[G_x]$ is the concentration of cello-oligosaccharide with DP of x and $[G_3]$ is the concentration of cellotriose measured in the first step. AG_x and AG_3 are area of peaks for respective cello-oligosaccharides. Rf_x is the response factor for individual cello-oligosaccharides in comparison to cellotriose. It was calculated by preparing a solution of cellotriose and cello-oligosaccharide

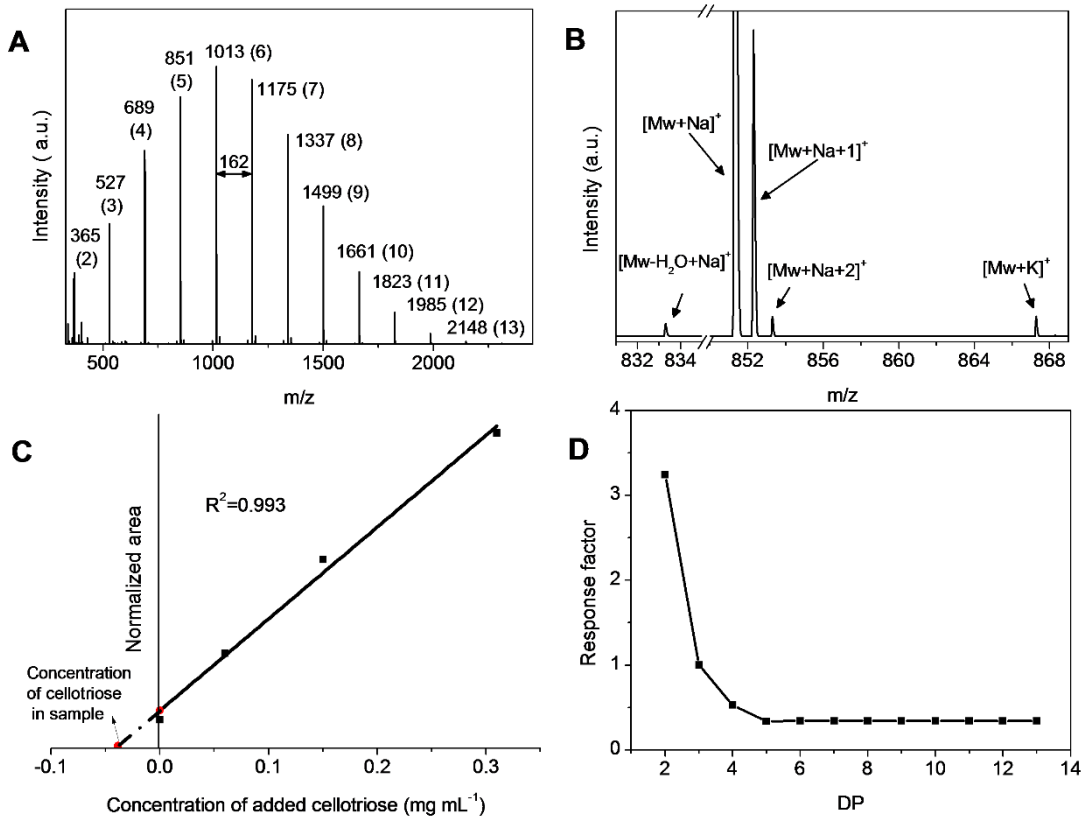

Figure 3. MALDI-TOF MS of cello-oligosaccharides. (A) mass spectrum of product solution, (B) expansion of the spectrum showing all peaks corresponding to cellopentaose, (C) the relationship of normalized area with concentration of added cellotriose, (D) plot showing relationship between response factor and DP of cello-oligosaccharides. Reaction conditions: 0.175 g (S/C = 6.5) MMC, 473 K, 40 min, 0.75 mL min^{-1} water, 1 h milling.

standards (G_2-G_6) with varying concentration ratio and determining the area in MALDI-TOF MS. A plot of the area ratio versus concentration ratio showed a linear relationship for all cello-oligosaccharides (Figure S6). The slope of the linear fit was set as Rf_x and used to calculate the yield of G_2 (9.9%), G_4 (9.0%), G_5 (8.8%) and G_6 (11%). Concentration of higher cello-oligosaccharides was calculated by assuming that Rf_x is constant for cello-oligosaccharides with DP more than 4 (Figure 3D). The result calculated by MALDI-TOF MS is shown in Table 1. The total yield of cello-oligosaccharides (69%) was in good agreement with the value calculated from HPLC (70%). The quantification by MALDI-TOF MS revealed that cello-oligosaccharides with DP less than 10 were the primary products.

The distribution of cello-oligosaccharide was controlled by altering the SV of the reaction. At a high SV of 70 h^{-1}, G_6 and G_7 were the major components. The SV was reduced by increasing the reactor volume, which also increased the sample loading. The decrease in SV did not prolong the time required for completion of the reaction (Figure S7). Cellotriose was obtained as a major product at SV of 20 h^{-1}. However, the composition remained unchanged by further reducing the SV to 13 h^{-1}. This result indicates that cello-oligosaccharides with lower DP undergo hydrolysis at a slower rate ascribed to their lower adsorption coefficient on carbon surface.[15]

The cello-oligosaccharide mixture was further characterized by FT-IR and proton nuclear magnetic resonance (^{1}H NMR) analysis after precipitation in ethanol. They were chemically identical to cellulose and lacked glycosidic bonds except β-1,4 linkages (supplementary text). Individual cello-oligosaccharides (G_3 to G_6) were separated by preparative HPLC and were also evaluated by ^{1}H NMR (Figure 4). The anomeric region showed a doublet at δ_H 4.50 (δ_H denotes ^{1}H chemical shift in ppm) which was assigned to the β-1,4 linked monomers with a non-reducing end (nr).[22] The dominant doublet at δ_H 4.52 was identified as β-1,4 internal linkages (i). As the length of cello-oligosaccharides chain increased, the resonances of β-1,4-i also increased in relative intensity. The doublets detected between δ_H 4.6 to 4.7 and δ_H 5.2 to 5.3 were characteristic of β reducing ends and α reducing ends, respectively. The chemical shifts around δ_H 5.0, indicative of branching α-1,6 linkages, were not detected.[12b] Furthermore, no unexpected peaks were observed in the full ^{1}H NMR spectra (Figure S8). These findings revealed that monomers in oligosaccharides were only linked by β-1,4 bonds. In addition, the number average DP (DP_n) of cello-oligosaccharides was calculated using equation 2

$$DP_n = \frac{A_i + A_{nr}}{A_\beta + A_\alpha} + 1 \qquad (2)$$

Where, A_i and A_{nr} are areas for peaks assigned to internal and non-reducing β-1,4 glycosidic bonds. A_β and A_α are areas for

Table 1. The yield of cello-oligosaccharides at different SV calculated by HPLC and MALDI-TOF MS.

Component	Yield (%)			
	HPLC	MALDI-TOF MS		
	SV = 70 h^{-1}	SV = 70 h^{-1}	SV = 20 h^{-1}	SV = 13 h^{-1}
Glucose	6.9			
G_2	5.3	9.9	14	15
G_3	7.9	8.4	14	13
G_4	-	9.0	11	11
G_5	-	8.8	7.7	7.4
G_6	-	11	7.5	7.1
G_7	-	11	6.1	5.8
G_8	-	9.0	4.3	4.3
G_9	-	6.0	2.7	2.6
G_{10}	-	3.3	1.3	1.3
G_{11}	-	1.4	0.10	0.40
G_{12}	-	0.50	0.03	0.10
G_{13}	-	0.10	0.01	0.10
G_3+[a]	70	69	55	53

[a] Total yield of G_3-G_{13} cello-oligosaccharides. Reaction conditions: MMC, 473 K, 20 min, 0.75 mL min^{-1}, 1 h mix-milling.

peaks assigned to the β and α reducing ends. The calculated DP_n of 3.2, 4.3, 5.4 and 6.5 for G_3-G_6, were in good agreement with the expected values.

In conclusion, we achieved high yield of cello-oligosaccharides from cellulose hydrolysis using weakly acidic carbon catalyst in conjugation with a semi-flow reactor. Presence of acidic functional groups on the carbon surface, adsorption of cellulose on catalyst and high space velocity contributed to maximize the product yield. ^{1}H NMR and FT-IR analysis confirmed the structure of β-1,4 linked straight chain cello-oligosaccharides and the absence of branching or impurities. We believe that this method can be used for large-scale synthesis of cello-oligosaccharides for application in agricultural and healthcare industries, especially by combining a clear understanding of their composition determined by MALDI-TOF MS. This process utilizes food grade activated carbon as catalyst which is oxidized using air. The avoidance of toxic chemicals during catalyst synthesis and the reaction ensures that the cello-oligosaccharide solution is compatible for use in agricultural and healthcare industries without the need for cost-intensive purification.

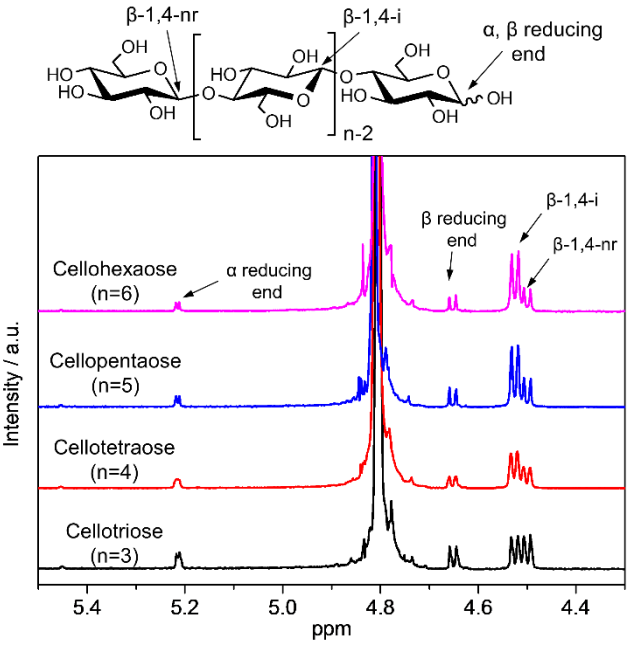

Figure 4. ^{1}H NMR spectra of the anomeric region of individual cello-oligosaccharides in D_2O.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: cellulose hydrolysis • cello-oligosaccharides • carbon catalyst • flow reactor • MALDI-TOF MS

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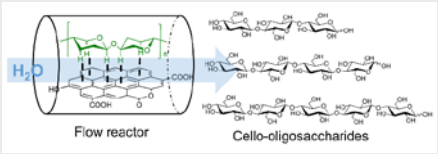

Benign carbon catalysts containing weakly acidic functional groups hydrolyze cellulose to produce food-grade cello-oligosaccharides. Adsorption of cellulose on the carbon surface and high space velocity of process maximized the cello-oligosaccharide yield. The quantification of individual cello-oligosaccharides was done by a method based on MALDI-TOF MS analysis, which showed dependence on the space velocity of the process.

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