

Article

Phosphoric Acid-Assisted Enzymatic Production of Water-Soluble Cellulosic Oligomers

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Abstract: Water-soluble cellulosic oligomers (WCOs) are increasingly recognized for their prebiotic benefits, but their efficient enzymatic production is hindered by the high crystallinity of cellulose, which limits enzyme accessibility. This study introduced an efficient and scalable strategy combining phosphoric acid pretreatment with enzymatic hydrolysis to produce high-purity WCOs. Microcrystalline cellulose treated with 85 wt% phosphoric acid at 10 °C exhibited significantly reduced crystallinity and crystallite size, improving its susceptibility to enzymatic degradation. Subsequent hydrolysis of the hydrated regenerated cellulose (HRC85-10) using Celluclast® at pH 7.0 for 1 h resulted in a WCO selectivity of 93.5%, with cellobiose and cellotriose identified as major oligomeric products via electrospray ionization mass spectrometry. Maintaining cellulose in a hydrated form significantly improved both the yield and selectivity of WCOs. In vivo studies further confirmed the prebiotic potential, with a significant increase in fecal *Lactobacillus* spp. and *Bifidobacterium* spp. ($p < 0.05$) following WCO supplementation. These findings demonstrated a practical and effective approach for producing functional WCOs for use in dietary and gut health applications.

Keywords: water-soluble cellulosic oligomers; phosphoric acid pretreatment; enzymatic hydrolysis; regenerated cellulose



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1. Introduction

Cellulose, the most abundant natural polymer on earth, is a renewable and sustainable resource widely utilized across the food, pharmaceutical, and biofuel industries. However, its high crystallinity significantly limits its functionality in food applications, where it is primarily used as an insoluble dietary fiber due to its poor solubility and limited digestibility. An emerging alternative is the production of water-soluble cellulosic oligomers (WCOs), short-chain cellulose fragments with a degree of polymerization (DP) typically below 12 [1], which offer enhanced functionality and notable health benefits. WCOs have demonstrated prebiotic effects by selectively promoting the growth of beneficial gut microbiota and stimulating the production of short-chain fatty acids, which are vital for gut health and metabolic regulation [2]. Additionally, clinical studies suggest that oral administration of WCOs may help reduce hepatic fat accumulation and total cholesterol levels, indicating their potential role in the prevention and management of lifestyle-related metabolic disorders [3].

Despite the promising applications of WCOs, their commercial production remains constrained by technical barriers, particularly in achieving high yield and selectivity. Conventional approaches to cellulose depolymerization, such as acid hydrolysis and enzymatic hydrolysis, present significant drawbacks [3]. Acid hydrolysis, though effective in breaking down cellulose, typically requires harsh reaction conditions and often results in the

formation of monosaccharides along with undesirable by-products such as hydroxymethylfurfural [1], compromising product purity and safety. Enzymatic hydrolysis, by contrast, offers a milder and more selective alternative with a well-defined DP distribution [4]; however, its efficiency is limited by the high crystallinity of cellulose, which hinders enzyme accessibility and necessitates elevated enzyme loading to achieve appreciable conversion.

To address the limitations of enzymatic hydrolysis, a range of pretreatment strategies—including ball milling, steam explosion, acid treatment, and ionic liquid dissolution—have been investigated to reduce cellulose crystallinity and improve enzymatic accessibility [5,6]. Among these, phosphoric acid pretreatment has emerged as a particularly effective and comparatively mild method compared to strong mineral acids [7]. It should be noted that phosphoric acid can be irritating and may pose environmental concerns, although it is generally considered less corrosive, easier to handle, and capable of producing highly digestible cellulose. It can effectively disrupt the crystalline structure without introducing substantial toxicity or operational complexity. Previous studies have shown that phosphoric acid can effectively amorphize lignocellulosic biomass, thereby enhancing its susceptibility to enzymatic hydrolysis and increasing oligomer yields [4]. However, many of these approaches depended on recombinant enzyme systems [4,6], which, although effective, were often associated with higher production costs and have limited accessibility for large-scale or food production.

Notably, most prior studies have focused on the enzymatic hydrolysis of dried pretreated cellulose [4,6,7], often overlooking the potential benefits of maintaining the substrate in a hydrated state. Our preliminary findings indicated that phosphoric acid-pretreated cellulose, especially in its wet form, significantly enhanced enzymatic hydrolysis efficiency compared to native cellulose. Moreover, it allowed for more selective production of WCOs by fine-tuning hydrolysis parameters such as pH and reaction time. Despite these promising observations, comprehensive studies that systematically integrate phosphoric acid pretreatment with enzymatic hydrolysis, particularly using hydrated cellulose and cost-effective enzymes, remain scarce. This highlights a critical gap in current research and underscores the need for simple and scalable strategies for WCO production without relying on complex recombinant enzyme cocktails, while minimizing glucose formation.

Therefore, this study aimed to develop an effective approach for the selective production of high-purity WCOs through phosphoric acid-assisted enzymatic hydrolysis, with a comparative evaluation of hydrolysis using endo-1,4- β -D-glucanase alone and the Celluclast[®] enzyme cocktail. We systematically examined the influence of key process variables—including phosphoric acid concentration, pretreatment temperature, hydration state of the cellulose, enzyme type, hydrolysis duration, and pH—on both the yield and selectivity of WCOs. Structural changes and product characteristics were analyzed using X-ray diffraction (XRD) and electrospray ionization mass spectrometry (ESI-MS). In addition, the prebiotic potential of the resulting WCOs was assessed in vivo using a mouse model, focusing on their ability to modulate gut microbiota composition by stimulating *Bifidobacterium* spp. and *Lactobacillus* spp. By addressing a critical gap in current literature, this work proposes a practical and efficient strategy for producing functional WCOs with promising applications in dietary supplementation.

2. Materials and Methods

2.1. Phosphoric Acid Pretreatment of Microcrystalline Cellulose

Commercial microcrystalline cellulose (MCC, Microcel[®] MC-101, Newmarket, ON, Canada) was moistened with deionized water at a ratio of 3:1 (water to cellulose by weight). Phosphoric acid solutions at concentrations of 77, 81, or 85 wt% were then gradually added to the pre-wetted MCC at a dosage of 10 g per 100 mL of acid. The mixture was incubated

at 10, 30, or 50 °C for 1 h in a temperature-controlled water bath (BBL-301, Yamato Scientific, Tokyo, Japan) to maintain the desired reaction temperature. To terminate the reaction, cold deionized water (ten times the volume of the reaction mixture) was added, followed by vigorous agitation to ensure thorough mixing and regeneration. The resulting precipitate, referred to as regenerated cellulose (RC), was neutralized by gradually adding 1 M NaOH until the pH reached 7, then dialyzed to remove residual impurities. After centrifugation to remove excess water, the RC was divided into two portions. One portion was stored in its wet form, designated as hydrated regenerated cellulose (HRC), and kept refrigerated for future use. The other portion was freeze-dried and ground into a fine powder, referred to as dried regenerated cellulose (DRC).

RC samples were labeled according to the concentration of phosphoric acid used and the pretreatment temperature. For example, DRC85-10 denotes dried RC pretreated with 85 wt% phosphoric acid at 10 °C, while HRC85-10 refers to its hydrated counterpart produced under the same conditions.

2.2. Enzymatic Hydrolysis of Cellulosic Biomass

Following the procedure described by He (2024) [8], the initial dry matter concentration of cellulosic biomass was set at 1% (*w/v*) for all hydrolysis experiments. Enzyme loading was set at 24 U/g of biomass for both endo-1,4-β-D-glucanase (Megazyme, International Ireland Ltd., Wicklow, Ireland) and Celluclast® 1.5L (Novozymes A/S, Bagsværd, Denmark). Hydrolysis reactions were carried out in 50 mL centrifuge tubes using 50 mM citrate-phosphate buffer and incubated in a shaking water bath at 50 °C and 120 rpm. At designated time points, depending on the experimental design, the reaction tubes were removed and heated at 90 °C for 15 min to inactivate the enzymes. The mixtures were then centrifuged at 2370× *g* for 15 min to separate the supernatants and unhydrolyzed residues, which were collected for subsequent analysis. All experiments were conducted in triplicate.

To evaluate the influence of crystallite structure and enzyme type on hydrolysis efficiency, MCC and all DRC samples described in Section 2.1 were used as substrates. Hydrolysis was performed using either endo-1,4-β-D-glucanase or Celluclast® 1.5L, with the buffer pH maintained at 4.8. Samples treated with endo-1,4-β-D-glucanase were incubated for 24 h, whereas those treated with Celluclast® 1.5L were incubated for 1 h.

To further optimize hydrolysis conditions for improved selectivity of WCOs, additional tests were performed using MCC, DRC, and HRC pretreated with 85 wt% phosphoric acid. These substrates were hydrolyzed using Celluclast® 1.5L for 1, 3, 6, and 24 h, with the reaction pH adjusted to either 4.8 or 7.0.

The solubilization ratio, representing the enzymatic hydrolysis efficiency of the samples, was calculated using the following equation:

$$\text{Solubilization ratio (mg/g biomass)} = M_{\text{WSC}} / M_{\text{biomass}}$$

where M_{WSC} represents the total dry mass of water-soluble content (mg) present in the supernatant, and M_{biomass} is the initial dry mass of cellulosic biomass used (g). The M_{WSC} , which included WCOs and glucose, was quantified using the gravimetric method and calculated as:

$$M_{\text{WSC}} \text{ (mg)} = M_{\text{biomass}} - M_{\text{residue}}$$

where M_{residue} represents the dry mass of unhydrolyzed residue (mg).

The glucose content in the supernatant was determined enzymatically using a peroxidase-coupled glucose assay [9]. In this method, β-D-glucose was oxidized by glucose oxidase, resulting in the formation of gluconic acid and hydrogen peroxide. The generated hydrogen peroxide then reacted with a chromogenic substrate in the presence of peroxidase,

producing a colored quinoneimine complex. The absorbance of this complex was measured spectrophotometrically at 500 nm. The glucose conversion (C_{Glc}) from cellulosic biomass was calculated using the following equation:

$$\text{Conversion of glucose } (C_{\text{Glc}}, \text{ mg/g biomass}) = M_{\text{Glc}} / M_{\text{biomass}}$$

where M_{Glc} denotes the mass of glucose produced (mg). The conversion of WCOs (C_{WCOs}) from cellulosic biomass and the selectivity of WCOs were calculated using the following formulas:

$$\text{Conversion of WCOs } (C_{\text{WCOs}}, \text{ mg/g biomass}) = (M_{\text{WSC}} - M_{\text{Glc}}) / M_{\text{biomass}}$$

$$\text{Selectivity of WCOs } (\%) = [C_{\text{WCOs}} / (C_{\text{WCOs}} + C_{\text{Glc}})] \times 100$$

2.3. X-Ray Diffraction

X-ray diffraction (XRD) was employed to evaluate the crystallinity and structural changes of cellulosic materials after different phosphoric acid treatments. The assessments included decrystallization, polymorphic transitions, crystallite size reduction, and inter-planar spacing modifications. MCC and all DRC samples described in Section 2.1 were analyzed using a high-resolution X-ray diffractometer (Bruker AXS, Karlsruhe, Germany) equipped with a Ni-filtered Cu K α radiation source ($\lambda = 1.5418 \text{ \AA}$), operated at 40 kV and 40 mA.

Scans were performed over a 2θ range of 5° to 45° , with a step size of 0.02° and a scanning speed of 1° per min. This range was selected to capture the characteristic diffraction peaks of cellulose I ($2\theta \approx 14.8^\circ$, 22.5° , and 34.5°) and cellulose II ($2\theta \approx 12^\circ$ and 20.2°). The crystallinity index (CrI) of cellulose was calculated using the following equation:

$$\text{Crystallinity Index } (\%) = [(I_{\text{crystalline}} - I_{\text{amorphous}}) / I_{\text{crystalline}}] \times 100$$

where $I_{\text{crystalline}}$ represents the intensity of the main crystalline peak (typically at 22.5° for cellulose I, and 20.2° for cellulose II), and $I_{\text{amorphous}}$ corresponds to the intensity of the amorphous background, measured at 18° . Crystallite size (D) was estimated using the Scherrer equation:

$$D = K\lambda / \beta \cos\theta$$

where K is the shape factor (typically 0.9), λ is the X-ray wavelength ($1.5418 \text{ \AA}$ for Cu K α), β is the full width at half maximum (FWHM) of the diffraction peak (in radians), and θ is the Bragg angle (in radians), typically 22.5° for cellulose I and 20.2° for cellulose II. The d-spacing (d) for diffraction plane was calculated using Bragg's Law:

$$d = \lambda / 2\sin\theta$$

2.4. Electrospray Ionization Mass Spectrometry

Electrospray ionization mass spectrometry (ESI-MS) was used to characterize the oligosaccharide profile of the water-soluble fraction obtained from the enzymatic hydrolysis of cellulosic biomass. The analysis was performed using an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with an electrospray ionization source. The instrument was operated in positive-ion mode to enable the detection of protonated and cation-adducted oligosaccharides.

Instrument settings included a spray voltage of 4 kV, with sheath and auxiliary gas flow rates maintained at 20 and 1 arbitrary units, respectively. The capillary temperature

was set at 275 °C, with a capillary voltage of 80 V and a tube lens voltage of 50 V to optimize ion transmission. The sample was introduced directly into the mass spectrometer in full-scan mode, covering a mass-to-charge (m/z) range of 150 to 2000. To improve the signal-to-noise ratio and clarify peak profiles, data were averaged over 20 consecutive scans, facilitating the detection of low-abundance oligomeric species with high mass accuracy.

2.5. In Vivo Evaluation of Prebiotic Potential of WCOs

Fifteen male ICR mice (six weeks old, initial weight: 30.3–32.2 g) were obtained from BioLASCO (Taipei City, Taiwan) and housed under controlled environmental conditions (22 ± 1 °C, 12 h light/dark cycle) with access to standard chow and water ad libitum. The study protocol was approved by the Animal Care and Use Committee of National Chung Hsing University (IACUC Approval No. 112–122), in accordance with the 3Rs principles.

Following a two-week acclimatization period, the mice were stratified into five weight classes, each consisting of three animals. Within each weight class, one mouse was randomly assigned to one of three groups: a control group ($n = 5$), which received no dietary supplementation, and two experimental groups ($n = 5$ each) receiving either a low dose (206 mg/kg/day) or a high dose (824 mg/kg/day) of WCOs. The WCOs were administered daily via oral gavage at a fixed time each day to maintain consistency across treatments. Body weight, food intake, and water intake were monitored and recorded daily throughout the 28-day feeding period.

At the end of the 28-day feeding period, fecal parameters—including pH and specific gut microbiota populations—were evaluated. Fresh fecal samples were collected under sterile conditions to prevent contamination from urine or residual feed. Fecal pH was measured in duplicate by homogenizing the samples in deionized water at a ratio of 1:4 (w/v), followed by centrifugation at $1006 \times g$ for 10 min.

To assess the modulation of gut microbiota, populations of *Lactobacillus* spp. and *Bifidobacterium* spp. were quantified using selective culture techniques. Fresh fecal samples were immediately diluted in anaerobic buffer (1:10 w/v) and homogenized. Serial dilutions were performed before plating onto selective media: Rogosa agar for *Lactobacillus* spp. and *Bifidobacterium* Iodoacetate Medium 25 (BIM-25) for *Bifidobacterium* spp. Anaerobic incubation was carried out at 37 °C, with *Lactobacillus* spp. cultured for 3 days and *Bifidobacterium* spp. for 5 days. Enumeration of *Bifidobacterium* spp. followed the method of Muñoa and Pares (1988) [10], while *Lactobacillus* spp. quantification adhered to standard microbiological protocols. Each bacterial count was determined in duplicate per animal.

2.6. Statistical Analysis

Statistical analyses were performed using SPSS software (Version 20.0; IBM Corp., Armonk, NY, USA). Data are expressed as mean $\pm$ standard deviation (SD), and statistical significance was defined as $p < 0.05$. Data collected from both enzymatic hydrolysis and in vivo experiments were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test for post hoc comparisons.

3. Results and Discussion

3.1. Structural Changes and Decrystallization of Cellulose Induced by Phosphoric Acid Pretreatment

In this study, we examined the effects of phosphoric acid concentration and treatment temperature across multiple conditions on the structural change of cellulose. Structural parameters—including crystallinity index, crystallite size, and interplanar spacing (d -spacing)—were evaluated using X-ray diffraction. All pretreatment reactions were conducted for a fixed duration of 1 h. Table 1 summarizes the CrI values, crystallite sizes, and

d-spacing. The XRD patterns of untreated microcrystalline cellulose (MCC) and phosphoric acid-pretreated DRC are shown in Figure 1. These analyses provided a comprehensive assessment of the structural transformations induced by different pretreatment conditions.

Table 1. Structural parameters of untreated MCC and phosphoric acid-pretreated DRC at different concentrations and temperatures.

Groups	Crystallinity Index (%)	Crystallite Size (nm)	d-Spacing (nm)
MCC	82.66	4.429	0.396
DRC77-10	79.16	4.290	0.396
DRC77-30	81.23	4.461	0.396
DRC77-50	82.48	4.452	0.396
DRC81-10	64.91	3.671	0.397
DRC81-30	77.19	4.181	0.396
DRC81-50	79.84	4.314	0.396
DRC85-10	36.57	2.051	0.438
DRC85-30	40.94	1.820	0.428
DRC85-50	62.50	3.445	0.400

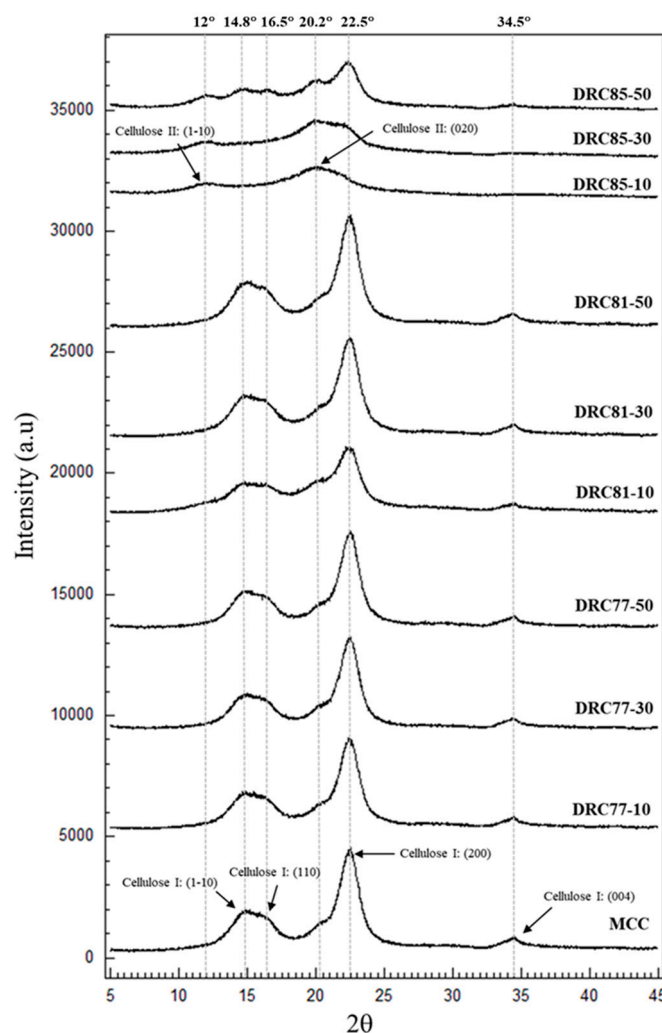


Figure 1. XRD patterns of untreated MCC and phosphoric acid-pretreated DRC at different concentrations and temperatures.

The XRD patterns of DRC77-10, DRC77-30, and DRC77-50 showed minimal deviations from untreated MCC, displaying characteristic diffraction peaks at $2\theta = 14.8^\circ$, 22.5° , and

34.5°, corresponding to the (1-10), (200), and (004) planes of cellulose I [7]. As shown in Table 1, the CrI values for these samples ranged from 79.16% to 82.48%, closely matching that of untreated MCC (82.66%). Similarly, crystallite sizes (4.290–4.461 nm) and d-spacing values (0.396 nm) remained largely unchanged. These results suggest that 77 wt% phosphoric acid lacked sufficient solvating power to disrupt the tightly packed crystalline regions of cellulose I or induce polymorphic transformation. This observation aligns with previous findings indicating that effective cellulose dissolution typically requires phosphoric acid concentrations exceeding 77.8 wt% [11].

More pronounced structural transformation was observed with 81 wt% phosphoric acid, particularly at 10 °C (DRC81-10). The CrI of DRC81-10 decreased substantially to 64.91%, accompanied by a reduction in crystallite size to 3.671 nm. Additionally, subtle but discernible new diffraction peaks emerged at $2\theta = 12^\circ$ and 20.2° , corresponding to the (1-10) and (020) planes of cellulose II [12], suggesting the onset of a partial polymorphic transformation. At elevated treatment temperatures (30 °C and 50 °C), this effect was less pronounced. The CrI values of DRC81-30 (77.19%) and DRC81-50 (79.84%) were comparable to those of untreated MCC, and their XRD patterns closely resembled cellulose I.

The most apparent structural alterations were observed in samples treated with 85 wt% phosphoric acid, particularly at 10 °C (DRC85-10). The CrI dropped drastically to 36.57%, accompanied by a substantial reduction in crystallite size (2.051 nm) and an increase in d-spacing (0.438 nm). These changes indicate severe disruption of the cellulose crystalline structure and a complete polymorphic transformation from cellulose I to cellulose II. A similar transformation was observed at 30 °C (DRC85-30), where the CrI remained low at 40.94%, the crystallite size further decreased to 1.820 nm, and the d-spacing measured 0.428 nm. In both DRC85-10 and DRC85-30, the characteristic diffraction peaks of cellulose II ($2\theta = 12^\circ$ and 20.2°) appeared prominently, while those of cellulose I progressively diminished. These results confirm that 85 wt% phosphoric acid effectively disrupts intermolecular hydrogen bonding at 10 °C and 30 °C, resulting in extensive amorphization and a robust polymorphic transition.

At 50 °C (DRC85-50), however, partial recovery of crystallinity was observed. The CrI increased to 62.50%, and the crystallite size grew to 3.445 nm. The simultaneous presence of diffraction peaks from both cellulose I (e.g., $2\theta = 14.8^\circ$, 22.5° , and 34.5°) and cellulose II (e.g., $2\theta = 12^\circ$ and 20.2°) suggests a dynamic and incomplete polymorphic transformation, indicating structural coexistence of both forms. This behavior highlights that the polymorphic transformation is not only temperature-dependent but is also influenced by the phosphoric acid concentration, revealing a complex interplay between these factors in modulating cellulose crystallinity.

Unlike previous studies where the appearance of cellulose II peaks (e.g., $2\theta = 12^\circ$ and 20.2°) was primarily attributed to time-dependent transformations with the gradual disappearance of cellulose I peaks (e.g., $2\theta = 14.8^\circ$, 22.5° , and 34.5°) [7], our study revealed a novel concentration-dependent polymorphic transformation modulated by reaction temperature. At lower temperatures (10 °C), a phosphoric acid concentration as low as 81 wt% was sufficient to initiate the emergence of cellulose II peaks along with the simultaneous attenuation of cellulose I peaks. At elevated temperatures (30 °C and 50 °C), a higher acid concentration (85 wt%) was required to induce similar structural transitions. These findings suggest that lower reaction temperatures can effectively reduce the acid concentration threshold necessary to trigger the cellulose I-to-II transformation. This insight presents a promising strategy for optimizing cellulose pretreatment under milder chemical conditions, potentially improving the efficiency and sustainability of industrial applications.

3.2. Influence of Crystallite Structure and Enzyme Type on the Enzymatic Hydrolysis of Cellulose

In this study, the solubilization ratio—defined as the extent of cellulosic biomass converted into water-soluble products—was used as a key indicator of enzymatic hydrolysis efficiency. Phosphoric acid-pretreated DRC samples were hydrolyzed at pH 4.8 using either endo-1,4- β -D-glucanase for 24 h or Celluclast® for 1 h. As shown in Figure 2, samples treated with Celluclast® (Figure 2b) consistently exhibited higher solubilization ratios than those treated with endo-1,4- β -D-glucanase (Figure 2a). This difference can be attributed to the broader enzymatic activity of Celluclast®, which contains endoglucanase, exoglucanase, and β -glucosidase, allowing for more complete conversion of cellulose into water-soluble products. This observation is consistent with prior studies, which have shown that the synergistic action of endoglucanase and exoglucanase is essential for efficiently releasing water-soluble sugars from cotton cellulose [13], rather than endoglucanase alone. Additionally, a negative correlation was observed between CrI and the solubilization ratio. Samples with a reduced CrI, such as DRC81-10 and DRC85-50, demonstrated higher solubilization efficiency under both enzymatic treatments compared to native MCC. In contrast, samples with CrI values comparable to MCC exhibited similar solubilization ratios, suggesting that the acid-induced reduction in crystallinity played a key role in enhancing enzyme accessibility and hydrolysis efficiency.

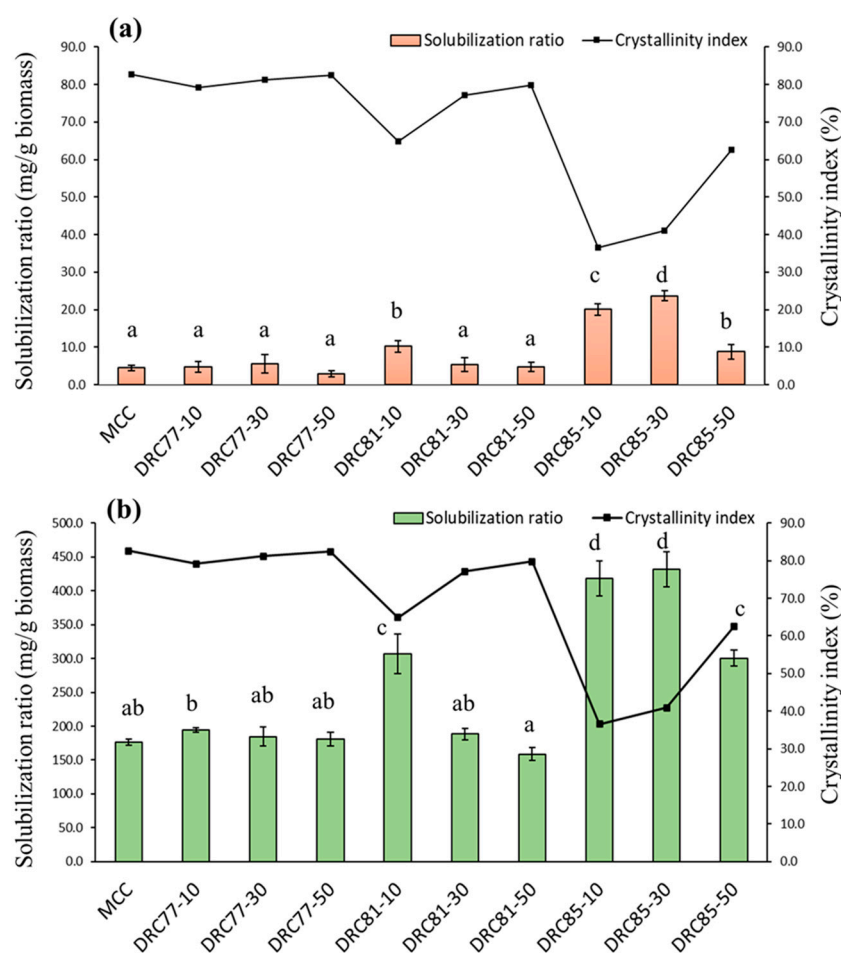


Figure 2. Solubilization ratio of phosphoric acid-pretreated DRC during enzymatic hydrolysis at pH 4.8 using (a) endo-1,4- β -D-glucanase for 24 h and (b) Celluclast® for 1 h. a–d: Means with different letters are significantly different in the bars ($p < 0.05$, $n = 3$).

Although DRC85-10 exhibited the lowest CrI (36.57%), its solubilization ratio under endo-1,4- β -D-glucanase hydrolysis (20.1 mg/g biomass) was not the highest. Instead,

DRC85-30 achieved a significantly greater solubilization ratio (23.7 mg/g biomass, $p < 0.05$), despite having a slightly higher CrI. Under Celluclast[®] hydrolysis, DRC85-30 again demonstrated the highest solubilization ratio (431.7 mg/g biomass), although the difference compared to DRC85-10 (418.5 mg/g biomass) was not statistically significant. These findings indicate that CrI alone does not fully account for enzymatic hydrolysis efficiency.

Rather, crystallite size appears to play a critical role. As shown in Table 1, DRC85-30 had a smaller crystallite size than DRC85-10, which likely contributed to its superior solubilization performance. The higher solubilization ratio of DRC85-30 suggests that reduced crystallite size may provide more cleavage sites for enzymatic action, thereby enhancing substrate fragmentation and improving overall enzyme accessibility. A similar phenomenon was reported on ionic liquid-pretreated biomass, where enzymatic digestibility was significantly improved due to reductions in crystallite size rather than CrI alone [14]. In that study, a 42.22% decrease in crystallite size and a 20.8% reduction in CrI resulted in a 10.3-fold increase in glucose recovery, emphasizing the synergistic contribution of both structural parameters in promoting efficient enzymatic hydrolysis.

The hydrolysate supernatants described above were further analyzed to quantify both WCO and glucose content. Figure 3 presents the conversion levels of WCOs and glucose following enzymatic hydrolysis at pH 4.8 using either endo-1,4- β -D-glucanase for 24 h or Celluclast[®] for 1 h. As shown in Figure 3a, no detectable glucose was present after hydrolysis with endo-1,4- β -D-glucanase, indicating that glucose concentrations were below the detection threshold. Thus, the measured water-soluble content could be attributed entirely to WCOs. Endo-1,4- β -D-glucanase primarily targets the amorphous regions of cellulose [13], and, therefore, samples with higher amorphous content, such as DRC85-10 and DRC85-30, exhibited greater yields compared to native MCC. Their yields of WCOs ranged from 20.1 to 23.7 mg/g biomass, exceeding that of MCC but remaining significantly lower than the yields obtained from samples hydrolyzed with Celluclast[®] (Figure 3b). Although Celluclast[®] hydrolysis led to higher overall WCO conversion than endo-1,4- β -D-glucanase, glucose yields were substantially greater than WCO yields. This result indicates that Celluclast[®] not only depolymerizes cellulose effectively but also hydrolyzes the resulting cello-oligosaccharides into glucose, limiting their accumulation. Consequently, the accumulation of WCOs was suppressed, while glucose production was significantly enhanced.

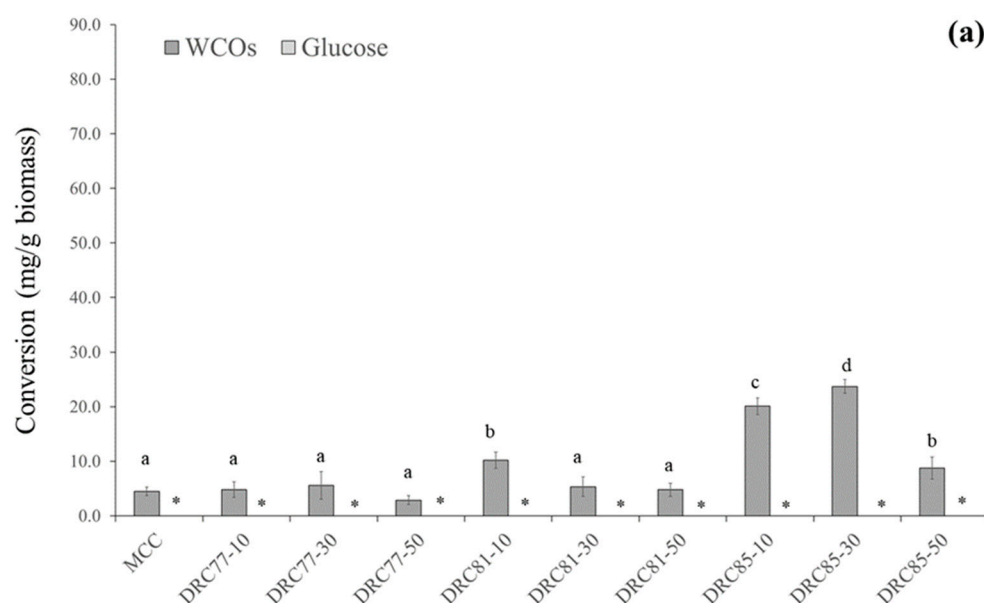


Figure 3. Cont.

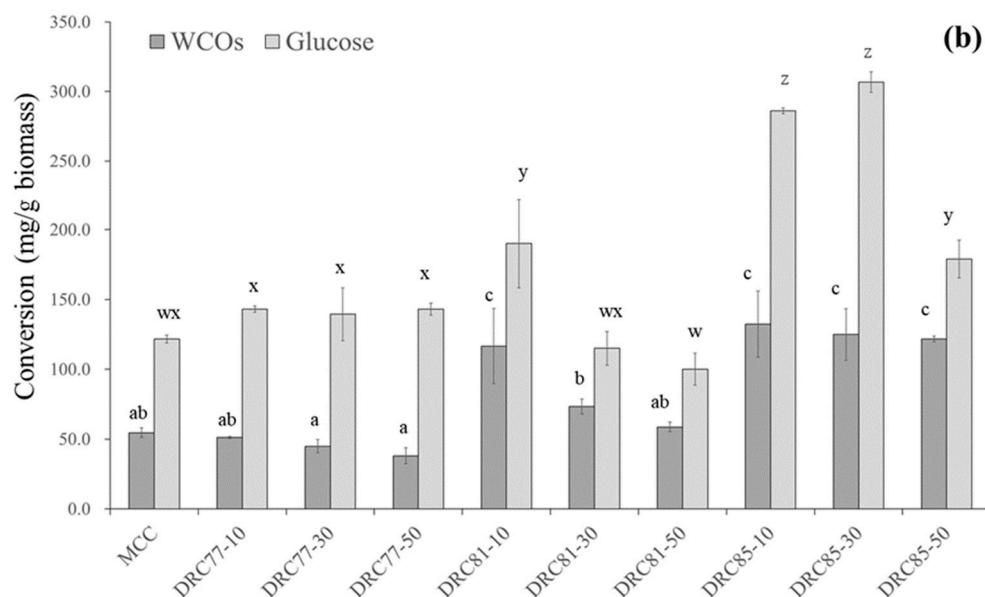


Figure 3. Conversion of WCOs and glucose during the enzymatic hydrolysis of phosphoric acid-pretreated DRC at pH 4.8 using (a) endo-1,4- β -D-glucanase for 24 h and (b) Celluclast® for 1 h. a–d: Means with different letters are significantly different among dark grey bars ($p < 0.05$, $n = 3$). w–z: Means with different letters are significantly different among grey bars ($p < 0.05$, $n = 3$). *: No detected.

3.3. Optimization of Hydrolysis Conditions for Enhanced Selectivity of WCOs

Interestingly, our investigation revealed that phosphoric acid-pretreated cellulose, particularly in its hydrated form (HRC), exhibited a significantly higher solubilization ratio ($p < 0.05$) compared to its dried counterpart (DRC), as shown in Table 2a,b. During Celluclast® hydrolysis at pH 4.8 (Table 2a), the solubilization ratios of HRC85-10, HRC85-30, and HRC85-50 increased by approximately 2.0-, 1.8-, and 1.5-fold, respectively, relative to their corresponding DRC samples. Although the overall solubilization ratios declined under neutral conditions (pH 7.0, Table 2b), the hydrated cellulose samples consistently demonstrated superior solubilization performance compared to the dried forms, suggesting that hydration likely enhances enzymatic accessibility.

This enhancement may be attributed to the increased structural flexibility of hydrated cellulose. For instance, cellulose II hydrate incorporates water molecules between its molecular sheets, which disrupts hydrogen bonding and improves enzyme accessibility [15]. Similarly, recent studies have shown that wet cellulose derived from ionic liquids exhibits reduced rigidity, owing to the lubricating effect of water molecules, which could facilitate better interaction with hydrolytic enzymes [16].

As shown in Table 3, both reaction time and pH apparently influenced the enzymatic hydrolysis of HRC85-10 by Celluclast®. At pH 4.8, WCO conversion decreased from 692.1 to 416.9 mg/g biomass ($p < 0.05$) as hydrolysis time increased from 1 to 24 h, while glucose production rose sharply from 132.5 to 529.5 mg/g biomass ($p < 0.05$). As a result, selectivity of WCOs declined from 83.9% to 44.1% ($p < 0.05$), indicating a temporal shift from oligomer formation toward glucose accumulation. Notably, after 1 h of hydrolysis at pH 4.8, selectivity of WCOs increased significantly from 31.5% for DRC85-10 to 83.9% for HRC85-10 ($p < 0.05$). It was inferred that this substantial enhancement was attributed to their differences in crystallinity. Although DRC85-10 exhibited relatively low crystallinity (~37%) due to phosphoric acid pretreatment, the HRC85-10 sample possessed a more amorphous and highly hydrated structure, with virtually no remaining crystallinity. These observations could be explained by the findings of Kendrick et al. (2022) [4], which indicated that the

yield of cellulosic oligomers from highly crystalline cellulose was limited by excessive degradation into glucose.

Table 2. (a) Solubilization ratios of DRC and HRC samples derived from 85% phosphoric acid pretreatment following 1 h of enzymatic hydrolysis with Celluclast® at pH 4.8. (b) Solubilization ratios of DRC and HRC samples derived from 85% phosphoric acid pretreatment following 1 h of enzymatic hydrolysis with Celluclast® at pH 7.0.

Groups	Solubilization Ratio (mg/g Biomass)
(a)	
MCC	176.4 ± 4.9 a
DRC85-10	418.5 ± 25.7 c
DRC85-30	431.7 ± 25.7 c
DRC85-50	300.7 ± 11.6 b
HRC85-10	824.7 ± 42.7 d
HRC85-30	790.7 ± 32.5 d
HRC85-50	455.1 ± 9.6 c
(b)	
MCC	82.4 ± 1.9 a
DRC85-10	143.6 ± 6.0 b
DRC85-30	129.5 ± 11.5 ab
DRC85-50	136.9 ± 14.6 ab
HRC85-10	527.8 ± 31.6 d
HRC85-30	503.6 ± 20.7 d
HRC85-50	251.3 ± 42.7 c

a–d: Means with different letters are significantly different ($p < 0.05$, $n = 3$).

Table 3. Effects of hydrolysis time and pH on WCO and glucose conversion, as well as WCO selectivity, of HRC85-10 during Celluclast® hydrolysis.

PH	Time (h)	WCOs (mg/g Biomass)	Glucose (mg/g Biomass)	WCO Selectivity (%)
4.8	1	692.1 ± 44.5 c	132.5 ± 18.0 a	83.9 ± 2.3 a
	3	662.0 ± 28.5 c	241.1 ± 36.3 b	73.3 ± 3.8 b
	6	544.4 ± 20.8 b	383.4 ± 17.5 c	58.7 ± 2.0 c
	24	416.9 ± 35.1 a	529.5 ± 61.2 d	44.1 ± 4.9 d
7.0	1	493.1 ± 24.5 b	34.8 ± 14.2 a	93.5 ± 2.3 a
	3	582.9 ± 51.6 c	58.5 ± 11.4 a	90.8 ± 2.0 a
	6	620.5 ± 38.6 c	119.1 ± 43.5 b	83.9 ± 5.8 b
	24	347.4 ± 71.1 a	429.4 ± 2.0 c	44.5 ± 5.2 c

a–d: Means with different letters are significantly different in same column ($p < 0.05$, $n = 3$).

At pH 7.0, glucose formation was further minimized, particularly within the first 3 h, resulting in even higher selectivity of WCOs ranging from 90.8% to 93.5%, surpassing values observed at pH 4.8. This outcome is consistent with previous studies indicating that Celluclast® exhibits limited β -glucosidase activity under neutral conditions, thereby favoring the retention of cello-oligosaccharides rather than their hydrolysis to glucose [17]. These findings highlight the importance of pH and reaction time in controlling the purity of WCOs. Specifically, lower pH and extended hydrolysis favored glucose production—likely due to the sustained activity of β -glucosidase—whereas neutral pH and shorter hydrolysis durations minimized glucose formation and promoted oligomer retention. Based on these results, HRC85-10 hydrolyzed with Celluclast® at pH 7.0 for 1 h was selected as the optimal condition for producing high-purity WCOs for subsequent in vivo evaluation.

Compared to previous studies, our approach demonstrated comparable or superior performance in both the yield and selectivity of WCOs, achieved under simplified reaction conditions. For instance, Leonarski et al. (2023) [18] reported that enzymatic hydrolysis of untreated bacterial cellulose using Celluclast[®] yielded high cellobiose selectivity (77–85%), but required prolonged hydrolysis durations (24–72 h) and achieved only moderate yields (260–350 mg/g biomass). In this study, a higher WCO yield of 493.1 mg/g biomass was obtained in just 1 h at pH 7.0, with superior selectivity of 93.5%.

Similarly, Kendrick et al. (2022) [4] achieved a cello-oligosaccharide yield of approximately 370 mg/g glucan with 91% selectivity from phosphoric acid-pretreated *Miscanthus*, but required a complex recombinant enzyme cocktail—including processive endoglucanases and cellobiohydrolases—which elevated production costs and operational complexity. It was interesting to note that this study employed only a single commercial enzyme product (Celluclast[®] 1.5L, Novozymes A/S, Bagsværd, Denmark), avoiding the complexity of recombinant enzyme mixtures while achieving comparable performance. This simplification enhanced the practicality and scalability of our method for industrial applications.

In sum, this study demonstrated that, at pH 4.8, 1 h hydrolysis yielded 692.1 mg/g biomass of WCOs, but with a lower selectivity (83.9%). Conversely, at pH 7.0, the yield decreased, whereas the selectivity increased to 93.5%. This inverse relationship suggested that optimizing WCO production requires balancing total yield against product purity. Regarding the trade-off between the yield and selectivity of WCOs in industrial applications, this balance should be tailored to the intended end use—whether prioritizing high-purity WCOs for prebiotic and functional food applications, or maximizing overall yield for bulk uses such as fermentation feedstocks.

3.4. Oligomeric Profile of Hydrolysate Supernatant from Enzymatic Hydrolysis

ESI-MS analysis of the hydrolysate supernatant from HRC85-10 treated with Celluclast[®] at pH 4.8 for 1 h in positive ion mode revealed a series of peaks corresponding to short-chain cellulosic oligomers and their derivatives (Figure 4). Notably, prominent peaks were observed at m/z 203.1, 365.2, 381.3, 527.2, 707.1, and 739.0, each of which was assigned to specific oligomeric species, confirming the occurrence of enzymatic hydrolysis.

The peak at m/z 203.1 was attributed to sodium-adducted glucose ($[M + Na]^+$), confirming the presence of free glucose in the hydrolysate. The peak at m/z 365.2 corresponded to sodium-adducted cellobiose ($[M + Na]^+$), while the adjacent peak at m/z 381.3 was assigned to potassium-adducted cellobiose ($[M + K]^+$), supporting the presence of disaccharide units as prominent hydrolysis products. The detection of a peak at m/z 527.2 indicated the presence of sodium adducted cellotriose ($[M + Na]^+$), confirming the release of trisaccharide structures.

These results are consistent with prior studies on the enzymatic hydrolysis of cellulose using Celluclast[®], in which endo-1,4- β -D-glucanase randomly cleaves β -1,4-glycosidic bonds, producing shorter oligosaccharides and increasing the number of reducing ends. Concurrently, exoglucanases hydrolyze cellulose chains (DP = 30–60) from both termini, predominantly releasing cellobiose [3]. This enzymatic specificity accounts for the high abundance of cellobiose detected in the hydrolysate, suggesting that it is a major component prior to its further conversion into glucose by β -glucosidase activity. Previous studies have shown that enzymatic hydrolysis of cellulosic biomass typically yields oligomers with DP 1 and 2, along with minor quantities of cellotriose and cellotetraose [17,19]. However, in the current analysis, cellotetraose was not detected in the hydrolysate after 1 h of reaction. It was inferred that this absence might be attributed to its further breakdown into smaller oligosaccharides, such as cellobiose or glucose. Notably, in our preliminary experiments

conducted at an earlier time point (0.5 h) (Supplementary Figure S1), cellotetraose (m/z 683.0) was observed along with glucose, cellobiose, and cellohexaose, suggesting that cellotetraose was a transient intermediate that underwent further hydrolysis during prolonged enzymatic treatment.

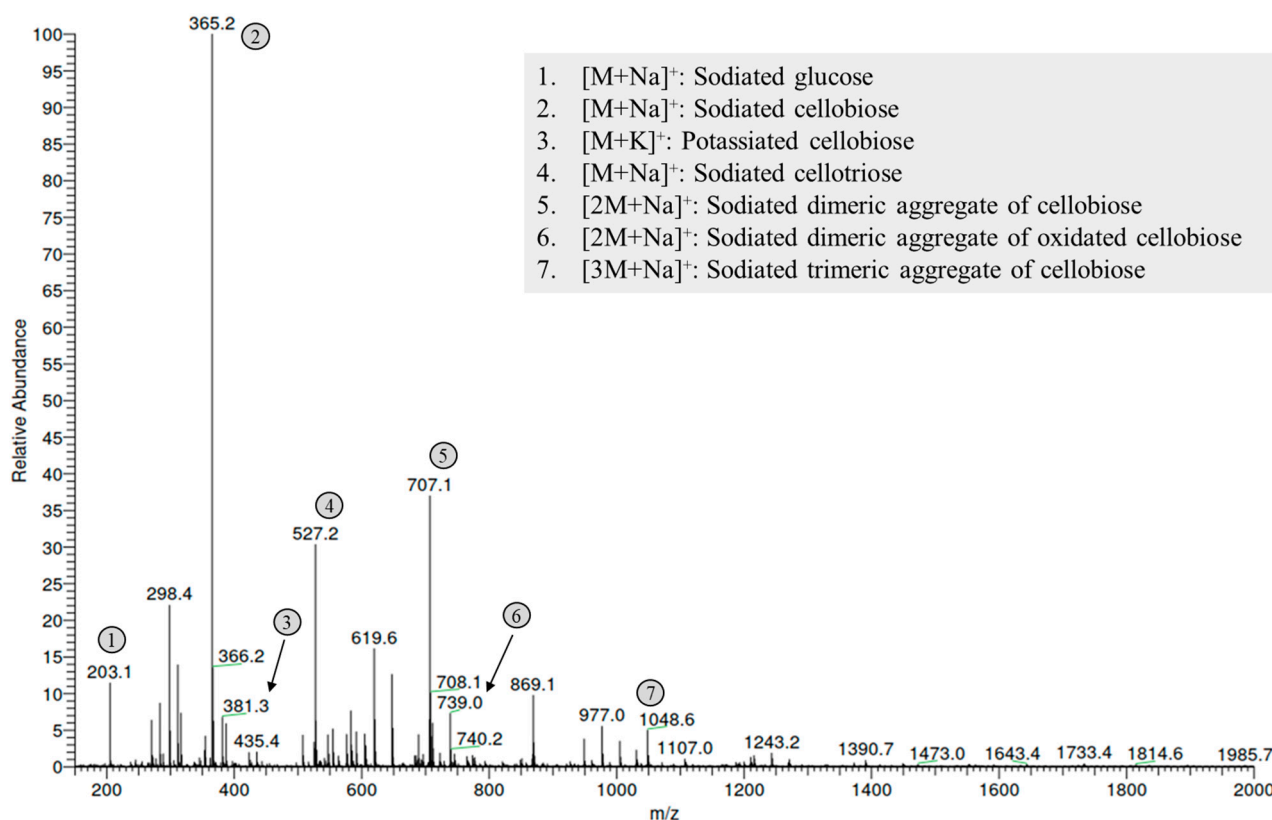


Figure 4. Mass spectrum of the hydrolysate supernatant. The hydrolysate supernatant was derived from HRC85-10 hydrolyzed with Celluclast® at pH 7 for 1 h. Green lines connect each m/z value to its corresponding peak to clearly indicate which peak the value refers to.

Based on the monoisotopic exact masses of molecular ion adducts commonly observed in ESI mass spectra, as reported by Huang and Siegel (1999) [20], the peaks at m/z 707.1 and 1048.6 were assigned to sodiated dimeric $[2M + Na]^+$ and trimeric $[3M + Na]^+$ aggregates of cellobiose, respectively. These findings provide direct evidence of spontaneous molecular self-assembly during the ionization process, a phenomenon consistent with prior observations reported by Chen et al. (2017) [21]. Moreover, the peak at m/z 739.0 was attributed to an oxidized dimer of cellobiose ($[2M + Na]^+$), indicating that partial oxidation may have occurred during ionization.

Together, these observations confirm the enzymatic release of WCOs with varying degrees of polymerization, primarily cellobiose and cellotriose, accompanied by glucose, while cellotetraose was not observed.

3.5. In Vivo Prebiotic Evaluation of WCOs Derived from Phosphoric Acid-Pretreated HRC

All mice remained healthy and active throughout the experimental period. During the experiment, mice in the treatment groups received daily intragastric administration of WCO solution at doses of 206 mg/kg body weight (low-dose group) and 824 mg/kg body weight (high-dose group), corresponding to human-equivalent doses of approximately 1 g/day and 4 g/day, respectively. The WCO solution—produced from phosphoric acid-pretreated HRC hydrolyzed at pH 7.0 for 1 h—had a high purity of approximately 93.5%. After 28 days of feeding, no significant differences were observed in final body weight

(36.1–37.2 g) and daily food intake (6.9–7.6 g/day) among the groups, indicating that WCO supplementation did not adversely affect general health or appetite.

To evaluate the prebiotic effects of WCOs, fecal pH and viable counts of *Lactobacillus* spp. and *Bifidobacterium* spp. were assessed. As shown in Table 4, *Lactobacillus* spp. counts increased significantly in the low-dose group ($p < 0.05$), rising from 6.33 log CFU/g (control) to 6.94 log CFU/g, and further increased to 7.24 log CFU/g in the high-dose group. In comparison, *Bifidobacterium* spp. exhibited a significant increase only in the high-dose group ($p < 0.05$), with counts rising from 7.09 log CFU/g (control) to 7.93 log CFU/g.

Table 4. Effects of WCO supplementation on fecal pH and viable counts of *Lactobacillus* spp. and *Bifidobacterium* spp. in mice.

Groups	Viable Counts [log (CFU/g)]		Fecal PH
	<i>Lactobacillus</i> spp.	<i>Bifidobacterium</i> spp.	
Control	6.33 ± 0.20 a	7.15 ± 0.15 a	6.99 ± 0.10 a
Low-dose	6.94 ± 0.25 b	7.53 ± 0.10 ab	6.49 ± 0.13 b
High-dose	7.24 ± 0.18 b	7.84 ± 0.43 b	6.56 ± 0.01 b

a–b: Means with different letters are significantly different in same column ($p < 0.05$, $n = 5$).

The prebiotic efficacy of WCOs derived from phosphoric acid-pretreated HRC likely stems from their resistance to digestion in the upper gastrointestinal tract and their subsequent fermentation by specific gut bacteria in the colon. Recent in vitro studies have demonstrated that certain *Lactobacillus* species, including *Levilactobacillus brevis* (formerly known as *Lactobacillus brevis*), *Lactobacillus plantarum*, and *Lactobacillus reuteri*, can metabolize β -(1→4)-linked cellobiose and, to varying extents, cellotriose [19,22]. This enzymatic capability enables these species to utilize cello-oligosaccharides as a carbon source, promoting their proliferation under favorable gut conditions. These species are commonly found in the gastrointestinal tracts of both humans and rodents, with *L. plantarum* and *L. reuteri* recognized as well-established gut commensals [23], while *L. brevis* is frequently introduced through dietary intake [24].

Both low-dose and high-dose WCO supplementation groups exhibited a significant reduction in fecal pH compared to the control group ($p < 0.05$), although no significant difference was observed between the two dosage levels. This reduction in fecal pH was likely attributable to microbial fermentation of WCOs by *Lactobacillus* spp., leading to the production of organic acids such as lactic acid and acetic acid [19,22].

Furthermore, *Bifidobacterium breve* UCC2003 has been shown to metabolize cellodextrins as a carbon source. Growth analysis revealed that this strain preferentially utilizes cellotriose over other cellodextrins, including cellobiose, cellotetraose, and cellopentaose, underscoring its efficient uptake and metabolic processing of shorter cello-oligosaccharides [25]. This observation aligns with our findings, where WCO supplementation significantly increased *Bifidobacterium* spp. counts in the gut. Collectively, these in vivo results support the hypothesis that WCOs possess substantial prebiotic potential by promoting beneficial gut bacteria and modulating the gut environment, as indicated by reduced fecal pH.

4. Conclusions

This study demonstrated an integrated phosphoric acid-assisted enzymatic strategy for the selective production of high-purity water-soluble cellulosic oligomers (WCOs) from microcrystalline cellulose. Pretreatment with 85 wt% phosphoric acid, particularly at 10 °C, effectively reduced cellulose crystallinity and crystallite size. Notably, maintaining cellulose in a hydrated state (HRC) further improved solubilization and WCO selectivity,

likely due to its fully amorphous, highly hydrated structure. Reaction conditions were critical in modulating hydrolysis outcomes; low pH and prolonged hydrolysis promoted glucose formation, whereas neutral pH with short hydrolysis (1 h) favored WCO retention, achieving up to 93.5% selectivity. ESI-MS analysis confirmed the presence of cellobiose and cellotriose as the major components of WCOs. In vivo evaluation revealed that high-purity WCOs stimulated the growth of *Lactobacillus* spp. and *Bifidobacterium* spp., supporting their prebiotic potential. These findings provide a practical and efficient approach for producing functional WCOs for dietary applications.

Beyond the current findings, this study opened up opportunities for broader applications and further investigation. The high-purity WCOs produced through this method have potential as functional ingredients in prebiotic formulations, particularly in the food and nutraceutical sectors. Future work should explore the scalability of the process, including phosphoric acid recovery and recycling strategies, to enhance both environmental and economic sustainability. Additionally, further studies are warranted to elucidate the fermentation behavior of these WCOs in the gut microbiota and to investigate their potential roles in other health-related applications.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pr13041245/s1>, Figure S1: ESI-MS spectrum of the hydrolysate derived from HRC85-10 treated with Celluclast® at pH 7.0 for 0.5 h, showing detectable signals corresponding to glucose, cellobiose, cellotetraose, and cellohexaose. Green lines connect each m/z value to its corresponding peak to clearly indicate which peak the value refers to.

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Abbreviations

The following abbreviations are used in this manuscript:

WCOs	Water-soluble cellulosic oligomers
MCC	Microcrystalline cellulose
RC	Regenerated cellulose
DRC	Dried regenerated cellulose
HRC	Hydrated regenerated cellulose
DP	Degree of polymerization
XRD	X-ray diffraction
ESI-MS	Electrospray ionization mass spectrometry
FWHM	Full width at half maximum
BIM-25	<i>Bifidobacterium</i> Iodoacetate Medium 25
ANOVA	One-way analysis of variance
CrI	Crystallinity index

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