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ORIGINAL ARTICLE

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Enzymatic saccharification of water-soluble portion after hot-compressed water treatment of Japanese beech with xylanase and β-xylosidase

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Abstract Enzymatic saccharifications of the hydrolysate collected as the water-soluble (WS) portion after hot-compressed water (HCW) treatment of Japanese beech with xylanase and β-xylosidase were performed to investigate the hydrolytic properties of these enzymes. The WS portions with different molecular weight distributions and initial concentrations of xylooligosaccharides were prepared as substrates, one of which contained mainly smaller xylooligosaccharides [degree of polymerization (DP) = 2 or 3], while the other contained larger xylooligosaccharides (DP ≥ 4). The highest xylose recovery was obtained from both of the WS portions treated with β-xylosidase rather than with xylanase. This suggests that β-xylosidase could have a higher activity than xylanase toward larger xylooligosaccharides (DP ≥ 4) as well as smaller xylooligosaccharides (DP = 2 or 3) recovered from HCW treatment. As a result, the use of β-xylosidase was found to be effective for saccharification of the WS portion, even with a short reaction time for the HCW treatment.

Key words Enzymatic saccharification · Water-soluble portion · Hot-compressed water treatment · Xylanase · β-Xylosidase

Introduction

Hot-compressed water (HCW) treatment of lignocellulosics has been studied for decades and is generally regarded as one of the pretreatments for saccharification of cellulose with cellulolytic enzymes. Other treatments include the use of steam, acid, or alkali.[1] HCW treatment is effective in solubilizing hemicellulose and lignin.[2] Under severe conditions of high reaction temperature or long reaction time, xylan dissolution increases and thus susceptibility of residual glucan with cellulolytic enzymes increases,[3] while xylan recovery decreases.[4] Saccharification of residual glucan into glucose with cellulolytic enzymes has been mainly targeted to ethanol production. Hemicellulolytic enzymes have also been employed in this process together with cellulolytic enzymes, because they enhance saccharifications of residual glucan by removing insoluble xylan to improve the accessibility of cellulolytic enzymes.[5] On the other hand, few studies have been made on saccharification of the water-soluble (WS) portion after HCW treatment, which mainly contains saccharides derived from xylan. From the viewpoint of cost effectiveness in ethanol production, conversion of all polysaccharides in lignocellulosic material into fermentable sugars is desirable. For HCW treatment, xylan is recovered in the form of oligosaccharides rather than monosaccharides.[6] Xylooligosaccharides in the WS portion could be saccharified with xylanolytic enzymes into xylose, which pentose-fermenting microorganisms could utilize to produce ethanol.

Xylanolytic enzymes are produced by many microorganisms accompanied with multiple isoenzymes, which may have diverse physicochemical properties, structures, specific activities, and so on, including typical examples of *Aspergillus niger* and *Trichoderma viride*.[7] It seems, therefore, that comprehensive understanding of the catalytic actions of these enzymes has been often hampered by their complexity. In practice, xylanase has been widely utilized in paper-making processes for enhancing delignification during chemical pulping and bleaching, in animal feed for the purpose of improving digestion of nutrients,[8] and in manufacturing of xylooligosaccharides for use in foods, cosmetics, and pharmaceuticals.[9] In general, xylanase cleaves xylan polysaccharide backbone randomly,[7] releasing oligosaccharides [degree of polymerization (DP) ≥ 4] initially and xylose, xylobiose, and xylotriose at a later stage.[8] In contrast, β-xylosidase cleaves xylooligosaccharides and xylobiose into xylose from the nonreducing ends, but does not hydrolyze xylan.[8] The activity of xylanase generally increases with increasing DP.[10] As for β-xylosidase, on the other hand, the best substrate is xylobiose and its affinity for xylooligosaccharides decreases with increasing DP.[8]

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In addition to hydrolytic activity, some xylanase[11] and β-xylosidase[12] have a transxylosylation activity. Due to the transxylosylation activity, xylanase[11] and β-xylosidase[12] can convert xylooligosaccharides partly into saccharides with higher DP. In addition to the hydrolytic actions of the single enzymes, synergisms between xylanase, β-xylosidase, and debranching enzymes such as acetyl xylan esterase[13] and α-glucronidase[14] on xylan degradation are important.

As mentioned above, the hydrolytic properties in saccharifications of the WS portion after HCW treatment with xylanolytic enzymes remain to be clarified. Relationships between the reaction conditions of HCW treatments and the enzymatic saccharifications of the WS portion after HCW treatment, for example, will provide additional insights into conversion efficiency of lignocellulosics. This could enable the evaluation of the reaction conditions of the HCW treatments in terms of the total recovery of fermentable pentose as well as hexose sugars. Therefore, in this study, enzymatic saccharifications of the WS portions after HCW treatment, which were prepared under two particular HCW conditions, were performed to investigate the hydrolytic properties of xylanase and β-xylosidase in the saccharifications.

Experimental

Materials and chemicals

Japanese beech (*Fagus crenata*) was subjected to HCW treatment. Wood chips of Japanese beech were milled into flours passing 80 mesh for use as raw materials. Xylobiose and xylotriose were purchased from Megazyme (Wicklow, Ireland). Distilled water of high-performance liquid chromatography (HPLC) grade, sodium acetate for high performance anion exchange chromatography (HPAEC) eluent, and 97% sulfuric acid for quantification of xylooligosaccharides in the WS portion were purchased from Nacalai Tesque (Kyoto, Japan). Sodium hydroxide solution (50% w/v) for HPAEC eluent was purchased from Wako (Osaka, Japan).

Enzymes

Xylanase was purchased from Sigma-Aldrich (St. Louis, MO, USA). According to the manufacturer's descriptions, xylanase was purified from *Trichoderma viride*, its activity was $80\,\mathrm{U\,mg^{-1}}$ containing cellulase, β-glucosidase, and β-xylosidase activities of less than 1%, 0.01%, and 0.002%, respectively. Lyophilized powder of xylanase was dispersed in acetate buffer and prepared as enzyme solution. One unit of xylanase was defined as the amount of enzyme that catalyzes the release of 1 µmol of xylose equivalents from xylan per minute at pH 4.5 at 30°C.

β-Xylosidase was also purchased from Sigma-Aldrich. According to the manufacturer's descriptions, β-xylosidase was purified from *Aspergillus niger*, its activity was $1.5\,\mathrm{U\,ml^{-1}}$ buffer solution containing β-*N*-acetylglucosaminidase, β-galactosidase, and α-galactosidase activities of less than 3%. One unit of β-xylosidase was defined as the amount of enzyme that hydrolyzes 1 µmol of *o*-nitrophenyl β-xyloside equivalents to *o*-nitrophenol and xylose per minute at pH 5.0 at 25°C.

HCW treatments

Japanese beech flour (450 mg) was sealed into a batch reactor[15,16] with 5.0 ml of distilled water. HCW treatments were initiated by soaking the reactor in a molten tin bath, which was preheated to approximately 10°C above the reaction temperature. The reaction temperature was set at 240°C, and reaction time at 45 s or 90 s. The reaction was stopped by immersing the reactor in a water bath. The reaction time was defined as the residence time for the reactor in the tin bath including 10 s of heating time until the inner temperature of the reactor reached 240°C. The pressure inside the batch reactor was 5.0 MPa when the inner temperature reached 240°C. The WS portions after the HCW treatments were separated from the residue by centrifugation and filtration with a 1.0-µm membrane.

Enzymatic saccharifications

To the WS portions after HCW treatments, xylanase or β-xylosidase were loaded at 0.03, 0.3, 1.5, $3\,\mathrm{U\,ml^{-1}}$ WS portions, respectively, after adjusting the pH to 5.0 with $Ca(OH)_2$. Then the WS portions were incubated at 30°C for 48 h with magnetic stirring. For analysis of the saccharides contained, the WS portions were withdrawn at treatment times of 1, 3, 6, 12, 24, and 48 h. The concentration of each saccharide was presented on the basis of the volume of the WS portions, because the concentrations of the saccharides were considerably diluted with enzyme solutions in cases of higher enzyme loading.

Analysis of the WS portion

Xylose, xylobiose, and xylotriose in the WS portions after HCW treatments and after enzymatic saccharifications were analyzed by HPAEC (ICS-3000, Dionex, Sunnyvale, CA, USA) with pulsed amperometric detection (Dionex, Sunnyvale, CA, USA) using a CarboPac PA-1 column (Dionex, Sunnyvale, CA, USA) connected with a CarboPac PA-1 guard column (Dionex, Sunnyvale, CA, USA). Sodium hydroxide solution (0.2 M), sodium acetate solution (1 M), and distilled water were used as mobile phase. Elution at a flow rate of $1.0\,\mathrm{ml\,min^{-1}}$ was performed isocratically with NaOH solution in distilled water over 12 min, followed by a combination of linear gradients of NaOH solution in distilled water to NaOH solution over 20 min, NaOH solution to CH_3COONa solution in NaOH solution over 20 min, and then isocratically with CH_3COONa solution in NaOH solution over 10 min. Before injections, each of the samples was filtered with a 0.45-µm filter and diluted 50-fold in distilled water.

Xylooligosaccharides in the WS portions after HCW treatments were quantified as follows. The WS portions with 4% sulfuric acid were autoclaved at 121°C for 1 h and filtered with a 0.45-µm filter and analyzed by HPLC (LC-10A, Shimadzu, Kyoto, Japan) using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) fitted with a Cation H cartridge (Bio-Rad, Hercules, CA, USA) in a column oven set at 45°C. Aqueous sulfuric acid (5 mM) was used as the mobile phase at a flow rate of 0.6 ml min^{-1}. Xylose was detected with a refractive index detector (RID-10A, Shimadzu, Kyoto, Japan). The concentrations of xylooligosaccharides with DP of 4 or greater were calculated by subtracting the summative concentrations of xylose, xylobiose, and xylotriose from the concentrations of xylose recovered in the quantification process.

Molecular weight distributions of the WS portions after HCW treatments were also analyzed using a gel permeation chromatography (GPC) column, Asahipak GS-220 HQ (Showa Denko, Tokyo, Japan), in a column oven set at 40°C with the same system and detector used for HPLC. Distilled water was used as the mobile phase at a flow rate of 0.6 ml min^{-1}.

Results and discussion

HCW treatments

GPC chromatograms of the WS portions obtained by HCW treatment of Japanese beech at 240°C for 45 s and 90 s are shown in Fig. 1. The concentrations of the monosaccharides and oligosaccharides of xylan recovered in the HCW treatments are shown in Table 1. The peak seen around 10–14 min in the chromatogram for the 45-s treatment (Fig. 1a) was thought to correspond to xylooligosaccharides (DP ≥ 4), which were mainly recovered in the HCW treatment for 45 s as in Table 1. This peak was found to be shifted to around 14–16 min in the chromatogram for the 90-s treatment (Fig. 1b), where the peaks correspond to xylotriose, xylobiose, and xylose, and a sharp xylose peak at around 16 min is observed. These xylooligosaccharides were the main components of the WS portion after the 90-s HCW treatment as in Table 1. Table 1 also shows that the recoveries of xylose, xylobiose, and xylotriose were higher for 90 s than for 45 s, whereas recovery of xylooligosaccharides with DP of 4 or greater was lower. From these results, it was deduced that xylooligosaccharides with DP of 4 or greater could be converted into xylose, xylobiose, and xylotriose by HCW treatment with the longer reaction time.

Hydrolytic properties of WS portions with xylanase or β-xylosidase

Effect of the amount of the enzymes loaded

The amount of enzyme loaded was optimized in order to maximize the recovery of xylose after enzymatic saccharification. Figure 2 shows the maximal concentrations of xylose, xylobiose, and xylotriose during 48 h of enzymatic saccharification of the WS portions obtained by HCW treatments of Japanese beech at 240°C for 90 s. Because the initial concentrations of the saccharides were considerably diluted with enzyme solutions in the cases of higher enzyme loading, the initial saccharification rate could be affected. For the saccharifications with xylanase (Fig. 2a), 0.3 U was sufficient to reach the highest maximal concentration of xylose. Loaded with excess amount of enzymes, the maximal concentrations of xylobiose and xylotriose increased although that of xylose did not change. For the saccharifications with β-xylosidase (Fig. 2b), 0.3 U was sufficient to reach the highest maximal concentration of xylose as well as with xylanase. However, loaded with excess enzyme, the maximal

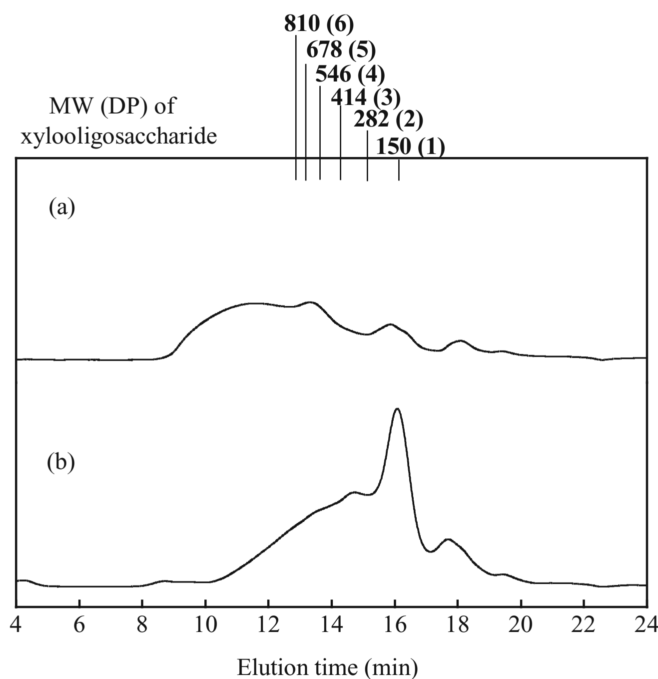

Fig. 1a,b. Gel permeation chromatograms of the water-soluble (WS) portions of Japanese beech at **a** 240°C for 45 s and **b** 240°C for 90 s. *MW*, Molecular weight, *DP*, degree of polymerization

Table 1. Concentrations of xylose, xylobiose, xylotriose, and xylooligosaccharides recovered in the water-soluble (WS) portions of Japanese beech

Reaction conditions	Concentration (g l^{-1})			
	Xylose	Xylobiose	Xylotriose	Xylooligosaccharides (DP ≥ 4)
240°C, 45 s	0.29	0.23	0.23	7.46
240°C, 90 s	2.50	1.30	0.95	4.63

DP, degree of polymerization

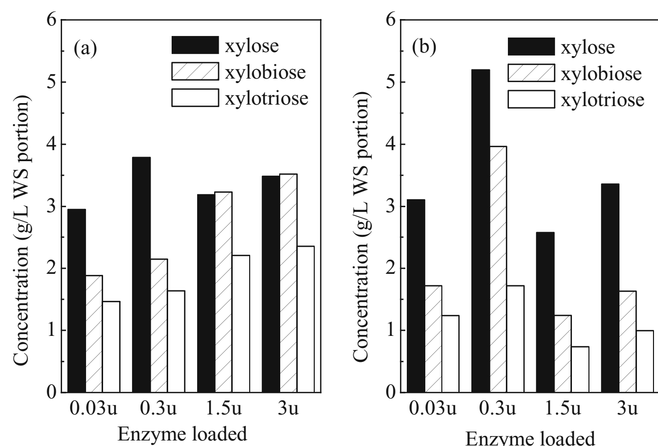

Fig. 2a,b. Maximum concentrations of xylose, xylobiose, and xylotriose during 48 h of enzymatic saccharification of the WS portions of Japanese beech at 240°C for 90 s with **a** xylanase and **b** β-xylosidase

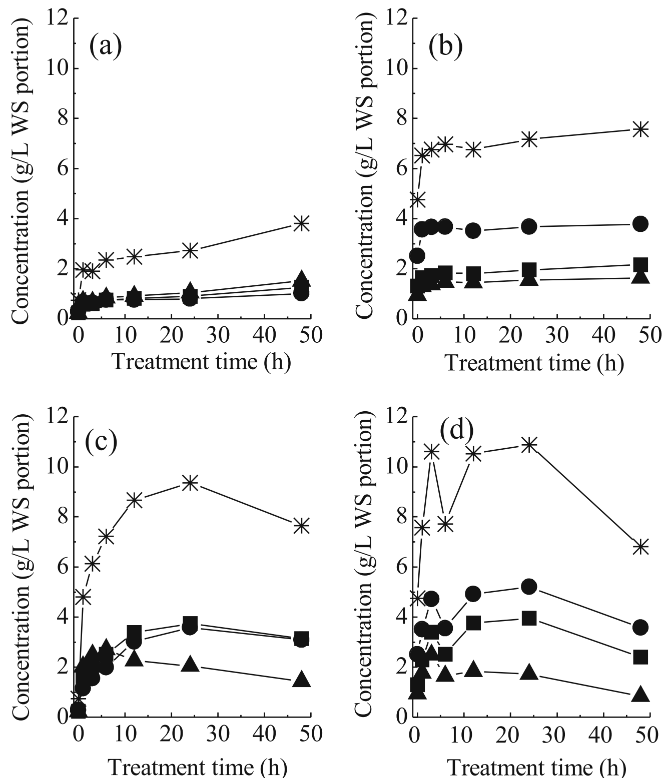

Fig. 3a–d. Time course of the concentrations of xylose, xylobiose, and xylotriose during enzymatic saccharifications of the WS portions of Japanese beech. **a** Hot-compressed water (HCW) treatment at 240°C for 45 s, xylanase 0.3 U; **b** HCW treatment at 240°C for 90 s, xylanase 0.3 U; **c** HCW treatment at 240°C for 45 s, β-xylosidase 0.3 U; **d** HCW treatment at 240°C for 90 s, β-xylosidase 0.3 U. *Circles*, xylose; *squares*, xylobiose; *triangles*, xylotriose; *asterisks*, sum of xylose, xylobiose, and xylotriose

concentrations of not only xylobiose and xylotriose but also xylose decreased in contrast with xylanase. This was probably due to transxylosylation with β-xylosidase. It could also be noted that xylobiose and xylotriose were not converted into xylose completely even with excess enzyme loadings for β-xylosidase. Apparently, an equilibrium state seemed to be reached within 48 h of enzymatic saccharification. This was possibly because enzymatic saccharifications with β-xylosidase were partly inhibited by xylose as previously reported.[17] The ratios of xylose, xylobiose, and xylotriose in the equilibrium states seemed to depend on the amount of β-xylosidase loaded.

Time-course profiles of enzymatic saccharifications

The time-course concentrations of xylose, xylobiose, xylotriose, and the sum of these saccharides during the 48-h enzymatic saccharifications with xylanase or β-xylosidase of the WS portions after HCW treatment at 240°C for 45 or 90 s are shown in Fig. 3. The amount of enzyme loaded was fixed to 0.3 U based on the results shown in Fig. 2.

The maximum concentrations of xylose, xylobiose, and xylotriose and the sum of them throughout the whole process of the HCW treatments and the enzymatic saccharifications were compared. The concentrations obtained during the enzymatic hydrolyses of the WS portions after HCW treatment for 90 s (Fig. 3b, d) were higher than those for the 45-s treatment (Fig. 3a, c) with either xylanase or β-xylosidase. However, this is partly because the initial concentrations of these saccharides contained in the WS portions were higher for 90 s than for 45 s.

It was interesting to find that the concentrations of the products increased and decreased during the saccharifications of the solutions treated with β-xylosidase (Fig. 3c, d). This type of hydrolytic property was also found in the saccharifications of the solutions treated for either 45 or 90 s with β-xylosidase, and even with xylanase for the lower amount of enzyme loaded (data not shown). This was probably due to the transxylosylation activity that both enzymes are reported to have.[11,12]

In addition, it is noteworthy that a state of equilibrium appeared to occur in each of the saccharifications shown in Fig. 3 as well as those mentioned above in relation to Fig. 2. The ratios of the concentrations of xylose, xylobiose, and xylotriose in the equilibrium states were also different between the solutions for the 45-s and 90-s treatments either with xylanase or β-xylosidase. This suggests that initial concentrations or the distribution of molecular weight of the saccharides contained in the WS portions could also affect the ratios in the equilibrium states, as well as the amount of enzyme loaded.

Net increases in the concentrations of xylose, xylobiose, and xylotriose and the sum of them during enzymatic saccharifications after 3 h and 48 h were calculated by subtracting the initial concentrations of these saccharides from the maximal concentrations of them within the periods, and indicated separately in Table 2. Initial saccharification rate was defined as the net increase in the concentration of xylose per hour. Firstly, the initial saccharification rates (xylose g l^{-1} h^{-1}) for the enzymatic saccharification using xylanase and β-xylosidase within 3 h were compared. The initial saccharification rates for the solution treated for 45 s were 0.11 g$l^{-1}h^{-1}$with xylanase and 0.42 g$l^{-1}h^{-1}$with β-xylosidase. For 90-s treatments, the rates were 0.38 g$l^{-1}h^{-1}$

Table 2. Net increases in concentrations of xylose, xylobiose, and xylotriose during enzymatic saccharifications of the WS portions of Japanese beech

Treatment		Net increase in concentration (g l^{-1} WS portion)								
		After 3 h					After 48 h			
		Xylose	Xylobiose	Xylotriose	Sum[a]	Initial rate[b] (xylose g l^{-1} h^{-1})	Xylose	Xylobiose	Xylotriose	Sum[a]
Xylanase	45 s	0.35	0.36	0.43	1.12	0.11	0.73	1.03	1.29	3.05
	90 s	1.15	1.60	0.42	2.01	0.38	1.28	0.85	0.69	2.82
β-Xylosidase	45 s	1.27	1.85	2.26	5.38	0.42	3.30	3.51	1.81	8.62
	90 s	2.22	2.10	1.55	5.85	0.74	2.70	2.66	0.77	6.13

[a] Sum of xylose, xylobiose, and xylotriose
[b] Initial saccharification rate

with xylanase and 0.74 g l^{-1} h^{-1} with β-xylosidase, indicating that the initial saccharification rates were higher for 90 s than for 45 s. This was probably because the initial concentrations of each saccharide contained in the WS portions were higher for 90 s than for 45 s. Comparing β-xylosidase with xylanase within 48 h, it was obvious that for both 45-s and 90-s HCW treatments, the net increases in the concentration of each saccharide and the sum for the solutions treated with β-xylosidase were much higher than those for the solutions treated with xylanase.

For saccharifications with β-xylosidase, it was found that the net increases in the concentration of each saccharide and the sum were higher for the WS portions treated for 45 s than for 90 s. It was unexpected that xylobiose and xylotriose increased to a higher extent with β-xylosidase than with xylanase from the solution treated for 45 s, which mainly contained xylooligosaccharides with DP of 4 and greater, as mentioned above. As reported in previous studies, xylanase has a higher affinity to larger xylooligosaccharides (DP ≥ 4), whereas β-xylosidase has a higher affinity to smaller xylooligosaccharides (DP = 2 or 3).[8,10] Nevertheless, in this study, more xylobiose and more xylotriose were recovered with β-xylosidase than with xylanase, and from the solutions treated for 45 s, rather than 90 s, with β-xylosidase.

This was possibly because β-xylosidase had a better activity toward larger xylooligosaccharides (DP ≥ 4) than xylanase in the WS portion recovered after the HCW treatment. A previous study found that activities of β-xylosidase rather than xylanase correlated well with saccharifications of the WS portion from steamed birchwood xylan with crude xylanolytic enzymes prepared from different *Trichoderma reesei* cultures. The study found that the purified β-xylosidase alone was, however, not capable of producing xylose from the WS portion.[18] The capability of β-xylosidase on the WS portion in this study is likely due to a difference in the molecular weight distribution of the xylooligosaccharides in the WS portion from that of the previous study. The WS portion was mainly composed of much larger xylooligosaccharides (DP > 10) in the previous study, and there may have been some differences between the steam and HCW treatments. However, it is also possible that in the present study, xylanase was more sensitive to the inhibitory compounds in the WS portion than β-xylosidase or that the level of inhibitory compounds in the WS portion treated for the 90-s treatment was much higher than for the 45-s treatment. Soluble phenolic compounds, for example, were reported to activate[19] or deactivate[20] xylanase activity. To the best of our knowledge, inhibitory effects on β-xylosidase by soluble compounds have not been reported. Further studies are required to elucidate the influence of inhibitory effects on these enzymes.

Conclusions

Hydrolytic properties of purified xylanase and β-xylosidase on WS portions after HCW treatment of Japanese beech at 240°C for 45 s and 90 s were investigated. The highest xylose recovery was obtained after the enzymatic saccharification of the WS portion after HCW treatment for 90 s with β-xylosidase. It was possible that β-xylosidase had a higher hydrolytic activity to the WS portions than xylanase, even when larger xylooligosaccharides (DP ≥ 4) recovered after HCW treatment for the shorter reaction time were the main components. Accordingly, it was suggested that using β-xylosidase, HCW treatments with the shorter reaction time could be an alternative for effective saccharifications. These results obtained in this study could be useful in evaluating the reaction conditions of HCW treatments or the subsequent enzymatic saccharifications to produce fermentable pentose sugars from the WS portions.

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