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Improving saccharide concentration by mixing octyl acetate during semi-flow, hot-compressed water treatment of woody biomass

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Abstract

Biomass-based ethanol or acetic acid can be produced by fermentation of saccharides following wood hydrolysis. Both high saccharide yield and concentration are essential for achieving an efficient hydrolysis process. However, there can be a trade-off between yield and concentration during semi-flow, hot-compressed water treatment, a candidate for wood hydrolysis. This trade-off is because the fast water flow rate is necessary for high saccharide yields, but the large amount of water dilutes the produced hydrolysate. In this study, we mixed octyl acetate, which is a water-immiscible solvent, during hot-compressed water treatment of beech wood. By adding octyl acetate, a high flow rate was achieved without increasing the water flow rate, and the saccharide concentration was thus improved, while suppressing the decrease in saccharide yield. Furthermore, lignin-derived oligomers, which have inhibitory effects on fermentation, were removed from hydrolysate because they can dissolve in octyl acetate. We performed acetic acid fermentation using the obtained hydrolysate and found that the fermentability was markedly improved compared with the hydrolysate obtained without octyl acetate.

Keywords

hot-compressed water; octyl acetate; lignocellulose; acetic acid fermentation

1. Introduction

Lignocellulosic resources, such as woody biomass, are abundant and do not compete with food demands; therefore, they are ideal raw materials for bioethanol. Lignocellulose is a complex material composed of cellulose, hemicelluloses, and lignin, and there are still many challenges for establishing bioethanol production technology. In typical bioethanol production systems [1], cellulose and hemicelluloses in lignocellulose are hydrolyzed into saccharides. These saccharides are fermented with yeast to produce an aqueous ethanol solution. The aqueous solution is then distilled and dehydrated to purify bioethanol.

For decades, many researchers have studied various hydrolysis techniques, including acid-based hydrolysis and enzymatic hydrolysis [2, 3]. However, these methods have drawbacks. Acid-catalyzed hydrolysis requires catalyst recovery or neutralization, and the enzymatic method is a high-cost process with low-productivity. Therefore, we have proposed a two-step, semi-flow, hot-compressed water treatment technique [4-8]. For this method, the first stage is performed at approximately 230 °C to decompose hemicelluloses and lignin; the second stage decomposes cellulose at approximately 270 °C. Semi-flow reactors can quench the reaction quickly to prevent undesired degradation of saccharides. Owing to the high ionic products of hot-compressed water, this method can efficiently hydrolyze various lignocelluloses without a catalyst, producing 40–50 wt% mono- and oligosaccharides on a dry weight basis of lignocellulose [4-8].

In general, both high saccharide yield and concentration are important for efficient bioethanol

production. Lower saccharide yields lead to lower ethanol yields. Additionally, low saccharide yield usually indicates that the saccharides were decomposed into other compounds, such as furans and organic acids, which inhibit alcoholic fermentation [9]. A low saccharide concentration occurs when a large amount of water is used in the reaction, which increases energy consumption, especially during distillation for water removal [10].

As noted above, the semi-flow, hot-compressed water treatment can efficiently hydrolyze lignocellulose. Increasing the water flow rate results in faster flow of hydrolysis products out of the reactor and higher saccharide yields. However, if the water flow rate increases, the saccharide concentration decreases. This trade-off relationship between yield and concentration is a concern for adopting this hydrolysis method for bioethanol production. In addition, our research group is studying bioethanol production via acetic acid fermentation instead of alcoholic fermentation [11]. Acetic acid fermentation has superior carbon utilization efficiency compared with alcoholic fermentation but requires both high saccharide yield and concentration. Lignin-derived products and degradation products of saccharides inhibit both acetic acid and alcohol fermentation [12].

Therefore, this research aimed to improve this trade-off relationship by adding a water-immiscible organic solvent during hot-compressed water treatment. High saccharide yield was expected because the total flow rate was increased by adding organic solvent. Because saccharides are hydrophilic and dissolve only in the water phase, a high-concentration aqueous saccharide solution could be obtained after phase separation. Additionally, if an organic solvent that dissolves

lignin-derived products is used, lignin-derived products will be removed from the water phase. Because lignin-derived products inhibit both alcohol [13, 14] and acetic acid fermentation [12], removal of these products is expected to improve the fermentability of the resulting water phase.

Thus, the following are requirements for the organic solvent: complete phase separation from water, solubility of lignin-derived products, and stability in hot-compressed water. Well-known solvents that phase separate from water include hydrocarbons, phenols and alkyl acetates. Hydrocarbons, such as alkanes and aromatics, are hydrophobic and separate well from water but do not well dissolve lignin-derived products that have hydrophilic characteristics. In fact, thermal decomposition of wood in various hydrocarbons tends to produce high amounts of char [15] because undissolved products are readily polymerized [16, 17]. Phenolic solvents, such as phenol and cresol, can dissolve lignin-derived products [18], but they are only slightly soluble in water because of the presence of hydroxyl groups. Anisoles are promising solvents for the same reasons as phenols but are also only slightly soluble in water. Alkyl acetates, such as ethyl acetate, are known as suitable solvents for the extraction of lignin-derived products. However, as described later, ethyl acetate can be hydrolyzed in hot-compressed water [19]. In preliminary experiments, acetates with longer alkyl groups exhibited better stability in hot-compressed water and better phase separation in water. As a result, *n*-octyl acetate was selected in this study because it fully satisfied the above requirements.

To clarify these effects, we added octyl acetate during the hot-water treatment of wood. The obtained aqueous saccharide solutions were subjected to acetic acid fermentation, and the cases with

and without octyl acetate mixing were compared. During the decomposition of lignocellulose with hot-compressed water, the yields and concentrations of saccharides conflicted, but no previous studies have improved this trade-off. This study aimed to improve the trade-off using the simple solution of adding a water-immiscible organic solvent, and the results provide important insights for biomass utilization.

2. Materials and methods

2.1 Wood sample and chemicals

The sapwood of Japanese beech (*Fagus crenata*) was milled in a Wiley mill (Thomas Scientific, NJ, USA.). The wood flour was sieved with mesh screens between 0.15 and 0.5 mm and extracted for 8 h with acetone (Extra pure reagent, Nacalai Tesque, Inc., Kyoto, Japan) using a Soxhlet apparatus. The extractive-free wood flour was dried at 105 °C for 24 h before the experiment. The lignin content, including acid-soluble lignin, was determined by the Klason method [20] to be 24.9 wt% on a dry wood basis, and the carbohydrate content (cellulose and hemicelluloses) was thus estimated to be 75.1 wt% (100 – lignin). Ion-exchange water was used for the two-step, semi-flow, hot-compressed water treatment with the addition of *n*-octyl acetate (purity >98%, Sigma-Aldrich Japan, Tokyo, Japan).

2.2 Two-step, semi-flow, hot-compressed water treatment

Using the system shown in Figure 1 [4-8], the two-step, semi-flow, hot-compressed water treatment was carried out at various conditions. For each experiment, approximately 0.5 g of wood flour was aliquoted to a sample basket, shown in the photo of Figure 1, which was then placed in the high-pressure resistant reaction vessel. After closing the vessel, ion-exchanged water and octyl acetate were independently supplied to the reaction vessel through preheaters by high-pressure pumps at designated flow rates; the pressure inside the vessel was maintained at 10 MPa by a back-pressure regulator. During the first treatment stage, the temperature was increased from 150 to 230°C in approximately 10 min and maintained at 230°C for 15 min using electric heaters. In the second treatment stage, the temperature was raised to 270°C in approximately 8 min and maintained for 15 min [4-8]. The total treatment time depended on the heating times, as described later. During this treatment, wood-derived products dissolved in the hot-compressed water and octyl acetate mixture were continuously flowed out of the reaction vessel, cooled in a cooling jacket, and then collected in glass vials. The water-soluble and octyl acetate-soluble portions were obtained after phase separation.

After cooling the system, the reaction vessel was opened, and the solid residue remaining in the sample basket was recovered as an insoluble residue by filtration with a membrane filter (0.45-μm pore size, ADVANTEC MFS, Inc., CA, USA) while rinsing with water. The insoluble residue was washed with methanol on the filter to remove the octyl acetate, oven-dried at 105 °C, and then weighed with an electric balance.

115 2.3 Acetic acid fermentation

116 Acetic acid fermentations of the water-soluble portions were performed with a co-culture of
117 *Clostridium thermocellum* (ATCC27405) and *Moorella thermoacetica* (ATCC39073). Although *C.*
118 *thermocellum* can hydrolyze cellulose and cellooligosaccharides, it produces various substances such as
119 acetic acid, lactic acid, ethanol, H_2, and CO_2 [21]. In contrast, *M. thermoacetica* has no cellulolytic
120 abilities, but can assimilate various monosaccharides, organic acids, ethanol, H_2, and CO_2 [22-24],
121 producing almost exclusively acetic acid. Because hydrolysates from hot-compressed water treatment
122 of wood contain oligosaccharides, monosaccharides, and their degradation products, we found
123 previously [25] that the co-culture of both microorganisms showed excellent fermentation abilities for
124 the selective production of acetic acid from wood hydrolysates. Freeze-dried strains were purchased
125 from the American Type Culture Collection (ATCC, VA, USA). Culture revival, preparation of
126 inoculums, and composition and sterilization of nutrients and buffers were performed according to our
127 previous methods [25].

128 For each fermentation, the water-soluble portion (45 mL) was the substrate and was poured
129 into a 50-mL vial. Nutrients and buffer were dissolved in the substrate. The *C. thermocellum* and *M.*
130 *thermoacetica* (2.5 mL each) inoculums were added, and the vial was sealed. These operations were
131 performed in a nitrogen-substituted glovebox because both microorganisms are considered anaerobic
132 organisms. The inoculated vials were placed in a thermostatic chamber and maintained at 60 °C with
133 magnetic stirring. During fermentation, 0.5 mL of the culture solution was sampled once daily, and the

concentration of acetic acid was determined. The same experiment was carried out for ion-exchange water instead of the water-soluble portion as a blank test. The fermentation of each water-soluble portion and blank test was conducted three times.

2.4 Analytical methods

For the determination of monosaccharides, the water-soluble portions were analyzed by high-performance anion-exchange chromatography (HPAEC) using a high-performance liquid chromatography (HPLC) system (Prominence, Shimadzu Corp., Kyoto, Japan) equipped with an electrochemical detector (DECADE Elite, Antec Scientific, Zoeterwoude, Netherlands) and the following conditions: CarboPac PA-1 column (4 mm x 250 mm, Thermo Fisher Scientific, MA, USA); 30 mM aqueous sodium hydroxide solution mobile phase; 1.0 mL min^{-1} flow rate; and 35 °C column temperature.

For oligosaccharide measurements, 4 ml of the water-soluble portion was hydrolyzed in an autoclave at 121 °C for 30 min by adding 0.09 ml of sulfuric acid (Extra pure reagent, Nacalai Tesque, Inc.) to convert oligosaccharides into monosaccharides and neutralized with an anion-exchange cartridge (OnGuard II A, Thermo Fisher Scientific). The hydrolysate was then analyzed by HPAEC to determine the total saccharide yield, i.e., sum of monosaccharides and oligosaccharides. The oligosaccharide yield was estimated by subtracting the monosaccharide yield from the total saccharide yield. The yields of oligosaccharides and monosaccharides were expressed as wt% based on the dry

153 weight of the treated beech wood.

154 For lignin-derived products, gel permeation chromatography (GPC) was performed with the
155 HPLC system with the following conditions: Shodex KF-801, KF-802, KF-802.5 and KF-803 columns
156 (Showa Denko, Tokyo, Japan) connected in series; tetrahydrofuran eluent; 0.6 mL min^{-1} flow rate;
157 50 °C column temperature; and ultraviolet light detector at 275 nm. For the mass calibration,
158 polystyrene standards (molecular weights: 162, 580, 1270 and 2960) were used.

159 Acetic acid in the fermentation broth or water-soluble portion was quantified by HPLC under
160 the following conditions: HPX-87H column (Bio-Rad Laboratories, Inc., CA, USA); 5 mM aqueous
161 sulfuric acid solution eluent; 0.6 mL min^{-1} flow rate; 45 °C column temperature; and refractive index
162 detector.

163

164 **3. Results and discussion**

165 3.1 Effect of octyl acetate

166 Table 1 summarizes the experimental results using various octyl acetate ratios and total flow
167 rates. In all experiments, treatment times for the first (230 °C) and second (270 °C) stages were fixed at
168 15 min each, but the heating periods tended to increase with increasing total flow rate or octyl acetate
169 ratio. Therefore, the total treatment time in Table 1 varied and ranged from 44 to 54 min. In our
170 previous study, 15-min hold times were long enough to degrade lignin and hemicelluloses at 230 °C,
171 and cellulose at 270 °C [4]. The slight variations of the heating periods might not affect the

decomposition of wood. However, because the volumes of water and octyl acetate varied depending on the total treatment time, the saccharide concentration was slightly affected. The saccharide yield was the sum of neutral monosaccharides, such as glucose and xylose, and their oligosaccharides.

In Table 1, nos. 1–5 show the cases with water only. When the flow rate was 20 mL min^{-1}, the saccharide yield was as high as 51.1 wt%. However, because the amount of water was as much as 0.94 L (20 mL min^{-1} × 47 min), the concentration was the lowest at 0.27 g L^{-1} (0.50 g of wood × 51.1 wt% ÷ 0.94 L). As the water flow rate decreased, the saccharide concentration increased, but the yield decreased. Thus, the trade-off relationship between yield and concentration was evident and as described in the introduction.

To show the effects of octyl acetate, some of the data in Table 1 (Nos. 2, 6, 8, 10–12, and 14) are summarized in Figure 2, where the ratio of octyl acetate ranged from 0 vol% (water only) to 100 vol% (octyl acetate only), and the total flow rate was fixed at 10 mL min^{-1}. In the treatment with water only, the insoluble residue (a) was approximately 10 wt%, indicating that approximately 90 wt% of the beech wood flour was decomposed and solubilized. The saccharide yield (b) and concentration (c) were 43.7 wt% and 0.47 g L^{-1}, respectively.

Even increasing the ratio of octyl acetate to 25, 50, and 75 vol% resulted in yields of insoluble residue of approximately 10 wt%. When octyl acetate was further increased, the insoluble residue increased rapidly. The saccharide yield did not substantially change until the octyl acetate reached 50 vol%; as octyl acetate was increased further, the yield dropped sharply. The saccharide

191 concentration increased with increasing octyl acetate because the amount of water decreased and
192 reached 2.15 g L^{-1} with 90 vol% octyl acetate. This concentration was 4.6 times higher than in the case
193 of water only (0.47 g L^{-1}). However, this condition was not considered optimal since the saccharide
194 yield was reduced to approximately 20 wt%. To improve the saccharide concentration without
195 impairing the saccharide yield, 50 vol% octyl acetate may be appropriate. For the condition of 50 vol%
196 octyl acetate, the saccharide concentration was 0.84 g L^{-1}, which was an improvement of approximately
197 1.8 times that of water only.

198 The insoluble residue increased when the ratio of octyl acetate exceeded 75 vol% and was
199 likely because the hydrolytic degradation of cellulose and hemicelluloses was reduced due to the
200 decrease in water. The insoluble residues after the treatment with over 90 vol% octyl acetate were
201 analyzed by the Klason method [20], and some amounts of cellulose and hemicelluloses remained in
202 the residues.

203 The proportions of monosaccharides and oligosaccharides are shown in Figure 3. When
204 treated with water only (0 vol%), most of the saccharides were oligosaccharides. In semi-flow reactors,
205 the residence time of decomposition products was generally short. Therefore, the oligosaccharides
206 dissolved in water quickly flowed from the reaction system, and the hydrolysis of oligosaccharides to
207 monosaccharides did not proceed much during this short residence time. Similarly, undesirable
208 degradation of saccharides was also prevented, and thus, the total saccharide yield was high. As octyl
209 acetate increased, the ratio of oligosaccharides decreased, and the total saccharide yield also decreased.

These behaviors may indicate that the residence time of saccharides in the reactor increased, despite the total flow rate of water and octyl acetate being fixed at 10 mL min^{-1}.

As the ratio of octyl acetate increased, the contact surface between water and wood decreased. Under this situation, the immediate dissolution of saccharides to the water phase could be inhibited by octyl acetate, increasing the residence time of the saccharides on the wood surface. Because the saccharides may have stayed in the reactor longer, both hydrolysis and thermal degradation progressed, and thus, both the oligosaccharide ratio and the total saccharide yield decreased. However, the mechanism remains unclear and further study is needed.

When the octyl acetate ratio was high (Nos. 9–14 in Table 1), the saccharide yield was relatively low. Only No. 9 had a high yield; however, in this condition (5 mL min^{-1} water and 15 mL min^{-1} octyl acetate), the total flow rate was 20 mL min^{-1}, which increased the energy consumption of the process. No. 8 appeared to be better than No.9 because the concentration was almost the same even though the total flow rate was half.

3.2 Stability of octyl acetate

Octyl acetate has the potential to be hydrolyzed in hot-compressed water and decomposed into acetic acid and *n*-octanol. For example, ethyl acetate, an alkyl ester similar to octyl acetate, is quickly hydrolyzed in hot-compressed water at approximately 250 °C to produce acetic acid and ethanol [19]. Acetic acid concentrations in the water-soluble portions were determined to examine the

229 stability of octyl acetate.

230 Figure 4 shows concentrations and amounts of acetic acid in the water-soluble portions from
231 treatments with various octyl acetate ratios (total flow rate, 10 mL min^{-1}). Acetic acid can be produced
232 not only by the hydrolysis of octyl acetate, but also by the decomposition of saccharides [26], beech
233 xylan, which contains acetyl groups, or both. However, because only a trace amount of acetic acid (~
234 0.01 g L^{-1}) was found in treatments without octyl acetate, such wood-derived acetic acid was negligible
235 in this study. However, when octyl acetate was added, some acetic acid was produced, which was likely
236 from the hydrolysis of octyl acetate. The acetic acid concentration increased with the increasing ratio of
237 octyl acetate and reached 1.34 g L^{-1} at 50 vol% and 5.03 g L^{-1} at 90 vol%. In contrast, the amount of
238 acetic acid (concentration × water volume) produced from octyl acetate decreased slightly as the octyl
239 acetate ratio increased. This may have been due to the decrease in the water ratio, which reduced the
240 hydrolysis of octyl acetate. Therefore, the increase in acetic acid concentration in Figure 4 was simply
241 due to the decrease in the water volume used.

242 For 50 vol% octyl acetate, the distribution of acetic acid to water and octyl acetate was 83:17,
243 which was estimated by extracting the octyl acetate-soluble portion with water. Thus, the total acetic
244 acid produced from octyl acetate corresponded to approximately 1.6 g L^{-1} (1.34 g L^{-1} ÷ 0.83). Because
245 the volumes of water and octyl acetate were 235 mL each (5 mL min^{-1} × 47 min) in this condition, it
246 was estimated that 0.38 g (0.006 mol, 1.6 g L^{-1} × 0.235 L) of acetic acid was produced from 235 mL of
247 octyl acetate (1.2 mol). That is, approximately 0.5 mol% (0.006 ÷ 1.2) of octyl acetate was decomposed

248 during this hot-compressed water treatment. Although the degree of decomposition seemed to be small,
249 greater decomposition suppression would be more optimal.

250 Acetic acid from octyl acetate may affect the decomposition of wood. Our previous study
251 reported that 1–30 g L^{-1} acetic acid enhanced the decomposition of cellulose and hemicellulose and
252 improved the saccharide yield to some extent [27]. Although the effect of acetic acid in the current
253 study remains unclear, it might not be as significant in the concentration range of 1–5 g L^{-1} shown in
254 Figure 3. However, the decrease in the saccharide yield in Figure 2 is not affected by produced acetic
255 acid.

256

257 3.3 Removal of fermentation inhibitors

258 The effect of octyl acetate was examined for the removal of lignin-derived products that are
259 potential fermentation inhibitors. Figure 5 shows GPC chromatograms of water-soluble portions
260 obtained under various conditions. Since the analysis was performed with an ultraviolet detector
261 (λ=275nm), substances that absorb ultraviolet, such as lignin-derived products and furans, were
262 detected, but saccharides were not. The broad peak from approximately 45 to 60 min was mainly
263 lignin-derived oligomers. Similar peaks have been observed for hot-compressed water treatment and
264 supercritical alcohol treatment of wood [28-30]. Elution time from approximately 60 to 67 min was a
265 region where mainly monomer substances, such as lignin-derived monomers, furfural, and
266 5-hydroxymethyl furfural (5-HMF), appeared. In fact, lignin-derived monomers such as coniferyl

alcohol and degradation products of saccharides such as 5-HMF and furfural were detected by HPLC analysis.

The oligomer peak was the highest for the treatment with water only at 5 mL min^{-1} (shown by the thick solid line). The monomer peak was also high for this treatment. When this water-soluble portion was washed with octyl acetate at a volume ratio of 1:1 (thick dashed line), the peaks in the oligomer and monomer regions were slightly reduced, which suggested that these substances were extracted by octyl acetate. For the treatment with water only at 10 mL min^{-1} (thin dashed line), the peak intensity became approximately half that of the water only treatment at 5 mL min^{-1}. This was because the amount of water doubled, and thus, the concentration was reduced to approximately half.

For the treatment with 5 ml min^{-1} water and 5 mL min^{-1} octyl acetate (thin solid line), the peak in the oligomer region was the lowest, and the oligomer intensity was lower than that of the 10 mL min^{-1} water only treatment, even though the amount of water was halved. Thus, the lignin-derived oligomers were largely removed from the water-soluble portion by mixing octyl acetate during the hot-compressed water treatment. Interestingly, the peak in the oligomer region became much smaller when octyl acetate was mixed during the treatment than when extracted after the treatment. While the reason is unclear, when octyl acetate is mixed, the decomposition and dissolution behavior of lignin might be different from that of the water only treatment. However, the peak in the monomer region was minimally reduced, even by the addition of octyl acetate. This may be because the slight decrease in saccharide yield increased monomer substances such as furfural, and it cancelled out the effect of

286 removal by octyl acetate.

287 To further remove lignin-derived products, it would be more effective to extract with ethyl
288 acetate. However, water and ethyl acetate cannot be completely phase-separated, and a certain amount
289 of ethyl acetate would in the water-soluble portion. Octyl acetate wholly separates from water and does
290 not remain in the water-soluble portion at all. For the complete phase separation, we can use a nonpolar
291 solvent such as hexane, but it does not appreciably dissolve lignin-derived substances. In general, since
292 the lignin-derived products have some polarity, the requirement of both complete phase separation from
293 water and good solubility for lignin is difficult to achieve. Therefore, octyl acetate is a rare solvent that
294 meets both requirements.

295

296 3.4 Acetic acid fermentation

297 Acetic acid fermentation was performed using the water-soluble portions obtained from the
298 conditions of (a) 10 mL min^{-1} water only, (b) 5 mL min^{-1} water only, and (c) 5 mL min^{-1} each water and
299 octyl acetate. As shown in Table 1, the saccharide concentrations in these water-soluble portions were
300 (a) 0.47, (b) 0.79, and (c) 0.84 g L^{-1}. The acetic acid concentration changes in the fermentation broth
301 are shown in Figure 6. Due to the dispersion of data, the fermentation and blank tests were conducted
302 three times each. Because acetic acid was contained in the inoculum solutions, acetic acid was detected
303 even in the blank tests (~ 0.5 g L^{-1}). Therefore, plots (filled circles) in the figure show values obtained
304 by subtracting the average of blank tests from the average of fermentation experiments. In addition,

upper and lower error bars were calculated from the difference between maximum and minimum results of blank tests and fermentation experiments. For the water-soluble portion (c), which included 1.34 g L^{-1} of acetic acid as a result of octyl acetate hydrolysis, this additional amount of acetic acid was further subtracted from the results.

During an ideal acetic acid fermentation, acetic acid is produced from a hexose, such as glucose, as follows [25].

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH \quad (1)$$

Pentoses, such as xylose, can also be fermented in a similar way. During alcoholic fermentation, carbon dioxide is produced simultaneously with the production of ethanol, and therefore, acetic acid fermentation has better carbon utilization efficiency. Thus, acetic acid was expected to be produced up to the saccharide concentration in the water-soluble portion (shown by the dashed line). For each water-soluble portion, the mean values μ and standard deviations σ of the acetic acid concentration ($\mu \pm \sigma$) after the fermentation for 115 h were (a) 0.34 ± 0.25 g L^{-1}, (b) 0.47 ± 0.09 g L^{-1}, and (c) 1.79 ± 0.10 g L^{-1}. Notably, the standard deviation of (a) was large.

Comparing (a) and (b) of Figure 6, the final acetic acid concentration was comparable. However, because the saccharide concentration of (b) (0.79 g L^{-1}) was approximately 1.7 times higher than (a) (0.47 g L^{-1}), the condition (b) should have produced more acetic acid. When fermentation efficiency was defined as acetic acid concentration ÷ saccharide concentration, 0.72 and 0.59 were obtained as mean values for (a) and (b), respectively. As the water-soluble portion of (b) contained

lignin-derived products in higher concentrations than (a), as shown in Figure 5, the lower-than-expected acetic acid production of (b) may have been due to inhibition of fermentation. Because of the large standard deviation of (a), an independent *t*-test was performed between the fermentation efficiencies of (a) and (b). The *p*-value was as large as 0.71, and thus, it could not be concluded that there was a statistically significant difference between (a) and (b).

In contrast, for the water-soluble portion (c) obtained by mixing octyl acetate, the fermentability was notably high, and acetic acid production reached approximately 1.8 g L^{-1} and exceeded the saccharide concentration (0.84 g L^{-1}). The mean fermentation efficiency for (c) was 2.1. As a result of *t*-tests comparing (c) with (a) and (b), *p*-values were only 0.049 and 8.7×10^{-5}, respectively. Therefore, the improvement of acetic acid fermentability was apparent statistically significant, which might be due to the removal of fermentation inhibitors (Figure 5). Furthermore, lignin-derived and saccharides-degraded substances inhibit acetic acid fermentation when the concentrations are high, but some of them can be fermented to form acetic acid if the concentrations are low [12]. Substances other than saccharides in water-soluble portions included 5-HMF, furfural, levoglucosan and glycolaldehyde as saccharides-degraded products and coniferyl and sinapyl alcohols as lignin-derived products [4]. Acetic acid production exceeding the saccharide concentration may be due to acetic acid production from such substances. For comparison, the concentration of total biomass-derived products is also shown by dotted lines in Figure 6. This was calculated by dividing the amount of decomposed beech wood by the amount of water used. In the case of (c), however, this line is only an indication because

some of the products were dissolved in octyl acetate. The final acetic acid concentration of (c) was close to the biomass concentration.

As described above, adding octyl acetate during the hot-compressed water treatment of wood improved the saccharide concentration and acetic acid fermentability of the resulting hydrolysates. The phase separation between the water and octyl acetate phases would work well even in full-scale plants because the separation was performed rapidly and completely without emulsions in the experiments. However, octyl acetate was slightly hydrolyzed and lost during the hot-compressed water treatment. Octyl acetate is not significantly expensive but is more costly than some other common organic solvents. Octyl acetate is produced from acetic acid and octanol, but octanol is synthesized from fossil resources (olefins). Therefore, it would be desirable to suppress the decomposition of octyl acetate as much as possible.

4. Concluding remarks

The current study demonstrated that saccharide concentration was improved by mixing octyl acetate during the semi-flow, hot-compressed water treatment of beech wood, without a significant decrease in saccharide yield. However, because the saccharide yield was reduced when the octyl acetate ratio was too high, the appropriate ratio was found to be approximately 1:1 octyl acetate to water. This suggests that the saccharide concentration should approximately double. In this study, the concentration increased 1.8 times. Furthermore, by mixing octyl acetate, the lignin-derived oligomers that cause

fermentation inhibition could be largely removed from the water-soluble portion, and acetic acid fermentability was thus greatly improved.

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442

443 **Figure caption**

444 Figure 1. Schematic diagram of the semi-flow, hot-compressed water treatment system

445

446 Figure 2. Yield changes of a) insoluble residue, b) total saccharides and c) the saccharide concentration
447 in the water-soluble portion of two-step, semi-flow, hot-compressed water treatment of beech wood
448 with various octyl acetate ratios (total flow rate = 10 mL min^{-1}) at 230 ℃ and then 270 ℃ for 15 min
449 each (Heating periods not included) at 10 MPa.

450

451 Figure 3. Changes in yields of monosaccharides and oligosaccharides in two-step, semi-flow,
452 hot-compressed water treatment of beech wood with various octyl acetate ratios (total flow rate = 10
453 mL min^{-1}) at 230 ℃ and then 270 ℃ for 15 min each (Heating periods not included) at 10 MPa.

454

455 Figure 4. Concentrations and amounts of acetic acid in the water-soluble portions obtained by two-step,
456 semi-flow, hot-compressed water treatment of beech wood with various octyl acetate ratios (total flow
457 rate = 10 mL min^{-1}) at 230 ℃ and then 270 ℃ for 15 min each (Heating periods not included) at 10
458 MPa.

459

460 Figure 5. GPC chromatograms of the water-soluble portions measured by ultraviolet light (λ=275nm)
461 as treated beech wood at 230 ℃ and then 270 ℃ for 15 min each (Heating periods not included) at 10

462 MPa with a) 5 mL min^{-1} water, b) 5 mL min^{-1} water then washed with octyl acetate, c) 10 mL min^{-1}

463 water, and d) 5 mL min^{-1} water and 5 mL min^{-1} octyl acetate.

464

465 Figure 6. Changes in acetic acid concentration during acetic acid fermentation with *C. thermocellum*

466 and *M. thermoacetica* at 60 °C for various water-soluble portions as treated beech wood at 230 °C and

467 then 270 °C for 15 min each (Heating periods not included) at 10 MPa with a) 10 mL min^{-1} water, b) 5

468 mL min^{-1} water, and c) 5 mL min^{-1} water and 5 mL min^{-1} octyl acetate.

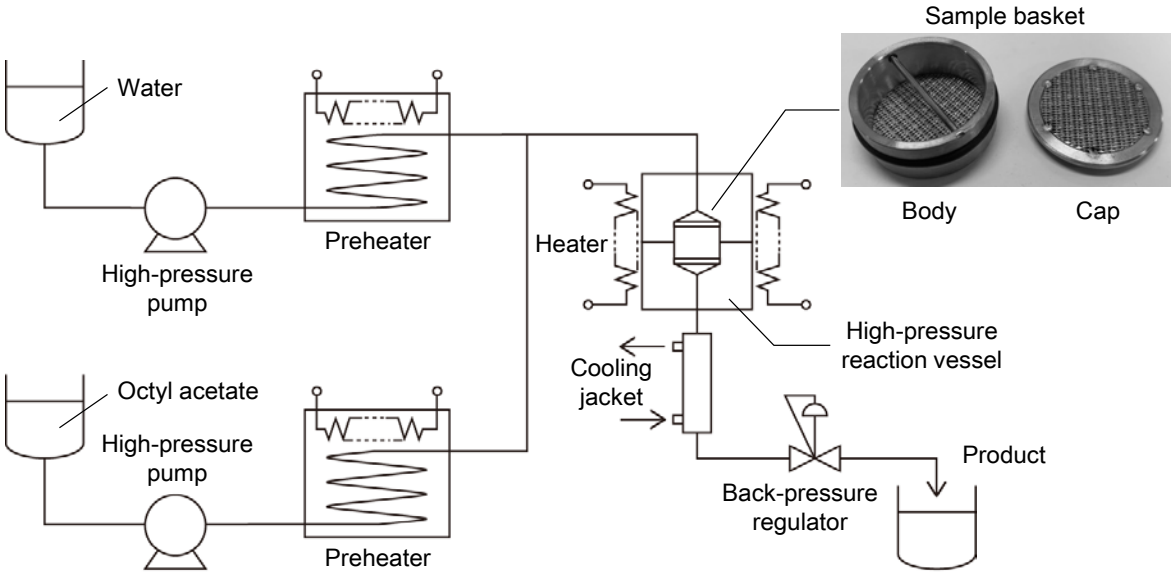

Fig.1

Minami, et al.

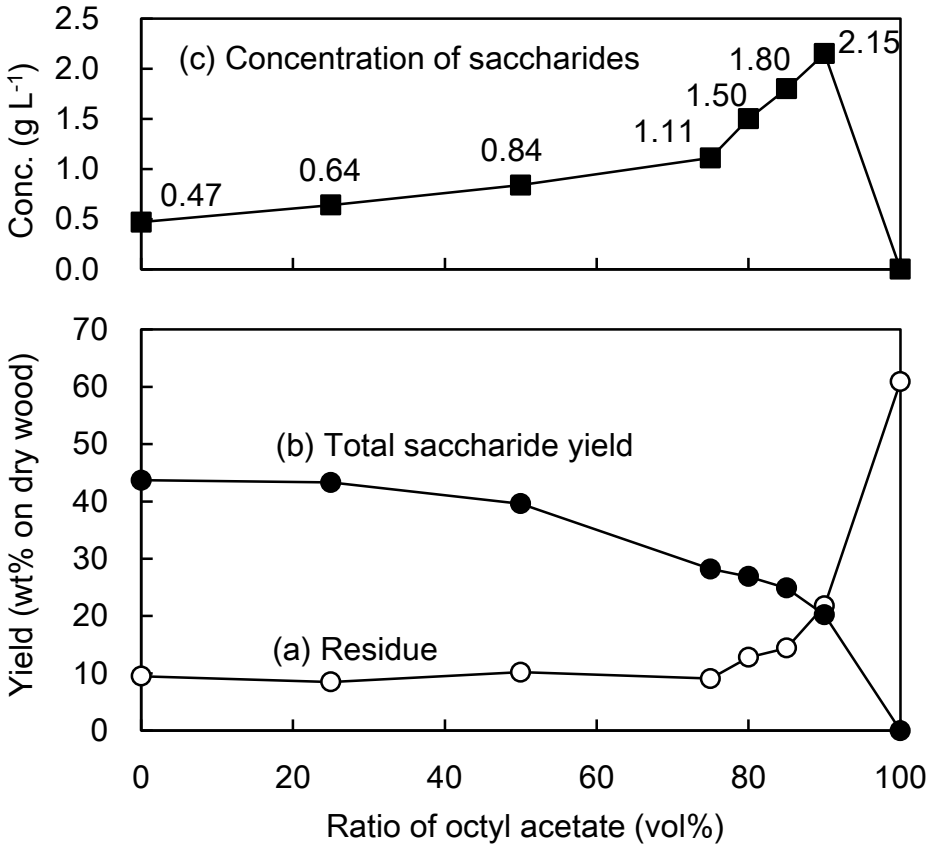

Fig.2

Minami, et al.

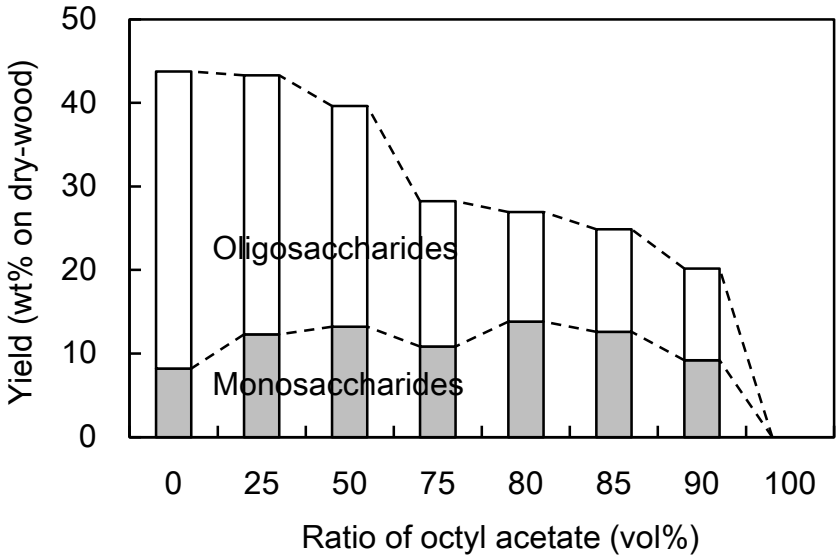

Fig.3
Minami, et al.

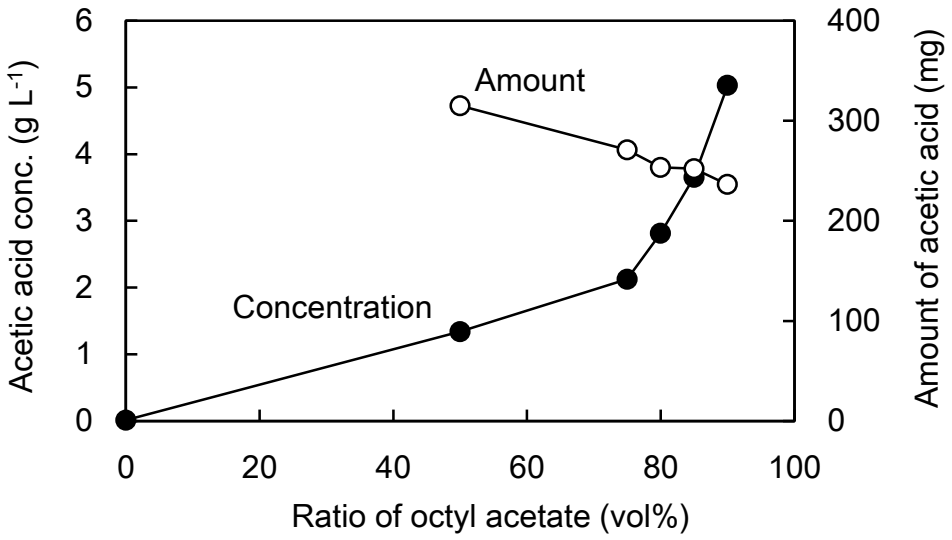

Fig.4
Minami, et al.

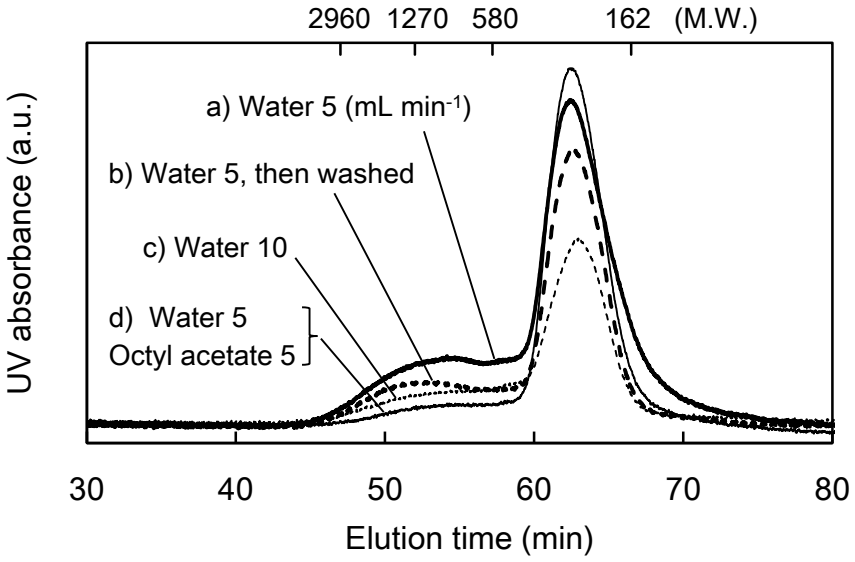

Fig.5
Minami, et al.

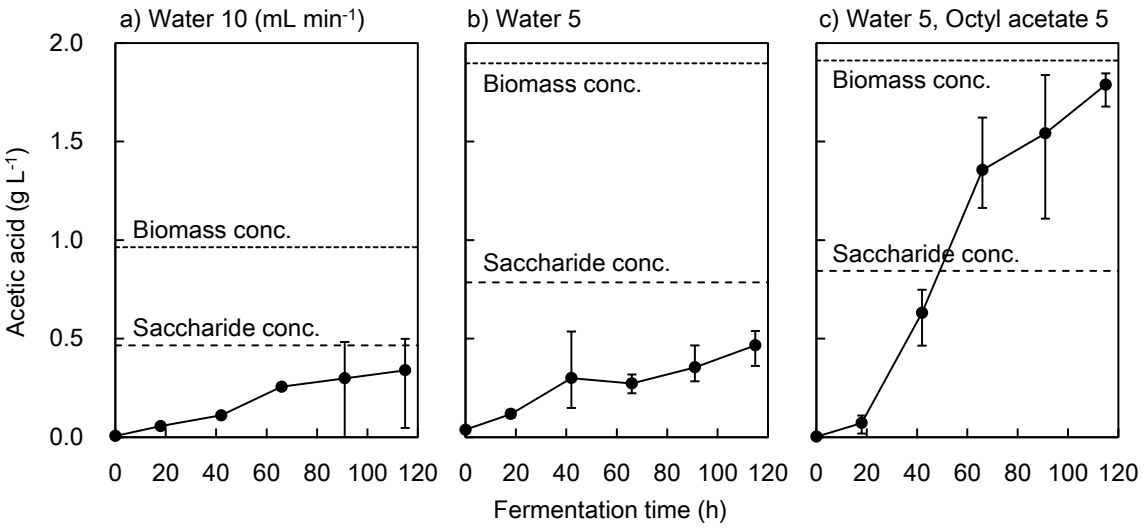

Fig.6

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Table 1. Experimental summary of the two-step, semi-flow, hot-compressed water treatment of beech wood with added octyl acetate at 230 °C and then 270 °C for 15 min each (Heating periods not included) at 10 MPa.

No.	Flow rate (mL min^{-1})		Treated beech (mg)	Treatment time (min)	Insoluble residue (wt%)	Saccharide conc. (g L^{-1})	Saccharide yield (wt%)
	Water	Octyl acetate					
1	20	-	500.6	47	2.6	0.27	51.1
2	10	-	500.5	47	9.5	0.47	43.7
3	5	-	500.0	45	14.6	0.79	35.4
4	2	-	500.7	44	12.0	1.55	27.3
5	1	-	501.3	44	10.8	1.88	16.5
6	7.5	2.5	500.4	45	8.5	0.64	43.3
7	10	10	500.2	45	12.4	0.51	45.5
8	5	5	500.5	47	10.2	0.84	39.6
9	5	15	500.7	50	13.5	0.90	45.0
10	2.5	7.5	500.4	51	9.1	1.11	28.2
11	2	8	500.6	45	12.8	1.50	26.9
12	1.5	8.5	500.4	46	14.4	1.80	24.9
13	2	18	500.6	54	22.8	1.16	25.1
14	1	9	500.2	47	21.8	2.15	20.2