Recovery of Soluble Sugars from Waste Medium for Enokitake (Flammulina velutipes) Mushroom Cultivation with Hydrothermal Reaction and Enzyme Digestion

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Abstract: Recovery of soluble sugars from waste medium for Enokitake mushroom cultivation was investigated using a hydrothermal reaction and enzyme treatment. The most suitable conditions for solubilization of hemicellulose fraction in the waste medium were found in the treatment with compressed hot water at 190°C, 1.8 MPa for 10 min. Under these conditions, a series of xylooligosaccharides from xylose to oligosaccharides with DP over 20 were detected in the soluble fraction. However, the yield of xylooligosaccharides decreased with an increase of the treatment temperature to over 190°C. The hydrothermal reaction at 190°C enhanced enzymatic digestibility and half of the residue was solubilized by cellulases, which was about eight times greater than enzymatic digestibility of non-treated medium. The combination of hydrothermal reaction and enzyme treatment made it possible to solubilize about 80% of waste medium, and about 20% of original waste medium remained, which was less than the sum of lignin and ash content.

Key words: hydrothermal reaction, waste medium, cellulase, hemicellulose, lignin

Human beings have paid much attention to environmental issues such as global warming, acid rain and pollution in the past ten years. The consumption of petroleum from carbon resources fixed in ancient ages promotes the state of global warming. Biomass resources are becoming important for sustainable development, as they are renewable and carbon-neutral materials synthesized by various living organisms. They are looked to, not only as energy sources, but also as chemical sources through biomass refining. Many researchers investigated the decomposition of plant biomass to constituent chemicals such as monosaccharides, which are the fermentable sugars, in the USA and Brazil, bioethanol was utilized as fuel for transportation, which bioethanol was made by fermentation from cornstarch or waste sugar syrup from sucrose production. However, alcohol production from plant biomass composed of lignocellulosic biomass cannot be put to practical use because of cost problems. There are several reasons why cellulosic biomass has not been utilized extensively. One of them is the high cost of collecting them in one place, because of widespread distribution. Another is the difficulty of conversion to constituent monosaccharides, as this treatment is costly.

For solving the above problems, various processes have been investigated. Acid hydrolysis has been studied but there are disadvantages in the production of byproducts that inhibit the enzymatic hydrolysis and recovery of the acid used. It is well known that enzymatic degradation has a great potential for biomass conversion as the energy consumed during the process can be reduced. However, pretreatment operations were essential for enzymatic treatment to achieve economically viable yields, as biomass contained hemicellulose and lignin in addition to cellulose and they inhibit the accessibility of cellulase to cellulose in plant cell walls. The comparative data were shown in the report of the project funded by the US Department of Agriculture Initiative for Future Agriculture and Food System Program using a single source of corn stover. In that report, Wyman and coworkers reported that the substantial differences in sugar release patterns in the pretreatment and enzymatic hydrolysis operations have implications for the choice of process, enzyme and fermentative organisms.

Supercritical water and sub-supercritical water treatments were investigated for crystalline cellulose degradation by some researchers. Cellulose can be degraded easily to carbon dioxide and water during an extremely short time, picoseconds, by supercritical water. Sub-supercritical water treatment has the capability to recover cellobiose oligosaccharides as cellulose is solubilized instantly in a several-hundred-millisecond treatment. In these cases pure microcrystalline cellulose was used for the treatment. Since most biomass species contain hemicellulose and lignin in addition to cellulose, it is not difficult to imagine that soluble behaviors and degradation mechanisms for

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Abbreviations: MALDI-TOFMS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; 2,5-DHB, 2,5-dihydroxy benzoic acid; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; CMC, carboxymethyl cellulose.
them are completely different from those of pure cellulose. In fact, most biomass contains three major components, hemicellulose, cellulose and lignin, not pure cellulose. It has been reported that a hydrothermal reaction was effective for the mixture of the three major components in biomass refinery. It is considered that a milder process is better in terms of the calorie-based yield than a severe process such as supercritical water treatment. Furthermore, the process without chemicals such as acid or alkaline is desirable for environmental reasons, because it is not necessary to treat the wastewater.

Recently mushroom cultivation has become popular in the agricultural industry, as functional foods are desired because of their ability to activate immunity. But after cultivation, most of waste medium has not been utilized except for compost. The amounts of waste medium are estimated to be over 300,000 tons in Nagano Prefecture. Therefore, we have been developing the fractionation of biomass components from waste medium to reuse industrial materials such as dietary fiber in food industry, bioethanol and biodegradable polymers. In this paper, the conditions for hydrothermal reaction to solubilize the hemicellulose rich fraction were investigated, and enzyme treatment of waste medium to solubilize cellulotic materials was also investigated. So the production of useful sugars from waste medium in mushroom cultivation during a hybrid process of hydrothermal reaction and enzymatic hydrolysis is reported.

MATERIALS AND METHODS

Waste medium. Waste medium from Enokitake (Flammulina velutipes) cultivation after harvesting the fruit bodies was obtained from Agricultural Technology Institute of Nagano Farmers’ Federation. The mushroom fungus, F. velutipes, was grown using corncob medium composed of corn cob (38%, w/w), rice bran (32%, w/w), wheat bran (10%, w/w), beet fiber (10%, w/w) and mineral component (10%, w/w, minerals and bean fiber). The waste medium was directly ground under wet conditions using a grinder (MKCA 6-3, Masuko Sangyo Co., Ltd.) so as to yield particles under 1 mm. This ground medium was used for compositional analysis and subjected to compressed hot water treatment and enzymatic digestion.

Hydrothermal reaction. A batch type-reactor constructed from a Hastelloy C-22 cell (Taiatsu Techno Co., volume: 10.77 mL) was heated in an electric heater to control the reaction time and temperature. Each cell contained 300 mg of waste medium with or without grinding and 8 mL of distilled water, resulting in 3.75% solid slurry. The pressure at each temperature was measured at 1.29 MPa (170°C), 1.48 MPa (180°C), 1.77 MPa (190°C), 2.14 MPa (200°C), 2.61 MPa (210°C), 3.20 MPa (220°C). Heat-up time was found to be around 15 min for all temperatures examined; thus a reaction time of 10 min at individual temperatures required a total time of 25 min. Cool-down was achieved by air circulation at room temperature, which caused the internal cell temperature to drop below 100°C in about 30 min. After the compressed hot water treatment, the slurry was fractionated by filtration to the solubilized fraction and precipitate. The precipitate was washed and then dried with vacuumed dedicator. The solubilization of waste medium was calculated from the weight of precipitate.

Enzyme reaction. A crude cellulase preparation, Driselase (Kyowa Hakko Co., Ltd.), was used throughout for enzymatic treatment. Crude enzyme was dissolved in 0.02 M acetate buffer and insoluble materials were removed. Then the enzyme solution was subjected to ultrafiltration to remove small molecules under 10,000 Da with an UF membrane (Asahi Kasei Co., ACP-0053). CMC degrading activity was measured from the reducing sugars produced, which reaction mixture was composed of 0.25% of CMC, 0.05 M of acetate buffer (pH 5.0) and the appropriate amount of enzyme. One unit for CMC degrading activity was defined as the amount of enzyme that produced reducing sugars corresponding to 1 µmol of glucose per min. Insoluble fractions from waste medium with the compressed hot water treatment were filtered and freeze-dried. Ten mg of residue (dry weight) was mixed with 0.1% (w/v) of enzyme (0.23 U/mL) solubilized in 0.02 M acetate buffer, pH 5.0 and incubated at 30°C for 48 h with mechanical shaking at 110 rpm. Reducing sugars from enzymatic hydrolysis were measured using the methods of Somogyi and Nelson.

Compositional analysis. Waste medium composition was determined by standard analytical procedure for wood components described by Yamaguchi. The ash content was determined by the weight of residue after burning at 600°C in the oven. The alcohol-benzene extract was determined by the Soxhlet extraction method. Lignin content was determined using 72% sulfuric acid by the method of JIS P8008; 1976. Holocellulose content was determined using chlorite by the method of JIS P8012; 1976. α-Cellulose content was determined by treatment with a 17.5% NaOH solution.

Sugar analysis. Sugar content in the soluble fraction obtained from waste medium with a hydrothermal reaction was measured by the phenol-sulfuric acid method. Sugar composition contained in the soluble fraction obtained from waste medium was analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Thirty µL of solution was spotted on a Silica gel plate developed with a solvent system of chloroform, methanol and water (90:65:15, v/v/v). The spots of sugars on the TLC plate were visualized by spraying with a 30% sulfuric acid solution and heating at 120°C for 15 min. The water-soluble fraction after compressed hot water treatment was deionized using Amberlite MB-3 resin (ORGANO Co.) to remove various salts and filtrated with 0.45 µm filters before HPLC analysis. A multi-solvent system (DS-4, Shodex) was coupled with a MCIGEL CK02S column (20 mm I.D. × 250 mm, Mitsubishi Chemical Co.) whose temperature was controlled at 85°C. Refractive index was monitored by a differential refractometer (RI-71, Shodex). The solvent system was water, pumped at 1.0 mL/min. For constituent sugar analysis, an HPX-87P column (7.8 mm I.D. × 300 mm, BioRad) was used. Column temperature was controlled at 85°C, and the solvent system was water, pumped at 0.6 mL/min.
MALDI-TOFMS analysis of xylose-oligomers and -polymer in soluble fraction. The water-soluble fraction after compressed hot water treatment was deionized using Amberlite MB-3 resin (ORGANO Co.) to remove various salts and then subjected to charcoal column chromatography to remove phenolic compounds. Then 1 µL of mixture of xyloooligosaccharides (0.6%, Brix degree) and 10 µL of matrix solution (10 mg of 2,5-DHB in 1 mL of distilled water) were shaken vigorously in a vortex mixer. One µL of solution was loaded onto the sample plate. The sample plate was allowed to dry and crystallize for about 10 min at room temperature and was subsequently loaded into Axima CFRplus (Shimadzu Biotech, 120 cm ion path length in linear mode). A nitrogen laser (337 nm) was used for the ionization.

Scanning electron microscopy of corncob surface characteristics. The morphology of the corncobs was observed by field emission scanning electron microscopy S-4100 (FE-SEM, Hitachi Ltd.) at low and high magnifications. The treated corncob was freeze-dried before being coated with gold particles using a Twin Coater (JEC-550, JEOL). The spattering of gold was carried out at 1.5 kVA for 2.5 min.

RESULTS AND DISCUSSION

Solubility of waste medium by compressed hot water treatment.

The components of the waste medium are shown in Table 1. We estimated cellulose and hemicellulose contents from α-cellulose and holocellulose contents analyzed by the wood component analysis method. The major component in the medium is hemicellulose (36.8%), which should be mainly xylan, as it is composed of corncobs and rice bran. About 20% of non-cellulosic components such as sugars, ash, lipid and protein were contained in the waste medium, which are shown as ash, crude lipid and others in the table. To solubilize the hemicellulose rich fraction, the conditions of the hydrothermal reaction were investigated (Fig. 1). The pH of the slurry solution treated with compressed hot water fell with the increase in the temperature. Finally it was almost constant at pH 4.1 when treated at over 200°C. It was reported that hydronium ions were generated from water ionization and from in situ generated acids (such as acetic acid coming from acetyl groups) when suitable raw materials were heated in an aqueous medium.9 Depending on the severity of the operational conditions, autohydrolysis can result in the formation of sugars and sugar degradation products. Furthermore, the solubility of the waste medium increased with the increasing temperature. On the other hand, Brix degree and total sugar content of solubilized solution were maximal at 190°C, and decreased with the increasing temperature. The solubility was correlated inversely with the pH of the slurry solution. From these results, the best conditions for compressed hot water treatment were 190°C for 10 min, from the point of view of the sugar recovery. It was reported that the optimized conditions for controlled pH liquid hot water pretreatment of 16% slurry of corn stover was found to be 190°C for 15 min.9 Compared with the conditions for treatment, the treatment temperature for corn stover was the same as that for the waste medium treatment as the waste medium was mainly composed of corncobs, because in both cases the constituent polysaccharide was xylan. However, the treatment time for corncobs was slightly shorter than that in case of corn stover.

Sugar composition of solubilized fraction with hydrothermal reaction.

Sugar composition contained in solubilized fraction for the hydrothermal reaction was analyzed with thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMASS). The waste medium originally contained glucose, trehalose and arabitol, which were extracted with boiling water. It is suggested that they were produced as intercellular components by mushroom fungus. After treatment with compressed hot water at 190°C, 1.8 MPa for 10 min, a series of xyloooligosaccharides were detected by TLC (Fig. 2), but in the case of 200°C treatment, xyloooligosaccharides with a DP of more than 5 disappeared, and cellobioseoligosaccharides of DP 2–4 were detected on the plate. Most xyl- and cello-oligosaccharides were not detected on the plate when treated at 220°C. The spots on the top of the plate was visualized with the increasing temperature, and it was thought they were decomposed to furfural under the over-200°C treatment. The constituent sugars in the solubilized solution of 190°C treatment were 69.9% of xylose, 16.0% of arabinose, 8.2% of glucose and 5.9% of galactose as neutral sugar when it was analyzed after complete hydrolysis by sulfuric acid. The solubilized fraction treated with compressed hot water at 190°C was also analyzed by using HPLC, because a higher molecular

Table 1. The components of waste medium for mushroom cultivation.

<table>
<thead>
<tr>
<th>Chemical composition</th>
<th>Relative content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash</td>
<td>7.0</td>
</tr>
<tr>
<td>Crude lipid</td>
<td>6.3</td>
</tr>
<tr>
<td>Lignin</td>
<td>19.2</td>
</tr>
<tr>
<td>Cellulose</td>
<td>23.4</td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>36.8</td>
</tr>
<tr>
<td>Others</td>
<td>7.3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100.0</strong></td>
</tr>
</tbody>
</table>

Fig. 1. Solubility of waste medium and pH, Brix degree and sugar content of solubilized solution with compressed hot water treatment.

\[ \text{brix degree}; \quad \text{sugar content}; \quad \text{solubility}; \quad \text{pH}. \]
weight fraction of oligosaccharides was detected on the TLC plate. As shown in Figs. 3 and 4, the oligosaccharides with higher DPs (about DP 10) were detected by HPLC, and further analyzed by MALDI TOF-MASS up to at least DP 20. It was found that xylooligosaccharides were detected as three ion species by mass spectrometry from alkali metal complexes such as Li, Na, K, as the difference in molecular weight between every three peaks was about 132 Da, which was coincident with the molecular weight of the xylose moiety. It was reported that insoluble glucose oligomers in decomposed cellulose were analyzed by MALDI TOFMS. In that report, mass differences between peaks were 162 Da, as expected for polymers composed of glucose units. As compared with these data, it is proposed that the soluble sugar is a polymer composed of xylose units. However we don’t know any reason why three ion species were detected in the case of xylan.

**Morphology changes in cell wall structure of corncob.**

We observed cell wall structure of corncobs in the waste medium with or without compressed hot water treatment using FE-SEM. The SEM image of a cross section of corncob showed that there were some small pores in the cell wall without treatment (Fig. 5A). On the other hand, large numbers of pores were observed in the cell wall of corncob shown by the arrow in Fig. 5B, and the thickness of cell wall also decreased with compressed hot water treatment. Moiser et al. reported that the pretreatment of corn stover by compressed hot water at 190°C for 15 min promoted the formation of larger pores and proposed that these pores may increase the enzyme accessible surface area, which increases the enzyme digestibility of the corn stover. Those morphological changes were observed in the corncob treated with compressed hot water at 190°C for 10 min in this experiment. It was suggested that most of the primary cell wall of corncob was solubilized by compressed hot water treatment and resultanty

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**Fig. 2.** Thin layer chromatogram of sugars in the solution solubilized with compressed hot water treatment at various temperatures.

S1–S4 represent the authentic sugars as follows: S1, trehalose; S2, arabinose; S3, xylooligosaccharides (X1–X4); S4, cellooligosaccharides (G1–G4). The waste medium was treated with compressed hot water at 170°C (1), 180°C (2), 190°C (3), 200°C (4), 210°C (5) and 220°C (6).

**Fig. 3.** Detection of xylooligosaccharides contained in soluble fraction after treatment with compressed hot water at 190°C.

HPLC conditions were described in MATERIALS AND METHODS. The arrows I and II in the figure show the retention time of xylose and xylo-decaose, respectively.

**Fig. 4.** MALDI TOF-MASS profiles of xylooligosaccharides produced from waste medium with hydrothermal reaction.

Horizontal arrows show the interval of repeating unit corresponding to xylose moiety.
the secondary cell wall was exposed. In fact cellulose microfibril could be seen in the cell wall of corncob after treatment. This observation is coincident with the data from sugar composition analysis in the soluble fraction because the constituent sugars of the primary cell wall were detected.

**Enzymatic degradation of residues after hydrothermal reaction.**

Separation of the cellulose fraction from residues after treatment that was contained in the secondary cell wall was investigated. A commercial crude cellulase preparation (Driselase) degraded insoluble cellulosic materials contained in the residues with its treatment and produced glucose and cellobiose (data not shown). As reported previously, Driselase caused powerful digesting activity on plant cell wall by various cellulases and xylanases.\(^{15-17}\) However, xylose was not produced from the residues with compressed hot water treatment at over 190\(\degree\)C. It was suggested that the hydrothermal reaction at over 190\(\degree\)C was sufficient for xylan extraction. The production of soluble sugars from the residues increased with the incubation time as shown in Fig. 6. The degradation rate of the residues after boiling water treatment was only 6.8\% by enzymatic digestion, while the residues after compressed hot water treatment at over 170\(\degree\)C were susceptible to enzymatic degradation. From the viewpoint of enzymatic susceptibility, the most suitable treatment was hydrothermal reaction at 190\(\degree\)C and the degradation rate was maximal at 50.1\% for 48 h incubation. This result was supported the morphology changes in which the primary cell of corncob was removed and the secondary cell wall was exposed. It is suggested, however, that excess treatment at over 200\(\degree\)C would inhibit the enzyme reaction because of production of inhibitors such as furfurals from xylose oxidative degradation.\(^{19}\)

**Estimation of total conversion rate.**

To separate the biomass components, a hybrid treatment was investigated, and the total yield of them is summarized in Fig. 7. Twenty percents of waste medium was solubilized with boiling water treatment, and the extract contained arabitol, glucose and trehalose, mainly that produced by mushroom fungus (*F. velutipes*). The sum of the solubilization rate with hydrothermal reaction and enzyme treatment reached a maximum at 190\(\degree\)C treatment, and decreased gradually with increased temperatures. The de-

![Fig. 5. Morphology changes in cell wall of corncob with compressed hot water treatment.](image)

Cell wall images of corncob before treatment (A) and after treatment (B). Bars in the images represent 60 \(\mu\)m. The arrow in the figure shows a pit in the cell wall of corncob.

![Fig. 6. Enzymatic hydrolysis of residues after compressed hot water treatment.](image)

Hydrolysis rate was calculated by the following equation:

\[
\text{Hydrolysis}(\%) = \frac{\text{mg of reducing sugars produced/mg of the residue after hydrothermal reaction}}{(162/180)}. \\
\]

- \(100^\circ\)C, \(\rightarrow\), \(170^\circ\)C, \(\leftarrow\), \(180^\circ\)C, \(\rightarrow\rightarrow\), \(190^\circ\)C, \(\leftarrow\leftarrow\), \(200^\circ\)C, \(\leftarrow\rightarrow\), \(210^\circ\)C, \(\rightarrow\), \(220^\circ\)C.

![Fig. 7. Changes in the amount of soluble fraction with hydrothermal reaction and enzyme digestion.](image)

Compressed hot water treatment was performed at 100\(\degree\)C (lane 1), 170\(\degree\)C (lane 2), 180\(\degree\)C (lane 3), 190\(\degree\)C (lane 4), 200\(\degree\)C (lane 5), 210\(\degree\)C (lane 6), 220\(\degree\)C (lane 7).

- \(\square\) compressed hot water soluble fraction; \(\square\) soluble fraction with enzyme treatment; \(\square\) residues after both treatments.
crease in the total extraction rate caused the decrease in the enzyme degradation, as the solubilization on the pretreatment of hydrothermal reaction at 190°C was almost the same as those at 200–220°C. As compared with the composition of waste medium (Table 1), the increase in solubilized fraction with hydrothermal reaction at 190°C could correspond to the rate of the hemicellulose fraction. In addition, xylooligosaccharides were not detected in the reaction mixture after enzyme degradation of the residues treated at 190°C. From these results, it is suggested that xylan can be completely solubilized from the waste medium in this pretreatment. It is proposed that the compressed hot water treatment could be effective for recovering the biomass component, but only a batch-type reactor was used in this study. Yang and Wyman reported the flowthrough and countercurrent reactors have an important potential advantage for pretreatment cellulosic biomass, including higher hemicellulose sugar yields, and enhanced cellulose digestibility.  

We expected the increases in the recovery of sugars when the flow type reactor was applied for the waste medium treatment.

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