The production of D-xylose by enzymatic hydrolysis of agricultural wastes

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Abstract Agricultural wastes, rich in D-xylose content, were hydrolyzed using the mixed crude enzymes produced by Penicillium sp. AHT-1 and Rhizomucor pusillus HHT-1. Shells of pistachio, peanut, walnut, chestnut, barley brans and sunflower seed peels, were used as raw or pretreated forms. Pretreatment was performed by milling or steam explosion. Enzymatic hydrolysis after steam explosion was more effective than milling processing. More than 13 g of D-xylose was produced from 100 g of milled pistachio shells, walnut shells, sunflower seed peels and peanut shells (less than 0.5 mm size) by the action of mixed enzyme solutions. A maximum of 36 g of D-xylose was produced from 100 g of milled pistachio shells when mixed enzyme solution, containing 3,000 U and 33 U per g of substrate with xylanase and β-xylosidase activities, respectively, was applied. The ratio of the enzymatic hydrolysis as compared to acid hydrolysis in this finding was 100%.

Keywords Enzymatic hydrolysis; pistachio shells; xylanase; D-xylose; b-xylosidase

Introduction
More than 50 billion tons of agricultural products are being produced in the world every year and about 10% becomes waste which is not economically utilized. Effective utilization of agricultural wastes will not only reduce the pollution burden but would also develop a new agro-based industry for the production of useful compounds such as xylose, xylitol, pulp and activated charcoal. Lignocellulose is abundant in various agricultural wastes. It mainly consists of cellulose (30–50%), hemimcellulose (20–50%) and lignin (15–35%) (Perego et al., 1990).

Lignocelluloses can be hydrolyzed to liberate sugars, with emphasis on large-scale production of useful compounds by means of acid or enzymatic hydrolysis. Many agro-industrial wastes contain hemimcellulose relatively in high amounts. In nature, hemimcellulose is the second most abundant constituent of lignocelluloses. Bioconversion of hemimcellulose produces the monosaccharide xylose. This xylose thus could be used as a substrate for fermentation to various useful compounds, such as butanol and xylitol. For example, xylitol, a sugar alcohol, has attracted much attention as a food sweetener because of its anticariogenic and cariostatic properties (Pepper et al., 1988; Aguirre-Zero et al., 1993), and is used for the treatment of diabetes and disorders in lipid metabolism (Touster, 1974; Emodi, 1978). The highest production yield from D-xylose was found by using Candida tropicalis (Horitsu et al., 1992; Yahashi et al., 1996; Yahashi et al., 1996).

In this study, we have tried enzymatic production of D-xylose from various agricultural wastes and found that 36 g D-xylose can be extracted from 100 g pistachio shells.

Material and methods

Materials
Raw and pretreated agricultural wastes, e.g. pistachio shells, barley brans, peanut shells, walnut shells, chestnut shells and sunflower seed peels were used in this study. Pretreatments were carried by milling (less than 0.5 mm size) or steam explosion at pressures ranging from 1.25 MPa to 2.79 MPa for 2 min.
Sugar analysis of wastes
Sugar composition of sixteen agricultural wastes were chemically analysed according to the method of Cho et al. (1999).

Fungal strains and culture conditions
Penicillium sp. AHT-1 and Rhizomucor pusillus HHT-1 isolated from Ibigawa river, Gifu Prefecture, Japan, and a jute compost in Bangladesh, respectively, were used in this study. Penicillium sp. AHT-1 was grown in liquid culture basal medium A containing oat spelt xylan 1.0%, K2HPO4 0.10%, MgSO4 0.05%, NaNO3 0.20%, KCl 0.05%, FeSO4 0.001%, polypepton 0.1% and pH 7.0. Cultivation was carried out in 2 L Erlenmeyer flask (total volume 420 ml) in a shaker at 150 rpm, 30°C. This strain was maintained on agar slants composed of the basal medium A and agar 1.50%. Strains were grown on this medium for 3 days at 30°C and then stored at 4°C.

Rhizomucor pusillus HHT-1 was grown in liquid culture basal medium B containing oat spelt xylan 1.0%, D-xylose 1.0%, KH2PO4 1.50%, (NH4)2SO4 0.12%, polypepton 0.60% and pH 5.5. Cultivation was carried out in 2 L Erlenmeyer flask (total volume 420 ml) in a shaker at 150 rpm, 40°C. This strain was maintained on agar slants composed of the basal medium B and agar 1.50%. Strains were grown on this medium for 3 days at 4°C and then stored at 4°C.

Inoculum and enzyme production
One loop of Penicillium sp. AHT-1 slant culture was inoculated in 70 ml basal medium A in 500 ml Sakaguchi flask, shaked at 30°C for a day at 150 rpm, and used as a preculture. After incubation, broth was poured into 350 ml of same medium in 2 L Erlenmeyer flask. The inoculated flask was incubated in a shaker at the same condition for 5 days. The fermentation slurry was centrifuged at 10,000 \( \times \) g for 30 min and then filtered using 0.30 \( \mu \)m membrane filter. The clear supernatant was used for the determination of enzyme activities and enzymatic hydrolysis. In case of Rhizomucor pusillus HHT-1 cultivation condition was similar to that of Penicillium sp. AHT-1 except for incubation temperature (40°C) and medium composition.

Enzyme, protein and D-xylose analysis
We have measured three major xylan-degrading enzyme activities: xylanase, β-xylosidase and α-L-arabinofuranosidase. Xylanases activity was determined using 1.0% birchwood xylan dissolved in 0.05 M phosphate at pH 6.8 as substrate (Bailey et al., 1992). The reaction mixture, containing 1.8 ml substrate and 0.2 ml of suitably diluted enzyme solution in the buffer, was incubated at 50°C for 5 min, and reaction was stopped by addition of 3 ml 3,5-dinitrosalicylic acid (0.0438 M DNS) reagent. The amount of reducing sugar was determined by DNS method (Miller et al., 1959) using D-xylose as a standard. One unit of xylanase activity was defined as the amount of enzyme liberating 1.0 \( \mu \)mol reducing sugar per minute and activity was expressed as U ml\(^{-1}\).

The β-xylosidase activity was determined by the following method. The reaction mixture consisting of 180 µl of 5 mM p-nitrophenyl β-D-xylopyranoside (dissolved in 50 mM citrate buffer pH 5.0), 20 µl of enzyme solution, was incubated at 50°C for 20 min, followed by the addition of 100 µl of Na2CO3 (1M), and absorbance at 414 nm was recorded. One unit of enzyme activity was defined as the amount of enzyme liberating 1.0 \( \mu \)mol of p-nitrophenol from the substrate per minute and activity was expressed as U ml\(^{-1}\). The α-L-arabinofuranosidase activity was determined by the similar method described for β-xylosidase except that the substrate was p-nitrophenyl α-L-arabinofuranoside. One unit of enzyme activity was defined as the amount of enzyme
liberating 1.0 µmol of p-nitrophenol from the substrate per minute and activity was expressed as U ml⁻¹.

The protein concentration was determined by the Lowry method (Lowry et al., 1951) at 280 nm. D-xylose was determined by high-performance liquid chromatography (Shimadzu LC-5A) using a Shodex SUGAR SZ-5532 column (Showa Denco, K .K) with a refractive index detector (Shimadzu RID-2A). The column was eluted with acetonitrile at a flow rate of 0.7 ml min⁻¹.

**Enzymatic hydrolysis**

Enzymatic hydrolysis was carried out with 50 ml crude concentrated enzyme solution and 2 g pretreated material in 500 ml Sakaguchi flask at 40°C with 150 rpm in a reciprocal shaker.

**Results and discussion**

We have chemically analyzed the sugar composition of 16 agricultural wastes to know the potential resource of D-xylose (Cho et al., 1999). The results are shown in Table 1.

From Table 1, pistachio shells, walnut shells, barley brans, sunflower seed peels, chestnut shells and peanut shells were found to be very good renewable sources of D-xylose. Therefore, these were used predominantly for the enzymatic hydrolysis experiment. The activities of the crude enzyme solutions prepared by *Penicillium* sp. AHT-1 and *Rhizomucor pusillus* HHT-1 are shown in Table 2. *Penicillium* sp. AHT-1 and *Rhizomucor pusillus* HHT-1 produces 60 and 2.4 U ml⁻¹ of xylanase, whereas 0.27 U ml⁻¹ of β-xylosidase was produced by the later strain. Optimum temperature for enzymatic hydrolysis was 40°C, which was confirmed additional studies on temperature effect (data not shown).

**Table 1** Sugar composition of agricultural wastes subjected to chemical pretreatment and acid hydrolysis

<table>
<thead>
<tr>
<th>Kinds</th>
<th>Hemi</th>
<th>Cell</th>
<th>Lig</th>
<th>Xyl</th>
<th>Ara</th>
<th>Glc</th>
<th>Gal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pistachio shells</td>
<td>48.9</td>
<td>39.5</td>
<td>11.6</td>
<td>36.0</td>
<td>0.8</td>
<td>2.2</td>
<td>3.7</td>
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<tr>
<td>Walnut shells</td>
<td>33.5</td>
<td>41.4</td>
<td>25.1</td>
<td>18.7</td>
<td>3.7</td>
<td>0.2</td>
<td>0.2</td>
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<tr>
<td>Sunflower seed peels</td>
<td>28.1</td>
<td>46.7</td>
<td>25.3</td>
<td>18.3</td>
<td>0.1</td>
<td>0.3</td>
<td>0.8</td>
</tr>
<tr>
<td>Barley brans</td>
<td>30.4</td>
<td>44.1</td>
<td>25.5</td>
<td>14.4</td>
<td>4.4</td>
<td>0.5</td>
<td>0.1</td>
</tr>
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<td>Peanut shells</td>
<td>22.9</td>
<td>50.7</td>
<td>26.4</td>
<td>13.0</td>
<td>1.0</td>
<td>2.8</td>
<td>ND</td>
</tr>
<tr>
<td>Chestnut shells</td>
<td>30.1</td>
<td>45.5</td>
<td>24.4</td>
<td>12.6</td>
<td>6.0</td>
<td>4.6</td>
<td>ND</td>
</tr>
<tr>
<td>Rice brans</td>
<td>38.3</td>
<td>35.1</td>
<td>26.6</td>
<td>10.7</td>
<td>0.9</td>
<td>0.2</td>
<td>ND</td>
</tr>
<tr>
<td>Ginkgo shells</td>
<td>45.1</td>
<td>32.4</td>
<td>22.5</td>
<td>8.7</td>
<td>2.9</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Rice straws</td>
<td>31.3</td>
<td>38.9</td>
<td>29.8</td>
<td>8.5</td>
<td>2.3</td>
<td>3.5</td>
<td>0.4</td>
</tr>
<tr>
<td>barley tea dregs</td>
<td>29.8</td>
<td>10.3</td>
<td>59.9</td>
<td>4.8</td>
<td>1.5</td>
<td>20.6</td>
<td>0.1</td>
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<tr>
<td>Japanese tea dregs</td>
<td>16.4</td>
<td>56.5</td>
<td>27.1</td>
<td>1.5</td>
<td>0.3</td>
<td>1.6</td>
<td>ND</td>
</tr>
<tr>
<td>Banana peels</td>
<td>8.2</td>
<td>49.9</td>
<td>41.9</td>
<td>1.4</td>
<td>1.5</td>
<td>2.6</td>
<td>ND</td>
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<tr>
<td>Orange peels</td>
<td>4.2</td>
<td>32.3</td>
<td>63.5</td>
<td>1.1</td>
<td>0.8</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>Tea dregs</td>
<td>21.8</td>
<td>51.4</td>
<td>26.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.5</td>
<td>1.2</td>
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<tr>
<td>carrot dregs</td>
<td>7.2</td>
<td>37.6</td>
<td>55.2</td>
<td>0.4</td>
<td>0.9</td>
<td>0.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Coffee dregs</td>
<td>10.8</td>
<td>64.1</td>
<td>25.1</td>
<td>0.2</td>
<td>0.3</td>
<td>1.4</td>
<td>1.1</td>
</tr>
</tbody>
</table>

**Table 2** Activities of crude enzymes of *Penicillium* sp. AHT-1 and *Rhizomucor pusillus* HHT-1

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Xylanase U ml⁻¹</th>
<th>β-Xylosidase U ml⁻¹</th>
<th>α-L-Arabinofuranosidase U ml⁻¹</th>
<th>Protein mg ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Penicillium</em> sp. AHT-1</td>
<td>60.0</td>
<td>0.05</td>
<td>0.02</td>
<td>1.88</td>
</tr>
<tr>
<td><em>Rhizomucor pusillus</em> HHT-1</td>
<td>2.40</td>
<td>0.27</td>
<td>0.60</td>
<td>1.70</td>
</tr>
</tbody>
</table>
Enzymatic hydrolysis of agricultural wastes by xylanase from Penicillium sp. AHT-1

Enzymatic hydrolysis of the pretreated agricultural wastes were performed with xylanase from *Penicillium* sp. AHT-1. Results are shown in Figure 1 and 2. In the case of enzymatic hydrolysis of the raw material which was only subjected to milling pretreatment (less than 0.5 mm size), considerable amounts of D-xylose, 10 and 7 g were produced from 100 g of pistachio shells and barley brans, respectively, while less than 0.5 g of xylose was produced from other materials (Figure 1). The xylanase applied was 1500 U per g of sample. On the contrary, in the case of enzymatic hydrolysis of steam exploded materials, 12 g, 8 g, 5.4 g, 4.9 g and 4.0 g of D-xylose were produced from pistachio shells, barley brans, peanut shells, walnut shells and sunflower seed peels, respectively, from 100 g of sample (Figure 2). From this hydrolysis experiment, it was found that steam explosion pretreatment was more effective for enzymatic hydrolysis than milling processing. It was supposed that reaction between enzyme and hemicellulose was more efficient because the structure of the cell wall was destroyed by steam explosion.

Effect of xylanase concentration on hydrolysis of pistachio shells is shown in Figure 3. Enzymatic hydrolyses were carried out with 430, 600, 1000, 1500 and 3000 U enzyme per g of milling pretreated pistachio shells. Four point four g of D-xylose was produced, when 430 U of xylanase per g of sample used. The value of D-xylose increased up to 11.6 g with 3000 U of xylanase. So it can be deduced that production of D-xylose is directly proportional to enzyme concentration. The comparative amount of D-xylose produced by enzymatic hydrolysis at this time was about 33% of that of D-xylose produced by acid hydrolysis (Table 1).

Enzymatic hydrolysis by mixed enzyme solution of *Penicillium* sp. AHT-1 and Rhizomucor pusillus HHT-1

D-xylose could hardly be produced when only milling treatment was applied to substrates except for pistachio shells. Xylo-oligosaccharides are produced by xylanase with D-xylose. It was considered that these xylo-oligosaccharides were further degraded into D-xylose by β-xylosidase resulting in the improvement of D-xylose production. Enzymatic hydrolysis were carried out by using mixed enzyme solution of xylanase and b-xylosidase from *Penicillium* sp. AHT-1 and *Rhizomucor pusillus* HHT-1. Results are shown in Figure 4. In these experiments, xylanase and b-xylosidase activity were 3,000 U and 19 U per g of sample.
substrate, respectively. The rate of enzymatic hydrolysis was increased from 45% to 67%.
Sixteen g and 9.7 g of D-xylose was produced from 100 g of pistachio shells and barley
brans, respectively. The production of D-xylose was hardly observed from walnut shell,
sunflower seed peels and peanut shells when only xylanase was used, while from
the catalytic action of mixed enzyme on substrates, 14.2 g, 14 g and 14.7 g of D-xylose were
liberated, respectively. This result is equivalent to 75% increase of production rate as com-
pared to that of acid hydrolysis (Table 1). From this experiment, it can be deduced that
xylo-oligosaccharides produced by xylanase from hemicellulose were further degraded
into D-xylose by $\beta$-xylosidase.

Effect of the amount of $\beta$-xylosidase on hydrolysis of pistachio shells was examined.
Enzymatic hydrolyses were carried out by using mixed enzyme solution containing 3,000
U of xylanase and 2 to 33 U per g of substrate with xylanase and $\beta$-xylosidase activity,
respectively. The results are shown in Figure 5. In the case of using enzyme from
Penicillium sp. AHT-1 (3,000 U and 2.2 U per g of substrate with xylanase and $\beta$-
xylosidase activity), about 10 g of D-xylose were produced from 100 g milled pistachio
shells (less than 0.5 mm size). A maximum of 36 g of D-xylose were produced with mixed
enzyme solution from Penicillium sp.

Figure 3 Effect of the crude xylanase concentra-
tion of Penicillium sp. AHT-1 on degradation of pis-
tachio shells

Figure 5 Effect of $\beta$-xylosidase concentration of mixed enzyme of Penicillium sp. AHT-1 and Rhizomucor
pusillus on degradation of pistachio shells
AHT-1 and *Rhizomucor pusillus* HHT-1 containing 3,000 U and 33 U per g of substrate of xylanase and β-xylosidase activity, respectively. So, it was understood that mixed enzyme enhanced the rate of hydrolysis 3 times more than that using enzyme from *Penicillium* sp. AHT-1 solely. D-xylose was perfectly extracted from pistachio shells by the mixed enzyme treatment as compared to the acid hydrolysis result (Table 1).

**Conclusions**

The amount of D-xylose is nearly equal to D-glucose in plant biomass. In the past there has been no efficient means for D-xylose extraction. In this study, we focused on the enzymatic method for producing D-xylose. The key findings of this experiment are summarized below.

1. Steam explosion for enzymatic hydrolysis was more effective than milling pretreatment.
2. Xylanase, β-xylosidase and α-L-arabinofuranosidase are effective to hydrolyze hemi-cellulose. Thirteen grams or more of D-xylose were produced from 100 g milled (less than 0.5 mm size) walnut shell, sunflower seed peel and peanut shells using mixed enzyme solutions of *Penicillium* sp. AHT-1 and *Rhizomucor pusillus* HHT-1.
3. The hydrolysis rates were proportional to the amount of xylanase and β-xylosidase. Thirty-six grams of D-xylose were produced when mixed enzyme solution of *Penicillium* sp. AHT-1 and *Rhizomucor pusillus* HHT-1 containing 3,000 U and 33 U per g of substrate with xylanase and β-xylosidase activity, respectively, was applied.

**References**


