Original article

Ethanol production with *β*-xylosidase, xylose isomerase and *Saccharomyces cerevisiae* from the hydrolysate of Japanese beech after hot-compressed water treatment

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Key words ethanol production・water-soluble portion・hot-compressed water treatment・*β*-xylosidase,・xylose isomerase

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Abstract

Ethanol was produced from the hydrolysate collected as the water-soluble (WS) portion after hot-compressed water (HCW) treatment of Japanese beech. The process involved saccharification with *β*-xylosidase followed by isomerization with xylose isomerase and fermentation with *Saccharomyces cerevisiae*. Several process schemes were compared to investigate the effect of process integration of saccharification, isomerization and fermentation. Higher ethanol yields were obtained for the processes that integrated isomerization and fermentation or saccharification and isomerization. Integration of isomerization and fermentation was effective in converting xylose into ethanol. Similarly, integration of saccharification and isomerization was effective in converting xylooligosaccharides into xylulose. It is presumed that the saccharification reaction toward xylose and the isomerization reaction toward xylulose were linked and therefore each reaction was enhanced.

Introduction

Ethanol production from lignocellulosic hydrolysate often encounters problems such as low efficiency of xylose fermentation and inhibitory effects of compounds in hydrolysates on enzymes and microorganisms. In order to address these problems, development of enzymes, microorganisms, and process strategies are required.

Efficient xylose-fermenting microorganisms have been found among natural and recombinant bacteria, yeasts, and fungi.[1] Another way of producing ethanol from xylose is first converting xylose to xylulose, the ketoisomer of xylose, with xylose isomerase followed by fermenting xylulose to ethanol.[1] Xylose isomerase is an enzyme capable of converting xylose into xylulose.[2] This enzyme is generally known as glucose isomerase and is employed for production of fructose syrups.[2] Isomerization of xylose is an equilibrium reaction between xylose and xylulose and lies toward the xylose product side. Performing the isomerization and fermentation simultaneously has the advantage of pulling the isomerization reaction toward xylulose. However, in the case of the simultaneous process, a compromise must be made between pH and temperature for the enzyme and microorganism.[3]

When xylose isomerase is applied to ethanol production from lignocellulosic hydrolysate, this enzyme is most applicable to low-salinity lignocellulosic hydrolysates such as enzymatically hydrolyzed steam-pretreated materials, because it is highly sensitive to salinity.[4] In our previous study, Japanese beech was solubilized by hot-compressed water (HCW), where the water-soluble (WS) portion contained xylose and xylooligosaccharides. Using β-xylosidase, xylooligosaccharides in the WS portion recovered after HCW treatment were converted into xylose, which is expected to be further converted into xylulose by xylose isomerase. The obtained xylulose is, subsequently, expected to be fermented to ethanol by *Saccharomyces cerevisiae*.

In this study, therefore, ethanol was produced from the hydrolysate collected as the WS portion after HCW treatment of Japanese beech using *β*-xylosidase, xylose isomerase, and *S. cerevisiae* yeast. In order to design an efficient whole process, this study focused on the integration of some of these processes, such as isomerization and fermentation, saccharification and isomerization, and also all of these processes together.

Experimental

Materials and chemicals

Japanese beech (*Fagus crenata*) was subjected to HCW treatment. Wood chips of Japanese beech were milled into flours passing through 80 mesh and were used as raw materials. Xylose and xylulose as standard monosaccharides were purchased from Nacalai Tesque (Kyoto, Japan) and Sigma-Aldrich (St. Louis, MO, USA). Xylobiose and xylotriose as standard xylooligosaccharides were purchased from Wako (Osaka, Japan). Distilled water and 97 % sulfuric acid in high-performance liquid chromatography (HPLC) grade, and sodium acetate for high-performance anion exchange chromatography (HPAEC) eluent were purchased from Nacalai Tesque (Kyoto, Japan). Sodium hydroxide solution (50%w/v) for HPAEC eluent was purchased from Wako.

HCW treatment

The HCW treatment of Japanese beech was conducted in a batch reactor at 240^{o}C for 90 s as described elsewhere.[5,6] The WS portion after the HCW treatment was separated from the residue by filtration with a 0.45-μm membrane and subjected to the subsequent process.

Enzymes

β-Xylosidase was purchased from MP Biomedicals, Inc. (Irvine, CA, USA). According to the manufacturer's descriptions, *β*-xylosidase was purified from *Trichoderma viride*, its activity was 5 U mg^{-1} solid. One unit of *β*-xylosidase was defined as the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol from *p*-nitrophenyl-α-D-xylopyranoside per minute at pH 4.0 at 37°C. *β*-Xylosidase solid (5 U) was dispersed in 0.1 M acetate buffer to be adjusted at 2.9 U ml^{-1} and supplied as enzyme solution.

Xylose isomerase, Sweetzyme T, was purchased from Novozymes (Bagsvaerd, Denmark). According to the manufacturer's descriptions, Sweetzyme T is an immobilized xylose isomerase produced from *Streptomyces murinus*, its activity is 350 U g^{-1}. One unit was defined as the amount of enzyme that converts glucose to fructose at an initial rate of 1μmol min^{-1} at pH 7.5 at 60°C.

Saccharification with *β*-xylosidase

Saccharification with *β*-xylosidase was carried out in a 6-ml glass vial containing 3 ml of the WS portion. *β*-Xylosidase was loaded at 0.03 U ml^{-1} WS portion. The pH was adjusted to 4.0 using solid $Ca(OH)_2$ and HCl solution and the temperature was set at 40°C.

Isomerization with xylose isomerase

After saccharification of the WS portion with *β*-xylosidase, isomerization with xylose isomerase was performed. Xylose isomerase was loaded at 12 U ml^{-1} solution after saccharification

with β-xylosidase. The pH was adjusted to 7.0 using solid $Ca(OH)_2$ and HCl solution and the temperature was set at 50°C. Although this temperature was below the standard condition for the enzyme, a compromise was made between activity and stability, because the stability of this enzyme was low above 50°C. This was particularly important given the prolonged treatment time in this study.

Fermentation

The yeast strain used was *Saccharomyces cerevisiae* (NBRC 0203). Inoculum was cultivated in a medium containing 10 g L^{-1} glucose, 3 g L^{-1} yeast extract, 3 g L^{-1} malt extract and 5 g L^{-1} peptone for 24 h at 28°C with shaking at 120 rpm. A nutrient broth was prepared containing 120 g L^{-1} yeast extract, 120 g L^{-1} malt extract and 200 g L^{-1} peptone. The medium was sterilized by autoclaving at 121°C for 20 min.

Fermentation was carried out without agitation in a 7-ml glass bottle equipped with a cannula for exhaust of carbon dioxide with 0.2 μm filter. The fermentation volume was 3ml, which contained 2.775 ml of the WS portion, 0.15 ml of the inoculums, and 0.075 ml of the nutrient broth. Prior to fermentation, pH was adjusted to 5.0 using solid $Ca(OH)_2$ and HCl solution and the temperature was set at 30°C.

Process integration

In order to study the effect of process integration, each of the processes of saccharification, isomerization, and fermentation was performed simultaneously as well as separately at various pH and temperatures. Process schemes are presented in Fig. 1. Xylooligosaccharides (XO) in the WS portion was the substrate for saccharification. Xylose (X) and xylulose (XL) were the substrates of the

subsequent isomerization and fermentation, respectively. Ethanol (EtOH) was the final product. Schemes 2, 3, and 4 were designed to integrate some of the processes; isomerization and fermentation for scheme 2, saccharification and isomerization for scheme 3, and all of the processes for scheme 4 were integrated. Because the optimum pH and temperature are different between each of the processes of saccharification, isomerization, and fermentation, a compromise condition and the optimum condition for each of the processes were adopted in terms of pH and temperature as in Fig. 1. In the case of scheme 2, for example, pH 6.0 and 40^{o}C (scheme 2c) was a compromised condition between isomerization and fermentation, whereas pH 7.0 and 50^{o}C (scheme 2a) and pH 5.0 and 30^{o}C (scheme 2b) were the optimum conditions for isomerization and fermentation, respectively.

Analytical methods

Xylose, xylulose and ethanol were analyzed by HPLC (LC-10A, Shimadzu, Kyoto, Japan) with a refractive index detector (RID-10A, Shimadzu, Kyoto, Japan) using a Shodex SUGAR KS-801 column (Showa Denko, Tokyo, Japan) The column oven was set at 80^{o}C with distilled water as the mobile phase at a flow rate of 1ml/min. Xylooligosaccharides larger than xylotriose were quantified based on the amount of xylose using an Aminex HPX-87H column, as described elsewhere.[7]

Xylobiose and xylotriose were analyzed by HPAEC (ICS-3000, Dionex, Sunnyvale, CA, USA) with a pulsed amperometric detector (Dionex) using a CarboPac PA-1 column (Dionex) connected with a CarboPac PA-1 guard column (Dionex). Sodium hydroxide solution (0.2M), sodium acetate solution (1M), and distilled water were used as mobile phase in a gradient mixture.[7] Before injections, each of the samples was filtered with a 0.45-μm filter and diluted in a 50-fold excess of water.

Molecular weight distributions of the WS portions after HCW treatments were also

analyzed by gel permeation chromatography (GPC) with and Asahipak GS-220 HQ column (Showa Denko, Tokyo, Japan) and the column oven set to 40^{o}C with the same system and detector as 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 treatment

With HCW treatment of Japanese beech at 240^{o}C for 90 s, xylose, xylobiose, xylotriose and larger xylooligosaccharides were recovered in the WS portion at 2.50 g L^{-1} , 1.30 g L^{-1} , 0.95 g L^{-1} and 4.63 g L^{-1} ,respectively. These saccharides were the substrates that were subjected to conversion into ethanol through saccharification, isomerization and fermentation.

Separate process of ethanol production

First, ethanol production from the WS portion with the separate processes of saccharification with β-xylosidase, isomerization with xylose isomerase, and fermentation with *Saccharomyces cerevisiae* was attempted. Figure 2 shows the time courses of the concentrations of xylose, xylulose, and ethanol during the process. β-Xylosidase, xylose isomerase, and *S. cerevisiae* were added at 0 h, 72 h, and 96 h, respectively.

After 72 h of saccharification, a xylose concentration of 3.74 g L^{-1} was obtained, which apparently decreased to a limited extent during the following isomerization and fermentation. This was probably due to an equilibrium state in the saccharification reaction with β-xylosidase as shown in the previous work.[7] Xylulose produced during isomerization was only 0.39 g L^{-1} and that limited the

ethanol concentration after fermentation to 0.20 g L^{-1} .

Isomerization of xylose into xylulose is a reversible reaction that equilibrates in favor of xylose and produces xylulose in a yield of only 10% to 30% xylose.[3] In this study, the xylulose yield during isomerization based on the initial xylose concentration was difficult to calculate because xylose was not only the substrate of the isomerization: it was also the product of the saccharification occurring at the same time, where the amount of xylose actually involved in the isomerization was more than that of the initial xylose. As shown in Fig. 2, 0.39 g L^{-1} of xylulose was produced during isomerization, even though no xylose appeared to be converted, probably due to an equilibrium state in the saccharification reaction as mentioned above. It appeared that additional xylose was supplied to the isomerization reaction. Supposing that an equivalent amount of xylose to xylulose produced was converted, an additional 0.39 g L^{-1} to the initial 3.74 g L^{-1} of xylose was assumed as the substrate in the isomerization reaction. Based on this assumption, the xylulose yield during isomerization was only about 9% on xylose. It is possible that the hydrolysate has detrimental effects on the activity and stability of the enzyme, as suggested in a previous report regarding isomerization of xylose in a bagasse hydrolysate.[2] The initial reaction rate obtained during the isomerization of the bagasse hydrolysate was slightly lower than that during the isomerization of pure xylose.[2] In order to improve the conversion efficiency of ethanol production, process integration was investigated as below.

Effect of process integration of isomerization and fermentation

Isomerization with xylose isomerase and fermentation with *S. cerevisiae* were applied to the WS portions after saccharification with *β*-xylosidase. Figure 3 shows the time-course concentrations of xylose, xylulose, and ethanol during separate (Fig. 3a) and simultaneous (Fig. 3b) processes. For the simultaneous process, the results obtained at a compromised condition of pH 6.0 and 40^{o}C

(scheme 2c) are shown. In a separate process (Fig. 3a), xylose isomerase was added at 0 h and *S. cerevisiae* was added at 24 h. In a simultaneous process (Fig. 3b), xylose isomerase and *S. cerevisiae* were added at 0h.

Ethanol production was higher for the simultaneous process (Fig. 3b) than for the separate process (Fig. 3a). This result is compatible with previous reports that stated simultaneous isomerization and fermentation is effective in converting xylose into ethanol by pulling the isomerization reaction of xylose toward xylulose.[3] It was found in the separate process (Fig. 3a) that ethanol concentration decreased after it reached the maximum. In the case of fermentation of xylulose by *S. cerevisiae* under aerated conditions, ethanol was produced only at the beginning of the fermentation, and then reassimilated into the system and the metabolism switched over to xylitol production.[8]

The ethanol yield for the simultaneous process (Fig. 3b) was calculated based on the assumption as outlined below. The initial 3.78 g L^{-1} of xylose was partly converted into ethanol to be leave 2.74 g L^{-1} , indicating that 1.04 g L^{-1} of xylose was apparently converted during the process. However, more of the initial xylose was assumed to be involved in this process, where additional xylose was supplied to the following reactions as mentioned in Fig. 2. The ethanol yield was 0.70 g L^{-1} which was 0.17 g L^{-1} above the theoretical ethanol concentration of 0.53g/L, and xylulose remained at 0.08g/L. Supposing that an equivalent amount of xylose to xylulose produced was converted and that a theoretical amount of xylulose to ethanol produced was converted, an additional 0.25g/L of xylose to the initial 3.78g/L of xylose was assumed as substrate in the simultaneous process. Based on this assumption, the ethanol yield was 17% . The yields for the optimum conditions for isomerization (scheme 2a) and for fermentation (scheme 2b) were 13% and 17% (data not shown). Fermentation of xylose in spent sulfite liquor with xylose isomerase has been reported,[4, 9] and the ethanol yields from xylose contained in the hydrolysate were estimated to be 28% and 20% according to the data shown.

The yields in this study were lower than these reports. Ethanol production might be affected by inhibitory effects on fermentation as well as on isomerization as mentioned above, or by the low initial concentration of xylose, because ethanol production rate in xylulose fermentation by *S. cerevisiae* is proportional to the substrate concentration.[10]

Effect of process integration of saccharification and isomerization

Saccharification with β-xylosidase and isomerization with xylose isomerase were applied to the WS portions. Figure 4 shows the time-course of concentrations of xylose, xylobiose, xylotriose, and xylulose during separate (Fig. 4a) and simultaneous (Fig. 4b) processes. For the simultaneous process, the results obtained at a compromise condition of pH 6.0 and 50^{o}C (scheme 3c) are shown. In the separate process (Fig. 4a), β-xylosidase was added at 0h and xylose isomerase was added at 72 h. In the simultaneous process (Fig. 4b), β-xylosidase and xylose isomerase were added at 0 h.

It was confirmed in preliminary experiments that the maximum concentration of xylulose was reached within 24 h of isomerization for the separate process (Fig. 4a) and 96h for the simultaneous process (Fig. 4b). The concentration of xylulose after isomerization for the simultaneous process (Figure 4b) was 0.84 g L^{-1}, which was higher than 0.39 g L^{-1} obtained for the separate process.

For both of the processes, the concentrations of xylose, xylobiose, and xylotriose increased and decreased during the treatments. This was presumably caused by hydrolytic activity of β-xylosidase accompanied by its transxylosilation activity to convert xylobiose and xylotriose into larger xylooligosaccharides.[11] Similar hydrolytic properties of β-xylosidase on the WS portion were found in our previous study, in which not only xylobiose and xylotriose but also xylooligosaccharides larger than xylotriose in the WS portion were found to be effectively hydrolyzed to reach an equilibrium state.[7] This is because xylobiose, xylotriose, and xylooligosaccharides larger than

xylotriose were not completely converted into xylose owing to transxylosilation and/or inhibition of xylose on β-xylosidase.[12] Based on these properties of β-xylosidase, it was assumed that the higher xylulose production for the simultaneous process (Fig. 4b) was because xylose produced in the saccharification was simultaneously converted into xylulose, which pulled the saccharification reaction toward xylose and/or mitigated the inhibition of xylose on β-xylosidase. Consequently, xylose concentration increased and pushed the following isomerization reaction toward xylulose.

However, no particular difference in the time-course concentrations of the products other than xylulose was found between the separate (Fig. 4a) and simultaneous processes (Fig. 4b). To investigate the difference in the product compositions after each of these processes, GPC chromatograms of the WS portions after these processes were compared as shown in Fig. 5. Major peaks were seen around 12 min and 16 min in the chromatogram for the separate processes (trace a), and around 13 to 14 mim and 16 min in the chromatogram for the simultaneous processes (trace b). The peak at around 16 min was assigned to xylose, while those around 13 to 14 min and around 12 min were assigned, respectively, to xylooligosaccharides with degree of polymerization (DP) of 3 to 6 and larger xylooligosaccharide with DP greater than 6. This suggests that more of the smaller xylooligosaccharides were recovered after the simultaneous process (Fig. 5b) than after the separate process (Fig. 5a) probably because the saccharification process was enhanced.

Comparison of the process schemes by ethanol yields

The process schemes were compared by ethanol yields (Table 1). The ethanol yield (percentage of theoretical yield) was defined as the produced ethanol concentration divided by the theoretical ethanol concentration of 4.78 g L^{-1}, which assumes the xylose and xylooligosaccharides initially contained in the WS portion were completely converted into ethanol.

The highest ethanol yields were obtained for the process shown in scheme 3 in which saccharification and isomerization were performed simultaneously. The yields for scheme 2 in which isomerization and fermentation were performed simultaneously were also higher than for the process shown in scheme 1. From these results, it was suggested that integrating saccharification and isomerization, or isomerization and fermentation, could be effective in ethanol production from the WS portion. Nevertheless, the yields for scheme 4 in which all the processes of saccharification, isomerization, and fermentation were performed simultaneously were lower than those for the process shown in schemes 2 and 3 except scheme 2a. This was possibly because the pH and temperature applied in scheme 4 were not optimal for each of the saccharification, isomerization, and fermentation processes. In each of the process schemes, differences in reaction conditions of pH and temperature affected the final ethanol yields to a limited extent except in the scheme 2a, where the temperature of 50^{o}C may be too high for the yeast, *S. cerevisiae*, to grow and facilitate fermentation.

Conclusions

Process integration of isomerization and fermentation was effective in ethanol production from the WS portion treated with β-xylosidase. A similar effect was found in the process integration of saccharification and isomerization for the xylulose production from the WS portion; it was assumed that the saccharification reaction was pulled toward xylose and the isomerization reaction was successively pushed toward xylulose. Owing to these effects, process schemes in which saccharification and isomerization or isomerization and fermentation were integrated could result in higher ethanol yields.

In general, ethanol yields on xylose fermentation with xylose isomerase and yeast (29%-73%)[1] have been lower than those with recombinant bacteria (86%-100%)[1], mainly because of

the low conversion efficiency of xylose to xylulose as mentioned above. By integrating saccharification and isomerization in this study, the conversion efficiency of xylose to xylulose was greatly improved to increase the ethanol yields by approximately four or five times. As a result, the ethanol yield was assumed to reach a level similar to those achieved with recombinant bacteria.

Acknowledgments

This work was supported by KAKENHI (19•6528), a Grant-in-Aid for Japan Society for the Promotion of Science (JSPS) Fellows

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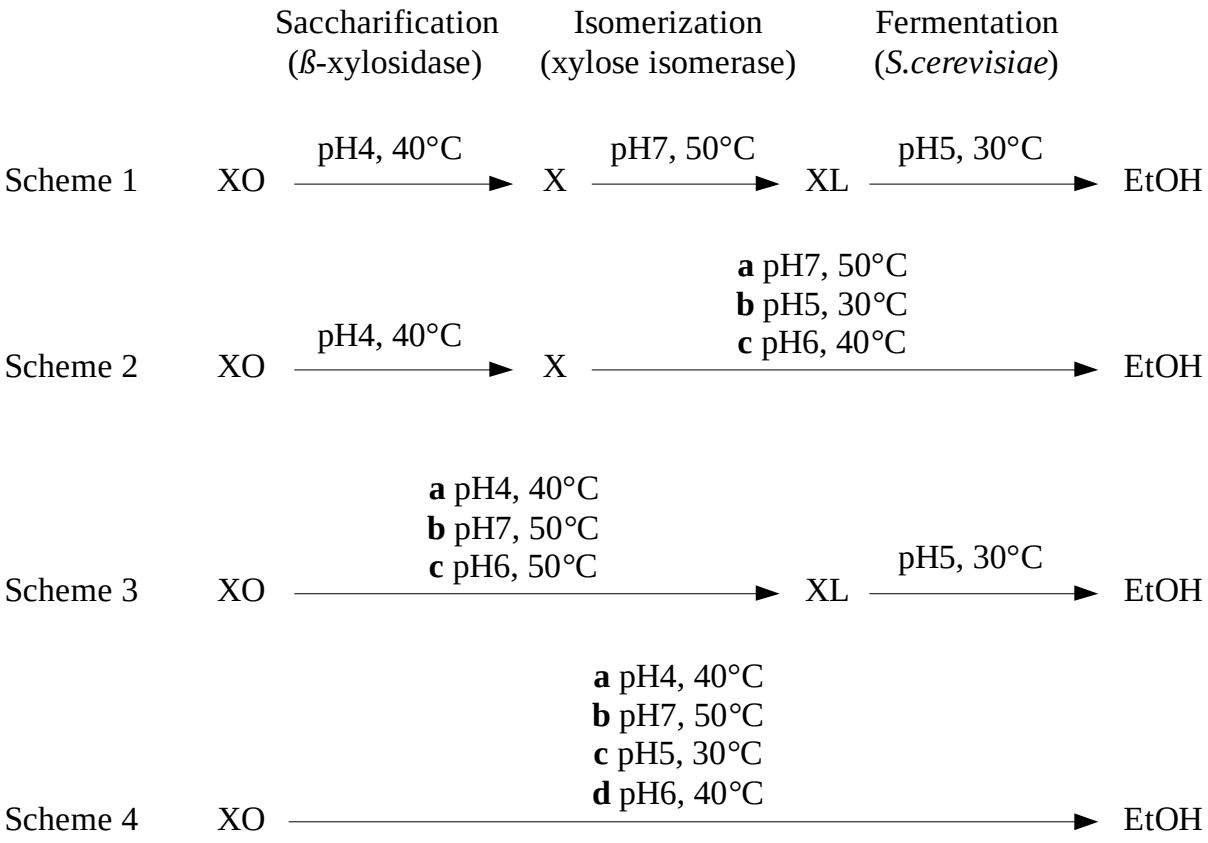

Fig. 1. Process schemes for ethanol production from xylooligosaccharides in the WS portion
XO: Xylooligosaccharide, X: Xylose, XL: Xylulose, EtOH: Ethanol

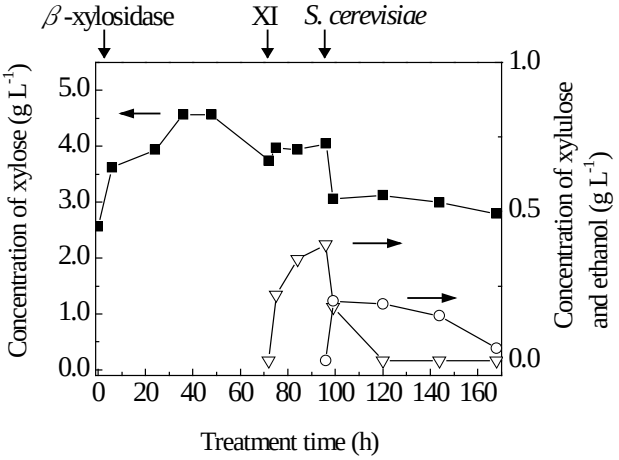

Fig. 2. Time-course concentrations of xylose (filled squares), xylulose (open triangles), and ethanol (open circles) in the WS portions during the separate processes of saccharification with β-xylosidase, isomerization with xylose isomerase (XI), and fermentation with *Saccharomyces cerevisiae*. Vertical arrows indicate time of addition to reaction; horizontal arrows indicate appropriate axes

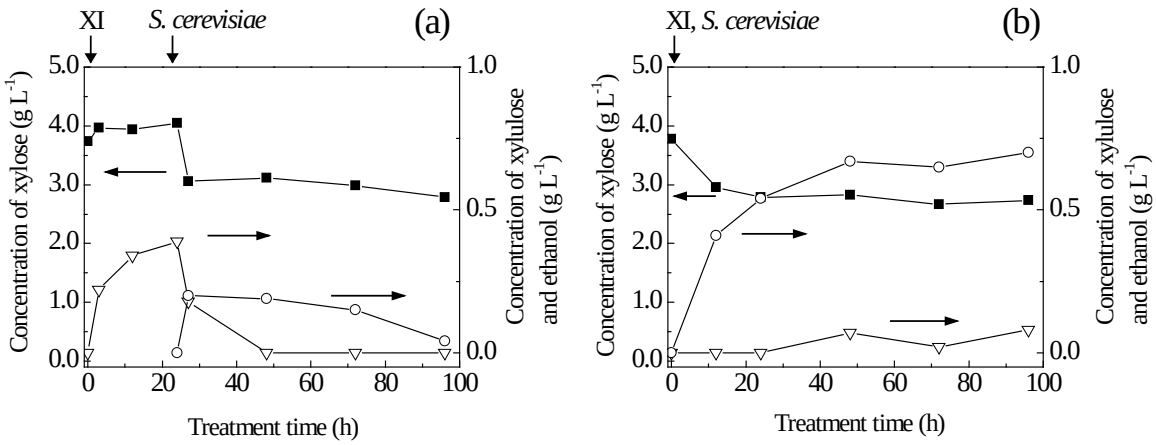

Fig. 3a, b. Time-course concentrations of xylose (filled squares), xylulose (open triangles), and ethanol (open circles) in the WS portions after saccharification followed by isomerization with xylose isomerase and fermentation with *S. cerevisiae* separately (a) and simultaneously (b). Vertical arrows indicate time of addition to reaction; horizontal arrows indicate appropriate axes

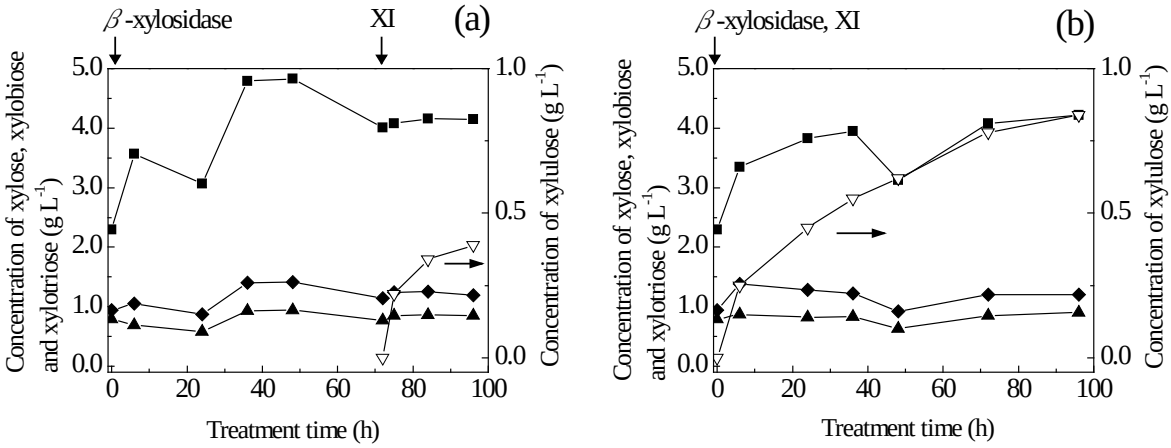

Fig. 4. Time-course concentrations of xylose (filled squares), xylobiose (filled diamonds), xylotriose (filled triangles), and xylulose (open triangles) in the WS portions treated with β-xylosidase and xylose isomerase separately (a) and simultaneously (b). Filled symbols, left axes; open symbols, right axes; vertical arrows, time of addition to reaction

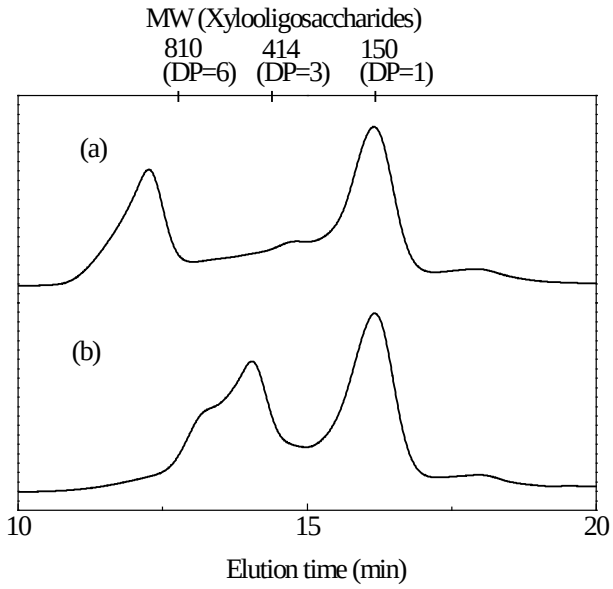

Fig. 5. Gel permeation chromatograms of the WS portions after 96 h of treatment with β-xylosidase and xylose isomerase separately (a) and simultaneously (b). MW, Molecular weight; DP, degree of polymerization

Table 1. Ethanol yields from adopted reaction schemes

Scheme[a]	Conditions	Ethanol produced (g/L)	Ethanol yield (%)[b]
Scheme 1		0.20	4.2
Scheme 2	**a** pH 7, 50°C	0.50	10.5
	b pH 5, 30°C	0.85	17.8
	c pH 6, 40°C	0.70	14.6
Scheme 3	**a** pH 4, 40°C	1.08	22.6
	b pH 7, 50°C	0.78	16.3
	c pH 6, 50°C	0.92	19.2
Scheme 4	**a** pH 4, 40°C	0.62	13.0
	b pH 7, 50°C	0.52	10.9
	c pH 5, 30°C	0.62	13.0
	d pH 6, 40°C	0.44	9.2

[a] Refer to Fig. 1 for descriptions of reaction schemes
[b] Percentage of theoretical yield