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Ethanol fermentation from lignocellulosic hydrolysate by a recombinant xylose- and cellooligosaccharide-assimilating yeast strain

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Abstract The sulfuric acid hydrolysate of lignocellulosic biomass, such as wood chips, from the forest industry is an important material for fuel bioethanol production. In this study, we constructed a recombinant yeast strain that can ferment xylose and cellooligosaccharides by integrating genes for the intercellular expressions of xylose reductase and xylitol dehydrogenase from *Pichia stipitis*, and xylulokinase from *Saccharomyces cerevisiae* and a gene for displaying β-glucosidase from *Aspergillus acleatus* on the cell surface. In the fermentation of the sulfuric acid hydrolysate of wood chips, xylose and cellooligosaccharides were completely fermented after 36 h by the recombinant strain, and then about 30 g/l ethanol was produced from 73 g/l total sugar added at the beginning. In this case, the ethanol yield of this recombinant yeast was much higher than that of the control yeast. These results demonstrate that the fermentation of the lignocellulose hydrolysate is performed efficiently by the recombinant *Saccharomyces* strain with abilities for xylose assimilation and cellooligosaccharide degradation.

Keywords Fermentation · Yeast · Xylose · Lignocellulosic hydrolysate · Ethanol · Cell surface display

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Introduction

Renewable lignocellulosic biomass, such as agricultural and forestry residues, waste paper, and industrial waste, is an attractive feedstock for bioethanol production. The utilization of bioethanol as fuel can significantly suppress the accumulation of greenhouse gasses (McMillan 1997; Claassen et al. 1999). Lignocellulose, which is composed of cellulose, hemicellulose, and lignin (Aristidou and Penttilä 2000; Saha 2003), is often hydrolyzed by acid treatment; the hydrolysate obtained is then used for ethanol fermentation by microorganisms such as yeast. Because such lignocellulose hydrolysate contains not only glucose, but also various monosaccharides, such as xylose, mannose, galactose, and arabinose, and oligosaccharides, microorganisms should be required to efficiently ferment these sugars for the successful industrial production of ethanol.

One of the most effective ethanol-producing yeasts, *Saccharomyces cerevisiae*, has several advantages owing to its high ethanol production from hexoses and high tolerance to ethanol and other inhibitory compounds in the acid hydrolysates of lignocellulosic biomass (Olsson and Hahn-Hägerdal 1993; Hahn-Hägerdal et al. 2001). However, because wild-type strains of this yeast cannot utilize pentoses, such as xylose and arabinose, and celloligosaccharides, ethanol production from a lignocellulose hydrolysate is inadequate. Accordingly, many researchers have engineered yeast capable of xylose fermentation (Ho et al. 1998; Eliasson et al. 2000; Hahn-Hägerdal et al. 2001, Jeffries and Jin 2004). Natural xylose-fermenting yeasts, such as *Pichia stipitis* (Verduyn et al. 1985), *Candida shehatae* (Ho et al. 1990), and *Candida parapsilosis* (Lee et al. 2003), can metabolize xylose via the action of xylose reductase (XR) to convert xylose to xylitol, and of xylitol dehydrogenase (XDH) to convert xylitol to xylulose. Therefore, ethanol fermentation from xylose can be successfully performed by recombinant *S. cerevisiae* carrying heterologous XR and XDH from *P. stipitis*, and xylulokinase (XK) from *S. cerevisiae* (Ho et al. 1999; Eliasson et al. 2000; Toivari et al. 2001).

On the other hand, for the fermentation of cellulosic materials to ethanol, various cellulases and β-glucosidases have been expressed in *S. cerevisiae* (Murai et al. 1998; van Rensburg et al. 1998; Cho and Yoo 1999; Fujita et al. 2004). Also, the hydrolysis of cellooligosaccharides (two to six glucose units) and an efficient ethanol production from cellobiose by a recombinant yeast strain displaying *Aspergillus aculeatus* β-glucosidase 1 (BGL1) on its cell surface have been achieved through cell-surface engineering (Murai et al. 1998; Fujita et al. 2002).

Although the fermentations of xylose and cellobiose are simultaneously performed by the thermotolerant methylotrophic yeast *Hansenula polymorpha* (Ryabova et al. 2003), the ethanol yield and productivity are insufficient. In this study, we constructed a recombinant *Saccharomyces* strain with xylose-fermenting ability by introducing genes of *P. stipitis* XR (*XYL1*) and XDH (*XYL2*), and *S. cerevisiae* XK (*XKS1*) for their intracellular expression, and with cellooligosaccharide-degrading ability by introducing the fusion gene of *A. aculeatus* BGL1 and 3'-half of α-agglutinin for its cell-surface display. Using this recombinant strain, we attempted ethanol fermentation from a lignocellulose hydrolysate, which was prepared by hydrolysing wood chips using concentrated sulfuric acid.

Materials and methods

Strains and media

All the microbial strains constructed and used in this study are listed in Table 1. The *Escherichiacoli* strain used as host strain for recombinant DNA manipulation in this study was NovaBlue {*endA1 hsdR17*($r_{K12}^{-}m_{K12}^{+}$) *supE44 thi-1 gyrA96 relA1 lac recA1/F'* [*proAB*$^{+}$*lacI*^{q}ZΔM15::Tn*10* (Tet^{r})]} (Novagen, Madison, Wisconsin, USA). *S. cerevisiae* MT8-1 (*MAT***a** *ade his3 leu2 trp1 ura3*) was used for cell-surface expression and fermentation (Tajima et al. 1985). *E. coli* was grown in Luria–Bertani medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l sodium chloride) containing 100 mg/l ampicillin. After precultivation, the yeast was aerobically cultivated at 30°C in synthetic medium [SD medium; 20 g/l glucose and 6.7 g/l yeast nitrogen base without amino acids (Difco Laboratories, Detroit, Michigan, USA) with appropriate supplements] containing 20 g/l casamino acids (SDC medium, Difco).

Table 1 Characteristics of strains and plasmids used in this study

Strain or plasmid	Relevant feature	Source or reference
Yeast strains		
S. cerevisiae MT8-1	*MAT***a** *ade his3 leu2 trp1 ura3*	Tajima et al. (1985)
S. cerevisiae		
MT8-1/Con	No expression (control strain)	This study
MT8-1/Xyl	Expressing XR, XDH, and XK	This study
MT8-1/BGL	Displaying BGL1	This study
Bacterial strains		
E. coli NovaBlue	*endA1 hsdR17*($r_{K12}^{-}m_{K12}^{+}$)*supE44 thi-I gyrA96 relA1 lac recA1/F'*[*proAB*$^{+}$*lacI*^{q}ZΔM15::Tn*10*(Tet^{r})]	Novagen
Plasmids		
pRS403	*HIS3* no expression (control plasmid)	Stratagene
pRS406	*URA3* no expression (control plasmid)	Stratagene
pIUX1X2XK	*URA3* intracellular coexpression of *XYL1*, *XYL2*, and *XKSI* genes	This study
pIBG13	*HIS3* surface expression *A. acleaus* β-glucosidase gene	This study

Lignocellulosic hydrolysate

The wood chip hydrolysate used was provided by the JGC (Yokohama, Japan). This hydrolysate was prepared by the modified concentrated sulfuric acid hydrolysis method, which was developed by Arkenol (Irvine, California, USA) (Farone and Cuzens 1997, 1998). The resulting hydrolysate was adjusted to pH 7.0 using $CaCO_3$, and used as lignocellulose hydrolysate for fermentation performance analysis. The wood chip hydrolysate contained 57.5 g/l glucose, 15.1 g/l mannose, 9.3 g/l galactose, 11.2 g/l xylose, and 10.2 g/l cellooligosaccharides.

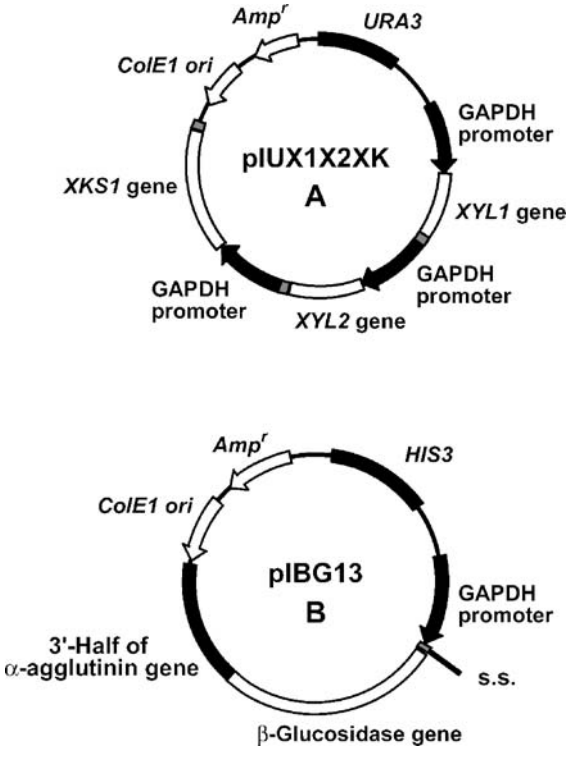

Fig. 1 Physical maps of pIUX1X2XK for intracellular coexpression of *XYL1*, *XYL2*, and *XKS1* (**a**), and expression plasmid pIBG13 for yeast cell surface displaying BGL1-α-agglutinin fusion protein (**b**). *s.s.* secretion signal sequence of *Rhizopusoryzae* glucoamylase gene

Construction of plasmids for cell surface display and intracellular expression

The plasmid pIUX1X2XK (Fig. 1a) used for the intracellular coexpression of the *XYL1*, *XYL2*, and *XKS1* genes was constructed as follows. The following fragments were prepared by polymerase chain reaction (PCR) using primers and plasmids as described by the previous study (Katahira et al. 2004): a 2.2-kbp *Kpn*I-*Xho*I DNA fragment composed of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter, *XYL1* gene, and the GAPDH terminator; a 2.3-kbp *Xho*I-*Not*I DNA fragment composed of the GAPDH promoter, *XYL2* gene, and the GAPDH terminator; and a 3.04-kbp *Not*I-*Sac*II DNA fragment composed of the GAPDH promoter, the *XKS1* gene, and the GAPDH terminator. These DNA fragments were introduced into the *Kpn*I-*Xho*I section, *Xho*I-*Not*I section, and *Not*I-*Sac*II section of the plasmid pRS406 (Stratagene, La Jolla, California, USA), and the resulting plasmid was designated pIUX1X2XK.

The plasmid pIBG13 for the cell surface display of *A. aculeatus* No. F-50 BGL1 was constructed as follows. The 2.5-kbp *Nco*I-*Xho*I DNA fragment encoding *bgl1* gene was prepared by PCR performed with primers 5′-GATCTCC ATGGCTGATGAACTGGCGTTCTCTCCTCCTTTC-3′ and 5′-TGGCGCTCGAGCCTTGCACCTTCGGGAGC GCCGCGTGAAG-3′ and with the plasmid pBG211 as template. This DNA fragment was digested with *Nco*I and *Xho*I and introduced into the *Nco*I-*Xho*I site of the cell-surface expression plasmid pIHCS (Fujita et al. 2002) containing the genes encoding the secretion-signal sequence of the glucoamylase gene from *Rhizopus oryzae* and the 3′-half region of the α-agglutinin gene (Lipke et al. 1989). The resulting plasmid was designated pIBG13 (Fig. 1b).

Yeast transformation

Yeast transformation was carried out by the lithium acetate method using the YEASTMAKER yeast transformation system (Clontech Laboratories, Palo Alto, California, USA). The plasmids pRS406 and pIUX1X2XK were digested with *Nco*I and *Pst*I, respectively, within the *URA3* gene, and the plasmids pRS403 and pIBG13 were digested with *Nde*I within the *HIS3* gene. Then, the linear products were used to transform *S. cerevisiae* MT8-1. The resulting yeast strains are summarized in Table 1.

Enzyme assays

Cell extracts for the assays of xylose metabolic enzymes were prepared as follows: After cultivation in SDC medium for 48 h at 30°C, cells were harvested by centrifugation at 6,000×*g* for 10 min at 4°C and washed with distilled water three times. Then, the cells were resuspended in 0.1 M sodium phosphate buffer with an equal volume of glass beads (0.5 mm diameter). The cells were disrupted for 15 min using 0.5 mm of glass beads. The lysate was centrifuged at 14,000×*g* for 5 min at 4°C, and the supernatant was analyzed for enzyme activities. Protein concentration was measured according to the Bradford method (Bradford 1976), with bovine serum albumin (BSA) as standard.

XR activity was measured spectrophotometrically by monitoring NADPH oxidation at 340 nm (Smiley and Bolen 1982; Walfridsson et al. 1997) in a reaction mixture with the following composition: 10 mM sodium phosphate buffer (pH 7.0) at 30°C, 0.2 M xylose, and 0.12 mM NADPH as substrate. XDH activity was measured spectrophotometrically by monitoring nicotinamide adenine dinucleotide (NAD^+) reduction at 340 nm (Smiley and Bolen 1982; Walfridsson et al. 1997) in a reaction mixture with the following composition: 0.1 M Tris–HCl (pH 7.0) at 30°C, 50 mM xylitol, and 2 mM NAD^+ as substrate. The XK reaction forming adenosine diphosphate was coupled with pyruvate kinase (PK) and lactate dehydrogenase (LDH) reactions. XK activity was measured spectrophotometrically by monitoring NADH oxidation by LDH at 340 nm (Shamanna and Sanderson 1979; Eliasson et al. 2000) in a reaction mixture of the following composition: 0.1 M Tris–HCl (pH 7.0), 2 mM $MgCl_2$, 8 mM NaF, 2 mM ATP, 0.2 mM phosphoenolpyruvate, 3 mM reduced glutathione, 10 U of LDH, 10 U of PK, 0.1 mM NADH, and 8.5 mM xylulose. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol each of NADPH and NADH reduced or oxidized from the substrate per minute.

β-glucosidase activity was measured as described previously (Fujita et al. 2002) with 50 mM sodium acetate buffer (pH 5.0) at 30°C with 1 mM *p*-nitrophenyl-β-D-glucopyranoside (pNPG) (Nacalai Tesque, Kyoto, Japan) as substrate. For activity assay, cultured yeast cells, were harvested by centrifugation at 6,000×*g* for 10 min at 4°C and washed with distilled water three times. The optical density at 600 nm (OD_{600}) of the reaction mixture was adjusted to 1. After the reaction, the supernatant was separated by centrifugation at 14,000×*g* for 5 min at 4°C, and the amount of *p*-nitrophenol released was determined by measuring absorbance at 400 nm. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol from the substrate per minute.

Analysis of substrates and products

Glucose, xylose, mannose, galactose, glycerol, and xylitol in the fermentation medium were analyzed by high performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan). A Shim-pack SPR-Pb column (Shimadzu) was used together with a refractive index detector (model RID-10A, Shimadzu). The HPLC system was operated at 80°C with water (flow rate, 0.4 ml/min) as mobile phase.

Ethanol concentration was measured by gas chromatography. A chromatograph (model GC-8A; Shimadzu) fitted with a flame ionization detector was operated under the following conditions: glass column (3.2 mm×2.0 m)

packed with Thermon-3000 (Shimadzu); temperature of column, injector, and detector, 180°C; and nitrogen carrier gas flow rate, 25 ml/min.

The concentration of cellooligosaccharides in the lignocellulose hydrolysate was determined as described below. The lignocellulose hydrolysate was hydrolyzed by commercially available cellulase and β-glucosidase (Sigma Chemical, St. Louis, MO, USA) in a reaction mixture with 50 mM sodium acetate buffer (pH 5.0) at 30°C. After 12 h incubation, the hydrolysate was analyzed by HPLC, as described above. The concentration of cellooligosaccharides in the lignocellulose hydrolysate was derived from the increase in glucose concentration.

Fermentation

The yeast transformants were aerobically cultivated in SDC medium for 48 h at 30°C. Their cells were collected by centrifugation at 3,000×*g* for 10 min at 4°C and washed with distilled water three times. Then, the cells were inoculated into the fermentation medium containing 6.7 g/l yeast nitrogen base without amino acids, 20 g/l casamino acids, and varieties of sugars as the sole carbon source. All fermentations were performed at 30°C with mild agitation at 100 rpm in 100-ml closed bottles equipped with a bubbling CO_2 outlet. The initial cell density in the fermentation medium was adjusted to an OD_{600} of 20.

During fermentation, cell growth was observed by monitoring optical density at 600 nm. Dry cell weight (DW) was determined as follows: the culture broth (10 ml) adjusted to various OD_{600} values was pelleted in preweighed 15-ml tubes by centrifugation at 3,000×*g* for 10 min. After resuspension in 10 ml of distilled water and further centrifugation, the pellets were freeze-dried with FreeZone FZ-1 (Labconco, Kansas, MO, USA). DW was calculated by reweighing the tubes. Cell concentration was estimated from the OD_{600}-to-DW correlation (0.31 g-DW/OD_{600} values). Specific ethanol production rate was determined as the coefficient of the linear regression of ethanol concentration vs the time integration of biomass concentration.

Result

Enzyme activities

In this study, we generated the xylose-fermenting yeast strain (MT8-1/Xyl), yeast strain displaying BGL1 on its cell surface (MT8-1/BGL), and xylose-fermenting and BGL1-displaying yeast strain (MT8-1/Xyl/BGL) by integratiing pIUX1X2XK for the coexpression of *XYL1*, *XYL2*, and *XKS1*, and pIBG13 for displaying BGL1 (Table 1).

The XR, XDH, and XK activities of all yeast strains were measured (Table 2). MT8-1/Xyl and MT8-1/Xyl/BGL with the three genes for xylose metabolism introduced into their chromosome showed similar XR, XDH, and XK activities, while MT8-1/Con and MT8-1/BGL showed no activity. The XR/XDH ratios of MT8-1/Xyl and MT8-1/Xyl/BGL were 0.10 and 0.13, respectively.

The BGL1 activity of the yeast strains displaying BGL1 was measured using both culture supernatant and cell pellet fractions. As shown in Table 2, the strains displaying BGL1 (MT8-1/BGL and MT8-1/Xyl/BGL) showed similar BGL activities [15.44 and 13.46 U/g (dry weight) of cells, respectively], while MT8-1/Con and MT8-1/Xyl showed no BGL1 activity. No BGL1 activity was detected in culture supernatant for all the strains (data not shown).

Ethanol fermentation from xylose and cellobiose

Ethanol fermentation from xylose was performed with 50 g/l xylose as carbon source using MT8-1/Xyl, MT8-1/Xyl/BGL, and MT8-1/Con (Fig. 2). The xylose-utilizing MT8-1/Xyl and MT8-1/Xyl/BGL produced about 18 g/l ethanol, and xylose was consumed at about 47 g/l at 72 h; no ethanol production or xylose consumption was observed in the control strain. The specific ethanol production rates of MT8-1/Xyl and MT8-1/Xyl/BGL were 0.064 and 0.058 g of ethanol per gram of DW per hour, respectively. The ethanol yield estimated from the grams of ethanol produced per gram of consumed xylose after 72 h fermentation by both MT8-1/Xyl and MT8-1/Xyl/BGL was 0.37 g per gram (Table 3). This ethanol yield corresponds to 72.5% of the theoretical yield (0.51 g of ethanol per gram of xylose). Xylitol accumulation in fermentation medium was maintained at a level lower than 2 g/l in both MT8-1/Xyl and MT8-1/Xyl/BGL (data not shown). Meanwhile, glycerol accumulation was observed in both strains (about 6 g/l at a maximum; data not shown). These yeast strains hardly grew during the fermentation experiments. The pHs of the fermentation medium of MT8-1/Xyl and MT8-1/Xyl/BGL decreased from 5.6 to 4.6 after 72 h fermentation.

Ethanol fermentation from cellobiose was performed with 50 g/l cellobiose as carbon source using the yeast

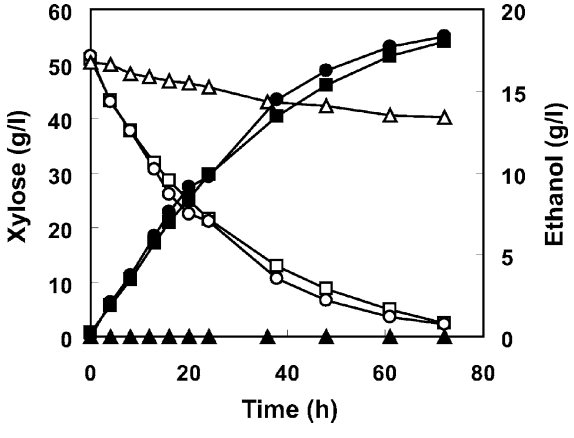

Fig. 2 Time course of ethanol fermentation from 50 g/l xylose as sole carbon source by recombinant yeast strains. *Triangle* MT8-1/Con; *circle* MT8-1/Xyl; and *square* MT8-1/Xyl/BGL. *Open* and *closedsymbols* show the xylose and ethanol concentrations, respectively. The *data points* are the average of two independent experiments

Table 2 Distribution of XR, XDH, and XK activities in cell extracts of cells, and BGL1 activity of cells

Strain	Enzyme activity (U/mg of protein)[a]			BGL1 activity in cells [U/g (dry weight) of cells][a]
	XR	XDH	XK	
MT8-1/Con	ND[b]	ND	ND	ND
MT8-1/Xyl	0.19	1.83	0.39	ND
MT8-1/BGL1	ND	ND	ND	15.44
MT8-1/Xyl/BGL1	0.23	1.75	0.37	13.46

[a]Values are average of three independent experiments. Derivations are always below 10% of the average
[b]*ND* not detected

strains displaying BGL1 on their cell surface, MT8-1/BGL and MT8-1/Xyl/BGL, and the control strain MT8-1/Con (Fig. 3). MT8-1/BGL and MT8-1/Xyl/BGL consumed cellobiose completely within 24 h and produced final ethanol concentrations of 19.3 and 18.4 g/l, respectively, whereas MT8-1/Con showed neither cellobiose reduction nor ethanol production in the medium. MT8-1/BGL and MT8-1/Xyl/BGL showed specific ethanol production rates of 0.10 and 0.08 g g^{-1} h^{-1}, respectively (Table 3). The ethanol yields from consumed cellobiose after 24 h fermentation by MT8-1/BGL and MT8-1/Xyl/BGL were 0.37 and 0.38 g per gram, respectively (Table 3). These ethanol yields correspond to 72.5 and 74.5% of the theoretical yield (0.51 g of ethanol per gram of glucose), respectively. Glucose accumulation was hardly observed during the fermentation (data not shown). Meanwhile, glycerol accumulation was observed in both strains (about 4 g/l at maximum; data not shown). Additionally, during the fermentation experiments, these strains grew gradually, and cell concentrations reached approximately 10 g/l DW after 24 h fermentation. The pHs of the fermentation medium of MT8-1/BGL and MT8-1/Xyl/BGL decreased from 5.6 to 4.3 and 4.2, respectively, after 24 h fermentation.

Table 3 Specific ethanol production rates and ethanol yields of recombinant strains in fermentation of xylose and cellobiose[a]

Strain	Specific ethanol production rate[b]		Ethanol yield[c]	
	Xylose	Cellobiose	Xylose	Cellobiose
MT8-1/Con	0	0	0	0
MT8-1/Xyl	0.06	0	0.37	0
MT8-1/BGL	0	0.10	0	0.37
MT8-1/Xyl/BGL	0.06	0.08	0.37	0.38

[a]Displayed values are the average of two independent experiments Deviations are always below 10% of the average
[b]Specific ethanol production rate is expressed in grams of ethanol/grams of dry weight of cells per hour
[c]Ethanol yeild is expressed in grams of produced ethanol/grams of consumed sugar after 72 h of fermentation in xylose and 24 h of fermentation

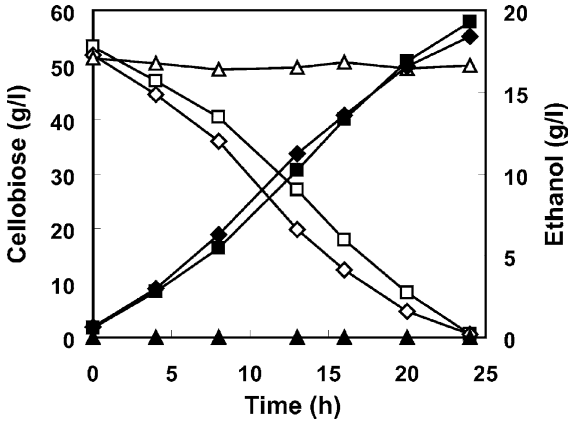

Fig. 3 Time course of ethanol fermentation from 50 g/l cellobiose as sole carbon source by recombinant yeast strains. *Triangle* MT8-1/Con; *diamond* MT8-1/BGL; and *square* MT8-1/Xyl/BGL. *Open* and *closed symbols* show the cellobiose and the ethanol concentrations, respectively. The *data points* are the average of two independent experiments

Ethanol fermentation from sugar mixture

A model sugar mixture reflecting the sugar composition of the lignocellulose hydrolysate was fermented by MT8-1/Xyl/BGL and MT8-1/Con. The fermentation medium contained 50 g/l glucose, 10 g/l mannose, 5 g/l galactose, 10 g/l xylose, and 10 g/l cellobiose. Glucose and mannose were rapidly fermented within 8 h by both strains. The recombinant strain MT8-1/Xyl/BGL degraded cellobiose and metabolized xylose (Fig. 4a), whereas the control strain utilized neither cellobiose nor xylose (Fig. 4b). MT8-1/Xyl/BGL metabolized 7.3 g/l xylose after 36 h fermentation, and completely hydrolyzed cellobiose within 12 h. Then, the highest ethanol concentration produced by MT8-1/Xyl/BGL reached approximately 32.5 g/l after 36 h fermentation, whereas the ethanol production by the control strain was less than 26 g/l after 16 h fermentation. MT8-1/Xyl/BGL produced 0.83 g/l xylitol at 36 h (data not shown). Meanwhile, galactose was slightly consumed by these strains. After 36 h fermentation, galactose remained at about 3 g/l in the medium of these strains (data not shown). This phenomenon suggests that the strain MT8-1 used in this study has a low galactose metabolic performance. MT8-1/Xyl/BGL and MT8-1/Con showed specific ethanol production rates of 0.41 and 0.40 g g^{-1} h^{-1}, respectively (Table 4). The ethanol yields of MT8-1/Xyl/BGL and MT8-1/Con estimated from the grams of ethanol produced per gram of total sugar added at the beginning were 0.39 and 0.32 after 36 h fermentation, respectively (Table 4). During the fermentation experiments, these strains grew gradually and cell concentrations of MT8-1/Con and MT8-1/Xyl/BGL reached 10.8 g and 11.2 g/l DW after 24 h fermentation, respectively (data not shown). The pHs of the fermentation medium of MT8-1/Con and MT8-1/Xyl/BGL decreased from 5.6 to 4.1 and 4.2, respectively, after 24 h fermentation.

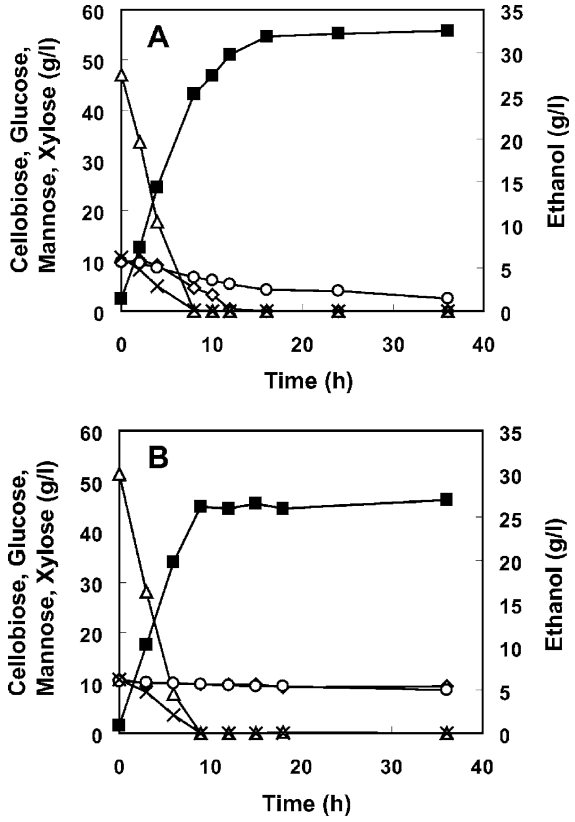

Fig. 4 Fermentation of sugar mixture by MT8-1/Xyl/BGL (**a**) and MT8-1/Con (**b**). *Closed square* ethanol; *open triangle* glucose; *open circle* xylose; *open diamond* cellobiose; and *cross* mannose. The *data points* are the average of two independent experiments

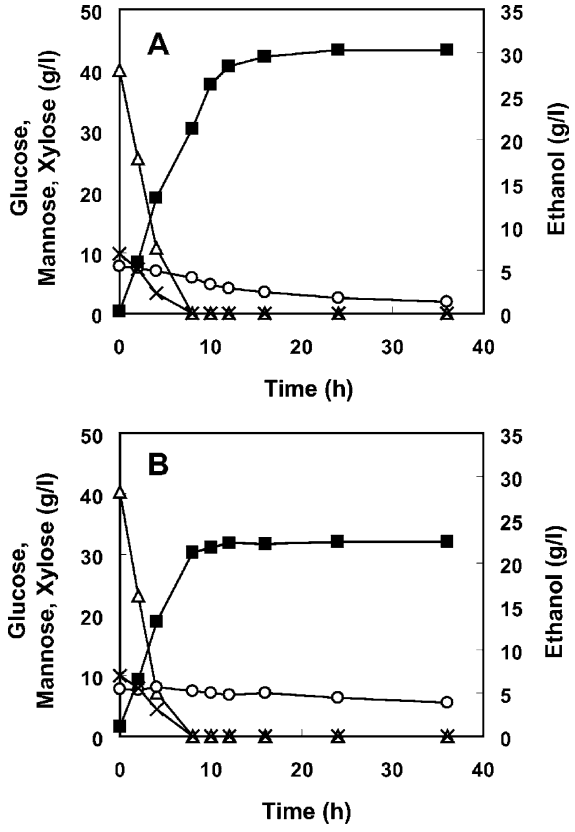

Fig. 5 Fermentation performances of MT8-1/Xyl/BGL (**a**) and MT8-1/Con (**b**) from lignocellulosic hydrolysate as carbon source. *Closed square* ethanol; *open triangle* glucose; *open circle* xylose; and *cross* mannose. The *data points* are the average of two independent experiments

Ethanol fermentation from lignocellulose hydrolysate

The lignocellulose hydrolysate was fermented by MT8-1/Xyl/BGL and MT8-1/Con. The fermentation medium contains 40.2 g/l glucose, 10.5 g/l mannose, 6.5 g/l galactose, 7.9 g/l xylose, and 7.2 g/l cellooligosaccharides. In the fermentation of the lignocellulose hydrolysate, glucose and mannose were rapidly fermented within 8 h by both strains (Fig. 5a,b). In the fermentation by MT8-1/Con as reference strain, the highest ethanol concentration was about 22 g/l after 12 h fermentation (Fig. 5b). Meanwhile, MT8-1/Xyl/BGL completely hydrolyzed and assimilated cellooligosaccharides and assimilated about 6 g/l xylose within 36 h, and achieved the highest ethanol concentration of 30.3 g/l after 36 h fermentation (Fig. 5a). A small amount of galactose was fermented within 36 h (data not shown). In the fermentation media of MT8-1/Xyl/BGL and the control strain, xylitol accumulation was hardly observed during the experiment (less than 1 g/l; data not shown). MT8-1/Xyl/BGL and MT8-1/Con showed specific ethanol production rates of 0.42 and 0.40 g g^{-1} h^{-1}, and ethanol yields of 0.41 and 0.31 g per gram, respectively (Table 4).

Table 4 Specific ethanol production rates and ethanol yields of recombinant strains in fermentation of sugar mixture and lignocellulosic hydrolysate[a]

Strain	Specific ethanol production rate[b]		Ethanol yield[c]	
	Sugar mixture	Hydrolysate	Sugar mixture	Hydrolysate
MT8-1/Con	0.40	0.40	0.32	0.31
MT8-1/Xyl/BGL	0.41	0.42	0.39	0.41

[a]Displayed values are the average of two independent experiments. Deviations are always below 10% of the average
[b]Specific ethanol production rate is expressed in grams of ethanol/grams of dry weight of cells per hour
[c]Ethanol yield is expressed in grams of produced ethanol per grams of total sugar after 36 h of fermentation

Discussion

We generated the recombinant yeast strains with xylose-assimilating and cellooligosaccharide-degrading abilities by integrating the genes for *XYL1*, *XYL2*, *XKS1*, and BGL1-α-aggulutinin in the yeast chromosome. Then, the fermentation performance of these recombinant strains was examined using xylose, cellobiose, the model sugar mixture, and the lignocellulose hydrolysate.

MT8-1/BGL and MT8-1/Xyl/BGL showed similar BGL1 activities. In its fermentation, cellobiose was completely hydrolyzed within 24 h by both strains. In addition, both strains showed similar specific ethanol production rates and ethanol yields. Meanwhile, in glucose fermentation, specific ethanol production rates were approximately 0.43 g g^{-1} h^{-1} for all the strains used in this study (data not shown). Specific ethanol production rate in fermentation of cellobiose was a quarter of that in the glucose fermentation. These results indicate that cellobiose hydrolysis by BGL1 is the rate-limiting step of ethanol fermentation from cellobiose. Thus, further work is needed to improve β-glucosidase activity for the enhancement of ethanol production rate.

The ethanol fermentation from xylose by these recombinant strains was performed efficiently without a marked xylitol accumulation, although a small amount of glycerol was accumulated (data not shown). As shown in Table 3, the ethanol yields of the xylose-assimilating strains (MT8-1/Xyl and MT8-1/Xyl/BGL) were higher than those of TMB3001 and 1400(pLNH32) (0.31 and 0.3, respectively), well-known xylose-fermenting *S. cerevisiae* strains (Ho et al. 1998; Eliasson et al. 2000; Jeppsson et al. 2002). The higher ethanol yields of MT8-1/Xyl and MT8-1/Xyl/BGL must be due to a very low xylitol accumulation (lower than 2 g/l). The low XR/XDH activity ratios of MT8-1/Xyl and MT8-1/Xyl/BGL (0.10 and 0.13) may contribute to a low xylitol accumulation (Walfridsson et al. 1997). Glycerol accumulation may be caused by the reoxidation of NAD^+, produced via NAD-dependent XDH, with glycerol formation, because of the intracellular redox imbalance caused by the difference in coenzyme specificity between XR and XDH. Recently, it has been reported that the coenzyme specificity of XDH from *P. stipitis* is completely changed from NAD^+ to nicotinamide adenine dinucleotide phosphate ($NADP^+$) by multiple-site direct mutagenesis (Watanabe et al. 2005). Moreover, the NADH-preferring XR from *C. parapsilosis* was observed (Lee et al. 2003). By using these enzymes, the principal stoichiometric redox problem might be solved.

Ethanol fermentation from xylose and cellobiose by wild-type strains of the thermotolerant methylotrophic yeast *H. polymorpha* was reported (Ryabova et al. 2003). *H. polymorpha* 356 produced more ethanol than *P. stipitis* in xylose fermentation at 37°C. However, the ethanol yield was approximately 0.12 g per gram after 60 h fermentation. Additionally, Ryabova et al. (2003) showed the growth of and ethanol production by *H. polymorpha* strains with cellobiose. *H. polymorpha* KT2 produced 5.3 g/l ethanol after 96 h fermentation. Although it is difficult to strictly compare our results with their results because of the difference in the experimental conditions used, the ethanol yields of MT8-1/Xyl/BGL in xylose and cellobiose fermentations were higher than those of yeast strains reported previously.

In the fermentation of the model sugar mixture, the ethanol yield of MT8-1/Xyl/BGL was considerably higher than that of the control strain (Table 4), although the specific ethanol production rates were similar in these strains. This was due to the consumption of xylose and cellobiose by MT8-1/pXyl/BGL. No consumption of these sugars by the control strain was observed. To our knowledge, this is the first report of ethanol fermentation from sugar mixture including cellobiose and xylose in a recombinant *S. cerevisiae* strain. In the fermentation of the lignocellulose hydrolysate of wood chip by concentrated sulfuric acid hydrolysis, as with the fermentation of the sugar mixture, MT8-1/Xyl/BGL showed a much higher ethanol yield than the control strain (Table 4). In addition, the specific ethanol production rate in the fermentation of lignocellulosic hydrolysate was similar to that in the fermentation of the model sugar mixture, indicating that there is no inhibitory effect of the components of lignocellulosic hydrolysate on ethanol fermentation. The concentrations of inhibitory compounds, such as furfural or hydroxymethylfurfural, were very low in the hydrolysate (data not shown). As a result, an effective ethanol fermentation from the lignocellulosic hydrolysate was realized using the xylose- and cellooligosaccharide-assimilating yeast strain.

In this study, we successfully demonstrated the efficient ethanol fermentation from a lignocellulose hydrolysate by the recombinant *S. cerevisiae* strain. Although we used a laboratory strain as a host in this study, the commercialization of lignocellulose hydrolysate fermentation will require strains that exhibit a high growth rate, a rapid fermentation rate, a high temperature tolerance, and a high resistance to ethanol and inhibitory substances (Olsson and Hahn-Hägerdal 1993; Hahn-Hägerdal et al. 2001). Typically, polyploid industrial strains are capable of rapid fermentation and are more resistant to the hydrolysate than the haploid laboratory strains (Sonderegger et al. 2004). Further studies using industrial strains as hosts of gene recombination and for the commercial production of ethanol from lignocellulose with low cost might be expected.

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